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Research Article

Antioxidant, Anti-cancer and Phytochemical Constituents of Hexane Extract Fractions of an Indian Medicinal Plant *Embelia ribes* Burm.

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ABSTRACT

Embelia ribes Burm. (Family: Myrsinaceae, Vidangam in Tamil) has been used as herbal drug for various diseases in Indian medicinal systems. Many research works have been focused on embelin (a high polar bioactive constituent), no study found on low polar compounds. Hence, in the present work we have analyzed the antioxidant and anticancer properties of three different fractions obtained from hexane extract of *E. ribes* fruit and also to reveal the volatile phytochemicals profile. Three fractions were obtained from hexane extract of *Embelia ribes* Burm using hexane-chloroform (80:20, V/V) in column chromatography. Fraction I (orange red colour), fraction II (brown colour) and fraction III (dark green colour) were analyzed for antioxidant (DPPH assay) and anticancer activities (EAC cell line model) and also the phytochemical profile was investigated using GC-MS. Among the three fractions investigated, fraction II exhibited higher level of antioxidant effect in terms of phosphomolybdate reducing power (IC50 0.21 mg/ml), DPPH radical scavenging activity (IC50 1.04 mg/ml) and superoxide radical inhibition activity (1.58 mg/ml). The anticancer assay also revealed that the fraction II was effective to control the growth of EAC cell lines (IC50 value 4.36 mg/ml) when compared to other fractions. GC-MS analysis revealed the presence of major volatile phytochemical constituents such as Dodecanoic acid and Ethyl tridecanoate, which could be responsible for the medicinal effects exhibited by the fraction II. Thus, fraction II of hexane extract of *Embelia ribes* with remarkable antioxidant and anticancer activities could be considered for further evaluation as anticancer drug in animal models.

Key words: Column fractions; DPPH assay; EAC cell line; GC-MS analysis; Vidamngam.

INTRODUCTION

Embelia ribes Burm. is a valuable medicinal plant belongs to the family Myrsinaceae. It is commonly known as Vidangam in Tamil, Bashmak or Krimigna in Sanskrit, and Baberung or Wawrung in Hindi[1]. It is a woody shrub sparsely distributed in the moist deciduous forests of Western Ghats of India, South Asia and Malaysia[2]. The dry fruit of *E. ribes* is one of the major ingredients in Ayurveda and Siddha formulations like *Vidangadya churna* and *Nellikkai ilakam*[3,4]. The fruit is bitter in taste, good appetizer, cures bronchitis, jaundice, brain tonic, mental disorders, dyspnoea, diseases of the heart, urinary discharges, scorpion-sting, snake bite, anthelmintic and tooth ache[5]. It has been reported to possess antioxidant and anti-inflammatory properties to relieve rheumatism and fever[6]. Anti-obesity potential of this plant was proved in high-fat diet induced rat model[7]. The anti-hyperglycemic activity, anti-dyslipidemia and lipid peroxidation inhibition potential of this plant extract were reported[8]. Anti-fertility action[9], cardioprotective effect[10], anti-fungal activity[11], nephroprotective potential[12], protective effect against ischemia-induced brain damage[13], anti-allergic, anti-anaphylactic and mast cell stabilizing activities of *E. ribes* were also demonstrated[14].

Embelin (2, 5-dihydroxy-3-undecyl-1, 4-benzoquinone) is an alkyl substituted benzoquinone bioactive molecule represents the major constituents of this plant[15]. It possess a wide spectrum of medicinal and pharmacological properties including anti-tumor, cardioprotective, antioxidant, anti-diabetic, anxiolytic, anti-bacterial, anti-fertility, anti-implantation, anti-spermatogenic, wound-healing hepatoprotective, anticonvulsant, ulcerative colitis, anti-depressant, anti-inflammatory and analgesic properties[16,17]. Toxicity studies revealed that embelin is safe to use and orally fed rats and mice did not show any toxic effects at doses of 10 mg to 3 g/kg[18]. Microwave-assisted extraction and TLC, HPTLC and HPLC based methods to detect embelin in herbal ingredients have been developed[19-22].

In addition to embelin, *E. ribes* fruits contain also contains an alkaloid (christembine) and a volatile oil (vilangin). Phytochemical analysis revealed the presence of stigmasterol, a novel and rare 1,4-dehydrated ceramide, embelamide and a new C-glycoalkaloid 1-(2′-deoxy-α-d-ribopyranosyl)-β-carboline from leaves of *E. ribes*[23,24]. An unusual nitrogen-containing 3-alkyl-1,4-benzoquinone derivative N-(3-carboxylpropyl)-5-amino-2-hydroxy-3-tridecyl-1,4-benzoquinone and a gomphilactone as well as daucosterol were isolated from the ethanolic extract of the

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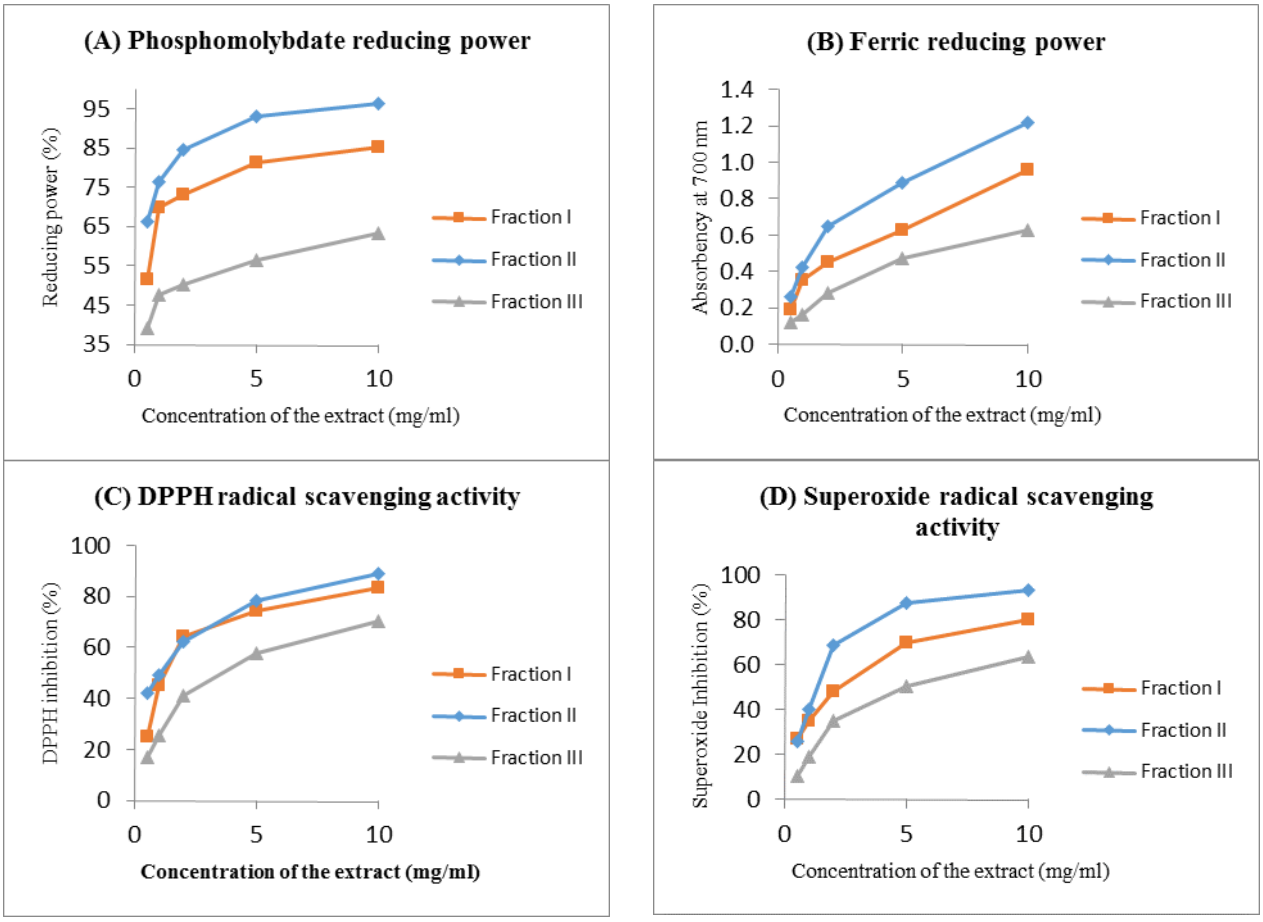

Figure 1: Antioxidant activity of different fractions of hexane extract of *Embelia ribes*

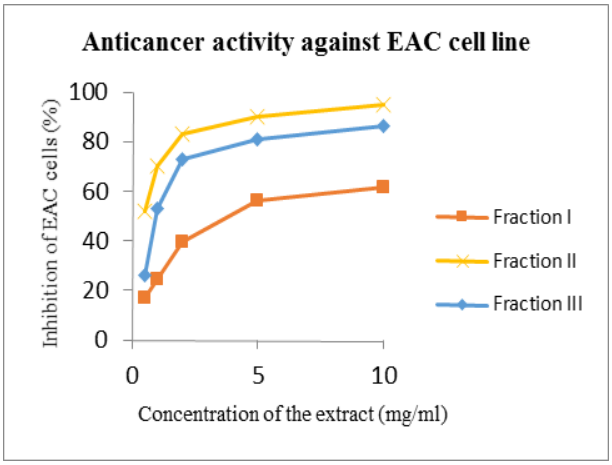

Figure 2: Anticancer activity of different fractions of hexane extract of *Embelia ribes*

roots of *E. ribes*[25]. Embelinol, embeliaribyl ester and embeliol were also identified in *E. ribes* seeds[26].

E. ribes is used traditionally in our Indian system of medicine for a long time and its several medicinal properties were proved scientifically, and few studies have also been conducted on its phytochemical composition. Even though certain well-known bioactive principles like embelin was investigated in detail, presence of other low-polar and volatile components and their bioactivities are not yet revealed. Hence, in the present study we have made an attempt to explore the antioxidant and anticancer activities of hexane extract fractions obtained from *E. ribes* fruits.

MATERIALS AND METHODS

Preparation of extract

The fruit samples of *E. ribes* was procured from the local market in Thanjavur, Tamilnadu, India and authenticated by Botanist Dr. Ravichandran, Centre for Advanced Research in Indian System of Medicine, SASTRA University. Powdered sample (100 g) was taken with 500 ml of hexane and kept on magnetic stirring for 5 h at room temperature. The extract was separated using Whatman No. 1 filter paper and then the solvent was evaporated to obtain a dry extract.

Fractionation

The slurry was prepared by taking 1 g dry hexane extract with silica and loaded on the glass column (60 x 3 cm) packed with silica (60-120 particle size). The extract was fractionated using different proportions of hexane and chloroform solvents and three fractions (Fraction I, orange red colour; Fraction II, brown colour; Fraction III, dark green colour) were eluted in hexane-chloroform (80:20, V/V) ratio. All the three fractions were collected separately and the solvents are evaporated on the water-bath at 50°C and then each fraction was re-dissolved in the same solvent at 10 mg/ml ratio and then analyzed for GC-MS profile and *in vitro* antioxidant and anticancer properties.

Antioxidant activity

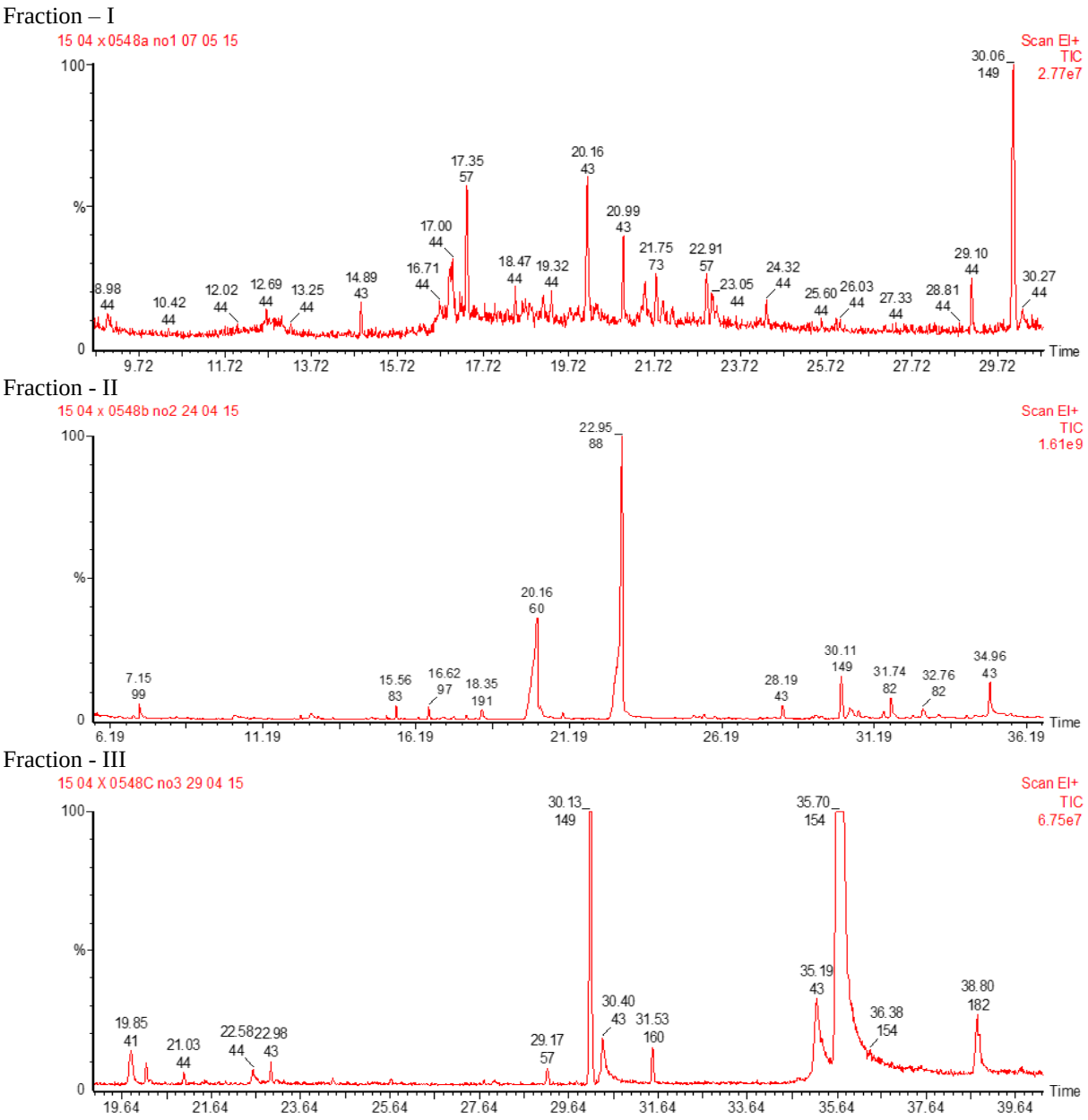

Figure 3: GC-MS profile of hexane extract fractions of *E. ribes*

The antioxidant activity of hexane extract fractions was evaluated in terms of phosphomolybdate assay[27], ferric reducing power[28], DPPH radical scavenging activity[29] and superoxide radical scavenging activity[30].

Anticancer activity

The anticancer activity of hexane extract fractions were evaluated in EAC cell line. The EAC cells were obtained from Swiss mice after 15 days of cancer induction. The peritoneal fluid containing EAC cells was collected aseptically using a sterile syringe and cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated foetal bovine serum. The anticancer effect of different concentrations of hexane extract fractions of *E. ribes* was analyzed by treating with EAC cells for 6 h and the cell viability will be checked using MTT assay.[31] The cells were dispersed in 96 well plate with a cell count of 9000 cells per well and incubated for 2 h. Then the solvent extract was added at different concentration and then again incubated for 5 h. At the end 20 µl of MTT reagent was added in each well and incubated for 3 h at 37 C in a water bath. Then 150 µl of acidic isopropanol was added and shaken for 30 min on a plate shaker under dark. The absorbance was measured at 540 nm and the percentage of inhibition was calculated.

GC-MS analysis

All the three fractions of hexane extract of *E. ribes* were analyzed for low polar and volatile phytochemical profile using Gas Chromatographic system coupled with Mass Spectrometry (Perkin Elmer, Model: Clarus-500). Silica capillary column (30 m x 0.25 mm, 0.25 µm film thickness, Elite-5 MS non-polar fused 5% Phenyl 95% dimethylpolysiloxane) was used. Oven temperature was programmed with an increase of 6°C/min from room temperature to 150°C and then an increase of 4°C/min from 150°C to 280°C was set. Injector temperature was

Table 1: Phytochemical constituents of hexane extract fractions of *E. ribes* identified through GC-MS analysis

S. No.	Fraction I	Fraction II	Fraction III
1.	Name: 3-Hexanone-2,4-dimethyl Formula: $C_8H_{16}O$ MW: 128, RT: 17.35 Peak area (%): 28.59	Name: 2-Pyrrolidinone-1-methyl Formula: C_5H_9NO MW: 99, RT: 7.15 Peak area (%): 1.94	Name: 4-Quinazolinone Formula: $C_8H_6N_2O$ MW: 146, RT: 19.85 Peak area (%): 1.45
2.	Name: Decane-6-ethyl-2-methyl Formula: $C_{13}H_{28}$ MW: 184, RT: 20.16 Peak area (%): 37.22	Name: Heptanoic acid-6-oxo Formula: $C_7H_{12}O_3$ MW: 144, RT: 12.77 Peak area (%): 1.63	Name: 2-Dodecanone Formula: $C_{12}H_{24}O$ MW: 184, RT: 20.19 Peak area (%): 0.53
3.	Name: Dodecanoic acid-1-methylethyl ester Formula: $C_{15}H_{30}O_2$, MW: 242, RT: 20.99 Peak area (%): 21.19	Name: Phenol-2,4-bis (1,1-dimethylethyl) Formula: $C_{14}H_{22}O$ MW: 206, RT: 18.35 Peak area (%): 1.90	Name: Isobutyl nitrite Formula: $C_4H_9NO_2$ MW: 103, RT: 21.03 Peak area (%): 0.24
4.	Name: 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione Formula: $C_{17}H_{24}O_3$ MW: 276, RT: 29.10 Peak area (%): 13.00	Name: Dodecanoic acid Formula: $C_{12}H_{24}O_2$ MW: 200, RT: 20.16 Peak area (%): 38.82	Name: Pentanoic acid Formula: $C_5H_{10}O_2$ MW: 102, RT: 22.58 Peak area (%): 0.68
5.	--	Name: Ethyl tridecanoate Formula: $C_{15}H_{30}O_2$ MW: 242, RT: 22.95 Peak area (%): 42.36	Name: Dibutyl phthalate Formula: $C_{16}H_{22}O_4$ MW: 278, RT: 30.13 Peak area (%): 10.78
6.	--	Name: Tetradecanoic acid-2-oxo ethyl ester Formula: $C_{16}H_{30}O_3$ MW: 270, RT: 28.19 Peak area (%): 2.09	Name: 2H-Isoindole-2-propanoic acid-1,3-dihydro-1,3-dioxo Formula: $C_{11}H_9NO_4$ MW: 219, RT: 31.53 Peak area (%): 0.91
7.	--	Name: Dodecanoic acid-2-hexen-1-yl ester Formula: $C_{18}H_{34}O_2$ MW: 282, RT: 32.76 Peak area (%): 2.02	Name: Cyclohexanone 4-ethyl-3,4-dimethyl Formula: $C_{10}H_{18}O$ MW: 154, RT: 35.71 Peak area (%): 82.42
8.	--	Name: 6,6,7-Trimethyl-octane-2,5-dione Formula: $C_{11}H_{20}O_2$ MW: 184, RT: 34.96 Peak area (%): 6.27	Name: Methanone (2-bromophenyl)-2-pyridinyl Formula: $C_{12}H_8BrNO$ MW: 261, RT: 38.80 Peak area (%): 2.96

280°C. Carrier gas was helium with the flow rate of 1 ml/min. Sample (1.0 µl) was injected with split ratio of 1:10. Ionization energy 70 ev was used in the electron ionization mode; ion source temperature was set at 160-200°C, mass was scanned in the range of 40-600 amu. The instrument was operated using Turbomass software version 5.2.0. The resulting mass spectrum was compared with inbuilt NIST library (2005) database and fragments of various compounds present in the extracts were identified.

RESULTS AND DISCUSSION

The fractionation of hexane extract of *E. ribes* in column chromatography with chloroform-hexane (80:20, V/V) has yielded three fractions: Fraction I (orange red colour), Fraction II (brown colour) and Fraction III (dark green colour). All these fractions were collected separately, dried and then reconstituted in the same solvent system and analyzed for antioxidant and anticancer activities using *in vitro* methods.

The phosphomolybdate reducing assay revealed that the fraction II of *E. ribes* hexane extract has higher reducing power with IC50 value of 0.21 mg/ml which is higher than that of fraction I and III (IC50 value 0.85 and 4.28 mg/ml, respectively) (Fig. 1A). When the molybdenum (VI) is reduced to Mo (V) by an antioxidant, it forms a green colored complex at acidic pH in the presence of phosphorous with the absorption maxima at 695 nm. This assay evaluate the reducing or electron donating power of the antioxidant to Molybdenum and the intensity of PMo(V) complex is proportional to antioxidant power of

the extract. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant. In ferric reducing assay, Fe (III) is reduced to Fe (II) by the antioxidant compound through electron transfer. The reduced Fe (II) forms the Pearl's blue complex, which can be measured at 700 nm. *E. ribes* extract fractions exhibited good ferric reducing power (Fig. 1B) in dose-dependent manner with higher reducing power shown by fraction II.

The DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity of presently investigated hexane extract fractions were shown in the Figure 1C. Fraction II revealed higher DPPH radical scavenging activity with IC50 value of 1.04 mg/ml when compared to fraction I (IC50 value 2.13 mg/ml) and fraction III (IC50 value 5.11 mg/ml). The evaluation of the antioxidant power by DPPH radical scavenging activity has been widely used for different plant extracts. DPPH is a stable radical, methanolic solution of which has dark purple colour with maximum absorption at 515 nm**.** Antioxidants can reduce DPPH through hydrogen transfer into its non-radical form (DPPH-H) and hence the absorption disappears at 515 nm. The decrease in absorbency at 515 nm may be due to the reaction between phytochemicals and DPPH, which indicates the antioxidant power of the hexane extract fractions of *E. ribes* fruit.

The superoxide radical scavenging activity of hexane extract fractions were investigated by generating superoxide through photo-induced reduction of riboflavin, which can generate superoxide radical in the presence of methionine. The generated superoxide radical reduce the NBT into purple colour formazan, which was measured at 560 nm. In presence of antioxidant, the generated superoxide radicals were scavenged and hence, formation of purple colour formazan is minimum or nil. Figure 1D illustrates the superoxide radical scavenging of hexane extract fractions of *E. ribes*. The superoxide radical scavenging activity of fraction II was higher (IC50 value 1.58 mg/ml), which is followed by fractions I and III (IC50 values 2.56 and 8.74 mg/ml, respectively).

The majority of most useful and curative anticancer drugs have been derived from plant sources. Plant extracts that contain several pharmacological compounds have been reported to act on multiple molecular and cellular targets and such approach is gaining support to fight against cancer. The anticancer effect of hexane extract fractions of *E. ribes* against EAC cell line was given in the Figure 2. In this experiment, EAC mouse cancer cells were treated with different concentrations of hexane extract fractions of *E. ribes* (0.5, 1, 2, 5 & 10 mg/ml) and incubated for 6 h. Then, the viability of the treated cells was analyzed by MTT assay and the percentage inhibition of cancer cells by extract fractions was calculated. Among the hexane fractions, fraction II revealed higher level of anti-cancer effect with IC50 value of 0.25, which is followed by fraction III (IC50 value 1.72 mg/ml) and fraction I (IC50 value 4.36 mg/ml). The results indicate that the fraction II of hexane extract of *E. ribes* is effective to control the proliferation of EAC cancer cells and hence it could be used for further studies to treat/prevent different types of mammalian cancer.

GC-MS analysis reveals that the Fraction I contains 3-Hexanone-2,4-dimethyl, Decane-6-ethyl-2-methyl-, Dodecanoic acid, 1-methylethyl ester and 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione as major compounds while Fraction II contains Dodecanoic acid and Ethyl tridecanoate and Fraction III contains Cyclohexanone 4-ethyl-3,4-dimethyl and Dibutyl phthalate and as the notable phytoconstituents (Figure 3 and Table 1). Remarkable level of antioxidant and anticancer activities observed in fraction II might be due to the presence of Dodecanoic acid and Ethyl tridecanoate compounds.

Dodecanoic acid (also known as lauric acid), is a saturated fatty acid with a 12-carbon atom chain falling into the medium chain fatty acids group. It is a component of triglycerides, comprises about half of the fatty acid content in coconut milk, coconut oil, laurel oil, and palm kernel oil[32]. It is also found in human breast milk (6.2% of total fat), cow's milk (2.9%), and goat's milk (3.1%)[33]. *In vitro* experiments have suggested that some fatty acids including lauric acid could be a useful component in a treatment for acne[34]. Lauric acid increases total serum cholesterol more than many other fatty acids. But most of the increase is attributable to an increase in high-density lipoprotein (HDL). As a result, lauric acid has been characterized as having a more favorable effect on total HDL cholesterol than any other fatty acids[35].

Similarly, ethyl tridecanoate could contribute the antioxidant and anticancer activities of fraction II of hexane extract of *E. ribes*. Presence of ethyl tridecanoate in Galangal rhizomes (*Alpinia galanga*) with antioxidant and antimicrobial properties[36]. It also found in the fruit pulp of mango[37], which could be responsible for the health benefits exhibited by this tropical fruit. It has also been reported as responsible component for the medicinal properties of *Hypnea musciformis* (red seaweed)[38].

CONCLUSIONS

In the present study, the hexane extract of *E. ribes* was fractionated into three distinct fractions I, II & III and also evaluated their antioxidant and anticancer activities using *in vitro* methods. The results indicated that fraction II of hexane extract has higher antioxidant power and anticancer effect when compared to fraction I and III. The major phytochemical constituents (Dodecanoic acid and Ethyl tridecanoate) present in the fraction II of hexane extract were identified using GC-MS analysis, which could be responsible for the observed biological activities of *E. ribes*. Further work on efficacy of this hexane extract fraction II against mammalian cancer in animal model could be useful to develop a plant-based, natural, safe and efficient anticancer drug with global acceptance.

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