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Phytochemical and *in vitro* antioxidant of an endemic medicinal plant species, *Elaeocarpus munronii* (wt.) mast. and *Elaeocarpus tuberculatus* Roxb. (Elaeocarpaceae)

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Abstract
Medicinal plants are imperative for the treatment of various human diseases. *Elaeocarpus* is a genus belonging to the family, Elaeocarpaceae. In Indian traditional system of medicine, different parts of rudraksha were taken for the alleviation of various health related problems such as mental disorders, headache, skin diseases and for healing wounds. The present study was undertaken to address phytochemical and *in vitro* antioxidant potential for the medicinal plant species, *Elaeocarpus munronii* and *Elaeocarpus tuberculatus*. Quantification of phytochemicals for various solvent systems viz., petroleum ether, ethyl acetate, ethanol and aqueous extracts and plant parts viz., leaf, stem, flower and fruit for the two medicinal plant species, *E. munronii* and *E. tuberculatus* were analyzed. Antioxidant and free radical scavenging potential in terms of DPPH, $ABTS^{\cdot+}$, reducing power, ferrous ion and superoxide radical scavenging activity were assessed using standard procedures. From the results obtained, the ethanolic leaf extracts of both the plant species of *Elaeocarpus* encompass significant activity. However, *E. munronii* determine appreciable activities than *E. tuberculatus* with excellent reputation in traditional Indian system of medicine. Furthermore studies are however required to elucidate their mechanism of action using molecular techniques.

Keywords: Phytochemical, antioxidant, ethanol, leaf

Introduction
Many plant derived molecules have shown promising effect in therapeutics (Lokhande *et al*., 2007) [22]. Plants are made up of secondary metabolites which are formed as products of primary metabolism produced for defense against predators. Several phytochemical surveys have been carried out for detecting diverse group of naturally occurring phytochemicals. The phytochemical research approach is considered effective in discovering bioactive profile of plants of therapeutic importance (Masih and Singh, 2012) [23]. Examples of such metabolites are tannins, flavonoids and alkaloids as they are known to be the brain behind the healing potentials of plants (Bhandary *et al.,* 2012) [6]. Antioxidants play an important role in protecting human body against alleviations of free radicals which cause oxidative stress in tissues of lungs, heart and cardiovascular system, kidney, liver, gastrointestinal tract, blood, eye, skin, muscle and brain (Huda *et al.,* 2009) [4]. In recent times, natural source of antioxidants are now being focused owing to the numerous side effects of synthetic antioxidants (Kumaran *et al.,* 2007) [21]. Hence in today's world, the use of natural source of antioxidants has been effective in reducing the severeity of diabetic complications (Roberts *et al.,* 2007) [27].

Elaeocarpus munronii (Wt.) Mast. and *E. tuberculatus* Roxb. belonging to the family Elaeocarpaceae is an evergreen tree where the ripe fruits contain a hard and highly ornamental stony endocarp known as bead or nut commonly termed as Rudraksha in India. It holds a great promise and unbreakable faith by experiments that it has confirmed medicinal uses apart from its attractive stones (Chopra *et al.,* 1956; Dadhich *et al.,* 2013) [9, 10]. Various species of *Elaeocarpus* have been known to exert antimicrobial (Indhiramuthu *et al.,* 2014) [18], antiarthritic (Geetha *et al.,* 2015a) [12] antidiabetic (Geetha *et al.,* 2015b; Geetha *et al.,* 2016) [13, 14] effects in various experimental studies. Based on the comprehensive literature survey and scientific evidences, the present study was addressed to analyze the phytochemical analysis, as well as to evaluate the antioxidant activities for the various plant parts and solvent systems of both the *Elaeocarpus* species via., the *in vitro* chemical models.

Materials and Methods

Collection of plant materials

Fresh plant parts of the study species, *E. munronii* and *E. tuberculatus* were collected from Megamalai hills, the Western Ghats, Theni district, Tamil Nadu, India. They were cleaned, shade dried and coarsely powdered in a Willy Mill to 60 mesh size for extraction.

Preparation of crude plant extracts

Fresh leaves of the study species was collected from Megamalai hills, Western Ghats, Theni district, Tamil Nadu, India. The authenticity of the selected plant materials were duly notorious and inveterate (vide no: BSI/SRC/5/23/2017/Tech/2994 & BSI/SRC/5/23/2017/Tech/2995) by comparison with reference specimen preserved at Botanical Survey of India, Southern Circle, Coimbatore. Fifty grams of coarsely powdered plant samples were extracted with successive solvent systems viz., petroleum ether, ethyl acetate and ethanol using soxhlet apparatus. Furthermore, the air dried residues were subjected to cold maceration with water. The extracts were filtered and concentrated to dryness under reduced pressure using rotary vacuum evaporator to remove traces of water molecules and the lyophilized powders were stored at 20 °C until used directly for the appraisal of various *in vitro* activities.

In vitro studies

Preliminary qualitative phytochemical analysis

Preliminary qualitative phytochemical analysis was carried out to identify the secondary metabolites present in various solvent extracts of leaf, stem, flower and fruit parts of *E. munronii* and *E. tuberculatus* (Trease and Evans, 2002; Harborne, 1984) [31, 17].

In vitro antioxidant activities

DPPH radical scavenging activity

The hydrogen donating capacity was assessed using the stable DPPH▪ method Blois, (1958) [7]. Briefly, a solution of 0.1mM DPPH• was prepared using methanol. The samples (50-250 µg/mL) were mixed with 5.0 mL of DPPH• solution. Reaction mixture was shaken, incubated at 27 °C for 20 min and the absorbance was measured at 517 nm. Results were compared with the activity of rutin, quercetin, BHA and BHT. Per cent DPPH▪ discolouration of the samples was calculated using the formula: DPPH radical scavenging activity (%) = [(Control OD – Sample OD)/Control OD] ×100. Antioxidant activities of the extracts were expressed as IC_{50}, these values were calculated from the linear regression of the percentage antioxidant activity versus concentration of the extracts (Ebrehimabadi *et al.*, 2010) [11]. A lower IC_{50} value indicates greater antioxidant activity.

Reducing power assay

The Fe3+ reducing power of the extract was determined according to the method suggested by Oyaizu, (1986) [26]. The plant extracts (100-500 µg/mL) were mixed with 5.0 mL of 0.2 M phosphate buffer of pH 6.6 and 5.0 mL of 1% K3 Fe (CN)6 and the mixtures were incubated at 50 °C for 20 min. The reaction was terminated by adding 5.0 mL of 10% TCA (w/v), and the mixture was centrifuged at 1000 rpm for 10 min. The upper layer of the supernatant (5.0 mL) was mixed with 5.0 mL of distilled water and 1.0 mL of 0.1% (w/v) FeCl3 and the absorbance was read at 700 nm. Rutin, quercetin, BHA and BHT served as the reference material. Increased absorbance indicates increased reductive capability.

Chelating ability for ferrous ions

The ferrous chelating potential of the extracts were assessed according to the method suggested by (Yamaguchi *et al.* 2000) [35]. The reaction was initiated with the sequential addition of 250 µg of sample extract, 0.25 mL of 1 mM FeSO4 solution, 1.0 mL of 0.2 M Tris–HCl buffer (pH 7.4), 1.0 mL of 2, 2' bipyridyl solution, 0.4 mL of 10% hydroxylamine hydrochloride and 2.0 mL of ethanol. The final volume was made up to 5.0 mL with deionised water and the absorbance was determined at 522 nm. EDTA was used to benchmark the chelating abilities. Lower absorbance of the reaction mixture indicated higher ferrous ion chelating ability.

Trolox equivalent antioxidant capacity assay

Antioxidant activity was performed using an improved ABTS▪+ method proposed by Siddhuraju and Manian (2007). The ABTS radical cation (ABTS▪+) was generated by a reaction of 7 mM ABTS▪+ and 2.45 mM potassium persulphate and the mixture was incubated for 12-16 h at room temperature in dark. Prior to assay, the solution was diluted in ethanol (about 1:89 v/v) and equilibrated to obtain an absorbance of 0.700 ± 0.02 at 734 nm. 10 µL/mL of sample was added to 1.0 mL of diluted ABTS•+ solution. After 30 min of incubation, absorbance was read at 734 nm. Trolox was used as a reference material.

Superoxide radical scavenging activity

Superoxide radicals were generated by the modified method of Beauchamp and Fridovich (1971) [5]. The assay was based on the capacity of the sample to inhibit formation by scavenging superoxide radicals generated by riboflavin-light-NBT in the system. Each 3 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mg NBT and various concentrations (200 - 1000µg) of sample extracts. Reaction was started by illuminating the reaction mixture with sample extract for 90 seconds. Immediately after illumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept in dark served as blank. The percentage inhibition of superoxide anion generation was calculated as:

Superoxide radical scavenging activity (%) = (control OD-sample OD / control OD) × 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

Statistical analysis

All the values were expressed as mean ± standard deviation(SD) of three determinations and were subjected to one way analysis of variance (ANOVA) followed by *post hoc* Duncan's multiple range test using SPSS (version 9, SPSS Inc., Chicago, USA). $P<0.05$ was chosen as the criterion for statistical significance.

Results

Qualitative estimations

Phytochemicals are the bioactive principles produced by plants in its various parts of the plants. The qualitative phytochemical screening was conducted for the different plant parts and solvent extracts of *E. munronii* and *E. tuberculatus* and it revealed the presence of a diverse class of phytochemical constituents, including alkaloids, flavonoids,

phenols, quinones, proteins, saponins, tannins, gums & mucilage etc., (Table 1 and 2). However, steroids, coumarins, glycosides, oils &fats and triterpenoids were found to be present in trace amount while terpenoids and carbohydrates were completely absent in both the studied species.

***In vitro* assay**

Dpph radical scavenging activity

DPPH, is a stable organic radical, widely used to test the ability of the compounds to act as free radical scavengers or hydrogen donors. It was visually noticeable by a drastic colour change from purple to yellow. DPPH radical scavenging activity for the two test species, *E. munronii* and *E. tuberculatus* were analysed and their IC_{50} values ranged between 21.1 to 86.3 µg/mL (Table 3). Among the samples investigated, the ethanolic leaf extract of *E. munronii* depicted the maximum value (21.1±0.1 µg/mL extract). Whereas, the ethyl aacetate extract of *E. munronii* stem registered markedly very low free radical scavenging activity (93.3±0.2 µg/mL extract).

Table 1: Quantitative phytochemical analysis of various solvent extracts of *Elaeocarpus munronii*

Tests	Secondary metabolites											
	PE			EA			ET			AQ		
	Leaf	Stem	Flower	Leaf	Stem	Flower	Leaf	Stem	Flower	Leaf	Stem	Flower
Alkaloids	+++	+	+++	+++	+++	++	+++	+++	+++	+++	+	-
Flavonoids	+++	-	+++	+++	++	+++	-	-	+++	-	-	+++
Steroids	-	-	-	-	-	-	-	+	-	++	-	++
Saponins	+++	+++	+	+++	-	-	+++	+++	+	+++	+++	+
Phenols	++	++	++	+++	-	+++	++	+	+	++	+	+
Quinones	+++	+	++		-	-	-	++	+	-	-	-
Coumarins	-	-	-	+	+	+	+	-	-	++	+	++
Terpinoids	-	-	-	-	-	-	-	-	-	-	-	-
Carbohydates	-	-	-	-	-	-	-	-	-	-	-	-
Glycosides	+++	-	-	+	+	++	+++	-	-	+++	-	-
Proteins	-	+	-	-	+	-	-	+	-	-	++	-
Oils and fats	+++	-	+	-	+	+++	-	-	+	-	-	+
Gums and mucilages	+++	+	++	+++	+++	++	+	+++	+	+++	+	+
Triterpinoids	+	++	++	+	+	+++	++	-	+	-	+	++
Tannins	+++	-	-	+++	+++	++	+++	-	-	+++	-	-

Legend: +++ (Much abundant), ++ (less abundant), + (minute), - (absent)

*Note: Refer Table.1 for abbreviations

Table 2: Qualitative phytochemical analysis of various solvent extracts of *Elaeocarpus tuberculatus.*

Tests	Secondary metabolites.											
	PE			EA			ET			AQ		
	Leaf	Stem	Fruit	Leaf	Stem	Fruit	Leaf	Stem	Fruit	Leaf	Stem	Fruit
Alkaloids	+	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++
Flavonoids	-	-	-	++	++	++	+++	+++	+++	+	-	-
Steroids	+++	-	-	-	+++	+++	++	-	-	+	-	-
Saponins	++	++	+	+++	+++	-	++	++	+++	+	+++	++
Phenols	+++	-	+	+	++	+	+++	+++	+++	++	+++	+++
Quinones	-	+++	-	-	-	-	-	++	+++	+	++	++
Coumarins	+++	-	-	-	+++	+++	+++	-	-	+	-	-
Terpinoids	-	++	++	-	-	-	-	++	+++	-	+++	+++
Carbohydates	-	-	-	-	-	-	-	-	-	-	-	-
Glycosides	-	-	-	++	++	-	-	-	+++	-	-	-
Proteins	+	-	++	++	-	-	-	-	-	-	-	-
Oils and fats	-	-	-	-	-	-	-	-	-	-	-	-
Gums and Mucilages	+++	+++	+++	+++	+++	+++	+++	+++	++	+	+++	+
Triterpinoids	-	-	-	++	-	-	+++	+++	+++	-	-	-
Tannins	+++	+++	+++	+++	+++	+++	+++	++	+++	+	-	-

Legend: +++ (Much abundant), ++ (less abundant), + (minute), - (absent)

*Note: Refer Table.1 for abbreviations

$ABTS^{\bullet+}$ scavenging activity.

In the evaluation of antioxidant capacity by $ABTS^{\bullet+}$ method, all the assessed sample extracts were able to quench $ABTS^{\bullet+}$ radical more effectively and their values ranged between99.3 and 629.1 µmol Trolox equivalent/g extract (Table 3). Among the samples investigated, the ethanolic leaf extract of *E. tuberculatus* (629.1±10.1 µmol/g extract) determined maximum value. While, the petroleum ether extract of *E. munronii* flower registered markedly very low activity (99.3±09.3 µmol/g extract).

Superoxide radical scavenging activity

The superoxide radical scavenging abilities for the various extracts of $O_2^{\bullet-}$ radical were determined and depicted in Table 3. Various plant part extracts of *E. munronii* and *E. tuberculatus* evidenced good superoxide radical scavenging abilities (18.3 to 81.2 µg/mL). However *E. munronii* leaf ethanolic extracts manifested high radical power activity; however, their activity was higher than that of *E. tuberculatus* and their IC_{50} values for the standard antioxidants in the order of : Rutin > Quercertin C > BHA > BHT respectively.

Chelating ability for ferrous ions

Ferrous ions are one of the most effective pro-oxidants and their interactions with hydrogen peroxide in biological systems can lead to the formation of highly reactive hydroxyl radicals. The Fe^{2+} chelating ability for both the plant species were examined and were presented in Table 3. Among the samples investigated, the ethanolic extracts of *E. munronii* leaf depicted markedly high metal chelating activity (99.3±0.9 mg EDTA/g extract) than the other said samples. While, the petroleum ether extract of *E. tuberculatus* fruit exhibited correspondingly very low ability for iron binding (12.9±0.1 mg EDTA/g extract).

Reducing power assay

In the reducing power assay, the presence of antioxidants in the samples would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. Table 3 depicts the dose dependent response for the two study plant species and their reductive abilities displayed an apparent linear relationship with concentration. The activity increases exponentially with increase in the concentration of the test drug. Where the ethanolic extracts of *E. munronii* leaf provided higher reductive power value (1.36±0.11 µg/ml) than the other said samples. While, the petroleum ether extract of *E. tuberculatus* stem exhibited correspondingly very low reducing power value (0.14±0.1 µg/ml). Interestingly, these values were higher and it even surpassed the efficiency of all the standard antioxidants tested (Table 3).

Table 3: DPPH, superoxide, $ABTS^{\bullet+}$, reducing power and ferrous ion chelating activities of *E. munronii* and *E. tuberculatus* plant parts.

Parts	Samples	Extracts	DPPH (IC_{50} µg/mL)	$O_2^{\bullet-}$ (IC_{50} µg/mL)	Reducing power activity (µg/mL)	$ABTS^{\bullet+}$ scavenging activity @	Ferrous Ion *
Leaf	*E. munronii*	PE	86.3±0.2^{w}	81.2±0.3^{u}	0.92±0.06^{c}	102.2±11.1^{s}	30.3±0.3^{l}
		EA	90.2±0.1^{x}	83.3±0.2^{v}	0.14±0.1^{w}	222.2±12.1^{k}	55.2±0.3^{e}
		ET	21.1±0.1^{c}	19.3±0.1^{b}	1.36±0.1^{a}	323.6±35.2^{f}	99.3±0.9^{a}
		AQ	75.3±0.2^{t}	21.3±0.3^{d}	0.59±0.1^{g}	112.3±16.3^{q}	81.3±0.8^{c}
	E. tuberculatus	PE	62.6 ±0.3^{p}	41.4 ±0.3^{o}	0.59 ±0.2^{g}	224.2±13.2^{j}	40.9±0.3^{i}
		EA	54.3±0.2^{n}	35.6±0.5^{l}	0.29±0.3^{o}	269.3±11.3^{i}	27.9±0.6^{n}
		ET	29.1 ±0.1^{d}	18.9 ±0.1^{a}	0.71 ±0.1^{d}	629.1±10.1^{a}	69.1±0.3^{d}
		AQ	51.1±0.3^{m}	29.2 ±0.2^{i}	0.52 ±0.1^{i}	444.4±20.1^{c}	41.3±0.2^{h}
Stem	*E. munronii*	PE	39.6±0.2^{h}	55.5±0.2^{s}	0.19±0.1^{v}	123.3±12.0^{p}	28.9±0.2
		EA	93.3±0.2^{y}	83.2±0.2^{v}	0.14±0.2^{w}	088.3±10.2^{v}	30.2±0.2^{l}
		ET	30.2±0.1^{e}	33.3±0.3^{k}	0.93±0.2^{b}	292.2±21.2^{h}	84.3±0.8^{b}
		AQ	78.8±0.1^{u}	25.5±0.3^{g}	0.55±0.3^{h}	110.3±16.3^{r}	44.3±0.5^{g}
	E. tuberculatus	PE	59.1±0.3^{o}	42.3 ±0.3^{p}	0.14 ±0.1^{w}	201.1±17.2^{l}	51.2±0.1^{f}
		EA	40.1±0.2^{i}	55.3±0.2^{s}	0.36±0.3^{n}	295.3±14.4^{g}	17.6±0.3^{r}
		ET	33.3±0.2^{f}	21.4 ±0.1^{d}	0.20 ±0.1^{u}	101.9±09.1^{t}	44.9±0.2^{g}
		AQ	69.7±0.3^{r}	31.3 ±0.2^{j}	0.42 ±0.1^{k}	397.9±14.1^{d}	23.9±0.2^{o}
Flower	*E. munronii*	PE	81.9±0.3^{v}	74.3±0.3^{t}	0.21±0.4^{t}	099.3±09.3^{u}	18.9±0.2^{q}
		EA	96.6±0.3^{z}	89.6±0.1^{w}	0.48±0.1^{j}	0.63±03.2^{w}	12.3±0.2^{s}
		ET	41.2±0.1^{j}	20.3±0.1^{c}	0.66±0.3^{f}	222.8±14.1^{k}	55.5±0.6^{e}
		AQ	66.3±0.2^{q}	18.3±0.1^{a}	0.15±0.5^{v}	195.3±12.9^{n}	36.3±0.3^{j}
Fruit	*E. tuberculatus*	PE	47.0±0.3^{l}	44.1 ±0.3^{q}	0.67±0.2^{e}	199.9±10.2^{m}	12.9±0.1^{s}
		EA	71.3±0.2^{s}	45.6±0.3^{r}	0.22±0.1^{s}	162.3±21.3^{o}	19.9±0.2^{p}
		ET	39.3±0.2^{h}	24.1 ±0.2^{f}	0.27±0.1^{p}	599.9±11.1^{b}	29.8±0.3^{m}
		AQ	42.4±0.1^{k}	37.7 ±0.3^{m}	0.37±0.1^{m}	325.4±21.1^{e}	34.9±0.2^{k}
Standard		Rutin	15.8±0.1^{a}	18.8 ±0.01^{a}	0.23±0.01^{r}	-	-
		Quercertin	20.7±0.1^{b}	23.0 ±0.07^{e}	0.38±0.07^{l}		
		BHA	21.4±0.1^{c}	26.4 ±0.51^{h}	0.24±0.01^{q}		
		BHT	34.7±0.3^{g}	39.5 ±0.63^{n}	0.24±0.05^{q}		

Values are mean ± SD of three independent experiments. Values not sharing a common letter in a column are significantly different (P<0.05).*Values expressed in mg EDTA/g extract; @Values expressed as TEAC (Trolox equivalent antioxidant capacity) in µmol/g extract.
$**Note**: PE-Petroleum ether; EA-Ethylacetate; ET-Ethanol; AQ-Aqueous; BHA-Butylated hydroxyanisole; BHT- Butylated hydroxytoluene.

Discussion

The worth of medicinal plants to mankind is well recognized, as numerous discoveries have exposed that the plant extracts contain not only minerals and primary metabolites, but also a wide range of secondary metabolites with huge healing efficiencies (Akinmoladun *et al*., 2007 and Cheikhyoussef *et al.*, 2015) [3, 1].

DPPH radical is one of the few stable organic nitrogen free radicals, which has been widely used to determine the free radical scavenging ability of the different samples (Williams *et al.*, 1995) [34]. Reducing properties are generally associated with the presence of reductons which have been shown to exert antioxidant action by breaking the free radical chain and by donating a hydrogen atom (Mohamed *et al.* 2012) [24, 33]. The ethanolic extract was found to be a prominent solvent for extracting phytochemicals owing to the presence of high polar solvents like phenolic and flavonoid compounds in it. Similar trend was observed for a lot of other plant extracts that have been deliberated (Katalinic *et al*., 2006) [20]. Iron is the chief peroxidant and is able to generate lipid peroxidation through the fenton reaction or by accelerating the dissociation of lipid hydroperoxides to their respective peroxy and alkoxy radicals (Gioti *et al.*, 2009) [15]. The ability of the extracts to bind Fe^{2+} in the presence of ferrozine was compared with that of EDTA (Chang *et al.*, 2012) [8]. $ABTS^{\bullet+}$ is a blue chromophore produced by the reaction between $ABTS^{\bullet+}$ and potassium sulphate (Vandita *et al*., 2012) [33]. Superoxide anion is an oxygen-centered radical with selective reactivity. It is biologically quite toxic. These precursor signals initiates free radicals to react with biological macromolecules and thereby inducing tissue damage (Gulcin *et al*., 2010) [16].

Findings from this study revealed that *E. munronii* and *E. tuberculatus* are the rich source of phytochemicals with proven antioxidant activities. Also, this plant can be a promising good source for further purification of phytochemical compounds. Further studies are necessary to strengthen the exact nature of the bioactive principles in the plant extracts that are responsible for its hypoglycemic effect. This can promote their use as natural products with the prospects of increasing the quality of life of diabetic sufferers.

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