

In Vitro Antioxidant and Anticancer Activity Studies on *Drosera Indica* L. (Droseraceae)

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ABSTRACT

Purpose: The aim of present *in vitro* studies was performed to examine the antioxidant and anticancer activities of ethanol and aqueous extracts of *Drosera indica* L. **Methods:** Different concentrations (5 – 640mcg/ml) of the ethanol (EEDI) and aqueous (AEDI) extracts of *D.indica* L were used in various antioxidant assay methods such as hydroxyl radicals, DPPH, super oxide radical scavenging activity, chelating ability of ferrous ion, nitric oxide radical inhibition, ABTS and reducing power. Ascorbic acid (AA) was used as the standard antioxidant for the free radical scavenging assays. Dalton's Ascitic Lymphoma (DAL) and Ehrlich Ascitic Carcinoma (EAC) cell lines were used as the *in vitro* cancer models for the tryphan blue dye and LDH leakage assays, where 5 to 250mcg /ml of both EEDI and AEDI were tested. **Results:** EEDI showed antioxidant activities with the minimum IC₅₀ values of 34.8±0.43 mcg/ml in scavenging of hydroxyl radical and moreover AEDI showed minimum IC₅₀ values of 94.51±0.84 mcg/ml in Fe²⁺ chelating assay. EEDI on the reducing power assay and ABTS showed higher IC₅₀ than standard AA. IC₅₀ values of AEDI on Fe²⁺ chelating assay and super oxide radical assay was lesser than IC₅₀ value of AA. Both extracts at 250mcg/ml dose showed remarkable increase in the percentage of dead cancer cells (90% by EEDI and 86% by AEDI in DAL model and 89% by EEDI and 80% by AEDI in EAC model). **Conclusion:** It is concluded from this study that *D.indica* L exhibited excellent antioxidant activity against the different *in vitro* antioxidant models and anticancer activity against the two different cell lines tested.

Introduction

Humans are exposed to free radicals in the environment through radiation and pollution. Free radicals are also produced naturally in the body through various metabolic reactions. These free radicals cause severe damage to cells, which can lead to degenerative diseases as well as premature ageing.¹ Antioxidants scavenge these free radicals and enable cells to rejuvenate or stabilize for the process of life.² Natural antioxidants increase the antioxidant capacity of the plasma and reduce the risk of certain diseases such as cancer, heart diseases and stroke.³ The secondary metabolites like phenolics and flavonoids from plants have been reported to be potent free radical scavengers. Various species under *Drosera* belong to this category because of the presence of flavonoids in them.⁴ *Drosera* is a cosmopolitan genus of insectivorous plants and consists of approximately 170 species. In India, *Drosera indica* L., *Drosera burmannii* Vahl and *Drosera peltata* J.E.Sm.ex Wild have been reported to be present at different location. These species are used

as vital components in the Ayurvedic preparation 'Swarnabhasma'(Golden ash). Swarnabhasma (gold ash) is used in several clinical manifestations including loss of memory, defective eyesight, infertility, overall body weakness and incidence of early aging. It is also used for the treatment of diseases like bronchial asthma, rheumatoid arthritis, diabetes mellitus and nervous disorders.⁵ The aim of the present work was to evaluate *in vitro* antioxidant and anticancer potentials of the (EEDI) and aqueous (AEDI) extracts of *D. indica* L.

Material and Methods

Plant material

The whole plant of *D. indica* L was collected from the forests of Savanadurga, Karnataka, India during November 2010. The plant material was identified and authenticated by Dr. S.N.Yoganarasimhan, Taxonomist and Research Coordinator at M. S. Ramaiah College of Pharmacy, Bangalore, Karnataka, India (Herbarium

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specimen no: SRIP/COGNOSY/2011-04). The material was washed, shade dried, powdered, passed through sieve no. 60 and stored in air tight containers for further experiments.

Preparation of the extracts

Alcohol extract

A weighed quantity of the air-dried powdered drug was extracted with ethanol (90 % v/v) in a soxhlet apparatus. The extract was concentrated in a rotary flash evaporator at a temperature not exceeding 50°C. The ethanol extract (EEDI) was suspended in distilled water for experimental use.

Aqueous extract

The marc from the ethanol extract was macerated with chloroform- water for 24h to obtain the aqueous extract (AEDI). AEDI was concentrated under vacuum and dissolved in distilled water for experimental studies. Both the extracts were stored in air tight containers.

In vitro antioxidant methods

All the assays were performed in triplicate.

DPPH (1, 1 diphenyl 2, picrylhydrazyl) Method

To the methanol solution of DPPH (1mM), an equal volume of the extracts dissolved in alcohol was added at various concentrations from 5 to 640mcg/ml to obtain a final volume of 1.0 ml. An equal amount of alcohol was added to the control. After 20 min, absorbance was recorded at 517nm.⁶

$$\text{Inhibition (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where A_0 is the absorbance of control and A_1 is the absorbance of test.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity. This method involves *in vitro* generation of hydroxyl radicals using Fe^{3+} / ascorbate/ EDTA/ H_2O_2 system by Fenton reaction. The hydroxyl radicals formed by the oxidation are made to react with DMSO (dimethyl sulphoxide) to yield formaldehyde. Formaldehyde formed produces intense yellow color with Nash reagent (2M ammonium acetate with 0.05M acetic acid and 0.02M acetyl acetone in distilled water), the intensity of which was measured at 412nm spectrophotometrically against reagent blank.⁷

$$\text{HRSA (\%)} = 1 - (\text{Differences in absorbance of sample} / \text{Difference in absorbance of blank}) \times 100$$

Nitric oxide radical scavenging

Sodium nitroprusside 5 mM was prepared in phosphate buffer pH 7.4. To 1 ml of various concentrations of test extracts, sodium nitroprusside 0.3 ml was added. The test tubes were incubated at 25 °C for 5 hours after which, 0.5 ml of Griess reagent was added. The absorbance of the chromophore was read at 546nm.⁸

$$\text{Inhibition (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where A_0 is the absorbance of control and A_1 is the absorbance of test.

Metal chelating activity

Various concentrations of EEDI and AEDI were added with 1 ml of 2mM FeCl_2 separately. The reaction was initiated by the addition of 5 mM ferrozine (1 ml). Absorbance was measured at 562nm after 10min.⁹

$$\text{Inhibition (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where A_0 is the absorbance of control and A_1 is the absorbance of test.

Reducing power Assay

1ml of the extracts were mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferric cyanide (30 mM) and incubated at 50°C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600 mM) was added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 0.5 ml of FeCl_3 (6 mM) and absorbance was measured at 700nm.¹⁰

$$\text{Inhibition (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where A_0 is the absorbance of control and A_1 is the absorbance of test.

Superoxide scavenging

The superoxide anion radicals were generated in a mixture containing 0.5 ml of NBT (0.3mM), 0.5 ml NADH (0.936 mM) solution, 1.0 ml extract and 0.5 ml Tris-HCl buffer (16 mM, pH 8.0). The reaction was started by the addition of 0.5 ml PMS (Phenazine methosulphate) solution (0.12 mM) to the mixture, which was then incubated at 25°C for 5 min and the absorbance was measured at 560 nm against a blank sample.⁸

$$\text{Inhibition (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where A_0 is the absorbance of control and A_1 is the absorbance of test.

ABTS radical scavenging assay

To the reaction mixture containing 0.3 ml of ABTS radical, 1.7 ml phosphate buffer and 0.5 ml extract was added at various concentrations from 2 to 500mcg/ml. Blank was carried out without drug. Absorbance was recorded at 734nm.¹¹

$$\text{Inhibition (\%)} = (A_0 - A_1 / A_0) \times 100$$

Where A_0 is the absorbance of control and A_1 is the absorbance of test.

In Vitro anticancer activity

Short term cytotoxicity was assessed by Trypan blue exclusion method and Lactate dehydrogenase (LDH) leakage assay.¹²

Trypan blue exclusion method

Trypan blue dye assay method^{13, 14} was carried out to evaluate the *in vitro* cytotoxicity potentials of both alcohol and aqueous extracts of *D. indica* L. EEDI and

AEDI were dissolved in distilled water. Different concentrations (5, 10, 50, 100, 150 and 250mcg/ml) of both extracts were prepared. In a test tube, 100µl of plant extract was mixed with 800µl of phosphate buffer saline and 100µl (1×10^6 in 1ml) of Dalton's Ascitic Lymphoma (DAL) was added. Similar method was followed with Ehrlich Ascitic Carcinoma (EAC) cell line also. Each concentration of the extracts was tested in triplicate. All the samples were incubated at 37°C in an incubator for 30min. About 100µl of trypan blue dye was added to all the test tubes and the number of dead cells was counted in a haemocytometer under a compound microscope. Percentage of cytotoxicity was calculated by the following formula.

$$\% \text{ dead cells} = \frac{\text{Number of dead cells}}{\text{Sum of dead cells and living cell}} \times 100.$$

Lactate Dehydrogenase (LDH) leakage assay

LDH leakage assay was carried out using LDH cytotoxicity detection kit by Sigma Aldrich Inc., USA, according to protocol in the user's manual. To determine IC_{50} , different concentrations of herbal extracts were incubated with 100 µl of DAL and EAC cell suspensions having 1×10^6 cell/ml in 96 well plates

and incubated at 37°C for 4 hrs in 5% CO_2 atmosphere. All the control and test substances were tested in triplicates and mean \pm SEM of the absorbance values were recorded to calculate the cytotoxicity.

LDH leakage (%) related to control wells containing cell culture medium without extracts was calculated by $[A]_{\text{test}} / [A]_{\text{control}} \times 100$. Where $[A]_{\text{test}}$ is the absorbance of the test sample and $[A]_{\text{control}}$ is the absorbance of the control sample.

Statistical Analysis

All the assays coming under in vitro antioxidant and anticancer assay were performed in triplicate and the results were expressed as mean \pm standard deviation.

Results

Concentrations ranging from 5 to 640 mcg/ml of the ethanol and aqueous extracts of *D.indica* L were tested for their antioxidant properties in different *in vitro* models. The percentage of inhibition was observed that free radicals were scavenged by the test compounds in a concentration dependent manner up to the given concentration in all the models (Table 1).

Table 1. *In vitro* Antioxidant assay of EEDI and AEDI

S no.	<i>In vitro</i> Model	Extract Concentration Used (mcg/ml)	IC_{50} Concentration of EEDI (mcg)	IC_{50} Concentration of AEDI (mcg)	IC_{50} Concentration of AA (mcg)
1	DPPH Assay		112.5 \pm 0.54	264.21 \pm 1.57	170 \pm 1.42
2	Hydroxyl Scavenging Activity		34.8 \pm 0.43	112.71 \pm 0.82	76.8 \pm 0.35
3	NO Scavenging Assay		58.41 \pm 1.3	180.59 \pm 0.46	85.71 \pm 0.82
4	Fe ²⁺ Chelating Assay	5,10,20,40,80,160,320,640	96.32 \pm 0.45	94.51 \pm 0.84	110.03 \pm 1.02
5	Reducing Power Assay		152.8 \pm 0.98	418.32 \pm 0.88	95.56 \pm 0.78
6	Super Oxide Radical Assay		88.76 \pm 0.79	105.37 \pm 0.72	160.78 \pm 0.26
7.	Radical Scavenging of ABTS		122.7 \pm 0.13	321.8 \pm 0.62	110.12 \pm 0.18

The percentage of scavenging effect on DPPH radical was increased with an increase in concentration of EEDI and AEDI. The percentage inhibition of EEDI was varying from 10% with 5mcg/ml of the extract to 85% with 640 mcg/ml of extract. Similarly the percentage inhibition of AEDI was varying from 6% with 5mcg/ml of the extract to 72% with 640mcg/ml of extract. The IC_{50} value of the EEDI and AEDI was calculated to be 112.5 \pm 0.54mcg/ml and 264.21 \pm 1.57mcg/ml while for the standard ascorbic acid was 170 \pm 1.42mcg/ml.

The hydroxyl radical scavenging activity also increased with increase in the concentrations of both extracts. The IC_{50} values of EEDI and AEDI were calculated to be 112.5 \pm 0.54 and 264.21 \pm 1.57mcg/ml, while that for ascorbic acid was 170 \pm 1.42mcg/ml.

The percentage of inhibition in nitric oxide scavenging assay was maximum (94% and 72%) at 640mcg/ml of EEDI and AEDI respectively. The IC_{50} value of the EEDI and AEDI was calculated to be 58.41 \pm 1.3 and

180.59 \pm 0.46mcg/ml while that for standard ascorbic acid was 85.71 \pm 0.82mcg/ml.

In reducing power assay, antioxidant compound forms a colored complex with potassium ferricyanide, trichloro acetic acid and ferric chloride, which is measured at 700nm, where in presence of reductants (antioxidants) in the plant extracts, causes the reduction of Fe^{3+} / Ferricyanide complex to the ferrous form. The IC_{50} values of EEDI (152.8 \pm 0.98) was found to be lesser than that of AEDI (418.32 \pm 0.88), indicating that EEDI has more reducing power than AEDI.

In metal chelating assay, ferrozine quantitatively forms complexes with Fe^{2+} . The absorbance of ferrozine- Fe^{2+} complex decreased linearly in a dose-dependent manner. Chelating agents that forms bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, and thereby stabilize the oxidized form of the metal ion. In the present work, EEDI was found to reduce inhibitory concentration (96.32 \pm 0.45) as compared to AEDI (94.51 \pm 0.84),

whereas standard ascorbic acid showed at a concentration of 110.03 ± 1.02 .

Superoxide free radicals were scavenged with the increase in the concentration of EEDI and AEDI. The IC_{50} were calculated to be 88.76 ± 0.79 mcg/ml and 105.37 ± 0.72 mcg/ml where the standard ascorbic acid was 160.78 ± 0.26 mcg/ml.

The principle behind the ABTS assay technique involves the reaction between ABTS and potassium persulphate to produce the ABTS radical cation ($ABTS^+$), a blue green chromogen. In the presence of extracts, the colored radical is converted back to colorless ABTS, the absorbance of which is measured at 734nm. The free radical scavenging activity by this method, showed significant inhibitory concentration with EEDI (122.7 ± 0.13) comparable with that of ascorbic acid (110.12 ± 0.18) whereas AEDI showed an inhibitory concentration of 321.8 ± 0.62 .

Anticancer activity of EEDI and AEDI against the test cells DAL and EAC by trypan blue exclusion and LDH leakage assay methods are shown in Figures 1 and 2 respectively. In trypan blue exclusion method, 250, 150, 100 mcg/ml of EEDI showed more significant effect against DAL than AEDI towards both the cell lines, whereas 50, 10 and 5 mcg/ml EEDB and AEDB showed less significant effects on DAL and EAC. The inhibition concentration was compared with that of control. A dose dependent increase in the % of LDH leakage was observed. A maximum leakage of LDH was observed at a concentration of 250mcg/ml. From figure 2, the % of LDH release was increased with increasing concentration of EEDI which is in direct proportion to the cell death. Maximum cell death after incubation was observed at 250 mcg/ml concentration.

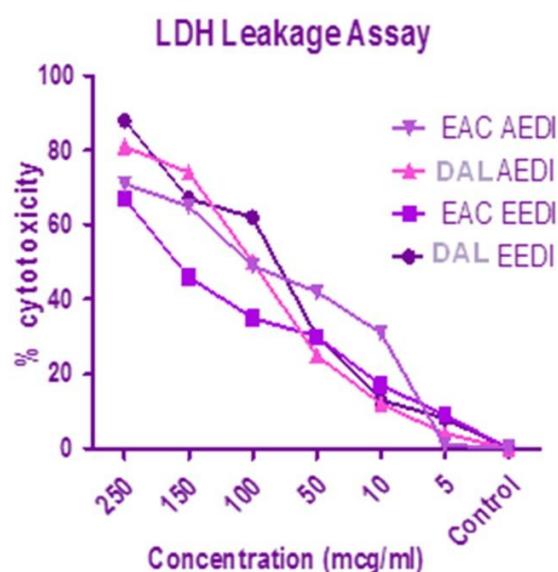


Figure 1. Anticancer activity of EEDI and AEDI against the DAL and EAC cells by LDH leakage assay

Discussion

Traditional Indian and Chinese medicinal herbs have been used in the treatment of different diseases in the

country for centuries. There have been claims that some traditional healers can successfully treat cancer using herbal drugs.¹⁵

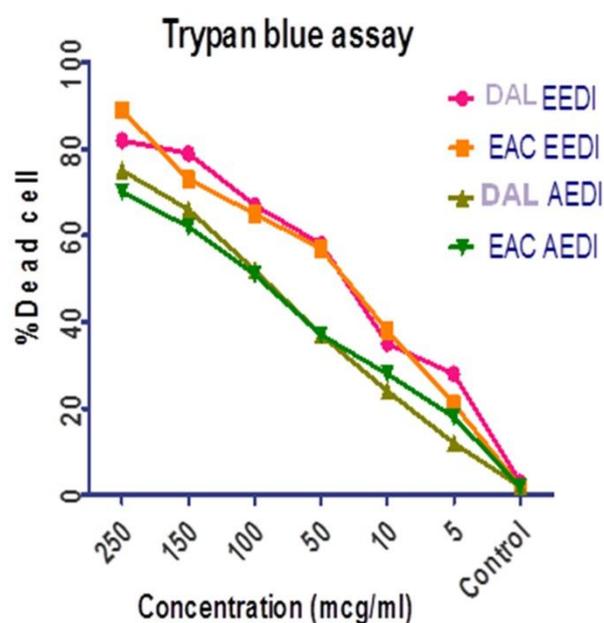


Figure 2. Anticancer activity of EEDI and AEDI against DAL and EAC cells by trypan blue exclusion method

In this study, it is evident that the extracts of *D.indica* L possess effective antioxidant and anticancer activities. This is due to the presence of phytochemicals like naphthoquinones, Quercetin etc. in this species.¹⁶ In vitro antioxidant activity of the ethanol and aqueous extracts of *D.indica* L was investigated in the present study by DPPH, hydroxyl, ABTS, nitric oxide, superoxide radical scavenging assays, reducing power and metal chelating assay. These methods have proved the antioxidant potential of the extracts in comparison with the reference antioxidant, ascorbic acid.

DPPH assay, which is based on the reduction of DPPH in methanol solution, is converted into DPPHH (non radical) in the presence of a hydrogen-donating antioxidant. This transformation results in a color change from purple to yellow, which is measured spectrophotometrically. The disappearance of the purple color is monitored at 517nm. The DPPH, is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant compounds.⁹ In the present study, the scavenging effect of different concentrations of the extracts from 5 to 640mcg/ml, on the DPPH radical is illustrated. The extracts had increasing significant scavenging effect with increase in the concentration of both extracts EEDI and AEDI. EEDI and AEDI showed significant scavenging effects on the hydroxyl radical, which increased with the increase in concentrations from 5-650 mcg/ml. The model used was ascorbic acid-iron-EDTA model of HO^{\cdot} generating system. This is a totally aqueous system in which ascorbic acid, iron and EDTA conspire with each other to generate hydroxyl radicals. It reacts

with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell. The formed hydroxyl radicals (HO[•]) ions chelate with the antioxidants, which results in the suppression of OH generation and inhibition of peroxidation processes of biological molecules.^{7,9}

In nitric oxide scavenging assay, the radicals generated from sodium nitroprusside in aqueous solution at physiological pH interact with oxygen to produce nitrite ions which is estimated with Griess reagent.¹⁷

The metal ion scavenging effect was increasing with an increase in the concentrations of extracts from 5-640mcg/ml. The high metal ion scavenging activity of the EEDI and AEDI was probably due to the chelating agents, which form sigma bonds with the metal and are effective as secondary antioxidants because they reduce the redox potential, thereby oxidized form of the metal ion.¹⁸

Reducing power assay is serving as a significant reflection of the antioxidant activity.¹⁹ Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants.²⁰

Super oxide is biologically important as it forms singlet oxygen and hydroxyl radical. Overproduction of super oxide anion radical contributes to redox imbalance and is associated with harmful physiological consequences. Numerous biological reactions generate superoxide anions which are highly toxic. In the PMS/NADH-NBT system, superoxide anion derived from dissolved oxygen from PMS/NADH coupling reaction reduces NBT.⁸ The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. From the results, it was found that the EEDI and AEDI showed potent free radical scavenging activity compared to the ascorbic acid (standard) at low IC₅₀.

LDH is a more reliable and accurate marker of cytotoxicity, because damaged cells are fragmented completely during the course of prolonged incubation with substances. In the present study, the LDH leakage increased significantly in high dose of when compared AEDI to the control cells. Extensive reports have documented on medicinal plant extract induced cytotoxicity to cancer cells.²¹ Hence, the LDH leakage in both cell lines may be due to the cytotoxic nature of the plant extract which confirms its antitumor activity.

Trypan blue is a vital stain used in the identification of dead tissue or cells. Living cells or tissues with intact cell membrane are not colored, because the dye is not absorbed through the intact cell membrane. However it traverses the membrane of dead cells. Hence dead cells are shown as a distinctive blue color under the microscope. In the trypan blue exclusion assay, there is a dose dependent inhibitory effect on both cancer cell lines treated with the extracts at increasing concentrations (5- 250mcg/ml) for 30min. After incubation with extracts significantly affected with

cytotoxic values at the maximum concentration of 250mcg/ml. The % of cytotoxicity was in the following order: EEDI against DLA (90%), EEDI against EAC (89%), AEDI against DLA (86%) and AEDI against EAC (80%).

Conclusion

The results of the present study indicate that the EEDI and AEDI of *D. indica* L possess significant antioxidant and anticancer activities when tested against different in vitro models. The antioxidant ability could be attributed to the phenolic compounds, especially flavonoids which possess antioxidant action.²² Thus, *D. indica* L extracts as promising natural sources of antioxidants and anticancer agent, can be used in nutritional or pharmaceutical fields for the prevention of free radical mediated diseases.

Conflict of Interest

There is no conflict of interest in this study.

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