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RESEARCH ARTICLE

Unlocking Bioactive Potential: A Comparative Analysis of Solvent Extraction on Phytochemicals and Antimicrobial Efficacy in *Eupatorium glandulosum* and *Eupatorium odoratum*

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Abstract

The purpose of this work was to explore and compare the phytochemical constitution, vitamin composition, antioxidant activity, and antimicrobial potential of leaf extracts from two Ethnomedicinal plants, *Eupatorium glandulosum* and *Eupatorium odoratum*, belonging to family Asteraceae collected from the Eastern Himalayan Darjeeling region and the plains of Jhargram in West Bengal, India. Four distinct solvents viz., 80 % aqueous ethanol, ethyl acetate, chloroform and n-hexane were used to prepare the leaf extracts. The identification and quantification of phytochemicals and water-soluble vitamins was conducted through HPLC. To evaluate antioxidant potential, 2,2-Diphenyl-1-picryl hydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), and Ferric Reducing Antioxidant Power (FRAP) assays were performed. The antimicrobial activity was investigated against Streptococcoccal strains (*S. oralis, S. mutans, S. pyogenes*). Both the plants exhibited a diverse array of identified phytochemicals. *E. glandulosum* contained five while *E. odoratum* presented an even richer composition of seven water-soluble vitamins. Remarkably, *E. odoratum* displayed the highest scavenging activity against DPPH radicals (70.08%), and notable reducing potential (10.74 AAE mg/gm). In contrast, *E. glandulosum* showcased pronounced scavenging potential against ABTS radicals (74.32%). Additionally, the ethyl acetate extract from *E. odoratum* displayed robust antimicrobial efficacy against, *S. oralis*, inducing an inhibition zone of 16.00 mm ± 2.82 while *E. glandulosum* exhibited an inhibition zone of 11.50 mm ± 0.707. These findings validate the therapeutic potential and ethnomedicinal use of these plants.

Keywords: Antimicrobial, Antioxidant, Ethnomedicine, Eupatorium, Phytocompounds, Vitamins

1. Introduction

As per World Health Organization (WHO) reports, natural resources, including plants and their extracts, fulfill the fundamental healthcare needs of approximately 80% of the global population[1,2]. The prominence of natural plant resources has been growing due to their cost-effectiveness, wide availability, minimal or negligible adverse effects, and notable efficacy in addressing diverse health issues, in contrast to synthetic drugs[3]. As a consequence of which there has been an increasing emphasis on leveraging the inherent advantages of these botanical resources. Plants are abundant reservoirs of various secondary bioactive compounds and have gained significant recognition for drug development across pharmaceutical industries. The reservoir of compounds present in traditional medicinal plants holds promising potential for addressing both chronic and infectious ailments.

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The *Eupatorium* genus, belonging to the Asteraceae family, encompasses almost 60 species, most of which have already been subject to extensive research, revealing their abundance in diverse bioactive compounds[4]. These bioactive constituents potentially underlie the significant pharmacological effects exhibited by these plants, including bactericidal, fungicidal, insecticidal, virucidal, anticancer, and pain-relieving properties[5-7]. Notably enriched in aromatic compounds, *Eupatorium* species possess fragrant attributes and serve as valuable sources of essential oils, thus offering considerable therapeutic potential with implications for drug development[8,9].

Regrettably, despite their promising qualities, *Eupatorium* species have evolved into invasive plants, establishing a reputation as noxious and invasive weeds across various regions. Their pervasive nature has rendered them prominent ruderal weeds, thriving in abandoned agricultural fields, wastelands, and roadside ditches. The invasive behavior of *Eupatorium* adversely impacts native biodiversity, leading to ecological disruptions, while simultaneously inflicting substantial agricultural losses[10,11].

Eupatorium glandulosum, commonly recognized by names such as Crofton weed, goat weed, sticky snakeroot, Nilgiri weed, and locally referred to as "*banmara*" (meaning "forest killer"), thrives predominantly in higher altitude regions. This herbaceous species holds significant importance in traditional medicine, where its leaves have been employed to address a range of health concerns, including wound healing, diabetes, jaundice, and dysentery. Additionally, it serves as a stimulant, astringent, and thermogenic agent. Remarkably, *E. glandulosum* has also demonstrated remarkable antioxidant and antiproliferative attributes[12]. It is noteworthy that this plant shows an extensive spectrum of antibacterial action against bacteria, both gram-positive and gram-negative bacteria. Indigenous communities residing in the Eastern Himalayan region, such as Darjeeling and Sikkim, have leveraged the extracts from its leaves for the treatment of cuts, wounds, and bruises, thereby attesting to its traditional medicinal value[13]. Owing to its substantial reservoir of bioactive phytochemicals, flavonoids, and phenolic compounds, *E. glandulosum* emerges as an auspicious candidate for drug development targeting a diverse array of ailments.

Eupatorium odoratum, commonly known as Siam weed, stands as another notable species within the same genus, primarily found in regions of lower altitudes. This plant has also exhibited antimicrobial attributes and has found application in traditional medicine for addressing cuts, wounds, bruises, and infections, thereby underlining its medicinal significance[14]. Beyond these uses, it has also earned recognition for its effectiveness against conditions like fever, malaria, toothache, and intestinal diseases[15]. Its versatile properties extend to astringent, antioxidant, anti-hypertensive, anti-diabetic, anti-inflammatory, and anti-spasmodic effects, as highlighted by studies[16]. Likewise, *E. odoratum* is rich in a diverse array of bioactive phytochemicals, including tannins, flavonoids, saponins, and steroids, which contribute to its remarkable medicinal attributes[17].

In response to the evolving nature of infections, researchers are actively exploring novel solutions. Ethnomedicinal plants have emerged as a prominent avenue for pioneering drug development, aligning with the dynamic shifts in infection patterns over time[3].

2. Materials and Methods

2.1 Chemicals

All solvents and chemicals were bought from Merck (Germany) and were of analytical grade. Folin–Ciocalteu reagent, DPPH reagent, were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Three standard strains of bacteria were purchased from the Microbial Type Culture Collection (MTCC, IMTECH, Chandigarh, India): *S. mutans* (MTCC 497), *S. oralis* (MTCC 2696), and *S. pyogenes* (MTCC 442).

2.2 Plant Materials

Fresh leaves of *E. glandulosum* Michx (Asteraceae) and *E. odoratum* L. (Asteraceae) were methodically collected in the month of July 2022. These collections were made from two distinct regions, the Eastern Himalayan region of Darjeeling hills and the plains of Jhargram in West Bengal, India. To ensure accuracy and reliability, the authenticity of these plant specimens was confirmed through authentication at the Botanical Survey of India (BSI), located in Howrah. Plant samples gathered were given unique register numbers in order to facilitate systematic recordkeeping: SXC/Microbiol/AB/NM 01

for *Eupatorium glandulosum* and SXC/Microbiol/AB/NM 02 for *Eupatorium odoratum*. Once collected, the leaves underwent a meticulous cleansing process to eliminate any dirt or dust particles. Subsequently, the leaves were gently air-dried under the shelter of shade. After drying, the leaves were finely pulverized to a suitable consistency and then securely stored within an airtight container, effectively preserving their attributes for future analysis.

2.3 Microbial Cultures

Three standard strains of bacteria were purchased from (MTCC, IMTECH, Chandigarh, India): *S. mutans* (MTCC 497), *S. oralis* (MTCC 2696), and *S. pyogenes* (MTCC 442). The antibacterial activity of the four distinct extracts (80 % aqueous ethanol, ethyl acetate, chloroform and n-hexane) of *E. glandulosum* and *E. odoratum* was determined against all three bacterial strains.

2.4 Plant Extracts Preparation

The pulverized leaf samples of *E. glandulosum* and *E. odoratum* underwent distinct extraction processes utilizing the sequential cold maceration method employing four distinct solvents: 80% aqueous ethanol, ethyl acetate, chloroform and n-hexane[18]. Each phase of extraction was conducted twice, involving a mixture of 30 grams of pulverized leaf samples with 300 ml of the respective solvent. Throughout this process, agitation was maintained at room temperature, sustained for a duration of 18 to 24 hours.

The extracts obtained from both initial and subsequent extraction rounds were meticulously pooled together. Subsequently, using Whatman No. 1 filter paper these combined extracts were filtered and concentrated using rotary evaporator. This method led to the formation of concentrated, viscous extracts. To achieve dehydration, freeze-drying was employed, resulting in the formation of desiccated extracts. For preservation, all dried extracts from each solvent were stored at a temperature of -20°C.

2.5 Estimation of Water-soluble Vitamins by HPLC

The analysis of water-soluble vitamin was carried out through HPLC as per the standard protocols with certain modifications[19]. Briefly, for mobile phase, acetonitrile (Solvent A) and 0.01% v/v aqueous trifluoroacetic acid (Solvent B) was used. An elution gradient was maintained by adjusting solvent A to solvent B ratio. Vitamins were detected at four distinct wavelengths (210, 245, 275, and 290 nm). All the investigated vitamins showed responses and were successfully separated at a wavelength of 275 nm. The observed absorption spectra which were equivalent for both standard vitamin stock and plant extracts helped identify the presence of vitamins in the extracts under study, which were expressed as mg/100g DPM (dry plant material).

2.6 Analysis of Total Flavonoid Content (TFC) and Total Phenolic Content (TPC)

The assessment of TPC and TFC of the four distinct extracts viz, 80 % aqueous ethanol (EE), ethyl acetate (EAE), chloroform (CE) and n-hexane (HE) of *E. glandulosum* (Eg) and *E. odoratum* (Eo) was determined for 100 µg/ml to 1000 µg/ml concentration range.

TPC was assessed through Folin-Ciocalteau (FC) procedure following standard method with minor modifications[20]. Briefly, 2.5 ml of the FC reagent and 2.5 ml of saturated sodium carbonate solution (7.5% w/v) were combined with 100 µl of the examined plant extracts. The reaction mix was kept for half an hour in dark. Using a Shimadzu UV 1800 UV visible spectrophotometer, absorbance was measured at 765 nm. Gallic acid equivalents (GAE) per gram of dry sample (mg GAE/g) was the unit of measurement used to express TPC.

TFC was determined spectrophotometrically through $AlCl_3$ method[21]. Briefly, 2 ml of ethanolic $AlCl_3$ (2% w/v) was combined with 2.0 ml of the examined plant extracts. The reaction mix was kept for one hour in dark. Using a Shimadzu UV 1800 absorbance was measured at 420 nm. Quercetin equivalents (QE) per gram of dry sample (mg QE/g) was the unit of measurement used to express TFC.

2.7 Quantification of Phenolics and Flavonoids by HPLC

The assessment procedure was executed following the standard approach with necessary modifications and following ICH and USP guidelines[19]. Briefly, the mobile phase encompassed methanol (Solvent A) and a 0.5% aqueous acetic acid solution (Solvent B). The

gradient elution approach was employed, with solvent A to solvent B ratio being modulated, and the volume injected was fixed at 20 µl. HPLC chromatograms of the diverse leaf extracts were captured at wavelengths of 272 nm, 280 nm, and 310 nm. Retention time analysis and spiking with standards were the two different approaches used to determine the TFC and TPC content of the assessed extracts. For the former, the retention time of established standards was juxtaposed with that of each compound in congruous conditions. Simultaneously, the chromatograms of the standard samples were juxtaposed with those of the leaf samples under evaluation, facilitating the identification of the sought-after compounds.

This comprehensive approach yielded precise insights into the quantum of phenolic and flavonoid constituents present within the analyzed leaf samples.

2.8 *In Vitro* Antioxidant Potential

Antioxidants are compounds that scavenge the free radicals present in the environment, thereby protecting the cell from various physiological harm. The total antioxidant potential of the two *Eupatorium* species was measured using DPPH radical scavenging assay, ABTS assay and FRAP assay.

2.8.1 DPPH Radical Scavenging Assay

Using the DPPH radical scavenging procedure with minor modifications, leaf extracts' antioxidant capacity was evaluated[22]. Briefly, 0.003 % w/v of DPPH solution was made in methanol. Stock solution of 1000 µg/ml of different leaf extracts was prepared. From the stock, 10 µl, 20 µl, 50 µl, 80 µl and 100 µl were pipetted out in five different test tubes. Methanol was added to each test tube until the total volume reached 100 µl, resulting in concentrations of 100, 200, 500, 800, and 1000 µg/ml, in that order. Each test tube was filled with 3.9 ml of newly prepared DPPH (0.003% w/v), and the reaction mix was then allowed to sit for half an hour in the dark. For control 3.9 ml of DPPH in methanol was combined with 100 µl methanol and allowed to sit for half an hour in the dark. The Optical Density (OD) of the reaction mixture and control was recorded at 517 nm against a blank in triplicates, by UV-visible spectrophotometer (Shimadzu UV 1800). Antioxidant potential was quantified as percentage inhibition of DPPH radical, using the following equation:

Percentage (%) inhibition of DPPH activity = $\{(Ac - At)/Ac\} \times 100$

Where, Ac = absorbance of DPPH radical + methanol and At = absorbance of DPPH radical + sample extract

2.8.2 ABTS Assay

Using the ABTS assay protocol with minor modifications ABTS scavenged (%) was determined[23]. To generate the ABTS radical, 2.45 mM potassium persulfate was mixed with 7 mM ABTS in water. To complete the process of radical production, the reaction mix was allowed to sit for 12–18 hours at room temperature in the dark. Methanol was used to adjust the solution's absorbance to 0.70 at 734 nm. To evaluate the scavenging activity, leaf extract was obtained at different concentrations (100, 200, 500, 800, and 1000 µg/ml). To 100 µl of sample extract 2.5 ml of diluted ABTS solution was added. Six minutes after the initial mixing, absorbance was measured at 734 nm using ethanol as the control.

The following equation was used to determine the percentage of inhibition: ABTS scavenged (%) = $\{(Ac - At)/Ac\} \times 100$

Where, Ac = absorbance of ABTS radical + methanol and At = absorbance of ABTS radical + sample extract.

2.8.3 FRAP Assay

Standard protocol with minor modifications was used for FRAP assay[24]. Various concentrations (100, 200, 500, 800 and 1000 µg/ml) of the assessed leaf extract was taken. 100 µl of the leaf extract was combined with 2.5 millilitres of phosphate buffer (0.2 M) and 2.5 millilitres of potassium ferrocyanide (1% aqueous). The reaction mix was allowed to sit for 20 minutes at 50°C. Post incubation 2.5 ml of (10% ethanolic) TCA (trichloroacetic acid) was added and centrifuged at 3000 rpm for 10 minutes. To 1 ml of the supernatant, 1 millilitres of deionized water and 0.5 millilitres of (0.1 % aqueous) ferric chloride was added. The reaction mixture was allowed to sit for 20 minutes in dark and optical density (OD) was measured at 700 nm against each concentration. Using the calibration curve equation: $y = 0.0096 x + 0.0244$, $R^2 = 0.9955$; where, y was the absorbance and x was the ascorbic acid equivalent (mg/g). Ascorbic Acid Equivalent (AAE) in milligram per gram (mg/g) of dry material

was the unit of measurement used to express the reducing power.

2.9 Antimicrobial Assay

The antimicrobial activity of the four-leaf extracts (80 % aqueous ethanol, ethyl acetate, chloroform and n-hexane) of *E. glandulosum* and *E. odoratum* was assessed by agar well diffusion assay with minor modifications[25]. The bacterial inoculum's turbidity was adjusted to 0.5 McFarland standard, which is equivalent to 0.08 optical density and 1.5 x 10^8cfu/ml. To sterile petriplates, 20 ml of sterilized Muller Hinton Agar (MHA) was poured and allowed to solidify. 100 µl of the bacterial inoculum adjusted to OD 0.08 was then spread on solidified MHA. A stock solution of 50 µg/µl was prepared by reconstituting the plant extracts in 0.1 % DMSO. 100 µl of four different concentrations viz., 5 µg/µl, 12.5 µg/µl, 25 µg/µl and 50 µg/µl was added to each well of 9 mm diameter and incubated at 37°C overnight.

3. Results

3.1 Extraction Yields

The calculation is done by the percentage yield of w/w of extracts obtained in different solvents using the powdered plant that has been air-dried as a reference. with reference to the air-dried plant powder. Among the two *Eupatorium* species and the extraction solvents used a significant difference in the mean yield percentages ($p < 0.016$) was observed and vary from 1.82 ± 0.04 % to 12.925 ± 0.24 %. While the highest percentage yield of 12.925 ± 0.24 % w/w was found in Eo-EE (*Eupatorium odoratum*-Ethanolic extract), 1.82 ± 0.04 % w/w the lowest found in Eg-HE (*Eupatorium glandulosum*-Hexane extract) as shown in Figure 1.

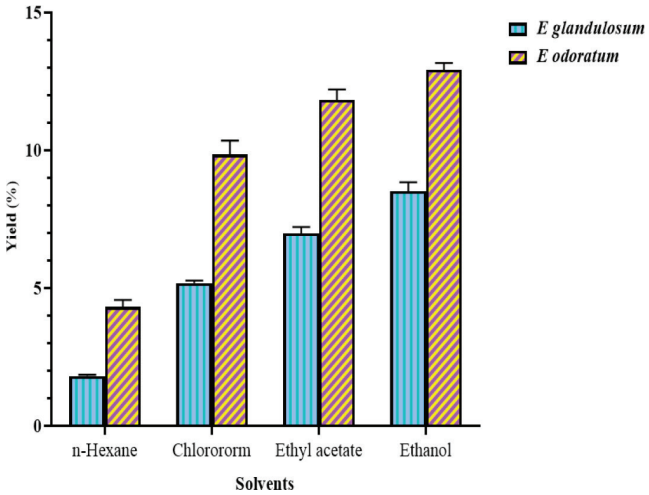


Figure 1. Percentage yield of w/w of extracts obtained in different solvents. All values are represented as mean ± SD (n=3).

3.2 Vitamin Analysis

In light of the extensive historical use of *E. glandulosum* and *E. odoratum* leaves for wound healing, our study was directed towards a thorough analysis of their vitamin content. The vitamin composition within *E. glandulosum* and *E. odoratum* is meticulously presented in Table 1, and these measurements were carried out in accordance with established protocols. Specifically, within *E. glandulosum*, the analysis revealed the presence of a total of five water soluble vitamins such as Vitamin C, Vitamin B2, Vitamin B6, Vitamin B5, and Vitamin B9. These vitamins were detected at concentrations of 7.45, 0.40, 0.36, 0.09, and 0.13 mg/100 gm of dry plant material, respectively. Likewise, in *E. odoratum*, the assessment unveiled the existence of a total of seven water soluble vitamins which includes Vitamin C, Vitamin B6, Vitamin B5, Vitamin B9, Vitamin B2, and Vitamin B1. The concentrations of these vitamins were determined to be 0.35, 0.42, 0.93, 0.96, 0.47, 0.31, and 0.09 mg/100 gm of DPM (dry plant material), respectively. These comprehensive findings underscore the role of *Eupatorium* species as a robust reservoir of essential vitamins, particularly Vitamin C, Vitamin B5, and Vitamin B9.

3.3 Analysis of Total Flavonoid Content (TFC) and Total Phenolic Content (TPC)

The TPC and TFC of the four different extracts, 80% aqueous ethanol (EE), ethyl acetate (EAE), chloroform

Table 1. Water-soluble vitamins analysed from *E. glandulosum* and *E. odoratum* leaves and expressed in mg/100 gm of dry plant material

Vitamins	*E. glandulosum*	*E. odoratum*
C	7.45	0.35
B6	0.40	0.42
B5	0.36	0.93
B9	0.09	0.96
B2	0.13	0.47
B3	-	0.31
B1	-	0.09

Table 2. The results of quantitative HPLC analysis of phenolic acids and flavonoids obtained from crude extracts of *E. odoratum* leaves, expressed in mg/100 gm of dry plant material

Polyphenolic compounds	n-Hexane	Chloroform	Ethyl-acetate	Ethanol
Gallic acid	-	1.302645	0.290	0.384913
Catechin	-	1.076456	0.123	0.06993
Chlorogenic acid	-	0.040468	0.085	8.593744
Vanillic acid	-	0.69546	0.241	0.022679
Caffeic acid	-	0.006232	0.141	-
Syringic acid	0.25112	1.19815	0.040	3.398727
p-Coumaric acid	0.101161	0.42864	0.143	4.33383
Ferulic acid	0.015669	0.056033	0.290	3.250016
Sinapic acid	-	0.120514	0.051	0.036907
Salicylic acid	-	-	0.014	-
Naringin	-	-	0.152	0.012518
Rutin	-	0.263711	4.564	0.049173
Ellagic acid	1.689231	2.860381	0.016	2.286907
Myricetin	0.025615	1.891919	1.184	0.055119
Quercetin	0.332969	2.629192	14.554	6.560998
Naringenin	3.688568	2.07103	19.316	6.772091
Apigenin	0.947054	8.834655	29.838	2.70683
Kaempferol	2.674412	4.931725	31.510	3.735032
Protocatechnic acid		0.285647		6.646279
Gentisic acid	-	-	-	1.606362
p-Hydroxy benzoic acid		0.148648		1.993368

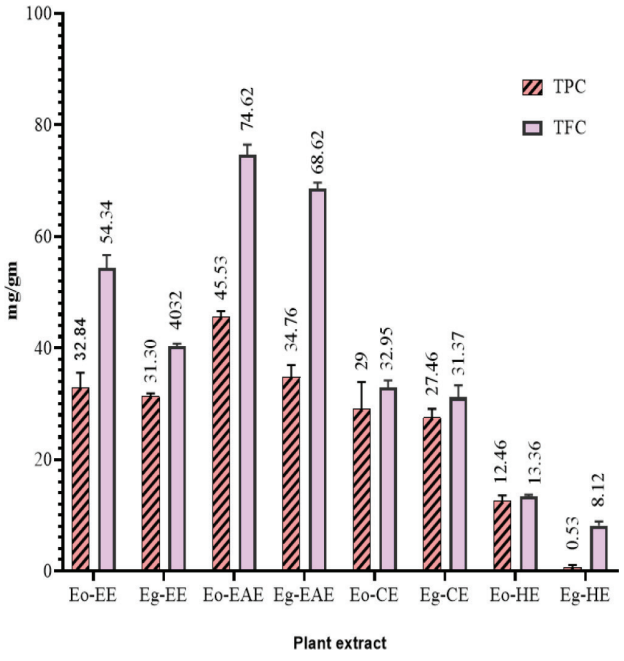
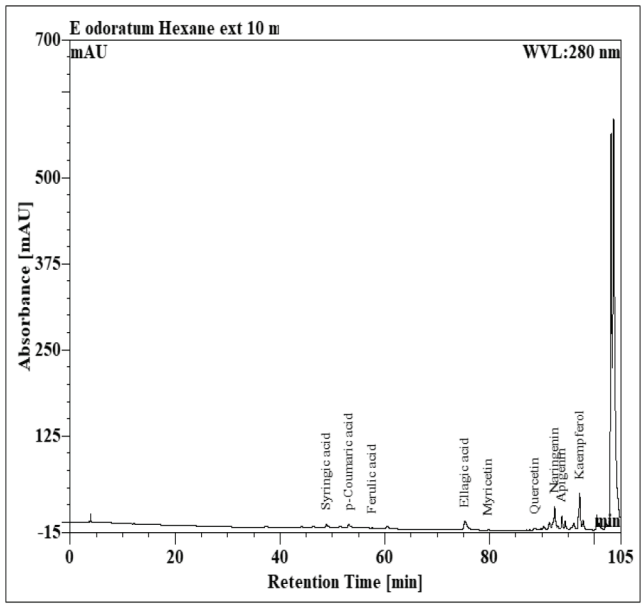


Figure 2. Comparative analysis of TPC and TFC present in the four different extracts of the two *Eupatorium* species. All values are represented as mean ± SD (n=3).

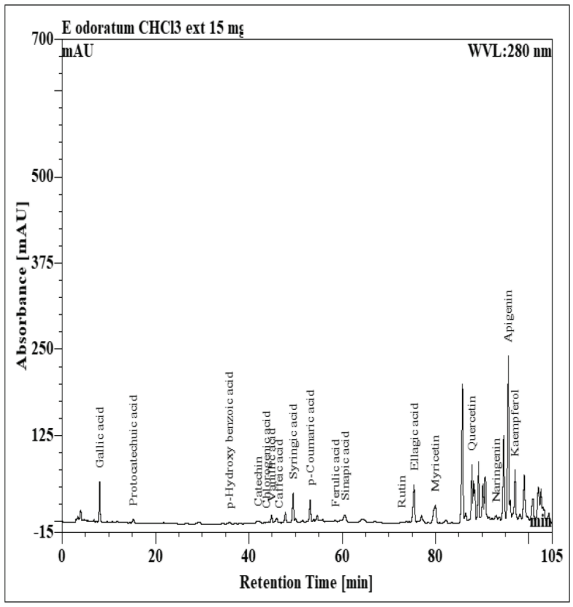
(CE) and n-hexane (HE)of *E. glandulosum* (Eg) and *E. odoratum* (Eo) was determined through Folin-Ciocalteau method and aluminium chloride ($AlCl_3$) method respectively. Using linear equations obtained from gallic acid (y = 0.0013x + 0.0498) and quercetin (y = 0.0353x + 0.0566) calibration curve, TPC and TFC was calculated respectively. Figure 2 represents the comparative amount of TPC and TFC present in the four different extracts of *E. glandulosum* and *E. odoratum*. The total amount of TPC and TFC obtained can be arranged by their mean values in the descending order: Eo-EAE >Eg-EAE > Eo-EE >Eg-EE >Eo-CE >Eg-CE >Eo-HE >Eg-HE. As determined by statistical analysis, a significant difference ($p < 0.05$) was observed in the TPC and TFC values between the two *Eupatorium* species and also between the extracts. All the four extracts of *E. odoratum* showed significant ($p < 0.05$) higher amount of TPC and TFC as compared to *E. glandulosum*. Moreover, Eo-EAE contained the highest TPC (45.53 ± 1.08 GAE mg/gm) and TFC (74.62 ± 1.84 QE mg/gm) as compared to those of Eg-EAE (34.76 ± 2.17 GAE mg/gm and 68.20 ± 1.04 QE mg/gm).

3.4 Analysis of Phytochemical Constituents by High-Performance Liquid Chromatography (HPLC)

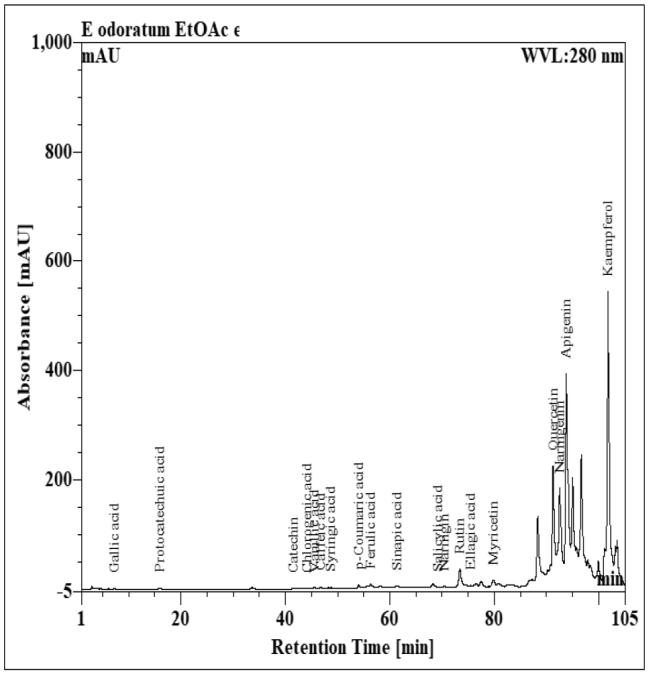
HPLC evaluation of the four distinct extracts (EE, EAE, CE, HE) derived from *E. odoratum* has revealed the presence of an array of phytochemical compounds, as meticulously detailed in Table 2, while the corresponding chromatograms are visually portrayed in Figure 3 (A, B, C, D). A comprehensive examination of the table underscores the consistent occurrence of syringic acid, ferulic acid, naringenin, myricetin, quercetin, apigenin, ellagic acid, p-coumaric acid, and kaempferol across all four extracts. Particularly noteworthy is the observation that EAE and EE encompass a more



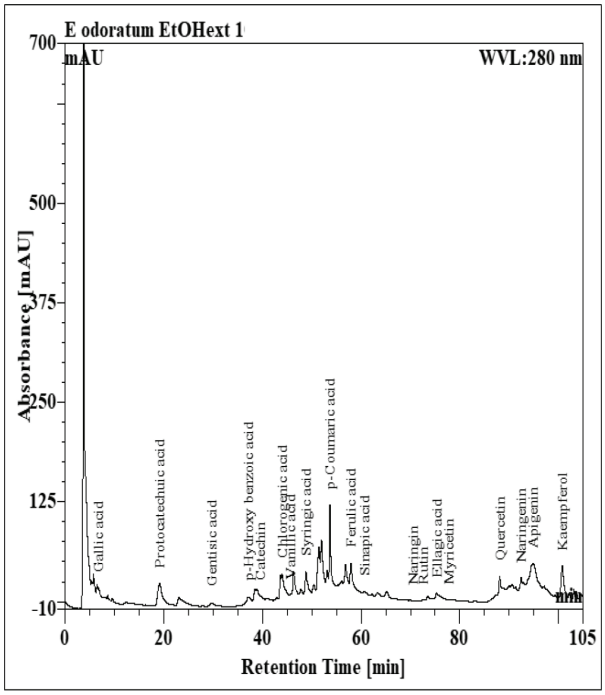
3A



3B



3C



3D

Figure 3 (A, B, C, D). HPLC chromatograms of the four distinct extracts (HE, CE, EAE, EE) derived from *E. odoratum*.

extensive assemblage of polyphenolic compounds in contrast to HE and CE. Among these compounds, the predominant polyphenolics include chlorogenic acid, apigenin, kaempferol, quercetin, ferulic acid, naringenin, rutin, syringic acid, p-coumaric acid, and protocatechuic acid.

Upon a closer analysis, it emerges that EAE is particularly enriched with apigenin, rutin, quercetin, naringenin, myricetin, and kaempferol. In contrast, EE showcases a prominence of chlorogenic acid, p-coumaric acid, ferulic acid, quercetin, naringenin, kaempferol, syringic acid, and protocatechuic acid. A comparative evaluation of the most active extract (EAE) from both *E. glandulosum* and *E. odoratum* reveals a more extensive repertoire of phytochemicals within *E. odoratum*, as elucidated in Table 3. Our experimental findings collectively attest to the superior phytochemical profile exhibited by *E. odoratum* vis-à-vis *E. glandulosum*. This enhanced profile is notably evident in the EAE and EE extracts, in contrast to HE and CE. The insights garnered from these findings not only deepen our comprehension of the intricate phytochemical composition but also provide invaluable insights into the potential bioactive compounds that these extracts harbour.

Table 3. Comparative data of results of quantitative HPLC analysis of phenolic acids and flavonoids obtained from the most active extracts (Ethyl-acetate) of *E. odoratum* and *E. glandulosum* leaves, expressed in mg/100 gm of dry plant material

Polyphenolic compounds	Ethyl-acetate *E. odoratum*	Ethyl-acetate *E. glandulosum*
Gallic acid	0.290	0.338
Catechin	0.123	-
Chlorogenic acid	0.085	-
Vanillic acid	0.241	-
Caffeic acid	0.141	0.941
Syringic acid	0.040	0.023
p-Coumaric acid	0.143	0.126
Ferulic acid	0.290	0.018
Sinapic acid	0.051	0.012
Salicylic acid	0.014	-
Naringin	0.152	-
Rutin	4.564	0.746
Ellagic acid	0.016	0.269
Myricetin	1.184	1.278
Quercetin	14.554	4.016
Naringenin	19.316	-
Apigenin	29.838	66.332
Kaempferol	31.510	5.645

3.5 Antioxidant Potential Determination

Five different doses (100 µg/ml, 200 µg/ml, 500 µg/ml, 800 µg/ml, and 1000 µg/ml) of the different leaf extracts (EE, EAE, CE, HE) of *E. glandulosum* and *E. odoratum* was used to determine the antioxidant potential using DPPH assay, free radical scavenging potential by ABTS assay and FRAP to determine reducing power potential. For positive control Ascorbic acid was used as the standard antioxidant. DPPH and ABTS assay revealed a concentration dependent rise in antioxidant potential as represented in Figure 4 and 5. Statistical analysis shows a significant difference ($p < 0.0015$) in the % inhibition values between EE and EAE and between the two *Eupatorium* species. The highest DPPH and ABTS scavenging % was observed for Eo-EAE (70.08%) and Eg-EE (74.32%) respectively.

The reducing power potential was assessed by the potential of the tested extracts to bring about reduction of ferrous ions as shown in Figure 6. A concentration dependent rise in antioxidant potential was observed similar to DPPH and ABTS. FRAP assay showed the

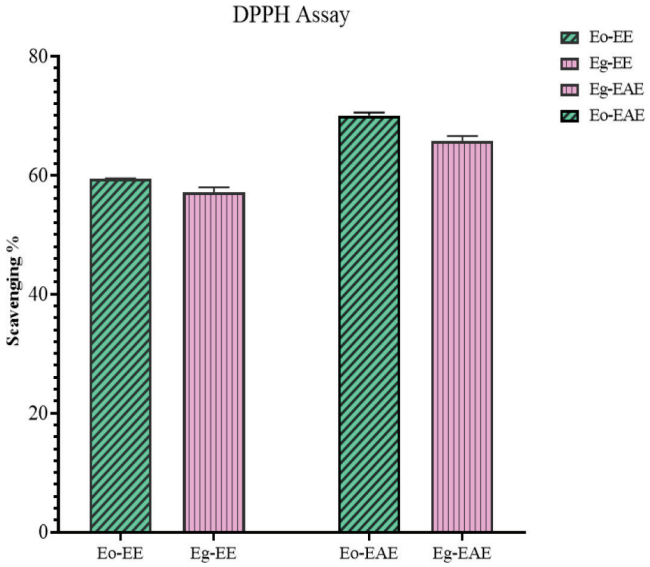


Figure 4. DPPH scavenging activity of ethanolic and ethyl acetate extract of the two *Eupatorium* species. All values are represented as mean ± SD (n=3).

reducing potential of the two *Eupatorium* species in the order Eo-EAE >Eg-EAE > Eo-EE >Eg-EE >Eo-HE >Eg-HE >Eo-CE >Eg-CE. The Eo-EAE showed maximum reducing potential of 10.74 AAE mg/gm of dry extract.

3.6 Antimicrobial Activity Determination

The antimicrobial activity determination was carried out through agar well diffusion assay employing a range of concentrations (5 µg/µl, 12.5 µg/µl, 25 µg/µl, and 50 µg/µl) of the four extracts derived from both *E. glandulosum* and *E. odoratum*. The results unveiled a concentration-dependent escalation in antimicrobial effectiveness, reflected in the expansion of inhibition zones. Figure 7 elegantly illustrates the outcomes of the agar well diffusion assay against the three Streptococcal strains, namely *S. oralis*, *S. mutans*, and *S. pyogenes*, at 25 µg/µl concentration.

For *E. glandulosum*, the ethanol extract (EE) exhibited maximum activity against *S. mutans* (12.25 mm ± 0.354), ethyl acetate extract (EAE) displayed superior activity against *S. mutans* (12.25 mm ± 0.354), chloroform extract (CE) showcased optimal activity against *S. oralis* (11.75 mm ± 0.354), and n-hexane extract (HE) presented heightened activity against *S. oralis* (11.25 mm ± 0.354). In parallel, within *E. odoratum*, the ethanol extract (EE) demonstrated the highest efficacy against *S. mutans* (15.25 mm ± 0.354), ethyl acetate extract (EAE) exhibited potent activity against *S. oralis* (16.00 mm ± 2.82), chloroform extract (CE) manifested notable activity against *S. mutans* (13.50 mm ± 0.707), and n-hexane extract (HE) showcased enhanced activity against *S. pyogenes* (11.50 mm ± 0.70).

In sum, the outcomes of the present study underscore that all four extracts sourced from both *E. glandulosum* and *E. odoratum* demonstrate commendable inhibitory activity against the three tested Streptococcal strains. Notably, the extracts originating from *E. odoratum* exhibit slightly heightened antimicrobial efficacy. The attribution of this antimicrobial prowess can be plausibly linked to the characteristic secondary metabolites that abound within the phytochemical profile of *Eupatorium* species. This observation accentuates the potential therapeutic significance of these plants and underscores the promise they hold as sources of natural antimicrobial agents.

3.7 Statistical Analysis

With GraphPad Prism version 8.0, ANOVA (the analysis of variance), standard error and average were assessed. The data are shown as means ± Standard Deviations (SDs), with each measurement carried

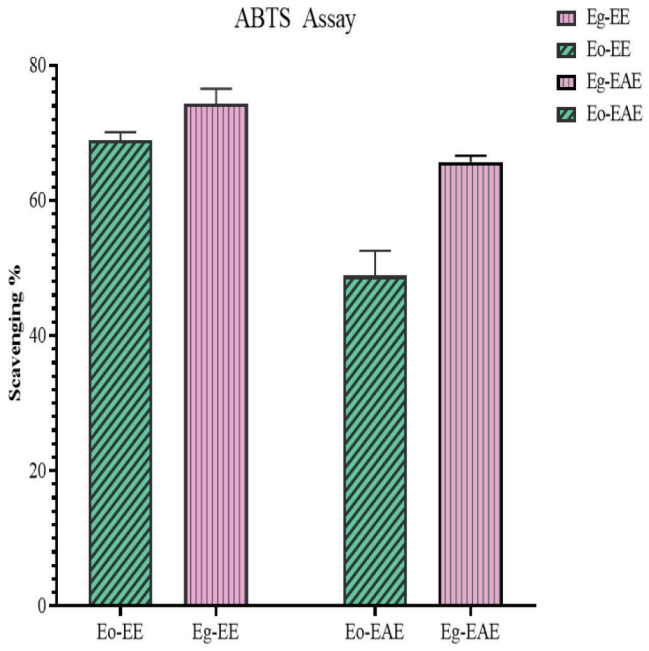


Figure 5. ABTS scavenging activity of ethanolic and ethyl acetate extract of the two *Eupatorium* species. All values are represented as mean ± SD (n=3).

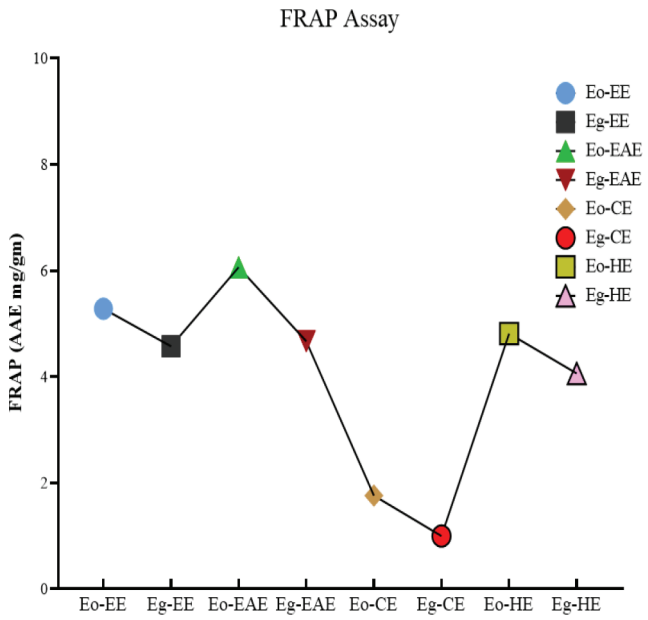


Figure 6. FRAP assay to determine reducing power potential in four different extracts of the two *Eupatorium* species. All values are represented as mean ± SD (n=3).

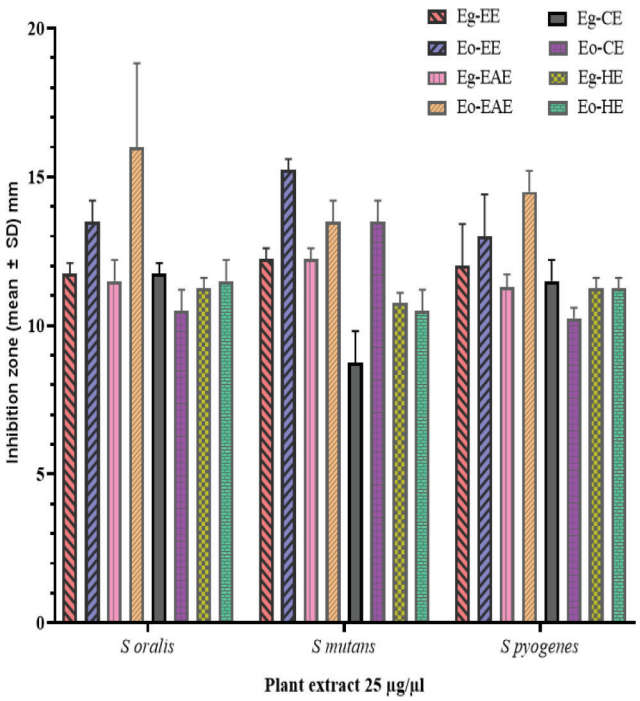


Figure 7. Antimicrobial inhibition zone shown by four different extracts of the two *Eupatorium* species. All values are represented as mean ± SD (n=3).

out in triplicate. P values less than 0.05 (<0.05) were deemed to have statistical significance.

4. Discussion

In regions where access to modern medicine remains limited, and natural resources have significantly served as medicinal alternatives within rural areas[12]. Among these, *Eupatorium* species from the Asteraceae family hold a global presence, being found across India, Pakistan, Malaysia, Nepal, China, eastern Australia, South Africa, and northern America[25]. These species have garnered a rich history of traditional medicinal use, particularly in the treatment of cuts, wounds, bruises, acting as analgesics, antiseptics, blood coagulants, and antipyretic agents according to native practices[26].

This study was conducted primarily to provide scientific substantiation for the traditional applications of *E. glandulosum* and *E. odoratum* leaves in medicinal practices. The focus was to chemically characterize the extracts obtained from these leaves, establishing a bridge between traditional knowledge and contemporary scientific understanding.

The leaves of *Eupatorium* exhibit a profusion of diverse phytochemical classes, encompassing phenolics, flavonoids, alkaloids, terpenoids, tannins, glycosides, and more, contributing significantly to their array of pharmacological attributes[27-29]. Remarkably, across all four extracts of *E. odoratum*, there was a notable and statistically significant increase ($p < 0.05$) in the content of TPC and TFC when juxtaposed with *E. glandulosum*. Particularly, Eo-EAE extract displayed the highest TPC content (45.53 ± 1.08 GAE mg/gm) and TFC content (74.62 ± 1.84 QE mg/gm), surpassing the levels observed in Eg-EAE extract (TPC: 34.76 ± 2.17 GAE mg/gm and TFC: 68.20 ± 1.04 QE mg/gm).

HPLC analysis revealed that both EAE and EE extracts exhibited a greater abundance of polyphenolic compounds in comparison to HE and CE extracts, irrespective of whether they belonged to *E. odoratum* or *E. glandulosum*. These polyphenolic constituents encompassed a diverse array of compounds including chlorogenic acid, apigenin, kaempferol, quercetin, syringic acid, naringenin, rutin, ferulic acid, p-coumaric acid, and protocatechuic acid. Evidently, *E. odoratum* emerged with a more pronounced and diversified phytochemical profile when juxtaposed with *E. glandulosum*. These findings collectively underscore the richer chemical composition and potential bioactivity within *E. odoratum* extracts.

The leaves of *Eupatorium* were subjected to an evaluation of their nutraceutical attributes. Within this context, the investigation revealed the presence of a noteworthy spectrum of water-soluble vitamins. *E. glandulosum* exhibited the presence of five water-soluble vitamins, while *E. odoratum* showcased the presence of seven. Of particular significance were the higher concentrations of certain vitamins, namely Vitamin C, Vitamin B5, and Vitamin B9, within both plant species. Vitamins play a vital role as essential organic constituents, integral for the proper functioning of biochemical processes. Humans, being unable to synthesize these compounds endogenously, acquire them via dietary sources. This critical role of vitamins in maintaining physiological health and supporting various bodily functions underscores their significance in our diet[1]. In essence, this study brings to light the nutritive value of *Eupatorium* leaves, reinforcing their potential to contribute to dietary vitamin intake and, consequently, overall health and wellbeing. Moreover,

it is worth noting that the choice of solvents employed for extraction plays a pivotal role in influencing both antioxidant and antimicrobial properties[1]. The assays conducted, encompassing DPPH, ABTS, and FRAP, all revealed a concentration-dependent escalation in activity, indicative of the potent antioxidant potential inherent in the extracts. Specifically, the maximum DPPH scavenging percentage was observed in *E. odoratum* ethyl acetate extract (Eo-EAE) at 70.08%, while *E. glandulosum* ethanol extract (Eg-EE) demonstrated the highest ABTS scavenging activity at 74.32%. The FRAP assay results indicated a hierarchical order of reducing potential across the *Eupatorium* species, with Eo-EAE leading, followed by Eg-EAE, Eo-EE, Eg-EE, Eo-HE, Eg-HE, Eo-CE, and Eg-CE. Of paramount significance, Eo-EAE manifested a robust reducing potential of 10.74 AAE mg/gm of dry extract. Additionally, all four extracts derived from both *E. glandulosum* and *E. odoratum* exhibited commendable inhibitory activity against the tested trio of Streptococcal strains such as *S. oralis, S. mutans,* and *S. pyogenes*. Notably, *E. odoratum* extracts showcased marginally elevated antimicrobial activity, with Eo-EAE demonstrating the highest inhibitory potential against *S. oralis* (16 ± 2.82), *S. mutans* (13.50 ± 0.70), and *S. pyogenes* (14.50 ± 0.70) when compared to *E. glandulosum*. The potential therapeutic value of the unique secondary metabolites found in the phytochemical profile of *Eupatorium* species can be explained by their ability to exhibit antibacterial effectiveness. The findings of this study underscore the remarkable richness in flavonoid and phenolic components, as well as an array of diverse phytocompounds, within the leaves of both *E. glandulosum* and *E. odoratum*. The presence of these constituents, coupled with the substantial vitamin content, has been identified as a potential source for the plants' pronounced antioxidant capacity and their wide-ranging effectiveness against various microbial strains. The overall collective profile of bioactive substances aligns with their possible use as cosmetic agents in foreseeable future, given their demonstrated attributes of antioxidant potential, anti-tyrosinase activity, anti-acne properties, and anti-inflammatory effects.

Furthermore, the scope of utilizing *Eupatorium* extracts extends to their integration into gel formulations, thereby presenting a novel avenue for wound healing and remediation of cuts and bruises. The abundant bioactive compounds present in these extracts suggest the feasibility of developing formulations that could facilitate enhanced healing and tissue regeneration. In light of these multifaceted attributes, the exploration of *Eupatorium* plants as valuable resources for cosmetic and therapeutic applications emerges as an exciting prospect, with the potential to yield innovative products with noteworthy benefits for both cosmetic and medical purposes.

5. Conclusion

The remarkable presence of abundant phytochemical and aromatic compounds within *Eupatorium* species imparts them with a fragrant quality and designates them as reservoirs of essential oils. Consequently, these attributes bestow upon them significant therapeutic properties, accentuating their potential role in drug development. Phytochemical analysis conducted on the leaves of these species has illuminated their richness in phytocompounds such as chlorogenic acid, apigenin, kaempferol, ferulic acid, quercetin, naringenin, rutin, p-coumaric acid, syringic acid, and protocatechuic acid, reinforcing their significance in the realm of natural compounds with therapeutic potential. The comprehensive exploration of different extracts from both *E. glandulosum* and *E. odoratum* has uncovered substantial phenol and flavonoid content, in tandem with robust antioxidant and antimicrobial activities. Particularly notable is the superior overall activity demonstrated by *E. odoratum* in comparison to *E. glandulosum*. This disparity in activity might be influenced by the differing altitudinal habitats of these species, adding a new dimension to the potential variables affecting their potency.

These findings resonate as a compelling call for further expansive research endeavours. The quest to scientifically authenticate the traditional medicinal uses of *Eupatorium* and to delve deeper into the efficacy of their extracts becomes even more pertinent in light of these results. By elucidating the potential mechanisms underlying their observed bioactivity, subsequent investigations could pave the way for harnessing the therapeutic potential of *Eupatorium* extracts with enhanced precision, ultimately fostering novel avenues in drug development and amplifying our understanding of the intricate interplay between plants and human health.

This investigation was conducted to primarily explore and compare the constituents of different phytocompounds, total flavonoid and phenolic content, and also carry out the evaluation of the antioxidant and antimicrobial properties of four distinct extracts obtained from *Eupatorium glandulosum* (Eg) *and Eupatorium odoratum* (Eo). Specifically, 80 % aqueous ethanol (EE), ethyl acetate (EAE), chloroform (CE) and n-hexane (HE) extracts were the subjects of analysis. In addition to these comparisons, the research delved into the influence of altitudinal variations and the diverse solvents employed on the biological and chemical characteristics of these extracts. The overarching objective was to establish a comprehensive foundation of knowledge, supporting the ethnomedicinal relevance of these plants, thus paving the way for continued investigation and prospective applications within the pharmaceutical sector.

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