The Pharma Innovation Journal 2021; 10(3): 84-88

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2021; 10(3): 84-88

www.thepharmajournal.com
Received: 04-01-2021
Accepted: 11-02-2021

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Quantification of total phenolic content, total flavonoid content and evaluation of *in vitro* free radical scavenging activities in *Ficus religiosa* Linn.

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Abstract
This present work was carried out to quantify the total phenolic and total flavonoid content and to investigate the antioxidant activity free radical scavenging activities of leaf of extracts of *Ficus religiosa* (Peepal). Leaf extracts of *Ficus religiosa* was prepared by Soxhlet extraction and various extracts were used for *in vitro* assays. The extraction yields of *Ficus religiosa* were ranged from 0.98- 10.15 g/50h (w/w) on dry weight basis. The qualitative phytochemical studies revealed presence of alkaloid, saponin, flavonoid, tannin, carbohydrates, and glycosides in different extracts of *Ficus religiosa* leaf. The results indicated the presence of higher phenolic and flavonoid content in aqueous leaf extracts of *Ficus religiosa*. It was observed that FRLE contained appreciable amount of TPC (19.97-62.93 µg GAE/mg) and TFC (29.88-135.95 µgQE/mg) as well as exhibited good DPPH radical scavenging activity (10.31-10.99 µg/ml) and nitric oxide radical scavenging activity (10.63-16.0199µg/ml). The results of the present investigation clearly demonstrated the significant variations in antioxidant properties of different solvents extract of *Ficus religiosa* leaves. It can be concluded from results that *Ficus religiosa* extracts were good source of natural antioxidants.

Keywords: *Ficus religiosa*, Antioxidant, TPC, TFC, IC_{50}, DPPH, NO radical scavenging activity

Introduction
Plants, the living chemical factories of nature, manufacture the important medicinal drugs. Many medicines prepared from the plants have been extensively investigated to evaluate their main as well as supplementary, complementary and synergistic action and to assess their place in the treatment of variety of clinical conditions and the maintenance of health. Although our body possess natural antioxidant defense mechanism to protect oxidative stress but antioxidants from natural sources could provide enhanced protection against diseases. Medicinal plants are rich source of phenolic compounds and have large number of biological effects including antioxidant activity which may help to protect the cells against the oxidative damage caused by free radicals (Berry 1996) [1].

Ficus religiosa (Peepal) belonging to family Moraceae is one of the most versatile medicinal plants having a wide spectrum of biological activity. It is used as antiulcer, antibacterial, anti-diabetic and skin diseases. Medicinal plants have curative properties due to the presence of different composition which are fond as secondary plant metabolites in one or more parts of plants. The leaves reported to have anti-venom activity and regulate menstrual cycle (Chandrasekhar 2010) [2]. The bark and leaves of *Ficus religiosa* have been used in indigenous system of medicine for different ailments. The *Ficus religiosa* is rich in phenols, flavonoids, tannins, saponin, sterols, terpenoids and carbohydrates (Sahoo and Nayak 2012) [3]. It is traditionally used to treat gonorrhoea, diarrhoea, and dysentery, menorrhagia for vaginal and other urogenital disorders, haemorrhoids and ulcers (Chandrasekhar 2010) [2]. Studies have also reported that the *Ficus religiosa* is rich source of antioxidant like vitamin C, polyphenols flavonoids and many other compounds help to reduce degenerative diseases such as heart disease, cancer and liver diseases (Devanesan *et al.*, 2018) [4].

Therefore, the present study was undertaken to evaluate phenolic and flavonoids content and antioxidant activity in different extracts of leaf of *Ficus religiosa* to study the beneficial effects in treatments of different disorders.

Materials and Methods

Different chemicals 2,2-diphenyl-1-picrylhydrazyl (DPPH), 3-(2- pyridyl)- 5,6-diphenyl-12,4-triazine-4, 4disulfonic acid, potassium ferricyanide, Quercetin, Gallic acid, ascorbic acid, Folin –Ciocalteu phenol reagent, and sodium carbonate were purchased from sigma chemicals co (St Louis MO USA). All the chemicals used in were of analytical grade.

Plant material and preparation of leaf extract

The fresh leaf of *Ficus Religiosa* was collected during March 2019 from Rajendranagar, Hyderabad, India. The plant species were authenticated by Scientist, Agricultural College, Hyderabad, India. The fresh leaf of FR was washed twice with distilled water and shade dried at room temperature for 40-45 days. Leaves were powdered using a mechanical blender and subjected to Soxhlet extraction. The extraction procedure was repeated successively with solvents of increasing polarity, petroleum ether, benzene, chloroform, acetone, hexane and aqueous (Deyab *et al.,* 2016) [5]. After extraction the extract was concentrated in a flash evaporation evaporator, solvent was recovered and the extract was dried in desiccators and the extracts were stored in brown bottles at room temperature.

$$\text{Percent extractability} = \frac{\text{Total amount of extract obtained}}{\text{Total weight of powder taken for extract}} \times 100$$

Qualitative Phytochemical screening

Crude extracts of leaves were examined for the presence of various secondary metabolites such as phenols, flavonoids, tannin, saponin, alkaloids, glycosides, phytosterol, steroids and carbohydrates as described by (Khan et al., 2016, Raman 2006) [6, 7].

Quantification of phytochemical constituents:

The total phenolic content of leaves extracts was estimated according to the Folin Ciocalteu method (Sembiring *et al*., 2018) [8] with minor modifications. Total phenolic content was calculated from calibration curve of Gallic acid (25-200ug/ml) and expressed in terms of Gallic acid equivalents (GAE) per gram of extracts. The standard solutions of Gallic acid concentrations 1.56-100ug/ml were prepared in water. 50ul of extract (1mg/ml) or standard solution were added to 50ul of distilled water. 50ul of 10% Folin Ciocalteu phenol reagent and 50ul 1M sodium carbonate solution were added to the mixture in a 96 well plate. Distilled water was used as blank. Reactions were incubated for 60 min at room temperature and protected from light. The absorbance was measured at 750nm with a microplate reader. Total phenolic contents were expressed as ug Gallic acid equivalents (GAE) per ml of plant extracts.

The total flavonoid content in different extract of leaves of *Ficus religiosa* were determined by using aluminium chloride method (Sembiring *et al*., 2018) [8]. The flavonoid content was calculated from standard curve of Quercetin (25-200ug/ml) and expressed as Quercetin equivalents (QE) per gram of extract. The total flavonoid content was determined by aluminium chloride calometric assay adopted from Sembiring [8]. Quercetin was used as a standard. Standard solutions of Quercetin of concentration 1.56-100 ug/ml were prepared in 80% ethanol. 50ul of extracts (1mg/ml) or standard solution was added to 10 ul of 10%aluminium chloride solution and followed by 150ul of 95% ethanol. 10ul of 1M sodium acetate was added to the mixture in a 96 well plate. 80% ethanol was used as reagent blank. All reagents were mixed and incubated for 40min at room temperature and protected from light. The absorbance was measured at 415nm with a microplate reader (Biotek, USA). Total flavonoid contents were expressed as ug Quercetin equivalents (QE) per ml of plant extracts.

Antioxidant scavenging activity

DPPH radical scavenging activity of different extracts was measured by Jose Prieto method (Jose Preito 2012) [9]. DPPH scavenging ability assay was used to evaluate the antioxidant activity of each extracts and the test was conducted in a 96 well plate with slight modifications. 20ul stock solution of extracts in different concentrations (1.075 to 200ug/ml) and 180ul of DPPH solution (0.147mM) was added to each well. After 30min of incubation at room temperature in dark room, absorbance was read at 517nm using microplate reader. Methanol was used as blank. Ascorbic acid was used as positive standard. All tests were performed in triplicate. Concentration of samples resulting in 50% inhibition on DPPH (IC_{50} value) was calculated. The scavenging ability (%) was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{Absorbance of standard-Absorbance of extract}}{\text{Absorbance of standard}} \times 100$$

The effective concentration required for 50% reduction of the DPPH radical (IC_{50}) was calculated by probit analysis using IBM SPSS version 20.0.

Nitric oxide scavenging assay was carried out using sodium nitroprusside method (10). Gallic acid was used as positive standard. The assay is the Nitric oxide radical scavenging assay. The extracts were prepared from a 10 mg/ml ethanol crude extract and were serially diluted with distilled water to make concentrations from 100−1000µg/ml of the plants extracts and the standard Gallic acid and stored at 4°C for later use. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.9% naphthyl ethylene diamine dihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 ml of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 ml of the different concentrations of the various extracts (100-1000 ug/ml) and incubated at 25 ^{0}C for 180 min. The extract was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the extracts but with an equal volume of buffer were prepared in a similar manner as was done for the test samples. The colour tubes contained ethanol extracts at the same concentrations with no sodium nitroprusside. A volume of 150µl of the reaction mixture was transferred to a 96-well plate. The absorbance was measured at 546 nm using a microplate reader. Gallic acid was used as the positive control. The percentage inhibition of the extract and standard was calculated and recorded. The percentage nitrite radical scavenging activity of the extracts and Gallic acid were calculated using the following formula: Percentage nitrite radical scavenging activity

$$\text{A control} \quad \frac{\text{A Test}}{\text{A control}} \quad \text{x100}$$

A control- Absorbance of control sample,
A Test - Absorbance in the presence of samples of extract or standards

Stastical Analysis

Inhibition of concentration and total phenolic and antioxidant were determined by regression analysis method which was used to calculate IC_{50}.

Result and Discussion

The extractive yields of different extracts of *Ficus religiosa* leaf were ranged from 0.98- 10.15g/50g.The highest percentage yield was in acetone whereas lowest in petroleum ether. Phytochemical screening of different extracts of FRL revealed the presence of alkaloids, flavonoids, glycosides, phenols, tannins and terpenoids in aqueous, benzene, chloroform and hexane leaf extracts, while there was absence of steroids and glycosides in acetone and petroleum ether extracts.

The total flavonoid content of *Ficus religiosa* leaf was expressed as ug GAE/mg extract and was ranged from 21.929-135.942 ug Quercetin equivalent (QE)/mg. Equation of calibration curve of Quercetin standard was y = 0.0156x-0.3419 R^2= 0.9416. The flavonoid contents were expressed as ug QE per mg of *Ficus religiosa* leaf extract. Among the six crude extracts, aqueous extracts contained the highest amount (135.942 ug QE/mg) of total flavonoid content followed by acetone (110.49 ug QE/mg), benzene (71.934 ug QE/mg), hexane (34.275 ug QE/mg), petroleum ether (29.880 ug QE/mg) and chloroform (21.929 ug QE/mg) from *Ficus religiosa* leaf extract.

Phenolic content of *Ficus religios*a leaf extract (FRLE) was expressed as ug GAE equivalent /mg extract. The total phenolic content of extracts of *Ficus religiosa* Linn ranged from 11.572 to 62.935 ug Gallic Acid Equivalents (GAE)/mg. Calibration curve from Gallic acid showed maximum absorbance at 765 nm wavelength (equation y= 0.2213x +0.082, R2= 0.984). The total phenolic content of six extracts of leaf of *Ficus religiosa* Linn determined by the Folin-Ciocalteau method were reported as Gallic acid equivalents (GAE). The aqueous extracts showed maximum total phenolic content of 62.935 ug GAE/mg, followed by acetone (43.967 ug GAE/mg), petroleum ether (33.924 ug GAE/mg), chloroform (29.221 ug GAE/mg), hexane (19.970 ug GAE/mg) and benzene (11.572 ug GAE/mg).

Phenolic compounds including flavonoids are considered as the important antioxidative components of plant materials because of the positive correlation between the concentration of plant phenolic and its total antioxidant capacity (1). DPPH free radical scavenging method was used to determine the concentration of extract at which they scavenge the 50% of the DPPH solution termed as IC50 values. Ascorbic acid was used as a standard for this purpose. In terms of percentage, the inhibitory activity (at 30 min) of ascorbic acid, aqueous, acetone, benzene, chloroform, hexane and petroleum ether extracts of *Ficus religiosa* leaf were found to be 45.99, 45.99, 45.49, 45.98, 47.48, 48.47 and 45.89%, respectively at the concentration of 10.87, 10.87, 10.99, 10.87, 10.53, 10.31 and 10.89 ug/ml. The maximum NO scavenging activity of aqueous, acetone, benzene, chloroform, hexane and petroleum ether was 41.44, 33.22, 41.44, 36.21, 31.23 and 47, respectively with IC_{50} values of 12.06, 15.05, 12.06, 13.80, 16.01 and 10.63 mg/ml.

Table 1: Extractive yield of different extracts of *Ficus Religiosa* leaf

Solvent	Extractive yield (g/50g) FRLE
Acetone	10.15
Aqueous	9.80
Benzene	2.44
Chloroform	2.71
Hexane	5.05
Petroleum ether	0.98

Table 2: Qualitative phytochemical screening of different extracts of *Ficus religiosa* leaf

Phytochemical constituent	Aqueous	Acetone	Benzene	Chloroform	Hexane	Petroleum ether
Alkaloids	+	+	+	+	+	+
Glycosides	+	-	-	+	+	-
Flavonoids	+	-	+	+	-	-
Phenols	+	+	-	+	+	-
Triterpenoids	+	-	+	+	+	-
Steroids	-	-	-	+	+	-
Saponins	-	-	+	-	-	-
Tannins	+	-	+	+	+	-
Carbohydrates	-	-	-	-	-	-
Proteins	-	-	-	-	-	-

Table 4: Quantification of total flavonoid and total phenolic content in different extracts of *Ficus religiosa* leaf:

Name of the extract	Total flavonoid content (µgQE/mg extract)	Total phenolic content (µgGAE/mg extract)
Aqueous	135.95	62.93
Acetone	110.49	43.96
Benzene	71.93	11.57
Chloroform	21.89	29.22
Hexane	34.27	19.97
Petroleum ether	29.88	33.92

Table 6: DPPH radical scavenging activity *of Ficus religiosa* leaf extract

Extract	IC_{50}(µg/ml) FRLE
Ascorbic acid	10.87
Acetone	10.87
Aqueous	10.31
Benzene	10.87
Chloroform	10.53
Hexane	10.99
Petroleum ether	10.89

Table 7: Nitric oxide radical scavenging activity *of Ficus religiosa (*FRLE*)* leaf extract

Extract	IC_{50}(µg/ml) FRLE
Gallic acid	14.26
Acetone	15.05
Aqueous	16.01
Benzene	12.06
Chloroform	13.8
Hexane	12.06
Petroleum ether	10.63

Natural antioxidants such as flavonoids and phenolic compounds are believed to possess antioxidant properties due to their reducing and chelating capabilities. Flavonoids and phenols are secondary metabolites with free radical scavenging abilities that are widely distributed in fruits, leaves, bark and other parts of plants. The total phenolic content of the extracts was determined by Folin Ciocalteu

reagent due to its high specificity for the polyphenolic compounds present in the plant extracts as it does not interact with the other phytochemicals due to due to complex formation between reducing species and phosphorus-molybdic tungstate (Khan *et al.,* 2016) [6]. It is evident from present study that leaf extracts of FR was good source of antioxidant.

FR showed highest percentage yield in acetone extract of FRLE, whereas the lowest was in petroleum ether. Acetone and aqueous leaf extracts of *Ficus religiosa* showed maximum yield (Sumitra 2012, Ruqaya *et al*., 2017) [11, 12]. Phytochemical screening of different extracts of FRLE revealed the presence of alkaloids, flavonoids, glycosides, phenols, tannins and terpenoids in aqueous, benzene, chloroform and hexane leaf extracts, while there was absence of steroids and glycosides in acetone and petroleum ether extracts. Our results are in agreement with the reports of earlier study who recorded the presence of phenolic compounds, tannins, sterols, saponins, terpenoids and glycosides in different extracts of *Ficus religiosa* leaf (Kapoor *et al.,* 2011, Damanpreet and Rajesh 2009) [13, 14].

FR was used as antioxidant (Parameswari *et al.,* 2012, Hima Bindu *et al*., 2013, Pal *et al*., 2018) [15-17]. It is evident from the present study that FR was good source of antioxidants. Among the six extracts of FRLE, aqueous extracts contained highest amount of total flavonoid content followed by acetone, benzene, hexane, petroleum ether and chloroform extracts. The aqueous extracts of FRLE showed the highest total phenolic content followed by acetone, petroleum ether, chloroform, hexane and benzene extracts. The aqueous extracts of FRLE showed the highest total phenolic content followed by acetone, petroleum ether, chloroform, hexane and benzene extracts.

The present study investigation demonstrated that the antioxidant potential of leaf extracts of FR through inhibition of generation of free radicals *in vitro*. The reactive oxygen species such as superoxide and hydroxyl radicals, hydrogen peroxide are often generated as product of biological reactions and damage the cells when present in excess. All the extracts of *Ficus religiosa* leaf showed significant DPPH scavenging activity in dose dependent manner. The DPPH method is a stable free radical system and a sensitive way to determine the *in vitro* antioxidant activity of plant extracts. The antioxidant efficacy is associated with their scavenging ability of stable free radicals (Kumar *et al*., 2011, Balakrishnan *et al*., 2014) [18, 19]. The DPPH assay suggests that the extracts of FR are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for the radical reactions (Charde *et al.,* 2010, Jung *et al*., 2008) [20, 21]. The highest DPPH radical scavenging activity was found in aqueous extract as compared to all other extracts. Maximum percentage scavenging of DPPH radicals was reported in aqueous extract of *Ficus religiosa* leaf.

The nitric oxide is another free radical, which acts as pleiotropic inhibitor of physiological processes and reacts with superoxide anion radicals to form a cytotoxic oxidant molecule, peroxynitrite. In the present investigation, FR effectively scavenged nitric oxide. The scavenging of NO by extracts was increased in a concentration-dependent manner and showed maximum activity in aqueous extracts (21). Phenolic compounds directly contribute to antioxidant action of the natural substances. It is reported that phenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans and polyphenols are potential protecting agents against lethal effects of oxidative stress. In the present investigation the aqueous leaf extract of FR had the highest phenolic content concomitant with high free radicals scavenging action. Hence high antioxidant potential of FR extract might be due to presence of more phenolic and flavonoid content. The total phenolic and flavonoid in aqueous extract of *Ficus religiosa* was more compared to that of other extracts. There is a direct correlation of phenolic and flavonoids with antioxidant activities in leaves which clearly indicate that phenolic compounds and flavonoids may be responsible for antioxidant activities of *Ficus religiosa*. The present study validates the use of leaves of *Ficus religiosa* for treatment of various ailments.

Acknowledgment

The first author acknowledges, Associate Dean, C. V. S., Hyderabad and Dept of veterinary pharmacology and toxicology, C. V. Sc., Rajendranagar, Hyderabad for providing necessary facilities for research work.

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