Research Article

PHYTOCHEMICAL SCREENING, GC-MS CHARACTERIZATION AND *IN VITRO* ANTIBACTERIAL ACTIVITY OF WHOLE PLANT *BLEPHARIS BOERHAVIFOLIA* PERS AGAINST MRSA

Akilandeswari Krishnan *, Ruckmani Kandasamy

Department of Pharmaceutical Technology, Bharathidasan Institute of Technology, Anna University, Tiruchirappalli, India

*Corresponding Author Email: akilakrishna79@gmail.com

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ABSTRACT

Blepharis boerhavifolia (Acanthaceae) commonly known as kozhimookan has been recognized as a part of traditional medication for the treatment of various diseases. This study was carried out with an objective to investigate phytochemical compounds, antibacterial activity and GC-MS analysis of *Blepharis boerhavifolia* (Acanthaceae). The crude extracts of *Blepharis boerhavifolia* revealed the presence of several biologically active phytochemicals with the highest quantity of alkaloids, flavonoids and phenols were performed for dichloromethane, chloroform, ethyl acetate, acetone and methanol extract. The antibacterial activity was evaluated against gram positive organism and gram negative organism *Enterococcus faecalis*, *Klebsiella pneumonia* NCIM 2719, *Enterobacter cloacae* NCIM 2164 and Clinical strain Methicillin resistant *staphylococcus aureus* (MRSA) by agar diffusion method. The antibacterial activity of ampicillin, ciprofloxacin and methanol extract showed maximum zone of inhibition 10 mm, 30.33 mm and 15 mm against MRSA respectively. In addition to that qualitative analysis of methanolic crude extract of *Blepharis boerhavifolia* was analyzed by using GC-MS showed that the presence of a ten different phytochemical compounds. The components were identified by compare their retention time and fragmentation patterns with those data stored in the National Institute of Standard and Technology (NIST) library. The reported chief constituents are Azulene derivatives, Cayophyllene, γ -Neoclovene, β -Humulene, Methanoazulene, Tetradecanoic acid and 13-Tetradecenal characterized. The GC-MS analysis revealed the presence of many bioactive compounds such as flavonoids, terpenoids, phenolics and steroids that can be postulated for antibacterial, antioxidant and anticancer activity.

Keywords: Phytochemical screening, *Blepharis boerhavifolia*, Acanthaceae, GC-MS analysis, Antibacterial activity, MRSA

INTRODUCTION

Blepharis boerhavifolia (BB), belonging to the family Acanthaceae, synonym of *Blepharis maderaspatensis* and commonly known as Kozhimookkan, Kooravaalchedi, has been used extensively as traditional medicine in many countries for the treatment of headache and bone fractures. It has also been used to suppress boils, diarrhoea, and induce lactation. In traditional practice, the leaves of BB and the yolks of two eggs are ground into a fine paste and are applied on fractured bones. The paste of the leaves is mixed with lime juice and applied on cuts. In addition, BB is reported to have antinociceptive, anti-inflammatory, antioxidant and antiproliferative properties¹⁻⁴.

Creeping *Blepharis* is a prostrate herb that creeps on the ground with wiry stems rooting at each nodes. Prostrate herbs. Leaves ternate, unequal, 3-5 x 2 cm, obovate, apex apiculate, puberulus, margins distantly serrate; petiole to 3 cm. It is found commonly on slopes, among rocks, poor croaky soil on the hills up to 1,400 m -1,600 m. Leaves occur in false whorls of four i.e., four leaves on each node. The flowers are white or cream in colour with purple veins. *Blepharis boerhavifolia* is mostly distributed throughout the world mostly tropical and sub-tropical regions. It is found in a wide variety of grassland, shrub and forest habitats, often in secondary and troubled areas. As the species name *maderaspatensis* indicates this plant was first found in Madeira Islands in Atlantic Ocean, near tropical Africa, South Africa, Madagascar, extending to Asia, specifically in the southern region of Tirunelveli¹⁻⁴.

Earlier reports suggest that *Blepharis boerhavifolia* has potential antimicrobial properties against both gram positive and gram negative bacteria. However, to date, there are no studies regarding the antibacterial activity of BB against MRSA clinical strain with various extracts.

MATERIALS AND METHODS

Collection and preparation of plant material

The entire parts of plant BB were collected from the natural environment in kanyakumari District of Tamilnadu, India. The plant was authenticated by Botanist Dr. V. Chelladurai, Research officer-Botany (Retd.), Central council for research in Ayurveda and Siddha, Government of India and the herbarium of was carefully examined and botanically identified as BB of the family Acanthaceae dated 20.06.2014 voucher specimen number BB001 has been deposited at the herbarium of Entomology research Institute, Loyola College, Chennai (India). The samples were washed thoroughly in running tap water to remove soil particles and adhered debris and finally washed with sterile distilled water. The cleansed plant sample was shade dried and pulverized by a mechanical grinder and passed through a 40 mesh sieve. The powdered sample material was then stored in air tight polythene bag until use⁵.

Extraction of Plant Material

Dried powdered whole parts of *Blepharis boerhavifolia* (500 g) was extracted with different solvents based on their increasing polarity (*i.e.*) petroleum ether, dichloromethane, chloroform, ethyl acetate, acetone, methanol and aqueous by hot continuous percolation method using soxhlet extractor. The shade dried coarsely powdered plants *B. boerhavifolia* was extracted separately with 2 L petroleum ether (40-60 °C). The marc obtained from the above extract was dried and extracted with 2L dichloromethane, 2 L chloroform, again extracted using 1.5 L of ethyl acetate (76-78°C), and further extracted with 2 L of methanol, and finally extracted with 1.5 L of distilled water. Extraction was accomplished with all solvents until the supernatant in the soxhlet turned into transparent and continued for 48-72 h. The solvents were then removed from each extract by evaporation under reduced pressure in a rotary vacuum evaporator (IKA RV10 DS 96, Germany). The individual extracts were weighed and stored in labeled screw capped air tight glass bottles and kept in vacuum desiccators until it is used. The extracts were then dissolved in water or DMSO prior to experimental use⁵.

Calculation of percentage yield

The percentage yield was calculated for the extracts and major compounds with reference to the crude material taken by using the formula and is given below.

Percentage yield with reference to crude plant material = $\frac{\text{Weight in grams of extracts obtained}}{\text{Weight in grams of plant material taken}} \times 100$

Qualitative phytochemical screening

Phytochemical analysis for identification of composited chemical constituents of the extracts that include, phenolic compounds, flavonoids, saponins, steroids, terpenoids, alkaloids, tannins and reducing sugars was carried out using following procedure⁶⁻¹⁰.

Tests for alkaloids

A small portion of the solvent free extract was stirred with a few drops of dilute hydrochloric acid and filtered. The filtrate was tested carefully with various alkaloid reagents such as:

Mayer's reagent: The filtrate was treated with Mayer's reagent (potassium mercuric iodide solution). The appearance of cream color indicated the presence of alkaloids.

Dragendorff's reagent: A little amount of the filtrate was treated with Dragendorff's reagent (potassium bismuth iodide solution). The appearance of reddish brown precipitate indicated the presence of alkaloids.

Hager's reagents: The test sample was treated with Hager's reagent (picric acid). The appearance of yellow precipitate indicated the presence of alkaloids.

Wagner's reagent: A little amount of the extract was treated with Wagner's reagent (iodine and potassium iodide solution). The appearance of reddish brown precipitate indicated the presence of alkaloids.

Test for carbohydrates

Dissolve a small quantity of the extract in 4 ml of distilled water and filtered. The filtrate was subjected to Molish's test for the presence of carbohydrate.

Molish's Test: To 2 ml of extract, 1 ml of α -naphthol solution was added and conc. Sulphuric acid was added through the sides

of the test tube. Formation of the violet color ring at the junction of two liquids indicated the presence of carbohydrates.

Tests for glycosides

Another small portion of the extract was hydrolyzed with diluted hydrochloric acid for a few minutes on a heating water bath and the hydrolysate was subjected to Legal's and Borntrager's tests to detect the presence of different glycosides.

Legal's test: To the hydrolysate, 1 ml of pyridine and few drops of sodium nitroprusside solution were added and then it was made alkaline with sodium hydroxide solution. The appearance of pink to red color shows the presence of cardenolide glycosides.

Borntrager's test: To the hydrolysate, 1 ml of chloroform was added and shaken well. The chloroform layer was separated and to this equal amount of dilute ammonia solution was added. The appearance of a pink color in the ammoniacal layer indicated the presence of anthraquinone glycosides.

Keller kiliani test: When a pinch of the extract was dissolved in glacial acetic acid, a few drops of ferric chloride solution was added, followed by the addition of concentrated sulphuric acid, formation of red ring at the junction of two liquids indicated the presence of glycosides.

Test for phytosterol

Libermann- Burchard's Test: A small amount of the extract was dissolved in a few drops of glacial acetic acid. 3 ml of acetic anhydride was added, followed by adding few drops of concentrated sulfuric acid. The appearance of bluish green color shows the presence of phytosterol.

Salkowski Test: Small quantity of the extract was dissolved in chloroform and the resulting solution was then shaken with a few drops of concentrated sulfuric acid. The appearance of bluish green color shows the presence of phytosterol.

Tests for fixed oils and fats

Saponification test: A few drops of 0.5 N alcoholic potassium hydroxide solution was added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on a water bath for 1-2 h. Formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

Spot test: A small quantity of the extract was pressed between two filter papers. Oil stains on the paper indicated the presence of fixed oil.

Test for saponins

Foam test: 1 g of the extract was shaken vigorously with 20 ml distilled water in a graduated cylinder for 15 min. 1 cm layer of foam indicated the presence of saponins.

Tests for tannins and phenolic compounds

A small quantity of the extract was dissolved in water, warmed and filtered. The resulting filtrate was used for the following tests

Ferric chloride test: A small amount of the filtrate was treated with neutral ferric chloride solution. The appearance of violet color indicated the presence of phenols.

Gelatin test: A small amount of the filtrate was treated with 1% w/v solution of gelatin in water containing 10 % sodium chloride. The appearance of cream precipitate indicated the presence of phenolic compounds.

Lead acetate test: A small amount of the filtrate was treated with 10% lead acetate solution. The appearance of white precipitate indicated the presence of phenolic compounds.

Tests for proteins and amino acids

A small quantity of the extract was shaken with few ml of water and the resulting mixture was subjected to the following tests:

Millon's test: When the test sample was treated with Millon's reagent (Mercuric nitrate solution), formation of white precipitate indicated the presence of proteins.

Ninhydrin test: When the test sample was treated with 0.1% w/v solution of ninhydrin in n-butanol, appearance of the violet or purple color indicated the presence of amino acid.

Biuret test: When the test sample was treated with equal volume of 5% sodium hydroxide solution and 1% copper sulphate reagent, appearance of the pink to purple color indicated the presence of proteins and free amino acids.

Test for flavonoids

A small quantity of the extract was shaken with a few ml of water and the resulting mixture was subjected to the following tests

Shinoda's test: A small quantity of test sample was dissolved in 5 ml of alcohol (95%) and treated with few drops of concentrated hydrochloric acid and 0.5 g of magnesium turnings. Development of a pink color within a minute indicated the presence of flavonoids.

Fluorescence test: A few mg of the extract was dissolved in alcohol and a drop of the resulting solution was placed on Whatmann filter paper and observed under UV light. The appearance of fluorescence indicated the presence of flavonoids.

Test for lignin

A small quantity of the extract was treated with few drops of phloroglucinol and hydrochloric acid. The appearance of pink or red color indicated the presence of lignin.

Tests for detection of triterpenes

a. Salkowski's test: A small quantity of the extract taken in a dry test tube, added a few tin granules and 1 ml of thionyl chloride and shaken well. The appearance of a pink color indicated the presence of triterpenes.

b. Libermann-Burchard's Test: To the solution of extract, 1 ml of acetic anhydride and acetic acid were added, followed by a few drops of concentrated sulfuric acid. A ray of colors from violet changing through purple to brown or blue indicated the presence of triterpenes⁶⁻¹⁰.

Bacterial strains

Klebsiella pneumonia NCIM 2719, *Enterobacter cloacae* NCIM 2164 were obtained from National collection of industrial micro organisms, Pune, clinical strains *Enterococcus faecalis* and *Staphylococcus aureus* were obtained from KAP viswanathan medical college, Tiruchirappalli.

Determination of antibacterial efficacy for the antibiotics and *B.boerhavifolia* methanol extract by zone of inhibition method

Determination of antibacterial efficacy for the antibiotics and methanol extracts were tested by studying the zone of inhibition by well diffusion technique. Plates were prepared using pre inoculated (one day old cultures of 0.5 McFarland standard turbidity) MHA, on the surface of which 4 wells were prepared using gel puncture and filled with different concentrations of test agents and its combinations with respect to the MIC values. The plates were incubated at 37°C for 18 h and the zone of inhibition of selected organisms was measured. The experiments were performed in triplicates and statistically evaluated^{11,12}.

GC-MS analysis

The Clarus 500 GC (Perkin Elmer) used in this analysis¹³. It employed a fused silica column packed with Elite – SMS (5%Diphenyl / 95% Dimethyl polysiloxane, 30mm x 0.25mm x0.25µm) and the components were separated using helium as carrier gas at a constant flow of 1 ml/min. The 2 µl sample extract injected into the instrument. It was detected by the turbo gold mass detector (Perkin Elmer) with the aid of Turbo mass 5.2 software. During the GC process the oven was maintained at a temperature of 110°C with 2 min holding. The injector temperature was set at 250°C. The different parameters involved in the operation of the Clarus 500 MS were also standardized. The inlet line temperature was 2000°C and source temperature was 200°C. Mass spectra were taken at 70 eV; a scan period of 0.5 s and fragments from 45–450 Da. The MS detection was completed in 36 min. The NIST ver. 2.0 year 2005 library was employed for detection¹³.

RESULTS

The various extracts of *B.boerhavifolia* were subjected to screening for its phytochemical constituents. The phytochemical screening results are shown in Table 1. The petroleum ether extract of *B.boerhavifolia* showed the presence of phytosterol, fixed oils and fats. Chloroform extract exhibited the presence of alkaloids, glycosides, phytosterol, fixed oils, tannins and proteins. Ethyl acetate extract showed the presence of carbohydrates, phytosterol, proteins, tannins, flavonoids and triterpenes. Methanolic extract depicted the presence of alkaloids, carbohydrates, saponins, tannins, proteins, flavonoids and triterpenes. Aqueous extract showed the presence of carbohydrates, saponins, proteins, flavonoids, lignin and triterpenes.

Table 1: Phytochemical analysis of various extracts from aerial parts of *B.boerhavifolia*

Name of the test	Extracts				
	Pet. ether	Chloroform	Ethyl acetate	Methanol	Aqueous
Test for Alkaloids					
a. Mayer's reagent	--	+	--	+	--
b. Dragendorff's reagent	--	+	--	+	--
c. Hager's reagent	--	+	--	+	--
d. Wagner's reagent	--	+	--	+	--

Test for Carbohydrates Molish's test	--	--	+	+	+
Test for Glycosides a. Legal's test b. Borntrager's test c. Keller kilani test	-- -- --	+ + +	+ + +	+ + +	-- -- --
Test for Phytosterol a. LibermannBurchard's test b. Salkowski's test	+ +	+ +	+ +	-- --	-- --
Test for Fixed oils and Fats a.Saponification test b.Spot test	+ +	+ +	-- --	-- --	-- --
Test for Saponins	--	+	+	+	+
Test for Tannins and Phenolic compounds	--	+	+	+	+
Test For Proteins and Free Amino Acids a. Millon's reagent b. Ninhydrin reagent c. Biuret test	-- -- --	+ + +	+ + +	+ + +	+ + +
Test for Flavonoids a. Shinoda's test b. Florescence test	-- --	+ +	+ +	+ +	+ +
Test for Lignin	--	--	--	--	+
Test for triterpenes a. Salkowski's test b.LibermannBurchard's test	-- --	+ +	+ +	+ +	+ +

+ indicates the presence of phytochemical compound -- indicates the absence of phytochemical compound

The properties of the collected extracts for different solvents are shown in Table 2. Typically, bioactive compounds of plants are produced as secondary metabolites, which are a group of compounds other than primary metabolites believed to help plant to increase their overall ability to survive and overcome local challenges by allowing them to interact with their surroundings.

Table 2: Properties of different extracts from whole parts of *B. boerhavifolia*

S.NO.	Extracts	Consistency	
		<i>B. boerhavifolia</i>	
1	Petroleum ether	Green	Semi solid
2	Chloroform	Pale green	Pasty
3	Ethyl acetate	Brownish green	Semi solid
4	Methanol	Brownish green	Semi solid
5	Acetone	Brown	Semi solid
5	Aqueous	Brownish orange	Solid

In Table 3 the disc diffusion tests demonstrated the inhibitory effects of ampicillin, ciprofloxacin and methanolic extract individually inhibited the microbial growth 10.0 mm, 30.33 mm and 15 mm respectively against MRSA.

Table 3: Antibacterial activity determined by Zone of inhibition method

S.No	Antibiotics and BB extract in ($\mu\text{g}/10\mu\text{l}$)	MRSA	<i>Enterococcus faecalis</i>	<i>Klebsiella pneumonia</i> NCIM 2719	<i>Enterobacter cloacae</i> NCIM 2164
1	Ampicillin 800($\mu\text{g}/10\mu\text{l}$)	10 $\pm$ 1	11.33 $\pm$ 2.1	9 $\pm$ 1.0	12.33 $\pm$ 2.1
2	Ciprofloxacin (5 $\mu\text{g}/10\mu\text{l}$)	30.33 $\pm$ 2.1	31.33 $\pm$ 2.1	29.67 $\pm$ 2.1	31 $\pm$ 2.1
3	70% Methanol extract	15 $\pm$ 1	15 $\pm$ 1	14 $\pm$ 1.3	14.67 $\pm$ 1

GC-MS showed that the presence of a ten different phytochemical compounds. The components were identified by compare their retention time and fragmentation patterns with those data stored in the National Institute of Standard and Technology (NIST) library. The reported chief constituents are Azulene derivatives, Cayophyllene, γ -Neoclovene, β -Humulene, Methanoazulene, Tetradecanoic acid and 13-Tetradecenal characterized and shown in Figure 1-3 and Table 4.

Figure 1: GC-MS chromatogram for methanol extract of *B.boerhavifolia* Figure 2: Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1 α ,7 α ,8 $\alpha\beta$)]-

Figure 3: 1H-Cyclopenta[a]pentalen-7-ol, decahydro-3,3,4,7a-tetramethyl-, 4-methylbenzenesulfonate- 3,3,4,7a-Tetramethylde

Table 4: Phytochemicals identified in the methanol extract of whole plant of *B.boerhavifolia* by GC-MS Peak

S.No	Name of the compound	Molecular formula	Retention index	Medicinal activity
1	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1 α ,7 α ,8 $\alpha\beta$)]-	C ₁₅ H ₂₄	1490	Anti-Inflammatory
2	1H-Cyclopenta[a]pentalen-7-ol, decahydro-3,3,4,7a-tetramethyl-, 4-methylbenzenesulfonate- 3,3,4,7a-Tetramethylde	C ₂₂ H ₃₂ O ₃ S	2723	—
3	1R,3Z,9S-2,6,10,10-Tetramethylbicyclo[7.2.0]undeca-2,6-diene	C ₁₅ H ₂₄	1499	—
4	Caryophyllene	C ₁₅ H ₂₄	1499	Anti-Oxidant, Local Anesthetic, Anti-leishmanial
5	Spiro[5.5]undec-2-ene, 3,7,7-trimethyl-11-methylene-,beta -Chamigrene-Spir	C ₁₅ H ₂₄	1507	-
6	Gamma-Neoclovene	C ₁₅ H ₂₄	1387	Anti-Bacterial, Anti-oxidant
7	Beta-Humulene	C ₁₅ H ₂₄	1530	Anti-inflammatory
8	Methanoazulene	C ₁₅ H ₂₆	1393	Anti-oxidant, Anti-bacterial, Anti-tumor
9	Tetradecanoic acid	C ₁₄ H ₂₈ O ₂	1769	Larvicidal and repellent
10	13-Tetradecenal	C ₁₄ H ₂₆ O	1591	Anti-oxidant, Anti-epileptic, Anti-bacterial

DISCUSSION

Extraction process is the preliminary step in medicinal plant research, since the preparation of crude extracts from the plants is the starting point for the evaluation of secondary metabolites present in the medicinal plants. During extraction, solvents diffuse into the solid plant material and solubilize compounds with similar polarity. The most common chemical procedure for obtaining organic constituents from dried plant tissues is to

continuously extract powdered material in a soxhlet apparatus with a range of solvents, starting with petroleum ether and chloroform and then using ethyl acetate and alcohol. Boiling ethanol is a good, all-purpose solvent for preliminary extraction¹⁰. The determination of extractable matter refers to the quantity of constituents present in a given medicinal plants. Here solvents like ethanol and ethyl acetate taken out more quantity of extractable matter when compared to other solvents in both plant extractions. Finally the prepared extracts were

subjected to preliminary phytochemical study. Among secondary metabolites some of these substances have an effect on biological systems which are considered as bioactive. Thus a simple definition of bioactive compounds in plants is secondary metabolites eliciting pharmacological or toxicological effects in human and animals¹⁴.

Literature review showed that the methanolic extracts of *B.boerhavifolia* have showed the presence of alkaloids, glycosides, proteins, amino acids, flavonoids, tannins and steroids¹⁵. It was reported that the petroleum ether, methanol and chloroform extracts of *B.boerhavifolia* leaves have showed the presence of alkaloids, flavonoids, saponins, phenols, tannins, cardiac glycosides, terpenoids¹⁶.

The preliminary phytochemical screening showed the presence of almost all compounds in whole parts of plant *B.boerhavifolia*. Especially, methanolic extract exhibited the presence of many important secondary metabolites like proteins, amino acid, flavonoids, tannins, phenolics and phytosterols. Methanolic extract showed the presence of variety of secondary metabolites and exhibited moderate to high yield when compared to other extracts. Hence the methanolic extracts of whole parts of plant *B.boerhavifolia* were subjected to *in vitro* antibacterial activity.

CONCLUSION

The results of the present study provided technical explanation for the use of the methanol extract of the whole plant of *Blepharis boerhavifolia*. The proportion of antibacterial activity indicated that the methanol extract is a possible source of bioactive compounds that could be useful for the development of new antibacterial agents capable of reducing the burden of drug resistance.

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