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Biochemical, HPLC Analysis and Pharmacological Activities of Methanol Extract of *Embelia ribes*

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Abstract

The methanol extract from the medicinal plant ***Embelia ribes* (*E.ribes*)** was biochemically analyzed for different bioactive components. Different biochemical properties of this plant extract were evaluated. It showed antioxidant and anticancer effect. It showed significant antimicrobial effect also against tested microbes. Antimicrobial activity against gram positive organisms such as *Staphylococcus aureus* MTCC 87and gram negative organisms like *Escherichia coli* MTTCC 41, *Proteus vulgaris* 426 (ATCC 6380), *Pseudomonas auriginosa* 424(ATCC 25619) and *Candida albicans* MTTCC 183(ATCC 2091) and found to have significant inhibitory effect. The effect of this extract was tested against the resistant strain Methicillin resistant *Staphylococcus aureus* also.

Introduction

Plants, especially medicinal plants have been explored continuously for therapeuticals. The plant products are widely classified mainly according to their chemical structure. The biological activities are related to the structure of the compound to an extent. *Embelia ribes* is medicinal plant belongs to the family *Myrsinaceae*. Vernacular names of the plant are 'Vezhalari' and 'Videga'[1]. It grows generally at mountain regions of India. Seeds are used commonly in the preparation of ayurvedic medicines. It shows properties as anti parasitic, anti helminthic, **anti leprosy** etc. It is also effective against the treatment of skin diseases. Bhandari et al., 2008 reported the cardioprotective activities of *Embelia ribes* The medicinal properties of *E.ribes* were evaluated in the present study. Most of the common pathogens develop drug resistance. Multidrug resistant *Staphylococcus aureus* is one of the common among them. *Staphylococcus aureus* is one of the major resistant pathogens. It is usually found in mucous membrane and human skin and extremely adaptable to antibiotics. Around 50% of infections were found to be due to MRSA in India. When some compounds are added to the common antibiotics used, it

can enhance the inhibitory effect on microbial growth. This can reduce the quantity of antibiotic consumed. This is an important strategy in combating antibiotic resistance. Use of mixture of extracts, plant derived compounds along with antibiotic etc is being studied at present to overcome the resistance developed by microbes[2].

Antioxidant activity

It is well known that highly reactive free radicals play a fundamental role in developing several diseases. Reactive nitrogen and oxygen species (RNS, ROS) have been intensively studied in recent years with regard to their relevant physiological and pathological importance along with its oxidative stress. The biochemical damage that they cause to the cells and tissues, lead to the diseases such as atherosclerosis, hyper tension, aging, cancer, diabetes mellitus, inflammation, renal failure, liver diseases, AIDS etc. [3,4,5]. The phenol substances present in the food and plants posses strong antioxidant properties and thus are being increasingly investigated. Phenol compounds widely distributed in the plant tissues include Flavanoids, tannins, hydroxyl cinnammate esters and lignin [6]. Some plant products have the anticancer property as well as antioxidant property and can be used as cancer preventive agents. Green tea phenols, which is a common food constituent is reported to be an antitumor agent. Plumbagin, Vincristine, Vinblastine and Coumarin are some of the plant derived anticancer agents.

Phenolic compounds are very important class of plant derived compounds. They can inactivate free radicals derived from fatty acid components and also prevent the decomposition of hydrogen peroxide into free radicals [7].

Flavanoids are one of the largest classes of naturally occurring plant poly phenolic compounds [8]. This was discovered by Albert Szent-Gyorgyi in 1924, along with vitamin C and called it as vitamin P4.These are used to treat the disorders of blood circulation, as anti-inflammatory, anti-allergic [9],to lower blood pressure [10] and as antimicrobial agent [11].

The various biological activities of plants against various illnesses are due to the active ingredients present in it. Medicinal plants contain wide range of bioactive components which are mainly responsible for their biological activities that help to treat chronic as well as infectious diseases. Microbes develop drug resistance due to different reasons. Continuous use of a particular drug creates drug resistance by affecting the common pathways of antibiotic actions like alteration in metabolic pathway, structural modification of the drug, changes in amino acid composition of the target protein etc. Most of the common pathogens develop drug resistance. Multidrug resistant *Staphylococcus aureus* is one of the common among them. It is reported recently in several parts of Delhi that *E. coli* which is very common in sewage samples gave rise to some antibiotic- resistant infections by the resistant strain called as New Delhi Metallo β- lactama superbug (NDM-1) [12,13].

Materials and methods

Dry plant material was purchased from local ayurvedic shop. The plant material was

identified from the Department of Botany, University of Calicut. Plant materials were washed, rinsed with distilled water and dried in air. About 10 to 20 gms of the dried materials were taken and powdered using a warring blender. This powder is then taken for extraction with different solvents.

Extraction of the plant material by Bioassay- guided fractionation [14].

In this method, plant extract was prepared using different solvents sequentially according to their increasing of order polarity. 10 gm of the powdered plant material (weight varies depending on plant material) was first mixed with the solvent of the least polarity (100ml) and the mixture was kept on an orbitory shaker at 200 rpm for 18 hrs. Supernatant was collected by filtering and evaporated to dryness. The residue obtained was weighed and stored. The residue obtained after the separation of supernatant was then mixed with solvent of next higher polarity and the process was repeated for different solvents, depending on its order of polarity. The different fractions thus collected on evaporation were dissolved in respective solvents at required concentration or in dimethylsulphoxide (DMSO) or in sterile water (if soluble). Concentration of the prepared extract was 10mg/ml (stock solution). The extract was stored under refrigeration (-20^{0}C) till its use for further analysis. The biological activities of the extract were tested after filter sterilization.

Polarity chart

n-Hexane
↓
Chloroform
↓
Benzene
↓
Diethyl ether
↓
Ethyl acetate
↓
Acetone
↓
Ethanol
↓
Methanol
↓
Water

The percentage yield of each extract was noted (Table1).

Test for phenolic compounds [15]: The presence of phenolic compounds was tested using Folin's - Ciocalteu (FC) reagent. In this method 1ml of the extract (1mg/ml)

was taken in a clean dry test tube and 5ml of FC reagent was added to it. 4 ml of 1.5% sodium carbonate was added and then kept for 30', at room temperature for incubation. Development of blue colour confirmed the presence of phenolic compounds. The intensity of blue colour was estimated using a colorimeter and the same was graded as + for low, ++ for medium and +++ for high intensities. The gradation of intensity represents the quantity of the phenolic compounds present in the extract .The results are tabulated in Table 2.

Tests for determining the presence of Flavanoids

Ferric chloride method [16]: The presence of flavanoid compounds in the extract was determined by ferric chloride method. In this method, 1 ml of the extract was taken in a test tube and a few drops of ferric chloride (0.1 % in distilled water) were added to the test tube. It is mixed well and kept at room temperature for few minutes. The presence of flavanoid compounds was indicated by the development of green colour. The intensity of the colour was noted using a colorimeter.

Biochemical estimation of total phenolic and flavanoid compounds

Biochemical estimations were done to determine the quantity of various secondary metabolites present in each extract.

Determination of total phenolic content: Total phenolic content of the extracts were estimated using Folin-Ciocalteu method [17].

High pressure liquid chromatography: HPLC

HPLC-profiling of the methanol extract of *E.ribes* was done using general method, changing the solvent system according to the plant material.

HPLC SYSTEM: HPLC unit with dual pump, rheo dyne injector, SPD photodiode array detector in combination with 6.12 SP5 integration software were used.

The following chromatographic conditions were given:

Column: Lichrosper RP 18 e 5µm (purchased from M/s. Merck, Mumbai),

Detector: SPD PDA, Flow rate: 1ml/min, Injection volume: 20µl.

The mobile system and wavelength were selected according to the sample.

The mobile phase used was Acetonitrile: methanol (95:5) for *E.ribes* (methanol extract).

1. The finger prints were recorded in different wavelength and shown as overlaid chromatogram. A PDA detector is used for recording. The number of peaks and area of the peaks were noted (Fig.4.) By analyzing this profile the sample was subjected for LC MS (Liquid chromatograpy mass spectroscopy) for identification of the compound.
2. The following procedure was used for LCMS analysis. The results are shown in figures, 16-22.
3. LC MS analysis: Column used : C-18

Probe used : APCI (Atmospheric pressure chemical ionisation)
Mode used : Positive (Which gives M+1 value
Negative (Which shows M-1 value)

Determination of antioxidant activity

DPPH method: DPPH (Diphenyl di picryl phenyl hydrazyl) method was used to determine the antioxidant activity of the extracts [18,19,20]

Antioxidants can scavenge the DPPH and reduce to hydrazine. The colour or absorbance of the DPPH will be reduced on reduction by an antioxidant compound.

500 µl of the sample was taken in a test tube. DPPH was prepared in methanol at a concentration of 0.1 Mm. 1 ml of the DPPH was then added to the extract and mixed well. It is kept at room temperature for 20 min., OD was measured at 517nm using a UV-VIS spectrophotometer.

Ascorbic acid was taken as control. Negative control was DPPH without the extract. Percentage activity was calculated using the formula given below

% activity =1-([A_{sample}/$A_{control}$] x 100).

The experiment was done in triplicate for each extract. Mean value ± SD was calculated. IC_{50} (Inhibitory concentration for 50% activity) value for each extract was determined utilizing linear regression formula by plotting graph with concentration on X axis and percentage of inhibition on Y-axis

Preparation of cell lines for MTT and cytotoxicity tests

The EAC and DLA cell lines were collected from Amala cancer research centre, Thrissur. The cells were induced to Balb /c mice.

The cells were aspirated from the peritoneal cavity of the tumour bearing mice and washed three times with PBS. The number of cells at 10^{-3} dilution was counted using a heamocytometer. The dilution was adjusted at 10^6 cells /ml.

Different dilutions (10^{-1}, 10^{-2}, 10 $^{-}$3) of the extract were made in sterile water (10mg/ml).

Incubated the different concentration of the extract like 0.0µg, 5.0, 10.0, 50.0, 100, 200, 500 and 1000 µg/ml with the cell lines at concentration of 10^6cells/ml per well. The final volume was made up to 1.0 ml with PBS and was incubated at 37^0c for 4hrs.

1 ml of 1% trypan blue (in distilled water) was added to each tube and mixed well. One drop was placed on each side of the haemocytometer and the number of dead cells were counted (10x) (Table.4.5).

% toxicity = (Number of dead cells/Total number of cells) x 100

The experiment was repeated for 12 extracts and % cyto toxicity was determined for the extract. Data obtained for DLA cell lines were plotted on a graph with concentration of extract on X-axis and % toxicity on Y-axis for calculating IC_{50} values of each extract (Fig).

Cytotoxicity testing using mice spleen cells [21]

Cytotoxicity of some extracts were tested using mice spleen cells cultured in RPM1 1640(Roswell Park Memorial Institute) medium. Extract at different concentration such as 0, 10, 50,100,200 and 500 μg/ml were added to spleen primary cell cultures (2 x 10^5 cells /well). It was then incubated for 72 hrs and observed for viability using tryphan blue. The number of viable cells was counted under microscope. Cell without

extract and one sample with DMSO were used as controls. % toxicity was determined as described above (Table 4.5)(Fig.).

Assay for testing anticancer activities of different plant extracts

MTT (Methylthiazolyldiphenyl-tetrazoleumbromide) assay for cell proliferation (Mosmann., 1983).

Methylthiazolyldiphenyl-tetrazoleumbromide, is water soluble and form a yellow coloured solution. Metabolically active, live cells can reduce MTT to formazon which makes the solution a blue or pink colour form a blue /pink colored solution. The change in colour is due to the formation of this insoluble product, formazon. This can be detected using a spectrophotometer.

DLA cells were collected and washed. It was then taken in fresh RPM1 medium. 500 µl of DLA cell lines in RPM1 medium was taken to load the wells at a concentration of 5000cells/well. The plates were then incubated at 37^0C and 5% CO_2 for 24 hrs. in a CO_2 incubator. 200µ l of the extract was added at a concentration of 0, 10, 20, 50, 100, 200, 500 and 1000µg/ml. 20 µ l MTT at a concentration of 5µg/ml was then added to each well. It was the incubated for 48 hrs at 37^0C and 5% CO_2 in a CO_2 incubator.

100µl of DMSO was added to each well and incubated at room temperature for 15 min.

Read OD at 545nm and blank at 630nm. Negative control is the well with cell lines and MTT but without extract.

The experiment was done in triplicate and average value of % inhibition was taken for calculating IC_{50} value.

% of the dead cells were calculated using the formula

% dead cells=100-[(OD of the well with drug/OD of the control) x 100] (Table 4.6).

The results were then analyzed using CHITEST and p value < 0.05 was taken as significant.

Testing of antimicrobial activity

Antimicrobial activity was tested using Baur and Kirby Method [22]. This method was developed by Heatley et al., in 1944. Bauer and Kirby modified it and it is being used as a standard method for antimicrobial assay. Antimicrobial susceptibility tests are approved by different agencies like National committee for clinical laboratory standard (NCCLS).

Microbial cultures

The following standard microbial strains were used in the present study.

Escherichia coli MTTCC 41(Massodi et al.,2008), *Proteus vulgaris* 426 (ATCC 6380) (Massodi et al.,2008), *Staphylococcus aureus* 87, (Massodi et al.,2008)*Pseudomonas auriginosa* 424(ATCC 25619) [8], *Candida albicans* MTTCC 183(ATCC 2091) [9,10]. These cultures were purchased from Institute of Microbial Technology (IMTECH), Chandigargh. *Klebsilla pneumonia and Aspergillus nige*r were collected from the Department of Life Sciences, University of Calicut.

Preparation of inocula.
Cultures were revived in 2 ml of sterile nutrient broth and sub cultured to fresh medium and incubated at 36^0C. Growth curve were prepared by monitoring OD at 600nm.The culture at log phase (i. e optical density 0. 5 to 0.8 or 6 x 10^5 CFU/ml) were taken for further inoculation either in the nutrient broth or plating on the nutrient agar plate.

Determination of Minimum Inhibitory Concentration (MIC) [23].
MICs for each extract against each microbial strains were determined using nutrient broth.10 ml of the medium was taken in a 50 ml conical flask and sterilized. The extract (stock diluted to 10mg/ml in DMSO) was then added to 10 ml of medium in a series of conical flasks to get a concentration of 10000, 1000, 100, 10, 0 µg/ml initially (10 times dilution). Then double dilution was used to determine the range of inhibitory activity(MIC value) of each extract. A loopfull of fresh culture at log phase (1 x 10^8 CFU/ml, turbidity equal to that of 0.5 MacFarland solution) was added to each flask [13] so as to have concentration of 5 x 10^5CFU/ml. It was then incubated at 36^0 for 18 to 24 hrs. The OD at 600nm was monitored at different concentrations of the drug in the culture media. One tube without microorganism was taken as blank. The least concentration at which the growth of the organism was completely inhibited was recorded as MIC of that extract, comparing the OD with that of the blank. It was then confirmed by inoculating to fresh agar plate. The experiment was done in triplicate and average value of MIC was then taken. It was then statistically analyzed and $p< 0.05$ was considered as significant. The experiment was repeated for different strains and extracts. The values were compared for gram positive and negative bacteria (Table)

Statistical analysis: All data were analyzed using SPSS version 16.0.

Results and Discussion

Traditional use of the plant showed that *Embelia ribes* is used in skin diseases, digestive and against head ache. In the present study, the methanol extract of *Embelia ribes* (ER1) showed moderate antimicrobial activity. ER1 has showed MIC of 187.5µg/ml to 500µg/ml against the microbes tested. Minimum MIC value was against *S.aureus.* and maximum was against *A.niger.* ER1 showed a significant difference in the activity against gram positive and gram negative organisms tested (p value< 0.01). ER1 extract also showed a good antimicrobial activity. ER1 has minimum inhibitory concentration of 234µg/ml against *E.coli* and *Acenetobacter.* A maximum value of MIC obtained against *Salmonella* was 350µg/ml. The MIC values obtained for clinical strains ranges from 125 to 1250 µg/ml when the various extracts are subjected to antimicrobial assay. The cardioprotective activity of *Embelia ribes* was reported by Bhandari et al., 2005[1]. Wound healing activity of embilin isolated from ethanol extract of *E.ribes* has been reported by Kumaraswamy et al., 2007.Phulan et al., 2004 reported the moderate activity of *E.ribes* extract on MRSA. Even though antioxidant and anti fertility activity of *Embelia ribes* were reported

earlier by Prakash et al., 1991, much reports are not available on its antimicrobial activity except moderate activity reported by Phulan et al., 2004[24,25,26].

ER1 showed the presence of phenolics (7.5 GAE), Flavanoids (2.2QE) and glycosides. It contains 0.98% of kaempherol and 0.025% of quercetin. A major peak at 254nm was observed in in the HPLC chromatogram of the methanol extract of E.ribes which has area of 3247303 with retention time 4.96. This corresponds to the major compound present in this extract. Conklin, et al., 2007 also reported the antimicrobial activity of quercetin. The same was reported earlier by Batyuk et al., 1985 and also by Moral et al., 2006 [27, 28]. So the antimicrobial activity of ER1 is due the presence of these compounds.

Methanol extract of *E.ribe*s has shown good DPPH scavenging activity of 35.44 µg/ml IC_{50} value indicating its strong antioxidant potential. The IC_{50}value obtained for cytotoxicity test was 568.88µg/ml. The therapeutical potential of the *E.ribes* was evaluated in the present study. It contains 0.98%quercetin.Quercetin is proved to be an anticancer agent which induces caspase activity[29]. So the anticancer effect exhibited by the methanol extract of *E.ribes* is due to the presence of quercetin.

Table 1.Percentage yield of the extract on bioassay guided fractionation

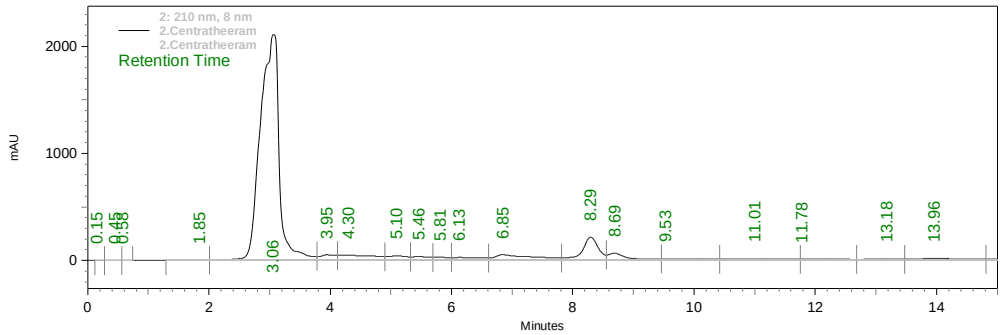
Name of the plant	Plant part used for the extraction	Solvent used	Dry mass (gm)	%yield	Extraction method used	Designated as
Embelia ribes	S	Methanol	3.13	15.25	A	ER1
		Ethanol	0.56	0.025		ER2
		Water	0.25	0.0125		ER3
		Chloroform	0.05	0.002		ER4

Table2.Results of the biochemical estimations for various metabolites are as follows

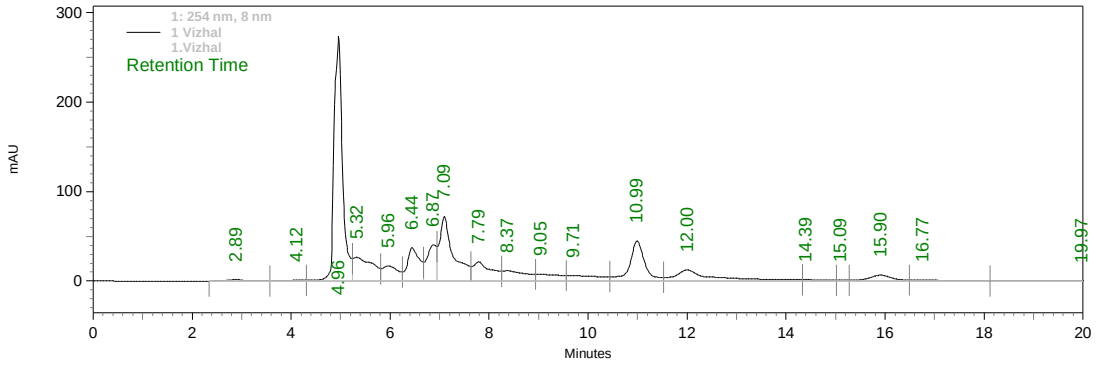
Name of the plant	Extract tested	Phenolic acids	Flavanoids	Alkaloids	Saponins	Glycosides	Terpenoids	Protein
Embelia ribes	ER1	+	++	-	-	+	-	-

Table 3.Total phenolics and flavonoids in the extract.

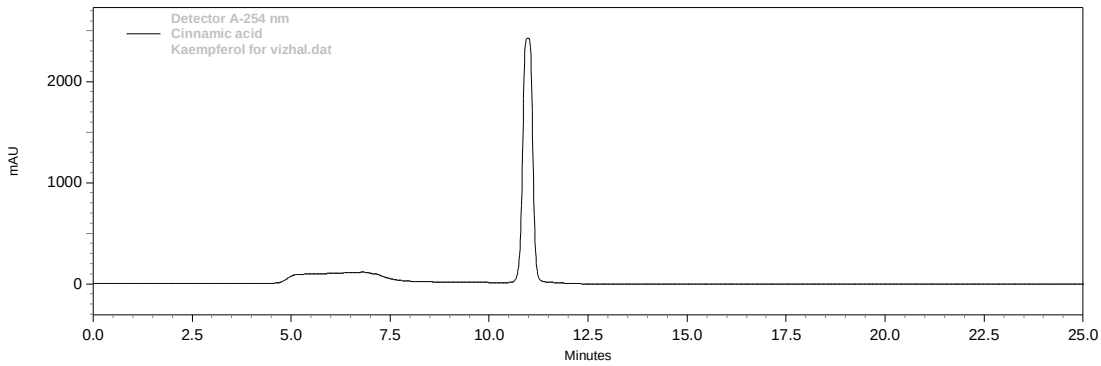
Name of the plant	Extract used for the estimation	Total phenolics as Gallic acid equivalents (GAE) (P)	Total flavonoids as Quercetin equivalents (QE) (F)	F/P ratio
1)Embelia ribes	ER1	7.50 ± 2.3	8.2± 0.5	1.09



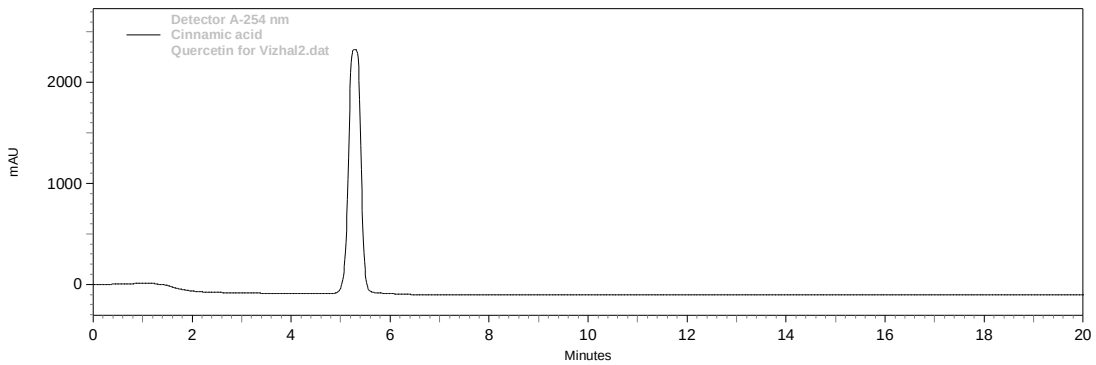
Quantification of kaempherol and quercetin in the methanol extract of Embelia ribes



Kaempherol



Quercetin



Quantification of Quercetin and Kaempherol in Embelia ribes

Method:

- Concentration of Sample: 25 mg/ml (wrt dry extract weight)
- Concentration of Std Quercetin: 100ug/ml (1 mg dissolved in 10ml)
- Concentration of Std Kaempherol: 100ug/ml (1 mg dissolved in 10ml)
- Retention time of Quercetin: 10.9
- Retention Time of Kaempherol: 4.9
- Percentage purity of injected Quercetin: 98%
- Percentage purity of injected Kaempherol: 99%
- Area given by Standard Quercetin in the standard profile: 54625463
- Area given by Standard Kaempherol in Standard profile: 74887452
- Area given by Quercetin in sample profile : 3247303
- Area given by Kaempherol in sample profile: 975535

Result:

- The percentage of Kaempherol in the sample : 0.98 %(w/w)
- The percentage of Quercetin in the sample : 0.025 %(w/w)

Table. 4 Detection of antioxidant activity of plant extract by DPPH method

Name of the plant	Extract used for the estimation	IC_{50}DPPH activity µg/ml	Reducing power method
Embelia ribes	ER1	35.044	48.14

Table 5: Cytotoxicity assay for various plant extract studied

Name of the plant	Extract used for the estimation100µg/ml	%Inhibition		IC_{50} (concentration for 50% inhibition)
		DLA cell lines	Normal Mice spleen cells	DLA cell lines
Embelia ribes	ER1	23	15	568.88

Table 6: MTT assay for cell proliferation of DLA cell lines using plant extracts under study.

Name of the plant	Extract used for the estimation	%viability(100µg/ml)
Embelia ribes	ER1	75±0.5

Table 7: MIC obtained for extracts against the microbes tested

Name of the plant	Extract used for the estimation	Escherichia coli µg/ml	Pseudomonas aeuroginosa	Klebsella pneumonia	Proteus vulgaris	Staphylococcus aureus	Candida albicans	Aspergillus niger
1)Embelia ribes	ER1	250	250	280	312	187.5	300	500
Gentamycin		20						
Streptomycin		40						
Penicillin		40						

Table 8: MIC results obtained against clinical strains tested

Name of the plant	Extract used	Shigella flexineri	Escherichia coli	Klebseilla pneumonia	Proteus vulgaris	Staphylococus aureus	Enterobacter	Acenetobacter bauminnii	Proteus mirabilis Salmonella rabis	
1)Embelia ribes	ER1	250	234	250	312.5	312.5	187.55	234.0	250	350
Gentamycin		50								
Streptomycin		60								
Penicillin		100								

Table 9: MIC obtained for MDR strains

Name of the plant	Extract used for the estimation	MRSA (µg/ml)
Embelia ribes	ER1	375

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