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Screening of *Embelia ribes* for Antifungal Activity

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ABSTRACT

The fruits of *Embelia ribes* reported to contain mainly benzoquinone derivatives such as Embelin (2, 5-dihydroxy-3-undecyl-2, 5-cyclohexadiene-1, 4-benzoquinone). Chemical structure of Embelin is having quite resemblance with the structure of natural Coenzyme Q10 (ubiquinones) and the role of this is well defined in various biochemical protective mechanism. Aim of the present study was to evaluate the antifungal activity of *Embelia ribes* (*Myrsinaceae*) plant extracts using standard *in vitro* antifungal susceptibility test methods like NCCLS M27- A2 protocol (The National Committee for Clinical Laboratory Standards , USA) and EUCAST (European Committee for Antifungal Susceptibility Tests). Values of the MIC_{50} obtained by NCCLS method revealed that Methanol extract and Embelin exhibited lowest MIC_{50} values against *C. albican* (183) which was 120 mg/L. Embelin's MIC_{50} values were below 700 mg/L for *C. albican, C. tropicalis, C. parapsilosis, C. albidus* and *A. flavus*. Diethyl ether extract, petroleum ether extract, methanol extract and embelin obtained MIC_{50} in range of 300-700 mg/L against *C. albican* and *C. parapsilosis*. Petroleum ether extract showed lowest MIC_{50} values for *C. parapsilosis* (250 mg/L); *C. laurintis* (360 mg/L); *I.orientalis* (180 mg/L) and *A. fumigatus*(170 mg/L).

Keywords: *Embelia ribes,* antifungal, NCCLS, EUCAST.

INTRODUCTION

Embelia ribes is most widely used in tradition herbal medicine in India. The fruits of *Embelia ribes* reported to contain mainly benzoquinone derivatives such as Embelin (2, 5-dihydroxy-3-undecyl-2, 5-cyclohexadiene-1, 4-benzoquinone) and Vilangin. The dried fruit has been used in India since ancient times as an anthelmintic. *Embelia ribes* has been shown to possess astringent [1], carminative, stimulant, antioxidant [2], anti-spermetogenic [3-7] anti-bacterial [8] and anticancer activity. [9-10]

The plant *Embelia ribes* contains embelin, quercitol, and fatty ingredients; an alkaloid, christembine, a resinoid, tannins and minute quantities of a volatile oil. [11-13] It is reported to be effective against tapeworm but not against roundworm or hookworm. [14] Embelin occurs in golden yellow needles insoluble in water and soluble in alcohol, chloroform and benzene. Embelin dyes silk and wool from an alcoholic solution. The dark colored fatty oil is reported to be similar to linseed and rapeseed oil in its properties. Chemical structure of Embelin is having quite resemblance with the structure of natural Coenzyme Q10 (ubiquinones) and the role of this is well defined in various biochemical protective mechanism. [15]

Antifungal screening of this plant is not studied in detail or if studied is not extended to the different spectrum of fungal which are causing human diseases and also the selective extracts were only investigated. [16-17]

Testing for antifungal activity of natural products, especially plant extracts, presents many challenges. The standardization of the in vitro antifungal susceptibility testing has advanced greatly in recent years. The National Committee for Clinical Laboratory Standards (NCCLS) has set the benchmark methodology by providing laboratory tested reproducible, consensus peer-reviewed standards. [18- 19] NCCLS M27- A2 standard for yeasts provides a broth microdilution test which could be a good screening method for plant extracts with its high through-put potential, considerable savings in media usage, and requirement of a small quantity of sample. [20-21]

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has standardized antimicrobial breakpoints and susceptibility testing in Europe [22- 23]; so that comparable results and interpretations are produced. The EUCAST method is a similar microdilution assay to the NCCLS method, but uses a larger inoculum size and higher glucose concentration in the medium as well as a spectrophotometric end-point determination. [24]

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We decided for antifungal screening on various extracts, isolated compounds and crude semi purified fractions of these plants on various fungal strains which have not been evaluated so far using standard broth dilution methods EUCAST and NCCLS as well as some modification in NCCLS method which used spectrophotometric determination of the end point.

MATERIALS AND METHODS

Plant-derived materials and preparation of samples

Embelia ribes powder was obtained from m/s LVG and Sons, (Ahmedabad, Gujarat, India) and it was authenticated by department of Pharmacognosy, S.K. Patel College of pharmaceutical education and research.

Fungal strains

The pathogenic fungal strains were obtained from Microbial Type Culture Collection, (MTCC, Sector 39-A, chandigarh-160036, India). which were used in study are: *Aspergillus fumigatus*-2550, *Aspergillus flavus*-871, *Candida albicans*-227 , *Candida albicans*-183, *Candida tropicalis*- 184, *Candida parapsilosis*-1744, *Issatchenkia orientalis*- 3020, *Cryptococcus albidus var. albidus* – 2661, *Cryptococcus layrentii var. laurentii* – 2898

Method for extraction

20 g of powdered plant material was taken in flask with 40 ml of the solvent. (Petroleum ether, Diethyl ether, methanol and water) the flask was allowed to saturation of drug powder for over night. Then after 24 h the solvent was filtrated by percolation method using Whatman filter paper. All extractive material was collected using fresh solvent until colour of the solvent become colour less. Collected extracts were evaporated to dryness in desiccators. The % yield of every extract was calculated.

Isolation of the Embelin

Isolation of the Embelin was carried out according to Indian herbal pharmacopoeia. 250 g of the powdered fruits of *Embelia ribes* was extracted with n-hexane using a soxhlet extractor for 6 hrs. The extract was evaporated on steam bath. Cold petroleum ether was added to the residue. It was stirred and filtered through buchner funnel under vacuum. The residue was washed with cold petroleum ether. The residue was dried and re-crystallized with chloroform to obtain pure Embelin.

Assessment of the MIC

The MIC values of the fungal strains under study were determined by use of NCCLS and EUCAST methods. [24-25] The freeze dried powders of the strains were sub cultured in their respective growth medium. Plates were prepared and subjected to read the absorbance in plate reader (MutliskanEX, Thermo, USA), after 24 h, 48 h and 72 h, at 530 nm. From absorbance data, the % growth was obtained using equation. To obtain the MIC the line graph, plot of concentration of the extract against percentage growth were prepared. Values of the MIC, MIC_{50} were obtained with the help of the Graphpad prism software (Version 4.0). MIC end-points were interpreted as the lowest sample concentration that remained blue (indicating no growth) or the first dilution that changed from blue to slightly purple (equivalent to prominent growth inhibition).

RESULTS AND DISCUSSION

As shown Fig. 1, the percentage growth decreased with the increase in the concentration of the plant extracts, except for the water extract. The line for the water extract remained linear at all concentrations. As shown in the chart, the MIC_{50} and MIC can be obtained easily.

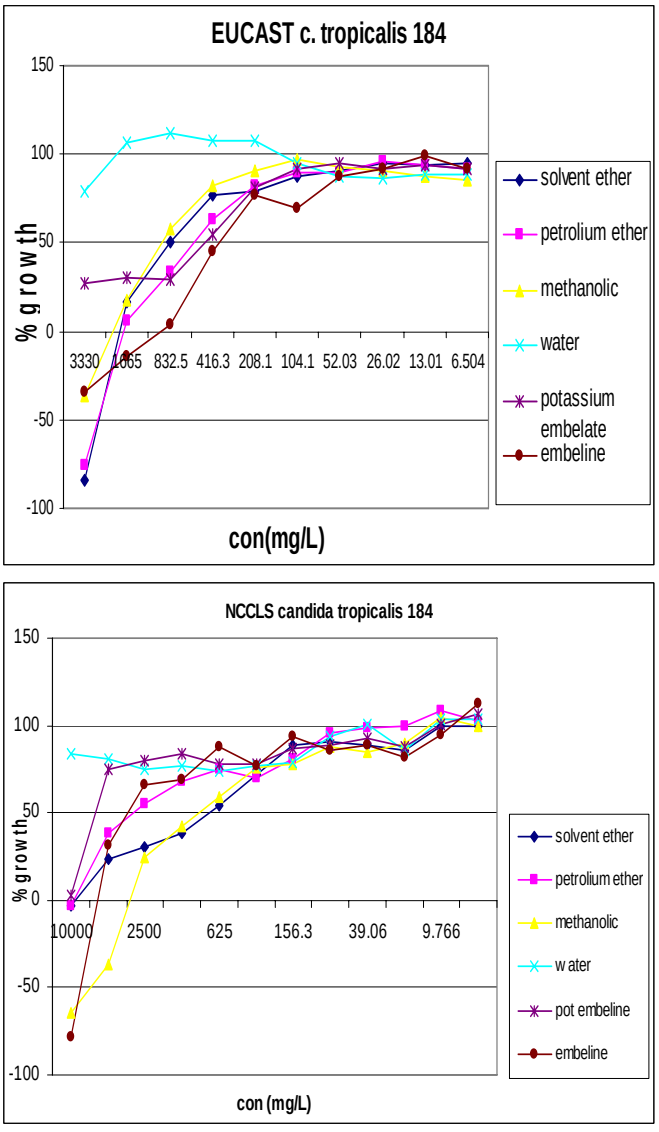

Fig. 1: Extract concentration against percentage growth of *C. tropicali* for EUCAST and NCCLS method.

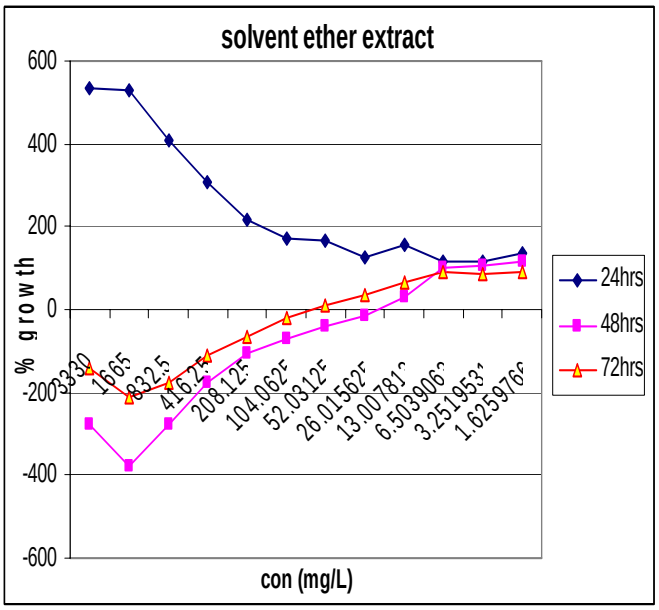

Fig. 2: Growth pattern of *Cryptococcus albidus*.2661 in presence of Diethyl ether extract.

Table1: Activity of different plant extracts of *Embelia ribes* against fungal strains using EUCAST method

Fungal strains (MTCC NO)	MIC 50% (mg/L)					
	Diethyl ether extract	Petroleum ether extract	Methanol extract	Water extract	Pot embelate	Embelin
C. albican 227	NA	740	340	1940	1650	NA
C. albican 183	65	32	520	2500	NA	NA
C. tropicalis 184	850	800	1080	>3330	1500	700
C. parapsilosis 1744	1520	600	1620	2230	760	NA
C. albidus 2661	NA	NA	NA	NA	NA	NA
C. laurantis 2898	NA	NA	1220	NA	NA	NA
I. orientalis 3020	>3330	750	>3330	2400	500	1650
A. flavus 871	NA	NA	NA	NA	970	NA
A. fumigatus 2550	NA	300	2160	1260	1500	NA

Table 2: Activity of different plant extracts of *Embelia Ribes* against fungal strains using NCCLS method

Fungal strains (MTCC NO)	MIC 50% (mg/L)					
	Diethyl ether extract	Petroleum ether extract	Methanol extract	Water extract	Pot embelate	Embelin
C. albican 227	330	700	430	>3330	780	350
C. albican 183	660	290	120	2140	>3330	120
C. tropicalis 184	930	1300	540	>3330	1660	680
C. parapsilosis 1744	370	255	840	3330	915	590
C. albidus 2661	870	450	460	NA	1660	325
C. laurantis 2898	1560	360	500	1200	750	1280
I. orientalis 3020	3330	180	1600	>3330	1660	1150
A. flavus 871	490	1560	>3330	NA	>3330	470
A. fumigatus 2550	>3330	170	1300	3330	1410	1015

Table 3: MIC values of the different plant extracts of *Embelia ribes*, potassium Embelate, Embelin and amphotericine –B (standard drug- as positive control) by both EUCAST and NCCLS method.

Fungal stains (MTCC NO)	MIC (mg/L)						
	Method	Diethyl ether extract	petroleum ether extract	Methanol extract	Water extract	Pot embelate	Embelin
C. albican 227	EUCAST	1670	1050	950	>3330	3200	NA
	NCCLS	1260	830	870	>3330	1280	832
C. albican 183	EUCAST	430	170	770	3330	NA	124
	NCCLS	860	480	500	1660	>3330	160
C. tropicalis 184	EUCAST	1260	1080	2330	>3330	>3330	1160
	NCCLS	2800	3060	1570	>3330	>3330	1050
C. parapsilosis 1744	EUCAST	2540	830	2630	3230	1140	NA
	NCCLS	830	660	1600	>3330	1650	960
C. albidus 2661	EUCAST	NA	NA	NA	NA	1660	NA
	NCCLS	2100	1010	700	NA	3070	832
C. laurantis 2898	EUCAST	NA	NA	1120	NA	NA	NA
	NCCLS	2970	710	1020	2330	1480	2300
I. orientalis 3020	EUCAST	>3330	1260	>3330	>3330	1140	3200
	NCCLS	>3330	2600	>3330	>3330	2500	3230
A. flavus 871	EUCAST	NA	NA	NA	NA	1104	NA
	NCCLS	1660	>3330	>3330	NA	>3330	960
A. fumigatus 2550	EUCAST	NA	380	2300	1660	1570	NA
	NCCLS	>3330	340	1660	>3330	1660	1900

EUCAST test results were taken for the three days (every 24 h, 48 h and 72 h) to determine the growth of the fungal stain in presence of the extracts. To evaluate the growth of the Fungal strain graphs of the percentage growth against extract concentration for each day were prepared for each fungal strain. Some species showed high growth at 24 h. While the growth was inhibited after 24 h and relatively less percentage growth was appeared at 48 and 72 h.

The growth of *Candida* species was inhibited before 24 h whereas growth of other species was inhibited after 24 h specifically with *Cryptococcus albidus* 2661, *C. laurantis* 2898 and *A. flavus* 871. The Petroleum ether extract, Diethyl ether extract and Methanol extract showed relatively less MIC_{50} values against the C*andida* species than the other species. Diethyl ether extract and Petroleum ether extract were found to be highly effective against the C. *albicans* 183 with the MIC_{50} values 65 mg/L and 32 mg/L respectively. The methanol extract were more active against C. *albican* species with 300-500 mg/L MIC_{50} value. The Diethyl ether extract, Petroleum ether extract, Methanol extract, Potassium embelate and Embelin exhibited the MIC_{50} values in range of 800-1600 mg/L against *C. tropicalis* 184 and *C. parapsilosis* 1744. The Petroleum ether extract and Potassium embelate was found to have MIC_{50} between range 300-700 mg/L against *C. parapsilosis* and *A. fumigatus*. Other extracts required higher concentrations against *C. parapsilosis* and *A. fumigatus*. Water extract was found to exert MIC_{50} values greater than 2000 mg/L against all fungus. Most of the results for the Embelin could not obtain by EUCAST method due higher fluctuation in readings for optical density.

Values of the MIC_{50} obtained in by NCCLS method revealed that Methanol extract and Embelin showed MIC_{50} values against *C. albican* (183) (120mg/L). Embelin's MIC_{50} values found to be greater than 700mg/L for *C. albican, C. tropicalis, C. parapsilosis, C. albidus* and *A. flavus*. Diethyl ether extract, petroleum ether extract, methanol extract and embelin found to have MIC_{50} in range of 300-700mg/L against *C. albican* and *C. parapsilosis*. Petroleum ether extract showed lowest MIC_{50} values for C. *parapsilosis* (250mg/L); *C. laurintis* (360mg/L); *I.orientalis* (180mg/L) and *A. fumigates* (170mg/L). Water extract was found to be inactive against all fungal species.

With NCCLS method, MIC_{50} values for most species were obtained. The Diethyl ether, Petroleum ether extract, methanol extract and Embelin were reported less MIC values for the C*andida* species. The growth of *A. fumigatus* 2550 was inhibited by petroleum ether extract at concentration 350 mg/L. Amphotericine B was found to be effective against all species in range of 0.4 -1.5 mg/L concentration.

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