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STUDY OF TOTAL FLAVONOIDS, PHYTOCHEMICAL SCREENING, STRUCTURAL ANALYSIS AND ANTIBACTERIAL ACTIVITY OF CRUDE EXTRACT OF *EXACUM PEDUNCULATUM L*

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ABSTRACT

Ethnomedicine falls back to ancient times, where plants have been used as an exemplary source of medicine. The bioactive constituents in plants have helped in formulating new and effective medicines. *Exacum pedunculatum* belonging to Gentianaceae family was used as febrifuge to treat fever and intestinal disorders by the tribal people in South India. Current study on this species gives an insight to the scientific exploration of the plant for its medicinal properties. The study was carried out with phytochemical screening, isolation of flavonoids and characterization of bioactive compounds present in the ethyl alcohol extract of *Exacum pedunculatum* using Thin Layer Chromatography, Ultraviolet- Visible spectroscopy and Fourier Transmission Infrared Spectroscopy (FTIR). UV-Visible profile of the plant extract showed different peaks ranging from 200-800 nm with different absorption. The FTIR spectrum confirmed the presence of phenols & alcohols, alkanes, carbonyls, carboxylic acids, nitro compounds and aromatic compounds in the extract. Antibacterial activity of the extract was carried out against reference strain bacteria: gram positive and gram negative. The results show that the extracts have good antibacterial activity.

INTRODUCTION

Ancient India primarily relied on herbal medicines to treat health problems. Plant based formulations are constantly being screened for their biological and pharmacological activities such as anti-diabetic, anti-oxidant, anti-microbial and anti-cancer activities [1-6]. Extensive use of herbal medicines is due to no side effects or less side effects. According to World Health Organisation, about 80% of the total population in Asian and African countries rely on traditional medicines for their primary health care. Medicinal herbs have a vast range of pharmacologically active components and each herb is unique in its combination and properties. These ingredients can be formulated as one drug with several pharmacological effects [7, 8]. Generally, many unidentified phytoconstituents play vital role in working against diseases, infections and disorders. Modern techniques for the identification and isolation of the bioactive components of plant origin can be implicated for its cost- effectiveness and rapid detection [9].

Gentianaceae is the native of northern temperate areas of the world [10] comprises of flowering plants with approximately 70-80 genera and 900-1200 species. Gentiana genus is very well known for their pharmacological properties. They are characteristic of intense bitter taste and therefore used as a remedy for digestive system ailments [11]. *Exacum pedunculatum,* belonging to Gentianaceae family is a phytochemically unexplored species. This preliminary study provides information of the bioactive components present in the plant and facilitates opportunities for further pharmacological investigations [12].

MATERIAL AND METHODS

Plant Material

Exacum pedunculatum Linn. (Gentianaceae) plant material was collected in monsoon season from Kodachadri Western Ghats of Karnataka, India and identified and authenticated based on its physical characteristics. The plant material was air dried in shade, crushed and powdered finely before following the extraction procedure.

Flavonoid extraction methods

200g of dried plant material of *Exacum pedunculatum* was immersed in 100ml ethyl alcohol (100%) for 24 hours at room temperature on magnetic stirrer [13].

The mixture was filtered using Whatman No. 1 filter paper and the process was repeated several times till 300ml of the residue with ethyl alcohol was obtained. Filtrate was added and treated with 100ml of 1% lead acetate for 4 hours for precipitation.

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The mixture was filtered; 250ml of acetone and 30ml of concentrated HCl was added to the mixture and filtered. The obtained pellet was freeze dried under vacuum for 12 hours. The extract was dissolved in ethyl alcohol and the extraction process was repeated, filtered and a red precipitate was procured. Finally, the filtrate was placed in a clean and dry petri dish at room temperature until deep red-brown powder was obtained.

Phytochemical Screening

The obtained dry extract of the plant was subjected to various phytochemical evaluations for the detection of the bioactive compounds [14, 15].

1. ***Flavonoids:*** Addition of 1% KOH to the alcoholic extract resulted in formation of yellow colour confirmed the presence of flavonoids.
2. ***Phenolic compounds:*** To the aqueous solution of the plant extract, 5 ml of 1% ferric chloride was added, resulting a blue-green colour formation indicating the presence of phenolic compounds.
3. Double bond test: Brown colour solution was formed on addition of $KMnO_4$ reagent, indicating the presence of double bond in the phytoconstituents.
4. ***Aldehyde and Ketone test:*** Formation of yellow colour precipitate on addition of 2, 4- dinitrophenyl hydrazine confirmed the presence of aldehydes and ketones.

Chemical Identification

Thin layer chromatography (TLC)

Crude extract was dissolved in ethyl alcohol and spotted on TLC plates. The plates were developed in chromatography chamber containing solvent mixture of petroleum ether, ethyl acetate and hexane in different concentrations. The developed plates were air dried and visualized under UV light.

The plates were placed in a chamber of saturated iodine vapours to observe spots with different colour. R_f values were calculated for isolated compounds. [16]

Structural analysis of major components

Ultraviolet-visible spectroscopy

UV-Visible absorption in the range of 200-800 nm of the crude extract was observed using UV-VIS Spectrophotometer model, Hitachi 3310, JAPAN and ethyl alcohol was used as the solvent. The spectrum showed maximum absorption at different wavelengths between the ranges of 200-800 nm. This helped to characterize the double bonds.

Infrared spectroscopy

The sample was analysed by FT-IR to find the functional group by KBr disc method using FT-IR Spectrometer, NICOLET 6700 model, USA in University Science Instruments Centre, Karnatak University, Dharwad, India.

Antibacterial activity

Preparation of the extract solution

The extract was dissolved in ethanol (100%) to a final concentration of 10 mg/ml for well diffusion method.

Bacterial Strains

The extract was individually tested against a set of human pathogenic microorganisms, including two Gram-positive bacteria: *Staphylococcus aureus*, *Bacillus subtilis*; three Gram-negative bacteria: *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia* isolated from different samples in the department of Biotechnology and Microbiology, Karnatak University, Dharwad, India. All the strains were confirmed by cultural and biochemical characteristics [19] and maintained in slants for further use.

The antimicrobial activities of the ethanolic extract from *Exacum pedunculatum* was determined by standard agar well diffusion method [17, 18, 20]. Mueller Hinton Agar (Hi Media) plates were swabbed (sterile cotton swabs) with 24 hour old - broth culture of bacteria. Wells of 6mm diameter and about 2 cm apart were made in each of these plates using sterile cork borer. About 100 µl of solvent extract was added with the help of sterile syringe into the wells and allowed to diffuse at room temperature for 2hrs. For each bacterial strain, controls were included that comprised pure solvents instead of the extract [21]. The plates were incubated overnight at 37°C for 24 hrs. Antibacterial activity was determined by measuring the diameter of the zone of inhibition (mm) surrounding bacterial growth. The experiments were repeated three times and the mean values are presented.

RESULT AND DISCUSSION

Extraction

In the present study, flavonoids were extracted from the whole plant material of *Exacum pedunculatum*, belonging to Gentianaceae family, using ethyl alcohol as a solvent. The obtained extract was deep brown colour. Table 1 shows physical properties and solubility of the plant extract.

Table 1 Physical properties and Solubility of the extract

Test	Flavonoid
Description	Red-brown crystals
pH	5.7
Solubility test	Soluble in ethanol, methanol, ethyl acetate, DMSO, acetone
Yield (%)	9.5%/100g dry weight

Results of the biochemical tests (Table 2) claims the presence of flavonoid in the plant extract which contains double bonds, ketone groups with phenolic groups.

Table 2 Results of preliminary qualitative tests

Tests	Result	Colour Reaction
Flavonoid	Positive	Yellow precipitate
Double bond test	Positive	Brown
Aldehyde & Ketone	Positive	Yellow precipitate
Phenol	Positive	Green

Results of TLC of the plant extract and their R_f values are given in the table (3) below.

The plates placed in the chamber of saturated Iodine vapours, showed spots with different R_f values as discussed in the above table. This study explains the presence of bioactive compounds which is to be further characterized.

Table 3 Retention values of the extract

Developing Solvent	Observation	Spot(s)	Rf Value (at 360 nm)
Petroleum ether	Naked eye, UV, Iodine Vapours	2	0.125 Yellow 0.2Light Brown
Ethyl acetate: Pet ether (80:20)		3	0.7 Yellow 0.8 Green 0.9 Brown
Ethyl acetate: Pet ether (50:50)		1	0.75 Green
Ethyl acetate		1	0.75 Green
Ethyl acetate: Pet ether (90:10)		1	0.75 Green

Results of UV-Visible spectroscopy

UV-Vis absorption in the range of 200-800 nm of the crude extract was observed using petroleum ether as the solvent. The spectrum shows maximum absorption at different wavelengths between the ranges of 200-800 nm. This helps us to characterize the double bonds.

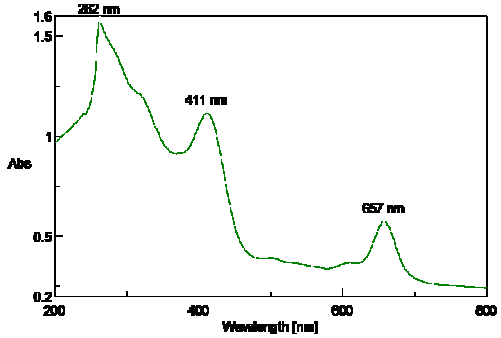

Fig 1 The UV-Visible spectrum shows three peaks of maximum absorption at 262nm, 411nm and 657 nm of petroleum ether extract due to $\sigma \rightarrow \pi^*$ transition which is characteristic of conjugated double bonds.

Results of Infrared spectroscopy

The sample was analyzed by FT-IR to identify the functional group. The study reveals the presence of phenolic and alcoholic groups, hydroxyl and carbonyl groups, nitro and aliphatic amines within the isolated compound.

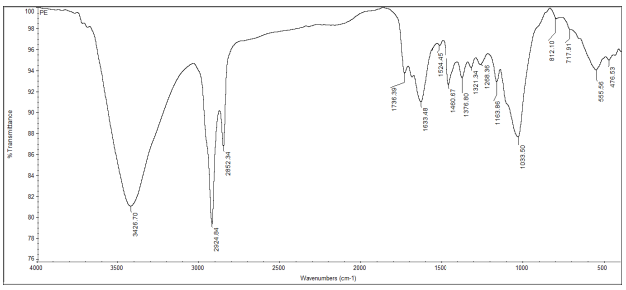

Fig 2 Infrared spectrum of the extract of *Exacum pedunculatum*

Table 4 Functional groups of the IR-spectrum of the petroleum ether extract

Wavenumber (cm^{-1})	Band shape	Bond	Functional group
3426.70	Strong, broad	O-H, free hydroxyl	Stretching of phenols & alcohols
2924.84	Medium	C-H stretch	Alkanes
1736.39	Strong	C=O	Carbonyls
1633.48	Strong	C-H	Carboxylic acids
1524.45	Strong	N-O asymmetric stretch	Nitro compounds
1321.34	Strong	C-N stretch	Aromatic amines

Antibacterial activity

The plant extract worked well on both Gram positive and Gram negative microorganisms showed, but showed higher activity on gram positive bacteria. The zone of inhibition of the extracted compound was higher on Gram positive bacteria than gram negative bacteria.

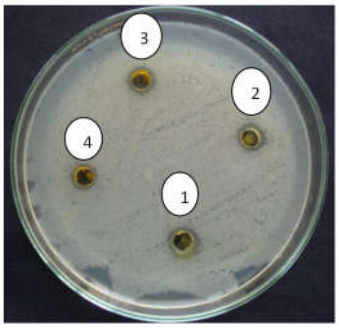

Figure 3 The antibacterial activity of the ethanolic extract of *Exacum pedunculatum* against selected human pathogenic bacteria. The no. 1 is showing the highest activity followed by 2, 3 and the 4th one showing minimum activity.

Table 5 Antibacterial activity of *Exacum pedunculatum* against human pathogenic bacteria

Test compound	Zone of inhibition in mm			
	B. subtilis	*E. coli*	*P. aeruginosa*	*K. pneumonia*
Ethanolic extract	11.2±0.41	9.2±0.53	8.1±0.47	6.1±0.63
Control	---	---	---	---
Streptomycin (Standard)	18± 0.45	13± 0.47	12± 0.32	15± 0.27

The *in vitro* antibacterial potential of the plant extract was carried out by agar well diffusion method and results were tabulated in Table 1. The extract showed significant antibacterial activity against *B. Subtilis* (11.2±0.41) followed by *E. coli* (9.2±0.53 mm) and moderate effect on growth of *P. aeruginosa* (8.1±0.47 mm)and very mild effect on *K. pneumonia* (6.1±0.63 mm).

Increased activity of the plant extract may be due to the presence of (-OH) group in the structure of the compound which helps in inhibiting the microbial growth and therefore these alcoholic compounds can be considered as antiseptic agents [22].

CONCLUSION

The above work explains the preliminary studies on the unexploited species *Exacum pedunculatum* belonging to Gentianaceae family. The results conclude that the species possess promising phytochemicals which can be further studied for its mechanism of action to use as therapeutic agents. Gentianaceae family is known for its potential phytoconstituents with bitter principles (secoirdoid glycosides) and xanthones widely distributed in the family.

Practise of herbal medicines falls back to the ancient times from Vedic medicine till date. The need for the day is to explore, identify and evaluate these natural compounds which can improve the quality of mankind. Hence, the plant source can be used as an add-on medication in treating the microbial infections. Further, the plant can be explored for various ailments and can be an additional source of medication along with various herbal medicines.

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