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Antioxidative properties of the leaves of *Daphniphyllum chartaceum* Rosenthal

Swarnendu Roy[1]* and Sukanta Majumdar[2,3]

[1]Department of Botany, Kurseong College, Dow Hill Road, Kurseong, West Bengal, India.
[2]Department of Botany, Darjeeling Government College, Darjeeling, West Bengal, India.
[3]Department of Botany, APC Roy Government College, Matigara, Siliguri-10, West Bengal, India.

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***In vitro* antioxidative properties of methanol, chloroform and diethyl ether extracts of the leaves of *Daphniphyllum chartaceum* Rosenthal. were examined spectrophotometrically. *In vitro* antioxidant activity of all the extracts were determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity assay, reducing power assay, hydrogen peroxide (H_2O_2) scavenging activity and total phenol content. Among all the three extracts, diethyl ether showed highest DPPH scavenging (32.78±0.11) at a concentration of 1000 µg/ml and reducing power activity (0.908±0.013). The methanolic extract exhibited higher phenolic content (0.19±0.013 mg/ml catechol equivalent) and as a result, there was an enhanced H_2O_2 scavenging activity (27.93±0.47), followed by the chloroform extract. The observations from this study suggest the potentiality of *D. chartaceum*, which could be commercially exploited by the pharmaceutical industry as a natural antioxidant.**

Key words: *In vitro* antioxidative activity, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay, reducing power assay, natural antioxidant.

INTRODUCTION

Daphniphyllum chartaceum Rosenthal., a type genus of the family Daphniphyllaceae is an evergreen dioecious tree growing up to above 6 m. It is locally known as dhodey-chandan. The plant is geographically distributed from Eastern Asia to Central Asia and Eastern Himalayas to Northern Burma in an altitude between 1200 to 3000 m (Gamble, 1922). The plants related to the genus *Daphniphyllum* are reported to be used in folklore medicines in South-East Asia and Southern China for the treatment of various ailments. Many of the plants of this genus are used in the treatment of asthma, cough, rheumatism, inflammation, fever, fractures and snake bites (Kothiyal et al., 2011). Recently, few members of the genus become famous for their anti-tumour, antioxidant, anti-platelet aggregation, vasorelaxant and insecticidal properties (Zhen et al., 2009). It also has some aesthetic and religious uses in this region (Avasthe et al., 2004). Over 200 *Daphniphyllum* alkaloids have been isolated from the different species of the genus which are biosynthesized from six molecules of mevalonic acid (Niwa et al., 1973).

All living organisms require oxygen for its existence, which is a highly reactive molecule that damages living organisms by producing reactive oxygen species (ROS) (Davis, 1995). Consequently, the organisms contain a complex network of antioxidant metabolites and enzymes that work together to prevent oxidative damage to cellular components such as DNA, proteins and lipids.

*Corresponding author. E-mail: swarnendu.kc@gmail.com. Tel: 0353 2344243, +919474514100.

In general, antioxidant systems either prevent these reactive species from being formed or remove them before they can damage vital components of the cell (Sies, 1997) which can otherwise lead to several human diseases such as cancer, stroke, myocardial infraction, diabetes and increase the rate of aging. In recent years, much attention has been devoted to natural antioxidant and their associations with health benefits (Arnous et al., 2007). Plants are potential sources of natural antioxidants. Natural antioxidants tend to be safer and they also possess antiviral, antiinflammatory, anti-cancer, anti-tumor and hepatoprotective properties (Arnous et al., 2007). However, the medicinal properties and antioxidative properties of the different species of the genus *Daphniphyllum* have been worked upon; there are no records for the species taken in the context of our study. Therefore, the aim of the study was to reveal and explore the *in vitro* antioxidative properties of the species.

MATERIALS AND METHODS

Leaves of *D. chartaceum* were collected from Dow Hill region of Kurseong, Darjeeling (Altitude – 1468mts), a part of the Eastern Himalayas. A flowering twig was dried and submitted to NBU herbarium (Accession No.-9623).

Preparation of plant extracts

The leaves were first detatched from the petioles and branches and cleaned with distilled water. The leaves were then shade dried for about 4 weeks and then powdered in a mortar and pestle. The coarsely powdered leaves were then exracted with three standard solvents: methanol, chloroform and diethyl ether following standard extraction protocol (Galvez et al., 2003). Briefly, about 5 gm of powdered leaves were extracted with 50 ml of the three solvents by Soxhlet for 48 h. After filtration, each extracts were concentrated to dryness under reduced pressure. The dried extracts thus obtained were weighed and dissolved in about 2 ml of the same solvents and stored in vials at 0°C for the analysis of antioxidative activity. The extractive values of the plant material for all the solvents were calculated by the formula as follows:

$$\text{Solvent extractive value (\%)} = \frac{\text{Weight of dry extract (gm)}}{\text{Weight of plant material taken for extractiont (gm)}} \times 100$$

Phytochemical screening

All the extracts were subjected to phytochemical screening for the presence of alkaloids, flavonoids, saponins, tannins, steroids and terpenes according to standard procedures (Trease and Evans, 1989).

Antioxidative property assay

Total phenol content

Total phenol content of the plant extracts were determined according to standardized method of Mahadevan and Sridhar (1986). To 1 ml of each extract at different concentrations (200, 400, 600, 800 and 1000 µg/ml), 1 ml of Folin-ciocalteau and 2 ml of 20% Na_2CO_3 reagent was added and mixed thoroughly. Mixtures were placed in boiling water bath for 1 min. These were then cooled under running tap water, the final volume was made up to 25 ml and the absorbance was measured at 670 nm in a colorimeter. The total phenol content is expressed as mg/ml catechol equivalent.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay

The antioxidant activity of the plant extracts were assessed on the basis of the radical scavenging effect of the stable DPPH free radical (Blois, 1958). About 100 µl of each extract at different concentrations (200, 400, 600, 800 and 1000 µg/ml) was added to 2.9 ml of DPPH in methanol (0.33%) in different test tubes. After incubation at 37°C for 30 min, the absorbance of each solution was determined at 517 nm using spectrophotometer (Hwang et al., 2001). Methanol was used as blank. The percentage of free radical scavenging activity was calculated as follows:

$$\text{\% inhibition of DPPH radical} = \frac{\text{Abs. of control} - \text{Abs. of test sample}}{\text{Abs. of control}} \times 100$$

Reducing power assay

Reducing power assay of the plant extracts were carried out as described previously (Yildirim et al., 2001). One (1) ml of each extract at different concentrations (200, 400, 600, 800 and 1000 µg/ml) was mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml 1% potassium ferricyanide [$K_3Fe(CN_6)$]. Then the mixture was incubated at 50°C for 20 min. After that, 2.5 ml of 10% trichloroacetic acid was added to the mixture, and then centrifuged at 1500 rpm for 10 min. Finally, to the supernatant solution 0.5 ml of 1%, $Fecl_3$ solution was added and absorbance was measured at 700 nm using a spectrophotometer. Phosphate buffer was used as blank solution. Increased absorbance indicates increased reducing power.

Hydrogen peroxide (H_2O_2) radical scavenging activity

The ability of the extract to scavenge H_2O_2 was determined according to the standardized method (Ruch et al., 1989). A solution of hydrogen peroxide (2 mmol/l) was prepared in phosphate buffer (pH 7.4). Two (2) ml of each extract at different concentrations (200, 400, 600, 800 and 1000 µg/ml) were added to H_2O_2 solution (0.6 ml). Absorbance of H_2O_2 at 230 nm was determined after 10 min against a control solution containing phosphate buffer without H_2O_2. The percentage scavenging activity

Table 1. Solvent extractive values of leaves of *D. chartaceum* extracts.

Type of extract	Solvent extractive value (%)
Methanol	4.4
Chloroform	2.2
Diethyl ether	2.8

Table 2. Phytochemical screening of *D. chartaceum* extracts.

Phytochemical test	Methanolic extract	Chloroform extract	Diethyl ether extract
Terpene/Sterols	+	-	-
Flavonoids	+	-	+++
Saponins	-	+	-
Alkaloids	++	+	+++
Tannins	+	-	++
Phenols	+++	+	+
Glycosides	+	-	-

+, Indicates presence of compound; -, indicates absence of compound.

Table 3. Total phenol content.

Conc. of plant extract (µg/ml)	Methanolic extract (mg/ml catechol equivalent)	Chloroform extract (mg/ml catechol equivalent)	Diethyl ether extract (mg/ml catechol equivalent)
200	0.041±0.003	0.007±0.004	0.0004±0.0003
400	0.107±0.008	0.008±0.003	0.0009±0.0003
600	0.15±0.013	0.012±0.002	0.0016±0.0005
800	0.18±0.015	0.015±0.003	0.0024±0.0003
1000	0.19±0.013	0.025±0.006	0.003±0.0029

$$\% \text{ inhibition of } H_2O_2 \text{ radical} = \frac{\text{Abs. of control} - \text{Abs. of test sample}}{\text{Abs. of control}} \times 100$$

of H_2O_2 by the extracts was calculated as:

RESULTS

The antioxidant activity of *D. chartaceum* was determined with reference to total phenol content, DPPH free radical scavenging activity, reducing power assay and H_2O_2 scavenging activity. The extractive value of different solvents was determined (Table 1). The phytochemical analysis (Table 2) showed the presence of terpenes, flavonoids, alkaloids, tannins, phenols and glycosides in methanolic plant extract, among which the intensity of phenolics was found to be higher as compared to the other two extracts. The chloroform extract showed the presence of saponins, alkaloids and phenols. The alkaloids, flavonoids and tannins were the main compounds in diethyl ether extract.

The total phenol content expressed as mg/ml catechol equivalent was found to be higher in case of methanolic extract as compared to chloroform and diethyl ether (Table 3). DPPH free radical scavenging assay was ideal for screening antioxidative activity because it is very sensitive to detect active ingredients even at low concentrations (Coyle et al., 1993). In the present study, diethyl ether extract was found to possess a higher DPPH radical scavenging activity followed by chloroform and methanol. The maximum percent DPPH radical scavenging (Table 4) was recorded to be 32.78±0.11 for diethyl ether extract at a concentration of 1000 µg/ml. At the same concentration, the value of DPPH scavenged by methanolic extract was 6.45±0.03 and for chloroformic extract was 11.08±0.03. Also, among the three extracts, diethyl ether extract exhibited better reducing power followed by chloroformic and methanolic extract. The maximum reducing power value (Table 5) of 0.908±0.013 was recorded for diethyl ether extract at a concentration of 1000 µg/ml. The H_2O_2 scavenging activity of all the 3 extracts was also determined (Table 6). The maximum

Table 4. DPPH free radical avenging assay.

Concentration (µg/ml)	Methanolic extract	Chloroform extract	Diethyl ether extract
200	1.2±0.009	8.47±0.12	18.76±0.02
400	2.18±0.03	8.68±0.1	21.63±0.2
600	4.7±0.04	9.87±0.05	27.35±0.07
800	5.03±0.007	10.52±0.09	29.84±0.14
1000	6.45±0.03	11.08±0.03	32.78±0.11

Table 5. Reducing power assay.

Concentration (µg/ml)	Methanolic extract	Chloroform extract	Diethyl ether extract
200	0.046±0.009	0.011±0.002	0.319±0.012
400	0.117±0.01	0.025±0.002	0.425±0.015
600	0.163±0.006	0.048±0.002	0.623±0.025
800	0.229±0.01	0.056±0.007	0.731±0.009
1000	0.344±0.01	0.074±0.009	0.908±0.013

Table 6. H_2O_2 scavenging assay.

Concentration (µg/ml)	Methanolic extract	Chloroform extract	Diethyl ether extract
200	21.53±0.7	14.32±0.35	0.72±0.13
400	23.57±0.74	15.38±0.21	1.34±0.06
600	24.28±0.5	15.87±0.11	2.5±0.09
800	25.48±0.38	16.69±0.31	5.01±0.01
1000	27.93±0.47	17.47±0.21	6.45±0.15

maximum percentage of H_2O_2 scavenged was recorded to be 27.93±0.47 for methanolic extract.

DISCUSSION

All the plant extracts evaluated in this study had potential *in vitro* antioxidative activity. Phytochemical analysis revealed that both methanolic and diethyl ether extract contains terpenes, flavonoids, alkaloids, tannins and phenols. Among the plant extracts, diethyl ether extract showed higher DPPH scavenging and reducing power activity. The probable reason of increased DPPH scavenging and reducing power exhibited by diethyl ether may be due to the presence of higher amounts of alkaloids, flavonoids, phenols and tannins compared to other two solvents. It has been previously reported that other species of the genus *Daphniphyllum* contain highly complex polycyclic alkaloids (Heathcock, 1996) and flavonoid glycosides (Gamez et al., 1998) that are capable of scavenging DPPH free radicals.

Polyphenolic compounds are potential antioxidant substances and protective agents against the development of human disease. On the other hand, there are also reports that describe the peroxidative properties of phenolic compounds (Goldman et al., 1999). A number of studies showed that antioxidant activity of plant extracts is correlated with total phenolics rather than with any individual phenolic compound (Frankel et al., 1995; Prior et al., 1999). From our study, it is evident that there is a positive correlation between the amount of phenolic compounds and H_2O_2 scavenging. In methanolic plant extracts, an increased percentage of H_2O_2 scavenging was found due to the presence of the different types of phenolic compounds in abundance.

The *in vitro* antioxidative assays in the present study establishes this species of *Daphniphyllum* as a potent source of natural antioxidant, that can be utilized for neutralizing different types of oxidative stress in biological systems. This study also provides a new lead as there was no previous record on the antioxidative activity of *D. chartaceum*. Therefore, further scientific investigation on this species may be carried out in the future that may lead to the development of a safe alternative source of therapeutic drugs against the harmful synthetic antioxidative drugs that are extensively used.

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