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Determination of Anti-oxidant activity of Different Extracts of *Ficus gibbosa* Blume, Isolation and Characterization of Flavonoid from Ethanol Extract by Column Chromatography.

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ABSTRACT

The current study involves the determination of antioxidant activity of aqueous and ethanolic extracts of the plant *Ficus gibbosa* Blume, isolation and characterization of flavonoid from the ethanolic extract by column chromatography. The antioxidant potential of the whole plant extracts were identified by DPPH assay, ABTS assay, hydroxyl radical scavenging activity, super oxide free radical scavenging activity, nitric oxide radical scavenging activity and total antioxidant activity methods. Among the two extracts, the aqueous extract shows better antioxidant activity. The ethanolic extract was subjected to column chromatography by gradient elution technique and the fraction 98-103 was found to be 3, 4', 5, 7- tetrahydroxy-6- methyl flavone.

Keywords: *Ficus gibbosa* Blume, column chromatography, DPPH assay, ABTS assay, hydroxyl radical scavenging activity, super oxide free radical scavenging activity, nitric oxide radical scavenging activity, total antioxidant activity, 3, 4', 5, 7- tetrahydroxy-6- methyl flavone.

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INTRODUCTION

The plant *Ficus gibbosa* Blume / *Ficus tinctoria* is a climbing strangler, forming a tree with prop-roots, to 25m. Alternately arrange leaves are often asymmetrical. Fruit is a fig, appearing in axils, usually paired, round to 1.5cm, ripening through orange to red or purple.

The fruit of a dye fig is the source of a red dye used in traditional fabric making in parts of Indonesia. The juice of the bark and leaves of dye fig or humped fig plant is used for grinding the pills and making a decoction in toxicology. Plant pacifies vitiated kapha, pitta, skin diseases, ulcers, hepatopathy, diabetes, ulcerative stomatitis, leucorrhoea and gynaecological problems [1].

Since ancient times, plants were used as a source of medicine for health benefits [2]. About 80-85% of global population rely on traditional medicines for their primary health care needs [3] because of their effectiveness, easy availability, low cost & comparatively less toxic effects [4]. In order to avoid the side effects of synthetic drugs [5, 6], lots of modern research have been carried out in herbal medicines for advancement in the treatment and control of various diseases. One of the limitation of the traditional medicine is the lack of standardization. The plants which contain phenolic and flavonoid compounds shows antioxidant activities and thus, prevent oxidative stress and there by related diseases[7].

Free radicals are molecular species accomplished of free existence that contains an unpaired electron in the outer shell of the molecule. The free radicals are highly reactive, unstable and can react with proteins, lipids, carbohydrates & DNA. Free radicals may be oxygen derived (reactive oxygen species) or nitrogen derived (reactive nitrogen species). O^{-}_{2} [superoxide], HO [hydroxyl], HO_2 [hydroperoxy], ROO [peroxyl], RO [alkoxyl] are oxygen derived free radical & H_2O_2 oxygen as non-radical. Whereas NO [nitric oxide], ONOO [peroxy nitrate], NO_2 [nitrogen dioxide] and N_2O_3 [dinitrogen trioxide] are nitrogen derived oxidant species [8, 9].

The uncontrolled generation of free radicals and oxidative stress results in diseases like Atherosclerosis, Cancer, Ulcer, Rheumatoid arthritis, Alzhiemers, Parkinsons disease, etc. Production of free radicals increases when there is an imbalance between oxidants and antioxidants. Antioxidants can prevent oxidative stress induced by free radicals by scavenging it [10].

The antioxidants work alone or in association with each other against various types of free radicals [11]. Nutritional antioxidant deficiency also leads to oxidative stress, which signifies the identification of natural anti-oxidants through the diet (eg:- Vit-C, E, polyphenols, etc.) [9, 12].

Plant material and Preparation of the Extract

Fresh plant of *Ficus gibbosa* Blume belongs to the family Moraceae was collected from the northern regions of Kerala, authenticated and identified by V.Chelladurai, Research Officer-Botany (Scientist – C), Central Council for Research in Ayurveda & Siddha, Government of India.

The whole parts of the plant were gabled for elimination of contaminants and then powdered. Air dried at room temperature and 150gm of the powdered part was successively extracted with Petroleum ether, Chloroform, Ethyl acetate, Ethanol and distilled water using Soxhlet extractor (gradient extraction). The extracts were concentrated in vacuum, weighed and properly labelled and stored in refrigerator until further use [13,14,15].

Determination of antioxidant activity

DPPH Assay Method

DPPH–(1, 1-diphenyl-2-Picryl hydrazyl)

The radical scavenging activities of different extracts were determined by using DPPH assay. The decrease in the absorption of the DPPH solution after the addition of an antioxidant was measured at 517nm. Ascorbic acid (10mg/ml DMSO) was used as reference. 1, 1-diphenyl-2-picryl hydrazyl is a stable free radical

with red colour which turns yellow when scavenged. The DPPH assay uses this character to show free radical scavenging activity. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as, DPPH + [H-A] →DPPH-H + (A). Antioxidants react with DPPH and reduce it to DPPH-H and as the consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds or extracts in terms of hydrogen donating ability. Different volumes (1.25-10µl) of plant extracts were made up to 40µl with DMSO and 2.96ml DPPH (0.1mM) solution was added. The reaction mixture incubated in dark condition at room temperature for 20 minutes. After 20 minutes, the absorbance of the mixture was read at 517nm.3ml of DPPH was taken as control [16].

$$\% \text{ Inhibition} = \frac{control - test}{control} X100$$

ABTS Assay Method

ABTS – (2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

The preformed radical monocation of 2,2'-azino-bis(3- ethyl benzothiazoline- 6- sulfonic acid) was generated by oxidation of ABTS with potassium persulfate (a blue chromogen) and was reduced in the presence of hydrogen donating antioxidants.

1ml of distilled water was added to 0.2ml of various concentration of the samples and 0.16ml of ABTS solution was added and make up to the final volume 1.36ml. Absorbance was measured after 20min at 734nm.
Solution I: ABTS, 20mM solution is prepared using H_2O
Solution II: Potassium persulfate, 17mM solution is prepared using H_2O

0.3ml of Solution II was added to 50ml of solution I. The reaction mixture was left to stand at room temperature overnight in dark before use [17].

$$\% \text{ inhibition} = \frac{control - test}{control} X100$$

Hydroxyl radical scavenging activity method

This assay was based on the quantification of the degradation product of 2 deoxy ribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+} -ascorbate- EDTA -H_2O_2 system (The Fenton reaction). The reaction mixture contained in the final volume of 1 ml - 2 deoxy 2 ribose (2.8mM), FeCl3 (100µM), EDTA (100µM), H_2O_2 (1.0mM), ascorbic acid (100µm) in KH_2PO_4-KOH buffer (20mM pH 7.4) and various concentrations (62.5 – 2000µg/ml) of the test sample. After incubation for 1hour at 37°C, 0.5 ml of the reaction mixture was added to 1ml of 2.8% TCA, then 1ml aqueous TBA was added and the mixture was incubated at 90°C for 15 minutes to develop the colour. After cooling the absorbance was measured at 532nm against a blank solution [18].

$$\% \text{ Inhibition} = \frac{control - test}{control} X100$$

Nitric oxide radical scavenging activity method

Griess reagent

Nitric oxide scavenging activity was measured spectrophotometrically. Sodium nitro prusside (5mmolL^{-1}) in phosphate buffered saline pH7.4, was mixed with different concentration of the extract (62.5-2000µg/ml) prepared in methanol and incubated at 25°C for 30minutes. A control without the test compound, but an equivalent amount of methanol was taken. After 30minutes, 1.5ml of the incubated solution was removed and diluted with 1.5ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diamine dihydrochloride). Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1 naphthyl ethylene diamine dihydrochloride

was measured at 546nm and the percentage scavenging activity was measured with reference to the standard [17].

$$\% \text{ Inhibition} = \frac{control - test}{control} X100$$

Super oxide free radical scavenging activity method

DMSO - Dimethyl sulfoxide

Superoxide is biologically important as it can form singlet oxygen and hydroxyl radical. Super oxide anion is generated in the riboflavin-NADH system by the oxidation of NADH and assayed by the reduction of NBT resulting in the formation of blue formazan product. Different concentrations of extracts (62.5-2000µg/ml), 0.05ml of Riboflavin solution (0.12mM), 0.2ml of EDTA solution [0.1M] and 0.1ml NBT (Nitro-blue tetrazolium) solution [1.5mM] were mixed in a test tube and the reaction mixture was diluted up to 2.64ml with phosphate buffer [0.067M]. The optical density of the solution was measured at 560nm using DMSO as blank after illumination for 5min and difference in OD was determined after 30minutes incubation in fluorescent light. Absorbance was measured after illumination for 30 min. at 560 nm on UV visible spectrophotometer [19].

$$\% \text{ Inhibition} = \frac{control - test}{control} X100$$

Total antioxidant activity method

0.3ml sample (extract) was obtained with 3ml of reagent solution (0.6ml H_2SO_4, 28mM sodium phosphate and 4mM ammonium molybdate). The tube containing the reaction solutions were incubated at 95^0C for 90 minutes. The absorbance of the solution was measured at 695nm against blank after cooling to room temperature (Methanol 0.3ml) in the place of extract was used as blank. The antioxidant activity is expressed as number of gram equivalent of ascorbic acid [17].

$$\% \text{ Inhibition} = \frac{control - test}{control} X100$$

Statistical Analysis of Data

Experimental results for all the extracts were expressed as mean ± SD. All measurements were replicated three times. The values were calculated from non-linear regression analysis.

Isolation and characterization of phytoconstituents of ethanol extract of *Ficus gibbosa* Blume

The ethanolic extract was subjected to column chromatography, by means of gradient elution technique [14].

RESULTS AND DISCUSSION

Determination of antioxidant activities of the selected extracts of *Ficus gibbosa* Blume

The in-vitro antioxidant activity by DPPH assay, ABTS assay, hydroxyl radical scavenging activity, super oxide radical scavenging activity, nitricoxide radical scavenging activity and total antioxidant activity methods were conducted as per the procedure mentioned. The antioxidant activity of plant extracts was carried at different concentrations (62.5-2000µg/ml) to determine the IC50 (50% growth inhibition). It was found that, the percentage of growth inhibition is increasing with increasing concentration of test compounds. The results were presented in Table 1-6 & graphically represented in Figure 1-6. Results of fractionation are presented in Table 7 & spectral datas are represented in figure 7-10.

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Table 1: Comparison of % inhibition of extracts of *Ficus gibbosa* Blume by DPPH assay method

Con(µg/ml)	%Inhibition	
	Ethanol	Water
62.5	43.6002	45.6650
125	46.6141	53.9888
250	52.6437	66.5120
500	59.1372	74.4898
1000	66.6048	85.96
2000	77.6901	93.84
IC50	159.7	91.28

Table 2: Comparison of % inhibition of extracts of *Ficus gibbosa* Blume by ABTS assay method

Con(µg/ml)	%Inhibition	
	Ethanol	Water
62.5	44.0630	46.6141
125	52.9220	53.9888
250	58.9981	67.0686
500	66.6048	77.6901
1000	78.2003	84.46
2000	83.3024	92.90
IC50	106.7	86.82

Table 3: Comparison of % inhibition of extracts of *Ficus gibbosa* Blume by hydroxyl radical scavenging activity method

Con(µg/ml)	%Inhibition	
	Ethanol	Water
62.5	38.67	44.34
125	42.27	53.5481
250	52.9924	65.7371
500	64.2536	78.5073
1000	74.9286	89.3658
2000	88.3870	95.2895
IC50	175.1	96.03

Table 4: Comparison of % inhibition of extracts of *Ficus gibbosa* Blume by nitric oxide radical scavenging activity method

Con(µg/ml)	%Inhibition	
	Ethanol	Water
62.5	24.9842	29.88
125	42.47	43.9274
250	55.6782	56.38
500	62.4605	66.71
1000	78.3911	85.96
2000	89.8264	93.84
IC50	208.00	174.7

Table 5: Comparison of % inhibition of extracts of *Ficus gibbosa* Blume by super oxide free radical scavenging activity method

Con(µg/ml)	%Inhibition	
	Ethanol	Water
62.5	33.2667	64.511
125	59.36	73.68
250	64.23	75.89
500	71.35	77.03
1000	76.4396	78.26
2000	80.9748	81.60
IC50	113.1	26.58

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Table 6: Comparison of % inhibition of extracts of *Ficus gibbosa* Blume by total antioxidant activity method

Con(µg/ml)	%Inhibition	
	Ethanol	**Water**
62.5	48.27	55.7387
125	54.4323	59.09
250	60.03	61.6796
500	64.63	67.7138
1000	69.26	71.6329
2000	77.8227	78.25
IC50	78.88	34.56

Table 7: Fractionation of ethanolic extract of *Ficus gibbosa* Blume by column chromatography

Fraction Number	Solvent ratio for column elution	NO. of spots	R_f value
1-3	100% P.E	-Nil-	-Nil-
4-6	P.E 90%: B 10%	-Nil-	-Nil-
6-8	P.E 80%: B 20%	-Nil-	-Nil-
9-11	P.E 70%: B 30%	-Nil-	-Nil-
12-14	P. E 60%:B 40%	Three	0.5,0.7,0.4
15-19	P. E 50%:B 50 %	One	0.8
20-23	P. E 40%: B 60 %	-Nil-	-Nil-
24-27	P.E 30%: B 70 %	-Nil-	-Nil-
28-33	P. E 20%: B 80 %	One	0.6
34-40	P. E 10%: B 90 %	-Nil-	-Nil-
40-47	100% B	-Nil-	-Nil-
48-56	B 90%:E.A 10%	One	0.9
57-60	B 80%: E.A 20%	-Nil-	-Nil-
61-64	B 70%: E.A 30%	-Nil-	-Nil-
65-68	B 60%: E.A 40%	-Nil-	-Nil-
69-72	B 50%: E.A 50%	-Nil-	-Nil-
73-78	B 40%: E.A 60%	-Nil-	-Nil-
79-84	B 30%: E.A 70%	-Nil-	-Nil-
85-91	B 20%: E.A 80%	-Nil-	-Nil-
92-97	B 10%: E.A 90%	-Nil-	-Nil-
98-103	100% E.A	One	0.4
104 – 108	E. A 90%:E 10%	-Nil-	-Nil-
109 – 113	E.A 80%: E 20%	-Nil-	-Nil-
114 – 117	E.A 70%: E 30%	-Nil-	-Nil-
118 – 121	E.A 60%: E 40%	-Nil-	-Nil-
122 – 126	E.A 50%: E 50%	-Nil-	-Nil-
127 – 130	E.A 40%: E60%	-Nil-	-Nil-
131 – 133	E.A 30%: E80%	-Nil-	-Nil-
134 – 137	E.A 20%: E 70%	-Nil-	-Nil-
138 – 141	E.A 10%: E90%	-Nil-	-Nil-
142 – 145	100% E	-Nil-	-Nil-

P.E.: Petroleum ether, B: Benzene, E.A.: Ethyl acetate, E: Ethanol

Physical Properties & Other details of Isolated Compound

- Colour : Pale white solid
- TLC single spot.
  1. Chloroform: Methanol (7:3) - Rf value 0.45
  2. Acetic acid: Water (5:5) - Rf value 0.63
- Shows the presence of **3, 4', 5, 7- tetrahydroxy-6- methyl flavone.**

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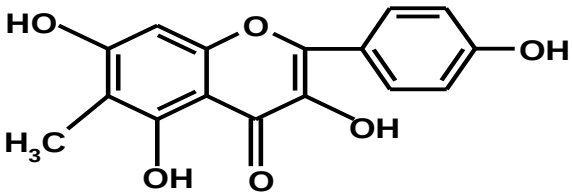

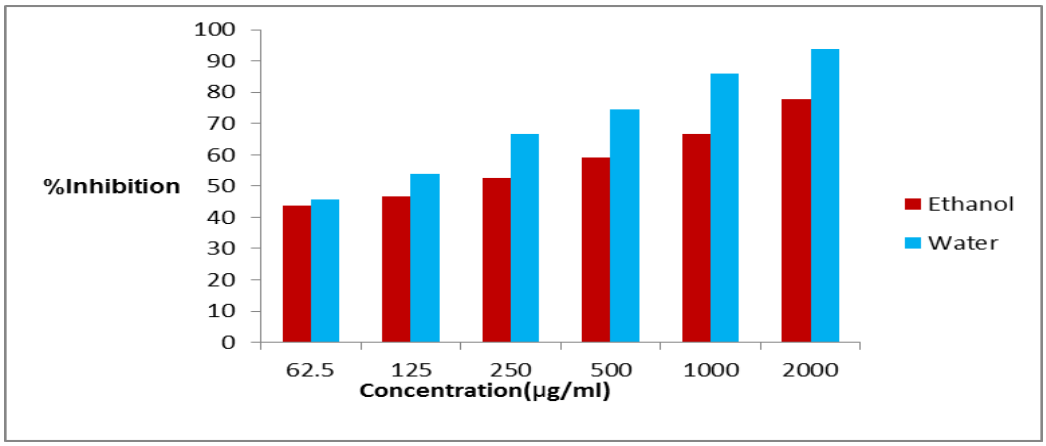

Figure 1: Comparison of % inhibition of extracts of *Ficus gibbosa* Blume by DPPH assay method

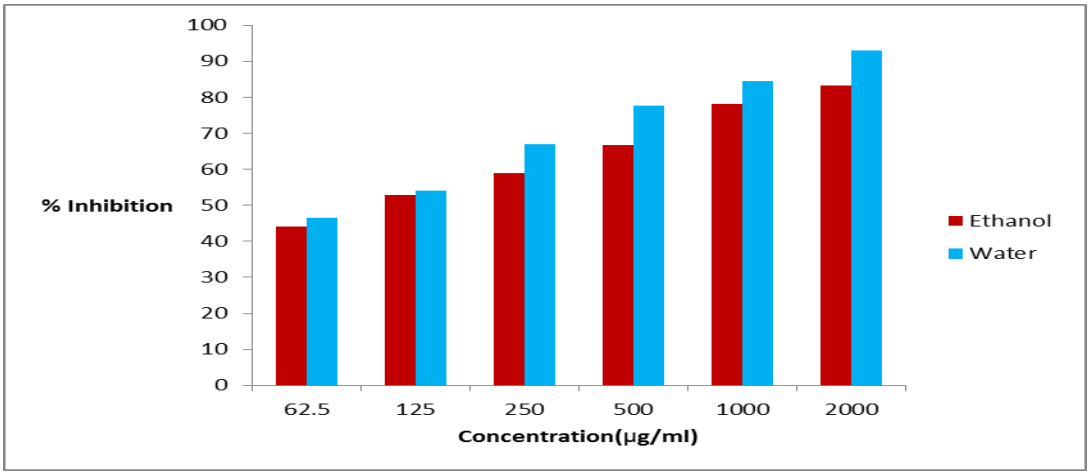

Figure 2: Comparison of % inhibition of extracts of *Ficus gibbosa* Blume by ABTS assay method

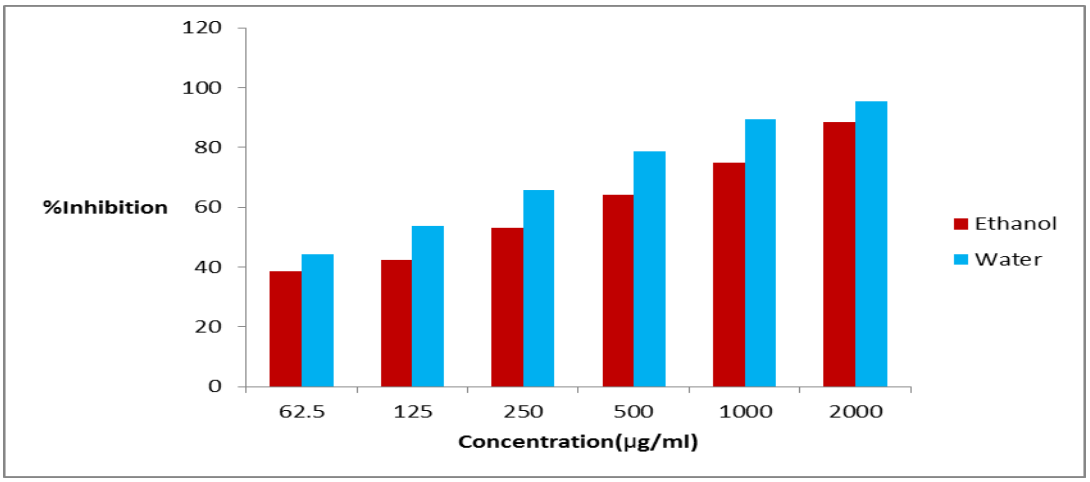

Figure 3: Comparison of % inhibition of extracts of *Ficus gibbosa* Blume by hydroxyl radical scavenging activity method

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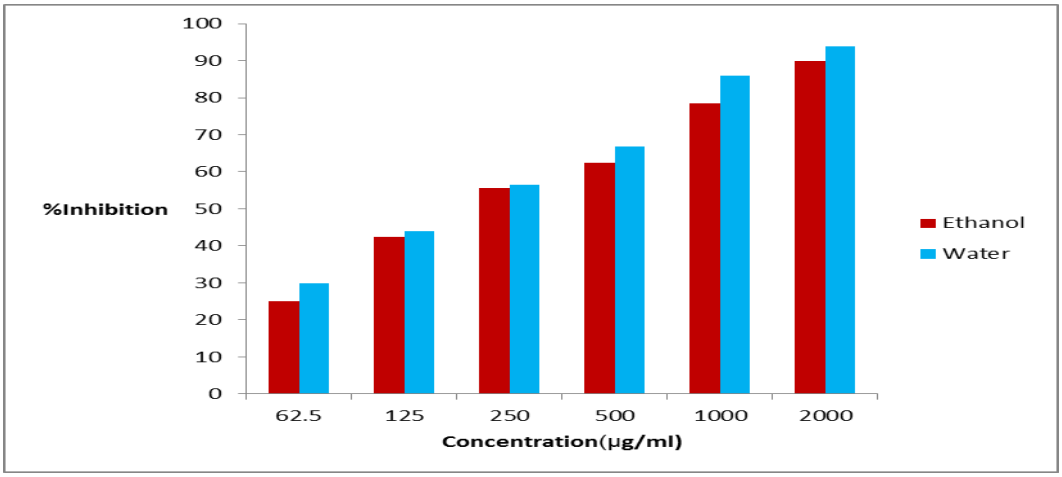

Figure 4: Comparison of % inhibition of extracts of *Ficus gibbosa* Blume by nitric oxide radical scavenging activity method

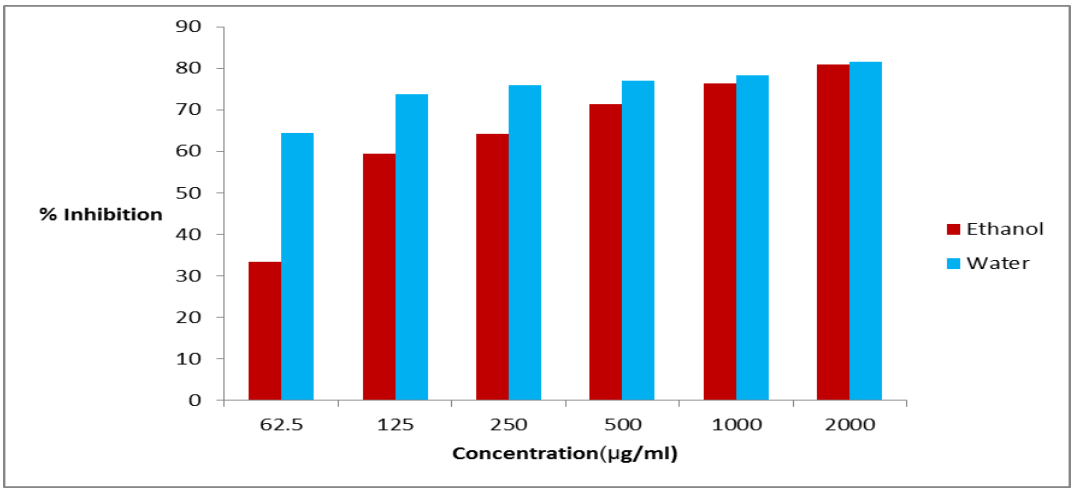

Figure 5: Comparison of % inhibition of extracts of *Ficus gibbosa* Blume by super oxide free radical scavenging activity method

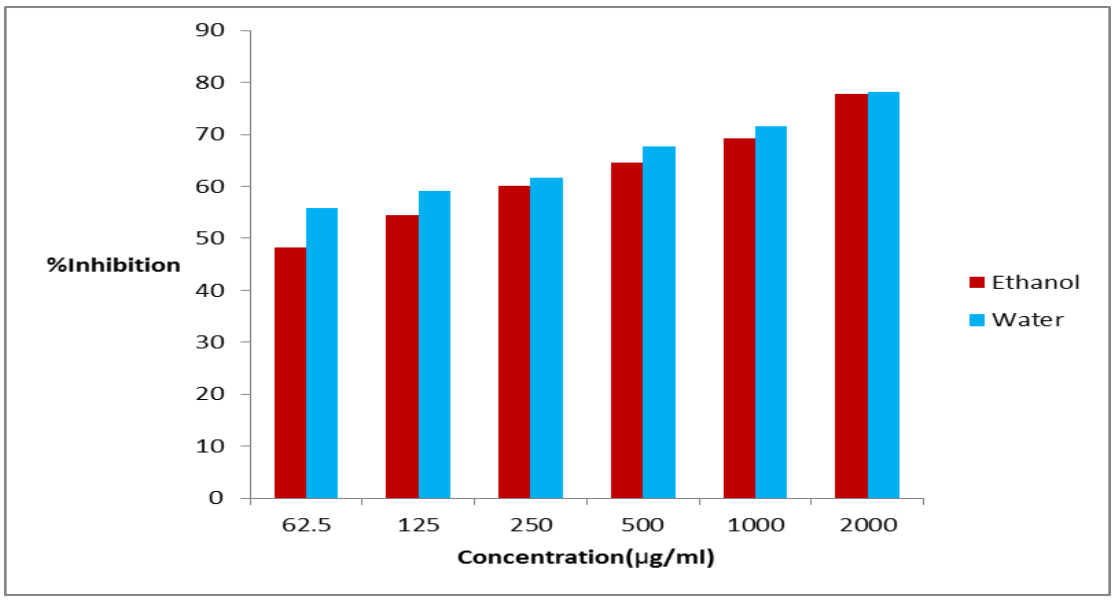

Figure 6: Comparison of % inhibition of extracts of *Ficus gibbosa* Blume by total antioxidant activity

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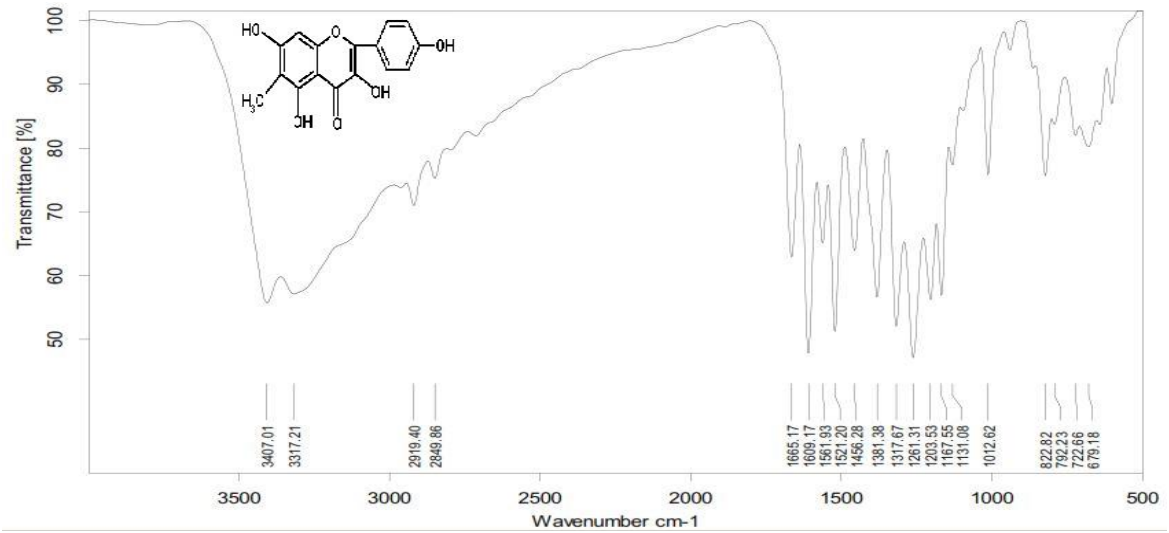

Figure 7: FT-IR Spectrum of isolated compound

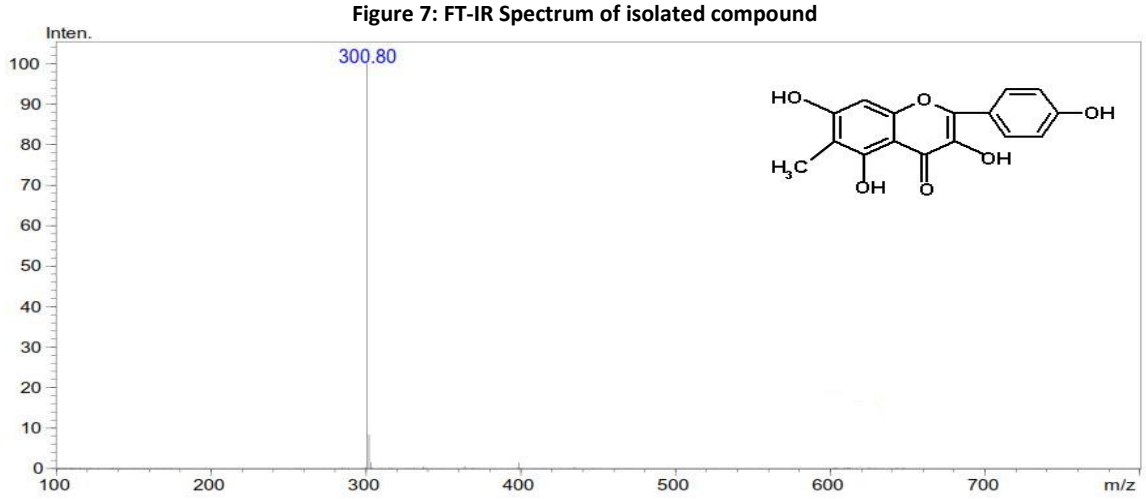

Figure 8: Mass spectrum of isolated compound

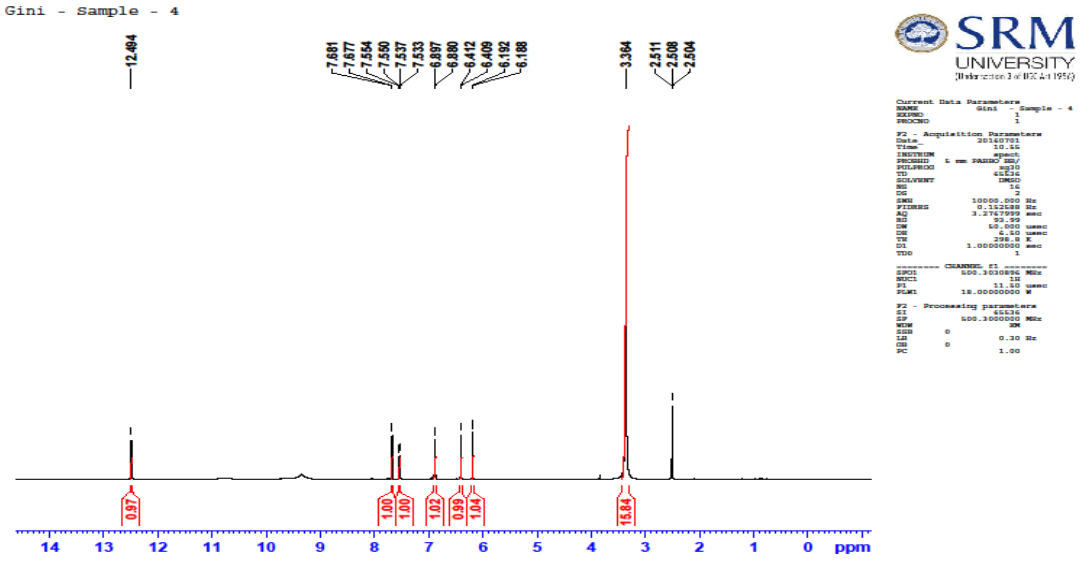

Figure 9: [1]HNMR Spectrum of isolated compound

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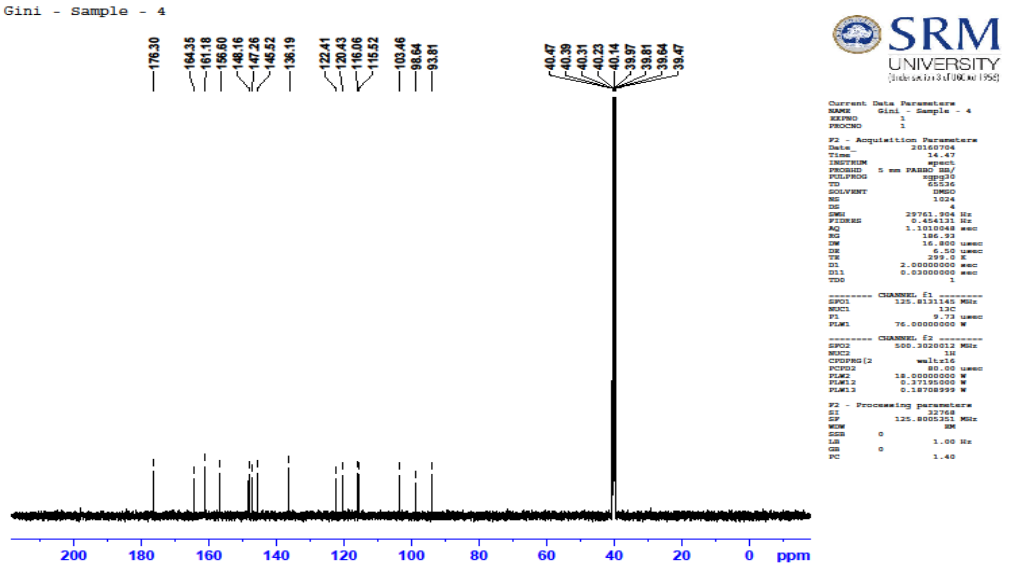

Figure 10: ^{13}C NMR Spectrum of isolated compound

Interpretation and observation of the isolated compound

The compound in its IR spectra exhibited absorption bands in the range 3407cm^{-1} & 3317cm^{-1} indicate the compound may contain hydroxylic group of phenolic in nature. Further, 2919cm^{-1} & 2849cm^{-1} represents that there will be an aliphatic – CH stretching. The prominent absorption at 1665cm^{-1} indicates C=O stretching vibrations of aromatic ketone. The bands at 1609cm^{-1}, 1561^{cm-1}, 1521cm^{-1} & 1456cm^{-1} represents the aromatic C=C stretching vibrations. The absorption band at 1261cm^{-1} represents C-CO-C stretching vibrations in the aromatic ring. The band at 1167cm^{-1} is due to –C-O-C stretching.

In the mass spectrum, the m/z value of the isolated compound of the molecular ion peak is found as 300.80(m^+) which corresponds to the molecular formula $C_{16}H_{12}O_6$.

In ^{1}HNMR, bands between δ6.1 – 6.8 shows aromaticity, δ7.5 – 7.6 shows presence of phenolic OH group, δ12.4 shows an alkyl group having substituents (R-CH_3), δ3.3 shows compound having ether linkage with an adjacent carbonyl group & δ2.50 – 2.51 shows the presence of aromatic ring having alkyl substituents (benzyl group).

In^{13} CNMR spectrum, a signal in the range δ100 – 160 shows Sp^2 hybridization (C=C), δ160 – 210 shows Sp^2 hybridization (ketones). A band in the range δ39 – 40 shows the presence of CH_3 (alkanes having Sp^3 hybridization) (Harborne 1975).

CONCLUSION

The present study concludes that the aqueous and ethanolic extract of the plant *Ficus gibbosa* Blume possess antioxidant activity. Among these the aqueous extract shows more antioxidant activity. So further studies were conducted for isolation and characterization of biologically active compounds by column chromatography. A compound 3, 4', 5, 7-tetrahydroxy-6-methyl flavone was isolated from the ethanolic extract(fraction 98-103). The traditional claims as well as literatures supported that the plant were being used for the treatment of skin diseases, ulcers, diabetes, inflammation and also it contains phytochemicals like flavonoids, alkaloids, glycosides, tannins and saponins. The drugs which possess antiulcer and antidiabetic activities may be antioxidant in nature. Since phenolic compounds are having good antioxidant activity, we concluded that the antioxidant activity may be due the presence of the compound 3, 4', 5, 7-tetrahydroxy-6-methyl flavone.

Abbreviations

DMSO	Dimethyl sulfoxide
TBA	Thiobarbituric acid
TCA	Total content of antioxidant
IC50	50% inhibitory concentration
NADH	Nicotinamide Adenine Dinucleotide – Hydrogen
EDTA	Ethylene diamine tetra acetic acid
SD	Standard deviation
Rf	Retention factor

REFERENCES

[1] *Gamble JS., Flora of the Presidency of Madras, Vol.I, Published under the authority of the Secretary of State for India in Council, 1935, pp.1000, 1365.*

[2] MdMizanur RM, MdSaifur R, Sayed KA, Syed MRD, Mahmodul H, Md Nurul A, Naim Uddin SM, Evaluation of cytotoxic and anthelmntic activities of the methanolic extract of Thevetia peruviana, *International Journal of Pharmacognosy and Phytochemical Research.*, 2013, 5(2), pp.92-95.

[3] OmPrakash, Amit K, Pawan K, Ajeet, Anticancer potential of plants and natural products : A review, *American Journal of Pharmacological Sciences,* 2013, 1(6), pp. 104 – 115.

[4] Agarwal SS, Paridhavi M, *Herbal drug technology*, University press Pvt. Ltd., Hyderabad, 2007, pp. 2.

[5] Kiranmayi G, Ramakrishnan G, Kothai R, Jaykar B, In-vitro anticanceractivity of methanolic extract of leaves of Argemone mexicana Linn., *International Journal of PharmTech Research,* 2011, 3(3), pp.1329-1333.

[6] Minu BP, Sivakumar T, Giriraj P, Preliminary phytochemical screening and anticancer potential of ethanolic extract of Madhuca neriifolia (Moon) H.J. Lam, *European Journal of Biomedical and Pharmaceutical Sciences,* 2014, 1(3), pp.542- 550.

[7] Minu BP, Sivakumar T, Kuppuswami S, Determination of antioxidant activity of the selected extracts of Mathuka neriifolia (Moon) H.J. Lam, isolation and characterization of phytoconstituents of the ethanol extract by column chromatography, *World Journal of Pharmaceutical Sciences,* 2015, 3(4), pp.716 – 728.

[8] Lobo V, Patil A, Phatak A, Chandra N, Free Radicals, antioxidants and functional foods: impact on human health, *Pharmacognosy Review,* 2010, 4(8), pp. 118-126.

[9] Badarinath AV, Mallikarjuna RK, Madhu Sudhana CC, Ramkanth S, Rajan TVS, Gnanaprakash K, A review on in-vitro antioxidant methods: comparisons, correlations and considerations, *International Journal of PharmTech Research*, 2010, 2(2), pp. 1276 – 1285.

[10] Rui HL, Potential Synergy of Phytochemicals in Cancer Prevention : Mechanism of Action, *The Journal of Nutrition,* https://www.ncbi.nlm.nih.gov, 2004, *pp. 18.*

[11] Tagne SR, ArmelHerve NK, FarahMukhtar, Medicinal Plants in Breast Cancer Therapy, *Journal of Diseases and Medicinal plants*, 2015, 1, pp. 19 – 23.

[12] Kuttan R, Kottarapat J, Vijayasteltar BL, Antitumour and cytotoxic activity of ginger essential oil (Zingiber officinale Roscoe), *International Journal of pharmacy And Pharmaceutical Sciences,* 2015, 7(8), pp. 341- 344.

[13] Seth AK, *Pharmaceutics-I,* Vikas Publications, Jalandhar, 2007, .2, pp.204.

[14] Treas GE, Evans WC, *Pharmacognosy*, 15th Edition, Sanders publications, London, 2002, pp.121-132,137-144.

[15] Kokkate CK, Purohit AP, Gokhale AP, *Pharmacognosy*, 46th edition, Nirali Prakashan., 2010, 1, pp.6.16-6.17.

[16] Halliwell B, Gutteridge JMC, Role of free radicals and catalytic metal ions in human disease: An overview, *Method in Enzymology,* 1990, 186, pp. 1-85.

[17] *Shirwaikar A, Kirti SP, Punitha ISR, In vitro antioxidant studies of Sphaeranthus indicus (Linn), Indian Journal of Experimental Biology, 2006, 44, pp. 993-996.*

[18] *Rao MNA, Elizabeth K, Oxygen Radical Scavenging Activity of Curcumin, International Journal of Pharmaceutics, 1990, 58, pp.237-240.*

[19] Valentao P, Fernandes E, Carvalho F, Andrade P B, Seabra RM, Bastos ML, Antioxidant activity of Centaurium erythraea infusion evidenced by its superoxide radical scavenging and xanthine oxidase inhibitory activity , *Journal of Agricultural and Food Chemistry*, 2001, 49, pp. 3476−3479.