Antioxidant and free-radical-scavenging effects of fruits of *Dregea volubilis*

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Abstract

This study evaluated the *in vitro* antioxidant potential of petroleum ether (60–80°C), chloroform, and methanol extract of the fruits of *Dregea volubilis* Benth (Asclepiadaceae). The different antioxidant assays, including total antioxidant activity, reducing power, free radical, super oxide anion radical, nitric oxide scavenging, lipid peroxidation, and total phenolic content were studied. The extracts exhibited potent total antioxidant activity that increased with increasing amount of extract concentration, which was compared with standard drug vitamin C at different concentrations as extracts. The different concentrations of all the extracts and vitamin C showed inhibition on lipid peroxidation. In addition, all the extracts had effective reducing power, free radical scavenging, super oxide anion scavenging, nitric oxide scavenging, lipid peroxidation, and total phenolic content depending on concentration. These various antioxidant activities were compared with standard antioxidant such as vitamin C at different concentration as different extracts.

Key words: *Dregea volubilis*, free-radical scavenging, *in vitro* antioxidant

INTRODUCTION

Antioxidants are provided to living organisms to protect them from damage caused by uncontrolled production of reactive oxygen species (ROS) and the concomitant lipid peroxidation, protein damage, and DNA-strand breaking. Current interest is focused on the potential role of antioxidants and antioxidant enzymes in the treatment and prevention of atherosclerosis, heart failure, neurodegenerative disorders, aging, cancer, diabetes mellitus, and several others diseases.[1]

Antioxidants are added to a variety of foods to prevent or deter free-radical-induced lipid peroxidation, which is responsible for the development of off-flavors and the undesirable chemical compounds in food.[2] These ROS cause destructive and irreversible damage to the components of a cell, such as lipids, proteins, and DNA. Although normal cells possess antioxidant-defense systems, ROS produced in the cells induces diseases such as cancer and aging.[3]

ROS are formed and degraded by all aerobic organisms. ROS can readily react with most biomolecules including proteins, lipids, lipoproteins, and DNA. Exogenous chemical and endogenous metabolic processes in the human body or in the food system might produce very effective ROS, which are capable of oxidizing biomolecules, resulting in tissue damage and cell death. When the mechanism of antioxidant protection becomes unbalanced by exogenous and endogenous factors, it results in inflammation, diabetes, genotoxicity, cancer, and accelerating aging.[4]

Antioxidant supplements or food-containing antioxidants may be used to help the human body reduce oxidative damage. The most commonly used antioxidants are BHA- Butylated hydroxyl anisole, BHT-Butylated hydroxyl toluene, propyl gallate, and *tert*-butyl-hydroquinone.[5] However, they have been suspected of being responsible for liver damage and carcinogenesis in laboratory animals. Therefore, the development and use of more effective antioxidants are desired.

Traditional medicine worldwide is being re-evaluated by extensive research on different plant species and their therapeutic principles. Plants produce antioxidants to control the oxidative stress caused by sunbeams and

oxygen; they can represent a source of new compounds with antioxidant activity.

Dregea volubilis Benth (Asclepiadaceae) is commonly known as "Jukti" in Bengal. It is a tall woody climber of 11 m of height and 95 cm in girth with densely lenticulate branches, occurring throughout the hotter parts of India and Car Nicober islands ascending to an altitude of 1500 m. In the traditional system of medicines, the juice of the plant is used as a sternutatory and leaves are employed in the application for boils and abscesses.[6] The roots and tender stalks are used as emetic and expectorant.[7] It is reported that an alcohol (50%) extract of the plant showed activity on the central nervous system as well as anticancer activity against Sarcoma 180 in mice. The maximum tolerated dose was found to be 500 mg/kg body weights of albino mice.[7] Two pentacyclic triterpenoids taraxerone and taraxerol isolated from this plant, showed antitumor activities against K562 leukemic cell line.[8]

This study evaluated the total antioxidant activity, reductive ability, free radical scavenging, super oxide anion radical scavenging, hydroxyl radical scavenging, and lipid peroxidation of petroleum ether, chloroform, and methanol extract of *D. volubilis*. An important objective of this research was to compare *in vitro* antioxidative potential of petroleum ether, chloroform, and methanol extract of *D. volubilis*.

MATERIALS AND METHODS

Plant material

The fruits of *D. volubilis* were collected during August 2008 from South 24 Paraganas, West Bengal, India. The plant material was taxonomically identified by Dr. Lakhmi Narashimhan, Scientist, Botanical Survey of India, Central National Herbarium, Howrah, West Bengal, India. The voucher specimen [CNH/I-I/(267)/2008/Tech.II/267] was maintained in our laboratory for future reference.

Preparation of plant extract

The plant material was shade-dried with occasional shifting and then powdered with mechanical grinder, passing through sieve no 40, and stored in a tight container.

The dried powder material (450 gm) of the plant *D. volubilis* was extracted with petroleum ether (60–80°C) for 72 h in the cone-shaped percolator at 33°C. The solvent was distilled in reduced pressure and resulting semisolid mass was vacuum dried using rotary flash evaporator to yield a solid residue (5.33%, w/w). The same plant part residue was then extracted with chloroform and methanol accordingly and was distilled in reduced pressure, and the resulting semisolid mass was vacuum-dried by using rotary flash evaporator to yield a solid residue. The extractive value of chloroform and methanol were subsequently (10.59%, w/w) and (20.29%, w/w). The preliminary phytochemical analysis was performed to identify the phytoconstituents present in the extract.[9]

Chemical

Ammonium thiocyanate was purchased from E Merck (Mumbai, India) and Sigma Chemical Co. Ltd. (Mumbai, India). Ferrous chloride, ferric chloride, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), Vitamin C, nitroblue tetrazolium (NBT), thiobarbituric acid (TBA), tricholoacetic acid (TCA), phenazine methosulfate (PMS), and potassium ferric cyanide were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). All other chemicals and reagent were of analytical grade.

Free-radical-scavenging activity measured by 1,1-diphenyl-2-picryl-hydrazil

The free-radical-scavenging activity of all of the extracts were measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH).[10] Briefly, an 0.1 mM solution of DPPH in methanol was prepared, and 1 ml of this solution was added to 3 ml of the all of the extracts solution respectively in petroleum ether and water at different concentrations (10, 20, 40, 80, 160 and 320 μg/ml). The mixture were shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm by using a UV–visible spectrophotometer (Genesys 10 UV: The location of Thermo Electron Corporation is USA). Lower absorbance values of reaction mixture indicate higher free-radical-scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0) \times 100],$$

where A_0 is the absorbance of the control reaction, and A_1 is the absorbance of presence of all of the extract samples and standard.

Total reduction capability by Fe^{3+}-Fe^{2+} transformation

The total reducing powers of all the extracts were determined[11] with different concentrations of all the extracts (10, 20, 40, 80, 160, 320 μg/ml) in 1 ml of distilled water mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (2.5 ml, 10%) was added to the mixture, which was centrifuged for 10 min at 1000 × *g* (Remi T8A, Mumbai, India). 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 using a UV–Vis spectrophotometer (Genesys 10UV: Thermo Electron

Corporation). Higher absorbance of the reaction mixture indicated greater reducing power.

Super oxide anion radical-scavenging activity in PMS-NADH system

Measurements of super oxide anion scavenging activity of all the extracts were based on the method[11] taking 1 ml of NBT solution (156 μM NBT in 100 mM phosphate buffer, pH 7.4), and various concentrations (10, 20, 40, 80, 160, 320 μg/ml) of sample solutions of all the extracts, respectively, in petroleum–ether, chloroform, and methanol were mixed. The reaction started by adding 100 μl PMS solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The mixtures were then incubated at 25°C for 5 min, and the absorbance at 560 nm was measured against blank samples. A decrease in the absorbance of reaction sample indicated increased super oxide anion scavenging activity and vitamin C was used as a standard drug taking at different concentrations as the different extracts.

Nitric oxide radical scavenging assay

In vitro nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which was measured[11] with reaction mixture (3 ml) containing sodium nitroprusside (10 mM) in phosphate-buffered saline (PBS) and various concentrations (20, 40, 80, 160, 320 μg/ml) of the plant extract were incubated at 25°C for 150 min. At the end of incubation, 0.5 ml of the reaction mixture was removed and 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4, and 0.1% naphthylethylene diamine dihydrochloride) was added. The absorbance of chromophore formed was measured at 546 nm. The percentage inhibition of nitrite oxide generated was measured by comparing the absorbance values of control and test compounds.

Determination of inhibition of lipid peroxidation

Rat liver homogenate was used as a source of polyunsaturated fatty acids for determining the extent of lipid peroxidation.[12] Rat was sacrificed by cervical dislocation. The liver was collected and was homogenized with 40 mM Tris–HCl buffer (pH 7.0) and centrifuged at 3000 × *g* for 10 min. Clear supernatant was taken and to it 100 μl of each of 0.15 M KCl, 15 mM $FeSO_4$, and 6 mM ascorbic acid were added, and the supernatant was incubated at 37°C for 1 h. One milliliter of TCA (10%) was added to the mixture and the sample was centrifuged at 3000 × *g* for 20 min at 4°C to remove the insoluble protein. Two milliliters of the supernatant was removed and 1.0 ml TBA (0.8%) was added to this fraction followed by heating at 90°C for 20 min in a water bath. After cooling, the colored TBA–MDA complex was extracted with organic solvent (2.0 ml butanol) and absorbance was measured at 532 nm.

Determination of total phenolic compounds

The total phenolic compounds in the fruits of *D. volubilis* at different concentrations were determined with Folin–Ciocalteu reagent by using pyrocatechol as a standard phenolic compound. Briefly, 1 mg of all the extracts solution (1000 μg of extract) was placed in a 100 ml Erlenmeyer flask diluted with distilled water (46 ml). Folin–Ciocalteu reagent (1 ml) was added and the contents of the flask were mixed thoroughly. After 3 min, 3 ml of Na_2CO_3 (2%) was added, then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm by a spectrophotometer (Genesys 10 UV; Thermo Electron Corporation).[12] The amount of total phenolic compounds in the fruits of *Dregea volubilis* was determined in micrograms of pyrocatechol equivalent, using the equation obtained from the standard pyrocatechol graph:

$$\text{Absorbance} = 0.001 \times \text{pyrocatechol } (\mu g) + 0.0033$$

Statistical analysis

Experimental results were mean ± S.E.M. of three parallel measurements. Statistical analysis was estimated by using Student's *t*-test followed by ANOVA method. The values for $P < 0.05$ were considered as significant and values for $P < 0.001$ as very significant.

RESULTS AND DISCUSSIONS

The qualitative chemical analysis of the fruits of *D. volubilis* showed positive results for the presence of steroids, triterpenoids, flavonoids, tannins, and glycosides. Antioxidant methods and modifications have been proposed to evaluate antioxidant characteristics and to explain how antioxidants function. Of these, antioxidant activity, reducing power, free radical scavenging, superoxide anion radical scavenging, and nitric oxide radical inhibition activities and estimation of phenol contents are most commonly used for the evaluation of the total antioxidant behavior of the extracts.

Effects on total antioxidant activity

The antioxidant activity of rat liver peroxidation due to the presence of phytoconstituents such as flavonoids and biflavones has been reported. Therefore, this study suggests that the antioxidant activity of all the extracts may be attributed to the reduction of free radicals, chelation of metal ions, or a combination thereof presence of phytoconstituents.

Effect on scavenging DPPH radical

The stable DPPH radical model is a widely used, relatively quick method for the evaluation of free

radical scavenging activity. The effects of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen-donating ability. DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The absorption maximum of a stable DPPH radical in methanol was 517 nm. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progressed, results in the scavenging of the radicals by hydrogen donation. It is visually noticeable that as a change in color from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the antioxidant activity of antioxidants.[12] It has been reported that oxidative stress, which occurs when free radical formation exceeds the body's ability to protect itself, forms the biological basis of chronic conditions such as arteriosclerosis.[13]

Based on the data obtained from this study, all the extracts were effective free radical inhibitor or scavenger, as well as a primary antioxidant that reacts with free radicals, which may limit free radical damage occurring in the human body. Table 1 shows a significant ($P < 0.001$) decrease in the concentration of DPPH radicals due to the scavenging ability of the extracts and standard. Free radical scavenging activity also increased with increasing concentration in the range of 10–320μg/ml.

Effect on reductive ability

For the measurement of the reductive, we investigated Fe^{3+}–Fe^{2+} transformation in the presence of extracts. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.[14] The antioxidant activity of putative antioxidants has been attributed to various mechanisms, among which the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides, the prevention of continued hydrogen abstraction, the reductive capacity, and radical scavenging are some examples.[15] Like the antioxidant activity, the reducing power of all the extracts increased with increasing concentration of samples. Table 2 shows the reductive capabilities of all the extracts compared with vitamin C. All the extracts concentrations tested showed higher activities than the control, and these differences were statistically significant ($P < 0.001$).

Effects on superoxide anion radical scavenging activity

Superoxide anion radical (O_2^-) are formed by activated phagocytes, such as monocytes, macrophages, eosinophils, and neutrophills, and the production of O_2^- is an important factor in the killing of bacteria by phagocytes. In the PMS–NADH–NBT system, superoxide anion, derived from dissolved oxygen from the coupling reaction of

Table 1: DPPH scavenging power of the different extracts of *D. volubilis* and of vitamin C as standard

Name of the extracts	Concentration (μg/ml)	Percentage (%) of DPPH scavenging activity, respectively	Mean ± S.E.M.
D. volubilis (petroleum ether)	10, 20, 40, 80, 160, and 320	41.46, 52.69, 68.78, 79.98, 88.95, and 94.32	71.03 ± 8.484
D. volubilis (chloroform)	10, 20, 40, 80, 160, and 320	40.62, 54.91, 69.38, 75.95, 87.47, and 90.65	69.83 ± 7.870
D. volubilis (methanol)	10, 20, 40, 80, 160, and 320	35.94, 42.29, 59.41, 60.39, 64.14, and 84.81	57.83 ± 7.065
Vitamin C	10, 20, 40, 80, 160, and 320	25.70, 32.10, 42.50, 92.20, and 97.23	57.946 ± 15.296

Table 2: Reducing power of the different extracts of *D. volubilis* and of vitamin C

Name of the extracts	Concentration (μg/ml)	Percentage (%) of reductive ability, respectively	Mean ± S.E.M.
D. volubilis (petroleum ether)	10, 20, 40, 80, 160, and 320	45.69, 58.06, 69.91, 78.69, 82.15, 94.89	71.565 ± 7.215
D. volubilis (chloroform)	10, 20, 40, 80, 160, and 320	33.16, 48.06, 59.82, 64.85, 73.59, 86.89	61.061 ± 7.719
D. volubilis (methanol)	10, 20, 40, 80, 160, and 320	34.19, 47.38, 58.37, 67.56, 76.79, 87.51	61.966 ± 7.960
Vitamin C	10, 20, 40, 80, 160, and 320	15.62, 38.75, 62.78, 79.87, 88.45, and 91.45	62.82 ± 1.348

Table 3: Super oxide scavenging power of the different extracts of *D. volubilis* and of vitamin C

Name of the extracts	Concentration (μg/ml)	Percentage (%) of super oxide scavenging activity, respectively	Mean ± S.E.M.
D. volubilis (petroleum ether)	10, 20, 40, 80, 160, and 320	49.67, 58.18, 69.29, 77.36, 89.49, and 92.78	72.795 ± 6.975
D. volubilis (chloroform)	10, 20, 40, 80, 160, and 320	36.18, 46.32, 55.48, 66.85, 76.81, and 87.52	61.526 ± 7.848
D. volubilis (methanol)	10, 20, 40, 80, 160, and 320	38.98, 46.69, 59.29, 69.28, 75.91, and 89.92	63.345 ± 7.718
Vitamin C	10, 20, 40, 80, 160, and 320	25.62, 35.25, 54.35, 69.23, 78.29, and 89.64	58.73 ± 10.90

PMS–NADH, reduces NBT.[16] The decrease in absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixtures, Table 3 shows the percent inhibition of superoxide radical generation by 20, 40, 60, 80, 100 μg/ml of all the extract compared with vitamin C. The concentrations of all the extracts have a significant level ($P < 0.001$) of superoxide radical scavenging activity compared with control.

Effects on nitric acid (NO-) scavenging activity

Nitric oxide and superoxide anion cause ischemic renal injury separately, and these radicals work together to bring further damage. The toxicity and damage caused by NO and O_2 is multiplies as they react to produce reactive peroxynitrite, which leads to serious toxic reactions with biomolecules, such as protein, lipids, and nucleic acids. High concentration of nitric oxide (NO) has deleterious effects, and therefore, it is necessary that the production of NO be tightly regulated.[17] When NO is produced by macrophages, the nitric oxide radical can be converted into peroxynitrites, which will cause diverse chemical reactions in a biological system including nitration of tyrosine residue of protein, triggering lipid peroxidation, inactivation of aconites, inhibition of mitochondrial electron transport and oxidation of biological thiol compound.[18]

Suppression of NO released may be partially attributed to direct NO scavenging, as concentrations of the extracts decreases; the amount of nitrite generated from the decomposition of sodium nitroprusside at physiologic pH was found to be inhibited by all the extracts. Table 4 shows the percentage inhibition of nitric oxide production. The extracts showed significant level ($P < 0.001$) of inhibition of nitric oxide production by activated peritoneal macrophages with *in vitro* conditions.

Effects on lipid peroxidation

All the extracts retard the peroxidation of linoleic acid (LH). The peroxidation of LH is well known to be a chain reaction in which the chains are carried by linoleylperoxyl radicals, LOO and the products are linoleyl hydroperoxides.[19] The retardation of LH peroxidation by the extracts had been found to be due to rapid chain termination via a very fast cross-reaction between HOO and LOO radicals. This antioxidant mechanism is completely different from the mechanism of antioxidant action of vitamin E. Since vitamin E becomes a pro-oxidant at high concentrations, the addition of the extracts to edible lipids may provide an alternative or supplementary strategy for obtaining large increases in their oxidative stability and shelf-life, something that cannot be achieved by simply adding more and more vitamin C. Table 5 shows the percentage inhibition of lipid peroxidation.

Table 4: Nitric oxide scavenging power of the different extracts of *D. volubilis* and of vitamin C

Name of the extracts	Concentration (μg/ml)	Percentage (%) of nitric oxide scavenging activity, respectively	Mean ± S.E.M.
D. volubilis (petroleum ether)	10, 20, 40, 80, 160, and 320	36.23, 41.59, 59.69, 65.61, 78.09, and 89.56	61.795 ± 8.401
D. volubilis (chloroform)	10, 20, 40, 80, 160, and 320	23.59, 35.18, 41.49, 59.18, 71.82, and 82.81	52.345 ± 9.298
D. volubilis (methanol)	10, 20, 40, 80, 160, and 320	29.13, 36.73, 53.62, 65.64, 75.62, and 88.09	58.138 ± 9.272
Vitamin C	10, 20, 40, 80, 160, and 320	28.40, 45.82, 64.47, 76.87, 86.50, and 95.32	66.23 ± 10.362

Table 5: Inhibition of lipid peroxidation of the different extracts of *D. volubilis* and of Vitamin C.

Name of the extracts	Concentration (μg/ml)	Percentage (%) of inhibition of lipid peroxidation, respectively	Mean ± S.E.M.
D. volubilis (petroleum ether)	10, 20, 40, 80, 160, and 320	43.69, 55.79, 69.18, 78.89, 86.19, and 95.61	71.66 ± 7.905
D. volubilis (chloroform)	10, 20, 40, 80, 160, and 320	19.65, 31.43, 43.96, 58.45, 69.65, 81.92	50.843 ± 9.617
D.volubilis (methanol)	10, 20, 40, 80, 160, and 320	29.63, 36.27, 45.68, 59.25, 79.82, and 89.36	56.66 ± 9.797
Vitamin C	10, 20, 40, 80, 160, and 320	27.54, 38.58, 48.98, 60.43, 75.19, and 82.34	55.51 ± 8.647

Effects of total phenolic compounds

Phenolic constituents are very important in plants because of their scavenging ability due to their hydroxyl groups.[20] A number of studies have focused on the biological activities of phenolic compounds, which are potential antioxidants and free scavengers. In addition, it has been reported that phenolic compounds are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation. One milligram of each concentration of all the extracts contained 95.03 μg of pyrocatechol equivalents of phenols. These phenolic compounds may contribute directly to the antioxidative action. It has been suggested that up to 1 g of polyphenolic compounds (from a diet rich in fruits and vegetables) ingested daily has inhibitory effects on mutagenesis and carcinogenesis in humans.[20]

CONCLUSIONS

Based on the results of this study, it is clear that all of the extracts have powerful *in vitro* antioxidants capacity

against various antioxidant systems. From the results, it can be concluded that the antioxidant activity of all the extracts were concentration dependent with inhibition of lipid peroxidation. From the above analyses, the possible mechanism of antioxidant activity of all the extracts include reductive ability, hydrogen-donating ability, and scavenging of superoxide, nitric oxide and free radicals, which may be due to the presence of phytoconstituents such as flavonoids and polyphenols present in the petroleum–ether, chloroform, and methanol extract of *D. volubilis*.

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