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PHYTOCHEMICAL ANALYSIS, ANTIOXIDANT AND ANTICANCER POTENTIAL OF LEAF EXTRACTS FROM EDIBLE GREATER YAM, *DIOSCOREA ALATA* L., FROM NORTH-EAST INDIA

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ABSTRACT

The medicinal role of dietary plants in promoting the healthy endurance of human beings is traditionally well documented through ancient and modern times all over the world. The purpose of this study was to delineate the differential antioxidant, ROS scavenging and iron chelation activities of the 70% methanolic extract (DALM) and aqueous extract (DALA) of *Dioscorea alata* L. leaves followed by the analysis of various phytochemicals as well as an attempt was made to clarify the active compounds through analytical High Performance Liquid Chromatography (HPLC). The present study is also aimed to investigate the potential anticancer activity of the extracts against human breast adenocarcinoma cell line (MCF-7). The study showed an impressive display of total antioxidant, reducing power, phenolic and carbohydrate contents by DALM followed by its excellent efficacy in scavenging the, singlet oxygen, hydroxyl chloride, and nitric oxide. DALA on the other hand showed fairly good scavenging activities of hydroxyl radical, singlet oxygen and hydroxyl chloride. DALM exhibited moderate cytotoxicity towards MCF-7 cells, whereas, DALA showed no or slight cytotoxicity. These results confirm the possible role of the 70% methanolic extract as a promising free radical scavenger and as a potent antioxidant and anticancer source.

Key Words: High performance liquid chromatography, Antioxidant, Free radicals, Phytochemicals, Cytotoxicity.

INTRODUCTION

Reactive oxygen species (ROS), formed during the incomplete reduction of oxygen, are believed to intercede in the toxicity of oxygen because of their higher reactivity as compared to the later, thereby displaying their capability of damaging biomolecules like proteins, nucleic acids and carbohydrates. Oxidative stress results when generation of ROS supersedes in competence with these cellular antioxidant defences, thus contributing as causative agent of many diseases like cancer, heart disease, diabetes mellitus cataract and congestive & neurodegenerative disorders (Qian *et al*., 2008).

It has been well documented in the last few decades, that antioxidant compounds are the major effectual agents that eliminate/scavenge free radicals hence inhibiting the oxidative stress and hinder the onset and advancement of many diseases as well as lipid peroxidation (Halliwell, 2000). The development and wide application of chemical antioxidants and anticancer drugs (Li *et al*., 2007) has been constrained due to negative side effects and escalating costs. A wide array of secondary metabolites of herbal origin such as the phenolic compounds (phenolic acids, flavonoids, coumarins, quinines and other polyphenols), nitrogen compounds (alkaloids and amines), vitamins, terpenoids and other secondary metabolites exist that have been

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proved for antioxidant as well as anticancer activities (Gul *et al*., 2011). The research in the field of natural products thus, eventually became more prominent and the search for new medicinal plants and explaining their dietary, nutritious and curative activities against many acute diseases caught the spotlight.

Dioscorea alata L. (Fam: *Dioscoreaceae*) commonly known as 'greater yam' and locally (in Assam) known as '*kath aloo*', are climbing perennial vines with heart-shaped leaves. Being well known for their edible tubers that serve as important staple foods and potential sources of ingredients for fabricated foods in South Asian countries, China, Africa and some parts of South America, they are rich in starch content (between 70% to 80% of dried matter) (Das *et al*., 2012; Huang *et al*., 2006). Among various yams, *D. alata* is also known for its high nutritional value, with a crude protein content of 7.4% and vitamin C content ranging from 13.0–24.7 mg per 100 grams (Osagie, 1992). The main constituents of *Dioscorea* spp. are dioscorin, saponins and dioscin having medicinal values which were well documented previously (Olayemi and Ajaiyeoba, 2007). Throughout decades much work has been done on various medicinal and clinical aspects of the *D. alata* tubers (Das *et al*., 2012; Chen *et al*., 2003), but no such substantial evidence exists on the leaves of the same plant.

The present work deals with the phytochemical analysis and a comparative assessment of possible antioxidant, free radical scavenging, iron chelation and anticancer activities of the 70% methanolic and aqueous extracts of *D. alata* leaves followed by the analysis of the availability and abundance of different phytochemicals.

MATERIALS AND METHODS

Chemicals

2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and cell proliferation reagent WST-1 were procured from Roche diagnostics, Mannheim, Germany and 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, ethylenediamminetetra acetic acid (EDTA), ascorbic acid, trichloroacetic acid (TCA), mannitol, nitro blue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), sodium nitroprusside (SNP), 1,10-phenanthroline, sulphanilamide, naphthylethylenediamine dihydrochloride (NED), L-histidine, lipoic acid, sodium pyruvate, quercetin and ferrozine were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. HPLC grade acetonitrile, ammonium acetate, hydrogen peroxide, potassium hexacyanoferate, Folin-ciocalteu reagent, sodium carbonate, mercuric chloride, potassium iodide, anthrone, vanillin, thiourea, 2,4-dinitro-phenylhydrazine, sodium hypochlorite, aluminium chloride, xylenol orange, butylated hydroxytoluene (BHT) and N,N- dimethyl-4-nitrosoaniline were taken from Merck, Mumbai, India. 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, (+) catechin, curcumin and RNAase A were obtained from MP Biomedicals, France. Catalase, reserpine and sodium bicarbonate, Dulbecco's Modified Eagle's Medium (DMEM), antibiotics and Amphotercin-B were obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Evans blue was purchased from BDH, England. D-glucose was procured from Qualigens Fine Chemicals, Mumbai. Diethylene-triamine-pentaacetic acid (DTPA) was obtained from Spectrochem Pvt. Ltd, Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemie, Mumbai, India. Fetal bovine serum was purchased from HyClone Laboratories, Inc., Utah, USA.

Plant material and preparation of extracts

Fresh leaves of *D. 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). The leaves 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 bottomed flask and concentrated under reduced pressure in a rotary evaporator followed by lyophilzation. The lyophilized powder was stored at -20°C until future use where the respective powders are dissolved in distilled water for the experiments. The aqueous and 70% methanolic extracts of the leaves of *D. alata*, were termed as DALA and DALM, respectively.

Phytochemical analysis

Standard qualitative methods as described previously (Harborne and Baxter, 1995; Kokate *et al*., 2003) were carried out in an order to investigate the resident phytochemicals like alkaloids, carbohydrates, flavonoids, glycosides, phenols, saponins, tannins, terpenoids, anthraquinones, and triterpenoids in both the extracts.

Determination of total phenolic content

According to a previously described protocol (Das *et al*., 2012), Folin-Ciocalteu (FC) reagent was used to determine the total phenolic content of DALA and DALM. 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 determined according to a previously discussed method (Das *et al*., 2012) using quercetin as a standard. The absorbance was measured at 510 nm. The flavonoid content was calculated from a quercetin standard curve.

Quantification of carbohydrate content

Carbohydrate contents of both the extracts were quantified was carried out using previously described method (Ghate *et al*., 2013a). Absorbance of the resultant dark green coloured solution was measured at 630 nm. All tests were performed six times. The carbohydrate content was evaluated from a glucose standard curve.

Quantification of alkaloid content

Quantification of alkaloid content for DALA and DALM was carried out using a method described earlier (Ghate *et al*., 2013a). The absorbance was taken at 500 nm and all tests were performed six times. The alkaloid content was evaluated from the reserpine standard graph.

Quantification of ascorbic acid content

This quantification was carried out according to the previously described method (Ghate *et al*., 2013a). All tests were performed six times. The ascorbic acid content was evaluated from a L-ascorbic acid standard curve.

Quantification of tannin content

This was assayed was performed as per a previously described method (Ghate *et al*., 2013a). The absorbance of the resulting magenta-pink colour was measured at 500 nm. All tests were performed six times. The tannin content was evaluated from a catechin standard graph.

High Performance Liquid Chromatography (HPLC) standardization of the extract

The method was carried out as previously described (Sarkar *et al*., 2013). 10 µg/ml stock solutions are prepared in mobile phase for the leaf extracts and the standards including tannin, catechin, reserpine, ascorbic acid, gallic acid & quercetin. The detection is carried out at 254 nm. The injection volume is 20 µl, and the sample and standards are analyzed in triplicates, with gradient elution for 80 min.

***In vitro* antioxidant and free radical scavenging assays**

Total antioxidant activity

Antioxidant capacities of both the leaf extracts (0.05–10 mg/ml) were evaluated by $ABTS^{\bullet+}$ radical cation decolourisation assay in comparison to trolox standard (Ghate *et al*., 2013a). Their absorbance was measured at 734 nm.

DPPH radical scavenging assay

A complementary data supporting the study of antioxidant potential of the yam leaf extracts was provided by the DPPH radical scavenging assay (*Das et al*., 2012) where different concentrations (0–100 µg/ml) of the extracts and the standard trolox were mixed with equal volume of ethanol followed by an addition of 50 µl of DPPH solution (1 mM) to each mixture. The sample sets were then spectrophotometrically measured at 517 nm after 2 min.

Hydroxyl radical scavenging assay

This assay, as described previously (Hazra *et al*., 2008) is based on quantification of the degradation product of 2-deoxyribose by condensation with TBA. Hydroxyl radical (HO•) was generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system (the Fenton reaction). Both the extracts in the dose range (0–200 µg/ml) were quantified for their respective hydroxyl radical scavenging activities spectrophotometrically at 532 nm. Mannitol, a classical OH• scavenger, was used as a positive control.

Superoxide radical scavenging assay

The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system generates superoxide ($O_2^{\bullet-}$) that reduce nitro blue tetrazolium (NBT) into a purple-coloured formazan. The scavenging activity of the superoxide radical was measured spectrophotometrically at 562 nm, for the leaf extracts (0–20 µg/ml) as described in a previously described method (Hazra *et al*., 2008). Quercetin was taken as a standard.

Nitric oxide radical scavenging assay

Nitric oxide (NO•) generated from SNP aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions which were measured by Griess Illosvoy reaction (Das et al. 2012). A pink coloured chromophore was generated through diazotization of sulphanilamide with nitrite ions and subsequent coupling with NED which was spectrophotometrically measured at 540 nm against the blank sample. Various doses of DALA and DALM (0–70µg/ml) along with the standard curcumin were assessed for their respective activities.

Peroxynitrite radical scavenging assay

Peroxynitrite ($ONOO^-$) was synthesized 12 h before the experiment, as described previously (Hazra *et al*., 2008). The percentage of peroxynitrite scavenging was measured spectrophotometrically at 611 nm with Evans blue bleaching and results were calculated by comparing those of the test (0–200 µg/ml) and blank samples. Gallic acid was used as reference compound.

Singlet oxygen radical scavenging assay

The production of singlet oxygen ($^1O_2$) was determined by monitoring *N*,*N*-dimethyl-4-nitrosoaniline (RNO) bleaching, using a previously reported method (Hazra *et al*., 2008). Singlet oxygen was generated by a reaction between NaOCl and H_2O_2 and the bleaching of RNO was read at 440 nm. The scavenging activities of the extracts (0–100 µg/ml) were compared with that of lipoic acid (reference compound).

Hypochlorous acid scavenging assay

This assay was carried out as described previously (Das *et al*., 2012). The scavenging activity of the leaf extracts (0–100 µg/ml) and the standard ascorbic acid was evaluated by measuring the decrease in absorbance of catalase at 404 nm.

Fe^{2+} chelation activity

The iron chelating capacities of DALA and DALM (0–300 µg/ml) are spectrophotometrically evaluated at 562 nm as described earlier (Das *et al*., 2012), by their ability to interrupt the formation of the violet coloured tris complex between ferrozine and Fe (II) resulting in the decrease in the characteristic violet colour. EDTA was used as a positive control.

Measurement of reducing power

The Fe^{3+} reducing power of the extract was determined by the method performed earlier (Hazra *et al*., 2008). Various concentrations (0–1.0 mg/ml) of the extracts were tested and their absorbance was measured at 700 nm against an appropriate blank.

Cell line and culture

Human breast adenocarcinoma (MCF-7) cell line was purchased from the National Centre for Cell Science (NCCS), India and maintained in the laboratory. MCF-7 were grown in DMEM. Media was supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml Penicillin G, 50µg/ml Gentamycin sulphate, 100 µg/ml Streptomycin and 2.5µg/ml Amphotericin B. The cell line was maintained at 37 ºC in a humidified atmosphere containing 5% CO_2 in CO_2 incubator.

Cytotoxicity assay

Cell proliferation and cell viability were quantified using the WST-1 Cell Proliferation Reagent, Roche diagnostics, according to the previous described method (Ghate *et al*., 2013b). In brief MCF-7 cells were seeded at 1 × 104 cells/well and were treated with DALA, DALM ranging from 0 - 200 µg/ml for 48 hours. After treatment, 10 µl of WST-1 cell proliferation reagent was added to each well followed by 3-4 hours of incubation at 37 ºC. Cell proliferation and viability were quantified by measuring absorbance of the formazan at 460 nm using a microplate ELISA reader MULTISKAN EX (Thermo Electron Corporation, USA).

Statistical analysis

All the above tests were performed six times and in each case the percentage of scavenging of the respective radical was calculated using the following equation:

% scavenging = $[(A_0 – A_1) / A_0] \times 100$

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. 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) and Origin professional 8.0, wherever necessary. 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

Phytochemical analysis

Qualitative analysis of the phytochemical contents of both DALA and DALM confirmed the presence of phenolic compounds, flavonoids, carbohydrates, alkaloids, tannins and ascorbic acid followed by a positive test for triterpenoids, only in case of DALM (Table 1). DALM displayed an impressive total phenolic content of 52.26±0.39 mg/100mg extract gallic acid equivalent whereas DALA showed a far lesser amount of only 1.59±0.39 mg/100mg extract gallic acid equivalent (Table 1). DALM again showed slightly more amounts of flavonoids when compared to DALA. In case of too the flaunted a greater amount when compared with that of its aqueous counterpart (Table 1). When quantified for carbohydrates, alkaloids, tannins and ascorbic acid the 70% methanolic extract showed much higher values in each case than DALA (Table 1).

HPLC analysis

When matched with the retention time peaks of the standard phytocompounds the HPLC chromatogram of DALA, tannic acid, reserpine, ascorbic acid and gallic acid were evident (Fig. 1a). In case of DALM confirmed the presence of phytocompounds tannic acid, quercetin, catechin, reserpine, ascorbic acid and gallic acid in the extract (Fig. 1b)

***In vitro* antioxidant and free-radical scavenging assays**

Total antioxidant activity

The total antioxidant activities of DALA and DALM were calculated (Table 2) by determining the TEAC (trolox equivalent antioxidant capacity) value

through ABTS•$^{+}$ radical cation decolourisation assay upon interaction with the leaf extracts or standard trolox thereby suppressing the absorbance of the cation at 734 nm. The results, expressed as percentage inhibition of absorbance, are shown in Fig. 2(a) and 2(b), respectively for the extracts and the standard.

DPPH radical scavenging activity

The complementary data of DPPH radical scavenging accessing antioxidant activity suggests that DALM showed a dose dependant increase in activity followed by a scavenging percentage of whereas DALA failed entirely to show any activity (Fig. 3). The IC_{50} values of DALM and the standard are provided in Table 2.

Hydroxyl radical scavenging activity

This assay shows the abilities of the leaf extracts and standard mannitol to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. Both DALA and DALM showed extremely good and better results as compared to standard mannitol (Fig. 4). The percentages of inhibition at the maximum dose of 200 µg/ml for DALA, DALM and mannitol were 36.84%, 40.90% and 21.90%, respectively. Table 2 shows their respective IC_{50} values.

Superoxide radical scavenging activity

The superoxide radicals generated from dissolved oxygen by PMS-NADH coupling were spectrophotometrically measured at 560 nm by their ability to reduce NBT. The decrease in absorbance with the plant extract and the reference compound quercetin indicates their abilities to quench superoxide radicals in the reaction mixture. DALM again showed excellent superoxide scavenging activity as compared to its aqueous counterpart and standard at all doses (Fig. 5; Table 2). DALA and quercetin both shared almost a same trend in increase of activity throughout the doses. DALM's scavenging activity at the maximum dose (120 µg/ml) was found to be 69.43% followed by 54.80% and 50.67% in case of DALA and quercetin, respectively.

Nitric oxide radical scavenging activity

When tested for their efficacies in scavenging the nitric oxide radicals, among both the extracts only DALM showed a moderate but substantial activity (Fig. 6). DALA exhibited a not so good activity as compared to the rest. Curcumin on the other hand showed an impressive dose dependent activity with a least IC_{50} value of 90.82 ± 4.75 µg/ml among the others (Table 2). Both the extracts showed maximum scavenging activities at the dose of 50 µg/ml, where DALM displayed a percentage of 23.48% and DALA showed 9.66%.

Peroxynitrite radical scavenging activity

DALM showed a moderate trend of activity in case of peroxynitrite scavenging followed by a far lesser activity of DALA (Fig. 7). Standard gallic acid not only showed an impressive trend in scavenging the radical but also showed the least IC_{50} value as compared to the rest (Table 2).

Singlet oxygen radical scavenging activity

None of the leaf extracts showed any substantial activity in scavenging singlet oxygen (Fig. 8). Both DALA & DALM showed extremely high IC_{50} values when compared to that of standard lipoic acid (Table 2).

Hypochlorous acid scavenging activity

Both DALA and DALM showed excellent efficacies in scavenging HOCl, where the later showed a better activity than the standard ascorbic acid at most of the doses (Fig. 9) and also a lower IC_{50} value than the others (Table 2). The maximum scavenging percentage at the highest dose of 100 µg/ml for ascorbic acid was although found to be 35.47% followed by a 32.92% scavenging with DALM and 25.15% in case of DALA.

Iron-chelation activity

Like in most cases, DALM showed a better dose dependent activity when challenged for iron-chelation (Fig. 10) followed by a threefold lesser IC_{50} value than DALA (Table 2).

Reducing power

DALM showed an excellent dose dependent Fe^{3+} reducing activity, whereas on the other hand DALA failed to exhibit any substantial role in the same assay (Fig. 11).

Table 1. Qualitative and quantitative phytochemical analysis of aqueous and 70% methanolic extracts of *D. alata* leaf

Tests	Samples	Phytochemicals									
		Phen	Flav	Carbo	Tan	Alka	Ter	Triter	Anth	Sap	Gly
Qualitative	DALA	+	+	+	+	+	-	-	-	-	-
	DALM	+	+	+	+	+	-	+	-	-	-
Quantitative	DALA	1.59±0.4	4.68±0	20.1±0.3	0.05±0	ND	ND	ND	ND	ND	ND
	DALM	52.26±0.4	5.92±0.2	31.4±0.4	1.42±0	ND	ND	ND	ND	ND	ND

Phen- Phenol, Flav- Flavonoid, Carbo- Carbohydrate, Tan.- Tannin, Alka- Alkaloid, Ter- Terpenoids, Triter- Triterpenoids, Anth-Anthraquinones, Sap-Saponin, Gly- Glycoside; Total phenolics (mg/100 mg extract gallic acid equivalent), Total flavonoids (mg/100 mg extract quercetin equivalent), Carbohydtrate (mg/100 mg extract glucose equivalent), Tannin (mg/100 mg extract catechin equivalent). "+" Represents presence of the phytoconstituent; "-" represents absence of the phytoconstituent.

Table 2. TEAC Values and IC_{50} values of DALA & DALM and standard compounds for ROS scavenging and iron chelating activity

Activity	Extract/Reference	IC_{50} (*)
TEAC Values	DALA	0.11±0.01
	DALM	0.50±0.01
*IC_{50} values of the extracts for free radical scavenging capacity for		
DPPH radical scavenging	DALA	--
	DALM	72.65 ± 1.55***
	Ascorbic acid	5.27 ± 0.27
OH• scavenging	DALA	458.57 ± 18.00***
	DALM	267.45 ± 4.65***
	Mannitol	571.45 ± 20.12
O_2^- scavenging	DALA	67.76 ± 1.66***
	DALM	37.30 ± 0.22***
	Quercetin	42.06 ± 1.35
NO• scavenging	DALA	696.72 ± 106.71***
	DALM	158.18 ± 8.10***
	Curcumin	90.82 ± 4.75
$ONOO^-$ scavenging	DALA	4430.60 ± 548.64***
	DALM	2930.22 ± 81.04***
	Gallic acid	876.24 ± 56.96
$^1O_2$ scavenging	DALA	2680.80 ± 658.80***
	DALM	23967.29±12652.67***
	Lipoic acid	46.15 ± 1.16
HOCl scavenging	DALA	305.57 ± 9.15***
	DALM	208.36± 6.77**
	Ascorbic acid	235.95 ± 5.75
Fe^{2+} Chelating Activity	DALA	1912.52 ± 60.36***
	DALM	645.66 ± 15.23***
	EDTA	1.27 ± 0.05

*IC_{50} values of all activities are determined in µg ml^{-1}.
Data expressed as mean ± S.D. (n=6). EDTA represents Ethylenediamine tetraacetic acid. ** p< 0.01. *** p< 0.001 vs. *Dioscorea alata*.

Fig 1. HPLC chromatogram of DALA (a) DALM (b) compared with different standard compounds

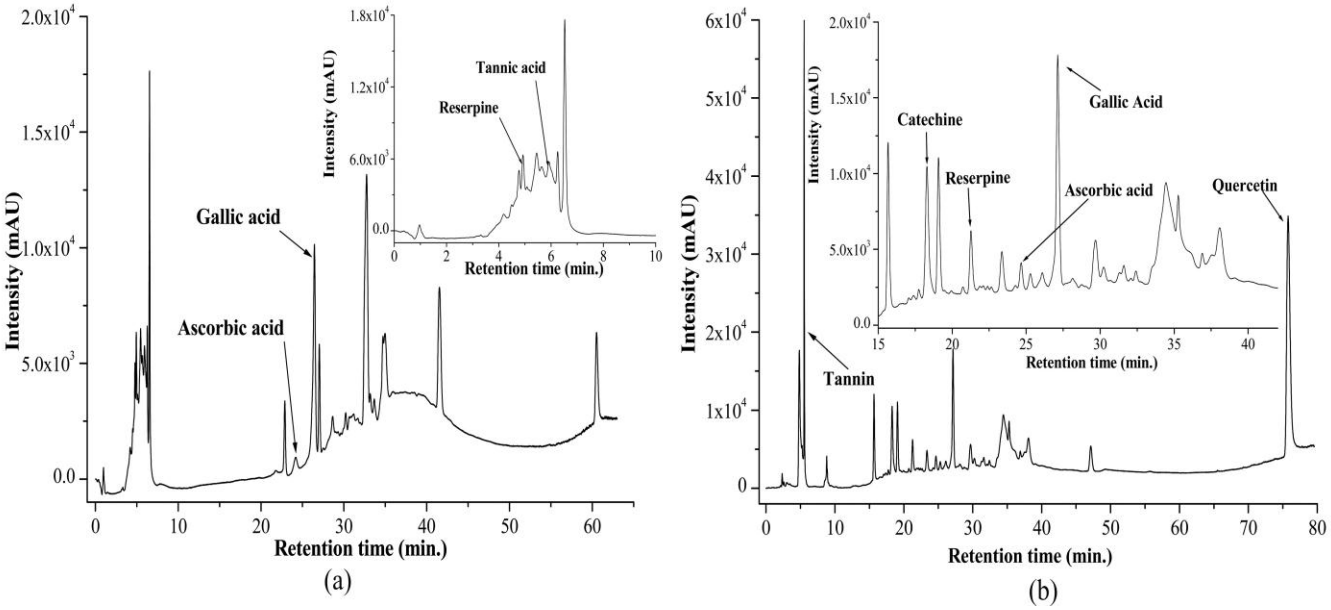
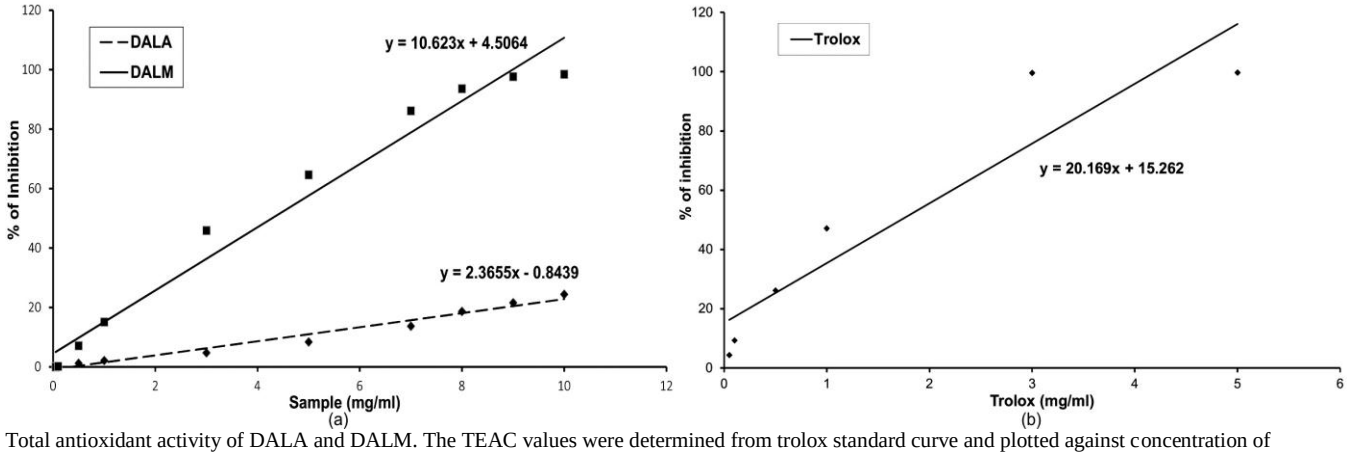
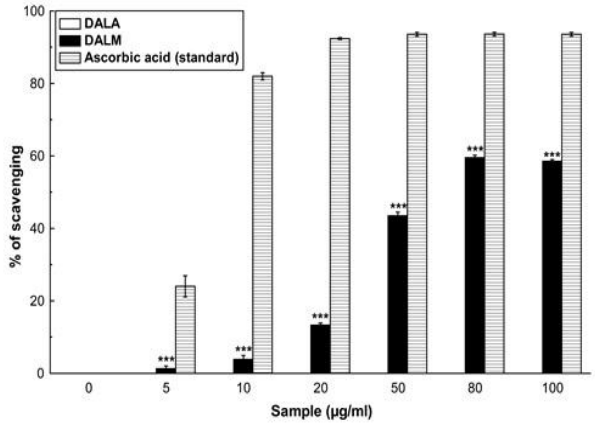


Fig 2. Total Antioxidant Activity



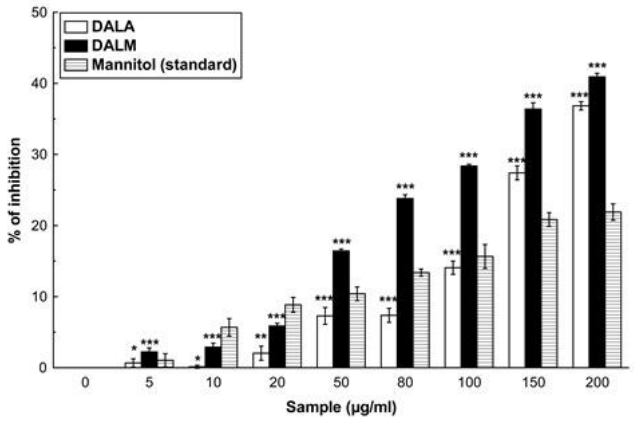
Total antioxidant activity of DALA and DALM. The TEAC values were determined from trolox standard curve and plotted against concentration of samples. All data are expressed as mean ± S.D. (n=6).

Fig 3. DPPH Radical Scavenging



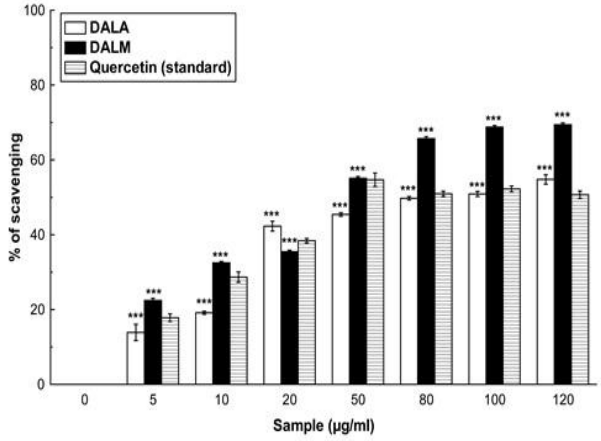
DPPH radical scavenging activities of the plant extracts and the reference compound ascorbic acid. The results are mean ± S.D. of six parallel measurements. ***p < 0.001 vs. 0 µg/ml

Fig 4. Hydroxyl Radical Scavenging



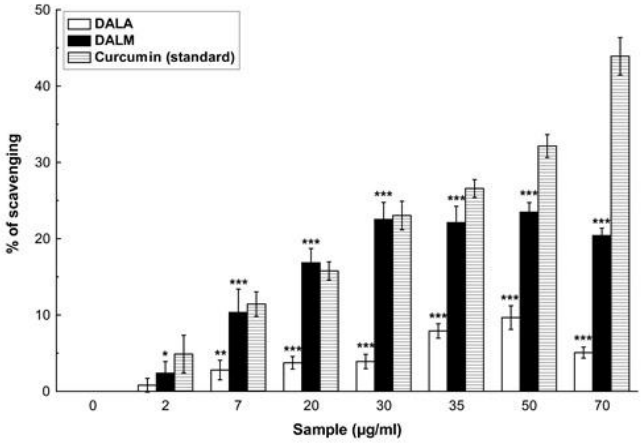
Hydroxyl radical scavenging activities of DALA, DALM and the reference compound mannitol. All data are expressed as mean ± S.D. (n=6). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. 0 µg/ml

Fig. 5 Superoxide Radical Scavenging



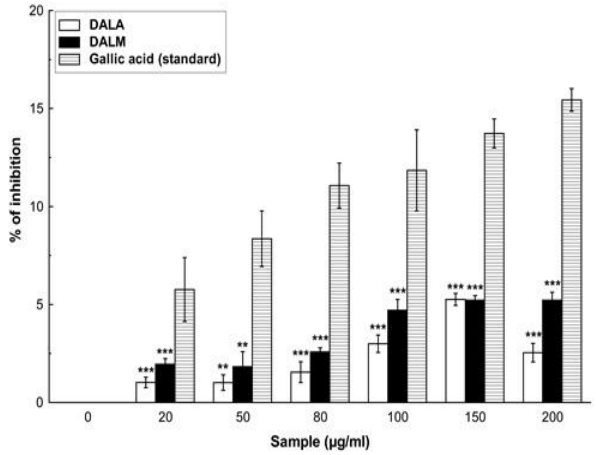
Scavenging effect of DALA, DALM and standard quercetin on superoxide radical. All data are expressed as mean ± S.D. (n=6). ***p < 0.001 vs. 0 µg/ml

Fig 6. Nitric oxide Radical Scavenging



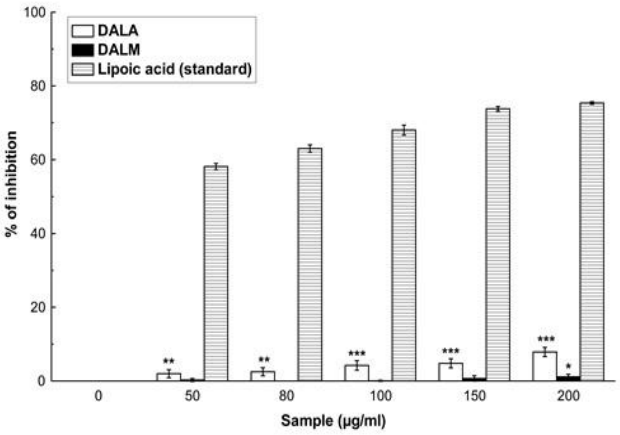
Nitric oxide radical scavenging activities of the extracts (DALA & DALM) and standard curcumin. Each value represents mean ± S.D. (n=6). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. 0 µg/ml

Fig 7. Peroxynitrite Anion Scavenging



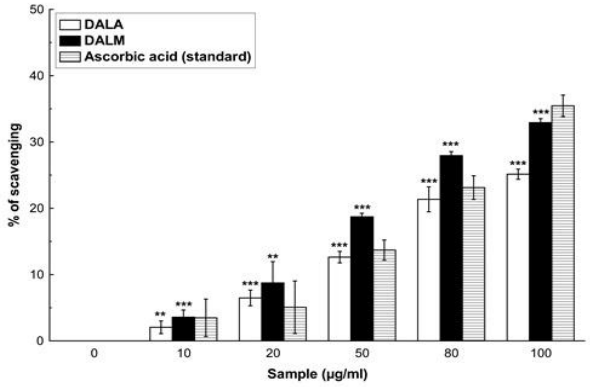
The peroxynitrite anion scavenging activities of DALA, DALM and standard gallic acid. Each value represents mean ± S.D. (n=6). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. 0 µg/ml

Fig 8. Singlet Oxygen Scavenging



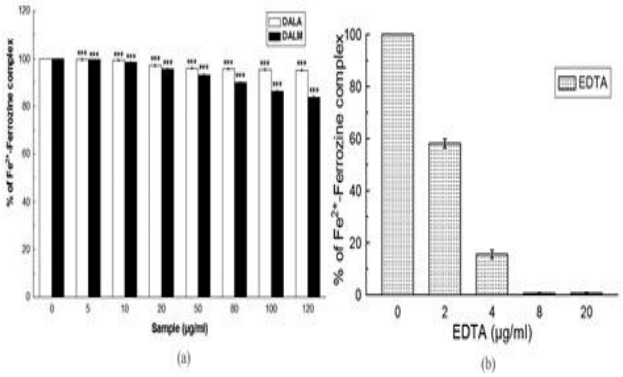
Effects of DALA, DALM and standard lipoic acid on the scavenging of singlet oxygen. The results are mean ± S.D. of six parallel measurements. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. 0 µg/ml.

Fig 9. HOCl Scavenging



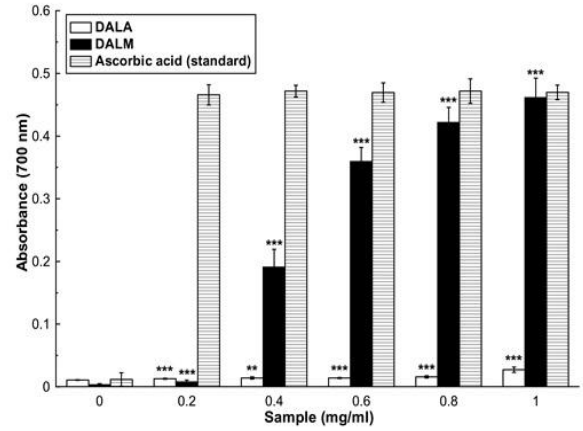
Hypochlorous acid scavenging activity of DALA, DALM and standard ascorbic acid. All data are expressed as mean ± S.D. (n=6). **p < 0.01 and ***p < 0.001 vs 0 µg/ml

Fig 10. Iron Chelation



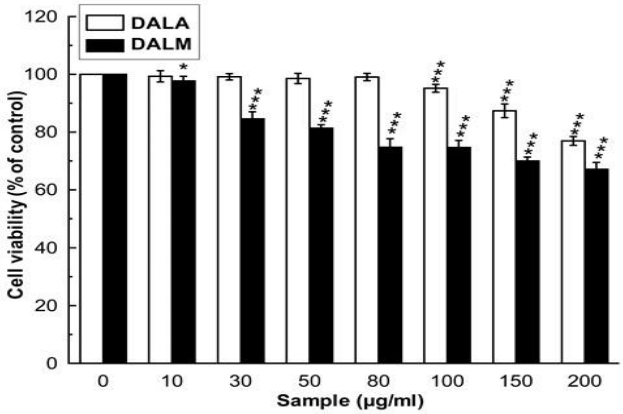
Iron chelation activity of DALA, DALM (a) and standard EDTA (b). All data are expressed as mean ± S.D. (n=6). ***p < 0.001 vs 0 µg/ml

Fig 11. Reducing Power



The reductive ability of DALA, DALM extracts and standard ascorbic acid. The absorbance (A_{700}) was plotted against concentration of sample. Each value represents mean ± S.D. (n=6). **p < 0.01 and ***p < 0.001 vs 0 mg/ml

Fig 12. Effect of DALA and DALM on cell proliferation and viability of MCF-7 cells.



Results were expressed as cell viability (% of control). All data is expressed as mean ± SD (n=6). *p < 0.05 and ***p < 0.001 vs. 0 µg/ml

Cytotoxicity Assay

The effect of DALA and DALM on cell viability was evaluated individually by WST-1 assay and the IC_{50} values were calculated from dose-dependent response studies assessed 48 hours post-treatment. DALM inhibited the growth of MCF-7 cells in a dose-dependent manner with an IC_{50} value of and 311.89±13.71 µg/ml. But DALA did not inhibit the cell proliferation significantly and IC_{50} calculated as 1039.32±70.21 µg/ml (Fig. 12).

DISCUSSION AND CONCLUSION

A decade old interest culminates in the research on plant derived food additives in the search for natural antioxidants to fight all kinds of oxidative stress due to the growing evidences of drawbacks in the established synthetic antioxidants. The research on the broad spectrum of various therapeutic and dietary aspects of the commodities of herbal origin shares the showcase with the 21st century allopathic drugs. The secondary metabolites generated in the plant systems act as major sources of dietary supplements and medicinal components for a human body. The carbohydrates for example act as energy source in a normal human diet. As it is evident from the results that both the aqueous and 70% methanolic extracts of the *D. alata* leaves have shown high amounts of carbohydrates, the later although showed a better percentage. Polyphenols, flavonoids and alkaloids from plants are believed to play imperative roles due to their reducing power, scavenging activities against free-radicals and metal chelating property (Rice-Evans *et al*., 1995; Kessler *et al*., 2003). The absorption of these compounds rich diet has remarkable effects on human nutrition and health. Based on the HPLC studies the presence of phenolic compounds like catechin, tannin and gallic acid along with quercetin, a flavonoid was confirmed in the 70% methanolic extract. Qualitative analysis revealed the presence of substantial amounts of total alkaloids in DALM & DALA, which were later indicated by the presence of reserpine, an indole alkaloid, through HPLC. Amounts of ascorbic acid were also evident whose quantitative analysis revealed its presence more in DALM than in DALA. It was a bit astonishing when none of the extracts indicated the presence of any saponin content though the tubers of *D. alata* along with many other species of the genus use to display substantial amounts of saponins (Sautour *et al*., 2007).

The natural antioxidants have the capacity to work as singlet and triplet oxygen quenchers, peroxide decomposers, as various enzyme inhibitors and synergists (Larson, 1988). The more the amount of total antioxidants present in the plant the more will its derived crude extracts show ROS scavenging potentials. When assayed for $ABTS^{\bullet+}$ scavenging both the extracts showed good total antioxidant capacities, where the 70% methanolic extract showed a four times better activity. The basic principle of the assay was the reduction of $ABTS^{\bullet+}$, a blue chromophore produced by the reaction between ABTS and potassium persulfate, to ABTS on a concentration dependant manner upon addition of any antioxidant.

DPPH scavenging activity, being a complementary assay for accessing the total antioxidant capacity was displayed well by DALM in a dose dependent manner. DALA as seen in the previous assay showed a far lesser activity than DALM, here too failed to show any scavenging activity at all. This proves the presence of a better cocktail of antioxidant compounds in the 70% methanolic extract of the leaf than the aqueous extract.

The hydroxyl radical, a noxious side product of the oxygen metabolism, serves a major role in oxidative stress leading to intracellular biomolecular devastation (Halliwell, 1991). The advantage with hydroxyl radicals is that, it's generation through the Haber-Weiss cycle requires iron in only catalytic (trace) amounts, and the resulting huge number of free radicals duly overload the cellular antioxidant defence mechanisms (Fubini and Hubbard, 2003), thereby indicating the absolute need of radical scavengers. DALM showed a brilliant dose dependent activity in scavenging the hydroxyl radical in an even better manner than the standard mannitol at most of the doses. DALA too followed the same trend as DALM and showed a better IC_{50} value than mannitol.

The ground state oxygen may be converted to a much more reactive species, the superoxide when electron transformation reactions with the former, intervene (Apel and Herbert, 2004). Due to its low chemical reactivity superoxide can interact and damage a limited number of specific cellular constituents but at the same time provides substrates for the Haber-Weiss reaction that leads to the formation of the highly reactive hydroxyl radicals (Benov, 2001) as described above. An excellent activity of superoxide radical scavenging was showcased by DALM which superseded the activities of the rest two. DALA and standard quercetin showed almost same potentials of scavenging.Nitric oxide radical (NO•) generated by the common biochemical pathway, the oxidation of arginine, is involved in the generation of nitrite (NO_2^-) in the presence of O_2 under physiological conditions. NO_2^- has the mutagenability to deaminate nitrogen bases and followed by various forms of nucleic acids under acidic conditions (Nguyen *et al*., 1992). To cease the production of nitrite, there is an upcoming need to scavenge NO• by potent scavengers. DALM along with the standard curcumin have shown good percentages of scavenging NO• at all doses, with the later showing better activity than the rest at higher doses. DALA although was not able to show any activity as good as the other two.

The reaction of NO• with superoxide radical eventually generates the species peroxynitrite ($ONOO^-$)

having a half-life of greater than oneunder physiological conditions, probably due to proton catalyzed decomposition and target molecule reaction (Deiana *et al*., 1999). Peroxynitrite has the capability of causing lipid peroxidation, oxidation of methionine and –SH residues in proteins, antioxidant depletion and deamination and nitration in nucleic acids (Deiana *et al*., 1999). In our case DALM showed a decent dose- dependent activity in scavenging the peroxynitrite radical *in vitro*; DALA on the other hand showed a comparatively lesser activity than DALM and the standard.

Singlet oxygen is generated by the transfer of energy to ground state (triplet) molecular oxygen by protein-bound or other chromophores, which are otherwise referred as the Type II reactions (Davies, 2003). In animals it is also metabolically produced during a phagocytic NADPH-oxidase catalyzed reduction of O_2 (Steinbeck *et al*., 1992). $^1O_2$ reacts with a wide range of biological targets including DNA, RNA, lipids and sterols but most importantly cellular proteins (Davies, 2003). Here none of the extracts showed any significant efficacy in scavenging $^1O_2$.

At sites of inflammation, activated monocytes and neutrophils generate HOCl from H_2O_2 and chloride ions in the presence of enzyme myeloperoxidase (Whiteman *et al*., 1999). The detrimental role of HOCl is described by its capability to oxidise various biomolecules like sulphydryl and thioether moieties, plasma membrane ATPases, collagen, ascorbate, proteins, nucleotides, and DNA repair enzymes and also cholesterol chlorination in cell membranes (Whiteman *et al*., 1999). Both DALM and DALA showed promising activities in scavenging HOCl. DALM showed the best efficacy to scavenge HOCl by showing the least IC_{50} value in the group including the standard ascorbic acid.

In human living systems, iron is present in various functional forms viz., haemoglobin, myoglobin, the cytochromes, enzymes with iron sulphur complexes, and other iron-dependent enzymes and maintained by the cells in optimum levels to uphold the balance between essentiality and toxicity (Sarkar *et al*., 2012). It's superfluous disruption although results in iron overload thereby instigating the oxidative stress induced disorders including anemia, heart failure, liver cirrhosis, fibrosis, diabetes, arthritis, depression, impotency, infertility and cancer (Sarkar *et al*., 2012). Therefore there is a need to remove the excess overloaded iron by potent iron chelating drugs. DALA and especially DALM indicated a trend of dose dependent iron chelation activity *in vitro*.

In a living system antioxidants work in an interactive and synergistic conduct in an order to prevent the damage caused by free radicals. Compounds with reducing power serve as primary and secondary antioxidants by virtue of their electron donating nature through which they can reduce the oxidized intermediates of lipid peroxidation processes (Jayanthi and Lalitha, 2011). The 70% methanolic extract showed a substantially good dose dependent activity as compared to its aqueous counterpart. This result, along with the total antioxidant capacity and DPPH radical scavenging assays indicate the fact that the cocktail of compounds in DALM act as potent antioxidants.

The cytotoxicity assay based on WST-1 assay showed DALM inhibited the growth of MCF-7 cells dose dependently. The viability of cells was reduced by 70% for MCF-7 upon 48 hours exposure to SPME dose dependently. By comparison, the cytotoxicity of DALM and DALA on human cancer cell lines it was found that DALM exhibited strong anticancer activity than DALA referring dose response curves and calculated IC_{50} values. This anticancer data also correlate the phytochemical component present and the antioxidant property of the extracts which showed DALM is more potent fraction of *D. alata* leaf.

On concluding remarks it can be well perceived that the 70% methanolic extract of the *D. alata* leaves displays promising radical scavenging activities and is an important source of natural antioxidants, which might be beneficial to prevent various oxidative stresses. The presence of promising amounts of phenolic compounds and a fair total flavonoid content percentage further supports the previous proclamation. The aqueous extract didn't quite ace in most of the *in vitro* assays, although it showed good scavenging activities against hydroxyl, superoxide and hypochlorous acid. This indicates a further investigation of any possible novel compounds in the *D. alata* leaf, being extracted with 70% methanol, for their structures and functional aspects as anticancer and radioprotective agents.

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