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- Dehimi K., Dahamna S., Speciale A., Cimino F., Cristani M. Biological properties of Algerian *Thymelaea microphylla* Coss. & Dur. extracts. FARMACOGNOSIA nuove opportunita terapeutiche dal mondo vegetale. 20-21, june 2014. Napoli, Italy.

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ملخص

Thymelaea microphylla Coss. and Dur. (المثنان) هو نبات طبي ذو طبيعة صحراوية، ينتمي إلى جنس *Thymelaea* المنتشر في حوض البحر الأبيض المتوسط. يستعمل هذا النبات في الطب التقليدي وذلك لخصائصه المضادة للسرطان، الالتهاب والسكري. تم في هذه الدراسة تقييم كل من النشاطية المضادة للأكسدة والمضادة للالتهاب لخمس مستخلصات حضرت من أوراق وأزهار نبات *T. microphylla* الذي جني بمنطقة ذراع الحاجة بولاية المسيلة بالجزائر، باستعمال عدة مذيبات عضوية ذات قطبية مختلفة. بالإضافة إلى ذلك، تم إجراء دراسات أولية حول التركيب الكيميائي واختبار سمية هذا النبات. تم بداية تقدير محتوى بعض المستقلبات الثانوية في المستخلصات، حيث وجد أن مستخلص الأسيتون يحتوي على أكبر كمية من الفلافونويدات (137.56 ميكروغ مكافئ الكاتشين/غ مستخلص) والفلافونولات (94.13 ميكروغ مكافئ الكرستين/غ مستخلص)، أما مستخلص أسيتات الإيثيل فقد كان الأغنى بالدباغ (29.23 ميكروغ مكافئ الكاتشين/غ مستخلص). أظهر تحليل المستخلصات بواسطة HPLC احتواءها على الأحماض الفينولية (حمض الغاليك، حمض الكافييك، حمض الفيروليك وحمض الكوماريك) خصوصا في المستخلص المائي، وعلى الفلافونويدات (لوتيولين وكامبفيرول) في المستخلصات العضوية. بالإضافة إلى ذلك، بين تقدير النشاطية المضادة للأكسدة/المزيحة للجذور الحرة والذي تم بواسطة عدة طرق *in vitro*، أن المستخلص المائي ومستخلص الأسيتون أظهرا الفعالية الأكبر باستعمال تقنية Folin، DPPH، FRAP، ABTS و ORAC، بينما في اختبار BCB، أظهرت النتائج أن مستخلص الأسيتون ومستخلص الهكسان قد يكونان الأغنى بمضادات الأكسدة القابلة للذوبان في الأوساط الدسمة. أما في اختبار SOD، فكذلك أظهر مستخلص الأسيتون النشاطية الأعلى (IC_{50}=1.81 مغ/مل). كما تم اختبار قدرة المستخلصات على تثبيط تكون AGEs ، *in vitro*، حيث كان المستخلص المائي الأكثر فعالية (IC_{50}=33.24 ميكروغ/مل). بينت نتائج تقدير النشاطية المضادة للالتهاب، والتي تمت بداية باستعمال اختبار التمسخ الحراري للألبومين، أن مستخلص الأسيتون كان الأكثر فعالية (IC_{50}=0.15 مغ/مل)، كما تم اختباره لاحقا باستعمال طرق *ex vivo* لتقدير نشاطه التثبيطي على انتاج prostaglandin E_2 و thromboxane B_2 في دم الإنسان، حيث أظهر هذا المستخلص نشاطية عالية مضادة للالتهاب ومثبطة لإنزيم cyclooxygenase، في حين لم يظهر أي سمية على خلايا دم الإنسان؛ بالإضافة إلى ذلك، أظهر هذا المستخلص قدرته على حماية خلايا البطانة الغشائية من الخلل الوظيفي الذي يسببه TNF-α، وهذا ما أثبته الانخفاض في مستويات كل من الموت الخلوي، انتاج E-selectin، التصاق كريات الدم البيضاء والحفاظ على مستوى GSH داخل الخلية. علاوة على ذلك، أظهر كل من مستخلص الإيثانول والمستخلص المائي سمية دالة إحصائيا على خلايا الدم أحادية النواة، بينما لم يسبب المستخلص المائي أي أعراض واضحة للسمية على الفئران ولم تسجل أي وفاة. هذه الدراسة التجريبية تدعم استخدام هذا النبات للعلاج الطبيعي والوقاية من الأمراض المرتبطة بالإجهاد التأكسدي والالتهاب.

الكلمات المفتاحية: Cyclooxygenase-2، الفلافونويدات، الالتهاب، الإجهاد التأكسدي، القدرة الإزاحية للجذور، *Thymelaea microphylla*، عامل نخر الورم ألفا.

Abstract

Thymelaea microphylla Coss. and Dur. (Methnane) is a medicinal plant with Saharan affinity, belonging to the Mediterranean genus *Thymelaea*. Leaves decoction is used in traditional medicine for anticancer, anti-inflammatory, and anti-diabetic properties. Herein, the antioxidant and anti-inflammatory activities of five extracts prepared using solvents with different polarities from leaves and flowers of Algerian *T. microphylla*, collected from the region of Draa El-Hadjja in M'sila, were evaluated. In addition, preliminary studies on phytochemical composition and toxicity estimation were realized. Extracts were first characterized for their content in some secondary metabolites, where acetone extract contained the highest amounts of flavonoids (137.56 µg Cat eq/mg) and flavonols (94.13 µg Que eq/mg), and ethyl acetate extract was the richest in tannins (29.23 µg Cat eq/mg). HPLC analysis revealed the presence of phenolic acids (gallic, caffeic, ferulic and p-coumaric acids) especially in aqueous extract, and flavonoids (luteolin and kaempferol) in organic extracts. Furthermore, antioxidant/free radical scavenging activity which was carried out by *in vitro* cell-free assays, showed that aqueous and acetone extracts exhibited the best potential in Folin, DPPH , FRAP, ABTS and ORAC assays, while in BCB, acetone and hexane extracts were very likely richer in antioxidants able to reach the lipophilic phase. In the same way, acetone extract was the most potent in SOD mimetic assay; with IC_{50} of 1.81mg/ml. Extracts were also tested for their inhibitory activity on *in vitro* AGEs formation, where aqueous extract was the most potent (IC_{50} = 33.24 µg/ml). The anti-inflammatory activity, which was first evaluated using albumin heat-induced denaturation assay, revealed that acetone extract was the most active with IC_{50} of 0.15 mg/ml, and it was further tested in *ex vivo* experiments, to estimate its inhibitory potential on prostaglandin E_2 and thromboxane B_2 release in human whole blood, where it showed excellent anti-inflammatory and cyclooxygenase-inhibitory activity, together with lack of toxicity on normal human blood cells; furthermore, it was able to protect human endothelial cells against dysfunction induced by TNF-α, as shown by decrease in cell death, E-selectin expression, leukocyte adhesion and prevention of depletion in GSH cell content. Moreover, ethanol and aqueous extracts had a significant cytotoxicity on peripheral blood mononuclear cells; however, aqueous extract did not cause mortality or any serious toxicity signs when tested *in vivo*. This experimental study support the use of this plant in phytotherapy and prevention of diseases related to oxidative stress and inflammation.

Keywords: Cyclooxygenase-2, flavonoids, inflammation, oxidative stress, radical scavenger activity, *Thymelaea microphylla*, tumor necrosis factor-α.

Résumé

Thymelaea microphylla Coss. et Dur. (Methnane) est une plante médicinale à affinité saharienne, appartenant au genre Méditerranéen *Thymelaea*. La décoction préparée des feuilles est utilisée en médecine traditionnelle pour ses propriétés anticancéreuse, anti-inflammatoire et antidiabétique. Dans ce travail, les activités antioxydante et anti-inflammatoire de cinq extraits préparés en utilisant des solvants de polarités différentes à partir des feuilles et fleurs de *T. microphylla* collectée de la région de Draa El-Hadjja à M'sila en Algérie, ont été évaluées. De plus, des études préliminaires sur la composition phyto-chimique et l'estimation de la toxicité ont été réalisées. Les extraits ont été d'abord caractérisés pour leur contenu en quelques métabolites secondaires, où l'extrait d'acétone contenait la plus grande quantité de flavonoïdes (137.56 µg Cat eq/mg) et de flavonols (94.13 µg Que eq/mg), alors que celui d'éthyle acétate était le plus riche en tannins (29.23 µg Cat eq/mg). L'analyse par HPLC a révélé la présence des acides phénoliques (acide gallique, caféique, férulique et p-coumarique), surtout dans l'extrait aqueux, et des flavonoïdes (lutéoline et kaempférol) dans les extraits organiques. En outre, l'activité anti-oxydante/anti-radicalaire, qui a été mesurée par des essais acellulaires *in vitro*, a montré que les extraits aqueux et acétonique avaient le meilleur potentiel dans les tests de Folin, DPPH, FRAP, ABTS et ORAC, tandis que dans le test BCB, les extraits d'acétone et d'hexane étaient probablement les plus riches en antioxydants capables d'atteindre la phase lipophile. De même, l'extrait d'acétone était le plus puissant dans le test du SOD ; avec IC_{50} de 1.81mg/ml. Les extraits étaient également testés pour leur pouvoir inhibiteur de la formation des AGEs *in vitro*, où l'extrait aqueux a montré la meilleure activité (IC_{50} = 33.24 µg/ml). L'activité anti-inflammatoire, qui a été d'abord évaluée en utilisant le test de la dénaturation thermique de l'albumine, a révélé que l'extrait d'acétone était le plus puissant avec IC_{50} de 0.15 mg/ml, cet extrait a été encore testé dans des expériences *ex vivo* pour estimer son potentiel inhibiteur sur la libération du prostaglandine E_2 et du thromboxane B_2 dans le sang entier humain, où il a montré une excellente activité anti-inflammatoire et inhibitrice de la cyclo-oxygénase, ainsi qu'il n'avait aucune toxicité sur les cellules sanguines humaines; De plus, cet extrait était capable de protéger les cellules endothéliales humaines contre le dysfonctionnement induit par le TNF-α, comme le montre la diminution de la mort cellulaire, l'expression de la sélectine E, l'adhésion aux leucocytes et la prévention de l'épuisement du GSH cellulaire. En outre, les extraits éthanolique et aqueux étaient significativement toxiques sur les cellules mononucléées du sang périphérique; Cependant, l'extrait aqueux n'a provoqué aucune mortalité ou autres signes graves de toxicité lorsque testé *in vivo*. Cette étude expérimentale supporte l'utilisation de cette plante en phytothérapie et la prévention des maladies reliées au stress oxydatif et à l'inflammation.

Mots clés: Cyclooxygénase-2, flavonoïdes, inflammation, stress oxydatif, activité anti-radicalaire, *Thymelaea microphylla*, facteur de nécrose tumorale-α.

Summary

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Abbreviations

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AGEs	advanced glycation end products
BCB	β-carotene bleaching assay
BSA	bovine serum albumin
CAT	catalase
COX	cyclooxygenase
DMSO	dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl radical
FL	fluorescein
FRAP	ferric reducing/antioxidant power assay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPx	glutathione peroxidase
GSH	reduced glutathione
GSSH	oxidized glutathione
HPLC	high performance liquid chromatography
HUVECs	human umbilical vein endothelial cells
IL	interleukin
LDL	low density lipoprotein
LOX	lipoxygenase
LPS	lipopolysaccharides
NADP	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-kappa B
NO•	nitric oxide
NOS	nitric oxide synthase
$^1O_2$	singlet oxygen
O2•-	superoxide anion
OH•	hydroxyl radical
ORAC	oxygen radical absorbance capacity
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PGE_2	prostaglandin E_2
RNS	reactive nitrogen species
ROS	reactive oxygen species
SOD	superoxide dismutase
TXB_2	thromboxane B_2
TNF-α	tumor necrosis factor-α

Introduction

Herbal medicines' use has remarkably increased in recent years, and there is a global trend of people returning to natural therapies. Currently, and according to the World Health Organization (WHO), about 80% of the world population still uses herbs for their primary health care needs, where medicinal plants have a big importance in public health maintenance, especially in developing areas such as African countries (Muthamizhe Selvan *et* al., 2013). According to the WHO, medicinal plants are probably the best source of a variety of drugs; in fact, about 40% of all medicines on the market today have been derived directly or indirectly from natural sources, of which 25% being from plants (Varalakshmi *et* al, 2011; Asare *et* al., 2012).

Oxidative stress consists of disturbed equilibrium between pro-oxidant and antioxidant homeostasis, which means overproduction of free radicals causing damage to biomolecules. Oxidative stress has become a major topic in all areas of medical knowledge, since it is an important etiologic factor of the pathologic process of many chronic diseases such as atherosclerosis, cancer, diabetics, rheumatoid arthritis, cardiovascular diseases, aging and other degenerative diseases in humans. There is also evidence that oxidative stress plays a crucial role in the development and perpetuation of inflammation, and thus contributes to development of chronic inflammation-related pathologies (Lugrin *et* al, 2014). Inflammation is, in fact, a part of the body's natural defense system against injury, however, chronic inflammation is believed to be, alongside with oxidative stress, a main contributing factor and a root cause of chronic degenerative diseases. Medicinal plants and derivative phytochemicals, especially polyphenols, have proven their ability to minimize oxidative stress damages and possess powerful antioxidant and anti-inflammatory activities (Srivastava and Mishra, 2015).

The Algerian Sahara is a place where climatic conditions are very extreme and severe. Plant species growing in this area have developed a very efficient secondary metabolism and are generally rich in polyphenols to cope with severe stress conditions. *T. microphylla* is a medicinal plant with Saharan affinity, very common in arid and desert pastures. The leaves decoction is used in folk medicine to treat abscesse, skin diseases, abdominal pain, cancer, inflammation and diabetes (Boukef 1986; Benhammou *et* al., 2009). Another similar species of *Thymelaea* genus (*Thymelaea hirsuta* Endl.), possessing the same vernacular name (Methnane), is traditionally used for its several and well documented antioxidant, anti-inflammatory and anticancer properties (Miyamae *et* al., 2009; Akrout *et* al., 2011; Trigui *et* al., 2013; Amari *et* al., 2014). However, only few data dealing with antibacterial and antioxidant activities of extracts from *T. microphylla* are available (Benhammou *et* al., 2009; Djeridane *et* al., 2010; Ladjel *et* al., 2011), as well as on its chemical composition (Mekhelfi *et* al. 2014, Kerbab *et* al., 2015).

The present work aims to investigate, by means of *in vitro* cell-free chemical assays, *ex vivo* experiments and cell culture based tests, the antioxidant/free radical scavenger and anti-inflammatory properties of several extracts (obtained by water, ethanol, acetone, ethyl acetate and hexane) from leaves and flowers of Algerian *Thymelaea microphylla*, and also to preliminary estimate phytochemical composition and toxic effect of these extracts. At this purpose prepared extracts were first screened for different phytochemicals which may be present in their structures, characterized for their total content in flavonoids, flavonols and tannins, and also analyzed using HPLC, to identify and quantify some of their flavonoids and phenolic acids. Extracts were then tested for their antioxidant potency by means of a battery of *in vitro* chemical assays differing in the mechanisms involved, the chemical environment used and the stressor applied (folin-Ciocalteau assay; bleaching of the stable DPPH radical; ABTS assay; oxygen radical absorbance capacity assay; beta-carotene bleaching test;

scavenging activity against superoxide anion and ferric reducing/antioxidant power assay). Extracts were also tested for their inhibitory effect on *in vitro* advanced glycation end products (AGEs) formation, which, alongside with ROS, have an important role in diabetic complications and cell damage. Anti-inflammatory effect of extracts was determined using prevention of heating-induced albumin denaturation assay, and on the basis of results in previous experiments and also taking into account the evidence of cytotoxicity shown by these extracts on *in vitro* cultured peripheral blood mononuclear cells (PBMCs), acetone extract was further investigated about its capability to inhibit cyclooxygenase (COX) activity, and consequently PGE_2 and TXB_2 release, in whole blood. The capacity of this extract to protect vessel endothelial cells against the damage induced by the proinflammatory cytokine tumor necrosis factor-α (TNF-α) was also evaluated in terms of cell viability and E-selectin expression which was estimated after RNA extraction and evaluation of E-selectin mRNA levels by means reverse transcription polymerase chain reaction (RT-PCR) technique. Leukocyte adhesion to endothelial cells and intracellular reduced glutathione (GSH) content were also evaluated. To further investigate the toxicity of *T. microphylla*, and view the use of its decoction in folk medicine, aqueous extract toxic effect was tested *in vivo* using male and female *Albino* mice.

Chapter 1
Literature Review

1. *Thymelaea microphylla*

1.1. *Thymelaeaceae* family

1.1.1. Morphology

Plants belonging to *Thymelaeaceae* family are shrubs or under-shrubs prevalent in tropical and moderate regions. This family contain up to 1200 species distributed in 67 genera. Species of this family are dicotyledons with persistent alternate leathery leaves. Flowers are hermaphrodites, dioecious or polygamous (4 to 5 merous), with tubular calyx and dry or drupaceous monospermous fruit. Perianth with welded parts at the base, often of coralline appearance more or less yellow or greenish. Stamina (8 to 10) are inserted in two rows (Quezel and Santa, 1962, 1963; Borris *et* al., 1988).

1.1.2. Phytochemical properties

Concerning phytochemical composition, many compounds were isolated from species belonging to this family. Among identified compounds groups, essential oils and monoterpenes confer to these plants aromatic properties, hence their use in perfumes fabrication. Diterpenes represent one of the most important chemical groups in this family; in general these compounds are derivative from daphnane and tigliane. Among characterizing compounds in *Thymelaeaceae* family, coumarins are present under different forms: simple coumarins such as daphnetin and umbelliferone, bis-coumarins like daphnoretin and tri-coumarins like edgeworoside. Many groups belonging to flavonoids are present in these plants, including flavones, flavonols, flavanones, biflavonoids and glycosylflavones; most common identified flavonoids are apigenin, luteolin, genkwanin, quercetin and kaempferol. Lignans also were identified in this family in free or linked form (Heywood, 1996; Julien, 2002).

1.1.3. Therapeutic interests

In folk medicine, plants belonging to *Thymelaeaceae* family have a large spectrum of use in treating different disorders. Prepared remedies from these species are used as vomitive, purgative, depurative, diuretic, against chronic rheumatism and skin diseases (Fournier, 1999a). Many species of this family were reported as toxic plants. In fact, as mentioned above, diterpenes which are known to have toxic properties are one of the most frequent compounds in *Thymelaeaceae* members, and responsible for their toxic effects (Borris *et* al., 1988; Fournier, 1999a).

1.2. The genus *Thymelaea*

The genus *Thymelaea* comprises thirty one species of annual xerophyllous shrubs and herbs (Kabbaj *et* al., 2013), with very small leaves and yellowish or greenish flowers (Fournier, 1999b). Among them, eight species are present in Algeria: *T. microphylla*, *T. hirsuta*, *T. passerina*, *T. velutina*, *T. virgata*, *T. nitida*, *T. virescens* and *T. tartonraira* (Quezel and Santa, 1962). Plants from this genus were found to be rich in flavonoids, phenolic acids, coumarins and lignans (Trigui *et* al., 2013; Ghanem *et* al., 2014; Kerbab *et* al., 2015). Many of these species are well-documented for their antioxidant, anti-inflammatory, anticancer and hypoglycemic effects (Diogo *et* al., 2009; Elamrani *et* al., 2009; Benhammou *et* al., 2009).

1.3. Description of the plant

1.3.1. Morphology

Thymelaea microphylla is an annual under shrub with dioic clusters. The leaves are very small (1 – 4 mm), ovoid, scattered and distant on the branches. Flowers are dioic and form glomeruli (2 – 5), with yellowish aspect. Branches are slender and canescent (Quezel and

Santa, 1963). *T. microphylla* has a Saharan affinity; it is very common in arid and desert pastures.

1.3.2. Classification

The vernacular name of *T. microphylla* is «Methnane». The classification of the plant is represented in table 1.

Table 1: Classification of *T. microphylla* Coss and Dur. (Quezel and Santa, 1962, 1963).

Kingdom	***Plantae***
Phylum	***Spermatophyta***
Sub- Phylum	***Angiospermae***
Class	***Dicotyledonae***
Sub- class	***Rosidae***
Order	***Malvales***
Family	***Thymelaeaceae***
Sub-family	***Thymelaeoideae***
Tribe	***Gnidieae***
Genus	***Thymelaea***
Species	***Thymelaea microphylla* Coss. and Dur.**

1.3.3. Traditional uses

T. microphylla is used in folk medicine for its anti-inflammatory, anticancer and antidiabetic properties. Decoction and natural compress prepared from aerial parts are used to treat abscess, skin diseases, abdominal pain and rheumatism; this plant is also used as antihelmentic (Boukef, 1986; Benhammou *et* al., 2009).

1.3.4. Chemical composition of the plant

Only few studies were reported in literature concerning the phytochemical composition of *Thymelaea microphylla*. In a study realized by Cheriti and Sekkoum in 1995, Oleanolic acid, β-sitosterol and 3-O-β-D-glucopyranosyl-β-sitosterol were detected in aerial parts of the plant (Dohou *et* al., 2003).

Other recent studies were reported about compounds isolated from this species. Among them, one study allowed isolation of two spiro-γ-lactone glycosides together with five biflavonoids (neochamaejasmin A, neochamaejasmin B, daphnodorin B, genkwanol A and stelleranol), one bis-coumarin (daphnoretin), two lignans (pinoresinol and matairesinol), one flavonoid glucoside (tiliroside) and a sinapyl alcohol glucoside (syringing), all isolated from ethyl acetate extracts of the aerial parts and roots of the plant (Ghanem *et* al., 2014).

Analysis of chloroform and ethyl acetate soluble parts of the aqueous-ethanol extract from aerial parts of *T. microphylla* led to the isolation of six compounds: vanillin, syringaresinol, daphnoretin, (Z)-8-hydroxylinalool, chrysoeriol and luteolin (Mekhelfi *et* al., 2014).

Another two studies (Kerbab *et* al., 2015; Noman *et* al., 2017) revealed the presence of monoterpene glucosides, phenolic acid derivatives (protocatechuic acid, chlorogenic acid butyl ester and ethyl gallate), phenylpropanoid glucosides, flavonoids (yuankanin, Kaempferol derivative and stenopalustroside A), lignans (such as matairesinol and prestegane B) and coumarins (such as umbelliferone and daphnoretin) in this species extracts.

2. Polyphenols

Phenolic compounds constitute one of the most extensive groups of chemicals in the plant kingdom. It is estimated that more than 8000 compounds have been isolated and described. These compounds are generally involved in plant defense against ultraviolet radiation or

aggression by pathogens. Their structure is characterized by the presence of, at least, an aromatic ring bearing one or more hydroxyl groups. Plant phenolic compounds have considerable significance as bioactive compounds with substantial health benefits, in fact, these polyhydroxylated phytochemicals are known to possess strong antioxidant, anti-inflammatory, anticancer, anti-diabetic and neuro-protective effects. Polyphenols may be classified into different groups as a function of the number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another (Hennebelle *et* al., 2004; Ramos, 2007; Pandey and Rizvi, 2009; Ameer *et* al., 2017). Herein, we report three sub-classes: flavonoids, phenolic acids and tannins.

2.1. Flavonoids

Flavonoids are considered as the most studied group of polyphenols, where more than 4000 compounds belonging to this sub-class were identified. In plants, flavonoids are more abundant in aerial parts, in which are responsible for attractive colors of flowers, fruits and leaves (Pandey and Rizvi, 2009). Basic chemical structure of flavonoids consists of a fifteen-carbon skeleton containing two benzene rings linked via a heterocyclic pyrane ring. According to the level of oxidation and pattern of substitution of the heterocyclic ring, flavonoids are divided to six groups: flavonols, flavones, flavanones, flavanols, anthocyanins and isoflavones (Fig. 1) (Kumar and Pandey, 2013). Flavonoids are associated with strong therapeutic properties, including antioxidant, anti-inflammatory and anticancer effects; in fact, there is an epidemiological evidence that a higher intake of flavonoids was associated with a lower risk of coronary heart disease mortality and cancer (Beecher, 2003; Castro-Vazquez *et* al., 2016; Suen *et* al., 2016). As antioxidants, and due to their phenolic hydroxyl groups attached to ring structures, flavonoids can act as reducing agents, hydrogen donators, singlet oxygen quenchers, superoxide radical scavengers and metal chelators; furthermore, these compounds can inhibit enzymes involved in ROS production, like xanthine oxidase, and

stimulate antioxidant enzymes (Tsao and Yang, 2003; Montoro *et* al., 2005). As anti-inflammatory agents, flavonoids can modulate the function of the immune system and inflammatory cells by affecting enzyme systems critically involved in the generation of inflammatory processes, especially tyrosine and serine-threonine protein kinases. Furthermore, flavonoids are able to inhibit expression of isoforms of inducible nitric oxide synthase, cyclooxygenase, and lipooxygenase, which are responsible for the production of a great amount of nitric oxide, prostanoids, leukotrienes and other mediators of the inflammatory process such as cytokines, chemokines, or adhesion molecules (D'Archivio *et* al., 2007; Kumar and Pandey, 2013).

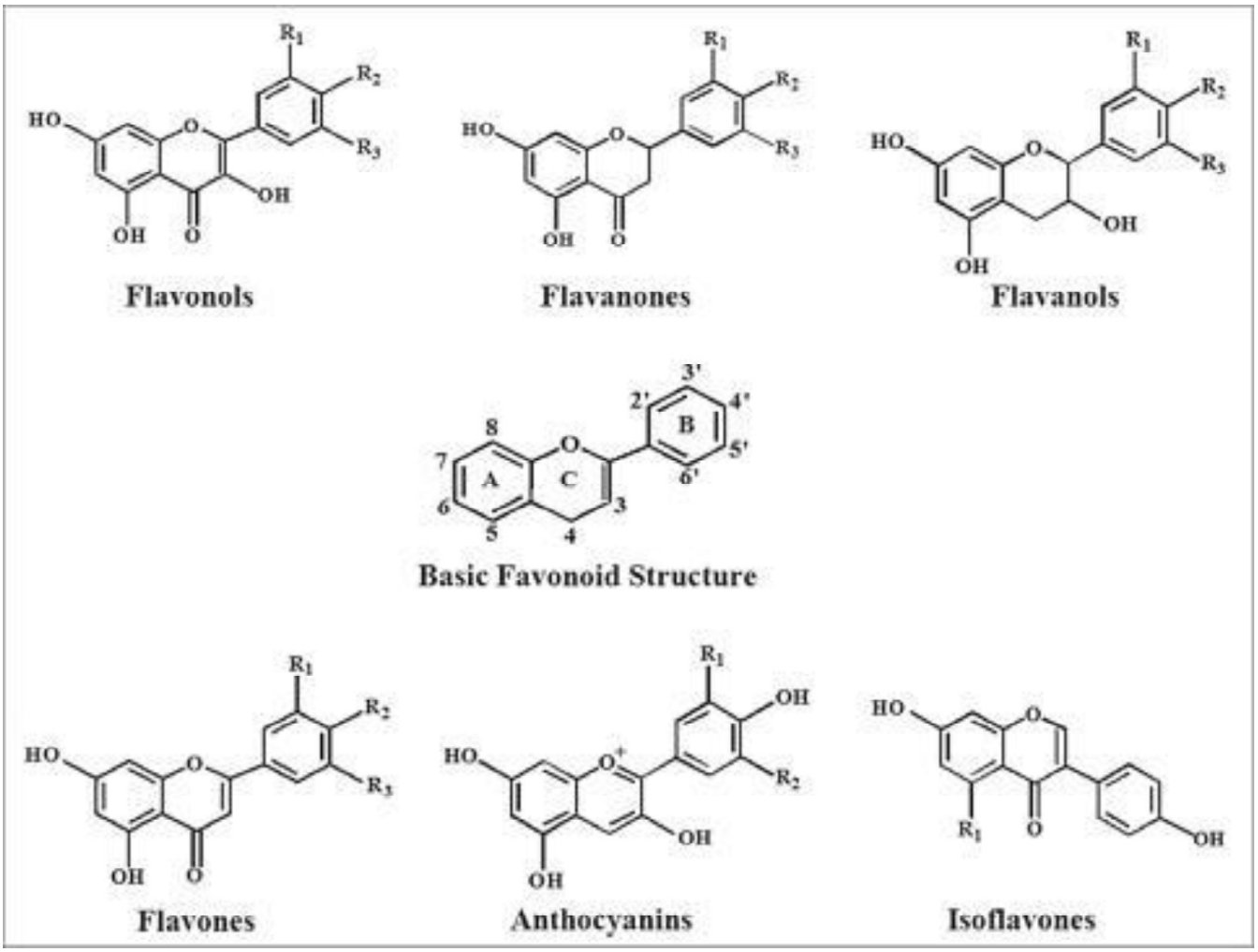


Fig. 1: Basic flavonoids structures (Pandey and Rizvi, 2009).

2.2. Phenolic acids

Phenolic compounds are a group of secondary metabolites in plants. These compounds are usually divided in two main groups (Fig. 2), the hydroxycinnamic acids, which form the largest class, comprise a three-carbon side chain (C6–C3) structure; as examples from this class, caffeic, ferulic, p-coumaric and sinapic acids are the most common. The second group is formed by hydroxybenzoic acids, which comprise a C6–C1 structure; it includes gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids. These metabolites can be found in free or derivative forms. They exhibit multiple physiological functions, such as antioxidant, anti-inflammatory, anti-microbial, anti-diabetic and anticancer activities. As antioxidants, phenolic acids counteract both ROS and RNS-induced cell damage by their direct free radical scavenging activity as well as the up-regulation of superoxide dismutase (SOD), and catalase (CAT). In inflammation, Chlorogenic acid was proven to possess antiplatelet and antithrombotic effects; furthermore, this phenolic acid was able to cause suppression of proinflammatory cytokines such as IL-1β, TNF-α, and IL-6, and suppress the production of NO and PGE_2 via down-regulating of the expression of iNOS and COX-2 enzyme (Robbins, 2003; Saibabu *et* al., 2015; Taofiq *et* al., 2017).

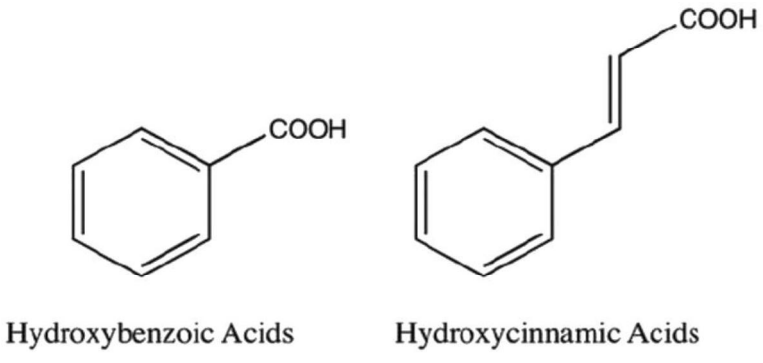


Fig. 2: Basic structures of phenolic acids (Khoddami *et* al., 2013).

2.3. Tannins

Tannins are complex phenolic compounds, which are widely distributed in the bark of trees, insect galls, leaves, stems and fruits. They are the chief plant constituents responsible for astringency. These compounds have a high molecular weight (500-5000), and are classified as hydrolysable tannins or condensed tannins (Fig. 3). Hydrolysable tannins are derived from simple phenolic acids, which are linked to sugar by esterification. Condensed tannins or proanthocyanidins are polymers of flavan-3-ols (catechins) and flavan-3,4-diols (leucoanthocyanins), they are only partially soluble in water and alcohol (Berthod *et* al., 1999; Pengelly *et* al., 2004). Most biological properties of tannins are linked to their ability to form complexes with macromolecules, particularly with proteins, in fact they have an enzymatic inhibitory effect on lipoxygenase and protein-kinase C; also these compounds can inhibit lipid peroxidation and are good free radical scavengers (Bruneton, 2002).

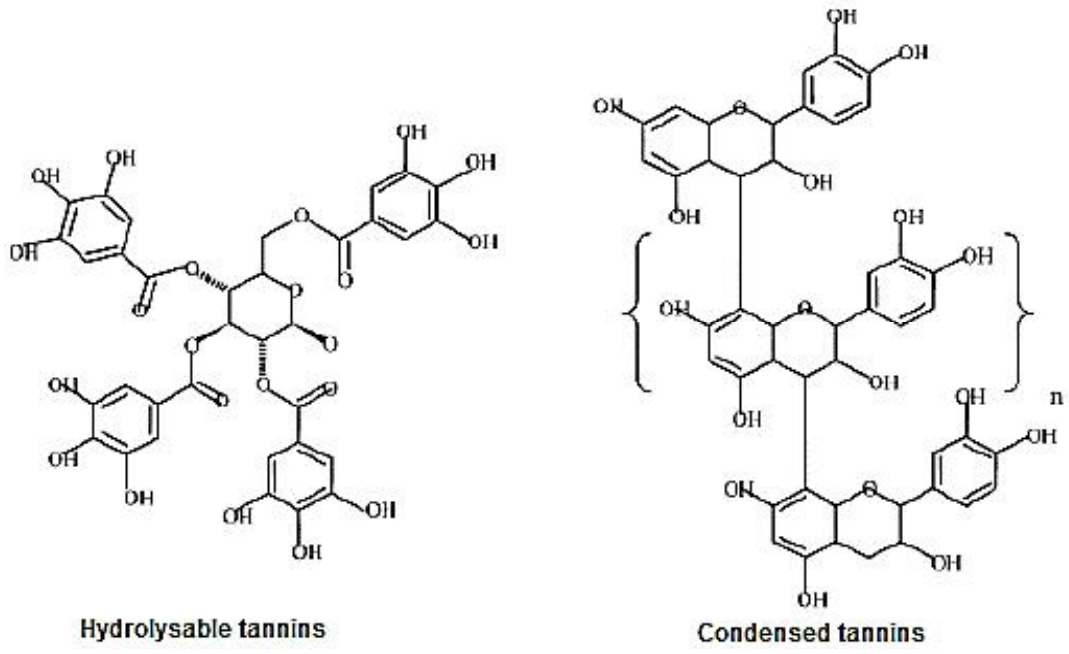


Fig. 3: Tannins chemical structure (Krause *et* al., 2005).

3. Oxidative stress and antioxidants

Oxidative stress results from an imbalance between oxidants and antioxidants in favor of the oxidants, which are normal products of aerobic metabolism, however, their excessive presence can lead to cell damage. The damaging effect of free radicals is counteracted by the action of antioxidants (Sies, 1997; Quiñonez-Flores *et* al., 2016).

3.1. Reactive oxygen species (ROS)

Free radicals are molecules with electrons that are unpaired, which make them unstable and reactive. These reactive species steal electrons from other stable molecules in order to become stable themselves, and so a destructive effect begins (Cooper, 1997). In fact, the term reactive species may refer, not only, to designate radicals characterized by an unpaired electron, but either to Non-radical oxygen derivatives, such as hydrogen peroxide H_2O_2, singlet oxygen $^1O_2$ and nitroperoxyde (ONOOH), which are also reactive and may be precursors of free radicals (Favier, 2003). Herein, we report some of the most important ROS and their sources.

3.1.1. Superoxide anion ($O_2^{\bullet-}$)

The uptake of one electron by molecular oxygen results in the formation of the superoxide anion radical (Marquardt *et* al., 1999).

$$O_2 + e^- \longrightarrow O_2^{\cdot -}$$

This reaction is mainly catalyzed by membrane NADPH oxidase. The superoxide anion may be formed in some cell organelles such as mitochondria where 2-5% of consumed oxygen is converted to superoxide radicals (Favier, 2003). $O_2^{\bullet-}$ is a primary ROS (the precursor of most ROS) and a mediator in oxidative chain reactions (Panglossi, 2006).

3.1.2. Hydroxyl radical ($OH^{\bullet}$)

It is the neutral form of the hydroxide ion. This radical is generated following interaction of Hydrogen peroxide (H_2O_2) with transition metal ions (Fenton-type reactions) (Marquardt *et* al., 1999; Eboh, 2014).

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH\cdot + OH^-$$

Or through reaction of Hydrogen peroxide with superoxide anion (reaction of Haber and Weiss) (Sorg, 2004).

$$H_2O_2 + O_2^{\cdot -} \xrightarrow{Fe(III)/Cu(II)} O_2 + OH^- + \cdot OH$$

Other reactions can lead also to the formation of hydroxyl radical, like the decomposition of peroxonitric acid and the reaction of hypochloric acid with superoxide anion (Bartosz, 2003).

The hydroxyl radical is a highly reactive oxidizing agent that can react with a wide variety of organic molecules (proteins, DNA, lipids), it can abstract Hydrogen atoms from essentially any Hydrogen-Carbon bond (Eboh, 2014).

3.1.3. Hydrogen peroxide (H_2O_2)

Hydrogen peroxide is a stable molecule generated as an end product of a variety of oxidative reactions in living cells. It may, in fact, act as both oxidizing and reducing agent. It is the least reactive molecule among reactive oxygen species and is stable under physiological pH and temperature in the absence of metal ions. H_2O_2 is produced by spontaneous or enzymatic dismutation of superoxide radical. Enzymatic dismutation is catalyzed by the superoxide dismutase (SOD) (Ahsan *et* al., 2003; Eboh, 2014; Nita and Grzybowski, 2016):

$$2\ O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

Additionally to SOD, there are other enzymes producing H_2O_2, such as oxidases present in peroxisomes. However, some of these enzymes like glycoxylate oxidase and D-aminoacide oxidase can directly catalyze the bivalent reduction of molecular Oxygen to give Hydrogen peroxide without formation of superoxide radical. Unlike the superoxide anion, the Hydrogen peroxide is capable to cross cells and organelles membranes causing damages away from his production site (Pham-Huy *et* al., 2008; Lobo *et* al., 2010).

3.1.4. Singlet Oxygen ($^1O_2$)

Although singlet Oxygen is not a free radical, it is a reactive oxygen species with strong oxidative properties; this molecule can quickly oxidize lipids and convert them into lipid peroxides. It is generated during some physiological and pathophysiological reactions (phagocytosis and prostaglandin biosynthesis), through oxidation reactions including those mediated by peroxidase and lipooxigenase between different ROS, or in the presence of light, oxygen and a photosensitizer (Sorg, 2004; Chen *et* al., 2012).

$$^3O_2 \xrightarrow{\text{light + photosensitizer}} {}^1O_2$$

$$O_2^{\cdot -} + M^{(n+1)+} \longrightarrow {}^1O_2 + M^{n+}$$

$$H_2O_2 + ONOO^- \longrightarrow {}^1O_2 + NO^{2-} + H_2O$$

$$H_2O_2 + ClO^- \longrightarrow {}^1O_2 + Cl^- + H_2O$$

3.1.5. Nitric oxide ($NO^{\bullet}$)

Nitric oxide is produced by oxidation of one of N-terminal atoms in L-arginine. This reaction is catalyzed by the Nitric oxide synthase enzyme (NOS) (Sorg, 2004):

$$O_2 + \text{Arginine} + NADPH \xrightarrow{NOS} NO^{\bullet} + \text{Citrulline} + H_2O + NADP^+$$

This production is important physiologically and plays a major role in neurotransmission, regulation of blood pressure, defense mechanism, smooth muscle relaxation, immune regulation (Valko *et* al., 2007). But at high concentrations, NO· is deleterious for cells in

particular when reacting with $O_2^{\cdot -}$ to form a powerful oxidizing agent which is the peroxynitrite (ONOO·) that can secondarily decompose and give other oxidants like NO_2 and OH· (Densiov and Afanas'ev, 2005).

Furthermore, various soluble enzymes generate free radicals by reducing molecular oxygen in their catalytic cycles. Membrane-bound enzymes and electron transport system can also release oxygen radicals and related ROS. Xenobiotic-metabolizing enzymes located in the endoplasmic reticulum and the nuclear membrane are also capable of activating molecular oxygen. This was shown to be the case for the cytochrome P450-dependent monooxygenases, the NADPH-dependent cytochrome reductases, and the flavin-dependent monooxygenases. Phagocytic cells are another major biological source of ROS (Table 2) (Marquardt *et* al., 1999; Phaniendra *et* al., 2015).

Table 2: Some biological systems capable of activating Molecular Oxygen (Marquardt *et* al., 1999).

Enzymes	Xanthine oxidase Aldehyde oxiodase Prostaglandin synthase Galactose oxidase Dopamine-β-hydroxylase Diamine oxidase Cytochrome P450-dependent monoxygenases
Autoxidation reactions	Catecholamines Thiols Hemoproteins Tetrahydropterines Flavins
Complex systems	Phagocyting cells Microsomal electron transport chain Mitochondrial electron transport chain

3.2. Biological roles of reactive oxygen species

It is well known since decades that living organisms use free radicals they produce in some fundamental physiological processes. Free radicals contribute in immune defense; phagocytosis of bacteria and parasites by macrophages is accompanied with a brutal and intense production of ROS. Inside phagosome, NADPH oxidase activation, superoxide dismutase (SOD) and NO synthase (NOS) actions, lead to a very corrosive mixture of $O_2^{\bullet-}$, H_2O_2, $OH^{\bullet}$ and ONOOH, besides the presence of HOCl and $^1O_2$ in the polynuclear; this reactive mixture destroys by oxidation the bacterial constituents. ROS can act also as signal molecules in apoptosis, metabolism, aging, and hypoxic signaling pathways, by interacting with some membrane receptors and activating them. The presence of ROS in extra-cellular medium causes the activation of some transcription factors, inducing then expression of corresponding genes. These biological functions of ROS confer to them an important role in cell cycle progression and cell proliferation (Favier, 2003; Delattre *et* al., 2005; Zhang *et* al., 2016).

3.3. Cell damages caused by reactive oxygen species

Reactive oxygen species may attack biological macromolecules, giving rise to protein, lipid, and nucleic acids damage. ROS, most importantly the OH•, can react with the nucleic acids attacking the nitrogenous bases and the sugar phosphate backbone and can evoke single- and double-stranded DNA breaks. The mitochondrial DNA is more vulnerable to the ROS attack than the nuclear DNA, because it is located in close proximity to the ROS generated place. In fact, RNA is subjected to more oxidative damage than DNA in humans, due to its single stranded nature, lack of an active repair mechanism for oxidized RNA, less protection by proteins than DNA and moreover cytoplasmic RNAs are located in close proximity to the mitochondria where loads of ROS are produced.

ROS attack also structural and enzymatic proteins and oxidize amino-acids present in their structure, causing formation of protein–protein cross linkages, results in the denaturing and loss of functioning of proteins, such as enzyme activity, function of receptors and transport proteins. Membrane lipids are also a target of reactive species; in fact, polyunsaturated fatty acids present in phospholipids are the most attacked by free radicals view the double bounds in their structures. This oxidation lead to loss of membrane functioning (inactivation of membrane bound enzymes and receptors) and formation of toxic end products from lipid peroxidation like malondialdehyde and 4-hydroxyl nonenal that cause damage to DNA and proteins. Furthermore, isoprostanes which are prostaglandin-like compounds, result from peroxidation of arachidonic acid, and are considered as markers of lipid peroxidation in oxidative stress.

Oxidative stress can then induce apoptosis, alters the growth signals and gene expression causing continuous proliferation of cells, alters the central nervous system due to the presence of high lipid content and causes atherosclerosis. All these conditions lead to several diseases such as cancer, neurodegenerative diseases like Alzheimer's and Parkinson's, cardiovascular diseases, rheumatoid arthritis, liver damage and diabetes complications (Phaniendra *et* al., 2015; Nita and Grzybowski, 2016).

3.4. Antioxidants

It has long been known that antioxidants fight the effects of free radicals and do much to slow down the aging process and prevent various types of diseases. Antioxidants are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. These molecules exhibit great structural diversity and ability to function in cooperation; when one antioxidant reacts with a free radical, another antioxidant is present to regenerate the first. There are several endogenous enzyme systems and substances

within the cells that scavenge free radicals. The importance of antioxidant systems is emphasized by the fact that compounds with other primary biological roles like albumin, glucose, uric acid, polyamines and fibrinogen, also can function as antioxidants. Cells can also attain antioxidants through the circulation after consumption of antioxidant rich beverages and food (Cooper, 1997; Olinescu and L.Smith, 2002; Panglossi, 2006).

3.4.1. Endogenous antioxidants

3.4.1.1. Glutathione

Glutathione has been termed as nature's master antioxidant. Reduced glutathione (GSH), a tripeptide (g-glutamylcysteinylglycine) with a free thiol group, is a major antioxidant in human tissues. It has been stated that low levels of glutathione may cause illness and premature death. It is instrumental in the detoxification of drugs and pollutants and for healthy liver function. Glutathione is important for strong immune system. It is also stated that boosting glutathione can possibly reverse age-related retardation of immune system. GSH provides reducing equivalents for the glutathione peroxidase (GPx) catalyzed reduction of hydrogen peroxide and lipid hydroperoxides to water and the respective alcohol. During this process GSH becomes oxidized glutathione (GSSG). GSSG is then recycled to GSH through interaction with the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), catalyzed by glutathione reductase (GR) (Panglossi, 2006; Bains and Bains, 2015):

$$H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADPH^+$$

3.4.1.2. Superoxide dismutase (SOD)

Since superoxide is the primary ROS produced in a variety of sources, its dismutation by SOD is of primary importance for each cell. SOD is an enzyme with a generalized presence in

the body, it has three variants, copper-zinc SOD found in the cytoplasm, manganese SOD located in the mitochondria and extra-cellular SOD. SODs catalyze transformation of superoxide anions to oxygen and hydrogen peroxide (view reaction above), protecting cells against the toxic effects of oxygen metabolism (Al-Dalaen and Al-Qtaitat, 2014; Jeeva *et* al., 2015).

3.4.1.3. Catalase (CAT)

Catalase is an antioxidant enzyme that acts as a catalyst for the conversion of hydrogen peroxide produced by the action of SODs or oxidases, such as xanthine oxidase, to molecular oxygen and water.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Catalase has one of the highest turnover rates of all enzymes; one molecule of catalase can convert approximately 6 million molecules of hydrogen peroxide to water and oxygen each minute (Panglossi, 2006; Al-Dalaen and Al-Qtaitat, 2014; Jeeva *et* al., 2015).

3.4.2. Dietary antioxidants

This group comprises a variety of structurally very different compounds that have been known to exhibit antioxidant properties. Diet, particularly fruits, vegetables, nuts and seeds form a rich source of antioxidant vitamins, phytochemicals and minerals (Panglossi, 2006).

3.4.2.1. Vitamins C and E

Vitamin C is a water-soluble antioxidant, which prevents oxidative damage to cells by scavenging free radicals, recycling vitamin E (alpha-tocopherol) and raising intracellular glutathione levels, and then plays an important role in protein thiol group protection against oxidation. However, vitamin E, which is a fat-soluble compound, is considered as major

powerful membrane-bound antioxidant, employed by the cell as a protection against lipid peroxidation; in fact, the anti-atherosclerotic activities of α-tocopherol have been related to its chain-breaking properties, preventing lipid peroxidation and formation of atherogenic oxidized LDL (Panglossi, 2006; Vance *et* al., 2013; Al-Dalaen and Al-Qtaitat, 2014).

3.4.2.2. Carotenoids

Carotenoids represent a very diverse group of natural pigments of the polyene type. They occur ubiquitously in all organisms capable of conducting photosynthesis. More than 700 carotenoids have been described, of which about 50 become constituents of the human diet. Carotenoids function as potent antioxidants and have also been shown to influence cell growth and induce apoptosis; some carotenoids serve as vitamin A precursors. Antioxidant activity of this group arises due to their ability to delocalize unpaired electrons, and thus quench singlet oxygen without degradation. β-carotene has been found to react with peroxyl (ROO.) to prevent damage in lipophilic compartments, hydroxyl ($OH^{\cdot}$), and superoxide (O_2-.) radicals (Vance *et* al., 2013; Al-Dalaen and Al-Qtaitat, 2014; Fiedor and Burda, 2014).

3.4.2.3. Minerals

Food derived minerals are crucial for antioxidant enzymes functions. Selenium is part of at least 25 different selenoproteins, such as glutathione peroxidase. This essential trace element is known for its antioxidant and anticarcinogenic activities. Furthermore, Iron, Copper, Zinc and Manganese are the co-factors of SODs, catalase, ceruloplasmin and metallothionein, which are implicated in free radicals removal. Deficiency of copper or zinc increases the cytochrome P_{450} activity in microsomes of liver and lungs, and thus enhances the ROS generation and iNOS expression (Panglossi, 2006; Noori, 2012; Vance *et* al., 2013).

3.4.3. Pro-oxidative effect

Some antioxidants may be actually becoming pro-oxidants under certain conditions; in fact, antioxidants function by being converted in the reaction to a live, but less reactive, free radical, and later are regenerated, such as vitamin E which is regenerated by vitamin C and glutathione. This is the case for most redox compounds that react as pro- or antioxidants depending on their environment and concentration. Some of the popular and well known antioxidants, flavonoids have been reported to act as pro-oxidants also when a transition metal is available, these have been found to be mutagenic in vitro (Olinescu and L.Smith, 2002; Rahal *et* al., 2014).

4. Inflammation

4.1. Definition

Inflammation is a vital part of the human immune system; it is a normal biological process in response to harmful stimuli, such as tissue injury, microbial pathogen infection, and chemical irritation. The primary functions of inflammation are to rapidly destroy or isolate the underlying source of the disturbance, remove damaged tissue, and then restore tissue homeostasis. Normal inflammation is rapid and self-limiting, but aberrant resolution and prolonged inflammation cause various chronic disorders (Kumar and Pandey, 2013; Saibabu *et* al., 2015).

4.2. Mechanisms

After detection of infection or damage via a set of receptors that recognize pathogen-associated molecular patterns (PAMPs) or via alarmins, inflammation is initiated by migration of immune cells from blood vessels and release of pro-inflammatory cytokines (via activation of NF-κB transcription factor), such as interleukin-1-beta (IL-1β), IL-6 and tumor necrosis

factor-alpha (TNF-α) which in conjunction with chemokines (attractants) such as PGE_2 (table 3), facilitate the recruitment of effector cells, such as monocytes and neutrophils, to the site of disturbance (Fig. 4). During this recruitment, an up-regulation of adhesion molecules is induced, to facilitate adhesion of inflammatory cells to endothelium. These cells release ROS, RNS, and various proteinases which are destructive to both foreign pathogens and hosts. The last step is resolution, which is set into motion by tissue-resident and recruited macrophages. During acute inflammation, these cells produce pro-inflammatory prostaglandins and leukotrienes, but rapidly switch to lipoxins, which block further neutrophil recruitment and instead favor enhanced filtration of monocytes important for wound healing (Zhang and An, 2007; Ashley *et* al., 2012).

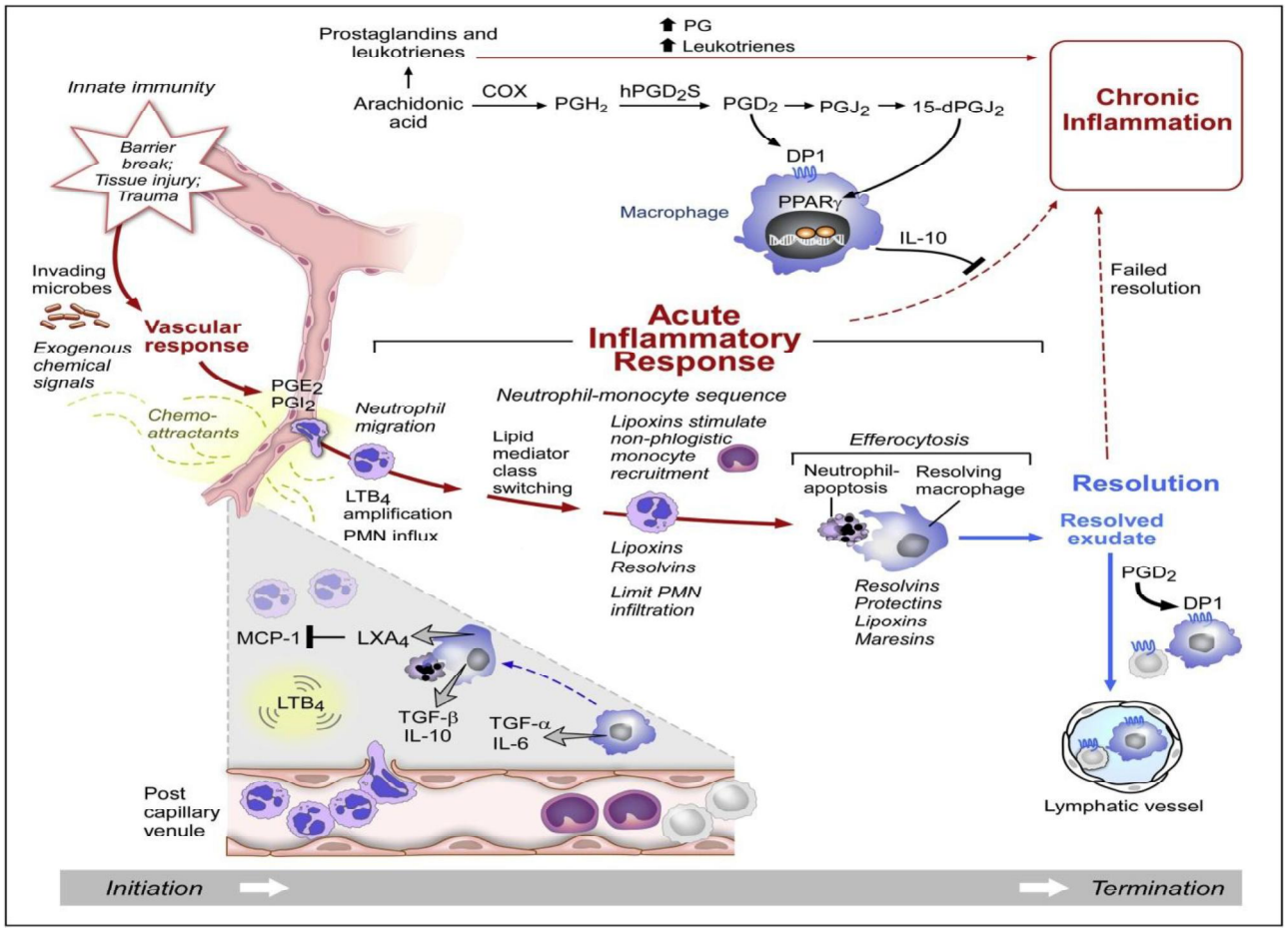


Fig. 4: Steps of acute inflammatory response (Buckley *et* al., 2014).

Table 3: Mediators in acute inflammatory response (Lawrence *et* al., 2002; Janeway *et* al., 2003).

Mediator classes	Mediators	Functions
Amines	histamine, bradykinin	Released by mast cells, cause vasodilation, increase capillary permeability
Lipid mediators	PGE_2, PGI_2, LTB_4, LTC_4	Vasodilators, attraction and activation of leukocytes
Adhesion molecules	E-selectin, ICAM1, VCAM1	Capture and adhesion of leukocytes to endothelial cells
Cytokines	TNF, IL-1β, IL-6	Released by macrophages, T cells and monocytes, their functions are cytokine production, cell proliferation and apoptosis
Chemokines	IL-8, MIP1α, MCP1	Released by macrophages, epithelial cells and endothelial cells, they have role in chemotaxis and angiogenesis

Among lipid mediators, prostaglandin E_2 (PGE_2) and thromboxane A_2 (TXA_2) are eicosanoids resulting from arachidonic acid metabolism, which is liberated from membranes by phospholipase A_2 (Fig. 5). TXA_2 has potentially pro-inflammatory actions; it is implicated in activation and aggregation of platelets and also in production of TNF-α and IL1-β in monocytes. The PGE_2 has actions which can be considered both pro- and anti-inflammatory, depending on cells and target receptors; it is implicated in vasodilatation and cause exudation but also can inhibit chemotaxis and production of O_2^- by neutrophils (James *et* al., 2001).

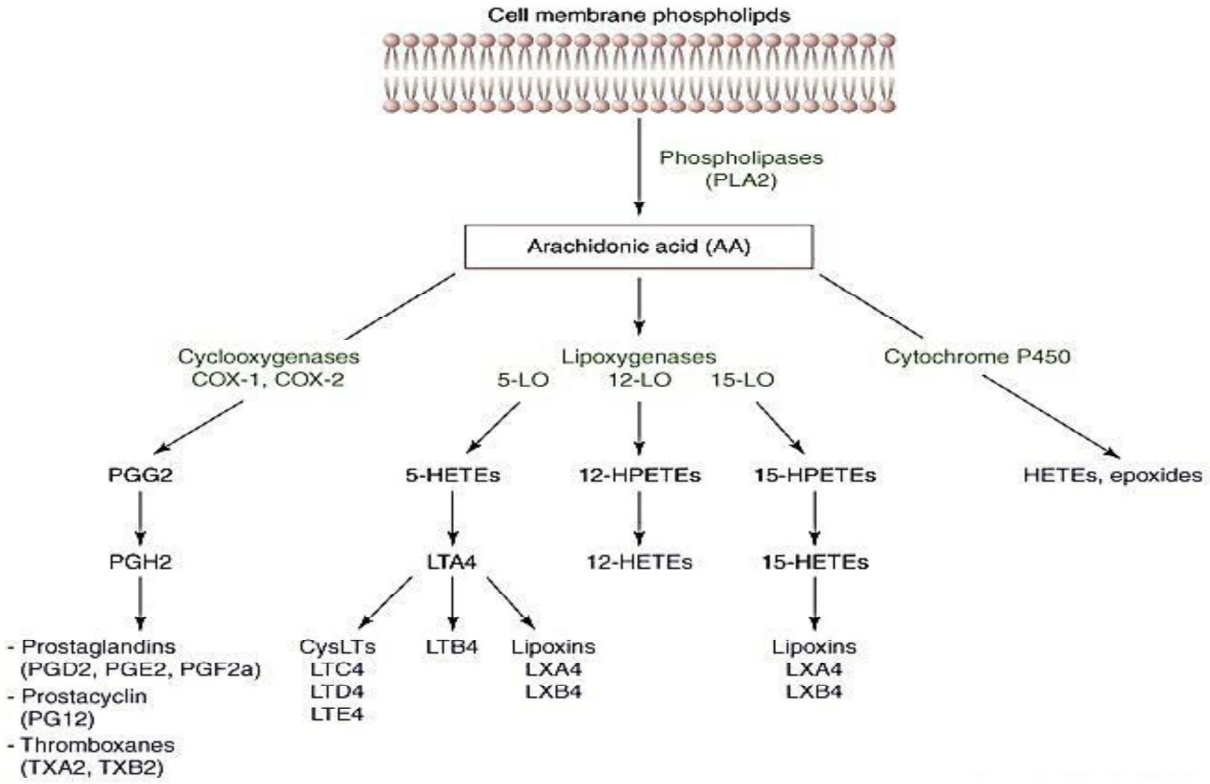


Fig. 5: Arachidonic acid metabolism (Harizi *et* al., 2008).

4.3. Inflammation related pathologies

Inflammation can become potentially deleterious if excessive or deregulated. In fact, the persistent chronic inflammation increases the development of the degenerative diseases such as cancer, metabolic disorder, type II diabetes, arthritis, autoimmune diseases, neurological diseases, multiple sclerosis, pulmonary diseases, and cardiovascular complications. These various disorders have been linked to increased expression of pro-inflammatory mediators which activates inflammatory cells by increasing the expression of pro-inflammatory cytokines, up-regulating genes that produce NF-κB, NADPH oxidase, phospholipase A_2, COX-1 and COX-2, 5-LOX, myeloperoxidase, iNOS, increasing oxygen consumption and producing many deleterious oxygen-free radicals (Iwalewa *et* al., 2007; Khansari *et* al., 2009; Prasad and Aggarwal, 2014).

4.4. Anti-inflammatory drugs

There are two main groups of anti-inflammatory drugs; non-steroidal and steroidal drugs. Non-steroidal anti-inflammatory drugs (NSAIDs) are a diverse group of compounds, able to inhibit prostaglandins production, by non-selective activity against COX-1 and COX-2 enzymes; NSAIDs can then eliminate the erythema, swelling, elevated temperature and pain (Vonkeman and Van de Laar, 2010). Steroidal anti-inflammatory drugs (SAIDs) are substances derived from cortisol, able to increase lipocortin production, and hence, inhibit phospholipase A_2 enzyme implicated in arachidonic acid release. Furthermore, these drugs decrease immune cells migration and release of cytokines and other inflammatory mediators. However, SAIDs are known for their numerous side effects, like hypertension, gastrointestinal disorders and osteoporosis (Payne and Adcock, 2001; Henzen, 2003). Anti-inflammatory effects of secondary metabolites from plants are also well documented, especially for flavonoids which exercise their activity via inhibition of tyrosine and serine-threonine protein kinases and COX/LOX enzymes. Besides flavonoids, other secondary metabolites exhibit also anti-inflammatory properties, such as phenolic acids, anthocyanins and tannins (Derbel and Ghedira, 2005; Kumar and Pandey, 2013).

Chapter 2
Materials and Methods

1. Materials

1.1. Chemicals

Pure HPLC reference standards were purchased from Extrasynthèse (Genay-France). Folin-Ciocalteau phenol reagent, analysis grade methanol, HPLC grade methanol, fluorescein sodium nitrite, and hydrochloride acid were obtained from Carlo Erba (Milan, Italy). HPLC grade water and acetonitrile, aluminium chloride anhydrous and potassium peroxodisulfate were from VWR. All other reagents, if not specified, were purchased from Sigma-Aldrich (Milan, Italy).

1.2. Animals

Sixty adult male and female *Albino* mice (average weight 25-30 g) were purchased from the institute of Pasteur, Algiers. Standard diet and water were available ad libitum. Animals were acclimated for two weeks before the experiment under the same laboratory conditions of photoperiod and room temperature (25 ± 2°C).

1.3. Plant material

Aerial parts of *Thymelaea microphylla* (Fig. 6) were collected in April 2011 from the region of Draa Elhadjja in M'sila – Algeria. The identification of the plant was based on the work of Quezel and Santa (1963), and validated by Pr. Laouar Hocine (laboratory of valorization of biological resources, university Ferhat Abbas Setif 1, Algeria). Samples were dried under shade at room temperature. Leaves and flowers were then separated from stems and used for extraction.

-A- **-B-**

Fig. 6: *Thymelaea microphylla* Coss. and Dur. (April 23rd, 2017).
A: Plant in environment. **B:** Leaves and flowers of the plant. (Photographed by author).

2. Methods

2.1. Extraction procedure

The extraction was realized according to the methods described by Gnanaprakash *et* al. (2010) and by Anushia *et* al. (2009) with slight modification. Five extracts (aqueous, ethanol, ethyl acetate, acetone and hexane) were prepared from dried leaves and flowers of *Thymelaea microphylla*. To obtain the aqueous extract, 10 g of the sample were put in contact with 100 ml of distilled water during 30 min at 70°C and then left for 2 days under occasional stirring. To obtain ethanol, ethyl acetate, acetone and hexane extracts, 10 g of sample were put in contact with 100 ml of each solvent during 48 hours with agitation in darkness. Then, the mixtures were filtered by Whatman N. 1 filter paper and the filtrates evaporated to dryness under vacuum by using a rotavapor. The residues were kept into brown vial in freezer until used.

2.2. Phytochemical study

2.2.1. Screening for phytoconstituents

Extracts were screened for the presence of phytoconstituents (tannins, saponins, flavonoids, steroids, alkaloids, terpenoids, cardiac glycosides and quinones) using the standard procedures described by Sunil H. Ganatra *et* al. (2012) and by Doughari *et* al. (2012).

2.2.2. Determination of total flavonoids content in plant extracts

The total content of flavonoids in *Thymelaea microphylla* extracts was determined by the method described by Tomaino *et* al. (2010). Fifty microliters of the solution containing the extracts to be tested (or catechin used as standard) were diluted with distilled water to a final volume of 0.5 ml, and 30 µl of 5% $NaNO_2$ were added. After 5 min the mixture was added with 60 µl of 10% $AlCl_3$, consequently after 6 min, with 200 µl of 1 M NaOH and 210 µl of distilled water. Absorbance was recorded at 510 nm using a Shimadzu UV-1601 spectrophotometer. Total flavonoids content was expressed as µg of catechin equivalents (CatE) per mg of dried extract (Fig. 7). All determinations were carried out in duplicate and repeated three times.

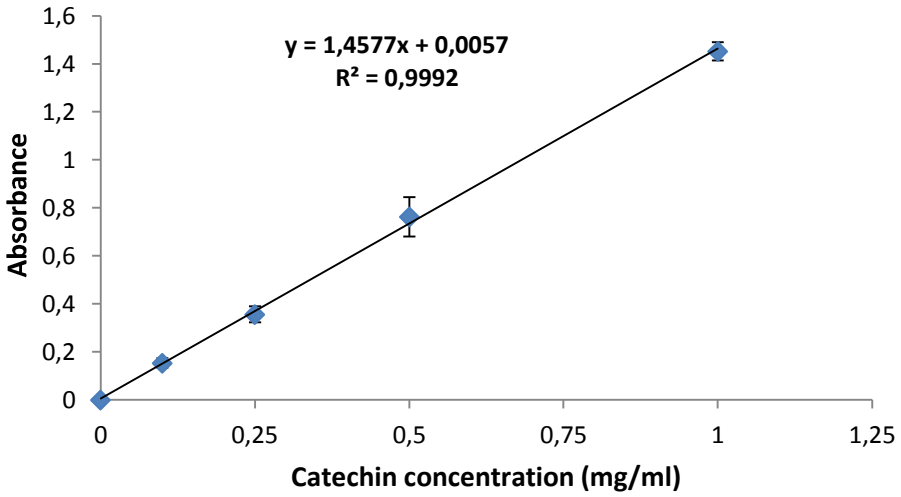


Fig. 7: Catechin calibration curve. Each point represents the mean ± SD (n=6).

2.2.3. Determination of total flavonols content in plant extracts

The flavonols content in extracts was measured according to the method described by Tomaino *et* al. (2010). 125 µl of each extract were added to an equal volume of $AlCl_3$ (2 mg/ml), and 750 µl of sodium acetate (50 mg/ml). After incubation during 2.5h, absorbance was recorded at 440 nm. Quercetin was used as standard with concentrations ranging from 2.5 to 100 µg/ml (Fig. 8). Total flavonols content was expressed as µg of quercetin equivalents (QE) per mg of dried extract. Each treatment was performed in duplicate and the whole experiment was repeated three times.

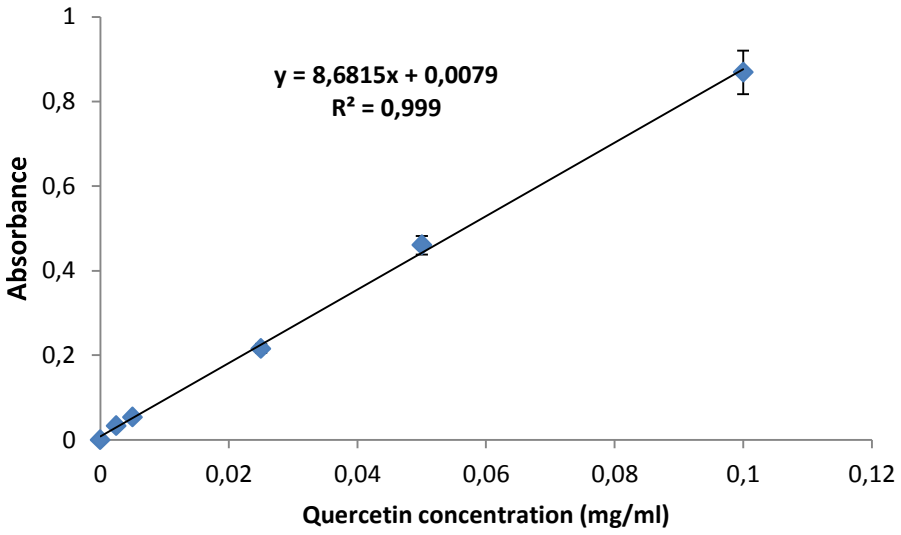


Fig. 8: Quercetin calibration curve. Each point represents the mean ± SD (n=6).

2.2.4. Determination of Condensed tannins content in plant extracts

The determination of condensed tannins content in extracts was carried out by the method described by Sun *et* al. (1998) and by Tounsi *et* al. (2009). 200 µl of extracts were added to 500 µl of vanillin methanolic solution (11 mg/ml) and 500 µl of H_2SO_4 (10%). After incubation for 15 min, absorbance was measured at 500 nm. Catechin was used as standard with concentrations ranging from 30 to 120 µg/ml (Fig. 9). Condensed tannins contents were expressed as µg of catechin equivalents (CatE) per mg of dried extract. Each determination was carried out in duplicate and repeated three times.

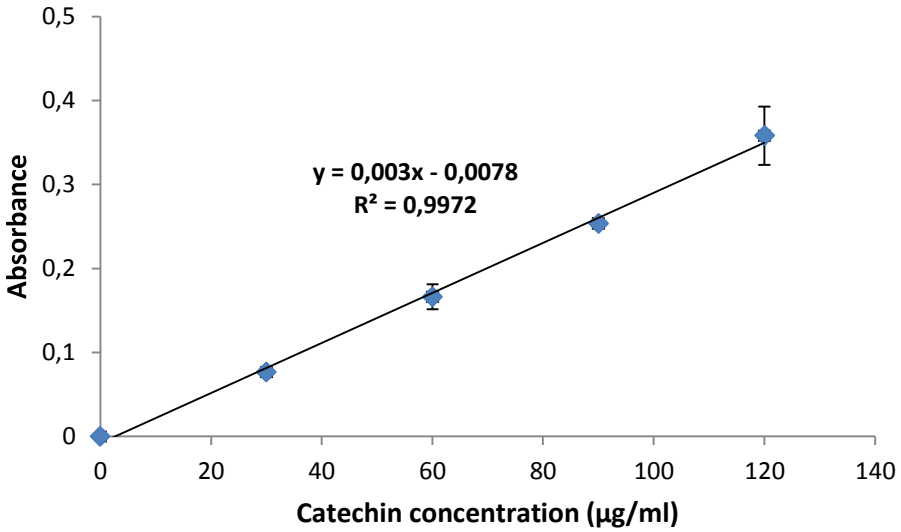


Fig. 9: Catechin calibration curve. Each point represents the mean ± SD (n=6).

2.2.5. Phenolic profile study using high performance liquid chromatography - diode array detector (HPLC-DAD)

Extracts from *Thymelaea microphylla* were analyzed using high performance liquid chromatography according to the method described by Siracusa *et* al. (2011). Qualitative and quantitative determination of phenolic profile of *Thymelaea microphylla* extracts was carried out using HPLC Varian Prostar system (Varian Prostar 220/230/240 pumps, Prostar 410 autosampler, Varian Prostar 325LC detector, Phenomenex Luna C18 column: 250 x 4.6 mm, 5 µm particle size). Extracts (5 mg/ml) and standards (50 µg/ml) were all dissolved in dimethylformamide (DMF) and analyzed using the following elution gradient of B (Acetonitrile) in A (2.5% formic acid in water): 0 min: 5% B, 10 min: 15% B, 30 min: 25% B, 35 min: 30% B, 50 min: 55% B, 55 min: 90% B, 57 min: 100% B, 65 min: 100% B, 70 min: 5% B. The flow rate was set to 1 ml/min, the temperature was kept at 25°C and the injector volume was 10 µl. UV-detection was performed at 280 and 370 nm for all samples.

Quantification of detected compounds was carried out at 280 nm and realized by means of the external standard method using corresponding compounds (Fig. 10 to 15). The results were

obtained from the average of three determinations and are expressed as mg/g dried extract ± percent relative standard deviation (% RSD).

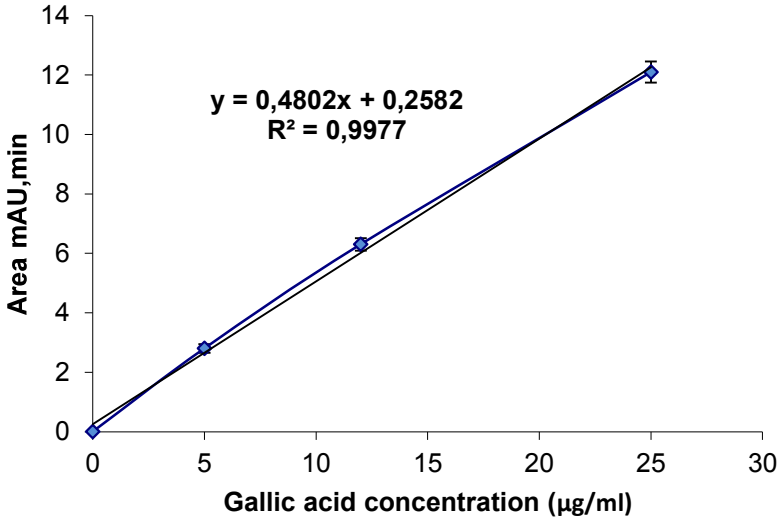


Fig. 10: Gallic acid calibration curve. Each point represents the mean ± SD (n=3).

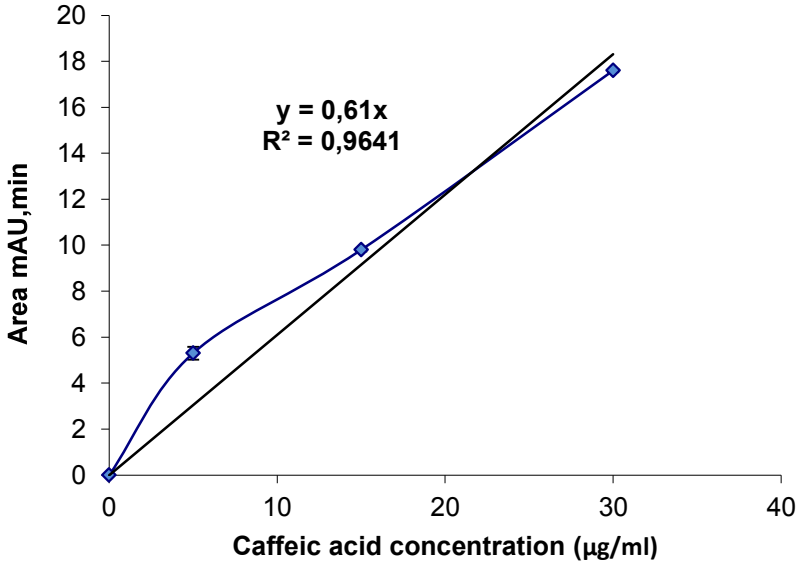


Fig. 11: Caffeic acid calibration curve. Each point represents the mean ± SD (n=3).

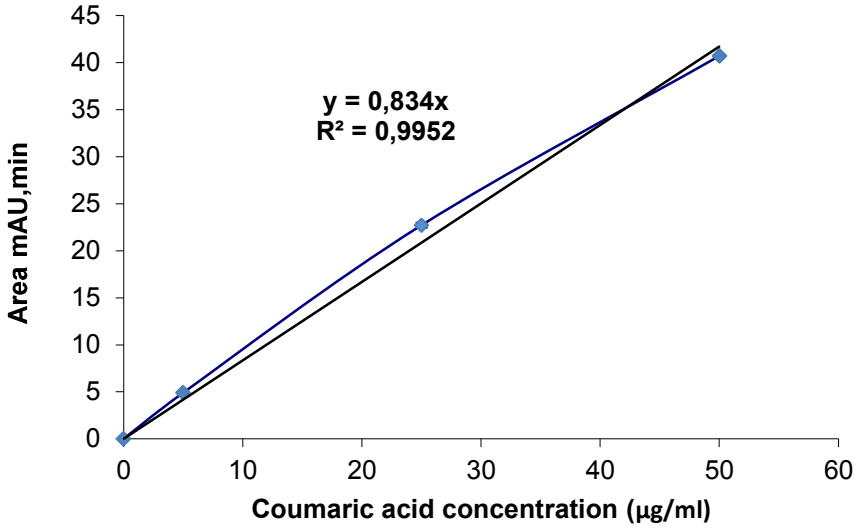


Fig. 12: Coumaric acid calibration curve. Each point represents the mean ± SD (n=3).

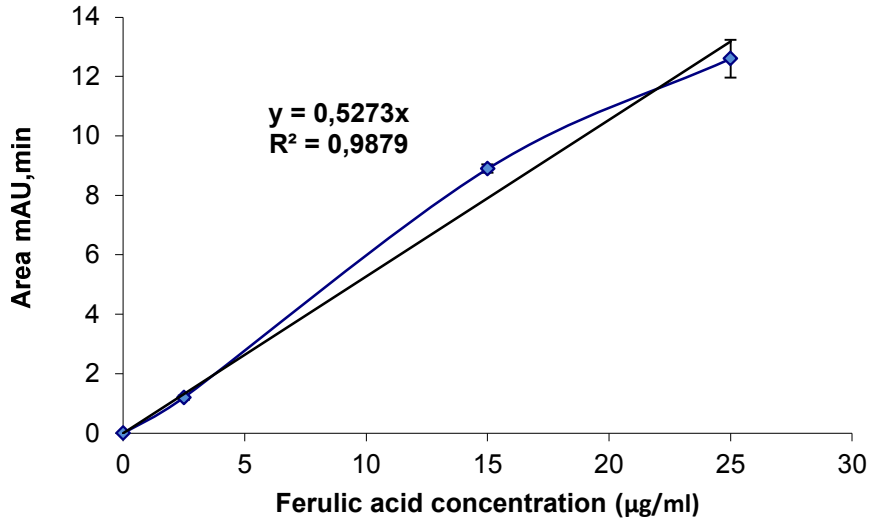


Fig. 13: Ferulic acid calibration curve. Each point represents the mean ± SD (n=3).

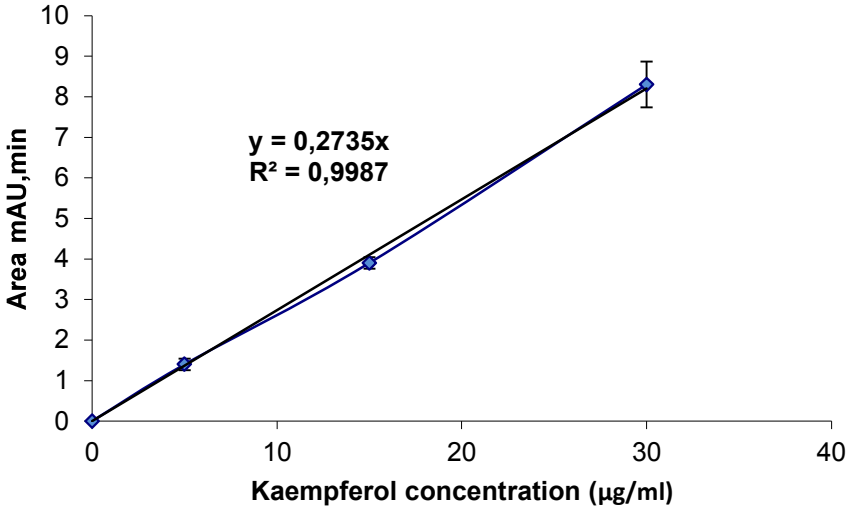


Fig. 14: Kaempferol calibration curve. Each point represents the mean ± SD (n=3).

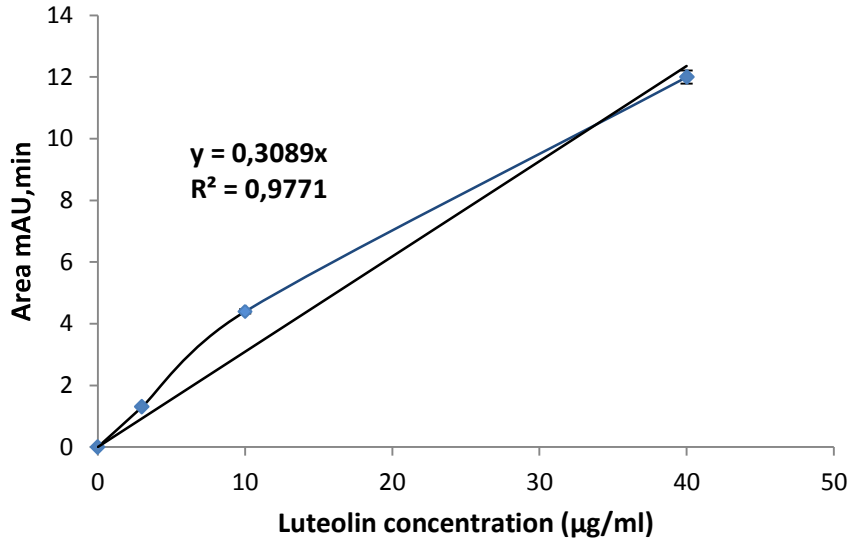


Fig. 15: Luteolin calibration curve. Each point represents the mean ± SD (n=3).

2.3. Antioxidant activity of plant extracts

2.3.1. Folin test

Antioxidant activity of extracts was first determined using the Folin–Ciocalteu colorimetric assay as described by Tuttolomondo *et* al. (2013). A volume of 50µl of each extract with different concentrations, or of the vehicle alone (DMSO), were added to 450 µl of deionized water, 500 µl of Folin–Ciocalteu reagent, and 500 µl of 10% aqueous sodium carbonate solution. The mixtures were allowed to stand at room temperature for 1 h. Absorbance was measured at 750 nm (UV-Vis Spectrophotometer, Shimadzu, Japan) against the blank containing 50 µl of the same solvent used to dissolve the extracts. Gallic acid was used as standard for the calibration curve (Fig. 16). Antioxidant activity was expressed as gallic acid equivalents in µg per mg of dried extract. Results are expressed as means ± SD from three experiments.

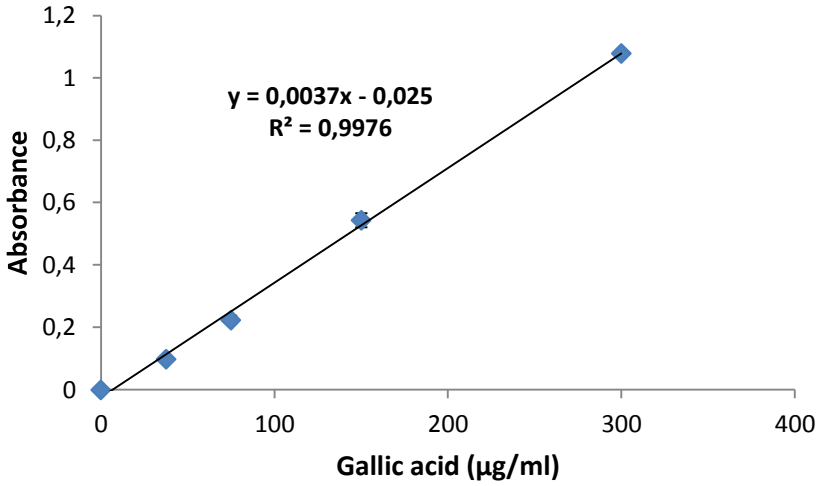


Fig. 16: Gallic acid calibration curve. Each point represents the mean ± SD (n=3).

2.3.2. 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

The antioxidant activity of tested extracts was evaluated through free radical scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. The determination was based on the

method described by Morabito *et* al. (2010) and Spagna *et* al. (1996). 37,5 µl of each extract with different concentrations were added to 1,5 ml of DPPH methanolic solution (100 mM). An equal volume of the solvent employed to dissolve extracts (DMSO) was added to control tubes. Mixtures were incubated at room temperature for 20 min and absorbance was then measured at 517 nm. Each determination was carried out in triplicate.

2.3.3. 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) quenching capacity

The ABTS assay measures the ability of studied extracts to quench ABTS•+ (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radicals. The test was realized according to the method described by Morabito *et* al. (2010). In our experiments, the ABTS·+ radical cation was produced by the oxidation of 1.7 mM ABTS with potassium persulfate (4.3 mM final concentration) in water. The prepared solution was left to stand in the dark at room temperature for 12–16 h before experiment realization, and then diluted with phosphate buffered saline (PBS) at pH 7.4 to give an absorbance of 0.7 ± 0.02 at 734 nm. Two milliliters of this mixture were added to 100 µl of a solution containing different concentrations of *Thymelaea microphylla* extracts to be tested or of the vehicle alone (DMSO). After an incubation period of 6 min in the dark at room temperature, absorbance of samples was measured at 734 nm in a UV-Vis spectrophotometer. Each determination was carried out in triplicate.

2.3.4. Ferric reducing/antioxidant power assay

The FRAP assay was realized according to the method described by Morabito *et* al. (2010) and Cimino *et* al. (2013). This test measures the change in absorbance at 593 nm caused by the formation of a blue colored FeII-tripyridyltriazine (TPTZ) complex when the colorless oxidized FeIII form is reduced by an electron donating antioxidant. Briefly, 50 µl of each extract solution or of the vehicle alone (DMSO), were added to 1 ml of freshly prepared and

pre-warmed (37 ºC) FRAP reagent. The FRAP reagent contained acetate buffer (300 mM, pH 3.6), 10 mM of 2,4,6 tripyridyl-s-triazine (TPTZ) prepared in 40 mM HCl and 20 mM of ferric chloride ($FeCl_3.6H_2O$), in the ratio of (10:1:1). The mixture of extracts and prepared reagent was then incubated at 20 ºC for 4 min before absorbance measuring. All determinations were performed in triplicates.

2.3.5. β-Carotene bleaching assay

The assay was carried out as described by Martorana *et* al. (2013) and Kulisic *et* al. (2004). A stock solution of β-carotene/linoleic acid was initially prepared by dissolving 1 mg of β-carotene in 10 ml of chloroform (HPLC grade). An aliquot of this solution (5 ml) was added to 40 µl of linoleic acid and 400 µl of Tween 40. Chloroform was then evaporated off using a rotary evaporator at 40°C for 5 min, and then 100 ml of distilled water were slowly added to the residue to form an emulsion. Aliquots of β-carotene/linoleic acid emulsion (5 ml) were mixed with 200 µl of a solution containing the extracts to be studied at different concentrations; the same volume of the solvent alone (DMSO) was used in control samples. As soon as the emulsified solution is added to the samples, zero time absorbance was measured at 470 nm against a blank, consisting of an emulsion without β-carotene. Then, the samples were placed in a water bath at 50°C, and the oxidation of the emulsion was monitored by measuring absorbance at 470 nm 120 min after the beginning of the reaction. The percentage of inhibition respect to the control was calculated using the following formula:

$$\% \text{ of inhibition} = ([A_t - C_t]/[C_0 - C_t])/100$$

Where A_t and C_t are the absorbance values measured for the sample to be tested and the control one, respectively, at $t = 120$ min, and C_0 is the absorbance value for the control measured at $t = 0$ min. Each determination was carried out in triplicate.

2.3.6. Scavenging activity against the superoxide anion (Superoxide dismutase assay)

The superoxide anion scavenging activity of *Thymelaea microphylla* extracts was measured using the method described by Tomaino *et* al. (2010) and Tuttolomondo *et* al. (2013), with slight modification. The superoxide anion radicals ($O_2^{\bullet -}$) were generated in 1.5 ml of Tris HCl buffer (16 mM, pH 8), containing 500 µl of Nicotinamide Adenine dinucleotide (NADH, 73 µM), 500 µl of nitroblue tetrazolium (NBT, 50 µM) and 500 µl of Phenazine methosulfate (PMS, 15 µM). The generated anions by means the PMS/NADH system reduce NBT to a purple formazan. Aliquots (20µl) of each extract solution, tested at different concentrations, or the vehicle alone (DMSO), were added to the previous mixture and absorbance was recorded at 560 nm after 2 min of incubation at room temperature. The concentration of each extract needed to inhibit 50% of NBT oxidation was calculated and expressed in mg/ml. Each treatment was at least carried out in triplicate.

2.3.7. Oxygen radical absorbance capacity assay

The Oxygen radical absorbance capacity (ORAC) of studied extracts was evaluated using the method reported by Dávalos *et* al. (2004) with modifications. The ORAC test is based on generation of free radicals using 2,2'- azobis (2-methylpropionamidine) dihydrochloride (AAPH) and measurement of decrease in fluorescence in the presence of free radical scavengers. In this assay, fluorescein (FL) was used as target free radical damage, AAPH as a peroxy radical generator and Trolox as standard control. The reaction was carried out in 75 mM phosphate buffer (pH 7.4), and the final reaction mixture was 2 ml. An aliquot of the solution containing different concentrations of the plant extracts to be tested (200 µl), or a same volume of the vehicle alone (DMSO, and 1.2 ml of FL solution (70 nM, final concentration) were placed in a cuvette. The mixture was pre-incubated for 15 min at 37 °C in the spectrofluorimeter (Shimadzu, RF-5301 PC). Freshly prepared AAPH solution (600 µL; 12 mM, final concentration) was rapidly added. Fluorescence was recorded every minute for

80 min at 37°C; the fluorescence conditions were as follows: excitation at 485 nm, and emission at 520 nm. A blank containing FL and AAPH but only the solvent employed to dissolve the extracts, and different calibration solutions using Trolox (1-7.5 μM, final concentration) as reference antioxidant, were also carried out in each assay. All the reaction mixtures were prepared in triplicate and at least three independent assays were performed for each sample. The area under the curve (AUC) was calculated for each sample by integrating the relative fluorescence curve. The net AUC was calculated by subtracting the AUC of the blank. The final ORAC values were determined by linear regression equation of Trolox concentrations, and expressed as mmol Trolox equivalents/g dried extract.

2.4. Inhibition of advanced glycation end products formation

Thymelaea microphylla extracts were tested for inhibitory effect on in vitro advanced glycation end products (AGEs) formation. The assay was realized as described by Harris *et* al. (2011). Briefly, bovine serum albumin (BSA, 1 mg/ml) was incubated with 100 mM glucose/100 mM fructose, in 100 mM phosphate-buffered saline (PBS, pH 7.4) with DMSO or methanol (negative control), or with different concentrations of tested extracts (0.5 – 10 mg/ml; experimental treatment), or with quercetin (50 – 750 μg/ml; positive control). Reagents were filtered before use and experiment was performed in sterile conditions to avoid contaminations. Control tubes for each treatment were prepared using the same reaction mixture without BSA (to control for analyte auto-fluorescence), and with BSA and vehicle (no sugar, to control for the fluorescence of BSA). Samples were then incubated on a mechanical shaker at 37°C for 7 days.

After this incubation time, fluorescence was recorded by means a spectrofluorometer (Shimadzu, RF-5301 PC) at excitation and emission wavelengths of 355 nm and 460 nm, respectively. Fluorescence readings for control and experimental treatments were blanked

against the BSA and appropriate treatment controls. The percent inhibition of AGEs formation was then calculated as follows:

$$\% \text{ inhibition} = [(F_{negative} - F_{experimental}) / (F_{negative})] \times 100\%$$

Where $F_{negative}$ is the corrected fluorescence for the negative control and $F_{experimental}$ is the corrected fluorescence for the experimental treatment.

Each treatment was repeated at least three times and results were expressed as IC_{50} values which are defined as the amount of each extract (mg/ml) required to reduce AGEs formation by 50 % relative to the negative control.

2.5. Anti-inflammatory potential of plant extracts

2.5.1. Inhibition of heat-induced Albumin denaturation

Denaturation of proteins is a well-documented cause of inflammation. As part of the investigation on anti-inflammatory activity of *Thymelaea microphylla* extracts, we studied their ability to inhibit heat-induced albumin denaturation according to the method described by Leelaprakash and Mohan Dass (2011). The reaction mixture consists of 100 µl of each tested extract or aspirin (used as positive control) with different concentrations, 1 ml of phosphate buffer 0.2M (pH=7.4) and 1 ml of bovine serum albumin solution (1%). The mixture was incubated at 37°C for 20 min and then heated to 51°C for other 20 min. After cooling, the turbidity of the samples was measured by reading the absorbance at 660nm, and then the mean percentage inhibition of protein denaturation was calculated. Each determination was carried out in triplicate.

2.5.2. Release of prostaglandin E_2 and thromboxane B_2 in whole blood

Among prepared extracts from *Thymelaea microphylla*, three extracts (water, ethyl acetate and acetone) were chosen to test their ability to act as *in vitro* selective inhibitors of

Cyclooxygenase-1 (COX-1) or of Cyclooxygenase-2 (COX-2) pathways, by decreasing release of thromboxane B_2 (TXB_2) and prostaglandin E_2 (PGE_2), respectively, in human whole blood. The assay was assessed as previously described by Siracusa *et* al. (2011), and Trombetta *et* al. (2014).

2.5.2.1. Blood samples collection

Peripheral blood samples were drawn from healthy volunteers who had taken no anti-inflammatory drug during the last two weeks before the study. Informed consent was obtained from each subject.

2.5.2.2. Release of PGE_2

To evaluate the effects of *Thymelaea microphylla* on PGE_2 release in whole blood, the plant studied extracts were dissolved in DMSO, and 2 µL of these solutions were put into test tubes with 1 ml of peripheral blood aliquots containing 10 IU of sodium heparin, to give a final concentration in whole blood ranging between 5 and 320 µg/mL. Samples were then incubated in the presence of lypopolysaccharide (LPS; 10 µg/mL) for 24 h at 37°C. The contribution of platelet prostaglandin endoperoxide synthase-1 was suppressed by adding aspirin (10 µg/mL) at time 0. The non-steroid anti-inflammatory drug, nimesulide (a selective COX-2 inhibitor), was used as reference drug. Plasma was then separated by centrifugation (10 min at 1600×g) and kept at −30°C until assayed for the content of PGE_2, which is an index of the COX activity of blood monocyte prostaglandin endoperoxide synthase-2.

2.5.2.3. Release of TXB_2

To evaluate changes in TXB_2 release, 1 mL of whole blood aliquots were immediately transferred into glass tubes containing 2µl of each tested extract with different concentrations or with indomethacin (a non-selective COX inhibitor) used as reference drug. Blood was allowed to clot at 37°C for 1 h. Serum was then separated by centrifugation (10 min at

1600×g) and kept at −30°C until assayed for TXB_2, which is an index of endogenously formed thrombin-stimulated COX activity of platelet prostaglandin endoperoxide synthase-1.

2.5.2.4. Enzyme immunoassay (EIA)

Plasma PGE_2 and serum TXB_2 levels in previous samples were measured by enzyme immunoassay using kits purchased from Cayman chemical, USA. The assay is based on competition between PGE_2 or TXB_2 and PGE_2-acetylcholinestrase (PGE_2 tracer) or TXB_2-acetylcholinesterase (TXB_2 tracer), respectively, for a limited number of target molecules' specific monoclonal antibodies. A substrate of acetylcholinesterase is added and the product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. Results were expressed as percentage decrease in PGE_2 and TXB_2 release with respect to control values and were reported as mean±S.D. of three experiments.

2.5.3. Protective effects on human umbilical vein endothelial cells exposed to tumor necrosis factor-α

The whole experiment was realized as described by Speciale *et* al. (2010, 2013). The capacity of acetone extract to protect human umbilical vein endothelial cells incubated in the presence of tumor necrosis factor-α was evaluated by cell viability assessment using Trypan blue assay.

2.5.3.1. Cell cultures

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly obtained human umbilical cords by collagenase digestion of the interior of the umbilical vein and were cultured in medium 199, supplemented with 20% fetal bovine serum, 1% L-glutamine, 20 mM HEPES, 100 units/mL penicillin/streptomycin, 50 mg/mL endothelial cell growth factor, and 10 μg/mL heparin, in gelatin-pretreated flasks. Cells were maintained in a humidified atmosphere containing 5% CO_2 in an incubator at 37°C. Cells used in this study were from the second to fourth passage.

2.5.3.2. Experiment procedure

The subconfluent cells were treated for 24 h in serum-free medium with various doses of acetone extract (20–40 μg/ml) whereas control cells were treated with 0.1% DMSO only. The final concentration of DMSO in the culture medium during different treatments was <0.1% (v/v). After incubation, cells were washed with PBS under sterile conditions and then incubated for 2 h with serum-free medium containing various doses of recombinant human TNF-α (20 ng/mL). Control cells were not exposed to TNF-α. At the end of the exposure time, cells were immediately processed and/or preserved at − 80°C until analysis as expected for each test. The cytotoxic effect of TNF-α on HUVECs pretreated or not with acetone extract was evaluated using a dye exclusion assay with Trypan blue. Briefly, 10 μL of cell suspension was mixed with 30 μL of Trypan blue isotonic solution (0.4% w/v) and loaded into a hemocytometer for both live and dead cells counting.

2.5.4. RNA isolation and quantitative RT-PCR analysis

Total cellular RNA was isolated from HUVECs according to the TRIzol protocol as described by Speciale *et* al. (2010, 2011). The quality of the RNA was tested in 1% formaldehyde-agarose gel stained with ethidium bromide (EtBr) and spectrophotometrically quantified. After reverse transcription (RT) with oligo (dT)15 primers, Polymerase Chain Reaction (PCR) was performed for identification of E-Selectin mRNA levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene for normalization. Gene expression was assessed by real-time PCR (Applied Biosystem 7300 Real-Time PCR System, Monza, Italy) coupled with the Sybr green JumpStart Taq ReadyMix kit. The specific primers set for the target genes were as follows: GAPDH, forward, 5'-GGC TCT CCA GAA CAT CAT CCC TGC-3', reverse, 5' -GGG TGT CGC TGT TGA AGT CAG AGG-3'; E-selectin, forward, 5'-CTG CCA AGT GGT AAA ATG TTC AAG-3', reverse, 5'-TTG GAC TCA GTG GGA GCT TCA-3'. Cycling conditions were 40 cycles of 94°C denaturation (15 s),

60°C annealing, and extension (1 min). A final dissociation stage was run to generate a melting curve for verification of amplification product specificity. Each sample was assayed at least three times from the same RNA. Data were collected and processed with SDS 1.3.1 software (Applied Biosystems, Monza, Italy) and given as threshold cycle (Ct). The fold increase compared with the control cells not treated and not exposed to TNF-α mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method. Primer efficiencies for the test genes were comparable to those for GAPDH (reference gene).

2.5.5. Leukocyte Adhesion assay

This test was performed as described by Mo *et* al. (2007). To evaluate the capacity of acetone extract to decrease adhesion level of isolated leukocytes to HUVECs, pre-treated endothelial cells with extract were co-cultured with isolated mononuclear cells in the presence of TNF-α, and adhesion levels were estimated.

2.5.5.1. Mononuclear cells and cocultures preparation

Mononuclear cells have been isolated from human whole blood with Histopaque-1077, following the procedure recommended by the manufacturer. Briefly, heparinized venous blood from healthy donors was centrifuged over Histopaque-1077; the mononuclear cell layer was collected, washed twice with Dulbecco's phosphate buffered saline (DPBS), suspended in medium 199, and immediately used. Two flasks with subconfluent HUVECs (prepared as described previously) were treated with various doses of acetone extract (20-40 μg/ml) and were incubated for 24 h at 37°C in a humid 5% CO2 atmosphere incubator. Two controls were treated with the medium containing only 0.1% DMSO. After this pretreatment, cells were washed with DPBS under sterile conditions and then cocultured with leukocytes (3x106 leukocytes/flask) and TNF-α 20 ng/mL for 2 h at 37°C with gentle shaking. Cells not exposed to TNF-α were used as controls. After this incubation time, medium was removed and cells were washed with DPBS. Cocultures were visualized under an inverted microscope and

photographed using a digital camera. Four areas for each flask were selected and used to count the number of adherent leukocytes. Increase in leukocyte adhesion upon stimulation of HUVECs with TNF-α was calculated in relation to the basal adhesion of leukocytes to unstimulated HUVECs, which was set to 1.

2.5.6. Determination of intracellular reduced glutathione (GSH) content

Intracellular GSH content was detected by HPLC (Shimadzu, Milan, Italy) coupled with a fluorescent detector (Hewlett-Packard 1046A) as described by Speciale *et* al. (2011, 2013). HUVECs treated as described above (first experiment) were lysed and centrifuged. The supernatant was then deproteinized with 2.5% 5-sulfosalicylic acid (SSA, w/v) and centrifuged again. Deproteinized supernatant was used directly for o-phthalaldehyde (OPA) derivatization. Quantization was performed using HPLC analysis of the GSH -o-phthalaldehyde (OPA) adducts by comparing derivative peak area to known standards. Results were normalized with protein content determined with the Bradford assay.

2.6. Preliminary toxicity evaluation of *Thymelaea microphylla* extracts

2.6.1. Evaluation of extracts toxicity on peripheral blood mononuclear cells

This assay was carried out as described by Cimino *et* al. (2013, 2014). Peripheral blood mononuclear cells (PBMCs, pool of 5 donors) have been isolated from whole blood with Histopaque-1077 (Sigma-Aldrich), according to the manufacturer's instructions, suspended in RPMI-1640 and immediately used. One milliliter of PBMC suspension ($1x10^6$ cells/mL) was exposed to *Thymelaea microphylla* extracts (dissolved in DMSO) and incubated at 37°C for 24 hours. Cell cultures exposed only to the same volume of the vehicle (DMSO; 0.1% final concentration) were used as controls. Changes in viability of PBMCs exposed to plant extracts were evaluated by means of the trypan blue exclusion assay as described above.

2.6.2. Estimation of aqueous extract toxicity in mice

This test was realized as described by Ntchapda *et* al. (2014). Six groups containing each 5 males and 5 females *Albino* mice were formed. Animals were fasted overnight with free access to water. The control group received distilled water, while the other groups received different doses of aqueous extract (2, 4, 6, 8 and 10 g/kg bw). The extract was administered orally and animals were observed for mortality, different symptoms and changes in behavior for 15 days later.

2.7. Statistical analysis

The Litchfield and Wilcoxon test was used to express the results as mean scavenging concentrations (SC_{50} for DPPH and ABTS assays) or as mean inhibition concentrations (IC_{50} for FRAP assay, SOD assay, BCB assay, AGEs inhibition formation, protein denaturation inhibition and PGE2 and TxB2 release inhibition) and 95% confidence limits (95% C.L.). Statistics were carried out using Pharm PCS-version4 software.

Results obtained in experiments on cell cultures and in PGE_2 and TXB_2 release assays are expressed as means ± SD from three experiments and were statistically analyzed by the ANOVA test, followed by Tukey's HSD, using the statistical software ezANOVA. Differences in groups and treatments were considered significant for $P < 0.05$.

Chapter 3
Results and Discussion

1. Extraction yield

In our study, five solvents of different polarities were used in extraction. The yields of the extractions, expressed as percentage, are resumed in table 4. The highest yield was obtained in aqueous extraction (10.44 %), which was prepared as a decoction by heating plant material in distilled water. It is well known that heating can enhance the solubility of some compounds in the solvent and hence, the extraction yield. The best yield in organic extractions (4.46 %) was obtained using ethanol as extraction solvent, followed by ethyl acetate (1.13 %), hexane (1 %) and acetone (0.95%).

Table 4: Yeild of aqueous and organic extractions from *Thymelaea microphylla* leaves and flowers.

Extract	Yield (%)
Aqueous	10.44
Ethanol	04.46
Acetone	00.95
Ethyl acetate	01.13
Hexane	01.00

Extraction is the crucial first step in the analysis of medicinal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization (Sasidharan *et* al., 2011). The efficacy of medicinal plants extracts is highly depended on the method of extraction, and the porpouse of finding the best extraction method is to enhance the bioavailability and hence, the efficacy of the plant material (Odey *et* al., 2012). Solvent extraction is most frequently used technique for isolation of plant active compounds. However, the extract yields and resulting activities of the plant materials are strongly dependent on the nature of extracting solvent, due to the presence of different compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent. Polar solvents are frequently employed for the recovery of polyphenols

from a plant matrix (Sultana *et* al., 2009). Besides the solvent nature and polarity, many other factors can affect extraction processes, such as matrix properties of the plant part, the ratio solvent to plant material, temperature, pressure and time (Azmir *et* al., 2013).

Preparation of aqueous extracts in a similar way to traditional decoctions helps, in fact, to target the same biomolecules existing in these decoctions and which have already proven their efficacy when used traditionally. Aqueous extracts are known to be rich in polar phenols such as highly polar flavonoids (di, tri, and tetra-glycosylated flavonoids) (Boussoualim, 2014). Ethanol has been extensively used to extract antioxidant compounds from various plants and plant-based foods; it is majorly used to extract tannins, flavonols and alkaloids. Acetone is also a frequently used solvent in plant extractions to prepare flavonoids' rich extracts (Azmir *et* al., 2013). Ethyl acetate can extract the less polar flavonoids like isoflavones, flavanones, methylated flavones, and flavonols (Andersen and Markham, 2006). Hexane can generally extract lipids and highly methoxylated aglycone flavonoids (Boussoualim, 2014).

2. Phytochemical profile of *Thymelaea microphylla* extracts

2.1. Preliminary phytochemical investigation

Phytochemical analyses revealed the presence of tannins and flavonoids in all studied extracts. Ethyl acetate and acetone extracts showed the presence of all tested compounds, except alkaloids which were absent in all extracts. Saponins were identified exclusively in aqueous extract. In addition to tannins and flavonoids, hexane fraction was characterized by the presence of terpenoids and cardiac glycosides, aqueous extract by terpenoids and quinones and ethanol extract by the presence of steroids. The results obtained in the present investigation are summarized in table 5.

Table 5: Phytochemicals investigation in *T.microphylla* aqueous and organic extracts.

	Extract				
Phytochemicals	**Aqueous**	**Ethanol**	**Acetone**	**Ethyl acetate**	**Hexane**
Tannins	+	+	+	+	+
Saponins	+	-	-	-	-
Flavonoids	+	+	+	+	+
Steroids	-	+	+	+	-
Alkaloids	-	-	-	-	-
Terpenoids	+	-	+	+	+
Cardiac glycosides	-	-	+	+	+
Quinones	+	-	+	+	-

+: Presence
-: Absence

The most common compounds usually identified in species belonging to the genus *Thymelaea* are polyphenols, especially flavonoids and phenolic acids, in addition to sterols, lignans and coumarins (Mekhelfi, 2016).

The preliminary screening of each extract was performed using standard methods, based on specific reactions of plant chemical compounds with chemical products. These methods are simple to realize and give a general idea about the different compounds which may be present in each plant fraction. Phenols are the most abundant secondary metabolites in plants; among investigated phytochemicals, flavonoids and tannins belong to phenols kingdom. Quinones are aromatic rings with two ketone substitutions known for their antimicrobial activity. Alkaloids are heterocyclic nitrogen compounds with toxic effect. Terpenoids are modified terpenes, and triterpenoids are precursors for the synthesis of steroids in plants. Saponins are amphipathic glycosides, mostly extracted with water and other polar solvents. Cardiac glycosides are toxic organic compounds in which the aglycone part is a steroid (Tiwari *et* al., 2011).

2.2. Total flavonoids, flavonols and condensed tannins contents in extracts

Aqueous and organic extracts were analyzed for their content in some secondary metabolites. Results indicated that acetone extract contained the highest amounts of both flavonoids

(137.56 µg Cat eq/mg extract) and flavonols (94.13 µg Que eq/mg extract), followed by ethyl acetate extract (74.14 µg Cat eq/mg extract and 76.27 µg Que eq/mg extract, respectively). Hexane fraction had the lowest quantities of flavonoids (31.72 µg Cat eq/mg extract), while aqueous extract showed the lowest content in flavonols (15.87 µg Que eq/mg extract). However, the quantification of tannins in extracts revealed that the highest amounts were found in ethyl acetate and hexane fractions by 29.23 and 25.81 µg Cat eq/mg of dried extract, respectively. Water and acetone extracts contained close amounts of tannins by 14.66 and 11.81 µg Cat eq/mg of dried extract, respectively, while the lowest value was found in ethanol fraction (9.82 µg Cat eq/mg extract) (Table 6).

Table 6: Total flavonoids, flavonols and condensed tannins contents in different extracts from *T. microphylla*.

Extract	Aqueous	Ethanol	Acetone	Ethyl acetate	Hexane
Total flavonoids µg [§]Cat eq/mg	41.05 ± 13.09	60.44 ± 11.11	137.56 ± 12.84	74.14 ± 16.33	31.72 ± 1.07
Total flavonols µg [#]Que eq/mg	15.78 ± 1.13	43.15 ± 2.56	94.13 ± 8.91	76.27 ± 12.06	31.31 ± 4.96
Condensed tannins µg [§]Cat eq/mg	14.66 ± 0.38	9.82 ± 0.09	11.81 ± 0.89	29.23 ± 2.32	25.81 ± 2.61

Results are expressed as mean ± S.D. (n=6).

[§] Cat eq: Catechin equivalents.
[#] Que eq: Quercetin equivalents.

Total flavonoids content in extracts was estimated using a colorimetric method involving the use of Sodium nitrite and catechin as standard. This procedure in the presence of $NaNO_2$ in alkaline medium seems to be specific for rutin, luteolin and catechins, but also phenolic acids exhibit considerable absorbance at 510 nm. This method is based on the nitration of any aromatic ring bearing a catechol group with its three or four positions unsubstituted or not sterically blocked. After addition of $AlCl_3$, a yellow solution of complex is formed, and then turned immediately to red solution after addition of NaOH, which absorb at 510 nm (Pekal

and Pyrzynska, 2014). Total flavonols contents were determined in extracts using Aluminum chloride method and quercetin as standard. This method is selective only for flavonols and flavones, which are the only category of flavonoids able to complex stably with Aluminum chloride (Chang *et* al., 2002; Pekal and Pyrzynska, 2014). The vanillin assay used to determin e condensed tannins contents is based on the colored reaction of vanillin with tannins in the presence of sulfuric acid which play a catalytic role (Sun *et* al., 1998).

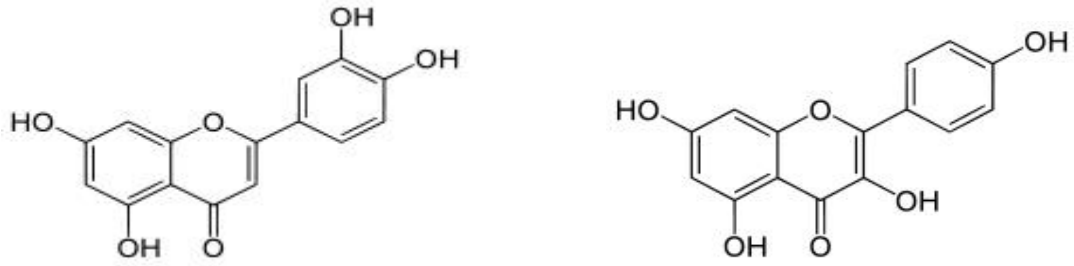
2.3. Extracts analysis using high performance liquid chromatography - diode array detector (HPLC-DAD)

In order to characterize the five extracts obtained from *T. microphylla*, an analyze using high performance liquid chromatography coupled to diode array detector was performed. Six compounds were identified in studied extracts, including four phenolic acids and two flavonoids (Table 7, Fig. 17 and 18). Aqueous extract was characterized by the presence of phenolic acids: gallic acid monohydrate, caffeic acid, p-coumaric acid and ferulic acid. The amount of each compound is shown in table 7 and expressed in microgram of identified compound per milligram of extract. Caffeic acid presented the highest concentration in aqueous extract with 2.81 µg/mg of extract. Gallic and p-coumaric acids were approximately present with the same proportions (2.29 and 2.31 µg/mg extract, respectively), while the concentration of ferulic acid was 1.70 µg/mg extract. In addition to ferulic acid, ethanol extract was characterized by the presence of considerable amounts of luteolin (a flavone, 6.60 µg/mg extract), and the presence of kaempferol (a flavonol), which was identified exactly with the same amount (1.96 µg/mg extract) in ethyl acetate extract. Both flavonoids were also present in acetone extract, while no peaks were identified in hexane fraction.

Table 7: HPLC-DAD identification and quantification of phenolic compounds contained in extracts obtained from *Thymelaea microphylla*.

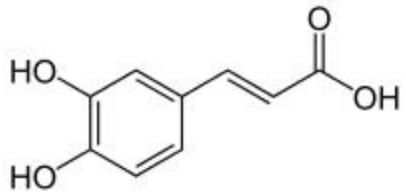
*Rt (min)	Compound	UV/Vis λ (nm)	Quantification (µg/mg extract ± % RSD)				
			Ethanol	Aqueous	Ethyl acetate	Acetone	Hexane
5.88	Gallic acid monohydrate	280	nd	2.29 ± 1.79	nd	nd	nd
15.51	Caffeic acid	280	nd	2.81 ± 0.34	nd	nd	nd
20.74	p-coumaric acid	280	nd	2.31 ± 0.55	nd	nd	nd
23.01	Ferulic acid	280	0.42 ± 3.08	1.70 ± 3.08	nd	nd	nd
39.35	Luteolin	280, 370	6.60 ± 1.48	nd	nd	4.12 ± 1.48	nd
44.16	kaempferol	280, 370	1.96 ± 4.64	nd	1.96 ± 4.64	2.19 ± 4.64	nd

*Rt: Retention time referred to the extract containing the highest amount of corresponding compound. Data are expressed as mean ± relative standard deviation (RSD) (n=3). nd: not detected.

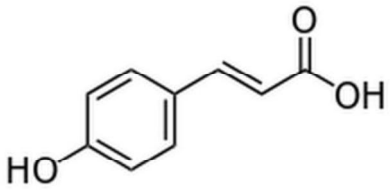


Luteolin Kaempferol

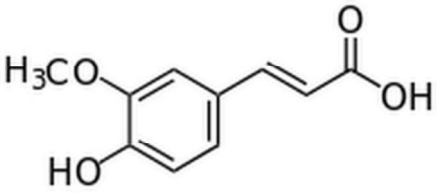
Fig. 17: Structures of flavonoids identified in *Thymelaea microphylla* extracts.



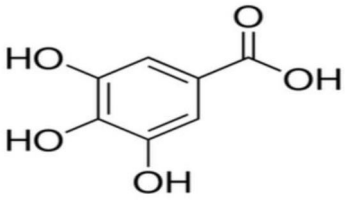
Caffeic acid



p-coumaric acid



Ferulic acid



Gallic acid

Fig. 18: Structures of phenolic acids identified in *Thymelaea microphylla* extracts.

HPLC is a versatile, robust, and widely used technique for the isolation, identification and quantification of natural products. Currently, this technique is gaining popularity among various analytical techniques as the main choice for fingerprinting study for the quality control of herbal plants (Boligon and Athayde, 2014).

Data obtained in the present study are in agreement with those previously reported by other authors (Mekhelfi *et* al., 2014) and (Kerbab *et* al., 2015), who evidenced the presence of flavonoids and phenolic acids in hydroalcoholic extracts from *Thymelaea microphylla* aerial parts. These studies confirmed the presence of luteolin and a derivative compound of kaempferol which is kaempferol 3-O- {3″,6″-di-O-[(E)-p-coumaroyl]-β-D-glucopyranoside in the plant extracts. Among previously identified phenolic acids in this plant, a derivative compound of gallic acid (ethyl gallate) was found, while caffeic, p-coumaric and ferulic acids are identified for the first time in the studied plant in this investigation.

Previous studies have also revealed the presence of monoterpene glucosides, phenylpropanoid glucosides, ionol glucosides, lignans and bis-coumarins in fractions prepared from *Thymelaea microphylla*. Moreover, a new study published in 2017 by Noman *et* al., demonstrated

isolation of a new compound (microphybenzimidazole) in addition of six known compounds (matairesinol, prestegane B, umbelliferone, daphnoretin, microphynolide A and microphynolide B).

3. Antioxidant potent

View that antioxidant activity should not be concluded based on a single antioxidant test model, various methods were used to investigate the antioxidant property of samples prepared from studied plant. The different redox-based assays used are Folin–Ciocalteu assay, bleaching of the stable radical DPPH, ABTS assay, FRAP assay, BCB test, ORAC assay and SOD assay. These methods are based on electron transfer and/or Hydrogen atom transfer.

3.1. Folin test

In this assay, Antioxidant activity was expressed as gallic acid equivalents in µg per mg of dried extract. Aqueous extract showed the best activity (60.448 ± 1.697 µg GaE/mg of extract), followed by acetone extract (47.590 ± 4.554 µg GaE/mg). Ethyl acetate extract (39.389 ± 2.978 µg GaE/mg) and ethanol extract (37.263 ± 1.207 µg GaE/mg) showed a close antioxidant values, while hexane fraction (16.560 ± 0.541 µg GaE/mg) had the lowest activity (Fig. 19).

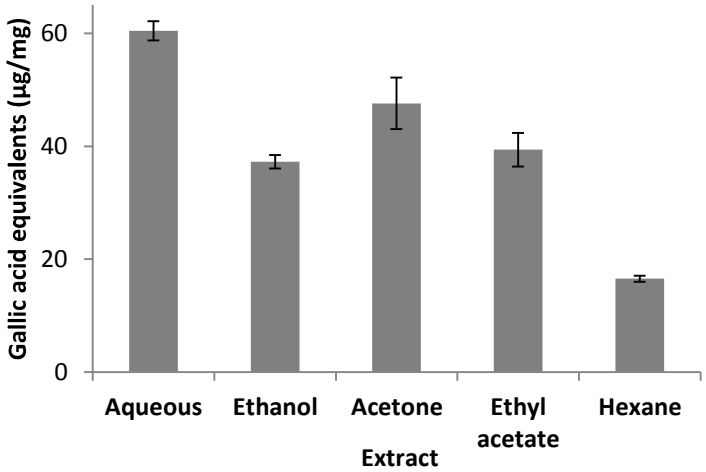


Fig. 19: Antioxidant potency of *T. microphylla* extracts expressed as gallic acid equivalents. Results are expressed as mean ± SD (n=3).

The Folin-Ciocalteau assay is today the most widely used overall antioxidant capacity test for natural products, although improperly referred as a measure of total phenolics content, and thus it gives results easily comparable with others reported in literature. The Folin-Ciocalteu assay relies on the transfer of electrons in alkaline medium from phenolic compounds to phosphomolybdic/phosphotungstic acid complexes to form blue complexes that are determined spectrophotometrically at 750 nm (Dai and Mumper, 2010; Berker *et* al., 2013).

3.2. DPPH radical scavenging activity of extracts

The results in this assay are expressed as scavenging concentration of 50% (SC_{50}) of DPPH radical for each extract (Fig. 20). According to the obtained results, aqueous and acetone extracts had the lowest SC_{50} values, and then the best free radical scavenging activity ($SC_{50\ Aqueous}$ = 3.54 mg/ml, $SC_{50\ Acetone}$ = 5.98 mg/ml). Ethyl acetate and ethanol extracts had a very close SC_{50} values (8.50 mg/ml and 8.67 mg/ml, respectively), while hexane extract showed a weak activity with SC_{50} of 47.97 mg/ml.

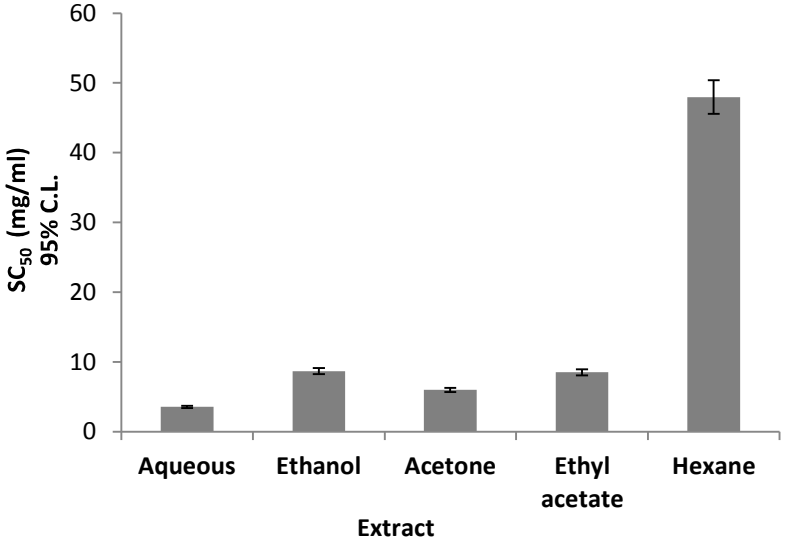


Fig. 20: DPPH radical scavenging activity of *T. microphylla* extracts.

Results are expressed as scavenging concentration of 50% of DPPH radical. Each determination is the mean with 95% C.L. (n=3).

DPPH• (2,2-diphenyl-1- picrylhydrazyl) is a stable free radical, due to the delocalization of the spare electron on the whole molecule. This delocalization determines the occurrence of a purple color, with an absorption band with a maximum around 520 nm (517 nm used in this study). When DPPH• reacts with a hydrogen donor, the reduced form of the radical is generated, accompanied by the disappearance of the violet color. Therefore, the absorbance diminution depends linearly on the antioxidant concentration (Pisoschi and Negulescu, 2011).

3.3. ABTS radical scavenging activity of extracts

This method, uses a diode-array spectrophotometer to measure the loss of color when an antioxidant is added to the blue–green chromophore ABTS·+ (2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid)). The ABTS· + cations are generated by oxidation of ABTS by potassium persulfate, and when antioxidants in samples are added, they reduce it again to ABTS and decolorize it. Antioxidant capacity in this assay is then measured as the ability of test compounds to decrease the color reacting directly with the ABTS·+ radical (Prior *et* al., 2005; Alam *et* al., 2013). Results of test compounds are expressed as concentration of each extract able to reduce 50% of ABTS·+ cations (Fig. 21).

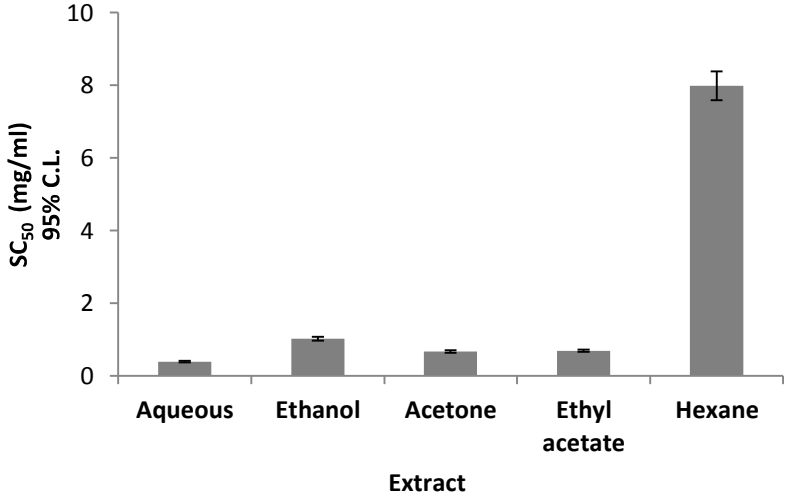


Fig. 21: Mean scavenging concentration (SC_{50}) of *T. microphylla* extracts against ABTS radicals. Results are expressed as mean with 95% C.L. (n=3).

Aqueous extract was found to possess the best activity with the lowest SC_{50} value (0.39 mg/ml). Acetone and ethyl acetate extracts had almost the same potency with SC_{50} of 0.67 mg/ml and 0.69 mg/ml, respectively. Ethanol extract had a higher SC_{50} of about 1.02 mg/ml, and hexane fraction showed the weakest activity with SC_{50} of 7.98 mg/ml.

3.4. Ferric reducing/antioxidant power assay

The ferric reducing/antioxidant power or FRAP assay is one of the methods utilizing single electron transfer reactions mechanism. This method measures the ability of antioxidants to reduce ferric iron (Fe^{3+}). It is based on the reduction of the complex of ferric iron and 2,4,6 tri -pyridyl-s-triazine (TPTZ) to the ferrous form at low pH. This reduction is monitored by measuring the change in absorption at 593 nm, using a diode-array spectrophotometer (Prior *et* al., 2005; Alam *et* al., 2013). Results are expressed as mean inhibitory concentration for each tested extract (Fig. 22).

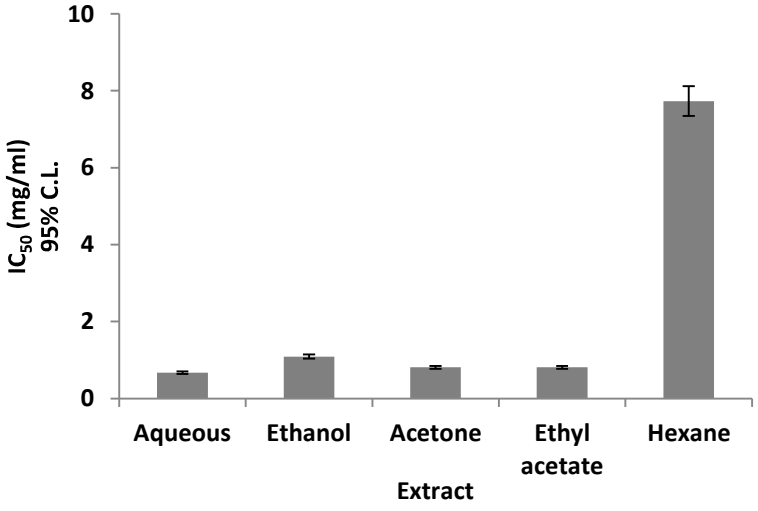


Fig. 22: Mean inhibitory concentrations of *T. microphylla* extracts obtained in FRAP assay. Results are expressed as mean with 95% C.L. (n=3).

In this test, the same potency order was observed where water extract had the best capacity with IC_{50} of 0.67 mg/ml, followed by acetone and ethyl acetate extracts with exactly the same potential (IC_{50}=0.81 mg/ml), then ethanol (IC_{50} = 1.09 mg/ml) and hexane (IC_{50} =7.73 mg/ml)

extracts.

3.5. Antioxidant activity of extracts using β-Carotene bleaching assay

This test is based on proton transfer reactions and on the principle that linoleic acid, which is an unsaturated fatty acid, gets oxidized by reactive Oxygen species produced by oxygenated water. The products formed will initiate the β carotene oxidation, which will lead to discoloration. Antioxidants present in tested extracts decrease the extent of discoloration (Kawsar *et* al., 2014). Results expressed as mean inhibition concentration for each extract are summarized in Fig. 23.

Unlike previous tests, in the BCB assay another potency order was observed. The best activity was shown by acetone extract with IC_{50} of 0.16 mg/ml, followed by hexane extract which showed also a good antioxidant effect with IC_{50} of 0.25 mg/ml. Ethyl acetate, ethanol and aqueous fractions had lower potential in comparison with previous extracts by IC_{50} of 0.43 mg/ml, 0.53 mg/ml and 1.29 mg/ml, respectively.

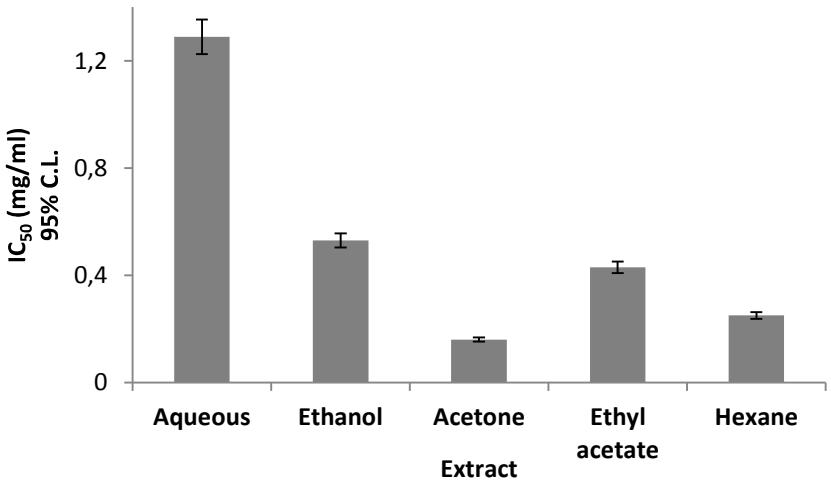


Fig. 23: Mean inhibition concentrations (IC_{50}) of different extracts from leaves and flowers of *T. microphylla* obtained in β-Carotene bleaching assay.

Results are expressed as mean with 95% C.L. (n=3).

3.6. Superoxide anion scavenging activity of extracts

In this assay, the superoxide anion derived from dissolved oxygen from PMS/NADH coupling reaction reduces NBT to a blue colored formazan that can be measured by spectrophotometry. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture (Chanda and Dave, 2009). The capacity of extracts to inhibit the color to 50% is measured in terms of IC_{50} for each extract. Obtained results are shown in Fig. 24.

Acetone and ethyl acetate extracts had the best scavenging activity against the superoxide anion, with very comparable values (IC_{50} =1.81mg/ml and IC_{50} = 1.89 mg/ml, respectively). Hexane fraction showed a good potential in this test also by a mean inhibitory concentration of 3.71 mg/ml, while aqueous and ethanol extracts had a weak activity with high values of IC_{50} (11.54 mg/ml and 7.24 mg/ml, respectively).

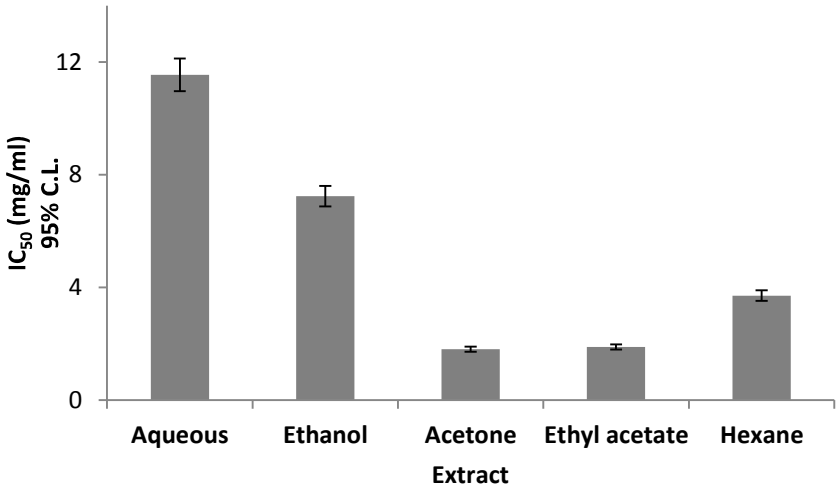


Fig. 24: Mean inhibitory concentrations of *T. microphylla* extracts against superoxide anion. Results are expressed as mean with 95% C.L. (n=3).

3.7. Oxygen radical absorbance capacity assay

The capacity of extracts under study to scavenge peroxyl radicals, generated by spontaneous decomposition of 2,2'- azobis (2-methylpropionamidine) dihydrochloride (AAPH), was

estimated in terms of trolox equivalents, using the ORAC assay. This assay measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects classical radical chain breaking antioxidant activity by Hydrogen atom transfer. It is based on the fact that peroxyl radical reacts with a fluorescent probe (fluorescein) to form a non-fluorescent product, which can be quantitated easily by fluorescence (Prior *et* al., 2005; Chanda and Dave, 2009).

In fact, the $ORAC_{FL}$ (using fluorescein as target free radical damage) is limited to measurement of hydrophilic chain breaking antioxidant capacity against only peroxyl radical; this ignores lipophilic antioxidants which are present in extracts prepared using less polar solvents. This was confirmed in our results (Fig. 25), where antioxidant capacity was proportional to extracts polarity, it was higher for aqueous (1.98 ± 0.07 mmol TE/g dried extract) and ethanol (1.39 ± 0.05 mmol TE/g) extracts, while less polar extracts had lower values (Acetone extract: 1.09 ± 0.01 mmol TE/g, Ethyl acetate extract: 0.84 ±0.04 mmol TE/g and Hexane extract: 0.22 ± 0.03 mmol TE/g).

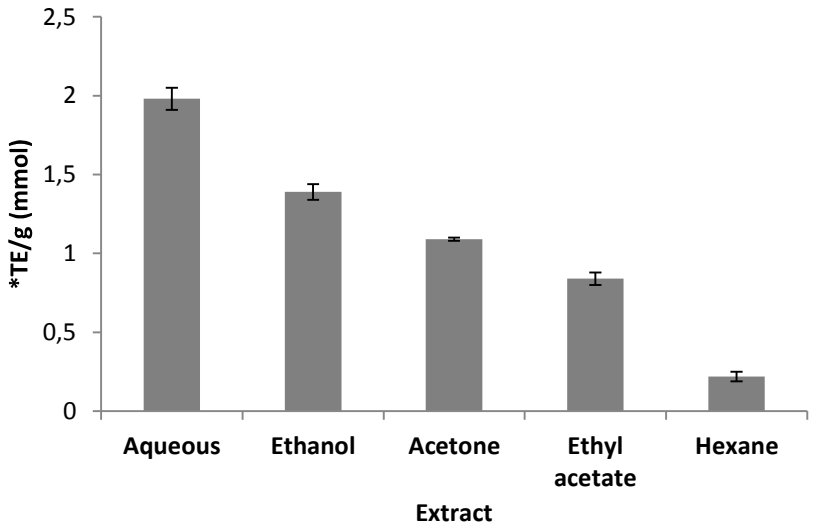


Fig. 25: Antioxidant capacity expressed as Trolox equivalents of extracts from *T. microphylla* as obtained in ORAC assay.

*TE: Trolox equivalents. Results are expressed as mean ± SD (n=9).

In general, these differences in results from an assay to another explain the necessity of using several methods to evaluate antioxidant power of plant extracts and that the use of one dimensional method to evaluate multifaceted antioxidants is not a complete analytical system. Plant extracts may contain different antioxidants which act by several mechanisms: by donating hydrogen to radicals, reducing power, free radical scavenging activity, metal chelating ability, inhibition of β-carotene bleaching and quenching singlet Oxygen. The assays used to evaluate antioxidant potency are differing in the mechanisms involved and the chemical environment used (Badarinath *et* al., 2010).

Concerning different mechanisms involved in assays used in this study, Folin and FRAP assays are a single electron transfer (ET) reaction based assays, DPPH and ABTS tests are classified as ET reactions; However, both of these radicals may also be neutralized by direct reduction via ETs or by radical quenching via Hydrogen atom transfer (HAT); while ORAC is classified as Hydrogen atom transfer reaction based assay.

These antioxidant potency evaluation methods differ also in the nature of chemical environment in which the assay is realized. For Folin, FRAP and ORAC assays, chemical environment is not suitable for measuring lipid-soluble antioxidants; conversely, the strong point of ABTS is that it can be applied for both water- and lipid-soluble antioxidants while DPPH is soluble only in organic solvents.

Unlike all the other assays, the BCB test is performed in a heterogeneous system; in fact, the presence of two phases, one hydrophilic and one hydrophobic might limit the antioxidant power of compounds unable to reach the organic phase (where lipo-peroxidation occurs) and/ or to interact with lipid micelles/biomembranes. The BCB test is a proton-transfer based assay given that the scavenging of the lipo-peroxyl radicals by an antioxidant is thought to be a proton-transfer based reaction, and thus it allows us to evaluate the capacity of a compound to act as a chain-breaking antioxidant in the process of lipid peroxidation, knowing that pro-

oxidant agent used in this assay is heating.

Although superoxide anion is a weak oxidant, it gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress (Chanda and Dave, 2009). The superoxide anion scavenging assay was carried out to investigate the antioxidant power of extracts under study, but also to evaluate the anti-inflammatory capability of these extracts. In fact, the superoxide anion O_2•- is the major reactive oxygen species and plays a pivotal role in inflammation; the enzyme superoxide dismutase (SOD) neutralizes O_2•- by transforming it into hydrogen peroxide, thereby preventing the formation of highly aggressive compounds such as peroxynitrite and hydroxyl radical.

All tested extracts showed a good antioxidant/free radical scavenger activity. In the ET-based assays (Folin, DPPH, FRAP and ABTS assays), the potency order was aqueous > acetone > ethyl acetate > ethanol >> hexane, while in the HAT-based assay (ORAC), the potency order was aqueous > ethanol > acetone > ethyl acetate >> hexane. These findings demonstrate that all the extracts of *T.microphylla* have a similar chemical profile as concerns the presence of antioxidants/radical scavengers acting by both HAT and ET. On the other hand, the acetone and hexane extracts are very likely richer, in comparison with the aqueous, ethanol and ethyl acetate extracts, of antioxidants able to reach the lipophilic phase; in fact, in the BCB assay, the potency order was acetone > hexane > ethyl acetate > ethanol >> aqueous (IC_{50} values ranging between 0.16 and 1.29 mg/ml). Interestingly, further in the SOD mimetic assay, the acetone, ethyl acetate and hexane extracts appeared to be endowed with the highest activity (acetone > ethyl acetate > hexane >> ethanol >> aqueous).

It is evident that antioxidant capacity shown by these extracts is related at least in part to their content in flavonoids and phenolic compounds previously identified and quantified in extracts These compounds are known for their antioxidant activity where they function as reducing

agents and free radical scavengers through the donation of electrons and hydrogen atoms, also can chelate transition metals and inhibit lipid peroxidation (Saxena *et* al., 2012; Banjarnahor and Artanti, 2014; Grzegorczyk-Karolak *et* al., 2016).

Only few studies were previously established concerning antioxidant activity of *T.microphylla* extracts . A previous study realized by Benhammou *et* al. (2009) on antioxidant capacity of methanolic and aqueous extracts from leaves and stems of the plant under study collected from the region of Naâma in Algeria, using DPPH and FRAP assays, showed that leaves extracts had better potency in comparison with those prepared from plant's stems in both assays. Another study published in 2015 by Kerbab *et* al. evaluated the free radical scavenging activity of a hydroalcoholic extract and some pure compounds isolated from the plant using DPPH assay, confirmed also the good antioxidant potency of *T. microphylla* extracts.

4. AGEs formation inhibition by *T. microphylla* extracts

The antiglycation activity of different extracts from *T. microphylla* was investigated by incubating each extract at different concentrations with albumin and sugars (glucose and fructose). Formation of fluorescent AGEs was measured by spectrofluorometry and mean inhibition concentration was calculated for extracts and quercetin which was used as a positive control. Among tested extracts, aqueous extract had the most important activity with IC_{50} of 33.24 µg/ml, which is the closest value to quercetin activity with IC_{50} of 4.34 µg/ml (Fig. 27). Ethyl acetate and acetone extracts had a moderate activity in comparison with the other extracts with IC_{50} values of 0.187 mg/ml and 0.238 mg/ml, respectively, while ethanol and hexane had a weak activity with high IC_{50} values (0.470 mg/ml and 1.131 mg/ml, respectively) (Fig. 26).

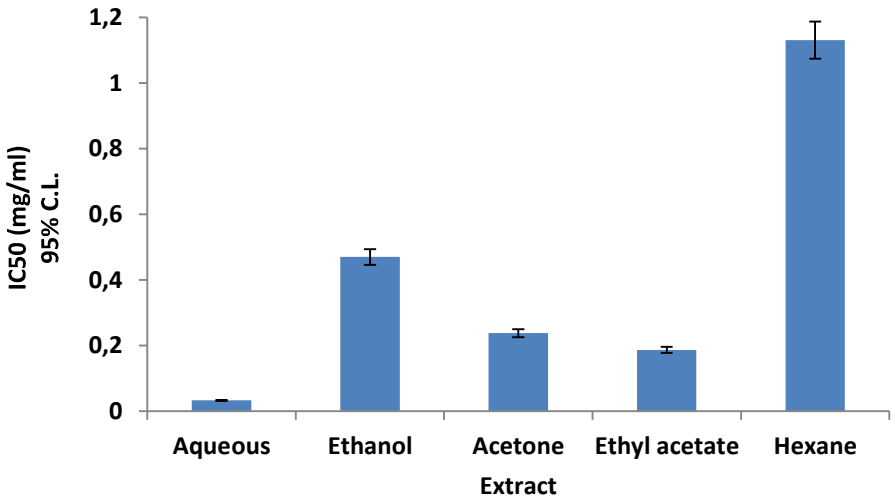


Fig. 26: Inhibition of Advanced Glycation End products formation by *T. microphylla* extracts. Each value represents the mean with 95% C.L. (n=3).

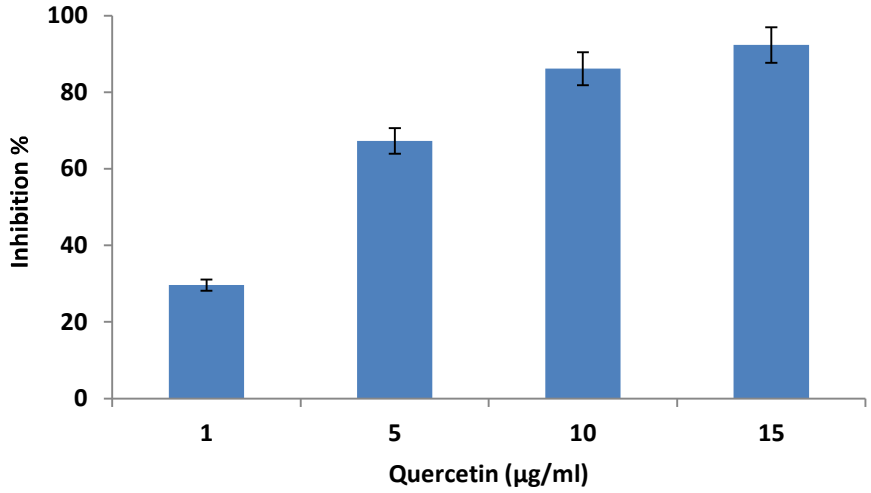


Fig. 27: Antiglycation activity of quercetin used as positive control in AGEs formation inhibition test. Each value represents the mean ± SD (n=3).

Diabetes predisposes to complications affecting various organs such as eyes, blood vessels, nerves and kidneys. Hyperglycemia, the hallmark of diabetes, plays an important role in the onset of complications in diabetic patients by promoting protein glycation and accumulation of advanced glycation end products (AGEs). The AGEs belong to a heterogeneous group of compounds resulting from non-enzymatic reactions (Maillard reaction or glycation) between sugars and amino groups of biological molecules such as proteins, lipids or nucleic acids. A

growing interest is currently focused on compounds that inhibit glycation because of their preventive or therapeutic potential (Guillet, 2010).

Reactive Oxygen species and free radicals have been reported to participate in AGEs formation and in cell damage (Dzib-Guerra *et* al., 2016). Glycation and oxidative stress are then closely linked, and are often referred to as "glycoxidation" processes. In fact, glycation is a major source of the reactive carbonyl and oxygen species generated by oxidative and non-oxidative pathways. Inhibitors of AGE products may act not only as quenchers of dicarbonyl intermediates, but also as antioxidants or metal ion chelators. Therefore, compounds with anti -oxidant activity could also inhibit the formation of AGEs. Antiglycation activities were positively correlated with total phenolic content and antioxidant potency in many studies (Nakagawa *et* al., 2002;Harris *et* al., 2011; Grzegorczyk-Karolak *et* al., 2016). This was the case in our study, where aqueous extract which had the best capacity in Folin, DPPH, FRAP, ABTS and ORAC tests shows also the best antiglycation activity. This extract was also tested in our previous studies for its hypoglycemic effect in rats, where it showed a good activity (Dahamna *et* al., 2015). Finally, natural products with antioxidant and anti-AGEs activity have then a great therapeutic potential in the treatment of diabetes and related complications.

5. Anti-inflammatory activity

Anti-inflammatory potential of extracts from leaves and flowers of *T. microphylla* was evaluated using different ex vivo assays and *in vitro* experiments on cell systems. This activity is evaluated for the first time in our study.

5.1. Albumin denaturation inhibition

In this assay, we tested the ability of extracts from *T. microphylla* to inhibit the denaturation of Albumin caused by heating. Results were expressed as mean inhibitory concentration for each extract. As depicted in Fig. 28, all extracts have shown a dose dependent ability to thermally

induced protein denaturation inhibition. The best inhibition activity was shown by acetone extract with IC_{50} value of 0.15 mg/ml. Ethyl acetate extract had a very close potency to previous extract with IC_{50} of 0.19 mg/ml, while ethanol and hexane showed also a good activity with IC_{50} values of 0.22 mg/ml and 0.24 mg/ml, respectively. Unlike previous extracts, water decoction didn't show a good inhibition activity and had the highest IC_{50} value (1.12 mg/ml). Aspirin, which was used as positive control, had an IC_{50} of 17.69 µg/ml.

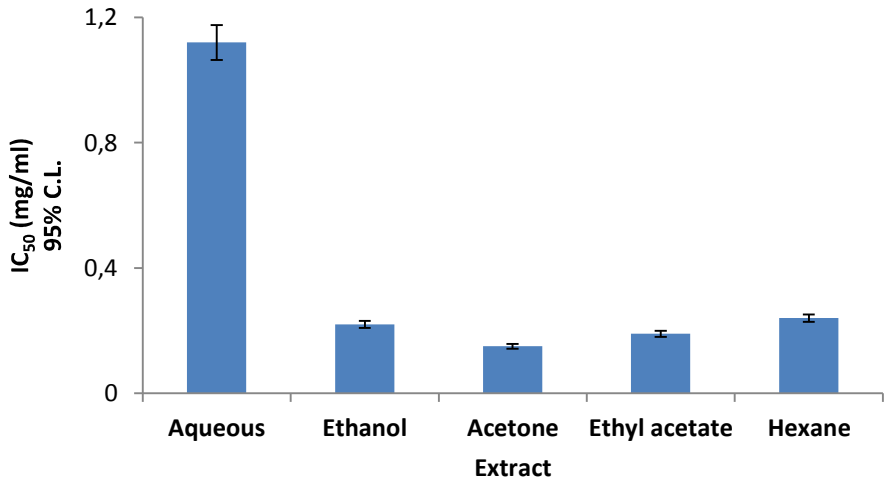


Fig. 28: Mean inhibition concentrations of extracts from *T. microphylla* obtained in heat induced protein denaturation assay.

Each value represents the mean with 95% C.L. (n=3).

Protein denaturation is a process in which proteins lose their tertiary and secondary structures, and consequently their function, by application of external stressors or compounds, such as strong acids or bases, concentrated inorganic salts, organic solvents or heat (Puglia *et* al., 2006). Denaturation of tissue proteins is one of the well-documented causes of inflammatory and arthritic diseases. Production of auto antigens in certain arthritic diseases may be due to denaturation of proteins *in vivo*. Agents that can prevent protein denaturation therefore, would be worthwhile for anti-inflammatory drug development (Chandra *et* al., 2012).

Although aqueous extract didn't seem to be active against protein denaturation, these findings still insufficient to judge its global anti-inflammatory potency, especially that it showed the

best antioxidant activity among all extracts tested; therefore, we chose this extract beside acetone and ethyl acetate fractions, which showed the best activity in albumin denaturation inhibition assay, to be tested in *ex vivo* experiments for the evaluation of anti-inflammatory activity.

5.2. Inhibitory activity on PGE_2 and TXB_2 release

Cyclooxygenase (COX) is an enzyme with two isomer forms, COX-1 and COX-2, which have an important role in inflammation and tumorigenesis. Monocytes and macrophages are the key players in inflammatory responses and are also the major sources of pro-inflammatory cytokines and enzymes including COX enzyme. COX is crucial in prostaglandin synthesis by converting arachidonic acid released from cell membrane, by means phospholipase A_2, into prostaglandin H_2, the precursor of various prostaglandins, prostacyclins and thromboxanes. Constitutive COX-1 is expressed in many tissues and plays a role in tissue homeostasis; its pathway is related to synthesis of thromboxane B_2 (TXB_2). The determination of serum TXB_2 production by platelets following blood coagulation is a specific and most common method for evaluation of COX-1 activity. COX-2 is inducible and the expression of which is stimulated by growth factors, inflammatory cytokines, carcinogens, and tumor promoters, implying a role for COX-2 in both inflammation and control of cell growth. COX-2 over-expression has been found in several types of human cancers and appears to control many cellular processes, thus, compounds that inhibit the activity or expression of COX-2 might be an important target for anti-inflammation or cancer chemoprevention. The COX-2 pathway is related to synthesis of prostaglandin E_2 (PGE_2) (Verma *et* al., 2010 ; Dominguez *et* al., 2010; Siracusa *et* al., 2011; Saadawi *et* al., 2012).

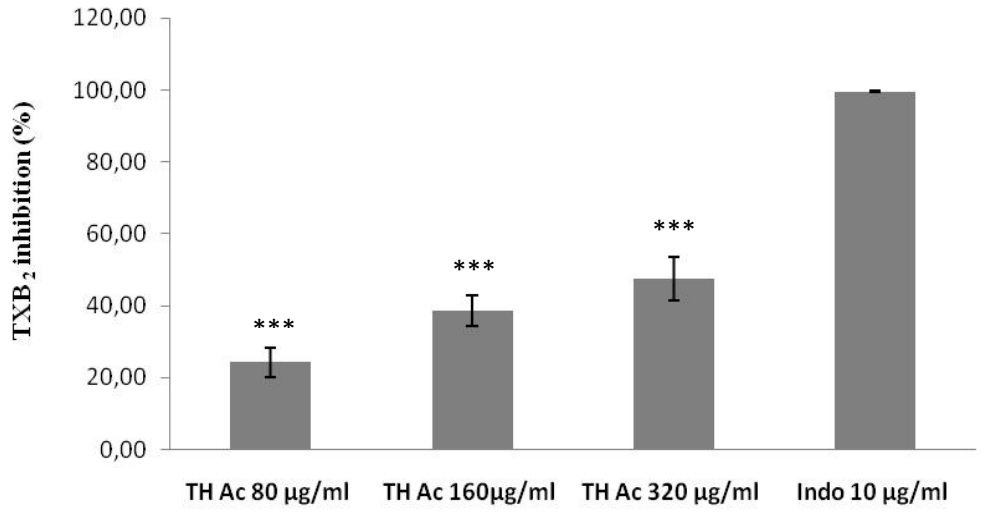
In our experiments, we assessed the ability of acetone, ethyl acetate and aqueous extracts to inhibit release of PGE_2 and TXB_2 in whole blood and then their ability to act as selective

inhibitors of COX-1 and COX-2 pathways. To check probable selective inhibition of COX-1 pathway by extracts, TXB_2 released by platelets was quantified in serum after blood coagulation. To check probable selective inhibition of COX-2 pathway by extracts, LPS which is an endotoxin from gram-negative bacteria was added to heparinized blood. LPS provokes inflammatory and immunological responses, it binds to LPS-binding protein in plasma and then be transferred to a trans-membrane signaling receptor. LPS stimulation of human monocytes activates several intracellular signaling pathways that include the COX-2 pathway (Huang *et* al., 2008). Furthermore, Aspirin (a non-steroidal anti-inflammatory agent) was added to irreversibly inhibit the COX-1 pathway. PGE_2 levels were then estimated by means enzyme immunoassay. Results obtained from both experiments for the three extracts are represented in Fig. 29, 30 and 31.

The *T. microphylla* acetone extract showed a good capability to inhibit release of both TXB_2 and PGE_2. However, even at the highest dose tested (320 µg/ml), the inhibition in TXB_2 release was not more than 50%. Conversely, it appeared more strongly to inhibit PGE_2 allowing us to calculate an IC_{50} of 15.12 µg/ml (95% CL: 12.84 –17.79 µg/ml). Thus, acetone extract could be able to inhibit the COX pathways. However, unlike tests performed on purified enzymes that only show a purely drug–enzyme interaction, it should be considered that other interactions can exist in an *ex vivo* test where the inhibitory potency is measured at a cellular level. In particular, the rate of PGE_2 production is related to COX-2 expression elicited by LPS. Several natural drugs are able to modulate cell signaling pathways and gene expression; thus, the effect of this extract could be due, partially at least, to a down-regulation of COX-2 expression. Similarly, ethyl acetate extract showed a better inhibition activity in PGE_2 release with IC_{50} of 16.94 µg/ml (95% CL: 13.15 – 21.81 µg/ml), while in the TXB_2 release test, it gives an inhibition of only 27.04% for the highest extract dose used (320 µg/ml). Conversely, the aqueous extract appeared unable, under these experimental

conditions, to inhibit TXB_2 release at all doses tested and showed only a weak capability to inhibit PGE_2 release, being the IC_{50} value of 348.45 µg/ml (95% CL: 262.96 – 562.16 µg/ml). Indomethacin and nimesulide used as positive controls in TXB_2 and PGE_2 tests, respectively, had the highest inhibition potency with more than 90% at a dose of 10 µg/ml.

(A)



(B)

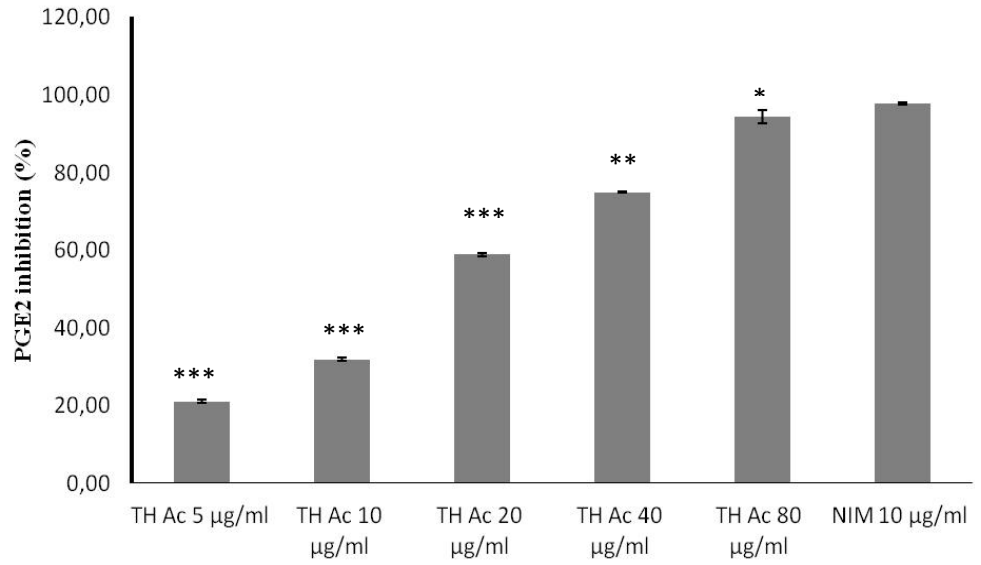
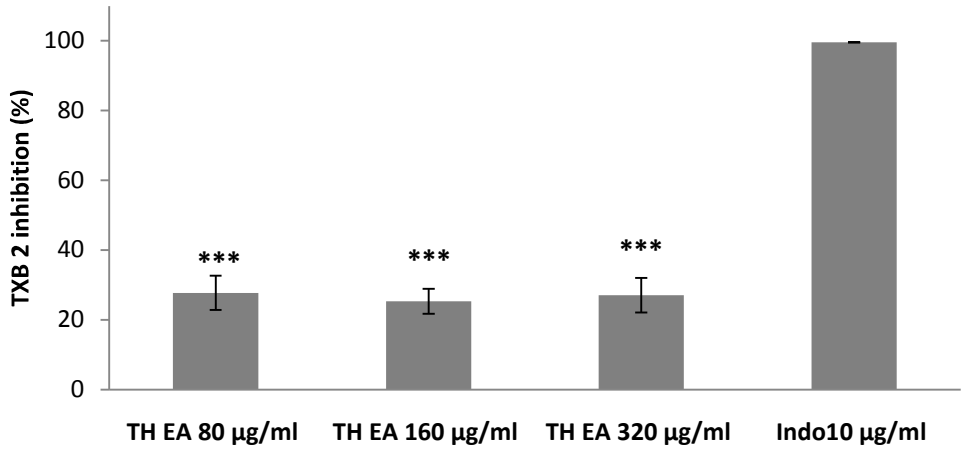


Fig. 29: Effect of *T. microphylla* acetone extract on release of (A) TxB_2 (in comparison with that of indomethacin) and of (B) PGE_2 (in comparison with that of nimesulide) in human whole blood. ($p<0.05$)*, ($p<0.01$)**, ($p<0.001$)***. Data are expressed as mean±SD (n=3).

(A)



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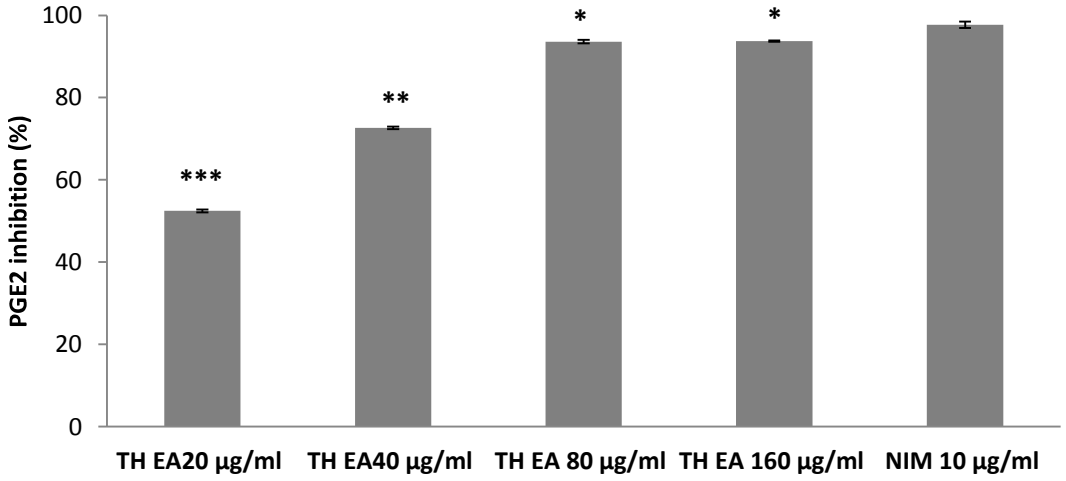
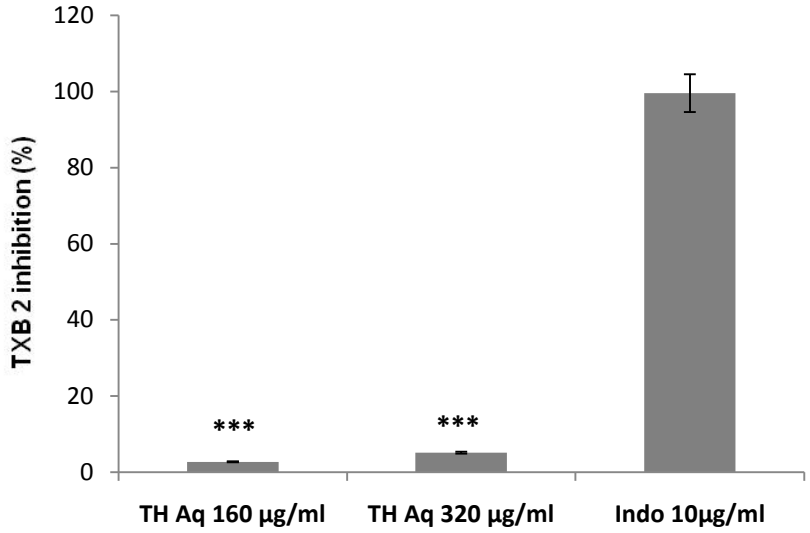


Fig. 30: Effect of *T. microphylla* ethyl acetate extract on release of A) TxB_2 (in comparison with that of indomethacin) and of B) PGE_2 (in comparison with that of nimesulide) in human whole blood. ($p<0.05$)*, ($p<0.01$)**, ($p<0.001$)***. Data are expressed as mean±SD (n=3).

(A)



(B)

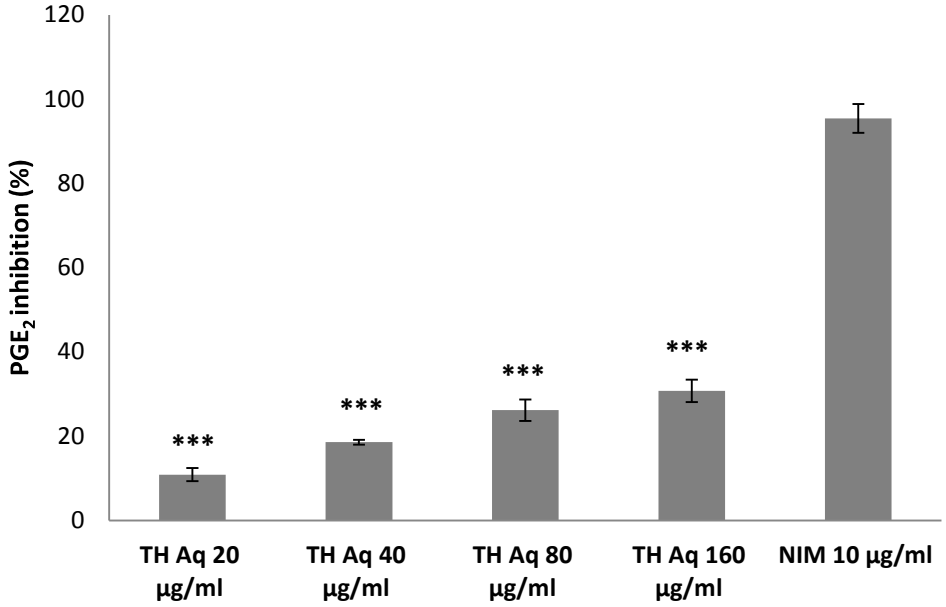


Fig. 31: Effect of *T. microphylla* aqueous extract on release of A) TxB_2 (in comparison with that of indomethacin) and of B) PGE_2 (in comparison with that of nimesulide) in human whole blood. ($p<0.05$)*, ($p<0.01$)**, ($p<0.001$)***. Data are expressed as mean±SD (n=3).

Plant derived compounds and especially flavonoids are well known for their anti-inflammatory properties, and for their ability to inhibit the COX enzyme, as well as other mediators of the inflammatory process (Ribeiro *et* al., 2015), thus, we can conclude that inhibitory activity on COX-1 and especially COX-2 by acetone and ethyl acetate extracts is at least partially due to their content in phenolic compounds and flavonoids. View that acetone extract showed the best anti-inflammatory potential, it was chosen to further activity investigation using *in vitro* cell-systems.

5.3. Acetone extract's protective effect on tumor necrosis factor-α-induced cytotoxicity

In this experiment, we studied the capability of acetone extract to protect human umbilical vein endothelial cells (HUVECs) against cytotoxic effect of TNF-α. HUVECs are a model system widely used to identify the effects of and targets for deleterious vascular risk factors. At this aim, cells were pretreated with the extract (20–40 µg/ml) and then exposed for 2 h to TNF-α (20 ng/ml). Cell viability was assessed using Trypan blue assay; results are presented in Fig. 32.

Our results indicate a clear cytotoxic effect of TNF-α when comparing cells viability values in presence and in absence of this cytokine. Cell pretreatment with acetone extract showed a dose-dependent protective effect against TNF-α-induced cell death. Furthermore, *T. microphylla* extract alone was unable to affect cell viability at the tested doses.

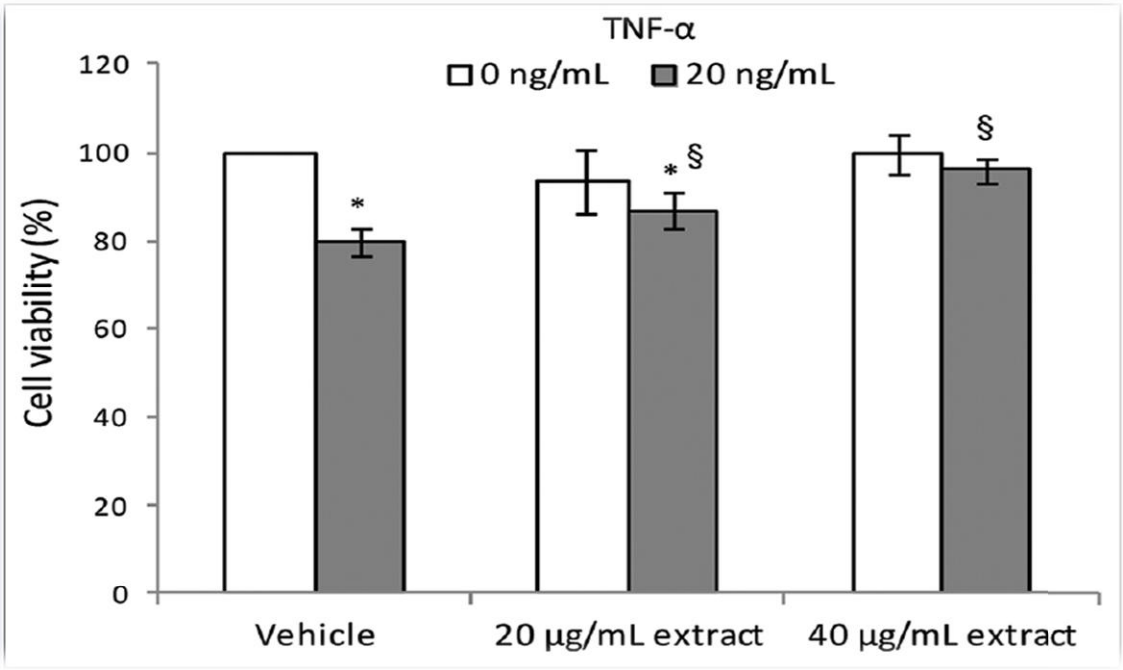


Fig. 32: Cell viability in human umbilical vein endothelial cells pretreated with *Thymelaea microphylla* acetone extract (20–40 µg/ml) for 24 h and then exposed for 2 h to tumor necrosis factor-α (20 ng/ml).

Cultures treated with the vehicle alone (dimethyl sulfoxide 0.1%) were used as controls. Data represent percentage of viable cells (mean percentage) calculated from the number of viable cells in treated samples versus control untreated and unexposed. **P* < 0.05 versus respective tumor necrosis factor-α 0 ng/ml; §*P* <0.05 versus vehicle exposed to tumor necrosis factor-α 20 ng/ml.

Since the *T. microphylla* acetone extract was the only extract showing a good chemical profile, significant antioxidant properties, and excellent anti-inflammatory and COX inhibitory activity, together with lack of toxicity on normal human blood cells (view results of toxicity on peripheral mononuclear cells experiment bellow), only this extract was tested about its protective effect on TNF-α-induced endothelial dysfunction.

The TNF-α is a pro-inflammatory cytokine which plays a pivotal role in various immune and inflammatory processes, including cellular activation, survival and proliferation, as well as cell death by necrosis and apoptosis. It is produced primarily by cells of hematopoietic origin, such as monocytes and macrophages, when stimulated by innate sensors, or by T and B lymphocytes in response to antigenic stimulation. An up-regulation of TNF-α was recorded in the majority of immune-mediated inflammatory diseases, which confirm its important role in causing dysregulations in inflammatory pathways leading to these disorders (Keystone and

Ware, 2010). TNF-α is known for its cytotoxic effect, in fact, many intracellular pathways are set in motion by the binding of TNF to its cell surface receptor (Larrick and Wright, 1990).

The vascular endothelium is a preferential target for therapy in various pathological conditions, including cardiovascular disease, neurodegenerative disease and cancer, all of which are underlying alterations of this tissue type. In endothelial cells, TNF-α cause dysfunction through its ability to promote intracellular ROS formation and activation of the redox-sensitive transcription factor, nuclear factor-kappaB (NF-κB).

5.4. E-selectin expression and leukocytes adhesion

Real-time PCR was used to evaluate E-selectin gene expression in endothelial cells as representative gene involved in cell adhesion. GAPDH, used as reference gene, is one of the most commonly used housekeeping genes used in comparisons of gene expression experiments.

According to the data reported in the literature, cell exposure to TNF-α significantly induced the surface expression of E-selectin, which was confirmed in our results (Fig. 33). TNF-α-induced upregulation of RNA expression of this adhesion molecule was significantly suppressed by pretreatment with acetone extract of the plant under study, with dose-dependent efficacy. This extract per se, without any kind of stimulus, had no effects on the basal expression of E-selectin gene.

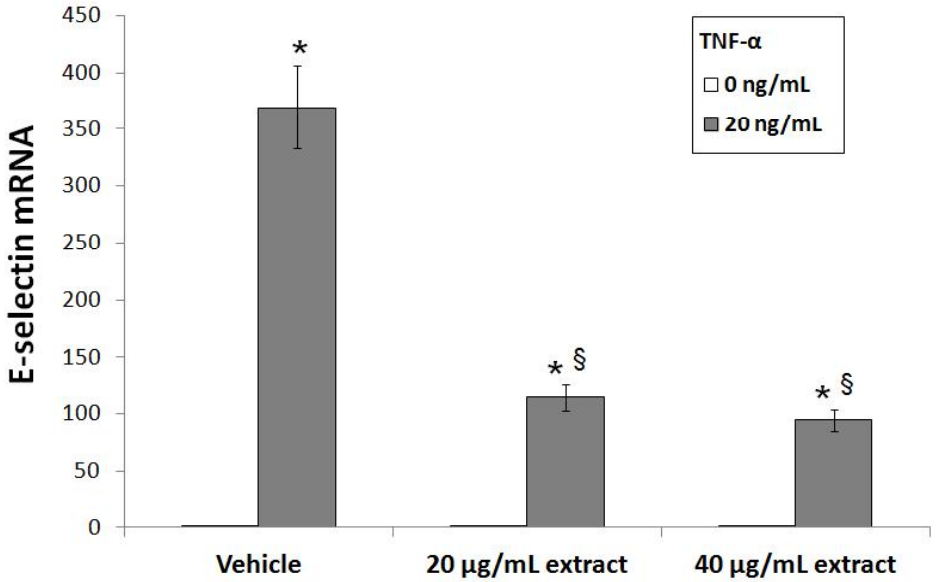


Fig. 33: Effect of *T. microphylla* acetone extract on TNF-α induced mRNA expression of E-selectin adhesion molecule, in HUVECs.

Cells were pretreated with *T. microphylla* extract (20-40 µg/ml) for 24 h and then exposed for 2 h to TNF-α (20 ng/ml). Cultures treated with the vehicle alone (DMSO 0.1%) were used as controls. Values are expressed as $2^{-\Delta\Delta Ct}$ normalized to control and reported as mean±SD (n=3).
*$p < 0.05$ vs respective TNF-α 0 ng/ml
§$p < 0.05$ vs vehicle exposed to TNF-α 20 ng/ml

Many studies have demonstrated that the increased adhesion of circulating monocytes to the injured endothelial layer is a critical early event in the development of atherogenesis. In fact, endothelial cells recruit monocytes by selectively expressing of various cell surface adhesion molecules such as E-selectin. Adhesion molecules are responsible for leukocyte adhesion to vascular endothelium, so promoting their migration into sub-endothelial space (Ferrari *et* al., 2017).

TNF-α is often present in chronic inflammatory diseases, exerting a prominent effect on the expression of pro-inflammatory genes in endothelial cells. This effect takes place predominantly through activation of intracellular signaling pathways involving NF-κB. In endothelial cells, NF-κB is involved in the regulation of the expression of several genes, including those encoding E-selectin and COX-2.

To confirm the inhibitory activity of acetone extract on endothelial activation induced by TNF-α, we investigated adhesion level of isolated leukocytes co-cultured with HUVECs.

Fig. 34 shows that the number of leukocytes adhered to the endothelial cells exposed to TNF-α was higher than that observed in controls, but it appeared to be reduced by pretreatment with the plant extract in a dose-dependent way. This effect on leukocytes adhesion to HUVECs is correlated, at least in part, to the down-regulation of E-selectin gene expression caused by the extract and which was illustrated in the previous experiment. In fact, previous studies demonstrated an association of E-selectin with the insoluble (cytoskeletal) fraction of endothelial monolayers that correlated with adhesion of leukocytes via an E-selectin-dependent mechanism (Yoshida *et* al., 1996). These results confirm the inhibitory activity of acetone extract on TNF-α-induced endothelial activation.

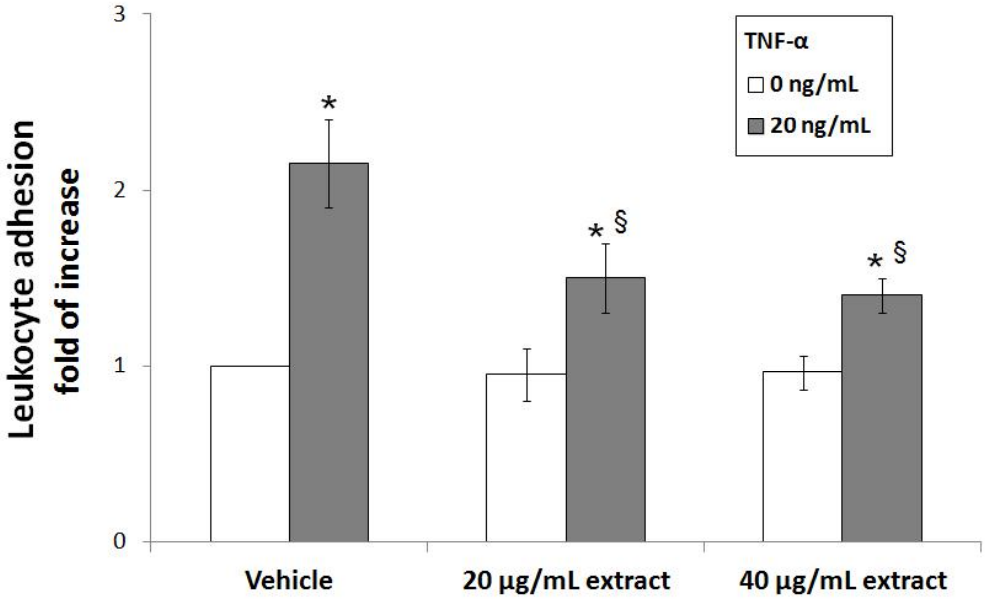


Fig. 34: Leukocyte adhesion in HUVECs pretreated with *T. microphylla* acetone extract (20-40 µg/ml) for 24 h and then exposed to TNF-α (20 ng/ml) for 2 h with gentle shaking. A flask containing the coculture and not exposed to TNF-α was used as control. Increase in leukocyte adhesion upon stimulation of HUVECs with TNF-α was calculated in relation to the basal adhesion of leukocytes to nonstimulated HUVECs (that was set to 1). Data are reported as mean±SD (n=3).
*$p < 0.05$ vs respective TNF-α 20 ng/ml
§$p < 0.05$ vs vehicle exposed to TNF-α 20 ng/ml

5.5. Intracellular GSH content

Increased oxidative stress is believed to be involved in the inflammatory response of endothelial vessels. In fact, ROS may play a role as signaling molecules modulating

inflammation pathways; for example, NF-κB and other transcription factors has been shown to be, at least in part, functionally dependent on cellular redox status (Na and Surh, 2006; Oliveira-Marques *et* al., 2009). It is known that TNF-α is able to promote intracellular ROS formation, which can then activate the redox-sensitive transcription factor NF-κB involved in the expression and release of chemotactic factors and additional cytokines that can each further contribute to inflammation.

On this basis, we studied the involvement of oxidative stress in endothelial alterations following TNF-α exposure and the capacity of acetone extract to modulate TNF-α-induced altered redox state in HUVECs. For this, we measured intracellular content of glutathione (GSH). In fact, cellular thiol redox status is critical for a variety of biological processes including transcriptional activation of various genes and regulation of cell proliferation, inflammation, and apoptosis. Thiols, particularly GSH, are also critical for cellular antioxidant defences, including protecting the cells from oxidant injury and inflammation.

In our present experiments, we found that GSH levels were significantly decreased in HUVECs exposed to TNF-α. As shown in table 8, the pretreatment with acetone extract was able to prevent the depletion in GSH cell content following exposure to TNF-α.

Table 8: Changes in intracellular GSH levels in HUVECs after 24 h of cell pretreatment with *T. microphylla* acetone extract (20-40 µg/ml) and then 2 h of TNF-α exposure.

	Cellular GSH (nmoles/mg prot)	
	TNF-α 0 ng/mL	**TNF-α 20 ng/mL**
Vehicle	42.4 ± 1.6	38.2 ± 1.3°
20 µg/ml extract	41.4 ± 2.1	41.5 ± 1.8*
40 µg/ml extract	43.1 ± 1.7	43.5 ± 2.1*

Cultures treated with the vehicle alone (DMSO 0.1%) were used as controls. Data are expressed as mean ± SD (n=3).
° $p < 0.05$ vs respective TNF-α 0 ng/ml.
*p< 0.05 vs vehicle exposed to TNF-α 20 ng/ml.

These findings contribute to confirm the hypothesis that several plant-derived polyphenols can suppress TNF-α activated inflammatory pathways (Gupta *et* al., 2014). In particular, both luteolin and kaempferol, two flavonoids recovered in this drug, have been previously demonstrated to protect endothelial cells against cytokine-induced proinflammatory status, whether by inhibition of reactive oxygen species, by attenuation of adhesion molecules expression, by their inhibitory activity on COX-2 protein levels or by their effect on NF-κB binding activity (Crespo *et* al., 2008; Jia *et* al., 2015).

6. Toxicity estimation of *T. microphylla* extracts

6.1. Cytotoxicity on blood mononuclear cells

To evaluate the cytotoxic effects of *T. microphylla* extracts, we used human fresh-isolated peripheral blood mononuclear cells (PBMCs) because these cells can be easily collected and cultured and the results obtained may be of great significance as biomarkers of toxic responses to chemicals (Cimino *et* al., 2014). Cell viability of PBMCs exposed to different doses of the extracts (20, 40, 80 μg/ml) was evaluated by the trypan blue exclusion assay.

As reported in Fig. 35, after 24 h of cell exposure to the plant extracts, only ethanolic extract, at all tested doses, and also the aqueous extract, at the highest dose tested, induced a significant cell death in a dose-dependent manner. On the contrary, the other extracts did not affect cell viability.

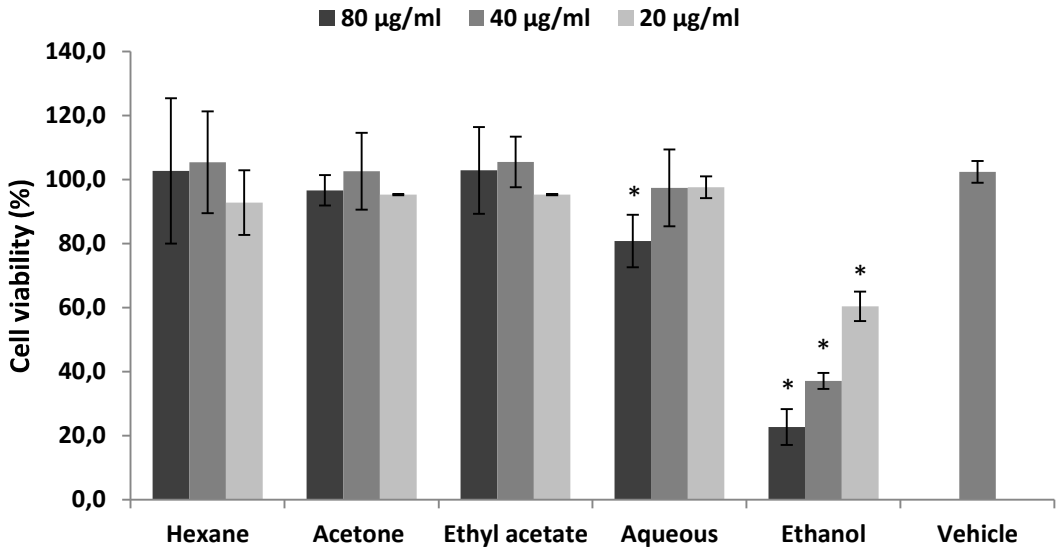


Fig. 35: Cell viability, evaluated in the trypan blue assay, of human PBMCs exposed for 24 h to three doses of *T. microphylla* extracts.

Cultures treated with vehicle alone were used as controls. Data represent percentage of viable cells (mean percentage) calculated from the number of viable cells in treated samples versus control (that was considered as 100%). Each point represents mean±SD (n=3). *$p < 0.05$ vs vehicle treated cells.

6.2. Toxicity of aqueous extract in mice

View the use of *Thymelaea microphylla* decoction orally in traditional therapy, aqueous extract was further investigated for its probable oral toxicity in mice. A single oral dose of extract at different concentrations (2, 4, 6, 8 and 10 g/Kg bw) was administered to *Albino* mice of both sexes. During 15 days of observation, no mortality was recorded in treated mice for all extract doses used, while some toxicity signs like slow motility, nausea and increased heart rate were noticed in mice treated with highest doses (8 and 10 g/Kg bw) in the first 24 h following extract administration.

Although high doses of *Thymelaea microphylla* leaves and flowers water extract did not cause any mortality in mice, the use of this plant should be always handled carefully regarding to the cytotoxic effect of this extract recorded in blood mononuclear cells, and also to toxicity signs observed in mice treated with high doses.

Conclusion & perspectives

This is the first time that leaves and flowers from *Thymelaea microphylla* are identified as a good source of bioactive compounds possessing significant antioxidant and anti-inflammatory properties, potentially effective in prevention and treatment of pathological conditions in which oxidative stress and inflammation play a significant role.

A preliminary phytochemical investigation revealed the rich composition of plant extracts in flavonoids, flavonols and tannins, besides, an analyze using HPLC allowed to identify two flavonoids (luteolin and kaempferol) and four phenolic acids (caffeic, ferulic, p-coumaric and gallic acids) in these extracts.

T. microphylla proved to be a strong antioxidant/free radical scavenging agent when tested in both ET and HAT-based assays; in fact, in Folin, DPPH, FRAP, ABTS and ORAC assays, aqueous and acetone extracts showed the best potential, while in BCB, acetone and hexane extracts were very likely richer, in comparison with the aqueous, ethanol and ethyl acetate extracts, of antioxidants able to reach the lipophilic phase. In the same way, acetone extract was the most potent in SOD mimetic assay.

Aqueous extract showed the highest Antiglycation effect when tested in vitro, in comparison with the other fractions. Besides, extracts could prevent *in vitro* heat-induced protein denaturation, which is a known cause of inflammatory disorders. In our experiments, *T. microphylla* appears able to decrease TXB_2 and PGE_2 release, very likely by an inhibition of COX activity, and further experiments could demonstrate a possible inhibitory effect of studied extracts selectively on the COX-1 and COX-2 pathways. Furthermore, due very likely to the mentioned bio-properties, acetone extract is able to protect against endothelial dysfunction, which is an early event in development of atherosclerosis and vascular diseases,

by protection against tumor necrosis factor-α-induced cytotoxicity, decreasing E-selectin expression and leukocytes adhesion, and preventing depletion in GSH cell content.

Cytotoxic effect of all studied extracts was tested on blood mononuclear cells, where only ethanol and aqueous extracts could cause a significant cell death in a dose-dependent manner. Aqueous extract, view the use of plant decoction in folk medicine, was further investigated for its toxic effect in mice, where no mortality was recorded.

Further studies are warranted to better understand both the chemical composition of this drug and the cellular mechanisms involved in its pharmacological activity.

Annexes

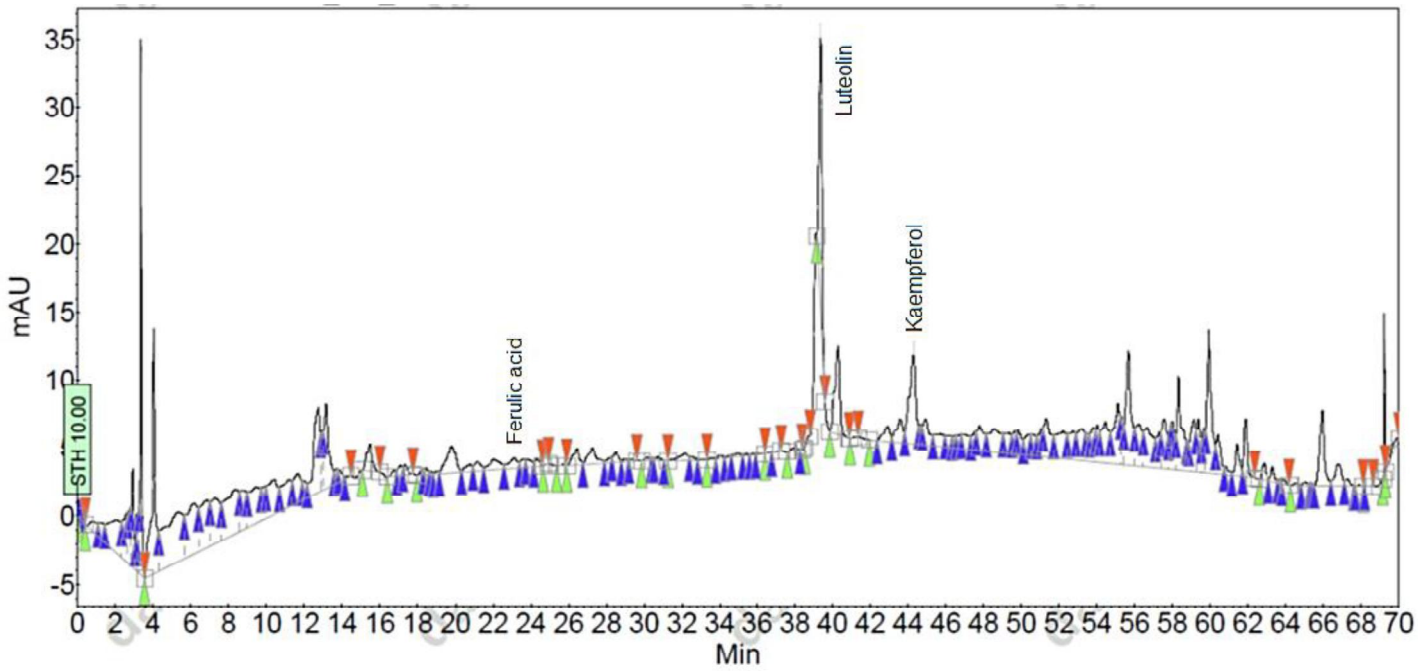


Fig. 36: HPLC chromatogram of ethanol extract.

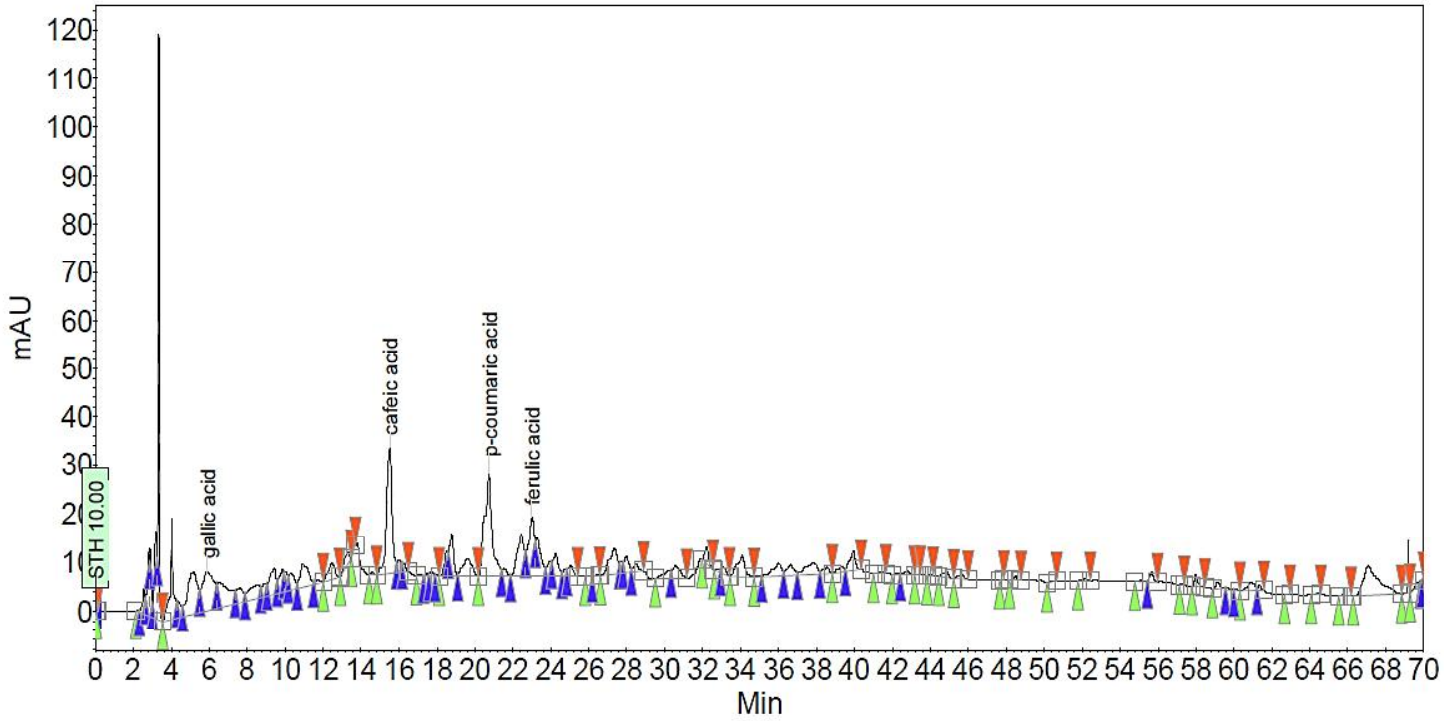


Fig. 37: HPLC chromatogram of aqueous extract.

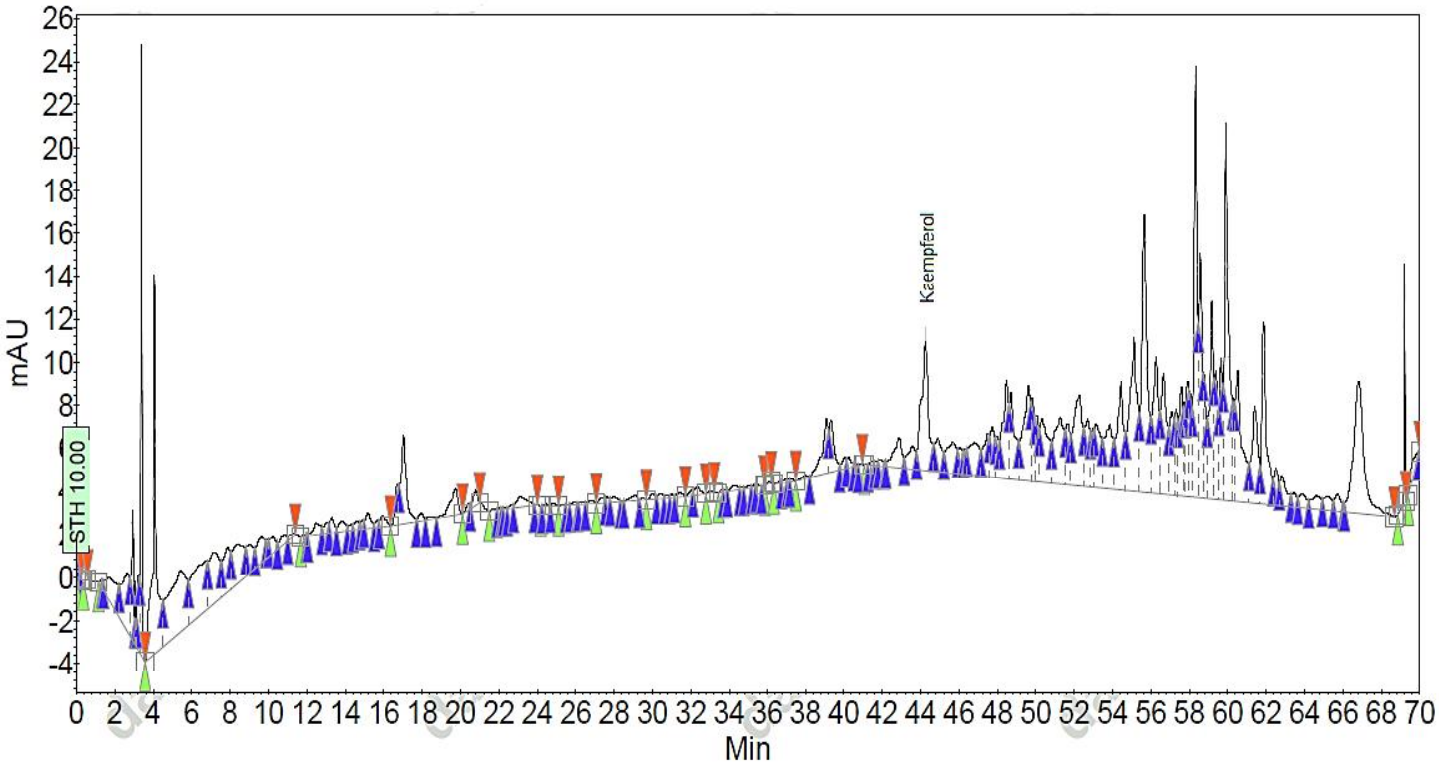


Fig. 38: HPLC chromatogram of ethyl acetate extract.

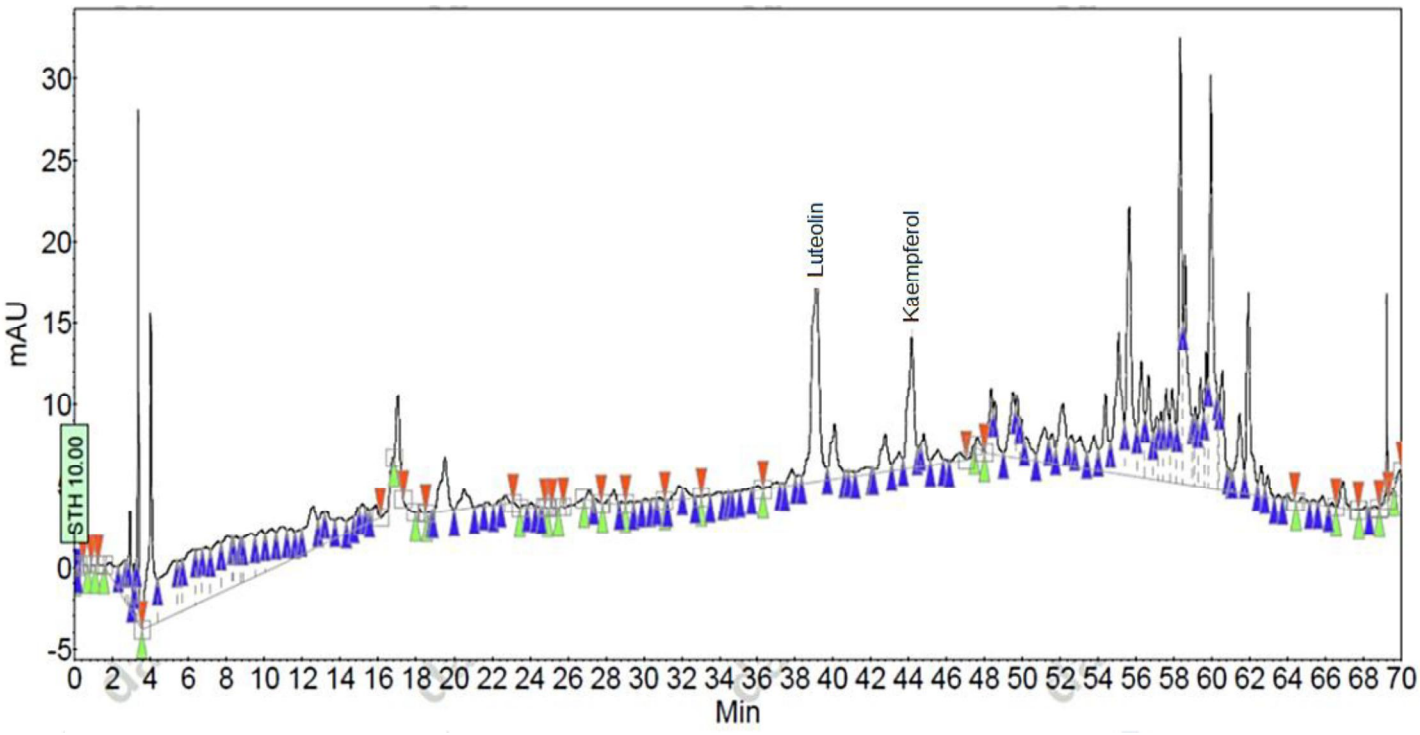


Fig. 39: HPLC chromatogram of acetone extract.

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ملخـص

Thymelaea microphylla Coss. and Dur. (المثنان) هو نبات طبي ذو طبيعة صحراوية، ينتمي إلى جنس*Thymelaea* المنتشر في حوض البحر الأبيض المتوسط. يستعمل هذا النبات في الطب التقليدي وذلك لخصائصه المضادة للسرطان، الالتهاب والسكري. تم في هذه الدراسة تقييم كل من النشاطية المضادة للأكسدة والمضادة للالتهاب لخمس مستخلصات حضرت من أوراق وأزهار نبات *T. microphylla* الذي جني بمنطقة ذراع الحاجة بولاية المسيلة بالجزائر، باستعمال عدة مذيبات عضوية ذات قطبية مختلفة. بالإضافة إلى ذلك، تم إجراء دراسات أولية حول التركيب الكيميائي واختبار سمية هذا النبات. تم بداية تقدير محتوى بعض المستقلبات الثانوية في المستخلصات، حيث وجد أن مستخلص الأسيتون يحتوي على أكبر كمية من الفلافونويدات (137.56 ميكروغ مكافئ الكاتشين/غ مستخلص) والفلافونولات (94.13 ميكروغ مكافئ الكرستين/غ مستخلص)، أما مستخلص أسيتات الإيثيل فقد كان الأغنى بالدباغ (29.23 ميكروغ مكافئ الكاتشين/غ مستخلص). أظهر تحليل المستخلصات بواسطة HPLC احتواءها على الأحماض الفينولية (حمض الغاليك، حمض الكافييك، حمض الفيروليك وحمض الكوماريك) خصوصا في المستخلص المائي، وعلى الفلافونويدات (لوتيولين وكامبفيرول) في المستخلصات العضوية. بالإضافة إلى ذلك، بين تقدير النشاطية المضادة للأكسدة/المزيحة للجذور الحرة والذي تم بواسطة عدة طرق *in vitro*، أن المستخلص المائي ومستخلص الأسيتون أظهرا الفعالية الأكبر باستعمال تقنية Folin، DPPH، FRAP، ABTS و ORAC، بينما في اختبار BCB، أظهرت النتائج أن مستخلص الأسيتون ومستخلص الهكسان قد يكونان الأغنى بمضادات الأكسدة القابلة للذوبان في الأوساط الدسمة. أما في اختبار SOD، فكذلك أظهر مستخلص الأسيتون النشاطية الأعلى (IC_{50}=1.81 مغ/مل). كما تم اختبار قدرة المستخلصات على تثبيط تكون AGEs ، *in vitro*، حيث كان المستخلص المائي الأكثر فعالية (IC_{50}=33.24 ميكروغ/مل). بينت نتائج تقدير النشاطية المضادة للالتهاب، والتي تمت بداية باستعمال اختبار التمسخ الحراري للألبومين، أن مستخلص الأسيتون كان الأكثر فعالية (IC_{50}=0.15 مغ/مل)، كما تم اختباره لاحقا باستعمال طرق *ex vivo* لتقدير نشاطه التثبيطي على انتاج E_2 prostaglandin و B_2 thromboxane في دم الإنسان، حيث أظهر هذا المستخلص نشاطية عالية مضادة للالتهاب ومثبطة لإنزيم cyclooxygenase، في حين لم يظهر أي سمية على خلايا دم الإنسان؛ بالإضافة إلى ذلك، أظهر هذا المستخلص قدرته على حماية خلايا البطانة الغشائية من الخلل الوظيفي الذي يسببه TNF-α، وهذا ما أثبته الانخفاض في مستويات كل من الموت الخلوي، انتاج E-selectin، التصاق كريات الدم البيضاء والحفاظ على مستوى GSH داخل الخلية. علاوة على ذلك، أظهر كل من مستخلص الإيثانول والمستخلص المائي سمية دالة إحصائيا على خلايا الدم أحادية النواة، بينما لم يسبب المستخلص المائي أي أعراض واضحة للسمية على الفئران ولم تسجل أي وفاة. هذه الدراسة التجريبية تدعم استخدام هذا النبات للعلاج الطبيعي والوقاية من الأمراض المرتبطة بالإجهاد التأكسدي والالتهاب.

الكلمات المفتاحية: Cyclooxygenase-2، الفلافونويدات، الالتهاب، الإجهاد التأكسدي، القدرة الإزاحية للجذور، *Thymelaea microphylla*، عامل نخر الورم ألفا.

Abstract

Thymelaea microphylla Coss. and Dur. (Methnane) is a medicinal plant with Saharan affinity, belonging to the Mediterranean genus *Thymelaea*. Leaves decoction is used in traditional medicine for anticancer, anti-inflammatory, and anti-diabetic properties. Herein, the antioxidant and anti-inflammatory activities of five extracts prepared using solvents with different polarities from leaves and flowers of Algerian *T. microphylla*, collected from the region of Draa El-Hadjja in M'sila, were evaluated. In addition, preliminary studies on phytochemical composition and toxicity estimation were realized. Extracts were first characterized for their content in some secondary metabolites, where acetone extract contained the highest amounts of flavonoids (137.56 µg Cat eq/mg) and flavonols (94.13 µg Que eq/mg), and ethyl acetate extract was the richest in tannins (29.23 µg Cat eq/mg). HPLC analysis revealed the presence of phenolic acids (gallic, caffeic, ferulic and p-coumaric acids) especially in aqueous extract, and flavonoids (luteolin and kaempferol) in organic extracts. Furthermore, antioxidant/free radical scavenging activity which was carried out by *in vitro* cell-free assays, showed that aqueous and acetone extracts exhibited the best potential in Folin, DPPH , FRAP, ABTS and ORAC assays, while in BCB, acetone and hexane extracts were very likely richer in antioxidants able to reach the lipophilic phase. In the same way, acetone extract was the most potent in SOD mimetic assay; with IC_{50} of 1.81mg/ml. Extracts were also tested for their inhibitory activity on *in vitro* AGEs formation, where aqueous extract was the most potent (IC_{50} = 33.24 µg/ml). The anti-inflammatory activity, which was first evaluated using albumin heat-induced denaturation assay, revealed that acetone extract was the most active with IC_{50} of 0.15 mg/ml, and it was further tested in *ex vivo* experiments, to estimate its inhibitory potential on prostaglandin E_2 and thromboxane B_2 release in human whole blood, where it showed excellent anti-inflammatory and cyclooxygenase-inhibitory activity, together with lack of toxicity on normal human blood cells; furthermore, it was able to protect human endothelial cells against dysfunction induced by TNF-α, as shown by decrease in cell death, E-selectin expression, leukocyte adhesion and prevention of depletion in GSH cell content. Moreover, ethanol and aqueous extracts had a significant cytotoxicity on peripheral blood mononuclear cells; however, aqueous extract did not cause mortality or any serious toxicity signs when tested *in vivo*. This experimental study support the use of this plant in phytotherapy and prevention of diseases related to oxidative stress and inflammation.

Keywords: Cyclooxygenase-2, flavonoids, inflammation, oxidative stress, radical scavenger activity, *Thymelaea microphylla*, tumor necrosis factor-α.