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FREE RADICAL SCAVENGING ACTIVITY AND GC-MS ANALYSIS OF METHANOLIC EXTRACT OF *FICUS RACEMOSA* L. LEAVES OF BUNDELKHAND REGION

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Ficus racemosa L. popularly known as the cluster fig has been found effective for the treatment of various disorders like diabetes, diarrhoea, inflammatory conditions, piles, respiratory, urinary diseases, etc. The present study is therefore aimed to investigate the phytochemical constituents, GCMS analysis, antioxidant potential, total phenolic & flavonoid contents in the leaf extracts of *F. racemosa*. A qualitative screening of aqueous and methanolic extracts showed the presence of Alkaloids, Flavonoids, glycosides, tannins, saponin and phenolic compounds. In TLC, total 5 spots were present in the methanolic extract with different Rf values. The total flavonoid and phenolic contents are 160.4 ± 48.51 mg QE/g & 40.68 ± 9.17 mg GAE/g respectively. The dose dependent antioxidant activity was found. GS-MS analysis was done based on retention time, peak area and molecular weight. The presence of secondary metabolites depends on the solvents used and the methods applied. Many biologically active components were present as analysed by GCMS. The highest peak area of isoamyl nitrite (4.205%) was observed. The antioxidant activity could be attributed due to the presence of phenolic and flavonoids contents. Thus the present study suggests that *F. racemosa* leaves extract could be used as a possible therapeutic agent for a variety of diseases.

Keywords: Antioxidant Activity, *F. racemosa*, GCMS, Total Phenolic contents, Total Flavonoids Contents

Introduction

India is noted for its wide variety of medicinal plants and is thus known as the world's botanical garden[1]. The incidence of natural products with medicinal properties has been traced to the widespread use of herbal remedies and healthcare preparations, such as those mentioned in ancient texts such as the Vedas and the Bible and obtained from widely used traditional herbs and medicinal plants[2]. The use of traditional medicine and medicinal plants in most developing countries, as a normative basis for the maintenance of good health, has also been widely observed[3]. The majority of medicines used in herbal medicine are derived from natural sources or semi-synthetic forms of natural products[4]. Today according to the world Health organization (WHO), as many as 80% of the world's people depend on traditional medicine for their primary healthcare needs. There are considerable economic benefits in the development of indigenous medicines and in the use of medicinal plants for the treatment of various diseases[5].

Ficus Racemosa L. is a moderate to large sized tree belongs to the family Moraceae and popularly known as the cluster fig tree with some other names like dimri, dumri, gular etc. Ficus is an exceptionally large pan-tropical genus with

over 700 species[6] distributed widely throughout the warmer parts of Asia, Africa, America, and Australia. It is retained as a single, large genus because it is well defined by its unique reproductive system, involving synconia fig and specialized pollinator wasps[7]. Several members of the genus *Ficus* (family: Moraceae) are being used traditionally in a wide variety of ethnomedical remedies. One among them, *Ficus racemosa* syn. *Ficus glomerata* (Gular; Udumbara)[8], is widely distributed all over India, northern Australia and other parts of Asia. All *Ficus* species possess latex-like material within their vasculatatures that provide defense and self healing from physical assaults[9]. Among various pharmacological properties, *F. racemosa* imparts vital role as anti-oxidant, anti cancer, antidiuretic, anti bacterial, anti-inflammatory, memory enhancing and gastro-protective agent etc[10]. Pharmacological studies have acknowledged the value of medicinal plants as a potential source of bioactive compounds[11]. Phytochemicals from medicinal plants serve as lead compounds in drug discovery and design[12].

Many medicinal plants shows antioxidant activities. Antioxidants are the substances that protect cells from the damage caused by reactive oxygen species (ROS) such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals, and peroxy nitrite[13,14]. In a variety of experimental models of carcinogenesis, antioxidant substances derived from dietary or medicinal plants have been shown to be efficient and versatile chemopreventive and antitumoral agents[15]. Cardiovascular disease, cancer, inflammatory disorders, asthma, liver disease, and macular degeneration are all associated to oxidative damage[16]. The potential of different plant extracts to induce anti-oxidant effects has been studied by many researchers[17&18]. Therefore the present investigation aimed to evaluate antioxidant activities, free radical scavenging potential, and GCMS of the methanolic extract of the *F. racemosa* leaves of Bundelkhand region.

Materials and Methods

Plant material collection:

F. racemosa leaves were collected from the Bundelkhand University campus, Jhansi (U.P.) in the month of March 2017. Firstly the collected leaves were washed with tap water for 3-4 times and followed by de-ionized water. After that, it kept in the dark for drying at room temperature, crushed with the help of electric grinder and stored for the extraction purpose.

Extraction Procedure:

Leaves of *F. racemosa* was extracted by two methods i.e. Aqueous and Methanolic extraction.

Different concentration of dry powder of *F. racemosa* leaves i.e. 5 gm and 10 gm in distilled water was extracted through water bath for 1 hour at 90°C, filtered this extract and stored at 4°C.

The powdered *F. racemosa* leaves were percolated using 80% of methanol in the soxhlet apparatus at 60-65°C. This extract was filtered and evaporated to dryness by using rota evaporator followed by desiccator, and stored in air tight bottles at 4°C temperature.

Phytochemical Analysis:

The screening for the presence or absence of secondary metabolites was described elsewhere[19] with some modifications.

Thin layer chromatography:

The extracts were tested using TLC analytical plates coated with silica gel-G of 0.2 mm thickness was used for the testing of methanolic extract of *F. racemosa* leaves. Here we used a solvent mixture (Butanol- acetic acid-water) at the ratio of 2:1:1 v/v as described by somewhere else[19]. Fully developed silica coated plate was air dried followed by heating for 20-25 minutes. The plate was sprayed with 0.2% freshly prepared ninhydrin solution to detect the bands, which were expressed by its retention factor (Rf).

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Phosphomolybdenum assay:
The total antioxidant capacity of the methanolic extract of *F. racemosa* leaves was done by the phosphomolybdenum complex formation carried out by Prieto et al.[20] with some of modifications. 0.2 mL of different concentrations of the extract was combined with 2 mL of reagent solution and incubated at 95°C for 90 min. After cooling at room temperature measured the absorbance at 695nm with the help of multi plate reader. And draw the calibration curve with the reference of ascorbic acid.
Nitric oxide radical scavenging assay:
Free radicals generated from sodium nitroprusside (SNP) were measured according to the earlier described method[21] with some modifications. Different concentration of reaction mixture containing SNP (15 mM) in PBS (pH 7.3) with and without sample, incubated at 25°C for 210 mins. Add Griess reagent followed by rest for 10 minutes at room temperature. BHT was used as a standard. The absorbance was measured at 560 nm using a UV-Vis microplate reader.
Superoxide anion scavenging assay:
The total antioxidant capacity of methanolic extract was based on the reduction of NBT according to a previously reported method[22] with some modification. The 1-mL reaction mixture contained phosphate buffer (20 mM, pH 7.4), PMS (60µM), NBT (156µM), and various concentrations of sample solution. After incubation for 5 min at 25°C temperature, the absorbance was taken at 560 nm against an appropriate blank solution. Ascorbic acid was used as positive control.
Total Phenolic Content (TPC):
The Folin- Ciocalteu method[23] was used to calculate the total phenolic material. Different concentrations were combined with 500 µl of water, then 100 µl of Folin-Ciocalteu reagent was added, mixture was left to stand for 6 minutes. Now, apply 1 mL of sodium carbonate and 500 µL of distilled water to the reaction mixture tubes, respectively. At room temperature, all of the tubes were incubated for 90 minutes. At 760 nm, the absorbance was estimated. Gallic acid in different concentrations was used as a standard. Calculate total phenolic content in gallic acid equivalents per gram (mg GAE/g).
Total Flavonoid Content (TFC):
To determine the total flavonoids content Aluminium chloride complex forming assay[24] was used for the methanolic extract of *F. racemosa* leaves. Flavonoid content was defined as a quercetin equivalent (mg QE/g). 100 µl of quercetin dilution was mixed with 500 µl of distilled water and then with 100 µl of 5% sodium nitrate for 6 minutes. Then 150 µl of 10% Aluminum chloride solution was added and allowed to stand for 5 minutes, after which 200 µl of 1M Sodium hydroxide solution was sequentially added. Absorption of this reaction mixture was recorded at 510 nm.
Gas Chromatography-Mass Spectrometry (GC-MS) Analysis:
On a Perkin Elmer Turbo Mass Spectrophotometer with a Perkin Elmer autosampler XLGC, GC-MS analysis of methanolic extract of *F. racemosa* leaves was performed. The column was a Perkin Elmer Elite - 5 capillary column with a film thickness of 0.25 mm and a length of 30 m. It was made of 95 percent dimethylpolysiloxane. At a flow rate of 0.5 ml/min, carrier gas helium (99.999 percent) was used as the carrier gas. As an injection length, a 1 l sample was used. The inlet temperature of the GC was maintained at 250 °C, with a programmed oven temperature of 110 °C (isothermal for 2 min), followed by a 10 °C/min increase to 200 °C, followed by a 5 °C/min increase to 280 °C, and a 5 °C/min increase to 280 °C, with a 5 °C/min isothermal at 280 °C. The GC took 30 minutes to run. The temperature of the MS transfer line is kept at 200°C, while the source temperature is kept at 180°C. The GCMS analysis was carried out using electron impact ionisation at 70eV, and Total Ion Count was used for data

evaluation of compound detection and quantification (TIC). The spectrum of the components was compared to the known components stored in the GC-MS library. For peak area measurement and data processing, Turbo-Mass OCPTVS-Demo SPL programme 19 was used.

Results and Discussion

Screening of the aqueous and methanolic extracts of *F. racemosa* leaves shows the presence of different secondary metabolites as determined by biochemical tests (Table 1). The presence or absence of these secondary metabolites depends on the solvent used as well as the qualitative detection methods. Secondary metabolites are essential for humans and animals alike. Two different concentrations i.e. 5 & 10 gm of plant material were used for aqueous extraction and it was observed that there is not much differences in the presence or absence of secondary metabolites. Further, aqueous and methanolic extract are equally important for the determination of secondary metabolites.

The presence of 5 spots on thin layer chromatography of methanolic extract of *F. racemosa* leaves was observed showing different Rf values i.e. 0.43, 0.55, 0.62, 0.73, and 0.81, respectively (Fig. 1). The Rf value of alanine amino acid, which we used as a reference, was 0.65. The dose dependent antioxidant activities were observed. The percent inhibition of methanolic extract was found to be 79.0% at a concentration of 1000 mg/ml whereas ascorbic acid, on the other hand, had a scavenging activity of 91.0% at the same concentration. As a result, the antioxidant potential is due to the phenolic and flavonoid material. The mean values of total phenolic and flavonoid contents were 40.68 ±9.17 mg GAE/g and 160.4 ±48.51 mg QE/g respectively (Figure 2 and Table 3). The nitric oxide and superoxide radical scavenging behaviour of the methanolic extract of *F. racemosa* leaves were dose dependent (Fig.3 and 4). The active principle analyzed by GCMS with molecular weight, molecular formula, peak area %, retention time, and composition of the bioactive components of methanolic

Table 1: Qualitative phytochemical analysis of the aqueous and methanolic extracts of *F. racemosa* leaves.

S No.	Phytochemical Test	Aqueous Extract		Methanolic Extract
		10 gm	5 gm	
1.	**Alkaloids Test**			
	Mayer's	+ ve	- ve	+ ve
	Wagner's	- ve	+ ve	+ ve
	Hager's	+ ve	+ ve	+ ve
2.	**Carbohydrates Test**			
	Molisch	+ ve	+ ve	+ ve
	Barfoed's	- ve	- ve	+ ve
3.	**Reducing Sugars Test**			
	Fehling's	+ ve	+ ve	- ve
	Benedict's	+ ve	+ ve	+ ve
4.	**Flavonoids Test**			
	Alkaline Reagent	+ ve	+ ve	+ ve
	Lead Acetate	+ ve	+ ve	+ ve
5.	**Glycosides Test**		- ve	+ ve
	Borntrager's	- ve	- ve	- ve
	Legal's	+ ve	- ve	- ve
	Keller-killiani	- ve		
6.	**Tannin & phenolic Test**		+ ve	+ ve
	Ferric Chloride	+ ve	- ve	+ ve
	Lead Acetate	+ ve	+ ve	+ ve
	Dilute iodine solution	+ ve		
7.	**Saponin Test**			
	Froth	- ve	- ve	+ ve
8.	**Protein & A.A. Test**			
	Ninhydrin	+ ve	+ ve	+ ve
	Biuret	+ ve	+ ve	+ ve
9.	**Triterpenoids &Steroids Test**			
	Salkowski's	+ ve	- ve	+ ve
10.	**Hydrolysable tannin Test**	+ ve	+ ve	+ ve

(+) indicates presence while, (–) indicates the absence of the components

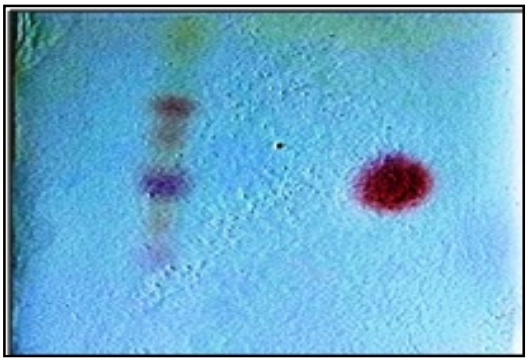

Fig. 1: TLC Plate showing spots having different Rf values (0.43, 0.55, 0.62, 0.73, 0.81) of Methanolic extract of *F. racemosa* leaves.

extract of *F. racemosa* leaves (Table 2). The GC-MS chromatogram of the identified compounds is shown in (Fig. 5). Many biologically active components were observed. The highest peak area of Isoamyl nitrite (4.205%), followed by 2-Penten-1-ol, (E)-(4.205%), 5-Hexynoic acid (3.587%), Alpha-l-rhamnopyranose (3.587%), 3-Nonenoic acid (3.587%), Decanal (3.587%), Quinic acid (3.587%), Tribehenin (3.146%), Phorbol 12,13-dihexanoate (3.146%), -Piperidineacetic acid, 1-acetyl-5-ethyl-2-[3-(2-hydroxyethyl)-1H-indol-2-yl]-à-methyl-, methyl ester (3.018%), 2,2'-Bithienyl, 5,5'-bis(trimethylstannyl)- (2.662 %), Cercosporin (2.423%), Rhodopin(2.107%), Lutein (2.107%), Phorbol 12,13,20-triacetate (1.908%), Acetic acid, (1,2,3,4,5,6,7,8-ctahydro-3,8,8-trimethylnaphth-2-yl)methyl ester (1.615%), Geranylgeraniol (1.505%), Squalene (1.505%), Hematoporphyrin (1.4594%), Linolenyl alcohol (1.296%), and Gamolenic Acid (1.296%).

Antioxidant, anticancerous, antimalerial and antileishmanial activities are shown by various identified compounds such as Cercosporin , Gamolenic Acid , Phorbol 12,13,20-triacetate , 4-Piperidineacetic acid, 1-acetyl-5-ethyl-2-[3-(2-hydroxyethyl)-1H-indol-2- yl]-à-methyl-, methyl ester , trans-Geranylgeraniol , Phorbol 12,13-dihexanoate , Hematoporphyrin, Squalene.

The genus *Ficus* constitutes an important group of trees with immense medicinal value. The medicinal plants are widely used by the traditional medical practitioners for curing various diseases in their day to day practice[10]. Medicinal plants and their products have been used extensively and safely for the treatment of medical problems[25]. However, herbal remedies have been used in developed countries since ancient times[26]. Secondary metabolites are the plant derived compounds which are non-nutritive but play an ecological and economical role. These metabolites plays vital role for all the living beings. Our results show the presence of various secondary metabolites both in aqueous and methanolic extracts of leaves of *F. racemosa.* Further, TLC of methanolic extract shows five different components.

Many industries use natural plants material for the purpose of manufacturing different type of drugs to treat the diseases. It is a good remedy for excessive appetite. The extract of *Ficus* fruit is used in diabetes, leucoderma, refrigerant, antiasthmatic, hepatoprotective, antioxidant, antiulcer and menorrhagia. It is used locally to relieve inflammation of skin wounds, lymphadenitis, in sprains and fibrositis[27]. The seeds are tiny, innumerable and grain-like. Outer surface of the bark consists of easily removable translucent flakes grayish to rusty brown, uniformly hard and non-brittle[28]. Following phytoconstituents have been found to contain in plant; bergapten, bergaptol, lanosterol, β-Sitosterol, Stigmasterol, lupen-3-one, β-sitosterol-d-glucoside (phytosterolin), vitamin k1[29, 30&31]. The bark contains tannin, wax, saponin, β-sitosterol, leucocyanidin-3-*O*-α-Lrhamnopyranoside, lupeol, ceryl behenate, lupeol acetate, α-amyrin acetate, leucocyanidin and leucoanthocyanin[32]. Leaves yield campestrol, stigmasterol, isofucosterol, α- amyrin, lupeol, tannic acid, arginine, serine, aspartic acid, glycine, threonine, alanine, proline, tryptophan, tyrosine, methionine, valine, isoleucine, leucine, n-nonacosane, nhentricontanen, hexa-cosanol, and n-octacosan[33, 34&35]. These leaves contained a variety of bioactive secondary metabolites, including alkaloids, flavonoids, tannins, steroids, and glycosides, according to GC-MS results. It can provide a variety of health benefits for living beings by protecting against oxidative stress when consumed on a regular basis.

A substance's ability to serve as an antioxidant is determined by its ability to minimise ROS by contributing hydrogen

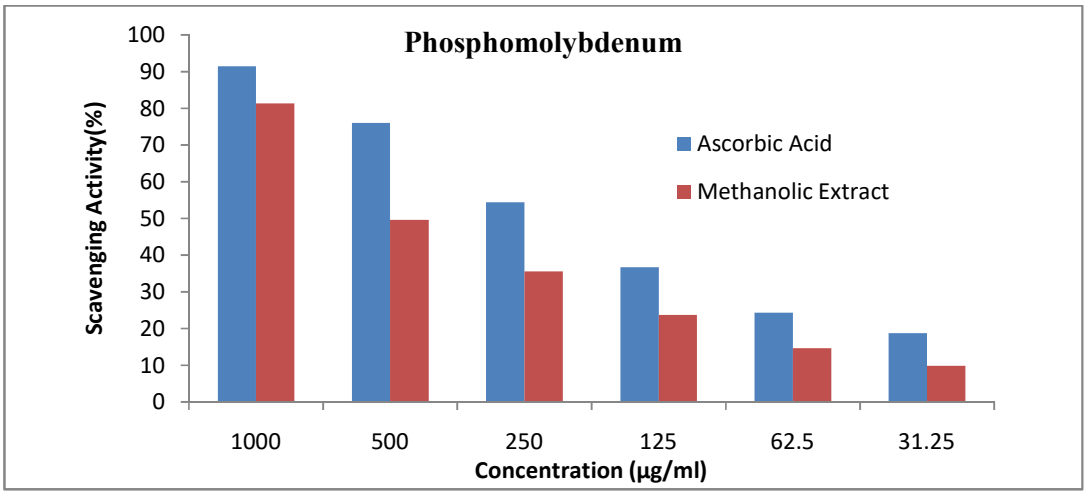

Fig. 2: The scavenging activity on phosphomolybdenum of methanolic extract of *F. racemosa* leaves

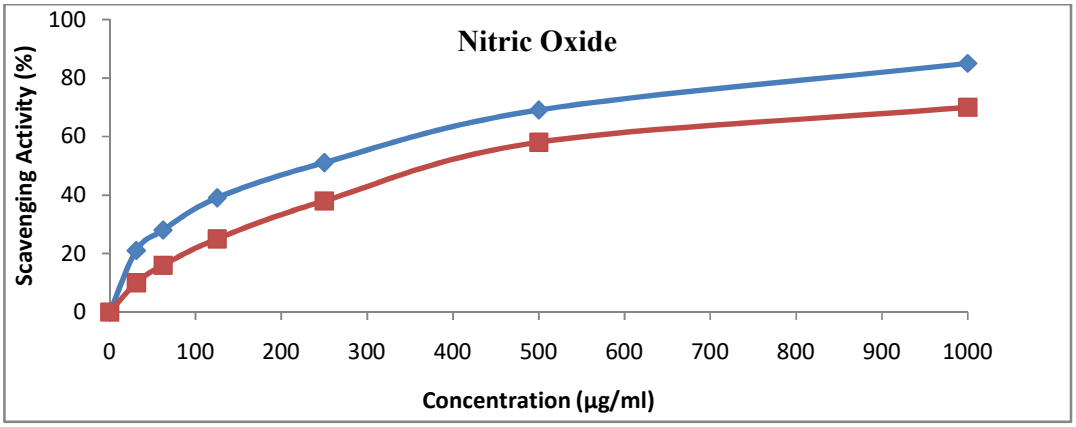

Fig. 3: Nitric oxide scavenging activity on methanolic extract of *F. racemosa* leaves

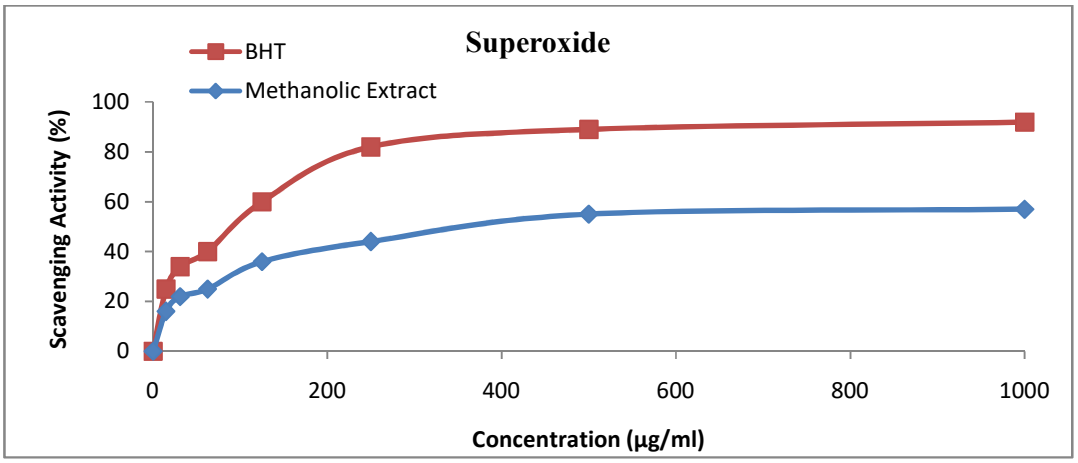

Fig. 4: The scavenging activity on superoxide radical of methanolic extract of *F. racemosa* leaves

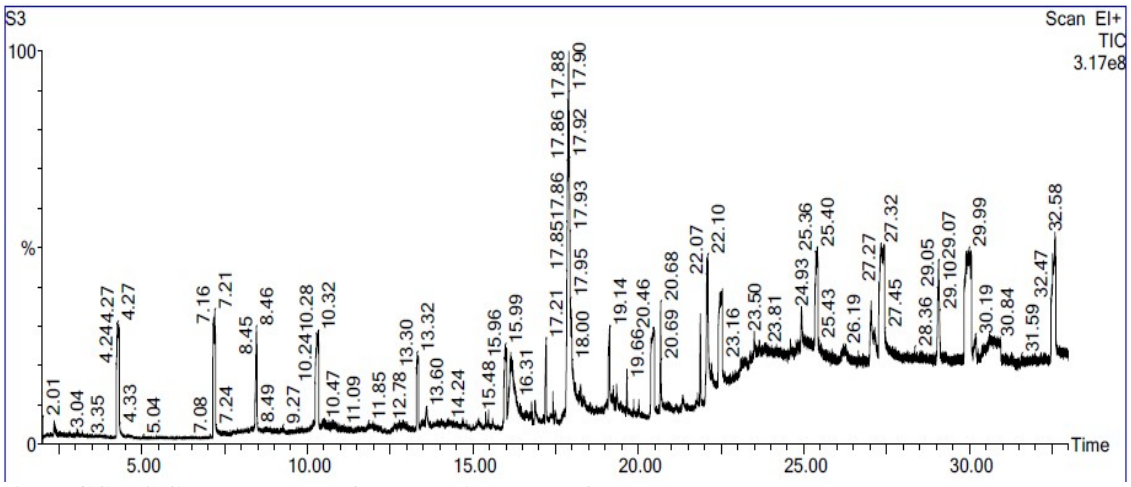

Fig. 5: GC-MS Chromatogram of methanolic extract of *F. racemosa* leaves

Table 2: Compounds identified by GC-MS analysis of methanolic extract of *F. racemosa* leaves

Sr. No	RT	Peak area %	Compound name	M.F.	MW	Biological Activities
1.	4.273	2.662	2,2'-Bithienyl, 5,5'-bis(trimethylstannyl)-	$C_{14}H_{22}S_2Sn_2$	491	Hazardous to the aquatic environment[38]
2.	10.335	2.423	Cercosporin	$C_{29}H_{26}O_{10}$	534	Antimalarial activity, Antileishmanial activity against Leishmania donovani promastigotes, Cytotoxicity against human SKOV3 cells Antimicrobial activity[39]
3.	17.886	3.587	5-Hexynoic acid	$C_6H_8O_2$	112	Skin corrosion Specific target organ toxicity[40]
4.	17.886	3.587	Alpha-l-rhamnopyranose	$C_6H_{12}O_5$	164	It is a known human metabolite of Quercitin[41]
5.	17.886	3.587	Decanal	$C_{10}H_{20}O$	156	Flavoring Agents, Make synthetic citrus oils, It is not broken down by light[42]
6.	17.886	3.587	Quinic acid	$C_7H_{12}O_6$	192	Cosmetics[43]
7.	17.903	4.205	Isoamyl nitrite	$C_5H_{11}NO_2$	117	Automotive Drug, Vasodilator[44] Cyanide poisoning antidote reduce myocardial oxygen consumption, Anti-angina Stimulate sexual desire[45]
8.	17.903	4.205	2-Penten-1-ol, (E)-	$C_5H_{10}O$	86	Flavoring Agents[46]
9.	17.978	1.615	Acetic acid, (1,2,3,4,5,6,7,8-ctahydro-3,8,8-trimethylnaphth-2-yl)methyl ester	$C_{16}H_{26}O_2$	250	HIV-1 RNase H Inhibition[47]
10.	22.099	1.296	Gamolenic Acid	$C_{18}H_{30}O_2$	278	Anti-inflammatory, Antithrombotic, Anticancerous, Antiproliferative Helps in Atherosclerosis, Diabetic complications, Skin disorders, Respiratory disorders, inhibitors of the malarial parasite[48]
11.	22.099	1.296	Linolenyl alcohol	$C_{18}H_{32}O$	264	Commercial Activity[49]
12.	27.336	3.146	Tribehenin	$C_{69}H_{134}O_6$	1059	Food Additives Drug, Personal care, cosmetics[50]
13.	27.336	3.146	Phorbol 12,13-dihexanoate	$C_{32}H_{48}O_8$	560	Antiviral activity against HIV1 3B. HIV2 ROD infected in MT4 cells Antiviral activity against chikungunya virus[51]
14.	27.427	1.459	Hematoporphyrin	$C_{34}H_{38}N_4O_6$	598	Therapeutically to treat psoriasis and various types of neoplasms, Anticancer[52]
15.	29.074	1.505	Squalene	$C_{30}H_{50}$	410	Personal care, Cosmetics, Eye care Antiinflammatory activity Antitubercular activity Antimycobacterial activity Antiamoebic activity[53]

16.	29.074	1.505	Geranylgeraniol	$C_{20}H_{34}O$	290	Inhibitors of Tau Fibril Formation Antiviral activity Inducers of the Endoplasmic Reticulum Stress Response (ERSR) in Human Glioma[54]
17.	29.907	1.908	Phorbol 12,13,20-triacetate	$C_{26}H_{34}O_9$	490	Antiviral activity[55]
18.	32.583	2.107	Rhodopin	$C_{40}H_{58}O$	554	Food Additives [56]
19.	32.583	2.107	Lutein	$C_{40}H_{56}O_2$	568	Food Additives Protect from oxidative stress and high-energy light[57]

Table 3: Total Flavonoid & Phenolic Content of methanolic extract of *F. racemosa* leaves

Total Flavonoid Content (mgQE/g)		**Total Phenolic Content (mgGAE/g)**	
Dilution (µg/ml)	**Gular (Leaves)**	**Dilution(µg/ml)**	**Gular (Leaves)**
1000	50	150	27.33
500	76	120	29.99
250	132	90	31.11
125	240	60	38.33
62.5	304	30	76.66
Mean of Content	**160.4 ± 48.51**	**Mean of Content**	**40.68 ± 9.17**

atoms[36]. The existence of reductants, which have antioxidative ability by splitting the free radical chain and contributing hydrogen atoms, defines a compound's reducing power[37]. When compared to ascorbic acid and BHT, *F. racemosa* whole leaf extracts showed reductive capacity. The extract's reducing ability was concentration dependent, and its antioxidant activities were comparable to those of ascorbic acid and BHT, well-known strong antioxidants.

Conclusion

In the present study, methanolic extract of *F. racemosa* leaves has most of the secondary metabolites and TLC results reveals five components. GCMS analysis shows many biologically active components with various biological roles. Further, antioxidant, super oxide and nitric oxide scavenging activity were observed which is comparable to the standard used. Therefore the methanolic extract of *F. racemosa* leaves has substantial antioxidant activity and could be used as a convenient source of natural antioxidants. Moreover it could be used for the treatment of many chronic diseases. Further, studies are needed to determine the role of individual components.

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