Evaluation of the phenolic content and antioxidant potential of *Althaea rosea* cultivated in Egypt

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Received 1 August 2013
Accepted 9 September 2013

Journal of the Arab Society for Medical Research 2013, 8:48–52

Objective

The aim of this study was to evaluate the total phenolic content (TPC) and antioxidant potential of *Althaea rosea* Cav., family Malvaceae, as well as to isolate and identify the flavonoid content of the methanolic extract of the aerial parts. In addition, a comparison between the TPC and antioxidant capacity of the methanolic extract of both aerial parts and flowers was carried out to discover new active constituents that can be utilized in drug industry.

Materials and methods

The extraction of the flavonoid compounds was carried out by percolation of the dried aerial parts of the plant under investigation with 70% methanol until exhaustion. The combined extract was then concentrated and defatted with petroleum ether (60–80°C). After separation of the lipoidal matter, the remaining extract was purified from mucilage and subjected to several column chromatographic techniques for isolation of the flavonoids. The identification of flavonoid compounds was carried out using physical, chemical, and spectral methods such as ultraviolet, ^{1}H NMR, and ^{13}C NMR. The antioxidant potential of the methanolic extracts of both aerial parts and flowers was determined using the stable 2, 2-diphenyl-1-picrylhydrazyl free radical scavenging activity method. Furthermore, their TPC was also determined using the Folin–Ciocalteu method.

Results

Five flavonoid compounds were isolated from the aerial parts of *A. rosea* Cav., which were identified as quercetin 3-*O*-β-D-glucuronopyranoside-8-C-β-D-glucopyranoside, kaempherol-3-*O*-β-D-rutinoside, kaempherol-4´-*O*-β-D-glucoside, kaempherol-3-*O*-β-D-glucoside, and kaempherol. The antioxidant activity was measured in terms of their IC_{50}. The IC_{50} values of the methanolic extracts of the aerial parts and flowers were 11 and 1 mg/ml, respectively, whereas the TPCs were 48 and 73 μg/ml, respectively.

Conclusion

The methanolic extracts of both aerial parts and flowers of *A. rosea* Cav. are rich in phenolic compounds and have a prominent antioxidant activity. The antioxidant activity of both extracts may be attributed to their phenolic content.

Keywords:
Althaea rosea, antioxidant activity, flavonoids, phenolic content

J Arab Soc Med Res 8:48–52
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1687-4293

Introduction

Malvaceae or the mallow family is a family of flowering plants containing over 200 genera with close to 2300 species. The largest genera in terms of species include *Hibiscus* (300 species), *Dombeya* (225 species), *Pavonia* (200 species), and *Sida* (200 species) [1,2], whereas the genus *Althaea* contains 50 species [3].

Althaea rosea (common name: Hollyhock; synonyms: *Althaea chinensis* Wall. and *Althaea ficifolia* Cav.) is an ornamental plant abundantly cultivated in gardens of Egypt [4]. Genus *Althaea* has been growing from the Mediterranean region to Central Asia and is widely distributed in the temperate regions of the world [3]. There are six species of *Althaea* growing in Egypt: *Althaea rosea* Cav., *Althaea ludwigii* L., *Althaea rufescens* Boiss., *Althaea acaulis* Cav., *Althaea striata* DC., and *Althaea apterocarpa* Del. [5]. *A. rosea* was imported in Europe from Southwestern China during the 15th century [6,7]. William Turner, a herbalist of that time, gave it the name Holyoke from which the English name derived [4]. The flowers have a range of colors from white to dark red including pink, yellow, and orange. The plant is easily grown from the seeds [8]. The Hollyhock flower is used in folk medicine for prophylaxis and therapy of diseases of respiratory, gastrointestinal, and urinary systems and also for relieving fever and thirst. The herb, roots, and seeds are used to treat cough and lung diseases. The root of the plant is described as a substitute for *Althaea officinalis* L. in cough and respiratory problems. Other uses include external application in skin inflammations and ulcers [9]. The current literature revealed that *A. rosea* Cav. is rich in its phenolic compounds and polysaccharides

(*Althaea* mucilage). High-molecular-weight acidic polysaccharides were isolated from the flowers, which consisted mainly of glucuronic acid, galacturonic acid, rhamnose, and galactose [10,11]. The phenolic compounds isolated from *A. rosea* were mainly phenolic acids and flavonoids. The phenolic acids include salicylic, vanillic, ferulic, syringic, caffeic, *p*-hydroxybenzoic, *p*-coumaric, and *p*-hydroxyphenylacetic acids [12]. The chemical composition of the flavonoid compounds of the flowers of *A. rosea* Cav. was investigated using various column chromatographic methods, and this led to the isolation of aglycones, namely kaempherol, apigenin, aromadendrin, and 4¢,5,7,8-tetrahydroxy-3-methoxyflavone. Flavonoid glycosides such as astragalin, saxifragin, choerospondin, and tiliroside were also isolated [13]. Anthocyanosides, isolated from the flowers, were mainly derivatives of malvidin, delphinidine, and petunidin [14].

In this study, the total phenolic content (TPC) and antioxidant activity, not previously studied, of the methanolic extract of both aerial parts and flowers of *A. rosea* Cav. cultivated in Egypt were evaluated. In addition, the flavonoid constituents of the aerial parts were isolated and identified using physical, chemical, and spectral techniques.

Materials and methods

Plant materials

The aerial parts and flowers of *A. rosea* Cav. were collected separately in June 2008 from a private garden in Cairo-Alexandria Desert Road and were authenticated by Mrs Terez Labeeb Youssef, Consultant of Plant Taxonomy, Orman Garden, Giza, Egypt. The plant was then cultivated in the Agricultural Research Station of the National Research Center, Giza, Egypt.

A voucher specimen of the plant was deposited in the Herbarium of the National Research Center. The aerial parts and flowers were separately air-dried and powdered. The powdered material was maintained in air-tight dark containers at room temperature until use.

Chemicals

All chemicals, shift reagents such as MeOH, $AlCl_3$, $AlCl_3$/HCl, AcONa, AcONa/H_3BO_4, solvents such as petroleum ether (60–80°C), methanol, *n*-butanol, and reagents for the assay of antioxidant activity [2, 2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid] and TPC (folin, gallic acid) were of analytical grade and purchased from E-Merck (Darmstadt, Germany).

Solvent systems

S_1 consisted of *n*-butanol, acetic acid, and water in the ratio of 4:1:5 (v/v, upper layer) and S_2 consisted of acetic acid and water in the ratio of 15:85 (v/v).

Apparatus

Portable ultraviolet (UV) lamp (254–365 nm) was used for localization of spots on paper and thin layer chromatograms. UV-visible spectrophotometer (Beckman DU7) and Shimadzu UV 240 (PIN-204-5800), and NMR (JEOL ECA 500 MHz Tokyo, Japan), were also used with dimethyl sulfoxide-d6 as solvent and trimethylsulfoxide (TMS) as internal standard. The data are expressed as δ-values in parts per million and *J*-values in Hertz.

Extraction and isolation of flavonoids

The air-dried aerial parts of *A. rosea* Cav. (750 g) were percolated with 70% methanol several times until exhaustion. The extract was concentrated and defatted with petroleum ether at 60–80°C (5 × 200 ml). The petroleum ether extracts were separated and the remaining mother liquor was purified from the mucilage content by adding slowly while stirring four times its volume of ethanol (95%). The precipitated mucilage was filtered and the remaining extract was evaporated at 40°C under reduced pressure to give a dark brown residue (130 g). An amount of 20 g of obtained residue was chromatographed on a polyamide column. Elution was started with water followed by decreasing polarity of the eluent through 10% increments in methanol until 100% methanol was reached. Fractions (150 ml each) were collected and evaporated under reduced pressure. All fractions were monitored using paper chromatography (Whatman no. 1) with S_1 and S_2 as developing systems, examined under UV lamp, and sprayed with $AlCl_3$ [15] before and after exposure to NH_3 vapors [16]. Similar fractions were combined to give four collective fractions (1, 2, 3, and 4).

Fraction 1, eluted with MeOH/H_2O (20–30%), was then rechromatographed on a subcolumn of Sephadex (LH-20) (ROTH. Bestellen sie zum Germany). Elution was performed using saturated butanol to yield compound 1.

Fraction 2, eluted with MeOH/H_2O (30–40%), was further purified on a column of Sephadex (LH-20) and eluted with MeOH to yield compound 2.

Fraction 3, eluted with MeOH/H_2O (40–50%), was then subjected to purification on a Sephadex (LH-20) column using MeOH as an eluent to yield two compounds – 3 and 4.

Fraction 4, eluted with 90–100% MeOH in water, was then rechromatographed on a Sephadex subcolumn (LH-20) and eluted with MeOH to yield compound 5.

All isolated compounds were structurally elucidated and confirmed through analysis of their UV data with methanol and complex shift reagents and using ^{1}H NMR and ^{13}C NMR. Their identity was further confirmed by comparing their data with those of authentic samples as well as reported literature [17–19].

Acid hydrolysis of glycosides [20]

An amount of 3 mg of each isolated glycosides was treated with 1.5 N HCl in aqueous 50% methanol for 2 h at 100°C. The samples were then withdrawn and tested chromatographically to ensure complete hydrolysis. The hydrolysate, in each case, was shaken with ether. The ether extracts were dried over anhydrous sodium sulfate, the solvent was evaporated, and the residues were crystallized from MeOH. The acidic mother liquor was used for the detection of the liberated sugars.

Identification of the sugars

The acidic mother liquor containing the sugar moieties was neutralized with barium carbonate and filtered. The filtrate was concentrated under reduced pressure, spotted separately alongside with authentic sugars on Whatman no. 1 sheets, developed in system S_1, dried, and sprayed with aniline hydrogen phthalate reagent [21], and then heated at 110°C until the color of the spots appeared.

Assay of Total Phenolic Content (TPC) [22,23]

The TPC was determined using the Folin–Ciocalteu reagent as described by Zheng and Wang [22]. The powdered aerial parts and flowers (0.5 g) were homogenized separately in 70% methanol (5 ml) and centrifuged for 20 min. The supernatant was used for the estimation of total polyphenols. A volume of 0.5 ml of both extracts, at a concentration of 1 mg/ml, was mixed with 2.5 ml of Folin–Ciocalteu reagent (diluted 10 times with water) and 2 ml of sodium carbonate (7.5% w/v). The tubes were vortexed, covered with parafilm, and incubated at 50°C for 5 min. Absorbance of the resultant blue colored solution was measured at 760 nm and compared with a gallic acid calibration curve prepared using serial dilutions of standard gallic acid. The results were expressed as milligram gallic acid equivalents (GAE)/g plant extract. Each assay was carried out in triplicate.

DPPH free radical scavenging activity assay [24]

Free radical scavenging activity was measured using the stable DPPH free radical, according to the method described by Brand-Williams and colleagues. Different dilutions of 70% methanolic extract of both aerial parts and flowers of *A. rosea* Cav. (5, 10, 20, and 50 mg/ml) were added to 2 ml solution of 200 mol/l DPPH in methanol, and the reaction mixture was shaken vigorously. After incubation at room temperature (28 ± 2°C) for 30 min, the absorbance (A) of DPPH for the positive control (ascorbic acid) and the samples was measured at 517 nm, and the radical scavenging activity of each extract was expressed as percentage inhibition of DPPH free radical.

%Inhibition=%Anti-radicalactivity=(Absorbance of control − Absorbance of sample/Absorbance of control) × 100

A standard curve was plotted between different concentrations of the 70% methanolic extract of the aerial parts and flowers and the corresponding percentages of inhibition. From this curve, the IC_{50} (the concentration that makes 50% inhibition) of both extracts and l-ascorbic acid (positive control) was calculated.

Results

Five flavonoid compounds were isolated from the defatted 70% methanolic extract of the aerial parts of *A. rosea* Cav. using different chromatographic techniques. Identification of the isolated compounds was achieved using physical, chemical, and spectral methods of analysis, including acid hydrolysis, chromatographic analysis, UV, ^{1}H NMR, and ^{13}C NMR and by comparison with published data [17–19].

Compound 1 was isolated as pale yellow crystals [15 mg, melting point (MP) 300°C]. Its R_f value was 46 and 78 using solvent systems S_1 and S_2, respectively. Hydrolysis of this compound liberated glucuronic acid and quercetin-8-C-β-D-glucopyranoside, which were identified through co-chromatography with authentic samples.

Compound 2 was isolated as yellow crystals (20mg, MP 200°C). Its R_f value was 50 and 69 on paper chromatography using S_1 and S_2, respectively. On hydrolysis, the compound liberated kaempherol aglycone and glucose and rhamnose sugars, which were identified through co-chromatography with authentic samples.

Compound 3 was isolated as yellow crystals (14 mg, MP 188°C). Its R_f value was 64 and 52 on paper chromatography using S_1 and S_2, respectively. Hydrolysis of this compound liberated the known kaempherol aglycone and glucose, which were identified through co-chromatography with authentic samples.

Compound 4 was isolated as yellow crystals (28 mg, MP 175°C). Its R_f value was 69 and 47 on paper chromatography using S_1 and S_2, respectively. Hydrolysis of this compound liberated kaempherol aglycone and glucose, which were identified through co-chromatography with authentic samples.

Compound 5 was isolated as yellow crystals (10 mg, MP 275°C). Its R_f value was 84 and 20 on paper chromatography using S_1 and S_2, respectively, which was identified as kaempherol through co-chromatography with authentic samples.

TPC

The TPC was expressed as GAE in μg/ml extract with reference to a pre-established standard calibration curve. The TPCs of the 70% methanolic extract of both flowers and aerial parts were 73 and 48 μg/ml, respectively.

DPPH radical scavenging activity

The results revealed that the 70% methanolic extract of both aerial parts and flowers of *A. rosea* Cav. exhibited a prominent antioxidant activity. The methanolic extract of the flowers had more potent activity than that of the aerial parts, and this was deduced from their percentages of inhibition (Table 1) as well as their IC_{50} values (1 and 10 mg/ml, respectively) compared with those of the positive control ascorbic acid (0.42 mg/ml).

Discussion

A. rosea Cav. is a hardy biennial or short-lived perennial popular garden ornamental plant. It can easily reach a height of 1–3 m. In folk medicine, the total plant is used for the treatment of gastritis, peptic ulcer, and constipation. It is also used topically for the treatment of dermatitis, skin lesions, and insect bites. Flower decoction is used to improve blood circulation and to treat constipation, dysmenorrhea, and hemorrhage. The root is astringent and demulcent, and it is crushed and applied as a poultice to ulcer [9].

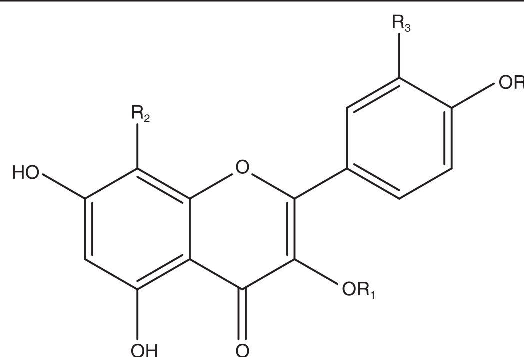
In this study, various column chromatographic methods using polyamide and Sephadex led to the isolation of five flavonoid compounds present in the 70% methanolic extract of the aerial parts of the plant. Identification of their chemical structure was carried out on the basis of their physical and chemical properties as well as different spectroscopic methods such as UV, ^{1}H NMR, and ^{13}C NMR. These compounds were identified as quercetin-3-*O*-β-D-glucuronopyranoside-8-C-β-glucopyranoside (compound 1), kaempherol-3-*O*-rutinoside [kaempherol-3-*O*-α-rhamnopyranosyl (1‴→6″)-β-glucopyranoside] (compound 2), kaempherol-4′-*O*-β-glucopyranoside (compound 3), kaempherol-3-*O*-β-D-glucoside (astragalin) (compound 4), and kaempherol (compound 5) (Fig. 1).

It is important to note that compound 4 (kaempherol-3-*O*-β-D-glucopyranoside) and compound 5 (kaempherol) were previously isolated from *A. rosea* Cav. [25], whereas compound 1 (quercetin-3-*O*-β-glucuronopyranoside-8-C-β-glucopyranoside), compound 2 (kaempherol-3-*O*-α-rhamnopyranosyl (1‴→6″)-β-glucopyranoside, and compound 3 (kaempherol-4′-*O*-β-D-glucoside) were isolated from this plant for the first time.

Some biological studies have found a positive association between the consumption of food containing kaempherol and a reduced risk of developing several disorders such as cancer and cardiovascular diseases. Numerous preclinical studies have shown that these compounds have a wide range of pharmacological activities such as antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, antiosteoporotic, anxiolytic, and antiallergic [26].

In this study, a comparison between the antioxidant capacity and the TPC of the 70% methanolic extract of both aerial parts and flowers of *A. rosea* Cav. was performed. Plant phenolics constitute one of the major groups of compounds acting as primary antioxidant or free radical terminators. Therefore, one of the aims of this study was to determine the TPC of both extracts. Flavonoids [13] are likely to be the most important natural phenolics because of their broad spectrum of chemical and biological activities, including antioxidant and free radical scavenging properties. The TPC was expressed as GAE in μg/g extract. The TPC of the 70% methanolic extract of both aerial parts and flowers was 48 and 73 μg GAE per gram extract, respectively.

Figure 1



Chemical structures of compounds 1–5. Compound 1: R_1=β-D-glucuronic acid, R_2=C-β-D-glucose, R_3=OH, R_4=H; compound 2: R_1=β-D-rutinoside, R_2=H, R_3=H, R_4=H; compound 3: R_1=H, R_2=H, R_3=H, R_4=β-D-glucose; compound 4: R_1=β-D-glucose, R_2=H, R_3=H, R_4=H; compound 5: R_1, R_2, R_3, R_4=H.

Table 1 Percentage of antioxidant activity of the methanolic extracts of the aerial parts and flowers of *Althaea rosea* Cav. (free radical scavenging activity)

Concentrations (mg/ml)	Percentage of inhibition	
	Aerial parts extract	Flower extract
5	36.99	45
10	44.07	60
20	75.23	75
50	82.67	88

The results displayed in Table 1 revealed that the 70% methanolic extract of both aerial parts and flowers has a wide range of free radical scavenging activity. Four different concentrations of the 70% methanolic extracts of the aerial parts and flowers were tested, and the values of inhibition percentages for the 70% extracts ranged from 36.99 to 82.67% and from 45 to 88%, respectively. The tested concentrations of both extracts were further used to determine their IC_{50} values. The extract of the flowers showed a higher DPPH radical scavenging activity (IC_{50} 1 mg/ml) than that of the aerial parts (IC_{50} 10 mg/ml) as compared with the positive control (ascorbic acid, IC_{50} 0.42 mg/ml). The variation of the free radical scavenging activity between the tested extracts may be due to the differences in their secondary metabolites [22]. This study showed that there is a correlation between the free radical scavenging activity and the TPC of both extracts, which may be attributed to their flavonoid compounds or to the presence of other antioxidant secondary metabolites in the extract that directly or indirectly contribute to this activity. Flavonoids are described as free radical scavengers and antioxidants [22]. This activity is attributed to their hydrogen donating ability. They also inhibit lipid peroxidation *in vitro* at an early stage by acting as scavengers of superoxide anion and hydroxyl radicals. They terminate chain radical reaction by donating hydrogen atom to peroxy radicals thus forming flavonoid radicals, which further react with the radicals and terminate the propagation reaction [22–24].

Conclusion

The methanolic extracts of both aerial parts and flowers of *A. rosea* Cav. are rich in phenolic compounds and have a prominent antioxidant activity. The antioxidant activity of both extracts may be attributed to their phenolic content. *A. rosea* Cav. can be considered as a good source of natural antioxidants.

Acknowledgements

The authors are thankful to the National Research Center, Cairo, Egypt, for supporting this work.

Conflicts of interest

None

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