CHEMICAL COMPOSITION, ANTIOXIDANT AND HEMOLYTIC ACTIVITIES OF SAGE (SALVIA FRUTICOSA MILLER) CULTIVATED IN LEBANON

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1. INTRODUCTION

*Salvia*, also known as sage, is one of the most important and widespread aromatic and medicinal genera of the lamiaceae family (Roby et al., 2013). This genus includes over 900 species that grow throughout the Mediterranean region, South-East Asia, and Central America (Sepahvand et al., 2014). Some *Salvia* species are economically important due to their use in the drug, flavor and food industry, and in the biosynthesis of various useful constituents such as terpenoids, flavonoids and other phenolics (Šulniute et al., 2017). Plants belonging to the *Salvia* family have been widely used in folk medicine for the treatment of different ailments (Dincer et al., 2013), and the identification of the bioactive constituents in these plants has been the subject of many pharmacognostic studies. One of the most important *Salvia* species for culinary and medicinal purposes is *Salvia fruticosa* Miller (*S. fruticosa* Miller), an aromatic perennial herb endemic to the Mediterranean region (Skoula et al., 1999; Koutsoulas et al., 2019). In folk medicine, the aerial parts of the plant have been used as herbal tea for the treatment of several ailments including, among others, colds, coughs, throat-aches, stomach-aches, tooth-aches, hypertension, rheumatism, and skin infections (Skoula et al., 1999; Süzgeç-Selçuk et al., 2021). Moreover, antimicrobial, anti-inflammatory, anti-cholinesterase, anti-proliferative, and antioxidant activities of *S. fruticosa* essential oils and aqueous or organic extracts have been reported in several studies (Pitarokili et al., 2003; Fu et al., 2013; Hani & Bayachou, 2014; Mrad et al., 2022; Dawra et al., 2023).

Lebanon is a Mediterranean country characterized by its rich plant biodiversity of more than 4,500 plant species. In particular, Lebanon hosts more than 300 medicinal and aromatic plants (MAPs), tens of which are endemic (GEF-UNDP-LARI, 2013). One of the most popular of these plants is *S. fruticosa* Miller. Unfortunately, this species suffers from depletion due to destructive unsustainable harvesting from the wild which currently constitutes the source of the plant, habitat loss caused by urbanization, fires, and climate change, and the extensive export of the plant to Jordan and other countries (GEF-UNDP-LARI, 2010). Therefore, the plant has been notified by the Ministry of Agriculture Decision 340/1, 1/8/1996 as protected against harvesting from the wild and its cultivation has been encouraged as an effective conservation measure for the prevention of further depletion of the plant, as well as an essential supportive measure for the Lebanese economy due to its high market value. As part of the efforts contributing towards encouraging the cultivation of commercially valuable MAPs and the conservation of vulnerable wild species, and understanding that the medicinal profile of herbs can be influenced by climatic and geographical factors (Prinsloo & Nogemane, 2018), we set out in the current study to assess the antioxidant activity of the essential oil, methanolic and aqueous extracts of *S. fruticosa* Miller cultivated at Beirut Arab University herbal garden in Bekaa, Eastern Lebanon. The phytochemical profile of the plant was deciphered by GC-MS, and the total phenolic, flavonoid, carbohydrate, and protein contents in the extracts were quantified. The cytotoxicity of the extracts against human erythrocytes was also assessed.

![Fig.1: S. fruticosa Miller cultivated at BAU herbal garden.](image-url)
2. Materials and Methods

2.1 Chemicals

β-carotene, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), Tween-80, Folin-Ciocalteau reagent, dinitrosalicilic acid (DNS), gallic acid, rutin, and aluminum chloride (AlCl₃) were purchased from Sigma Aldrich (Germany). Linoleic acid, Tris-base, nicotinamide adenine dinucleotide (NADH) and phenazinemethosulfate (PMS) were obtained from Acros Organics (Belgium). Quercetin, ferrous sulfate heptahydrate (FeSO₄·7H₂O), methanol, ethanol and chloroform were purchased from Fluka (Switzerland). Potassium ferricyanide (K₃[Fe(CN)₆]), concentrated sulfuric acid, and trichloroacetic acid were bought from Merck (Germany). Ferric chloride (FeCl₃), sodium hydroxide and dextrose were purchased from Alpha chemika. Nitrobluetetrazolium (NBT) was obtained from Fisher Scientific (Germany). Ethylenediaminetetraacetic acid (EDTA), ascorbic acid and sodium carbonate (Na₂CO₃) were obtained from Alieha. Ferrozine was purchased from HiMedia (India). Phenol was purchased from Riedel-de Haën (Germany).

2.2 Plant Material

Fresh aerial parts of *S. fruticosa* plant were collected during the flowering period from Beirut Arab University herbal garden in Bekaa at an altitude of 890 m above sea level. The plant was identified by Prof. Nelly Arnold from USEK. A voucher specimen was deposited at the Research Center of Environment and Development at BAU campus in Bekaa (herbarium number: ps-14-12).

2.3 Essential Oil Isolation

Fresh aerial flowering parts (1.6 Kg divided in 400 g quantities) of *S. fruticosa* cultivated at BAU herbal garden, Bekaa were collected and hydrodistilled by Clevenger-type apparatus for three hours. The essential oil (19.3 mL) was collected and stored in sterile sealed vials in the dark at 4°C until analysis.

2.4 Preparation of Extracts

10 g of crushed leaves or stems (dry) were suspended in 500 mL of methanol or deionized water in a 1000 mL round bottomed flask, and the suspension was heated at 60°C for 18 hours. These conditions were chosen to achieve maximal extraction of phytochemicals while avoiding/minimizing its decomposition. The mixture was then cooled to room temperature and filtered by suction filtration. Volatiles in the filtrate were evaporated under vacuum on a rotary evaporator, and the resulting residue was dried in a vacuum oven for 4 h. The resulting residue was stored in dark at -20°C until the time of analysis.

2.5 Determination of the Total Phenolic Content

Total phenolic content of the extracts was determined using Folin-Ciocalteau assay according to the method of Singleton and Rossi with some modifications (Singleton & Rossi, 1965). 100 µL of sample was diluted with 5 mL of deionized water and mixed with 500 µL of Folin-Ciocalteau phenol reagent. The mixture was shaken vigorously using a vortex shaker for 5 minutes. 2.5 mL of 5% Na₂CO₃ solution were then added to the mixture. The total volume was adjusted to 12 mL with deionized water. The solution mixture was incubated for 60 minutes at 25°C, and the absorbance was then measured at 750 nm. Gallic acid was used as standard and the results were expressed as g GAE/100 g DW (GAE).

2.6 Determination of the Total Flavonoid Content

The total flavonoid content of the extracts was determined by aluminum nitrate chromogenic method according to the method of Yu and coinvestigators with some modifications (Yu et al., 2013). 1 mL of sample was diluted with 4.2 mL of 70% ethanol solution, followed by adding 400 µL of 5% NaNO₂ solution. The solution was shaken and allowed to stand for 6 minutes. 400 µL of 10% Al(NO₃)₃ solution were added to the solution mixture and the mixture was allowed to stand for another 6 minutes. 4 mL of 4% NaOH
solution were added to the mixture. The mixture was then incubated for 15 minutes at room temperature, and the absorbance of the mixture was measured at 410 nm. Rutin was used as a standard and the results were expressed as g of rutin per 100g of dry sample (g RE/100 g dw).

2.7 Determination of the Total Carbohydrate Content

Total carbohydrate content of the extracts was determined using the phenol-sulfuric acid method as described by Dubois et al. with some modifications (Dubois et al., 1956). 1 mL of different sample solutions was pipetted into a 15 mL glass test tube. 1 mL of 5% phenol solution and 4 mL of concentrated sulfuric acid (98%) were added to the sample. The contents of the test tube were mixed thoroughly and allowed to stand for 10 minutes. The test tubes were placed in a water bath at 25-30˚C for 15 minutes. The absorbance of the resulting solutions was measured at 490 nm. Glucose was used as a standard and the results were expressed as grams of glucose in 100 g of sample (g-glucose/100 g-sample).

2.8 Determination of the Total Protein Content

Crude protein content of the crushed leaves and stems (powder) and extracts was calculated by multiplying the total nitrogen content of the different samples by a factor of 6.25 (AOAC, 1990). The total nitrogen content of the different samples was measured using a Kjeldahl analyzer.

2.9 DPPH Radical Scavenging Activity Assay

The antioxidant potential of the essential oil, aqueous and methanolic extracts of S. fruticosa to scavenge the DPPH radical was evaluated according to the method described by Moualek et al. (2016). 2 mL of an extract solution were mixed with 2 mL of 60 mM DPPH solution in sealed vials wrapped with aluminum foil. The mixture was incubated for 30 minutes at room temperature. The absorbance was then measured at 517 nm. Quercetin was used as a positive control. A mixture of DPPH and solvent was used as a negative control. The percentage of DPPH scavenging activity expressed as percentage inhibition of DPPH reduction was calculated according to the following equation:

\[
\% \text{ Inhibition} = \left(1 - \frac{A_s}{A_c} \right) \times 100
\]

Where \(A_c\) is the absorbance of the control, and \(A_s\) is the absorbance of the sample.

2.10 β-Carotene Bleaching Assay

The ability of oil and extracts to inhibit β-carotene oxidation was measured according to the method of Amiri with some modifications (Amiri, 2014). An emulsion was prepared by mixing 2 mL of 0.5 mg/mL β-carotene solution, 140 μL linoleic acid solution, and 2.4 mL Tween-80 solution (prepared by dissolving 400 μL tween-80 in 2 mL chloroform) in a 100 mL round bottomed flask. Chloroform was evaporated on a rotary evaporator at 40°C, followed by gradual addition of 100 mL of distilled water to the residue. The mixture was sonicated for 2 min to obtain a stable emulsion. Another emulsion devoid of β-carotene was prepared for blank measurements by following the same procedure described above. 200 μL of the different extract solutions were mixed with 5 mL of the emulsion in glass test tubes, and the mixture was shaken and incubated in an oven at 50°C for 2 h. Absorbance was measured at 470 nm after 120 minutes. Quercetin was used as a positive control. The percentage inhibition was calculated according to the following equation:

\[
\% \text{ Inhibition} = \left\{1 - \left[\frac{A_s(0) - A_c}{A_s(120) - A_c(120)}\right]\right\} \times 100
\]

Where \(A_s(0)\) is the absorbance of the negative control before incubation; \(A_c(120)\) is the absorbance of the negative control after incubation; and \(A_s\) is the absorbance of the sample after incubation.
2.11 Reducing Power Assay

The reducing power ability of the extracts was evaluated using the method described by Oyaizu et al. (1986) with some modifications. The extracts solutions were mixed with phosphate buffer (0.2 M, pH=6.6) and potassium ferricyanide (1% w/v), and incubated for 20 min at 50°C in an oven. Trichloroacetic acid (10% w/v) was added, and the mixture was shaken on a vortex mixer. Distilled water and ferric chloride (0.1% w/v) were then added to the mixture and the absorbance was measured at 700 nm using a spectrophotometer. Ascorbic acid was used as a positive control.

2.12 Superoxide Radical Scavenging Assay

The ability of oil and extracts to scavenge the superoxide radical ($O_2^-$) was evaluated according to the method of Robak and Gryglewski (1988) with some modifications for the oil samples. 0.5 mL of 0.3 mM NBT solution, 0.5 mL of 0.936 mM NADH solution, 0.5 mL of 16 mM Tris-HCl buffer solution were mixed. 1 mL of sample was pipetted to the mixture. The reaction was initiated by adding 0.5 mL of 0.12 mM PMS solution. The solution mixture was shaken and incubated for 5 min at 25°C. The absorbance was then measured at 560 nm. Ascorbic acid was used as a positive control. All the reagents were freshly prepared and placed in well-sealed volumetric flasks wrapped with aluminum foil. The percentage of superoxide radical scavenging activity was calculated according to the following equation:

$$\text{% Inhibition} = \left[\frac{(A_c - A_s)}{A_c}\right] \times 100$$

Where $A_c$ is the absorbance of the control, and $A_s$ is the absorbance of the sample.

2.13 Metal Chelating Activity Assay

The metal chelating activity of the samples was evaluated according to the method of Dinis et al. (1994). 2 mL of 0.2 mM ferrous sulfate heptahydrate solution and 400 μL of 2.5 mM ferrozine solution were added to 200 μL of the sample solution. The mixture was vigorously shaken and incubated for 10 minutes at room temperature. The absorbance was then measured at 562 nm. EDTA was used as a positive control. The percentage of metal chelating activity, expressed as percentage inhibition of complex formation, was calculated according to the following equation:

$$\text{% Inhibition} = \left[\frac{(A_c - A_s)}{A_c}\right] \times 100$$

Where $A_c$ is the absorbance of the control, and $A_s$ is the absorbance of the sample.

2.14 Hemolysis Assay

Hemolysis assay was performed according to the method of Henkelman et al. (2009) with some modifications. 5 mL of blood were obtained from a healthy volunteer and placed in tubes containing EDTA to prevent blood coagulation. The blood sample was first centrifuged at 2500 rpm for 5 minutes and the supernatant (plasma) was removed. The erythrocytes (lower layer) were washed with 1X phosphate buffer saline (PBS) until the supernatant became clear. 2% erythrocyte suspension in PBS was prepared and used for analysis. The erythrocyte solution was stored at 4°C and washed as described above before each use. 100 μL of the blood sample was mixed with 150 μL of each sample solution. The tubes were gently shaken and the reaction mixture was incubated at 37°C for 1 hour. The tubes were gently shaken after 30 min of incubation. After one hour of incubation, the tubes were centrifuged at 2500 rpm for 3 min. Absorbance of the supernatant was measured using 96-well plate reader. 2% Triton X-100 was used as a positive control. Measurements were performed as triplets for each sample, and the average hemolytic activity was calculated according to the following equation:

$$\text{% H} = \left[\frac{A_s}{A_c}\right] \times 100$$
Where, H represents hemolytic activity; A_s is the absorbance of the sample; A_c is the absorbance of the positive control.

2.15 Statistical Analysis

Data was analyzed using IBM SPSS statistics 21. The data was normally distributed based on the Shapiro-Wilk test. One-way ANOVA was done to test for the significant difference between groups under each assay, followed by the Bonferroni Post Hoc test. Pearson correlation was performed to test the relationship between phytochemicals content and antioxidant activity.

3. RESULTS AND DISCUSSION

3.1. GC-MS Analysis

The GC-MS analysis of *S. fruticosa* Miller essential oil revealed the presence of ten major constituents. 2-Methylene-2-vinylcyclopentane was the most abundant (39.54%), followed by 1,8-cineole (10.81%), camphor (9.66%), camphene (8.73%) and α-pinene (7.08%) (Table 1). All compounds identified in the essential oil belonged to the terpenes family. Several studies reported the presence of such phytochemicals in the essential oil of *S. fruticosa* Miller (Pitarokili et al., 2003; Cvetkovikj et al., 2015; Sarroua et al., 2016; Karik et al., 2018; Zgheib et al., 2019).

<table>
<thead>
<tr>
<th>Table 1: The major constituents of <em>S. fruticosa</em> extracts as identified by GC-MS analysis.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical constituent</td>
</tr>
<tr>
<td>Essential oil of <em>S. fruticosa</em></td>
</tr>
<tr>
<td>1. α-Pinene</td>
</tr>
<tr>
<td>2. Camphene</td>
</tr>
<tr>
<td>3. β-Phellandrene</td>
</tr>
<tr>
<td>4. 1,8-Cineole</td>
</tr>
<tr>
<td>5. 2-Methylene-2-vinylcyclopentane</td>
</tr>
<tr>
<td>6. 1-Methyl-1-cyclopent-3-one</td>
</tr>
<tr>
<td>7. 1,6-Dimethyl-1,5-cyclooctadiene</td>
</tr>
<tr>
<td>8. 1-Ethylcyclohexene</td>
</tr>
<tr>
<td>9. Camphor</td>
</tr>
<tr>
<td>10. α-Terpineene</td>
</tr>
<tr>
<td>Methanolic extract of <em>S. fruticosa</em></td>
</tr>
<tr>
<td>1. Eucalyptol</td>
</tr>
<tr>
<td>2. Thujone</td>
</tr>
<tr>
<td>3. Camphor</td>
</tr>
<tr>
<td>4. Terpineol</td>
</tr>
<tr>
<td>5. δ3-Carene</td>
</tr>
<tr>
<td>6. Caryophyllene</td>
</tr>
<tr>
<td>7. α-Gurjunene</td>
</tr>
<tr>
<td>8. Spathulenol</td>
</tr>
<tr>
<td>9. Caryophyllene oxide</td>
</tr>
<tr>
<td>10. Methyl linolelaidate</td>
</tr>
<tr>
<td>11. Eicosane</td>
</tr>
<tr>
<td>12. 2,3,4,5-Tetramethyl-1,4-hexadiene</td>
</tr>
<tr>
<td>13. Pentaethylstylene</td>
</tr>
<tr>
<td>14. 4-Methyldocosane</td>
</tr>
</tbody>
</table>
On the other hand, 31 major constituents were identified in the methanolic extract of *S. fruticosa*. The major constituent was pentaethylstyrene (11.32%), followed by eicosane (9.37%), eucalyptol (3.98%), and caryophyllene (5.97%) (Table 1). Finally, ten major constituents were identified in the aqueous extract of the plant, where 10-heneicosene was the most abundant (23.25%), followed by 1,54-dibromotetrapentac tone (22.83%), and 4-methyldocosane (20.37%) (Table 1). The nature and quantity of the extracted bioactive phytochemicals vary with the polarity of the extracting solvent. In fact, there is no universal solvent that can be recommended for extraction as optimal extraction yields depend on the plant being extracted and the bioactive compounds contained in it that can have different solubility in different solvents. Therefore, customized and optimized extraction conditions (solvent type, temperature, etc.) should be established for individual plants (Truong et al., 2019).
3.2. Total Phenolic and Total Flavonoid Contents

Phytochemicals are plant secondary metabolites involved in the treatment and prevention of several human diseases. These compounds are considered as the bioactive constituents in plants and are responsible for the plants’ biological activities (Setchell & Cassidy, 1999). Hence, metabolite profiling is an essential step in the evaluation of the biological properties of medicinal plants. There is a large body of evidence describing the direct correlation between the antioxidant activity of medicinal plants and their content of secondary metabolites, notably the total phenolic content. Among the different phenolic compounds, flavonoids have gained a great interest due to their significant antioxidant and free radical scavenging activities.

The total phenolic content of the extracts (methanolic and aqueous) was determined using Folin-Ciocalteau method. The results are expressed as gallic acid equivalents (GAE) (grams of gallic acid per 100 grams of dry weight sample) and are summarized in Table 2. The methanolic extract exhibited the highest phenolic content (24.26 g GAE/100 g DW), followed by the aqueous extract (12.57 g GAE /100 g DW). High total phenolic content in the methanolic extract of S. fruticosa was reported previously (Boukhary et al., 2016; Sarroua et al., 2016; Karik et al., 2018; Zgheib et al., 2019). For example, a recent study reported the phenolic content of the extracts of S. fruticosa plant collected from the southern Mediterranean region at altitudes ranging from 200 to 400 m at the littoral of Beirut, Lebanon. The methanolic extract showed a phenolic content of 122.67 ± 0.44 mg GAE/g, which is lower than the value reported in our study for methanolic extract sample (equivalent to 242.6 mg GAE/g). The authors reported a weak positive correlation between the total phenolic content of the different extracts and antioxidant activity (R^2=0.0559, r=0.24) (Boukhary et al., 2016).

The total flavonoid content of the extracts (methanolic and aqueous) was determined using the aluminum nitrate chromogenic method. Results were expressed as rutin equivalents (RE) (grams of rutin per 100 g of dry weight sample), and are summarized in Table 2. The methanolic extract showed a flavonoid content of 122.67 ± 0.44 mg GAE/g, which is lower than the value reported in our study for methanolic extract sample (equivalent to 242.6 mg GAE/g). The authors reported a weak positive correlation between the total phenolic content of the different extracts and antioxidant activity (R^2=0.0559, r=0.24) (Boukhary et al., 2016).

The total flavonoid content of the extracts (methanolic and aqueous) was determined using the aluminum nitrate chromogenic method. Results were expressed as rutin equivalents (RE) (grams of rutin per 100 g of dry weight sample), and are summarized in Table 2. The methanolic extract showed a flavonoid content of 35.24 mg RE/100 g DW, followed by the aqueous extract (31.173 mg RE/100 g DW). However, the difference between the methanolic and aqueous extracts was not statistically different. Several factors affect the levels of phytochemicals in S. fruticosa such as extraction methods employed, harvesting year, geographical location, climate, soil characteristics, and other ecological conditions (Hamouz et al., 2010). In addition, Dincer et al. found higher levels of total phenols in cultivated S. fruticosa compared to wild samples, while higher levels of total flavonoids were detected in the latter (Dincer et al., 2012). On the other hand, Erdoğan et al. reported The total phenolic and flavonoid contents of the methanolic extracts of the S. fruticosa samples ranged between 0.666 and 1.17 g/100 g GAE and 1.943 and 0.664 g/100g CE (catechin equivalents), respectively. This range is much lower than the value we obtained for our methanolic extract sample (35.24 g/100 g RE equivalents) (Erdoğan et al., 2014).

Table 2: Phytochemical composition of Lebanese S. fruticosa methanolic and aqueous extract.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total phenolic content (g GAE/100 g DW)</th>
<th>Total flavonoid content (g RE/100 g DW)</th>
<th>Total carbohydrate content (g GE/100 g DW)</th>
<th>Total protein content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanolic</td>
<td>24.26 ± 1.352a</td>
<td>35.24 ± 4.677a</td>
<td>23.42 ± 3.41a</td>
<td>9.29</td>
</tr>
<tr>
<td>Aqueous</td>
<td>12.57 ± 2.011b</td>
<td>31.173 ± 5.863a</td>
<td>29.08 ± 0.47b</td>
<td>9.84</td>
</tr>
</tbody>
</table>

* Measurements were done in triplicates, and values are presented as mean ± standard deviation.
* Different letters in the same row means significant difference at p<0.05.
3. Total Carbohydrate Content and Total Protein Contents

Although the medicinal properties of *S. fruticosa* and other Salvia species have been extensively investigated, the nutritional value of these species is barely studied. In the current study, we evaluated the total carbohydrate and total protein contents in methanolic and aqueous extracts of *S. fruticosa* using sulfuric acid-phenol method. The results are expressed as grams of glucose equivalence per 100 g of dry weight sample (g GE/100 g DW) and are summarized in table 2. The aqueous extract exhibited the highest carbohydrate content with a (29.08 g GE/100 g DW), followed by the methanolic extract (23.42 g GE/100 g DW). Therefore, it can be concluded that the aqueous extract is considered a better source of carbohydrates than the methanolic extract. The higher extraction efficiency can be attributed to the superior solubility of the polar carbohydrate molecules in the more polar water solvent. The total carbohydrate levels determined in this study were comparable to those reported in seven *Salvia hispanica* populations which ranged between 20.8 and 25.5 g GE/100 g (de Falco et al., 2017), thus highlighting the nutritious value of *S. fruticosa* investigated in the current study.

Among the twenty amino acids that build up major proteins, nine amino acids known as essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, threonine and valine) are not produced by the human body and must be obtained from the diet. Different plant foods contain different percentages of these essential amino acids, and therefore necessary amounts can be provided by a well-planned plant-based diet. In fact, research findings linked plant-based diets to lower risks of several pathologies including cardiovascular diseases, hypertension, diabetes, obesity and certain types of cancer (Hughes et al., 2014). Hence, relying on plant-derived proteins instead of animal proteins has been a nutrition trend in recent years. The total protein content of *S. fruticosa* methanolic and aqueous extracts was determined using the universal Kjeldahl method. The methanolic and aqueous extracts contained comparable content of protein with values of 9.29% and 9.84%, respectively (Table 2). These values suggested that extracts of *S. fruticosa* cultivated at BAU can be considered an important source of proteins. The total protein content determined for *S. fruticosa* essential oil falls within the range (12-16%) reported for *Salvia hispanica* seeds which are known for their significant protein content (de Falco et al., 2017). On the other hand, the values determined for the methanolic and aqueous extracts were below this range.

3.4. Assessment of Antioxidant Activity

The use of a multiple approach in the study of antioxidant activity is recommended since plants contain different classes of antioxidants that may act by different mechanisms of action. In the current study, the *in vitro* antioxidant activity of extracts *S. fruticosa* cultivated at BAU herbal garden in Bekaa was assessed through DPPH radical scavenging assay, β-carotene-linoleic acid bleaching assay, superoxide radical scavenging assay, reducing power activity assay, and metal chelating activity assay.

3.4.1. DPPH radical scavenging activity

The DPPH radical scavenging assay is a simple and sensitive method intensively used for the evaluation of total antioxidant activity of liquid and solid samples (Alam et al., 2013 Fayoumi et al., 2022a). The IC₅₀ value, defined as the concentration of the sample required to scavenge 50% of the DPPH radicals initially present in the solution, was determined for the different samples and used to assess its total antioxidant activity (Table 3). The methanolic extract showed the highest antioxidant activity with a very low IC₅₀ value of 3.25 µg/mL, followed by the aqueous extract with an IC₅₀ value of 12.9 µg/mL and the essential oil with an IC₅₀ value of 38 mg/mL (Table 3). It is remarkable that the activity of the oil is much lower than that of the methanolic and aqueous extracts. Moreover, the free radical scavenging activity of the methanolic extract is very close to that of quercetin (IC₅₀=1.28 µg/mL), a well-known natural antioxidant used as a positive control in this assay (Table 3).
Several studies relied on the DPPH assay to assess the antioxidant activity of *S. fruticosa*. For instance, Tundis et al. assessed the antioxidant activity of the methanolic extract of *S. fruticosa* subsp. *Thomasii* collected from Calabria, Italy (Tundis et al., 2017). The obtained IC$_{50}$ of 33.6 µg/mL for the methanolic soluble fraction of the extract suggested that the methanolic extract in the current study possessed superior free radical scavenging potential. Similar results were demonstrated by Duletić-Laušević et al. (2018) who reported IC$_{50}$ values of 36.37 µg/mL and 48.11 µg/mL for the methanolic and aqueous extracts of Libyan *S. fruticosa*, respectively. The enhanced activity observed for our extracts can be attributed, at least in part, to the superior levels of total phenolic and total flavonoids contents.

The antioxidant activity of the essential oil of wild *S. fruticosa* from Libya was evaluated using the DPPH radical scavenging assay (Giweli et al., 2013). The IC$_{50}$ value of 15.53 mg/mL was lower than that obtained for the essential oil in our study (35 mg/mL). The authors analyzed the chemical composition of the essential oil by GC and GC-MS. 1,8-cineole was found to be the most abundant compound, followed by camphor, β-pinene, myrcene and α-pinene. Several studies reported 1,8-cineole as the major constituent of *S. fruticosa* essential oil, while few studies reported α-thujone and camphor as the major constituents (Sarroua et al., 2016). 1,8-cineole and camphor, which are major constituents in the essential oil tested in the current study, exhibit moderate antioxidant activity (Giweli et al., 2013). In addition, the low levels of total phenolic and total flavonoid contents in the essential oil can also justify the weak antioxidant activity of the oil compared to the other extracts in our study.

<table>
<thead>
<tr>
<th>Table 3: Antioxidant activity of <em>S. fruticosa</em> extracts and comparison to positive controls.</th>
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<tbody>
<tr>
<td><strong>DPPH activity:</strong></td>
</tr>
<tr>
<td><strong>IC$_{50}$ (µg/mL)</strong></td>
</tr>
<tr>
<td><strong>Essential oil</strong></td>
</tr>
<tr>
<td><strong>Methanolic extract</strong></td>
</tr>
<tr>
<td><strong>Aqueous extract</strong></td>
</tr>
<tr>
<td><strong>Quercetin</strong></td>
</tr>
<tr>
<td><strong>Ascorbic acid</strong></td>
</tr>
<tr>
<td><strong>EDTA</strong></td>
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</table>

* Measurements were done in triplicates and the values are expressed as mean ± standard deviation. Different letters in the same row means significant difference at *p*<0.05.

### 3.4.2. β-carotene bleaching assay

In the human body, lipid peroxides are involved in the initiation of lipid peroxidation chain reactions that are associated with the disturbance of cell membranes and rearrangement of the membrane structure (Montuschi et al., 1999; Wood et al., 2000). The β-carotene bleaching assay is a rapid method for the estimation of the ability of samples to scavenge the linoleic acid peroxides. The ability of the test samples to inhibit β-carotene discoloration is monitored spectrophotometrically at 470 nm. The IC$_{50}$ values, defined as the concentration of the sample required for 50% inhibition of β-carotene discoloration in comparison to the negative control, were determined for the extracts and used to evaluate the lipid peroxide radical scavenging activity of the different samples. In this assay, the methanolic extract showed the highest antioxidant activity with a low IC$_{50}$ value of 0.553 mg/mL, followed by the aqueous extract with an IC$_{50}$ value of 2.86 mg/mL and
the essential oil with an IC$_{50}$ value of 10.5 mg/mL (Table 3). These results suggested that the methanolic extract has the highest potential to inhibit β-carotene oxidation and consequently to scavenge the lipid peroxide radicals, followed by the aqueous extract and the oil. The antioxidant activity of the methanolic extract in this assay was comparable to that of quercetin, which exhibited an IC$_{50}$ value of 0.54 mg/mL. This highlights the promising antioxidant activity of the methanolic extract. Tundis et al. reported an IC$_{50}$ of 56 μg/mL for the methanolic extract of S. fruticosa (Tundis et al., 2017). Duletić-Laušević et al. evaluated the ability of S. fruticosa extracts to inhibit β-carotene bleaching at a fixed concentration of 0.5 mg/mL. The methanolic extract showed only 12% inhibition of β-carotene bleaching, compared to 49% at similar concentration in our study. On the other hand, the aqueous extract showed 70% inhibition compared to 25% in the current study (Duletić-Laušević et al., 2018). Essential oil of S. fruticosa Mill subsp. Thomassii, an endemic sage of Southern Italy, displayed IC$_{50}$ values ranging between 0.095-0.130 mg/mL, and therefore was more active in inhibiting lipid peroxidation compared to the essential oil tested in our study (Tundis et al., 2017).

3.4.3. Reducing power

Although it is difficult to give one specific definition of the term ‘antioxidant’, it is conventional that, in mechanistic terms, an antioxidant is a hydrogen donor or an electron donor. Thereby, several single electron transfer-based methods have been developed to assess the ability of samples to transfer electrons and thus to reduce metals, carbonyl groups and radicals (Miguēl, 2010a). The reducing power assay is a method that evaluates the antioxidant activity of samples by measuring their ability to reduce the ferric cation into the ferrous cation. The EC$_{50}$ values, defined as the sample concentration that produces an absorbance of 0.5, were determined for the different samples (Miguēl, 2010b). The reducing powers of the extracts were concentration-dependent. This observation was in accordance with the report of Urek et al. who reported similar trend for the aqueous extract of S. fruticosa collected from Turkey (Urek et al., 2008). Similar to the trend observed in the DPPH and β-carotene bleaching assays, the methanolic extract showed the highest reducing power with the lowest EC$_{50}$ (17.9 μg/mL), followed by the aqueous extract with an EC$_{50}$ value of 149 μg/mL, and the essential oil with an EC$_{50}$ value higher than 60 mg/mL (Table 3). These values indicated that the methanolic extract is the most active sample in reducing the ferric cation into the ferrous cation. Interestingly, the methanolic extract displayed higher reducing power ability than ascorbic acid, which was used as the positive control and showed an EC$_{50}$ value of 79.3 μg/mL. The reducing power of the extracts can be correlated to the presence of good electron donors such as the phenolic compounds, thus converting reactive radicals into stable unreactive species (Fayoumi et al., 2022b).

3.4.4. Superoxide radical scavenging activity

The superoxide radical (O$_2^-$) is a highly reactive species that can interact with other molecules directly, through metal-catalyzed, or through enzyme-catalyzed processes to produce ROS involved in several pathogenesis due to its interaction with vital targets in vivo, including lipids, proteins and DNA (Lu et al., 2003). Thus, scavenging superoxide radicals is one of the modes of action utilized by natural antioxidants to exert its antioxidant activity. In this method, O$_2^-$ reduces the yellow dye (NBT$^{2+}$) to produce the blue formazan, which is measured spectrophotometrically at 560 nm. Thus, the assay measures the ability of the extracts to neutralize O$_2^-$ radicals and thus blue formazan formation. The reaction was monitored by the decrease in absorbance at 560 nm in comparison to the negative control (Nimse and Pal, 2015). The IC$_{50}$ for each sample, defined as the concentration of the sample required to scavenge 50% of the O$_2^-$ radicals initially present in the solution, were determined and used to evaluate the superoxide radical scavenging capacity of the different samples. In this assay, the aqueous extract showed the highest potential in scavenging the superoxide radical with an IC$_{50}$ value of 0.27
mg/mL, followed by the methanolic extract with an IC$_{50}$ of 1.89 mg/mL. On the other hand, the essential oil showed the least activity with a much higher IC$_{50}$ of 70.73 mg/mL (Table 3). Notably, the antioxidant activity of the aqueous extract was significantly non different from that of the potent antioxidant ascorbic acid which displayed with an IC$_{50}$ value of 0.20 mg/mL.

3.4.5. Metal chelating activity

Transition metals can play various roles in stimulating lipid peroxidation. They can generate initiative species, accelerate peroxidation and decompose lipid hydroperoxides. Therefore, it is important to study the metal chelating activity potential of antioxidant samples (Viuda-Martos et al., 2010). The metal chelating activity assay assessed the capability of the *S. fruticosa* essential oil and extracts to chelate ferrous cations and prevent the red complex formation between the ferrous cation and ferrozine. The reaction was monitored by the decrease in the absorbance of the mixture at 562 nm in comparison to a negative control. All extracts exhibited weak-to-moderate metal chelating activity, and therefore IC$_{25}$ values, corresponding to sample concentration required for inhibiting 25% complex formation compared to the negative control, were determined (Table 3). The methanolic and aqueous extracts were significantly more effective than the essential oil. The metal chelating activity of all extracts was lower than that of EDTA, a well-known chelating agent used as the positive control in the assay.

Reports describing the metal chelating activity of Salvia species in general and *S. fruticosa* in particular, are scarce. The findings of the current study are comparable to those of Topcu et al. who reported metal chelation inhibition of 40% (Topcu et al., 2010). A recent study on the chemical composition of extracts of three Salvia species, namely *S. blepharochlaena*, *S. euphratica var. leiocalycina*, and *S. verticillata* subsp. *Amasiaca*, assessed the antioxidant activity of the dichloromethane, methanolic and aqueous extracts of these species (Ozkan et al., 2010). Among the studied species, only *S. euphratica* methanolic extract showed higher total phenolic content and DPPH radical scavenging activity than that of the aqueous extract. Rosmarinic acid constituted the major phenolic compound in the different samples. In agreement with our findings, the authors demonstrated that the aqueous extracts exhibited a stronger metal chelating activity than the methanolic extracts of the different samples.

3.5. Correlation between Antioxidant Activities and Phytochemicals Content

Pearson’s correlation coefficients (r) were calculated between total phenolic, flavonoid, carbohydrate, and protein contents and antioxidant activities of the extracts. According to Taylor et al. (1990), $r < 0.35$, $0.36 < r < 0.67$, and $0.68 < r < 1$ represent weak, moderate, and strong correlations, respectively ($r$ in absolute value). Strong negative correlation was found between inhibition constants in DPPH, β-carotene, SOD and metal chelating assays, and the total phenolic, total flavonoids, and total carbohydrate contents, indicating that as the levels of such phytochemicals increases in extracts, the antioxidant activity increases. Our findings are in agreement with other studies which showed that antioxidant activity of rosemary and sage, both belonging to the family Lamiaceae, is mostly manifested by presence of the phenolic acids, terpenoids, flavonoids, and other phenolic (Lu and Foo, 2001; Kamatou et al., 2010; Orhan et al., 2012; Alimpić et al., 2015). The differences in the antioxidant activity between our samples and other populations of *S. fruticosa* or Salvia species can be attributed to genetic, geographical and/or environmental conditions, in addition to the effect of different storage conditions on the chemical profiles and biological activities of the plant species under study (Dincer et al., 2012). In general, our results were in agreement with the previously published studies on the phenolic content and antioxidant activity of different Salvia species.
Table 4: Linear correlation coefficient (r) of antioxidant activities versus total phenolic, flavonoid, carbohydrate, and protein contents.

<table>
<thead>
<tr>
<th></th>
<th>Total phenolic content</th>
<th>Total flavonoids content</th>
<th>Total carbohydrate content</th>
<th>Total protein content</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH</td>
<td>-0.897</td>
<td>-0.994</td>
<td>-0.978</td>
<td>0.998*</td>
</tr>
<tr>
<td>β-Carotene bleaching</td>
<td>-0.973</td>
<td>-0.993</td>
<td>-0.908</td>
<td>0.988</td>
</tr>
<tr>
<td>Reducing power</td>
<td>-0.898</td>
<td>-0.995</td>
<td>-0.978</td>
<td>0.998*</td>
</tr>
<tr>
<td>SOD</td>
<td>-0.888</td>
<td>-0.992</td>
<td>-0.982</td>
<td>0.996</td>
</tr>
<tr>
<td>Metal Chelating</td>
<td>-0.871</td>
<td>-0.987</td>
<td>-0.988</td>
<td>0.992</td>
</tr>
</tbody>
</table>

* Significant at \( p < 0.05; \) a \( r < 0.35 \) weak correlation; \( b \) \( 0.36 < r < 0.67 \) moderate correlation; \( c \) \( 0.68 < r < 1 \) strong correlation

### 3.6. Hemolytic Activity

Knowing that many plants may have toxic effects on the human health, it is critical to assess the toxicity of medicinal plants along with their pharmacological properties. One of the simplest methods used to assess toxicity of plant formulations is to study their hemolytic activity. To the best of our knowledge, the hemolytic activity of *S. fruticosa* has not been assessed before. In the current study, the hemolytic activity of *S. fruticosa* essential oil, methanolic and aqueous extracts against human erythrocytes was evaluated. No significant hemolytic activity was detected for the essential oil, methanolic and aqueous extracts at concentrations up to 0.88, 1, and 5 mg/mL, respectively. Higher concentrations of the essential oil (1.77 mg/mL) resulted in major hemolytic activity (34%), while moderate hemolysis (16%) was recorded for the methanolic extract at a concentration of 2 mg/mL. The aqueous extract did not show hemolytic activity within the tested concentration range (0.625-5 mg/mL), and thus considered safe to use within the studied range. On the other hand, cautions should be taken when using the methanolic extract and essential oil for pharmacological applications or in food industry. It has been reported that lipophilic substances have the capability to interact with membrane phospholipids leading to the lysis of erythrocytes and release of hemoglobin (de Carvalho Selvati Rezende et al., 2017). Based on our findings, the hemolytic activity exhibited by our oil sample can be attributed to the high content of lipophilic components in the oil. Likewise, methanol is a better solvent for lipophilic substances than water and a higher amount of lipophilic components is expected to be present in the methanolic extract compared to the aqueous extract. This can explain the moderate hemolytic activity of the methanolic extract at high concentrations and the absence of any hemolytic effect of the aqueous extract even at higher concentrations than those used for the oil and methanolic extract. Furthermore, 1,8-cineole which has been reported as the major constituent of our essential oil sample showed significant levels of hemolytic activity at concentrations exceeding 10 mM (Mendanha et al., 2013). This can also justify the hemolytic activity exhibited by our essential oil sample. Nevertheless, a complete screening of the phytochemicals in the samples used in this study would help in a more relevant analysis of the obtained data. The determination of the cytotoxicity of the samples through assays other than the hemolysis assay is also critical to determine any adverse effects that might be accompanied with the use of this plant material.
4. CONCLUSION

The current study assessed the antioxidant activity, deciphered the phytochemical profile, and evaluated the toxicity of *S. fruticosa* Miller cultivated in the Bekaa district, Eastern Lebanon. GC-MS analysis of the different plant extracts revealed phytochemicals commonly encountered in the *S. fruticosa*. The high levels of total phenolic compounds and total flavonoids detected in the plant extracts were strongly correlated to its prominent antioxidant activity. The observed levels of total carbohydrates and proteins suggested that the plant constitutes a reliable nutritious source of these phytochemicals. The hemolytic activity assay demonstrated that the plant extracts were safe for consumption at the studied concentrations. The collective analysis of the data offered an encouraging evidence for the cultivation of commercially valuable MAPs such as *S. fruticosa* Miller as a supportive measure for the Lebanese economy. Investing in the establishment of *S. fruticosa* nurseries holds huge promise for startups. In fact, *S. fruticosa* does not need special maintenance practices as it is generally resistant to infections. In addition, the climate in Lebanon is suitable to grow a produce that is rich in bioactive compounds. The worldwide popularity of the plant as a medicinal herb should constitute an incentive for local investors to cultivate *S. fruticosa* on an industrial scale due to the large demand for the plant that does not meet the market need (local and international). *S. fruticosa* based-products can be manifested in different forms. Dried plant parts can be sold directly for consumption as herbal tea, while fresh plant can be hydrodistilled for the production of essential oil that can be marketed to pharmaceutical companies to use in many formulations.

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Conflict of Interest Statement

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