Antioxidant activities of *ficus glomerata* (moraceae) leaf gall extracts

Ravi Shankara Birur Eshwarappa[1,2,3], Shanthi Iyer[4], Sundara Rajan Subaramaihha[2], S Austin Richard[5], Bhadrapura Lakkappa Dhananjaya[6]

[1]Department of Chemistry, School of Graduate Studies, [2]Research Unit in Vrukshayurveda, A Division of Centre for Advanced Studies in Biosciences, [4]Department of Life Science, Sri Bhagawan Mahaveer Jain College, Jain University, Bangalore, [3]Post Graduate Department of Studies and Research in Biotechnology, Kuvempu University, Shankarghatta, [5]Department of Biochemistry, University of Mysore, Mysore, [6]Toxinology/Toxicology and Drug Discovery Unit, Center for Emerging Technologies, Jain University, Kanakpura Taluk, Ramanagara, Karnataka, India

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ABSTRACT

An excess production or decreased scavenging of reactive oxygen species (ROS) has been implicated in the pathogenesis of diverse metabolic disorders such as diabetes, cancer, atherosclerosis and neurodegeneration. Hence the antioxidant therapy has gained an utmost importance in the treatment of such diseases linked to free radicals. The medicinal properties of plants have been investigated and explored for their potent antioxidant activities to counteract metabolic disorders. This research highlights the chemical composition and antioxidant potential of leaf gall extracts (aqueous and methanol) of *Ficus glomerata* (*F. glomerata*), which is extensively used in the preparation of traditional medications to treat various metabolic diseases. The presences of phenolics, flavonoids, phytosterols, terpenoids and reducing sugars were identified in both the extracts. In comparison to the aqueous extract, the methanol extract had the highest total phenolic and flavonoid content at 370 ± 3.2 mg of gallic acid equivalent per gram of dry weight (mg GAE/g dw) and 155 ± 3.2 mg of quercetin equivalent per gram of dry weight (mg QUE/g dw), respectively. The antioxidant activities of leaf gall extracts were examined using diphenylpicrylhydrazyl (DPPH), Nitric oxide scavenging, hydroxyl scavenging and ferric reducing power (FRAP) methods. In all the methods, the methanolic extract showed higher antioxidant potential than the aqueous extract. A higher content of both total phenolics and flavonoids were found in the methanolic extract and the significantly high antioxidant activity can be positively correlated to the high content of total polyphenols/flavonoids of the methanol extract. The results of this study confirm the folklore use of *F. glomerata* leaf gall extracts as a natural antioxidant and justify its ethnobotanical use. Further, the results of antioxidant properties encourage the use of *F. glomerata* leaf gall extracts for medicinal health, functional food and nutraceuticals applications. Future work will be interesting in knowing the chemical composition and better understand the mechanism of action of the antioxidants present for development as drug for its therapeutic application.

Key words: Antioxidant, drug, *Ficus glomerata*, gallic acid, galls, metabolic diseases, plants

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INTRODUCTION

Reactive oxygen species (ROS) are highly reactive ions and "free radicals" involving oxygen molecules. ROS include oxygen ions (O_2) free radicals (superoxide [O_2^-] and hydroxyl [OH] radicals), and peroxides [hydrogen peroxide (H_2O_2)]. They are the products of normal oxygen consuming metabolic process in the body. During times of cell stress ROS levels can greatly increase. Because of their highly reactive nature, ROS can cause oxidative damage to proteins, lipids, enzymes, and DNA molecules.[1] While our immune system possesses powerful scavenging potential to avoid excess ROS-induced cellular injury, but with ageing and under influence of external stresses, these mechanisms become inefficient leading to metabolic distress. Hence, free radicals are implicated in several metabolic diseases that include heart diseases, acquired immunodeficiency syndrome, diabetes mellitus, arthritis, cancer, ageing, liver disorder etc., the antioxidant therapy has gained an

Address for correspondence:
Dr. Bhadrapura Lakkappa Dhananjaya,
Jain University, Bangalore, Karnataka - 560 001, India.
E-mail: chandu_greeshma@rediffmail.com

utmost importance in the treatment of these diseases. The World Health Organization (WHO) estimates that 4 billion people (80% of the World's population) use herbal medicines in some aspects of primary healthcare and there is a growing tendency to "Go Natural".[2] In these aspects, all round the world, the medicinal properties of plants have been investigated and explored for their potent antioxidant activities to counteract metabolic disorders, that are with no side effects and with high economic viability.[3-5]

Ficus glomerata Roxb. *(*syn *Ficus racemosa)* is a species of plant in the Moraceae family. It is an evergreen, moderate to large sized deciduous tree found throughout greater part of India and is often cultivated in villages for its edible fruit. It is usually planted around temple and known to be a member of '*ksirivrkas*' that is plants that are beneficial for human health. It is commonly known as Cluster Fig tree, Indian Fig Tree or Goolar (Gular). *F. glomerata* has been valued in ayurveda and unani system of medication for possessing variety of therapeutic properties and widely used in folk medicine for the treatment of various diseases. It consists of gall-like excrescences formed by insects on the leaves, petioles and branches of the plant by the insect *Pauropsylla depressa* (Homoptera). These galls are, commonly known as *Karkatshringi* in Sanskrit, is extensively used in Ayurveda and Indian traditional medicine. *Karkatshringi* is used in indigenous system of medicine (Ayurveda, Unani and Siddha) as a remedy for cough, asthma, fever, respiratory and in liver disorders.[4,6-8] *Karkatshringi* also finds usage in the treatment of children's ear infections, suppress hemorrhage from gums and used to suppress bleeding from nose.[5,9,10] Hakims consider galls useful in pulmonary infections, diarrhea and vomiting.[11]

F. glomerata extracts have also been reported to possess significant medicinal and pharmacological properties like hepatoprotective, gastroprotective, hypoglycemic, anti-microbial and anti-ulcer activities.[12-17] Historically, galls are used in some of the ayurvedic formulations like '*Chvyanprash avaleha*', '*KumariAsava'*, '*KumariKalp'* etc., and prescribed for weakness as rejuvenating agent and tonic.[18] The use of leaf galls as a rejuvenator may be attributed to antioxidant property.[19] As the ethanomedical uses of galls of *F. glomerata* suggests that it might poses antioxidant activities. Therefore in the present study, the photochemical constituents and the antioxidant potential of leaf galls of *F. glomerata* are determined to exemplify its further potential development and use as drug against metabolic diseases.

MATERIALS AND METHODS

Materials

Folin-Ciocalteu (FC) reagent and quercetin were obtained from Qualigens, Mumbai, India. Ascorbic acid, Gallic acid, Quercetin, L-ascorbic acid, potassium thiocyanate, ethylene diamine tetra acetic acid (EDTA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-deoxyribose, thiobarbituric acid (TBA), sodium nitroprusside, N-(1-Naphthyl) ethylenediamine dihydrochloride, potassium hexacyanoferrate ($K_3Fe(CN)_6$), trichloroacetic acid (TCA) and ferric chloride were procured from SRL Chemicals, India. All the other reagents and solvents were of analytical grade.

Plant material

Gall induced leaves of *Ficus glomerata* were collected and authenticated By Dr. S. Sundararajan at Center for Advanced Studies in Biology, Jain University, Bangalore, India, and the voucher specimen (JU-RUV-74) was conserved in the herbarium. The galls were cleaned with distilled water, dried and crushed into fine powder by using electric grinder.

Preparation of extract

The coarsely powdered gall materials were sequentially extracted with methanol (Me-OH) and aqueous solvents in Soxhlet apparatus for 24 h. The extracts were evaporated to dryness under reduced pressure using a Rotavapor (BuchiFlawil, Switzerland) and a portion of the residue was used for the antioxidant assays.

Phytochemical analysis

Qualitative analysis

The preliminary qualitative phytochemical analyses of carbohydrates, saponins, alkaloids, flavonoids, fixed oils and fats, phenolic and tannins, glycosides, phytosterols and triterpenoids in the extracts were carried out using the standard methods as described earlier.[4,20-23]

Quantitative analysis

Determination of total phenolic content

The total phenolics were determined in the *F. glomerata* leaf gall extracts (Methanol and Aqueous) using FC reagent method, employing gallic acid as standard.[24] Briefly, 200 ml of both methanol and aqueous extracts (2 mg/ml) were made up to 3 ml with distilled water, then mixed thoroughly with 0.5 ml of FC reagent. After mixing for 3 min, 2 ml of 20% (w/v) sodium carbonate was added and allowed to stand for a further 60 min in the dark. The absorbance of the reaction mixtures was measured at 650 nm, and the results were expressed as mg of gallic acid equivalent (GAE)/g of dry weight.

Determination of total flavonoid content

Total flavonoid content of both crude extracts and essential oils was determined using the aluminium chloride (AlCl3) colorimetric method as described by Chang *et al.* (2002).[25] In brief, 50 μl of methanol and aqueous extracts (2 mg/ml) were made up to 1 ml with

methanol then mixed with 4 ml of distilled water and subsequently with 0.3 ml of 5% $NaNO_2$ solution. After 5 min of incubation, 0.3 ml of 10% AlCl3 solution was added and then allowed to stand for 6 min, followed by adding 2 ml of 1 M NaOH solution to the mixture. Then water was added to the mixture to bring the final volume to 10 ml and the mixture was allowed to stand for 15 min. The absorbance was measured at 510 nm. Total flavonoid content was calculated as quercetin from a calibration curve. The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5-100 mg ml^{-1} in methanol. The result was expressed as mg quercetin equivalent (QUE)/g of dry weight.

Evaluation of in vitro antioxidant and free radical scavenging potential of F. glomerata leaf gall extract

Antioxidant and free radical scavenging potential of *F. glomerata* leaf gall extracts (methanol and aqueous) is evaluated by using DPPH, FRAP, Nitric Oxide and hydroxyl radical assays.

DPPH free radical scavenging activity

Quantitative measurement of radical scavenging properties of *F. glomerata* leaf gall extracts was carried out according to the method of Blois, (1958).[26] Briefly, a 0.1 mM solution of 2, 2-diphenyl-1-picryl-hydrazyl (DPPH*) in methanol was prepared and 1 ml of this solution was added to 3 ml of the both methanol and aqueous extracts at different concentration (1-250 μg/ml). Ascorbic acid was used as a positive control. After incubation for 30 min in the dark, the discoloration was measured at 517 nm. Measurements were taken in triplicate. The capacity to scavenge the DPPH* radical was calculated and expressed as percent inhibition using the following equation:

$$I\ \% = (\text{Absorbance of control} - \text{Absorbance of test}/\text{Absorbance of control}) \times 100$$

The IC_{50} values (concentration of sample required to scavenge 50% of free radicals) were calculated from the regression equation prepared from the different concentrations of both the methanol and aqueous extracts.

Ferric reducing/antioxidant power activity (FRAP)

Ferric reducing/antioxidant power (FRAP) was determined following the method reported by Zhao *et al.* (2008).[27] *F. glomerata* leaf gall extracts (methanol and aqueous) at various concentrations (1-500 μg/ml) was mixed with 2.5 ml of 200 mM phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. Mixtures were incubated at 50ºC for 20 min, 2.5 ml of 10% trichloroacetic acid was added and the tubes were centrifuged at 10,000 rpm for 10 min. Five milliliters of the upper layer of the solution was mixed with 5.0 ml distilled water and 1 ml of 0.1% ferric chloride. The absorbance of the reaction mixtures was measured at 700 nm. Ascorbic acid was taken as standard and the final results were expressed as mg ascorbic acid equivalent/g of dry weight.

Hydroxyl Radical Scavenging activity

Quantitative measurement of hydroxyl radical scavenging properties of *F. glomerata* leaf gall extracts was carried out by measuring the competition between deoxyribose and the extract for hydroxyl radicals generated from the Fe^{3+}/Ascorbate/EDTA/H_2O_2 system.[28] *F. glomerata* leaf gall extracts (methanol and aqueous) at various concentrations (1-150 μg/ml) was mixed with the reaction mixture containing 0.1 ml of 3.0 mM deoxyribose, 0.5 ml of $FeCl_3$ (0.1 mM), 0.5 ml of EDTA (0.l mM), 0.5 ml of ascorbic acid (0.1 mM), 0.5 ml of H_2O_2 (1 mM) and 0.8 ml of phosphate buffer (20 mM, pH 7.4) and made up to final volume of 3.0 ml. The reaction mixture was incubated at 37ºC for 1 h. A 1 ml portion of the incubated mixture was mixed with 1 ml of 10% trichloroacetic acid and 1.0 ml of 0.5% thiobarbituric acid to develop pink chromogen that is measured at 532 nm. The hydroxyl radical scavenging capacity was calculated and expressed as % inhibition of deoxyribose degradation using following equation:

$$I\ \% = (\text{Absorbance of control-Absorbance of test}/\text{Absorbance of control}) \times 100$$

The IC50 values (concentration of sample required to scavenge 50% of free radicals) were calculated from the regression equation prepared from the different concentrations of both the methanol and aqueous extracts.

Nitric Oxide radical Assay

Quantitative measurement of nitric oxide radical scavenging properties of *F. glomerata* leaf gall extracts was carried out where at physiological pH, the nitric oxide generated from aqueous sodium nitroprusside solution, which interacts with oxygen to produce nitrite ions solution, is quantified by the Griess Illosvoy reaction.[29] *F. glomerata* leaf gall extracts (methanol and aqueous) at various concentrations (1150 μg/ml) was mixed with 2 ml of sodium Nitroprusside (10 mM) in standard phosphate buffer saline (50 mM, pH 7.4) and incubated at room temperature for 3 hours. After the incubation period, samples were diluted with of 0.5 ml of Griess reagents. The absorbance of the color developed during diazotization of Nitrite with sulphanilamide and its subsequent coupling with N-(1-Naphthyl) ethylenediamine dihydrochloride was measured at 550 nm on spectrophotometer. Ascorbic

acid was used as a standard. Nitric oxide radical scavenging capacity was calculated and expressed as percent inhibition using the following equation:

$$I\,\% = (\text{Absorbance of control} - \text{Absorbance of test}/\text{Absorbance of control}) \times 100$$

The IC_{50} values (concentration of sample required to scavenge 50% of free radicals) were calculated from the regression equation prepared from the different concentrations of both the methanol and aqueous extracts.

Statistical analysis

The experiments were carried out in triplicate and results are given as the mean ± standard deviation. The data in all the experiments were analyzed (Microsoft Excel 2007) for statistical significance using Students *t*-test and differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

As antioxidant therapy is gaining importance in the treatment of several metabolic diseases (diabetes mellitus, arthritis, cancer, ageing, liver disorder etc.), where free radicals are implicated. All over the world, scientifically developmental programs have aimed at investigating the medicinal properties of plants for their potent antioxidant properties.[3,4] In these lines, the antioxidant potential of aqueous and methanol extracts of leaf galls of *F. glomerata* is evaluated and its phytochemical constituents determined. In the phytochemical screening, the qualitative presence of phenolics, flavonoids, phytosterols, terpenoids and reducing sugars were identified in both the aqueous and methanol extracts of leaf galls of *F. glomerata* [Table 1]. The antioxidant activities of plant/herb extracts are often explained by their total phenolic and flavonoid contents.[30] The total amount of phenolic and flavonoid content of aqueous and methanol extracts of leaf galls of *F. glomerata* is presented in Table 2. The results obtained indicate that in comparison with the aqueous exact, the methanol extract had the highest total phenolic and flavonoid content at 370 ± 3.2 mg of GAE/g (dw) and 155 ± 3.2 mg of QUE/g (dw), respectively. These results show that the methanol extract possessed significant activity in releasing most of the secondary metabolites from leave galls of *F. glomerata*. This may be due to the fact that phenolic and flavonoid compounds are often extracted in higher amounts by using polar solvents such as aqueous methanol/ethanol.[31] Differences in the polarity of the extracting solvents could result in a wide variation in the polyphenolic and flavonoid contents of the extract.[4,32] Phenolic antioxidants are products of secondary metabolism in plants, and their antioxidant activity is mainly due to their redox properties and chemical structure, which can play an important role in chelating transitional metals and scavenging free radicals.[33] Similarly, the mechanisms of action of flavonoids are also through scavenging or chelating processes.[34] In addition, compounds such as flavonoids, which contain hydroxyl functional groups, are responsible for the antioxidant effects of plants.[35]

The higher amount of total phenolic and flavonoid content of leaf galls extracts of *F. glomerata* suggested that it possesses high antioxidant activity. DPPH is a stable free radical, which has been widely accepted as a tool for estimating free radical-scavenging activities of antioxidants.[36] The percentage inhibition of DPPH in the presence of aqueous and methanol extracts of *F. glomerata* are shown in Figure 1. The aqueous extract exhibited an inhibition of 59%, whereas methanol extract showed 39.45% inhibition at the concentration of 250 μg/ml. Therefore, in the present study the methanol extract exhibited higher DPPH scavenging activity, when compared to the aqueous leaf gall extract of *F. glomerata*. The IC_{50} values of aqueous and methanolic extract were found to be 315 ± 05 μg/ml and 220 ± 03 μg/ml, respectively [Table 3]. The results indicate that the methanol extract (220 ± 03 μg/ml) processes potent DPPH scavenging activities. These results indicate that the methanol extracts have a noticeable effect on scavenging free radicals and can be related to the high phenolic constituents present [Table 2]. Phenolic antioxidants are products of secondary metabolism in plants, and the antioxidant activity is mainly due to their redox properties and chemical structure, which can play an

Table 1: Phytochemical evaluation of extracts of *F. glomerata* insect gall

Phytochemical analysis	*F. glomerata*	
	Aqueous extract	*Methanolic extract*
Carbohydrates	+	+
Glycosides	-	-
Saponins	+	-
Alkaloids	+	-
Flavonoids	+	+
Phenolics and Tannins	-	-
Phytosterols and Triterpenoids	-	-
Fixed oils and fats	-	-

(+) Indicates present, (-) Indicates absent

Table 2: Total phenolic and total flavonoid content of *F. glomerata* leaf gall extracts

Gall extracts	Total phenolic [mg of GAE/g (dw)]	Total flavonoids [mg of QUE/g (dw)]
Aqueous extract	40±2.5	Negligible
Methanolic extract	370±3.2	155±3.2

GAE=Gallic acid equivalent; QUE=Quercetin equivalent

important role in chelating transitional metals, inhibiting lipoxygenase and scavenging free radicals.[37] Phenolic compounds are also effective hydrogen donors, which makes them good antioxidants.[38]

Nitric oxide is a reactive molecule and causes severe cytotoxicity in living cells, thus removing nitric oxide is of prime importance in antioxidant therapy. The percentage inhibition of nitric oxide scavenging potential of aqueous and methanol extracts of *F. glomerata* are shown in Figure 2. The methanolic extract of *F. glomerata* is found to be effective in scavenging nitric oxide radicals than the aqueous extract of *F. glomerata*. Therefore, in the present study the methanol extract exhibited higher nitric oxide scavenging activity, when compared to the aqueous leaf gall extract of *F. glomerata*. The scavenging activity in terms of IC_{50} value of aqueous and methanolic extracts of *F. glomerata* gall are calculated as 172.37 ± 02 μg/ml and 163.37 ± 04 μg/ml, respectively [Table 3]. These results indicate that the methanol extracts have a noticeable effect on scavenging free radicals and can be related to the high phenolic constituents present [Table 2].

Table 3: IC_{50} Values of *F. glomerata* gall extracts and standard ascorbic acid

Samples	IC_{50} Values (μg/ml)		
	DPPH assay	NO scavenging assay	Hydroxyl radical scavenging assay
F. glomerata (Aqueous extract)	315±05	172.37±02	328.2±06
F. glomerata (Methanolic extract)	220±03	163.37±04	142.53±02
Ascorbic Acid	17.58±04	18.81±02	15.67±01

Each value is expressed as mean±SD

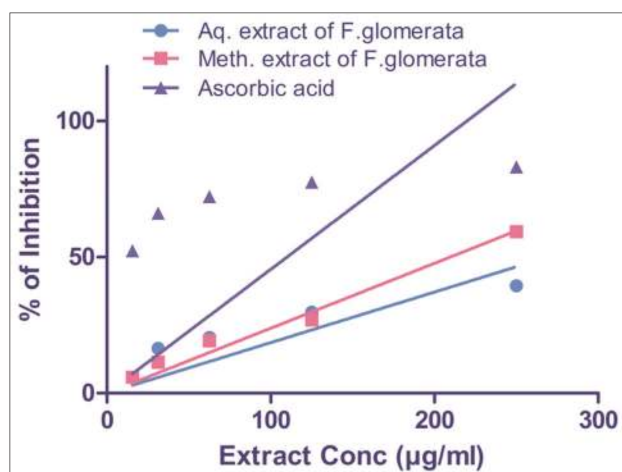

Figure 1: Free radical scavenging activity of aqueous and methanol extract of leaf galls of *F. glomerata*. ascorbic acid is included as positive control. Activity was measure by the scavenging of DPPH radicals and expressed as percent inhibition. Each value is expressed as the mean ± standard deviation

The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins. The hydroxyl radical scavenging activity of leaf galls of *F. glomerata* is as shown in Figure 3. The percentage of inhibition of aqueous and methanol extract of *F. glomerata* gall was found to be at 31.67% and 42.31%, respectively at a concentration of 125 μg/ml. Therefore, the methanol extract exhibited higher hydroxyl radical scavenging activity, when compared to the aqueous leaf gall extract of *F. glomerata*. The IC_{50} values of aqueous and methanolic extract were found to be 328.2 ± 06 μg/ml and 142.53 ± 02 μg/ml, respectively [Table 3]. The results indicate that the methanol extract (142.53 ± 02 μg/ml) processes potent hydroxyl radical scavenging activities which can be related to the high phenolic constituents present [Table 2].

The reducing capacity of a compounds or extracts may serve as a significant indicator of its potential antioxidant activity. The presence of a reductant, such as the antioxidant substances in plant extracts, causes the reduction of Fe^{3+} ferricyanide complex to the ferrous form, Fe^{2+}. The reduction capabilities of aqueous and methanol extracts of leaf galls of *F. glomerata* in comparison with standard ascorbic acid is indicated as in Figure 4. In comparison with the aqueous extract, the methanol extract had better reducing power at a concentration of 500 μg/ml [Figure 4]. The ferric reducing power of leaf galls of *F. glomerata* may be attributed to the high phenolic and flavonoid contents of the extracts [Table 2]. The ability to reduce Fe (III) may be attributed to the hydrogen donation from phenolic compound, which is related to the presence of a reducing agent.[39,40] In addition, the number and position of hydroxyl group of phenolic compounds also govern their antioxidant activity.[38]

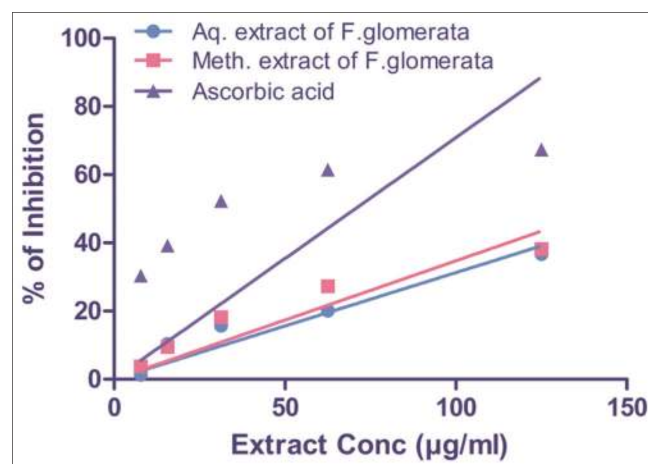

Figure 2: Nitric oxide scavenging activity of aqueous and methanol extract of leaf galls of *F. glomerata*. Ascorbic acid is included as positive control. Activity was measure and expressed as percent inhibition. Each value is expressed as the mean ± standard deviation

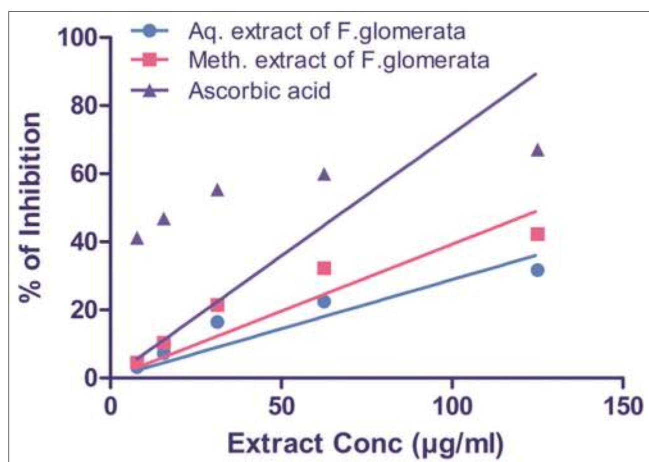

Figure 3: Hydroxyl radical scavenging activity of aqueous and methanol extract of leaf gall of *F. glomerata*. Ascorbic acid is included as positive control. Activity was measure and expressed as percent inhibition. Each value is expressed as the mean ± standard deviation

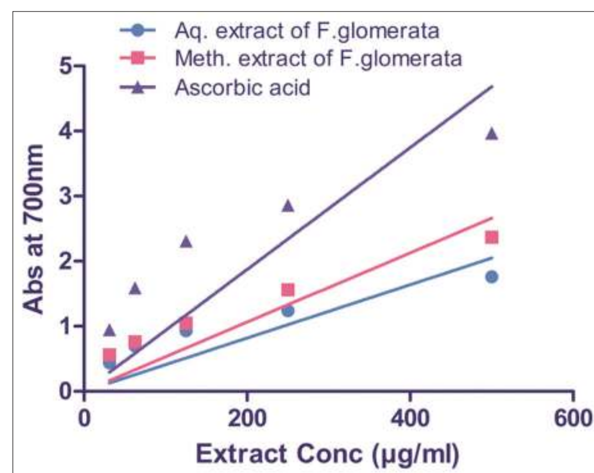

Figure 4: Ferric reducing power of aqueous and methanol extract of leaf gall of *F. glomerata*. Ascorbic acid is included as positive control. Activity is expressed at absorbance at 700 nm. Each value is expressed as the mean ± standard deviation

As exemplified earlier, the antioxidant activities of plant/herb extracts are often explained by their total phenolic and flavonoid contents. In this study, it was observed that there was a strong correlation of antioxidant activities with that of total phenolic and flavonoid content in the leaf gall extracts of *F. glomerata*. *Ficus* species are reported to be very rich in tannins, flavonoids, essential oils, anthocyanins and others phenolic constituents.[41,42] The results given in this investigation showed that the phenolic and flavonoid content was higher in polar extracts (methanol) and subsequently its higher antioxidant potential. Therefore, it seems clear that the presence of polar phenolics is fundamental in the evaluation of free radical-scavenging activity.[43] The activity of antioxidant has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging.[44] These observations confirm the folklore use of *F. glomerata* leaf gall extracts as a natural antioxidant and justify the ethnobotanical approach in the search for novel bioactive compounds.

CONCLUSION

The findings of this study support the view that the extracts obtained using a high polarity solvent (methanol) were considerably more effective radical scavengers. Further, it was observed that there was a strong correlation of higher antioxidant activities with that of high total phenolic and flavonoid content in the methanolic leaf gall extracts of *F. glomerata*. The results of this study confirm the folklore use of *F. glomerata* leaf gall extracts as a natural antioxidant and justify the ethnobotanical approach in the search for novel bioactive compounds. Further, the results support the use of gall extracts as promising sources of potential antioxidants that may be effective as preventive agents in the pathogenesis of some metabolic diseases. Therefore, the results encourage the use of *F. glomerata* leaf gall extracts for medicinal health, functional food and nutraceuticals applications, due to their antioxidant properties. Future work will be interesting to know the chemical composition and better understand the mechanism of action of the antioxidants present in the extract for development as drug for therapeutic application.

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