Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(2): 2181-2184
Received: 19-01-2018
Accepted: 20-02-2018

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Phytochemical Analysis and antimicrobial activites of *Embelia robusta*

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Abstract
Objective: To determine the Phytochemical composition and antimicrobial activites of *E.robusta* seeds extract (Myrsinaceae).
Methods: Phytochemical screening was conducted using standard Qualitative methods and antimicrobial analysis was determinated by agar well diffusion method.
Results: Preliminary phytochemical screening of the seed crude extract revealed the presence of alkaloids, flavonoids, Glycosides, Tannins, Saponins, Steriods & Triterpenoids. The presence of these bioactive constituents is related to the antibacterial activity of the seed extract. Agar well diffusion method exposed high degree of antimicrobial activity.
Conclusion: The results confirm that the *E.robusta* seeds extract have Phytochemical composition and antimicrobial activities, so it can be used as a source of drugs to fight against infection causing microorganisms. Further study is necessary for isolation and characterization of the active seed extract compound.

Keywords: *Embelia robusta*, phytochemical, antimicrobial, agar well diffusion

Introduction
Indian plant kingdom is a richest source of medicinal plants. Since ancient times, plants have been utilized as therapeutic agents for various kinds of ill health [1, 2]. These medicinal plants contain thousand of phytochemical constituents and are valuable sources of new and biologically active molecules, which can be divided chemically into a number of groups which are alkaloids, flavonoids, Glycosides, Tannins, Saponins, Steriods & Triterpenoids [3, 6].
Plant extract has been tested *in vitro* and *in vivo* for potential exploitation of their therapeutic activity in various diseases, including anticancer, antidiabetic, anti-inflammatory and antimicrobial properties [7]. The ultimate aim of recent research in this area has been the development of alternative control strategies to reduce dependency on synthetic bactericides, fungicides. These group of compounds serve as plant defence mechanism against pathogenic microorganism attack and predation by animals. These drugs from plants are easily available, inexpensive, safe, environmental-friendly, efficient and rarely accompanied by side effects [8].
Embelia robusta (Family-Myrsinaceae) commercially known as Vidang or Baibirang is one of the important Indian medicinal plant. This plant is a climber with slender branches and long internodes. The leaves are elliptic, covered with minute glands. The flowers are small, white racemes arranged in panicle inflorescence at the end of the branches. The fruits are like berries, round, red to black colour and tipped with style. The seed resembles so much to pepper and often referred as false pepper. The fruits are used as an antihelmintic, diuretic, carminative, contraceptive, anti-bacterial, anti-inflammatory, anti-astringent and also fruit decoction is useful in fever and diseases of chest and skin [9].
This study presents a preliminary phytochemical investigation of *E.robusta*, which is responsible for the biological activity of the extracts of the seed on both antibacterial and antimicrobial activity.

Material and Methods
Collection of plant material
Mature *E.robusta* seeds were collected from local firm near Hyderabad, identified by Department of Botany, Osmania University for women, Koti, Hyderabad.

Material required for phytochemical screening and antimicrobial activity
Dilute Hydrochloric Acid, Mayer's reagent (Potassium Mercuric lodide), Wagner's Reagent (Iodine in Potassium lodide), Distilled water, Lead acetate solution, Sodium hydroxide solution, Gelatin solution, Glacial acetic acid, ferric chloride, Conc.

Sulphuric acid, Chloroform, acetic anhydride, Sulphuric acid and potato dextrose Agar (PDA) Nutrient agar (NA)**.** All chemicals were of analytical grade and were purchased from commercial sources.

Preparation of seed extract

Healthy and fresh *E.robusta* seeds were collected, washed with tap water for several times and shade dried, Air-dried seed powder was extracted twice with 1.5 liters of methanol for 72hr at 60°c in a Soxhlet apparatus. The seed extract was further concentrated in under rotary evaporator; residues were weighed and stored in sterile glass bottles at room temperature until screened. Different concentrations of stock solutions were prepared by dissolving the plant extract in dimethyl sulphoxide (DMSO).

Preliminary phytochemical screening

The phytochemical studies were performed as described by Harborne JB (1998), Kokate KC (1997) [10, 11] for the presence of alkaloids, flavonoids, saponins, tannins, glycosides.

Test for alkaloids

Mayer's test: Filtrate extract was treated with Mayer's reagent and formation of a yellow colored precipitate indicates the presence of alkaloids.

Wagner's test: Filtrate extract was treated with Wagner's reagent and formation of brown/reddish precipitate indicates the presence of alkaloids.

Test for flavonoids

Lead acetate test: Extract was treated with few drops of lead acetate solution and formation of yellow colour precipitate indicates the presence of flavonoids.

Alkaline reagent test: Extract was treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which become colourless on addition of dilute acid, indicates the presence of flavonoids.

Test for Saponins

Foam test - Extract was mixed with water and shaken by hand for 15 min. A foam layer was obtained on the top of the test tube. This foam layer indicates the presence of saponins.

Test for Tannins

Gelatin Test – Test solution when treated with gelatin solution gave white precipitate indicating the presence of tannins.

Test for Glycosides

Keller Killiani Test – Test solution was treated with few drops of glacial acetic acid and ferric chloride solution and mixed. Concentrated sulphuric acid was added, and observed for the formation of two layers. Lower reddish brown layer and upper acetic acid layer which turns bluish green would indicate a positive test for glycoside.

Test for Steroids/Triterpenoids: Extract was treated with chloroform, then acetic anhydride was added and sulphuric acid was added along the sides of the tube. Colour formation at the junction is noted that the appearance of blue-green colour indicates the presence of steroids, the appearance of red, pink, violet colour at the junction indicates the presence of triterpenoids.

Culture and maintenance of microorganisms

The standard strains of *Bacillus subtilis* (ATCC 633), *Staphylococcus aureus* (ATCC 9144), *Escherichia coli* (ATCC25922) and *Pseudomonas Aeruginosa* (ATCC25619) although standard strains of *Aspergillus niger* (MTCC 281), *Rhizopus oryzae* (MTCC 262), *Aspergillus terreus* (MTCC 1281), *Cladosporium species* (MTCC 1003), *Colletotricum crassipes* (MTCC 2223), *Collectotricum capsici* (MTCC 2071), *Armillaria mellea* (MTCC 409), *Candida albicans* (MTCC 183). Pure cultures of all experimental bacteria and fungi were obtained from the Institute of Microbial Technology, Chandigarh. The Pure bacterial cultures were maintained on nutrient agar medium and fungal culture on potato dextrose agar (PDA) medium. Each bacterial and fungal culture was further maintained by sub culturing regularly on the same medium and stored at 4^0C before use in experiments.

Media Preparation and sterilization

The 39g of ready-made potato dextrose Agar (PDA) and Nutrient agar (NA) media (Hi media) was weighed and dissolved in 900 ml of distilled water, stirred well and made up to one litre with addition of distilled water. The media was autoclaved at 121^0c at 15 PSI for 15 minutes.

Determination of antimicrobial activity by agar well diffusion method

Agar well-diffusion method was employed for determining the antimicrobial activity of seed extract. Potato dextrose Agar (PDA) and Nutrient agar (NA) media were poured in petriplates. The media was poured into Petri dishes under aseptic conditions in a laminar flow chamber and left to solidify.

Seven day old broth Cultures of both selected bacteria and fungi organism (0.5ml) were inoculated onto the medium. 8 mm wells were made in each of these plates using sterile cork borer. Stock solution of seed extract at a concentration of 1mg/ml was prepared and different concentrations of test solutions were added into this wells and allowed to diffuse for 2hrs at room temperature. Then the plates were incubated at 37^0C for 24 h for bacterial and 28^0C 48 h for fungal pathogens. Triplicates were maintained and the experiment was repeated thrice and for each replicates readings and average values were recorded.

Table 1: Phytochemical Screening of *Embelia robusta.*

Secondary metabolites	Test	Methanol	Aqueous	Ethanol	Hexane	Chloroform
Alkaloids	Mayer's test	+	+	+	-	+
	Wagner's test	+	+	+	-	+
Glycosides	Killer killiani test	+	-	-	-	-
Flavanoids	Lead acetate test	++	-	-	-	-
	Alkaline reagent test	++	-	+	+	++
Tannins	Gelatin test	+++	-	+	-	+
Saponins	Foam test	+	+	+	-	++
Steriods Triterpenoids	Liebermann Burchard test	-	-	+	-	-

Table 2: Antibacterial activity of *Embelia robusta*

Concentration Methanol	Ampicilion	DMSO	*Pseudomonas Aeruginosa*	*Bacillus subtilis*	*Klbsiellae pneumonia*	*Escherichia coli*	*Staphylococcus aureus*
10	5	-	4	4	4	3	4
25	6	-	5	5	4	4	5
50	8	-	5	6	5	6	6
100	8	-	6	7	5	7	7
150	10	-	7	9	6	8	8
Concentration Hexone							
10	5	-	-	-	-	-	-
25	6	-	-	-	-	-	-
50	8	-	-	-	-	-	-
100	8	-	-	-	-	-	-
150	10	-	-	-	0.5	0.5	-
Concentration Chloroform							
10	5	-	-	-	-	-	-
25	6	-	-	-	-	-	-
50	8	-	-	-	-	-	-
100	8	-	-	-	-	-	-
150	10	-	-	0.5	0.5	0.5	-

Table 3: Antifungal activity of *Embelia robusta*

Concentration Methanol	Bavistin	DMSO	*Aspergillus niger*	*Rhizopus oryzae*	*Aspergillus terreus*	*Cladosporium species*	*Colletotricum crassipes*	*Collectotricum capsici*	*Armillaria mellea*	*Candida albicans*
10	5	-	-	-	-	-	-	-	-	-
25	6	-	-	-	-	-	-	-	-	-
50	8	-	2	4	4	6	5	5	5	5.5
100	10	-	3	6	4.5	6.5	7	5.5	6	6
150	12	-	3	10	5	7	10	6.5	8	6
Concentration Hexone										
10	5	-	-	-	-	-	-	-	-	-
25	6	-	-	-	-	-	-	-	-	-
50	8	-	1	-	-	1	-	-	-	-
100	10	-	1.5	-	-	2	-	0.5	1	0.5
150	12	-	2	0.5	-	2.5	1	1	1.5	1
Concentration Chloroform										
10	5	-	-	-	-	-	-	-	-	-
25	6	-	-	-	-	-	-	-	-	-
50	8	-	0.5	5	5	4	5	4	3	2
100	10	-	2	6	6.5	5	5.5	4.5	4	2
150	12	-	3	8	7	5.5	6	5	4	4

Results and Discussion

The Preliminary phytochemical screening of crude extract of *E.robusta* revealed the presence of active phytoconstituents such as alkaloids, flavanoids, tannins, saponins, steroids, triterpenoids, using methanol, hexone, chloroform were analysed and investigated were summarized in table 1. The presence of alkaloids is interesting, as significant quantities are used as antimalarials, analgestics, and stimulants [12]. The flavonoids, which are known to inhibit tumor growth and also used to protect against gastrointestinal infections and are of pharmacognostic importance thus giving confirmation to the plant ethnomedicine [13].

The antibacterial activity of seed extracts was proved to possess potential antimicrobial activity which was estimated using standard conventional methods against the four bacterial strains. Among them two were Gram positive namely *Bacillus subtilis*, *Klbsiellae pneumonia* and three were negative bacteria namely *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas Aeruginosa* as well as standard strains of fungi *Aspergillus niger*, *Rhizopus oryzae*, *Aspergillus terreus*, *Cladosporium species*, *Colletotricum crassipes*, *Collectotricum capsici*, *Armillaria mellea*, *Candida albicans.*

The methanolic seed extract of *E.robusta* exhibited moderate antimicrobial activity against the test organism at all the concentrations of 10µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/m. The maximum zone of inhibition i,e. 12mm for *Bacillus subtilis*, 10mm for *Staphylococcus aureus*, 9mm for *Escherichia coli* and 10mm for *Pseudomonas Aeruginosa* were observed at the highest concentration of 150 µg/m.

However, hexane extract of *E.robusta* showed the zone of inhibition against two pathogens viz., *Klbsiellae pneumonia* (20mm), *Escherichia coli* (20mm), and chloroform extracts showed the zone of inhibition against three *Bacillus subtilis*, *Klbsiellae pneumonia*, *Escherichia coli* pathogens at the highest concentration of 150 µg/m respectively, chloroform and hexane extract of *E.robusta* could not inhibit the growth of pathogens at all concentrations according to the results given in the (Table 2).

On the other hand, standard antibiotic ampicillin showed more significant antibacterial activity against all tested gram positive and gram negative bacteria showed the zone of inhibition in methanol, ethanol, chloroform seed extract at concentration 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/m showed the zone of inhibition 5 mm, 25 mm, 50 mm, 100 mm, 150 mm respectively, in comparison with negative control DMSO has not shown zone of inhibition for respective organisms.

The antifungal activity of seed extracts *E.robusta* exhibited moderate antimicrobial activity. 13mm for *Aspergillus niger*, *Rhizopus oryzae*, *Aspergillus terreus*, *Cladosporium species*, *Colletotricum crassipes*, *Collectotricum capsici*, *Armillaria mellea*, *Candida albicans* at concentrations 50 µg/ml, 100

µg/ml, 150 µg/m of methanolic seed extract of *E.robusta* showed small potential at most of the concentration against all fungal strains (Table 3). *Aspergillus niger, Cladosporium species* at the concentration of 50 µg/ml showed small potential antifungal activity against all bacterial strains and at the concentration 100 µg/ml of seed extracts *E.robusta* showed in *Aspergillus niger, Aspergillus terreus, Cladosporium species, Collectotricum capsici, Armillaria mellea, Candida albicans. Aspergillus terreus* did not show any activity at the concentration 150 µg/ml of seed extract against all bacterial strains. The chloroform crude extract showed potential activity at concentrations 50 µg/ml, 100 µg/ml, 150 µg/ml against all the fungal pathogen. Chloroform crude extract showed activity at all concentrations and the results are given in table 3.

At the concentrations of 10 µg/ml, 25 µg/ml there was no activity in solvents like methanol, hexone and chloroform against in all fungal pathogens.

The inhibition zone of methanol, ethanol, chloroform seed extract in comparison with standard positive control at concentration 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/m showed the zone of inhibition 5 mm, 25 mm, 50 mm, 100 mm, 150 mm respectively, in comparison with negative control DMSO has not shown zone of inhibition for respective organisms.

In vitro antimicrobial efficacy of the crude extract of *E.robusta* was quantitatively assessed on the basis of zone of inhibition. Antimicrobial activity of *E.robusta showing* varing degree of inhibitaroy effect against the selected bacterial and fungal pathogen. It has been shown that when solvents like methanol, hexane, chloroform are used to extract the active compound, most of them are able to exhibit inhibitory effect on both gram negative and gram positive bacterial and fungal pathogen [14].

Conclusion

The present study with preliminary Phytochemical screening and antimicrobial activity of the methanolic seed extract of *E.robusta* showed that the presence of alkaloids, flavonoids, Glycosides, Tannins, Saponins, Steriods & Triterpenoids and this crude extract could serve as potential sources of new antimicrobial activites. However, the mechanism of the action of these plant constituents is not fully known it is clear that the effectiveness of the extract largely depends on the type of solvent used. Phytochemical and pharmacological studies are required to under the nature of the antibacterial agents present in seed extract. Further research is needed towards isolation and identification of active principles present in the extract which could be used for pharmaceutical.

Acknowledgement

The authors are thankful to UGC-RFSMS, New Delhi for providing fellowship, the Central facilities for research and development and head, Department of Genetics, Osmania University, Hyderabad for providing all the facilities for our research work.

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