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**Development of Personal Care Products with Active  
Ingredients Obtained from Natural Products without  
Added Value**

Thesis Submitted in Fulfillment of the Requirements to Obtain the PhD Degree in  
Pharmaceutical Sciences - Pharmaceutical Technology Speciality

Tese do 3.º Ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências  
Farmacêuticas na Especialidade de Tecnologia Farmacêutica

**Work developed under supervision of Professor M.<sup>a</sup> Beatriz P.P. Oliveira and co-  
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**March 2016**

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Francisca Pinto Lisboa Martins Rodrigues Sarmiento

*“Beauty is a harmonious relation between something in our nature and the quality of the object which delights us”.*

Blaise Pascal

# Development of Personal Care Products with Active Ingredients Obtained from Natural Products without Added Value

This work was financially supported by research grants from:

- Foundation for the Science and Technology (Portugal) through a PhD scholarship (SFRH/BDE/51385/2011), financed by POPH-QREN and subsidized by European Science Foundation;
- Fourmag, Lda;
- European Union (FEDER funds through COMPETE) and National Funds (FCT, Foundation for the Science and Technology), through project UID/ QUI/50006/2013 - POCI/01/0145/FERDER/007265, with financial support from FCT/MEC through national funds and co-financed by FEDER, under the Partnership Agreement PT2020.



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## **Dedication**

*I dedicate this thesis to the most important persons in my life, my family, Bruno and our lovely daughter, Maria Francisca.*

## Acknowledgements

I would like to formally express my deep gratitude to the following people and institutions - who (and which) have meant a lot to me during my PhD program and made it possible:

Professor Beatriz Oliveira for all the guidance and support, in the easiest and most difficult moments. All the friendship and trust placed in me to perform this and other works and for all the opportunities provided to make me grow as a researcher. Also for all the scientific discussions, comments and corrections performed to the common publications and the present thesis.

Professor Helena Amaral for all the support and all the comments and corrections performed to the common papers and the present thesis.

My most sincerely acknowledges to Fourmag Lda., for the crucial collaboration in my PhD course, the indubitable hospitality and for providing facilities and logistical to best support my studies. Particularly, a special thank to Ana Almeida, Elisabete Sousa and Carla Miranda, for all the moments of relax and fun that we had! Also, a special thank to Sr. Rogério Cardoso and Sr. Mário Cardoso, for trust in me and accept most of the suggestions that I made during my PhD.

Laboratory of Bromatology and Hydrology for provide me conditions and equipments to develop my work and for all kindness and constant availability cooperation.

The Director of Department of Drug Sciences, Laboratory of Pharmaceutical Technology, from Faculty of Pharmacy University of Porto, Professor Sousa Lobo, for accepting me as PhD student during my doctoral program.

The members of Laboratory of Bromatology and Hydrology, particularly, Elsa Vieira, Sónia Soares, Joana Santos, Olga Cardoso, Anabela Costa, Filipa Pimentel, Antónia Nunes and Ivone Pinto, for their friendship, help assistance, scientific discussions and moments of relax in the laboratory, in an almost daily basis.

Bruno Sarmiento Research Team for the equipment for *in vitro* toxicity and stability evaluation and all support. Particularly, Dr. José das Neves from INEB, for



assistance in the initial *in vitro* toxicity studies, and Carla Pereira, from INEB, for the assistance in preparing histology slides.

Ana Palmeira de Oliveira, from Faculty of Health Sciences - University of Beira Interior, for the collaboration in antimicrobial evaluation and all scientific discussion that we had.

The Inovapotek Pharmaceutical Research members, particularly Rita Matias and Rita Figueiredo, for teach me the techniques of *in vivo* experiments.

Ana Catarina, Cláudia Nunes and Professor Salette Reis, Department of Chemical Sciences from Faculty of Pharmacy University of Porto, for their support, scientific discussion and knowledge transmission that had awakened my interest for a new area.

Ana Isabel Oliveira, from Escola Superior de Tecnologia da Saúde do Porto, for the collaboration in the use of some equipments.

José das Neves for his friendship and for his valuable scientific and personal advices and the motivation he gave me to take my work further.

All my volunteers that participated in the *in vivo* assays. Thank you for trust in me and my project.

The Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde & Instituto Universitário de Ciências da Saúde, CESPU, for the available cooperation during this project.

BICAFÉ for kindly provide me samples of coffee silverskin.

DS-Produtos Químicos for all raw materials that had send me during my PhD, particularly during the scale up process.

My closest friends for their friendship, support, companionship, complicity, and for putting me up in the most difficult moments. Particularly, an extra smile for those who appears late in our life to show us that we are always in time to find truly friendships!!

A particular word to Cristina Rodrigues, one of my old cousins (I know you hate this word) to all enthusiastic efforts to recruit most of her friends for my studies. Thanks for your friendship and all lunches together! You are my favourite cousin!

Those I most love in life, my mother and my father, that continuously encourage and support me and without whom I would not be who I am. As you say daddy, to you took me to kindergarten, to primary school, to basic and secondary education, to the university, to my marriage, and finally to my dream: my PhD!! Thank you so much for all your support and for believe in me!! Mummy, you put my feet on the ground when my dreams go too high. You are my one-story reference, my anchor!!

My beloved husband, Bruno, for his truly love and for share with me those four amazing years of my live!! You are my love, my best friend and my truly partner! You teach me to go, feet by feet, in the tortuous route of life and science. Sometimes you see me fly to high and push me down...Sometimes you know that I am completely disappointed and you make me smile!! In those moments you gave me all those thinks that make me believe in me (especially chocolates and cakes!!)...I love you my sweetheart and you are part of me!!

Last, the most important person in my life: my daughter Maria Francisca!!! Girl, I love you so much so much so much that I can destroy you with kisses!!! You are my sunshine!! I dream with you all my life!! Since I was a child I want to be a mother and you are the baby of my dream!!

To Foundation for the Science and Technology (Portugal) for the PhD grant SFRH/BDE/51385/2011 financed by POPH-QREN and subsidized by European Science Foundation. This grant permitted timely development of my research program, as well as participation in several international scientific meetings to complement my training and sharing my results. This work received financial support from the European Union (FEDER funds through COMPETE) and National Funds (FCT, Foundation for the Science and Technology) through project LAQV/UID/QUI/50006/2013. The work also received financial support from the European Union (FEDER funds) under the framework of QREN through Project NORTE-07-0124-FEDER-000069. To all financing sources the author is greatly indebted.



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## Abstract

Currently, there is an increasing interest of cosmetic industry on natural extracts. The inclusion of antioxidants in topical formulations can contribute to minimize oxidative stress in the skin, which has been associated with aging. Agro-industrial by-products have the potential to be used with different purposes, providing economical advantage to otherwise disposable residues. In particular, the field of skin care products and cosmetics may benefit from these remaining materials. Also, questions of sustainability are leading to the study of new cosmetic ingredients obtained from food by-products. Coffee Silverskin (CS) is the most abundant solid by-product generated during coffee roasting. *Medicago* is a leguminous crop used as animal feeding, normally discarded as waste. Thus, this work aims to explore the use of food by-products extracts as cosmetic active ingredients.

Hydroalcoholic extracts of seven species of *Medicago* (*M. minima*, *M. tornata*, *M. truncatula*, *M. rigidula*, *M. scutelata*, *M. segitalis* and *M. sativa*) and aqueous, hydroalcoholic and alcoholic extracts of CS were characterized for total phenolic and flavonoid contents (TPC and TFC, respectively), antioxidant activity (by 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP), antimicrobial activity and cytotoxicity in two skin cell lines (fibroblasts and keratinocytes). For both antioxidant activity methods CS extracts displayed the highest antioxidant activity. The TPC of the different extracts were in the same range, varying from 18 to 36 mg GAE/g db sample, being the highest TPC obtained for *M. minima*. The highest TFC were obtained with *M. rigidula*. All extracts demonstrated a low cytotoxicity. Preliminary assays for antimicrobial potential showed that extracts display antibacterial activity, particularly CS extracts. Regarding the isoflavones content in *Medicago* extracts, two glycosides (daidzin and genistin) and six aglycones (daidzein, glycitein, genistein, formononetin, prunetin and biochanin A) were determined by HPLC/DAD. In all samples genistin, daidzein and genistein were the most abundant isoflavone compounds.

An *in vitro* skin and ocular irritation assay using reconstructed human epidermis, EpiSkin™, and human corneal epithelial model, SkinEthic™ HCE, and an *in vivo* assay were performed for CS extracts. The *in vitro* results showed that extracts were not classified as irritant and the histological analyses proved that extracts did not affect the both models structure. The content of caffeine (CAF), 5-hydroxymethyl furfural (HMF) and chlorogenic acid (CGA) was quantified after the epidermal assay. The *in vivo* test carried out with the most promising extract (hydroalcoholic) in 20 volunteers proved

that extracts can be regarded as safe for topical application. Therefore, different formulations with *M. sativa* and/or CS extract were developed and characterized. Formulations showed stable physical properties and antioxidant activity during 180 days of storage at 25 °C/ 65% relative humidity (RH) and 40 °C/ 75% RH. *In vitro* toxicity was screened in two skin cell lines (fibroblasts and keratinocytes) and any toxicity was reported. A sensorial test was carried out for all formulations and the consumer acceptance was very high, with more than 90% of volunteers classifying them as very pleasant. An *in vivo* test carried out showed that, with respect to irritant effects, formulations can be regarded as safe for topical application and the skin hydration improved after 30 days of its use.

A facial formulation with CS and the same cream enriched with hyaluronic acid were evaluated *in vivo* and compared. Formulations were applied twice a day by volunteers (n=20 for each formulation) during 28 days. The influence on skin hydration and viscoelastic properties were investigated with validated devices (Corneometer<sup>®</sup> and Cutometer<sup>®</sup>). Wrinkles depth, roughness, volume of cavities and Visioface<sup>®</sup> images were analysed at time 0 and after 28 days. Volunteers were asked about efficacy perception. Significant changes in skin hydration and viscoelastic parameters were detected for both formulations, without differences between them. However, no differences were observed regarding wrinkles depth, roughness and volume of cavities for both formulations. CS represents an effective ingredient for cosmetic creams which are intended to increase skin hydration and firmness. These natural extracts, which toxicology, safety and effectiveness were evaluated *in vitro* and *in vivo* according to the international guidelines, proved to be promising active ingredients for cosmetics. It can be also concluded that this study highlights the need to explore new food wastes as active ingredients to be used on skin care.

Finally, nanostructured lipid carriers (NLCs) loaded with caffeine obtained from CS were developed and evaluated as a new approach. The produced NLCs were within the nanosized range ( $\approx 200$  nm) with relatively low polydispersity index ( $< 0.25$ ) and zeta potential values around - 30 mV. NLCs demonstrated storage stability at 25 °C/ 65% RH and 40 °C/ 75% RH up to 180 days. The association efficiency of caffeine was about 30% at time of production and after the storage period. The *in vitro* skin permeation study demonstrated that caffeine loaded into NLCs was similarly permeable through skin compared to non encapsulated caffeine.

**Key-words:** Coffee silverskin; *Medicago*; Cosmetic; 3-D *in vitro* models; Skin biophysical techniques; *In vivo* studies

## Resumo

Há, atualmente, um interesse crescente da indústria cosmética na utilização de extratos naturais. A inclusão de antioxidantes em formulações tópicas contribui para minimizar o stresse oxidativo na pele, associado ao envelhecimento cutâneo. Os resíduos agroindustriais têm sido estudados como fonte de compostos bioativos para diferentes finalidades. Os produtos cosméticos e de higiene corporal (PCHC) podem beneficiar destas matérias-primas. Acresce ainda o fato de questões de sustentabilidade conduzirem ao estudo de novos ingredientes ativos obtidos a partir de produtos sem valor acrescentado. A pele de prata (CS) é um subproduto do café gerado durante a torrefação. Por outro lado, o género *Medicago* é recorrentemente utilizado na alimentação animal, sendo o excesso normalmente descartado. Este trabalho tem como objetivo avaliar o potencial uso de subprodutos alimentares na obtenção de novos ingredientes ativos para PCHC.

Com efeito, avaliou-se o teor total de polifenóis e flavonóides (TPC e TFC, respetivamente), a atividade antioxidante (DPPH e FRAP), a atividade antimicrobiana e a citotoxicidade (em fibroblastos e queratinócitos) de extratos hidro-alcoólicos de sete espécies de *Medicago* (*M. minima*, *M. tornata*, *M. truncatula*, *M. rigidula*, *M. scutellata*, *M. segitalis* e *M. sativa*) e de extratos aquosos, hidro-alcoólicos e alcoólicos de CS. Constatou-se que os extratos de CS apresentaram uma maior atividade antioxidante. O TPC dos diferentes extratos encontra-se na mesma ordem de grandeza (18-36 mg amostra GAE/ db g), sendo o mais elevado obtido com o *M. mínima*. O *M. rigidula* apresentou os valores mais elevados de TFC. Todos os extratos mostraram uma reduzida citotoxicidade. Ficou igualmente comprovada a capacidade antibacteriana dos extratos, em particular dos extratos de CS. Em relação ao teor de isoflavonas, estas foram determinadas por HPLC/DAD nos extratos de *Medicago*, sendo a genistina, daidzeína e genisteína as mais abundantes.

Avaliou-se igualmente a irritação cutânea e ocular, respetivamente, com um modelo de epiderme humana reconstruída, EpiSkin<sup>TM</sup>, e um modelo de epitélio da córnea humana, SkinEthic<sup>TM</sup> HCE. Efetuou-se também um ensaio *in vivo* de irritação cutânea em 20 voluntários. Os teores de cafeína (CAF), 5-hidroximetil furfural (HMF) e ácido clorogénico (CGA) foram quantificados após o ensaio epidérmico. Os resultados *in vitro* permitiram classificar os extratos como não irritantes. A avaliação *in vivo* realizada com o extrato mais promissor (hidroalcoólico) assegurou que este pode ser considerado seguro para aplicação tópica. Procedeu-se, assim, ao desenvolvimento e caracterização de diferentes formulações de PCHC com *M. sativa* e/ou extrato de CS.

Nenhuma das formulações apresentou toxicidade em fibroblastos ou queratinócitos. As formulações demonstraram ser estáveis quer nas propriedades físicas, quer na atividade antioxidante, durante 180 dias, quando armazenadas a 25 °C/ 65% de humidade relativa (HR) e 40 °C/ 75% HR. A avaliação sensorial foi realizada para todas as formulações e de um modo geral, a aceitação do consumidor foi muito elevada, tendo sido classificadas por mais de 90% dos voluntários como muito agradáveis. O ensaio *in vivo* realizado assegurou que as formulações podem ser considerados como seguras para aplicação tópica, aumentando a hidratação da pele após 30 dias de utilização.

De seguida, efetuou-se a avaliação e comparação *in vivo* de um creme de rosto com CS e a mesma formulação enriquecida com ácido hialurónico. As formulações foram aplicadas de manhã e à noite durante 28 dias por 20 voluntários (para cada formulação). A influência sobre a hidratação e as propriedades viscoelásticas da pele foram avaliadas recorrendo ao Corneometer® e ao Cutometer®. A profundidade das rugas, o volume das cavidades e a rugosidade da pele foram analisadas no tempo 0 e após 28 dias. No final realizou-se um questionário de perceção de eficácia. Os resultados mostraram diferenças significativas na hidratação da pele e nos parâmetros viscoelásticos para ambas as formulações, mas sem diferenças entre elas. No entanto, não foram observadas diferenças em relação à profundidade das rugas, rugosidade e volume das cavidades para ambas as formulações. A CS surge, deste modo, como um novo ingrediente eficaz para aumentar a hidratação e a firmeza da pele. A toxicidade, segurança e eficácia destes extratos foram demonstradas de acordo com as diretrizes internacionais.

Por fim, foram igualmente desenvolvidos e caracterizados transportadores lipídicos nanoestruturados (NLC) de cafeína obtida a partir de CS. Os NLC produzidos estavam dentro da gama nanométrica ( $\approx 200$  nm), apresentando um baixo índice de polidispersão ( $<0,25$ ) e valores de potencial zeta em torno de -30 mV. Demonstraram igualmente estabilidade quando armazenados a 25 °C/ 65% HR e 40 °C/ 75% HR durante 180 dias. A eficiência de associação da cafeína foi de cerca de 30% imediatamente após a produção, tendo-se mantido após o período de armazenamento. No entanto, o estudo *in vitro* de permeação em pele de porco demonstrou que a cafeína encapsulada teve uma permeação semelhante à cafeína no extrato de CS.

**Palavras-chave:** Pele de prata; *Medicago*; Cosmético; Modelos *in vitro* 3-D; Técnicas de biofísica para avaliação da pele; Estudos *in vivo*

## List of Publications

This thesis is organized in 8 chapters, closely reflecting the development of my research work. All chapters were related to each other and the aims and methodology chosen in each chapter were indeed dependent on the conclusions brought about in previous one(s).

Overall, the work described in this thesis encompasses the development and characterization of a cosmetic line with active ingredients obtained from two different food by-products: Coffee silverskin and *Medicago sativa*.

This dissertation is based on the following publications, which are referred to in the text by their respective roman numerals (I-):

### Publications in international peer-review Journals

**I** **Francisca Rodrigues**, Ana Palmeira-de-Oliveira José das Neves, Bruno Sarmento, M. Helena Amaral, M. Beatriz P.P. Oliveira, *Medicago* spp. extracts as promising ingredients for skin care products, *Industrial Crops and Products*, 49:634-644, 2013

**II** **Francisca Rodrigues**, Ivone Almeida, Bruno Sarmento, M. Helena Amaral, M. Beatriz P.P. Oliveira, Study of the isoflavone content of different extracts of *Medicago* spp. as potential active ingredient, *Industrial Crops and Products*, 57:110-115, 2014

**III** **Francisca Rodrigues**, Ana Palmeira-de-Oliveira, José das Neves, Bruno Sarmento, M. Helena Amaral, M. Beatriz P.P. Oliveira, Coffee silverskin: A possible valuable cosmetic ingredient, *Pharmaceutical Biology*, 4:1-9, 2014

**IV** **Francisca Rodrigues**, Carla Pereira, Filipa B. Pimentel, Rita C. Alves, Marta Ferreira, Bruno Sarmento, M. Helena Amaral, M. Beatriz P.P. Oliveira, Are coffee silverskin extracts safe for topical use? An *in vitro* and *in vivo* approach, *Industrial Crops and Products*, 63:167-174, 2015

**V** **Francisca Rodrigues**, Carlos Gaspar, Ana Palmeira-de-Oliveira, Bruno Sarmento, M. Helena Amaral, M. Beatriz P.P. Oliveira, Application of Coffee silverskin in cosmetic formulations: Physical / antioxidant stability studies and cytotoxicity effects, *Drug Development and Industrial Pharmacy*, 42(1): 99-106, 2016

**VI Francisca Rodrigues**, Bruno Sarmento, M. Helena Amaral, M. Beatriz P.P. Oliveira, Exploring the antioxidant potentiality of two food by-products into a topical cream: Stability, *in vitro* and *in vivo* evaluation, Drug Development and Industrial Pharmacy, 2016, *in press*

**VIII Francisca Rodrigues**, Rita Matias, Marta Ferreira, M. Helena Amaral, M. Beatriz P.P. Oliveira, *In vitro* and *in vivo* comparative study of cosmetic ingredients Coffee Silverskin and hyaluronic acid Rodrigues, Experimental Dermatology, 2016, *in press*

**IX Francisca Rodrigues**, Ana Catarina Alves, Cláudia Nunes, Bruno Sarmento, M. Helena Amaral, Salette Reis, M. Beatriz P.P. Oliveira, Permeation of topically applied caffeine from a food by - product in cosmetic formulations: a nanoscale *in vitro* approach, International Journal of Nanomedicine, submitted

#### **Book Chapters**

**IX Francisca Rodrigues**, M. Antonia Nunes, M. Beatriz P.P. Oliveira, Applications of recovered bioactive compounds in cosmetics and other products, “Olive Mill Wastewater: Recent Advances for the Sustainable Management of Olive Oil Industry“ (Ed. Charis M. Galanakis), Elsevier, 2016, *in press*.

**X Francisca Rodrigues** and M. Beatriz P.P. Oliveira, Cell-based *in vitro* models for dermal permeability studies, “Concepts and Models for Drug Permeability Studies” (Ed. Bruno Sarmento), Woodhead Publishing, 155-167, 2016.

The following articles are the result of cooperation with other scientific groups, but not a part of this thesis:

**I Francisca Rodrigues**, M. Helena Amaral, M. Beatriz P.P. Oliveira, Indústria portuguesa de produção de produtos cosméticos e de higiene corporal: realidade e perspectivas de evolução, Cosmetics and Toiletries Brasil, 25: 56-64, 2012



**II Francisca Rodrigues**, M. João Gonçalves, José das Neves, Bruno Sarmento, M. Helena Amaral, M. Beatriz P.P. Oliveira, Vaginal suppositories containing *Lactobacillus acidophilus*: Development and characterization, *Drug Development and Industrial Pharmacy*, 53: 386-394, 2015

**III** Bruno Sarmento, Fernanda Andrade, Sara Batista, **Francisca Rodrigues**, José das Neves, Domingos Ferreira, Cell-based *in vitro* models for predicting drug absorption, *Expert Opinion On Drug Metabolism and Toxicology*, 8: 607-621, 2012

**III** Nair Braga, **Francisca Rodrigues**, M. Beatriz P.P. Oliveira, *Castanea sativa* by-products: a review on added value and sustainable application, *Natural Product Research*, 29: 1-18, 2014

**IV Francisca Rodrigues**, Joana Santos, Filipa B. Pimentel, Nair Braga, Ana Palmeira de Oliveira, M. Beatriz P.P. Oliveira, Promising new applications for *Castanea sativa* shell: nutritional composition, antioxidant activity, amino-acids and vitamin E profile, *Food & Function*, 6: 2854-2860, 2015

**V Francisca Rodrigues**, Filipa B. Pimentel, M. Beatriz P.P. Oliveira, Olive by-products: Challenge application in cosmetic industry, *Industrial Crops and Products*, 70: 116-124, 2015

**VI** Ivone M. C. Almeida, **Francisca Rodrigues**, Bruno Sarmento, Rita C. Alves, M. Beatriz P.P. Oliveira, Isoflavones in food supplements: chemical profile, label accordance and permeability study in Caco-2 cells, *Food & Function*, 6: 938-946, 2015

**VII** M. Guadalupe Soto-Zarazúa, **Francisca Rodrigues**, Filipa B. Pimentel, M.M. Bah, M. Beatriz P.P. Oliveira, Isoflavone content of two new alfalfa-derived products for instant beverages preparation, *Food & Function*, 7: 364-371, 2016

## **Awards**

Gold Premium (1st place) Seed Project - Quem semeia conhecimento, colhe desenvolvimento, Grupo JMV, "A Pele de Prata (subproduto do café) como Ingrediente Ativo de Produtos Cosméticos e de Higiene Corporal", 2014

# Table of Contents

Dedication .....	v
Acknowledgements .....	vi
Abstract .....	ix
Resumo .....	xi
List of Publications.....	xiii
Table of Contents .....	xvi
Table of Contents .....	xvi
List of Figures .....	xxvii
List of Tables.....	xxxii
Abbreviations and Acronyms .....	xxxvi
GENERAL INTRODUCTION.....	1
Chapter   1 State-of-Art .....	3
1. Sustainability in Cosmetic Industry .....	4
2. Coffee Industry .....	5
2.1 Coffee processing techniques.....	7
2.2 Coffee industry by-products .....	9
3. <i>Medicago</i> .....	11
4. Re-Use of Food by-Products .....	12
4.1 Skin morphology: Structure and composition .....	13
4.2 Antioxidant activity .....	20
4.3 Isoflavones .....	23
4.4 Moisture.....	24

5.	How to Evaluate a New Cosmetic Ingredient/Product? .....	24
5.1	Cell-based <i>in vitro</i> models for dermal permeability studies.....	27
5.1.1	Human skin and dermal permeability .....	28
5.1.2	Drug permeability <i>in vitro</i> models .....	29
5.1.2.1	Two-dimensional (2-D) models .....	30
5.1.2.2	Three-dimensional (3-D) models.....	31
5.1.3	Reconstructed dermal equivalents .....	33
5.1.3.1	EpiSkin™ .....	33
5.1.3.2	Epiderm™ .....	35
5.1.3.3	SkinEthic™ .....	36
5.1.4	Reconstructed "full thickness models".....	37
5.1.4.1	StrataTest™ .....	37
5.1.4.2	Epiderm FT™ .....	38
5.1.4.3	epiCS™ .....	38
5.2	Models limitations .....	39
5.3	Skin biophysical techniques .....	39
5.3.1	Hydratation.....	40
5.3.2	Transepidermal water loss (TEWL).....	41
5.3.3	pH .....	43
5.3.4	Skin color .....	43
5.3.5	Mechanical properties of skin .....	44
5.3.6	Skin surface topography.....	46
	<b>Chapter   2 Aims and Goals .....</b>	<b>49</b>
	<b>EXPERIMENTAL PART .....</b>	<b>52</b>
	<b>Chapter   3 Formulations .....</b>	<b>54</b>
1.	Hand Cream.....	55
2.	Body Cream .....	56

3. Body Scrub.....	57
4. Facial Cream.....	58
5. Cleansing Shower Cream.....	59
<b>Chapter   4.....</b>	<b>60</b>
<b>I. Evaluation of <i>Medicago</i> as New Ingredient for Skin Care Products.....</b>	<b>60</b>
1. Introduction .....	61
2. Materials and Methods .....	63
2.1 Chemicals and reagents .....	63
2.2 Plant materials.....	64
2.3 Preparation of hydroalcoholic extracts .....	64
2.4 Determination of total phenolic content .....	64
2.5 Determination of total flavonoid content .....	65
2.6 <i>In vitro</i> antioxidant activity.....	65
2.6.1 DPPH free radical scavenging assay .....	65
2.6.2 Ferric reducing antioxidant power (FRAP) assay .....	66
2.7 Antimicrobial assay.....	66
2.7.1 Microorganisms strains .....	66
2.7.2 MIC by broth dilution assay .....	66
2.8 Keratinocytes and fibroblasts cytotoxicity assay.....	67
2.8.1 Cell Lines and Culture Conditions .....	67
2.8.2 MTS assay.....	68
2.8.3 LDH assay .....	68
2.9 Statistical analysis of data.....	69
3. Results and Discussion .....	69
3.1 Determination of total phenolic content .....	69

3.2	Determination of total flavonoid content .....	71
3.3	<i>In vitro</i> antioxidant activity .....	72
3.3.1	DPPH free radical scavenging assay .....	72
3.3.2	Ferric reducing antioxidant power (FRAP) assay .....	73
3.4	Correlation between phenolic composition and antioxidant activity .....	75
3.5	Correlation between flavonoids and antioxidant activity .....	76
3.6	Antimicrobial assay .....	76
3.7	Keratinocytes and fibroblasts cytotoxicity assay.....	78
3.7.1	MTS assay .....	79
3.7.2	LDH assay .....	81
4.	Conclusion .....	82
<b>II. Evaluation of <i>Medicago</i> as New Ingredient for Skin Care Products.....</b>		<b>84</b>
1.	Introduction .....	85
2.	Materials and methods .....	87
2.1	Chemicals and Solutions .....	87
2.2	Samples.....	87
2.3	Extracts preparation.....	87
2.4	HPLC equipment .....	88
2.5	Statistical analysis .....	89
3.	Results and Discussion .....	89
4.	Conclusion .....	96
<b>Chapter   5.....</b>		<b>98</b>
<b>I. Coffee Silverskin: A Possible New Cosmetic Ingredient? .....</b>		<b>98</b>
1.	Introduction .....	99

2.	Materials and Methods .....	100
2.1	Materials .....	100
2.2	Preparation of coffee silverskin extracts.....	101
2.3	Determination of total phenolic and flavonoid contents .....	101
2.4	Antioxidant capacity of coffee silverskin extracts.....	102
2.5	Antimicrobial activity of coffee silverskin extracts.....	102
2.5.1	Microorganisms strains .....	102
2.5.2	Determination of minimal inhibitory concentrations.....	103
2.6	Cytotoxicity and cell viability of coffee silverskin extracts .....	103
2.6.1	Cell lines and culture conditions .....	103
2.6.2	Cell viability and toxicity assays .....	104
2.7	Statistical analysis .....	104
3.	Results .....	105
3.1	Determination of total phenolic content (TPC).....	105
3.2	Determination of total flavonoids content (TFC) .....	106
3.3	<i>In vitro</i> antioxidant activity .....	106
3.4	Antimicrobial activity .....	106
3.5	Keratinocytes and fibroblasts cytotoxicity assay.....	107
4.	Discussion .....	109
5.	Conclusion .....	113
<b>II. Coffee Silverskin: A Possible New Cosmetic Ingredient? .....</b>		<b>115</b>
1.	Introduction .....	116
2.	Materials and Methods .....	117
2.1	Chemicals.....	117

2.2	Preparation of extracts.....	118
2.3	<i>In vitro</i> models assays .....	118
2.3.1	Skin irritation test.....	118
2.3.2	Ocular irritation test .....	119
2.3.3	MTT assay .....	119
2.3.4	IL-1 $\alpha$ assay .....	120
2.3.5	Histology .....	120
2.4	Analysis of CAF, HMF and CGA content in EpiSkin <sup>TM</sup> .....	120
2.5	<i>In vivo</i> skin irritation .....	121
2.5.1	Patch Test.....	121
2.5.2	Visual and instrumental assessments .....	121
2.6	Statistical analysis .....	122
3.	Results and Discussion .....	122
3.1	Skin irritability tests .....	122
3.2	Analytical methods.....	125
3.3	Ocular irritability tests.....	126
3.4	<i>In vivo</i> test .....	128
3.4.1	Skin compatibility by evaluation of primary skin irritation .....	129
3.4.2	Transepidermal water loss variation .....	130
4.	Conclusion .....	131
<b>Chapter   6.....</b>		<b>133</b>
<b>I. <i>In Vitro</i> and <i>In Vivo</i> Evaluation.....</b>		<b>133</b>
1.	Introduction .....	134
2.	Materials and Methods .....	135
2.1	Preparation of extracts.....	135

2.2	Preparation of the formulation .....	135
2.3	Cytotoxicity .....	136
2.3.1	Cell lines and culture conditions .....	136
2.3.2	MTS assay .....	136
2.3.3	LDH assay .....	137
2.4	Long-term stability evaluation .....	137
2.4.1	Particles morphology.....	137
2.4.2	pH .....	138
2.4.3	Accelerated stability by centrifugation .....	138
2.4.4	Color .....	138
2.4.5	Rheological analysis .....	138
2.4.6	Texture analysis .....	138
2.5	Microbiological analysis .....	139
2.6	Antioxidant activity .....	139
2.6.1	Determination of total phenolic content (TPC) .....	139
2.6.2	Determination of antioxidant activity .....	139
2.7	Statistical analysis .....	140
3.	Results .....	140
3.1	Keratinocytes and fibroblasts cytotoxicity assay.....	140
3.1.1	MTS assay .....	140
3.1.2	LDH assay .....	141
3.2	Long term stability study .....	141
3.3	Antioxidative properties.....	146
4.	Discussion .....	147
5.	Conclusion .....	149



<b>II. <i>In Vitro</i> and <i>In Vivo</i> Evaluation.....</b>	<b>150</b>
1. Introduction .....	151
2. Materials and Methods .....	152
2.1 Materials .....	152
2.2 Preparation of extracts.....	153
2.3 Formulation.....	153
2.4 Long-term stability evaluation .....	155
2.4.1 pH .....	155
2.4.2 Accelerated stability by centrifugation .....	155
2.4.3 Color .....	155
2.4.4 Rheological analysis .....	155
2.4.5 Microbiological analysis.....	156
2.4.6 Determination of total phenolic content (TPC).....	156
2.4.7 Determination of antioxidant activity .....	156
2.5 <i>In vitro</i> evaluation.....	157
2.5.1 Keratinocytes and fibroblasts cytotoxicity assays .....	157
2.5.2 Cell Lines and culture conditions.....	157
2.5.3 MTS assay .....	158
2.5.4 LDH assay .....	158
2.6 <i>In vivo</i> skin irritation .....	159
2.6.1 Patch test.....	159
2.6.2 Visual and instrumental assessments .....	159
2.6.3 Long term hydration effect.....	160
2.6.4 Sensory evaluation.....	160
2.7 Statistical analysis .....	160
3. Results .....	161

3.1	Stability .....	161
3.2	TPC content and antioxidant activity .....	164
3.3	<i>In vitro</i> toxicity .....	166
3.4	<i>In vivo</i> evaluation .....	167
4.	Conclusion .....	171
<b>III. <i>In Vitro</i> and <i>In Vivo</i> Evaluation.....</b>		<b>173</b>
1.	Introduction .....	174
2.	Materials and Methods .....	175
2.1	Materials .....	175
2.2	Coffee silverskin extract preparation .....	176
2.3	Preparation of formulations .....	176
2.4	<i>In vitro</i> caffeine permeability .....	176
2.5	<i>In vivo</i> extract hydration effect .....	177
2.6	Formulations cytotoxicity.....	177
2.6.1	Cell lines and culture conditions .....	178
2.6.2	MTS assay .....	178
2.6.3	LDH assay .....	179
2.7	Clinical studies.....	179
2.7.1	Study design .....	179
2.7.2	Biophysical techniques.....	180
2.8	Sensory evaluation .....	181
2.9	Statistical analysis .....	181
3.	Results and Discussion .....	181
3.1	<i>In vitro</i> skin permeation.....	181

3.2	<i>In vivo</i> extract hydration effect .....	182
3.3	Formulation cytotoxicity .....	183
3.4	<i>In vivo</i> evaluation .....	184
3.4.1	Safety and efficacy studies.....	184
4.	Conclusion .....	193
<b>Chapter   7 Nanoencapsulation of Coffee Silvserkin: A Future Approach? .....</b>		<b>194</b>
1.	Introduction .....	195
2.	Materials and Methods .....	196
2.1.	Materials .....	196
2.2.	Preparation of coffee silverskin extract .....	197
2.3.	Preparation of NLCs .....	197
2.4.	Determination of association efficiency .....	198
2.5.	Determination of particle size, surface charge and physical stability .....	199
2.6.	Morphology .....	199
2.7.	Stability studies.....	199
2.8.	Cell viability and toxicity .....	200
2.8.1	Cell lines and culture conditions .....	200
2.8.2	Cell viability assay .....	200
2.9.	<i>In vitro</i> skin permeation study .....	201
2.10.	Statistical analysis .....	201
3.	Results .....	202
3.1	CS-loaded NLCs characterization: particle size, PDI, surface charge and morphology.....	202
3.2	Stability studies.....	205

3.3	Cell viability studies .....	208
3.4	<i>In vitro</i> permeation study.....	209
4.	Conclusion .....	211
<b>CONCLUSIONS &amp; FUTURE PROSPECTS.....</b>		<b>212</b>
<b>Chapter   8 General Conclusions &amp; Future Prospects .....</b>		<b>214</b>
<b>References .....</b>		<b>218</b>

# List of Figures

## Chapter | 1 State-of-Art

Figure 1.1 - The three pillars of sustainability.....	5
Figure 1.2 - Coffee cherry (Available from <a href="http://hiloliving.blogspot.pt/2009_07_01_archive.html">http://hiloliving.blogspot.pt/2009_07_01_archive.html</a> - accessed on 01.02.16). .....	6
Figure 1.3 - Coffee curing processes. ....	7
Figure 1.4 - Cross-section of a coffee cherry (Adapted from <a href="https://pt.pinterest.com/pin/545709679817527226/">https://pt.pinterest.com/pin/545709679817527226/</a> - accessed on 01.02.16).....	9
Figure 1.5 - Schematic picture of the native skin that is sub-classified into three main compartments: epidermis, dermis and subcutis (hypodermis) (Adapted with permission from Macmillan Publishers Ltd: Nature Reviews Immunology ([58]), 3792130776332 (2016))......	14
Figure 1.6 - Morphology of human skin and different <i>in vitro</i> dermal permeation models (A) normal human skin epidermis, (B) EpiSkin™ model, (C) EpiDerm™ model, (D) StrataTest™, (E) SkinEthic™ RHE model, (F) Epiderm FT™ and (G) epiCS™ by Hematoxylin and Eosin (HE). ....	31
Figure 1.7 – Corneometer® probe (Available from <a href="http://www.courage-khazaka.de/index.php/en/faq-en/faq-scientific-devices/61-corneometer">http://www.courage-khazaka.de/index.php/en/faq-en/faq-scientific-devices/61-corneometer</a> ; accessed on 25.01.16). ....	41
Figure 1.8 - Tewameter® probe (Available from <a href="http://www.courage-khazaka.de/index.php/en/products/scientific/139-tewameter">http://www.courage-khazaka.de/index.php/en/products/scientific/139-tewameter</a> ; accessed on 25.01.16). .....	42
Figure 1.9 - Skin-pH-meter® probe (Available from <a href="http://www.courage-khazaka.de/index.php/en/products/scientific/132-skinphmeter">http://www.courage-khazaka.de/index.php/en/products/scientific/132-skinphmeter</a> ; accessed on 25.01.16). ....	43
Figure 1.10 – Cutometer® probe (Available from <a href="http://www.courage-khazaka.de/index.php/en/faq-en/faq-scientific-devices/69-cutometer">http://www.courage-khazaka.de/index.php/en/faq-en/faq-scientific-devices/69-cutometer</a> ; accessed on 25.01.16). ....	45

Figure 1.11 – Graphical scheme of a skin deformation curve obtained with the Cutometer® .....	45
Figure 1.12 – The PRIMOS® equipment (Available from <a href="http://www.in-cosmetics.com/__novadocuments/34659?v=635120853501200000">http://www.in-cosmetics.com/__novadocuments/34659?v=635120853501200000</a> ; accessed on 24.01.16). .....	48

## Chapter | 4 Evaluation of Medicago as New Ingredient for Skin Care Products

Figure 4.1 - Total polyphenol content in seven hydroalcoholic <i>Medicago</i> spp. extracts. Values are expressed as means $\pm$ SD (n = 6). GAE, gallic acid equivalents. Different letters (a, b, c) indicate significant differences between mean values ( $p < 0.05$ ). .....	69
Figure 4.2 - Total flavonoid content in seven hydroalcoholic <i>Medicago</i> spp. extracts. Values are expressed as means $\pm$ SD (n = 6). CAE, catequin equivalents. Different letters (a, b, c) indicate significant differences between mean values ( $p < 0.05$ ). .....	71
Figure 4.3 - Antioxidant activities of seven hydroalcoholic extracts of <i>Medicago</i> spp. species based on their abilities to scavenge DPPH free radicals. Values are expressed as means $\pm$ SD (n = 6). Different letters (a, b, c) indicate significant differences between mean values ( $p < 0.05$ ). .....	73
Figure 4.4 - Antioxidant activities of seven hydroalcoholic extracts of <i>Medicago</i> spp. species based on their abilities to reduce ferric iron ( $\text{Fe}^{3+}$ ) to ferrous iron ( $\text{Fe}^{2+}$ ) (n = 6, error bars represent SD). Different letters (a, b, c) indicate significant differences between mean values ( $p < 0.05$ ). .....	74
Figure 4.5 - Effect of hydroalcoholic extracts of <i>Medicago</i> spp. exposure on the viability of HaCaT cells at different concentrations, as measured by the MTS assay. Values are expressed as means $\pm$ SD (n=6). * $p < 0.05$ versus positive control (cells exposed to medium). .....	79
Figure 4.6 - Effect of exposure to different extracts of <i>Medicago</i> on the metabolic activity of HFF-1 cells at different concentrations, as measured by the MTS assay. Values are expressed as means $\pm$ SD (n=6). * $p < 0.05$ versus positive control (cells exposed to medium). .....	80

Figure 4.7 - Cytotoxicity (%) of LDH in HaCaT cells after 24 h treatment with different seven hydroalcoholic *Medicago* spp. extracts. The cytotoxicity of LDH was determined using fluorescence with an excitation wavelength of 490 nm and an emission wavelength of 690 nm. Values are expressed as means  $\pm$  SD (n=6). ..... 81

Figure 4.8 - Chromatograms (254 nm) of a standard mixture (1 mg/mL) (A) and of hydroalcoholic extract of *Medicago minima* (B) and UV absorption spectra of daidzin (1), genistin (2), daidzein (3), glycitein (4), genistein (5), formononetin (6), prunetin (7) and biochanin A (8). ..... 90

## **Chapter | 5 Coffee Silverskin: A Possible New Cosmetic Ingredient?**

Figure 5.1 - Hematoxylin-eosin (HE) staining of the EpiSkin™ tissues. (a) After topical application of 10  $\mu$ l of hydroalcoholic extract of CS: no irritation signs. (b) Negative control (100% cell viability): no irritation signs. (c) Positive control (5% SDS): marked epidermolysis. .... 124

Figure 5.2 - Chromatograms of aqueous extract (a), hydroalcoholic extract (b) and alcoholic extract (c) of CS and a standard mixture (1 mg/mL) (d) of HMF (1), CQA (2) and CAF (3). .... 125

Figure 5.3 - Hematoxylin-eosin (HE) staining of the SkinEthic™ HCE tissues. After topical application of 10  $\mu$ l of alcoholic (a) extract of CS: irritation signals. (b) Negative control (100% cell viability): no irritation signs. (c) Positive control (5% SLS): marked epidermolysis. .... 128

Figure 5.4 - Frequency of clinical scores attributed to positive control (PC - SLS), negative control (NC - purified water) and hydroalcoholic extract of CS (HA) regarding erythema and edema after patch remove.\* means a significant difference (p<0.05). 130

Figure 5.5 - Transepidermal water loss (TEWL) variation from basal values of negative control (NC), positive control (PC) and hydroalcoholic extract (HA). The left boxes represent values at time 0 and the right boxes represent values 2 h after patch removal. Outliers are presented as circles.\* means a significant difference (p<0.05).131

## Chapter | 6 *In Vitro* and *In Vivo* Evaluation

- Figure 6.1 - Effect of exposure to BC and CS formulations on the viability of HaCaT and HFF-1 cells at different concentrations, as measured by the MTS assay. Values are expressed as means  $\pm$  SD (n = 6). \*p<0.05. .... 141
- Figure 6.2 - Microphotographs of the BC (A) and the CS formulations (B) at time 0 and after 180 days of storage at different conditions - 40 x magnification. .... 144
- Figure 6.3 - Comparison of the rheograms of both formulations evaluated initially (A) and after 6 months of storage (B) at 25 °C/ 65% RH and 40 °C/ 75% RH..... 145
- Figure 6.4 - Color variation during the stability test conducted at 25 °C/ 65% RH and 40 °C/ 75 % RH for F (A) and CSMS (B) formulations. L – lightness, a – red to green, b – yellow to blue. \* means a statistical difference (p<0.05). .... 162
- Figure 6.5 - Rheograms of formulations evaluated initially and after 180 days of storage at 25 °C/ 65% RH and 40 °C/ 75% RH (A – Formulation F at 25 °C/ 65% RH; B – Formulation F at 40 °C/ 75% RH; C – CSMS at 25 °C/ 65% RH; D – CSMS at 40 °C/ 75 % RH)..... 163
- Figure 6.6 - Frequency of clinical scores attributed to positive control (PC - SLS), negative control (NC— purified water) and formulation (CSMC) regarding erythema (A) and edema (B) after patch removal. \* means a significant difference (p<0.05). .... 168
- Figure 6.7 - Transepidermal water loss (TEWL) variation from basal values of negative control (NC), positive control (PC) and formulation (CSMS). The left boxes represent values at time 0 and the right boxes represent values 2 h after patch removal. Outliers are presented as circles. \* means a significant difference (p<0.05)..... 169
- Figure 6.8 – Radar plot of hedonic sensory evaluation of the body cream formulation from different characteristics. .... 171
- Figure 6.9 - Permeation of caffeine of CS extract through pig ear skin over 8 hours. Each value represents the mean  $\pm$  SD (n = 3). .... 182
- Figure 6.10 - Illustrative images obtained with the digital photography imaging system Visioface<sup>®</sup> at time 0 and after 28 days of Formulation A application. .... 185
- Figure 6.11 - Illustrative images obtained with the digital photography imaging system Visioface<sup>®</sup> at time 0 and after 28 days of Formulation B application. .... 185



Figure 6.12 - Photos of periorcular area acquired with PRIMOS<sup>®</sup> equipment of one of the volunteers who exhibited reduced depth of wrinkles at time 0 and after 28 days of Formulation A application. (A) Photography. (B) Representation of relief in a colored image, where each color corresponds to a height (positive values) or depth (negative values) with respect to the reference plane, showing a decrease of wrinkle depth throughout the study. .... 188

Figure 6.13 - Three-dimensional representation of the periorcular area acquired with PRIMOS<sup>®</sup> equipment of one of the volunteers who exhibited reduced depth of wrinkles. .... 188

Figure 6.14 - Illustrative box plot graphs of the depth values of wrinkles at baseline and 28 days after using the test products. (A) Formulation A; (B) Formulation B. .... 189

Figure 6.15 - Illustrative box plot graphs of volume of cavities at baseline and 28 days after using the test products. (A) Formulation A; (B) Formulation B. .... 190

Figure 6.16 - Illustrative box plot graphs of the roughness parameters at baseline and 28 days after using the test products. (A) Formulation A; (B) Formulation B. .... 190

## **Chapter | 7 Nanoencapsulation of Coffee Silverskin: A Future Approach?**

Figure 7.1 - NLC and NLC-CS images. Cryo-scanning electron microscopy (cryo-SEM) images at time 0 and after 180 days of storage at 25 °C/ 65% RH and 40 °C/ 75% RH. The scale indicated below the pictures is 2 µm. Amplification: 30,000 x. .... 204

Figure 7.2 – Effect of exposure of NLC and NLC-CS with different concentrations of caffeine (CAF) on the metabolic activity of HaCaT cells. Cell viability was determined by the MTT assay. Each value represents the mean ± SD (n = 3). .... 208

Figure 7.3 - Permeation of caffeine of NLC-CS and CS extract through pig ear skin over 8 hours. Each value represents the mean ± SD (n = 3). .... 210

## List of Tables

### Chapter | 1 State-of-Art

Table 1.1 - Coffee by-products generated during coffee processing. ....	9
Table 1.2 - Changes observed in aged skin. ....	19
Table 1.3 - Allowed assays for cosmetic ingredients. ....	26
Table 1.4 - Summary of commercially available human skin equivalents. ....	32

### Chapter | 4 Evaluation of Medicago as New Ingredient for Skin Care Products

Table 4.1 - Antibacterial activity of hydroalcoholic extracts of <i>Medicago</i> species expressed as MIC in $\mu\text{g/mL}$ . ....	77
Table 4.2 - Isoflavone contents (mg/Kg db) in <i>Medicago</i> species (n = 3). Mean $\pm$ SD. n.d. - not determined. Values in the same line followed by different letters (in the different tables) indicate significant differences ( $p < 0.05$ ). Values in the same column followed by different numbers indicate significant differences ( $p < 0.05$ ). Different numbers in different species name indicate significant differences between them ( $p < 0.05$ ). ....	91

### Chapter | 5 Coffee Silverskin: A Possible New Cosmetic Ingredient?

Table 5.1 - Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Ferric Reducing Antioxidant Potential Assay (FRAP) and 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH*) scavenging assay of coffee silverskin extracts (CS). ....	105
Table 5.2 - Antibacterial activity of different extracts of CS expressed as the Minimal Inhibitory Concentration (MIC). Data expressed as mean of the replicates (n=3). ....	107
Table 5.3 - Effect on the metabolic activity of HaCaT cells after the exposure to CS extracts at different concentrations, measured by the MTS assay. Values are expressed as the means $\pm$ SD (n=6). ....	108

Table 5.4 - Effect on the metabolic activity of HFF-1 cells after the exposure to CS extracts at different concentrations, measured by the MTS assay. Values are expressed as the means $\pm$ SD (n=6).....	108
Table 5.5 - EpiSkin™ tissues were exposed for 15 min to controls and extracts. Cell viability was assessed by MTT while the post-exposure basal media was analyzed for IL-1 $\alpha$ release (pg/mL).....	123
Table 5.6 - Quantification of HMF, CGA and CAF in aqueous (W), hydroalcoholic (WA) and alcoholic (A) extracts of CS medium after EpiSkin™ assay.....	126
Table 5.7 - SkinEthic™ HCE model was exposed for 10 min to controls and extracts. Cell viability was assessed by MTT while the post-exposure basal media was analyzed for IL-1 $\alpha$ release (pg/mL).....	127

## **Chapter | 6 *In Vitro* and *In Vivo* Evaluation**

Table 6.1 - Physical and chemical characteristics of formulations at time 0 and 180 days under different storage conditions. * means a statistical difference (p<0.05).....	143
Table 6.2 - Color variation during the stability test conducted at 25 °C/ 65% RH and 40 °C/ 75% RH for BC and CS formulation. * means a statistical difference (p<0.05).....	144
Table 6.3 - Total polyphenol content (TPC) and DPPH assay of BC and CS formulations at time 0 and after 180 days of storage at 25 °C/ 65% RH and 40 °C/ 75% RH. Different letters (a, b, c) in the same column indicate significant differences between mean values (p<0.05).....	146
Table 6.4 - Components of both formulations under study.....	154
Table 6.5 - Physical characteristics of formulations at time 0 and 180 days under different storage conditions. * means a statistical difference (p<0.05).....	161
Table 6.6 - Total polyphenol content and antioxidant activity of formulations at time 0 and after 180 days of storage at 25 °C/ 65% RH and 40 °C / 75 % RH. Different letters (a, b, c) in the same column indicate significant differences between mean values (p<0.05). ....	164

Table 6.7 - Effect of body care formulations on viability of HFF and HaCaT cells at different concentrations, measured by MTS assay. Values are expressed as means $\pm$ SD (n=6). * p<0.05 versus positive control (cells exposed to medium).....	166
Table 6.8 - Hydration variation from basal values of negative control (NC) and CS extract (CS) at time 0 and 2 h after patch removal following a 48h occlusion period.....	183
Table 6.9 - Effect on the metabolic activity of HaCaT and HFF-1 cells after exposure to Formulation A and B at different concentrations, measured by the MTS assay. Values are expressed as mean $\pm$ SD (n=6). .....	183
Table 6.10 - Hydration variation from basal values of Formulation A and Formulation B at time 0 and after 28 days of study. ....	186
Table 6.11 - Effects of topical application of Formulation A and B on mechanical properties of skin. ....	186
Table 6.12 - Sensory evaluation of both formulations considering different characteristics such as color, smell, penetration rate, efficacy and overall quality. ....	192

## **Chapter | 7 Nanoencapsulation of Coffee Silverskin: A Future Approach?**

Table 7.1 - Composition of the developed NLCs formulations (NLC - empty formulation; NLC-CS- coffee silverskin formulation). ....	197
Table 7.2 - Characterization of NLC and NLC-CS.....	202
Table 7.3 - Storage stability. Effect of time of storage at 25 °C/ 65% RH and 40 °C/ 75% RH on particle size of NLC and NLC-CS. All data represent the mean $\pm$ standard deviation (n = 4). Different letters (a,b,c,d) indicate significant differences between mean values during storage (p<0.05).....	205
Table 7.4 - Storage stability. Effect of time of storage at 25 °C/ 65% RH and 40 °C/ 75% RH on zeta potential of NLC and NLC-CS. All data represent the mean $\pm$ standard deviation (n = 4). Different letters (a,b,c,d) indicate significant differences between mean values during storage (p<0.05). ....	206

Table 7.5 - Association efficiency (%) into NLC-CS with time under different storage conditions. Values are mean of three assays. Within each formulation along the time, data were not statistically different, for  $p < 0.05$ . .....207

## Abbreviations and Acronyms

2-D - Two-dimensional model

3-D - Three-dimensional model

ABTS - 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid

AHA - Alpha hydroxyl acid

AE - Association efficiency

AO - Antioxidant

ATCC - American Type Culture Collection

AP - Activator Protein

AU - Arbitrary Units

CAF - Caffeine

CEQ - Catechin Equivalents

CGA - Chlorogenic Acid

CIE - Commission Internationale de l'Eclairage

CLSI - Clinical and Laboratory Standards Institute

CPM - Cohesive Polydensified Matrix

CS - Coffee Silverskin

DAD - Diode-array Detector

DMEM - Dulbecco's modified Eagle's medium

DMSO - Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

DPPH - 2,2-diphenyl-1-picrylhydrazyl

ECM - Extracellular Matrix

ECVAM - European Center for the Validation of Alternative Methods

EEMCO - Efficacy Measurements on Cosmetics and Other Topical Products

ELISA - Enzyme-linked immunosorbent assay

ELS - Electrophoretic Light Scattering

ERT - Estrogen Replacement Therapy

EU - European Union

EURL - European Union Reference Laboratory

FBS - Fetal Bovine Serum

FRAP - Ferric Reducing Antioxidant Power

GAE - Gallic Acid Equivalents

GAG - Glycosaminoglycans

HCE – Human Corneal Epithelium

HE - Haematoxylin–eosin

HMF - 5-hydroxymethyl furfural

HPLC - High-performance Liquid Chromatography

HRT - Hormone Replacement Therapy

ICO - International Coffee Organization

IL - Interleukin

ISO - International Organization for Standardization

LDH - Lactate Dehydrogenase

LED - Light Emitting Diode

MIC - Minimal Inhibitory Concentration

MH - Mueller-Hilton

MHT - Menopausal Hormone Therapy

MMP - Matrix Metalloproteinase

mRNA - Messenger Ribonucleic Acid

MTS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

MTT - 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide

NLC - Nanostructured Lipid Carrier

OECD - Organization of Economic Cooperation and Development

PBS - Phosphate Buffered Saline

PCHC - Produtos Cosméticos e de Higiene Corporal

PDI - Polydispersivity index

PRIMOS - Phaseshift Rapid In-vivo Measurement of Skin

PTFE - Polytetrafluoroethylene

REACH - Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals

RH - Relative Humidity

RHE - Reconstructed Human Epidermis

ROS - Reactive Oxygen Species

RPM - Rotations per Minute

SC - *Stratum corneum*

SCCS - Scientific Committee on Consumer Safety

SCP - Sustainable Consumption and Production

SD - Standard Desviation

SEM - Scanning Electron Microscopy

SIP - Sustainable Industrial Policy



SLN - Solid Lipid Nanoparticle

SLS - Sodium Lauryl Sulphate

SPSS - Statistical Package for the Social Sciences

TEWL - Transepidermal Water Loss

TFC - Total Flavonoid Content

TGF - Transforming Growth Factor

TPC - Total Phenolic Content

UV - Ultraviolet

UV-Vis – Ultraviolet - Visible

# **GENERAL INTRODUCTION**

“Recomeça...  
Se puderes,  
Sem angústia e sem pressa.  
E os passos que deres,  
Nesse caminho duro  
Do futuro,  
Dá-os em liberdade.  
Enquanto não alcances  
Não descanses.  
De nenhum fruto queiras só metade.  
E, nunca saciado,  
Vai colhendo  
Ilusões sucessivas no pomar  
E vendo  
Acordado,  
O logro da aventura.  
És homem, não te esqueças!  
Só é tua a loucura  
Onde, com lucidez, te reconheças”

*Miguel Torga*

## State-of-Art

The information presented in this chapter was partially published in the following publications:

- **Francisca Rodrigues** and M. Beatriz P.P. Oliveira, Cell-based *in vitro* models for dermal permeability studies, “Concepts and Models for Drug Permeability Studies” (Ed. Bruno Sarmiento), Woodhead Publishing, 155-167, 2016

- **Francisca Rodrigues**, M. Antonia Nunes, M. Beatriz P.P. Oliveira, Applications of recovered bioactive compounds in cosmetics and other products, “Olive Mill Wastewater: Recent Advances for the Sustainable Management of Olive Oil Industry”(Ed. Charis M. Galanakis), Elsevier, 2016, *in press*

## 1. Sustainability in Cosmetic Industry

The global population has reached 7 billion and is projected to reach 9 billion by 2050 [1]. There is a general agreement that the planet's resources cannot support with such rise in human population, especially at this consumption rates [1]. Simultaneously, consumers become more informed, demanding more benefits from the products they buy [1]. The rising education levels and the World Wide Web make consumers more aware, leading them to questioning product origins, production methods, ecological implications, as well as safety issues. This rise in ethical consumerism is having a major impact on the cosmetics industry. A core component of industry responsibility is its commitment to sustainable development.

Human history has always been closely linked to the control, extraction and use of natural resources [2]. However, over the past decades, demand for natural resources has accelerated to the extent that it is now widely considered a serious threat to the well-functioning of economies and societies due to associated environmental problems such as climate change, biodiversity loss, desertification, and ecosystem degradation [2]. At a time when eco-consumerism is increasingly focusing end-user attention on all aspects of products like raw material sourcing, manufacturing and disposal, the beauty product sector has been accused of numerous environmentally unfriendly practices [3]. According to Cosmetics Europe - The Personal Care Association, each day 450 million Europeans use a variety of cosmetic products such as soap, shampoo, hair conditioner, toothpaste, deodorant, shaving cream, skin care, perfume, make-up, etc [4]. These products do not only help with personal hygiene, and thus contribute to the improvement of public health; for most people, using cosmetics improves their self-esteem and self-confidence, therefore, their general well-being [4]. The European cosmetic industry provides employment for some 1.7 million people and is the world's largest exporter of cosmetic products, thereby significantly contributing to economic growth in Europe [4].

In fact, within the rapidly growing market for personal care products, a number of studies show that the highest environmental impact of cosmetic products is at the consumer level. The consumers concerns range from unsustainable sourcing of raw materials, pollution both in the manufacturing phase and the disposal of packaging and products, to animal testing [3]. In response to that, companies have attempted to address these issues holistically by focusing on sustainable raw material sourcing and greener formulations, as well as through packaging reductions and lower overall resource consumption [3]. However, these laudable practices face both consumer scepticism that

often views these activities as “green washing” and the reality of the market, where in a global economy, there is little desire for premium pricing to cover the increased costs of these products compared to traditional ones [3].

Sustainability has many interpretations. A widely accepted definition is that of the Brundtland Commission of the United Nations which refers to sustainability in the concept of sustainable development: ‘meeting the needs of the present without compromising the ability of future generations to meet their own needs’ [5]. The three pillars or dimensions of sustainability are environmental, social and economic dimension, as depicted in Figure 1.1.

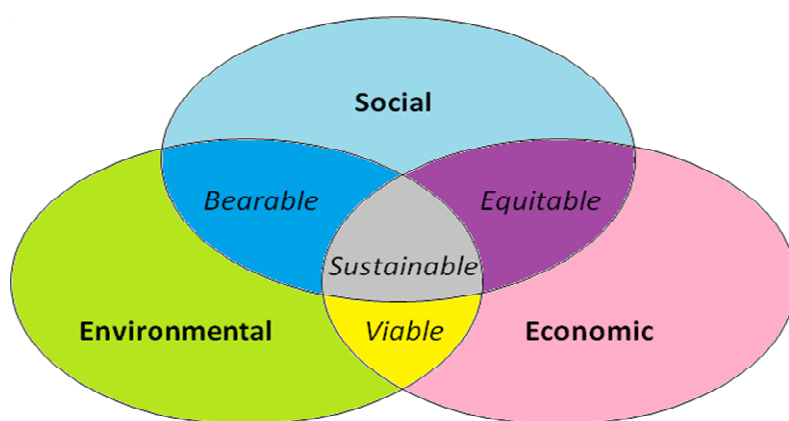


Figure 1.1 - The three pillars of sustainability.

It is against this backdrop that the idea of this research came about. Is it possible to develop new cosmetic ingredients based on food by-products? With growing scarcity of resources and rising ethical consumerism, how can the food industry, associated with the cosmetic industry, become more sustainable? What are the best ways to improve this food by-products use? Are they safe? This PhD thesis aims to address such questions.

## 2. Coffee Industry

Coffee, one of the most popular beverages, is consumed by millions of people every day, being highly appreciated in Portugal. The coffee fruit (also called berry or cherry - Figure 1.2) consists of a smooth, tough outer skin or pericarp, usually green in

unripe fruits but turns red-violet or deep red when ripe (even yellow or orange in particular genotypes) [6].



Figure 1.2 - Coffee cherry (Available from [http://hiloliving.blogspot.pt/2009\\_07\\_01\\_archive.html](http://hiloliving.blogspot.pt/2009_07_01_archive.html) - accessed on 01.02.16).

The economic importance of coffee is mainly due to the coffee brew or beverage, an infusion prepared from the roasted and ground beans. Most coffee beverage consumed around the world is produced by the species *Coffea arabica* (Arabica) and *Coffea canephora* (Robusta) [6]. According to the International Coffee Organization (ICO) in their last report, the total coffee production in 2013/14 was estimated at 146.8 million 60 kg bags, very slightly down on the 147.5 million bags produced in 2012/13 [7]. Also, the United States Department of Agriculture has released its latest estimates for annual global coffee production, predicting a 1.5 million total drop to 147.8 million bags in 2014/15, despite rising demand [8]. World coffee exports amounted to 9.10 million bags in August 2015, compared with 9.35 million bags in August 2014 [8]. The total demand has been growing at around 2.1% per annum for the last four years. This growth has mostly been driven by increased demand in exporting countries and new consuming countries, which have registered average annual growth rates of 3.1% and 2.7%, respectively, since 2010 [7]. Portugal is a consumer country with a high number of roasting companies distributed all over the territory.

The coffee tree is part of the subkingdom of plants known scientifically as the Angiosperm, which means that the plant reproduces by seeds that are enclosed in a box-like compartment, known as ovary, that are the base of the flower. It belongs to the botanical family Rubiaceae and to the genus *Coffea*.

Although widely consumed, during production this beverage originates a lot of food by-products that are still poorly characterized, especially in what concerns to its bioactive compounds and sustainable re-uses.

## 2.1 Coffee processing techniques

Coffee is internationally traded as green coffee which is produced by either wet or dry processing. Both processes are summarized in Figure 1.3.

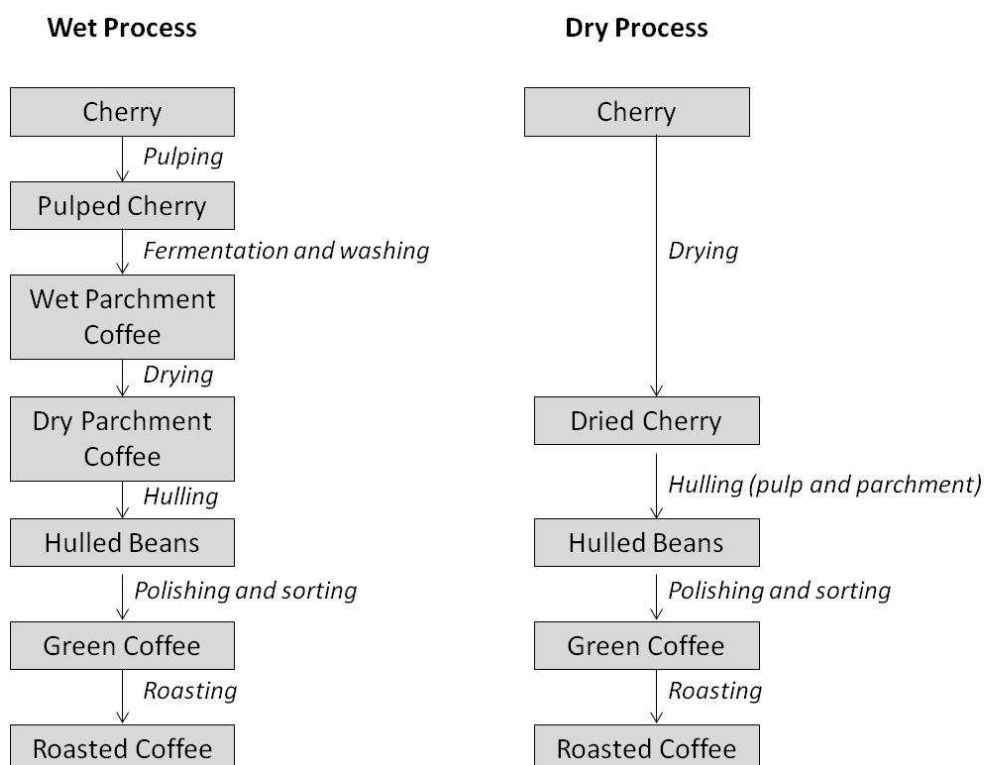


Figure 1.3 - Coffee curing processes.

### - **Wet method**

Coffee processed by wet method is also called washed or parchment coffee [9]. The process is called wet because water is the primary means to both moving the coffee through the process and to making the extraction of the seed possible. Basically, the coffee outer fruit skin is removed, floated in water (to remove defective beans), fermented (to break down the fruit mucilage layer), washed (to remove the fruit) and dried. At this point, green coffee seed is inside an outer parchment shell, rested for a period of time and dried into the green bean. Wet processing often produces a brighter, cleaner flavour profile, with lighter body than dry



process coffees or the hybrid pulp natural process. This process is also referred as washed coffees or fully washed. The coffee seed is not fermented in this process, just the other fruit layer between the skin and the parchment shell, in order to avoid the loss of bean color and moisture absorption by the beans. The fermentation occurs to break down the mucilage layer on the parchment into simple non-sticky substances, being digested. This is a natural action of peptic enzymes in the coffee. The skin and most pulp of the sunken fruits are mechanically removed by pressing the fruit in water through a screen (by using a pulper) [6]. Pulp remnants and the mucilage layer have to be removed in a following step. This can be conducted through “controlled” fermentation (for 12 - 48 h) and wash in concrete tanks, or through mechanical scrubbing (aqua pulping) [6]. The possible mechanical removal of the pulp reduces the amount of water used and, in consequence, the waste polluted water, and allows recovering the mucilage fraction [6, 9]. After washing, is usual to soak the parchment in clean water for 12 h in order to improve the visual appearance of the beans and the quality by removing diterpenes and polyphenolic substances which impart hardness to brew [9]. Coffee beans are dried until the moisture reaches around 10% [9]. Normally beans are dried in the sun or by machine but in most cases it is dried in the sun to 12-13% moisture and brought down to 10% by machine. Ecologically sensitive farms reprocess the wastewater along with the shell and mucilage as compost to be used in soil fertilization programs. After the drying process (in the sun and/or through machines), the parchment skin is thoroughly dry and crumbly, and easily removed in the hulling process.

- ***Dry method***

The dry-process (also known as unwashed or natural method) is the oldest method of processing coffee, producing a heavy in body, sweet, smooth, and complex coffee. This process is often used in countries where rainfall is scarce and long periods of sunshine are available to dry the coffee properly such as Indonesia, Ethiopia, Brazil or Yemen. In this method, the freshly harvested fruits are spread evenly to a thickness of about 8 cm on clean drying yard [9]. They are stirred and ridged once every hour and normally get fully dried in 12-15 days under bright weather conditions [9]. Drying rate of parchment is dependent on the initial moisture of the parchment, ambient air temperature, humidity, thickness of the spread and periodicity of stirring [9].

## 2.2 Coffee industry by-products

The industrial processing of coffee cherries occurs to separate coffee powder by removing shell and mucilaginous part from the cherries. This process generates large amount of coffee by-products. The cherry structure is shown in Figure 1.4.



Figure 1.4 - Cross-section of a coffee cherry (Adapted from <https://pt.pinterest.com/pin/545709679817527226/> - accessed on 01.02.16).

The processing of coffee generates expressive amounts of agricultural waste. Depending upon the method of coffee cherries processing, roasting and brewing solid residues like pulp, husk, silverskin, parchment or mucilage are obtained. Table 1.1 summarizes the residues obtained from the different coffee processes.

Table 1.1 – Coffee by-products generated during coffee processing.

Process	Type of by-product	Amount (for every 1 ton of green bean processed)
Pulping	Pulp	Nearly 1 ton of pulp is generated
Washing	Mucilage & parchment	40 m <sup>3</sup> of water with mucilage and parchment are generated
Milling	Mucilage & parchment	105 kg of dry parchment and 44 kg of dry mucilage
Polishing	Silverskin	The amount varies due to the thickness of upper layers

### **Coffee husks**

The main by-product of the dry processing is composed by the skin, pulp, mucilage and parchment, all together in a single fraction (coffee husks), that are probably the major

residues from the handling and processing of coffee [6]. According to Saenger *et al.*, for a ton of coffee beans produced, approximately 1 ton of husks are generated during dry processing, whereas for wet and semi-wet processing this residue amounts to more than 2 ton [10]. Murthy *et al.* consider that coffee husk encloses the coffee beans and represents about 12% of the berry on dry-weight basis [9]. The main composition of coffee husks are carbohydrates, being viewed as a potential raw material for bio-ethanol production [11]. Proteins, fibers and minerals are also present in huge amounts [12]. Other proposed alternative uses include employing this solid residue as a supplement for animal feed, direct use as fuel, fermentation for the production of a diversity of products (enzymes, citric acid and flavoring substances), use as a substrate for growth of mushrooms and as adsorbents [11].

### **Coffee pulp**

Coffee pulp have a similar composition to that of the husks: protein (7.5-15.0%), fat (2.0-7.0%) and carbohydrates (21-32%), representing about 29% on dry-weight of the whole berry [9, 13]. The organic components present in coffee pulp (dry weight) includes tannins (1.80-8.56%), total pectic substances (6.5%), reducing sugars (12.4%), non-reducing sugars (2.0%), caffeine (1.3%), chlorogenic acid (2.6%) and total caffeic acid (1.6%) [9].

### **Coffee mucilage**

The coffee mucilage fraction remains adhered to the coffee bean in the wet processing after depulping without enzymatic degradation [6]. According to Belitz *et al.* water is the main component of coffee mucilage (84.2%), followed by protein (8.9%) and sugar (4.1%) [14].

### **Coffee parchment**

Coffee parchment is the fibrous endocarp that covers both hemispheres of the coffee seed and separates them from each other [6]. In the dry processing, the parchment is separated from the green coffee beans together with the peel and pulp, in a single step, while in the wet process, the parchment is removed after drying and hulling, in separate steps [14]. The composition is similar to coffee mucilage [6].

### **Coffee silverskin**

Coffee silverskin (CS) is obtained after coffee beans roasting, being completely removed and releasing the ground roasted coffee beans for coffee beverage production. Different authors had intensively studied this coffee by-product. Regarding its macronutritional composition, is well documented its high content in dietary fiber (50-60%, including 85% and 15% of soluble and insoluble fiber, respectively), protein (16.2-19%) and minerals (4-5%) [15-17]. However, the exact mineral composition of CS have not been clarified [18]. Different authors had also demonstrated the high antioxidant capacity, probably due to the concentration of phenolic compounds of CS [15, 19-22].

### **3. *Medicago***

Legumes are important agricultural and commercial crops consumed in large amounts by both humans and animals. Nowadays, an integrated and sustainable exploitation of plant resources is a must as only few amounts are used for actual human consumption. Waste and by-products from vegetable origin are currently rising, driven by both a net increase in plant products consumption and the changing consumer trend toward ready-to-use products [23].

The Leguminosae taxonomic class includes a variety of plants with significant economic value, including soybean, alfalfa, clover, pea, peanut and various beans, often proposed as potential natural sources of specific bioactive components, with emphasis in phenolic compounds [24]. The genus *Medicago* is part of the botanical family of Leguminosae and includes about 56 different species mainly distributed in Mediterranean climatic conditions areas [25]. Alfalfa (*Medicago sativa*) is the main *Medicago* species widely grown throughout the world, predominantly as a source of high quality forage for livestock, renewable energy production, phytoremediation, and as a source of phytochemicals [26-27]. It is also used as a human food ingredient, consumed as sprouts in salads, in sandwiches or soups [28], as leaf protein concentrates [29], or as food supplements [30]. Despite this use, alfalfa have pharmacological activities, being used in some human health dysfunctions, such as anaemia, diabetes, endometriosis, stomach ulcers, osteoporosis, menopausal symptoms, breast and prostate cancers and low bone density [31-32]. Also, it is considered that the main applications of *Medicago* species are grazing food for cattle and other livestock. Like other plants of the same family, it was reported that high levels of isoflavones are desirable on lamb feeding, that gain weight more quickly, as well as in milk production, but, in some cases, reproductive

disorders have also been reported in sheep and cattle fed on forages with high isoflavone content [33-34]. Thus, depending on the final objective for cattle, *Medicago* could not be desired for farmers, originating a high amount of by-products.

*Medicago* species are important sources of phytochemicals, including carotenoids, saponins or phytoestrogens, which are known to act as antimicrobial agents, phytoanticipins, phytoalexins, structural barriers, modulators of pathogenicity, plant defence genes activators or fungitoxic agents [24, 27, 35-36]. Over this panoply of compounds, phytoestrogens are the most interesting for cosmetic products. Phytoestrogen is a general term used to define a wide variety of compounds that are non-steroidal and are either of plant origin or derived from the *in vivo* metabolism of several plants used as food [37]. Isoflavones are phenolic compounds with antioxidant activity and structural similarity to estradiol molecule [38-39], being primarily found in plants of the Fabaceae family, including soy, lentils, bean plant, chickpeas, alfalfa and red clover [40]. A number of epidemiological studies associate the consumption of isoflavone-rich foods with low incidence of the major hormone-dependent cancers [41], cardiovascular diseases [42], osteoporosis [39], and climateric complaints [43]. Because of their structural similarity to  $\beta$ -estradiol, health benefits of isoflavones have been evaluated in age-related and hormone-dependent diseases [44]. These compounds are largely described in redclover, a Leguminosae plant [45-48]. Biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) and formononetin (7-hydroxy-4'-methoxyisoflavone) are methylated precursors of genistein and daidzein, respectively, and existing high amounts in red clover [45, 49].

Therefore, estrogens can slow down the aging process of the skin in postmenopausal women. For this reason, isoflavones are also very interesting compounds for cosmetic formulations. When incorporated in creams, natural estrogens could result in morphological modifications to the aged skin, characterized mainly by an increased number of fibroblasts and glycosaminoglycans (GAGs), and, consequently, collagen and hyaluronic acid.

#### **4. Re-Use of Food by-Products**

Very few studies report possible uses for these agro-industrial wastes [50-51]. Cosmetic application could be a new way to reuse them. As it is well known, human skin is a complex organ that regulates body heat and water loss, whilst preventing the entry of toxic substances and microorganisms. Natural ingredients, phytonutrients, microbial

metabolites, dairy derived actives, minerals and animal protein components have long been believed to benefit healthy skin aging [52]. Indeed, from a sustainable point of view, this new application could provide, in the near future, a way of recycling for food companies, developing cost-efficient processing methods, decreasing the negative impacts of wastes on the environment and providing other economical advantages for companies. But, how could these new ingredients act on skin?

#### 4.1 Skin morphology: Structure and composition

Skin has historically been used for the topical delivery of compounds. The human skin is the largest organ of human body, forming the outermost biological barrier between the human body and the external environment. Among the multiple complex functions of mammalian skin, one of its major roles is to prevent invasion of the organism by acting as a defensive barrier to threats from the external environment [53]. In the 1960s the tremendous influence of the thickness of the *stratum corneum* (SC) became obvious [54]. It consists of different layers exhibiting individual composition and physiology [55]. The barrier function of the skin consists of a cornified layer of protein-rich dead cells (corneocytes) embedded in a lipid matrix, namely the SC [56]. The corneocytes are held together by corneodesmosomes, which help to form a tough outer layer by maintaining cellular shape and regular packing [53]. The lipid-enriched component consists predominantly of ceramides (about 50% by weight), as well as cholesterol and free fatty acids [57]. This layer is formed at the end of a balanced differentiation process, beginning at the basal layer of the epidermis, where progenitor cells divide and newly emerged keratinocytes are pushed towards the apical side of the epidermis [56]. Keratinocytes undergo a process of keratinisation, in which the cell differentiates and moves upward from the basal layer (*stratum basale*), through the *stratum spinosum* and *stratum granulosum*, to the outermost layer, the *stratum corneum*, where they anucleated and flattened and are eventually sloughed off [53]. Interspersed amongst the keratinocytes, in the viable epidermis are cells with roles such as melanin production (melanocytes), sensory perception (Merkel cells) and immunological function (Langerhans and other cells) [53]. The water content at normal skin surface is low (10-15%) [54]. Deeper skin layers are constituted by the viable epidermis (50-100  $\mu\text{m}$ ) with the basal membrane (consisting of at least one member of the protein family of laminin, type IV collagen and nidogen, and the proteoglycan perlecan) as well as by the dermis (1-2 mm), where

appendices like sweat glands and hair follicle are located [54-55]. The dermis is divided into an upper papillary layer containing loosely arranged collagen fibers and a reticular layer with dense collagen fibers arranged in parallel to the surface of the skin. As well as collagen, the dermal matrix comprises a high amount of elastin, to provide the elastic properties of the skin [56]. This matrix is produced by fibroblasts, which are the main cell type of the dermis. Dermis is pervaded by blood and lymph vessels. Nerves, sweat and sebaceous glands as well as hair follicles and shafts are embedded in the dermis. Beneath the dermis lies the subcutis, also known as the hypodermis. The subcutis functions as both an insulator, conserving the body's heat, and as a shock-absorber. Next to fibroblasts, adipocytes are the most prominent cells type in this compartment [56]. Figure 1.5 summarizes the skin structure.

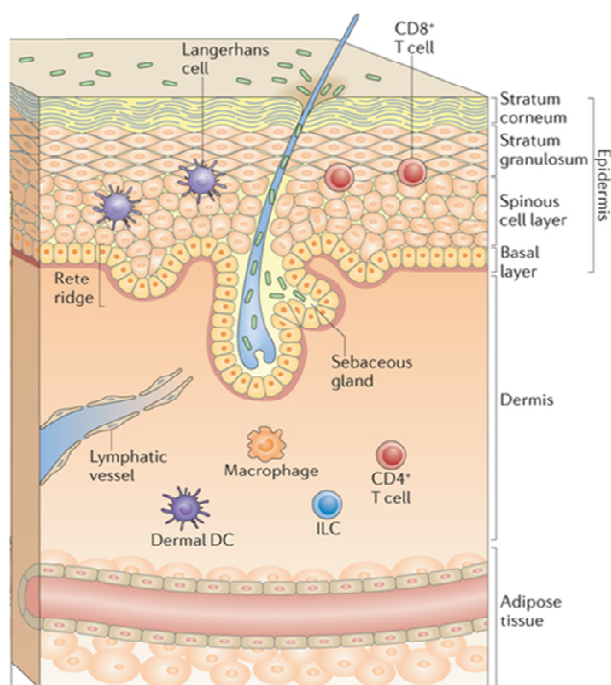


Figure 1.5 - Schematic picture of the native skin that is sub-classified into three main compartments: epidermis, dermis and subcutis (hypodermis) (Adapted with permission from Macmillan Publishers Ltd: Nature Reviews Immunology ([58]), 3792130776332 (2016)).

In addition to the structured cellular components of skin, there are appendages including the pilosebaceous units (hair follicles and associated sebaceous glands), apocrine and eccrine sweat glands [53].

The surface of the skin has long been recognised to be acidic, with a pH of 4.2-5.6, being described as the acid mantle [59]. This acid mantle has a number of diverse functions such as antimicrobial defence, maintenance of the permeability barrier by effects on extracellular lipid organisation and processing, preservation of optimal corneocyte integrity and cohesion, regulated by pH-sensitive proteolytic enzymes, and restriction of inflammation by inhibiting the release of pro-inflammatory cytokines [60].

The transport of substances across the SC mainly occurs by passive diffusion and based on the dual-compartment bricks and mortar structure of the SC, interrupted by appendages. Three possible routes are considered: the transcellular, the intercellular and the appendageal routes [53]. Polar solutes normally takes the transcellular route and more lipophilic solutes go via the intercellular lipids [53]. The delivery of drugs or particles via the appendageal route is regarded as a realistic alternative to delivery across the SC, covering only 0.1% of the human skin surface area.

Disorders of the skin can occur by several means, such as by an imbalance in molecular events that govern communication between the cells, infections, neoplastic transformation, immunological and non-immunological responses to foreign agents and autoimmune events, venous insufficiency and pressure or shear on the skin, as it is the case for certain ulcers, as well as by physical and chemical external insults [56].

Epidermal regeneration is initiated by epidermal stem cells, which are present in different compartments of the skin, i.e. in interfollicular compartments as well as in epidermal appendages, such as sweat glands and hair follicles with their associated sebaceous glands [56].

In the last few decades, owing to a rapid and unprecedented expansion of the elderly population, skin aging has emerged as a field of the utmost importance. Skin aging is thought to be a complex biological process that is traditionally classified as intrinsic and extrinsic aging, affected by a variety of internal (like genetic predisposition, hormonal disorders, vitamin deficiencies) and external factors (such as ultraviolet (UV) radiation, environmental pollution, improper care) [61-64]. This complex and multifactorial process whose baseline rate is genetically determined, may be accelerated by environmental, mechanical, or socioeconomic factors [62]. Traditionally, the clinical signs of skin aging are thinning skin with exaggerated expression lines, wrinkles, age spots and actinic keratoses [61]. Until now, the number of published works about the histological structure of wrinkles is not very large. There are salient features exhibited by aged skin, regardless



of the cause(s) of skin aging. The changes undergone by skin as it ages occur throughout the epidermis, dermis and subcutaneous tissue and can manifest in discrete and broad alterations in skin topography [65].

- **Epidermis**

Epidermis manifests some important changes related to aging. Some studies related that aged skin is characterized by a thinner epidermis, however, is general the concept that SC does not change with age [65]. In aged skin the intersection of the epidermis and dermis, known as dermal-epidermal junction, is altered, being flatter and diminishing the connecting area surface [65]. This lower surface contact could contribute to the increase fragility of skin observed in aged people and may also lead to the reduction of nutrient transfer between dermal and epidermal layers [65]. A decrease in the cell turnover is also observed in the epidermal layer. According to Kilgman *et al.* the SC transit time is of 20 days in young adults and 30 or more days in older adults [66]. This slow turnover leads to a protracted SC replacement rate, epidermal atrophy, slower wound healing and often less effective desquamation [65].

The molecular basis for epidermal aging is only beginning to be studied systematically. On a cellular level, apoptosis and decreased proliferative capacity, two key features of aging, affect epidermal structure and function, while on a molecular level, telomere shortening and reactive oxygen species (ROS), respectively, account for the characteristic structural and functional changes of chronologically aged skin [67]. Telomeres, which shorten with chronological aging, are repetitive deoxyribonucleic acid (DNA) sequences, in all mammals TTAGGG and its complement, that cap the ends of chromosomes [68]. Telomeres do not encode genes, but rather protect the proximal genes and regulatory sequences in several ways [69]. Since terminal base pairs are lost with each round of mitosis, progressive chromosome shortening occurs with each somatic cell division. Destabilization of the loop structure by progressive depletion with chronological aging, exposes the telomeric repeat sequence. Then, an as-yet-unidentified sensing mechanism interacts with the overhang to initiate a signal cascade that can lead to cell cycle arrest, premature senescence or apoptosis [68].

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- ***Dermis***

Approximately 20% of dermal thickness disappears as people become elderly [65]. Even more striking changes occur in the dermis: massive elastosis (deposition of abnormal elastic fibres), collagen degeneration, and twisted, dilated microvasculature [70]. As the three primary structural components of the dermis, collagen, elastin and GAGs have been the subjects of the majority of anti-aging research pertaining to the skin [65]. The cosmetic approach to wrinkles includes cleansing, photoprotection and active ingredients [71].

Collagen is the primary structural component of the dermis and the most abundant protein found in humans, comprising about 25-35% of total body protein content, being responsible for strength and support of human skin [72]. This protein comprises approximately 70% to 80% of the dry weight of the dermis [73]. Human skin contains over 14 types of collagen, of which 80% is type I - the collagen that is responsible for skin strength - and 15% is type III - the collagen that is responsible for the elastic properties of skin [72]. Each collagen molecule consists of three polypeptide chains (particularly rich in four amino acids: lysine, proline, hydroxylysine and hydroxyproline), each containing about 1000 amino acids in their primary sequence [73]. Alterations in collagen play an integral role in the aging process. This, in turn, partly explains the popularity of collagen-containing products intended for anti-aging purposes [65]. The ratio of collagen types found in human skin also changes with age [65]. During aging process, a marked loss of fibrillin-positive structures as well as a reduced content of collagen VII is observed, weakening the bond between dermis and epidermis [74-75]. In young skin, collagen I comprises 80% and collagen III comprises about 15% of total skin collagen; in older skin, the ratio of type III to type I collagen has been shown to increase, due significantly to an appreciable loss of collagen I [76-77]. In older skin, collagen looks irregular and disorganized [76]. The overall collagen content per unit area of the skin surface is known to decline approximately 1%/year [78]. Although collagen I is the most abundant and significant collagen type found in the skin, the effects of aging are seen in other types of collagen in human dermis. For example, an integral constituent of the dermal-epidermal junction is collagen IV that imparts a structural framework for other molecules and plays a key role in maintaining mechanical stability [65]. Significantly lower levels of collagen IV have been identified at the base of wrinkles.

Dermal fibroblasts make precursor molecules called procollagen, which is converted into collagen. Fibroblast collapse, due to the accumulation of degraded collagen fibres that prohibit construction of a healthy collagen matrix, causes the ratio of collagen synthesis to collagen degradation to become deranged in a self-perpetuating cycle [79]. There are two important regulators of collagen production: transforming growth factor (TGF)- $\beta$ , a cytokine that promotes collagen production, and activator protein (AP-1), a transcription factor that inhibits collagen production and up regulates collagen breakdown by up-regulating enzymes called matrix metalloproteinases (MMPs) [80]. In aged skin there is elevation of AP-1 as compared to young skin [81]. MMP activity is increased in aged human skin, being associated with dramatic increased levels of degraded collagen [67]. Also, synthesis of type I and III procollagen is reduced in aged human skin [82]. This combination results in an overall decrease in collagen levels in the dermis.

MMP are a family of ubiquitous endopeptidases playing a role in many different physiological and pathological processes in the skin, including cutaneous aging [83-84]. These zinc-containing proteinases family are responsible for degradation of extracellular matrix (ECM) proteins, which form skin dermal connective tissue. MMPs are classified as collagenases, gelatinases, stromelysins, and membrane-type MMPs according to their substrate specificities and whether they are secreted soluble proteins or bound to cell surface membrane. Four MMPs in particular are thought to be important in matrix degradation in the skin [85]. The combined actions of collagenase (MMP1), 92 kDa gelatinase (MMP2), 72 kDa gelatinase (MMP9) and stromelysin 1 (MMP3) can fully degrade collagen and components of the elastic network of skin [85-86]. Together with collagen, elastin and elastic fibers, MMP are essential for strengthening of muscles, tendons and joints [87]. Degradation of these vital tissues and/or oxidative damage of DNA lead to loosening of the skin and eventually formation of wrinkles [87]. Coupled with these changes, elastic gene expression is markedly reduced after the age of 40-50 [85].

Functional elastin also declines in the dermis with age, as elastin becomes calcified in aged skin and elastin fibers degrade [88]. The turnover also declines. GAGs, the primary dermal skin matrix constituents assisting in binding water and an important contributor to the skin structure, declines with age [76]. The same occurs with the amount of hyaluronic acid produced by fibroblasts and the amount of interfibrillary ground substance, also a component of a healthy dermal matrix [89].

- **Hypodermis**

The overall volume of subcutaneous fat typically diminishes with age, as well as fat distribution. The physiological significance may be to increase thermoregulatory function by further insulating internal organs [90].

Table 1.2 summarizes the changes in aged skin.

Table 1.2 – Changes observed in aged skin.

<b>Observed effect of skin</b>	
Lower lipid content	
<b>Epidermis</b>	<ul style="list-style-type: none"> <li>- Dermal–epidermal junction flattens</li> <li>- ↓ Number of enzymatically active melanocytes</li> <li>- ↓ Number of Langerhans cells</li> <li>- ↓ Capacity for re-epithelization</li> <li>- ↑ Number of pores</li> </ul>
<b>Dermis</b>	<ul style="list-style-type: none"> <li>- Thickness reduced (atrophy)</li> <li>- ↓ Vascularity and cellularity</li> <li>- ↓ Collagen synthesis</li> <li>- Pacinian and Meissner’s corpuscles degenerate</li> <li>- Structure of sweat glands becomes distorted,</li> <li>- ↓ number of functional sweat glands</li> <li>- Elastic fibers degrade</li> <li>- ↓ Number of blood vessels</li> <li>- ↓ Number of nerve endings</li> </ul>
<b>Hypodermis</b>	<ul style="list-style-type: none"> <li>- Distribution of subcutaneous fat changes</li> <li>- Overall volume decreases</li> </ul>
<b>Appendages</b>	<ul style="list-style-type: none"> <li>- Hair loses normal pigments</li> <li>- Hair thins</li> <li>- ↓ Number of sweat glands</li> <li>- Nail plates become abnormal</li> <li>- ↓ Sebum production</li> </ul>

Thus, wrinkle cosmetic treatments are exerted by a variety of active functional ingredients. The modern anti-aging cosmetics go well beyond the simple moisturizing function of traditional anti-wrinkle creams, by exerting a more complex function in protecting the skin from external injuries, nourishing it, and removing its superficial layers [71]. The efficacy of active ingredients is related to their diffusion through the barrier and their concentration in the formulation [91]. Small soluble molecules with lipophilic and hydrophilic properties have greater ability to cross the SC than particles, polymers, or highly lipophilic substances [91]. There are numerous synthetic skincare formulations containing active ingredients such as alpha hydroxyl acids (AHAs), hyaluronic acid, cohesive polydensified matrix (CPM), etc., that have adverse reactions; for instance, allergic contact dermatitis, irritant contact dermatitis, phototoxic and photo-allergic reactions [87]. Plant-derived polyphenolic substances such as alloin, catechin, epicatechin, curcumin, myricetin, quercetin, etc., are beneficial as anti-aging ingredients [87]. Many herbal polyphenolic substances have found to be effective in reducing the rate and intensity of wrinkle formation. But, how these ingredients work? How can they reach the final function desired in aging?

#### **4.2 Antioxidant activity**

The skin naturally relies on antioxidants (AOs) to protect it from oxidant stress generated by sunlight and pollution [92]. Both the cutaneous permeability and the antioxidant barriers largely reside in the outer layers of the epidermis [68]. ROS, whether generated as by-product of cellular metabolism or of environmental origin, can modify amino acid structure sufficiently to result in loss of function [68]. To counteract oxidative injury, human skin is equipped with a network of enzymatic and non-enzymatic antioxidant systems. An imbalance between ROS and antioxidant protection mechanisms leads to oxidation of macromolecules, including DNA, lipids and proteins [68]. The oxidative modifications could result in loss of structural and/or functional integrity of components of epidermal barrier.

Antioxidants are a very heterogeneous class of functional substances: they include vitamins, minerals, and essential amino acids, all endowed with a specific property, among others, of fighting free radicals [71]. The antioxidant activity is an excellent example of a functional benefit that plant extracts can deliver. Cosmetic treatments of the latest generation, developed against wrinkles, rely on antioxidant properties of some

ingredients, especially those derived from plants [71]. Plants are known to contain a variety of natural antioxidants that protect and preserve themselves [93]. Many of these compounds and extracts from plants are emerging as candidates for moderating the effects of the aging process on skin by limiting biochemical consequences of oxidation [93]. Skin is a shield protecting us from harmful exposure to UV radiation and air pollutants and naturally uses antioxidants to protect itself from photodamage [86, 94]. At low concentrations, ROS exert essential intracellular functions, as second messengers, gene regulators, and mediators for cell activation (kinases and transcription factors), and could also play a key role in body's defense against infectious organisms [95]. Due to the high metabolic activity, human skin present a high risk of undergoing oxidative stress that can be brought on by internal and external conditions [96]. Oxidative stress is defined by an imbalance between ROS and antioxidants, with ROS being in excess [95]. Of the three types of UV rays, mid-range UVB (290 nm to 320 nm) and long-wave UVA (320 nm to 400 nm) rays are widely known to be the chief causes of premature photoaging, immune dysfunction and certain skin cancers. UVB rays are absorbed by epidermal chromophores such as melanin and urocanic acid and lead to direct molecular damage while also generating ROS [95]. UVA rays penetrate more deeply in the dermis, increase the production of ROS, and contribute to long-term actinic damage [95]. Symptoms of cutaneous damage, including wrinkling and pigmentation, develop earlier in sun-exposed skin than in unexposed skin, a phenomenon referred to as photoaging [97]. Both UVA and UVB induce the activation of a wide range of transcription factors in skin cells, which in turn may increase the production of MMPs, degrading collagen and elastin [95]. This exposure results in direct damage to the nucleus of the cell itself and in formation of ROS and other free radicals that subsequently react with important molecules in connective tissue and cell membranes [94]. These photochemical reactions can result in DNA damages, including oxidation of nucleic acids, with oxidative reactions also modifying proteins and lipids, leading to changes in its function and destabilizing the membranes of keratinocytes [86]. The human body is well equipped to deal with oxidative stress, using enzymatic and non-enzymatic antioxidants to decrease these changes. Many antioxidants come into a control mechanism to keep *in vivo* generated ROS balanced, being involved endogenous (e.g. superoxide dismutase, superoxide reductase, catalase, and glutathione peroxidase) and exogenous antioxidants (vitamins, trace elements, and phytoantioxidants) [86, 95].

An increasing importance is given to the formation of ROS inside the epidermal cell, not only by solar rays but also by the oxireduction reactions that take place inside the cell [96]. This modifies the phospholipids in the membrane, by peroxidation of their polyunsaturated fatty acids, with cell destruction and eventual cell death [96]. The skin's susceptibility to peroxidation is strictly connected to the presence or absence of an adequate antioxidant system represented by: the enzymes superoxide-dismutase and glutathione peroxidase;  $\alpha$ -tocopherol,  $\beta$ -carotene, and squalene; and the phenol compounds [96]. Free radical peroxidation is a theory that explains aging. Oxygen free radicals cause progressive damage to biologic membranes and subcellular organelles, impairing their functionality, and modifying DNA replication [96]. According to Viola *et al.* biologic membrane sensitivity to peroxidative phenomena increases with aging [96]. A progressive loss of functionality, with increased activity of phospholipase A2 enzyme, which hydrolyzes phospholipids and release arachidonic acid, is described [96]. By the action of cicloxygenase, this leads to an increased synthesis of thromboxane with consequent vasoconstriction, increased platelet aggregation, and hypertension, and the subsequent reduction of nutrients and oxygen in the tissues [96]. After lipoperoxidation, the skin forms lipofuscin (an indicator of skin aging) and cancer risk is increased [96].

In the cosmetic field, anti-aging products are one of the main goals. Nowadays, all consumers are focused on their health without forgetting the appearance, creating a great demand for anti-aging cosmetic products. In the field of cosmetic treatments, antioxidants are innovative ingredients for topical applications. Because low-molecular-weight antioxidants protect skin against oxidative stress, undergoing depletion in the process, it should be desirable to apply the antioxidants directly to skin as an available source for protection [86]. Diet and oral supplementation can supply skin with antioxidants. However, the metabolic process limits the amount that can be delivered into skin. Skin is increasingly used for topical administration of substances. Thus, the application of exogenous antioxidants might be proficuous on reducing the effects of free radicals. Topical use of antioxidants can provide additional protection to neutralize ROS from both endogenous and exogenous sources [98]. Since many relatively simple bioassays are readily available to assess antioxidant activity, a large number of plant compounds and extracts have been shown to act as antioxidants *in vitro*, and many have also demonstrated the capacity to reduce oxidative stress in skin *in vivo*, as well as in skin cells *in vitro* [93]. However, very few studies reported the use of by-products as antioxidant sources for cosmetic field [22-23, 99-103]. According to these researches, the majority of

these antioxidants are polyphenols and isoflavones. According to the composition previously reported for coffee silverskin, in an environmental and economic point of view, they could be considered a promising cosmetic ingredient source. Nevertheless, to guarantee the safety of these new ingredients stability and toxicity assays should be performed in order to avoid the presence of irritant constituents [102]. The cytotoxicity of these compounds can be evaluated using MTS and LDH assays in different monolayer's skin cell lines. Also, a number of *in vitro* three-dimensional models tests have been developed to assess the potential skin or eye irritants by cosmetic industry, such as the reconstructed human epidermis test (EpiSkin™) or the Human Corneal Epithelial Model (SkinEthic™ HCE), respectively.

### 4.3 Isoflavones

Changes in skin aging and function occur at variable rates and are also influenced by hormonal factors unique to each individual. Over time, the skin experiences a progressive increase in extensibility and a reduction in elasticity, thereby becoming more frail and susceptible to trauma. This in turn leads to an increased risk of skin injury (e.g., lacerations, tears, ulcerations, bruising), and an impairment of wound healing [72]. Since the average woman in a developed nation spends about one third of her life after the onset of menopause, the benefits and risks of estrogen replacement therapy (ERT) - also known as hormone replacement therapy (HRT), and menopausal hormone therapy (MHT) - have become major areas of focus for research [72]. ERT increases skin collagen content and preserves thickness, thereby reducing wrinkling. Skin moisture content improves with ERT, as it increases the skin's hyaluronic acid, acid mucopolysaccharides, and sebum levels, and possibly maintains stratum corneum barrier function [72]. Collagen becomes progressively sparse, disordered, and atrophied as skin ages - one of the chief reasons for the skin transformations resulting from aging. Copious studies establish menopause leads to estrogen deficiency, and research over the past 60 years demonstrates skin thickness, estrogen content, and skin collagen are closely correlated [72]. Topical estrogen administration could preserve the dermal collagen with significant improvement in skin appearance [104]. Estrogens have significant effects on skin physiology and modulate epidermal keratinocytes, dermal fibroblasts and melanocytes, in addition to skin appendages including the hair follicle and the sebaceous gland [104]. Many of the effects of estrogen on human skin are based on the changes that are seen in



post-menopausal women, with a number of studies documenting the differences seen following the menopause, although there is also a variation in skin thickness during the menstrual cycle, with skin thickness lowest at the start of the menstrual cycle, when estrogen and progesterone levels are low, which then increases with the rising levels of estrogen [104]. In skin tissue estrogen increase vascularization and show effects at various levels of dermal tissue [105]. Estrogen compounds were shown to be effective in the treatment of skin aging symptoms of perimenopausal women, increasing epidermal hydration, skin elasticity, skin thickness, and also reducing skin wrinkles and improving the content and quality of collagen and the level of vascularization [105-107].

#### **4.4 Moisture**

The presence of water is crucial to the maintenance of skin elasticity and integrity. The loss of moisture, which leads to dry skin, is closely linked to age-related skin condition [72]. The topical and systemic estrogen therapies probably preserve skin moisture probably due to an increase of acid mucopolysaccharides and hyaluronic acid in the dermis (high water holding capacity), higher sebum levels and water-holding capacity of the SC, and changes in the corneocytes surface area [72].

But how could a new cosmetic ingredient be evaluated and classified as safe? Which studies should be performed? *In vitro?* *In vivo?*

### **5. How to Evaluate a New Cosmetic Ingredient/Product?**

The safety evaluation of a new cosmetic ingredient is a complex matter. In order to make sure that a cosmetic ingredient or product is safe for its intended use, a multidisciplinary team is completely requested. Cosmetics and personal care products companies uses a multi-tiered scientific approach to extensively evaluate the safety of cosmetic products and ingredients. First of all the definition of cosmetic ingredient should be highlighted. According to Scientific Committee on Consumer Safety (SCCS) a cosmetic ingredient is “any chemical substance or mixture of synthetic or natural origin, used in the formulation of cosmetic products. A cosmetic ingredient may be: 1- a chemically well-defined single substance with a molecular and structural formula, 2- a complex mixture, requiring a clear definition and often corresponding to a mixture of substances of unknown

or variable composition and biological nature, 3- a mixture of 1 and 2, used in the formulation of a finished cosmetic product” [108]. Also, the definition of a cosmetic product is of extremely importance: “Any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly to cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours” [109]. In a simple way of view, the key components of this strict and methodical safety process include not only the evaluation and testing of human health impacts, but also the examination of cumulative exposure to the human body and the test and evaluation performed by scientists trained in product safety [110].

Following a thorough review of each ingredient in a cosmetic formulation, additional safety data on ingredients and finished product are reviewed. These may include cell culture (*in vitro*) and clinical (human) tests conducted on the final product and on products similar in composition to the product being evaluated. The potential for ingredient interactions within the product leading to unexpected adverse effects is also considered. Confirmatory testing of cosmetic product compatibility and acceptability on human volunteers (clinical testing) is often undertaken with informed consent and with the appropriate safeguards to detect any undesirable effects that could occur. Factors to be considered in determining exposure levels for personal care products and ingredients include: product type, amount used per application, frequency of application, site of body contact, duration of product contact, concentration of individual ingredients in the final product, use by sensitive subpopulations (such as infants, elderly, and pregnant women), method of application, external factors (such as sunlight exposure and variation in use related to weather), local or temporal habits, trends and cultural considerations, and possible conditions of foreseeable misuse [108, 110]. To ensure the reliability of testing, cosmetic product safety studies are designed and monitored, and the results are interpreted and evaluated by scientists who are specially trained and experienced in toxicology and safety evaluation. These scientists have a fundamental understanding of cosmetic, personal care and fragrance products and of tests being used.

The assessment of the toxicological potential is the first step in the hazard evaluation of a ingredient and consists in a series of distinct toxicity studies, specific for distinct toxicological end points. Besides, phototoxicity studies have to be performed for some specific cases [111]. Furthermore, animal testing in the European Union is already prohibited since 2004 for cosmetic products and since 2009 for cosmetic ingredients

('testing ban') [112]. As from March 2009, it is also prohibited to market in the European Union cosmetic products containing ingredients which have been tested on animals ('marketing ban'). For the most complex human health effects (repeated-dose toxicity, including skin sensitisation and carcinogenicity, reproductive toxicity and toxicokinetics) the deadline for the marketing ban was extended to 11 March 2013 [112]. For these endpoints no alternative *in vitro* methods are available. This approved delay has now expired. Table 1.3 summarizes the evolution on assays allowed for cosmetic ingredients.

Table 1.3 – Allowed assays for cosmetic ingredients.

EU Cosmetic Ingredient tested before March 2009	EU Cosmetic Ingredient tested after March 2009	EU Cosmetic Ingredient tested after March 2013
Physico-chemical Identification	Physico-chemical Identification	Physico-chemical Identification
Acute Toxicity		
Skin Corrosivity and Irritation	Skin Corrosivity and Irritation	Skin Corrosivity and Irritation
Eye Irritation	Eye Irritation	Eye Irritation
Skin Sensitisation	Skin Sensitisation <sup>a</sup>	
<i>In vitro</i> Dermal Absorption	<i>In vitro</i> Dermal Absorption	<i>In vitro</i> Dermal Absorption
Repeated Dose Toxicity	Repeated Dose Toxicity <sup>a</sup>	
Mutagenicity/ Genotoxicity <i>in vitro</i> and <i>in vivo</i>	Mutagenicity/ Genotoxicity <i>in vitro</i>	Mutagenicity/ Genotoxicity <i>in vitro</i>
Reproductive Toxicity	Reproductive Toxicity <sup>a</sup>	
Carcinogenicity	Carcinogenicity <sup>a</sup>	
Chronic Toxicity	Chronic Toxicity <sup>a</sup>	
Toxicokinetic Studies	Toxicokinetic Studies <sup>a</sup>	
<i>In vitro</i> Photo-induced Toxicity	<i>In vitro</i> Photo-induced Toxicity	<i>In vitro</i> Photo-induced Toxicity
Human Data	Human Data	Human Data

<sup>a</sup> - animals tests developed outside the European Union

The point is: How to evaluate these new ingredients? There are any *in vitro* models that could be used for dermal permeability? And which *in vivo* assays should be performed?

## 5.1 Cell-based *in vitro* models for dermal permeability studies

Skin absorption processes are useful to evaluate/understand safety aspects of chemicals, xenobiotics and cosmetic formulations. The main aim of skin absorption is the opportunity to deliver drug substances to the skin and further to the systemic circulation [113]. Therefore, the knowledge of dermal absorption phenomena is relevant for safety issues as well as for therapeutic aspects. Changes in regulatory requirements and social views on animal testing have incremented the development of reliable alternative tests for predicting skin absorption potential of new compounds and to evaluate dermal permeability. Although some procedures on conducting skin permeation studies are reported, no formal standardization is available [114-116]. The assessment of dermal permeation is one of the primary procedures to evaluate a substance and classify it as hazard, particularly, in cosmetics or pharmaceutical industries [117]. Skin is a multilayered biomembrane with particular absorption characteristics, answering differently to different substance classes.

Skin is a dynamic, living tissue and as such its absorption characteristics are susceptible to constant changes. Upon contact with the skin, the molecules penetrate into the dead SC and can subsequently reach the viable epidermis, the dermis and the vascular network [114]. Dermal absorption is influenced by many factors such as physicochemical properties of the substance, the vehicle, possible occlusion, the substance concentration, the exposure pattern or even the skin particularities in the different body parts [114].

A vast number of studies in the past have compared the *in vitro* with *in vivo* methods for measuring dermal absorption. They concluded, if properly conducted, that *in vitro* measurements can be used to predict *in vivo* absorption [118]. Organization of Economic Cooperation and Development (OECD) Guidance document 28 for the conduct of skin absorption studies allows the use of reconstructed human skin, if its application leads to absorption results similar to the ones obtained with excised human skin [118].

Also, in line with the 7th amendment deadlines, European Union bans the *in vivo* skin permeation assessment on ingredients for cosmetic purposes. Alternative methods are needed and require validation by European Center for the Validation of Alternative Methods (ECVAM). In response to that, OECD adopted a guideline for assessment of skin corrosion based on reconstructed human epidermis (RHE) [119]. In 2004, a technical

guidance of OECD also states skin equivalents that can be used for skin permeation assays [118].

For ethical reasons, fundamental skin absorption data cannot normally be obtained by conducting *in vivo* studies [113]. According to European Union and Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) guidelines, animal testing for skin studies should only be performed if all other approaches have failed to provide a conclusive result. Therefore, other techniques must be used to obtain the desired information such as *in vitro* penetration and permeation studies. Concerns about the test's predictivity and reproducibility, plus animal welfare and political pressure in Europe, have prompted a search for alternative test methods [120]. Since the early 90's, several models of the skin have been developed, characterized, validated and accepted as valid replacement method for animal experimentation.

Thus, all *in vitro* models based on skin equivalents available in the market for permeation studies are described below.

### **5.1.1 Human skin and dermal permeability**

Human skin performs a wide range of protective, perceptive, and regulatory functions, being the role as protective barrier, the most critical for survival [121]. Skin, the largest organ of the human body, is organized into an elaborated layered structure consisting mainly of the outermost epidermis and the underlying dermis. A subcutaneous adipose-storing hypodermis layer and various appendages such as hair follicles, sweat glands, sebaceous glands, nerves, lymphatics and blood vessels are also present in the skin. These multiple components of the skin ensure survival by providing critical functions in protection, thermoregulation, excretion, absorption, metabolic functions, sensation, evaporation management and aesthetics [122].

The barrier function of skin is performed by the epidermis, which is comprised mainly by keratinocytes [121]. The epidermis is a keratinized stratified squamous epithelium without a vascular system. The highly hydrophobia comes from differentiated non-nucleated cells, the corneocytes. This non-viable layer is the rate limiting step for skin absorption of most molecules [123]. Keratinocytes accounts for about 80% of epidermal cells [122], but epidermis is also composed of the pigment-producing melanocytes, Merkel cells, which are thought to play also a sensory role [124-125], and specialized dendritic

Langerhans cells (which have an essential role in the skin immune defense system) [126-127]. One of the most important criteria for evaluation of the performance of any *in vitro* skin culture system is the formation of a competent permeability barrier [128]. The human skin permeability resides in the uppermost layer of the epidermis, the *stratum corneum*, that consists of protein-enriched corneocytes embedded in a lipid-enriched, intercellular matrix [128]. The extracellular lipids are organized in multiple bilayers originating from small organelles, the so-called lamellar bodies, that are synthesized in the spinous and granular cells [128]. The *stratum corneum* provides the greatest barrier function against hydrophilic compounds, whereas the viable epidermis is most resistant to highly lipophilic compounds [114]. The lack of correlation in transdermal permeation of molecules between species or from different application sites in the same animal model is due mainly to variations in *stratum corneum* thickness, in the composition of intercellular *stratum corneum* lipids and in the number of skin shafts [123].

### 5.1.2 Drug permeability *in vitro* models

The limited availability and poor reproducibility of *ex vivo* human skin samples have led various investigators to develop three-dimensional models to allow the metabolic behaviour of human skin [129]. To determine dermal absorption by *in vitro* experiments, the freshly prepared human skin is nearest to the human *in vivo* situation. However, supply of fresh human skin by surgical departments is minimal and irregular, with considerable difficulties associated with legal and ethical issues, linked to the use of human tissues for commercial purposes. Also, the access to human material is limited and their freezer storage may influence its properties. Furthermore, high variability is possible due to donor or preparation techniques, which led to increased efforts to develop high reproducible and cost effective skin models equivalents. The development of genetically controlled and well-characterized skin models can have important implications, not only for scientists and physicians, but also for manufacturers, consumers, governing regulatory boards and animal welfare organizations [122]. Therefore, since 1970's a considerable interest has focused on three-dimensional reconstructed human skin models. Nowadays, for dermal penetration studies epidermal models (containing keratinocytes) and "full thickness" models (containing keratinocytes and fibroblasts) are commercially available.

### 5.1.2.1 Two-dimensional (2-D) models

Monolayer cultures of human cells as human derived cell lines have been used for many years. Data of the past 30 years demonstrated the significant limitations of traditional two-dimensional (2-D) cell monolayers in predicting the behaviour of cells in living organisms [130]. 2D monolayer models not only fail to reproduce the complex and dynamic environments of *in vivo* tissues, but can also trigger false results by forcing cells to adapt to an artificial, flat and rigid surface [122]. The chemically and spatially defined three-dimensional (3-D) network of tissue-specific architecture, mechanical and biochemical signals, and cell–cell communication are lost. All these mechanisms that governs differentiation, proliferation and function of cells *in vivo* are, in fact, lost under the simplified 2-D conditions [130]. Interactions between individual cells, its immediate neighbors and the extracellular matrix are responsible for the control of cell behavior, which means that cells in 2-D mono layers cannot capture the relevant complexity of the *in vivo* microenvironment [130]. Keratinocytes in monoculture produce only a thin epidermal layer and without mesenchymal support undergo apoptosis after about 2 weeks in culture [131]. They are also influenced by dermal fibroblasts that induce the production of basement membrane proteins or melanogenic factors by secretion of soluble factors that diffuse to the overlying epidermis, being another limitation of 2-D models [131-132]. Dermal fibroblasts promote not only keratinocyte proliferation, but also the development of identifiable keratinocytes layers [122]. Consequently, properly stratified epithelia fails to form in simple 2-D feeder-layer co-cultures, upon combination of post mitotic dermal fibroblasts (feeder cells) and epidermal keratinocytes [122], submerged cultures restrict the testing to materials that can be dissolved in aqueous media. Thus, oil-soluble ingredients and finished products such as creams, lotions, or powders cannot be adequately tested in submerged culture systems. Furthermore, submerged culture systems of skin cells do not possess differentiated functions and barrier properties of stratified skin tissues and thus do not accurately reflect the *in vivo* physiology of the skin. However, as they are easy and convenient to set up, they still represent the most popular models for *in vitro* studies, as the 3-D models are very expensive [130].

Therefore, strategies that allow the reconstitution of artificial human skin equivalents in a 3-D setting provide versatility as well as answers to physiological questions that cannot be answered in the context of monolayer tissue culture [122]. Introducing skin models also used for skin absorption, beside skin corrosion and skin

irritation, would allow comparing results for specific chemicals across endpoints more easily.

### 5.1.2.2 Three-dimensional (3-D) models

Skin equivalents have been used to measure percutaneous absorption [123], being able to reproduce specific tissue-like structures and to mimic functions and responses of real tissues in a way that is more physiologically relevant than what can be achieved through traditional 2-D cell monolayers. Thus, 3-D cell culture represents a potential bridge to cover the gap between animal models and human studies. *In vivo*, cells develop and grow within the 3-D architecture of tissues and organs [130]. Since the 1970s the design of a 3-D microenvironment to assess dermal permeability has been proposed. Today a variety of 3D *in vitro* reconstructed human skin models for dermal permeation are commercially available on a routine basis, with a very similar structure to native skin (Figure 1.6).

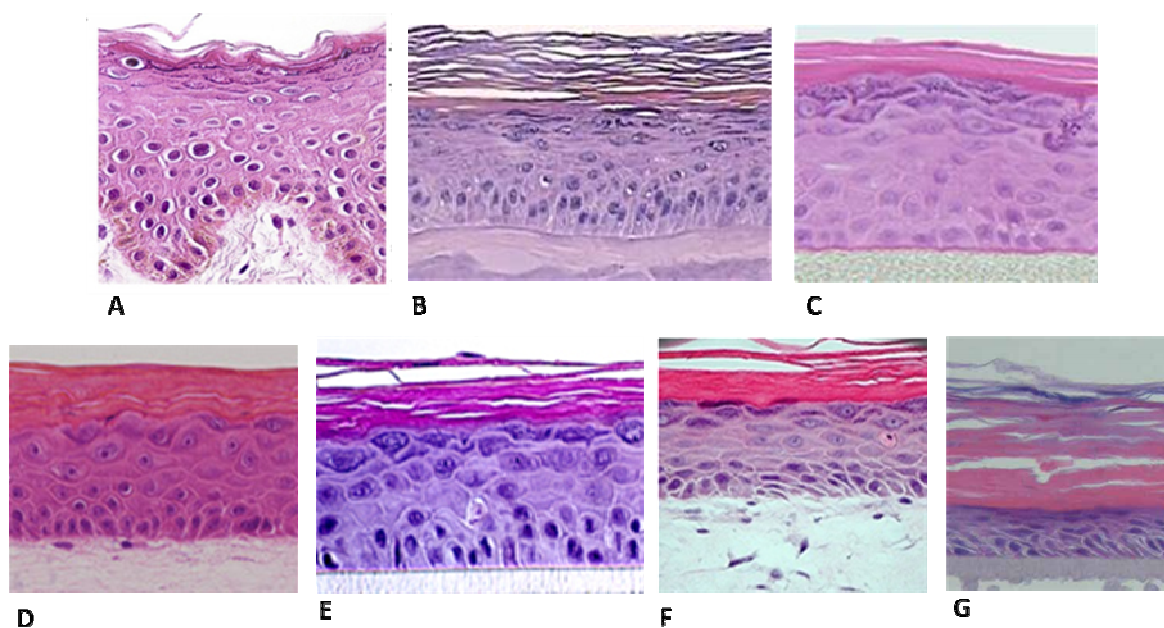


Figure 1.6 - Morphology of human skin and different *in vitro* dermal permeation models (A) normal human skin epidermis, (B) EpiSkin™ model, (C) EpiDerm™ model, (D) StrataTest™, (E) SkinEthic™ RHE model, (F) Epiderm FT™ and (G) epiCS™ by Hematoxylin and Eosin (HE).

Table 1.4 summarizes the 3-D models available in the market for dermal permeation studies.



Table 1.4 - Summary of commercially available human skin equivalents.

<b><i>In vitro</i> model</b>	<b>Company</b>	<b>Product description</b>	<b>Membrane</b>	<b>Biological origin of cells</b>
SkinEthic™ Rhe (Reconstructed Human Epidermis)	SkinEthic	Human keratinocytes cultured on an inert polycarbonate filter at the air-liquid interface in chemically defined medium	Polycarbonate	Single neonatal donor - foreskin or abdomen
EpiSkin™	SkinEthic	Human keratinocytes cultured on a collagen base which permit terminal differentiation and reconstruction of the epidermis with a functional stratum corneum	Collagen I sheet	Several donors - abdomen
Epiderm™	MatTek	Neonatal human-derived epidermal keratinocytes (NHEK) cultured to form a multi-layered, highly differentiated model of the human epidermis	Collagen coated	Single donor (neonatal or adult) - foreskin or abdomen
Epiderm FT™	MatTek	Neonatal human-derived dermal fibroblasts (NHFB) and NHEK co-cultured to form a multi-layered, highly differentiated model of the human dermis and epidermis	Collagen matrix	Single donor (neonatal or adult) - foreskin or abdomen
epiCS™	CellSystems Biotechnologie GmbH	Reconstructed epidermal model made from primary human keratinocytes; it comprises a fully differentiated epidermis with viable and cornified cell layers	Polycarbonate	Single neonatal donor - foreskin or abdomen
StrataTest™	StrataTech	Full thickness skin model where a near-diploid human keratinocytes cell line, NIKS, was utilized.	Collagen I sheet	-

These skin models constitute a platform for cosmetic, photoaging and cancer models, as well as an excellent system for pharmacological analyses [122]. Moreover, they represent time and cost-effective alternatives to the use of laboratory animals that are currently being used for such studies [122].

According to different authors, cells growing in 3-D have different cell surface receptor expression, proliferative capacity, extracellular matrix synthesis, cell density, and metabolic functions [130, 133-134]. The performance of an *in vitro* skin culture system could be evaluated by the formation of a competent permeability barrier, which resides in the upper layer of the epidermis, the *stratum corneum*, that consists of protein-enriched corneocytes embedded in a lipid-enriched, intercellular matrix [128]. To be accepted as a valid human skin model, several criteria must be met. According to Schaefer *et al.*, the artificial skin must compromise a functional *stratum corneum*, as well as the viability of the epidermis [113]. The chemicals to be tested are applied up to 4 h as a liquid or a wet powder onto the skin model. Afterwards, careful washing has to be performed before sequential analyses.

However, these models still have some deficiencies such as the absence of skin appendages including pilosebaceous units, hair follicles and sweat glands [135]. Moreover, these tissues provide much lower barrier properties than the whole skin due to their structure and lipid composition. For this reason, the kinetic parameters of skin permeation obtained overestimate flux across human skin [135].

Different types of reconstructed human skin models can be produced, depending on the substrate used: reconstructed epidermis and reconstructed "full thickness models". Both types are described in detail below.

### **5.1.3 Reconstructed dermal equivalents**

#### **5.1.3.1 EpiSkin™**

The EpiSkin™ model (L'Oréal, Lyon, France) is one of the most used in skin permeation/absorption studies [135]. Research work with skin models is carried out at the research center of L'Oréal, leading to the development of artificial skin models. The EpiSkin™ model was first developed by E. Tinois and was bought by L'Oréal in April 1997 [135]. According to the manufacture, it consists of a dermal substrate generated on type I

bovine collagen matrix, representing the dermis, with a film of type IV human collagen, upon which is laid, after 13 days in culture, stratified differentiated epidermis derived from second passage human keratinocytes. If a more suitable model for studying drug penetration is required, keratinocytes are cultured for 20 days prior transferring to the collagen substrate [136].

The EpiSkin™ model contains the epidermal layers of native skin. In general, the overall ultrastructural appearance of the epidermis is highly similar to native tissue, with seven cell layers (38-48 µm) in penetration skin models [128]. It is also possible to observe the presence of a thick *stratum corneum* of about 60-100 layers (73-102 µm) [128]. All major epidermal strata, including *stratum basale*, *stratum spinosum*, *stratum granulosum* and *stratum corneum*, are present. However, according to Ponec *et al.* it is not always possible to distinguish the different strata of the viable cell layers (*basale*, *spinosum* and *granulosum*) in EpiSkin™ penetration models [128].

The *stratum corneum* present a significantly increased number of cell layers compared to most native *stratum corneum* samples, being thicker [135]. The cells of the viable portion of the EpiSkin™ model are organized somewhat differently than in native epidermis. Also, sudden changes in cell shape in the suprabasal compartment occurred and basal cells tend to be cubical in shape, while the upper cell layers are relatively flat. Granular cells with keratohyalin are present but with irregular shape. Regarding the lipid composition, all major epidermal classes are present. The phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine and cholesterol contents are closed to native tissue [128]. Some differences are found regarding lanosterol, di-/triglycerides and free fatty acids as well as phospholipids amounts which are considerably lower. Also, the ceramide amounts in the EpiSkin™ models differed from native epidermis. The basal cell layer had a more irregular appearance, with lipid droplets short keratin filaments in the cytoplasm of basal cells [128]. Regarding *stratum spinosum*, cells could be irregular in shape [128]. In *stratum granulosum* numerous lamellar bodies were ubiquitously present at amounts similar to native tissue [128].

Currently, EpiSkin™ kit has 12 well plates and the assay mimics the normal route of exposure. The model is adapted to safety or efficacy assays from products for several applications. Regarding safety assays, at the moment the model is validated by OECD for skin corrosion (OECD 431) [119] and irritation (OECD 439) [137]. The protocol was based on a short treatment time (15 min) with the compound, placed on the top of the

model, followed by an extended 42 hour post treatment incubation period. The prediction model is mainly based on the validate cell viability measurement (MTT) as endpoint. Complementary assays could be done such as LDH, cytokine release, histology analyses or even gene arrays. It should also be noted the low intra-batch variation, but a greater inter-batch variation.

Different authors had used EpiSkin™ *in vitro* model to evaluate permeation of different compounds. Rozman *et al.* evaluated the absorption of vitamins C and E from topical microemulsions using reconstructed human epidermis as skin model [138]. In this particular study, RHE were placed on static Franz diffusional cells and the formulations placed on the donor compartment [138]. Dreher *et al.* investigated caffeine's and  $\alpha$ -tocopherol's cutaneous bioavailability from cosmetic vehicles such as a water-in-oil emulsion, an oil-in-water emulsion, a liposome dispersion and a hydrogel applied at finite dose using the reconstructed human skin models EpiDerm™ and EpiSkin™ [139].

### 5.1.3.2 EpiDerm™

The EpiDerm™ model (MatTek Corporation, MA, USA) was introduced in commercial market in 1993. The dermal penetration of drugs can be easily predicted by this model. It is composed by human epidermal keratinocytes as fully-differentiated epidermal tissue (fully-differentiated natural epidermis displaying a basement membrane, proliferating keratinocytes and a *stratum corneum*) after air-liquid interface cultivation on inert polycarbonate membranes. The model tissue consists on metabolica and mitotically active cells organized into a basal, spinous and granular layers, along with a multi-layered *stratum corneum* [136].

Regarding their composition, EpiDerm™ cultures have an epidermal thickness of about six to eight layers (28-43  $\mu\text{m}$ ) and a *stratum corneum* with 16-25 layers (12-28  $\mu\text{m}$ ) [128]. *Stratum basale* have columnar to round in shape basal cells and lamellar bodies and lipid droplets were found in *stratum spinosum* [128]. In both native skin and EpiDerm™ model, *stratum granulosum* is characterized by two cytoplasmic organelles: stellate shaped keratohyalin granules and lamellar bodies.

Regarding *stratum corneum*, EpiDerm™ cultures are characterized by intracellularly located lipid droplets in corneocytes [128]. The total lipid content was the highest in EpiDerm™ models and similar to native skin ( $0.997 \pm 0.06 \text{ mg/cm}^2$ ,

representing 35%) [128]. Lipid analyses revealed that the major barrier lipids are synthesized *in vitro* but in different proportions of native skin. It has a low content of fatty acids and an incomplete profile of glucosphingolipids and ceramides. The relative amount of individual phospholipid classes is similar to EpiSkin™.

An evaluation of dermal penetration with EpiDerm™ used two hair dye ingredients, p-aminophenol and p-phenylenediamine [140]. Both compounds were applied topically or from the bottom of the tissue via the culture media. More recently, Lombardi Rogia *et al.* compared six commercial prednicarbate preparations for topical use using Epidermal™ model [141].

### 5.1.3.3 SkinEthic™

The SkinEthic Reconstructed Human Epidermis (RHE)™ model was developed by M. Rosdy in 1990 and became commercially available in 1993 by SkinEthic Laboratories, acquired by L'Oreal in 2006. The model is generated on inert polycarbonate filters where normal human keratinocytes are cultured. The epidermis consisted of five to nine cell layers (23-59 µm) and 14-24 layers (15-32 µm) in the *stratum corneum* [128]. The presence of layer hemidesmosomes could be demonstrated [128]. *Stratum basale* present columnar to round in shape basal cells and lipid droplets in the cytoplasm [128]. The RHE expresses the major differentiation markers (filaggrin and involucrin in granular cell layers, transglutaminase I and keratin 10 in supra basal cell layers and loricrin in upper granular cell layers), as well as expressing the basement membrane markers (type IV collagen; integrin alpha 6, integrin beta 4, antigen BP, laminin I and laminin V). Free fatty acids and ceramides are detected in the lipid profile. The ultra-structural features show secretion and normal arrangement of bi-layered lipid content into the intercellular spaces of the cornified cell layers (formation of normal permeability barrier).

An advantage of RHE is the possibility of incorporating various additional cell types, such as melanocytes, in combination with keratinocytes or the creation of pathological models.

#### 5.1.4 Reconstructed "full thickness models"

##### 5.1.4.1 StrataTest™

The StrataTest™ human skin model has been developed to simulate the architecture and biological responses of human skin [142]. The model was developed and commercialized by Stratatech Corporation (Madison, WI, USA), founded in 2000, under the same standards as the company's flagship StrataGraft™ human skin substitute, which recently was evaluated in a human clinical safety trial.

This model is constituted by pathogen-free NIKS™ keratinocyte progenitors - a human keratinocytes cell line that produces living tissue nearly identical to the native human skin. It is a fully-stratified tissue containing epidermal and dermal components that possesses barrier function. According to the manufacturer, NIKS™ reproduce normal human skin tissue architecture and barrier function when cultured appropriately [142]. These novel cells possess the ability to undergo normal epidermal differentiation, generating a fully-stratified epithelium similar to that of native human epidermis [143]. The full-thickness model is produced in individual cell culture inserts with a surface area of 0.6 cm<sup>2</sup>, where normal human dermal fibroblasts are embedded in type I collagen and NIKS™ cells seeded onto dermal equivalents and later lifted to the air interface to promote terminal differentiation. Histological and indirect immunofluorescent analysis confirmed the formation of epidermal tissue with the appropriate expression of proteins, essential for the integrity, structure, and function of human skin. The analysis of the full-thickness StrataTest™ human skin model confirmed the formation of tissue typical of the interfollicular epidermis, as well as the expression and appropriate localization of proteins essential to cell–cell adhesion, basement membrane formation, and epidermal function [142]. The model is supplied in 24 well plates format not yet validated by OECD or ECVAM. Nevertheless, according to the manufacturers, the main advantage of this model is the use of NIKS™ cells that yields a skin model with a high degree of consistency, advantageous over other *in vitro* models such as EpiDerm™, EpiSkin™ or EST1000. The later ones must periodically change the source of donor cells or are manufactured using cells pooled from multiple donors [142].

#### 5.1.4.2 EpiDerm FT™

Recently, MatTek has developed EpiDerm FT™, a full thickness skin model. It contains normal, epidermal keratinocytes and dermal fibroblasts human-derived, cultured to form a highly differentiated model of the human dermis and epidermis. Both cell lines are cultured on specially prepared cell culture inserts using serum free medium. Ultrastructurally, the EpiDerm FT™ closely parallels human skin, being mitotic and metabolically active, providing a useful *in vitro* way to assess dermal irritancy and toxicity.

The model present organized basal, spinous, granular, and cornified epidermal layers and a dermal compartment composed by a collagen matrix containing viable normal human dermal fibroblasts. A set of markers proves the epidermis-specific differentiation. Immunohistochemical analysis shows the presence of basement membrane structure and signaling proteins including collagen IV, Laminin, collagen VII and integrin  $\alpha 6$ .

#### 5.1.4.3 epiCS™

epiCS™ (CellSystems, Germany), available since 2004, consists of normal human epidermal keratinocytes from a single neonatal donor. A submerge culture of the keratinocytes is followed by a culture at air-liquid interphase. This “airlift culture” under defined media conditions stimulates the differentiation of the cells and the generation of the physiological epidermal layers. The model presents basement membrane, proliferating keratinocytes and a *stratum corneum* with an intact barrier function on a polycarbonate membrane. The epidermis model is supplied in convenient 24-well formats. Until recently the epiCS™ test method was formerly known under the name EST-1000 and was recently renamed.

epiCS™ (CellSystems, Germany) is validated for the classification of compounds concerning skin corrosion according to the OECD test guideline 431 for testing of chemicals and ECVAM has accepted this method to be used for distinguishing between corrosive and non-corrosive chemicals [144].

## 5.2 Models limitations

The *in vitro* 3-D models are close to reproducing human skin due to their general histological architecture and physiological properties. Although their similarity to human native skin, some important limitations could be reported: models are considerably more permeable than human skin and the barrier function is weak. However, the culture models appear to be more consistent in permeability and responsiveness with less variability. Thus, the major goal of manufacturers is to fit skin models with a barrier similar to human skin *in vivo*. Nevertheless, it should be noted the constant development of commercially available skin models, representing a considerable improvement over the years. As alternative methods, they are useful for the development of new products by the pharmaceutical and cosmetics industries. Further researches and studies with *in vitro* models still represent an important challenge. Also, development of 3-D tissue models and the validation of applications based on those tissues will need to occur in the future.

## 5.3 Skin biophysical techniques

About twenty years ago cosmetic products promotion took more importance and advertising claims became more aggressive and closer to the limit of what could be scientifically shown and consumer perceived [145]. In order to monitor the claims made about cosmetic products and protect the consumer against misleading advertisement, European Commission have issued rules under the form of laws, or directives, to ensure that proper substantiation of claims exists [145]. In the European Union, cosmetic claims substantiation is regulated since 1997, stating that cosmetics and toiletries manufacturers making claims for their products have to demonstrate the proof of their claims [109].

Skin biophysical techniques are used to support claims. Claims on cosmetic products are extremely varied and often depend on the product, the market, and the current trends. However, several claims have been used on different product types for many years. Different claims could be evaluated such as safety-related claims (e.g., mildness, sensitive skin-designed products, non comedogenicity claims), some efficacy claims (e.g., skin-hydration effect, smoothing and anti-wrinkle effect) or sensory claims. The application of biophysical techniques in the study of cosmetics efficacy is of huge importance. Biophysical instruments are objective and quantitative tools for characterization of different skin conditions and have widely been used in the evaluation of the effect of formulations of topical use, mainly due to the fact of facilitating the



evaluation of products in its real use conditions, that is to say, directly in the human skin. The confirmation of the effects of cosmetic products and its action mechanism, by means of experimental protocols properly elaborated using the skin biophysics is of fundamental importance. In the later 20 years, skin engineering techniques have been considerably improved especially since the implementation of the European Directive 2003/15/EC2 prohibiting animal experiments for testing cosmetic ingredients and products [146]. *In vivo* skin mechanical testing was primarily developed for the dermocosmetic industry [146]. There are numerous biophysical methodologies available that allow the non-invasive evaluation of different skin properties. Through these techniques it is possible to assess the effectiveness of cosmetic products as well as to ascertain the normal functioning of the skin. The skin biophysical techniques comprises a set of methods that, through not invasive instruments, evaluates the physicochemical properties of the skin, such as sebum, hydration, pH, mechanical properties, transepidermal water loss, pigment or temperature [147-148]. In this brief section, the biophysical methods commonly used in the assessment of the effects of the skin care formulations will be discussed.

### 5.3.1 Hydration

Cosmetic and dermato-pharmaceutical products often claim skin-moisturizing benefits. The measurement of hydration in the surface layer of the skin, *stratum corneum*, gives important information on the biophysical properties and function of the skin barrier [149]. With an adequate amount of water in the SC the skin maintains its intact barrier function, feels soft and flexible, looking smooth and healthy. Different methods could be used for determining the hydration state of SC. The degree of hydration is most frequently determined by measuring electrical properties of skin [150]. Infrared and photoacoustic spectroscopy have also been used [150]. Measurement of skin permeability to alternating electric current (impedance) reflects electromagnetic interaction with skin dipoles (proteins) and electrolytes.

Since 80<sup>th</sup> decade, many types of instruments have been developed and used widely for diagnosis and the development of moisturizing agents and other topical drugs [151]. The equipments commonly used are based on the measurement of electrical capacitance, impedance and conductance. Inside these, the most used equipments are based on electrical capacitance measurements. By definition, electrical impedance corresponds to the opposition that skin offers when is subjected to an alternating current.

In turn, capacitance is an electrical quantity determined by the amount of electrical energy that can be accumulated in the skin and the amount of alternating current there through at a certain frequency. Finally, the conductance measurement principle, also known as the electrodermal response (and in older terminology as "galvanic skin response"), is known to measure the water binding capacity of the *stratum corneum*. In this phenomenon skin momentarily becomes a better conductor of electricity when either external or internal stimuli occur that are physiologically arousing. Different equipments are available in the market for hydration evaluation. The most commonly used is Corneometer<sup>®</sup> (Courage & Khasaka, Köln, Germany) that is based on a capacitive measurement principle - Figure 1.7.



Figure 1.7 – Corneometer<sup>®</sup> probe (Available from <http://www.courage-khazaka.de/index.php/en/faq-en/faq-scientific-devices/61-corneometer>; accessed on 25.01.16).

The probe is made of two finger-type metal plates close to each other, with a measurement depth of approximately 40  $\mu\text{m}$  [152]. The capacitance measurement is based on the difference between the dielectric constant of water and other substances (the constant value dielectric is, in most cases, less than 7) [152]. The measurement is performed by placing the head of the probe on the skin surface. During the measurement, an electric field penetrates the skin and allows the determination of the difference between dielectric constants [152]. The modification of the registered capacitance by Corneometer<sup>®</sup> in contact with the SC is a function of the water content. The hydration of the SC values is expressed in arbitrary units (AU).

### 5.3.2 Transepidermal water loss (TEWL)

Measurement of transepidermal water loss (TEWL), expressed in grams per squaremeter and per hour, is used for studying the water barrier function of the human

skin. Based on the estimation of the water vapor gradient in an open chamber, it is being used to support claims of cosmetics including product mildness, reduction of skin irritation reactions, skin hydration, skin repair, protective effect against UV damage and others [153]. TEWL measurement can also screen ingredients that have a beneficial effect on the barrier function and offer the possibility to monitor *in vivo*, in human skin, the effect of topical treatment in an objective and non-invasive way [153]. This method uses an electric hygrometer to measure the amount of water being lost slowly from the body through the *stratum corneum*. The measurement principle is based on Fick's diffusion law, defining the mass of water transported per unit area of skin over a period of time. A high number of variables affecting TEWL measurements have been identified. Because the measurement is affected by perspiration, it is performed at an ambient temperature of 22°C or less and under conditions that cause no sweating [151]. According to Martini *et al.* the average flow of water that evaporates at the skin surface is 5 g/m<sup>2</sup>/hour, corresponding to a water loss of 300 and 400 ml/24 hours [154]. However, despite being a permanent evaporation, water is renewed by a process of diffusion from the deeper layers of skin and is also offset by a nutritional support, calling up this process by TEWL [154]. The Standardization Group of the European Society of Contact Dermatitis had published guidelines on exposure testing with sodium lauryl sulfate (SLS) [155].

Different equipments are available in the market: ServoMed<sup>®</sup> (SeroMed AB, Stockholm, Sweden), Tewameter<sup>®</sup> (Courage & Khazaka, Köln, Germany), AquaFlux<sup>®</sup> (Biox Systems Ltd, London, United Kingdom) or VapoMeter<sup>®</sup> (Delfin Technology, Kuopio, Finland). The most used equipment for the TEWL evaluation is Tewameter<sup>®</sup>. According to Courage & Khazaka the Tewameter<sup>®</sup> probe measures the density gradient of the water evaporation from the skin indirectly by the two pairs of sensors (temperature and relative humidity) inside the hollow cylinder (Figure 1.8) [156].



Figure 1.8 - Tewameter<sup>®</sup> probe (Available from <http://www.courage-khazaka.de/index.php/en/products/scientific/139-tewameter>; accessed on 25.01.16).

### 5.3.3 pH

The pH (hydrogen ionic concentration) determination of cutaneous surface is seen as an important functional indicator of the skin, since it arises from the production of lactic acid which confers the so-called "cutaneous acid mantle" [157]. Healthy skin presents a slightly acid pH (4.6-5.8), which contributes to the bactericidal and fungicidal protection of its surface [157]. Furthermore, the cutaneous secretions present an appreciable buffering capacity, which are extremely important when skin pH is altered as a consequence of use of inappropriate topical products. Hence, the determination and control of cutaneous pH, from the cosmetic and/or dermatological point of view, are of extreme importance.

Skin-pH-meter<sup>®</sup> (Courage & Khazaka, Köln, Germany) uses the direct potentiometry method through a special electrode for measurement of this variable and allows sensitivity in the order of 0.1 of the pH unit. According to the manufacture, both glass H<sup>+</sup> ion sensitive electrode and additional reference electrode are placed in one housing that is connected to a probe handle containing the measurement electronics [158]. Figure 1.9 shows a Skin-pH-meter<sup>®</sup> probe.

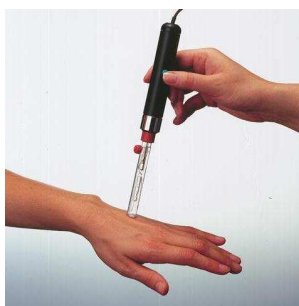


Figure 1.9 - Skin-pH-meter<sup>®</sup> probe (Available from <http://www.courage-khazaka.de/index.php/en/products/scientific/132-skinphmeter>; accessed on 25.01.16).

### 5.3.4 Skin color

The measurement of the redness, whiteness, and pigmentation of the skin has been showing extremely remarkable progress. Most color measurements of the skin surface are based on reflectance colorimetry instruments, such as Chromameter (Minolta, Tokyo, Japan), erythema index, Erythema meter (Diastron, Hampshire, United Kingdom), Mexameter<sup>®</sup> (Courage & Khazaka, Köln, Germany) or Deraspectrometer (Cortex

Technology, Hadsund, Denmark). One of the most notable developments is Minolta Chromameter<sup>®</sup>. These instruments are based on the same optical principle, namely, measurements of light absorption and reflection of respectively the melanin and hemoglobin components of the skin. The specific absorption of melanin and hemoglobin in the visible (green and red) and in the near infrared is determined and these instruments quantify redness by a relative erythema index. The erythema index is proportional to the hemoglobin content of the upper layers of the dermis. The Minolta Chromameter<sup>®</sup>, considered by many investigators as a sort of reference instrument, quantifies skin surface color using the three-dimensional CIE color representation with the L\*a\*b\* system.

### 5.3.5 Mechanical properties of skin

Skin and its subcutaneous layer represent a complex composite of tissues, whose mechanical characteristics depend upon the mutual interdependence of their constituent parts. The stratum corneum, the association between the living epidermis and papillary dermis, the reticular dermis and the hypodermis have each their own intimate structures whose tensile functions are ideally balanced to respond adequately to the casual mechanical demands [159]. The viscoelastic functions of aging skin can be tested by altering the orientation and magnitude of imposed stresses and strains over time [160]. The assessment of tensile functions of skin can be performed by distinct approaches mainly characterized by the orientation and magnitude of the imposed stress and strain over time. Testing methods are basically grouped into six major classes: tensile, torsional, acoustic shear wave, indentation, impact and elevation [160-161]. This topic was extensively addressed by the Efficacy Measurements on Cosmetics and Other Topical Products (EEMCO) group [159-160].

Non invasive suction devices were employed for the study of the elastic properties of the skin. Different equipments are available in the market considering the major classes mentioned: Frictiometer<sup>®</sup> (Courage & Khazaka, Köln, Germany) that uses torsional tests; Reviscometer<sup>®</sup> (Courage & Khazaka, Köln, Germany) with acoustic shear wave; or Cutometer<sup>®</sup> (Courage & Khazaka, Köln, Germany) and Dermaflex<sup>®</sup> (Cortex Technology, Hadsund, Denmark) with indentation.

In the present work, Cutometer<sup>®</sup> was the device selected. Cutometer<sup>®</sup> is a commercially available device that comprehensively measures the mechanical and

physical properties of the skin, such as extensibility and viscoelasticity [151]. This instrument applies suction to the skin and measures its displacement and time to recovery [151]. Briefly, the measuring principle is based on the suction method [162]. A negative pressure (a maximum of 500 mbar) is created in the device, the skin is drawn into the aperture of the probe, and after a defined time released again. Inside the probe, the penetration depth is determined by a non-contact optical measuring system. This optical measuring system consists of a light source and a light receptor, as well as two prisms facing each other, which project the light from transmitter to receptor. The light intensity varies due to the penetration depth of the skin. Figure 1.10 represents a Cutometer®.



Figure 1.10 – Cutometer® probe (Available from <http://www.courage-khazaka.de/index.php/en/faq-en/faq-scientific-devices/69-cutometer>; accessed on 25.01.16).

The resistance of the skin to the negative pressure (firmness) and its ability to return into its original position (elasticity) are displayed as curves (penetration depth in mm/time) in real time during the measurement - Figure 1.11.

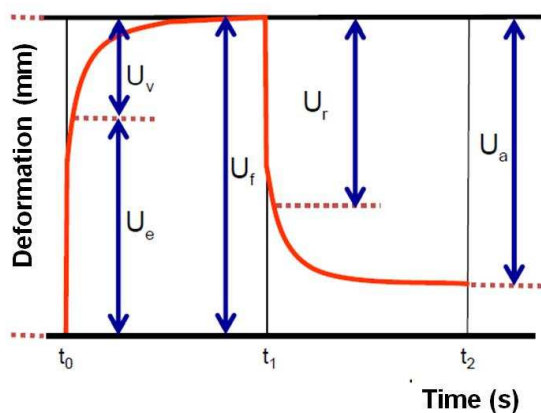


Figure 1.11 – Graphical scheme of a skin deformation curve obtained with the Cutometer®.

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With these curves different parameters could be obtained, such as:

$U_f$  ( $U_e+U_v$ ) - maximum amplitude and represents the passive behavior of the skin to force (firmness).

$U_r$  - The ability of the skin to return to its original state (minimum amplitude after relaxation).

$U_a$  - Complete relaxation after the pressure is cut off.

$U_a/U_f$  - Gross elasticity: resistance *versus* ability of returning.

$U_r/U_e$  - Net elasticity: elastic portion of the suction part *versus* the elastic portion of the relaxation part.

$U_v/U_e$  - Portion of the viscoelasticity of the curve.

$U_r/U_f$  - Portion of the elasticity compared to the complete curve.

This measurement principle allows getting information about the elastic and mechanical properties of skin surface and enables to objectively quantify skin aging [162].

### 5.3.6 Skin surface topography

Wrinkles can be easily visualized and many clinical studies have involved the use of ranking scales that rely on subjective assessments by expert graders. To make possible to achieve this goal, fast and exactly working 3D *in vivo* measurement methods are necessary, which permit a direct access to the three-dimensionality of the human skin surface.

Evaluation of wrinkles using skin replicas has also been used along with the visual evaluation [163]. The most common approach for measuring the geometric properties of human skin is to transfer the details of the skin's topography to a material suitable for the selected method of measurement [164]. Various measuring instruments and systems have been developed to gauge these replicas of human skin [164]. The most widely used method for detailed observation of skin surface topography seems to be the replica (microrelief) method [151]. The surface topography of the skin is transferred to a soft nitrocellulose or silicone rubber material, transferred further from it to a resin or other appropriate material, and then observed under the light or electron microscope. Many

techniques have been developed to analyze replicas according to different principles [163]. Those techniques include profilometric methods [165-166], two-dimensional image analysis [167] and scanning electron or confocal microscopic methods [168]. Therefore, methods using replicas are quite greatly useful and convenient to evaluate wrinkle levels in subjects or the wrinkle smoothing efficacy of products or cosmetic surgery [163]. However, replicas do not reflect the morphology of the skin perfectly because accurate morphological information of the skin might be lost during the replica process [163].

Attempts have been made to visualize the surface topography in 2-dimensional representation and to achieve 3-dimensional reconstruction [151, 169-170]. At present, specimens are usually observed under oblique light and the resulting shadows are read into an image analyzer connected to a computer, which calculates the area of detected shadows. Three-dimensional *in-vivo* optical skin imaging provided a rapid and quantitative assessment of surface topography and facial fine lines. With the digital fringe projection based on micromirror projectors from the Texas Instruments company and the PRIMOS<sup>®</sup> technology, respectively, a new optical 3D measurement method is presented, which makes it possible to measure human skin surfaces fast and very accurately, both in the micro and macro-ranges and to document treatment results objectively.

The PRIMOS<sup>®</sup> optical three-dimensional *in vivo* skin measurement device deploys a parallel stripe pattern imaging technique that is projected onto the skin surface and depicted on the charge coupled device chip of a high resolution camera [170]. Light patterns are created by a digital micromirror projector. The use of micromirror-based digital light projectors is advantageous when applied to optical 3D *in vivo* skin measurement because the light intensity is high, exposure time is short and the light can be controlled point and/or pixel wise [170]. The 3D effect is achieved by means of minute elevation differences on the skin surface deflecting the parallel projection stripes to produce a qualitative and quantitative measurement of the skin's profile [170]. Images are digitized and transferred for computer assisted quantitative evaluation and measurement. Mathematical algorithms embedded in the analytical software reconstruct the data into a highly precise 3D profile of the skin.

A 3D optical scanner using fringe projection is used to acquire a 3D surface image of an area and its surrounding skin. For this thesis the PRIMOS<sup>®</sup> 3D portable scanner shown in Figure 1.12 was used.





Figure 1.12 – The PRIMOS® equipment (Available from [http://www.in-cosmetics.com/\\_novadocuments/34659?v=635120853501200000](http://www.in-cosmetics.com/_novadocuments/34659?v=635120853501200000); accessed on 24.01.16).

Three-dimensional *in vivo* optical imaging provided higher resolution of the skin topography than analysis by clinical photography or skin replicas changes, and was in agreement with clinical photography or skin replicas changes, clinical assessment and objective 3D *in vivo* imaging. Its acquisition time is <100 ms with a lateral and vertical resolution of 28  $\mu\text{m}$  and 2  $\mu\text{m}$ , respectively.

The application of skin biophysical techniques has enabled researchers to grasp numerically the properties of the skin that cannot be evaluated by the human senses. This approach has become essential to understand not only the changes caused by skin diseases, but also the characteristics of normal skin reflecting age, sex, and anatomical location, as well as the effectiveness of cosmetic products.

## **Chapter | 2**

### **Aims and Goals**

In recent years, issues of environmental and economic sustainability are subject of great attention from civil and business society. Production and sustainable processing of cosmetics are intended to significantly reduce the waste of raw materials and minimize pollution, becoming mandatory components of the business model. Indeed, at 16<sup>th</sup> July 2008, the European Commission (EU) released the EU Commission Action Plan on Sustainable Consumption and Production (SCP) and Sustainable Industrial Policy (SIP), which includes a number of proposals on eco-design standards for wide range of products, energy and environmental labelling, incentives rewarding eco-friendly products (including green public procurement work with retailers), a retailer forum, environmental industry support, environmental technologies, green public procurement, and promotion of an international sustainable industry consumption and production, to contribute to an improvement in performance. It also aims to encourage EU industries for innovation opportunities. Sustainable production and consumption are, therefore, a challenge and an opportunity, maximizing the business potential for turn environmental challenges into economic opportunities. The ability to minimize environmental impacts, coupled with the ability to manage the re-introduction of underutilized by-products and products in the production chain, can be the guarantee in the future of the balance between economics, politics and environmental conservation. Thus, manage value added products such as red clover or coffee silverskin, introducing them in the chain of active ingredients used in cosmetic industry, could be an added value for green chemistry and environmental ecology.

The main objectives undertaken on the present thesis were:

- i) Development and characterization of extracts obtained from food by-products. Concerning questions of sustainability, different solvents and temperatures were tested;
- ii) Development and characterization of a cosmetic line (with and without extracts) containing five different products: shower bath, scrub gel, hand cream, body cream and a face cream. Formulations were developed taking into account appropriate characteristics for skin compatibility;
- iii) Assess the *in vitro* biological biocompatibility of extracts and cosmetic formulations. Skin cell lines (keratinocytes and fibroblasts) were used to study the safety;

- iv) Evaluate the skin and ocular compatibility of extracts considering 3-D models. The selected models are validated alternatives by European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM);
- v) Evaluate the *ex vivo* skin permeation of extracts in Franz cells in semi-solids formulations and encapsulated into lipid nanoparticles;
- vi) Evaluate the *in vivo* efficacy and safety of the formulations developed. Thereunto, formulations were used and evaluated by human volunteers.

## **EXPERIMENTAL PART**

“Um horizonte, - a saudade  
Do que não há-de voltar;  
Outro horizonte, - a esperança  
Dos tempos que hão-de chegar;  
No presente, - sempre escuro, -  
Vive a alma ambiciosa  
Na ilusão voluptuosa  
Do passado e do futuro.”

*Machado de Assis*

**Chapter | 3**

**Formulations**

Along the laboratorial development of this PhD thesis different formulations (with and without extracts) were elaborated. The processing steps controlled different characteristics of the final formulation such as viscosity, pH and stability, and also the market target, leading to the presented final formulations composition. For all of them, stability studies and safety assessment reports, as well as sensorial analysis, were performed. At the moment, five of them are commercialized and the other are in the final stage of production for commercialization. The different formulations are:

### 1. Hand Cream

Ingredients	Composition(%) / Formulations				Function
	W	X	Y	Z	
A	77.73	75.23	75.23	75.23	Solvent
B	10.00	10.00	10.00	10.00	Emulsifier, emollient, viscosity controller
C	5.00	5.00	5.00	5.00	Skin protector, humectant
D	3.00	3.00	3.00	3.00	Emollient, skin conditioner
F	2.00	2.00	2.00	2.00	Emollient, skin conditioner
Coffee silverskin Extract	-	2.5	1.25	-	Active Ingredient
<i>Medicago sativa</i> Extract	-	-	1.25	2.5	Active Ingredient
G	0.90	0.90	0.90	0.90	Skin conditioner
H	0.50	0.50	0.50	0.50	Emulsifier
I	0.50	0.50	0.50	0.50	Preservative
J	0.30	0.30	0.30	0.30	Buffer
K	0.07	0.07	0.07	0.07	Parfum



**2. Body Cream**

Ingredients	Composition(%) / Formulations		Function
	X	Y	
A	73.10	70.60	Solvent
B	6.80	6.80	Emollient, skin protector
C	5.00	5.00	Emollient, skin conditioner
D	4.00	4.00	Skin protector, humectant
E	3.80	3.80	Emulsifier
F	2.20	2.20	Emulsifier
G	1.50	1.50	Emulsifier
Coffee silverskin Extract	-	1.25	Active Ingredient
<i>Medicago sativa</i> Extract	-	1.25	Active Ingredient
H	0.80	0.80	Emulsifier
I	0.70	0.70	Skin conditioner
J	0.60	0.60	Preservative
K	0.50	0.50	Skin conditioner
L	0.30	0.30	Viscosity controller
M	0.30	0.30	Buffer
N	0.20	0.20	Parfum
O	0.10	0.10	Opacifier
P	0.10	0.10	Buffer

### 3. Body Scrub

Ingredients	Composition(%) / Formulations		Function
	X	Y	
A	86.7712	84.2712	Solvent
B	6.00	6.00	Abrasive
C	3.00	3.00	Humectant
D	1.80	1.80	Emulsifier, foam booster
Coffee silverskin Extract	-	1.25	Active Ingredient
<i>Medicago sativa</i> Extract	-	1.25	Active Ingredient
E	0.80	0.80	Emulsifier
F	0.60	0.60	Preservative
G	0.50	0.50	Foam booster
H	0.25	0.25	Emulsifier
I	0.16	0.16	Parfum
J	0.068	0.068	Buffer
K	0.05	0.05	Chelator
L	0.0008	0.0008	Colorant

#### 4. Facial Cream

Ingredients	Composition(%) / Formulations		Function
	X	Y	
A	76.20	73.70	Solvent
B	8.00	8.00	Emulsifier
C	6.00	6.00	Emollient
D	3.00	3.00	Emollient
Coffee silverskin Extract	-	2.50	Active Ingredient
E	2.00	2.00	Emulsifier, emulsion stabilizer
F	2.00	2.00	Humectant
G	1.50	1.50	Skin conditioner, moisturiser
H	0.70	0.70	Parfum
I	0.60	0.60	Preservative

## 5. Cleansing Shower Cream

Ingredients	Composition(%) / Formulations		Function
	X	Y	
A	66.57	64.07	Solvent
B	14.00	14.00	Emulsifier
C	6.00	6.00	Humectant
D	4.00	4.00	Emulsifier, emollient, foam booster
E	3.00	3.00	Foam booster
F	2.30	2.30	Emulsifier
Coffee silverskin Extract	-	1.25	Active Ingredient
<i>Medicago sativa</i> Extract	-	1.25	Active Ingredient
G	1.00	1.00	Cleanser, foam booster
H	0.80	0.80	Skin conditioner
I	0.65	0.65	Viscosity controller
J	0.63	0.63	Emulsifier
K	0.30	0.30	Viscosity controller
L	0.20	0.20	Parfum
M	0.20	0.20	Skin conditioner, skin protector
N	0.15	0.15	Preservative
O	0.10	0.10	Preservative
P	0.10	0.10	Buffer

## I. Evaluation of *Medicago* as New Ingredient for Skin Care Products

The information presented in this chapter section was published in the following publication:

- **Francisca Rodrigues**, Ana Palmeira-de-Oliveira José das Neves, Bruno Sarmento, M. Helena Amaral, M. Beatriz P.P. Oliveira, *Medicago* spp. extracts as promising ingredients for skin care products, *Industrial Crops and Products*, 49:634-644, 2013

## 1. Introduction

In recent years there is an increased demand for natural plant extracts to be included into cosmetics, either as fragrances or for their medicinal properties. Indeed, these have been increasingly used in skin care products with claims of cosmeceutical effects [171-172]. Given the inherent economic potential in the exploitation of natural resources in ecosystems, herbal extracts have been used by cosmetic science in order to beautify and maintain the physiological balance of the human skin. The desirable features of cosmetic ingredients are efficacy, safety, novelty, formulation stability, easy metabolism in the skin and low cost. The referred characteristics can be attributed to herbal cosmetics with consequent increase in public demand. Furthermore, compared to synthetic cosmetic products, herbal products are mild and biodegradable, and simultaneously present low toxicity [173]. On the other hand, by-products of plant food processing represent a major disposal problem for the industry concerned. But some of them could be also promising sources of compounds due to their favourable technological or nutritional/medicinal properties [174]. Nowadays, millions of tons of by-products are produced during food processing in agro industries without economic value but potentially recoverable as new ingredients for the cosmetic and pharmaceutical industries. Therefore, one can use these wastes to obtain new topical natural antioxidants, maximizing agro-industrial residues, protecting the environment and also maximizing resources. The current challenges are to identify new bioactive natural compounds with potential application and/or replacement of synthetic substances, especially for food, pharmaceutical and cosmetic industries [175].

It has been well established that free radicals can cause oxidation and damage to biological molecules while phenolic compounds, due to their antioxidant activity, are able to neutralize their action [176]. Several plants have been studied as sources of potentially safe natural antioxidants for the food industry and various compounds have been isolated, many of them polyphenols [174, 177]. Topical natural antioxidants may also be a useful strategy for the prevention of photo aging and oxidative stress mediated skin diseases [178]. Many herbal agents used in cosmetics have been selected by a process of 'trial and error' and, thus, are used based on experience rather than experimental investigation. There is now, however, growing scientific evidence that plants possess a vast and complex arsenal of active ingredients able not only to calm or smooth but also actively restore, heal and protect the human skin [179-181].

In addition, plant extracts may contain compounds with antimicrobial or other beneficial properties, influencing the formulation of natural and non-chemical cosmetic products. The public trend towards natural and sustainable products is on-going. However the contamination of cosmetic products is an inherent risk. Natural products, due to their composition, are more susceptible to microbial contamination [182]. Due to this drawback, formulators are looking for novel approaches to keep cosmetic products microbiologically stable. The evaluation of antimicrobial activity of the natural extracts is now included in the challenge. Thus, the study of plant extracts intended for cosmetic purposes, especially as preservatives, or to justify their use as antimicrobial agents, is very important. Indeed, some bacteria, which showed resistance to certain antibiotics, can be sensitive to extracts [183-184]. Natural extracts are part of a group of substances considered as “tolerated” but not admitted as additives in a strictly legal way [182]. Vegetal extracts would be included in the additive group classified as “aromatic and flavoring substances”, in which are included all the natural products and relevant synthetic products that can be used on all animal species, without any restriction regarding age or product dosage. Due to the fact that these products are well accepted by consumers, they are a promising alternative to antibiotics (not being the final product considered as medicinal), and the search of new substances represents an important research area.

Although the term plant extract inherently purports their beneficial and benign properties, these could have adverse reactions in individuals. Therefore, it is essential to attend the issue of ensuring quality and safety of this extracts in cosmetic products before embarking on the more arduous task of ensuring efficacy. In view of this underlying principle, the screening of natural plant extracts with scavenging activity for pro-oxidant reactive species is a primary requirement for the development of new topical antioxidant formulations [178]. Also, during the development of new topic formulations, skin irritation potential is investigated prior to human exposure, in order to identify chemicals which might induce adverse skin reactions [172].

In this context, the study of the antioxidant and antimicrobial properties of extracts, associated with the study of their cytotoxicity in different skin cell types, like keratinocytes or fibroblasts, should be considered basic studies.

The genus *Medicago* is part of the botanical family of Leguminosae and includes about 56 different species mainly distributed in areas presenting Mediterranean climatic conditions [25]. Alfalfa (*Medicago sativa*), is the main *Medicago* species widely grown

throughout the world, predominantly as a source of high quality forage for livestock, renewable energy production, phytoremediation, and as a source of phytochemicals [26]. It is also used as a human food ingredient, consumed as sprouts in salads, in sandwiches or soups [28], as leaf protein concentrates [29], or as food supplements [30]. Alfalfa may help in some human health disturbances, such as anaemia, diabetes, endometriosis, stomach ulcers, osteoporosis, menopausal symptoms, breast and prostate cancers and low bone density [31-32]. To our best knowledge, there is no published data on the antioxidant and antimicrobial activity, or cytotoxicity potential, of any extract of *Medicago* spp. Therefore, the evaluation of these properties in different species of *Medicago* remains an important challenge in understanding the possible benefits of their application in cosmetic products.

## 2. Materials and Methods

### 2.1 Chemicals and reagents

Ascorbic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH<sup>\*</sup>) free radical, catechin, Folin–Ciocalteu's reagent, gallic acid, iodine, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble derivative of vitamin E (Trolox) and  $\alpha$ -tocopherol, were all purchased from Sigma-Aldrich (Steinheim, Germany). Ethanol reagent grade, sodium acetate, sodium carbonate decahydrate, sodium nitrite, aluminium chloride and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX<sup>™</sup>-I, fetal bovine serum (FBS), streptomycin, penicillin and amphotericin B were from Invitrogen (Carlsbad, CA, USA). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2,4-sulfophenyl)-2H-tetrazolium (MTS) assay kit (CellTiter 96<sup>®</sup> Aqueous One Solution Cell Proliferation Assay) was purchased from Promega (Madison, WI, USA). Lactate dehydrogenase (LDH) colorimetric cytotoxicity assay kit (LDH Cytotoxicity Detection Kit) was purchased from Takara Bio Inc. (Shiga, Japan). Ultra-pure water was obtained in-house using a Milli-Q water purification system (TGI Pure Water Systems, USA).



## 2.2 Plant materials

Leaves of six species of *Medicago* (*M. minima*, *M. tornata*, *M. truncatula*, *M. rigidula*, *M. scutellata* and *M. segitalis*) were sown at the Experimental Field of the University of Porto at the Agrarian Station of Vairão. Samples were collected from February to July 2009. *M. sativa* was sown and collected in Azurara, Vila do Conde, Portugal, in February 2012. The plant leaves were randomly selected, dried at 65 °C during 72 h and stored, protected from light and moisture, at room temperature (25-28 °C) until the preparation of extracts.

## 2.3 Preparation of hydroalcoholic extracts

*Medicago* spp. leaves were milled at particle size of approximately 0.1 mm using an A11 basic analysis mill (IKA Wearke, Staufen, Germany) and stored in plastic tubes at room temperature.

Powdered samples (1 g) were submitted to solvent extraction by maceration with 20 mL of 50% ethanol at 40 °C for 30 minutes. Extracts were filtered through Whatman No. 1 filter paper, concentrated under vacuum at 37 °C and kept under refrigeration (4 °C) prior to use.

## 2.4 Determination of total phenolic content

Total phenolic content (TPC) was determined spectrophotometrically according to the Folin-Ciocalteu procedure [185] with minor modifications [186]. Briefly, 500 µL of extract was mixed with 2.5 mL of Folin–Ciocalteu reagent (10 x dilution) and allowed to react for 5 min. Then 2.5 mL of Na<sub>2</sub>CO<sub>3</sub> 7.5% solution was added and allowed to stand for 15 min at 45 °C and 30 min at room temperature, before the absorbance being determined at 765 nm using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). A calibration curve for the standard gallic acid was used to obtain a correlation between sample absorbance and standard concentration (linearity range = 5-100 µg/mL,  $r^2 > 0.998$ ). The total polyphenol content (TPC) of the extracts was expressed as mg of gallic acid equivalents (GAE) per gram of plant material on dry basis (db).

## 2.5 Determination of total flavonoid content

Total flavonoid content (TFC) was determined by a colorimetric assay based on the formation of flavonoid-aluminum compound according to Barroso *et al.* [187]. Briefly, 1 mL of a diluted extract was mixed with 4 mL of ultrapure water and 300  $\mu\text{L}$  of 5% (w/v)  $\text{NaNO}_2$  solution. After 5 min, 300  $\mu\text{L}$  of  $\text{AlCl}_3$  solution (0.1 g/mL) were spiked, and after 1 min, 2 mL of 1 mol/L NaOH and 2.4 mL of ultrapure water were also added. The absorbance was read at 510 nm using the Synergy HT Microplate Reader. Catechin was used as reference to plot the standard curve (linearity range = 0-400  $\mu\text{g}/\text{mL}$ ,  $r^2 > 0.999$ ). Total flavonoid concentration (TFC) was expressed as milligrams of catechin equivalents (CAE) per gram of plant material on db.

## 2.6 *In vitro* antioxidant activity

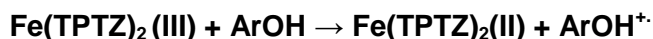
The antioxidant activity of the samples was evaluated by DPPH<sup>\*</sup> radical-scavenging activity and ferric reducing antioxidant power (FRAP) as detailed in the following.

### 2.6.1 DPPH free radical scavenging assay

DPPH is known as a stable free radical which possesses a characteristic maximum absorption between 515 and 517 nm. In the presence of antioxidant compounds, the DPPH<sup>\*</sup> is reduced to the corresponding hydrazine when reacts with hydrogen donors, such as an antioxidant, and lower absorbance values of the reactive mixture indicate higher free radical scavenging activity. This method is an essential tool to access the antioxidant potential, more specifically, the antiradical activity of extracts. The reaction mixture on 96 wells plate consisted of a solution by well of the different samples concentrations (30  $\mu\text{L}$ ) and methanol solution (270  $\mu\text{L}$ ) containing DPPH radicals ( $6 \times 10^{-5}$  mol/L). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm [188]. A calibration curve was prepared with Trolox (linearity range: 2.5-100  $\mu\text{g}/\text{mL}$ ,  $r^2 > 0.996$ ). The antioxidant capacity based on the DPPH free radical scavenging ability of the extract was expressed as  $\mu\text{mol}$  Trolox equivalents per gram of plant material on db.

## 2.6.2 Ferric reducing antioxidant power (FRAP) assay

The relevant chemical reaction of the FRAP method involves a single electron reaction between Fe (TPTZ)<sub>2</sub> (III) and a single electron donor ArOH:



FRAP assay was carried out by the method of Benzie and Strain [189] with minor modification. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe<sup>3+</sup>-TPTZ) to the ferrous form (Fe<sup>2+</sup>-TPTZ). An aliquot (90 µL) of an extract (with appropriate dilution, if necessary) was added to 2,7 mL of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM TPTZ solution and 1 part of 20 mM FeCl<sub>3</sub> · 6H<sub>2</sub>O solution) and the reaction mixture was incubated at 37 °C. The increase in absorbance at 592 nm was measured after 30 min. Solutions of known Fe (II) concentrations (FeSO<sub>4</sub>·7H<sub>2</sub>O) were used for calibration. A calibration curve was prepared with ferrous sulphate (linearity range: 150-2000 µM, r<sup>2</sup>>0.996).

## 2.7 Antimicrobial assay

### 2.7.1 Microorganisms strains

Eight microorganisms (seven bacteria and one yeast) were included in the study. Both American Type Culture Collection (ATCC) and clinical isolates were selected, corresponding to *Staphylococcus aureus* (ATCC 6538 and a clinical isolate), *S. epidermidis* (clinical isolate), *Escherichia coli* (ATCC 1576 and a clinical isolate), *Klebsiella pneumoniae* (ATCC 4352), *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 10231). Clinical isolates were identified to species-level with Vitek-2 identification cards (Biomérieux, France) and all strains were kept frozen in Brain Heart Infusion (Difco Laboratories, USA) with 20% glycerol (Sigma) at -70° C until testing. For each experiment, microorganisms were subcultured twice in Nutrient agar (Difco Laboratories, USA) for bacteria and Sabouraud dextrose agar (SDA; Difco Laboratories, USA) for *C. albicans*, in order to access culture viability.

### 2.7.2 MIC by broth dilution assay

The antibacterial activity was tested according to the Clinical and Laboratory Standards Institute (CLSI) M7-A6 micromethod and the antifungal activity was tested

according to M27-A3 micromethod from the same protocol, after 48 h of incubation at 37 °C [190-191]. Briefly, two-fold serial dilutions of extracts were performed in Mueller Hinton broth (MH; Difco Laboratories, USA) for bacteria and in RPMI (Biochrom, Germany) for yeast. Concentrations ranging from 3.90 µg/mL to 500 µg/mL were tested. Microorganism's growth was visually compared for each concentration with the growth control (without plant extract). Minimal inhibitory concentration (MIC) was defined as the lowest extract concentration able to completely inhibit microorganism growth, corresponding to 100% MIC value. All determinations were performed in duplicate for each assay and three independent experiments were run with concordant results.

## **2.8 Keratinocytes and fibroblasts cytotoxicity assay**

To ensure the quality of extracts, it is very important to assess their biological activity and cytotoxic potential. Any study has been done regarding different extracts of *Medicago*.

Two different assays were used to assess cell viability and cytotoxicity of the extracts: (1) monitoring the uptake and metabolism of the vital mitochondrial dye, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) by cell mitochondria and (2) determining the leakage of the cytosolic enzyme, lactate dehydrogenase (LDH) into the cell medium (LDH assay). In both tests, triplicate wells were incubated with fresh medium in the absence or presence of extracts dissolved in cell culture medium containing 0.1, 1, 10, 100 and 1000 µg/mL of extracts. Extracts dissolved in culture medium were filtered on 0.45 µm Millex GV filters (Millipore, Nepean, ON, Canada) and exposed to keratinocytes (HaCaT) and fibroblasts (HFF-1) cells during 24 h.

### **2.8.1 Cell Lines and Culture Conditions**

Human immortalized non-tumorigenic keratinocyte cell line HaCaT (Ethnicity, Caucasian; Age, 62 years; gender, Male and tissue, skin) was acquired from CLS Cell Lines Service, Germany. Human foreskin fibroblasts (HFF-1) were purchased from ATCC (ATCC Number: SCRC-1041; ATCC, Manassas, VA, USA). Passage 17-19 of HFF-1 and passage 77-79 of HaCaT cells were used for both MTS and LDH assay. HaCaT and HFF-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™-I (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B, and maintained in a 5% CO<sub>2</sub>

environment at 37 °C. At 90-95% confluence, both cell lines were harvested using trypsin (Invitrogen), viability assessed using the trypan blue dye (Gibco) exclusion assay, and replated in T75 flasks.

### 2.8.2 MTS assay

The MTS assay was performed according to the manufacturer instructions. Briefly, cells were cultured in 96-well microtiter plate at a density of  $25 \times 10^3$  cells per mL culture medium for 24 h. The cells were incubated with 0.1 µg/mL - 1 mg/mL plant extract for 24 h at 37 °C. Following the removal of extracts from the wells, the cells were washed with phosphate buffered saline (PBS pH 7.4). Then the medium was changed to fresh medium containing different concentrations of *Medicago* spp. extracts. The cells were then incubated at 37 °C for 24 h. Then medium was rejected and the number of viable cells evaluated by adding, the MTS reagent in medium to each well and incubating for 4 h at 37 °C. Control was determined incubating cells with culture medium. The absorbance was measured at 490 nm with background subtraction at 630 nm. Each concentration was tested in triplicate in three independent experiments.

### 2.8.3 LDH assay

Lactate dehydrogenase (LDH), a stable cytoplasmic enzyme found in all cells, is quickly released upon damage to the plasma membrane. This assay can detect as few as 100 cells, and strongly correlates with the number of lysed cells. Briefly, cell lines were seeded in 96-well plates (5,000 cells for LDH) and cultured for 48 h. Serial dilutions of the different extracts were prepared in appropriate medium and added to the cells. Media without FBS were used in the case of LDH. After incubation (37 °C/ 5% CO<sub>2</sub>), cells were washed twice with PBS and the kit was used according to the instructions of the manufacturers. The maximum LDH release (high control) from cells was determined using 1% (w/v) Triton X-100 (Boehringer Mannheim). Spontaneous LDH release (low control) was determined by incubating the cells with plain medium. Absorbance was measured at 490 nm with background subtraction at 690. Each concentration was tested in triplicate in three independent experiments.

## 2.9 Statistical analysis of data

Data were reported as mean  $\pm$  standard deviation of at least triplicate experiments. Statistical analysis of the results was performed with SPSS 19.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to investigate the differences between different species for all assays. Post hoc comparisons of the means were performed according to Tukey's HSD test. In all cases,  $p < 0.05$  was accepted as denoting significance.

## 3. Results and Discussion

### 3.1 Determination of total phenolic content

The TPC of the plant extracts was determined using the Folin-Ciocalteu phenol reagent. It is a simple and widespread method, although it presents some limitations as there are some interfering substances, such as sugars, aromatic amines, sulphur dioxide and ascorbic acid [192]. Results are presented in Figure 4.1. The TPC of the different extracts varied from 21.96 mg to 36.41 mg GAE per g db sample, being the highest TPC obtained for *M. minima* and the lowest for the *M. scutelata*. The order of TPC of different extracts was: *M. scutelata* < *M. sativa* < *M. segitalis* < *M. truncatula* < *M. tornata* < *M. rigidula* < *M. minima*.

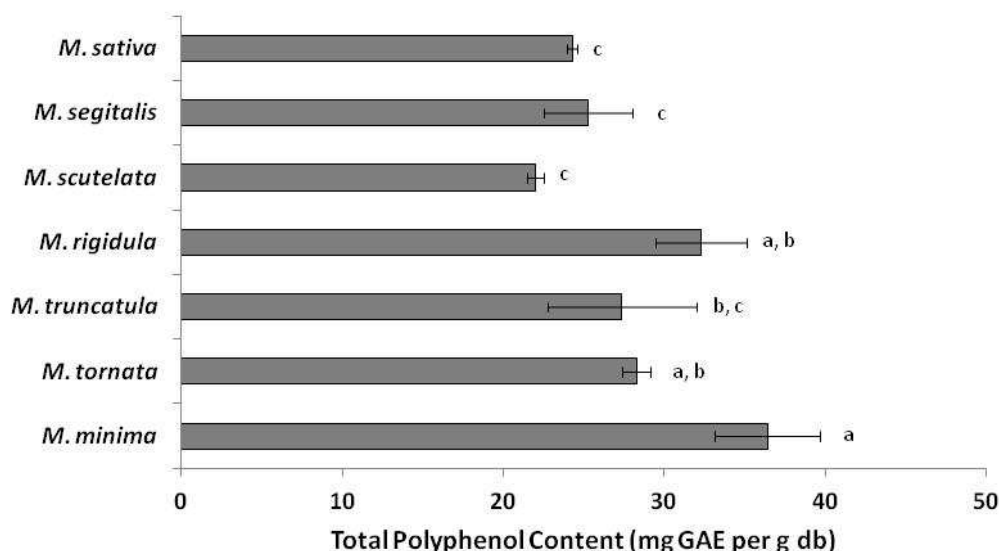


Figure 4.1 - Total polyphenol content in seven hydroalcoholic *Medicago* spp. extracts. Values are expressed as means  $\pm$  SD (n = 6). GAE, gallic acid equivalents. Different letters (a, b, c) indicate significant differences between mean values ( $p < 0.05$ ).

The Folin-Ciocalteu phenol reagent is used to obtain a crude estimate of the amount of phenolic compounds present in an extract. Generally, extracts that contain a high amount of polyphenols also exhibit high antioxidant activity [193]. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the reagent. However, the assay has been shown to non-specific to polyphenols [194-195], and other substances could be oxidized by the Folin reagent. The higher phenol content of the extracts might account for the better results in their reducing power and radical scavenging effect on DPPH radicals. As expected *M. minima* high TPC values will correspond to a high antioxidant activity of this *Medicago* species.

Comparing with different extracts that has been study for cosmetic application like *Castanea sativa*, *Quercus* sp or *Trifolium* sp (this from the same family of *Medicago* - Leguminosae), the importance of *Medicago* extract can be emphasized. In addition, these botanical extracts are considered by Committee on Herbal Medicinal Products of European Medicines Agency as safety [196-197]. Also, according to different authors, extracts of *C. sativa* can be obtained from fruits or leaves, which are most rich in TPC and antioxidants [198-201]. Comparing with *Medicago* extracts, the content in antioxidant compounds and the antioxidant activity of the nuts showed low TPC and discrete antioxidant activity [202]. The TPC ranges from 9.6 to 19.4 mg/g db of the fruit, being considerably lower than *Medicago* extracts [201-202]. Previous studies have shown that total phenolics of chestnut fruit range between 11.0 and 13.6 mg/g db [198, 202]. In the same way, *Juglans regia* has also demonstrated their potential as promising antioxidants [203-205]. The TPC of *J. regia* is higher than *Medicago*, ranging from 58.78 to 95.06 mg GAE/g extract [206-209]. *Aesculus hippocastanum*, vulgarly known as horse chestnut, is a native European plant which seeds and bark of young branches have been study to be incorporated in new cosmetic products [205]. Studies carried out with seed extract showed some others beneficial effects such as strong active oxygen-scavenging activity and cell-protective effects *in vitro* linked to anti-aging properties of antioxidants [210]. The extracts of leaves revealed a higher amount of phenolics, 75 mg GAE/g extract [204]. Thus, the potential source of polyphenols of *Medicago* is in the same range of other vegetable sources.

### 3.2 Determination of total flavonoid content

Flavonoids are a group of phenolic compounds widely distributed in the medicinal plants, vegetables, fruit juices and a variety of beverages (tea, coffee, wines and fruit drinks) [211-214]. Flavonoids, and particularly quercetin derivatives, have received a lot of attention as dietary constituents during the last few years. Experimental studies demonstrated that they possess numerous beneficial effects on human health, including cardiovascular protection, anticancer activity, antiulcer effects, and antiallergic, antiviral, and anti-inflammatory properties [215-216]. As the biological activity of flavonoids is intimately related to their mixtures and the amount of individual flavonoids in fruits and plants is usually low, they have often been recorded unspecifically as ‘total phenolics’. TFC was measured using the aluminium chloride colorimetric method.

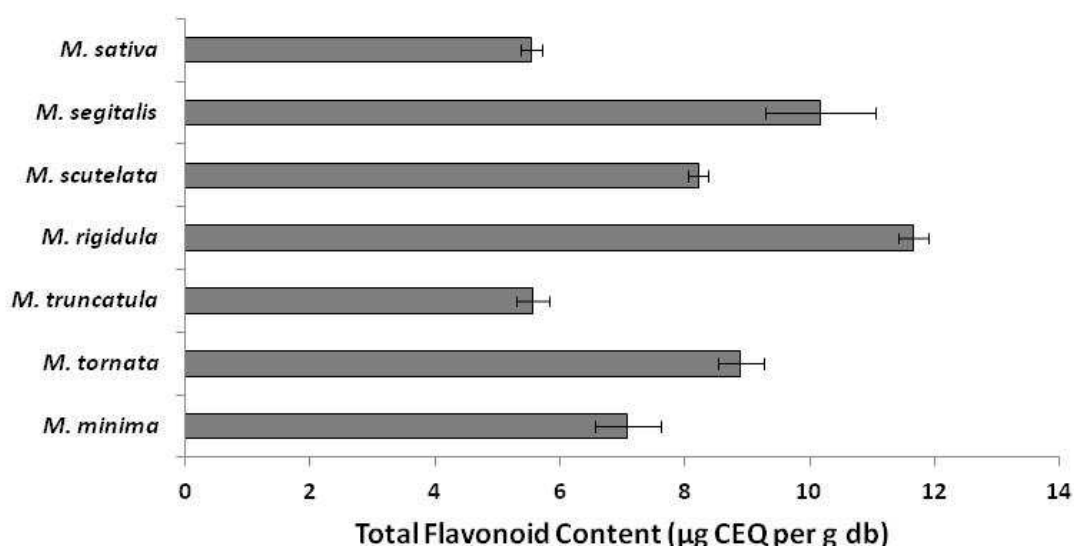


Figure 4.2 - Total flavonoid content in seven hydroalcoholic *Medicago* spp. extracts. Values are expressed as means  $\pm$  SD (n = 6). CAE, catequin equivalents. Different letters (a, b, c) indicate significant differences between mean values (p<0.05).

The results depicted in Figure 4.2 show that TFC varied from 5.54 to 11.67 µg in terms of CAE per g extract db. The order of TFC was: *M. sativa* < *M. truncatula* < *M. minima* < *M. scutelata* < *M. tornata* < *M. segitalis* < *M. rigidula*. Figure 4.2 also show that extracts can be grouped according to its TFC contents (p<0.05). This is the case of *M. rigidula*, the richest one, followed by *M. segitalis*, *M. tornata* and *M. scutelata*. *M. minima*, *M. truncatula* and *M. sativa* presented the lowest levels. Even though it is apparent that



the flavonoids are an important phenolic compounds group, contributing to the antioxidant activity, other compounds with similar chemical behaviour can be included.

Comparing the TFC of *Medicago* with other vegetable extracts, it was observed to be in the same concentration range. For *C. sativa*, the values ranging from 26.6 to 67.2 mg CAE/g db [201] and for *J. regia* extracts from 61 to 124 mg CAE/100 g [206], although differences in the extraction procedure was observed, being difficult to clearly identify TFC differences.

### 3.3 *In vitro* antioxidant activity

Antioxidant activity of the extracts is related to the compounds ability to protect a biological system against the potential harmful effect of oxidative processes. Antioxidants are essential to preserve the biological system from free radicals damage to biological molecules [217]. In this study, the antioxidant activity of the different *Medicago* spp. extracts was evaluated by scavenging activity on DPPH and the reducing power.

#### 3.3.1 DPPH free radical scavenging assay

The stable radical DPPH has been widely used for the determination of primary antioxidant activity, that is, the free radical scavenging activities of pure antioxidant compounds, plant and fruit extracts and food materials [193]. The assay is based on the reduction of DPPH radicals in methanol which causes an absorbance drop at 517 nm. In this study, the antioxidant activity was expressed as Trolox equivalents per gram of plant material on a db as it is a more meaningful and descriptive expression than assays that express antioxidant activity as the percentage decrease in absorbance. As such, the results provide a direct comparison of the antioxidant activity with Trolox. The DPPH free radical scavenging activity of 7 different hydroalcoholic plant extracts of *Medicago* spp. is shown in Figure 4.3. *M. rigidula* showed the lowest DPPH free radical scavenging activity ( $127.18 \pm 1.96$   $\mu\text{mol}$  Trolox equivalent per g db) while *M. segitalis* had the highest activity ( $214.49 \pm 6.69$   $\mu\text{mol}$  Trolox equivalent per g db) but with similar values to those of *M. tornata*. Other group of extracts (*M. sativa*, *M. scutelata* and *M. truncatula*) showed relatively high antioxidant activity. The order of antioxidant activity according to DPPH assay was *M. rigidula* < *M. minima* < *M. sativa* < *M. truncatula* < *M. scutelata* < *M. tornata* < *M. segitalis*.

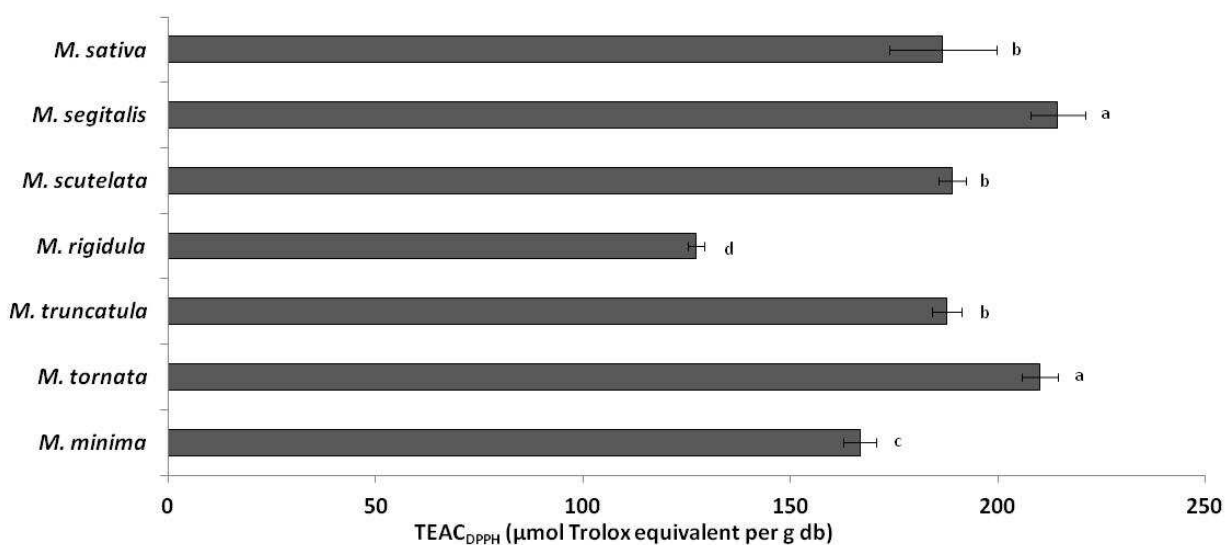


Figure 4.3 - Antioxidant activities of seven hydroalcoholic extracts of *Medicago* spp. species based on their abilities to scavenge DPPH free radicals. Values are expressed as means  $\pm$  SD ( $n = 6$ ). Different letters (a, b, c) indicate significant differences between mean values ( $p < 0.05$ ).

The high total phenolic content associated with a high antioxidant activity is a good evidence of hydroalcoholic extracts of *Medicago* spp. Nevertheless, it is clear that some species have values due to the presence of compounds not quantified in this study [218], not quantified as polyphenolic compounds. One possibility can be isoflavones, which will further increase the chances of use of this extracts in skin care products [218].

### 3.3.2 Ferric reducing antioxidant power (FRAP) assay

The ability of the plant extracts to reduce ferric ions was determined using the FRAP assay [219]. The ferric reducing antioxidant power (FRAP) assay measures the reduction of ferric ( $\text{Fe}^{3+}$ ) to ferrous iron ( $\text{Fe}^{2+}$ ) in the presence of antioxidants, which are reductants with half-reaction reduction potentials above  $\text{Fe}^{3+}/\text{Fe}^{2+}$ .

The antioxidant activity averages of the different extracts based on FRAP assay are given in Figure 4.4. *M. segitalis* showed the highest level of antioxidant activity based on FRAP assay ( $120.84 \pm 1.86 \mu\text{mol per mg db}$ ) and *M. minima* showed the lowest one ( $58.05 \pm 6.09 \mu\text{mol per mg db}$ ). The order of antioxidant activity based on FRAP assay was: *M. minima* < *M. tornata* < *M. scutelata* < *M. sativa* < *M. rigidula* < *M. truncatula* < *M. segitalis*. The analysis of variance (ANOVA) showed differences between the different

extracts ( $p < 0.05$ ), indicating different behaviours and consequently compositional differences presented by the evaluated extracts.

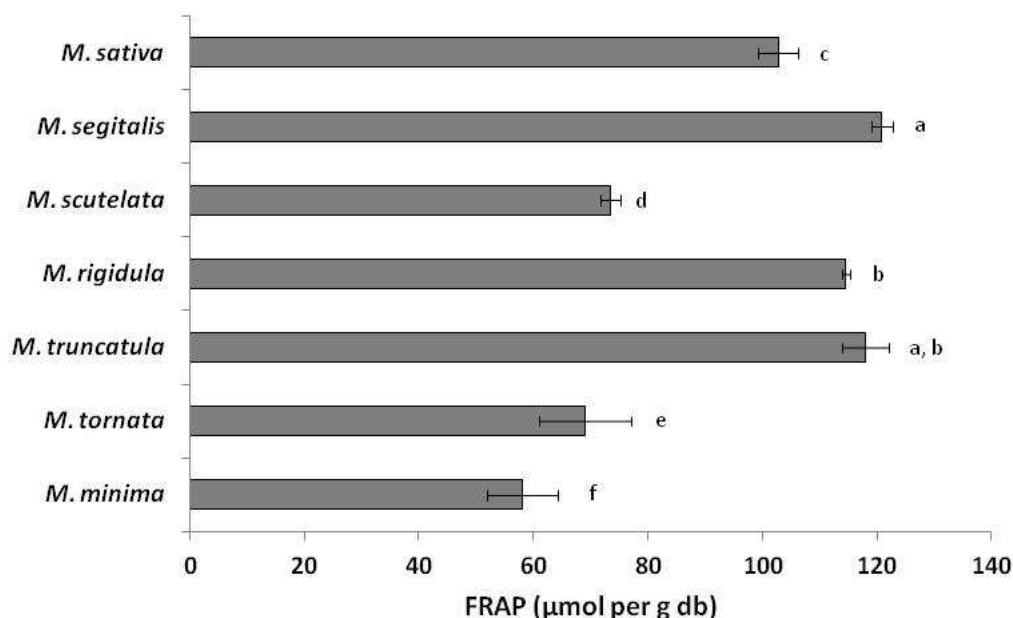


Figure 4.4 - Antioxidant activities of seven hydroalcoholic extracts of *Medicago* spp. species based on their abilities to reduce ferric iron ( $Fe^{3+}$ ) to ferrous iron ( $Fe^{2+}$ ) ( $n = 6$ , error bars represent SD). Different letters (a, b, c) indicate significant differences between mean values ( $p < 0.05$ ).

The two assays of antioxidant capacity used in this study were spectrophotometry-based methods. The differences in the antioxidant activity measurements between assays could be expected, as each assay has a different mechanism of action or different reaction conditions. DPPH is a method based on the measurement of the reducing ability of antioxidants toward 1,1-diphenyl-2-picrylhydrazyl ( $DPPH^{\bullet}$ ). The ability can be evaluated by electron spin resonance or by measuring the decrease of its absorbance. Although both  $DPPH^{\bullet}$  and FRAP have been widely used to measure the antioxidant capacity of natural extracts based on their ability to reduce the radical cation, the reaction of DPPH with free radical scavengers present in the test sample occur rapidly and can be assessed by following the decrease in the absorbance at 517 nm. The reaction time of the improved DPPH assay is only 30 min, while the FRAP assay measures the reducing capability by increased sample absorbance based on the ferrous ions released, being thus more time consuming. On the other hand, the DPPH assay is preferred to the ABTS assay because it is commercially available and does not have to be generated before assay like  $ABTS^{\bullet+}$  [192]. The ABTS radical cation is generated by the oxidation of ABTS with potassium

persulfate, and its reduction in the presence of hydrogen-donating antioxidants is measured spectrophotometrically at 734 nm [220]. According to Prior *et al.* [192], the assay may not be complete even for several hours after the reaction started, such that a single end-point of the reaction cannot be determined. On the other hand, FRAP assay has some drawbacks, such as interference, reaction kinetics and quantification methods [221]. Various factors such as variety, growing condition, maturity, season, fertilizer, soil type, storage conditions and amount of sunlight received, among others, might be responsible for the observed differences. Thus, DPPH results are more feasible and probably better reflect the real antioxidant activity of the extracts.

### 3.4 Correlation between phenolic composition and antioxidant activity

Polyphenols have been reported as responsible for the antioxidant activity of herbal extracts. This activity may be related to several properties like redox properties, which allow them to act as reducing agents or hydrogen-atom donors. Also, the ability to chelate metals inhibits lipoxygenase and scavenges free radicals. Both DPPH free radical scavenging and FRAP assays have been used to measure antioxidant activity and the results of DPPH and FRAP assays should correlate with those of TPC. However, the correlation between the phenolic compounds and antioxidant potential is not consensual. Some authors showed the existence of significant correlations between the phenolic compounds and antioxidant potential [222-225]. Nevertheless, in other studies no correlation was observed [226].

When a regression analysis was performed between the values of  $\mu\text{mol Trolox}$  equivalents per g db sample or  $\mu\text{mol FRAP}$  per mg db in the antioxidant evaluation and the total phenolic content, an extremely negative correlation ( $r^2 = -0.5827$ ;  $p < 0.01$ ) and a negative correlation ( $r^2 = -0.2218$ ;  $p < 0.001$ ) were established, respectively. Thus, in this work, no correlations between phenolics content and antioxidant capacity were established. Nevertheless, the interpretation of results needs to be done with precaution, as the final effects of a complex extract results from the combinatory and synergic actions of all its constituents. Other compounds, belonging to other chemical classes, may also contribute to the observed effects. Further work is in progress in our laboratory to elucidate the identity of compounds responsible for the antioxidant activity.

### 3.5 Correlation between flavonoids and antioxidant activity

When a regression analysis was performed between the values mg CAE equivalents per g db sample and the total phenolic content, a weak positive correlation ( $r^2=0,1718$ ;  $p<0.01$ ) was established. As demonstrated by other authors, it is not mandatory a strong positive correlations between these two contents [226]. In this way, further work is necessary to elucidate about the flavonoid compounds.

### 3.6 Antimicrobial assay

The minimum inhibitory concentrations (MICs) for the active plant extracts were determined using modified methods of the CLSI as mentioned above. In this study, microorganisms were selected to cover Gram-positive bacteria (*S. aureus* and *S. epidermidis*), Gram-negative bacteria (*E. coli*, *K. pneumoniae*, *P. aeruginosa*) and yeasts (*Candida albicans*). According to different authors, the 'normal' resident skin flora includes *S. epidermis*, *S. aureus* and *P. aeruginosa* [227-228]. *Staphylococcus* is one of the most representative bacterial genera present in human skin and *S. epidermidis* is the most prevalent specie [227, 229-230]. *S. aureus* is usually thought of as a pathogen, but the methicillin-resistant strain is increasingly found colonizing moist skin sites of healthy people, being very important the evaluation of extracts effectiveness on this specie [230]. On the skin, *P. aeruginosa* occasionally causes dermatitis or deeper soft-tissue infections [231]. *E. Coli* and *K. pneumonia* are two important species in nosocomial infections [232-233]. *Candida* species are the most common fungal pathogens of humans, being the causative agents of candidiasis, and *C. albicans* is the most pathogenic *Candida* species [234]. According to that, the evaluation of extracts in these species can be considered of high importance.

The antibacterial activity of the seven hydroalcoholic extracts was evaluated by the determination of MIC values (Table 4.1).

Table 4.1 - Antibacterial activity of hydroalcoholic extracts of *Medicago* species expressed as MIC in µg/mL.

Bacterial Strain	MIC (µg/mL)*						
	<i>M. minima</i>	<i>M. tornata</i>	<i>M. truncatula</i>	<i>M. rigidula</i>	<i>M. scutelata</i>	<i>M. segitalis</i>	<i>M. sativa</i>
<i>S. aureus</i> ATCC 6538	31.3	62.5	31.3	31.3	31.3	62.5	31.3
<i>S. aureus</i> MRSA	250	500	250	500	250	500	250
<i>S. epidermidis</i> B21	31.3	125	62.5	31.3	125	62.5	62.5
<i>E. coli</i> ATCC 1576	125	250	125	125	125	250	250
<i>E. coli</i> B26	250	250	125	125	125	250	125
<i>K. pneumoniae</i> ATCC 4352	31.3	125	31.3	31.3	31.3	62.5	62.5
<i>P. aeruginosa</i> ATCC 9027	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000
<i>C. albicans</i> ATCC 10231	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000	> 1000

\* Data expressed as mean of three data points (n=3)

Hydroalcoholic extracts of the different *Medicago* species presented MICs in the range of 31.3 µg/mL-500 µg/mL against *S. aureus*, *S. epidermidis*, *E. coli* and *K. pneumoniae*. All plant extracts were active against the tested microorganisms, except for *P. aeruginosa* and *C. albicans*. The inefficacy of these extracts against these microorganisms can be related with the presence of *P. aeruginosa* extracellular matrix and the complex yeast cell wall, preventing extracts to contact with cell structures. *S. aureus* and *K. pneumoniae* from ATCC were the most susceptible strains to all the extracts exhibiting MIC values between 31.3 and 125 µg/mL. Interestingly, *S. aureus* clinical isolate was notoriously more resistant to these natural products than the collection strains, and the same tendency was observed for *E. coli* clinical isolate compared to the ATCC strain for some extracts.

These results are of great importance, particularly in the case of *S. aureus*, which are well-known for being resistant towards some antibiotics and for their production of several enterotoxins that cause many multiresistant skin infections [235-236]. According to Fabry *et al.* [237] for crude solvent extracts of plants to be considered as potentially useful therapeutically, they should have MIC values < 8mg/mL, whilst Gibbons [238] suggests that isolated phytochemicals should have MIC < 1mg/mL. The MIC values determined for these extracts were bellow these values, thus indicating a potential antimicrobial effect which may be advantageous for cosmetic products. In particular, these extracts may provide self-preservative properties to the formulations in which they are incorporated. The antibacterial activity of all extracts from *Medicago* spp. could be related to the presence of high amounts of phenolic and flavonoid compounds in their composition, as referred above.

### **3.7 Keratinocytes and fibroblasts cytotoxicity assay**

The effects of the extracts on the growth of keratinocyte (HaCaT) and fibroblast (HFF-1) cells were investigated by MTS and LDH assays. These cell lines are regarded as suitable *in vitro* models for testing the toxicity potential of substances or products intended for dermatological use [239-243].

### 3.7.1 MTS assay

*Medicago* spp. extracts were added to HaCaT and HFF-1 cells to analyse their effects on cell viability (Figures 4.5 and 4.6). All experiments are carried out up to 1000 µg/mL of extract.

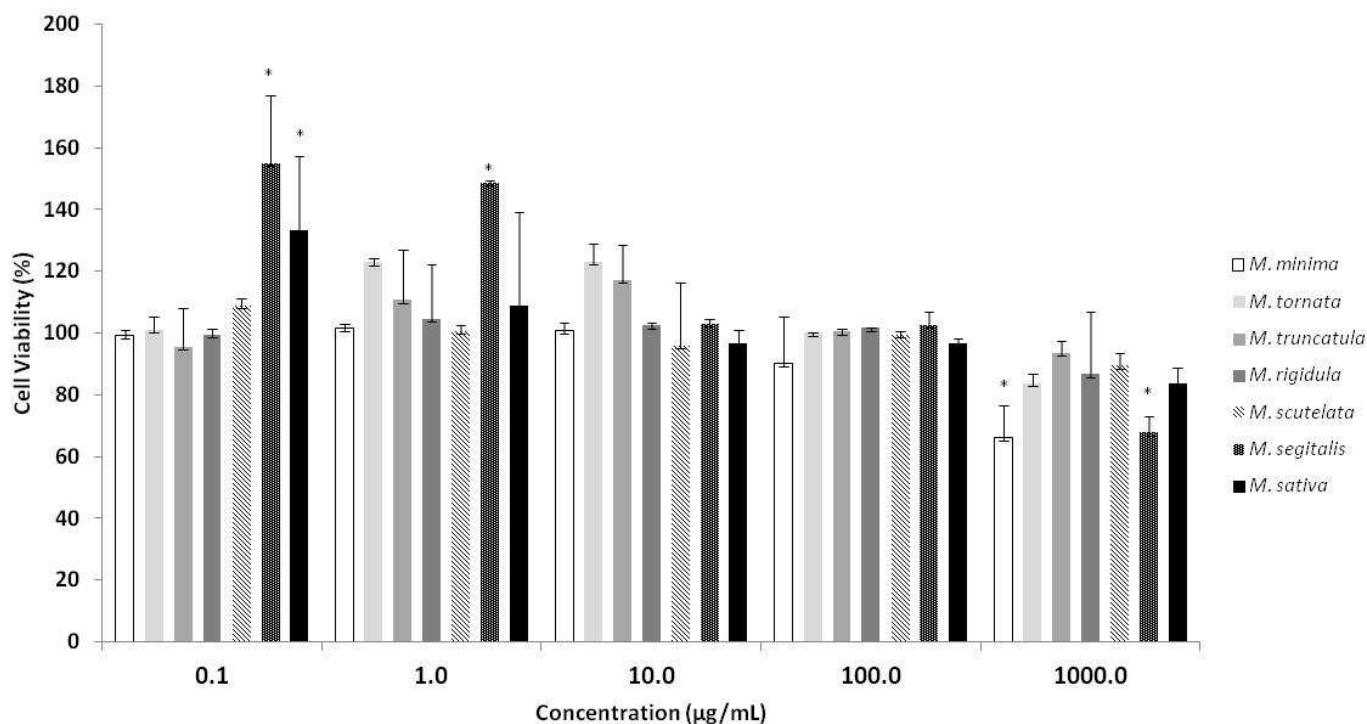


Figure 4.5 - Effect of hydroalcoholic extracts of *Medicago* spp. exposure on the viability of HaCaT cells at different concentrations, as measured by the MTS assay. Values are expressed as means  $\pm$  SD (n=6). \* $p < 0.05$  versus positive control (cells exposed to medium).

According to the obtained results, none of the extracts exhibited cytotoxicity against HaCaT cells (Figure 4.5) at concentrations up to 100 µg/mL. At concentration of 1000 µg/mL there is a decrease in cell viability of keratinocytes, particularly with *M. minima* (66.2%) and *M. segitatis* (67.9%) extracts. Between this two species there is no significant statistic difference. The evidence of cytotoxicity of the extracts at doses above 100 µg/mL implies that caution must be taken when using extracts of these plants, since viability of cells may be affected at higher concentrations. These data allowed selecting the optimal range of non-cytotoxic concentrations of each plant extract (up to 100 µg/mL), used in further experiments. However, no statistical differences were observed between the other extracts ( $p < 0.05$ ).



Figure 4.6 presents the toxicity data of the seven hydroalcoholic extracts assessed in HFF-1 cells. The number of viable fibroblast cells was similar at all concentration of *Medicago* spp. extract (0.1, 1.0, 10, 100, 1000  $\mu\text{g/mL}$ ), except for *M. minima*, *M. tornata* and *M. scutelata* extracts, with considerably inferior viability for all concentrations tested. The values ranged from 66.1% for *M. minima* (1000  $\mu\text{g/mL}$ ) to 79.7% for *M. scutelata* (1000  $\mu\text{g/mL}$ ). These results also indicate that fibroblasts are more sensitive to all extracts as compared to HaCaT cells.

These results are in accordance with those obtained by different authors, who observed that keratinocytes were more resistant than fibroblasts when exposed to different substances [172, 240, 244-246].

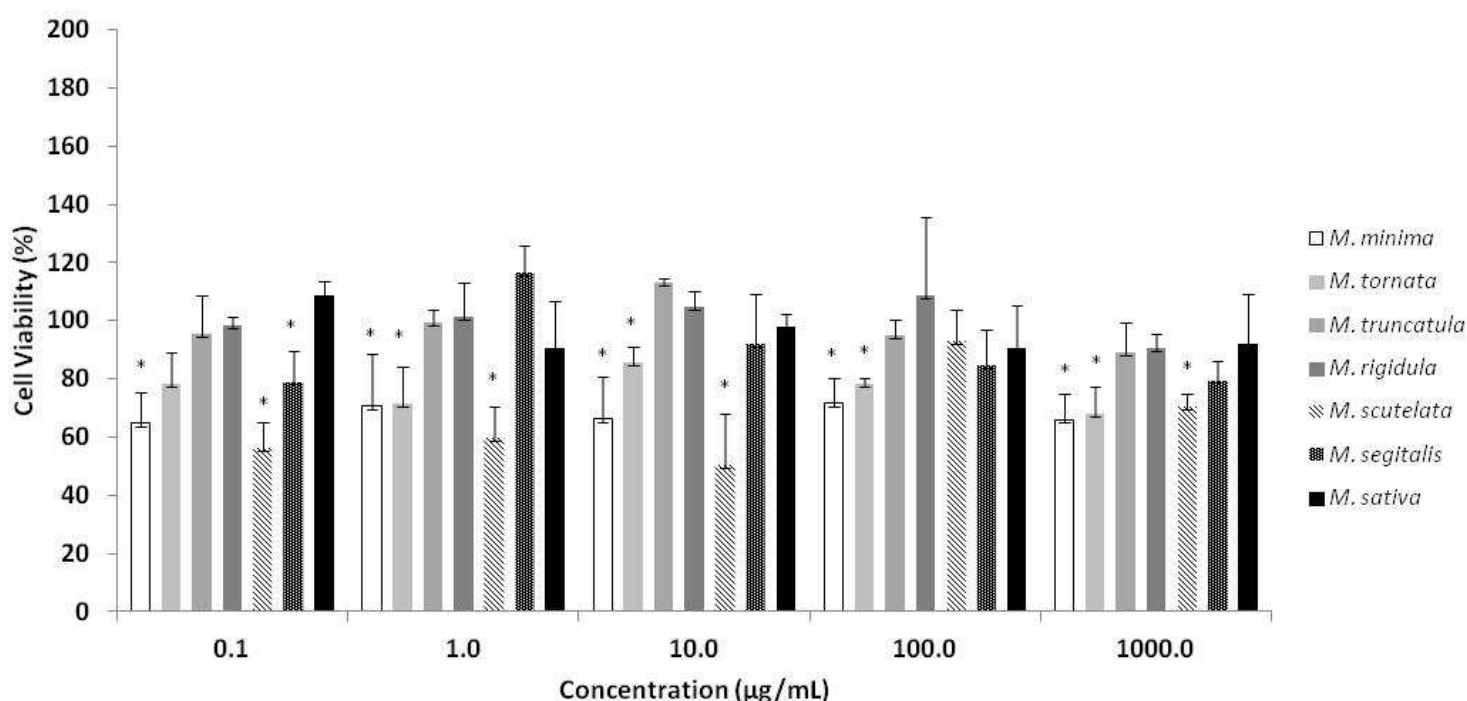


Figure 4.6 - Effect of exposure to different extracts of *Medicago* on the metabolic activity of HFF-1 cells at different concentrations, as measured by the MTS assay. Values are expressed as means  $\pm$  SD (n=6). \* $p < 0.05$  versus positive control (cells exposed to medium).

Furthermore, these results suggested that hydroalcoholic extract of *M. truncatula*, *M. rigidula*, *M. segitalis* and *M. sativa*, at concentrations  $< 1000 \mu\text{g/mL}$  have no adverse effect on the viability of fibroblast cells *in vitro*. These results could indicate a probable protective effect of the extracts on the cell line, but further studies are needed to confirm that.

Cell viability was higher for the HaCaT cells when exposed to extracts in comparison to fibroblasts, at concentrations from 0.1 to 1000 µg/ml. Therefore, in light of these viability values, and having no cytotoxic effects at these higher concentration ranges, we could assume no risk of application of this extracts in skin cells.

### 3.7.2 LDH assay

An important indicator of cell damage in oxidative stress is the increase in membrane permeability. In this study, potential cytotoxic effects of different extracts of *Medicago* spp. were investigated by examining membrane integrity of HaCaT and HFF-1 cells using a LDH release assay.

In HaCaT cells (Figure 4.7), at all concentrations tested, *M. rigidula*, *M. scutelata*, *M. segitalis* and *M. sativa*, did not originate any effects on the LDH leakage following 24 hours of incubation, which means that all these extracts are potentially safe to HaCaT cells. The evidence of possible absence of cytotoxicity of this four hydroalcoholic extracts at five different concentrations, namely, 0.1, 1.0, 10, 100 and 1000 µg/mL, implies that these extracts present low toxicity to these cells. However, *M. minima*, *M. tornata* and *M. truncatula* extracts increased cellular LDH efflux for all concentrations tested.

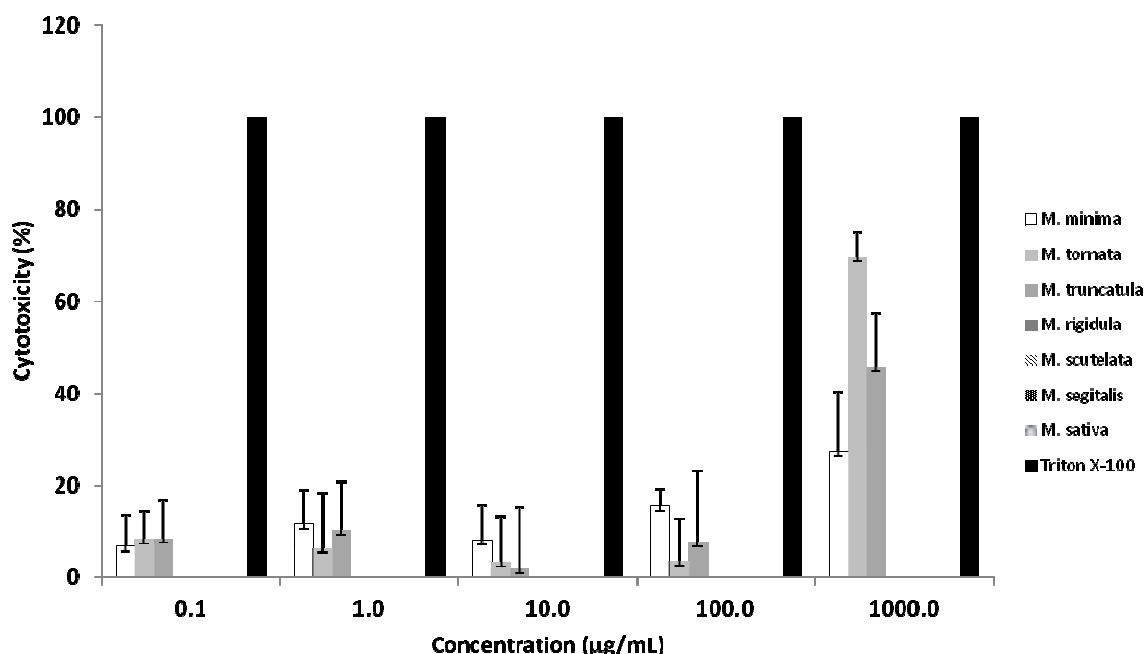


Figure 4.7 - Cytotoxicity (%) of LDH in HaCaT cells after 24 h treatment with different seven hydroalcoholic *Medicago* spp. extracts. The cytotoxicity of LDH was determined using fluorescence with an excitation wavelength of 490 nm and an emission wavelength of 690 nm. Values are expressed as means ± SD (n=6).

Test on cytotoxicity of the extract on fibroblasts was conducted at 0.1, 1.0, 10, 100 and 1000 µg/mL by quantification of LDH-release. No significant LDH-increase was observed, indicating the absence of cytotoxicity in this dose range on human fibroblasts for all extracts tested.

Using these assays, we found that the seven hydroalcoholic extracts presented low toxicity potential towards HaCaT, with the exception of those prepared from *M. minima*, *M. tornata* and *M. truncatula* at the higher concentration tested, and any cytotoxicity for fibroblast cells.

*Quercus* leaves extracts are also being study as a new source of antioxidants as promising active ingredients for cosmetics [247-248]. Different authors had reported the radical scavenging activity of lyophilised extracts of different species of *Quercus* leaves and their effect on proliferation and survival of different cells [248-250]. *Glycyrrhiza uralensis* is another specie of the same family of *Medicago* (Leguminosae) which antioxidant properties have been study [251]. The study of cytotoxicity showed that the extract is safe and did not show cytotoxicity in the range of 4 - 100 µg/mL, which is in agreement with the present results [251]. Carvalho *et al.* studied the antiproliferative effectiveness of *J. regia* extract using human renal cancer cell lines A-498 and 769-P and the colon cancer cell line Caco-2, and proved a strong antiproliferative capacity of walnut, showing cytotoxicity for these cells [207]. Park *et al.* proved that *J. mandshurica* leaf extract protects skin fibroblasts from damage by regulating the oxidative defense system, being safety used in cosmetic preparations [252]. Darmani *et al.* studied the effect of aqueous extracts of *J. regia* and concluded that they enhance the growth of fibroblasts and inhibit the growth of cariogenic bacteria [253]. The results of cytotoxicity of *A. hippocastanum* indicated that the extract was not able to generate lethal DNA lesions [204-205], being expected the same effect for *Medicago* extracts.

#### 4. Conclusion

The use of by-products of fruit and vegetable processing as a source of functional compounds and their application in cosmetics is a promising field which requires interdisciplinary research of cosmetic technologists, chemists and toxicologists. The bioactivity, bioavailability and toxicity of phytochemicals needs to be carefully assessed by *in vitro* and *in vivo* studies [174]. Agro-industrial by-products are good sources of phenolic compounds, and have been explored as a source of natural antioxidants [254].

In this work, *Medicago* spp. residue extracts presented antioxidant and antimicrobial activity, and low cytotoxic potential for skin cells. *Medicago* spp. extracts may be useful for developing skin care products.

The lack of correlation observed between the two *in vitro* assays carried out to assess antioxidant activity (DPPH and FRAP) may be due to differences in sensitivity of the tests and characteristics of the extracts. Evaluation of antimicrobial activity reveals that these extracts are effective at concentrations of 500 µg/mL against important strains of skin, particularly *S. aureus*. These results are also good indicators of their possible use in skin products. Last but not least important, the absence of toxicity of the extracts in keratinocytes is indicative of its potential safe use. With regard to fibroblasts, it was found that *M. minima*, *M. scutelata* and *M. tornata* possess some toxicity and, depending of the concentration used, can be unsuitable for skin use. On the other hand, *M. minima*, *M. tornata* and *M. truncatula* extracts increased cellular LDH efflux for all concentrations tested and so, depending on the concentrations used, can be toxic. Indeed, we can say that *M. sativa*, *M. rigidula* and *M. segitalis* extracts can be more safely used in cosmetics.

It is, thus, suggested that *Medicago* spp. extracts possess non-toxic, antimicrobial and antioxidant activities which may render them suitable as potential active ingredients for use in skin cosmetics. Further investigations are now needed to establish the exact mechanism of action and identify the active bio-ingredient(s) of each extract in order to explain their therapeutic efficacy. Future assays will be performed to study the probable protective effect of the extracts in skin-derived cells and also to identify and quantify all compounds.

## II. Evaluation of *Medicago* as New Ingredient for Skin Care Products

The information presented in this chapter section was published in the following publication:

- **Francisca Rodrigues**, Ivone Almeida, Bruno Sarmento, M. Helena Amaral, M. Beatriz P.P. Oliveira, Study of the isoflavone content of different extracts of *Medicago* spp. as potential active ingredient, *Industrial Crops and Products*, 57:110-115, 2014

## 1. Introduction

Legumes are important agricultural and commercial crops consumed in large amounts by both humans and animals. The Leguminosae taxonomic class includes a variety of plants with significant economic value, including soybean, alfalfa, clover, pea, peanut and various beans [25]. Nowadays, an integrated and sustainable exploitation of plant resources is a must as only few amounts are used for actual human consumption. Waste and by-products from vegetal origin are currently rising, driven by both a net increase in plant products consumption and the changing consumer trend towards ready-to-use products. In this way, the importance of exploiting natural compounds from by-products or no added value plants increased in recent years. They are considered to be attractive due to their low cost and easy availability, in large quantity, as raw material. Presently, most are taken advantage as animal feed and plant fertilizers [255]. After specific pre-treatments, by-products might provide valuable natural compounds of enormous interest to pharmaceutical, cosmetic or food industries [256].

The concept of sustainability should also be taking into account and encompasses the type of chemicals and solvents used for the extraction of interesting compounds. For this reason, and baring in mind the question of green chemical, the extraction process should be designed as simple as possible and, simultaneously, considering the effective costs for industries.

Phytoestrogen is a general term used to define a wide variety of compounds that are non-steroidal and are either of plant origin or derived from the *in vivo* metabolism of several plants used as food [37]. Phytoestrogens possess estrogen properties due to their structural similarities to the hormone estradiol, the most common female hormone [38]. Isoflavones are recognized to possess a wide range of human health benefits such as prevention of certain cancers [257-258], cardiovascular diseases [259-260], osteoporosis [261] and alleviation of menopausal symptoms [262]. Because of their structural similarity to  $\beta$ -estradiol, health benefits of isoflavones have been evaluated in age-related and hormone-dependent diseases [44]. These compounds are abundant in the Leguminosae family (Fabaceae) and largely described in soy and red clover [45-46, 48, 263-264]. Soybean has attracted considerable attention worldwide for their health-promoting, disease-preventing effects, mainly attributed to isoflavones [265]. In fact, soybean isoflavones are the most studied and largely used as ingredients of food supplements. Glycitein (4',7-dihydroxy-6-methoxyisoflavone), together with genistein (4',5,7-

trihydroxyisoflavone), daidzein (4',7-dihydroxyisoflavone) and genistin (Genistein 7-O-β-D-glucoside) are the most plentiful and the best characterized compounds of this group and appears in large amounts in soybean [266-267]. Biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) and formononetin (7-hydroxy-4'-methoxyisoflavone) are methylated precursors of genistein and daidzein, respectively, and exist in high amounts in red clover [45, 49].

The genus *Medicago* is part of the botanical family of Leguminosae and includes about 56 different species mainly distributed in Mediterranean climatic conditions areas [25]. Alfalfa (*Medicago sativa*) is the main *Medicago* species widely grown throughout the world, predominantly as a source of high quality forage for livestock, renewable energy production, phytoremediation, and as a source of phytochemicals [26-27]. It is also used as a human food ingredient, consumed as sprouts in salads, in sandwiches or soups [28], as leaf protein concentrates [29] or as food supplements [30]. Despite this use, alfalfa have pharmacological activities, being used in some human health disfunctions, such as anaemia, diabetes, endometriosis, stomach ulcers, osteoporosis, menopausal symptoms, breast and prostate cancers and low bone density [31-32].

It should be also considerate that the main applications of *Medicago* species are grazing food for cattle and other livestock. Like other plants of the same family, it was reported that high levels of isoflavones are desirable on lamb feeding, that gain weight more quickly, as well as in milk production, but, in some cases, reproductive disorders have also been reported in sheep and cattle fed on forages with high isoflavone content [33-34]. Thus, depending on the final objective for cattle, *Medicago* could not be desired for farmers, originating a high amount of by-products.

To the best of our knowledge, there is no published data on the isoflavones profile of any species of *Medicago* spp. Therefore, the aim of this study was to ascertain the influence of the *Medicago* species and the solvent extractor on the extract content of daidzin, genistin, daidzein, glycitein, genistein, formononetin, prunetin and biochanina A. Therefore, the evaluation of these compounds in different extracts of *Medicago* species remains an important challenge to understanding the possible advantages of their application in pharmaceutical and cosmetic industries.

## 2. Materials and methods

### 2.1 Chemicals and Solutions

Standards of biochanin A ( $\geq 97\%$ ), prunetin ( $\geq 98\%$ ), daidzin ( $\geq 95\%$ ), daidzein ( $\geq 98\%$ ), genistin ( $\geq 95\%$ ), glycitein ( $\geq 97\%$ ) and genistein ( $\geq 98\%$ ) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions (1 g/L) were prepared in DMSO and stored at  $-20\text{ }^{\circ}\text{C}$  in amber glass vials. A composite stock standard solution of multiple isoflavones (20 mg/L of biochanin A, puerarin, glycitein, daidzein, daidzin, prunetin, genistein, genistin, formononetin, pseudobaptigenin, pratensein and irilone) was prepared in  $\text{H}_2\text{O}$ : methanol (25:75, v/v). The internal standard (I.S.) 2-methoxyflavone was obtained from Sigma. A working I.S. solution was prepared in methanol at 1 g/L and stored at  $-20\text{ }^{\circ}\text{C}$  in amber glass vials when not in use.

Methanol and formic acid (both of HPLC grade) were from Merck (Darmstadt, Germany), and HPLC water was purified with a “Seradest LFM 20” system (Seral, Ransbach-Baumbach, Germany). The eluents were filtered through  $0.45\text{ }\mu\text{m}$  filters and degassed under reduced pressure. Disposable filter PTFE  $0.45\text{ }\mu\text{m}$  was from Macherey-Nagel (Düren, Germany).

### 2.2 Samples

Leaves of six species of *Medicago* (*M. minima*, *M. tornata*, *M. truncatula*, *M. rigidula*, *M. scutellata* and *M. segitalis*) were sown at the Experimental Field of the University of Porto at the Agrarian Station of Vairão. *M. sativa* was sown and collected in Azurara, Vila do Conde, Portugal. The plant leaves were randomly selected, dried at  $65\text{ }^{\circ}\text{C}$  during 72h and stored, protected from light and moisture, at room temperature ( $25\text{-}28\text{ }^{\circ}\text{C}$ ).

### 2.3 Extracts preparation

The leaves were milled at particle size of approximately 0.1 mm using an A11 basic analysis mill (IKA Wearke, Staufen, Germany) and stored in plastic tubes at room temperature. Samples (1 g) were submitted to solvent extraction by maceration with 20 mL of three different solvents: ethanol, ethanol:water (1:1) and distilled water for 30 minutes at  $40\text{ }^{\circ}\text{C}$ . Extracts were filtered through Whatman No. 1 paper filter and collected. Aqueous extracts were freeze-dried and hydroalcoholic and alcoholic ones were reduced



to residue using a rotary evaporator set to 37 °C. All extracts were stored at 4 °C until analysis. For quantification of isoflavones, the extracts were reconstituted with the original solvent and filtered through a PTFE membrane before HPLC analysis. The yields of each extract was calculated and varied between for 18.74-20.3 % for aqueous extracts, 16.9-18.1 % for hydroalcoholic extracts and 13.8-15.2 % for alcoholic extracts.

#### 2.4 HPLC equipment

The HPLC method used in this study to separate and quantify daidzin, genistin, daidzein, glycitein, genistein, formononetin, prunetin and biochanin A in *Medicago* extracts was based on the work of Visnevschi-Necrasov *et al.* [268].

The chromatographic analysis was performed in a HPLC integrated system (Jasco, Japan) equipped with a PU-2080 quaternary pump and a Jasco AS-950 automatic sampler with a 20 µL loop. Detection was performed with a Jasco model MD-2010 multiwavelength diode-array detector (DAD). Chromatographic separation of compounds was achieved with a Luna 5U C<sub>18</sub> (5 µm, 150 x 4.60 mm) column (Teknokroma, Spain) operating at 40 °C. The eluent was a gradient of 0.1% formic acid (A) and 0.1% formic acid in methanol (B), at a flow rate of 1 mL/min, with a gradient as follows: 0 min 33% B, 7 min 45% B, 15 min 50% B, 25 min 60% B, 30 min 70% B, 35 min 0% B, 37 min 33% B, maintaining these conditions for 10 min and returning to the initial ones within 3 min.

Analytes were monitored at 254 nm, and quantified on the basis of the internal standard method. Chromatographic data were analyzed using the Borwin-PDA Controller Software (JMBS Developments, Le Fontanil, France). Calibration curves were prepared for daidzin, genistin, daidzein, glycitein, genistein, formononetin, prunetin and biochanin A, all at concentrations ranging from 12.5 to 200.0 mg/mL. Each extract and eight solutions of each standard were injected three times. Confirmation of isoflavones identities was performed by comparing retention times and coelution with authentic standards and by UV absorption spectral analysis. Correlation coefficients of calibration curves were >0.99.

## 2.5 Statistical analysis

Data were reported as mean  $\pm$  standard deviation of at least triplicate experiments. Statistical analysis of the results was performed with SPSS 22.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to investigate the differences between different species for all assays. Post hoc comparisons of the means were performed according to Tukey's HSD test. In all cases,  $p < 0.05$  was accepted as denoting significance.

## 3. Results and Discussion

The recycling of by-products derived from various industries is crucial, as many studies have reported that they contain abundant health-beneficial compounds [101, 269]. According to that, the present work studied the effect of *Medicago* species and the solvent used in the obtained profile and content of isoflavones.

**Variability within *Medicago* Species:** *M. sativa* is the most popular species of the genus *Medicago* due to its peculiar characteristics: the plant is used as animal feed and easily available with low economic value. However, many other species complete this genus. Chromatograms of a hydroalcoholic extract of *M. minima* (A) and standards (B) are depicted in Figure 4.8.

The determined amounts of daidzin, genistin, daidzein, glycitein, genistein, formononetin, prunetin and biochanina A, in all species studied, and with the different extraction solvents, are presented in Table 4.2.

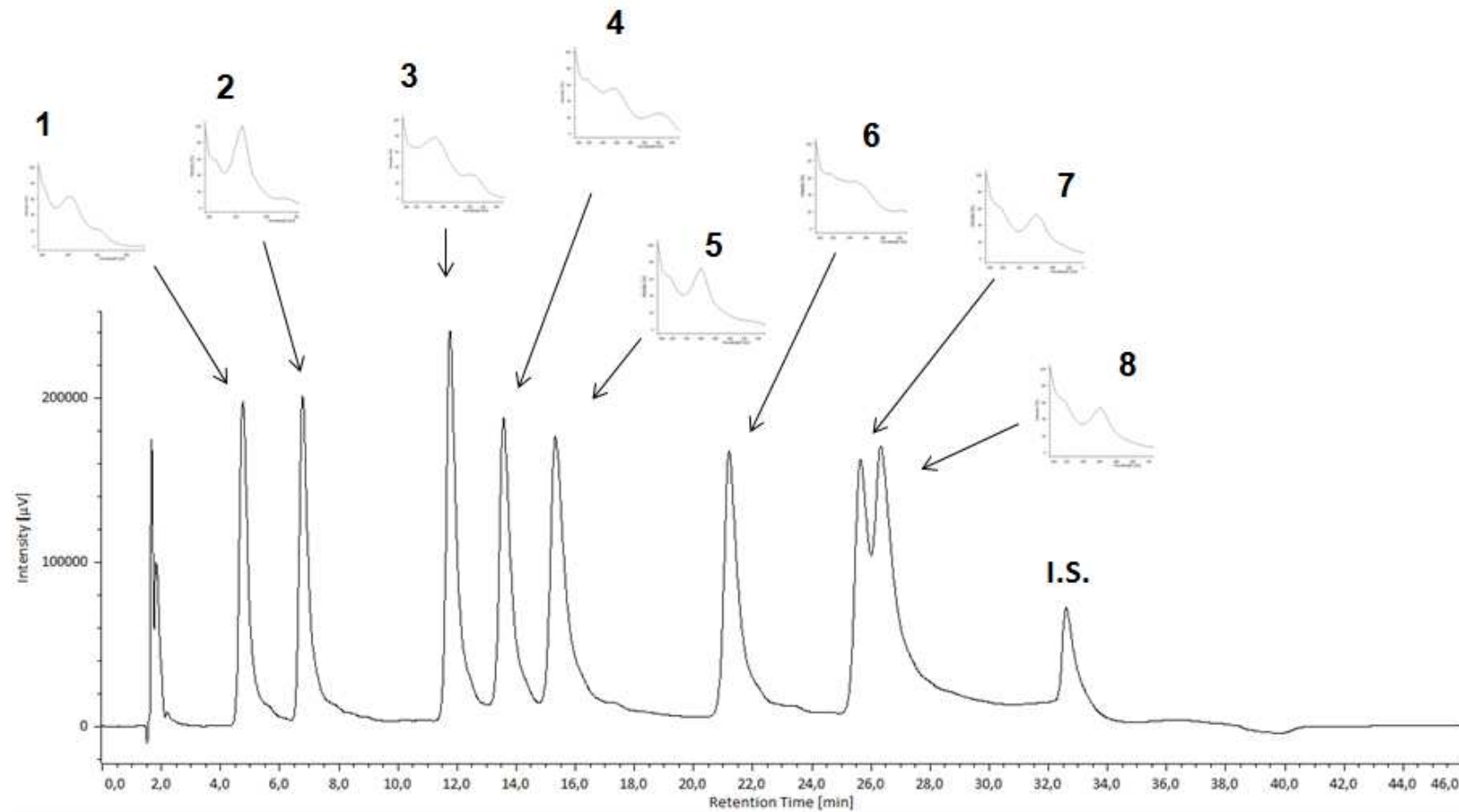


Figure 4.8 - Chromatograms (254 nm) of a standard mixture (1 mg/mL) (A) and of hydroalcoholic extract of *Medicago minima* (B) and UV absorption spectra of daidzin (1), genistin (2), daidzein (3), glycitein (4), genistein (5), formononetin (6), prunetin (7) and biochanin A (8).

Table 4.2 - Isoflavone contents (mg/Kg db) in *Medicago* species (n = 3). Mean  $\pm$  SD. n.d. - not determined. Values in the same line followed by different letters (in the different tables) indicate significant differences ( $p < 0.05$ ). Values in the same column followed by different numbers indicate significant differences ( $p < 0.05$ ). Different numbers in different species name indicate significant differences between them ( $p < 0.05$ ).

	<i>M. minima</i> <sup>12</sup>		
	Aqueous <sup>2</sup>	Hydroalcoholic <sup>2</sup>	Alcoholic <sup>12</sup>
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Daidzin	1.70 $\pm$ 0.05 <sup>ed4</sup>	n.d.	n.d.
Genistin	2.42 $\pm$ 0.01 <sup>fg<sup>h3</sup></sup>	6.52 $\pm$ 0.26 <sup>c1</sup>	2.82 $\pm$ 0.54 <sup>efgh<sup>3</sup></sup>
Daidzein	3.89 $\pm$ 0.10 <sup>d2</sup>	2.26 $\pm$ 0.1 <sup>gh2</sup>	3.36 $\pm$ 0.00 <sup>def<sup>3</sup></sup>
Glycitein	1.07 $\pm$ 0.00 <sup>f5</sup>	1.49 $\pm$ 0.06 <sup>e3</sup>	0.42 $\pm$ 0.02 <sup>h5</sup>
Genistein	3.51 $\pm$ 0.16 <sup>de1</sup>	1.72 $\pm$ 0.05 <sup>ghi<sup>3</sup></sup>	9.31 $\pm$ 0.14 <sup>b1</sup>
Formononetin	n.d.	n.d.	4.33 $\pm$ 0.00 <sup>c2</sup>
Prunetin	n.d.	n.d.	3.03 $\pm$ 0.01 <sup>b3</sup>
Biochanin A	n.d.	n.d.	1.62 $\pm$ 0.17 <sup>b4</sup>
<b>Total</b>	12.59	11.99	24.89

A

	<i>M. tornata</i> <sup>12</sup>		
	Aqueous <sup>2</sup>	Hydroalcoholic <sup>12</sup>	Alcoholic <sup>2</sup>
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Daidzin	1.60 $\pm$ 0.01 <sup>e3</sup>	5.28 $\pm$ 0.07 <sup>a2</sup>	1.82 $\pm$ 0.11 <sup>d3</sup>
Genistin	2.40 $\pm$ 0.05 <sup>fg<sup>h2</sup></sup>	8.81 $\pm$ 0.26 <sup>b1</sup>	3.15 $\pm$ 0.07 <sup>efgh<sup>1</sup></sup>
Daidzein	3.91 $\pm$ 0.35 <sup>d1</sup>	3.72 $\pm$ 0.01 <sup>de3</sup>	2.57 $\pm$ 0.03 <sup>fg<sup>2</sup></sup>
Glycitein	0.41 $\pm$ 0.01 <sup>h4</sup>	n.d.	n.d.
Genistein	0.72 $\pm$ 0.00 <sup>i4</sup>	3.28 $\pm$ 0.04 <sup>e4</sup>	1.03 $\pm$ 0.07 <sup>hi<sup>4</sup></sup>
Formononetin	n.d.	n.d.	n.d.
Prunetin	n.d.	n.d.	3.11 $\pm$ 0.04 <sup>b1</sup>
Biochanin A	n.d.	n.d.	n.d.
<b>Total</b>	12.14	22.03	11.68

B

	<i>M. truncatula</i> <sup>12</sup>		
	Aqueous <sup>2</sup>	Hydroalcoholic <sup>12</sup>	Alcoholic <sup>12</sup>
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Daidzin	1.58 $\pm$ 0.00 <sup>e3</sup>	n.d.	n.d.
Genistin	2.15 $\pm$ 0.01 <sup>h12</sup>	2.42 $\pm$ 0.44 <sup>fg<sup>h3</sup></sup>	2.67 $\pm$ 0.12 <sup>efgh<sup>3</sup></sup>
Daidzein	2.74 $\pm$ 0.35 <sup>efg<sup>2</sup></sup>	4.09 $\pm$ 0.01 <sup>de2</sup>	7.13 $\pm$ 0.55 <sup>c1</sup>
Glycitein	4.56 $\pm$ 0.36 <sup>b1</sup>	7.39 $\pm$ 0.53 <sup>a1</sup>	0.53 $\pm$ 0.01 <sup>g1</sup>
Genistein	n.d.	7.08 $\pm$ 0.28 <sup>c1</sup>	2.82 $\pm$ 0.38 <sup>fg<sup>3</sup></sup>
Formononetin	2.55 $\pm$ 0.10 <sup>e2</sup>	3.18 $\pm$ 0.00 <sup>d23</sup>	5.46 $\pm$ 0.12 <sup>b2</sup>
Prunetin	n.d.	n.d.	n.d.
Biochanin A	n.d.	n.d.	n.d.
<b>Total</b>	13.58	24.16	18.61

C

	<i>M. rigidula</i> <sup>2</sup>		
	Aqueous <sup>2</sup>	Hydroalcoholic <sup>12</sup>	Alcoholic <sup>12</sup>
	Mean ± SD	Mean ± SD	Mean ± SD
Daidzin	n.d.	n.d.	1.88 ± 0.01 <sup>d1</sup>
Genistin	2.22 ± 0.05 <sup>gh2</sup>	5.31 ± 0.25 <sup>d2</sup>	3.62 ± 0.10 <sup>be2</sup>
Daidzein	n.d.	12.21 ± 0.10 <sup>b1</sup>	8.10 ± 0.11 <sup>c5</sup>
Glycitein	n.d.	2.28 ± 0.02 <sup>d34</sup>	n.d.
Genistein	5.67 ± 0.24 <sup>d1</sup>	1.84 ± 0.89 <sup>gh34</sup>	6.41 ± 0.17 <sup>cd34</sup>
Formononetin	n.d.	2.63 ± 0.41 <sup>e34</sup>	6.91 ± 0.36 <sup>a4</sup>
Prunetin	n.d.	3.22 ± 0.01 <sup>b3</sup>	5.92 ± 0.22 <sup>a3</sup>
Biochanin A	n.d.	1.57 ± 0.03 <sup>b1</sup>	1.88 ± 0.15 <sup>a1</sup>
<b>Total</b>	<b>13.58</b>	<b>24.16</b>	<b>18.61</b>

D

	<i>M. scutellata</i> <sup>12</sup>		
	Aqueous <sup>2</sup>	Hydroalcoholic <sup>2</sup>	Alcoholic <sup>1</sup>
	Mean ± SD	Mean ± SD	Mean ± SD
Daidzin	2.47 ± 0.07 <sup>c1</sup>	2.36 ± 0.17 <sup>c2</sup>	3.52 ± 0.05 <sup>b4</sup>
Genistin	n.d.	5.16 ± 0.56 <sup>d1</sup>	9.60 ± 0.27 <sup>a3</sup>
Daidzein	n.d.	n.d.	19.20 ± 0.57 <sup>a1</sup>
Glycitein	n.d.	n.d.	3.92 ± 0.02 <sup>b4</sup>
Genistein	n.d.	n.d.	16.09 ± 0.26 <sup>a2</sup>
Formononetin	n.d.	n.d.	n.d.
Prunetin	n.d.	n.d.	n.d.
Biochanin A	n.d.	n.d.	n.d.
<b>Total</b>	<b>2.47</b>	<b>7.52</b>	<b>52.33</b>

E

	<i>M. segitalis</i> <sup>12</sup>		
	Aqueous <sup>2</sup>	Hydroalcoholic <sup>2</sup>	Alcoholic <sup>2</sup>
	Mean ± SD	Mean ± SD	Mean ± SD
Daidzin	n.d.	n.d.	n.d.
Genistin	2.78 ± 0.16 <sup>efgh1</sup>	3.32 ± 0.07 <sup>efg2</sup>	3.68 ± 0.30 <sup>e1</sup>
Daidzein	1.87 ± 0.05 <sup>gh2</sup>	3.67 ± 0.18 <sup>de1</sup>	2.18 ± 0.04 <sup>gh2</sup>
Glycitein	n.d.	n.d.	n.d.
Genistein	n.d.	n.d.	n.d.
Formononetin	n.d.	n.d.	n.d.
Prunetin	n.d.	n.d.	n.d.
Biochanin A	n.d.	n.d.	n.d.
<b>Total</b>	<b>4.65</b>	<b>6.99</b>	<b>5.86</b>

F

	<i>M. sativa</i> <sup>1</sup>		
	Aqueous <sup>2</sup>	Hydroalcoholic <sup>2</sup>	Alcoholic <sup>2</sup>
	Mean ± SD	Mean ± SD	Mean ± SD
<b>Daidzin</b>	n.d.	n.d.	n.d.
<b>Genistin</b>	3.32 ± 0.40 <sup>ef1</sup>	2.29 ± 0.14 <sup>gh1</sup>	3.14 ± 0.51 <sup>efgh1</sup>
<b>Daidzein</b>	n.d.	n.d.	1.47 ± 0.05 <sup>h3</sup>
<b>Glycitein</b>	n.d.	n.d.	0.45 ± 0.01 <sup>g4</sup>
<b>Genistein</b>	n.d.	n.d.	0.93 ± 0.13 <sup>i234</sup>
<b>Formononetin</b>	n.d.	n.d.	2.40 ± 0.06 <sup>e2</sup>
<b>Prunetin</b>	n.d.	n.d.	n.d.
<b>Biochanin A</b>	n.d.	n.d.	n.d.
<b>Total</b>	3.32	2.29	8.39

**G**

A large variability in the isoflavones content was observed within all species and obtained extracts. The total mean content of isoflavones ranged between 2.3 and 52.3 (mg/kg/db) for hydroalcoholic and alcoholic extracts from *M. sativa* and *M. scutelata*, respectively. Despite this variation, the compounds profile was relatively similar for all species, with genistin present in almost species (except aqueous extract of *M. scutelata*) and in higher amounts, followed by daidzein and genistein, by this order. However, the comparison of the individual compounds among the seven species (Table 4.2 - A, B, C, D, E, F and G) revealed some significant differences ( $p < 0.05$ ). The highest daidzin level was observed in extracts of *M. tornata* (5.3 mg/kg/db, hydroalcoholic extract). The only species that presented daidzin in all extracts were *M. tornata* and *M. scutelata*, being the hydroalcoholic extract the richest for *M. tornata* while for *M. scutelata* was the alcoholic. Some species do not contained this glycoside (*M. segitalis* and *M. sativa*) and in others only was detected in the alcoholic (*M. rigidula*) or in the aqueous extract (*M. minima* and *M. tornata*).

The highest concentration of genistin was observed in hydroalcoholic and alcoholic extracts of *M. scutelata*, in amounts 1.8-4.47 fold higher than the other samples evaluated ( $p < 0.05$ ). Differently of the other species, the aqueous extract does not have this isoflavone in detectable amounts. Daidzein, the aglycone form of daidzin, is also present in a high amount in *M. scutelata* (19.2 mg/kg db, alcoholic extract). However, it should be highlighted that this specie presented the highest values in alcoholic extract (compared with the other species), but for aqueous and hydroalcoholic was the poorest.

Glycitein is also present in most of species but the content was lower than daidzein or genistein. The results showed that genistein was present in several species at

concentrations ranging from 0.7 (aqueous extract of *M. tornata*) to 9.3 mg/kg/db (alcoholic extract of *M. minima*). Formononetin, prunetin and biochanin A are absent in most species and their contents were considerably lower than the contents of other isoflavones.

In terms of total isoflavones content per species, results show a significantly difference ( $p < 0.05$ ) between *M. rigidula* and *M. sativa* (Table 4.2 - D and G), being the total content higher in *M. rigidula* and lower in *M. sativa*.

Comparing the isoflavones content in *Medicago* spp. with other plants of the Leguminosae family, literature reports that the highest concentrations of isoflavones occur in soybeans, red clover and kudzu root [31]. Concentrations of total isoflavones (sum of daidzein, genistein, and glycitein in aglycone equivalents) determined in different types of soy flour range from 60 to 265 mg/100 g [270]. At the moment, soy is reported as the richest source of daidzein and genistein, up to 3800 mg/kg wet weight of soybean [271-272]. Isoflavones can also be extracted from red clover, another Leguminosae, in huge amounts. Different authors determined a large amount of isoflavones in this plant, which are mainly available as malonate conjugates, e.g. glycoside malonates of formononetin, irilone and biochanin A, in concentration ranging between 0.042 µg/g to 6 mg/g [45, 273-274]. The values obtained for *Medicago* can also be compared with the values obtained by Liggins *et al.* in raw vegetables and vegetable products and also in fruits and nuts, being higher [271-272]. Daidzein and genistein were found in a variety of fruits and nuts with concentrations ranging from 1 to 2250 µg/kg [272]. Also according to these authors, legumes contained between 20 and 5750 mg/kg wet weight of food, with an average of 620 mg/kg [271]. The daidzein and genistein contents in vegetables such as potatoes, cabbages or cucumber were about 8 µg/kg, considerable lower than *Medicago* [271]. On the other hand, coffee is the most important food commodity worldwide [6, 275] and has been intensively studied as a potential source of compounds with functional properties. As so, it seems interesting to compare *Medicago* values with those ones. Kuhnle *et al.* determined the phytoestrogen contents in coffee and tea and the values are lower (7-20 µg/100 g) when compared with *Medicago* [275]. Also, it should be take in consideration that the main use of all these comparable plants are as food ingredients and not as active ingredients for different industries. As this, reutilization of food by-products could be seen as a new valorisation of products without economic interest, not using food that is necessary over the world, and so *Medicago* could be a good promising source of these compounds. Rodrigues *et al.* described a detailed study about this subject [101].

This is, to the best of our knowledge, the first published description of these eight isoflavones in these extracts of *Medicago* species. It should be taken into consideration that the concentrations of isoflavones in *Medicago* are probably also dependent on a variety of factors besides genetic differences. These compounds are affected by different kind of factors as the growing conditions, including climate, soil, sunlight, postharvest conditions and age, among others [31, 276-277]. With the expectation that not only the species but also the solvent used for extraction (water, water: alcohol and alcohol) could affect the final content of isoflavones, the influence of solvent extractor was evaluated.

**Influence of solvent extractor:** Three solvents (water, water: ethanol and ethanol) were used as extractors to embrace the most effective one, regarding the total isoflavones content. The main goal of this study was to found a new promising use for these by-products, taking into account ecological and economic sustainability of the process. The obtained results are discriminated in Table 4.2.

The influence of the solvent extractor was clear demonstrated. Generally the alcoholic extracts were the most rich (*M. minima*, *M. rigidula*, *M. scutelata* and *M. sativa*). However, *M. tornata*, *M. truncatula* and *M. segitalis* showed best results with hydroalcoholic solvent while *M. sativa* and *M. minima* had similar results for aqueous and hydroalcoholic extracts. For *M. segitalis* the different used solvents had similar extraction capacity, a characteristic behavior of this specie.

When the three solvent extracts were analyzed individually by species, a general increase of genistin levels in alcoholic extracts was noted. For daidzein, the increase is not evident, being the sample of *M. truncatula* and *M. scutelata* exceptions, with higher contents in hydroalcoholic extracts (Table 4.2 - C and E). For genistein, the result was variable. These different behaviors seem to result from different factors, namely, genetics of species and an unexpected extractability variation. In a general way, the compounds extractability significantly increased ( $p < 0.05$ ) from aqueous to alcoholic extracts. Exceptions were *M. segitalis* and *M. sativa* (Table 4.2 - F and G), for which no significant differences ( $p < 0.05$ ) in total isoflavones extractability were detected with different solvents. The results also show that, although the extractability of the individual isoflavones varied with the sample, in a general way, genistin, daidzein and genistein are present at higher levels than the other isoflavones, especially at *M. minima* (alcoholic extract), *M. tornata* (hydroalcoholic extract), *M. truncatula* (hydroalcoholic extract), *M. rigidula* (hydroalcoholic and alcoholic extract) and *M. scutelata* (alcoholic extract).



However, as already referred, in plants, isoflavones are usually bound to other molecules that modify their solubility properties [278]. Aglycones of phenolic compounds are hydrophobic compounds, however they have an intermediate solubility (such as flavonoids) [279-280]. The higher concentration of daidzein in extracts is a pattern that has been observed in other species of Leguminosae and, probably, is related to the biosynthetic pathways of these compounds. Formononetin, prunetin and biochanin A most of time were not determined in samples and, if so, they were not the main isoflavones and are more abundant in alcoholic extracts.

An increase in the total isoflavones levels of *Medicago* species was observed as the solvent polarity decreases, for each individual sample. Independent of the species, aqueous extraction always contained inferior amounts of isoflavones. The use of ethanol as extraction solvent is very interesting if it is desired the extract application in food or cosmetic products, for example. The use of organic solvents in the manufacturing process to obtain cosmetic ingredients is regulated [109], and from the perspective of cosmetic safety it is preferable to use solvents such as ethanol or water: ethanol mixtures since they are in compliance with good manufacturing practices. Methanol is usually a good extraction solvent but it has a toxic character. Therefore, the extracts produced with methanol arise serious issues for application in food and pharmaceutical products, according to the present legislation [281].

#### 4. Conclusion

Our investigation gives for the first time a comprehensive picture on the isoflavone pattern of seven *Medicago* species (*M. minima*, *M. tornata*, *M. truncatula*, *M. rigidula*, *M. scutellata*, *M. segitalis* and *M. sativa*) extracts (aqueous, hydroalcoholic and alcoholic). This information may be of interest to its use in cosmetic or pharmaceutical preparations. As part of this work, eight isoflavone, including daidzin, genistin, daidzein, glycitein, genistein, formononetin, prunetin and biochanin A, were detected and quantified for the first time in extracts of *Medicago*. Genistin, daidzein and genistein were the main isoflavones extracted, while formononetin, prunetin and biochanin A appears in small amounts and in few extracts. In terms of total isoflavones content per specie, they have different contents being *M. rigidula* the richest and *M. sativa* the poorest. Comparing with other plants of the same family like *Trifolium*, *Medicago* showed a lower content of isoflavones. However, raw vegetables like potatoes presented a lower content than

*Medicago*. The same occurs with coffee. The influence of solvent extractor was also studied, and, despite the alcohol present very high values of isoflavones in some species, the use of a hydroalcoholic solvent must be taken into account, since it originated promising values and was less toxic.

The results reported here will contribute to databases of the phytoestrogen content of diets applied to the dietary intake of foods of free living individuals and is the sole source of such information in the literature at the present time for *Medicago* species.

## **I. Coffee Silverskin: A Possible New Cosmetic Ingredient?**

The information presented in this chapter section was published in the following publication:

- **Francisca Rodrigues**, Ana Palmeira-de-Oliveira, José das Neves, Bruno Sarmento, M. Helena Amaral, M. Beatriz P.P. Oliveira, Coffee silverskin: A possible valuable cosmetic ingredient, *Pharmaceutical Biology*, 4:1-9, 2014

## 1. Introduction

Alongside rising interest in natural products for health and personal care applications, there is a trend towards the use of environmentally-friendly products obtained by sustainable resources. By-products valorisation is a relatively new concept in the field of industrial residues management, promoting the principle of sustainable development. Application of agro-industrial residues in bioprocesses, on one hand, provides alternative substrates with potential market value, while on the other hand helps solving pollution problems, of otherwise disposable remainders [282]. Food processing by-products enables reduced waste and the recovery of extracts for the production of precious metabolites via chemical and biotechnological processes. After specific pre-treatments with physical and biological agents followed by tailored recovery procedures, they might provide valuable natural antioxidants, antimicrobial agents, vitamins, etc., along with macromolecules, such as cellulose, starch, lipids, proteins, plant enzymes and pigments, of enormous interest to pharmaceutical, cosmetic and food industries [256]. In particular, agro-industrial by-products are good sources of phenolic compounds and have also been explored as source of natural antioxidants [254]. While the use of naturally occurring phenolic compounds as food antioxidants is particularly interesting, practical aspects should be considered as the extraction efficiency, availability of raw material and safety.

Coffee beans contain several classes of health related chemicals such as phenolic compounds, diterpenes, xanthines and vitamin precursors [283-284]. Coffee phenolics have attracted much interest in recent years due to their strong antioxidant and metal-chelating properties [285]. These properties are believed to provide *in vivo* protection against free radical damage and reduce the risk of degenerative diseases associated with oxidative stress. Advances in industrial biotechnology offer potential opportunities for economic utilization of agro-industrial residues of coffee such as coffee pulp, coffee husk or coffee silverskin [282]. Usually, two coffee beans are found in each fruit, each bean covered with a thin closely fitting skin called silverskin (CS), outside of which is a looser, yellowish skin called the parchment, the whole being encased in a pulp which forms the flesh of the cherry [10]. The CS is a tegument of coffee beans that constitutes a by-product of the roasting procedure and is the most abundant solid by-product generated during the coffee process. CS has no commercial value and is currently discarded as a solid waste, being used, in some cases, as fertilizers or simply burned [10]. This has negative effects on the environment, and so, the disposal of CS requires proper

management. Alternatively, the transformation of this by-product into valuable products for other purposes is attractive. However, few works have been performed on the properties of CS, in particular the antioxidant capacity [15-16, 21].

According to Narita & Inouye [21] consumers generally prefer natural antioxidants to synthetic ones because of the higher safety. Recently, it has been reported that CS is a rich source of antioxidants constituents [15, 21], but to the best of our knowledge, there are no reports on the use of CS for cosmetic applications. A recent work carried out in our laboratory showed that antioxidant content of CS extracts depends of temperature and time of extraction [20]. We consider that lower temperatures may be more attractive from an economical and industrial point of view. Thus, our current goal involves the extraction of active compounds from biological waste at relatively low temperatures. The objective of this work was to evaluate the antioxidant activity of aqueous, hydroalcoholic and alcoholic extracts of CS obtained at 40 °C. Also, the antimicrobial properties and cytotoxicity of extracts were assessed, as relevant characteristics to the use of these ingredients in cosmetics.

Thus, the present study was designed: (a) to quantify the antioxidant activity of different CS extracts by DPPH and FRAP assay and evaluate the total polyphenol and flavonoid content; (b) to evaluate the antimicrobial activity of CS extracts against bacteria and yeast; (c) to analyse the cytotoxicity profile of CS extracts in keratinocytes and fibroblasts from skin; (d) to choose the best extract to be used in cosmetic applications.

## 2. Materials and Methods

### 2.1 Materials

Coffee silverskin samples were provided by Bicafé (BICAFÉ – Torrefacção e Comércio de Café Lda, Portugal), located in Porto, Portugal. Ascorbic acid, DPPH<sup>\*</sup> (1,1-diphenyl-2-picrylhydrazyl) free radical, catechin, Folin-Ciocalteu's reagent, gallic acid, iodine, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble derivative of vitamin E) and  $\alpha$ -tocopherol, were purchased from Sigma-Aldrich (Steinheim, Germany). Reagent grade ethanol, sodium acetate, sodium carbonate decahydrate, sodium nitrite, aluminum chloride and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX<sup>™</sup>-I, fetal bovine serum (FBS), streptomycin, penicillin and amphotericin B were from

Invitrogen (Carlsbad, CA, USA). MTS assay kit (CellTiter 96<sup>®</sup> Aqueous One Solution Cell Proliferation Assay) was purchased from Promega Co. (Madison, WI, USA). Lactate dehydrogenase (LDH) colorimetric cytotoxicity assay kit (LDH Cytotoxicity Detection Kit) was purchased from Takara Bio Inc. (Shiga, Japan). Deionized water was obtained using Mili-Q water purification system (TGI Pure Water Systems, USA).

## 2.2 Preparation of coffee silverskin extracts

CS was milled to particle size of approximately 0.1 mm using a A11 basic analysis mill (IKA Wearke, Staufen, Germany) and stored in silicone tubes at room temperature until extraction. Three samples of 1 g were submitted to maceration for 30 min at 40 °C with 20 mL of ethanol, ethanol: water (1:1) or distilled water. Extracts were filtered through Whatman No. 1 paper filter and the filtrates collected. Aqueous filtrates were freeze-dried and hydroalcoholic and alcoholic filtrates were reduced to residue using a rotary evaporator set to 37 °C. Samples were stored at 4 °C until analysis. For determination of the chemical parameters the extracts were reconstituted in the solvent used for extraction.

## 2.3 Determination of total phenolic and flavonoid contents

Total phenolic content (TPC) was spectrophotometrically determined according to the Folin-Ciocalteu procedure [185] with minor modifications. Briefly, 500 µl of extract (1 mg/mL) was mixed with 2.5 mL of Folin-Ciocalteu reagent (10x dilution) and allowed to react for 5 min. Then, 2.5 mL of Na<sub>2</sub>CO<sub>3</sub> 7.5% (w/v) solution was added and after 15 min at 45°C, and 30 min at room temperature, the absorbance was measured at 765 nm using a microplate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA). A calibration curve of gallic acid with linearity range of 5-100 µg/ml and  $r^2 > 0.998$  was used. The TPC of the extract was expressed as mg of gallic acid equivalents per g of plant material on dry basis (mg GAE/g db). The assay was done in triplicate.

Total flavonoid content (TFC) was determined by a colorimetric assay based on the formation of flavonoid-aluminum compound according to Barroso *et al.* [187]. Briefly, 1 mL of extract (1mg/mL) was mixed with 4 mL of water and 300 µl of NaNO<sub>2</sub> solution (5 % w/v). Then, 300 µL of AlCl<sub>3</sub> solution (0.1 g/mL) were added, and after 1 min, 2 mL of

NaOH (1 M) and 2.4 mL of water. The absorbance was determined at 510 nm using the microplate reader. A calibration curve of catechin with linearity range of 0-400 µg/mL and  $r^2 > 0.999$  was used. The TFC of the extract was expressed as µg of catechin equivalents per g of plant material on dry basis (µg CAE/g db). The assay was done in triplicate.

## 2.4 Antioxidant capacity of coffee silverskin extracts

The antioxidant activity of the samples was evaluated by DPPH radical-scavenging activity and ferric reducing antioxidant power (FRAP). DPPH is known as a stable free radical which possesses a characteristic maximum absorption between 515 and 517 nm. The reaction mixture on 96 wells plate consisted of one solution per well for different sample concentrations (30 µL) and methanol solution (270 µL) containing DPPH radicals ( $6 \times 10^{-5}$  mol/L). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm [188] using the microplate reader. The antioxidant capacity based on the DPPH free radical scavenging ability of the extract was expressed as µmol Trolox equivalents per g of plant material on dry basis (µmol Trolox /g db). A calibration curve of Trolox with linearity range of 2.5-100 µg/mL and  $r^2 > 0.996$  was prepared.

Ferric Reducing Antioxidant Power (FRAP) was carried out according to Benzie & Strain [189] with minor modifications. Briefly, 90 µL of different sample concentrations were added to 2.7 mL of FRAP reagent (10:1:1 of 300 mM sodium acetate buffer at pH 3.6, 10 mM TPTZ and 20 mM  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) and the reaction mixture was incubated in a water bath at 37 °C. The increase in absorbance at 592 nm was measured after 30 min using a microplate reader. A calibration curve of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  with linearity range of 150-2000 µM and  $r^2 > 0.996$  was used. The results were expressed as µmol  $\text{Fe}^{2+}$  equivalents per g of material on dry basis (µmol  $\text{Fe}^{2+}$  /g db).

## 2.5 Antimicrobial activity of coffee silverskin extracts

### 2.5.1 Microorganisms strains

To test the antimicrobial activity, eight microorganisms (seven bacteria and one yeast) were enrolled in the study. Both American Type Culture Collection (ATCC) and clinical isolates were selected, corresponding to two *Staphylococcus aureus* (ATCC 6538

and one clinical isolate), *Staphylococcus epidermidis* (clinical isolate), two *Escherichia coli* (ATCC 1576 and one clinical isolate), *Klebsiella pneumoniae* (ATCC 4352), *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 10231). Clinical isolates were identified as to species-level with Vitek-2 identification cards (Biomérieux, France) and all strains were kept frozen in Brain Heart Infusion (Difco Laboratories, USA) with 20% glycerol (Sigma) at -70°C until testing. For each experiment, microorganisms were subcultured twice in Nutrient agar (Difco Laboratories, USA) for bacteria and Sabouraud dextrose agar (SDA; Difco Laboratories, USA) for yeasts, to assess culture viability.

### **2.5.2 Determination of minimal inhibitory concentrations**

The antibacterial activity was tested according to CLSI M7-A6 microdilution [191] and the antifungal activity was tested according to M27-A3 microdilution from the same protocol, after 48 h of incubation at 37 °C and 35 °C, respectively [190]. Briefly, two-fold serial dilutions of extracts were performed in Mueller Hinton broth (MH; Difco Laboratories, USA) for bacteria and with RPMI (Biochrom, Germany) for yeast. Concentrations ranging from 3.9 to 500 µg/mL were tested. Microorganisms growth was visually compared for each concentration with the negative control (without extracts). Minimal inhibitory concentration (MIC) was defined as the lowest extract concentration able to completely inhibit microorganisms growth, corresponding to 100% MIC value. All determinations were performed in triplicate for each assay and three independent experiments were run with concordant results.

## **2.6 Cytotoxicity and cell viability of coffee silverskin extracts**

### **2.6.1 Cell lines and culture conditions**

Human immortalized non-tumorigenic keratinocyte cell line (HaCaT) and human foreskin fibroblasts (HFF-1) were purchased from CLS Cell Lines Service (Germany) and ATCC (Manassas, VA, USA), respectively. HaCaT and HFF-1 cells were individually maintained in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™-I (Invitrogen), 10% inactivated fetal calf serum (FBS; Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen), and 0.25 µg/mL amphotericin B (Invitrogen), in a 5% CO<sub>2</sub> environment at 37 °C. The number of viable cells was periodically assessed by the trypan blue exclusion assay. After attachment, they were incubated in serum-free medium



containing 0.1 µg/mL to 1 mg/mL extracts for 24 h at 37 °C. Plant extracts were dissolved in cell culture medium.

### **2.6.2 Cell viability and toxicity assays**

Two different assays were used to assess cell integrity and cytotoxicity of the extracts: (1) monitoring the uptake of the vital mitochondrial dye, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) by cell mitochondria and (2) determining the leakage of the cytosolic enzyme, lactate dehydrogenase (LDH) into the cell medium (LDH assay). In both cases, cells (HFF-1 or HaCaT) were seeded in 96-well plates ( $5 \times 10^3$  cells/well) and incubated with medium for 24h (37 °C/ 5% CO<sub>2</sub>). HFF-1 and HaCaT cells were used at passages 17-19 and 77-79, respectively. Then, triplicate wells were incubated with extracts dissolved in cell culture medium pre-filtered by 0.45 µm Millex GV filters (Millipore, Nepean, ON, Canada) at different concentrations (0.1-1000 µg/mL). Cells were exposed to extracts for 24 h. Medium without FBS was used in the case of LDH. Afterwards, MTS and LDH assays were performed according to manufacturers' instructions. Briefly, cell supernatants were collected for the LDH assay and cells were washed twice with phosphate buffered saline pH 7.4 (PBS) before being incubated with 120 µL of MTS in medium for 4 h (37 °C/ 5% CO<sub>2</sub>). The absorbance was measured for each well at 490 nm with background subtraction at 630 nm using a microplate reader. In the case of LDH, absorbance after 30 min/room temperature incubation of cell supernatants with LDH reagent was measured at 490 nm with background subtraction at 690 nm using a microplate reader. Also, the maximum release of this enzyme (high control) was determined by incubating cells with 1% (w/v) Triton X-100 (Boehringer Mannheim), while spontaneous LDH release (low control) was assessed by incubating the cells with DMEM alone. In MTS assay the positive control was the medium and the negative control was Triton X-100. In all cases, each concentration was tested in triplicate in three independent experiments.

### **2.7 Statistical analysis**

Data were reported as mean ± standard deviation of at least triplicate experiments. Statistical analysis of the results was performed with SPSS 19.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to investigate the differences between different

extracts for all assays. Post hoc comparisons were performed according to Tukey's HSD test. In all cases,  $p < 0.05$  was accepted as denoting significance.

### 3. Results

#### 3.1 Determination of total phenolic content (TPC)

The broad range of effects of free radicals in biological systems has drawn the attention of many experimental works. It has been proved that these mechanisms may be important in the pathogenesis of certain diseases and aging.

The TPC of the different CS extracts was determined using the Folin-Ciocalteu reagent. Results are presented in Table 5.1.

Table 5.1 - Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Ferric Reducing Antioxidant Potential Assay (FRAP) and 1,1-diphenyl-2-picrylhydrazyl hydrate (DPPH•) scavenging assay of coffee silverskin extracts (CS).

	Aqueous Extract		Hydroalcoholic Extract		Alcoholic Extract	
	Mean	SD	Mean	SD	Mean	SD
<b>TPC (mg GAE/g)</b>	18.33 <sup>b,c</sup>	0.52	30.50 <sup>a,c</sup>	0.25	35.25 <sup>a,b</sup>	0.25
<b>TFC (µg CAE/g)</b>	1.08 <sup>b,c</sup>	0.23	2.47 <sup>a</sup>	0.06	2.61 <sup>a</sup>	0.15
<b>DPPH (µmol Trolox/g)</b>	206.00 <sup>b,c</sup>	3.72	242.28 <sup>a,c</sup>	5.42	386.35 <sup>a,b</sup>	5.10
<b>FRAP (µmol Fe<sup>2+</sup>/g)</b>	95.95 <sup>c</sup>	1.50	134.0 <sup>c</sup>	1.50	216.40 <sup>a</sup>	2.70

"a-c" Denote a significant difference between mean values, where "a" denotes a significant difference from CS Aqueous Extract, "b" a significant difference from CS Hydroalcoholic Extracts and "c" a significant difference from CS Alcoholic Extracts. Repeated measures ANOVA followed by Turkey's post-hoc test,  $n=3$  independent experiments. SD=standard deviation.

The largest amount of total phenolic components ( $35.25 \pm 0.25$  mg) extracted from g of CS was determined with ethanol as solvent. It was almost two times higher than aqueous extract ( $18.33 \pm 0.52$  mg).

### 3.2 Determination of total flavonoids content (TFC)

Flavonoids, which constitute the largest group of plant phenolics accounting for over half of the eight thousand naturally occurring phenolic compounds [254], were present in all CS extracts. Results varied between 1.08 mg (water extract) to 2.61 mg (ethanol extract) of CAE/g of CS (Table 5.1). The results clearly showed that the highest content of flavonoids was obtained with ethanol.

### 3.3 *In vitro* antioxidant activity

The antioxidant potential of the CS extracts was determined by two methods based on different approaches, namely the DPPH radical scavenging method and the FRAP assay, which have been extensively used to determine the antioxidant potential of various plant extracts and natural products [101]. Irrespective of the solvent used for extraction, all the extracts obtained from CS showed antioxidant potential (Table 5.1). Among them, the extracts obtained with ethanol had the highest antioxidant activity by both DPPH and FRAP methods.

Similar to the DPPH results, the highest antioxidant activity in FRAP was also obtained in alcoholic extract (Table 5.1). A range of 95.95, 134.0 and 216.40  $\mu\text{mol Fe}^{2+}/\text{g}$  for aqueous, hydroalcoholic and alcoholic samples were obtained, respectively.

### 3.4 Antimicrobial activity

Microorganisms such as fungi and bacteria are important factors that cause oxidative processes during storage of cosmetic products and consequent deterioration. Despite several previous studies on the antimicrobial properties of coffee against many human's pathogens, to the date there is not research on the antifungal and antibacterial activity of CS [286].

The antibacterial activity of CS extracts was evaluated by the determination of MIC values (Table 5.2). In this study, microorganisms were selected to cover Gram-positive bacteria (*S. aureus* and *S. epidermidis*), Gram-negative bacteria (*E. coli*, *K. pneumoniae*, *P. aeruginosa*) and yeasts (*Candida albicans*). The consistent and reproducible results

obtained showed that CS extracts exhibited an interesting antimicrobial potential (Table 5.2).

Table 5.2 - Antibacterial activity of different extracts of CS expressed as the Minimal Inhibitory Concentration (MIC). Data expressed as mean of the replicates (n=3).

Bacterial strains	MIC ( $\mu\text{g/mL}$ )		
	Aqueous Extract	Hydroalcoholic Extract	Alcoholic Extract
<i>S. aureus</i> ATCC 6538	31.3	125	125
<i>S. aureus</i> MRSA	250	250	250
<i>S. epidermidis</i> B21	62.5	62.5	62.5
<i>E. coli</i> ATCC 1576	62.5	125	125
<i>E. coli</i> B26	125	125	125
<i>K. pneumoniae</i> ATCC 4352	31.3	31.3	31.3
<i>P. aeruginosa</i> ATCC 9027	>1000	>1000	>1000
<i>C. albicans</i> ATCC 10231	>1000	>1000	>1000

Aqueous extracts of CS presented MICs in the range of 31.3-250  $\mu\text{g/mL}$  against *S. aureus*, *S. epidermidis*, *E. coli* and *K. pneumoniae*. The hydroalcoholic and alcoholic extract showed similar activity against the microorganisms tested. Both presented MICs in the range of 31.3  $\mu\text{g/mL}$ -250  $\mu\text{g/mL}$  against those tested microorganisms. The values varied from 31.3  $\mu\text{g/mL}$  to 1000  $\mu\text{g/mL}$  of culture medium, and the lowest and highest mean MICs among the three groups were observed against *K. pneumoniae* (31.3  $\mu\text{g/mL}$ ) and *C. albicans* / *P. aeruginosa* (>1000  $\mu\text{g/mL}$ ), respectively. Concerning antifungal test, the analysed extracts failed to show any activity against *C. albicans*.

### 3.5 Keratinocytes and fibroblasts cytotoxicity assay

The effects of the extracts on the growth of keratinocyte (HaCaT) and fibroblast (HFF-1) cells were investigated by MTS and LDH assays. These cell lines are regarded as suitable *in vitro* models for testing the toxicity potential of substances or products intended for cosmetic use [242].

According to the obtained results for the MTS assay, none of the extracts exhibited cytotoxicity against HaCaT cells (Table 5.3) at all concentrations up to 1000  $\mu\text{g/mL}$ .

Table 5.3 - Effect on the metabolic activity of HaCaT cells after the exposure to CS extracts at different concentrations, measured by the MTS assay. Values are expressed as the means  $\pm$  SD (n=6).

Concentration ( $\mu\text{g/mL}$ )	Cell Viability (%)					
	Aqueous Extract		Hydroalcoholic Extract		Alcoholic Extract	
	Mean	SD	Mean	SD	Mean	SD
0.1	99.79	1.09	96.11	3.90	103.46 <sup>(2,3)</sup>	2.21
1.0	91.31 <sup>c</sup>	3.14	93.43 <sup>c</sup>	2.82	103.46 <sup>(1,3,4,5)a,b</sup>	2.49
10	96.11 <sup>b,c</sup>	0.46	92.58 <sup>a,c</sup>	1.11	100.78 <sup>(1,2)a,b</sup>	0.4
100	99.51 <sup>b,c</sup>	4.56	97.67 <sup>a</sup>	1.04	100.78 <sup>(2)a</sup>	1.5
1000	103.60	5.89	101.55	4.04	101.91 <sup>(2)</sup>	0.42

“a-c” Denote a significant difference between mean values, where “a” denotes a significant difference from aqueous extract, “b” a significant difference from hydroalcoholic extract and “c” a significant difference from alcoholic extracts. Repeated measures ANOVA followed by Turkey's post-doc test, n=3 independent experiments. “1-5” Denote a significant difference between mean values, where 1 denotes a significant difference from concentration 0.1  $\mu\text{g/mL}$  inside an extract and 2 denotes a significant difference from concentration 1.0  $\mu\text{g/mL}$  inside the same extract, etc. Repeated measures ANOVA followed by Turkey's post-doc test, n=3 independent experiments. SD = standard deviation.

Table 5.4 presents the cytotoxicity data of the three different extracts of CS against HFF-1 cells and as determined by the MTS assay.

Table 5.4 - Effect on the metabolic activity of HFF-1 cells after the exposure to CS extracts at different concentrations, measured by the MTS assay. Values are expressed as the means  $\pm$  SD (n=6).

Concentration ( $\mu\text{g/mL}$ )	Cell Viability (%)					
	Aqueous Extract		Hydroalcoholic Extract		Alcoholic Extract	
	Mean	SD	Mean	SD	Mean	SD
0.1	91.64 <sup>(4,5)b,c</sup>	7.90	130.53 <sup>(2,3,5)a</sup>	5.89	132.88 <sup>(3,4,5)a</sup>	3.08
1.0	98.90 <sup>(4,5)b,c</sup>	5.87	147.08 <sup>(1,4,5)a,c</sup>	0.81	130.79 <sup>(3,4,5)a,b</sup>	4.23
10	96.35 <sup>(4,5)b</sup>	8.60	144.00 <sup>(1,5)a,c</sup>	2.95	105.31 <sup>(1,2,5)b</sup>	5.06
100	122.87 <sup>(1,2,3)b,c</sup>	1.05	128.63 <sup>(2,5)a,c</sup>	9.10	112.22 <sup>(1,2,5)a,b</sup>	4.36
1000	120.14 <sup>(1,2,3)b,c</sup>	5.38	96.92 <sup>(1,2,3,4)a</sup>	7.68	96.85 <sup>(1,2,3,4)a</sup>	5.36

“a-c” Denote a significant difference between mean values, where “a” denotes a significant difference from aqueous extract, “b” a significant difference from hydroalcoholic extracts and “c” a significant difference from alcoholic extracts. Repeated measures ANOVA followed by Turkey's post-doc test, n=3 independent experiments. “1-5” Denote a significant difference between mean values, where 1 denotes a significant difference from concentration 0.1 µg/mL inside an extract and 2 denotes a significant difference from concentration 1.0 µg/mL inside an extract, etc. Repeated measures ANOVA followed by Turkey's post-doc test, n=3 independent experiments. SD = standard deviation.

The number of viable fibroblast cells was similar for all extracts tested. The values ranged from 91.64% for aqueous extract (0.1 µg/mL), 96.92% for hydroalcoholic extract and 96.85% for alcoholic extract (1000 µg/mL).

An important indicator of cell damage in oxidative stress is the increase in cell membrane permeability. In this study, potential cytotoxicity effects of different extracts of CS were investigated by examining membrane integrity of HaCaT and HFF-1 cells using a LDH release assay. In HaCaT cells, at all concentrations tested, CS extracts did not affect LDH leakage following 24 h incubation. The evidence of possible absence of cytotoxicity of this extracts at five different concentrations, namely, 0.1, 1.0, 10, 100 and 1000 µg/mL, implies that these extracts present low toxicity to these cells. Similarly, results for fibroblasts were comparable to those of HaCaT cells, with no significant increase in LDH being observed.

The results between MTS and LDH are in concordance, which is in agreement with Issa *et al.* and Fotakis *et al.* who showed that both assays are inversely proportional [287-288].

#### **4. Discussion**

In this work, a new procedure was proposed in order to maximize the extraction results and to define the best conditions for antioxidant compounds extraction from CS to be used as new active ingredient for different kind of industries, especially cosmetic industries. In particular, aqueous, hydroalcoholic and alcoholic solvents were studied. Thirty minutes was fixed as the extraction time since the use of longer periods could not be economically advantageous. The extraction temperature was fixed at 40 °C, considering also economical reasons and since no significant advantage in extraction efficiency was observed when using higher temperatures, as previously demonstrated by

our group [20]. Statistical data analysis revealed a significant influence of all the studied solvents on the total phenolics content and antioxidant activity, which were improved when the ethanol was the solvent.

The TPC of the CS extracts obtained with different solvents increased with decreasing polarity of solvent systems. This might be due to the fact that phenolic compounds are often more soluble in organic solvents, less polar than water [289]. Moreover, the obtained values are in agreement with the content reported in other important antioxidant sources by-products that are being study for antioxidant activity, such as spent coffee grounds (13 to 35 mg GAE/g dry matter) [281, 285], blackberry (50-80 mg GAE/g dry matter) [290], brewer's spent grains (9.9 mg GAE/g dry matter) [289], almond shells (38.0 mg GAE/g dry matter) [291] or apple pomace, which was below 15 mg GAE/g dry matter [292]. When comparing the results obtained for CS with the ones for coffee, it is apparent that the coffee waste has similar phenolic content [293]. Naidu *et al.* studied the antioxidant composition of green coffee and conclude that the total polyphenol content from extraction with isopropanol plus water was around 32 mg GAE/g.

On average, the flavonoid content was increased by about 2-fold, respectively, when water was replaced by ethanol, which can be attributed to an on-average higher affinity of these compounds for ethanol than for pure water. Data examination reveals that the solvent had a significant effect on the extraction efficiency. According to some authors, the addition of water to polar organic solvents such as acetone, methanol and ethanol, creates a more polar medium that facilitates the extraction of phenolic compounds [294].

The content of total flavonoids was in high correlation with the content of total phenols ( $r^2=0.963$ ). According to Hečimović *et al.* [295], the TFC in coffee varies between 12-20 mg CAE/g. Comparing with flavonoid content in CS, the obtained results indicate that roasting affects the polyphenolic compounds of CS. Comparing with other coffee by-products like spent coffee grounds, the TFC is similar (2.11 to 8.03 mg CAE/g dry waste) [285].

The DPPH radical scavenging activity of different solvents of the extract of CS was measured and the  $\mu\text{mol}$  Trolox equivalents determined. As indicated by the DPPH values in Table 5.1, for each waste material the use of ethanol as extraction solvent resulted in higher antioxidant activity. The results showed that DPPH radical scavenging activity efficiencies were in the order of alcoholic > hydroalcoholic > aqueous extract. The DPPH

values range from 206.0 to 386.35  $\mu\text{mol}$  Trolox equivalents, for water and ethanol, respectively. This can be attributed to the higher total polyphenolic content of CS. A good correlation between the total polyphenol content and DPPH scavenging radical activity was expected. In addition, plotting the DPPH value of the various extracts against their corresponding total phenolic content showed that these values were correlated. However, a mean  $r^2$  value of 0.69 was determined. This discrepancy could be due to a larger natural variation in the polyphenolic content. Also, the low correlation observed may be due to compounds that do not respond to the used reagent but also have antioxidant activity, such as caffeine or isoflavones. Furthermore, DPPH radical scavenging activity depends on the hydrogen donating ability of the extracted antioxidants [192].

Comparing the total phenolic content and ferric reducing capacity of the samples a positive correlation ( $r^2=0.80$ ) was observed, as expected. Even though both the FRAP and DPPH assays were used to determine the antioxidant activity of the CS extracts, results are different due to the nature of each antioxidant assay. DPPH is a method based on the measurement of the reducing ability of antioxidants toward 1,1-diphenyl-2-picrylhydrazyl (DPPH $^{\bullet}$ ). This ability can be evaluated by electron spin resonance or by measuring the decrease of its absorbance. The reaction of DPPH with free radical scavengers present in the test sample occurs rapidly and can be assessed by following the absorbance decrease at 517 nm. The reaction time of the improved DPPH assay is only 30 min, while the FRAP assay measures the reducing capability by increased sample absorbance based on the ferrous ions released, being thus more time consuming. On the other hand, the DPPH assay is preferred to the ABTS assay because it is commercially available and does not have to be generated before assay like ABTS $^+$  [192]. It is worth noting that the extracts produced with water presented low antioxidant potential and also low content of total phenols when compared with most of the extracts produced with organic solvents, like ethanol. Similar results have been reported during the extraction of antioxidant compounds from other raw materials such as spent coffee grounds [281, 285], grape by-products [296] or almond [291].

The use of ethanol as extraction solvent is very interesting if it is desired the extract application in cosmetic products, for example. The use of organic solvents in the manufacturing process to obtain cosmetic ingredients is regulated, and from the perspective of cosmetic safety it is preferable to use solvents such as ethanol or ethanol: water mixtures since they are in compliance with good manufacturing practice. Methanol is usually a good extraction solvent but it has a toxic character. Therefore, the extracts



produced with methanol arises serious issues for application in food and pharmaceutical products, according to the present legislation [281].

Analyzing the antimicrobial activity, the inefficacy of the extracts against *P. aeruginosa* may be related with the presence of the extracellular matrix. For *C. albicans*, the complex yeast wall is responsible for preventing extracts to contact with cell structures. Generally, Gram-negative are more resistant to bactericidal polyphenols than Gram-positive bacteria [297]. According to Fabry *et al.*, for crude extracts of plants to be considered as potentially useful therapeutically they should have MIC values < 8 mg/mL [237]. Gibbons suggests that isolated phytochemicals should have MIC < 1 mg/mL [238]. The MIC values herein determined for CS extracts were below these values, except for *C. albicans* and *P. aeruginosa*, thus indicating a potential antimicrobial effect which may be advantageous for cosmetic products. In particular, these extracts may provide self-preservative properties to the formulations in which they are incorporated.

As CS is the outer layer of the roasted coffee beans, it is conceivable that some of the properties described for coffee brews, namely antimicrobial and antioxidant properties, are also maintained in CS. So, we expect that CS has similar antioxidant and antimicrobial activities to coffee brews. The latter is probably a consequence of the high contents of melanoidins generated during roasting, because CS has a lower content of free phenol compounds [6, 10, 15]. Also, antibacterial activity of the extracts could be related to the presence of high amounts of phenolic and flavonoid compounds in their composition, as referred above. As shown, plants are rich in a wide variety of secondary metabolites, such as polyphenols and flavonoids, which have been found to have antimicrobial properties *in vitro* [298]. These compounds have been suggested to interact with microorganism's cell membrane causing protein membrane damages and consequently inducing its disruption. This effect leads to leakage of cellular content and causes interference with active transport and metabolic enzymes, as also as cytoplasm coagulation [298-300]. This cascade effects result in cell death. According to Dorman and Deans the high phenolic antibacterial activity could also be explained in terms of phenol nucleus alkyl substitution [301]. Its alkylation alters the distribution ratio between the aqueous/nonaqueous phases by reducing the surface tension or altering the species selectivity. This alkyl substituted phenolic compounds form phenoxy radicals which interact with isomeric alkyl substituent's [302]. The hydroxyl group relative position seems to exert an influence upon the components effectiveness against Gram-negative and Gram-positive bacteria [301].

The results of previous reports about coffee, along with our findings in CS, can confirm the inhibitory effects of CS extracts on growth and development of fungi and bacteria. Further studies, to identify the responsible molecules for antibacterial activity observed, are being developed in our laboratory.

The cytotoxicity results appear to indicate that fibroblasts are more sensitive to all extracts as compared to HaCaT cells, as previously mentioned by other authors to similar compounds [172, 245]. Furthermore, these results suggested that all extract of CS, at concentrations < 1000 µg/mL, have no adverse effect on the viability of fibroblast cells *in vitro*. These results could indicate a probable protective effect of the extracts on the cell line, but further studies are needed to confirm that. Ekmekcioglu *et al.* studied the effect of coffee in Caco-2 cells and no cytotoxicity was observed [303]. Other by-products, like brewers' spent grain, a protein-rich by-product of the brewing industry, has been tested for their cytotoxicity effect [304]. McCarthy *et al.* studied the cytotoxicity effect of Brewers' spent grain, against U937 and Jurkat T cells and concluded that cytotoxicity is low [304]. Over the three peel ethanolic extracts analyzed (*Punica granatum* (pomegranate), *Nephelium lappaceum* (rambutan), and *Garcinia mangostana* (mangosteen)) only rambutan may be considered potentially useful as a source of natural antioxidants for food or drug product because of its high antioxidant activity and non-toxic property to normal cells, at concentrations above 100 µg/mL [305]. In the same way, Khonkarn *et al.* studied the cytotoxicity of fruit peel extracts from rambutan, mangosteen and coconut against KB (human epidermal carcinoma of the mouth with HeLa contaminant) and Caco-2 cells and proved that coconut showed a high cytotoxic effect towards KB cells for concentration above 100 µg/mL [306]. This means, that most of by-products extracts have some cytotoxicity. However, CS showed no cytotoxicity for concentrations above 1000 µg/mL. For the best of our knowledge, this is the first study that reports the effects of CS in skin cell lines. On another hand, most of the studies of cell toxicity of by-products are not done in fibroblast or keratinocytes. The unique study that reports the use of these cells with by-products was done by Rodrigues *et al.*, who studied the effect of *Medicago* in fibroblast and keratinocytes cells, and, as well, no cytotoxicity was observed [101].

## 5. Conclusion

The CS extracts showed high polyphenol content, mainly in the ethanolic extract that also presented high flavonoid content. The antioxidant activity was high and positive correlations with polyphenol content were observed. Also, a good antimicrobial activity

was observed against *K. pneumoniae*, *S. epidermidis* and *S. aureus*, with no cytotoxicity of all extracts to fibroblasts and keratinocytes at tested concentrations.

In conclusion, CS ethanol extract can be considered the best one, as it expressed high antioxidant and antimicrobial potential with no toxicity. In this way, CS extracts may constitute a promising source of good antioxidative agents. However, investigation of the active constituents, associated with further purification, may provide useful comparative information in the future.

## II. Coffee Silverskin: A Possible New Cosmetic Ingredient?

The information presented in this chapter section was published in the following publication:

- **Francisca Rodrigues**, Carla Pereira, Filipa B. Pimentel, Rita C. Alves, Marta Ferreira, Bruno Sarmiento, M. Helena Amaral, M. Beatriz P.P. Oliveira, Are coffee silverskin extracts safe for topical use? An *in vitro* and *in vivo* approach, *Industrial Crops and Products*, 63:167-174, 2015

## 1. Introduction

In recent years, sustainable approaches have become an essential challenge for different industries. Answering to this issue, the present study was undertaken to investigate the potential use of Coffee Silverskin (CS) extracts in cosmetic products. Usually, per coffee fruit are found two coffee beans, each one covered by a thin closely skin called silverskin [10]. CS is a main by-product of the coffee roasting procedure and has no commercial value, being discarded as a solid waste [10]. This may have negative effects on the environment requiring proper management. Recent advances in industrial biotechnology leads to potential opportunities for economic valorisation of this by-product [282]. Some work has been performed on the properties of CS, in particular its antioxidant behaviour, which reveals a good content [15-16, 20-22, 285]. An example is chlorogenic acid (CGA), which is a highly valuable natural polyphenol compound used in medicine and industries [307]. However, some process steps in coffee production could affect CGA content, such as roasting, decaffeination and/or blending [308]. Also, the caffeine (CAF) content should be very high and similar to that of coffee beans [16, 19, 21]. These compounds are believed to provide *in vivo* protection against free radical damage. As coffee beans, CS contains several classes of health compounds such as phenolics, diterpenes, xanthines, and vitamin precursors [283-284]. Caffeine is being increasingly used in cosmetics due to its high biological activity and ability to penetrate the skin barrier [309]. A number of claims, as anti-cellulite properties, are based on the implicit assumption that this bioactive substance is effectively released from the formulation into epidermis and probably through epidermis into the dermal and subcutaneous tissues, preventing the excessive accumulation of fat in cells, and providing a slimming effect [309-310]. This alkaloid stimulates the degradation of fats during lipolysis through inhibition of the phosphodiesterase activity and has potent antioxidant properties [309]. However, studies assessing the skin absorption of caffeine released from extracts are extremely rare in the literature. Another compound that should be taken in consideration regarding CS is 5-hydroxymethyl furfural (HMF), which is formed during coffee roasting [311]. HMF is cytotoxic, irritating to the eyes, skin and mucous membranes at high concentrations, being very important to quantify its presence in CS extracts [312].

The assessment of irritation is one of the primary procedures to evaluate and hazards classify a substance, particularly, in cosmetics or pharmaceuticals [117]. In line with the 7th amendment deadlines, European Union bans the *in vivo* skin irritation assessment on ingredients for cosmetic purposes, regarding concerns about the test's

reproducibility, plus animal welfare and political pressures [313]. A number of *in vitro* tests to assess potential skin or eye irritants have been developed. The reconstructed human epidermis EpiSkin™ test method was validated by European Union Reference Laboratory for alternatives to animal testing (ECVAM) as replacement test for the prediction of acute skin irritation. According to Cotovio *et al.*, the assessment of ocular irritation is also one of the primary procedures to evaluate and hazards classify a new substance [117]. The *in vivo* Draize irritation rabbit eye test continues to be described in the current OECD test guidelines, but ethical questions are leading to the development of *in vitro* alternative tests, such as Human Corneal Epithelial Model (SkinEthic™ HCE), which are still under validation by ECVAM.

The aim of this study is to evaluate the *in vitro* and *in vivo* irritation potential of three CS extracts. For both EpiSkin™ and SkinEthic HCE™ assays, MTT and IL-1 $\alpha$  were used as endpoints. After the extract contact with the model, the histology of the model was evaluated. To ensure the possible content of CAF, CQA and HMF that pass through RhE, an HPLC assay was performed. *In vivo* skin irritation potential observed after single application under occlusion was assessed along with sodium lauryl sulfate (SLS) solution (2%, w/v) as irritant model (positive control) and water as non-irritant (negative control).

## 2. Materials and Methods

### 2.1 Chemicals

CS samples were provided by a national coffee roaster company (BICAFÉ – Torrefação e Comércio de Café Lda, Portugal). EpiSkin™ and SkinEthic™ HCE model were purchased from SkinEthic Laboratories (Lyon, France). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and sodium lauryl sulfate (SLS; purity > 99%) were purchased from Sigma-Aldrich Chemical (St Quentin Fallavier, France). ELISA Quantikin kit for IL-1 $\alpha$  measurements was from R&D Systems (Lille, France). Deionised water was obtained using Mili-Q water purification system (TGI Pure Water Systems, USA). Ethanol was obtained from Panreac (Barcelona, Spain). HPLC-grade acetonitrile was from Fluka (Madrid, Spain). 5-(Hydroxymethyl)furfural, caffeine and chlorogenic acid standards were from Sigma-Aldrich (Steinheim, Germany). Metaphosphoric acid, isopropanol, phosphate buffered saline (PBS; pH 7.4) and paraformaldehyde were from Sigma - Aldrich (St. Louis, Missouri, USA).

## 2.2 Preparation of extracts

CS was milled to particle size of approximately 0.1 mm using a A11 basic analysis mill (IKA Wearke, Staufen, Germany) and stored in silicone tubes at room temperature until extract preparation, as described by different authors [22]. Samples (1 g) were submitted to solvent extraction by maceration with 20 mL of water, ethanol: water (1:1 v/v) or ethanol for 30 min at 40 °C. The three different extracts obtained were filtered through Whatman No. 1 paper filter and the filtrates collected [20].

## 2.3 *In vitro* models assays

In order to determine the irritant potential of CS extracts on skin and eye, *in vitro* tests were performed. Three different extracts mentioned above were tested.

The EpiSkin™ model (large model) was obtained from a standardized large-scale production certified by the ISO 9001 standard. EpiSkin™ units were delivered to the laboratory within 24 h. Upon arrival, tissues were transferred to 12 well plates containing 37 °C pre-warmed maintenance media (2 mL/well) and incubated overnight at 37 °C/ 5% CO<sub>2</sub> and 95% relative humidity.

The SkinEthic™ HCE model is composed of immortalized human corneal epithelial cells cultured in a chemically defined medium and seeded on a synthetic membrane at the air-liquid interface. The tissue is represented by a multilayered epithelium, with 5-7 cell layers and a surface area of 0.5 cm<sup>2</sup>. The assay was performed according to the manufacturer instructions [117, 314]. Upon arrival, tissues were transferred to 24 well plates containing 37 °C pre-warmed maintenance media (1 mL/well) and incubated 24 hours at 37 °C, 5% CO<sub>2</sub> and 95% relative humidity.

### 2.3.1 Skin irritation test

The *in vitro* reconstructed human skin tissue (EpiSkin™) method, proposed to replace animal testing for skin corrosivity and skin irritation [315], is based on determining cell viability, and cytokine release (IL-1 $\alpha$ ) as an additional endpoint. The reconstructed 0.38 cm<sup>2</sup> skin inserts were used and the assay was performed according to the manufacturer instructions and protocol. Negative (PBS-treated) and positive controls (5% (w/v) of SLS) were used. After exposing to the extracts and controls for 15 min, the epidermis samples were washed with sterile PBS and then incubated in the maintenance

medium. After 42 h, the medium was collected and frozen at -20 °C for further determination of IL-1 $\alpha$ . Cell viability was determined by the MTT assay. Viability was calculated, considering 100% for the negative control. The histology of models was also evaluated as described below.

### 2.3.2 Ocular irritation test

The SkinEthic™ HCE model was used and the assay performed according to the manufacturer instructions and protocol. Negative (PBS-treated) and positive controls (ethanol) were used. After exposing to the extracts and controls for 10 min, the epidermis samples were washed with sterile PBS and then incubated in the maintenance medium for 16 h. After this time, the medium was collected and frozen at -20 °C for further determination of IL-1 $\alpha$ , and the cell viability was determined by the MTT assay. Viability was calculated, considering 100% for the negative control. The model was also analysed regarding the histological aspects.

### 2.3.3 MTT assay

Immediately after rinsing, the tissues were evaluated for cell viability using the MTT assay, where yellow MTT is reduced to purple formazan primarily by enzymes (reductases) located in the mitochondria of living cells. A stock solution of MTT was prepared in maintenance media (provided with tissues) just prior to use and warmed to 37 °C in a water bath. Tissues were transferred to 24-well plates (Corning Inc., Corning, New York) containing 300  $\mu$ L MTT medium per well and placed in the 37 °C, 5% CO<sub>2</sub> humidified incubator. After 3 h incubation, the tissues were removed from the MTT medium and any residual MTT media on the exterior of the tissue insert was blotted with absorbent pads. The formazan salt was extracted from the tissues by transferring them to 24-well plates containing isopropanol. The submerged tissues were incubated 3 h at room temperature protected from light. The plates were shaken for approximately 15 min once the extraction was complete. The optical density of the extracted formazan was determined by transferring 200  $\mu$ L of each extraction solution into a clean 96-well plate. Isopropanol was used as a blank. The plates were read using a spectrophotometer (Synergy HT Microplate Reader; BioTek Instruments, Inc., Winooski, VT, USA) at 570 nm. Relative cell viability was calculated for each tissue as percent of the mean of the negative control tissues.



For both models, if the percentage of viability was  $> 50\%$ , the substance was predicted as Non-Irritant (EU classification: No label; GHS classification: No category); if the percentage of viability was  $\leq 50\%$ , the substance was predicted as Irritant (EU classification: R38, R41 or R36, depending on the model; GHS classification: Category 1 or Category 2) [117].

#### **2.3.4 IL-1 $\alpha$ assay**

For assessing the release of IL-1 $\alpha$ , the culture media underneath the tissues were collected at the end of the post-incubation period and kept at  $-20\text{ }^{\circ}\text{C}$  until measurement. The quantification of IL-1 $\alpha$  was performed using the commercial enzyme-linked immunosorbent assay (ELISA) kit Quantikine<sup>®</sup> DLA50 (R&D Systems, UK) according to the manufacturer's instructions. Mean concentrations (pg/mL) were obtained using duplicate measurements per tissue. IL-1 $\alpha$  released was expressed as absolute data.

#### **2.3.5 Histology**

After incubating in the presence of CS extracts, EpiSkin<sup>™</sup> and SkinEthic<sup>™</sup> HCE samples (three samples per extract) were fixed with 2% paraformaldehyde during 1 h and embedded in Richard-Allan Scientific<sup>™</sup> Neg-50<sup>™</sup> Frozen Section Medium (ThermoScientific<sup>™</sup>) before cryosections (7  $\mu\text{m}$ ) preparation (Microm HM550 cryostat; ThermoScientific<sup>™</sup>). Samples were stained with haematoxylin–eosin–safran (HE) and epidermal thickness was measured using image analysis with Axiovision software (Zeiss, Sartrouville, France). Each image was studied for changes to the epidermis and especially to cell morphology.

### **2.4 Analysis of CAF, HMF and CGA content in EpiSkin<sup>™</sup>**

The HPLC method used in this study to quantify HMF, CQA and CAF in CS extracts was based on the work of Lemos *et al.* and Rivelli *et al.* [316-317]. The chromatographic analysis was performed in a HPLC integrated system (Jasco, Japan) equipped with a PU-980 pump and a Jasco AS-950 automatic sampler with a 20  $\mu\text{L}$  loop. Detection was performed with a Jasco model MD-2010 multiwavelength diode-array detector (DAD). The column employed was a Luna 5U C18 (5  $\mu\text{m}$ , 150 x 4.60 mm) chromatographic column (Teknokroma, Spain). The mobile phase adopted was aqueous metaphosphoric acid solution: acetonitrile (82:18 v/v) with a flow rate of 1 mL/min and a

column temperature of 23 °C. Analytes were monitored at 275, 285 and 330 nm, respectively, for CAF, HMF and CGA, and quantification was performed on the basis of the internal standard method. Chromatographic data were analyzed using the ChromNAV Software (Jasco, Japan). Calibration curves were prepared for HMF (0.36 - 93.0 mg/mL), CGA (5.0 - 160.0 mg/mL) and CAF (4.25 - 136.0 mg/mL). Analyses were carried out in duplicate. Confirmation of compounds identities was performed by comparing retention times and co-elution with authentic standards and also by UV absorption spectra.

## **2.5 *In vivo* skin irritation**

### **2.5.1 Patch Test**

A single blinded study was done in order to evaluate the *in vivo* skin irritation. Twenty healthy individuals (15 women and 5 men) with a mean age of  $30 \pm 5$  years, without known dermatological diseases or allergy to substances in topical products, participated in the study, in accordance with Declaration of Helsinki. Informed consent was obtained from all volunteers. The volunteers were asked to not apply any topical products in the forearms 24 h before the beginning and throughout the test period. Additionally, solar exposure was forbidden. Along with extracts, SLS (2%, w/v, aqueous solution) and purified water were also assayed, respectively, as positive and negative control. Five sites were marked in the inner forearms of volunteers. Before application of patch tests, the areas on the ventral part of each forearm were marked using a skin marker, and basal values were obtained by non-invasive measuring method. After basal measures were taken, the patches were applied. Fifty microlitres of the test solutions were applied on a filter paper disc (12 mm, Filter Paper Discs, SmartPractice, Phoenix, USA) and occlusion was achieved with aluminium chambers (12 mm, Finn Chambers, SmartPractice, Phoenix, USA). Patches were removed after 48 h, and following a period of 2 h of observation patch test areas were evaluated by non-invasive measuring methods and visual scoring.

### **2.5.2 Visual and instrumental assessments**

The visual assessment of the degree of irritation was graded according to the following scale, previously used by Agner *et al.*: 0 - no reaction; 1 - weak, spotty erythema; 2 - well perceptible erythema covering the total exposure area; 3 - moderate erythema or severe erythema that covers the total exposure area [318]. The same scale

was used for edema: 0 - no reaction; 1 - weak, spotty edema; 2 - well perceptible edema covering the total exposure area; 3 - moderate or severe edema that covers the total exposure area [318].

Non-invasive biophysical measurements were also performed. All measurements were made with controlled temperature (20-22 °C) and relative humidity (45-55%). Transepidermal water loss (TEWL) is traditionally used to assess skin barrier function [319]. Barrier function was evaluated by TEWL measurements, carried out with a Tewameter® TM 210 (Courage & Khazaka, Köln, Germany), which measures the relative humidity percentage that diffuses from the dermis to the skin surface (g/cm<sup>2</sup> h).

## 2.6 Statistical analysis

Data were reported as mean ± standard deviation of at least triplicate experiments. Statistical analysis of the results was performed with the software SPSS 22.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to investigate the differences between different extracts for all assays. Post hoc comparisons were performed according to Tukey's HSD test. In all cases,  $p < 0.05$  was accepted as denoting significance.

## 3. Results and Discussion

### 3.1 Skin irritability tests

Irritation is defined as the “production of reversible damage to the skin following the application of a test substance for up to 4 h”, therefore, the more significant the initial injury (cell death), the stronger the irritant effect [320]. In order to confirm the potential of the extracts for topical use, irritability assays with EpiSkin™ test were performed. EpiSkin™ is an alternative *in vitro* method efficient in predicting epidermal alterations *in vivo* caused by irritants. Data for the cell viability and the IL-1 $\alpha$  release from *in vitro* assays with reconstituted human epidermis are shown in Table 5.5.

Table 5.5 - EpiSkin™ tissues were exposed for 15 min to controls and extracts. Cell viability was assessed by MTT while the post-exposure basal media was analyzed for IL-1 $\alpha$  release (pg/mL).

Extract	Viability (%)	IL-1 $\alpha$ (pg/mL)
<b>W</b>	117.7 $\pm$ 11.7 <sup>a</sup>	8.5 $\pm$ 0.1 <sup>c</sup>
<b>WA</b>	105.9 $\pm$ 15.9 <sup>a</sup>	76.4 $\pm$ 2.0 <sup>b</sup>
<b>A</b>	117.8 $\pm$ 9.8 <sup>a</sup>	17.6 $\pm$ 3.2 <sup>c</sup>
<b>PC (SLS)</b>	11.6 $\pm$ 2.4 <sup>b</sup>	522.9 $\pm$ 32.1 <sup>a</sup>
<b>NC (PBS)</b>	100 $\pm$ 9.7 <sup>a</sup>	28.4 $\pm$ 2.6 <sup>b,c</sup>

Water (W), hydroalcoholic mixture (WA), alcohol (A), a positive control (PC: SLS – sodium lauryl sulfate) and a negative control (NC: PBS - phosphate buffered saline) were used. Values are expressed as mean  $\pm$  SD (n = 3). Values in the same column followed by different letters indicate significant differences (p<0.05).

The method distinguishes between irritants and non-irritants. Irritant chemicals are identified by their ability to decrease cell viability below defined threshold levels of 50%. The three extracts are not skin irritants for topical use in cosmetic formulations as the viability in all cases is higher than 50%, with values ranging between 105.9  $\pm$  15.9 and 117.8  $\pm$  9.8. This potential use was also confirmed by the low release of IL-1 $\alpha$  as endpoint of the irritation process (in the range of negative control), produced by keratinocytes. IL-1 $\alpha$  is a highly active and pleiotropic pro-inflammatory cytokine that play a key role in inflammation, being the biological mirror of skin irritation [321]. Keratinocytes produces IL-1 $\alpha$  and IL-1 $\beta$  mRNA *in vitro*, but only IL-1 $\alpha$  biological activity has been identified in keratinocytes cultures [322]. According to Zhang *et al.*, the concentration of IL1- $\alpha$  released by keratinocytes in cultured medium increased significantly follower the exposure to different irritants [323].

The IL-1 $\alpha$  content, expressed as pg/mL, was 28.4  $\pm$  2.6 for PBS, 522.9  $\pm$  32.1 for SLS, and 8.5  $\pm$  0.1, 76.4  $\pm$  2.0 and 17.6  $\pm$  3.2, respectively, for aqueous, hydroalcoholic and alcoholic extract of CS. The absence of skin-irritant effects in extracts tested indicated that CS extracts could be safe for topical use.

The morphology alteration that occurs after the contact between the extracts and the model was evaluated for all samples and controls. Figure 5.1 shows the effects of hydroalcoholic extract and controls on the morphology of the treated tissues. No morphological differences were observed for the treated tissues with extracts as

compared to the negative control (PBS). In contrast, the adverse effects observed with positive control in all epidermal layers confirm the viability measurements observed. Results suggest that histological analysis did not assess alterations to the epidermis in human skin samples after exposure to CS extracts.

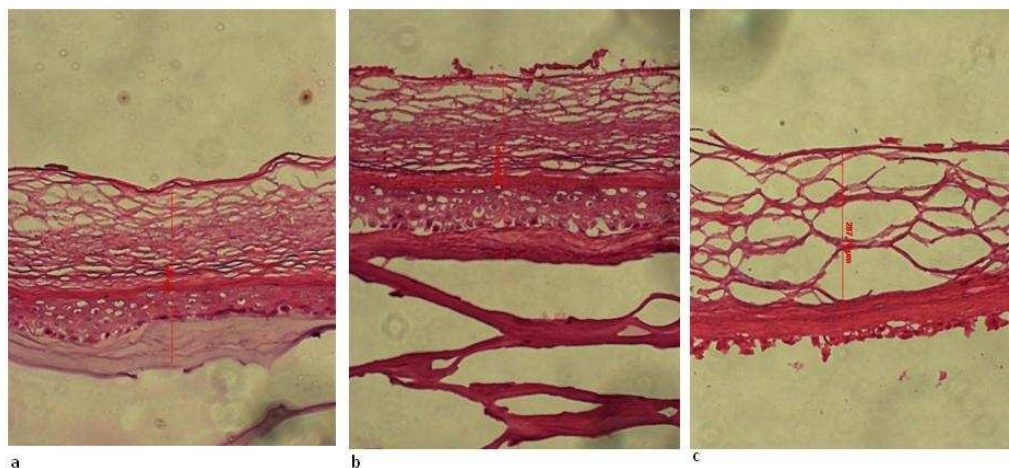


Figure 5.1 - Hematoxylin-eosin (HE) staining of the EpiSkin™ tissues. (a) After topical application of 10  $\mu$ l of hydroalcoholic extract of CS: no irritation signs. (b) Negative control (100% cell viability): no irritation signs. (c) Positive control (5% SDS): marked epidermolysis.

There are very few reports of irritation potential in skin models using extracts as this model was recently validated by ECVAM. Balboa *et al.* evaluated the *in vitro* antioxidants properties and the skin irritant effects of natural extracts obtained from underutilized and residual vegetal and macroalgal biomass [321]. Extracts did not affect the human reconstructed epidermis but the cell viability was lower than in this study. Regarding the IL-1 $\alpha$  release, the irritation process seems to be lower. This could be justified by the different solvents used in both studies. Considering the extract composition, reported by Rodrigues *et al.*, it is also expected no effect on skin cells, since the identified compounds did not interact with cells, presenting an high antioxidant activity and absence of cytotoxicity in keratinocytes and fibroblast [22]. Also, washing procedures could not remove all materials and even lead to mechanical damage to the tissues, which results in an impaired prediction of the true skin irritation potential of the materials [324].

### 3.2 Analytical methods

A further part of the study was concerned with the analysis of CAF, CGA and HMF in the medium after the skin *in vitro* model assay for the three extracts. Chromatograms of CS extracts and standards are depicted in Figure 5.2. Compounds were numbered according to their elution order.

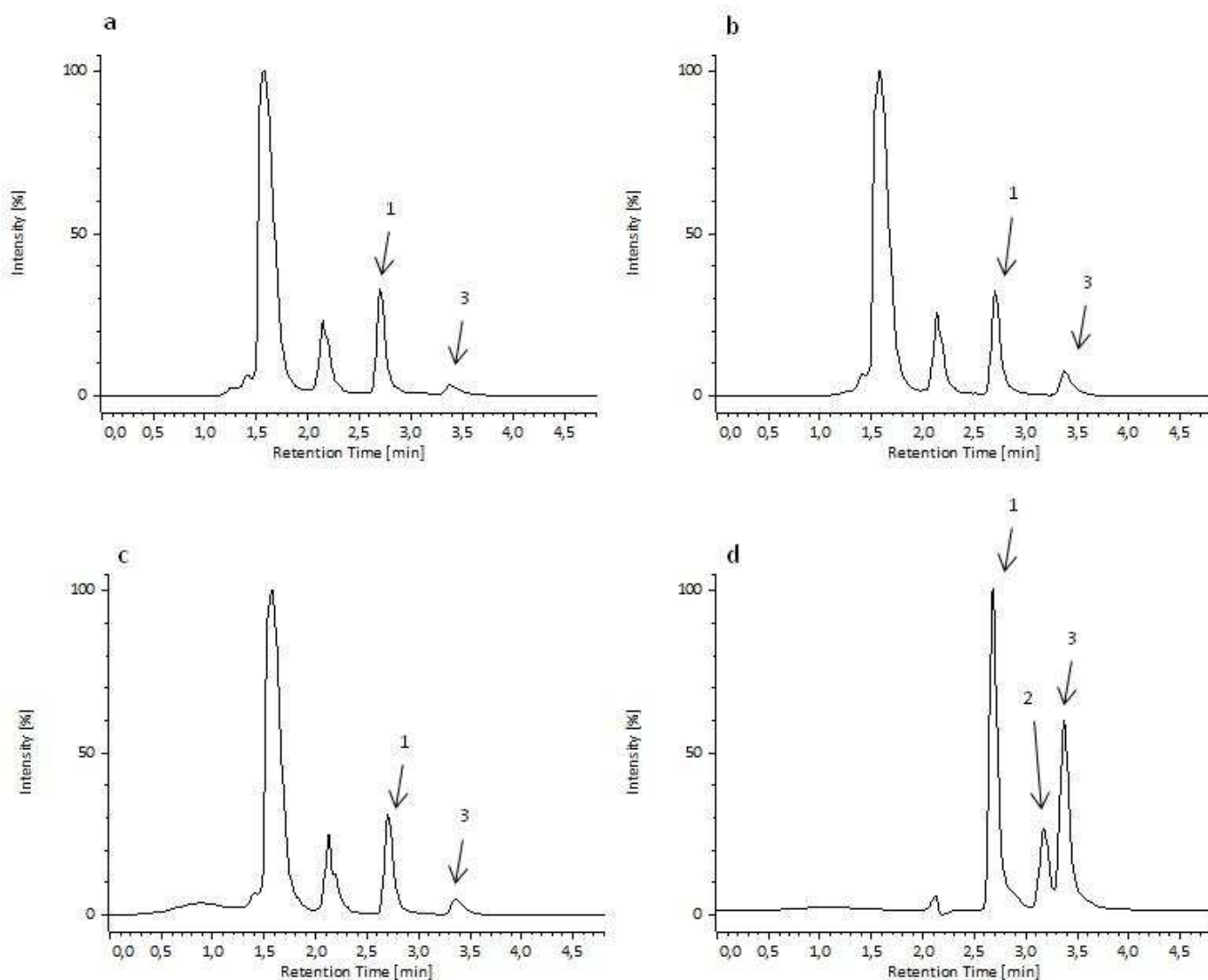


Figure 5.2 - Chromatograms of aqueous extract (a), hydroalcoholic extract (b) and alcoholic extract (c) of CS and a standard mixture (1 mg/mL) (d) of HMF (1), CQA (2) and CAF (3).

HPLC analysis of the CS extracts indicated the presence of CAF and HMF and the absence of CGA for all extracts. The quantification of CAF and HMF is reported in Table 5.6.

Table 5.6 - Quantification of HMF, CGA and CAF in aqueous (W), hydroalcoholic (WA) and alcoholic (A) extracts of CS medium after EpiSkin™ assay.

Extract	HMF (µg/mL extract)	CGA (µg/mL extract)	CAF (µg/mL extract)
W	2.28 ± 0.18	-	1.26 ± 0.06
WA	2.39 ± 0.27	-	1.54 ± 0.14*
A	2.26 ± 0.09	-	1.40 ± 0.07

Values are expressed as mean ± SD (n = 3). \* means a significant difference (p<0.05).

CAF content in CS extracts ranged between 1.26 ± 0.06 and 1.54 ± 0.14 µg/mL for aqueous and hydroalcoholic ones, respectively. There is a statistical difference between the CAF content of hydroalcoholic extract and the other extracts. As previous mentioned, determination of CAF in CS is very important, since they have a great effect on the final quality of the extracts used for cosmetic proposes. Previously work done in our laboratory, evaluated the CGA content in CS hydroalcoholic extract and results demonstrated the presence of this compound [325]. The absence observed in the present study is justified by the retention effect of the epidermal model, which prevents the compounds passage. For the best of our knowledge, this is the first study that reports the content of CAF after an EpiSkin™ assay using extracts.

### 3.3 Ocular irritability tests

The assessment of ocular irritation is part of the early testing procedure for the evaluation of new cosmetic ingredients [117]. The use of cosmetic products can produce adverse effects on the ocular surface, ranging from mild discomfort to vision-threatening conditions [326]. These complications can be related to allergy or toxicity [326]. In this field, numerous non animal test systems have been developed over the years [327-328]. SkinEthic™ HCE is one of the two reconstructed human tissue that are currently available for the purpose of eye irritation and is under validation by ECVAM [314].

In order to evaluate the eye irritation potential of CS extracts, a SkinEthic™ HCE test was done. Table 5.7 summarizes the results for cell viability and release of IL-1 $\alpha$  observed with extracts and controls.

Table 5.7 - SkinEthic™ HCE model was exposed for 10 min to controls and extracts. Cell viability was assessed by MTT while the post-exposure basal media was analyzed for IL-1 $\alpha$  release (pg/mL).

<b>Extract</b>	<b>Viability (%)</b>	<b>IL-1<math>\alpha</math> (pg/mL)</b>
<b>W</b>	132.6 $\pm$ 21.3 <sup>a</sup>	41.4 $\pm$ 2.5 <sup>c</sup>
<b>WA</b>	123.6 $\pm$ 13.7 <sup>a</sup>	34.2 $\pm$ 1.6 <sup>c</sup>
<b>A</b>	48.0 $\pm$ 7.5 <sup>c</sup>	82.2 $\pm$ 22.9 <sup>b</sup>
<b>PC (Ethanol)</b>	2.9 $\pm$ 0.5 <sup>d</sup>	114.4 $\pm$ 2.5 <sup>a</sup>
<b>NC (PBS)</b>	86.0 $\pm$ 6.1 <sup>b</sup>	23.7 $\pm$ 1.0 <sup>c</sup>

Water (W), hydroalcoholic mixture (WA), alcohol (A), a positive control (PC) and a negative control (NC: PBS - phosphate buffered saline) were used. Values are expressed as mean  $\pm$  SD (n = 3). Values in the same column followed by different letters indicate significant differences (p<0.05).

The viability of cells with aqueous and hydroalcoholic extracts were higher than 50%, with percentages of 132.6  $\pm$  21.3 and 123.6  $\pm$  13.7, respectively, which means that the extracts were classified as non irritants for the eyes. Ethanolic extract presented a percentage of viability of 48.0  $\pm$  7.5, being in the borderline between irritant and non irritant, which is justify by the higher amount of alcohol used. Regarding the IL-1 $\alpha$  release, no significant differences were observed between negative control and aqueous or hydroalcoholic extracts. However, the release of IL-1 $\alpha$  slightly increased when using the positive control and ethanol extract as compared to the negative control. As mentioned by manufacture, ethanol was used as positive control.

The histological images obtained after topical application of the aqueous and hydroalcoholic extracts of CS also confirms that both are not irritant, being good candidates for cosmetic ingredients. Regarding alcoholic extract, results are very



different. As it is possible to confirm, this extract leads to histological alterations in SkinEthic™ HCE tissues, similarly to positive control (Figure 5.3).

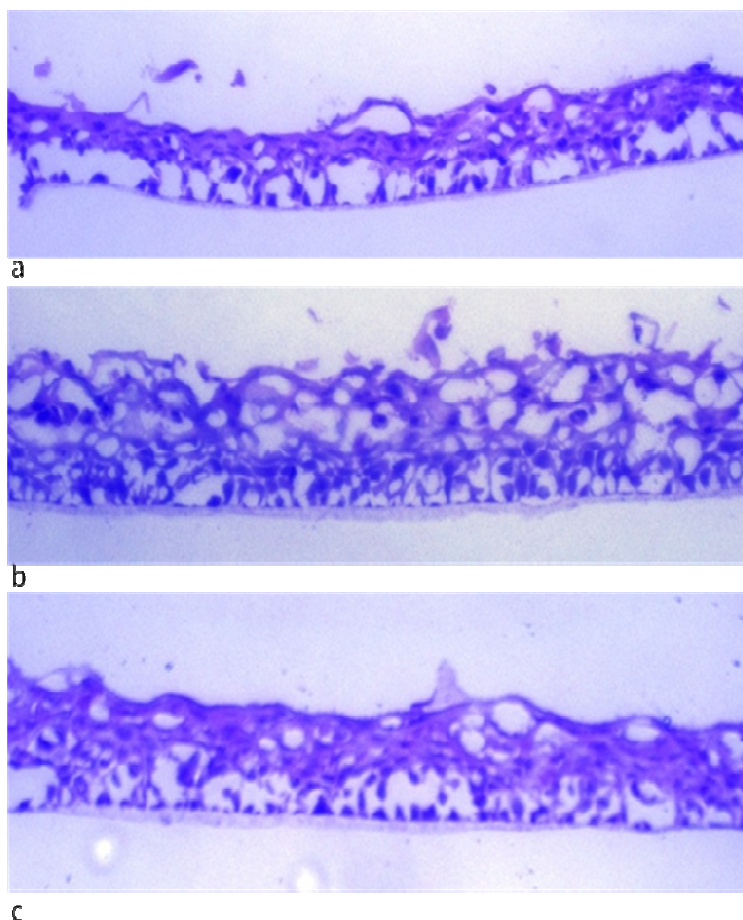


Figure 5.3 - Hematoxylin-eosin (HE) staining of the SkinEthic™ HCE tissues. After topical application of 10  $\mu$ l of alcoholic (a) extract of CS: irritation signals. (b) Negative control (100% cell viability): no irritation signs. (c) Positive control (5% SLS): marked epidermolysis.

As far as we know, this is also the first time that SkinEthic™ HCE is used to evaluate the ocular irritation for food by-products extracts in order to incorporate them in cosmetics as active ingredients.

### 3.4 *In vivo* test

According to Miles *et al.*, reconstructed human epidermis like EpiSkin™ has some limitations [329]. This model is composed of a highly differentiated multi-layer human epidermis cells, but are totally lacking in any network of the pilo-sebaceous units, which regulate dermal homeostasis [329]. Also, the hydrolipid film cannot be totally reproduced

regarding its bio-physiological properties of components (urea, glycolic acid) neither to the occlusive effect of the film's lipid component (ceramides) [329]. Some cells normally present in human skin are not present in this model such as dendritic Langerhans' cells. The higher penetration rate of these tissues makes these skin models more sensitive to test compounds [330], which could result in over-prediction of strong irritants [331]. Thus, to ensure that the extract is completely safe, some other assays like *patch test* should be done. Patch testing after a single application is a widely used procedure to evaluate acute irritant reactions in human volunteers.

The previous assays led us to select the hydroalcoholic extract as the best one to be incorporated in cosmetic products, as it did not affect the morphology of skin and ocular models, with high cell viability for both assays and low IL-1 $\alpha$  release. Also the CAF content was higher than in the other extracts evaluated and, according to Rodrigues *et al.*, the antioxidant content and antimicrobial activity is higher [22]. Considering not only these results but also sustainability questions and other previous works regarding the antioxidant activity of these extracts, hydroalcoholic extract seems to be the best one, being selected for the final patch test [19-21].

### **3.4.1 Skin compatibility by evaluation of primary skin irritation**

Initially, evaluation of irritancy testing was based on visual score. Results obtained by visual analyse are shown in Figure 5.4. Results of patch testing demonstrated that hydroalcoholic provoked a slight erythema in three volunteers 2 h after the patch test removal. However, when statistical analyses were applied regarding negative control, no differences were found. Regarding the positive control, it is possible to observe statistical differences when comparing with negative control and hydroalcoholic extract ( $p < 0.05$ ).

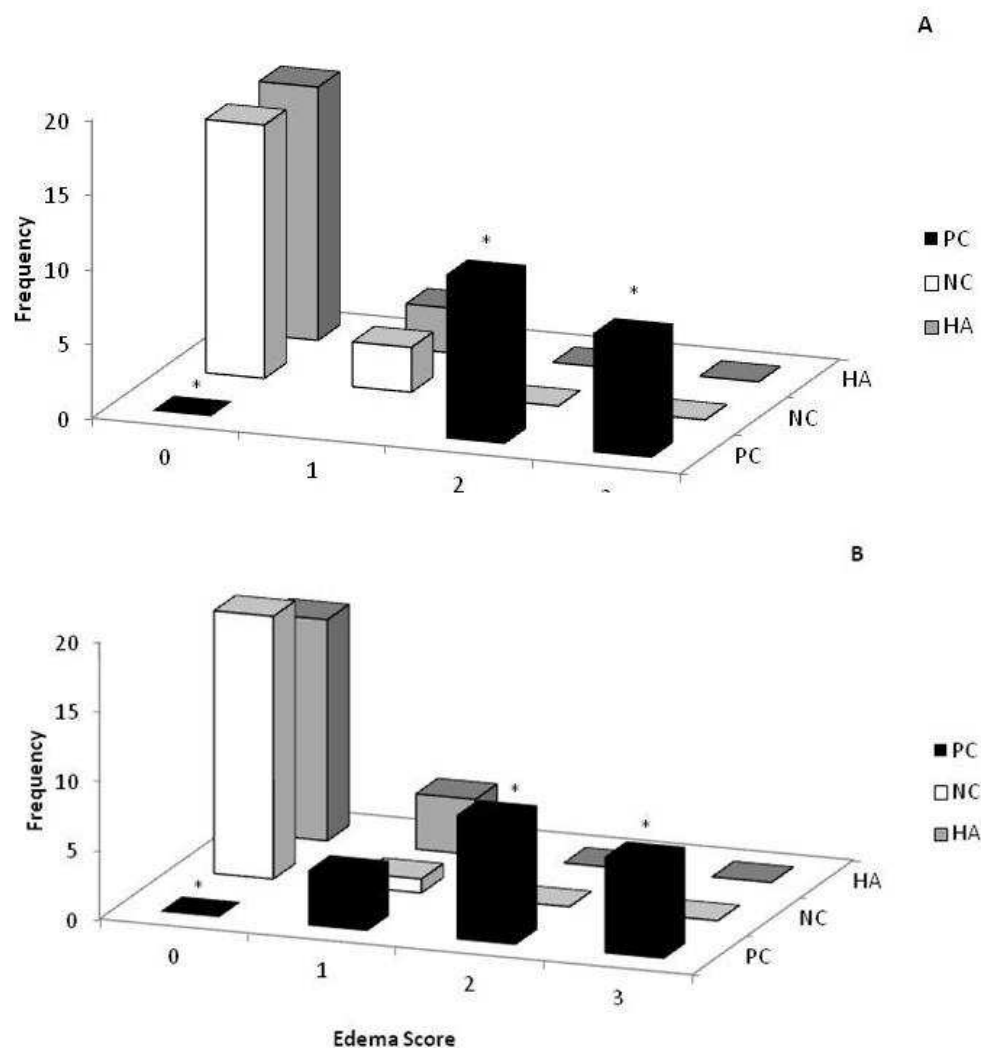


Figure 5.4 - Frequency of clinical scores attributed to positive control (PC - SLS), negative control (NC - purified water) and hydroalcoholic extract of CS (HA) regarding erythema and edema after patch remove.\* means a significant difference ( $p < 0.05$ ).

### 3.4.2 Transepidermal water loss variation

The measurement of transepidermal water loss (TEWL) is a well established method in dermatology to assess the integrity of the skin barrier *in vivo* and an important measure of epidermal barrier function [332]. When skin is damaged, the barrier function is impaired which results in a higher water loss. The extract did not alter the skin barrier function, as TEWL values were not changed, when compared to the positive control (Figure 5.5).

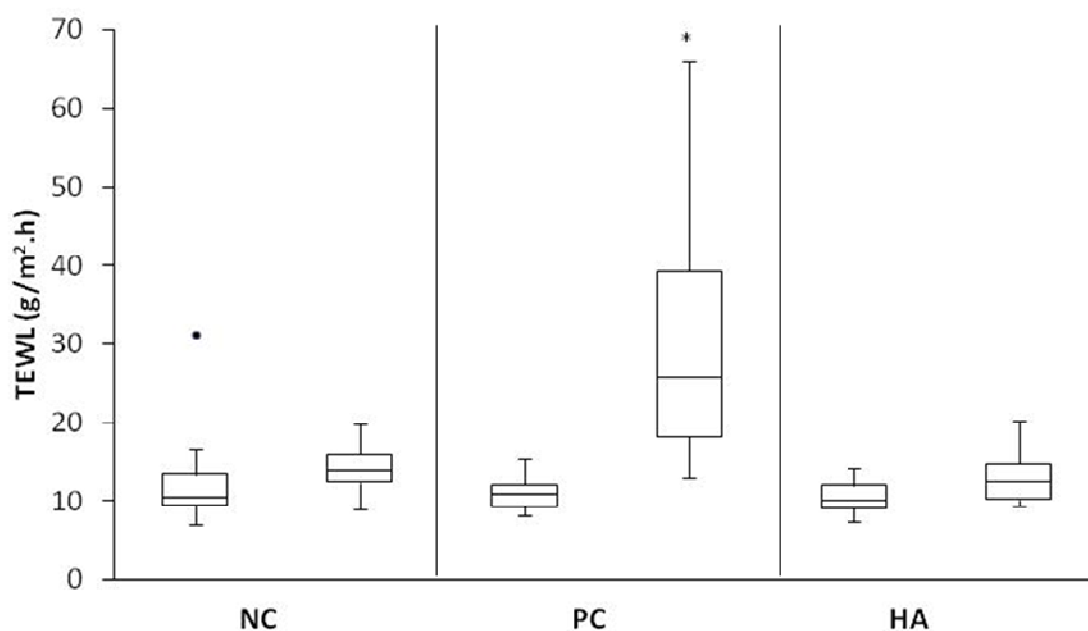


Figure 5.5 - Transepidermal water loss (TEWL) variation from basal values of negative control (NC), positive control (PC) and hydroalcoholic extract (HA). The left boxes represent values at time 0 and the right boxes represent values 2 h after patch removal. Outliers are presented as circles. \* means a significant difference ( $p < 0.05$ ).

No significant differences were observed for TEWL measurements, in comparison with purified water, 2 h after patch removal ( $p < 0.05$ ), which indicates the absence of barrier disruption. Consequently, CS hydroalcoholic extract is not potentially irritating. The only effect on TEWL values was probably due to the eventual formation of a greasy film by the lipophilic components of the extract. Comparing with other extracts that are under investigation, CS could be considered as safe for cosmetic purposes [333-334]. Unlike arnica and *Calendula officinalis*, CS did not originate skin irritation [335]. As expected, SLS (PC) leads to an increase of TEWL values.

#### 4. Conclusion

This study constitutes an objective evaluation of the safety of CS extract as cosmetic ingredient and contributes to the elucidation of its mechanism of action. It is an overview of the effect of CS extracts in a validated human skin model for irritancy and eye model under validation. *In vitro* studies on ocular and dermal irritation were carried out with three different extracts of CS. The *in vivo* study was carried out with the extract that revealed the best results for the *in vitro* assays. Results of *in vitro* studies revealed that

CS extracts are safe regarding skin and ocular irritancy as cell viability was equal to the negative control in both assays and the IL-1 $\alpha$  was under 50%. The histological analysis demonstrated that extracts did not affect the skin neither the ocular model. Quantitative chromatographic investigations revealed that the three extracts contained CAF and HMF, but there were no traces of CGA. The *in vivo* patch tests proved that the hydroalcoholic extract did not cause skin irritation. The next steps to evaluate the safety of the extract would undergo sensitization and tolerance studies in normal condition of use.

## I. *In Vitro* and *In Vivo* Evaluation

The information presented in this chapter section was published in the following publication:

- **Francisca Rodrigues**, Carlos Gaspar, Ana Palmeira-de-Oliveira, Bruno Sarmento, M. Helena Amaral, M. Beatriz P.P. Oliveira, Application of coffee silverskin in cosmetic formulations: Physical / antioxidant stability studies and cytotoxicity effects, *Drug Development and Industrial Pharmacy*, 2015, *in press*

## 1. Introduction

Agro-industrial by-products have the potential to be used with different purposes, providing economical advantage to otherwise disposable residues [101]. In particular, the field of skin care products and cosmetics may benefit from these remaining materials [101]. During the last decade consumers have widely expressed their interest in natural products forcing the industry and the scientific community to search for alternative sources of raw materials. The desirable features of cosmetic ingredients are efficacy, safety, novelty, formulation stability, easy metabolism in the skin and low cost [101]. One of the most important reason for this change is the appearance of allergies and skin irritations due to synthetic preservatives (e.g. parabens), colourants, stabilizers, etc., that have not yet been fully tested in the long run for their consequences on consumers' health [336].

Coffee is one of the popular beverages of the world and second largest traded commodity after petroleum [9]. Coffee dispensation requires an elevated degree of processing know-how and produces large amounts of processing by-products such as coffee pulp, husk and silverskin, which have limited applications such as fertilizer and livestock feed [9-10]. Advances in industrial biotechnology offer potential opportunities for economic utilization of agro-industrial residues of coffee [282]. Coffee silverskin is a tegument of coffee beans that constitutes a by-product of the roasting procedure and is the most abundant solid by-product generated during the coffee process. Different authors evaluated the coffee silverskin properties, in particular its antioxidant properties and cytotoxicity against epidermal cells, but also the inhibitory activity on hyaluronidase [15-16, 21-22]. According to different authors, coffee silverskin extracts are rich in polyphenol compounds, particularly chlorogenic acids and caffeine [21-22]. Thus, the use of coffee silverskin for topical application is a new procedure. Caffeine has been used in pharmaceutical and cosmetic preparations for a long time on the basis of its many favourable effects on the skin [337]. As proved by Bresciani *et al.*, coffee silverskin contains the same amounts of caffeine as coffee beans [19]. However, cosmetic formulations containing coffee silverskin have to be submitted to studies of chemical stability in order to evaluate their quality, since the integrity of the extract must be kept constant during the formulation shelf-life. Stability prediction is usually performed by accelerated storage conditions, such as temperature variation, to induce rapid chemical alterations in formulations. Also, the cytotoxicity of the final formulation should be evaluated previously of the *in vivo* studies.

Often, the antioxidant potential of either plant extracts with active compounds or the pure isolated compounds is evaluated, but very few reports are available on the antioxidant properties of final formulations. In the present study, the hydroalcoholic extract of coffee silverskin, rich in antioxidant compounds and low cytotoxicity, were incorporated into a hand cream. This extract is easily dispersed, non-oily and can carry hydrosoluble active principles, for antioxidant potential assessment [22]. For the best of our knowledge, there are no studies analyzing the stability of this extract when vehiculated in topical formulations and their cytotoxicity against different skin cell lines.

## **2. Materials and Methods**

### **2.1 Preparation of extracts**

Hydroalcoholic extract was prepared according to the method described by Rodrigues *et al.* [9]. Briefly, coffee silverskin was milled to particle size of approximately 0.1 mm using a A11 basic analysis mill (IKA Wearke, Staufen, Germany) and stored in silicone tubes at room temperature until extraction. Samples (1 g) were submitted to solvent extraction by maceration with 20 mL of ethanol: water (1:1) for 30 minutes at 40 °C. Extracts were filtered through Whatman No. 1 p aper filter and the filtrates collected.

### **2.2 Preparation of the formulation**

A hand cream was chosen for the experiments. The extract was added as antioxidant in the cosmetic preparation (CS). The hand cream was formulated with the following components: water, cetostearyl alcohol, dimethicone, jojoba oil, lactic acid, glycerin, sodium laureth sulfate, triethanolamine (if the mixture had acid pH), parfum and preservative. The base cream represents an oil-in-water semi-solid emulsion containing the same ingredients except the extract (BC). The extract was added in the final concentration of 2.5% and dissolved in the mixture after reaching the room temperature. The formulation was rested for 24 h at 20 °C before measurements. Formulation was developed taking into account the final acceptance for consumers and after a scale up study in industry, being prepared batches both at laboratory and in industrial machines.



## 2.3 Cytotoxicity

Two different assays were used to assess cell viability and cytotoxicity of the extracts: (1) monitoring the uptake and metabolism of the vital mitochondrial dye, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) by cell mitochondria and (2) determining the leakage of the cytosolic enzyme, lactate dehydrogenase (LDH) into the cell medium (LDH assay). In both tests, triplicate wells were incubated with fresh medium in the absence or presence of both formulations dissolved in cell culture medium containing 1, 5 and 10 mg/mL of formulations. Formulations dissolved in culture medium were filtered on 0.45 µm Millex GV filters (Millipore, Nepean, ON, Canada) and exposed to keratinocytes (HaCaT) and fibroblasts (HFF-1) cells during 24 h.

### 2.3.1 Cell lines and culture conditions

Human immortalized non-tumorigenic keratinocyte cell line HaCaT (ethnicity, Caucasian; age, 62 years; gender, male and tissue, skin) was acquired from CLS Cell Lines Service, Germany. Human foreskin fibroblasts (HFF-1) were purchased from ATCC (ATCC Number: SCRC-1041; ATCC, Manassas, VA, USA). Passage 17-19 of HFF-1 and passage 77-79 of HaCaT cells were used for both MTS and LDH assay. HaCaT and HFF-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™-I (Invitrogen) supplemented with 10% FBS, 100 µg/mL penicillin and 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B, and maintained in a 5% CO<sub>2</sub> environment at 37 °C. At 90-95% confluence, both cell lines were harvested using trypsin (Invitrogen), viability assessed using the trypan blue dye (Gibco) exclusion assay, and re-plated in T75 flasks.

### 2.3.2 MTS assay

The MTS assay was performed according to the manufacturer instructions. Briefly, cells were cultured in 96-well microtiter plate at a density of  $25 \times 10^3$  cells per mL culture medium for 24 h at 37 °C. Following the removal of medium from the wells, the cells were washed with phosphate buffered saline (PBS pH 7.4). Then the medium was changed to fresh medium containing different concentrations of both formulations, namely 1, 5 and 10 mg/mL. Cells were then incubated at 37 °C for 24 h. Then medium was rejected and the number of viable cells evaluated by adding the MTS reagent in medium to each well and

incubating for 4 h at 37 °C. The positive control was the medium and the negative control was Triton X-100. The absorbance was measured at 490 nm with background subtraction at 630 nm. Each concentration was tested in triplicate in three independent experiments.

### **2.3.3 LDH assay**

Lactate dehydrogenase (LDH), a stable cytoplasmic enzyme found in all cells, is quickly released upon damage to the plasma membrane. Cell lines were seeded in 96-well plates (5000 cells for LDH) and cultured for 48 h. Different concentration of the formulations, namely 1, 5 and 10 mg/mL, were prepared in appropriate medium and added to the cells. Media without FBS were used in the case of LDH. After incubation (37 °C/ 5% CO<sub>2</sub>), cells were washed twice with PBS and the kit was used according to the instructions of the manufacturers. The maximum LDH release (high control) from cells was determined using 1% (w/v) Triton X-100 (Boehringer Mannheim). Spontaneous LDH release (low control) was determined by incubating the cells with plain medium. Absorbance was measured at 490 nm with background subtraction at 690. Each concentration was tested in triplicate in three independent experiments.

## **2.4 Long-term stability evaluation**

Batches of the formulation were prepared as earlier described and physical stability was evaluated by submitting the formulation to storage, for a period of 6 months, at two different conditions: 25 °C/ 65% relative humidity (RH) and at 40 °C/ 75% RH. Physical, chemical and microbiological properties were evaluated in samples from each formulation container at the initial time and after 30, 60, 90 and 180 days of storage using the following methods:

### **2.4.1 Particles morphology**

Morphological characterization was performed using an optical microscope (Eclipse E400, Nikon, Tokyo, Japan) equipped with a camera at 40x resolution (Coolpix 950, Nikon, Tokyo, Japan).

### **2.4.2 pH**

One gram of the formulation was diluted with 3 mL of neutral water and the pH of the aqueous mixture was analyzed with a pHmeter (691, Mettler, Greifensee, Switzerland) equipped with a glass electrode. The measurements were performed in triplicate for each sample.

### **2.4.3 Accelerated stability by centrifugation**

Samples (5 g) were centrifuged at 3000 rpm for 30 min (Centrifuge 5804, Eppendorf, Hamburg, Germany) in order to assess their stability.

### **2.4.4 Color**

Color was evaluated with a colorimeter (CR-400, Konica Minolta, Tokyo, Japan) and results were expressed according to the color space CIE 1976 L\*a\*b\*, defined by the Commission Internationale de l'Eclairage (CIE). The three coordinates represent the lightness of the color (L\*), its position between red and green (a\*) and its position between yellow and blue (b\*).

### **2.4.5 Rheological analysis**

Apparent viscosity was measured using a Brookfield rotational viscometer (Brookfield DV E, Cologne, Germany). Viscosity determinations were performed using a spindle number 5, sequentially increasing the values of shear rate in the range 3 - 50 rpm to obtain the ascending curve. The procedure was repeated in reverse with progressively slower rates (50 - 3 rpm) to obtain the descending curve. Temperature was kept constant at  $20 \pm 0.5$  °C and all samples were equilibrated at room temperature prior to viscosity measurement.

### **2.4.6 Texture analysis**

The textural analysis was performed in the compression mode using a texturometer (Stable Micro Systems TA-XT2i, Godalming, UK), by carrying out a penetration test (T=20 °C, penetration distance of 5 mm, test speed of 3 mm/s, probe with a diameter of 13 mm, load cell of 5 kg). From the graph force *versus* distance, the maximum force (N) corresponds to the firmness and the negative area (N.mm) corresponds to the adhesiveness.

## 2.5 Microbiological analysis

At time 0, 30, 60, 90 and 180 days, batches were evaluated for microbiological quality. Dilutions were performed with buffered peptone sterile solution and 3% w/v of polysorbate 80 was added to culture media to neutralize the preservative. A total colony count was performed using Tryptic soy agar (Oxoid Ltd, Hampshire, UK). Incubation was carried out during 5 days at 30-35 °C. For yeast and molds counting, sabouraud agar (bioMérieux, France) was used and incubation was carried out during 5 days at 25 °C. Additionally at time 0 and at time 180 days the preservative stability was tested according to the ISO 11930:2012 procedures.

## 2.6 Antioxidant activity

### 2.6.1 Determination of total phenolic content (TPC)

Total phenolic content (TPC) was spectrophotometrically determined according to the Folin-Ciocalteu procedure [185] with minor modifications. Briefly, 500 µL of formulation with proper dilution were mixed with 2.5 mL of Folin-Ciocalteu reagent (10 x dilutions) and allowed to react for 5 min. Then, 2.5 mL of Na<sub>2</sub>CO<sub>3</sub> 7.5% (w/v) solution was added and allowed to stand for 15 min at 45 °C, and then 30 min at room temperature, following the absorbance of the reaction mixture determined at 765 nm using a microplate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA). A calibration curve for the standard gallic acid was used to obtain a correlation between sample absorbance and standard concentration (linearity range = 5-100 µg/ml,  $r^2 > 0.998$ ). The TPC of both formulations were expressed as mg of gallic acid equivalents per gram of formulation. The assay was done in triplicate.

### 2.6.2 Determination of antioxidant activity

The antioxidant activity of the samples was evaluated by DPPH radical-scavenging activity. DPPH is known as a stable free radical which possesses a characteristic maximum absorption between 515 and 517 nm. In the presence of antioxidant compounds, the DPPH<sup>•</sup> is reduced to the corresponding hydrazine when reacts with hydrogen donors, such as an antioxidant, and lower absorbance values of the reactive mixture indicate higher free radical scavenging activity. This method is an essential tool to access the antioxidant potential, more specifically, the antiradical activity of formulations.

The reaction mixture on 96 wells plate consisted of one solution per well for different sample concentrations (30  $\mu\text{L}$ ) and ethanolic solution (270  $\mu\text{L}$ ) containing DPPH radicals ( $6 \times 10^{-5}$  mol/L). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm [188] using the microplate reader. The antioxidant capacity based on the DPPH free radical scavenging ability of the formulation was expressed as  $\mu\text{mol}$  Trolox equivalents per gram of formulation. The formulations were dissolved in isopropanol: DMSO (50:50) thorough shaking as in some cases interferences were observed due to non-transparent solutions that impair this photometric assay. A calibration curve was prepared with Trolox (linearity range: 2.5-100  $\mu\text{g}/\text{mL}$ ,  $r^2 > 0.996$ ).

## 2.7 Statistical analysis

Data were reported as mean  $\pm$  standard deviation of at least triplicate experiments. Statistical analysis of the results was performed with SPSS 22.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to investigate the differences between different formulations for all assays. Post hoc comparisons were performed according to Tukey's HSD test. In all cases,  $p < 0.05$  was accepted as denoting significance.

## 3. Results

### 3.1 Keratinocytes and fibroblasts cytotoxicity assay

The effects of both formulations on the growth of keratinocyte (HaCaT) and fibroblast (HFF-1) cells were investigated by MTS and LDH assays.

#### 3.1.1 MTS assay

Both formulations were added to HaCaT and HFF-1 cells to analyze their effects on cell viability. Experiments were carried out up in three different concentration of the final formulations: 1, 5 and 10  $\text{mg}/\text{mL}$ . Figure 6.1 summarizes the results obtained in HaCaT and HFF-1 cells for both formulations at the same concentration.

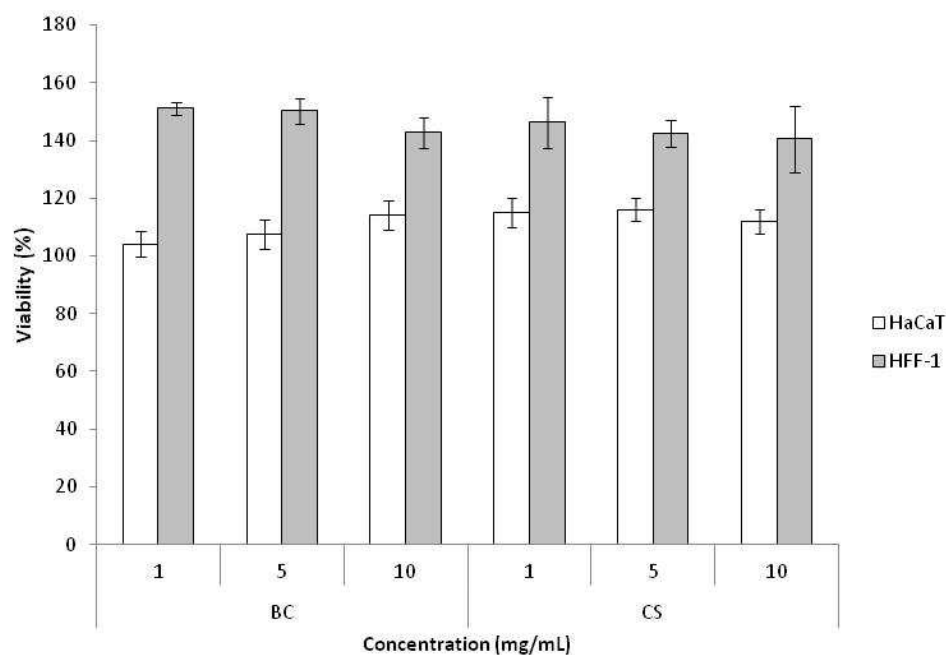


Figure 6.1 - Effect of exposure to BC and CS formulations on the viability of HaCaT and HFF-1 cells at different concentrations, as measured by the MTS assay. Values are expressed as means  $\pm$  SD ( $n = 6$ ). \* $p < 0.05$ .

### 3.1.2 LDH assay

In this study, potential cytotoxic effects of both formulations were investigated by examining membrane integrity of HaCaT and HFF-1 cells using a LDH release assay. In HaCaT cells, at all concentrations tested, BC and CS formulation did not originate any effects on the LDH leakage following 24 h incubation, which means that these formulations are potentially safe to HaCaT cells. The evidence of possible absence of cytotoxicity of these formulations was tested at three different concentrations, namely, 1, 5 and 10 mg/mL.

Test on cytotoxicity of both formulations on fibroblasts was conducted at the same concentrations. Also, no significant LDH-increase was observed, indicating the absence of cytotoxicity of these formulations on human fibroblasts.

### 3.2 Long term stability study

The extracts used were produced from underutilized sources and processed by previously optimized technologies [20, 22, 101]. Physical and chemical stability tests

included measurements of pH, color, and precipitation during the storage period. These characteristics were observed at  $25 \pm 2$  °C/  $65 \pm 5\%$  RH and at  $40 \pm 2$  °C/  $70 \pm 5\%$  RH for 180 days of storage. The chemical and physical properties of both formulations are shown in Table 6.1. The organoleptic characteristics were also evaluated.

It is possible to observe that the addition of coffee silverskin extract to the formulations did not caused decreases in their pH values. The pH values showed minimal changes ( $< \text{pH } 1$ ) within ranges for the storage conditions.

The accelerated stability centrifugation test (Table 6.1) is of major interest since it provides fast information about comparable stability properties. No phase separation after centrifugation was seen in the tested formulations kept at  $25 \pm 2$  °C/  $65 \pm 5\%$  RH neither at  $40 \pm 2$  °C/  $70 \pm 5\%$  RH, during the entire period of study, which means that both formulations (with and without extract) are stable.

Firmness and adhesiveness of both formulations along storage time are also summarized in Table 6.1. Along time a decrease of firmness is observed, for both formulations at 40 °C, with statistical differences. The same occurs for adhesiveness.

Regarding the organoleptic characteristics of the formulations, it was observed that the addition of the extract confers to the formulation a characteristic appearance, color and smell.

Optical microscopy pictures showed the classical appearance of emulsions, a dispersion of spherical droplets (Figure 6.2). As it is possible to visualize, the CS formulation present a higher amount of spherical droplets, which are constituted by the incorporated extract. In the case of BC, 180 days of storage at 40 °C led to an increase in droplets amount. For CS formulation the opposite occurs, as it is possible to observe a decrease in the amount of droplets.

Table 6.1 - Physical and chemical characteristics of formulations at time 0 and 180 days under different storage conditions. \* means a statistical difference ( $p < 0.05$ ).

Physical and chemical characteristics	Formulations							
	BC				CS			
	25 °C / 65% RH		40 °C / 75% RH		25 °C / 65% RH		40 °C / 75% RH	
Time (days)	0	180	0	180	0	180	0	180
<b>pH</b>	5.44 ± 0.02	4.81 ± 0.05	5.44 ± 0.02	5.31 ± 0.02	5.82 ± 0.01	5.75 ± 0.00	5.82 ± 0.01	5.67 ± 0.01
<b>Precipitate</b>	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
<b>Firmness (N)</b>	0.36 ± 0.01	0.30 ± 0.00	0.36 ± 0.01	0.08 ± 0.00*	0.18 ± 0.01	0.07 ± 0.00*	0.18 ± 0.01	0.08 ± 0.00*
<b>Adhesiveness (N.mm)</b>	-1.66 ± 0.20*	-0.86 ± 0.04	-1.66 ± 0.20*	-0.30 ± 0.02	-0.83 ± 0.05*	-0.35 ± 0.01	-0.83 ± 0.05*	-0.32 ± 0.01

Values are means ± SD (n=3).



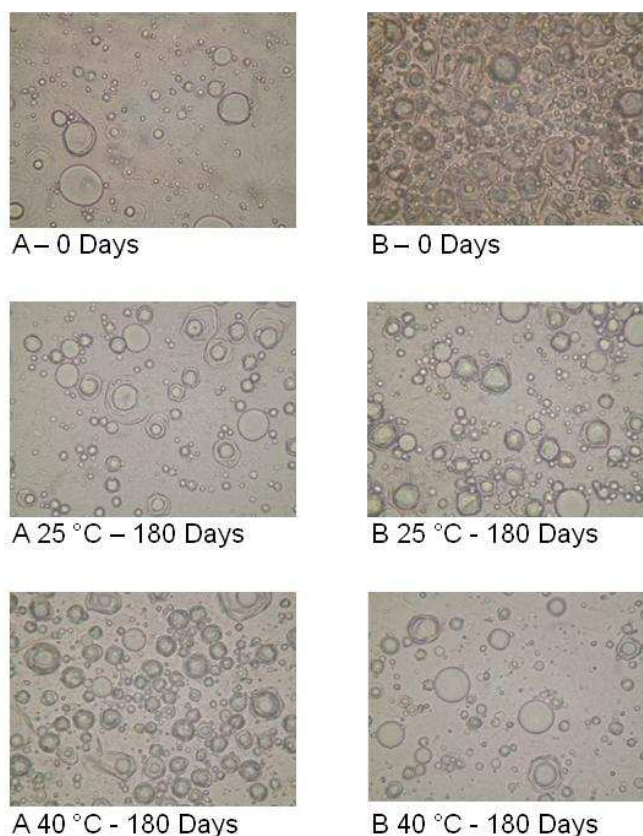


Figure 6.2 - Microphotographs of the BC (A) and the CS formulations (B) at time 0 and after 180 days of storage at different conditions - 40 x magnification.

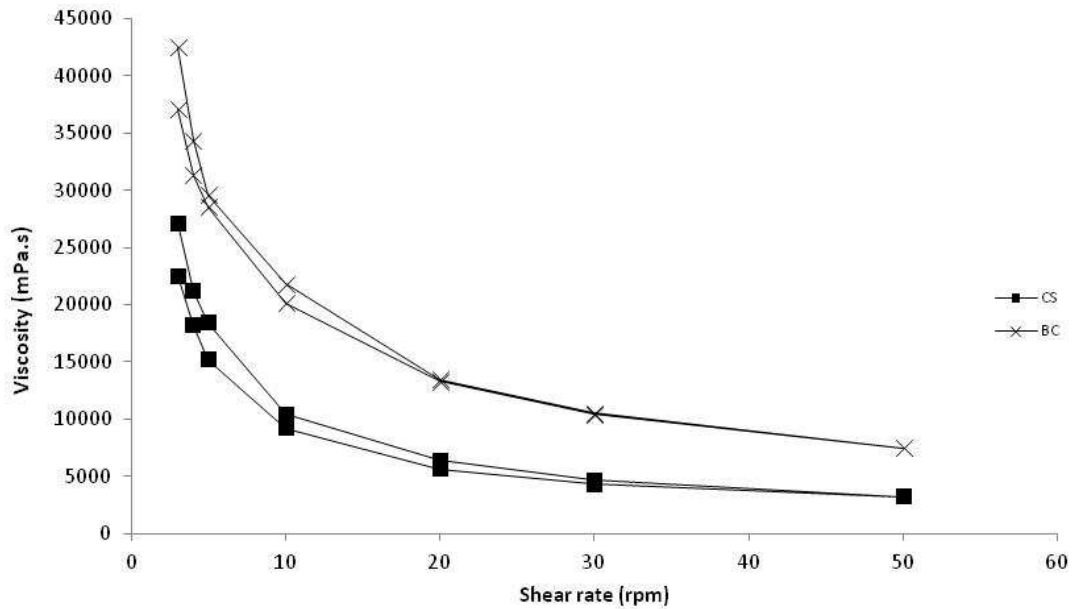
Color changes of the formulations from the stability study are summarized in Table 6.2.

Table 6.2 - Color variation during the stability test conducted at 25 °C/ 65% RH and 40 °C/ 75% RH for BC and CS formulation. \* means a statistical difference ( $p < 0.05$ ).

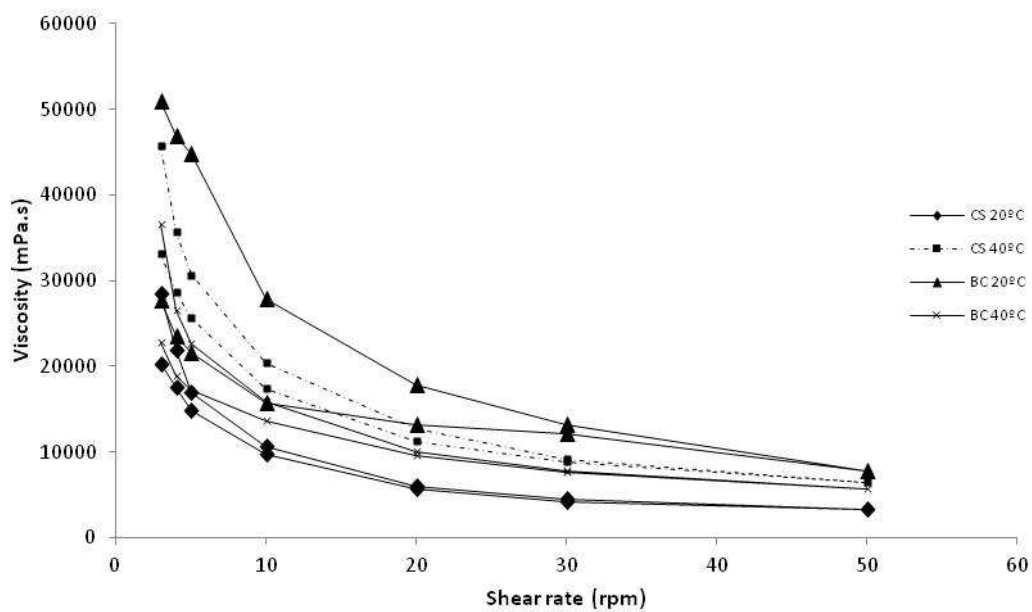
Formulation	Time (days)	Temperature (°C)	L*	a*	b*
BC	0	25	77.98 ± 0.07	-6.11 ± 0.01	3.89 ± 0.01
	180	25	82.56 ± 0.01	-1.08 ± 0.01*	-1.86 ± 0.01*
		40	76.93 ± 0.08	-1.68 ± 0.00*	-1.86 ± 0.02*
CS	0	25	80.75 ± 0.03	-5.85 ± 0.03	9.83 ± 0.01
	180	25	81.21 ± 0.01	-0.87 ± 0.02*	4.86 ± 0.02*
		40	80.45 ± 0.02	-1.73 ± 0.01*	4.22 ± 0.01*

The CS formulation hadn't significantly lower lightness after 180 days ( $p>0.05$ ). A reduction of the green color ( $a^*$ ) and the yellow color ( $b^*$ ) were detected with time.

Both samples presented a rheofluidificant or shear thinning behavior, since apparent viscosity decreased with increasing shear rate (Figure 6.3).



**A**



**B**

Figure 6.3 - Comparison of the rheograms of both formulations evaluated initially (A) and after 6 months of storage (B) at 25 °C/ 65% RH and 40 °C/ 75% RH.

The BC formulation shows an increase in the hysteresis area, which means that the thixotropy of this formulation increased with time (the apparent viscosity decreased with time after 180 days of storage). The CS cream also showed thixotropy, which is a beneficial characteristic of formulations for skin application.

Microbiological quality was analyzed at time 0, 30, 60, 90 and 180 days after storage. The total colony count was < 10/g for all formulations and < 100/g for mould and yeast which did not revealed signs of microbial contamination, being an indicator of the use of a proper preservative system. Also, challenge test results at time 0 and after 180 days of storage at different temperatures showed the efficacy of the preservative system used as no grows was observed and the product was classified as class A for all the tested microorganisms.

### 3.3 Antioxidative properties

Table 6.3 shows the TPC for all formulations. As it is possible to observe, the TPC of CS formulation is slightly higher than those of the normal one, although there were a considerable decrease of the TPC after 180 days of storage. The TPC of the CS formulation at time 0 (polyphenols: 2163.11 µg/mL) was significantly higher ( $p < 0.05$ ) compared to those of the BC (polyphenols: 1132.84 µg/mL). The same occurs after 180 days.

Table 6.3 – Total polyphenol content (TPC) and DPPH assay of BC and CS formulations at time 0 and after 180 days of storage at 25 °C/ 65% RH and 40 °C/ 75% RH. Different letters (a, b, c) in the same column indicate significant differences between mean values ( $p < 0.05$ ).

Formulation	Time (days)	Temperature (°C)	Antioxidant activity	
			TPC (µg GAE eq./mL)	DPPH (µmol Trolox eq./g)
BC	0	-	1132.84 ± 1.64 <sup>a</sup>	1722.47 ± 178.44 <sup>a</sup>
	180	25	45.90 ± 1.68 <sup>b</sup>	1417.66 ± 143.11 <sup>b</sup>
	180	40	114.41 ± 4.27 <sup>c</sup>	1458.12 ± 189.45 <sup>b</sup>
CS	0	-	2163.11 ± 2.15 <sup>a</sup>	1896.03 ± 177.13 <sup>a</sup>
	180	25	439.37 ± 18.98 <sup>b</sup>	2036.23 ± 128.86 <sup>b</sup>
	180	40	285.33 ± 6.55 <sup>c</sup>	1296.25 ± 55.16 <sup>c</sup>

#### 4. Discussion

Skin is a shield protecting us from harmful exposure to solar ultraviolet (UV) radiation and air pollutants, being continually exposed to the oxidative injury caused by free radicals [94]. The topical use of antioxidant can protect and possibly correct oxidative skin damage by neutralizing free radicals [338]. Indeed, is necessary to use an appropriate vehicle and excipients in order to obtain good formulation stability and the maintenance of the antioxidant activity. An oil-in-water emulsion was developed taking in account the consumer acceptance and the final scale-up process.

The main concept and novelty of the present study is the sustainable use of new compounds as active ingredients. Skin and pulp represent almost 45% w/w of the whole fruit [6]. According to the International Coffee Organization (ICO), in 2014, the world coffee production has been around 141620 million bags of 60 kg, which means that about  $3,823,74 \times 10^7$  kg of these wastes were produced [8]. In this way, the re-use of these wastes has a considerable impact in the environment all over the world. Also, there are an increase number of reports in the literature related to the use of plant extracts incorporated into topical bases but none of them explore the use of wastes in cosmetics [339-340]. Thus, it is of extremely importance for the cosmetic industry the demonstration of the potentially of new active compounds obtained from food waste.

According to the obtained results, none of the formulations, at any tested concentration, exhibited cytotoxicity against HaCaT cells (Figure 6.3). Also, no statistic differences were observed between the tested concentrations and both formulations ( $p < 0.05$ ). The number of viable fibroblast cells was similar at all concentration for both formulations. These cell lines are regarded as suitable *in vitro* models for testing the toxicity potential of substances or products intended for dermatological use [239-240, 242]. According to the results obtained for MTS and LDH assays, it is possible to conclude that both formulations presented low toxicity potential toward both cell lines. Considering the results obtained by Rodrigues *et al.* regarding the cytotoxicity of coffee silverskin against these cell lines, it was not expected any effect [22]. However the final formulation has new excipients that should be taken in consideration and the final effect in these cell lines should be evaluated. Regarding the incorporation of food by-products in topical formulations, very few studies were done in this field [321]. For the best of our knowledge this is the first study that evaluates the cytotoxicity of a final formulation against keratinocytes and fibroblasts.

It was observed that the pH of formulations was stable until the period of 180 days in all storage conditions evaluated. Skin pH values range from 5.0 to 6.0 and a pH of 5.5 is considered to be the average pH of the skin. Although pH values changed slightly and are low, they stabilized during this period. Also, all formulations showed stable organoleptic characteristics during the time and conditions of storage (data not shown). Regarding color, comparing with the BC, CS formulation had similar lightness, however, as the extract color varies between green and brown, the final formulation had different redness. When coffee silverskin extract undergo aging, sugar and amino acid elements produce melanoidin, a dark-colored substance that is responsible for the color of the extract [341-342].

The ability of the CS formulation to scavenge DPPH radicals was significantly higher than that of the base formulation ( $p < 0.05$ ), as shown in Table 6.3. When coffee silverskin extract was added to the formulation, its anti-radical efficiencies were increased. However, after 180 days of storage at 40 °C/ 75% RH its anti-radical efficiencies considerably decrease for values below BC. This fact could be justified by the high temperature and humidity that leads to the destruction of antioxidant compounds [343-345]. The TPC and DPPH assay have been widely used to determine the free radical scavenging activity of various plants and pure compounds, including coffee silverskin extract [22, 101]. The formulation of a suitable base for antioxidants is challenging due to their recognized instability in aqueous systems and sometimes extracts lose their functionality after incorporation into a base. As so, is very important to evaluate the antioxidant content after storage. Chlorogenic and caffeic acid are the main free phenols present in coffee silverskin that contribute for TPC [15]. However, according to Borrelli *et al.*, the concentration of free phenolic compounds is much lower than that detectable in roasted beans which is justified with the higher temperature experienced by the outer layer of the beans during roasting, that causes fast degradation of phenol compounds [15]. Furthermore, melanoidins formed during roasting are rich in phenolic groups [346]. This high amount of antioxidant and polyphenols could be used in aging defence. According to Darvin *et al.*, the utilization of a balanced mixture of antioxidants can substantially decrease the oxidative activity of free radicals generated in cosmetic formulations, providing an excellent protection against oxidation, but also the optimal antioxidative protection of the skin against free radicals [347].

The stability of antioxidant formulations has been subject of various studies [336, 338, 348-351], but few of them evaluated the use of food by-products extracts [321]. Our

results are in agreement with Balboa *et al.* who evaluated the inclusion of different by-products extracts in different preparations and concluded that in comparison to synthetic antioxidants, the extracts used could be added at higher levels due to their non-toxic characteristics, showing strong antioxidant activity and concomitant bioactivities [321].

## 5. Conclusion

In this work, the physical, chemical and microbiological stability of a semisolid formulation containing coffee silverskin extract, a food by-product, was proven. This study concluded that a formulation containing 2.5% coffee silverskin extract retained physical stability and also had high anti-radical efficiencies. Accelerated stress conditions did not promoted modifications in the tested formulation, suggesting that any specific storage conditions should be recommended. Moreover, it is possible to suggest that this formulation may be safe against keratinocytes and fibroblasts. Nevertheless, the results must be interpreted carefully and further *in vivo* studies are needed in order to evaluate the *in vivo* antioxidant efficacy of formulations containing coffee silverskin extract.

## II. *In Vitro* and *In Vivo* Evaluation

The information presented in this chapter section was published in the following publication:

- **Francisca Rodrigues**, Bruno Sarmiento, M. Helena Amaral, M. Beatriz P.P. Oliveira, Exploring the antioxidant potentiality of two food by-products into a topical cream: stability, *in vitro* and *in vivo* evaluation, Drug Development and Industrial Pharmacy, 2015, *in press*

## 1. Introduction

A new tendency in cosmetic formulations is the use of ingredients with antioxidant, immunomodulatory or photoprotective purposes, obtained from food by-products or natural products without added value, taking in account questions of sustainability [22-23, 99, 101]. Millions of tons of by-products are produced during food processing in agro industries without economic value but potentially recoverable as source of new ingredients for the cosmetic and pharmaceutical industries. An example is Coffee Silverskin (CS), a waste of coffee roasting industry [22]. CS is a thin closely skin that covers the coffee beans. Some of the properties described for coffee brews, due to the presence of several classes of health related chemicals (as phenolic compounds, diterpenes, xanthines, and vitamin precursors), namely antimicrobial and antioxidant properties [283-285], are also present in CS extracts, as described by different authors [20, 22, 102]. Also, previous studies reported the skin compatibility and safety of CS extracts. Evaluation of CS extracts with *in vitro* skin and ocular irritation assays, using reconstructed human epidermis, EpiSkin™, and human corneal epithelial model, SkinEthic™ HCE, respectively, were performed by Rodrigues *et al.* [102]. An *in vivo* assay was also described by the same research group aiming to guarantee the extract safety. The *in vitro* results demonstrated that extracts were not classified as irritants and the histological analyses proved that both models structure were not affected after exposures. The *in vivo* test carried out with the most promising extract (hydroalcoholic) showed that, with respect to irritant effects, it can be regarded as safe for topical application.

Another product intensively studied is *Medicago sativa* (MS), used as spout in food salads or as forage for livestock. Recent reports, demonstrated that this plant is a potential source of natural compounds with antioxidant activity, low cytotoxicity for skin cells and ability to potentially prevent skin microbial infections [101]. Its isoflavones content is also relatively high when compared to other food wastes [23].

Nowadays, research in cosmetics aims to create innovative and well accepted products to perfectly come up to the sensory expectations of consumers [352]. Indeed, consumers generally choose a cosmetic cream for its functions, or its promise of efficiency, but are mostly seduced by the pleasure it brings to them, especially what concerns texture. The number of studies to found a correlation between instrumental and sensory properties of cosmetic products still remains limited and most of them only highlight a basic proportionality between sensory and instrumental parameters [353].



Thus, it seems very important to incorporate both extracts in a topical formulation and evaluate not only the *in vitro* toxicity, but also the *in vivo* efficacy and safety. Some studies showed that antioxidants could maintain or restore a healthy skin barrier [86]. Based on these considerations, the dermatological effects of a body cream formulation containing CS and MS extracts was herein evaluated in order to improve skin conditions and to promote healthy skin. The efficacy perception by volunteers was also considered. The *in vitro* effect of the final formulations against keratinocytes and fibroblasts was also assessed. As stability is one of the main concerns regarding topical formulation, a stability study was carried out under different climatic conditions. Finally, considering that the natural ingredients under study can present antioxidant and hydrating effect, this research contributed to a better understanding of its effects on human skin when applied topically. Also, this study demonstrated the skin compatibility (by primary skin irritation) and efficacy of cosmetic formulations supplemented with both extracts.

## 2. Materials and Methods

### 2.1 Materials

CS samples were provided by a national coffee roaster company (BICAFÉ – Torrefação e Comércio de Café Lda, Portugal). *Medicago sativa* was sown and collected in Azurara, Vila do Conde, Portugal.

Sodium lauryl sulfate (SLS; purity > 99%), 1,1-diphenyl-2-picrylhydrazyl (DPPH') free radical, Folin-Ciocalteu's reagent and gallic acid were purchased from Sigma–Aldrich Chemical (St Quentin Fallavier, France). Deionised water was obtained using Mili-Q water purification system (TGI Pure Water Systems, USA). Ethanol was obtained from Panreac (Barcelona, Spain).

Sodium carbonate decahydrate was purchased from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™-I, fetal bovine serum (FBS), streptomycin, penicillin and amphotericin B were from Invitrogen (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-4-sulfophenyl)-2H-tetrazolium (MTS) assay kit (CellTiter 96® Aqueous One Solution Cell Proliferation Assay) was purchased from Promega (Madison, WI, USA). Lactate dehydrogenase (LDH) colorimetric cytotoxicity assay kit (LDH Cytotoxicity Detection Kit) was purchased from Takara Bio Inc. (Shiga, Japan).

## 2.2 Preparation of extracts

MS leaves were randomly selected, dried at 65 °C during 72 h and stored, protected from light and moisture, at room temperature until the milling process [101]. CS and MS were milled to particle size of approximately 0.1 mm using a A11 basic analysis mill (IKA Wearke, Staufen, Germany) and stored in silicone tubes at room temperature until extract preparation, as described by different authors [22, 101]. Samples (1 g) were submitted to solvent extraction by maceration with ethanol: water (1:1, v/v) for 30 min at 40 °C. Extracts obtained were filtered through Whatman No. 1 paper filter and the filtrates collected [22].

## 2.3 Formulation

A body cream formulation (F) was developed and supplemented with both extracts (CSMS), as described in Table 1.

Briefly, the oil and water phase were separately heated up to  $75 \pm 1$  °C. W/O emulsions were prepared by adding the aqueous phase to the oily phase under continuous stirring by the mechanical mixer for 15 min until the aqueous phase was added completely. Then, the mixer speed was reduced for a period of 10 min for complete homogenization until the emulsion cooled to room temperature. At this temperature the extract were added to the CSMS formulation. In case of base reference sample (galenic base) no extract was added.

Table 6.4 - Components of both formulations under study.

Components	Function	Percentage of components in each formulation (w/w)	
		F	CSMS
Purified water	Solvent	73.1	70.6
Paraffinum liquidum	Emollient	6.8	6.8
Decyl Oleate	Emollient	5.0	5.0
Dimethicone	Skin conditioner	4.0	4.0
Glycerin	Humectant	3.8	3.8
Polyglyceryl-3 Methylglucose Distearate	Emulsifier	2.2	2.2
Glyceryl Stearate	Emulsifier	1.5	1.5
<i>Medicago sativa</i> hydroalcoholic extract	Moisturizer / Antioxidant	-	1.25
Coffee Silverskin hydroalcoholic extract	Moisturizer / Antioxidant	-	1.25
Cetearyl alcohol	Viscosity controller	0.8	0.8
Lanolin	Emollient	0.7	0.7
2-Phenoxyethanol /2-Methyl-2H-isothiazol-3-one	Preservative	0.6	0.6
Panthenol	Skin conditioner	0.5	0.5
Carbomer	Rheology Modifier	0.3	0.3
Lactic acid	Humectant	0.3	0.3
Parfum	Fragrance	0.2	0.2
Titanium dioxide	Opacifier	0.1	0.1
Triethanolamine	Buffer	0.1	0.1

## **2.4 Long-term stability evaluation**

Batches of both formulations (F and CSMS) were prepared and physical stability evaluated by submitting formulations to storage, for a period of 180 days, at two different conditions: 25 °C/ 65% relative humidity (RH) and 40 °C/ 75% RH [354-355]. Physical, chemical and microbiological properties were evaluated in formulations packaged in a suitable container at the initial time and after 30, 60, 90 and 180 days of storage using the following methods:

### **2.4.1 pH**

One gram of the formulation was diluted with 3 mL of neutral water and the pH of the aqueous mixture was analyzed with a pHmeter (691, Mettler, Greifensee, Switzerland) equipped with a glass electrode. The measurements were performed in triplicate for each sample.

### **2.4.2 Accelerated stability by centrifugation**

Samples (5 g) were centrifuged at 3000 rpm for 30 min (centrifuge 5804, Eppendorf, Hamburg, Germany), and the eventual phase separation was analysed in order to assess their stability.

### **2.4.3 Color**

Color was evaluated with a colorimeter (CR-400, Konica Minolta, Tokyo, Japan) and results were expressed according to the color space CIE 1976 L\*a\*b\*, defined by the Commission Internationale de l'Eclairage (CIE). The three coordinates represent the lightness of the color (L\*), its position between red and green (a\*) and its position between yellow and blue (b\*).

### **2.4.4 Rheological analysis**

Apparent viscosity was measured using a Brookfield rotational viscometer (Brookfield DVE, Cologne, Germany). Viscosity determinations were performed using a spindle number 5, sequentially increasing and decreasing values of shear rate in the range 3-50 rpm in order to obtain the ascending and descending curves. Temperature was kept constant at 20.0 ± 0.5 °C and all samples were maintained at room temperature prior to viscosity measurements.

### 2.4.5 Microbiological analysis

At time 0, 30, 60, 90 and 180 days, batches were evaluated for microbiological quality. Dilutions were performed with buffered peptone sterile solution and 3% (w/v) of polysorbate 80 was added to the culture media to neutralize the preservative. A total colony count was performed using Tryptic soy agar (Oxoid Ltd, Hampshire, UK). Incubation was carried out during 5 days at 30-35 °C. For yeast and molds counting, Sabouraud agar (bioMérieux, France) was used and incubation was carried out during 5 days at 25 °C. Additionally at time 0 and at time 180 days the preservative stability was tested according to the ISO 11930:2012 procedures.

### 2.4.6 Determination of total phenolic content (TPC)

Total phenolic content (TPC) was spectrophotometrically determined according to the Folin-Ciocalteu procedure [86] with minor modifications. Briefly, 500 µL of formulation with proper dilution was mixed with 2.5 mL of Folin-Ciocalteu reagent (10 x dilutions) and allowed to react for 5 min. Then, 2.5 mL of Na<sub>2</sub>CO<sub>3</sub> 7.5% (w/v) solution was added and allowed to stand for 15 min at 45 °C, and then 30 min at room temperature. After that the absorbance of the reaction mixture was determined at 765 nm using a microplate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA). A calibration curve for the standard gallic acid was used to obtain a correlation between sample absorbance and standard concentration (linearity range = 5-100 µg/ml,  $r^2 > 0.998$ ). The TPC of formulation was expressed as mg of gallic acid equivalents per gram of formulation. The assay was performed in triplicate.

### 2.4.7 Determination of antioxidant activity

The antioxidant activity of the samples was evaluated by DPPH radical-scavenging activity. The reaction mixture on 96 wells plate consisted of one solution per well for different sample concentrations (30 µL) and ethanolic solution (270 µL) containing DPPH radicals ( $6 \times 10^{-5}$  mol/L). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm [101] using the microplate reader. The antioxidant capacity based on the DPPH free radical scavenging ability of the formulation was expressed as µmol Trolox equivalents per gram of formulation. The formulations were dissolved in isopropanol: DMSO (50:50)

through shaking as in some cases interferences were observed due to opalescent solutions that impair this photometric assay. A calibration curve was prepared with Trolox (linearity range: 2.5-100 µg/mL,  $r^2 > 0.996$ ).

## **2.5 *In vitro* evaluation**

### **2.5.1 Keratinocytes and fibroblasts cytotoxicity assays**

Two different assays were used to assess cell integrity and cytotoxicity of the final cosmetic formulation: (1) monitoring the uptake of the vital mitochondrial dye, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) by cell mitochondria and (2) determining the leakage of the cytosolic enzyme, lactate dehydrogenase (LDH) into the cell medium (LDH assay). For the cell treatment emulsions were diluted in water and Dulbecco's Modified Eagle's Medium (DMEM). Emulsions were prepared in a range of concentrations from 1 to 10 mg/ml. In both tests, triplicate wells were incubated with fresh medium in the absence or presence of formulation. Formulation was exposed to keratinocytes (HaCaT) and fibroblasts (HFF-1) cells during 24 h.

### **2.5.2 Cell Lines and culture conditions**

Human immortalized non-tumorigenic keratinocyte cell line HaCaT (Ethnicity, Caucasian; Age, 62 years; gender, Male and tissue, skin) was supplied by CLS Cell Lines Service, Germany. Human foreskin fibroblasts (HFF-1) were purchased from ATCC (ATCC Number: SCRC-1041; ATCC, Manassas, VA, USA). Passage 17-19 of HFF-1 and passage 77-79 of HaCaT cells were used for both MTS and LDH assay. HaCaT and HFF-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™-I, 10% inactivated fetal calf serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B, pH 7.4 and maintained in a O<sub>2</sub>:CO<sub>2</sub> (95:5 v/v) environment at 37 °C. At 90-95% confluence, both cell types were trypsinized and plated in microtiter dishes. The viable cells were counted using trypan blue dye (Gibco) in hemocytometer. After attachment, they were incubated in serum-free medium containing 1 mg/mL, 5 mg/mL and 10 mg/mL of emulsion for 24 h at 37 °C.

### 2.5.3 MTS assay

Cell viability was assessed using a commercially available kit based on the bioreduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) by metabolically active cells (CellTiter 96<sup>®</sup> Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA), further referred to as MTS assay. The MTS assay was performed according to the manufacturer instructions. MTS assay was used to assess cell damage by formulation.

Cells were cultured in 96-well micro titter plate at a density of  $25 \times 10^3$  cells per mL culture medium for 24 h. The cells were incubated with 1 mg/mL - 10 mg/mL formulation for 24 h at 37 °C. After the removal of formulation from the wells, cells were washed in phosphate buffered saline. Then the medium was changed to fresh control medium. The cells were then incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C for 24 h. To evaluate the number of viable cells, 120 µl of MTS solution was added into each well and incubated for 4 h at 37 °C in the dark. The absorbance was measured at 490 nm with background subtraction at 630 nm by a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

### 2.5.4 LDH assay

Briefly, cell lines were seeded in 96-well plates (5,000 cells for LDH) and cultured for 48 h. Formulation at different concentrations were prepared in appropriate medium and added to the cells. Media without FBS were used in the case of LDH. After incubation (37 °C / 5% CO<sub>2</sub>), cells were washed twice with PBS (pH 7.4) and the kits were used according to the instructions of the manufacturers. To determine maximum LDH release (high control), some cells were solubilised with a final concentration of 1% (w/v) Triton X-100 (Boehringer Mannheim). Spontaneous LDH release (low control) was determined by incubating formulation with DMEM-gent. Absorbance was measured at 490 nm with background subtraction at 690 nm by a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). Each concentration was tested in triplicate in three independent experiments.

Results were expressed as:

$$\% \text{ Cytotoxicity} = [(\text{Experimental value} - \text{Low control} / \text{High control} - \text{Low control}) \times 100]$$

## 2.6 *In vivo* skin irritation

### 2.6.1 Patch test

In order to evaluate the *in vivo* skin irritation a single blinded study was performed. Twenty healthy individuals (15 women and 5 men) with a mean age of  $30 \pm 5$  years, without known dermatological diseases or allergy to substances in topical products, participated in the study, in accordance with Declaration of Helsinki. Written informed consent was obtained from all volunteers. The volunteers were asked to not apply any topical products in the forearms 24 h before the beginning and throughout the test period. Additionally, solar exposure was forbidden. Along with formulation, SLS (2%, w/v, aqueous solution) and purified water were also assayed, respectively, as positive and negative control. Sites were marked in the inner forearms of volunteers. Before application of patch tests, the areas on the ventral part of each forearm were marked using a skin marker, and basal values were obtained by non-invasive biometric method. After basal measurements, the patches were applied. A pre-determined amount of formulation (300 mg), and controls were applied on skin and occlusion was achieved with aluminium chambers (12 mm, Finn Chambers, SmartPractice, Phoenix, USA). Patches were removed after 48 h of occlusion, and following a period of 2 h of observation patch test areas were evaluated by non-invasive measuring methods and visual scoring.

### 2.6.2 Visual and instrumental assessments

The visual assessment of the degree of irritation was graded according to the following scale, previously used by Agner *et al.*: 0 - no reaction; 1 - weak, spotty erythema; 2 - well perceptible erythema covering the total exposure area; 3 - moderate erythema or severe erythema that covers the total exposure area. The same scale was used for edema: 0 - no reaction; 1 - weak, spotty edema; 2 - well perceptible edema covering the total exposure area; 3 - moderate or severe edema that covers the total exposure area [318].

Non-invasive biophysical measurements were also performed. All measurements were performed at controlled temperature (20-22°C) and relative humidity (45-55%). Transepidermal water loss (TEWL) is traditionally used to assess skin barrier function [319]. TEWL measurements were carried out with a Tewameter<sup>®</sup> TM 210 (Courage &



Khazaka, Köln, Germany), evaluating the relative humidity percentage which diffuses from the dermis to the skin surface ( $\text{g}/\text{cm}^2 \text{ h}$ ).

### **2.6.3 Long term hydration effect**

The same volunteers were asked to apply once a day the body cream formulation. After 30 days of use, the skin hydration was evaluated, for both formulations. All measurements were performed on forearms. Volunteers acclimatized for at least 30 minutes under standard atmospheric conditions previously reported. Skin hydration levels were measured using a Corneometer CM 825 (Courage & Khazaka, Köln, Germany) according to European Group for Efficacy Measurements on Cosmetics and Other Topical Products (EEMCO) guidelines [356]. Three measurements at least were taken per area.

### **2.6.4 Sensory evaluation**

Thirty days after the beginning of the study volunteers were asked to answer a questionnaire about the formulation, in order to have information about their sense and perception concerning the cosmetic qualities (appearance, texture, smell, spreadability, penetration rate and overall quality). For the different categories the scale used was very pleasant, pleasant, unpleasant and very unpleasant.

## **2.7 Statistical analysis**

Data were reported as mean  $\pm$  standard deviation of at least triplicate experiments. Statistical analysis of the results was performed with the software SPSS 22.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to investigate the differences between different formulations for all assays. Post hoc comparisons were performed according to Tukey's HSD test. In all cases,  $p < 0.05$  was accepted as denoting significance.

### 3. Results

#### 3.1 Stability

Physical and chemical stability tests included measurements of pH, color and phase separation during the storage period. These characteristics were observed at 25 °C/ 65% RH and at 40 °C/ 75% RH at time 0, 30, 60, 90 and 180 days of storage. Table 6.5 summarizes the pH values and the behaviour in what concerns phase separation of formulations.

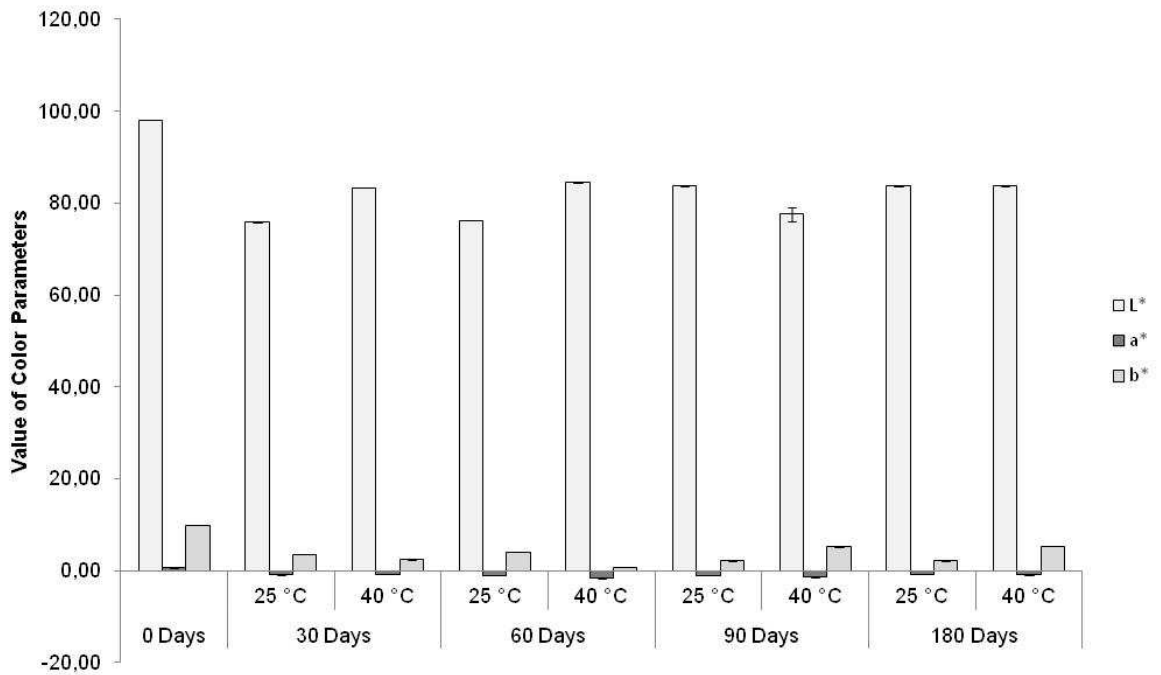
Table 6.5 – Physical characteristics of formulations at time 0 and 180 days under different storage conditions.  
\* means a statistical difference ( $p < 0.05$ ).

Formulation								
Time (days)	F				CSMS			
	25°C	40°C	25°C	40°C	25°C	40 °C	25°C	40 °C
	pH		Phase separation		pH		Phase separation	
0	4.36±0.02		-	-	4.87±0.02			
30	4.52±0.02	4.55±0.01	-	-	5.26±0.01	5.26±0.01	-	-
60	4.46±0.01	4.34±0.02	-	-	5.32±0.01	5.25±0.01	-	-
90	4.67±0.01	4.18±0.01	-	-	5.40±0.01	5.24±0.01	-	-
180	4.55±0.01	4.45±0.01	-	-	5.35±0.00	5.15±0.01	-	-

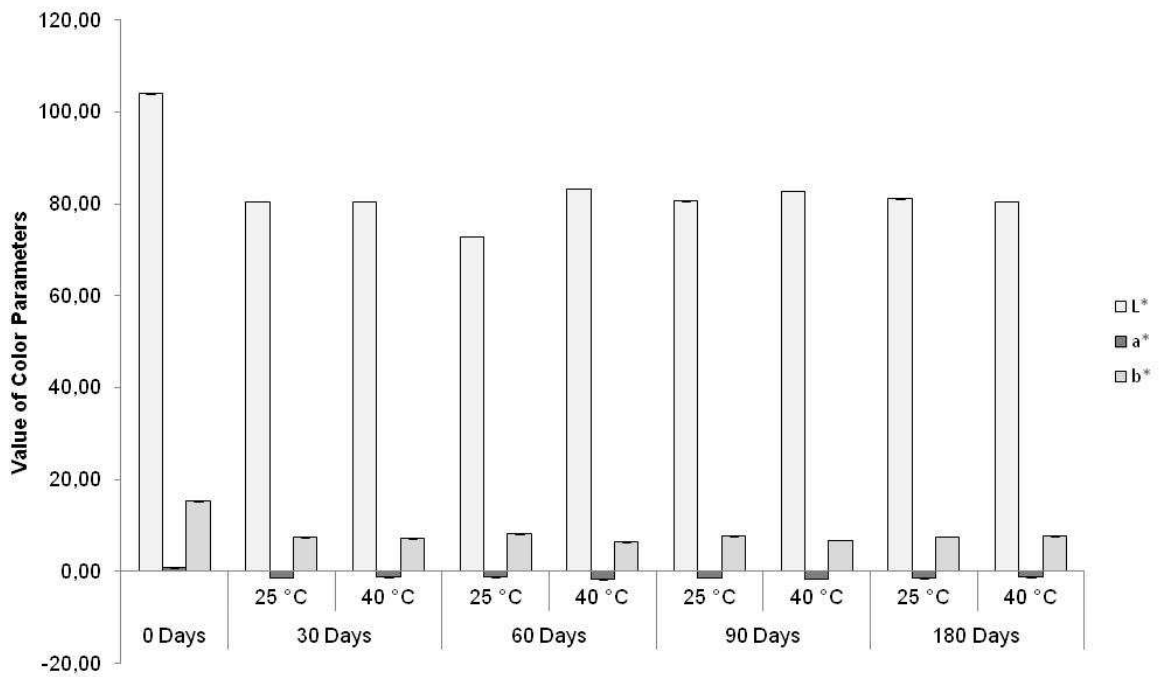
As it is possible to observe, the pH of formulations did not decrease over time. The pH values showed minimal changes ( $< \text{pH } 1$ ) within ranges for the storage conditions. As it is possible to observe, the pH of CSMS formulation is higher during all stability study, probably due to the presence of both extracts.

The accelerated stability centrifugation test (Table 6.5) is of major interest since it provides fast information about stability properties. No phase separation, after centrifugation, was verified in both tested formulations kept at  $25 \pm 2$  °C/  $65 \pm 5\%$  RH and at  $40 \pm 2$  °C/  $70 \pm 5\%$  RH, during the study, meaning that both formulations are stable.

The organoleptic characteristics were also evaluated, showing no significant variation during all study, maintaining not only the initial appearance but also color and smell. Color changes are summarized in Figure 6.4.



**A**



**B**

Figure 6.4 - Color variation during the stability test conducted at 25 °C/ 65% RH and 40 °C/ 75 % RH f or F (A) and CSMS (B) formulations. L – lightness, a – red to green, b – yellow to blue. \* means a statistical difference (p<0.05).

As it is possible to observe, both formulations had a significantly lower lightness after 30 days of storage at both conditions. However, after 180 days there is no statistical difference when compared to 30 days of storage values. A similar reduction color parameter  $a^*$  was detected with time. The color parameter  $b^*$  did not change significantly during 180 days of storage. A possible explanation for this color variation could be the fact that extracts undergo aging. CS extract has sugar and amino acid elements that during aging originate melanoidin, a dark-colored substance that is responsible for the color of the extract [341-342]. Also, slight changes of pH and the evolution of phenolic compounds under aging (that suffer chemical oxidation and/or polymerization) could contribute to this color changes observed.

Regarding rheological studies, all the samples presented a rheofluidificant or shear thinning behaviour, since apparent viscosity decreased with increasing shear rate (Figure 6.5).

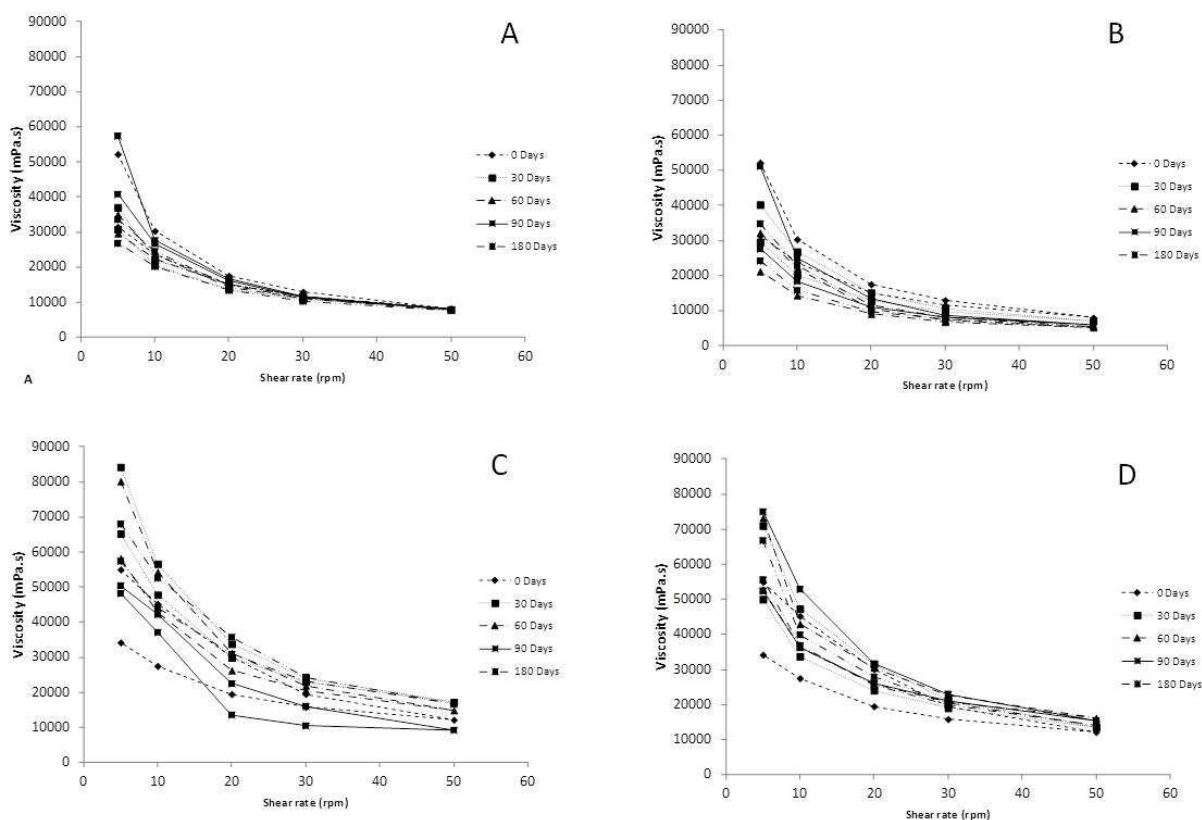


Figure 6.5 - Rheograms of formulations evaluated initially and after 180 days of storage at 25 °C/ 65% RH and 40 °C/ 75% RH (A – Formulation F at 25 °C/ 65% RH; B – Formulation F at 40 °C/ 75% RH; C – CSMS at 25 °C/ 65% RH; D – CSMS at 40 °C/ 75 % RH).

Formulations show an increase in the hysteresis area along the time of storage, at both temperatures, which means that the thixotropy increased with time (the apparent viscosity decreased with time after 180 days of storage), being a beneficial characteristic of formulations for skin application. The viscosity of F is slight low at time 0, but after 180 days of storage viscosity of both formulations is similar under different conditions.

Microbiological quality was analyzed at time 0, 30, 60, 90 and 180 days after storage. Results did not revealed signs of microbial contamination, being an indicator of the use of a proper preservative system. Also, challenge test results at time 0 and after 180 days of storage at different temperatures showed the efficacy of the preservative system used.

### 3.2 TPC content and antioxidant activity

The TPC and DPPH values for both formulations at time 0 and after 180 days of storage at different temperatures are summarized in Table 6.6.

Table 6.6 – Total polyphenol content and antioxidant activity of formulations at time 0 and after 180 days of storage at 25 °C/ 65% RH and 40 °C / 75 % RH. Different letters (a, b, c) in the same column indicate significant differences between mean values ( $p < 0.05$ ).

Time (days)	Temp. (°C)	Formulations			
		F		CSMS	
		TPC (µg GAE eq./mL)	DPPH (µmol Trolox eq./g)	TPC (µg GAE eq./mL)	DPPH (µmol Trolox eq./g)
0	-	1979.22±28.58 <sup>a</sup>	216.37±25.77 <sup>c</sup>	4192.95±50.25 <sup>a</sup>	312.85±17.35 <sup>c</sup>
180	25	1318.83±118.28 <sup>b</sup>	482.48±54.51 <sup>a</sup>	3022.03±231.31 <sup>b</sup>	780.28±60.58 <sup>a</sup>
180	40	1130.31±112.19 <sup>c</sup>	349.80±34.73 <sup>b</sup>	2918.71±90.42 <sup>b</sup>	470.52±107.25 <sup>b</sup>

The TPC and DPPH assays have been widely used to determine the free radical scavenging activity of different plants extracts and pure compounds, including CS and MS extracts. For these compounds the TPC determined were about 25 mg GAE/g dry basis and 30 mg GAE/g dry basis, respectively, for *Medicago sativa* and coffee silverskin [22, 101]. The formulation of a suitable base for antioxidants is challenging due to their recognized instability in aqueous systems. Sometimes extracts loss their functionality after

incorporation into a base. As so, it is very important to evaluate the antioxidant capacity and content after storage. As it is possible to observe, the TPC of both formulations decreases significantly after 180 days of storage. For both cases, the TPC at time 0 was significantly higher ( $p < 0.05$ ) compared to the values after 180 days, at both temperatures. Also, after 180 days of storage there are no statistical differences between storage at 25 °C / 65% RH or 40 °C / 75% RH.

Considering antioxidant activity, the ability of the formulation to scavenge DPPH radicals was significantly higher after 180 days of storage ( $p < 0.05$ ), as shown in Table 6.6. When extracts were added to the formulation, the anti-radical efficiencies were increased. This could be observed for both formulations but, particularly, for CSMS formulation, after storage at 25 °C.

The differences in TPC content and antioxidant activity observed between formulations could be explained based on the composition of extracts used in CSMS. Chlorogenic and caffeic acid are the main free phenols present in CS that contributes for TPC. This high amount of antioxidants and polyphenols could be used in aging defence. However, according to Borrelli *et al.*, the concentration of free phenolic compounds is much lower than that detectable in roasted beans, which is justified with the higher temperature experienced by the outer layer of the beans during roasting that causes fast degradation of phenol compounds [15]. Furthermore, melanoidins formed during roasting are rich in phenolic groups [346]. According to Darvin *et al.*, the utilization of a balanced mixture of antioxidants can substantially decrease the oxidative activity of free radicals generated in cosmetic formulations, providing an excellent protection against oxidation, but also the optimal antioxidative protection of the skin against free radicals [347]. These facts could easily explain the polyphenol content observed for CSMS formulation.

Regarding *M. sativa*, as previously reported by us, its antioxidant activity could be considered high when compared to other food by-products [101]. However, few studies were reported regarding their exact composition. Silva *et al.* reported their rich composition in phenolic compounds, fatty acids and volatiles that could justify their high antioxidant activity [27].

As it is possible to observe, the antioxidant activity increases over 180 days of storage, for both temperatures of storage. However, at 25 °C the increase is higher, with statistical differences to 40 °C ( $p < 0.05$ ). This higher antioxidant activity could be justified by reactions between excipients and extracts or even between excipients that generate new

compounds with antioxidant activity. Also, new compounds formed could react with DPPH. Previously works developed by Rodrigues *et al.* using coffee silverskin extract incorporated into a hand cream showed the same antioxidant profile after 180 days of storage, which means that probably the extract originate compounds that react with DPPH reagent [100].

### 3.3 *In vitro* toxicity

The MTS and LDH assays results in keratinocytes and fibroblasts were obtained at different concentrations of the final formulations. Table 6.7 summarized cellular viability obtained, since the IC<sub>50</sub> could not be estimated due to the low cytotoxicity of the emulsion.

Table 6.7 - Effect of body care formulations on viability of HFF and HaCaT cells at different concentrations, measured by MTS assay. Values are expressed as means  $\pm$  SD (n=6). \* p<0.05 versus positive control (cells exposed to medium).

Concentration (mg/mL)	Cell Viability (%)			
	HFF-1		HaCaT	
	F	CSMS	F	CSMS
1	133.45 $\pm$ 4.83	147.26 $\pm$ 5.39	122.46 $\pm$ 1.68	136.35 $\pm$ 3.63
5	180.81 $\pm$ 8.21	146.19 $\pm$ 7.51	118.50 $\pm$ 2.79	133.75 $\pm$ 4.15
10	173.47 $\pm$ 15.06	129.17 $\pm$ 8.21	121.72 $\pm$ 4.44	124.69 $\pm$ 7.00

According to the obtained results, both formulations did not have significant effect on cell viability. These results are in agreement with previously results obtained for MS and CS extracts, which were tested against the same cell lines [22, 101].

The data obtained from both MTS and LDH experiments together suggest that formulation are potentially non-toxic to fibroblasts and HaCaT at concentrations from 1 to 10 mg/mL and can be suitable for cosmetic use. The concentrations used to determine their irritation potential was extremely high because lower concentrations did not provide expressive results. These cell lines are regarded as suitable *in vitro* models for testing the

toxicity potential of substances or products intended for dermatological use [239-240, 242].

An important indicator of cell damage in oxidative stress is the increase in cell membrane permeability. In this study, a potential cytotoxicity effect of the final formulations was investigated by examining membrane integrity of HaCaT and HFF-1 cells using a LDH release assay. In keratinocytes and fibroblasts, at all concentrations tested, formulations did not affect LDH leakage following 24 h of incubation, which means that formulations are safe on the concentration range studied.

### **3.4 *In vivo* evaluation**

Taking in consideration the referred above, an *in vivo* evaluation of CSMS formulation supplemented with extracts was developed.

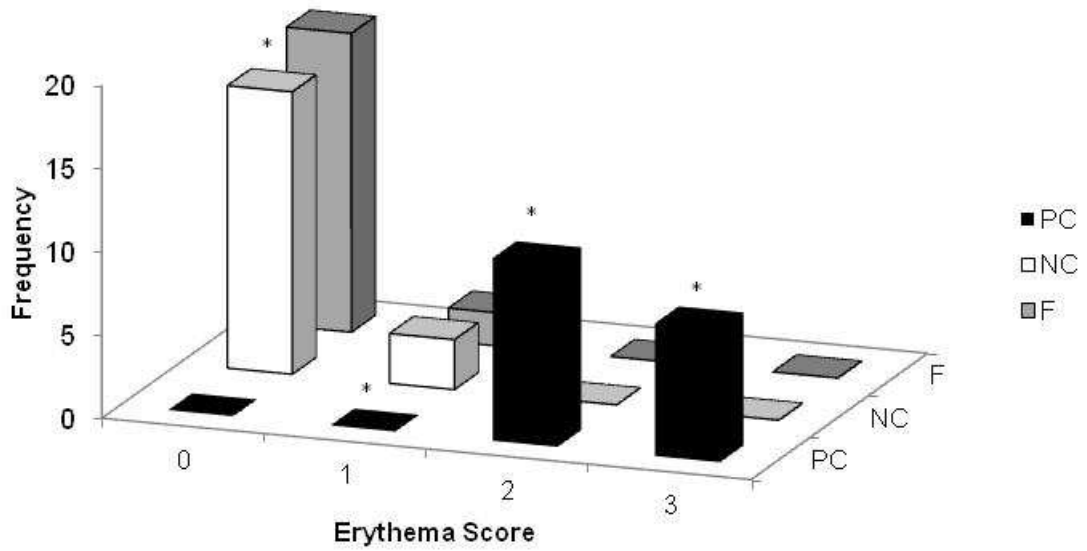
#### **- Skin compatibility by evaluation of primary skin irritation**

To ensure that a formulation is completely safe, some assays such as patch test should be performed. Patch testing after a single application is a widely used procedure to evaluate acute irritant reactions in human volunteers. The evaluation of irritancy after patch testing was initially based on visual score. Results obtained by visual analysis are shown in Figure 6.6.

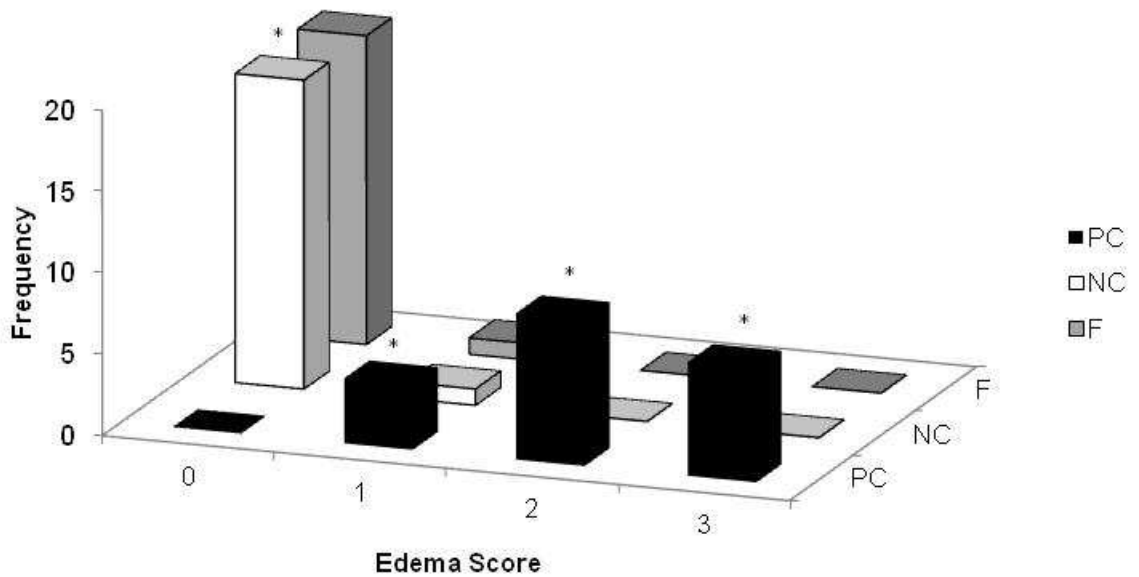
According to the Figure 6.6, patch test demonstrated that formulation provoked a slight erythema in two volunteers 2 h after the patch test removal. However, when statistical analyses were applied regarding negative control, no differences were found. Regarding the positive control, it is possible to observe statistical differences when comparing negative control and CSMS formulation ( $p < 0.05$ ).

Regarding edema, only one volunteer showed a slight edema and no statistical differences were observed between the formulation developed and the negative control ( $p < 0.05$ ).





**A**



**B**

Figure 6.6 - Frequency of clinical scores attributed to positive control (PC - SLS), negative control (NC-- purified water) and formulation (CSMC) regarding erythema (A) and edema (B) after patch removal. \* means a significant difference ( $p < 0.05$ ).

### - Transepidermal water loss variation

The measurement of transepidermal water loss (TEWL) is traditionally used to assess skin barrier function [319, 332]. When skin is damaged, its barrier function is impaired resulting in higher water loss. According to Figure 6.7 the formulation did not alter the skin barrier function, as TEWL values were not changed, when compared to the positive control ( $p < 0.05$ ). No significant differences were observed for TEWL measurements, in comparison with purified water, 2 h after patch removal ( $p < 0.05$ ), which indicates the absence of barrier disruption. Consequently, formulation could be considered as no potentially irritating.

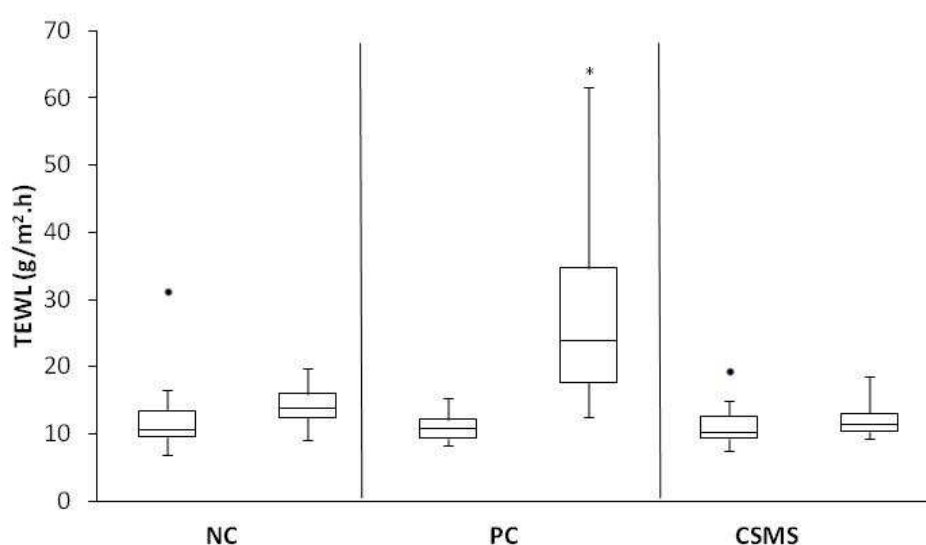


Figure 6.7 - Transepidermal water loss (TEWL) variation from basal values of negative control (NC), positive control (PC) and formulation (CSMS). The left boxes represent values at time 0 and the right boxes represent values 2 h after patch removal. Outliers are presented as circles. \* means a significant difference ( $p < 0.05$ ).

### - Long term hydration effect

Still, reliable research has shown that adequate hydration of the *stratum corneum* (SC) is an effective method to prevent or treat hypertrophic scars or keloids [357]. The decrease of SC hydration can cause the weakening of the skin barrier function. The electrical capacitance examination showed that there was significant change in SC hydration value before and after 30 days of application of the formulation ( $p > 0.05$ ). After 30 days of application of body cream, once a day, the skin hydration was measured and compared with day 0. Results were statistically different. At time 0 the hydration was

34.36 ± 3.95 corneometer units, while at time 30 days the value was 49.46 ± 6.16 corneometer units, for body cream containing the extracts. Regarding formulation F, the hydration was 35.97 ± 3.09 corneometer units and 42.39 ± 3.34 corneometer units, respectively, at 0 and 30 days of application.

The topical hydration increased with the daily applications of the formulation, as shown by the slight increase in the values of capacitance in the SC. The results of the present study are consistent with the literature data [358-361]. In these studies different authors evaluated the occlusive and hydration properties of different cosmetic formulations. In particular, the results are consistent with those of Wanitphakdeedecha *et al.*, who showed that a cream containing 0.1% of mucopolysaccharide polysulphate improves the hydration of the face skin after multiple applications during 28 days and of the skin of the volar side of the forearm after a single application [361].

#### - **Sensory evaluation**

The acceptance of personal care products for the consumer often depends on the perceived sensory properties during use. Sensory analysis could be defined as the examination of a product through the evaluation of the attributes perceptible by the five sense organs (organoleptic attributes), such as color, odor, taste, touch, texture and noise, allowing the establishment of the organoleptic profile of diverse products, including cosmetics [362]. An important advantage of the use of sensorial analysis in the quality control of a cosmetic product is that it yields a complex analysis in relation to all sensorial attributes that a product could present. The volunteer who participates on the sensorial analysis should be able to give information about the fragrance, the sensation, the appearance, the consistence, and other features experienced when use such product. According to Rose *et al.* the association of data obtained from sensorial analysis and instrumental analysis (especially physicochemical analysis) provides great information and a more complete profile of the product [363]. Figure 6.8 shows the average results of the hedonic sensory evaluation on a radar plot for the body formulation tested. The improvement in the conditions of the application area was apparent from the objective epidermic hydration measurements, on the one hand, and from the subjective judgment, on the other hand. The attributes of smell and texture appearance were mostly classified as very pleasant, penetration rate and appearance were tried as pleasant. The overall quality of the formulation was also considered very pleasant for most volunteers.

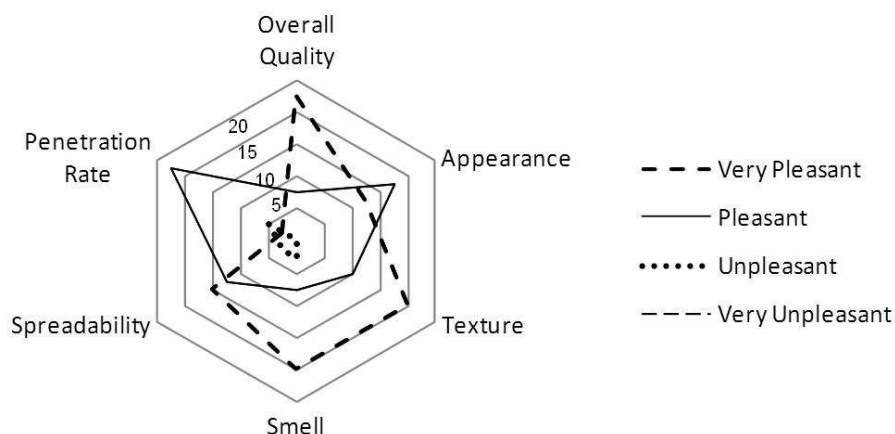


Figure 6.8 – Radar plot of hedonic sensory evaluation of the body cream formulation from different characteristics.

Finally, the results of this work contribute to the knowledge of consumers' perception regarding formulations containing food by-products extracts. The use of these food by-products extracts in cosmetic products can be indicated in night skin care and in dry skin products, as well as in products for consumers living in countries with a cold climate as it can form an oily film that has a protective effect on skin [364-365].

#### 4. Conclusion

Under the experimental conditions of this work, it is possible to conclude that CS and MS extracts, food by-products, influenced the sensory properties of a body care formulation.

The stability studies carried out suggested a shelf-life of about 12 months, considering international guidelines. After 180 days of storage at 25 °C / 65% RH and 40 °C / 75% RH the physical stability remains constant. Also, the antioxidant properties of formulation were satisfactory.

Results of *in vitro* studies revealed that the body cream formulation containing CS and MS extracts is safe in contact with human skin cells, namely, fibroblasts and keratinocytes. The *in vivo* patch test proved that this formulation did not cause skin

irritation. Also objective measurements, which are an important tool in clinical efficacy studies of these products, were performed. In the clinical studies on human volunteers, formulation containing the extracts showed significant improvement effects on skin hydration. The improvement in skin conditions shown by the efficacy studies confirms through objective analyses how effective are cosmetic formulations containing these extracts when the objective is the skin hydration and protection.

In addition, the results contribute to the understanding of cosmetic stability and efficacy in formulations containing food by-products, as there are no studies evaluating the stability and effects of these wastes when incorporated in topical formulations.

Finally, due to its good stability and efficacy, this formulation is a real option cosmetic product to improve skin conditions.

### **III. *In Vitro* and *In Vivo* Evaluation**

The information presented in this chapter section was published in the following publication:

- **Francisca Rodrigues**, Rita Matias, Marta Ferreira, M. Helena Amaral, M. Beatriz P.P. Oliveira, *In vitro* and *in vivo* comparative study of cosmetic ingredients Coffee Silverskin and hyaluronic acid, *Experimental Dermatology*, 2016, *in press*

## 1. Introduction

Facial wrinkles are one of the major features of aging. A key wrinkle-prone region of concern is the periorbital area, where the first signal of aging usually appear [366]. Over the last decades an increasing interest in reduction of aging appearance was observed [367]. Also, researchers aim to create innovative and well accepted cosmetics to perfectly come up to the sensory expectations of consumers. Traditionally, hyaluronic acid is used as a potent anti-wrinkle compound. HyaCare<sup>®</sup> Filler CL is a new anti-aging ingredient with a unique cross-linked polysaccharide made from fermentation-derived hyaluronic acid that contributes instantly to the reduction of facial fine lines and wrinkles, as well as increase skin elasticity [368]. Also, because of its high water-binding and strong short-term moisturization properties, HyaCare<sup>®</sup> Filler CL supports effectively the hydration of the skin [368]. Therefore, it is able to act as topical dermal filler, quickly reducing the appearance of wrinkles after application [368].

Recently the same purposes were made for caffeine. The topical use of caffeine is not a new procedure. It has been used in pharmaceutical and cosmetic preparations for a long time on the basis of its many favorable effects on the skin, namely, anti-cellulite and anti-aging effects. According to different authors, extracts from the two most common species of coffee directly combat UV damage improving strength, resilience and elasticity of facial skin [369]. *Coffea arabica* seed oil significantly improves collagen and elastin production while *Coffea robusta* has a high concentration of chlorogenic acid (which reduces redness associated with excessive sunlight exposure) and caffeine (which limits photodamage, decreases skin roughness and wrinkle formation, and reduces the appearance of crow's feet) [369]. However, to the best of our knowledge, any *in vivo* study in human volunteers was performed until now.

Nowadays, a new tendency in cosmetic formulations is the use of raw materials obtained from food by-products that normally are discarded as waste, with antioxidant, immunomodulatory or photoprotective purposes [22-23, 99, 101-102, 321, 370]. The use of these ingredients in cosmetic formulations answers to sustainability concerns [22-23, 99, 101-102]. Millions of tons of by-products are discarded during food processing in agro-industries, without economic value, but being a potential source of new ingredients for several industries, namely cosmetic and pharmaceutical [100]. Coffee Silverskin (CS) is a main by-product of the coffee roasting procedure and has no commercial value, being normally discarded as waste or used as fertilizer. Recent studies performed on the properties of CS reveals its antioxidant potential, huge content in caffeine and low

cytotoxicity against keratinocytes and fibroblasts [20, 22]. CS displays a diverse range of bioactive secondary metabolites with important antioxidant ability that could prevent free radicals deleterious actions. The *in vitro* irritancy of these extracts in skin and ocular 3-D models (validated and under validation by ECVAM) have been performed, according to the European Commission guidelines [102]. Rodrigues *et al.* evaluated the CS extracts behavior in *in vitro* skin and ocular irritation assays, using reconstructed human epidermis, EpiSkin™, and human corneal epithelial model, SkinEthic™ HCE, respectively [102]. An *in vivo* assay was also described by the same research group aiming to guarantee the extract safety [102]. The *in vitro* results demonstrated that the extract was not classified as irritant and the histological analyses proved that both models structure were not affected after exposure. The *in vivo* test carried out with the most promising extract (hydroalcoholic) showed that, with respect to irritant effects, it can be regarded as safe for topical application [102]. Also, other studies showed that some antioxidants acting as photoprotectives could maintain or restore a healthy skin barrier [86, 102].

In this study, a cream containing CS extract (Formulation A) as active ingredient was compared to an equal formulation supplemented with 1.5% of HyaCare® Filler CL (Formulation B). Based on these considerations, an *ex-vivo* assay in pig ear skin was performed with CS extract in order to quantify the caffeine content that cross the skin barrier. Also, an *in vitro* assay in keratinocytes and fibroblasts was performed with both formulations to access their safety in skin cell lines. The dermatological effects (skin efficacy and sensorial analysis) of both formulations in order to improve skin anti-wrinkles effect and to promote healthy skin were determined.

## 2. Materials and Methods

### 2.1 Materials

CS samples were provided by Bicafé (BICAFÉ – Torrefação e Comércio de Café Lda, Portugal), located in Porto, Portugal. Sabowax AE (Glyceryl Stearate, Cetearth-20, Cetearth-12, Cetearyl), Sabonal C16-18 (Cetearyl alcohol), Saboderm AB (C12-15 Alkyl Benzoate), Cyclopentasiloxane, Glycerol and Hyacare® Filler CL were provided by DS Produtos Químicos (Lisbon, Portugal). Methylisothiazolinone and Methylchlorisothiazolinone were obtained from Thor (Barcelona, Spain). Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™-I, fetal bovine serum (FBS), streptomycin, penicillin, and amphotericin B were from Invitrogen (Carlsbad, CA). MTS assay kit (CellTiter 96® Aqueous One Solution Cell Proliferation Assay) was purchased



from Promega Co. (Madison, WI). Lactate dehydrogenase (LDH) colorimetric cytotoxicity assay kit (LDH Cytotoxicity Detection Kit) was purchased from Takara Bio Inc. (Shiga, Japan). Deionized water was obtained using Mili-Q water purification system (TGI Pure Water Systems, St Lincoln, NE).

## **2.2 Coffee silverskin extract preparation**

The CS extract was prepared according to the method described by Rodrigues *et al.* [102]. Briefly, the sample (1 g) was submitted to solvent extraction by maceration with 20 mL of ethanol: water (1:1, v/v) for 30 min at 40 °C, filtered through Whatman No. 1 paper filter and collected.

## **2.3 Preparation of formulations**

Two formulations were prepared: the first one with 2.5% of CS extract (Formulation A) and another one with 2.5% CS and 1.5% of Hyacare<sup>®</sup> Filler CL (Formulation B). Briefly, Glyceryl Stearate, Ceteareth-20, Ceteareth-12, Cetearyl Alcohol, Cetyl Palmitate, Cetyl stearyl alcohol (50/50), Ciclopentasiloxane, Hyacare<sup>®</sup> Filler CL and C12 - C15 Alkyl Benzoate were heated at 70 °C, under magnetic stirring. Water and preservatives (Methylisothiazolinone and Methylchlorisothiazolinone) were mixed and heated at the same temperature. Water phase was slowly added to oil phase with mechanical stirring and allowed to reach 30 °C. At this temperature CS extract was added, the mixture homogenized and the formulation rested at least 24 h (20 °C).

## **2.4 *In vitro* caffeine permeability**

The skin permeation of caffeine from CS was evaluated using a Franz cell assembly (9 mm unjacketed Franz Diffusion Cell with 5 mL receptor volume, o-ring joint, clear glass, clamp and stir-bar; PermeGear, Inc., USA) and pig ear skin as barrier. The donor medium consisted of 0.3 mL CS extract. The actual concentration of caffeine in donor was 151 µg/mL. The receptor medium (5 mL) was HEPES buffer in order to maintain skin conditions. The available diffusion area between cells was 0.785 cm<sup>2</sup>. The stirring rate and temperature of receptor were kept respectively at 600 rpm and 37 °C. At

appropriate intervals, 0.6 mL aliquots of the receptor medium were withdrawn and immediately replaced with equal volumes of fresh buffer. The cumulative amount of caffeine was determined by UV-VIS spectrophotometry (Jasco V-660 Spectrophotometer, USA) at 275 nm.

### **2.5 *In vivo* extract hydration effect**

A single blinded study was performed in order to evaluate the *in vivo* skin hydration effect of CS. Twenty healthy individuals (15 women and 5 men) with a mean age of  $30 \pm 5$  years, without known dermatological diseases or allergy to substances in topical products, participated in the study, in accordance with the Declaration of Helsinki. Informed consent was obtained from all volunteers. They were asked to not apply any topical products in the forearms 24 h before the beginning and throughout the test period. Additionally, solar exposure was forbidden. Along with extracts, purified water was also assayed, respectively, as negative control. Before application of patch tests, the areas on the ventral part of each forearm were marked using a skin marker and basal values of hydration were obtained by non-invasive measuring method. The test solutions were applied, after basal measures were taken. Fifty microlitres of the test solutions (CS extract and the negative control (water )) were applied on a filter paper disc (12 mm, Filter Paper Discs, SmartPractice, Phoenix, USA) and occlusion was achieved with aluminium chambers patches (12 mm, Finn Chambers, SmartPractice, Phoenix, USA). Patches were removed after 48 h of occlusion. 2h after patches removal the levels of skin hydration were measured using a Corneometer<sup>®</sup> CM 825 (Courage & Khasaka, Köln, Germany) according to the European Group for Efficacy Measurements on Cosmetics and Other Topical Products (EEMCO) guidelines [371]. All measurements were performed at controlled temperature (20-22 °C) and relative humidity (45-55%). Three measurements at least were taken per area.

### **2.6 Formulations cytotoxicity**

To assess cell viability and cytotoxicity of the formulations, two different assays were used: (1) monitoring the uptake and metabolism of the vital mitochondrial dye, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) by cell mitochondria and (2) determining the leakage of the cytosolic enzyme,

lactate dehydrogenase (LDH) into the cell medium (LDH assay). In both tests, triplicate wells were incubated with fresh medium in the absence or presence of both formulations (1, 5 and 10 mg/mL) dissolved in cell culture medium. The dissolved formulations were filtered on 0.45 µm Millex GV filters (Millipore, Nepean, ON, Canada) and exposed to keratinocytes (HaCaT) and fibroblasts (HFF-1) cells during 24 h.

### 2.6.1 Cell lines and culture conditions

Human immortalized non-tumorigenic keratinocyte cell line HaCaT (ethnicity, Caucasian; age, 62 years; gender, male; tissue, skin) was acquired from CLS Cell Lines Service, Germany. Human foreskin fibroblasts (HFF-1) were purchased from ATCC (ATCC Number: SCRC-1041; ATCC, Manassas, VA, USA). Passage 17-19 of HFF-1 and passage 77-79 of HaCaT cells were used for both MTS and LDH assays. HaCaT and HFF-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™-I (Invitrogen) supplemented with 10% FBS, 100 µg/mL penicillin and 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B, and maintained in a 5% CO<sub>2</sub> environment at 37 °C. At 90-95% confluence, both cell lines were harvested using trypsin (Invitrogen), their viability assessed using the trypan blue dye (Gibco) exclusion assay, and re-plated in T75 flasks.

### 2.6.2 MTS assay

The MTS assay was performed according to the manufacturer instructions. Briefly, cells were cultured in 96-well microtiter plate at a density of  $25 \times 10^3$  cells per mL culture medium for 24 h at 37 °C. Following the removal of medium from the wells, the cells were washed with phosphate buffered saline (PBS pH 7.4). After the change of medium it was added fresh one with different concentrations of both formulations (1, 5 and 10 mg/mL) and cells were incubated at 37 °C for 24 h. After that medium was rejected and the number of viable cells evaluated by adding the MTS reagent in medium to each well and incubating for 4 h at 37 °C. The positive control was the medium and the negative control was Triton X-100. The absorbance was measured at 490 nm with background subtraction at 630 nm. Each concentration was tested in triplicate in three independent experiments.

### 2.6.3 LDH assay

Lactate dehydrogenase (LDH), a stable cytoplasmic enzyme found in all cells, is quickly released to the plasma membrane upon damage. Cell lines were seeded in 96-well plates (5000 cells for LDH) and cultured for 48 h. Different concentrations of the formulations, namely 1, 5 and 10 mg/mL, were prepared in appropriate medium and added to the cells. Media without FBS were used in the case of LDH. After incubation (37 °C/ 5% CO<sub>2</sub>), cells were washed twice with PBS and the kit was used according to the instructions of the manufacturers. The maximum LDH release (high control) from cells was determined using 1% (w/v) Triton X-100 (Boehringer Mannheim). Spontaneous LDH release (low control) was determined by incubating the cells with plain medium. Absorbance was measured at 490 nm with background subtraction at 690. Each concentration was tested in triplicate in three independent experiments.

## 2.7 Clinical studies

The present study was conducted in accordance to the principles of the Declaration of Helsinki and its amendments and in compliance with independent ethics committee requirements.

### 2.7.1 Study design

For each formulation, twenty female volunteers with clearly wrinkles signals, aged 35 - 55 years (average  $42.15 \pm 6.28$ ) and skin types II–IV, were randomly enrolled in the study, between November 2014 and March 2015. Informed consent was obtained from each volunteer. The volunteers' skin wrinkles were classified according to Fitzpatrick Wrinkle Scale [372]. Inclusion criteria were as follows: ocular wrinkles, voluntary participation and agreement not to use other creams during the study. Exclusion criteria were as follows: pregnancy, active skin diseases, atopy, allergic contact dermatitis, cancer areas, retinoid therapy, hormonal therapy, current participation in another clinical study, ultraviolet light exposure and lack of cooperation.

Formulations were applied to the face of each volunteer, twice a day, during 28 days. Before starting the measurements, the subjects stayed during 30 min in a temperature and humidity controlled environment (20-22 °C and 45-55%, respectively) for acclimatization. Measurements were performed before application (basal values) and 28 days later (long term effect).

## 2.7.2 Biophysical techniques

### Visual Evaluation (VisioFace Quick)

In order to guarantee standardized photo documentation a VisioFace Quick<sup>®</sup> machine (Courage & Khasaka, Köln, Germany) was used. The Visioface<sup>®</sup> digital photography imaging system consists of a cabin attached to a high resolution digital camera (10 megapixels) and 200 white LED and 400 UV LEDs. The visual evaluation of treatment results was carried out by trained professional staff at time 0 and after 28 days of study, in order to visualize final aspect effects.

### Corneometer<sup>®</sup>

Skin hydration was investigated using a Corneometer<sup>®</sup> CM 825 (Courage & Khasaka, Köln, Germany) coupled to a Multi Probe Adapter<sup>®</sup> MPA 5 (Courage & Khasaka, Köln, Germany). Capacitance changes, depending almost solely upon the water content in the stratum corneum, are detected and evaluated. Different capacitance changes are converted into a digital measured value (arbitrary units), which is proportional to the skin humidity [25]. Three measurements were performed in each testing area on the periorbital wrinkles.

### Cutometer<sup>®</sup>

The viscoelastic properties of the skin were investigated with a Cutometer<sup>®</sup> SEM 575 (Courage & Khasaka, Köln, Germany). The measuring principle is suction/elongation. An optical system detects the decrease in infrared light intensity depending on the distance the skin is being sucked into the probe. The strain time-mode was applied in this study. A probe with a 2 mm opening was used, and a pressure of 450 mbar was applied in order to suck the skin into the probe. Each measurement consisted of five suction cycles (3 s of suction followed by 3 s of relaxation) on the corner eye. The parameters  $U_f$  (maximum deformation during the first cycle),  $U_r/U_e$  (neto-elasticity of the skin) and  $U_r/U_f$  (biological elasticity) were evaluated.

### Profilometry (PRIMOS<sup>®</sup>)

The profilometric measurements were carried out using the PRIMOS<sup>®</sup> Premium ("*Phase Shift Rapid Imaging of Skin*"; GfMesstechnik, Germany) method in the area around the lateral corner of the eye (crow's feet area). Three measurements were taken on days 0 and 28. Three parameters were evaluated: wrinkle depth, volume of cavities

and roughness. To calculate the skin profile and determine the depth of wrinkles, PRIMOS<sup>®</sup> software V5.7, 2010 was used.

## 2.8 Sensory evaluation

Twenty eight days after the beginning of the study, volunteers were asked to answer a questionnaire about the formulation and sensorial effects, in order to have information about their sense and perception concerning the cosmetic qualities and efficacy results. 4-option answers were used for the questionnaire evaluation of the attributes of penetration rate, color, smell, efficacy and overall quality after application (strongly agree, agree, slightly agree and disagree).

## 2.9 Statistical analysis

Data were reported as mean  $\pm$  standard deviation of at least triplicate experiments. Statistical analysis of the results was performed with the software SPSS 22.0 (SPSS Inc., Chicago, IL, USA). In all cases,  $p < 0.05$  was accepted as denoting significance.

# 3. Results and Discussion

## 3.1 *In vitro* skin permeation

When a cosmetic product is topically applied, an active agent must be released from the carrier (vehicle) and be able to penetrate into the *stratum corneum* and lower layers of the skin [373]. In the particular case of CS extracts, caffeine and other phytopharmaceuticals present in the extract are able to carry out pharmacological activities, and even the expected activity may be the result of synergic action of several compounds acting together. For cosmetic products, it is important that the active compounds stay in the skin, penetrate sufficiently deep but not too deep leading to systemic availability [374].

The caffeine permeation into pig ear skin treated with CS extract was analyzed. Franz diffusion cells were used and pig ear skin was selected as model barrier, considering its similarity to human skin. Results are presented in Figure 6.9.

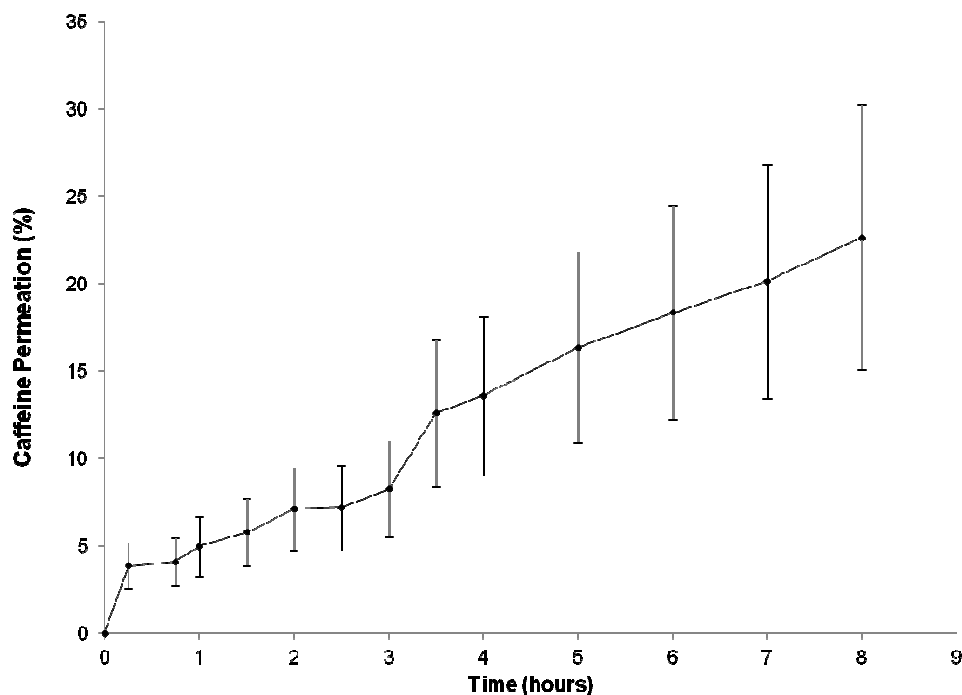


Figure 6.9 - Permeation of caffeine of CS extract through pig ear skin over 8 hours. Each value represents the mean  $\pm$  SD ( $n = 3$ ).

After 8 hours, only a very small amount of caffeine crossed the skin, respectively, about 20% of the extract content. The delivery of active substances to the skin is not easy, since this organ is not organized to favor the absorbance of substances from the external environment, but rather to provide a protective barrier [375]. Epidermis is an effective self-renewing barrier on the surface, largely composed by hyperkeratinized cells, while dermis is a deeper layer of metabolically active cells and extracellular matrix. The delivery of active components that should address specific physio-pathological conditions, such as compounds endowed with anti-aging properties, should be able to efficiently pass these compartments. Until the present, and to the best of our knowledge, there is no published research works concerning the permeability of CS extracts.

### 3.2 *In vivo* extract hydration effect

The measurement of hydration is a well-established method in dermatology to assess the skin effect of a new ingredient. Results of CS on skin are summarized in Table 6.8.

Table 6.8 - Hydration variation from basal values of negative control (NC) and CS extract (CS) at time 0 and 2 h after patch removal following a 48h occlusion period.

Corneometer Units		
	T0 hours	T48 hours
NC	35.98±7.06	36.48±6.58
CS	35.98±7.07	46.56±7.08*

\* means a significant difference ( $p < 0.05$ ).

According to Table 6.8, the extract clearly improves skin hydration after 48 hours of a patch test when compared to the negative control used ( $p < 0.05$ ). Probably, this effect is due to the well detailed extract composition [22, 102-103].

### 3.3 Formulation cytotoxicity

The effects of both formulations on keratinocyte (HaCaT) and fibroblast (HFF-1) cell lines growth were determined by MTS and LDH assays. In the MTS assay both formulations were added to HaCaT and HFF-1 cells to analyze their effects on cell viability (Table 6.9).

Table 6.9 - Effect on the metabolic activity of HaCaT and HFF-1 cells after exposure to Formulation A and B at different concentrations, measured by the MTS assay. Values are expressed as mean  $\pm$  SD ( $n=6$ ).

	Cell Viability (%)					
	HaCaT			HFF-1		
	1 mg/mL	5 mg/mL	10 mg/mL	1 mg/mL	5 mg/mL	10 mg/mL
<b>Formulation A</b>	135.05±6.89	124.37±4.09	124.37±7.56	136.40±4.24	127.33±3.77	119.09±8.12
<b>Formulation B</b>	125.44±3.02	163.75±2.52	152.46±6.29	116.25±4.74	152.39±4.04	153.46±2.70

In this study, potential cytotoxic effects of both formulations were also investigated by examining membrane integrity of HaCaT and HFF-1 cells using a LDH release assay. In keratinocytes, at all concentrations tested, both formulations did not originate any effects on the LDH leakage following 24 h incubation, which means that these



formulations are potentially safe. The evidence of possible absence of cytotoxicity was tested at three different concentrations (1, 5 and 10 mg/mL). The cytotoxicity test of both formulations on fibroblasts was conducted at the same concentrations and also no significant LDH-increase was observed, indicating the absence of cytotoxicity of these formulations on human fibroblasts. These results are in line with the cytotoxicity of CS extract previously evaluated by our research group, that also demonstrated a low toxicity against keratinocytes and fibroblasts cells lines [22].

### **3.4 *In vivo* evaluation**

#### **3.4.1 Safety and efficacy studies**

The purpose of this study was to evaluate the safety and cosmetic efficacy of a new cosmetic ingredient, namely CS extract, incorporated in a facial cream and compares its efficacy with the same formulation containing a well known ingredient with anti-wrinkles effect, Hyacare<sup>®</sup> Filler CL. With the aim to standardize templates, all subjects were asked to wash off any make-up before photographic sessions and rest sitting for 15 minutes at constant thermal comfort room parameters: temperature (20-22 °C) and relative humidity (45-55%). At the end of each photographic session an *in vivo* 3D photographic quantitative evaluation of skin wrinkles was performed.

Standardized clinical photographs were taken at baseline and after 28 days using a Visioface<sup>®</sup>, in order to obtain standardized images in terms of brightness, image size and field of vision. Figure 6.10 and 6.11 are examples of clinical photographs obtained at time 0 and after 28 days of application of Formulation A and B, respectively.



Figure 6.10 - Illustrative images obtained with the digital photography imaging system Visioface<sup>®</sup> at time 0 and after 28 days of Formulation A application.



Figure 6.11 - Illustrative images obtained with the digital photography imaging system Visioface<sup>®</sup> at time 0 and after 28 days of Formulation B application.

## Hydration

As it is well known, skin hydration is very important for normal cutaneous metabolism and to prevent skin alterations and early aging. The skin moisture content was measured at time 0 and after 28 days of the study period. Results for both formulations are summarized in Table 6.10.

Table 6.10 - Hydration variation from basal values of Formulation A and Formulation B at time 0 and after 28 days of study.

	Corneometer Units	
	T0 Days	T28 Days
Formulation A	54.74 ± 6.57	65.54 ± 5.67*
Formulation B	55.76 ± 5.98	62.08 ± 9.20*

\* means a significant difference ( $p < 0.05$ ).

As it is possible to observe the hydration improvement was similar for both creams, with statistical differences regarding time 0. No statistical differences were observed between Formulations A and B.

## Mechanical properties of skin

The elasticity is one of the basic measurements for all cosmetic products that are intended to be claimed as anti-aging. Different parameters from the different portions of the measurement curve could be obtained. Table 6.11 summarized the obtained results.

Table 6.11 - Effects of topical application of Formulation A and B on mechanical properties of skin.

	Day	Cutometer parameters		
		Uf	Ur/Ue	Ur/Uf
Formulation A	0	0.37±0.09	0.50±0.13	0.23±0.04
	28	0.35±0.06	0.57±0.04*	0.26±0.04*
Formulation B	0	0.36±0.09	0.48±0.15	0.22±0.04
	28	0.35±0.08	0.58±0.13*	0.26±0.04*

\* means a significant difference ( $p < 0.05$ ).

The Cutometer<sup>®</sup> parameter UF (maximum deformation during the first cycle) gives information about the firmness of the skin. Lower amplitude correlates with firmer skin. During the application period, UF decreased for Formulation A, from 0.37 mm (SD 0.09) to 0.35 mm (SD 0.06), and Formulation B, from 0.36 mm (SD 0.09) to 0.35 mm (SD 0.13). However, no significant differences ( $p < 0.05$ ) were detected at time 28 days. Therefore, the tightening effects on the skin are negligible. Also, it has to be critically remarked that the inter-individual differences of this absolute parameter were well-pronounced. In order to make valuable statements on effects which influence the viscoelasticity of the skin, relative parameter seem to be useful instead of absolute parameters. The relative Cutometer<sup>®</sup> parameter  $Ur/Ue$  stands for the neto-elasticity of the skin. The initial values were similar for both formulations (Table 6.11). The application period of 28 days lead to an increase of  $Ur/Ue$  from 0.50 mm (SD 0.13) to 0.57 mm (SD 0.04) for Formulation A and from 0.48 mm (SD 0.15) to 0.58 mm (SD 0.13) for Formulation B (Table 6.11). Both formulations increased the neto elasticity of the skin to a certain extent with statistically differences. Also, there was no difference in effectiveness between the formulations. Regarding the  $Ur/Uf$  parameter, that allows for biological elasticity, it is also possible to observe an increase for both formulations. Formulation A presented an increase from 0.23 mm (SD 0.04) to 0.26 mm (SD 0.04), while Formulation B had an increase from 0.22 mm (SD 0.05) to 0.26 (SD 0.05). Once again, inside both formulations it is possible to observe statistical differences at time 28 days. However, no statistical differences were observed between Formulations A and B. Using the chosen conditions, it was possible to monitor an increase in elasticity on the skin for both formulations. The comparison of Formulation A with other formulations containing extracts or even nanoparticles, led us to conclude that CS extract present clear effects on skin firmness [351, 376].

### **Effect on skin relief**

PRIMOS<sup>®</sup> is an optical system that produces three-dimensional measurements of the skin surface. A pattern consisting of parallel stripes is projected onto the skin surface by micro-mirrors contained within a digital projector. The PRIMOS<sup>®</sup> test was conducted at the corner of the eye, for technical reasons, and allows a precise evaluation of depth, roughness and volume of a wrinkle. The resulting wrinkle profile enables quick and efficient visualization of changes in the analyzed wrinkle [63]. The detailed skin surface analyses were carried out and one example is shown in Figure 6.12. The photographs obtained with PRIMOS<sup>®</sup> (Figure 6.12 and Figure 6.13) were also utilized to evaluate different parameters: wrinkle depth, volume of cavities and roughness.

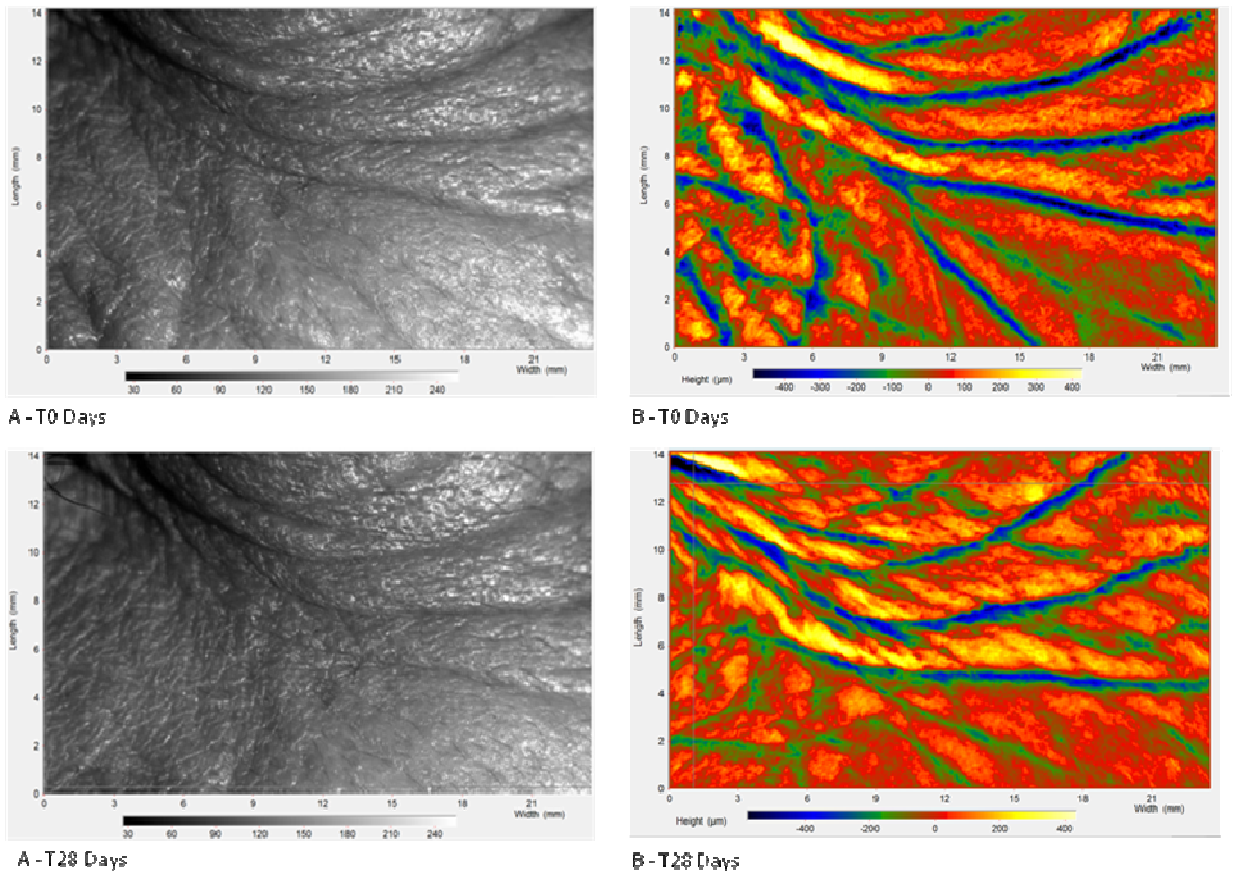


Figure 6.12 - Photos of periorcular area acquired with PRIMOS<sup>®</sup> equipment of one of the volunteers who exhibited reduced depth of wrinkles at time 0 and after 28 days of Formulation A application. (A) Photography. (B) Representation of relief in a colored image, where each color corresponds to a height (positive values) or depth (negative values) with respect to the reference plane, showing a decrease of wrinkle depth throughout the study.

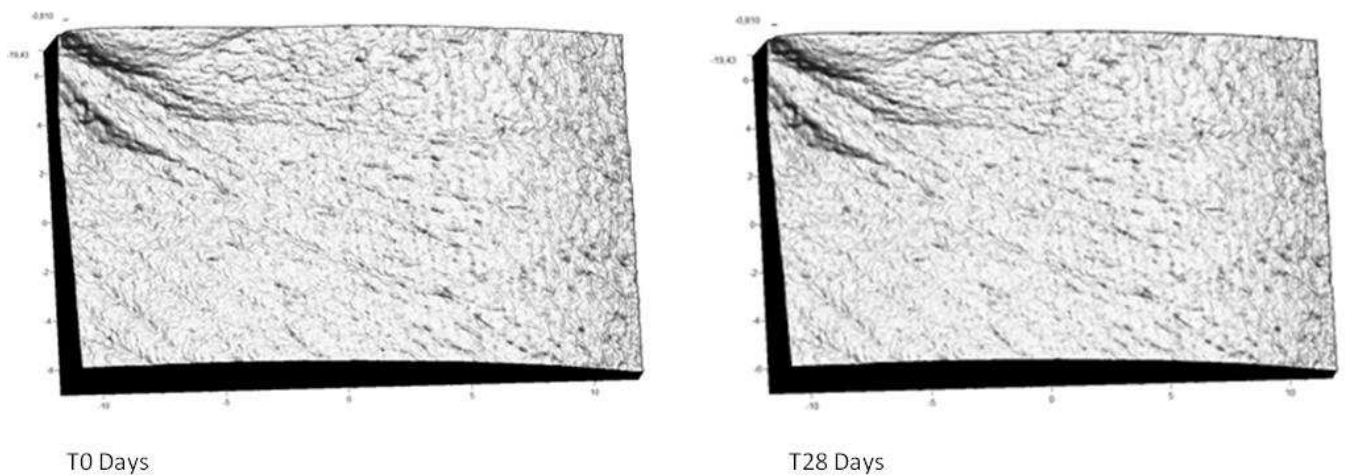


Figure 6.13 - Three-dimensional representation of the periorcular area acquired with PRIMOS<sup>®</sup> equipment of one of the volunteers who exhibited reduced depth of wrinkles.

Figure 6.12 reveals a gradual refilling of an eye wrinkle during the treatments. As shown, the analyzed wrinkle depth decreased from 206  $\mu\text{m}$  to 179  $\mu\text{m}$  after an application period of 28 days of Formulation A. However, no statistical differences were observed considering all volunteers. Also, between Formulation A and Formulation B no differences were observed. The volume of the wrinkle did not change after 28 days of treatment, but a slight decrease could be observed, with no statistical differences (Figure 6.14). Also, results revealed that older volunteers presented a clear improvement of wrinkles depth, unlike the younger volunteers, where it was possible to observe a decrease not significant. The percent of changes in the values for volunteers were calculated and summarized in Figures 6.14, 6.15 and 6.16.

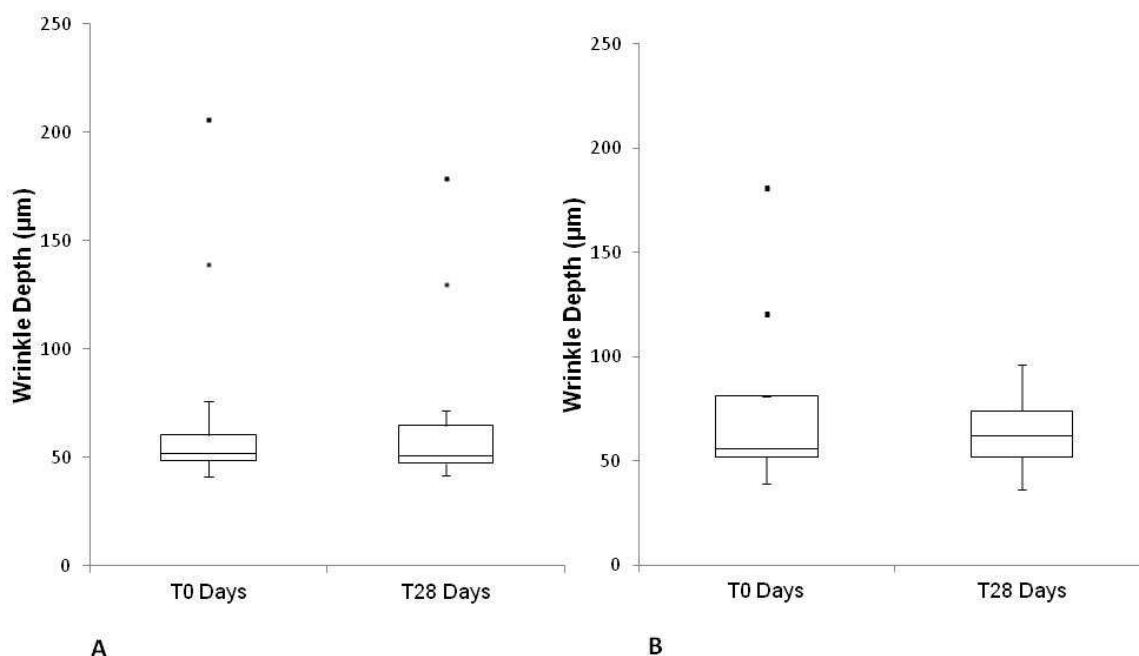


Figure 6.14 - Illustrative box plot graphs of the depth values of wrinkles at baseline and 28 days after using the test products. (A) Formulation A; (B) Formulation B.

Regarding volume of cavities, it is also possible to observe a slight decrease for both formulations, but no statistical differences were observed at time 0 and 28 (Figure 6.15).

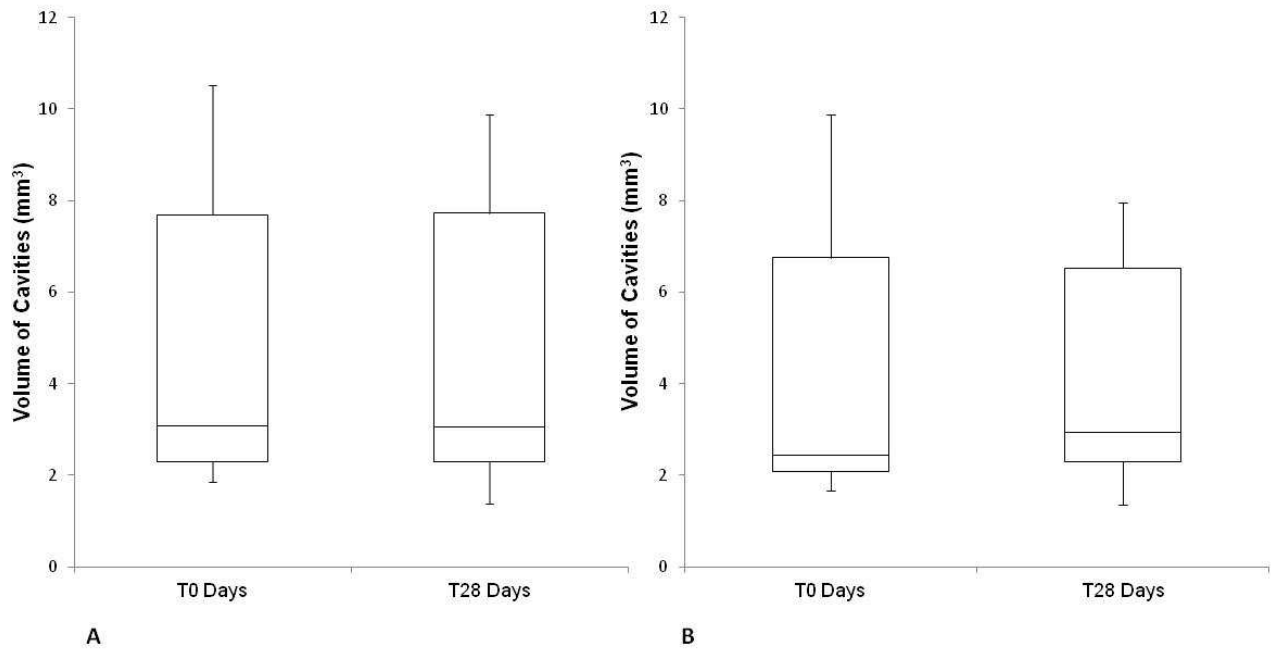


Figure 6.15 - Illustrative box plot graphs of volume of cavities at baseline and 28 days after using the test products. (A) Formulation A; (B) Formulation B.

Also, for roughness the scenario is the same, with no statistical differences for any formulation (Figure 6.16).

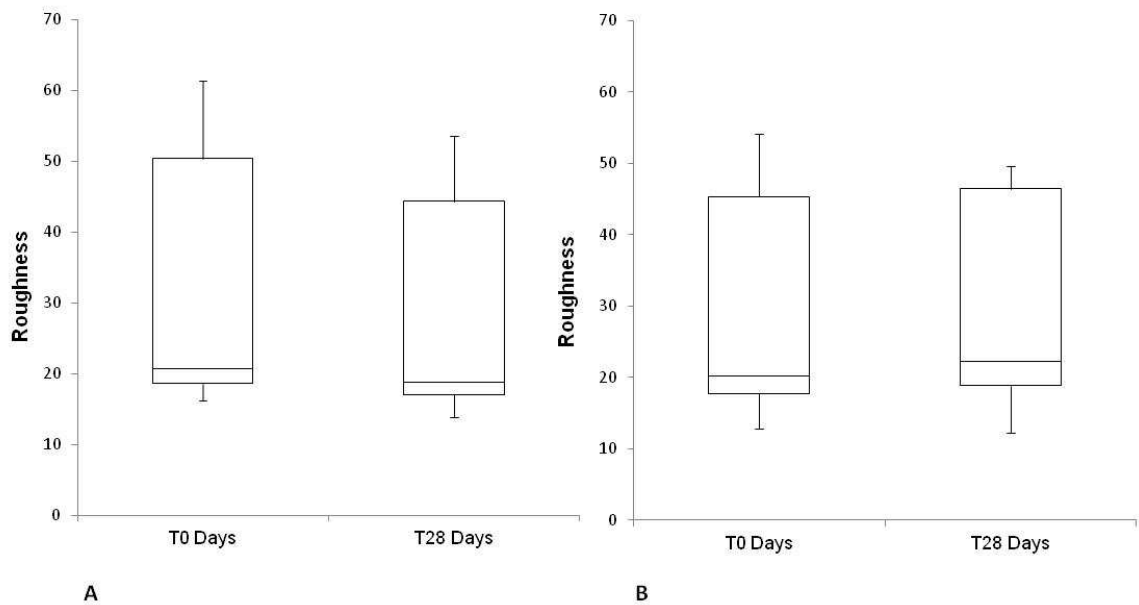


Figure 6.16 - Illustrative box plot graphs of the roughness parameters at baseline and 28 days after using the test products. (A) Formulation A; (B) Formulation B.

It has to be remarked that the volunteers had middle initial values of roughness, wrinkle depth and volume of cavities. Due to the fact that they were  $42.15 \pm 6.28$  years old, volunteers probably did not have the so-called 'aged' skin. This is supported by the fact that older volunteers presented statistical differences between time 0 and 28. Also, a longer study period, 56 days for example, could determine statistical differences between time 0 and time 56. At this point, it is possible to conclude that there are no statistical differences between time 0 and time 28 intra and inter-formulation.

The results in this report are relevant and confirm observations from other authors, showing the efficacy of anti-aging formulations containing antioxidants, such as vitamins and flavonoids, and an improvement in skin microrelief, objectively or determined in a subjective manner as a perception of efficacy [334, 351, 377-378]. A complete efficacy study of cosmetic formulations containing CS and/or other active substances such as hyaluronic acid is of fundamental importance in order to obtain conclusive results and as an indicator of adequate components to be included in stable and efficient products. According to the results obtained in efficacy studies, CS was shown to be an effective ingredient, with similar results to hyaluronic acid, in the improvement of skin conditions, particularly, skin hydration and firmness. In addition, according to Rodrigues *et al.* [22, 102] the extract had a good antioxidant activity and low cytotoxicity in skin cell lines. Also, the compound is described as a stable substance in topical formulations, which is very important in terms of efficacy [100, 103]. However, these formulations did not improved skin microrelief, especially in relation to wrinkle depth, volume of cavities and roughness. A possible justification for the lack of results could be the young age of the volunteers. In fact, old volunteers presented statistical differences at time 28 in all microrelief parameters. Also, a longer study period could probably lead to considerable differences. No statement can be made regarding the influence of both test formulations on biomechanic skin properties.

### **Sensorial evaluation**

Nowadays, the actual development of cosmetics include sensory analysis to find the best formulation for a certain product from a number of candidates. Table 6.12 summarizes the obtained results in sensorial evaluation.



Table 6.12 - Sensory evaluation of both formulations considering different characteristics such as color, smell, penetration rate, efficacy and overall quality.

	<b>Formulation A</b>			
	<b>Strongly agree</b>	<b>Agree</b>	<b>Slightly agree</b>	<b>Disagree</b>
I liked this cosmetic product.	94.74	5.26	0.00	0.00
I liked the cosmetic product color.	94.74	5.26	0.00	0.00
I liked the cosmetic product smell.	73.68	21.05	5.26	0.00
The cosmetic product had a good penetration rate.	88.89	5.56	5.56	0.00
I believe that the cosmetic product was effective.	57.89	26.32	15.79	0.00
I would like to continue using this cosmetic product.	68.42	21.05	10.53	0.00
I would buy this cosmetic product.	63.16	26.32	10.53	0.00
	<b>Formulation B</b>			
I liked this cosmetic product.	68.75	25.00	6.25	0.00
I liked the cosmetic product color.	87.50	12.50	0.00	0.00
I liked the cosmetic product smell.	68.75	31.25	0.00	0.00
The cosmetic product had a good penetration rate.	68.75	25.00	0.00	6.25
I believe that the cosmetic product was effective.	75.00	18.75	6.25	0.00
I would like to continue using this cosmetic product.	50.00	37.50	6.25	6.25
I would buy this cosmetic product.	50.00	31.25	6.25	12.50

Based on responses to the administered questionnaire it was determined that:

- 58% of trial participants felt that Formulation A was effective at reducing their wrinkles, comparing with 75% for Formulation B;

- 89% of women felt that the penetration rate of Formulation A was good, comparing with 69% for Formulation B;
- 100% of women were likely to recommend Formulation A to a friend, while for Formulation B was 94%.

In a general way it is possible to observe that almost all volunteers appreciated both formulations and classified them in a positive way regarding effectiveness and characteristics, with no differences.

#### 4. Conclusion

*In vitro* permeation studies using Franz diffusion cells demonstrates that CS extract permeate into pig ear skin and, therefore, presumably should permeate human skin *in vivo* if incorporated in a cosmetic product. The amount of caffeine that penetrated was about 20% after 8 hours.

Two formulations (supplemented or not with hyaluronic acid) were developed and *in vitro* and *in vivo* evaluations were performed. The *in vitro* cytotoxicity assay in fibroblasts and keratinocytes demonstrated that both formulations are safe. Also, the *in vivo* efficacy studies on human skin, in twenty volunteers per each formulation, demonstrated that both formulations enhanced *stratum corneum* moisture content when compared with the baseline values.

The viscoelastic-to-elastic ratio after 28 days of application improved for Formulation A and B. For both parameters, no differences were found between formulations.

Regarding microrelief, a slightly decrease in wrinkle depth, roughness and volume of cavities could be observed after 28 days of study. However, no statistical differences were detected. Probably a longer clinical trial with evaluation after 56 days could originate a clear decrease in these parameters. These results are similar for both formulations.

In the sensorial evaluation, all volunteers expressed satisfaction with the product. Both formulations were classified as safe and effective, in a consumer perspective.

In conclusion, it can be stated that there are no differences between coffee silverskin extract and the well documented hydration ingredient hyaluronic acid. Also, according to CS composition, this could be considered as a new effective ingredient for cosmetic purposes.

# Nanoencapsulation of Coffee Silverskin: A Future Approach?

The information presented in this chapter was partially published in the following publications:

- **Francisca Rodrigues**, Ana Catarina Alves, Cláudia Nunes, Bruno Sarmento, M. Helena Amaral, Salette Reis, M. Beatriz P.P. Oliveira, Permeation of topically applied caffeine from a food by-product in cosmetic formulations: a nanoscale *in vitro* approach, International Journal of Nanomedicine, *submitted*

## 1. Introduction

Over the last years increasing research interest has been focused on natural compounds and their therapeutic potential. For most topically applied pharmaceuticals and cosmetics, permeation through the skin barrier is essential for exert their effects [379].

The use of caffeine in topical application is not a new procedure. It has been used in pharmaceutical and cosmetic preparations for a long time on the basis of its many favorable effects on the skin, namely, anti-cellulitis and anti-aging effects. According to different authors, extracts from the two most common species of coffee plant directly combat UV damage, by improving the strength, resilience and elasticity of facial skin [369]. *Coffea arabica* seed oil significantly improves collagen and elastin production while *Coffea robusta* has a high concentration of chlorogenic acid (which reduces redness associated with excessive sunlight exposure) and caffeine (which limits photodamage, decreases skin roughness and wrinkle formation, and reduces the appearance of crow's feet) [369]. Also, its high biological activity and ability to penetrate the skin barrier had lead to a wide use [309]. Recently, there is also some evidence that dermally applied caffeine can protect the skin from cancer caused by sun exposure [380]. Caffeine and its derivatives are used in a number of commercial anti-cellulite creams as an additive ingredient. However, there are limited direct evidences to support their role in cellulite reduction. A number of claims, as anti-cellulite properties, are based on the implicit assumption that this bioactive substance is effectively released from the formulation into the epidermis and then passes to the dermal and subcutaneous tissues, preventing the excessive accumulation of fat in cells, and providing a slimming effect [309-310, 381]. According to Herman *et al.* this alkaloid stimulates the degradation of fats during lipolysis through inhibition of the phosphodiesterase activity and has potent antioxidant properties [309]. However, studies assessing the skin absorption of caffeine released from extracts are extremely rare in the literature.

In recent years, sustainable approaches have become an essential challenge for different industries, particularly cosmetic industry, looking for new active ingredients, always taking in account questions of sustainability and green chemistry. Coffee Silverskin (CS) is a main by-product of the coffee roasting procedure and has no commercial value, being discarded as a solid waste [10]. Some work has been performed on CS properties, particularly regarding its antioxidant and caffeine content, cytotoxic effects and isoflavones profile [15, 19-20, 22, 102]. According to Bresciani *et al.* caffeine content in CS is about to 10 mg/g of product [19]. Recent changes in regulatory requirements and social views on

animal testing have incremented the development of reliable alternative tests for predicting skin and ocular irritation potential of products based on new raw materials [102]. Thus, we evaluated the *in vitro* and *in vivo* safety of this new possible cosmetic ingredient. An *in vitro* skin and ocular irritation assay (using reconstructed human epidermis, EpiSkin™, and human corneal epithelial model, SkinEthic™ HCE) and an *in vivo* assay (patch test) were performed with very good results.

Lipid nanoparticles, namely solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) are among the popular research topics for the delivery of drugs [382]. Overall, lipid nanoparticles present many advantages such as low production costs, easily to scale up and additionally low toxicity, since organic solvents are not required for the synthesis of these nanoparticles [383]. NLCs represent the second generation of lipid nanoparticles and are composed of a blend between solid and liquid lipids which allows the formation of an overall amorphous nanostructure with many imperfections within its matrix, providing NLCs with higher drug loading capacity and lesser degree of drug expulsion during storage comparing with SLNs.

In order to improve the permeation of caffeine into skin, the incorporation of CS in NLCs could be a new approach. The aim of this work intends to evaluate NLCs formulations potential for the dermal treatment of different skin conditions, such as aging or cellulite, with caffeine obtained from a coffee by-product, comparing with the extract directly applied into skin.

## 2. Materials and Methods

### 2.1. Materials

CS samples were provided by Bicafé (BICAFÉ - Torrefação e Comércio de Café Lda, Portugal), located in Porto, Portugal. Precifac® ATO (cetyl palmitate) was a kindly gift from Gattefossé (Cedex, France). Miglyol® 812 was obtained from Acofarma (Madrid, Spain). Tween® 60 (Polysorbate 60) was purchased from Merck KGaA (Darmstadt, Germany). All chemicals and solvents were of analytical grade. Aqueous solutions were prepared with double-deionized water (Arium Pro Sartorius AG, Göttingen, Germany, conductivity less than 0.1  $\mu\text{S cm}^{-1}$ ).

## 2.2. Preparation of coffee silverskin extract

CS was milled to particle size of approximately 0.1mm using a A11 basic analysis mill (IKA Wearke, Staufen, Germany) and stored in silicone tubes at room temperature until extraction. Samples of 1 g were subjected to maceration for 30 min at 40 °C with 20 mL of ethanol: water (1:1). Extracts were filtered through Whatman No. 1 paper filter and the filtrate collected. Samples were stored at 4 °C until analysis [22, 102].

## 2.3. Preparation of NLCs

The method for the preparation of nanoparticles was based on hot homogenization followed by sonication. Briefly, the excipients (lipids and surfactant) were mixed together and kept at a temperature above the lipids melting point (70 °C) to promote their mixture. Extract was added to the lipid phase. The primary emulsion was poured in a hot aqueous phase to obtain a nanoemulsion with sonication (Vibra-Cell™; Sonics and Materials, Inc, Newtown, CT, USA). The sonicator adjustable frequency used was 70% during 20 minutes, after which was cooled to room temperature, allowing the inner oil phase to solidify and forming NLCs dispersed in an aqueous phase.

To optimize the caffeine association in the NLCs and to determine the best concentration of the different excipients in order to obtain a mean size and polydispersity index (PDI) of almost 200 nm and below 0.250, respectively, different concentrations of excipients were tested. The choice of the best rate was based on the analyses of the size and zeta potential. The final composition of the formulations is shown in Table 7.1.

Table 7.1 - Composition of the developed NLCs formulations (NLC - empty formulation; NLC-CS- coffee silverskin formulation).

Formulation Code	Polysorbate 60	Miglyol 812	Precifac ATO	CS	Water
NLC	10	3	7	-	80.0
NLC-CS	10	3	7	2.5	77.5

Amounts expressed in g/100 g of formulation.

## 2.4. Determination of association efficiency

The association efficiency (AE) was determined by quantifying the difference between the total amount of caffeine used to prepare the systems and the amount of caffeine that remained in the aqueous phase after NLC isolation, determined by HPLC. A 1:5 dilution of NLC formulations in double-deionized water was subsequently centrifuged (Heraeus™ Multifuge™ x 1R Centrifuge, USA) through centrifugal filter units (Amicon® Ultra Centrifugal Filters, Ultracel - 50 KDa, Darmstadt, Germany) at 4500 rpm and 20 °C, during 45 min or until complete separation between the NLCs retained in the filter unit and the aqueous phase corresponding to the filtrate. The filtrate was used to quantify the amount of non-incorporated caffeine.

The HPLC method used in this study to quantify caffeine was based on the work of Lemos *et al.* and Rivelli *et al.* [316-317]. The chromatographic analysis was performed in a HPLC integrated system (Jasco, Japan) equipped with a PU-980 pump and a Jasco AS-950 automatic sampler with a 20 µL loop. Detection was performed with a Jasco model MD-2010 multiwave length diode-array detector (DAD). The column employed was a Luna 5U C18 (5 µm, 150 mm × 4.60 mm) chromatographic column (Teknokroma, Spain). The mobile phase adopted was aqueous metaphosphoric acid solution: acetonitrile (82:18, v/v) with a flow rate of 1 mL/min and a column temperature of 23 °C. Caffeine was monitored at 275 nm and quantification was performed on the basis of the internal standard method. Chromatographic data were analysed using the ChromNAV Software (Jasco, Japan). Calibration curves were prepared (0.244 - 750 µg/mL) and analyses carried out in duplicate. Confirmation of compounds identities was performed by comparing retention times and co-elution with authentic standards and by UV absorption spectra.

Taking into account the drug initially added to the NLCs formulation and subtracting the free caffeine remaining in the filtrate, it was possible to determine the amount of drug incorporated in the NLCs and thus the association efficiency by the following equation:

$$AE = \frac{\text{Total amount of Caffeine} - \text{Free caffeine in supernatant}}{\text{Total amount of Caffeine}} \times 100$$

## **2.5. Determination of particle size, surface charge and physical stability**

The particle size and PDI were determined by dynamic light scattering (DLS), while the zeta potential (surface charge) was evaluated by electrophoretic light scattering (ELS), using a ZetaPALS zeta potential analyzer (Brookhaven Instruments Corporation, Holtsville, NY, USA). Samples were diluted with Milli-Q-water to suitable concentration to determine particle size and PDI at 25 °C. All measurements were performed in triplicate. For each sample, the corresponding mean diameter standard deviation values were obtained from six determinations and calculated by the multimodal analysis.

The zeta potential Smoluchowski mathematical model was used to obtain the corresponding measurements of surface charge analysis. For each sample, the corresponding mean  $\pm$  standard deviation values were obtained from six runs of ten cycles. For each final formulation, at least three batches were analysed for mean particle size, polydispersity and zeta potential as described.

## **2.6. Morphology**

Cryogenic scanning electron microscopy (cryo-SEM) was performed in all dispersions in order to obtain information about particle size, shape and surface morphology, at the Materials Centre of the University of Porto, Portugal (CEMUP). A 1:500 dilution of NLCs in double-deionized water was dropped on a support and rapidly frozen in liquid nitrogen. Cryofractures were then performed using an ALTO 2500 (Gatan Alto 2500 (Pleasanton, CA, USA), with subsequent sublimation and coating with Au/Pd by sputtering for 35 s. The samples were then observed at -150 °C using a JSM 6301F microscope (JEOL, Tokyo, Japan). The cryo-SEM was performed at time 0 and after stability studies.

## **2.7. Stability studies**

Stability studies are important for providing evidence on formulation quality during time, under influence of different conditions. Parameters such as size, zeta potential and AE were measured over 180 days under different conditions (25 °C/ 65% relative humidity (RH) and 40 °C/ 75% RH). Particle size determination during time at different conditions allows the possibility to observe the aggregation and sedimentation tendency. The particle



charge determination is also important to predict stability, since highly charged nanoparticles will be more stable, given that the electric repulsion prevents nanoparticle aggregation [384]. The NLC characterization (particle size and zeta potential) was done on the production day and 30, 60, 90 and 180 days later. The particle size and zeta potential were assessed by DLS and electrophoretic light scattering, respectively, as previously described.

In order to evaluate NLC stability, AE was also evaluated after 180 days of storage, in all three batches, to analyze possible compounds expulsion from the NLCs, according to the methodology previously described.

## **2.8. Cell viability and toxicity**

### **2.8.1 Cell lines and culture conditions**

Human immortalized non-tumorigenic keratinocyte cell line (HaCaT) was purchased from CLS Cell Lines Service (Eppelheim, Germany). HaCaT cells were individually maintained in Dulbecco's modified Eagle's medium (DMEM) with GlutaMAX™-I (Invitrogen, Grand Island, NY), 10% inactivated fetal calf serum (FBS; Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, Grand Island, NY) and 0.25 µg/mL amphotericin B (Invitrogen, Grand Island, NY), in a 5% CO<sub>2</sub> environment at 37 °C. The number of viable cells was periodically assessed by the trypan blue exclusion assay.

### **2.8.2 Cell viability assay**

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was used to assess cell integrity and cytotoxicity of NLC and NLC-CS formulations. Cells (HaCaT) were seeded in 96-well plates (10x10<sup>3</sup> cells/well) and incubated with medium for 24 h (37 °C/ 5% CO<sub>2</sub>). HaCaT cells were used at passages 86-88, respectively. Then, triplicate wells were incubated with formulations dissolved in cell culture medium pre-filtered by 0.45 µm Millex GV filters (Millipore, Nepean, ON, Canada) at different concentrations. Cells were exposed to formulations for 24 h. Afterwards, MTT assay was performed according to manufacturers' instructions. Briefly, cells were washed twice with phosphate buffered saline pH 7.4 (PBS) before being incubated with 120 µL of MTT in medium for 3 h (37 °C/ 5% CO<sub>2</sub>). The absorbance was measured for each well at 490 nm with background subtraction.

### **2.9. *In vitro* skin permeation study**

The skin permeation of caffeine in CS and NLC-CS was evaluated using Franz cells assembly (9 mm unjacketed Franz Diffusion Cell with 5 mL receptor volume, o-ring joint, clear glass, clamp and stir-bar; PermeGear, Inc., USA) and pig ear skin as barrier. The donor medium consisted of 300  $\mu$ L of vehicle systems. The actual concentration of caffeine in donor was 151  $\mu$ g/mL and 63  $\mu$ g/mL, for extract and NLCs, respectively. The receptor medium (5 mL) was HEPES buffer in order to maintain sink conditions. The available diffusion area between cells was 0.785 cm<sup>2</sup>. The stirring rate and temperature of receptor were respectively kept at 600 rpm and 37 °C. At appropriate intervals, 600  $\mu$ L aliquots of the receptor medium were withdrawn and immediately replaced with equal volumes of fresh buffer. The cumulative amount of caffeine was determined by UV-Vis spectrophotometry (Jasco V-660 Spectrophotometer, USA) at  $\lambda$  max 275 nm.

### **2.10. Statistical analysis**

Data were reported as mean  $\pm$  standard deviation of at least triplicate experiments. Statistical analysis of the results was performed with the software SPSS 22.0 (SPSS Inc., Chicago, IL, USA). The Student's t-test (two-tailed) was used to evaluate the statistical significance of differences in mean values in the experimental groups. The one-way analysis of variance-test was used to assess the differences in means between formulations in the *in vitro* skin permeation. Post hoc comparisons were performed according to Tukey's HSD test. In all cases,  $p < 0.05$  was accepted as denoting significance.

### 3. Results

#### 3.1 CS-loaded NLCs characterization: particle size, PDI, surface charge and morphology

For the characterization of NLCs, particle size and surface charge (zeta potential) were analyzed and the results showed that nanoparticles loaded with extract present a mean size and PDI of almost 200 nm and below 0.250, respectively, as desired to achieve delivery into the skin. The physicochemical characteristics of NLCs are depicted in Table 7.2.

Table 7.2 – Characterization of NLC and NLC-CS.

Formulation	Particle Size (nm)	PDI	Zeta Potential (mV)	AE (%)
NLC	178 ± 8	0.182 ± 0.008	-30 ± 3	-
NLC-CS	183 ± 15	0.19 ± 0.01	-23.9 ± 0.5	33 ± 3

\*p<0.05, Student's t-test showed mean particle size to be statistically equal between NLC and NLC-CS.

It is possible to observe that after associate CS extract, the mean particle was maintained. This indicates that the incorporation of this ingredient does not have a great influence on nanoparticles size. The particles present an overall size beneath of 200 nm, which should be suitable for a topical administration. In fact, according to Kohli *et al.*, a desirable value of particle size for NLCs is established as < 500 nm, in order to permeate the skin epithelium [385].

The results obtained from zeta potential measurements reveal that the NLCs were negatively charged. The zeta potential is an important parameter that allows predictions on the physical stability of colloidal dispersions. In theory, higher values of zeta potential, either positive or negative, tend to stabilize the suspension and aggregation phenomena are less likely to occur for charged particles with pronounced zeta potential (>|30|mV), due to the electrostatic repulsion between particles with the same electrical charge [386]. Also, the measured slight negative zeta potential is in agreement with literature and result from the surface coverage by polysorbate 60, giving them a steric stabilization at the

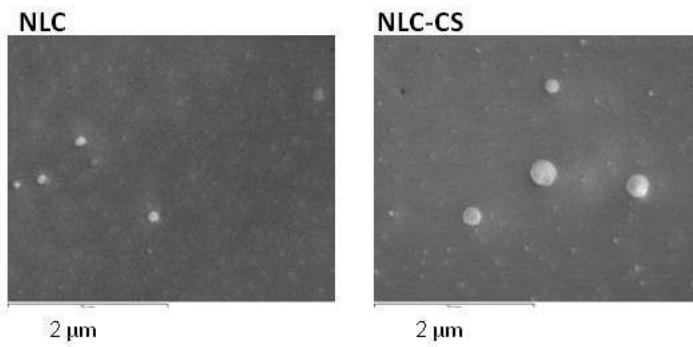
particle/water interface [387-388]. As skin is negatively charged slight interactions can be expected [389], as positively charged and neutral particles of all sizes do not penetrate [385]. The NLC without extract presented charges around -30 mV at time 0, which indicates that the nanoformulations had good physical stability. However, NLC-CS had a charge of about -24 mV, indicating some possible instability of the formulation along time.

AE is another important parameter to characterize nanoparticles. This should be high enough to avoid losing compounds during the production and also to avoid the need of using a higher concentration of compounds. Percentages obtained on the day of production show that about 30% of caffeine was incorporated. Caffeine is a hydrophilic compound - a behavior that does not favor the interaction with lipids - which explains these results.

At the time of production, both formulations presented a PDI below 0.20. Mitri *et al.* suggested that polydispersity values below 0.25 reveals homogeneous nanoparticles, with minimum predisposition to aggregation [390]. The values obtained also indicate a fairly narrow and monomodal particle size distribution. Statistical analysis indicated no differences between the polydispersity index of NLC and NLC-CS ( $p>0.05$ ).

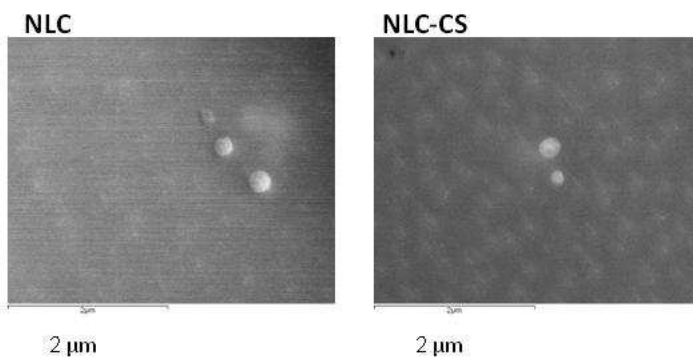
Morphology of the produced nanoparticles was analyzed by cryo-SEM after production and along time (Figure 7.1) in order to obtain more information about the particle size and morphology of NLCs. All formulations exhibited a spherical shape and a smooth surface. The sizes of the NLCs observed by cryo-SEM were in the range of 150-200 nm, with no observable difference being noted between NLC and NLC-CS.

**0 Days**



**180 Days**

**25°C / 65 % RH**



**40°C / 75 % RH**

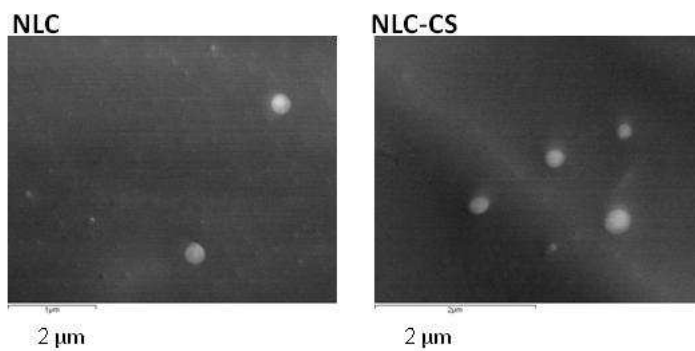


Figure 7.1 - NLC and NLC-CS images. Cryo-scanning electron microscopy (cryo-SEM) images at time 0 and after 180 days of storage at 25 °C/ 65% RH and 40 °C/ 75% RH. The scale indicated below the pictures is 2 μm. Amplification: – 30,000 x.

### 3.2 Stability studies

To evaluate the stability of the formulations in study, particle size and surface charge, were measured over 15, 30, 60, 90 and 180 days of storage at different conditions, according to the ICH Guidelines [355]. Also, the AE over 180 days of stability was measured.

#### Particle size

Table 7.3 shows the mean particle size of NLC and NLC-CS evaluated at different times over 180 days of storage.

Table 7.3 - Storage stability. Effect of time of storage at 25 °C/ 65% RH and 40 °C/ 75% RH on particle size of NLC and NLC-CS. All data represent the mean ± standard deviation (n = 4). Different letters (a,b,c,d) indicate significant differences between mean values during storage (p<0.05).

	Particle Size			
	NCL		NCL-CS	
	25 °C/ 65% RH	40 °C/ 75% RH	25 °C/ 65% RH	40 °C/ 75% RH
0 Days	178 ± 8 <sup>b,c</sup>	178 ± 8 <sup>a,b</sup>	183 ± 15 <sup>a</sup>	183 ± 15 <sup>a</sup>
15 Days	189 ± 8 <sup>c</sup>	181 ± 8 <sup>a</sup>	182 ± 14 <sup>a</sup>	182 ± 11 <sup>a</sup>
30 Days	186.6 ± 13 <sup>c</sup>	185 ± 9 <sup>a</sup>	183 ± 7 <sup>a</sup>	182 ± 7 <sup>a</sup>
60 Days	185.4 ± 12 <sup>c</sup>	179 ± 11 <sup>a,b</sup>	171 ± 12 <sup>a,b</sup>	172 ± 7 <sup>a</sup>
90 Days	160.6 ± 0.9 <sup>a,b</sup>	154.8 ± 0.9 <sup>c</sup>	148 ± 6 <sup>b</sup>	148 ± 6 <sup>b</sup>
180 Days	155 ± 7 <sup>a</sup>	157.9 ± 0.9 <sup>b,c</sup>	150 ± 17 <sup>a,b</sup>	148 ± 8 <sup>b</sup>

The results show that mean particle size decrease slowly over the observation period for both formulations (both loaded and unloaded), with sizes in the range of 155 to

178 nm for NLC, and 148 to 183 nm for NLC-CS. However, no statistical differences were observed between both stability conditions inside the same formulation, meaning that temperature did not affect particle size. The mean sizes of all formulations were, over 180 days, almost 200 nm as desired for dermal application.

### Zeta potential

Regarding the determination of the zeta potential, results are summarized in Table 7.4.

Table 7.4 - Storage stability. Effect of time of storage at 25 °C/ 65% RH and 40 °C/ 75% RH on zeta potential of NLC and NLC-CS. All data represent the mean ± standard deviation (n = 4). Different letters (a,b,c,d) indicate significant differences between mean values during storage (p<0.05).

	<b>Zeta Potential</b>			
	<b>NCL</b>		<b>NCL-CS</b>	
	<b>25 °C/ 65% RH</b>	<b>40 °C/ 75%RH</b>	<b>25 °C/ 65% RH</b>	<b>40 °C/ 75%RH</b>
0 Days	-30 ± 3 <sup>a</sup>	-30 ± 3 <sup>a</sup>	-23.9 ± 0.5 <sup>a</sup>	-23.9 ± 0.5 <sup>a</sup>
15 Days	-21 ± 2 <sup>b</sup>	-21.5 ± 0.3 <sup>b,c</sup>	-22 ± 3 <sup>a,b</sup>	-22 ± 5 <sup>a,b</sup>
30 Days	-24 ± 2 <sup>a,b</sup>	-16 ± 3 <sup>d</sup>	-20 ± 4 <sup>a,b,c</sup>	-21.25 ± 3.73 <sup>a,b</sup>
60 Days	-23 ± 2 <sup>a,b</sup>	-19 ± 1 <sup>b,c,d</sup>	-18.2 ± 0.3 <sup>b,c</sup>	-19.5 ± 0.4 <sup>b</sup>
90 Days	-22.1 ± 0.3 <sup>a,b</sup>	-24 ± 4 <sup>b</sup>	-17 ± 4 <sup>bc</sup>	-12 ± 2 <sup>c</sup>
180 Days	-17.6 ± 0.2 <sup>b</sup>	-16.9 ± 0.6 <sup>c,d</sup>	-15 ± 3 <sup>c</sup>	-17.6 ± 0.6 <sup>b</sup>

It is possible to observe that these values were always negative, but a clear decrease is observed for both formulations. Once again, the storage temperature did not influence these results. Both formulations presented zeta-potential values between -12 and -30 mV during storage period at both conditions, which predicts some long-term instability.

### Association efficiency

The AE of a delivery system such as NLCs is one of the most important features to be controlled since will give us information about the percentage of caffeine from CS that was associated to the lipid and produced the NLC-CS. AE is defined as the percentage of compound incorporated into the lipid nanoparticles relative to the total compound added. In the present study, the AE was evaluated immediately after production and after 180 days of storage at different temperatures and relative humidity.

Table 7.5 shows the results of the NLC-CS association efficiency at time 0 and over 180 days of investigation.

Table 7.5 - Association efficiency (%) into NLC-CS with time under different storage conditions. Values are mean of three assays. Within each formulation along the time, data were not statistically different, for  $p < 0.05$ .

<b>Association Efficiency (%)</b>	
0 Days	$33.20 \pm 3.11$
180 Days / 25 °C 65% RH	$34.24 \pm 0.48$
180 Days / 40 °C 75% RH	$33.90 \pm 2.58$

The amount of caffeine present in the NLCs after the storage period was identical to the values determined with fresh produced formulation. The percentages of AE were relatively low, at time 0 and after 180 days of storage, with no statistical differences, meaning that the caffeine association did not change throughout storage time. Regarding association efficiency, values are low, but relatively high, considering that this extract is hydrophilic.



With regard to CS entrapped in NLCs, a good chemical stability of all formulations was observed, i.e. the percentage of active ingredient remaining within the systems was equal when stored at 25 °C/ 65% RH and 40 °C/ 75% R H during 180 days. The results suggest that caffeine from CS is a stable molecule and did not degrade easily at studied temperatures.

### 3.3 Cell viability studies

Different concerns have been raised regarding the potential toxicity of NLCs. *In vitro* models have been shown useful in providing important guidance towards the understanding of the toxicity potential of different compounds and drug vehicles intended for cosmetic preparations. To understand the role of different concentration of NLC and NLC-CS formulations in terms of cytotoxicity, the cell viability in human keratinocytes was investigated. As it is well documented, the cytotoxicity of NLC might be influenced by both the nature of the lipid-core material used as well as by the surfactant. However, the specific contribution of these components is difficult to extrapolate from the literature due to the lack of reports dealing with the comparison of NLC formulations. Figure 7.2 summarizes the cytotoxicity results.

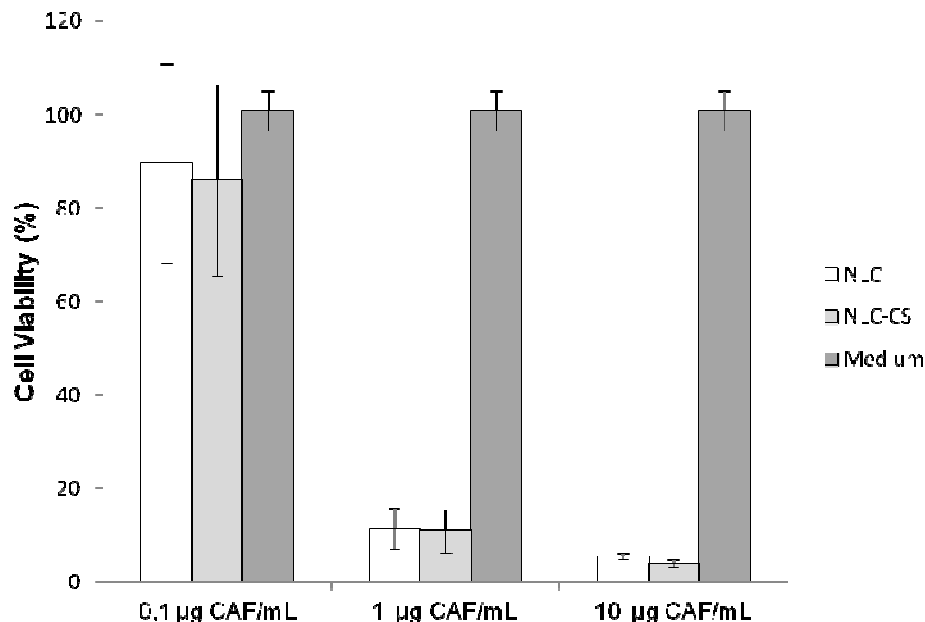


Figure 7.2 – Effect of exposure of NLC and NLC-CS with different concentrations of caffeine (CAF) on the metabolic activity of HaCaT cells. Cell viability was determined by the MTT assay. Each value represents the mean ± SD (n = 3).

Cell viability for NLC-CS was 85.9 %, 10.8 % and 3.9 %, respectively, to 0.1, 1.0 and 10.0 µg CAF/mL. For NLC results were similar. Cell viability decreases with increasing nanoparticles concentration of caffeine, which indicates a concentration depending effect. For the lower studied concentration cell viability was not affected. However, the high concentration tested indicates a significant cell viability decrease for NLC-CS and NLC. No difference was observed between formulations with or without caffeine. The results indicate that both formulations have cell viability decrease in high concentrations, probably due to nanoparticles composition and not CS. These results are consistent with cytotoxicity tests previously reported for these CS extract [22, 102].

### 3.4 *In vitro* permeation study

When synthetic or natural compounds are applied topically on the skin, an active agent must be released from the carrier (vehicle) and be able to penetrate into the *stratum corneum* and lower layers of the skin [373]. In the particular case of CS extracts, caffeine and other phytochemicals present are able to carry out pharmacological activities, and even the expected activity may be the result of synergic action of several compounds acting together. For cosmetic products, it is important that the active compounds stay in the skin, penetrate sufficiently deep but not too deep leading to systemic availability [374]. Indeed, penetration should be sufficient to lead to a cosmetic but not a pharmaceutical effect.

According to Muller *et al.*, considering their submicron-size and pearl-like nature, NLCs could be a promising help for penetration of some compounds in certain layers of the skin [374, 391]. Caffeine, as it is well known, is a hydrophilic compound. However, as we are using a natural extract with a caffeine content of about one-third of coffee beans, the incorporation into NLCs could guarantee that huge amounts are delivered into fat cells. The caffeine permeation into pig ear skin treated with CS extract and NLC-CS was analyzed. The extract not encapsulated was also evaluated in order to compare the skin permeation. Franz diffusion cells were used and pig ear skin was selected as model barrier, considering its similarity to human skin. Results are presented in Figure 7.3.

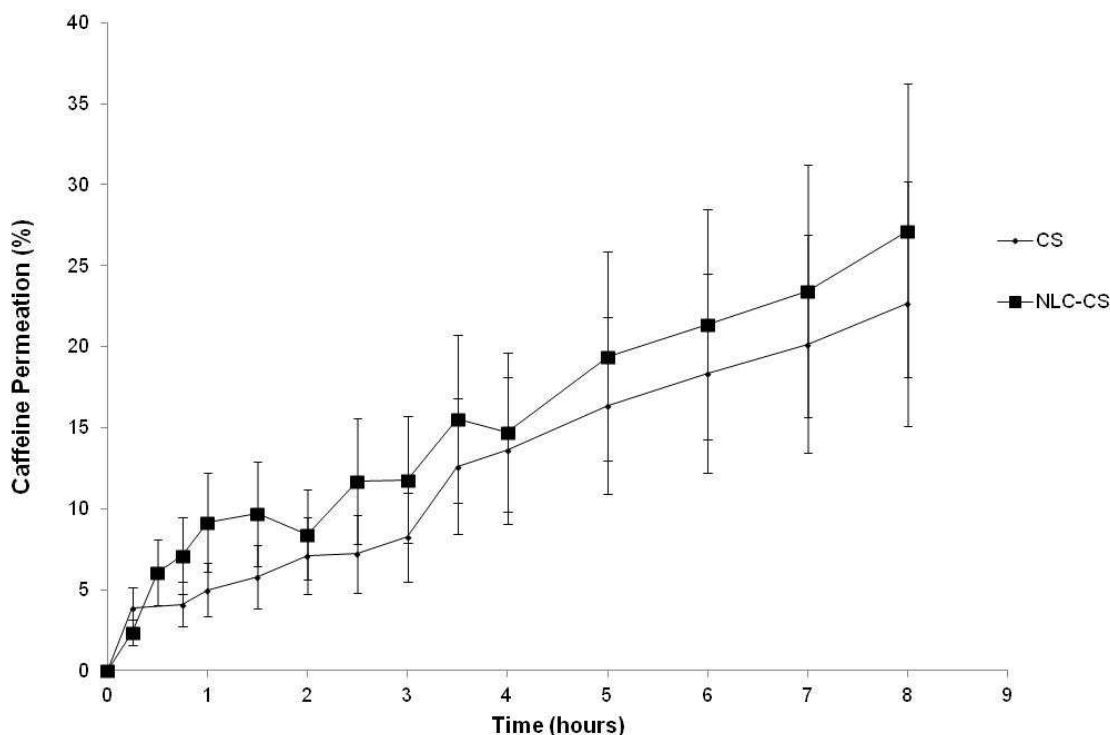


Figure 7.3 - Permeation of caffeine of NLC-CS and CS extract through pig ear skin over 8 hours. Each value represents the mean  $\pm$  SD (n = 3).

Under the experimental conditions, the release profile of caffeine from NLC was almost found in the first eight hours of study. Probably this is due to the high hydrophilicity of caffeine. The data show an improved permeation of caffeine from NLC-CS when compared to CS extract. After 8 hours, a small quantity of caffeine crossed the skin, respectively, about 20% for extract and 25% for NLC-CS. A possible way to promote permeation is the application of permeation enhancers. It has been demonstrated that ethanol can enhance skin permeation of drugs when simultaneously applied with compounds [392-393]. Ethanol seems to be an effective carrier of caffeine topically-applied, penetrating the tissue model. However, the permeation enhancing effect of ethanol on the permeation of caffeine appears to be rapidly attenuated, by evaporation or dissipation into the skin, or perhaps as ethanol changes the hydration state. Nevertheless, it should always be considered that caffeine is a hydrophilic compound.

The caffeine permeation was assessed by UV/VIS. A possible justification for some variability in the results could be due to the fact that the skin used for experiments was from different ears and pigs, which introduces variance. However, to decrease the

analytical error, *in vivo* studies should be performed in future experiments to circumvent practical problems of *in vitro* permeation experiments such as complete dehydration of the porcine ear skin samples with longer residence times.

Also, it should be considered that the delivery of active substances to the skin is not easy, since this organ is not optimized to favor the absorbance of substances from the external environment, but rather, to provide a protective barrier [375]. Epidermis is an effective self-renewing barrier on the surface, largely composed of hyperkeratinized cells, while dermis is a deeper layer of metabolically active cells and extracellular matrix. The delivery of active components that should address specific physio-pathological conditions, such as compounds endowed with anti-cellulite properties, should be able to efficiently pass these compartments. Nanolipid carriers could represent an effective strategy to maximize this delivery. Until the present date, and to the best of our knowledge, there are no published research works concerning the use of CS extracts encapsulated in NLCs.

#### **4. Conclusion**

Through this work, it was possible to successfully produce NLCs loaded with CS extracts, rich in caffeine, which possess characteristics that would render them as a promising alternative for the topical treatment of cellulite. Nanostructured lipid carriers were prepared by a double emulsion. Furthermore, previous *in vivo* studies indicate safety of the extracts for topical application as well as for final cosmetic formulations. The produced NLCs had acceptable dimensions (<200 nm), accompanied by high absolute zeta potential levels, low values of PDI and relatively high values of association efficiency for hydrophilic compounds, with a spherical shape and smooth surface in terms of morphology at time of production. The formulations stability was also confirmed after storage during 180 days at different temperatures and relative humidities.

The *in vitro* evaluation of compound release from the produced NLCs in a simulated physiological environment revealed a biphasic compound release profile, characterized by a quick initial phase release followed by a phase with prolonged release until 8 h. Evaluation of the *in vitro* skin permeation of caffeine in CS extracts showed its capability to go through the skin barrier when loaded within nanoparticles.

Overall, the results of the present work confirm some potential of NLCs as carriers for caffeine from CS extracts and their feasibility for topical delivery.

## **CONCLUSIONS & FUTURE PROSPECTS**

"Apesar das ruínas e da morte,  
Onde sempre acabou cada ilusão,  
A força dos meus sonhos é tão forte,  
Que de tudo renasce a exaltação  
E nunca as minhas mãos ficam vazias. "

*Sophia de Mello Breyner Andresen*

## **General Conclusions & Future Prospects**

The importance of new active ingredients for cosmetics obtained from sustainable sources, such as food by-products, gained a new breath in the last years. This is a result of the important advances in the field of food by-products and the common sense of sustainability from industries and consumers, which, in turn, allowed the development of new and improved cosmetic ingredients. Also, new insights on the understanding of extracts composition and mechanics boosted the development of innovative ingredients obtained with eco-friendly solvents and adapted for the challenges imposed by the new formulations. Still, the development of efficient and safe formulations to topical delivery of ingredients obtained from food by-products imposes numerous challenges.

In this thesis it was pursued to achieve formulations for topical administration of new cosmetic ingredients, based on food by-products. Two food by-products, CS and *M. sativa*, were initially selected to be characterized, owing to huge amounts disposable in the region behind the enterprise Fourmag.

In a first step both wastes were evaluated with different sustainable solvents and temperatures in order to obtain extracts with a cosmetic profile. From the different conditions tested, the combination of water and ethanol mixed in equal proportions, and temperature of 40 °C, were selected to produce extracts with an interesting composition. Both were characterized regarding antioxidant, flavonoid and polyphenol content, antimicrobial activity and cytotoxicity against different skin cell lines (keratinocytes and fibroblasts). *Medicago* extracts from different species were also evaluated and compared regarding isoflavones composition. Results showed that these food wastes are rich in antioxidant and polyphenols, presenting antimicrobial activity and low cytotoxicity against skin cell lines. Also, *M. sativa* extracts were founded as good source of isoflavones. Interestingly, the content of caffeine in CS extracts was in the same concentration range of the caffeine of coffee beans.

According to the European Community guidelines, that had banned the animal tests, skin and ocular irritation potential of CS extracts were evaluated in 3D models, using reconstructed human epidermis, EpiSkin™, and human corneal epithelial model, SkinEthic™ HCE. EpiSkin™ was validated by ECVAM and SkinEthic™ HCE is under validation. The histology of the models after extracts applications was analysed. The *in vitro* results demonstrated that extracts were not classified as irritants and the histological analysis proved that they did not affect both models structure. The content of caffeine, HMF and CGA was quantified after the epidermal assay. The *in vivo* patch test carried out



with the most promising extract (hydroalcoholic) showed that, with respect to irritant effects, these extracts can be regarded as safe for topical application. The hydration effect of CS extract on skin was also improved after 48 hours of a patch test.

Different cosmetic formulations (with and without extracts) were developed and characterized considering different parameters such as viscosity, pH and color. The formulations were a facial cream, a body cream, a body scrub, a hand cream and a shower gel. *In vitro* toxicity was screened in two skin cell lines (fibroblasts and keratinocytes) for all formulations and any toxicity was reported.

Regarding stability, all formulations were stored at 25 °C and 40 °C for 6 months and the pH, color, viscosity and antioxidant activity were analyzed over time. No significant differences on the formulation characteristics were observed up to six months. Additionally, the antioxidant activity was maintained.

Besides characterization and toxicological relevance of extracts and formulations were guaranteed by *in vitro* model systems, the study of the effective biologic potential required a minimum amount of *in vivo* experiments. Thus, *in vivo* effects of formulations were assessed in volunteers. A facial formulation with CS (Formulation A) and the same cream enriched with hyaluronic acid (Formulation B) were compared. Formulations were applied twice a day by volunteers (n=20 for each formulation) during 28 days. The influence on skin hydration and viscoelastic properties were investigated with validated devices (Corneometer® and Cutometer®). Wrinkles depth, roughness, volume of cavities and Visioface® images were analysed at time 0 and after 28 days. Volunteers were asked about efficacy perception. Both formulations significantly improved the skin hydration and viscoelastic parameters. However, no differences were observed regarding wrinkles depth, roughness and volume of cavities for both formulations. CS represents an effective ingredient for cosmetic creams which are intended to increase skin hydration and firmness.

Regarding body cream, it can be classified as safe for topical application and the skin hydration improved after 30 days of application. Also, considering the consumer acceptance, more than 90% of volunteers classified it as very pleasant.

The development of these new formulations with active ingredients obtained from food by-products provided a further insight on cosmetic industry, benefiting from the huge amounts of these products without added value and providing new potential applications

for these wastes. However, in research, there is always another experiment to be done. At this point, the performed work may be a good contribution to the food by-products and cosmetic fields.

Finally, as a new perspective, NLCs were developed and characterized with CS in order to observe if the amount of caffeine that crosses the skin was higher or not. Additionally, *in vitro* release studies using Franz cells with pig ear skin were performed. The produced NLCs were within the nanosized range ( $\approx 200$  nm) with relatively low polydispersity index ( $< 0.25$ ) and zeta potential values around  $-30$  mV. Also, NLCs demonstrated storage stability at  $25$  °C/  $65\%$  RH and  $40$  °C/  $75\%$  RH up to 180 days. The AE was about 30% at time of production and after storage period. Cryo-SEM images showed the spherical shape of the empty and caffeine-loaded NLCs. The *in vitro* skin permeation study demonstrated that caffeine loaded into NLCs was similarly permeable through skin compared to non encapsulated caffeine.

Future works may be extended to encapsulation of extracts and *in vivo* efficacy studies, in order to compare them with the present one. Also, the evaluation of other food by-products with interesting composition for the cosmetic industry, such as olive by-products, should be consider and investigated. Nevertheless, an interesting field could be the isolation of specific ingredients from food by-products using green techniques, such as, supercritical fluids, to be re-used.

## References

1. Sahota A, Introduction to Sustainability, In: Sahota A, editor. Sustainability: How the Cosmetics Industry is Greening Up. London: Wiley; 2014. p. 1-16.
2. Behrens A, Giljum S, Kovanda J, Niza S. The material basis of the global economy: Worldwide patterns of natural resource extraction and their implications for sustainable resource use policies. *Ecol Econom* 2007; 64: 444-453.
3. McPhee D, Jain R. Price, performance, supply and sustainability all-in-one - The Amyris case. *H&PC Today* 2015; 10(5): 71-72.
4. Cosmetics Europe - The Personal Care Association: Sustainable Development. Available from: <https://www.cosmeticseurope.eu/responsible-industry-the-european-cosmetic-cosmetics-association/sustainable-development.html> [accessed on 05.12.2015].
5. Drexhage J, Murphy D, editors. Sustainable Development: From Brundtland to Rio 2012. High Level Panel on Global Sustainability, New York. United Nations Headquarters; 2010.
6. Esquivel P, Jiménez VM. Functional properties of coffee and coffee by-products. *Food Res Int* 2012; 46: 488-495.
7. International Coffee Organisation Annual Review 2013/14. Available from: <http://www.ico.org/documents/cy2014-15/annual-review-2013-14-electronic-e.pdf> [accessed on 02.02.2015].
8. International Coffee Organisation. Available from: <http://www.ico.org> [accessed on 02.02.2015].
9. Murthy PS, Madhava Naidu M. Sustainable management of coffee industry by-products and value addition - A review. *Resour Conserv Recy* 2012; 66: 45-58.
10. Saenger M, Hartge EU, Werther J, Ogada T, Siagi Z. Combustion of coffee husks. *Renew Energ* 2001; 23: 103-121.
11. Gouvea B, Torres C, Franca A, Oliveira L, Oliveira E. Feasibility of ethanol production from coffee husks. *Biotechnol Lett* 2009; 31: 1315-1319.
12. Brand D, Pandey A, Rodriguez-Leon JA, Roussos S, Brand I, Soccol CR. Packed bed column fermenter and kinetic modeling for upgrading the nutritional quality of coffee husk in solid-state fermentation. *Biotechnol Prog* 2001; 17: 1065-70.
13. Ulloa Rojas JB, Verreth JAJ, van Weerd JH, Huisman EA. Effect of different chemical treatments on nutritional and antinutritional properties of coffee pulp. *Anim Feed Sci Technol* 2002; 99: 195-204.
14. Belitz HD, Grosch W, Schieberle P. Coffee, Tea, Cocoa, In: Belitz HD, Grosch W, Schieberle P, editors. *Food Chemistry*. Berlin: Springer; 2009. p. 938-970.
15. Borrelli RC, Esposito F, Napolitano A, Ritieni A, Fogliano V. , Characterization of a new potential functional ingredient: Coffee silverskin. *J Agr Food Chem* 2004; 52: 1338-1343.
16. Pourfarzad A, Mahdavian-Mehr H, Sedaghat N. Coffee silverskin as a source of dietary fiber in bread-making: Optimization of chemical treatment using response surface methodology. *LWT - Food Sci Technol* 2013; 50: 599-606.
17. Napolitano A, Fogliano V, Tafuri A, Ritieni A. Natural occurrence of ochratoxin A and antioxidant activities of green and roasted coffees and corresponding byproducts. *J Agr Food Chem* 2007; 55: 10499-10504.
18. Narita Y, Inouye K. Review on utilization and composition of coffee silverskin, *Food Res Int* 2014; 61: 16-22.
19. Bresciani L, Calani L, Bruni R, Brighenti F, Del Rio D. Phenolic composition, caffeine content and antioxidant capacity of coffee silverskin. *Food Res Int* 2014; 61: 196-201.
20. Costa ASG, Alves RC, Vinha AF, Barreira SVP, Nunes MA, Cunha LM, et al. Optimization of antioxidants extraction from coffee silverskin, a roasting by-product, having in view a sustainable process. *Ind Crop Prod* 2014; 53: 350-357.

21. Narita Y, Inouye K. High antioxidant activity of coffee silverskin extracts obtained by the treatment of coffee silverskin with subcritical water. *Food Chem* 2012; 135: 943-949.
22. Rodrigues F, Palmeira-de-Oliveira A, das Neves J, Sarmiento B, Amaral MH, Oliveira MBPP. Coffee silverskin: A possible valuable cosmetic ingredient. *Pharm Biol* 2015; 53: 386-394.
23. Rodrigues F, Almeida I, Sarmiento B, Amaral MH, Oliveira MBPP. Study of the isoflavone content of different extracts of *Medicago* spp. as potential active ingredient. *Ind Crop Prod* 2014; 57: 110-115.
24. Barreira JCM, Visnevschi-Necrasov T, Nunes E, Cunha SC, Pereira G, Oliveira MBPP. *Medicago* spp. as potential sources of bioactive isoflavones: Characterization according to phylogenetic and phenologic factors. *Phytochem* 2015; 116: 230-238.
25. Farag MA, Huhman DV, Lei Z, Sumner LW, Metabolic profiling and systematic identification of flavonoids and isoflavonoids in roots and cell suspension cultures of *Medicago truncatula* using HPLC–UV–ESI–MS and GC–MS. *Phytochem* 2007; 68: 342-354.
26. Nunes C, de Sousa Araújo S, da Silva JM, Feveireiro MPS, da Silva AB. Physiological responses of the legume model *Medicago truncatula* cv. Jemalong to water deficit. *Environ Exp Bot* 2008; 63: 289-296.
27. Silva LR, Pereira MJ, Azevedo J, Gonçalves RF, Valentão P, de Pinho PG, et al. *Glycine max* (L.) Merr., *Vigna radiata* L. and *Medicago sativa* L. sprouts: A natural source of bioactive compounds. *Food Res Int* 2013; 50: 167-175.
28. Facciola S. *Cornucopia - a source book of edible plants*. 1<sup>st</sup> ed. Vista CA: Kampong Publications; 1990.
29. Koschuh W, Povoden G, Thang VH, Kromus S, Kulbe KD, Novalin S, et al. Production of leaf protein concentrate from ryegrass (*Lolium perenne* x *multiflorum*) and alfalfa (*Medicago sativa* subsp. *sativa*). Comparison between heat coagulation/centrifugation and ultrafiltration. *Desalination* 2004; 163: 253-259.
30. Takyi EEK, Kido Y, Rikimaru T, Kennedy DO. Possible use of alfalfa (*Medicago sativa*) as supplement in infant nutrition: Comparison of weight gained by rats fed on alfalfa and a popular weaning diet. *J Sci Food Agric* 1992; 59: 109-115.
31. Mortensen A, Kulling SE, Schwartz H, Rowland I, Ruefer CE, Rimbach G, et al. Analytical and compositional aspects of isoflavones in food and their biological effects. *Mol Nutr Food Res* 2009; 53: S266-S309.
32. Rostagno MA, Villares A, Guillamón E, García-Lafuente A, Martínez JA. Sample preparation for the analysis of isoflavones from soybeans and soy foods. *J Chromatogr A* 2009; 1216: 2-29.
33. Sabudak T, Guler N. *Trifolium* L. - A review on its phytochemical and pharmacological profile. *Phytother Res* 2009; 23: 439-446.
34. Sivesind E, Seguin P. Effects of the environment, cultivar, maturity, and preservation method on red clover isoflavone concentration. *J Agr Food Chem* 2005; 53: 6397-6402.
35. Visnevschi-Necrasov T, Barreira JCM, Cunha SC, Pereira G, Nunes E, Oliveira MBPP. Phylogenetic insights on the isoflavone profile variations in Fabaceae spp.: Assessment through PCA and LDA. *Food Res Int* 2015; 76: 51-57.
36. Von Baer D, Saelzer R, Vega M, Ibieta P, Molina L, Von Baer E, et al. Isoflavones in *Lupinus albus* and *Lupinus angustifolius*: Quantitative determination by capillary zone electrophoresis, evolution of their concentration during plant development and effect on anthracnose causing fungus *Colletotrichum lupini*. *J Chil Chem Soc* 2006; 51: 1025-1029.

37. Brzezinski A, Debi A. Phytoestrogens: the “natural” selective estrogen receptor modulators?. *Eur J Obstet Gynecol Reprod Biol* 1999; 85: 47-51.
38. Bacaloni A, Cavaliere C, Faberi A, Foglia P, Samperi R, Laganà A. Determination of isoflavones and coumestrol in river water and domestic wastewater sewage treatment plants. *Anal Chim Acta* 2005; 531: 229-237.
39. Tham DM, Gardner CD, Haskell WL. Potential health benefits of dietary phytoestrogens: A review of the clinical, epidemiological and mechanistic evidence. *J Clin Endocrinol Metab* 1998; 83: 2223-2235.
40. Fletcher RJ. Food sources of phyto-oestrogens and their precursors in Europe. *Br J Nutr* 2003; 89: S39-S43.
41. Adlercreutz H. Phytoestrogens: epidemiology and a possible role in cancer protection. *Environ Health Perspect* 1995; 103: 103-112.
42. Clarkson TB, Anthony MS, Hughes Jr CL. Estrogenic soybean isoflavones and chronic disease: Risks and benefits. *Trends Endocrinol Metab* 1995; 6: 11-16.
43. Adlercreutz H, Hämäläinen E, Gorbach S, Goldin B. Dietary phyto-oestrogens and the menopause in Japan. *The Lancet* 1992; 339: 1233.
44. Moraes AB, Haidar MA, Soares Júnior JM, Simões MJ, Baracat EC, Patriarca MT. The effects of topical isoflavones on postmenopausal skin: Double-blind and randomized clinical trial of efficacy. *Eur J Obstet Gynecol Reprod Biol* 2009; 146: 188-192.
45. Wu Q, Wang M, Simon JE. Determination of isoflavones in red clover and related species by high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection. *J Chromatogr A* 2003; 1016: 195-209.
46. Delmonte P, Perry J, Rader JI. Determination of isoflavones in dietary supplements containing soy, red clover and kudzu: Extraction followed by basic or acid hydrolysis. *J Chromatogr A* 2006; 1107: 59-69.
47. Klejdus B, Vitamvásová-Sterbová D, Kubán V. Identification of isoflavone conjugates in red clover (*Trifolium pratense*) by liquid chromatography-mass spectrometry after two-dimensional solid-phase extraction. *Anal Chim Acta* 2001; 450: 81-97.
48. Mazur WM, Duke JA, Wahala K, Rasku S, Adlercreutz H. Isoflavonoids and lignans in legumes: Nutritional and health aspects in humans. *J Nutr Biochem* 1998; 9: 193-200.
49. Krenn L, Unterrieder I, Ruprechter R. Quantification of isoflavones in red clover by high-performance liquid chromatography. *J Chromatogr B* 2002; 777: 123-128.
50. Martinez-Saez N, Ullate M, Martín-Cabrejas MA, Martorell P, Genovés S, Ramón D, et al. A novel antioxidant beverage for body weight control based on coffee silverskin. *Food Chem* 2014; 150: 227-234.
51. Mussatto SI, Machado EMS, Carneiro LM, Teixeira JA. Sugars metabolism and ethanol production by different yeast strains from coffee industry wastes hydrolysates. *Appl Energy* 2012; 92: 763-768.
52. Prakash L, Majeed M. Natural ingredients for anti-ageing skin care. *H&PC Today* 2009; 2: 44-46.
53. Prow TW, Grice JE, Lin LL, Faye R, Butler M, Becker W, et al. Nanoparticles and microparticles for skin drug delivery. *Adv Drug Deliv Rev* 2011; 63: 470-491.
54. Schäfer-Korting M, Mehnert W, Korting H-C. Lipid nanoparticles for improved topical application of drugs for skin diseases. *Adv Drug Deliv Rev* 2007; 59: 427-443.
55. Franzen L, Windbergs M. Applications of Raman spectroscopy in skin research - From skin physiology and diagnosis up to risk assessment and dermal drug delivery. *Adv Drug Deliv Rev* 2015; 89: 91-104.

56. Mathes SH, Ruffner H, Graf-Hausner U. The use of skin models in drug development. *Adv Drug Deliv Rev* 2014; 69-70: 81-102.
57. Schurer NY, Elias PM. The biochemistry and function of *stratum corneum* lipids. *Adv Lipid Res* 1991; 24: 27-56.
58. Pasparakis M, Haase I, Nestle FO. Mechanisms regulating skin immunity and inflammation. *Nat Rev Immunol* 2014; 14: 289-301.
59. Schmid-Wendtner MH, Korting HC. The pH of the skin surface and its impact on the barrier function. *Skin Pharmacol Physiol* 2006; 19: 296-302.
60. Honari G, Maibach H. Skin Structure and Function. In: Honari HM, editor. *Applied Dermatotoxicology*. Boston: Academic Press; 2014. p. 1-10.
61. Longo C, Casari A, Beretti F, Cesinaro AM, Pellacani G. Skin aging: *In vivo* microscopic assessment of epidermal and dermal changes by means of confocal microscopy. *J Am Acad Dermatol* 2013; 68:73-82.
62. Farage MA, Miller KW, Elsner P, Maibach HI. Structural characteristics of the aging skin: A Review. *Cutan Ocul Toxicol* 2007; 26: 343-357.
63. Dzwigałowska A, Sołyga-Żurek A, Dębowska RM, Eris I. Preliminary study in the evaluation of anti-aging cosmetic treatment using two complementary methods for assessing skin surface. *Skin Res Technol* 2013; 19: 155-161.
64. Farage MA, Miller KW, Elsner P, Maibach HI. Intrinsic and extrinsic factors in skin ageing: a review. *Int J Cosmetic Sci* 2008; 30: 87-95.
65. Baumann L. Skin ageing and its treatment. *J Pathol* 2007; 211: 241-251.
66. Kligman AM. Perspectives and problems in cutaneous gerontology. *J Invest Dermatol* 1979; 73: 39-46.
67. Fisher GJ, Kang S, Varani J, Bata-Csorgo Z, Wan Y, Datta Z, et al. Mechanisms of photoaging and chronological skin aging. *Arch Dermatol* 2002; 138: 1462-1470.
68. Thiele J, Barland CO, Ghadially R, Elias PM. Permeability and antioxidant barriers in aged epidermis. In: Gilchrist BA, Krutmann J, editors. *Skin Aging*. New York: Springer; 2006. p. 65-79.
69. Krutmann J, Gilchrist BA. Photoaging of skin, In: Gilchrist BA, Krutmann J, editors. *Skin Aging*. New York: Springer; 2006. p. 33-44.
70. Gilchrist BA. A review of skin ageing and its medical therapy. *Br J Dermatol* 1996; 135: 867-75.
71. Rona C, Vailati F, Berardesca E. The cosmetic treatment of wrinkles. *J Cosmet Dermatol* 2004; 3: 26-34.
72. Thompson Z, Maibach HI. Biological effects of estrogen on skin. In: Farage MA, Maibach HI, editors. *Textbook of Aging Skin*. Berlin: Springer; 2010. p. 361-367.
73. Waller JM, Maibach HI. A quantitative approach to age and skin structure and function: Protein, glycosaminoglycan, water, and lipid content and structure. In: Barel AO, Paye M, Maibach HI, editors. *Handbook of Cosmetic Science and Technology*. New York: Informa Healthcare USA, Inc.; 2009. p. 243-260.
74. Watson REB, Craven NM, Kang S, Jones CJP, Kielty CM, Griffiths CEM. A short-term screening protocol, using fibrillin-1 as a reporter molecule, for photoaging repair agents. *J Invest Dermatol* 2001; 116: 672-678.
75. Contet-Audonneau JL, Jeanmaire C, Pauly G. A histological study of human wrinkle structures: comparison between sun-exposed areas of the face, with or without wrinkles, and sun-protected areas. *Br J Dermatol* 1999; 140: 1038-1047.
76. Ganceviciene R, Liakou AI, Theodoridis A, Makrantonaki E, Zouboulis CC. Skin anti-aging strategies. *Dermatoendocrinol* 2012; 4: 308-319.
77. Oikarinen A. The aging of skin: Chronoaging versus photoaging. *Photodermatol Photoimmunol Photomed* 1990; 7: 3-4.
78. Shuster S, Black MM, McVitie E. The influence of age and sex on skin thickness, skin collagen and density. *Br J Dermatol* 1975; 93: 639-643.

79. Fisher GJ, Varani J, Voorhees JJ. Looking older: Fibroblast collapse and therapeutic implications. *Arch Dermatol* 2008; 144: 666-72.
80. Kang S, Fisher GJ, Voorhees JJ. Photoaging and topical tretinoin: Therapy, pathogenesis and prevention. *Arch Dermatol* 1997; 133: 1280-4.
81. Chung JH, Kang S, Varani J, Lin J, Fisher GJ, Voorhees JJ. Decreased extracellular-signal-regulated kinase and increased stress-activated MAP kinase activities in aged human skin *in vivo*. *J Invest Dermatol* 2000; 115: 177-82.
82. Varani J, Warner RL, Gharaee-Kermani M, Phan SH, Kang S, Chung JH, et al. Vitamin A antagonizes decreased cell growth and elevated collagen-degrading matrix metalloproteinases and stimulates collagen accumulation in naturally aged human skin. *J Invest Dermatol* 2000; 114: 480-6.
83. Sárdy M. Role of matrix metalloproteinases in skin ageing. *Connect Tissue Res* 2009; 50: 132-138.
84. Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM. Matrix metalloproteinases: Biologic activity and clinical implications. *J Clin Oncol* 2000; 18(5): 1135.
85. Cinque B, Palumbo P, La Torre C, Melchiorre E, Corridoni D, Miconi G, et al. Probiotics in Aging Skin. In: Farage MA, Miller KW, Maibach HI, editors. *Textbook of Aging Skin*. Berlin: Springer; 2010. p. 811-820.
86. Pinnell SR. Cutaneous photodamage, oxidative stress, and topical antioxidant protection. *J Am Acad Dermatol* 2003; 48: 1-22.
87. Pimple BP, Badole SL. Polyphenols: A remedy for skin wrinkles. In: Watson RR, Preedy VR, Zibadi S, editors. *Polyphenols in human health and disease*. San Diego: Academic Press; 2014. p. 861-869.
88. Boss GR, Seegmiller JE. Age-related physiological changes and their clinical significance. *Western J Med* 1981; 135: 434-440.
89. Sudel KM, Venzke K, Mielke H, Breitenbach U, Mundt C, Jaspers S, et al. Novel aspects of intrinsic and extrinsic aging of human skin: beneficial effects of soy extract. *Photochem Photobiol* 2005; 81: 581-587.
90. Farage MA, Miller KW, Maibach HI. Degenerative changes in aging skin. In: Farage MA, Miller KW, Maibach HI, editors. *Textbook of Aging Skin*. Berlin: Springer; 2010. p. 25-35.
91. Rawlings AV, Matts PJ. *Stratum corneum* moisturization at the molecular Level : An update in relation to the dry skin cycle. *J Investig Dermatol* 2005; 124: 1099-1110.
92. Thiele JJ, Dreher F, Packer L. Antioxidant defense systems in skin. *J Toxicol Cutan Ocul Toxicol* 2002; 21: 119-160.
93. Angerhofer CK, Maes D, Giacomoni PU. The use of natural compounds and botanicals in the development of anti-aging skin care products. In: Dayan N, Kromidas L, editors. *Skin Aging Handbook*. Norwich, NY: William Andrew Publishing; 2009. p. 205-263.
94. Burke KE. Prevention and treatment of aging skin with topical antioxidants. In: Dayan N, editor. *Skin Aging Handbook*. Norwich, NY: William Andrew Publishing; 2009. p. 149-176.
95. Pouillot A, Polla LL, Tacchini P, Neequaye A, Polla A, Polla B. Natural antioxidants and their effects on the skin. In: Dayan N, Kromidas L, editors. *Formulating, packaging and marketing of natural cosmetic products*. New Jersey: John Wiley & Sons, Inc.; 2011. p. 239-257.
96. Viola P, Viola M. Virgin olive oil as a fundamental nutritional component and skin protector. *Clin Dermatol* 2009; 27: 159-165.
97. Rodrigues F, Pimentel FB, Oliveira MBPP. Olive by-products: Challenge application in cosmetic industry. *Ind Crop Prod* 2015; 70: 116-124.



98. Chen L, Hu JY, Wang SQ. The role of antioxidants in photoprotection: A critical review. *J Am Acad Dermatol* 2012; 67: 1013-1024
99. Braga N, Rodrigues F, Oliveira MBPP. *Castanea sativa* by-products: a review on added value and sustainable application. *Nat Prod Res* 2014; 29: 1-18.
100. Rodrigues F, Gaspar C, Palmeira-de-Oliveira A, Sarmiento B, Amaral MH, Oliveira MBPP. Application of Coffee silverskin in cosmetic formulations: Physical/antioxidant stability studies and cytotoxicity effects. *Drug Dev Ind Pharm* 2016; 42: 99-106.
101. Rodrigues F, Palmeira-de-Oliveira A, das Neves J, Sarmiento B, Amaral MH, Oliveira MBPP. *Medicago* spp. extracts as promising ingredients for skin care products. *Ind Crop Prod* 2013; 49: 634-644.
102. Rodrigues F, Pereira C, Pimentel FB, Alves RC, Ferreira M, Sarmiento B, et al. Are coffee silverskin extracts safe for topical use? An *in vitro* and *in vivo* approach. *Ind Crop Prod* 2015; 63: 167-174.
103. Rodrigues F, Sarmiento B, Amaral MH, Oliveira MBPP. Exploring the antioxidant potentiality of two food by-products into a topical cream: Stability, *in vitro* and *in vivo* evaluation. *Drug Dev Ind Pharm*, *in press*.
104. Stevenson S, Thornton J. Effect of estrogens on skin aging and the potential role of SERMs. *Clin Interv Aging* 2007; 2: 283-297.
105. Schmidt JB, Binder M, Demschik G, Bieglmayer C, Reiner A. Treatment of skin aging with topical estrogens. *Int J Dermatol* 1996; 35: 669-674.
106. Sator PG, Schmidt JB, Sator MO, Huber JC, Honigsmann H. The influence of hormone replacement therapy on skin ageing: A pilot study. *Maturitas* 2001; 39: 43-55.
107. Phillips TJ, Demircay Z, Sahu M. Hormonal effects on skin aging. *Clin Geriatr Med* 2001; 17: 661-72.
108. SCCS. The SCCS's notes of guidance for the testing of cosmetic substances and their safety evaluation. Scientific Committee on Consumer Safety, editor. Brussels: European Commission; 2012.
109. EC. Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on Cosmetic Products. Official Journal of the European Union, editor. Brussels: European Parliament and Council of European Union; 2009.
110. Cosmetic Info. Available from: <http://www.cosmeticsinfo.org/safety-cosmetics> [accessed on 02.01.2016].
111. Guidelines for the safety assessment of a cosmetic product. COLIPA - The European Cosmetic, Toiletry and Perfumery Association, editor. Brussels: COLIPA; 1997.
112. Directive 2003/15/EC of the European Parliament and of the Council, Official Journal of the European Union 2003; 46: 26-35.
113. Schaefer UF, Hansen S, Schneider M, Contreras JL, Lehr CM. Models for skin absorption and skin toxicity testing. In: Ehrhardt C, Kim K-J, editors. *Drug Absorption Studies: In Situ, In Vitro and In Silico Models*. Boston: Springer US; 2008. p. 3-33.
114. EC. Guidance Document on Dermal Absorption, Health & Consumer Protection Directorate-General, editor. Brussels: European Commission (EC); 2004.
115. EC. Basic criteria for the *in vitro* assessment of dermal absorption of cosmetic ingredients, Scientific Committee on Consumer Safety, editor. Brussels: European Commission (EC); 2006.
116. FDA, Guidance for industry: SUPAC-SS In vitro release testing and in vivo bioequivalence documentation, U.S. Department of Health and Human Services, editor. Rockville, MD: Food and Drug Administration (FDA); 1997.

117. Cotovio J, Grandidier M-H, Lelièvre D, Bremond C, Amsellem C, Maloug S, et al. *In vitro* assessment of eye irritancy using the Reconstructed Human Corneal Epithelial SkinEthic HCE model: Application to 435 substances from consumer products industry. *Toxicol in Vitro* 2010; 24: 523-537.
118. OECD, Guidance document for the conduct of skin absorption studies. OECD series in testing and assessment, editor. Paris: OECD Publishing; 2004.
119. OECD. Test No. 431: In vitro skin corrosion: Human skin model test., OECD Guidelines for the Testing of Chemicals, editor. Paris: OECD Publishing; 2004.
120. Spielmann H, Hoffmann S, Liebsch M, Botham P, Fentem JH, Eskes C, et al. The ECVAM international validation study on *in vitro* tests for acute skin irritation: report on the validity of the EPISKIN and EpiDerm assays and on the Skin Integrity Function Test. *Altern Lab Anim* 2007; 35: 559-601.
121. Supp DM, Boyce ST. Engineered skin substitutes: practices and potentials. *Clin Dermatol* 2005; 23: 403-412.
122. Brohem CA, Cardeal LB, Tiago M, Soengas MS, Barros SB, Maria-Engler SS. Artificial skin in perspective: Concepts and applications. *Pigment Cell Melanoma Res* 2011; 24: 35-50.
123. Godin B, Touitou E. Transdermal skin delivery: Predictions for humans from *in vivo*, *ex vivo* and animal models. *Adv Drug Deliv Rev* 2007; 59: 1152-1161.
124. Boyce ST, Warden GD. Principles and practices for treatment of cutaneous wounds with cultured skin substitutes. *Am J Surg* 2002; 183: 445-456.
125. Feliciani C, Gupta AK, Saucier DN. Keratinocytes and cytokine/growth factors. *Crit Rev Oral Biol Med* 1996; 7: 300-318.
126. Regnier M, Duval C, Schmidt R. Potential cosmetic applications of reconstructed epidermis. *Int J Cosmet Sci* 1999; 21: 51-58.
127. Régnier M, Patwardhan A, Scheynius A, Schmidt R. Reconstructed human epidermis composed of keratinocytes, melanocytes and langerhans cells. *Med Biol Eng Comput* 1998; 36: 821-824.
128. Ponc M, Boelsma E, Weerheim A, Mulder A, Bouwstra J, Mommaas M. Lipid and ultrastructural characterization of reconstructed skin models. *Int J Pharm* 2000; 203: 211-25.
129. Roguet R. The use of standardized human skin models for cutaneous pharmacotoxicology studies. *Skin Pharmacol Physiol* 2002; 15: 1-3.
130. Mazzoleni G, Di Lorenzo D, Steimberg N. Modelling tissues in 3D: The next future of pharmaco-toxicology and food research?, *Genes Nutr* 2009; 4: 13-22.
131. Wong T, McGrath JA, Navsaria H. The role of fibroblasts in tissue engineering and regeneration. *Br J Dermatol* 2007; 156: 1149-1155.
132. Balasubramani M, Kumar TR, Babu M. Skin substitutes: a review. *Burns* 2001; 27: 534-544.
133. Grinnell F. Fibroblast mechanics in three-dimensional collagen matrices. *J Bodyw Mov Ther* 2008; 12: 191-193.
134. Horning JL, Sahoo SK, Vijayaraghavalu S, Dimitrijevic S, Vasir JK, Jain TK, et al. 3-D tumor model for *in vitro* evaluation of anticancer drugs. *Mol Pharm* 2008; 5: 849-862.
135. Netzlaff F, Lehr CM, Wertz PW, Schaefer UF. The human epidermis models EpiSkin®, SkinEthic® and EpiDerm®: An evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity and substance transport. *Eur J Pharm Biopharm* 2005; 60: 167-178.
136. Sarmiento B, Andrade F, Silva SB, Rodrigues F, das Neves J, Ferreira D. Cell-based *in vitro* models for predicting drug permeability. *Expert Opin Drug Metab Toxicol* 2012; 8: 607-621.

137. OECD. Test No. 439: *In vitro* skin irritation - Reconstructed human epidermis test method, OECD Guidelines for the Testing of Chemicals, editor. Paris: OECD Publishing; 2013.
138. Rozman B, Gasperlin M, Tinois-Tessoneaud E, Pirot F, Falson F. Simultaneous absorption of vitamins C and E from topical microemulsions using reconstructed human epidermis as a skin model. *Eur J Pharm Biopharm* 2009; 72: 69-75.
139. Dreher F, Fouchard F, Patouillet C, Andrian M, Simonnet JT, Benech-Kieffer F. Comparison of cutaneous bioavailability of cosmetic preparations containing caffeine or  $\alpha$ -tocopherol applied on human skin models or human skin *ex vivo* at finite doses. *Skin Pharmacol Physiol* 2002; 15: 40-58.
140. Hu T, Bailey RE, Morrall SW, Aardema MJ, Stanley LA, Skare JA. Dermal penetration and metabolism of *p*-aminophenol and *p*-phenylenediamine: Application of the EpiDerm™ human reconstructed epidermis model. *Toxicol Lett* 2009; 188: 119-129.
141. Lombardi Borgia S, Schlupp P, Mehnert W, Schäfer-Korting M. *In vitro* skin absorption and drug release - A comparison of six commercial prednicarbate preparations for topical use. *Eur J Pharm Biopharm* 2008; 68: 380-389.
142. Rasmussen C, Gratz K, Liebel F, Southall M, Garay M, Bhattacharyya S, et al. The StrataTest® human skin model, a consistent *in vitro* alternative for toxicological testing. *Toxicol In Vitro* 2010; 24: 2021-2029.
143. Allen-Hoffmann BL, Schlosser SJ, Ivarie CAR, Sattler CA, Meisner LF, O'Connor SL. Normal growth and differentiation in a spontaneously immortalized near-diploid human keratinocyte cell line, NIKS. *J Investig Dermatol* 2000; 114: 444-455.
144. EC. EURL ECVAM progress report on the development, validation and regulatory acceptance of alternative methods, Joint Research Center, editor. Luxembourg: European Commission (EC); 2013.
145. Paye M, Barel AO. Introduction to the proof of claims. In: Barel AO, Paye M, Maibach H I, editors. *Handbook of Cosmetic Science and Technology*. New York: CRS Press; 2001. p. 797-806.
146. Houcine A, Delalleau A, Heraud S, Guiraud B, Payre B, Duplan H, et al. How biophysical *in vivo* testing techniques can be used to characterize full thickness skin equivalents. *Skin Res Technol* 2015, *in press*.
147. Elkhyat A, Agache P, Zahouani H, Humbert P. A new method to measure *in vivo* human skin hydrophobia. *Int J Cosmet Sci* 2001; 23: 347-352.
148. Wiechers JM, Barlow T. Skin moisturisation and elasticity originate from at least two different mechanisms. *Int J Cosmet Sci* 1999; 21: 425-435.
149. Alanen E, Nuutinen J, Nicklén K, Lahtinen T, Mönkkönen J. Measurement of hydration in the *stratum corneum* with the MoistureMeter and comparison with the Corneometer. *Skin Res Technol* 2004; 10: 32-37.
150. Kajs TM, Gartsein V. Review of the instrumental assessment of skin: Effects of cleansing products. *J Soc Cosmet Chem* 1991; 42: 249-279.
151. Tagami H. Development of skin measurement instruments. *Japan Med Assoc J* 2004; 47: 495-500.
152. Information and Operating Instructions for the Multi Probe Adapter MPA®. Corneometer. Available from: <http://www.courage-khazaka.de/index.php/en/component/content/article/59-english/products/scientific/55-corneometer> (accessed on 14.12.2015).
153. Rogiers V. EEMCO Guidance for the assessment of transepidermal water loss in cosmetic sciences. *Skin Pharmacol Physiol* 2001; 14: 117-128.
154. Martini MC. Hidratación cutánea y productos hidratantes. In: Martini MC, editor. *Introducción a la dermatofarmacia y a la cosmetología*. Zaragoza: Acribia Editorial; 2005. p. 39-45.

155. Tupker RA, Willis C, Berardksca E, Lee CH, Fartasch M, Atinrat T, et al. Guidelines on sodium lauryl sulfate (SLS) exposure tests. *Contact Derm* 1997; 37: 53-69.
156. Information and Operating Instructions for the Multi Probe Adapter MPA®. Tewameter. Available from: <http://www.courage-khazaka.de/index.php/en/component/content/article/59-english/products/scientific/139-tewameter> (accessed on 14.12.2015).
157. Leonardi GR, Gaspar LR, Campos PM. Study of pH variation on the skin using cosmetic formulations with and without vitamins A, E or ceramide: By a non-invasive method. *An Bras Dermatol* 2002; 77: 563-569.
158. Information and Operating Instructions for the Multi Probe Adapter MPA®. Skin-pH-Meter. Available from <http://www.courage-khazaka.de/index.php/en/component/content/article/59-english/products/scientific/132-skinphmeter> (accessed on 14.12.2015).
159. Piérard GE. EEMCO Guidance to the *in vivo* assessment of tensile functional properties of the skin. *Skin Pharmacol Physiol* 1999; 12: 352-362.
160. Rodrigues L. EEMCO Guidance to the *in vivo* assessment of tensile functional properties of the skin. *Skin Pharmacol Physiol* 2001; 14: 52-67.
161. Hermanns-Lê T, Uhoda I, Smitz S, Piérard GE. Skin tensile properties revisited during ageing. Where now, where next?. *J Cosmet Dermatol* 2004; 3: 35-40.
162. Information and Operating Instructions for the Multi Probe Adapter MPA®. Cutometer. Available from: <http://www.courage-khazaka.de/index.php/en/component/content/article/59-english/products/scientific/140-cutometer> (accessed on 14.12.2015).
163. Fujimura T, Haketa K, Hotta M, Kitahara T. Global and systematic demonstration for the practical usage of a direct *in vivo* measurement system to evaluate wrinkles. *Int J Cosmet Sci* 2007; 29: 423-436.
164. Jaspers S, Hopermann H, Sauermann G, Hoppe U, Lunderstädt R, Ennen J. Rapid *in vivo* measurement of the topography of human skin by active image triangulation using a digital micromirror device. *Skin Res Technol* 1999; 5: 195-207.
165. Akazaki S, Nakagawa H, Kazama H, Osanai O, Kawai M, Takema Y, et al. Age-related changes in skin wrinkles assessed by a novel three-dimensional morphometric analysis. *Br J Dermatol* 2002; 147: 689-695.
166. Jacobi U, Chen M, Frankowski G, Sinkgraven R, Hund M, Rzany B, et al. *In vivo* determination of skin surface topography using an optical 3D device. *Skin Res Technol* 2004; 10: 207-214.
167. Camarasa JG, Anthoine P, Boixareu MJT. Demonstration of the anti-wrinkle efficacy of a cosmetic product. Correlation between clinical observations and instrumental methods. *J Appl Cosmetol* 1997; 15: 13-20.
168. Grover R, Grobbelaar AO, Morgan BDG, Gault DT. A quantitative method for the assessment of facial rejuvenation: a prospective study investigating the carbon dioxide laser. *Brit J Plast Surg* 1998; 51: 8-13.
169. Lagarde JM, Rouvrais C, Black D, Diridollou S, Gall Y. Skin topography measurement by interference fringe projection: A technical validation. *Skin Res Technol* 2001; 7: 112-121.
170. Friedman PM, Skover GR, Payonk G, Kauvar AN, Geronemus RG. 3D *in-vivo* optical skin imaging for topographical quantitative assessment of non-ablative laser technology. *Dermatol Surg* 2002; 28: 199-204.
171. Reuter J, Wölfle U, Korting HC, Schempp C. Which plant for which skin disease? Part 2: Dermatophytes, chronic venous insufficiency, photoprotection, actinic

- keratoses, vitiligo, hair loss, cosmetic indications. *J Dtsch Dermatol Ges* 2010; 8: 866-873.
172. Zanatta CF, Ugartondo V, Mitjans M, Rocha-Filho PA, Vinardell MP. Low cytotoxicity of creams and lotions formulated with Buriti oil (*Mauritia flexuosa*) assessed by the neutral red release test. *Food Chem Toxicol* 2008; 46: 2776-2781.
  173. Chanchal D, Swarnlata S. Novel approaches in herbal cosmetics. *J Cosmet Dermatol* 2008; 7: 89-95.
  174. Schieber A, Stintzing FC, Carle R. By-products of plant food processing as a source of functional compounds - recent developments. *Trends Food Sci Tech* 2001; 12: 401-413.
  175. Capecka E, Mareczek A, Leja M. Antioxidant activity of fresh and dry herbs of some Lamiaceae species. *Food Chem* 2005; 93: 223-226.
  176. Baydar NG, Baydar H. Phenolic compounds, antiradical activity and antioxidant capacity of oil-bearing rose (*Rosa damascena* Mill.) extracts. *Ind Crop Prod* 2013; 41: 375-380.
  177. Moure A, Cruz J, Franco D, Domínguez M, Sineiro J, Domínguez H, et al. Natural antioxidants from residual sources. *Food Chem* 2001; 72: 145-171.
  178. Almeida IF, Fernandes E, Lima JLFC, Costa PC, Bahia MF. Protective effect of *Castanea sativa* and *Quercus robur* leaf extracts against oxygen and nitrogen reactive species. *J Photochem Photobiol B* 2008; 91: 87-95.
  179. Abbasi AM, Khan MA, Ahmad M, Zafar M, Jahan S, Sultana S. Ethnopharmacological application of medicinal plants to cure skin diseases and in folk cosmetics among the tribal communities of North-West Frontier Province, Pakistan. *J Ethnopharmacol* 2010; 128: 322-335.
  180. Saikia AP, Ryakala VK, Sharma P, Goswami P, Bora U. Ethnobotany of medicinal plants used by Assamese people for various skin ailments and cosmetics. *J Ethnopharmacol* 2006; 106: 149-157.
  181. Wang K-H, Lin R-D, Hsu F-L, Huang Y-H, Chang H-C, Huang C-Y, et al. Cosmetic applications of selected traditional Chinese herbal medicines. *J Ethnopharmacol* 2006; 106: 353-359.
  182. Antignac E, Nohynek GJ, Re T, Clouzeau J, Toutain H. Safety of botanical ingredients in personal care products/cosmetics. *Food Chem Toxicol* 2011; 49: 324-341.
  183. Ahmad I, Aqil F. *In vitro* efficacy of bioactive extracts of 15 medicinal plants against ES $\beta$ L-producing multidrug-resistant enteric bacteria. *Microbiol Res* 2007; 162: 264-275.
  184. Zampini IC, Cuello S, Alberto MR, Ordoñez RM, Almeida RD, Solorzano E, et al. Antimicrobial activity of selected plant species from "the Argentine Puna" against sensitive and multi-resistant bacteria. *J Ethnopharmacol* 2009; 124: 499-505.
  185. Singleton VL, Rossi JAJ. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Vitic* 1965; 16: 144-158.
  186. Alves R, Costa A, Jerez M, Casal S, Sineiro J, Núñez M, et al. Antiradical activity, phenolics profile, and hydroxymethylfurfural in espresso coffee: Influence of technological factors. *J Agr Food Chem* 2010; 58: 12221-12229.
  187. Barroso MF, Delerue-Matos C, Oliveira MBPP. Evaluation of the total antioxidant capacity of flavored water and electrochemical purine damage by sulfate radicals using a purine-based sensor. *Electrochim Acta* 2011; 56: 8954-8961.
  188. Guimarães R, Barros L, Barreira JCM, Sousa MJ, Carvalho AM, Ferreira ICFR. Targeting excessive free radicals with peels and juices of citrus fruits: Grapefruit, lemon, lime and orange. *Food Chem Toxicol* 2010; 48: 99-106.

189. Benzie IFF, Strain JJ. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. In: Lester P, editor. *Methods in Enzymology*. New York: Academic Press; 1999. p. 15-27.
190. Clinical Laboratory Standards Institute, Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. NCCLS: Wayne, Pennsylvania; 2008.
191. Clinical Laboratory and Standard Institute, Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. NCCLS: Wayne, Pennsylvania; 2003.
192. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J Agr Food Chem* 2005; 53: 4290-4302.
193. Wong SP, Leong LP, William Koh JH. Antioxidant activities of aqueous extracts of selected plants. *Food Chem* 2006; 99: 775-783.
194. Escarpa A, González MC. Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. *Anal Chim Acta* 2001; 427: 119-127.
195. Singleton VL, Orthofer R, Lamuela-Raventós RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In: Lester P, editor. *Methods in Enzymology*. New York: Academic Press; 1999. p. 152-178.
196. EMA. Assessment report on *Juglans regia L., folium*. Committee on Herbal Medicinal Products, London: European Medicines Agency (EMA); 2013.
197. EMA. Community herbal monograph on *Quercus robur L., Quercus petraea.(Matt.) Liebl., Quercus pubescens Willd., cortex*. Committee on Herbal Medicinal Products. London: European Medicines Agency (EMA); 2010.
198. Barreira JCM, Ferreira ICFR, Oliveira MBPP, Pereira JA. Antioxidant potential of chestnut (*Castanea sativa L.*) and almond (*Prunus dulcis L.*) by-products. *Food Sci Technol Int* 2010; 16: 209-216.
199. Barros L, Oliveira S, Carvalho AM, Ferreira ICFR. *In vitro* antioxidant properties and characterization in nutrients and phytochemicals of six medicinal plants from the Portuguese folk medicine. *Ind Crop Prod* 2010; 32: 572-579.
200. Díaz-Reinoso B, Moure A, Domínguez H, Parajó JC. Membrane concentration of antioxidants from *Castanea sativa* leaves aqueous extracts. *Chem Eng J* 2011; 175: 95-102.
201. Dinis LT, Oliveira MM, Almeida J, Costa R, Gomes-Laranjo J, Peixoto F. Antioxidant activities of chestnut nut of *Castanea sativa* Mill. (cultivar 'Judia') as function of origin ecosystem. *Food Chem* 2012; 132: 1-8.
202. Neri L, Dimitri G, Sacchetti G. Chemical composition and antioxidant activity of cured chestnuts from three sweet chestnut (*Castanea sativa* Mill.) ecotypes from Italy. *J Food Compost Anal* 2010; 23: 23-29.
203. Almeida IF, Fernandes E, Lima JLFC, Costa PC, Bahia MF. Walnut (*Juglans regia*) leaf extracts are strong scavengers of pro-oxidant reactive species. *Food Chem* 2008; 106: 1014-1020.
204. Celep A, Yilmaz S, Coruh N. Antioxidant capacity and cytotoxicity of *Aesculus hippocastanum* on breast cancer MCF-7 cells. *J Food Drug Anal* 2012; 20: 692-698.
205. Felipe MBMC, de Carvalho FM, Félix-Silva J, Fernandes-Pedrosa MF, Scortecchi KC, Agnez-Lima LF, et al. Evaluation of genotoxic and antioxidant activity of an *Aesculus hippocastanum L.* (Sapindaceae) phytotherapeutic agent. *Biomed Prev Nutr* 2012; 3: 261-266.

206. Akbari V, Jamei R, Heidari R, Esfahlan AJ. , Antiradical activity of different parts of Walnut (*Juglans regia* L.) fruit as a function of genotype. *Food Chem* 2012; 135: 2404-2410.
207. Carvalho M, Ferreira PJ, Mendes VS, Silva R, Pereira JA, Jerónimo C, et al. Human cancer cell antiproliferative and antioxidant activities of *Juglans regia* L. *Food Chem Toxicol* 2010; 48: 441-447.
208. Fernández-Agulló A, Pereira E, Freire MS, Valentão P, Andrade PB, González-Álvarez J, et al. Influence of solvent on the antioxidant and antimicrobial properties of walnut (*Juglans regia* L.) green husk extracts. *Ind Crop Prod* 2013; 42: 126-132.
209. Pereira JA, Oliveira I, Sousa A, Ferreira ICFR, Bento A, Estevinho L. Bioactive properties and chemical composition of six walnut (*Juglans regia* L.) cultivars. *Food Chem Toxicol* 2008; 46: 2103-2111.
210. Masaki H, Sakaki S, Atsumi T, Sakurai H. Active-oxygen scavenging activity of plant extracts. *Biol Pharm Bull* 1995; 18: 162-166.
211. Biglari F, AlKarkhi AFM, Easa AM. Antioxidant activity and phenolic content of various date palm (*Phoenix dactylifera*) fruits from Iran. *Food Chem* 2008; 107: 1636-1641.
212. Celli GB, Pereira-Netto AB, Beta T. Comparative analysis of total phenolic content, antioxidant activity, and flavonoids profile of fruits from two varieties of Brazilian cherry (*Eugenia uniflora* L.) throughout the fruit developmental stages. *Food Res Int* 2011; 44: 2442-2451.
213. Costa ASG, Nunes MA, Almeida IMC, Carvalho MR, Barroso MF, Alves RC, et al. Teas, dietary supplements and fruit juices: A comparative study regarding antioxidant activity and bioactive compounds. *LWT - Food Sci Technol* 2012; 49: 324-328.
214. Niseteo T, Komes D, Belščak-Cvitanović A, Horžić D, Budeč M. Bioactive composition and antioxidant potential of different commonly consumed coffee brews affected by their preparation technique and milk addition. *Food Chem* 2012; 134: 1870-1877.
215. Chen AY, Chen YC. A review of the dietary flavonoid, kaempferol on human health and cancer chemoprevention. *Food Chem* 2013; 138: 2099-2107.
216. Shaghaghi M, Manzoori JL, Jouyban A. Determination of total phenols in tea infusions, tomato and apple juice by terbium sensitized fluorescence method as an alternative approach to the Folin-Ciocalteu spectrophotometric method. *Food Chem* 2008; 108: 695-701.
217. Mishra N, Dubey A, Mishra R, Barik N. Study on antioxidant activity of common dry fruits. *Food Chem Toxicol* 2010; 48: 3316-3320.
218. Visnevschi-Necrasov T, Phylogenetic and chemical study of portuguese forage leguminous: application of molecular biology techniques and composition assessment in isoflavones by HPLC / DAD [PhD Dissertation]. Porto: Faculty of Pharmacy University of Porto; 2013.
219. Benzie IFF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP Assay. *Anal Biochem* 1996; 239: 70-76.
220. Krishnaiah D, Sarbatly R, Nithyanandam R. A review of the antioxidant potential of medicinal plant species. *Food Bioprod Processing* 2011; 89: 217-233.
221. Ou B, Huang D, Hampsch-Woodill M, Flanagan JA, Deemer EK. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. *J Agr Food Chem* 2002; 50: 3122-3128.
222. Ferreira ICFR, Baptista P, Vilas-Boas M, Barros L. Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: Individual cap and stipe activity. *Food Chem* 2007; 100: 1511-1516.

223. Malheiro R, Sá O, Pereira E, Aguiar C, Baptista P, Pereira JA. *Arbutus unedo* L. leaves as source of phytochemicals with bioactive properties. *Ind Crop Prod* 2012; 37: 473-478.
224. Malheiro R, Sousa A, Casal S, Bento A, Pereira JA., Cultivar effect on the phenolic composition and antioxidant potential of stoned table olives. *Food Chem Toxicol* 2011; 49: 450-457.
225. Velioglu YS, Mazza G, Gao L, Oomah BD. Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. *J Agr Food Chem* 1998; 46: 4113-4117.
226. Fernandes F, Ramalhosa E, Pires P, Verdial J, Valentão P, Andrade P, et al. *Vitis vinifera* leaves towards bioactivity. *Ind Crop Prod* 2013; 43: 434-440.
227. Kong HH. Skin microbiome: genomics-based insights into the diversity and role of skin microbes. *Trends Mol Med* 2011; 17: 320-328.
228. Rosenthal M, Goldberg D, Aiello A, Larson E, Foxman B. Skin microbiota: Microbial community structure and its potential association with health and disease. *Infect Genet Evol* 2011; 11: 839-848.
229. Gao Z, Perez-Perez GI, Chen Y, Blaser MJ. Quantitation of major human cutaneous bacterial and fungal populations. *J Clin Microbiol* 2010; 48: 3575-81.
230. Scharschmidt TC, Fischbach MA. What lives on our skin: ecology, genomics and therapeutic opportunities of the skin microbiome. *Drug Discov Today Dis Mech* 2013; 10: 83-89.
231. Cogen AL, Nizet V, Gallo RL. Skin microbiota: a source of disease or defence?. *Br J Dermatol* 2008; 158: 442-55.
232. Drews MB, Ludwig AC, Leititis JU, Daschner FD. Low birth weight and nosocomial infection of neonates in a neonatal intensive care unit. *J Hosp Infect* 1995; 30: 65-72.
233. Mylotte JM, Graham R, Kahler L, Young L,, Goodnough S. Epidemiology of nosocomial infection and resistant organisms in patients admitted for the first time to an acute rehabilitation unit. *Clin Infect Dis* 2000; 30: 425-432.
234. Naglik JR, Moyes DL, Wächtler B,, Hube B. *Candida albicans* interactions with epithelial cells and mucosal immunity. *Microb Infect* 2011; 13: 963-976.
235. Adetutu A, Morgan WA, Corcoran O. Antibacterial, antioxidant and fibroblast growth stimulation activity of crude extracts of *Bridelia ferruginea* leaf, a wound-healing plant of Nigeria. *J Ethnopharmacol* 2011; 133: 116-119.
236. Hajji M, Jarraya R, Lassoued I, Masmoudi O, Damak M,, Nasri M. GC/MS and LC/MS analysis, and antioxidant and antimicrobial activities of various solvent extracts from *Mirabilis jalapa* tubers. *Process Biochem* 2010; 45: 1486-1493.
237. Fabry W, Okemo PO, Ansorg R. Antibacterial activity of East African medicinal plants. *J Ethnopharmacol* 1998; 60: 79-84.
238. Gibbons S. Plants as a source of bacterial resistance modulators and anti-infective agents. *Phytochem Rev* 2005; 4: 63-78.
239. Groeber F, Holeiter M, Hampel M, Hinderer S, Schenke-Layland K. Skin tissue engineering - *In vivo* and *in vitro* applications. *Adv Drug Deliv Rev* 2011; 63: 352-366.
240. MacNeil S. Progress and opportunities for tissue-engineered skin. *Nature* 2007; 445: 874-80.
241. Menon G, Nólen L. *Stratum corneum* ceramides and their role in skin barrier function In: Leyden JJ, Rawlings AV, editors. *Skin Moisturization*. New York: Markel Dekker, Inc.; 2002. p. 31-60.
242. Pillai S, Cornell M, Oresajo C. Epidermal barrier. In: Draelos ZD, editor. *Cosmetic Dermatology - Products and Procedures*. West Sussex: Blackwell Publishing Ltd; 2010. p. 3-12.



243. Moharamzadeh K, Van Noort R, Brook IM, Scutt AM, Cytotoxicity of resin monomers on human gingival fibroblasts and HaCaT keratinocytes. *Dent Mater* 2007; 23: 40-44.
244. Kempf M, Kimble RM, Cuttle L. Cytotoxicity testing of burn wound dressings, ointments and creams: A method using polycarbonate cell culture inserts on a cell culture system. *Burns* 2011; 37: 994-1000.
245. Neamnark A, Sanchavanakit N, Pavasant P, Rujiravanit R, Supaphol P. *In vitro* biocompatibility of electrospun hexanoyl chitosan fibrous scaffolds towards human keratinocytes and fibroblasts. *Eur Polym J* 2008; 44: 2060-2067.
246. Rehders M, Grosshäuser BB, Smarandache A, Sadhukhan A, Mirastschijski U, Kempf J, et al. Effects of lunar and mars dust simulants on HaCaT keratinocytes and CHO-K1 fibroblasts. *Adv Space Res* 2011; 47: 1200-1213.
247. Dudonné S, Vitrac X, Coutière P, Woillez M, Mérillon JM. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *J Agr Food Chem* 2009; 57: 1768-1774.
248. Rocha-Guzmán N, Gallegos-Infante JA, González-Laredo Rn, Reynoso-Camacho Ra, Ramos-Gómez M, Garcia-Gasca T, et al. Antioxidant activity and genotoxic effect on HeLa cells of phenolic compounds from infusions of *Quercus resinosa* leaves. *Food Chem* 2009; 115: 1320-1325.
249. Almeida IF, Valentão P, Andrade PB, Seabra RM, Pereira TM, Amaral MH, et al. Oak leaf extract as topical antioxidant: free radical scavenging and iron chelating activities and *in vivo* skin irritation potential. *Biofactors* 2008; 33: 267-279.
250. Lee S, Koh J-S, Ha B-J, Boo Y. *Quercus glauca* extract and rutin inhibit the UVB-induced expression of matrix metalloproteinase-1 in human dermal fibroblasts. *J Korean Soc Appl Biol Chem* 2010; 53: 677-684.
251. Lee SE, Hwang HJ, Ha J-S, Jeong H-S, Kim JH. Screening of medicinal plant extracts for antioxidant activity. *Life Sci* 2003; 73: 167-179.
252. Park G, Jang DS, Oh MS. *Juglans mandshurica* leaf extract protects skin fibroblasts from damage by regulating the oxidative defense system. *Biochem Biophys Res Commun* 2012; 421: 343-348.
253. Darmani H, Nusayr T, Al-Hiyasat AS. Effects of extracts of miswak and derum on proliferation of Balb/C 3T3 fibroblasts and viability of cariogenic bacteria. *Int J Dent Hyg* 2006; 4: 62-66.
254. Balasundram N, Sundram K, Samman S. Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chem* 2006; 99: 191-203.
255. Gaiker Centro Tecnológico, editor. Handbook for the prevention and minimization of waste and valorisation of by-products in european agro-food industries. AWARENET; 2004.
256. Federici F, Fava F, Kalogerakis N, Mantzavinos D. Valorisation of agro-industrial by-products, effluents and waste: concept, opportunities and the case of olive mill wastewaters. *J Chem Technol Biot* 2009; 84: 895-900.
257. He FJ, Chen JQ. Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: Differences between chinese women and women in western countries and possible mechanisms. *Food Sci Human Wellness* 2013; 2: 146-161.
258. Hyun J, Shin SY, So KM, Lee YH, Lim Y. Isoflavones inhibit the clonogenicity of human colon cancer cells. *Bioorg Med Chem Lett* 2012; 22: 2664-2669.
259. Mann GE, Bonacasa B, Ishii T, Siow RCM. Targeting the redox sensitive Nrf2-Keap1 defense pathway in cardiovascular disease: protection afforded by dietary isoflavones. *Curr Opin Pharmacol* 2009; 9: 139-145.

260. Watanabe S, Uesugi S, Kikuchi Y. Isoflavones for prevention of cancer, cardiovascular diseases, gynecological problems and possible immune potentiation. *Biomed Pharmacother* 2002; 56: 302-312.
261. Taku K, Melby MK, Nishi N, Omori T, Kurzer MS. Soy isoflavones for osteoporosis: An evidence-based approach. *Maturitas* 2011; 70: 333-338.
262. Jou HJ, Wu SC, Chang FW, Ling PY, Chu KS, Wu WH. Effect of intestinal production of equol on menopausal symptoms in women treated with soy isoflavones. *Int J Gynecol Obstet* 2008; 102: 44-49.
263. Campos MG, Matos MP, Câmara MT, Cunha MM. The variability of isoflavones in soy seeds and the possibility of obtaining extracts for over the counter tablet preparations that can be standardized. *Ind Crop Prod* 2007; 26: 85-92.
264. Klejduš B, Mikelová R, Petrlová J, Potesil D, Adam V, Stiborová M, et al. Determination of isoflavones in soy bits by fast column high-performance liquid chromatography coupled with UV-visible diode-array detection. *J Chromatogr A* 2005; 1084: 71-79.
265. Nara K, Nihei Ki, Ogasawara Y, Koga H, Kato Y. Novel isoflavone diglycoside in groundnut (*Apios americana* Medik). *Food Chem* 2011, 124: 703-710.
266. Huang ZR, Hung CF, Lin YK, Fang JY. *In vitro* and *in vivo* evaluation of topical delivery and potential dermal use of soy isoflavones genistein and daidzein. *Int J Pharm* 2008; 364: 36-44.
267. Shinkaruk S, Durand M, Lamothe V, Carpaye A, Martinet A, Chantre P, et al. Bioavailability of glycitein relatively to other soy isoflavones in healthy young Caucasian men. *Food Chem* 2012; 135: 1104-1111.
268. Visnevschi-Necrasov T, Barreira JCM, Cunha SC, Pereira G, Nunes E, Oliveira MBPP. Advances in isoflavone profile characterisation using matrix solid-phase dispersion coupled to HPLC/DAD in *Medicago* species. *Phytochem Anal* 2015; 26: 40-46.
269. Amin I, Mukhrizah O. Antioxidant capacity of methanolic and water extracts prepared from food-processing by-products. *J Sci Food Agric* 2006; 86: 778-784.
270. USDA. Database on the Isoflavone Content of Foods. 2002; Available from: <http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav.html> (accessed on 06.04.2012).
271. Liggins J, Bluck LJC, Runswick S, Atkinson C, Coward WA, Bingham SA. Daidzein and genistein contents of vegetables. *Br J Nutr* 2000; 84: 717-725.
272. Liggins J, Bluck LJC, Runswick S, Atkinson C, Coward WA, Bingham SA. Daidzein and genistein content of fruits and nuts. *J Nutr Biochem* 2000; 11: 326-331.
273. de Rijke E, Zafra-Gómez A, Ariese F, Brinkman UAT, Gooijer C. Determination of isoflavone glucoside malonates in *Trifolium pratense* L. (red clover) extracts: quantification and stability studies. *J Chromatogr A* 2001; 932: 55-64.
274. Peng YY, Ye JN. Determination of isoflavones in red clover by capillary electrophoresis with electrochemical detection. *Fitoterapia* 2006; 77: 171-178.
275. Kuhnle GGC, Dell'Aquila C, Aspinall SM, Runswick SA, Mulligan AA, Bingham SA. Phytoestrogen content of beverages, nuts, seeds, and oils. *J Agr Food Chem* 2008; 56: 7311-7315.
276. Caldwell CR, Britz SJ, Mirecki RM. Effect of temperature, elevated carbon dioxide, and drought during seed development on the isoflavone content of dwarf soybean [*Glycine max* (L.) Merrill] grown in controlled environments. *J Agr Food Chem* 2005; 53: 1125-1129.
277. Edwards R, Tiller SA, Parry AD. The effect of plant age and nodulation on the isoflavonoid content of red clover (*Trifolium pratense*). *J Plant Physiol* 1997; 150: 603-610.

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278. Alves RC, Almeida IMC, Casal S, Oliveira MBPP. Isoflavones in coffee: Influence of species, roast degree, and brewing method. *J Agr Food Chem* 2010; 58: 3002-3007.
279. Committee on Toxicity of Chemicals in Food, Consumer Products and the Environmental. Working Group on Phytoestrogens. Chemistry of Phytoestrogens and overview of Analytical Methodology (PEG/2000/06). London: Food Standards Agency; 2000.
280. Setchell KDR, Cassidy A. Dietary isoflavones: Biological effects and relevance to human health. *J Nutr* 1999; 129: 758.
281. Mussatto SI, Ballesteros LF, Martins S, Teixeira JA. Extraction of antioxidant phenolic compounds from spent coffee grounds. *Sep Purif Technol* 2011; 83: 173-179.
282. Pandey A, Soccol CR, Nigam P, Brand D, Mohan R, Roussos S. Biotechnological potential of coffee pulp and coffee husk for bioprocesses. *Biochem Eng J* 2000; 6: 153-162.
283. Alves RC, Casal S, Oliveira MBPP. Tocopherols in espresso coffee: Analytical method development and validation. *Food Chem* 2009; 115: 1549-1555.
284. Ludwig IA, Sanchez L, Caemmerer B, Kroh LW, De Peña MP, Cid C. Extraction of coffee antioxidants: Impact of brewing time and method. *Food Res Int* 2012; 48: 57-64.
285. Panusa A, Zuorro A, Lavecchia R, Marrosu G, Petrucci R. Recovery of natural antioxidants from spent coffee grounds. *J Agr Food Chem* 2013; 61: 4162-4168.
286. Martínez-Tomé M, Jiménez-Monreal A, García-Jiménez L, Almela L, García-Diz L, Mariscal-Arcas M, et al. , Assessment of antimicrobial activity of coffee brewed in three different ways from different origins. *Eur Food Res Technol* 2011; 233: 497-505.
287. Issa Y, Watts DC, Brunton PA, Waters CM, Duxbury AJ. Resin composite monomers alter MTT and LDH activity of human gingival fibroblasts *in vitro*. *Dent Mater* 2004; 20: 12-20.
288. Fotakis G, Timbrell JA. *In vitro* cytotoxicity assays: Comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett* 2006; 160: 171-177.
289. Meneses NGT, Martins S, Teixeira JA, Mussatto SI. Influence of extraction solvents on the recovery of antioxidant phenolic compounds from brewer's spent grains. *Sep Purif Technol* 2013; 108: 152-158.
290. Aybastier Ö, Işık E, Şahin S, Demir C. Optimization of ultrasonic-assisted extraction of antioxidant compounds from blackberry leaves using response surface methodology. *Ind Crop Prod* 2013; 44: 558-565.
291. Sfahlan AJ, Mahmoodzadeh A, Hasanzadeh A, Heidari R, Jamei R. Antioxidants and antiradicals in almond hull and shell (*Amygdalus communis* L.) as a function of genotype. *Food Chem* 2009; 115: 529-533.
292. Reis SF, Rai DK, Abu-Ghannam N. Water at room temperature as a solvent for the extraction of apple pomace phenolic compounds. *Food Chem* 2012; 135: 1991-1998.
293. Naidu M, Sulochanamma G, Sampathu SR, Srinivas P. Studies on extraction and antioxidant potential of green coffee. *Food Chem* 2008, 107: 377-384.
294. Spigno G, Tramelli L, De Faveri DM. Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape marc phenolics. *J Food Eng* 2007; 81: 200-208.
295. Hečimović I, Belščak-Cvitanović A, Horžić D, Komes D. Comparative study of polyphenols and caffeine in different coffee varieties affected by the degree of roasting. *Food Chem* 2011; 129: 991-1000.

296. Mendoza L, Yañez K, Vivanco M, Melo R, Cotoras M. Characterization of extracts from winery by-products with antifungal activity against *Botrytis cinerea*. *Ind Crop Prod* 2013; 43: 360-364.
297. Dhaouadi K, Raboudi F, Funez-Gomez L, Pamies D, Estevan C, Hamdaoui M, et al. Polyphenolic extract of barbery-fig (*Opuntia ficus-indica*) syrup: RP–HPLC–ESI-MS analysis and determination of antioxidant, antimicrobial and cancer-cells cytotoxic potentials. *Food Anal Methods* 2013; 6: 45-53.
298. Cowan MM. Plant products as antimicrobial agents. *Clin Microbiol Rev* 1999; 12: 564-582.
299. Burt S. Essential oils: their antibacterial properties and potential applications in foods - a review. *Int J Food Microbiol* 2004; 94: 223-253.
300. Tiwari BK, Valdramidis VP, O' Donnell CP, Muthukumarappan K, Bourke P, Cullen PJ. Application of natural antimicrobials for food preservation. *J Agr Food Chem* 2009; 57: 5987-6000.
301. Dorman HJD, Deans SG. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J Appl Microbiol* 2000; 88: 308-316.
302. Pauli A, Knobloch K. Inhibitory effects of essential oil components on growth of food-contaminating fungi. *Z Lebensm Unters Forsch* 1987; 185: 10-13.
303. Ekmekcioglu C, Strauss-Blasche G, J. Leibetseder V, Marktl W. Toxicological and biochemical effects of different beverages on human intestinal cells. *Food Res Int* 1999; 32: 421-427.
304. McCarthy AL, O'Callaghan YC, Connolly A, Piggott CO, FitzGerald RJ, O'Brien NM. *In vitro* antioxidant and anti-inflammatory effects of brewers' spent grain protein rich isolate and its associated hydrolysates. *Food Res Int* 2013; 50: 205-212.
305. Okonogi S, Duangrat C, Anuchpreeda S, Tachakittirungrod S, Chowwanapoonpohn S. Comparison of antioxidant capacities and cytotoxicities of certain fruit peels. *Food Chem* 2007; 103: 839-846.
306. Khonkarn R, Okonogi S, Ampasavate C, Anuchapreeda S. Investigation of fruit peel extracts as sources for compounds with antioxidant and antiproliferative activities against human cell lines. *Food Chem Toxicol* 2010; 48: 2122-2129.
307. Sato Y, Itagaki S, Kurokawa T, Ogura J, Kobayashi M, Hirano T, et al. *In vitro* and *in vivo* antioxidant properties of chlorogenic acid and caffeic acid. *Int J Pharm* 2011; 403: 136-138.
308. Mills CE, Oruna-Concha MJ, Mottram DS, Gibson GR, Spencer JPE. The effect of processing on chlorogenic acid content of commercially available coffee. *Food Chem* 2013; 141: 3335-3340.
309. Herman A, Herman AP. Caffeine's mechanisms of action and its cosmetic use. *Skin Pharm Physiol* 2013; 26: 8-14.
310. Bolzinger MA, Briançon S, Pelletier J, Fessi H, Chevalier Y. Percutaneous release of caffeine from microemulsion, emulsion and gel dosage forms. *Eur J Pharm Biopharm* 2008; 68: 446-451.
311. del Campo G, Berregi I, Caracena R, Zuriarrain J. Quantitative determination of caffeine, formic acid, trigonelline and 5-(hydroxymethyl)furfural in soluble coffees by <sup>1</sup>H NMR spectrometry. *Talanta* 2010; 81: 367-371.
312. Capuano E, Fogliano V. Acrylamide and 5-hydroxymethylfurfural (HMF): A review on metabolism, toxicity, occurrence in food and mitigation strategies. *LWT - Food Sci Technol* 2011; 44: 793-810.
313. Draize JH, Woodward G, Clavery HO. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. *J Pharmacol Exp Ther* 1944; 82: 377-390.

314. Alépée N, Bessou-Touya S, Cotovio J, de Smedt A, de Wever B, Faller C, et al. Cosmetics Europe multi-laboratory pre-validation of the SkinEthic™ reconstituted human corneal epithelium test method for the prediction of eye irritation. *Toxicol In Vitro* 2013; 27: 1476-1488.
315. Tornier C, Amsellem C, Fraissinette AB, Alépée N. Assessment of the optimized SkinEthic Reconstructed Human Epidermis (RHE) 42 bis skin irritation protocol over 39 test substances. *Toxicol In Vitro* 2010; 24: 245-256.
316. Lemos G, dos Santos JS, dos Santos MLP. Method validation to HMF determination in honey by HPLC-UV and its influence on the product quality. *Quim Nova* 2010; 33: 1682-1685.
317. Rivelli DP, Ropke CD, Silva VV, Miranda DV, Almeida RL, Sawada TCH, et al. Simultaneous determination of chlorogenic acid, caffeic acid and caffeine in hydroalcoholic and aqueous extracts of *Ilex paraguariensis* by HPLC and correlation with antioxidant capacity of the extracts by DPPH· reduction. *Braz J Pharm Sci* 2007; 43: 215-222.
318. Agner T, Held E, West W, Gray J. Evaluation of an experimental *patch test* model for the detection of irritant skin reactions to moisturisers. *Skin Res Technol* 2000; 6: 250-254.
319. Wa CV, Maibach HI. Mapping the human face: biophysical properties. *Skin Res Technol* 2010; 16: 38-54.
320. GHS. Skin corrosion/irritation. In: United Nations, editor. Globally harmonized system of classification and labelling of chemicals (GHS). New York: United Nations Economic Commission for Europe; 2011.
321. Balboa EM, Soto ML, Nogueira DR, González-López N, Conde E, Moure A, et al. Potential of antioxidant extracts produced by aqueous processing of renewable resources for the formulation of cosmetics. *Ind Crop Prod* 2014; 58: 104-110.
322. Mizutani H, Black R, Kupper TS. Human keratinocytes produce but do not process pro-interleukin-1 (IL-1) beta. Different strategies of IL-1 production and processing in monocytes and keratinocytes. *J Clin Invest* 1991; 87: 1066-1071.
323. Zhang Q, Dai T, Zhang L, Zhang M, Xiao X, Hu H, et al. Identification of potential biomarkers for predicting acute dermal irritation by proteomic analysis. *J Appl Toxicol* 2011; 31: 762-772.
324. Molinari J, Eskes C, Andres E, Remoué N, Sá-Rocha VM, Hurtado SP, et al. Improved procedures for *in vitro* skin irritation testing of sticky and greasy natural botanicals. *Toxicol In Vitro* 2013; 27: 441-450.
325. Pereira S. Caracterização da pele de prata no contexto da valorização de subprodutos da indústria agroalimentar [Master Dissertation]. Porto: Faculty of Pharmacy University of Porto; 2012.
326. Coroneo MT, Rosenberg ML, Cheung LM. Ocular effects of cosmetic products and procedures. *Ocul Surf* 2006; 4: 94-102.
327. OECD. Test No. 437: The bovine corneal opacity and permeability (BCOP) test method for identifying ocular corrosives and severe irritants, OECD Guidelines for the Testing of Chemicals, editor. Paris: OECD Publishing; 2009.
328. OECD, Test No. 438: The isolated chicken eye (ICE) test method for identifying ocular corrosives and severe irritants, OECD Guidelines for the Testing of Chemicals, editor. Paris: OECD Publishing; 2009.
329. Miles A, Berthet A, Hopf NB, Gilliet M, Raffoul W, Vernez D, et al. A new alternative method for testing skin irritation using a human skin model: A pilot study. *Toxicol In Vitro* 2014; 28: 240-247.
330. Perkins MA, Osborne R, Rana FR, Ghassemi A, Robinson MK. Comparison of *in vitro* and *in vivo* human skin responses to consumer products and ingredients with a range of irritancy potential. *Toxicol Sci* 1999; 48: 218-229.

331. Welss T, Basketter DA, Schröder KR. In vitro skin irritation: facts and future. State of the art review of mechanisms and models. *Toxicol In Vitro* 2004; 18: 231-243.
332. Pinnagoda J, Tupkek RA, Agner T, Serup J. Guidelines for transepidermal water loss (TEWL) measurement. *Contact Dermatitis* 1990; 22: 164-178.
333. Almeida IF, Valentão P, Andrade PB, Seabra RM, Pereira TM, Amaral MH, et al. *In vivo* skin irritation potential of a *Castanea sativa* (Chestnut) leaf extract, a putative natural antioxidant for topical application. *Basic Clin Pharmacol Toxicol* 2008; 103: 461-467.
334. Dal'Belo SE, Gaspar LR, Maia Campos PM. Moisturizing effect of cosmetic formulations containing *Aloe vera* extract in different concentrations assessed by skin bioengineering techniques. *Skin Res Technol* 2006; 12: 241-246.
335. Reider N, Komericki P, Hausen BM, Fritsch P, Aberer W. The seamy side of natural medicines: contact sensitization to arnica (*Arnica montana* L.) and marigold (*Calendula officinalis* L.). *Contact Dermatitis* 2001; 45: 269-272.
336. Glampedaki P, Dutschk V. Stability studies of cosmetic emulsions prepared from natural products such as wine, grape seed oil and mastic resin. *Colloids Surf A Physicochem Eng Asp* 2014; 460: 306-311.
337. Rubio L, Alonso C, Coderch L, Parra JL, Martí M, Cebrián J, et al. Skin delivery of caffeine contained in biofunctional textiles. *Tex Res J* 2010; 80: 1214-1221.
338. Di Mambro VM, Fonseca MJV. Assays of physical stability and antioxidant activity of a topical formulation added with different plant extracts. *J Pharm Biomed Anal* 2005, 37: 287-295.
339. Almeida IF, Maleckova J, Saffi R, Monteiro H, Góios F, Amaral MH, et al. Characterization of an antioxidant surfactant-free topical formulation containing *Castanea sativa* leaf extract. *Drug Dev Ind Pharm* 2015, 41: 148-155.
340. Zanatta CF, de Faria Santos AM, de Camargo FB Jr, Campos PM, Rocha-Filho PA. Rheological behavior, zeta potential, and accelerated stability tests of Buriti oil (*Mauritia flexuosa*) emulsions containing lyotropic liquid crystals. *Drug Dev Ind Pharm* 2010; 36: 93-101.
341. Vignoli JA, Bassoli DG, Benassi MT. Antioxidant activity, polyphenols, caffeine and melanoidins in soluble coffee: The influence of processing conditions and raw material. *Food Chem* 2011; 124: 863-868.
342. Jaiswal R, Matei MF, Golon A, Witt M, Kuhnert N. Understanding the fate of chlorogenic acids in coffee roasting using mass spectrometry based targeted and non-targeted analytical strategies. *Food Funct* 2012; 3: 976-984.
343. Ayala-Zavala JF, Wang SY, Wang CY, González-Aguilar GA. Effect of storage temperatures on antioxidant capacity and aroma compounds in strawberry fruit. *LWT - Food Sci Technol* 2004; 37: 687-695.
344. Carmona-Jiménez Y, García-Moreno MV, Igartuburu JM, Garcia Barroso C. Simplification of the DPPH assay for estimating the antioxidant activity of wine and wine by-products. *Food Chem* 2014; 165: 198-204.
345. Larrauri JA, Sánchez-Moreno C, Saura-Calixto F. Effect of temperature on the free radical scavenging capacity of extracts from red and white grape pomace peels. *J Agr Food Chem* 1998; 46: 2694-2697.
346. Borrelli RC, Visconti A, Mennella C, Anese M, Fogliano V. Chemical characterization and antioxidant properties of coffee melanoidins. *J Agr Food Chem* 2002; 50: 6527-6533.
347. Darvin ME, Sterry W, Lademann J. Resonance raman spectroscopy as an effective tool for the determination of antioxidative stability of cosmetic formulations. *J Biophotonics* 2010; 3: 82-88..

348. Anchisi C, Maccioni AM, Sinico C, Valenti D. Stability studies of new cosmetic formulations with vegetable extracts as functional agents. *Farmaco* 2001; 56: 427-431.
349. Guaratini T, Gianeti MD, Campos PM. Stability of cosmetic formulations containing esters of vitamins E and A: Chemical and physical aspects. *Int J Pharm* 2006; 327: 12-16.
350. Kim SH, Jung EY, Kang DH, Chang UJ, Hong Y-H, Suh HJ. Physical stability, antioxidative properties, and photoprotective effects of a functionalized formulation containing black garlic extract. *J Photochem Photobiol B* 2012; 117: 104-110.
351. Maia Campos PM, Gianeti MD, Camargo FB Jr, Gaspar LR. Application of tetra-isopalmitoyl ascorbic acid in cosmetic formulations: Stability studies and *in vivo* efficacy. *Eur J Pharm Biopharm* 2012; 82: 580-586.
352. Gilbert L, Savary G, Grisel M, Picard C. Predicting sensory texture properties of cosmetic emulsions by physical measurements. *Chemometr Intell Lab* 2013; 124: 21-31.
353. Lee I-S, Yang H-M, Kim J-W, Maeng Y-J, Lee C-W, Kang Y-S, et al. Terminology development and panel training for sensory evaluation of skin care products including aqua cream. *J Sens Stud* 2005; 20: 421-433.
354. AFSSAPS, Recommandations relatives a l'estimation de la periode apres ouverture (PAO), Département d'évaluation des produits cosmétiques, editor. Saint-Denis: Agence française de sécurité sanitaire des produits de santé (AFSSAPS); 2006.
355. EMA, ICH Q1A(R2) - Guideline on stability testing: stability testing of new drug substances and products, ICH Expert Working Group, editor. London: International Conference on Harmonisation (ICH); 2003.
356. Lévêque JL. EEMCO guidance for the assessment of skin topography. *J Eur Acad Dermatol* 1999; 12: 103-114.
357. Hoeksema H, De Vos M, Verbelen J, Pirayesh A, Monstrey S. Scar management by means of occlusion and hydration: A comparative study of silicones versus a hydrating gel-cream. *Burns* 2013; 39: 1437-1448.
358. Zhang HY, Ji J, Tan YM, Zhang LL, Wang XJ, Wang PR, et al. Evaluation of 5-aminolevulinic acid-mediated photorejuvenation of neck skin. *Photodiagnosis Photodyn Ther* 2014; 11: 498-509.
359. Fujioka N, Hibino S, Wakahara A, Kawagishi T, Taku K, Mizuno S, et al. Effects of various soap elements on skin. *J Anti Aging Med* 2009; 6: 109-118.
360. Hori M, Kishimoto S, Tezuka Y, Nishigori H, Nomoto K, Hamada U, et al. Double-blind study on effects of glucosyl ceramide in beet extract on skin elasticity and fibronectin production in human dermal fibroblasts. *J Anti Aging Med* 2009; 7: 129-142.
361. Wanitphakdeedecha R, Eimpunth S, Manuskiatti W. The effects of mucopolysaccharide polysulphate on hydration and elasticity of human skin. *Dermatol Res Pract* 2011; 807906.
362. Piana ML, Oddo LP, Bentabol A, Bruneau E, Bogdanov S, Declerck CG. Sensory analysis applied to honey: State of the art. *Apidologie* 2004; 35: S26-S37.
363. Ross CF. Sensory science at the human-machine interface. *Trends Food Sci Tech* 2009; 20: 63-72.
364. Parente ME, Gámbaro A, Ares G. Sensory characterization of emollients. *J Sens Stud* 2008; 23: 149-161.
365. Wagemaker TAL, Carvalho CRL, Maia NB, Baggio SR, Guerreiro Filho O. Sun protection factor, content and composition of lipid fraction of green coffee beans. *Ind Crop Prod* 2011; 33: 469-473.

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366. Kaczvinsky JR, Griffiths CEM, Schnicker MS, Li J. Efficacy of anti-aging products for periorbital wrinkles as measured by 3-D imaging. *J Cosmet Dermatol* 2009; 8: 228-233.
367. Hunt K, Hung S, Ernst E. Botanical extracts as anti-aging preparations for the skin. *Drug Aging* 2010; 27: 973-985.
368. Evonik. Hyacare Filler CL. Available from: <http://corporate.evonik.com/en/products/search-products/Pages/product-details.aspx?pid=34979&pfsearch=H&pfcmd=letter> [accessed on 21.10.2015].
369. Velazquez Pereda Mdel C, Dieamant Gde C, Eberlin S, Nogueira C, Colombi D, Di Stasi LC, et al. Effect of green *Coffea arabica* L. seed oil on extracellular matrix components and water-channel expression in in vitro and ex vivo human skin models. *J Cosmet Dermatol* 2009; 8: 56-62.
370. Balboa EM, Li YX, Ahn BN, Eom SH, Domínguez H, Jiménez C, et al. Photodamage attenuation effect by a tetraprenyltoluquinol chromane meroterpenoid isolated from *Sargassum muticum*. *J Photochem Photobiol B* 2015; 148: 51-58.
371. Berardesca E. EEMCO guidance for the assessment of *stratum corneum* hydration: electrical methods. *Skin Res Technol* 1997; 3: 126-132.
372. Goldman MP, Fitzpatrick RE. *Cutaneous Laser Surgery - the art and science of selective photothermolysis*. 2<sup>nd</sup> ed. St Louis: Mosby Inc;1999.
373. Marquele-Oliveira F, Fonseca YM, de Freitas O, and Fonseca MJV. Development of topical functionalized formulations added with propolis extract: Stability, cutaneous absorption and *in vivo* studies. *Int J Pharm* 2007; 342: 40-48.
374. Müller RH, Radtke M, Wissing SA. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. *Adv Drug Deliv Rev* 2002; 54: S131-S155.
375. Brugè F, Damiani E, Marcheggiani F, Offerta A, Puglia C, Tiano L. A comparative study on the possible cytotoxic effects of different nanostructured lipid carrier (NLC) compositions in human dermal fibroblasts. *Int J Pharm* 2015; 495: 879-885.
376. Wissing SA, Müller RH. The influence of solid lipid nanoparticles on skin hydration and viscoelasticity - *in vivo* study. *Eur J Pharm Biopharm* 2003; 56: 67-72.
377. Gaspar LR, Camargo FB Jr, Gianeti MD, Maia Campos PM. Evaluation of dermatological effects of cosmetic formulations containing *Saccharomyces cerevisiae* extract and vitamins. *Food Chem Toxicol* 2008; 46: 3493-3500.
378. Campos PM, Gonçalves GMS, Gaspar LR. *In vitro* antioxidant activity and *in vivo* efficacy of topical formulations containing vitamin C and its derivatives studied by non-invasive methods. *Skin Res Technol* 2008; 14: 376-380.
379. Trauer S, Patzelt A, Otberg N, Knorr F, Rozycki C, Balizs G, et al. Permeation of topically applied caffeine through human skin - a comparison of *in vivo* and *in vitro* data. *Br J Clin Pharmacol* 2009; 68: 181-186.
380. Shakeel F, Ramadan W. Transdermal delivery of anticancer drug caffeine from water-in-oil nanoemulsions. *Colloids Surf B Biointerfaces* 2010; 75: 356-362.
381. Velasco MV, Tano CT, Machado-Santelli GM, Consiglieri VO, Kaneko TM, Baby AR. Effects of caffeine and siloxanetriol alginate caffeine, as anticellulite agents, on fatty tissue: histological evaluation. *J Cosmet Dermatol* 2008; 7: 23-9.
382. Das S, Ng WK, Tan RBH. Are nanostructured lipid carriers (NLCs) better than solid lipid nanoparticles (SLNs): Development, characterizations and comparative evaluations of clotrimazole-loaded SLNs and NLCs?. *Eur J Pharm Sci* 2012; 47: 139-151.
383. Wang CL, Al-Suwayeh SA, and Fang JY. Nanostructured lipid carriers (NLCs) for drug delivery and targeting. *Recent Pat Nanotechnol* 2013; 7: 41-55.



- 
384. Mehnert W, Mäder K. Solid lipid nanoparticles: Production, characterization and applications. *Adv Drug Deliv Rev* 2011; 47: 165-196.
  385. Kohli AK, Alpar HO. Potential use of nanoparticles for transcutaneous vaccine delivery: effect of particle size and charge. *Int J Pharm* 2004; 275: 13-17.
  386. Gonzalez-Mira E, Egea MA, Garcia ML, Souto EB. Design and ocular tolerance of flurbiprofen loaded ultrasound-engineered NLC. *Colloids Surf B Biointerfaces* 2010; 81: 412-421.
  387. Friedrich RB, Dimer FA, Guterres SS, Beck RCR, Pohlmann AR. Nanoencapsulation of tacrolimus in lipid-core nanocapsules showed similar immunosuppressive activity after oral and intraperitoneal administrations. *J Biomed Nanotechnol* 2014; 10: 1599-1609.
  388. Venturini CG, Jäger E, Oliveira CP, Bernardi A, Battastini AMO, Guterres SS, et al. Formulation of lipid core nanocapsules. *Colloids Surf A Physicochem Eng Asp* 2011; 375: 200-208.
  389. Honary S, Zahir F. , Effect of zeta potential on the properties of nano-drug delivery systems - A review (Part 1), *Trop J Pharm Res* 2013; 12: 255-264.
  390. Mitri K, Shegokar R, Gohla S, Anselmi C, Müller RH. , Lipid nanocarriers for dermal delivery of lutein: Preparation, characterization, stability and performance. *Int J Pharm* 2011; 414: 267-275.
  391. Souto EB, Müller RH. Cosmetic features and applications of lipid nanoparticles (SLN<sup>®</sup>, NLC<sup>®</sup>). *Int J Cosmet Sci* 2008; 30: 157-165.
  392. Lee CK, Uchida T, Noguchi E, Kim NS, Goto S. Skin permeation enhancement of tegafur by ethanol/panasate 800 or ethanol/water binary vehicle and combined effect of fatty acids and fatty alcohols. *J Pharm Sci* 1993; 82: 1155-1159.
  393. Horita D, Todo H, Sugibayashi K. Effect of ethanol pretreatment on skin permeation of drugs. *Biol Pharm Bull* 2012; 35: 1343-1348.
  394. Rao J, Gold MH, Goldman MP. A two-center, double-blinded, randomized trial testing the tolerability and efficacy of a novel therapeutic agent for cellulite reduction. *J Cosmet Dermatol* 2005; 4: 93-102.
  395. Pierard GE. Commentary on cellulite: skin mechanobiology and the waist-to-hip ratio. *J Cosmet Dermatol* 2005; 4: 151-152.
  396. Rawlings AV. Cellulite and its treatment. *Int J Cosmet Sci* 2006; 28: 175-190.

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