Universidade do Minho
Escola de Ciências

Ana Rita Leite Araújo

Plant extracts: isolation, characterization, nanoencapsulation in lipids, cytotoxicity in AGS cells and synthesis of analogues

Outubro 2018

Plant extracts: isolation, characterization, nanoencapsulation in lipids, cytotoxicity in AGS cells and synthesis of analogues

Ana Rita Leite Araújo

UMinho | 2018

Universidade do Minho
Escola de Ciências

Ana Rita Leite Araújo

Plant extracts: isolation, characterization, nanoencapsulation in lipids, cytotoxicity in AGS cells and synthesis of analogues

Tese de Mestrado

Mestrado em Química Medicinal

Trabalho efetuado sob a orientação de

Professor Doutor António Gil Fortes

Professora Doutora Maria do Sameiro Torres Gonçalves

Outubro de 2018

DECLARATION

Name: Ana Rita Leite Araújo

E-mail: pg31364@alunos.uminho.pt

Telephone: +351 919136183

ID Number: 14172473

Title of Master's Thesis:

Plant extracts: isolation, characterization, nanoencapsulation in lipids, cytotoxity in AGS cells and synthesis of analogues

Supervisors:

Professor Doutor António Gil Fortes

Professora Doutora Maria do Sameiro Torres Gonçalves

Reception Institutions:

University of Minho (Department of Chemistry)

Conclusion year: 2018

Master's Degree:

Master's in Medicinal Chemistry

Minho University, 31/10/2018

Assinatura: Rita Araújo

ACKNOWLEDGEMENTS

In concluding this project, I cannot fail to thank some people who, directly or indirectly, were essential and helped me at such an important stage as this. Therefore, I send special thanks to my project supervisors, Professor Dr. Maria Sameiro Gonçalves and Professor Dr. António Gil Fortes for the patience, encouragement and wise advice that guided me in the preparation of this work both in the experimental component and in the structuring of the project. I would also like to thank Professor Dr. Elisabete Coutinho and Professor Dr. David Pereira for the availability, support and sympathy.

I would also like to thank the entire Department of Chemistry/Chemistry Centre of the University of Minho, which provided me with all the means for such experiments, and also the Laboratory of Pharmacognosy of the Department of Chemical Sciences of the Faculty of Pharmacy/REQUIMTE of the University of Porto and the Department of Physics/Physics Centre of the University of Minho. Thanks are also due to *Fundação para a Ciência e Tecnologia* (FCT-Portugal) and FEDER through the project PTDC/ASP-AGR/30154/2017.

To Dr. Elisa Pinto and Dr. Vânia Azevedo for the professionalism shown in the realization of the Nuclear Magnetic Resonance spectra.

To my parents and sister, for all the patience, psychological and monetary support. For the motivation they have always given me to overcome all the obstacles that appeared and for always believing in my abilities. The immense gratitude I feel I can never convey in words.

To my laboratory colleagues, Rui Sousa and Mariana Monteiro. Mentioning a special thanks to my partner in this adventure, who accompanied all my work almost as if it were hers, for all the laughter even in the most inopportune moments. Thank you Mariana.

And finally, to all my friends Joana Parente, Diogo Lopes, David Freitas, Marta Ferreira, Vânia Sousa, Salete Teixeira, Margarida Fontaínhas and João Bans for being with me through all this journey and for being the best of the best.

ABSTRACT

There is growing interest in plants as a potential source of bioactive molecules with application in different areas, from cosmetics to pharmaceuticals, as well as in the food industry. It is possible to extract phytochemicals by different extraction techniques, as well as to isolate and determine their composition using characterization techniques.

Despite their high interest, many of the phytochemicals have some limitations on their potential use due to high volatility and/or easy degradation when exposed to air. In this perspective, nanoencapsulation of these compounds allows their preservation and controlled release in the body are currently of interest.

In this work three different species, *Ginkgo biloba*, *Foeniculum Vulgare* and *Chelidonia majus*. were subjected to Soxhlet extraction and the extracts obtained were purified and characterized. Ginkgolic acid, estragole and coptisine have been identified in *Ginkgo Biloba*, *F. Vulgare* and *Chelidonia majus*, respectively. In order to obtain derivatives of the isolated compounds reactivity studies were performed using ginkgolic acid and estragole. Methylated ginkgolic acid and dioxinone, as well as epoxide derivatives were obtained from ginkgolic acid and estragole, respectively. Characterization of the synthesized compounds was carried out using NMR (^{1}H and ^{13}C), and IR spectroscopies, HPLC-DAD and mass spectrometry.

Extracts from *F. vulgare* and *C. majus* as well as estragole and coptisine were subjected to encapsulation assays using soybean lecithin by ethanolic injection method.

Furthermore biological activity studies have been performed to assess the toxicity of the extracts/compounds obtained using AGS cell line through MTT and LDH assays.

KEY WORDS: PLANTS EXTRACT, SYNTHESIS, BIOLOGICAL ACTIVITY, NANOENCAPSULATION

RESUMO

É cada vez maior o interesse nas plantas como potencial fonte de moléculas bioativas com aplicação em diferentes áreas, desde a cosmética aos fármacos, bem como na indústria alimentar. É possível extrair fitoquímicos por diferentes técnicas de extração, bem como isolar e determinar a sua composição recorrendo a técnicas de caracterização.

Apesar do seu elevado interesse, muitos dos fitoquímicos apresentam algumas limitações quanto ao seu potencial, devido à elevada volatilidade, assim como à fácil degradação quando expostos ao ar. Nesta perspetiva, técnicas de nanoencapsulamento deste tipo de compostos que permitam a sua preservação e libertação controlada no organismo são atualmente alvo de interesse.

Neste trabalho, três espécies diferentes, *Ginkgo biloba*, *Foeniculum vulgare* e *Chelidonia majus* foram submetidas à extração por Soxhlet e os extratos obtidos foram purificados e caracterizados. O ácido ginkgólico, o estragole e a coptisina foram identificados nos extratos de *Ginkgo Biloba*, *F. vulgare* e *Chelidonia majus*, respetivamente. De modo a obter derivados dos compostos isolados, realizaram-se estudos de reatividade utilizando o ácido ginkgolico e o estragole. O ácido ginkgólico metilado e a dioxinona, assim como o epóxido foram obtidos a partir do ácido ginkgólico e do estragole, respectivamente. A caracterização dos compostos sintetizados foi realizada utilizando as espectroscopias de RMN (^{1}H e ^{13}C), IV, HPLC-DAD e espectrometria de massa.

Extratos de *F. vulgare* e *C. majus*, bem como estragol e coptisina, foram submetidos a ensaios de encapsulamento utilizando lecitina de soja por injeção etanólica.

Foram ainda realizados estudos de atividade biológica para avaliar a toxicidade dos extratos/compostos obtidos utilizando a linha celular AGS através de ensaios de MTT e LDH.

PALAVRAS CHAVE: EXTRACTOS VEGETAIS, SÍNTESE, ATIVIDADE BIOLÓGICA, NANOENCAPSULAMENTO

INDEX

FIGURES LIST

SCHEMES LIST

TABLES LIST

ABREVIATION LIST

δ	chemical shift
η	yield
λ	wavelength
ε	molar absorption coefficient
ν_{max}	maximum wavenumber (expressed in cm^{-1})
Abs	absorption
ACN	acetonitrile
AGS	gastric adenocarcinoma cells
d	doublet
DCC	*N*,*N*'-dicyclohexylcarbodiimide
DCM	dichloromethane
dd	doublet of doublets
DMAP	4-dimethylaminopyridine
DME	dimethoxyethane
DMEM	Dulbecco's modification of Eagle medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
EOs	essencial oils
eq.	equivalent
ESI	electrospray ionization
et al.	*et alia* (from Latin, reference to other people)
EtOH	etanol
FBS	fetal bovine serum
g	grams
HBSS	Hank's Balanced Salt Solution
HRMS	High resolution mass spectrometry

HOBt	hydroxybenzotriazole
Hz	Hertz
IC_{50}	half maximal inhibitory concentration
J	coupling constant (Hertz)
LPs	liposomes
m	multiplet
***m*-CPBA**	*meta*-chloroperoxybenzoic acid
MAP	microwave-assisted process
MeOH	methanol
NMR	nuclear magnetic resonance nuclear magnetic resonance
ppm	parts per million
s	singlet
sl	large singlet
SNL	solid lipid nanoparticle
t	triplet
TBAI	tetrabutylammonium iodide
TLC	thin layer chromatography
UV	ultraviolet
vis	visible

COMMUNICATION

Araújo, R. L.; Castanheira, E. M. S., Gil Fortes, A.; Gonçalves, M. S. T.. "Plant extracts: isolation, characterization and nanoencapsulation", poster communication (P2), *V* Encontro em Técnicas de Caracterização e Análise Química, Braga, Portugal, 4th may 2018

CHAPTER 1: INTRODUCTION

1.1. PLANTS AS SOURCE OF PHYTOCHEMICALS

Plants are an inexhaustible source of natural products, many of them secondary metabolites, which humans use in a wide range of areas, including the food, cosmetics and perfumery industries. The group of compounds of secondary metabolism includes various pharmacologically active substances regularly employed as pharmaceuticals. Many of these natural substances are extracted from plants in the form of relatively complex extracts.

The metabolites found in plants can be divided into different classes of compounds: phenolics, alkaloids and terpenoids.

1.1.1. Phenolic compounds

Phenolic compounds are one of the most widely occurring groups of phytochemicals, being of considerable physiological and morphological importance in plants. These type of compounds play an important role in growth and reproduction, providing protection against pathogens and predators[1], in addition to their contribution towards the color and sensory characteristics of fruits and vegetables[2].

Structurally, phenolic compounds comprise an aromatic ring, bearing one or more hydroxyl substituents, and range from simple phenolic molecules to highly polymerised compounds.[3]

This class is divided into subclasses such as flavonoids, phenolic acids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and others based on the number of phenolic rings and structural elements that link these rings.[4]

The classes listed below are the predominant ones in the composition of plant species, and some of them are-studied in this work.

1.1.1.1. Flavonoids

Flavonoids lie in a group that occurs naturally in plants and there are more than 4,000 phenolic compounds described.[5] These compounds usually have the basic skeleton of phenylbenzopyrone structure (C_6-C_3-C_6) **1** consisting of two aromatic rings (A- and B- rings) linked by three carbons that are usually in an oxygenated central pyran ring, C - ring (Figure 1).[6] According to the saturation level and opening of the central pyran ring, they are categorized mainly into flavones (basic structure, B ring binds to the 2 - position) **2**, flavonols (having a hydroxyl group at the 3 - position) **3**, flavanones (dihydroflavones) **4** and flavanonols (dihydroflavonols; 2 - 3 bond is saturated) **5**, flavanols (flavan-3-ols **6** and flavan-3,4-diols; C-ring is 1-pyran), anthocyanins (anthocyanidins **7**; C-ring is 1-pyran, and 1–2 and 3–4 bonds are unsaturated).[7] (Fig. 1).

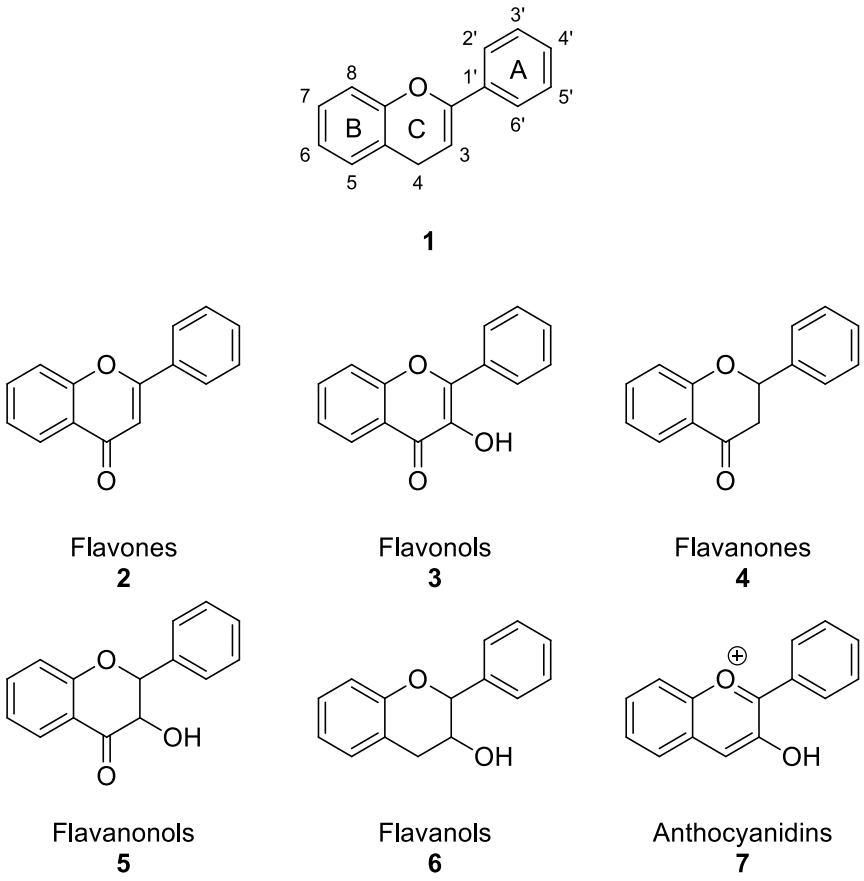
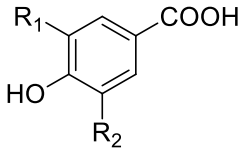


Figure 1. Structures of major groups/subgroups of flavonoids.

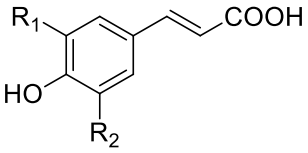
Since they are known to be synthesized by plants in response to microbial infection[8], it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and also with bacterial cell walls, as described for quinones. More lipophilic flavonoids may also disrupt microbial membranes.[9]

1.1.1.2. Phenolic Acids

Phenolic acids are a major class of phenolic compounds, widely occurring in the plant kingdom. As shown in Figure 2, predominant phenolic acids include hydroxybenzoic acids (e.g., *p*-hydroxybenzoic acid **8**, protocatechuic acid **9**, gallic acid **10**, vanillic acid **11**, and syringic acid **12**) and hydroxycinnamic acids (e.g., *p*-coumaric acid **13**, caffeic acid **14**, ferulic acid **15** and sinapic acid **16**).[10]



8 - 12



13 - 16

R_1 = H, R_2 = H: *p*-hydroxybenzoic acid **8**
R_1 = H, R_2 = OH: protocatechuic acid **9**
R_1 = OH, R_2 = OH: gallic acid **10**
R_1 = OCH_3, R_2 = H: vanillic acid **11**
R_1 = OCH_3, R_2 = OCH_3: syringic acid **12**

R_1 = H, R_2 = H: *p*-coumaric acid **13**
R_1 = H, R_2 = OH: caffeic acid **14**
R_1 = H, R_2 = OCH_3: ferulic acid **15**
R_1 = OCH_3, R_2 = OCH_3: sinapic acid **16**

Figure 2. Chemical structures of common phenolic acids.

Phenolic acids are highlighted as a subclass of phenolic compounds and they are one of the classes present in the species treated in this study.

1.1.1.3. Tannins

Tannins are natural, water-soluble, polyphenolic compounds with molecular weight ranging from 500 to 4,000, usually classified into two classes: hydrolysable tannins (gallo-**17**and ellagi-tannins **18**) and condensed tannins (proanthocyanidins **19**), Figure 3.[11]

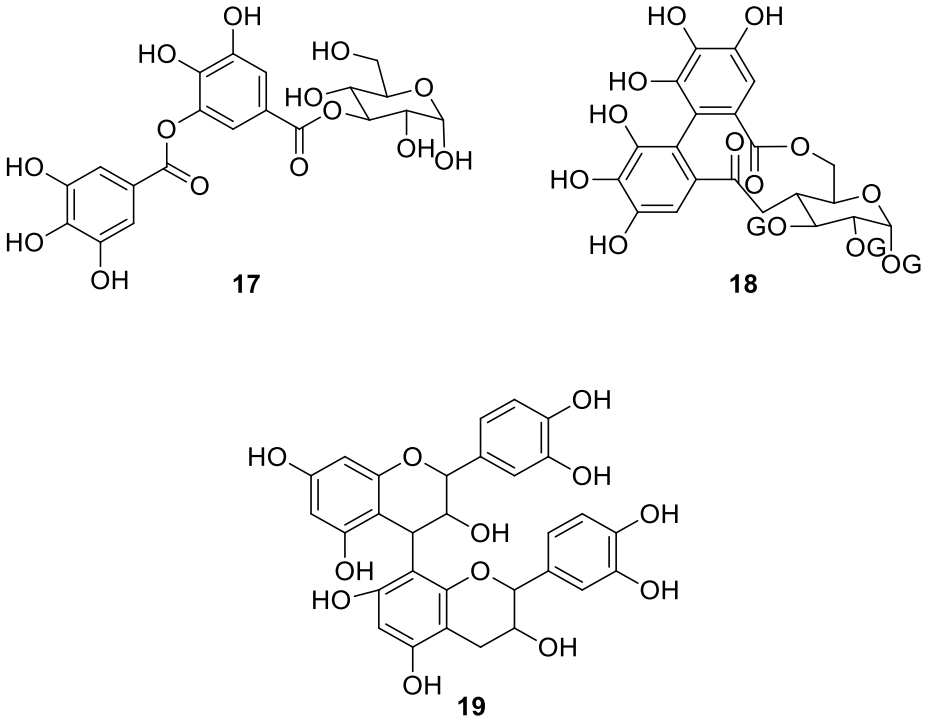


Figure 3. The basic skeletons and structures of hydrolysable **17** (di-*O*-galloyl-β-D-glucose, gallotannins), **18** (tellimagrandin II, ellagitannins) and condensed **19** (Procyanidin B_2) tannins.

Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumor activity, and a wide range of anti-infective actions have been assigned to tannins.[12]

1.1.2. Alkaloids

Alkaloids are amongst the isolated pure compounds from plants, a diverse class of nitrogen containing heterocyclic compounds with alkali-like chemical reactivity and pharmacological activity. Many alkaloids are toxic and consequently are used by plants to protect themselves against the aggression from other organisms, having this instinct action an important ecological function.[13]

The alkaloids can be divided into different subclasses such as isoquinoline **20**, indole **21**, amide **22**, quinolone **23**, purine **24**, monoterpene **25** and pyrrolidine **26** alkaloids (Figure 4).[14]

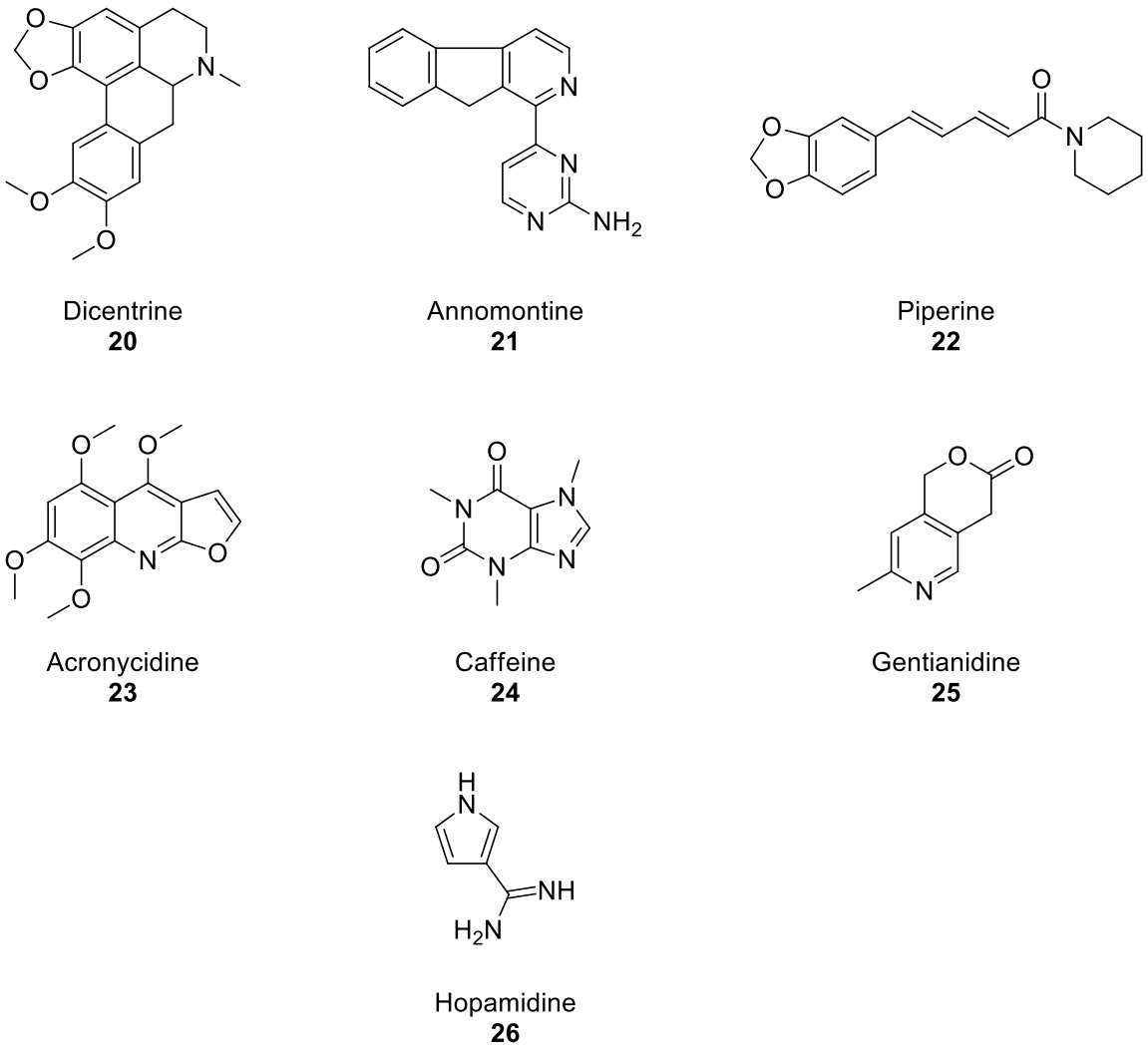


Figure 4. Structure of isoquinoline **20**, indole **21**, amide **22**, quinoline **23**, purine **24**, monoterpene **25** and pyrrolidine **26** alkaloids.

1.1.3. Terpenoids

Terpenes, also referred to as terpenoids or isoprenoids, constitute the largest class of natural products with more than 55,000 known compounds structurally diversified.[15] These chemicals are part of the secondary metabolism of vegetal and animal species and are derived from C5 isoprene, whose molecular formula is C_5H_8. Typical structures contain carbon skeletons represented by (C5)*n* and are classified as hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterpenes (C25), triterpenes (C30) and tetraterpenes (C40) (Figure 5).[16] A terpene containing oxygen is called a terpenoid.

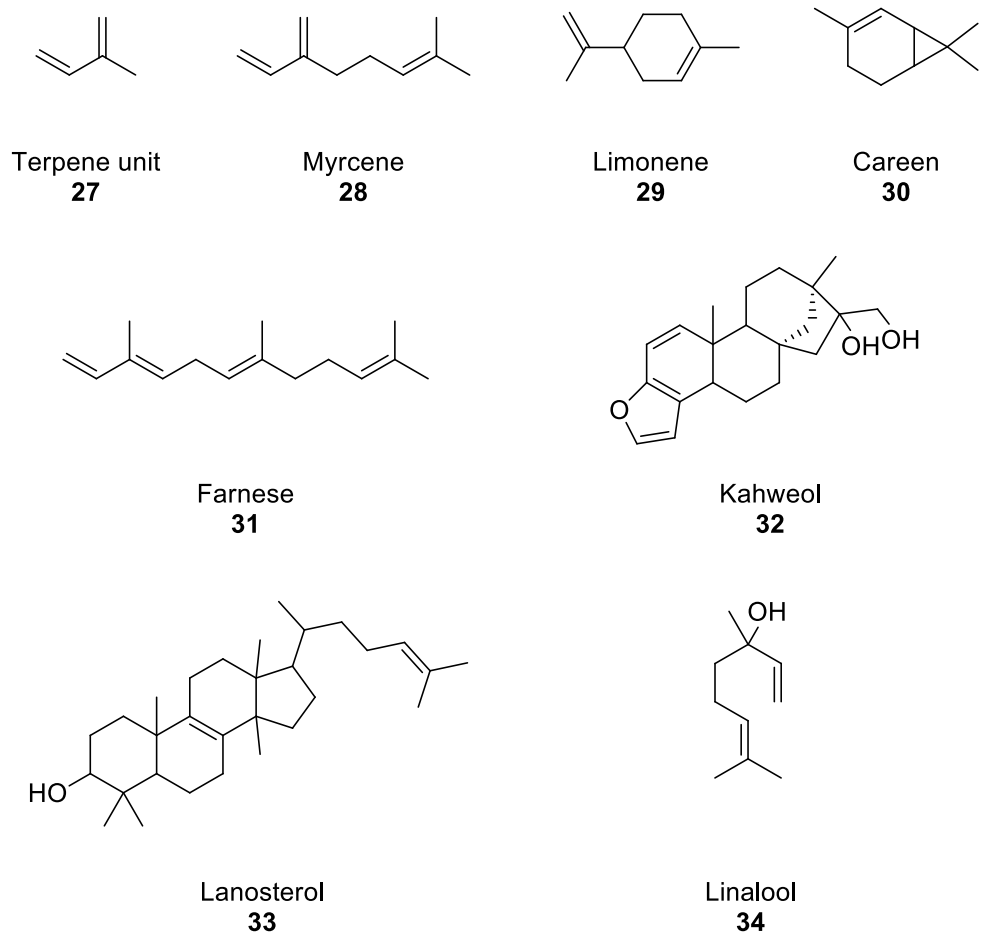


Figure 5. Structures of terpenes and terpenoids: isoprene (terpene unit **27**), acyclic monoterpenes (myrcene **28**), cyclic monoterpenes (limonene **29**), bicyclic monoterpenes (careen **30**), sesquiterpenes (farnesene **31**), diterpenes (kahweol **32**), triterpenes (lanosterol **33**), terpenoid (linalool **34**).

Growing interest in the clinical application of these compounds is assigned to their broad range of the biological properties that have been described, including cancer chemopreventive effects,[17] antimicrobial, antifungal,[18] antiviral,[19] antihyperglycemic,[20] analgesic, anti-inflammatory[21] and antiparasitic activities.[22]

1.2. ESSENTIAL OILS

Essential oils (EOs), aroma or essence are the volatile odoriferous principles produced by plants and used since ancient times not only for their medicinal properties but also for their importance in the perfume and flavor industry.[23] These are essentially produced by glandular trichomes and are synthesized for the purpose of protecting plants from various pathogens.[24]

Essential oils are found in various parts of plants, such as leaves, roots, barks, seeds, fruits and stalks, among others. They provide the essence, odor or taste of the plant and can be extracted by using various techniques, namely soxhlet extraction, hydrodistillation and steam distillation. The chemical composition of the essential oils can be complex, due to the number of different components, which may have promising chemical and biological properties.[25] These natural mixtures may contain at about 20 to 60 components in very different concentrations. They are characterized by two or three major components in fairly high concentrations (20-70%) compared to other components present in trace amounts.[26]

Numerous literature publications on the composition of the various EOs, and their main components of economic interest are summarized by Bauer *et al.* 2001.[27] The detailed compositional analysis of EOs is obtained through gas chromatography (GC) and mass spectroscopy.[28]

Table 1 and Figure 6 show the major components of several EOs and some selected structures, respectively.

Table 1. Major components of selected EOs.[a]

Common name of EO	Latin name	Major components	Composition (%)[b]	References
Cilantro	*Coriandrum sativum*	Linalool *E*-2-decanal	26% 20%	Delaquis *et al.*, 2002[29]
Cinnamon	*Cinnamomum zeylandicum*	*t*-cinnamaldehyde	65%	Lens-Lisbonne *et al.*, 1987[30]
Oregano	*Origanum vulgare*	Carvacrol Thymol γ-Terpinene *p*-Cymene	Trace-80% Trace-64% 2-52% Trace-52%	Lawrence, 1984[31]; Daferera *et al.*, 2000[32]; Demetzos and Perdetzoglou, 2001[33]; Marino *et al.*, 1999[34]
Rosemary	*Rosmarinus officinalis*	α-Pinene Bornyl acetate Camphor 1,8-Cineole	2-25% 0-17% 2-14% 3-89%	Daferera *et al.*, 2000, 2003[35]; Pintore *et al.*, 2002[36]

[a] EOs which have been shown to exert antibacterial properties in vitro or in food models and for which the composition could be found in the literature.
[b] Percentages of total volatiles rounded up to the nearest whole number.

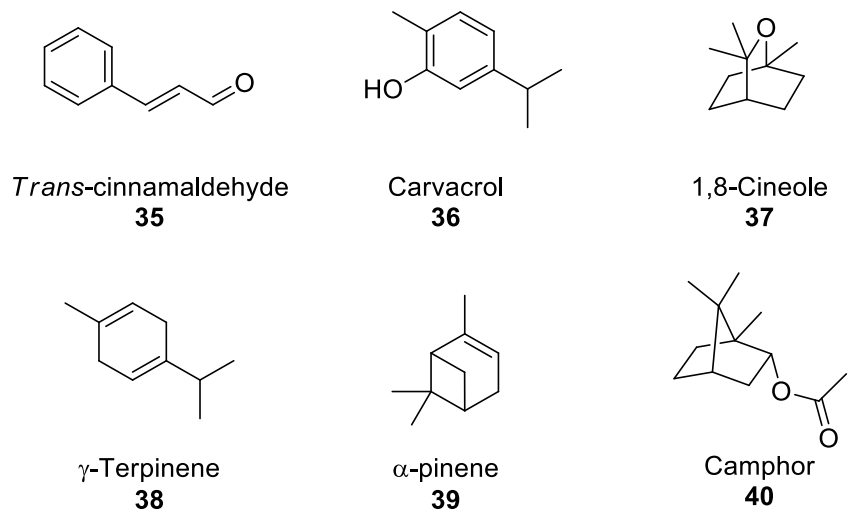


Figure 6. Chemical structures of some selected components of EOs, *trans*-cinnamaldehyde **35**, carvacrol **36**, 1,8-cineole **37**, γ-terpinene **38**, α-pinene **39** e camphor **40**.

Essential oils, which can be obtained by hydrodistillation or extraction with solvents, are usually liquids at room temperature, not miscible with water, but miscible with organic solvents. In general, EOs are a mixture of compounds with their own physicochemical characteristics which combined give the oil a particular odor. The different aroma of the oils is fundamentally due to the variations in volatility and the relative concentration of their constituents.[23].

The amount of EOs will depend on several factors, such as the organ where these compounds are produced, age, vegetative state, climate and soil composition. Oxidation, resinification, polymerization, ester hydrolysis and interaction of functional groups are some of the deterioration processes of the essential oils. These processes are activated by factors such as heat, oxygen, moisture and light.[37]

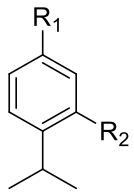
1.3. Selected Plants Species

1.3.1. *Ginkgo biloba L.*

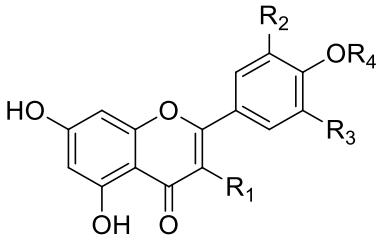
Ginkgo biloba L. (Figure 7) is one of the most widely used plants in the world in herbal medicines, dietary supplements or plant protection products. Although its natural habitat is China, Japan and Korea, it is believed to originate in the remote mountain valleys of eastern China's Zhejiang province.[38,39] This plant is the only surviving species of the *Ginkgoaceae* family being often referred to as a "living fossil".[40]

Figure 7. *Ginkgo biloba L*. tree and corresponding leaf.

Several classes of constituents have been identified in this species, and a number of secondary metabolites have been reported, including terpenoids, polyphenols, allyl phenols, organic acids, carbohydrates, fatty acids, lipids, amino acids and inorganic salts.[41] However, presenting as main constituents bioactive terpenes and flavonoids as shown in Figure 8. [42,43]



41 - 43



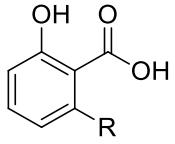
44 - 47

	R_1	R_2
p - Cymene **41**	CH_3	H
Isopropylphenol **42**	H	OH
Thymol **43**	CH_3	OH

	R_1	R_2	R_3	R_4
Kaempferol **44**	OH	H	H	H
Quercetin **45**	OH	OH	H	H
Mirecetin **46**	OH	OH	OH	H
Luteonin **47**	H	OH	H	H

Figure 8. Terpenes **41** - **43** and flavonoids **44** - **47** present in the extract of *G. biloba*.

Among the most controversial compounds present in *Ginkgo biloba* are ginkgolic acids (GA) due to their diversity of properties (Figure 9). Ginkgolic acids are natural derivatives of salicylic acid with long chain alkyl substituents present in the *ortho* position relative to the carboxylic group, which usually contains 13 to 17 carbon atoms and between 0 to 2 double bonds.[44] Due to the significant risk factors of ginkgolic acids, the content of these undesirable components in the standardized extracts of *G. biloba* is currently limited to <5 ppm in the European and North American pharmacopoeia.[45]



48

GA (13:0) R = $C_{13}H_{27}$
GA (15:0) R = $C_{15}H_{31}$
GA (15:1) R = $C_{15}H_{29}$
GA (17:1) R = $C_{17}H_{33}$
GA (17:2) R = $C_{17}H_{31}$

Figure 9. Structures of the most abundant ginkgolic acids.

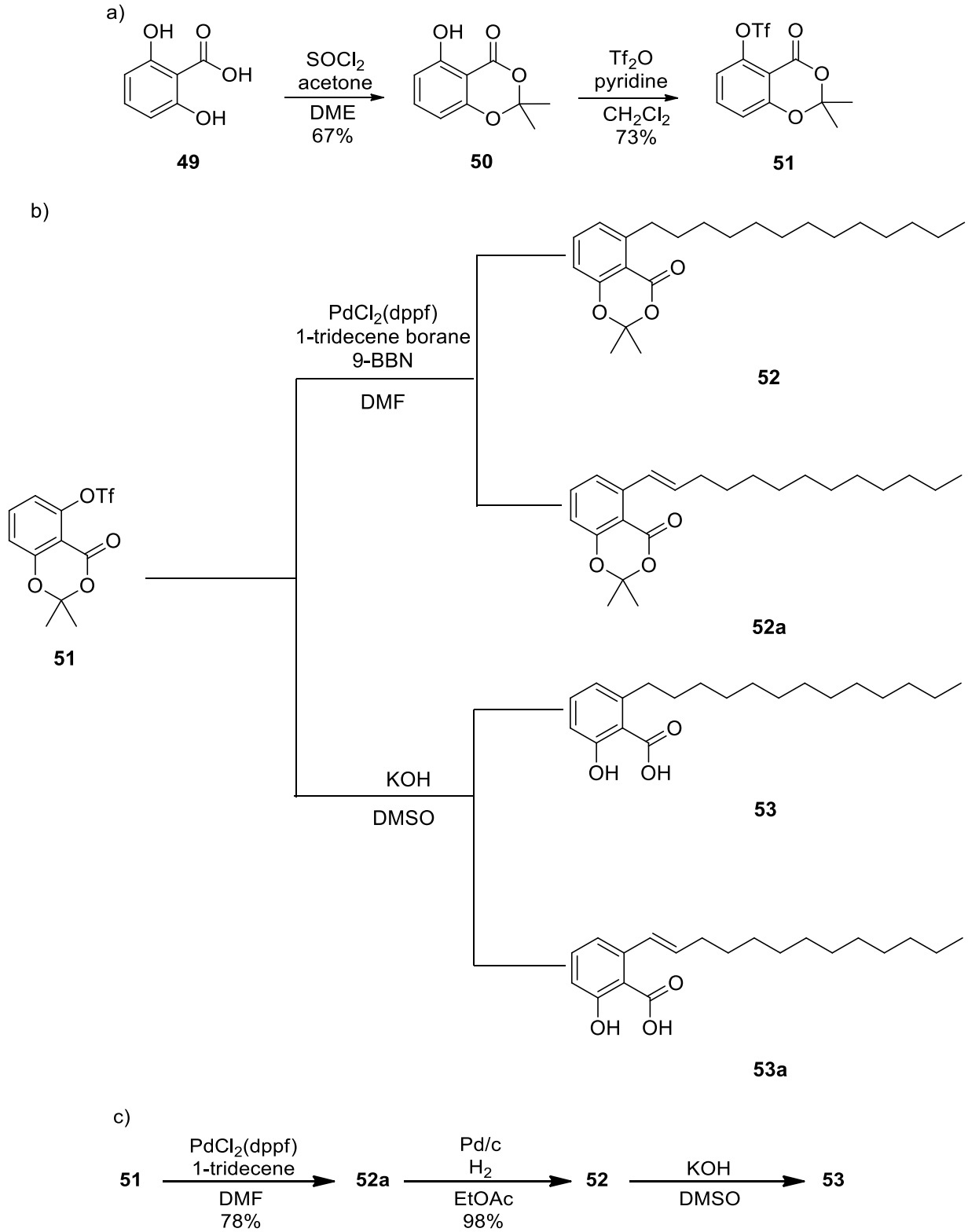
Thus, it is necessary to have a simple, sensitive and valid analytical method for the detection and quantification of each of the alkylphenols in commercial products, such as herbal medicines and dietary supplements. Numerous analytical techniques, including HPLC, GC, HPTLC, ELISA and NMR, have been proposed for the analysis of GAs. Comprehensive reviews of the chemical analysis (including alkylphenols) of *G. biloba* leaves, extracts and phytopharmaceuticals by van Beek criticized the various analytical approaches to GAs by 2009.[46,43] The main challenges for GAs analysis are (i) the low concentrations (<5 ppm) imposed by regulation for standardized extracts, (ii) the complex matrix of *G. biloba* plant samples and dietary supplements, (iii) the difficulties to obtain pure and authentic standards for identification and quantification purposes, and (iv) rigorous resolution requirements for the analysis of alkylphenol isomers. Often only the total concentration of all GAs in a given sample is determined. However, the exact role of the various isomeric GAs in the pharmacological effects of *G. biloba* have never been determined. Thus, any attempt to correlate a given pharmacological effect with a specific GA isomer will require an analytical method that allows the quantification of all GA isomers. Liquid chromatography is currently the analytical method of choice for GAs.[45]

Over the years the different activities of this class of GAs have been reported. Among the activities considered beneficial are antitumor, antidepressant, anti-HIV, anti-stress, antifungal, miticidal, insecticide and larvicidal.[47,48] However, GAs can trigger allergic contact, cytotoxic, embryotoxic, immunotoxic, mutagenic and neurotoxic properties.[49,50]

Despite the broad spectrum of biological activities of GAs, their natural availability is very limited. As a result, many research studies at the synthesis level have been carried out in order to develop an efficient strategy to obtain the GAs for subsequent studies of biological activity.[44] Tyman *et al.*[51,52] applied a sequential alkylation and carboxylation of 2-fluoroanisole with alkylithium and carbon dioxide, followed by demethylation with boron trichloride,

affording GA (15:0) in a low yield. Then, the strategy of chain elongation of 6-methylsalicylate was used and resulted in higher yield of GA (15:0). Fürstner *et al.* concisely prepared GA (15:0) using the Suzuki cross-coupling reaction and directly constructed the side chain.[53] Satoh *et al.* successfully synthesized GA (15:0)[54,55] and GA (15:2)[56] by annelation reaction of isoxazoles with ethyl acetoacetate and a Wittig reaction.

Yuanqing Fu *et al.*,[44] after all previous findings, proceeded to GA synthesis (13:0) by two different synthetic pathways. A novel synthesis was successfully developed from 2,6-dihydroxybenzoic acid involving a palladium catalyzed cross coupling reaction and catalytic hydrogenation in a total yield of 34% in five steps (Scheme 1).

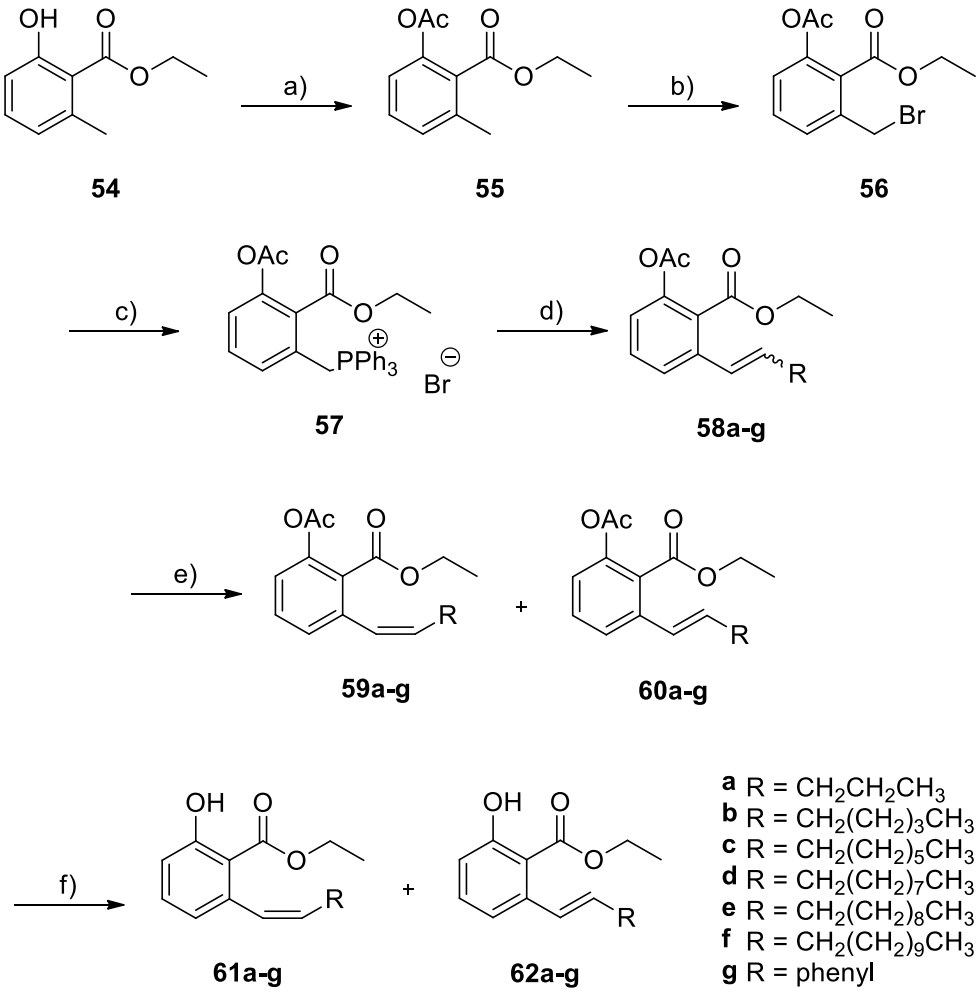


Scheme 1. (a) Synthesis of trifluoromethanesulfonic acid 2,2-dimethyl-4-oxo-4*H*-1,3-benzodioxin-5-yl ester **51**. (b) Synthesis of GA (13: 0) **53** with 9-BBN. (c) Synthesis of GA (13: 0) without 9-BBN.

The preparation of compound **50** started with the ketalization of 2,6-dihydroxybenzoic acid **49** according to a procedure described by Hadfield *et al*.[57] The remaining hydroxyl group of compound **50** was readily converted to triflate **51** as a colorless solid by treatment with trifluoromethanesulfonic anhydride and pyridine in anhydrous dichloromethane following the procedure set by Uchiyama *et al*. [58] GA (13:0) was obtained by two different routes, one involving 9-borabicyclo (3.3.1) nonane (9-BBN) and another without using 9-BBN. In the synthesis involving the 9-BBN a Suzuki cross-coupling reaction was attempted according to the Fürstner method.[53] 1-Tridecene borane was hydroborated with 9-BBN and 1-tridecane borane was generated *in situ*. The resulting alkylborane was treated with potassium carbonate to form the activated boron complex which then participated in the palladium catalyzed Suzuki coupling reaction as the nucleophile and coupled to triflate **51**. The reaction in absence of 9-BBN involved the direct coupling of 1-tridecene and triflate, followed by catalytic hydrogenation. As expected, coupling of 1-tridecene and triflate proceeded gently to give compound **52a**, in the presence of potassium carbonate and catalytic palladium chloride (dppf) under nitrogen in anhydrous DMF (Scheme 1 c). The reaction was completed in 12h.

A second method was achieved by Zhang *et al.* in order to understand the structure-activity relationship for these compounds and to improve their molluscicidal activity. In their study a series of *Z*/*E* isomers of GA analogues with different chain lengths (5 ≈ 13 carbon atoms) and phenyl rings based on one double bond have been synthesized from 6-methylsalicylic acid.[59]

The unhydrolyzed mixtures of *Z* and *E* isomers **58a**-**g** were synthesized from ethyl 6-methylsalicylate according to the reaction sequence delineated in Scheme 2. These were separated by column chromatography on silica gel. Target compounds **61a**-**g**, **62a**-**g** were obtained by ester hydrolysis and extracted from acidic solution with chloroform.



Scheme 2. Synthesis of GA analogues from 6-methylsalicylate. a) $(CH_3CO)_2O$, H_2SO_4, 15 min, 95%; b) CCl_4, NBS, AIBN, 90%); c) Ph_3P, 0 to 130ºC, $CHCl_3$, $(CH_3CH_2)_2O$, 98%; d) R-CHO, Et_3N, DMSO, 125ºC, 21-26 h, 46.4 ≈ 80%, e) separated by silica gel column chromatography with gradient mixtures of n-hexane-acetone (30:1); f) EtOH, NaOH. Reflux, 5 ≈ 8h,83 ≈ 96%.

Concerning the importance of *Ginkgo biloba,* it displays a number of diverse biological activities, some of them presented in the following table (Table 2).

Table 2. Biological activities of *Ginkgo biloba* extract.

Biological activity	Reference
Anti-Alzheimer Effective on presenile and senile primary degenerative dementia of the Alzheimer type and multi-infarct dementia according to DSM-III-R.	Kanowski *et al.*[60]
Efficacy in cerebral insufficiency Reduce corticosteroid production, improve cerebral blood flow, increase glucose uptake and utilization, ATP production, mitochondrial metabolism, and intra and extra-cellular ionic gradients	Kleijnen *el at.*[61]
Increase of blood flow Improve blood flow by increasing red blood cell deformability and decreasing red cell aggregation	Dubey *et al.*[62]
Antagonism and Anti-inflammatory Effect Possess very specific and potent antagonist activity against platelets aggregation factor, which may result in increased peripheral blood flow	Braquet *et al.*[63]
Anxiolytic activity. Ginkgolic acid exhibited anxiolytic activity	Satyan *et al.*[64]
Antioxidant Topically applied Ginkgo biloba leave extract induced superoxide dismutase and catalase enzyme activities locally in the epidermis and systemically in the liver, heart, and kidney of Sprague-Dawley rats	Lin *et al.*[65]

1.3.2. *Foeniculum vulgare Mill.*

Foeniculum vulgare Mill., commonly known as fennel, is a medicinal and aromatic plant belonging to the family *Apiaceae* (*Umbelliferaceae*). It is a tough herb with yellow flowers and feathery leaves (figure 10).[66]

Figure 10. Flowers and leave of F. vulgare.

F. vulgare is well known for its essential oil, which is responsible for its aniseed characteristic odor, excellent as flavoring agent in bakery products, food industry, ice cream and alcoholic beverages.[67] The major components of this essential oil have been reported as *trans*-anethole, fenchone, limonene and estragole whose molecular structures are represented in Figure 11 and the remaining constituents are shown in Table 3.[68]

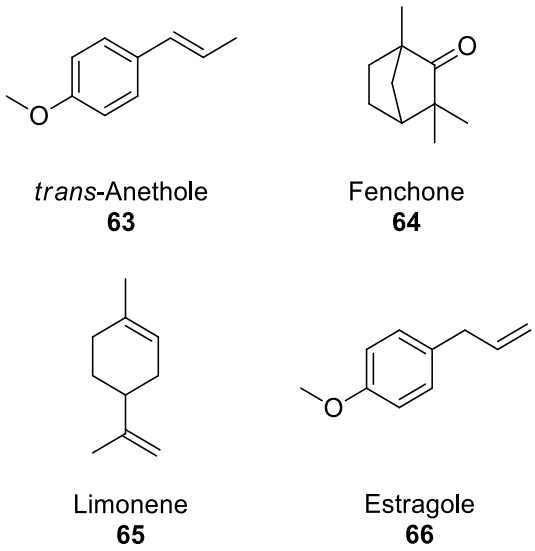


Figure 11. Molecular structures of the main components of essential oil of *Foeniculum vulgare.*

Table 3. Extraction time and composition of essential oil of *Foeniculum* vulgare seed (5 kg), collected from cultivated plants in Sétif region (Eastern Algeria, 1096 m above sea level) during May 2007, through a hydrodistillation in a Clevenger - type apparatus.

Compound	**Composition (%)**				
Extraction time (h)	2	3	4	6	8
α-Pinene	0.59	0.78	0.82	0.89	0.93
Camphene	0.09	0.12	0.13	0.14	0.14
β-Phellandrene	0.23	0.27	0.27	0.28	0.28
β-Myrcene	0.39	0.43	0.44	0.46	0.45
α-Phellandrene	0.08	0.09	0.09	0.10	0.09
1-methyl-4-(1-methylethyl)benzene	0.07	0.09	0.08	0.08	0.09
Limonene	5.82	6.19	6.12	6.43	6.68
3,7-dimethyl-(*E*)-1,3,6-octatriene	0.37	0.38	0.39	0.41	0.41
3-Carene	0.10	0.11	0.11	0.12	0.12
Fenchone	11.13	11.35	11.04	10.04	10.29
Camphor	0.20	0.21	0.21	0.18	0.18
Estragole	4.75	4.95	4.93	4.58	4.93
Fenchyl acetate	0.12	0.11	0.11	0.13	0.12
trans-Anethol	76.00	74.86	75.16	75.97	75.11
Apiol	0.03	-	-	0.04	0.03
n-Hexadecanoic acid	-	0.04	0.06	0.09	0.12

The relative concentration of these compounds varies considerably depending on the morphological status and origin of the fennel.[69] The composition of the essential oil of *F. vulgare* exhibits considerable chemical diversity depending on the method of extraction and geographical origin. The accumulation of these volatile compounds within the plant is variable, appearing practically in any of its parts such as roots, stem, shoots, flowers and fruits.[70]

This herb is traditionally used as a treatment for colic, irritable bowel, kidneys, spleen, liver, lungs, suppress appetite, increase breasts, promote menstruation, improve digestive system, increase urine and milk flow.[71] Several tests have shown that extracts of the fennel

fruit obtained with organic solvents, as well as its essential oil are beneficial to humans due to the various phytochemicals that can cure different diseases.[72] Essential oil of this herbal plant was found to have different medicinal and antioxidant properties against some diseases. It has been shown that can be used as an antimicrobial agent against other species display an effect against *Candida albicans* and some other bacterial infections. Different pharmacological experiments *in vitro* and *in vivo* models have convincingly demonstrated the ability of the fennel to exhibit antifungal, antibacterial and antioxidant properties. [73,74]

1.3.3. *Chelidonium majus L.*

The greater celandine, *Chelidonium majus L.* of the family of *Papaveraceae,* which is widely distributed in Europe and Asia is a plant of great interest due to its use in the treatment of various diseases in European countries and in Chinese herbal medicine (Figure 12).[75]

Figure 12. Leaves and flower of *C. majus.*

Its main constituents include isoquinoleic alkaloids, benzophenanthridine type (chelidonin **67**, sanguinarine **68**, chelerythrine **69**, berberine **70**, coptisine **71**, didehydrochelidonine **72** and nitidine **73**) from 0.3 to 1%; organic acids (malic **74**, chelidonic **75** and citric **76** acids), flavonoids (rutin **77** and quercetin **78**), tectin, saponin and carotenoids (Figure 13).[76]

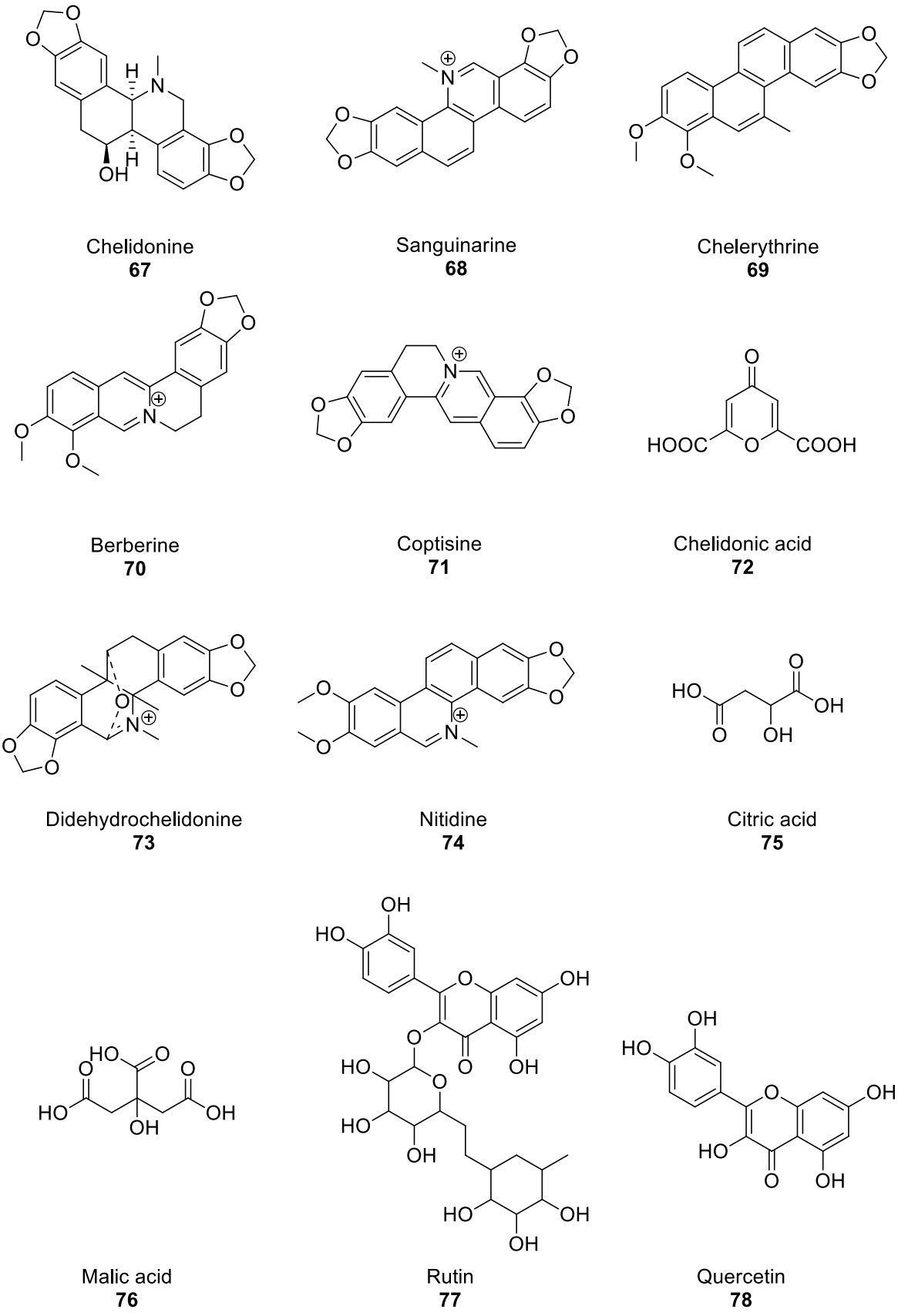


Figure 13. Selected molecular structures of the main components of *C. majus*.

In Chinese herbal medicine, the *C. majus* is used to treat whooping cough, blood stasis, chronic bronchitis, asthma, jaundice, gallstones and gallbladder pains as well as to promote diuresis in edema and ascites.[77,78] In homeopathic medicine, ultra-high dilutions (potencies) of *C. majus* extract are reputedly used against different forms of liver disorders including liver cancer.[79]

Table 4 shows biological activities of *C. majus*, as well as the constituent responsible.

The alkaloids present in *C. majus* have been thoroughly studied and their potential application as anticancer agents has been reported. [80,81] The antiproliferative effects of *C. majus* were evaluated *in vitro* in human keratinocyte cell lines of rapid multiplication, resulting in IC_{50} of 1.9 µg/mL for dry extract containing 0.68% of alkaloids expressed in chelidonine. Sanguinarine, chelerythrine and chelidonine showed IC_{50} values of 0.2, 3.2 and 3.3 µM, respectively, whereas berberine presented only low potency with an IC_{50} of 30 µM. The lactate dehydrogenase assay showed cytostatic activity rather than cytotoxic activity.[82] Most *in vitro* studies suggest that sanguinarine, chelidonine, cheleritrine and berberine are responsible for the antitumor effect of *C. majus* extract. The strongest antitumor agent found of the foregoing compounds was the sanguinarine, which strongly intercalates with the DNA. Chelidonine, chelerythrine and berberine are also active but less potent than sanguinarine.[83]

Table 4. Biological activities of some greater constituents present in *C. majus.*

Constituents	Biological activity	References
Chelidonine **67**	Anticancer	El-Ready *et al.*[84]
	Hepatopretective	Paul *et al.* [85]
	Anti-alzheimer	Cahlíková *et al.*[86]
Chelerythrine **69**	Anti-inflammatory	Li *et al.*[87]
	Anti-alzheimer	Brunhofer *et al.*[88]
	Anticariogenic	Cheng *et al.*[89]
	Nephroprotective	Parlakpinar *et al.*[90]
	Antimycobacterial	Liang *et al.*[91]
	Gastroprotective	Li *et al.*[92]
Dihydrochelerythrine **75**	Antifungal	Feng *et al.*[93]
	Anticancer	Vrba *et al*.[94]
Sanguinarine **68**	Antibacterial	Beuria *et al.*[95]
	Anticancer	Vrba *et al.*[96]
	Anti-inflammatory	Niu *et al*.[97]
	Antiplatelet	Jeng *et al*.[98]
	Anti-alzheimer	Kuznetsova *et al*.[99]
Berberine **70**	Antimicrobial	Schmeller *et al*.[100]
	Anticancer	Hattori *et al*.[101]
	Antiangiogenic	Hamsa *et al*.[102]
	Hepatoprotective	Domitrovic *et al*.[103]
	Antiviral	Hayashi *et al*.[104]
	Anti-alzheimer	Kuznetsova *et al*.[105]
	Anti-inflammatory	Hamsa *et al*.[102]
Coptisine **71**	Anti-inflammatory	Guo *et al.*[106]
	Cardioprotective	Guo *et al.*[106]
	Antinephritic	Hatori *et al.*[107]
	Vasorelaxant	Gong *et al.*[108]

1.4. EXTRACTION TECHNIQUES OF VEGETABLE MATTER

Several methods using organic and/or aqueous solvents are employed in the extraction of natural products. Solvent extraction relies on the principle of either liquid–liquid or solid–liquid extraction. In solid–liquid extraction, the plant material is placed in contact with a solvent. Although the whole process is dynamic, it can be simplified by dividing it into different steps. In the first instance, the solvent diffuse into cells, in the following step it has to solubilize the metabolites, and finally it diffuse out of the cells enriched in the extracted metabolites.[109]

1.4.1. Maceration

The process of maceration is a very simple extraction technique based on the dissolution of the sample in a solvent, by stirring the closed container where the sample was inserted.[110]

Extraction stops when the equilibrium between the concentration of metabolites in the extract and in the plant material is reached. After extraction, the residual material of the plant should be separated from the solvent (decantation and filtration).

The disadvantage of maceration is that the process is quite time-consuming, taking several hours or even several weeks. Exhaustive maceration can also consume large volumes of solvent and can lead to potential loss of metabolites or plant material.[109]

1.4.2. Steam Distillation

In steam distillation the sample is placed on a column where the lower part is connected to a flask with water under heating and the upper part is connected to a condenser.[111] The vapor which is produced passes through the sample, carrying with it the essential oils to the condenser, which will cause condensation of the water-oil mixture. At the end, these two phases can be separated by liquid-liquid extraction (Figure 14).

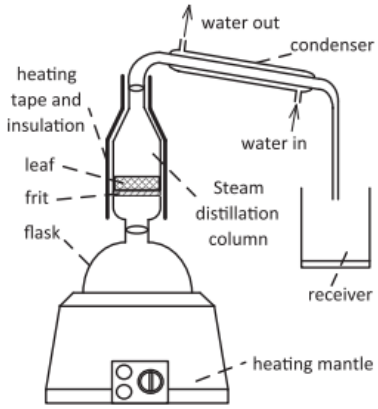


Figure 14. Experimental setup used in steam distillation.[112]

1.4.3. Hydrodistillation

Unlike steam distillation, hydrodistillation is characterized by the direct contact between the solvent and the plant material (Figure 15), that is, the raw material is submerged in water, which is boiling.[113] Thus, the cell walls will be broken and the oil will be evaporated along with the water, and then condensed into a mixture of water vapor and volatile compounds of the vegetable raw material. However, since these two phases (volatile compounds and water) are immiscible, it is possible to make a further separation according to the difference in density.[114]

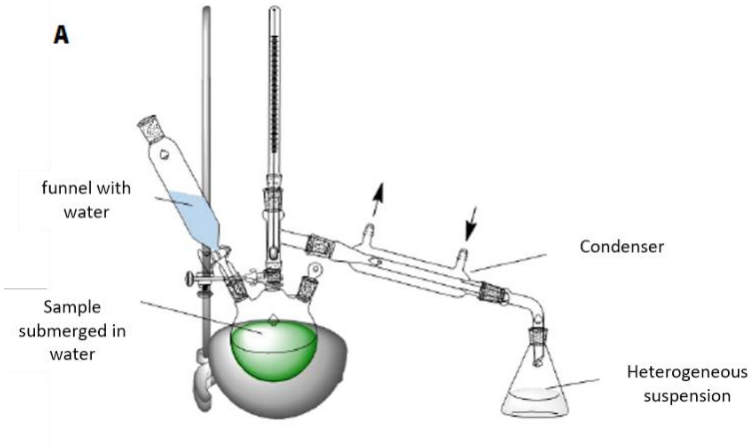


Figure 15. Schematic representation of hydrodistillation.

This technique proved to be inexpensive but, in contrast, it is not selective, since it causes the waste of large amounts of compound in the solvent, that is, part of the extract can be lost in the aqueous phase, giving lower yields.[115,116]

1.4.4. Soxhlet Extraction

In the Soxhlet extraction technique, the sample is not placed directly in contact with the heat source, but in the Soxhlet extractor (Figure 16), being in contact with the organic solvent when it is evaporated. As a result, this extraction method allows the sample to be permanently in contact with a given quantity of fresh solvent and, at the end of the process, it is not necessary to carry out a filtration, leading to higher yields.[117]

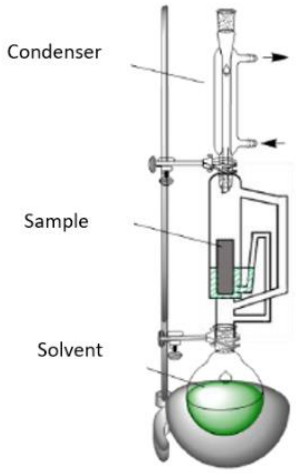


Figure 16. Schematic representation of extraction by Soxhlet.

However, in comparison to the other traditional methods, this is a time-consuming method that causes large quantities of solvent to be wasted, since it does not allow agitation that would speed up the process. The main counterpart of using organic solvents is that they may cause chemical changes or cause toxic effects on the final product, and final extract may have undergone changes in its taste or aroma.[114]

1.4.5. Other extraction techniques

In addition to the above mentioned extraction techniques, in recent years more innovative techniques have been applied, being the highlighted microwave assisted processes (MAP) and the extraction with supercritical carbon dioxide.

In the MAP the microwaves are used to excite the molecules of water existing in the vegetal tissues, resulting in the breakdown of cells and, consequently, in the release of essential oils.[118]

The extraction with supercritical carbon dioxide is promising, since the carbon dioxide is an inexpensive solvent with little odor or taste and, in addition, is capable of penetrating into the vegetable samples in an easy way and then being removed without leaving any kind of waste.

Compared to other extraction techniques, the latter method, while requiring a high investment in the necessary equipment, proved to have lower energy costs in the long run.[119]

1.5. NANOENCAPSULATION

The application of essential oils focuses mainly on the food, pharmaceutical and cosmetic industries. However, these compounds are extremely sensitive to environmental factors such as oxygen, light, moisture and heat.[120,121] These disadvantages can be avoided by incorporating the compounds into nanoencapsulation systems, allowing the preservation and controlled release of these compounds. The encapsulation of molecules with biological activity thus increases their life span and changes the way they interact with the surrounding environment, increasing thermal stability and chemical protection.[122]

Nanoencapsulation of active compounds involves the formation of particles with a diameter between 1 and 1000 nm. The nanoparticles can be either solid (e.g. solid lipid nanoparticles, SLN), or soft condensed (e.g. vesicles, microemulsions), have a submicron size and may or may not be biodegradable. The term nanoparticle encompasses nanospheres and nanocapsules (Figure 17). The nanospheres have a matrix-like structure and the active

compounds can be adsorbed on the surface of the bead or encapsulated within the particle. On the other hand, nanocapsules are usually vesicular systems in which the active compound is immersed in a liquid, within a cavity, surrounded by a polymeric membrane, or transported in the membrane.[123,124]

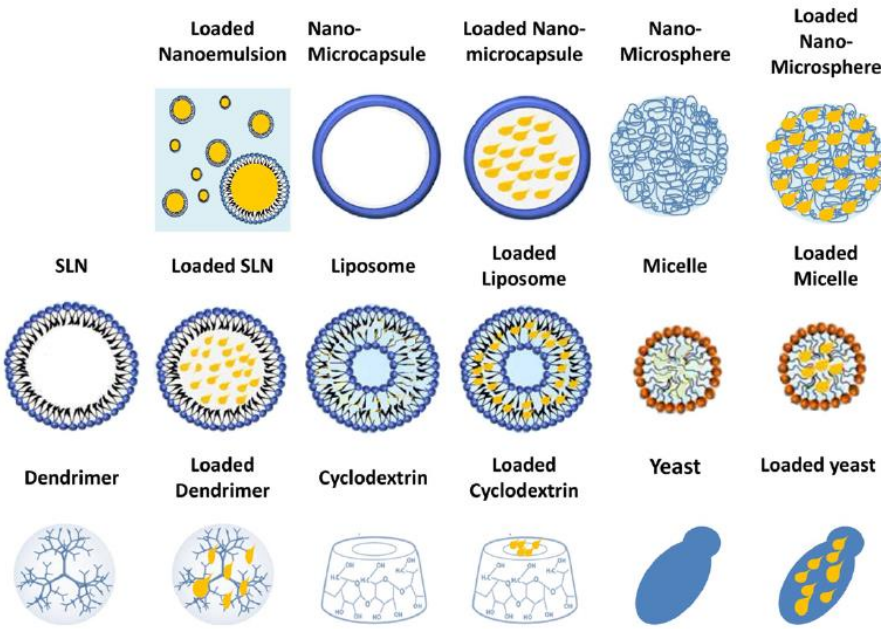


Figure 17. Different encapsulation structures for bioactive oils, comparing loaded and unloaded nanosystems.[125]

The system used to encapsulate a sample depends on its size, shape and nature, as well as the intended end use. As such, lipid-based or chitosan-based nanosystems are two of the most used encapsulation systems for essential oils (Figure 18).

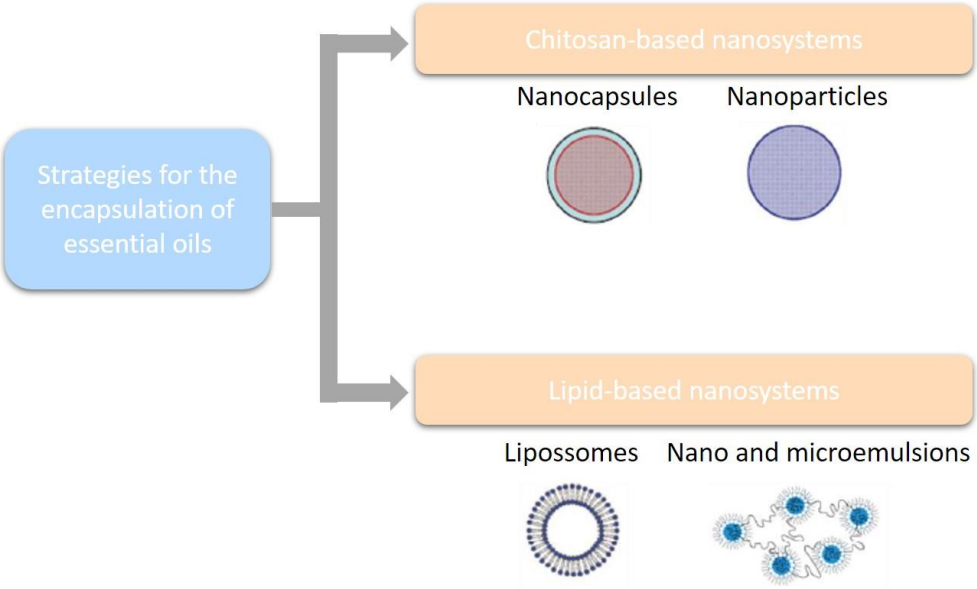


Figure 18. Schematic illustration of lipid-based and chitosan-based nanosystems for encapsulation of essential oils.[120]

Nanoparticles are receiving increasing interest for release of bioactive agents, and they have several advantages over microparticles and liposomes. According to the literature, they allow a greater therapeutic efficacy occurring controlled release of drugs/compounds, a safe administration and lower doses, have low toxicity and high selectivity, remain in circulation longer and can incorporate both hydrophilic and lipophilic substances. Submicron size allows for greater intracellular absorption compared to microparticles. In addition to size, composition and charge also influence the absorption through the intestinal epithelium.[123,124]

The growing interest in the development of nanoparticles lies in the fact that they require little toxic reagents and simple processes of formation. Moreover, they are safe and allow high efficiency in the delivery of the drug to its target.

1.5.1. Lipid-based nanosystems

Within lipid-based nanosystems, there are different structures, the most used being liposomes (LPs) and nano- or microemulsions. Liposomes (LPs) are an association of amphiphilic lipids that spontaneously organize into bilayer vesicles. Thus, in this case, the

stored bioactive compounds are protected from degradation and their solubility may be enhanced.

Another case is the nano- or microemulsions, which are homogeneous dispersions of two immiscible liquids, being stabilized by a film of surfactants. These particles do not require large amounts of energy to form the emulsion, since they form spontaneously when they encounter aqueous or oily components. By contrast, their formation requires high concentrations of surfactant, which may cause toxicity.

1.5.2. Chitosan-based nanosystems

Chitosan is a polysaccharide consisting of a combination of monosaccharides (2-amino-2-deoxy-β-D-glucan) by β1 → 4 glycosidic bonds and, given the primary amine groups it possesses, it has a wide interest in pharmaceutical applications.[121]

In the encapsulation process, these nanoparticles control the release of bioactive compounds, dispense the use of organic solvents (as they are soluble in acidic aqueous solutions) and retain multivalent anions, thanks to their cationic nature. In addition, the characterization of chitosan can be made by its molecular weight, which provides better control of the formation of nanoparticles.

CHAPTER 2: RESULTS AND DISCUSSION

2.1. INTRODUCTION

Plants are a major source of natural products, with a large application in many industries, such as food and cosmetics, and due the presence of many biological active substances, they are also of great interest for medicinal and agriculture industries.

The present project consisted in the extraction of various less studied vegetable species, characterization of the chemical composition of the extracts obtained, and evaluation of their potential biological activity. Studies on the encapsulation of most promising extracts were carried out, as well as the synthesis of analogues of ginkgolic acid, a very toxic and less available compound isolated from *G. biloba* leaves.

This work is part of a recently funded FCT project (PTDC/ASP-AGR/30154/2017), which main application interests are related to environment-friendly preservatives and biopesticides preparation.

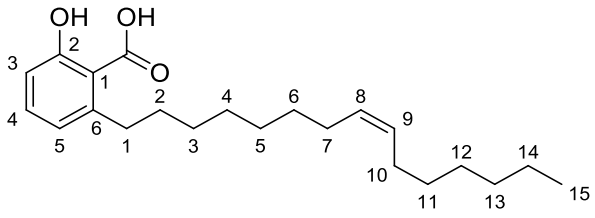
2.2. EXTRACTIONS

Among the various techniques used in the extraction of natural compounds from vegetable matter, Soxhlet extraction was the selected technique.

The method of extraction by Soxhlet, as already mentioned, uses specific equipment called Soxhlet extractor, consisting of three main sections: a percolator (distillation flask and reflux condenser), where solvent reflux occurs; a cellulose cartridge, whose function is to retain the solid particles (vegetable matter); and a siphon, which empties periodically the chamber where the cartridge is placed (Figure 16).

2.2.1. Extraction of *Ginkgo biloba* components

The fresh *Ginkgo Biloba* leaves collected in Braga were subjected to Soxhlet extraction, using dichloromethane as solvent, affording an extract as a greenish oil. The ^{1}H NMR spectrum analysis revealed the presence of a 15:1 ginkgolic acid, trivial name for 2-hydroxy-6-(pentadec-8-en-1-yl)benzoic acid **79**, in its composition as a major constituent (Figure 19).



79

Figure 19. Chemical structure of 2-hydroxy-6-(pentadec-8-en-1-yl)benzoic acid **79**.

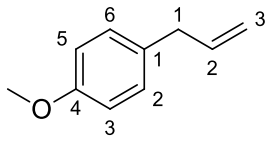
^{1}H NMR spectra showed signals corresponding to the aliphatic side chain such as the terminal methyl group as a multiplet (δ0.86 - 0.94 ppm), the methylene groups as multiplets (δ1.26-1.35, 1.54-1.68 and 1.99-2.08 ppm), the protons to the double bond present at C8 - C9 as a triplet (δ5.35 ppm, with *J* = 4.8 Hz), as well as the aromatic protons, namely H-5 and H-3 as a doublets of doublets (δ6.77 ppm, with *J* = 7,6 and 0.8 Hz; δ6.87 ppm, with *J* = 8.4 and 1.2 Hz, respectively), and H-4 as a triplet (δ7.36 ppm, with *J* = 0.8 Hz). In addition the proton of hydroxyl group of the carboxylic acid was also visible as a broad singlet (δ11.12 ppm).

^{13}C NMR spectra showed the presence of signals corresponding to the aliphatic side chain, such as the terminal methyl group (δ13.97 ppm), the methylene groups (δ29.23, 29.47, 29.58, 29.62, 29.68, 29.75, 29.80, 31.95 ppm), the C8-C9 of the double bond (δ129.87, 129.89 ppm), as well as the aromatic carbons, namely C-5, C-3 and C-4 (δ122.66, 115.77 and 135.21 ppm, respectively), in addition to the signal of carbonyl group (δ175.25 ppm)

The presence of GA was also confirmed by high resolution mass spectrometry (ESI), where a fragment corresponding to the exact mass (347.25779) was obtained.

2.2.2. Extraction of *Foeniculum vulgare Mill*. components

Fresh *F. vulgare* collected in Braga was subjected to Soxhlet extraction using dichloromethane as the solvent. The extract was obtained as a greenish oil. The analysis of the ^{1}H NMR spectrum revealed the existence of a mixture of compounds, which was further purified by dry flash chromatography. It was possible to isolate one of the major components of the fennel, the estragole **66** (trivial name for 1-allyl-4-methoxybenzene) (Figure 20).



66

Figure 20. Chemical structure of 1-allyl-4-methoxybenzene **66**.

The main characteristics of the ^{1}H NMR spectrum of compound **66** are the presence of a singlet (δ 3.80 ppm) corresponding to the methoxy protons, two multiplets (δ 5.02 - 5.11 ppm, CH_2; 5.90 - 6.02 ppm, CH) of the protons of the double bond of the side chain, and the aromatic protons H-3 and H-5 (δ 6.85 ppm, as a doublet with *J* = 8.4 Hz), as well as H-2 and H-6 as a doublet (δ 7.11 ppm with *J* = 8.4 Hz).

A GC-APCI-QTOF analysis of the DCM extract of *F. vulgare* was performed (Figure 21). Three main components are present in this sample, with m/z of 153.1269, 149.0958 and 445.1200. After comparison with the components identified in the literature, it is possible to affirm that peak 1, with mass 153.126 corresponds to fenchone **64** and peak 2, with m/z 149.06, corresponds to estragole **66**.

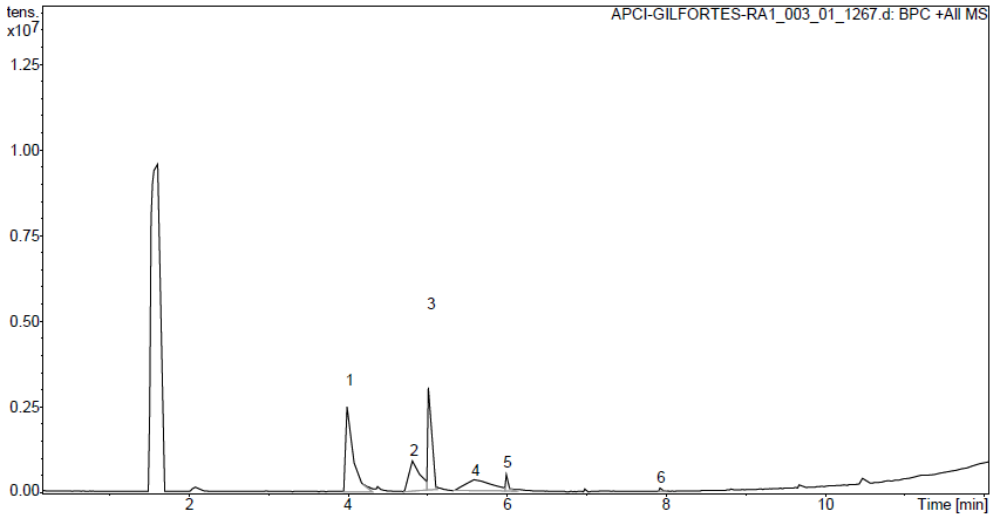


Figure 21. Chromatogram of *F. vulgare* extract in GC-APCI-QTOF analysis.

2.2.3. Extraction of *Chelidonium majus* components.

In the case of *C. majus*, this was initially dried in a greenhouse at a temperature of 40-45°C over a period of 24 hours. The material was then grounded and sieved with a sieve with a pore size of less than 910 µm, and used for subsequent extractions. Two distinct strategies of Soxhlet extraction were carried out based on different polarity solvent types (Figure 22).

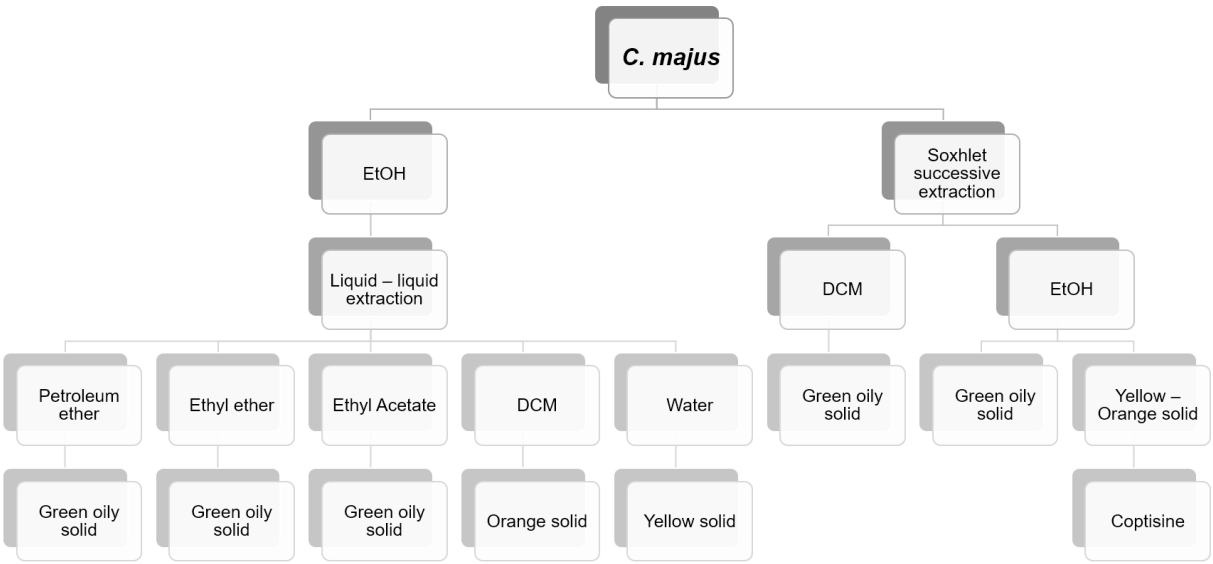
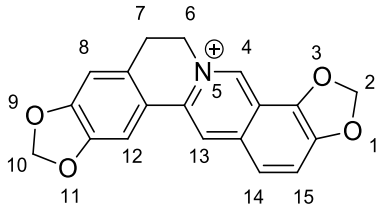


Figure 22. Scheme of extractions by Soxhlet performed with *C. majus*.

Firstly, extraction was carried out through Soxhlet using ethanol as solvent, and a green oily solid **E_1** was obtained. As a complex mixture of compounds was determined by ^{1}H NMR and TLC, it was decided to carry out a method of selective extraction with solvents of increasing polarities, in order to obtain purer extracts. The obtained ethanolic extract was suspended in distilled water and the solvent was added (petroleum ether, ethyl ether, ethyl acetate and dichloromethane) to a separation funnel. The first solvent used was petroleum ether, as the less polar, affording a green oily solid. This procedure was repeated with ethyl ether and ethyl acetate to give also green oily solids. Finally, dichloromethane was used affording a yellow solid. The remaining aqueous phase obtained after the washings, afforded a yellow solid. All obtained extracts were analyzed by ^{1}H NMR but showed to correspond to mixtures, which was also confirmed by TLC analyses.

Considering the results obtained, a Soxhlet selective extraction of *C. majus* was performed. For this, a Soxhlet extraction of the dried vegetable matter with dichloromethane was carried out, obtaining a green oily solid extract **E_2** containing less polar components. Then, using the same cartridge of vegetable matter, a new extraction was carried out using a more polar solvent, ethanol, giving also a green oily solid extract **E_3** (Table 5). The ^{1}H NMR spectra of both different extracts were carried out and after analyzing and comparing them with the TLC it was decided to proceed to a chromatography column of the ethanol extract **E_3** as it

appeared to have a smaller number of components and a better separation thereof. However, before the chromatography dichloromethane was added to the residue and it was found that the portion of the extract not soluble in dichloromethane was a yellow solid, which remained attached to the flask. The solid was separated and since its TLC seemed promising it was chosen for purification by column chromatography. Compound **71**, coptisine, was isolated with an eluent mixture of DCM: EtOH (95:5) as a yellow - orange solid, which was fully characterized by ^{1}H and ^{13}C NMR spectroscopy and high resolution mass spectrometry.



71
Coptisine

Figure 23. Coptisine **71**, isolated through *C. majus* extract.

^{1}H NMR spectra showed signals of the methylene groups in position 7 and 6 as triples (δ2.49 and 4.85 ppm, respectively), as well as positions 2 and 10 of oxolo rings, as singlets (δ 6.15 and 6.51 ppm). In addition all the aromatic protons are visible, with H-8 and H-12 appearing as singlets (δ7.06 and 7.76 ppm, respectively), H-15 and H-14 shown as doublets (δ 8.4 and 8.8 ppm, respectively), and H-13 and H-4 as singlets (δ8.91 and 9.9 ppm, respectively).

^{13}C NMR spectra is also compatible with structure **71**, showing the carbons of methylene groups in positions 7, 6, 2 and 10 of the heterocyclic system (δ26.35, 55.22, 102.17 and 104.56 ppm). In addition, the aromatic carbons are also visible, C-12, C-8, C-14, C-13, C-15 and C-4 (δ105.39, 108.52, 120.58, 121.07, 121.85, 144.62 ppm, respectively).

The presence of compound **71** was also confirmed by high resolution mass spectrometry (ESI), where a fragment corresponding to the exact mass (320.0917) was presented.

Table 5. Data of Soxhlet extractions *C. majus:* solvent, mass of the vegetable matter, mass obtained, percentage of mass obtained and color of the different materials obtained.

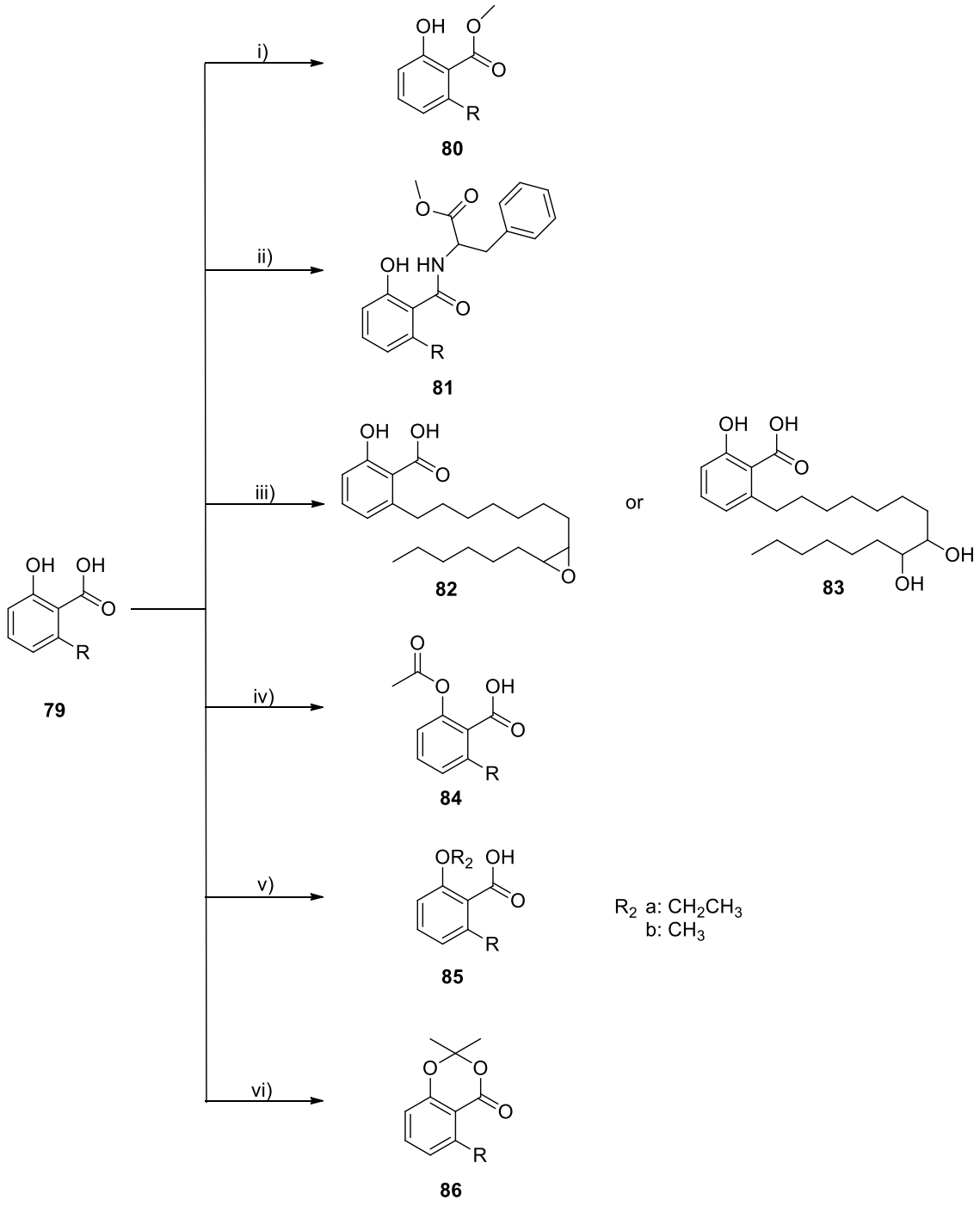
Solvent	Mass of vegetable matter (g)	Obtained mass (g)	% Mass	Color
E_1	8.024	2.935	37	Green oily solid
E_2		0.722	8.9	Green oily solid
E_3	8.088	0.182	2.3	Yellow solid
		0.155	1.9	Green solid

2.3. SYNTHESIS OF ANALOGUES

2.3.1. Synthesis of ginkgolic acid analogues

GA is known to be very toxic[126] which for most purpose is a disadvantage but could represent a good choice as a starting material for the synthesis of analogues with potential application as biopesticides for example.

Despite the broad spectrum of biological activities of GA, its natural availability as well as its synthetic derivates are very limited. Considering these facts, the present work aimed to functionalize the GA previously isolated from *G. Biloba* for the exploration of the reactivity of different parts of the molecule in order to obtain derivatives. In this sense, reactions involving the carboxylic acid (esterification and coupling), the double bond present in the side chain (epoxidation) and the hydroxyl group (acylation and alkylation) were selected for the preparation of analogues (Scheme 3).

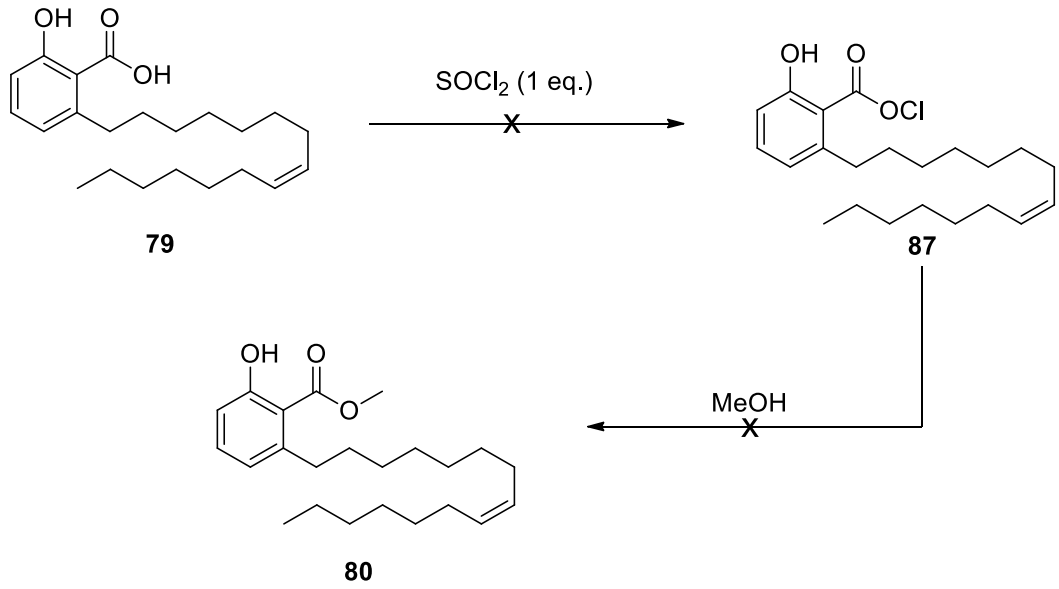


Scheme 3. Reaction scheme of the desired GA analogues. i) esterification, ii) coupling, iii) epoxidation, iv) acetylation, v) alkylation and vi) protection.

The analogues obtained are intended to use in biological assays and consequently correlate the structural changes with cellular viability. In the future, this work is aimed at adding molecular modelling chemistry in order to determine which functionalization will be more favorable for the selected application area.

2.3.1.1. Attempting esterification of ginkgolic acid

Initially, reactions involving the carboxylic acid functionalization were performed. In a first attempt thionyl chloride and the methanol were used, but the corresponding methyl ester was not formed, as confirmed by ^{1}H NMR that showed a complex mixture. Since the reactivity of the carboxylic acid function is low and consequently its substitution is difficult due to the presence of an acidic proton and a weak leaving group, its conversion in the acyl chloride is preferable in the synthesis of ester derivatives. Thus, the strategy is to react GA with thionyl chloride to form the corresponding chloride compound **87**. The compound formed *in situ* would then be reacted with methanol, whereupon the substitution occurs and the final esterified compound **80** would be formed.



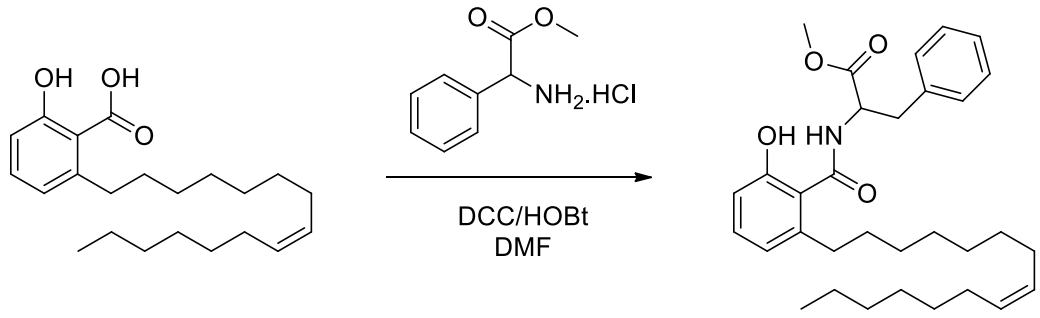
Scheme 4. Attempt to esterify ginkgolic acid using thionyl chloride and methanol.

However, the esterification reaction under these conditions was not efficient and the esterified GA was not obtained. Attempts were made to optimize the conditions, and the reaction was firstly carried out using 2 eq. of thionyl chloride instead of 1 eq. and the temperature was increased to 20°C. As the reaction was not efficient, 4 eq. of thionyl chloride but again the product was not formed. As a last attempt, the reaction was performed using

triethylamine, with the initial reaction conditions, but the reaction did not work as confirmed by ^{1}H NMR which indicated a complex mixture.

2.3.1.2. Attempting coupling of ginkgolic acid using DCC

The reaction between the ginkgolic acid and the phenylalanine methyl ester hydrochloride was then attempted (Scheme 5), using *N, N'*-dicyclohexylcarbodimide (DCC)/1-hydroxybenzotriazole (HOBt) as coupling agents. ^{1}H NMR data showed that product **81** was not obtained.



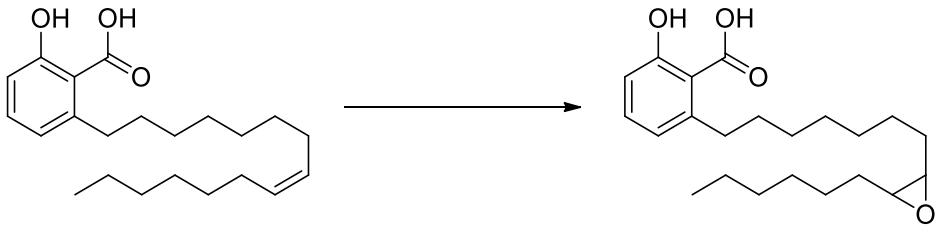
79 **81**

Scheme 5. Coupling attempt of ginkgolic acid **79** with phenylalanine methyl ester hydrochloride.

2.3.1.3. Attempting epoxidation of ginkgolic acid

After the failure of previous attempts of functionalization of the carboxylic acid, it was decided to study the reactivity of the double bond of the lateral chain of GA.

Epoxidation reaction of the double bond present in the side chain was performed (C8-C9), using *meta*-chloroperoxybenzoic acid (*m*-CPBA) as the oxidizing agent. The reaction was allowed to stir at 60°C for a period of 24 hours, but after work-up there was no formation of the desired product (Scheme 6).



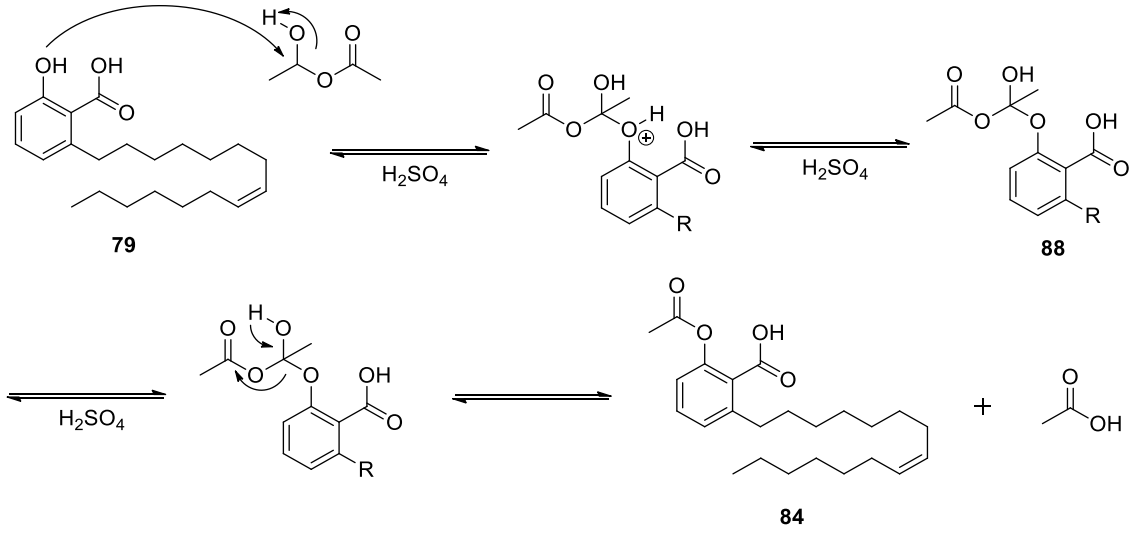
79 **82**

Scheme 6. Attempted epoxidation of ginkgolic acid **79**.

2.3.1.4. Attempting acetylation of ginkgolic acid

As the reactions in the previous functional groups did not succeed, hydroxyl group was the next part of the molecule to be studied, namely through acetylation and alkylation reactions.

In the case of the acetylation, GA was reacted with acetic anhydride in the presence of concentrated sulfuric acid. This reaction was reported using salicylic acid as the starting material. In the case of GA, the alkylated product was not obtained as found by ^{1}H NMR, which indicated a complex mixture of compounds.



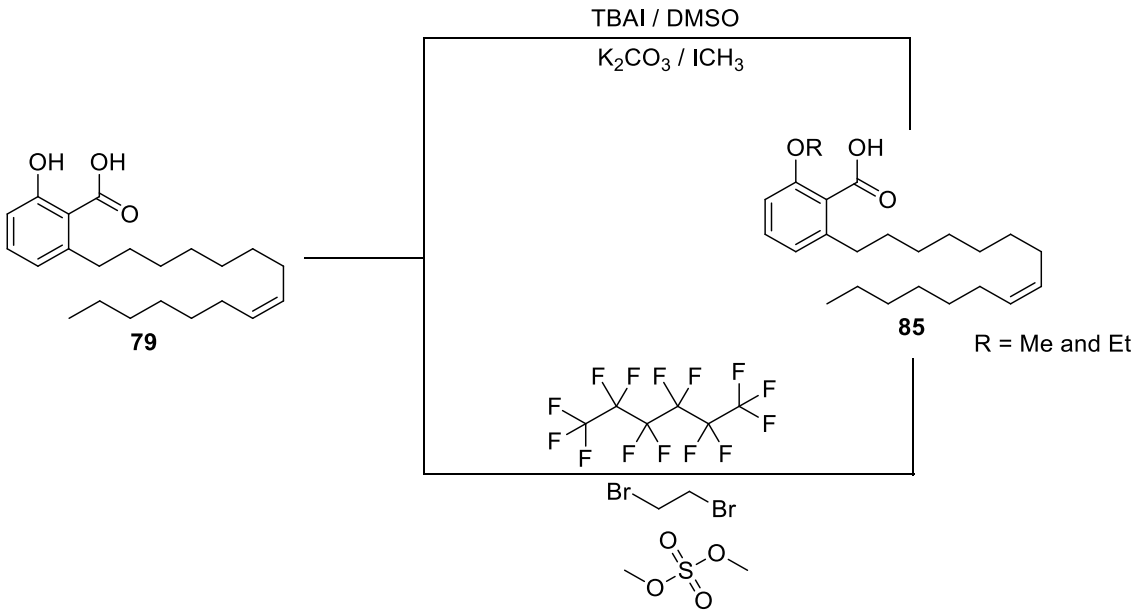
79 **88** **84**

Scheme 7. Attempted epoxidation of ginkgolic acid **79**.

2.3.1.5. Alkylation of ginkgolic acid

Two alkylations were then carried out by distinct methods, since one of the alkylations was not achieved.

Initially, in order to obtain compound **85** the alkylation, described by Kazemi *et al.*, of GA was performed using tetrabutylammonium iodide (TBAI) and DMSO followed by the addition of potassium carbonate and methyl iodide. At the end of the reaction time, it was found that the achievement of the desired compound did not succeed (Scheme 8).



Scheme 8. Alkylation of ginkgolic acid **79**.

With the results obtained and after some bibliographical research, a different GA alkylation approach was attempted.

Jana *et al*.,[127] performed an alkylation reaction of the salicylic acid forming two distinct phases in the reaction mixture, thereby obtaining the respective alkylated derivative in 90% yield.

GA alkylation reaction was performed using this method. Initially, perfluorohexane, 1,2-dibromoethane and dimethyl sulfate were added to form two distinct phases in the reaction mixture. After checking the formation of this separation, the GA and triethylamine were added. The product obtained was then purified by dry flash column chromatography and

the compound **85** was isolated using a mixture of petroleum ether: dichloromethane (1:1) as eluent.

The ^{1}H NMR spectrum of the product obtained shows the presence of a mixture of compounds. Main features are a duplication of signals in different parts of the spectrum, namely two singlets corresponding to COO*H* (δ 11.11 and 10.97 ppm), as well as two triplets corresponding to the Ph-CH_2 protons (δ 2.96 and 2.89 ppm), indicating the presence of two structurally similar compounds in a ratio 2:1. It is also possible to observe the presence of a singlet, which should correspond to the OCH_3 (δ 3.96 ppm), compatible with the expected product. ^{13}C NMR spectrum shows also a duplication of most signals, supporting also the presence of two compounds structurally similar.

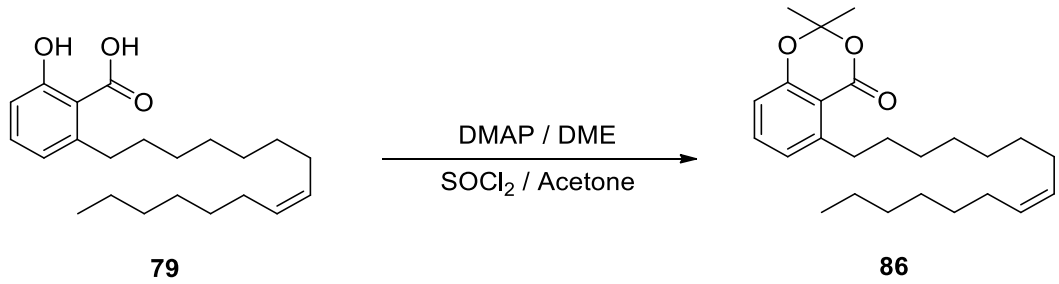
TLC analysis of the reaction mixture confirmed the absence of starting material showing only a major spot (R_f = 0.59 in DCM) even with different types of eluent, suggesting that both compounds should have similar polarity. Further analytical data, namely HPLC-DAD and HRMS are needed for confirmation of the compounds present.

2.3.1.6. Ginkgolic acid protection reaction

A possible explanation for the unsuccess of the previous attempts may be related with the formation of hydrogen bonds between the OH group and COOH group, forming a 6-membered ring and avoiding further reactivity under standard conditions. Another reason may be the size of the side chain containing a total of 15 carbons, presenting only a double bond, which may decrease the reactivity of the CO_2H and OH. Facing these results, a possible strategy to overcome this lack of reactivity is the protection of these groups in order to allow the reactivity on other parts of the molecule, particularly on the double bond.

Fu *et al*.[44] suggested, as discussed above, a method of reactional pathways to obtain GA in the laboratory. This same reaction was attempted in this work using GA (15:1). This reaction consists of a reaction involving GA, DMAP and DME followed by the addition of thionyl chloride and acetone. After the reaction, the product obtained was subjected to a silica gel column chromatography and the protected derivative of the ginkgolic acid was isolated

(elution with petroleum ether: ethyl ether (9: 1) (Scheme 9). Compound **86** was obtained as colorless oil in 51% yield.



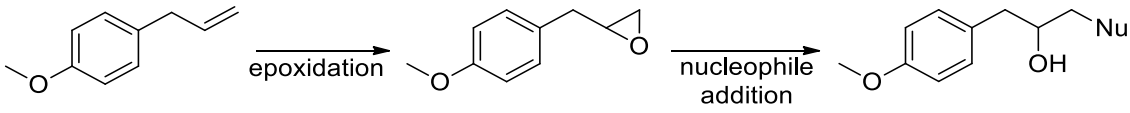
79 **86**

Scheme 9. Ginkgolic acid **79** protection reaction.

Structural information about compound **86** was obtained using ^{1}H- and ^{13}C- NMR. Concerning the signals referring to the ^{1}H spectrum, the main features are the signal at 1.70 ppm, which corresponds to a total of 6 protons, for the two methyl groups of the acetal, function matching the disappearance of the signals corresponding to the hydroxyl proton of the carboxylic acid group. It is also possible to observe in the ^{13}C spectrum the 25.63 ppm signal corresponding to the carbons of two methyl groups.

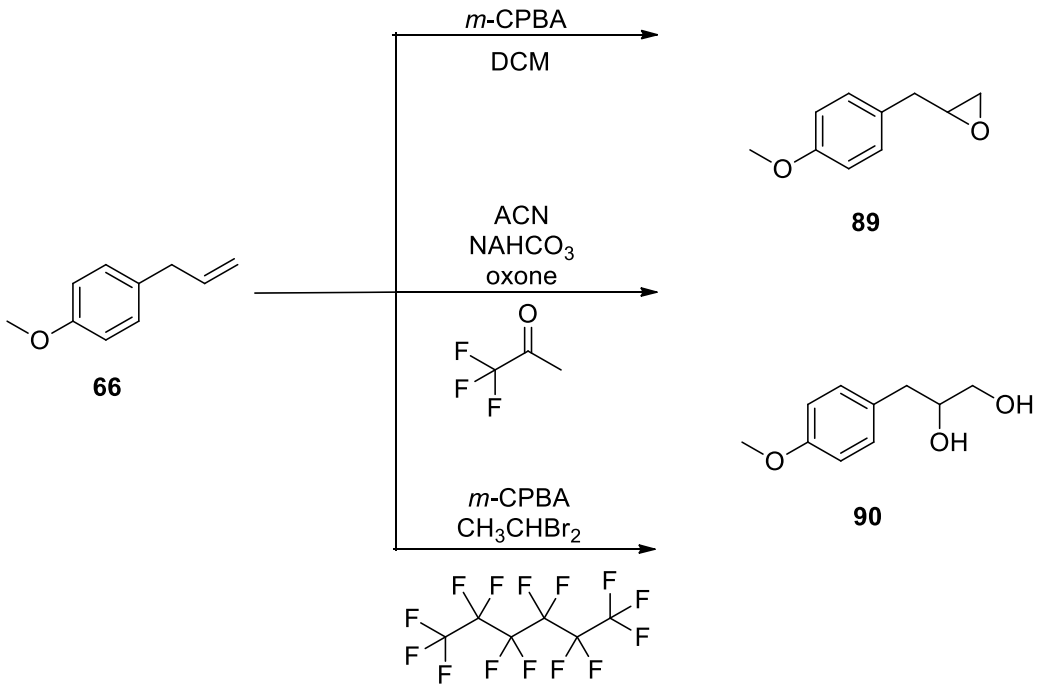
2.3.2. Estragole

Estragole was the major compound obtained from fennel; structurally estragole is a phenylpropene, having a double bond, which has potential for further functionalization, namely through epoxidation reaction. The epoxide itself can also be reacted with various nucleophiles (O-, N- and S-) in order to obtain the corresponding analogues, according to Scheme 10.



Scheme 10. Synthesis of estragole analogues through epoxidation followed by nucleophilic attack.

2.3.2.1. Epoxidation of estragole



Scheme 11. Epoxidation of estragole.

The first epoxidation reaction of estragole was carried out using *m*-CPBA as the oxidizing agent, at 60°C for 24 hours. TLC showed that no reaction occurred.

As an alternative to *m*-CPBA, oxone was used as epoxidizing agent. Initially the estragole was dissolved in ACN and water, followed by the addition of trifluoroacetone, sodium hydrogen carbonate and oxone. ^{1}H NMR, after dry flash chromatography, confirmed that no reaction occurred due to the existence of complex mixtures.

A third epoxidation attempt was carried out using *m*-CPBA in the presence of perfluorohexane. The reaction product was subjected to a dry flash chromatography and the

product was eluted with ethyl ether-ethyl ether (1:1). The ^{1}H NMR spectra of the product shows the presence of the 2-(4-methoxybenzyl)oxirane **89** through the signals of the CH of oxirane as a multiplet (δ 3.12-3.11 ppm), and two new signals as doublet of doublets (δ 2.88 and 2.44 ppm).

2.4. NANOENCAPSULATION STUDIES

Encapsulation assays were performed using liposomes of a natural lipid mixture, soy lecithin (Sternchemie) and the ethanolic injection method for lipid incorporation in aqueous media. The natural and economical choice of lecithin has already demonstrated good potential in the encapsulation of textile dyestuffs for dyeing.[128,129] Lecithin has emulsifying properties and is a surfactant. It can be totally metabolized by humans and is therefore well tolerated and non-toxic when ingested. In aqueous solution, their phospholipids can form liposomes, micelles or lamellar structures, depending on hydration and temperature. Lecithin is present, among other foods, in eggs, milk, sunflower and soybeans, with lecithin being the lipid used in this work for the encapsulation of the compounds.[130]

The method used in this work for the encapsulation of the compounds was the ethanolic injection. This is a method previously proven as a methodology that allows high encapsulation efficiencies of hydrophobic/lipophilic compounds.[131]

2.4.1. *F. vulgare*-related nanoencapsulation

In the case of *F. vulgare*, encapsulation tests of its extract in dichloromethane as well as of the isolated compound from the extract, were carried out. For this, solutions of the two samples were initially prepared and diluted to $8x10^{-4}$ M. The absorption and fluorescence spectra were then plotted for each of the samples (Figure 24).

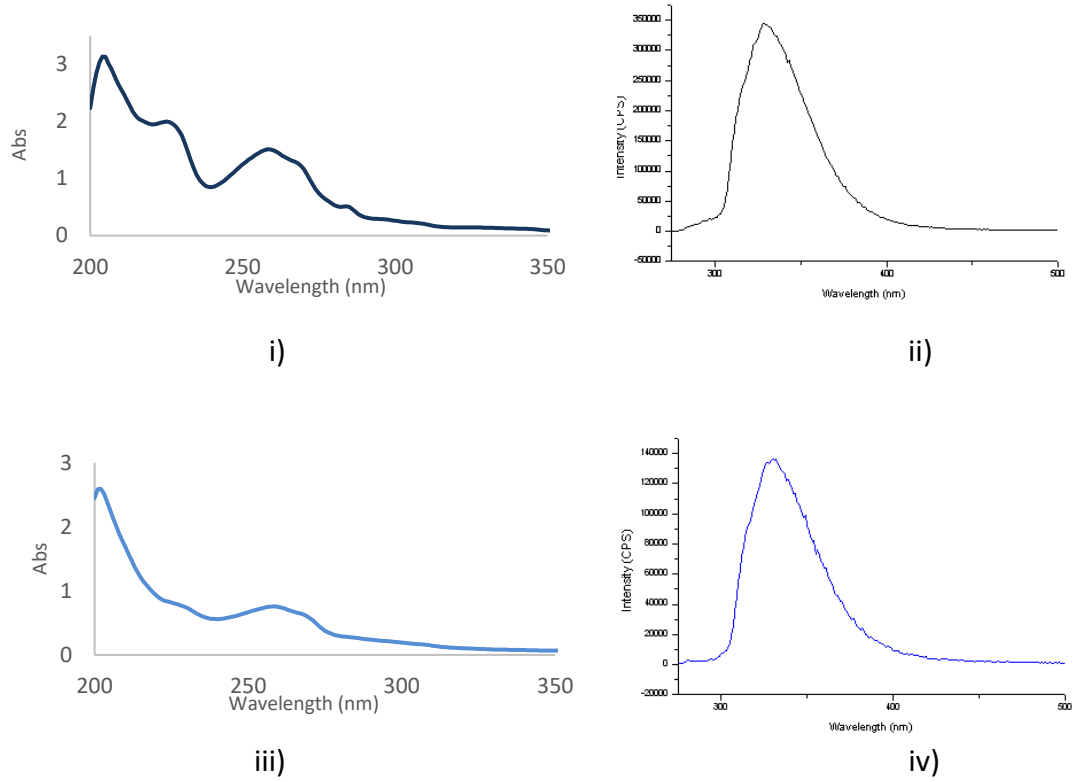


Figure 24. i) Absorption spectrum of *F. vulgare* extract; ii) Fluorescence spectrum of *F. vulgare* (excitation at 262 nm) at concentration 5x10^{-5} M. iii) Absorption spectrum of the estragole **66**; iv) Fluorescence spectrum of the estragole **66** (excitation at 260 nm) at concentration 5x10^{-5} M.

The absorption and fluorescence spectra in the UV region are characteristic of the aromatic moieties of estragole presented in the *F. vulgare* extracts.

Dilutions were then carried out at concentrations of 1.0×10^{-6} - 4.5×10^{-5} M for calculation of the calibration curves and determination of the encapsulation efficiency (Figure 25). The concentration of the encapsulated extract was determined by the Beer-Lambert law:

$$A = \varepsilon\, l\, c$$

where A is the absorbance, ε is the molar absorption coefficient, l is the cuvette optical path (1 cm, in this case) and c is the concentration of the sample.

The encapsulation efficiency was determined by following equation and the results are summarized in Table 6 for all the extracts used.

$$EE(\%) = \frac{Total\ amount\ -\ Amount\ of\ nonencapsulated\ extract}{Total\ amount} \times 100$$

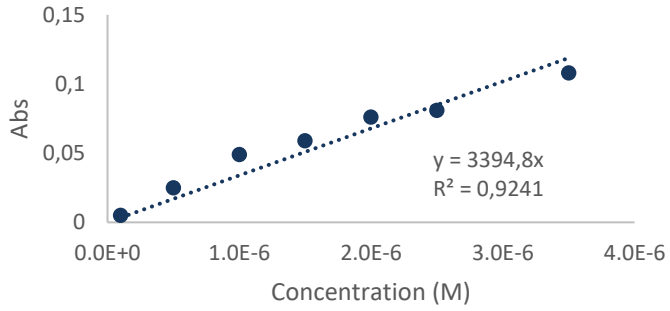


Figure 25. Calibration curve for *F. vulgare* (absorbance vs. concentration).

Table 6. Results obtained through the encapsulation of the *F. vulgare* extract: wavelength, absorptivity and encapsulation efficiency.

Extract	Wavelength (nm)	ε (M^{-1} cm^{-1})	Encapsulation efficiency (%)
F. vulgare	250	3395	62

As can be observed, the *F. vulgare* extract has an encapsulation efficiency of 62% using soybean lecithin as lipid; in the case of estragole **66** the test was also carried out, but it was not conclusive. For the latter sample, other nanoencapsulation systems must be tested, like the chitosan-based microemulsions. These assays will be carried out in the near future.

2.4.2. *Chelidonium majus*-related nanoencapsulation

Regarding *C. majus*, the photophysical studies (UV/visible absorption spectra and fluorescence spectra) of three samples related to the successive extension by Soxhlet, were carried out, these being the extract in dichloromethane **E_2**, the ethanol extract **E_3** and the compound isolated from the ethanol extract of the *C. majus*, coptisine **71**.

These samples were also dissolved in methanol (spectroscopic grade) and the solutions were diluted to the following concentrations: 4.17×10^{-4} M in the case of the extract **E_2**, 5.66×10^{-4} M in the case of extract **E_3** and 7.09×10^{-4} M in the case of isolated compound **71**.

The absorption and fluorescence spectra were then measured for each of the samples and are shown in Figure 26.

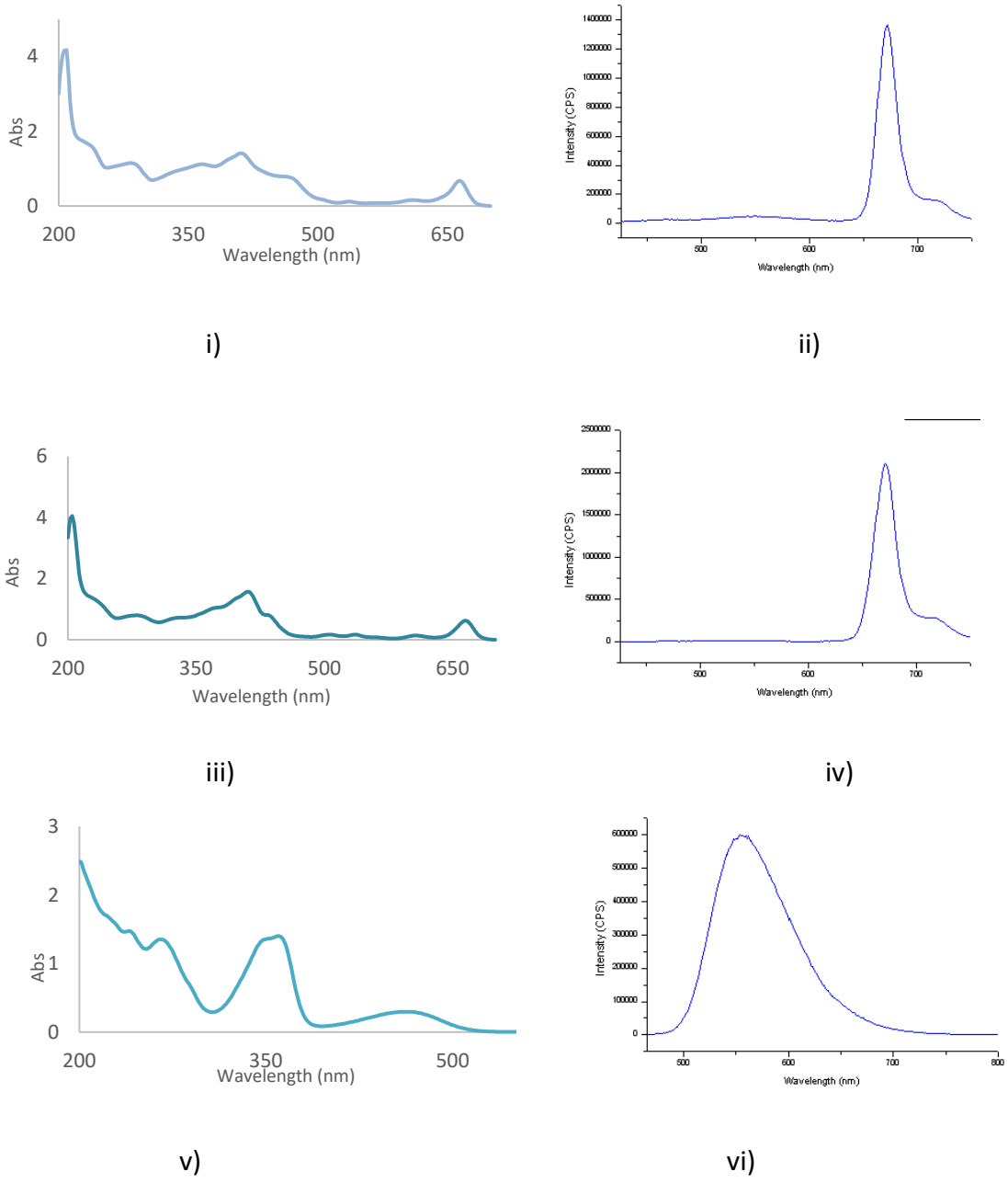


Figure 26. i) Absorption spectrum of *C. majus* extract in DCM **E_2**; ii) Fluorescence spectrum of *C. majus* extract in DCM **E_2** (excitation at 411 nm) at 5×10^{-5} M. iii) Absorption spectrum of *C. majus* extract in EtOH **E_3**, iv) Fluorescence spectrum of *C. majus* extract in EtOH **E_3** (excitation at 411 nm) at 5×10^{-5} M. v) Absorption spectrum of the isolated compound of *C. majus* extract **71**, vi) Fluorescence spectrum of the isolated compound of *C. majus* extract in EtOH **71** (excitation at 450 nm) at 5×10^{-5} M.

In the first two cases (spectra i) to iv)), absorption and fluorescence are dominated by chlorophyll, exhibiting its characteristic absorption bands, with maxima at 440 nm (blue) and 665 nm[132] (red), and a strong fluorescence emission at 690 nm.[132] This is not surprising, because these two extracts were isolated as green oils. Regarding spectra v and vi, they correspond to the isolated compound **71**, being located in the usual regions for absorption and emission of aromatic penta- or hexacyclic compounds, justifying the yellow color. A strong resonance effect is anticipated from the large and unstructured fluorescence band.

For the determination of calibration curves, dilutions of the prepared solutions in the range of 0.1×10^{-5} - 4.5×10^{-5} M were also performed, as above. In the first two cases, it was used the band of chlorophyll red absorption (around 665 nm). In the case of the isolated compound **71**, it was chosen the band centered at *ca.* 350 nm. The encapsulation efficiency was measured for the three samples and the data can be found in table below (Table 7).

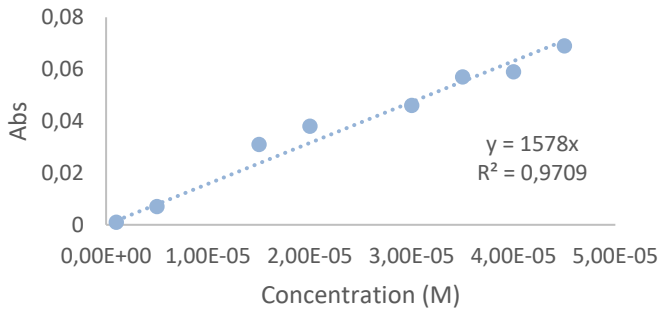


Figure 27. Calibration curve for the extract **E_2** of *C. majus* (absorbance *vs*. concentration) at 665 nm.

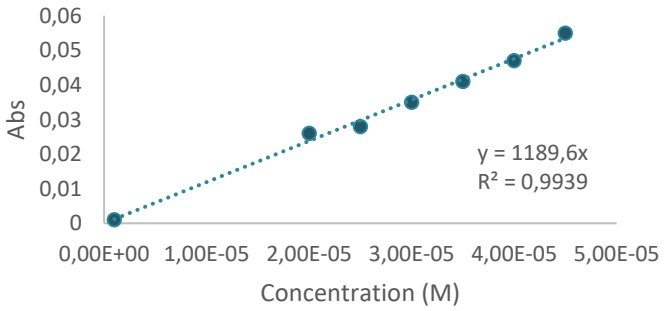


Figure 28. Calibration curve for the extract **E_3** of *C. majus* (absorbance *vs*. concentration) at 665 nm.

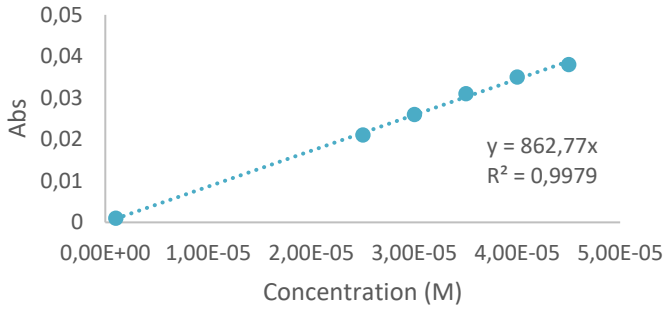


Figure 29. Calibration curve for the coptisine **71** from *C. majus* (absorbance *vs*. concentration) at 460 nm.

Table 7. Results obtained for the encapsulation of the different extracts: wavelength, molar extinction coefficient and encapsulation efficiency.

Extract	Wavelength (nm)	ε (M^{-1} cm^{-1})	Encapsulation efficiency (%)
E_2	665	1578	99.5
E_3	665	1190	96
71	460	863	65.5

After obtaining the results of encapsulation efficiency, it was possible to conclude that the extracts of the *C. majus* in dichloromethane **E_2** and in ethanol **E_3** present high efficacy values with 99.5% and 96%, respectively, giving indication that the liposomes of commercial soybean lecithin prepared by ethanolic injection method are promising nanosystems for the encapsulation of these extracts. For the isolated compound, and giving the very reasonable encapsulation efficiency obtained, however lower than in previous cases, the thin film hydration method could be an alternative for the same lipid formulation. The use of a purified lecithin mixture also appears as an alternative to explore.

Although no encapsulation assays of the remaining extracts/compounds were performed, UV/vis and fluorescence measurements were performed for **79**, **85** and **86** (Figure 30).

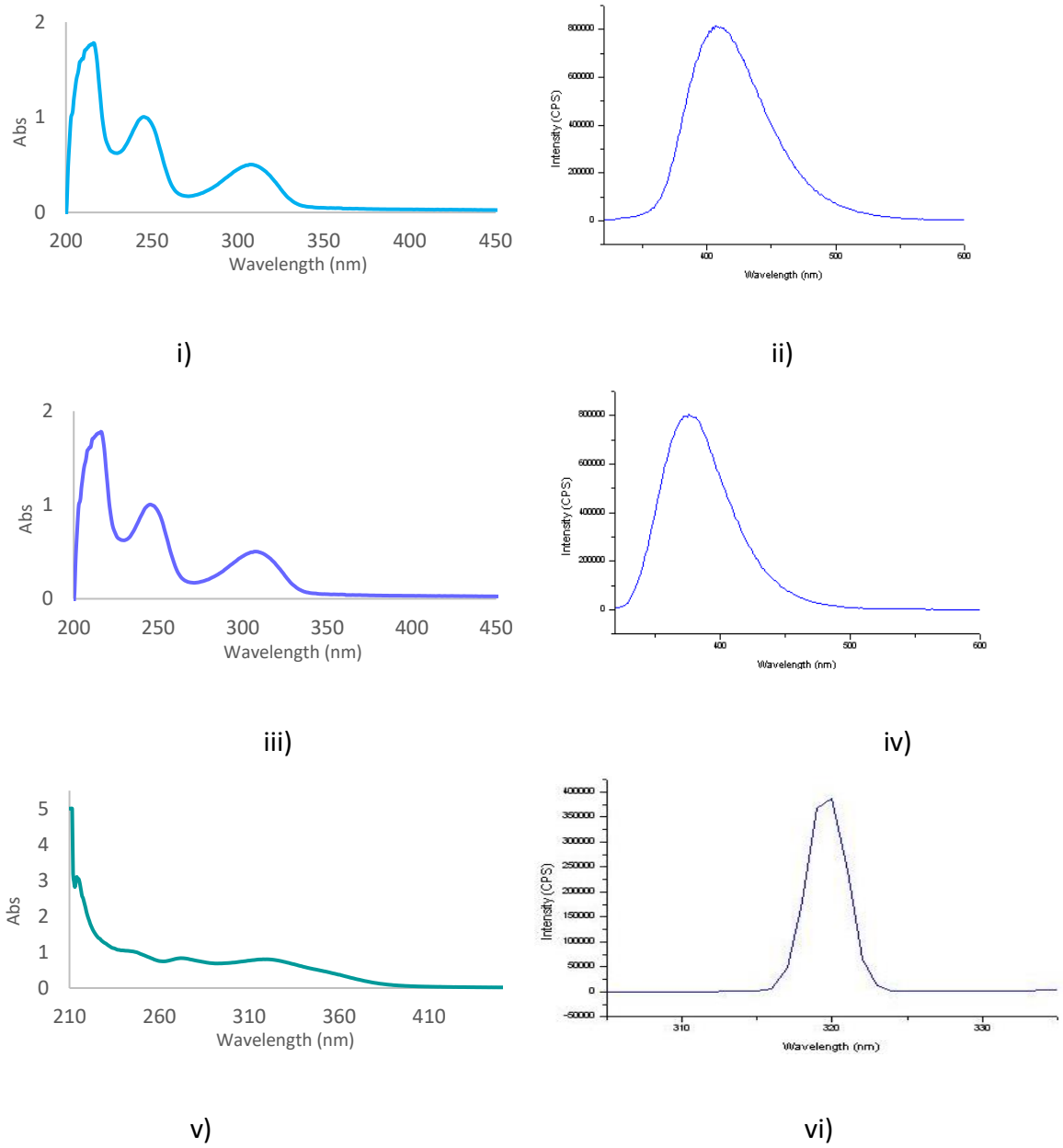


Figure 30. i) Absorption spectrum of GA **79**; ii) Fluorescence spectrum of GA (excitation at 305 nm) at 5×10^{-5} M. iii) Absorption spectrum of **86**, iv) Fluorescence spectrum of **86** (excitation at 304 nm) at 5×10^{-5} M. v) Absorption spectrum of **85**, vi) Fluorescence spectrum of **85** (excitation at 315 nm) at 1×10^{-4} M.

In the case of the *Ginkgo biloba* related spectra, carried out at 5.0 x 10^{-5} M, it was found that GA **79** shows an λ_{abs} value of 216 nm with a λ_{emi} of 411 nm. After the protection reaction of **79**, compound **86** still exhibit an λ_{abs} of 216 nm and a λ_{emi} of 376 nm. Compound **85** displayed λ_{abs} value at 210 nm and λ_{emi} at 320 nm (Table 8).

Table 8. Absorption and fluorescence maximum wavelengths for compounds **79**, **85** and **86**.

Compound	λ_{abs} (nm)	λ_{emi} (nm)
79	216	411
85	210	320
86	216	376

2.5. BIOLOGICAL ASSAYS

Several toxicity assays involving GA (15:1) using MTT and different cell lines such as $HepG_2$ (liver hepatocellular cells), MC-3T3-E1 (mouse osteoblastic cell line) and Tac8113 (human tongue squamous carcinoma cell line) have already been performed. The results for $HepG_2$ showed that 60 µM GA decreased viability to 90%.[133] Another representative assay showed that GA in cellular Tac8113 decreased cell viability, these same results compared to the nontumorigenic MC-3T3-E1 demonstrated that GA can present selectivity to tumor cells since it only has effects on the viability of tumor cells.[134]

In relation to coptisine, an assay evaluating its toxicity to the LoVo (human colon adenocarcinoma), HT 29 (human colorectal adenocarcinoma) and L-1210 (mouse lymphocytic leukemia) cell lines is highlighted. This compound showed to be quite toxic to HT 29 cells and to have an antiproliferative effect on the LoVo cells. To the L-1210 cell line, this compound did not show as active as the others.[135]

Regarding the results presented before, and since the AGS cell line is not reported in any of the MTT assays for any of the three species under study, in this work biological assays were performed using AGS cell line. This line is made up of gastric adenocarcinoma cells and, since the route of administration of pharmacological drugs is the oral via as well as the use of pesticides can be an important factor for the quality of human life, it gives the stomach an important role as a model to consider for the reasons presented above.

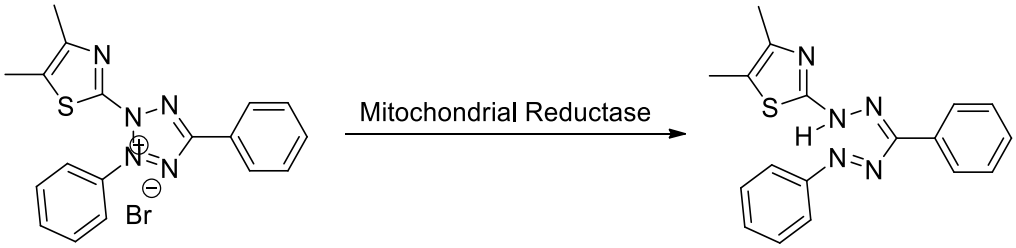
In this way, the cytotoxicity was evaluated for four extracts obtained (ginkgo biloba extract, fennel extract, *C. majus* dichloromethane extract and the ethanol extract of the *C. majus*), for two isolated compounds (estragole and compound isolated from the *C. majus*) and three achieved reactions (GA alkylation **85**, GA protection **86** and the product of epoxidation

of the estragole **89**) using the AGS cell line. For this, two simultaneous tests were performed for the nine samples under study: MTT assay and LDH assay.

2.5.1. Cell viability

2.5.1.1. MTT Assay

The MTT reduction assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) has been widely used to quantify cell viability. This type of assay is based on the metabolic potential, in this case, assessing the mitochondrial enzymatic activity of the cells. Cells with active metabolism convert MTT to formazan, with purpura color and a maximum absorption around 570 nm. When cells die, they lose the ability to convert MTT and thus, color formation serves as an indicator of cell viability.



Scheme 12. Mitochondrial reduction of MTT to formazan.

2.5.1.2. LDH Assay

In order to estimate the toxic potential of the compounds under study, the lactate dehydrogenase (LDH) release test was performed. The cytotoxic effects of the compound are associated with the integrity of the cell membrane and, thus, the release of intracellular substances into the medium serves as an indication of this integrity. LDH is a cytosol marker enzyme and its frequently used to estimate the integrity of the cell membrane.

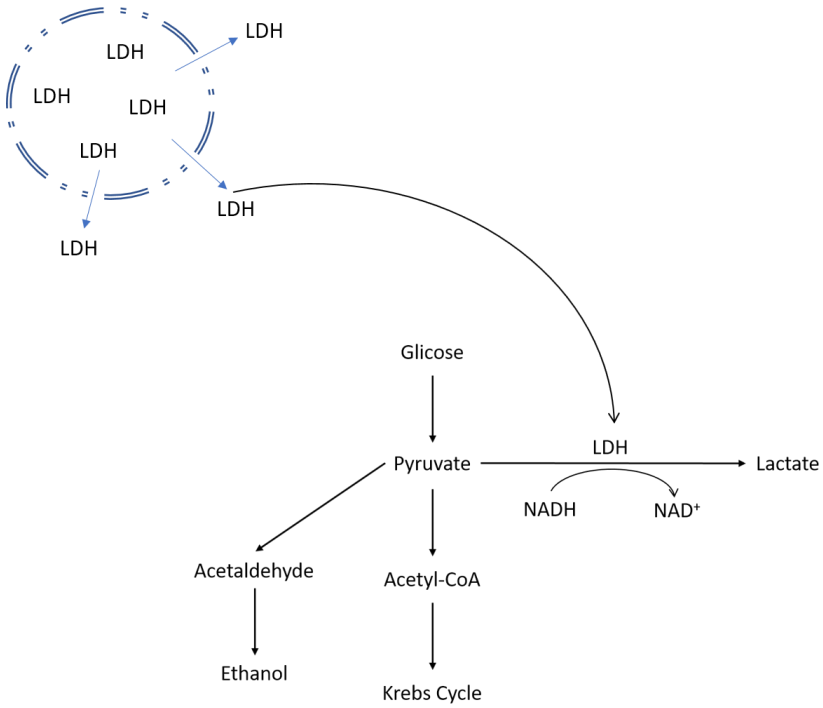


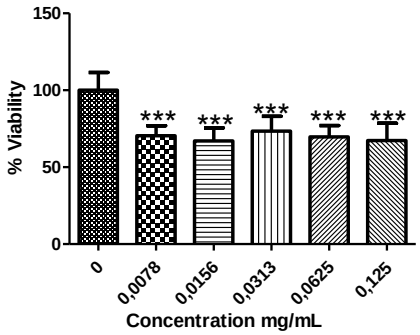
Figure 31. Schematic representation of the reduction of NADH to NAD^+ by the LDH enzyme with lactate formation.

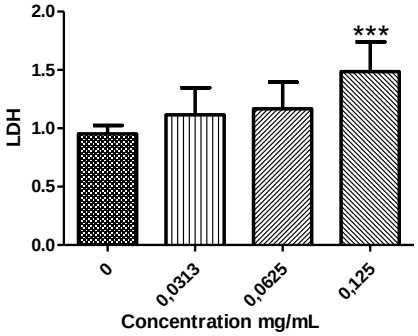
2.5.2. Cell viability Results

As noted above, cytotoxicity was evaluated using the AGS cell line. For this purpose extracts, isolated components and analogues obtained through synthetic routes were used in the biological assays. The assays were performed using concentrations in the range of 0.0625-1 mg/mL, except in the case of the estragole and DCM extract of *C. majus*. This is because, through an initial assay, it was found that at the concentration of 0.125 mg/mL both samples already had a high toxicity, and it was necessary to adjust the concentrations to the range of 0.0078-0.125 mg/mL. Due to the low solubility of these compounds in the medium used, except for the estragole, DMSO was added to stock solutions in the required volume so after dilution they had only 0.5% DMSO in solution. The results obtained in the MTT and LDH assays for all the study samples are shown in Figure 32.

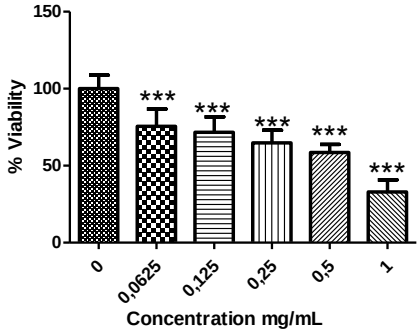
(A) (B)

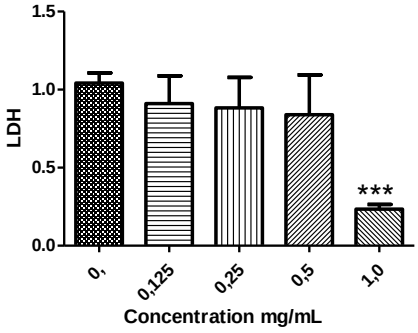
F. Vulgare extract



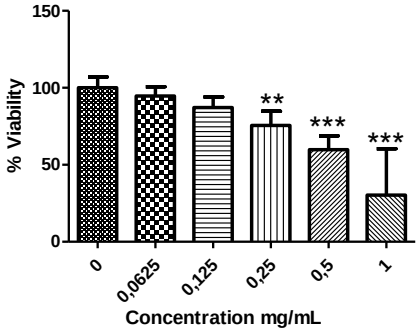


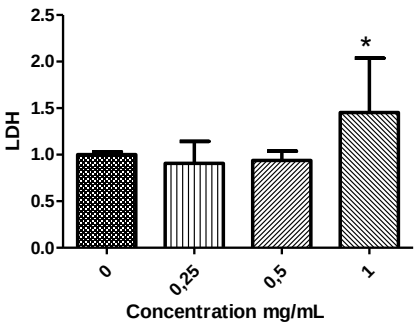
66



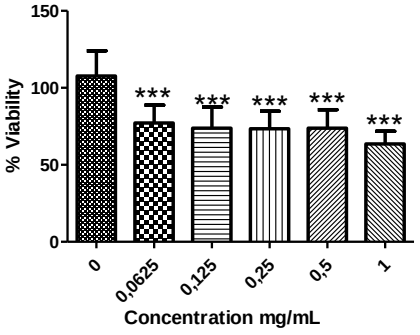


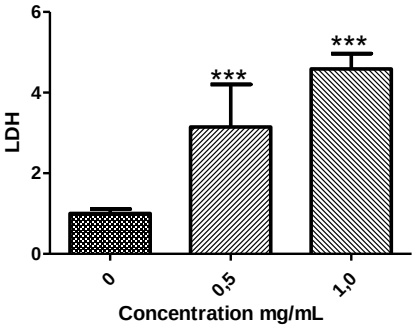
89



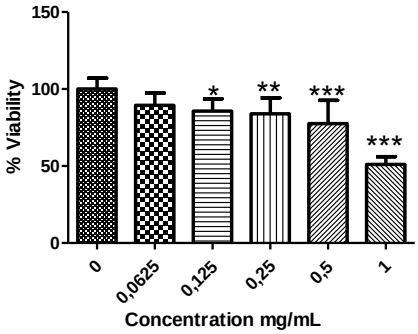


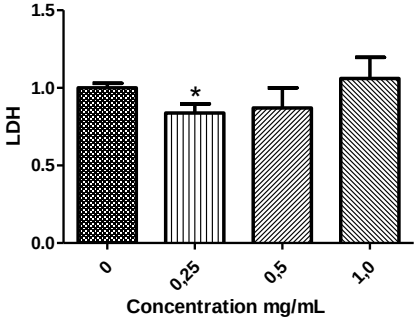
79



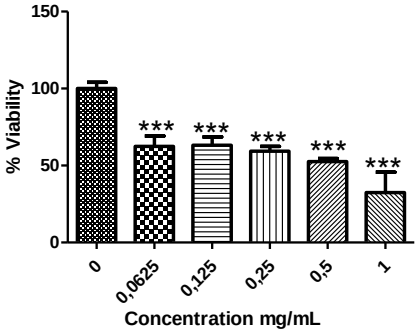


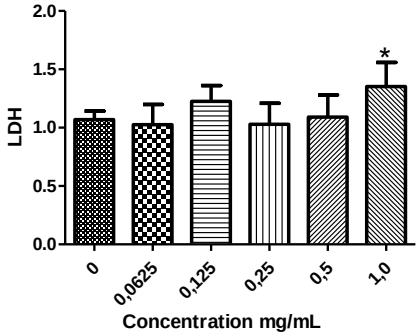
85


% Viability
150
100
50
0
*
**
***
***
0
0,0625
0,125
0,25
0,5
1
Concentration mg/mL

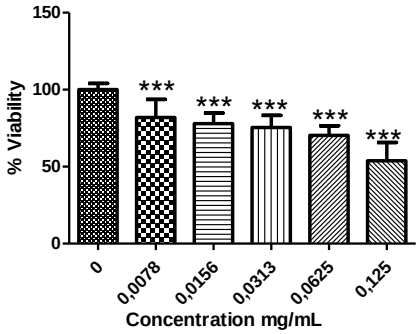

LDH
1.5
1.0
0.5
0.0
*
0
0,25
0,5
1,0
Concentration mg/mL

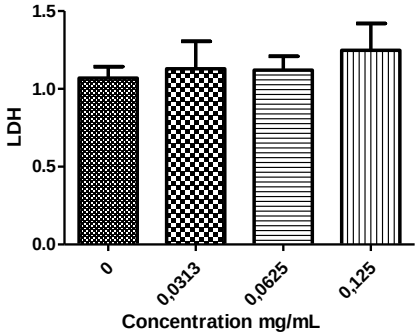
86


% Viability
150
100
50
0
***
***
***
***
***
0
0,0625
0,125
0,25
0,5
1
Concentration mg/mL

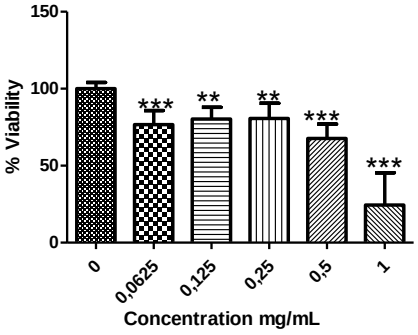

LDH
2.0
1.5
1.0
0.5
0.0
*
0
0,0625
0,125
0,25
0,5
1,0
Concentration mg/mL

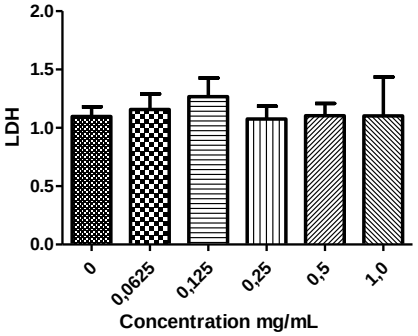
E_2


% Viability
150
100
50
0
***
***
***
***
***
0
0,0078
0,0156
0,0313
0,0625
0,125
Concentration mg/mL


LDH
1.5
1.0
0.5
0.0
0
0,0313
0,0625
0,125
Concentration mg/mL

E_3


% Viability
150
100
50
0
***
**
**
***
***
0
0,0625
0,125
0,25
0,5
1
Concentration mg/mL


LDH
2.0
1.5
1.0
0.5
0.0
0
0,0625
0,125
0,25
0,5
1,0
Concentration mg/mL

71

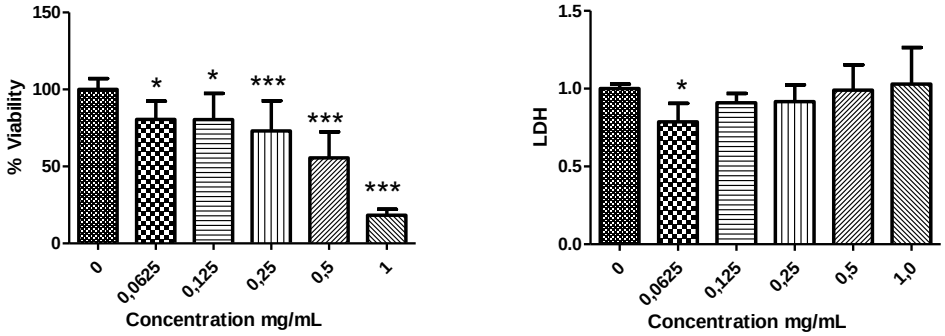


Figure 32. Effect of the samples related to *F. vulgare*, *Ginkgo Biloba* and *C. majus* on viability (A) and integrity of the cell membrane (B) in the AGS cell line. Cells were incubated with the different concentrations presented for each of the samples at 37 ° C for 24 hours. *p<0.05, p**<0.01, ***p<0.001 compared to the respective control.

For the concentrations used we can verify that the extract in DCM of the *F. vulgare* does not present a relevant toxicity in the range of concentrations selected and the highest concentration used was 0.125 mg/mL. In the case of the estragole **66**, compound isolated from the previous extract, at a concentration of 1mg/mL presents on average 41.1% of cell viability. The assay of the epoxidation of the estragole **89** was also carried out and, as we can see, it does not present a significant decrease in the viability as its concentration increases as in the case of the estragole. We conclude that the estragole **66** will be more toxic to the cell line under study compared to the epoxidized analogue.

Relatively to *G. biloba*, the DCM extract **79** (with its main constituent GA), the protected GA **86** and the alkylated GA **85** were tested. In the case of GA **79**, there were no major changes in cell viability, even at concentrations of 1 mg/mL. The viability study of the protected GA **86** presents significant changes in relation to GA **79**. At the lowest concentration used in the assay, 0.0625 mg/mL, compound **86** already exhibits a cell viability of 65% and at the highest concentration, 1 mg/mL, has cell viability of 33%. The toxicity provoked by the alkylated GA **85**, did not present a toxicity equivalent to the compound **86**.

Finally, the toxicity of the DCM extract of *C. majus* $\mathbf{E_2}$, such as its ethanol extract $\mathbf{E_3}$ and the compound isolated coptisine **71**, was tested. The assay realized for extract $\mathbf{E_2}$ was performed at low concentrations. And it showed 50% of viability at the concentration of 0.125 mg/mL. In the case of the ethanolic extract of *C. majus* $\mathbf{E_3}$, this presents a little variation as the

concentration of the sample increases. However, the compound isolated from this extract, coptisine **71,** shows a high toxicity at 1mg/mL, it was possible to verify about 20% of cell viability for the AGS cell line.

Evaluating the results obtained from the LDH assays we concluded that, except for the estragole **66** and *C. majus* ethanol extract **E_3**, the study samples present positive LDH. That is, LDH release occurred due to rupture of the membrane, evidencing cellular necrosis. Since the compound **66** and extract **E_3** did not show the LDH released at the concentrations tested in the study, its toxicity appears to be unrelated to processes leading to cell necrosis and consequently the release of LDH to the outside of cell by rupture of the cellular membrane.

CONCLUSIONS AND FUTURE PERSPECTIVES

Extracts from species *Ginkgo Biloba*, *F. vulgare*, and *C. majus* were obtained by Soxhlet extraction and their chemical composition was partial confirmed using various analytical techniques (NMR, IR, UV-Vis, HPLC and mass spectrometry). In the case of the extract obtained from *Ginkgo Biloba* leaves, ginkgolic acid was isolated as a major compound. Considering its biological potential, the synthesis of analogues was carried out. In the case of *F. vulgare,* estragole was isolated and it was used in the synthesis of the corresponding epoxide.

Lipidic nanoencapsulation and evaluation of biological activity of some extracts were carried out. Encapsulation studies showed high efficiencies up to 99% in soybean lecithin, with *C. majus* displaying the most promising results. Biological assays indicated that *F. vulgare* and *C. majus* show higher toxicity/lower viability in AGS cell line.

Future work should include full chemical characterization of the extracts and synthesis of derivatives of isolated compounds supported by molecular modelling studies. In particular, synthesis of GA derivatives will be pursued in order to obtain compounds with increased biological activity. Biological activity studies of extracts before and after nanoencapsulation will also be under study.

CHAPTER 3: EXPERIMENTAL

3.1. MATERIALS AND INSTRUMENTS

Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker Avance III 400 apparatus at a frequency of 400 MHz to ^{1}H and 100.6 MHz to ^{13}C, using the solvent peak as an internal reference, at 25°C. The chemical shifts are reported in ppm, using the value δ_H Me_4Si = 0 ppm as reference, and the coupling constants (*J*) are in Hz. The assignment of the ^{1}H and ^{13}C signals was effected by comparison of chemical shifts, multiplicity of peaks and *J* values, and it was also used double-decoupling techniques, as well as two-dimensional heteronuclear spectroscopic correlation, namely HMQC and HMBC. The deuterated solvent used was chloroform with deuteration degree greater than 99.8% of Eurisotop®. When necessary the vegetable matter was dried in an oven. TLC analyzes were performed on 0.25 mm thick silica gel plates (Merck Fertigplatten Kieselgel 60F254) and in the development, visual detection and ultraviolet light (λ_{max} 254 nm) were used in a CN-6 chamber. Column and dry flash chromatographies used Kieselgel Merck 60 (230-400 mesh). The ultraviolet/visible absorption spectra of the compounds/extracts for the nanoencapsulation study were obtained on a Shimadzu UV/2501 PC spectrophotometer. For the encapsulation, a commercial lipid mixture, soybean lecithin (Sternchemie) containing (% mol/mol) 22% phosphatidylcholine, 20% phosphatidylethanolamine, 20% phosphatidylinositol and 10% phosphatidic acid were as main components, with a concentration of 1×10^{-3} M. The amicons used were from Amicon Ultra, Millipore. The solvents used were p.a. products supplied by Fisher Chemical, Panreac or Carlo Erba. The samples were also sent to Santiago de Compostela (Spain) at the CACTUS building in the unit of Mass Spectrometry and Proteomics and analyzed by GC-APCI-QTOF (timsTOF).

3.2. PLANT MATERIAL

The *Gingko Biloba* leaves were collected in Tenões-Nogueiró, Braga, from September of 2017 to May of 2018. Leaves of green and yellow color were used due to the changes of seasons. For extraction only the leaves and fresh material were used.

The plant material corresponding to the *F. vulgare* was collected on a wild plot in Aveleda, Braga. For extraction only the stem and fresh material were used. Material was collected from November 2017 to May 2018.

Plant materials of *Chelidonium majus* (leaves, stems and flowers) were collected from Paderne Melgaço in mid-September of 2017. The material was dried in the oven (Heracus) at a temperature of 40-45°C for 24 hours. Then the dried material was crushed using a shredder (Moulinex) and then sieved through a sieve with a porosity of less than 910 μm.

3.3. GENERAL EXTRACTION PROCEDURE WITH SOXHLET

To a flask containing the solvent indicated was coupled a Soxhlet having a cellulose cartridge where the plant material was placed (*Ginkgo Biloba*, *F. vulgare*, *Chelidonium majus*). The extraction system was assembled (Figure 33) and the solvent was refluxed, and the contents of the cartridge were sometimes replaced with new plant material to obtain a larger amount of extract. At the end the solvent was evaporated to give the different extracts as solids or oils. After determination of the mass of each extract when appropriate, column or dry flash chromatography on silica gel was performed.

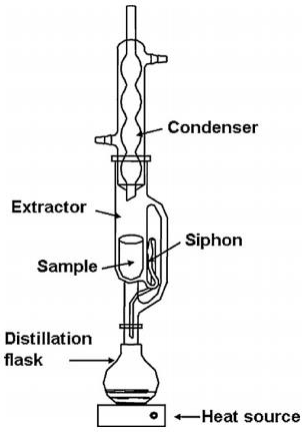
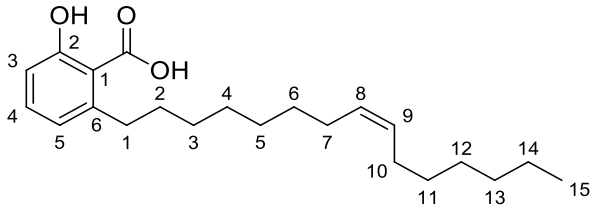


Figure 33. Schematic representation of extraction by Soxhet.[136]

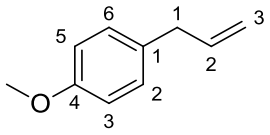
3.3.1. Extraction of *Ginkgo Biloba* components



79

Ginkgo Biloba (60.898 g) and DCM (750 mL), reflux time of 4 hours; the 2-hydroxy-6-(pentadec-8-en-1-yl) benzoic acid **79** was obtained as a green oily solid (1,0284 g). R_f = 0.39 (DCM:MeOH, 9:1). ν_{max} (neat): 2950, 2852, 2348, 1782, 1709 cm^{-1}. ^1H NMR δ_H($CDCl_3$, 400 MHz): 11.12 (sl, 1 H, CO_2H), 7.36 (t, *J* = 0.8 Hz, 1H, H-4), 6.87 (dd, *J* = 8.4 e 1.2 Hz, 1H, H-3), 6.77 (dd, *J* = 7.6 and 0.8 Hz, 1H, H-5), 5.35 (t, *J* = 4.8 Hz, 2 H, *H*C=C*H*), 2.98 (t, *J* = 8.0 Hz, 2H, Ph-CH_2), 1.99-2.08 (m, 4H, 2×CH_2), 1.54-1.68 (m, 2H, CH_2), 1.26-1.35 (m, 16H, 8 x CH_2), 0.86-0.94 (m, 3 H, CH_3) ppm. ^{13}C NMR δ_C($CDCl_3$, 100.6 MHz): 175.25 (*C*O_2H), 163.55 (C-2), 147.64 (C-6), 135.21 (C-4), 129.89 (H*C*=*C*H), 129.87 (H*C*=*C*H), 122.66 (C-5), 115.77 (C-3), 110.50 (C-1), 36.46 (Ph*C*H_2), 31.95 (CH_2), 29.80 (CH_2), 29.75 (CH_2), 29.68 (CH_2), 29.62 (CH_2), 29.58 (CH_2), 29.47 (CH_2), 29.23 (CH_2), 22.67 (*C*H_2CH_3), 13.97 (CH_3) ppm. HRMS m/z (ESI): calc for $C_{22}H_{35}O_3$ [M^+ +1] 347.25807; found 347.25779.

3.3.2. Extraction of *F. vulgare* component



66

Using fennel (70.8849 g) and DCM (350 mL), reflux time of 4 hours, extract obtained as a greenish oily solid (0.964 g). Flash chromatography (DCM): three fractions were obtained, being the whitish solid identified as 1-allyl-4-methoxybenzene (0.0792 g); R_f = 0.75 (TLC: DCM). ν_{max} (Nujol): 3403, 1738, 1609 cm^{-1}. ^1H NMR δ_H($CDCl_3$, 400 MHz): 7.11 (d, *J* = 8.4 Hz, 2H, H-2 and H-6), 6.85 (d, *J* = 8.4 Hz, 2H, H-3 and H-5), 5.90-6.02 (m, 1H, C*H*=CH_2), 5.02-5.11 (m, 2H, CH=C*H*$_2$), 3.80 (s, 1H, OC*H*$_3$), 3.34 (d, *J* = 6.8 Hz, 2H, PhC*H*$_2$). ^{13}C NMR δ_C($CDCl_3$): 157.95 (C-1), 137.86 (*C*H=CH_2), 132.08 (C-4), 129.47 (C-3 and C-5), 115.38 (CH=*C*H$_2$), 113.87 (C-2 and C-6), 55.24 (O*C*H$_3$), 39.31 (Ph*C*H$_2$) ppm.

3.3.3. Extraction of *Chelidonium majus* components

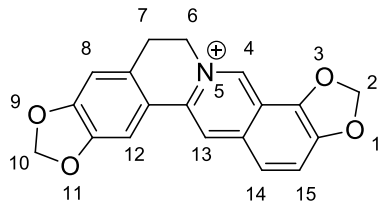
3.3.3.1. Procedure 1

Using *C. majus* (8.0235 g) and EtOH (350 mL), reflux time of 4 hours, the extract was obtained as a green oily solid **E_1** (2.9347 g). The extract was suspended in distilled water (50 mL) transferred to a separatory funnel, and a liquid-liquid extraction was carried out using petroleum ether (30 mL x 4). The organic solution was dried with anhydrous magnesium sulfate, and evaporated to give a solid material. The aqueous phase was sequentially subject to others liquid-liquid extraction with ethyl ether (30 mL x 4), ethyl acetate (30 mL x 4) and dichloromethane (30 mL x 4), following the same procedure as described above to give solid materials. The resulting aqueous phase obtained was evaporated to give also a solid material. R_f (initial extract in ethanol): 0.86 and 0.45.

Table 9. Results obtained in the successive washes of the ethanol extract of *C. majus*.

Solvent	Obtained mass (g)	Colour
Petroleum ether	0.151	Green
Ethyl ether	0.349	Green
Ethyl acetate	0.030	Green
Dichloromethane	0.288	Orange
Water	0.182	Yellow

3.3.3.2. Procedure 2



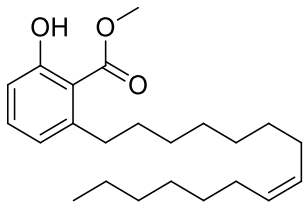
71

Using *C. majus* (8.0876 g) and DCM (350 mL), reflux time of 4 hours, the extract was obtained as a green oily solid **E_2** (0.722 g). Vegetable material was maintained in the cartridge and was subjected to a new extraction with EtOH (350 mL) with reflux time of 4 hours the extract obtained as a green oily solid **E_3** (0.337 mg). Addition of DCM result in two solids of different colors and polarities. A green solid soluble in DCM (0.155 g), which showed a complex mixture by TLC (MeOH) and a yellow solid which was only soluble in EtOH (0.182 g). The yellow solid was subjected to column chromatography (DCM/EtOH, mixture of increasing polarity). Using DCM:EtOH (90:10) as eluent, coptisine **71** (6,7-dihydro-[1,3]dioxolo[4',5':7,8]isoquinolino[3,2-a][1,3]dioxolo[4,5-g]isoquinolin-5-ium) was isolated as a yellow / orange solid (0.029 g). R_f = 0.20 (MeOH). ν_{max} (neat): 3379, 2947, 2834, 2524, 2345, 2222, 2045, 1724, 1660 cm^{-1}; ^1H NMR δ_H($CDCl_3$, 400 MHz): 9.90 (s, 1H, H-4), 8.91 (s, 1H, H-13), 8.01 (d, *J* = 8.8 Hz, 1H, H-14), 7.82 (d, *J* = 8.4 Hz, 2H, H-15), 7.76 (s, 1H, H-12), 7.06 (s, 1H, H-8), 6.51 (s, 2H, CH_2-2 or CH_2-10), 6.15 (s, 2H, CH_2-2 or CH_2-10), 4.85 (t, *J* = 6.0 Hz, 2H, CH_2-6), 2.49

(t, *J* = 6.0 Hz, 2H, CH_2-7). ^{13}C NMR δ_C($CDCl_3$, 100.6 MHz): 149.87 (Ar-C), 147.79 (Ar-C), 147.16 (Ar-C), 144.62 (C-4), 143.94 (Ar-C), 136.93 (Ar-C), 132.42 (Ar-C), 130.65 (Ar-C), 121.85 (C-15), 121.11 (Ar-C), 121.07 (C-13), 120.58 (C-14), 111.75 (Ar-C), 108.52 (C-8), 105.39 (C-12), 104.56 (C-2 or C-10), 102.17 (C-2 or C-10), 55.22 (C-6), 26,35 (C-7) ppm. HRMS m/z (TOF ESI): calc for $C_{19}H_{14}NO_4[M^+]$ 320.0917 found 320.0917.

3.4. SYNTHESIS OF ANALOGUES

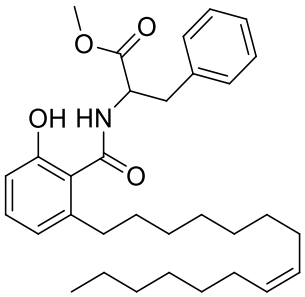
3.4.1. Attempting esterification of ginkgolic acid



80

To the ginkgolic acid **79** (0.4 mmol, 0.170 g) in an ice bath thionyl chloride (2 mL) was added. The mixture was heated at 60°C and kept under stirring for 8 hours. Then, dried MeOH (2 mL) was added dropwise and the mixture was stirred at room temperature for 48 hours. After solvent evaporation, an oil was obtained, which, ^1H NMR ($CDCl_3$) did not correspond to the expected compound.

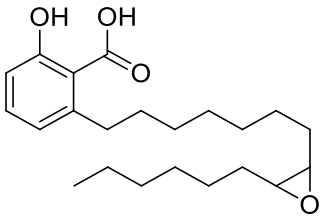
3.4.2. Attempting coupling of ginkgolic acid using DCC



81

To a solution of ginkgolic acid **79** (0.2 mmol, 0.070 g) in DMF (1 mL) was added 1-hydroxybenzotriazole (1 eq, 0.2 mmol, 0.031 g) and stirred for 10 minutes, at room temperature. *N*,*N*'-dicyclohexylcarbodiimide (1.1 eq, 0.22 mmol, 0.044 g) was then added and the mixture was stirred again for 10 minutes. A mixture of phenylalanine methyl ester hydrochloride (1 eq, 0.2 mmol, 0.043 g) in DMF (0.55 mL) and triethylamine (2 eq, 0.4 mmol, 55 mL) was added dropwise, at low temperature (ice bath), the reaction mixture was allowed to stand for 24 hours at room temperature. The reaction was monitored by TLC (DCM), and the ^{1}H NMR that showed that the reaction didn't occur, due the fact the signals of the starting GA were evident, such as of the reagents used in the reaction.

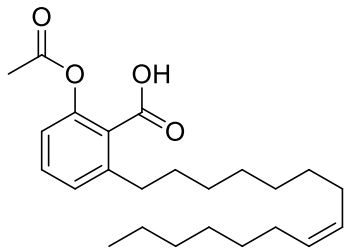
3.4.3. Attempting epoxidation of ginkgolic acid



82

To a solution of ginkgolic acid **79** (0.2 mmol, 0.070 g) in DCM (3 mL) at 0°C (ice bath), under stirring, *meta*-chloroperoxybenzoic acid (5eq, 1 mmol, 0.173 g) was added and the mixture was kept in these conditions for about 1 hour. Then the mixture was heated at 60°C, kept for 21 hours and the reaction was monitored by TLC. ^{1}H NMR ($CDCl_3$) showed that the brown oil obtained was not the expected compound, showing a complex mixture being impossible to identify the signals of the starting material and reagents used.

3.4.4. Attempting acetylation of ginkgolic acid

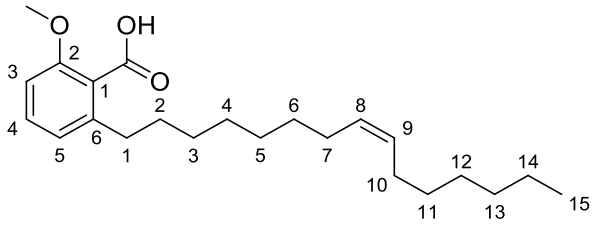


84

To a flask containing ginkgolic acid **79** (0.2 mmol, 0.070 g) were added acetic anhydride (1 eq., 0.2 mmol, $3.87x10^{-2}$ mL), and concentrated sulfuric acid (0.05 mL). The reaction mixture was stirred gently and heated at 50 to 60°C for 15 minutes.

The mixture was poured into distilled water (0.276 mL) and the precipitate formed was filtered out under reduced pressure, washed with cold distilled water and dried in the oven (40°C), to give a brown oil. ^{1}H NMR ($CDCl_3$) showed that the brown oil obtained was not the expected compound due to the complexity of the signals from the obtained mixture.

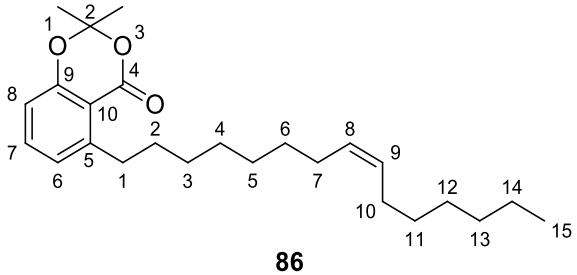
3.4.5. Synthesis of 2-methoxy-6-(pentadec-8-en-1-yl)benzoic acid 85



85

To a test tube containing perfluorohexane (0.428 mL), a solution of 1,2-dibromoethane (0.428mL) and dimethyl sulfate (0.055 mL) were added. After 5 minutes, when two layers remained, ginkgolic acid **79** (5.8×10^{-4} mol, 0.200 g) and NEt_3 (0.162 mL) dissolved in acetone (0.856 mL) were carefully added to the test tube from the uppermost layer. The tube was covered with aluminium foil and the bottom layer was stirred at room temperature for 2 days. The reaction was monitored by TLC (DCM). The acetone was evaporated, and the residue was dissolved with water (30 mL) and washed with DCM (3 x 30 mL). The organic phase was dried with anhydrous magnesium sulfate and the solvent removed by evaporation. R_f = 0.59 (DCM). ν_{max} (neat) 3322, 2921, 2852, 2348, 1854, 1730, 1660, 1607 cm^{-1}. ^1H NMR δ_H($CDCl_3$, 400 MHz): 11.13 (s, 1H), 10.97 (s, 1H), 7.34-7.30 (m, 2H), 6.89 (m, 2H), 6.79-6.72 (m, 2H), 5.36 (t, *J* = 4.4 Hz, 1H), 4.71 (t, *J* = 6 Hz, 1H), 3.96 (s, 1H), 2.96 (t, *J* = 8 Hz, 1H), 2.88 (t, *J* = 8 Hz, 1H), 1.31 (m, 58 H), 0.85 (m, 9H) ppm.

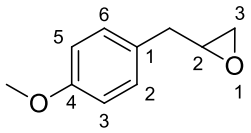
3.4.6. Synthesis of 2,2-dimethyl-5-(pentadec-8-en-1-yl)-4*H*-benzo[*d*][1,3] dioxin-4-one 86



86

Acetone (35 eq., 10.4 mmol, 0.22 mL) and thionyl chloride (2.4 eq., 0.69 mmol, 0.055 mL) were added to a solution of ginkgolic acid **79** (0.29 mmol, 0.100 g) and DMAP (0.015 mmol, 0.002 g) in 1,2-dimethoxyethane (0,217 mL). The reaction mixture was kept under stirring at 0 °C for 1 hour, and at room temperature for 24 hour. Saturated aqueous sodium hydrogen carbonate solution was added to the mixture, and the aqueous solution was extracted with DCM (25 mL × 3). The organic extract was dried over anhydrous magnesium sulfate, and evaporated under reduced pressure. Compound **86** was obtained as a colorless oil (0.057 g). R_f = 0.63 (DCM). ν_{max} (neat) 2950, 2852, 2348, 1782, 1709 cm^{-1}. ^1H NMR δ_H($CDCl_3$, 400 MHz): 7.40 (t, *J* = 7.6, 1H, H-4), 6.93 (d, *J* = 7.6 Hz, 1H, H-3), 6.80 (d, *J* = 8.0 Hz, 1H, H-6), 5.40-5.38 (m, 1H, *H*C=CH), 4.11-4.18 (m, 1H, *H*C=CH), 3.16-3.07 (m, 2H, Ph-CH_2), 1.70 (s, 6H, C(CH_3)$_2$), 1.65-1.25 (m, 24H, 11 x CH_2), 0.95-0.91 (m, 3H, CH_3) ppm. ^{13}C NMR δ_C($CDCl_3$, 100.6 MHz): 160.22 (*C*O), 157.10 (C-9), 148.52 (C-5), 148.48 (Ar-H), 135.02 (C-7), 125.06 (C-8), 115.03 (C-6), 112.07 (C-4a), 68.99 (H*C*=*C*H), 68.50 (H*C*=*C*H), 25.63 (CH_3 x 2), 13.88 (CH_3) ppm. HRMS m/z (ESI-TOF): calc for $C_{25}H_{39}O_3$ [M^+ +1] 387.2894 found 387.2893.

3.4.7. Synthesis of 2-(4-methoxybenzyl)oxirane 89



89

To perfluorohexane (0.50 mL) in a test tube was added *meta*-chloroperoxybenzoic acid (1.5 eq, 0.173 g) dissolved in dibromoethane (0.67 mL). The estragole **66** (0.100 g, 6.7 x 10^{-4} mol) was dissolved in DCM (0.50 mL) and added to the reaction mixture, which was left under constant stirring at room temperature, covered with aluminum foil for 48 hours.

The obtained mixture was diluted in water (30 mL) and washed with DCM (3 x 30 mL). The organic phase was dried with anhydrous magnesium sulfate, filtered and the solvent was removed by evaporation. Compound **89** was obtained as a white oily solid (0.046 g). Rf =0.51 (DCM). ν_{max} (neat): 2916, 2848, 1726, 1710 cm^{-1}. ^1H NMR δ_H($CDCl_3$, 400 MHz): 7.18 (d, *J* = 8.4 Hz, 2H, H-2 and H-6), 6.88 (d, *J* = 8.8 Hz, 2 H, H-3 and H-5), 3.81 (s, 3H, OCH_3), 3.15-3.11 (m, 1H, CH_2CH), 2.88 (dd, *J* = 14.4 and 5.2 Hz, 1H), 2.80-2.75 (m, 2H, $PhCH_2$), 2.54 (dd, 1H, *J* = 5.2 and 2.8 Hz, 1H) ppm.

3.5. NANOENCAPSULATION STUDIES

For nanoencapsulation studies, the extracts obtained from Soxhlet of *F. vulgare* and *C. majus* were used. Absorption spectra measurements were performed in the UV-Visible range to determine the absorption region of each of the selected extracts. Dilutions were then performed at concentrations of 0.1×10^{-5}-4.5×10^{-5} M for the measurement of calibration curves and determination of the encapsulation efficiency. Liposomes were prepared by the ethanolic injection method,[137,138] using a commercial lipid mixture, soybean lecithin (Sternchemie), containing (% mol/mol) 22% phosphatidylcholine, 20% phosphatidyl-ethanolamine, 20% phosphatidylinositol and 10% of phosphatidic acid as the major

components at a concentration of 1×10^{-3} M. Extracts/compounds at 2×10^{-5} M concentration were added to the lipid mixture and then pooled dropwise, under vortex, in a buffer solution of Tris-HCl pH = 7.3. The formulations were placed in Amicon™ filter tubes and centrifuged for 10 min. at 3000 rpm. During centrifugation, there was a separation of the encapsulated and non-encapsulated compound. The non-encapsulated fraction was evaporated, and 2 mL of ethanol were added. The absorbance of three independent assays for each compound was measured, and the concentration of compound that was not encapsulated was determined using the Lambert-Beer law (equation 1), through a calibration curve previously obtained

$$A = \varepsilon \times l \times c \quad (1)$$

where A is the absorbance, ε is the molar absorption coefficient, l is the optical path and c is the concentration of the sample. The encapsulation efficiency, EE (%), was obtained through equation 2.

$$EE(\%) = \frac{Total\ amount - Amount\ of\ nonencapsulated\ extract}{Total\ amount} \times 100 \quad (2)$$

3.6. CELL CULTURE

Cells were thawed in DMEN(1x)+GlutaMax™-1 (Dulbecco's Modified Eagle Medium) (45 mL) culture medium for AGS, enriched with 1% penicillin/streptomycin and 10% FBS (fetal bovine serum) at 37%. After centrifugation of 210*g* for 5 minutes, the supernatant was discarded and the deposit was resuspended in 15 ml of medium at 37°C. To proceed to a 2D culture column, a volume of cell suspension suitable for the desired growth was placed in a 300 mL flask, with culture medium (15 mL). The conditions described were adapted from da Silva *et al.*, 2017[139] and from Pereira *et al.*, 2014.[140]

3.6.1. Cell viability MTT

After obtaining about 80% confluency from the flask, the medium was discarded and the flask washed twice with of HBSS (Hank's balanced salt solution) (8 mL) purchased from Gilbco. Thereafter, 0.25% Trypsin (25 mL) was placed and the flask was brought to 37°C and 5% carbon dioxide for 3 minutes. Then medium (7 mL) was added and the entire volume of the flask was collected and centrifuged at 1300 rpm for 3 minutes at 37°C. The supernatant was discarded and the deposit resuspended in medium (4 mL).

In one eppendorf were placed trypan blue (0.090 mL) and the cell suspension (0.010 mL). A Neubauer chamber was filled with this solution to count the number of cells present in the cell suspension.

After 24 hours, successive dilutions of the test compounds were prepared, using up to 0.5% DMSO (except estragole). The medium from each well was aspirated and each dilution of the compound (0.100 mL) in triplicate was placed as described in previously reported work.[139–141]

The MTT substrate was prepared in a solution with the medium and added to the cultured cells in each well after removal of the test compound in a 1:10 portion. An incubation was carried out for 2 hours at 37°C, after which the MTT solution was removed. The formazan formed was dissolved in a mixture of DMSO:isopropanol (3:1) (0.200 mL). The absorbance was then measured at 570 nm using a Thermo Scientific ™ Multiskan ™ GO microplate reader.

3.6.2. Cell viability LDH

The solution present in each well (0.020 mL) was withdrawn into a new 96 well plate after 24 hours of incubation of the test compound as previously described by Pereira, *et al*. 2015, and Silva *et al.*, 2017.[139,142] The pyruvate solution (0.025 mL) and a solution of NADH (0.230 mL), both in 3 mg/20 mL concentrations, were added. These two solutions were prepared in phosphate buffer at pH 7.4 and 1% Triton X-100 was used as the positive control.

The integrity of the cell membrane was measured by the absorbance recorded at 340 nm in a microplate reader Thermo Scientific ™ Multiskan ™ GO.

CHAPTER 4: BIBLIOGRAPHY

4.1. BIBLIOGRAPHY

(1) Bravo, L. Polyphenols: Chemistry, Dietary Sources, Metabolism, and Nutritional Significance. *Nutr. Rev.* **1998**, *56*, 317–333.

(2) Alasalvar, C., Grigor, J. M., Zhang, D., Quantick, P. C., & Shahidi, F. Comparison of Volatiles, Phenolics, Sugars, Antioxidant Vitamins, and Sensory Quality of Different Colored Carrot Varieties. *J. Agric. Food Chem.* **2001**, *49*, 1410–1416.

(3) 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* (1), 191–203.

(4) Fresco, P.; Borges, F.; Diniz, C.; Marques, M. P. M. New Insights on the Anticancer Properties of Dietary Polyphenols. *Med. Res. Rev.* **2006**, *26* (6), 747–766.

(5) Ren, W.; Qiao, Z.; Wang, H.; Zhu, L.; Zhang, L. Flavonoids: Promising Anticancer Agents. *Med. Res. Rev.* **2003**, *23* (4), 519–534.

(6) Cai, Y.; Luo, Q.; Sun, M.; Corke, H. Antioxidant Activity and Phenolic Compounds of 112 Traditional Chinese Medicinal Plants Associated with Anticancer. *Life Sci.* **2004**, *74* (17), 2157–2184.

(7) Cai, Y. Z.; Mei Sun; Jie Xing; Luo, Q.; Corke, H. Structure-Radical Scavenging Activity Relationships of Phenolic Compounds from Traditional Chinese Medicinal Plants. *Life Sci.* **2006**, *78* (25), 2872–2888.

(8) Dixon, R. A., P. M. Dey, and C. J. L. *Phytoalexins: Enzymology and Molecular Biology*; 1983.

(9) Tsuchiya, H.; Sato, M.; Miyazaki, T.; Fujiwara, S.; Tanigaki, S.; Ohyama, M.; Tanaka, T.; Iinuma, M. Comparative Study on the Antibacterial Activity of Phytochemical Flavanones against Methicillin-Resistant Staphylococcus Aureus. *J. Ethnopharmacol.* **1996**, *50* (1), 27–34.

(10) Huang, W. Y.; Cai, Y. Z.; Zhang, Y. Natural Phenolic Compounds from Medicinal Herbs and Dietary Plants: Potential Use for Cancer Prevention. *Nutr. Cancer* **2010**, *62* (1), 1–20.

(11) Schofield, P., Mbugua, D., & Pell, A. Analysis of Condensed Tannins: A Review. *Anim.*

Feed Sci. Technol. **2001**, 21–40.

(12) Haslam E. Natural Polyphenols (Vegetable Tannins) as Drug: Possibles Modes of Action. *J. Nat. Prod.* **1996**, *59*, 205–215.

(13) Karuppusamy, S. A Review on Trends in Production of Secondary Metabolites from Higher Plants by in Vitro Tissue, Organ and Cell Cultures. *J. Med. Plants Res.* **2009**, *3* (13), 1222–1239.

(14) Barbosa-Filho, J. M.; Piuvezam, M. R.; Moura, M. D.; Silva, M. S.; Lima, K. V. B.; da-Cunha, E. V. L.; Fechine, I. M.; Takemura, O. S. Anti-Inflammatory Activity of Alkaloids: A Twenty-Century Review. *Rev. Bras. Farmacogn.* **2006**, *16* (1), 109–139.

(15) Chang, T.-H.; Hsieh, F.-L.; Ko, T.-P.; Teng, K.-H.; Liang, P.-H.; Wang, A. H.-J. Structure of a Heterotetrameric Geranyl Pyrophosphate Synthase from Mint (Mentha Piperita) Reveals Intersubunit Regulation. *Plant Cell* **2010**, *22* (2), 454–467.

(16) Dewick, P. M. *Medicinal Natural Products: A Biosynthetic Approach*; Sons, J. W. &, Ed.; 2002.

(17) Holubec, H.; Baines, A. T.; Bayse, J. L.; Waite, S.; Bhattacharyya, A. K.; Clark, L. C.; Payne, C. M.; Earnest, D. L.; Nelson, M. A. Chemoprevention of Colon Carcinogenesis by Dietary Selenomethionine. *Gastroenterology* **1998**, *114* (4 PART 2), A612.

(18) Trombetta, D.; Castelli, F.; Sarpietro, M. G.; Venuti, V.; Cristani, M.; Daniele, C.; Saija, A.; Mazzanti, G.; Bisignano, G.; Grazia, M. Mechanisms of Antibacterial Action of Three Monoterpenes Mechanisms of Antibacterial Action of Three Monoterpenes. *J. Antimicrob. Agents Chemother.* **2005**, *49* (6), 2474–2478.

(19) Khan, M. T. H.; Ather, A.; Thompson, K. D.; Gambari, R. Extracts and Molecules from Medicinal Plants against Herpes Simplex Viruses. *Antiviral Res.* **2005**, *67* (2), 107–119.

(20) Wong, K.-L.; Lin, J.-W.; Liu, J.-C.; Yang, H.-Y.; Kao, P.-F.; Chen, C.-H.; Loh, S.-H.; Chiu, W.-T.; Cheng, T.-H.; Lin, J.-G.; et al. Antiproliferative Effect of Isosteviol on Angiotensin-II-Treated Rat Aortic Smooth Muscle Cells. *Pharmacology* **2006**, *76* (4), 163–169.

(21) Peana, A. T.; Aquila, P. S. D.; Panin, F.; Serra, G.; Pippia, P.; Moretti, M. D. L. Anti-Inflammatory Activ Ity of Linalool and Linaly l Acetate Constituents of Essential Oils. **2002**, 721–726.

(22) Ziegler, H. L.; Jensen, T. H.; Christensen, J.; Stærk, D.; Hägerstrand, H.; Sittie, A. A.; Olsen, C. E.; Staalsø, T.; Ekpe, P.; Jaroszewski, J. W. Possible Artefacts in the in Vitro

Determination of Antimalarial Activity of Natural Products That Incorporate into Lipid Bilayer: Apparent Antiplasmodial Activity of Dehydroabietinol, a Constituent of Hyptis Suaveolens. *Planta Med.* **2002**, *68* (6), 547–549.

(23) Figueiredo, A. C.; Pedro, L. G.; Barroso, J. G. PLANTAS AROMÁTICAS E MEDICINAIS - Óleos Essenciais e Voláteis. *Rev. da APH* **2007**, *114*, 29–33.

(24) El-baz, F. K.; Mahmoud, K.; El-senousy, W. M.; Darwesh, O. M.; Elgohary, A. E. Eucalyptus Camaldulensis Essential Oils. **2015**, *31* (50), 262–268.

(25) Peixoto, L. R.; Rosalen, P. L.; Ferreira, G. L. S.; Freires, I. A.; de Carvalho, F. G.; Castellano, L. R.; de Castro, R. D. Antifungal Activity, Mode of Action and Anti-Biofilm Effects of Laurus Nobilis Linnaeus Essential Oil against Candida Spp. *Arch. Oral Biol.* **2017**, *73*, 179–185.

(26) Bakkali, F.; Averbeck, S.; Averbeck, D.; Idaomar, M. Biological Effects of Essential Oils - A Review. *Food Chem. Toxicol.* **2008**, *46* (2), 446–475.

(27) Bauer, K., Garbe, D., Surburg, H. *Common Fragrance and Flavor Materials: Preparation, Properties and Uses*; 2001.

(28) Burt, S. Essential Oils: Their Antibacterial Properties and Potential Applications in Foods—a Review. *Int. J. Food Microbiol. 94*, 223 – 253.

(29) Delaquis, P. J.; Stanich, K.; Girard, B.; Mazza, G. Antimicrobialactivity of Individual and Mixedfractions of Dill, Cilantro, Coriander and Eucalyptus Essential Oils. *Int. J. Food Microbiol.* **2002**, *74*, 101–109.

(30) Lens-Lisbonne, C., Cremieux, A., Maillard, C., Balansard, G. Methodes d'evaluation de l'activite Antibacterienne Des Huiles Essentielles: Application Aux Essences de Thym et de Cannelle. *J. Pharm. Belg. 42* (5), 297– 302.

(31) Lawrence, B. M. The Botanical and Chemical Aspects of Oregano. *Perfum. Flavorist* **1984**, *9*, 41– 51.

(32) Daferera, D. J.; Ziogas, B. N.; Polissiou, M. G. GC-MS Analysis of Essential Oils from Some Greek Aromatic Plants and Their Fungitoxicity on Penicillium Digitatum. *J. Agric. Food Chem.* **2000**, *48* (6), 2576–2581.

(33) Demetzos, C.; Perdetzoglou, D. K.; Tan, K. Composition and Antimicrobial Studies of the Oils of Origanum Calcaratum Juss. and O. Scabrum Boiss. et Heldr. from Greece. *J. Essent. Oil Res.* **2001**, *13* (6), 460–462.

(34) Marino, M.; Bersani, C.; Comi, G. Antimicrobial Activity of the Essential Oils of Thymus Vulgaris L. Measured Using a Bioimpedometric Method. *J. Food Prot.* **1999**, *62* (9), 1017–1023.

(35) Daferera, D. J.; Ziogas, B. N.; Polissiou, M. G. The Effectiveness of Plant Essential Oils on the Growth of Botrytis Cinerea, Fusarium Sp. and Clavibacter Michiganensis Subsp. Michiganensis. *Crop Prot.* **2003**, *22* (1), 39–44.

(36) Pintore, G.; Usai, M.; Bradesi, P.; Juliano, C.; Boatto, G.; Tomi, F.; Chessa, M.; Cerri, R.; Casanova, J. Chemical Composition and Antimicrobial Activity of Rosmarinus Officinalis L. Oils from Sardinia and Corsica. *Flavour Fragr. J.* **2002**, *17* (1), 15–19.

(37) Angioni, A.; Barra, A.; Coroneo, V.; Dessi, S.; Cabras, P. Chemical Composition, Seasonal Variability, and Antifungal Activity of Lavandula Stoechas L. Ssp. Stoechas Essential Oils from Stem/Leaves and Flowers. *J. Agric. Food Chem.* **2006**, *54* (12), 4364–4370.

(38) Kubitzki K. In: Kramer K, Greech PS, E. *The Families and Genera of Vascular Plants—pteridophytes and Gymnosperms*; 1990.

(39) Hori T. In: Ridge RW, Tulecke W, Del Tredici P, Tremouillaux Guiller, Tobe JH, E. *Ginkgo Biloba— a Global Treasure. From Biology to Medicine.*; 1997.

(40) Rimmer, C. A.; Howerton, S. B.; Sharpless, K. E.; Sander, L. C.; Long, S. E.; Murphy, K. E.; Porter, B. J.; Putzbach, K.; Rearick, M. S.; Wise, S. A.; et al. Characterization of a Suite of Ginkgo-Containing Standard Reference Materials. *Anal. Bioanal. Chem.* **2007**, *389* (1), 179–196.

(41) Singh, B.; Kaur, P.; Gopichand; Singh, R. D.; Ahuja, P. S. Biology and Chemistry of Ginkgo Biloba. *Fitoterapia* **2008**, *79* (6), 401–418.

(42) Vanbeek, T. A.; Lelyveld, G. P. Concentration of Ginkgolides and Bilobalide in Ginkgo-Biloba Leaves in Relation To the Time of Year. *Planta Med.* **1992**, *58* (5), 413–416.

(43) van Beek, T. A. Chemical Analysis of Ginkgo Biloba Leaves and Extracts. *J Chromatogr A* **2002**, *967* (1), 21–55.

(44) Fu, Y.; Hong, S.; Li, D.; Liu, S. Novel Chemical Synthesis of Ginkgolic Acid (13:0) and Evaluation of Its Tyrosinase Inhibitory Activity. *J. Agric. Food Chem.* **2013**, *61* (22), 5347–5352.

(45) Fuzzati, N.; Pace, R.; Villa, F. A Simple HPLC-UV Method for the Assay of Ginkgolic Acids in Ginkgo Biloba Extracts. *Fitoterapia* **2003**, *74* (3), 247–256.

(46) van Beek, T. A.; Montoro, P. Chemical Analysis and Quality Control of Ginkgo Biloba Leaves, Extracts, and Phytopharmaceuticals. *J. Chromatogr. A* **2009**, *1216* (11), 2002–2032.

(47) Hemshekhar, M.; Sebastin Santhosh, M.; Kemparaju, K.; Girish, K. S. Emerging Roles of Anacardic Acid and Its Derivatives: A Pharmacological Overview. *Basic Clin. Pharmacol. Toxicol.* **2012**, *110* (2), 122–132.

(48) Lü, J. M., Yan, S., Jamaluddin, S., Weakley, S. M., Liang, Z., Siwak, E. B. Ginkgolic Acid Inhibits HIV Protease Activity and HIV Infection in Vitro. *edical Sci. Monit. Int. Med. J. Exp. Clin. Res.* **2012**, *18* (8), 293–298.

(49) Hausen, B. M.; Berger, M. The Sensitizing Capacity of Coumarins (III). *Contact Dermatitis* **1989**, *21* (3), 141–147.

(50) Lepoittevin, J.-P.; Benezra, C.; Asakawa, Y. Allergic Contact Dermatitis to Ginkgo Biloba L.: Relationship with Urushiol. *Arch Dermatol Res* **1989**, *281*, 227–230.

(51) Durrani, B. A. A.; Tyman, J. H. P. Synthesis of 6-n-Alkylsalicylic Acids (and Isomeric Acids) from Fluoroanisoles with Alkyl-Lithium. *J. Chem. Soc* **1979**, *8*, 2079−2087.

(52) Tyman, J. H. P.; Visani, N. Synthesis of Saturated Anacardic Acids, and Alkenyl and Alkynyl Analogues. *J. Chem. Res. - Part S* **1997**, No. 1, 14–15.

(53) Fürstner, A.; Seidel, G. Shortcut Syntheses of Naturally Occurring 5-Alkylresorcinols with DNA-Cleaving Properties. *J. Org. Chem.* **1997**, *62* (8), 2332–2336.

(54) Unversity, T. B. NII-Electronic Library Service. **2008**.

(55) Satoh, M.; Takeuchi, N.; Fujita, T.; Yamazaki, K.; Nishimura, T.; Tobinaga, S. Improved Synthesis of Anacardic Acids, 6-Pentadecylsalicylic Acid and 6-[8(Z)-Pentadecenyl]Salicylic Acid, from Aldehyde and Acetoacetate. *Chem. Pharm. Bull. (Tokyo).* **1999**, *47* (8), 1115–1116.

(56) Satoh, M.; Takeuchi, N.; Nishimura, T.; Ohta, T.; Tobinaga, S. Synthesis of Anacardic Acids, 6-[8(*Z*),11(*Z*)-Pentadecadienyl]Salicylic Acid and 6-[8(*Z*),11(*Z*),14-Pentadecatrienyl]Salicylic Acid. *Chem. Pharm. Bull.* **2001**, *49* (1), 18–22.

(57) Hadfield, A.; Schweitzer, H.; Trova, M. P.; Green, K. Practical, Large-Scale Synthesis of 2,2-Dimethyl-5-Hydroxy-4-Oxo-Benzo-1,4-Dioxin. *Synth. Commun.* **1994**, *24* (7), 1025–1028.

(58) Uchiyama, M.; Ozawa, H.; Takuma, K.; Matsumoto, Y.; Yonehara, M.; Hiroya, K.;

Sakamoto, T. Regiocontrolled Intramolecular Cyclizations of Carboxylic Acids to Carbon-Carbon Triple Bonds Promoted by Acid or Base Catalyst. *Org. Lett.* **2006**, *8* (24), 5517–5520.

(59) Zhang, P.; Pan, J.; Duan, W.; Li, X.; Zhang, Y.; Zhou, Y.; Jiang, Q.; Mao, Z.; Yu, P. Synthesis of Ginkgolic Acid Analogues and Evaluation of Their Molluscicidal Activity. *Molecules* **2011**, *16* (5), 4059–4069.

(60) Kanowski, S.; Herrmann, W. M.; Stephan, K.; Wierich, W.; Hörr, R. Proof of Efficacy of the Ginkgo Biloba Special Extract EGb 761 in Outpatients Suffering from Mild to Moderate Primary Degenerative Dementia of the Alzheimer Type or Multi-Infarct Dementia. *Pharmacopsychiatry* **1996**, *29* (2), 47–56.

(61) Kleijnen, J.; Knipschild, P. Ginkgo Biloba for Cerebral Insufficiency. *Br. J. Clin. Pharmacol.* **1992**, *34* (4), 352–358.

(62) Dubey, A. K., Shankar, P. R., Upadhyaya, D., & Deshpande, V. Y. Ginkgo Biloba--an Appraisal. *Kathmandu Univ. Med. J. (KUMJ)* **2004**, *2*, 225–229.

(63) Braquet, P.; Hosford, D. Ethnopharmacology and the Development of Natural PAF Antagonists as Therapeutic Agents. *J. Ethnopharmacol.* **1991**, *32* (1–3), 135–139.

(64) Satyan, K. S.; Jaiswal, A. K.; Ghosal, S.; Bhattacharya, S. K. Anxiolytic Activity of Ginkgolic Acid Conjugates from Indian Ginkgo Biloba. *Psychopharmacology (Berl).* **1998**, *136* (2), 148–152.

(65) Lin, S. Y., & Chang, H. P. Induction of Superoxide Dismutase and Catalase Activity in Different Rat Tissues and Protection from UVB Irradiation after Topical Application of Ginkgo Biloba Extracts. *Methods Find. Exp. Clin. Pharmacol.* **1997**, *19* (6), 367–371.

(66) Pavela, R.; Žabka, M.; Bednář, J.; Tříska, J.; Vrchotová, N. New Knowledge for Yield, Composition and Insecticidal Activity of Essential Oils Obtained from the Aerial Parts or Seeds of Fennel (Foeniculum Vulgare Mill.). *Ind. Crops Prod.* **2016**, *83*, 275–282.

(67) Rather, M. A., Dar, B. A., Sofi, S. N., Bhat, B. A., & Qurishi, M. A. Foeniculum Vulgare: A Comprehensive Review of Its Traditional Use, Phytochemistry, Pharmacology, and Safety. *Arab. J. Chem.* **2016**, *9*, S1574–S1583.

(68) Zoubiri, S., Baaliouamer, A., Seba, N., & Chamouni, N. Chemical Composition and Larvicidal Activity of Algerian Foeniculum Vulgare Seed Essential Oil. *Arab. J. Chem.* **2014**, *7*, 480–485.

(69) Díaz-Maroto, M. C.; Pérez-Coello, M. S.; Esteban, J.; Sanz, J. Comparison of the Volatile Composition of Wild Fennel Samples (Foeniculum Vulgare Mill.) from Central Spain. *J. Agric. Food Chem.* **2006**, *54* (18), 6814–6818.

(70) Gross, M.; Lewinsohn, E.; Tadmor, Y.; Bar, E.; Dudai, N.; Cohen, Y.; Friedman, J. The Inheritance of Volatile Phenylpropenes in Bitter Fennel (Foeniculum Vulgare Mill. Var. Vulgare, Apiaceae) Chemotypes and Their Distribution within the Plant. *Biochem. Syst. Ecol.* **2009**, *37* (4), 308–316.

(71) Delaram, M., Kheiri, S., Hodjati, M. R. Comparing the Effects of Echinop Hora-Platyloba, Fennel and Placebo on Pre-Menstrual Syndrome. *J. Reprod. Infertil.* **2011**, *12* (3), 221–226.

(72) Kooti, W., Ghasemiboroon, M., Asadi-Samani, M., Ahangarpoor, A.; Abadi, A., Noori, M. The Effects of Hydro-Alcoholic Extract of Celery on Lipid Profile of Rats Fed a High Fat Diet. *Adv. Environ. Biol.* **2014**, *8*, 325–330.

(73) Ostad, S. N.; Soodi, M.; Shariffzadeh, M.; Khorshidi, N.; Marzban, H. The Effect of Fennel Essential Oil on Uterine Contraction as a Model for Dysmenorrhea, Pharmacology and Toxicology Study. *J. Ethnopharmacol.* **2001**, *76* (3), 299–304.

(74) Pai, M. B., Prashant, G. M., Murlikrishna, K. S., Shivakumar, K. M., & Chandu, G. N. Antifungal Efficacy of Punica Granatum, Acacia Nilotica, Cuminum Cyminum and Foeniculum Vulgare on Candida Albicans: An in Vitro Study. *Indian J. Dent. Res.* **2010**, *21*, 334.

(75) Yao, J. Y., Li, X. L., Shen, J. Y., Pan, X. Y., Hao, G. J., Xu, Y., ... & Liu, X. L. Isolation of Bioactive Components from Chelidonium Majus L. with Activity against Trichodina Sp. *Aquaculture* **2011**, *318*, 235–238.

(76) Cunha, A. P., & Roque, O. R. *Plantas Medicinais de Farmacopeia Portuguesa, Constituintes, Controlo, Farmacologia e Utilização*; 2008.

(77) Ćirić, A.; Vinterhalter, B.; Šavikin-Fodulović, K.; Soković, M.; Vinterhalter, D. Chemical Analysis and Antimicrobial Activity of Methanol Extracts of Celandine (Chelidonium Majus L.) Plants Growing in Nature and Cultured in Vitro. *Arch. Biol. Sci.* **2008**, *60* (1).

(78) Gilca, M.; Gaman, L.; Panait, E.; Stoian, I.; Atanasiu, V. Chelidonium Majus - An Integrative Review: Traditional Knowledge versus Modern Findings. *Forsch. Komplementarmed.* **2010**, *17* (5), 241–248.

(79) Biswas, S. J., & Khuda-Bukhsh, A. R. Effect of a Homeopathic Drug, Chelidonium, in Amelioration of p-DAB Induced Hepatocarcinogenesis in Mice. *BMC Complement. Altern. Med.* **2002**, *2*, 4.

(80) Barnes, J., Anderson, L., Philipson, D. *Herbal Medicines: A Guide for Healthcare*; 2007.

(81) Colombo, M. L., & Bosisio, E. Pharmacological Activities of Chelidonium Majus l.(Papaveraceae). *Pharmacol. Res.* **1996**, *33*, 127–134.

(82) Vavreckova, C., Gawlik, I., Muller, K. Benzophenanthridine Alkaloids of Chelidonium Majus; I. Inhibition of 5- and 1 2-Lipoxygenase by a Non-Redox Mechanism. *Planta Med* **1996**, *62* (1), 397–401.

(83) Rica Capistrano, I.; Wouters, A.; Lardon, F.; Gravekamp, C.; Apers, S.; Pieters, L. In Vitro and in Vivo Investigations on the Antitumour Activity of Chelidonium Majus. *Phytomedicine* **2015**, *22* (14), 1279–1287.

(84) El-Readi, M. Z.; Eid, S.; Ashour, M. L.; Tahrani, A.; Wink, M. Modulation of Multidrug Resistance in Cancer Cells by Chelidonine and Chelidonium Majus Alkaloids. *Phytomedicine* **2013**, *20* (3–4), 282–294.

(85) Paul, A.; Das, J.; Das, S.; Samadder, A.; Khuda-Bukhsh, A. R. Poly (Lactide-Co-Glycolide) Nano-Encapsulation of Chelidonine, an Active Bioingredient of Greater Celandine (Chelidonium Majus), Enhances Its Ameliorative Potential against Cadmium Induced Oxidative Stress and Hepatic Injury in Mice. *Environ. Toxicol. Pharmacol.* **2013**, *36* (3), 937–947.

(86) Cahlíková, L., Opletal, L., Kurfürst, M., Macakova, K., Kulhánková, A., & Hostálková, A. Acetylcholinesterase and Butyrylcholinesterase Inhibitory Compounds from Chelidonium Majus (Papaveraceae). *Nat. Prod. Commun.* **2010**, *5*, 1751–1754.

(87) Li, W., Fan, T., Zhang, Y., Niu, X., & Xing, W. Effect of Chelerythrine against Endotoxic Shock in Mice and Its Modulation of Inflammatory Mediators in Peritoneal Macrophages through the Modulation of Mitogen-Activated Protein Kinase (MAPK) Pathway. *Inflammation* **2012**, *35*, 1814–1824.

(88) Brunhofer, G.; Fallarero, A.; Karlsson, D.; Batista-Gonzalez, A.; Shinde, P.; Gopi Mohan, C.; Vuorela, P. Exploration of Natural Compounds as Sources of New Bifunctional Scaffolds Targeting Cholinesterases and Beta Amyloid Aggregation: The Case of Chelerythrine. *Bioorganic Med. Chem.* **2012**, *20* (22), 6669–6679.

(89) Cheng, R. B., Chen, X., Liu, S. J., & Zhang, G. H. Effect of Chelerythrine on Glucosyltransferase and Water-Insoluble Glucan of Streptococcus Mutans. *Shanghai kou qiang yi xue= Shanghai J. Stomatol.* **2007**, *16*, 324–327.

(90) H, P.; S, T.; A, P.; A, B.-K.; N, V.; Al., U. M. Protective Effect of Trans-Resveratrol on Gentamicin-Induced Nephrotoxicity. *Antioxid Redox Signal* **2002**, *4* (6), 893–898.

(91) Liang J, Zeng F, Guo A, Liu L, Guo N, L. L. et al. Microarray Analysis of the Chelerythrine-Induced Transcriptome of Mycobacterium Tuberculosis. *Curr. Microbiol.* **2011**, *62*, 1200–1208.

(92) Li, W. F.; Hao, D. J.; Fan, T.; Huang, H. M.; Yao, H.; Niu, X. F. Protective Effect of Chelerythrine against Ethanol-Induced Gastric Ulcer in Mice. *Chem. Biol. Interact.* **2014**, *208* (1), 18–27.

(93) Feng, G.; Zhang, J.; Liu, Y. Q. Inhibitory Activity of Dihydrosanguinarine and Dihydrochelerythrine against Phytopathogenic Fungi. *Nat. Prod. Res.* **2011**, *25* (11), 1082–1089.

(94) Vrba, J.; Doležel, P.; Vičar, J.; Modrianský, M.; Ulrichová, J. Chelerythrine and Dihydrochelerythrine Induce G1 Phase Arrest and Bimodal Cell Death in Human Leukemia HL-60 Cells. *Toxicol. Vitr.* **2008**, *22* (4), 1008–1017.

(95) Beuria, T. K.; Santra, M. K.; Panda, D. Sanguinarine Blocks Cytokinesis in Bacteria by Inhibiting FtsZ Assembly a Bundling. *Biochemistry* **2005**, *44* (50), 16584–16593.

(96) Vrba, J.; Doležel, P.; Vičar, J.; Ulrichová, J. Cytotoxic Activity of Sanguinarine and Dihydrosanguinarine in Human Promyelocytic Leukemia HL-60 Cells. *Toxicol. Vitr.* **2009**, *23* (4), 580–588.

(97) Niu, X.; Fan, T.; Li, W.; Huang, H.; Zhang, Y.; Xing, W. Protective Effect of Sanguinarine against Acetic Acid-Induced Ulcerative Colitis in Mice. *Toxicol. Appl. Pharmacol.* **2013**, *267* (3), 256–265.

(98) Jeng, J. H.; Wu, H. L.; Lin, B. R.; Lan, W. H.; Chang, H. H.; Ho, Y. S.; Lee, P. H.; Wang, Y. J.; Wang, J. S.; Chen, Y. J.; et al. Antiplatelet Effect of Sanguinarine Is Correlated to Calcium Mobilization, Thromboxane and CAMP Production. *Atherosclerosis* **2007**, *191* (2), 250–258.

(99) Kuznetsova, L. P., Nikol'Skaia, E. B., Sochilina, E. E., & Faddeeva, M. D. The Inhibition Enzymatic Hydrolysis of Acetylthiocholine by Acetylcholinesterase Using Principal

Alkaloids Isolated from Celandine and Macleya and Their Derivatives. *Tsitologiia* **2001**, *43*, 1046–1050.

(100) Schmeller, T., Latz-Brüning, B., & Wink, M. Biochemical Activities of Berberine, Palmatine and Sanguinarine Mediating Chemical Defence against Microorganisms and Herbivores. *Phytochem. Lett.* **1997**, *47*, 257–266.

(101) Hattori T, Furuta K, Nagao T, Nagamatsu T, I. M.; Y., S. Studies on the Antinephritic Effect of Plant Components (4): Reduction of Protein Excretion by Berberine and Coptisine in Rats with Original-Type Anti- GBM Nephritis. *Jpn. J. Pharmacol.* **1992**, *59*, 159–169.

(102) Hamsa, T. P.; Kuttan, G. Antiangiogenic Activity of Berberine Is Mediated through the Downregulation of Hypoxia-Inducible Factor-1, VEGF, and Proinflammatory Mediators. *Drug Chem. Toxicol.* **2012**, *35* (1), 57–70.

(103) Domitrović, R.; Jakovac, H.; Blagojević, G. Hepatoprotective Activity of Berberine Is Mediated by Inhibition of TNF-α, COX-2, and INOS Expression in CCl4-Intoxicated Mice. *Toxicology* **2011**, *280* (1–2), 33–43.

(104) Hayashi, K.; Minoda, K.; Nagaoka, Y.; Hayashi, T.; Uesato, S. Antiviral Activity of Berberine and Related Compounds against Human Cytomegalovirus. *Bioorganic Med. Chem. Lett.* **2007**, *17* (6), 1562–1564.

(105) Kuznetsova, L. P., Nikol'Skaia, E. B., Sochilina, E. E., & Faddeeva, M. D. The Inhibition Enzymatic Hydrolysis of Acetylthiocholine by Acetylcholinesterase Using Principal Alkaloids Isolated from Celandine and Macleya and Their Derivatives. *Tsitologiia* **2001**, *43* (11), 1046–1050.

(106) Guo, J., Wang, S. B., Yuan, T. Y., Wu, Y. J., Yan, Y., Li, L., ... & Du, G. H. Coptisine Protects Rat Heart against Myocardial Ischemia/Reperfusion Injury by Suppressing Myocardial Apoptosis and Inflammation. *Atherosclerosis* **2013**, *231*, 384–391.

(107) Hattori, T., Furuta, K., Nagao, T., Nagamatsu, T., Ito, M., & Suzuki, Y. Studies on the Antinephritic Effect of Plant Components (4): Reduction of Protein Excretion by Berberine and Coptisine in Rats with Original-Type Anti-GBM Nephritis. *Jpn. J. Pharmacol.* **1992**, *59*, 159–169.

(108) Gong, L. L., Fang, L. H., Qin, H. L., Lv, Y., & Du, G. H. Analysis of the Mechanisms Underlying the Vasorelaxant Action of Coptisine in Rat Aortic Rings. *Am. J. Chin. Med.* **2012**, *40* (02), 309–320.

(109) *Natural Products Isolation*; Satyajit D. Sarker, Zahid Latif, A. I. G., Ed.; Springer Science & Business Media, 2006.

(110) Ncube, N. S.; Afolayan, A. J.; Okoh, A. I. Assessment Techniques of Antimicrobial Properties of Natural Compounds of Plant Origin: Current Methods and Future Trends. *African J. Biotechnol.* **2008**, *7* (12), 1797–1806.

(111) Boutekedjiret, C.; Bentahar, F.; Belabbes, R.; Bessiere, J. M. Extraction of Rosemary Essential Oil by Steam Distillation and Hydrodistillation. *Flavour Fragr. J.* **2003**, *18* (6), 481–484.

(112) Yani, S.; Gao, X.; Grayling, P.; Wu, H. Steam Distillation of Mallee Leaf: Extraction of 1,8-Cineole and Changes in the Fuel Properties of Spent Biomass. *Fuel* **2014**, *133*, 341–349.

(113) Silva, M. G. F. Atividade Antioxidante e Antimicrobiana in Vitro de Óleos Essenciais e Extratos Hidroalcóolicos de Manjerona (Origanum Majorana l .) e Manjericão (Ocimum Basilicum L .). *Trab. conclusão curso, apresentado à Comissão Diplomação do Curso Bacharelado em Química da Univ. Tecnológica Fed. do Paraná (UTFPR), Campus Pato Branco, como requisito parcial para obtenção do título Bacharel em Química.* **2011**, *1* (1), 01-70.

(114) Sartor, R. B. *Modelagem, Simulação e Otimização de Uma Unidade Industrial de Extração de Óleos Essenciais Por Arraste a Vapor*; 2009.

(115) Ozel, M. Z.; Kaymaz, H. Superheated Water Extraction, Steam Distillation and Soxhlet Extraction of Essential Oils of Origanum Onites. *Anal. Bioanal. Chem.* **2004**, *379* (7–8), 1127–1133.

(116) Masango, P. Cleaner Production of Essential Oils by Steam Distillation. *J. Clean. Prod.* **2005**, *13* (8), 833–839.

(117) Luque de Castro, M. D.; García-Ayuso, L. E. Soxhlet Extraction of Solid Materials: An Outdated Technique with a Promising Innovative Future. *Anal. Chim. Acta* **1998**, *369* (1–2), 1–10.

(118) Chiasson, H.; Bélanger, A.; Bostanian, N.; Vincent, C.; Poliquin, A. Acaricidal Properties of Artemisia Absinthium and Tanacetum Vulgare (Asteraceae) Essential Oils Obtained by Three Methods of Extraction. *J. Econ. Entomol.* **2001**, *94* (1), 167–171.

(119) Moyler, D. A. Extraction of Essential Oils with Carbon Dioxide. *Flavour Fragance J.* **1993**, *8* (January), 235–247.

(120) Fang, Z. & Bhandari, B. Encapsulation of Polyphenols - Review. *Trends Food Sci. Technol.* **2010**, *21*, 510–523.

(121) Agnihotri, S. A.; Mallikarjuna, N. N.; Aminabhavi, T. M. Recent Advances on Chitosan-Based Micro- and Nanoparticles in Drug Delivery. *J. Control. Release* **2004**, *100* (1), 5–28.

(122) Pinho, E.; Grootveld, M.; Soares, G.; Henriques, M. Cyclodextrins as Encapsulation Agents for Plant Bioactive Compounds. *Carbohydr. Polym.* **2014**, *101* (1), 121–135.

(123) Reis, C. P., Neufeld, R. J., & Veiga, F. *Nanoencapsulation I. Methods for Preparation of Drug-Loaded Polymeric Nanoparticles. Nanomedicine: Nanotechnology, Biology and Medicine*; 2006.

(124) Ezhilarasi, P. N.; Karthik, P.; Chhanwal, N.; Anandharamakrishnan, C. Nanoencapsulation Techniques for Food Bioactive Components: A Review. *Food Bioprocess Technol.* **2013**, *6* (3), 628–647.

(125) Rodríguez, J.; Martín, M. J.; Ruiz, M. A.; Clares, B. Current Encapsulation Strategies for Bioactive Oils : From Alimentary to Pharmaceutical Perspectives. *FRIN* **2016**, *83*, 41–59.

(126) Ndjoko, K.; Wolfender, J. L.; Hostettmann, K. Determination of Trace Amounts of Ginkgolic Acids in Ginkgo Biloba L. Leaf Extracts and Phytopharmaceuticals by Liquid Chromatography-Electrospray Mass Spectrometry. *J. Chromatogr. B Biomed. Sci. Appl.* **2000**, *744* (2), 249–255.

(127) Jana, N. K.; Verkade, J. G. Phase-Vanishing Methodology for Efficient Bromination, Alkylation, Epoxidation, and Oxidation Reactions of Organic Substrates. *Org. Lett.* **2003**, *5* (21), 3787–3790.

(128) De Sousa, I. S. C.; Castanheira, E. M. S.; Rocha Gomes, J. I. N.; Real Oliveira, M. E. C. D. Study of the Release of a Microencapsulated Acid Dye in Polyamide Dyeing Using Mixed Cationic Liposomes. *J. Liposome Res.* **2011**, *21* (2), 151–157.

(129) Azure, C. De; Gualtar, C. De. Lipid Interaction with Textile Fibres in Dyeing Conditions. *Colloid Polym. Sci.* **2004**, 88–93.

(130) Peters, S. E.; Brain, C. H. Benefits of a Soy Lecithin Based Nanotechnology for the Animal and Human Food Industry. *ACS Symp. Ser.* **2009**, *1007*, 183–197.

(131) Jaafar-Maalej, C.; Diab, R.; Andrieu, V.; Elaissari, A.; Fessi, H. Ethanol Injection Method for Hydrophilic and Lipophilic Drug-Loaded Liposome Preparation. *J. Liposome Res.*

2010, *20* (3), 228–243.

(132) Greg Mitchell, B.; Kiefer, D. A. Chlorophyll α Specific Absorption and Fluorescence Excitation Spectra for Light-Limited Phytoplankton. *Deep Sea Res. Part A, Oceanogr. Res. Pap.* **1988**, *35* (5), 639–663.

(133) Liu, Z. H.; Zeng, S. Cytotoxicity of Ginkgolic Acid in HepG2 Cells and Primary Rat Hepatocytes. *Toxicol. Lett.* **2009**, *187* (3), 131–136.

(134) Zhou, C.; Li, X.; Du, W.; Feng, Y.; Kong, X.; Li, Y.; Xiao, L.; Zhang, P. Antitumor Effects of Ginkgolic Acid in Human Cancer Cell Occur via Cell Cycle Arrest and Decrease the Bcl-2/Bax Ratio to Induce Apoptosis. *Chemotherapy* **2010**, *56* (5), 393–402.

(135) Colombo, M. L.; Bugatti, C.; Mossa, A.; Pescalli, N.; Piazzoni, L.; Pezzoni, G.; Menta, E.; Spinelli, S.; Johnson, F.; Gupta, R. C.; et al. Cytotoxicity Evaluation of Natural Coptisine and Synthesis of Coptisine from Berberine. *Farmaco* **2001**, *56* (5–7), 403–409.

(136) Luque de Castro, M. D.; Priego-Capote, F. Soxhlet Extraction: Past and Present Panacea. *J. Chromatogr. A* **2010**, *1217* (16), 2383–2389.

(137) Batzri, S.; Korn, E. D. Single Bilayer Liposomes Prepared without Sonication. *BBA - Biomembr.* **1973**, *298* (4), 1015–1019.

(138) Kremer, J. M. H.; Esker, M. W. J. V. D.; Pathmamanoharan, C.; Wiersema, P. H. Vesicles of Variable Diameter Prepared by a Modified Injection Method. *Biochemistry* **1977**, *16* (17), 3932–3935.

(139) da Silva, D. C.; Andrade, P. B.; Valentão, P.; Pereira, D. M. Neurotoxicity of the Steroidal Alkaloids Tomatine and Tomatidine Is RIP1 Kinase- and Caspase-Independent and Involves the EIF2α Branch of the Endoplasmic Reticulum. *J. Steroid Biochem. Mol. Biol.* **2017**, *171*, 178–186.

(140) Pereira, D.; Correia-da-Silva, G.; Valentao, P.; Teixeira, N.; Andrade, P. GC-MS Lipidomic Profiling of the Echinoderm Marthasterias Glacialis and Screening for Activity Against Human Cancer and Non-Cancer Cell Lines. *Comb. Chem. High Throughput Screen.* **2014**, *17* (5), 450–457.

(141) Silva, T. C.; de Andrade, P. B.; Paiva-Martins, F.; Valentão, P.; Pereira, D. M. In Vitro Anti-Inflammatory and Cytotoxic Effects of Aqueous Extracts from the Edible Sea Anemones Anemonia Sulcata and Actinia Equina. *Int. J. Mol. Sci.* **2017**, *18* (3).

(142) Pereira, R. B.; Taveira, M.; Valentão, P.; Sousa, C.; Andrade, P. B. Fatty Acids from Edible

Sea Hares: Anti-Inflammatory Capacity in LPS-Stimulated RAW 264.7 Cells Involves INOS Modulation. *RSC Adv.* **2015**, *5* (12), 8981–8987.