Antimicrobial and Antioxidant Potential of *Astragalus psilocentros*

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Astragalus psilocentros Fisch. is a medicinal plant belongs to family Papilionaceae and is used to cure various diseases such as cancer, diabetes, liver diseases, hypertension, cardiac ischemia, heart failure, nephritis etc. The aim of this study was to identify the antimicrobial activity for controlling pathogens and antioxidant efficacy. The methanolic extract of *A. psilocentros* was dissolved in water and partitioned with hexane, chloroform, ethyl acetate and *n*-butanol successively. Phytochemical screening revealed the presence of alkaloids, terpenoids, tannins, phenolics, sugars, saponins and flavonoids. Antimicrobial activity was carried out against four bacterial (*Bacillus subtilis*, *Pasturella multocida*, *Escherichia coli*, *Staphylococcus aureus*) and two fungal (*Aspergillus niger* and *Alternaria fusarium*) strains. The chloroform, ethyl acetate and methanol extracts were effective against these tested microbes. The antioxidant potential of all these fractions and remaining aqueous fraction was evaluated by seven methods i.e. DPPH free radical scavenging activity, total antioxidant activity, FRAP assay, TPC assay, inhibition of lipid peroxidation, ABTS assay and super anion radical scavenging activity. All the fractions exhibited significant antioxidant potential. The results revealed that ethyl acetate showed the highest % inhibition by DPPH radical (183.92 ± 0.17), highest FRAP value of (56.86 ± 0.39), highest TPC value (53.69 ± 0.68) and highest inhibition by lipid peroxidation value (44.65 ± 0.44). The chloroform soluble fraction showed the highest TAA of (0.7005 ± 0.05). The GC-MS analysis of hexane soluble part of ethyl acetate fraction identified few potential markers responsible for the activity e.g., 1,3-bis-(trimethylsiloxy) benzene, diethyl benzoylmethylphosphonate, nonadecyl alcohol and 1,2-benzene dicarboxylic acid.

Key Words: Phytochemical screening, Antimicrobial activity, Antioxidant potential, GC-MS analysis, 1,3-Bis-(trimethylsiloxy)benzene, Diethyl benzoylmethylphosphonate, Nonadecyl alcohol, 1,2-Benzene dicarboxylic acid.

INTRODUCTION

It is evident from several studies that medicinal and aromatic plants are major source of nutrient and non nutrient molecules, which exhibit antioxidant and antimicrobial properties have ability to protect the human body against both cellular oxidation reactions and pathogens^{1,2}. Our group is working to characterize medicinal plants for their antioxidant and antimicrobial potential as an initial study of extracting pure natural metabolites responsible for that particular activity³. Plant produces antioxidative compounds that include free radicals such as superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) and non free-radical species such as H_2O_2 or singlet oxygen (1O_2) to counteract reactive oxygen species (ROS) in order to survive⁴. Phenolics have been suggested to play a contraceptive role in the development of cancer, heart disease⁵ and help in reducing cellular injury and aging process⁶. Lipid peroxidation of food due to reactive oxygen species responsible for formation of potentially toxic compounds that

are responsible for the rancid odours and flavours during processing and storage, decreasing the nutritional quality and effect food safety. Thus the shelf life of foods is enhanced by addition of antioxidants in them^{7,8}. The antioxidant potential can be evaluated by directly determining the strength of reactive oxygen species or by determining level of antioxidants present against reactive oxygen species. Different antioxidant assay are performed under different reaction conditions e.g. acidic, basic, neutral or buffer stabilized, that is because of stability of radical cations under these conditions.

Astragalus psilocentros Fisch. belongs to family papilionaceae and is widely distributed in Pakistan as well in Jammu and Kashmir, India⁹. *Astragalus* is regarded as a potent tonic for increasing energy levels and stimulating the immune system. It has also been employed effectively as a diuretic, a vasodilator and as a treatment for respiratory infections¹⁰. Fresh leaves of plants from this family commonly used by natives in stomach ulcers and act as an antibacterial, increase number of white blood cell, strengthens the body's resistance, reduce

debilitating conditions, helps to improve digestion and energies the human body¹¹.

To the best of our knowledge no studies on antioxidant and antimicrobial properties have been reported yet on *Astragalus psilocentros*. Thus, it is important to report *in vitro* antioxidant potential of aqueous and organic fractions of this species by using the ferric reducing antioxidant power, superoxide anion radical scavenging activity, total antioxidant activity by using different assays. These antioxidant assays can give an idea about the chemistry of the plant. For the antimicrobial study various bacterial and fungal pathogens were used which include *Bacillus subtilis*, *Pasturella multocida*, *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger* and *Alternaria fusarium*.

EXPERIMENTAL

The plant *Astragalus psilocentros* was collected from Kotly in October 2010 and identified by Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore and voucher specimen was deposited (Voucher No GC.Bot.Herb.987).

Extraction and fractionation of plant: The shade-dried ground whole plant (5 kg) was exhaustively extracted with methanol (12 L $\times$ 4) at room temperature. The extract was evaporated to yield the residue methanolic extract (815 g), which was dissolved in distilled water (1.5 L) and partitioned with *n*-hexane (1 L $\times$ 4), chloroform (1 L $\times$ 4), ethyl acetate (1 L $\times$ 4) and *n*-butanol (1 L $\times$ 4), respectively. These organic fractions and remaining water fraction was concentrated separately on rotary evaporator.

DPPH[•] (1,1-diphenyl-2-picrylhydrazyl 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).

Phytochemical analysis of the plant extract: Qualitative tests for indicating presence of phytochemical components like tannins, saponins, flavonoids, sugars, terpenoids, alkaloids and phenols in *Astragalus psilocentros* were screened using the methods reported by Sofowora, Trease and Evans^{12,13}. The intensity of colour produced was used for estimating particular secondary metabolite in the plant.

Test for terpenoids (Salkowski test): 2 mL of chloroform was added to 0.5 g of each extract followed by 3 mL of conc. sulphuric acid that was added carefully to form a layer. Presence of terpenoids were indicated by preparation of a reddish brown layer at the interface of the organic and aqueous layer.

Test for flavonoids: We used two methods for flavonoids identification. In first test 5 mL of dilute ammonia solution (10 %) was added to a portion of aqueous filtrate of plant extracts. 1 mL concentrated sulphuric acid was added afterward. A yellow colouration that disappears on standing indicates the presence of flavonoids.

In another method few drops of 1 % solution of aluminium chloride solution were added to another portion of plant

extracts. A persistent yellow colouration indicates the presence of flavonoids.

Test for saponins: 0.5 g of plant extracts were added to 5 mL distilled water in a test tube. Stable persistent froth that is observed after vigorous shaking mixed with 3 drops of olive oil forms an emulsion indicates presence of saponins.

Test for tannins: For indicating presence of tannins in plant extracts, about 0.5 g of the extracts were boiled in 10 mL of water and then filtered. If observed brownish-green or a blue-black colouration after adding few drops of 0.1 % ferric chloride to this filtrate indicates presence of tannins in the extracts.

Test for alkaloids: Alkaloidal portion first extracted by diluting 0.5 g of plant extracts to 10 mL with 1 % aqueous hydrochloric acid solution, boiled and filtered. To 5 mL of the filtrate added 2 mL of dilute ammonia solution and alkaloidal portion was extracted by 5 mL chloroform, separated and divided into two portions. Mayer's reagent was added to one portion and Dragendorff's reagent to the other. The formation of a cream (with Mayer's reagent) or reddish brown precipitate (with Dragendorff's reagent) indicated presence of alkaloids.

Test for phenolics: Neutral solution of ferric chloride was added to ca. 0.5 g of each extract. The bluish green colour indicated the presence of phenolics.

Test for sugars: 1 mL of water and 5-8 drops of Fehling's solution was added to 0.5 g of each plant extract. It is heated and observed for brick red precipitate, indicating presence of sugars.

Antioxidant activity

Sample preparation for antioxidant assays: The sample solutions of different extracts were prepared by dissolving 0.05 g of the extract in 10 mL of the respective solvents (5000 µg/mL).

Total antioxidant activity by phosphomolybdenum method: The total antioxidant activities of various fractions of plant were evaluated by phosphomolybdenum complex formation method¹⁴. In brief, 500 µL of volatile fraction was mixed with 4 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in sample vials. The blank solution contained 4 mL of reagent solution. The vials were capped and incubated in water bath at 95 °C for 1.5 h. After the samples had been cooled to room temperature, the absorbance of mixture was measured at 695 nm against blank. The antioxidant activity was expressed relative to that of butylated hydroxytoluene. All determinations were assayed in triplicate and mean values were calculated.

Ferric thiocyanate assay: The antioxidant activities of various fractions of plant on inhibition of linoleic acid peroxidation were assayed by thiocyanate method¹⁵. The 0.1 mL of each sample solution was mixed with 2.5 mL of linoleic acid emulsion (0.02 M, pH 7.0) and 2.0 mL of phosphate buffer (0.02 M, pH 7.0). The linoleic emulsion was prepared by mixing 0.28 g of linoleic acid, 0.28 g of Tween-20 as emulsifier and 50.0 mL of phosphate buffer. The reaction mixture was incubated for 5 days at 40 °C. The mixture without extract was used as control. The 0.1 mL of the mixture was taken and mixed with 5 mL of 75 % ethanol, 0.1 mL solution of 30 % ammonium thiocyanate and 0.1 mL

solution of 20 mM ferrous chloride in 3.5 % HCl and allowed to stand at room temperature. Precisely, 3 min after addition of ferrous chloride to the reaction mixture, absorbance was recorded at 500 nm. The antioxidant activity was expressed as percentage inhibition of peroxidation (IP %):

$$\text{IP \%} = \{(1 - \text{abs. of sample}) / (\text{abs. of control})\} \times 100$$

The antioxidant activity of butylated hydroxytoluene was assayed for comparison as reference standard.

ABTS⁺ Decolorization Assay: Total antioxidant activity in terms of Trolox equivalent antioxidant activity (TEAC) was measured using an improved ABTS method as described by Re *et al.*¹⁶. The ABTS radical cation (ABTS⁺) solution was prepared through the reaction of 7 mM ABTS and 2.45 mM potassium phosphate. The reaction mixture was incubated at room temperature in the dark for 12-16 h. The ABTS⁺ solution was diluted with phosphate buffer saline (pH 7.4) to obtain an absorbance of 0.700 ± 0.02 at 734 nm and equilibrated at 30 °C. After addition of 10 µL of sample or standard solution in 3 mL of ABTS⁺ solution ($A_{734 \text{ nm}} = 0.700 \pm 0.02$) to 100 µL of diluted sample solutions, the absorbance reading was taken at 30 °C exactly after 1 min initial mixing up to 6 min. Appropriate solvent blanks were run in each assay. The percentage inhibition of absorbance at 734 nm was calculated using the following formula:

$$\% \text{ Inhibition} = \{(1 - A_f) / A_o\} \times 100$$

where A_o and A_f are the absorbance's of ABTS⁺ solution at 734 nm before and after the addition of plant extract or standard solution respectively. The percentage inhibition was plotted as a function of concentration of antioxidants and Trolox for the standard reference data.

Diphenyl-1-picrylhydrazyl radical scavenging capacity assay: The DPPH radical scavenging activities of various fractions of plant were examined by comparison with that of known antioxidant *i.e.* butylated hydroxytoluene following the method of Lee and Shibamoto¹⁷. Briefly, various amounts of the samples (1000, 500, 250, 125 mg/mL) were mixed with 3 mL of methanolic solution of DPPH (0.1 mM). The mixture was shaken vigorously and allowed to stand at room temperature for 1 h. Then, absorbance was measured at 517 nm against methanol as a blank in the spectrophotometer. Lower absorbance of the spectrophotometer indicated higher free radical scavenging activity. The readings are noted at 517 nm over a time period till IC_{50} was obtained. Upon reduction, the colour of the solutions is faded.

The per cent of DPPH decolouration of the samples was calculated according to the formula:

$$\text{Antiradical activity} = \{(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}\} \times 100$$

Each sample was assayed in triplicate and mean values were calculated.

Total phenolic content assay (TPC): The concentrations of total phenolic contents were calculated from the procedure reported by Singleton and Rossi¹⁸. Briefly, 40 µL aliquots from each of the replicates were mixed with 3.16 mL of distilled H₂O and 200 µL of 0.2 N Follin-Ciocalteu's reagent. After 8 min, 600 µL of saturated sodium carbonate (75 g/L) was added. The absorbance of the resulting blue solution was measured at 765 nm after incubation at 40 °C for 0.5 h with intermittent shaking. Quantitative measurements were performed based on a six point standard calibration curve of 20, 100, 200, 300,

400, 500 mg/L of gallic acid in 80 % methanol. The total phenolic contents were expressed as gallic acid equivalents in milligrams per gram dry plant material.

$$\text{TPC} = 0.118x + 0.0824 \text{ mg/L (as Gallic acid equivalent)}$$

Ferric reducing antioxidant power assay: The ferric reducing antioxidant power assay was done using the method of Benzie and Strain¹⁹. The stock solutions included 300 mM acetate buffer (3.1 g CH₃COONa.3H₂O and 16 mL CH₃COOH, pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl and 20 mM FeCl₃.6H₂O solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL solution of TPTZ and 2.5 mL solution of FeCl₃.6H₂O and warmed at 37 °C before using. The solutions of plant samples and that of trolox were prepared in methanol (250 µg/mL). 10 mL each of sample and butylated hydroxytoluene solutions were taken in separate test tubes and 2990 mL of ferric reducing antioxidant power solution was added in each to make total volume up to 3 mL. The plant samples were allowed to react with ferric reducing antioxidant power solution in the dark for 0.5 h. Readings of the coloured product [ferrous tripyridyl-triazine complex] were then taken at 595 nm. The ferric reducing antioxidant power values were determined as micromoles of Trolox equivalents per mL of sample by computing with standard calibration curve constructed for different concentrations of trolox. Results were expressed in Trolox equivalent (mg/mL).

Superoxide anion radical scavenging activity: Superoxide anion radical scavenging activity was determined according to the method of Nikishimi and Rao²⁰. The PMS-NADH system was used for the generation of superoxide radicals. The reaction mixture contained 100 µL extract, 200 µM NBT, 624 µM NADH and 80 µM phosphate buffer (pH 7.4). After 2 min of incubation; absorbance was measured using UV/VIS spectrophotometer at 560 nm. The percentage scavenging is calculated by the formula:

$$\text{Scavenging (\%)} = [(1 - A_s) / A_b] \times 100$$

where A_s and A_b are the absorbances of sample and blank solutions at 560 nm, respectively.

Determination of antimicrobial activity

Antimicrobial assay: The pure bacterial and fungal strains were obtained from the biological division of the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. The bacterial culture used were *Bacillus subtilis*, *Pasturella multocida*, *Escherichia coli*, *Staphylococcus aureus* and the fungal strains were *Aspergillus niger* and *Alternaria fusarium*. Bacterial cultures were incubated overnight at 37 °C in nutrient agar and were used for microbial activity determination by disk diffusion method. Fungal strains were incubated over two nights at 30 °C in potato dextrose agar and were used for antifungal activity determination by disk diffusion method.

Disc diffusion method: The antimicrobial activity of the plant extracts were determined by the disc diffusion method²¹. The disc (6 mm in diameter) were impregnated with 100 µL dilution, where each mL contains 10 mg/mL of the plant extract (100 µg/disc) placed on the inoculated agar. Chloramphenicol (50 µg/disc) was used as the positive control. Disc without sample was used as a negative control. The growth was indicated by the presence of a white pellet on the well bottom.

All the measurements were done in triplicate and statistical analysis was performed by Microsoft excel 2003. Results are presented as average $\pm$ SEM.

GC-MS analysis: A Shimadzu 2010 (Shimadzu, Japan) gas chromatograph equipped with a split-splitless auto-injector model AOCi, an auto sampler model AOC-20s and a MS-QP 2010 (Shimadzu, Japan) series mass selective detector was used for the analysis of the essential oil studied. A fused silica capillary column (J & W DB 5MS®), 5 % phenyl polysiloxane as non-polar stationary phase (30 m $\times$ 0.25 mm i.d.) and 0.25 μ m film thickness, supplied by Agilent (Palo Alto, CA, USA) was used for the GC separation, with helium as carrier gas at a constant flow at 1.27 mL/min. The temperature program used was as follows: initial temperature 40 °C held for 5 min, then at the rate of 10 °C/min to 180 °C and 3 °C/min to 240 °C and then maintaining this temperature for 5 min. The temperature of the injection port was 200 °C and a 1 μ L volume was injected in split mode with 90 % split ratio. Mass selective detector was operated in electron impact (EI) ionization mode with an ionizing energy of 70 eV, scanning from m/z 40 to 950 at 0.5 s per scan. The ion source temperature was 250 °C and the MS transfer line temperature was 205 °C. The electron multiplier voltage (EM voltage) was maintained at 1000 V and the solvent delay of 3 min was employed^{22,23}. All the compounds were identified by comparison of their GC retention data and mass spectra with those of pure and authentic samples and also by comparing fragmentation patterns of mass spectra with those stored in the spectrometer data base (NIST 147) and bibliography^{24,25}.

RESULTS AND DISCUSSION

The phytochemical analysis conducted on *Astragalus psilocentros* extracts revealed the presence of tannins, flavonoids, sugars, alkaloids, terpenoids, phenolics saponins in methanolic, *n*-hexane, chloroform, ethyl acetate, butanol extracts.

The amount of common bioactive components like terpenoids, alkaloids, phenolics, flavonoids and saponins was concentrated in medium polar and polar fractions (*i.e.* chloroform, ethyl acetate and butanol) as expected, while the quantity of sugars was good in aqueous fraction. The results were tabulated in Table-1.

Antioxidant potentials of the plant extracts were summarized in Table-2. Antioxidants exert their mode of action by suppressing the formation of reactive oxygen species either by inhibition of enzymes or by chelating trace elements. Different antioxidant activities were performed in different reaction conditions *i.e.* acidic, basic or neutral pH and maintained with

help of buffer solutions. The reaction conditions govern the certain type or class of compounds to act as antioxidant. For example DPPH is widely used to evaluate the free radical scavenging effect of natural antioxidant. DPPH is a stable free radical at room temperature and neutral pH, which produces a violet solution (at 517 nm) in ethanol. The violet coloured DPPH radical after accepting an electron or hydrogen atom from the antioxidant compounds is converted into a colourless or somewhat yellow diamagnetic DPPH molecule resulting in decreased absorbance at this particular wavelength (517 nm). The bleaching of DPPH molecules can be correlated with the number of available hydroxyl groups. It may suggests that the good activity of the extract may be probably due to abundantly available flavonoids or phenolic compound. It was found that ethyl acetate fraction of this plant had the highest radical-scavenging activity of (183.92 ± 0.17) followed by the order of chloroform fraction (238.54 ± 0.29), butanol fraction (272.72 ± 0.39), methanol fraction (312.66 ± 0.14), *n*-hexane fraction (672.70 ± 0.84) and aqueous fraction (710.89 ± 0.73).

TABLE-1
RESULTS OF PHYTOCHEMICAL SCREENING

Extracts	Terp	Alk	Tan	Phen	Sug	Sap	Flav
Methanol	+++	+++	++	+++	+++	+++	+++
N-hexane	++	+	+	-	-	-	-
Chloroform	++	++	+	++	+	++	+++
Ethyl acetate	++	+++	++	+++	++	++	+++
Butanol	+	+	-	++	++	+++	++
Aqueous	-	+	-	+	+++	+	-

Abbreviations: Terp = Terpenoids, Alk = Alkaloids, Tan = Tannins, Phen. = Phenolics, Sug = Sugars, Sap = Saponins, Flav = Flavonoids
Key: +++ Strong; ++ medium; +poor Presence; -: absence, repeated the experiments three times for each replicates, Classification was based on observation of colour intensity and amount of precipitate

Total antioxidant capacity of the fractions was measured spectrophotometrically by phosphomolybdenum method, which is based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of green phosphate/Mo(V) compounds with a maximum absorption at 695 nm. The phosphomolybdenum method usually detects antioxidants such as ascorbic acid, some phenolics, tocopherols and carotenoids. From results, it has been observed that chloroform soluble fraction has highest total antioxidant activity (0.7005 ± 0.05) followed by, ethyl acetate fraction (0.603 ± 0.007), *n*-butanol fraction (0.528 ± 0.008) *n*-hexane fraction (0.519 ± 0.04), methanol fraction (0.3475 ± 0.01) and aqueous fraction (0.2655 ± 0.003) relative to butylated

TABLE-2
ANTIOXIDANT POTENTIAL OF DIFFERENT FRACTIONS OF *Astragalus psilocentros*

S. No.	Sample	IC ₅₀ of DPPH assay (g/mL) $\pm$ S.E.M)	Total antioxidant Activity $\pm$ S.E.M	FRAP value TE (g/mL) $\pm$ S.E.M	Total phenolics (GAE mg/g of sample) $\pm$ S.E.M	Inhibition of lipid Peroxid-ation (%) $\pm$ S.E.M	ABTS ⁺ Assay	Superanion Radical Scavenging Activity
1	Methanol	312.66 $\pm$ 0.14	0.519 $\pm$ 0.01	43.76 $\pm$ 0.68	40.82 $\pm$ 0.15	30.52 $\pm$ 0.81	9.41 $\pm$ 0.63	41.58 $\pm$ 1.26
2	<i>n</i> -hexane	672.70 $\pm$ 0.84	0.3475 $\pm$ 0.01	19.16 $\pm$ 0.92	16.76 $\pm$ 0.81	19.94 $\pm$ 0.36	4.56 $\pm$ 0.70	12.15 $\pm$ 1.09
3	Chloroform	238.54 $\pm$ 0.29	0.7005 $\pm$ 0.05	45.97 $\pm$ 0.54	47.32 $\pm$ 0.29	40.38 $\pm$ 0.12	15.29 $\pm$ 0.71	55.84 $\pm$ 1.53
4	Ethylacetate	183.92 $\pm$ 0.17	0.603 $\pm$ 0.007	56.89 $\pm$ 0.39	53.69 $\pm$ 0.68	44.65 $\pm$ 0.44	21.83 $\pm$ 1.15	48.71 $\pm$ 1.21
5	Butanol	272.72 $\pm$ 0.39	0.528 $\pm$ 0.008	36.96 $\pm$ 0.52	39.73 $\pm$ 0.73	36.49 $\pm$ 0.87	9.98 $\pm$ 1.05	31.92 $\pm$ 1.03
6	Aqueous	710.89 $\pm$ 0.73	0.265 $\pm$ 0.003	24.42 $\pm$ 0.18	22.43 $\pm$ 0.18	12.78 $\pm$ 0.93	6.42 $\pm$ 0.99	24.79 $\pm$ 1.07

hydroxytoluene, a reference standard having total antioxidant activity 0.928 ± 0.09 . It showed that chloroform fraction has highest phenolic content while aqueous fraction has lowest phenolic content.

The ABTS assay is based on the reaction of $K_2S_2O_8$ forms a greenish blue radical cation. Standard and sample antioxidants that are able to transfer an electron to ABTS radical cation scavenge the colour of the solution proportionate to their amount. The extent of scavenging depends upon the concentration of AO and the time duration for the reaction. This method is used to measure the antioxidant capacity of both lipophilic and hydrophilic standard antioxidants and plasma samples by measuring the extent of scavenging of ABTS radical cation on addition of the sample solution at 734 nm for a time period of 8 min for standard antioxidants and plant extracts. The extent of scavenging ABTS radical anion by the samples is compared with the standard Trolox, a water soluble analogue of vitamin E. The % inhibition values of extract samples with standard Trolox curve are calculated by plotting a bar graph. It was found that the highest antioxidant capacity is present in the ethyl acetate fraction (21.83 ± 1.15) of the plant followed by, chloroform (15.29 ± 0.71), butanol (9.98 ± 1.05), methanol (9.41 ± 0.63), aqueous (6.42 ± 0.99) and *n*-hexane (4.56 ± 0.70) fraction having the least activity.

Phenolic compounds contain hydroxyl groups in their structures so that they act as powerful antioxidants. The polyphenolic compounds can also be detected by TPC assay. The high phenolic contents were found in the following order: ethyl acetate (53.69 ± 0.68), chloroform (47.32 ± 0.29), methanol (40.82 ± 0.15), butanol (39.73 ± 0.73), aqueous (22.43 ± 0.19) and *n*-hexane fraction (16.76 ± 0.81).

The ferric reducing antioxidant power (FRAP) assay involves a single electron reduction of the Fe (TPTZ) $_2^{3+}$ complex (pale yellow) to the Fe(TPTZ) $_2^{+2}$ complex (blue) by sample antioxidant at acidic pH. This reduction is monitored spectrophotometrically at 593 nm. Appearance of intense blue colouration indicates reducing components in the sample. The absorbance readings, therefore, were taken at a 6 min interval after addition of sample to TPTZ reagent allowing the reaction to reach a steady state. The ferric reducing antioxidant power values of the fractions were measured by comparison with a calibration curve obtained using iron (+3) sulfate as the standard reductant. The FRAP values was highest in the ethyl acetate fraction (56.89 ± 0.39) while the other fractions had the following order chloroform (45.97 ± 0.54), methanol (43.76 ± 0.68), butanol (39.69 ± 0.52), aqueous (24.42 ± 0.18) and *n*-hexane fraction (19.16 ± 0.92) had the least activity.

The superoxide anion radical scavenging involves a superoxide radical that acts as a free radical. In cellular oxidation the superoxide radical is formed first and it produces other kinds of cell-damaging free radicals and oxidizing agents. Basically superoxide radical participates in the initiation of lipid peroxidation. The percentage of scavenging activity was found in the following order: chloroform (55.84 ± 1.53), ethyl acetate (48.71 ± 1.21), methanol (41.58 ± 1.26), butanol (31.92 ± 1.03), aqueous (24.79 ± 1.07) and *n*-hexane (12.15 ± 1.09).

The ferric thiocyanate method is used to determine the ROO $^{\cdot}$ scavenging activity. The ROO $^{\cdot}$ formed due to oxidation of linoleic acid have ability to oxidize Fe $^{2+}$ to Fe $^{3+}$. The

peroxidation value is noted at the absorbance of 500 nm. In case of the sample solutions the antioxidants will try to lower the peroxidation value by slowing down or inhibition of oxidation of linoleic acid. Lipid peroxidation values are determined with the function of time. Trolox is used as a control. The ethyl acetate has the maximum activity of 44.65 ± 0.44 , chloroform 40.38 ± 0.12 , butanol 36.49 ± 0.87 , methanol 30.52 ± 0.81 , *n*-hexane 19.94 ± 0.36 and aqueous 12.78 ± 0.93 .

The antimicrobial activity of the herbal extracts is summarized in the Table-2. The results revealed that the extracts showed moderate and high as well as poor antimicrobial activity against all the tested microbial strains. Among the various solvent extracts studied at a concentration of 10 mg/mL showed that the chloroform extracts showed the highest degree of inhibition.

Bacillus subtilis exhibited the highest inhibitory activity for methanolic and chloroform fractions, while the *n*-hexane, ethyl acetate, butanol, aqueous extracts showed almost equal activity but less than methanol and chloroform fractions. Chloroform fraction had the largest zone of inhibition against *Pasturella multocida*. Butanol, aqueous and ethyl acetate exhibited almost same activity, methanol had slight susceptibility and *n*-hexane showed no activity for this microbe. Chloroform, ethyl acetate, butanol and aqueous fractions possess maximum antibacterial activity against *E. coli* while *n*-hexane and methanol fractions showed equal but less activity than other four fractions. *S. aureus* exhibited best inhibitory activity against methanolic fraction. It showed medium activity for chloroform and ethyl acetate, while it was inhibited by *n*-hexane, butanol and aqueous fractions of the plant. The fungi *Aspergillus niger* exhibited similar zone of inhibition for all the fractions. *Alternaria fusarium* exhibited best activity against methanolic fraction and had medium activity against ethyl acetate, chloroform, *n*-hexane fractions, while it showed resistance against butanol and aqueous extracts. There zone of inhibitions are listed in Table-3.

TABLE-3
ZONE OF INHIBITIONS (mm) FOR DIFFERENT EXTRACTS OF PLANTS AGAINST BACTERIAL AND FUNGAL SPECIES

Extracts	Zone of Inhibition (mm) against bacterial and fungal strains					
	B.S.	P.M.	E.C.	S.A.	A.N.	A.F.
Methanol	20	9	15	30	11	20
<i>n</i> -hexane	10	R	18	R	11	10
Chloroform	20	30	30	20	12	10
Ethyl acetate	10	16	30	20	10	12
Butanol	10	20	30	R	10	R
Aqueous	10	20	10	R	10	R
Chlormphincol (+ve std)	20	20	20	30	20	20

R = Resistant, B.S. = *Bacillus subtilis*, P.M. = *Pasturella multocida*, E.C. = *Escherichia coli*, S.A. = *Staphylococcus aureus*, A.N. = *Aspergillus niger*, A.F. = *Alternaria fusarium*

For all these analysis ethyl acetate fraction showed maximum activity. The hexane extracted part of ethyl acetate fraction was subjected to GC-MS analysis for evaluation of its volatile constituents. The complete chromatogram exhibit presence of at least ten distinct components but by using NIST dictionary we are able to identify just four out of them that are 1,3-bis-(trimethylsiloxy) benzene (r.t. 4.153, 25.17 %), diethyl

benzoylmethylphosphonate (r.t. 7.561, 1.17 %), Nonadecyl alcohol (r.t. 10.008, 10.19 %) and 1,2-benzenedicarboxylic acid (r.t. 13.997, 36.94 %).

All these components already identified from different plants²²⁻²⁵ however we are reporting them first time from this plant. One of the compounds 1,2-benzene dicarboxylic acid, is identified in the flower of *Spathodea campanulata* and from roots of *Plumbago zeylanica* L. by GC-MS analysis and reported to show moderate to good antibacterial activity against both gram positive and gram negative bacteria²⁶⁻²⁸. Six other components are also identified by GC-MS but their structures are not confirmed through our database. Isolation of secondary metabolites from these extracts using different chromatographic techniques is in progress, some of them may be an addition to scientific database.

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