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In vitro antioxidant activity of *Litsea quinqueflora* (Dennst.) Suresh

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Abstract
Litsea quinqueflora (Dennst.) Suresh (Loraceae) is a medicinally important plant used by the traditional healers to cure inflammatory ailments. The present study focused on the antioxidant activity of ethyl methyl ketone and methanol extracts of the leaves and quantification of phenols and flavonoids. Antioxidant activity of the leaf extract was assessed by DPPH, CUPRAC and PFRAP assays using L-ascorbic acid as standard. Total phenols were estimated by Folin- Ciocalteu's method and flavonoids by aluminium chloride method. In DPPH assay, IC 50 value for methanol extract (38.6567µg/ml) was lower than that of ethyl methyl ketone extract (56.8µg/ml). But in CUPRAC and PFRAP assay, EC 50 values are higher for ethyl methyl ketone extract than that of methanol extract. Statistical analysis by means of one way ANOVA with Post hoc Tukey tests gave significant P values in each assay. All measured antioxidant potential values were significant when compared with the standard. Quantification yielded considerable amount of phenolics and flavonoids with gallic acid and quercetin as standards respectively. The above results supported the effective role of leaf extracts of *Litsea quinqueflora* (Dennst.) Suresh as an antioxidant and could be recommended as an alternative for synthetic antioxidants.

Keywords: *Litsea quinqueflora*, DPPH, CUPRAC, PFRAP, antioxidant, phenolics, flavonoids

Introduction
Biochemical reactions in the living system are too numerous to mention. Such reactions when leave a molecule or atom with an unpaired electron, free radicals will be generated. In order to attain stability, free radicals always salvage other atoms to catch electrons. This process goes on and forms many free radicals [1]. They were formed not only due to biochemical reactions but also with some external factors such as exposure to X- rays, ozone, cigarette smoking, industrial chemicals and air pollution [2, 3]. Many essential elements become enemy to our body in some cases at relatively higher concentrations. For example, oxygen is very crucial to living things but at the level of reactive oxygen species, they are the most deleterious [2, 3, 4]. At moderate levels, free radicals are effectively involved in many physiological functions of body especially immunity, cellular signaling pathways, mitogenic response and redox regulation [1, 5, 6]. But when present in higher concentrations, free radicals cause the damage of cells and cell membranes, mutation and are also the contributors of many degenerative diseases such as cancer, cardiovascular diseases, neurodegenerative disorders and other chronic conditions [7, 8, 9, 10]. In this context, the role of antioxidants is relevant. Antioxidants natively originate in living systems as antioxidant enzymes, metal-binding proteins and supplemented from phyto-constituents in the form of diet [11]. Toxicology problem of synthetic antioxidants leads to the discovery of many naturally purified antioxidants. Powerful antioxidants without side effects are an emerging need of this era. Medicinal plants are the main source of natural antioxidants. Synthetic ones are powerful but may adversely affect liver and lungs. Antioxidants play a vital role in food preservation. Examples of commercially used antioxid arts include butylated hydroxyanisole (BHA), butylated hydroxyl toluene (BHT), propyl gallate (PG), tert-butylhydroquinone (TBHQ) etc [12]. Though BHT and BHA are effective antioxidants, their safety is questionable. Therefore, food industry is also demanding innocuous antioxidants with natural origin.In this context, the need for natural plant derived antioxidants are very relevant [13, 14, 15].

Litsea quinqueflora (Dennst.) Suresh. (Syn. *L. ligustrina*) belongs to the family Lauraceae and is used by traditional healers of Kerala in treating many ailments related to inflammatory disorders [16]. It is considered to be endemic in Western Ghats and rarely present in South Sahyadri, Palakkadu hills, Wayanadu, Kottayam and Thiruvananthapuram districts of Kerala and is reported as rare, endemic to South Western Ghats [17]. There are no research reports on antioxidant activities of this plant. Therefore this study was aimed at analysing *in vitro* antioxidant activities of methanolic (MeOH) and ethyl methyl ketone (EMK) leaf extracts of *L.*

Quinqueflora using DPPH (2, 2- diphenyl-1-picrylhydrazyl radical) scavenging, CUPRAC (Cupric Ion Reducing Antioxidant Capacity) [18, 19] and PFRAP (*Potassium Ferricyanide* Reducing Power method) [19, 20] assays with L-Ascorbic acid as standard.

Materials and Methods

Chemicals

2,2- diphenyl-1-picrylhydrazyl radical (DPPH) purchased from Sigma Chemical (Sigma-Aldrich Co., St. Louis, MO, USA), Cupric chloride, Ammonium acetate, Neocuprioin, Pottacium ferricyanide, Sodium Phosphate, Trichloro acetic acid, L- ascorbic acid, Gallic acid, Folin-Ciocalteu's phenol reagent and Quercetin were obtained from Merk, USA and all other chemicals used were of highest analytical purity.

Plant Material

Leaves of *L. quinqueflora* were collected from Kurianad, Kottayam District, Kerala, India. The taxonomical identification of the plant was carried out at Kerala Forest Research Institute, Peechi, Thrissur, Kerala and voucher specimen was deposited in the herbarium with accession No: KFRI. 13057. The collected leaves were washed under running tap water and shade dried in open air for two weeks. The dried leaves were powdered using a mechanical blender.

Preparation of extracts

Twenty five grams of dried leaf powder was sequentially extracted with different solvents in the order of increasing polarity such as petroleum ether, ethyl acetate, ethyl methyl ketone and finally methanol. The extracts were filtered well and dried through vacuum evaporation. Since phenolic compounds were absent in other extracts after preliminary screening, the methanol (MeOH) and ethyl methyl ketone (EMK) extracts were used for further antioxidant analysis.

Total phenolic content (TPC)

The quantity of phenolic content in methanol and ethyl methyl ketone fractions was measured using Folin Ciocalteu's phenol reagent (FCR). To 2.5 ml of 10 % FCR reagent in distilled water, 0.5 ml of the extract was added. Reaction mixture was incubated for 5 min in dark and added 3 ml of 7.5 % sodium carbonate. The absorbance was measured at 765 nm after 30 min. The phenolic compounds were quantified as Gallic acid equivalents from standard Gallic acid graph [21, 22].

Total flavonoid content (TFC)

Aluminium chloride spectrophotometric method [23, 24] was used to measure the total flavonoid content in the samples where quercetin was used as standard. Plant extracts (0.5 ml) were mixed with 0.15 ml of 5% sodium nitrite. After six minutes, added 0.15 ml 10 % aluminium chloride. After six minutes of incubation, 2 ml of sodium hydroxide (1 N) was added and the fixed volume was made up to 5 ml with distilled water. It was then incubated for 15 minutes and the absorbance was measured at 510 nm.

In vitro antioxidant assays

DPPH free radical scavenging assay

The free radical scavenging assay of L. *quinqueflora* against DPPH was determined spectrometric ally by the method of Brand Williams *et al* with necessary modifications [25]. A reaction mixture containing 1 ml of 0.1 mmol/L DPPH, and various concentrations of extracts (20, 40, 60, 80 and 100 µg/ml) were made up to 3 ml using ethanol. After dark incubation for 20 min, the deep violet color became yellow on the basis of activity and was measured at 517 nm. L- Ascorbic acid was used as a standard [26].

Cupric Ion Reducing Antioxidant Capacity (CUPRAC)

CUPRAC assay was carriedout by the method of Apak [18]. 1 ml of extract was mixed with 1ml 10mM cupric chloride ($CuCl_2$), 1ml 7.5mM neocuprioine, 1ml 1M ammonium acetate buffer (pH 7.4) and 1 ml absolute ethanol. These reaction mixtures were incubated for 30 minutes at room temperature and absorbance was measured spectrophotometrically at 450 nm and compared it with the standard L- ascorbic acid [27].

Potassium ferricyanide Reducing Power method (PFRAP)

The reducing power of plant extracts were measured by the standard method of Oyaizu [28] with slight modifications. 0.5 ml extracts with different concentrations were taken and mixed with 0.5 ml phosphate buffer (0.2 M, pH 6.6) and 0.5 ml *Potassium ferricyanide* (1%) and kept at 50^0 C for 20 minutes. Cooled reaction mixture was diluted with 4 ml distilled water after adding 1.5 ml Trichloro acetic acid (10%) and allowed to react with 0.5 ml ferric chloride (0.1 %). Then spectrophotometrically measured it at 700 nm. Then compared it with L- ascorbic acid [29].

Statistical analysis

Each analysis was triplicated and the results were expressed in the form of mean ± standard deviation and statistically compared with the standard using the method of ANOVA followed by post-hoc Tukey test in Graphpad Instat for windows. Microsoft excel 2007 with its data analysis add in was used to calculate IC 50 and EC 50 values of each sample extract from linear regression analysis of graph.

Results

Total Phenolic content

Total phenolic content was measured through Folin-Ciocalteu's phenol reagent method and quantified as gallic acid equivalent. The amount of phenolic content in each extract was assessed from the linear regression equation ($y=0.007x-0.009$, $R^2=0.998$) which obtained from the standard curve of gallic acid. It was observed that phenolic content in ethyl methyl ketone extract is higher than that of methanol extract (Table 1).

Total flavonoid content

Aluminium chloride method was used to calculate the amount of total flavonoid in the samples. Flavonoid contents were measured and made equivalent with quercetin, the standard. The flavonoids present in the sample is quantified from regression linear equation ($y=0.003x+0.018$, $R^2=0.985$) of standard graph of quercetin measured in µg/ml. Ethyl methyl ketone extract has high flavonoid content when compared with methanol extract.

Antioxidant activity of methanol and ethyl methyl ketone extracts

Antioxidant capacity of different extracts in DPPH assay was expressed as inhibitory concentration showed 50% activity, IC 50 value (µg/ml) (Table 1) and that of CUPRAC and PFRAP assays were as effective concentration at which activity was 50%, EC 50 value (µg/ml) (Table 1). Both values were calculated by linear regression analysis from the graph

of each extract. The antioxidant potential was inversely proportional to IC 50 and EC 50 values.

DPPH Assay

Reaction of DPPH with the sample extracts resulted in the disappearance of its violet color whose intensity was measured at 517 nm. Based on the IC 50 value, MeOH extract showed higher activity than that of EMK extract. When compared with the standard, both extracts were having significant values (Table 1). The percentage of inhibition increased with increasing concentration of the extrct. IC 50 value was calculated from the regression equation showed in the figure 1. (y = 0.402x + 47.08, R^2=0.975 for methanol extract and y=0.286x+36.59, R^2 =0.993 for ethyl methyl ketone extract).

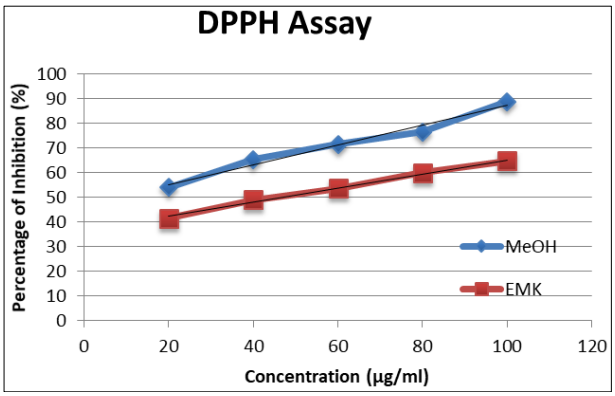

Fig 1: Percentage of inhibition and regression linear equation of DPPH Assay

CUPRAC Assay

CUPRAC assay expressed the reduction of Cu (II) to Cu (I) through the action of antioxidant components of the extracts was measured. The conversion of color from blue green to yellow was quantified from the absorbance at 450 nm. EMK extract exhibited highest activity than that of MeOH extract. The antioxidant potential of EMK extract is less significant in statistical analysis. The potential of MeOH extract was the most significant (Table 1). EC 50 value was calculated from the graph showed in the figure 2. (y = 1.024x + 18.72, R^2=0.977 for methanol extract and y=1.2x+31.14, R^2 =0.969 for ethyl methyl ketone extract).

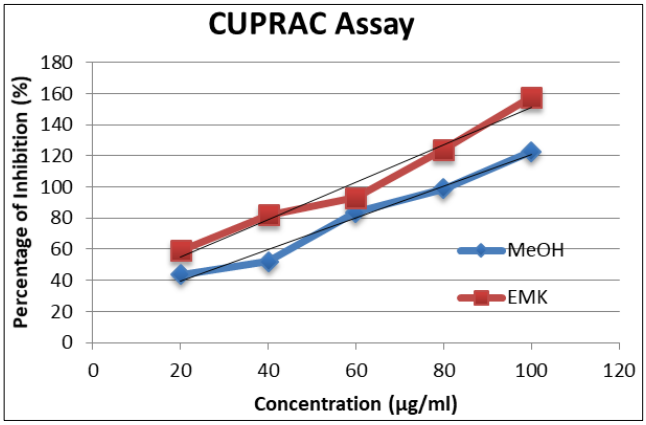

Fig 2: Percentage of inhibition and regression linear equation of CUPRAC Assay

PFRAP Assay

Antioxidant compounds present in the extracts reacted with *potassium ferricyanide* and formed potassium ferrocyanide. Potassium ferrocyanide reacts with ferric chloride to form blue coloured ferric ferricyanide whose absorbance was read at 700 nm. EMK extract shows high activity than MeOH extract and lowest EC 50 value (Table 1). EC 50 value was calculated from the graph (Figure 3). (y = 1.996x +10.85, R^2=0.998 for methanol extract and y=2.169x+18.73, R^2 =0.98 for ethyl methyl ketone extract).

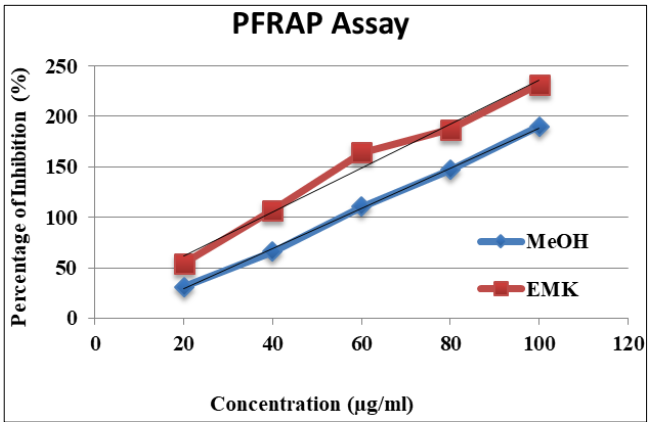

Fig 3: Percentage of inhibition and regression linear equation of PFRAP Assay

Table 1: Antioxidant capacities (µg/ml) and TPC (mg Gallic acid/g), TFC (mg Quercetin/g)

Sample	DPPH (IC50 µg/ml)	CUPRAC (EC50 µg/ml)	PFRAP (EC 50 µg/ml)	TPC	TFC
MeOH extract	38.66±1.86***	29.81± 1.98***	30.4±3.22***	115.92±1.1	97.1±0.69
EMK extract	56.8 ± 3.03***	15.573± 3.57**	14.4±1.83**	122.35±1.6	115.3±1.77
L –Ascorbic acid	2.145 ± 1.2	2.845 ± 0.19	0.46 ± 0.26		

Since P << 0.05, it is significant. *** P<0.001, **P<0.01

Discussion

Plants served as promising sources of medicine and were continuously exploited for the isolation, characterization and synthesis of novel compounds with very less side effects. Among the various activities exhibited by plant derived drugs antioxidant, anti-inflammatory, anti-cancerous and antimicrobial properties are of great importance to health sector. Antioxidant assays can beeither Hydrogen Atom Transfer (HAT) based or Single Electron Transfer (SET) based. In HAT based assays, reactions occur between antioxidant agents and peroxyl radicals. In the present investigation, three SET based assays such as DPPH, CUPRAC and PFRAP was executed among which DPPH was HAT based also. The antioxidants present in the plant extracts react with oxidizing agents and their potential to reduce the oxidizing agents was evaluated spectrophotometrically [30].

The genus *Litsea* was reported to contain various phytochemicals with promising bioactivities [31]. The antioxidant potential of methanolic and methyl ethyl ketone extracts of Litsea *quinqueflora* (Dennst.) Suresh was evaluated by measuring the colour intensity change on reduction. Decolonization occurred in DPPH assay. But in CUPRAC and PFRAP assays color shifts from one to another [32].

In *in vitro* antioxidant assays, the antioxidant components present in the sample neutralize free radicals and prevent it

from damaging other molecules [2, 33]. Antioxidant activity of the extracts can safely inhibit free radicals and block the chain reaction before cellular damage. Certain antioxidants such as glutathione, ubiquinol and uric acid were produced during normal metabolism of living body [2, 34]. Vitamin E (α-tocopherol), vitamin C (ascorbic acid) and β- carotene are effective micronutrients present in the diet, but cannot be synthesized by the body [2, 35]. In the present investigation, L-ascorbic acid was used as standard for comparison with the test samples. Various phytochemicals such as alkaloids, flavonoids, coumarins, terpenoids, anthraquinones, phenols etc were reported in *Litsea quinqueflora* (Dennst.) Suresh [16]. Hence, an enquiry into the antioxidant property has been undertaken since there were no reports available.

Aluminium chloride colorimetric method was used for the estimation of total flavonoid content in the extracts of *L. quinqueflora*. Aluminium chloride reacts with C-4 keto groups and C-3 or C-5 hydroxyl group of flavones and flavonols present in the samples. It also reacts with orthohydroxyl groups in the A- or B- ring of flavonoids and forms acid labile complexes [36] and produce an orange color. The range of color was measured as optical density value and estimated the total flavonoid content.

Rapid oxidation reaction of phenols in the sample with an alkali, normally sodium carbonate, results in phenolate ions. The phenolates reduce yellow Folin Ciocalteu reagent into blue coloured compound [37]. Quantification of phenols was obtained from the absorbance taken at 765 nm. There were different concentrations mentioned for alkali in literature but we used 7.5 % of sodium carbonate which gave good results in the present experiment [27]. The phenolic content of extracts of *L. quinqueflora* was higher than that of flavonoid content which can be attributed towards the antioxidant potential.

In DPPH assay, the stable free radical 1, 1- Diphenyl-2-picrylhydrazyl (DPPH) reacts with the antioxidant components present in the extracts and undergone reduction. This reduction leads to decrease in deep purple color and this radical is used in many in vitro antioxidant studies [38, 39]. Methanol fraction illustrated free radical scavenging activity even in reduced concentrations [40, 41]. The DPPH radical scavenging activity in a concentration dependent manner was reported in the methanolic extracts of *Oxalis corniculata* Linn. [42]. Simillar studies were also described in *Litsea eliptica* Blume and *Litsea resinosa* [43]. In the present study, the DPPH scavenging activity was found to be higher than the above reports with an IC 50 value of 38.66 µg/ ml and was significant at 5%.

In CUPRAC assay, copper (II) neocuproine reagent is the chromogenic oxidizing agent and can be considered as more accurate since it was performed at physiological pH (7.4). The reduction of neocuproine reagent leads to the production of bis (neocuproine) copper (I) chelate. The light blue copper (II) neocuproine cation changed to orange yellow copper (I) neocuproine cation [30]. The level of intensity of color was measured at 450 nm. Absorbance increased with increasing concentration and showed high antioxidant potential (low EC 50 value). Ethyl methyl ketone extract was superior to methanolic extract and both showed concentration dependent antioxidant potential. Simillar results were published on the basis of cupric ion reducing activity of alchoholic leaf extact of *Spatholobus parviflorus* (Roxb. Ex. Dc.) Kuntze [22].

PFRAP is a SET based assay which depends on the strength of an antioxidant to reduce free radicals by donating an electron. This leads to a color change in the solution. In the present study, the sample shows a high activity or percentage of inhibition in concentrations less than 100µg/ml. Percentage of inhibition of ethyl methyl ketone extract was almost similar to standard drug when compared to methanol extract. In this study reducing ability of extracts of *L. quinqueflora* to change Fe^{3+} to Fe^{2+} was expressed as EC 50 value, although the results of many studies on reducing power of plant extracts were denoted as standard ascorbic acid or Trolox equivalent. But in reducing power assay of hydromethanolic extracts of *Haloxylon articulatum* Boiss, *Solenostemma oleifolium* Bull. & Bruce and *Echium pycnanthum* Pomel [44], andalso in another study of methanol extracts of wild edible mushroom species, *Lactarius delicious* (L.) Gray and *Tricholoma portentosum* (Fr.) Que'l [45], reducing power was expressed as EC 50 value. Reducing power of extracts of *L. quinqueflora* was higher than that of above mentioned plant extracts.

Conclusion

The *in vitro* antioxidant studies on *Litsea quinqueflora* (Dennst.) Suresh showed significant results. Since this plant's antioxidant potential is high, this can be considered as a promising source of commercial antioxidant compounds.

Conflict of Interest

The authors have no conflict of interest.

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