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PHYTOCHEMICAL QUANTIFICATION, ANTIMICROBIAL AND ANTIOXIDANT POTENTIAL OF *SWERTIA CHIRAYITA* FROM PAKISTAN

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Abstract
The present study was carried out to determine various phytochemicals from *Swertia chirayita* and to evaluate antimicrobial and antioxidant properties of the extracted phytochemicals. Phytochemical determination i.e., flavonoids, tannins, *β*-carotene and lycopene, and alkaloids was carried out using different methods. Among the phytochemicals, flavonoids i.e., 67.4 mg/ml were present in higher concentrations, tannins i.e., 38.2 mg/ml in moderate concentrations, while *β*-carotene i.e., 0.678 mg/50 ml, lycopene i.e., 0.150 mg/50 ml and alkaloids i.e., 0.073 mg were found in trace amounts. Among various extracts, acetone extract had shown antimicrobial activity against *Klebsiella pneumonia, Haemophilus influenza, Acinetobacter baumanii* and *Morganella morganii*, while it was inactive against *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa*. Ethanol extract showed activity only against *Haemophilus influenza* and all other microorganisms showed resistance against ethanol extract. Other all the tested extracts showed no notable activity against the microorganisms. Flavonoid and tannin showed significant antioxidant activity and among the extracts ethanol and methanol showed higher antioxidant activity, *n*-hexane, acetone and chloroform showed moderate activity followed by butanol and water showing the lowest activity. Thus, *S. chirayita* possesses significant antimicrobial and antioxidant properties and can be used to treat pathogenic infections.

Keywords: *Swertia chirayita,* phytochemicals, antimicrobial activity, antioxidant property

1. Introduction

Swertia chirata commonly known as *Chirayita* belongs to the genus *Swertia* and family Gentianaceae which has about 80 genera and 700 species. The plant is indigenous to temperate Himalayas having a habitat at an altitude of 1200-3000 m (4000-10,000 ft) from Kashmir to Bhutan and also in the Khasi hills at 1200-1500 m (4000-5000 ft) (PaoloScartezzini, 2000). Since various *Swertia* species have been used as crude drugs in traditional medicine systems in curing different diseases, but *S. chirayita*

is considered as the most important high-value medicinal plant having immense potential and is used as an appetizer, as a febrifuge and for the treatment of asthma and liver diseases (Goutam Brahmachari S. M., 2004). Since, various *Swertia* species such as *S. paniculata, S. dilatata, S. alata, S. bimaculata, S. nervosa, S. minor, S. angustifolia* have close relation and less bitter adulterance with the true *Chirayita* (Kunjani, 2008), so to distinguish *Chirayita* from other *Swertia* species, mangiferin can be used as an effective biomarker (Satyendra Suryawanshi, 2007).

Medicinal plants have substances useful for therapeutic purposes or are precursors to synthesize useful drugs. Being a medicinal plant, all parts of *Chirayita* are used in traditional medicine systems; however the root is considered to have most powerful therapeutic properties. Herbal medicines like Ayush 64, Mensturyl syrup, Diabecon and Melicon ointment contain different quantities of *S. chirayita* extracts (Joshi, 2008). *Chirayita* contains amarogentin, swerchirin, swertiamarine and other active compounds which are responsible for the bitterness, hypoglycemic, anthelmintic and antipyretic properties (Michael Keil B. H., 2000). It also has been reported for possessing antitumor, antioxidant, antiviral, and analgesic properties (Alam KD, 2010). *S. chirayita* is used either individually or in combination with other herbs for the preparation of medicines in different traditional medicine systems. When combined with cardamom, turmeric and kutki, it is used for the treatment of gastrointestinal infections and along with ginger is used for curing fever (Michael Keil B. H., 2000). In Ayurveda industry, *Chirayita* is an active ingredient of anti-cancer drugs and skin tonic 'Safi'. Skin soaps and cosmetic products also contain *Chirayita* extracts (Susanna Phoboo, 2010).

Chirayita also contains several important phytochemicals including flavonoids, iridoids, xanthons, xanthone glycosides and triterpenoids (Goutam Brahmachari S. M., 2004). Xanthone derivatives such as mangostin, mangostin triacetate and isomangostin are considered to have significant anti-inflammatory activities. There are also reports which show that several xanthone derivatives have potent anti-cancer, anti-fungal, anti-platelet, CNS stimulant and antimalarial properties (SHIVAJI BANERJEE, 2000). The alkaloids found in *Chirayita* are gentianine and gentiocrucine and two bitter glycosides are chiratine and amarogentin. Besides these substances, it also contains a bitter yellow acid known as ophelic acid and a yellow crystalline substance which is used in dyeing (Anonymous). Keeping in mind the strong medicinal background, *Chirayita* was investigated in the present study to determine phytochemicals and to explore its antimicrobial, and antioxidant properties by using various extracts.

2. Materials and methods

2.1 Collection and preparation of sample

Sample of *Chirayita* was collected from the Abbottabad (Thandyani also called Shimla pahari) for phytochemical determination and evaluation of antioxidant and antimicrobial activities. The entire *Chirayita* plant was air-dried and grounded to fine powder form by an electric blender. Samples were preserved in fine plastic bags at 4 °C for further analysis.

2.2 Isolation and determination of bioactive compounds

2.2.1 Determination of total flavonoid contents

Total flavonoid contents determination was carried out by spectrophotometric assay as reported (Ferreira, 2007). Briefly, 5 g of samples were dissolved in 50 mL of aqueous ethanol (80% v/v), and mixtures were placed in shaking incubator for 24 h. After 24 h, the extracts were centrifuged (at 3,000 g for 15 min), pellets were discarded and supernatants were kept in 50 mL falcon tube at 4 °C. A 250 µl extract containing flavonoid was mixed in 1.25 mL of distilled water and 75 µl of $NaNO_2$ solution (5.0% w/v). After 5 min, 150 µl of 10% $AlCl_3.H_2O$ was added and incubated for 6 min. After this, 500 µl of 1M NaOH and 275 µl of distilled water were added to the mixture and absorbance of the solution was measured at OD_{415}.

2.2.2 Determination of *β*-carotene and lycopene

Previously reported method of (M.P.Revuelta, 1997) was adopted for *β*-carotene and lycopene determination. Ten gram of the sample was dissolved in 100 mL of methanol and solution was kept

in temperature controlled shaker for 24 h. The extract was centrifuged, filtered (Whatman filter paper No. 1) and supernatant was kept in hot water-bath for solvent evaporation. The dried evaporated sample was extracted with acetone: *n*-hexane mixture (4: 6). The reaction mixture was analyzed spectrophotometrically for *ß*-carotene and lycopene at 453, 505, 645 and 663 nm. *ß*-carotene and lycopene were calculated by using the following equations:

Lycopene (mg/50 ml) = $0.0458A_{663} + 0.372A_{505} - 0.0806A_{453}$

ß-carotene (mg/50 mL) =$0.216\ A_{663} – 0.304A_{505} + 0.452\ A_{453}$

2.2.3 Determination of tannins

Tannins were estimated by the methodology described by (Harinder P S Makkar, 1993). Different concentrations of tannic acid (3-50 mg) were made by diluting it serially from stock solution (50 mg/100 mL 70% acetone). The tannin extract (50 µl) was mixed with 950 µl of distilled water followed by addition of 0.5 ml of Foline Ciocalteu's phenol and 2.5 ml of 20% $NaCO_3$ solution with continuous agitation. Before measuring the absorbance at 725 nm, the solution was incubated at room temperature for 40 min. 70% acetone was used as blank and treated in the same way as that of positive control.

2.2.4 Determination of alkaloids

Alkaloids were extracted by using acid base shifting method as described by (H O Edeoga, 2005). According to the method dried sample was dissolved in ethanol (1:10) and was left on shaking for 24 h. Extract was filtered and concentrated near to dryness in oven and was re-dissolved in ethanol with addition of 1% HCl. The mixture was placed in refrigerator for three days. The solution was filtered and pH was maintained 8-10 and was extracted with chloroform by using separating funnel. Chloroform layer was recovered and ethanol layer was discarded whereas the solution was heated in hot water bath for evaporation. After that the sample was dried in oven to constant weight. Alkaloid contents were calculated on the basis of weight obtained and weight used.

2.3 Antimicrobial activity

Antimicrobial activity was tested against *Escherichia coli, staphylococcus aureus, Pseudomonas aeruginosa, Klebsella pneumonia, Acinetobacter baumanii, Morganella morganii* and *Haemophilus influenza* by using agar well diffusion method by agar well diffusion method. Lauria-Bertani agar media was prepared and autoclaved at 121 ℃ for 15 min which was then cooled and poured in Petri plates under sterilized condition of laminar flow hood. The wells of 9 mm were bored in each plate and the plates were inoculated with 75 µl of inoculum. Each of sample 100 µl was pipetted in each well and plates were incubated at 37 ℃ for 24 h. After 24 h, zones of inhibition were measured and expressed in millimeter.

2.4 Antioxidant activity

The ferric ion reducing power capability of sample extract was determined by using a modified method of (Gow-Chin Yen, 1995). The extract (750 µl) of each sample was mixed with an equal amount of phosphate buffer (0.2 M, pH 6.6) and 1% potassium ferricyanide (a source of ferric ions). The mixture was incubated at 50 °C for 20 min followed by addition of (750 µl) trichloroacetic acid (10%) to stop the reaction and was then centrifuged at 3000 rpm for 10 minutes. Two layers were formed, pellet and the supernatant. Upper layer (1.5 mL) was separated and mixed with an equal amount of distilled water and 0.1 mL $FeCl_3$ solution (0.1%). The same procedure was repeated with different concentrations of isolate phytochemicals. A blank was also prepared by using same procedure and the absorbance was measured at 700 nm as the reducing power. Higher the absorbance higher will be the reducing power capability of phytochemical. In parallel ascorbic acid (vitamin C) was used as standard positive control.

2.5. DPPH Radical Scavenging Assay

The antioxidant activity of plant extracts against stable 2, 2-diphenyl-1-picrylhydrazyl hydrate (DPPH) was assayed following standard method with slight modifications [19]. Briefly, 2ml of 0.1mM DPPH solution in ethanol was mixed with 1ml of plant extract at a concentration range of 62.5*µg/ml-1000µg/ml.* Equivalent positive control was prepared using L-Ascorbic acid (1-100 µg/ml) as standard. A negative control was prepared by mixing 1ml ethanol and 2ml DPPH solution. Likewise a negative control was prepared for aqueous extract. The reaction mixture was allowed to incubate for 5min at room temperature in the dark and the scavenging activity of each plant extract were quantified by decolourization at 517 nm using UV-Vis spectrophotometer (*Hitachi U-2900* ,*Tokyo, Japan*).The reaction was carried out in triplicate and percentage of free radical scavenging activity of each extract was expressed as percent inhibition from the given formula.

% Inhibition of DPPH radical = Ac-As/Ac×100

Where Ac is the absorbance of the control As is the absorbance of the sample

2.7 Statistical analysis

All experiments were performed in 3 different sets with each set in triplicates. The data are expressed as mean ± SEM (standard error of the mean). The IC_{50} values for alpha amylase inhibition and DPPH scavenging activity were calculated from plots of log inhibitor concentration versus percentage inhibition in Prism 6.01 software (GraphPad, La Jolla, CA, USA) using a non-linear regression analysis.

3.0 Results and Discussion

3.1 Bioactive compound Determination

Figure 1 portrays the results of bioactive compounds determination such as alkaloids, tannins, flavonoids, *β*-carotene and lycopene of *S. chirayita*. Results showed that flavonoids were found in higher concentration (67.4 mg/ml) followed by tannins (38.2 mg/ml), *β*-carotene (0.678 mg/50 ml) and lycopene (0.150 mg/50 ml) and alkaloids (0.073 mg). Phytochemical studies have shown that *Swertia chirayita* contains secondary metabolites which are biologically active compounds (In-Kyoung Lee, 2006).

The phytochemical analysis of the extracts of *Swertia chirayita* had revealed that it contains flavonoids, xanthones, iridoids, secoiridoid glycosides, terpenoids and saponin. Among these, flavonoids are well-known for their ability to inhibit pain perception. Due to their inhibitory effects on enzymes involved in the production of the chemical mediator of inflammation, flavonoids also have anti-inflammatory properties. Flavone and its methoxy derivatives exhibited significant dose dependent analgesic activity. Due to the presence of flavonoids, *Swertia chirayita* extracts also have been reported to exhibit Ca^{2+} antagonist activities (Victor B.Owoyele, 2005).

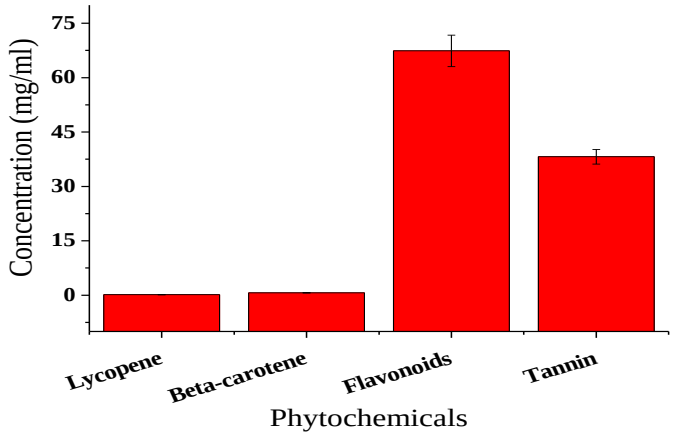

Figure 1: Determination of phytochemicals (lycopene, *β*-carotene, flavonoids and tannins) in *S. chirayita*

3.2 Antimicrobial activity of *S. chirayita* extracts

The antimicrobial activity of different extracts (n-hexane, acetone, chloroform, ethanol, methanol and water) of *S. chirayita* was tested against different pathogens namely *E. coli, S. aureus, K. pneumonia, H. influenza, A. baumanii, P. aeruginosa and M. morganii* (Table 1). Results showed that acetone and ethanol extracts exhibited inhibitory effect against the tested microorganisms. The n-hexane extract was found to be inactive against all the tested microbes as it showed no zone of inhibition. Acetone extract showed activity only against *K. pneumonia (*18 mm zone of inhibition*), H. influenza* (15 mm), *A. baumanii* (15 mm) and *M. morganii* (16 mm), while other three microorganisms including *E. coli, S. aureus* and *P. aeruginosa* showed resistance against acetone extract. Ethanol extract showed activity only against *H. influenza*, while all other microorganisms showed resistance against ethanol extract. The *n*-hexane, chloroform, methanol and water extracts did not show any activity against all the microbes.

Gram positive bacteria are more susceptible to inhibition by plant extracts as compared to Gram negative bacteria that may probably be due the morphological differences in cell wall compositions. In Gram-negative organisms, the presence of lipopolysaccharide layer may prevent the diffusion of extracts to the peptidoglycan layer of cell wall leading to resistance of these organisms towards most of the extracts (M. Kaneria, 2009).

Table 1 Antimicrobial activity of various extracts of *S. chirayita*

Tested microorganisms	*n*-Hexane	Acetone	Chloroform	Ethanol	Methanol	Water
	Diameter of inhibition zone (mm)					
E. coli	ND	ND	ND	ND	ND	ND
S. aureus	ND	ND	ND	ND	ND	ND
k. pneumonia	ND	18± 2.12	ND	ND	ND	ND
H. influenza	ND	15± 1.41	ND	15 ± 2.83	ND	ND
A. baumanii	ND	15± 0.54	ND	ND	ND	ND
P. aeruginoa	ND	ND	ND	ND	ND	ND
M. morganii	ND	16± 1.32	ND	ND	ND	ND

3.3 Antioxidant activity

3.3.1 Antioxidant activity of phytochemicals

In-vitro antioxidant activity of different phytochemicals i.e. tannins and flavonoids of *S. chirayita* was evaluated using ascorbic acid as a standard. Assay involved the use of $FeCl_3/K_3Fe(CN)_6$ as a source of ferric ions which may reduce to ferrous ion in the presence of phytochemical compounds. This results in the production of green color complex whose intensity was measured spectrophotometrically and increase in absorbance indicates increased antioxidant activity (Nethravathi Guthalu Puttaraju, 2006). Results of antioxidant activity of the standard ascorbic acid, and of the phytochemicals i.e., tannins and flavonoids are shown in Figure 2. The test samples showed different antioxidant activities at different concentrations, and it was found to be increased with increasing the concentration of the sample. Among the phytochemicals, tannins had shown the highest antioxidant activity, while flavonoids showed the moderate activity. Upon comparison, tannin having 3.00 absorbance (A_{700}) at 750 µg concentration was more active than the standard ascorbic acid having 1.65 absorbance (A_{700}) at 7500 µg concentration while flavonoids having 1.19 absorbance (A_{700}) were less active than ascorbic acid. Since flavonoids and tannins are polyphenolic compounds which donate their hydrogen atom to ferric ions and convert them to their reduced form resulting in the production of intense green color and greater absorbance. These polyphenolic compounds act as antioxidant agent and may protect the body from oxidative damage. Oxidative stress in the body can cause serious damage to macromolecules including protein, carbohydrate, lipid and DNA which may lead to various degenerative diseases including cardiovascular diseases, cancer, immune system decline and cataracts. Natural antioxidant compounds such as flavonoids and tannins interact with the production of free radicals and inactivate them by quenching singlet and triplet oxygen, decompose hydrogen peroxide and inhibit enzymes (Timothy Johns P. B., 2006).

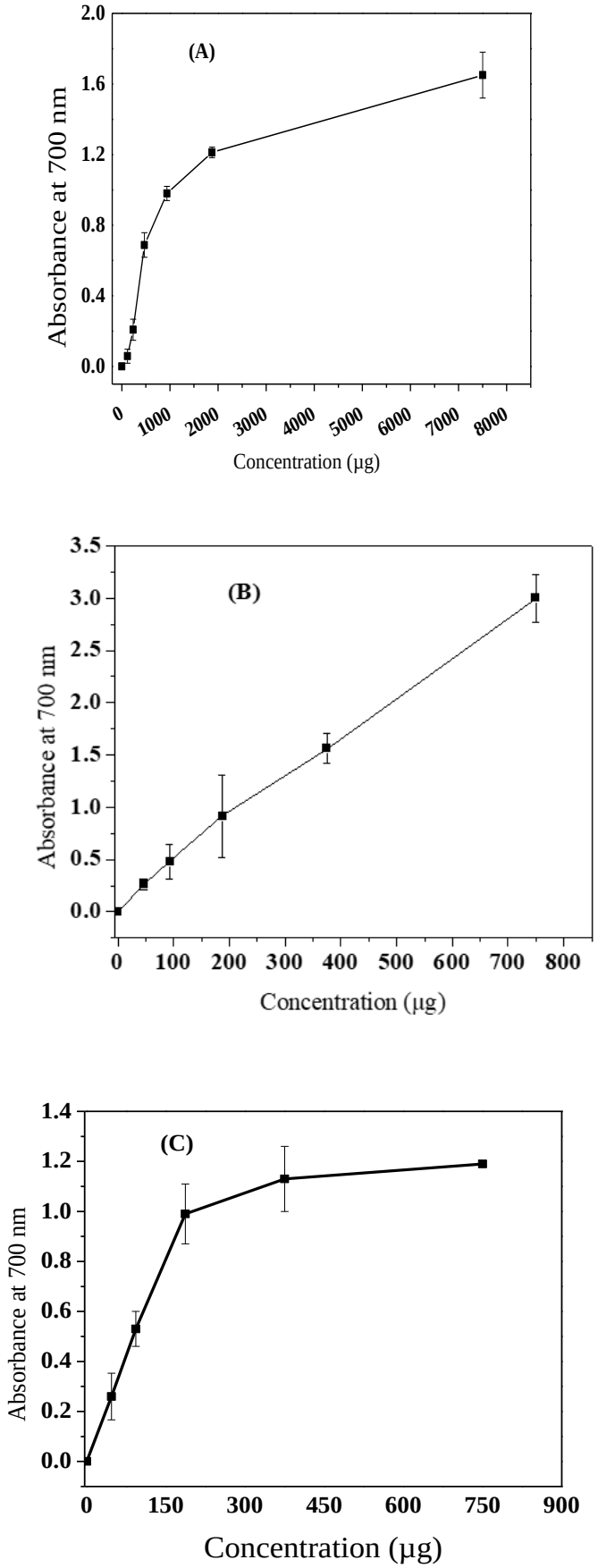

Figure 2 Antioxidant activities of A) ascorbic acid, B) tannin and C) flavonoid of *S. chirayita* using different concentrations

3.3.2 Antioxidant activity of various extracts of *S. chirayita*

Figure 3 illustrates the antioxidant activity of various extracts of *S. chirayita*. Among all the extracts, methanol and ethanol had shown the higher antioxidant activity while *n*-hexane, acetone and chloroform had shown moderate activity followed by butanol and water showing the lowest activity. Results had also shown that antioxidant activity of methanol extract having 1.53 absorbance (A_{700}) at the concentration of 750 µg was higher than that of the standard ascorbic acid having 1.65 absorbance (A_{700}) at the concentration of 7500 µg. Antioxidant activity of ethanol extract with 1.35 absorbance (A_{700}) is comparable with that of the standard, while the antioxidant activity of other extracts like *n*-hexane i.e., 0.63 absorbance (A_{700}), acetone i.e., 0.52 absorbance (A_{700}), chloroform i.e., 0.61 absorbance (A_{700}), butanol i.e., 0.33 absorbance (A_{700}) and water i.e., 0.48 absorbance (A_{700}) was very low as compared to the standard ascorbic acid. From the results, it is concluded that methanol extract possesses significant antioxidant activity so, it is recommended to isolate and purify active compounds from methanol extract of *S. chirayita*. Natural compounds act as antioxidant agent and may protect the body from oxidative damage. Oxidative stress in the body can cause serious damage to macromolecules including protein, carbohydrate, lipid and DNA which may lead to various degenerative diseases including cardiovascular diseases, cancer, immune system decline and cataracts. Natural antioxidant compounds interact with the production of free radicals and inactivate them by quenching singlet and triplet oxygen, decompose hydrogen peroxide and inhibit enzymes (Timothy Johns P. B., 2006).

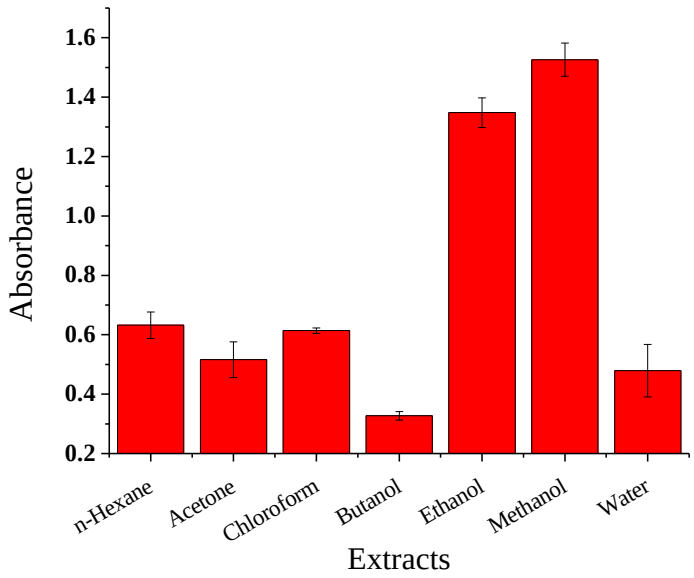

Figure 3 Antioxidant activities of various extracts of *Swertia chirayita*

Antioxidant activity by DPPH Radical Scavenging Assay

In another series of experiments the antioxidant potential was also evaluated using DPPH radical scavenging assay. At 25 °C DPPH is a stable free radical and accepts a proton to become a stable diamagnetic molecule and purple color of DPPH solution become change to yellowish color. At 517 nm the DPPH solution shows maximum absorbance. The decrease in absorbance values show the reduction capability of the DPPH radical, caused by antioxidants. The reaction between antioxidant molecule and free radical results in radical scavenging by hydrogen donation. It is visually noticeable as a change in color from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the antioxidants activity. The results for DPPH scavenging activity are portrayed in Figure 4 and percentage inhibitions were calculated using absorbance value. Results showed that the percentage inhibition increased with increase in concentration of plant samples.

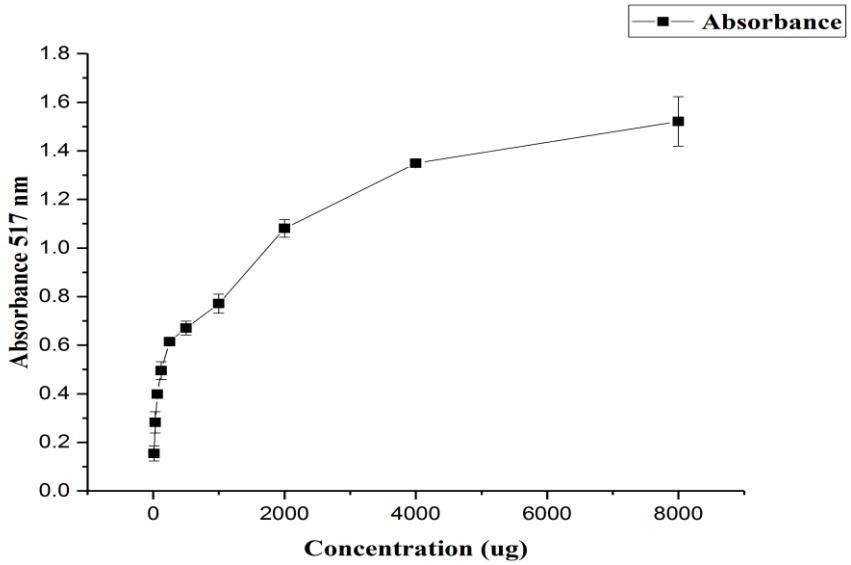

Figure 4: Antioxidant activity of *Swertia chirayita* by DPPH method

4. Conclusions

This study reports the phytochemical determination, antimicrobial activity and antioxidant activity of *S. chirayita*. Phytochemical analysis showed that various phytochemicals including flavonoids, tannins, *β*-carotene, lycopene and alkaloids were found in *Chirayita* plant. Only acetone and ethanol extracts of *Chirayita* exhibited the noticeable antimicrobial activity, however, the n-hexane, chloroform, methanol and water extracts showed no antimicrobial activity. Antioxidant profile revealed that tannins showed highest antioxidant activity as compared to flavonoids and among the extracts, ethanol and methanol showed highest activity. So, it is concluded that the most active compounds of *S. chirayita* are polar in nature as ethanol and methanol extracts showed highest antimicrobial and antioxidant activities. It is of great significance to isolate and purify active compounds from methanol extract of *S. chirayita* to cure infectious diseases.

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