

Antioxidant, Antibacterial and Antiproliferative Activities of Areal Parts of *Swertia chirata* (Bush Ham) Plant Extracts Using *In Vitro* Models

SYED ALI RAZA NAQVI^{1,*}, QURAT-UL-AIN¹, ZULFIQAR ALI KHAN¹, ZAIB HUSSAIN²,
SOHAIL ANJUM SHAHZAD³, MUHAMMAD YAR⁴, ABDUL GHAFFAR¹, NASIR MAHMOOD⁵ and SAMINA KOUSAR²

¹Department of Chemistry, Government College University, Faisalabad-38000, Pakistan

²Institute of Chemistry, University of the Punjab, Lahore-54000, Pakistan

³Department of Chemistry, COMSATS Institute of Information Technology, Abbottabad-22060, Pakistan

⁴Interdisciplinary Research Center in Biomedical Materials, COMSATS Institute of Information Technology, Lahore-54000, Pakistan

⁵Department of Allied Health Sciences, University of Health Sciences, Lahore-54000, Pakistan

*Corresponding author: E-mail: a_naqvi313@yahoo.com

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Aerial parts of *Swertia chirata* was investigated for its medicinal values. Like many other plants and herbs, *Swertia chirata* has also been used for treating diseases particularly liver disorder. Anti-disease functions of most of the plants or herbs extracts have been associated with antioxidant and antibacterial potential. This study was performed to explore the antioxidant and antibacterial activity of the *Swertia chirata* (Bush ham). This was done by measuring possible constituents responsible for its antioxidant activity such as total phenolic and flavonoid contents. Antioxidant potential was measured using authentic *in vitro* assay models such as DPPH free radical scavenging assay and per cent inhibition of linoleic acid peroxide. The antibacterial and antiproliferative activity was measured against four different strains of gram positive and gram negative bacteria and MDBK cancer cell line, respectively. Promising concentration of total phenolics and flavonoids resulted encouraging DPPH free radical scavenging (84.32 ± 2.78 and 82.54 ± 3.12 %) and linoleic acid peroxide inhibition (94.04 ± 4.23 and 92.86 ± 4.78 %) and consequently credible antioxidant potential. Antibacterial and antiproliferative data of extracts indicated *Swertia chirata* might be a good member for fighting against infectious and chronic diseases.

Key Words: Antioxidants, *Swertia chirata*, HPLC, Antibacterial activity, Total phenolics, Free radical scavenging.

INTRODUCTION

The medicinal plants have a successful history to combat different chronic diseases. Plants contain a wide range of biological active compounds with great deal of interest in food, cosmetics and pharmaceutical industries. Due to no or lesser side effects of medicinal plant extracts, approximately 60-80 % of the world's population, especially in developing countries, still relies on folk medicines for the treatment of more than 87 % common and serious illnesses^{1,2}. Medicinal plants have a set of compounds that work together against the attack of disease causing factors. Most commonly these compounds behave directly or indirectly as antioxidants, antiinfection and anticancer agents.

Oxidative stress is considered to involve in key phenomena of living bodies. The stress is generated by different factors including reactive oxygen species (ROS). Reactive oxygen species and immune system both together attack to kill pathogens³. However, the common trend is that ROS, such as hydrogen peroxide, superoxide anion and hydroxyl radical that produce

in living system are responsible for damaging cell structure, lipids, DNA and proteins⁴. Reactive oxygen species work through free radical chain mechanism which increase the risk of more than 30 different disease processes, including cancer⁵, stroke, Alzheimer's disease⁶, heart diseases⁷, Parkinson's disease (PD)⁸, Schizophrenia⁹, arteriosclerosis¹⁰ etc. Naturally occurring antioxidants have strong and effective mechanism to scavenge these species.

Pakistan has great diversity in seasons in different areas and also has world's rich land of naturally occurring flora. The diversity in seasons, through out the year imparts very useful change in biological active constituents in plants. Most of the herbs indigenous to Pakistan are being used to manage oxidative stress and infectious diseases. Very promising ratio of medicinal and biological active constituents in medicinal plants play golden role to treat those pathogenic infections which have shown resistance to synthetic antibiotics. This might be the reason that leading medicinal research groups are actively engaged for revaluing antioxidant and antimicrobial activities of plants and herbs indigenous to their countries.

Swertia chirata (Fig. 1) belong to family Gentianaceae, which is considered of great traditional medicinal values. The plant is indigenous to temperate Himalaya. The entire plant is considered medicinally important particularly roots. The extract of this plant is being used in number of herbal medicines¹¹ as antihypoglycaemic, antifungal, antipyretic and antiinfectant¹. Despite a descent hold in the herbal industry, insufficient research has been undertaken to explore its antioxidant, antimicrobial and anticancer values. Further it is also not clear whether the plant behaves differently with varying climatic conditions or genotypes.



Fig. 1. Areal parts of the *Swertia chirata* plant

The focus of this study was to investigate the antioxidant, antimicrobial and anticancer potential of methanolic and ethanolic extracts of *Swertia chirata* collected from northern areas of Pakistan. The antioxidant activity was determined by estimating total phenolic contents, total flavonoid contents and using different free radical scavenging assays. The antimicrobial and anticancer study was done against four different bacterial strains and MDBK cancer cell line.

EXPERIMENTAL

Methanol, ethanol, ethyl acetate (extra pure) were purchased from sigfrom/merk. Water was twice distilled and filtered (FH-0.45 lm, Advantec MFS Inc., Japan) using a decompressing pump (Division of Millipore, Waters, USA). 2,2-Diphenyl-1-picrylhydrazil (DPPH), ascorbic acid (vitamin C), Folin-Ciocalteu reagent, butylated hydroxytoluene (BHT), gallic acid, butylated hydroxy anisole (BHA) and all phenolic standards were purchased from Sigma-Aldrich chemical Co. A Millipore type filter (pore size 0.22 and 0.45 μm) was obtained from Sartorius, USA. Absorption in UV-visible range of samples was measured using double beam U-2800 UV-visible spectrometer, HITACHI, Japan. Bacterial strains and MDBK cancer cell line was obtained from University of Health Sciences, Lahore.

Plant materials: The complete plants were collected from northern areas of Pakistan and identified at the Botany Department, Government College University, Faisalabad. Voucher Specimens were deposited at the Department of Botany, Government College University, Faisalabad.

Preparation of plant sample and extraction: Leave, stems and flowers after cleaning with distilled water dried at normal room temperature. The dried plants were chopped into

small pieces and ground to fine homogenous powder with electrical blender (National®, Japan). The fine homogenous plant powder was soaked and shaken for 6 h at orbital shaker either in 95 % ethanol or 80 % methanol at room temperature. The mixture was then filtered using whatman 1 filter paper and the supernatant was then subjected to rotary evaporator (EYELA, N-N Series, Rikakikai Co. Ltd. Tokyo, Japan) to evaporate the solvent at 40 °C. The dried supernatant was then stored in refrigerator at 4 °C till further analysis.

Total phenolic content (TPC): Amount of total phenolic content was calculated using Folin-Ciocalteu reagent as described by Chaovanalikit and Wrolstad¹² with slight modification. Briefly, 1 mL of extract solution (0.05 g/mL ethanol) was mixed with 200 μL of Folin-Ciocalteu reagent and 3 mL deionized water and the mixture was kept at room temperature for 10 min and then 0.75 mL of 20 % sodium carbonate (w/v) was added. The mixture was heated in a water bath at 40 °C for 20 min and then cooled in an ice-bath. Finally absorbance was taken at 755 nm. Amount of total phenolic content was calculated using a calibration curve for gallic acid (10-130 ppm). The results were expressed as gallic acid equivalents (GAE) g/100 g dry extract matter. Sample was analyzed thrice and results were averaged.

Total flavonoid content (TFC): Total flavonoid content was determined following the procedure by Dewanto *et al.*¹³. One milliliter of extract solution containing 0.01 g/1 mL of dry matter was placed in a 10 mL volumetric flask and then 5 mL of distilled water was added followed by 0.3 mL of 5 % NaNO_2 . After 5 min, 0.6 mL of 10 % AlCl_3 was added and for another 5 min interval, 2 mL of 1 M NaOH was added and volume was made up to mark with distilled water. Absorbance was measured at 510 nm. Total flavonoid concentration was expressed as catechin equivalents g/100 g of dry matter. Sample was analyzed thrice and results were averaged.

Free radical scavenging activity: Free radical scavenging activity of extracts was assessed according to the method reported by Bozin *et al.*¹⁴. Sample solution of dried extract was made by dissolving 0.01 g in 10 mL methanol. Took equal volume of sample solution and 90 μM DPPH solution (2 mL of each) to make a 4 mL stock solution. Stock solution was then put in dark for 0.5 h. Blank solution was made by mixing 2 mL of 90 μM DPPH solution in 2 mL of methanol. The absorbance at 515 nm of the resulting solutions and the blank were recorded at room temperature using a spectrophotometer (U-2001, Hitachi Instruments Inc. Tokyo, Japan). Butylated hydroxytoluene (BHT) was used as a positive control. For each sample, three replicate absorbance data was recorded. Per cent scavenging of DPPH free radical was calculated using following formula:

$$\text{Scavenging (\%)} = \left(\frac{A_{\text{Blank}} - A_{\text{Sample}}}{A_{\text{Blank}}} \right) \times 100$$

Per cent inhibition in linoleic acid system: The antioxidant potential of *Swertia chirata* extracts was determined in terms of measurement of per cent inhibition of peroxidation in linoleic acid system¹⁵. To a solution mixture of linoleic acid (0.05 mL), 99 % ethanol (4 mL) and 4 mL of 0.1 M sodium phosphate buffer (pH 7), 2 mg dried plant extract was added

and total mixture was diluted to 10 mL with distilled water. The solution was incubated at 40 °C. The degree of oxidation was measured following the thiocyanate ion method¹⁶. Briefly, 10 mL of ethanol (75 %; v/v), 0.2 mL of an aqueous solution of ammonium thiocyanate (30 %), 0.2 mL of sample solution and 0.2 mL of ferrous chloride (FeCl₂) solution (20 mM in 3.5 % HCl; v/v) were added sequentially. After 3 min of stirring, the absorption values of mixtures measured at 500 nm were taken as peroxide contents. Synthetic antioxidants; butylated hydroxytoluene (BHT) was used as a positive control. The minimum peroxidation level of blank was observed at 75 h (3 days) time point that contained no antioxidant component (used as a test point). Per cent inhibition of linoleic acid per oxidation (I %) was calculated using following relation:

Inhibition (%)

$$= \left[100 - \left(\frac{\text{Abs. increase of sample at 75 h}}{\text{Abs. increase of control at 75 h}} \right) \times 100 \right]$$

Antibacterial assay: The antibacterial potential of *Swertia chirata* methanolic and ethanolic extracts was measured as described earlier Nasir *et al.*¹⁷. The liquid cultures of four bacterial strains (100 µL) with optical density (OD) value 1.4-1.6 at 600 nm were used to inoculate 10 mL LB broth medium containing 12.5, 25, 37.5, 50 and 0 µg (as control) of each extract. All tubes containing bacterial cultures and extracts were incubated for 24 h at 37 °C. The bacterial growth inhibition was measured by taking optical density at 600 nm.

Antiproliferative assay: The antiproliferative bioassay was carried as described earlier Mosmann¹⁸. MDBK cell line was used for assay and extracts were diluted five folds in labelled wells while maintaining the cell number constant in each well of 96-wells plate *i.e.*, 2000 cells/well. GMEM medium was used for the growth of cells amended with 5 % FBS and 5 % CO₂ supply while incubating at 37 °C for 48 h. After incubation added 20 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) stock solution of concentration 5 mg/mL. The plate was again placed in 5 % CO₂ incubator at 37 °C for 3 h for the formation of crystals of purple formazan. Then Added 100 µL of DMSO in each well to dissolve the crystals and OD value was measured at 570 nm with reference to control. Experiments were repeated thrice to obtain mean values.

RESULTS AND DISCUSSION

Per cent yield: Extraction in 80 % methanol and ethanol revealed methanol is more favourable solvent for improving per cent yield of compounds of interest. Twenty per cent increase in yield was noted with methanol (10.3 g/100 g dried plant sample) solvent was used as compared to ethanol (8.3 g/100 g dried plant sample).

Total phenolic and flavonoid contents: Both, methanolic and ethanolic extracts were subjected to estimate phenolics and flavonoids. Phenolics or polyphenolics are considered to involve in various biological defence *i.e.* as an antioxidant, antiatherogenic, antiproliferative, antimutagenic and cardio-protective vectors¹⁹⁻²⁵. Fruits, cereals and vegetables contain effective amount of polyphenolics and are extensively used to combat oxidative stress diseases and pathogenic infections^{26,27}.

Total phenolics found in methanolic extract was higher (272.67 ± 5.09 mg gallic acid equivalents/g of dry weight extract of *Swertia chirata*) than obtained using ethanol (189.20 ± 3.98 mg gallic acid equivalents/g of dry weight extract of *Swertia chirata*) and same trend in concentration was observed while estimating total flavonoids *i.e.*, 3.38 ± 0.56 mg catechin equivalents/g of dry weight of *Swertia chirata* and 3.17 ± 0.31 mg catechin equivalents/g of dry weight of *Swertia chirata*, respectively (Table-1). Yue *et al.*²⁵ reported antioxidant study of ethanolic extract of *Swertia chirata*. They reported approximately similar contents of phenolics and flavonoids.

TABLE-1
PER CENT YIELD, TOTAL PHENOLIC CONTENTS
AND TOTAL FLAVONOID CONTENTS OF METHANOLIC
AND ETHANOLIC EXTRACTS OF *Swertia chirata*

S. No.	<i>Swertia chirata</i> Extract	Yield (%)	Total phenolic contents (TPC) (g/100 g)	Total flavonoid contents (TFC) (g/100 g)
1	Methanolic	10.45 ± 1.08	27.2 ± 2.84	3.38 ± 0.23
2	Ethanolic	8.23 ± 1.12	18.9 ± 2.43	3.17 ± 0.19

Means of three replicates ± SD (standard deviation).

Antioxidant behaviour of the extracts: Fig. 2 expresses the promising scavenging of DPPH free radical. DPPH is a stable free radical that has commonly been used to test the antioxidant potential of natural extracts. The antioxidant property of the natural product is evaluated by measuring the decrease in absorbance power of DPPH on addition of particular concentration of antioxidant test sample. The two parameters, absorbance and the antioxidant activity, are inversely proportional to each other. Methanolic and ethanolic extracts of areal parts of the plant having concentration 200 µg/mL were showed significant inhibition of DPPH free radical *i.e.*, 84.32 ± 2.78 and 82.54 ± 3.12 %, respectively while at the same concentration of standard (BHT) showed 93.72 ± 3.22 % inhibition. At the same concentration of aqueous extract of *Swertia chirata*, Vivek *et al.*^{5a} reported 59.80 % scavenging of DPPH free radical.

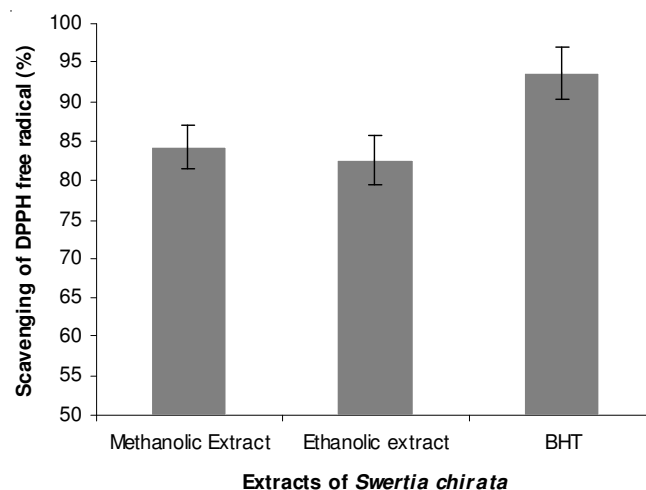


Fig. 2. Methanolic and ethanolic *Swertia chirata* extract potential to scavenge DPPH free radical

Inhibition of linoleic acid peroxidation assay was also used to evaluate the antioxidant activity of the test sample. Fig. 3 shows the results obtained by this assay. Linoleic acid is a poly unsaturated fatty acid, upon oxidation, peroxide are formed which oxidized Fe^{2+} - Fe^{3+} ions. The later state of peroxides form complex with thiocyanate ions. The intensity of the coloured complex is measured at 500 nm. Per cent inhibition of linoleic acid peroxidation of methanolic and ethanolic extract was measured 94.04 ± 4.23 and 92.86 ± 4.78 %, respectively. Anyhow, standard BHT showed 97.81 ± 3.07 % for the same concentration.

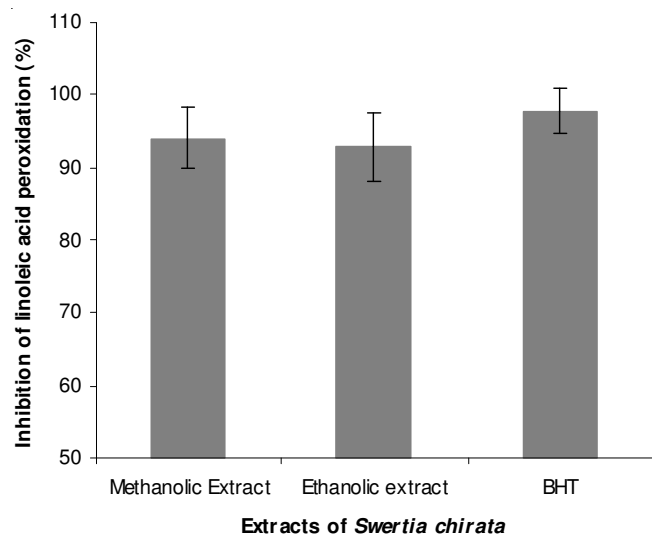


Fig. 3. Methanolic and ethanolic *Swertia chirata* extract potential to inhibit the linoleic acid peroxidation

Antibacterial study of extracts: Methanolic and ethanolic extracts of *Swertia chirata* showed strong antibacterial potential with increased concentration from 0-50 μ g against two bacterial species viz., *Streptococcus* sp. and *E. coli*. Maximum reduction of bacterial growth was observed at 50 μ g concentration with reference to control (0 μ g) devoid of any extract. In case of *Bacillus* sp. and *Staphylococcus* sp. no any significant decline in bacterial growth was observed as was in case of other two bacterial strains (Table-2). These results are in agreement with Ganjewala *et al.*²⁸ and Shinwari *et al.*²⁹ who reported the same facts while working on extract of *Lantana camara* and *Justicia adhatoda* while Joshi and Dhawan¹¹ also reported the microbial growth inhibition potential of aqueous extract of *Swertia chirayita*.

Antiproliferative potential of extracts: The antiproliferative assays of methanolic and ethanolic extracts of *Swertia chirata* were carried out by using MDBK cell line in order to explore the anticancerous potential of these extracts. Both extracts have not shown significant effect regarding inhibition of cancerous cells growth with reference to control which was devoid of plant extract (Fig. 4). The current study depicts the direct effect of both extracts on MDBK cells whose growth was not inhibited in response to these extracts which was not in agreement with Saha *et al.*³⁰ who reported the anticancerous effect of *Swertia chirata* by using mouse model.

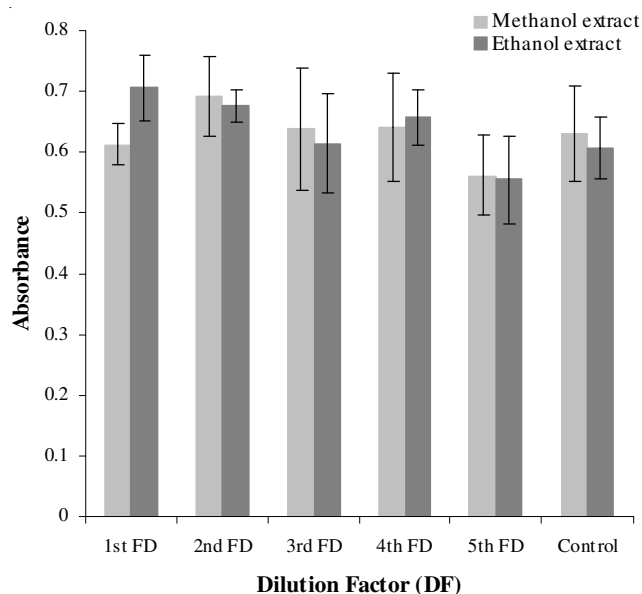


Fig. 4. Antiproliferative assay of methanolic and ethanolic extracts of *Swertia chirata*

Conclusion

There is a strong correlation among polyphenolics, flavonoids and antioxidant property. It also has been reported many times that if a plant has antioxidant potential possibly will carry antibacterial potential. Methanolic and ethanolic extracts of the areal parts of *Swertia charata* showed significant antioxidant activity. Methanolic extract was slightly more active as compared to ethanolic extract to inhibit oxidation process. However, regarding antibacterial potential both extract showed promising inhibition of *Streptococcus* sp. and *E. coli* growth while in case of *Bacillus* sp. and *Staphylococcus* sp., it

TABLE-2
ANTIBACTERIAL ACTIVITY OF METHANOLIC AND ETHANOLIC
EXTRACTS OF AREAL PART OF *Swertia chirata* PLANT EXTRACTS

Microorganism	0 μ g	12.5 μ g	25 μ g	37.5 μ g	50 μ g
<i>Swertia chirata</i> methanolic extract					
<i>Bacillus</i> sp.	2.1 ± 0.10	1.63 ± 0.21	2.41 ± 0.32	1.34 ± 0.16	1.06 ± 0.13
<i>Staphylococcus</i> sp.	1.9 ± 0.15	1.89 ± 0.19	2.16 ± 0.27	1.12 ± 0.09	1.23 ± 0.10
<i>Streptococcus</i> sp.	2.4 ± 0.09	2.37 ± 0.28	1.80 ± 0.11	1.10 ± 0.15	0.42 ± 0.07
<i>E. coli</i>	2.5 ± 0.11	2.22 ± 0.22	1.65 ± 0.18	1.24 ± 0.17	0.86 ± 0.11
<i>Swertia chirata</i> ethanolic extract					
<i>Bacillus</i> sp.	2.1 ± 0.10	1.75 ± 0.13	1.56 ± 0.21	1.50 ± 0.21	1.33 ± 0.25
<i>Staphylococcus</i> sp.	1.9 ± 0.15	2.05 ± 0.20	2.42 ± 0.24	1.41 ± 0.15	1.05 ± 0.14
<i>Streptococcus</i> sp.	2.4 ± 0.09	2.38 ± 0.19	1.71 ± 0.13	1.23 ± 0.11	0.62 ± 0.09
<i>E. coli</i>	2.5 ± 0.11	2.45 ± 0.24	1.82 ± 0.16	1.41 ± 0.14	0.83 ± 0.18

Means of three replicates ± SD (standard deviation).

was unable to inhibit growth prominently. Antiproliferative study results of *Swertia chirata* extracts against MDBK cancer cell line also not encouraging. It is therefore concluded that methanolic and ethanolic extracts of *Swertia chirata* could be used against oxidative stress diseases and bacterial infections.

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