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In vitro Antioxidant assay of *Ficus microcarpa* Linn. Leaf extract

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Abstract: *Ficus microcarpa Linn. of Moraceae is a tall tree characterized by rust-colored aerial roots, lanceolate stipules, glabrous petiole, narrow elliptical leaf blade with entire margin and male gall and female flowers are arranged within same fig, the hypanthodium inflorescence.*
The present investigation is aimed to evaluate the antioxidant activity of methanol extract of leaves of F. microcarpa in in vitro condition. The results indicated that the extracts possess some anti-oxidant constituents. The methanol extracts of leaves of F. microcarpa contained phenolics in the concentration range of 20.50 mgTAE/g to 36.75 mg TAE/g. The DPPH scavenging activity of the methanol extracts (0.5 -1.5 mg/ml) exhibited concentration-dependent free radical scavenging activity. The extracts (0.5-1.5 mg/ml) and the standard antioxidant n-propyl gallate (0.003-0.03 mg/ml) caused a concentration – dependent reduction of Fe3+ to Fe2+. The extracts (0.5 – 1.5 mg/ml) and n-propyl gallate (0.003 - 0.03 mg/ml) caused a concentration-dependent inhibition of linoleic acid autoxidation. The per cent inhibition of lipid peroxidation by methanol extract increased with increase in concentration of methanol extract. At concentration of 1.5 mg/ml concentration the methanol extract caused maximum inhibition of lipid peroxidation (85.9±0.21%).
Key words: *Ficus microcarpa, Methanol extract, Antioxidant, DPPH, TAE, n-Propyl gallate*

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I. Introduction

Oxidation process is one of the important routes for production of free radicals and these high energy molecules may abruptly interfere with the normal metabolic activities of the body causing immense damage to the normal tissues [1]. There is a close relationship between diabetes and oxidative stress and it has been observed that the free radicals are produced in the form of ROS (reactive oxygen species) which cause mitochondrial DNA mutation thus resulting in hypoglycemic memory [2]. Free radicals generated during diabetes interfere with vital organ tissues and may lead to cardiovascular complications, diabetic nephropathy, diabetic retinopathy, erectile dysfunction and diabetic neuropathy [3]. Several plants are known for their efficacy to overcome these complications by enhancing the *in vivo* anti oxidant defense and provide protection against oxidative tissue damage [4]. The SOD (Superoxide dismutase), CAT (Catalase), Vitamin E and C are some of the antioxidants which provide protection to the diabetic tissues [5] and their level of defense can be assessed by measuring the MDA concentration which is the end product of lipid peroxidation [6].

Metabolic processes in the body generate highly reactive species, known as free radicals, which injure cellular molecules. Free radicals are highly reactive atomic or molecular species that contain an unpaired electron [7] which contributes to their high reactivity. Free radicals react quickly with the nearest stable molecule to capture the electron they need to gain stability. The „injured" molecule loses its electron, becoming a free radical itself. They can damage vital cellular components like nucleic acids, cell membranes and mitochondria, resulting in subsequent cell death. As all aerobic organisms utilize oxygen during cellular respiration and normal metabolism, the generation of free radicals by biochemical cellular reactions and from the mitochondrial electron transport chain is inevitable [8]. The free radicals include reactive oxygen and nitrogen species such as superoxide ($O2^{\bullet-}$), hydroxyl ($OH^{\bullet}$)-, peroxyl (ROO-), peroxinitrite ($\cdot ONOO^{-}$) and nitric oxide ($NO\cdot$) radicals. All these are produced through oxidative processes within the mammalian body [9]. They may also be generated through environmental pollutants such as cigarette smoke, automobile exhaust fumes, radiation, air pollution and pesticides [10, 11]. To protect the cells and organ systems of the body against reactive oxygen and nitrogen species, humans have evolved a highly sophisticated and complex antioxidant protection system, that functions interactively and synergistically to neutralize free radicals. These antioxidants are capable of stabilizing or deactivating, free radicals before they attack cells. Antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase destroy toxic peroxides. In addition to antioxidant enzymes, non-enzymatic molecules play important roles in antioxidant defence systems. These

non- enzymatic molecules are of an exogenous nature and are obtained from foods. They include *α*-tocopherol, *β*-carotene, and ascorbic acid, and such micronutrient elements as zinc and selenium [10]. Normally, there is a balance between free radical generation and scavenging [12]. Oxidative stress results from an imbalance between excessive generation of oxidant compounds and insufficient anti-oxidant defence mechanisms [1]. When the natural antioxidant mammalian mechanism becomes inadequate, the excess of free radicals can damage both the structure and function of cell membranes in a chain reaction leading to degenerative diseases and conditions such as Alzheimer‟s disease, cataracts, acute liver toxicity, arteriosclerosis, nephritis, diabetes mellitus, rheumatism and DNA damage which can lead to carcinogenesis [9].

All cells in eukaryotic organisms contain powerful antioxidant enzymes. Endogenous antioxidants made in the body are believed to be more potent in preventing free radical damage than exogenous antioxidants. The major classes of endogenous antioxidant enzymes are the superoxide dismutases, catalases and glutathione peroxidases [1], *α*-lipoic acid and coenzyme Q10. In addition, there are numerous specialized antioxidant enzymes reacting with and, in general, detoxifying oxidant compounds.

Superoxide dismutases are present in almost all aerobic cells and in extracellular fluids [13]. Superoxide dismutase enzymes contain metal ion cofactors that, depending on the isozyme, can be copper, zinc, manganese or iron. They catalyse the breakdown of the superoxide anion into oxygen and hydrogen peroxide [14]. Catalases, on the other hand, are enzymes that catalyse the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor [15].

Ficus microcarpa Linn. of Moraceae is a tall tree characterized by rust-colored aerial roots, lanceolate stipules, glabrous petiole, narrow elliptical leaf blade with entire margin, apex obtuse, fig (hypanthodium) axillary yellow to slightly red, depressed globose; male gall and female flowers within same fig, male flowers scattered, sessile or pedicellate, gall and female flowers broadly ovate with lateral style, stigma short and clavate.

The bark, leaves and aerial roots of *F. microcarpa* are known to possess antioxidant, antimicrobial, antisecretary, antiulcer, anti-inflammatory, antihistamine, antidiabetic and antihyperlipidemic activities [16-19].

The phytochemical constituents so far isolated from the plant are (2S, 3S, 4R) -2-[(2'R) -2' -hydroxypentracosanoylamino]- heptadecane -1, 3, 4-triol, 12, 20 (30) -ursa-dien-3alpha-ol, epifriedelanol, alpha-amyrin acetate, beta-sitosterol, betadaucosterol, hexacosanoic acid, heneicosanoic acid [20], ficuscarpanoside A, guaiacylglycerol 9-*O*-β-Dglucopyranoside, erythro-guaiacylglycerol 9-*O*-β-Dglucopyranoside, guaiacylglycerol, erythro-guaiacylglycerol, 4-methoxy guaiacylglycerol 7-*O*-β-D-glucopyranoside, and 3-(4-hydroxy-3-methoxy phenyl) propan-1,2-diol, ficuscarpanoside B, (7*E*,9*Z*)-dihydrophaseic acid 3-*O*-β-dglucopyranoside, ficuscarpanic acid, 2,2'-dihydroxyl ether, [(7*S*,8*R*)-syringoylglycerol, (7*S*,8*R*)-syringoylglycerol-7-*O*-β- d-glucopyranoside and icariside D2 from the aerial roots.

The phytochemistry of the bark of *Ficus microcarpa* shows presence of triterpenoids, fatty alcohol, steroids, coumarin, flavane, 4-hydroxybenzoates, and a carotenoid-like compound [21]. The antioxidant and antibacterial potential plant phenolics such as protocatechuic acid, catechol, *p*-vinylguaiacol, syringol, *p*-propylphenol, vanillin, and syringaldehyde have been identified in bark of *F. microcarpa* [22]. The latex of the plant has been reported to contain chitinase, giving rise to antifungal properties [23]. Liu *et al*., [24] reported antitussive and expectorant potential of *F. microcarpa*.

In the present investigation antioxidant activity of methanol extract of leaves of *F. microcarpa* was assayed *in vitro.*

II. Materials and Methods

Preparation of methanol extract

Freshly harvested leaves of *Ficus microcarpa* were washed under running tap water, blotted with filter paper and dried in shade at room temperature for 7 days. The dried leaves were powdered using a mixture grinder and stored in air-tight container for future use. Methanol was used for preparation of solvent extracts. The dried sample was soaked separately with methanol under reflux condition for the solvent extract preparation. About 1 gm of the dried sample was added into the test tubes containing 5 ml of solvent and was extracted at room temperature. The sample was homogenized with extraction buffer. The supernatant was collected after three rounds of extraction. The solvent was evaporated under reduced pressure in a rotary evaporator at 40^0C. To this thick paste colloidal silicon dioxide was added and dried in vacuum tube dryer. The methanol extracts of leaves thus obtained were then stored separately in deep freezer at -20^0C until further test.

Antioxidant assay

***In vitro* qualitative DPPH test**

The qualitative test for antioxidant activity was performed using the rapid DPPH radical scavenging assay [25] (Cuendet *et al.,* 1997). 10 µl of the leaf extracts and inflorescence extracts were applied on silica gel

plates 60 F254 (Merck, 0.25 mm thick) and allowed to dry completely. The plate was then sprayed with a solution of 2% DPPH in methanol. A pale yellow to white spot over a purple background indicated a radical scavenging activity of the particular extract.

Quantitative antioxidant assays of extracts

Reducing power assay

Reducing activity of all the extracts was assayed as follows. Different concentrations (0.25 – 2.0 mg/ml) of the extracts as well as the standard drug *n*-propyl gallate (3.75 – 30 µg/ml) were prepared in aqueous methanol (50% v/v) and 1 ml each taken into test tubes in triplicates. To the test tubes, 2.5 ml of sodium phosphate buffer (pH=7) and 2.5 ml of 1% potassium ferric cyanide solution was added. The contents were mixed well and incubated at 50 °C for 20 minutes. After incubation, 2.5 ml of 10% trichloroacetic acid was added and the mixture centrifuged at 3000 revolutions per minute for 10 minutes. After centrifugation 2.5 ml of supernatant was added to 2.5 ml of distilled water. To this about 1 ml of 0.1% ferric chloride was added. The absorbance was then taken at 700 nm. A graph of absorbance was then plotted against the concentration of the extracts. Increase in absorbance indicates higher reducing power of the extract.

Scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical assay

The free radical scavenging activity was determined as follows. 1 ml each of the extracts (0.25, 0.5, 1.0 and 2 mg/ml in methanol) was added to 3 ml methanolic solution of DPPH solution (20 mg/l) in a test tube. The reaction mixture was kept at 25°C for 30 mins. The absorbance of the residual DPPH was determined at 517 nm in a spectrophotometer (Cecil CE 7200 spectrophotometer, Cecil instrument limited, Milton Technical Centre, England). One milliliter (1 ml) methanol (50%) was added to 3 ml DPPH solution, incubated at 25 °C for 30 minutes and used as control. *n*-propyl gallate (3.75-30 µg/l) was used as a standard free radical scavenger. The absorbance decreases with increasing free radical scavenging ability. Results were expressed as percentages of blank (100%). The concentration required to cause a 50% decrease in the absorbance was calculated (EC50). Each test was carried out using three replicates. The % DPPH scavenging effect (% of control) of the antioxidant was calculated as follows:

$$\% \text{ DPPH scavenging effects} = (Ac - At)/Ac \times 100$$

Where
Ac = Absorbance of the control
At = Absorbance of the test drug/ extracts

Total antioxidant capacity assay

The assay is based on the reduction of molybdenum, Mo +6 to Mo +5, by the extracts and subsequent formation of a green phosphate-molybdate (Mo +5) complex at acidic pH [26]. Test tubes containing l ml each of the extracts (0.25-2 mg/ml) and 3 ml of the reagent solution (0.6 M sulphuric acid, 28 mM disodium phosphate and 4 mM ammonium molybdate) were incubated at 95^0C for 90 minutes. After the mixture had cooled to room temperature, the absorbance of the solution was measured at 695 nm. Four concentrations of ascorbic acid (0.025, 0.05, 0.1 and 0.2 mg/ml) was used to construct a calibration curve. A blank solution was prepared by adding every other solution but without extract or standard drug. The antioxidant capacity was expressed as mg of ascorbic acid equivalent (AAE) per g of extract. This procedure was used for all the extracts.

Determination of total phenolic content

The presence of phenol in the extract was determined qualitatively using ferric chloride test. An intense positive colouration indicating the presence of phenols led to further quantification of total soluble phenols in the extract. The total phenol in the extract was determined by spectrophotometric assay using the Folin-Ciocalteu‟s reagent as described by Singleton *et al.,* (1999) [27] using tannic acid as standard.

1ml of the extracts (0.25-2 mg/ml) in distilled water was added to 1 ml Folin-Ciocalteu‟s reagent in a test tube. The content of the test tube was mixed and allowed to stand for five minutes at 25^0 C in an incubator. 1 ml of sodium bicarbonate solution (2%) was added to the mixture. The reaction mixture was allowed to stand for 2 hours with shaking at 25^0C in an incubator. The mixture was then centrifuged at 3000 rpm for 10 minutes and absorbance of the supernatant determined at 760 nm. Three replicates were prepared for each concentration of tannic acid and extracts. 1 ml distilled water was added to 1 ml Folin-Ciocalteu‟s reagent processed in the same way as the test drugs and used as blank. Tannic acid was used as reference. Four concentrations of tannic acid (0.025, 0.05, 0.1, 0.2 mg/ml) were used to construct a calibration curve and the total phenols expressed as mg of tannic acid equivalents (TAE)/g of extract.

This method depends on the reduction of Folin-Ciocalteau reagent by phenols to a mixture of blue oxides which have a maximal absorption in the region of 760 nm. The reaction equation is as follows:
Folin: Mo^{+6} (yellow) + è (from antioxidant) → Mo^{+5} (blue)
Where the oxidizing reagent is a molybdophosphotungstic heteropolyacid comprised of $3H_2O{\cdot}P_2O_5{\cdot}13WO_3{\cdot}5MoO_3{\cdot}10H_2O$, in which the hypothesized active centre is Mo^{+6}.

Linoleic acid auto-oxidation assay

The extracts (0.25-2 mg/ml) in absolute alcohol were compared with *n*-propyl gallate (3.75-30 µgm/l) in absolute alcohol as a reference antioxidant. 2 ml of the extract, 2 ml of 2.5% linoleic acid in absolute ethanol, 4 ml of 0.05 M phosphate buffer (pH =7) and 1.9 ml of distilled water were put into test tubes with a screw cap and placed in an oven at 40^0C in the dark for 7 days. After the seven day period, 2 ml each of the extracts and standard drug was added to 20 % aqueous trichloroactetic acid solution and 1 ml of 0.6 % aqueous thiobarbituric acid solution. This mixture was placed in boiling water bath for 10 minutes and after cooling, was centrifuged at 3000 rpm for 10 minutes. The absorbance of the supernatant was measured 535 nm. Each test was carried out in three replicates. Percentage inhibition of lipid peroxidation by the test drugs was assessed by comparing the absorbance of the drug test with that of the control (linoleic acid mixture without any drug). Data was presented as percentage inhibition of lipid peroxidation against concentration. The % inhibition of linoleic acid autoxidation was calculated as follows:

$$\% \text{ inhibition} = \left(1 - \frac{D - D_O}{C_O - C} \right)$$

Where
Co= (Full reaction mixture) is the degree of lipid peroxidation in the absence of antioxidant
C= is the underlying lipid peroxidation before the initiation of lipid peroxidation
D= is any absorbance produced by the extract/ linoleic acid mixture
Do= is the absorbance produced by the extract alone

III. Results

Antioxidant Activity

Qualitative DPPH test

The methanol extracts of *Ficus microcarpa* caused bleaching of the purple DPPH radical, thus giving pale spots over a purple background. This indicates that the extracts possess some anti-oxidant constituents.

Quantitative antioxidant assay of extracts

The methods used to determine quantitative antioxidant activity of the methanol extracts of leaves of *F. microcarpa* included total phenolic content, total anti-oxidant capacity, reducing power, DPPH radical scavenging activity and linoleic acid autoxidation assays.

1. The total phenolic content of the extracts was determined using the Folin- Ciocalteu's reagent and tannic acid as standard. The total phenolic content of the extracts was expressed as mg of tannic acid equivalents (TAE) per g of extract. The four different concentrations of methanol extracts were used for quantitative assay. The total phenolic content in methanol extracts has been presented in Table-1 and Fig-1.

Table-1: Total Phenolic content of leaf extracts of *F. microcarpa*

Concentration of methanol extract (mg/ml)	***F. microcarpa***
	Mean mgTAE/g ±SE
1.0	**20.50 ±0.51**
1.5	**25.50 ±0.41**
2.0	**30.65±0.47**
2.5	**36.75±0.38**

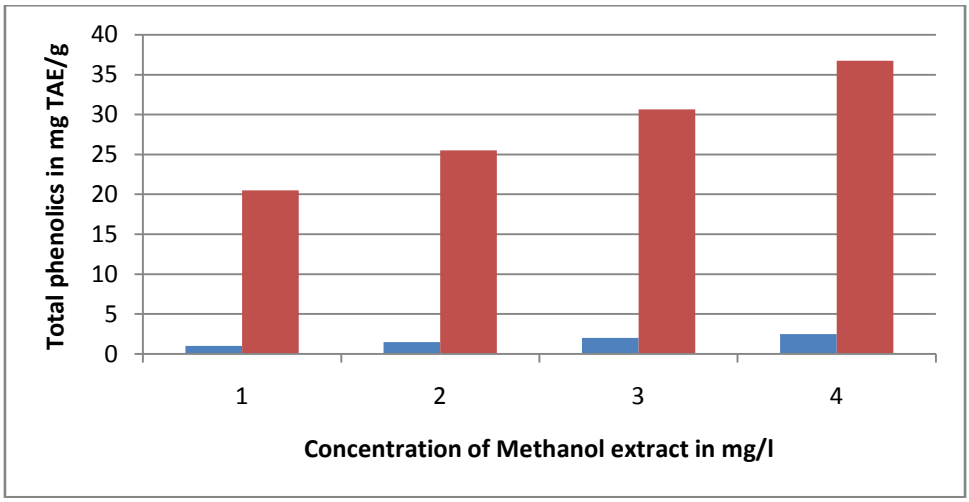

Fig-1: Total phenolics in the methanol extract of *F. microcarpa* (mg TAE/g)

From the results it is clear that the methanol extracts of leaves of *F. microcarpa* contained phenolics in the concentration range of 20.50 mgTAE/g to 36.75 mg TAE/g depending on the concentration of methanol extracts.

2. Free Radical Scavenging Activity

The results of the free radical scavenging potential of methanol extracts of *F. microcarpa* using DPPH free radical scavenging method are depicted in Table-2 and Fig-2. The DPPH scavenging activity of the methanol extracts (0.5 -1.5 mg/ml) exhibited concentration-dependent free radical scavenging activity (Table- 2; Fig-2). The methanol extract of leaves of *F. microcarpa* exhibited highest free radical scavenging activity.

Table-2: DPPH scavenging activity of methanol extracts of *F. microcarpa*

Dose of extract in mg/ml	***F. microcarpa***
	IC50 (µg/ml) ±SEM
0.5	**78.5 ±0.025**
1.0	**230.5 ±0.095**
1.5	**560.7 ±0.087**

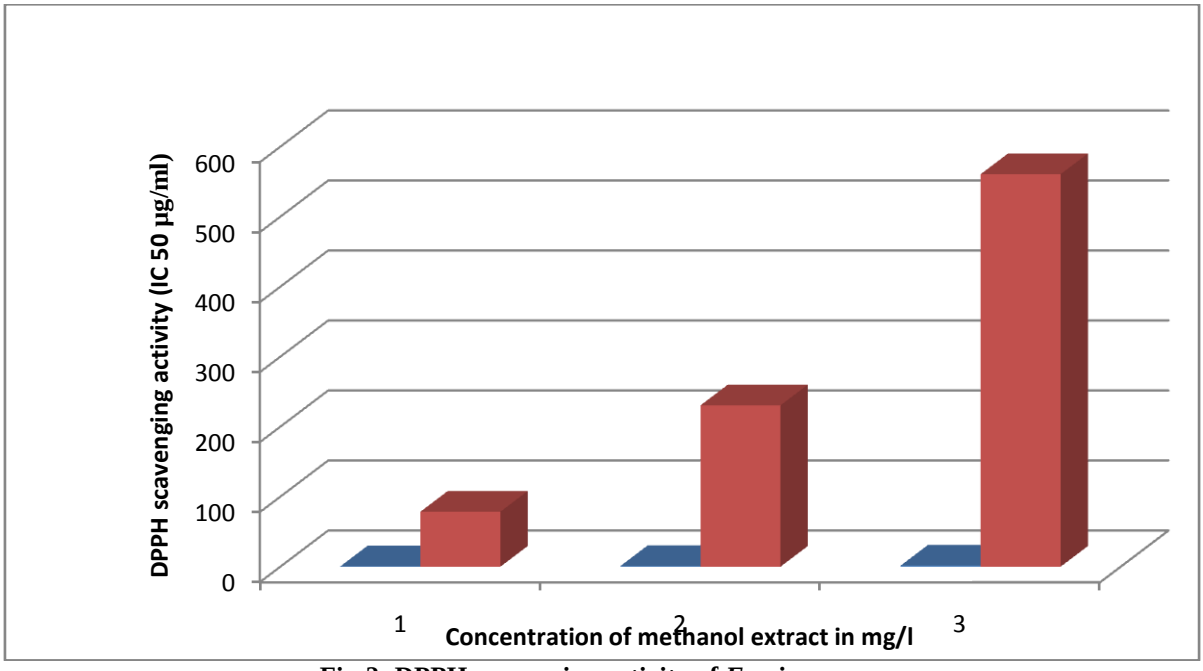

Fig-2: DPPH scavenging activity of *F. microcarpa*

3. Reducing power

The extracts (0.5-1.5 mg/ml) and the standard antioxidant *n*-propyl gallate (0.003-0.03 mg/ml) caused a concentration – dependent reduction of Fe3+ to Fe2+. From the IC50 values (Table-3; Fig-3), the methanol extract showed the highest reducing power.

Table-3: Reducing power of methanol extracts of *F. microcarpa*

Dose of extract in mg/ml	*F. microcarpa*
	IC50 (µg/ml) ±SEM
0.5	187 ±0.015
1.0	215.17 ±0.017
1.5	567.725±0.025
n-PG	70.25±0.003

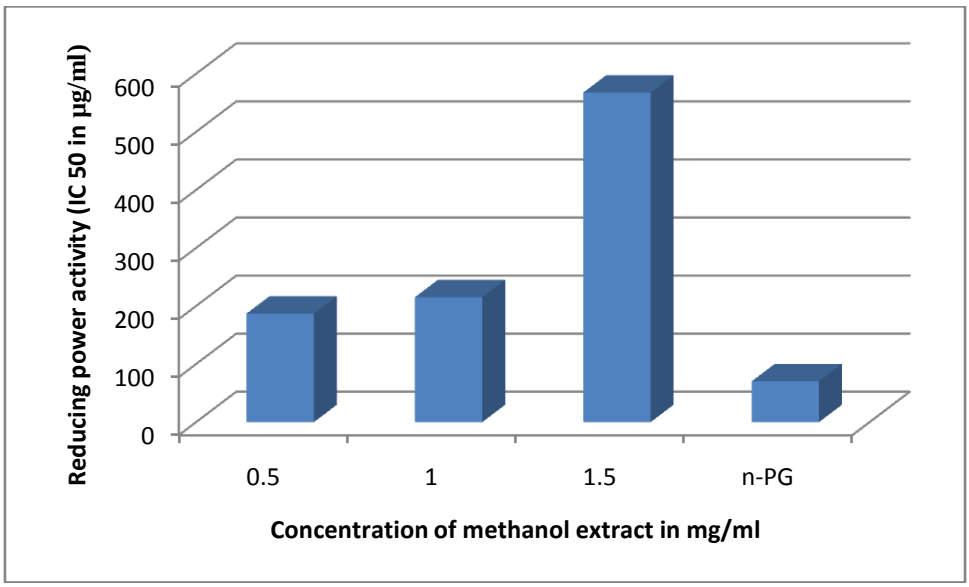

Fig-3: Reducing power activity in terms of IC 50 µg/ml

4. Lipid peroxidation

The ability of the extracts and test drug to inhibit linoleic acid auto-oxidation was investigated. The extracts (0.5 – 1.5 mg/ml) and *n*-propyl gallate (0.003 - 0.03 mg/ml) caused a concentration- dependent inhibition of linoleic acid autoxidation (Table-4; Fig-4). The per cent inhibition of lipid peroxidation by methanol extract of *S. asper* increased with increase in concentration of methanol extract. At concentration of 1.5 mg/ml concentration the methanol extract caused maximum inhibition of lipid peroxidation (85.9±0.21%).

Table-4: % inhibition of lipid peroxidation by methanol extract of *F. microcarpa*

Dose of extract in mg/ml	*F. microcarpa*
	% inhibition of lipid peroxidation ±SEM
0.5	30.5±0.15
1.0	55.7 ±0.13
1.5	85.9±0.21
n-PG	65.5±0.12

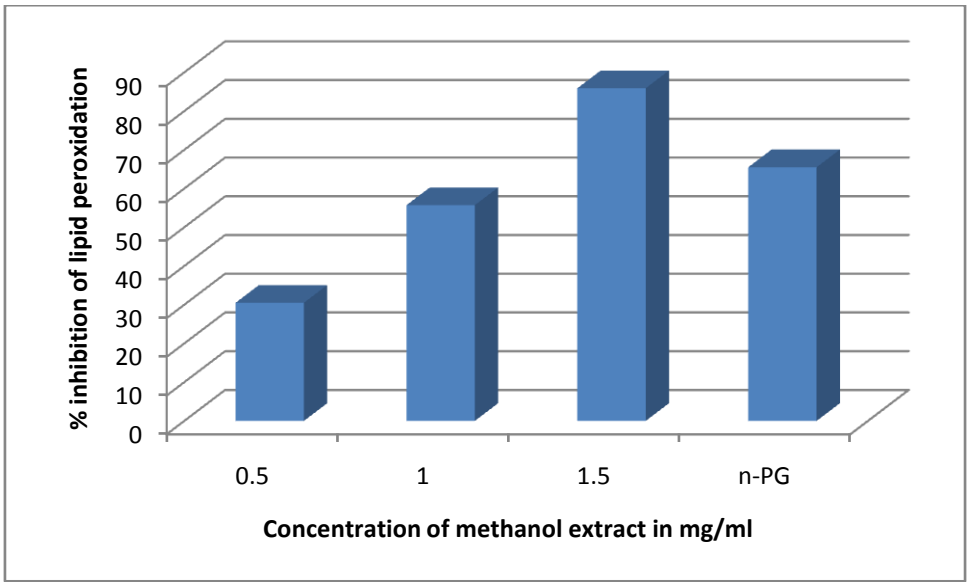

Fig-4: Per cent inhibition of lipid peroxidation at different concentration of methanol extract of F. microcarpa (mg/ml)

IV. Discussion

In the present investigation, antioxidant activity of the methanol extracts of leaves of *Ficus microcarpa* were assayed by total antioxidant capacity, total phenolic content, DPPH scavenging activity, reducing power and lipid peroxidation activity. In all these assays, the antioxidant activity increased with increasing concentration of the extracts of *Ficus microcarpa* (Table-2; Fig-2 and Table-3; Fig-3). The total methanol extract showed higher reducing power and inhibited linoleic acid lipid peroxidation (Table-4; Fig-4) considerably than the standard antioxidant *n*-propyl gallate (IC50 = 65.5± 0.12 µg/ml). The present findings gains support from the work of Javanmardia *et al.*, [28] who found a more or less similar antioxidative pattern of leaf extracts of *Ficus exasperata*. Antioxidant activity of plant extracts is not limited to phenolic compounds. Pratima et al., [29] also reported a more or less similar antioxidant activity of methanol extract of *Streblus asper*. Activity may also be due to the presence of other antioxidant secondary metabolites, such as volatile oils, carotenoids and vitamins. Thus the present study has shown that the leaves of *F. microcarpa* possess significant antioxidant properties and may contribute to the retardation of the inflammatory process. This is because inflammatory tissue injuries are mediated by reactive oxygen metabolites from phagocytic leukocytes (e.g neutrophils, monocytes, macrophages and eosinophils) that invade the tissues and cause injury to essential cellular components [30]. Compounds that have scavenging activities toward these radicals have been found to be beneficial in inflammatory diseases [31, 32]. Also the ability of the leaf extracts to inhibit the peroxidation of linoleic acid supports the use of the leaves of *F. microcarpa* in the preservation of palm oil in indigenous societies [33]. The antioxidant activity of the extract may also support its traditional use for wound healing. This is because in acute and chronic wounds, oxidants cause cell damage and thus inhibits wound healing [34]. The administration of antioxidants or free radical scavengers is reportedly helpful, notably to limit the delayed sequale of thermal trauma and to enhance the healing process [34].

V. Conclusion

Plants are sources of new natural products used in pharmaceutical, cosmetic and food production. An *in vitro* antioxidant assay provides scientific evidence to prove the traditional claims to the *Ficus microcarpa* of family Moraceae. On the basis of the present results it can be concluded that the methanolic leaf extracts of this plant possess significant antioxidant activity. Presence of adequate amount of phenolics and flavonoids account for this fact. So the present investigation suggests that *F. microcarpa* is a potential source of natural antioxidant. The active phytochemicals responsible for antioxidant activity and their mechanism of action *in vivo* as well as *in vitro* require further investigation at scientific level.

Conflict of Interest: Authors state no conflict of interest.

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