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HPTLC finger print profiling and evaluation of anti-inflammatory and antioxidant properties of fractions of leaf extract of *Litsea quinqueflora* (Dennst.) Suresh

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ABSTRACT
Folklore medicines are well-known among local people and have been used for curing different ailments. Plant-derived drugs are a potent source of many therapeutically active compounds. The isolation, purification and characterization of such compounds using chromatography and advanced spectrophotometric methods followed by their bioactivity studies has opened a new era in the field of herbal medicine. Litsea quinqueflora has been used by traditional healers of Kerala as an anti-inflammatory agent. The different fractions of methanol extract of leaves were used for the present study. HPTLC fingerprint profiling followed by derivatization with aluminium chloride revealed the presence of flavonoids. Anti-inflammatory properties of hexane, chloroform, ethyl acetate and methanol fractions were assessed through inhibition of protein denaturation assay. Ethyl acetate and methanol fractions showed IC50 values of 113.29µg/ml and 66µg/ml respectively. Antioxidant studies using methanol fraction clearly indicated its free radical scavenging activity with IC50 values 69.25µg/ml (DPPH) and 56.37µg/ml (ABTS) and total antioxidant capacity of 0.139±0.47 GAE/g. These results scientifically support the traditional use of L. quinqueflora as a natural anti-inflammatory and antioxidant agent.
Keywords*: Litsea quinqueflora, HPTLC, flavonoids, protein denaturation, DPPH, ABTS.*

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INTRODUCTION

Phytochemical constituents of herbal plants are the key factors behind their medicinal attributes. Medicinal plants and their wide applications were prevalent in traditional systems of medicine even during the prehistoric period. But their phytochemical compositions remained hidden for many years and became popular with the advent of science and technology. The study of the chemistry of medicinal plants has gained attention as there is an urge to synthesize natural as well as synthetic drugs with fewer side effects and affordable prices. Isolation of the active principle present in plants will help in the production of pure and active natural components [1]. These chemical constituents are not meant for their immediate survival and are produced by secondary metabolism. Secondary metabolites are byproducts of primary metabolism [2], produced incidentally and considered as the immune system of plants.

Herbal formulations have been used as preventive medicines and are capable of fighting against many diseases [3]. In this context, the selection and identification of a well-known traditional medicinal plant and extraction of its phytochemical constituents are crucial. Phytochemical components are non-nutritive chemical compounds synthesized by plants and have been used as an effective anti-inflammatory, antioxidant, antimicrobial, anti-diabetic, anticancer, anti-aging, and antidepressant agents [4].

In the present study *Litsea quinqueflora* (Dennst.) Suresh of the family Lauraceae has been selected to scientifically validate its traditional use as an anti-inflammatory agent. The genus *Litsea* is a group of plants with effective medicinal properties and renowned drugs has been used by the local people to cure many inflammatory disorders and lack scientific evidence [5]. They possess different bioactive compounds such as alkaloids, flavonoids, sesquiterpenes, monoterpenes, diterpenes, amides, steroids, lignans and fatty acids with different biological activities [6]. There exist many lacunae in the proper assessment through bioassay-guided phytochemical analysis and purification of bioactive components. *L. quinqueflora* was reported to contain many pharmacologically effective compounds viz. decanal, β-elemene, β- caryophyllene and α- caryophyllene and *L. deccanensis* harbours germacra-3,9,11-triene, β- caryophyllene, caryophyllene epoxide, bicyclogermacrene, limonene and α-humulene. Among them, β-caryophyllene is biologically more active with anti-inflammatory, antibiotic, antioxidant, anticarcinogenic and anaesthetic properties [7]. The

antioxidant and anti-inflammatory activiti es of leaf extracts of *L. quinqueflora* was reported in earlier studies [8, 9]. In the present investigation, methanolic extract of the leaves of *L. quinqueflora* is fractionated and analysed by HPTLC followed by antioxidant and anti-inflammatory studies. The phytochemical constituents were extracted by a soxhlet followed by fractionation through the liquid-liquid partition. HPTLC fingerprint profiling was performed for the separation and identification of the active phytochemical compounds. Bioactivity based assays such as inhibition of protein denaturation, total antioxidant activity and scavenging of free radicals such as ABTS and DPPH were also done.

MATERIALS AND METHODS

Collection of plant and its processing

Leaves of *Litsea quinqueflora* (Dennst.) Suresh were collected from Kurianad area of Kottayam district, Kerala, India. Plant identification was done at Kerala Forest Research Institute and voucher were deposited with accession number KFRI 13057. Leaves were separated from twigs and cleaned under running water. The leaves were then shade dried and made into a fine powder using an electric blender.

Preparation of extract and fractions

The direct methanol extract (LMeOH) was obtained by dissolving 50g of leaf powder in 250 ml methanol in a soxhlet apparatus. The extract was collected and separated into different fractions by liquid-liquid partition using a separating funnel using different solvents such as hexane, chloroform and ethyl acetate. Each solvent was mixed well with the extract at least three consecutive times and collected their corresponding fractions. The remaining fraction was collected with the addition of methanol and it was taken as a modified extract of methanol and used for further studies. Thus the hexane (LHF), chloroform (LCF), ethyl acetate (LEF) and modified methanol fractions (LMF) of direct methanolic extract (LMeOH) was obtained and used for further studies after drying.

High-Performance Thin Layer Chromatography (HPTLC) fingerprint profiling

The HPTLC fingerprint profiling of fractions obtained via liquid-liquid partition was carried out using the instrument (Camag, Muttenz, Switzerland) and consisted of Linomat-V automatic TLC applicator and spotter having a 100µl syringe connected to a nitrogen cylinder, a twin-trough developing chamber, a derivatization chamber, and a viewing cabinet with dual-wavelength UV lamps (254 nm and 366 nm). The stationary phase contained aluminium plates precoated with silica gel F_{254} (Merck, Germany). The prepared extracts were filtered through a fine mesh and applied to the stationary phase. The sample was spotted on TLC plates as a narrow band of 8mm width and 10mm distance from the bottom. This application of sample was under a continuous flow of nitrogen gas in order to regulate the sample application process. A twin trough glass chamber was saturated with mobile phase and followed the development of sample carrying TLC plate. The TLC plate was allowed to be in the trough till the mobile phase reached a distance of 80mm from the point of application and then dried using an air drier.

Proper mobile phases were selected for each phytochemical on the basis of standard procedures proposed by Wagner and Bladt, [10] and Reich and Schibli [11]. Sample (2µl) was applied on TLC plates. The chromatogram was developed for LM, LMeOH and different fractions, LHF, LCF, LEF and LMF using the mobile phase- Toluene: Ethyl acetate: Methanol (7:3:1). Derivatisation was done using aluminium chloride, specific for flavonoids.

In vitro anti-inflammatory screening: Inhibition of protein denaturation assay was performed for different fractions of crude methanolic extract. It was the screening technique used to identify the anti-inflammatory potential of *L. quinqueflora*. The method of Mizushima and Kobayashi [12] was used to frame the assay with slight modifications [13].

Antioxidant assays

Antioxidant activity of fractions LMF was evaluated using DPPH (1, 1 diphenyl-2-picryl hydrazyl) radical scavenging assay and $ABTS^{\cdot+}$ [2, 2' – azino – bis (3- ethylbenzothiazolin – 6 – sulfonic acid)] assay and total antioxidant capacity (TAC) was analyzed through phosphomolybdenum assay.

DPPH (1, 1 diphenyl-2-picryl hydrazyl) radical scavenging assay

Evaluation of radical scavenging activity of PLE against DPPH was done with 0.1mmol/L of DPPH based on the method of Brand Williams [14]. The reaction was done with 1ml DPPH and 0.5 ml of LMF of various concentrations (12.5, 25, 50 and 100µg/ml) and test control was taken without sample. The reaction solution was finally adjusted to 3 ml by adding ethanol and incubated in dark for 20 minutes and then measured the absorbance at 517nm.

ABTS [2, 2' – azino – bis (3- ethylbenzothiazolin – 6 – sulfonic acid)] assay

This decolourization assay was done according to the method of Re [15]. The reaction solution was prepared by mixing 7mM ABTS and 2.45mM Pottassium persulfate (1:1) in distilled water. It was then incubated in dark at room temperature for 12–16 hours. The prepared ABTS solution was diluted with methanol in order to obtain an absorbance of 0.700 at 734nm. Test solution (100µl) was prepared with

different concentrations of LMF (12.5, 25, 50, 100, 200µg/ml) and was added to 900µl of ABTS solution and incubated at room temperature for 1 minute. The absorbance was measured at 734nm where methanol served as blank and ascorbic acid as standard.

Total antioxidant capacity (TAC) assay

Phosphomolybdenum method of Prieto *et al*. [16] was used to assess the total antioxidant activity of LMF. LMF solution (100µg/100µl) was mixed with 3ml of reagent solution containing 0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate and incubated at 95 ºC for 90 minutes. 100µl of solvent was added to the blank solution instead of LMF and absorbance was measured at 695nm. A standard curve was plotted using ascorbic acid and the equivalent antioxidant concentration of the sample was determined. The experiments were repeated three times and the final result was expressed in terms of ascorbic acid equivalents.

Statistical analysis

Anti-inflammatory and antioxidant assays were performed in triplicates and results were expressed in mean with standard deviation. The inhibitory percentages obtained with each concentration of each assay were statistically analyzed through one-way analysis of variance (ANOVA) followed by post-hoc Duncan test using IBM SPSS statistics 25. Regression equations and IC 50 values were obtained in Microsoft Office Excel 2013.

RESULTS AND DISCUSSION

HPTLC fingerprint profiling

LHF and LCF were not much prominent in the case of flavonoids, but LEF and LMF were presenting specific bands before and after derivatization. The chromatograms of LEF and LMF before and after derivatization, densitometric scanning image and three-dimensional representation were given in figures (1 & 2). The detailed report of Rf values and area percentage of LEF and LMF are shown in tables (I & II). LEF shows 11 bands under 254nm and 13 bands under 366nm and its derivatization with aluminium chloride displayed 13 bands under 366nm. TLC plates correspond to LMF produced 4 bands. Derivatized plates of both LMF and LEF denoted the presence of flavonoids in the samples with yellowish fluorescent bands. LMF gave a clearer picture and 4 consistent bands under all conditions. So this can be the purified fraction and identified as modified methanol fraction of direct methanol extract of leaves of *L. quinqueflora.* The yellowish fluorescence was noted between Rf 0.1 and 0.35 of LMF. Rf 0.18 and 0.27 were the maximum peak points of LMF with area percentages of 53.96 and 41.95 respectively. LEF gave yellowish fluorescence at Rf 0.17 with an area percentage of 35.63 and at 0.27 with 48.57% of the area.

Table I: HPTLC finger print data of LEF

Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Assigned substance
1	0.01	0.0	0.02	17.3	0.94	0.03	0.6	167.8	0.35	unknown*
2	0.04	0.8	0.07	16.9	0.91	0.08	5.4	397.4	0.82	unknown*
3	0.11	1.3	0.16	238.3	12.89	0.20	0.4	8687.0	17.99	unknown*
4	0.21	0.3	0.25	242.6	13.12	0.26	222.8	5050.6	10.46	unknown*
5	0.26	226.6	0.27	466.9	25.25	0.29	20.9	5504.6	11.40	unknown*
6	0.29	21.0	0.30	23.8	1.29	0.32	11.8	373.2	0.77	unknown*
7	0.32	11.8	0.3	23.9	1.29	0.37	0.1	517.2	1.07	unknown*
8	0.50	15.1	0.55	37.9	2.05	0.57	12.3	1397.9	2.89	unknown*
9	0.61	8.3	0.65	24.5	1.32	0.67	13.8	775.3	1.61	unknown*
10	0.72	18.9	0.75	38.1	2.06	0.75	36.8	738.8	1.53	unknown*
11	0.76	36.1	0.79	54.8	2.96	0.81	33.7	1743.0	3.61	unknown*
12	0.81	33.7	0.84	79.1	4.28	0.87	47.5	2583.2	5.35	unknown*
13	0.88	47.9	0.95	585.2	31.64	0.99	3.4	20355.3	42.15	unknown*

Table II: HPTLC finger print data of LMF

Peak	Start Rf	Start height	Max Rf	Max height	Max %	End Rf	End height	Area	Area %	Assigned substance
1	-0.02	107.8	-0.02	112.6	19.4	-0.01	2.7	661.5	3.19	unknown*
2	0.08	0.8	0.18	239.1	41.3	0.21	42.8	11179.7	53.96	unknown*
3	0.22	44.1	0.27	211.7	36.6	0.31	1.2	8691.5	41.95	unknown*
4	0.89	3.0	0.90	15.8	2.72	0.91	5.1	184.6	0.89	unknown*

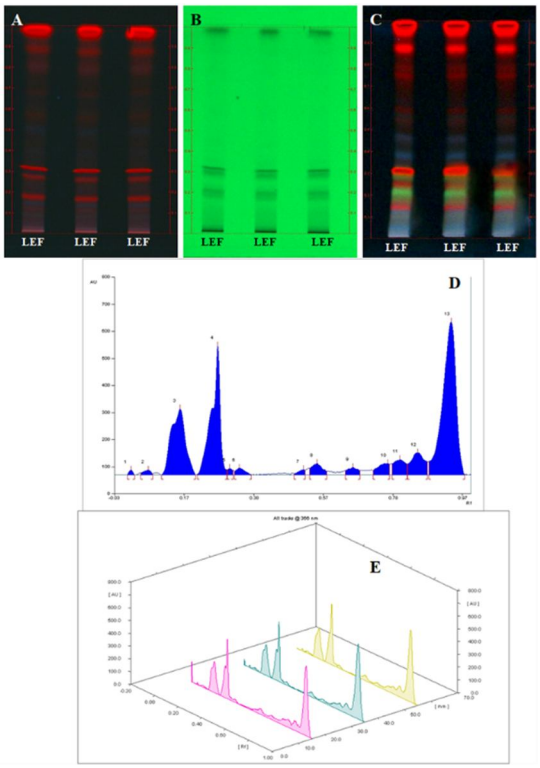

Figure 1: HPTLC profile of LEF (A) Before derivatization at UV 366 nm (B) Before derivatization at UV 254 nm (C) After derivatization at UV 366 nm (D) Peak densitogram of LEF (E) Three-dimensional representation of chromatogram.

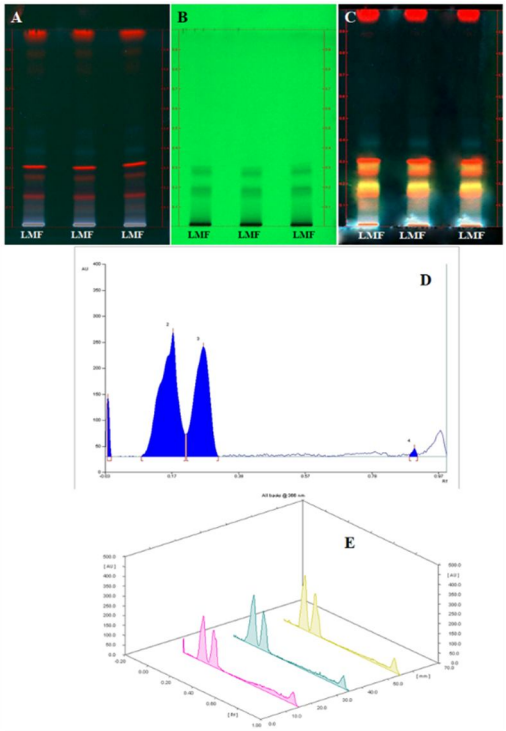

Figure 2: HPTLC profile of LMF (A) Before derivatization at UV 366 nm (B) Before derivatization at UV 254 nm (C) After derivatization at UV 366 nm (D) Peak densitogram of LMF (E) Three dimensional representation of chromatogram.

The specific bands of LEF and LMF obtained before and after derivatization led to an idea of clear cut presence of polyphenols especially the flavonoids in the samples. Among them, LMF gave four consistent bands and hence considered as more purified fraction. The HPTLC analysis of hexane, ethyl acetate and

ethanol extracts of heart wood and small branches of *L. chinensis* produced similar banding pattern in which ethanol extract showed clearer bands than others [17]. The HPTLC finger print profiling of flavonoids of stem extract of *Solena amplexicaulis* [18] leaf and root extracts of *Hypochaeris radicata* [19] fruit extracts of *Carissa bispinosa, Ficus sycomorus* and *Grewia bicolor* [20] used aluminium chloride as spraying reagent in derivatization of plates and reported the presence of flavonoids. The principle behind the use of aluminium chloride as derivatization agent lies in the capacity of the aluminium ion, Al (III) to react with carbonyl and hydroxyl groups of flavones and flavonols that resulted into the formation of a yellow complex [21].

***In vitro* anti-inflammatory screening:**

Fractions of methanol extract exhibited significant inhibition against protein denaturation. Inhibitory percentage was directly proportional to the increase in concentrations of each sample. The IC50 value of LHF, LCF, LEF and LMF were 1092.29µg/ml (y=0.045x+0.847; R^2=0.951), 749.88µg/ml (y=0.058x+6.507; R^2=0.998), 113.29µg/ml (y=0.076x+41.39; R^2=0.833) and 66µg/ml (y=0.078x+44.85; R^2=0.829) respectively and obtained inhibitory percentages were displayed in table III. LMF can be selected as best inhibitor against protein denaturation on the basis of IC50 values and inhibitory percentage.

Table III: Percentage of inhibition of leaf extracts of *L. quinqueflora* in protein denaturation assay

Concentration (µg/ml)	LHF	LCF	LEF	LMF	DS
62.5	2.01±0.52***	9.81±0.88***	38.78±0.68***	42.29±0.7***	39.18±1.54***
125	6.74±0.67***	13.78±0.78***	54.94±0.73***	58.25±0.46***	74.02±1.19***
250	15.07±0.94***	21.6±0.87***	67.26±0.84***	72.28±1.07***	83.48±1.35***
500	22.59±0.99***	35.32±0.9***	75.93±0.43***	80.37±1.06***	93.83±1.63***

Inflammation can be go with gradual denaturation of proteins especially in some inflammatory conditions such as arthritis, diabetes and cancer. Denaturation of proteins can be due to different stimuli such as heat, acid, alkali, detergents, urea etc. [22]. In the present study, high temperature was used as a stimulus. Denaturation of proteins changes them from native folding state to unfolding state and thus the breakage of secondary, tertiary and quaternary structure of proteins and ultimately cell death [23]. This leads to the disruption of proteins through breakage of attractive forces between monomers of proteins. Denatured protein can act as antigen and affect immunity of our system and leads to chronic inflammatory conditions like rheumatoid arthritis [24]. Hence, a drug with anti-denaturant activity can be considered as an alleviator in inflammatory conditions. Many plant extracts exhibit anti denaturant activity where polyphenols, especially flavonoids, are mainly considered as the anti-inflammatory agents [25]. Bovine serum albumin (BSA) with similar properties, structure and functionalities of human serum albumin (HAS) was selected as protein candidate and was subjected to heat induced denaturation at 57 ºC [26]. The anti-denaturant activity of methanol fractions of leaves of *L. quinqueflora* can be compared with that of different fractions of roots of *Ribes alpestre* [27]. LMF, LEF and LCF exhibited higher inhibitory percentage than that of methanol extract of ginger, a well- known anti-inflammatory agent [28]. On the basis of inhibitory percentages of samples at each concentration level, LMF as well as LEF observed with higher inhibitory potential. Among the fractions of LMeOH, LMF exhibited higher inhibitory potential on the basis of IC 50 value and is selected for further antioxidant studies. Even though LHF and LCF were identified with less activity, both exhibited significant results with considerable IC 50 values. The earlier report of inhibitory potential of ethyl acetate and methanol (LM) leaf extracts of *L. quinqueflora* [29] was less than that of LEF and LMF. Hence, LEF and LMF can be considered as more active fractions than that of extracts. Silver nano particles synthesized through the mediation of methanol extract of *L. quinqueflora* [13] also exhibited less inhibitory potential than that of LMF.

Antioxidant assays

DPPH (1, 1 diphenyl-2-picryl hydrazyl) radical scavenging assay

The IC 50 value or inhibitory concentration at 50% of LMF in DPPH assay was 69.25µg/ml. IC 50 value was measured from the straight line regression equation (y = 0.2985x + 29.328; R^2 = 0.9513). The inhibitory percentage was directly proportional to concentration of sample which was shown in table IV. Color of the reaction mixture was gradually changed from violet to yellow with increase in concentration of drug. The potential of LMF as reducing agent was compared against standard ascorbic acid.

ABTS [2, 2' – azino – bis (3- ethylbenzothiazolin – 6 – sulfonic acid)] assay

This cation reduction assay was highlighted with decolourization in which colour of the reaction mixture reduced from intense blue colour to colourless. The control solution retained blue colour and the test solutions showed reduction in blue colour from lower concentration to higher concentrations. The inhibitory percentage increased with increase in concentration of LMF and was confirmed by reading the

color density in the spectrophotometer at 734nm (Table IV). The IC 50 value 56.37µg/ml was calculated from linear graph regression equation (y = 0.3671x + 29.308, R^2=0.836).

Table IV: Antioxidant activity of LMF and AA

Concentration (µg/ml)	DPPH (% of Inhibition)		ABTS (% of Inhibition)	
	AA	LMF	AA	LMF
3	66.22±1.22		35.06±0.66	
6.25	78.82±0.84		79.45±0.57	
12.5	85.91±1.66	25.94±0.9***	88.87±0.54	18.17±0.74***
25	92.11±1.42	38.24±0.83***	93.16±0.59	40.94±1.2***
50		47.87±0.97***		53.99±0.8***
100		64.56±1.1***		81.72±0.9***
200		85.69±0.86***		93.97±1.68***

Values represent mean ± standard deviation of triplicate determination. ***p < 0.001

Total antioxidant capacity (TAC) assay

Phosphomolybdenum assay enabled to calculate the total antioxidant capacity and was observed from standard graph (Figure 3) plotted using ascorbic acid with different concentrations and ascorbic acid standard equation (y = 0.0193x - 0.0475; R^2 = 0.9992) was obtained. It was measured as ascorbic acid equivalent (AAE) and LMF has TAC of 0.139±0.47gAAE/g.

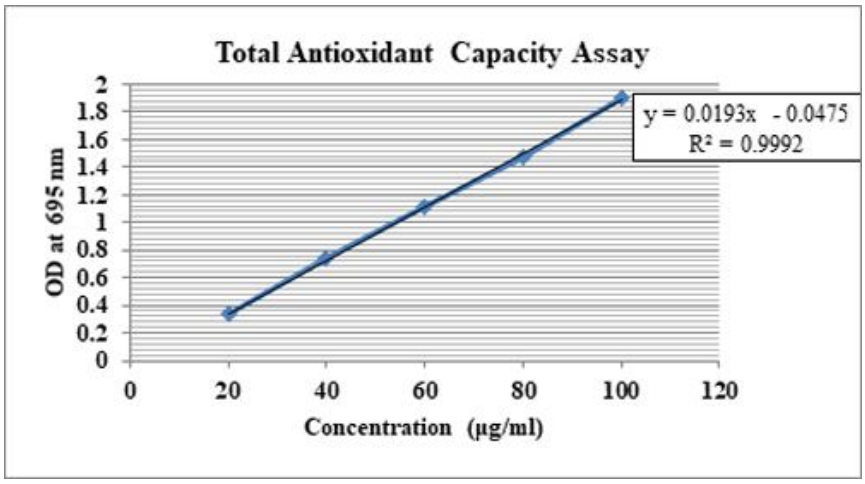

Figure 3: Standard graph of ascorbic acid obtained in total antioxidant assay of LMF

Many biomolecules especially phenolic compounds, were associated with antioxidant activity of herbal plants. This free radical scavenging mechanism can be involved with removal of free radicals, inhibition of enzymes related with free radical synthesis, enhance the level biological antioxidants etc. [30]. Free radical scavenging activity of LMF can be attributed to the flavonoids presented in the sample and it was observed in derivatized HPTLC plate. Radical scavenging activity against DPPH of LMF was higher than that of methanol extract of ginger [28] whereas solvent extracts of *Enteromorpha intestinalis* scavenged DPPH free radicals [31] more effectively than that of all the fractions of *L. quinqueflora*. On the basis of IC50 value and inhibitory percentage, root and leaf extracts of *Raphanus sativus* were reported with less free radical scavenging activity with DPPH and ABTS [32]. The study on leaf extracts of *Globularia alypum* [33] exhibited scavenging activity against DPPH and ABTS radicals in which ethyl acetate extract was reported with higher inhibitory activity and chloroform extract was with higher IC50 value than that of LMF. Inhibitory percentage of LMF increased with increase in concentration and can be considered as effective antioxidant to protect cells from damage caused by free radicals.

Phosphomolybdenum assay was a quantitative method which enabled to calculate the total antioxidant capacity and associated with the reduction of molybdate ions and diminishing of blue green colour. Total antioxidant capacity can be calculated in terms of different antioxidants such as ascorbic acid, carotenoids, α- tocopherol etc. The present investigation focused on ascorbic acid and calculated the TAC as ascorbic acid equivalent [16]. Total antioxidant capacity of LMF was quite high when compared with the solvent extract of seeds and pods of *Calycotome villosa* [34], root extracts of *Anchomanes difformis* [35] and almost similar to the root and bark extracts of *Vitex grandifolia* [36]. TAC of stem extracts of *Crotalaria pallida*

expressed as AAE [37] and showed higher concentrations than that of LMF. Hence on the basis of DPPH, ABTS and TAC assays, LMF can be considered as a natural antioxidant.

CONCLUSION

HPTLC finger print profiling of LEF and LMF fractions of direct methanol extract clearly indicated the presence of flavonoids. The anti-inflammatory activity of hexane, chloroform, ethyl acetate and methanol fractions were calculated through inhibition of protein denaturation assay. Ethyl acetate and methanol fractions showed significant activity with IC50 values of 113.29µg/ml and 66µg/ml respectively. Antioxidant studies using methanol fraction clearly indicated its free radical scavenging activity with IC50 values 69.25µg/ml (DPPH) and 56.37µg/ml (ABTS) and total antioxidant capacity of 0.139±0.47 GAE/g. The role of *L. quinqueflora* as a folklore medicine in inflammatory conditions can be credited to the antioxidant and anti-inflammatory property of the methanolic fractions of *L.quiqueflora*.

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COMPETING INTERESTS

The authors report no competing interest.

AUTHORS' CONTRIBUTIONS

Authors designed the experiment. First author carried out the work. Both authors read and approved the final manuscript.

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