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Research Article

STUDY OF ANTIOXIDANT AND REACTIVE OXYGEN SPECIES SCAVENGING ACTIVITY OF THE EDIBLE TUBER OF "GREATER YAM" (*DIOSCOREA ALATA* L.) FROM NORTH-EAST INDIA

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ABSTRACT

Antioxidants of plant origin can play a critical role in the prevention of diseases caused due to oxidative stress by reactive oxygen species. The present work reflects a comparative study between the aerial and underground tubers of *Dioscorea alata* L. for their antioxidant potentials by ABTS and DPPH methods, scavenging activities for various radicals including hydroxyl, nitric oxide, peroxynitrite, hypochlorous acid etc.; and iron chelating properties along with their total phenolic and flavonoid contents.
The aerial tuber showed considerable scavenging of hydroxyl and peroxynitrite radicals. The underground tuber showed a comparatively greater phenolic content than aerial tuber and a higher protection against hypochlorous acid damage at low doses and better reducing power. The underground tuber showed a moderate activity in cases of other radicals like, DPPH, superoxide and singlet oxygen, when compared to aerial part. The scavenging activity for other radicals was found to be moderate with both the tubers.

Keywords: Oxidative stress, Reactive oxygen Species, Hydroxyl radical, phenolic content, peroxynitrite

INTRODUCTION

Reactive oxygen species (ROS) is a collective term describing the chemical species that are formed upon incomplete reduction of oxygen and comprises short-lived diffusible entities such as hydroxyl (•OH), alkoxyl/peroxyl (RO•/ROO•) radicals along with some radical species of medium lifetime such as superoxide ($O_2^{\bullet-}$) and nitric oxide (NO•). Some non-radicals like hydrogen peroxide (H_2O_2), peroxynitrite ($ONOOH/ONOO^-$), organic hydroperoxides (ROOH) and hypochlorous acid (HOCl), also fall in the ROS family, since upon disintegration, they too lead to the formation of the aforementioned radicals. ROS are generated through a plethora of enzymatic reactions in various metabolic life processes[1] and are recruited as secondary messengers in signalling pathways [2, 3, 4]. However, oxidative stress initiated by free radicals that seek stability through electron pairing with biological molecules in healthy cells and cause protein and DNA damage along with lipid peroxidation[5] often leads to irreversible imbalance in cellular organisation which is a major problem of concern. These changes are implicated in the pathogenesis of various human diseases such as myocardial and cerebral ischemia, arteriosclerosis, diabetes, rheumatoid arthritis, inflammation, cancer-initiation, and ageing process [6, 7, 8]. On the other hand, antioxidant enzymes of aerobic life system, including superoxide dismutases (SOD), catalase (CAT), glutathione peroxidise (GSH-Px) etc., along with other intrinsic repair systems sometimes fail to entirely prevent the damage. Some synthetic antioxidants have been developed in the past few years, for potential use in preventing or limiting the damage induced by free radicals[9], but their wide application has been restricted due to negative side effects. Thus, the interest in activities of natural antioxidants of plant origin showing lesser or no side effects has increased considerably[10, 11, 12].

The flora of North-Eastern part of India is rich in diversity and among them, many are found to be of immense medicinal value[13]. Among these, *Dioscorea alata* L., locally known as '*Kath aloo*' and commonly known as "greater yam", is a favoured plant for dietary and medicinal reasons. It is popular mostly for its edible yams which grow underground as well as aerially. It grows both domestically and wildly in various regions of Assam and Meghalaya. Most species contain steroid saponins (sometimes with a yield >2%) along with sapogenins, such as diosgenin, which is the starting material of industrial interest in the synthesis of many steroids which are on the market as anti-inflammatory, androgenic, estrogenic, and contraceptive drugs. Besides, this class of compounds are experimentally inferred to possess cytotoxic, antitumor, antifungal, immunoregulatory, hypoglycemic, and cardiovascular properties[14].

In China, yams are used in treatment of poor appetite, chronic diarrhoea, asthma, dry cough, frequent or uncontrollable urination, diabetes and emotional instability[15]. It is applied externally to ulcers, boils and abscesses[16, 17]. Tuber extracts from Chinese yam were shown to have diverse physiologic functions, such as anti-diabetic activity[18, 19], anti-neoplastic activity[20, 21], anti-hypercholesterolaemic activity[22], anti-osteoporotic activity[23], anti-microbial activity[24], anti-hepatonephrotoxicity against acetaminophen[25], anti-obesity activity[26] and immune cell-stimulating activity[27]. Among other species of *Dioscorea*, Diosgenin-3-*O*-α-l-rhamnopyranosyl-(1→4)-β-d-glucopyranoside (DRG), a pentacyclic triterpene glycoside from the rhizomes of *Dioscorea futschauensis*, has shown a stronger anticancer activity than that of the positive control Cisplatin when tested on some human cancer cell lines and it has conferred by morphological observation, DNA ladder detection and flow cytometric analysis, that on HCT-15 cell line DRG induces apoptosis[28]. The petroleum ether extract of *Dioscorea bulbifera* was found to considerably increase the life span of HepA ascites bearing mice[29].

In the present study, an attempt has been made to compare the 70% methanolic extracts of aerial and underground tubers of *Dioscorea alata* for their anti-oxidant potential, radical scavenging and iron chelation activity. The extracts were thus examined for scavenging activities of ROS including hydroxyl, superoxide, nitric oxide, hydrogen peroxide, peroxynitrite, singlet oxygen and hypochlorous acid, and for phenolic and flavonoid contents, iron chelating capacity and total antioxidant activity.

MATERIALS AND METHODS

Chemicals

2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Roche diagnostics, Mannheim, Germany. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Fluka, Buchs, Switzerland. Potassium persulfate ($K_2S_2O_8$), 2-deoxy-2-ribose, ethylenediamminetetraacetic acid (EDTA), ascorbic acid, trichloroacetic acid (TCA), mannitol, nitro blue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), sodium nitroprusside (SNP), sulphanilamide, naphthylethylenediamine dihydrochloride (NED), L-histidine, lipoic acid, sodium pyruvate quercetin and ferrozine were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Hydrogen peroxide, potassium hexacyanoferate, Folin-ciocalteu reagent, sodium carbonate, sodium hypochlorite, aluminium chloride, xylenol orange, butylated hydroxytoluene (BHT) and N,N-

dimethyl-4-nitrosoaniline were obtained from Merck, Mumbai, India. 1,1-diphenyl-2-picrylhydrazyl (DPPH), Gallic acid and curcumin were obtained from MP Biomedicals, France. Catalase was obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Evans blue was purchased from BDH, England. Diethylene-triamine-pentaacetic acid (DTPA) was obtained from Spectrochem Pvt. Ltd, Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemie, Mumbai, India.

Plant Material

The aerial and underground tubers of *Dioscorea alata* were collected from "Sonitpur" district of Assam, India during the months of October-December and authenticated by Botanical Survey of India, Shillong, Meghalaya, India (accession number 78051).

Preparation of Plant extract

The tubers (underground and aerial) of *D. alata* were dried at room temperature, finely powdered and used for extraction. The powder (100 g) was mixed with 1000 ml methanol:water (7:3) using a magnetic stirrer for 15 h, then the mixture was centrifuged at 2850 × *g* and the supernatant was aspirated out. The pellet was mixed again with 1000 ml methanol-water and the entire extraction process was repeated. The supernatants collected from the two phases were mixed in a round bottom flask and concentrated under reduced pressure in a rotary evaporator. The concentrated extract was then lyophilized. The lyophilized powder was stored at -20°C until future use where the respective powders are dissolved in distilled water for the experiments. The methanolic extracts of the aerial and underground tubers of *D. alata*, were marked as DAAM and DAUM, respectively.

Total antioxidant capacity

Antioxidant capacity of both the extracts was evaluated by $ABTS^{\bullet+}$ radical cation decolourisation assay in comparison to trolox standard[30]. The $ABTS^{\bullet+}$ radical cation was generated by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate and incubating for 12–16 h in the dark at room temperature. The absorbance of the $ABTS^{\bullet+}$ solution was equilibrated to 0.70 (±0.02) by diluting with water at room temperature, then 1 ml was mixed with 10 µl of each of the tuber extracts (0.05–10 mg/ml) and their absorbance was measured at 734 nm after 6 min. All experiments were repeated six times.

DPPH radical scavenging assay

The study for the antioxidant capacity of the tuber extracts was compared by the DPPH scavenging assay according to Mahakunakorn et al.[31], with minor modification. Different concentrations (0-100 µg/ml) of the extracts and the standard ascorbic acid were mixed with equal volume of ethanol. Then 50 µl of DPPH solution (1 mM) was pipetted into the previous mixture and stirred thoroughly. The optical density (OD) of the resulting solution was immediately measured at 517 nm. All experiments were repeated six times. The percentage radical scavenging activity was calculated from the following formula:

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

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard.

Hydroxyl radical scavenging assay

This assay was performed as described previously[5]. The assay is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (the Fenton reaction). The reaction mixture in a final volume of 1 ml contained 2-deoxy-2-ribose (2.8 mM); KH_2PO_4-KOH buffer (20 mM, pH 7.4); $FeCl_3$ (100 µM); EDTA (100 µM); H_2O_2 (1.0 mM); ascorbic acid (100 µM) and various concentrations (0–200 µg/ml) of the plant extracts or reference compound. After incubation for 1 h at 37°C, 0.5 ml of the reaction mixture was added to 1 ml 2.8% TCA, then 1 ml 1% TBA was added and the mixture was incubated at 90°C for 15 min to develop the colour. The absorbance was measured at 532 nm against an appropriate blank solution after the samples were cooled to room temperature. All tests were performed six times. Mannitol, a classical OH• scavenger, was used as a positive control. Percentage inhibition was evaluated by comparing the test and blank solutions.

$$\% \text{ inhibition [OH•]} = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard.

Superoxide radical scavenging assay

The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide radicals that reduce nitro blue tetrazolium (NBT) into a purple-colored formazan. Measurement of scavenging activity of the superoxide radical was done spectrophotometrically at 562 nm, according to a previously described method[30]. Equal volumes of phosphate buffer (20mM, pH 7.4), NADH (73 µM), NBT (50 µM), PMS (15 µM) and various plant extract concentrations or standard quercetin (0–50 µg/ml) were mixed to make a final volume of 1 ml reaction mixture followed by an incubation at room temperature for 5 min. All tests were performed six times and the percent of inhibition was calculated accordingly:

$$\% \text{ inhibition } [O_2^{\bullet-}] = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard.

Nitric oxide radical scavenging assay

Nitric oxide generated from SNP aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess Illosvoy reaction[5]. The pink coloured chromophore generated through diazotization of sulphanilamide with nitrite ions and subsequent coupling with NED was spectrophotometrically measured at 540 nm against the blank sample. The 3 ml reaction mixture contained 10-mM SNP, phosphate buffered saline (pH 7.4) and various doses (0–70 µg/ml) of test solution. After incubation for 150 min at 25°C, 1-ml sulfanilamide (0.33% in 20% glacial acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 min. Then 1 ml of napthylethylenediamine dihydrochloride (NED) (0.1% w/v) was added and the mixture was incubated for 30 min at 25°C. All tests were performed six times and curcumin was used as a standard. The percentage inhibition of nitric oxide radical generation was calculated using the following formula:

$$\% \text{ inhibition [NO•]} = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the samples and standard.

Peroxynitrite radical scavenging assay

Peroxynitrite ($ONOO^-$) synthesis was done 12 h before the experiment, as described by Beckman et al[32]. Acidic solution (0.6 M HCl) of 15 ml H_2O_2 (0.7 M) was mixed with 15 ml of 0.6 M KNO_2 on an ice bath for 1 s and 15 ml of ice-cold 1.2 M NaOH was added to the reaction mixture. Excess H_2O_2 was adsorbed on granular MnO_2 and the reaction mixture was left at -20°C. The concentration of the peroxynitrite solution was measured spectrophotometrically at 302 nm (ε = 1670 M^{-1} cm^{-1}). Evans blue bleaching assay was used to measure the peroxynitrite scavenging activity.

The percentage of scavenging of $ONOO^-$ was calculated by comparing the results of the test and blank samples. All tests were performed six times. Gallic acid was used as reference compound. The percentage of scavenging of peroxynitrite anion was calculated using the following equation:

$$\% \text{ scavenging [ONOO}^-\text{]} = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 was the absorbance of the control, and A_1 was the absorbance in the presence of the plant extracts and standard.

Singlet oxygen radical scavenging

The production of singlet oxygen ($^1O_2$) was determined by monitoring *N*,*N*-dimethyl-4-nitrosoaniline (RNO) bleaching, using a

previously reported spectrophotometric method[33]. Singlet oxygen was generated by a reaction between NaOCl and H_2O_2 and the bleaching of RNO was read at 440 nm.

The reaction mixture contained 45 mM phosphate buffer (pH 7.1), 50 mM NaOCl, 50 mM H_2O_2, 50 mM L-histidine, 10 µM RNO and various concentrations (0–100 µg/ml) of sample in a final volume of 2 ml. It was incubated at 30°C for 40 min and decrease in the absorbance of RNO was measured spectrophotometrically. The scavenging activity of sample was compared with that of lipoic acid, used as a reference compound. All tests were performed six times. The percentage of radical scavenging was calculated by the following equation:

$$\% \text{ scavenging } [^1O_2] = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 was the absorbance of the control, and A_1 was the absorbance in the presence of the plant extracts and standard.

Hypochlorous acid scavenging assay

This assay was carried out as described by Aruoma and Halliwell[34] with minor changes. This assay involves an observation of the absorption spectrum of catalase, an anti-oxidant enzyme, in the presence of hypochlorous acid that inactivates catalase as a consequence of heme prosthetic group destruction. Hypochlorous acid (HOCl) was freshly prepared prior to the experiment by adjusting the pH of a 10% (v/v) solution of NaOCl to 6.2 with 0.6 M H_2SO_4 and the concentration of HOCl was determined by using the absorbance at 235 nm and the molar extinction coefficient of 100/M cm.

The scavenging activity was evaluated by measuring the decrease in absorbance of catalase at 404 nm. The reaction mixture contained, in a final volume of 1 ml, 50 mM phosphate buffer (pH 6.8), bovine liver catalase (7.2 µM), HOCI (8.4 mM) and increasing concentrations (0–200 µg/ml) of plant extract. The assay mixture was incubated at 25°C for 20 min and the absorbance was taken against an appropriate blank. All tests were performed six times. Ascorbic acid was used as the reference compound. The percent scavenging activity was calculated by the following equation:

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

Where A_0 was the absorbance of the control, and A_1 was the absorbance in the presence of the plant extracts and standard.

Hydrogen peroxide scavenging assay

This assay was done according to a previously described method[5] with some modifications. An aliquot of 20 mM H_2O_2 and various concentrations (0–2 mg/ml) of samples were mixed (1:1 v/v) and incubated for 30 min at room temperature. After incubation, 100 µl of the sample solution was mixed with 900 µl FOX reagent (prepared in advance by mixing 9 volumes of 4.4 mM BHT in HPLC grade methanol with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H_2SO_4). The reaction mixture was then vortexed and incubated at room temperature for 30 min. The absorbance of the ferric-xylenol orange complex was measured at 560 nm. Sodium pyruvate was used as the reference compound. All tests were carried out six times and the percentage of scavenging of hydrogen peroxide anion was calculated using the following equation:

$$\% \text{ scavenging } [H_2O_2] = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 was the absorbance of the control, and A_1 was the absorbance in the presence of the sample of fruit extracts and standard.

Fe^{2+} chelation activity

The chelating activity of the extracts for ferrous ion was evaluated as previously described[30] with minor changes. Spectrophotometric evaluation confirms the formation of a violet coloured tris complex between ferrozine and Fe (II). The visible absorption spectrum of the ferrous complex of ferrozine exhibits a single sharp peak with maximum absorbance at 562 nm. In the presence of chelating agents like EDTA and the plant extracts with iron chelating capacity, the complex formation is interrupted and as a result the violet colour of the complex is decreased. The reaction was carried out in HEPES buffer (20 mM, pH 7.2). Briefly, various concentrations of plant extracts (0–300 µg/ml) were added to 12.5 µM ferrous sulphate solution and the reaction was initiated by the addition of ferrozine (75 µM). The mixture was shaken vigorously and incubated for 20 min at room temperature prior to measurement of absorbance. All tests were performed for six times. EDTA was used as a positive control.

Reducing Power assay

The Fe^{3+} reducing power of the extract was determined by the method of Oyaizu[35] with a slight modification. Different concentrations (0–1.0 mg/ml) of extract (0.5 ml) were mixed with 0.5 ml phosphate buffer (pH 6.6) and 0.5 ml 0.1% potassium hexacyanoferrate, followed by incubation at 50°C in water bath for 20 min. After incubation, 0.5 ml 10% TCA was added to stop the reaction. 1 ml supernatant of the solution was mixed with 1 ml of distilled water and 0.1 ml 0.01% $FeCl_3$ solution was added. The reaction mixture was left for 10 min at room temperature and the absorbance was measured at 700 nm against appropriate blank solution. All tests were performed six times. A higher absorbance of the reaction mixture indicated greater reducing power. Ascorbic acid was used as a positive control.

Inhibition of lipid peroxidation

The tuber extracts were employed to measure inhibition of lipid peroxidation, following a method previously described[36]. Brain isolated from Swiss Albino mice (20±2 gm) was homogenated with 50 mM phosphate buffer (pH 7.4) and 120 mM KCl, and centrifuged at 3000 rpm for 10 min. A 100 µl aliquot of the supernatant homogenate was mixed with plant extract of various concentrations (2.5-25 µg/ml), followed by addition of 0.1 mM $FeSO_4$ and 0.1 mM ascorbic acid, each of 100 µl and incubated for 1 hr at 37°C. 500 µl 28% TCA was used to stop the reaction and then 380 µl 2% TBA was added with heating at 95°C for 30 min, to generate the colour. The reaction mixtures were then brought to room temperature, centrifuged at 8000 rpm for 2 min and the absorbance of the supernatant was taken at 532 nm. All tests were repeated six times. Trolox was used as the standard.

Determination of total phenolic content

Total phenolic content was determined using Folin-Ciocalteu (FC) reagent according to the method of Singleton and Rossi[37] with minor modification. Briefly, the plant extracts (0.1 ml) was mixed with 0.75 ml of FC reagent (previously diluted 1000-fold with deionised water) and incubated for 5 min at 22°C. 0.06 % Na_2CO_3 solution was added afterwards followed by incubation at 22°C for 90 min. Absorbance was measured at 725 nm. All tests were performed six times. The phenolic content was calculated from a gallic acid standard curve.

Determination of total flavonoids

Total flavonoid content was decided according to a known method[38] using quercetin as a standard. The plant extract of 0.1 ml was added to 0.3 ml distilled water followed by 0.03 ml 5% $NaNO_2$. After 5 min at 25°C, 0.03 ml 10% $AlCl_3$ was added. After another 5 min, the reaction mixture was treated with 0.2 ml 1 mM NaOH. Finally the reaction mixture was diluted to volume (1 ml) with water. Then the absorbance was measured at 510 nm. The flavonoid content was calculated from a quercetin standard curve.

Statistical Analysis

All data were reported as the mean ± SD of six measurements. The statistical analysis was performed by KyPlot version 2.0 beta 15 (32 bit). The IC_{50} values were calculated by the formula,

$$Y = 100 \times A_1/(X + A_1)$$

where $A_1 = IC_{50}$, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The IC_{50} values were compared by paired t test (two-sided) and one-sided ANOVA, wherever required. $p<0.05$ was considered significant.

RESULTS

Total antioxidant activity

The percentage inhibition of absorbance was calculated and plotted as a function of the concentration of standard and sample to determine the trolox equivalent antioxidant concentration (TEAC). To calculate the TEAC, the gradient of the plot for the sample was divided by the gradient of the plot for trolox. The total antioxidant activities of the tuber extracts (DAAM & DAUM) and trolox, as shown in FIG. 1(a) and (b), respectively were calculated from the spectral data of decolourisation of $ABTS^{\bullet+}$, obtained at 734 nm. The TEAC value of DAAM was found to be 0.273±0.013 and that of DAUM was 0.391±0.024 (Table 1).

Table 1: TEAC and other phytochemical values of the aerial and underground tubers of *D. alata* (DAAM & DAUM)

Plant extract	Trolox equivalent antioxidant capacity (TEAC)	Phenolic content per 100 mg extract (gallic acid equivalent) (mg)	Flavonoid content per 100 mg (quercetin equivalent) (mg)
DAAM	0.273 ± 0.013	33.902 ± 0.879	20.058 ± 0.148
DAUM	0.391 ± 0.024	59.066 ± 0.649	20.902 ± 0.171

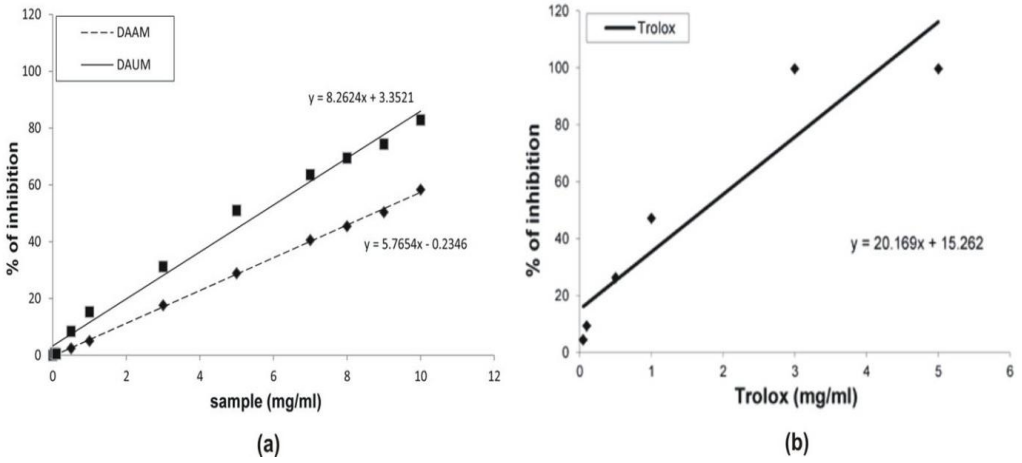

Fig 1: Total Antioxidant Activity

Total antioxidant activity of DAAM and DAUM. The TEAC values were determined from trolox standard curve and plotted against concentration of samples. All data are expressed as mean ± S.D. (n=6).

DPPH radical scavenging activity

The results shown in FIG. 2 is a comparison of the DPPH radical scavenging activities of both the tuber extracts and the standard ascorbic acid. The IC_{50} values (Table 2) of DAAM and DAUM were found to be 244.45±10.10 µg/ml and 139.57±29.19 µg/ml, respectively and that of the standard (ascorbic acid) was 5.29±0.28 µg/ml. The percentage of scavenging of DPPH radical at 100 µg/ml for DAAM, DAUM and ascorbic acid are 27.94%, 42.01% and 93.58 %, respectively.

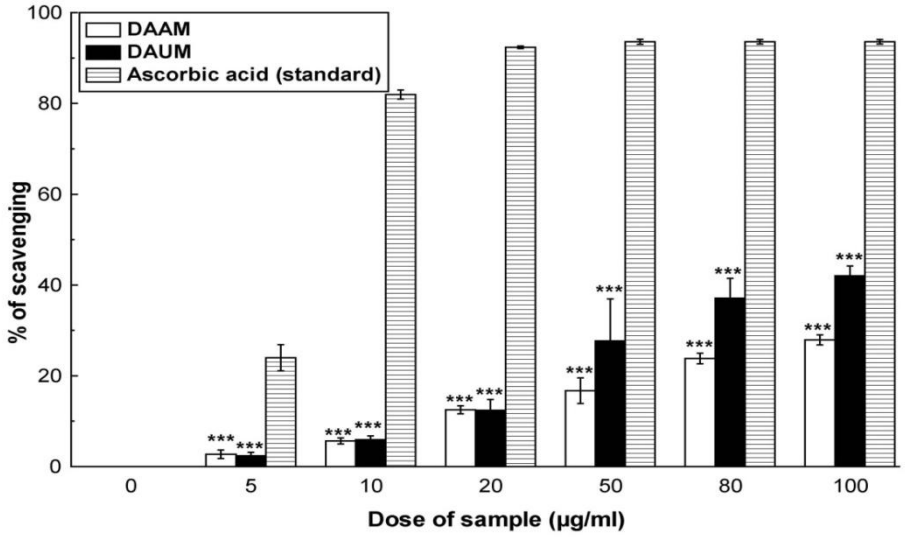

Fig 2: DPPH Radical Scavenging

DPPH radical scavenging activities of plant extracts and the reference compound ascorbic acid. The results are mean ± S.D. of six parallel measurements. ***p < 0.001 vs. 0 µg/ml.

Table 2: IC_{50} values of the aerial and underground tubers of *D. alata* (DAAM & DAUM) and standard compounds for ROS scavenging and iron chelating activity

Activity	Extract/Reference	IC_{50} (#)
DPPH radical (DPPH•) scavenging	DAAM	244.45 ± 10.10 ***(6)
	DAUM	139.57 ± 29.19 ***(6)
	Ascorbic acid	5.29 ± 0.28 (6)
Hydroxyl radical (OH•) scavenging	DAAM	517.30 ± 10.70 ***(6)
	DAUM	544.85 ± 10.46 **(6)
	Mannitol	579.96 ± 24.35 (6)
Superoxide anion ($O_2^{\bullet-}$) scavenging	DAAM	397.38 ± 20.56 ***(6)
	DAUM	277.30 ± 17.33 ***(6)
	Quercetin	60.51 ± 2.09 (6)
Nitric oxide radical (NO) scavenging	DAAM	223.75 ± 27.91 ***(6)
	DAUM	209.42 ± 52.53 **(6)
	Curcumin	96.49 ± 4.99 (6)
Peroxynitrite ($ONOO^-$) scavenging	DAAM	855.38 ± 24.06 NS(6)
	DAUM	1630.05 ± 64.19 ***(6)
	Gallic acid	879.25 ± 59.73 (6)
Singlet oxygen ($^1O_2$) scavenging	DAAM	749.98 ± 33.67 ***(6)
	DAUM	439.41 ± 11.92 ***(6)
	Lipoic acid	46.16 ± 1.16 (6)
Hypochlorous acid (HOCl) scavenging	DAAM	404.36 ± 23.43 ***(6)
	DAUM	310.28 ± 38.37 **(6)
	Ascorbic acid	235.96 ± 5.75 (6)
Iron Chelating Activity	DAAM	225.93 ± 3.62 ***(6)
	DAUM	577.39 ± 21.04 ***(6)
	EDTA	1.28 ± 0.05 (6)
Inhibition of Lipid Peroxydation	DAAM	117.26 ± 3.43 ***(6)
	DAUM	161.35 ± 9.54 ***(6)
	Trolox	13.52 ± 0.33 (6)

 # IC_{50} values of all activities are determined in µg/ml.Data expressed as mean ± S.D. Data in parenthesis indicate number of independent assays.EDTA represents Ethylenediamine tetraacetic acid.* p< 0.05; ** p< 0.01; *** p< 0.001 vs. *Dioscorea alata.*

Hydroxyl radical scavenging activity

This assay shows the abilities of the extracts and standard mannitol to scavenge hydroxyl radical, as shown in FIG. 3. The IC_{50} values (Table 2) for DAAM and DAUM are found to be 517.30±10.70 µg/ml and 544.85±10.46 µg/ml respectively. The standard showed an IC_{50} value of 579.96±24.35 µg/ml. The percentages of hydroxyl radical scavenging at the highest dose, 200 µg/ml, are found to be 30.97%, 28.68% and 21.9% for DAAM, DAUM and standard mannitol, respectively.

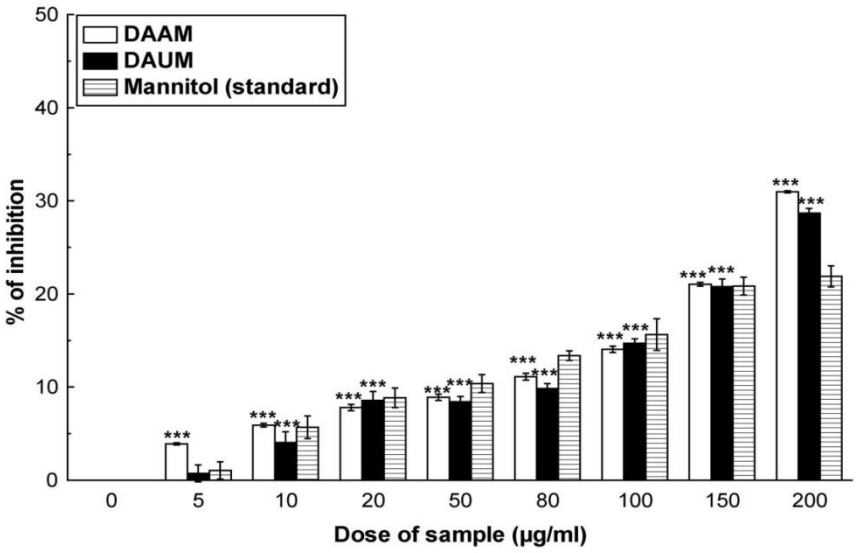

Fig 3: Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activities of DAAM, DAUM and the reference compound mannitol. All data are expressed as mean ± S.D. (n=6). ***p < 0.001 vs. 0 µg/ml.

Superoxide radical scavenging activity

FIG. 4 shows the abilities of the yam extracts and the reference compound quercetin to quench superoxide radicals in the PMS-NADH reaction mixture. The IC_{50} values of the DAAM and DAUM are found to be 397.38±20.56 µg/ml and 277.30±17.33 µg/ml (Table 2). Quercetin on the other hand showed 60.51±2.09 µg/ml as its IC_{50} value. The DAAM and DAUM extracts showed a percentage of superoxide radical scavenging activity as 22.00% and 29.73%, respectively at the maximum dose 120 µg/ml, whereas Quercetin showed 50.67% as the same at the highest dose.

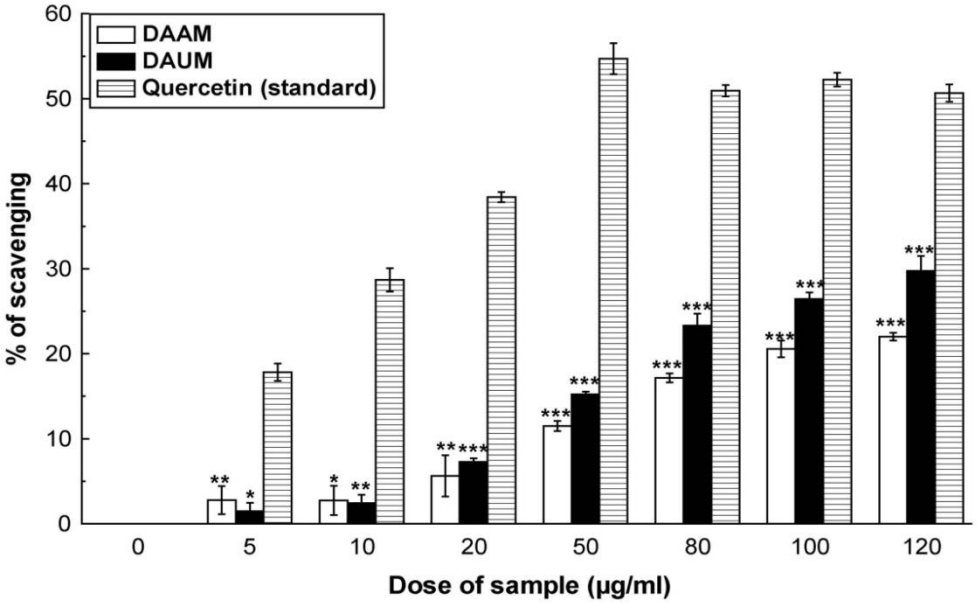

Fig 4: Superoxide Radical Scavenging

Scavenging effect of DAAM, DAUM and standard quercetin on superoxide radical. All data are expressed as mean ± S.D. (n=6). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. 0 µg/ml.

Nitric oxide radical scavenging activity

Both extracts also caused a moderate scavenging of nitric oxide (FIG. 5) with IC_{50} values of 223.75±27.91 µg/ml and 209.42±52.53 µg/ml for DAAM and DAUM, respectively (Table 2). DAAM showed a dose dependent effect unlike DAUM. Curcumin, the reference compound showed IC_{50} of 96.49±4.99 µg/ml. DAAM and DAUM showed a scavenging percentage of 20.94% and 20.71%, respectively at the highest dose of 70 µg/ml. At the same dose, standard curcumin showed a percentage of 43.91% as its scavenging potential.

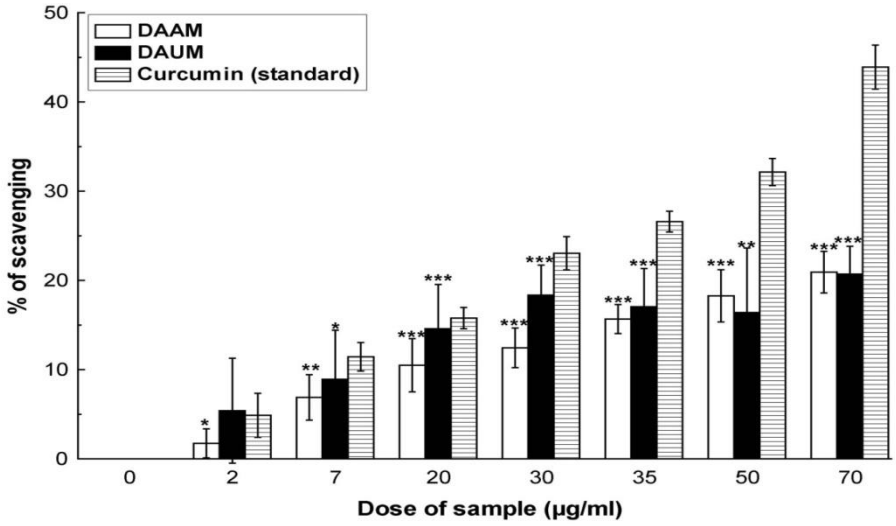

Fig 5: Nitric oxide Radical Scavenging

Nitric oxide radical scavenging activities of the extracts (DAAM & DAUM) and standard curcumin. Each value represents mean ± S.D. (n=6). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. 0 µg/ml.

Peroxynitrite anion scavenging activity

As illustrated in FIG. 6, extract from the aerial part showed a good scavenging activity for peroxynitrite anion similar to the standard gallic acid, which is also supported by the IC_{50} values (Table 2) of DAAM (855.38±24.06 µg/ml) and gallic acid (879.25±59.73 µg/ml). The IC_{50} value for the DAUM on the other hand was inferred as 1630.05±64.19 µg/ml. DAAM showed a scavenging percentage of 16.93% at the highest dose 200µg/ml, which was nearly similar to that of the standard gallic acid (15.44%). DAUM showed a lesser percentage of scavenging, 8.81% at the same dose.

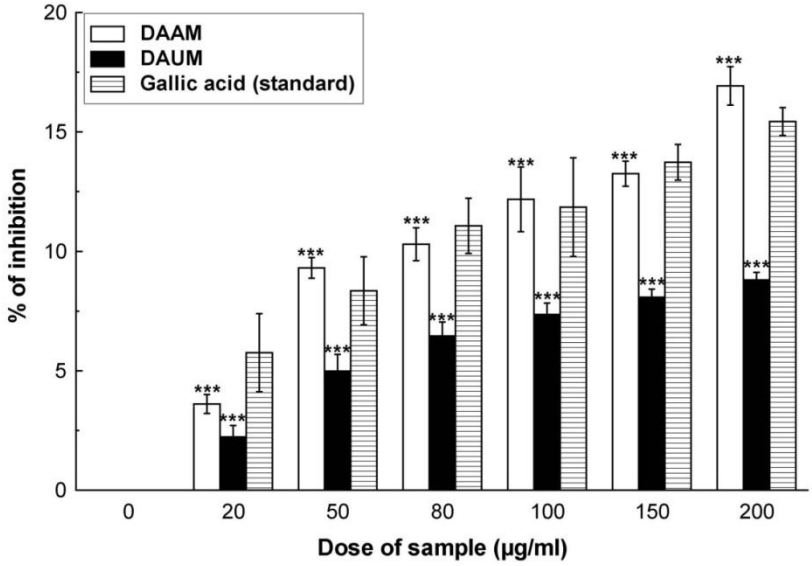

Fig 6: Peroxynitrite Anion Scavenging

The peroxynitrite anion scavenging activities of DAAM, DAUM and standard gallic acid. Each value represents mean ± S.D. (n=6). ***p < 0.001 vs. 0 µg/ml.

Singlet oxygen scavenging activity

Both extracts showed low but dose-dependent activity as scavengers of singlet oxygen (FIG. 7) in comparison to the standard, lipoic acid. Here DAUM showed comparatively better activity than DAAM. The IC_{50} values (Table 2) of DAAM & DAUM were 749.98±33.67 µg/ml and 439.41±11.92 µg/ml whereas that of lipoic acid was 46.16±1.16 µg/ml. The percentages of scavenging of DAAM, DAUM and standard lipoic acid were evaluated as 24.10%, 29.46% and 75.39 %, respectively at the highest dose 200 µg/ml.

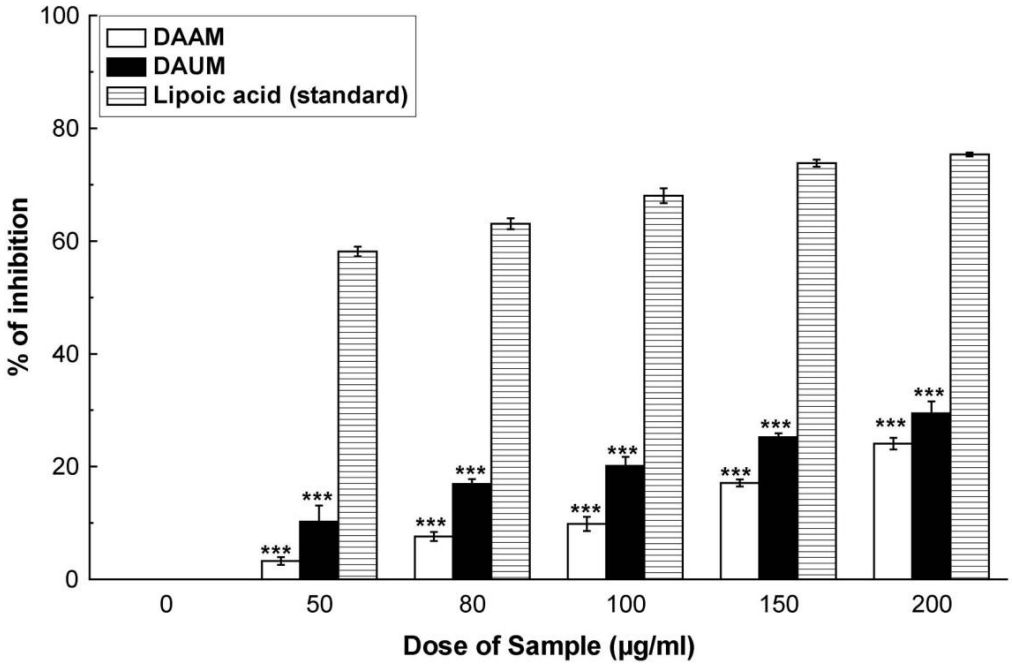

Fig 7: Singlet Oxygen Scavenging

Effects of DAAM, DAUM and standard lipoic acid on the scavenging of singlet oxygen. The results are mean ± S.D. of six parallel measurements. ***p < 0.001 vs. 0 µg/ml.

HOCl scavenging activity

DAUM showed a considerable dose-dependent HOCl scavenging activity as compared to ascorbic acid, at lower doses; DAAM showing slightly lesser activity than DAUM and standard (FIG. 8). The IC_{50} values appeared as 404.36 ± 23.43 µg/ml, 310.28 ± 38.37 µg/ml and 235.96±5.75 µg/ml of aerial extract, underground extract and standard ascorbic acid, respectively (Table 2). The percentages of scavenging were evaluated as 19.23%, 23.63% and 35.47% for aerial & underground parts and standard ascorbic acid, respectively at the highest dose 100 µg/ml.

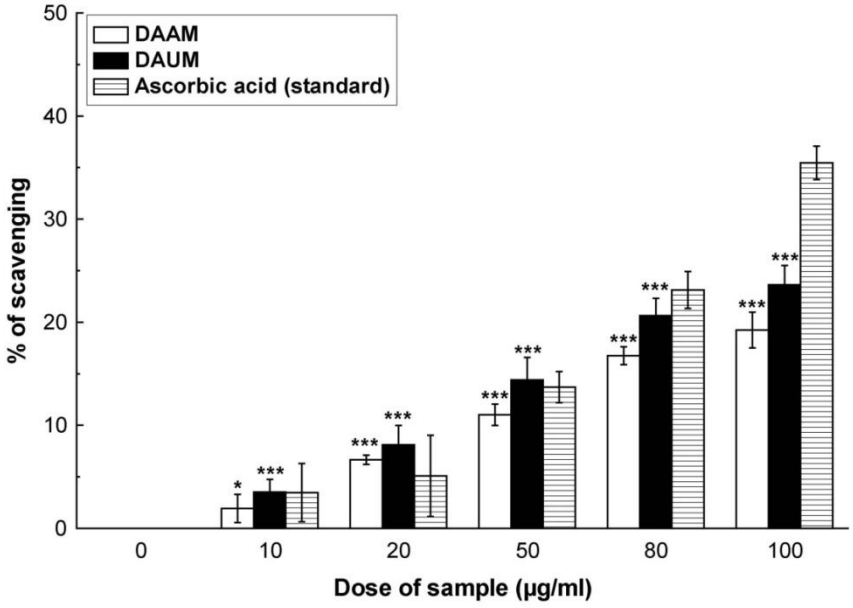

Fig 8: HOCl Scavenging

Hypochlorous acid scavenging activity of DAAM, DAUM and standard ascorbic acid. All data are expressed as mean ± S.D. (n=6). *p < 0.05 and ***p < 0.001 vs 0 µg/ml

Hydrogen peroxide radical scavenging activity

Hydrogen peroxide scavenging was assayed by the FOX reagent method and it was found that the result was not competent to be represented; and so no figure was provided.

Fe^{2+} chelating activity

The Fe^{2+} chelating activity is evaluated by the spectrophotometric analysis of the decrease in characteristic violet colour formation due to inhibition of ferrozine–Fe^{2+} complex. The extracts showing minor iron chelating properties as compared to the standard EDTA (FIG. 9) and IC_{50} values of DAAM, DAUM and EDTA are calculated to be 225.93±3.62 µg/ml, 577.39±21.04 µg/ml and 1.28±0.05 µg/ml (Table 2).

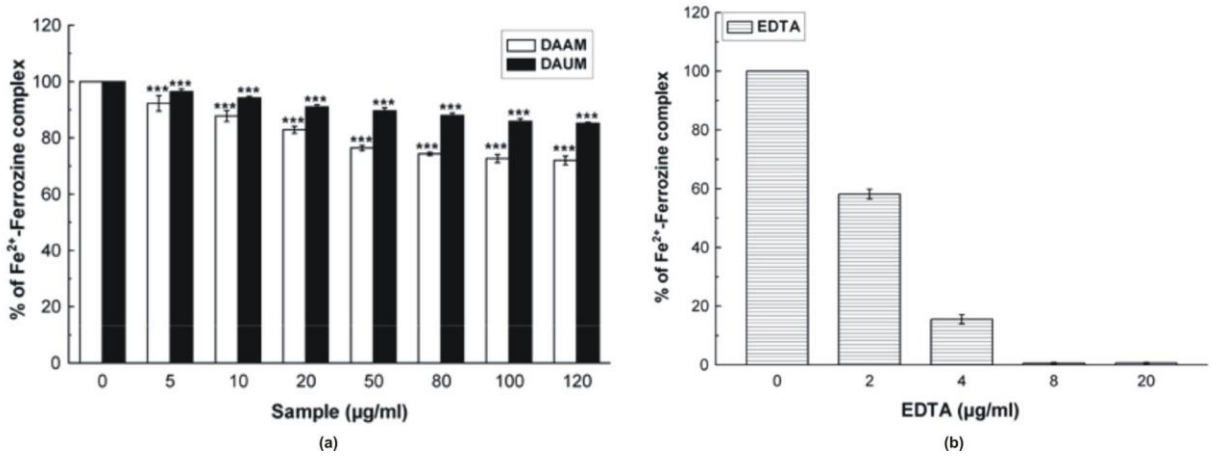

Fig 9: Iron Chelation

Effect of (a) DAAM & DAUM and (b) standard EDTA on ferrozine-Fe^{2+} complex formation. The results are mean ± S.D. of six parallel measurements. ***p < 0.001 vs. 0 µg/ml.

Reducing power

Reducing power of a compound is determined by its ability to facilitate the transformation of Fe^{3+} to Fe^{2+}. As witnessed by the optical density graphs (FIG. 10), DAUM showed a dose dependent and considerably greater reducing activity than the standard ascorbic acid at higher doses (1.0–1.5 mg/ml). On the other side the DAAM showed a dose dependent but lesser activity of reduction with all the doses, throughout.

Inhibition of lipid peroxidation

Both DAAM and DAUM exhibited suppressive effect on the lipid peroxidation with an IC_{50} value of 234.53±6.86 µg/ml and 161.35±9.54 µg/ml, respectively (FIG. 11). The reference compound trolox though showed a very high inhibitory effect with an IC_{50} value of 13.52±0.33 µg/ml (Table 2). The percentages of inhibition were evaluated as 17.53%, 13.14% and 78.87% for DAAM & DAUM and standard trolox, respectively at the highest dose 25 µg/ml

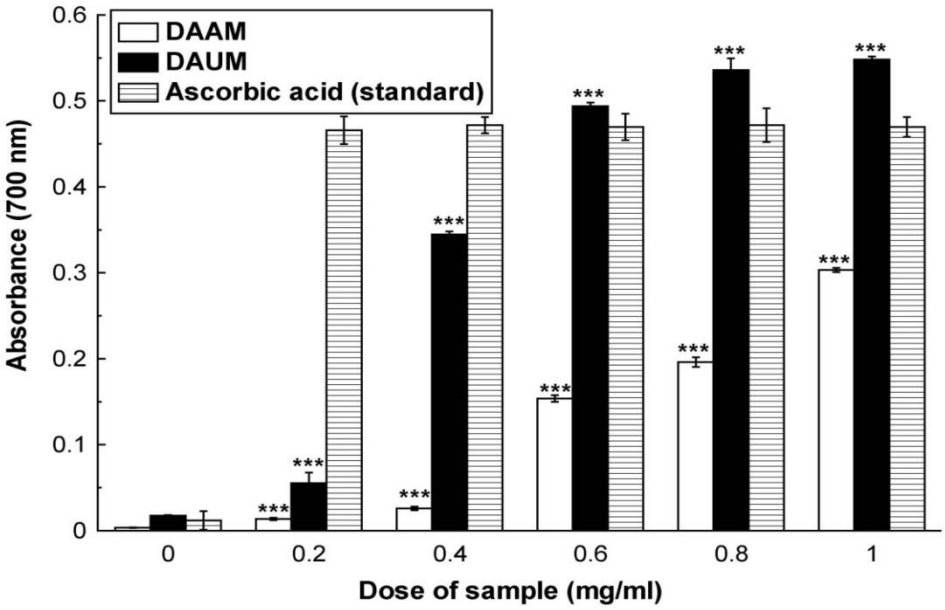

Fig 10: Reducing Power

The reductive ability of DAAM, DAUM extracts and standard ascorbic acid. The absorbance (A_{700}) was plotted against concentration of sample. Each value represents mean ± S.D. (n=6). ***p < 0.001 vs 0 mg/ml.

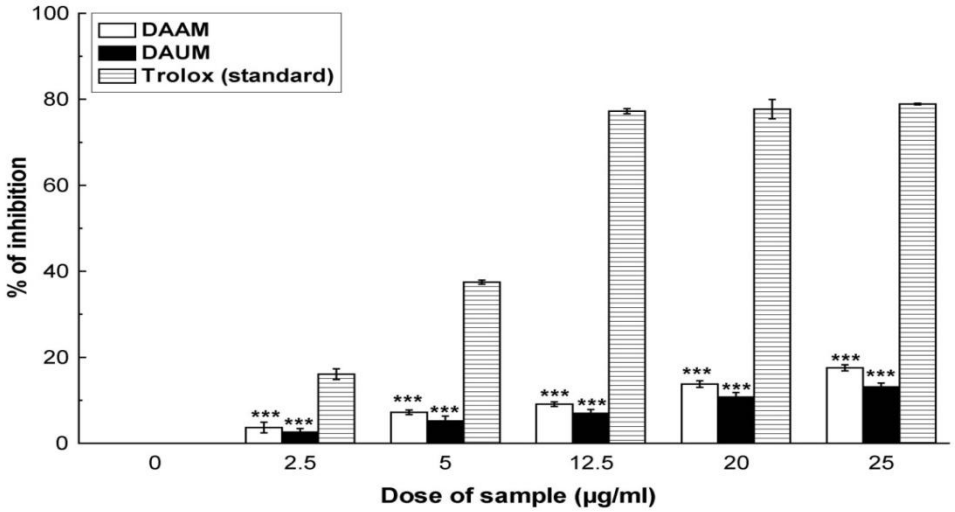

Fig 11: Inhibition of Lipid Peroxidation

Lipid peroxidation inhibiting capacity of the DAAM and DAUM extracts and the standard trolox. Each value represents mean ± S.D. (n=6). *** p < 0.001 vs. 0 µg/ml.

Total phenolic content

The total phenolic content in a plant extract may directly be related to its anti-oxidant action. DAAM showed a total phenolic content of 33.90±0.88 mg (Table 2) gallic acid equivalent per 100 mg of the extract. DAUM on the other hand showed 59.07±0.65 mg gallic acid equivalent per 100 mg extract as its total phenolic content (Table 1).

Total flavonoids

The total flavonoid content of the DAAM and DAUM extracts are found to be 20.06±0.15 and 20.90±0.17 mg quercetin equivalent per 100 mg extract, respectively.

DISCUSSION

Oxidative stress occurs when there is an excessive production of reactive oxygen species or when total antioxidant capacity decreases[9]. The mitochondrial electron transport chain (ETC) is mainly recognized as major cellular generators of ROS, where some electrons leak out of the chain to molecular oxygen (O_2) to form superoxide. The production of ROS by mitochondria is believed to be important in the ageing process and in the pathogenesis of neurodegenerative diseases like Parkinson's disease[39]. In support of the naturally occurring defence mechanism against oxidative stress, recent years have seen the development of many antioxidants, of plant origin which are naturally preferred for their limited or no side effects as compared to their synthetic counterparts[40]. *Dioscorea alata* tuber extracts have shown to be effective in various ailments[14], an information which have initiated the impetus for this study of its antioxidant, radical scavenging and iron chelation activities.

$ABTS^{\bullet+}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate which is reduced to ABTS on a concentration dependant manner upon addition of the 70% methanolic extracts. The results are compared with trolox and the TEAC value demonstrates the extracts as a potent antioxidant, where the TEAC value of underground tuber is more than that of the aerial tuber.

The role of the tuber extracts on DPPH radical scavenging furthermore provides a strong ground supporting the fact that *D. alata* tuber extracts can exhibit a smooth antioxidant activity when required, since the study on TEAC and DPPH scavenging can be observed as complementary to each other[41]. The underground tuber again showed a greater affinity to scavenge DPPH radicals as compared to the aerial part.

Perhaps the most detrimental of all the free radicals formed in the biological systems is the hydroxyl radical that has the potential to bring enormous damage to biomolecules[42]. The addition of the tuber extracts and standard mannitol to the Fenton's reaction has shown that they effectively scavenge the resulting hydroxyl radicals, which if implemented in biological system, may block the deoxyribose damage. Although if compared, both the tubers have shown good affinity towards the •OH radical scavenging against the standard mannitol, the aerial part has shown slightly but better activity than the extract from the underground tuber.

Superoxide anion is also considered as a potent ROS leading to extensive damage to components[43] indirectly by causing initiation of lipid oxidation by virtue of singlet oxygen generation. The experimental data for the assay confirms the fact that both the extracts show a dose dependent activity (the underground part of the plant showing more affinity towards radical scavenging than its aerial counterpart), although not as efficiently as the standard Quercetin.

Direct tissue toxicity and vascular collapse associated with septic shock, may result from a sustained production of the nitric oxide radical; moreover chronic expression of the radical contributes in many carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis[44]. Both extracts showed a moderate NO• scavenging activity where aerial tuber showed a dose dependent effect. The standard curcumin although showed a better activity as compared to the extracts.

The formation of highly reactive peroxynitrite ($ONOO^-$), in inflamed tissues by diffusion-limited reaction of NO• with superoxide anion, in excess amounts, contribute in many pathological conditions. The scavenging activity of DAAM was found to be remarkably good, and similar to the standard gallic acid (FIG. 7). Both the extracts showed a dose dependent activity, although DAUM showed a comparatively lesser activity.

Singlet oxygen, a high energy form of oxygen, is generated in the skin upon UV-irradiation and is responsible for causing hyperoxidation, oxygen cytotoxicity and decrease in antioxidant activity[45]. The assay of singlet oxygen scavenging showed a dose dependent increase in the scavenging activity; DAUM showing a better effect than the DAAM. On the other hand standard Lipoic acid showed an excellent scavenging activity with an inhibition percentage of 75.39 %.

Hypochlorous acid is another harmful ROS. At the sites of inflammation, the oxidation of Cl- ions by the neutrophil enzyme myeloperoxidase results in its production, which breaks down the heme prosthetic group and inactivates the antioxidant enzyme catalase. At lower doses, the activity of DAUM was noteworthy and substantially better than the standard ascorbic acid. DAAM though showed a dose-dependent effect of scavenging, similar but less than its underground counterpart, both being less than the standard.

The capacity of iron chelation has high perceptibility in living system as it reduces the concentration of the transition metals that catalyze processes like lipid peroxidation. In case of lipid peroxidation it is stimulated by iron through Fenton reaction and by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can propagate the chain reaction. The extracts showed a concentration dependent decrease in the characteristic violet colour formation, indicating their iron chelating property; DAAM showed a better activity of chelation as compared to DAUM. Although standard EDTA showed a tremendously high activity of iron chelation as compared to both the extracts (FIG. 9).

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The optical density graphs from our assay confer the reducing power activity of DAUM with DAAM showing very little but dose dependent effect on the same. At higher doses the underground extract showed better reducing power than the standard ascorbic acid.

Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids in the cell membranes that generates a number of degradation products. The inhibition of $FeSO_4$-ascorbic acid induced TBARS formation in the brain homogenate by the extracts, indicating their lipid peroxidation inhibitory activities. Both extracts were found to inhibit lipid peroxidation in a dose dependent manner but not as efficiently as the standard trolox.

Plants produce an astonishing diversity of phenolic metabolites which contribute as one of the principal agents acting as primary antioxidants or free radical terminators. The total phenolic content of DAUM was found to be more than DAAM. The flavonoid content of both the tubers was however found to be similar. The phenolic content as can be correlated directly with the antioxidant capacity, the flavonoids mostly facilitate scavenging or chelation activities.

CONCLUSION

Dioscorea alata tubers hereby showed a promising display of antioxidant & radical scavenging properties. Both the tubers however showed unalike activities. The aerial tuber showed an extremely good tendency towards scavenging of hydroxyl and peroxynitrite radicals. On the other hand, the underground tuber with a comparatively greater phenolic content, showed an almost similar tendency for hydroxyl radical scavenging, a higher protection against hypochlorous acid damage at low doses and a greater reducing power. In cases of other radicals like, DPPH, superoxide and singlet oxygen the underground tuber showed a moderate but comparatively better activity than the aerial tuber. As a future goal, the analysis of the active compounds from both the tubers will further give a clear picture of the mechanism of action.

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