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Characterisation of *In Vitro* Produced Embelin and Evaluation of its Antifungal Properties

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ABSTRACT: Traditionally used toxic chemical wood preservatives are being replaced with eco-friendly biocidal wood protectants. Many plant extractives are being investigated for their potential to control biodegradation of wood, and a single organic compound effective against a broad range of wood rot fungi is yet to be identified. Embelin, the main active component of the extremely valuable medicinal plant, *Embelia ribes*, is effective against a wide spectrum of fungi causing human diseases and is therefore used in the formulation of anti-fungal drugs. However, there is no prior report on its effect against wood degrading fungi. *In vitro* production of Embelin through callus or cell cultures can be adopted to obtain this compound at a shorter duration in large amounts. In this study, Embelin was extracted in chloroform from leaf derived callus and seeds of *E. ribes*, which were characterised by TLC and FT-IR analyses. Assays against wood rot fungi using the extracted Embelin revealed that it can be used effectively as a wood preservative. The outcome of this study can efficiently decrease the load on natural populations of *E. ribes* for production of Embelin for medicinal uses, and also to replace harmful chemical wood preservatives.

Keywords: Embelin, antifungal, *Embelia ribes*, bioreactor, wood rot fungi.

INTRODUCTION

Embelia ribes Burm. f. is a highly valuable medicinal plant, belonging to the *Myrsinaceae* family. It is a woody climbing shrub growing at an altitude of 1,500m in the semi-evergreen and deciduous forests of India (Asadulla *et al.*, 2011). It is also found in other Asian countries like China, Srilanka, Malaya and Singapore (Harish *et al.*, 2012). It is used in around 75 Ayurvedic preparations and in various forms like asava, churna, aristha, lauha and taila, and is generally called as false black pepper or "Vidanga", its Sanskrit trade name (Lal and Mishra, 2013; Patwardhan *et al.*, 2014). Embelin, being the major bioactive constituent of *E. ribes*, is produced along with other constituents like christembine, quercitol, vilangin and resinoid (Lal and Mishra, 2013; Radhakrishnan *et al.*, 2011). Embelin does not cause unwanted physiological effects, as it is non-steroidal and non-hormonal (Asadulla *et al.*, 2011). The anti-oxidant, hypo-glycemic, anti-microbial, anthelmintic, anti-inflammatory, anti-tumour/anti-cancer, anti-spermatazoal, anti-androgenic, enzyme inhibitory, anti-hyperlipidemic, anti-convulsant, chemopreventive, anti-ulcer, anti-angiogenesis, carminative and wound healing properties of Embelin are well documented, and hence *E. ribes* is used as a drug in Ayurveda, Siddha and Unani (Mhaskar *et al.*, 2011; Othman *et al.*, 2020; Radhakrishnan and Gnanamani, 2014; Xavier and Kani, 2021).

Owing to its medicinal value, the natural populations of *E. ribes* have been overexploited, leading to its red-listed status as vulnerable in red list data book (Ravikumar *et al.*, 2000). Since the natural populations and conventional means of propagation of *E. ribes* are insufficient to meet its commercial demands, plant tissue culture was explored for its conservation, propagation, and also for production of the active component Embelin. Earlier studies have showed that Embelin can be potentially produced in large scale through callus or cell culture techniques, without depending on extraction from natural populations (Raghu *et al.*, 2006; Dhavala and Rathore, 2010; Raghu *et al.*, 2011; Sinha *et al.*, 2014). In our study, Embelin was produced from *E. ribes* through callus cultures and the isolated compound was characterised by TLC and FT-IR analyses. The antifungal effect of the extract against wood rot fungi was also analysed.

MATERIALS AND METHODS

A. In vitro Embelin production

Callus was initiated from *in vitro* grown leaves of *E. ribes* in MS medium containing hormones and additives (Dhavala and Rathore, 2010), and multiplied by subculturing onto fresh medium of the same composition. Embelin was extracted from seeds and leaf induced callus of *E. ribes* with chloroform following the method described by Chauhan *et al.* (1999) with slight modifications.

B. Thin Layer Chromatography

5µl of crude Embelin extract was applied on precoated silica gel G aluminium plate, which was then developed in mobile phase containing Ethyl acetate: Chloroform: Methanol:Formic acid (5:4:1:0.5). The chromatogram was visualised in white light, and also under UV light at 365nm, for the identification of the separated compounds. The R_f value for each sample was determined, and the presence of Embelin was checked against that of standard Embelin (Chauhan *et al*., 1999).

C. Fourier Transform Infrared (FT-IR) analysis

FT-IR analysis was performed employing KBr pellet method (Radhakrishnan *et al*., 2011) for confirming the presence of Embelin in the *E. ribes* extract. The Embelin, separated by thin layer chromatography, was removed along with the silica gel and dissolved in chloroform, and allowed to stand till the silica gel settled completely, after which, the solvent containing Embelin was subjected to FT-IR analysis. The peaks obtained for the samples were compared with the peaks of standard Embelin.

D. Assay of antifungal activity

The antifungal activity of *E. ribes* extract was assessed by poisoned food method (Balouiri *et al*., 2016). The crude extracts of *E. ribes* were dissolved in DMSO and vortexed to dissolve the compound completely. 1ml extract was added to 100ml of sterile malt agar medium, and fungal discs of *Trimates hirsuta* and *Oligoporus placentus* were placed on the center after solidification. DMSO was used as negative control and the positive control was DMSO containing Bavistin, and 3 replicates were taken for each treatment. The colony diameter was measured after incubation at 28°C for three days, and the percentage inhibition of mycelial growth was calculated as,

Antifungal activity (%) = ((Dc-Ds)/Dc) × 100,

where 'Ds' is the colony diameter of poisoned plate (with extracted Embelin), and 'Dc' is the colony diameter of non poisoned plate (with only DMSO).

E. Statistical analysis

All the studies were performed under well-defined aseptic conditions for callus induction and multiplication, and for antifungal assay. Completely Randomised Design (CRD) was employed for each experiment and the data recorded were analysed using ANOVA.

RESULTS AND DISCUSSION

A. Identification and purification of Embelin by TLC

The Embelin standard and those in the samples appeared as light violet-coloured spots at the same R_f when observed under visible light. When observed under UV light of 365nm the spots appeared dark blue in colour (Fig. 1). Saraf *et al.* (2016) performed HPTLC to quantify Embelin and anisaldehyde-sulphuric acid was used as the visualization agent. Whereas, Vijayan and Raghu (2021) observed pink colour bands of Embelin with Rf value of 0.6 when Propanol:Butanol:Ammonia (7:3:7) was used as mobile phase. In the present study, R_f value of standard Embelin was found to be 0.75 using Ethyl acetate: Chloroform: Methanol: Formic acid (0.5:0.4:0.1:0.05) as mobile phase. Presence of Embelin in test samples was confirmed by the appearance of identical spots with similar R_f values. Kumaraswamy *et al*. (2007) isolated Embelin from *E. ribes* leaves by column chromatography using ethanol:chloroform (1:1) for elution.

B. Characterisation of Embelin by FT-IR analysis

Kaur *et al.* (2015) isolated Embelin from berries of *E. ribes* which was characterised by melting point, TLC, partition coefficient, solubility, FT-IR, NMR and Mass Spectroscopy. In this study, FT-IR analysis was performed to characterise the purified Embelin.

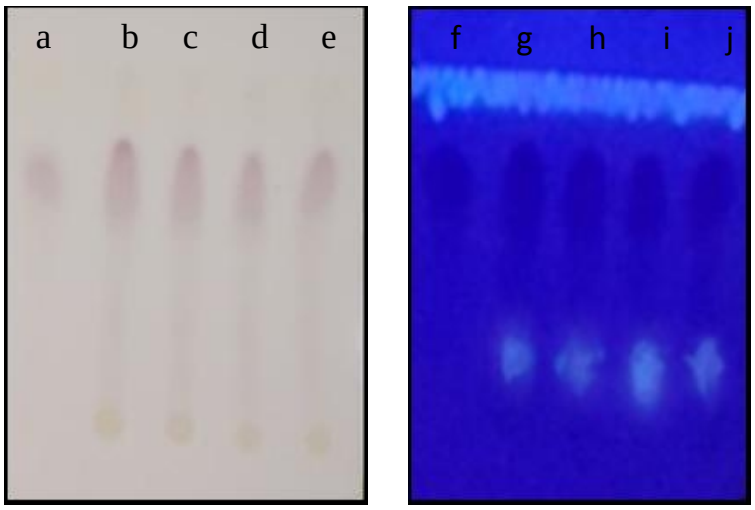


Fig. 1. TLC profiles of Embelin standard under (a) visible light and (f) 365nm; *E. ribes* callus extract of different genotypes (S, S2, S3, Ag) under visible light (b, c, d & e) and 365nm (g, h, i & j).

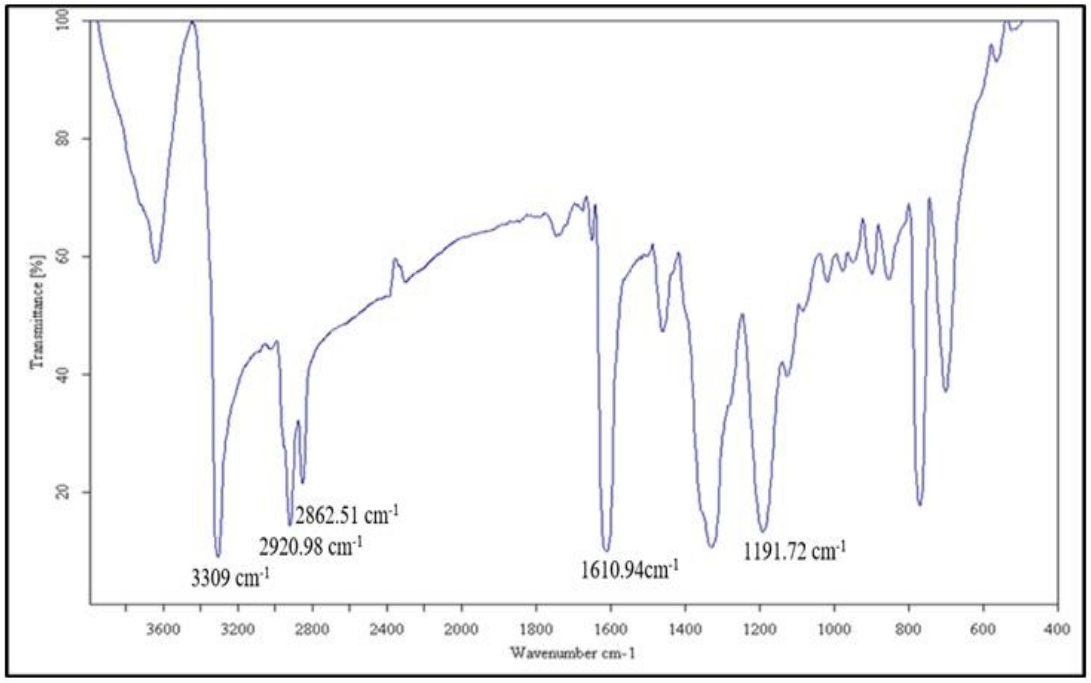
FT-IR analysis of callus extract showed transmittance peaks at wave numbers similar to that of standard Embelin (Table 1 and Fig. 2). The peaks obtained at 800cm^{-1} and 1300cm^{-1} correspond to the solvent, i.e., chloroform. The peaks for hydroxyl- and carbonyl-groups were present at the same wave numbers reported by Kumaraswamy *et al*. (2007).

C. Antifungal activity of Embelin extract

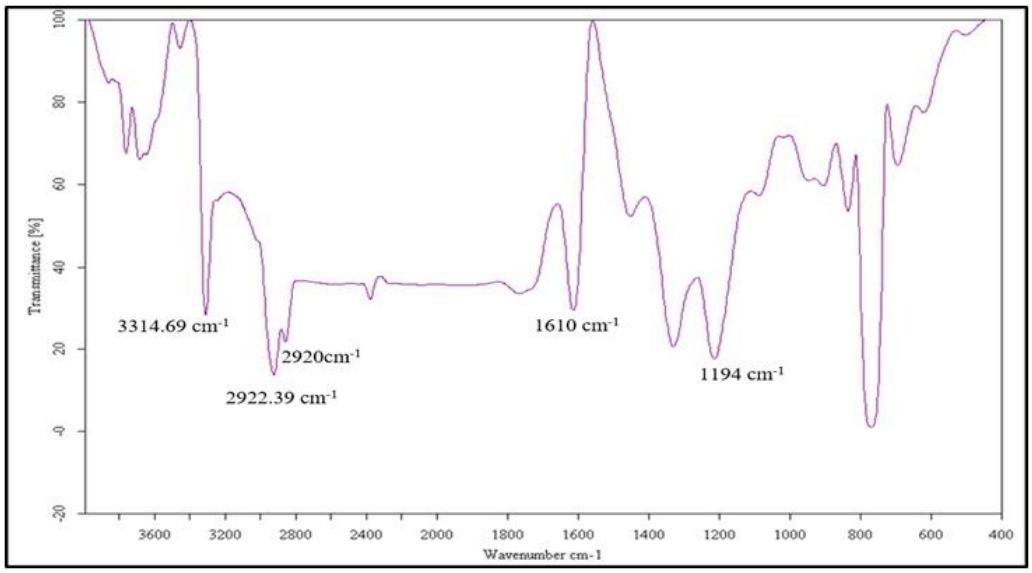
Earlier, the antifungal activity of different types of *E. ribes* extract was evaluated for four *Candida* spp. by Rathi *et al*. (2009). In our study, the crude extract of *E. ribes* callus exhibited antifungal activity against both *Oligoporus placentus* (Fig. 3) and *Trametes hirsuta* (Fig. 4), as compared to the negative control. The diameter of mycelial growth decreased with increase in concentration of *E. ribes* extracts, showing that embelin in the extracts was effective in controlling fungal growth (Fig. 5). The seed extracts of *E. ribes* showed better antifungal activity against *O. placentus*, as compared to *T. hirsuta.* Whereas, the callus extracts were observed to be more effective against *T. hirsuta*. Differences in the antifungal activity of crude extracts of seed and callus of *E. ribes* against different wood rot fungi, could be due to the combined effect of Embelin with other phytochemicals. Pure Embelin at 10mg/l showed 6.2% and 7.9% antifungal activity for *T. hirsuta* and *O. placentus* respectively. This is the first report on the effectiveness of *E. ribes* extracts in controlling the growth of wood decaying fungi.

Table 1: Wave number (cm^{-1}) at which peaks were obtained during FT-IR analysis.

S. No.	Standard Embelin	Embelin from seeds	Embelin from callus
1.	1191.72 (C-O bond)	1192	1194
2.	1610.94 (Stretching vibration of C=O)	1612	1610
3.	2862.51 (Stretching vibration of methyl C-H)	2863	2922.39
4.	3309 (Stretching vibration of -OH group)	3314	3314.69
5.	2920.98 (Aromatic C-H stretching)	2919.26	2920



(a) Embelin standard



(b) Callus extract.

Fig. 2. FT-IR spectra.

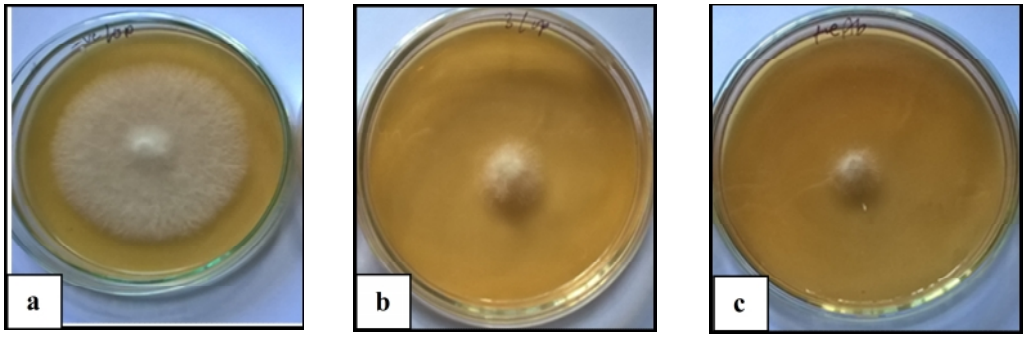


Fig. 3. Antifungal activity of *E. ribes* on *O. placentus*: (a) Negative control (DMSO), (b) 100mg/l callus extract, and (c) Positive control (50mg/l Bavistin).

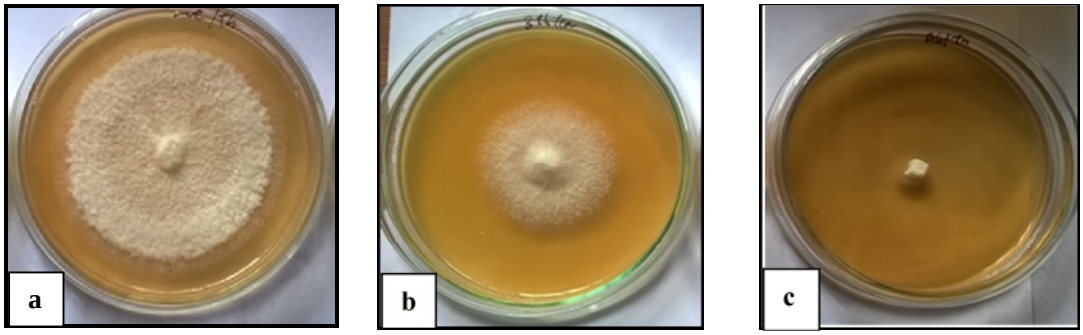


Fig. 4. Antifungal activity of *E. ribes* on *T. hirsuta*: (a) Negative control (DMSO), (b) 100mg/l callus extract, and (c) Positive control (50mg/l Bavistin).

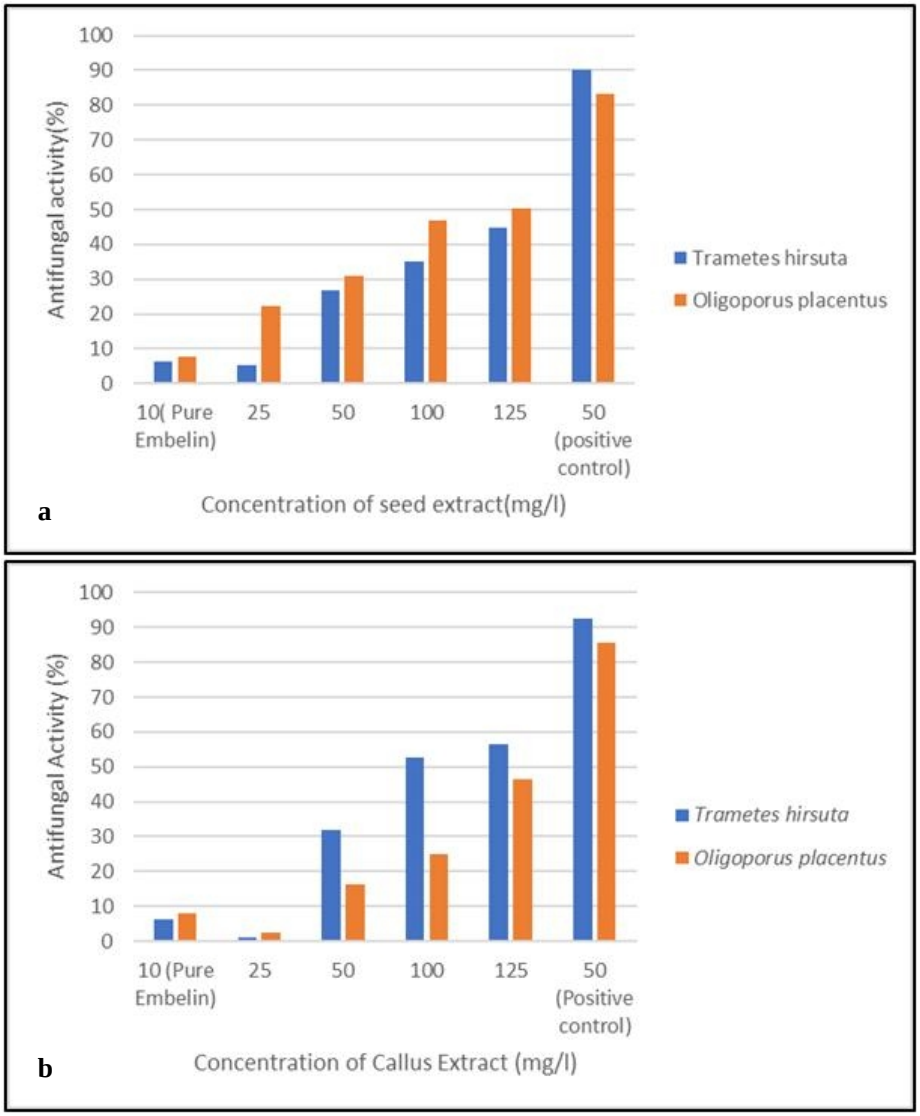


Fig. 5. Antifungal activity of (a) *E. ribes* seed extract, and (b) *E. ribes* callus extract.

CONCLUSION

The production of secondary metabolites of medicinally important compounds using plant tissue culture technology has become feasible due to the enhanced understanding about metabolic pathways and advancements in metabolic engineering. We have earlier showed the feasibility of using callus culture as an alternative for Embelin production. In the present study, the evaluation of the antifungal efficacy of extracted and characterised Embelin is reported. The Embelin in callus extract, seed extract and *ex vitro* leaf extract was confirmed by TLC and FT-IR. This study has also shown that, Embelin is effective against wood rotters such as *Trimates hirsuta* and *Oligoporus placentus* at concentrations above 25mg/l for seed and callus extract. Therefore, Embelin can also be used as an alternative to chemical wood preservatives. Also, there is a possibility of developing *E. ribes* as an important source of biopesticide and a potential antifungal agent. However, further studies are needed for isolation and purification of all the bioactive constituents in *E. ribes*.

Our study has revealed that *E. ribes* extracts can be utilised as alternative to wood preservative chemicals, against common wood decay fungi. The production of Embelin in large quantities is required to meet market demands, given its varied medicinal properties coupled with the need to preserve *E. ribes*. Further optimisation of the *in vitro* techniques developed, to enhance the yield of Embelin from callus cultures, can lead to large-scale Embelin production through cell suspension culture in bioreactors, without depleting the natural sources for its extraction.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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