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Effect of solvent extraction system on the antioxidant activities of three invasive alien species and quantification of phenolic compounds by HPLC

Sudeshna Datta, BK Sinha, Soumen Bhattacharjee and Tapan Seal

Abstract
The present study aimed to investigate the antioxidant activities of three invasive alien species e.g. *Scoparia dulcis*, *Pilea microphylla* and *Alternanthera paronychioides* collected from West Bengal, India using four different solvent extraction system. The plants were evaluated by using 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, ABTS radical scavenging ability, reducing power capacity, metal chelating activity, lipid peroxidation assay, estimation of total phenolic content and flavonoid content. The solvent systems used were benzene, chloroform, methanol and 70% aq. ethanol. The different levels of antioxidant activities were found in the solvent systems used. The quantitation of phenolic acids and flavonoids in these plants were carried out by HPLC using Acclaim C 18 column (5µm particle size, 250 x 4.6 mm), Dionex Ultimate 3000 liquid chromatograph and detection was carried out in photo diode array (PDA) detector. The HPLC analysis also showed the presence of phenolic acids and flavonoids in various amounts in these plant which could be utilized as natural antioxidant.

Keywords: Invasive alien species, antioxidant activity, solvent extraction system, HPLC

1. Introduction
Plants are commonly to play a beneficial role in health care. It can synthesize a large variety of chemical substances such as phenols, flavonoids, alkaloids, tannins, vitamins, carotenoids etc. which protect cell against the damaging effect of reactive oxygen species (ROS), reduce the oxidative stress in cell and useful in the treatment of many diseases such as cancer, arthritis, aging process, neurodegenerative disorder, cardiovascular disease and diabetic [1]. Therefore the study of plant as a source of medicine has become more important in the context of present global scenario, where oxidative stress is found to be one of the major causes of health hazards. Medicinal plants are considered to play a key role in Health care [2]. About 40% of the Indian flora is alien and 25% of which are Invasive Alien Species (IAS) predominantly of neotropic origin [3-5]. The invasive alien species are ready colonizers in disturbed areas and cause considerable ecological damage to India's natural areas, speed the disappearance of threatened and endemic species, reduce the carrying capacity of pastures, increase the maintenance costs of croplands. At the same time these IAS also contribute as ethnomedicne. Amongst the different IAS identified in West Bengal [3, 6-8] the following three plants *viz*. *Alternanthera paronychioides*, *Pilea microphylla* and *Scoparia dulcis* are being studied for their antioxidant potential.
Leaves of *S. dulcis* have been used as one of remedies for diarrhoea [9] bronchitis and diabetes [10]. Leaf decoction of *A. paronychioides* is used in the treatment of diarrhoea [10]. *P. microphylla* is used on inflammation [11] and to relief pain [12]. It also posses anti-microbial activity and applied on wounds [13]. This paper reports the evaluation of the antioxidant activities of the aerial part of *A. paronychioides*, *P. microphylla* and *S. dulcis* along with a HPLC based identification of phenolic compounds and relate them to the medicinal uses.

2. Materials and Methods
2.1 Plant Materials
The fresh plants (Aerial parts) of *A. paronychioides*, *P. microphylla* and *S. dulcis* were collected from various locations of Kolkata, India and identifications were authenticated from Botanical Survey of India, Howrah. The voucher specimens were preserved in our office. The plant materials taken in our laboratory were shed-dried, pulverized and stored in an airtight container to evaluate the antioxidant properties using four different solvent extraction systems.

2.2 Chemicals

The standards chemicals like phenolic acids (Gallic, caffeic, syringic, p-coumaric, ferulic, and sinapic), flavonoids (catechin, rutin, myricetin, quercetin, apigenin and kaempferol), Ferrozine, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), TPTZ (2, 4, 6-tripyridyl triazine) were purchased from Sigma Chemical Co. (St. Louis, MO, USA), Folin-Ciocalteus's phenol reagent, linoleic acid, hydrochloric acid, sodium hydrogen phosphate, disodium hydrogen phosphate, potassium ferricyanide, potassium per sulphate, aluminium chloride, ferrous chloride, ferric chloride, ammonium thiocyanate and sodium carbonate and the HPLC-grade solvents such as acetonitrile, methanol, water were purchased from Merck (Germany). All the chemicals used including the solvents, were of analytical grade.

2.3 HPLC Equipment

HPLC analyses were performed using Dionex Ultimate 3000 liquid chromatograph including a diode array detector (DAD) with 5 cm flow cell and with Chromeleon system manager as data processor. Separation was achieved by a reversed-phase Acclaim C18 column (5 micron particle size, 250 × 4.6 mm). 20 μL of sample was introduced into the HPLC column.

2.4 Antioxidant activities of the plants in different solvent extraction system

2.4.1 Extraction of plant material

One gram of each plant material was extracted with 20 ml each of benzene, chloroform, methanol and 70% aq. ethanol separately with agitation for 18–24 h at ambient temperature. The extracts were filtered and diluted to 25 ml and aliquot were analyzed for their total phenolic and flavonoid content, reducing power and their *in vitro* radical scavenging capacity.

2.4.2 Estimation of total phenolic content (TPC)

The total phenolic content of plant extracts was determined according to Folin-Ciocalteu procedure [14]. 100 µl of the tested samples were introduced into test tubes; 2.5 ml of Folin-Ciocalteu reagent and 2.5 ml of sodium carbonate (7.5%) were added, incubated for 30 mins in dark. Absorption was measured at 765 nm (UV-VIS spectrophotometer, Shimadzu UV 1800). The total phenolic content was calculated as gallic acid equivalent (GAE) in mg/g dry weight of extract using the following equation based on the calibration curve y = 0.0013x + 0.0498, R^2 = 0.999, where y was the absorbance and x was the Gallic acid equivalent (mg/g).

2.4.3 Estimation of total flavonoid content (TFC)

Total flavonoid content was estimated using the method of Ordonez *et al*. [15]. To 2 ml of sample, 2 ml of 2% $AlCl_3$ ethanol solution was added and incubated for an hour, at room temperature, the absorbance was measured at 420 nm (UV-VIS spectrophotometer, Shimadzu UV 1800). Total flavonoid contents were calculated as rutin equivalent (RE) mg/ g dry weight using the following equation based on the calibration curve: y = 0.0353x + 0.0566, R^2 = 0.9985, where y was the absorbance and x was the quercetin equivalent (mg/g).

2.4.4 Measurement of reducing power

The reducing power of the extracts was determined according to the method of Oyaizu [16]. 100 µl of sample was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. 2.5 ml of 10% trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant was mixed with distilled 2.5 ml water and a freshly prepared 0.5 ml, 0.1% ferric chloride solution. The absorbance was measured at 700 nm (UV-VIS spectrophotometer, Shimadzu UV 1800). Reducing power is calculated as ascorbic acid equivalent (AAE) in mg/ g of dry material using the following equation based on the calibration curve: y = 0.0023x - 0.0063, R^2 = 0.9955, where y was the absorbance and x was the ascorbic acid equivalent (mg/g).

2.4.5 Ferric reducing antioxidant power (FRAP) assay

The ability to reduce ferric ions was measured using the method described by Benzie and Strain [17]. The FRAP working solution was prepared by mixing 300 mM sodium acetate buffer (pH 3.6), 10.0 mM TPTZ (2, 4, 6-tripyridyltriazine) solution in 40 mM HCl and 20 mM $FeCl_3$ solution in a ratio of 10:1:1 (v/v/v). 1 ml of plant extract was then added to 2.85 ml of FRAP working solution. The reaction mixture was vortexed and incubated at 37 °C for 30 min away from light. The increase in absorbance at 593 nm was measured (UV-VIS spectrophotometer, Shimadzu UV 1800). FRAP working solution with deionized water was used as blank. The antioxidant capacity based on the ability to reduce ferric ions of sample is calculated as trolox equivalent (TE) in mg/ g dry material using the following equation y = 0.084x - 0.168, R^2 = 0.996, where y was the absorbance and x was the trolox equivalent (mg/g).

2.4.6 Determination of DPPH radical scavenging activity

The free radical scavenging activity of the plant samples was determined using the stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl) [18]. 100 µl of the tested sample (or water for the control), were placed in test tubes and 3.9 ml of freshly prepared DPPH solution (25 mg L^{-1}) in methanol was added in each test tube and mixed. 30 min later, the absorbance was measured at 517 nm (UV-VIS spectrophotometer, Shimadzu UV 1800). The capability to scavenge the DPPH radical was calculated, using the following equation:

$$\text{DPPH scavenged (\%)} = \{(Ac - At)/Ac\} \times 100$$

where Ac is the absorbance of the control reaction and At is the absorbance in presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value is defined as the concentration in mg of dry material per ml (mg / ml) that inhibits the formation of DPPH radicals by 50%. Each value was determined from regression equation

2.4.7 Scavenging activity of ABTS radical cation

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation ($ABTS^{\cdot+}$) scavenging activity was measured according to the method described by Re *et al* [19]. ABTS was dissolved in water to a 7 mM concentration. The ABTS radicals were produced by adding 2.45mM potassium persulphate (final concentration).The completion of radical generation was obtained in the dark at room temperature for 12–16 h. This solution was then diluted with ethanol to adjust its absorbance at 734 nm to 0.70 ± 0.02. To determine the scavenging activity, 1 ml of diluted $ABTS^{\cdot+}$ solution was added to 100 µl of plant extract (or water for the control), and the absorbance at 734 nm (UV-VIS spectrophotometer, Shimadzu UV 1800) was measured 15 mins after the initial mixing, using ethanol as the blank. The percentage of inhibition was calculated by the equation:

ABTS scavenged (%) = {(Ac – At)/Ac} × 100

where A_c and A_s are the absorbencies of the control and of the test sample, respectively. From a plot of concentration against % inhibition, a linear regression analysis was performed to determine the IC_{50} value of the sample.

2.4.8 Metal Chelating activity

For determination of metal chelating activity, the process of Lin *et al*. [20] was followed. One ml of plant extract was added to 20 µl of 2 mM $FeCl_2$. The reaction was initiated by the addition of 5 mM Ferrozine and the whole reaction mixture was shaken vigorously. The mixture was incubated for 10 min in dark and absorbance was measured at 562 nm (UV-VIS spectrophotometer, Shimadzu UV 1800). The inhibition percentage of ferrozine–Fe^{+2} complex formation was calculated by using the formula given below:

Chelating ability (%) = {(Ac – At)/Ac} × 100

where Ac is the absorbance of the control reaction and At is the absorbance in presence of the sample of the extracts.

2.4.9 Anti-lipid peroxidation in linoleic acid system

Anti-lipid peroxidation was assayed following method of Amabye [21], with modifications. One ml of plant extract was added to a solution of linoleic acid (0.13 ml), 99.8% ethanol (10 ml) and 0.2M sodium phosphate buffer (pH 7, 10 ml). the mixture was made upto25 ml and incubated at 40^0 C upto 360 hours. Extent of oxidation was measured by thiocyanate method. 75% ethanol (10 ml), 30% aq. Solution of ammonium thiocyanate (0.2 ml), 30% sample solution (0.2 ml) and 20mM (in 3.5% HCl) ferrous chloride added sequentially. After 3 mins absorbance was measured using spectrophotometer at 500 nm. A negative control was maintained that contained all the reagents except the extract. Inhibition of peroxidation was calculated using the formula

Inhibition of lipid peroxidation (%) = {(Ac – At)/Ac} × 100

where Ac is the increase of absorbance of the control reaction and At is the increase of absorbance in presence of the sample of the extracts.

All assays were carried out in triplicate and values were obtained by calculating the average of three experiments and data are presented as Mean ± SEM.

2.5 Estimation of phenolic acids and flavonoids by HPLC

2.5.1 Preparation of standard solutions of flavonoids and phenolic acids

The stock solution of concentration 1mg / ml was prepared by dissolving 10 mg gallic acid in 1 ml HPLC-grade methanol followed by sonication for 10 min and the resulting volume was made up to 10 ml with the solvent for the mobile phase (methanol and 0.5% aq. acetic acid 1:9). The same method was followed to prepare the standard stock solutions of the phenolic acids and the flavonoids *viz*. protocatechuic acid, gentisic acid, chlorogenic acid, p-hydroxy benzoic acid, vanillic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, sinapic acid, salicylic acid and ellagic acid, catechin, rutin, myricetin, quercetin, naringin, apigenin and kaempferol. The working standard solutions of concentrations 20, 40, 60, 80 and 100 µg/ml were prepared by further dilution of the standard solution with the mobile phase solvent system. The standard and working solutions were filtered through 0.45 µm PVDF-syringe filter and the mobile phase was degassed before the injection of the solutions.

2.5.2 Chromatographic analysis of phenolic acids and flavonoids

HPLC analyses for the quantification of phenolic acids and flavonoids in the extract was performed following method of Seal *et al*. [22] using Dionex Ultimate 3000 liquid chromatograph including a diode array detector (DAD) with 5 cm flow cell and with Chromeleon system manager as data processor. Separation was achieved by a reversed phase Acclaim C18 column (5 micron particle size, 250 x 4.6 mm). 20 µL of sample was introduced into the HPLC column. The method was validated according to the USP and ICH guidelines. The mobile phase contains methanol (Solvent A) and 0.5% aq. acetic acid solution (Solvent B) and the column was thermostatically controlled at 25ºC and the injection volume was kept at 20 µl. A gradient elution was performed by varying the proportion of solvent A to solvent B. The gradient elusion was 10 % A and 90% B with flow rate 1ml/min to 0.7 ml/min in 27 min, from 10 to 40 % A with flow rate 0.7 ml/min for 23 min, 40% A and 60% B with flow rate 0.7 ml/min initially for 2 min and then flow rate changed from 0.7 to 0.3 ml/min in 65min, from 40 to 44% A with flow rate 0.3 to 0.7ml/min in 70 min, 44% A with flow rate 0.7 to 1ml/min for 10 min duration, solvent A changed from 44% to 58 % with flow rate 1ml/min for 5 min, 58 to 70% A in 98 min at constant flow rate 1 ml/min. The mobile phase composition back to initial condition (solvent A: solvent B: 10: 90) in 101 min and allowed to run for another 4 min, before the injection of another sample. Total analysis time per sample was 105 min. HPLC chromatograms were detected using a photo diode array UV detector at three different wavelengths (272, 280 and 310 nm) according to absorption maxima of analysed compounds. Each compound was identified by its retention time and by spiking with standards under the same conditions. The quantification of phenolic acids and flavonoids in the extracts were carried out by the measurement of the integrated peak area and the contents were calculated using the calibration curve by plotting peak area against concentration of the respective standard sample. The data were reported with convergence limit in triplicate.

3. Results and Discussion

3.1 Extraction yield, total phenolics and flavonoids content

The percentage yields of the four different solvent extracts of *P. microphylla, S. dulcis* and *A. paronychioides* are shown in Table 1. The extraction yield of these plants varied from 0.813% to 4.866 % (Table 1). The 70 % hydro-ethanol followed by methanol proved to be better solvent for extraction of antioxidant compounds from the plants under study. The process of extraction solubilises the phenolic compounds of the plant sample studied and hence it is an essential part of phenol estimation. In this study four different solvent systems of varying polarity *viz*. 70% aq.ethanol, methanol, chloroform and benzene were used as extracting solvent. The solvent polarity and the chemical properties of the sample are two most important factors affecting extraction yield [23]. Based on our results reported here, the highest extraction yield was found with 70 % hydro-alcoholic solvents. This indicates that most of the components in *P. microphylla, S. dulcis* and *A. paronychioides* are water-soluble. The results confirmed that chloroform and benzene were least effective solvent for extraction of antioxidant compounds from the plants studied. Comparative better yield from chloroform extract of *P. microphylla* suggests presence of antioxidant compounds of moderate polarity or extraction

of pigments. The addition of water to organic solvent increases polarity and facilitates the extraction of antioxidant compounds of wide range of polarity [24]. Hydro-alcohol has revealed to be more efficient in extracting phenolic constituents than compared to pure solvent system [25]. High content of antioxidant compounds was obtained with 80 % aq. methanol from plant materials like rice bran, wheat bran, coffee beans, citrus peel and guava leaves [26]. Methanol proved to be a better extracting solvent in case of wild leafy vegetables [27] and wild edible fruits [28]. In the present analysis the differences in the extract yields from the plant materials might be ascribed to the different availability of extractable components [29]. The amount of the antioxidant components extracted from a plant is affected by the plant parts used, extraction procedure and efficiency of the extracting solvent to dissolve the antioxidant compounds [30-31].

Table 1 summarizes the total phenolic content and is expressed as mg gallic acid equivalent (GAE)/ g dry mass (dm). The 70% aq. ethanol extracts of *S. dulcis* (140.319 ± 0.131 mg GAE/g dm), *A. paronychioides* (95.983 ± 0.180 mg GAE/g dm) and *P. microphylla* (50.585 ± 0.268 mg GAE/g dm) were found to contain the highest amount of phenolic content followed by methanol extracts of these plants. The phenolic content can be correlated to the % extractive yield using different solvents. Phenolics are plant secondary metabolites that are very important for their antioxidant activity by chelating redox-active metal ions, inactivating lipid free radical chains and preventing hydroperoxide conversions into reactive oxyradicals [32-33]. It is also suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis [34]. Therefore, it was essential to find the total phenolic content of these plants. The maximum phenolic content in the hydro ethanol extract of *S. dulcis* indicates the high polarity of the phenolics. Results of the present study showed that among all the solvent extracts; the hydro-ethanol extracts had the highest phenolic content

Table 1: Extractive value (%), Total phenolic content (mg GAE/ g dm) and Flavonoid content (mg RE/ g dm) of *P. microphylla*, *S. dulcis* and *A. paronychioides* in different solvent system

	P. microphylla				***S. dulcis***				***A. paronychioides***			
Parameter	**70% aq. ethanol**	**Methanol**	**Chloroform**	**Benzene**	**70% aq. ethanol**	**Methanol**	**Chloroform**	**Benzene**	**70 % aq. ethanol**	**Methanol**	**Chloroform**	**Benzene**
Extractive Value (%)	4.778 ± 0.102	4.038 ± 0.609	3.998 ± 0.117	2.533 ± 0.088	4.866 ± 0.093	4.125 ± 0.128	1.652 ± 0.113	0.813 ± 0.108	3.274 ± 0.838	2.493 ± 0.115	0.986 ± 0.112	1.968 ± 0.080
TPC (mg GAE/ g dm)	50.585 ± 0.268	40.577 ± 0.555	30.705 ± 0.160	24.769 ± 0.444	140.319 ± 0.131	80.997 ± 0.142	61.282 ± 0.641	18.718 ± 1.282	95.983 ± 0.180	68.103 ± 0.256	66.410 ± 0.641	29.359 ± 0.320
TFC (mg RE/ g dm)	9.840 ± 0.033	8.027 ± 0.225	6.623 ± 0.050	5.706 ± 0.018	22.666 ± 0.061	21.608 ± 0.020	17.747 ± 0.137	15.806 ± 0.092	21.708 ± 0.020	18.655 ± 0.048	16.465 ± 0.242	15.925 ± 0.255

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM
TPC : Total Phenolic content ; TFC : Total flavonoid content ; GAE : Gallic acid equivalent ; RE : Rutin equivalent ; dm : Dry mass

Table 2: Reducing activity (mg AAE/ g dm), FRAP (µ mole TE/ g dm) and Metal chelating activity (% inhibition/ g dm) of *P. microphylla*, *S. dulcis* and *A. paronychioides* in different solvent system

	P. microphylla				***S. dulcis***				***A. paronychioides***			
	70% aq. ethanol	**Methanol**	**Chloroform**	**Benzene**	**70% aq. ethanol**	**Methanol**	**Chloroform**	**Benzene**	**70 % aq. ethanol**	**Methanol**	**Chloroform**	**Benzene**
Reducing power (mg AAE/ g dm)	46.980 ± 0.263	36.938 ± 0.479	22.455 ± 0.091	9.551 ± 0.145	25.392 ± 0.074	21.006 ± 0.322	17.717 ± 1.255	16.594 ± 0.725	25.400 ± 0.102	12.014 ± 0.145	9.022 ± 0.628	4.692 ± 0.181
FRAP (µ mole TE/ g dm)	1.498 ± 0.002	1.434 ± 0.002	0.787 ± 0.005	0.615 ± 0.001	2.090 ± 0.003	2.017 ± 0.004	1.789 ± 0.003	0.663 ± 0.001	2.029 ± 0.001	2.017 ± 0.003	1.783 ± 0.004	0.577 ± 0.003
Metal chelating activity (% inhibition/ g dm)	24.324 ± 0.142	18.780 ± 0.141	10.712 ± 0.179	4.973 ± 0.139	50.696 ± 0.164	49.919 ± 0.163	37.577 ± 0.257	13.756 ± 0.139	49.795 ± 0.433	32.602 ± 0.081	15.615 ± 0.148	4.179 ± 0.211

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM
AAE : Ascorbic acid equivalent ; TE : Trolox equivalent.

and it is reduced with decreasing polarity. Phenolics are often extracted in higher amounts in more polar solvents such as aqueous methanol/ethanol as compared with absolute methanol/ethanol [26-28, 30-31]. Studies have also demonstrated the efficacy of ethyl acetate to extract phenolic compounds from onion and citrus peel [35-38].

The flavonoid content is expressed as mg rutin equivalent (RE)/ g dm. The flavonoid content ranged between 22.666 mg RE in 70% aq. ethanol extract *S. dulcis* to 5.706 mg RE in benzene extract of *P. microphylla* (Table 1). Flavonoids are widely distributed group of plant phenolic compounds responsible for the antioxidant activity of the plants. It has been proven to display a wide range of pharmacological and biochemical activities including radical scavenging properties [39-41]. The flavonoid content in *S. dulcis* was maximum in 70% aq. ethanol and it decreased with solvent polarity. Similar observation was made in case of *Torilis leptophylla* where the highest amount of flavonoid content was analyzed in ethanolic extract, followed by that in methanol and benzene [42]. *S. dulcis* contained greater amount of flavonoids in all the extracts studied. The quantity of flavonoids from the solvent extract correlates to the nature of flavonoid present in the plant sample. Non polar solvents extract aglycone flavonoids whereas polar solvents are useful in extracting glycoside flavonoids.

3.2 Reducing property and FRAP

Reducing property is expressed as mg ascorbic acid equivalent (AAE)/ g dm. The reducing property of the solvent extract can be ranked as 70% ethanol > Methanol >

Chloroform> Benzene for all the plants under study (Table 2). The reducing property was recorded to be maximum in *P. microphylla* hydro ethanol extract (46.980 ± 0.263 mg AAE). Reducing property was also studied by FRAP and is expressed as µ mole trolox equivalent (TE)/ g dm and is represented in Table 2. The results indicate that FRAP for both hydro ethanol and methanol extract of all the plants under study were comparable. In the reducing power assay, the presence of antioxidants in the sample results in the reduction of Fe+3 to Fe^{+2} and it reflects the antioxidant potential of the sample. They are electron donors and can reduce the intermediates of lipid peroxidation processes [43]. Highest reducing property is observed in the hydro alcoholic extracts of all the plants which can be attributed to its high content of phenolics in those extracts. The reducing power generally corresponds to high phenolic content [44].

3.3 Metal chelating property

Metal chelating ability is represented as % inhibition of metal ions/ g dm and is represented in Table 2. The hydro-ethanol extracts of both *S. dulcis* (50.696 ± 0.164 %) and *A. paronychioides* (49.795 ± 0.433 %) showed promising metal chelating property (Table 2). Metal ions can initiate lipid peroxidation and start a chain reaction that leads to the deterioration of food [45]. The catalysis of metal ions also correlates with incidents of cancer and arthritis [46]. Ferrous ions, the most effective pro-oxidants, are commonly found in food systems [47]. In the present study, the chelating ability of *P. microphylla, S. dulcis*and *A. paronychioides* extracts was investigated. The chelating capacity of the extracts decreased with the decreasing polarity. Chelating ability can be attributed to the high phenol and flavonoid content in these extract.

3.4 Radical scavenging property using DPPH and ABTS

The antioxidant capacities using DPPH and ABTS assays of various extracts from *P. microphylla, S. dulcis* and *A. paronychioides* were shown in Tables 3. In the DPPH method, the free radical scavenging capacities of the plant studied ranged between 3 – 51% for *S. dulcis*, 4 – 45% for *A. paronychioides* and 4 – 13% for *P. microphylla*. In the ABTS method, the free radical scavenging capacities of the plant studied ranged between 5 – 74% for *S. dulcis*, 13 – 69% for *A. paronychioides* and 1 – 51% for *P. microphylla.* Maximum radical scavenging activity was observed with 70% aq. ethanol and the activity decreased with decreasing polarity. Methanol extracts have previously been reported to have high scavenging activity, followed by acetone and ethanol extracts of fruit powder of citron and blood orange radical scavenging activity [48]. It was also shown that methanol extract exhibited maximum radical scavenging activity (92.5%) at 100 ppm concentration, followed by acetone of green coffee extracts [49]. The data suggests that polar components like phenol and flavonoids of the plant studied contributed to the radical scavenging activity.

3.5 Lipid peroxidation assay

The lipid peroxidation assays of these three plants were studied in 70 % aq. ethanol and methanol extract. The maximum inhibition of lipid peroxidation was observed in *A. paronychioides* represented in Table 4. Amongst the several factors that lead to deteriorated food products, one of the most concerned is lipid auto-oxidation. Lipid peroxidation leads to rapid development of rancid and stale flavours, and it is considered as a primary mechanism of quality deterioration in lipid foods [50]. To protect food against oxidative degradation has prompted the usage of food additives. Synthetic antioxidants, e.g., BHA, are added in food during processing to suppress lipid peroxidation and resulting improved food quality and stability. In addition, lipid peroxidation of cell membrane is associated with various pathological events such as atherosclerosis, inflammation and liver injury [51]. The phenolic compounds and other chemical components may suppress lipid peroxidation through.

Table 3: Radical scavenging activity (% inhibition/ g dm) of *P. microphylla, S. dulcis* and *A. paronychioides* in different solvent system using DPPH and ABTS

	P. microphylla				***S. dulcis***				***A. paronychioides***			
	70 % aq. ethanol	**Methanol**	**Chloroform**	**Benzene**	**70% aq. ethanol**	**Methanol**	**Chloroform**	**Benzene**	**70% aq. ethanol**	**Methanol**	**Chloroform**	**Benzene**
DPPH	13.780 ± 0.691	9.794 ± 0.304	4.313 ± 0.107	4.153 ± 0.040	51.880 ± 0.332	41.571 ± 0.127	31.705 ± 0.127	3.496 ± 0.0.048	45.373 ± 0.263	44.471 ± 0.105	13.882 ± 0.173	4.853 ± 0.278
ABTS	51.092 ± 0.126	30.589 ± 0.105	13.206 ± 0.105	1.949 ± 0.105	74.972 ± 0.111	67.917 ± 0.053	48.025 ± 0.072	5.698 ± 0.139	69.030 ± 0.301	60.009 ± 0.280	28.610 ± 0.468	13.720 ± 0.315

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

Table 4: Anti-lipid peroxidation assay (% inhibition/ g dm) of *P. microphylla, S. dulcis* and *A. paronychioides* in different solvent system

	P. microphylla		***S. dulcis***		***A. paronychioides***	
	70% aq. ethanol	**Methanol**	**70% aq. ethanol**	**Methanol**	**70% aq. ethanol**	**Methanol**
Anti-lipid peroxidation assay	26.555 ± 0.674	24.401 ± 0.058	42.006 ± 0.797	23.836 ± 0.273	54.646 ± 0.089	32.158 ± 0.005

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

different chemical mechanisms, including free radical quenching, electron transfer, radical addition or radical recombination [52]. Though phenolic content in 70% aq. ethanol extract of *S. dulcis* was higher than *A. paronychioides*, the percentage of inhibition of lipid peroxidation was observed to be higher in *A. paronychioides*, this may be due to the chemical structure of phenolic compounds and the availability of phenolic hydroxyl groups which have the capacity to donate their electron or hydrogen thereby forming stable end product.

4. Identification and quantification of different phenolic acids and flavonoids in 70% aq. ethanol extracts of the plants

The 70% aq. ethanol extract of *P. microphylla* showed the presence of quercetin apigenin and kaempferol whereas the 70% ethanol extract of *S. dulcis* contained catechin, chlorogenic acid, caffeic acid, ferulic acid, sinapic acid, rutin, quercetin, apigenin and kaempferol in varying amounts and the presence of chlorogenic acid, syringic acid, ellagic acid, apigenin and kaempferol were detected in 70% aq. ethanol extract of *A. paronychioides*. The quantification data shows greater amount of phenolics and flavonoids in *S. dulcis* compared to *A. paronychioides* and *P. microphylla* as represented in table 5.

Kaempferol possess an array of antioxidant activity and can also prevent the oxidation of low-density lipid proteins indicating a potential protective role in atherosclerosis, consumption of food with kaemferol reduces the chances of gastric cancer [53]. Apigenin, a flavone, reduce the risk of cardiac ailments, neurological syndromes, mutagenesis [54]. *P. microphylla* contains maximum amount of both kaempferol (0.752 ± 0.001 mg/ 100g dm) and apigenin (1.866 ± 0.00 mg/ 100 g dm) compared to *S. dulcis* and *A. paronychioides.*

Chlorogenic acid, an ester of caffeic acid and quinic acid is found in coffee and coffee beans and also found in higher plants. It has been reported to reduce blood sugar levels and potentially exert an anti-diabetic effect [22]. The presence of chlorogenic acid in *S. dulcis* (0.601 ± 0.002 mg/ 100 g dm) would therefore justify its use as anti-diabetic plant ethno medicinally. Chlorogenic acid content in *A. paronichioides* (2.452 ± 0.002 mg/ 100g dm) is higher but there has been no report of its use as an anti-diabetic agent, therefore its potential can further be explored. The consumption of plants containing chlorogenic acid is associated with a lower risk of liver cirrhosis and liver cancer [55].

Table 5: Comparative account of phenolic acids and flavonoids (mg/ 100 g dm) in 70% aq. ethanol extract of *P. microphylla*, *S. dulcis* and *A. paronychioides* by HPLC

Phenolic acids and flavonoids	*P. microphylla*	*S. dulcis*	*A. paronychioides*
Amount mg/100gm dry mass (dm)			
Gallic acid	ND	ND	ND
Protocatechuic acid	ND	ND	ND
Gentisic acid	ND	ND	ND
p-Hydroxy benzoic acid	ND	ND	ND
Catechin	ND	4.989 ± 0.003	ND
Chlorogenic acid	ND	0.601 ± 0.002	2.452 ± 0.002
Vanillic acid	ND	ND	ND
Caffeic acid	ND	0.325 ± 0.001	ND
Syringic acid	ND	ND	5.187 ± 0.002
p-Coumaric acid	ND	ND	ND
Ferulic acid	ND	1.117 ± 0.001	ND
Sinapic acid	ND	0.138 ± 0.002	ND
Salicylic acid	ND	ND	ND
Naringin	ND	3.852 ± 0.002	ND
Rutin	ND	0.234 ± 0.002	ND
Ellagic acid	ND	ND	0.044 ± 0.001
Myricetin	ND	ND	ND
Quercetin	0.114 ± 0.001	0.293 ± 0.002	ND
Naringenin	ND	ND	ND
Apigenin	1.866 ± 0.001	0.217 ± 0.001	0.716 ± 0.001
Kaempferol	0.752 ± 0.001	0.234 ± 0.001	0.669 ± 0.002

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM ND: Not detect

Quercetin display anti-cancer [56] anti-histamine, as also anti-inflammatory activities [57] which mostly follow its antioxidant traits. The dietary sources of quercetin include citrus fruits, apples, onions, parsley, sage, tea and red wine. *P. microphylla* (0.114 ± 0.001 mg/ 100g dm) and *S. dulcis* (0.293 ± 0.002 mg/ 100g dm) both contain quercetin which potentially contributes to the anti-inflammatory action of these plants and supports the ethnomedicinal uses recorded.

Syringic acid is found in fruits and is well known for its anti-cancer, anti-proliferative, and hepato-protective actions [22]. Ellagic acid possess antioxidant and antiproliferative activity [58] and acts as a chemopreventive agent [59]. *A. paronychioides* contains syringic acid (5.187 ± 0.002 mg/ 100 gdm) and ellagic acid (0.044 ± 0.001 mg /100 g dm) which justifies itsantioxidant property and also signifies the need of a detailed study on its anti-cancer and anti-proliferative activity.

Catechin is detected only in *S. dulcis* (4.989 ± 0.003 mg/ 100g dm) in high amount which may contribute to its antioxidant property. Caffeic acid is a well- known antioxidant [60], controls lipid levels in blood and anti-mutagenic [22]. The present study showed that only *S. dulcis* to contain caffeic acid. Ferulic acid is present in both the plant extract and is known for its anti-microbial, anti-inflammatory, anti-cancer activities etc [61]. Sinapic acid showed antioxidant, anti-microbial, anti-inflammatory, anticancer, and anti-anxiety activity [57]. Naringin has been proven to have anti-infammatory and anticancerous activity [62]. Ferulic acid (1.117 ± 0.001 mg/ 100g dm) and sinapic acid (0.138 ± 0.002 mg/ 100 g dm) are detected only in *S. dulcis* in appreciable amount and also justifies the use of this plant in cough and cold and other bronchial complaints. Rutin is a flavonol with biological effects, such as antidiabetic effect [63] and anticancer activity [64] and can potentially be used as a therapeutic agent.

5. Conclusion

To conclude, the findings of the present study support that the crude ethanolic extract of aerial parts of *S. dulcis*, *P. microphylla* and *A. paronychioides* indicated strong antioxidant activity which might be helpful in preventing or

slowing the progress of various oxidative stresses induce diseases such as ulcer, diabetes, which would be beneficial to the human health. This may be related to the high amount of phenolic, flavonoid and tannin compounds present in this plant extract. Further studies are needed to clarify the *in vivo* potential of this plant in the management of human diseases resulting from oxidative stress.

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7. References

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