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ESTIMATION OF BIOMASS CONTENTS AND PHYTOCONSTITUENT ANALYSIS OF *ENICOSTEMMA LITTORALE* BLUME.

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ABSTRACT

Enicostemma littorale Blume is a medicinal plant commonly called as whitehead. Traditionally, the plant is used in the treatment of large number of human ailments such as in hypoglycemic, lowering the blood glucose level, as a potent anti-diabetic and antioxidant activity. Currently, the scientific research is going on to prove its medicinal value. Hence, to evaluate the phytoconstituents and to estimate the total biomass content of chlorophyll, protein and flavonoid present in the aerial part of the plant has been aimed at. The result shows the presence of protein, flavonoid, carbohydrate, saponins, steroids, alkaloids, etc, and also the significant quantity of chlorophyll and protein in the fresh leaves, and flavonoid in the fresh fruit extracts. Thus, from the result, it has been proved that the plant *E.littorale* is rich in phytoconstituents and to possesses significant quantity of flavonoid, chlorophyll and protein accompanying its medicinal value.

KEYWORDS: ***Enicostemma littorale*** **Blume, Phytoconstituents, Flavonoids, Protein and chlorophyll.**

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INTRODUCTION

Plants, besides providing nutrition, have always formed an important source of chemical compounds as secondary metabolites, which can be used for medicinal purposes[1]. It has been reported that about 64% of the total global population remain dependent on traditional medicine and medicinal plants for provision of their health-care needs[2]. In recent years, traditional medicine has become of interest to both scientists and the general population for a number of reasons, which include high price of allopathic drugs beyond the reach of the poorer segments of society in almost every country, lack of access to medical clinics and hospitals by the rural population of developing countries, the side-effects and toxicities of modern synthetic drugs, and the realization that phytochemicals present in plants can be effective therapeutic agents by themselves or serve as effective adjunct therapies to modern drugs[3]. Perhaps, although India is reported to be rich in herbal resources, not more than 30-40 % of them are either analyzed or characterized. Hence, in this perception, an uncharacterized herb *E.littorale* was chosen as a target for investigation.

Enicostemma littorale (Gentianaceae) is a glabrous perennial herb with sessile lanceolate leaves and is found throughout India. Various Ayurvedic formulations containing *E. littorale* as one of the ingredients have produced the antihyperglycemic activity in hyperglycemic rat models[4]. Ethnomedical studies have revealed the use of hot aqueous extract of *E. littorale* to treat diabetes, fever, stomach pain, dyspepsia and malaria[5]. It is also reported for its anticancer[6], hypolipidaemic effect in *p*-dimethylaminobenzene (*p*-DAB) induced hepatotoxic animals[7] and anti-inflammatory properties[8].

Phytochemicals such as saponins, terpenoids, flavonoids, alkaloids, tannins, carotenoids, flavonoids, phytoestrogens and non-digestible carbohydrate possess hypoglycemic activities[9], hypocholesterolemic and antidiabetic properties[10], whereas Steroids and triterpenoids show the analgesic properties[11] and are also responsible for central nervous system activities[12] and anti-inflammatory effects[13]. From clinical studies, it is shown that terpenoids strengthen the skin, increase the concentration of antioxidants in wounds and restore inflamed tissues by increasing blood supply. The terpenoids have also shown to decrease blood sugar levels in animal studies[14]. It has been proved that plant polyphenols such as steroids, terpenoids, flavonoids as a potential are antioxidants under *in vitro* studies[15]. The tannins and resins are employed as astringent both in gastro-intestinal tract and on skin abrasions whereas the macronutrients such as proteins, carbohydrates are reported to involve in the energy giving and body building functions[16]. Tannins are also reported to have various physiological effects like anti-irritant, antisecretolytic, antiphlogistic, antimicrobial and antiparasitic effects. Phytotherapeutically, tannin containing plants and plants which are rich in alkaloids, glucosides, glycosides, steroids, flavanoids, fatty oils, phenols, resins, phosphorous and calcium are used to treat nonspecific diarrhea, inflammations of mouth and throat and slightly injured skins[17].

Photosynthesis is the most important biochemical process occurring in plants and chlorophyll (Chl) is the key pigment that absorbs solar light energy and provides mechanisms for its utilization in photosynthetic reactions[18]. Chlorophyll is an indirect indicator of nitrogen status and is used in optical reflectance- based variable rate chemical application technology[19]. The ability to accurately estimate plant chlorophyll content and concentration provide growers with valuable information on the crop yield potential and nitrogen (N) management[20]. Since, chlorophyll content of leaves is a useful indicator of general plant vigour[21, 22] and change in the amount of it would indicate its adaptive responses[23]. It is measured spectrophotometrically[24].

Proteins are important class of biological macromolecules found in all organisms. They are made from elements such as carbon, hydrogen, oxygen, nitrogen and sulphur. Protein deficiency is one of the major nutritional problems in the developing world. The most disastrous consequences occur in children where protein malnutrition manifests itself in forms of two notorious diseases: Marasmus and kwashiorkor. The process of photosynthesis is the only non depletable protein source and can supply some essential amino acids as well as provide adequate nitrogen in the diet for synthesis of non essential amino acids in addition to vitamins and minerals[25]. Based on their size (1-100 nm), proteins are nanoparticles[26]. All proteins are polymers of amino acids. The polymers (known as polypeptides) consist of sequences of 20 different L-□-amino acids (referred to as residues). For chains fewer than 40 residues, the term peptidesis frequently used instead of proteins. To be able to perform their biological functions, proteins must possess at least 50 residues. Proteins exist as primary, secondary, tertiary and quarternary structures. Plant proteins act as plant defense agent against the microbial infection.

Flavonoids are the large groups of polyphenolic compounds including anthocyanin and other phenolic compounds present relatively at high concentrations in plant tissues and vegetables providing health effects to the consumers. They are water soluble and commonly occur in vacuoles, membrane-enclosed structures within cells which also store water and nutrients; however its synthesis is induced by the photoreceptive plant pigments such as phytochrome and flavins. Chemists have identified more than 5,000 naturally occurring flavonoids[27], which are placed into 12 different major classes and the best known of them are the anthocyanins, flavonols, and flavones. All flavonoids have 15 carbon atoms and consist of two 6-carbon rings connected to one another by a carbon atom which contains an oxygen atom and occur as O-glycosides with one or more sugars bound at the C3 position bound to one or more sugar molecules. Small changes in a flavonoid's structure can cause large change in its color and directly influence the metabolic activities. Moreover, it is reported for its antiplatelet[28]; antitumoral[29, 30, 31]; antilipoperoxidant[32]; anti-ischemic[33]; anti-inflammatory and anti-allergic effects[34, 35]; anticarcinogenic[36, 30, 37, 38] and antimutagenic[39, 40]. They also have antioxidant properties and inhibit the oxidation of LDL[41, 42]. Hence, UV-absorbing flavones and flavonols present in the leaves of many species protect plants by screening out harmful ultraviolet radiation from the Sun. Thus, realizing the importance of these, the present study was aimed to estimate the total content of the chlorophyll, protein and flavonoid in the different plant parts exposed to various drying treatment. And the leaves was analyzed for the presence of trace elements such as aluminium (Al), cadmium (Cd), mercury (Hg), lead (Pb) and Silver (Ag). A number of minerals essential to human nutrition are accumulated in different parts of plants and it includes metals such as cadmium (Cd), cobal (Co) and silver (Ag), which are of unknown direct benefit to the plant[43]. The evaluation of the macro and microelements present in medicinal plants have picked up as these are probably related to the pharmacodynamic action of the plant extracts. Perhaps the investigation of the effect of elements in medicinal herbs is an important topic for contemporary medicine and life sciences, for it provides a scientific basis for the medical mechanism of action, toxic effects and quality control.

MATERIALS AND METHODS

Chemicals and Reagents

Folin-Ciocalteau Reagent (1N), Ethanol (80%), Sodium Carbonate (20%), Catechol, Methionine, Riboflavin, Ethylene Diamine Tetra Acetic acid (EDTA), Nitroblue Tetrazolium (Nbt), Xylene, 2,2`-Dipyridyl, Ferric Chloride, Pyrogallol, DL-α-Tocopherols, Hydrogen Peroxide.

Plant Materials

The wild plant of *Enicostemma littorale* grown at sub-tropical regions of Thirunelveli and Salem districts of Tamilnadu was collected in the month of August and September (2009) at the end of the flowering season by uprooting method. The species identification was examined by comparing its morphological features and microscopic examination of the anatomy as per the standard methodologies at Botanical Survey of India, Tamil Nadu Agricultural University (TNAU), Coimbatore, Tamil Nadu, India (Ref No.BSI/SC/5/23/09-09/tech.-1842). The collected material was brought for investigation to the Phytomatics Laboratory, Department of Bioinformatics, Bharathiar University, Coimbatore, Tamil Nadu, India.

Phytochemical Analysis

The methanolic extract of E.littorale was subjected for phytochemical analysis for the presence of alkaloids, flavonoids, Carbohydrates, proteins, phenols, terpenoids, steroids, glycosides, tannins and saponins.

Test for Proteins

To a small amount of methanolic extract, 5-6 drops of Million's reagent was added to form a white precipitate which turned red on heating that indicated the presence of proteins[44].

Test for Carbohydrates

The 0.5 mL of powdered sample of extract, 5 mL of Benedict's reagent was added and boiled for 5 min. Formation of bluish green color showed the presence of carbohydrate while the presence of flavonoid was indicated by reddish pink or dirty brown color[44].

Test for Phenols

Total phenol estimation of methanolic extract was carried out with the Folin-Ciocalteau reagent using the standard Gallic acid as described by[44].

Test for Alkaloids

About 0.2 g of methanolic extract was warmed with 1% of aqueous hydrochloric acid for two minutes. The mixture was filtered and few drops of Dragedorff's reagent was added which turned reddish-brown with turbidity indicating the presence of alkaloids[45].

Test for Flavonoids

About 0.2 g of the extracts was dissolved in 1 mL 10% of sodium hydroxide (NaOH) and Hydrochloric acid (HCl) each respectively. A yellow solution that turned colorless on addition of HCl indicated the presence of flavonoids[46].

Test for Terpenoids

5 mL of methanolic extract was mixed with 2 mL of $CHCl_3$ in a test tube. 3 mL of concentrated H_2SO_4 was carefully added to the mixture to form a layer. An interface with a reddish brown coloration indicated the presence of terpenoids[45].

Test for Steroids

The test for steroids was done by the Lieberman acid test. A portion of the extract was treated with drops of acetic anhydride. Concentrated H_2SO_4 was carefully added to the side of the test tube. The presence of a brown ring at the boundary of the mixture was taken as positive result[47].

Test for Glycosides

The test method was referred to as Lieberman's acid test. A small quantity of the extracts was dissolved in 2 mL of acetic anhydride and cooled in ice. Concentrated H_2SO_4 was then carefully added. The color change from violet to blue to green indicated the presence of a glycoside[48].

Test for Tannins

5 mg of the powdered extracts was stirred with 10 mL of hot distilled water, filtered and 5 mL ferric chloride was added to the filtrate and observed for blue-black, blue-green or green precipitate[45].

Test for Saponin

About 2.5 g of dried powdered sample was extracted with boiling water. After cooling the extract was shaken vigorously to froth and then allowed to stand for 15-20 min and classified for

saponin content as follows. No froth = negative; forth less than 1 cm = weakly positive; froth 1.2 cm high = positive and froth greater than 2 cm high = strongly positive[49].

Determination of Plant Weight and Moisture

To assess the weight and moisture content, calculations of plant parts such as root, leaf, stem, flowers, fruits and whole plant fresh weight, dry weight and moisture contents were determined before and after oven drying the samples at 80˚C for 24 hrs.

The moisture content of the sample was calculated using the following equation:

$$\%W = \frac{A - B}{A} \times 100$$

Where:

%W = Percentage of moisture in the sample,
A = Weight of wet sample (grams), and
B = Weight of dry sample (grams)

Experimental Design

The Plant materials obtained was separated into two groups; one used fresh and the other was subjected to drying conditions namely, shade and sun dry. However for all the three strategies, approximately five hundred grams of the fresh plant material was washed, drained and used. For shade dry, the prewashed and drained plant material was placed on a filter paper (90 cm × 60 cm) at room temperature (27±1°) for three days. For sun dry, the fresh material was placed in greenhouse for 3 days. Once the drying process was over, the dry weights were measured to calculate the percentage of water loss and powdered using a laboratory blender and stored for further work. From the storage, approximately fifty milligram of the material was drawn to perform the analysis[50].

Chlorophyll

Chlorophyll was extracted according to [51], where to about 1g of plant material was added 20 mL of 80% acetone and vortexed well. It was centrifuged at 5000 rpm for 5 minutes to obtain the supernatant and the extraction was repeated until the residue was colorless. The supernatants collected were pooled and the absorbance of the green colored solution was read at 645 and 663nm against the solvent (80% acetone) blank.

$$\text{Mg total chlorophyll} = 20.2\,(A_{645}) + 8.02\,(A_{663}) \times \frac{V}{1000 \times W}$$

Estimation of Total Proteins

Total protein estimation was performed by a method described by [52] where, 500 mg of plant material was mixed with 10 ml distilled water, vortexed for two minutes and was centrifuged for 10 min at 3000rpm. A volume of 0.05 ml supernatant was pipetted out into a test tube and made up to 1 ml with distilled water, 3 ml of reagent A (2% sodium carbonate in 0.1N sodium hydroxide) and 1 ml of reagent B (0.5% copper sulphate in 1% potassium sodium tartarate) and 0.2ml of Folin-Ciocalteau reagent were added and incubated for 30 min at room temperature. Bovine Serum Albumin was used as a reference standard in a range of 10- 250 µg/ml. All the samples and standards were prepared in triplicates and absorbance was measured at 600nm against a blank having all the reagents except the sample. Total proteins were calculated from linear regression equation, obtained from the standard curve.

Estimation of Total Flavonoids

The assay for total flavonoid was conducted according to the method of [53], where 1 ml of extract was added with 4 ml of distilled water and 0.3 ml 5% sodium nitrate. After 5 min, 0.3 ml of 10% alluminium chloride, 2 ml of 1 M sodium hydroxide was added. Then the mixture was further diluted to 10 ml by adding 2.4 ml distilled water. After thorough mixing the absorbance was measured at 510 nm and calibrated against the plot prepared with quercetin solutions at concentrations from 12.5 to 100 g ml^{-1} in methanol.

Determination of Elements by ICP-OES

ICP-OES (Inductive Coupled Plasma Optical Emission Spectroscopy) measurements were carried out using a Vista MPX instrument with charge coupled devices (CCD) simultaneous detection systems (Varian Inc., Victoria, Australia). Plasma torch alignment was performed by using a Mn solution (5µg g^{-1}) at emission line 257.61 nm. During measurements, chemical attack of instrumental parts such as nebulizer, spray chamber and plasma torch as well as residue formation should be minimized. As the sample solutions contained HBF_4 which is corrosive to quartz, an HBF_4 resistant InertV-groove nebulizer (Varian Inc.) was used. Similarly, an inert Ertalyt® (Polyetlylene Terephtalate) Sturman-Masters spray chamber (Varian Inc.) was employed to improve plasma stability and to minimize matrix interferences.

Statistical Analysis

All the assays were done in triplicate for differently exposed plants. The data obtained was subjected to statistical One Way Analysis of Variance (ANOVA) and the significant difference among the means were compared with Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$ level using the SPSS/PC + Student Ware software (version 17.1.2).

RESULTS

Preliminary Phytochemical Investigation

Qualitative analysis carried out for methanolic extract of the aerial parts of *Enicostemma littorale* showed the presence of biomolecules such as alkaloids, phenols and few major groups of phytochemicals (Table 1). Slight presence of Saponin was confirmed by foam test in the methanolic aerial parts. Qualitative phytochemical studies of carbohydrate and Glycoside showed a good characteristic color and precipitate in the tested reagent. Phenolic compounds and flavonoids were abundantly present in the extract. The formation of yellow precipitate or turbidity revealed the presence of alkaloids in the Mayer's test. The observation of specific color change with the addition of respective reagents represents the presence of carbohydrates, tannins, steroids and terpenoids in the extract.

Table 1
Screening for the biomolecules in methanolic extract of Enicostemma littorale Blume

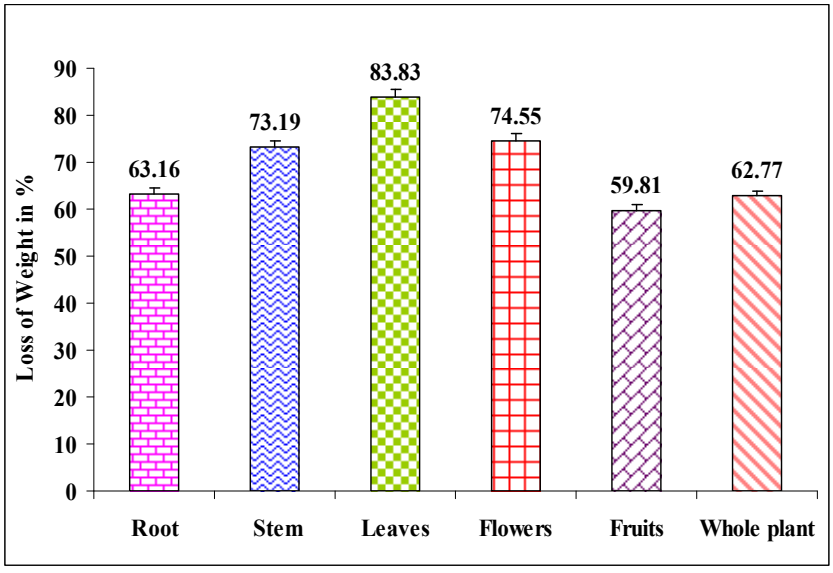
Phytochemical Constituents	Methanol extract
Phenols	+
Flavonoids	+
Alkaloids	+
Steroids	+
Terpenoids	+
Tannins	+
Saponin	+
Glycosides	+
Carbohydrate	+
Protein	+

+ represents the presence of Phytochemical constituents

Loss on Drying

The loss of weight before and after drying was observed and the percentage was calculated (Fig. 2). Weight loss varied in different plant parts showed the higher percentage of loss in the leaves (83.83%) followed by flowers (74.55%) and stem (73.19%). Less percentage of weight loss was observed in fruits (59.81%), while the root (63.16%) and whole plant (62.77%) was observed to possess closely related percentage of weight loss on drying.

Figure. 2
Determination of moisture content in E. littorale



Total Chlorophyll

The total chlorophyll content of the aerial parts of *E. littorale* extracted as fresh, sun dried, and shade dried was observed and compared with each other. It was revealed to exhibit as high as 1.85 ± 0.152 mg/g in the leaves of the fresh plants followed by shade dried plants (1.19 ± 0.195mg/g) which was found to be 57.98% less 37.3% high respectively when compared to fresh and sun dried materials respectively (Table 2). However, the least amount was found in the floral parts 0.03 ± 0.008 mg/g of fresh plant and stems of the sun dried plants respectively 0.02 ± 0.055 mg/g.

Total Protein

Total protein was found significantly high in fresh leaves (310.2 ± 0.002 mg/g) of the plant followed by the floral parts (297.5 ± 0.003mg/g). Protein content was 45% and 36% high in fresh leaves compared to the shade dried leaves (266.9 ± 0.003 mg/g) and flowers (141.3 ± 0.002 mg/g). It was found that fresh leaves show 86% higher protein content than the sun dried leaves (113.8 ± 0.003 mg/g). The least protein content was observed in sun dried stem of 43.8 ± 0.001 mg/g.

Total Flavonoid

Flavonoid content was observed to be significant in fruits of fresh plant (86.2 ± 0.002 mg/g) followed by flowers (48.6 ± 0.003 mg/g). It was found 56% high in fruits compared to the flowers of fresh plants. The shade dried fruit (65.9 ± 0.004 mg/g) shows 45% high flavonoid content compared to flowers (29.7 ± 0.001 mg/g) of shade dried plant. The least flavonoid content was found in the fruits of sun dried 21.4 ± 0.003 mg/g plants which were 25% lower than the fresh fruits (Fig.3). The least flavonoid content was observed in the stem of the sun dried plants of 1.5 ± 0.001 mg/g.

Table 2
Preliminary biochemical assay on Enicostemma littorale blume

***Enicostemma littorale* under different condition**

Plant part	Fresh			Shade dry			Sun dry		
	Total Chlorophyll mg/g	Protein mg/g	Flavonoid mg/g	Total Chlorophyll mg/g	Protein mg/g	Flavonoid mg/g	Total Chlorophyll mg/g	Protein mg/g	Flavonoid mg/g
Root	-	249.4 ± 0.003	8.6 ± 0.001	-	128.1 ±0.002	5.7±0.003	-	44.5 ± 0.002	2.7 ± 0.002
Stem	0.11 ± 0.020	141.3± 0.001	5.7 ± 0.001	0.2 ± 0.003	66.3 ± 0.001	2.6 ± 0.003	0.02 ± 0.055	43.8 ± 0.001	1.5 ± 0.001
Leaves	1.85 ± 0.152*	310.2 ± 0.002*	35.9 ± 0.002	1.19 ± 0.195	266.9±0.003*	26.3 ±0.002	0.69± 0.394	113.8±0.003	6.8 ± 0.001
Flowers	0.28 ± 0.041	297.5 ± 0.003	48.6 ± 0.003	0.03 ± 0.008	141.3 ± 0.002	29.7 ±0.001	0.24 ± 0.047	142.5±0.003	26.2 ± 0.001
Fruits	0.14 ± 0.015	143.8 ± 0.002	86.2 ± 0.002*	0.12 ± 0.026	66.3 ± 0.003	65.9 ±0.004	0.05 ± 0.035	44.4±0.002	21.4 ± 0.003
Whole	1.06 ± 0.115	233.8 ± 0.002	37.4 ± 0.002	0.53 ± 0.075	162.5± 0.002	25.5 ±0.002	0.50± 0.213	36.3±0.002	13.9 ± 0.004

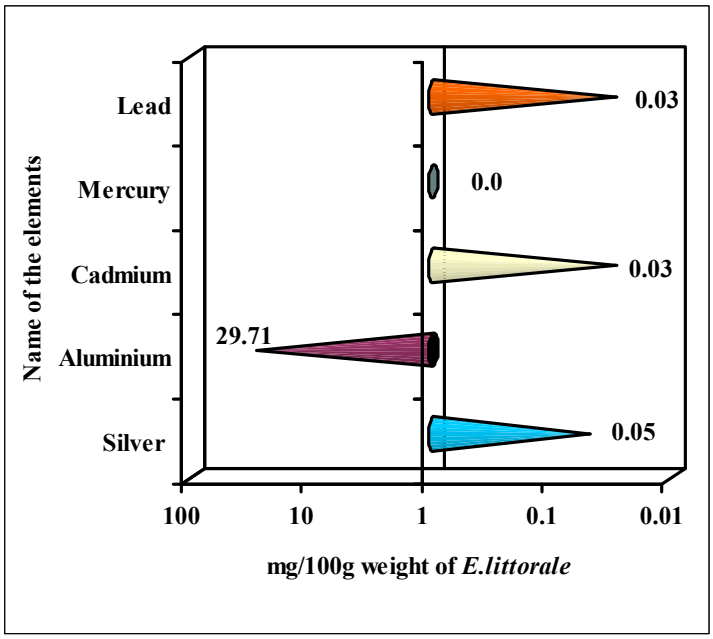
Data expressed as mean ± SEM of triplicates and values in * indicates the significant difference (P<0.05).

Elemental Analysis

The concentration of Ag, Al, Cd, Hg and Pb in *Enicostemma littorale* plant is appended (Fig.2). Elemental analysis of the plant leaf powder indicates the presence of Silver (Ag), Aluminium (Al), Cadmium (Cd) and Lead (Pb). The quantity of Cd and Pb was observed in very low quantity about 0.03 mg/100g, where as Al was observed to be present in higher range about 29.71 mg/100g. Mercury (Hg) was not detected in the leaf powder and a silver nanoparticle observed was about 0.05 mg/100g.

Figure. 2

Elemental analysis in Enicostemma littorale Blume plant leaf powder by using ICP-OES



DISCUSSION

The plants form secondary metabolites for protection against pests, as colouring, scent, or attractants and boost the immune system, protect the body from free radicals and kill pathogenic germs and much more. [54] we investigated the extracts of roughly 19 plants for the presence of various phytochemicals which indicated their potentials as bioactive compounds because if they are properly and extensively studied they could provide many chemically interesting and biologically active drug candidates that may include some with potential antitumor and anti-proliferative properties. It has been reported that flavonoids are known to be synthesized by plants in response to microbial attack when *Persea americana* seed extracts was tested *in vitro* against a wide array of microorganisms[55] of this property and could have been probably due to the their ability to react with extracellular and soluble proteins and to complex with bacterial cell walls leading to the death of the bacteria as reported [56]. According to [57], several plant species rich in flavonoids were reported to have disease prevention and therapeutic properties. This observation is of particular importance since flavonoids are ingredients of many vegetables and fruits and the association of vegetable and fruit consumption with reduced cancer risk has been reported[58]. According to [59] flavonoids and tannins in all the plants is likely to be responsible for the free radical scavenging effects observed which act as primary antioxidants or free radical

scavengers[60]. Polyphenols such as apigenin and luteolin[61], kaempferol[62], myricetin and quercetin[63], genistein and diadzein[64] etc have antiobesity effects, similar to the phytoconstituents such as phytoalexins, carotenoids and coumarin derivatives which prevent diseases by its lipid metabolism[65].

Phytochemical screening of methanol extracts of *E. littorale* aerial parts used in this study revealed that the crude extracts contained phenols, alkaloids, flavonoids, tannins, saponins, proteins, carbohydrates and terpenoids. The presence of flavonoids, alkaloids and glycosides in this plant was already reported by [66], however an indication as to which part of the plant is not revealed. Hence, an effort was made on this to check for the availability in specific leaf part of the plant. Qualitative analysis of the ash revealed the presence of minerals like iron, potassium, sodium, calcium, magnesium, silica, phosphate, chloride, sulphate and carbonate, in addition to five alkaloids, two sterols and volatile oil, have been reported by [67].

Monoterpene alkaloids like enicoflavin and gentiocrucine were also isolated[68, 69]. The presence of catechins, saponins, steroids and triterpenoids were reported by earlier workers[70], Betulin, a triterpene sapogenin (m.p. 252-254 $^{\circ}$C) was also isolated by earlier workers[71]. The plant has been reported to possess the alkaloid gentianine and the bitter glycoside swertiamarin [72]. Steroidal compounds present in almost all of the medicinal plants are noted for its importance in pharmacy[73]. Qualitative analysis of *E. littorale* aqueous extract showed the presence of phenols, tannins, flavonoids, glycosides, anthroquinones and sterols[74], because the biological actions and pharmacological properties are primarily due to these phytocomponents[17].

Chlorophyll concentration relates strongly to the photosynthetic potential of a plant while pigment content and composition are related to physiological and metabolic status[19] which plays an important role in the development of the assimilating system of plants. Its concentration varies with age and maturing, day length, season and environment. The chlorophyll content of the sun dried and shade dried plant was less compared to the fresh plants which could be attributed to the varied pigment ratios of sun and shade leaves in response to nutrients and soil conditions[75]. The chlorophyll content in leaf gives an indication of the efficiency of leaf to prepare food through photosynthesis. Significant differences were recorded for the chlorophyll content in leaf. Higher chlorophyll was noticed in shoots of the plant and complete absent in the roots of the plant.

The protein content was found significantly high in the fresh plant parts. Plants have been tested as production systems for a range of therapeutic proteins that can be used either directly in foods or after purification[76]. Protein content also determines the aminoacid content of the plant. As the major component of most food which contains nitrogen is protein, if the amount of nitrogen is multiplied by a factor depending on the kinds of protein expected in the food the protein can be determined and this value is known as the “crude protein” content.

Flavonoids appear to play vital roles in defense against pathogen and predators and contribute to physiological function such as seed maturation and dormancy[77]. Perhaps, impart color to flowers and fruits and attract insects for pollination provides resistance to plants and thus have been ever used as chemotaxonomic markers. It has been predicted that average intake of all flavonoids is several grams per day[77]. Numerous epidemiological studies confirm signinificant relationship between the high dietary intake of flavonoids and the reduction of cardiovascular and carcinogenic risk[78]. The formulation of preventive and healthy nutrition requires information about phenolic and flavonoid composition in plant foods[53]. The ingesetion of natural antioxidants has been associated with reduced risks of cancer, cardiovascular disease, diabetes, and other diseases associated with ageing, and in recent years, there has been a worldwide trend towards the use of the natural phytochemical present in berry crops, teas, herbs, oil-seeds, beans, fruits

and vegetables[79]. In several biosystematical and evolutionary studies, secondary compounds like flavonoids have been very valuable for obtaining information about relationships and the hybrid origin of taxa[80].

Flavonoids play a vital role in the physiology of plants by producing the red and purple anthocyanin pigments[81], while the non-pigmented flavonoids also play central roles in the biology of plants, serving as signals for pollinators and for other beneficial organisms, participating in plant hormone signaling, facilitating pollen-tube germination, protecting plants from UV-B and functioning as phytoalexins and allelopathic compounds[82, 83]. However, the exogenous application of flavonoids reports plant growth regulation[84].

Medicinal herbs are easily contaminated by metals and microbial growth during growth, development and processing due to factors such as environment, pollution, atmosphere, soil, harvesting and handling[85]. After collection and transformation into dosage form the heavy metals confined in plants finally enter the human body and may disturb the normal functions of central nervous system, liver, lungs, heart, kidney and brain, leading to hypertension, abdominal pain, skin eruptions, intestinal ulcer and different types of cancers. Hence, the *E.littorale* leaves was analyzed for the presence of the heavy metals where in the present study, the concentration of heavy metals determined was well found below the critical limit especially lead (Pb) (0.03 mg/100g), cadmium (Cd) (0.03 mg/100g) and silver (Ag) (0.05 mg/100g), however surprisingly mercury (Hg) was not detected. Among the heavy metals detected aluminium (Al) (29.71 mg/100g) was found to be higher.

Lead (Pb) and cadmium are non-essential trace elements having functions neither in human body nor in plants. They induce various toxic effects in humans at low doses. The typical symptoms of Pb poisoning are colic, anemia, headache, convulsions and chronic nephritis of the kidneys, brain damage, vascular and immune system and central nervous system disorders, aluminium may cause strong effects including dysuria, discomfort, cataract and neurotoxicity[86], if intake is more than recommended values. Cadmium accumulation in human body could damage mainly the kidneys and liver, causing both acute and chronic poisoning[87]. Likewise, Mercury (Hg) has several effects on human such as damage to brain functions, DNA damage and chromosomal damage, allergic reactions, resulting in skin rashes, tiredness and headaches, disruption of nerve system, negative reproductive effects, such as sperm damage, birth defects and miscarriages.

WHO recommends that medicinal plants which form the raw materials for the finished products may be checked for the presence of heavy metals, further it regulates maximum permissible limits of toxic metals like arsenic, cadmium and lead, which amount to 1.0, 0.3 and 10 ppm, respectively[88]. Furthermore the European commission has established the lead, cadmium and mercury limits in food supplements that have been in force since March 2001.

There are several acts to provide healthy food and drugs, whereas in 2003 the Food and Drug Administration (FDA) proposed regulation that would make dietary supplement manufacturing, packaging and storage be in compliance with current good manufacturing practices (cGMPs). The heavy metal recommendation level varies for babies from adults both in preparation of food materials and medicine[89]. [88] prescribed limit for Pb contents in herbal medicine is 10 ppm while the dietary intake limit for Pb is 3 mg/week. The lowest level of Cd which can cause yield reduction is 5-30 ppm, while the maximum acceptable concentration for food stuff is around 1 ppm[90]. The implication of findings may be taken into consideration whilst using the herbs for human consumption. Therefore, the results of elemental analysis suggest that the plant may be used for human consumption or for preparation of herbal products and standardized extracts should be collected from an unpolluted natural habitat.

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