

Phytochemistry and biological activities of selected Lebanese plant species (Crataegus azarolus L. and Ephedra campylopoda)

Hany Kallassy

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Phytochemistry and biological activities of selected Lebanese plant species (Crataegus azarolus L. and Ephedra campylopoda)

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ABSTRACT

Plants have long been known for their natural arsenal, serving as an important source of nutrients and therapeutic components. Since about 600,000 years ago, humans used plants as medicines. Plant medicines correspond to the preparations issued from those plants. Today, plant medicines are widely worldwide where about 80% of the world's population uses herbs as primary medicines. This medicinal value is mainly attributed to the fact that plants are rich in bioactive phytochemicals. Lebanon, due to its geographical location and important environmental characteristics, is endowed with a rich flora. Hundreds of Lebanese plants have been defined in terms of their chemical composition and medicinal value where many other species are yet to be characterized.

In this study, we aimed at characterizing the phytochemical content and therapeutic value of two Lebanese plant species, *Crataegus azarolus L* and *Ephedra campylopoda*. Fresh leaves, derived from each plant species, were dissolved in three different solvents distilled water, ethanol, and methanol. The phytochemical composition of different extracts issued from the two plant species was examined using high performance liquid chromatography (HPLC) and the essential oil content was determined by gas chromatography (GC) coupled with mass spectrometry (MS). DPPH radical scavenging and Fe^{2+} chelating activity assays were used to assess the antioxidant potential. Anti-inflammatory potential was evaluated by measuring the secreted amounts of the pro-inflammatory mediator PGE_2 using ELISA technique, as well as by assaying the mRNA levels of the pro-inflammatory cytokines (IL-α, IL-β and IL-6), chemokines (CCL3 and CCL4) and inflammation-sensitive COX-2 and iNOS using quantitative RT-PCR (qRT-PCR). XTT viability assay was carried out to determine the anti-proliferative effect of each extract. For both plant species, we observed an important phytochemical content with the alcoholic (methanol and ethanol) extracts being more rich in bioactive molecules. In parallel, the two plant species exhibited significant biological activities with the alcoholic extracts exerting important, *in vitro*, antioxidant, anti-inflammatory and anti-proliferative effects.

Collectively, our observations suggest a promising potential for *Crataegus azarolus L* and *Ephedra campylopoda* during treatment of diseases associated with oxidative stress, aberrant inflammatory responses or uncontrolled cell proliferation. However, further *in vivo* characterization of these effects is indispensable.

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List of abbreviations

CAM	Complementary and alternative medecine
COX	Cyclooxygenase
DNA	Deoxyribo-nucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
GSH	Glutathione
HDL	High density lipoprotein
IFN-ϒ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	C-Jun-terminal kinase
LDL	Low density lipoprotein
LOX	Lipoxygenase
LPS	Lipopolysaccharides
MAPK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NANC	non adrenergic non cholinergic
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAIDs	Nonsteroidal anti-inflammatory drugs
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factors
PG	Prostaglandin
PRX	Peroxiredoxines
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
TCM	Traditional chinese medecine
TLR	Toll like receptor
TNF-α	Tumor necrosis factor α
TRX	Thioredoxins
UV	Ultra violet
VEGF	Vascular endothelial growth factor
WHO	World health organization

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Introduction

1.1 Overview about traditional medicine

Thousands of years ago, herbs and plant products were used in folk medicine for treating a broad spectrum of ailments and diseases. Folk remedies are usually prepared as powders, poultices, ointments, baths, decoctions, infusions and teas. The interest in examining the biological activities of traditional medicinal plants as well as isolating their bioactive components for therapeutic applications has been increasing worldwide and comprehensive screening programs have been established [1]. Floristic analysis showed that there are about 500,000 plant species on our planet. Out of these, about 120,000 plant species can be used to create biologically active products, which, in turn, might be used in disease treatment [2]. Today, a great number of different medicinal teas and other plant products are available on market including cosmetics and pharmaceuticals, which contain biologically active substances [3]. Recently, some products of plant origin were shown to be effective sources of chemotherapeutic agents showing strong biological activity without exerting undesirable side effects. This drew the attention of many scientists and encouraged them to screen the constituents of plants and study their biological activity and pharmacological benefits [4]. New systematic methods for separation, identification and determination of chemical constituents have developed, in parallel to different biological activity tests. Most of the new phytochemical studies follow similar methodologies including plant collection, chemical components extraction, biological tests, chemical analysis and statistical analysis [5].

1.2 Phytochemicals in plants

Plants have evolved secondary biochemical pathways for the synthesis of chemicals in response to environmental stimuli such as herbivore, pathogen deterrence and nutrient shortage. These secondary metabolites, which are called phytochemicals, do not interfere with primary metabolism, have relatively low or nontoxic nature and play a significant therapeutic importance such as to reduce or prevent some types of cancer and inhibit the development and spread of inflammatory diseases [6]. Those chemical compounds include flavonoids and quinones.

1.2.1 Phenolic compounds – Flavonoids

Phenolic compounds are widely distributed in the plant kingdom with over 10,000 types identified so far. These compounds share at least one aromatic hydrocarbon ring

along with one or more hydroxyl groups in their structure (Table 1). The three most abundant phenolic compounds are phenolic acids, flavonoids and polyphenols.

Table 1: The major classes of phenolic compounds in plants

S.N.	Number of carbon atom	Basic skeleton	Class
1.	6	C6	Simple phenols benzoquinones
2.	7	C6-C1	Phenolic acids
3.	8	C6-C2	Acetophenones tyrosine derivatives
4.	9	C6-C3	Hydroxycinnamic acid, coumarins
5.	10	C6-C4	Naphthoquinones
6.	13	C6- C1-C6	Xanthones
7.	14	C6- C2-C6	Stilbenes
8.	15	C6- C3-C6	Flavonoids
9.	18	(C6- C3)2	Lignans
10.	30	(C6- C3-C6) 2	Bioflavonoids
11.	N	(C6- C3-C6)n	Condensed tannins

For instance, phenolic acids are the esters of polyols such as glucose. Caffeic acid is a phenolic acid compound normally found in esterified forms such as chlorogenic acid in coffee and vegetables (Figure 1). Tannins are derivatives of phenolic acids [7], since they are a heterogeneous group of high molecular weight polyphenolic compounds with the

capacity to form reversible and irreversible complexes with proteins (mainly), polysaccharides (cellulose, hemicellulose, pectin, etc.), alkaloids, nucleic acids and minerals. On the basis of their structural characteristics it is therefore possible to divide the tannins into four major groups: gallotannins, ellagitannins, complex tannins, and condensed tannins (Figure 2). Tannins are commonly found in fruits such as grapes, persimmon, blueberry, tea, and chocolate.

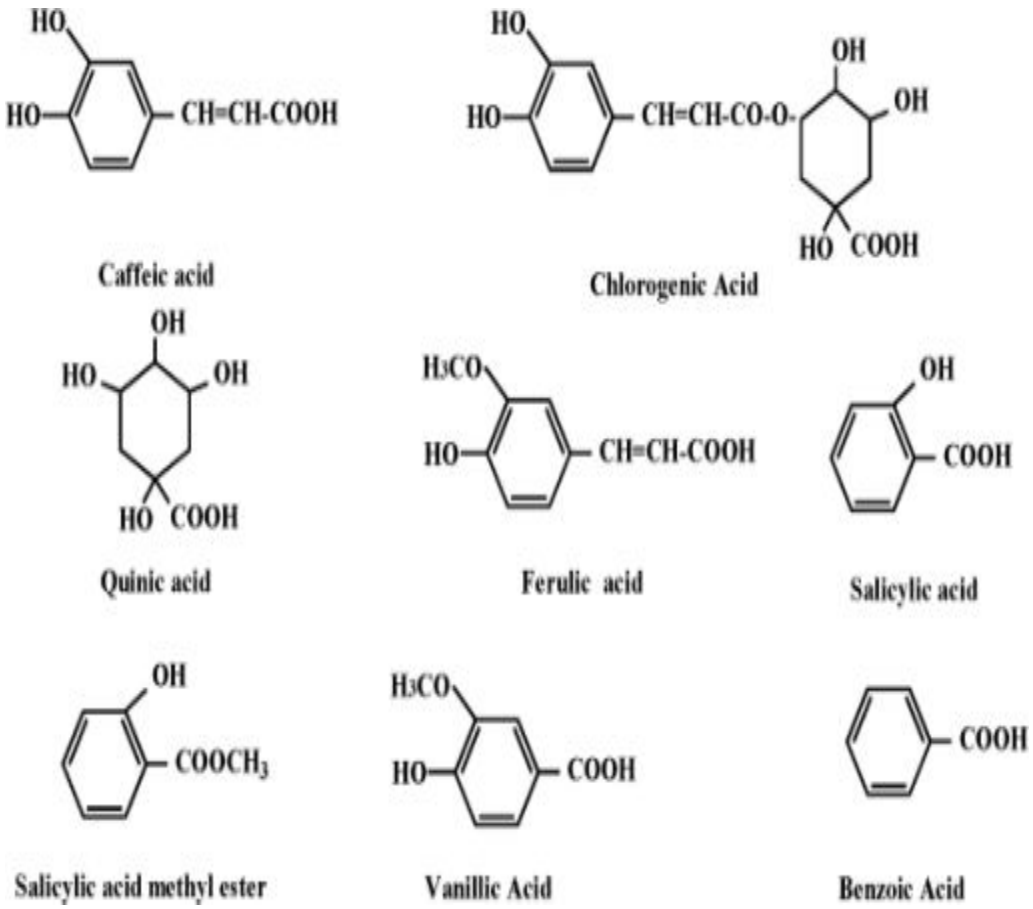


Figure 1: Structures of the important naturally occurring phenolic acids

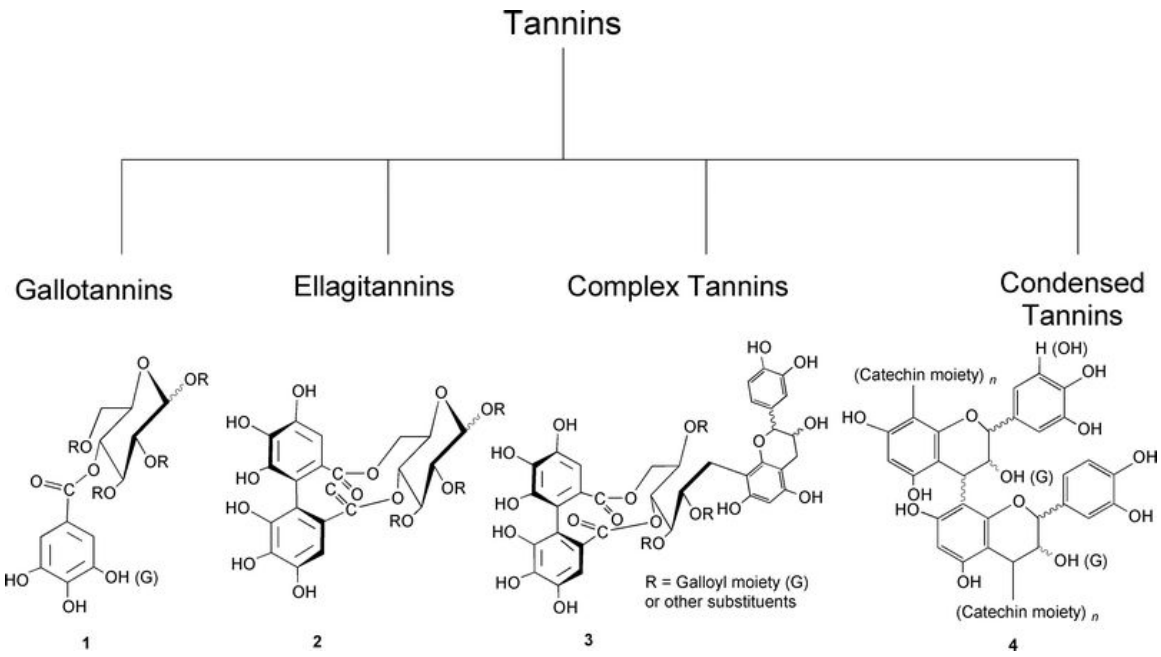


Figure 2: Classification of tannins

Among the phenolic compounds, flavonoids are the largest group with over 6000 types. These are benzopyran derivatives phenyl substituted chromones and consist of a 15- carbon basic skeleton which contain two 6- carbon rings with a 3-carbon bridge acting as third ring. Following modifications of this skeleton, basic flavonoid skeleton molecule give rise to several classes of flavonoids such as flavones, isoflavones, flavonols, flavanones, flavan-3-ols and anthocyanidins [6] (Figure 3).

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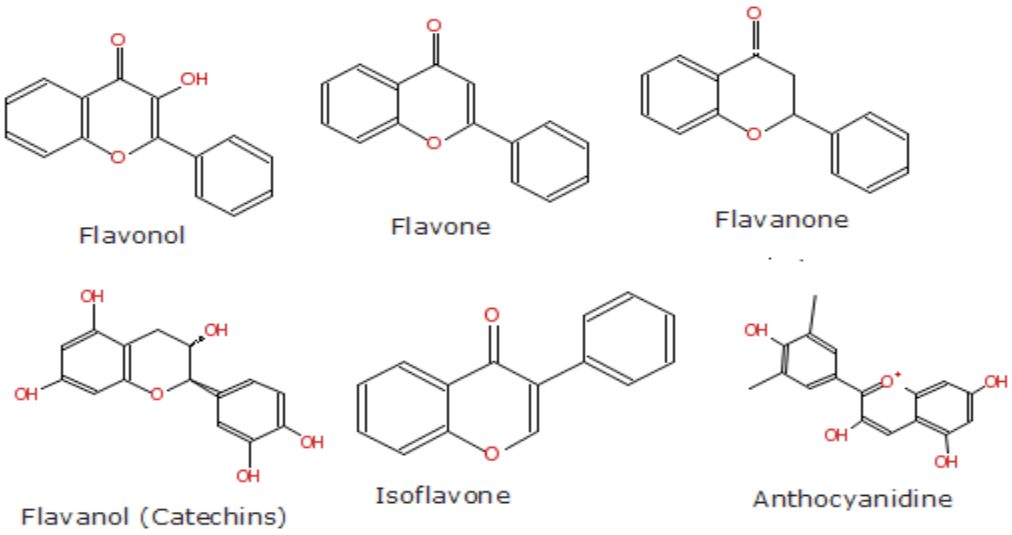
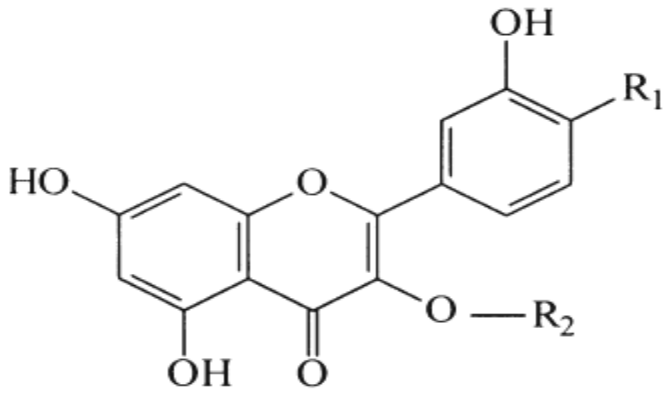


Figure 3: Basic structures of flavonoid subclasses.

Purified flavonoids are found to affect several aspects of atherogenesis including low density lipoprotein (LDL) oxidation, chemotaxis, cell adhesion, inflammation, smooth muscle cell proliferation and platelet aggregation and thus several flavonoids are identified as having positive effects on cardiovascular disease prevention. Flavonoids inhibit a number of cytochrome P450 isoforms and thus have potentials for altering drug properties [8].

1.2.2 Flavonols

Major sources of dietary flavonols (Figure 4) are fruits, vegetables, tea and red wine. However, dietary intake of flavonols is influenced by species, growth rate, season, light availability, ripeness, food preparation and processing [9]. Decreased cardiovascular disease mortality has been reported in free living populations having high dietary intake of flavonols [10]. Quercetin is an important flavonol which is recommended at a daily dose of 1 g but normal dietary intake remains 10–100 mg [11]. Other important flavonols include kaempferol and myricetin. Quercetin and kaempferol inhibit calcium channel blockers such as nifedipine and felodipine and this was clinically significant, since patients experienced increased heart rate, decreased diastolic blood pressure and headaches



Flavonol	R_1	R_2
Rutin	OH	Rutinose
Quercitrin	OH	Rhamnose
Quercetin	OH	H
Kaempferol	H	H
Isorhamnetin	OCH_3	H

Figure 4: Different flavonol structures.

1.2.3 Flavones

Flavones, which have a very close structural relationship to flavonols (Figure 3), are generally less abundant in fruits and vegetables as compared to flavonols. Parsley and celery are the primary sources of flavones. Important flavones include luteolin and apigenin.

Luteolin (5,7,3',4'-tetrahydroxyflavone) is usually found in parsley, celery, peppers, onion leaves, cabbage, broccoli, and apple skin. It is used to treat hypertension, inflammatory conditions, and cancer because of its anti-inflammatory, anti-carcinogenic, antioxidant, apoptosis promoting, anti-angiogenesis and anti-metastasis properties [12].

Chrysin (5,7-dihydroxyflavone) is a potent aromatase inhibitor and thus enhances testosterone levels in men. As such, chrysin is very poorly bioavailable but its methylated forms are stable and are highly bioavailable [13].

1.2.4 Flavanones

The primary sources of flavanones are citrus fruit, which contain naringenin, eriodictyol and hesperidin. Naringin is citrus flavonoid-glycoside, which produces the bitter taste in grapefruit. Naringin is hydrolyzed into naringenin by gut microflora. Naringenin possess antioxidant, antitumor and radioprotective activities and thus affects cancer, cardiovascular diseases, hyperlipidemia, neurological disorders and skin disorders [14]. Hesperidin exhibits antioxidant, anti-inflammatory, anticarcinogenic, antimicrobial and radioprotective activities [15].

1.2.5 Isoflavones

Isoflavones are the predominant constituent of the diet. Most common in human consumption are genistein (4',5,7-trihydroxyisoflavone), diadzein (4',7-dihydroxyisoflavone), and glycitein (7,4'-dihydroxy-6-methoxyisoflavone). These chemicals possess estrogenic activity upon binding the estrogen receptors ER-β. Genistein exhibit significant antiproliferative and apoptotic activities and thus acts as chemopreventive agent [16]. Genistein is described to improve blood flow by affecting vasodilation and this effect can be reversed by NO synthase inhibition [17].

1.2.6 Flavan-3-ols

Flavan-3-ols like catechin, gallocatechin, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate, are found in grapes and other food sources. These compounds can enhance the release of nitric oxide (NO) and decrease superoxide production during platelet aggregation and thus limit this process. These phytochemicals also reduce the release of pro-inflammatory mediators from platelets and thus inhibit inflammation in arteries. These components also suppress LDL / HDL oxidation [11]. LDL are the major carrier of cholesterol in the blood; if LDL level is increased, those compounds can promote atherosclerosis upon oxidizing the LDL in artery walls and narrowing it with cholesterol particles. On the other hand, HDL act to reverse cholesterol transport and inhibit LDL-induced cytotoxicity.

1.2.7 Anthocyanidin

Anthocyanidins exhibit a range of pro-health effects including antioxidant, anti-inflammatory, antimicrobial, anti-carcinogenic, neuroprotective, vision improving, and apoptosis inducing properties. Anthocyanidins such as pelargonidin, cyanidin,

delphinidin, peonidin, malvidin, malvidin 3-glucoside, and malvidin 3,5-diglucosides are found to be strong inhibitors of NO production *in vitro* without exerting harmful effects [18]. Anthocyanins, cyanidins and delphinidins potently inhibit platelet derived growth factor (PDGF)-induced vascular endothelial growth factor (VEGF) expression in vascular smooth muscle cells by preventing p38 mitogen activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways. Thus, the ability of the active anthocyanin compounds to maintain endothelial cells in a quiescent state by preventing the vascular expression of VEGF might contribute to a decreased rate of development of atherosclerotic lesions [19].

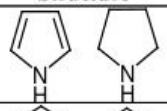
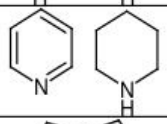
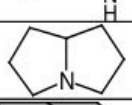
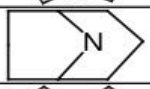
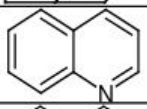
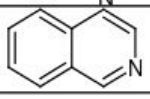
1.2.8 Alkaloids

Alkaloids are a diverse class of cyclic nitrogenous compounds found in about 20% of plant species. Alkaloids exist in many categories such as indole, benzylisoquinoline, tropane, pyrrolizidine, or purine alkaloids (Table 2). For over 5000 years, alkaloids have been used as poisons, neurotoxins, and psychedelics. Caffeine, cocaine, ephedrine, morphine, nicotine, atropine, physostigmine, scopolamine are common alkaloids that affect many health conditions including behaviour [6]. Bioprotective alkaloids in plants and plant-endophyte associations include pyrrolizidines, ergot alkaloids, indole diterpenes, pyrrolopyrazines. Such alkaloids increase fitness of plants and host plants (which supplies food resources and acts as a substrate for commensalist insects or other fauna) against the exploitation by animals and make them more resistant and competent to drought. Ergot alkaloids are the main sources of toxicities to livestock [20]. The majority of polyhydroxylated alkaloids such as pyrrolidine, piperidine, and pyrrolizidine inhibit glycosidic bond hydrolysis by interfering with glycosidases, which have highly important functions in primary metabolism. Various polyhydroxylated alkaloids are used for therapeutic uses such as being anticancer, antiviral, and antidiabetic agents, immune stimulants and are also used in treating glycosphingolipid lysosomal storage diseases [21].

Table 2: Chemical classification of alkaloids [22].

Classification of alkaloids

Chemical classification

A) True alkaloids			
Sr. no.	**Type**	**Structure**	**Examples**
1.	Pyrrole and pyrrolidine		e.g. Hygrine, coca species
2.	Pyiridine and piperidine		e.g. Arecoline, anabasine, lobeline, conine, trigonelline
3.	Pyrrolizdine		e.g. Echimidine, senecionine, seneciphylline
4.	Tropane		e.g. Atropine, hyoscine, hyoscyamine, cocaine, pseudopelletirine
5.	Quinoline		e.g. Quinine, quinidine, cinchonine, cupreine, camptothecine
6.	Isoquinoline		e.g.Morphine, codeine, emetine, cephaline, narcotine, narceine, d-tubocurarine

1.2.9 Quinones

Quinones are aromatic compounds that are usually derived from benzene or naphthalene (Figure 5). The major groups of natural 2-methyl-3-oligoisoprenyl quinones are vitamin K, coenzyme Q (ubiquitones), and tocopherol quinones which are produced from the oxidation of vitamin E. Ubiquitone is a prenylated benzoquinone, which plays basic role in electron shuttle in the electron transport chain. It also participates in the mitochondrial oxidation of dihydroorotate as well as in the biosynthesis of pyrimidines [23]. Antiproliferative and cytotoxic properties of many quinones and hydroquinones derived from sponges have created promising opportunities for the development of antitumor agents and many quinones exhibit significant anti-tuberculosis, antimalarial, antifungal and cardiotonic activities [24].

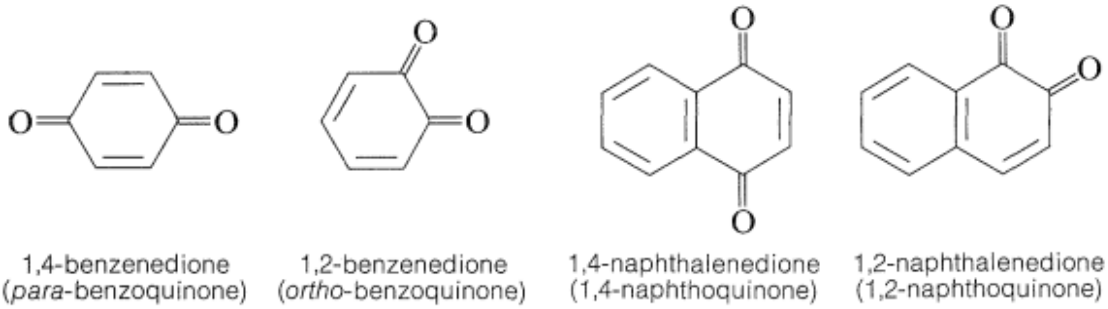


Figure 5: Different quinones structures.

1.3 Phytochemicals and health effects

Phytochemicals are in use for almost every pathological condition and many of their efficacious roles are well documented in animal models as well as in humans. Results of *in vitro* experiments cannot be treated as preclinical evidence for clinical trials as *in vitro* tests are conducted under completely controlled conditions while a host of factors are needed to be taken into account before testing a bioactive chemical on humans. On the other hand, *in vivo* experiments can substantiate the findings of *in vitro* studies as a multitude of factors can alter the path of bioactive compound under test [16]. Preliminary studies have provided impetus for well controlled and randomized trials to evaluate the efficacies and as more evidence gathers, scientific and medical knowledge of the therapeutic potentials of phytochemicals will become conclusive. Natural products are well known, nowadays, to exhibit an extensive spectrum of biological activities such as stimulation of the immune system, antibacterial, antiviral, anti-hepatotoxic, anti-ulcer, anti-inflammatory, antioxidant, anti-mutagenic, anti-cancer effects, and induction of apoptosis [25] (Table 3), and medicinal plants are still used despite the advantages of modern synthetic drugs. Moreover, medicinal plants are more natural and more accessible than manufactured drugs, so people believe that the use of the medicinal plants for the treatment of the diseases is more safe [26].

More than 70% of the developing world's population depends, today, on traditional medicinal system, otherwise known as complementary or alternative systems of medicine [27, 28]. For instance, 30-75% of cancer patients in the USA uses the medicinal plants as complementary and alternative medicine [29]. This encourage the

researchers to search for possible anticancer agents from plants in different countries. Indeed, the use of medicinal plants by people in their food systems will open the window for many important new pharmaceuticals. In the last 20 years, more than 25% of drugs have been directly derived from plants, while other 25% have been chemically altered natural products [30]. Pharmacologically active chemicals present in medicinal plants are now proving potential for use in treating inflammatory diseases as well as cancer [28].

Table 3: Medicinal plants used to treat distinct ailments based on traditional Arab medicine.

Plant species	Preparation	Uses
Allium cepa	Bulb juice	Diabetes, liver diseases, and coughing
Arum palaestinum	Foliage decoction	bacterial infection, poisoning, and circulatory system
Peganum harmala	Roots and seeds	anti-bacterial activity, cytotoxicity antitumoral activity, and anti-oxidant activity.
Crataegus azarolus	Fruit and flower decoction	Cardiovascular diseases, diabeties, and sexual diseases
Quercus calliprinos	Fruit and bark decoction	Ulcer, diabetes, and skin diseases
Zea mays	Kernel and fiber decoction	Blood pressure, joint inflammation, and weight loss
Triticum aestivum	Shoot decoction	Anemia, and skin diseases

1.4 Anti-cancer properties of phytochemicals

Cancer is a major health problem worldwide, with more than 20% of all deaths among the world's population being attributed to this disease [31-33]. The body maintains a system of checks and balances on cell growth so that cells divide to produce new cells only when new cells are needed or when normal cells grow old or get damaged. Disruption of this system results in an uncontrolled division and proliferation of cells that

are build up an extra cells often forms a mass of tissue known as a tumor, which grow or proliferate throughout the tissues of the body and it may progress and cause death [34].

Tumors can be benign or malignant, where the term “cancer” refers usually to malignant tumors. Benign tumors can usually be removed and do not spread to other parts of the body and usually don’t need to be removed. However, malignant tumors, grow aggressively and invade other tissues of the body and often can be removed but may grow back, allowing entry of tumor cells into the bloodstream or lymphatic system and then to other sites and organs in the body such as bone, brain and liver, where they overwhelm these sites by consuming their oxygen, nutrients, and space. This process of spread is termed metastasis, where the areas of tumor growth at these distant sites are called metastases. There are different causes of cancer, among which are chemicals, radiation, smoking, viral infection, dietary factors, and environmental factors. Physicians and researchers need to find a comprehensive cancer treatment that is based on the increased awareness of the role of traditional and complementary medicine [35]. According to number of global deaths, the most frequent types of cancer worldwide are lung, stomach, liver, colon (colorectal) and prostate, among men, while among women, they are breast, lung, stomach, colorectal and cervical [36].

There are many strategies to treat cancer, among which is a process called chemotherapy. Chemoprevention is defined as the use of non-toxic chemical substances or their mixtures to inhibit, retard or delay the overall process of multi-stage carcinogenesis. A wide array of compounds, of both synthetic and natural origin, have been reported to exert anti-mutagenic and anti-carcinogenic effects in numerous animal and cell culture systems. Chemotherapy may be used alone, with radiation therapy, or after surgery. Chemotherapy uses drugs to kill cancer cells. When radiation therapy and chemotherapy are given at the same time, the side effects may be worse. Cancer chemotherapy has faced dramatic problems. Poor selectivity of anticancer agents, kills both malignant and normal cells [37]. Contentious treatment with chemotherapy may lead to drug-resistant [38]. This in turn justifies the interest in search of possible anticancer agents from the flora of different countries [39]. Some products of plant origin have strong biological activity and can be used as an effective sources of chemotherapeutic

agents without side effects. This attracted the attention of many scientists to screen plants and to study their chemical, pharmacological and biological activity [40]. In studies conducted in the Middle East, during chemotherapy treatment [41], about half of the cancer patients uses the complementary and alternative medicine (CAM) in Turkey [42], and 35% in Jordan uses CAM [43]. CAM is also used by patients with hematological, malignancies, gynecological [44] and pediatric disorders [45].

Many nutritional agents are believed to be critical in carcinogenesis [46]. Evidences from epidemiologic studies indicate that diets that contain high fruits and vegetables such as cabbage, broccoli, tomatoes, apples and grapes [47], are associated with a lower risk of different cancers [48], such as prostate, oral cavity, lung, breast and colon [49, 50]. For instance, a high consumption of soybean products in Asian countries reduce the incidence of colon cancer [51], a high consumption of vegetables reduces the risk of colon cancer mortality [52], and recently, medicinal plant extracts have been demonstrated to be able to control the proliferation of prostate cancer cells [53]. Moreover, several organizations, such as National research council of the national academy of sciences [46], the national cancer institute [54], and the American cancer society [55], encourage the increase intake of citrus fruits, green and yellow vegetables.

1.5 Anti-oxidant properties of phytochemicals

1.5.1 Free radicals, endogenous defense and the role of oxidative stress in human

1.5.1.1 Definition of free radicals

"Any species (atoms, molecules or ions) that contain one or more unpaired electrons in their orbitals are known as free radicals [56]. In other words, they possess an open shell configuration [57], where an open shell is a valence shell [58], which is not completely filled with electrons, or all the electrons contained in it are not in chemical bonding with other atoms or molecules. Free radicals may carry positive, negative or zero charge [59]. The free electrons present in the open shells make these radicals highly reactive species. These free radicals play a considerable role in the processes of polymerization [60] and combustion [61] and many chemical reactions of plasma chemistry, atmospheric chemistry [62] and biochemistry [63]. In living organisms,

intermediary metabolism [64] of many biological compounds takes place via these reactive species. In a process referred to as redox signaling [65], free radicals serve as messengers.

Free radicals play a very important role in a number of chemical reactions, which occur in our body. Usually, our body exploits a number of free radical physiological and biochemical reactions, and keeps them under tight control [56]. However, unfortunately, these processes may go out of control leading to a chain of reactions that are damaging to surrounding tissue. They, therefore, could play constructive as well as destructive roles in the body.

1.5.1.2 Origins and targets of free radicals

Each day, our body produces, during metabolism, numerous amount of free radicals which in turn react with other molecules in several of ways [56, 66]. Mitochondria is the most important cellular source for free radicals and deal with regulatory and toxic reactions accomplishing different physiological processes including cell cycle, proliferation and cell death. Mitochondria is the major site of generation of primary free radicals such as superoxide anion (O_2^-) and nitric oxide ($\cdot NO$) along with hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) as termination products (Figure 6) [67]. Electron transport chain directly produces superoxide anion radical and then hydrogen peroxide which in turn gives rise to hydroxyl radical [68]. As free radicals contain unstable or free electrons in their shells, they tend to be more reactive than non-radical species, although they possess a wide range of reactivity. When two radicals come together, they share their unpaired electrons through the formation of a covalent bond.

Hydroxyl free radicals are highly reactive and can attack any of the molecules found in the living cells causing a damage to the system. A hydroxyl radical can give birth to a chain of reactions in the cell and if it attacks DNA, it causes chemical changes in the DNA bases and even breaks the linkages between these bases, activating oncogenes and carcinogenesis.

In addition to DNA, proteins are considered as the initial cell targets of free radicals [69]. Upon exposure to hydroxyl free radicals, proteins are described to be oxidized before lipids [70].

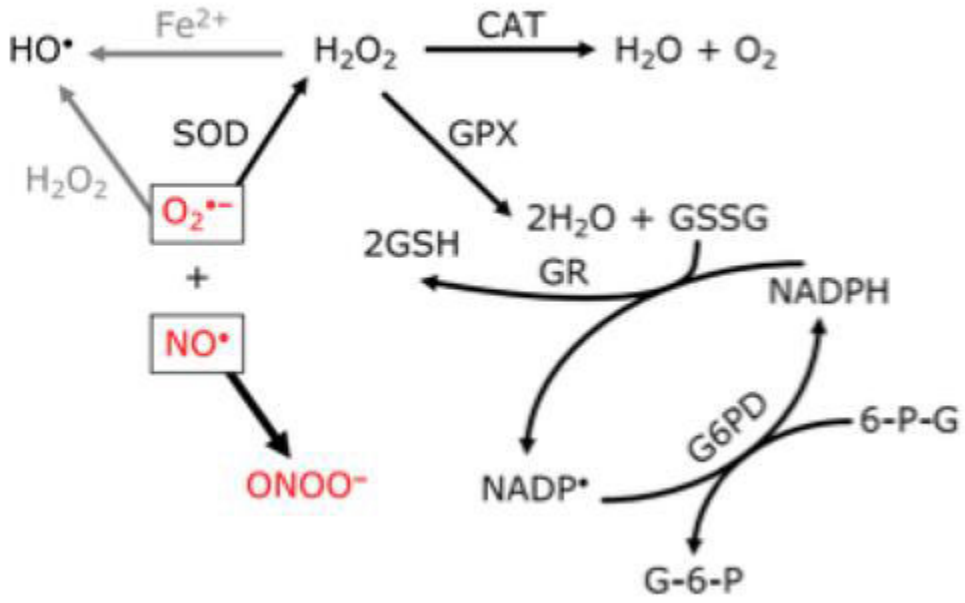


Figure 6: Generation of primary free radicals by mitochondria.

1.5.1.3 Source of free radicals

As mentioned above, the source of free radicals is our body itself as well as the environment. Mitochondrial electron transport chain is an adventitious source of free radicals during normal metabolism [71] in addition to other physiological factors [72]. Thus, sources of free radicals can be either internal or external.

Enzymatic reactions occurring in the body serve as a source of free radicals. These reactions are involved in the electron transport chain, phagocytosis, cytochrome P450 systems and prostaglandin synthesis. Mitochondria, phagocytes, peroxisomes, xanthine oxidase, arachidonate pathways, ischemia, inflammation and exercise are described as internal sources of free radical generation [73].

Similarly, non-enzymatic reactions of oxygen with organic compounds and the reactions initiated by ionizing radiations serve as an external source of free radicals. Additionally, cigarette smoke, environmental pollutants, ozone, drugs, pesticides, UV light, industrial solvents and electronic pollutants (extra low frequencies from computers, power lines, television, microwaves, ...) are some other external sources of free radicals [74]. Physiological factors like stress, emotions, anxiety and other disease conditions add to the generation of free radicals and put the body under oxidative stress [75].

1.5.1.4 Formation of reactive oxygen species and action *in vivo*

Reactive oxygen species (ROS) are chemically highly reactive molecules with one unpaired electron in their valance shell derived from molecular oxygen [76]. ROS are generated during normal metabolism of oxygen by endogenous systems as well as exposure to physiochemical and patho-physiological conditions, and play a key role in cell signaling and homeostasis. ROS include superoxide, hydroxyl radical, hydrogen peroxide, peroxyl radical, organic hydro peroxide, singlet oxygen and ozone [76]. These reactive oxygen species are produced in the body with controlled mechanisms. Enzymes such as α -1-microglobulin, catalase, superoxide dismutase (SOD), glutathione peroxidase and lactoperoxidase defend living cells against oxidative damage by ROS [77] (Table 4). If these mechanisms get out of control, free radicals can adversely affect proteins, lipids, DNA and may lead to a number of human diseases [78].

Table 4: Reactive oxygen species (ROS) [79, 80].

Reactive Oxygen Species	Symbol	Reactivity
Superoxide	$O_2^{-}\bullet$	Produced in mitochondria and cardiovascular system
Hydroxyl radical	•OH	Highly reactive, generated in iron overload and attack every molecule of the living cells
Hydrogen peroxide	H_2O_2	By product of several body reactions and gives rise to potent $^{\bullet}OH$
Peroxyl radical	$RO_2^{\bullet}$	Reactive and generated during oxidative damage to lipids, proteins, DNA and sugar
Organic hydroperoxide	ROOH	Reacts with transient metal ions
Singlet oxygen	$^1O_2$	Highly reactive, generated in photosensitization and other chemical reactions
Ozone	$\bullet O_3$	An environmental pollutant, reacts with living molecules and yields singlet oxygen

Among ROS, the hydroxyl radical is the most reactive and potent damaging species which can affect each and every molecule found in the living cells. *In vivo*, it has been shown that hydroxyl radical can lead to formation of DNA-protein linkages, base damages, DNA strand breaks, protein fragmentation and lipid peroxidation [79].

1.5.2 Antioxidants

Antioxidants are defined as the substances which at low concentration can significantly inhibit or delay the oxidative process, while often being oxidized themselves [81]. Endogenous and exogenous antioxidants are used to neutralize free radicals and protect the body from free radicals by maintaining redox balance [82]. The human body is equipped with a variety of endogenous antioxidants that serve to counterbalance the effect of oxidants. These are divided into two categories: enzymatic and non-enzymatic antioxidants.

1.5.2.1. Enzymatic endogenous antioxidants

The major enzymatic antioxidants are superoxide dismutase, catalase, GSH (glutathione) peroxidase and GSH reductase. In addition to these major enzymes, other antioxidants, including glutathione S-transferase (GST), heme oxygenase-1, and redox proteins, such as thioredoxin (TRX), peroxiredoxins (PRXs) and glutaredoxins, are also described to play crucial roles during antioxidant defenses [80, 83].

1.5.2.2. Non-enzymatic endogenous antioxidants

Non-enzymatic antioxidants include glutathione (GSH), uric acid, bilirubine and others.

1.5.2.2.1. Glutathione

GSH is a tripeptide (L-g-glutamyl-L-cysteinyl-Lglycine) and the major soluble antioxidant being highly abundant in all cell compartments. It also serves as a cofactor for several detoxifying enzymes, such as GSH-Px and GSH transferase, and has a role in converting vitamin C and E back to their active forms. GSH protects cells against apoptosis by interacting with pro-apoptotic and anti-apoptotic signaling pathways. In addition, reduced glutathione donates protons to membrane lipids and protects them from oxidant attacks [80, 84].

1.5.2.2.2. Uric acid

It is a highly abundant aqueous antioxidant, considered as the main contributor for the antioxidant capacity in the plasma. It is able to quenche HO• and ONOO- and prevent lipid peroxidation [84]. The scavenging of ONOO- by uric acid is critically enhanced in the presence of vitamin C and cysteine. Uric acid can also serve as a chelator of iron in extracellular fluids [84].1.5.2.2.3. Bilirubin

It represents the end-product of heme catabolism. Uric acid functions as a chain breaking antioxidant where low circulating bilirubin levels are considered a risk factor for cardiovascular diseases. Plasma albumin, the most abundant plasma protein, is also considered as an antioxidant due to its sulfhydryl groups and its ability to scavenge MPO-derived chlorinated reactive species and ROO• radicals [84].

1.5.2.3. Exogenous antioxidant

Exogenous antioxidants include vitamin E, vitamin C, carotenoids, polyphenols and others which are the principal dietary antioxidants from fruits, vegetables and grains.

1.5.2.3.1. Vitamin E

It is a lipid-soluble vitamin concentrated in the hydrophobic interior site of cell membrane and the principal defense system against oxidant-induced membrane injury. Vitamin E donates electron to peroxyl radical, which is generated during lipid peroxidation. α-tocopherol represents the most active form of vitamin E and the major membrane-bound antioxidant in cell. Vitamin E can trigger apoptosis of cancer cells and inhibits free radicals formation via converting O_2•- and HO• to less reactive forms [80].

1.5.2.3.2. Vitamin C

Ascorbic acid is a water-soluble vitamin that provides intracellular and extracellular aqueous-phase antioxidant capacity primarily by scavenging ROS and lipid hydroperoxides. It also converts vitamin E free radicals back to vitamin E.

1.5.2.3.3. Carotenoids

They are pigments found in plants. Primarily, β-carotene is described to react with peroxyl (ROO•), hydroxyl (•OH), and superoxide (O_2•-) radicals. Carotenoids exert their antioxidant effects in conditions of low oxygen partial pressure but might have pro-oxidant effects at higher oxygen concentrations. Both carotenoids and retinoic acids (RAs) are capable of regulating transcription factors. In addition, β-carotene inhibits the oxidant-induced NF-κB activation and interleukin (IL)-6 and tumor necrosis factor α (TNF-α) production. Carotenoids has also been reported to affect apoptosis of cells [80, 85].

1.5.2.3.4. Polyphenols

Polyphenols are naturally available from various dietary sources, and they are increasingly recommended as a daily supplement to prevent oxidative stress. Plants are reported to produce more than 8000 different polyphenols as secondary metabolites. Chemically, polyphenols are compounds bearing one or more hydroxyl groups attached to a benzene ring. They can be classified as simple phenolics, phenolic acids (derivatives of cinnamic and benzoic acids), coumarins, flavonoids, stilbenes, tannins, lignans and lignins. Among these, flavonoids have the most potent antioxidant potential [86, 87].

1.6 Anti-inflammatory properties of phytochemicals

1.6.1 Inflammation

Inflammation is a process being implicated in the pathophysiology of an increasing number of diseases including cardiovascular disease, cancer, diabetes, age-related macular degeneration, Parkinson's disease, Alzheimer's disease, and possibly depression [88].

1.6.2 Overview over the inflammatory process

Inflammation is a rapid and non-specific response to cellular injury or infection. The inflammatory response is produced and controlled by complex interactions between different cellular and plasma protein components. The cellular components involve intercellular communication effected by a range of cytokines. The causes of inflammation are apparently causes of diseases; it can be induced upon bacterial infections by compounds including lipopolysaccharides (LPS), as well as by viruses, which are detected by Toll-like receptors (TLRs), expressed by immune cells like macrophages. Besides, inflammation can be triggered by physical injuries (i.e., UV) or chemical compounds (i.e., reactive oxygen species). It is characterized by five cardinal signs: redness, heat, swelling, pain, and loss of function [89].

1.6.3 Acute and chronic inflammation

Inflammation is classified crudely based on duration of the lesion and histological appearances into acute and chronic inflammation. Acute inflammation usually takes place first in response to the attack of the risk factor (s). As soon as the risk factor (s) is removed, the acute inflammatory response will stop. However, when the risk factor (s) persist, the acute inflammation will progress to chronic inflammation. It is the chronic inflammation that plays the major role in the pathogenesis of all inflammatory diseases. By monitoring soluble markers corresponding to the sequential events of both acute and chronic inflammation it is possible not only to determine whether an individual is currently exposed to risk factor (s) of inflammation but also the extent of overall inflammation reaction.

All phases of inflammatory reaction are sustained and controlled by a number of extracellular mediators and regulators including cytokines, chemokines, growth factors,

eicosanoids (prostaglandins, leukotrienes, etc), complement and peptides. These extracellular events are matched by equally complex intracellular signaling control mechanisms, with the ability of cells to assemble and disassemble an almost bewildering array of signaling pathways as they move from inactive to dedicated roles within the inflammatory response and site (Table 5) [89] .

Table 5: Different types of Cytokines found in acute and chronic inflammations [89].

Cytokines and Chemokines

Cytokine	Principal Sources	Principal Actions in Inflammation
IN ACUTE INFLAMMATION		
TNF	Macrophages, mast cells, T lymphocytes	Stimulates expression of endothelial adhesion molecules and secretion of other cytokines; systemic effects
IL-1	Macrophages, endothelial cells, some epithelial cells	Similar to TNF; greater role in fever
IL-6	Macrophages, other cells	Systemic effects (acute-phase response)
Chemokine	Macrophages, endothelial cells, T lymphocytes, mast cells, other cell types	Recruitment of leukocytes to sites of inflammation; migration of cells to normal tissues
IN CHRONIC INFLAMMATION		
IL-12	Dendritic cells, macrophages	Increased production of IFN-γ
IFN-γ	T lymphocytes, NK cells	Activation of macrophages (increased ability to kill microbes and tumor cells)
IL-17	T lymphocytes	Recruitment of neutrophils and monocytes

The inflammatory response commences with a brief constriction of arterioles followed by vasodilatation and exudation of protein-containing plasma and blood cells into the injured tissue. This results in swelling and edema. Meanwhile, leukocytes adhere to vessel walls and cause the endothelial cells to contract, creating enough space between these cells, thus allowing leukocytes to enter the extra-vascular tissue. Increased vascular permeability is maintained until the inflammatory state is resolved, and it is the interplay between blood cells and plasma proteins in the affected tissue that controls the

inflammatory response and interacts with part of the immune response (Figure 7). Inflammation is orchestrated by different cell types including mast cells, endothelial cells, phagocytic leukocytes (polymorph nuclear neutrophils, macrophages, and eosinophils), and platelets which secrete different regulatory molecules such as cytokines, chemokines, prostaglandins and NO.

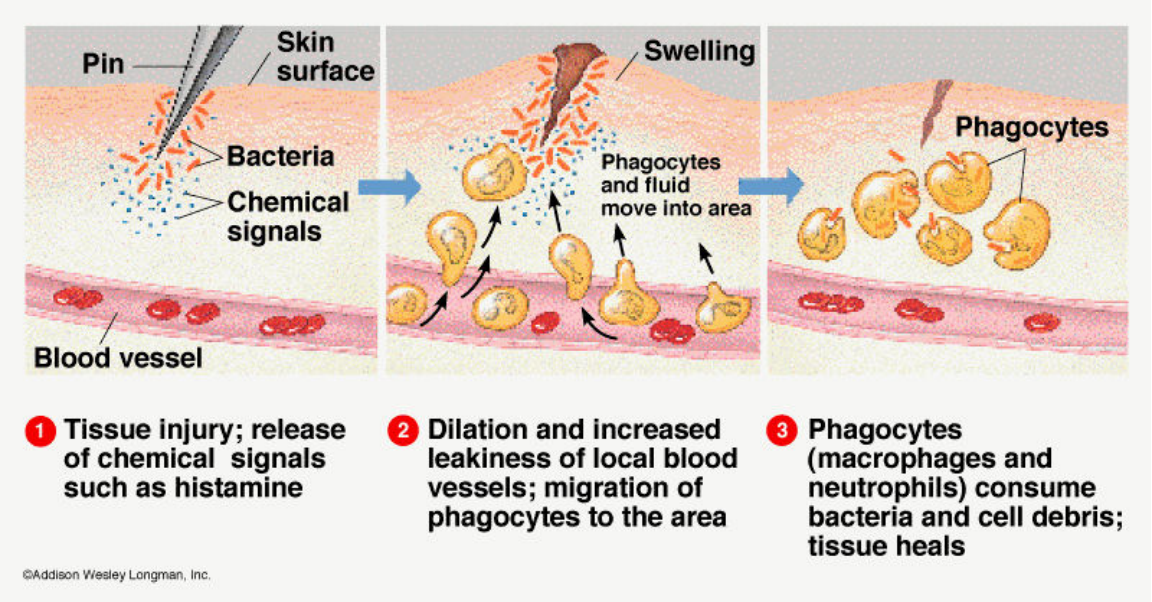


Figure 7: Inflammation response steps [90].

1.6.4 Prostaglandins and cyclooxygenase (COX)

The prostaglandins (PGs) correspond to a group of cyclic, 20-C unsaturated fatty acids, which all share a double-bond at C13-C14. Based on structural differences, PGs are classified into nine groups, named A-I. PGs are known to be synthesized and released in virtually all body tissues. As no tissue (except seminal fluid) can store PGs, the rate of release reflects the rate of biosynthesis. PGs are generally produced in response to a variety of stimuli, including inflammation, allergic responses and trauma, and they usually exert their action locally, close to their site of release.

PGI_2 (prostacyclin) is the predominant PG in the vascular endothelium, where it inhibits platelet aggregation and causes vasodilation. PGE_2 is the prevailing PG in the kidney [91]. PGE_2 has several pro-inflammatory activities, including vasodilation and

increasing vascular permeability, inducing fever, and enhancing pain and edema caused by other mediators such as bradykinin and histamine. Large amounts of PGE_2 and PGF_2 can be produced by stimulated monocytes and macrophages, whereas stimulated neutrophils produce moderate amounts of PGE_2 [92].

PGs are products of arachidonic acid metabolism. Arachidonic acid is released from the cell membrane by phospholipase enzymes, in particular phospholipase A_2, and the free fatty acid can be metabolized to eicosanoids by cyclooxygenase (COX) and lipoxygenase (LOX). Metabolism catalyzed by COX gives rise to PGs of the 2-series as well as thromboxanes, while LOX metabolism leads to the formation of leukotrienes (Figure 8).

COX exist in two isoforms; these are known as COX-1 and COX-2 and their respective protein products show differential distribution. COX-1 is constitutively expressed in most tissues, where it catalyzes the biosynthesis of eicosanoids (PGs and thromboxanes) that regulate numerous cellular processes. In contrast, COX-2 activity is generally undetectable in most tissues, but the expression of COX-2 can be rapidly induced in inflammatory cells in response to stimulation by pro-inflammatory cytokines or by growth factors [92] (Figure 9).

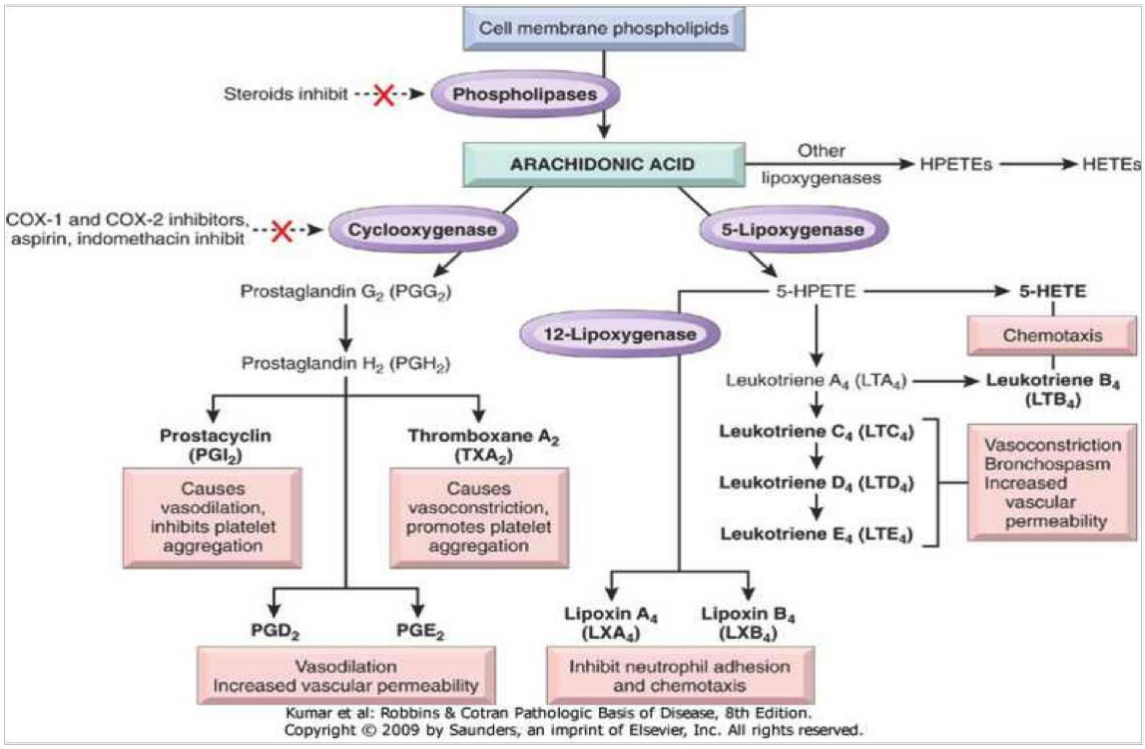


Figure 8: Schematic representation of the arachidonic acid metabolism catalysed by cyclooxygenase (COX) and lipoxygenase (LOX) [93].

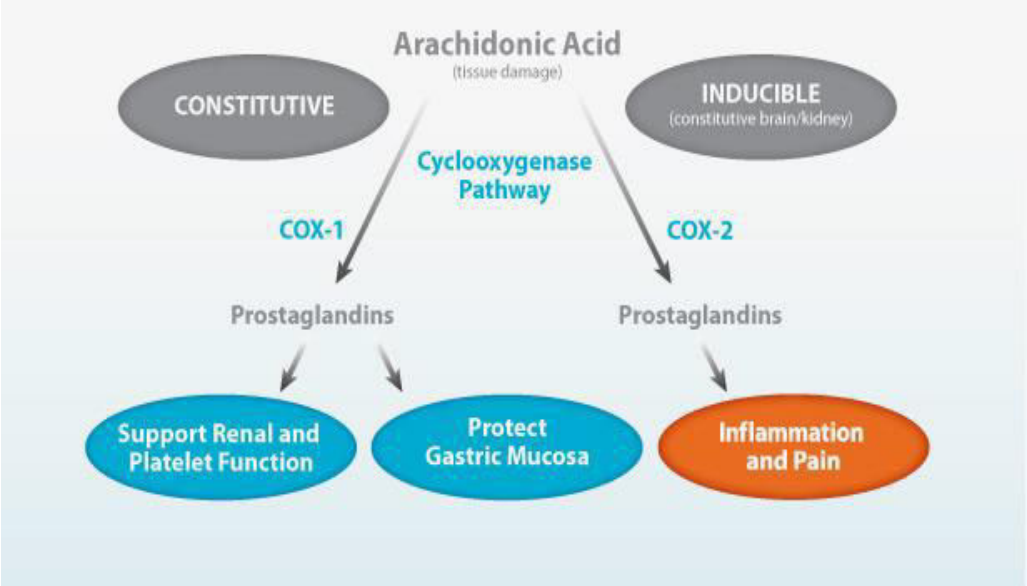


Figure 9: Cyclooxygenase isoforms and their corresponding functions [94] .

A new class of anti-inflammatory and analgesic agents that are selective COX-2 inhibitors were developed in the 1990s (e.g. celecoxib, rofecoxib). The rationale for this drug development was that selective COX-2 inhibitors were not expected to interfere with homoeostatic physiological processes and should therefore be less likely than non-selective COX inhibitors to cause the unwanted side effects typical of traditional NSAIDs [94].

1.6.5 Nitric oxide (NO) and nitric oxide synthase (NOS)

NO, short-lived free radical, is a small gaseous signaling molecule that diffuses freely within cells from site of formation to site of action. NO is implicated in distinct physiological and pathophysiological conditions and can have detrimental or beneficial effects in the human body. During inflammation, NO modulates various vascular and cellular responses. It regulates vasorelaxation and platelet aggregation. NO also regulates neutrophil activation and cell growth and induces apoptosis. NO has also an important role in host defense mechanisms. It mediates the toxicity of natural killer cells and regulates T cell activation. It acts as a neurotransmitter in NANC (non adrenergic, non cholinergic) nerves and in the central nervous system. Nitrosylation of proteins by NO driven radicals regulates protein activity and function. [94]

NO can be generated by three different forms of nitric oxide synthases (NOS): inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS). These enzymes catalyze L-arginine into L-citrulline and nitric oxide in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) (Figure 10).

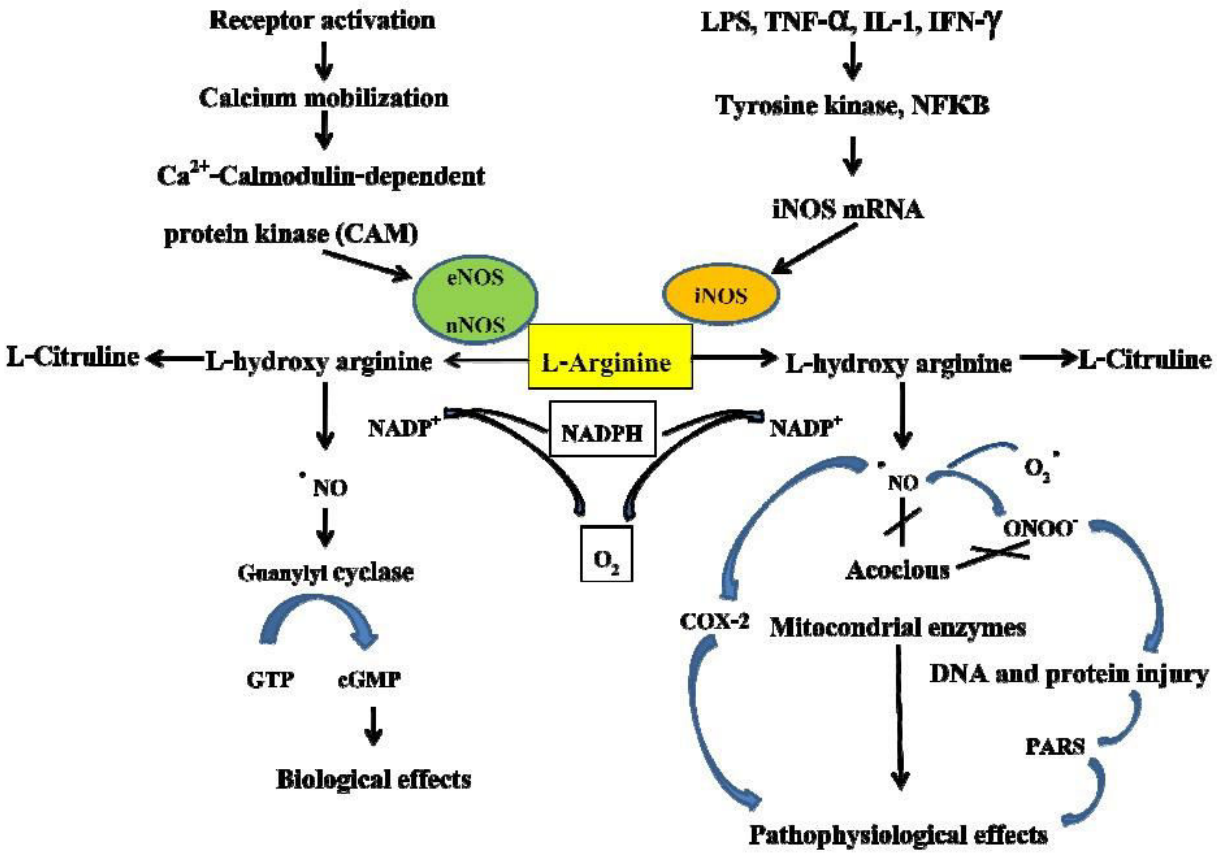


Figure 10: Synthesis of nitric oxide by NOS [95].

eNOS is present in endothelium, and is involved in the dynamic control of vascular tone. nNOS is mainly present in neural tissue. iNOS is induced by either bacterial lipopolysaccharide (LPS) or a number of cytokines including TNF-α and interferon-γ (IFN-γ) in macrophages, hepatocytes and endothelial cells. iNOS and nitric oxide are involved in host defense and immunity and modulate the inflammatory response [94]. Key similarities and dissimilarities among the three NOS isoforms (nNOS, eNOS and iNOS) are summarized in Table 6.

Table 6: Biological comparison of three known isoforms of nitric oxide synthases

Characteristics	nNOS	eNOS	iNOS
Primary tissue or cellular source	Brain, peripheral (PNS) and central nervous system (CNS), and skeletal muscles.	Vascular endothelial cells, cardiomyocytes	Macrophages
Known functions	Neuromodulator in brain neurotransmittor in PNS regulation of smooth muscle activity and neuroendocrine functions in skeletal muscles	Vasodilator regulation of blood flow and pressure	Activation of APCs against infections and microbes cell death
Expression	Constitutively present	Constitutively present	Inducible
Concentration	In picomolar range Calcium dependence	In picomolar range Ca^{2+} dependent Utilized only small	In nanomolar range $^{Ca2+}$ dependent Quantities of Ca^{2+}. Mostly considered as Ca^{2+} independent
Sub-cellular location	Cytosol (not yet comfirmed)	Found in caveolae of membrane bound to calveolin-1	Cytoplasm
Association of dysregulated levels of nitric oxide with disorders	Dysregulation associated with neurodegenerative disorders	Hypertension, hypercholesterolemia diabetes, heart failure. At low concentrations prevents apoptosis of endothelial cells, inhibits platelet aggregation, and smooth muscle proliferation	Infections, rheumatoid, arthritis, Crohn's disease, asthma, septic shock

1.6.6. Cytokines

Cytokines control a broad range of physiological processes including immune responses, inflammation, haemopoiesis, cell proliferation, bone formation, wound healing, and the response to injury [96]. They are peptides of low molecular weight,

typically between 15 and 30 kD, and can be secreted by immune and non-immune cells in response to various stimuli. The cytokine family of regulatory peptides includes interleukins, tumor necrosis factors, interferons, lymphokines, colony stimulating factors, transforming growth factors, chemotaxins, and some growth factors. Table 7 lists different cytokines that play an important role in inflammation.

Table 7: Different cytokines involved in inflammation.

Cytokine	Source	Main actions relevant to inflammation
IL-1	Mainly macrophages	Inflammatory mediator; increases prostaglandin production
IL-6	Monocytes, macrophages, T and helper T cells	Stimulates inflammatory response
IL-9	Helper T cells	T cell and mast cell growth factor
IFN-γ (IL-18)	T and helper T cells, NK cells	Activates macrophages
TNF-α	Macrophages, mast cells, lymphocytes	Increases other cytokines; increases inflammatory and immune responses
Macrophage colony stimulating factor	Inflamed endothelial cells, monocytes, lymphocytes, fibroblasts	Macrophage growth factor
Transforming growth factor-β	Macrophages, platelets, lymphocytes	Chemotactic for macrophages; increases IL-1 production

1.6.7. Chemokines

Chemokines are soluble, low molecular weight (8-14 kDa) chemotactic cytokines that bind to their cognate seven trans-membrane G-protein coupled receptors (GPCRs) in order to mediate cellular migration. To date, there are over 40 characterized human chemokines, all of which have two to four highly conserved cysteine residues (Table 8) as well as a number of virally encoded chemokine-like proteins. The chemokine superfamily can be separated into four sub-families based on the presence and relative positioning of the first two cysteine residues at the N-terminus. The cysteine residues in

the CXC (or α) family are separated by a non-conserved amino acid, while in the CC (or β) family, these cysteine residues are adjacent to each other. The XC (or γ) chemokines have only a single cysteine residue, while the CX3C (or δ) chemokine, CX3CL1, has three non-conserved amino acids between the first two cysteine residues.

Chemokines are also functionally classified depending on whether they are constitutively produced or are inducible. Constitutive, or homeostatic, chemokines are involved in basal leukocyte migration and development, whereas inducible, or inflammatory, chemokines control the recruitment of effector leukocytes during an immunological insult [97]. It is noteworthy that this classification is not absolute, given that several chemokines cannot be assigned unambiguously to either one of the two functional categories. CXCL9, for instance, is a "dual-function" chemokine that is up-regulated under inflammatory conditions, but also participates in T cell lymphopoiesis [98]. Most chemokines are secreted from the cell, with the exception of CXCL16 and CX3CL1, which are membrane-bound proteins [99]. These proteins can also exist as soluble glycoproteins upon protease cleavage of their trans-membrane stalks. Thus far, 47 human chemokines have been described, many of which bind to several of the 18 described human chemokine receptors. Although there is considerable redundancy in the chemokine system, chemokine ligand-receptor binding does not usually cross the CC versus CXC chemokine boundaries. This redundancy also ensures sufficient levels of robustness within the system such that essential processes are not compromised by chance mutations.

Table 8: The chemokine superfamily and classification

Systematic Names	Alternate Names	Receptor(s)	Expression Profile
CXC Chemokines			
CXCL1	Groα/MGSAα	CXCR2, CXCR1	Inducible
CXCL2	Groβ/MGSAβ	CXCR2	Inducible
CXCL3	Groγ	CXCR2	Inducible
CXCL4	PF4	CXCR3b	Inducible

CXCL5	ENA-78	CXCR2	Inducible
CXCL6	GCP-2	CXCR1, CXCR2	Inducible
CXCL7	NAP-2	CXCR2	Inducible
CXCL8	IL-8	CXCR1, CXCR2	Inducible
CXCL9	MIG	CXCR3, CXCR3b	Dual-function
CXCL10	IP-10	CXCR3, CXCR3b	Dual-function
CXCL11	I-TAC	CXCR3, CXCR3b,CXCR7	Dual-function
CXCL12	SDF-1α/β	CXCR4, CXCR7	Constitutive
CXCL13	BLC, BCA-1	CXCR5	Constitutive
CXCL14	BRAK,Bolekine	Unknown	Constitutive
CXCL15	None	Unknown	Constitutive
CXCL16	None	CXCR6	Dual-function
CXCL17	DMC	Unknown	Unknown

Systematic Names	**Alternate Names**	**Receptor(s)**	**Expression Profile**
CC Chemokines			
CCL1	I-309	CCR8	Dual-function
CCL2	MCP-1	CCR2	Inducible
CCL3	MIP-1α/LD78	CCR1, CCR5	Inducible
CCL4	MIP-1β	CCR5	Inducible
CCL5	RANTES	CCR1, CCR3, CCR5	Inducible
CCL7	MCP-3	CCR1, CCR2, CCR3	Inducible
CCL8	MCP-2	CCR1, CCR2, CCR3,CCR5	Inducible
CCL11	Eotaxin	CCR3	Inducible
CCL13	MCP-4	CCR1, CCR2, CCR3	Inducible
CCL14	HCC-1	CCR1	Inducible
CCL15	HCC-2/LKN1/MIP-1γ	CCR1, CCR3	Inducible
CCL16	HCC-4/LEC/LCC-1	CCR1, CCR3	Dual-function
CCL17	TARC	CCR4	Dual-function
CCL18	DC-CK1/PARC/AMAC-1	Unknown	Constitutive
CCL19	MIP-3β/ELC	CCR7	Constitutive
CCL20	MIP-3β/LARC	CCR6	Dual-function
CCL21	SLC/6Ckinase	CCR7	Constitutive
CCL22	MDC/STCP-1	CCR4	Dual-function
CCL23	MPIF/CKβ8	CCR1	Constitutive
CCL24	Eotaxin-2/ MPIF-2	CCR3	Inducible

CCL25	TECK	CCR9	Dual-function
CCL26	Eotaxin-3	CCR3	Inducible
CCL27	CTACK/ILC	CCR10	Inducible
CCL28	MEC	CCR3, CCR10	Inducible

Systematic Names	**Alternate Names**	**Receptor(s)**	**Expression Profile**
C Chemokines			
XCL1	Lymphotactin/ SCM-1α	XCR1	Inducible
XCL2	SCM-1β	XCR1	Inducible
CX3C Chemokines			
CX3CL1	Fractalkine	CX3CR1	Inducible

1.7. Medicinal plants in Lebanon

Long time ago, plants have been the source of medicines for the treatment of many diseases. Nowadays, plants remain an important part of the health care in many countries, mainly the developing ones [100]. In 2000, the World Health Organization reported that a big percentage of the world's population depends on plants as the main source for the treatment of many diseases in the primary health care [101].

In the last years, herbal medicine has been gaining interest in the scientific research, specifically, regarding cancer prevention or treatment [102]. It is still highly used in the Mediterranean region, where a high percentage of individuals collect and consume wild edible plants as part of their traditional source of food with low health risks [35]. In fact, epidemiological studies provide robust evidence for a protection effect of the Mediterranean diet against cardiovascular disease and cancer [97].

Lebanon is endowed with a very large flora consisting of 783 genera and 2,607 species, out of which 78 are endemic [103, 104]. Its geographical location, eastern to the Mediterranean Sea, has contributed to a varied topography with different elevations, a

diversity of soil types, and a variety of climatic zones as well as microclimates ranging from dry and hot to temperate and humid [103, 105]. All of these factors resulted in a relatively small geographical area with a very diverse flora adapted to several habitats. Being at the “meeting point of three continents” [103], Lebanon has had an important role in the Middle East as a trade center, and as a result obtained good knowledge and experience in herbal medicine. Old Lebanese generations relied heavily on herbal medicine, and herbs such as sage, thyme, rosemary, lavender, mint and chamomile, were commonly grown in gardens [106]. A report from Abou Chaar [107] listed the use of 138 plants commonly used in herbal medicine in Lebanon. Although a number of Lebanese plants have been described for their medicinal value, many other plants are yet to characterized for their biological activities and therapeutic potential.

Aim of the study

Plants could contain potent biochemical elements and components of phytomedicine. Plant-based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits and seeds. Plants are usually considered as more natural and more accessible source for medicine than manufactured drugs. Besides their nutritional value, a large number of plants have been found to contain ingredients that have antibacterial, antifungal, antioxidant, anti-inflammatory and anticancer activities. Lebanon is characterized by a large flora where a number of plant species has been reported to have important biological activities and therapeutic value for treating distinct ailments. Despite of that, many other Lebanese plants are yet to be characterized in terms of their chemical composition and medicinal value. In this study, two Lebanese plants, *Crataegus azarolus L* and *Ephedra campylopoda*, were examined in terms of their phytochemical content and biological properties upon achieving the following specific aims:

1- Perform phytochemical screening which will allow, through conventional testing, a qualitative evaluation of different compounds present in different parts (leaves and stems) of the two selected plants.
2- Determination of polyphenols and flavonoids contents in different plant extracts using different chromatographic techniques.
3- Evaluate the antioxidant activity through several tests such as: DPPH radical scavenging and Fe^{2+}-chelating activity assays.
4- Investigate the anti-inflammatory capacity by evaluating RAW 264.7 murine macrophage cells-mediated secretion of PGE_2, using ELISA technique, and quantifying the mRNA levels of the proinflammatory cytokines (IL-α, IL-β and IL-6), chemokines (CCL3 and CCL4) and inflammation-inducible COX-2 and iNOS using quantitative RT-PCR (qRT-PCR).
5- Evaluate the antiproliferative potential of these plant extracts by using the XTT viability assay.

Results

Chapter 1

Chemical composition, antioxidant, anti-inflammatory and antiproliferative activities of Lebanese *Crataegus azarolus* L. plant

Crataegus species (*Rosaceae*), known as "Hawthorn" comprise approximately 280 species which are native to Mediterranean region, North Africa, Europe and Central Asia. The genus name *Crataegus* is derived from a Greek word Kratos meaning hardness of wood [108]. Most species of Hawthorn have prominent, long, straight and sharp thorn, ranging from 1-5 inches. In Mediterranean region, the predominant species of the genus *Crataegus* is *Crataegus azarolus L.* (*C. azarolus*) that populates the mountains of these areas [109]. In Lebanon, *Crataegus azarolus L.* is particularly localized in Khourbet-Qanafar, Kifraya, Rachaiya, Jabal Moussa, Baalchmay, Arz Ain-Zhalta and Rihane [110].

Azarole tree (*C. azarolus L.*) is a deciduous tree, growing up to 5-8 m high, with felty young branches showing a brown bark and a thorny tip. The leaves grow spirally arranged on long shoots, and in clusters on spur shoots on the branches or twigs. The leaf corresponds to a triangular cunate, and its base narrows gradually towards the petiole. Its end is cleaved into 3-5 lobes. It is 3-5 cm long, and its texture is rather leathery. The flowers are white, typical of the *Rosaceae*, and are arranged in dense clusters of 5-10 flowers. The calyx is campanulate, and its five lobes are rolled back. The corolla has 5 separate rounded leaves. It has a diameter of 1.5 cm and contains 2 styles and 5-25 stamens. The fruit is called the azerole; it is up to 25 mm in diameter and contains 1-3 large seeds. When it matures, its skin color changes from white cream to yellow (Figure 11) [111].



A B C

Figure 11: Different parts of *C. azarolus L.*: A: leaves, B: flowers and C: fruits

Crataegus species have been traditionally used for treating cardiovascular diseases including congestive heart failure, angina, hypertension, peripheral vascular disease, hyperlipidaemia and diabetes mellitus [112]. In Turkey, they are used as therapeutic agent for treatment of cough, flu, asthma, stomach ache, rheumatic pain, nephritis, hemorrhoids, cardiac diseases [113]. As the fruits and flowers of this plant have a hypotensive effect, they are especially useful in the treatment of heart conditions associated with high blood pressure. In Algeria, the leaves of *C. azarolus* are recommended as hypotensive agent [114].

Different phytochemical studies focused on identifying the major components in hawthorn revealed that this plant contains: polyphenols, catechins, mainly (-)-epicatechin, oligomeric proanthocyanidins such as the prominent B2 dimeric procyanidin, and flavonoids such as hyperoside (flowers and fruits) and vitexin-2”-O-rhamnoside (leaves)). Many of these phenolic compounds are described to be cytoprotective upon reducing oxidative stress. HPLC analysis of flowers buds and open flowers of *C. azarolus* grown in Tunisia revealed the presence of two flavan-3-ols ((-)-epicatechin and procyanidin B2 dimer), the cholinergic acid and five flavonoids (hyperoside, rutin, spiraeoside, isoquercitrin and quercetin) [115].

Crataegus species possess immense medicinal applications, but a few species have been screened for their biological activities. In this study, we characterized the chemical composition and biological activities of Lebanese *C. azarolus L* plant.

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Chemical Composition, Antioxidant, Anti-Inflammatory, and Antiproliferative Activities of the Plant Lebanese *Crataegus Azarolus* L

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Data Interpretation D
Manuscript Preparation E
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Background: In the present study, phytochemical screening, antioxidant, anti-inflammatory, and antiproliferative capacities of 3 extracts from leaves of Lebanese *Crataegus azarolus* L. were evaluated.

Material/Methods: Fresh leaves were dissolved in 3 different solvents: distilled water, ethanol, and methanol. The chemical composition was determined using high-performance liquid chromatography (HPLC) and the content of essential oil of this plant was examined by gas chromatography (GC) coupled with mass spectrometry (MS). The antioxidant potential was evaluated using DPPH radical scavenging and Fe^{2+} chelating activity assays. Anti-inflammatory effect was investigated by measuring the secreted amounts of the proinflammatory mediator PGE_2 using ELISA technique, as well as by assaying the mRNA levels of the proinflammatory cytokines (IL-α, IL-β, and Il-6), chemokines (CCL3 and CCL4) and inflammation-sensitive COX2 and iNOS enzymes using quantitative real-time PCR (qRT-PCR). The antiproliferative effect was evaluated using the XTT viability assay.

Results: The obtained results show that alcohol (methanol and ethanol) extracts were rich in bioactive molecules with medical relevance and exerted substantial antioxidant, anti-inflammatory, and antiproliferative capacities. On the other hand, aqueous extract contained fewer chemical components and exhibited less therapeutic efficiency.

Conclusions: Our observations indicate that *Crataegus azarolus* L. could be used for treating diseases related to oxidative stress, inflammatory reactions, and uncontrolled cell growth.

MeSH Keywords: Anti-Inflammatory Agents • Antioxidants • Chemical Fractionation

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Background

It is well established that reactive oxygen species (ROS), such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), as well as reactive nitrogen species (RNS), including nitric oxide (NO) and nitric dioxide (NO_2), play a dual role as both beneficial and deleterious chemical components. When present in moderate amounts, ROS and RNS could play a beneficial role upon serving as signaling messengers regulating a number of physiological processes, including gene expression, cell growth, and orchestration of immune responses [1–4]. However, excess of these reactive molecules generates oxidative stress, a harmful process that can damage all biological macromolecules and cell structures [4–7]. Oxidative stress is generally considered as a risk factor triggering the development of various critical pathologies, including cancer, arthritis, atherosclerosis, diabetes, autoimmune disorders, and cardiovascular and neurodegenerative diseases [8,9]. Endogenous antioxidants (fabricated by the body), as well as exogenous ones (supplied through diet), are chemicals that interact with and neutralize the ROS and RNS molecules, thus preventing their toxic effect [8,10]. Historically, plants are well known for their medicinal value, mainly related to their phytochemical component content, including phenolic compounds, flavonoids, alkaloids, tannins, and other stress-responsive products [11–14]. Indeed, plant-derived antioxidants, especially polyphenolic compounds, have proven success in minimizing the levels of toxic free radicals and relieving different oxidative stress-mediated diseases [13,15,16]. In addition, daily intake of natural antioxidants has been correlated with reduced occurrence of different diseases, including cancer, diabetes, and cardiovascular diseases [17]. Moreover, the phenolics and flavonoids of various medicinal plants exhibit potent anti-inflammatory and antiproliferative capacities [12,18–20]. *Crataegus* (Hawthorn), belonging to the Rosacea family, comprises about 280 species that are mainly distributed in the northern temperate zones of North America, East Asia, Central Asia, and Europe [21]. Interestingly, hawthorn fruits have long been used in traditional medicine to treat different health concerns, mainly those related to the heart and blood vessels [22]. The pharmacological potential of hawthorn has been attributed to its important chemical composition, including proanthocyanidins, flavonoids, tannins, vitamin C, and glycosides [21,23]. Although the chemistry of different *Crataegus* species has already been described, the chemical composition of many other species is yet to be characterized. *Crataegus*, known as Zaarour in Lebanon, is represented by 3 species in the Lebanese flora: *C. azarolus* L., *C. monogyna* (Jacq), and *C. sinaica* (Boiss). So far, neither analytical nor biological studies have been performed on the Lebanese *Crataegus* species. In this study, we characterized the phytochemical component content, antioxidant, anti-inflammatory, and antiproliferative capacities of 3 extracts (water, ethanolic, and methanolic) prepared from fresh leaves of *Crataegus azarolus* L. grown in Lebanon.

Material and Methods

Plant collection and preparation of powders

Fresh leaves were gathered from southern Lebanon at 350 m altitude in spring season between March and May in 2011, and the biological authentication was carried out by Professor George Tohme, president of CNRS of Lebanon. After harvesting, they were well washed, cut into small pieces, and dried in the shade at room temperature, away from sunlight. After this period, the dried leaves were crushed and ground to a homogeneous fine powder by use of a grinder and then kept in the dark at room temperature until use in different studies.

Apparatus and chemicals

All of the used chemicals were of analytical grade. Absolute ethanol, methanol, n-hexane, sodium hydroxide, ethyl acetate, and dichloromethane were purchased from BDH England. Aluminium chloride and $FeSO_4 \cdot 7H_2O$, silica gel was purchased from Merck Germany. Sodium carbonate and hydrogen peroxide were purchased from Unichem India. Ascorbic acid, gallic acid, rutin, Folin-Ciocalteau reagent, EDTA, ferrozine, and DPPH were purchased from Sigma Aldrich, USA. PBS was purchased from Gibco, UK. MS spectra were recorded on an Agilent series device and MSMS spectra were recorded on a Shimadzu series device.

Preparation of crude extracts using water, ethanol, and methanol as solvents

Powdered leaves (100 g) were deposited into a flask with 500 ml of the selected solvent (distilled water, ethanol, or methanol). After a period of maceration and stirring for 1 week at room temperature, the macerate was collected and filtered using filter paper. Extracts were then concentrated using a rotary evaporator at 40°C under reduced pressure (for ethanol and methanol extracts). The aqueous extract was prepared using the same steps as the alcoholic extraction except the temperature of the extraction was 60°C and the filtrates were then frozen before being lyophilized to obtain powders.

Phytochemical screening

To determine the chemical composition of the different extracts from leaves of *C. azarolus*, qualitative tests were done to detect the presence of primary and secondary metabolites as shown in Table 1. These tests are useful to estimate biological activities that might be due to the presence of secondary metabolites in the leaves of this plant.

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Table 1. Detection of primary and secondary metabolites in leaves of *Crataegus azarolus* L.

Metabolites	Added reagent	Expected result
Alkaloids [36]	Dragendorff reagent	Red or Orange precipitate
Tanins [36]	$FeCl_3$ (1%)	Blue coloration
Resines [36]	Acetone + water	Turbidity
Saponines [37]	Agitation	Formation of foam
Phenols [36]	$FeCl_3$ (1%) + $K_3(Fe(CN)_6)$ (1%)	Green-blue coloration
Terpenoids [37]	Chloroform + H_2SO_4 conc	Reddish brown coloration
Flavonoids [38]	KOH (50%)	Yellow coloration
Carbohydrates [37]	α-naphtol + H_2SO_4	Purple ring
Reducing sugars [37]	Fehlings (A+B)	Brownish-red precipitate
Quinones [39]	HCl conc	Yellow precipitate
Sterols & steroids [37,38]	Chloroform + H_2SO_4 conc	Red color (surface) + fluorescence Greenish-yellow
Cardiac glycosides [37,38]	Glacial acetic acid + $FeCl_3$ (5%) + H_2SO_4 conc	Ring
Diterpenes [36]	Copper acetate	Green coloration
Anthraquinones [38]	HCl (10%) + chloroform + Ammonia (10%)	Pink coloration
Proteins & aminoacids [40]	Ninhydrin 0.25%	Blue coloration
Lignines [40]	Safranine	Pink coloration
Phlabotannins [41]	HCl (1%)	Blue coloration
Anthocyanines [42]	NaOH (10%)	Blue coloration
Flavanones [42]	H_2SO_4 conc	Bluish-red Coloration
Fixed oils and fats [37]	Spot Test	Oil stain

Gas chromatography – mass spectrometry (GC/MS) analysis

The GC/MS analysis was performed on an Agilent 7890A-GCMS device. In the separation and identification by GC/MS technique, components were identified on the basis of the retention time and spectral index from the NIST and WILEY library. The instrument specifications and analysis conditions adjusted are given below in Tables 2 and 3.

Liquid chromatography – mass spectrometry (LC/MS/MS) analysis

The LC/MS/MS analysis was performed on a Shimadzu-AB Sciex LCMSMS device for the detection. In the separation and identification by LC/MS/MS technique, components were identified on the basis of the retention time and mass spectral characteristics. The instrument specifications and analysis conditions adjusted are given below in Table 4.

Biological activities

DPPH radical scavenging assay

The antioxidant activity was assessed according to the method of Farhan et al. [24] using free radical DPPH. Increasing concentrations of extracts (0.05, 0.1, 0.2, 0.4, and 0.5 mg/ml) were prepared. We added 1 ml of each prepared dilution of each extract to 1 ml of DPPH reagent [0.15 mM]. The solutions were incubated in the dark at room temperature for 30 min and the absorbance was measured at 517 nm using a Gene Quant 1300 UV-Vis spectrophotometer. The DPPH-scavenging ability of leaf extracts was calculated according to the following equation:

$$\% \text{ scavenging activity} = [(\text{Abs control} - \text{Abs sample}) / \text{Abs control}] \times 100$$

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Table 2. The instrument specifications and analysis conditions for water extracts.

GC Program	
Oven Maximum Temperature	325 °C
Hold time	1 min
Post Run	50 c
Program	8c/min – 290 c – 11 min
Equilibration Time	3 min
Injection volume	1 µl
Front SS Inlet Mode	Split
Injector temperature	280 °C
Pressure	52.76 psi
Total Flow	6 ml/min
Split Ratio	5: 1
Split Flow	5 ml/min
Column	DB-5MS: 30 m×250 µm×0.25 µm
MS Source	230 c maximum 250 c
MS Quad	150 c maximum 200 c
Acquisition Mode	Scan
Solvent Delay	2.5 min
Low Mass	33
High Mass	500

Table 3. The instrument specifications and analysis conditions for methanol and ethanol extracts.

GC Program	
Oven Temperature Set point	35°C
Hold time	2 min
Post Run	60 c
Program	3 c/min – 320 c – 1 min
Equilibration Time	0.5 min
Injection volume	1 µl
Back SS Inlet Mode	Split
Injector temperature	300 °C
Pressure	11.192 psi
Total Flow	504 ml/min
Split Ratio	500: 1
Split Flow	500 ml/min
Column	TG-5MS: 30 m×250 µm×0.25 µm
MS Source	230 c maximum 250 c
MS Quad	150 c maximum 200 c
Acquisition Mode	Scan
Solvent Delay	2 min
Low Mass	1.6
High Mass	450

The control was prepared by mixing 1 ml DPPH with 1 ml of selected solvent. The blank was composed of 1 ml of the selected solvent.

Metal chelating activity

The chelation of ferrous ions by extracts was estimated by the method of Dinis et al. [24]. Briefly, 50 µl of $FeCl_2$ (2 mM) was added to 1 ml of different concentrations of the extract (500, 750, 1000, 1250, and 1500 µg/ml). The reaction was initiated by the addition of 0.2 ml of ferrozine solution (5 mM). The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured at 562 nm.

Anti-inflammatory activity

RAW 264.7, a murine monocyte/macrophage cell line, was grown in DMEM medium supplemented with 10% defined FBS and 1% penicillin G-streptomycin in an atmosphere containing 5% CO_2/95% air at 37°C. The macrophages were seeded in 12-well plates (1×10^6 cells/well) using fresh medium. After preincubation for 24 h, plates were cotreated with LPS at 100 ng/ml and 2 different concentrations of the drugs (100µg/ml and 50 µg/ml) in DMEM without FBS for 24 h (for RNA extraction and COX-2 activity).

PGE_2 immunoassay

PGE_2 amounts in culture medium were quantified in supernatants by enzyme immune assay using ELISA kits (R&D Systems), following manufacturer's guidelines.

Cell viability

Jurkat cells, corresponding to human leukemic T cell line, were seeded in 96-well plates (8×10^3 cells/well). The following day, cells were treated with the different extracts at concentrations

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Table 4. The instrument specifications and analysis conditions.

HPLC/Pump	Shimadzu/LC20AD
Mass spectrometer	API 4000/AB Sciex instruments
Component Name	Triple Quadrupole LC/MS/MS Mass Spectrometer
Source Temperature (at set point)	300°C
LC system Equilibration time	2 min
LC system Injection Volume	10 µl
Pumping Mode	Low pressure Gradient: Time (min) Module Events Parameter 0.01 Pumps ACN+ 0.1% Formic acid 0.0 0.10 Pumps ACN+ 0.1% Formic acid 20 6.00 Pumps ACN+ 0.1% Formic acid 90 9.00 Pumps ACN+ 0.1% Formic acid 90 9.50 Pumps ACN+ 0.1% Formic acid 0 12.00 System Controller Stop
Total Flow	0.3 ml/min
Autosampler model	SIL-20A/HT
Column	C18 (15 cm*0.2 mm*3.5 um)

ranging from 5 to 200 µg/ml for 24, 48, and 72 h and cell viability was detected using Cell Proliferation Assay, XTT (Gentaur, Belgium) as previously described [25]. The XTT (sodium 3'-1 (phenylaminocarbonyl)-3,4-tetrazolium-bis (4-methoxy- 6-nitro) benzene sulfonic acid) cell proliferation assay is an effective method to measure cell growth and drug sensitivity in tumor cell lines. XTT is a colorless or slightly yellow compound that when reduced becomes bright orange. Briefly, XTT is cleaved by the mitochondrial dehydrogenase in metabolically active living cells to form an orange formazan dye. The absorbance of each sample was measured with a spectrophotometer at a wavelength of 450 nm.

Quantitative real-time PCR

Total RNA was extracted with Trizol reagent according to the manufacturer's guidelines (Invitrogen, Merelbeke, Belgium) and first-strand cDNAs were synthesized by reverse transcription (Superscript First-strand Synthesis System for RT-PCR kit; Invitrogen, Merelbeke, Belgium). Quantitative mRNA expression for the different genes was measured by real-time PCR with the PRISM 7900 sequence detection system (Applied Biosystems, Gent, Belgium), and the SYBR Green Master mix kit with β-actin mRNA was used as an internal control. The primers used for the amplification of each of the genes are indicated in Table 5. The program used for amplification was: 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. All qPCR reactions were performed in triplicate. The expression levels ($2^{-\Delta\Delta Ct}$) of mRNAs were calculated as described previously [26].

Table 5. List of primers used in this study.

Primers	Sequence (5'-3')
IL-1α-FO	GAATGACgCCCTCAATCAAAGT
IL-1α-RE	TCATCTTGGGCAGTCACATACA
IL-1β-FO	CCTTCCAGGATGAGGACATGA
IL-1β-RE	TGAGTCACAGAGGATGGGCTC
IL-6-FO	GAGGATACCACTCCCAACAGACC
IL-6-RE	AAGTGCATCATCGTTGTTCATACA
CCL3-FO	CAGCCAGGTGTCATTTTCCT
CCL3-RE	CTGCCTCCAAGACTCTCAGG
CCL4-FO	AAAACCTCTTTGCCACCAATACC
CCL4-RE	GAGAGCAGAAGGCAGCTACTAG
COX2-FO	CAGACAACATAAACTGCGCCTT
COX2-RE	GATACACCTCTCCACCAATGACC
iNOS-FO	GCAGAATGTGACCAT CATGG
iNOS-RE	ACAACCTTG GTGTTGAAG GC

Statistical analysis

The data are presented as means ±SEM of at least 3 independent experiments and analyzed using Student's *t*-test to determine any differences between group means, using SPSS for

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Table 6. Phytochemical screening of *C. azarolus*. L. leaf extracts. Key: –, absent; +, low in abundance; ++, moderate in abundance; +++, high in abundance.

	Aqueous	Methanol	Ethanol
Alkaloids	–	+++	+
Tannins	–	–	–
Resins	+	+++	+++
Saponins	+++	–	–
Phenol	+++	+++	+++
Terpenoids	+++	–	–
Flavonoids	+++	+	+
Quinones	–	+++	+++
Coumarin	–	–	–
Carbohydrates	+	+	–
Amino acids	+++	–	–
Sterols + Steroides	–	++	++
Cardiac Glycosides	++	+	+
Diterpenes	–	+++	+++
Anthraquinones	–	–	–
Reducing sugars	+++	+	–
Phlobatannins	+	+	–
Anthocyanins	–	–	–
Flavones	+	+	+
Lignin	+++	+++	+++
Fixed oil + Lipids	–	–	–

Windows (Version 21). P-Values <0.05 (*), <0.01 (**), <0.001 (***) were considered significant.

Results

Phytochemical screening of the leaves of *Crataegus azarolus* L.

Phytochemical screening of *C. azarolus* L. fresh leaf crude extract indicated the presence of some important bioactive components, which are listed in Table 6. The aqueous crude extract showed high concentrations of saponins, phenols, terpenoids, flavonoids, amino acids, reducing sugars, and lignin; moderate concentrations of cardiac glycosides; low concentrations of resins, carbohydrates, phlobatannins, and flavones; and absence of alkaloids, tannins, quinone, coumarin, sterols/steroids, diterpenes, anthraquinones, anthocyanin, and fixed oils and lipids. On the other hand, the methanolic crude extract showed high abundance of alkaloids, resins, phenol, quinones, diterpenes, and lignin; moderate abundance of sterols/steroids; low abundance of flavonoids, carbohydrates, cardiac glycosides, reducing sugars, phlobatannins and flavones; and absence of tannins, saponins, terpenoids, coumarin, amino acids, anthraquinones, anthocyanin, and fixed oils and lipids. The ethanolic crude extract exhibited high amounts of resins, phenol, quinones, diterpenes and lignin; moderate amounts of sterols/steroids; low amounts of alkaloids, flavonoids, cardiac glycosides and flavones; and absence of tannins, saponins, terpenoids, coumarin, carbohydrates, amino acids, anthraquinones, reducing sugars, phlobatannins, anthocyanin, and fixed oils and lipids. Altogether, these observations indicate that the different solvents used had preferential extraction of some phytochemicals from *C. azarolus* leaves.

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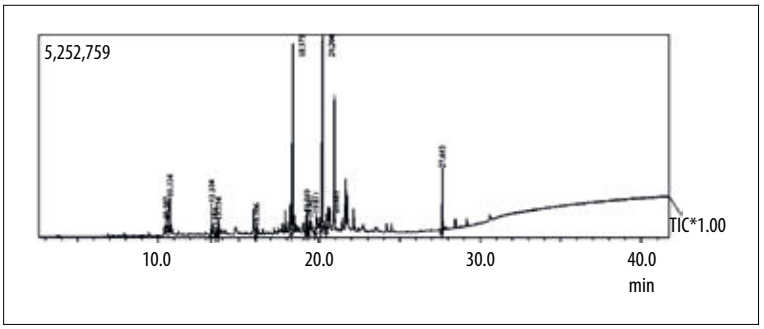


Figure 1. GC chromatogram of the water extract of *C. azarolus* L. leaf.

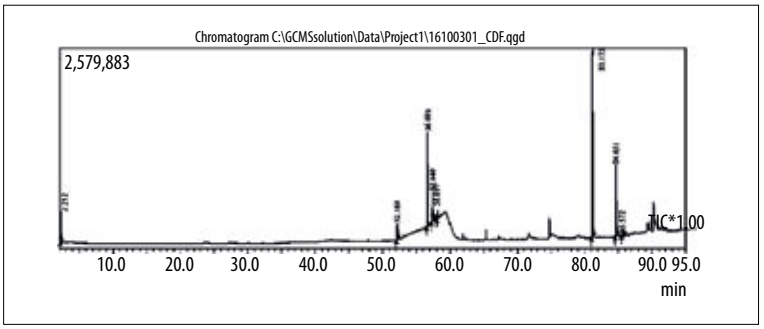


Figure 2. GC chromatogram of the ethanol extract of *C. azarolus* L. leaf.

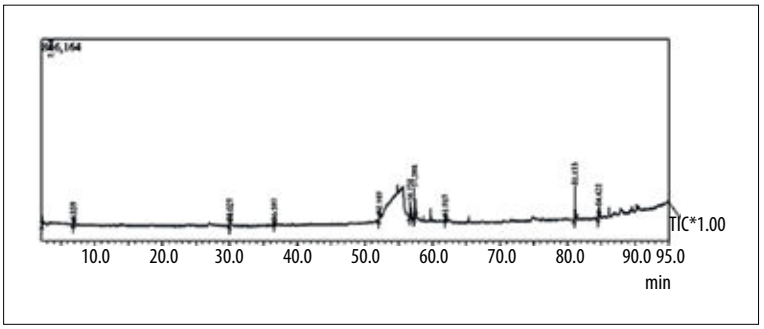


Figure 3. GC chromatogram of the methanol extract of *C. azarolus* L. leaf.

GC/MS Analysis of essential oil obtained from the *C. azarolus*. L leaf extracts

The GC spectrum of the water, ethanolic, and methanolic extracts are shown in Figures 1–3, respectively. A total of 11 compounds present in the water extract, 7 compounds present in the ethanolic extract, and 8 compounds present in the methanolic extract were determined by the chromatographic method with the help of NIST and WILEY library as shown in Tables 7–9, respectively. In the case of water extract, pluchidiol compound was found to be in the highest concentration (33.62%) and other compounds were found in trace amounts (Table 7). In the case of ethanolic extract, γ-tocopheryl methyl compound was found to be in the highest concentration (43.73%) followed by phytol isomer (20.47%), and other compounds were found in trace amounts (Table 8). In the case of methanolic extract, α tocopherol-beta-d-mannoside (21.87%), and ethyl linolinate (18.79%) compounds were found to be in the highest concentration and other compounds were found in trace amounts (Table 9).

The LC/MS/MS analysis for *C. azarolus* L leaf extracts

The LC spectrum results of the *C. azarolus* leaf extracts are shown in Table 10. A total of 2, 6, and 6 compounds were present in the water, ethanolic, and methanolic extracts, respectively, were determined by the chromatographic method based on the retention time and mass characteristics.

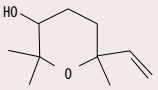
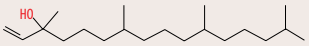
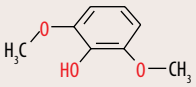
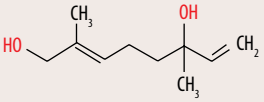
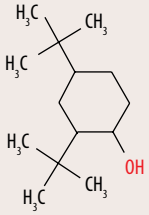
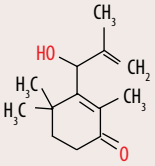
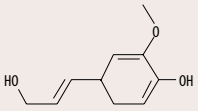
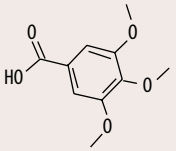
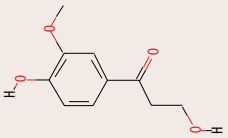
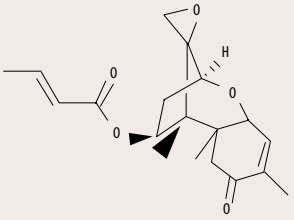
Antioxidant activity of *C. azarolus* L leaf extracts

The antioxidant activity of the aqueous, methanolic and ethanolic crude extracts was evaluated using 2 different assays: (1) DPPH free radical scavenging assay and (2) an assay assessing the iron (II) chelating ability. Our obtained results demonstrated that the different extracts displayed significant antioxidant activities and their scavenging effects on DPPH radical were in the following order: ethanolic extract (IC_{50}=50±5.2 µg/ml) >methanolic extract (IC_{50}=55±2.8 µg/ml) > water extract (IC_{50}=60±2.2 µg/ml) (Table 11).

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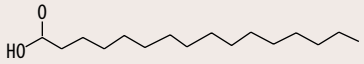
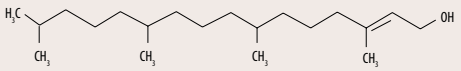
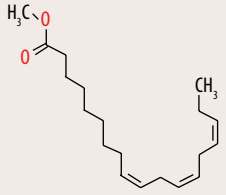
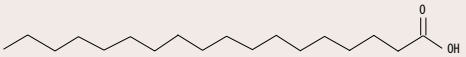
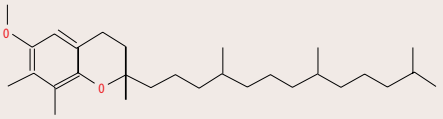
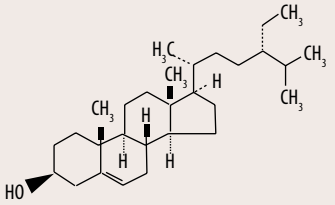
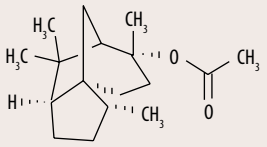
Table 7. Results of the GC-MS analysis of the water extract of the *C. azarolus.* L. leaf.

Peak#	RT	Name	MW	Structure	Molecular formula	Area %
1	10.507	Epoxylinalol	170.25		$C_{10}H_{18}O_2$	4.73
2	13.324	Isophytol	296.54		$C_{20}H_{40}O$	3.03
3	13.584	Syringol	154.163		$C_8H_{10}O_3$	0.58
4	13.754	8-Hydroxylinalool	170.24		$C_{10}H_{18}O_2$	1.46
5	16.106	2,4- DI-T-Butylphenol	206.324		C14$H_{22}O$	0.85
6	19.240	4-Oxo-Beta-Isodamascol	208.297		$C_{13}H_{20}O_2$	3.04
7	19.422	Gamma-Hydroxyisoeugenol (Coniferol)	180.201		$C_{10}H_{12}O_3$	2.35
8	19.811	Gallic acid trimethyl ether	212.2		$C_{10}H_{12}O_5$	2.82
9	20.204	Pluchidiol	208	-----	$C_{13}H_{20}O_2$	33.62
10	20.483	Beta-Hydroxypropiovanillone	196.202		$C_{10}H_{12}O_4$	2.16
11	27.663	Trichothecin	332.39		$C_{19}H_{24}O_5$	5.67

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Table 8. Results of the GC-MS analysis of the ethanol extract of the *C. azarolus*. L. leaf.

Peak#	RT	Name	MW	Structure	Molecular formula	Area%
1	52.169	Palmitic acid	256.42		$C_{16}H_{32}O_2$	3.99
2	56.691	Phytol Isomer	296.53		$C_{20}H_{40}O$	20.47
3	57.440	9,12,15-Octadecatrienoic acid	278.436		$C_{18}H_{30}O_2$	7.44
4	58.077	Stearic acid	284.48		$C_{18}H_{36}O_2$	0.95
5	81.175	Gamma-Tocopheryl methyl	416.69		$C_{28}H_{48}O_2$	43.73
6	84.651	Gamma-Sitosterol	414.71		$C_{29}H_{50}O$	19.2
7	85.572	Cedryl acetate	264.41		$C_{17}H_{28}O_2$	0.72

Ferrous ion (Fe^{2+}) is a major preoxidant that upon interaction with hydrogen peroxide can lead to the generation of highly reactive hydroxyl radicals. The Fe^{2+} chelating assay is based on the principle that ferrozine can quantitatively form colored complexes with Fe^{2+}. However, when other chelating agents are present, the complex formation is disrupted and the extent of color reduction allows determination of the chelating activity of the coexisting chelator. Using the Fe^{2+} chelating assay, the antioxidant potential of aqueous, methanolic, and ethanolic crude extracts derived from fresh leaves was assessed upon determination of their abilities to bind Fe^{2+} in the presence of ferrozine. Methanolic extract had the highest Fe^{2+} chelating capacity (IC_{50}=0.5±0.08 mg/ml), followed by ethanolic extract (IC_{50}=1±0.08 mg/ml) and then aqueous extract (IC_{50}=1.5±0.07 mg/ml) (Table 11). Our observations indicate that the alcoholic extracts show more efficient antioxidant capacity than aqueous extract.

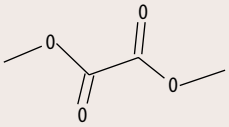
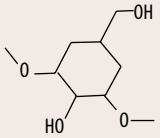
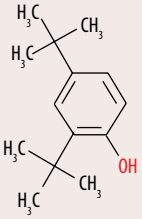
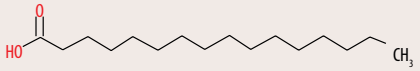
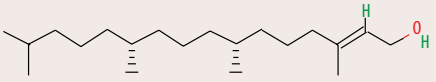
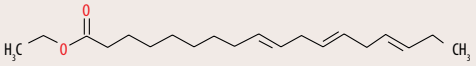
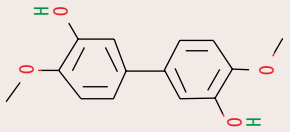
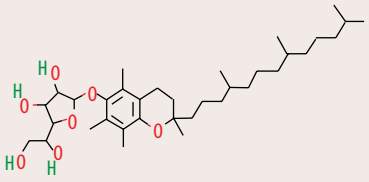
Anti-inflammatory activity of *C. azarolus* leaf extracts

Inflammatory response is a host's defensive mechanism against pathogens and is triggered by various microbial products such as lipopolysaccharide (LPS) [27]. Among the most important immune cells involved in this process are macrophages. Indeed, LPS can stimulate macrophages to produce large amounts of proinflammatory cytokines (such as IL-1α, IL-1β, and IL-6) and chemokine (including CCL3 and CCL4) [27] as well as other proinflammatory mediators including nitric oxide (NO) and prostaglandin E_2 (PGE_2) that are fabricated by the inflammation-inducible isoforms of NO synthase (iNOS) and cyclooxygenase-2 (COX-2) enzymes [28,29]. To assess the potential anti-inflammatory properties of *C. azarolus*. L leaf extracts, RAW 264.7 murine macrophage cells were used. These are capable of producing PGE_2 upon stimulation with LPS. Cells were treated for 24 h with either LPS (100 ng/ml) alone (control) or LPS together with different concentrations (50

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Table 9. Results of the GC-MS analysis of the methanol extract of the *C. azarolus*. L.leaf.

Peak#	RT	NAME	MW	Structure	Molecular formula	Area%
1	6.859	Oxalic acid dimethyl ester	118.09		$C_4H_6O_4$	1.58
2	30.027	Syringol	184.19		$C_9H_{12}O_4$	1.62
3	36.597	2,4 di-tert-butylphenol	206.324		$C_{14}H_{22}O$	1.51
4	52.103	Cetylic acid	256.42		$C_{16}H_{32}O_2$	2.56
5	56.738	Phytol	296.53		$C_{20}H_{40}O$	13.3
6	57.388	Ethyl linolinate	308.50		$C_{20}H_{34}O_2$	18.79
7	61.917	4,4'- biguaiacol	246.26		$C_{14}H_{14}O_4$	1.37
8	81.133	alpha-tocopherol-beta-d-mannosid	592.858		$C_{35}H_{60}O_7$	21.87

or 100 µg/ml) of aqueous, methanolic, and ethanolic crude extracts derived from fresh leaves. In a first step, and upon using quantitative real-time PCR (qRT-PCR), relative iNOS and COX-2 mRNA levels in leaf extract-treated RAW264.7 cells versus non-treated control cells were determined. In the case of COX-2, 100 µg/ml of aqueous extract were required to trigger about 50% reduction in mRNA levels (Figure 4A). Ethanolic and methanolic extracts were more potent in terms of inhibition of COX-2 transcription since 50 µg/ml of either extract was sufficient to reduce COX-2 mRNA levels by about half (Figure 4A). Interestingly, COX-2 transcription was nearly lost upon treating cells with 100 µg/ml of either ethanolic or methanolic extract (Figure 1A). In the case of iNOS, the 3 different extracts exhibited high efficiency in terms of impairing iNOS transcription, with ethanolic extract being the most potent, followed by methanolic extract and then aqueous extract (Figure 4B).

In a second step, and upon using ELISA technique, relative PGE_2 amounts present in the cell culture media were evaluated. Interestingly, all extracts were highly potent in terms of impairing PGE_2 production, with methanolic extract being the most efficient (Figure 4C).

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Table 10. Results of LC/MS/MS technique of *C. azarolus*. L. leaf.

C. azarolus L.	Compounds names	Retention time	Q1 Mass (Da)	Q3 Mass (Da)	CE	DP (V)
Water	Vitexin	4.96	431	341	–30	–40
	Hyperoside	4.97	463	301	–38	–40
Ethanol	Prunin	5.23	433	271	–20	–40
	Quercetin	5.87	301	179	–35	–40
	Rutin	4.89	609	301	–35	–40
	Vitexin	4.94	431	341	–30	–40
	Hyperoside	4.99	463	301	–38	–40
	Isoorientin	4.88	447	429	–30	–40
Methanol	Prunin	5.23	433	271	–20	–40
	Quercetin	5.88	301	179	–35	–40
	Rutin	4.89	609	301	–35	–40
	Vitexin	4.94	431	341	–30	–40
	Hyperoside	4.99	463	301	–38	–40
	Isoorientin	4.88	447	429	–30	–40

Table 11. DPPH free scavenging capacity (IC_{50}, µg/ml) and Ferrous-ion (Fe^{2+}) chelating ability (IC_{50}, mg/ml) of aqueous, methanol or ethanol extracts derived from fresh *C. azarolus* leaves. IC_{50} value corresponds to the effective concentration of sample required to scavenge DPPH radical or Ferrous-ion by 50%. Each value represents a mean ±SD (n=3).

		DPPH assay	Fe^{2+} chelating assay
	Extract	IC^{50}, µg/ml	IC_{50}, mg/ml
Fresh leaves	Aqueous	60±2.28	1.5±0.07
	Methanol	55±2.886	0.5±0.08
	Ethanol	50±5.207	1±0.08

In a third step, and upon using qRT-PCR, the relative expression of the proinflammatory cytokines IL-1α, IL-1β, and IL-6 was assessed. In the case of IL-1α, neither of the 2 utilized aqueous extract concentrations was able to significantly impair IL-1α transcription (Figure 4D). On the other hand, 100 µg/ml of either ethanolic or methanolic extract dramatically reduced IL-1α mRNA levels (Figure 4D). For IL-1β, aqueous extract was again not efficient in terms of reducing the mRNA levels (Figure 4E). On the other hand, 100 µg/ml of ethanolic extract reduced IL-1β mRNA levels to less than half their level in control cells (Figure 4E). Methanolic extract was more efficient in impairing IL-1β transcription, since minimal IL-1β mRNA levels were detected in cells treated with 100 µg/ml of methanol extract (Figure 4E). In the case of IL-6, no striking alteration in mRNA levels was detected in cells treated with 50 µg/ml or 100 µg/ml aqueous extract (Figure 4F). IL-6 mRNA levels were not largely reduced in cells treated with 50 µg/ml of ethanolic extract but was lost following treatment with 100 µg/ml (Figure 4F). The effect of methanolic extract was more robust since about 75% of IL-6 mRNA amount was lost in cells treated with 50 µg/ml (Figure 4F). IL-6 transcription was lost in cells treated with 100 µg/ml of methanolic extract (Figure 4F).

In a fourth step, qRT-PCR was carried out to determine the transcription profiles of the proinflammatory chemokines CCL3 and CCL4. These 2 chemokines exhibited comparable transcription profiles under the different studied conditions (Figure 4G, 4H). Neither CCL3 nor CCL4 transcription was lowered following treatment with either concentration of aqueous extract (Figure 4G, 4H). Where no reduction in neither CCL3 nor

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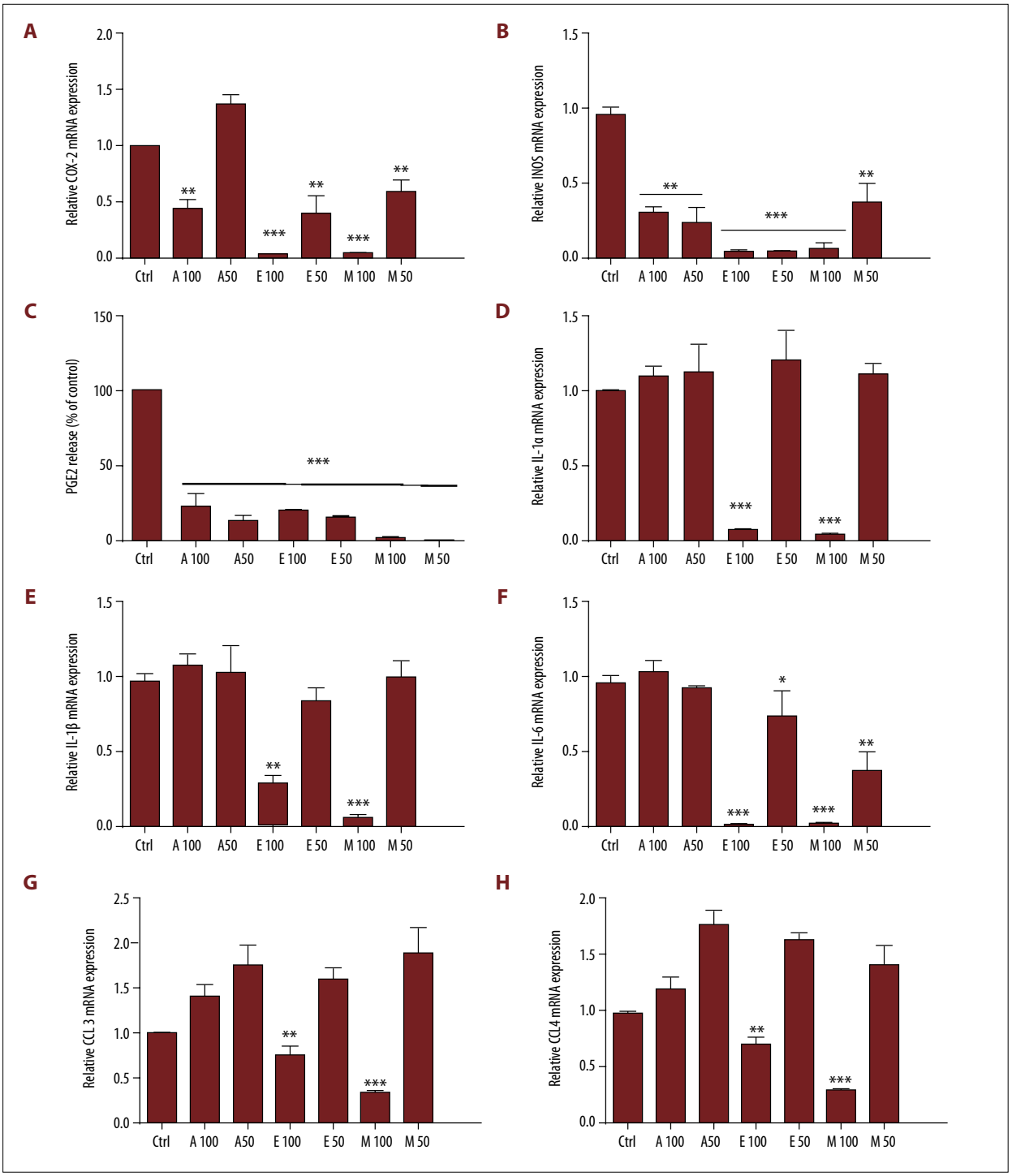


Figure 4. Effects of *C. azarolus* leaf extracts on LPS-Induced iNOS, COX-2, PGE_2, IL-1α, IL-1-β, IL-6, CCL3, and CCL4 levels in RAW 264.7 cells. Cells were treated for 24 h with 100 ng/ml LPS in the absence or presence of 50 or 100 µg/ml of either aqueous (A), ethanol (E), or methanol (M) extract. Total RNA was prepared and qRT-PCR was performed to quantify the mRNA levels of COX2 (**A**), iNOS (**B**), IL-1α (**D**), IL-1β (**E**), IL-6 (**F**), CCL3 (**G**), and CCL4 (**H**). The presented data correspond to the relative mRNA levels (values obtained in: RAW 264.7 cells treated with both LPS and extract/RAW 264.7 cells treated with only LPS). (**C**) Cell-free supernatants were collected and assayed for PGE_2 content via ELISA. The data correspond to the relative percentage of PGE_2. Reported values represent the averages ±SEM of 3 independent experiments (n=3) each done in triplicate. * $p<0.05$; ** $p<0.01$, *** $p<0.001$ *vs.* control untreated cells (Student's *t*-test).

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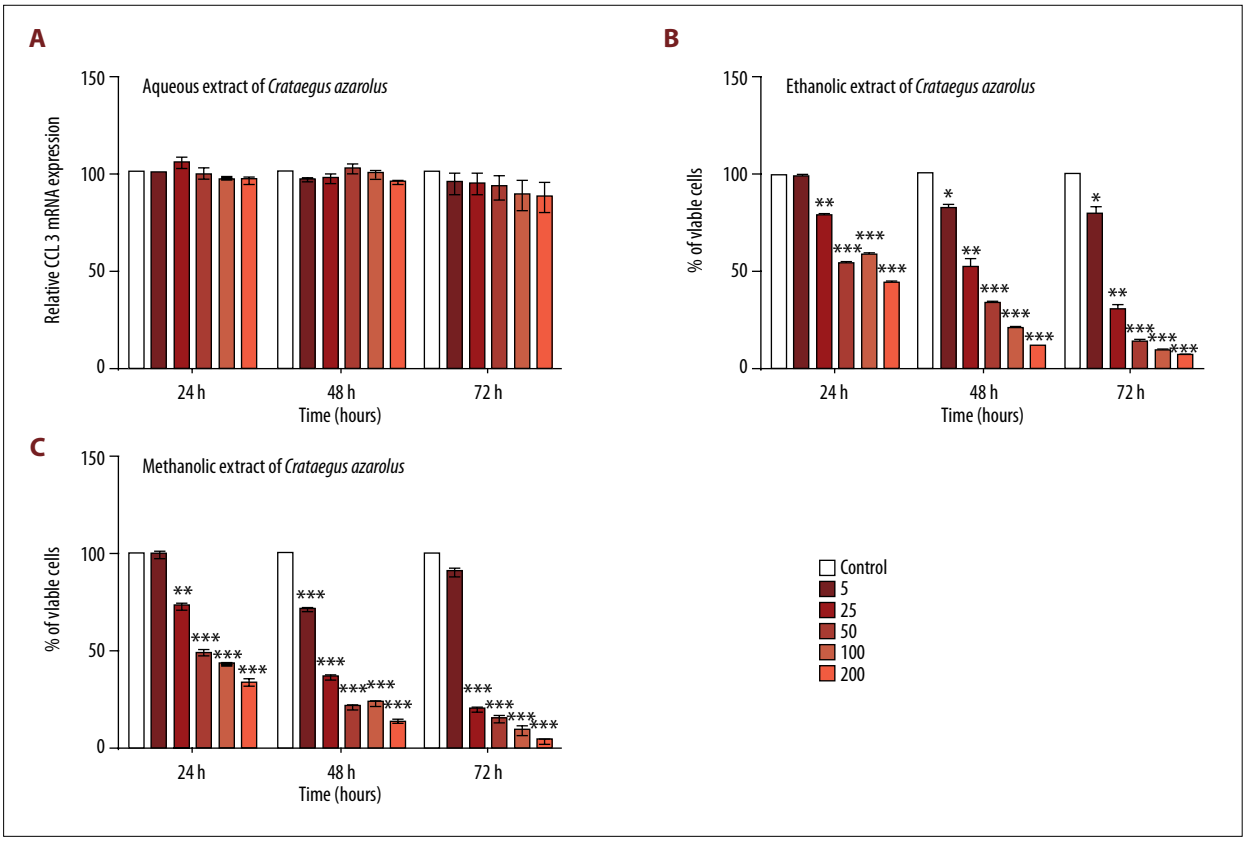


Figure 5. Effects of *C. azarolus* L leaf extracts on Jurkat cancer cell proliferation. Cells were treated with various concentrations (0, 5, 25, 50, 100, 200 µg/ml) of extracts for 24, 48, and 72 h and antiproliferative activities were measured by XTT assay. Each value represents a mean ± SEM for 3 independent experiments (n=3) each done in triplicate. Fresh leaf-derived aqueous extract (**A**), ethanol extract (**B**), and methanol extract (**C**). * $p<0.05$; ** $p<0.01$, *** $p<0.001$ *vs.* control untreated cells (Student's *t*-test).

CCL4 transcription was detected upon treating cells with 50 µg/ml of either ethanolic or methanolic extract, 100 µg/ml of the ethanolic extract reduced the mRNA levels of either chemokine by about 30%, and 100 µg/ml of methanolic extract decreased the mRNA levels by about 75% (Figure 4G, 4H).

Antiproliferative activity of *Crataegus azarolus* L. leaf extracts

To determine if *C. azarolus* L leaf extracts affect cell viability, the XTT assay was carried out. This is a colorimetric assay during which the yellow water-soluble substrate XTT is reduced to a highly colored formazan product by succinate dehydrogenase enzymes in metabolically active cells. This conversion takes place only in viable cells; therefore, the amount of the formed formazan is proportional to the concentration of viable cells in the sample. Jurkat cancer cells were treated with different concentrations (5–200 µg/mL) of either aqueous, methanolic, or ethanolic crude extracts for different periods of time (24, 48, or 72 h). The aqueous extract exerted no significant effect on cell viability in all tested conditions (Figure 5A), while ethanolic and methanolic extracts exerted dose- and time-dependent inhibitory effects (Figure 5B, 5C). In the case of ethanolic extracts, the IC_{50} value (dose required to inhibit cell growth by 50%) corresponded to 150±4.4 µg/ml after 24 h (Figure 5B), and prolonged treatment for 48 h and 72 h caused a more striking inhibition of cell growth, as the IC_{50} values were 25±1.6 µg/ml and 15±2.88 µg/ml, respectively (Figure 5B). Methanolic extract showed a more potent inhibitory effect than ethanolic extract; after 24 h of treatment with methanolic extract, the IC_{50} value corresponded to only 50 µg/ml and prolonged treatment reduced this value to 22±1.6 µg/ml (after 48 h) and 13±1.8 µg/ml (after 72 h) (Figure 5C).

Discussion

Plants have been used throughout history to cure human diseases. Worldwide, people still use medicinal plants for healing and relieving physical suffering. Many modern medicines have been derived either directly or indirectly from medicinal plants [30]. Many studies still focus on identifying new medicinal plants.

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In this study, we identified the chemical content of *Crataegus azarolus* L leaf extracts and assessed their therapeutic value. Interestingly, our phytochemical analysis showed the presence of various important medicinal components in the tested plant, including alkaloids, resins, phenol, quinones, diterpenes, lignin, sterols/steroids, flavonoids, carbohydrates, cardiac glycosides, reducing sugars, phlobatannins, and flavones. In agreement with previous observations [31–33], our data showed that the alcoholic solvents (ethanol and methanol) were more efficient than the aqueous one in extracting those bioactive compounds. Identifying such important medicinal components in *C. azarolus* species is not surprising since various *Crataegus* species studied so far contained valuable therapeutic chemical compounds and are widely used in clinical applications [23]. Despite the identified chemical components, the Lebanese *Crataegus azarolus* L plant could carry much more compounds in them. In this study, different parameters account for the limited number of identified components. For instance, the output of the GC/MS analysis, aimed to identify essential oils, is affected by the type of solvent used during the extraction method. Moreover, the limited number of chemical elements identified by LC/MS/MS analysis is due to the fact that this analysis was targeted towards only 11 flavonoid compounds, and the presence/absence of many other elements was not checked. Further, the chemical composition of this plant could also be affected by environmental and geographical parameters, including the year and the season of harvest, exposure to sunlight, the altitude, and the region where the plant was harvested.

To characterize the medicinal potential of the Lebanese *C. azarolus* species, we determined their antioxidant, anti-inflammatory, and antiproliferative effects.

Oxidative stress occurs when the number of free radicals and reactive biomolecules in a body exceeds the body's ability to neutralize and eliminate them. Oxidative stress has deleterious effects on human health since it forms the basis for a variety of critical diseases affecting the heart, brain, kidney, liver, lungs, eyes, blood, skin, and joints [34]. Identifying new natural antioxidants is therefore highly valuable. In this study, 2 different methods, DPPH-scavenging ability and Fe^{2+}-chelating activity assays, identified an antioxidant potential of *C. azarolus* extracts, with varied efficiency according to the type of used solvent. Indeed, and in both assays, extracts prepared using the alcoholic solvents showed more potent antioxidant activity than aqueous extracts. This could be related to the prominent chemical content in the alcohol- *vs.* water-prepared extracts.

In addition to their cytotoxic effect, the reactive biomolecules might also trigger the initiation and/or amplification of inflammation via upregulation of different genes encoding for proinflammatory cytokines and molecules. Inflammation is believed to be associated with nearly all known chronic diseases, including heart diseases, diabetes, neurodegenerative disorders, autoimmune pathologies, and cancer [27,35]. Suppression of the inflammatory responses is therefore indispensable for treating these diseases. Interestingly, in the present study we evaluated the inhibitory potential of the indicated plant on both the secreted amounts of PGE_2 and the transcription levels of the proinflammatory cytokines (IL-α, IL-β and Il-6) and chemokines (CCL3 and CCL4), as well as COX-2 and iNOS enzymes. The different plant extracts showed varied anti-inflammatory capacities in a manner dependent on the type of solvent. Although the aqueous extract showed only moderate anti-inflammatory capacity, the alcoholic extracts strongly suppressed all of the mentioned proinflammatory mediators, with methanolic extract being more efficient than ethanolic extract. This robust anti-inflammatory potential is in agreement with their chemical arsenal and antioxidant activity. *Crataegus azarolus L* leaves could be then used to treat inflammatory diseases or serve as a promising resource for developing inflammatory-suppressive drugs.

In this study, the antiproliferative activity of the different extracts from leaves of *Crataegus azarolus L* was investigated in Jurkat cancer cells by XTT viability assay. In contrast to the aqueous extract, which failed to inhibit Jurkat cells proliferation, the alcoholic extracts substantially suppressed cell growth, with methanolic extract showing a more potent suppressive capacity than ethanolic extract. This inhibitory effect was time- and dose-dependent. The rich phytochemical arsenal identified in the alcoholic extracts might explain their robust antiproliferative potential. However, the molecular mechanisms accounting for this cytotoxicity are still unclear. Whether components of the apoptotic pathway are involved remains to be investigated. Moreover, whether *Crataegus azarolus L* leaf extracts could suppress the proliferation of different cancer cell types will be addressed in our future work.

Conclusions

In conclusion, the present study revealed the presence of different medicinal compounds in the leaves of Lebanese *Crataegus azarolus L*. Due to its substantial antioxidant, anti-inflammatory, and antiproliferative activities, this plant might offer a novel promising therapy that is beneficial for general health.

Acknowledgments

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Conflict of interest

None.

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Chapter 2

Chemical composition, antioxidant, anti-inflammatory and antiproliferative activities of Lebanese *Ephedra campylopoda* plant

Ephedra corresponds to a genus of gymnosperms including over 40 species of tropical and subtropical, small, much-branched shrubs founds in the dry regions of both hemispheres. *Ephedra* is the only member of the family *Ephedraceae*. These plants, somewhat similar to the horsetails in appearance, are evergreen and their branches remain green for several seasons [116]. In Mediterranean region, *Ephedra campylopoda* is mainly found in Palestine and Jordan. In Lebanon, it is especially localized in Aandqet, Thoum, Monsef, Jiddayel, Chouane, Hazmieh, Saadiyat. [110].

Ephedra campylopoda is a clumbing shrubby tree in bushes beinvery ramified (Figure 13). The branches are long and distorted, the twigs are whorled often retrorse. The male and female plants are independent: male flowers have 5-6 anthers in globulous heads and female plants have 2-3 glabrous involucral bracts. *Ephedra* plants are dioecious, each cone or fertile shoot bearing one or more spore cases. The cones are ovoid or subspherical and are made up of several crossed pairs of bracts borne on short axes. The fruits may be berry-like and red (Figure 12), and those of some species are edible or used for medicinal purposes [116].



A B

Figure 12: Different parts of *Ephedra campylopoda*: A: stems, B: fruits.

For at least 5,000 years, the *Ephedra* species have been dispensed in traditional Chinese medicines (TCM) [117], where dried stems are used to alleviate symptoms caused by common cold, influenza, asthma, bronchitis, nasal congestion and hay fever. They were also used for a treatment of arthritis, fever, hives, lack of perspiration, headache, aching joints and bones, wheezing, and low blood pressure [118]. It is noteworthy that the tissue used in TCM is the dried green stem of one of three *Ephedra* species (*Ephedra sinica*, *E. equisetina* and *E. intermedia*), which are usually boiled in water and administered as a hot tea. In contrast to the diaphoretic uses and properties of ma-huang (stem part), the root and rhizome of *Ephedra* species, called mahuanggen, have antiperspirant property and are employed to treat spontaneous and night sweating [119]. In Western medicine, ephedrine is used for the treatment of nasal congestion due to hay fever, allergic rhinitis, asthma, and common cold [120]. Ephedrine salts are also prescribed in the form of nasal sprays to relieve congestion and swelling. When injected subcutaneously, ephedrine prevents hypotension during anesthesia. Orally administered ephedrine has been used in treating certain forms of epilepsy, nocturnal enuresis, myasthenia gravis, and urticaria accompanying angioneurotic edema. Pseudoephedrine, taken orally, is an effective nasal decongestant [117].

The major active ingredients of *Ephedra* are alkaloids that constitute 0.5 to 2.5 percent of the total mass, and are referred to as ephedrine-type alkaloids [22]. The six optically active alkaloids that have been isolated from *Ephedra* species are (-)-ephedrine and (+)-pseudoephedrine, (-)-N-methylephedrine, (+)-N-methylpseudoephedrine, (-)-norephedrine, (+)-norpseudoephedrine. Usually, (-)-ephedrine is the major isomer that comprises 30 to 90 percent of total alkaloid fraction accompanied by (+)-pseudoephedrine, with trace amount of other ephedrine-type alkaloids [22]. The total content of ephedrine-type alkaloids depends on the species of *Ephedra*, time of year of harvest, weather conditions, and altitude where the plant grows This variation according to environmental conditions explains why some *Ephedra* containing dietary supplements of the same brand often show alkaloid content markedly differing from label claims and also variation among lots when analyzed chemically [121].

Till date, neither chemical content nor the therapeutic potential of Lebanese *Ephedra campylopoda* has been characterized. In this study, we assessed the chemical composition and the biological activities of this plant.

Chemical composition, antioxidant, anti-inflammatory and antiprolferative activities of Lebanese Ephedra Campylopoda plant

Pharmacological potential of Ephedra Campylopoda

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All authors declare no conflict of interest

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Abstract

Background: This study aims to identify the phytochemical content and evaluate the antioxidant, anti-inflammatory and antiproliferative capacities of various solvent extracts of Ephedra Campylopoda stems.

Material and Methods: Fresh stems were suspended in three different solvent systems including distilled water, ethanol, and methanol. The chemical composition was determined using high performance liquid chromatography (HPLC) and the content of essential oil of this species was determined by gas chromatography (GC) coupled with mass spectrometry (MS). Antioxidant activity was determined using DPPH radical scavenging and Fe^{2+}-chelating activity assays. Anti-inflammatory capacity was estimated by both evaluating RAW 264.7 murine macrophage cells-mediated secretion of PGE_2, using ELISA technique, and quantifying the mRNA level of the proinflammatory cytokines (IL-α, IL-β and IL-6), chemokines (CCL3 and CCL4) and inflammation-inducible COX-2 and iNOS enzymes using quantitative Real-time PCR (qRT-PCR). The antiproliferative potential was determined using the XTT viability assay.

Results: Our results showed that the alcoholic extracts were better than the aqueous one in terms of their chemical composition. In parallel, the alcoholic extracts showed more potent antioxidant, anti-inflammatory and antiproliferative capacities than aqueous extract.

Conclusion: Our observations suggest that Ephedra Campylopoda plant could be a promising resource of natural products with antioxidant, anti-inflammatory and antiproliferative capacities.

Keywords: *Ephedra Campylopoda*, chemical composition, antioxidant activity, anti-inflammatory activity, antiproliferative effect

Background

Oxidative and inflammatory processes are described to be associated with a number of chronic diseases including atherosclerosis, Alzheimer's disease, cardiovascular diseases, neurodegenerative disorders as well as various human cancers [1]. Inflammatory response is a major defensive mechanism against infection, during which, inflammatory cells, such as macrophages, produce reactive oxygen species (ROS) and nitric oxide (NO) [2]. At low concentrations, these molecules serve important physiological role upon acting as second messenger in cell signaling. However, at higher amounts, these components can damage cellular lipids, proteins and DNA, leading to cell death [3–6]. To neutralize the toxic effect of these bioactive molecules, human body has evolved different defense mechanisms including the generation of antioxidants [7,8]. Oxidative stress is a result of imbalance between ROS formation and endogenous antioxidant capacity due to excessive ROS production and/or impaired antioxidant system. Identifying exogenous sources of antioxidants and anti-inflammatory molecules is therefore of great importance. Plants have traditionally been used, for thousands of years, for treating inflammation and oxidative stress related disorders. Plants' medicinal value is mainly attributed to their phytochemical component content especially phenolic compounds and flavonoids that can exert potent antioxidant and anti-inflammatory effects [9–14]. In the Middle East region, herbal medicines are extensively used and a growing interest in identifying medicinal plants has been noted during the past years. Indeed, more than hundered of plant species, known for their medicinal value, have been isolated from this region [15,16] where many other plants are yet to be characterized. Lebanon, thanks to its geographic location, varied topography, distinct soil types and climatic variations, is characterized by a relatively large flora consisting of about 2607 species distributed over 783 genera. Among these, few hunderd species are used to treat various diseases including

gastrointestinal disorders, kidney and urinary diseases, cardiovascular diseases, diabetes, asthma, sexual disoders, hair problems and various tumours [17,18]. Nowadays, as many studies are focused on characterizing the therapeutic value of Lebanese plants, the list of Lebanese medicinal plants is expected to enlarge. The genus Ephedra Campylopoda belongs to the Ephedraceae family of plants. Those plants are small, leafless, highly branched shrubs, distributed in the dry regions of both hemisphers [19]. In Lebanon, Ephedra Campylopoda is found in different regions mainly rocky ones. In this study, we screened the phytochemical component content and characterized the antioxidant, anti-inflammatory and antiproliferative capacities of three extracts from the stems of Ephedra Campylopoda.

Materials and Methods

Plant collection and preparation of powders

Fresh plant was gathered from South of Lebanon at 350 m of altitude on spring season between March and May in 2011 and the biological authentication was carried out by Professor George Tohme, president of C.N.R.S of Lebanon. After that, they were well washed, cut into small pieces and dried in the shade at room temperature, away from sun light. After this period, the dried stems were crushed up and ground to get homogeneous fine powder by a grinder and then kept in a dark place at room temperature till use in different studies.

Apparatus and chemicals

All of the used chemicals were of analytical grade. Absolute ethanol, methanol, n-hexane and sodium hydroxide, ethyl acetate, dichloromethane were purchased from BDH England. Aluminium chloride and $FeSO_4 \cdot 7H_2O$, silica gel was purchased from Merck Germany.

Sodium carbonate and hydrogen peroxide were purchased from Unichem India. Ascorbic acid, gallic acid, rutin, Folin-Ciocalteau reagent, EDTA, Ferrozine and DPPH were purchased from Sigma Aldrich, USA. PBS was purchased from Gibco, UK. MS spectra were recorded on Agilent series, MSMS spectra were recorded on Shimadzu series.

Preparation of crude extracts using aqueous, ethanol and methanol as solvents

Powdered stems (100 g) were deposited into a flask with 500 ml of the selected solvent (distilled water, ethanol or methanol). After a period of maceration and stirring for 1 week at room temperature, the macerate was collected and filtered using a filter paper. Extracts were then concentrated using a rotary evaporator at 40 °C under reduced pressure (for ethanol and methanol extracts). The aqueous extract has been prepared using the same steps of the ethanolic extraction except the temperature of the extraction should be 60 °C and the filtrates were then frozen before being lyophilized to obtain powders.

Phytochemical Screening

In order to study the chemical composition of the different extracts from the stems of the studied plant, qualitative tests have been done to detect the presence or the absence of primary and secondary metabolites as shown in table 1. These tests are useful to estimate some biological activities that might be due to the presence of some secondary metabolites in the stems of this plant.

Gas chromatography–mass spectrometry (GC/MS) Analysis

The GC/MS analysis was performed on Agilent 7890A-GCMS. In the separation and identification by GC/MS technique, components were identified on the basis of the retention time and spectral index from the NIST and WILEY library.

Liquid Chromatography–Mass Spectrometry (LC/MS/MS) Analysis

The LC/MS/MS analysis was performed on Shimadzu- AB Sciex LCMSMS for the detection. In the separation and identification by LC/MS/MS technique, components were identified on the basis of the retention time and mass spectral characteristics.

Biological analysis

DPPH radical scavenging assay

The antioxidant activity was practiced according to the method of Farhan et al. [20] using free radical DPPH. Increasing concentrations of extracts (0.05, 0.1, 0.2, 0.4, 0.5 mg/ml) were prepared. 1 ml of each prepared dilution of each extract was added to 1 ml of DPPH reagent. The solutions were incubated in the dark at room temperature for 30 minutes and the absorbance was measured at 517 nm by a Gene Quant 1300 UV-Vis spectrophotometer. The DPPH scavenging ability of peels extracts was calculated according to the following equation:

% scavenging activity = [(Abs control-Abs sample) / Abs control] ×100

Control was prepared by mixing 1 ml DPPH with 1 ml of selected solvent.

The blank was composed of 1 ml of the selected solvent.

Metal chelating activity

The chelation of ferrous ions by extracts was estimated by method of Dinis et al [21]. Briefly, 50 µl of $FeCl_2$ (2 mM) was added to 1 ml of different concentrations of the extract (500, 750, 1000, 1250, 1500 µg/ml). The reaction was initiated by the addition of 0.2 ml of ferrozine solution (5 mM). The mixture was vigorously shaken and left to stand at room temperature for 10 minutes. The absorbance of the solution was thereafter measured at 562 nm.

Anti-inflammatory evaluation of the extracts

RAW 264.7, a murine monocyte/macrophage cell line, was grown in DMEM medium supplemented with 10% defined FBS and 1% penicillin G-streptomycin under the atmosphere containing 5% CO2/95% air at 37 °C. The macrophages were seeded in 12 well plates (1×10^6 cells/well) using fresh medium. After preincubation for 24 h, plates were cotreated with LPS at 100 ng/ml and two different concentrations of the drugs (100 μg/ml and 50 μg/ml) in DMEM without FBS for 24h (for RNA extraction and COX-2 activity).

PGE_2 immuno assay

PGE2 amounts in culture medium were quantified in supernatants by enzyme immune assay, ELISA kits (R&D Systems), following manufacture's guidelines.

Cell viability

Jurkat cells, corresponding to human leukaemic T cell line, were seeded in 96-well plates (8×10^3 cells/well). The following day, cells were treated with the different extracts at concentrations ranging from 5 to 200 μg/ml for 24, 48, and 72 hours and cell viability was detected using Cell Proliferation Assay, XTT (Gentaur, Belgium) as previously described [22]. The XTT (sodium 3'-1 (phenylaminocarbonyl)-3,4-tetrazolium-bis (4-methoxy- 6-nitro) benzene sulfonic acid) cell proliferation assay is an effective method to measure cell growth and drug sensitivity in tumor cell lines. XTT is a colorless or slightly yellow compound that when reduced becomes brightly orange. Briefly, XTT is cleaved by the mitochondrial dehydrogenase in metabolically active living cells to form an orange formazan dye. The absorbance of each sample was measured with a spectrophotometer at a wavelength of 450 nm.

Quantitative Real-Time PCR

Total RNA was extracted with Trizol reagent according to the manufacturer's guidelines (Invitrogen, Merelbeke, Belgium) and first-strand cDNAs were synthesized by reverse transcription (Superscript First-strand Synthesis System for RT-PCR kit; Invitrogen, Merelbeke, Belgium). Quantitative mRNA expression for the different genes was measured by real-time PCR with PRISM 7900 sequence detection system (Applied Biosystems, Gent, Belgium), and the SYBR Green Master mix kit with β-actin mRNA used as an internal control. The program used for amplification was: 10 min at 95°C followed by 40 cycles of 15 s at 95°C, 1 min at 60°C. All qPCR reactions were performed in triplicate. The expression levels ($2^{-\Delta\Delta Ct}$) of mRNAs were calculated as described previously [23].

Statistical analysis

The data are presented as means ± SEM of at least three independent experiments and analyzed using Student's *t*-test to determine any differences between group means, using SPSS for Windows (Version 21). P-Values < 0.05 (*), < 0.01(**), < 0.001(***) were considered significant.

Results

Phytochemical screening of stems of Ephedra Campylopoda.

Phytochemical screening of the aqueous, ethanolic and methanolic extracts of fresh Ephedra Campylopoda stems identified the presence of different medically active compounds (Table 2). The aqueous crude extract showed high abundance of saponins, phenols, reducing sugars and lignin; average abundance of flavonoids, carbohydrates and amino acids; low abundance of quinones, cardiac glycosides and diterpenes; but absence of alkaloids, tannins, resins, terpenoids, coumarins, sterols/steroids, anthraquinones, phlobatannins, anthocyanin, flavones, fixed oils and lipids. On the other hand, the ethanolic crude extract exhibited high amounts of phenols and lignin; moderate amounts of flavonoids, quinones, carbohydrates, amino acids and sterols/steroids; low amounts of alkaloids, coumarins and diterpenes; but absence of tannins, resins, saponins, terpenoids, cardiac glycosides, anthraquinones, reducing sugars, phlobatannins, anthocyanin and fixed oils and lipids. Differently from the aqueous and ethanolic extracts, more constituents were present in the methanolic extract which displayed high presence of phenols, carbohydrates, sterols/steroids, flavones and lignin; moderate presence of tannins, quinones, amino acids, cardiac glycosides and phlobatannins; low presence of resins, terpenoids, flavonoids, coumarins, reducing sugars and anthocyanins; but absence of only alkaloids, saponins, anthraquinones and fixed oils and lipids. Altogether, these observations indicate that methanol was the best solvent, in comparison to aqueous and ethanolic solvents, to extract bioactive compounds present in the Ephedra Campylopoda stem.

GC/MS Analysis of essential oil obtained from the Ephedra Campylopoda stem extracts

The GC spectrum of the aqueous, ethanolic and methanolic extracts are shown in figures 1, 2 and 3, respectively. A total of 7 compounds present in the aqueous extract, 6 compounds present in the ethanolic extract and 7 compounds present in the methanolic extract were

determined by the chromatographic method with the help of NIST and WILEY library as shown in Tables 3, 4 and 5, respectively. In the case of ethanolic extract, Linolenic acid methyl ester compound was found to be in the highest concentration (77.97%) whilst other compounds were found in trace amount (Table 4). In the case of methanolic extract, trans-phytol compound was found to be in the highest concentration (39.17%) whilst other compounds were found in trace amount (Table 5).

The LC/MS/MS Analysis of the Ephedra Campylopoda stem extracts

The LC spectrum results of the *E. Campylopoda* stem extracts are shown in the Table 6. A total of 1, 6, and 6 compounds present in the aqueous, ethanolic and methanolic extracts respectively were determined by the chromatographic method based on the retention time and mass characteristics.

Antioxidant activity of Ephedra Campylopoda stem extracts

To investigate the antioxidant activities of the aqueous, methanolic and ethanolic crude extracts-derived from fresh stems, DPPH free radical scavenging assay was carried out in a first step. As antioxidants can react with the violet coloured stable free radical DPPH, and convert it into a yellow coloured α,α-diphenil-β-picrylhydrazine, this assay is based on quantifying the change of the reaction mixture color as a readout of the scavenging capacity of antioxidants towards DPPH. The different extracts showed varied antioxidant potential and their DPPH scavenging capacities were in the following order: ethanolic extract (IC_{50}=125 ± 4.4 µg/ml) > methanolic extract (IC_{50}=150 ±5.1 µg/ml) > aqueous extract (IC_{50}=300 ± 4.4 µg/ml) (Table 7).

Despites its beneficial roles as being required for oxygen transport, respiration and enzyme activity, iron is a highly reactive metal that can cause oxidative changes in proteins, lipids and

other structural components. Accordingly, in a second step, we characterized the antioxidant activities of the aqueous, methanolic and ethanolic crude extracts of E. Campylopoda stem by performing ferrous (Fe^{2+}) chelating activity assay. As Ferrozine can combine with Fe^{2+} to form coloured complex and since other chelating agents, when present, can disrupt the complex formation and thus reduces the extent of color, the ferrous (Fe^{2+}) chelating assay is based on determination of the change of the reaction mixture color as a readout of the chelating activity of the coexisting chelator. Our results showed that methanolic extract was the most efficient Fe^{2+} chelating capacity (IC_{50}=1 ±1.2 mg/ml) in comparison to both the ethanolic and the aqueous extracts which presented IC_{50} values of more than 1.5 mg/ml (Table 7).

Anti-inflammatory activity of Ephedra Campylopoda stem extracts

Inflammation is one form of host's defense strategies to combat against pathogenic intruders. Inflammation occurs when different immune cells, mainly macrophages, detect pathogen-associated molecular patterns (PAMPs) such as microbial lipopolysaccharide (LPS) [24]. Once activated, macrophages can then induce the expression of different pro-inflammatory cytokines (including IL-1α, IL-1β and IL-6), chemokine (such as CCL3 and CCL4) and other pro-inflammatory mediators including nitric oxide (NO) and prostaglandin E_2 (PGE_2) that are respectively synthesized by the induced isoforms of NO synthase (iNOS) and cyclooxygenase-2 (COX-2) enzymes [24]. To characterize the anti-inflammatory capacity of E. Campylopoda stem extracts, we used RAW 264.7 murine macrophage cells, which upon being stimulated with LPS, can produce PGE_2. Cells were treated, for 24 h, with either LPS (100ng/ml) alone (control), or LPS together with varying concentrations (50 or 100 µg/ml) of either aqueous, methanolic or ethanolic crude extracts-derived from fresh stems. In a first step, quantitative Real-Time PCR (qRT-PCR) was used to assay the relative iNOS and COX-

2 mRNA transcription in extract-treated RAW264.7 cells versus non-treated control cells. In the case of COX2, the mRNA levels were not significantly lowered in response to either of the utilized aqueous extract concentrations (Figure 4, Panel A). Interestingly, about 50-60% of COX2 mRNA levels were lost in response to either of the added ethanolic extract concentrations (Figure 4, Panel A). However, the methanolic extract was less efficient as only about 15% of COX2 mRNA levels were absent in response to 50 µg/ml but not 100 µg/ml of extract (Figure 4, Panel A). In the case of iNOS, the three different extracts were efficient in terms of impairing iNOS transcription with methanolic extract being the most efficient, followed by ethanolic extract and finally aqueous extract (Figure 4, Panel B).

In a second step, ELISA technique was used to assess the relative PGE_2 amounts present in the cell culture media. Intriguingly, the different extracts showed prominent capacity to dampen PGE_2 production (Figure 4, Panel C). Indeed, about 75%, 60% and 78% reduction in secreted PGE_2 levels were observed upon treating cells with 50 µg/ml of aqueous, ethanolic and methanolic extracts, respectively (Figure 4, Panel C). These levels were further increased to reach 85%, 99% and 95% in response to 100 µg/ml of aqueous, ethanolic and methanolic extracts, respectively (Figure 4, Panel C).

In a third step, qRT-PCR was applied to assay the relative expression of the pro-inflammatory cytokines IL-1α, IL-1β and IL-6. In the case of aqueous extract, neither of the two utilized concentrations was able to significantly reduce IL-1α (Figure 4, Panel D), IL-1β (Figure 4, Panel E) or IL-6 (Figure 4, Panel F). Interestingly, the ethanolic extract was highly efficient in impairing the transcription of the three tested cytokines. Indeed, treating cells with 50 µg/ml of ethanolic extract caused about 75% reduction in mRNA levels of either cytokine whereas 100 µg/ml of extract resulted in complete loss of transcription of the different cytokines (Figure 4, Panels D, E and F). Methanolic extract also showed efficiency, eventhough less than that of ethanolic extract, in lowering IL-1α, IL-1β and IL-6 transcription

levels. For instance, upon treating cells with 50 µg/ml of methanolic extract, the mRNA levels of IL-1α, IL-1β and IL-6 decreased by 60%, 10% and 60% respectively (Figure 4, Panels D, E and F). However, only minimal transcription of either cytokines was detected upon treating cells with 100 µg/ml of methanolic extract (Figure 4, Panels D, E and F).

In a fourth step, and upon performing qRT-PCR, the mRNA levels of the pro-inflammatory chemokines CCL3 and CCL4 were evaluated. In the case of CCL3, 50 µg/ml of either extract was enough to loose about 75% of mRNA levels (Figure 4, Panel G). Although this % was only slighltly increased to reach about 78% upon treating cells with 100 µg/ml of aqueous extract, it was more strikingly enhanced to reach about 99% and 95% in the case of ethanolic and methanolic extracts, respectively (Figure 4, Panel G). In the case of CCL4, aqueous extract moderately reduced CCL4 transcription as about 45% of mRNA levels were lost upon exposure of cells to 50 but not 100 µg/ml of extract (Figure 4, Panel H). On the other hand, both ethanolic and methanolic extracts exhibited prominent efficiency in terms of impairing CCL4 trasncription. Indeed, about 75% and 70% of CCL4 mRNA levels were absent following cell treatment with 50 µg/ml of ethanolic and methanolic extracts, respectively (Figure 4, Panel H). Moreover, only residual transcription was observed upon treating cells with 100 µg/ml of either extract (Figure 4, Panel H).

Antiproliferative activity of Ephedra Campylopoda stem extracts

In order to evaluate the antiproliferative capacity of Ephedra Campylopoda stem extracts, XTT assay was performed. This colometric assay measures cell viability upon determining mitochondrial activity. Indeed, XTT, yellow water-soluble substrate, can be converted, by mitochondrial succinate dehydrogenase enzymes, to a highly colored formazan product.This reaction occurs in viable but not dead cells and thus, the quantity of the generated formazan is proportional to the amount of viable cells in the sample. Jurkat cells were treated with various

fresh stem-derived aqueous, methanolic or ethanolic extract concentrations (in range from 5 to 200 µg/mL) for different times (24, 48 or 72 h). Neither of the utilized aqueous extract concentrations exerted a significant effect on cell viability at any of the indicated time periods (Figure 5, Panel A). However, a dose-dependent inhibitory effect was detected in the case of ethanolic and methanolic extracts (Figure 5, Panels B and C). Time-impact on the observed inhibition was not striking since, for both extracts, the IC_{50} values (dose required to inhibit cell growth by 50%) calculated at the different time intervals were comparable. For instance, in the case of ethanolic extract, the IC_{50} values obtained after 24, 48 and 72 h were 40±2.88, 38 ± 1.54 and 35 ±1.54 µg/ml, respectively (Figure 5, Panel B) whilst in the case of methanolic extract, the IC_{50} values corresponded to 40±1.45, 48 ±3.51 and 50 ±7.73 µg/ml after 24, 48 and 72 h, respectively (Figure 5, Panel C).

Discussion

Humans have used plants as medicine throughout the history. People all around the world still rely on herbs to relieve pain and heal sickness. Today, medicinal plants play an important role in modern medicine development since a large number of modern drugs are simple copies or synthetic modifications of natural chemical substances found in plants [25]. Nowadays, substantial research investments are skewed toward identifying and characterizing new medicinal plants. In the present study, we aimed at screening the chemical content of Ephedra Campylopoda stem extracts and characterizing their pharmaceutical value. Our phytochemical analysis identified the presence of various important medicinal compounds, such as phenols, flavonoids, carbohydrates, proteins, diterpenes and lignins in stem extracts being prepared using aqueous, ethanolic or methanolic solvents. Coumarins, sterols/steroids and flavones were found in both of the alcoholic solvents but not the aqueous one. Alkaloids were detected only in the ethanolic extracts whilst tannins, resin, terpenoids, reducing sugars, phlobatannins and anthocyanin were detected in the methanolic extracts, specifically. Consistently with

previous reports [26–28], our data indicate that the methanolic and ethanolic solvents extract are more rich, than aqueous one, in plant bioactive components. As the medicinal value of Ephedra Campylopoda species is yet not fully defined, we therefore assessed their antioxidant, anti-inflammatory and anti-proliferative activities.

Although they can mediate beneficial physiologic roles, overload of free radicals and reactive biomolecules that cannot be balanced or destroyed generates oxidative stress. This process is harmful for humans since it plays a major role in the initiation and development of chronic and degenerative pathologies such as cardiovascular and neurodegenerative diseases, autoimmune disorders, aging and cancer [29]. Identifying plants with potent antioxidant potential is therefore of great interest. Here, we assessed the antioxidant capacity of Ephedra Campylopoda extracts upon evaluating their ability to scavenge free DPPH radicals or chelate Fe^{2+} ions. Our data showed a varied antioxidant potential of Ephedra Campylopoda extracts, in a manner dependent on the type of utilized solvent. In fact, the alcoholic solvents appeared to exert more potent antioxidant effect than the aqueous extract. This observation parallels the observed more important chemical content in the alcoholic- than water-derived extracts.

Nowadays, a tight association is established between oxidative stress and inflammation since free radicals can be a result or a cause of inflammation. Inflammation has been identified as a primary cause of numerous chronic diseases including arthritis, atherosclerosis, Alzheimer's, diabetes, heart disease and cancer [30,31]. Immune signaling via proinflammatory cytokines and mediators is the major mechanism for inflammation initiation and amplification. Inhibiting the expression of these pro-inflammatory molecules, is therefore, a requisite to suppress the inflammatory responses. In this study, we evaluated the ability of the mentioned plant extracts to inhibit PGE_2 secretion and suppress the transcription of the proinflammatory cytokines (IL-α, IL-β and IL-6), chemokines (CCL3 and CCL4) and inflammation-responsive COX-2 and iNOS enzymes. Varied plant extract-mediated suppressive capacities on these

different pro-inflamatory components was observed. Indeed, alcoholic extracts were more efficient than aqueous one in impairing PGE_2 secretion and inhibiting IL-α, IL-β, IL-6, CCL3, CCL4, COX-2, and iNOS transcription. The prominent anti-inflammatory effect exerted by alcoholic extracts parallels their rich chemical arsenal as well as their substantial antioxidant activity. These observations highlight Ephedra Campylopoda as a putative promising resource for designing novel inflammatory suppressive drugs and treating inflammatory disorders.

In the present work, we assessed, using XTT viability assay, the ability of Ephedra Campylopoda stem extracts to inhibit Jurkat cancer cell proliferation. In line with their low phytochemical content and moderate antioxidant and anti-inflammatory capacities, aqueous extract was inefficient in terms of suppressing cell growth. However, the alcoholic extracts exerted prominent cytotoxic effect that appeared to be dose-dependent. This robust antiproliferative potential could be attributed to the prominent phytochemical content detected in the alcoholic solvents. So far, a mechanistic understanding of this cytotoxic effect is still lacking. A possible involvement of the apoptotic pathway in the observed impaired cell growth should be addressed in future studies. Moreover, whether Ephedra Campylopoda stem extracts are cytotoxic for cancer cells and thus can be used in tumour therapy is the objective of our ongoing work.

In conclusion, the present study identified the presence of various medicinal constituents in Ephedra Campylopoda stem extracts. Moreover, this plant exhibited prominent antioxidant, anti-inflammatory and antiproliferative capacities, thus can be suggested as a novel promising therapy for a wide array of human diseases.

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Tables

Table 1 : Detection of primary and secondary metabolites in stems of Ephedra Campylopoda

Metabolites	Added reagent	Expected result
Alkaloids [32]	Dragendorff reagent	Red or Orange precipitate
Tanins [32]	$FeCl_3$ (1%)	Blue coloration
Resines [32]	Acetone + water	Turbidity
Saponines [33]	Agitation	Formation of foam
Phenols [32]	$FeCl_3$ (1%) + $K_3(Fe(CN)_6)$ (1%)	Green-blue coloration
Terpenoids [33]	Chloroform + H_2SO_4 conc	Reddish brown coloration
Flavonoids [34]	KOH (50%)	Yellow coloration
Carbohydrates [33]	α-naphtol + H_2SO_4	Purple ring
Reducing sugars [33]	Fehlings (A+B)	Brownish-red precipitate
Quinones [35]	HCl conc	Yellow precipitate
Sterols & Steroids *(Khandelwal, 2005* ; [33]	Chloroform + H_2SO_4 conc	Red color (surface) + fluorescence Greenish-yellow
Cardiac glycosides *(Khandelwal, 2005* ; [33]	Glacial acetic acid + $FeCl_3$ (5%) + H_2SO_4 conc	Ring
Diterpenes [32]	Copper acetate	Green coloration
Anthraquinones [34]	HCl (10%) + chloroform + Ammonia (10 %)	Pink coloration
Proteins & aminoacids [36]	Ninhydrin 0.25%	Blue coloration
Lignines [36]	Safranine	Pink coloration

Phlabotannins [37]	HCl (1%)	Blue coloration
Anthocyanines [38]	NaOH (10%)	Blue coloration
Flavanones [38]	H_2SO_4 conc	Bluish-red Coloration
Fixed oils and fats [33]	Spot Test	Oil stain

Table. 2. Phytochemical screening of Ephedra Campylopoda stem extract using aqueous, methanol or ethanol as extraction solvents. Key: –, absent; +, low in abundance; ++, moderate in abundance; +++, high in abundance.

	Aqueous extract	Methanol extract	Ethanol extract
Alkaloids	-	-	+
Tannins	-	++	-
Resins	-	+	-
Saponins	+++	-	-
Phenol	+++	+++	+++
Terpenoids	-	+	-
Flavonoids	++	+	++
Quinones	+	++	++
Coumarin	-	+	+
Carbohydrates	++	+++	++
Amino acids	++	++	++
Sterols + Steroids	-	+++	++
Cardiac Glycosides	+	++	-
Diterpenes	+	++	+
Anthraquinones	-	-	-
Reducing sugars	+++	+	-
Phlobatannins	-	++	-
Anthocyanins	-	+	-
Flavones	-	+++	+
Lignin	+++	+++	+++
Fixed oil + Lipids	-	-	-

Table 3. Results of the GC-MS analysis of the water extract of the *E. Campylopoda* stem

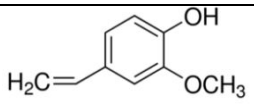
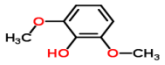
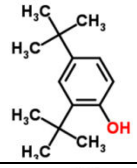
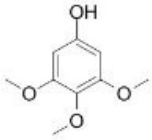
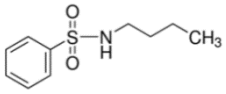
Peak#	RT	Name	MW	Structure	Molecular formula	Area%
5	12.981	Vinyl guaiacol	150.17		$C_9H_{10}O_2$	1.47
7	13.591	Syringol	154.163		$C_8H_{10}O_3$	3.48
8	16.107	di-tert-butylphenol	206.324		$C_{14}H_{22}O$	0.62
9	17.558	Antiarol	184.2		$C_9H_{12}O_4$	3.28
11	20.038	N-Butylbenzenesulfonamide	213.30		$C_{10}H_{15}NO_2S$	0.83
12	20.153	Pluchidiol	208	-----	$C_{13}H_{20}O_2$	7.85
13	22.149	Cetylic acid	256.42		$C_{16}H_{32}O_2$	4.8

Table 4. Results of the GC-MS analysis of the ethanolic extract of the *E. Campylopoda* stem

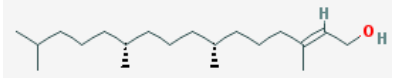
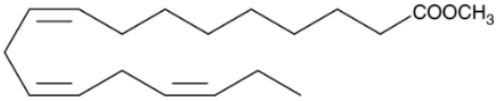
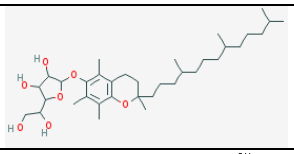
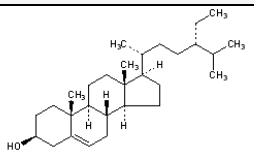
Peak#	RT	NAME	MW	Structure	Molecular formula	Area%
2	52.165	Palmitic acid	256.42		$C_{16}H_{32}O_2$	2.17
3	56.680	Phytol	296.53		$C_{20}H_{40}O$	5.04
4	57.222	Linoleic	280.452		$C_{18}H_{32}O_2$	0.7
5	57.450	Linolenic acid methyl ester	294.479		$C_{19}H_{34}O_2$	6.33
6	81.142	α-tocopherol-β-d-mannosid	592.858		$C_{35}H_{60}O_7$	2.82
7	84.636	γ-sitosterol	414.71		$C_{29}H_{50}O$	4.97

Table 5. Results of the GC-MS analysis of the methanolic extract of the *E. Campylopoda* stem

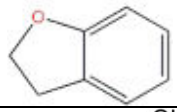
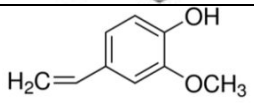
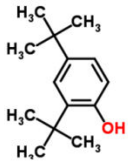
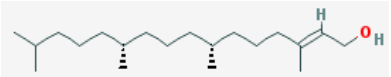
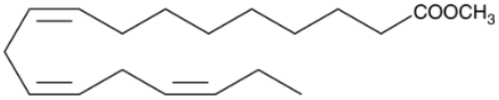
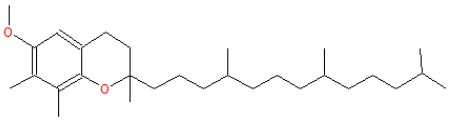
Peak#	RT	NAME	MW	Structure	Molecular formula	Area%
2	24.327	Coumaran	120.15		C_8H_8O	6.04
3	28.406	Vinylguaiacol	150.17		$C_9H_{10}O_2$	4.85
4	36.608	2,4-di-tert-butylphenol	206.324		$C_{14}H_{22}O$	4.15
5	52.114	Palmitic acid	256.42		$C_{16}H_{32}O_2$	4.19
6	56.677	Trans-phytol	296.53		$C_{20}H_{40}O$	39.17
7	57.383	Linolenic acid methyl ester	294.479		$C_{19}H_{34}O_2$	9.6
8	81.15	Vitamin E	430.717		$C_{29}H_{50}O_2$	3.31

Table 6. Results of LC/MS/MS technique of *E. Campylopoda* plant

E. CAMPYLOPODA	Compounds names	RETENTION TIME	Q1 Mass (Da)	Q3 Mass (Da)	CE	DP (V)
WATER	Vitexin	4.96	431	341	-30	-40
ETHANOL	Prunin	5.22	433	271	-20	-40
	Quercetin	5.88	301	179	-35	-40
	Rutin	4.86	609	301	-35	-40
	Vitexin	4.93	431	341	-30	-40
	Hyperoside	4.98	463	301	-38	-40
	Isoorientin	4.74	447	429	-30	-40
METHANOL	Prunin	5.24	433	271	-20	-40
	Quercetin	5.89	301	179	-35	-40
	Rutin	4.91	609	301	-35	-40
	Vitexin	4.96	431	341	-30	-40
	Hyperoside	5.02	463	301	-38	-40
	Isoorientin	4.79	447	429	-30	-40

Table. 7. DPPH free scavenging capacity (IC_{50}, µg/ml) and Ferrous-ion (Fe^{2+}) chelating ability (IC_{50}, mg/ml) of aqueous, methanol or ethanol extracts derived from fresh stems of Ephedra Campylopoda. IC_{50} value represents the concentration of sample required to scavenge DPPH radical or Ferrous-ion by 50%. Each value represents a mean ± SD (n = 3).

		DPPH assay	**Fe^{2+} chelating assay**
	Extract	**IC_{50}, µg/ml**	**IC_{50}, mg/ml**
Fresh stems			
	Aqueous	300 ±4.4	>1.5
	Methanol	150 ±5.1	1 ±1.2
	Ethanol	125 ±4.4	>1.5

Figure legends

Figure 1. GC chromatogram of the water extract of *E. Campylopoda* stem.

Figure 2. GC chromatogram of the ethanol extract of *E. Campylopoda* stem.

Figure 3. GC chromatogram of the methanol extract of E. Campylopoda stem.

Figure 4. Impact of Ephedra Campylopoda stem extracts on LPS-Induced iNOS, COX-2, PGE_2, IL-1α, IL-1-β, IL-6, CCL3 and CCL4 levels in RAW 264.7 cells. Cells were treated, for 24 h, with 100 ng/ml LPS in the absence or presence of 50 or 100µg/ml of either aqueous (A), ethanol (E) or methanol (M) extract. Total RNA was isolated and qRT-PCR was carried out to quantify the mRNA levels of COX-2 **(*Panel A*)**, iNOS **(*Panel B*)** IL-1α **(*Panel D*)**, IL-1β **(*Panel E*)**, IL-6 **(*Panel F*)**, CCL3 **(Panel G)** and CCL4 **(Panel H)**. The presented data correspond to the relative mRNA levels (values obtained in: RAW 264.7 cells treated with both LPS and extract/ RAW 264.7 cells treated with only LPS). ***Panel C.*** Cell-free supernatants were harvested and assayed for PGE_2 content via ELISA. The data correspond to the relative percentage of PGE_2. Reported values represent the averages ± SEM of three independent experiments (n=3) each done in triplicate. *p<0.05; **p<0.01, ***p<0.001 *vs.* control untreated cells (Student's *t*-test).

Figure 5. Impact of Ephedra Campylopoda stem extracts on Jurkat cells proliferation. Cells were treated with various concentrations (0, 5, 25, 50, 100, 200 µg/ml) of stem extracts for 24, 48 and 72 h and XTT assay was used to assess their antiproliferative potential. Each value represents a mean ± SEM for three independent experiments (n=3) each done in triplicate. Fresh stem-derived aqueous extract (Panel A), ethanol extract (Panel B) and methanol extract (Panel C). *p<0.05; **p<0.01, ***p<0.001 *vs.* control untreated cells (Student's *t*-test).

Figure 1

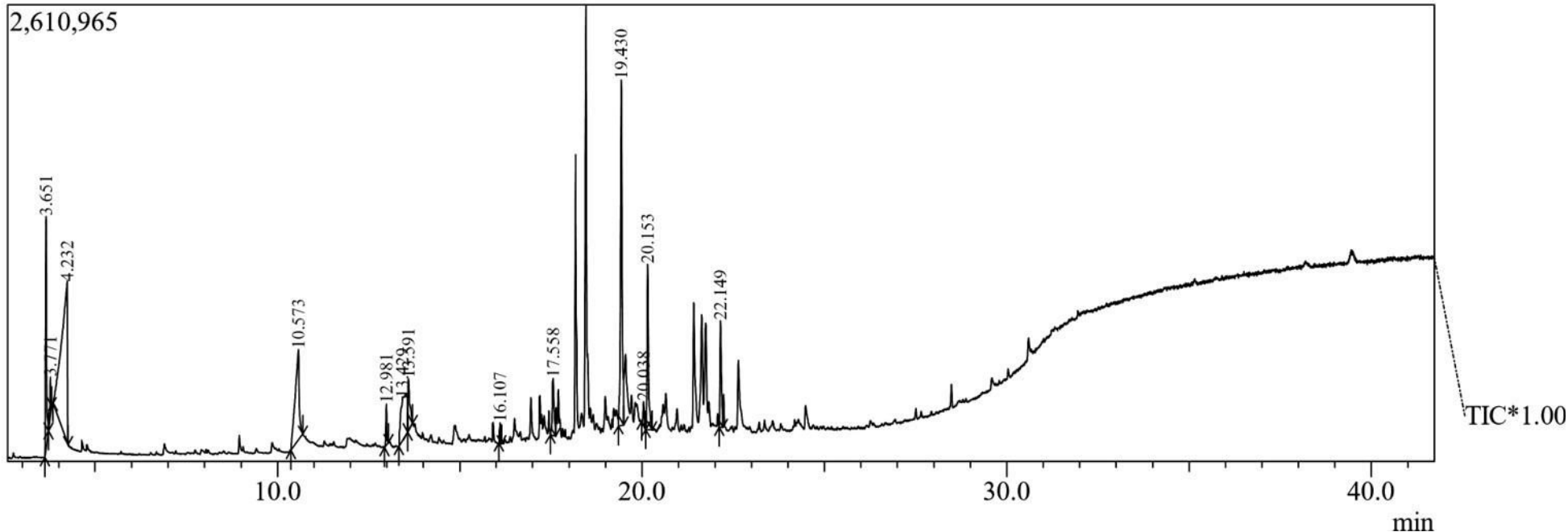

2,610,965
3.651
4.232
10.573
12.981
13.429
13.591
16.107
17.558
19.430
20.153
20.038
22.149
TIC*1.00
10.0
20.0
30.0
40.0
min

Figure 2

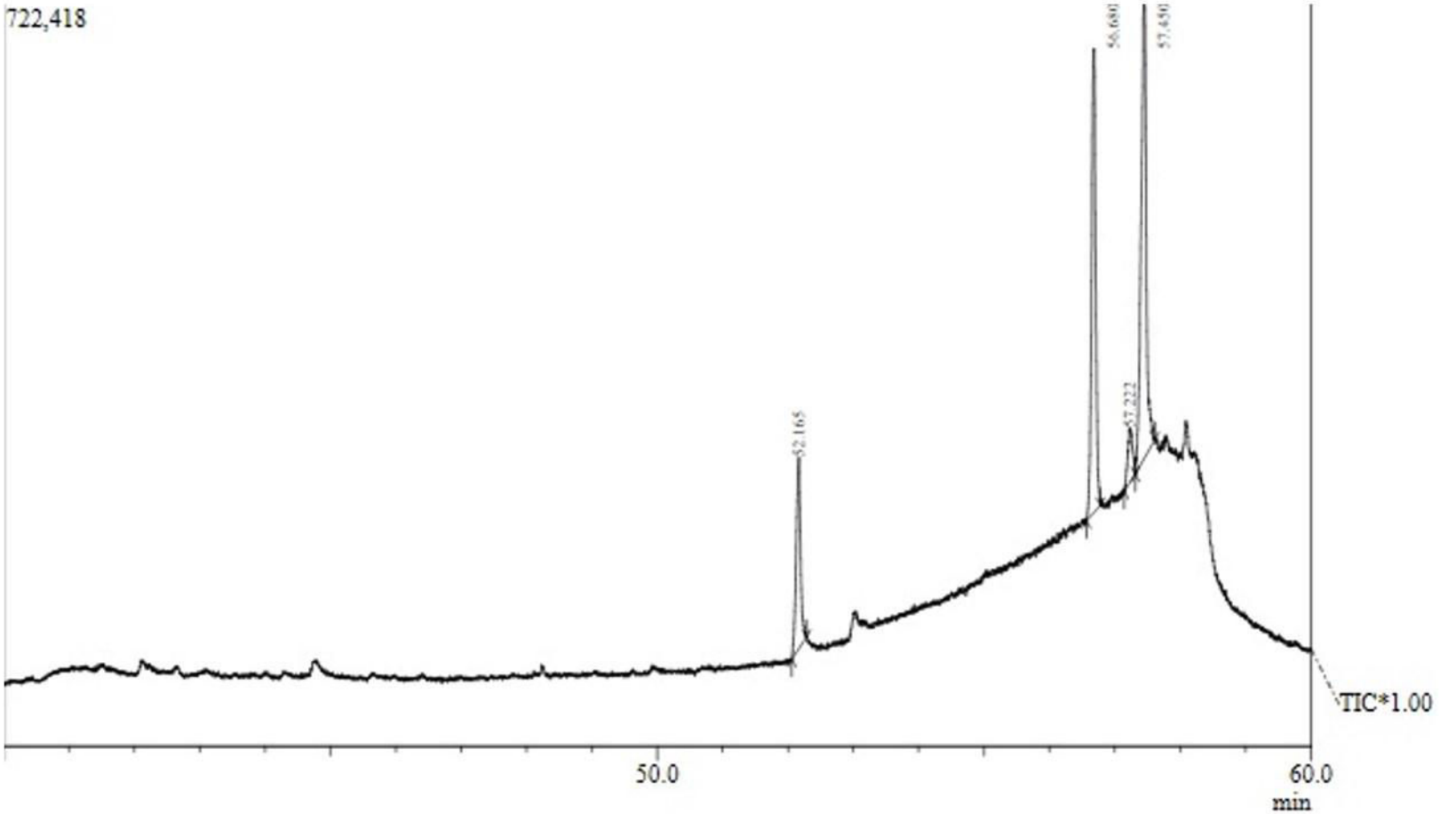


Figure 3

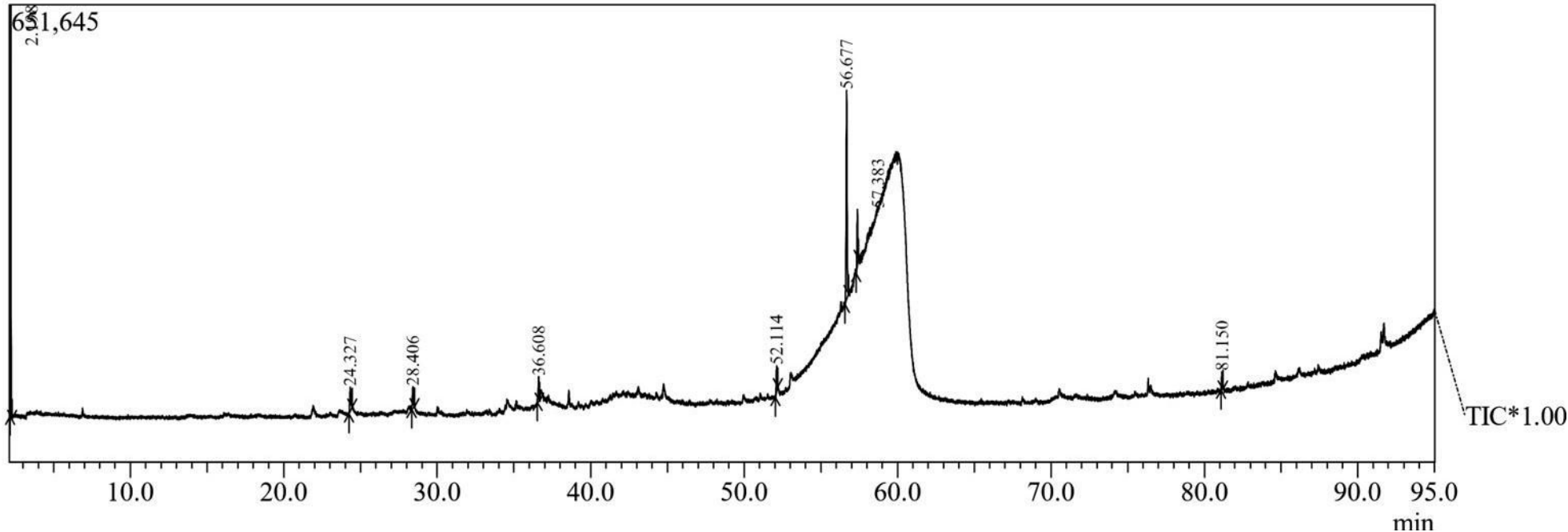

651,645
2.511
24.327
28.406
36.608
52.114
56.677
57.383
81.150
TIC*1.00
10.0
20.0
30.0
40.0
50.0
60.0
70.0
80.0
90.0
95.0
min

Figure 4

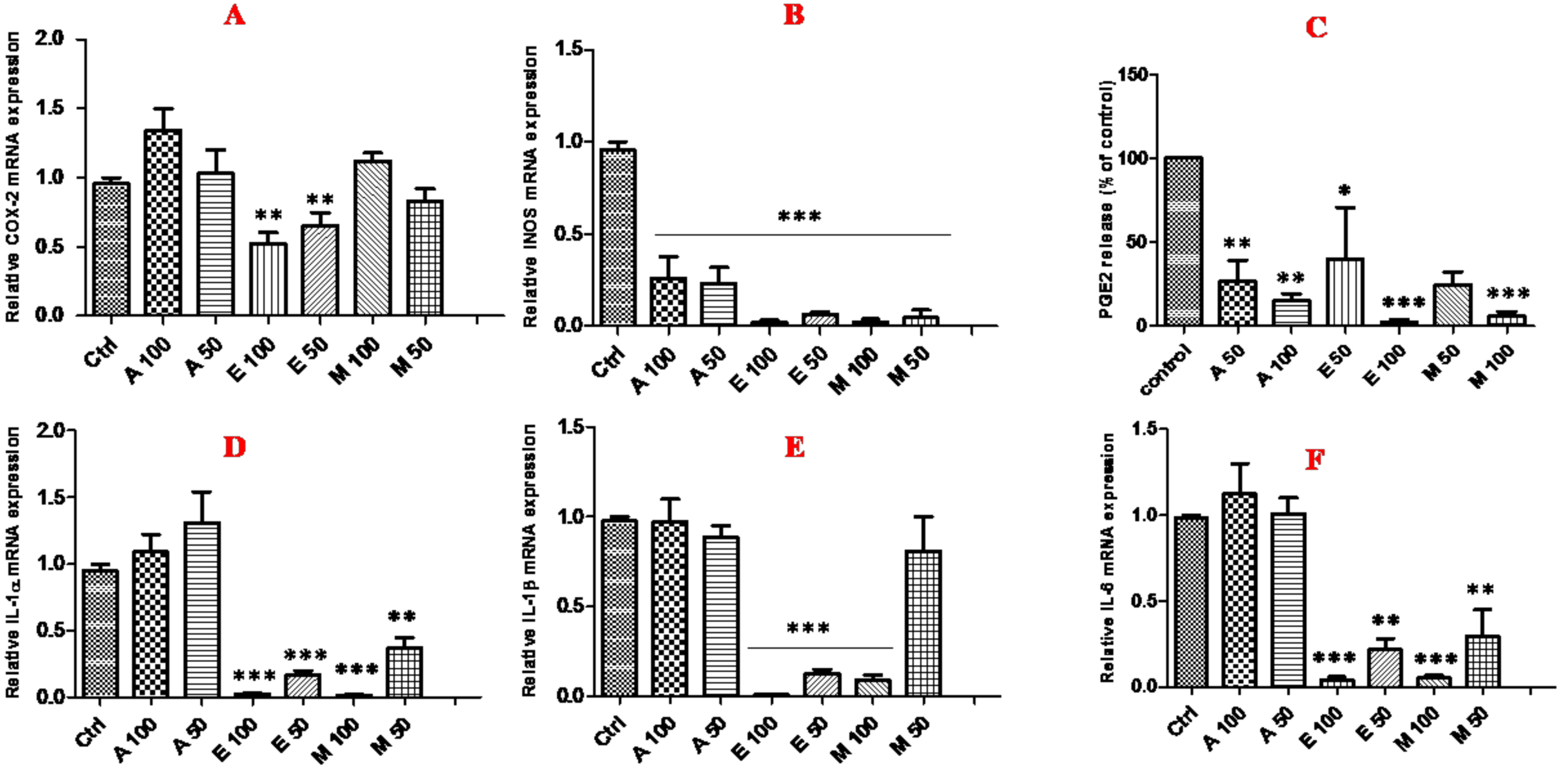

A
Relative COX-2 mRNA expression
2.0
1.5
1.0
0.5
0.0
**
**
Ctrl
A 100
A 50
E 100
E 50
M 100
M 50
B
Relative INOS mRNA expression
1.5
1.0
0.5
0.0
***
Ctrl
A 100
A 50
E 100
E 50
M 100
M 50
C
PGE2 release (% of control)
150
100
50
0
**
**
*
***
***
control
A 50
A 100
E 50
E 100
M 50
M 100
D
Relative IL-1α mRNA expression
2.0
1.5
1.0
0.5
0.0
***
***
***
**
Ctrl
A 100
A 50
E 100
E 50
M 100
M 50
E
Relative IL-1β mRNA expression
1.5
1.0
0.5
0.0
***
Ctrl
A 100
A 50
E 100
E 50
M 100
M 50
F
Relative IL-8 mRNA expression
1.5
1.0
0.5
0.0
***
**
***
**
Ctrl
A 100
A 50
E 100
E 50
M 100
M 50

Figure 4

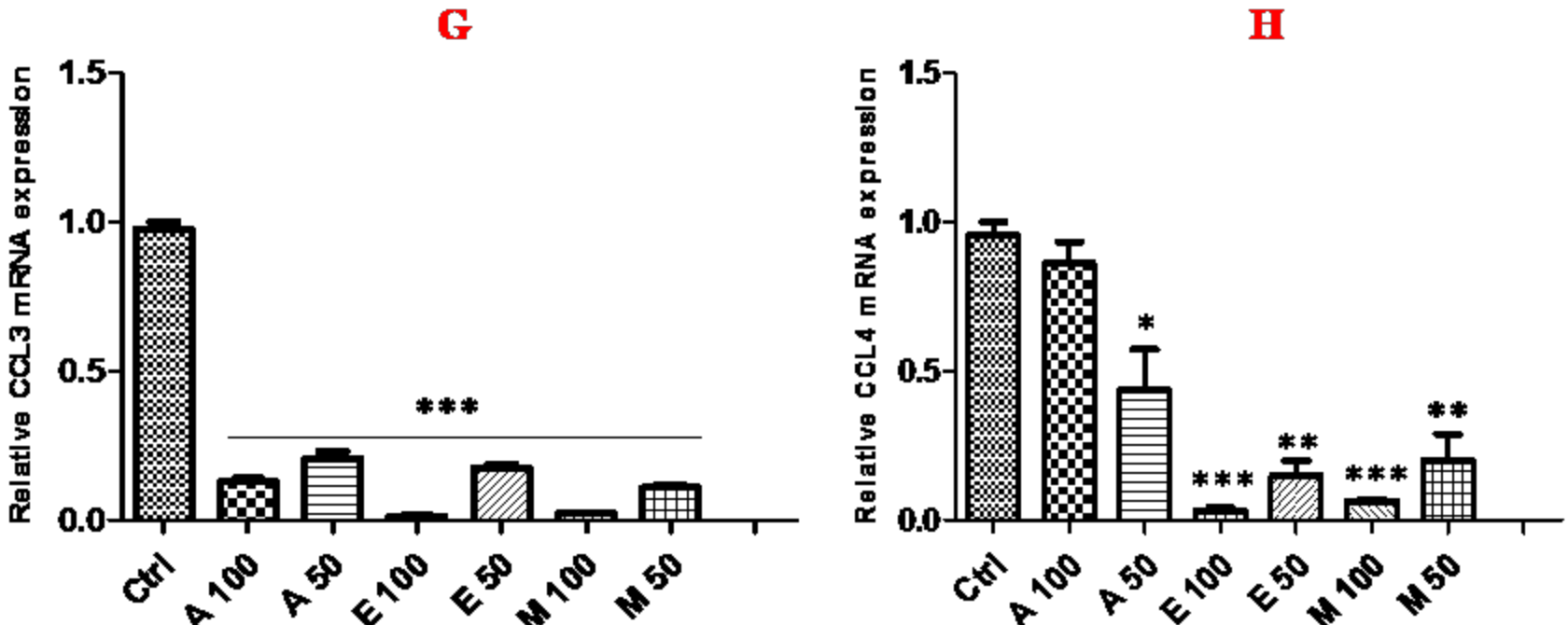
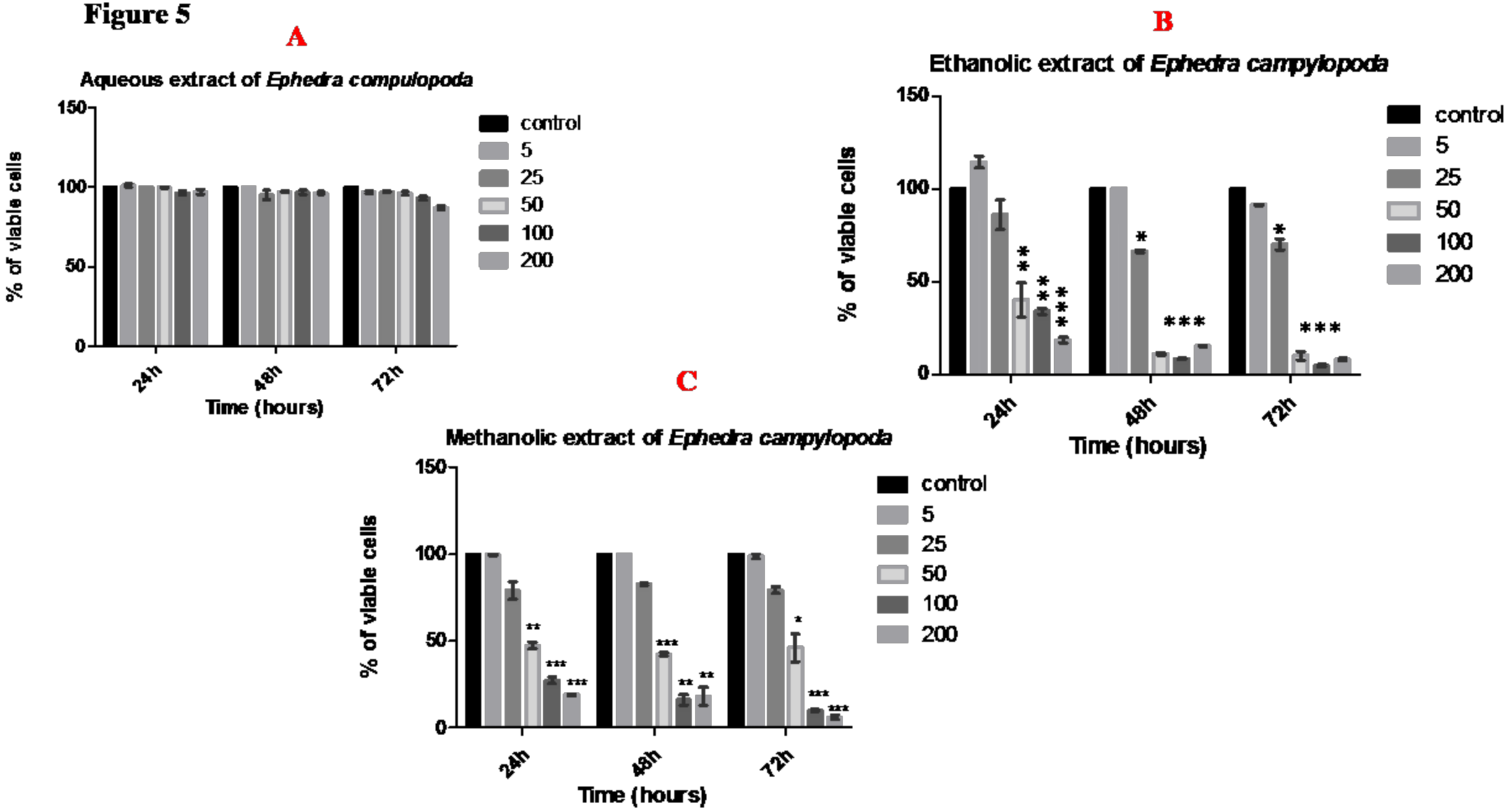

G
H
Relative CCL3 mRNA expression
Relative CCL4 mRNA expression
1.5
1.0
0.5
0.0
***
*
**
Ctrl
A 100
A 50
E 100
E 50
M 100
M 50

Figure 5


A
Aqueous extract of Ephedra compulopoda
% of viable cells
150
100
50
0
24h
48h
72h
Time (hours)
control
5
25
50
100
200
B
Ethanolic extract of Ephedra campylopoda
% of viable cells
150
100
50
0
24h
48h
72h
Time (hours)
control
5
25
50
100
200
C
Methanolic extract of Ephedra campylopoda
% of viable cells
150
100
50
0
24h
48h
72h
Time (hours)
control
5
25
50
100
200

General discussion

Approximately 80% of the world population depend exclusively on plants for their health and healing. In the developed world, reliance on surgery and pharmaceutical medicine is more usual but in the recent years, more and more people are complementing their treatment with natural supplements. Furthermore, motivation of people towards herbs is increasing due to their concern about the side effect of drugs, especially those that are prepared from synthetic materials [122].

Phytochemicals are biologically active, naturally occurring chemical compounds found in plants, and are involved in protecting plant cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack. Besides protecting plants from disease and damage, phytochemicals contribute to the plant's color, aroma and flavor [123]. Remarkably, phytochemicals were identified to serve important roles in the protection of human health, when their dietary intake is significant. More than 4,000 phytochemicals have been catalogued and are classified by protective function, physical characteristics and chemical properties where 150 phytochemicals have been studied in details [124].

Phytochemicals can exist in different parts of the plants, such as in the roots, stems, leaves, flowers, fruits or seeds [125]. Many phytochemicals, particularly the pigment molecules, are often concentrated in the outer layers of the various plant tissues. Phytochemical levels vary from plant to another depending upon the variety, processing, cooking and growing conditions [126]. Phytochemicals compounds are known as secondary plant metabolites and have biological properties such as antioxidant activity, antimicrobial effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property.

In the Middle East, a growing interest in medicinal plants has been noted. Several ethnopharmacological surveys on the medicinal herbs used in the region, mainly in Jordan, have been conducted [127, 128]. They showed that more than 100 plant species are used in herbal medicine and that there is still a flourishing and big demand on

traditional drugs, namely of plant origin [127]. However, such extensive ethnopharmacological studies have not been conducted in Lebanon, which has the richest flora in the Middle East, and there is little information about which medicinal plants are still used by Lebanese herbalists who are decreasing in number. In order to preserve this heritage of plant usage in Lebanon, we selected during this study the following two plants: *Crataegus azarolus*, and *Ephedra campylopoda*. We then screened the phytochemical component content and characterized the antioxidant, anti-inflammatory and antiproliferative capacities of three extracts (water, ethanol, methanol) from both the stems of *Ephedra campylopoda* and the leaves of *Crataegus azarolus*.

In this study, we identified an important phytochemical arsenal in the two studied plants. This chemical composition was associated with important biological activities where each of these two plants exhibited significant antioxidant capacity, anti-inflammatory effect and antiproliferative potential. The molecular mechanisms underlying the observed anti-inflammatory effect are yet to be determined. In future work, we will try to evaluate the involvement of different signaling pathways such as the NF-κB pathway in mediating this effect. Moreover, the signaling events leading to the anti-proliferative effect are still vague. Accordingly, we will also address the requirement of different signaling cascades, such as ones involved during apoptosis, to ensure this behavior. Further, we will evaluate the anti-proliferative effect of these extracts on other cancer types including breast and colon cancer. Since the observed anti-inflammatory and anti-proliferative effects are observed *in vitro*, it will be indispensable, before claiming any therapeutic value, to examine the *in vivo* potential of these plant extracts. Further, other biological activities such as anti-bacterial, anti-fungal and anti-viral effects of these plant extracts are still to be addressed.

Finally, a survey conducted on medicinal herbs used by Lebanese herbalists revealed that 128 plant species were used for treating a number of diseases. Most plants were used to treat more than one medical condition.

This study highlights a medicinal potential for the two studied plant species and the list can be even more enlarged upon further and deep investigations of different Lebanese species for assessing their chemical and biological effects.

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