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Research Article

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Quantification of phytochemical constituents and in-vitro antioxidant activity of *Althaea rosea* seeds

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ABSTRACT

To investigate the quantification of total phenolic and in-vitro antioxidant activity of distilled water, ethanol, butanol and chloroform extracts of A. rosea seeds. The quantification of the total phenolic content was estimated by taking gallic acid as a standard. Antioxidant activities were studied via different methods that including DPPH assay, superoxide anion scavenging assay, hydroxyl radical scavenging assay and superoxide dismutase activity assay. The results showed that the distilled water extracts of A. rosea seeds have more phenolic content than other extracts. The four solvent extracts were found to possess concentration dependent scavenging radical's activity. With current findings, A. rosea seeds might be used as potential natural antioxidant.

Keywords: Total phenolic content, In-vitro, Antioxidant activity, DPPH assay, Superoxide dismutase.

INTRODUCTION

Althaea rosea Linn. (Family Malvaceae), commonly known as hollyhock, is an ornament herb and distributed in Sichuan, Xinjiang, Shanghai, Jiangsu, and Fujian region of China [1]. In the Uyghur medicinal system, *A. rosea* flowers and roots are prescribed as an expectorant and are known to relieve demulcent and febrifuge. The seeds of this plant are regularly used by folk physicians for the treatment of kidney and uterus inflammation. *A. rosea* contained high-molecular-weight acidic polysaccharides known as mucilages were isolated from the flowers and leaves, which composed mainly of glucuronic acid, galacturonic acid, rhamnose, and galactose [2]. The phenolic compounds isolated from A. rosea flower were mainly including salicylic, vanillic, ferulic, syringic, caffeic, p-coumaric, p-hydroxybenzoic and p-hydroxyphenylacetic acids [3]. The current literature revealed that aqueous extract of the seeds of *A. rosea* seeds is rich in alkaloids, carbohydrates, fatty acids, phenolic compounds, glycosides and flavonoids [4]. The fatty acids compounds include linoleic acid, oleic acid, palmitic acid, stearic acid, and linolenic acid [5]. However, to our knowledge, few studies were devoted to the extraction of *A. rosea* seeds the total phenolic content (TPC) and antioxidant activity.

In the body, excess production of free radicals affects lipid cell membranes to produce lipid peroxides and reactive oxygen species (ROS) which leads to decline in membrane fluidity and many biological changes [6,7]. Reactive oxygen species (ROS) play an important role in living organisms and in the forms of superoxide anion radical ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), and hydrogen peroxide (H_2O_2) [8-10]. Excessive production is implicated in aging and in many diseases, including inflammation, cancer, cardiovascular disease, osteoporosis, and degenerative diseases. Antioxidants are very important for human health; antioxidant supplements and foods that contain antioxidants are useful in helping the human body reduce oxidative damage [11]. The polyphenols of plants have received much attention as sources of biological active substances including antioxidants, due to their potent against several disease, no side effects and economic viability. A polyphenol antioxidant is a type of antioxidant containing a polyphenolic or natural phenol substructure [12]. Numbering over 4,000 distinct species, many of these compounds have antioxidant activity in vitro. The regulation theory considers a polyphenol antioxidant's ability to scavenge free

radicals and up regulate certain metal chelation reactions [13]. Various reactive oxygen species, such as singlet oxygen, peroxynitrite and hydrogen peroxide, must be continually removed from cells to maintain healthy metabolic function.

Thus, the aim of the present study was to evaluate a potential new source of natural antioxidants from different fraction of *A. rosea* seeds which will prove beneficial for maintenance of optimal health and may increase the demand of these bioactive substances by food, cosmetic and pharmaceutical industries. The four methods commonly used in antioxidant activity assays are 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, superoxide anion scavenging activity, hydroxyl radical scavenging activity, and superoxide dismutase (SOD)-like activity has been used by investigators to assay the antioxidant activity of *A. rosea* seeds.

EXPERIMENTAL SECTION

Chemicals

2, 2-diphenyl-1-picrylhydrazyl (DPPH), Ortho-oxybenzoic acid was purchased from Sigma Co. (USA) , Superoxide dismutase (SOD) kit was purchased from Nanjing Jiancheng Bioengineering Institute（China, 20131201). Galic acid were obtained from the Tianjin Reagent Co. (Tianjin, China). All other solvents and chemicals were analytically graded and purchased from Tianjin Fu-Yu Chemical Ltd., Co. (Tianjin, China).

Plant material

The samples of *A. rosea* seeds were purchased from Xinjiang QiKang Habo Uygur medicine Co. Ltd., and were authenticated by Yonghe Li, a chief apothecary of Chinese medicine at the Hospital of Xinjiang. Voucher specimens were deposited in the Department of Traditional Chinese Medicine Ethnical Herbs Specimen Museum Of Xinjiang Medical University. The seeds were dried at room temperature, washed thoroughly with tap water to remove all sand particles and epiphytes, smashed into powder with a pulverizer, passed through a 60-mesh sieve and stored in a dry place at room temperature until used.

Sample extraction

The powdered material of *A. rosea* seeds (10 g) was separately extracted three times in a soxhlet apparatus for 6 h successively with distilled water, ethanol, butanol and chloroform. Each extract was left to cool at room temperature, filtered; the supernatant solutions were condensed to about 200 mL by using a rotating evaporator under vacuum at 45□. The solutions were kept in the dark at 4℃ until tested.

Quantification of total phenolic content

Estimation of the total polyphenol content of *A. rosea* seeds was performed according to the Folin-Ciocalteu method. Briefly, 0.5 mL of each extracts was added with 1 mL of Folin Ciocalteu's reagent and 2 mL of 20% (w/v) sodium carbonate and 60% methanol were added successively. The mixture solutions were shaken and allowed to incubate at room temperature for 30 min. After incubation the mixture was centrifuged at 4000r for 3 min and the absorbance of the supernatant was measured with a spectrophotometer at 765 nm. The calibration curve was plotted using gallic acid (0-100 mg/mL) as standard and the result of polyphenol content was expressed in milligrams of gallic acid equivalent per gram of sample (GAE/g of sample). All assays were conducted in triplicate.

In-vitro antioxidant activity

DPPH radical scavenging activity assay

The free radical scavenging activity of plant extracts were measured using the stable radical DPPH according to a literature procedure with a few modifications [9]. DPPH radical in ethanol (5mM) was prepared and shaken vigorously and then immediately incubated in darkness 70 min. Briefly, 2 mL of DPPH solution was allowed to react with 4 mL sample solution at different concentration ranging from 40 to 400 μg/mL. The mixture was left in the dark for 30 min and the absorbance was then measured at 517 nm. Ascorbic acid was used as a positive reference.

The DPPH radical scavenging rate (%) was calculated as follows:

$$\text{Scavenging activity}\,(\%) = \frac{A_{\text{blank517}} - A_{\text{sample517}}}{A_{\text{blank517}}} \times 100$$

where $A_{blank517}$ and$_{sample517}$ the absorbances of sample solution/ascorbic acid and DPPH solution, respectively.

Superoxide anion radical scavenging activity assay

The superoxide radical scavenging abilities of all the sample solutions were assessed based on the pyrogallol

autoxidation method. Briefly, 3 mL of 0.2 mol/L Tris–HCl buffer (pH 8.2) was allowed to react with 0.5 mL sample solution at different concentration ranging from 40 to 400 μg/mL and 3 mL of pyrogallic acid. The mixture was shaken quickly and incubated at room temperature for 5 min and the reaction was terminated by adding 1 mL of concentrated HCl. The absorbance was determined at 320 nm by a spectrophotometer against blank samples and ascorbic acid was used as a positive control. The antioxidant activity of extracts on superoxide anion radical was calculated as follows:

$$\text{Scavenging activity}(\%) = \frac{A_{blank320} - A_{sample320}}{A_{blank320}} \times 100$$

where $A_{blank320}$ and $A_{sample320}$ the absorbances of extracts/ascorbic acid and Tris-HCl buffer solution, respectively.

Hydroxyl radical scavenging activity assay

The hydroxyl radical-scavenging activity of polysaccharides was determined according to Fenton method. Briefly, 1 mL of $FeSO_4$ (9 mmol/L) was allowed to react with 1 mL of salicylic acid-ethanol solution (9 mol/L), 1 mL sample solution at different concentration ranging from 40 to 400 μg/mL and 1 mL of H_2O_2. The mixture was shaken quickly and incubated at 37 ◦C for 30 min. The absorbance of the mixture was determined absorbance at 510 nm against a blank by spectrophotometer. The antioxidant activity of extracts on hydroxyl radical was calculated as follows:

$$\text{Scavenging activity}(\%) = \frac{A_{blank510} - A_{sample510}}{A_{blank510}} \times 100$$

where $A_{blank510}$ and $A_{sample510}$ the absorbances of extracts/ascorbic acid and hydroxyl radical solution, respectively.

Superoxide dismutase activity assay

SOD on the organism oxidation and antioxidation balance plays a vital role, this enzyme is capable of scavenging superoxide anion free radical, protect cells from damage. SOD enzyme in the *Althaea rosea* seeds extracts has a specific inhibitory effect on superoxide anion free radical, the formation of nitrite reduction, resulting in the absorbance value lower than the absorbance of the care value, calculated by the formula which can calculate the SOD activity in samples. All analysis was performed according to the operating instructions.

$$\text{Activity rate}(\%) = \frac{A_{blank550} - A_{sample550}}{A_{blank550}} \times 100$$

where $A_{blank550}$ and $A_{sample550}$ the absorbances of extracts/ascorbic acid and SOD solution, respectively.

RESULTS AND DISCUSSION

Quantification of total phenolic content

Plant polyphenols are considered as a major source of biological active substances that contributed to the antioxidant activity. The TPC from different fraction of *A. rosea* seeds were evaluated according to the Folin-Ciocalteu method, which proved to be a convenient, simple, and rapid method for the estimation of total phenolic content of plant polyphenols. Table 1 showed a significant difference in total phenolics was noticed between different solvents used for extraction. The standard curve prepared using gallic acid was *A = 6.375C + 0.0129*, *r = 0.9993* (A: absorbance, C: concentration), which shows good linearity at concentrations ranging from 0.002 mg/mL to 0.1 mg/mL. The phenolic contents of *A. rosea* seeds extracts were ranging from 3.11 ± 0.17 to 9.53 ± 0.13 (mg/g). The TPC of *A. rosea* seeds extracts in distilled water were 9.53 ± 0.13 mg/g, which were much higher than others. The lowest TPC was in butanol which was only 3.11 ± 0.17 mg/g. The TPC is arranged as following sequence: distilled water > ethanol > chloroform > butanol.

Table 1 Contents of total phenol in diferent solvent extracts from *A. rosea* seeds. (x±s，n=3)

The extract	Total phenolic content（mg/g）
Distilled water	9.53 ± 0.13
Ethanol	6.37 ± 0.25
Butanol	3.11 ± 0.17
Chloroform	4.57 ± 0.34

In-vitro antioxidant activity

In the present study, the distilled water, ethanol, butanol and chloroform extracts of *A. rosea* seeds were found to possess concentration dependent scavenging radical's activity and the results were given in Figure 1. The effects

were evaluated for its antioxidant activity on different in vitro models like DPPH radical scavenging, superoxide anion radical scavenging activity, hydroxyl radical scavenging activity and superoxide dismutase activity.

DPPH radical scavenging activity assay

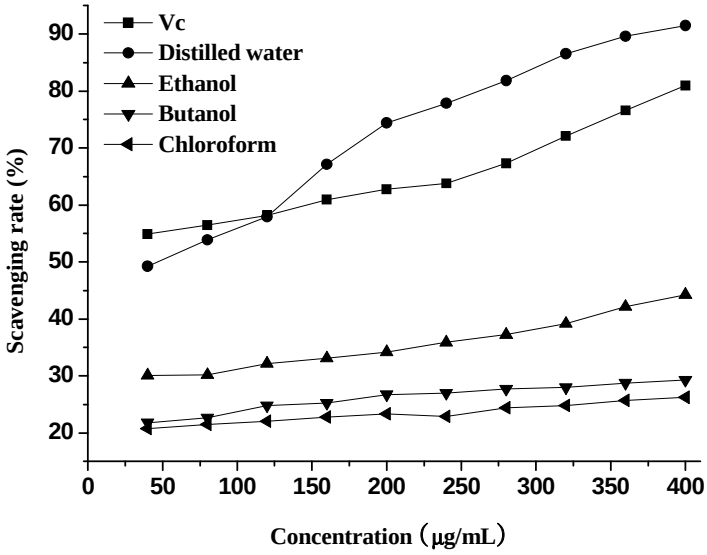

Fig. 1 The activity of scavenging DPPH· free radical

Fig. 1 showed that distilled water, ethanol, butanol and chloroform extracts possessed DPPH radical scavenging activities which in a concentration dependent manner, and the abilities were followed by Vc, distilled water, ethanol, butanol, and chloroform. The scavenging effects of the four extracts were remarkable at all tested concentrations, and well correlated with increased concentration up to 400 μg/mL. Chloroform extracts had the weakest activity. At 400 μg/mL, the scavenging activities of distilled water, ethanol, butanol, chloroform extracts were 91.48%, 44.24%, 29.31% and 26.23%, respectively. In summary, Vc showed significantly higher ability on DPPH radical scavenging activities than distilled water, ethanol, butanol and chloroform extracts of *A. rosea* seeds.

Superoxide anion radical scavenging activity assay

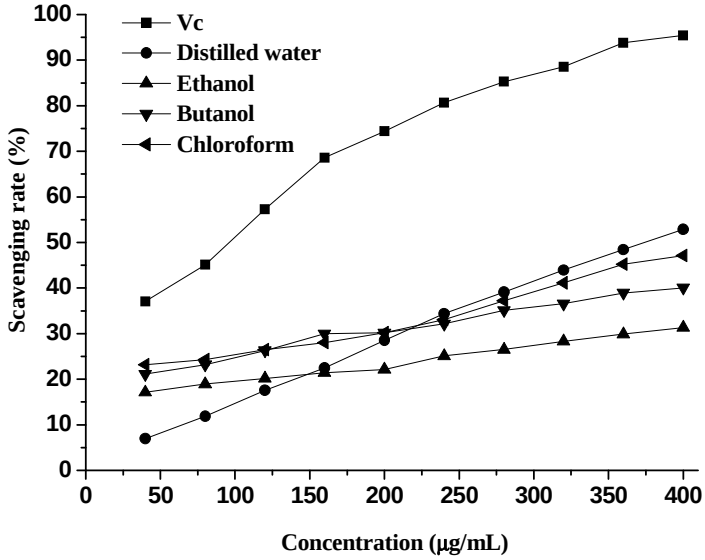

Fig. 2 The activity of scavenging superoxide anion

Fig. 2 showed the results of superoxide radical scavenging activity. The scavenging activity increased with increase

in the concentration of distilled water, ethanol, butanol, chloroform extracts and Vc. Although the activity of *A. rosea* seeds extracts was weaker than that of Vc, it can scavenge superoxide anion well in higher concentrations. At 400 μg/mL, the scavenging activities of distilled water, ethanol, butanol, chloroform extracts were 52.89%, 31.33%, 40.06% and 47.12%, respectively. Therefore, *A. rosea* seeds extracts had an appreciable power on superoxide radicals.

Hydroxyl radical scavenging activity assay

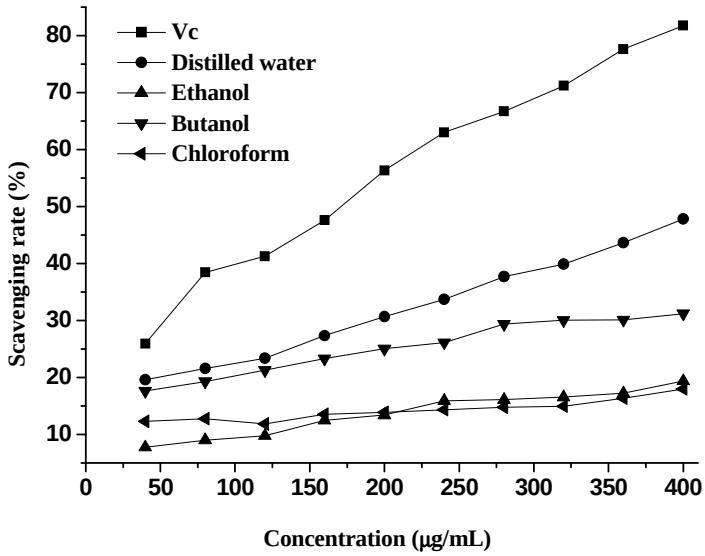

Fig. 3 The activity of scavenging hydroxyl radical

Fig. 3 showed the scavenging activities of distilled water, ethanol, butanol, chloroform extracts and Vc against the hydroxyl radical. The hydroxyl radical scavenging activity of the four extracts was notable at all tested concentrations, and positively correlated with increased concentration up to 400 μg/mL. Obviously, hydroxyl radical scavenging activity of Vc was significantly higher than that of *A. rosea* seeds extracts. Ethanol extracts had the weakest activity. At the concentration of 400 μg/mL, the scavenging activities of distilled water, ethanol, butanol, chloroform extracts and Vc were 47.82%, 19.38%, 31.22%, 17.98% and 81.77%, respectively. These data on hydroxyl radical scavenging activity assay indicated that *A. rosea* seeds could act as antioxidant to reduce the formation of free radical.

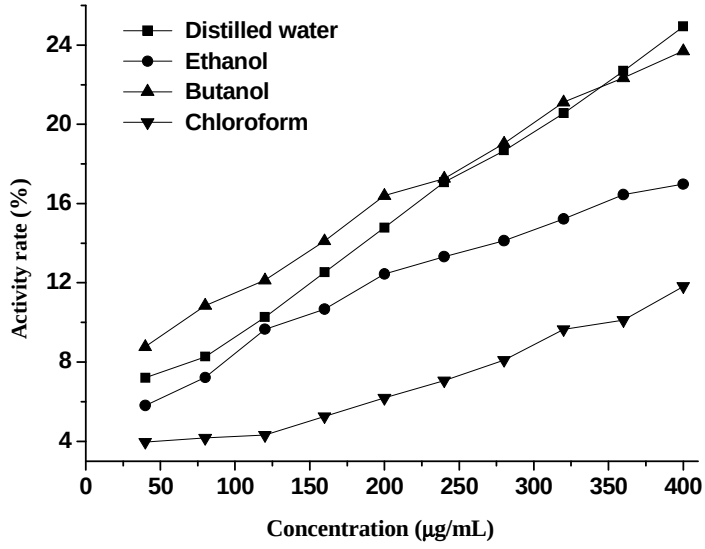

Fig. 4 The activity rate of SOD

Superoxide dismutase activity assay

Fig. 4 showed the superoxide dismutase activity of distilled water, ethanol, butanol, chloroform extracts against the superoxide anion free radical. Distilled water extracts had the strongest inhibition capacity. At 400 μg/mL, the inhibition of distilled water, ethanol, butanol, chloroform extracts was 24.94%, 16.98%, 23.70% and 11.83%, respectively. Obviously, although the *A. rosea* seeds extracts had strong inhibition against superoxide anion free radical, Vc bested them all. This result indicated that the *A. rosea* seeds was a good scavenger for superoxide anion radicals.

CONCLUSION

The results from this study indicate that *A. rosea* seeds is a rich source of polyphenols which may had an appreciable power contribute to its antioxidative properties. In the present study, significant antioxidant activities of four solvent extracts from *A. rosea* seeds were estimated that including DPPH assay, superoxide anion scavenging assay, hydroxyl radical scavenging assay and superoxide dismutase activity assay. The data all showed significant antioxidant activities in a concentration dependent manner, distilled water extracts showed good antioxidant activity. Hence, *A. rosea* seeds are natural antioxidants, and may be potential for functional food ingredients.

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