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Valorization of *Acacia melanoxylon* extracts for Biorefinery: phenolic content, antioxidant and anti-inflammatory activities.

Mestrado em Química
Especialização em Química

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2015

Acknowledgements

I want to thank to who shared this part traveled, contributing directly and indirectly, for me to perform this research, helping me and giving me strength at times when most needed.

My gratitude, first, goes to my advisers, PhD Amelia Pilar and PhD Luísa Bivar Roseiro, for having provided me unique "learning" through the great encouragement and guidance that I have been granted during this journey. Thanks also for all the patience, good humor and essentially the availability in helping me.

Thanks equally to Professor Alice Martins, who also provided me with great learning, not only for this work and to my future life. Thanks also for the constant joy, patience and availability, always very helpful.

Colleagues from the Laboratório dos Glúcidos, of Faculdade de Ciências da Universidade de Lisboa, for the welcome, availability, and the material conditions necessary to carry out the experimental part of this work.

To all the Laboratório Nacional de Geologia e Energia, particularly for Unidade Biológica, for all the mentoring that has always given to me, for the encouragement and motivation that always instilled in me by always demonstrating goodwill and above all for your friendship.

I thank especially to my parents that, despite the distance, supported me and motivated me to not give up, believing that I could give them this joy. Mom and Dad were tireless and always gave me support and love.

To my sister, my brother-in-law and my nephew for all the support, sense of humor and patiente to put up with my tantrums.

To all the family, grandfather, uncles, aunts and cousins, who cheered and believed in the completion of this course, I am very grateful.

To my friends, for the friendship and affection they have always shown me, the words of encouragement throughout my life and confidence that has always given me.

The whole school team work, Escola do II Ciclo do Ensino Secundário do Lubango; especially the directors, for the trust and opportunity they have given me for the dismissal of my work activities to make it possible the realization of this Master Degree.

To all my colleagues and fellow students for having supported the studing and working with me, and for helping me when I needed. Thank you!

Resumo

A floresta portuguesa ocupa hoje aproximadamente 3154800 hectares (ha), ou seja, cerca de 35 % do território continental. Estima-se que a produção total de resíduos seja de 5630 mil toneladas secas/ano e a biomassa produzida sem utilização é de 2628 mil toneladas secas/ano, provenientes principalmente da floresta e agricultura. Desta forma, o desperdício da biomassa vegetal tem-se mostrado cada vez mais uma preocupação, pois, para além de estar disponível em grandes quantidades, é potenciadora de fogos florestais, pelo que a sua valorização surge com uma alternativa bastante viável e urgente.

Os resíduos de muitas espécies florestais têm na sua composição compostos com potencial de valor acrescentado como por exemplo propriedades biológicas e funcionais, tornando-os adequados para serem utilizados nos mais diversos produtos, tais como produtos da indústria química, farmacêutica, cosmética e/ou alimentar.

O objectivo do presente trabalho centrou-se na avaliação do potencial bioativo de extratos de biomassa vegetal, e na obtenção de compostos naturais de interesse industrial, tais como compostos antioxidantes e anti-inflamatórios da espécie *Acacia melanoxylon* R. Br., valorizando assim estes resíduos e tornando-os economicamente viáveis.

A *Acacia melanoxylon* R. Br., vulgarmente conhecida como acácia-da-austrália, acácia-negra-da-austrália, acácia-negra e acácia-austrália, é também uma árvore da família Fabaceae (Leguminosae) nativa do Sudeste da Austrália, Tasmânia. É uma árvore de crescimento rápido que pode atingir 15-20 metros de altura. As suas folhas são ligeiramente em forma de foice e as flores apresentam uma cor amarela ou esbranquiçadas. Os frutos são vagens castanho-avermelhadas, compridas, contorcidas, cujas sementes se encontram completamente rodeadas por um funículo alaranjado.

A espécie *Acacia melanoxylon* R. Br. foi introduzida em Portugal em meados do século XIX, estando atualmente distribuída por todo o território continental, arquipélago dos Açores e arquipélago da Madeira. Esta espécie foi inicialmente introduzida no território português para fins ornamentais, tendo sido cultivada como espécie florestal, árvore de sombra e fixadora de solos. A madeira proveniente da casca da árvore foi desde sempre utilizada para a marcenaria, no fabrico de lanças, boomerangs, escudos e cordas nas regiões de origem. Atualmente, na Austrália, as aplicações da espécie *Acacia melanoxylon* R. Br. expandem-se ao fabrico de mobílias, devido à qualidade e aparência da madeira.

A *Acacia melanoxylon* R. Br. é uma das espécies mais invasoras em Portugal, Espanha, França e Itália. Apresenta uma elevada capacidade de invasão em ambientes poluídos, áridos, húmidos, ventosos e sujeitos a temperaturas extremas. Produz muitas sementes que podem permanecer no solo durante mais de 50 anos. Estas podem ser disseminadas pela água, vento, aves e roedores. Esta espécie forma povoamentos muito densos impedindo o desenvolvimento da vegetação nativa.

No sentido de complementar as linhas de investigação já descritas para esta espécie de *Acacia*, teve-se em conta uma forte componente de pesquisa em artigos científicos. Alguns estudos apontam para possíveis aplicações da espécie *Acacia melanoxylon* R. Br. na área da indústria. Resultados obtidos permitiram qualificar a madeira da *Acacia melanoxylon* R. Br. como uma potencial fonte de material lenhoso para a indústria nacional de serração e construção. Outros estudos mostram a potencial aplicação da madeira da *Acacia melanoxylon* R. Br. na produção de pasta de papel. Apesar das características biométricas do material fibroso da pasta de *A. melanoxylon* R. Br. serem significativamente diferentes das do eucalipto, nomeadamente maior colapsabilidade, os resultados experimentais demonstraram que a *Acacia melanoxylon* R.Br. pode ser utilizada na produção da pasta de papel quando os níveis de resistência mecânica exigidos ao papel não forem muito elevados.

No entanto, vários estudos noutras espécies de *Acacia* permitiram identificar alguns compostos fenólicos com ação anti-inflamatória, e também a existência de atividade inibidora da enzima xantina oxidase (XO), deixando em aberto a importância de continuar estes estudos no sentido de identificar compostos bioativos. Pretendeu-se ainda associar os resultados obtidos à informação disponível, de forma a fazer a correspondência com as demais espécies de *Acacia*.

Iniciou-se o trabalho experimental recorrendo-se ao método de hidrodestilação por Clevenger das partes aéreas (casca, folhas e ramos) da referida espécie, com o objectivo de obter óleos essenciais. As águas de decocção residuais deste processo foram estudadas para caracterização do perfil de compostos fenólicos. Avaliaram-se as composições em compostos fenólicos totais, não-taninos e taninos pelo método de Folin-Ciocalteau, utilizou-se o ácido gálico como padrão, expressando os resultados em GAE (Gallic Acid Equivalents). A actividade antioxidante foi determinada pelo método de captura do radical livre $DPPH^{\bullet}$ e pelo método $ABTS^{\bullet+}$, utilizando-se para ambos os métodos o Trolox como padrão e os resultados foram expressos em percentagem de inibição dos radicais e em TEAC (Trolox Equivalent Antioxidant Capacity). A determinação da capacidade de inibição de radicais livres, utilizando o radical estável $DPPH^{\bullet}$, é um método simples e rápido de avaliar a capacidade antioxidante. Com este método é possível determinar o poder antiradical de um antioxidante, através da medição do decréscimo da absorvância do radical $DPPH^{\bullet}$ a 515 nm. Outro método igualmente utilizado é o método $ABTS^{\bullet+}$, usado para avaliar a capacidade antioxidante total.

Após a avaliação e determinação da actividade antioxidante procedeu-se à análise e ao fracionamento dos extratos recorrendo à cromatografia em camada fina e à cromatografia em coluna. Analisaram-se as frações obtidas por CZE, tendo-se identificado diferentes compostos fenólicos, desde estruturas simples a complexas, alguns dos quais nunca reportados para o género *Acacia*. Utilizando o *software* de análise disponível, foi possível determinar as percentagens de aproximação (*matching*) dos dados das amostras analisadas com os dados dos padrões de compostos fenólicos disponíveis na base de dados do sistema.

Por fim, procedeu-se à determinação da actividade anti-inflamatória pelo método da inibição da desnaturação da albumina bovina (BSA), utilizando-se o ácido acetilsalicílico como padrão. Os resultados foram expressos em percentagem de inibição da albumina bovina. Não existem

na literatura estudos de actividade anti-inflamatória em espécies de acácias recorrendo a este método. No entanto, está descrita a determinação da actividade anti-inflamatória por outras metodologias noutras espécies de *Acacia* que não a *Acacia melanoxylon* R. Br., que referem o papel dos compostos fenólicos, particularmente das catequinas e ácidos fenólicos nas propriedades anti-inflamatórias destas espécies. Estes resultados suportam a pesquisa levada a cabo neste trabalho para a *Acacia melanoxylon* R. Br.

De acordo com o estado da arte, foram identificados pela primeira vez neste trabalho ácidos fenólicos e flavonóides nos extratos aquosos das folhas e ramos e da casca de *Acacia melanoxylon* R. Br., assim como se refere pela primeira vez a presença de ácido *O*-acetilsalisilico em espécies de *Acacia*.

A realização deste trabalho permitiu, não só complementar o estudo fitoquímico existente para esta espécie, como também avaliar o potencial bioativo dos compostos fenólicos identificados nesta espécie, potencializando assim a valorização da biomassa como uma fonte abundante de compostos naturais com actividade biológica, uma vez que os seus resíduos florestais são utilizados apenas como lenha. Espera-se futuramente poder dar continuidade a estas linhas de investigação para o desenvolvimento de produtos de valor acrescentado com base nestes compostos bioativos, úteis na indústria alimentar, farmacêutica e química.

O presente trabalho revelou o potencial de valorização dos resíduos florestais endógenos, nomeadamente da biomassa de *Acacia melanoxylon* R. Br., sendo essa valorização uma mais-valia tanto para a floresta portuguesa, reduzindo a quantidade de sobrantes, como para a economia portuguesa, através da obtenção de produtos de valor acrescentado e a respetiva comercialização. No caso do presente estudo, ficou demonstrado que essa valorização poderá passar pela obtenção de extratos ricos em compostos fenólicos com atividades antioxidante e anti-inflamatória.

Palavras-chave

Acacia melanoxylon R. Br.; biomassa; compostos fenólicos; actividades biológicas; produtos de valor acrescentado.

Abstract

The purpose of this study focused on the assessment of the potential of natural extracts from vegetable biomass of the species *Acacia melanoxylon* R. Br. to obtain compounds of industrial interest, such as antioxidants and anti-inflammatory compounds, enhancing these wastes and making them economically viable.

The experimental work used the hydrodistillation method with a Clevenger apparatus for the aerial parts (bark, leaves and branches) of *Acacia melanoxylon* species, to obtain essential oils. The residual decoction waters were evaluated for their phenolic composition. The decoction waters were evaluated for the total phenolic compounds, tannins and non-tannins by the Folin-Ciocalteau method using gallic acid as standard, expressing the results in GAE (gallic acid equivalents). The antioxidant activity was determined by the free radical $DPPH^{\bullet}$ capture method and the $ABTS^{\bullet+}$ method, using Trolox as standard.

Further characterization was done by fractionation of the extracts using column chromatography and thin layer chromatography. Fractions obtained were analyzed by CZE, having identified different phenolic compounds from simple to complex structures, some of which have never been reported for the genus *Acacia*.

Finally, the anti-inflammatory activity was determined by the bovine serum albumin (BSA) inhibition method, using acetyl salicylic acid as standard.

To the best of our knowledge, this is the first time that phenolic acids and flavonoids were identified in the aqueous extract of leaves/branches and bark of *Acacia melanoxylon*, and that the presence of *O*-acetylsalisylic acid is referred in *Acacia* species.

This work allowed, not only to supplement the existing phytochemical study for this species, but also to evaluate the potential of the bioactive phenolic compounds identified, enhancing thus the valorization of biomass as an abundant source of natural compounds with pharmacological activity. It is expected to continue these lines of research for development of value added products based on these bioactive compounds useful for the food, pharmaceutical and chemical industries.

Keywords

Acacia melanoxylon R. Br.; biomass; phenolic compounds; biological activities; value-added products.

List of Abbreviations

Abs – Absorbance

ABTS – [2,2-azinobis(3-ethylbenzotthizoline-6-sulphonic acid) diammoniun salt]

ASA – Acetyl salicylic acid

Bk – Bark

Brc – Branches

BSA – Bovine serum albumin

C – Bark

CC – Column chromatography

CE – Capillary electrophoresis

CZE – Capillary zone electrophoresis

DPPH – 2,2-diphenyl-1-picrylhydrazyl

dwb – Dry weight basis

EXT – Extract

FR – Leaves and Branches

GAE – Gallic acid equivalents

Lvs – Leaves

n.i. – No inhibition

n-T – Non-tannins

PVPP – Polyvinilpolypyrrolidone

RT – Room temperature

StdD – Standard deviation

T – Tannins

TEAC – Trolox equivalent antioxidant capacity

TLC – Thin layer chromatography

TP – Total phenolics

Trolox – (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid

UV – Ultra violet

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1. Introduction

As well as provide us with oxygen, food, clothing, perfumes and building blocks for numerous chemicals, plants are a source of a great diversity of compounds with medicinal and pharmaceutical application (Figueiredo et al. 2007). Thus, the search in plants for drugs against various diseases has been described since ancient civilizations. A widely exploited source of plants and its therapeutic application has been done until the present. Medicinal plants have always played an important role in the healing of various diseases throughout the history of mankind, and sometimes, as the only alternative (Neto and Caetano, 2005).

However, an interest in determining which were the active compounds of medicinal plants began only in the nineteenth century. A major achievement of these studies was the discovery of quinine obtained from the bark of chinchona tree (*Chinchona officinalis* L.) found in South America, which in popular medicine would be used in the treatment of malaria (Phillipson, 2001). In recent decades there has been a resurgence of interest in folk medicine and how this practice can combine with traditional medicine. The demand for more biologically active natural substances, was encouraged by their use as natural antimicrobials and antioxidants in food (Figueiredo et al., 2007).

The medicinal use of plants has assumed an important role in society because they have many properties that allow a cheaper and healthier treatment for some diseases compared to those produced by synthesis (Pires, 2010). Today, the benefits of natural products from plants are involved in various areas of human health, such as dietary supplements and in functional foods. Recently, the major impact of drugs derived from plants emerged in the antitumoral area where, for example, taxol, vinblastine, vincristine, and camptothecin that contributed to the effectiveness of chemotherapy against fatal cancers (Raskin et al., 2002).

Natural products include primary metabolites and secondary metabolites. The secondary metabolites are synthesized by only a few species of living beings, including plants and fungi, which may or may not become directly involved in their growth and development. These are mainly present in higher plants and have a restricted distribution, i.e. they are specific to certain species and their roles in metabolism are still unknown (Pires, 2010; Ribeiro, 2012).

The phenolic compounds are originated by the secondary metabolism of plants and are essential for their growth and reproduction. Also, these are formed under stress conditions as infections, injuries, ultraviolet radiation, and other (Naczk and Shahidi, 2004).

Phenolic compounds are a very diverse group of phytochemicals derivatives of phenylalanine and tyrosine. Phenolic compounds in plants are essential to their growth and reproduction, as already mentioned above. They also play a role as anti-pathogenic and pigmenting agents (Naczk and Shahidi, 2004). In food phenolic compounds are responsible for the color, astringency, aroma (Peleg et al., 1998) and oxidative stability (Naczk and Shahidi, 2004).

Chemically, phenolic compounds are defined as substances with aromatic ring with one or more hydroxyl substituents, which also includes their functional groups (Lee et al., 2005). Its variable structure gives phenolic compounds multifunctionality. According to Shahidi and Naczk (2004) there are about five thousand phenols, highlighting the flavonoids, phenolic acids, simple phenols, coumarins, tannins, lignins and tocopherols. Further, they may be in their free form or bound to sugars (glycosides), and proteins (Bravo, 1998).

The beneficial health effects that have been attributed to phenolic compounds present in fruits, vegetables, teas and wines are varied. Epidemiological studies have demonstrated multiple biological effects related to phenolic compounds, such as antioxidant activity, anti-inflammatory, antimicrobial, among others.

Phenolic acids are secondary metabolites and represent an important and dominant role in the human diet daily, in particular in foods derived from plants such as fruits and vegetables (Barros et al., 2009). They are characterized for presenting a benzene ring, a carboxylic acid group and one or more hydroxyl groups and/or methoxyl in the molecule to confer antioxidant activity (Soares, 2002).

This group includes benzoic acid, cinnamic acid and its derivatives, hydroxyl patterns and differing in methylation of the aromatic rings.

The most common hydroxycinnamic acids are caffeic acid, p-coumaric, ferulic and sinapic acids, which occur frequently with an ester linkage to small molecules (quinic and tartaric acids) or structural components such as cellulose, lignin and protein. The most abundant hydroxybenzoic acids are *p*-hydroxybenzoic acid, vanillic, protocatechuic, gallic and syringic, often being components of a complex structure such as lignins and hydrolysable tannins, or in the form of glycosides, and organic acids (Barros et al., 2009).

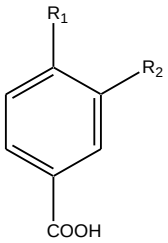
Table 1 shows the structures of the mentioned group of compounds.

Figure 1 - Chemical struture of phenolic acids. Phenolic acids found whith two substitutions on the:

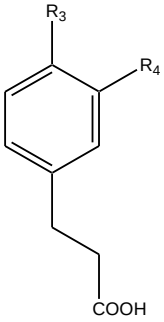
A. Phenyl ring on the 3- and 4- positions;

B. Some less commonly found with triple subtituions on the 3-, 4- and 5- positions

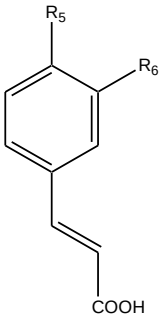
A



	R_1	R_2
benzoic acid	H	H
4- or *p*-hydroxybenzoic acid	OH	H
3- or *m*-hydrobenzoic	H	OH
protocatechuic acid	OH	OH
vanillic acid	OH	OCH_3
isovanillic acid	OCH_3	OH

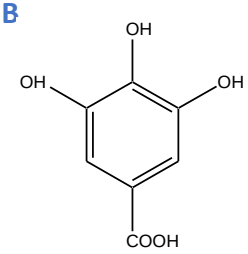


	R_3	R_4
phenylproplonic acid	H	H
4- or *p*-hydroxyphenypropionic acid	OH	H
3- or *m*-hydroxyphenypropionic acid	H	OH
3,4-dihydroxyphenypropionic acid	OH	OH
dihydroferulic acid	OH	OCH_3
dihydroisoferulic acid	OCH_3	OH

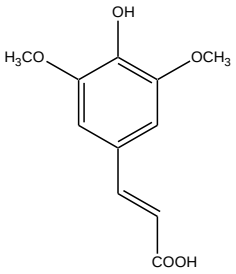


	R_5	R_6
cinnamic acid	H	H
p-coumaric	OH	H
m-coumaric	H	OH
caffeic acid	OH	OH
ferulic acid	OH	OCH_3
isoferulic acid	OCH_3	OH

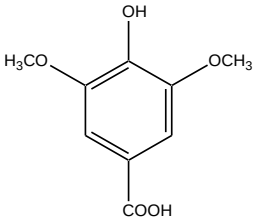
B



Gallic acid



Sinapic acid



Syringic acid

In the vast family of phenolic compounds, phenolic acids have attracted significant interest in recent years due to the potential benefits to human health. As polyphenolic compounds, these

are powerful antioxidants and it has been reported by its antibacterial activity, antiviral, anti-cancer, anti-inflammatory and vasodilatory actions (Mattila and Hellström, 2007).

Flavonoids are compounds widely distributed in the plant kingdom, present in fruits, leaves, seeds and other parts of plants as glycosides or aglycones. These compounds have low molecular weight, consisting of 15 carbon atoms arranged in a C6-C3-C6 configuration (Bravo, 1998). Its chemical structure consists of two aromatic rings, ring A and B, joined by three carbons form a heterocyclic ring, C-ring (Figure 1).

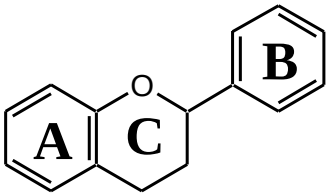


Figure 1 - Basic flavonoid structure.

The variations by replacing the standard C ring originate important classes of flavonoids such as flavonols, flavones, flavanones, catechins, anthocyanins and isoflavones (Hollman and Katan, 1999). In turn, the substitutions of the A and B rings give rise to different compounds within each class of flavonoids (Hollman and Katan, 1999).

As previously mentioned, the major structural categories of flavonoids are flavones, flavanones, flavonols, chalcones, catechins, anthocyanidins, isoflavones, isoflavanones and aurones whose structure are shown in Figure 2.

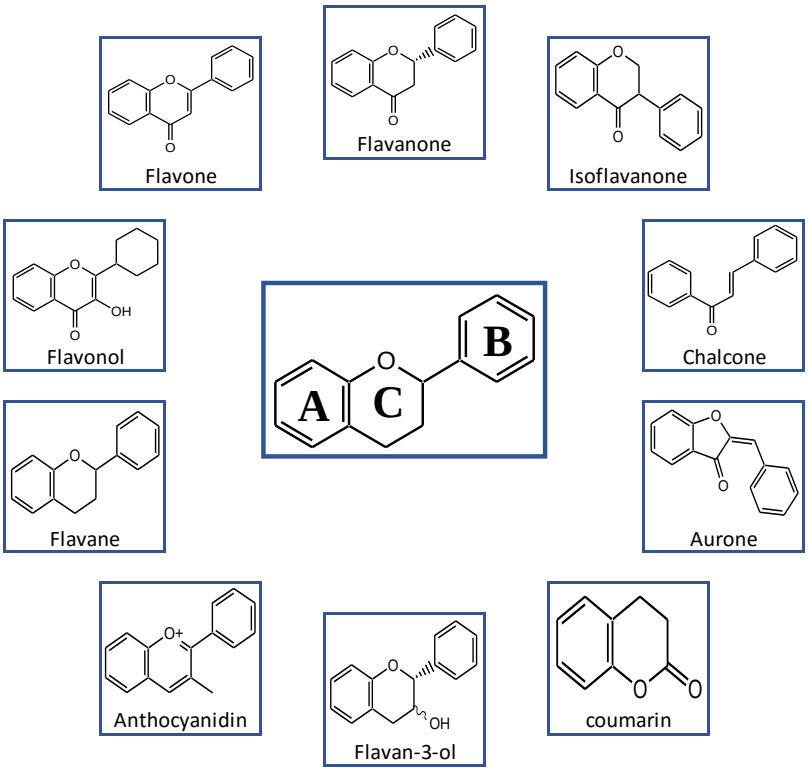


Figure 2 - Structures of the main flavonoid subgroups

1.1. Biomass and Biorefinery

Ecologically, biomass is the total amount of living matter present in an ecosystem or a plant or animal population. In power generation, the definition of biomass does not account for traditional fossil fuels, while also being derived from plant or animal life. This can still be considered as a renewable natural resource, unlike fossil fuels.

A biorefinery is a facility that integrates biomass conversion processes in biofuels, chemical inputs, materials, food, feed and energy. This includes several conversion routes (biochemical, microbial, chemical and thermochemical), looking for better use of the biomass and the energy contained therein.

The chemicals developed from co-products and residues are those with the greatest potential to add value to the supply chains of biomass, due to the strategic involvement of the chemical industry in the supply of inputs and final products to various sectors of the economy, as petrochemical, pharmaceutical, building, cosmetic, among others.

The abundant existence of raw materials and the disadvantages that invasive plants have the general direction of forest resources, argues that priority should be given to creating a market for the fuel based on plants, promoting the use of residual biomass and reducing the cleaning costs of the areas of interest (Direcção Geral de Recursos Florestais, 2006). Since the alien species are more adapted to the summer weather, such as *Acacia*, these are most promising for the production of biomass. Therefore, the costs involved in the control of invasive plants, mainly of Acacias in Portugal, are high and it takes several follow-up measures of the shares, hence the use of these as biomass material for energy production and/or bioproducts can be a very feasible and advantageous alternative (Santos et al., 2004).

The plant biomass, vegetable and forest waste resulting from industrial activity, is an abundant and renewable source of a great variety of phenolic compounds with potential bioactive activities. In the present work, the biomass of an *Acacia* species (namely the bark, branches and leaves) was used to study the possibility of valorizing this residue through value added compounds with biological activity that could be used in the food, pharmaceutical and chemical industries.

1.2. Gender *Acacia*

1.2.1. Botanic Characterization and Distribution

The *Acacia* are a gender of trees in the *Fabaceae* family, subfamily *Mimosoideae*, and is the second largest genus in the *Fabaceae* family, after the Genus *Astragalus*. They grow naturally in tropical climates, subtropical and warm temperate, as in Africa (144 species), Asia (89 species), America (185 species) and the Pacific region (993 species) (World Wide Wattle, 2015). It is composed of more than 1,400 species, of which more than 1,200 of these species are native to Australia. The taxonomic classification of this gender has gone through some changes along

the times, since Bentham (1675) to Maslin et al (2003) (Fernandes and Fernandes, 2012; Maslin et al., 2003).

Acacias from Australia were introduced in southwestern Europe during the nineteenth century, during a time of exaltation of exotic plants, where Australia was seen as the promised land of gardening (Fernandes and Fernandes, 2012). Several species were introduced in Portugal for their ornamental interest and economic potential as a source of raw materials (Breton et al., 2008; Kull et al., 2011). One of the promoters of Acacias in Portugal was Tait, a businessman who started in 1880 a plantation of *Acacia* and *Eucalyptus* trees on the outskirts of Abrantes. Therefore, Acacias from Australia have been used in Portugal since the nineteenth century in public forests, and coastal dune systems were fixed with the aid of various species, such as the *Acacia melanoxylon* (Fernandes and Fernandes, 2012).

One of the problems associated with the *Acacia* is its facility to spread, being considered in some regions, and particularly in Portugal, as an invasive species. By definition, an invasive plant is a naturalized plant that produces fertile and abundant offspring, with strong potential for dispersion of the parental plant (Lourenço, 2009; Richardson et al., 2000). Acacias threaten native habitats by competing with indigenous vegetation, replacing grass communities, reducing native biodiversity and increasing water loss from riparian zones. In addition to its great colonizing capacity, it typically dominates the colonized site, with understory species scarce and present only with low cover. At present, the species *Acacia dealbata*, *Acacia melanxylon* and *Acacia longifolia* are the most prolific invaders in France, Italy, Portugal and Spain, especially in conservation areas (Lorenzo et al., 2010).

Figure 3 - Some examples of plants from the genus *Acacia*.
A. *Acacia dealbata.*
B. *Acacia melanoxylon*

1.2.2. Ethnobotanical Uses

Ethnobotany attempts to understand the relationship between plants and people, studying how plants, in some way, are used by civilizations. In Portugal, the ethnobotanical studies are rare. Garcia da Orta had a very important role with his trip to India, where he practiced medi-

cine and developed studies on the use of plants in medicine (Rodrigues, 2001). The climate conditions in Portugal are favorable to a large number of species with high value for the pharmaceutical and cosmetic industries (Rodrigues, 2001).

Currently, the traditional applications of *Acacia* are preserved due to the growing interest of the scientific community for this gender.

Several authors tried to analyze the timber from *Acacia* for the production of paper. Santos et al. (2007) found that the paper produced by the *Acacia* has greater resistance to tearing.

In Portugal there are many spontaneous stands of *Acacia* species, including *Acacia dealbata* and *Acacia melanoxylon*. Although this is an invasive species, *Acacia melanoxylon* has not yet been considered as an ecological problem, despite adapting well the Portuguese conditions. Therefore, it is considered as a producer of alternative raw material for sawmills and paper industry, and can also minimize the risk of fire (Santos et al., 2007).

1.2.3. Phytochemical Characterization

Phytochemical research of plants generally involves the following steps: plant selection, collection, identification and authentication, extraction, extracts fractionation/separation, isolation of pure compounds, structural characterization, investigation of the biosynthetic pathways of a particular compound, quantitative assessments and pharmacological activities (Visht and Chaturvedi, 2012).

Despite the scarce information, there are some research papers reporting compounds that have been isolated from the aerial parts (bark, leaves, branches and flowers) from *Acacia* species, in particular phenolic compounds.

Reports regarding the isolation of phenolic groups in *Acacia melanoxylon* are rare, but Polya and Foo (1994) extracted catechins from this species.

In the various species of *Acacia,* different flavonoids can be found. Table 2 shows some compounds already disclosed and isolated from different species of *Acacia*.

Figure 2 - Description of flavonoid compounds identified in different species of *Acacia*

Acacia Species	Flavonoids Identified	Ref.
A. longifólia	naringerin 5-β-D-galactosyl-naringenin 5-β-D-glucosyl-naringenin aurone 4-*O*-galactosyl aureusidin gallo-catechin 5,2',5'-trihydroxy-6,7-dimetoxiflavanone	Anam (1997); Kerber (1993); Peitz (2003); Silva (2001); Trindade & Roux (1969)
A. catechu	kaempferol quercetin iso-rhamnetin dihydrokaempferol fisetin quercetagetin catechin	Azuine & Bhide (1992); Deshpande & Patil (1981); Hathway & Seakin (1957)
A. cyanophylla	quercetin 3-*O*-glucoside 4,2',4',6'-tetrahydroxichalcone-4-glycoside quercitrin kaempferol	Imperato (1978); Salem et al. (1997)
A. dealbata	rutin quercetin robinetin myricetin naringenin-5-diglycoside 6'-*O*-glycosyl-naringeninchalcone 6'-di-*O*-glycosyl-naringenin-chalcone	Imperaro (1982)
A. mearnsii	myricetin myricetin-3-rhamnoside quercitrin quercitrin-3-rhamnoside catechin galocatequin isoquercitrin mearnsetin myricetin-3-glucoside	Drewes & Isley (1968); Mackenzie (1967); Saayman & Roux (1965)
A. papova	rutin (quercetin-3-*O*-rutinoside)	Nauchini (1963)
A. auriculiformis	flavan-3,4-diol auriculoside 7,3',5'-trihydroxy-4'-methoxyflavan-3'-glucoside	Drewes & Roux (1966); Sahai (1980)

A. obtusifolia	teracacidine isoteracacidine	Clark_Lewis & Dainis (1961)
A. nilotica	naringenin catechin catechin-5-galoil ester	Khalid et al. (1986)
A. melanoxylon	3-D-galactosyl-quercetin di-hidroflavonois (-)2,3-cis-3',4',7,8-tetrahydroxydihydroflavonol (+)2,3-trans-3',4',7-trihydroxy-5-methoxy-dihydroflavonol (+)2,3-trans-3',4',7,8-tetrahydroxydihidroflavonol 2,3-cis-leucoantocianidin flavan-3,4-diol luteolin apigenin quercetin-3-glucoside	Falco & Vries (1964); Foo (1987); Gonzalez et al. (1995)
A. saligna	quercitrin astragalin (3-glycoside kaempferol) myricitrin 7-*O*-D-glycosyl naringenin naringenin 6-C-D-glucosyl-naringenin quercetin di-hydrokaempferol-8-C-glycoside, luteolin 7-*O*-glucoside 7-*O*-rutinoside apigenin 7-*O*-neo-hesperidoside myricetin-3-*O*-rhamnoside isorhamnetin-3-*O*-galactoside	El Sissi & El Sherbeiny (1967); El Sawi (2000)
A. ixiophylla	rhamnitrin quercetin di-glucoside quercitrin quercetin rhamnetin apigenin apigenin 7,4'-dimethyl ether (-) - epicatechin	Clark Lewis & Dai-nis (1961)
A. nigrescens	2,3-trans-flavan-3,4-cis-diol 3',4',7,8-tetra hydroxy-3-methoxyflavone 4',7,8-trihydroxy-3,3'-dimethoxyflavone 4',7,8-trihydroxy-hidroxiflavona	Fourier et al. (1974); Malan (1993)

A. farnesiana	apigenin diosmethin farnesin naringenin quercetin kaempferol	Thieme & Khogali (1974)
A. neovernicosa	2',4'-dihydroxy chalcone 4'-hydroxy-2'-methoxy chalcone 2',4'-dihydroxy-3'-methoxy chalcone 2',4',4-trihydroxy-chalcone-7-hydroxyflavanone isoliquiritigenin pinocembrin chrysin	Wollenweber & Seugler (1982)
A. gerrardii	(+) - catechin-3'-gallate (+) - catechin-4'-gallate (+) - catechin-7'-gallate (+) - catechin-4',7-digallate (+) - catechin 3',7-digallate	Malan & Pienaar (1987)
A. leucophloea	myricetin quercetin 3'-hydroxy-7-methoxyisoflavone apigenin apigenin 8-C-glycoside quercitrin kaempferol rutin	Rao et al. (1991) Valsakumari & Sulochana (1991)

1.2.4. Biological Activities

From an early age, *Acacia* species have been used by different populations in the world. In Australia, for example, the aborigines used various species of *Acacia* as their food, taking advantage of different parts of the same. The gums were collected and chewed, while the sap was harnessed to make a sweet drink (Australian Plants Online, 2015).

Multiple biological activities have been investigated in the extracts and compounds isolated from various *Acacia* species. For example, Dhawan and colleagues (1980) report that auriculoside showed a depressant activity of the central nervous system. Some species have flavonoids that have a molluscicidal activity against some parasites, and other exhibit antioxidant effects by the presence of polyphenols, such as flavonoids and tannins (Chang et al., 2001).

Chang et al. (2001) differentiated applications of the species according to the extracted compounds, as follows:

- The ethanol extracts of some *Acacia* species have a hypoglycemic action;
- The catechin derivatives inhibit protein kinases;
- The aqueous extracts exhibit anti-inflammatory, analgesic and antipyretic activities;
- The ethyl acetate extract have hapatoprotective properties;
- Some flavonoid groups decreased tumor cell proliferation.

Phenolic derivatives, flavonoids, tannins and coumarins have bioactive activities such as antimicrobial, antioxidant and anti-inflammatory activities. The inflammatory process is the body response to an injurious stimulus evoked by a wide variety of agents, e.g. infections, and diseases like atherosclerosis, diabetes, and others, are now considered to be conditions with a strong inflammatory component (Manohara Reddy et al., 2011). Medicines for the management of inflammation are non-steroidal anti-inflammatory drugs (NSAIDs), which are reported to have several adverse side-effects. Natural products have contributed significantly towards the development of modern therapeutically agents and traditional medicine is being re-evaluated worldwide, by extensive research on different plant species and their active principles. This fact is supported by the very recent Nobel Prize 2015 for medicine to the Chinese scientist Youyou Tu, for her discovery of Artemisinin (from the Chinese herb *Artemisia annua*) as an alternative to the standard drug chloroquine used to malaria cure.

Antioxidants are natural substances that may stop or limit the damage caused by free radicals, protect and reverse the damage caused by oxidation to some extent. Antioxidants are found in many foods, including fruits and vegetables. Antioxidants are compounds with enzymatic activity capable of blocking the onset of oxidation, i.e., enzymes that remove reactive oxygen species. Moreover, antioxidants without enzymatic activity can be found, where the molecules interact with the radical species and are consumed during the reaction. In this latter group are included natural antioxidants such as phenolic compounds (Bonoli et al., 2004).

Thus, the plant kingdom can represent an effective source of new compounds with less adverse effects, low costs and significant antioxidant and anti-inflammatory properties. Dafallah and Al-Mustafa (1996) found that some species of *Acacia* trees showed an inhibitory effect of edema in mice, and also observed a significant increase in hot chemical reaction located in these animals. Both reactions which represent the anti-inflammatory activity of the plants were attributed not only to the presence of flavonoids, but also to polysaccharides and organic acids. These results were also obtained by other authors that also performed experiments on mice (Dongmo et al., 2005).

The antioxidant assay may be performed by several techniques, for example, the ABTS and DPPH scavenging radicals. These assays include testing with pre-formed radicals, as the cationic moiety of the acid 2-2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) in the case of the ABTS technique, and the radical 2,2-diphenyl-1-picrylhydrazyl in the case of DPPH technique, which are often expressed as Trolox Equivalent Antioxidant Capacity (TEAC) (Prior et al., 2005).

The anti-inflammatory activity can be assayed by a test of inhibition of denaturation of albumin, since some anti-inflammatory agents inhibit the denaturation of proteins (Bhaskar and Mohite, 2010). Protein denaturation is the cause and consequence of the inflammatory response, and anti-inflammatory agents possess the ability to inhibit the denaturation of proteins. The inhibition of bovine albumin technique is based on protein denaturation using bovine serum albumin.

Thus, a major objective of the present work is to evaluate the potential of a forestry by-product, the bark, leaves and branches of *Acacia melanoxylon*, as a source of new ingredients with antioxidant and anti-inflammatory activities.

1.3. *Acacia melanoxylon* R. Br.

1.3.1. Botanical Characterization

The *Acacia* concerned, with the scientific name *Acacia melanoxylon* R. Br., was characterized by Robert Brown for the first time, and is native to southeastern Australia, Tasmania. It prefers granite terrain, avoiding limestone, and is commonly known as: Acacia-of-Australia, black wattle-of-Australia, Blackwood and wattle Australian Acacia. It takes place over a wide range of soil and climatic conditions, developing best in colder climates, with a strong invasiveness in polluted environments, dry, wet, windy and extreme temperatures.

Figure 4 - *Acacia melanoxylon* R. Br

A. Tree **B.** Flowers **C.** Trunk **D.** Seeds

It is an evergreen tree, 15m on average, reaching up to 45m; dark brown rhytidome deeply split; young leaves blended or reduced to phyllodes; adult leaves reduced to laminar phyllodes, slightly sickle, with 3-5 longitudinal ribs; pale yellow flowers, gathered in chapters with about 10-12 mm in diameter; flat pod twisted, reddish-brown; funiculus orange seed surrounded by a double fold.

1.4. Analytical Methodologies

The structural diversity and complexity of natural products demand for specific separation and analytical methodologies. Thus, new techniques for separation and analysis have been developed to isolate and identify compounds of different natures (Visht and Chaturvedi, 2012).

According to what is to be isolated the most suitable separation techniques must be selected. The criteria to consider when choosing the method and material to be used are as follows:

- Availability;
- Adaptability;
- Total capacity of the method;
- Analysis time;
- Recovery facility;
- Costs;
- Sensitivity and / or specificity;
- Accuracy and precision;
- Repeatability and reproducibility;
- Detection and quantification limits.

The main objective of the present work was the valorization of *Acacia melanoxylon* R. Br. biomass, through the estimation of essential oils and the separation and characterization of phenolic compounds from the aerial parts, aiming to identify compounds with biological activities. For that purpose, essential oils were estimated by the Clevenger method and the phenolic compounds rich extracts were obtained from the decoction waters resulting from the essential oil extraction These water extracts were concentrated by freeze-drying, and the resulting dried extracts were characterized for their phenolic profile and biological activities.

1.4.1. Estimation of Essential Oil by Clevenger Apparatus

Essential oils have numerous applications in fields like medicine, cosmetic and food industry. Essential oils are complex mixtures of volatiles, usually with odor and colorless. These are soluble in nonpolar solvents and have limited solubility in water, but sufficient for flavoring aqueous solutions. Essential oils evaporate quickly when exposed to ambient temperature. (Simoes, 2001).

Essential oils have various biological activities, having high importance in the pharmaceutical industry. Among these other characteristics have antioxidant properties and, since they are natural, they have been proposed as an alternative to synthetic antioxidants. According to the various species, the antioxidant properties are assigned to different active substances thereof.

The essential oils include volatile fraction obtained by distillation entrained by steam, responsible for the characteristic aroma of plant origin. Technologically, the essential oil is an aromatic product obtained by distillation with water steam or fractional distillation of plant material.

The separation of the matrix oils by the action of water vapor is the most frequent and economical method, and it uses a Clevenger apparatus. In this technique the process agent is water that is heated in a container where the plant material is. It is the heat of steam which causes the opening of the cell walls, causing the oil present between the cells to evaporate along with the water and go into the cooling tube. The oily phase is not miscible with the aqueous phase, whereas essential oils are lighter and are concentrated on the water layer, making its separation easy. The Clevenger apparatus can only be applied to small-scale productions.

Figure 5 - Clevenger apparatus.

The average yield of essential oil distilled from plants is usually very low, and consequently, a considerable amount of solid residues is generated. These residues are especially rich in phenolic compounds and can be exploited as value-added compounds, and thus enhance the overall profitability of the plants.

1.4.2. Phenolic Compounds Extraction

The extraction of phenolic compounds is a critical step, since its effectiveness depends on several variables such as the type of sample, type of analytes to be extracted, location of the analytes in the sample, type of extractor, solvent extraction method and temperature, among others.

When the samples are solid, it is first necessary to make the transfer of analytes into the liquid phase, using the appropriate solvent. This process is called leaching or solid-liquid extraction, and is one of the oldest methods of extraction techniques (Luque de Castro and Priego-Capote, 2010).

The time of extraction of phenolic compounds is very variable, as are the solvents used. The solvents with better efficacy are hydroalcoholic mixtures with methanol and ethanol, with concentrations varying between 50% and 80%. Ethanol has been used more often, compared to methanol, because it is a solvent with low toxicity (Rodríguez-Rojo et al., 2012).

Costa and Koblitz (2010) analyzed the extraction techniques used by different researchers, and observed that the temperature may vary from 4°C to 90°C and the extraction time may vary from 20 minutes to overnight (Table 3) (Silva et al., 2010).

Therefore, before proceeding to the selection of extraction methodology, it should be considered all the methodologies described so far, and the available material and characterization of the initial sample, trying to select the one that best meets the needs.

Figure 3 - Comparison between different extraction techniques*

Solvents	**Extraction Time**	**Extraction Temperature**	**Other Information**
Ethanol	-	-	Soxhlet followed by column chromatography purification on silica gel and activated carbon (1: 0.5)
Ethanol	6h	-	Soxhlet, purification by partition with oil and acetone followed by precipitation with lead acetate ether.
Methanol	-	room temperature (RT)	Magnetic stirrer , partition with hexane and clarification with barium hydroxide and zinc sulfate
Acetone and deionized water (0%, 25%, 50%, 75% and 100%)	Overnight	4°C a- 10°C	-
Acetone 70°c	20min	4°C	Centrifugation 4200x*g*
Ethyl ether	1h	RT	Magnetic stirring and vacuum filtration
100% methanol	12h	RT	Magnetic stirring
96 % n-hexane, acetone, ethyl acetate, ethanol and methanol	15h	-	Soxhlet
Ethanol - water (80/20) and methanol-water (50/50)	2h	-	Orbital shaker extract recovery by vacuum filtration and evaporation of the solvent under rotary evaporator
Water and aqueous Na_2SO_3 2.5%	1h	90°C	Glass reactor with mechanical agitation

*Adapted from Costa and Koblitz (2010).

In the case of the present work, the extraction was performed using 100% water at boiling temperature, as a consequence of the Clevenger methodology for estimating the presence of essential oil in the species under study.

1.4.3. Capillary Zone Electrophoresis

Capillary zone electrophoresis (CZE) is the simplest form of capillary electrophoresis (CE). CE is electrophoresis performed in a capillary tube. In this mode sample is applied as a narrow zone (band), which is surrounded by the separation buffer. As an electric field is applied, each component in the sample zone migrates according to its own apparent mobility. It is the most efficient separation technique available for the analysis of both large and small molecules. CE analyses are usually very fast, use little sample and reagents, and cost much less than chromatography or conventional electrophoresis. Modern CE has demonstrated tremendous potential for a wide range of applications. Qualitative analysis in CE provides information about the identity of a peak in an electropherogram. The simplest way to identify a CE peak is to compare its migration time and UV spectrum with those of a known compound. (Xu, 1996).

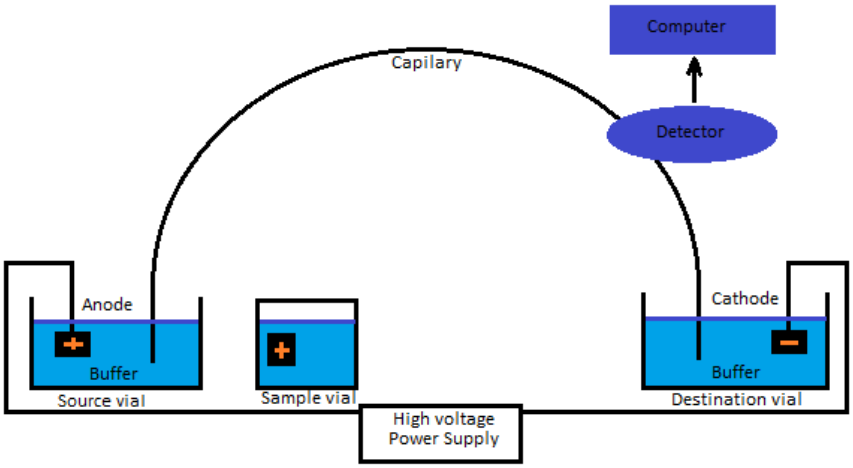


Figure 6 - Diagram of a CE system

1.4.4. Chromatographic Methods

Plant extracts are a complex mixture of chemicals that need to be analyzed, fractionated and purified. These steps can be accomplished by analytical and preparative chromatographic techniques, namely thin layer chromatography (TLC) and column chromatography (CC). In the present work, TLC will be used to screen crude extracts composition and to monitor the chemical profile of fractions obtained by CC. A clean-up step of crude extracts will be performed by CC filled with a resin, through an ion-exchange process, aiming at the separation of sugars from phenolic compounds. Then, size exclusion chromatography, also known by gel permeation chromatography, will be used to separate phenolic compounds according to their molecular size.

2. Materials and Methods

2.1. Plant Material

The biomass of *Acacia melanoxylon*, R. Br. was obtained in the Lisbon region and provided by LNEG Bioenergy Unit, after drying in a cool and dry place, out of direct sunlight, and at room temperature. Only the aerial parts of the plant – leaves (Lvs), branches (Brc) and bark (Bk) – were used for this study.

2.2. Solvents

The purity of the solvents used for this study was selected depending on the objectives associated with each experimental technique.

2.3. Extraction of Bioactive Compounds from *Acacia melanoxylon*

2.3.1. Evaluation of Essential Oils

2.3.1.1. Hydrodistillation with Clevenger

The aerial parts of *Acacia melanoxylon* (leaves, branches and bark) were cut into smaller pieces and subjected to hydrodistillation in a Clevenger apparatus (Figure 5), individually, in order to evaluate the presence of essential oils. For this, 150 g of bark or leaves/branches, respectively, were weighed and placed into a 3 L flask and 1.5 L of distilled water was added, proceeding to the extraction for 3 hours (T).

2.3.1.2. Aqueous extracts of phenolic compounds

As a consequence of the hydrodestillation for the essential oils, the respective decoction waters rich, in phenolic compounds, were separated from the solid residue by filtration, then frozen and lyophilized in a freeze-drier (Heto Dry Power LL3000, THERMO) for further analysis.

2.3.2. Phenolic Profile by Capillary Zone Electrophoresis

2.3.2.1. Preparation of samples and standards

Solutions of all extracts, fractions and standard compounds were prepared at 1 mg/mL in water:methanol (50:50). Thereafter, the samples were filtered prior to analysis.

2.3.2.2. Experimental analysis conditions

The separation by CZE method was performed using a CE system (Agilent Technologies) equipped with a network diode array detector (DAD), capillary "bubble" (increased sensitivity) fused-silica uncoated with 50 micron internal diameter and 64.5/60 cm in length (Agilent). The used buffer solution (electrolyte) was sodium tetraborate decahydrate (50 mM) prepared in

10% methanol (organic solvent used to improve the efficiency of separation and selectivity implemented method). This solution was adjusted to pH 9.3 with hydrochloric acid. The voltage was selected for 25 kV with an increasing ramp 0.5 minutes (avoiding a sudden separation) and a current 120 µA. During the separation, it was maintained at a temperature of 30 °C inside the capillary. The samples (extracts obtained and phenolic compounds solutions) were pre-filtered with a membrane (0.45 mM) and injected directly under 50 mbar pressure for 15 seconds at the anode (positive electrode). The capillary was pre-conditioned between runs for 3 minutes with a solution of NaOH (0.1 M) and then with the buffer solution for another 3 minutes. The electropherograms were recorded at 200, 280 and 375 nm. The whole system was controlled by ChemStation software (Agilent Technologies), which was also used for the treatment of the results. The compounds in the extracts were identified by comparison of UV spectra and migration times with UV spectra and migration times of the standards of phenolic compounds available that are recorded in the equipment spectra library.

2.3.3. Quantification of Total Phenolics, Tannins and Non-tannins

Total phenolics (TP), tannins (T) and non-tannins (n-T) were determined by using the Folin-Ciocalteau colorimetric method according to Roseiro et al. (2013) with slight modifications. Briefly, The extract sample (or water for blank) (0.1mL) was made up to 0.5 mL with distilled water and mixed with 1/1 (v/v) diluted Folin-Ciocalteau reagent (0.25 mL) and 20 % $Na_2CO_3 \times 10H_2O$ (1.25 mL). A calibration curve of gallic acid was prepared according to Table 4 below. For each sample, blanck or gallic acid solution, 250 µL were transferred into a 96 microplate well, and absorbance was measured at 725 nm after 40 min incubation at room temperature, in a Multiskan™ GO Microplate Spectrophotometer. Total phenolics were expressed as mg GAE (gallic acid equivalents)/ mL by comparison to the gallic acid standard curve and converted to g GAE/100 g of extracts on dry weight basis (dwb). Total tannin content was determined as above, after removal of tannins by their adsorption on insoluble matrix (polyvinylpolypyrrolidone, PVPP). Insoluble cross-linked PVPP (100 mg) was weighed into test tubes, to which distilled acidified water (pH 3) (1.0 mL) and tannin-containing extract sample (1.0 mL) were added. After 15 min at 4 °C, tubes were vortexed, transferred into Eppendorf tubes and centrifuged for 10 min at 3000×g (mini spin® Eppendorf centrifuge, Eppendorf AG, Hamburg, Germany). Aliquots (0.1 mL) of supernatant were transferred into test tubes and non-adsorbed phenolics (non-tannins) determined as previously described. Calculated values were subtracted from total phenolics contents and total tannin contents expressed as g GAE/100 g kibbles on dry weight basis (dwb). Experiments were carried out in triplicate when possible.

Figure 4 - Preparation of solutions for the construction of standard curve of gallic acid.

Tube	Gallic Acid 0,1mg/mL (µL)	Gallic Acid (µg)	Distilled Water (µL)	Folin (µL)	Na_2CO_3 (µL)
Blank	00	0	500	250	1250
T1	20	2	480	250	1250
T2	40	4	460	250	1250
T3	80	8	420	250	1250
T4	100	10	400	250	1250
T5	120	12	380	250	1250
T6	140	14	360	250	1250
T7	180	18	320	250	1250

2.3.4. Determination of Antioxidant Activity

2.3.4.1. DPPH radical scavenging Method

Radical scavenging activity of plant extracts against stable DPPH• (2,2-diphenyl-2-picrylhydrazyl hydrate) was determined spectrophotometrically. When DPPH• reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The changes in colour (from deep-violet to light-yellow) were detected at 515 nm.

A solution of DPPH• 0.06mM (60 µM) was prepared and transferred to an appropriately labeled dark bottle. A stock solution of 1.5 mM Trolox (a standard antioxidant analogous to vitamin E) was prepared in order to construct a standard curve by pipetted 100; 200; 400; 1000; 1400; and 1700 µL of the stock solution and made up to 2000 µL of EtOH, according to the following table:

Figure 5 - Preparation of solutions for the construction of standard curve of Trolox – DPPH.

Trolox stock solution (µL)	EtOH (µL)	Final concentration (mM)
100	1900	0.08
200	1800	0.15
400	1600	0.30
700	1300	0.53
1000	1000	0.75
1400	600	1.05
1700	300	1.28

Radical scavenging activity of extracts was measured according to Roseiro et al. (2013), with slight modifications. The solution of DPPH• in ethanol (60 µM) was prepared daily, before UV measurements. This solution (1950 µL) was mixed with 50 µL of Trolox solution for the

calibration curve, or the extract solution (50 µL) and vortexed. Each sample (250 µL) was transferred into a 96 microplate well, kept in the dark for 30min at room temperature and then the decrease in absorption was measured in a Multiskan™ GO Microplate Spectrophotometer. Absorption of blank sample containing the same amount of ethanol and DPPH$^{\bullet}$ solution was prepared and measured daily. The decreasing of the DPPH$^{\bullet}$ solution absorbance indicated an increase of the DPPH radical-scavenging activity. The experiment was carried out in triplicate. Radical scavenging activity (%) was calculated by the following formula:

$$\%DPPH^{\bullet} \text{ inhibition} = [(Abs_b - Abs_f) / Abs_b] \times 100$$

Where Abs_b is the absorption of blank sample (t = 0 min) and Abs_f is the absorption of tested extract solution (t = 30 min).

2.3.4.2. ABTS cation radical decolourisation assay

ABTS [2,2-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)diammonium salt] radical cation decolourisation test is also a spectrophotometric method widely used for the assessment of antioxidant activity of various substances. The experiments were carried out using the method described by Roseiro et al. (2013) with slight modifications. ABTS$^{+\bullet}$ was generated by oxidation of ABTS with potassium persulphate. The ABTS radical cation (ABTS$^{+\bullet}$) solution was prepared by mixing 7 mM ABTS (5 mL) and 140 mM potassium persulphate (88µL) leading to a 2.45 mM final concentration, and the mixture was incubated in the dark at room temperature for 16 h. The ABTS$^{+\bullet}$ solution was then diluted with 80 % (v/v) ethanol to obtain an absorbance of 0.700 ± 0.005 at 734 nm. A stock solution of 3.0 mM Trolox (a standard antioxidant analogous to vitamin E) was prepared each day of analysis, in order to construct a standard curve by pipetted 50; 200; 300; 500; 700 and 800 µL initial Trolox solution and diluted to 1000 µL with EtOH to obtain 0.15; 0.6; 0.9; 0.9; 1.5; 2.1; and 2.4 mM final concentration, respectively, as the Table 6 below:

Figure 6 - Preparation of solutions for the construction of standard curve of Trolox – ABTS.

Trolox Standard Solution (µL)	EtOH (µL)	Final Concentration (mM)
50	950	0.15
200	800	0.6
300	700	0.9
500	500	1.5
700	300	2.1
800	200	2.4

Aliquots of the extracts or Trolox solutions (30 µL) were added to the radical solution (3000 µL) and then 250 µL were transferred to a 96 microplate well, and the decrease in absorption was measured at zero and after 6 min in a Multiskan™ GO Microplate Spectrophotometer at 734

nm. All determinations were carried out in triplicate. The percent absorbance reduction was determined as follows:

$$\%ABTS^{\bullet} \text{ inhibition} = [(Abs_b - Abs_f) / Abs_b] \times 100$$

Where Abs_b is the absorption of blank sample (t = 0 min) and Abs_f is the absorption of tested extract solution (t = 6 min).

2.3.5. Determination of EC50

The EC50 is the concentration of extract capable of reducing to 50 % the initial amount of the radical. This is inversely proportional to the antioxidant activity parameter, i.e. the lower the EC50 value, the higher the antioxidant activity. For each extract, four different concentration solutions were prepared in distilled water. Then, triplicate readings were obtained, according to the procedure described in sections 2.3.4.1 and 2.3.4.2 for $DPPH^{\bullet}$ and $ATBS^{+\bullet}$ methods, respectively.

2.3.6. Fractionation and Purification of Phenolic Compounds

For the fractionation and purification of phenolic compounds present in the water decoction of the aerial parts of *Acacia melanoxylon*, various chromatographic techniques were used, particularly column chromatography (CC) and thin layer chromatography (TLC).

2.3.6.1. Preliminary Analysis of Extracts

Thin layer chromatography (TLC) was used to analyze the chemical profile of crude extracts, fractions and pure compounds from natural and synthetic origin. In the present work, silica-gel TLC plates (ALUGRAM® Xtra SIL G / UV254, 20x20 cm, 0.20 mm thickness, Macherey-Nagel, lot 407195) were used. The plates were cut into different sizes, depending on the number of samples to be analyzed.

Samples were dissolved in the minimum amount of methanol and then, they were deposited, with a Pasteur pipette, at c.a. 1 cm from the bottom of the TLC plate base. After drying the application points, the elution was held in CAMAG glass chambers with adequate dimensions to the size of the plates. The chromatographic chamber was previously filled with the chosen elution system, which consisted of mixtures of dichloromethane and ethanol/methanol of increasing polarity. The chromatograms were developed to approximately 0.5 cm of the plate upper end and then, plates were removed from elution chamber, dried with a stream of nitrogen, and spots visualized under a UV lamp (CAMAG), at λ_{254} and λ_{336} nm. Finally, the spots were revealed by spraying with a solution of H_2SO_4 (10 %) in ethanol, and by heating at 120 ºC with a heat gun.

2.3.6.2. Phenolics extraction

Amberlite XAD-2 (Supelco, Bellafonte, USA) was used to perform a cleaning step of crude extracts and column was previously packed following the manufacturer instructions. Samples were mixed with 5 parts of distilled water acidified with HCl (pH 2), homogenized in an

ultrasound bath and filtered through cotton wood to remove solid particles. The filtrate was then passed through a glass column (25 x 3.0 cm) containing Amberlite XAD-2, as reported by (Andrade et al., 1998). The column was washed with water (100 mL, pH 2 with HCl) and then with distilled water (300 mL). Sugars and other polar compounds were eluted with water, while the phenolic fraction remained in the column. Phenolics were then eluted with methanol (c.a. 300 mL) and the methanol extract was concentrated to dryness under reduced pressure at 40 °C, in a rotary evaporator. Phenolic profile was evaluated by TLC as described previously in 2.3.6.1. Aqueous fractions were frozen and lyophilized in a HetoPower Dry 3000 apparatus for further characterization by CZE.

2.3.6.3. Phenolics fractionation

Sephadex LH-20 (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden) was used for phenolics fractionation. The gel was previously swelled for 21 hours in the solvent used to dissolve extracts (10 g gel/100 mg extract) and packed into a glass chromatographic column (2.0 x 40 cm). Phenolic extracts were dissolved in a small volume of solvent, introduced in the top of the column and eluted with ethyl acetate, acetone, ethanol and methanol (Annex A). Fractions (5-20 mL) were collected and their chemical profile monitored by TLC at 254 and 366 nm under a UV lamp (2.3.6.1). Eluates showing the same composition were pooled into major fractions. After solvent evaporation, dry residues were characterized by CZE and the most promising ones screened for their anti-inflammatory activity.

2.3.7. Determination of *In Vitro* Anti-inflammatory Activity

The anti-inflammatory activity of *Acacia melanoxylon* extracts and fractions was evaluated through their capacity to inhibit albumin denaturation, as reported previously by Mizushima and Kobayashi (1968). In the present work it was followed the methodology described by Leelaprakash and Dass (2011), with slight modifications. Briefly, an aqueous solution (1 %) of bovine serum albumin (BSA, Sigma-Aldrich, Barcelona, Spain, Ref. A3912) was prepared, and extracts and acetyl salicylic acid (ASA, Sigma-Aldrich, Barcelona, Spain, Ref. A5376), the standard drug, were dissolved in distilled water in the concentrations of 500 and 100 µg/mL. Test solutions (1 mL) containing extracts and ASA were mixed with BSA solution (1 mL) and incubated at 29 ± 1 °C, for 20 min, in a water bath. BSA denaturation was induced by keeping the reaction mixture also in a water bath at 60 ± 1 °C, for 3 min. Then, samples were cooled at room temperature, and turbidity was measured at 660 nm in a UV-Vis. spectrophotometer Spectroquant Pharo 100, Merck. Each experiment was done in triplicate.

The percentage inhibition of protein denaturation was calculated as follows:

$$\% \text{ Inhibition} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

3. Results and Discussion

3.1. Essential Oils

Several authors have reported the presence of essential oils in some *Acacia* species, namely, from different plant parts of *Acacia cyanophylla* (El Ayeb-Zakhama et al., 2015), from the leaves and bark of *Acacia mearnsii* (Avoseh et al., 2015), from the bark of *Acacia nilotica* and *Acacia albida* (Okeniyi et al., 2010) and from the leaves of *Acacia tortilis* (Ogunwande et al., 2008). However, in the present study it was observed that nor the leaves/branches, nor the bark of *Acacia melanoxylon* revealled the presence of essential oils. In fact, little is known about the volatile composition of most species of *Acacia*, and what is known is mainly from the flowers of some *Acacia* species (Siegler, 2003), mostly used in perfumery, but no reference was found to the presence of essential oils in *Acacia melanoxylon*.

3.2. Profile of Phenolic Compounds by CZE

The CZE phenolic profile of the decoction waters from the hydrodestillation of bark and leaves/branches from *Acacia melanoxylon* were similar and revealed the presence of gallic acid, rutin and epigallocatechin gallate, among other non identified compounds. Figure 7 shows the phenolic profile for the decoction water for the leaves and branches as an example. By using the software of the CZE equipment it was possible to determine the matching percentage of the compounds analyzed in relation to data of standard phenolic compounds available, based on the migration time and the UV spectram of the sample compounds and standards.

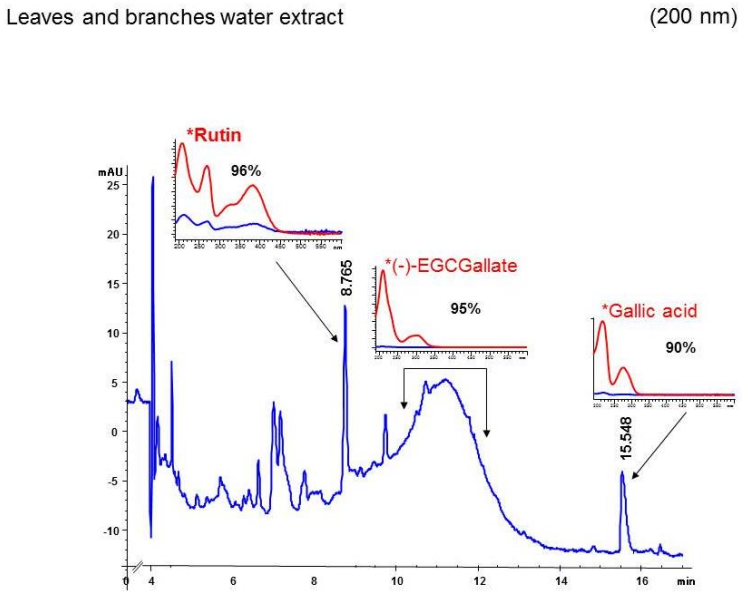


Figure 7 - CZE electropherogram of leaves and branches decoction water.

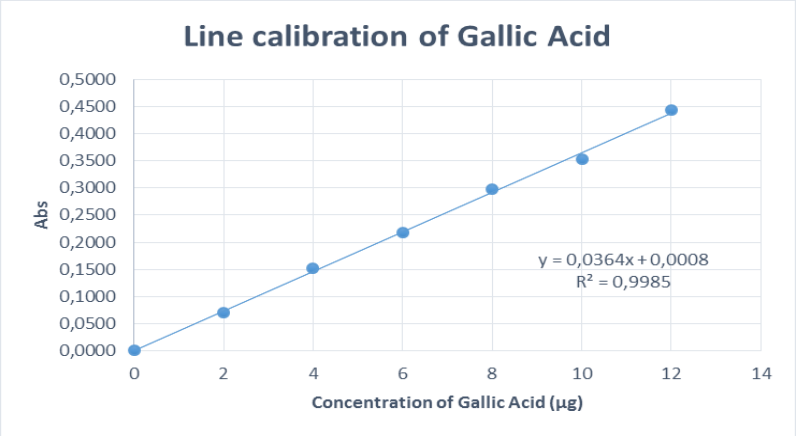
It can be seen that the profile is complex in terms of phenolic composition, showing a large band matching the epigallocatechin gallate standard with 95% proximity, revealing a rich

presence of different catechins and catechin gallate compounds in the sample. The most evident substances in many *Acacia* species are complex phenolic compounds (hydrolysable and condensed tannins), but only few species have been examined in any detail for these substances (Siegler, 2003). Zhang et al. (2010) have found that the bark of *Acacia mangium* has very high concentrations of phenolic compounds, condensed tannins in particular. Lee et al. (2000) have also identified three new flavonol galloylglycosides from the leaves of *Acacia confusa*, and Nyila et al. (2012) isolated epicatechin and epigallocatechin from the leaves of *Acacia karroo*. Maldini et al. (2011), refer to the complexity of catechin derivatives and the fact that the investigation of this class of natural compounds has been limited by difficulties in their separation. These authors have also found evidence of the presence of galloylated catechin- and gallocatechin derivatives, along with galloylated glucose derivatives in the 80 % ethanol extract of *Acacia nilotica* pods.

A further chromatographic separation of these samples was done for better CZE profiling and characterization of the compounds. These results are presented in section 3.6.

3.3. Content in Phenolic Compounds, Tannins and Non-tannins

The graph below (Graph 1) shows the gallic acid standard curve, obtained for determining the content of phenolic compounds.



Graph 1 - Calibration curve of gallic acid.

From the absorbances measured and standard calibration curve (Graph 1), the amount of total phenolics, tannins and non-tannin phenolics was calculated as gallic acid equivalents (GAE). The results were expressed in grams of gallic acid equivalents per 100 gram extract (GAE g/100 g ext) (Annex B). It proceeded in the same way to calculate total phenolics for the remaining trials (see annex C). The results for leaves/branches (Lvs+Brc) and bark (Bk) decoction waters obtained at a concentration of 1 mg/mL are shown in Table 7 (mean values n=9):

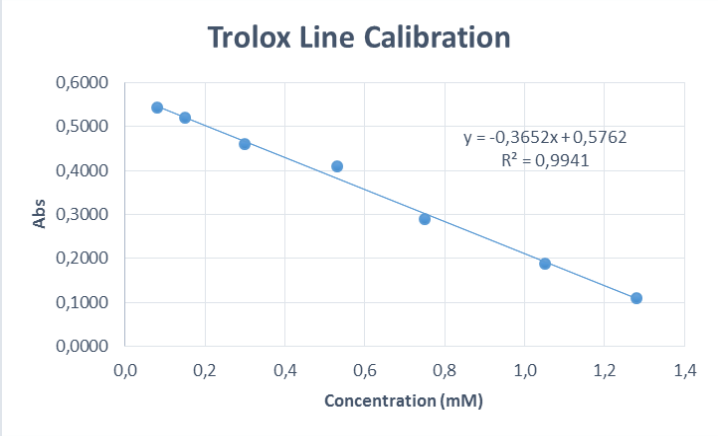
Figure 7 - Total phenols, no-tannins and tannins in g GAE/100g ext ± SD.

Sample	TP	n-T	T
Lvs + Brc	16.11 ± 0.60	4.47 ± 1.24	11.64 ± 1.56
Bk	26.99 ± 1.48	6.77 ± 5.16	20.21 ± 2.04

3.4. Determination of Antioxidant Activity

3.4.1. DPPH Scavenging Assay

Graph 2 shows the calibration curve for the standard Trolox used for the DPPH method.



Graph 2 - Calibration curve for Trolox.

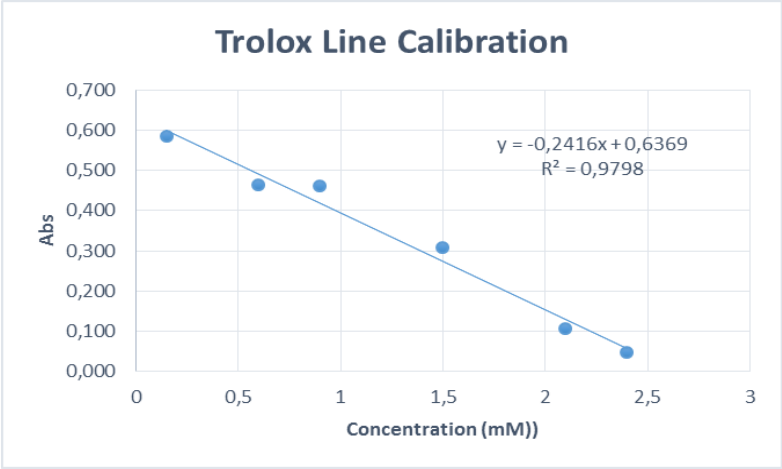
After monitoring the consumption of the free radical DPPH, results were expressed as inhibition % of the DPPH radical and as mM TEAC (Trolox Equivalent Antioxidant Capacity), (see Annex D).

Figure 8 - Antioxidant activity expressed as mM TEAC and% inhibition for DPPH•.

Sample	mM TEAC	%I
Lvs + Brc	1.06 ± 0.02	70.82 ± 0.01
Bk	1.23 ± 0.03	82.37 ± 0.02

3.4.2. ABTS Method

Graph 3 shows the calibration curve for the standard Trolox used for the ABTS•+method.



Graph 3 - Calibration curve for Trolox.

After monitoring the consumption of the free radical $ABTS^{\bullet+}$, results were expressed as inhibition % of the $ABTS^{\bullet+}$ radical and as mM TEAC (Trolox Equivalent Antioxidant Capacity), (see Annex D), and are shown in Table 9.

Figure 9 - Antioxidant activity expressed as mM TEAC and % inhibition for $ABTS^{\bullet+}$.

Sample	mM TEAC	%I
Lvs + Brc	1.56 ± 0.01	60.45 ± 0.02
Bk	2.42 ± 0.01	92.51 ± 0.01

3.4.3. EC50

The EC50 values for the $DPPH^{\bullet}$ method were 0.67mg/mL for leaves /branches and 0.41 mg/mL for bark. The EC50 values for the $ABTS^{\bullet+}$ method were 0.75 mg/mL for leaves and branches and 0.38 mg/mL for bark.

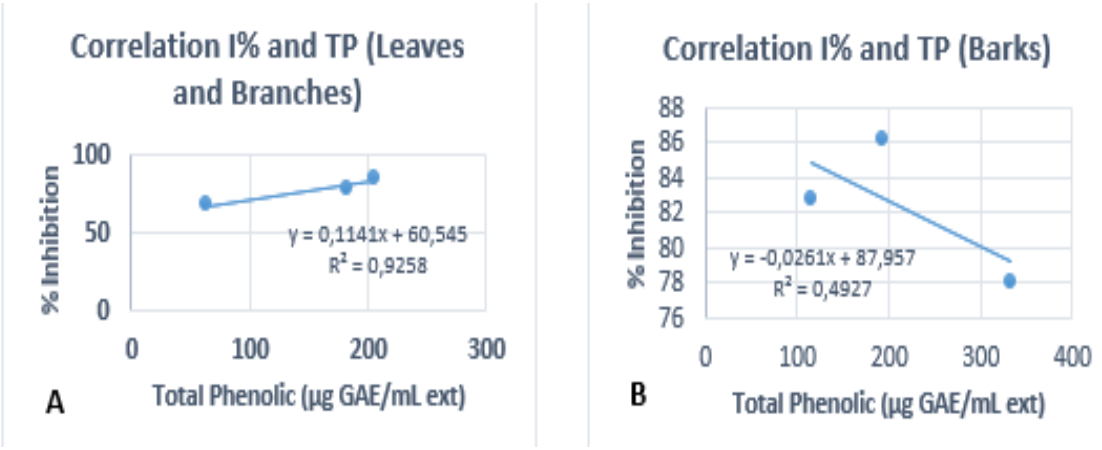
To the best of our knowledge, these are the first results for total phenolic content and antioxidant activity for extracts of *Acacia melanoxylon*, as no results were found in the literature for this specie. It can be observed from Tables 7 - 9 that results obtained for bark are higher than the ones obtained for the leaves and branches. These are in agreement with the work of Devi & Prasad (1991), who determined the total phenol and tannins content of water extracts from the leaves, bark and pod of ten *Acacia* species biomass (but not for *Acacia melanoxylon*), showing that, in general, bark presented higher values. However, these values are not comparable with the ones in Table 7 obtained for *Acacia melanoxylon* because total phenols and tannins were determined according to the method Folin-Denis and casein precipitation, respectively. Tung *et al*. (2007), found the total phenolic content of the water fraction of the 70 % ethanol extract from *Acacia confusa* bark to be 40 g GAE/100 g, by the Folin-Ciocalteau method. Andrade et al. (2007) found for the total phenols from the ethanol extracts of flowers of *Acacia podalyriifolia,* the values of 20.6 g GAE/100 g, 24.0 g GAE/100 g and 33.8 g/100 g for the ethanol, dichloromethane and ethyl acetate fractions, respectively.

It can also be observed that the majority of the phenolic compounds are tannins. These results are in agreement with the few results of the literature for other *Acacia* species, which refer that, in general, *Acacia* species are rich in tannin compounds (Siegler, 2003). Alam *et al*. (2007) revealed the total phenol and tannin content of the leaves and pods from *Acacia nilotica* to be 20.08 g GAE /100 g and 13.61 g GAE /100 g, respectively. These results are in close aggreement with the ones obtained for *Acacia melanoxylon* (Table 7). Singh *et al*. (2007) found the phenol content of the water fraction of the methanol extract obtained by decreasing solvent polarity of bark powder from *Acacia auriculiformis* to be 8.8 g/100 g GAE. These authors also observed that the water fraction exhibited maximum free radical-scavenging activity of 74.1 % at 100 µg/mL concentration by the DPPH radical scavenging assay. For the decoction water of bark from *Acacia melanoxylon*, this value was 82.37 % at a concentration of 1 mg/mL, by the DPPH radical scavenging assay (Table 8). Maldini *et al*. (2011) determined the antioxidant activity of galloylated catechin- and gallocatechin derivatives along with galloylated glucose derivatives in the 80 % ethanol extract of *Acacia nilotica* pods, ranging from 0.16 to 3.11 mM TEAC by the ABTS method. The values obtained for the decoction waters of bark from *Acacia melanoxylon*, are within these referred values (Table 9). From the results obtained, it seems that decoction

waters of *Acacia melanoxylon* are a rich source of polyphenolic constituents, containing probably different galloylated derivatives of catechin and gallocatechin (Figure 7). Also, both water extracts from leaves/branches and bark exhibit a strong free radical scavenging activities.

3.4.4. Correlation Between Total Phenolics and Antioxidant Activity

The previous results were used to calculate a correlation between the content in phenolic compounds and the antioxidant activity. For the DPPH• method, for example, a correlation of 0.92 was found for leaves and branches. However, for bark this value was only 0 .49.



Graph 4 - Correlation between inhibition of total phenolics by DPPH•.

Some studies have shown a relationship between antioxidant activity and the content of total phenolic compound, but generally, this value is very variable. For example, Yizhong Cai et al. (2004) found a correlation between the total antioxidant capacity and phenolics of 0.97 in Chinese herbs, and Tawaha et al. (2007) found a correlation of 0.89 between antioxidant capacity and phenolics compounds in some species of Jordan.

The correlation between total phenolic content and antioxidant activity by DPPH• method of leaves and branches was quite significant; in turn, the bark had a low value. These low correlation values, as verified by various authors, can occur because the Folin-Ciocalteau method is not a selective method, that is by the Folin-Ciocalteau assay for total phenols is affected by several interfering substances such as ascorbic acid, sugar, organic acids and non-phenolic organic substances that react with the Folin-Ciocalteau reagent. In the particular case of bark, the results for the phenolic compounds can be influenced by the amount of sugar extract from *Acacia melanoxylon* decoction water. They may also be justified by the fact that not all the phenolic compounds having antioxidant activity, or even because different phenolics present different answers with the Folin-Ciocalteau reagent, for example, different flavonoids present low absorption which leads to an underestimation of the various compounds (Zhang et al., 2010).

3.5. Extracts Analysis and Fractionation

The chemical profile of crude extracts from *Acacia melanoxylon* (Lvs+Brc, Bk) were previously analyzed by TLC which denoted some complexity on their composition. So, a cleaning step was

performed by using CC, filled with Amberlite XAD-2 (Figure 8 – A) aiming at to separate sugars from the desired phenolic compounds. For each crude extract, 4 fractions were obtained (Annex E). Then, the methanol fraction (Figure 8 – B) containing the phenolic compounds was concentrated to dryness and submitted to a separation according compounds molecular size, through a Sephadex column (Figure 8 – C).

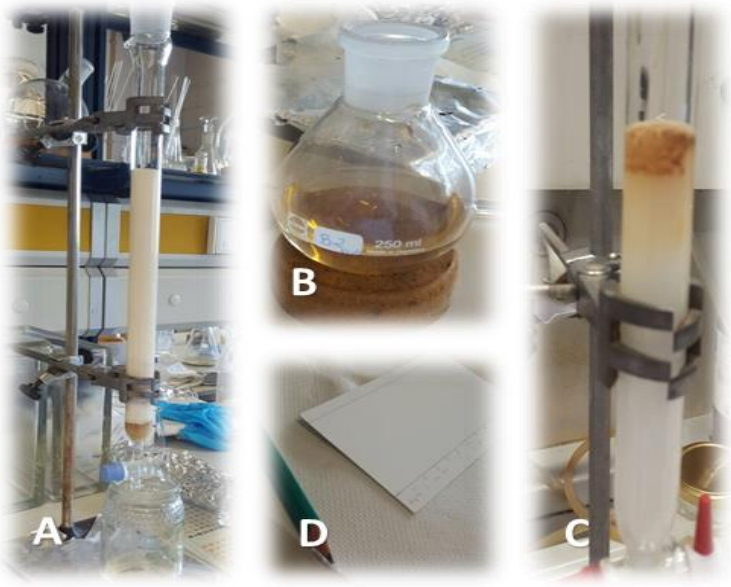


Figure 8 - Extracts analysis.

A total of 57 fractions were obtained from the methanol extracts of bark and leaves/branches (Annex F). The composition of fractions obtained from both columns was monitored by TLC (Figure 8 – D), and fractions showing the same chemical profile were pooled into major fractions as depicted in Table 10.

Figure 10 - TLC profile of MeOH/H_2O and MeOH fractions from *Acacia melanoxylon*

Samples	Fractions	Colour	CZE Code
Lvs + Brc	I	Cream	11FR
	II	Greenish yellow	12FR
	III	Greenish brown	13FR
	IV	Opaque brown	14FR
	V	Lilac	15FR
	VI	Brown	16FR
	VII	Intense pink	17FR
	VIII	Pink	18FR
	IX	Light pink	19FR
	X	Pinkish brown	20FR
	XI	Colorless	21FR
Bk	I	Colorless	4C
	II	Greenish brown	5C
	III	Green-brown	6C
	IV	Light brown	7C
	V	Colorless	8C
	VI	Cream	9C
	VII	Colorless	10C

All fractions were completely evaporated to dryness and subjected to CZE for compounds identification. Those which revealed a promising phenolic composition were tested through an *in vitro* assay regarding their anti-inflammatory activity.

3.6. Phenolic Profile of Chromatographic Fractions

3.6.1. CZE

The analysis of the profile of phenolic compounds by CZE was performed for all the fractions obtained after column chromatography and TLC evaluation (Annex G). The profile of the most promising fractions, two from the bark (6 C and 7 C) and two from the leaves and branches (13 FR and 14 FR) are shown in figures 10 and 11, respectively.

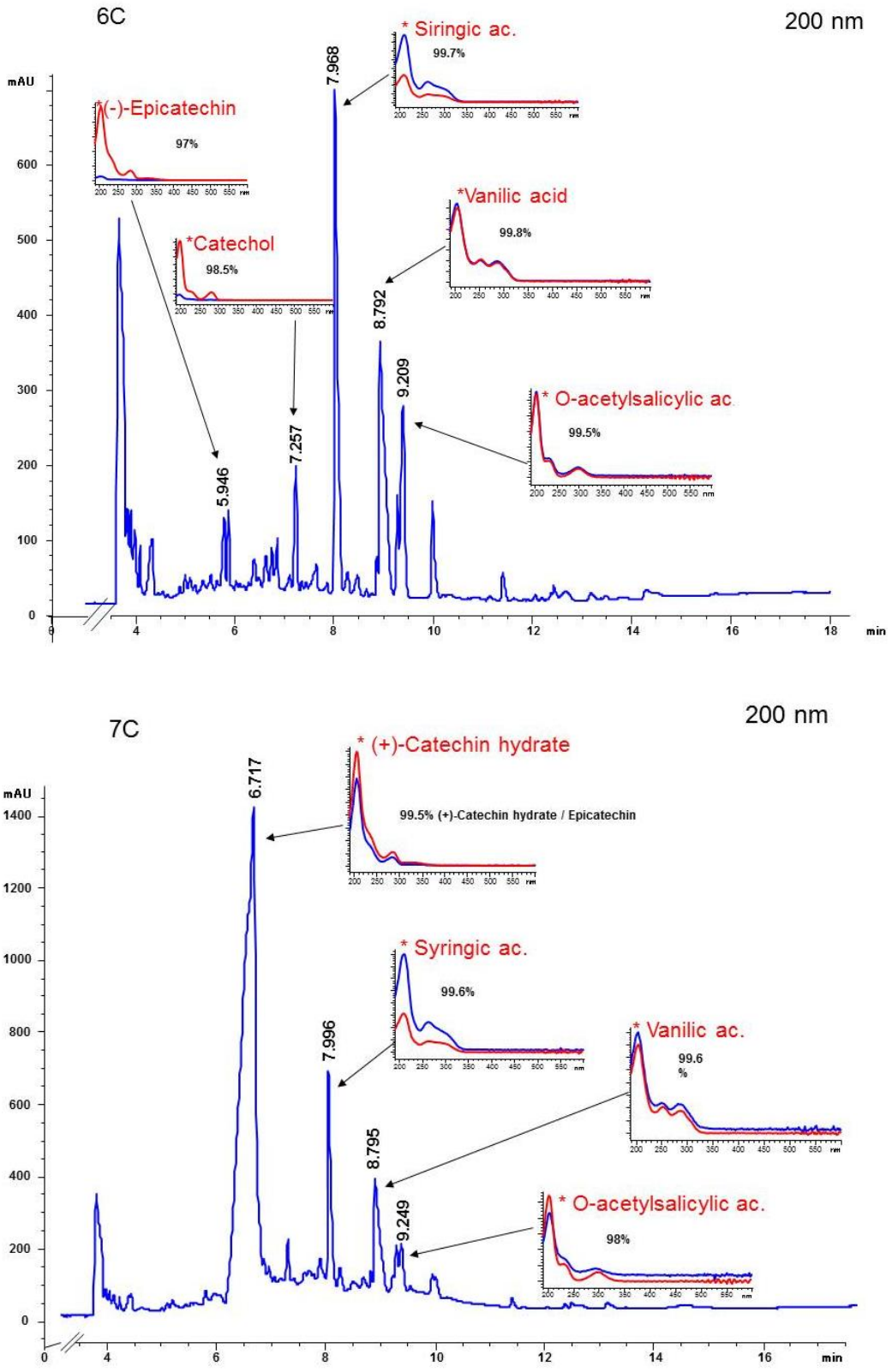


Figure 9 - CZE electropherogram of bark 6C and 7C.

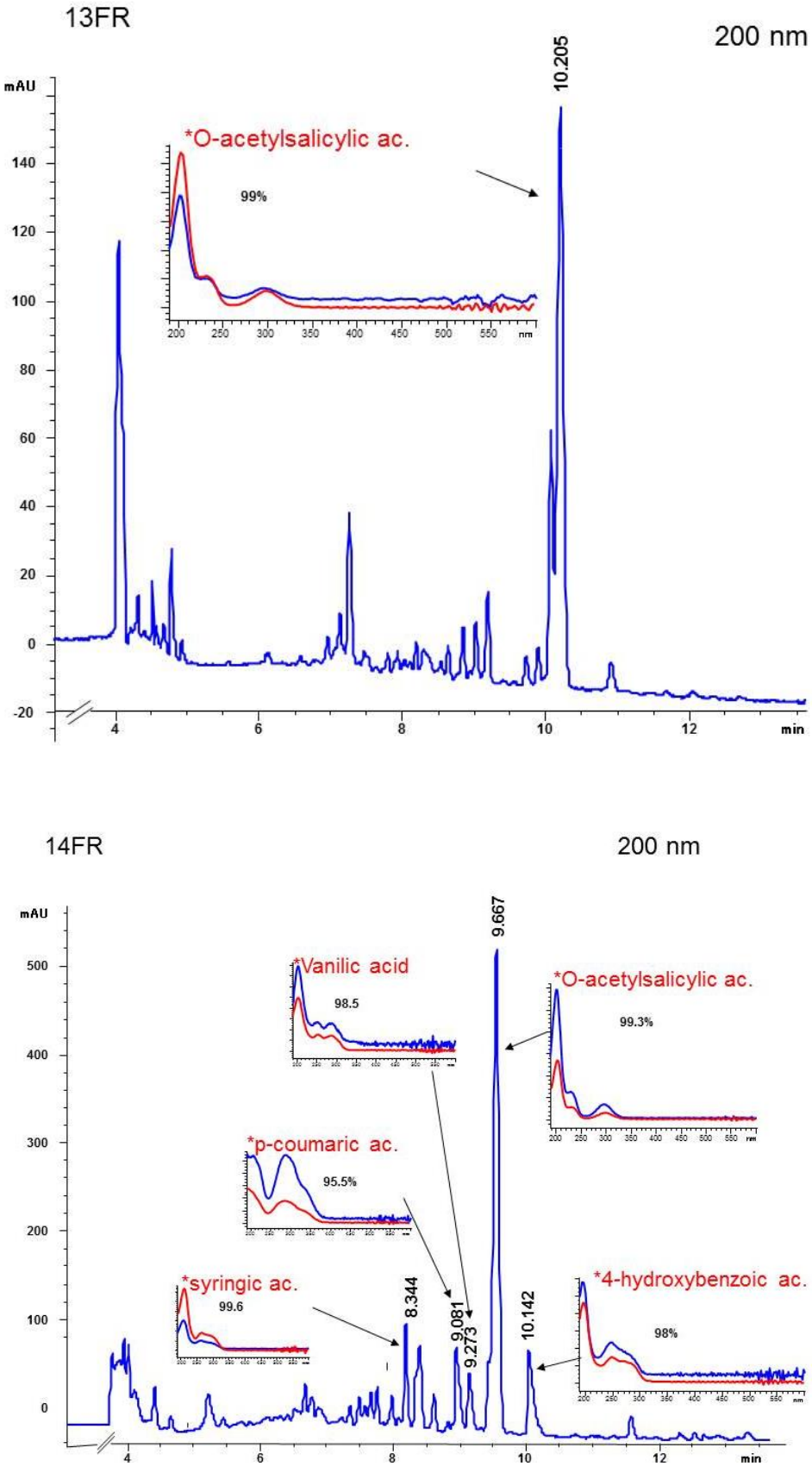


Figure 10 - CZE electropherogram of leaves and branches.

After the clean-up procedure, several other compounds could be identified, such as epicatechin and catechin hydrate, but mainly phenolic acids and, in particular *O*-acetylsalisylic acid. This compound is recognized as having anti-inflammatory activity and is the active ingredient of aspirin, which was first isolated from the bark of the willow tree, in 1763, by Edward Stone of Wadham College, University of Oxford (Stone, 1763). To the best of our knowledge, only two studies were so far performed for the characterization of the phenolic composition of *Acacia melanoxylon*, and only one of these studies was done in the diethyl ether extracts of the leaves and bark. González et al. (1995) identified apigenin, 4-hydroxy-3-methoxybenzyl alcohol, ferulic acid, quercetin-3-glycoside (rutin), vanillic acid, vanillin and luteolin in the leaves, and luteolin, apigenin, vanillin, ellagic acid and ellagitannins in the bark.

Hussain et al. (2011) have characterized the flower and phyllode MeOH/H_2O extracts of *A. melanoxylon* and confirmed the presence of gallic acid, protochatecuic acid, *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vannilic acid, syringic acid, *p*-coumaric acid, ferulic acid, rutin, quercetin, luteolin, apigenin and catechin. Most of the referred compounds are also present in the decoction waters, but, apparently, *O*-acetylsalisylic acid has not been identified before. To the best of our knowledge, this is the first time that phenolic acids and flavonoids are identified in the aqueous extract of leaves/branches and bark of *Acacia melanoxylon*, and that the presence of *O*-acetylsalisylic acid is reported in *Acacia* species. Only few publications refer to the phenolic composition of *Acacia* species (vide Table 2 for references). Tung et al. (2009; 2007), for example, have also identified some phenolic compounds in the ethanolic extracts of bark and hot water extracts of leaves from *Acacia confusa*, namely, phenolic acids in the bark, and gallic acid, (+)-catechin, (-)-epicatechin, myricetin-3-glucopyranoside and myricetin-3-rhamnopyranoside in the leaves, respectively.

3.7. Anti-inflammatory Activity

The capacity of crude extracts and fractions obtained from the bark and leaves/branches of *Acacia melanoxylon* to inhibit bovine serum albumin (BSA) heat denaturation was compared to the standard drug, acetylsalicylic acid (ASA).

Some of the tested fractions (500 µg/mL) were effective in inhibiting BSA denaturation (Table 11). Inhibition (%) was observed for fractions 6 C (14.64 %), 7 C (40.80 %), 13 FR (13.39 %) and 14 FR (39.25 %).

Figure 11 - Anti-inflammatory activity expressed as % inhibition of BSA denaturation.

Sample	Abs_{660nm}	%I
Blank	0.69 ± 0.01	-
ASA	0.01 ± 0.00	99.09 ± 0.00
6C	0.40 ± 0.00	14.64 ± 0.00
7C	0.28 ± 0.01	40.80 ± 0.01
13FR	0.80 ± 0.03	13.39 ± 0.01
14FR	0.56 ± 0.00	39.25 ± 0.02

These results can be explained and related to the chemical composition of the tested fractions (Figures 9 and 10), as these fractions are rich in phenolic acids (acetylsalicylic acid, vanillic, *p*-coumaric, and syringic acids) known to have anti-inflammatory activity. Besides the well documented activity of acetyl salicylic acid, one of the main constituents of *Acacia melanoxylon*, there are also some reports confirming the *in vitro* and *in vivo* anti-inflammatory properties of vanillic (Calixto-Campos et al., 2015), syringic (Fernandez et al., 1998), and *p*-coumaric acids (Pragasam and Rasool, 2013). Differences in the percentage inhibition are related to the concentration of the referred compounds in the samples analysed, being fractions 7 C and 14 FR the ones presenting the higher percentages in line with the absorbance level. The standard drug ASA (100 µg/mL) showed an inhibition ≥ 98.26 %, when compared to the control (Table 11). Once it is a pure compound it is expected that ASA inhibition percentage is higher than that observed for the bioactive fractions, which have a more complex composition and minor relative concentrations of each bioactive constituent. On the contrary, fractions 15/20 FR (Annex G) didn't show relevant inhibition of BSA denaturation. Although their main constituent was catechin derivatives, it is possible that some tannin based compounds, e.g. galloyl catechins and gallic acid, promoted albumin precipitation and no activity was observed. The same behavior was denoted by the crude extracts, where gallocatechins, gallates and gallic acid were also predominant. The relationships of chemical structure of catechins and their binding affinities to human serum albumin (HSA) were studied by Trnková et al. (2011). These authors confirmed the importance of galloyl moiety on the C-ring in the binding affinity of catechins to HSA. Catechins lacking the galloyl moiety in their structure (catechin, epicatechin, gallocatechin and epigallocatechin) caused only negligible alterations in the protein molecule, while the galloylated catechins showed significantly higher binding ability than the non-galloylated catechins, promoting protein precipitation.

There are no reports in the literature regarding the application of the *in vitro* BSA denaturation assay to evaluate the anti-inflammatory activity of extracts from *Acacia* species, nor to the phenolic compounds identified in *Acacia melanoxylon*. However, the anti-inflammatory activity of *Acacia hydaspica* (Afsar et al., 2015) and *Acacia catechu* (Stohs and Bagchi, 2015) were recently reported by a different methodology. Both authors refer the role of phenolic compounds, including catechins and phenolic acids, on the anti-inflammatory properties of such extracts *in vivo* models, while Garcia-Lafuente et al. (2014) reported an *in vitro* assay evidencing the importance of catechins on the anti-inflammatory activity of extracts from two beans species. These reports add further support to our results, evidencing the potential of *Acacia melanoxylon* as a source of new add-value ingredients with antioxidant and anti-inflammatory properties.

Conclusions and Future Prospects

In this study, it was revealed for the first time that the aerial parts and bark from *Acacia melanoxylon* biomass do not present essential oils. However, the decoction waters thereof are rich in phenolic compounds, particularly tannins, and possess strong antioxidant activity based on the DPPH and ABTS assays. Some of the compounds identified in *Acacia melanoxylon* are responsible for the strong radical scavenging activity and also for the anti-inflammatory proprieties, namely, catechin derivatives and *O*-acetylsalicylic acid, which was here identified for the first time in *Acacia* species.

Through this work we have tried to explore the value added compounds and the therapeutic potential of the biomass of *Acacia melanoxylon*, concluding to be a promising route for new, safe, biodegradable and renewable source of drugs with high therapeutic index. Within the concept of biomass refining, *Acacia melanoxylon* biomass can thus be assessed for the co-production of polyphenols, tannins, timber and firewood.

References

Afsar, T., Khan, M. R., Razak, S., Ullah, S. and Mirza, B. 2015. Antipyretic, anti-inflammatory and analgesic activity of Acacia hydaspica R. Parker and its phytochemical analysis. BMC Complement Altern Med 15:136.

Alam, M. R., Amin, M. R., Kabir, A. K. M. A., Moniruzzaman, M. and McNeill, D. M. 2007. Effect of Tannins in Acacia nilotica, Albizia procera and Sesbania acculeata foliage determined in vitro, in sacco, and in vivo. Asian-Australasian Journal of Animal Sciences 20:220-228.

Anam, E. M. 1997. A flavanone from the roots of Acacia longifolia (Leguminosae). Indian Journal of Heterocyclic Chemistry 7:63-66.

Andrade, C. A., Costa, C. K., Bora, K., Miguel, M. D., Miguel, O. G. and Kerber, V. A. 2007. Determinação do conteúdo fenólico e avaliação da atividade antioxidante de Acacia podalyriifolia A. Cunn. ex G. Don, Leguminosae-mimosoideae. Rev Bras Farmacogn 17:231-235.

Andrade, P. B., Carvalho, A. R. F., Seabra, R. M. and Ferreira, M. A. 1998. A previous study of phenolic profiles of quince, pear, and apple purees by HPLC diode array detection for the evaluation of quince puree genuineness. Journal of Agricultural and Food Chemistry 46:968-972.

Avoseh, O. N., Oyedeji, O. O., Aremu, K., Nkeh-Chungag, B. N., Songca, S. P., Oluwafemi, S. O. and Oyedeji, A. O. 2015. Chemical composition and anti-inflammatory activities of the essential oils from Acacia mearnsii de Wild. Nat Prod Res 29:1184-8.

Azuine, M. A. and Bhide, S. V. 1992. Protective single/combined treatment with betel leaf and turmeric against methyl (acetoxymethyl) nitrosamine-induced hamster oral carcinogenesis. Int J Cancer 51:412-5.

Barros, L., Duenas, M., Ferreira, I. C., Baptista, P. and Santos-Buelga, C. 2009. Phenolic acids determination by HPLC-DAD-ESI/MS in sixteen different Portuguese wild mushrooms species. Food Chem Toxicol 47:1076-9.

Bhaskar, V. H. and Mohite, P. B. 2010. Design, synthesis, characterization and biological evaluation of some novel 1,5 disubstituted tetrazole as potential anti-inflammatory agents. J Optoelectronics Biomedical Materials 2:231-237.

Bonoli, M., Verardo, V., Marconi, E. and Caboni, M. F. 2004. Antioxidant phenols in barley (Hordeum vulgare L.) flour: comparative spectrophotometric study among extraction methods of free and bound phenolic compounds. J Agr Food Chem 52:5195-5200.

Bravo, L. 1998. Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. Nutr Rev 56:317-33.

Breton, C., Guerin, J., Ducatillion, C., Médail, F., Kull, C. A. and Bervillé, A. 2008. Taming the wild and 'wilding' the tame: Tree breeding and dispersal in Australia and the Mediterranean. Plant Sci 175:197-205.

Cai, Y., Luo, Q., Sun, M. and Corke, H. 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci 74:2157-84.

Calixto-Campos, C., Carvalho, T. T., Hohmann, M. S. N., Pinho-Ribeiro, F. A., Fattori, V., Manchope, M. F., Zarpelon, A. C., Baracat, M. M., Georgetti, S. R. and Casagrande, R. 2015. Vanillic acid inhibits inflammatory pain by inhibiting neutrophil recruitment,

oxidative stress, cytokine production, and NFκB activation in Mice. J Nat Prod 78:1799-1808.

Chang, S. T., Wu, J. H., Wang, S. Y., Kang, P. L., Yang, N. S. and Shyur, L. F. 2001. Antioxidant activity of extracts from Acacia confusa bark and heartwood. J Agric Food Chem 49:3420-4.

Clark-Lewis, J. W., Katekar, G. F. and Mortimer, P. I. 1961. Flavan derivatives. Part IV. Teracacidin, a new leucoanthocyanidin from *Acacia intertexta*. J Chem Soc:499-503.

Dafallah, A. A. and al-Mustafa, Z. 1996. Investigation of the anti-inflammatory activity of Acacia nilotica and Hibiscus sabdariffa. Am J Chin Med 24:263-9.

Deshande, V. & Patil, A. 1981. Flavonoid of *Acacia catechu*, heartwood. Ind J Chem Soc Sect B 20B (7):628.

Devi, S. R. and Prasad, M. N. V. 1991. Tannins and related polyphenols from ten common *Acacia* species of India. Bioresour technol 36:189-192.

Direcção Geral de Rrecursos Florestais 2006. Estratégia Nacional para as Florestas. DGRF. Lisboa.

Djeridane, A., Yousfi, M., Nadjemi, B., Boutassouna, D., Stocker, P. and Vidal, N. 2006. Antioxidant activity of some algerian medicinal plants extracts containing phenolic compounds. Food Chem 97:654-660.

Dongmo, A. B., Nguelefack, T. and Lacaille-Dubois, M. A. 2005. Antinociceptive and anti-inflammatory activities of *Acacia pennata* Wild (Mimosaceae). J Ethnopharmacol 98:201-6.

Drewes, S. E. and Ilsley, A. H. 1968. Isolation of 3-methoxyfisetin from *Acacia mearnsii*. Chem Comm (London):1246-1247.

Drewes, S. E. and Roux, D. G. 1966. A new flavan-3, 4-diol from *Acacia auriculiformis* by paper ionophoresis. Biochem. J 98:493-500.

El Ayeb-Zakhama, A., Sakka-Rouis, L., Bergaoui, A., Flamini, G., Ben Jannet, H. and Harzallah-Skhiri, F. 2015. Chemical composition and allelopathic potential of essential oils obtained from *Acacia cyanophylla* Lindl. Cultivated in Tunisia. Chem Biodivers 12:615-626.

El Sawi, S. A. 2000. A new rare 8-C-glucosylflavonoid and other eight flavonoids from the molluscicidal plant *Acacia saligna* Wendl. Pharm Pharmacol Lett 11:30-33.

El Sissi, H. I. and El Sherbeiny, A. E. A. 1967. The flavanoid components of the leaves of *Acacia saligna*. Qualit Plant Mater Veg 14:257-266.

Falco, M. R. and Vries, J. X. 1964. Isolation of hyperoside (quercetin-3-D-galactoside) from the flowers of *Acacia melanoxylon*. Naturwissenschaften 51:462-463.

Fernandes, J. R. and Fernandes, M. M. 2012. Cadernos: Curso de Doutoramento em Geografia. Universidade do Porto: Porto.

Fernandez, M. A., Saenz, M. T. and Garcia, M. D. 1998. Natural Products: anti-inflammatory activity in rats and mice of phenolic acids isolated from *Scrophularia frutescens*. J Pharm Pharmacol 50:1183-1186.

Figueiredo, A. C., Barroso, J. G. and Pedro, L. G. 2007. Plantas aromáticas e medicinais. Factores que afectam a produção. Pages 1-18 in: Potencialidades e aplicações das plantas aromáticas e medicinais. Curso teórico-práctico. Faculdade de Ciências da Universiade de Lisboa - Centro de Biotecnologia Vegetal: Lisboa.

Foo, L. Y. 1987. Configuration and conformation of dihydroflavonols from *Acacia melanoxylon*. Phytochem 26:813-817.

Fourie, T. G., Ferreira, D. and Roux, D. G. 1974. 8-*O*-methyl-and the first 3-*O*-methylflavan-3, 4-diols from *Acacia saxatilis*. Phytochem 13:2573-2581.

Garcia-Lafuente, A., Moro, C., Manchon, N., Gonzalo-Ruiz, A., Villares, A., Guillamon, E., Rostagno, M. and Mateo-Vivaracho, L. 2014. In vitro anti-inflammatory activity of phenolic rich extracts from white and red common beans. Food Chem 161:216-23.

González, L., Souto, X. C. and Reigosa, M. J. 1995. Allelopathic effects of *Acacia melanoxylon* R.Br. phyllodes during their decomposition. Forest Ecol Manag 77:53-63.

Hathway, D. E. and Seakins, J. W. T. 1957. Enzymic oxidation of catechin to a polymer structurally related to some phlobatannins. Biochem J 67:239.

Hollman, P. C. and Katan, M. B. 1999. Dietary flavonoids: intake, health effects and bioavailability. Food Chem Toxicol 37:937-42.

Hussain, M. I., González, L., Souto, C. and Reigosa, M. J. 2011. Ecophysiological responses of three native herbs to phytotoxic potential of invasive *Acacia melanoxylon* R. Br. Agroforest Syst 83:149-166.

Imperato, F. 1978. A new chalcone glucoside and isosalipurposide from *Acacia cyanophylla*. Phytochem 17:822-823.

Kerber, V. A. and Silva, G. A. A. B. 1993. Flavonóides da *Acacia longifolia* (Andr.) Willd. Leguminosae Mimosoideae. Rev Bras Farm 74:16-18.

Khalid, S. A., Farouk, A., Geary, T. G. and Jensen, J. B. 1986. Potential antimalarial candidates from African plants: an *in vitro* approach using *Plasmodium falciparum*. J Ethnopharmacol 15:201-209.

Kull, C. A., Shackleton, C. M., Cunningham, P. J., Ducatillon, C., Dufour-Dror, J. M., Esler, K. J., Friday, J. B., Gouveia, A. C., Griffin, A. R., Marchante, E., Midgley, S. J., Pauchard, A., Rangan, H., Richardson, D. M., Rinaudo, T., Tassin, J., Urgenson, L. S., Von Maltitz, G. P., Zenni, R. D. and Zylstra, M. J. 2011. Adoption, use and perception of Australian Acacias around the world. Diversity Distrib 17:822-836.

Lee, S. J., Umano, K., Shibamoto, T. and Lee, K. G. 2005. Identification of volatile components in basil (*Ocimum basilicum* L.) and thyme leaves (*Thymus vulgaris* L.) and their antioxidant properties. Food Chem 91:131-137.

Lee, T. H., Qiu, F., Waller, G. R. and Chou, C. H. 2000. Three new flavonol galloylglycosides from leaves of *Acacia confusa*. J Nat Prod 63:710-712.

Leelaprakash, G. and Dass, S. M. 2011. *In vitro* anti-inflammatory activity of methanol extract of *Enicostemma axillare*. Int J Drug Dev & Res 3:89-196.

Lorenzo, P., González, L. and Reigosa, M. J. 2010. The genus *Acacia* as invader: the characteristic case of *Acacia dealbata* Link in Europe. Ann Forest Sci 67:101.

Lourenço, D. C. G. R. 2009. Avaliação de áreas invadidas por espécies de *Acacia* na Paisagem Protegida da Arriba Fóssil da Costa de Caparica in: Departamento de Ciências e Engenharia Ambiente. Universidade Nova de Lisboa: Faculdade de Ciências e Tecnologia: Lisboa.

Luque de Castro, M. D. and Priego-Capote, F. 2010. Soxhlet extraction: Past and present panacea. J Chromatogr A 1217:2383-9.

Mackenzie, A. 1967. Mearnsitrin: a new [methoxylated] flavonol glycoside [a rhamnoside] from the leaves of *Acacia mearnsii.* Tetrahedron Lett 26:2519-2550.

Malan, E. & Penaar, D. 1987. (+)-Catechin-galloyl esters from the banrk of *Acacia gerrardii.* Phytochem 26 (7):2049-2051.

Malan, E. 1993. 7,8,4'-trihydroxy-3,3'-dimetoxy flavone from the heartwood of *Acacia nigrescens*. Phytochem 33 (3):733-734.

Maldini, M., Montoro, P., Hamed, A.I., Mahalel, U.A., Oleszek, W., Stochmal, A., Piacente, S. 2011. Strong antioxidant phenolics from *Acacia nilotica*: Profiling by ESI-MS and

qualitative-quantitative determination by LC-ESI-MS. J Farm Biomed Analysis 56:228-239.

Manohara Reddy, S. A., Mudgal, J., Bansal, P., Vasanthraju, S. G., Srinivasan, K. K., Rao, C. M. and Gopalan Kutty, N. 2011. Antioxidant, anti-inflammatory and anti-hyperglycaemic activities of heterocyclic homoprostanoid derivatives. Bioorg Med Chem 19:384-92.

Maslin, B. R., Orchard, A. E. and West, J. G. 2003. Nomenclatural and classification history of *Acacia* (Leguminosae: Mimosoideae), and the implications of generic subdivision. Unpublished paper available online at http://www.worldwidewattle.com/infogallery/taxonomy/nomen-class.pdf.

Mattila, P. and Hellström, J. 2007. Phenolic acids in potatoes, vegetables, and some of their products. J Food Composit Anal 20:152-160.

Mizushima, Y. and Kobayashi, M. 1968. Interaction of anti-inflammatory drugs with serum proteins, especially with some biologically active proteins. J Pharm Pharmacol 20:169-173.

Naczk, M. and Shahidi, F. 2004. Extraction and analysis of phenolics in food. J Chromatogr A 1054:95-111.

Nauchini, Y. 1963. Rutin from the flowers of Japanese: *Acacia papoya.* Tr. Vich. Inst. Kranifelns Vkujona Plom-Plovdjv. 10:233-300.

Neto, P. A. S. P. and Caetano, L. C. 2005. Plantas medicinais: do popular ao científico. Edições UFAL: Maceió, Alagoas, Brazil.

Nyila, M.A., Leonard, C.M., Hussein, A.A., Lall, N. 2012. Activity of South African medicinal plants against Listeria monocytogenes biofilms, and isolation of active compounds from *Acacia karroo*. South African J Botany 78:220-227.

Ogunbinu, A. O., Okeniyi , S., Flamini , G., Cioni ,P. L., Ogunwande, I. A., Babalola, I. T. 2010. Essential oil composition of *Acacia nilotica* Linn., and *Acacia albida* Delile (Leguminosae) from Nigeria, J Essent Oil Res 22:540-542.

Ogunwande, I.A., Matsui, T, Matsumoto, K., Shimoda, M., Kubmarawa, D. 2008. Constituents of the essential oil from the leaves of Acacia tortilis (Forsk.) Hayne. J. Essent.Oil Res. 20, 116-119.

Peleg, H., Bodine, K. K. and Noble, A. C. 1998. The influence of acid on astringency of alum and phenolic compounds. Chem Senses 23:371-8.

Phillipson, J. D. 2001. Phytochemistry and medicinal plants. Phytochemistry 56:237-43.

Pires, A. P. M. 2010. Composição química e actividade antioxidante de folhas de diferentes castas de videira in: Escola Superior Agrária. Instituto Politécnico de Bragança: Bragança, Portugal.

Plants on-line. 2015. Australian Native Plants Societs In: http://anpsa.org.au/a-pod.html (consulted in 17-05-2015).

Polya, G. M. and Foo, L. Y. 1994. Inhibition of eukaryote signal-regulated protein kinases by plant-derived catechin-related compounds. Phytochemistry 35:1399-405.

Pragasam, S. J. and Rasool, M. 2013. Dietary component p-coumaric acid suppresses monosodium urate crystal-induced inflammation in rats. Inflammation Research 62:489-498.

Prior, R. L., Wu, X. and Schaich, K. 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 53:4290-302.

Rao, J. et al. 1991. Chemical investigation of *Acacia leucopholea* flowers. Indian J Nat Prod 7 (1):20-21.

Raskin, I., Ribnicky, D. M., Komarnytsky, S., Ilic, N., Poulev, A., Borisjuk, N., Brinker, A., Moreno, D. A., Ripoll, C., Yakoby, N., O'Neal, J. M., Cornwell, T., Pastor, I. and Fridlender, B. 2002. Plants and human health in the twenty-first century. Trends Biotechnol 20:522-31.

Ribeiro, M. A. 2012. Estudo da composição polifenólica de extratos da biomassa de *Acacia melanoxylon*, R. Br. e avaliação do potencial bioactivo in: Departamento de Química e Bioquímica. Faculdade de Ciências da Universiade de Lisboa: Lisboa, Portugal.

Richardson, D. M. and Rejmánek, M. 2011. Trees and shrubs as invasive alien species - a global review. Divers Distrib 17:788-809.

Richardson, D. M., Pyšek, P., Rejmánek, M., Barbour, M. G., Panetta, F. D. and West, C. J. 2000. Naturalization and invasion of alien plants: concepts and definitions. Divers Distrib 6:93-107.

Rodrigues, J. S. C. 2001. Contributo para o estudo etnobotânico das plantas medicinais e aromáticas no parque natural da serra de S. Mamede. Rep. FCUL: Lisboa.

Rodríguez-Rojo, S., Visentin, A., Maestri, D. and Cocero, M. J. 2012. Assisted extraction of rosemary antioxidants with green solvents. J Food Eng 109:98-103.

Roseiro, L. B., Tavares, C. S., Roseiro, J. C. and Rauter, A. P. 2013. Antioxidants from aqueous decoction of carob pods biomass (Ceretonia siliqua L.): Optimisation using response surface methodology and phenolic profile by capillary electrophoresis. Ind Crops and Prod 44:119-126.

Saayman, H. & Roux, D. 1965. The origin of tannins and flavonoids in black-wattle barks and heartwoods, and their associated ‘‘non-tannin’’ components. Biochem J 97:794–801.

Sahai, R., Agarwal, S. K. and Rastogi, R. P. 1980. Auriculoside, a new flavan glycoside from *Acacia auriculiformis*. Phytochem 19:1560-1562.

Salem, H. B., Nefzaoui, A., Salem, L. B., Ferchichi, H. and Tisserand, J. L. 1997. Intake and digestion in sheep given fresh or air-dried *Acacia cyanophylla* Lindl foliage. Pages 361-374 in: Annales de Zootechnie.

Santos, A., Simões, R., Pereira, H. and Anjos, O. 2004. Alternative species for the forest industry as forms of diversify the landscape.

Santos, A., Teixeira, A., Anjos, O., Simões, R., Nunes, L., Machado, J. S. and Tavares, M. 2007. Utilização potencial do lenho de Acacia melanoxylon a crescer em povoamentos puros ou mistos com Pinus pinaster pela indústria florestal portuguesa. Silva Lusit 15:57-77.

Seigler, D. S. 2003. Phytochemistry of *Acacia - sensu lato*. Biochem Syst Ecol 31:845-873.

Silva, M. L. C., Costa, R. S., Santana, A. S. and Koblitz, M. G. B. 2010. Compostos fenólicos, carotenóides e atividade antioxidante em produtos vegetais. Semina: Ciências Agrárias 31:669.

Silva, V. C. and Keber, V. A. 2003. Análise dos flavonoides da *Acacia longifolia* (Andr.) Willd. Leguminosae-Mimosoideae. Visão Acadêmica 4.

Simoes, C. M. O. 2001. Farmacognosia: da planta ao medicamento. UFRGS; Florianópolis: UFSC.

Singh, R., Singh, S., Kumar, S., Arora, S. 2007. Studies on antioxidant potential of methanol extract/fractions of *Acacia auriculiformis* A. Cunn. Food Chem 103:505-511.

Soares, S. E. 2002. Ácidos fenólicos como antioxidantes. Rev. Nutr 15:71-81.

Stohs, S. J. and Bagchi, D. 2015. Antioxidant, anti-inflammatory, and chemoprotective properties of *Acacia catechu* heartwood extracts. Phytotherapy Research.

Stone, E. 1763. An account of the success of the bark of the willow in the cure of agues. In a letter to the Right Honourable George Earl of Macclesfield, President of RS from the

Rev. Mr. Edmund Stone, of Chipping-Norton in Oxfordshire. Philosophical Transactions 53:195-200.

Tawaha, K., Alali, F., Gharaibeh, M., Mohammad, M. and Elelimat, T. 2007. Antioxidant activity and total phenolic content of selected Jordanian plant species. Food Chem 104:1372-1378.

Thieme, H. & Khogali, A. 1974. Isolation of apigenin 6-8- BIS (C-b-D-glucopiranoside) from leaves of *Accia farnesiana*. Harmazie Berlin. 29 (5): 352.

Tindale, M. & Roux, D. 1969. A Phytochemical survey of the Australian species of *Acacia*. Phytochem. 8 (9): 1713-1727.

Trnkova, L., Bousova, I., Stankova, V. and Drsata, J. 2011. Study on the interaction of catechins with human serum albumin using spectroscopic and electrophoretic techniques. J Molec Struct 985:243-250.

Tung, Y. T., Wu, J. H., Kuo, Y. H. and Chang, S. T. 2007. Antioxidant activities of natural phenolic compounds from *Acacia confusa* bark. Bioresour Technol 98:1120-3.

Tung, Y-T., Wu, J-H, Hsieh, C-Y., Chen, P-S., Chang, S-T. 2009. Free radical-scavenging phytochemicals of hot water extracts of *Acacia confusa* leaves detected by an on-line screening method. Food Chem 115:1019-1024.

Valsakumari, M. & Sulochana, N. 1991. Chemical examination of *Acacia leucophloea*. J Indian Chem Soc 68 (12):673-674.

Visht, S. and Chaturvedi, S. 2012. Isolation of Natural Products. Current Pharm Research.

Wollenweber, E. & Seigler, D. 1982. Flavonoids from the exsudate of *Acacia neovernicosa*. Phytochem. 21 (5):1063-1066.

World Wide Wattle (2015) Distribution and phytogeography of Acacia. In: http://www.worldwidewattle.com/ (consulted in 18/05/2015).

Xu, Y. 1996. Tutorial: capillary electrophoresis. The Chem Educ 1:1-14.

Zhang, L., Chen, J., Wang, Y., Wu, D., Xu, M. 2010. Phenolic extracts from *Acacia mangium* bark and their antioxidant activities. Molecules 15:3567-3577.

Annexes

Annex A - Eluents used for the Sephadex columns

Sample	Collected Fractions	Solvents
FR	01-02	Ethyl acetate
	03-09	Acetone
	10-13	Ethanol
	14-37	Methanol
C	01-15	Ethyl acetate
	16-18	Acetone

Annex B - Calculation for the determination of total phenolics in GAE% in the extracts.

100ul Test	Dilution	Sample	Abs 1	Abs 2	Abs 3	Mean Abs	StdD	Y Mean-Blank	y=mx+b	Dilution	ug GA/ml ext	mg GA/ml ext	FT mg GA/mg ext	% gGAE/100g ext
1	TQ	**FR**	0,7129	0,7140	0,7364	0,7211	0,0133	0,6546	17,96		179,6	0,180	0,18	**18,0**
	1/2 50mg/50ml	**FR**	0,3961	0,4040	0,3957	0,3986	0,0047	0,3321	9,10	18,20	182,0	0,182	0,18	**18,2**
	TQ	**C**	1,2329	1,2300	1,2378	1,2336	0,0039	1,1671	32,04		320,4	0,320	0,32	**32,0**
	1/2 50mg/50ml	**C**	0,6789	0,6764	0,6662	0,6738	0,0067	0,6073	16,66	33,33	333,3	0,333	0,33	**33,3**
2	1/2 50mg/50ml	**FR**	0,2100	0,2162	0,2432	0,2131	0,0044	0,1459	3,14	6,28	62,8	0,063	0,06	**6,3**
	1/2 50mg/50ml	**C**	0,3206	0,3882	0,3536	0,3541	0,0338	0,2869	5,80	11,59	115,9	0,116	0,12	**11,6**
3	TQ	**FR**	0,6177	0,6167	0,6035	0,6126	0,0079	0,5596	20,54		205,4	0,205	0,21	**20,5**
	1/2 50mg/50ml	**FR**	0,3254	0,3388	0,3356	0,3333	0,0070	0,2803	9,71	19,42	194,2	0,194	0,19	**19,4**
	1/2 50mg/50ml	**C**	0,5439	0,5515	0,5473	0,5476	0,0038	0,4946	18,02	36,04	360,4	0,360	0,36	**36,0**

Annex C - Calculation for the determination of total phenolics, tannins and not tannins (mg GAE/mg ext).

100ul Test	Dilution	Sample	Abs 1	Abs 2	Abs 3	Mean Abs	StdD	Y Mean-Blank	y=mx+b	Dilution	ug GA/ml ext	mg GA/ml ext	FT mg GA/mg ext	FT	NT	T mg GAE/mg extr
1	1/2 50mg/50ml	FR	0,2091	0,1731	0,2096	0,209	0,0004	0,143	3,902	15,61	52,03	0,052	0,052	0,182	0,052	0,130
	1/2 50mg/50ml	C	0,1889	0,2479	0,3143	0,218	0,0417	0,152	4,151	16,60	55,35	0,055	0,055	0,333	0,055	0,278
2	1/2 50mg/50ml	FR	0,2091	0,1731	0,2096	0,209	0,0004	0,142	3,071	12,28	40,94	0,041	0,041	0,063	0,041	0,022
	1/2 50mg/50ml	C	0,1889	0,2479	0,3143	0,218	0,0417	0,151	3,241	12,96	43,21	0,043	0,043	0,116	0,043	0,073
3	TQ	FR	0,2166	0,1914	0,1808	0,196	0,0184	0,143	4,402	8,80	22,01	0,022	0,022	0,205	0,022	0,183
	1/2 50mg/50ml	FR	0,1705	0,1631	0,2854	0,206	0,0686	0,153	4,792	19,17	63,89	0,064	0,064	0,194	0,064	0,130
	1/2 50mg/50ml	C	0,3085	0,2641	0,2825	0,285	0,0223	0,232	7,842	31,37	104,57	0,105	0,105	0,360	0,105	0,256

Annex D - Calculations for determining the antioxidant activity expressed in mM TEAC and % inhibition (DPPH).

Method	Test	Sample	Abs			Mean	StdD	AA (TEAC) mM	% Inhibition
			Abs 1	Abs 2	Abs 3				
DPPH	1	FR	0,1912	0,1618	0,1987	0,18	0,02	1,07	65,65
		C	0,0725	0,0716	0,0773	0,07	0,00	1,38	86,21
	2	FR	0,2133	0,2285	0,1663	0,22	0,01	1,04	68,15
		C	0,1213	0,1171	0,0743	0,12	0,00	1,26	82,81
	3	FR	0,0825	0,1051	0,116	0,10	0,02	1,06	78,66
		C	0,0582	0,1881	0,0656	0,10	0,07	1,05	78,08
ABTS	1	FR	0,2503	0,1912	0,2655	0,26	0,01	1,56	60,44
		C	0,0451	0,0455	0,0559	0,05	0,01	2,42	92,51

Annex E - Eluents used in CC.

Collected Fractions	Solvents
I	Distilled Water
II	Water (pH=2)
III	Methanol/Water (pH=2)
IV	Methanol

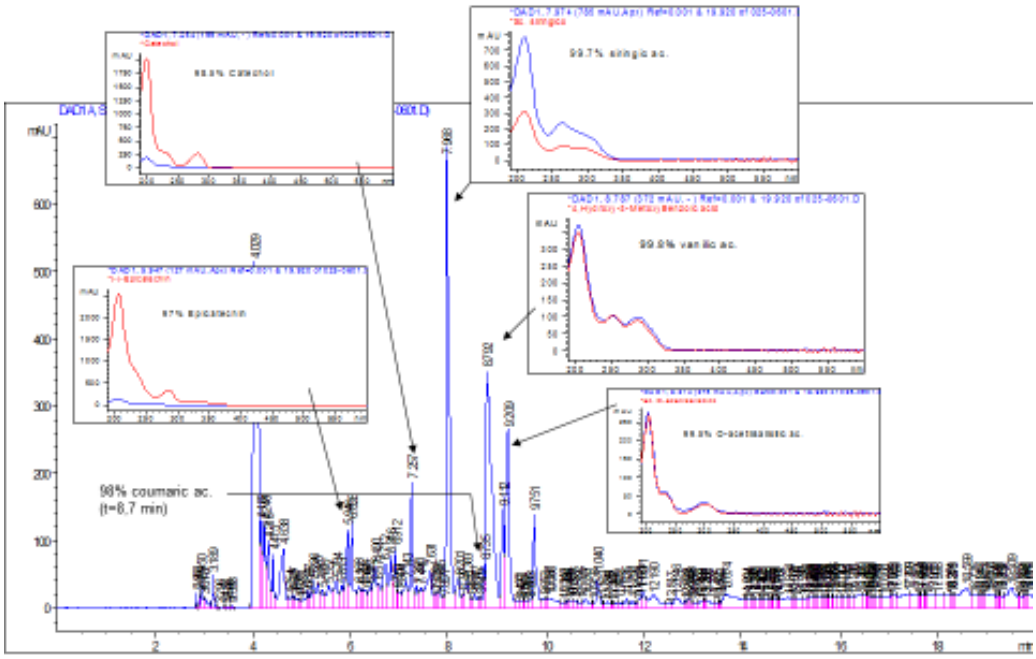
Annex F - Extract fractions obtained from CC.

Sample	Collected Fractions	Solvents	Fractions	CZE Code
FR	1	Ethyl Acetate	I	11FR
	2	Ethyl Acetate	II	12FR
	3	Acetone	III	13FR
	4-5	Acetone	IV	14FR
	6-8	Acetone	V	15FR
	9-10	Ethanol	VI	16FR
	11	Ethanol		
	12-13	Ethanol	VII	17FR
	14-17	Methanol	VIII	18FR
	18-23	Methanol	IX	19FR
	24-33	Methanol	X	20FR
	34-39	Methanol	XI	21FR
C	1	Methanol	I	4C
	2	Methanol	II	5C
	3	Methanol	III	6C
	4-5	Methanol	IV	7C
	6-9	Methanol	V	8C
	10	Methanol	VI	9C
	11-14	Methanol	VII	10C
	15-18	Acetone		

Annex G - Electropherograms CZE.

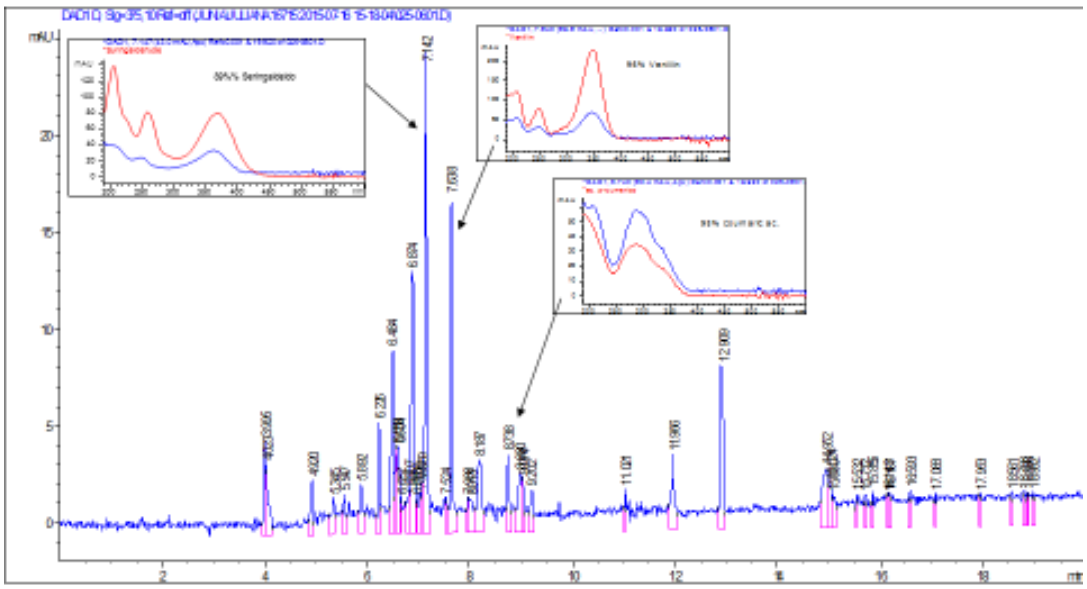
6C – fracção sephadex da casca A. Melanoxylon (águas de decocção após Clevanger)

200 nm

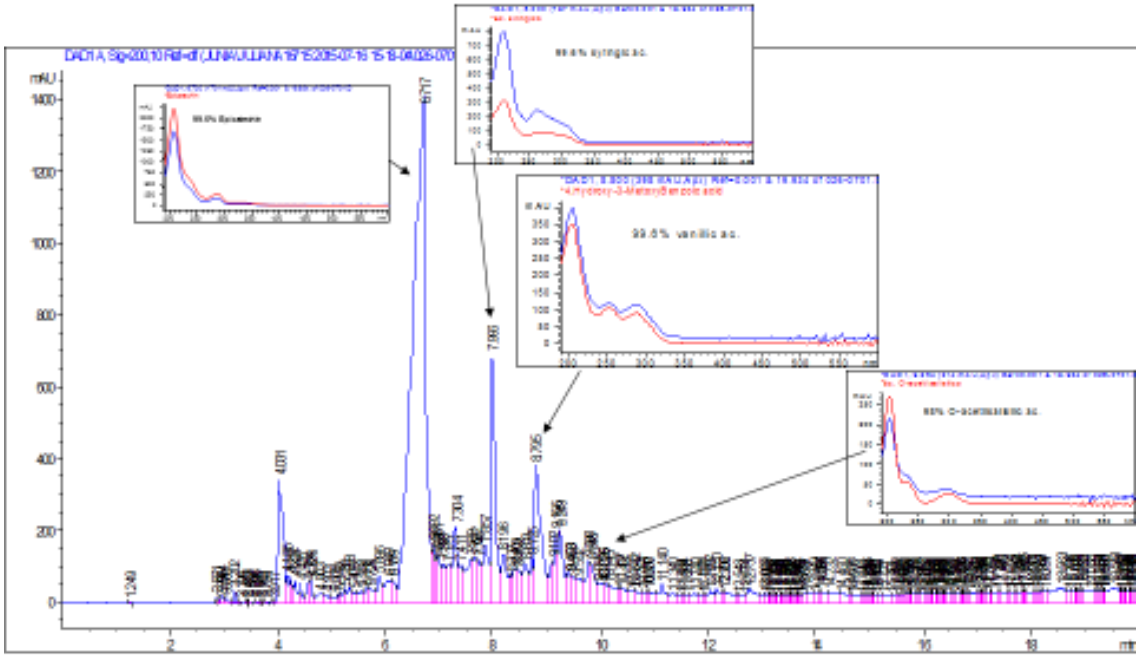


6C – fracção sephadex da casca (águas de decocção após Clevanger)

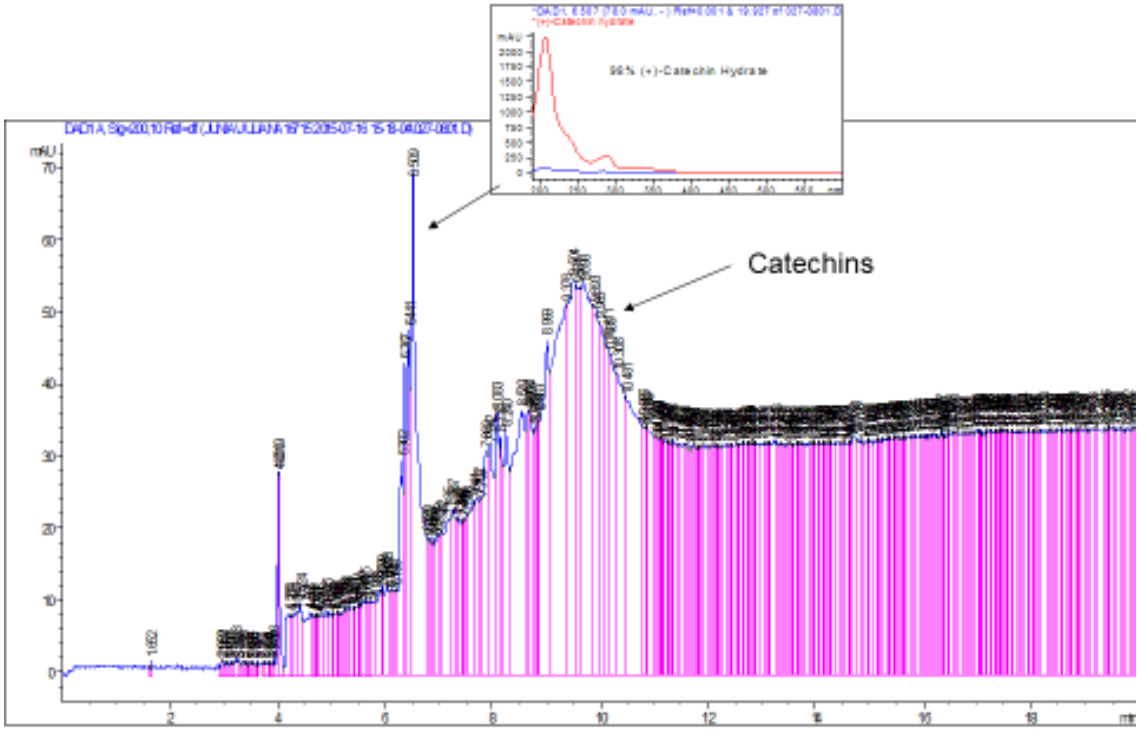
375 nm



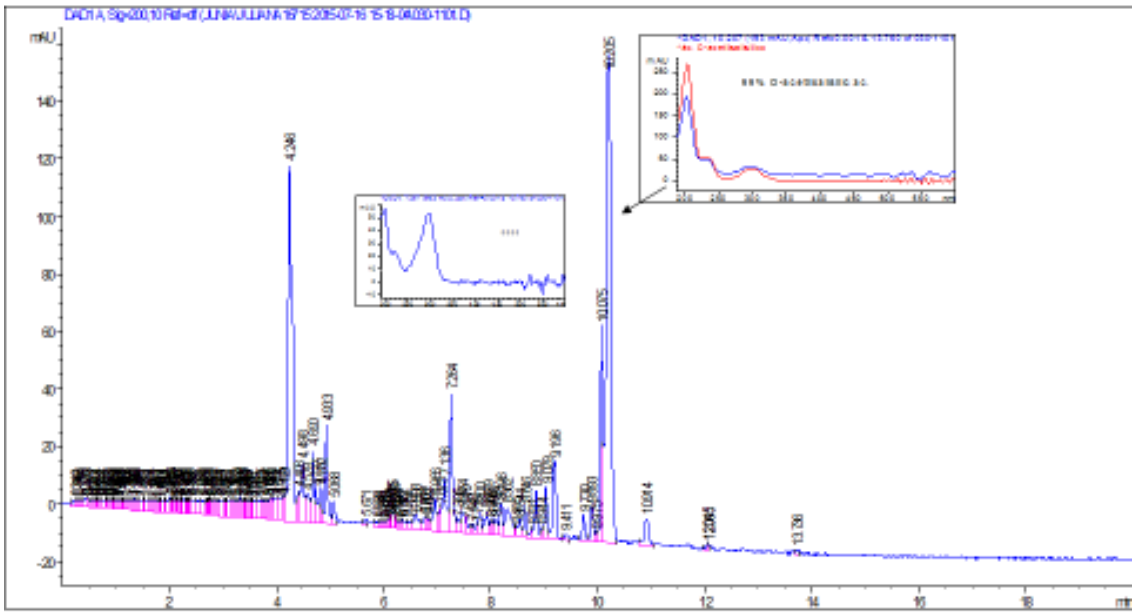
7C – fracção sephadex da casca (águas de decocção após Clevanger)



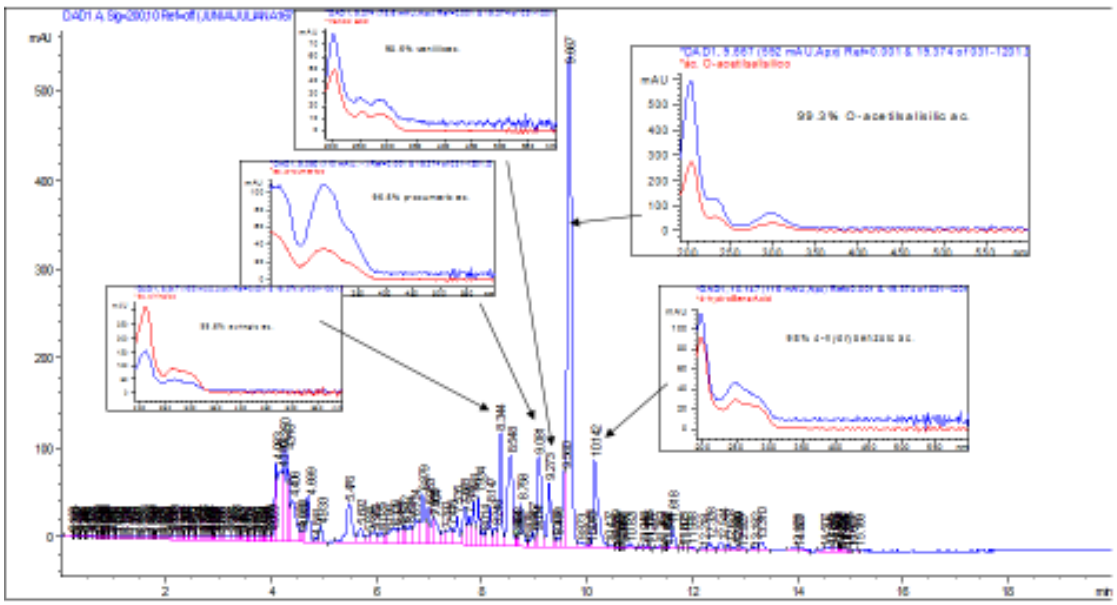
8C – fracção sephadex da casca (águas de decocção após Clevanger)



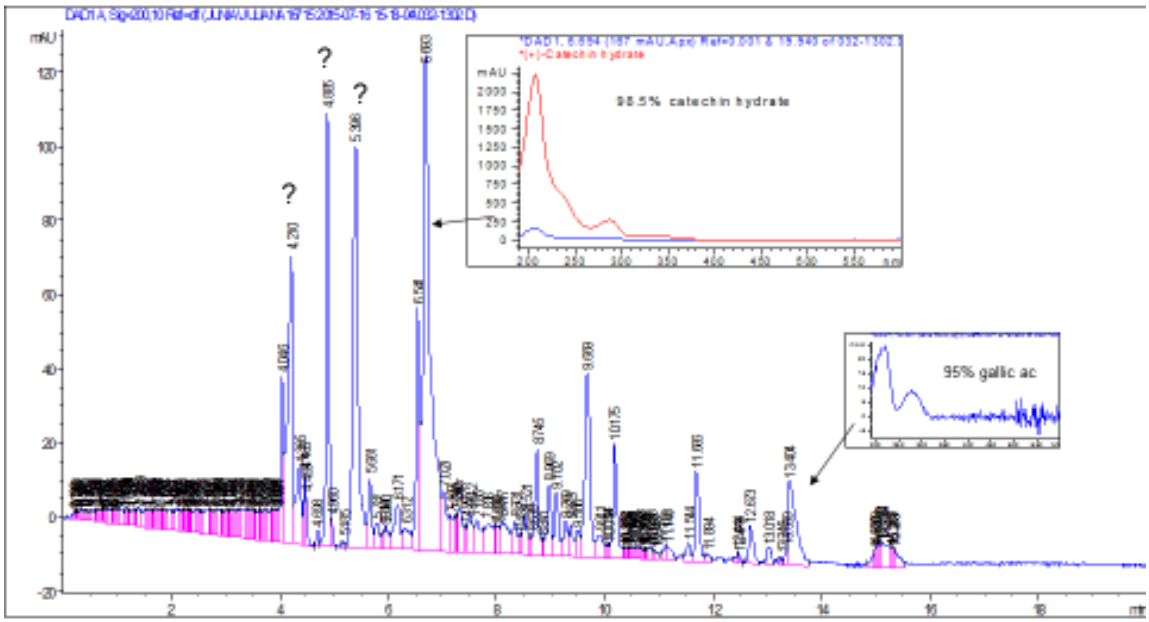
13FR – fracção sephadex das folhas e ramos (águas de decocção após Clevanger)



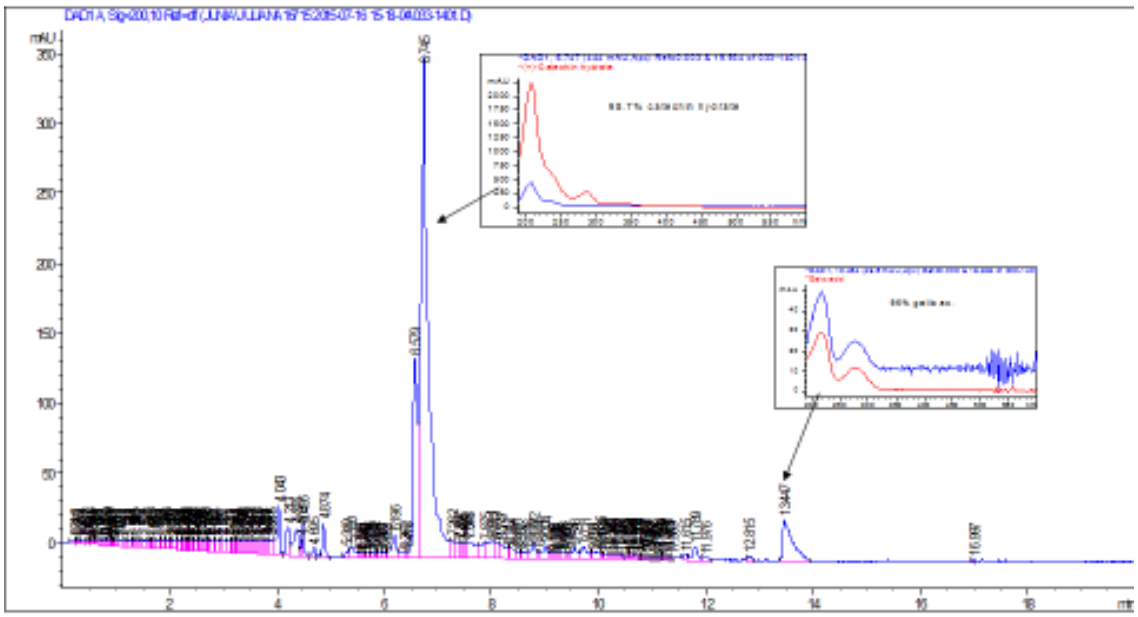
14FR – fracção sephadex das folhas e ramos (águas de decocção após Clevanger)



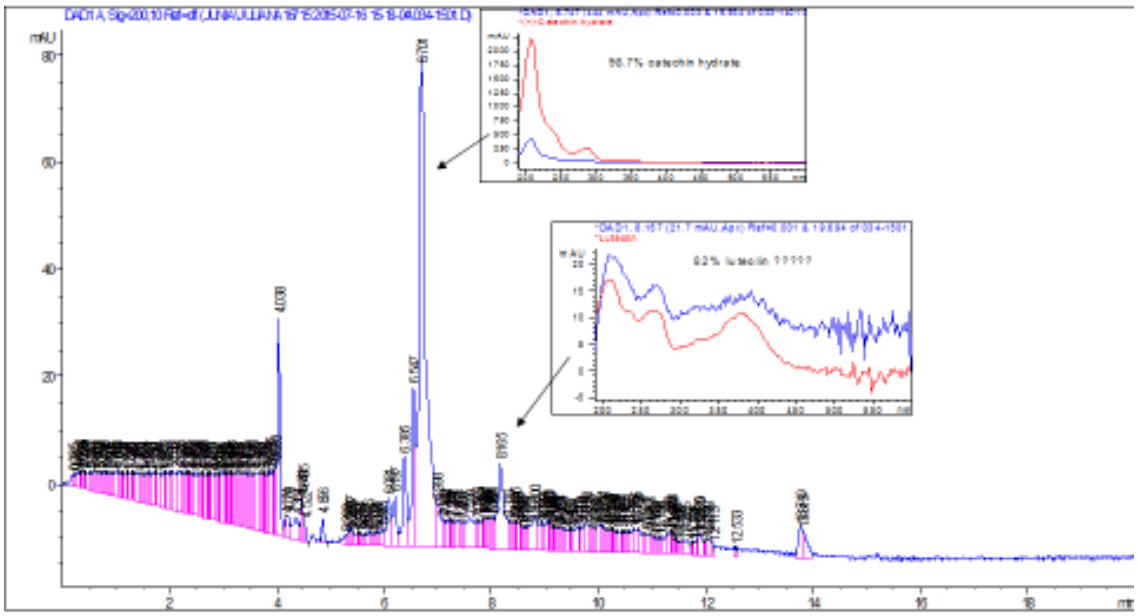
15FR – fracção sephadex das folhas e ramos (águas de decocção após Clevanger)



16FR – fracção sephadex das folhas e ramos (águas de decocção após Clevanger)

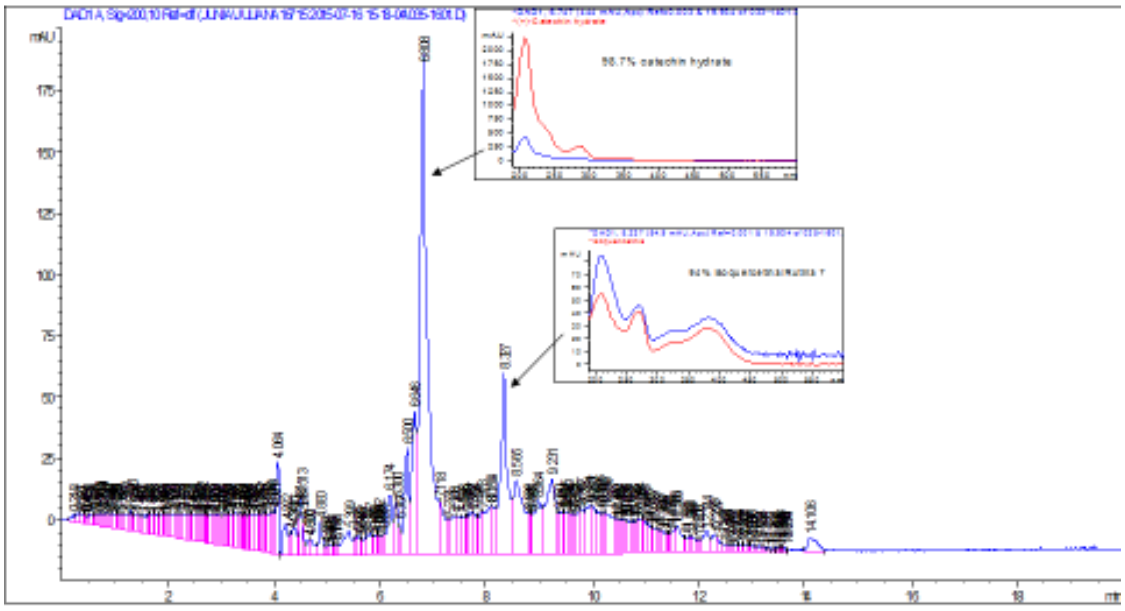


17FR – fracção sephadex das folhas e ramos (águas de decocção após Clevanger)



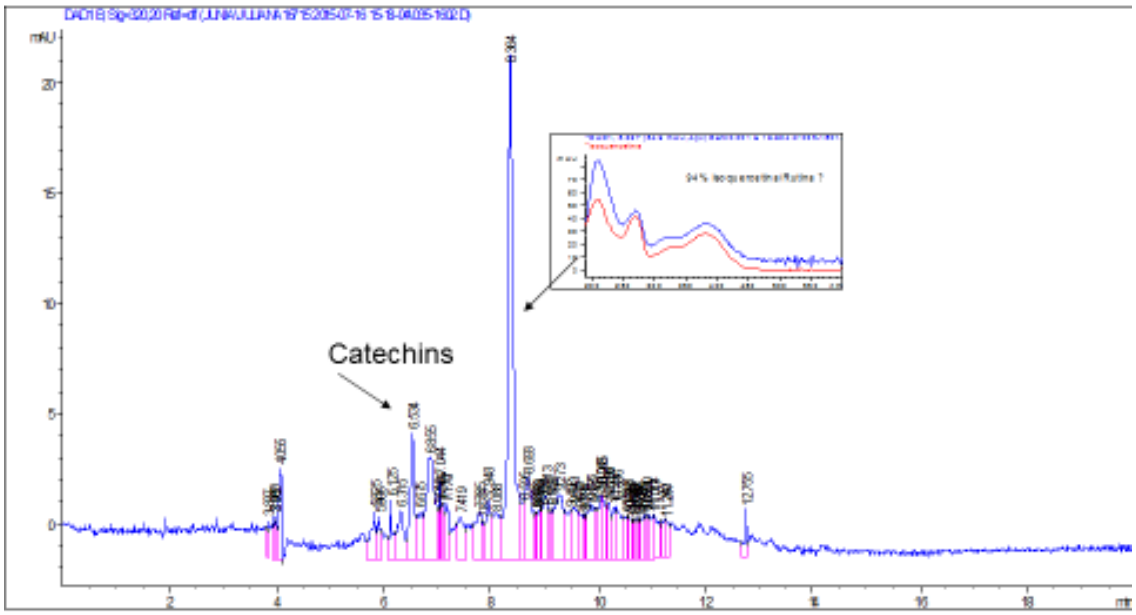
18FR – fracção sephadex das folhas e ramos (águas de decocção após Clevanger)

200 nm



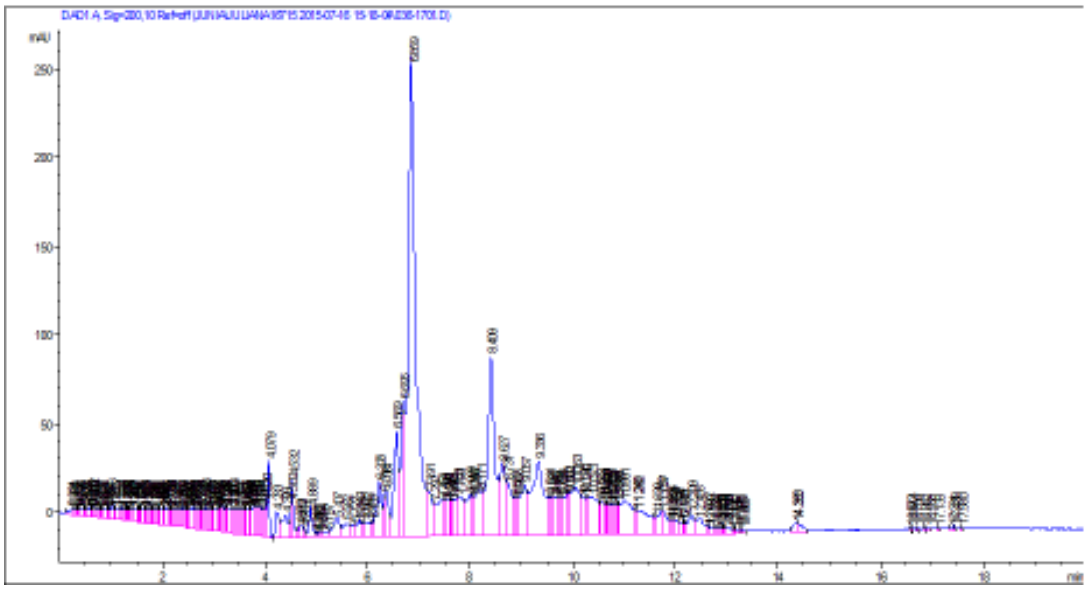
18aFR – fracção sephadex das folhas e ramos (águas de decocção após Clevanger)

320 nm



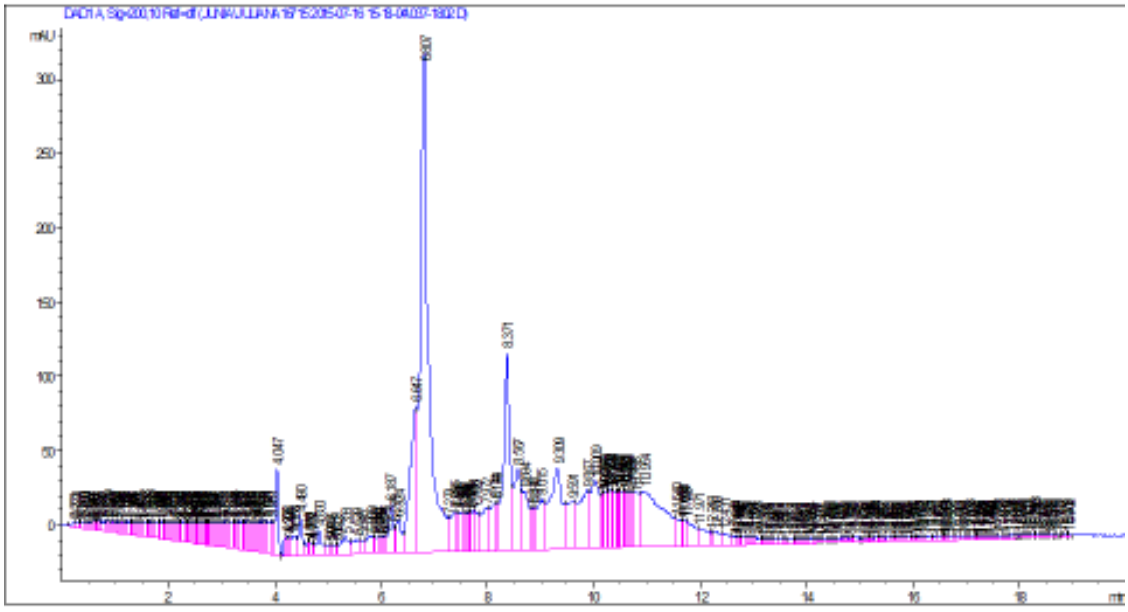
19FR – fracção sephadex das folhas e ramos (águas de decocção após Clevanger)

19FR igual a 17FR e 18FR: juntar estas fracções numa única



20FR – fracção sephadex das folhas e ramos (águas de decocção após Clevanger)

20FR igual a 17FR- 19FR: juntar estas fracções numa única



Annex H - Anti-inflammatory activity

Sample	Abs			Mean	StdD	% Inhibition
	Abs 1	Abs 2	Abs 3			
Blank	0,5270	0,4680	0,4610	0,4645	0,0049	------
ASA	0,0020	0,0010	0,0000	0,0003	0,0007	99,93
EXT C	0,5730	0,7230	0,6460	0,6095	0,0516	n.i.
6C	0,3960	0,4380	0,3970	0,3965	0,0007	14,64
7C	0,2970	0,2690	0,2810	0,2750	0,0085	40,80
13FR	0,8170	0,7740	0,9920	0,7955	0,0304	13,39
14FR	0,5580	0,6140	0,5580	0,5580	0,0000	39,25
15FR	1,1660	1,0960	1,1790	1,1725	0,0092	n.i.
16FR	1,4950	1,5710	1,5950	1,5830	0,0170	n.i.
17-20FR	1,1650	0,9610	1,0700	1,0653	0,0672	n.i.