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Preliminary phytochemical, HPLC and GC-MS profile of *Embelia ribes* burm. f.– An vulnerable species

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Abstract
Embelia ribes Burm. F. have shown many phytochemicals which contributes to the medicinal activity and pharmaceutically important compounds detected through GC-MS analysis are: Phenol, 2,4-Bis(1,1-Dimethylethyl)-, Dodecanoic Acid, Cyclohexanone, 2-Isopropyl-2,5-Dimethyl-, Diphenylamine, 1-Tetradecanol, Eicosane, Tetra Decanoic Acid, Dodecane, 1,1-Dimethoxy-, 3-Pentanol, 3-Ethyl-, Cyclohexanol, 1-Butyl-, Octadecanoic Acid, Methyl Ester, Beta.-Sitosterol. These compounds are responsible for antifungal, antimicrobial, antioxidant, and anti-inflammatory in this plant.

Keywords: *Embelia ribes*, FT-IR, phytochemical, HPLC, GC-MS

Introduction
Genus *Embelia* belongs to family Primulaceae consist of about 130 species (Angiosperm Phylogeny Group IV 2016). *Embelia ribes* Burm. f. is one of the oldest Indian traditional medicinal species and reported to be vulnerable and red-listed species in Western Ghats of India. It is mainly use in ayurvedic system of medicine in various forms like churna, asava, aristha, lauha and taila. It is an Indo-Malaysian species. Many research of Botany and Ayurvedha have correlated this species was included as official drug in Indian Pharmacopoeia. The main component is Embelin, (chemically 2, 5-dihydroxy-3-undecyl-1, 4- benzoquinone) is one of the major bioactive constituents and a wide spectrum of biological activities of antibacterial, antioxidant, wood healing, antitumor and antibacterial.

Materials and Methods
Collection and authentication
Plant materials of *E. ribes* (Stem) were collected from the Western Ghats of Tamil Nadu and Kerala. They were authenticated by Dr. S. John Britto, Director and Head, The Rapinat Herbarium and Centre for Molecular Systematics, St. Joseph's College (Autonomous) Tiruchirappalli, Tamil Nadu, S. India. (Voucher specimen no: RHT 68490).

Preparation of extract
The powdered plant parts (stem) 10 g of each were extracted in 100 ml of Acetone, ethanol, methanol, aqueous with continuous shaking on mechanical shaker for 24/hrs at room temperature. The extracts were then filtered through what mann No.1 filter paper and stored airtight.

Preliminary phytochemical analysis
Qualitative phytochemical tests for the identification of alkaloids, flavonoids, steroids and terpenoids were carried out for all the extracts by the method described by Mukherjee (2002) [3].

Detection of Alkaloids
Solvent free extract 50 mg was stirred with few ml of dil. HCl and filtered. The filtrate was tested carefully with various alkaloidal reagents as given below.

a. Mayer's Test
To a few ml of filtrate, one or two drops of Mayer's reagent were added by the side of the test tube. A white milky cream precipitate indicated the test as positive.

b. Wagner's Test
To a few ml of filtrate, few drops of Wagner's reagent were added by the side of the test tube. A reddish brown precipitate confirmed the test as positive.

c. Hager's Test
To a few ml of filtrate, 1 or 2 ml of Hager's reagent (Saturated aqueous Solution of picric acid) was added. A prominent yellow precipitate indicated the test as positive.

Detection of Carbohydrates and Glycosides
The extract of 100mg was dissolved in 5 ml water and filtered. The filtrate was subjected to the following tests

a. Molish's Test
To 2 ml of filtrate two drops of alcoholic solution of α-napthol was added, the mixture was shaken well and 1 ml of con H_2SO_4 was added slowly, along the sides of the test tube and allowed to stand. A violet ring indicated the presence of carbohydrates.

b. Benedict's Test
To 0.5 ml of filtrate, 1 ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 mins. A characteristic coloured precipitate indicated the presence of sugar.

Detection of Glycosides

50 mg of extract was hydrolysed with concentrated HCl for 2 hours on a water bath, filtered and the hydrolysate was subjected to the following tests:

a. Borntrage's Test

To 2 ml of filtered hydrolysate, 3 ml of chloroform is added and shaken, chloroform layer was separated and 10% ammonia solution was added to it. Pink colour indicated the presence of glycosides.

b. Legal's Test

50 mg of the extract was dissolved in pyridine. Sodium nitro prusside solution was added and made alkaline using 10% NaOH. Presence of glycosides was indicated by pink colour.

Detection of Saponins

To 1ml of extract, add 2ml of distilled water and shaken vigorously and allowed to stand for 10 min. There is the development of foam on the surface of the mixture. Then shake for 10 minutes, it indicates the presence of saponins.

Detection of Proteins and Amino Acids

The extract (100 mg) was dissolved in 10 ml of distilled water and filtered through whatman no: 1 filter paper and filtrate was subjected to tests for proteins and amino acids.

a. Millon's Test

To 2 ml filtrate, few drops of millon's reagent were added. A white precipitate indicated the presence of proteins.

b. Biuret Test

An aliquot of 2 ml of filtrate was heated with 1 drop of 2 % $CuSO_4$ solution. To this 1 ml of ethanol (95%) was added, followed by excess of KOH Pellets. Pink colour in the ethanolic layers indicated the presence of proteins.

c. Ninhydrin Test

2 drops of Ninhydrin solution (10 mg of Ninhydrin in 200 ml of acetone) was added to 2 ml of aqueous filtrate. A characteristics purple colour indicated the presence of amino acids.

Detection of Phytosterols

a. Libermann – Burchard's Test

The extract (50 mg) was dissolved in 2 ml acetic anhydride. To this one or two drops of concentrated H_2SO_4 were added slowly along the sides of the test tube. An array of colour change showed the presence of phytosterols.

Detection of Fixed Oils and Fats

a. Spot Test

A small quantity of extract was pressed between two filter papers. Oil stain on the paper indicated the presence of fixed oil.

b. Saponification Test

A few drops of 0.5N alcoholic KOH solution were added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on water bath for 2 hours. Formation of soap or partial neutralization of alkali indicated the presence of fixed oils and fats.

Detection of Phenolic Compounds and Tannins

a. Ferric Chloride Test

The extract (50 mg) was dissolved in 5 ml of distilled water. To this few drops of neutral 5% ferric chloride solution was added. A dark green colour indicated the presence of phenolic compounds.

b. Lead Acetate Test

The extract (50 mg) was dissolved in distilled water and to this 3 ml of 10% lead acetate solution was added. A bulky white precipitate indicates the presence of phenolic compounds.

c. Gelatin Test

The extract (50 mg) was dissolved in 50 ml of distilled water 2 ml of 1% solution of gelation containing 10% sodium chloride was added to it. White precipitate indicated the presence of phenolic compounds.

d. Alkaline Reagent Test

An aqueous solution of the extract was heated with 10% NH_4OH solution. Yellow fluorescence indicated the presence of flavonoids.

e. Magnesium and Hydrochloric Test

The extract (50 mg) was dissolved in 5 ml of alcohol and few fragments of magnesium ribbon and concentrated HCl were added (dropioire). If any pink to crimson developed, presence of flavanol glycoside was inferred.

Test for starch

To mix 3 ml of the extract was added a few drops of dilute iodine solution. Blue colour indicated the presence starch. Colour disappears on boiling and reappears on cooling.

Test for flavonoids

a. *Shinoda test*

To 2ml of the extract and a few fragments of magnesium ribbon were added and to it con. Sulphuric acid was added drop wise. Pink scarlet or crimson red appeared.

b. *Zinc chloride reduction test*

To 2ml of the extract a mixture of zinc dust and con. HCl were added. A red colour was obtained after few minutes.

c. *Alkaline reagent test*

To 2ml of the extract sodium hydroxide solution was added to give a yellow or red colour.

FT-IR Spectrometric Analysis

Fixed amount of plant specimen was mixed with KBr salt, using a mortar and pestle, and compressed into a thin pellet. Infrared spectra were recorded as KBr pellets on a Thermo scientific Nicot iS5 iD1 transmission, between 4000-400 cm^{-1} 21. The powdered sample of the plant extract was treated for FT-IR spectroscopy. The result was analyzed based on peak obtained.

HPLC Analysis

The underlying principle of HPLC is that the sample mixture (mobile phase) is pumped at high pressure (up to 400 atm.) through a column with chromatographic packing material (stationary phase). All chromatographic separations, including HPLC operate under the same basic

principle. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column. Sample retention time varies based on the interaction between the stationary phase, the molecules being analysed, and the solvent. As the sample passes through the column, it interacts between the two phases at different rate due to different polarities in the molecules. The molecules that have the least amount of interaction with the stationary phase will exit the column faster.

Specification and Procedure

For HPLC analysis those ethanolic extracts of the selected plant materials were used in Shimadzu HPLC System (Model SPD-20A UV-VIS Detector). The conditions and specifications were adopted from with a slight modification given below:

Communication Module: CBM-20A Shimadzu

Detector: SPD-20A Shimadzu at 254nm

Pump A: LC-8A Shimadzu pumps HPLC distilled water

Pump B: LC-8A Shimadzu pumps acetonitrile

Mobile Phase: Acetonitrile: Distilled Water (80:20

Injection Volume: 20µl

Flow Rate: 1ml/min

Column Temperature: 25℃

Column Pack: LC-18 column (25cm × 4.6mm)

Run Time: 20-30 minutes based on sample type

Application Software: LC Solution Version 1.24 SP1

Results and Discussion

Preliminary phytochemical Tests

The results of the preliminary phytochemical screening are tabulated in (Table 1). The stem extract showed the presence of alkaloids, carbohydrates, cardiac glycosides, quinones, phenols and tannins. Starch and saponins were absent in the stem. However, the stem showed the presence of cardiac glycosides in high concentration. They also exhibited the presence of reducing sugars.

FT-IR spectrometric analysis

The stem powder of *E. ribes* exhibited 11 characteristic bands. The highest band occurred at 3412.63 cm^{-1} indicating the presence of functional groups like alcohols and phenols having O–H stretch and H–bonded groups, 2975.45 cm^{-1} indicating the presence of alkane (C–H stretch) group, 1734.43 cm^{-1} indicating the presence of aromatic (C=O stretch), 1417.11cm^{-1} indicating the presence of aromatic (C-C stretch), 1051.93cm^{-1} indicating the presence of aliphatic amine (C-N stretch), 622.26cm^{-1}indicating the presence of alkyl halide (C–C1 stretch).

Table 1: Preliminary Phytochemical Analysis of *E. ribes* stem extract

	Test	Acetone	Ethanol	Methanol	Aqueous
Alkaloids	Wager's	+	+	++	+
	Hager's	+	++	++	+
	Mayer's	+	+	+	++
Flavonoids	Pew's	-++	+	++	+
	Shinoda	+	++	++	+
	NaOH	+	-	++	-
	Con.H_2SO_4		++	++	-
Phenols & Tannins	$FeCl_3$	++	++	++	++
	$K_2Cr_2O_7$	+	++	++	+
	Lead Acetate	-	-	-	+
	Braymers	++	+	+	+
Saponins	Foam	-	+	+	-
	$NaHco_3$	+	+	+	+
Glycosides	Keller-Kiiani	+	+	++	+
	Glycosides	++	++	+++	++
	Libermann	++	+	++	+
Carbohydrates	Molish	+	+	++	+
	Benedict's	+	+	+	+
	Emodins	-	+	+	-
Anthocyanin	Borntrager's	-	-	+	-
	Quinones	-	-	+	-
Sterols	Salkowski's	+	+	+	-
	Triterpenoids	+	+	++	-
Protein	Biuret	++	++	+++	++
	Con. H_2SO_4	+++	+++	+++	+
	Xanthoprotein	++	+	+	+
	Terpenoids	+	+	+	+

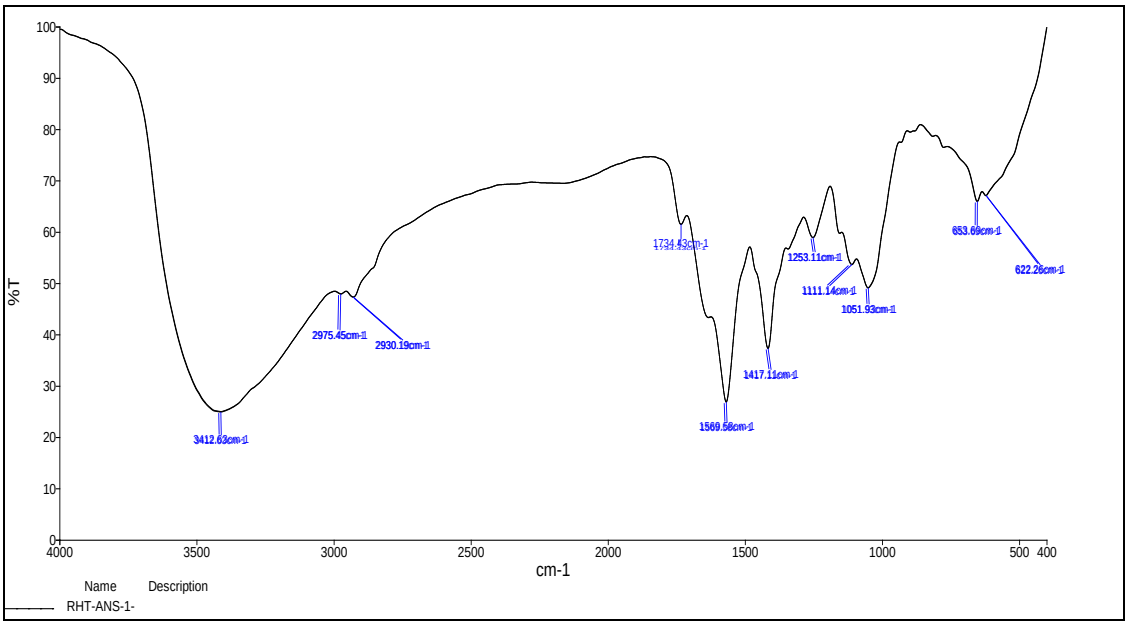


Fig 1: FT-IR analysis of *E. ribes* stem extract

Table 2: FT-IR analysis of *E. ribes* stem extract

Frequency (cm-1)	Type of vibration	Functional group	Intensity
3412.63	O-H stretch, H-bonded	Alcohol	Strong, broad
2975.45	C-H stretch	Alkane	Strong
2930.19	C-H stretch	Alkane	Strong
1734.43	C=O stretch	Aldehydes	Strong
1569.58	N-H bending	Amide	Strong
1417.11	C-C stretch	Aromatic	Medium
1253.11	C-O stretch	Alcohol	Strong
1111.14	C-O stretch	Alcohol	Strong
1051.93	C-N stretch	Aliphatic amine	Medium
653.69	-C=C-H:C-H bending	Alkyne	Strong, broad
622.26	C-C1 stretch	Alkyl Halide	Medium

HPLC analysis

The HPLC analysis of ethanolic extract of produced two peaks with retention time 4.309 and 6.741 with area of 99.167 and 0.833 indicating high concentration of two bioactive compounds (Fig. 2). The first peak showing greater 99% of area coverage is an indicative of the presence of highest concentration in the stem of *E. ribes.*

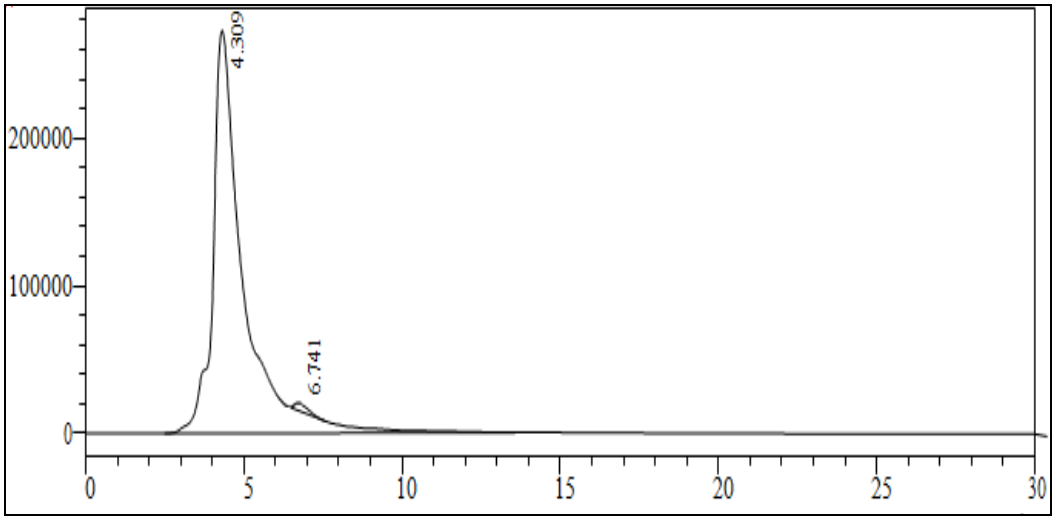


Fig 2: HPLC analysis of *E. ribes* stem extract

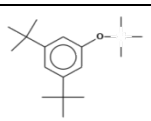
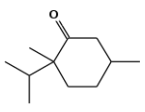
Table 3: HPLC analysis of *E. ribes* stem extract

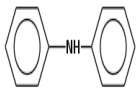
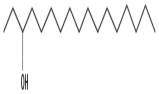
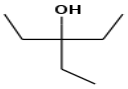
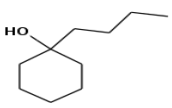
Peak#	Ret. Time	Area	Height	Area%	Height%
1	4.309	18356513	273022	99.167	98.218
2	6.741	154206	4954	0.833	1.782
Total		18510719	277976	100.000	100.000

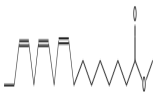
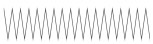
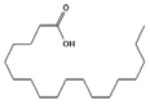
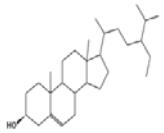
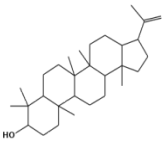
GC-MS analysis

E. ribes have shown many phytochemicals which contributes to the medicinal activity and pharmaceutically important compounds detected through GC-MS analysis are: Phenol, 2,4-Bis(1,1-Dimethylethyl)-, Dodecanoic Acid, Cyclohexanone, 2-Isopropyl-2,-Dimethyl-, Diphenylamine, 1-Tetradecanol, Eicosane, Tetra Decanoic Acid, Dodecane, 1,1-Dimethoxy-, 3-Pentanol, 3-Ethyl-, Cyclohexanol, 1-Butyl-, Octadecanoic Acid, Methyl Ester, Beta.-Sitosterol. These compounds are responsible for antifungal, antimicrobial, antioxidant, and anti-inflammatory in this plant.

Table 4: GC-MS analysis of *E. ribes* stem extract

Si No	RT	Name Of The Compound	Molecular formula	Molecular Weight	Peak Area %	Structure	Activity
1	9.760	Phenol, 2,4-Bis(1,1-Dimethylethyl)-	$C_{17}H_{30}OSi$	278.5050	5.67		Antioxidant
2	10.375	Dodecanoic Acid	$C_{12}H_{24}O_2$	200.3178	0.36		Acne treatment, increases high-density lipoprotein
3	10.499	Cyclohexanone, 2-Isopropyl-2,5-Dimethyl-	$C_{11}H_{20}O$	168.2759	0.19		As a flavor component of cognac and in grapefruit peel oil, star fruit, corn mint oil and spearmint oil.

4	11.258	Diphenylamine	$C_{12}H_{11}N$	169.2224	0.25		Industrial antioxidant, dye mordant and reagent and is also employed in agriculture as a fungicide and antihelmintic.
5	11.775	1-Tetradecanol	$C_{14}H_{30}O$	214.3874	0.18		organic synthesis, plasticizers, antifoaming agent, intermediate, perfume fixative for soaps and cosmetics
6	12.040	Eicosane	$C_{20}H_{42}$	282.5475	0.36		candles and paraffin waxes with solar energy storage capacity
7	12.700	Tetra Decanoic Acid	$C_{14}H_{28}O_2$	228.3709	0.40		Soaps & Cosmetics; Surfactant; Cleansing Agent; Emulsifier; lubricant
8	13.262	3-Heptadecanol	$C_{17}H_{36}O$	256.4671	0.49		NR
9	13.417	Hexadecanal	$C_{16}H_{34}O$	242.4406	0.17		Emulsifier in cosmetics and pharmaceuticals
10	13.463	Isopropyl Myristate	$C_{17}H_{34}O_2$	270.4507	0.33		Flavouring Agents in food additives, mouth wash, Solvent in perfume, flea and tick products for pets.
11	13.650	Neophytadiene	$C_{20}H_{38}$	278.5157	0.92		antidiabetic, anti-inflammatory, antiarthritic and anticancer activities
12	13.917	Pentadecanoic Acid	$C_{15}H_{30}O_2$	242.3975	0.46		Adhesives and sealant, lubricants and lubricant additives
13	14.079	Dodecane, 1,1-Dimethoxy-	$C_{14}H_{30}O_2$	230.3868	0.09		NR
14	15.110	3-Pentanol, 3-Ethyl-	$C_7H_{16}O$	116.2013	0.21		NR
15	15.307	Hexadecanoic Acid	$C_{16}H_{32}O_2$	256.4241	10.78		Soaps, cosmetics, food additives, antioxidant, hypocholesterolemic
16	16.592	Heptadecanoic Acid	$C_{17}H_{34}O_2$	270.4507	0.31		Surfactant, adhesives, sealant lubricants and lubricant additives
17	16.819	Cyclohexanol, 1-Butyl-	$C_{10}H_{20}O$	156.2652	0.17		NR

18	17.141	Methyl Linolenate	$C_{19}H_{32}O_2$	292.4562	0.47		Skin whitening agent with anti-melanogenesis activity.
19	17.303	Phytol	$C_{20}H_{40}O$	296.5310	1.40		antinociceptive and antioxidant activities as well as anti-inflammatory and antiallergic effects
20	17.474	Hexatriacontane	$C_{36}H_{74}$	506.9728	0.33		Antioxidant, antineoplastic, antimicrobial
21	17.517	Octadecanoic Acid, Methyl Ester	$C_{19}H_{32}O_2$	292.4562	0.26		NR
22	18.059	Octadecanoic Acid	$C_{18}H_{36}O_2$	284.4772	1.51		Flavouring, soaps, cosmetics, detergents, lubricants, insecticide, herbicide
23	22.900	Beta.-Sitosterol	$C_{29}H_{50}O$	414.7067	3.25		Reduces prostatic hyperplasia and blood cholesterol
24	27.817	Moretenol	$C_{30}H_{50}O$	426.7174	6.65		Painkiller
25	30.577	Squalene	$C_{30}H_{50}$	410.7180	0.55		Antioxidant, wound healing

Discussion

The phytochemical studies of *E. ribes* by FT-IR, HPLC and GC-MS from the shown satisfactory results of proving that these species contain high level chemical properties for curing the related ailments in humans.

Conclusion

Based on these results it may be concluded that the components of further studies carried out to the future.

Acknowledge

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