ISSN 0976 – 3333

Available Online at www.ijpba.info

International Journal of Pharmaceutical & Biological Archives 2017; 8(6): 48-54

RESEARCH ARTICLE

Novel evaluation of *In vitro* Anti-inflammatory and Free Radical Scavenging activities of *Ficus krishnae*

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Received 30 Sep 2017; Revised 20 Oct 2017; Accepted 12 Nov 2017

ABSTRACT

Ficus krishnae also known as "Makkhankathor" belongs to family Moraceae **a**nd is reported to be highly medicinal in India. As a folk medicine, the plant is used in treatment of broad range of diseases and disorders. In the present study the methanol and aqueous extracts of *Ficus krishnae* was evaluated for its total phenolic, Flavonoid content, *in vitro* anti-inflammatory and free radical scavenging activity of *Ficus krishnae*. Invitro anti-inflammatory activity by inhibition of protein denaturation method and antioxidant potential of extract was carried out by free radical scavenging activity using 2, 2 diphenyl 1-picryl hydrazyl (DPPH), ABTS radical scavenging activity, reducing power assay and phosphomolybdenum assay by using ascorbic acid and BHT as reference standards. Total phenol and flavonoid content of methanol extracts found to be 0.34mg/g and 0.60mg/g. whereas aqueous extract was to be 0.14mg/g and 0.10mg/g. Invitro anti-inflammatory study of polar solvent extracts has showed that the crude methanol extract of *F.krishnae* is a potential source of natural inhibition of protein denaturation and antioxidants this justified its uses in folkloric medicine.

Keywords: Phenol, flavonoid**,** Anti-inflammatory, Antioxidant, DPPH, ABTS, *Fius krishnae.*

INTRODUCTION

Inflammation is a local response of body defense reactions in living mammalian tissues to the injury, infection or destruction it is characterised by heat, redness, pain, swelling and disturbed physiological functions, which eliminate or limit the spread of injurious agents [1].The commonly used drug for management of inflammatory conditions are non-steroidal anti-inflammatory drugs, which have several adverse effects especially gastric irritation leading to formation of gastric ulcers [2].

Antioxidants are compounds capable to either delay or inhibit the oxidation processes which occur under the influence of atmospheric oxygen or reactive oxygen species. They are used for the stabilization of polymeric products, of petrochemicals, foodstuffs, cosmetics and Pharmaceuticals. These basically involved in the defense mechanism of the organism against the pathologies associated to the attack of free radicals. Endogenous and exogenous antioxidants are the two types of antioxidants, whereas the endogenous antioxidants are enzymes like, superoxide dismutase, catalase, glutathione peroxidase or nonenzymatic compounds, such as uric acid, bilirubin, albumin, metallothioneins. Exogenous antioxidants can be derived from natural sources (vitamins, flavonoids, anthocyanins and some mineral compounds), but can also be the synthetic compounds, like butylhydroxyanisole, butylhydroxy toluene, gallates, etc[3].Recently, antioxidants have attracted considerable attention in relation to radicals and oxidative stress, cancer prophylaxis and therapy, also longevity [4]. Phenols and polyphenols are the target analytes in many such cases; they may be detected by enzymes like tyrosinase or other phenol oxidases, or even by plant tissues containing these enzymes [5]. *Ficus krishnae* is a very large, fast growing; evergreen tree grow up to 30 m tall, with spreading branches and aerial roots belongs to the family Moraceae. [6, 7]The unique feature of the leaves have a pocket-like structure at the base. All parts of the plants are used to cure diseases of 'Kapha' also useful in treatment of ulcers,vomiting, vaginal complaints, fever, inflammations and leprosy. Latex is

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aphrodisiac, tonic; useful in piles and gonorrhea. The aerial root is styptic, useful in syphilis, biliousness, dysentery, inflammation of liver, anti-diabetic and antihyperlipidemic activity. [8, 9]

MATERIAL AND METHODS

Collection of plant materials:

The *Ficus krishnae* stem bark was collected from the dev dev vana of Bidar botanical garden, Bidar, Karnataka, India. In the month of July every year.

Extract preparation

Stem bark of *Ficus krishnae* were washed under running tap water to remove dust particles and dried in shade for 3-4 weeks. The shade dried stem bark was finely powdered, and stored in aseptic condition for further use. The powdered stem bark (100 g) was successively extracted by four different solvents (500 mL each), from non-polar to polar, i.e., petroleum ether, chloroform, methanol and aqueous, using Soxhlet extraction. The successive extracts of different solvents were dried, weighed and stored at - 4° c for further use.

Determination of total phenolic content:

Total phenolic content was estimated according to the method of Stankovic, M. S., (2011)[11]

Using FolinCiocalteu reagent (FCR) and gallic acid as standard. Extract (1mg/ml) was taken in different concentration (100, 200, 400, 600, 800 and 1000µg) in a test tube, then add with 2.5ml of 10% FCR reagent, add 2ml of 7.5% NaHCO3, then add distilled water. After 15mins incubation, the solution absorbance was measured at 765nm reagent. Total phenol content was expressed in Gallic acid equivalent (GAE) of each gm extract of dry weight. Concentration of phenolic compound was calculated according to the fallowing equation against standard graph obtained from Gallic acid graph (1).

Absorbance = 0.001×-0.073(R2=0.995)

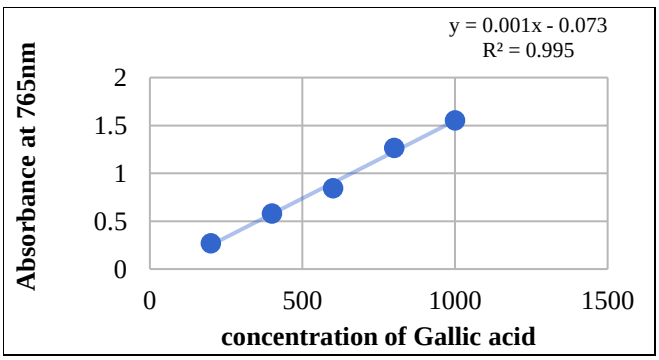


Fig 1: Standard Gallic acid curve (phenol)

Estimation of total flavonoids content:

The concentration of flavonoid was determined according to the method of Pallab K, (2013) [12] quercetin as a standard. The extract (1mg/ml) was taken in different concentration (100, 200, 400, 600, 800 and 1000µg) in test tube, add 4ml of distilled water then add 0.3ml of 5% sodium nitrite. After incubation of 5 mins .0.3ml of 10% $AlCl_3$ was added to the test tube again incubate for 6 mins add 2ml of 1M NaOH and read the absorbance at 510nm. Concentration of flavonoid content was calculated according to the following equation obtained from standard quercetin graph (2).

Absorbance = 0.000x0.011 (R2= 0.984).

In vitro anti-inflammatory activity

Inhibition of protein denaturation

The reaction mixture (5 ml) consisted of 0.2 ml of egg albumin (from hen's egg), 2.8 ml of phosphate buffered saline (PBS, pH 6.4) 2 ml extract of *Ficus krishnae* stem bark of varying concentrations of methanol and Aqueous and Diclofenac sodium (used as reference drug) [13] so that final concentration become 62.5, 125, 250, 500, 1000 µg/ml. similar volume of double distilled water has served as control. Then the mixtures were incubated at 37±2 °C for 15 mins and then heated at 70 °C for 5 mins. After cooling, their absorbance was measured at 660 nm, by using vehicle as blank. The percentage inhibition of protein denaturation was calculated by the following formulae.

% inhibition 100× (Vt/Vc-1)

Where, Vt=absorbance of test sample

Vc=absorbance of control

The extract drug concentration for 50 % inhibition (IC50) was determined by plotting percentage inhibition with respect to control against concentration.

In vitro antioxidant activity

The free radical scavenging activity of the petroleum ether and chloroform extract were determined by using various *in vitro* assays such as DPPH, ABTS, Phosphomolibdate assay Reducing power and Hydrogen peroxide assay.

DPPH radical scavenging activity

Free radical scavenging activity of the methanol and aqueous extracts were determined by using a stable 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH). DPPH is a free radical of violet color. The antioxidants in the sample scavenge the free radicals and turn it into yellow color. The change of colour from violet to yellow is proportional to the radical scavenging activity. Briefly, the assay contained 0.9 ml of DPPH solution (0.004% w/v)

was prepared in 95% methanol and various concentrations of methanol and aqueous extracts and standards with the stock solutions (10 mg/mL) in the same solvent and made up to 1 ml with ethanol. The contents were mixed well immediately and then incubated for 15 min at room temperature (24–30° C). The degree of reduction of absorbance was recorded in UV–Vis spectrophotometer at 517 nm. Ethanol (95%), DPPH solution and ascorbic acid (AA) were used as blank, control and reference standard respectively.

DPPH radical scavenging activity (%) = (Ac − As)/Ac × 100,

Where Ac is the absorbance without the samples and As is the absorbance in the presence of the samples.

ABTS radical scavenging activity

ABTS radical cat ion decolorization activity was assayed by the method of Thoo et al. (2013) and Surveswaran *et al.* (2007) [14, 15] with a slight modification. ABTS radical cat ions were generated by reacting 7 mM ABTS with 2.45 mM potassium per sulfate (1:1). The mixture was left to stand for 12 to 16 h in the dark at room temperature. The ABTS radical cat ion solution (100 µL) was then diluted with ethanol (3.9 ml) to give an absorbance of 0.700 ±0.02 at 734 nm. Different concentrations of the methanol and aqueous extracts were mixed with diluted ABTS radical cat ion solution (1 ml). The mixture was vortexed and left to stand at room temperature for 6 min. The absorbance of the resulting solution was measured at 734 nm using a UV-visible spectrophotometer. The radical scavenging activity is calculated as fallows.

$$\text{ABTS Scavenging Activity} = \frac{As - Ai}{As} \times 100$$

Where ‘As is the absorbance of pure ABTS mixture and Ai is the absorbance of ABTS mixture in the presence of extract.

Phosphomolybdenum assay method

The assay was based on the reduction of MO (VI) - MO (V) by the extract and subsequent formation of a Mo (V) complex (green color) at acidic PH, 0.3ml of extract (5, 10, 20, 40, 80 and 100µg/ml) were combined with 3ml of reagent solution [0.6M sulphuric acid, 28mM sodium phosphate and 4Mm ammonium molybdate] and incubated at 950˚c for 90 min. then the absorbance of solutions were measured at 695nm using spectrophotometer against blank.

Reducing Power Method

Different concentrations of extract of *Ficus krishnae (*05, 10, 20, 40, 80, 100µg/ml) in distilled water were mixed with phosphate buffer (0.2M, PH 6.6) and potassium ferricyanide (1%) and incubated at 50˚c for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5ml) was mixed with freshly prepared ferric chloride (0.1%) and the absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicates the reducing power.

Statistical analysis

Data were expressed as mean ± SD. Analyses of variance (ANOVA) were done for the comparison of results using Fischer's test. Statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Quantitative estimation of Phenols and Flavonoid content:

The Quantitative analysis of methanol extract for the presence of phenol and flavonoid is carried out and found to be 0.34mg/g and 0.60mg/g. whereas aqueous extract found to be 0.14mg/g and 0.10mg/g respectively. The antioxidant property of plant is due to the presence of phenol and flavonoid compounds [16, 17]. Phenols and flavonoids are secondary metabolites distributed throughout the plant kingdom and associated with colour, sensory qualities, nutritional, anti inflammatory and antioxidant properties of plant[18].

Invitro anti-inflammatory activity

Inhibition of protein denaturation

There are certain ethical issues for using animals in experimental pharmacological research, due to this reason other alternative *in-vitro* methods are available or could be investigated. Hence, in the present study the protein denaturation bioassay was selected for in vitro assessment of anti-inflammatory property of methanol and aqueous extract of stem bark . Denaturation of tissue proteins is one of the processes in causing of inflammatory and arthritic diseases. The main principle of anti-inflammatory is to prevent the protein denaturation; Agents that can prevent protein denaturation would become an new anti-inflammatory drug in discovery. The absorbance of test samples with respect to control indicated stabilization of protein i.e. inhibition of heat-induced protein (albumin) denaturation by

methanol and aqueous extracts reference drug diclofenac sodium [18]. From the IC50 values it becomes evident that both extracts were more active than diclofenac sodium, being effective in lower concentrations.

DPPH Radical scavenging assay

DPPH is stable nitrogen centered free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Shukla *et al*. 2009)[19]. The reduction capability of DPPH was determined by the decrease in its absorbance at 517 nm, induced by antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized, which can be quantitatively measured from the changes in absorbance, result in a decrease in absorbance. The antioxidant activity of different extracts of *Ficus krishnae* was calculated according to the percentage inhibition in DPPH free radical scavenging assay shown in figur 2.

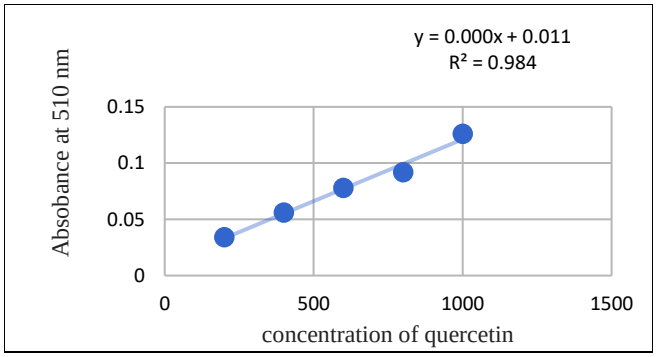


Fig 2: Standard Quercetin curve (flavonoid)

The radical scavenging activity of methanol extract was found to be 46.97 %, 56.54 %, 79.18%, 94.23%, 95.21 and 97.74% at 5, 10, 20, 40, 80 and 100 µg/mL of F. krishnae methanol extract (IC50 = 6.5 µg/mL) respectively. Whereas aqueous extract were shown to be 39.07 %, 44.16%, 49.5%, 55.41%, 59.21% and 72.01 % at 5, 10, 20, 40, 80 and 100 µg/mL of aqueous extract (IC50 = 20 µg/mL) respectively. Although the scavenging effect of the extracts was less than that of ascorbic acid and BHT the study revealed that the methanol extract had comparatively significant antioxidant activity as that of the standards. The higher concentration of total phenols and flavonoids in the methanol extracts may be the reason for high antioxidant property. The decrease in the absorbance of the DPPH radical reported for phenolic compounds are the key inducer for the reaction between antioxidant molecules and radicals, resulting in the scavenging of the radical by hydrogen donation, which is visualized as a discoloration from purple to yellow (Meir *et al*. 1995)[20].

ABTS radical scavenging activity

ABTS radical scavenging assays are performed to estimate the free radical scavenging activity of sample and are determined by the decrease in its absorbance at 745nm induced by reduction of free radicals. The methanol and aqueous extracts of Ficus krishnae stem bark extracts at different concentration ranging from 5-100µg/mL have scavenged the ABTS free radicals in concentration dependent manner. The Fig. 3 depects the ABTS radical scavenging activity of methanol and aqueous extracts of *Ficus krishae* using Ascorbic acid and BHT as standards.

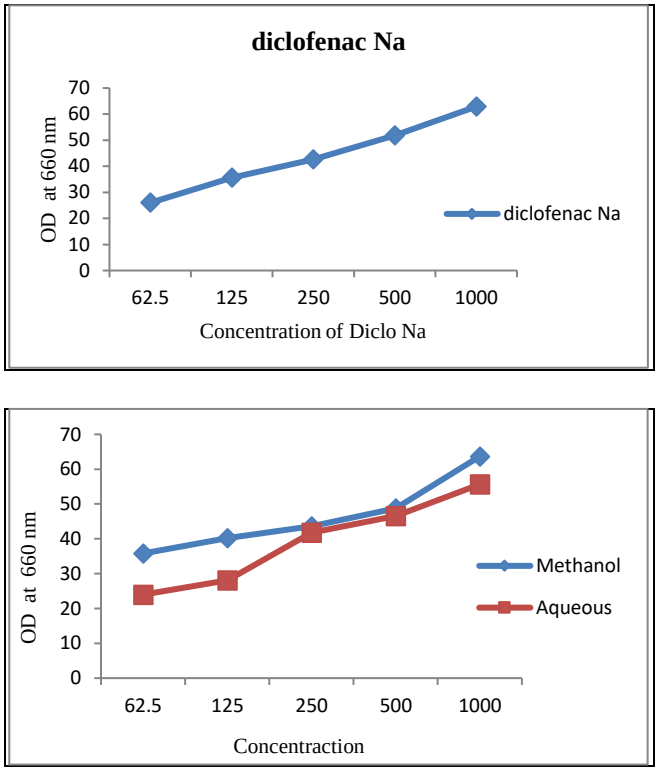


Fig 3:Anti-inflammatory activity of standard Diclofenac sodium, methanol and aqueous extract of *Ficus krishnae*.

The percentage of inhibition of ABTS radical scavenging activity of methanol is increased from 56% to 99.42%, whereas for aqueous extract the percentages of inhibition free radical ranging from 50.85% to 84.42%. The IC_{50} was found to be 5±0.5 µg/mL for both methanol and aqueous extracts of *Ficus krishnae* stem bark. Scavenging effect of both the exracts indicates the potential antioxidant property compared with standard. This antioxidant activity of the plant extract may be due to the presence in the extract phenolic content was correlated (Awika et al) [21].

Phosphomolybdenum assay

Phosphomolybdenum assay based is on the reduction of MO (VI)-MO (V) by the extract and formation of a MO (V) complex at acidic pH and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm. The result revealed the reducing power of the crude methanol and aqueous extracts of *Ficus krishnae* stem bark. Increase in absorbance was observed by standard and extract. The methanol and aqueous extract showed substantial antioxidant activity compared with BHT. At the concentration of 5µg/ml, the absorbance of methanol, aqueous and BHT were 0.046, 0,019 and 0.036 respectively. The extract was found to be increased in phosphomolybdate antioxidant assay with increase in concentration. At 100±0.5 µg/ml concentration, the absorbance of methanol, aqueous and BHT were 0.397, 0.068 and 0.678 respectively illustrated in Fig 4.

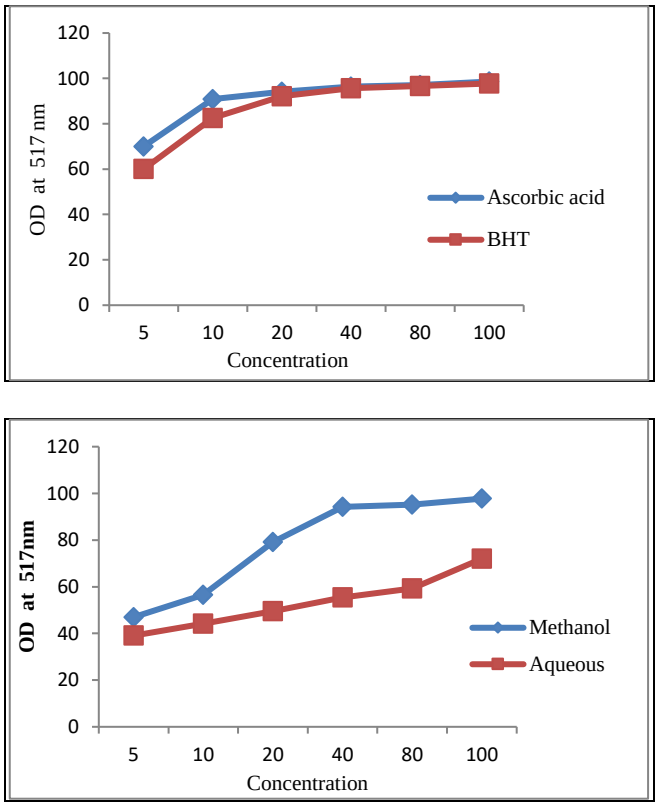


Fig 4: DPPH scavenging activity of Methanol and aqueous extracts, Ascorbic acid and BHT. Value are means ± SD (n = 3).

Reducing power assay

In reducing power assay iron (Fe^{+3}) the ferric chloride is converted to ferrous (Fe^{+2}) by anti oxidant compounds in extract resulting in conversion of yellow colour of the test solution to green, the intensity of colour directly proportion to the reducing power of the sample (Muhammad, et al)[22]. Increase in the absorbance of reaction mixture at 700nm indicates that increased reducing power of the sample is principally due to hydrogen donating ability of antioxidants of the free radicals (Gordon, M, 1990) [23]. Fig 5 depicts the reducing power of the methanol, aqueous, Ascorbic acid and BHT at different concentration ranges from 5-100µg/ml. At lower concentration 5µg/ml the absorbance of methanol, aqueous, Ascorbic acid and BHT are 0.147, 139, 0.458 and 0.172 respectively. The concentration of extract effect on absorbance at higher concentration 100µg/ml of methanol, aqueous, ascorbic acid and BHT was found to be 0.907, 0.315, 1.849 and 0,997 respectively.

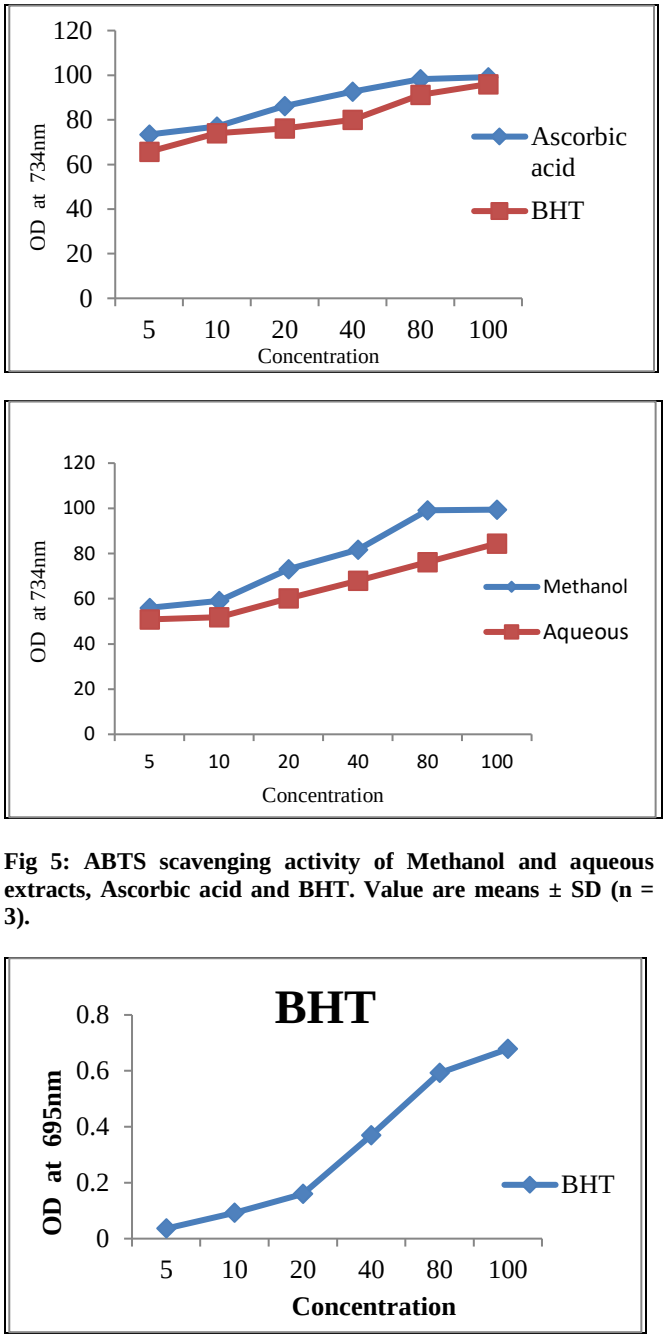


Fig 5: ABTS scavenging activity of Methanol and aqueous extracts, Ascorbic acid and BHT. Value are means ± SD (n = 3).

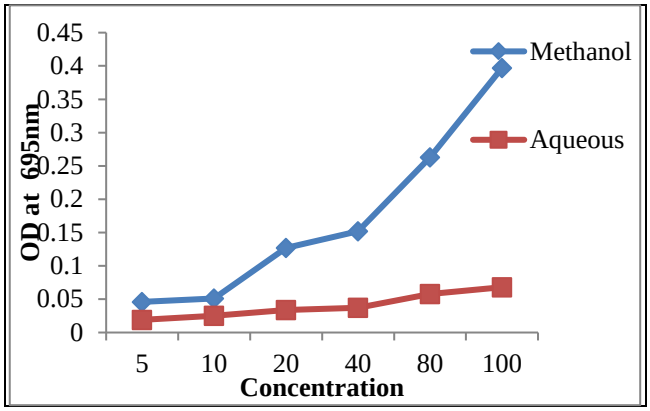
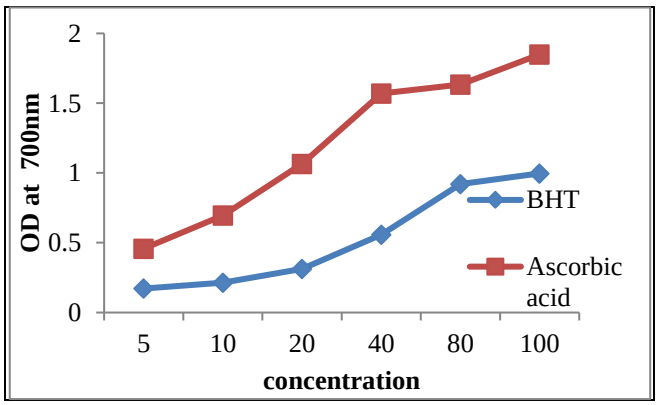


Fig 6: Radical scavenging activity by Phosphomolybdate assay of Methanol and aqueous extracts and BHT. Value are means ± SD (n = 3).



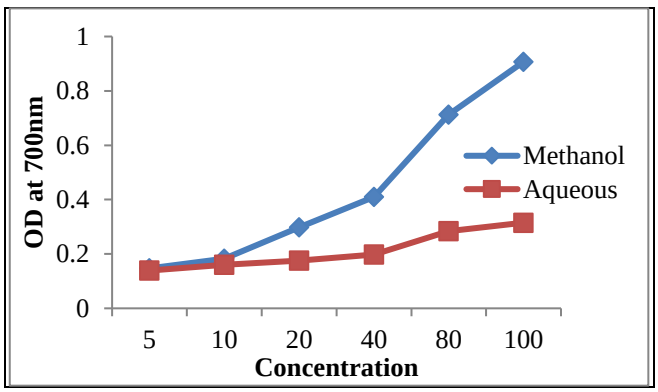


Fig 7: Radical scavenging activity by Reducing power assay of Methanol and aqueous extracts and BHT. Value are means ± SD (n = 3).

Acknowledgment

The author's thanks the University Grant Commission (UGC), New Delhi for providing financial support to carry out this research work under Rajiv Gandhi national fellowship (RGNF) scheme.

CONCLUSION

In the present study, analysis of the antioxidant activities of polar extracts (methanol and aqueous) of *Ficus krishnae* stem bark by number of in vitro protocols using biologically accepted models has been carried out. The presence of phenol and flavonoid contens in the methanol and aqueous extracts of *F. krishnae* stem bark reveals it can be a potent source of natural antioxidants. The high phenolic content and their significant linear correlation with antioxidant activity indicated that these compounds may be responsible for its antioxidant properties. Whereas, methanol and aqueous extracts have high flavonoids content in them and these are the main natural agents to inhibit the anti inflammatory activity. Efforts are in progress in our laboratory to isolate and purify the active principle from this medicinal plant.

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