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ANTIMICROBIAL EVALUATION OF PHENOLIC COMPOUNDS ISOLATED FROM EUGENIA JAMBOLANA STEM BARK.

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ABSTRACT

Phytochemical investigation of the stem bark of Eugenia jambolana yielded four different phenolic subfractions characterised as 3,4,5-trihydroxybenzoic acid (gallic acid), (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-chromen-4-one (quercetin), 3,5,7-trihydroxy-2-(4-hydroxyphenyl) chromen-4-one(kaempferol) and 5,7-dihydroxy-2-phenyl,chromen-4-one (chrysin). The isolated compounds were assayed for antibacterial activity against bacterial strain Staphylococcus aureus, Protens Mirabilis (G+ve), E. coli, Klessiella pneumonia (G-ve) and antifungal activity against the fungal strain Aspergilus niger and Candida albican. All four compounds were observed with moderate to good antimicrobial activity. Better antibacterial and antifungal activity were observed with Quercetin and Kaempferol that showed excellent inhibitory activity against all the micro organisms tested. The strongest inhibitory activity effect was observed with Kaempferol against Aspergillus niger and with quercetin against Klessiella pneumonia. The results of present study provide scientific basis for the use of the plant extract in the future development as antifungal, antibacterial, antioxidant and anti-inflammatory agent.

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KEYWORDS *: Eugenia jambolana, Stem bark extracts, Phytochemical Screening, Antimicrobial Activity.*

INTRODUCTION

Medicinal plants have been associated with the prevention of degenerative diseases such as cancer and cardiovascular diseases. The presence of wide range of phytochemicals such as phenolics, thiols, carotenoids, anthocyanins and tocopherol have been suggested to exert chemopreventive and cardio protective effects as well as protecting the human body against oxidative damage by free radicals.[1] Natural phytochemicals derived from fruits, vegetables and herbs have been reported to possess a wide range of biological effects, including antioxidant, antimicrobial and anti-inflammatory actions.[2] Among them, phenolic acids and

flavonoid compounds have attracted considerable interest in the past few years due to their many potential health benefits. As polyphenols, phenolic acids and flavonoids are powerful antioxidants and have been reported to demonstrate antibacterial, antiviral, anticarcinogenic, anti-inflammatory and vasiodilatory actions. [3,4]

The plant *Eugenia jambolana* Lam. (Syn. *Syzygium cumini* Skeels or *Syzynium jamolana)* belongs to the family Myrtaceae is a large evergreen tree up to 30meters height and a girth of 3.6 m with a bole upto 15 meters, found throughout India upto an altitude of 1,800 m.[5] Most of the plant parts of *E. jambolana* are used in traditional system of medicine in India. Stem bark of *Syzygium cumini* contains betulinic acid, ß-sitosterol, friedelin, epi-friedelanol,[6] and new ester of epi-friedelanol (eugenin).[7] It also contains ß-sitosterol-D-glucoside, kaempferol-3-o-glucoside, quercetin,[8] myricetin, astragalin and gallic acid.[9]

Aerial parts of *Syzygium cumini* (Jamun) have hypoglycaemic[10,11,12], anti-inflammatory[13], antipyretic[14], psychopharmacological[15], hypolipidaemic[16], and antioxidant [17,18,19] activities. The literature survey revealed that there are no scientific studies carried out regarding antifungal activity of phenolic acids and flavonoids the stem barks of *Eugenia jambolana*. Hence, the present study is focused to evaluate the antifungal potentials of phenolic acid and flavonoid compounds present in *Eugenia jambolana* stem bark.

MATERIALS AND METHODS:

All the chemicals used for isolation were of laboratory grade and purchased from S.D. Fine Chemicals and Sigma Aldrich. All the reactions were carried out under prescribed laboratory conditions. Melting points of the synthesized compounds were determined by open capillary and are uncorrected. The purity of the compounds was checked using precoated TLC plates (MERCK, 60F) using chloroform: methanol (8:2) solvent system. The developed chromatographic plates were visualized under UV at 254nm. IR spectra were recorded using KBr on Josco FTIR model 8400 spectrophotometer, 1H NMR spectra in DMSO on a BRUKER FT-NMR instrument using TMS as internal standard. FAB mass spectra were recorded on JEOL SX 102 (DA-6000 mass Spectrometer) data system using Argon (6KV.10MA) as the FAB gas.

Plant materials:

The fully mature *Eugenia jambolana* stem barks were collected in February 2012 from Kengal village in Channapatna of Karnataka, India from a single tree. The stem bark was identified and authenticated at Department of Plant Biology and Plant Biotechnology, Presidency College, Chennai.

Extraction of phenolic compounds:

The sun-dried stem barks (1kg) of *Eugenia jambolana* were coarsely powdered and extracted exhaustively in soxhlet apparatus with methanol for 72 hrs. The methanolic extract was concentrated to dryness under reduced pressure to obtain dark brown viscous mass (23g). The concentrated extract was successively partitioned with Petroleum ether, chloroform and ethyl acetate. Each of the steps was repeated three times to ensure complete extraction in each case. The combined organic layer of each partition was evaporated to dryness under reduced pressure to afford Petroleum ether, chloroform ethyl acetate and methanol fractions. The weights of individual extracts are given in Table no 1.

Table no. 01: Weights of the *Eugenia jambolana* stem bark extracts:

Extract	Weight (g)	Consistency
Petroleum ether fraction	1.2	Light brown
Chloroform fraction	2.3	Dark brown
Ethyl acetate fraction	6.1	Brownish yellow
Methanol extract	10.6	Dark brown

Phytochemical screening:

All the extracts obtained as above were tested for the following qualitative chemical tests for the identification of various phytoconstituents.

- **Tests for alkaloids:** Dragendorff's test, Mayer's test, Hager's test.
- **Tests for steroids and sterols:** Libermann-Burchard test and Salkowaski test.
- **Tests for glycosides:** Legal test, Baljet test, Borntrager's test and Keller-Kiliani test.
- **Test for flavonoids:** Shinoda test.
- **Tests for tannins:** Lead acetate test and gelatin test.
- **Test for phenolics:** Ferric chloride test.

The individual extracts have shown positive tests for following phytoconstituents.

Petroleum ether fraction: Steroids and sterols
Chloroform fraction: Alkaloids
Ethyl acetate fraction: Glycosides, phenolics, flavonoids
Methanol fraction: Glycosides, phenolics, flavonoids, tannins

Petroleum ether and chloroform fractions were rejected since they are not containing phenolic compounds. An ethyl acetate and methanol fraction are combined since having similar phytoconstituents and was fractionated on silica gel column chromatography using an increasing gradient of ethyl acetate in chloroform up to 100%, followed by an increasing gradient of methanol up to 100%. The various fractions from column chromatography are tabulated in Table no 2.

Table no. 02: Column fractions of combined ethyl acetate and methanol extract of *Eugenia jambolana* stem bark:

Fraction no.	Eluent	Residue on evaporation
1-6 (A)	Chloroform (100)	No residue
7-12 (B)	Chloroform:Ethyl acetate (75:25)	Light brown residue *
13-18 (C)	Chloroform:Ethyl acetate (50:50)	Brown residue
19-24 (D)	Chloroform:Ethyl acetate (25:75)	Brown residue
25-30 (E)	Ethyl acetate (100)	Yellow residue
31-36 (F)	Ethyl acetate:Methnaol (75:25)	Dark brown residue
37-42 (G)	Ethyl acetate:Methnaol (50:50)	Dark brown residue
43-48 (H)	Ethyl acetate:Methnaol(25:75)	Light brown residue *
49-54 (I)	Methanol (100)	Light brown residue *

* Quantity not sufficient for further analysis and hence rejected.

Purification of C (40.0 mg) on Sephadex column chromatography starting with chloroform- ethyl acetate (7.5:2.5) followed by increasing gradient of ethyl acetate up to 70% afforded residue C_1 (19.0 mg). Then C_1 is further purified by subjecting to Preparative thin layer chromatography (PTLC) using Glass plates (20 x 20 cm) thickly coated (0.4-0.5 nm) with silica gel 'G' (45 gm/80 ml water) Mobile phase Toluene: Ethyl Acetate: methanol [7:5:1v/v/v], Developed plates were air dried and visualized under UV light. The fluorescent spots were marked. The marked spots were scrapped and collected separately along with the silica gel 'G' and extracted with Ethyl acetate. The extract was then crystallized to give **compound 1** (Chrysin) (6mg). The purified material was subjected to its spectral analysis.

Fraction D (120 mg) was purified on Sephadex column chromatography starting with chloroform-ethyl acetate (5:5) followed by increasing gradient of ethyl acetate up to 100% afforded residue D_1 (40.0 mg). It was hydrolyzed by refluxing with 7% H_2SO_4 (10 ml/gm residue) for 5 hours. The mixture was filtered and the filtrate extracted with ethyl acetate in a separating funnel. The ethyl acetate layer was washed with distilled water till neutrality and dried in vacuum Then it is further purified by subjecting to Preparative thin layer chromatography (PTLC) using Glass plates (20 x 20 cm) thickly coated (0.4-0.5 nm) with silica gel 'G'

(45 gm/80 ml water) Mobile phase ethyl acetate: glacial acetic acid: formic acid: water [100:11:11:25,v/v/v/v/]. Developed plates were air dried and visualized under UV light. The fluorescent spots were marked. The marked spots were scrapped and collected separately along with the silica gel 'G' and extracted with Ethyl acetate. The extract was then crystallized to give **compound 2**(Kaempferol) (5mg). The purified material was subjected to its spectral analysis.

Fraction E (100 mg) was purified on Sephadex column chromatography starting with chloroform-ethyl acetate (2.5:7.5) followed by increasing gradient of ethyl acetate up to100% and ethyl acetate-methanol up to (2.5:7.5) afforded residue E_1 (35.0 mg). It was hydrolyzed by refluxing with 7% H_2SO_4 (10 ml/gm residue) for 5 hours. The mixture was filtered and the filtrate extracted with ethyl acetate in a separating funnel. The ethyl acetate layer was washed with distilled water till neutrality and dried in vacuum Then it is further purified by subjecting to Preparative thin layer chromatography (PTLC) using Glass plates (20 x 20 cm) thickly coated (0.4-0.5 nm) with silica gel 'G' (45 gm/80 ml water) Mobile phase ethyl acetate: glacial acetic acid: formic acid: water [100:11:11:25,v/v/v/v/v]. Developed plates were air dried and visualized under UV light. The fluorescent spots were marked. The marked spots were scrapped and collected separately along with the silica gel 'G' and extracted with Ethyl acetate. The extract was then crystallized to give **compound 3**(Quercetin) (5mg). The purified material was subjected to its spectral analysis.

Fraction F and G are combined (500 mg) was purified on Sephadex column chromatography starting with ethyl acetate–methanol (9:1) followed by increasing gradient of methanol up to 90% afforded residue FG_1 (200 mg). Then FG_1 is further purified by subjecting to Preparative thin layer chromatography (PTLC) using Glass plates (20 x 20 cm) thickly coated (0.4-0.5 nm) with silica gel 'G' (45 gm/80 ml water) Mobile phase Toluene: Ethyl Acetate: Formic Acid [7:5:1 v/v], Developed plates were air dried and visualized under UV light. The fluorescent spots were marked. The marked spots were scrapped and collected separately along with the silica gel 'G' and extracted with Ethyl acetate. The extract was then crystallized to give **compound 4**(Gallic acid) (15mg). The purified material was subjected to its spectral analysis.

Antimicrobial activity:

The isolated compounds were assayed for antibacterial activity against bacterial strain *Staphyloccoccus aureus, Protens Mirabilis (G+ve), E. coli, Klessiella pneumonia (G-ve) and* antifungal activity against the fungal strain *Aspergilus niger and Candida albican.* Stock solutions of the isolated compounds and standard drug used were prepared in dimethyl formamide taken in the concentration of 50 & 100 µg/ml for antibacterial activity and 250 & 500µg/ml for antifungal activity. Ciprofloxacin is used as standard antibacterial agent and Fluconazole as antifungal agent.

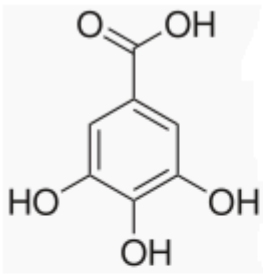
The Petri dishes were washed thoroughly and sterilized in hot air oven at 160^{O} C for one hour. 30 ml of sterile nutrient agar media for bacteria and potato dextrose agar media for fungi was poured in to sterile Petri dishes and allowed to solidify. The plates were incubated at 37^{O} C for 24 hours to check for sterility. The medium was seeded with the organism by spread plate method using sterile cotton swabs. Bores were made on the medium using sterile borer and 2 mg/ml of Ciprofloxacin for bacteria and 2 mg/ml Fluconazole for fungi were taken as standard reference while the sample preparation were made 0.1 mg/ml and 1 mg/ml in DMSO. The Petri dishes were kept in refrigerator at 4^{O} C for 15 minutes, allowing diffusion to take place. The Petri plates were incubated at 37^{O} C for 24 hours and zone of inhibition were observed and measured using a scale. Antimicrobial activities of all the compounds were carried out against all six microorganisms.

Chemical studies:

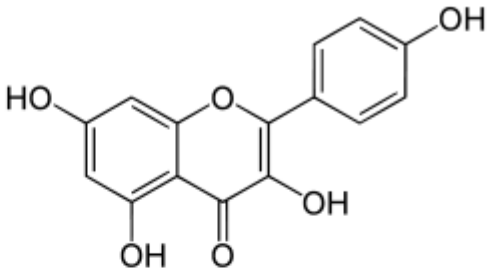
Preliminary phytochemical screening of *Eugenia jambolana* extracts revealed the presence of Steroids, sterols alkaloids, glycosides, phenolics,

flavonoids and tannins. (Table 1). Four phenolic sub fractions were isolated from the extracts and identified as Gallic acid, Quercetin, Kaempferol and Chrysin. The structures of isolated compounds are given in figure 1.

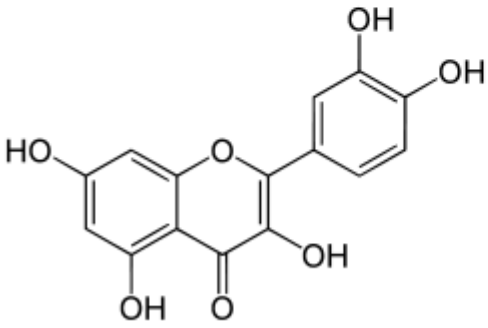
Figure 1: Structures of isolated compounds.



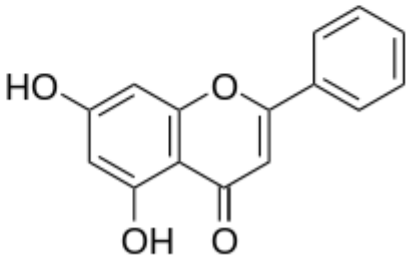
3,4,5-trihydroxybenzoic acid



3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one



2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-chromen-4-one



5,7-dihydroxy-2-phenyl,chromen-4-one

Physical properties and spectral data of isolated compounds:

Compound 1: Chrysin (5,7-dihydroxy-2-phenyl,chromen-4-one). Yellowish white crystalline solid. 0.38 g, m.p. 285°C - 286°C, Rf; 0.76, uv λmax 270, 329 nm. ^{1}H NMR (300 MHz, DMSO-d$_6$):12.15 (1H, s, 5-OH), 10.80 (1H, s, 7-OH), 9.60 (1H, s, H-4'), 7.32 (2H, d, H-2', H-6'), 6.81 (2H, d, H-3', H-5'), 5.90 (2H, s, H-6, H-8), 5.43 (1H, d, H-2), 3.26 (1H, d, H-3ax), 2.69 d, H-3eq); ^{13}C-NMR (100MHZ, DMSO-d6) 196.6 (C-4), 166.8 (C-7), 163.4 (C-5), 162.1 (C-9), 157.8 (C-4'), 129.4 (C-1'), 128.8 (C-2', C-6'), 115.6 (C-3',5'), 102.2 (C-10), 96.1 (C-6), 95.4 (C-8), 78.9 (C-2), 42.4 (C-3). MS m/z,330 [M$^+$]. The spectra data were in agreement with that of chrysin reported in the literature.

Compound 2: Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one). Yellow amorphous powder, 0.38 g, m.p. 276-278°C, Rf; 0.50, uv λmax 394, 341 nm. ^{1}H NMR (400 MHZ, DMSO-d$_6$) 7.62(1H, d, J=2.2HZ, H-2`), 7.53(1H, d, J=8.4, 2.2 HZ, H-6`), 6.78(1H, d, J=8.4 HZ, H-5`), (1H, d, J=1.8 HZ, H-8), 6.07(1H, d, J=1.8HZ, H-6). $^{13}$C-NMR (100MHZ, DMSO-d6) 146.2 (C-2), 135.2(C-3), 175.1(C- 4),159.4 (C-5), 98.1 (C-6), 163.2 (C-7), 93.0(C-8), 122.2(C-1`), 115.3(C-2`), 145.2(C-3`), 147.2(C-4`), 115.3(C-5`) and 120.2(C-6`). MS m/z, 330 [M$^+$]. The spectra data were in agreement with that of kempferol reported in the literature.

Compound 3: Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-chromen-4-one). Yellow crystalline powder, 0.38 g, m.p. 310-317 °C, Rf; 0.40, uv λmax 394, 341 nm. ^{1}H NMR (300 MHz, DMSO-d$_6$): 6.18 (1H, d, J = 1.8 Hz, H-6), 6.40 (1H, d, J = 2.1 Hz, H-8), 6.89 (1H, d, J = 8.4 Hz, H- 5'), 7.54 (1H, dd, J = 2.1 and 8.7 Hz, H-6'), 7.67 (1H, d, J = 2.1 Hz, H- 1'), 12.49 (1H, s, 5-OH). ^{13}C-NMR (75 MHz, DMSO- d$_6$). 146.8 (C2), 135.8 (C3), 175.9 (C4), 160.7 (C5), 98.2 (C6), 163.9 (C7), 93.4 (C8), 156.2 (C9),

103.0 (C10), 122.0 (C1`), 115.1 (C2`), 145.1 (C3`), 147.7 (C4`), 115.6 (C5`), 120.0 (C6`). MS m/z, 330 [M^+]. The spectra data were in agreement with that of quercetin reported in the literature.

Compound 4: Gallic acid (3,4,5-trihydroxybenzoic acid). White to off white crystalline powder, 0.38 g, m.p. 250-252°C, Rf; 0.39, uv λmax 220, 271 nm. ^{1}H NMR (90 MHz, DMSO-d_6):7.11(2H, s, H-2,H-6), 8.1(3H, s, 3-OH,4-OH,5-OH), 12.8(1H, s, 1'-OH). ^{13}C-NMR (22.5 MHz, DMSO- d_6).108.1(C1), 120.45(C2, C6), 137.12(C3, C5), 145.4(C4), 168.32(C1`). MS m/z, 330 [M^+]. The spectra data were in agreement with that of gallic acid reported in the literature.

RESULTS:

The antimicrobial activity (zone of inhibition) of the isolated compounds is listed in table no 03 (antibacterial activity) and table no 04 (antifungal activity).All four compounds were observed with moderate to good antimicrobial activity. Better antibacterial and antifungal activity were observed with Quercetin and Kaempferol that showed excellent inhibitory activity against all the micro organisms tested. The strongest inhibitory activity effect was observed with Kaempferol against *Aspergillus niger* and with quercetin against *Klessiella pneumonia*.

Table no. 03: Anti-bacterial activity data of isolated compounds:

Sl. no	Compound	Concentration μg/ml	Zone of inhibition(mm)			
			E.coli	S.Aureus	P.Mirabilis	K. Pneumonia
1	Chrysin	50	9	9	10	8
		100	11	10	11	10
2	Kaempferol	50	10	11	10	9
		100	11	13	12	12
3	Quercetin	50	8	9	10	13
		100	10	11	12	15
4	Gallic acid	50	6	7	7	6
		100	9	10	8	9
5	Ciprofloxacin	50	24	20	22	26

Table no. 04: Anti-fungal activity data of isolated compounds:

Sl. no.	Compound	Concentration μg/ml	Zone of inhibition(mm)	
			Candidaalbicans	Aspergius niger
1	Chrysin	250	10	9
		500	11	11
2	Kaempferol	250	10	12
		500	12	14
3	Quercetin	250	9	9
		500	11	10
4	Gallic acid	250	8	9
		500	10	10
5	Fluconazole	250	21	20

DISCUSSION:

All the steps in isolation were monitored by TLC. The TLC plates were visualized by viewing in UV chamber. Structure and purity of the anticipated compounds were characterized by physical constant and FTIR spectral studies initially followed by NMR and Mass spectroscopy.

From the literature survey it reveals

that phenols and flavonoids have been reported for number of pharmacological activities. All four isolated compounds were screened for anti bacterial activity using DMF as a solvent against the organisms, *Staphylococcus aureus, Protens Mirabilis* and *E. coli, Klessiella pneumonia*. And Antifungal activity using *Aspergilus niger* and *Candida albicans.* By Cup plate method on nutrient agar media. The standard drug used was Ciprofloxacin for antibacterial and Fluconazole as standard for antifungal activity. All the isolated compounds shown good activity against the microorganisms tested.

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