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Phytochemical compound identification and evaluation of antimicrobial activity of *Eugenia Bracteata* Roxb

Kavitha Alli, Lakshmi Narasu Mangamoori*.

Centre for Biotechnology, IST,
Jawaharlal Nehru Technological University, Hyderabad 500 072, India.
** email id: mangamoori@gmail. com phone no: 919490173899*

Abstract

There has been a renewed interest in naturally-occurring phytochemicals from fruits, vegetables and plants. Early studies showed antioxidant properties in *Eugenia Bracteata*. This study was aimed at identifying phytochemical compounds present in the extracts of the *Eugenia bracteata*. Powdered materials of leaves, stem and root were subjected to antimicrobial sensitivity test and Minimum inhibitory concentration test after extraction using rotavapor. Leaf extracts were further investigated by GC-MS to identify chemical compounds and analyze their medicinal activity to ascertain antimicrobal activity observed during antimicrobial sensitivity test. Methanol extract of leaves showed better antimicrobial activity compared to other extracts. 30 compounds identified from all extracts are known to possess antimicrobial, antioxidant and anti-inflammatory activities. Major compounds found in methanol extract like Caryophyllene, Tetradecanoic acid, alpha-bergamotene, Copaene validated its better antimicrobial activities. Antimicrobial activity of extracts and presence of terpenes and fatty acids among phytochemicals identified proved medicinal values of *Eugenia Bracteata*. This is the first report on antimicrobial activity and identification of phytochemicals from *Eugenia Bracteata*.

Key words: Eugenia Bracteata, Zone of inhibition, Agar well diffusion method, Minimum inhibitory concentration, phytochemical components, GC-MS analysis, traditional medicine.

Abbreviations:
MIC: minimum inhibitory concentration.
MBC: minimum bactericidal concentration

GC-MS: Gas Chromatography Mass Spectroscopy
NIST: National institute of standards and Technology

INTRODUCTION

For ages nature has gifted us plenty of herbs and plants which form the main source of traditional medicines used to help in relief from illness and are still widely used all over the world. Herbal treatment is still used for many health problems. Medicinal plants are safe, less toxic, economical and a reliable key natural resource of drugs all over the world[1]. Medicinal plants contain active principles which can be used as a substitute to despicable and valuable herbal drugs against ordinary bacterial infections. Indiscriminate use of man-made antibiotics has led to an alarming increase in antibiotic resistance among microorganisms [2-5], thus necessitating the need for development of novel antimicrobials [6-7]. Researchers are increasingly turning their attention to the medicinal plants and it is estimated that, plant materials are present in, or have provided the models for 25-50% Western drugs [8-10]. Development of a safer antimicrobial principle from medicinal plants include investigation of antimicrobial activity of extract, preliminary phytochemical analysis, isolation, characterization, identification and biological studies of potential compounds[11-12].
The aim of this study was to investigate the antimicrobial activity of extracts from medicinal plant used in folk medicine. *Eugenia Bracteata*Roxb(Wild) family: Myrtaceaecommonly known as kundanereduis a shrub of 2-4 tallwith pretty white flowers and red berries, bark yellowish grey smooth, wood grey hard, and close grained distributed along the hilly areas and most commonly inthe coastal regions of Andhra Pradesh, India [13]. *Eugenia Bracteata* was known to possess antimicrobial properties based on its usage in remedies followed by tribes of venadu (Andhra Pradesh, India) region, but there was no scientific evaluation of its antimicrobial properties till now. Preliminary phytochemical screening and agar well diffusion methods were used to investigate the presence of secondary metabolites and antimicrobial activity.

MATERIALS and METHODS

Collection of plant material:

Plants were collected from venadu island area of Andhra Pradesh, India and the plant was identified by Dr. Rasingham of the Botanical Survey of India, Hyderabad, India.

Chemicals:

Methanol, acetone, ethyl acetate, double distilled water. Muller-Hinton Agar and Muller-Hinton broth were bought from Himedia Mumbai, India.

Preparation of crude plant extract:

The fresh leaves, stem and root were taken and washed with the free-flowing, clean water and later cleansed further with distilled water. The washed leaves, stem and root were then, shade-dried to retain the active components of the plant material. After

drying, the plant material was chopped into small pieces and then, powdered using the mortar and pestle. Thirty grams of powdered material was dissolved in 300 mL of the solvent in a glass stoppered round bottomed flask. The mixture was shaken well and kept at room temperature in a shaking incubator for 72 hrs. The extracts were filtered by using Whatman No. 1 filter paper. Then, the extracts were concentrated in a rotavapor at reduced pressure below 40oC and evaporated to dryness in a vacuum oven at 40oC. The extracts obtained were stored at 4oC until further use.

Microorganisms:
All the microorganisms used for antimicrobial screening were procured from. A total of ten microorganisms were selected for screening, of which three (*Bacillus Subtilis* (MTCC 2391), *Pseudomonas aeruginosa* (MTCC 6642), *Escherichia coli* (MTCC 1563)) were procured from MTCC, chandigarh, India and rest of pathogens (*Staphylococcus aureus, Enterococcus faecalis and Micrococcus luteus, Salmonella typhimurium, Klebsiella pneumoniae, Enterobacter cloacae* and *Proteus vulgaris*) are clinical isolates from Global Hospitals, Hyderabad, India.. Bacterial stock cultures were maintained on Muller Hinton agar slants and were stored at 4^{o}C.

Antimicrobial activity
***Sensitivity test by agar well diffusion method*:**
Testing for antimicrobial activity was done on different microorganisms (four gram positive, six gram negative) according to the method described by [14-15]. Similar method was followed to measure the zone of inhibition for the antibiotic chloramphenicol at a concentration of 1mg/mL.

Minimum Inhibitory Concentration (MIC) :
The tube broth dilution assay described by [16] was followed for determining MIC. The two-fold serial dilution assay was performed in Muller-Hinton broth (MHB). This assay was repeated three times and final MIC was determined based on mean of the three values.

Minimum Bactericidal Concentration (MBC) :
For determination of MBC, a portion of 5µL from each tube that showing no turbidity was diluted serially with drug-free MHB and incubated at 37oC for 48h. The lowest concentration of the tube with no visible growth was regarded as MBC. MBC assay was also performed in triplicate.

GC-MS analysis:
The bioactive compounds in leaf extracts of *Eugenia Bracteata*were analyzed by GC-MS using an Agilent 6890 N gas chromatograph coupled to an Agilent 5975 N mass spectrometer. An HP-5MS capillary column (30m X 0. 25mm ID, 0. 25µm film thickness) was used for gas chromatographic separation. The instrument was set to an initial temperature of 60oC and maintained at this temperature (isothermal) for 2 minutes. The GC oven temperature was programmed from 60oC to 280oC at a rate of 10oC/min and ending with a 9min isothermal at 280oC. The injection volume was

2μL with a split ratio of 1:10, the injection temperature was held at 220oC. Helium (99. 999%) was used as carrier gas at 1mL/min. The mass spectrometer was operated in electron impact (EI) mode with ionization energy of 70 eV and source temperature was held at 280oC. MS spectra were obtained in the mass range of m/z 43-450. Interpretation of mass spectrum of GC-MS was done using database of NIST. Compound identification was done by comparing the retention times with those of authentic compounds and the spectral data obtained from NIST Ver2. 0 MS library data of the corresponding compounds.

RESULTS AND DISCUSSION

Antimicrobial sensitivity test:

The methanol, acetone and ethyl acetate extracts of leaves, stem and root has shown inhibition effects on all the ten microorganisms. Methanol extract of leaves showed the maximum zone of inhibition of 24mm. Methanol extracts have shown zone of inhibition ranging from 16mm to 24mm, acetone extracts have shown zone of inhibition from 12mm to 23mm and ethyl acetate has zone of inhibition from 10mm to 20mm. Chloramphenicol has shown Zone from 21mm to 24mm. All the values of zone of inhibition are shown in Table 2. Results also showed that the leaf had shown higher zone of inhibition compared to stem, root against most of the microorganisms, and leaf extract was found to be more effective against *Enterococcus faecalis*and *Salmonella typhimurium* and stem extract is found to be more effective against *Klebsiella pneumonia, Enterococcus faecalis*and *Salmonella typhimurium*.

This study recorded a notable susceptibility of these resistance strains, especially to leaf extract, suggesting that the components contained in that particular extract may provide an alternate strategy for combating these organisms and thus could improve the treatment of infections caused by these organisms.

MIC and MBC

Comparison of MIC, MBC values shows the extent of inhibitory activities of different extracts of leaves, stem. The inhibitory activities of methanol extracts of leaf, stem on *Pseudomonas aeruginosa* were close to that of standard drug Chloramphenicol than other extracts. The MIC's of leaves ranged from 0. 078mg/mL to 0. 625mg/mL. Stem ranged from 0. 07806mg/mL to 1. 250mg/mL and root ranged from 0. 3125mg/mL to 1. 250mg/mL.

The MIC, MBC values varied from one microorganism to another, but showed the same trend as that of zone of inhibition in most of the cases. Good results with methanol extract of leafs and acetone extract of stem indicate a difference in chemical nature of the constituents due to their solubility in different solvents.

GC-MS analysis

GCMS analysis of each sample contained 55-75 compounds that matched NIST library data. GC-MS phytochemical screening results of all extracts showed presence of 130 compounds whose peak area were greater than 1. Table 2 gives the retention times, relative area of the compounds with known medicinal activity. Some of the

main compounds like Caryophyllene, Tetradecanoic acid, alpha-bergamotene, Copaene, n-Hexadecanoic acid, phytol and some more fatty acids, heterocyclic acids were seen to be present in all the extracts.

Table 1: Antimicrobial sensitivity of all extracts and chloramphenicol – Zone is in mm and extracts is 10mg/mL

Pathogenic organism	Part tested	Aqueous	Methanol	Acetone	Ethyl acetate	Chloramphenicol 1mg/mL
S.aureus	Leaf	NIL	20-0.3125 (0.625)	22-0.625 (1.25)	18-0.3125 (0.625)	21-0.0125 (1.25)
	Stem	NIL	21-0.3125 (0.625)	22-0.3125 (0.625)	15-0.625 (1.25)	
	Root	NIL	20-0.625 (1.25)	19-0.625 (1.25)	12-1.25 (1.25)	
B.subtilis	Leaf	NIL	20-0.3125 (0.625)	21-0.156 (0.3125)	15-0.625 (1.25)	22-0.020 (0.020)
	Stem	NIL	19-0.3125 (0.625)	18-0.156 (0.3125)	14-0.625 (1.25)	
	Root	NIL	18-0.625 (1.25)	19-0.156 (0.3125)	10-1.25 (1.25)	
E.faecalis	Leaf	NIL	24-0.078 (0.3125)	23-0.3125 (0.625)	20-0.3125 (0.625)	21-0.006 (0.078)
	Stem	NIL	22-0.078 (0.3125)	23-0.156 (0.3125)	16-0.625 (1.25)	
	Root	NIL	18-0.3125 (0.625)	19-0.3125 (0.625)	10-0.625 (1.25)	
M.luteus	Leaf	NIL	20-0.3125 (0.625)	20-0.3125 (0.625)	16-0.3125 (0.625)	23-0.031 (0.312)
	Stem	NIL	20-0.3125 (0.625)	19-0.156 (0.3125)	15-0.625 (1.25)	
	Root	NIL	18-0.625 (1.25)	15-0.3125 (0.625)	10-1.25 (1.25)	
P.aeuruginosa	Leaf	NIL	21-0.078 (0.3125)	22-0.625 (0.625)	14-0.156 (0.3125)	23-0.078 (0.625)
	Stem	NIL	20-0.156 (0.3125)	18-0.156 (0.3125)	13-0.3125 (0.625)	
	Root	NIL	17-0.3125 (0.625)	18-0.156 (0.3125)	10-0.625 (1.25)	
E.coli	Leaf	NIL	20-0.3125 (0.625)	22-0.3125 (0.625)	13-0.3125 (0.625)	21-0.006 (0.312)
	Stem	NIL	20-0.3125 (0.625)	17-0.156 (0.3125)	12-0.3125 (0.625)	

	Root	NIL	16-0.625 (1.25)	12-0.3125 (0.625)	10-1.25 (1.25)	
S.typhimurium	Leaf	NIL	23-0.078 (0.156)	20-0.625 (1.25)	13-0.625 (0.625)	23-0.003 (0.312)
	Stem	NIL	23-0.156 (0.3125)	20-0.156 (0.3125)	13-0.625 (1.25)	
	Root	NIL	20-0.3125 (0.625)	19-0.3125 (0.625)	11-1.25 (1.25)	
K.pneumonia	Leaf	NIL	21-0.625 (0.625)	20-0.3125 (1.25)	14-0.3125 (0.625)	22-0.012 (0.156)
	Stem	NIL	20-0.625 (1.25)	23-0.3125 (0.625)	15-0.625 (0.625)	
	Root	NIL	17-1.25 (1.25)	19-0.3125 (0.625)	10-1.25 (1.25)	
E.cloacae	Leaf	NIL	19-0.3125 (0.625)	18-0.625 (1.25)	10-0.625 (0.625)	23-0.012 (0.312)
	Stem	NIL	20-0.625 (1.25)	19-1.25 (1.25)	16-1.25 (1.25)	
	Root	NIL	19-0.625 (1.25)	17-1.25 (1.25)	15-1.25 (1.25)	
P.vulgaris	Leaf	NIL	20-0.3125 (0.625)	20-1.25 (1.25)	16-0.625 (0.625)	21-0.039 (0.078)
	Stem	NIL	21-0.625 (0.625)	19-0.3125 (0.625)	13-1.25 (1.25)	
	Root	NIL	16-0.625 (1.25)	14-0.625 (1.25)	10-1.25 (1.25)	

Analysis also revealed presence of terpenes like Bicyclo[3. 1. 1] heptane, 2, 6, 6-trimethyl-, phytol, Hexadeca-2, 6, 10, 14-tetraen-1-ol,, 7, 11, 16-tetramethyl-, (E, E, E), Hexadeca-2, 6, 10, 14-tetraen-1-ol,, 7, 11, 16-tetramethyl-, (E, E, E)-, Bicyclo[3. 1. 1]heptane, 2, 6, 6-trimethyl-, [1R-(1α, 2β, 5α)]-. Samples contained small quantities of phenols. Some of the GC-MS peaks remained unidentified because lack of authentic samples and library data of corresponding compounds. The compounds with known medicinal properties were found in all extracts of leaf were Caryophyllene, n-Hexadecanoic acid, Copaene, 9, 12, 15-Octadecatrien-1-ol, (Z, Z, Z) – and. gamma.-Elemene. Results showed that more no of compounds were present in methanol extract with medicinal properties. This report is the first of its kind to analyze the chemical constituents responsible for the antimicrobial activity of *Eugenia Bracteata*. GC-MS analysis results showed the same trend observed in zone of Inhibition and MIC results.

The samples contained fatty acids and carboxylic acids which were known to possess antimicrobial properties. Samples also showed presence of terpenes, phenols, sterols, Quinone's, siloxanes, alkanes, alkaloids and small quantities of aromatic alcohols, proteins.

Fatty acids like n-Hexadecanoic acid, Tetradecanoic acid, 9, 12, 15-Octadecatrienoic acid, methyl ester, (Z, Z, Z)-were present in good quantities in most of the extracts. These components have reported anti-inflammatory, antimicrobial activities. Caryophyllene, linolenic acid, bergamotene and phytol have reported antioxidant, antimicrobial and anticancer activities. Better antimicrobial properties of methanol extract of leaf, acetone extract of stem can be attributed to fatty acids, terpenes and other phytochemicals with known antimicrobial activities. These extracts also had phytochemicals with known medicinal properties like anti-inflammatory, anti-oxidant and anti-cancer activities. However phytochemical composition of each extract is different from the other extracts. Studies on *Eugenia Bracteata* done till now [17] (Bharat and Suryanarayana 2014) were mostly focused on antioxidant activities [18] (Hemalatha et al 2008).

Table 3: Bioactive phytocomponents with peak area in each extract of leaves of *Eugenia Bracteata* by GC-MS analysis

S. No	Compound name	RT	Methanol	Ethyl acetate	Acetone	Medicinal values	Family	other name
1	Caryophyllene	9.26	16.67	11.28	14.66	anti-bacterial, anti-oxidant, anti-proliferative,	Terpene	
2	9, 12, 15-Octadecatrien-1-ol, (Z, Z, Z)-	16.85	8.5	1.24	1.5	antibacterial, antiviral, drugs of urinary, nervous system disorders	Fatty alcohol	Linoleyl alcohol
3	9, 12, 15-Octadecatrienoic acid, methyl ester, (Z, Z, Z)-	16.85	8.5	1.24	1.5	Anti-inflammatory, Cancer preventive, Anti-coronary,	Fatty acids	linolenic acid
4	Caryophyllene oxide	11.25	7.21	8.28	7.17	antifungal, antibacterial, antioxidant, anti-proliferative	Sesquiterpene	
5	Cycloisolongifolene	10.19	6.39			Anti-proliferative	Terpene	
6	trans-.alpha.-Bergamotene	9.43	5.21	4.15		Anti-oxidant, anti-proliferative, anti-inflammatory	Terpene	

7	.alpha.-Caryophyllene	9.67	4.61	3.73	4.75	Anti-inflammatory, antibacterial, antioxidant, anti-proliferative,	Terpene	
8	n-Hexadecanoic acid	15.21	1.99	1.34	1.38	Antioxidant, 5-Alpha reductase inhibitor	Fatty acid	palmistic acid
9	Tetradecanoic acid	15.21	1.99		1.38	antibacterial, cleansing, emulsifying agent	Fatty acid	myristic acid
10	2-Cyclohexen-1-one, 4-(1-methylethyl)-	11.54	1.94		1.92	Antimicrobial	Terpene	
11	Copaene	11.91	1.93	1.24	1.6	antimicrobial, antioxidant, anti-cancer, anti-inflammatory, anti-plasmodial	Sesquiterpene /alkene	
12	Eudesma-4(14), 11-diene	12.05	1.56			Antimicrobial	Terpene	
13	5-Methyl-5, 8-dihydro-1, 4-naphthoquinone	14.76	1.49		0.87	antibacterial, anti-inflammatory, and antipyretic	Quinone	
14	Isoaromadendrene epoxide	13.69	1.45		1.53	antimicrobial, anticancer, antioxidant	Sesquiterpene	
15	.gamma.-Elemene	10.92	1.43	1.72	1.55	anti-proliferative	Sesquiterpene	
16	Cyclohexane, 1-ethenyl-1-methyl-2-(1-methylethenyl)-4-(1-methylethylidene)-	10.92	1.43		1.55	anti-proliferative	Sesquiterpene	
17	alpha.-Cubebene	8.33	1.29	1.24	1.6	antiseptic, mouth wash		

18	Succinimide, N-phenyl-3-(2-vinylcyclohexyl)-	12.73	1.28			anesthetic, anticonvulsant, neuroprotective		
19	Succinimide, 3-(3-cyclohexen-1-yl)-N-phenyl-	12.73	1.28			anesthetic, anticonvulsant, neuroprotective		
20	Succinimide, 3-cyclohexyl-N-phenyl	12.73	1.28			anesthetic, anticonvulsant, neuroprotective		
21	Dibenzo[b, f][1, 4]thiazepine-11(10H)-thione	17.97	1.16	0.25		Antipsychotic, antimicrobial, anticancer,	Heterocyclic	
22	Phytol	16.62		0.68	1.59	Antimicrobial, Anticancer, Anti-inflammatory	Diterpenes	
23	Guaia-3, 9-diene	12.07			1.54	Used in cure for kidney, bladder diseases	Terpenoid	
24	4-Hexadecen-6-yne, (E)-	16.88			1.5	antimicrobial, antimalarial, cytotoxic		
25	2-Pyridylacetamide	17.41		1.8	0.69	Anticancer		

CONCLUSION

Presence of various phytochemicals with known antimicrobial activities confirms the antimicrobial activity of *Eugenia Bracteata*and shows that methanol extracts of leaf, acetone extract of stem shows better activity against all the test microorganisms than all other extracts. Leaf and stem of *Eugenia Bracteata*has better antimicrobial properties than roots and their effectiveness varied from pathogen to pathogen based on extract used.

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Conflict of Interest:
The authors declare that they have no conflict of interest

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