

# An Accurate Embelin Extraction Method for Limited Biomass of *Embelia* Species

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## ABSTRACT

The natural benzoquinone, embelin, from the *Embelia* species has therapeutic benefits in a wide range of diseases. Although several extraction methods and solvents have been explored, consensus on the economic use of material and time was ambiguous. The purpose of this study was to devise a protocol for the rapid estimation of embelin. Chloroform, ethyl acetate and acetone extracts were prepared using soxhlet, microwave, sonication and cold extraction methods. The bioactivity of the chloroform extracts was assayed using the DPPH radical scavenging and the Reducing Power Assays. The embelin content in chloroform and ethyl acetate extracts were better in some extraction methods. The chloroform extracts exhibited antioxidant activity which remained unaffected regardless of the extraction technique. The microwave extraction technique yielded quick and accurate results. This technique could be adopted for rapid screening of samples with limited availability of biomass.

**KEY WORDS:** ANTIOXIDANT ACTIVITY, *EMBELIA* SP., EMBELIN, EXTRACTION METHODS, MICROWAVE EXTRACTION.

## INTRODUCTION

*Embelia basaal* is a large shrub and *Embelia ribes* is a rare woody climber or shrub of the family Primulaceae. *E. ribes* is located up to an altitude of 1750 meters in the western and eastern ghats of India, Sri Lanka, Singapore, China and Malaysia (Annapurna et al. 2013; Patwardhan et al. 2014). The brittle pericarp of the dried berries of *E. ribes* encloses a reddish, single seed with spots of embelin on its surface and is covered by a thin membrane (Sudhakaran 2016). The dried berries of *E. basaal* appear reddish-black with vertical striations on its surface and can be easily differentiated from the grey to black, wrinkled berries of *E. ribes* (Fig. 1) (Nayak et al. 2009). Traditionally *Embelia* has been used extensively for impaired digestion, colon diseases, ulcers, skin diseases, as an anthelmintic, for the treatment of abdominal pain, mental disorders, jaundice, heart diseases and bronchitis (Bhishagratna 1911; Atal et al. 1984; Choudhary et al. 2021).

Embelin has clinically proven its value as an antimicrobial, antioxidant, anti-tumour, analgesic, anti-inflammatory, anti-androgenic, anti-hyperglycaemic, anthelmintic agent and in healing wounds (Radhakrishnan and Gnanamani 2014).

Different parts of the plant were used in the form of paste, decoction, oil and powder for treating various illnesses. *Embelia ribes* (Vidanga) has been used in the preparation of about 75 ayurvedic formulations (Bhishagratna 1911; Patwardhan et al. 2014). Pharmacological applications aside, it has also been used as a dyeing agent for cotton, nylon, wool and silk (Radhakrishnan et al. 2011a). A recent study of embelin indicated an antiviral property against Covid-19 (Caruso et al. 2020b). The medicinal value of *E. basaal* and *E. ribes* can be ascribed to its active component embelin a yellow-orange crystalline compound (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) that constitutes about 1 to 5% of the berries (Lu et al. 2016; Prabhu et al. 2018; Ferreria and Laddha 2013) (Pandey and Ojha 2011; Nagamani et al. 2013 Ferreria and Laddha 2013; Lu et al. 2016; Prabhu et al. 2018). Bioprospecting or evaluation of the best medium composition for desired results would demand rugged and accurate protocols with an economical investment of biomass and time. The plant biomass has been extracted using various solvents and extraction techniques and the determination of the biochemical activities of phytochemicals in the crude extracts have been regularly explored for better alternatives to the existing options (Altemimi et al. 2017; Tlili et al. 2019; Kamble et al. 2020; Vijayan and Raghu 2021).

Extraction of embelin has been reported using several extraction methods and solvents. However, these studies did not actively explore their efficacy in embelin extraction

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from small samples. The present study aims to optimize the embelin extraction from small samples of the berries of two species of *Embelia* using different solvents and extraction methods while retaining their bioactivity.

## MATERIAL AND METHODS

The analytical grade solvents were procured from SD Fine-Chem (India) and Merck. The other reagents used for analysis were sourced from Sigma-Aldrich, HiMedia (India), SRL (India) and SD Fine-Chem (India). *Embelia basaal* berries were collected from Supegaon village, Raigad District, Maharashtra. A specimen was deposited in Blatter Herbarium, St. Xaviers College, Mumbai and was identified as *Embelia basaal* (Roem & Schult) A. DC. The berries of *Embelia ribes* were collected from Shimoga District, Karnataka. A specimen was submitted to 'Botanical Survey of India', Pune and was identified as *Embelia ribes* Burm f. The berries of both species were air-dried and stored at room temperature for further analysis. The berries of *E. basaal* and *E. ribes* were crushed, using a mortar and pestle, enough to separate the pericarp from the seeds and expose sufficient seed surface for extraction of the entire embelin content (Ferreria and Laddha 2013). The pericarp and the seeds were subjected to extraction using the solvents chloroform, ethyl acetate, acetone and four extraction methods. The sample to solvent ratio was maintained at 2% (w/v) in each method. The extractions were carried out in triplicate and the embelin content was expressed as mean  $\pm$  standard error of the mean (SEM).

For Soxhlet Extraction (SE), Coarse ground berries (1 g) of *Embelia* species were refluxed in 50 ml of extraction solvent in a soxhlet apparatus (J-SIL company, India) for 1 h by which time the extract in the siphon arm of the apparatus was colourless. Microwave extraction (ME): The sample (0.2 g) was extracted with 5 ml of solvent for 3 min in a microwave (LG, India) set at 180 W. The sample was heated for 50 s, three times, in a cycle. A resting period of 10 s was given between each spurt of 50 s heating. The solvent was then filtered through Whatman filter paper no. 1 and collected in a beaker. Fresh solvent (5 ml) was added to the sample residue and a second extraction was carried out for 50 s twice, with a resting period of 10 s as described above, followed by filtration. The filtrates obtained were pooled together. Ultrasonication extraction (UE): The sample (0.2 g) in 5 ml of solvent was subjected to sonication using a water bath sonicator (Pure Enterprises, India) for 10 min, followed by filtration. Fresh solvent (5 ml) was added to the sample which was sonicated for another 5 min and filtered. The filtrates obtained were pooled together. For cold extraction (CE), the sample (0.2 g) with 10 ml of solvent was placed on an orbital shaker (Remi RS 12 PLUS) at 200 rpm for 17 h. The samples were then filtered. The extracts obtained by the four extraction methods using various solvents were oven-dried at 40°C and the residues were weighed. The dried extracts were dissolved in chloroform for quantification by UV-VIS spectrophotometer (Shimadzu Scientific Instruments Inc.).

Standard embelin was procured from Sigma-Aldrich and a stock solution (1 mg/ml) was prepared in chloroform.

Dilutions were prepared such that the final concentrations ranged between 4 to 24  $\mu$ g/ml. The UV absorbance at 290 nm of each solution was recorded using a UV-VIS spectrophotometer. A standard graph of absorbance vs concentration of embelin was plotted and the resultant equation was used for calculating the concentration of embelin in the samples. The embelin from samples was also quantified by High Performance Liquid Chromatography (HPLC) for the bioactivity experiments.

Four concentrations of standard embelin in methanol (10, 20, 30 and 40  $\mu$ g/ml) were run through an HPLC column (Shim-pack GIST C18, 5 $\mu$ m, 20 x 250 of the Shimadzu HPLC system). The mobile phase, 0.1% formic acid in acetonitrile: deionized water (90:10; v/v), was degassed for 30 min. The flow rate was maintained at 0.5 ml/min in isocratic elution mode and the chromatogram was run for 20 min. The volume of sample injected each time was 20  $\mu$ l. The area under the peak was considered to generate a standard graph for the estimation of embelin in samples. The dried chloroform extracts of the *Embelia* berries, obtained by the four extraction methods were reconstituted in methanol (1 mg/ml) by sonication for 5 min and filtered through a 0.22  $\mu$ m nylon filter. These extracts would be further referred to as reconstituted extracts. The embelin content in these samples was determined by the HPLC analysis described above and the antioxidant activity was confirmed by the following DPPH and RPA assays.

The DPPH assay method described by Brand-Williams et al. (1995) was adopted with some modifications. The assays included ascorbic acid as a reference standard, a methanol control and the test samples. The DPPH reagent (100  $\mu$ M) was prepared in chilled methanol and the initial U.V. absorbance of the reagent was approximately 1 (1.0542). Two-fold dilutions of the test samples dissolved in methanol were prepared with final concentrations that ranged from 800  $\mu$ g/ml to 25  $\mu$ g/ml. Similarly, standard ascorbic acid solutions were prepared in the range between 40  $\mu$ g/ml to 1.25  $\mu$ g/ml. Aliquots (500  $\mu$ l of standard/ sample) were added to 2.5 ml of DPPH solution and incubated in dark for 30 min before noting the U.V. absorbance at 517 nm. The percentage of radical scavenging activity was calculated by the formula:

$$\text{Radical Scavenging activity (\%)} = \frac{A_o - A_t}{A_o} \times 100$$

Where  $A_o$  = absorbance of the control and  $A_t$  = absorbance of the test sample.

EC<sub>50</sub> values (50% effective concentration value - the value obtained by plotting percentage radical scavenging activity vs concentration of extract) were calculated for each sample and standard using GraphPad Prism 6.0 software. The reducing power assay (RPA) method described by Muruhan et al. (2013) was adopted with some modifications. A range of two-fold dilutions of standard solution (80  $\mu$ g/ml to 1.25  $\mu$ g/ml) and sample (800  $\mu$ g/ml to 25  $\mu$ g/ml) was prepared.

Each concentration of the standard or sample was reacted individually with 1.25 ml of sodium phosphate buffer (pH 6.6) and 1.25 ml potassium ferricyanide. This mixture was incubated at 50°C for 20 min. The reaction was stopped with the addition of 10 % trichloroacetic acid (TCA). The solutions were then centrifuged at 3000 rpm for 10 min. The resultant supernatants (2.5 ml) were mixed with 2.5 ml of distilled water and 0.5 ml of ferric chloride reagent. The U.V. absorbance of these reaction mixtures was recorded at 700 nm and its reducing potential was expressed as

ascorbic acid equivalents. All data obtained were subjected to appropriate statistical treatment and analysis. Two-way analysis of variance (ANOVA) followed by Tukey's test was performed using GraphPad Prism 6.0 software wherever required.

## RESULTS AND DISCUSSION

**Yield of Extract:** The residue obtained after the evaporation of solvent from the extract (yield) varied across the different extraction methods and the solvent used. This ranged from 5% to 7.8% (w/w) for *E. basaal* and 3.5% to 6.2% (w/w) for *E. ribes*. Two-way ANOVA followed by Tukey's test revealed that the yield of the ME - chloroform extract was significantly higher than that of the other methods (*E. basaal*) whereas, for *E. ribes*, the CE - chloroform extract showed maximum yield. The yields obtained by different extraction methods tested in this study were variable but inconsistent with their embelin content (Table 1). Similarly, the Supercritical carbon dioxide extraction resulted in a lower yield than the SE but had a higher flavonoid content (Bimakr et al. 2011).

**Legend:** The letters and symbols across the cells for each species indicates the statistical significance at  $p \leq 0.05$ . CH: chloroform; EA: ethyl acetate; AC: acetone; The equation and the regression coefficient (squared) of embelin standard graph is as follows:  $y = 0.063x - 0.059$  and  $r^2 = 0.9963$ .

**Figure 1: The dried berries of *E. basaal* (A) and *E. ribes* (B); The brittle testa removed to expose the seeds with spots of embelin of *E. basaal* (C) and *E. ribes* (D).**

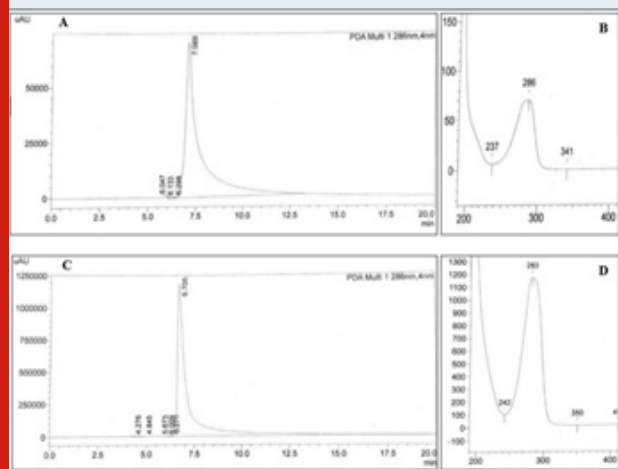


**Table 1. The yield, embelin content and time required for extraction from berries of *E. basaal* and *E. ribes***

<i>E. basaal</i>				<i>E. ribes</i>			
	Yield (mg/g)	Embelin content (mg/g)	Time (min)		Yield (mg/g)	Embelin content (mg/g)	Time (min)
SE				SE			
CH	71.37 ± 3.76 <sup>φ</sup>	54.69 ± 0.96 <sup>A</sup>	60	CH	39.50 ± 1.50 <sup>αβ</sup>	18.17 ± 0.25 <sup>ab</sup>	60
EA	59.90 ± 2.47 <sup>φ</sup>	57.96 ± 1.52 <sup>A</sup>	60	EA	41.13 ± 1.86 <sup>αβ</sup>	19.34 ± 0.69 <sup>abcd</sup>	60
AC	64.37 ± 0.67 <sup>φ</sup>	44.94 ± 1.84 <sup>ABC</sup>	60	AC	40.13 ± 0.68 <sup>αβ</sup>	17.05 ± 1.03 <sup>a</sup>	60
ME				ME			
CH	78.83 ± 3.90 <sup>ψ</sup>	54.22 ± 2.65 <sup>A</sup>	5	CH	53 ± 0.0.29 <sup>γ</sup>	21.28 ± 0.57 <sup>bcd</sup>	5
EA	55.33 ± 5.18 <sup>φ</sup>	53.06 ± 5.30 <sup>AB</sup>	5	EA	40 ± 1.32 <sup>αβ</sup>	21.94 ± 0.14 <sup>cd</sup>	5
AC	65.83 ± 5.73 <sup>φ</sup>	40.30 ± 2.53 <sup>ABC</sup>	5	AC	43.33 ± 1.67 <sup>β</sup>	18.78 ± 0.18 <sup>abc</sup>	5
UE				UE			
CH	63.33 ± 3.03 <sup>φ</sup>	53.30 ± 2.08 <sup>AB</sup>	15	CH	55 ± 0.76 <sup>γ</sup>	21.18 ± 0.76 <sup>bcd</sup>	15
EA	50.67 ± 2.89 <sup>φ</sup>	49.45 ± 2.46 <sup>AB</sup>	15	EA	37.50 ± 2.25 <sup>αβ</sup>	21.25 ± 0.57 <sup>bcd</sup>	15
AC	54.50 ± 7.01 <sup>φ</sup>	35.32 ± 7.31 <sup>BC</sup>	15	AC	35.50 ± 1.00 <sup>α</sup>	18.63 ± 0.48 <sup>ab</sup>	15
CE				CE			
CH	75.67 ± 7.86 <sup>φ</sup>	47.20 ± 1.34 <sup>AB</sup>	1020	CH	62.50 ± 0.50 <sup>δ</sup>	22.06 ± 0.60 <sup>d</sup>	1020
EA	63.50 ± 5.20 <sup>φ</sup>	49.41 ± 6.02 <sup>AB</sup>	1020	EA	42.67 ± 0.88 <sup>β</sup>	22.51 ± 0.89 <sup>d</sup>	1020
AC	63 ± 7.25 <sup>φ</sup>	28.88 ± 1.67 <sup>C</sup>	1020	AC	44 ± 1.32 <sup>β</sup>	19.81 ± 0.64 <sup>abcd</sup>	1020

**Estimation of embelin content by spectrophotometry:** Variations in the embelin content in berries could be attributed to its geographical location (1.2 to 4.9%) and/or stage of maturation (1 to 5.2%) (Pandey and Ojha 2011; Nagamani et al. 2013). The embelin content of extracts would also depend on the extraction method and the solvent which was significant in this study of both *Embelia* species. The embelin content in *Embelia ribes* extracts, reported using different extraction methods, ranged from 1.9% to 3.8% (SE), 5% (ME), 0.84% to 23.71 % (UE) and 1.77% (CE) (Madhavan et al. 2011; Radhakrishnan et al. 2011b; Alam et al. 2015; Sathe and Dixit 2015; Kamble et al. 2020). The embelin content of the various extracts in the current study of *E. ribes* was determined as 1.7% to 1.93% (SE), 1.87% to 2.19% (ME), 1.86% to 2.12% (UE) and 1.98% to 2.25% (CE) with different solvents. These values for *E. basaal* ranged from 4.49% to 5.79% (SE), 4.03% to 5.42% (ME), 3.53% to 5.33% (UE) and 2.88% to 4.94% (CE). The highest extracted embelin content in this study was 5.79% from *E. basaal* berries. *E. ribes* was reported to be the best source of embelin when compared to the species tested in a previous study (Vijayan and Raghu 2021).

**Figure 2: The HPLC chromatogram of standard embelin (A),  $\lambda_{\max}$  of the standard peak from the chromatogram (B), HPLC chromatogram of Sample (*E. basaal* - UE) (C), and  $\lambda_{\max}$  of the sample peak obtained in the chromatogram (D).**



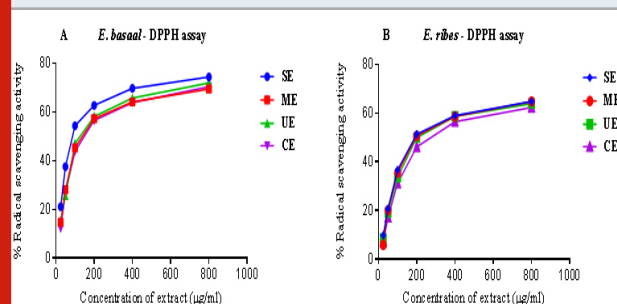
**Table 2. Embelin content of reconstituted extracts by HPLC**

	Embelin content by HPLC (µg/ml)			
	SE	ME	UE	CE
<i>E. basaal</i>	807.12 <sup>a</sup>	621.58 <sup>ab</sup>	617.07 <sup>ab</sup>	601.55 <sup>ab</sup>
<i>E. ribes</i>	508.77 <sup>ab</sup>	450.58 <sup>ab</sup>	374.3 <sup>ab</sup>	268.96 <sup>b</sup>

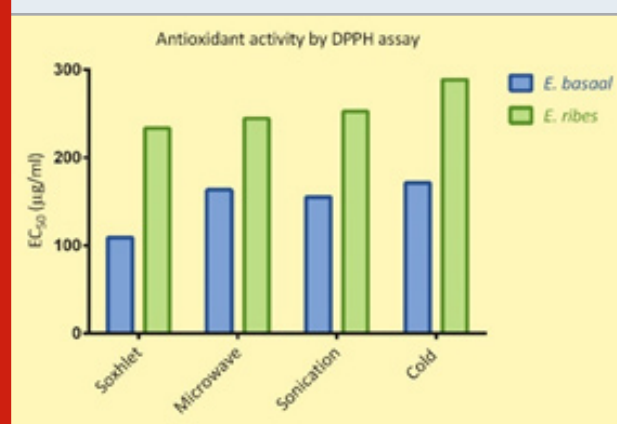
A selection of conventional (SE and CE) and modern (ME and UE) methods of extraction were compared in this study. The SE and ME embelin contents were better in *E. basaal*

extracts whereas the SE of *E. ribes* was comparatively inefficient than the other extraction methods tested. Solvents used for extraction of secondary metabolites may include a pure organic solvent, a mixture of solvents, water or a percentage of a solvent in water (Gupta et al. 2012).

**Figure 3: Percentage of DPPH radical scavenging activity of *E. basaal* (A) and *E. ribes* (B) extracts at different concentrations (µg/ml) obtained using selected extraction techniques. SE: Soxhlet extraction; ME: Microwave extraction; UE: Ultrasonication extraction; CE: Cold extraction.**



**Figure 4: The EC<sub>50</sub> values of the reconstituted extracts of *E. basaal* and *E. ribes*, using various extraction methods.**



*Embelin* has a polar benzoquinone ring and a nonpolar alkyl saturated chain. The isolated and purified embelin from *E. ribes* is insoluble in water, freely soluble in organic solvents such as DMSO, slightly soluble in methanol and ethanol (Kaur et al. 2015; Caruso et al. 2020a). Previous studies preferred hexane, diethyl ether, chloroform, ethyl acetate, acetone and methanol for extraction of embelin (Madhavan et al. 2011; Alam et al. 2015; Pundarikakshudu et al. 2016), (Vijayan and Raghu 2021). Even so, the results of these experiments failed to reach a consensus on the best solvent for embelin extraction.

Chloroform extraction by maceration, ethyl acetate extraction by sonication and acetone extraction by microwave-assisted extraction method of *E. ribes* yielded



maximum embelin (Latha 2007; Alam et al. 2015; Pundarikakshudu et al. 2016). The most promising solvents were investigated for the development of the rapid analysis protocol. The statistical analysis of this study however indicated that the solvents chloroform and ethyl acetate were found to be marginally more efficient in the extraction of embelin (Table 1). Chloroform was also found to be suitable for the extraction of benzoquinone in *Ficus foveolata* (Meerungrueang and Panichayupakaranant 2015). Methanol was discontinued after the initial extraction process as the residue obtained was sticky, which was also observed by (Pundarikakshudu et al. 2016). Another factor that should be considered is the efficacy of the bioactive principle(s) following an extraction method. The chloroform extracts using all four extraction methods from both species were further analysed for their bioactivity.

**Estimation of embelin in reconstituted extracts using HPLC:** The current solvent system was inspired by the existing literature and modified for better resolution (Ferreria and Laddha 2013). The standard embelin chromatogram exhibited one sharp peak at a retention time that ranged from 7.082 to 7.16. The spectrophotometric analysis of this peak showed a  $\lambda_{\text{max}}$  at 286 nm. Hence the chromatograms of the samples were monitored at 286 nm and all samples showed one peak with retention times between 6.66 and 7.17 with the  $\lambda_{\text{max}}$  that ranged between 277 to 283 nm (Fig. 2). The embelin content of the reconstituted *E. basaal* and *E. ribes* extracts ranged between 602 to 807  $\mu\text{g/ml}$  and 269 to 509  $\mu\text{g/ml}$ , respectively. A significant difference was noticed between the embelin content of *E. basaal* and *E. ribes* extracts. However, all extraction methods were comparatively efficient (Table 2). Contrary to the current observation, a study described that the embelin content varied with different extraction methods (Kamble et al. 2020). The differences in embelin content among species of *Embelia* have been reported in previous studies (Vijayan and Raghu 2021).

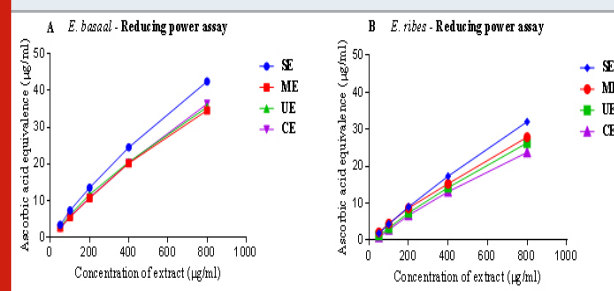
**Legend:** SE: Soxhlet extraction; ME: Microwave extraction; UE: Ultrasonication extraction; CE: Cold extraction. The equation determined for the embelin estimation by the HPLC method was  $y = 69697x - 38593$  with the  $r^2$  value 0.9972.

**Antioxidant Assays;** Antioxidants are chemical substances that halt the chain reaction of free radical species generation and protect the cells from cellular damage. The antioxidant activity of plants is generally attributed to the flavonoid, phenolic and tannin content in the extract (Mosquera et al. 2009). Earlier reports of the antioxidant activity of extracts from *E. basaal* and *E. ribes* prompted testing for their antioxidant activity (Ansari et al. 2008; Kamble et al. 2011).

**DPPH assay;** Antioxidant activity of the chemically synthesized embelin, a benzoquinone, was studied by Joshi

et al. (2007) and its activity determined by the DPPH assay was found to be  $2.55 \times 10^{-4} \text{ mol dm}^{-3}$ . Purified embelin extracts displayed an  $\text{IC}_{50}$  of 27.92  $\mu\text{g/ml}$  and an  $\text{EC}_{50}$  of 27  $\mu\text{g/ml}$  (Mahendran et al. 2011; Mohapatra and Basak 2017). Ascorbic acid (standard) was reported to have an  $\text{IC}_{50}$  that ranged from 3.028 to 4.92  $\mu\text{g/ml}$  (Kamble et al. 2011; Mahendran et al. 2011). The  $\text{IC}_{50}$  of crude embelin extracts from various *Embelia* species ranged from 9.87 to 50  $\mu\text{g/ml}$  whereas the  $\text{EC}_{50}$  reported from *E. tsjeriam cottam* was 11  $\mu\text{g/ml}$  (Kamble et al. 2011; Barbade and Datar 2015; Mohapatra and Basak 2017). The radical scavenging activity (the  $\text{EC}_{50}$ ) of the ascorbic acid in the present study was 12.15  $\mu\text{g/ml}$  and that of the samples were 109 to 171  $\mu\text{g/ml}$  (*E. basaal*) and 233 to 289  $\mu\text{g/ml}$  (*E. ribes*). Statistical evaluation of the calculated  $\text{EC}_{50}$  values indicated that the antioxidant activity of *E. basaal* extracts were significantly higher than those of *E. ribes* and this was more pronounced in the SE and CE extracts of the species. The variation between the extraction methods was insignificant (Fig. 3 and 4).

**Figure 5: The Reducing Power Assay using the reconstituted extracts of *E. basaal* (A) and *E. ribes* (B), at different concentrations ( $\mu\text{g/ml}$ ) obtained using different extraction techniques and expressed as the equivalence of antioxidant activity of ascorbic acid. SE: Soxhlet extraction; ME: Microwave extraction; UE: Ultrasonication extraction; CE: Cold extraction. The equation obtained for the reducing power assay using ascorbic acid as standard was  $Y = 0.0174 X + 0.0204$  ( $r^2 = 0.9975$ ) and was used to calculate the ascorbic acid equivalence antioxidant activity of samples.**



**Reducing power assay:** RPA is another standard assay to confirm the antioxidant activity of extracts. High values of UV absorbance indicate high antioxidant capacity. The results of the RPA have been shown in Fig. 5. The average antioxidant activity of the reconstituted extracts (1 mg/ml) expressed as the equivalence of ascorbic acid ranged from 64.33 to 50.52  $\mu\text{g/ml}$  and 41.85 to 27.84  $\mu\text{g/ml}$  for *E. basaal* and *E. ribes* respectively. The statistical analysis of this data (normalized to 1 mg/ml) revealed that there is a significant difference between the reducing power of the *E. basaal* and *E. ribes* extracts. The reducing power of extracts obtained using different extraction methods was insignificant within each species. This is the first report as yet that compared the antioxidant activities of different *Embelia* species. Genotypes of the plants collected from different localities

and solvents have influenced variations in the antioxidant activities of *E. ribes* (Kamble et al. 2020).

## CONCLUSION

The findings of the present study indicated that the preferred solvents for extraction of embelin would be chloroform or ethyl acetate. The embelin content of ME extracts was comparable to conventional methods. Antioxidant studies with the reconstituted chloroform extracts confirmed that the bioactivity was retained regardless of the extraction method. Thus, ME has the added advantage of the speed and economic use of biomass. This protocol would be valuable for the screening and selection of plants or callus with high embelin content. This protocol could be adopted for the screening of other important phytoconstituents as well.

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**Conflict of Interests:** Authors declare no conflicts of interests to disclose.

**Data Availability Statement:** The database generated and /or analysed during the current study are not publicly available due to privacy, but are available from the corresponding author on reasonable request.

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