Available online at www.rajournals.in

RA JOURNAL OF APPLIED RESEARCH
ISSN: 2394-6709
10.31142/rajar/v4i4.14
Volume: 04 Issue: 04 April-2018

International Open Access
ICV- 74.25
Page no.- 1649-1655

In-Vitro Antioxidant Activities of Aqueous and Methanol Extracts of *Enicostemma littorale* Blume

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ARTICLE INFO

Published Online:
28 April 2018

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ABSTRACT

The total phenolic and flavonoid contents and antioxidant potential of hot aqueous and methanol extracts of whole plant of *Enicostemma littorale* Blume was investigated. The percentage yield of hot aqueous extractive value (36.3%) was greater than the percentage yield of hot methanol extractive value (21.8%) and the total phenolic and flavonoid contents in hot methanol extract were found to be higher than in the hot aqueous extract of *Enicostemma littorale*. Overall results of in-vitro antioxidant activity assays indicated that in comparison to the standard trolox, *Enicostemma littorale* showed low antioxidant activity in DPPH, ABTS and FRAP methods. Iron chelating activity not found at highest possible concentration of both extracts. Although the total phenolic and flavonoids components were detected in the hot methanol and aqueous extracts, these does not appear to be reported a comparison between the observed in-vitro antioxidant activity and extractive values with total phenolic and flavonoids contents of *Enicostemma littorale*.

KEYWORDS: *Enicostemma littorale*, in- vitro antioxidant, Aqueous, Methanol, Extracts

I. INTRODUCTION

Antioxidants are substances which reduce oxidative damage often by inactivating free radicals. Antioxidant protect key cell components in biological systems by neutralizing the damaging effects of free radicals and increase the shelf life of lipid containing foods by delaying, retarding or preventing the development rancidity or other flavor deterioration due to the lipid oxidation [1]. According to a survey conducted by W.H.O., traditional healers treat 65% patients in Sri Lanka and 80 % in India [2].Plants are potential sources of natural antioxidants. During the past decade, researches conducted in many laboratories have shown that plants are very important sources of antioxidant and radical scavenging components [3] [4] [5].

Enicostemma littorale (*E. littorale*) is widely used in Siddha system of medicine under the name "*vellarugu"* [6] [7]. This plant is used in folk medicine to treat diabetes mellitus, control arthritis, rheumatism, constipation, abdominal ulcers, swelling, skin diseases and insect poisoning [8] [9] [10]. In Sri Lanka, it is found in on open, sandy places among sparse grass close to the beach throughout the dry zone particularly from northwestern to northeastern coastal belt [11].It is a rainy season herb, growing on moist, damp and shady ridgesand slopes of the borders of cultivated fields [12].

II. MATERIALS AND METHODS

A. Plant material

Whole plants of *E. littorale* were collected during the month of January 2012 in and around Jaffna District, Sri Lanka. The botanical identity of this plant was authenticated and a voucher specimen (Assess no. 2454) has been deposited in the Bandaranayaka Ayurveda Memorial Research Institute (BMARI), Nawinna, Maharahama, Sri Lanka.

B. Preparation of herbal medicine

The collected *E. littorale*whole plants were washed with tap water. The plants were cut in to small pieces and air-dried thoroughly under shade (at room temperature) for 2-3 weeks to avoid direct loss of phytoconstituents from sunlight. The shade dried materials were powdered using the pulverizer and sieved up to 80 meshes. It was then homogenized to fine powder and stored in airtight container for further analysis.

C. Chemicals

All chemicals used were of analytical grade. Folin-Ciocalteau reagent, gallic acid, quercetinkrist ($C_{15}H_{10}O_7$. $2H_2O$),6-hydroxy-2-5-7-8-tetramethylchroman-2-carboxylic acid (Trolox), 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical,2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulphate, 2,4,6-tripyridyl-s-triazine (TPTZ), 4,4'-disulfonic acid sodium salt (ferrozine) and Ethylenediamine tetra acetic acid disodium

salt dehydrate (EDTA) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Other reagents were obtained from Fisher Scientific (Springfield, NJ, USA).

All the analyses were carried out using High- throughput 96-well micro-plate reader (Spectra Max Plus384, Molecular Devices/ U.S.A) at Bioactivity Lab/ Herbal Technology Section, Industrial Technology Institute, Colombo- 07, Sri Lanka.

D. Preparation of freeze-dried extracts

Ten gram of powder material was suspended in 150ml distilled water and refluxed three hours in a round bottom flask on heating mantle. The raw extract was pooled, filtered, and evaporated to dryness in a rotary vacuum evaporator at 40^0C. The dried crude aqueous extract was weighed and 1 g portion was freeze dried and stored at 4^0C for further investigation.

Same procedure was followed using 95% (v/ v) methanol to dried crude methanol extract. The extracts were dissolved in DMSO (Dimethyl sulfoxide) prior to use. The extractable matters were calculated as the content of in mg per g of air-dried material according to the way of wet and dry basis.

E. Assaying methods

i. Total Phenolic Contents Determination:

The total phenolics content in hot aqueous and methanol extracts of *E. littorale* were estimated according to the Folin– Ciocalteu method [13]. The freeze dried aqueous extract was re-dissolved with DMSO and distilled water using Vortex machine (VIBROFIX VF1 Electronic) and the concentrations of each extract adjusted to 1mg/ ml with distilled water. The methanol extract was re-dissolved with DMSO and methanol (Anala R* grade) to a concentration of 1mg/ ml. After 10 minutes, 40 µL of each extracts, and 110 µL of 10-fold diluted 2N Folin–Ciocalteu reagent (Sigma - UK) were combined in a micro plate as triplicate and then mixed well using a Vortex mixture (Biocote - stuart). The mixture was allowed to react for 5 minute then 70µl of 10% Sodium carbonate (Na_2CO_3) solution was added and mixed well. The solution was incubated at room temperature (27 ^{0}C) in the dark for 30 minutes. The absorbance was measured at 765nm against a methanol blank, using a Micro Plate Reader (Molecular Deviser – Spectra Max 384 Plus). Gallic acid (Sigma- Aldrich Chemical, USA) (0.125- 1 mg/ ml) was used as a standard to prepare a calibration curve and the values are presented as means of triplicate analyses. The total phenolic content was expressed in mg of Gallic acid equivalents (GAE)/ g of extract using the following equation:

$$y = 3.535x + 0.011$$

ii. Total Flavonoids Contents Determination:

The total flavonoids content in hot aqueous and methanol extracts of *E. littorale* were estimated according to the $AICl_3$ method [14]. Each dried aqueous and methanol extracts were re-dissolved in methanol to a final concentration of 5mg/ ml. 100µl of 2% Aluminium chloride ($AlCl_3$ Y $6H_2O$) in methanol mixed with 100µl of 2.5 times and 5 times diluted each extracts in methanol (2.5 mg/ml and 5mg/ ml) were combined in a micro plate as triplicate and then mixed well using a Vortex mixture. The mixture was allowed to react for 10 minutes. The absorbance was measured at 415nm against a methanol blankusing a Micro Plate Reader. The results were determined using a standard curve prepared with Quercetin (Sigma- Aldrich Chemical, USA) 1mg/ ml, 6-fold diluted with MeOH} as the standard and the values are presented as means of triplicate analyses. The total flavonoid contents are expressed as mg of quercetin equivalent (QE)/ g of extract using the following equation:

$$y = 0.999x + 4E\text{-}05$$

iii. DPPH Radical Scavenging Assay:

The ability of the extracts to scavenge DPPH free radicals was determined by the standard method [15]. A 100µl various concentrations of each extract was mixed with 100µl methanol buffer and 50 µl of 0.05mM 1, 1-diphenyl-2-picrylhydrazyl in methanol. The controls contained all the reaction reagents except the extract or positive control substance. After 20 min incubation in darkness and at 25°C, the resultant absorbance was recorded at 517nm. Linear graph of concentration vs. percentage inhibition was prepared and IC50 values were calculated from equation of line obtained by plotting a graph of concentration versus % inhibition. The values are presented as the mean of triplicate analyses. Trolox was used as positive control. The antioxidant capacity based on the DPPH free radical scavenging ability of the extracts were expressed as µmol Trolox equivalents per gram of plant material on dry basis. The % inhibition was calculated according to the following equation:

$$\text{DPPH (\% inhibition)} = \{(\text{A control} - \text{A test})/ \text{A control}\} \times 100.$$

iv. Ferric Reducing Antioxidant Potential Assay

The ability to reduce ferric ions was measured using a modified version of the method described by Benzie and Strain [16]. A 20µl various concentrations of each extract was mixed with 30µl of 300mM, pH 3.6 acetate buffer and 150µl of FRAP reagent (10 parts of 300mM sodium acetate buffer at pH 3.6, 1 part of 10mM TPTZ solution in 40mM HCl and 1 part of 20mM FeCl3. 6H2O solution) and the reaction mixture was incubated in a water bath (WISE BATH®, WISD Laboratory Instrument) at 37 ^{0}C for 10 min. The increase in absorbance at 600 nm was measured at 30 min. against a blank that was prepared using acetate buffer. Trolox was used as positive control. The antioxidant capacity based on the ability to reduce ferric ions of the extract was expressed as µmol Trolox equivalents per gram of plant material on dry basis.

v. ABTS Radical Scavenging Assay

ABTS (2, 2'-azinobis (3-ethylbenzothiozoline-6-sulphonic acid) diammonium salt, assay is based on the scavenging of

light by ABTS radicals. ABTS radical cation (ABTS.+) was produced by reacting ABTS solution (7mM) with 2.45mM potassium persulfate and the mixture was allowed to stand in dark at 25±2° C for 12- 16 h before use. For this study, different concentrations (100µg/ ml) of extracts (150µl) were mixed with 120µl of 5mM, pH 7.4 phosphate buffers and 200µl of diluted ABTS solution and the reaction mixture was incubated at 25°C for 10 min. The absorbance was read at 734nm and the experiment was performed in triplicate. Linear graph of concentration vs. percentage inhibition was prepared and IC50 values were calculated. The antioxidant capacity based on the ABTS free radical scavenging ability of the extracts were expressed as µmol Trolox equivalents per gram of plant material on dry basis [17].

vi. Iron Chelating Activity:

The chelation of iron (II) ions by the different extracts was carried out as described by standard methods [14] [18].One hundred micro-liters (100µl) of each extract (5 concentrations) were added to 40 µl water in the micro plate as triplicate and the pre plate reading at 562nm was recorded using a Micro Plate. Then 20 µl of 1mM $FeSO_4$ was added in to the different concentrations of the extracts. The controls contained all the reaction reagents except the extract or positive control substance. After 5 min incubation, the reaction was initiated by the addition of 40µl of 1mM ferrozine solution. After a 10 min. equilibrium period, the absorbance at 562nm was recorded. The increased sensitivity obtained from the stable magenta colour of the iron-ferrozine complex makes it possible to monitor iron cheating activity of extracts. EDTA was used as positive control. The values are presented as the means of triplicate analyses. The iron chelation activities were calculated from the absorbance of the control (Ac) and of the sample (As) using Equation and expressed as EDTA equivalents (mg EDTA/ g extract) using the following equation:

Inhibition % = {(Ac - As) / Ac} x 100.

F. Statistical analyses

All analyses were performed in triplicate, and the data are expressed as the Mean ± standard deviation (SD). Data were analysed by statistical software-Statistical Package for Social Sciences (SPSS) version 17.

III. Results and Discussions

A. Extractive values

In this present study, the percentage yield of hot aqueous extractive value (36.3%) of *E. littorale* was greater than the percentage yield of methanol extractive value (21.8%). Gite, et al., mentioned that the extractive values are valuable to estimate the specific constituents soluble present in the particular solvent. The water soluble extractive value was indicating the presence of sugar, acids and inorganic compounds and alcohol soluble extractive values indicated the presence of polar constituents [19].

B. Total Phenolic and Flavonoids Contents

Phenolic and flavonoids component have been reported to exert significant antioxidant activity [20] [21]. Therefore, the obtained total phenolic contents of the hot methanol and aqueous extracts of *E. littorale* was represented in Figure 1 and the content of total phenols is expressed as Gallic acid (Figure2) equivalents (mg GAE/g dry extract).

Comparing with the phenolic content of each extracts of *E. littorale*, it was observed that, the total phenolic contents in hot methanol extract (44.41±1.26 mg GAE/g) was found to be higher than in the hot aqueous extract (20.11±0.92 mg GAE/g) of *E. littorale*.

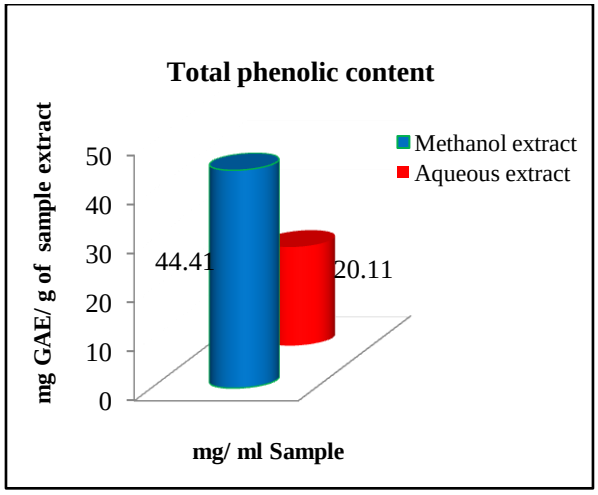

Fig.1 Total phenolic contents of hot methanol and aqueous extracts of *E. littorale*

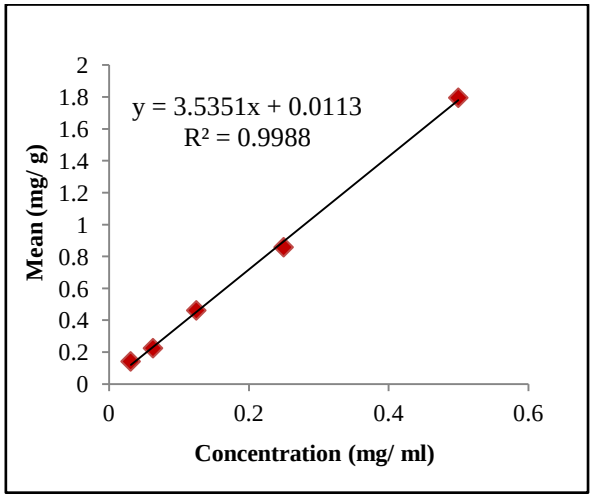

Fig.2 Standard curve for Gallic acid in phenolic activity

Further, the obtained total flavonoids contents of the hot methanol and aqueous extracts of *E. littorale* was represented in Figure III and the content of total flavonoids is expressed as quercetin (Figure 4) equivalents (mg QE/ g dry extract).

As evident from Figure 3, the total flavonoid contents in hot methanol extract (174.44±9.32 mgQE/g) was found to be higher than in the hot aqueous extract (42.74±2.82 mg QE/g).

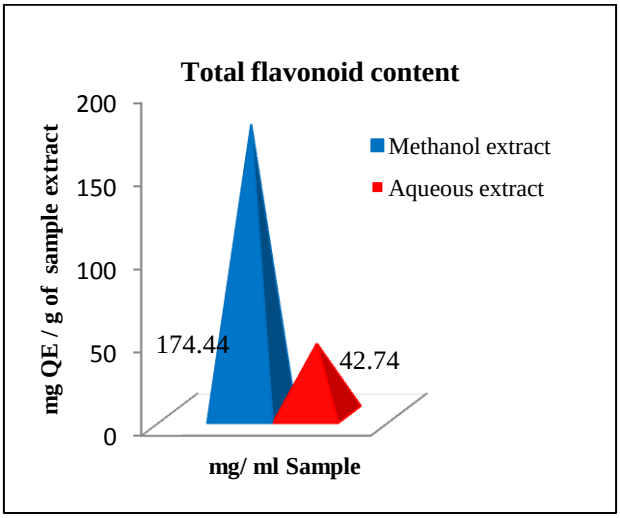

Fig. 3 Total flavonoids contents of hot methanol and aqueous extracts of *E. littorale*

The present study has revealed that the total phenolic and flavonoids contents of the methanol extract were higher thanin the aqueous extract of *E. littorale.*

Aqueous extract of *E. littorale* had the lowest content of total phenolic and flavonoid contents. This may be due to polarity of the solvent. The extraction yields, nature of the compounds, and the materials from which the compounds were extracted strongly depend on the solvents due to the presence of different concentration of bioactive compounds with different polarities [22].

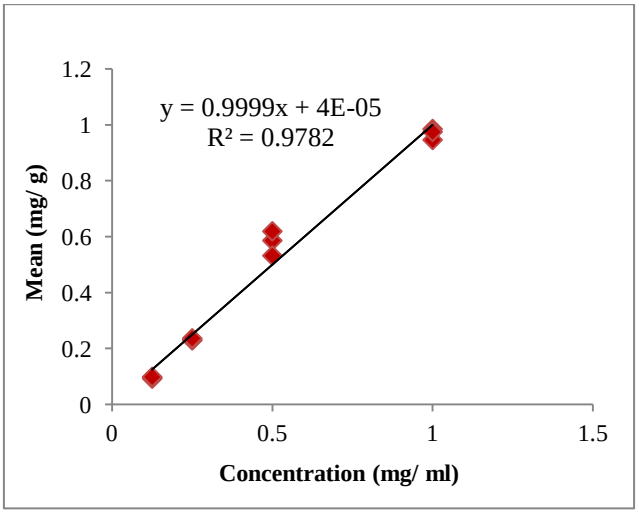

Fig. 4 Standard curve for Quercetin in flavonoid activity

However, results of the present study were not supported by the previous studies conducted by other researchers. Thus, Jaishree et al revealed that the total phenolic and flavonoid contents were 97.33±4.80 and 31.33±2.19 respectively in the methanol extract of *E. axillare* [23]. Sathiskumar et al found that the total phenolic content was 1.95 mg/ g and 1.82 mg/ g in distilled water boiled and methanol extract of the shade dried sample of the *E. littorale* respectively [24].These differences may be due to the preparation method or seasonal variation in the environment or geographical variations and or extraction procedures.

C. DPPH Radical Scavenging Activity

In this study, the antioxidant activity was expressed as Trolox equivalents per gram of plant material on a dry basis. Table I shows the DPPH radical scavenging activity for the hot aqueous and methanol extracts of the *E. littorale.*

TABLE I: DPPH RADICAL SCAVENGING ASSAY OF THE *E. LITTORALE*

Extracts	DPPH antioxidant activity µ mole TE/ g
Hot aqueous extract	23.84 ± 01.24
Hot methanol extract	134.11 ± 20.70

Values expressed as mean ± S.D., n=5 different concentrations with three replicates.

Antioxidant activity expressed as Trolox equivalents per gram of plant material on a dry basis.

The DPPH radical scavenging activity of hot methanol extract (134.11 ± 20.70µ mole TE/ g) was higher than the hot aqueous extract (23.84 ± 01.24µ mole TE/ g) of *E. littorale*.

The IC_{50} values (DPPH) of the hot methanol and aqueous extracts of *E. littorale* were represented in Fig. 5. Maximum activity was shown by methanol extract and minimum by aqueous extract of *E. littorale.* The radical scavenging potential of the preparations used here were does dependent that on increasing the concentration of extracts (156- 625µg/ ml). The highest % of inhibition shown by methanol extract was 71.98% at 625µg/ ml whereas highest % of inhibition shown by aqueous extract of *E. littorale*29.43% (Fig. 6). When considering in this assay, the DPPH activity of the methanol extract was higher than that of aqueous extract of *E. littorale.*

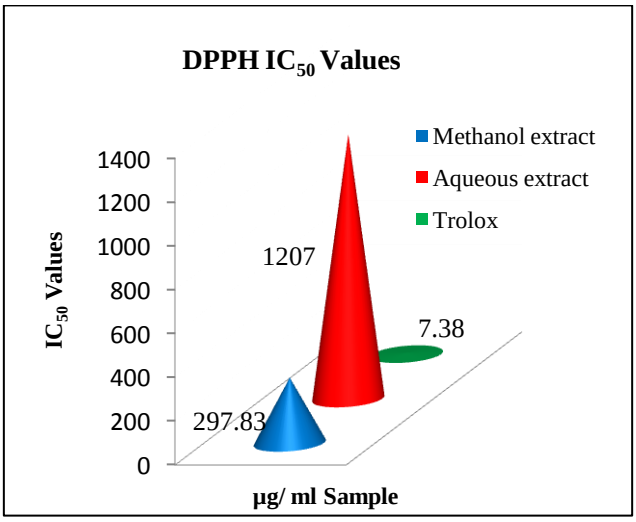

Fig. 5 IC_{50} values of the hot methanol and aqueous extracts of *E. littorale*

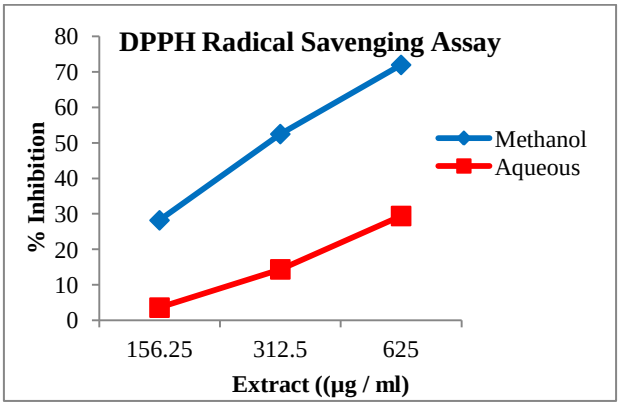

Fig. 6 DPPH radical scavenging activity of the hot methanol and aqueous extracts of *E. littorale*

D. FRAP Assay

Here the FRAP showed the results of aqueous and methanol extracts that of µ mole equivalent to Trolox (TE)/ g of sample.

At low pH, measuring the change in absorption at 600 nm can monitor reduction of a ferric complex to the ferrous form, which has an intense bluish green color. The change in absorbance is directly related to the combined or "total" reducing power of the electron-donating antioxidants present in the reaction mixture. The FRAP values ranged from 677 -1266 µ mole TE/ g of dry mater are shown in Table II.

TABLE II: FRAP ACTIVITY OF THE *E. LITTORALE*

Extracts	FRAP antioxidant activity µ mole TE/ g
Hot aqueous extract	677 ± 24
Hot methanol extract	1266 ± 22

Values expressed as mean ± S.D., n=5 different concentrations with three replicates.

Antioxidant activity expressed as Trolox equivalents per gram of plant material on a dry basis.

The FRAP activity of the hot methanol and aqueous extracts of *E. littorale* was represented in Fig. 7. Although the methanol and aqueous extracts of *E. littorale* showed weak FRAP activity in comparison to the standard Trolox, the antioxidant activity of the hot methanol extract was higher than that of hot aqueous extract.

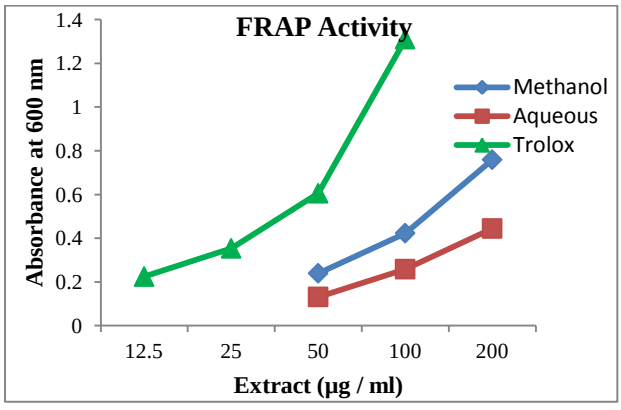

Fig. 7 FRAP activity of the hot methanol and aqueous extracts of *E. littorale*

E. ABTS Activity

A more appropriate format for the assay is a decolorization technique in that the radical is generated directly in a stable form prior to reaction ns with putative antioxidants [17].The ABTS assay ability measured by direct production of the blue/ green $ABTS^{\bullet+}$ chromospheres through the reaction between ABTS and potassium per sulfate. Table III summarizes the ABTS activity of the hot methanol and aqueous extracts of *E. littorale.*

TABLE III: ABTS RADICAL CATION DECOLORIZATION ASSAY OF THE *E. LITTORALE*

Extracts	ABTS antioxidant activity µ mole TE/ g
Hot aqueous extract	88.42 ± 18.52
Hot methanol extract	389.07 ± 25.56
Trolox as standard	

Values expressed as mean ± S.D., n=5 different concentrations with three replicates.

Antioxidant activity expressed as Trolox equivalents per gram of plant material on a dry basis.

The IC_{50} values and ABTS radical activity of the hot methanol and aqueous extracts of *E. littorale* were represented in Fig. 8 and Fig. 9 respectively.

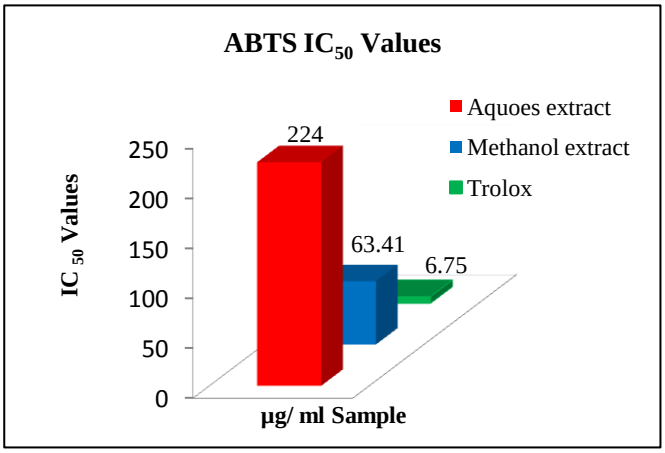

Fig. 8 IC_{50} values of the hot aqueous and methanol extracts of *E. littorale*

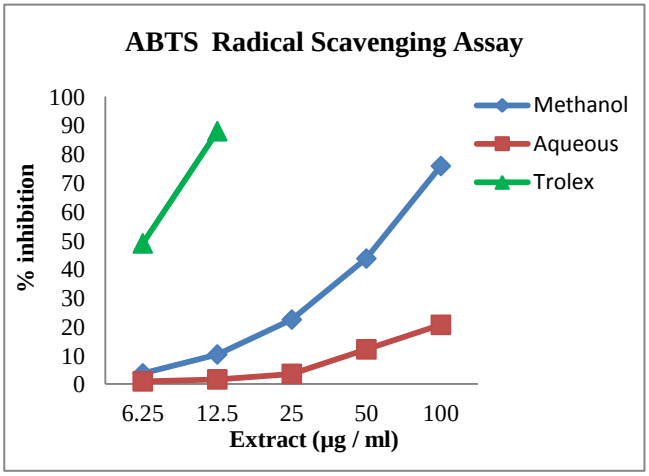

Fig. 9 ABTS activity of the hot methanol and aqueous extracts of *E. littorale*

Hot methanol extract showed highest activity (IC_{50}: 63.41 µg/ml) while aqueous extract of *E. littorale* showed the weakest (IC_{50}: 224 µg/ml). The IC_{50} value for Trolox was 6.5 mg/ ml. The methanol and aqueous extracts of *E. littorale* showed lower antioxidant activities in comparison to the standard Trolox.

F. Iron Chelating Activity

In this present activity the magenta colour of the complex was not decreased and values were lower than limit. Comparison to the total phenolic and flavonoid contents, DPPH, FRAP and ABTS activities, the hot aqueous and methanol extracts of *E. littorale*, interfered with the formation of ferrous and ferrozine complex, suggesting that these both extracts have not iron chelating activity at 1.75 mg/ml concentration (highest possible concentration).

The overall results of present study revealed that,hot methanol extract contains higher concentrations of total phenolic and flavonoid contents than hot aqueous extract and can also exert greater antioxidant activity than hot aqueous extract of *E. littorale*, although the in-vitro antioxidant (ABTS, FRAP and DPPH) assays of hot methanol and aqueous extracts demonstrated were lower than the positive control Trolox. Although the total phenolic and flavonoids components were detected in the hot methanol and aqueous extracts, these does not appear to be reported a comparison between the observed in-vitro antioxidant activity and extractive values with total phenolic and flavonoids contents of *E. littorale*.

Further, a number of plant alkaloids [25] and flavonoids [26] [27] have been shown to possess antioxidant properties. Phenols and poly phenolic compounds also have been shown to possess significant antioxidant activities [20] [21]. This finding may account for the observed antioxidant potential in *E. littorale*. The overall antioxidant activity was not correlated with the total phenolic and flavonoid contents of the methanol and aqueous extracts of *E. littorale*.

Present study findings are also supported by findings of earlier in-vitro antioxidant studies carried out with *E. littorale* by other investigators which show, that it possesses a strong free radical scavenging activity and ferric reducing property indicating it to be a good potential source of natural antioxidants to prevent free radical mediated oxidative damages. This study indicated that methanolic extracts of dried plant materials possessed lower antioxidant properties than fresh samples [24]. Another study has also stated that, in the ABTS method, four successive extracts (petroleum ether, chloroform, ethyl acetate, and methanol) of the whole plant of *E. axillare* showed potent antioxidant activity with IC_{50} values ranging from 13.26 to 24.36 µg/ ml. Although, the total phenolic and flavonoid contents in the methanol extract were found to be higher than in other extracts, the methanol extract was found to have the least antioxidant activity among the four extracts [23]. The in vitro antioxidant activity of aqueous, hydro alcoholic, methanolic, chloroform and ethyl acetate extract of leaves of this plant has been evaluated. The possible mechanism involved was investigated by using different model covering nitric oxide and DPPH method. The result indicated efficacy of extracts for antioxidant activity in following sequence: methanol > hydro alcoholic > aqueous > chloroform [28].

At the same time, in the present study result was different to that reported by Sathishkumar et al., [29].They have reported that, different drying treatments (fresh, shade dry, sundry, and microwave, especially microwave treatment) of plant material results in a significant reduction ($P \leq 0.05$) in antioxidant properties of *E. littorale* in methanolic extracts as compared to that of boiling water extracts, which appeared to exhibit significantly stronger antioxidant potentials even in dried plant materials due to greater solubility of compounds, breakdown of cellular constituents as well as hydrolysis of tannins.

These differences may be due to variations in the preparation procedure, and / or storage method ofplant, seasonal or geographical variations in the environment from which plant material was collected, or standardization method used.

IV. CONCLUSION

Although, this in-vitro antioxidant activities demonstrated by the hot methanol and aqueous extracts of *E. littorale* was very low, further research work of other standard antioxidant activities {electron transfer (ET)and hydrogen atom transfer (HAT) based assays} of different extracts and or active constituents should be carried out in future.

ACKNOWLEDGMENT

The financial support provided Higher Education Twenty first Century (HETC) Project, Ministry of Higher Education, Sri Lanka, and the assistance given by Staff, Bioactivity Lab/ Herbal Technology Section, Industrial Technology Institute (ITI), Colombo-07 to conduct the antioxidant activity studies are gratefully acknowledged.

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