Phytochemical Screening and In vitro Antioxidant Activities of an unexploited wild Piper

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Abstract

The present investigation has been undertaken to study the preliminary phytochemical screening and antioxidant activity of *Piper schmidtii* leaf and stem. The different extracts such as petroleum ether, chloroform, ethyl acetate and ethanol were subjected for quantification (total phenolics, tannin, flavonoids) and *in vitro* antioxidant assay such as DPPH, Phosphomolybdenum, Superoxide radical scavenging assay and reducing power assay. Among the various solvents, ethanol extract of leaves displayed maximum total phenolic (267.86 mg GAE/g extract), tannin (250.14 mg GAE/g extract) and flavonoid contents (294.04 mg RE/g extract). Results of *in vitro* antioxidant studies revealed that the ethanol extract of leaf possessed an efficient 2,2-diphenyl-1-picryl-hydrazyl (DPPH•) (IC_{50} 15.09 µg/mL), Phosphomolybdenum (52.93 mg AAE/g extract), superoxide (64.64%) radical scavenging activities and Reducing power possesses higher activity. Therefore, the results indicate that the leaf of *Piper schmidtii* can serve as a potential preliminary phytochemical and antioxidant activity.

Keywords: *Piper schmidtii,* phytochemical screening and antioxidant

INTRODUCTION

Piper belong to family piperaceae it contains about 3,600 species which are distributed in 13 genera, there are two major genera's namely Piper (2000 species) and Peperomia (1600 species) that are considered to be highly medicinal. From ancient times traditional system of medicine is been used to treat many diseases. Piper consists of a principal alkaloid Piperine (1-piperoyl piperidine) which is a main constituent of black and long peppers which are used world-wide (Vijayakumar *et al*., 2004). This compound is reported to play an important role in curing many disorders such as cough, bronchitis, asthma, palsy, gout, lumbago and used as analgesic [1].

Oxidative stress play an major role in building up various diseases as these are reactive molecules involved in many physiological process and have been associated with many diseases such as cancer, arthritis and liver injury. The over production of ROS and RNS can result in tissue injury and has been implicated in disease progression and oxidative damage of nucleic acids, proteins and lipids [2]. Antioxidants are substances

that inhibit oxidation. Naturally, there is a dynamic balance between the amount of free-radicals generated in the body and antioxidants to quench and scavenge them and protect the body against their deleterious effects [3]. When there is a lack of antioxidants to quench the excess reactive free radicals, cardiovascular, cancer, neurodegenerative, Alzheimer's and inflammatory diseases may develop in the body [4]. Thus Plants are considered to be over all source of antioxidant since these hold abundant compounds such as phenolic and flavonoid compound. Thus, the aim of this study was to phytochemical profiling, *in vitro* antioxidant activity of all the extract of *Piper schmidtii* leaf and stem.

MATERIALS AND METHODS

Collection and Identification of Plant Material

The fresh plant materials of *Piper schmidtii* were collected during the month of August, 2018 from Coonoor, The Nilgiris district, Tamil Nadu, India. The taxonomic identity of the plant was confirmed from the Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu. The plant materials were washed under running tap water to remove the surface pollutants and the different parts such as leaf, and stem were separated mechanically. The separated plant parts were air dried under shade. The dried samples were powdered and used for further studies.

Chemicals

2,2-diphenyl-1-picryl-hydrazyl (DPPH), potassium persulfate, 2,2'azinobis (3-ethylbenzothiozoline-6-sulfonic acid) dimmonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), linoleic acid, ferrous chloride, ammonium thiocyanate, ferric chloride, 2,4,6-tripyridyl-*s*-triazine (TPTZ), polyvinyl polypyrrolidone (PVPP) were obtained from Himedia (Mumbai, Maharashtra, India), Merck (Hyderabad, Andhra Pradesh, India) and Sigma (Thane, Maharashtra, India). All other reagents used were of analytical grade.

Extraction of Plant Material

The powdered plant materials such as leaf and stem were packed in small thimbles separately and extracted successively with different solvents such as petroleum ether, chloroform, ethyl acetate and ethanol in the increasing order of polarity using Soxhlet apparatus. Each time before extracting with the next solvent, the thimble was air dried. The different solvent extracts were concentrated by rotary vacuum evaporator (Yamato RE300, Japan) and then air dried. The dried extract obtained with each solvent was weighed. The percentage of yield was calculated in terms of the air dried weight of extract from each solvent. The stock solution of the extract obtained was prepared (1 mg/mL of respective organic solvents) and used for further analysis.

Extract Recovery Percentage

The amount of crude extract recovered after successive extraction was weighed and the percentage of yield was calculated by the following formula,

$$\text{Extract recovery percent} = \frac{\text{Amount of extract recovered (g)}}{\text{Amount of plant sample (g)}} \times 100$$

Qualitative Phytochemical Screening

The leaf and stem extracts of *P.schmidtii* were analyzed for the presence of major phytochemicals such as Carbohydrates, Proteins, Amino acids, Alkaloids, Saponins, Phenolic compounds, Tannins, Flavonoids, Glycosides, Flavanol glycosides, Cardiac glycosides, Phytosterols, Fixed oils & fats and Gums &mucilages according to standard methods [5].

Quantification Assays

Quantification of Total Phenolics

The total phenolics of the different plant extracts were determined according to the method described by [6]. In this method 500µL of different plant extracts were taken into a series of test tubes and made up to 1 mL with distilled water. A test tube with 1 mL of distilled water served as the blank. Then, 500 µL of Folin – Ciocalteau Phenol reagent (1 N) was added to all the test tubes including the blank. After 5 minutes, 2.5 mL of sodium carbonate solution (20%) was added to all the test tubes. The test tubes were vortexed well to mix the contents and incubated in dark for 40 minutes. The formation of blue colour in the incubated test tubes indicated the presence of phenolics. Soon after incubation the absorbance was read at 725 nm against the reagent blank. Gallic acid standard was also prepared and the results were expressed as Gallic acid equivalents (GAE). The analyses were performed in triplicates.

Quantification of Tannins

The total phenolics contain both tannin and non-tannin phenolics. The amount of total tannins was calculated by subtracting the total non-tannin phenolics from total phenolics. For the determination of total non-tannin phenolics [6], 500 µL of each plant samples were incubated with 100 mg of polyvinyl polypyrrolidone (PVPP) and 500 µL of distilled water taken in a 2 mL eppendorf tube for 4 hours at 4º C. After incubation the eppendorf tubes were centrifuged at 4000 rpm for 10 minutes at 4º C. The supernatant contains only the non-tannin phenolics since the tannins would have been precipitated along with PVPP. The supernatant was collected and the non–tannin phenolics were determined by the same method described for the quantification of total phenolics. The analyses were also performed in triplicates and the results were expressed in Gallic acid equivalents. From these two results, the tannin content of the plant samples were calculated as follows,

Tannins = Total phenolics - Non tannin phenolics

Quantification of Total Flavonoids

The flavonoid contents of all the extracts were quantified according to the method described by [6]. About 200 µL of all the plant extracts were taken in different test tubes and 2 mL of distilled water was added to each test tube. A test tube containing 2.5 mL of distilled water served as blank. Then, 150 µL of 5% $NaNO_2$ was added to all the test tubes followed by incubation at room temperature for 6 minutes. After incubation, 150 µL of 10% $AlCl_3$ was added to all the test tubes including the blank. All the test tubes were incubated for 6 minutes at room temperature. Then 2 mL of 4% NaOH was added to all the test tubes which were then made up to 5 mL using distilled water. The contents in all the test tubes were vortexed well and they were allowed to stand for 15 minutes at room temperature. The pink colour developed due to the presence of flavonoids was read Spectrophotometrically at 510 nm. Rutin was used as the standard for the quantification of flavonoids. All the experiments were done in triplicates and the results were expressed in Rutin Equivalents (RE).

***In vitro* Antioxidant Assays**

DPPH˙Scavenging Activity

The antioxidant activity of the extract was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH**˙**, according to the method of [6]. Sample extracts at various concentrations were taken and the volume was adjusted to 100 µL with methanol. About 3 mL of 0.004 % methanolic solution of DPPH was added to the aliquots of samples and standards (BHT and Rutin) and shaken vigorously. Negative control was prepared by adding 100 µL of methanol in 3 mL of methanolic DPPH solution. The tubes were allowed to stand for 30 minutes at 27°C. The absorbance of the samples and control were measured at 517 nm against the methanol blank. Radical scavenging activity of the samples was expressed as IC_{50} which is the concentration of the sample required to inhibit 50% of DPPH˙ concentration.

Phosphomolybdenum Assay

The antioxidant activity of samples was evaluated by the green phosphomolybdenum complex formation according to the method of [6]. An aliquot of 100 µL of samples and standards (BHT and rutin) were taken into a series of test tubes and were made up to 300 µL with methanol. About 300 µL methanol taken in a test tube was considered as the blank. All the test tubes were added with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and vortexed well to mix the contents. The mouth of the test tubes were covered with foil and incubated in a water bath at 95°C for 90 minutes. After the samples were cooled to room temperature, the absorbance of the mixture was measured at 695 nm against the reagent blank. Ascorbic acid was used as the reference standard and the results were expressed as milligrams of ascorbic acid equivalents per gram extract.

Superoxide Radical Scavenging Activity

The assay was based on the capacity of various extracts to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin–light–NBT system [6]. About 3 mL of reaction mixture containing 50 mM sodium phosphate buffer (pH-7.6), 20 µg riboflavin, 12 mM EDTA and 0.1 mg NBT was added to 100 µL sample solution, BHT and rutin. Reaction was started by illuminating the reaction mixture with samples for 90 seconds. The illuminated reaction mixture without sample was used as the negative control. Immediately after illumination, the absorbance was measured at 590 nm against the blank (unilluminated reaction mixture without plant sample). The scavenging activity on superoxide anion generation was calculated as:

Scavenging activity (%) = [(Control OD – Sample OD)/Control OD] x 100

Reducing Power Assay

The reducing power of sample extracts was determined according to the method of [6]. Different aliquots of extracts (50–250 mg) and standards (BHT and rutin) were taken into a series of test tubes and were made up to 1ml with methanol. A test tube with 1 mL of methanol served as the blank. Then each test tube including the blank was added with 2.5 ml of 0.2M phosphate buffer (pH-6.6) and 2.5ml of 1% potassium ferric cyanide. The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 650 x g for 10 minutes at room temperature. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Statistical Analysis

All the experiments were done in triplicates and the results were expressed as Mean ± SD.

RESULTS AND DISCUSSION

Extract Recovery Percent

The percentage yield of *Piper schmidtii* leaf and stem extracts of different solvents are presented. The maximum yield for both leaf and stem were obtained in chloroform extract, 1.88 and 1.18%, respectively. These results conclude that the polar solvents could dissolve more constituents in both leaf and stem parts of the plant *P. schmidtii*.

Qualitative Phytochemical Screening

The qualitative phytochemical screening of leaf and stem of *P. schmidtii* for major primary and secondary phytochemicals are shown in Table 1. The results revealed that the primary metabolites such as carbohydrates and proteins showed positive result in all the extracts of both leaf and stem samples.

The results of the qualitative phytochemical screening showed the presence of all the phytochemical constitution of the present study such as alkaloid, tannin, saponins, phenol, flavonol glycosides, cardiac glycosides, phytosterol, fixed oils and fats, gums and mucilage in both the samples. The ++ sign indicates high

concentration of particular secondary metabolites which was indicated by the high intensity of the colour developed.

Table 1: Preliminary Phytochemical Analysis of *P. schmidtii*

Sample	Leaf				Stem			
Extract / Phyto Chemical	P.E	C.F	E.A	E	P.E	C.F	E.A	E
Carbohydrate	+	+	+	++	+	+	+	++
Protein	+	+	+	++	+	+	+	++
Amino acid	+	+	+	++	+	+	+	++
Alkaloids	+	+	++	+	+	+	++	+
Saponins	+	+	+	+	+	+	+	+
Phenol	++	+	+	+	+	+	++	+
Flavonoid	+	+	+	++	+	+	+	++
Glycoside	+	+	+	+	+	+	+	+
Flavonos glycosides	+	+	+	+	+	+	+	+
Cardiac glycoside	++	+	+	+	++	+	+	+
Phyto sterol	+	++	++	++	+	++	++	+
Fixed oils & fats	++	+	+	+	++	+	++	+
Gums &mucilages	+	+	+	+	+	+	++	+

(+): Presence of chemical compound, (-): Absence of chemical compound
(+) < (++) < (+++): Based on the intensity of characteristic colour
P.E-Petroleum ether: C.F-Chloroform: E.A-ethyl acetate: E-Ethanol

Ash values represent the presence of inorganic content in the drug and also determine the purity and quality of drug [7]. The presence of high amount of carbohydrates and proteins in the *P. schmidtii* leaf and stem revealed that it can also be used for nutritional purposes. Alkaloids are known to be the most potent anti-inflammatory agents of the naturally occurring products of secondary metabolism [8]. Saponins are reported to have antibiotic activities [9]. Antifungal activities and antiviral activity [10]. Tannin are reported to have possible anti-carcinogenic effects [11], and are also suggested to play a major role in plants defense against fungi and insects. Apart from the inhibition of digestion, tannins breakdown to produce fatty liver and Gallic acid in the presence of esterase's [12].

Quantitative Analysis

Determination of Total Phenolics and Tannin Contents of *P. schmidtii*

The amount of total phenolics in leaf and stem extracts of *P. schmidtii* were analyzed and the results were shown in Table 2. The total phenolics were found to be higher in ethanol extract of both leaf and stem samples (267.86mg GAE/g extract and 129.75 mg GAE/g extract).The tannins were found to be higher in

ethanol extract of leaf (250.14 mg GAE/g) which is followed by chloroform extract (130.79 mg GAE/g extract). In case of stem the maximum tannin content was reported in ethanol extract (119.3 g GAE/g extract).

Table 2: Total Phenolic and Tannins Contents in *P. schmidtii*

Samples	Extracts	Total Phenolics (mg GAE/g extract)	Tannins (mg GAE/g extract)
Leaf	Petroleum ether	87.2±4.00	83.32±4.6
	Chloroform	140.53±4.16	130.79±3.92
	Ethyl acetate	230.53±4.16	221.3±4.48
	Ethanol	**267.86±3.05**	**250.14±1.78**
Stem	Petroleum ether	34.31±3.15	30.43±3.31
	Chloroform	69.75±0.60	64.25±0.54
	Ethyl acetate	98.17±2.64	90.55±3.16
	Ethanol	**129.75±2.64**	**119.3±4.19**

GAE – Gallic Acid Equivalent

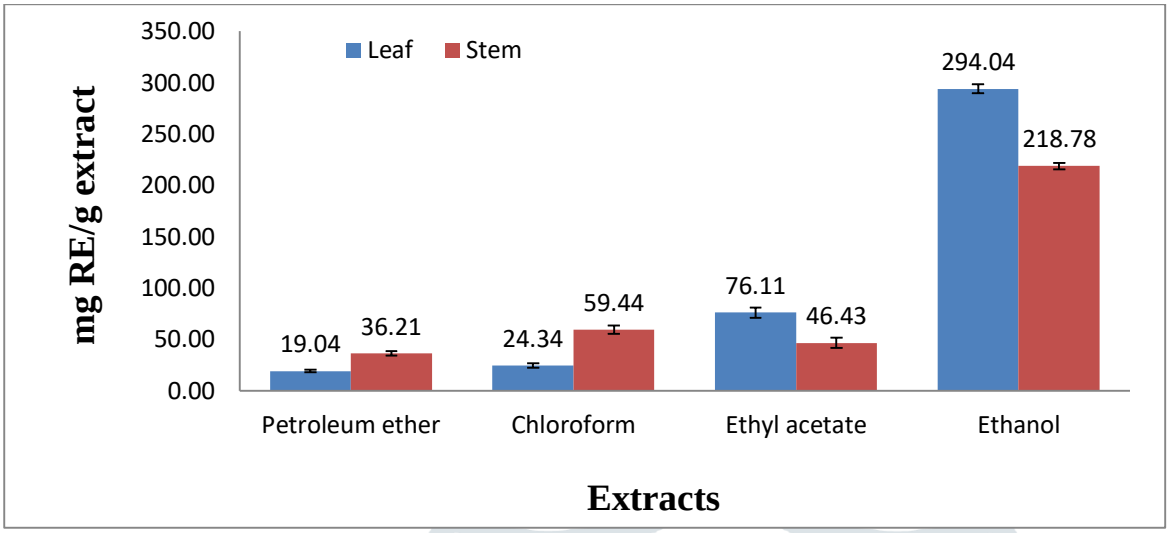
Values are mean of triplicate determination (n=3) ± standard deviation

In many scientific papers it has been discussed that antioxidant capacity can be influenced by total phenolic and anthocyanin content, maturity, and a variety of plant species. The phenolic compounds are the dominant antioxidant components which support strong antioxidant activity and stress response in the many tested plants [13]. Phenolic compounds, tannins and flavonoids have been reported to have multiple biological effects, including antioxidant and anti-inflammatory properties [14]. The higher amount of phenolics and tannin in the ethanol extracts of *P. schmidtii* leaf and stem could be due to its higher solubility and it could be significantly contribute to the antioxidant capacity of that species.

Quantification of Flavonoids

Flavonoids have been intensively studied for their free radical scavenging and antioxidant properties. Flavonoids are reported to possess strong free radical scavenging activities based on their ability to act as hydrogen or electron donors and chelate transition metals [15]. The flavonoid contents in leaf and stem of *P. schmidtii* were analyzed and were presented in Fig.1 and it was found that the ethanol extracts of leaves revealed maximum amount of flavonoid content (294.04 RE/g) followed by ethanol extract of stem (218.78 mg RE /g). Since *P. schmidtii* possess good flavonoid content in leaf and stem, it could be assumed that it can have a higher free radical scavenging activity which involves the transfer of electron or hydrogen atom from flavonoids to free radicals.

Fig. 1: Flavonoid Contents of *P. schmidtii* Leaf and Stem Extracts



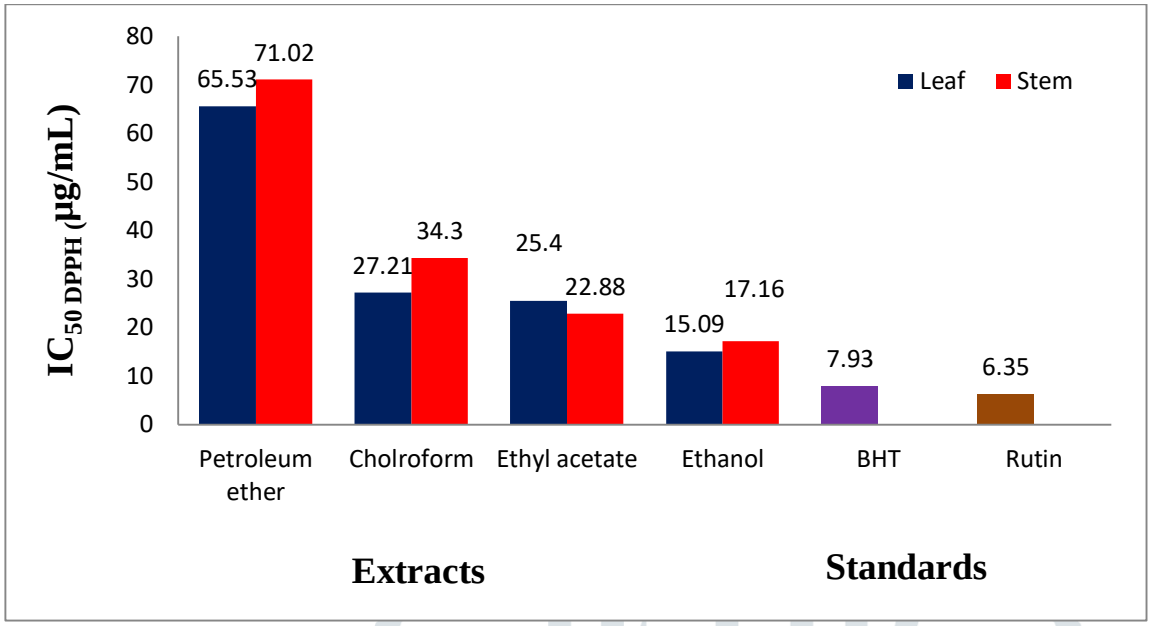
Values are mean of triplicate determination (n=3) ± standard deviation

***In vitro* Antioxidant Assays**

DPPH˙ Scavenging Activity

The DPPH free radical is a stable free radical that has been widely accepted as a tool for estimating the free radical scavenging activity of antioxidant [16]. In the DPPH test, the antioxidants were able to reduce the stable DPPH radical to the yellow-colored diphenylpicryhydrazine. The effect of antioxidant on DPPH radical scavenging was conceived to their hydrogen-donating ability [17]. The DPPH radical scavenging activities of different extracts of leaf and stem of *P. schmidtii* is shown in Fig. 2. The lower value of IC_{50} indicates higher antioxidant activity of the extracts. Among the different parts studied, ethanol extracts of leaf showed appreciable activity (15.09 µg/mL). The IC_{50} of plant extracts were also found to be comparable with the standard Rutin and BHT. The enhanced activity of ethanol extract may be due to the polar nature and extracting ability of phenolic compounds from the parts under study. Thus DPPH assay confirms that the plant *P. schmidtii* contains high amount of antioxidants which can be isolated and purified.

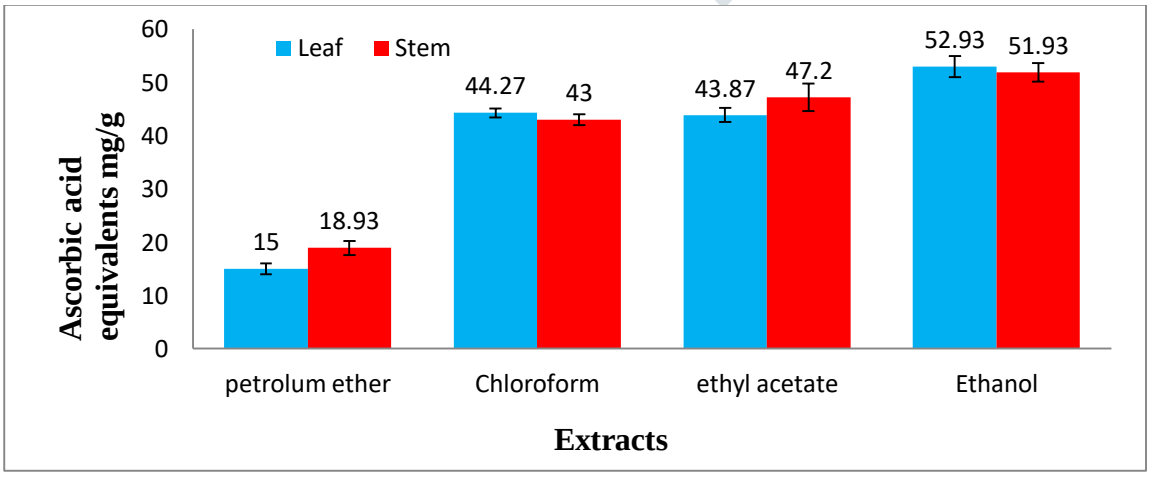
Fig 2: DPPH Radical Scavenging Activity of *P. schmidtii*



Phosphomolybdenum Assay

The phosphomolybdenum assay is successfully used to determine the ability of extracts to reduce Mo (VI) to Mo (V) and subsequent formation of green phosphate/Mo (V) complex at an acid pH. It was also used to quantify vitamin E, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend to plant extracts to analyze the potential of phosphomolybdenum reducing ability [18]. The total antioxidant capacity of different solvent extracts of leaf and stem of *P. schmidtii* were analyzed and shown in Fig.3. The better antioxidant capacity was shown by ethanol extract of both leaf and stem (52.93 mg AAE/g extract and 51.93 mg AAE/g extract).

Fig. 3: Phosphomolybdenum Reduction assay of *P. schmidtii*

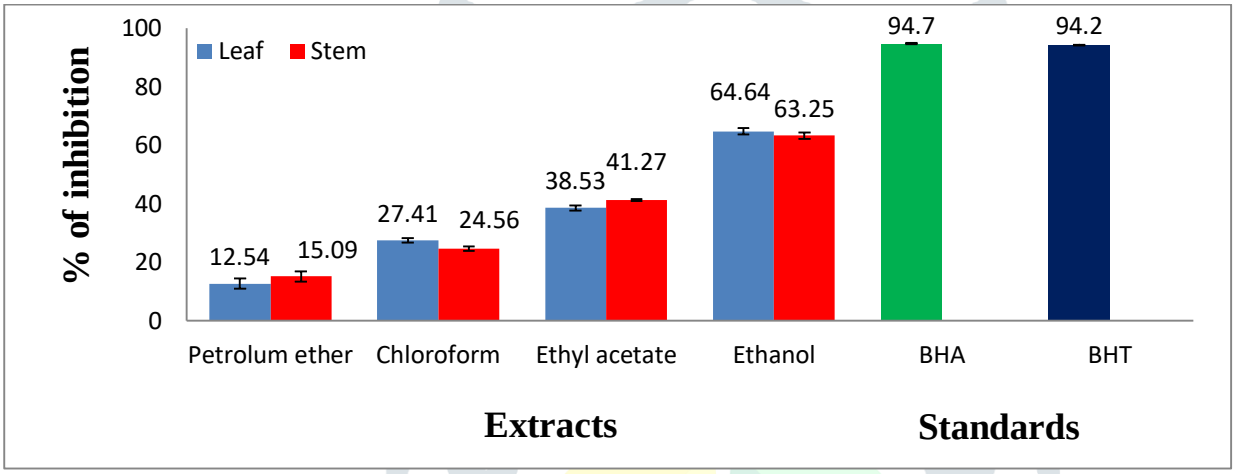


Values are mean of triplicate determination (n=3) ± standard deviation

Superoxide radical Scavenging Activity

The extracts were found to be an efficient scavenger of superoxide radical generated in riboflavin- NBT- light system *in-vitro*. The scavenging activity of ethanol extract of leaf and stem were found to be 64.64% and 63.25%, respectively, whereas, ethyl acetate extracts of leaf and stem were found to be 38.53% and 41.27%, respectively. The ethanol and ethyl acetate extracts showed moderate free radical scavenging activity when compared to the synthetic antioxidants BHT and BHA. From this assay, using different extracts of *P. schmidtii*, it is noted that the extracts inhibited the formation of blue formazan and also the percentage inhibition are directly proportional to the concentration of the plant extract. The percentages of inhibition of all the extracts are showed in the Fig 4. These results clearly suggest that the antioxidant activity of the plant *P. schmidtii* also related to scavenge the superoxide radicals.

Fig. 4: Superoxide Radical Scavenging Activity of *P. schmidtii*



Values are mean of triplicate determination (n=3) ± standard deviation

Superoxide radical acts as a precursor of more reactive oxygen species like hydrogen peroxide, hydroxyl and singlet oxygen and is known to be a very harmful species to cellular components [19]. Although they cannot directly initiate lipid oxidation, superoxide radical anions are potent precursors of highly reactive species such as hydroxyl radical and thus the study of scavenging of this radical is important [20]. Since the methanol extract of leaf and stem of *P. schmidtii* showed appreciable percentage of scavenging activity against superoxide radical, it can be used against adverse effects caused by superoxide radical in the body. The active principles in the plant extracts may eliminate the radical by its reduction to attain the octant stage or through the formation of water molecule.

Reducing Power Assay

The reducing power assay is based on the measurement of reducing potential of crude extract or compound. The reducing power ability (electron donating capacity) is based on the transformation of ferric to ferrous ion in the presence of crude extract or compound [21]. It was suggested that the electron donating capacity is associated with antioxidant activity [22]. The reducing power ability of various extracts and ascorbic acid is summarized in Fig. (5& 6).The reducing power of all the extracts showed a concentration dependent activity from 100-500 µg/mL of samples. Increase in absorbance of the reaction mixture indicated the reducing power of the samples. The reductions present in the extract acts by preventing the peroxide formation or

reacting with free radicals and terminating the free radical chain reaction.In the present study such termination could greatly be contributed by the ethanol extracts.

Fig.5: Reducing Power Assay of *P. schmidtii* Leaf Extract

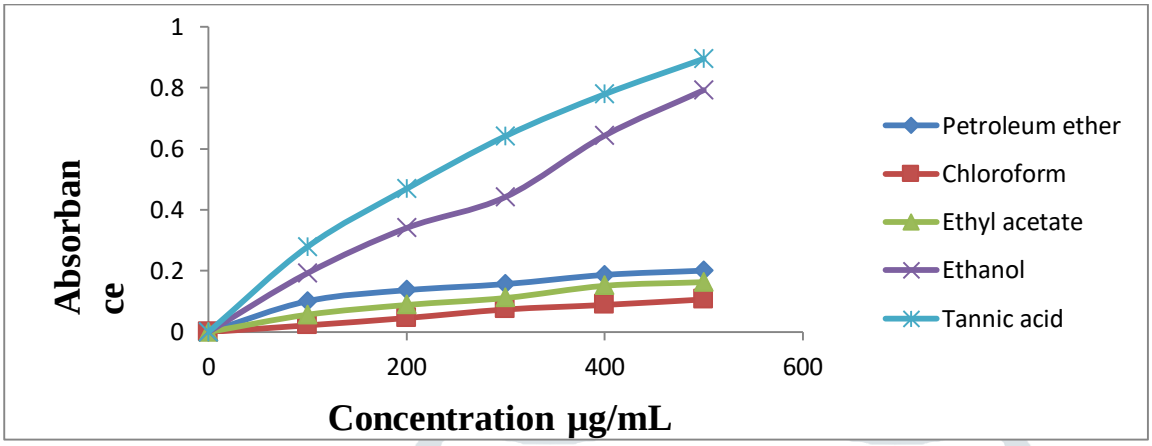
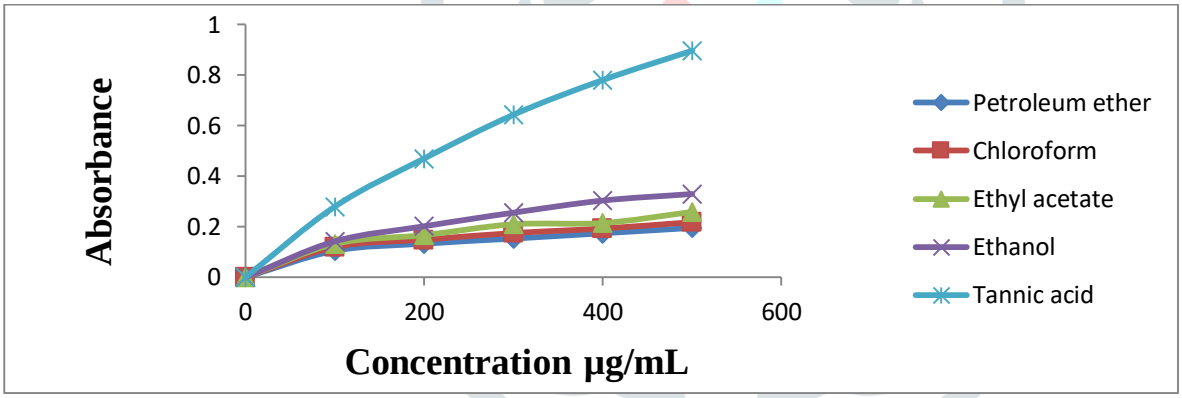


Fig.6: Reducing Power Assay of *P. schmidtii* Stem Extract



Conclusion

The antioxidant activity of *P. schmidtii* has shown to be promising as a food additive to replace synthetic antioxidants. Thus, the study ascertains the value of plants used traditionally, which could be of considerable interest to the development of new drugs. Further studies are warranted for the isolation and identification of individual bioactive compounds and also *in vivo* studies are needed for understanding their mechanism of action as an antioxidant drug which can be a cost effective and reliable source of medicine for the human welfare.

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