THE BROAD SPECTRUM OF BIOACTIVE PROPERTIES OF PHENOLIC EXTRACTS: A PROSPECTIVE STUDY IN THREE DIFFERENT PLANTS

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List of Abbreviations

λ max	Wavelengths of maximum absorption
5-LOX	5-Lipooxygenase
A	Absorbance of the control
B	Absorbance of the sample solution
A_{DPPH}	Absorbance of DPPH solution
A_S	Absorbance of sample
CBI	β-Carotene bleaching inhibition
CE	Collision energy
CES	Collision energy spread
COX-2	Cyclooxygenase-2
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DMEM	Dulbecco's modified Eagle's minimum essential medium
DMSO	Dimethyl sulfoxide
DP	Declustering potential
EC_{50}	Extract concentration achieving 50% of antioxidant/anti-inflammatory activity
ECACC	European collection of animal cell culture
EMS	Enhanced mass spectrometer
EPI	Enhanced product ion
FBS	Fetal bovine serum
GI_{50}	Extract concentration achieving 50% of growth inhibition in cell lines
HBSS	Hank's balanced salt solution
HepG2	Hepatocellular carcinoma cells
HeLa	Human cervical carcinoma cell line
HPLC	High performance liquid chromatography
IC_{50}	Extract concentration achieving 50% of antioxidant/anti-inflammatory activity
ICAM-1	Intercellular adhesion molecule-1
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
LPI	Lipid peroxidation inhibition

LPS Lipopolysaccharide
MCF-7 Breast carcinoma cells
MDA Malondialdehyde
MS Mass spectrometer
NCI-H460 Non-small cell lung cancer cells
NED N-(1-napthyl) ethylenediamine hydrochloride
NF-κβ Nuclear factor-κB
NO Nitric oxide
PDA Photodiode array detector
PGE2 Prostaglandin E2
PI3K Phosphoinositide 3-kinase
PLP2 Non-tumor liver cells
RNS Reactive nitrogen species
ROS Reactive oxygen species
RP Reducing power
RSA Radical-scavenging activity
RSS Reactive sulphur species
Rt Retention time
SD Standard deviation
SRB Sulforhodamine B
TCA Trichloroacetic acid
TNF-α Tumour necrosis factor α
UV Ultraviolet
WHO World health organization

Abstract

Medicinal plants have received great attention in the last years because of their richness in bioactive compounds responsible for different biological activities. In the present study, hydroethanolic extracts of *Hypericum androseamum* L., *Equisetum giganteum* L. and *Tilia platyphyllos* Scop., were chemically characterized in terms of phenolic composition and evaluated for their antioxidant, antitumor and anti-inflammatory properties. All three medicinal plants displayed antioxidant activity as free radical scavengers but mainly as lipid peroxidation inhibitors. The extracts were also effective in inhibiting nitric oxide production, as an indicator of the anti-inflammatory potential, displayed antitumor properties in breast carcinoma (MCF-7), non-small cell lung cancer (NCI-H460), cervical carcinoma (HeLa) and hepatocellular carcinoma (HepG2) cell lines and, up to the maximal tested concentration, did not show any cytotoxic effects for non-tumor cells. 5-*O*-Caffeoylquinic acid and 3-*O*-caffeoylquinic acid were the most abundant phenolic compounds identified in the *H. androsaemum* extract, and might be responsible for the observed bioactivity. Protocatechuic acid and epicatechin were most abundant in the *T. platyphyllos* extract, while kaempferol-*O*-glucoside-*O*-rutinoside was the most abundant phenolic compound in the *E. giganteum* extract. The results showed that these medicinal plants displayed very interesting bioactivities, which are related with phenolic composition and quantity. However, it would be interesting to evaluate the *in vivo* efficacy of these plant extracts, as also their mechanisms of action, in order to establish real therapeutic doses.

1 MEDICINAL PLANTS

1.1 Medicinal plants

1.1.1 The extended use of medicinal plants

Natural resources like plants are currently used all over developed and under developed countries of the world as traditional home remedies and are promising agents for drug discovery as they play crucial role in traditional medicine. The use of plants for medicinal purpose usually varies from country to country and region to region because their use depends on the history, culture, philosophy and personal attitudes of the users (Ahmad et al., 2015).

The use of plants and plant products as drugs predates the written human history (Hayta et al., 2014). Plants are a very important resource for traditional drugs and around 80% of the population of the planet use plants for the treatment of many diseases and traditional herbal medicine accounts for 30-50% of the total medicinal consumption in China. In North America, Europe and other well-developed regions over 50% of the population have used traditional preparations at least once (Dos Santos Reinaldo et al., 2015).

Medicinal plants have been used over years for multiple purposes, and have increasingly attract the interest of researchers in order to evaluate their contribution to health maintenance and disease's prevention (Murray, 2004). Recently between 50,000 and 70,000 species of plants are known and are being used in the development of modern drugs. Plants were the main therapeutic agents used by humans from the 19th century, and their role in medicine is always topical (Hayta et al., 2014).

The studies of medicinal plants are rapidly increasing due to the search for new active molecules, and to improve the production of plants or bioactive molecules for the pharmaceutical industries (Rates, 2001). Several studies have been reported, but numerous active compounds directly responsible for the observed bioactive properties remain unknown, while in other cases the mechanism of action is not fully understood.

According to the WHO 25% of all modern medicines including both western and traditional medicine have been extracted from plants, while 75% of new drugs against infective diseases that have arrived between 1981 and 2002 originated from natural sources, it was reported that the world market for herbal medicines stood at over US $60 billion per year and is growing steadily (Bedoya et al., 2009). Traditional medicine has an important economic impact in the 21st century as it is used worldwide, taking advantage on the low cost, accessibility, flexibility and diversity of medicinal plants (Balunas & Kinghorn, 2005).

1.1.2 The particular case of *Hypericum androsaemum* L., *Tilia platiphyllos Scop*. and *Equisetum giganteum* L.

1.1.2.1 *Hypericum androsaemum* L.

Hypericum androsaemum L. (Hypericaceae family), commonly known as "Tutsan", is a medicinal plant species growing in damp or shady places in Europe (Valentão et al., 2004). Several studies on anti-depressive, anti-inflammatory, antitumor, antimicrobial, antioxidant and antiviral benefits of *Hypericum perforatum* L. have been reported (Rainha et al., 2011; Nogueira et al., 2013).

Figure 1. *Hypericum androsaemum* L. (http://luzcardoso.blogspot.pt/2009/02/hipericao-do-geres-hypericum.html).

However, based on recent findings, other *Hypericum* species have also revealed very interesting functional properties. *Hypericum androsaemum* L. **(Figure 1)**, commonly known as sweet-amber, has a popular use as cholagogue, hepatoprotector, nephroprotective and diuretic (Guedes et al., 2003). Hypericum genus (Hypericaceae) includes 18 species, among which 15 have spontaneous grow, and can be found in three regions in Portugal (three in Azores, one in Madeira and eleven in mainland) (Ramalhete et al., 2016). *H. androsaemum* has been used in traditional herbal medicine by different cultures. In the Portuguese folk medicine, the plant known locally as "Hipericão do Gerês", is known for its diuretic effect and leaf infusions are used for liver, kidneys and bladder diseases. In Britain and France, Tutsan means "all-healthy", or "all heal" due to its medicinal properties. In England the pounded leaves are mixed with lard to get an ointment for wounds and dressing cuts (Caprioli et al., 2016).

1.1.2.2 *Tilia platiphyllos Scop*.

Tilia platiphyllos Scop. is a flowering plant in the family of *Tiliaceae*, native to most of the temperate Northern Hemisphere, Asia, Europe and North America; covers virtually the entire continent of Europe except for large parts of Scandinavia and it is absent in western North America. *T. platyphyllos* (**Figure 2**) is often planted as an ornamental tree in parks and as a shade or lawn tree. They are resistant to drought, dry winds and low temperatures; they are suitable for planting and trade protection (Radoglou et al., 2008).

Figure 2. *Tilia platiphyllos* Scop. (http://eattheplanet.org/wp-content/uploads/2013/09/Tilia_americana_NRCS-1.jpg).

T. platiphyllos, is one of lime flower species used as a traditional medicinal plant in Europe and Latin America mainly as sedatives and tranquilizers. Based on its medicinal benefit, infusions of their inflorescences are used to prepare tea (Cotrim et al., 1999). It has been reported to have antispasmodic properties, being used since ancient times for treatment of diseases such as hypertension associated with atherosclerosis and nervous tension (Karioti et al., 2014), migraine, cold fever, hysteria and blood pressure (Ropiak et al., 2016). Also because of their soothing and hydrating properties, extracts of this plant may find application in the phytocosmetic industry (Karioti et al., 2014).

1.1.2.3 *Equisetum giganteum* L.

*Equisetum gigante*um L. (Equisetaceae Hippocrates subgenus), also called “cavalinha”, “caballo de cola”, “horsetail” or “giant horsetail” (Francescato et al., 2013). It is a lower vascular plant found in Southern and Central America (Danielski et al., 2007).

Figure 3. *Equisetum giganteum*L. (http://gardenofeaden.blogspot.pt/2013/06/the-giant-horsetail-equisetum-giganteum.html).

This species is used mainly in Mexico, Guatemala, Venezuela, Argentina and other countries as traditional medicine. The aerial parts have been widely used as a diuretic in herbal medicine with no oral toxicity observed, to treat liver and urinary disorders and as food supplements. *E. giganteum* (**Figure 3)** was characterized for the first time in Argentina and was commonly used as a diuretic, hemostatic and astringent. The plant infusion is also recommended for diarrhea, gonorrhea and to cure kidney stones as well as for weight loss in Brazil and Argentina (Francescato et al., 2013). Due to its high mineral contents and its remineralizing properties, the plant is also recommended for the revitalization of fingernails and lifeless hair, among other applications (Calvo et al., 2015).

1.2 Phenolic compounds as bioactive molecules

1.2.1 Chemical aspects of phenolic compounds

Plants produce secondary metabolites with bioactive properties, and phenolic compounds represent an important group of these compounds. They are usually found in fruits, vegetables, herbs, roots, leaves, and seeds and they play vital roles such as structure, innate defence system, reproduction or sensorial properties (colour, bitterness, taste and flavour) and the amount of phenolic compounds released may increase due to exposure to UV radiation, infection by pathogens and parasites and exposure to extreme temperatures (Soto et al., 2015).

Phenolic compounds are secondary metabolites which are synthesized through the pentose phosphate, shikimate and phenylpropanoid pathways (Adeboye et al., 2014). Phenolic compounds are the most common secondary metabolites in the plant kingdom and the term “Phenol” denotes a chemical structure comprising a phenyl ring bearing one or more hydroxyl substituents (Lattanzio, 2013). Phenolic compounds consist of more than one aromatic ring bearing and one or more hydroxyl functional groups (Min et al., 2015).

Phenolic compounds include a diversified group of molecules with a wide range of chemical structures identified as monomeric, dimeric, and polymeric phenolics. Several classes of phenolic compounds (**Table 1**) have been categorized on the basis of their basic carbon skeleton ranging from phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, and quinones (**Figure 4**) and the amount of phenolic compound in each plant usually depends on several factors such as cultivation techniques, cultivar, growing conditions, ripening process, processing and storage conditions (Lattanzio, 2013).

Table 1. The major classes of phenolic compounds in plants (Soto et al., 2015).

Phenolic compound	Basic skeleton	Examples
Simple phenol, Benzoquinones	C6	Catechol, hydroquinone
Phenolic acids	C6—C1	Gallic, salicylic acids
Acetophenone, Phenylacetic acid	C6—C2	Tyrosol, *p*-hydroxyphenylacetic
Hydroxycinnamic acid, Coumarins, Phenylpropanes, Chromones	C6—C3	Caffeic, ferulic, myristicin, eugenol, eugenin, bergenon
Naphthoquinones	C6—C4	Juglone, plumbagin
Xanthones	C6—C1—C6	Mangiferin
Stilbenes, Anthraquinones	C6—C2—C6	Emodin resveratrol
Flavonoids, Isoflavonoids, Neoflavonoids	C6—C3—C6	Quercetin, cyanidin, Genistein
Bi, tri, flavonoids	(C6—C3—C6)2,3	Amentoflavone
Lignans, Neolignans	(C6—C3)2	Pinoresinol, eusiderin
Lignins, Catechol melanins	(C6—C3)n, (C6)n	Guaiacyl lignins
Condensed tannins	(C6—C3—C6)n	Procyanidins, propelargonidins

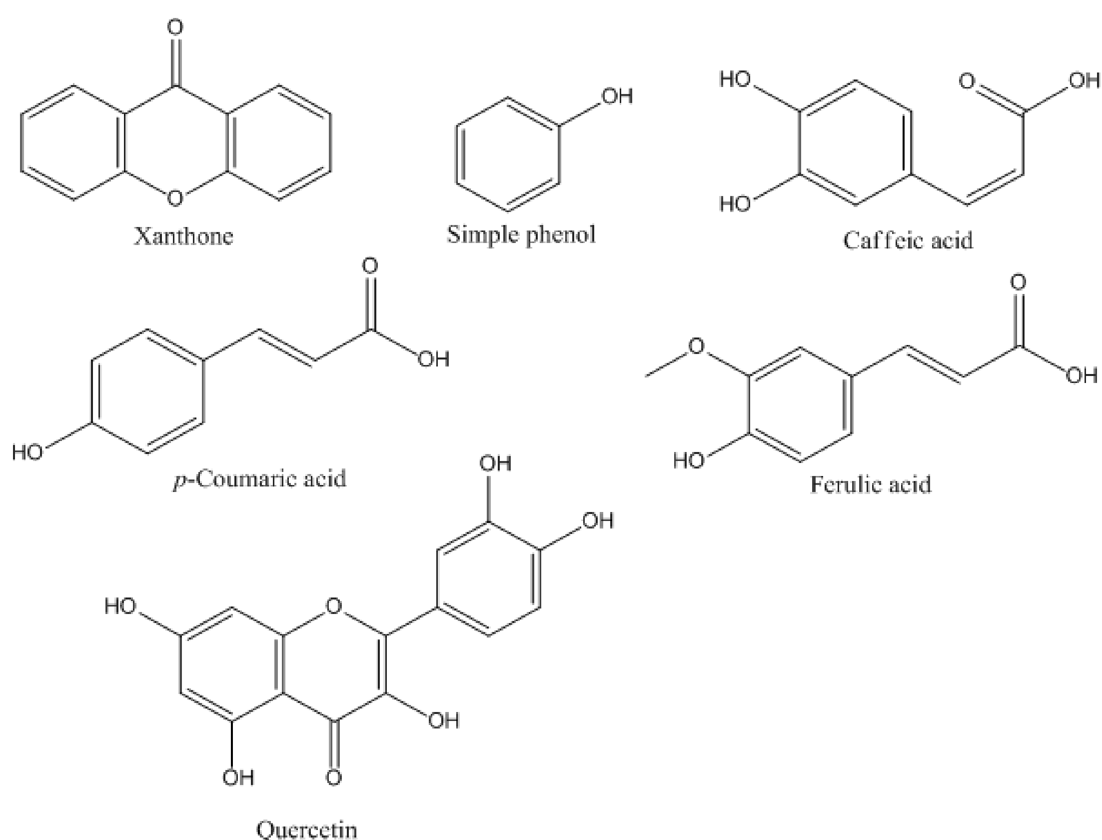


Figure 4. Common phenolic compounds found in medicinal plants.

1.2.2 Reported bioactive properties

As previously mentioned, phenolic compounds and their derivatives are one of the most important groups of secondary metabolites in plants, with several various physiological functions (Vidović et al., 2015). Many phenolic compounds have been reported to have potent anticarcinogenic, antimutagenic, antiatherosclerotic, antihypoglycemic, antibacterial, antifungal, antiviral, anti-thrombotic, cardioprotective and vasodilatory effects as well as antioxidant, antitumor and anti-inflammatory activities (Wojdyło et al., 2007; dos Santos Reinaldo et al.,2015; Touati et al., 2015).

1.2.2.1 Antioxidant activity of phenolic compounds

Phenolic acids are composed mainly of hydroxycinnamic and hydroxybenzoic acid derivatives. They are usually present in plant materials as esters and glycosides. Their antioxidant potential has been known to be due to their free radical scavenging property over hydroxyl, peroxyl radical, superoxide anion and peroxynitrites and also due to their chelating effect. Hydroxybenzoic acid derivatives include gallic acid and several tannins while hydroxycinamic derivatives and many others include coumaric, caffeic and ferulic acids (Carocho & Ferreira, 2013a). Phenolic compounds have been reported by Martins et al. (2016) to display promising antioxidant potential and the study of antioxidant properties of phenolic extracts of plant origin is one of the hottest subjects for researchers. They also reviewed several phenolic compounds (commercial and isolated compounds) from plant and concluded that anthocyanins, hydroxybenzoic and hydroxycinnamic acids derivatives are the most studied phenolic compounds. Flavonoids have also shown a wide range of biochemical and anti-allergic effects, but the most important biological activity of these compounds is the capacity to act as antioxidants (Karakaya & Nehir, 1999).

1.2.2.2 Antitumor activity of phenolic compounds

Phenolic compounds represent one of the most studied compounds worldwide because of their reported beneficial effect in promoting and maintaining good health. Several *in vitro* and *in vivo* studies have reported the important role of phenolic compounds in the fight against cancer and among them are gallic acid, ellagic acid, ferulic acid, caffeic acid, cinnamic acid, courmarin, 7-hydroxycoumarin dicoumarol, β-mangostin, arbutin, forbesione, gambogic acid, resveratrol, kraft lignins, quercetin, flavone acetic acid, catechin and genistein (Carocho & Ferreira, 2013b). Some of the mechanisms of antitumor activity have been known although some are still not fully understood. Carocho & Ferreira (2013b) reviewed the anticancer

activity of several phenolic compounds, measured *in vitro* or *in vivo*, and reported the individual mechanism of antitumor action. Most of the compounds were reported to prevent activation of PI3K/Akt pathway usually associated with colon cancer, inhibit growth of cells by decreasing the hyper phosphorylation of the protein retinoblastoma, apoptosis and DNA damage of the cancer cells and finally causing tumor inhibition. Some of these compounds have been isolated and separated from natural products and have been clinically tried without negative effect or toxicity to the patient but the procedure is faced with several challenges allowing for more and more studies to be conducted.

1.2.2.3 Anti-inflammatory activity of phenolic compounds

Natural products such as plants, mushrooms, rhizomes and marine algae are an important source for isolating and developing potential therapeutic compounds that display anti-inflammatory effect with less toxicity. Some of these matrices have been known to display activity but it is also important to identify the bioactive metabolites that are responsible for this activity. Polysaccharides, terpenes, phenolic compounds, steroids and fatty acids are among the highlighted bioactive compounds. In particular, some of the phenolic compounds reported to display anti-inflammatory activity are benzenoids, syringaldehyde, syringic acid, cinnamic acid, protocathechuic acid and ethynylbenzene (Taofiq et al., 2016).

The NF-κB is a transcription factor that regulates the expression of several pro-inflammatory cytokines and enzymes such as IL-1β, TNF-α, iNOS, and COX-2 that are important in the inflammatory process. The mechanism of anti-inflammation by this natural compounds has been associated with a reduction in the release of nitric oxide (NO) and other inflammatory mediators such as interleukins (IL 1β, IL-6, IL-8), tumor necrosis factor (TNF-α) and prostaglandin E2 (PGE2) from inflammatory cells. Several phenolic compounds such as flavonoids have been reported to display anti-inflammatory activity by inhibition of NF-κB pathway by preventing phosphorylation of IkB protein thereby causing reduction in cytokine release (Taofiq et al., 2016). These natural inhibitors of NF-κB are becoming a target for researchers and can be further studied and used to treat and prevent various inflammatory diseases.

1.2.3 The presence of these compounds in *Hypericum androsaemum* L., *Tilia platiphyllos Scop*. and *Equisetum giganteum* L.

The composition of *H. androsaemum* in terms of phenolic compounds has been reported by several authors, namely in aqueous (Valentão et al., 2004) and methanolic (Porzel et al., 2013) extracts from leaves and aerial parts of the plant (Rainha et al.,2011; Ramalhete et al., 2016). Many studies of *H. androsaemum* reported the presence of several phenolic compounds such as flavonoids, phenolic acids (Caprioli et al., 2016) and xanthones which are usually present in the roots and its leaves (Valentão et al., 2002).

There are also reports on *T. platyphyllos* phenolics namely flavonol glycosides that together with mucilage components have been pointed out as the active ingredients responsible for bioactive/medicinal properties of this plant (Toker et al., 2001; Stef et al., 2010; Karioti et al., 2014).

E. giganteum shows the presence of phenolic compounds derived from caffeic and ferulic acids and flavonoid glycosides such as kaempferol and quercetin derivatives, in addition to styrylpyrone (Alavarce et al., 2015). Several studies report the presence of high amounts of phenolic compounds, such as flavones, isoflavones, flavonols and flavanols in this plant (Danielski et al., 2007).

1.3 Bioactive properties of the plants under study

1.3.1 Antioxidant properties

Free radicals are atoms, molecules or ions with unpaired electrons that are highly unstable and active towards chemical reactions with other molecules. They are derived from three elements: oxygen, nitrogen and sulphur, thus creating reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive sulphur species (RSS). Antioxidants provide a balance between the production and scavenging of ROS and when there is a disruption in this balance, there is over overproduction of ROS and the cells start to suffer the consequences of oxidative stress (Carocho & Ferreira, 2013a).

Oxidative stress is a term that defines an imbalance between oxidants antioxidants and pro-oxidants potentially leading to damage of cell components and has been suggested as the main cause of aging, cancer and other diseases in humans. Now modern medicine considers the

balance between oxidation and antioxidation a crucial concept in maintaining healthy biological system (Rahman et al., 2012).
The antioxidant activity of plant extracts is of particular interest because of their beneficial physiological activity on human health and due to the great potential to replace synthetic antioxidants used in medicines and food supplements (Stef et al., 2010).
Previous studies on *H. androsaemum* infusion showed strong antioxidant activity against several free radicals namely against superoxide anion radical, hydroxyl radical and hypochlorous acid (Valentão et al., 2004). The antioxidant potential of *H. androsaemum* aqueous extracts prepared from the whole plant (Hernandez et al., 2010; Ramos et al., 2013), aerial parts (Rainha et al., 2011) and leaves (Valentao et al., 2002) has been described. In addition, the hepatoprotective effects of aqueous extracts obtained from leaves were also reported, which were directly correlated with the antioxidant potential of this plant (Valentão et al., 2004).

The radical scavenging capacity of ethanolic extracts of *T. platyphyllos* was also previously reported (Stef et al., 2010). Water and ethanolic extracts of *E. giganteum* were also previously studied and show remarkable antioxidant activity that are explained by the presence of high amounts of phenolic compounds (Nagai et al., 2005). *In vitro* and *in vivo* antioxidant activities of aqueous extract from leaves and flowers of *T. platyphyllos* were previously reported and related with the abundance of phenolic compounds among them are quercetin and kaempferol derivatives (Majer et al., 2014; Yayalaci et al., 2014).

1.3.2 Antitumor properties

Cancer is characterized by uncontrolled cell growth and acquisition of metastatic properties. In this process, the activation of oncogenes or the inactivation of tumor suppressor genes lead to cell cycle arrest and apoptotic pathway (Han et al., 2015). Cancer cells are known to have alterations in multiple cellular signalling pathways, and because of the complex communications between these signalling networks, the cure of most human cancers remains a great challenge. It is the world's second biggest killer after cardiovascular disease and there is an increase attention on isolation of biologically active compounds from plant for anti-cancer treatment (Chatterjee et al., 2011). Approximately 60% of drugs currently used for cancer treatment have been isolated from natural products with a composite mixture of compounds present in the whole plant and they have shown potent anticancer properties with less cytotoxicity effect compared to conventional drugs (Solowey et al., 2014).

The antitumor potential of *H. androsaemum* aqueous extracts was previously studied (Xavier et al., 2012) through the evaluation of its anti-proliferative and pro-apoptotic effects in human colorectal cancer cells. Also Dias et al. (2000) reported that *H. androsaemum* displayed potent antitumor activity *in vivo*. Recent studies show that water extracts of this plant have protective effect against oxidative damage of DNA responsible for the induction of colon carcinogenesis (Ramos et al., 2013). No reports could be found for the antitumor activity of extracts of *T. platyphyllos* and *E. giganteum.*

1.3.3 Anti-inflammatory properties

Inflammation is a complex biological response to remove harmful stimuli such as pathogens, damaged cells, or irritation from the body and this is usually associated with many diseases such as atherosclerosis, obesity, metabolic syndrome, and diabetes (Taofiq et al., 2015).When body cells get in contact with immune stimulants such as pathogens, inflammatory cells like macrophages, monocytes secrete cytokines and other mediators, which initiate the inflammation process. The common inflammatory mediators are interleukins (IL-1β, IL-6, IL-8), tumour necrosis factor (TNF-α), nuclear factor-κB (NF-κB), intercellular adhesion molecule-1 (ICAM-1), inducible type cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), 5-lipooxygenase (5-LOX), and inducible nitric oxide synthase (iNOS) that leads to the production of reactive nitrogen species such as nitric oxide (NO). Overproduction of these inflammatory mediators leads to different kinds of cell damage (Kanwar et al., 2009). Research studies are focused on finding plant extracts and their bioactive compounds that have the ability to suppress the production of inflammatory mediators through down regulation of the gene expression of different types of inflammatory mediators (Souza et al., 2015).

Some studies have shown that ethanol extracts of the aerial parts of *H. androsaemum* possess anti-inflammatory activity (Šavikin et al., 2007). *E.giganteum* decoction and infusion are widely used in traditional medicine in inflammatory conditions (Nagai et al., 2005; Farinon et al., 2013; Alavarce et al., 2015). No reports could be found for the anti-inflammatory activity of extracts of *T. platyphyllos*.

1.4 Objectives

The main objective of the present study was to perform a prospective study of the bioactive compounds and properties in plants used in the traditional medicine, and contribute to increase scientific evidences of their uses.

The specific objectives were:

To evaluate the *in vitro* antioxidant, antitumor and anti-inflammatory properties of hydroethanolic extracts of *Hypericum androsaemum* L., *Tilia platiphyllos* Scop., and *Equisetum giganteum* L.;

To chemically characterise the extracts in terms of phenolic compounds and correlate the composition with the observed bioactive properties.

2 MATERIALS AND METHODS

2.1 Standards and reagents

HPLC grade acetonitrile 99.9% and analytical grade purity ethanol were from Fisher Scientific (Lisbon, Portugal). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Formic acid, trolox (6-hydroxy-2,5,7, 8-tetramethylchroman-2-carboxylic acid), sulforhodamine B, trypan blue, trichloroacetic acid (TCA) and Tris were from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium, Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), L-glutamine, trypsin-EDTA, penicillin/streptomycin solution (100 U/mL and 100 mg/ mL, respectively), were purchased from Gibco Invitrogen Life Technologies (California, USA). RAW264.7 cells were purchased from ECACC ("European Collection of Animal Cell Culture") (Salisburg, UK), lipopolysaccharide (LPS) from Sigma and DMEM medium from HyClone. The Griess Reagent System Kit was purchased from Promega, and dexamethasone from Sigma. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2 Extracts preparation

Aerial parts of *Equisetum giganteum* L., *Tilia platyphyllos* Scop. And *Hypericum androsaemum* L. (**Figure 5**) were provided by "Cantinho das Aromáticas", an organic and certified farm from Vila Nova de Gaia, Portugal. The dried samples were reduced to powder and submitted to the following extraction procedure in three independent experiments: 4 g were stirred with 30 mL of ethanol: water (80:20, *v/v)* for 1 h, filtered and re-extracted in the same conditions. Ethanol was removed in a rotary evaporator (Büchi R-210, Flawil, Switzerland), while water was removed by lyophilisation. Finally, stock solutions of each hydroethanolic extract were prepared: 5 mg/mL in water/ethanol for antioxidant activity assays; 8 mg/mL in water for antitumor and anti-inflammatory assays, from which several dilutions were performed; and 2.5 mg/mL in ethanol: water (80:20, *v/v*) for phenolic compound analysis (the extract was filtered through a 0.45 µm Whatman syringe filter and transferred to amber color HPLC vial).

Figure 5. Dried samples of *Equisetum giganteum* L.,*Hypericum androsaemum* L. and *Tilia platyphyllos Scop*.

2.3 Evaluation of the antioxidant activity

Four different assays were used to assess the *in vitro* antioxidant potential of plant extracts: scavenging effects on DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals (RSA), Reducing power, measured by ferricyanide Prussian blue assay (RP), β-carotene bleaching inhibition (CBI) and lipid peroxidation inhibition (LPI).

2.3.1 DPPH radical-scavenging activity assay

This methodology was performed using a 96 well Microplate. The reaction mixture on the plate consists of 30 μL of extract solutions with different concentrations and 270 μL methanol containing DPPH radicals (6×10^{-5} mol/L). The mixture was left to stand for 1hour in the dark, and the absorbance was read at 515 nm in the ELX800 Microplate Reader (Bio-Tek Instruments, Inc; Winooski, VT, USA) (**Figure 6**). The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: $\%RSA= [(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where AS is the absorbance of the solution containing the sample, and A_{DPPH} is the absorbance of the DPPH solution (Fernandes et al., 2014).

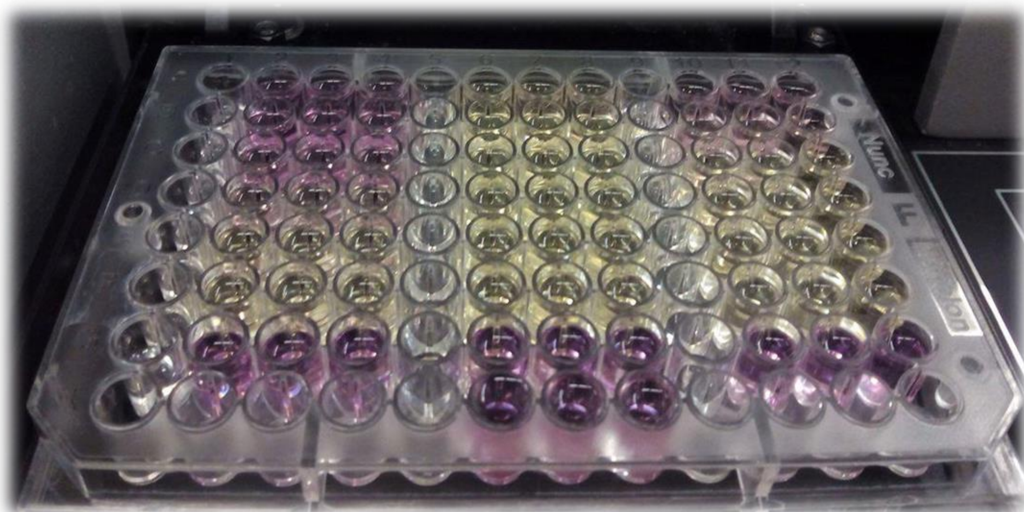


Figure 6. Microplate showing the DPPH assay.

2.3.2 Reducing power by ferricyanide/Prussian blue assay

Extract with different concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mmol/L, pH 6.6, 0.5 mL) and potassium ferricyanide (1% *w*/*v*, 0.5 mL). The mixture was incubated at 50 ºC for 20 min, and trichloroacetic acid (10% *w*/*v*, 0.5 mL) was added. The mixture (0.8 mL) was poured in the 48 wells plate. Deionized water (0.8 mL) and ferric chloride (0.1% *w*/*v*, 0.16 mL) were added and the absorbance was read at 690 nm in ELX800 Microplate Reader (**Figure 7**) (Bio-Tek Instruments, Inc; Winooski, VT, USA) (Fernandes et al., 2014).

Figure 7. Microplate showing the reducing power assay.

2.3.3 Inhibition of β-carotene bleaching

A β-carotene solution was prepared by dissolving β-carotene (2 mg) in chloroform (10 mL). Afterwards, 2 mL of this solution were pipetted into a round-bottom flask and the chloroform was removed at 40 ºC under vacuum and a linoleic acid (40 mg), Tween 80 emulsifier (400 mg), and distilled water (100 mL) were added to the flask with vigorous shaking. Aliquots (4.8 mL) of this emulsion were transferred into test tubes containing (0.2 mL) extract solutions with different concentrations. A control was prepared in which extraction solvent was added instead of the mushroom extract. The tubes were shaken and incubated at 50 ºC in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm (**Figure 8**). The absorbance of the reaction mixture was finally read after 2 h and the β-carotene bleaching inhibition was calculated using the following equation: (absorbance after 2 h of assay/initial absorbance) × 100 (Fernandes et al., 2014).

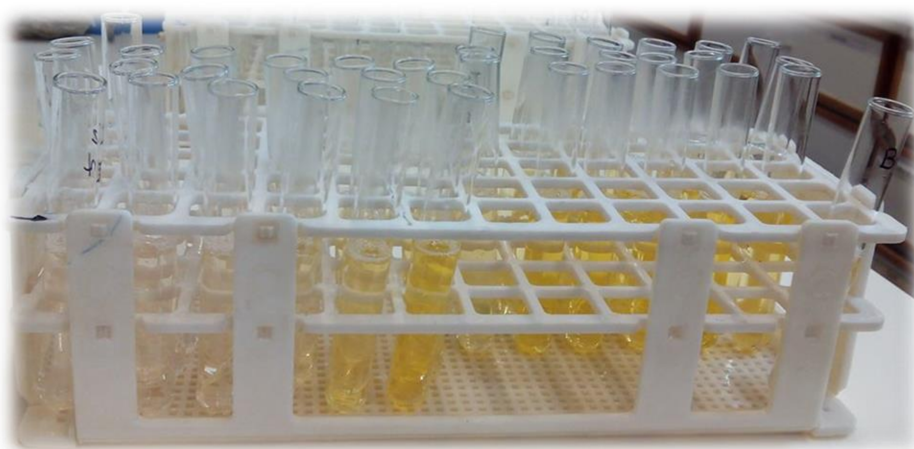


Figure 8. Test tubes showing the β-carotene bleaching assay before absorbance measurement.

2.3.4 Thiobarbituric acid reactive substances (TBARS) assay

Porcine brains were obtained from official slaughtered animals, dissected, and homogenized with Polytron in an ice cold Tris-HCl buffer (20 mM, pH 7.4) to produce a 1:2 *w/v* brain tissue homogenate which was centrifuged at 3000 × g for 10 min. An aliquot (100 µL) of the supernatant was incubated with the different concentrations of the sample solutions (200 µL) in the presence of $FeSO_4$ (10 mM; 100 µL) and ascorbic acid (0.1 mM; 100 µL) at 37 ºC for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% *w/v*, 500 µL), followed by thiobarbituric acid (TBA, 2%, *w/v*, 380 µL), and the mixture was then heated at 80 ºC for 20 min. After centrifugation at 3000 × *g* for 10 min to remove the precipitated

protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was measured by its absorbance at 532 nm (**Figure 9**). The inhibition ratio (%) was calculated using the following formula: inhibition ratio (%) = [(A − B)/A] × 100%, where A and B were absorbance of the control and the sample solution, respectively (Fernandes et al., 2014).

The results from each assay were expressed as EC_{50} values, corresponding to the extracts concentration that provides 50% of antioxidant activity, or 0.5 of absorbance in the case of reducing power assay. Trolox was used as positive control.

Figure 9. Test tubes showing the TBARS assay before absorbance measurement.

2.4 Evaluation of the anti-inflammatory activity

2.4.1 Cells treatment

The anti-inflammatory activity was carried out according to the procedure reported by (Taofiq et al., 2015). The mouse macrophage-like cell line RAW264.7 was cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, glutamine and antibiotics at 37 °C under 5% CO_2, in humidified air. For each experiment, cells were detached with a cell scraper. A cell density of 5×10^5 cells/mL was used, and the proportion of dead cells was less than 5%, according to Trypan blue dye exclusion test. Cells were seeded in 96-well plates at 150,000 cells/well and allowed do attach to the plate overnight. Then, cells were treated with the different concentrations of each one of the extracts for 1 h. Dexamethasone (50 μM) was used as a positive control for the experiment. The following step was the stimulation with LPS (1 μg/mL) for 18 h. The effect of all the tested samples in the absence of LPS was also evaluated, in order to observe if they induced changes in nitric

oxide (NO) basal levels. In negative controls, no LPS was added. Both extracts and LPS were dissolved in supplemented DMEM.

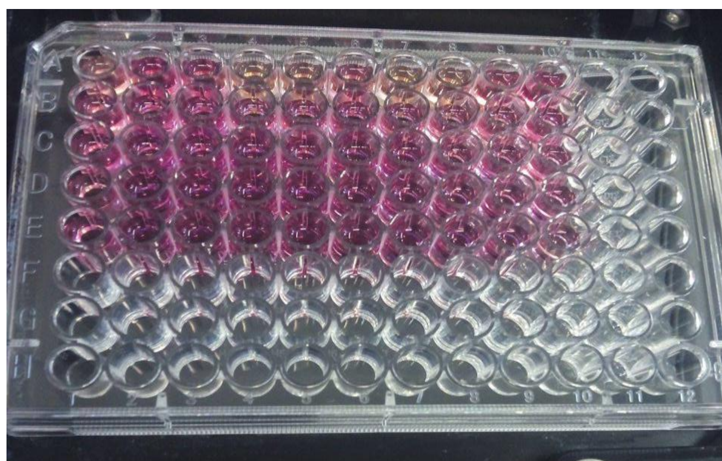


Figure 10. Microplate showing the anti-inflammatory activity assay.

2.4.2 Nitric oxide determination

For the determination of nitric oxide, Griess Reagent System kit was used, which contains sulphanilamide, N-(1-napthyl) ethylenediamine hydrochloride (NED) and nitrite solutions. A reference curve of the nitrite (sodium nitrite 100 μM to 1.6 μM; y = 0.0066x + 0.1349; R^2 = 0.9986) was prepared in a 96-well plate. The cell culture supernatant (100 μL) was transferred to the plate and mixed with sulphanilamide and NED solutions, 5–10 min each, at room temperature. The nitric oxide produced was determined by measuring the absorbance at 540 nm (microplate reader ELX800 Biotek), and by comparison with the standard calibration curve **(Figure 10)**.The final results expressed in EC_{50} values, i.e. the extract concentration that inhibited 50% of the NO production.

2.5 Evaluation of the cytotoxic activity

2.5.1 In tumor cell lines

The human tumor cell lines used were: HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer). Each of the cell lines were plated in a 96-well plate, at an appropriate density (7.5×10^3 cells/well for MCF-7 and NCI-H460 and 1.0×10^4 cells/well for HeLa and HepG2) and were allowed to attach for 24 h. Afterwards, various extract concentrations were added to the cells and incubated during 48 h. Afterwards, cold trichloroacetic acid (TCA 10%, 100 µL) was used in order to bind the adherent cells and further incubated for 60 min at 4 ºC. After the incubation period, the plates were washed with deionised water and dried and sulforhodamine B solution (SRB 0.1% in 1% acetic acid, 100 µL) was then added to each plate well and incubated for 30 min at room temperature. The plates were washed with acetic acid (1%) in order to remove the unbound SRB and air dried, the bounded SRB was solubilised with Tris (10 mM, 200 µL) and the absorbance was measured at 540 nm using an ELX800 microplate reader (Bio-Tek Instruments, Inc; Winooski, VT, USA) (Abreu et al., 2011).The obtained results were expressed in GI_{50} values, i.e. the extract concentration that inhibited 50% of the net cell growth. Ellipticine was used as positive control.

2.5.2 In non-tumor porcine liver cells (PLP2)

Liver tissues were washed in Hank's balanced salt solution, containing 100 U/mL of penicillin and 100 µg/mL of streptomycin, and then divided into 1×1 mm^3 explants. Some of these explants were placed in 25 cm^3 tissue flasks, containing DMEM and supplemented with 10% fetal bovine serum, 2 mM of non-essential amino acids, 100 U/mL of penicillin and 100 mg/mL of streptomycin. Subsequently were incubated at 37 ºC, in a humidified atmosphere containing 5% CO_2. This medium was changed every 2 days, and the cultivation of cells continued under direct monitoring every 2-3 days, by using a phase contrast microscope. Prior to confluence, cells were sub-cultured and plated in 96-well plates, with a final density of 1.0×10^4 cells/well and cultivated in DMEM medium, consisting of 10% FBS, 100 U/mL of penicillin and 100 µg/mL of streptomycin (Abreu et al., 2011). Finally, cells were treated for 48 h with different diluted sample solutions of each plant extract tested (**Figure 11**). The obtained results were expressed in GI_{50} values, i.e. the extract concentration that inhibited 50% of the net cell growth. Ellipticine was used as positive control.

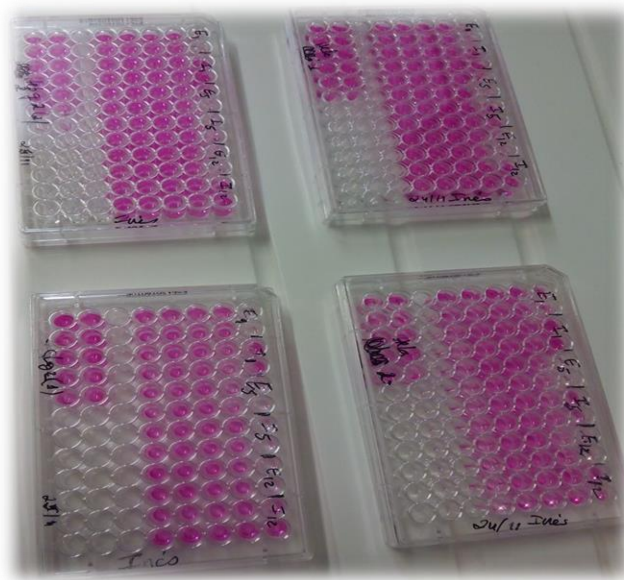


Figure 11.Microplate showing the cytotoxic evaluation assay.

2.6 Chemical characterization of the extracts

HPLC-DAD–ESI/MSn analyses was performed using a Hewlett-Packard 1100 chromatograph (Hewlett-Packard 1100, Agilent Technologies, Santa Clara, CA, US) with a quaternary pump and a diode array detector (DAD) coupled to an HP Chem Station (rev. A.05.04) data-processing station and connected via the cell outlet to a MS detector API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) through an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by the Analyst 5.1 software. Separation was achieved on a Waters Spherisorb S3 ODS-2 C_{18}, (3 µm, 4.6 mm × 150 mm) column thermostatted at 35 °C was used. The mobile phase consisted of (A) 0.1% formic acid in water, (B) acetonitrile at a flow rate of 0.5 mL/min (injection volume 100 µL). Gradient elution was carried out using the following timetable: 15% for 5 min, 15% B to 20% B over 5 min, 20-25% B over 10 min, 25-35% B over 10 min, 35-50% B for 10 min. The resulting total run time was 55 min, followed by column reconditioning of 10 minutes. The chromatogram was recorded at several wavelengths, characteristic of different classes of polyphenols, such as 280 nm for phenolic acids, flavan-3-ols and flavanones, 330 nm for hydroxycinnamic acids and 370 nm for flavonols and flavones.

For MS detection a zero grade air served as the nebulizer gas (30 psi) and turbo gas for solvent drying (400 ºC, 40 psi). Nitrogen served as the curtain (20 psi) and collision gas

(medium). The quadrupols were set at unit resolution. The ion spray voltage was set at -4500V in the negative mode. The MS detector was programmed for recording in two consecutive modes: Enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, so as to obtain an overview of all of the ions in sample. Settings used were: declustering potential (DP) -450 V, entrance potential (EP) -6 V, collision energy (CE) -10V. EPI mode was performed in order to obtain the fragmentation pattern of the parent ion(s) in the previous scan using the following parameters: DP -50 V, EP -6 V, CE -25V, and collision energy spread (CES) 0 V. Spectra were recorded in negative ion mode between *m/z* 100 and 1700 (Souza et al., 2015).

The phenolic compounds were identified by comparing their retention times, UV-vis and mass spectra with those obtained with standard compounds, when available. Otherwise, compounds were tentatively identified comparing the obtained information with available data reported in the literature. For quantitative analysis, a calibration curve for each available phenolic standard: (+)-catechin (y=134.92x+32.987; R^2=0.999); caffeic acid (y=359x+488; R^2=0.998); chlorogenic acid (y=304x-248; R^2=0.999); (-)-epicatechin (y=163.23x+39.586; R^2=0.999); kaempferol-3-*O*-glucoside (y=236x+70; R^2=0.999); kaempferol-3-*O*-rutinoside (y=183x+97; R^2=0.999); naringenin (y=539,98x+161,46; R^2=0.994); protocatechuic acid (y= 258.17x+328.45; R^2=0.998); quercetin-3-*O*-glucoside (y=336x+358; R^2=0.998) and quercetin-3-*O*-rutinoside (y=281x+374; R^2=0.998), was constructed based on the UV signal. For the identified phenolic compounds for which a commercial standard was not available, the quantification was performed through the calibration curve of another compound from the same phenolic group and results were expressed as mg per g of extract.

2.7 Statistical analysis

Three samples were used for each preparation and all the assays were carried out in triplicate. The results are expressed as mean values ± standard deviation (SD). The results were analyzed using a Student´s *t*-test to determine the significant difference among two different samples, with $\alpha = 0.05$. This treatment was carried out using SPSS v. 22.0 program.

3 RESULTS AND DISCUSSION

3.1 Studies with *H. androsaemum*

3.1.1 Antioxidant activity

Numerous studies have been conducted to identify effective antioxidant compounds, including their active concentration and related modes of action being currently considered one of the highest hot topics among the scientific community (Dato et al., 2013; Espín, García-Conesa, & Tomás-Barberán, 2007). In fact, a relation between premature aging, longevity impairment and oxidative stress status is known to exist, despite the intensive investigation a general consensus was not established (Halliwell, 2012). On this field, phenolic compounds seem to be promissory antioxidant agents (Carocho & Ferreira, 2013a; Fernandez-Panchon et al., 2008). Thus, aiming to put forward alternative sources of phenolic compounds, the present study was carried out to evaluate the antioxidant potential of *H. androsaemum* hydroethanolic extract as an alternative source of phenolic compound using four different *in vitro* assays. The results are given in **Table 2**.

Table 2. *In vitro* antioxidant, antitumor and anti-inflammatory properties of *H. androsaemum* ethanol: water extract (mean ± SD).

	H. androsaemum	*Positive control
Antioxidant activity (EC_{50}, µg/mL)		
DPPH scavenging activity	96±3	41±1
Reducing power	103±1	41±2
β-carotene bleaching inhibition	155±5	18±1
TBARS inhibition	36±1	23±1
Antitumor activity(GI_{50} values, µg/mL)		
MCF-7 (breast carcinoma)	106±11	0.91±0.04
NCI-H460 (non-small cell lung cancer)	215±13	1.03±0.09
HeLa (cervical carcinoma)	205±18	1.91±0.06
HepG2 (hepatocellular carcinoma)	100±7	1.1±0.2

Hepatotoxicity (GI_{50} values, μg/mL)		
PLP2	>400	3.2±0.7
Anti-inflammatory activity (EC_{50} values, μg/mL)		
NO production inhibition	179±17	16±1

*Trolox, ellipticine and dexamethasone for antioxidant, antitumor and anti-inflammatory activity assays, respectively. EC_{50} values correspond to the extract concentration achieving 50% of antioxidant/anti-inflammatory activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the extract concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2.

The most significant antioxidant effect was observed in the LPI assay, followed by RSA, RP and CBI assays, which means that the studied *H. androsaemum* extract acts mainly as lipid peroxidation inhibitor, followed by radical scavenger and inhibitor of linoleic acid oxidation. Rainha et al. (2011) also evaluated the antioxidant potential of aqueous extracts from the aerial parts of different *Hypericum* species and the result are in agreement with the ones reported in the present work and the authors concluded that *H. androseamum* exerted strong radical scavenger effect and moderate inhibitory effect of linoleic acid oxidation. Also, Valentão et al. (2002) evaluated the antioxidant potential of water extracts prepared from *H. androseamum* leaves and reported their antioxidant action mainly as strong scavengers of reactive oxygen species (mainly superoxide radicals). Similar radical scavenging effects of *H. androseamum* aqueous extracts were described by Hernandez et al. (2010) and Ramos et al. (2013), being also reported by the last authors a significant ability to repair DNA damages. Lastly, Valentão et al. (2004) aiming to directly correlate the hepatoprotective effects of *H. androseamum* with its antioxidant potential, concluded that those effects mainly derived from its strong ability to inhibit lipid peroxidation, which was in part attributed to its phenolic composition.

3.1.2 Cytotoxic activity in tumor and non-tumor cells

The cytotoxicity of *H. androseamum* was evaluated in non-tumor porcine liver cells (PLP2) as also in tumor cell lines (breast carcinoma- MCF-7, non-small cell lung carcinoma- NCI-H460, cervival carcinoma- HeLa and hepatocellular carcinoma- HepG2), and the results are given in **Table 2**. Up to 400 μg/mL, the *H. androseamum* extract showed no toxicity in non-tumor liver cells (PLP2), while ellipticine used as control showed high hepatotoxicity.

Otherwise, a relative low concentration of the extracts was necessary to cause 50% of growth inhibition in human tumor cell lines. *H. androseamum* was found to be most potent in MCF-7 and HepG2 cell lines with GI_{50} values of 106.03±10.60 µg/mL and 100.27±6.53 µg/mL, respectively, while a significant effect was also found against NCI-H460 and HeLa cells. Xavier et al. (2012) reported the *in vitro* anti-proliferative and pro-apoptotic effects of *H. androsaemum* aqueous extracts in human colon carcinoma-derived cell lines, and described an IC_{50} concentration of 65 µg/mL. It is to note that chlorogenic acid was the most abundant phenolic compound in the extract studied by those authors (Xavier et al., 2012), which is in agreement with the herein tested ethanol:water extract, where two chlorogenic acids, 5-*O*-caffeoylquinic acid and 3-*O*-caffeoylquinic acid were the majority compounds.

3.1.3 Anti-inflammatory activity

Inflammation is in the origin of numerous degenerative and metabolic disorders, among which the most tremendous is cancer. Notwithstanding, the inflammatory process is a very complex process, and in most of the cases the triggering factor cannot be identified, although free radicals overproduction might be one of the reasons (Aruoma, 2003; Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005). Thus, and considering the observed antioxidant potential of the *H. androseamum* ethanol:water extract of *H. androseamum* was evaluated for inhibition of nitric oxide (NO) production. The results are presented in **Table 2** and the *H. androseamum* extract inhibited NO with an EC_{50} value of 178.78±16.60 µg/mL while dexamethasone used as positive control inhibited NO production with an EC_{50} value of 15.70±1.1 µg/mL. As far as we know, this would be the first report on the anti-inflammatory potential of *H. androseamum* extracts.

3.1.4 Chemical characterization

The chromatographic profile of the *H. androsaemum* ethanol:water extract recorded at 280 nm is shown in **Figure 12**, and peak characteristics, tentative identities and phenolic compounds quantification are presented in **Table 3**. Compounds 2 (protocatechuic acid), 4 (5-*O*-caffeoylquinic acid), 6 (epicatechin), 11 (taxifolin), 12 (quercetin-3-*O*-glucuronide) and 13 (quercetin-3-*O*-glucoside) were positively identified by comparison with authentic standards, as also by their MS fragmentation pattern, retention time and UV-vis characteristics. 5-*O*-Caffeoylquinic acid and quercetin derivatives have been reported in methanol extracts from the aerial parts (Rainha et al., 2011) and leaves (Valentão et al., 2003; Porzel et al., 2014) of

H. androsaemum, and in aqueous extracts from the leaves (Valentão et al., 2002; Valentão et al., 2004).

Compound 1 ($[M-H]^-$at *m/z* 353) was identified as 3-*O*-caffeoylquinic acid based on its fragmentation pattern, yielding a base peak at *m/z* 191 (deprotonated quinic acid) and an ion at *m/z* 179 $[\text{caffeic acid-H}]^-$ with an intensity of 47% of the base peak, as reported by Clifford et al. (2003, 2005). Similarly, compound 3 ([M-H]- at m/z 337) was tentatively identified according to its MS^2 fragmentation as 3-*O*-*p*-coumaroylquinic acid (Clifford et al. 2003, 2005). These compounds have been already reported in aqueous (Valentão et al., 2002; Valentão et al., 2004) and methanolic (Valentão et al., 2003; Porzel et al., 2014) extracts from leaves and in aerial parts (Rainha et al., 2011) of *H. androseamum*.

Compounds 5, 7, 8 and 10 showed UV spectra with λ_{max} 280 nm, characteristic of proanthocyanidins. Peaks 5 and 10 presented pseudomolecular ions corresponding to (epi)catechin dimers ($[M-H]^-$ at *m/z* 577) and compounds 7 ($[M-H]^-$ at *m/z* 865) and 8 ($[M-H]^-$ at *m/z* 1153) to a trimer and a tetramer, respectively. The positive identification in the extract of epicatechin as the only flavan-3-ol monomer would suggest an epicatechin-based identity for those peaks. Thus, the compounds might correspond to the B-linked epicatechin series, i.e., procyanidin dimer B2 (epicatechin-4,8-epicatechin, peak 5), trimer C1 (epicatechin-4,8-epicatechin-4,8-epicatechin, peak 7) and the (4,8)-linked epicatechin tetramer (peak 8), whereas the late elution of peak 10 suggests a (4,6) linkage (i.e., epicatechin-4,6-epicatechin; dimer B5). These identities would also be coherent with their expected relative order of elution (Santos-Buelga, García-Viguera, & Tomás-Barberán, 2003). This identification differs from the one recently reported by Ramalhete et al., (2016) who indicated the presence of (+)-catechin and related proanthocyanidins in methanol extracts obtained from the aerial parts of this plant. Compounds 14 ($[M-H]^-$ at *m/z* 463) and 15 ($[M-H]^-$ at *m/z* 433) presented UV spectra (λ_{max}354-356 nm) and the production of a unique fragment ion at *m/z* 301 ($[\text{quercetin-H}]^-$), which allowed their identification as quercetin-*O*-hexoside and quercetin-*O*-pentoside, respectively. Finally, compound 9 ($[M-H]^-$ at *m/z* 449) was tentatively identified as eriodictyol-*O*-hexoside, based on its UV spectrum and the product ion observed at *m/z* 287. No further conclusion about the nature and position of the substituting sugar of these compounds can be made from the obtained LC-DAD-MS data. Similar quercetin glycosides have been indicated in aqueous (Valentão et al., 2002; Valentão et al., 2004) and methanol (Valentão et al., 2003; Porzel et al., 2014; Rainha et al., 2011) preparations of the plant,

whereas, to the author's best knowledge, the presence of eriodictyol-*O*-hexoside has not been previously reported.

Overall, 5-*O*-caffeoylquinic acid followed by 3-*O*-caffeoylquinic acid were the majority phenolic compounds present in the *H. androsaemum* ethanol:water extract, and they should account for the observed antioxidant, antitumor and anti-inflammatory activities.

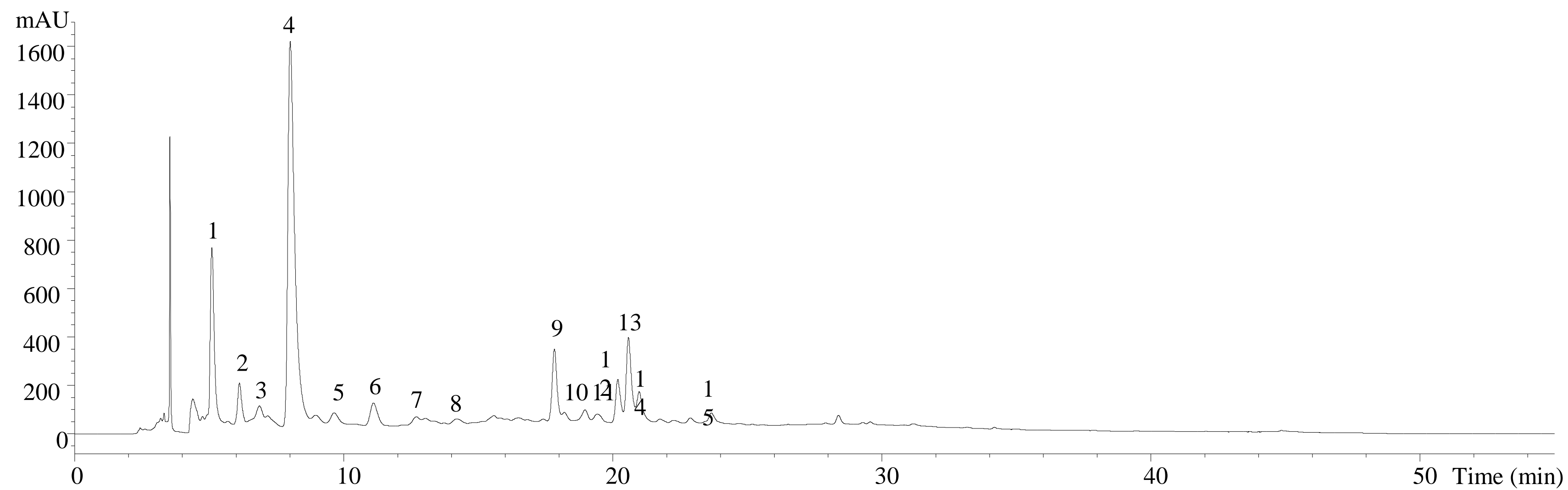


Figure 12. Phenolic compounds profile of the *H. androsaemum* ethanol:water extract recorded at 280 nm. The numbering correspond to the peak number presented in Table 3.

Table 3. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, identification and quantification (mean ± SD) of phenolic compounds in the *H. androsaemum* ethanol:water extract.

Compound	Rt (min)	λ_{max} (nm)	Pseudomolecular ion [M-H]$^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g extract)
1	5.1	328	353	191(100),179(47),161(5),135(38)	3-*O*-Caffeoylquinic acid	11.6±0.1
2	6.1	260,294sh	153	109(100)	Protocatechuic acid	4.29±0.001
3	6.9	312	337	191(25),173(10),163(100),119(65)	3-*O*-*p*-Coumaroylquinic acid	0.88±0.01
4	8.0	328	353	191(100),179(3),161(6),135(4)	5-*O*-Caffeoylquinic acid	40.1±0.1
5	9.0	280	577	451(25),425(50),407(100),289(63),287(8)	Procyanidin B2	5.6±0.1
6	11.1	280	289	245(18),203(11),187(4),161(7),137(8)	(-)-Epicatechin	6.9±0.2
7	13.1	280	865	739(4),713(3),577(8),575(4),425(19),407(20),289(23),287(8)	Procyanidin C1	3.4±0.2
8	14.2	280	1153	865(9),863(12),577(25),575(42),289(5),287(9)	Epicatechin tetramer	4.4±0.2
9	17.9	286,336sh	449	287(100)	Eriodictyol-*O*-glucoside	3.7±0.2
10	19.0	280	577	451(67),425(61),407(100),289(82),287(14)	Procyanidin B5	4.8±0.5
11	19.4	288,340sh	303	285(100),199(12),177(8),150(15),125(35)	Taxifolin	3.4±0.1
12	20.2	350	477	301(100)	Quercetin-3-*O*-glucuronide	4.71±0.01
13	20.6	354	463	301(100)	Quercetin-3-*O*-glucoside	10.69±0.02
14	21.0	354	463	301(100)	Quercetin-*O*-hexoside	4.41±0.05
15	23.7	356	433	301(100)	Quercetin-*O*-pentoside	1.33±0.02
					Total phenolic acid derivatives	56.9±0.2
					Total flavonoids	53±1
					Total phenolic compounds	110±0.5

3.2 Studies with *T. platyphyllos* and *E. giganteum*

3.2.1 Antioxidant activity

The results of the antioxidant activity of hydroethanolic extracts of *T. platyphyllos* and *E. giganteum* are shown in **Table 4**. In general, *T. platyphyllos* evidenced a higher antioxidant potential than *E. giganteum*, both as free radical scavenger and also as lipid peroxidation inhibitor, being those results clearly confirmed, respectively, by the lowest RSA, RP, CBI and LPI EC_{50} values. Not least interesting to highlight is that this biological activity seems to be directly correlated with the relative abundance in phenolic compounds: for the plant extract with higher antioxidant effects, i.e. *T. platyphyllos*, a high concentration of phenolic compounds was found **Table 6**. Thus, it is feasible to infer that the higher antioxidant potential of *T. platyphyllos* was mainly attributed to their richness in phenolic compounds, particularly flavonoids (quercetin and kaempferol derivatives), as shown in **Table 6**.

Majer et al. (2014) reported the *in vitro* antioxidant potential (singlet oxygen scavenging) of *T. platyphyllos* leaf flavonoids and concluded that the sun leaves possess higher myricetin content than shade leaves, and that quercetin and myricetin derivatives, mainly occurring as rhamnosides, exerted much higher singlet oxygen scavenger effects than kaempferol glycosides. In fact, a higher content in quercetin derivatives was observed in *T. platyphyllos*, in detriment of kaempferol derivatives; furthermore, in *E. giganteum* only vestigial amounts of quercetin were determined, being kaempferol the most abundant. On the other hand, Yayalaci et al. (2014) evaluating the *in vivo* antioxidant potential of *T. platyphyllos* flowers against ethanol-induced oxidative stress, concluded that linden flowers were able not only to prevent oxidative damage in the studied tissues, but also to inhibit the production of ethanol-induced free radicals in rats. Thus, and considering the obtained results, it is feasible to assume that the antioxidant activity of *T. platyphyllos* is directly correlated with the relative abundance in phenolic compounds, mainly quercetin derivatives.

3.2.2 Cytotoxic activity in tumor and non-tumor cells

The cytotoxicity of *T. platyphyllos* and *E. giganteum* extracts was evaluated in non-tumor porcine liver cells (PLP2) as also in tumor cell lines (breast carcinoma- MCF-7, non-small cell lung carcinoma- NCI-H460, cervival carcinoma- HeLa and hepatocellular carcinoma- HepG2), and the results are given in **Table 4**. Neither, *E. giganteum* nor *T. platyphyllos*

extracts have shown toxicity in non-tumor liver cells (PLP2). Otherwise, a relative low concentration of the extracts was necessary to cause 50% of growth inhibition in human tumor cell lines. Once again, *T. platyphyllos* revealed more potency than *E. giganteum*, for all the tested tumor cell lines and *T. platyphyllos* was found to be most potent against HepG2 with a GI_{50} value of 173±13 µg/mL while *E. giganteum* also displayed the best activity against HepG2 with a GI_{50} value of 239±18 µg/mL. Cytotoxic properties of the mentioned extracts were not previously mentioned in literature and it is interesting to highlight that this is the first report on the cytotoxicity of *T. platyphyllos* and *E. giganteum* extracts.

Table 4. *In vitro* antioxidant, antitumor and anti-inflammatory properties of *T. platyphyllos* and *E. giganteum* ethanol: water extracts (mean ± SD).

	E. giganteum	*T. platyphyllos*	*t*-Students test *p*-value
Antioxidant activity (EC_{50} values, µg/mL)			
DPPH scavenging activity (RSA)	123±5	105±1	<0.001
Reducing power (RP)	136±1	123±7	<0.001
β-carotene bleaching inhibition (CBI)	202±3	167±2	<0.001
TBARS inhibition (LPI)	57.4±0.5	55.9±0.4	0.024
Antitumor activity(GI_{50} values, µg/mL)			
MCF-7 (breast carcinoma)	250±15	224±19	0.058
NCI-H460 (non-small cell lung cancer)	258±13	247±22	0.380
HeLa (cervical carcinoma)	268± 16	195±15	0.001
HepG2 (hepatocellular carcinoma)	239±18	173±13	0.002
Hepatotoxicity (GI_{50}values, µg/mL)			
PLP2	>400	>400	-
Anti-inflammatory activity (EC_{50} values, µg/mL)			
Nitric oxide (NO) production	239±20	225±22	0.314

The antioxidant activity was expressed as EC_{50} values, what means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: Extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. Trolox EC_{50} values: 41 µg/mL (reducing power), 42 µg/mL (DPPH scavenging activity), 18 µg/mL (β-carotene bleaching inhibition) and 23 µg/mL (TBARS inhibition). Results of the anti-inflammatory activity are expressed in EC_{50} values: sample concentration providing 50% of inhibition of nitric oxide (NO) production. Dexamethasone EC_{50} value: 16±2 µg/mL. Cytotoxicity results are expressed in GI_{50} values corresponding to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2. Ellipticine GI_{50} values: 1.2 µg/mL (MCF-7), 1.0 µg/mL (NCI-H460), 0.91 µg/mL (HeLa), 1.1 µg/mL (HepG2) and 2.3 (PLP2).

3.2.3 Anti-inflammatory activity

The anti-inflammatory potential of *T. platyphyllos* and *E. giganteum* extracts was evaluated through measurement of nitric oxide (NO) production, and the obtained results are presented in **Table 4**. Once again, phenolic composition seems to be most important contributor to the anti-inflammatory potential of plant extracts. *T. platyphyllos* inhibited NO production and presented an EC_{50} value of 225±22 μg/mL which correspond to the concentration of plant extract that is necessary to achieve 50% inhibition of NO production and it appears to be more effective than *E. giganteum* with an EC_{50} value of 239±20 μg/mL. As previously mentioned, only Farinon et al. (2013) reported the *in vivo* anti-inflammatory potential of *E. giganteum*; otherwise, to the author's best knowledge, no studies were previously reported on the anti-inflammatory potential of *T. platyphyllos*. From the present results anti-inflammatory effect of *T. platyphyllos* was markedly higher than that of *E. giganteum* and therefore, it may be considered a potential source of anti-inflammatory agents.

3.2.4 Chemical characterization

Table 5 and **6** present the characteristics of the identified phenolic compounds, tentative identities and quantification of *E. giganteum* and *T. platyphyllos*. The chromatographic profile of *E. giganteum* and *T. platyphyllos* can be observed in **Figure 13**. *Equisetum giganteum* presented fourteen phenolic compounds (two phenolic acids and twelve flavonol glycoside derivatives), while *T. platyphyllos* presented to be a more complex matrix in its phenolic composition, revealing the presence of thirty-three compounds (three phenolic acids, six flavan-3-ol derivatives, twenty flavonol glycoside derivatives, two flavanone and flavone derivatives). Compounds 2^{Tp} (protocatechuic acid), 4^{Eg} (caffeic acid), 3^{Tp} (catechin), 4^{Tp} (5-*O*-caffeoylquinic acid), 6^{Tp} (epicatechin), 15^{Tp} (quercetin-3-*O*-rutinoside; rutin), 17^{Tp} (quercetin-3-*O*-glucuronide), 10^{Eg} and 20^{Tp} (quercetin-3-*O*-glucoside; isoquercitrin), 23^{Tp} and 11^{Eg}(kaempferol-3-*O*-rutinoside) and 13^{Eg} (kaempferol-3-*O*-glucoside) were positively identified by comparison with authentic standard, as also to their MS fragmentation pattern, retention time and UV-vis characteristics. Kaempferol-3-*O*-glucoside has been reported in hydroethanolic extracts of aerial parts of *E. giganteum* (Francescato et al., 2013; Alavarce et al., 2015) as also a quercetin-hexoside (Alavarce et al., 2015) Protocatechuic acid, catechin, epicatechin and quercetin-3-*O*-glucoside have also been found in commercial samples of *T. platyphyllos* from Syria and Tuscany (Karioti et al., 2014).

Besides the already mentioned phenolic acid derivatives, 3-*O*-caffeoylquinic acid (peak 11^{Tp}) was also tentatively identified based on its MS^2 fragmentation pattern, according to the systematic keys provided by Clifford et al (2003) for the identification of chlorogenic acids. Phenolic acid derivatives represent 19% of the total phenolic composition of *T. platyphyllos*.

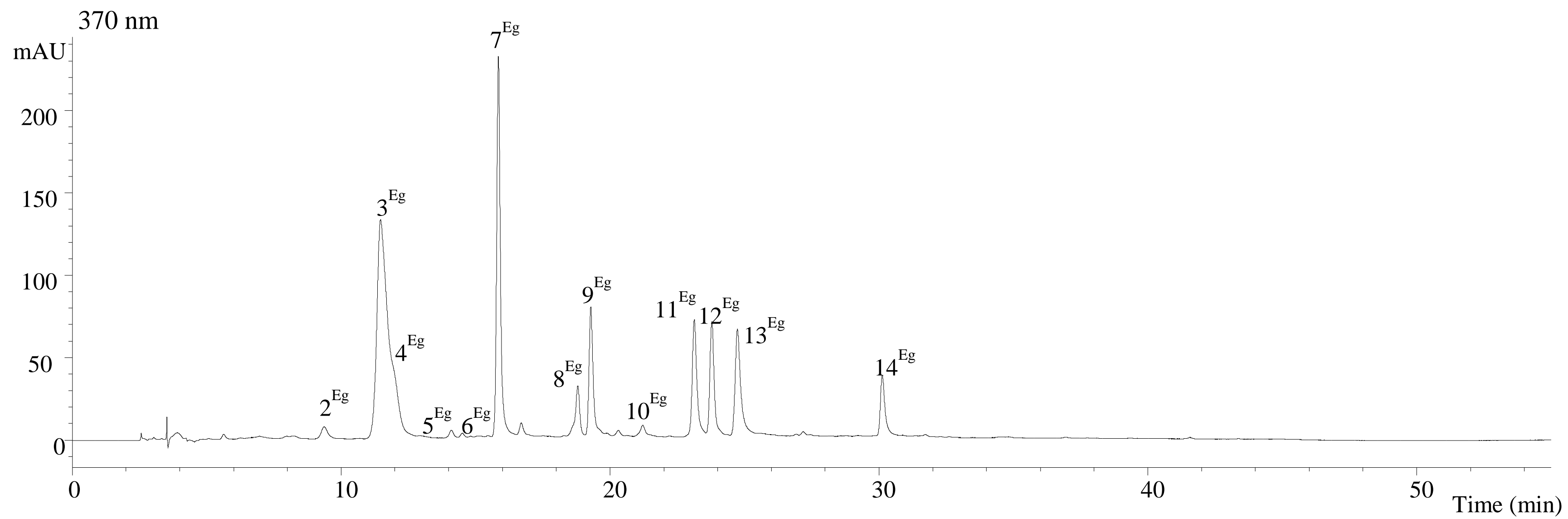
The sample of *T. platyphyllos* presented four compounds 5^{Tp} ($[M-H]^-$ at *m/z* 577), 7^{Tp} ($[M-H]^-$ at *m/z* 865), 8^{Tp} ($[M-H]^-$ at *m/z* 1153) and 9^{Tp} ($[M-H]^-$ at *m/z* 1441), with characteristic of proanthocyanidins (UV spectra with λ_{max} 280 nm), being assigned as procyanidin dimer, trimer, tetramer and pentamer, respectively. Comparison with our library database allowed identifying peak 5^{Tp} as procyanidin dimer B2, also reported by Negri et al. (2013) in hydroethanolic extracts from leaves of *Tilia cordata*. These compounds have been reported as major compounds in samples of *T. platyphyllos* by Karioti et al. (2014), in the present study these compounds were also present in high amounts, representing 34% of the total phenolic content, including (epi)catechin, being (-)-epicatechin the most abundant compound found (6.4 mg/g extract).

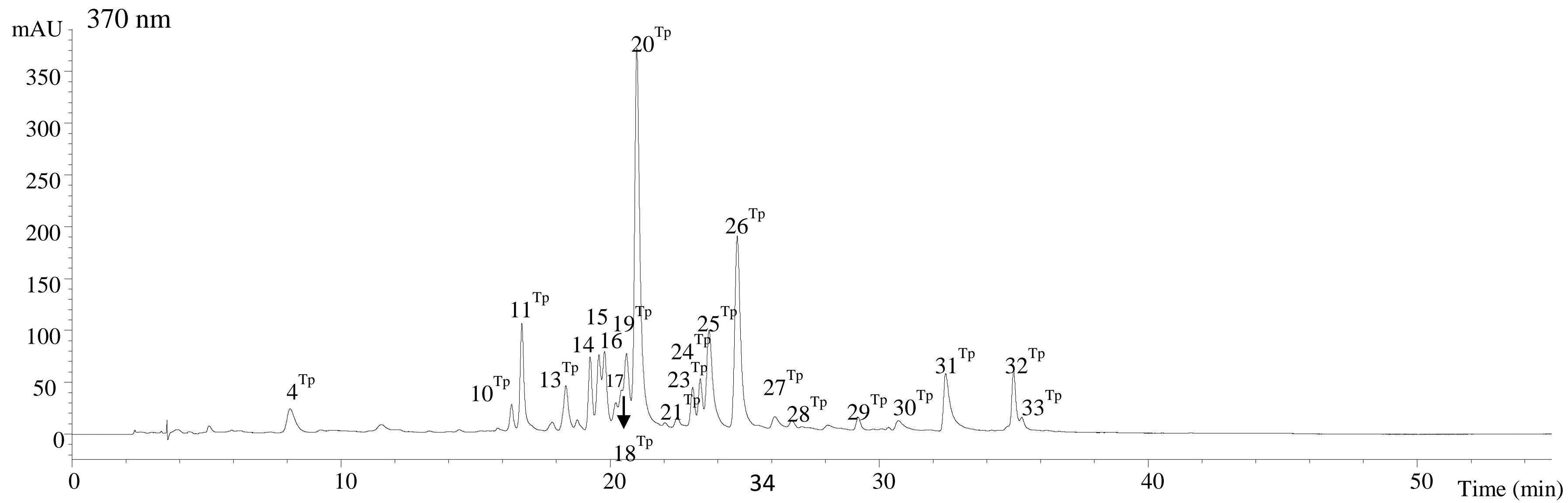
Table 5. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, identification and quantification (mean ± SD) of phenolic compounds in *Equisetum giganteum* extract.

Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g extract)
1^{Eg}	7.2	330	341	179(100)	Caffeic acid hexoside	0.131±0.003
2^{Eg}	9.4	352	625	463(100),301(32)	Quercetin-3,7-di-*O*-glucoside	Tr
3^{Eg}	11.5	348	755	593(100),447(10),285(38)	Kaempferol-*O*-glucoside-*O*-rutinoside	7.6±0.2
4^{Eg}	11.9	330	179	135(100)	Caffeic acid	4.86±0.03
5^{Eg}	14.1	348	651	489(33),447(17),285(17)	Kaempferol-*O*-acetylglucoside-*O*-glucoside	0.087±0.005
6^{Eg}	14.5	352	667	505(100),301(42)	Quercetin-*O*-acetylglucoside-*O*-glucoside	Tr
7^{Eg}	15.8	348	651	489(38),447(55),285(56)	Kaempferol-*O*-acetylglucoside-*O*-glucoside	5.01±0.05
8^{Eg}	18.8	336	739	593(77),431(10),285(27)	Kaempferol-*O*-rhamnoside-*O*-rutinoside	0.67±0.04
9^{Eg}	19.2	348	593	447(41),431(51),285(58)	Kaempferol-*O*-glucoside-*O*-rhamnoside	1.86±0.02
10^{Eg}	21.2	358	463	301(100)	Quercetin-3-*O*-glucoside	Tr
11^{Eg}	23.1	348	593	285(100)	Kaempferol-3-*O*-rutinoside	1.95±0.03
12^{Eg}	23.8	350	635	489(13),431(55),285(31)	Kaempferol-*O*-acetylglucoside-*O*-rhamnoside	1.77±0.06
13^{Eg}	24.7	350	447	285(100)	Kaempferol-3-*O*-glucoside	1.81±0.03
14^{Eg}	30.1	350	489	285(100)	Kaempferol-*O*-acetylglucoside	0.88±0.03
					Total phenolic acids	4.98±0.03
					Total flavonoids	21.7±0.4
					Total phenolic compounds	26.6±0.3

Tr-traces.

A





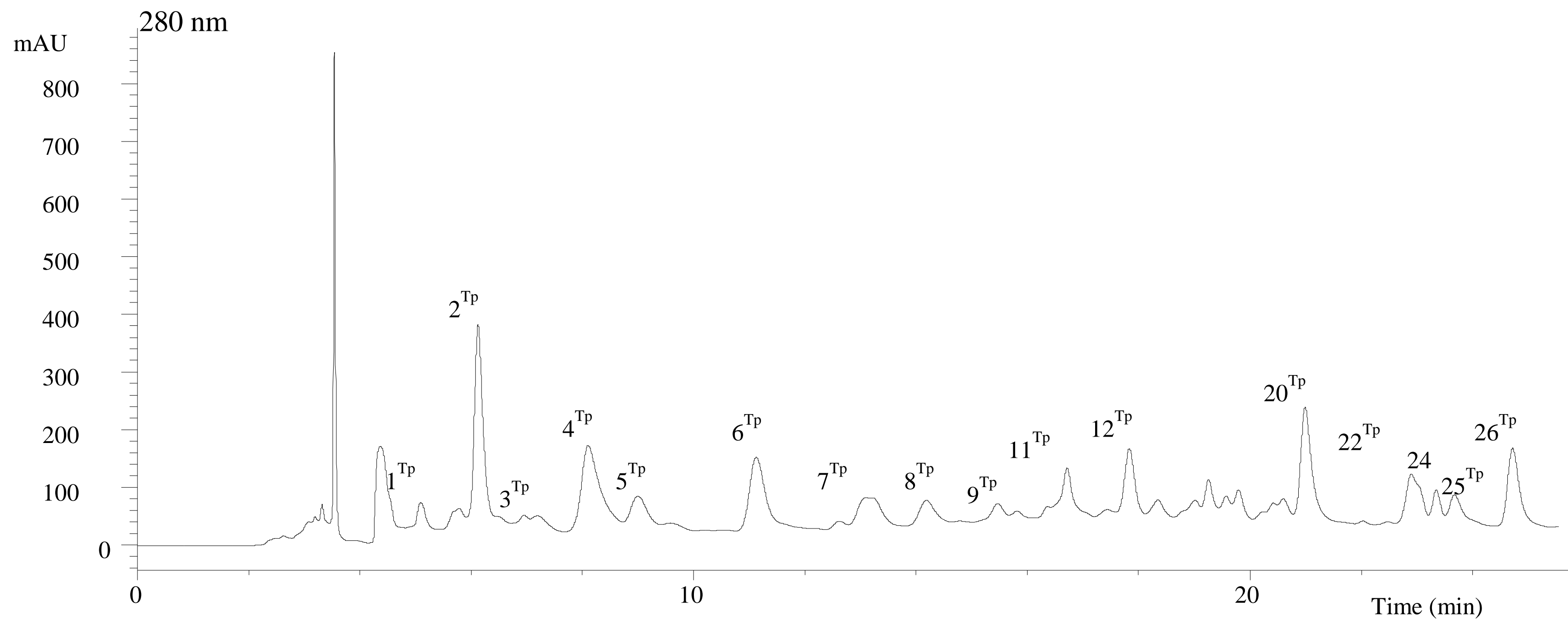


Figure 13. Phenolic compounds profile of *E. giganteum* recorded at 370 nm (A) and *T. platyphyllos* recorded at 370 and 280 nm (B). The numbering correspond to the peak number presented in Tables 5 and 6.

Table 6. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, identification and quantification (mean ± SD) of phenolic compounds in *Tilia platyphyllos* extract.

Compound	Rt (min)	λ_{max} (nm)	Molecular ion $[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g extract)
1^{Tp}	5.1	328	353	191(100),179(44),161(5),135(38)	3-*O*-Caffeoylquinic acid	0.64±0.03
2^{Tp}	6.1	260,294sh	153	109(100)	Protocatechuic acid	6.3±0.1
3^{Tp}	7.2	280	289	245(12),203(20),187(10),161(5),137(5)	(+)-Catechin	1.6±0.1
4^{Tp}	8.1	328	353	191(100),179(5),135(6)	5-*O*-Caffeoylquinic acid	4.7±0.1
5^{Tp}	9.0	280	577	451(26),425(37),407(100),289(69),287(7)	Procyanidin dimer B2	4.2±0.1
6^{Tp}	11.1	280	289	245(8),203(22),187(11),161(7),137(7)	(-)-Epicatechin	6.41±0.01
7^{Tp}	13.1	280	865	739(10),713(18),577(34),575(21),425(14),407(25), 289(15),287(20)	Procyanidin trimer	2.54±0.02
8^{Tp}	14.2	280	1153	865(6),863(17),577(61),575(17),289(42),287(10)	Procyanidin tetramer	3.9±0.1
9^{Tp}	15.5	280	1441	1153(9),865(9),577(31),289(96),287(30)	Procyanidin pentamer	2.4±0.2
10^{Tp}	16.3	356	609	463(9),447(27),301(12)	Quercetin-*O*-hexoside-*O*-deoxyhexoside	0.13±0.01
11^{Tp}	16.7	356	609	463(20),447(45),301(31)	Quercetin-3-*O*-glucoside-7-*O*-rhamnoside	1.4±0.1
12^{Tp}	17.8	286,336sh	449	287(100)	Eriodictyol-*O*-hexoside	1.3±0.1
13^{Tp}	18.3	358	579	447(64),433(10),301(33)	Quercetin-*O*-pentoside-*O*-rhamnoside	0.65±0.05
14^{Tp}	19.2	348	593	447(41),431(54),285(33)	Kaempferol-3-*O*-glucoside-7-*O*-rhamnoside	1.52±0.02
15^{Tp}	19.5	356	609	301(100)	Quercetin-3-*O*-rutinoside (rutin)	0.96±0.02
16^{Tp}	19.8	352	593	446(53),301(39)	Quercetin-3,7-di-*O*-rhamnoside	1.1±0.1
17^{Tp}	20.2	350	477	301(100)	Quercetin-3-*O*-glucuronide	0.21±0.05
18^{Tp}	20.5	348	563	431(66),417(26),285(39)	Kaempferol-*O*-pentoside-*O*-deoxyhexoside	0.76±0.05
19^{Tp}	20.6	358	463	301(100)	Quercetin-3-*O*-galactoside (hyperoside)	1.01±0.02

20^{Tp}	21.1	356	463	301(100)	Quercetin-3-*O*-glucoside (isoquercitrin)	6.36±0.04
21^{Tp}	22.5	360	505	463(12),301(100)	Quercetin-3-*O*-acetylglucoside	0.090±0.004
22^{Tp}	22.9	284,334sh	433	271(100)	Naringenin-*O*-hexoside	0.90±0.05
23^{Tp}	23.1	346	593	285(100)	Kaempferol-3-*O*-rutinoside	1.03±0.01
24^{Tp}	23.4	346	577	431(72),285(78)	Kaempferol-3,7-di-*O*-rhamnoside	1.15±0.03
25^{Tp}	23.7	356	433	301(100)	Quercetin-*O*-pentoside	1.8±0.1
26^{Tp}	24.8	350	447	301(100)	Quercetin-3-*O*-rhamnoside (quercitrin)	3.5±0.1
27^{Tp}	26.1	348	447	285(100)	Kaempferol-*O*-hexoside	0.55±0.03
28^{Tp}	26.8	352	417	285(100)	Kaempferol-*O*-pentoside	0.38±0.03
29^{Tp}	29.2	346	431	285(100)	Kaempferol-*O*-rhamnoside	0.39±0.03
30^{Tp}	30.7	350	431	285(100)	Kaempferol-*O*-rhamnoside	0.45±0.04
31^{Tp}	32.5	332	283	269(100)	Methyl apigenin	2.07±0.01
32^{Tp}	35.0	316	593	447(10),285(79)	*trans*-Tiliroside	1.38±0.03
33^{Tp}	35.3	326	593	447(10),285(79)	*cis*-Tiliroside	0.27±0.03
					Total phenolic acids	11.65±0.05
					Total flavonoids	50.4±0.4
					Total phenolic compounds	62.0±0.4

Peaks 10^{Tp}, 11^{Tp}, 13^{Tp}, 16^{Tp}, 19^{Tp}, 21^{Tp}, 25 and 26^{Tp} were identified as quercetin derivatives owing to the product ion observed at *m/z* 301 and their UV spectra (λ_{max} around 350-358 nm). Peaks 10^{Tp} and 11^{Tp} both presented the same pseudomolecular ion ($[M-H]^-$ at *m/z* 609) as peak 15^{Tp} (quercetin-3-*O*-rutinoside), but a different fragmentation pattern, as in those compounds product ions from the alternative loss of hexosyl (*m/z* at 447; -162 u) and deoxyhexosyl (*m/z* at 463; -146 u) were observed, indicating their location on different positions of the aglycone. Compounds with similar features were reported in *T. platyphyllos* and *T. cordata* (Negri et al., 2013; Karioti et al., 2014) and identified as quercetin-3-*O*-glucoside-7-*O*-rhamnoside, identity that was tentatively assumed for peak 11^{Tp} in our sample owing to its greater concentration, whereas peak 10^{Tp} remained just assigned as quercetin-*O*-hexoside-*O*-deoxyhexoside. Peak 16^{Tp} showed a pseudomolecular ion $[M-H]^-$ at *m/z* 593, yielding two fragment ions from consecutive loss of two deoxyhexosyl residues; it was tentatively identified as quercetin-3,7-di-*O*-rhamnoside, a compound commonly reported in *Tilia* species (Toker et al., 2001; Aguirre-Hernández et al., 2010; Negri et al., 2013; Ieri et al., 2015) including *T. platyphyllos* (Karioti et al., 2014), whereas peak 13^{Tp} ($[M-H]^-$ at *m/z* 579) was tentatively assigned as quercetin-*O*-pentoside-*O*-rhamnoside, as reported in *T. cordata* by Negri et al. (2013). Compounds 19^{Tp} ($[M-H]^-$ at *m/z* 463), 25^{Tp} ($[M-H]^-$ at *m/z* 433) and 26^{Tp} ($[M-H]^-$ at *m/z* 447), presented MS^2 fragments corresponding to distinct losses of hexosyl (-162 u), pentosyl (-132 u) and deoxyhexosyl (-146 u) moieties, and an elution order coherent with the type of sugar substituents, according to their expected polarity. Although the position and nature of the sugar moieties could not be identified, an identity as quercetin-3-*O*-rhamnoside (quercitrin) was assumed for compound 26^{Tp}, previously described in *T. platyphyllos* (Karioti et al., 2014) and other *Tilia* species (Aguirre-Hernández et al., 2010; Negri et al., 2013; Ieri et al., 2015). Similarly, peak 19^{Tp} was tentatively assigned as quercetin-3-*O*-galactoside (hyperoside) owing to its previous identification in *Tilia tomentosa* (Ieri et al., 2015) and *T. cordata* (Kosakowska et al., 2015) that identity would also be supported by its close elution to quercetin-3-*O*-glucoside (peak 20^{Tp}), since galactosides typically elute before the equivalent glucosides in RP-HPLC (Santos-Buelga et al., 2003). Peak 21^{Tp} ($[M-H]^-$ at *m/z* 505), with a molecular weight 42 u (acetyl residue) higher than compounds 19^{Tp} and 20^{Tp} was tentatively identified as quercetin-3-*O*-acetylglucoside, assuming that it could derive from the more abundant precursor.

Peaks 14^{Tp}, 18^{Tp}, 24^{Tp}, 27^{Tp}, 28^{Tp}, 29^{Tp}, 30^{Tp}, 32^{Tp} and 33^{Tp} were identified as kaempferol glycosides based on their UV spectra (λ_{max} around 348 nm) and the production of an MS^2

fragment ion at *m/z* 285. Tentative identities of these compounds were assigned based on their pseudomolecular ions using a similar reasoning as for quercetin derivatives. Thus, peaks 14^{Tp} ($[M-H]^-$ at *m/z* 593) and 24^{Tp} ($[M-H]^-$ at *m/z* 577) should correspond to kaempferol-3-*O*-glucoside-7-*O*-rhamnoside and kaempferol-3,7-*O*-dirhamnoside, respectively, compounds that have been reported in *T. platyphyllos* (Karioti et al., 2014) and other *Tilia* species (Aguirre-Hernández et al., 2010; Ieri et al., 2015; Negri et al., 2013; Toker et al., 2004). Peaks 32^{Tp} and 33^{Tp} possessed the same pseudomolecular ion ($[M-H]^-$ at *m/z* 593) as peaks 14^{Tp} and 23^{Tp} (kaempferol-3-*O*-rutinoside), but eluted at a longer retention time. The presence of tiliroside (kaempferol-3-*O*-(6-*p*-coumaroyl)-glucoside; 6’’-*O*-(4-hydroxycinnamoyl)-astragalin) has been consistently reported in different species of *Tilia* (Toker et al., 2004; Aguirre-Hernández et al., 2010; Ieri et al., 2015), including *T. platyphyllos*. The observation in their fragmentation of a product ion at *m/z* 447, from the loss of *p*-coumaroyl residue (146 u) would also be coherent with that identity, as well as the late elution, since the presence of the hydroxycinnamoyl residue implies a decrease in polarity. The detection of two peaks could be explained by the existence of *trans*- and *cis*- isomers of the *p*-coumaric acid, as also noted by Karioti et al. (2014). The more abundant peak 32^{Tp} can be assigned as *trans*-tiliroside taking into account that the form *trans* is the usual one for *p*-coumaric acid. Peak 27^{Tp} ($[M-H]^-$ at *m/z* 447) presented the same pseudomolecular ion as kaempferol-3-*O*-glucoside (astragalin), a compound that was commonly detected in *Tilia* spp. (Exarchou, Fiamegos, Beek, Nanos, & Vervoort, 2006; Aguirre-Hernández et al., 2010; Negri et al., 2013; Karioti et al., 2014; Kosakowska et al., 2015), but it eluted at a different retention time, as compared with a commercial standard; thus, the detected compound was just assigned as kaempferol-*O*-hexoside. Peaks 29^{Tp} and 30^{Tp}, with the same pseudomolecular ion ($[M-H]^-$ at *m/z* 431) coherent with kaempferol bearing as deoxyhexosyl moiety. Two undefined kaempferol rhamnosides were found in *T. tomentosa* by Ieri et al. (2015), while the presence of kaempferol-3-*O*-rhamnoside was reported in different *Tilia* spp. (Aguirre-Hernández et al., 2010; Karioti et al., 2014; Negri et al., 2013) that could correspond to one of the compounds detected in our study. However, since no sufficient information to assign that identity to any of the two detected peaks could be obtained, both compounds remain identified as kaempferol-*O*-rhamnoside. The remaining two kaempferol derivatives, i.e., peaks 18^{Tp} and 28^{Tp}, were respectively assigned as kaempferol-*O*-pentoside-*O*-hexoside and kaempferol-*O*-pentoside, respectively, based on their mass spectral characteristics. As far as we are aware, none of those compounds has been previously reported in *Tilia* spp. Flavonols accounted for

41% of the content of total phenolic compounds, being quercetin-3-*O*-glucoside the main molecule of this group.

Compounds 12^{Tp} ($[M-H]^-$ at *m/z* 449) and 22^{Tp} ($[M-H]^-$ at *m/z* 433) presented UV spectra characteristics of flavanones and MS^2 fragments at *m/z* 287 (eriodictyol) and at *m/z* 271 (naringenin), respectively, indicating the loss of an hexosyl residue (162 u), so that they were tentatively assigned as eriodictyol-*O*-hexoside and naringenin-*O*-hexoside. Finally, peak 31^{Tp} showed a pseudomolecular ion $[M-H]^-$ at *m/z* 283 that released an MS^2 fragment at *m/z* 269 (apigenin, $[M-H-14]^-$) indicating the loss of a methyl group; this observation together with its UV spectrum, characteristic of a flavone, suggested that it might correspond to a methyl apigenin. To the best of our knowledge, neither flavanones nor flavones have been described in *T. platyphyllos*.

Equisetum giganteum mainly presented kaempferol derivatives, accounting to 81% of the total phenolic content. Thus, in addition to peaks 11^{Eg} and 13^{Eg}, positively identified as kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucoside, peaks 3^{Eg}, 5^{Eg}, 7^{Eg}, 8^{Eg}, 9^{Eg}, 12^{Eg} and 14^{Eg} were also assigned as kaempferol-derived compounds based on their UV spectra (λ_{max} around 348 nm) and the observation of a product ion at *m/z* 285; tentative identities were attributed from their molecular weight and fragmentation patterns.

Compound 3^{Eg} ($[M-H]^-$ at *m/z* 755) MS^2 fragments revealed the alternative loss of hexosyl (*m/z* at 593; -162 u) and deoxyhexosyl-hexoside (*m/z* at 447; -308 u) residues, indicating location of each residue on different positions of the aglycone. The positive identification of kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucoside may suggest the presence of glucosyl and rutinosyl as sugar substituents, so that the compound was tentatively assigned as kaempferol-*O*-glucoside-*O*-rutinoside. This molecule was the most abundant phenolic compound (7.6 mg/g extract) present in this sample. Similarly, compound 8^{Eg} ($[M-H]^-$ at *m/z* 739) was assigned as kaempferol-*O*-rhamnoside-*O*-rutinoside.

Peaks 5^{Eg} and 7^{Eg}, with the same $[M-H]^-$ at *m/z* 651 and similar fragmentation pattern, with production of fragment ions at *m/z* 489 and 447, from the alternative loss of hexosyl (-162 u) and acetylhexosyl residues (-204 u), were assigned as kaempferol-*O*-acetylglucoside-*O*-glucoside. The observation of two compounds might be explained by different substitution positions of either each of the two residues on the aglycone or the acetyl moiety on the glucose. Compound 9^{Eg} ($[M-H]^-$ at *m/z* 593) showed the same pseudomolecular ion as kaempferol-3-*O*-rutinoside but a different fragmentation pattern. The observation of product

ions at *m/z* 447 (-146 u) and 431 (-162 u) revealed the alternative loss of deoxyhexosyl and hexosyl residues, allowing its identification as kaempferol-*O*-glucoside-*O*-rhamnoside, compound that was reported in extracts of the aerial parts of *Equisetum telmateia* by Correia et al. (2005). Peak 12^{Eg} ($[M-H]^-$ at *m/z* 635) presented a molecular weight 42 u greater than peak 9^{Eg} and MS^2 fragment ions at *m/z* 489 (-146 u, loss of a rhamnosyl residue) and 431 (-204 u, loss of an acetylhexosyl residue), being identified as kaempferol-*O*-acetylglucoside-*O*-rhamnoside. Peak 14^{Eg}, with an $[M-H]^-$ at *m/z* 489 yielding a unique fragment ion at *m/z* 285, from the loss of an acetylhexosyl residue, was assigned as kaempferol-3-*O*-acetylglucoside, compound also reported by Correia et al. (2005) in *E. telmateia*.

Peaks 2^{Eg} and 6^{Eg} were assigned as quercetin derivatives based on their absorption spectra and the production of a fragment ion at *m/z* 301. Based on their fragmentation features and following a similar reasoning as for the previous kaempferol derivatives, they were assigned as quercetin-di-*O*-glucoside and quercetin-*O*-acetylglucoside-*O*-glucoside, respectively. Peak 2^{Eg} was tentatively identified as quercetin-3,7-di-*O*-glucoside, owing to the previous description of such compound in *E. giganteum* by Francescato et al. (2013) and Alavarce et al. (2015).

Finally, peak 1^{Eg} was identified as a caffeic acid hexoside according to its UV and mass characteristics.

With the exception of quercetin-3-*O*-glucoside and kaempferol-3-*O*-rutinoside, reported in *E. giganteum* by Francescato et al. (2013) and Alavarce et al. (2015), none of the compounds detected here in had been previously described in this species.

The total phenolic compounds, phenolic acids and flavonoids were highly correlated with the biological activity of both samples, presenting high correlation factors, especially for DPPH scavenging activity (R^2=0.89), reducing power (R^2=0.66), β-carotene bleaching inhibition (R^2=0.98), TBARS (R^2=0.73), and for the human tumor cell lines HeLa (R^2=0.94), HepG2 (R^2=0.93) and MCF-7 (R^2=0.62). Confirming the statements mentioned above.

4 CONCLUSION

Medicinal plants have been utilised for several years because of their therapeutic and health promoting benefits. The present study revealed that medicinal plants contain a lot of bioactive compounds that can be very beneficial in the pharmaceutical industry because of their health benefits.

Ethanol:water extracts of *Hypericum androseamum*, *Equisetum giganteum*, and *Tilia platyphyllos* revealed:

i) Antioxidant activity both as free radical scavengers and lipid peroxidation inhibitors;
ii) Antitumor activity in breast carcinoma (MCF-7), non-small cell lung cancer (NCI-H460), cervical carcinoma (HeLa) and hepatocellular carcinoma (HepG2)
iii) Anti-inflammatory activity, through NO production inhibition;
iv) Up to 400 µg/mL, the extracts did not show toxicity in non-tumor porcine liver cells.

It was interesting to study the anti-inflammatory activity of *H. androseamum* as no previous studies have been reported. 5-*O*-caffeoylquinic acid and 3-*O*-caffeoylquinic acid, two chlorogenic acid were the most abundant phenolic compounds in the *H. androseamum* extract, while phenolic acid derivatives represent 19% of the total phenolic composition in *T. platyphyllos* extract. Protocatechuic acid and epicatechin were most abundant in *T. platyphyllos* extract while kaempferol-*O*-glucoside-*O*-rutinoside was the most abundant phenolic compound in *E. giganteum* and kaempferol derivatives represented 81% of the total phenolic content. *E. giganteum* presented fourteen phenolic compounds (two phenolic acids and twelve flavonol glycoside derivatives), while *T. platyphyllos* proved to be a more complex matrix in its phenolic composition, revealing the presence of thirty-three compounds.

The results showed that these medicinal plants displayed very interesting bioactive properties which are related with their phenolic composition. However, further *in vivo* studies should be conducted to determine and validate the mechanism of action of these plant extracts and their safety, as also to be able to determine real therapeutic doses.

References

Abreu, R. M. V., Ferreira, I. C. F. R., Calhelha, R. C., Lima, R. T., Vasconcelos, M. H., Adega, F., Chaves, R., Queiroz, M. J. R. P. (**2011**). Anti-hepatocellular carcinoma activity using human HepG2 cells and hepatotoxicity of 6-substituted methyl 3- aminothieno [3,2-b] pyridine-2-carboxylate derivatives: In vitro evaluation, cell cycle analysis and QSAR studies. European Journal of Medicinal Chemistry, 46, 5800–5806.

Adeboye, P. T., Bettiga, M., & Olsson, L. (**2014**). The chemical nature of phenolic compounds determines their toxicity and induces distinct physiological responses in *Saccharomyces cerevisiae* in lignocellulose hydrolysates. AMB Express. 2014, 4-46.

Aguirre-Hernández, E., González-Trujano, M. E., Martínez, A. L., Moreno, J., Kite, G., Terrazas, T., & Soto-Hernández, M. (**2010**). HPLC/MS analysis and anxiolytic-like effect of quercetin and kaempferol flavonoids from *Tilia americana* var. mexicana. Journal of Ethnopharmacology, 127, 91–97.

Ahmad, L., Semotiuk, A., Zafar, M., Ahmad, M., Sultana, S., Liu, Q. R., Zada, P. M., Ul-Abidin, S. Z., & Yaseen, G. (**2015**). Ethnopharmacological documentation of medicinal plants used for hypertension among the local communities of DIR Lower, Pakistan. Journal of Ethnopharmacology, 175, 138–146.

Alavarce, R. A. S., Saldanha, L. L., Almeida, N. L. M., Porto, V. C., Dokkedal, A. L., & Lara, V. S. (**2015**). The Beneficial Effect of *Equisetum giganteum* L. against *Candida* Biofilm Formation: New Approaches to Denture Stomatitis. Evidence-Based Complementary and Alternative Medicine, 2015, 1-10.

Aruoma, O. I. (**2003**). Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 523-524, 9–20.

Balunas, M. J., & Kinghorn, A. D. (**2005**). Drug discovery from medicinal plants. Life sciences, 78, 431–41.

Bedoya, L. M., Bermejo, P., Abad, M. J. (**2009**). Anti-infectious activity in the *Cistaceae* family in the Peninsula. Mini-Reviews in Medicinal Chemistry, 9, 519–525.

Calvo, M. I., & Cavero, R. Y. (**2015**). Medicinal plants used for neurological and mental disorders in Navarra and their validation from official sources. Journal of Ethnopharmacology, 169, 263–268.

Caprioli, G., Iannarelli, R., Cianfaglione, K., Fiorini, D., Giuliani, C., Lucarini, D., Papa, F., Sagratini, G., Vittori, S., & Maggi, F. (**2016**). Volatile profile, nutritional value and secretory

structures of the berry-like fruits of *Hypericum androsaemum* L. Food Research International 79, 1–10.

Carocho, M., & Ferreira, I. C. F. R. (**2013a**). A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food and Chemical Toxicology, 51, 15–25.

Carocho, M., & Ferreira, I. C. F. R. (**2013b**).The role of phenolic compounds in the fight against cancer – A review. Anti-Cancer Agents in Medicinal Chemistry, 13, 1236–1258.

Chatterjee, D., Sahu, R. K., Jha, A. K., & Dwivedi, J. (**2011**). Evaluation of antitumor activity of Cuscuta *reflexa roxb* (Cuscutaceae) against Ehrlich ascites carcinoma in swiss albino mice. Tropical Journal of Pharmaceutical Research 10, 447–454.

Clifford, M. N., Johnston, K. L., Knight, S., & Kuhnert, N. (**2003**). Hierarchical scheme for LC-MSn identification of chlorogenic acids. Journal of Agricultural and Food Chemistry, 51, 2900–2911.

Clifford, M. N., Johnston, K. L., Knight, S., & Kuhnert, N. A. (**2005**). Discriminating between the six isomers of dicaffeoylquinic acid by LC-MSn. Journal of Agricultural and Food Chemistry, 53, 3821–3832.

Correia, H., González-Paramás, A., Amaral, M. T., Santos-Buelga, C., & Batista, M. T. (**2005**). Characterization of polyphenols by HPLC-PAD-ESI/MS and antioxidant activity in *Equisetumtelmateia*. Phytochemical Analysis, 16, 380–387.

Cotrim, M. D., Figueiredo, I. V., Cavadas, C., Proenca da Cunha, A., Caramona, M. M. & Macedo, T. R. A. (**1999**). Pharmacological properties of *Tilia europeae* aqueous extract: screening anxiolytic/sedative activity in mice. Aquatic pathology journal, XXXI, 23-29

Danielski, L., Michielin, E. M. Z., & Ferreira, S. R. S. (**2007**). Horsetail (*Equisetum giganteum* L.) oleoresin and supercritical CO_2: experimental solubility and empirical data correlation. Journal of Food Engineering, 78, 1054–1059.

Dato, S., Crocco, P., D’Aquila, P., Rango, F., Bellizzi, D., Rose, G., & Passarino, G. (**2013**). Exploring the role of genetic variability and lifestyle in oxidative stress response for healthy aging and longevity. International Journal of Molecular Sciences, 14, 16443–16472.

Dias, A. C. P., Seabra, R. M., Andrade, P. B., Ferreres, F., & Ferreira, M. (**2000**). Xanthone biosynthesis and accumulation in calli and suspended cells of *Hypericum androsaemum*. Plant Science. 150, 93–10.

dos Santos Reinaldo, R. C. P., Santiago, A. C. P., Medeiros, P. M., & Albuquerque, U. S. (**2015**). Do ferns and lycophytes function as medicinal plants? A study of their low representation in traditional pharmacopoeias. Journal of Ethnopharmacology, 175, 39–47.

Espín, J. C., García-Conesa, M. T., & Tomás-Barberán, F. A. (**2007**). Nutraceuticals: facts and fiction. Phytochemistry, 68, 2986–3008.

Exarchou, V., Fiamegos, Y. C., Beek, T. A. van, Nanos, C., & Vervoort, J. (**2006**). Hyphenated chromatographic techniques for the rapid screening and identification of antioxidants in methanolic extracts of pharmaceutically used plants. Journal of Chromatography A, 1112, 293–302.

Farinon, M., Lora, P. S., Francescato, L. N., Bassani, V. L., Henriques, A. T., Xavier, R. M., & de Oliveira, P. G. (**2013**). Effect of Aqueous Extract of Giant Horsetail (*Equisetum giganteum* L.) in Antigen-Induced Arthritis. The Open Rheumatology, 7, 129-133.

Fernandes, Â., Barreira, J.C., Antonio, A.L., Oliveira, M.B., Martins, A., & Ferreira, I.C.F.R. (2014). Effects of gamma irradiation on chemical composition and antioxidant potential of processed samples of the wild mushroom *Macrolepiota procera*. Food Chemistry, 149, 91–98.

Fernandez-Panchon, M. S., Villano, D., Troncoso, A. M., Garcia-Parrilla M. C. (**2008**). Antioxidant activity of phenolic compounds: from in vitro results to *in vivo* evidence. Critical Reviews in Food Science and Nutrition 48, 649–671.

Francescato, N. L., Debenedetti, L. S., Schwanz, G. T., Bassani, L. V., & Henriques, T. A. (**2013**). Identification of phenolic compounds in *Equisetum giganteum* by LC-ESI-MS/MS and a new approach to total flavonoid quantification. Talanta, 105, 192–203.

Guedes, A. P., Amorim, L. R., Vicente, A. M. S., Ramos, G., & Fernandes- Ferriera, M. (**2003**). Essential oils from plants and *in vitro* shoots of *Hypericum androsaemum* L. Journal of Agricultural and Food Chemistry, 51, 1399-1404.

Halliwell, B. (**2012**). Free radicals and antioxidants: updating a personal view. Nutrition Reviews, 70, 257–265.

Han, G., Xia, J., Gao, J., Inagaki, Y., Tang, W., & Kokudo N. (**2015**). Anti-tumor effects and cellular mechanisms of resveratrol. Drug Discoveries & Therapeutics, 9, 1–12.

Hayta, S., Polat, R., & Selvi, S. (**2014**). Traditional uses of medicinal plants in Elazig (Turkey). Journal of Ethnopharmacology 154, 613–623.

Hernandez, M. F., Fale, P. L. V., Araujo, M. E. M., Serralheiro, M. L. M. (**2010**). Acetylcholinesterase inhibition and antioxidant activity of the water extracts of several *Hypericum* species. Food Chemistry 120, 1076–1082.

Ieri, F., Innocenti, M., Possieri, L., Gallori, S., & Mulinacci, N. (**2015**). Phenolic composition of “bud extracts” of *Ribes nigrum* L., *Rosa canina* L. and *Tilia tomentosa* M. Journal of Pharmaceutical and Biomedical Analysis, 115, 1–9.

Kanwar, J. R., Kanwar, R. K., Burrow, H., & Baratchi, S. (**2009**). Recent advances on the roles of NO in cancer and chronic inflammatory disorders. Current Medicinal Chemistry, 16, 2373–2394.

Karakaya, S., & Nehir, S. (**1999**). Quercetin, luteolin, apigenin and kaempferol contents of some foods. Food Chemistry, 66, 289-292.

Karioti, A., Chiarabini, L., Alachkar, A., Chehna, M. F., Vincieri, F. F., & Bilia, A. R. (**2014**). HPLC–DAD and HPLC–ESI-MS analyses of *Tiliae flos* and its preparations. Journal of Pharmaceutical and Biomedical Analysis, 100, 205–214.

Kosakowska, O. K., Baczek, K., Przybył, J. L., Ejdys, M., Kuźma, P., Obiedziński, M., Weglarz, Z. **(2015).** Intraspecific variability in the content of phenolic compounds, essential oil and mucilage of small-leaved lime (*Tilia cordata* Mill.) from Poland. Industrial Crops and Products, 78, 58–65.

Lattanzio, V. (**2013**). Phenolic Compounds: Introduction. In Handbook of Natural Products; Ramawat, K.G., Merillon, J.M., Eds.; Springer-Verlag: Berlin Heidelberg, Germany, 1543 1580.

Majer, P., Neugart, S., Krumbein, A., Schreiner, M., Hideg, É. **(2014).** Singlet oxygen scavenging by leaf flavonoids contributes to sunlight acclimation in *Tilia platyphyllos*. Environmental and Experimental Botany. 100, 1–9.

Martins, M., Barros, L., & Ferreira, I. C. F. R. (**2016**). *In vivo* antioxidant activity of phenolic compounds: Facts and gaps. Trends in Food Science & Technology 48, 1-12.

Min, K., Freeman, C., Kang, H., & Choi, S. U. (**2015**). The Regulation by Phenolic Compounds of Soil Organic Matter Dynamics under a Changing Environment. BioMed Research International, 2015, 1-12.

Murray, M. T. (**2004**).The healing power of herbs, Random House, New York, NY, 2nd edition.

Nagai, T., Myoda, T., & Nagashima, T. (**2005**). Antioxidative activities of water extract and ethanol extract from field horsetail (tsukushi) *Equisetum arvense* L. Food Chemistry, 91, 389–394.

Negri, G., Santi, D., & Tabach, R. (2013). Flavonol glycosides found in hydroethanolic extracts from *Tilia cordata*, a species utilized as anxiolytics. Brazilian Journal of Medicinal Plants, 15, 217–224.

Nogueira, T., Augusta Medeiros, M., João Marcelo-Curto, M., García-Pérez, B. E., Luna-Herrera, J., & Céu Costa, M. **(2013)**. Profile of antimicrobial potential of fifteen *Hypericum* species from Portugal. Industrial Crops and Products, 47, 126–131.

Porzel, A., Farag, M. A., Mülbradt, J., & Wessjohann, L. A. **(2013)**. Metabolite profiling and fingerprinting of *Hypericum* species: a comparison of MS and NMR metabolomics. Metabolomics, 1–15.

Radoglou, K., Dobrowolska, D., Spyroglou, G., & Nicolecu, V. N. **(2008)**. A review on the ecology and silviculture of limes. (*Tilia cordata* Mill.,*Tilia platyphyllos* Scop. and *Tilia tomentosa* Moench.) in Europe, 1 –29.

Rahman, T., Hosen, I., Islam, M. M. T., & Shekhar, H. U. **(2012)**. Oxidative stress and human health. Advances in Bioscience and Biotechnology, 3, 997-1019.

Rainha, N., Lima, E., & Baptista, J. **(2011)**. Comparison of the endemic Azorean *Hypericum foliosum* with other *Hypericum* species: Antioxidant activity and phenolic profile. Natural Product Research, 25, 123–135.

Ramalhete, N., Machado, A., Serrano, R., Gomes, E. T., Mota-Filipe, H., & Silva, O. **(2016)**. Comparative study on the *in vivo* antidepressant activities of the Portuguese *Hypericum foliosum, Hypericum androsaemum* and *Hypericum perforatum* medicinal plants. Industrial Crops and Products, 82, 29–36.

Ramos, A. A., Marques, F., Fernandes-Ferreira, M., & Pereira-Wilson, C. **(2013)**. Water extracts of tree *Hypericum sps*. protect DNA from oxidative and alkylating damage and enhance DNA repair in colon cells. Food and Chemical Toxicology 51, 80-86

Rates, S. **(2001)**. Plant as source of drugs. Toxicon 39, 603-613.

Ropiak, H. M., Ramsay, A., & Mueller-Harvey I. **(2016)**.Condensed tannins in extracts from European medicinal plants and herbal products. Journal of Pharmaceutical and Biomedical Analysis, 120, 225–231.

Savikin, K., Dobric, S., Tadic, V., & Zdunic, G. **(2007)**. Antiinflammatory activity of ethanol extracts of *Hypericum perforatum* L., *H. barbatum* Jacq., *H. hirsutum* L., *H. richeri* Vill. and*H. androsaemum* L. in rats. Phytotherapy Research, 21, 176–180.

Santos-Buelga, C., García-Viguera, C., & Tomás-Barberán, F. A. (2003). On-line identification of flavonoids by HPLC coupled to diode array detection. In C. Santos-Buelga & G. Williamson (Ed.), Methods in polyphenol analysis (pp. 92–127). Cambridge, UK: Royal Society of Chemistry.

Soobrattee, M. A., Neergheen, V. S., Luximon-Ramma, A., Aruoma, O. I., & Bahorun, T. (**2005**). Phenolics as potential antioxidant therapeutic agents: Mechanism and actions. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 579, 200–213.

Solowey, E., Lichtenstein, M., Sallon, S., Paavilainen, H., Solowey, E., & Lorberboum-Galski, H. (**2014**). Evaluating medicinal plants for anticancer activity. Scientific World Journal, 2014, 1-12.

Soto, M. L., Falqué, E., & Domínguez, H. (**2015**). Relevance of Natural Phenolics from Grape and Derivative Products in the Formulation of Cosmetics- Review. Cosmetics, 2, 259-276.

Souza, A. H .P., Corrêa, R. C. G., Barros, L., Calhelha, R. C., Santos-Buelga, C., Peralta, R. M., Bracht, A., Matsushita, M., & Ferreira, I. C. F. R. (**2015**). Phytochemicals and bioactive properties of *Ilex paraguariensis*: An *in-vitro* comparative study between the whole plants, leaves and stems. Food Research International, 78, 286–294.

Stef, D. S., Iosif, G., Ioan-Trasca, T., Stef, L., Pop, C., Harmanescu, M., Biron, R., & Pet, E. (**2010**). Evaluation of 33 medicinal plant extracts for the antioxidant capacity and total phenols. Journal of Food, Agriculture & Environment, 8, 207-210.

Taofiq, O., Calhelha, R. C., Heleno, S. A., Barros, L., Martins, A., Santos-Buelga, C., Queiroz, M. J. R. P., & Ferreira, I. C. F. R. (**2015**). The contribution of phenolic acids to the anti-inflammatory activity of mushrooms: Screening in phenolic extracts, individual parent molecules and synthesized glucuronated and methylated derivatives. Food Research International, 76, 821–827.

Taofiq, O., Martins, A., Barreiro, M. F., & Ferreira I. C. F. R. (**2016**). Anti-inflammatory potential of mushroom extracts and isolated metabolites. Trends in Food Science & Technology, 50, 193-210.

Touati, R., Santos, S. A. O., Rocha, S. M., Belhamel, K., & Silvestre, A. J. D. (**2015**). The potential of cork from *Quercus suber* L. grown in Algeria as a source of bioactive lipophilic and phenolic compounds. Industrial Crops and Products 76, 936–945.

Toker, G., Aslan, M., Yesilada, E., Memisoglu, M., Ito, S. (**2001**). Comparative evaluation of the flavonoid content in officinal *Tiliae flos* and Turkish lime species for quality assessment. Journal of Pharmaceutical and Biomedical Analysis 26, 111–121.

Valentao, P., Fernndes, E., Canvalho, E., Andrade, P. B., Seabra, R. M., Bastos, M. L., (**2002**). Antioxidant activity of *Hypericum androsaenium* infusion scavenging effect on superoxide radical, hydroxyl radical and hypochlorous acid.Biological and Pharmaceutical Bulletin 25, 1324–1327.

Valentão, P., Carvalho, M., Carvalho, F., Fernandes, E., das Neves, R. P., Pereira, M. L., Andrade, P. B., Seabra, R. M., & Bastos, M. L. (**2004**). *Hypericum androsaemum* infusion increases tert-butyl hydroperoxideinduced mice hepatotoxicity *in vivo*. Journal of Ethnopharmacology, 94, 345–351.

Vidović, M., Morina, F., Milić, S., & Jovanović, S. V. (**2015**). An improved HPLC-DAD method for simultaneously measuring phenolics in the leaves of *Tilia platyphyllos* and *Ailanthus altissima*. Botanica Serbica, 39, 177-186.

Wojdyło, A., Oszmianski, J., & Czemerys, R. (**2007**). Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chemistry, 105, 940-949.

Xavier, C. P., Lima, C. F., Fernandes-Ferreira, M., & Pereira-Wilson, C. (**2012**). *Hypericum androsaemum* water extract inhibits proliferation in human colorectal cancer cells through effects on MAP kinases and PI3K/Aktpathway. Food and Function, 3, 844–852.

Yayalaci, Y., Celik, I., Bati, B. (**2014**). Hepatoprotective and antioxidant activity of linden (*Tilia platyphyllos* L.) infusion against ethanol-induced oxidative stress in rats. Journal of Membrane Biology, 247, 181–188.