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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; 10(9): 05-09

www.thepharmajournal.com
Received: 05-07-2021
Accepted: 13-08-2021

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Antibacterial potential and phytochemical evaluation of *Ficus racemosa* leaf extracts

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Abstract
The need of the plant derived medicine for the animal and human use in recent years is so much increasing. In present study the antibacterial property of *Ficus racemosa* leaf extracts having the four solvents ie. Aqueous, chloroform, methanol and acetone were evaluated using the disc diffusion method against the test bacteria *Escherichia coli*; 10mg, 20mg and 50mg concentrations were taken, the standard antibiotic used were Gentamicin (50 µg). Qualitative phytochemical analysis of four extracts and the extract having most potent antibacterial activity was evaluated using Liquid chromatography-mass spectrometry method for phytochemicals. In disc diffusion method the Aqueous extract was found to be most potent as showed highest zone of inhibition as compared to the other extracts against *Escherichia coli*. Liquid chromatography-mass spectrometry analysis of Aqueous extract showed presence of eleven phytochemical compounds, which could be the probable cause of the present antibacterial activity.

Keywords: *Ficus racemosa*, leaf extracts, disc diffusion method, phytochemical analysis

Introduction
Escherichia coli is a bacterial commensal of the intestinal microflora of a variety of animals, including humans, which causes colibacillosis in creatures and birds. *Escherichia coli* is a gram-negative, rod-shaped, and motile bacteria. In any case, not all *E. coli* strains are harmless, as some can cause infections in people just as in animals and birds [4, 6]. The treatment part of the gram-negative microorganisms incorporates the use of aminoglycosides which shows bactericidal action and covers the wide range of gram-negative bacilli [8]. Considering the bacterial resistance, side and unfavorable impact of the allopathic antimicrobial medications, the medicinal plants possessing the antibacterial properties serve as alternatives for modern allopathic antibiotics. Keeping these point in view in present research work the use of *Ficus racemosa* leaf extract against the gram-negative bacteria *Escherichia coli* were detected by *in vitro* method, qualitative phytochemical evalution and by Liquid chromatography-mass spectrometry (LCMS) done as well.

Material and Methods
The present study was carried out in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Udgir, a constituent college of Maharashtra Animal and Fishery Sciences University, Nagpur. The facilities required for this work were provided by the Department of Veterinary Pharmacology and Toxicology, Department of Veterinary Microbiology and Department of Biochemistry, College of Veterinary and Animal Sciences, Udgir. Whole fresh leaves of plant of *Ficus racemosa* were collected from campus of College of Fisheries Sciences, Udgir. The leaves of the plant were dried under shade and the powder of dried leaves was prepared by mechanical grinder. The dried powder was further passed through the mesh sieve to obtain the fine powder. The powder were kept in plastic box stoppered tightly and were placed in refrigerator.
Disc diffusion method:

Materials Used
Nutrient agar (NA), Nutrient broth (NB), Mueller Hinton agar (MHA), Mueller Hinton broth, standard discs of gentamicin (50µg) and dimethyl sulfoxide (DMSO) supplied by Hi-media Laboratories Ltd., Mumbai.

Bacterial Strains
The standard strains of Gram negative bacteria namely *E. coli* (ATCC 43888) were selected for the study.
The sticks of bacterial cultures were revived as per the instructions given by American Type Culture Collection (ATCC).

Preparation of media and plates
The agar was prepared as per the guidelines given by Hi-media. The dehydrated powdered media of Mueller Hinton agar (MHA) and Nutrient Agar were weighed accurately, taken in sterile conical flask and were added with appropriate amount of distilled water. The flask was kept for autoclaving at 15 *psi* pressure for 15 minutes at 121^0C. About 10 millilitres of autoclaved liquid agar media was poured into the sterile petri plates and were allowed to solidify under laminar flow. After solidification of agar, the plates were kept in incubator at 37ºC for 12 hrs to ensure sterility and avoid any contamination of plates. Plates with no visible growth were used for performing antibiotic sensitivity test.

Preparation of bacterial inoculums
Two sterile test tubes were taken and 2-3 millilitres of nutrient broth were added in each tube. A loop of single, isolated colony of *E. coli* (ATCC 43888) were inoculated into tubes simultaneously and denoted with strain number on tubes. Inoculated tubes were incubated for 4 to 5 hours. After incubation, the tubes were spectrophotometrically matched at 620 nm by using nutrient broth to get final optical density of 0.08 which exactly yields starting number of 1 - 2 × 10^8 CFU/ml bacteria [3]. The spectrophotometrically adjusted inoculum were used within 15 minutes of standardization in order to prevent change in number of bacteria.

Preparation of discs
A stock solution of extract was prepared by dissolving 0.1 gm of extract with 100ml of their DMSO (Dimethyl sulfoxide) to produce final concentration of 100mg/ml. The stock solution was then diluted to concentrations of 10, 20 and 50 mg/ml. 20 µl of each dilution was impregnated on sterile blank discs of 6 mm in diameter. 5 µl of extract solution was spotted alternately on both sides of the discs and allowed to dry before the next 5µl of was spotted to ensure precise impregnation [12]. The DMSO loaded discs were also prepared and were used as negative control. The discs were air dried for 3 to 4 hr to evaporate the solvent completely before placing them on agar plates. Discs of gentamicin (50 µg) were used as a positive control.

Phytochemical study for qualitative analysis of certain active principles
The preliminary qualitative phytochemical analysis was done to detect presence or absence of various phytoconstituents namely alkaloid, glycosides, tannins, saponins, protein and amino acids, phytosterols, phenolic compounds, resins, reducing sugars and flavonoids in aqueous, chloroform, methanol and acetone leaf extracts of *Ficus racemosa*(11).

Phytochemical analysis of extract having potent antibacterial activity by LC-MS (Liquid chromatography Mass spectrometry)
LC-MS (Liquid chromatography Mass spectrometry) was used to determine the phytochemical constituent in the extract having potent antibacterial activity. The facilities for the phytochemical analysis by LC-MS were made available at Triyat Genomics, Wardha Road, Nagpur. The details of the instrument used for the study are as below.

Instrument Details
A) LC-MS
The liquid chromatography LCMS (Agilent Technologies-1260 Infinity) system consisted of the Quaternary pump, Autosampler and MS detector (6120 Quadrupole).

B) Column: Agilent- Eclipse plus C-18 4.6 x 250 mm

C) Mobile phase
Acetonitrile:10Mm ammonium acetate in water (60:40); Injection Volume –10µL; Flow rate -0.6ml/ mint Run time-42 mint.; Drying gas Flow-12 (l/mint); Nebulizer Pressure(psig)-50; Drying gas temperature-350℃; Capillary Voltage-4000V; Polarity-positive

D) Sample Preparation: 0.3g sample dissolved in 10 mL Methanol+Filter+Injected to LC-MS.

The data were statistically analysed by IBM SPSS software using method of ANOVA single factor and Duncan's Multiple Range Test (DMRT).

Result and Discussion
Disc diffusion method: The aqueous, chloroform, methanol, and acetone leaf extracts of *Ficus racemosa* were screened for *In vitro* antibacterial property by disc diffusion method. against *Escherichia coli.* The antibacterial activity was compared with that of the standard antibacterial drug Gentamicin (50µg). The antibacterial activity of the aqueous, chloroform, methanol, and acetone leaf extracts of plant were assessed and compared with that of the Gentamicin (50µg).

Table 1: Zone of Inhibitions produced by different *Ficus racemosa* leaf extracts at various concentrations

Extracts	Zone of Inhibitions(mm)				
	10 mg	20mg	50mg	Gentamicin (50µg)	P value
Aqueous	11.46±0.74^a	12.80±1.33^a	13.63±0.85^a	21.60±0.30^b	0.0002**
Chloroform	9.50±0.28^a	12.16±0.60^b	13.16±0.60^b	20.93±0.03^c	0.000 **
Methanol	10.50±1.04^a	12.66±1.08^a	13.70±0.90^a	20.53±0.27^b	0.0002**
Acetone	9.00±0.29^a	11.50±0.76^b	13.80±0.15^c	21.43±0.26^d	0.000 **

** Highly Significant (P< 0.001)
Mean with different superscripts differ significantly from each other

The zone of inhibitions produced by the different *Ficus racemosa* leaf extracts at various concentrations were shown in table 1. The mean zone of inhibitions observed at 10mg, 20mg and 50mg concentrations of aqueous leaf extract of *Ficus racemosa* were 11.46±0.74, 12.80±1.33 and 13.63±0.85 mm respectively against *E.coli.* While the Gentamicin (50µg) produced significantly the highest mean zone of inhibition (21.60±0.30mm) in comparison with that of the mean zone of inhibitions produced by the aqueous leaf extract at different concentrations against *E.coli.* Whereas, there was a non-significant difference (P< 0.01) between the zone of inhibition exhibited by 10mg (11.46±0.74 mm), 20mg (12.80±1.33 mm) and 50mg (13.63±0.85 mm) concentrations. In similar work it is also recorded the antibacterial activity of *Ficus racemosa* aqueous fruit leaf extract against *E.coli* [10]. Similarly, evaluated the antibacterial activity of aqueous bark leaf extract of *Ficus religiosa* and *Ficus benghalensis* against enterotoxigenic *E.coli* and observed that both plants bear

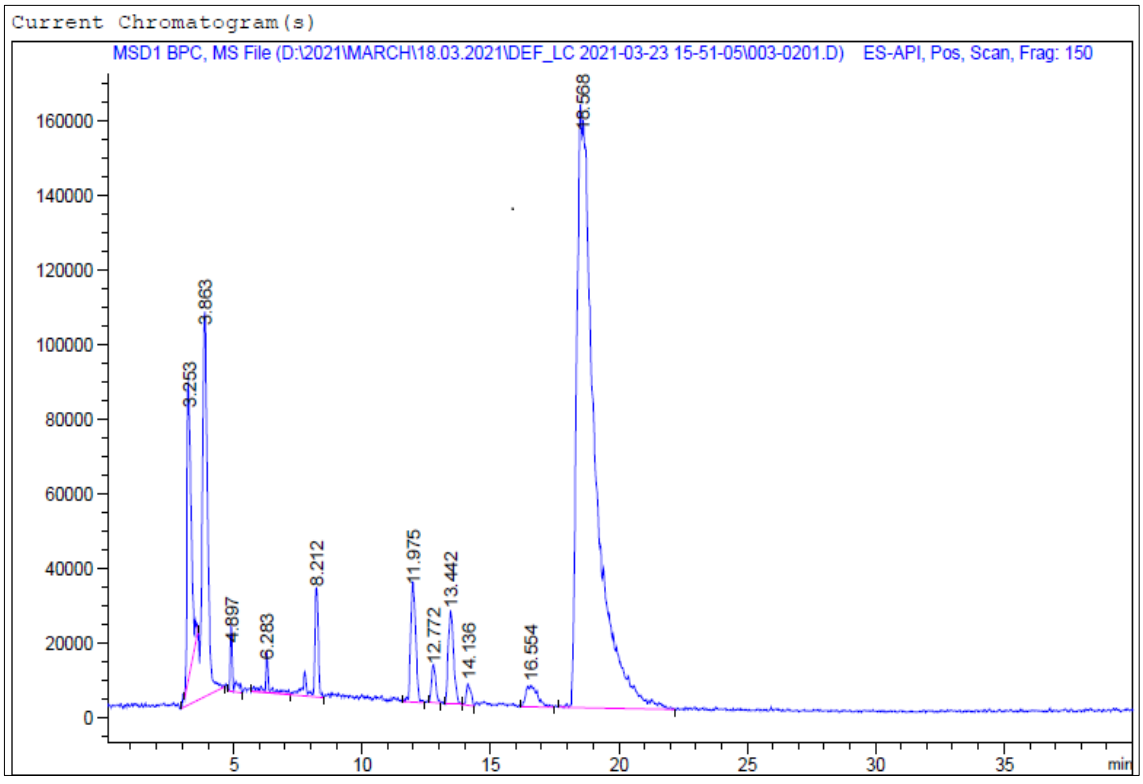
antibacterial properties [14]. The mean zone of inhibitions produced by 10 mg, 20 mg, 50mg concentrations of aqueous leaf extract of *Syzygium cumini* were 9.06±0.52mm, 11.16±1.30 mm and 14.26±0.54 mm respectively against *E.coli.* While the zone of inhibition produced by Gentamicin (50µg) was found to be significantly greater (21.33±0.29mm) against *E.coli.* The observations indicate the zone of inhibitions produced by 10 mg, 20mg and 50mg of methanolic leaf extract of *Ficus racemosa* ranged between 10.50±1.04 mm and 13.70±0.90 mm. The zone of inhibitions produced by 10 mg (10.50±1.04 mm), 20mg (12.66±1.08 mm) and 50mg (13.70±0.90 mm) concentrations did not differ significantly. While, the zone of inhibition exhibited by standard antibiotic Gentamicin (20.53±0.27 mm) differ significantly with a zone of inhibition. produced by 10 mg (10.50±1.04 mm), 20mg (12.66±1.08 mm) and 50mg (13.70±0.90 mm) leaf extracts. Similarly, on the same analogy, in research work conducted it also found leaves, bark and stem of *Ficus racemosa* to possess the *in vitro* antibacterial properties against *Escherichia coli*[1],[7]. The Mean zone of inhibition values recorded for 10mg, 20mg, and 50mg acetone leaf extract was 7.6±0.30 mm, 11.50±0.76 mm and 13±1.04 mm respectively, whereas the zone of inhibition was 21.9±0.05 mm for Gentamicin (50µg). The observations show that there was a significant difference between the zone of inhibitions produced by 10mg (7.6±0.30 mm) and 20 mg (11.50±0.76 mm) and an insignificant difference between the zone of inhibitions produced by 20 mg (11.50±0.76 mm) and 50mg (13±1.04 mm). The highest zone of inhibition (21.9±0.05 mm) was exhibited by Gentamicin (50µg). From the obtained results it is evident that there was an insignificant difference between the zone of inhibitions produced at 20 mg (11.50±0.76 mm) and 50mg (13±1.04 mm). The results of the present study correlate with the findings of a research who also revealed antibacterial properties of *Ficus racemosa* stem bark using acetone extract [9].

Qualitative phytochemical analysis

Qualitative phytochemical tests for the phytochemical, the observations expected for the respective test and the results obtained are shown in table 2 related to various phytochemical tests of the aqueous, chloroform, Methanol and acetone leaf extracts of the *Ficus racemosa.*

LC-MS analysis

The crude aqueous extract of *Ficus racemosa* leaf was subjected to Liquid Chromatography mass spectrophotometry analysis. The chromatogram showing peaks of the phytochemicals observed in *Ficus racemosa* are presented in Graph 1. The identified active principles, area, retention time (RT) and area (%) of the crude extract of *Ficus racemosa* represented in table 3. Possible Interpretations regarding the LC-MS analysis of the aqueous extract shows the compounds Phloretin (64.165%) and Choline (11.933%) with higher percentage area. As per the findings of LC-MS analysis it was observed that, Choline (Amino acids), Citronellol (Terpenoids), Gallocatechin-4beta-ol (flavnoid), 1,11-Undecanedicarboxylic acid, xi-p-Menth-3-ene (terpenoids), gamma-Nonalactone (flavnoids), Adonirubin (amine), Delcosine (terpenoids), Amitenone (terpenoids), Phloretin **(**Phenolics or polyphenol) etc compounds were found in the aqueous leaf extracts of the *Ficus racemosa.* In one of the Similar work conducted phytochemical analysis of plants under *Ficus* species by LCMS. They also detected various phytochemicals like catechin, epicatechin, catechin glucosides quercetin-3-glucoside, kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside, rutin and chlorogenic acid in the plants under *Ficus* species. They are of the opinion that the antimicrobial properties of the plants under *Ficus* species are due to the presence of these phytochemicals [3]. The possible cause of the inhibition of bacteria in present study also may be due to the various phytochemicals traced out in the aqueous extract of *Ficus racemosa*



Graph 1: LC-MS spectra of aqueous extract of *Ficus racemosa* (Chromatogram).

Table 2: Qualitative test for detection of phytochemicals present in the Aqueous, Chloroform, Methanol and Acetone Leaf Extracts of *Ficus racemosa*

				Result			
Sr. No.	**Phytochemical**	**Phytochemical test**	**Expected Observations**	**FRAq**	**FRC**	**FRM**	**FRAc**
01.	Alkaloid	Wagner's reagent test	Formation of Brown flocculent precipitate	+	+	+	+
		Dragendroff's reagent test	Formation of Prominent yellow precipitate	+	+	+	+
02.	Glycosides	Benedict's reagent test:	Formation of Coloured precipitate	+	+	+	+
		Fehlings reagent test:	Formation of Red precipitate	+	+	+	+
03.	Tannins	Lead acetate test	Formation of precipitate	+	+	+	+
		Ferric chloride test	Dark green colour formation	+	+	+	+
04.	Saponins	Foam test	Formation of foam	+	-	+	+
05.	Protein and amino acids	Xanthoprotein	Formation of white precipitate	+	+	+	+
		Biurets	Violet/pink colour Formation	-	-	-	-
06.	Phytosterols	Salkowski reaction	Formation of Red and yellow colour in chloroform and lower layer respectively	+	-	-	+
07.	Phenolic compounds	Ferric chloride	Dark green colour formation	+	+	+	+
08.	Resins	Petroleum ether and Alcohol test	Appearance of turbidity	-	-	+	-
09.	Reducing sugars	Benedict's reagent test	Formation of Brown to red color precipitates	+	+	+	+
		Foiln Wu copper reagent test	Red color formation	-	-	-	-
10.	Flavonoids	Lead acetate test	Yellow precipitate formation	+	-	+	-
11.	Terpenoids	-	A grey coloured solution	+	+	+	-

FRAq: *F. racemosa* Aqueous; FRC: *F. racemosa* chloroform; FRM: *F. racemosa* Methanol; FRAc: *F. racemosa* acetone

Table 3: Phytochemicals identified in the Aqueous extract of *Ficus racemosa* by LC-MS.

Sr.no.	Time	Area	Area%	Identified compounds	Type of active principle
1	3.253	1210162	9.658	9-Fluoro-11beta-hydroxy-16alpha-methylandrosta-1,4-diene-3,17-dione	-
2	3.863	1495151	11.933	Choline	Amino acids
3	4.897	133001.3	1.061	a) (-)-Citronellol	Terpenoids
				b) 2,4,6-Triethyl-1,3,5-trioxane	
4	6.283	126618.2	1.011	a) Gallocatechin-4beta-ol	Flavonoids
				b) 1,11-Undecanedicarboxylic acid	
				d) 4-Phospho-D-erythronate	
5	8.212	347718.7	2.775	a) Isopropyl hexanoate	Plant metabolites
				b) (2xi,6xi)-7-Methyl-3-methylene-1,2,6,7-octanetetrol	
6	11.975	438396.9	3.499	xi-p-Menth-3-ene	Terpenoids
7	12.772	117688.8	0.939	a) gamma-Nonalactone	Flavonoids
				b) (+/-)-trans- and cis-4,8-Dimethyl-3,7-nonadien-2-ol	
				c) nonan-1-ol	
8	13.442	374478.9	2.989	a) Phoenicoxanthin/Adonirubin/ 3-Hydroxycanthaxanthin	Protein / Amino acids
				b) Dihydropteroic acid	
9	14.136	67079.7	0.535	a) 3,7-Dihydroxy-25-methoxycucurbita-5,23-dien-19-al	Diterpenoids, Glycoside phosphate
				b) Epothilone A	
				c) Delcosine	
				d) 5"Phosphoribostamycin	
10	16.554	179670.7	1.434	a) Uridine	Amino acids, Diterpenoids
				b) Amitenone	
11	18.568	8039747	64.165	Phloretin	Phenolics or polyphenol

Conclusion

The antibacterial activity of the aqueous leaf extract of *Ficus racemosa* was highest as compared to the antibacterial activity of chloroform, methanol and acetone leaf extracts of *Ficus racemosa.* The preliminary qualitative phytochemical study showed that in general, the aqueous, chloroform, methanol and acetone leaf extracts of both *Ficus racemosa* contains alkaloid, glycosides, tannins, saponins, protein and amino acids, phytosterols, phenolic compounds, resins, reducing sugars and flavonoids. The presence of different active phytochemicals as evident in LC-MS analysis gives a large scope for isolation of active compounds and to study their antibacterial properties.

Acknowledgement

I am very grateful to the Respected Dean, and HoD Department of Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Udgir for giving me chance to work.

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