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

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Statistical Analysis C  
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<sup>2+</sup> chelating activity assays. Anti-inflammatory effect was investigated by measuring the secreted amounts of the proinflammatory mediator PGE<sub>2</sub> using ELISA technique, as well as by assaying the mRNA levels of the proinflammatory cytokines (IL- $\alpha$ , IL- $\beta$ , 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**

**Full-text PDF:** <https://www.basic.medscimonit.com/abstract/index/idArt/905066>

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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-Ciocalteu 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.

**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 <sub>3</sub> (1%)	Blue coloration
Resines [36]	Acetone + water	Turbidity
Saponines [37]	Agitation	Formation of foam
Phenols [36]	FeCl <sub>3</sub> (1%) + K <sub>3</sub> (Fe(CN) <sub>6</sub> ) (1%)	Green-blue coloration
Terpenoids [37]	Chloroform + H <sub>2</sub> SO <sub>4</sub> conc	Reddish brown coloration
Flavonoids [38]	KOH (50%)	Yellow coloration
Carbohydrates [37]	α-naphthol + H <sub>2</sub> SO <sub>4</sub>	Purple ring
Reducing sugars [37]	Fehlings (A+B)	Brownish-red precipitate
Quinones [39]	HCl conc	Yellow precipitate
Sterols & steroids [37,38]	Chloroform + H <sub>2</sub> SO <sub>4</sub> conc	Red color (surface) + fluorescence Greenish-yellow
Cardiac glycosides [37,38]	Glacial acetic acid + FeCl <sub>3</sub> (5%) + H <sub>2</sub> SO <sub>4</sub> 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
Phlobatannins [41]	HCl (1%)	Blue coloration
Anthocyanines [42]	NaOH (10%)	Blue coloration
Flavanones [42]	H <sub>2</sub> SO <sub>4</sub> 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:

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

**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<sub>2</sub> (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<sub>2</sub>/95% air at 37°C. The macrophages were seeded in 12-well plates (1×10<sup>6</sup> 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<sub>2</sub> immunoassay**

PGE<sub>2</sub> 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<sup>3</sup> cells/well). The following day, cells were treated with the different extracts at concentrations

**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<sup>-ΔΔCt</sup>) 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	TCATCTGGGCAGTCACATACA
IL-1β-FO	CCTCCAGGATGAGGACATGA
IL-1β-RE	TGAGTCACAGAGGATGGGCTC
IL-6-FO	GAGGATACCACTCCCAACAGACC
IL-6-RE	AAGTCATCATCGTTGTCATACA
CCL3-FO	CAGCCAGGTGTCATTTTCCT
CCL3-RE	CTGCCTCAAGACTCTCAGG
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

**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.

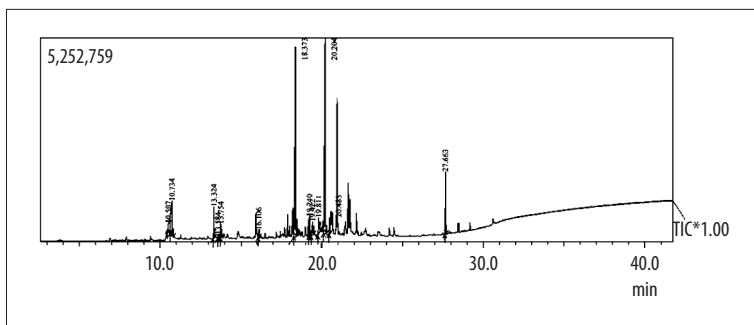
## Results

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

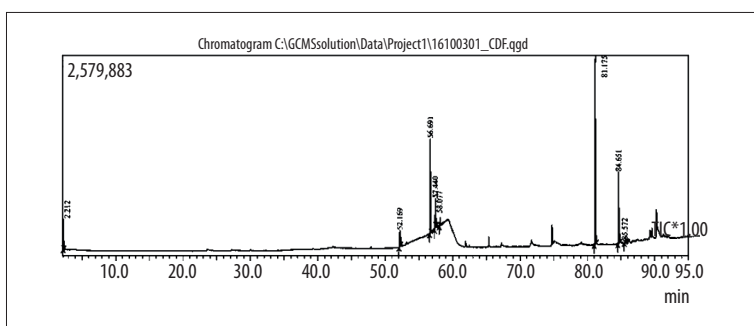
Phytochemical screening of *C. azarolus* L. fresh leaf crude extract indicated the presence of some important bioactive components, which are listed in Table 6. The aqueous crude extract showed high concentrations of saponins, phenols, terpenoids, flavonoids, amino acids, reducing sugars, and lignin; 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.

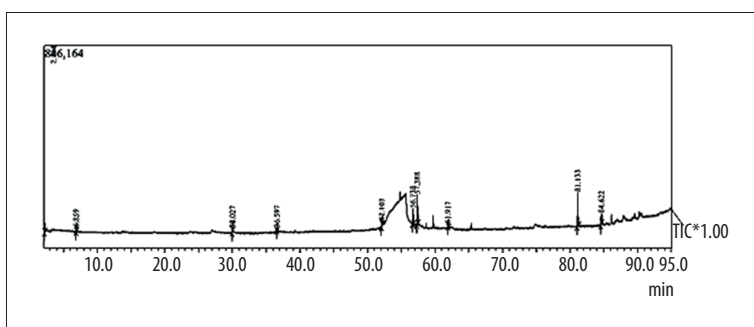




**Figure 1.** GC chromatogram of the water extract of *C. azarolus* L. leaf.



**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, pluchidol 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,  $\gamma$ -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,  $\alpha$  tocopherol-beta-d-mannoside (21.87%), and ethyl linolate (18.79%) compounds were found to be in the highest concentration and other compounds were found in trace amounts (Table 9).

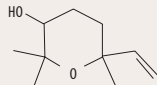
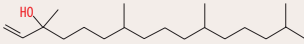
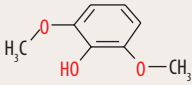
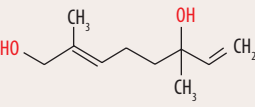
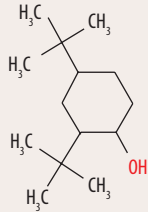
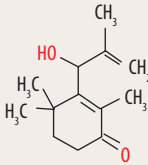
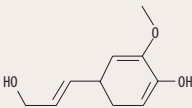
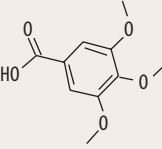
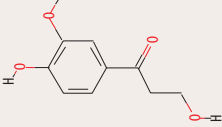
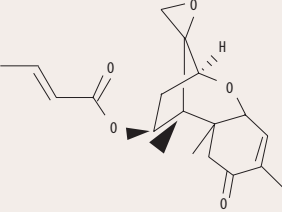
**The LC/MS/MS analysis for *C. azarolus* L leaf extracts**

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**

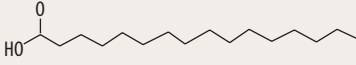
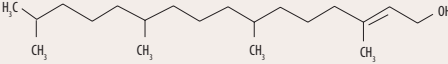
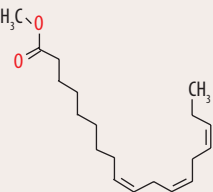
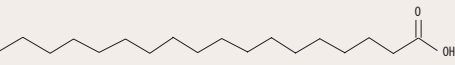
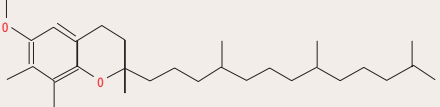
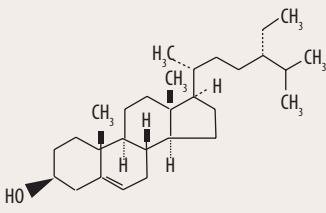
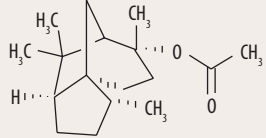
The antioxidant activity of the aqueous, methanolic and ethanolic crude extracts was evaluated using 2 different assays: (1) DPPH free radical scavenging assay and (2) an assay assessing the iron (II) chelating ability. Our obtained results demonstrated that the different extracts displayed significant antioxidant activities and their scavenging effects on DPPH radical were in the following order: ethanolic extract ( $IC_{50}=50\pm5.2 \mu\text{g/ml}$ ) > methanolic extract ( $IC_{50}=55\pm2.8 \mu\text{g/ml}$ ) > water extract ( $IC_{50}=60\pm2.2 \mu\text{g/ml}$ ) (Table 11).

**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	Epoxylinolol	170.25		C <sub>10</sub> H <sub>18</sub> O <sub>2</sub>	4.73
2	13.324	Isophytol	296.54		C <sub>20</sub> H <sub>40</sub> O	3.03
3	13.584	Syringol	154.163		C <sub>8</sub> H <sub>10</sub> O <sub>3</sub>	0.58
4	13.754	8-Hydroxylinolol	170.24		C <sub>10</sub> H <sub>18</sub> O <sub>2</sub>	1.46
5	16.106	2,4- DI-T-Butylphenol	206.324		C <sub>14</sub> H <sub>22</sub> O	0.85
6	19.240	4-Oxo-Beta-Isodamascol	208.297		C <sub>13</sub> H <sub>20</sub> O <sub>2</sub>	3.04
7	19.422	Gamma-Hydroxyisoeugenol (Coniferol)	180.201		C <sub>10</sub> H <sub>12</sub> O <sub>3</sub>	2.35
8	19.811	Gallic acid trimethyl ether	212.2		C <sub>10</sub> H <sub>12</sub> O <sub>5</sub>	2.82
9	20.204	Pluchidiol	208	----	C <sub>13</sub> H <sub>20</sub> O <sub>2</sub>	33.62
10	20.483	Beta-Hydroxypropiovanillone	196.202		C <sub>10</sub> H <sub>12</sub> O <sub>4</sub>	2.16
11	27.663	Trichothecin	332.39		C <sub>19</sub> H <sub>24</sub> O <sub>5</sub>	5.67



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

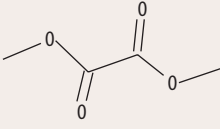
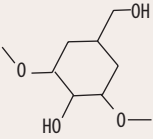
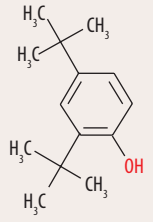
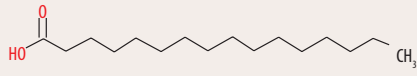
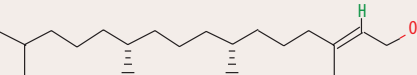
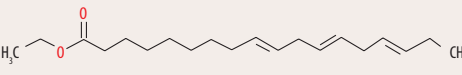
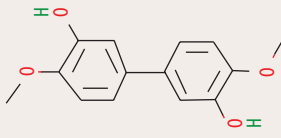
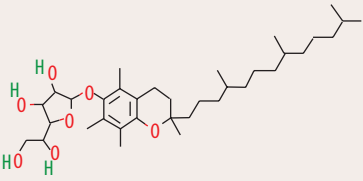
Peak#	RT	Name	MW	Structure	Molecular formula	Area%
1	52.169	Palmitic acid	256.42		C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	3.99
2	56.691	Phytol Isomer	296.53		C <sub>20</sub> H <sub>40</sub> O	20.47
3	57.440	9,12,15-Octadecatrienoic acid	278.436		C <sub>18</sub> H <sub>30</sub> O <sub>2</sub>	7.44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

Ferrous ion (Fe<sup>2+</sup>) is a major prooxidant that upon interaction with hydrogen peroxide can lead to the generation of highly reactive hydroxyl radicals. The Fe<sup>2+</sup> chelating assay is based on the principle that ferrozine can quantitatively form colored complexes with Fe<sup>2+</sup>. 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<sup>2+</sup> 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<sup>2+</sup> in the presence of ferrozine. Methanolic extract had the highest Fe<sup>2+</sup> chelating capacity (IC<sub>50</sub>=0.5±0.08 mg/ml), followed by ethanolic extract (IC<sub>50</sub>=1±0.08 mg/ml) and then aqueous extract (IC<sub>50</sub>=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<sub>2</sub> (PGE<sub>2</sub>) 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<sub>2</sub> 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

**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 <sub>4</sub> H <sub>6</sub> O <sub>4</sub>	1.58
2	30.027	Syringol	184.19		C <sub>9</sub> H <sub>12</sub> O <sub>4</sub>	1.62
3	36.597	2,4 di-tert-butylphenol	206.324		C <sub>14</sub> H <sub>22</sub> O	1.51
4	52.103	Cetylic acid	256.42		C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	2.56
5	56.738	Phytol	296.53		C <sub>20</sub> H <sub>40</sub> O	13.3
6	57.388	Ethyl linolate	308.50		C <sub>20</sub> H <sub>34</sub> O <sub>2</sub>	18.79
7	61.917	4,4'- biguaiacol	246.26		C <sub>14</sub> H <sub>14</sub> O <sub>4</sub>	1.37
8	81.133	alpha-tocopherol-beta-d-mannosid	592.858		C <sub>35</sub> H <sub>60</sub> O <sub>7</sub>	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<sub>2</sub> amounts present in the cell culture media were evaluated. Interestingly, all extracts were highly potent in terms of impairing PGE<sub>2</sub> production, with methanolic extract being the most efficient (Figure 4C).

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

<i>C. azarolus</i> 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
	Isororientin	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
	Isororientin	4.88	447	429	-30	-40

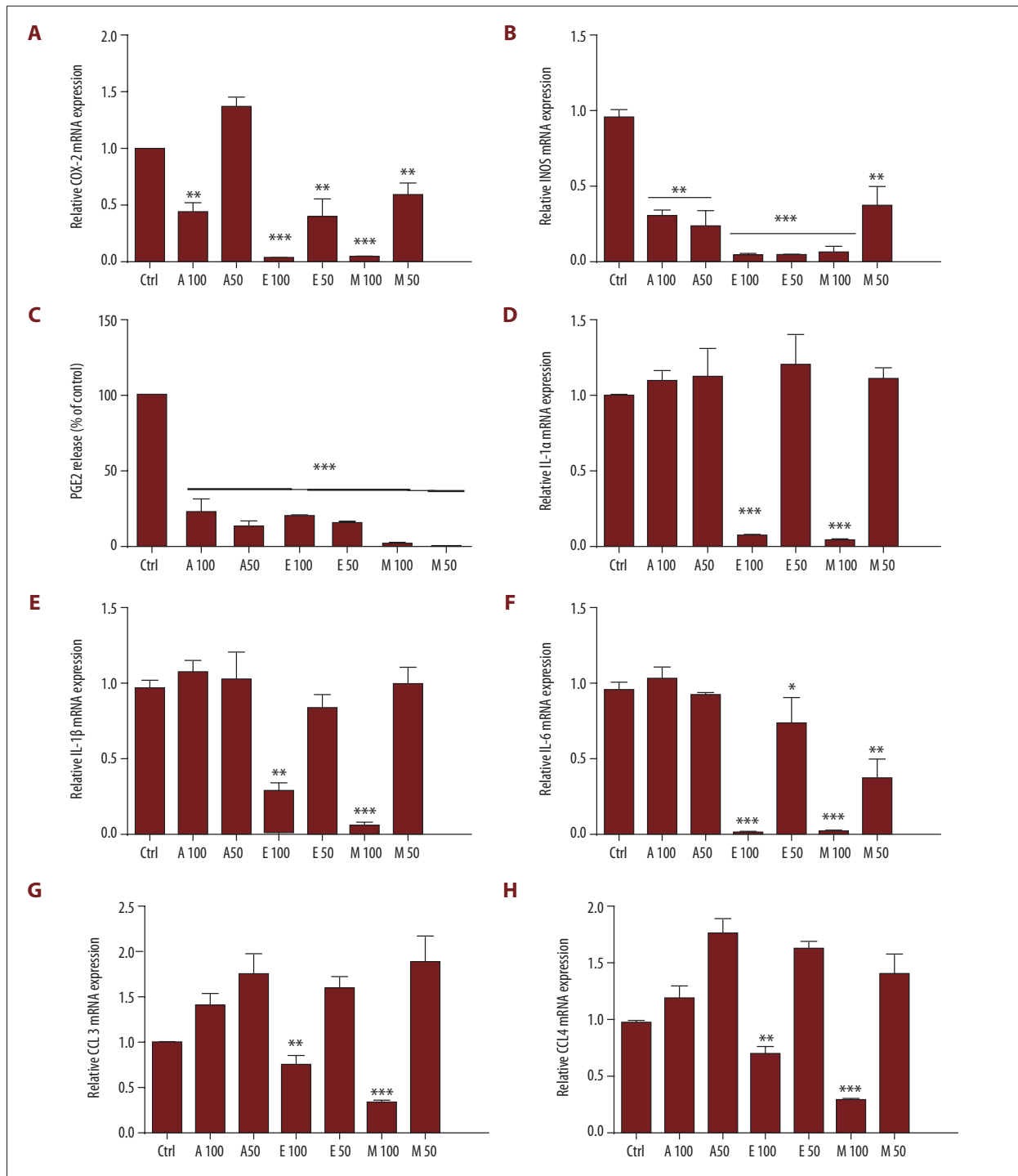
**Table 11.** DPPH free scavenging capacity ( $IC_{50}$ ,  $\mu\text{g/ml}$ ) and Ferrous-ion ( $\text{Fe}^{2+}$ ) chelating ability ( $IC_{50}$ ,  $\text{mg/ml}$ ) of aqueous, methanol or ethanol extracts derived from fresh *C. azarolus* leaves.  $IC_{50}$  value corresponds to the effective concentration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

Extract	DPPH assay	$\text{Fe}^{2+}$ chelating assay
	$IC_{50}$ , $\mu\text{g/ml}$	$IC_{50}$ , $\text{mg/ml}$
Fresh leaves	Aqueous	$60 \pm 2.28$
	Methanol	$55 \pm 2.886$
	Ethanol	$50 \pm 5.207$
		$1 \pm 0.08$

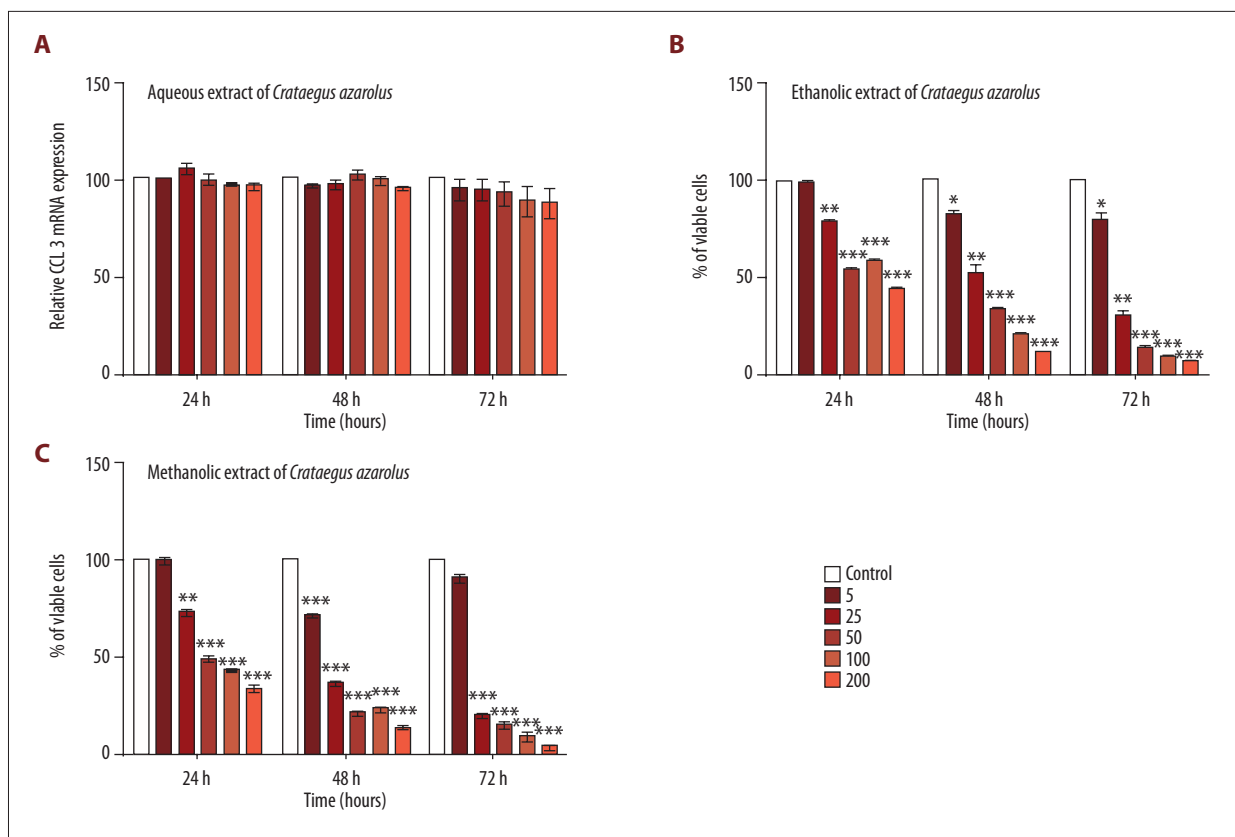
In a third step, and upon using qRT-PCR, the relative expression of the proinflammatory cytokines IL-1 $\alpha$ , IL-1 $\beta$ , and IL-6 was assessed. In the case of IL-1 $\alpha$ , neither of the 2 utilized aqueous extract concentrations was able to significantly impair IL-1 $\alpha$  transcription (Figure 4D). On the other hand, 100  $\mu\text{g/ml}$  of either ethanolic or methanolic extract dramatically reduced IL-1 $\alpha$  mRNA levels (Figure 4D). For IL-1 $\beta$ , aqueous extract was again not efficient in terms of reducing the mRNA levels (Figure 4E). On the other hand, 100  $\mu\text{g/ml}$  of ethanolic extract reduced IL-1 $\beta$  mRNA levels to less than half their level in control cells (Figure 4E). Methanolic extract was more efficient in impairing IL-1 $\beta$  transcription, since minimal IL-1 $\beta$  mRNA levels were detected in cells treated with 100  $\mu\text{g/ml}$  of methanol extract (Figure 4E). In the case of IL-6, no striking alteration in mRNA levels was detected in cells treated with 50  $\mu\text{g/ml}$  or 100  $\mu\text{g/ml}$

aqueous extract (Figure 4F). IL-6 mRNA levels were not largely reduced in cells treated with 50  $\mu\text{g/ml}$  of ethanolic extract but was lost following treatment with 100  $\mu\text{g/ml}$  (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  $\mu\text{g/ml}$  (Figure 4F). IL-6 transcription was lost in cells treated with 100  $\mu\text{g/ml}$  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



**Figure 4.** Effects of *C. azarolus* leaf extracts on LPS-Induced iNOS, COX-2, PGE<sub>2</sub>, IL-1α, IL-1β, 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<sub>2</sub> content via ELISA. The data correspond to the relative percentage of PGE<sub>2</sub>. 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).



**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  $150 \pm 4.4$  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  $25 \pm 1.6$  µg/ml and  $15 \pm 2.88$  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  $22 \pm 1.6$  µg/ml (after 48 h) and  $13 \pm 1.8$  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.

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<sup>2+</sup>-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<sub>2</sub> and the transcription levels of the proinflammatory cytokines (IL- $\alpha$ , IL- $\beta$  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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