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IN VITRO ANTIOXIDANT AND CYTOTOXIC ACTIVITY OF LEAVES AND STEM EXTRACTS OF *ECBOLIUM LINNEANUM*

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ABSTRACT

Ecbolium linneanum is used conventionally to treat various diseases like jaundice, rheumatism and also used as anti-inflammatory agents. Pharmacological properties of the leaf and stem of *E. linneanum* were evaluated by antioxidant potential and cytotoxic activity. Ethanol, acetone, dichloromethane and petroleum ether extracts of leaf and stem of *E. linneanum* were prepared to study the total phenolic content, radical scavenging activity, reducing power assay, total antioxidant capacity and cytotoxic by multiple *in vitro* assays. The total phenolic content, analysed by using Folin- Ciocalteau reagent, varied from 33.3 to 129.00 mg/g dry weight. The results showed that leaf and stem extracts of *E. linneanum* possesses antioxidant and free radical scavenging properties. Moreover, cytotoxicity was also examined *in vitro* in HeLa cervical cancer cell line by trypan blue assay and ethanolic extract of leaf exhibited over 80% cell death. This study revealed that the activities observed could be attributed to the presence of some phytochemicals which have been associated with antioxidant and cytotoxic property.

KEY WORDS; ***Ecbolium linneanum*, antioxidant, cytotoxicity, DPPH, HeLa**

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INTRODUCTION

Antioxidants are defined as any substance, when present at low concentration compared to that of oxidizable substrate, significantly delays or inhibits the oxidation of that substrate[1]. Free radicals play a key role in many degenerative diseases of ageing such as cancer, cardiovascular disease, cataracts, weak immune system and brain dysfunction[2]. Recently, there is a great deal of interest in newer bioactive molecules from nature with health-promoting potential[3]. Due to the recent trends in nutrition towards development of healthy foods in the form of 'functional foods', one of the desirable properties in a dietary component is considered to be have antioxidant effect[4]. Many plant compounds (notably phenolics) are antioxidants and function by scavenging free radicals such as hydroxyl (-OH) and hydrogen superoxide (-H_2O_2) free radicals (ROS)[1].

Antioxidant bioactive compounds from plant sources are commercially promoted as neutraceutical and have been shown to reduce the incidence of diseases[5]. Although plants may possess pharmacological properties, they may also be toxic or mutagenic. The toxic effects of most widely used medicinal plants are not well documented. Moreover, one might expect plants used in traditional medicine to be safe over a long period[6]. Hence, an evaluation of the toxicity, cytotoxicity and mutagenicity are important in all scientific studies[7].

Ecbolium linneanum belongs to the family *Acanthaceae* commonly referred to as Blue Fox Tail or Blue Justicia in English, Neel Kantha in Bengali, Udajati in Hindi and Nilambari in Tamil. It is an indigenous Indian plant that grows naturally along the Eastern parts of India. It has been also found in Africa and tropical Asia. *E. linneanum* has been reported as a curative in jaundice, menorrhoea, rheumatism[8] and also has anti-inflammatory activity[9]. The plant is used to treat gout and dysuria; decoction of leaves for stricture[10] and their root juice is used as anti-helmintic and also to treat premenstrual colic[11]. To the best of our knowledge, scientific evidences are not available on antioxidant and cytotoxicity activity of *E. linneanum*. Therefore, the present study is undertaken to assess *in vitro* antioxidant and cytotoxic activities of leaf and stem extracts of *E. linneanum.*

MATERIALS AND METHODS

(i)Chemicals

All solvents ethanol, acetone, dichloromethane and petroleum ether were purchased from Merck, India. All chemicals used in the study were of analytical grade.

(ii)Collection and processing of plant samples

Healthy, disease free leaf and stem of *E. linneanum* were collected in the month of May 2011 in and around the villages of Bankura district of West Bengal, India. Plants with complete herbarium was identified and authenticated from Post Graduate and Research Department of Botany, PSGR Krishnammal College for Women, Coimbatore, South India. The collected leaf and stem were washed properly in the tap water followed by detergent and finally rinsed with distilled water until no foreign material remained (damaged leaves were removed). The fresh materials were left to dry in a closed room (25-28ºC) for approximately ten days. The parts of the dried plant were pulverized by using sterile electrical blender to obtain a powder. The powdered samples were stored in air tight container, protected from sunlight for further use.

(iii)Extract Preparation

Twenty five grams of powdered plant materials were continuously extracted with different solvents like ethanol, acetone, dichloromethane and petroleum ether for successive solvent extraction based on polarity using soxhlet extraction apparatus at the boiling point of the respective solvents for 12-16 h or until the colour of the extracted solvent became clear. Different extracts were concentrated under reduced pressure using rotary evaporator and they were poured into a pre-weighed vial, further dried in a desiccating

chamber until a constant dry weight was obtained. The residues were stored at 4ºC for further studies.

(iv)In vitro Antioxidant Assays

(a)Determination of Total Phenolic Content (TPC)

The total phenolic content was determined by the Folin- Ciocalteau method[12]. 0.5ml of extract was mixed with Folin-Ciocalteau reagent (5 ml, 1:10 diluted with distilled water) for 5 min and then aqueous Na_2CO_3 (4 ml, 1 M) was added. The mixture was allowed to stand for 15 min and the phenols were determined by spectrophotometric method at 765 nm. The standard curve was prepared by 0, 50, 100, 150, 200, and 250 µg/ml solutions of Gallic acid in 50% methanol. The total phenolic content was expressed as Gallic acid (GA) equivalent (mg GA /g dry weight)

(b)DPPH Free Radical Scavenging Assay

The DPPH free radical scavenging assay was carried out by the method of Chang et al.[13] One ml of 0.1mM DPPH (in ethanol) was added to different concentrations of plant extracts. The reaction mixture was well shaken and incubated in dark for 30 minutes. Absorbance was checked at 517 nm against a blank (ethanol). Ascorbic acid was taken as the standard. Percentage of scavenging activity is inversely proportional to the absorbance of the reaction mixture. The percentage of inhibition of free radicals was determined by the formula

$$\% \text{ Inhibition} = [(\text{Control OD} - \text{Sample OD}) / \text{Control OD}] \times 100$$

where control was prepared as above without extract.

(c)Reducing Power Assay

The reducing power assay was carried out by the method of Makari et al.[14] (2008), Koleva et al.[15] (2002). 500 µl of different concentration of plant extracts were separately mixed with 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (0.1%). The mixture was incubated at 50º C for 20 minutes. 2.5 ml of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of the supernatant solution was mixed with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (0.01%) and absorbance measured at 700nm in UV-Visible Spectrophotometer. Ascorbic acid was used as standard and phosphate buffer as blank solution. Increased absorbance of the reaction mixture indicates stronger reducing power.

(d)Total Antioxidant Capacity

The total antioxidant capacity was assayed following the method of Preito et al.[16] (1999). 0.1 ml of different concentrations (100, 400, 800, 1200 µg/ml) of plant extracts were mixed with 1 ml of reagent solution (0.6 M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate; mixed in 1:1:1 ratio) respectively. The tubes were capped and incubated in a thermal block at 95°C for 90 min. After cooling to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Ascorbic acid was used as the standard and the total antioxidant capacity was expressed as equivalents of ascorbic acid.

(v)Statistical Analysis

All the grouped data were statistically evaluated by using student's't' test with SPSS/16 software. Values are presented as the mean ± S.D. of each three replicates in each experiment.

(vi)Cytotoxicity

Cytotoxicity of the plant extracts was determined by Trypan Blue Dye Exclusion method.

(a)Cell line and condition

HeLa cervical cancer cell line was obtained from NCCS (National Centre for Cell Sciences), Pune, India. Cells were cultured in Eagle essential medium and supplemented with 10% fetal bovine serum from Hi-Media and 20 µg/ml gentamicin procured from Nicholas Piramal, India Ltd. Routinely the cells were maintained under 97% humidity in a biological incubator at 37°C with 5% CO_2. All cell culture procedures were carried out in a laminar flow cabinet containing a UV light. Once the 80% confluence was reached, the

cells were ready for assay. Cell numbers were determined by counting with the haemocytometer and the viability was assessed using trypan blue dye exclusion.

(b)Cell Viability using Trypan Blue Dye Exclusion Assay

The cell numbers were adjusted to 1×10^6 cells per ml by diluting with PBS. Sterile test tube containing varying concentrations of plant extract (0.25, 0.5, 1, 2mg/ml) in PBS (0.8ml) were treated with $1x10^6$ cells/ml (0.1ml). The seeded test tubes were incubated in the biological incubator at 37°C with 5% CO_2 and 97% humidity for three hours. On termination, 0.1 ml of 0.4% trypan blue: deionised water (1:1) was added to the test as well as control tubes in order to estimate the dead HeLa cells. The cell viability was estimated by using a haemocytometer. Dead cells appear stained blue, while live cells are unstained (translucent). Cell mortality was expressed as the percentage of trypan blue positive cells was compared to the total number of cells. The percentage viability was determined using the formula:

$$\% \text{ Cell mortality} = \frac{\text{Number of dead cells (stained cells)}}{\text{Total number of cells (stained and unstained cells)}} \times 100$$

RESULTS

1.In vitro antioxidant activity

Determination of total phenolic content (TPC) of E. linneanum leaf and stem extracts Total phenolic content of leaf and stem extracts are given in table 1. Phenolic content varied from 37.33±0.58 to 129.00±1.00 mg/g for *E. linneanum* extracts. The acetone extract of stem of *E. linneanum* possessed the highest phenolic content when compared with ethanol, dichloromethane and petroleum ether extracts. Generally, the phenolic content of all the extracts was considerably high, which could be a major contributing factor to the strong antioxidant activity of these extracts.

Table 1
Total phenolic content of E. linneanum leaf and stem extracts

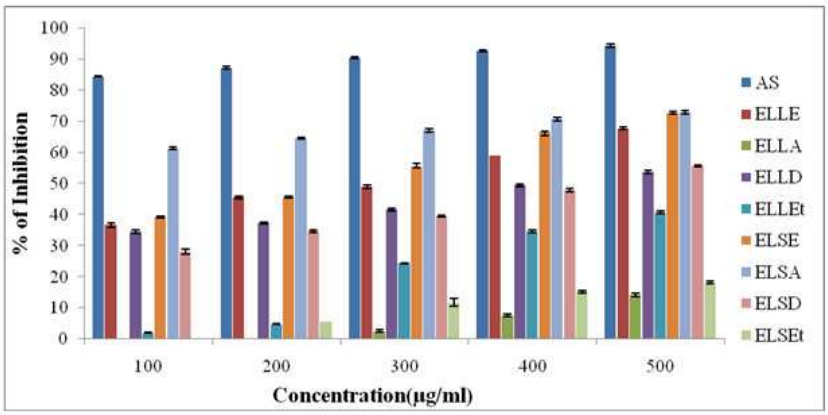
Plant Parts	Phenol Content (mgGA/g extract)
E. linneanum Leaf Ethanol extract (ELLE)	87.33±0.58
E. linneanum Leaf Acetone extract (ELLA)	63.33±1.15
E. linneanum Leaf Dichloromethane extract (ELLD)	56.67±1.52
E. linneanum Leaf Petroleum ether extract (ELLEt)	50.33±0.58
E. linneanum Stem Ethanol extract (ELSE)	111.00±1.73
E. linneanum Stem Acetone extract (ELSA)	129.00±1.00
E. linneanum Stem Dichloromethane extract (ELSD)	77.33±0.58
E. linneanum Stem Petroleum ether extract (ELSEt)	37.33±0.58

(Values are mean ± SD n=3)

DPPH radical-scavenging activity

Positive DPPH test recommends that the leaf and stem extract were free radical scavenger. The DPPH activity of the extracts was found to increase in dose dependent manner. When compared among the leaf extracts, ELLE and ELLD exhibited more than 50% scavenging activity at 500µg/ml, whereas low scavenging activity was observed for ELLA and ELLEt. Among the stem extracts, ELSA and ELSE exhibited potential effect of DPPH activity as percentage of free radicals scavenger with 73.1 and 72.7% at 500µg/ml respectively, whereas low scavenging activity was

observed against ELSEt. The radical scavenging activity of leaf and stem extracts were compared with standard ascorbic acid (Fig. 1) and all the extracts exhibited lower activity than ascorbic acid. This may be due to the occurrence of reactive concentration of bioactive constituents and mixture of other nutrients in the extract.

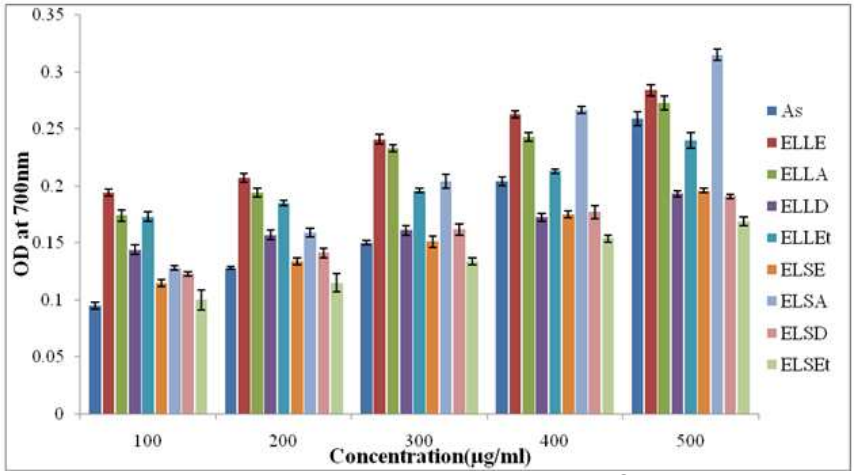


As: Ascorbic Acid. (Values are mean ± SD n=3)

Figure 1
DPPH free radical scavenging assay of leaf and stem extracts of E. linneanum

Reducing power assay

The reductive ability of the extracts increased proportionally with the absorbance. The figure recommends that at lower concentrations, the association between concentration and the decrease in the absorbance is linear. The trend for ferric ions reducing activities of the extracts did not differ distinctly from their DPPH free radical scavenging activities, when a relationship between Figs. 1 and 2 was made. Interestingly, reducing power was consistently higher than those obtained for DPPH scavenging for all the extracts. The reducing power activities of the extracts increased with increasing concentration (Fig. 2). When compared with standard ascorbic acid, it was found that ELSA, ELLE and ELLA exhibited higher activity, whereas activity of ELLEt was similar to that of ascorbic acid.

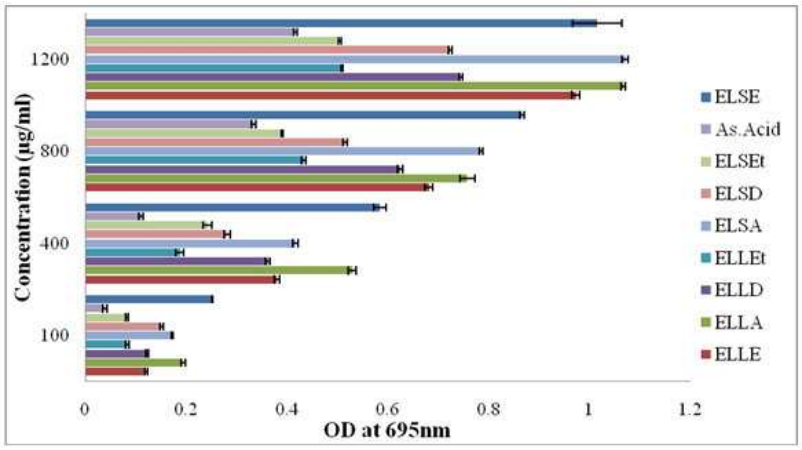


As: Ascorbic Acid. (Values are mean ± SD n=3)

Figure 2
Reducing power assay of leaf and stem extracts of E. linneanum

Total antioxidant assay

The total antioxidant capacity of the extracts was calculated based on the formation of the phosphomolybdenum complex. Total antioxidant assay of *E. linneanum* leaf and stem extracts was compared with ascorbic acid (Fig. 3). The antioxidant activity determined by this method was different according to the extracts analysed, but in general it was also higher in the acetone extract of stem, thus coinciding with the results of other assays. The total antioxidant potential was also higher in acetone extracts of leaf and stem. The lowest antioxidant activity was always exhibited by the petroleum ether extract irrespective of plant part. The antioxidant activity was increased in a dose dependent manner. The results clearly indicated that all the extracts possessed higher level of antioxidant activity than standard ascorbic acid.



As. Acid: Ascorbic Acid. (Values are mean ± SD n=3)

Figure 3
Total antioxidant assay of E. linneanum leaf and stem extracts

2. In vitro cytotoxicity assay

Initial screenings for cytotoxicity of ethanolic leaf and stem extracts of *E. linneanum* were performed on the HeLa cell line using the trypan blue assay. The result of the cytotoxic activity of crude extracts is depicted in figure 4. Among the plant extracts, ELLE induced over 80% death of Hela cells at a treatment concentration of 2mg/ml (LC_{50}-460µg/ml) and ELSE (78%, LC_{50}-490µg/ml). The cell viability data obtained in these extracts allow us to predict their potential not only because of the cystostatic effect, but in terms of potential for tumor reduction.

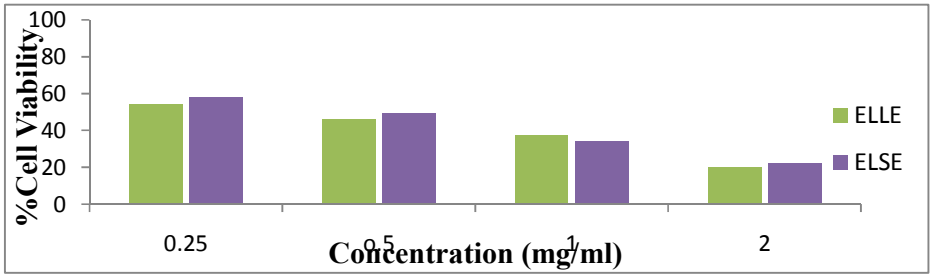


Figure 4
Cytotoxicity assay of leaf and stem extracts of E. linneanum

DISCUSSION

The antioxidant capacities of selected leaf and stem of medicinal plants were studied by multiple *in vitro* assays. Superoxide anion is a free radical generated by one electron transfer and plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems[17]. Antioxidants through their scavenging power are useful for the management of diseases like neurodegenerative, cancer and AIDS. It can also react with nitric oxide and form peroxynitrite, which can generate toxic radicals such as hydroxyl radical[18].

In the present study, the antioxidant activity of the plant extracts was evaluated by four *in vitro* methods. The antioxidant activity of phenolic compounds is mainly due to their redox properties which make them act as reducing agents, hydrogen donors, and singlet oxygen quenchers including metallic chelating potential[19]. Phenols and polyphenolic compounds, such as flavonoids, are widely found in food products derived from plant sources, and also known to possess significant antioxidant activities[20]. DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts[15]. When the odd electron becomes paired off in the presence of a free radical scavenger, the absorption reduces and the DPPH solution is decolourised as the colour changes from deep violet to light yellow. The degree of reduction in absorbance measurement indicated the free radical scavenging (antioxidant) activity of the extracts[21]. High concentration of phenols present in the extracts may explain their high antioxidative activities as it was observed in the current study. ELSA showed high phenolic content as well as highest percentage of inhibition in DPPH scavenging. ELSEt exhibited minimum % of inhibition in DPPH scavenging because of the lower level of phenolic content. This result showed that DPPH scavenging was totally relative to the total phenolic content of the plant extracts.

The reductive property of plant extracts generally depends on the presence of phyto-reductants, which have exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom[22]. Fe (III) reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action[20]. Presence of reducers causes the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. The reducing power of various extracts of leaf and stem of *E. linneanum* was very potent and increased with the increasing concentration.

Total antioxidant capacity of various extracts of the plant was expressed as the number of equivalents of ascorbic acid. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compounds and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm[23]. The study also reveals that the antioxidant activity of the extract exhibited increasing trend with the increasing concentration of the plant extracts. According to the present study, *E. linneanum* clearly exhibited higher antioxidant activity and contain significantly more phenolics than the common vegetables and fruits (nutritional plants).

Cytotoxic activity is very sensitive to a wide range of compounds and may be due to one or many number of phytochemicals within the crude preparation[24]. In this study, the cytotoxic activity of leaf and stem extracts (ELLE, ELSE) of *E. linneanum* was evaluated and concluded that the ethanolic extract of *E. linneanum* leaf showed stronger cytotoxic activity than stem. All of them had values greater than 75% mortality at the highest concentrations tested (2mg/ml). In addition, it is known that some anticancer agents might exhibit their moderate or no cytotoxic activity *in vitro* but it may reveal good *in vivo* antitumor activity. This phenomenon is due to immune modulation by the compound which could lead to antitumor activity *in vivo*[25]. Re-evaluation of the active antioxidant principle in the crude extract need to be undertaken to determine if the cytotoxicity is due to the

compound itself or some other chemical constituent.

It was noticed in this study that the parts of this plant are strong radical scavengers and can be considered as good sources of natural antioxidants for medicinal and commercial uses. The activity of the plant extracts on cancer cell line suggests that this agent can act as a potential candidate for cancer therapy. On the basis of this study, it can further be studied *in vivo* to confirm the advantageous quality of these extracts. Further studies are necessary to identify and characterize the active components from this plant that could provide potential natural sources of antioxidant compounds as well as anticancer agent.

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