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# *In vitro* assessment of the protection from oxidative stress by various fractions of *Artemisia incisa* Pamp.

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*Abstract*: The methanolic extract of *Artemisia incisa* Pamp. was dissolved in distilled water and successively partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol. The antioxidant potential of all these fractions and remaining aqueous fraction was evaluated by four methods, *i.e.*, the scavenging activity of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), the total antioxidant activity, the ferric reducing antioxidant power (FRAP) assay and the ferric thiocyanate assay. In addition, the total phenolics was determined. The obtained results revealed that among the studied fractions the ethyl acetate soluble fraction showed the most potent DPPH-radical scavenging activity with an $IC_{50}$ value of 5.30±0.71 μg $mL^{-1}$, which is even more effective than the standard antioxidant butylated hydroxytoluene (BHT) ($IC_{50}$ value of 12.10±0.29 μg $mL^{-1}$). The ethyl acetate fraction also showed the highest *FRAP* value (3677.13±0.60 μg TE $mL^{-1}$), inhibition of lipid peroxidation (60.93±1.15 % at 500 μg $mL^{-1}$) and total phenolic content (95.50±0.58 μg GAE $g^{-1}$) as compared to other fractions. However, the remaining aqueous fraction was found to possess the highest antioxidant activity of all the fractions.

*Keywords*: *Artemisia incisa* Pamp.; DPPH assay; total antioxidant activity; FRAP value; total phenolics; inhibition of lipid peroxidation.

## INTRODUCTION

Lipid peroxidation is one of the main reasons for the deterioration of food products during processing and storage. Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and *tert*-butylhydroquinone (TBHQ) are widely used as food additives to increase the shelf life, especially of lipids and lipid-containing products, by retarding the process of lipid peroxidation. However, BHT and BHA are known to have not only toxic and carcinogenic effects on humans, but also to have abnor-

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mal effects on enzyme systems. Therefore, the interest in natural antioxidants, especially of plant origin, has greatly increased in recent years.[1] Several reports indicate that the antioxidant potential of medicinal plants may be related to the concentration of their phenolic compounds, which include phenolic acids, flavonoids, anthocyanins and tannins.[2,3] These compounds are of great value in preventing the onset and/or progression of many human diseases, such as cancer, heart disease, hypertension and stroke.[4] The health promoting effect of antioxidants from plants is thought to arise from their protective effects by counteracting reactive oxygen species (ROS).[3]

*Artemisia* is a quite large genus within the family of Asteraceae (Compositae), with almost 200 individual species known, which are usually found in dry areas, invariably as small fragrant shrubs or herbs and most yield essential oils. Some of these oils have found applications in perfumery and medicine (for example, vermifuges, stimulants, *etc*.), whereas the leaves of some species are used as culinary herbs. The plants themselves are popular among gardeners as cultivated ornamentals. In Pakistan, this genus is represented by almost twenty five species.[5] The plants of this genus have been used for a wide range of specific ailments including coughs, colds, sore throat, heartburn, haemorrhoids, fever, malaria, asthma, diabetes mellitus, enhancing eyesight, enhancement of the immune system and cardiovascular health, as well as to decrease the risk of atherosclerosis, cancer, arthritis and gastrointestinal disorders. Phytochemical analyses of various species showed that they are rich sources of terpenoids (artemisinin), flavonoids including apigenin, cirsimaritin, and various novel compounds.[6–9] but to the best of our knowledge, there is little in the literature on the employment of *Artemisia incisa* Pamp. as a natural antioxidant. Therefore, the present study was undertaken to investigate the *in vitro* antioxidant potential of various fractions of this plant.

## EXPERIMENTAL

### *Plant material*

The plant *Artemisia incisa* Pamp. was collected from Kotly, Azad Kashmir in March 2009, and identified by Mr. Muhammad Ajaib (taxonomist), Department of Botany, Government College University, Lahore. A voucher specimen (G.C.Herb.Bot.858) is deposited in the Herbarium of the Botany Department of the same university.

### *Extraction and fractionation of antioxidants*

Shade-dried ground whole plant (6.8 kg) was exhaustively extracted with methanol (4×12 L) at room temperature. The solvent was removed in a rotary evaporator (Laborta 4000--efficent Heidolph) at 40 °C under vacuum to yield the residue (615 g), which was dissolved in distilled water (1.5 L) and successively partitioned with *n*-hexane (4×1 L), chloroform (4×1 L), ethyl acetate (4×1 L) and *n*-butanol (4×1 L) to obtain four organic fractions. The organic fractions as well as the remaining water fraction were all concentrated separately on a rotary evaporator (the *n*-hexane-partitioned fraction at 40 °C, the chloroform-partitioned fraction at 40 °C, the ethyl acetate-partitioned fraction at 43 °C, the *n*-butanol-partitioned fraction at 50

°C and the remaining water fraction at 60 °C under vacuum). The thus obtained residues for each fraction were used to evaluate their *in vitro* antioxidant potential.

*Chemicals and standards*

1,1-Diphenyl-2-picrylhydrazyl radical (DPPH·), 2,4,6-tripyridyl-*s*-triazine (TPTZ), trolox, gallic acid, Folin–Ciocalteu; BHT was obtained from the Sigma Chemical Company Ltd. (USA) and the organic solvents (*n*-hexane, chloroform, ethyl acetate and *n*-butanol), sulphuric acid, sodium phosphate, ammonium molybdate, ferric chloride and ferrous chloride from Merck (Pvt.) Ltd. (Germany).

*DPPH radical scavenging activity*

The DPPH radical scavenging activities of the various fractions of the plant were examined by comparison with that of a known antioxidant (BHT) using a reported method.[10] Briefly, various amounts of the samples (1000, 500, 250, 125, 60, 30, 15 and 8 μg $mL^{-1}$) were mixed with 3 mL of a methanolic solution of DPPH (0.1 mM). The mixture was shaken vigorously and allowed to stand at room temperature for one hour. Then the absorbance was measured at 517 nm against methanol as a blank in a spectrophotometer (CECIL Instruments CE 7200 Cambridge, England). A lower absorbance of the reaction mixture indicated a higher free radical scavenging activity.

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

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

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

*Total antioxidant activity by the phosphomolybdenum method*

The total antioxidant activities of the various fractions of the plant were evaluated by the phosphomolybdenum complex formation method.[11] Briefly, 500 μg $mL^{-1}$ of each sample 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 a water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against the blank. The antioxidant activity was expressed relative to that of BHT. All determinations were assayed in triplicate and mean values were calculated.

*Ferric reducing antioxidant power (FRAP) assay*

The FRAP assay was realised according to Benzie and Strain[12] with some modifications. The stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM hydrochloric acid and 20 mM ferric chloride hexahydrate solution. The fresh working solution (FRAP solution) was prepared by mixing 25 mL of the acetate buffer, 2.5 mL of the TPTZ solution and 2.5 mL of the ferric chloride hexahydrate solution and then warmed at 37 °C before use. Solutions of the plant samples and that of trolox were prepared in methanol (250 μg $mL^{-1}$). 10 μL of each of sample solution were taken in separate test tubes and 2990 μL of FRAP solution were added to each to make the total volume 3 mL. The plant samples were allowed to react with the FRAP solution in the dark for 30 min. The absorbance of the coloured product (the ferrous tripyridyltriazine complex) was measured at 593 nm. The *FRAP* values are expressed as μmol of trolox equivalents (*TE*) per mg of the sample using a standard curve constructed for different concentrations of trolox. The results are expressed as *TE* in μg $mg^{-1}$.

*Ferric thiocyanate (FTC) assay*

The antioxidant activities of the various fractions of plant on the inhibition of linoleic acid peroxidation were assayed by the thiocyanate method.[14] Samples (0.1 mL, 0.5 mg $mL^{-1}$) were 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 the control. The mixture (0.1 mL) was taken and mixed with 5.0 mL of 75 % ethanol, 0.1 mL of 30 % ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5 % hydrochloric acid and allowed to stand at room temperature. Precisely 3 minutes after addition of ferrous chloride to the reaction mixture, absorbance was recorded at 500 nm. The antioxidant activity is expressed as follows:

$$\text{Inhibition of lipid peroxidation (\%)} = 100\{1 - (A_{\text{sample}}) / (A_{\text{control}})\}$$

The antioxidant activity of BHT as a reference standard was assayed for comparison.

*Total phenolic content*

Total phenolics of the various fractions of plant were determined by a reported method.[14] A 0.1 mL aliquot of the sample solution (0.5 mg $mL^{-1}$) was combined with 2.8 mL of a 10 % sodium carbonate solution and 0.1 mL of 2 M Folin–Ciocalteu reagent. After 40 min, the absorbance at 725 nm was measured using a UV–Vis spectrophotometer. The total phenolics were determined as milligrams of gallic acid equivalents per gram of sample. These were determined with the help of standard calibration curve, which was constructed for different concentrations of gallic acid. The standard gallic acid curve was linear in the concentration range 50–400 μg $mL^{-1}$. The results are expressed in μg GAE $mL^{-1}$.

*Statistical analysis*

All the measurements were performed in triplicate and the statistical analysis was realised using Microsoft Excel 2003. The results are presented as mean ± *SEM*.

## RESULTS AND DISCUSSION

The scavenging of the stable DPPH radical model is a widely used method to evaluate antioxidant activities in a relatively short time. The addition of the extracts to a DPPH solution caused a rapid decrease in the optical density at 517 nm. The degree of decolouration is an indication of the scavenging capacity of the extracts. Free radicals cause autoxidation of unsaturated lipids in food.[15] The effect of an antioxidant on DPPH radical scavenging is thought to be due to its hydrogen donating ability or radical scavenging activity.[16] Antioxidants cease the free radical reaction by donating a hydrogen from the phenolic hydroxyl groups. Thus, the formation of a stable end-product does not permit further oxidation of the lipid.[17] The decrease in the absorbance of the stable radical DPPH, accompanied with colour changes from violet to yellow, was measured for three concentrations, and the results are given in Table I. According to the obtained results, the ethyl acetate soluble fraction showed the highest DPPH scavenging activity of 87.74±1.75 % at a concentration of 125 μg $mL^{-1}$, while the *n*-hexane soluble fraction revealed the lowest scavenging value of 68.10±1.70 % at a con-

centration of 1000 μg mL$^{-1}$. For each fraction, the $IC_{50}$ value was calculated and is presented in Table II. The $IC_{50}$ value is the concentration which causes a 50 % inhibition of the DPPH radicals. Different concentrations of the fractions were taken such that some concentrations had an inhibition above 50 % and some below 50 %. The lower the $IC_{50}$ value, the greater is the antioxidant activity. The $IC_{50}$ value of the ethyl acetate soluble fraction was very low (5.30±0.71 μg mL$^{-1}$) and hence it showed the highest antioxidant activity as compared to the other fractions. The $IC_{50}$ values for other fractions increased in order chloroform soluble fraction < *n*-butanol soluble fraction < remaining aqueous fraction < *n*-hexane soluble fraction (Table II). The total antioxidant capacity of all fractions was measured spectrophotometrically by the 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.[18] The aqueous fraction displayed the highest total antioxidant activity value (1.56-fold greater than BHT), followed by the slightly less potent ethyl acetate, chloroform and *n*-hexane soluble fractions (from 1.45 to 1.47-fold greater activity than BHT), while the *n*-butanol soluble fraction had the lowest total antioxidant activity (1.35-fold greater than BHT).

TABLE I. 1,1-Diphenyl-2-picryl hydrazyl radical (DPPH) radical scavenging activity of the various fractions of *Artemisia incisa* Pamp.

| Sr. No. | Sample | Concentration in assay, μg ml$^{-1}$ | Scavenging of DPPH, %±*SEM*[a] |
|---|---|---|---|
| 1 | *n*-Hexane soluble fraction | 1000 | 68.10±1.70[b] |
| | | 500 | 59.30±1.17[b] |
| | | 50 | 36.30±0.61[b] |
| 2 | Chloroform soluble fraction | 125 | 87.71±1.75 |
| | | 60 | 67.43±1.14 |
| | | 30 | 40.77±1.14[b] |
| 3 | Ethyl acetate soluble fraction | 125 | 87.74±1.13 |
| | | 60 | 75.90±1.73 |
| | | 30 | 58.30±1.76[b] |
| 4 | *n*-Butanol soluble fraction | 125 | 87.60±0.99 |
| | | 60 | 61.25±1.17[b] |
| | | 30 | 44.10±1.17[b] |
| 5 | Remaining aqueous fraction | 250 | 72.65±1.55[b] |
| | | 125 | 61.95±0.68[b] |
| | | 50 | 49.8±1.02[b] |
| 6 | BHT[c] | 60 | 91.17±1.20 |
| | | 30 | 72.14±1.14 |
| | | 15 | 65.86±2.32 |

[a]All results are presented as mean ± standard mean error of three assays; [b]$p < 0.05$ when compared with the negative control, *i.e.*, blank/solvent ($p < 0.05$ is taken as significant); [c]standard antioxidant. Note: $IC_{50}$ should be calculated by the linear regression method of plots of the percentage of antiradical activity against the concentration of the tested samples. Hence, the concentrations of the fractions were taken in such a way that some concentrations show % inhibition above 50 % and some below 50 %

TABLE II. $IC_{50}$, total phenolics, total antioxidant activity, *FRAP* values and lipid peroxidation inhibition values of different fractions of *Artemisia incisa* Pamp.; all results are presented as mean ± standard mean error of three assays

| Sr. No. | Sample | $IC_{50}$ μg mL$^{-1}$ | Total antioxidant activity | *FRAP* value μg TE mg$^{-1}$ | Inhibition of lipid peroxidation, %[a] | Total phenolics mg GAE g$^{-1}$ |
|---|---|---|---|---|---|---|
| 1 | *n*-Hexane soluble fraction | 379.10±9.48 | 1.45±0.01[b] | 179.80±0.46[b] | 47.06±0.59[b] | 51.20±0.46[b] |
| 2 | Chloroform Soluble fraction | 37.90±13.59 | 1.46±0.01[b] | 1571.30±0.49[b] | 51.37±0.56[b] | 54.50±0.59[b] |
| 3 | Ethyl acetate soluble fraction | 5.30±0.71 | 1.47±0.01[b] | 3677.13±0.60[b] | 60.93±1.15 | 95.50±0.58[b] |
| 4 | *n*-Butanol soluble fraction | 39.80±12.65 | 1.35±0.02[b] | 2136.40±0.34[b] | 49.60±0.99[b] | 59.75±1.17[b] |
| 5 | Remaining aqueous fraction | 44.40±6.60 | 1.56±0.01[b] | 620.50±0.52[b] | 54.20±0.88[b] | 69.25±0.59[b] |
| 6 | BHT[c] | 12.10±0.29 | 1.2186±0.01 | – | 62.91± 1.16 | – |
| Blank[d] | | – | – | 2.30 | – | 1.15[b] |

[a]Tested concentration at 500 μg mL$^{-1}$; [b]$p < 0.05$ when compared with the negative control, *i.e.*, blank/solvent ($p < 0.05$ is taken as significant); [c]expressed relative to BHT; [d]expressed relative to blank

The FRAP assay is a simple and inexpensive procedure that measures the total antioxidant levels in a sample. It utilizes the reducing potential of the antioxidants to react with the ferric tripyridyltriazine (Fe(III)-TPTZ) complex and produce the coloured ferrous tripyridyltriazine (Fe(II)-TPTZ) complex.[19,20] Among all the *Artemisia incisa* fractions, the ethyl acetate soluble fraction showed the highest *FRAP* value (3677.13±0.60 μg TE mg$^{-1}$) and then the other fractions which followed the order *n*-butanol soluble fraction (2136.40±0.34 μg TE mg$^{-1}$) > > chloroform soluble fraction (1571.30±0.49 μg TE mg$^{-1}$) > aqueous fraction (620.50±0.52 μg TE mg$^{-1}$) while the *n*–hexane soluble fraction revealed the lowest *FRAP* value (179.80±0.46 μg TE mg$^{-1}$).

The ferric thiocyanate method measures the amount of peroxide generated during the initial stage of linoleic acid emulsion during incubation. Here, the peroxide reacted with ferrous chloride to form ferric chloride, which in turn reacted with ammonium thiocyanate to produce ferric thiocyanate, a reddish pigment. The low absorbance values measured *via* the FTC method indicated high antioxidant activity.[21] The obtained results revealed that the ethyl acetate soluble fraction showed the maximal inhibition of lipid peroxidation (60.93±1.15 %) and the *n*-hexane soluble fraction the minimal (47.06±0.59 %) at a concentration of

500 μg $mL^{-1}$; the following order for the different fractions of this plant was observed: ethyl acetate soluble fraction > remaining aqueous fraction > chloroform soluble fraction > *n*-butanol soluble fraction > *n*-hexane soluble fraction. The inhibition of lipid peroxidation by BHT (standard) was 62.91±0.60 % at 500 μg $mL^{-1}$ (Table II). Phenolic compounds are very important plant constituents because of their antioxidant activities.[22] It has been revealed that various phenolic antioxidants, such as flavonoids, tannins, coumarins and procyanidins scavenge radicals dose-dependently. Thus, they are viewed as promising therapeutic drugs for free radical pathologies.[23] According to the present results, the ethyl acetate soluble fraction possessed the highest amount of total phenolics, having a value of 95.5±0.58 mg GAE $g^{-1}$, followed by the remaining aqueous fraction (69.25±0.59 mg GAE $g^{-1}$), the *n*-butanol soluble fraction (59.75±1.17 mg GAE $g^{-1}$), the chloroform soluble fraction (54.5±0.59 mg GAE $g^{-1}$) while the *n*-hexane soluble fraction exhibited the lowest total phenolic content (51.20±0.46 mg GAE $g^{-1}$) (Table II).

## CONCLUSIONS

The results showed that among the studied fractions, the ethyl acetate soluble fraction showed the most potent DPPH-radical scavenging activity with an $IC_{50}$ value of 5.30±0.71 μg $mL^{-1}$ and it was even more effective than the standard antioxidant BHT ($IC_{50}$ = 12.10±0.29 μg $mL^{-1}$). The ethyl acetate fraction also showed the highest FRAP value (3677.13±0.60 μg TE $mg^{-1}$), inhibition of lipid peroxidation (60.93±1.15 % at 500 μg $mL^{-1}$) and total phenolic content (95.50±0.58 μg GAE $g^{-1}$) when compared to the other fractions. However, the remaining aqueous fraction was found to possess the highest total antioxidant activity among all the fractions. Hence, it was concluded that the ethyl acetate soluble fraction of this plant is rich in strong antioxidants but it is also promising to be able to conclude that, except for the *n*-hexane soluble fraction, all the other fractions of this plant are potentially valuable sources of natural antioxidants and might show effectiveness against diseases caused by the over-production of radicals. Thus, further phytochemical studies are required to isolate these potent natural antioxidants.

## ИЗВОД

### АНТИОКСИДАТИВНА АКТИВНОСТ ЕКСТРАКАТА БИЉКЕ *Artemisia incisa* Pamp.

AZIZ-UR-REHMAN[1], SEHRISH GULZAR[1], MUHAMMAD A. ABBASI[1], TAYYABA SHAHZADI[1], TAUHEEDA RIAZ[1], SABAHAT Z. SIDDIQUI[1] и MUHAMMAD AJAIB[2]

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Метанолни екстракт биљке *Artemisia incisa* Pamp. је, након упаравања растварача, растворен у дестилованој води и подвргнут подеоној екстракцији *n*-хексаном, хлороформом, етил-ацетатом и *n*-бутанолом. Антиоксидативна активност фракција је одређивана следећим

методама: а) реакцијом хватања 1,1-дифенил-2-пикрилхидразил радикала (DPPH), б) мерењем укупне антиоксидативне активности, в) тестирањем способности редукције фери јона (FRAP тест) и г) извођењем фери-тиоцијанатног теста. Такође је одређена укупна количина фенолних једињења. Резултати су показали да је етил-ацетатни екстракт показао највећу способност хватања DPPH радикала, његова $IC_{50}$ вредност је била 5,30±0,71 μg $mL^{-1}$ и био је ефикаснији од стандардног антиоксиданса BHT, чија је $IC_{50}$ вредност била 12,10±0,92 μg $mL^{-1}$. Етил-ацетатна фракција је имала и највећу способност редукције фери јона (FRAP вредност је била 3677,13±27,1 μg TE $mL^{-1}$), способност инхибиције липидне пероксидације (60,93±0,84 % при концентрацији 500 μg $mL^{-1}$), а и садржај фенолних једињења је био највећи (95,50±0,05 μg GAE $g^{-1}$). Водена фракција, заостала после екстракције органским растварачима, имала је највећу укупну антиоксидативну активност.

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