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Determination of *In Vitro* Antioxidant and Radical Scavenging Activities of *Alcea rosea*

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ABSTRACT

Background: *Alcea rosea* belongs to family Malvaceae and is used to treat various diseases on folklore levels in Kashmir valley. Objective of the study is to explore *in vitro* antioxidant potential of *Alcea rosea*. **Materials and Methods**: Antioxidant potential of extracts was evaluated by means of total phenolics, DPPH, reducing power and superoxide radical scavenging activity by using standard procedures. **Results**: The highest phenolic content of 1157.43 ± 57.87mg GAE/g was observed in methanol extract followed by ethyl acetate (690 ±22.6 mg GAE/g) extract. At concentration of 700 µg/mL, DPPH radical scavenging activity of methanol extract was (88±4.72%) IC_{50} (368 µg/ml) and ethyl acetate (80±3.05%) IC_{50} (393 µg/ml). The reducing power of the extracts increased in a concentration dependent manner. Superoxide radical scavenging activity of *Alcea rosea* extracts increased in a dose dependent manner with IC_{50} values 31.10 µg/ml (methanol) and 36.76 µg/ml (ethyl acetate extract).**Conclusion**: These results clearly indicate that *Alcea rosea* extracts possesses the free radical scavenging activity as such can be active as potential antioxidant agent against various oxidative stress related pathological conditions.

Key words: In vitro antioxidant, *Alcea rosea*, DPPH, superoxide radical.

INTRODUCTION

During the process of respiration, harmful intermediates known as reactive oxygen species (ROS) are formed including superoxide anions, hydroxyl radicals, hydrogen peroxide, and alkoxyl radicals which cause damage to biological macromolecules. (DNA, proteins, lipids,

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carbohydrates etc.) which further leads to various degenerative diseases like cancer, atherosclerosis, cardiovascular diseases (Murphy, 2009). Antioxidation and oxidation balance is thought to be a crucial element for sustaining a healthy biological system(Gandhi & Abramov, 2012).Antioxidants in the diet can help to boost cellular defences and protect cellular components from oxidative damage (Chiva-Blanch & Visioli, 2012). Because of the possible health risks and toxicity of synthetic antioxidants, a broad consensus was required to replace synthetic antioxidants with natural antioxidants (P.Evans & B.Halliwell, 2007). As a result, attempts have been undertaken to uncover novel natural resources for active antioxidant molecules and the hunt for antioxidants from natural sources has attracted a lot of attention (Wong *et al.*, 2006).

*Alcea rosea (*Malvaceae) popularly known as Holyhock is extensively cultivated in gardens and parks throughout Southern Europe and Asia. Numerous pharmacological studies have reported that this plant possesses anti-inflammatory, antibacterial and analgesic effects.*(Dar et al.*, 2017; Mert *et al*., 2010). In Iranian traditional medicine, the roots of *Alcea rosea* have been used to treat a variety of diseases including bronchitis, diarrhoea, constipation, inflammation, severe coughs and angina.(Ahmadi *et al.,* 2012).The results of ethnobotanical and ethnopharmacological studies of different species *Alcea rosea* indicate the potential use of these plants for the treatment of a large variety of diseases. Due to the increasing interest in the relationship between antioxidants and diseases, it is important to measure the overall antioxidant activity of *Alcea rosea*. Therefore, the objective of this study was to evaluate the antioxidant and free radical scavenging activity as well as total protein contents of different extracts of *Alcea rosea.*

Materials and methods

Plant Material Collection and Extraction

The whole plant of *Alcea rosea* was collected from badamwari Srinagar area of Jammu and Kashmir during the month of June, identified by the Centre of Plant Taxonomy, Department of Botany, University of Kashmir, and authenticated by Akhter Hussain Malik. A reference specimen has been retained in the herbarium under reference number KASH-Bot/ku/AR-705-IA.

The authentically identified plant material was shade dried under room temperature at 30 ± 2°C. The dried plant material was grind into powder using mortar and pestle and sieved with a sieve of 0.3mm aperture size. The powder obtained was successively extracted in hexane, ethyl acetate, absolute ethanol, methanol and aqueous solvents by using Soxhlet extractor (60-80°C). The extracts were then concentrated with the help of rotary evaporator under reduced pressure, and the solid extract was stored in refrigerator for further use.

Experimental methods

DPPH radical scavenging activity

The DPPH assay was performed by using the method of (Ahmad *et al*., 2015).Various concentrations of plant extracts (100-700µg/ml was added to 1ml of the 0.004% methanol solution of DPPH, and the mixture was vortexed vigorously. The tubes were then incubated at room temperature for 30 minutes in dark, and the absorbance was taken at 517nm. Lower

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absorbance of the reaction mixture indicates higher free radical scavenging activity. BHT was taken as known free radical scavenger. IC_{50} value (the concentration required to scavenge 50% DPPH free radicals) was also calculated and the percentage inhibition activity was calculated by using the formula.

$$\% \text{ inhibition} = [(A_0-A_1)/A_0] \times 100$$

Where A_0 was the absorbance of the control and A_1 was absorbance in the presence of *Alcea rosea* extracts/known antioxidant.

Reducing power

The reducing power of *Alcea rosea* extracts were evaluated according to (Oyaizu, 1986). Different concentrations of the plant extracts were mixed with 2.5ml of 0.2 M phosphate buffer (pH 6.6), and 2.5ml of 1% potassium hexacyano ferrate II. The mixture was incubated at 50^{o}C for 20 minutes, 2.5ml of 10% TCA was added to the mixture and centrifuged at 3000rpm for 10 minutes. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml) and $FeCl_3$ (0.5ml, 0.1%), and the absorbance was measured at 700nm. BHT was taken as the known standard. The percentage reduction of the sample as compared with BHT was calculated by using formula:

$$\text{Reduction (\%)} = [1- (1- A_C/A_S)] \times 100$$

Where, AC is absorbance of standard at maximum concentration tested and AS is absorbance of sample.

Total phenolics

The total phenolics of *Alcea rosea* (methanolic and ethyl acetate extracts) were determined by using Folin-Ciocalteau reagent according to the protocol of (Chandler & Dodds, 1983). One hundred milligram of extracts was dissolved in 100 ml of methanolic/water (60:40, v/v, 0.3% HCl). To 1ml of sample, add 1ml of 95% ethanol, 5ml of distilled water and 0.5ml of 50% Folin- Ciocalteau reagent. The mixture was allowed to react for 5 minutes and 1ml of 5% sodium carbonate was added and mixed completely. After one hour incubation at room temperature, the absorbance of the solution at 765 nm was measured with spectrophotometer. Quantitation was based on the standard curve of gallic acid (10mg %), which was dissolved in methanol/water (60:40, v/v, 0.3% HCl). The concentration of polyphenols was expressed in terms of mg/100ml of sample.

Assessment of superoxide anion radical scavenging property

Superoxide anion radical generated by the Xanthine/Xanthine oxidase system was spectrophotometrically determined by monitoring the product of nitroblue tetrazolium (NBT) using the method of (Qian *et al*., 2008). A reaction mixture containing 1.0ml of 0.05M phosphate buffer (pH 7.4), 0.04ml of 0.15% BSA, 0.04ml of 15.0mM NBT and various concentrations of (plant extracts and known antioxidants) was incubated at 25^{o}C for 10min, and the reaction was then started by adding 0.04ml of 1.5U/ml Xanthine oxidase and again incubated at 25^{o}C for 20min. The absorbance of the reaction mixture was measured at 560nm. Decreased absorbance of the reaction mixture indicates increased superoxide anion radical scavenging activity.

The scavenging activity of the plant extracts on Superoxide anion radical was expressed as:

$$\% \text{ inhibition} = [(A_0-A_1)/A_0] \times 100$$

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Where A_0 was the absorbance of the control and A_1 was absorbance in the presence of *Alcea rosea* root extracts/ known antioxidant.

RESULTS

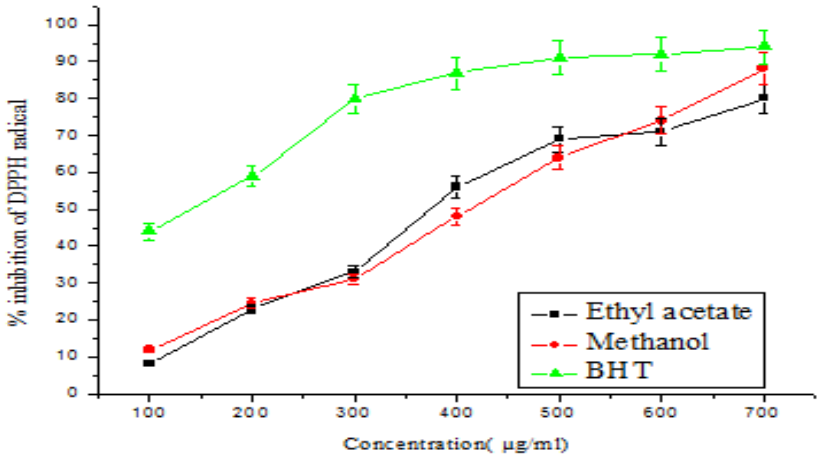

Figure 1: Percent inhibition of DPPH free radical by different extracts of *Alcea rosea*. The results represent mean± SD of 3 separate experiments.

DPPH radical scavenging activity is one of the most extensively used method for screening the antioxidant activity of plant extracts. 100-700µg/ml extract of *Alcea rosea* was used and the percentage inhibition was recorded in a concentration dependent manner. The methanol and ethyl acetate extracts of *Alcea rosea* showed concentration dependent inhibition of DPPH free radical with methanol extract showing maximum inhibition of 88±4.72% at 700µg/ml concentration followed by ethyl acetate with 80±3.05% (Fig.1) The IC_{50} value of ethyl acetate and methanol extract is 393.05±30.05 and 368.83±29.4µg/ml respectively. The results are represented relative to butylated hydroxytoluene (BHT), a reference standard with IC_{50} value of 52.84±2.05 µg/ml.

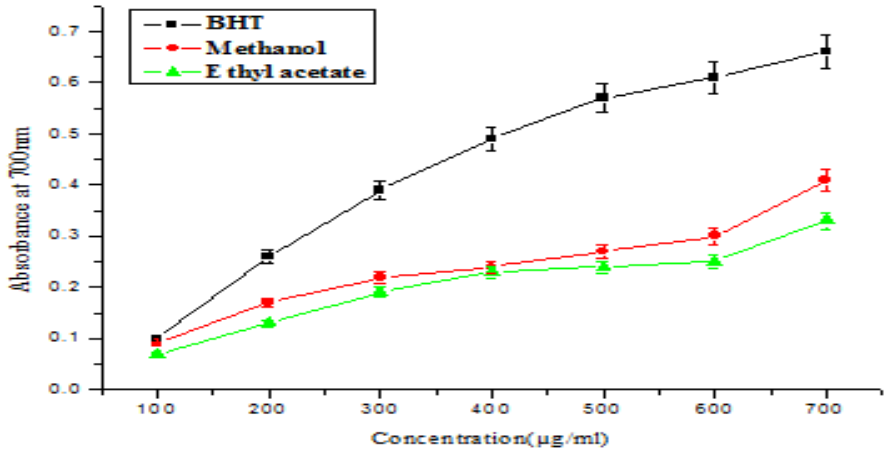

Figure 2: Reducing power of different extracts of *Alcea rosea* represented by increase in absorbance with increase in concentration. The results represent mean± SD of 3 separate experiments.

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The reducing power of different extracts of *Alcea rosea* followed a concentration dependent manner as shown in Fig.2. Higher the value of absorbance, strong is the reducing activity of the sample. The absorbance at 700nm increased with 100-700µg/ml from 0.09 to 0.41 for methanol extract. Likewise the absorbance for same concentrations with ethyl acetate extracts increased from 0.07 to 0.33 as shown in Fig. 2.

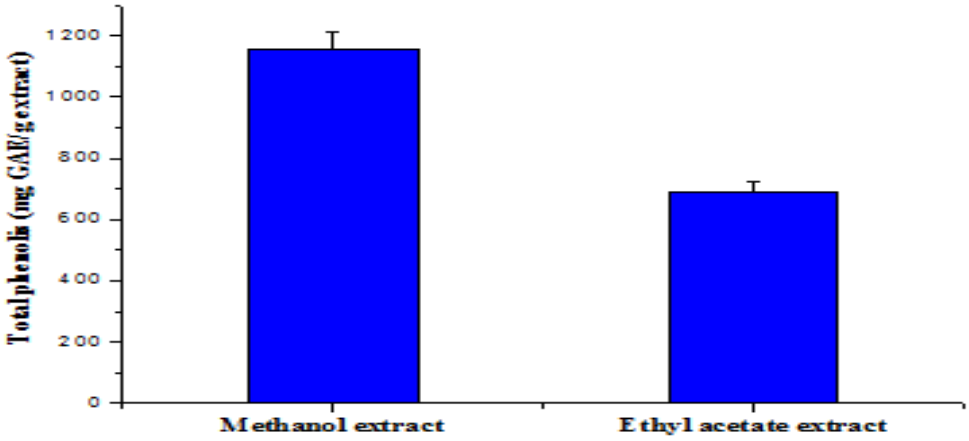

Figure 3: Total phenolic content of different extracts of *Alcea rosea*

Total phenolic content was determined by Folin Ciocalteu method and expressed as mg/ g of gallic acid equivalent. The methanolic extract showed the highest phenolic content. The methanol and ethyl acetate extracts of *Alcea rosea* were found to be 1157.43 ± 57.87mg GAE/g and 690 ±22.6 mg GAE/g extract. It is clear from the results that the phenolic content was found in huge amount in all the extracts. Among all the extracts tested methanol extract exhibited high quantity of phenolic compounds compared to other extracts (Fig. 3). Phenols are very important plant constituents and because of the presence of hydroxyl groups they have a great scavenging ability. Phenolic compounds are therefore, known to be powerful chain breaking antioxidants.

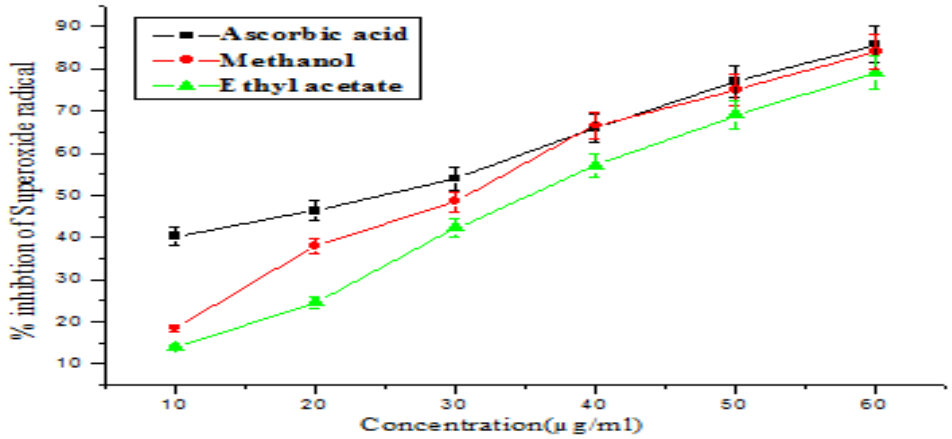

Figure 5: Percent inhibition of Superoxide radical by different extracts of *Alcea rosea* . The results represent mean± SD of 3 separate experiments.

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Methanol extract of *Alcea rosea* was found to scavenge the superoxide radicals much efficiently than the ethyl acetate extract. *Alcea rosea* extracts also showed concentration dependent inhibition of superoxide radical with methanol extract showing maximum percent inhibition of 84±4.2% at 60µg/ml concentration. However, ethyl acetate extract showed percent inhibition of 79.08±3.95% at 60µg/ml (Fig. 5.B). The IC_{50} values of the methanol and ethyl acetate extracts of *Alcea rosea* were found to be 31.10±0.1and 36.76±0.16µg/ml respectively (Table 4). Ascorbic acid a known antioxidant, taken as reference standard showed 85.75% inhibition at the same concentration.

Discussion

Alcea rosea is used ethno pharmacologically for the cure of various complaints. The beneficial value of medicinal plants is usually contributed to their antioxidant properties. Antioxidants protect us from a variety of ailments by fighting free radicals. They work by either scavenging reactive oxygen species or by preserving the antioxidant defence systems (Sharma *et al.,* 2018).In this study, *in vitro* antioxidant activity of different extracts of *Alcea rosea* were tested for their antioxidant activity using DPPH, reducing power, total phenolic content and super oxide radical scavanging assays. The ability of antioxidants to donate hydrogen is assumed to be the reason for their action on DPPH (Letelier *et al.,* 2008). Radical scavenging activities are very significant to inhibit the deleterious role of free radicals in different diseases including cancer.The DPPH free radical scavenging method is widely used to test the antioxidant properties of plant extracts. The addition of the extract to a violet-colored DPPH solution reduces it to a yellow-colored product, diphenylpicryl hydrazine, in a concentration-dependent manner in the DPPH assay. This method has been used widely to predict antioxidant activities because of the relatively short time required for analysis. Our results revealed that the methanolic extract of *Alcea rosea* had a similar free radical scavenging activity when compared with standard BHT (Fig. 1). Because of their hydrogen-donating capabilities, polyphenols and tocopherols scavenge DPPH radicals (Baumann, 1979; Huang *et al.,* 2005).The results obtained in this study suggest that all the extracts from *Alcea rosea* showed radical scavenging activity by their electron transfer or hydrogen donating ability. The quantity of total polyphenols and the activity of antioxidants that scavenge free radicals are highly connected(Abdel-Hameed, 2009).

Reducing power is also commonly employed to assess plant polyphenol antioxidant activity. The presence of reductants, which act as antioxidants by breaking free radical chains by donating a hydrogen atom, is often associated with the existence of reducing power. The presence of reductants in the antioxidant sample converts the Fe3+/ferricyanide complex to the Fe2+/ferrous form in this assay. Hence, the reducing power of the sample can be checked by measuring the formation of Perl's Prussian blue at 700 nm (Oktay *et al.,* 2003) . In this study, the iron reducing capacity of the methanolic and ethyl acetate extracts of *Alcea rosea* was estimated from their ability to reduce the Fe^{3+}-ferricyanide complex to the ferrous form by donating an electron. The reducing ability of the extracts was in the range of 0.09 to 0.41 for methanol and for ethyl acetate 0.07 to 0.33 at the absorbance of 700nm (Fig.2). All the extracts exhibited a good reducing power capacity, which was concentration-dependent

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(Fig.2). At this point, we assume that the antioxidant activity and reducing power capacity of the extracts was likely due to the presence of polyphenols, which can act as free radicals scavenger by donating an electron or hydrogen.

Plant phenolic compounds are potent free radical scavengers that can prevent lipid peroxidation by neutralising peroxyl radicals produced during lipid oxidation(Ardestani & Yazdanparast, 2007).The TPC of the different extracts of *Alcea rosea* was assayed by the Folin-Ciocalteu method using gallic acid as standard. It was found that the TPC of different extracts was in the descending order of methanol >ethyl acetate extract (Figure 3).The highest TPC of 1157.43 ± 57.87mg GAE/g was obtained in methanol, whereas the lowest TPC of 690 ±22.6 mg GAE/g was achieved in ethyl acetate extract. It is worthwhile to mention that the TPC of ethyl acetate extract was lower than that of methanol extract, which may be the result of enhancement of the phenolic components in the extracts.

Superoxide anion is one of the most significant agents of free radicals. It serves as a precursor to more reactive oxidative species such single oxygen and hydroxyl radicals, which have the capacity to react with biological macromolecules and cause tissue damage, as well as a key part in lipid peroxidation (Sangameswaran *et al.*, 2009).Methanol extract with IC_{50} value of 31.10±0.1µg/ml showed strong super oxide radical scavenging activity than ethyl acetate extract, however the values remain below the BHT used as known superoxide radical scavenger.

Conclusion

Antioxidants are gaining popularity as a result of the health benefits that natural resources bring. This entails preventing the occurrence of oxidative stress-related disorders as a result of free radical attacks on various bio-components in the human body. Our findings demonstrated *Alcea rosea* potential as a natural antioxidant source, and the plant could be a promising agent in scavenging free radicals and treating diseases caused by free radical reactions, as well as being used in the pharmaceutical industry. Therefore, this study can be a guideline for further biological activities of investigation.

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