IN VITRO STUDY OF THE ANTIOXIDANT POTENTIAL OF SOME TRADITIONALLY USED MEDICINAL PLANTS OF NORTH-EAST INDIA AND ASSESSMENT OF THEIR TOTAL PHENOLIC CONTENT

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Summary

The antioxidant activity of four traditionally used medicinal plants found in the North East region of India was determined. The methanol and aqueous extracts of the plants were evaluated for their *in vitro* scavenging effects on 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and the reactive oxygen species (ROS), hydroxyl ion (OH•), superoxide anion ($O_2^{\bullet -}$) free radicals and hydrogen peroxide (H_2O_2). The methanol extracts exhibited varying DPPH and H_2O_2 scavenging activity with *Osbeckia chinensis* (IC_{50}= 12.74 ± 0.35 µg/ml) showing the highest activity against DPPH and *Potentilla fulgens* (IC_{50}= 40.5 ± 1.78 µg/ml) against H_2O_2. The aqueous extract of *Potentilla fulgens* was found to be the most active in scavenging $O_2^{\bullet -}$ radicals (IC_{50}= 14.68 ± 1.07 µg/ml) while the aqueous extract of *Flemingia macrophylla* comparatively exhibited the highest scavenging activity against OH• radicals (IC_{50} = 190.64 ± 4.4 µg/ml). The total phenolic content of these extracts determined by the Folin Ciocalteu method ranged between 12.78 ± 0.22 and 529.3 ± 18.5 mg GAE/g dry weight of extract. *Potentilla fulgens* had the highest total phenolic content in both methanol and aqueous extracts with 529.3 ± 18.5 and 412.4 ± 1.6 GAE/g dry weight of extract, respectively. Results indicate that these plants have significant albeit variable antioxidant activity and phenolic content. There was no definite correlation between their antioxidant activity and total phenolic content.

Keywords: Antioxidant activity, reactive oxygen species, free radical scavenging effects, total phenolic content.

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Introduction

Reactive oxygen species (ROS) including superoxide (O_2^-) radicals, hydroxyl (OH·) radicals and hydrogen peroxide (H_2O_2) are generated as byproducts of biological reactions such as the mitochondrial respiratory chain or from exogenous factors or environmental stresses [1,2]. They are continuously produced during normal physiological processes and removed by antioxidant defense mechanisms [3] thereby establishing a balance between generation of ROS and their removal by the antioxidant system in an organism. Mammalian cells possess elaborate defense mechanisms for free radical detoxification involving metabolic enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which destroy toxic peroxides [1]. Non-enzymatic molecules, including thioredoxin, thiols, vitamins E, C and trace metals, such as selenium function as direct scavengers of ROS [4]. Under pathologic conditions, ROS are overproduced causing damage to cell membranes and DNA, inducing oxidation that causes membrane lipid peroxidation, decreased membrane fluidity, and DNA mutations leading to cancer [5-7] and many age-related chronic diseases including atherosclerosis, diabetes mellitus, rheumatoid arthritis and neurodegenerative diseases [8,9]. Oxidative stress occurs when the rate of ROS generation exceeds the capacity of the cellular mechanisms for their removal.

Antioxidant- based drugs and formulations for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer have appeared during the last three decades [10,11]. This has attracted a great deal of interest in natural antioxidants. Natural antioxidants have been reported to have a wide range of biochemical activities, including inhibition of ROS generation, direct or indirect scavenging of free radicals, and alteration of intracellular redox potential [12]. Further, many of the natural antioxidants are present in everyday food constituting a part of the developing nation's daily dietary intake which is within the reach of the majority. Therefore, in recent years, considerable effort has been directed towards identifying naturally occurring substances and food components that can protect against oxidative stress [13] as in the case of curcumin [14]. In addition, natural extracts with proven antioxidant activity have been reported to contain compounds with a phenolic moiety, for example, coumarins,

flavonoids, tocopherols, catechins [15,16]. Organic acids, carotenoids, protein hydrolysates and tannins can also be present and act as antioxidants or have a synergistic effect with phenolic compounds [17].

Plants selected for this study are commonly found in the North East region of India and have been used as folk remedies by local practitioners for a variety of ailments. We have earlier reported the anti-diabetic activity of *P. fulgens* [18], *O. chinensis* [19] and *F. macrophylla* [20] showing their hypoglycemic and anti-hyperglycemic properties in mice. Medicinal properties of various species of the genus *Solanum* [21,22] have also been reported. This investigation was undertaken to screen the methanol and aqueous extracts of these plants for their antioxidant activity using four *in vitro* tests, the DPPH free radical scavenging activity, hydroxyl radical (OH•), superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide scavenging methods. In addition, the total phenolic content of the plant (s) methanol and aqueous extracts was determined and a possible correlation with their antioxidant activity was examined.

Materials and methods

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), thiobarbituric acid (TBA), Folin-Ciocalteu reagent, rutin and gallic acid were procured from Sigma-Aldrich (St. Louis, USA), while all other chemicals used were of analytical grade obtained from SRL, Hi-media and Rankem, India.

Plant materials

P. fulgens was collected from Shillong peak area of Meghalaya; *O. chinensis*, *F. macrophylla* and *S. kurzii* were collected from NEHU campus, Shillong, Meghalaya. Each plant specimen of *P. fulgens* (voucher no. 464), *O. chinensis* (voucher no. 71), *F. macrophylla* (voucher no. 5452) and *S. kurzii* (voucher no. 2260) was submitted and identified by herbarium curator Dr. P.B. Gurung, Department of Botany, NEHU, Shillong.

Preparation of plant extracts

Plant(s) extracts were prepared in accordance with the method used earlier [18-20]. The roots of *P. fulgens* and *O.* chinensis; leaves of *F. macrophylla* and fruits of *S. kurzii* were separately washed, shredded and dried in the shade and then powdered using a grinder. One half of the dried powder was repeatedly extracted with 10 volumes of aqueous-methanol solution (1:4) [23]. The mixture was filtered and the filtrate was dried under vacuum in a rotary evaporator (Yamato RE800) yielding the crude methanol extract. The other half of the respective powdered sample was extracted with 10 volumes of distilled water, mixture was filtered and the filtrate was lyophilized to obtain the dry crude aqueous extract. Both methanol and aqueous extracts were accordingly used for investigation.

DPPH radical scavenging method

The DPPH radical scavenging potential of the plant extracts was measured using the method described by Brand-Williams et al. (1995) [24] with some modifications. 1 ml of the methanol extract of the plant of varying concentrations (5-1600 µg/ml) was added to 2 ml of a solution of DPPH in methanol (0.004%). The mixture was shaken vigorously and allowed to stand for 30 min at room temperature. The absorbance (Abs_{sample}) of the resulting solution was measured at λ 517 nm against a blank solution that contained 2 ml methanol and 1 ml plant extract (Abs_{blank}). A solution containing 2 ml DPPH and 1 ml methanol was used as the control ($Abs_{control}$). Ascorbic acid, gallic acid and rutin were used as standards.

The absorbance of the solution was converted into percentage of antioxidant activity (AA%) using the formula:

$$AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\}$$

The radical scavenging activity was expressed in terms of the amount of antioxidants necessary to decrease the initial DPPH absorbance by 50% (IC_{50}). A lower IC_{50} value corresponds with a higher antioxidant power.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging potential of the plant extracts was determined using the deoxyribose assay [25,26]. The reacting mixture in a final volume of 1 ml contained the following reagents: 200 µl KH_2PO_4-KOH (100 mM), 200 µl deoxyribose (15 mM), 200 µl $FeCl_3$ (500 µM), 100 µl EDTA (1 mM), 100 µl aqueous extract of the plant at various concentrations (10-1000 µg/ml), 100 µl H_2O_2 (10 mM) and 100 µl ascorbic acid (1 mM). Reaction mixtures were incubated at 37ºC for 1h.

After incubation 1 ml of 1% (w/v) thiobarbituric acid (TBA) was added to each mixture followed by the addition of 1 ml 2.8 % trichloroacetic acid (TCA). The solutions were heated in a water bath at 80ºC for 20 min to develop the pink coloured malondialdehyde, MDA-$(TBA)_2$ adduct. The solutions were cooled and absorbance was measured at λ 532 nm against an appropriate blank solution. Mannitol was used as the control.

Percent inhibition of deoxyribose degradation by hydroxyl radical to MDA was calculated and the IC_{50} value was determined.

$$\% \text{ Inhibition} = 1 - (\text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100$$

Scavenging of superoxide anion radical

The superoxide scavenging activity was measured using the method of Gulcin et al. (2006) [27] by monitoring the superoxide-induced reduction of NBT to the blue chromogen diformazan. $O_2^{\bullet-}$ radicals were generated by the phenazine methosulphate (PMS)/NADH system. In these experiments, superoxide radicals were generated in 3 ml of Tris-HCl buffer (16 mM, pH 8.0) containing 1 ml of NBT (50 µM) solution, 1 ml NADH (78 µM) solution and various concentrations of the aqueous extract (1.25-750 µg/ml) dissolved in water. The reaction was triggered by adding 1 ml of PMS solution (10 µM) into the mixture. The reaction mixture was incubated at 25ºC for 5 min, and the absorbance at λ 560 nm was measured against a blank sample. Ascorbic acid and gallic acid were used as the reference standards. Decreased absorbance of the reaction mixture indicates increased superoxide anion radical scavenging activity.

The percentage inhibition of superoxide anion generation was calculated using the formula:

$$\% \text{ Inhibition} = 100 - [(\text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100]$$

where $\text{Abs}_{\text{sample}}$ is the absorbance of the solution containing the plant extract and $\text{Abs}_{\text{control}}$ is the absorbance of the solution containing all reagents without the extract.

Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging potential of the plant extracts was determined using the method described by Jayaprakasha et al. (2004) [28]. A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffer saline (PBS, pH 7.4). Different concentrations of the extract (10-800 µg/ml) in methanol (1 ml) were added to 2 ml of hydrogen peroxide solution in PBS. After 10 min the absorbance was measured at λ 230 nm against a blank solution that contained extracts in PBS without hydrogen peroxide. The control contained hydrogen peroxide solution without the extract.

The percentage of H_2O_2 scavenging of the plant extracts was calculated as follows:

$$\% \text{ scavenged } [H_2O_2] = [(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{control}}] \times 100$$

Estimation of total phenolic content

Total phenolic content was determined using a Folin-Ciocalteu reagent according to the procedure described by Singleton and Rossi (1965) [29] with some modifications. The extract (1 ml) was mixed with 5 ml Folin-Ciocalteu reagent (previously diluted 1:10 with distilled water). After a period of 3-8 min, 4 ml of 7.5% sodium carbonate solution was added. The solution was kept in the dark at room temperature for 2h; the absorbance was then measured at λ 740 nm. A calibration curve of gallic acid was prepared and the results were expressed as mg GAE (gallic acid equivalent)/ g dry weight of extract.

Results and Discussion

The various plant(s) extracts exhibited a wide range of scavenging activity against the ROS tested, represented by their corresponding IC_{50} values (Table 1).

DPPH radical scavenging activity

The method based on the scavenging activity of the stable radical 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) has been used extensively to predict the antioxidant activities of extracts of plants [24,30]. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [31]. Methanol extracts of the plants showed varying DPPH scavenging activity with *O. chinensis* exhibiting the highest scavenging activity with an IC_{50} value of 12.74 ± 0.35 µg/ml. Although the activity was comparable but it was not as high as that of the reference standards used. Activity was found to be concentration dependent. The scavenging capacity of the extracts against DPPH under the experimental conditions was found to decrease in the following order: *O. chinensis* > *P. fulgens* > *F. macrophylla* > *S. kurzii.*

Hydroxyl radical scavenging activity

Hydroxyl radicals are extremely reactive species having been implicated as highly damaging in free radical pathology, capable of damaging almost every molecule found in living cell [32]. They form lipid peroxides, organic radicals and add directly to compounds [2] like nucleotides in DNA and causes strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity [33]. In this assay, OH• radicals generated by a Fenton system ($FeCl_2$/EDTA/H_2O_2) attack the sugar deoxyribose and set off a series of reactions that result in the formation of malondialdehyde (MDA) [25]. The ability of the extracts to protect sugar deoxyribose from degradation to MDA by scavenging OH• radicals was in the following decreasing order: *F. macrophylla* > *S. kurzii* > *O. chinensis* > *P. fulgens*. The aqueous extract of *F. flemingia* with an IC_{50} value of 190.64 ± 4.4 µg/ml showed to be the most potent in scavenging OH• radicals. Its value was lower than that of the

standard mannitol (324.8 ± 23.85 µg/ml), indicating a higher antioxidant power.

Superoxide anion radical scavenging activity

Superoxide anions ($O_2^{\bullet-}$) are precursors for more active free radicals that have potential for reacting with biological macromolecules and thereby inducing tissue damage [27]. It has been reported that antioxidant properties of some flavonoids are effective mainly by scavenging $O_2^{\bullet-}$ radicals [34]. In the assay, superoxide radicals were generated in PMS-NADH system by oxidation of NADH and assayed by the reduction of NBT. Antioxidants are able to inhibit the blue NBT formation. All plant(s) extracts with the exception of *S. Kurzii* exhibited significant scavenging activity with the highest activity shown by *P. fulgens* having an IC_{50} value of 14.68 ± 1.07 µg/ml comparable to the reference standards, ascorbic acid (11.53 ± 0.33 µg/ml) and gallic acid (9.15 ± 0.17 µg/ml). The activity in decreasing order observed in this assay was *P. fulgens* > *F. macrophylla* > *O. chinensis* > *S. kurzii*.

Hydrogen peroxide scavenging activity

Hydrogen peroxide, although actually not a radical, is a weak oxidizing agent that is classified as an ROS because it can generate the very reactive OH• radicals in the presence of transition metal ions. There is increasing evidence that H_2O_2, either directly or indirectly via its reduction product, OH•, can act as a messenger molecule in the synthesis and activation of several inflammatory mediators [35]. Thus its removal is important for antioxidant defense in cells. In the assay against H_2O_2 , the methanol extract of *P. fulgens* was found to be the most active in the hydrogen peroxide scavenging assay with an IC_{50} value of 40.5 ± 1.78 µg/ml, which was more potent than even the standards used. The scavenging activity of the extracts decreased in the order of *P. fulgens* > *O. chinensis* > *F. macrophylla* > *S. kurzii.*

Plants	IC_{50} values ± SEM (µg/ml)			
	DPPH	OH·	$O_2^{\bullet-}$	H_2O_2
P. fulgens	21.9 ± 0.1	840.7 ± 43.87	14.68 ± 1.07	40.5 ± 1.78
O. chinensis	12.74 ± 0.35	662.3 ± 3.03	31.63 ± 0.24	42.05 ± 0.55
F. macrophylla	113.43 ± 1.4	190.64 ± 4.4	25.12 ± 0.072	87.06 ± 0.84
S. kurzii	1243.88 ± 1.54	316.69 ± 10.4	303.5 ± 2.8	540.22 ± 4.92
Reference Standards				
Ascorbic acid	7.31 ± 0.015	—	11.53 ± 0.33	227.8 ± 6.8
Gallic acid	3 ± 0.09	—	9.15 ± 0.17	58.64 ± 0.42
Rutin	10.15 ± 0.05	—	—	77.91 ± 0.48
Mannitol	—	324.8 ± 23.85	—	—

Table 1. *In vitro* antioxidant activity of the four medicinal plants. Values are reported as mean ± SEM. All experiments were carried out in triplicates. The procedure for methanol and aqueous extraction has been described in the materials and methods section.

Total phenolic content

The comparative evaluation of total phenolic content (TPC) in both methanol and aqueous extracts of the plants is shown in Figure 1. All the plants showed higher TPC in methanol extracts than in aqueous extracts. The highest total phenolic content was observed in the methanol extract of *P. fulgens* (529.3 ± 18.5 mg GAE/g dry weight of extract); elevated level was also seen in *O. chinensis* (427.21 ± 2.88 mg GAE/g dry weight of extract), moderate level in *F. macrophylla* (209.8 ± 3.79 mg GAE/g dry weight of extract) and significantly low level in *S. kurzii* (20.91 ± 0.09 mg GAE/g dry weight of extract). Aqueous extracts of the plants comparatively showed lower concentration of total phenolics ranging between 12.78 ± 0.22 and 412.4 ± 1.6 mg GAE/g dry weight of extract (Figure 1).

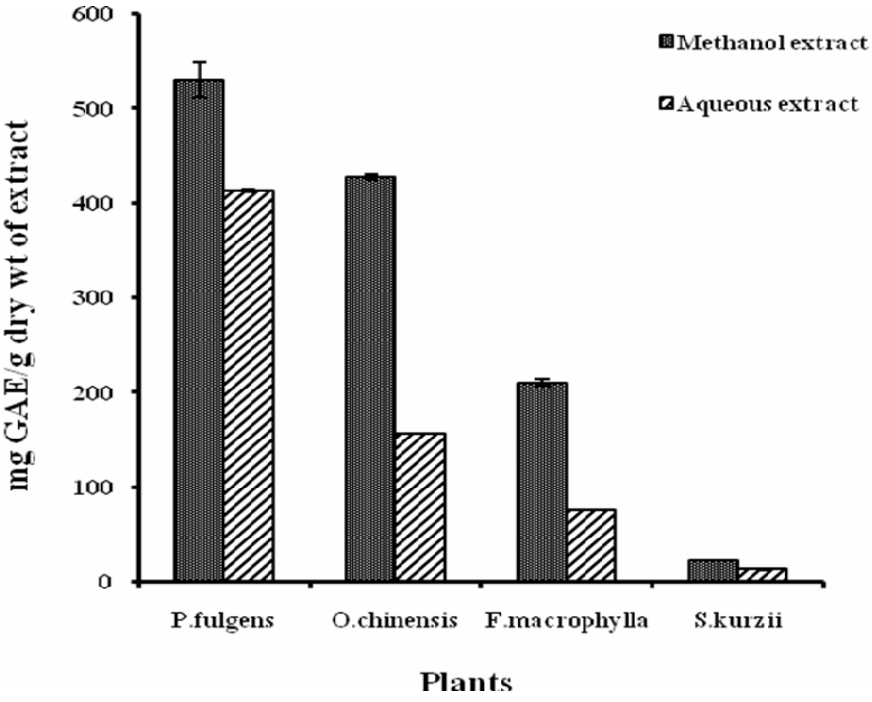

Figure 1. Total phenolic content (TPC) of the methanol and aqueous extracts of the plants. Results are expressed as mg GAE/g dry weight of extract. Values are reported as mean ± SEM. All experiments were carried out in triplicates.

Conclusion

From the results, it is evident that the extracts of the four plants tested show considerable antioxidant potential *in vitro*. Having reported earlier the anti-diabetic properties of *O. chinensis* and *F. macrophylla* [19,20], their antioxidant activities observed in this study is a strong indication of their medicinal potential. Given that *S. kurzii* exhibited a greater scavenging capacity against OH• radicals compared to that of *P. fulgens* and *O. chinensis*, a further study of this plant is required. Our earlier studies on *P. fulgens* have indicated its anti-diabetic, anti-tumor and anti-hyperlipidemic properties in mice [18,36,37]. The results of this study on *P. fulgens* strongly favour this plant for being considered as a potential source for a plant-derived medicinal compound. It has been earlier reported that the antioxidant activity of many compounds of botanical origin is proportional to the TPC, suggesting a causative relationship

between TPC and antioxidant activity [38]. Our study shows no clear correlation between antioxidant activity and TPC of these plants, however the higher TPC in methanol extracts of *P. fulgens* and *O. chinensis* as a contributing factor to their antioxidant activity against DPPH and H_2O_2 cannot be ruled out at this stage. The results of this study suggest that all four plant species may be added to the list of wild edible plants with antioxidant potential and therefore merit further phytochemical analysis.

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