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A potential antibacterial agent Embelin, a natural benzoquinone extracted from *Embelia ribes*

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Abstract

Herbal medicine has been used for the prevention and treatment of various health ailments from time immemorial. In the present work, embelin from *Embelia ribes* berries of Indian origin was extracted and characterized by UV, NMR, thermal and X-ray diffraction analyses. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of embelin against both Gram +ve and Gram -ve bacteria were studied using micro dilution method and agar plate method by sub-culturing 10 μ l of the test dilutions from MIC tubes on to fresh Mueller-Hinton agar plates. About 1.9 \pm 0.1 gram of pure embelin was obtained from 100 gram of powdered berries (*E.ribes*). The characteristics studied reveal the properties are on par with the standard embelin received from Sigma (USA). With regard to antibacterial activity, embelin showed bactericidal activity against Gram +ve organisms, and bacteriostatic against Gram -ve organisms. Thus, embelin finds application as potent antibacterial agent.

Keywords: Embelin; Bactericidal activity; Bacteriostatic activity; Differential scanning calorimeter (DSC).

Introduction

In India, 70% of population use traditional medicine for primary healthcare. The present annual turnover of herbal medicinal products manufactured by large companies in India is estimated to be approximately US \$ 300 million, compared to the turnover of US \$ 2.5 billion for modern drugs. In folk medicine, researchers are looking for new leads, which will help to develop better drugs against microbial infections. About three quarter of the world's population relies on plants and plant extracts for healthcare (Srinivasan *et al.*, 2001). Therefore, the discovery of novel active compounds against new targets is always in limelight of research.

Quinone constitutes one of the well-known groups of naturally occurring organic compounds. One of the major attractions among researchers towards quinone compounds is their color and biological activities (Nohl *et al.*, 1986). Benzoquinones are the simplest representatives of quinone group. Embelin (2, 5-dihydroxy-3-undecyl-p-benzoquinone), is found to be the active principle of *Embelia ribes* and reported to possess a wide spectrum of biological activities. Chemical structure of embelin is having quite resemblance with that of natural Coenzyme Q10 (ubiquinones) and the role of this is well defined in various biochemical protective mechanism (Kobaisy *et al.*, 2008).

E.ribes is widely used as traditional herbal medicine in India. Especially, the berries

of *E.ribes* are reported to contain mainly benzoquinone derivatives such as embelin (2,5-dihydroxy-3-undecyl-2,5-cyclohexadiene-1,4-benzoquinone) and vilangin. In addition, dried berries have been used in India since ancient times as an antihelminthic. In addition, dried berries are also reported to inhibit enzymes such as pancreatic lipase (Gowadia and Vasudevan, 2000), alpha amylase (Prashanth *et al.*, 2001) and trypsin (Vijaya and Vasudevan, 1994). Embelin, as such, evaluated against *Heligmosomoides polygyrus* in mice and found to reduce the total worm count (Githiori *et al.*, 2004). Rathinam *et al.* (1976) reported embelin as potent oral contraceptive having 85.7% anti-implantation activity in rats when administered at 50 mg kg⁻¹ for 7 days and it also inhibited pregnancy at single dose regimen. In addition, embelin inhibited pregnancy and possessed anti-estrogenic and weak progestational activity (Johri *et al.*, 1991). Embelin also reported to have anti-inflammatory (Chitra *et al.*, 1994), antibacterial (Chitra *et al.*, 2003), antitumor (Chitra *et al.*, 2004), antioxidant and free radical scavenging activities (Joshi *et al.*, 2007).

However, minimum inhibitory concentration index (MIC index) and minimum bactericidal concentration (MBC) for embelin have not been reported so far. Therefore, the present work deals with extraction, characterization and identification of minimum inhibitory concentration (MIC) and

bactericidal/bacteriostatic activities of embelin against both Gram +ve and Gram -ve bacteria.

Materials and Methods

Plant material

E.ribes berries were obtained from M/s Abirami Botanical Corporation (Tuticorin, Tamil Nadu, India) and authenticated by Dr. Anandan, Research Officer, Anna Hospital, Chennai.

Extraction of Embelin

Extraction of embelin was carried out according to Indian Herbal Pharmacopoeia (2002). 100 g of the powdered berries of *E.ribes* was extracted with n-hexane using a Soxhlet extractor for 6 hrs. The extract was then evaporated on rotator and recrystallized using ethanol and chloroform and characterized using UV-Visible, FT-IR, NMR, DSC, TGA and X-ray diffraction analyses according to the standard methods. UV-Visible spectrum was recorded using UV-2450 from Shimadzu (Japan) in the wavelength range between 200-800 nm. FT-IR spectral measurement was made using Spectrum One (Perkin-Elmer Co., USA). Proton and carbon NMR spectrum was obtained from JEOL (500 MHz, DMSO-d₆). Thermal analysis was recorded using DSC Q200 (V23.10 Build 79), TGA Q50 (V20.6 Build 31) and finally, powder X-ray diffraction analysis was done using Miniflux (Rigaku Diffractometer, Japan).

Microorganisms

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of the embelin was tested individually on ATCC Gram +ve and Gram -ve bacterial strains obtained from MicroBioLogics, USA. *Bacillus cereus* (ATCC 10876), *Micrococcus luteus* (ATCC 4698) and *Staphylococcus aureus* (ATCC 6538) were the Gram +ve bacterial strains, *Escherichia coli* (ATCC 4157), *Klebsiella pneumoniae* (ATCC 4352), *Proteus mirabilis* (ATCC 7002), *Shigella flexneri* (ATCC 9199) and *Pseudomonas aeruginosa* (ATCC 9027) were the Gram -ve bacterial strains, used in the present study. Bacterial strains were maintained on nutrient agar at 4 °C and sub-cultured every month.

Determination of minimum inhibitory concentration

A clear solution containing 1.0 mg of embelin dissolved in 1.0 ml of DMSO was used as the stock concentration and DMSO alone acts as control. Minimum inhibitory concentration (MIC) was determined by the microdilution method, using 2.0 ml of liquid broth with different concentration of embelin prepared from the stock concentration. Tubes were inoculated with a microorganism suspension of cell density of 1×10^5 cells/ml and were incubated at 37 °C for 24 h. Turbidity of the solution was read at 600 nm against Mueller Hinton broth (blank solution) and the absorbance was measured. Standard drug Silver Sulfadiazine (SSD) (1 mg dissolved in one ml of DMSO) was used as standard for comparisons.

Determination of minimum bactericidal concentration

A minimum bactericidal concentration (MBC) is the lowest concentration of an antibiotic required to kill a microorganism. MBC was determined by sub-culturing 10 µl of the test dilutions from MIC tubes on to fresh Mueller-Hinton agar plates. Plates were incubated for 18-24 h. The highest dilution that yielded no single bacterial colony on the plates was recorded as MBC.

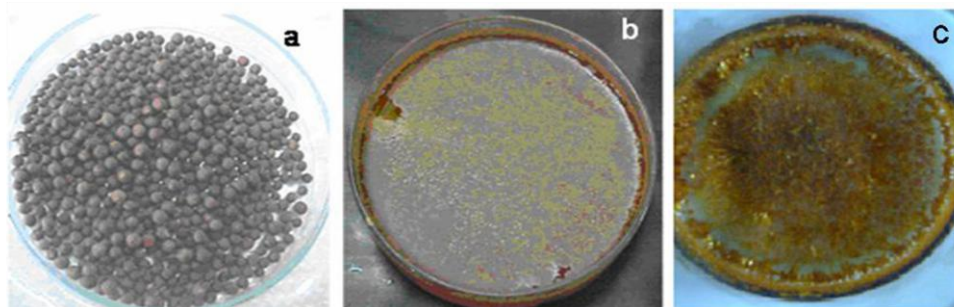
MIC index

The MIC index (MIC/MBC) was determined whether an extract is bactericidal (MIC/MBC <4) or bacteriostatic (MIC/MBC >4) on growth of bacterial organisms. MIC index values of greater than 4 and less than 32 are considered as bacteriostatic.

Results

Figure 1a illustrates the physical appearance of *E.ribes* used in the present study. The seeds were highly coloured and strong, pure and not adulterated with other materials. Extraction of embelin from 100 gm of *E.ribes* using hexane provided 1.9 ± 0.1 gram of pure embelin. Figure 1b and 1c depict the physical appearance of embelin extracted under laboratory conditions and the recrystallization steps provided embelin crystals with high purity.

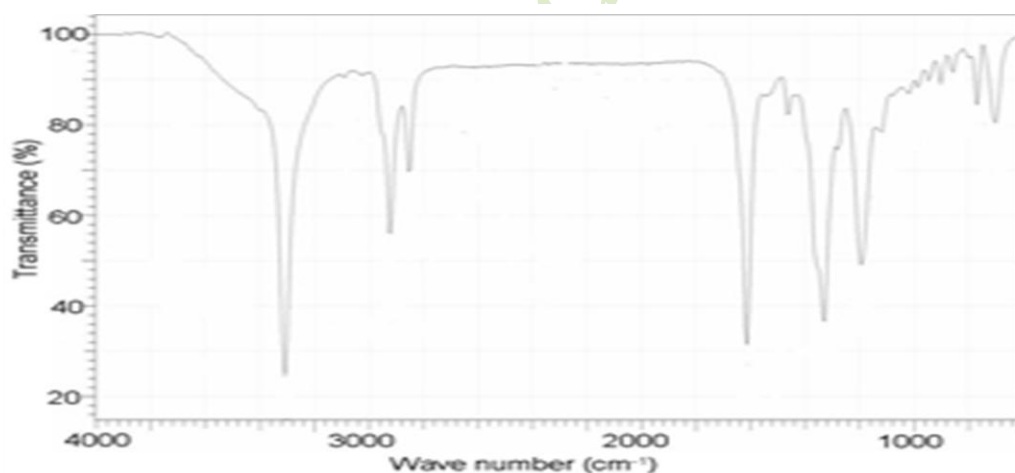
Figure 1 (a-c): Represents the *Embelia ribes* berries, formation of embelin crystals and crystalline form of embelin.



UV spectrum exhibits λ_{max} at 289 nm.
IR spectrum displays absorption frequency at 3310 cm^{-1} due to hydroxyl group, 1630 cm^{-1} for

carbonyl stretching frequency as shown in figure 2.

Figure 2: Represents FT-IR spectrum of embelin.

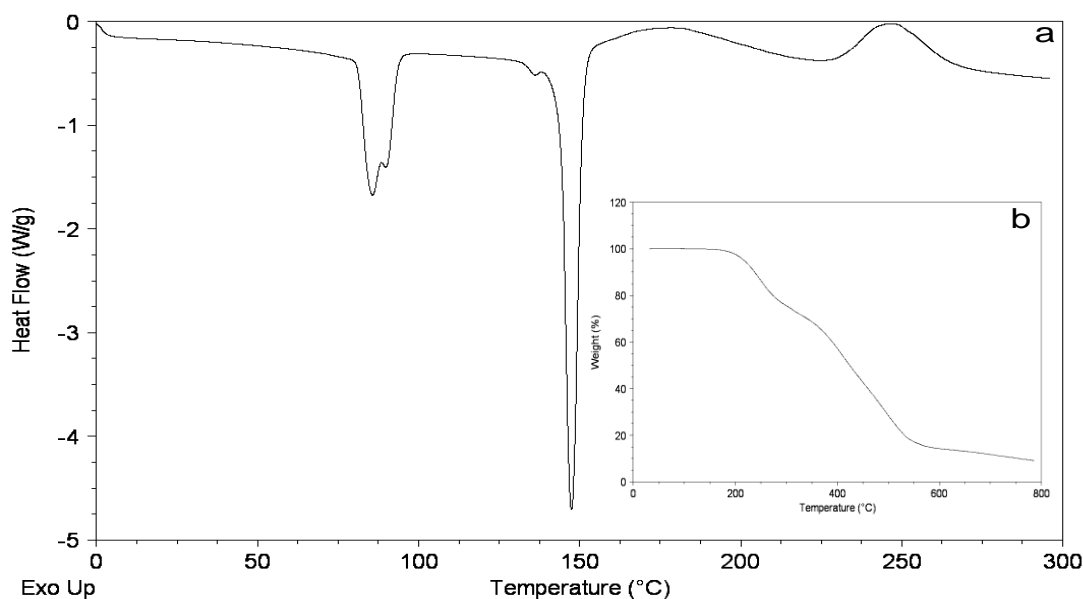


Proton NMR (^1H NMR- 300 MHz, DMSO- d_6) demonstrates chemical shifts at δ 11.08 (2H, broad spectra, 2xOH), 5.78(1H, singlet, H-3), 2.28(2H, triplet, J 7.2 Hz, H-7), 1.32 (18H, unresolved multiplet, H-8- H-16), 0.85 (3H, triplet, J 6.6 Hz, H-17) and Carbon NMR (^{13}C NMR-500 MHz, DMSO- d_6) showed shifts at δ 117.88(C-6), 104.35(C-3), 31.83(C-15), 29.57-22.63 (C-9-C14), 14.47 (C-17). A chemical shift observed at δ 160-175 ppm corresponds to the resonance within the ring

and for the carbon attached to hydroxyl and carbonyl groups.

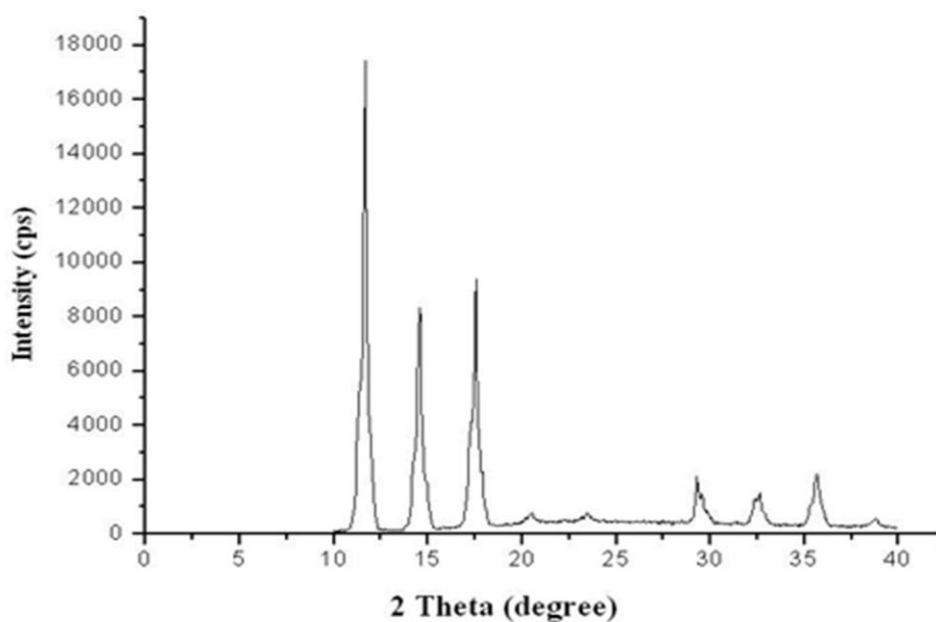
With regard to thermal analysis, Differential scanning calorimeter (DSC) of embelin exhibited three endothermic peaks at 85.70°C , 147.73°C and 224.64°C resulting from loss of water, melting point peak and decomposition (Figure 3a). Thermo gravimetric analysis (TGA) showed thermal stability of embelin as represented in figure 3b.

Figure 3 (a, b): Represents the thermograms of embelin.



The XRD of embelin showed that the sharp peaks of embelin representing the crystalline nature of the embelin (Figure 4).

Figure 4: Represents the XRD diffractogram of embelin.



Followed by the characterization of embelin, assessment on evaluation of MIC, MBC for

Gram +ve and Gram -ve organisms was made and the results (n=3) are shown in Table 1.

Table 1: Minimum inhibition concentration (MIC), Minimum bactericidal concentration (MBC) and MIC index of embelin.

S. No.	Organisms	MIC (in $\mu\text{g/ml}$)	Std drug-SSD* (in $\mu\text{g/ml}$)	MBC (in $\mu\text{g/ml}$)	MIC Index (MBC/MIC)
1.	<i>Bacillus cereus</i> (ATCC 10876)	20	23	75	3.75
2.	<i>Bacillus subtilis</i> (ATCC11774)	20	24	75	3.75
3.	<i>Staphylococcus aureus</i> (ATCC 6538)	20	22	75	3.75
4.	<i>Micrococcus luteus</i> (ATCC 4698)	25	23	100	4.00
5.	<i>Escherichia coli</i> (ATCC 4157)	45	27	325	7.22
6.	<i>Shigella flexneri</i> (ATCC 9199)	30	25	175	5.83
7.	<i>Klebsiella pneumoniae</i> (ATCC 4352)	50	29	400	8
8.	<i>Pseudomonas aeruginosa</i> (ATCC 9027)	25	20	125	5
9.	<i>Proteus mirabilis</i> (ATCC 7002)	25	17	150	6

SSD* - Silver Sulfadiazine.

Embelin showed bactericidal activity (MIC index is 4 or less than 4) against Gram +ve organisms, whereas against Gram -ve organisms it showed bacteriostatic activity (MIC index values greater than 4 and less than 32).

Discussion

In the present study, extraction of embelin was carried out with hexane as a solvent. However, extraction with other solvents namely carbon tetrachloride, diethyl ether, chloroform, acetone, ethyl acetate, propanol and methanol was also reported by Ganesan *et al.* (2010) and Madhavan *et al.* (2010). In addition, Latha (2006) reported selective extraction of embelin from *E. ribes* using aromatic hydrotropes such as sodium n-butyl benzene sulfonate (NaNBBS) and sodium cumene sulfonate (NaCS) while, Kumaraswamy *et al.* (2007) reported extraction of embelin from leaves of *E. ribes* using ethanol solvent. Followed by extraction the characterization studied of embelin revealed identical spectral details similar to the results of Madhavan *et al.* (2010), Kumaraswamy *et al.* (2007), Pathan and Bhandari (2010) and Cherutoi *et al.* (2005). NMR spectra details of embelin are found to identify with that of Ganesan *et al.* (2009). With respect of DSC analysis of embelin, similarly, Pathan and Bhandari (2010) reported single endothermic peak at 147 °C, while Singh *et al.* (2007) reported five endothermic peaks for embelin-

borax complex. With regard to XRD analysis of embelin, the results are similar to the observation made by Abraham *et al.* (2003) and Pathan and Bhandari (2010).

With reference to the antibacterial activity, Chitra *et al.* (2003) reported that embelin (100 μg) exhibited significant antibacterial activity against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Shigella flexneri*, *Shigella sonnei* and *Pseudomonas aeruginosa*, however, it showed moderate zone of lysis against *Salmonella typhi*, *Shigella boydii* and *Proteus mirabilis*. Feresin *et al.* (2003) reported that embelin inhibited both methicillin-sensitive and methicillin-resistant strains of *Staphylococcus aureus* with MICs of 250 and 62 $\mu\text{g/ml}$, respectively. While the MIC for the methicillin-sensitive strain of *Staphylococcus aureus* was 250 $\mu\text{g/ml}$. Moreover, the MIC for *Escherichia coli* was 50 $\mu\text{g/ml}$. Areekul *et al.* (2009) reported no antimicrobial activity (6.08%) for crude *E. ribes* extracts, while Schrader *et al.* (2010) reported antibacterial activity against *Edwardsiella ictaluri* bacteria which cause enteric septicemia in channel catfish (*Ictalurus punctatus*) with MIC of >294.4 mg/ml. Khan *et al.* (2010) reported minimum bactericidal concentration (MBC) of *E. ribes* (ethanolic extract) as 18.5, 17, 18, 17 and 16 (in mg/ml) for organisms such as *Streptococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa*

respectively. Our observations exhibit that embelin has potential bactericidal activity against all the tested Gram +ve strains.

Conclusion

Thus, in the present study embelin offers a remarkable bactericidal activity against Gram +ve organisms, whereas it showed bacteriostatic activity against Gram -ve organisms. Based on these results, embelin finds potential application in pharmaceutical and cosmetic product industries.

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