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RESEARCH ARTICLE

EVALUATION OF PHYTOCHEMICAL CONSTITUENTS AND ANTIBACTERIAL ACTIVITY FROM LEAF AND CALLUS EXTRACTS OF *EUPATORIUM TRIPLINERVE*

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ABSTRACT

The present study involves the phytochemical evaluation and antibacterial activity from leaf and callus extracts of *Eupatorium triplinerve* (Asteraceae). Phytochemical screening in various extracts such as aqueous, ethanol, chloroform, acetone and petroleum ether of leaf and callus reveals the presence of tannins, saponins, phenols, flavonoids, cardiac glycosides, terpenoids, alkaloids and steroids. The leaf and callus extracts were quantitatively evaluated for tannin content with tannic acid as standard. The optimum yield of tannins was found in ethanol extract of callus was 7.82 ± 0.3 mg tannic acid Equivalents (TAE) / g) followed by ethanol extract of leaf was 6.71± 0.3mg tannic acid Equivalents (TAE) / g). Different concentrations of ethanolic extracts in leaf and callus were tested for the anti-bacterial activity against *Bacillus subtilis, Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* using agar disc diffusion technique. The ethanolic callus extract shows maximum zone of inhibition in *Bacillus cereus* followed by *Pseudomonas aeruginosa*. It was concluded that the powerful antibacterial effect was attributed to the greater amount of tannin compounds in the ethanolic callus extracts of *Eupatorium triplinerve*.

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INTRODUCTION

Plants are the richest resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs. The Ayurvedic and Unani systems of medicines are widely used by the people of Indian subcontinent. In spite of the recent domination of the synthetic chemistry as a method to discover and produce drugs, the potential of bioactive plants or their extracts to provide new and novel products for disease treatment and prevention is still enormous (Hammer *et al.*, 1999). The different plant derivatives, secondary metabolites have been proven to be the most important group of compounds that showed wide range of antibacterial and antifungal activity (Raskin *et al*., 2002, kareem *et al.*, 2010). Tannins have high polyphenolic compounds present in plants, foods, and beverages, soluble in water and polar organic solvents. Tannins may also bind to bacterial enzymes or form indigestible complexes with cell wall carbohydrates reducing the cell wall digestibility (Ahmed *et al.*, 1999, Rahman *et al.*, 1999). In recent years, tannins have been investigated to possess high antioxidants (Barry *et al*., 1986), free radical scavenging activity Reed *et al*., 1990), antimicrobial (Amarowicz *et al*., 2004) , gastro-protective and anti-ulcerogenic activities (Koleckar *et al*., 2008). Due to these therapeutic properties tannins can be used in the treatment of various diseases to improve human health. *Eupatorium triplinerve* Vahl belonging to the family Asteraceae, commonly known as Ayapana, is a native of South America, particularly the Amazon region of Brazil (Trang *et al*., 1993). It has also been found in Hawaii, India, Vietnam, and the Mascarene Islands (Gauvin-Bialecki and Marodon, 2009). This plant grows up to 1m high and is an ornamental erect perennial herb that is semi-woody at the base. The leaves (4.5-1o.5cm long and 0.8-1.7 cm wide) are aromatic, smooth, simple, opposite, sub-sessile, 3-nerved, acuminate, glabrous, and lanceolate. The stems are reddish brown. The many flowering heads are each 6-13 mm long and bear approximately 40 pink flowers (Gauvin-Bialecki and Marodon, 2009). *Eupatorium triplinerve* is widely used in folk medicine and its analgesic, anticoagulant, antianorexic, antiparasitic, anthelmintic, sedative, antifungal, and antibacterial properties have been reported (Bose *et al*., 2007; Chaurasia and Kher, 1978; Garg & Nakhare, 1993; Gupta *et al*., 2002; Jelager *et al*., 1998; Kokate *et al*., 1971; Verpoorte and Dihal, 1987; Yadava and Saini, 1990). In addition, the plant extract is used as antiseptic, and in the treatment of various ulcers and haemorrhages (Ghani 1998). Hence in this present study, the leaf and callus of *Eupatorium triplinerve* were screened for phytochemical constituents, tannins content and the antibacterial activity against various human pathogens.

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MATERIALS AND METHODS

Collection and Authentication of Plant Material

The healthy plants of *Eupatorium triplinerve* were collected from Azhiyar (Coimbatore). The collected plants were identified by Prof.P.Jayaraman, Director, and Plant Anatomy Research Centre (PARC) Chennai-45.

Plant Material

The collected plant material was separated as leaves, shade dried for 15to20 days and grounded into fine powder and stored separatively in an air tight container.

Preparation of leaf extract

About 1g of leaf dried powder of *Eupatorium triplinerve* plant materials were extracted with 20 ml ethanol (75%), acetone, chloroform, aqueous and petroleum ether (Merck, extra pure) for 1 min using an Ultra Turax mixer (13,000 rpm) and soaked overnight at room temperature. The sample was then filtered through Whatman No. 1 paper in a Buchner funnel. The filtered solution was evaporated under vacuum in a rota-evator at 40 °C to a constant weight and then dissolved in respective solvents. The concentrated extracts were stored in an airtight container in the refrigerator below 10°C.

Initiation of callus

Healthy and disease free young green leaves and nodal explants of *Eupatorium triplinerve* were collected from four months old mother plants and the explants were cultured on MS basal media containing various concentrations of 2, 4-D (1.13, 2.26, 4.52, 6.78 and 9.04 µM); NAA (1.342, 2.68, 5.37, 8.05 and 10.74 µM) and BA (1.10, 2.22, 4.44, 6.66 and 8.88µM) for callus induction. Primary callus was established from cotyledonary leaf explants. For secondary callus production, a small portion of primary callus was excised using sterile knife holder and was sub-cultured periodically once in three weeks. The secondary callus was used for all the experimental studies.

Phytochemical screening from leaf and callus extract of *Eupatorium triplinerve*

The phytochemical screening of leaf and callus extract was assessed by standard method as described by Savithramma *et al.,* (2011) and Selvaraj *et al*., (2014). Phytochemical screening was carried out on the leaf extracts using different solvents to identify the major natural chemical groups such as tannins, saponins, flavonoids, phenols, terpenoids, alkaloids, glycosides, cardiac glycosides, coumarins and steroids. General reactions in these analyses revealed the presence or absence of these compounds in the leaf extracts tested.

Extraction of active compound from leaf and callus extract using

Column chromatography - (Azhiyar- accession)

The mixture of compounds can be separated using column chromatography. The concentrated ethanol extracts of leaves and callus of *Eupatorium triplinerve* was separated and analysed by column chromatography technique as per standard methods (Ebenezer, 2013). The Concentrated ethanol extracts of *in vitro* leaf and callus of *Eupatorium triplinerve* (10mg /ml) was carefully transferred on to the upper surface of silica gel. The mobile phase used was methanol: chloroform in 2:1 ratio. The mobile phase was slowly passed through the column and a total of nine fractions were collected at an interval of five minutes at a flow rate of 1 ml /minute. The collected fractions were subjected to quantitative antioxidant activity with BHT as standard.

Duration taken for collection of each fraction

Fraction I : (0 – 5 mins)	Fraction VII : (30 -35 mins)
Fraction II : (5 – 10 mins)	Fraction VIII : (35 - 40 mins)
Fraction III : (10 - 15 mins)	Fraction IX : (40 - 45 mins)
Fraction IV : (15 -20 mins)	
Fraction V : (20 -25 mins)	
Fraction VI : (25-30 mins)	

Antibacterial activity of In vitro -leaf and callus extracts

The best fractions collected from leaf - *In vitro* (fraction- III) and Callus (fraction – IV) extracts of *Eupatorium triplinerve* plant were used for antibacterial study (Ozkan *et al*., 2004; Janarthanam, B. and Sumathi, 2010). Different concentration of (50mg, 100mg and 150mg /ml) the concentrated fraction extracts was tested for its antimicrobial activity against pathogenic Bacterial strain such as *Bacillus cereus, Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa and Escherichia coli.* Antibacterial activity was measured using the standard method of diffusion disc plates on agar (Britto *et al*., 2011). For antimicrobial assay, all bacterial strains were grown in Mueller Hinton Broth Medium (Himedia) (Erturk *et al*., 2006) for 24 hours at 37° C and plated on Mueller Hinton Agar (Himedia) for agar diffusion experiments. Then 0.1ml of each culture of bacteria was spread on agar plate surfaces. Sterile disc (Hi Media, 6mm in diameter) were placed on the agar medium to load 20µl of different concentration (50 -150mg /ml) of ethanolic leaf and callus extracts of *Eupatorium triplinerve* were tested. Inhibition diameters were measured after incubation for 24 hours at 37° C. Blanks of solvent only (processed in the same way), were also tested for antibacterial activity.

RESULTS AND DISCUSSION

The medicinal plants are useful for healing as well as for curing of human diseases because of the presence of phytochemical constituents. This study has revealed the presence of phytochemicals considered as active medicinal chemical constituents. In the present study, screening of phytochemicals on the leaf and callus extracts of *Eupatorium triplinerve* shows the availability of natural compounds such as phenols flavonoids, alkaloids, terpenoids, saponins, coumarins, cardiac glucosides and tannins. The ethanolic leaf and callus extract of *Eupatorium triplinerve* was shown more positive for the presence of natural chemical constituents followed by other solvents namely acetone, aqueous, petroleum ether and chloroform (Table 1). The result of the present study recorded highest tannins content in the callus extract of *Eupatorium triplinerve* and the tannins content was expressed as mg tannic acid equivalent (TAE) per gram of the sample. The optimum yield of tannins was found to be 7.82 mg TAE / g dry weight from callus followed by 6.71 mg TAE / g dry weight from leaf of *Eupatorium triplinerve* (Table 2).

Table 1. Phytochemical Screening from leaf and callus extract of *Eupatorium triplinerve*

S.No.	Phytochemical test	Leaf					Callus				
		Aq	Et	Ac	Ch	P	Aq	Et	Ac	Ch	P
1.	Alkaloids (Wagner's test)	+	+	-	-	-	+	+	-	-	-
2	Tannins	+	++	-	-	-	+	++	-	-	-
3.	Saponins (Foam Test)	++	++	+	+	-	++	++	+	+	-
4.	Phenols (Ferric chloride)	++	++	+	+	+	++	++	+	+	+
5.	Flavonoids (Lead Acetate)	+	++	+	-	-	+	++	+	-	-
6.	Terpenoids (Salwoski's Test)	+	++	+	+	+	+	++	+	+	+
7.	Glycosides	-	-	-	-	-	-	-	-	-	-
8	Cardiac glycosides	+	++	+	-	-	+	++	+	-	-
9.	Beta cyanin	+	+	-	-	-	+	+	-	-	-
10.	Anthocyanin (Hcl and NH_3)	-	-	-	-	-	-	-	-	-	-
11.	Coumarins	+	++	-	-	+	+	++	-	-	+
12	Quinones	+	++	++	+	+	+	++	++	++	+
13	Steroids	+	+	++	+	+	+	++	++	+	+

+ = positive, ++ = strong positive, - = negative,
Aq- Aqueous, Et- Ethanol, Ac- Acetone, Ch - Chloroform, P – Petroleum ether

Table 2. Quantitative estimation of tannin content from leaf and callus extract of *Eupatorium triplinerve*

S.No.	Ethanol extract of leaf	Ethanol extract of callus
1	6.71±0.3 mg/g	7.82±0.3 mg/g

Table 3. Antibacterial activity of best fractions collected from leaf – *in vitro* (fraction- III) extracts of *Eupatorium triplinerve*

Micro-organisms Tested	Ethanolic Leaf extract inhibition zone in diameter(mm)*		
Leaf - *In vitro* (fraction- III) *Eupatorium triplinerve*	50mg/ml	100mg/ml	150mg/ml
Bacillus subtilis MTCC No. 10224	.00±.00[a]	8.33±0.58[b]	10.33±0.58[c]
Bacillus cereus MTCC No. 10211	.00±.00[a]	8.33±0.58[b]	11.33±0.58[c]
Pseudomonas aeruginosa MTCC No. 14676	.00±.00[a]	9.67±0.58[b]	12.33±0.58[c]
Staphylococcus aureus MTCC No. 9542	.00±.00[a]	7.67±0.58[b]	10.33±0.58[c]
Escherichia coli MTCC No. 1563	.00±.00[a]	9.67±0.58[b]	12.67±0.58[c]

Note: 1. **denotes significant at 1% level
2.Different alphabets among leaf (fraction-III) extracts of *Eupatorium triplinerve* denote signification's at 5% level using Duncan Multiple Range Test (DMRT).

Table 4. Antibacterial activity of best fractions collected from callus (fraction – IV) extracts of *Eupatorium triplinerve*

Micro-organisms Tested	Ethanolic callus extract inhibition zone in diameter(mm)*		
Callus (fraction- IV) *Eupatorium triplinerve*	50mg/ml	100mg/ml	150mg/ml
Bacillus subtilis MTCC No. 10224	.00±.00[a]	8.33±0.58[a]	10.33±0.58
Bacillus cereus MTCC No. 10211	.00±.00[a]	8.67±0.58[b]	13.33±0.58
Pseudomonas aeruginosa MTCC No. 14676	.00±.00[a]	7.67±0.58[d]	18.33±0.58
Staphylococcus aureus MTCC No. 9542	.00±.00[a]	8.33±0.58[c]	16.33±0.58
Escherichia coli MTCC No. 1563	.00±.00[a]	9.67±0.58[b]	12.67±0.58

Note: 1. **denotes significant at 1% level
2.Different alphabets among callus (fraction-IV) extracts of *Eupatorium triplinerve* denote signification's at 5% level using Duncan Multiple Range Test (DMRT).

The effect of ethanol on extraction of tannins from *Eupatorium triplinerve* callus extracts was good followed by leaf extract. The results corroborates with the findings of (Singh *et al.,* 2012) who has reported the maximum yield of tannins from ethanolic extract of *Artemisia absinthium.* Ethanol has been found to be the most commonly used solvent for the extraction of tannins rather than other organic solvents (Salah *et al*.,1995). Tannins have stringent properties, hasten the healing of wounds and inflamed mucous membranes (Obon *et al*., 2007). The data presented in (Table 3 & Table 4), indicate that the leaf and callus extracts of *Eupatorium triplinerve* inhibit the growth of some microorganism to various concentration. The concentrations of 50mg/ml - 150mg/ml ethanolic extract showed antimicrobial activity against *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Pseudomonas aeruginosa* and inactivity against *Escherichia coli* .The maximum clear zone of inhibition was found at 150mg/ml of 75% ethanolic callus extract of *Eupatorium triplinerve* than leaf extract**.**

Tannin compounds present in many medicinal plants inhibit the growth of many fungi, yeasts, bacteria and viruses (Sodipo *et al*., 2000). In both the case of leaf and callus extracts, no zone of inhibition was found in lower concentration (50mg/ml). Similar results were obtained on ethanol extracts from leaves of *Sida acuta and Acalypha wilkesiana* which exhibited antibacterial activity (Gotep *et al*., 2010). The antimicrobial activities of ethanol extract may be due to the presence of tannins, triterpenoids and flavonoids. Tannin compounds present in many medicinal plants inhibit the growth of many fungi, yeasts, bacteria and viruses (Stary, 1998).

Conclusion

Thus the preliminary screening tests may be useful in the detection of the bioactive principles, leading to drug discovery and development. Further, these tests facilitate their quantitative estimation and qualitative separation of pharmacologically active chemical compounds.

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