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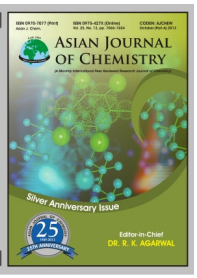

Phytochemical Screening and Antioxidant Evaluations of Different Fractions of *Argyrolobium roseum*

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The present investigations were carried out to establish the medicinal value of *Argyrolobium roseum* synonym *Cytisus roseum*. It is a medicinal plant, belongs to family Leguminoseae. The antioxidant potentials of the different fractions of *A. roseum* were screened by using $ABTS^{\bullet+}$ assay, DPPH free radical scavenging activity, ferric reducing antioxidant power (FRAP) assay, total antioxidant activity by phosphomolybdenum method, total phenolic contents determination, total flavonoid contents and metal chelating activity of ethyl acetate, *n*-hexane, chloroform, methanol and aqueous fractions. $ABTS^{\bullet+}$ assay results reveal that ethyl acetate soluble fraction demonstrated the good antioxidant activity having the highest Trolox equivalent antioxidant capacity (TEAC) value *i.e.*, 152.43 ± 0.43 mM/µL Trolox equivalent and the highest total flavonoid contents having total flavonoid content value 931.91 ± 0.06 mg/g QE, *n*-hexane soluble fraction showed the highest percentage of metal chelating activity 61.85 ± 0.9 % bound iron and highest total phenolic contents 792.5 ± 0.55 gallic acid equivalent mg/g whereas aqueous extract indicated the ferric reducing antioxidant power value 70.48 g/mL. It was concluded from antioxidant screening that ethyl acetate soluble fraction was rich in strong radical scavenging active components but chloroform soluble fraction and aqueous extract were valuable resources of phenolics and flavonoids.

Key Words: Phytochemical screening, *Argyrolobium roseum*, Antioxidant potential, $ABTS^{\bullet+}$ assay, FRAP assay.

INTRODUCTION

The world health organization (WHO) defined traditional medicines as comprising therapeutic practices that have been in existence for hundreds of years. Even today WHO estimates that up to 80 % of the world's population still relies mainly on traditional remedies such as herbs for their medicines[1]. Medicinal plants are one of the important sources of pharmaceuticals and healthcare products. A whole range of plant derived dietary supplements, phytochemicals and pro-vitamins have shown beneficial therapeutic potentials in maintaining good health and combating disease due to the presence of functional ingredients and nutraceuticals.

The role of medicinal plants in disease prevention and control is due to the presence of antioxidant properties of their constituents usually associated to a wide range of amphipathic molecules; broadly termed polyphenolic compounds[2]. Our group is trying to characterize medicinal plants for their phytochemical constituents, antioxidant potentials and extraction of pure metabolites responsible for bioactivities[3,4]. These compounds are commonly found in both edible and inedible plants and have multiple applications in food, cosmetics and pharmaceutical industries[5]. Antioxidant potential is mainly due to the presence of flavones, flavonoids, isoflavones, anthocyanin, lignans, coumarin, catechins and isocatechins[6]. Free radicals such as hydroxyl radicals $(OH)^-$ and superoxide anion $(O_2)^-$ and reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) are produced as normal products of cellular metabolism[7]. Mitochondrion is the chief site of metabolism. Rapid production of free radicals can lead to oxidative damage to biomolecules including proteins, lipids and purines, pyrimidine bases and sugar moiety of nucleic acid[8]. Free radicals contribute to more than hundred disorders in human beings including atherosclerosis, arthritis, ischemia, reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS[9]. Due to environmental pollutants, radiation, chemicals, toxins, deep fried and spicy foods as well as physical stress cause free radicals depletion of immune system, change in expression and induce abnormal proteins[10]. Synthetic antioxidants like butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT), tertiary butylated hydroquinone and gallic acid esters have

been suspected to produce negative health effects with low solubility. Hence their application is strongly restricted and there is a trend to substitute them with naturally occurring antioxidants[11]. The present study was conducted to evaluate the antioxidant activities of various extracts of *A. roseum* of different polarities. Because of the stability of radical cations under acidic, basic, neutral or in buffers solutions, assays are carried out under different reaction conditions.

Argyrolobium roseum belongs to a large family Leguminoseae/ Fabaceae and is widely distributed in Pakistan. This family is the third largest family amongst the flowering plants. *A. roseum* (Cambers Jaub and Spauch) belongs to sub family Papilionaceae which contains about 482 genera and 7,200 species. It is a sexually reproducing, rare herb that grows in tropical and sub-temperate tracts of the north western Himalayan region located in Indian sub-continent and Pakistan[12]. Plants belonging to this family have high medicinal values. Many alkaloids possess curative properties[13] *i.e*., natural hallucinogenic and analgesic potentiator used as astringent (*Butea monosperma*) in the treatment of leucoderma and leprosy (*Psordea corylifortia*). Infusion of leaves of *Cajanus cajan* L. commonly known as yellow Dhal, pigeon pea is used for curing anemia, hepatitis, diabetes, urinary infections and yellow fever[14]. Cytisine is also an alkaloid present among the plants of this family and used for the cigarette cessation[15].

To the best of our knowledge, no detailed work has been done on various polar and non-polar fractions of this whole plant. We are reporting here for the first time the *in vitro* antioxidant capacities of organic fractions and aqueous extract of *A. roseum*. As the purpose of this study was to determine new potential sources of natural products, various antioxidant methods like DPPH radical scavenging activity, ABTS radical cation scavenging activity, FRAP assay, total phenolic contents (TPC), total flavonoid contents (TFC) along with determination of their total antioxidant activity by phosphomolybdenum complex method relative to conventionally used standards were employed[16].

EXPERIMENTAL

DPPH (1-1-diphenyl-2-picrylehydrazyl radical), TPTZ (2,4,6-tripyridyl-*s*-triazine), Trolox, gallic acid, Follin Ciocalteu reagent and butylated hydroxytoluene were obtained from Sigma Chemical Company Ltd. (USA) and organic solvents (*n*-hexane, chloroform, ethyl acetate, *n*-butanol), sulphuric acid, sodium phosphate ammonium molybdate, ferric chloride and ferrous chloride from Merck (Pvt.) Ltd. (Germany).

Plant *Argyrolobium roseum* was collected from Kotly in April, 2010 and was identified by Muhammad Ajaib (Taxonomist), Department of Botany, of GC University Lahore, Pakistan. Voucher specimen was deposited (Voucher No. GC.Bot.Herb.1397) in the same university.

Extraction and fractionation of plant material: 2.5 kg of *Argyrolobium roseum* was shade dried and air currents were allowed to pass through in order to avoid fungal deterioration. Whole plant was ground into fine powder and exhaustively extracted with methanol (5 L × 3 L) at room temperature. Three extracts were mixed and concentrated to obtain dark green semi-solid methanolic extract. It was dissolved in *ca.* (1.5 L) distilled water and treated with *n*-hexane (1.5 L) to yield non polar fraction, with chloroform (1.5 L), ethyl acetate (1.5 L) and *n*-butanol (1.5 L) to obtain polar fractions, respectively. Organic fractions and aqueous fractions were then concentrated on a rotary evaporator.

Phytochemical analysis of the plant extracts: Qualitative tests for the investigation of phytochemical components like terpenes, phenols, saponins, flavonoids, alkaloids, tannins, sugars and cardiac glycosides in *Argyrolobium roseum* were detected by using the methods reported by Sofowora, Trease and Evans[17,18]. Presence of a particular secondary metabolite is estimated from the intensity of colour.

Test for Terpenes (Salkowski test): Presence of terpenes in *A. roseum* was confirmed by performing two tests. Placed the spots of different extracts on the TLC card, it was then sprayed with ceric sulphate solution and heated up to 105 ºC in order to develop the spots on a TLC plate heater. Presence of terpenes was indicated by the appearance of brown coloured spots on the card. 2.0 mL of chloroform was added to 0.5 g of each of the fraction in a test tube followed by the careful addition of concentrated H_2SO_4. Appearance of reddish brown colour suggested the presence of terpenes (Salkowski Test).

Tests for phenols: To 0.5 g of each of plant fraction, neutral solution of $FeCl_3$ was added and the appearance of bluish-green colour indicated the presence of phenols.

Tests for saponins: Saponins can be detected by vigorous shaking of 0.5 g of plant fraction with 0.5 mL distilled water; development of persistent froth indicated the presence of saponins. Formation of an emulsion on shaking after the addition of few drops of olive oil further confirmed their presence.

Tests for flavonoids: Two methods were also applied for the determination of flavonoids. 10 % of ammonia solution was added to 5.0 mL of each plant fraction afterwards 1.0 mL of conc. H_2SO_4 was added. Appearance of yellow colouration which disappeared on standing indicated the presence of flavonoids. Appearance of persistent yellow colour on addition of 1 % aluminum chloride to another portion of plant fractions indicated the presence of flavonoids.

Tests for alkaloids: Alkaloids were tested for their presence by conventional methods. Spots of different fractions were placed on a piece of TLC card and dried. Then sprayed with Dragon Dorff's reagent, the development of reddish-brown colour indicated the presence of alkaloid. To a small portion of each fraction Mayer's reagent confirmed the presence of alkaloids.

Test for tannins: Tannins were indicated by boiling 0.5 g of each fraction in 10 mL distilled water, after filtration few drops of 1 % $FeCl_3$ solution were added, appearance of brownish-green or bluish-black colouration confirmed the presence of tannins.

Tests for reducing sugars: To 0.5 mL of the sample fraction, 0.5 mL of Fehling solution A and Fehling solution B were added; reaction mixture was strongly heated for 1 min. The appearance of the red precipitates confirmed the presence of reducing sugars.

Tests for cardiac glycosides: A violet or brown coloured ring formation showed the presence of cardiac glycosides when

1 mL of glacial acetic acid was added having 1 drop of $FeCl_3$ followed by the addition of 1 mL of concentrated sulphuric acid.

Antioxidant activity: Following assays were performed to determine the antioxidant activity.

$ABTS^{\bullet+}$ assay: Total antioxidant activity was measured in terms of Trolox equivalent antioxidant capacity (TEAC) method described by Re *et al.*[19]. 7.0 mM solution of ABTS was prepared in double distilled water to generate $ABTS^{\bullet+}$ on reacting with 2.45 mM potassium persulphate, after 24 h in dark at room temperature. $ABTS^{\bullet+}$ solution diluted with TBS buffer at pH 7.4 to obtain an absorbance of 0.70 ± 0.02 at 734 nm. 10 µL of sample was added to 2.29 mL of diluted solution of $ABTS^{\bullet+}$. Absorbance reading was taken exactly after 1.0 min of initial mixing up to 6.0 min at 300 ºC, blank reading was taken in each assay. The percentage inhibition of absorbance at 734 nm was determined using the formula:

$$\text{Inhibition (\%) (at 734 nm)} = \left(1 - \frac{A_f}{A_o}\right) \times 100$$

A_o is the absorbance of ABTS radical cation and A_f is the absorbance after sample addition.

DPPH assay: DPPH radical scavenging activities of different organic fractions and aqueous extract of the plant under investigation were determined by comparing the results with that of the already known antioxidant *i.e.*, butylated hydroxytoluene (BHT) using the reported method. Different concentrations of samples were prepared as 1000, 500, 250, 125, 60, 30, 15 and 8.0 µg/mL were mixed with 3.0 mL of methanolic solution of DPPH of 0.1 mM strength. After the addition of DPPH solution, each of the mixtures was vigorously shaken at room temperature and allowed to stand for *ca.* 1.0 h. Methanol was taken as for blank. UV visible spectrophotometer (CECIL instrument CE 7200 Cambridge England) was used to measure the absorbance at 517 nm. Higher free radical scavenging activity of the fraction was indicated by lower absorption of spectrophotometer. The percentage of DPPH radical scavenging was calculated for each value[20] by using the formula:

$$\text{Ab. (\%)} = \left[\frac{\text{(Ab. of pure DPPH - Ab. of sample}}{\text{(Ab. of pure DPPH}}\right] \times 100$$

Ferric reducing antioxidant power: 25 mL of 300 mM acetate buffer of pH 3.5 and 2.5 mL of 10 mM TPTZ solution in 40 mM HCl and 20 mM $FeCl_3$ (2.5 mL) were mixed to prepare the FRAP reagent, then to 100 mL of each sample, 3 mL of FRAP reagent and 300 µL of distilled water were mixed to measure the absorbance at 593 nm. FRAP values were calculated from standard curve of iron(II) sulphate[21].

Phosphomolybdate assay for total antioxidant activity: To determine the antioxidant activity, reagent solution was prepared by mixing 0.6 M H_2SO_4, 4.0 mM ammonium molybdate and 28.0 mM of sodium phosphate. 4.0 mL of this reagent was taken in three test tubes and 100, 300 and 500 µL of samples were mixed in each test tube. A blank was also prepared containing only 4.0 mL of the stock solution. Test tubes were capped and placed to incubate at 95 ºC for 90 min. Absorbance was measured at 695 nm against the blank after cooling. Each sample was run in triplicate, mean values were calculated and the antioxidant activity was expressed compared with butylated hydroxytoluene.

Total phenolics contents: 40 µL from each of the sample were mixed with 3.16 mL of distilled water and 200 µL of 0.2 N Follin Ciocalteu reagent. 600 µL of super saturated sodium carbonate (75 g/L) was added after 8.0 min, solution turned blue. Absorbance was measured at 765 nm after incubation at 40 ºC for 0.5 h with intermittent shaking. Total phenolic contents were calculated from the standard curve expressed as mg/g equivalents of gallic acid (GAE)[22].

Total flavonoid content determination: TFC contents of various fractions were determined by the standard method[23]. 0.25 mL of plant sample and quercetein standard solution were mixed with 1250 µL of distilled water taken in a test tube, 75 µL of $NaNO_2$ were added followed by 0.5 mL of 1 M NaOH solution after 5.0 min and volume was raised to 2.5 mL by adding distilled water. Absorbance was measured at 510 nm. TFC contents were determined from the standard curve of quercetein expressed as milligrams of quercetein equivalents per gram of sample.

Metal chelating activity: The reaction mixture was prepared by adding 100 mL of sample in 0.05 mL of $FeSO_4$ and 0.2 mL of 5.0 mM ferrozine and total volume was made up to 4.0 mL with double distilled ethanol, after 10 min absorbance was noted at 562 nm[24]. Percentage of bound iron was calculated from the formula in terms of EDTA standard.

$$\text{Bound iron (\%)} = \left[\frac{(A_{\text{Control}} - A_{\text{Sample}})}{A_{\text{Control}}}\right] \times 100$$

RESULTS AND DISCUSSION

Phytochemical screening to find out the biologically active constituents confirmed the presence of terpenoids, phenols, saponins, flavonoids, alkaloids, reducing sugars and cardiac glycosides. Results revealed that ethyl acetate soluble fraction was rich in phenols, flavonoids and cardiac glycosides whereas traces of tannins, saponins and terpenoids were present. Alkaloids and cardiac glycosides were detected in considerable amounts in chloroform fraction but phenols, tannins and saponins were totally absent. Crude methanolic extract was rich in tannins, flavonoids and cardiac glycosides but alkaloids, reducing sugars and terpenoids were also present in moderate amounts. Aqueous fraction contained good amounts of saponins, alkaloids, cardiac glycosides and terpenoids but lack of tannins. Overall results are listed in Table-1.

Antioxidant evaluations were performed by using the conventional methods. $ABTS^{\bullet+}$ scavenging activities assay was calibrated by using an alpha tocopherol analog 'Trolox' as standard. The results of $ABTS^{\bullet+}$ were expressed in terms of TEAC values as shown in Table-2. TEAC is a measure of effective antioxidant activity of the substance and stands for "Trolox equivalent antioxidant capacity". High TEAC value referred to a greater antioxidant potential of the sample as they have scavenged $ABTS^{\bullet+}$. Ethyl acetate fraction showed highest activity. Aqueous fraction had slightly less activity. Methanolic fraction and chloroform fraction indicated greater activities and *n*-hexane fraction showed moderate activities. Results are presented in Table-2. The TEAC values showed the following trend:

TABLE-1
RESULTS OF PHYTOCHEMICAL SCREENING OF *Argyrolobium roseum*

Extracts	*n*-Hexane Fr.	Ethyl acetate Fr.	Chloroform Fr.	Methanol Fr.	Aqueous Fr.
Terpenes	+	+	+	+++	+++
Phenols	–	+++	–	+++	++
Saponins	+	+	–	+	+++
Flavonoids	–	+++	++	+++	+++
Alkaloids	–	–	+++	+	++
Tannins	–	+	–	+++	–
Reducing sugars	–	+	+	++	++
Cardiac glycosides	–	+++	+	+++	+++

+++ Strong; ++ medium; + poor presence; - absence, repeated the experiment three times for each replicate.
Classification was based on observation of colour intensity and amount of precipitates.

Ethyl acetate > aq. extract>methanol>chloroform > *n*-hexane

DPPH assay is widely used to evaluate the radical scavenging activity of natural antioxidants in a shorter time. It is a stable free radical at room temperature and neutral pH, produces a violet colour in ethanol at 517 nm and is converted in a colourless or somewhat yellow diamagnetic DPPH, after accepting an electron or hydrogen from an antioxidant. DPPH at this stage results in decrease in absorbance at 517 nm. The bleaching in colour is correlated with the number of hydroxyl groups. It is concluded that a good antioxidant activity is probably due to the abundant availability of flavonoid or phenolic compounds. It was observed that ethyl acetate fraction of *A. roseum* had the highest radical scavenging activity with IC_{50} 24.33 ± 0.14 as presented in Table-2. The order of activity is as follows:

Ethyl acetate>chloroform>*n*-hexane>methanol > aq. fraction

Ferric reducing antioxidant power involved the reduction of ferric tripyridyltriazine Fe(III)-$(TPTZ)_2Cl_3$ pale yellow in colour to Fe(II)-$(TPTZ)_2Cl_2$ blue in colour when it come in contact with an antioxidant at 593 nm absorbance. Results were expressed in FRAP units. A higher value referred to a high reducing power as given in Table-2. The absorbance reading was taken just 6.0 min interval after mixing TPTZ to the sample. FRAP value was highest for aqueous extract, slightly less in ethyl acetate fraction, *n*-hexane and chloroform fraction showed moderate activities. Phenolic contents of the plant materials were suggested to correlate directly with antioxidant activities. In stabilizing lipid oxidation polyphenolic compounds play an important role. Order of reactivity is;

Aq.fraction>ethyl acetate > methanol>chloroform>*n*-hexane

Phosphomolybdenum method was employed spectroscopically in order to find out the total antioxidant activity of *A. roseum*. Order of reactivity of different fractions is as;

n-hexane > chloroform>aq. fraction>ethyl acetate>methanol

Method is based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of green phosphate/Mo(VI) compounds which absorbed at 695 nm. Antioxidant such as ascorbic acid, some phenolics, tochopherols and carotenoids can be detected by this method. Results are shown in the following Table-2.

It showed that *n*-hexane fraction had the highest phenolic contents while methanolic extract had lowest phenolic contents relative to butylated hydroxy-toluene, a reference standard having total antioxidant activity 0.928 ± 0.09. The powerful antioxidants are phenolic compounds having hydroxyl group in their structures. They can be detected by TPC assay. Results are illustrated in Table-2. It was inferred that *n*-hexane fraction had GAE mg/g of sample. Total phenolics in different fractions were found in the following order;

n-Hexane>ethyl acetate>chloroform>methanol>aq. fraction

TABLE-2
ANTIOXIDANT EVALUATIONS OF DIFFERENT FRACTIONS OF *Argyrolobium roseum*

	n-Hexane	Chloroform	Ethyl acetate	Methanol	Aqueous fraction	BHT
			$ABTS^+$ Assay			
TEAC value (mM) of Trolox equivalent	88.58	127.9	152.5	130.37	147.6	–
			DPPH radical scavenging activity (IC_{50}; ?g/mL) ± S.E.M)			
IC_{50} µg/mL	129.93 ± 0.12	49.01 ± 0.34	24.33 ± 0.14	–	–	12.54 ± 0.89
			Ferric reducing antioxidant power			
FRAP value of TE (g/mL)	15.09	42.6	60.36	44.89	70.48	–
			Total antioxidant activity ± S.E.M			
	0.784 ± 0.001	0.669 ± 0.201	0.258 ± 0.008	0.206 ± 0.003	0.276 ± 0.005	–
			Total phenolic contents			
GAE (mg/g) of sample	792.5	662.5	705.8	622.11	247.5	–
			Total flavonoid contents			
TFC (mg/g QE)	750.09	470.09	931.91	633.73	378.27	–
			Metal chelating activity			
Bound iron (%)	61.85	52.14	24.74	52.48	21.11	–

S.E.M. = Standard mean error of three assays, BHT = Standard antioxidant.

The principle for the determination of total flavonoids is that aluminum chloride forms acid stable complexes with the C-4 keto group and either the C-4 or C-5 hydoxyl group of flavones or flavonoles. In addition, aluminum chloride forms acid labile complexes with the ortho-dihydroxyl group in the A- or B-ring of flavonoids which absorbs maximally at 400 nm. Results are shown in Table-2. Order of reactivity of fractions is:

Ethyl acetate > *n*-hexane > methanol > chloroform > aq.fr.

Iron is known to generate free radicals through the Fenton and Haber-Weiss reaction. Metal ion chelating activity of an antioxidant molecule prevents oxy-radical generation as a result oxidative damage also reduces the concentration of the catalyzing transition metal. Results of this assay were expressed in percentage of bound iron. Iron(II) formed a coloured complex with ferrozine which can be determined at 562 nm. The results are presented in Table-2. *n*-Hexane fraction had shown a maximum value of percentage bound iron.

n-Hexane>methanol>chloroform > ethyl acetate > aq. extract

It was concluded that ethyl acetate soluble fraction, methanolic and aqueous extracts are rich in terpenes, phenols, flavonoids, tannins, saponins and cardiac glycosides. Ethyl acetate soluble fraction showed good activity due to the presence of such bioactive compounds whereas methanol soluble fraction showed moderate to good activity so these fractions are valuable sources of bioactive compounds as well as good antioxidants. Alkaloids are also present in appreciable amounts. Due to the presence of antioxidant properties in its constituents this plant may play an important role in disease prevention and control.

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