

Screening of Antimicrobial Potential of Flavonoids Extracted from *Erythrina indica*

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ABSTRACT

Context: Antibiotic resistance has become a global concern. There has been an increasing incidence of multiple resistances in human pathogenic microorganism in recent years, largely due to indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases. Flavonoids are group of naturally occurring chemical compounds, which are known to have antimicrobial properties. **Aim:** Present work was carried out for screening of the antimicrobial potential of flavonoids extracted from *E.indica* against some multidrug resistant pathogenic bacteria and fungi. **Material and Methods:** Different plant parts (stem, root, and leaves) of *E.indica* were collected and air dried and then Soxhlet extracted by using standard method for flavonoids extraction. After this, extracts were tested for their antimicrobial activity using well diffusion method. Minimum inhibitory concentration and total activity were also calculated. **Statistical Analysis:** Mean value and Standard Deviation were calculated for the test bacteria and fungi. Data were analyzed by one way analysis of variance and P values were considered significant at $P < 0.05$. **Results:** *P. aeruginosa* was found to be the most susceptible organism followed by *B.subtilis*, *S. aureus*, and *P.funiculosum*. *A. niger* was observed resistant as none of the tested extract show activity against this fungi. The range of MIC was found to be 0.019-0.625 mg/ml. **Conclusion:** Result of the present study reveals that extracts of *E.indica* are showing antimicrobial potential against tested microorganism and can be used for future antimicrobial drug.

Keywords: Flavonoids, minimum inhibitory concentration, total activity, well diffusion method.

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INTRODUCTION

Plant-based antimicrobials represent a vast untapped source for medicines and further exploration of plant antimicrobials is needed as antimicrobials of plant origin have enormous therapeutic potential. They may act as lead compounds for the pharmaceutical industry or as the base for the development of new antimicrobials [1, 2].

Today, world is gradually turning to herbal formulations, which are known to be effective against a large repertoire of diseases and ailments. More importantly they are not known to cause any notable derogatory effects and are readily available at affordable prices [3]. In the developing countries synthetic drugs are not only expensive and inadequate for the treatment of diseases, but are also often with adulterations and side-effects, as a result,

different remedies evolved in different regions of the world [4]. The World Health Organization has reported that more than 80% of the world's population in developing countries depends primarily on herbal medicine for basic health-care needs. Hence, there is a need to search plants of medicinal value.

Due to indiscriminate use of antimicrobial drugs, the microorganisms have developed resistance to many antibiotics. They have created immense clinical problem in the treatment of infectious diseases [5]. This situation has risen to an alarming level. Plants used in the traditional medicine contain a vast array of active substances that can be used to treat many human diseases [6]. Plant extracts have been proposed to be used as antimicrobial substances [7]. To determine the potential

and to promote the use of herbal medicines, it is essential the study of medicinal plants that find place in folklore [8].

The experimental plant *E.indica* belongs to family *Fabaceae*, is a medium sized deciduous small tree with prickly stem and branches. This plant contains very important medicinal properties. Its leaves are used in fever, inflammation and joint pain. Leaves juice along with honey is used to kill tapeworm, roundworm and threadworm. Antibacterial activity of isoflavonoids isolated from *Erythrina* against methicillin resistant *S.aureus* has been studied. Erycristagallin and orientanol B isolated from root extract shows antibacterial activity against methicillin resistant *S.aureus* [9].

Hence, in the present work an extraction and screening for antimicrobial activity of the flavonoids of *E.indica* has been undertaken.

MATERIALS AND METHODS

Various plant parts of *E.indica* (leaves, stem, and roots) were collected from Kapoor chand koolish garden, J.L.N road, Jaipur and authenticated. The voucher (RUBL* No. 211320) of experimental plant was deposited in the Herbarium of Department of Botany, University of Rajasthan, Jaipur. Plant parts were separated, cleaned and oven dried at 35°C for 30 min and then at 25°C till constant weight was achieved and powdered.

Preliminary Detection of Flavonoids

Two methods were used to determine the presence of flavonoids in each sample (root, stem, and leaf) of *E.indica* [10,11]. The aqueous extracts were prepared by soaking 50 g of dried powdered sample in 100 ml of distilled water for 12 h. The extracts were filtered using Whatman filter paper No. 1.

Dilute ammonia solution (5 ml) was added to a portion of the aqueous filtrate of each part of the plant, followed by addition of concentrated H₂SO₄. A yellow color observed in each extract indicated the presence of flavonoids. The yellow coloration disappeared on standing.

Few drops of NaOH solution was added to each test extract. A yellow color observed in each extract. The color disappeared after addition of dilute acid, indicate the presence of flavonoids.

Extraction of Flavonoids

Different parts of *E.indica* (root, stem, and leaf) were subjected for flavonoids extraction, following the well established method [12]. Hundred grams of finely powdered plant part was Soxhlet extracted with hot 80% methanol (500 ml) and filtered. Filtrate was re-extracted successively with petroleum ether (fraction I), ethyl ether (fraction II) and ethyl acetate (fraction III) using separating funnel. Petroleum ether fraction was discarded due to being rich in fatty substances, whereas ethyl ether and ethyl acetate fractions were analyzed for free and bound flavonoids, respectively. Ethyl acetate fraction was hydrolyzed by refluxing with 7% H₂SO₄ for 2 h (for removal of bound sugars from the flavonoids). Resulting mixture was filtered and filtrate was extracted with ethyl acetate in separating funnel. Ethyl acetate extract thus obtained was washed with distilled water till neutrality. Ethyl ether (free flavonoids) and ethyl acetate fraction (bound flavonoids) were dried in vacuo and weighed.

Selected Test Microorganisms

Pathogenic microorganisms selected for the study included four bacteria, namely, *P. aeruginosa* (MTCC 1934), *S. aureus* (MTCC 3160), *B. subtilis* (MTCC 121), *S. pneumoniae* (2672), four fungal strains, namely, *A. niger* (MTCC 282), *P. funiculosum* (2552), *T. reesei* (3929), *F. moniliforme* (6576). The selected microorganisms were procured from IMTECH, Chandigarh, India. The bacterial strains were grown and maintained on 'Muller-Hinton Agar Medium' (Beef extract 2.0 g; Peptone 17.5 g; Starch 1.5 g; Agar 17.0 g; in 1000 ml of distilled water; Final pH 7.4±0.2 at 37±2°C), while fungal strains were kept on 'Sabouraud Dextrose Agar Medium' (Peptone 10 g; Dextrose 20; Agar 20 g in 1000 ml of distilled water; pH adjusted to 6-8-7.0 at 27±2°C).

Determination of Antibacterial Assay

Antibacterial activity of the crude methanol extract was studied against gram positive and negative bacterial strains by the agar well diffusion method [13]. Mueller Hinton Agar No. 2 (Hi Media, India) was used as the bacteriological medium. The extracts were diluted in 100% dimethylsulphoxide at the concentrations of 5 mg/ml. The Mueller

Hinton agar was melted and cooled to 48.50 °C and a standardized inoculum (1.5×10^8 CFU mL⁻¹) (0.5 mcfarland) was then added aseptically to the molten agar and poured into sterile petridishes to give a solid plant. Wells were prepared in the seeded agar plates. The test compound (100 µl) was introduced in the well (6 mm). The test compound (100 µl) was introduced in the well (6 mm). The plates were incubated overnight at 37°C. The antimicrobial spectrum of the extract was determined for the bacterial species in terms of zone sizes around each well. The diameters of zone of inhibition produced by the agent were compared with those produced by the commercial control antibiotic streptomycin and ampicillin. For each bacterial and fungal strain, controls were maintained where pure solvents were used instead of the extract. The control zones were subtracted from the test zones and the resulting zone diameter was measured with antibiotic zone reader to nearest mm. the experiment was performed in triplicate to minimize the error and the mean values are presented.

Determination of Antifungal Assay

Antifungal activity of the experimental plant was investigated by agar well diffusion method [14]. The yeasts and saprophytic fungi were sub cultured on Sabouraud's Dextrose Agar (SDA; Merck, Germany) medium and respectively incubated at 37°C for 24 h and 25°C for 2-5 days. Suspensions of fungal spores were prepared in sterile PBS (Phosphate buffered saline) and adjusted to a concentration of 10^6 cells mL⁻¹. Dipping a sterile swab into the fungal suspension was rolled on the surface of the agar medium. The plates were dried at room temperature for 15 min. Wells of 100 mm in diameter and about 7 mm apart were punctured in the culture media using sterile glass tube. 0.1mL of several dilutions of fresh extracts was administered to fullness for each well. Plates were incubated at 37 °C. After incubation of 24h, bioactivities were determined by measuring the diameters of inhibition produced were compared with those of standard antifungal agent. All the experiments were performed in triplicate All the experiments were performed in triplicate and mean values were taken.

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) was determined for the plant extract showing antimicrobial activity against test pathogens in a disc diffusion assay. The broth micro-dilution method was followed for determination of the MIC values[15]. Plant extracts were re-suspended in acetone (which has no activity against test microorganisms) to make a 10 mg/ml final concentration, and this was added to the broth media in 96-wells of microtitre plates using a two-fold serial dilution. Thereafter, a 100 µl inoculum of standard size was added to each well. Bacterial and fungal suspensions were used as negative controls, while broth containing the standard drug was used as a positive control. The microtitre plates were incubated at 37±2°C for 24 hours for bacteria, 27±2°C for 48 hours for yeast, and 27±2°C for five to seven days for fungi. Each extract was assayed in duplicate and each time two sets of microtitre plates were prepared; one was kept for incubation, while another set was kept at 4°C for comparing the turbidity in the wells of the microtitre plate. The MIC values were taken as the lowest concentration of the extracts in the well of the microtitre plate that showed no turbidity after incubation. The turbidity in the well in the microtitre plate was interpreted as a visible growth of microorganisms.

Total activity (TA)

Total activity (TA) is the volume at which the test extract can be diluted without losing the ability to kill microorganisms. It is calculated by dividing the amount of extract from 1 g of plant material by the MIC of the same extract or an isolated compound, and is expressed in ml/g [16]. In mathematical terms it can be expressed as :
Total activity = Amount extracted from 1 g plant material/MIC of the ext

Statistical Analysis

Mean value and standard deviation were calculated for each test bacteria and fungus. Data were analyzed by one-way ANOVA and P. values were considered significant at $P < 0.05$.

RESULTS**Qualitative and Quantitative Estimation**

All the parts (Stem, roots, and leaves) of *E.indica* showed positive response in the preliminary detection test of flavonoids. Flavonoid content estimated in each gram of dried plant material was recorded [Table1].Content of free flavonoids were

obtained maximum in leaves (8.25 mg/g.d.w)and minimum in roots (4.75mg/g.d.w) whereas bound flavonoids was maximum in stem (0.55 mg/g.d.w) and minimum in leaves (0.35mg/g.d.w).Total flavonoids yield was observed maximum in leaves (8.60 mg/g.d.w) whereas minimum in roots (5.25 mg/g.d.w).

Table 1: Quantitative Estimation of Flavonoids of *E.indica*

Parts	Flavonoids (mg/g.d.w.)		BOUND (mg/g.d.w.)	TOTAL (mg/g.d.w.)
	FREE			
Stem	5.0		0.55	5.55
Root	4.75		0.50	5.25
Leaf	8.25		0.35	8.60

Antimicrobial Activity

Flavonoid extracts of *E.indica* were screened for antibacterial activity (Table -2) and antifungal activity (Table-3) against selected test microorganisms. Result indicated that all four extracts showed significant antibacterial activity and found active against all tested bacteria while no extract show activity against *A. niger*. Maximum antibacterial activity were recorded for leaf bound flavonoids against *P.aeruginosa* (IZ = 15 and AI = 0.75 ± 0.05) and against *S. aureus* (IZ = 15 and AI = 0.625 ± 0.041),for root extract against *P. aeruginosa* (IZ= 14 and AI = 0.70± 0.04) and

for stem extract against *S.aureus* (IZ = 14 and AI = 0.583 ± 0.042).Among free flavonoids only leaf free flavonoids show maximum activity against *S.aureus* (IZ=13 and AI=0.541±0.04). Highest antifungal activities were recorded for stem bound flavonoids against *P.funiculosum* (IZ = 16 for AI = 0.72 ± 0.04), for root extract against *P. funiculosa* (IZ =13,AI = 0.590 ± 0.05),for leaf extract against *F. moniliformae*(IZ=13 and AI=0.72±0.05). Among all extracts only stem free flavonoids show antifungal activity against *P. funiculosum* (IZ=12 and AI=0.54±0.04).

Table 2: Antibacterial Activity of Flavonoids of *E.Indica* Against Some Pathogenic Bacteria

Plant Parts	Extract	Test microorganism							
		<i>Bacillus subtilis</i>		<i>Pseudomonas aeruginosa</i>		<i>Staphylococcus aureus</i>		<i>Streptococcus pneumoniae</i>	
		IZ mm	AI	IZ mm	AI	IZ mm	AI	IZ mm	AI
Stem	E1	-	-	11	0.55± 0.045	-	-	-	-
	E2	13	0.65± 0.05	13	0.65± 0.045	14	0.583±0 .042	11	0.50±0. 045
Root	E1	-	-	-	-	-	-	-	-
	E2	9	0.45± 0.05	14	0.70± 0.04	11	0.458± 0.040	-	-
Leaf	E1	-	-	12	0.60± 0.05	13	0.541±0 .041	-	-
	E2	10	0.50± 0.05	15	0.75± 0.05	15	0.625±0 .041	13	0.590±0 .045

E1 = Free flavonoid;

E2 = Bound flavonoid;

±SEM (Standard Error Mean), (-): No inhibition

Standards: Streptomycin for bacteria; IZ - inhibition zone; AI- Activity index

Table 3: Antifungal Activity of Flavonoids of *E.Indica*

Plant Parts	Extract	Test microorganism							
		<i>A. niger</i>		<i>T. reesie</i>		<i>F.moniliforme</i>		<i>P. funiculosum</i>	
		IZ mm	AI	IZ mm	AI	IZ mm	AI	IZ mm	AI
Stem	E1	-	-	-	-	-	-	12	0.54± 0.045
	E2	-	-	12	0.66± 0.06	14	0.77± 0.05	16	0.72± 0.40
Root	E1	-	-	-	-	-	-	-	-
	E2	-	-	-	-	-	-	13	0.590±0 .05
Leaf	E1	-	-	-	-	-	-	-	-
	E2	-	-	11	0.611± 0.055	13	0.72± 0.05	-	-

E1 = Free flavonoid;

E2 = Bound flavonoid;

± SEM (Standard Error Mean), (-): No inhibition,

Standards: Ketokenozol for fungus; IZ - inhibition zone; AI- Activity index

MIC Value

MIC values were evaluated for the active extracts [Table -4]. The range of MIC of extracts recorded was 0.019-0.625 mg/ml. In the present investigation, lowest MIC value (0.019 mg/ml) were recorded for root bound flavonoids against *B. subtilis*, leaf

bound flavonoids against *B.subtilis*. Highest values of MIC (0.625 mg/ml) were recorded for leaf bound flavonoids against *S. aureus*, and *P.aeruginosa* and for stem bound flavonoids against *P. funiculosum*. Among all extract stem and leaf extracts show strong Antibacterial and Antifungal potential.

Table 4: Minimum Inhibitory Concentration of Flavonoids of *E.Indica*

Plant parts	Extract	<i>B. subtilis</i>	<i>P. aerugi nosa</i>	<i>S. aureus</i>	<i>S. pneum oniae</i>	<i>A. niger</i>	<i>T. reesie</i>	<i>F. monilifo rme</i>	<i>P. funiculosum</i>
		MIC	MIC	MIC	MIC	MIC	MIC	MIC	MIC
Root	E1	-	-	-	-	-	-	-	-
	E2	0.019	0.312	0.078	-	-	-	-	0.312
Leaf	E1	-	0.039	0.045	-	-	-	-	-
	E2	0.019	0.625	0.625	0.312	-	0.078	0.312	-
stem	E1	-	-	-	-	-	-	-	0.156
	E2	0.078	0.078	0.156	0.078	-	0.156	0.312	0.625

All figures are in mg/ml unit;

E1: Free flavonoids;

E2: Bound flavonoids;

(-) Not determined since there was no activity;

MIC - minimum inhibitory concentration.

Total Activity

Total activity indicated the volume at which extract can be diluted with still having ability to kill microorganism. Total activity for all flavonoids was calculated & Tabulated [Table-5]. Maximum total activity

values were calculated for leaf free flavonoids against *P.aeruginosa* (211.53 ml/g), and *S. aureus* (183.33 ml/g). For bound flavonoids maximum activity was observed for root against *B.subtilis* (26.31mg/g).

Table 5: Total Activity of Flavonoids of *E.indica*

Plant Parts	Extract	Total activity (ml/g)							
		<i>B. subtilis</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	<i>S. pneumoniae</i>	<i>A. niger</i>	<i>T. reesie</i>	<i>F.monili forme</i>	<i>P. funiculo sum</i>
Root	E1	-	-	-	-	-	-	-	-
	E2	26.31	1.60	6.41	-	-	-	-	1.60
Leaf	E1	-	211.53	183.33	-	-	-	-	-
	E2	18.42	0.56	0.56	1.12	-	4.48	1.12	-
stem	E1	-	-	-	-	-	-	-	3.52
	E2	7.05	7.05	3.52	7.05	-	3.52	1.76	0.88

E1: Free flavonoids;

E2: Bound flavonoids;

(-) Not determined since there was no activity

DISCUSSION

Medicinal plants may be a viable alternative source to costly antibiotic, as most of the medicinal plants are safe with little or no side effects, cost effective and have ability to affect a wide range of antibiotic resistant microorganism.

Screening of plant under investigation (*E.indica*) so far has not been worked out for flavonoids. Mostly the crude extracts have been screened of the whole aerial part, without MIC and TA determination. Such studies could only indicate their antimicrobial potential but are not helpful in establishing them as an antibiotic, hence cannot replace the existing antibiotics. Extract under study showed activity against both gram positive and negative bacteria. Although it is considered that Gram negative bacteria are less sensitive to plant extract, possibly as a result of their extra lipo-polysaccharide and protein cell wall that provides permeability barrier to the antibacterial agent [17]. Activity of tested extracts against both gram positive and negative bacteria suggests its wide range of effectiveness against pathogens. In the light of the fact that microorganism are becoming resistant to the drugs in use, the present study is of great significance, as far as the future drugs are concerned and uses of selected plant by the pharmaceutical industries for preparing plant based antimicrobials drugs.

CONCLUSION

The present finding can be of commercial interest to both pharmaceutical companies and research institutes in the production of new antimicrobial drugs. More importantly,

there have been no side effect reports from many years on this plant. There is still a lot of scope for further research, especially towards the mechanism of biological activity of phytochemicals from this plant.

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