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Total Phenolic, Flavonoid Contents and *in vitro* Antioxidant Activity of *Dioscorea alata* l. Tuber

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Abstract
Objective: To investigate the *in vitro* antioxidant activity of tuber of *Dioscorea alata* L.
Method: Total phenolic content was estimated using the Folin-ciocalteau method. The flavonoid content was determined by Aluminium chloride method. *In vitro* antioxidant activity viz., DPPH, hydroxyl, superoxide, ABTs radical cation scavenging activity and reducing power of petroleum ether, benzene, ethyl acetate, methanol and ethanol of tuber of *Dioscorea alata* were determined using standard methods.
Results: The total phenolics and flavonoids in methanol extract were found to be 0.68 g 100 g^{-1} and 1.21 g 100 g^{-1} respectively. The methanol extract of tuber showed potent hydroxyl, superoxide, ABTs radical cation scavenging activities. Ethanol extract of tuber showed strong DPPH radical scavenging activity. The maximum inhibitory concentration (IC_{50}) in all models viz., DPPH, hydroxyl, superoxide and ABTs radical cation scavenging activity of tuber of *Dioscorea alata* were found to be 27.16, 26.12, 30.65 and 25.53µg/mL respectively at 1µg/mL concentration.
Conclusions: The findings of the present study confirmed the presence of total phenolics and flavonoids and possess *in vitro* antioxidant activity.
Keywords: *In vitro* antioxidant activity, flavonoid, DPPH, ABTs

INTRODUCTION

Free radicals are produced continuously in various metabolic processes and exist in biological systems. They are important for maintaining normal physiological function. Reactive oxygen species (ROS) (e.g. superoxide anion, hydroxyl radical and hydrogen peroxide) increases the possibility of a wide range of common degenerative diseases. The free radical may cause lipid peroxidation, aggregation of protein and degradation of DNA, protein and polysaccharide [1], which eventually destroys cell membranes and kills cells [2].
Recently, more investigations are about the antioxidant activity of medicinal plant extracts, which are commonly used in clinical and folk medicine and in cosmetics. Since plants or herbs contain a diverse range of bioactive molecules, many of which have antioxidant properties [3-5].
Dioscorea which is commonly known as yam and a large genus in the family Dioscoreaceae is one of the staples in many tropical countries. Yam is widely grown in many West African countries. Some yams are used as medicines in oriental countries to prevent diarrhea and diabetes. Studies have shown that yam extracts can reduce blood sugar [6-15], inhibit microbial activity [16] and show antioxidative activity [17].
In view of the above medicinal properties of genus *Dioscorea*, the present study was designed to evaluate the *in vitro* antioxidant activity of petroleum ether, benzene, ethylacetate, methanol extracts of tuber of *Dioscorea alata* L.

MATERIALS AND METHODS

Collection of plant sample

Tuber of *Dioscorea alata* L. was collected from Agasthiarmalai Biosphere reserve, Western ghats, Tamil Nadu. With the help of local flora, voucher specimens were identified and preserved in the Ethnopharmacology Unit, Research Department of Botany, V.O.Chidambaram College, Tuticorin, Tamil Nadu for further references.

Plant sample extraction

Tubers were cleaned, shade dried and pulverized to powder in a mechanical grinder. Required quantity of powder was weighed and transferred to Stoppard flask and treated with methanol until the powder is fully immersed. The flask was shaken every hour for the first six hours and then it was kept aside and again shaken after 24hours. This process was repeated for three days and then the extract was filtered. The extract was collected and evaporated to dryness by using vacuum distillation unit. The final extract thus obtained was used for *in vitro* antioxidants activity.

Estimation of total phenolic content

Total phenolic content was estimated using the Folin-Ciocalteu method [18]. Samples (100µL) were mixed thoroughly with 2 ml of 2% Na_2CO_3. After 2 min. 100 µL of Folin-Ciocalteu reagent was added to the mixture. The resulting mixture was allowed to stand at room temperature for 30 min and the absorbance was measured at 743 nm against a blank. Total phenolic content was expressed as gram of gallic equivalents per 100 gram of dry weight (g $100g^{-1}$DW) of the plant samples.

Estimation of Flavonoids

The flavonoids content was determined according to Eom *et al.*[19]. An aliquot of 0.5ml of sample (1mg/mL) was mixed with 0.1ml of 10% aluminium chloride and 0.1ml of potassium acetate (1M). In this mixture, 4.3ml of 80% methanol was added to make 5mL volume. This mixture was vortexed and the absorbance was measured spectrophotometrically at 415nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

DPPH radical scavenging activity

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component.

This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H[20].

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method [20]. Briefly, an 0.1mm solution of DPPH in methanol was prepared, and 1mL of this solution was added to 3 ml of the solution of all extracts in methanol at different concentration (125,250,500 &1000µg/mL).The the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition) = $\{(A_0 - A_1)/A_0)*100\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Hydroxyl radical scavenging activity

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell et al (1987) [21]. Stock solutions of EDTA (1mM), FeCl3 (10mM), Ascorbic Acid (1mM), H_2O_2 (10mM) and Deoxyribose (10 mM), were prepared in distilled deionized water.

The assay was performed by adding 0.1mL EDTA , 0.01mL of $FeCl_3$,0.1mL H_2O_2, 0.36mL of deoxyribose, 1.0mL of the extract of different concentration (125, 250, 500 &1000µg/mL)dissolved in distilled water,0.33mL of phosphate buffer (50mM , pH 7.9), 0.1mL of ascorbic acid in sequence . The mixture was then incubated at 37^0c for 1 hour. 1.0mL portion of the incubated mixture was mixed with 1.0mL of 10%TCA and 1.0mL of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532nm. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation is calculated by using the following equation

Hydroxyl radical scavenging activity= $\{(A_0 - A_1)/A_0)*100\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Superoxide radical scavenging activity

The superoxide anion scavenging activity was measured as described by Srinivasan et al [22]. The superoxide anion radicals were generated in 3.0 ml of Tris – HCL buffer (16 mM, p^H 8.0), containing 0.5 mL of NBT (0.3mM), 0.5 ml NADH (0.936mM) solution, 1.0 mL extract of different concentration (125,250,500 &1000µg/ml), and 0.5 mL Tris – HCl buffer (16mM, P^H 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12mM) to the mixture, incubated at 25^oC for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by using the following equation

Superoxide radical scavenging activity= $\{(A_0 - A_1)/A_0)*100\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Antioxidant Activity by Radical Cation (ABTS. +)

ABTS assay was based on the slightly modified method of Huang et al [23]. ABTS radical cation (ABTS+) was produced by reacting 7mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS + Solution were diluted with ethanol to an absorbance of 0.70+0.02 at 734 nm. After addition of 100µL of sample or trolox standard to 3.9 mL of diluted ABTS+ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC).

ABTS radical cation activity = $\{(A_0 - A_1)/A_0)*100\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged.

Reducing Power

The reducing power of the extract was determined by the method of Kumar and Hemalatha (2011) [24]. 1.0 mL of solution containing 125, 250, 500 & 1000µg/mL of extract was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH6.6) and potassium ferricyanide (5.0 mL, 1.0%): The mixture was incubated at 50^oC for 20 minutes. Then 5mL of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5^oC) in a refrigerator centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride and absorbance read at 700 nm. The experiment was performed thrice and results were averaged.

Statistical analysis

Antioxidant activities like DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical activity, ABTS radical cation scavenging activity and reducing powers were estimated in triplicate determinations. Data were analyzed using the statistical analysis system SPSS (SPSS software for windows release 17.5; SPSS Inc., Chicago IL, USA) Estimates of mean, standard error for aforesaid parameters were calculated.

RESULTS

The total phenolic and flavonoid contents of methanol extract of *Dioscorea alata* tuber were found to be 0.68 g 100 g^{-1} and 1.21 g 100 g^{-1} respectively. The result of DPPH radical scavenging activity of the extract and the standard ascorbic acid were presented in figure1. The extract exhibited potent radical scavenging activity. The IC_{50} values of the extract were comparable to the standard. Figure2 showed the

hydroxyl radical scavenging activity of methanol extract of *Dioscorea alata* tuber and compared with ascorbic acid. At a concentration of 1000µg/ml, the hydroxyl scavenging activity of the methanol extract of tuber reached 59.09% while at the same concentration that the ascorbic acid was 69.73%. Superoxide radical scavenging activity of the methanol extract of *Dioscorea alata* tuber (1000µg/ml) exhibited the maximum superoxide scavenging activity of 96.16% which is slightly higher than the standard ascorbic acid whose scavenging activity effect is 95.34%. ABTs radical cation scavenging potential of the methanol extract of *Dioscorea alata* tuber was presented in figure4. The percentage of scavenging activity and IC_{50} value of the investigated extract at 1 minute of the reaction time was calculated. The highest percentage of activity of 81.11% (1000µg/ml) was found for methanol extract of tuber. Figure 5 showed the reducing capacity of the plant extract compared to ascorbic acid. The reducing power of methanol extract of *Dioscorea alata* tuber was very potent and the power of the extract was increased with the quality of sample. At a concentration of 1000µg/ml, reducing power of methanol extract of tuber was 0.7764 OD. The IC_{50} values of DPPH, hydroxyl, superoxide and ABTs radical scavenging activity showed in figure 6.

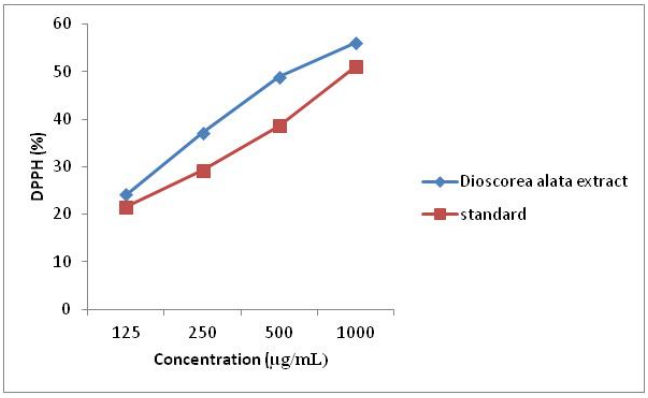

Fig 1: DPPH radical scavenging activity of different solvent extracts of Tuber of *Diascorea alata*

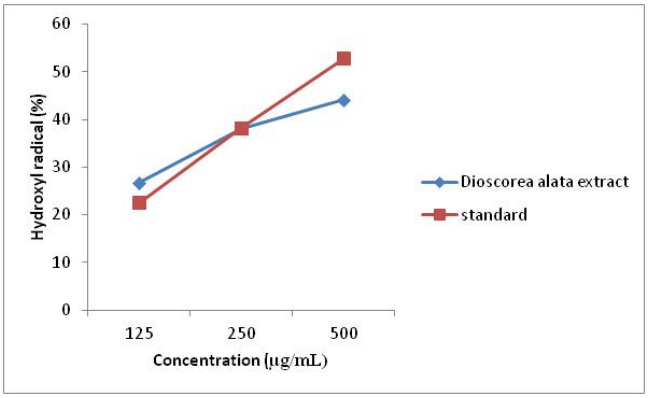

Fig 2: Hydroxyl radical scavenging activity of different solvent extracts of Tuber of *Dioscorea alata*

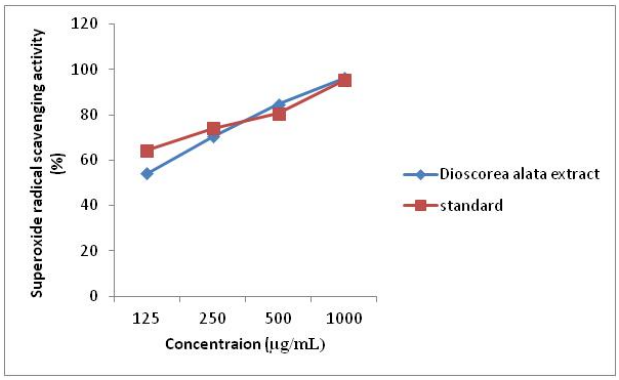

Fig 3: Superoxide radical scavenging activity of different solvent extracts of Tuber of *Diascorea alata*

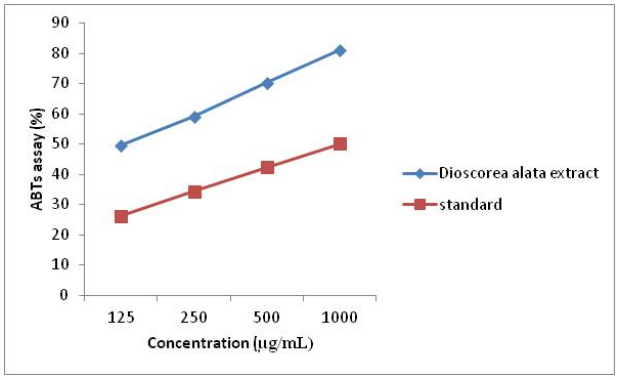

Fig 4: ABTS radical cation scavenging activity of different solvent extracts of Tuber of *Diascorea alata*

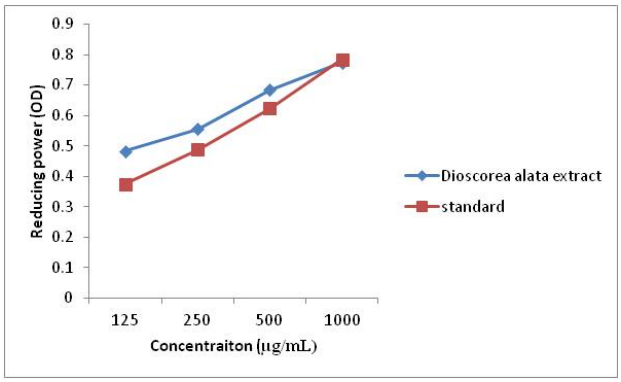

Fig 5: Reducing power ability of different solvents extract of Tuber of *Diascorea alata*

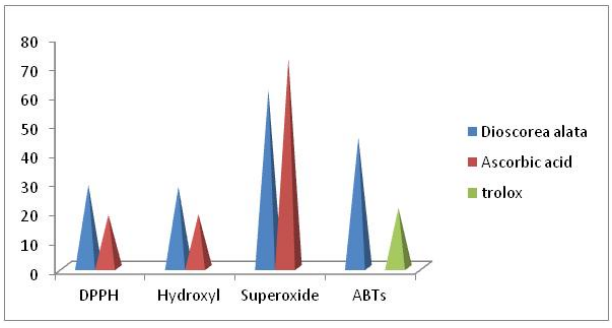

Fig 6: IC_{50} values of different solvents extract of Tuber of *Diascorea alata*

DISCUSSION

Phenolics are secondary metabolites that play a role in the maintenance of the human body. The presence of phytoconstituents, such as phenols, flavonoids and tannin in plants, indicates the possibility of antioxidant activity and this activity will helps in preventing a number of diseases through free radical scavenging activity. Phenolic compounds are commonly found in plants and have been reported to have several biological activities including antioxidant properties. Many studies focused on the biological activities of phenolic compounds, which have potential antioxidants and free radical scavengers [25]

Phenolic compounds are well known as antioxidant and scavenging agents free radicals associated with oxidative damage. Phenolic compounds have attracted much interest recently because *in vitro* studies suggest that they have a variety of beneficial biological properties like anti-inflammatory, antitumor and antimicrobial activities. Studies have attributed that antioxidant properties are due to the presence of phenols and flavonoids [26, 27]

Antioxidant activity of phenolic compounds is based on their ability to donate hydrogen atoms to free radicals. In addition, they possess ideal structural properties for free radical scavenging properties. Flavonoids are important secondary metabolites of plant modulating lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis. It has been confirmed that pharmacological effects of flavonoids is correlating with their antioxidant activities [28]

Free radicals and other reactive species are thought to play an important role in many human diseases. Radical scavenging activities are very important due to the deleterious role of free radicals in biological systems. Many secondary metabolites which include flavonoids, phenolic compounds etc serve as sources of antioxidants and do scavenging activity [29]. In this study, it is evident that the extract of the study species, *Dioscorea alata* leaf possess effective antioxidant activity. *Dioscorea alata* leaf extract exhibited potent *in vitro* antioxidant activity in DPPH radical scavenging assay, hydroxyl radical scavenging assay, superoxide free radical scavenging assay, ABTs radical cation scavenging activity and reducing power in comparison to the known antioxidants such as ascorbic acid and trolox.

The DPPH test provided information on the reactivity of test compounds with a stable free radical. Because of its odd electron, DPPH gives a strong absorption band at 517nm in visible spectroscopy. The efficacies of antioxidants are often associated with their ability to scavenge stable free radicals [30]. In the present study methanol extract exhibited more DPPH radical scavenging activity with IC_{50} value 18.26µg/mL compared to ascorbic acid (18.26µg/ml). From the result in the present study a dose dependent relationship in the DPPH radical scavenging activity.

Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell [31]. In the present study, *Dioscorea alata* tuber extract showed potent hydroxyl radical scavenging activity which is comparable to the standard used. The hydroxyl activity may be due to the presence of various phytochemicals including phenolics and flavonoids in methanol extract of *Dioscorea alata.*

Although superoxide anion is a weak oxidant, it gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress [32]. Numerous biological reactions generate superoxide anions which are highly toxic species. The IC_{50} values were found to be 61.37µg/mL and 78.08µg/mL respectively for methanol extract of *Dioscorea alata* tuber and ascorbic acid. The results clearly indicate that the *Dioscorea alata* leaf extracts have a noticeable effect as scavenging superoxide radical.

The decolorization of ABTs radical reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical species. The ABTs radical cation is generated from the reaction of ABTs with potassium persulfate overnight in water [33]. Result of the present study revealed that methanol extract possesses superior antioxidant activity. The extract showed potent antioxidant activity in ABTs method which is higher than standard trolox.

The reducing capacity of a compound may serve as an important indicator of its potential antioxidants activity [34]. The reducing activity of a compound generally depends on the presence of reductases which have been exhibited antioxidant potential by breaking the free radical chain, donating a hydrogen atom [35]. The reducing capacity of *Dioscorea alata* tuber is a significant indicator of this potential antioxidant activity.

The methanol extracts of *Dioscorea alata* leaf showed strong antioxidant activity in various *in vitro* systems tested. The antioxidant effect of *Dioscorea alata* leaf may be due to the phenolics and flavonoids present in it. In conclusion, the high antioxidant activity exhibited by the extracts of *Dioscorea alata* leaf provided justification for the therapeutic use of this plant in folkloric medicine due to the phytochemical constituents. The present study suggests that this extract could be of great importance for the treatment of radical related diseases and age associated diseases.

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