Annexure

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COMPARITIVE PHARMACOGNOSTICAL, PHYTOCHEMICAL AND ANTIOXIDANT PROPERTIES OF THREE DIFFERENT *EUPATORIUM* SPECIES

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ABSTRACT

The leaves of three different Eupatorium species such as Eupatorium glandulosum, Eupatorium odoratum and Eupatorium triplinerve belongs to the family Asteraceae, were screened in order to study the pharmacognostical, phytochemical and antioxidant property. The Physicochemical parameters such as Loss on drying, total ash, Acid insoluble ash, Water soluble ash and percentage of solubility was calculated. The leaf powders of different species were extracted with different solvents like petroleum ether, benzene, chloroform, acetone, methanol and water and the results revealed that polar solvents showed higher extractive value than non-polar solvents. The results of qualitative phytochemical analysis confirmed the presence of alkaloids, anthraquinone glycosides, phenols, glycosides, protein, amino acids, flavonoids, fats, fixed oils and saponins in the ethanol and water extracts. The leaves were also screened for the antioxidant property using standard ascorbic acid as a control and found that they possess good antioxidant property. Thus, the preliminary studies of the three-plant species could help us to understand the medicinal properties of the plant and also help us to construct monograph of the plant.

KEY WORDS

Pharmacological, phytochemical, antioxidant, Eupatorium glandulosum, Eupatorium odoratum, Eupatorium triplinerve

I. INTRODUCTION

Plants are an essential source of medicines and play a key role in world health. The contribution of medicinal plants is important to the global economy as approximately 85% of traditional medicine preparations involve the use of plants or plant extracts [1]. Natural products derived from plants are the essential sources of reliable bioactive compounds such as secondary metabolites and antioxidants. They are concentrated at different parts of plant such as leaves, flower, stem, bark, fruit, roots and seeds. Many plants contain several secondary metabolites have very important applications in the fields of agriculture, human health and veterinary medicine [2].

Standardization of herbal drugs is an essential factor in order to assess the quality, purity, safety and efficacy of drugs. Development of standards for plant-based drugs being a challenging task, it needs innovative and creative approaches. The quality of plant will be analysed by different parameters such as identification, organoleptic, pharmacognostic, physiochemical and phytochemical properties [3].

E. glandulosum belongs to the family Asteraceae, is a native of Mexico, introduced as an ornamental shrub in several countries. In India, the tribes of Nilgiris use the leaves of the plant to heal wounds and small injuries [4]. In folklore medicine it is used as an astringent, thermogenic and stimulant [5].

The plant *E. odoratum* is a fast-growing perennial shrub, native of Central and South America has spread in tropical and subtropical regions of the world. The tribes of Indonesia used the leaf extract to cure skin diseases, poison bites, wounds, burns, cough, diabetes, diarrhea, fever, inflammation and rheumatism. The boiled roots are used to cure urinary disorders [6, 7, 8].

E triplinerve is a tropical American shrub commonly called as Ayapana is an ornamental erect perennial herb having aromatic leaves. In tribal medicine, it is used to cure fever with convulsions, pneumonia, indigestion, and cough [9].

Thus, to consider the importance these plants, the present study is aimed to investigate the comparative account of physicochemical, phytochemical and antioxidant properties of three different species of *Eupatorium*.

II. MATERIALS AND METHODS

Collection of plant material

The leaves of *E. glandulosum, E. odoratum* and *E. triplinerve* were collected from Nilgiri Hills of Western Ghats, Coimbatore plain and Kanjikode in Kerela respectively and certified by Botanical survey of India in Coimbatore, Tamil Nadu. The collected leaves were washed thoroughly, dried, powdered and stored in air tight container for further study.

Extraction of plant material

The leaf powder of selected plant materials were extracted with different solvents like Petroleum ether, acetone, benzene, chloroform, ethanol and water.

Physicochemical parameters [10, 11]

The plant powder was subjected to calculate total ash, acid insoluble ash and water-soluble ash, loss on drying, solubility percentage in alcohol and water and extractive values.

Loss on drying

Freshly collected and pre-weighed samples were dried in Hot air oven at 45°C until it reaches a constant weight.

Total ash

3gm of leaf powder was taken in silica crucible and ignited in an electric muffle furnace at 100°C until the sample free from carbon. The percentage of total ash was calculated with reference to the air-dried sample.

$$\text{Percentage of ash value} = \frac{\text{Weight of fresh sample} - \text{Weight of dried sample}}{\text{Initial weight of the sample}} \times 100$$

Acid insoluble ash

Total ash obtained was heated with 25ml of diluted hydrochloric acid for 10 minutes, filtered in ash less filter paper (Whatman No.1) and the residue was incinerated in the furnace to get a constant weight. The weight of the insoluble matter was subtracted from the weight of total ash, represents the acid insoluble ash. The percentage of acid insoluble ash was calculated with reference to the air-dried drug.

Water soluble ash

The total ash obtained above was boiled with 25 ml of distilled water for 5 minutes. The insoluble matter was collected on an ash less filter paper, washed with hot water and dried to get constant weight at low temperature. The weight of the insoluble matter was subtracted from the weight of total ash, represents the water-soluble ash. The percentage of water-soluble ash was calculated with reference to the air-dried drug.

Solubility percentage

Alcohol

1gm of powdered material was mixed with 20ml ethyl alcohol and shaken frequently for 6 hours and kept undisturbed overnight. The extract was concentrated and the solubility percentage was calculated on dry weight basis.

Water

The procedure adopted for solubility percentage of alcohol, is used to calculate the solubility percentage of water.

Extractive values

The powdered materials were extracted with different solvents like petroleum ether, benzene, chloroform, acetone, methanol and water in a soxhlet apparatus. The extracts were concentrated and the extractive values were calculated on dry weight basis.

Fluorescent analysis

Powdered plant materials were treated with different solvents and their illuminations were observed under ordinary and Ultra-violet light conditions [12].

Qualitative phytochemical analysis

Qualitative phytochemical analysis was done by using the procedure of Kokate [13]. Presence of alkaloids, flavonoids, glycosides, tannins, phenols, fixed oils, fats and saponins were analysed qualitatively.

Alkaloids (Mayer's Test)

Plant extract was treated with Mayer's reagent and formation of yellow colour precipitate indicates the presence of alkaloids.

Glycosides (Fehling's test)

Fehling reagent mixed with plant extract and gave red colour precipitate indicates the presence of glycosides.

Phenols and Tannins (Ferric Chloride Test)

Plant extracts were treated with 3-4 drops of 5% ferric chloride solution. Formation of bluish black colour indicates the presence of phenols.

Flavonoids (Alkaline Reagent Test)

Few drops of sodium hydroxide solution were added to the plant extract. Initially Formation of intense yellow colour, which becomes colourless on addition of dilute acid which indicates the presence of flavonoids.

Proteins and Amino acids (Ninhydrin test)

Ninhydrin reagent was added to the plant extract and boiled for few minutes. Formation of blue colour indicates the presence of proteins.

Anthroquinone Glycosides

To the plant extract, 5% potassium hydroxides solution was added. Appearance of red color indicates the presence of anthroquinones.

Fats and fixed oils

The extracts are treated with 0.5N alcoholic potassium hydroxide along with a drop of phenolphthalein and then heated on water bath for few minutes. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

Phytosterols (Salkowski's Test)

The plant extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Con. Sulphuric acid, shaken and allowed to stand for few minutes. Appearance of golden yellow colour indicates the presence of phytosterols.

Saponins

1ml of the plant extract diluted with distilled water and made up to 20 ml and shaken in a test tube for 15 minutes. Formation of foamy layer indicates the presence of saponins.

Antioxidant activity

Preparation of standard solution

The standard solution was prepared by dissolving 1mg of Ascorbic acid in 1ml of methanol to obtain various concentrations such as 20, 40, 60, 80 and 100 µg/ml.

Preparation of test sample

The ethanol and water extracts were dried and about 10mg of dried extract were dissolved in 10ml of methanol to give concentration of mg/ml.

DPPH Free Radical Scavenging Assay [14]

A solution of the radical is prepared by dissolving 2.4 mg DPPH in 100 ml methanol. A test solution of various concentrations such as 20, 40, 60, 80 and 100µl were added to 3.98, 3.96, 3.94, 3.92 and 3 ml of DPPH respectively. The mixture was shaken vigorously and kept at room temperature for 20 min in the dark. All the determinations were performed in triplicate. The DPPH reagent itself served as control. After 20 minutes the absorbance was measured at 515nm in spectrophotometer. The scavenging percentage of the extract was calculated using the following formula:

$$\% \text{ of scavenging} = \frac{\text{OD of control} - \text{OD of test}}{\text{OD of control}} \times 100$$

Hydrogen peroxide scavenging activity [15]

The leaf powder extracts (4ml) were prepared using distilled water at various concentrations. To the plant extract, 0.6 ml of 4mM Hydrogen peroxide solution which is prepared in 0.1M phosphate buffer (pH-7.4) was added. The mixture was incubated for 10 minutes and absorbance was read at 230 nm in UV visible spectrophotometer. Ascorbic acid (control) was used as standard reference.

Hydrogen peroxide scavenging activity of powder extracts were calculated using the following formula,

$$\% \text{ scavenged } (H_2O_2) = \frac{(A_{control} - A_{sample})}{(A_{control})} \times 100$$

Where,

$A_{control}$ - Absorbance of standard reference (Ascorbic acid)

A_{sample} - Absorbance of the powder extract.

III. RESULTS

Physicochemical analysis

The leaf powder of three different *Eupatorium* species were screened for analytical values like moisture content, total ash, acid insoluble ash, water soluble ash and solubility percentage of ash in alcohol and water. The results observed that moisture content of *E. odoratum* was 19% and in *E. glandulosum* 24 %. The value of total ash and acid insoluble ash in *E. glandulosum* was found to be 2.1% and 0.4% respectively. The percentage of water-soluble ash content was almost equal in all the three samples. Among the three plants, the leaf powder of *E. odoratum* showed maximum solubility percentage in both water (24%) and ethanol (25%) (Table 1). Comparing the results of two solvents, plant powder of selected three plants extracted with ethanol showed maximum solubility percentage than water.

Extractive Value

The leaf powders of three plants were subjected to successive solvent extraction using different solvents in the soxhlet apparatus. The extractive values were observed to be better in polar solvents (water and ethanol) than non-polar solvents (Table 2). The extractive value was found to be maximum in ethanol (3.99%) and water (3.31%) extract of *E. odoratum* compared to other two plants.

Fluorescence analysis

The fluorescence behaviour of the powdered plant materials were studied by treating them with various solvents and observed under normal and UV light. No significant variations were observed among the three plants except *E. odoratum*. It revealed characteristic variation when it treated with ethanol (Table 3).

Phytochemical analysis

The preliminary screening of leaf extracts of all the three plants showed maximum phytoconstituents in ethanol and water extract (Table 4). The extract of *E. glandulosum* showed the presence of all the phytoconstituents tested except fats and fixed oils. Whereas in *E. odoratum,* phytosterols was absent in ethanol and water extract (Table 5) and in *E. triplinerve*, anthroquinone glycosides, fats, fixed oils and phytosterols were absent (Table 6).

Antioxidant activity

Antioxidant studies were carried out to find out the antioxidant's properties of the selected plant materials by hydrogen peroxide scavenging method and DPPH method. Methanol and water extracts were used for the study. In DPPH method, the antioxidant activity was higher in all the extracts tested compared to the Hydrogen peroxide scavenging activity. The antioxidant activity of standard (ascorbic acid) increased with increasing concentration. In DPPH method, the ethanolic extract of *E. odoratum* and *E. glandulosum* exhibited higher antioxidant activity of about 81.35% and 75.87% respectively. Whereas *E. triplinerve* showed minimum antioxidant acitivity compared to other two plants. The scavenging percentage of plant extracts were increase with increasing concentration. (Figure 1 & 2).

IV. DISCUSSION

The leaves of three different *Eupatorium* species were analysed to identify their physicochemical, phytochemical and antioxidant property.

The physicochemical parameters help us to check the quality, standard and adulterants in the plant powder. Moisture is one of the major factors responsible for the deterioration of the drugs and affecting their shelf life [16]. In the present investigation, the moisture content of the leaves of all the three plants was found to be very low. Determination of ash value provides criteria for judging the purity of the drug [17]. A high ash value is an indicator of contamination, substitution, adulteration or carelessness in drug preparation or drug formulation for marketing. In the present investigation, ash value was found to be very low in all the selected plants. The acid insoluble ash content was ranging between 0.3-0.4% in all the three plants which was found to be very low when comparing to the earlier report available in the leaves of *Sesbania grandiflora* and *Wedelia trilobata* [18, 19]. The remaining ash content showed the existence of inorganic components in the plant sample which shows the purity of the samples.

Extractive value plays an important role in evaluation of crude drugs. Less extractive value indicates addition of exhausted material, adulteration or incorrect processing during drying or storage or formulation [20]. In the present study, the extractive values were observed to be better in polar solvents than non polar solvents. The ethanol and water extracts showed highest percentage of extractive value in Ethanol extract

of *E. odoratum* (3.99%) and *E. glandulosum* (3.21%) indicated the quality of the drugs.

The fluorescence analysis is a sensitive and enables the precise and accurate determination of drug. The fluorescence colour is specific for each compound. A non-fluorescent compound may fluoresce if mixed with impurities which are fluorescent. The colour of the plant powder treated with different organic and inorganic solvents was observed under ordinary and UV light and found that there was no fluorescent compounds and not much colour variation among the species were observed.

The phytochemical screening of the three selected plants confirmed the existence of alkaloids, glycosides, flavonoids, saponins, tannins and phenols were observed in ethanol and water extract of all the three leaves. This result resembles the earlier reports on the leaves of *E. glandulosum* [21, 22], *E. odoratum* [23, 24, 25, 26] and *E.triplinerve* [27].

Antioxidant protects the body against the damaging effects of free radicals produced naturally within the body. These free radicals' production could cause damage to proteins, DNA and the genetic material within the cells [28]. In the present study, ethanol and water extracts of all the three plants showed good antioxidant activity. Among the three plants ethanol extracts of *E. odoratum* (81.35%) and *E. glandulosum* (75.87%) revealed maximum antioxidant activity tested by DPPH method. This is due to the presence of flavonoids in plants and act as antioxidants. According to earlier report, the antioxidant activity of flavonoids has the ability to reduce free radical formation and scavenge the free radicals [29, 30]. In the present study, all the plant extracts showed the presence of flavonoids which are the reason behind the better antioxidant property. This finding resembles the earlier report available in *E. odoratum* [31, 32].

V. CONCLUSION

From the above findings, it is concluded that *E. glandulosum* , *E. odoratum* and *E.triplinerve* were subjected to various analysis such as physicochemical, phytochemical and antioxidant properties and found that there is no distinct variation among *E. glandulosum* and *E. odoratum* in physicochemical and phytochemical properties. But in *E.triplinerve,* it showed less antioxidant properties and lack of fats, fixed oils and phytosterols.

Table 1 Physicochemical analysis

S. No	Parameter studied	Value expressed in % (W/W)		
		E. glandulosum	***E. odoratum***	***E. triplinerve***
1	Moisture content	24	19	25
2	Total ash	2.1	1.9	1.5
3	Acid insoluble ash	0.4	0.3	0.3
4	Water insoluble ash	0.2	0.2	0.3
5	Solubility %			
	1) Water	20	24	18
	2) Alcohol	23	25	21

Table 2 Extractive values

S.No	Solvent	Yield (%)		
		E. glandulosum	***E. odoratum***	***E. triplinerve***
1	Petroleum ether	0.91	0.89	0.77
2	Benzene	1.02	1.19	0.93
3	Chloroform	1.67	1.56	1.19
4	Acetone	2.13	2.29	1.87
5	Ethanol	3.21	3.99	2.25
6	Water	3.12	3.31	2.42

Table 3 Fluorescence analysis

S. No	Solvents	*E. glandulosum*		*E. odoratum*		*E. triplinerve*	
		Normal light	UV light	Normal light	UV light	Normal light	UV light
1	Powder as such	Green	Dark green	Green	Dark green	Green	Dark green
2	Con.H_2SO_4	Dark green	Dark green	Green	Dark green	Green	Dark green
3	Con.HCl	Green	Blackish green	Pale green	Blackish green	Pale green	Dark green
4	Con.HNO_3	Dark green	Fluroscent green	Light green	Dark green	Pale green	Dark green
5	1N NaOH	Green	Dark green	Green	Dark green	Green	Dark green
6	Ethanol	Light green	Blackish green	Green	Reddish brown	Dark green	Blackish green
7	Water	Dark green	Reddish green	Green	Pale green	Dark green	Blackish green

Table 4 Qualitative phytochemical studies on *Eupatorium glandulosum*

S.No	Constituents	Petroleum Ether	Benzene	Chloroform	Acetone	Ethanol	Water
1	Alkaloids	+	+	+	+	+	+
2	Glycosides	-	+	-	+	+	+
3	Phenols & tannins	+	+	+	+	+	+
4	Flavonoids	-	-	-	+	+	+
5	Protein & amino acids	+	+	-	+	+	+
6	Anthroquinone Glycosides	-	+	-	+	+	+
7	Fats & fixed oils	-	-	-	-	-	-
8	Phytosterols	-	-	-	+	+	+
9	Saponins	-	-	-	-	+	+

+=Presence; -=Absence

Table 5 Qualitative phytochemical studies on *Eupatorium odoratum*

S.No	Constituents	Petroleum Ether	Benzene	Chloroform	Acetone	Ethanol	Water
1	Alkaloids	+	+	+	+	+	+
2	Glycosides	-	-	+	+	+	+
3	Phenols & tannins	+	+	+	+	+	+
4	Flavonoids	+	+	+	+	+	+
5	Protein & amino acids	-	-	-	-	+	+
6	Anthroquinone Glycosides	-	-	-	-	+	+
7	Fats & fixed oils	-	-	+	+	+	-
8	Phytosterols	-	-	-	-	-	-
9	Saponins	-	-	-	-	+	+

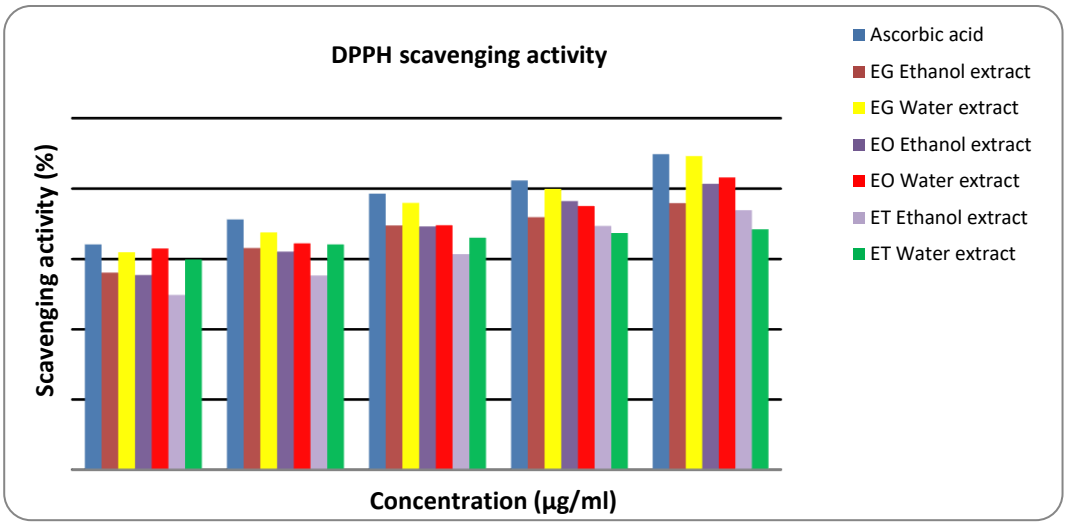
+=Presence; -=Absence

Table 6 Qualitative phytochemical studies on *Eupatorium triplinerve*

S.No	Constituents	Petroleum Ether	Benzene	Chloroform	Acetone	Ethanol	Water
1	Alkaloids	-	-	+	+	+	+
2	Glycosides	-	-	-	+	+	+
3	Phenols & tannins	-	+	+	+	+	+
4	Flavonoids	-	-	-	+	+	+
5	Protein & amino acids	-	-	+	+	+	+
6	Anthroquinone Glycosides	-	-	-	-	-	-
7	Fats & fixed oils	+	+	-	-	-	-
8	Phytosterols	-	-	-	-	-	-
9	Saponins	-	-	-	+	+	+

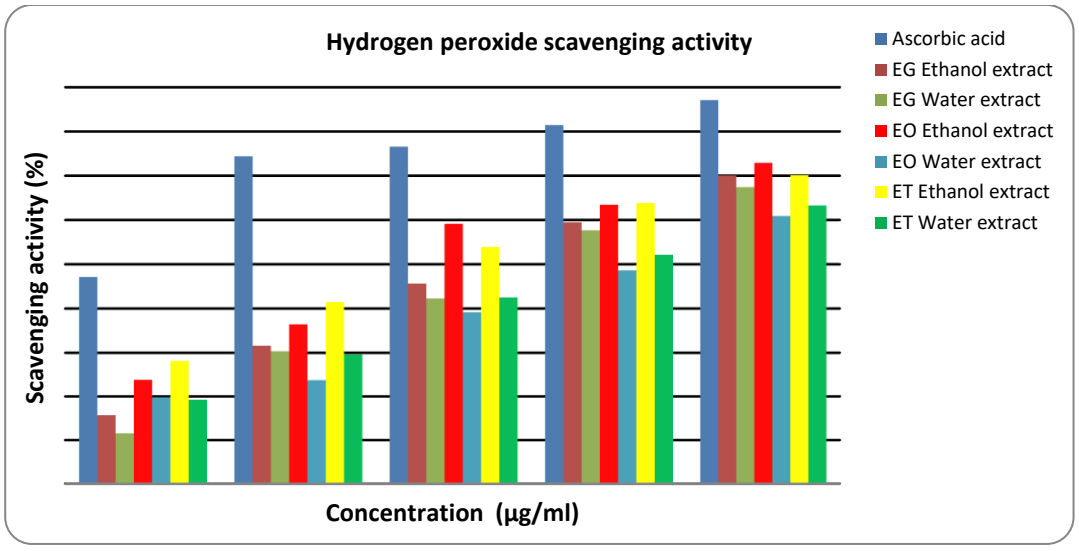
+=Presence; -=Absence

Figure 1: Antioxidant activity using DPPH method



EG-*E. glandulosum*, EO- *E. odoratum*, ET-*E. triplinerve*

Figure 2: Antioxidant activity using Hydrogen peroxide scavenging method



EG-*E. glandulosum*, EO- *E. odoratum*, ET-*E. triplinerve*

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GC-MS analysis of leaf and callus extracts of *Eupatorium glandulosum*

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ABSTRACT

Eupatorium glandulosum (Family: Asteraceae), an important plant curing various diseases was collected from the wild and the *in vitro* grown callus were analysed for phytoconstituents using Gas Chromatography Mass Spectroscopy (GC-MS). In the study, 20 compounds were identified in the leaf extract and 16 compounds in the callus extract. Six unique compounds were identified in the callus extract which were not present in the parent plant. Thus the technique confirmed the existence of new compounds in callus which may be useful for mass production in pharmaceutical industry (**Keywords:** *Eupatorium glandulosum,* GC-MS, callus extract).

INTRODUCTION

The genus *Eupatorium* comprises of about 1200 species distributed in the tropical countries like America, Europe, Africa and Asia. *E. glandulosum* is a native of Mexico and introduced into several parts of the world as an ornamental plant growing in higher elevations (hilly regions). It is used in the treatment of stomach aches and to prevent bleeding. In India, the leaves of the plant are used for curing wounds, ulcer, jaundice, gum and tooth infection by the tribes of Nilgiris (Desingh *et al*., 2014). It is also reported to have antimicrobial, antioxidant, anti-inflammatory, antipyretic, analgesic, therapeutic and anticoccidal properties (Vasanthi and Gopalakrishnan, 2013; Desingh *et al*., 2014; Harish Kumar *et al.,* 2014).

The secondary metabolites such as alkaloids, steroids, flavonoids, tannins, saponins, terpenoids, anthroquinone glycosides and phenolic compounds are also present in the plant (Iyeswarya *et al.*, 2013; Zhang *et al.*, 2013; Rajeswary and Govindarajan, 2013). There are also reports on secondary metabolites in higher concentrations in *in vitro* grown materials than the mother plant. A wide variety of various secondary metabolites are being produced. There is an increased demand for natural products in the current market (Karuppusamy, 2009). Therefore, considering the importance of secondary metabolites, the present study was carried out to compare the phytoconstituents present in the leaves and callus of *E. glandulosum* using GC-MS analysis.

MATERIALS AND METHODS

Preparation of plant extract

The leaves of *E. glandulosum* were collected from the Nilgiri Hills of the Western Ghats and certified by Botanical Survey of India (BSI), Coimbatore (Vocher No: BSIR/RC/5/23/2017/Tech/338). The leaves were then shade dried, powdered and extracted using ethanol in soxhlet apparatus for 8h. The extract was concentrated and dissolved in methanol and filtered using filter paper.

Preparation of callus extract

The fresh leaves of *E. glandulosum* were inoculated in MS medium containing various concentrations of auxin and cytokinin for callus induction. The four week old friable callus was collected, dried in oven at 50°C, powdered and extracted with ethanol for 8 h in a soxhlet apparatus. The extract was filtered using Whatmann No.41 filter paper and used for the study.

Gas Chromatography-Mass Spectrometry analysis (GC-MS)

The GC-MS study was carried out in SITRA (South India Textile Research Association), Coimbatore. The test was performed in ethanol extract of leaves of *E. glandulosum* and the callus obtained from leaf explants using Thermo GC Trace Ultra Version 5.0 equipment with a run time of 35:32 min and the Mass Spectrometry (MS) was achieved using Thermo MS DSQ II equipment. Auto sampler and gas

chromatograph interfaced to a mass spectrometer (GC-MS) instrument with the following conditions was used: column TR 5-MS capillary standard non-polar column (30 Mts, ID : 0.25mM, FILM : 0.25 µm); Helium carrier gas at a constant flow of 1mL min^{-1} and an injection volume of 1µL was employed (split ratio of 10:1) under injector temperature 250ºC; ion-source temperature 280ºC. Mass spectra were analysed using electron impact ionization at 80 eV and scanned for every 0.5 seconds fragments from 40 to 550 Da. The compounds were identified by their retention times and mass fragmentation patterns using data of standards at NIST (National Institute of Standard Technology) library.

RESULTS AND DISCUSSION

The nature of compound present in a plant determines its medicinal value which can be assessed by the phytochemical screening of the compound. In the present study, GC-MS analysis revealed the presence of new compounds from the GC fractions of the leaf and callus extract of *E. glandulosum* (Figures 1 and 2).

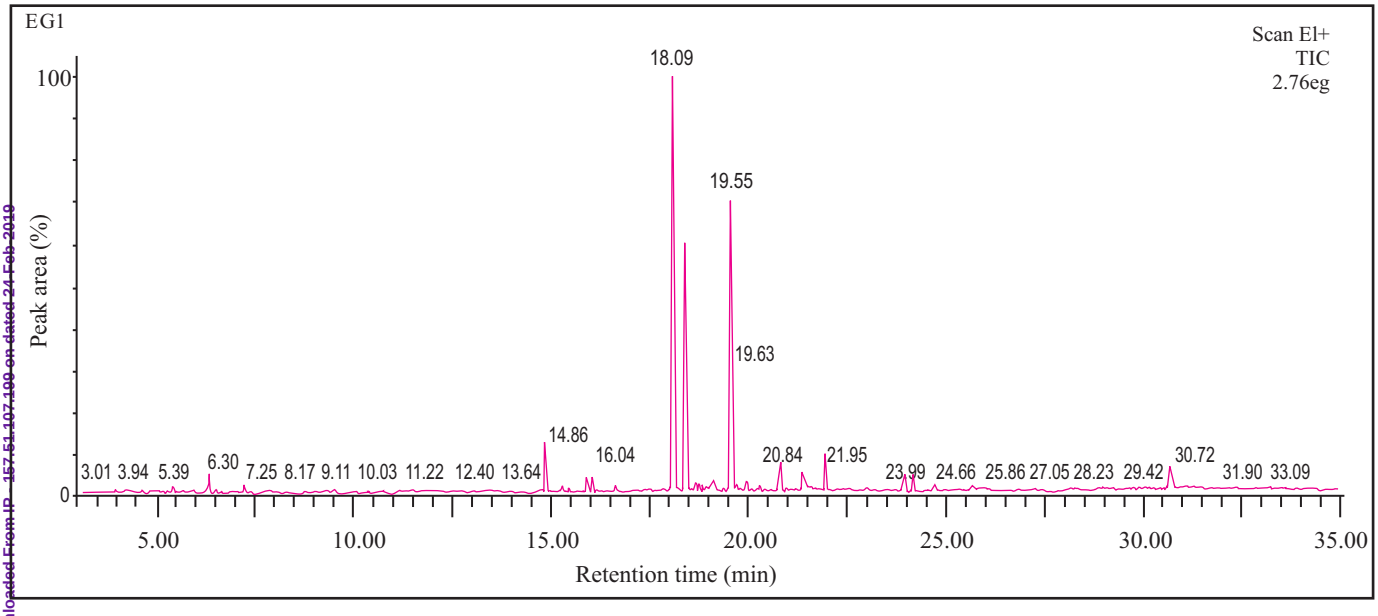

Figure 1. GC-MS chromatogram of leaf extract of *E. glandulosum*

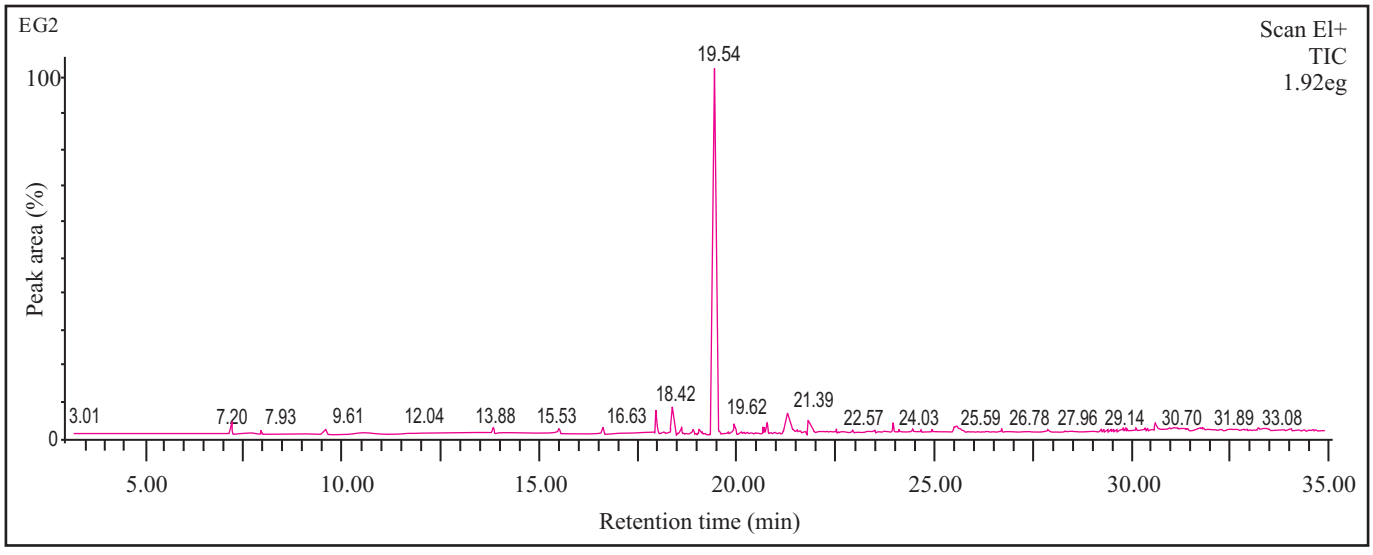

Figure 2. GC-MS chromatogram of callus extract of *E. glandulosum*

GC-MS analysis revealed the presence of 20 compounds in leaf extract and 16 compounds in callus extract of *E. glandulosum* (Tables 1 and 2), of which, ten compounds were present in both the extracts. These compounds were obtained at same retention time in leaf and callus extract (1,2,3,4,6,7,8,8a-Octahydronaphthalene-6,7-diol, 5,8a-dimethyl-3-isopropenyl-, cyclic carbonate, trans; 2(3H)-Naphthalenone, 4,4a,5,6,7,8-hexahydro-4a,7,7-trimethyl; 1,4,4,7a- Tetramethyl-2,4,5,6,7,7a-hexahydro-1Hindene-1,7-diol; 1-Naphthalenemethanol,1,4,4a,5,6,7,8,8aoctahydro-2,5,5,8a-tetramethyl; á-Cyclocostunolide; Bicyclo(5.1.0)octan-2-one, 4,6-diisopropylidene-8,8-dimethyl; Benzofuran, 7-cyclohexyl-2,3-dihydro-2-methyl; 2(1H)Naphthalenone,3,5,6,7,8,8a-hexahydro-4,8adimethyl-6-(1-methylethenyl); Di-isooctyl phthalate; Hexa-t-butylselenatrisiletane). In leaf extract, 2(3H)-Naphthalenone, 4,4a,5,6,7,8-hexahydro-4a,7,7-trimethyl compound showed the highest peak area percentage (22.431) at the retention time of 18.09. Whereas, in callus extract, Bicyclo(5.1.0) octan-2-one, 4,6-diisopropylidene-8, 8-dimethyl- obtained at Rf 19.54 showed the highest peak area percentage (32.261).

Some compounds of callus extract showed the highest peak area percentage than the leaf extract. For example, the peak area percentage of Bicyclo (5.1.0) octan-2-one, 4,6-diisopropylidene-8,8-dimethyl was 14.420 and 32.261 in leaf and callus extract, respectively, which reflected the higher production of secondary metabolites in the callus than the leaf extract. The callus also produced new compounds such as 1H-Indene, 1-methylene, Cyclopropanebutanoic acid, 2-[[2- [[2- [(2- pentyl cyclopropyl) methyl] cyclopropyl] methyl] cyclo propyl]methyl]-, methyl ester, (E)-5-Octadecene, 8-Pentadecanone and Squalene, which were not found in the mother plant.

Table 1. Phytoconstituents identified in ethanolic extract of leaves of *E. glandulosum* by GC-MS

S. No.	Compound name	Retention time (min)	Peak area (%)
1	Pyrrolidine, 2-(methoxymethyl)-	6.30	0.641
2	1H-Cycloprop[e]azulen-7-ol, decahydro-1,1,7- trimethyl-4-methylene-, [1ar-1aà,4aà,7á,7aá,7bà)]-	13.64	0.335
3	1-Naphthalenol, 1,2,3,4,4a,7,8,8a-octahydro-1,6-dimethyl-4-(1-methylethyl)-	14.86	2.432
4	2H-Cyclopropa[a]naphthalen-2-one, 1,1a,4,5,6,7, 7a,7b-octahydro-1,1,7,7a-tetramethyl-,(1aà,7à,7aà,7bà)-	16.04	0.669
5	1,2,3,4,6,7,8,8a-Octahydronaphthalene-6,7-diol, 5, 8a-dimethyl-3-isopropenyl-, cyclic carbonate, trans-	16.63	0.411
6	2(3H)-Naphthalenone, 4,4a,5,6,7,8-hexahydro-4a,7,7-trimethyl	18.09	22.431
7	1,4,4,7a-Tetramethyl-2,4,5,6,7,7a-hexahydro-1Hindene-1,7-diol	18.43	13.578
8	1-Naphthalenemethanol, 1,4,4a,5,6,7,8,8aoctahydro- 2,5,5,8a-tetramethyl	18.68	0.335
9	á-Cyclocostunolide	19.10	0.808
10	Bicyclo(5.1.0)octan-2-one, 4,6-diisopropylidene-8,8-dimethyl	19.55	14.420
11	1-Propyl-3-(propen-1-yl)adamantine	19.63	4.164
12	Benzofuran, 7-cyclohexyl-2,3-dihydro-2-methyl-	19.99	0.739
13	Hexadecanoic acid	20.74	0.667
14	2(1H)Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8adimethyl-6-(1-methylethenyl)-	20.84	1.383
15	2aS,3aR,5aS,9bR)-2a,5a,9-Trimethyl-2a,4,5,5a,6,7,8,9b-octahydro-2H-naphtho[1,2-b]oxireno[2,3-c]furan	21.95	1.893
16	9,12,15-Octadecatrienoic acid, methyl ester	23.99	0.923
17	Phytol	24.66	1.013
18	trans-Farnesol	25.86	0.604
19	Di-isooctyl phthalate	30.72	1.807
20	Hexa-t-butylselenatrisiletane	31.90	0.274

Table 2. Phytoconstituents identified in ethanolic extract of callus of *E. glandulosum* by GC-MS

S. No.	Compound name	Retention time (min)	Peak area (%)
1	1H-Indene, 1-methylene-	7.20	0.840
2	Cyclopropanebutanoic acid, 2-[[2-[[2-[(2- pentylcyclopropyl)methyl]cyclopropyl]methyl]cyclo propyl]methyl]-, methyl ester	12.04	0.372
3	(E)-5-Octadecene	13.88	0.331
4	8-Pentadecanone	15.53	0.440
5	1,2,3,4,6,7,8,8a-Octahydronaphthalene-6,7-diol, 5, 8a-dimethyl-3-isopropenyl-, cyclic carbonate, trans-	16.63	0.529
6	2(3H)-Naphthalenone, 4,4a,5,6,7,8-hexahydro-4a,7,7-trimethyl	18.08	2.127
7	1,4,4,7a-Tetramethyl-2,4,5,6,7,7a-hexahydro-1Hindene- 1,7-diol	18.42	2.617
8	1-Naphthalenemethanol, 1,4,4a,5,6,7,8,8aoctahydro-2,5,5,8a-tetramethyl-	18.68	0.479
9	á-Cyclocostunolide	19.10	0.377
10	Benzofuran, 7-cyclohexyl-2,3-dihydro-2-methyl	19.98	0.878
11	Bicyclo(5.1.0) octan-2-one, 4,6-diisopropylidene-8,8-dimethyl-	19.54	32.261
12	2(1H)Naphthalenone, 3,5,6,7,8,8a-hexahydro-4,8adimethyl- 6-(1-methylethenyl)-	19.62	4.194
13	n-Hexadecanoic acid	21.39	3.713
14	Squalene	25.59	2.156
15	Di-isooctyl phthalate	30.70	1.854
16	Hexa-t-butylselenatrisiletane	31.89	0.329

According to earlier reports, Cadinane sesquiterpenes, monoterpenes, diterpenes, coumarins, steroids, propanol, coumaric acid, phenyl proponoids and quercetagetin were found in the leaves of *E. glandulosum* (Mukherjee *et al.,* 2001; Samuel *et al.,* 2013). However, in the present study many new compounds have also been identified in both leaf and callus extract. The identified compounds in the leaf and callus extract have been reported to possess various biological properties. The compound 2(3H)-Naphthalenone4,4a,5,6,7,8-hexahydro-4a-methyl was reported in aerial parts of *Cordia retusa* and it demonstrated anti-inflammatory activity (Amudha and Rani, 2014). n-Hexadecanoic acid, Phytol and Squalene were reported in the leaves of *Cassia italica* and *Aloe vera* and they showed antioxidant, hypocholesterolemic nematicide, lubricant, antiandrogenic, antimicrobial and anticancer activity (Arunkumar and Muthuselvam, 2009; Sermakkani and Thangapandian, 2012; Gunes, 2013; Sheeja *et al.,* 2016). Rowshanul Habib and Rezaul Karim (2009) reported the presence of Di-isooctyl phthalate in flowers of *Calotropis gigantea* which showed antimicrobial and cytotoxic activity. α-Cyclocostunolide and E-5-Octadecene showed antimicrobial and antioxidant properties (Cantrell *et al*., 2001; Yassa *et al.,* 2009; Yogeswari *et al.,* 2012). Sunita *et al.* (2017) reported the presence of 8-Pentadecanone in *Cenchrus ciliaris* and was in the treatment of Demyelination Conjunctivitis and also showed hepatotoxic activity.

CONCLUSION

GC-MS analysis of leaf and callus extract of *E. glandulosum* confirmed the presence of various phytoconstituents. Callus extract revealed some unique compounds which were not present in the mother plant. The identified compounds were reported to have medicinal properties such as anti-inflammatory, antimicrobial, antioxidant, anticancer activity, etc. Therefore the plant callus with potential bioactive phytochemicals can be commercially used for pharmaceutical applications.

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ESTIMATION OF QUERCETIN CONTENT IN THREE DIFFERENT SPECIES OF EUPATORIUM BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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Keywords:

HPTLC, *Eupatorium glandulosum, Eupatorium odoratum, Eupatorium triplinerve*, quercetin

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ABSTRACT: Three different species of *Eupatorium* namely *E. glandulosum, E. odoratum* and *E. triplinerve* belongs to the family Asteraceae are selected for the analysis of quercetin content. Leaves are extracted with ethanol and water and used for the analysis of quercetin by HPTLC technique using the mobile phase containing toluene: ethyl acetate: formic acid: methanol (5.5:4:1:0.5). Determination of quercetin content was performed by densitometric scanning under 254 nm, and the quercetin was detected at the R_f value of 0.54. The quantity of the quercetin content in plant extract was estimated by the calibration curve obtained from the standard quercetin. The result showed that the ethanolic extracts of *E. glandulosum* showed a high amount of quercetin (17.44 mg/g) followed by *E. odoratum* (13.4 mg/g) and *E. triplinrve* (9.29 mg/g).

INTRODUCTION: Plants produce a high diversity of secondary metabolites which are helpful in the defense mechanism of plants played against abiotic stress, and many of them have some medicinal importance. Quercetin is a flavonol, proved to be a potent antioxidant among poly-phenols [1, 2, 3]. It possesses antiviral, antibacterial, anticarcinogenic and anti-inflammatory effects [4, 5]. As per the literature, quercetin was reported in *E. glandulosum* [6], *E. perfoliatum* [7] and *E. cannabinum* [8]. In the present study, three different species of Eupatorium such as *E. glandulosum*, *E. odoratum* and *E. triplinerve* have been selected for the estimation of quercetin using High-Performance Thin Layer Chromatography.

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E. glandulosum belongs to the family Asteraceae is a native of Mexico, introduced as an ornamental shrub in several countries. In India, the tribes of Nilgiris use the leaves of this plant to heal wounds and small injuries [9]. *E. odoratum* is a fast growing perennial shrub, native of Central and South America has spread in tropical and subtropical regions of the world. The tribes of Indonesia used the leaf extract to cure skin diseases, poison bites, wounds, burns, cough, diabetes, diarrhea, fever, inflammation, and rheumatism. The boiled roots are used to cure urinary disorders [10, 11, 12].

E. triplinerve commonly called as Ayapana is an ornamental, erect, perennial herb having aromatic leaves. In tribal medicine, it is used to cure a fever with convulsions, pneumonia, indigestion, and cough [13]. Hence, to consider the medicinal importance of the above-said plants, the present study is undertaken with the objective of estimation of the quantity of quercetin content in three different species of *Eupatorium* using HPTLC technique.

MATERIALS AND METHODS:

Collection of Plant Material: The leaves of *E. glandulosum*, *E. odoratum*, and *E. triplinerve* were collected from Nilgiri Hills of Western Ghats, Coimbatore and Kanjikode Kerela respectively and certified by Botanical Survey of India, Coimbatore, Tamil Nadu. The plant materials are maintained in BSI Coimbatore under Voucher no: BSIR/RC/ 5/23/2017/Tech/338, BSIR/RC/5/23/2017/Tech/339 and BSIR/RC/5/23/2017/Tech/340. The leaves were washed thoroughly, dried, powdered and stored in air tight container for the study.

Preparation of Standard Solution: Quercetin (1mg/10ml) was prepared by dissolving 1 mg of quercetin in 10 ml of methanol in a standard flask.

Preparation of Plant Extracts: The leaf powder was defatted with petroleum ether and extracted with Ethanol (70 ºC) and water (100 ºC) in a Soxhlet apparatus. The extract was then dried and dissolved in a required amount of methanol.

Chromatography and Detection of Quercetin: Chromatography was performed on a 10 × 20 cm precoated HPTLC Silica gel 60 F_{254} plates (E-Merck, Mumbai, India). Aliquots of each of the extracts were separately applied (Samples and standard) to the plate as 6 mm wide band with an automatic TLC applicator Linomat-5 applicator (CAMAG, Switzerland), 5 mm from the bottom. The mobile phase consisted of Toluene: Ethyl acetate: Formic acid: methanol (5.5:4:1:0.5) was used per chromatography. The twin glass chamber was saturated with the mobile phase for about 30 minutes. The plate was developed up to 10 cm in twin glass horizontal developing chamber at the room temperature. Plates were air dried, and scanning was performed on a Camag TLC Scanner at 254 nm.

Calibration curve of Quercetin: The quercetin compound was determined by using a calibration curve established with a standard concentration ranging from 40 to 320 ng/spot. A stock solution of standard quercetin (l mg/ml) was prepared in methanol. The different volumes of stock solution 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8 and 3.2 ml were spotted on HPTLC plate to obtained concentration 40, 80, 120, 160, 200, 240, 280 and 320 ng/spot, respectively (bandwidth 6 mm, distance between tracks 7 mm) using automatic sample spotter. Peak areas were recorded for quercetin, and the calibration curve was obtained by plotting peak area against the concentration of quercetin.

RESULTS AND DISCUSSION: HPTLC fingerprinting of plant species is not only helps in the identification and quality control of a species but also to provide basic information useful for the isolation, purification, characterization, and identification of marker chemical compounds of the species. In the present study, methanol and water extracts of leaf powder of *E. glandulosum*, *E. odoratum* and *E. triplinerve* are used for analysis of quercetin content. Different concentrations of standard quercetin and leaf extracts were applied on HPTLC plates and developed in a solvent system consisting of toluene: ethyl acetate: formic acid: methanol (5.5: 4: 1: 0.5) and dried in air and scanned densitometrically **Fig. 1, 2a** and **2b**. The calibration curve of quercetin was found to be linear in the range of 4 to 32 mg/spot. A good linear relationship of standard quercetin was found to be R^2 = 0.998 concerning the concentration and peak area **Fig. 3** and **4**. The regression equation was found to be Y = 1268x + 1578 with respective to concentration.

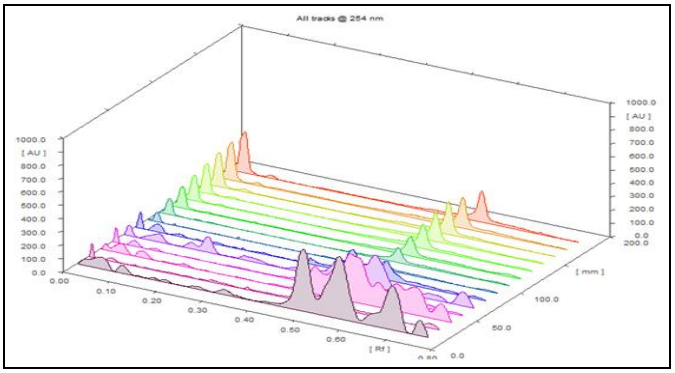

FIG. 1: DENSITOMETRIC CHROMATOGRAM OF QUERCETIN AND LEAF EXTRACTS (3D VIEW)

The total number of peaks was found to be 10, 12 and 12 in ethanol extracts of *E. glandulosum*, *E. odoratum*, *and E. triplinerve* respectively **Fig. 5**, **6** and **7**. The water extracts of *E. glandulosum*, *E. odoratum and E. triplinerve* showed 9, 7 and 10 peaks respectively **Fig. 8, 9** and **10**. The R_f value of standard quercetin was determined as 0.54 **Fig. 3**. In the plant samples, the ethanol and water extracts showed the peak at the R_f values of 0.54 and 0.55 **Fig. 5, 6, 7, 8, 9** and **10**.

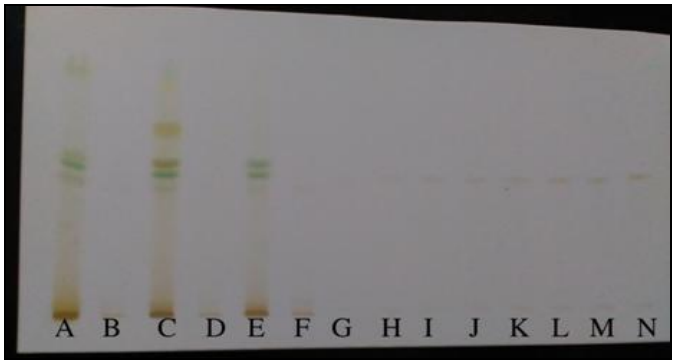

FIG. 2A: HPTLC PHOTOGRAPH OF SAMPLES AND VARIOUS CONCENTRATIONS OF QUERCETIN UNDER NORMAL LIGHT

FIG. 2B: HPTLC PHOTOGRAPH OF SAMPLES AND VARIOUS CONCENTRATIONS OF QUERCETIN UNDER UV LIGHT

A- Ethanol extract of *E. glandulosum*; B- Aqueous extract of *E. glandulosum*; C- Ethanol extract of *E. odoratum*
D- Aqueous extract of *E. odoratum*; E- Ethanol extract of *E. triplinerve*; F- Aqueous extract of *E. triplinerve*
G-N- Different concentration of quercetin

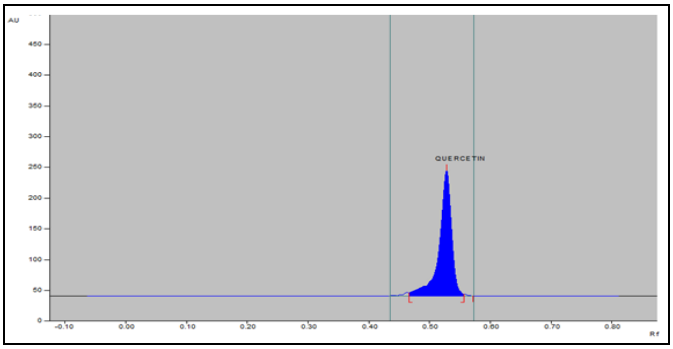

FIG. 3: HPTLC CHROMATOGRAM OF STANDARD QUERCETIN

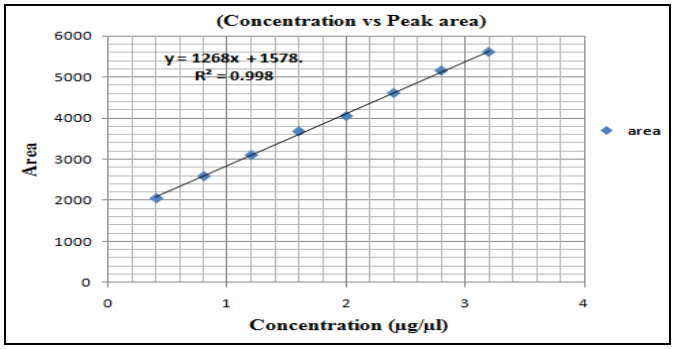

FIG. 4: CALIBRATION CURVE FOR STANDARD QUERCETIN

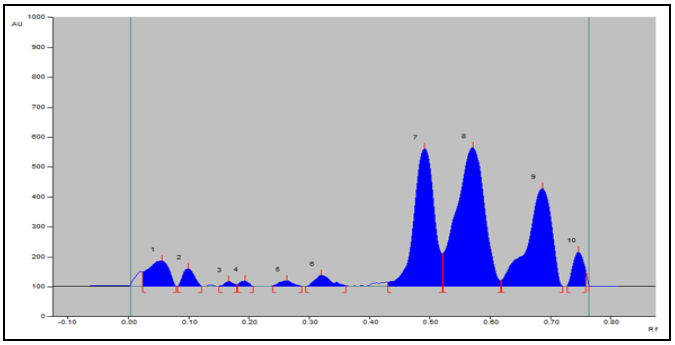

FIG. 5: HPTLC CHROMATOGRAM OF ETHANOL EXTRACT OF *EUPATORIUM GLANDULOSUM*

PEAK TABLE

Peak no.	R_f	Peak Area	Area (%)	Chemical substance
1	0.08	6176	12.85	Unknown
2	0.10	3091	5.90	Unknown
3	0.18	1346	2.52	Unknown
4	0.19	1529	2.79	Unknown
5	0.25	1348	2.52	Unknown
6	0.32	1861	2.89	Unknown
7	0.49	17016	28.50	Unknown
8	0.55	21395	37.74	Quercetin
9	0.69	13018	20.61	Unknown
10	0.75	6541	13.89	Unknown

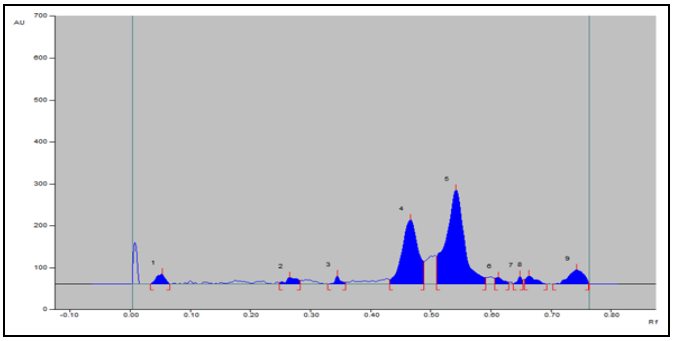

FIG. 6: HPTLC CHROMATOGRAM OF AQUEOUS EXTRACT OF *EUPATORIUM GLANDULOSUM*

PEAK TABLE

Peak no.	R_f	Peak Area	Area (%)	Chemical substance
1	0.05	2904	5.78	Unknown
2	0.26	492	0.85	Unknown
3	0.35	125	0.17	Unknown
4	0.47	5846	10.31	Unknown
5	0.54	6258	13.57	Quercetin
6	0.61	391	0.72	Unknown
7	0.64	101	0.10	Unknown
8	0.68	568	0.94	Unknown
9	0.75	3148	6.45	Unknown

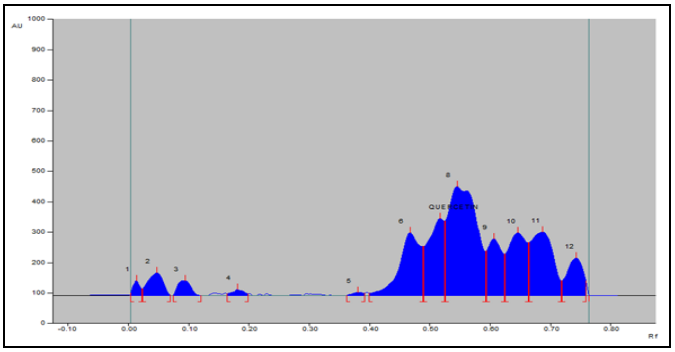

FIG. 7: HPTLC CHROMATOGRAM OF ETHANOLIC EXTRACT OF *EUPATORIUM ODORATUM*

PEAK TABLE

Peak no.	R_f	Peak Area	Area (%)	Chemical substance
1	0.01	381	0.70	Unknown
2	0.05	546	0.84	Unknown
3	0.08	421	0.74	Unknown
4	0.19	184	0.32	Unknown
5	0.39	116	0.13	Quercetin
6	0.48	9724	17.52	Unknown
7	0.52	10485	19.21	Unknown
8	0.55	14157	23.42	Quercetin
9	0.62	8485	15.96	Unknown
10	0.65	9834	17.64	Unknown
11	0.69	9842	17.91	Unknown
12	0.73	3461	6.85	Unknown

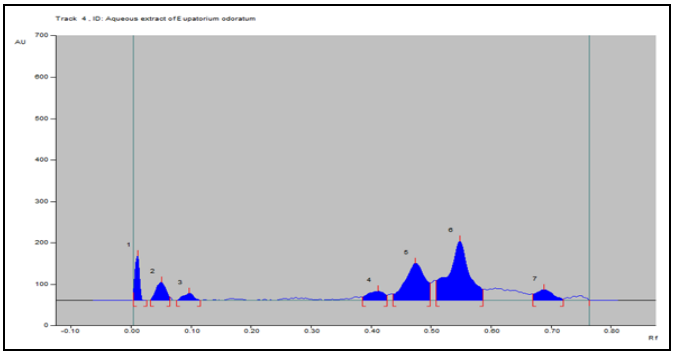

FIG. 8: HPTLC CHROMATOGRAM OF AQUEOUS EXTRACT OF *EUPATORIUM ODORATUM*

PEAK TABLE

Peak no.	R_f	Peak Area	Area (%)	Chemical substance
1	0.01	495	0.86	Unknown
2	0.05	542	0.92	Unknown
3	0.09	263	0.41	Unknown
4	0.42	932	1.40	Unknown
5	0.48	2663	5.41	Unknow
6	0.55	4411	7.85	Quercetin
7	0.69	945	1.53	Unknown

Among the selected three plants, the ethanol extract of *E. glandulosum* showed the maximum amount of quercetin (17.44 mg/g) followed by *E. odoratum* (13.40 mg/g) and *Eupatorium triplinerve* (9.29 mg/g). Water extracts showed a minimum amount of quercetin (2.67 mg/g) in *E. triplinerve* **Table 1** and **2**.

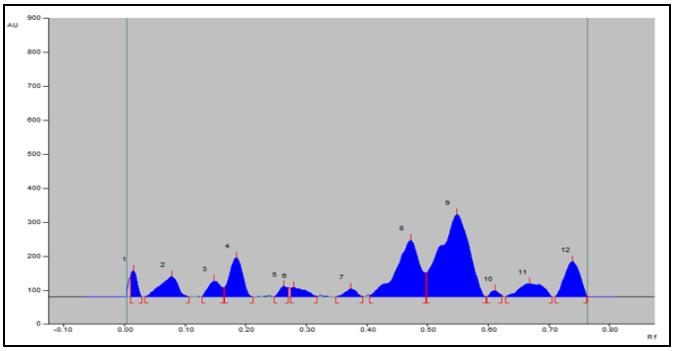

FIG. 9: HPTLC CHROMATOGRAM OF ETHANOLIC EXTRACT OF *EUPATORIUM TRIPLINERVE*

PEAK TABLE

Peak no.	R_f	Peak Area	Area (%)	Chemical substance
1	0.01	452	0.77	Unknown
2	0.08	2016	5.02	Unknown
3	0.15	942	1.52	Unknown
4	0.19	5249	10.16	Unknown
5	0.27	352	0.64	Unknown
6	0.28	395	0.75	Unknown
7	0.38	241	0.37	Unknown
8	0.48	8321	15.36	Unknown
9	0.55	10655	19.42	Quercetin
10	0.61	132	0.24	Unknown
11	0.66	236	0.36	Unknown
12	0.73	5245	10.15	Unknown

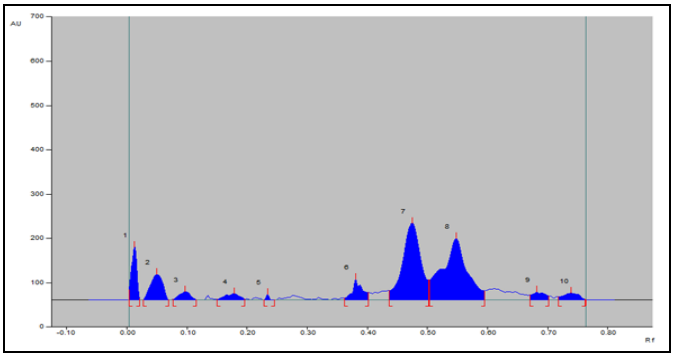

FIG. 10: HPTLC CHROMATOGRAM OF AQUEOUS EXTRACT OF *EUPATORIUM TRIPLINERVE*

PEAK TABLE

Peak no.	R_f	Peak Area	Area (%)	Chemical substance
1	0.01	2165	5.18	Unknown
2	0.05	2542	5.39	Unknown
3	0.10	598	0.96	Unknown
4	0.18	456	0.79	Unknown
5	0.23	57	0.02	Unknown
6	0.38	1064	2.37	Unknown
7	0.48	5946	10.73	Unknown
8	0.55	5013	10.02	Quercetin
9	0.69	325	0.64	Unknown
10	0.73	418	0.73	Unknown

Apart from quercetin, all the leaf extracts showed many numbers of peaks at different R_f values which shows the presence of various other compounds.

Similarly, the ethanolic extract of *Calamus rotang* showed the R_f value of quercetin at 0.54 [14,] but in contrast, a polyherbal syrub zymodyne and methanolic leaf and flower extract of *Moringa oleifera* showed the presence of quercetin at the R_f value of 0.86 and 0.35 respectively [15, 16]. The HPTLC analysis of an aqueous extract of *Eruca sativa* was found to be 17.94 [17] which is equal to *E. glandulosum*.

TABLE 1: PEAK TABLE OF QUERCETIN IN LEAF EXTRACTS

S. no.	Plant name	Solvent name	Total number of peaks	Peak no.	R_f	Peak area	Area (%)
1	*Eupatorium glandulosum*	Ethanol	10	8	0.55	21395	37.74
2	*Eupatorium glandulosum*	Aqueous	9	5	0.54	6258	13.57
3	*Eupatorium odoratum*	Ethanol	12	8	0.55	14157	23.42
4	*Eupatorium odoratum*	Aqueous	7	6	0.55	4411	7.85
5	*Eupatorium triplinerve*	Ethanol	12	9	0.55	10655	19.42
6	*Eupatorium triplinerve*	Aqueous	10	8	0.55	5013	10.02

As per the literature, quercetin possesses biological and therapeutic effects including anti-cancer, anti-oxidative, anti-microbial and anti-inflammatory, cardioprotective and hepatoprotective activities [18, 19, 20]. Hence, from the above findings, it is confirmed that the leaf possesses a good amount of quercetin content.

TABLE 2: QUERCETIN CONTENT IN PLANT EXTRACTS

S. no.	Name of the plant extract	Amount of Quercetin (mg/g)	
		Ethanol extract	Water extract
1	*E. glandulosum*	17.44	2.92
2	*E. odoratum*	13.40	3.74
3	*E. triplinerve*	9.29	2.67

CONCLUSION: The quantitative estimation of quercetin was analyzed in three different species of *Eupatorium* leaves by HPTLC fingerprinting technique. The quercetin content was found to be maximum in the ethanolic extract of all the three plants than water extract. The ethanolic extract of *E. glandulosum* leaves showed maximum quercetin content than other two species studied. Since, the leaves possess the promising amount of quercetin, it can be used for curing various ailments.

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CONFLICT OF INTEREST: The authors declare no conflicts of interest.

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