https://doi.org/10.31925/farmacia.2022.3.13 **ORIGINAL ARTICLE**

CHEMICAL COMPOSITION AND THERAPEUTIC ACTIVITY OF LEBANESE ROSE GERANIUM (*PELARGONIUM* HYBRID) EXTRACTS

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Manuscript received: November 2021

Abstract

The phytochemical constituents were identified, and the antioxidant, antiinflammatory, antiacetylcholinesterase (AChE), cytotoxic, and anti-haemolytic properties were assessed for the leaves and stem extracts of Lebanese rose geranium extracted by four solvents with different polarity (ethanol, water, ethyl acetate and chloroform). The highest amounts of total phenolics (237 GAE mg/g), total flavonoids (356 RE mg/g) and condensed tannins content (441 LE mg/g) were detected in the ethanolic leaves extracts. The antioxidant activity was estimated by four methods, namely DPPH radical scavenging, hydrogen peroxide scavenging, reducing power and β-carotene bleaching assays and the ethanolic leaves extract exhibited the superior antioxidant potential. On the other hand, the chloroform extracts revealed good anti-inflammatory activity. The ethanol and ethyl acetate leaves and stem extracts demonstrated the highest anti-AChE activity with 80% inhibition achieved at 5 mg/mL. The cytotoxic activity of the extracts was evaluated against HCT 116 colon cancer cells, with the chloroform leaves extract being the most potent with an IC_{50} of 0.4 mg/mL. The toxicity of the extracts was assessed by testing its ability to induce haemolysis of red blood cells, and the water leaves extract was the least toxic.

Rezumat

Au fost identificați constituenții fitochimici din frunzele și tulpina unui hibrid de *Pelargonium spp*., cărora le-au fost evaluate acțiunile antioxidantă, antiinflamatoare, anti-acetilcolinesterazică (AChE), citotoxică și antihemolitică, pe extracte extrase cu patru solvenți cu polaritate diferită (etanol, apă, acetat de etil și cloroform). Cele mai mari cantități de fenoli totali (237 GAE mg/g), flavonoide totale (356 RE mg/g) și taninuri condensate (441 LE mg/g) au fost detectate în extractele etanolice din frunze. Activitatea antioxidantă a fost estimată cu ajutorul a patru metode, și anume: DPPH, transformarea peroxidului de hidrogen, puterea reducătoare și testul β-carotenului. Extractul etanolic din frunze a prezentat un efect antioxidant superior, iar extractele în cloroform au evidențiat o bună activitate antiinflamatoare. Extractele etanolice și cele în acetat de etil din frunze și tulpină au demonstrat cea mai mare activitate anti-AChE cu o inhibiție de 80% la 5 mg/mL. Activitatea citotoxică a extractelor a fost evaluată pe celule HCT 116, extractul de frunze în cloroform dovedindu-se cel mai puternic cu o IC_{50} de 0,4 mg/mL. Toxicitatea extractelor a fost evaluată prin testarea capacității lor de a induce hemoliza globulelor roșii, iar extractul apos din frunze s-a dovedit cel mai puțin toxic.

Keywords: Lebanese rose geranium, HPLC, phenolic compounds, heat induced haemolysis, albumin denaturation, radical scavenging activity

Introduction

Medicinal plants have long been considered a valuable renewable natural source for the discovery of novel therapeutic molecules or for the identification of lead entities that can be optimized through structure activity relationship (SAR) studies into commercial drugs. The therapeutic activity of medicinal plants is attributed to the presence of secondary metabolites that act as natural defence system against infections [1]. One of the plants that received considerable attention in traditional medicine is *Pelargonium* genus (*Geraniaceae*), an aromatic plant native to South Africa encompassing more than 250 species and is cultivated worldwide including the Middle East. Plants of this genus are characterized by scented leaves of various odours ranging from pleasantly fruity to rather oppressively balsamic [2]. A worth noting plant in this class is rose geranium (commercially referred to as a hybrid of *Pelargonium graveolens* L'Hér.). Rose geranium is a hybrid between *P. graveolens* or

P. radens and *P. capitatum*, although it is erroneously and almost consistently referred to in scientific literature as *P. graveolens* L'Hér. [3-5]. While the correct nomenclature of the plant has yet to be determined, we refer to it as *Pelargonium* cv. Rosé in this paper. Rose geranium is a perennial evergreen plant with lobed leaves and distinctive texture, being hairy with deeply-cut blades and aroma odour [6]. The edible part of this plant is their leaves, which was used as a treatment of throat infections, inflammation and in aromatherapy in traditional medicine [6]. While the essential oil of rose geranium (commercially known as geranium oil) was heavily investigated in scientific literature because of its pharmacological and industrial value, the leaves and stem organic extracts did not receive a matching attention. Previous studies reported the presence of secondary metabolites in leaves extracts of rose geranium such as flavonoids, tannins and phenolic compounds [7], which may have contributed to the observed biological properties of the plant. The alcoholic and water leaves and flower extracts of Tunisian rose geranium exhibited α-amylase and acetylcholinesterase (AChE) inhibitory, antibacterial, antioxidant and phylotoxic activities [8]. These findings corroborated those of Boukhris *et al.* who also reported significant antioxidant and antibacterial potential of leaf water and methanol extracts of Tunisian rose geranium [9]. Another recent study investigated the phenolic content and biological benefits of organic extracts of rose geranium growing in Morocco [10]. The authors highlighted that the methanolic extract contained the highest amounts of total phenolic, flavonoids, flavonols and condensed tannins, and demonstrated strong antioxidant, enzyme inhibitory and antimicrobial activities. In a study that evaluated the methanolic leaves extract of Iraqi rose geranium, the plant displayed prominent antioxidant and cytotoxic activities [11].

Lebanon is a Middle Eastern country with rich biodiversity. As the phytochemical and pharmacological profile of plants of the same species is affected by geographical origin [5], we aimed in the current study to investigate the phytochemical profile and assessing the biological properties of rose geranium cultivated in Lebanon. In specific, the chemical composition, antioxidant, antiinflammatory, anti-AChE, cytotoxic and haemolytic activity of the ethanol, water, ethyl acetate and chloroform extracts of leaves and stems were investigated. To the best of our knowledge, no reports have described the chemical composition and therapeutic potential of Lebanese rose geranium extracts.

Materials and Methods

Materials

All chemical were used as received without further purification. 1,1-Diphenyl-2-picrylhydrazl (DPPH), linoleic acid and β-carotene were purchased from Acros Organics, USA, while rutin was obtained from Merck, USA. Potassium ferricyanide, trichloroacetic acid, ferric ammonium sulfate and ferric chloride were purchased from Alpha Aesar, USA. Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, quercetin, sodium thiosulfate, ammonium molibdate, potassium iodide, ferric ammonium sulfate and sulfuric acid were purchased from Fluka, Germany. S-Acetylthiocholine iodide (ATCI), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), diclofenac sodium, Folin-Ciocâlteu reagent, hydrogen peroxide, Tween-80, chloroform, gallic acid, butanol, N1-naphthylethylenediamine dichloride and bovine serum albumin were purchased from Sigma Aldrich, USA. Triton X-100, sodium carbonate, sodium bicarbonate, sodium nitropruside and sulphanilamide were obtained from Himedia, India.

Sample collection and preparation

Rose geranium was collected in April 2017 from Bechamoun area, southern Beirut, Lebanon (33°47'20.3"N 35°29'51.9"E). The leaves and stems were separated, dried in open air in the dark for 7 days, grounded into fine powder, and stored in refrigerator for further use. Stems and leaves were extracted by stirring 10 g of sample in 125 mL of different solvents at 50°C for 4 h. Four solvents were used for extraction, namely ethanol, ethyl acetate, water and chloroform, and eight different extracts were obtained.

Phytochemical analysis

Total phenolics content. The total phenolic content in the plant extracts was determined using the Folin-Ciocâlteu reagent as described by Singleton *et al*. with slight modification [12]. In brief, 50 μL of plant extract were mixed with 2 mL water and 100 μL of Folin-Ciocâlteu reagent, and the mixture was shaken for 10 min. 3 mL of 2% Na_2CO_3 was added next, and the mixture was stored in the dark for one hour at room temperature. The absorbance was then read at 750 nm using a UV spectrophotometer. The concentration of phenolic compounds was determined by employing the equation obtained from the curve plotted using gallic acid as a standard. The results were expressed in mg gallic acid equivalent *per* g of extract (mg GAE/g).

Total flavonoid content. Aluminium chloride chromogenic method was used for determining the total flavonoid content as previously described [13]. In brief, 100 μL of plant extract was mixed with 5 mL of 70% ethanol, followed by the addition of 0.5 mL of 2% methanolic aluminium chloride solution. The solution was incubated for 6 min, and then diluted to 10 mL by 70% ethanol. The absorbance was measured at 415 nm spectrophotometrically. The flavonoids concentration was calculated according to the equation obtained from the calibration curve using rutin as a standard and the results are reported in mg rutin equivalent *per* g of extract (mg RE/g).

Condensed tannins. The butanol-HCl assay was used as described by Porter *et al*. [14]. In brief, 0.5 mL of extract solution, 3 mL of butanol:HCl (95:5 v/v) reagent, and 100 µL of ferric reagent (2% ferric ammonium sulfate in 2 N HCl) were mixed. The test combination was mixed with Vortex and placed in a boiling water bath for 60 min. A blank was prepared by mixing the plant extract with butanol-HCl reagent and ferric reagent without heating. Absorbance was read at 550 nm using a UV-vis spectrophotometer against blank. Condensed tannin was calculated as leucocyanidin equivalents using the formula developed by Porter [14].

Antioxidant activity

DPPH radical scavenging activity. The ability of the extracts to scavenge DPPH radicals was assessed according to the method of Sharma *et al*. with some modification [15]. In brief, 2 mL of ethanolic DPPH solution (0.06 mM) were mixed with 2 mL of different concentrations of the extract, and the mixture was incubated in dark at 37°C for 30 min. Absorbance was measured at 517 nm using UV-Vis spectrophotometer against blank comprised of 1:1 mixture of solvent and plant extract. Ascorbic acid was used as positive control. A mixture of DPPH and solvent was used as a negative control. A DPPH solution plus solvent was used as a negative control. The percent inhibition of DPPH scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = ((Ac - As)/Ac) * 100,$$

where, Ac is absorbance of negative control and As is the absorbance of test sample.

Reducing power assay. The ferric reducing capacity of the extracts was tested by the ferricyanide/Prussian blue method as described by Işıl Berker *et al*. [16]. In brief, 2.5 mL of phosphate buffer (0.2 M, pH 6.6), 2.5 mL of 1% potassium ferricyanide solution, and 1 mL of different concentrations of the plant extract were mixed and incubated at 50°C for 20 min. After incubation, 2.5 mL of trichloroacetic acid (1%) were added, and the mixture was mixed vigorously. Finally, 2.5 mL of the reaction mixture were mixed with 2.5 mL water and 0.5 mL $FeCl_3$ (0.1%). The absorbance of the solution was measured at 700 nm using UV-Vis spectrophotometer. The sample concentration providing 0.5 of absorbance (IC_{50}) was calculated by plotting absorbance against the corresponding sample concentration.

Hydrogen peroxide (H_2O_2) scavenging activity. Hydrogen peroxide scavenging activity of the extracts was estimated by replacement titration as reported by Sharma *et al*. with some modifications [15]. The working hydrogen peroxide solution was prepared by diluting 0.2 mL H_2O_2 in 100 mL water, followed by diluting 10 mL of the prepared solution in 100 mL of water. Aliquots of 2 mL of the working hydrogen peroxide solution were mixed with 2 mL of different concentrations of the extracts, followed by the addition of 2 drops of 3% ammonium molybdate, and 10 mL of 2 M H_2SO_4. The mixture was incubated for 15 min. 10.0 mL of 10% KI were then added to the reaction mixture. The mixture was titrated with 5.09 mM sodium thiosulfate until the disappearance of the yellow colour using starch as indicator. Ascorbic acid was used as positive control. Percentage of H_2O_2 scavenging was calculated using the following formula:

$$\% \text{ Inhibition} = ((V_0 - V_1)/V_0) *100,$$

where, V_0 is the volume of $Na_2S_2O_3$ solution used in the titration of H_2O_2 without extract (Blank); V_1 is the volume of $Na_2S_2O_3$ solution used in the titration of H_2O_2 in presence of extract.

β-carotene bleaching assay. The β-carotene bleaching assay was performed according to the method of Miraliakbari *et al*. with some modification [17]. In brief, 2 mL of β-carotene solution (0.2 mg/mL in chloroform), 44 µL of linoleic acid and 377 µL of Tween 80 were mixed in a round bottom flask. The solvent was evaporated on a rotary evaporator, and 100 mL of oxygenated deionized water were added to the mixture. The solution was emulsified for 15 min in a sonicator to form an emulsion. Aliquots of 5 mL of this emulsion were transferred into a series of test tubes containing 200 µL of different concentrations of the extracts. Optical density (OD) at 470 nm was determined for all samples immediately (t = 0) and after incubation for 2 h at 50°C. A second emulsion was also prepared and used as blank. Quercetin was used as positive control. The percentage inhibition was calculated according to the following formula:

$$\% \text{ Inhibition} = [(A_{a(120)} - A_{C(120)})/(A_{C(0)} - A_{C(120)})] \times 100,$$

where, $A_{a(120)}$ is the absorbance of the sample at t = 120 min, $A_{C(120)}$ is the absorbance of the control at t = 120 min and $A_{C(0)}$ is the absorbance of the control at t = 0 min.

Anti-AChE activity

The activity of acetylcholinesterase enzyme (AChE) was determined following the Ellman's method with some modifications [18]. Experimental procedures were conducted according to the guidelines set by BAU Institutional Review Board (IRB). The IRB provides independent and timed decisions based on adherence to the guidelines of the Animal Welfare Act12 and the Canadian Council on Animal Care's (CCAC) Guide to the Care and Use of Experimental Animals. Male Sprague-Dawley rats (weighing 350 g, 8 weeks old) were housed in cages at an ambient temperature of 21°C with 50 - 60% relative humidity in a 12 h light/dark cycle. The animals had free access to the standard pellet diet and drinking water during the experiments. Rats were sacrificed by chloroform euthanasia and brains were extracted. One g of rat brain was washed with saline solution (0.9 M), mixed with 9 mL of PBS (pH = 7.4), homogenized and centrifuged at 3000 rpm and 4°C for 10 min. The

supernatant was collected and stored at -20°C for further use. In a 96-well plate, 20 µL of different concentrations of rose geranium extracts dissolved in DMSO were mixed with 130 µL of PBS (pH 8). 20 µL of DMSO was added in the control wells. 20 µL of AChE enzyme were added to the wells, and the plate was incubated for 45 min at room temperature. 10 µL of DTNB (4 mg/mL in PBS) were added, and the reaction was initiated by adding 10 µL of the substrate ATCI (17.4 mg/mL in PBS). Absorbance was then measured at 405 nm after 2 min for two times. The percentage of AChE enzyme inhibition was calculated according to the following equation:

$$\% \text{ Inhibition} = ((Ac - \Delta A)/Ac) * 100,$$

where, Ac is the absorbance of control and ΔA is the difference of absorbance between two consecutive readings.

Antiinflammatory activity

Heat induced haemolysis. This assay was performed according to the method described by Abou-Ellela with some modifications [19]. Blood samples, obtained from a healthy volunteer, were placed in tubes containing EDTA to prevent blood coagulation and centrifuged at 2500 rpm for 5 min. RBCs were collected and repeatedly washed by PBS (pH 7.4) until the supernatant becomes clear. 10% of erythrocyte suspension was prepared in PBS and restored at 4°C for further use. Equal volumes of erythrocyte and different concentration of rose geranium extracts were mixed in a test tube. The resulting solution was incubated at 56°C for 30 min, then cooled to room temperature and centrifuged at 2500 rpm for 10 min. The supernatant was collected, and absorbance was measured at 540 nm using ELISA plate reader. Diclofenac sodium was used as positive control, while DMSO was the negative. The stability of RBCs was determined using the following equation:

$$\% \text{ stability of HRBC} = ((Ac - As)/Ac) * 100,$$

where, Ac is the absorbance of negative control solution and As is the absorbance of samples.

Albumin denaturation. The ability of rose geranium extracts to prevent albumin denaturation was performed according to the method of Leelaprakash and Dass with some modification [20]. In brief, 100 µL of BSA (1%) dissolved in PBS (0.1 M) were mixed with 100 µL of different concentrations of rose geranium extracts in a 96-well plate. A mixture of 100 µL of BSA and 100 µL of DMSO was used as a negative control. The plate was incubated at 37°C for 20 min, then at 57°C for additional 20 min. The plate was then cooled to room temperature, and absorbance was measured on an ELISA plate reader at 620 nm. The percentage inhibition was calculated using the following equation:

$$\% \text{ Inhibition of albumin denaturation} = ((A_c - A_s)/A_c) \times 100,$$

where, A_c is the absorbance of the control and A_s is the absorbance of the sample.

Nitric oxide (NO) scavenging activity. The NO scavenging activity of rose geranium extracts was assessed according to the method of Sharma and Singh with some modifications [15]. In brief, 50 µL of sodium nitroprusside (10 mM in PBS) were mixed with 50 µL of different concentrations of the extracts. The mixtures were incubated in the dark at 25°C for 30 min. 100 µL of Griess reagent were then added, mixed well, and incubated at room temperature for 10 min. Absorbance was finally measured at 540 nm using an ELISA plate reader. A negative control was prepared similarly without the plant extract. The percentage of nitric oxide inhibition was calculated as follows:

$$\% \text{ Inhibition of NO} = ((A_c - A_s)/A_c) \times 100,$$

where, A_c is absorbance of the negative control and A_s is absorbance of the test sample.

Cytotoxic activity

HCT 116 colon cancer cells, cultured in DMEM medium supplemented with 10% heat-inactivated FBS and 100U penicillin/streptomycin, were seeded in 96-well plate (3×10^4 cells/well), and incubated at 37°C and 5% CO_2 in a humidified chamber for 24 h. The culture media was then aspirated, and cells were treated with different concentrations of rose geranium extracts dissolved in 10% DMSO in DMEM, and incubated for 48 h. 10 µL of cell proliferation reagent WST-1 were added to the wells, and the plate was incubated for 2 h. Absorbance at 480 nm was measured on an ELISA plate reader. Wells containing culture media and WST-1 reagent without cells were used as blank, while wells containing different concentration of extracts and WST-1 reagent were used as background. Cells that did not receive plant extracts were used as negative control. The percentage of cell viability was calculated as follows:

$$\% \text{ cell viability} = ((A_s - A_b)/(A_c - B)) * 100,$$

where, A_s is the absorbance of the sample, A_b is the absorbance of background, A_c is the absorbance of the control and B is the absorbance of the blank.

Haemolysis

The ability of rose geranium extracts to induce haemolysis of red blood cells was tested by mixing 50 µL of erythrocyte (10%), 75 µL of different concentrations of the extracts, and 100 µL of PBS (pH = 7.4) in centrifuge tubes. The mixture was incubated at 37°C for 1 h during which the tubes were gently shaken every 20 min. The tubes were then centrifuged at 2500 rpm for 3 min. The absorbance of the supernatant was measured using 96-well plate at 540 nm using an ELISA plate reader. 2% of Triton X-100 in PBS was used as positive control, and PBS was used as negative control. The haemolytic activity of the extracts was calculated as follows:

$$\% \text{ Haemolytic} = (A_s/A_c) \times 100,$$

where, A_s is the absorbance of the supernatant of the test sample and A_c is the absorbance of the supernatant of the positive control.

Statistical analysis

All experimental results were performed in triplicate, and the data were recorded as mean ± standard deviation.

Results and Discussion

Chemical composition of rose geranium extracts

It is widely acknowledged that the chemical composition of rose geranium is influenced by changes in environmental factors such as country of cultivation, soil, temperature, harvesting period, harvested parts, etc. Therefore, it is very important to investigate the chemical composition of the extracts of rose geranium grown in Lebanon.

The presence of phenolic compounds such as phenolic acids, flavonoids and tannins significantly influence the biological profile and therapeutic spectrum of plant extracts. In the current study, the total phenolics, total flavonoids, and condensed tannins contents in the eight extracts were determined spectrophotometrically, and results are summarized in Table 1. The total phenolic content was estimated using Folin-Ciocâlteu reagent, and the results were expressed as gallic acid equivalent *per* gram of dry extract (mg GAE/g) using a gallic acid calibration curve. Interestingly, the leaves extracts contained higher phenolic content compared to the stem extracts, regardless of the extraction solvent, with values ranging between 237.0 ± 7.0 GAE mg/g for the ethanolic leaves extract and 9.89 ± 0.20 GAE mg/g for chloroform stem extract. Polar solvents, such as ethanol and water, were more efficient in extracting phenolic compounds. These results are in agreement with multiple reports [21-23] who reported the methanol is the best solvent for the recovery of phenolic compounds compared to water or nonpolar solvents such as hexane.

The total flavonoids content was determined by using the aluminium chloride method, and results were reported as rutin equivalent (RE) *per* grams of dry extract (mg RE/g) using the regression equation for rutin standard curve. The total flavonoid content ranged between 356.0 ± 5.0 mg RE/g for ethanolic leaves extract and 73.1 ± 0.5 mg RE/g for water stem extract. These results are in agreement with the findings of Boukharis *et al*. who reported that polar solvents such as methanol are more suitable for the extraction of flavonoids compared to water [9]. On the other hand, our findings were contradictory to those reported by Ben Hsouna and Hamdi who showed that water was a better extraction solvent of flavonoids from leaves of rose geranium compared to methanol, ethyl acetate and hexane [21]. It should also be noted that, and in agreement with Hsouna and Hamdi [21], high phenolic content is not always accompanied by high flavonoid concentrations.

Both hydrolysable and non-hydrolysable tannins are synthesized in substantial amounts in many species of the genus *Pelargonium* [24]. In the current study, the butanol-HCl assay was used to determine the condensed tannins content, and values were expressed as leucocyanidin equivalents (LE) *per* grams of dry extract (mg LE/g). The highest amounts of condensed tannin were found in the ethanol leaves extract (441.0 ± 2.02 mg LE/g), and lowest levels were detected in chloroform stem extracts (13.8 ± 2.0 mg LE/g). Such observation is recurring in literature where significantly higher amounts of condensed tannins were extracted by polar solvents such as methanol compared to non-polar ones such as hexane and dichloromethane [10]. Factors influencing the difference in levels of the phytochemical constituents in the extracts evaluated in this study from literature reports can be multi-fold such extraction technique, polarity of the extraction solvents, environmental factors (climate, soil, etc.), cultivation and harvesting processes [7, 21].

Table I

Total flavonoid, total phenolic, and condensed tannins contents in the leaves and stems extracts of *Pelargonium* cv. Rosé grown in Lebanon

Extract	Total flavonoid content (mg RE/g)		Total phenolic content (mg GAE/g)		Condensed tannins (mg LE/g)	
	Leaves	Stem	Leaves	Stem	Leaves	Stem
EtOH	356.0 ± 5.0	336.0 ± 3.3	237.0 ± 7.0	186.0 ± 13.0	441.0 ± 2.02	201.0 ± 4.0
Water	156.0 ± 0.9	73.1 ± 0.5	105.0 ± 7.0	49.0 ± 9.0	83.0 ± 0.4	36.9 ± 1.0
EtOAc	299.0 ± 3.3	278.3 ± 3.0	69.0 ± 8.0	25.0 ± 3.0	23.0 ± 0.75	14.0 ± 0.3
$CHCl_3$	312.0 ± 3.9	292.3 ± 6.5	91.0 ± 6.0	9.89 ± 0.20	17.0 ± 0.6	13.8 ± 2.0

(mg GAE /g): mg of gallic acid equivalent *per* g of dry plant extract; (mg RE/g): mg of rutin equivalent *per* g of dry plant extract; (mg LE/g): mg of leucocyanidin equivalent *per* g of dry plant extract

Antioxidant activity

The use of multiple approaches to study antioxidant activity is recommended since plants contain different classes of antioxidants that may act by different mechanisms. In the current report, the antioxidant activity of the rose geranium extracts was assessed by four different approaches, namely DPPH radical scavenging activity, β-carotene bleaching assay, reducing power assay and hydrogen peroxide scavenging activity [25].

DPPH radical scavenging activity. The ability of the eight rose geranium extracts to scavenge free radicals was tested by the DPPH scavenging assay. DPPH has a stable free radical with violet colour which turns colourless upon reduction (gain of a hydrogen atom) by an antioxidant. Such activity is dose dependent, i.e. increasing the concentration of antioxidant promotes the discoloration of DPPH radical. IC_{50} values, defined as the concentration of the extract that inhibits 50% of DPPH discoloration, are summarized in Table II. Among the leaves extracts, ethanol showed the highest DPPH radical scavenging activity with IC_{50} value of 7.88 ± 0.28 µg/mL, while chloroform was the least active with IC_{50} value of 233.1 ± 11.0 µg/mL. A similar trend was observed for the stem extracts with the ethanolic extract having an IC_{50} value of 10.0 ± 0.3 µg/mL while the chloroform extract had IC_{50} value of 453.2 ± 55.1 µg/mL. Overall, the ethanolic leaves extracts showed higher DPPH radical scavenging activity compared to the stem extracts. These results are similar to those reported in literature. For example, Ben ElHaj Ali *et al.* showed that ethanolic leaves extracts of rose geranium cultivated in Tunisia exhibited higher antioxidant activity (IC_{50} = 14.68 ± 0.44 µg/mL) compared to the aqueous leaves extract (IC_{50} = 39.45 ± 1.32 µg/mL) [8]. Interestingly, the ethanolic and aqueous extracts of rose geranium cultivated in Lebanon demonstrated better DPPH radical scavenging activity. In a study that evaluated the biological properties of extracts of rose geranium grown in Morocco, El Aanachi *et al.* reported that the methanolic extracts of aerial parts of the plant showed higher antioxidant activity compared to the nonpolar extracts (hexane and dichloromethane) [10]. Al-Saffar *et al*. revealed that the methanolic extracts of rose geranium grown in Iraq exhibited higher antioxidant activity when assessed by the DPPH assay with an IC_{50} of 292 µg/mL. Sompaga *et al*. studied the antioxidant activity of organic extracts of rose geranium grown in India, and reported that the methanolic extract showed higher DPPH radical scavenging activity compared to the ethyl acetate fraction [26].

Table II

Antioxidant activity of leaves and stem extracts of *Pelargonium* cv. Rosé grown in Lebanon

Extract	DPPH IC_{50} (µg/mL)		H_2O_2 IC_{50} (µg/mL)		Reducing power $A_{0.5}$ (µg/mL)		β-Carotene bleaching IC_{50} (µg/mL)	
	Leaves	Stems	Leaves	Stems	Leaves	Stems	Leaves	Stems
EtOH	7.88 ± 0.28	10.0 ± 0.30	2533 ± 76	3550 ± 130	143.3 ± 5.0	137.2 ± 10.3	78.3 ± 2.8	533.4 ± 70.7
Water	10.1 ± 0.12	28.3 ± 2.9	ND*	ND*	316.4 ± 15.2	795.5 ± 80.4	103.0 ± 23.1	410.2 ± 10.3
EtOAc	26.5 ± 1.3	141.6 ± 3.0	ND*	3720 ± 160	ND**	ND**	300 ± 49	376.4 ± 40.1
$CHCl_3$	233.1 ± 11.0	453.2 ± 55.1	ND*	ND*	ND**	ND**	400 ± 10	573.3 ± 23.2
Quercetin	0.757 ± 0.023		-		-		55.8 ± 3.8	
Ascorbic acid	-		22.0 ± 2.6		28.0 ± 0.5		-	

ND* and ND** = not determined.

Reducing power assay. The reducing power assay is a rapid method for assessing the ability of an antioxidant to donate electrons by reducing Fe^{3+} to Fe^{2+} ions. In the presence of a reducing agent (electron donor), a ferric cyanide complex (Fe^{3+}) is reduced to the ferrous cyanide form (Fe^{2+}), leading to the formation of a Prussian blue coloured ferric ferro-cyanide complex in the presence of $FeCl_3$ with a characteristic absorption at 700 nm in the UV-Vis spectrum. The stronger the reducing power of the antioxidant, the more intense is the generated blue colour and consequently the higher is the measured absorbance [27]. Among with the reducing activities of various rose geranium leaves and stem extracts in comparison with ascorbic acid as positive control, the concentrations of the extracts that produced an absorbance of 0.5 ($A_{0.5}$) are summarized in Table II [10]. The methanolic extracts of the leaves and stems exhibited the strongest reducing power, followed by the water, ethyl acetate and chloroforms extracts. On the other hand, ascorbic acid displayed higher reducing power compared to any of the rose geranium extracts. It is possible that natural bioreductants are extracted to polar solvents more efficiently compared to less polar solvents. The order of reducing power is in agreement with the levels of total phenolic, total flavonoid and condensed tannins contents determined for the various extracts. The richness of these phyto-chemicals with phenolic hydroxyl groups capable of stabilizing the radical species generated following electron donation renders them powerful bioreducing agents [28].

H_2O_2 scavenging activity. The antioxidant activity of rose geranium extracts was further assessed by evaluating its ability to decompose H_2O_2. It has been reported that the accumulation of H_2O_2 in the body can lead to adverse effects through the direct inactivation of enzymes by oxidizing the thiol (SH) group of cysteine, or by crossing cell membranes to interact with intracellular ferrous ions to form hydroxyl radicals which cause irreversible damage to vital biomolecules such as genetic material, proteins and lipids. Therefore, it is highly advantageous for cells to control the amount of H_2O_2 and prevent its accumulation [15].

The results depicted the hydrogen peroxide scavenging activity of rose geranium leaves and stem extracts relative to ascorbic acid used as the positive control.

All extracts displayed a weak dose-dependent activity. IC_{50}, defined as the concentration of the sample required to scavenge 50% of the H_2O_2 initially present in the solution, was reached only by ethanolic leaves and stem extracts as well as ethyl acetate stem extract, with values of 2.5, 3.6 and 3.6 mg/mL, respectively (Table II). On the other hand, ascorbic acid demonstrated a high activity with IC_{50} value of 0.022 mg/mL in the same assay. The water leaves and stem extracts showed the weakest activity of all extracts.

β-Carotene bleaching assay. Unsaturated fatty acids, such as linoleic acid and arachidonic acid, found in the bilayer lipid cell membrane are prone to oxidation through a process known as lipid peroxidation. Lipid peroxides are involved in the initiation of lipid peroxidation chain reactions resulting in the disturbance of cell membranes and rearrangement of the membrane structure [29, 30]. The β-carotene bleaching assay is one of the most common experimental models for the estimation of the ability of samples to scavenge the linoleic acid peroxides. The oxidation of linoleic acid produces free radicals due to the removal of hydrogen atom from diallylic methylene groups of linoleic acid that leads to the degradation of β-carotene and the loss of its characteristic orange colour [31]. However, the presence of antioxidant constituents could prevent the bleaching of β-carotene. The ability of the test samples to inhibit β-carotene discoloration is monitored spectrophotometrically at 470 nm. The IC_{50} values for the extracts, defined as the concentration of the sample required for 50% inhibition of β-carotene discoloration in comparison to the negative control, were determined and used to evaluate the lipid peroxide radical scavenging activity of the different samples (Table II). In general, the leaves extracts demonstrated better inhibition of β-carotene bleaching compared to the stem extracts. The ethanolic leaves extract exhibited the highest activity with an IC_{50} value of 0.0783 mg/mL which was comparable to that of the natural antioxidant quercetin (0.0558 mg/mL). The prominent activity can be attributed to the high levels of phenolic compounds in the ethanolic extracts. It has been reported that phenolic compounds have important role in preventing lipid peroxidation [32], and a positive linear correlation was found between antioxidant capacity and total phenolic contents of medicinal herbs [33, 34].

AChE inhibition assay

Alzheimer's disease (AD) is the most common form of dementia in elderly people. It is characterized by loss of basal forebrain cholinergic neurons and the eventual the decrease in the levels of the neurotransmitter acetylcholine (ACh) [35]. Current treatments of AD is dominated by inhibitors of acetylcholinesterase enzyme (AChE) that is present in the brain and acts to hydrolyse ACh into choline and acetic acid leading to memory loss in AD patients. These inhibitors act to prevent ACh decomposition and restore synaptic levels of the neurotransmitter [36]. In addition, AChE is implicated in the pathogenesis of AD as a facilitator for β-amyloid plaque deposition which is considered the hallmark of AD [37, 38]. Natural products have already proven to be promising sources of useful AChE inhibitors. In fact, two of the currently available drugs for AD, namely rivastigmine and galanthamine, are of natural origin [39]. Therefore, the development of novel natural AChE inhibitors holds huge promise for AD treatment [40].

Herein, the anti-AChE activity of rose geranium extracts was tested and results are depicted in Figure 1 and compared to the donepezil, a synthetic acetylcholinesterase inhibitor approved for AD treatment. IC_{50} values, defined as the concentration of the extract required to inhibit 50% of AChE activity, was derived from the plots and results are summarized in Table III. The ethanol and ethyl acetate leaves and stem extracts demonstrated the highest anti-AChE activity with 80% inhibition achieved at 5 mg/mL of the extract. The specific activity of AChE decreased with the increase of the extracts' concentrations. This reveals that these extracts can act as AChE inhibitors. These findings are consistent with the results reported recently by Ben ElHaji Ali *et al*. who found that the ethanolic leaves extracts of rose geranium were 3-times more potent as anti-AChE inhibitors compared to the water leaves extracts due that the presence of higher levels of phenolic acids, flavonols and flavonoids compounds [8]. Another study by Ennaifer *et al*. reported that the decoction water extracts of rose geranium cultivated in Tunisia possessed good anti-AChE activity with 93% inhibition achieved at 2.5 mg/mL [41]. Jazayeri *et al*. reported that the maceration extract of rose geranium in aqueous methanol (1:1) was a strong AChE inhibitor with IC_{50} of 197 µg/mL, and attributed the anti-AChE activity to the presence of phytochemicals such as polyphenols, flavonoids and alkaloids [42].

Antiinflammatory activity

Inflammation is the natural response of our body to stimuli such as physical injuries, heat, microbial infections and noxious chemical irritations. During inflammation, vascular permeability, protein denaturation and membrane alteration are augmented leading to redness, heat, swelling, pain and disturbance of physiological functions. Non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen and diclofenac sodium, commonly prescribed for treatment of pain and inflammatory conditions are not devoid of side effects where gastrointestinal bleeding and suppression of the immune system were reported upon the use of these medications [43]. Natural products with anti-inflammatory activity have long been used in folk medicine for the treatment of inflammatory conditions such as fevers, pain, migraine and arthritis [44, 45]. The richness of natural products in phenolic compounds, flavonoids and terpenoids contributed to its anti-inflammatory action. Therefore, we set out to assess

the anti-inflammatory activity of rose geranium extracts through three different assays.

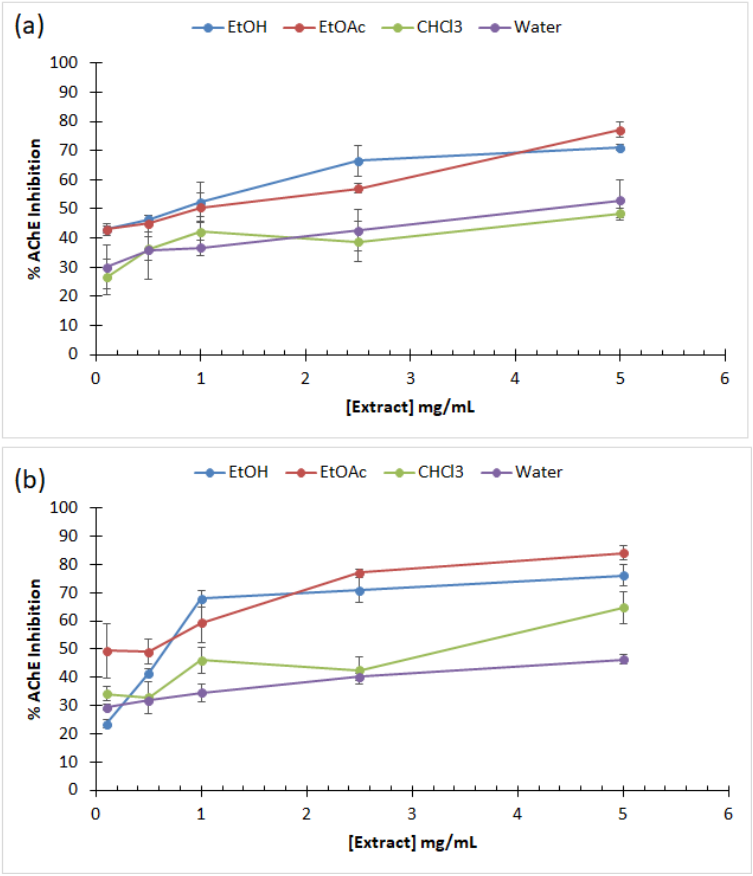

Figure 1.

AChE inhibitory activity of (a) leaves and (b) stems extracts of *Pelargonium* cv. Rosé grown in Lebanon

Experiments were done in triplicates and presented as mean ± standard deviation

Albumin denaturation. Protein denaturation is a process where proteins lose their secondary, tertiary, or quaternary structures under the effect of external stimuli, and is regarded as a marker for inflammatory diseases. In addition to its inhibitory action on cyclo-oxygenase, the ability of NSAIDs to prevent protein denaturation is a well-documented mechanism for its antiinflammatory action [46]. Thus, assessing the tendency of rose geranium extracts to inhibit protein denaturation can be used as an indirect measure for asserting its antiinflammatory potential. All extracts demonstrated a dose dependent inhibition of heat induced albumin denaturation with the stems chloroform extract showing the highest activity (IC_{50} = 0.86 mg/mL) and the water stem extract the weakest (> 20 mg/mL) (Table III, Figure 2). Interestingly, the chloroform extract had an albumin denaturation inhibition activity higher than diclofenac (IC_{50} = 3.77 mg/mL). Several studies have proven that many flavonoids contributed significantly to antiinflammatory activities of many plants [43, 47].

Table III

AChE inhibitory, antiinflammatory (in albumin denaturation and heat induced haemolysis), cytotoxic and haemolytic activity of leaves and stem extracts of *Pelargonium* cv. Rosé grown in Lebanon

Solvent	AChE inhibition (IC_{50} mg/mL)		Albumin denaturation (IC_{50} mg/mL)		Heat induced haemolysis (IC_{50} mg/mL)		Cytotoxicity (IC_{50} mg/mL)		Haemolysis (IC_{50} mg/mL)	
	Leaves	Stem	Leaves	Stem	Leaves	Stem	Leaves	Stem	Leaves	Stem
EtOH	0.63 ± 0.151	0.65 ± 0.05	5.3 ± 1.18	ND*	0.32 ± 0.029	1.53 ± 0.46	1.10 ± 0.05	1.03 ± 0.3	10.5 ± 0.85	21.6 ± 1.1
EtOAc	1.0 ± 0.52	0.84 ± 0.49	2.23 ± 0.25	1.38 ± 0.43	0.63 ± 0.029	0.48 ± 0.14	1.90 ± 0.53	2.16 ± 0.25	9.5 ± 4.0	4.76 ± 0.15
$CHCl_3$	5.0 ± 0.11	3.3 ± 0.5	2.5 ± 0.6	0.86 ± 0.15	0.21 ± 0.01	1.39 ± 0.35	0.40 ± 0.01	1.00 ± 0.26	6.1 ± 1.2	12.36 ± 0.28
Water	4.0 ± 0.8	4.2 ± 2.8	5.63 ± 0.55	ND*	1.0 ± 0.1	5.0 ± 0.1	1.52 ± 0.94	3.46 ± 1.61	ND*	14.56 ± 0.58
Donepezil	0.27		-		-		-		-	
Diclofenac	-		3.76 ± 0.57		1.23 ± 0.25		-		-	

ND* = not determined

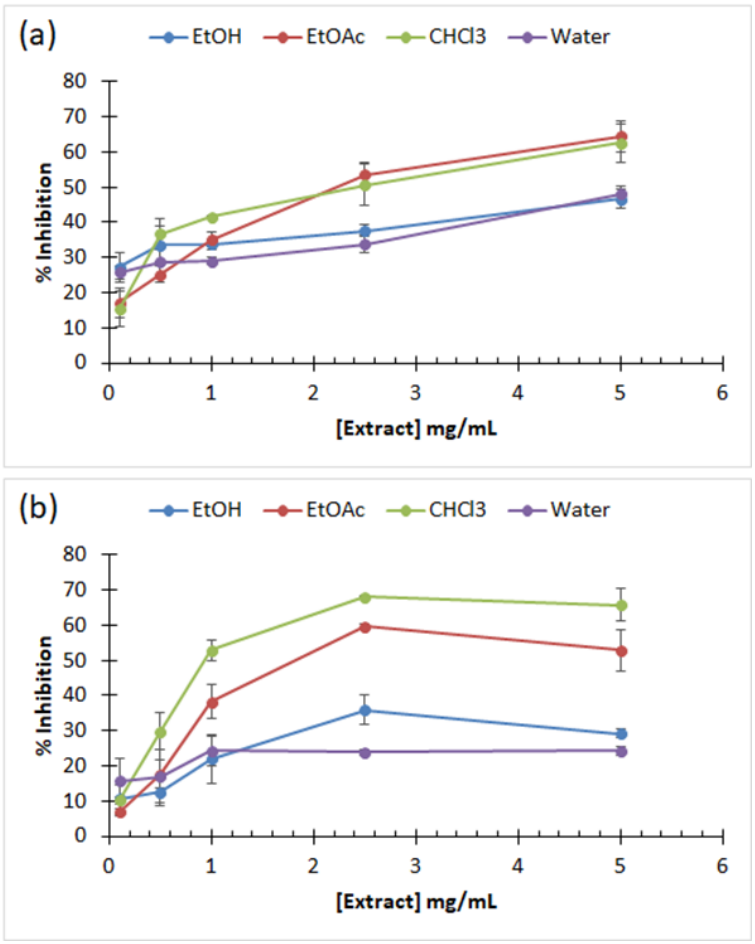

Figure 2.
Albumin denaturation inhibitory activity of (a) leaves and (b) stems extracts of rose geranium grown in Lebanon
Experiments were done in triplicates and presented as mean ± standard deviation

We have demonstrated in this study that the chloroform extracts were rich in flavonoids (292 - 312 µg RE/g) while the water extracts contained the lowest levels among all extracts (73 - 156 µg RE/g). This might explain, at least in part, the poor albumin denaturation inhibition activity of the water leaves and stems extracts.

Heat induced haemolysis. The disruption of lysosome membrane in human cells by any infection (bacteria, injury) leads to the secretion of hydrolytic enzymes that mediate the hydrolysis of phospholipids to produce inflammatory mediators. Therefore, membrane stabilization will stop the release of the lysosomal constituents of neutrophils at the site of inflammation and prevent the progression of inflammation. It has been reported that the human red blood cells (HRBC) membrane are structurally similar to lysosomal membranes. Therefore, we studied the ability of the various rose geranium extracts to inhibit heat induced haemolysis of HRBC membranes as a model for membrane stabilization. Results are displayed in Figure 3, and the derived IC_{50} values, defined as the concentration of the extract to inhibit 50% HRBC membrane lysis, are summarized in Table III. The results of this study reflected the significant membrane stabilizing properties of the extracts. In specific, the leaves chloroform and ethanol extracts demonstrated remarkable stabilization of the HRBC membranes with IC_{50} values of 0.21 and 0.32 mg/mL, respectively. These extracts outperformed the commercial antiinflammatory drug diclofenac that exhibited IC_{50} value of 1.23 ± 0.25 mg/mL in the same assay. While the exact mechanism of membrane stabilization is not fully understood, it is believed that plant extracts with membrane stabilizing properties exert its effect by interacting with membranes to prevent the release of inflammatory mediators such as phospholipases, or by affecting the surface area-to-volume ratio of the cells by expanding or shrinking the membrane *via* interaction with membrane proteins [47-49].

NO scavenging activity. Nitric oxide (NO) is a signalling molecule produced upon the conversion of arginine into citrulline by inducible nitric oxide synthase (iNOS) and is believed to play a role in the pathogenesis of inflammation. NO exhibit anti-inflammatory activity under normal physiological conditions [50]. However, the accumulation of large amounts of NO at the site of inflammation alters the structural and functional

behaviour of many cellular components and promotes inflammation [51]. Therefore, inhibitors of NO production constitute a viable strategy for the management of inflammation. Nitric oxide assay determines the ability of the extract to inhibit nitrite formation by competing with oxygen to react with nitric oxide generated from sodium nitroprusside as an index for NO synthesis [52, 53].

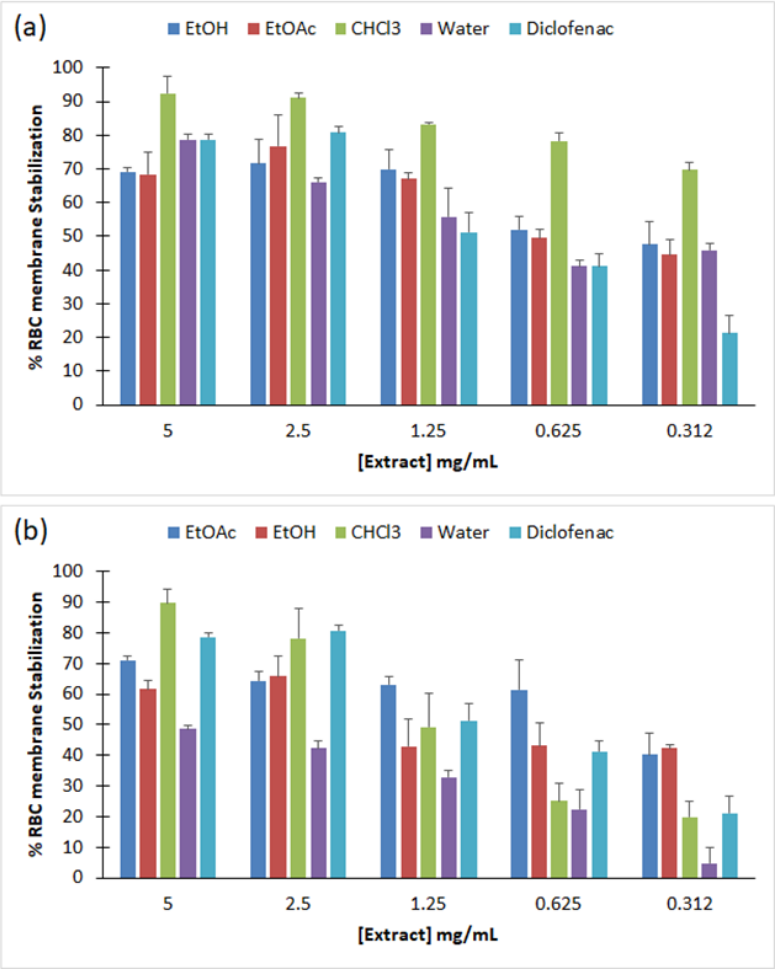

Figure 3.
Heat induced haemolysis inhibition activity of (a) leaves and (b) stems extracts of *Pelargonium* cv. Rosé grown in Lebanon
Experiments were done in triplicates and presented as mean ± standard deviation

The extracts displayed moderate NO scavenging activity with the chloroform extracts showing the highest activity of 23% inhibition achieved at 5 mg/mL. It has been reported that flavonoids are potent NO scavengers [54]. In particular, rutin suppressed the production of inflammatory mediators and pro-inflammatory cytokines *in vitro*, including NO [55]. To the best of our knowledge, no studies evaluated the antiinflammatory activity of rose geranium extracts in terms of albumin denaturation, heat induced haemolysis and NO scavenging, and therefore, direct comparison was not possible. However, Martins *et al*. tested the antiinflammatory potential of different extracts of rose geranium as a function of prostanoid inhibition in LPS-induced RAW 264.7 cells, and reported moderate activity for extracts with the highest inhibition observed with the ethyl acetate extract [56].

Cytotoxic activity

The cytotoxic activity of rose geranium extracts was assessed by WST-1 cell proliferation assay where the cell viability of HCT 116 colon cancer cells was measured in response to different concentrations of the extracts, and results are depicted in Figure 4. All extracts demonstrated good dose dependent anti-proliferative activity with IC_{50} values ranging between 0.4 mg/mL, for the chloroform leaves extract, and 3.46 mg/mL, for the water stem extract (Table III). The highest cytotoxic activity was attained at higher concentrations of the extracts. The chloroform leaves extract exhibited the most cytotoxic potential with an IC_{50} value of 0.4 mg/mL, indicating that the nonpolar active phytochemicals are responsible for most of the anti-proliferative activity [57]. The observed cytotoxic activity can be attributed to the presence of bioactive

constituents with demonstrated anti-proliferative activities such as gallic acid [58], rutin [59], quercetin [60], phenolic compounds [61] and flavonoids [62]. To the best of our knowledge, no study has reported the cytotoxic activity of rose geranium extracts against HCT 116 colon cancer cells, and therefore, direct comparison with literature reports was not possible. However, Al-Saffar *et al*. tested the cytotoxic activity of rose geranium methanolic extract against MCF-7 breast cancer cell line and demonstrated that the extract was highly cytotoxic with IC_{50} of 0.288 mg/mL [11]. Another study by Halees *et al*. evaluated the *in vitro* anti-proliferative activity of rose geranium methanol, water and dichloromethane extracts against MCF-7, T47D and EMT6/P breast cancer cell lines. The authors found that the water extract was the most potent against all tested cell lines, followed by the dichloromethane and methanol extracts.

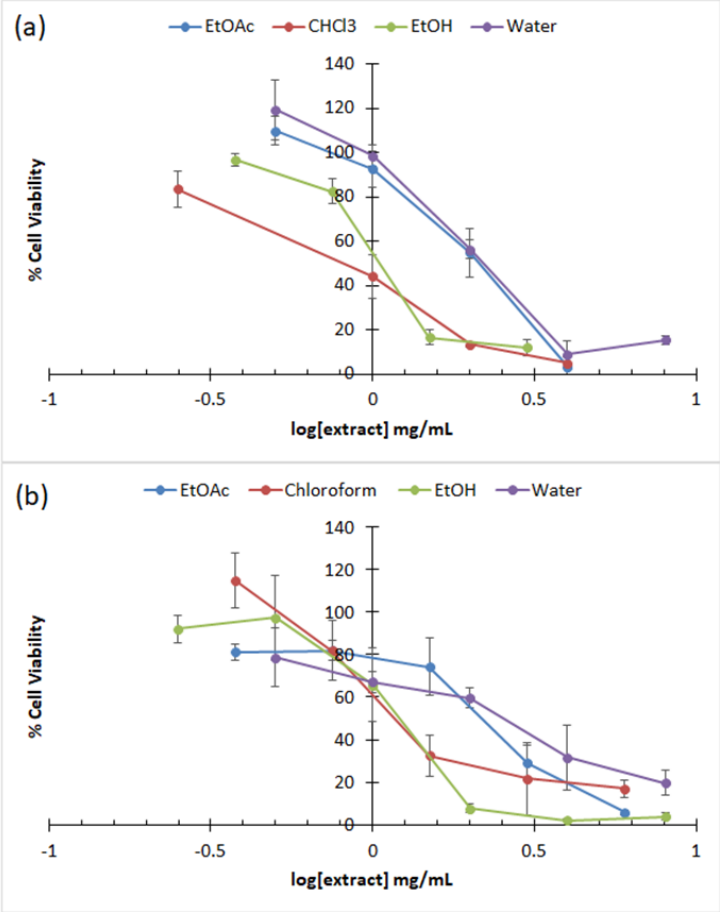

Figure 4.
Cytotoxic activity of (a) leaves and (b) stems extracts of rose geranium grown in Lebanon against HCT 116 colon cancer cells
Experiments were done in triplicates and presented as mean ± standard deviation

Haemolysis

An equally important aspect in evaluating the pharmacological profile of natural products is to assess its toxicity to human body. In this regard, the *in vitro* RBC haemolysis assay presents a simple, low cost and effective method for testing the cytotoxicity of plant formulations against human erythrocytes. Chemical constituents of plants, especially lipophilic substances, have the tendency to interact with membrane phospholipids leading to the lysis of erythrocytes and release of haemoglobin causing several complications such as haemolytic anaemia, multiple organ failure and even death [63]. Table III summarizes IC_{50} values defined as the extract concentration to prevent haemolysis of 50% of HRBC. A dose dependent increase in haemolysis of RBC was observed for all extracts. The chloroform leaves extract displayed the highest haemolytic activity with significant haemolysis (~ 17.7%) occurring at 2.5 mg/mL and increased to 70% at 10 mg/mL of the extract. On the other hand, the water leaves extract did not have major impact on the erythrocytes membrane with 19% haemolytic activity occurring at 20 mg/mL of extract.

Conclusions

The current study describes the phytochemical constituents and pharmacological profile of the crude extracts of Lebanese *Pelargonium* cv. Rosé leaves and

stems. Wide variations were found in the chemical composition of the extracts as a function of the extraction solvent polarity where the ethanolic extracts contained the highest levels of phenolic compounds. The ethanolic leaves and stem extracts demonstrated the highest antioxidant and AChE inhibition activities. On the other hand, the chloroform extracts exhibited the best antiinflammatory and cytotoxic capacities, thus highlighting the significance of assessing various extraction solvents for different applications. Our findings highlight the importance of the Lebanese rose geranium extracts as a nutritional source rich in phenolic compounds, which may alleviate various oxidative stress-related diseases such as Alzheimer's and cancer, and support the large-scale cultivation of the plant for potential applications in the pharmaceutical and food industries.

Acknowledgement

We are grateful to the Doctoral School of Science and Technology at the Lebanese University for the support in conducting some assays, and to Prof. Nelly Arnold-Apostolides from the Holy Spirit University of Kaslik, Lebanon, for identifying the plant.

Conflict of interest

The authors declare no conflict of interest.

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