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Phytochemical screening and evaluation of antioxidant potential of seeds extract from *Embelia ribes*

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Abstract
Embelia ribes, commonly known as Vayuvilanga, is a medicinally valuable, woody climber, a well-known drug in Ayurvedic system. The dried powdered seeds were subjected to sequential extraction by solvents like acetone, petroleum ether, water, methanol, and chloroform in increasing order of their polarity. The phytochemical screening of seed extracts revealed that the seeds were rich source of secondary metabolites. The results of DPPH and ABTS assays for antioxidant activity showed great free radical scavenging activity under low concentrations for methanolic extracts. Upon quantification of methanol and acetone extract showed the highest amount of flavonoid and phenolic compounds respectively. The presence of flavonoids and phenolics are responsible for the antioxidant activity as they are free radical scavengers *in vivo*.

Keywords: *Embelia ribes*, Antioxidant activity, ABTS assay DPPH assay

1. Introduction
Human use of plants as medicines could be dated back to the middle Palaeolithic age, which is about 60, 000 years ago, according to fossil records [1].
Plants are a source of large amount of drugs consists of different groups such as antispasmodics, emetics, anti-cancer, antimicrobials etc. Many plants are claimed to possess the antibiotic properties in the traditional system and are also used extensively by the tribal people worldwide. The curing ability of plant/extract is attributed to the presence of phytochemical constituents within them. Phytochemicals naturally occur in the leaves, seeds, stem bark, fruits and roots of medicinal plants that have defence mechanism and protect from various diseases. Natural products from plants called secondary metabolites are the end products of primary metabolites which include alkaloids, steroids, flavonoids, terpenoids, glycosides, saponins, tannins, phenolic compounds etc.
Many plants contain antioxidant compounds where it protect cells against the damaging effects of reactive oxygen species (ROS) such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite which results in oxidative stress leading to cellular damage [2]. Cell damage caused by reactive oxygen species appears to be a major contributor to aging and to degenerative diseases of aging such as cancer, cardiovascular disease, cataracts, immune system decline, and brain dysfunction [3].
The plant *E. ribes* is identified and recognized by Medicinal Board, Government of India, New Delhi [4] and is one of the red list plant mainly found in semi evergreen and deciduous forests at an altitude of 400 to 1500 m of Northern Western Ghats of India, Sri Lanka, China [5].

2. Material and methods
2.1 Extraction and qualitative analysis of the secondary metabolites
2.1.1 Collection of seed material
Dried seeds of *E. ribes* were collected from a locally situated medicinal practitioners and Ayurveda outlets.

2.1.2 Preparation of seed extracts
The dried seeds were pulverized to get coarse powder and it was extracted by continuous hot percolation method using soxhlet apparatus in various solvents such as Petroleum ether, Ethyl acetate, Acetone and Methanol according to their increasing strength of polarity. The extract obtained with each solvent was weighed and the percentage yield was calculated in terms of dried weight of the plant material.

2.1.3 Qualitative analysis
The extracts were subjected to qualitative tests to detect the presence of various Phytochemicals such as alkaloids, steroids, tannins, flavonoids, diterpenes, glycosides, saponins, phenols, proteins and amino acids.

2.2 Analysis of Antioxidant activity
The antioxidant activity was analysed by Di Phenyl Picryl Hydrazine (DPPH) radical scavenging activity as well as by 2, 2´-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) assay. The samples extracts were in DMSO and were mixed with DPPH and ABTS respectively and incubated in dark chamber. The absorbance was taken at 517nm for DPPH assay and 734nm for ABTS assay using spectrophotometer. A standard gallic acid (100mg/mL) was used as positive control and a graph of percentage of radical scavenging activity versus concentration was plotted and 50% Inhibitory Concentration (IC50) values were determined for each extract in both assays using the formula

$$\% \text{ Inhibition} = \frac{\text{Control Absorbance} - \text{Sample Absorbance}}{\text{Control Absorbance}} \times 100$$

2.3 Quantitative estimation of secondary metabolites by HPLC
Quantitative estimation of phenols and flavonoids in seed extracts of *E. ribes* were done using HPLC. Acetonitrile and water in the ratio of 7:3 was used as mobile phase. The Standards Rutin (Flavonoid) and Gallic Acid (Phenols) were prepared in mobile phase (10mg/mL). 20µL of each standard sample was injected into HPLC separately and the chromatograms were obtained at 272nm and 252nm for flavonoids and phenols respectively.
The amount of flavonoids and phenols present in the extract was calculated by using following formula:

$$\text{Amount} = \frac{Sample area}{Standard area} \times Standard amount \times Dilution \times \frac{Dilution}{Sample amount} \times Mean weight of sample$$

3. Results
3.1Extraction of secondary metabolites
High percentage of extract yield (4.2%) was obtained for aqueous extract whereas ethyl acetate extraction yielded less (0.562%) (Table 1).

Table 1: Extraction of phytochemicals from *E.ribes* Seeds

Type of Extract	Amount of Extract (gm)	Yield (%W/W)
Petroleum ether extract	0.963	1.926
Ethyl acetate extract	0.281	0.562
Acetone extract	0.791	1.582
Methanolic extract	0.663	1.326
Aqueous extract	1.054	4.216

3.2 Phytochemical Screening
The methanol extract and acetone extract contained the most of the phytochemicals compounds such as alkaloids, steroids, tannins, flavonoids and diterpenes in methanol extract and alkaloids, steroids, phenols, sapon ins and glycosides in acetone extract whereas, petroleum ether and ethyl acetate extract showed the least number of phytochemicals. This can be implied that the phytochemical compounds in *E. ribes* dissolved better in methanol solvent (Table 2).

Table 2: Phytochemical screening of various extracts of *E. ribes* seeds

Phytochemical screening tests	Petroleum Ether extract	Ethyl acetate extract	Acetone extract	Methanol extract	Water extract
Alkaloids	+	+	+	+	-
Tannins	-	-	-	+	+
Flavonoids	-	-	-	+	+
Steroids	+	+	+	+	+
Phenols	-	-	+	-	-
Diterpenes	-	-	-	+	+
Saponins	-	-	+	-	-
Glycosides	+	+	+	-	+
Proteins and amino acids	+	+	+	+	+

3.3 DPPH Assay
All the solvent extracts scavenged DPPH free radical molecules in a concentration dependent manner. Methanol extract showed the highest antioxidant activity with an IC50 value of 78.077µg/mL (Table 3; Fig 1). The lower IC50 value indicates the solvent extract has a higher the antioxidant activity. The lowest antioxidant activity was obtained by the petroleum ether extract with an IC 50 value 312µg/mL.

Table 3: DPPH assay of methanol extract

Sl. No	Sample (µL)	Concentration of Sample (µg/mL)	DPPH reagent (mL)		Absorbance at 517nm	% of inhibition	IC 50 Value
1	10	100	3	Incubate at dark chamber for 15 min	0.6	40	78.077
2	20	200	3		0.239	76.1	
3	30	300	3		0.2	80	
4	40	400	3		0.129	87.4	
5	50	500	3		0.1	90	

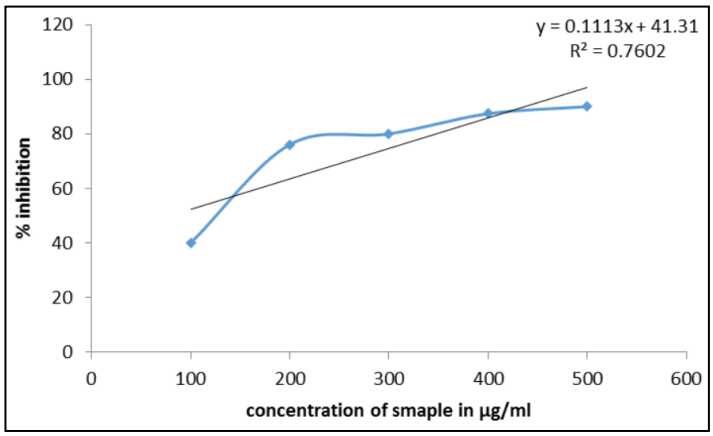

Fig 1: DPPH assay of methanol extract

3.4 ABTS assay

All the solvent extracts scavenged ABTS free radical molecules in a concentration dependent manner. Methanol extract showed the highest antioxidant activity with an IC50 value of 56.466µg/mL (Table 4; Fig 2). The lower IC50 value indicates the solvent extract has a higher the antioxidant activity. The lowest antioxidant activity was obtained by the petroleum ether extract with an IC 50 value of 135.88µg/mL.

Table 4: DPPH assay of methanol extract

Sl. No	Sample (µL)	Concentration of Sample (µg/mL)	DPPH reagent (mL)		Absorbance at 517nm	% of inhibition	IC 50 Value
1	10	100	3	Incubate at dark chamber for 15 min	0.446	55.4	56.466
2	20	200	3		0.404	59.6	
3	30	300	3		0.304	69.6	
4	40	400	3		0.275	72.5	
5	50	500	3		0.12	88	

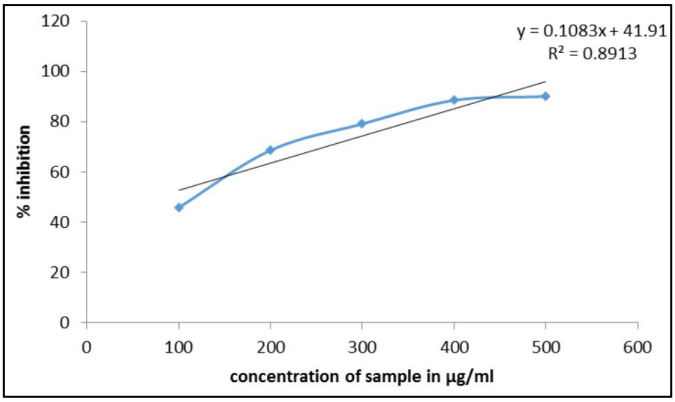

Fig 2: ABTS assay of methanol extract

3.5 Quantitative estimation of phytochemicals by HPLC

Highest amount of flavonoid obtained from methanol extract (2.83mg) as compared to aqueous extract (1.54mg). Amount of phenols obtained from acetone extract was found to be 1.0731mg.

4. Discussion

It is proven that sequential extraction of secondary metabolites with increasing order of polarity index of solvents is able to extract a wide range of secondary metabolites compared with single of solvent system [6]. Hence, the sequential extraction with increasing polarity index of solvent was used in this study. The study revealed that both acetone and methanol are the best solvents for phytochemical extraction from seeds of *E. ribes*. Petroleum ether and ethyl acetate extracts have the capacity to extract less phytoconstituents due to their nonpolar nature.

Gorinstein *et al.* [7] has reported that the high antioxidant activity of plant extracts were due to the presence of high phenolic and flavonoids compounds which are polar compounds in the 50% of methanol extract. In addition, Guha [8] has conducted a study that showed that the polar solvent extract (methanol and aqueous) possessed a higher antioxidant activity compare to non-polar extract (hexane and chloroform) over 56 different type of plants.

Flavonoids are potential antioxidant agent that acts as free radical scavenger. The example of flavonoids that have antioxidant properties are flavonols, flavones, anthocyanins, isoflavonoids, flavanols and flavanones [9]. Phenolic compounds are usually contributed to the antioxidant activity due to the presence of hydroxyl functional groups in their chemical structures [10]. High concentration of tannins also shows antioxidant activity [11, 12].

5. Conclusion

The phytochemical screening of secondary metabolites from the sequential extracts of *E. ribes* showed that the seeds are rich source of various classes of important constituents phytochemical. The methanol extract of *E. ribes* showed highest antioxidant activity and is able to scavenge high percentage of free radicals even at lower concentrations.

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