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Effect of Drying Treatment on the Content of Antioxidants in *Enicostemma littorale* Blume

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Abstract: The Total Phenolic Content (TPC) and antioxidant activity of fresh and dried materials of *Enicostemma littorale* Blume were evaluated using the Folin-ciocalteau method, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and Ferric Reducing Antioxidant Power (FRAP) assays. Different drying treatments especially, microwave treated plant material led to significant reduction ($p \leq 0.05$) in antioxidant properties of *E. littorale* in methanolic extracts as compared to that of the boiling water extracts, which appeared to exhibit significantly stronger antioxidant potentials ($p \leq 0.05$) even in dried plant materials due to greater solubility of compounds, breakdown of cellular constituents as well as hydrolysis of tannins. A strong free radical scavenging activity in the chosen plant material suggests that it has great potential in the food industry as functional food ingredient.

Key words: Antioxidant activity, Folin-ciocalteau method, *Enicostemma littorale*, phenolic content, DPPH free radical scavenging, ferric reducing, functional food ingredient

INTRODUCTION

Enicostemma littorale (Gentianaceae) also called as Chota chirayata in Hindi, Mamejavo in Gujarati, Nagajivha in Bengal and Vellarugu in Tamil is a glabrous perennial herb with sessile lanceolate leaves and is found throughout India up to a height of 1500 ft. Qualitative analysis of the ash content of aerial parts of the plant revealed the presence of minerals like iron, potassium, sodium, calcium, magnesium, silica, phosphate, chloride, sulphate and carbonate. Monoterpene alkaloids like-enicoflavin and gentiocrucine were also isolated. In addition to the steroids, triterpenoids including catechins, saponins, betulin, were also isolated (Rai and Thakar, 1966).

Various Ayurvedic formulations containing *E. littorale* as one of the ingredients have been shown to produce antihyperglycemic activity in hyperglycemic rat models (Gupta and Seth, 1962). Ethnomedical studies of North Gujarat (India) revealed the use of hot aqueous extract of *E. littorale* by the tribal inhabitants for the treatment of diabetes, fever, stomach pain, dyspepsia and malaria (Murali *et al*., 2002). This herb is also known for its anticancer property (Kavimani and Manisenthilkumar, 2000) and hypolipidaemic effect in *p*-dimethylaminobenzene (*p*-DAB) induced hepatotoxic animals (Gopal *et al*., 2004) and is also anti-inflammatory (Vasu *et al*., 2005).

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Free radicals are chemical species, which contains one or more unpaired electrons due to which they are highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. Reactive Oxygen Species (ROS) such as superoxide anion, hydroxyl radical and hydrogen peroxide formed *in vivo*, are highly reactive and potentially damaging transient chemical species. These are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function. The ROS are regulated by endogenous superoxide dismutase, glutathione peroxidase and catalase but due to over-production of reactive species, induced by exposure to external oxidant substances or a failure in the defense mechanisms, damages cell structures, DNA, lipids and protein contents, thereby increasing risk of more than 30 divergent disease processes (Valko *et al*., 2007). Oxidative damages caused by free radicals to living cells mediate the pathogenesis for many chronic diseases, such as atherosclerosis, Parkinson's disease, Alzheimer's disease, stroke, arthritis, chronic inflammatory diseases including, malaria, acquired immunodeficiency syndrome diabetes, anemia and cardiovascular diseases (Agbor *et al*., 2007).

Some herbs or crops are perishable in their fresh state and may deteriorate within a few days after harvest. One way to preserve these plant products is to dry them in order to conserve their desirable qualities, reduce storage volume and to extend their shelf life. Drying functions to inactivate the enzymes polyphenol oxidases and can either be performed by traditional sun/shade drying or microwave drying/oven drying. However, enzymatic and/of non-enzymatic processes that may occur during drying fresh plant tissues may lead to significant changes in the composition of phytochemicals (Capecka *et al*., 2005). Generally, these processes may cause negative attribute to the final food product, however studies by Nicoli *et al*. (1999) proved that the overall antioxidant properties of certain foods may instead be enhanced due to the formation of Milliard Reaction Products (MRPs),which results from a condensation reaction between amino acids (or proteins) and reducing sugars or lipid oxidation products. These MRPs exhibited antioxidant activity as measured by 1, 1-diphenyl-2-picrylhydrazyl and α-carotene bleaching assays, however, the reducing power and iron-chelating abilities of MRPs were also reported (Chawla *et al*., 2009) to increase upon irradiation to scavenge hydroxyl and superoxide anion radicals under *in vitro* conditions.

Phenolic compounds possess a wide spectrum of biological effects including antioxidant and free radical scavenging (Pellati *et al*., 2004). Phenolics are classified into two groups such as polyphenols and simple phenols (Marinova *et al*., 2005). Most of the antioxidant properties in plants are also due to polyphenols, flavanoids and vitamin C. Polyphenols function by trapping and scavenging free radicals and also regulate nitric oxide, decrease leukocyte immobilization, inhibit cell proliferation and angiogenesis AND exhibit phytoestrogenic activity (Arts and Hollman, 2005). Poly Pheonl Oxidases (PPOs), also referred to as catecholoxidases, are copper-containing enzymes comprised of catechol oxidase and laccase that catalyzes the aerobic oxidation of variety of phenolic substrates in the plant material into o-quinones with a concomitant O_2 reduction thus causing browning of damaged fruits or vegetables.

2, 2-diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical and is used to measure the radical scavenging activity (Koleva *et al*., 2002). Moreover, the total antioxidant activity could also be measured by Ferric Reducing Antioxidant Power (FRAP), which depends upon the reduction of ferric tripyridyltriazine (Fe (III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe (II)-TPTZ) by a reductant at low pH, (Reka and Ilona, 2002). Therefore, the objective of this study is to evaluate the effects of various drying methods on the total phenol contents and antioxidant properties such as DPPH and FRAP activities of the herb *Enicostemma littorale* under different solvent.

MATERIALS AND METHODS

Chemicals and Reagents

Gallic acid, Folin-Ciocalteu's reagent, linoleic acid, iron (III) chloride and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma, Potassium dihydrogen phosphate, di-potassium hydrogen phosphate, potassium ferricyanide and ascorbic acid were obtained from Merck (Germany). Solvents used were from Fisher Chemicals (Springfield, NJ).

Plant Materials

The wild plant of *Enicostemma littorale* growing at sub-tropical regions of Thirunelveli and Salem districts, Tamilnadu was collected in the month of August and September (2008) 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 to the Phytomatics Laboratory, Department of Bioinformatics, Bharathiar University, Coimbatore, Tamil Nadu, India.

Experimental Design

The Plant materials obtained were separated into two groups; one fresh and another to be subjected to various drying conditions. The whole plants were subjected to three different drying conditions namely, shade, sun and microwave oven dry, however for all the three strategies, approximately five hundred grams of the fresh plant material washed, drained and used. For shade dry, the pre washed and drained plant material was placed on a filter paper (90×60 cm) at room temperature (27±1°C) for 3 days. For sun dry, the fresh material was placed in to the greenhouse for 3 days. For microwave oven drying, the plant material was placed in the middle of the turntable of a commercial microwave oven (LG Model MG-555F; 900W) for 4 min. Once, the drying process was over, the dry weights were measured to calculate the percentage of water loss and were powdered using a laboratory blender and stored for further work. From the storage, approximately fifty milligram of the material was drawn to extract the metabolites under different solvents, which was further used for analyzing the antioxidant contents. However, similar strategy was also adopted for the fresh plant material to concentrate for different solvent extraction and thereafter for antioxidants assay (Lim and Murtijaya, 2006).

Sample Extraction

Fifty-milligrams of each treated powder were crushed with 1 mL each of methanol (100%), distilled water and distilled boiling water (100°C) separately. It was allowed to stand for 30 min with out any disturbance at room temperature and then swirled with a vortex for 5 min after which was centrifuged at 10,000 rpm for 10 min to collect the supernatant. This extract was stored at -20°C until further use (Lim and Murtijaya, 2006).

Determination of Total Phenolic Content

The Total Phenolic Content (TPC) of the plant extracts was determined spectrophotometrically using Folin-Ciocalteau's reagent according to the modified method of Kahkonen *et al.* (1999). Fifty microlitter of the samples in triplicate was added into the test tubes followed by 1.5 mL of 2 N Folin-Ciocalteau reagent (diluted 10 times) and 1.2 mL of 20% sodium carbonate. The contents of the tubes were mixed thoroughly and stored at dark for

30 min. Phenols react with phosphomolibdic acid of Folin-Ciocalteau's reagent in alkaline medium and produce blue colored complex, was measured at 765 nm and expressed as mg Gallic acid per gm of plant material with Gallic acid as the standard.

Determination of Dpph (2, 2-diphenyl-1-picrylhydrazyl) Radical Scavenging Activity

Free radical scavenging activity against 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical was measured according to the modified method of Oboh (2005). To about 50 μL of the extract, 1.5 mL of 0.1 mM DPPH was added and vortexed for 15 to 30 sec and allowed to stand with out any disturbance for 30 min at room temperature. Indication in the activity of DPPH was observed with a change in the colour from purple to yellow and was measured by reading the absorbance at 517 nm. Ascorbic acid was used as the standard, while the inhibition ratio for DPPH scavenging activity was calculated from the equation:

$$AA(\%) = \frac{A_c - A_s}{A_c} \times 100$$

Where:
AA = Ascorbic acid
A_c = Absorbance of control
A_s = Absorbance of test sample

Determination of Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing property of the extracts was determined using assay described by Yen and Chen (1995). To about 50 μL of the extract, 1.5 mL of 0.1 mM FRAP solution (0.2 M Potassium phosphate buffer, pH 6.6 and 10 mM 2, 4, 6- Tris (2-pyridyl)-S-Triazine) was added and vortexed for 15 to 30 sec and incubated at 50°C for 20 min. In order to terminate the reaction, 2.5 mL of 10% trichloroacetic acid and equal volume of ultra pure water was added to the mixture after which was added 0.5 mL of $FeCl_3$ (1 g L^{-1}). The procedure was carried out in triplicate and allowed to stand for 30 min before measuring the absorbance at 593 nm. The absorbance obtained was converted to Gallic acid equivalents in milligrams per gram fresh material (mg g^{-1}) using a Gallic acid standard.

Statistical Analysis

All the assay such as Total phenol content, DPPH and FRAP were done in triplicate for all the treated plants extracted under different solvents. 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

The results of antioxidants such as Total Phenol Content, DPPH and FRAP gave an interesting observation. In general, three different drying methods were involved. The results seemed to be higher in fresh samples; this may be due to the reason that they had been a loss of moisture content approximately 38, 23.55 and 23.37% during the process of shade, sun and microwave dry, respectively. However, drying resulted in considerable shrinkage thereby making the plant material crispier and ease to powder coarsely for further analysis. Although,

Table 1: Effect of drying treatments on total phenol content of *E. littorale*

	Total phenol content							
	Fresh		Sun dry		Shade dry		Micro wave	
Plant part	Phenol (mg g^{-1})	Mean±SEM	Phenol (mg g^{-1})	Mean±SEM	Phenol (mg g^{-1})	Mean±SEM	Phenol (mg g^{-1})	Mean±SEM
Methanol	2.08	0.218±0.0035	1.93	0.204±0.0035	1.82	0.192±0.0046	0.45	0.045±0.0046
Distilled water	2.05	0.215±0.0087	1.54	0.159±0.0041	1.77	0.187±0.0069	0.35	0.039±0.0041
Distilled boiled water	2.15	0.232±0.0032*	1.91	0.201±0.0041	1.95	0.207±0.0056	0.55	0.059±0.0291

Data expressed as Mean±SEM of triplicates. *Indicates the significant difference ($p \leq 0.05$)

the biochemical analysis performed with different solvents such as 100% methanol, distilled water (at room temperature) and boiled water (100°C), invariably, the samples extracted fresh showed higher activity for all the analyzed antioxidants.

Total Phenolic Content (TPC)

Highly significant (2.150±0.003 mg g^{-1}) phenol content was recorded with freshly used plant material extracted with boiling distilled water (100°C), which revealed to be approximately 3.36% higher compared to the methanol (2.080±0.004 mg g^{-1}) extract of the fresh material, however the same in distilled water yielded only(2.050±0.009 mg g^{-1}). Among the drying conditions, both sun and shade dried extracts recovered good percentage of TPC when compared to microwave dried extract. Extract with boiled distilled water yielded (1.950±0.006 and 1.930±0.004 mg g^{-1}) of phenol, respectively.

However, this was 10.25% lesser compared to the extracts of boiled distilled water extraction with fresh material. Microwave dried sample yielded very poor content of TPC and was revealed to be lesser approximately 20.93, 16.27 and 25.58% in extracts of methanol, distilled water and distilled boiled water, respectively, when compared to the high significant values (Table 1).

DPPH (2, 2-diphenyl-1-picrylhydrazyl)

The activity of DPPH under different conditions was observed and compared in *E. littorale*. In contrast to the availability of TPC, the activity of DPPH declined in the freshly extracted sample. Methanolic extraction of Microwave sample showed significantly increasing value of DPPH activity with a (89.362±0.409 mg AA g^{-1}) and highlighting activity of 2.77 and 8.79% compared to the extract from distilled water and distilled boiled water, respectively. Extraction of sundried plant material ranked second in the order of DPPH activity. It showed an activity of (76.579±0.383 mg AA g^{-1}) was 38.6% in methanolic extract which was revealed to be lesser to the microwave dried methanolic extract.

However, the least activity of (8.410±0.387 mg AA g^{-1}) (Table 2) was recorded in the extract of freshly used plant material, which was revealed to differ about 90.59% especially in extracts obtained from distilled water.

FRAP (Ferric Reducing Antioxidant Power)

Proposionate to the phenolic content, the ferric reducing activity of *E. littorale* was increased in the extracts obtained from fresh materials. However, extract from boiling water showed significant Ferric reducing activity of (6.080±0.006 mg g^{-1}). which was revealed to be 9.54 and 17.23% greater than extracts from methanol and distilled water, respectively. Among the drying process, great variation was observed, shade dried extracts proved to be

Table 2: Effect of drying treatments on DPPH activity of *E. littorale*

	DPPH radical scavenging activity			
	Fresh		Sun dry	
Plant part	DPPH activity (mg AA g^{-1})	Mean±SEM	DPPH activity (mg AA g^{-1})	Mean±SEM
Methanol	10.990	10.990±0.3579	76.579	76.579±0.3825
Distilled water	8.410	8.4100±0.3868	72.006	72.006±0.4218
Distilled boiled water	10.122	10.122±0.3531	62.827	62.827±0.4623
	DPPH radical scavenging activity			
	Shade dry		Micro wave	
Plant part	DPPH activity (mg AA g^{-1})	Mean±SEM	DPPH activity (mg AA g^{-1})	Mean±SEM
Methanol	64.473	64.473±0.4077	89.362	89.362±0.4087*
Distilled water	62.823	62.823±0.4123	88.707	88.707±0.4443
Distilled boiled water	54.334	54.334±0.3082	82.141	82.141±0.3040

Data expressed as Mean±SEM of triplicates. *Indicates the significant difference ($p \leq 0.05$)

Table 3: Effect of drying treatments on ferric reducing activity of *E. littorale*

	Ferric reducing antioxidant power			
	Fresh		Sun dry	
Plant part	FRAP activity (mg g^{-1})	Mean±SEM	FRAP activity (mg g^{-1})	Mean±SEM
Methanol	5.55	0.8390±0.0105	3.22	0.494±0.0535
Distilled water	4.95	0.0754±0.0052	3.99	0.595±0.0062
Distilled boiled water	6.08	0.9110±0.0059*	4.06	0.607±0.0069
	Ferric reducing antioxidant power			
	Shade dry		Micro wave	
Plant part	FRAP activity (mg g^{-1})	Mean±SEM	FRAP activity (mg g^{-1})	Mean±SEM
Methanol	3.94	0.588±0.0049	0.56	0.090±0.0038
Distilled water	3.70	0.565±0.0046	0.45	0.066±0.0041
Distilled boiled water	3.99	0.593±0.0038	0.71	0.096±0.0040

Data expressed as Mean±SEM of triplicates. *Indicates the significant difference ($p \leq 0.05$)

more effective in FRAP compound then to sundry and microwave treatments. Among the shade dried material, boiled water and methanolic extract produced the higher activity of (0.593±0.004 and 0.588±0.005 mg g^{-1}) (Table 3), respectively and showed a negligible difference between them. However, it was approximately 4.9% higher than the extracts in distilled water. Nevertheless, microwave treatment totally declined the activity and proved to be fatal for the examination.

DISCUSSION

In the present study the activity of certain antioxidants such as TPC, DPPH and FRAP were evaluated in *Enicostemma littorale* under different conditions using various solvents. This systematic comparison of the antioxidant activities revealed the fact that *E. littorale* could posses 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.

First of all, drying treatments have played an important role in the loss of total phenolic content, which might have been attributed by the deactivation of the degradative enzymes such as polyphenol oxidases; that could degrade phenolic compounds before the plant materials are completely dried especially during sun drying. Moreover sun drying could also lead to an uneven loss of TPC as it is affected by climatic factors as evidenced by Mueller-Harvey (2001). Microwave heating, brought about by absorption of water molecules by microwave energy, is more energy efficient than conventional heating because heat generated is more intensive, thus, could have inactivated the degradative enzymes, thereby indicating decline in TPC, which has been in accordance with the view of Lim and Murtijaya (2006), according to whom, the phenolic compounds decomposed rapidly in direct sunlight or at elevated temperatures in *Pyllanthus amarus*.

It has also been evidenced by Tomaino *et al*. (2004) that drying process would generally result in a depletion of naturally occurring antioxidants because, intense and/or prolonged thermal treatment may be responsible for a significant loss of natural antioxidants, as most of these compounds are relatively unstable as against some compounds (carotenoids or Lycopene), which are heat stable (Nicoli *et al*., 1999). However, other causes of depletion of antioxidants could also be due to operations such as peeling, cutting and slicing as they induce a rapid enzymatic oxidation of natural antioxidants as reported by Oboh *et al*. (2009), according to whom, sun drying of green leafy vegetables could cause a significant increase in TPC, reducing property AND free radical scavenging activity, even though there was a significant decrease in vitamin C content. Another investigation by Capecka *et al*. (2005) also reported that air drying could result in a considerable increase of TPC in oregano and peppermint leaves but no significant difference was observed for lemon balm. These researches clearly explains that the TPC obtained after drying process may be higher or lower based on the type of phenolic compounds present and their location in the cell.

Apart from drying, other factor viz., kind of solvents used could also be considered for the difference in the pattern of antioxidants obtained. The solvents used to extract were methanol, distilled boiling water and distilled water at room temperature. Methanolic extracts of fresh sample revealed higher activity of both TPC and antioxidant such as DPPH scavenging activity and (FRAP) Ferric reducing activity, compared to distilled water extracts and this may be due to the fact evidenced by Lim and Murtijaya (2006), according to whom, methanol possessed the property of denaturing the polyphenols oxidases. Moreover, methanol being an organic and volatile solvent is more efficient in plant cell wall degradation; therefore could extract a greater amount of endocellular materials than water.

Perhaphs, its soluble substances were involved in this scavenging process as also evidenced by Mahnaz *et al*. (2009). Similarly boiling water extraction of dried *E. littorale* also resulted in higher level of TPC and activities of antioxidants mainly due to the fact that boiling water could completely activate the degradative enzymes present in fresh plant materials as against the distilled water. This significance is in concomitance with the literature of Oboh (2005), who attributed that the tannin breakdown to simple phenols when, plant materials are exposed to a high temperature during extraction process, thus result in increased number of compounds with free hydroxyl groups.

However, in dried plant materials, where polyphenol oxidases have been inactivated, methanol extract was still found to yield lower recovery of TPC and their antioxidant properties than both distilled boiled water and distilled water. Since, water is a polar solvent, helped in extracting the polar compounds, while the heat generated by boiling have aided in releasing the cell wall phenolics in an unbound manner due to the breakdown of cellular constituents, thus causing more polyphenols to be extracted as indicated by Toor and

Savage (2004). Additionally, high temperature has also resulted in increased solubility of phenols as evidenced by Amin *et al.* (2006) and has led to the extraction of more polyphenols from the dried plant samples under boiling condition. The polyphenolic compounds are known to have antioxidant activity in the methanolic extracts of leaves and stem of *Celtis africana* was believed to be mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Adedapo *et al.*, 2009). However, Adedapo *et al.* (2008) also indicated that the increase in antioxidant potentials of plants such as *Adenia gummifera* and *Acokanthera oppositifolia* were proportional to the polyphenol contents, hence interpreted a high positive relationship between the two.

In conclusion, this study indicated that methanolic extracts of dried plant materials possessed lower antioxidant properties than fresh samples. In general, plant phenolic compounds and their antioxidants present at different binding status would depend on plant species. Perhaps, data on the effects of drying and solvent usage on the antioxidant properties of herbs and vegetables, although are conflicting due to several factors may show variation in their activities in response to the mode of extraction. Since, most medicinal herbs are prepared for consumption, the compounds extracted by simple boiling would benefit better rather than methanolic solvents. Hence, when an easier and conventional methods are available, more attempt on this perception is desirous to find use of their extract as a dietary supplements in nutraceutical and/or cosmoceutical preparations for protection against the complications arising from the oxidative stress. In this direction, further attempt is made to isolate the potential antioxidants and to quantify them using high through put technologies.

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