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Research Article

PROTECTIVE EFFECT OF *FICUS RELIGIOSA* ON CYCLOPHOSPHAMIDE INDUCED OXIDATIVE STRESS IN BRAIN

Shalini Balakrishnan[1], Soumya Shrivastava[1], MS. Karchulli[2] and Megha Jha[2]*

[1]Kalyan Post Graduate College, Department of Biotechnology, Bhilainagar, Chhattisgarh, India.

[2]Pinnacle Biomedical Research Institute, Bhopal, Madhya Pradesh, India.

ABSTRACT

Objective: Protective effect of *Ficus religiosa* leaf extract on cyclophosphamide induced oxidative stress on brain. **Material and Method:** *In* vitro antioxidative analysis for the exploration of the antioxidative properties of *Ficus religiosa* leaf extract (FRL) and *Ficus religosa* bark (FRB) was done. To analysis the *In vivo* antioxidant property of extracts on the oxidative stress induced by the cyclophosphamide. Oral administration of leaf and bark extracts (0.1ml/kg of body weight/day) for 7 days prior to an acute dose of cyclophosphamide (75 mg/kg) on 5th day which significantly ($P< 0.05$) augmented level of malondieldehyde in the rat brain. Later animal were sacrificed and brain was taken out and homogenate were prepared in Tris buffer (pH 7.4) in cold condition. And taken for biochemical estimation involving Superoxide dismutase, Lipid peroxide and Reduced Glutathion test. **Result and conclusion**: The cyclophosphamide cause declination in the level of glutathione, *superoxide dismutase* and increase in lipid peroxidation which was with the administration of *Ficus religiosa* extract significantly ($P< 0.05$) prevented in brain of rat. Analysis of different biochemical parameters, results clearly indicated the prophylactic action of FRLand FRB extract against cyclophosphamide induced oxidative stress in brain.

Keywords: Cyclophosphamide, *Ficus religiosa*, FRL, FRB ,oxidative stress, rat brain.

INTRODUCTION

Oxidative stress is resulted by excessive free radicals. Brain is a highly specialized organ any damage or form of stress experienced in this part of the body may have serious impact on the entire organism[1]**.** The brain consumes 20% of their metabolic oxygen. The brain and nervous system are particularly vulnerable to oxidative stress due to limited antioxidant capacity[2]. Have only moderate levels of both enzymatic and nonenzymatic scavengers of reactive oxygen species[3]**.** The most prominent and natural way to defense against the oxidative stress is the antioxidants. In particular flavonoids have been found to possess anti-inflammatory, antioxidant, antiallergic, neuro protective, antithrombotic and anti-carcinogenic effects[4,5].Cyclophosphamide is an antineoplastic agent metabolized to active alkylating metabolites, widely used alkylating agents that damage normal cells while killing cancerous cells in vivo by slowing or stopping cell growth. It also possesses marked immunosuppressant properties. It is widely used to treat various types of cancer, in combination with other agents[6-8]. The use of CP in treating cancer patients is limited due to its severe toxicities induced mainly oxidative stress[9]**.**Cyclophosphamide can initiate oxidative stress by inactivating endogenous antioxidant enzymes and also deplete antioxidant metabolites such as glutathione with a resultant disruption of cell membrane, mutation of DNA and loss of structure and function of protein[10]. The secondary metabolites like phenolics and flavonoids from plants have been reported to be potent free radical scavengers, found in all parts of plants such as leaves, fruits, seeds, roots and bark[11]**.** *Ficus religiosa* is a sacred tree native to India. In Ayurveda, *F. religiosa* belongs to a class of drugs called rasayana (rejuvenators, antioxidants and relieve stress in the body[12].

The bark and leaves are taken for the treatment of diarrhoea, dysentery, mumps; the latex is used to treat warts. The bark is used in diabetes, diarrhoea, nervous disorders and other urinogenital disorders. The seeds and fruits are cooling, laxative and refrigerant[13].Therefore, a more extensive investigation was undertaken to understand effect of *Ficus religiosa* leaves (FRL)and *Ficus religosa* bark (FRB) on Cyclophosphamide induced oxidative stress in swiss albino mice brain.

MATERIALS AND METHODS

Sample collection

Ficus religiosa were collected from locality of Shyamla Hills, Bhopal and authenticated from the Department of Botany, Safia Science College, Bhopal (M.P.). The voucher specimen number was Ref: 461/Bot/Safia/13.

Extract preparation

The powdered *Ficus religiosa* leaves (FRL) and *Ficus religosa* bark (FRB) was successively extracted with soxhlet extraction using solvents of increasing polarity; petroleum ether, chloroform, ethyl acetate and ethanol. The solvents were removed under reduced pressure on rotary evaporator until it became complete dry. The percentage yield for each extract was determined and the ethanol (70%) crude extract was subjected to further *in-vivo*oxidative assay[14].

Solubility testing and Phytochemical screening

Crude extract of FRL and FRB was subjected to solubility test in various solvent. Detailed phytochemical testing was performed to identify presence or absence of different phytoconstituents[15].

Spectrophotometric quantification

Total Flavonoid Content

Total flavonoids of FRL andFRB extract were measured by a colorimetric assay[16].An aliquot of diluted sample treated with 75µl of $NaNO_2$ and mixed for 6 minutes. 0.15ml of $AlCl_3$ (10%) was added and after 5 minutes 0.5ml of NaOH was added. The volume was adjusted to 2.5 ml with distilled water and mixed. Absorbance was determined at 510 nm. The total flavonoid content was calculated as rutin equivalent.

Total Phenolic Content test

The amount of total phenolic content of the extract was determined by the reported method[17] with the Folin Ciocalteu reagent. A concentration of 0.1 and 1mg/ml of both extracts were oxidized with 2.5ml of 10 fold dilute Folin Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. Incubated at 30ºC for 40 min and taken absorbance at 765 nm.

IN VITRO ANTIOXIDANTASSAY

DPPH radical scavenging assay

The free radical scavenging ability of FRL and FRB extract was determined against 1, 1-diphenyl-2 picryl hydrazyl free radical described[18]. 1ml of DPPH methanol solution (0.4mM) was added to 3 ml of extract solutions at different concentration (50-250µg/ml) and mixtures were allowed to stand for 30 min. at room temperature in dark condition, absorbance was taken at 517 nm spectrophotometrically. Control tube (containing no sample or standard) was also noted like these of samples.

Free Radical Scavenging Activity

$$\% (DPPH\bullet) = [1-(As / Ac)] \times 100$$

Here, A_c is the absorbance of the control and A_s is the absorbance of the samples (extracts) or standards.

Reducing power assay

A spectrophotometric method was used for the measurement of reducing power. For this 2.5 ml of each of the extracts was mixed with 2.5ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide (10 mg/ml). The mixture of both extract FRL and FRB was incubated at 50 ºC for 20 min separately, and then rapidly cooled, mixed with 2.5 ml of 10% trichloroacetic acid and centrifuged at 6500 rpm for 10 min. An aliquot (2.5ml) of the supernatant was diluted with distilled water (2.5ml) and then ferric chloride (0.5ml, 0.1%) was added and allowed to stand for 10 min. the absorbance was read spectrophotometrically at 700 nm. Butylated hydroxy toluene (BHT) was used as standard for construction of calibration curve[19].

$$\text{Reducing Power (\%)} = (As / AB) \times 100$$

Here, A_B is the absorbance of control (BHT) and A_s is the absorbance of samples (extracts) or standards.

H_2O_2 radical scavenging activity

The activity of the FRL and FRB extracts to scavenge the hydrogen peroxide was determined according to the method[20]. Extracts of FRL and FRB were prepared in distilled water of concentration (50- 250µM). 1 ml of each solution of both extract was mixed with 2.4 ml of 0.1M phosphate buffer solution

and 600µlof 40mM H_2O_2 solution separately. After 10 min absorbance of each extracts solutions were taken at 230 nm. The percentage of scavenging the hydrogen peroxide of extracts and standards were calculated using the following equation:

Scavenged % (H2O2) = [1 - (As/Ac)] × 100

Here, A_c is the absorbance of the control and A_s is the absorbance in the presence of the samples (extracts) or standards.

***In vivo* study**

Animals

The experiments were carried out on either sex of Wistar rats (weight 150±25 gm). Animals were housed in separate cages under controlled conditions of temperature (22±2°C). All animals were given standard diet (golden feed, New Delhi) and water ad libitum. Animals were further randomly divided in six groups with four animals in each group. All animal experiments were approved by Institutional Animal Ethics Committee (IAEC) of Pinnacle Biomedical Research Institute (PBRI) Bhopal (Reg No. 1283/c/09/CPCSEA). Protocol Approval Reference No. PBRI/13/IAEC/PN-320.

Acute oral toxicity (OECD 423)

FRL and FRB extracts was dissolved in distilled water and administered orally at 5 mg/kg, 50 mg/kg, 300 mg/kg and 2000 mg/kg to animals as per the guidelines.

Treatment schedule

Rats were divided into six experimental groups of 6 animals each. Group 1: Control, Group 2: Vehical orally for 7 days + CYP (75 mg/kg) intraperitoneally on 5th day, Group 3: CYP + FRL(200mg/kg) orally for 7 days, Group 4: CYP + FRL(400mg/kg) orally for 7 days, Group 5: CYP + FRB(200mg/kg) orally for 7 days and Group 6: CYP + FRB(400mg/kg) orally for 7 days.

Tissue Preparation

After treatment period, animals were sacrificed by cervical dislocation. Brains were immediately taken out and washed with ice cold saline to remove blood and kept at -80°C. Various regions of the brain viz., cerebral cortex, hippocampus and cerebellum were rapidly dissected from the intact brain carefully on ice plate. The right and the left cerebral cortices and hippocampus were pooled to make one sample of the tissue. The cerebral cortex, hippocampus and cerebellum were homogenized individually in Tris buffer (pH 7.4). The tissue homogenate (10%) was made (w/v), which was centrifuged at 4000g for 5 min. at 4°C. All processes were carried out in cold conditions.

Biochemical Estimation of FRL and FRB

Lipid peroxidation assay[21]

The TBA reacts with MDA in acidic medium to form TBARS. The assessment of serum total thiobarbituric acid-reactive substances (TBARS) provides a measure of total serum lipid peroxidation, an indicator of whole-body free radical activity.Result is expressed in MDA nano mole per millilitre (nM/ml).

Reduced glutathione (GSH)[22]

In this assay, DTNB (Dithiobis-(2-nitrobenzoic acid) reduced by SH-groups, in presence of GSH to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance's can be measured at 412 nm, calculated as mg/g tissue.

Superoxide anion scavenging activity[23]

The production of superoxide from O_2 **(**using reduced b-nicotinamide adenine dinucleotide (NADH) as a reductant, and phenazine methosulphate (PMS) as a catayst**)** in the presence of an indicator, nitro blue tetrazolium (NBT), which turns blue when reduced by superoxide. The color change can be monitored at 560 nm, calculated as U/gm tissue.

Statistical analysis

The data were expressed as Mean ± deviation (SD). Statistical differences between the values were analyzed through one way analysis of variance (ANOVA). Differences between the test and the control group were evaluated by least significant difference method at $P < 0.05$.

RESULTS AND DISSCUSSION

Phytochemical screening of FRL and FRB in various solvents showed that it contains flavonoids, phenolic compound, terpenoids, alkaloids etc. (Table. 1)

The total phenolic content of FRL was 73.4 µg/mg equiv. to Gallic acid and in FRB it was 163.8 µg/mg equiv. to Gallic acid. The FRB extract contained 474 µg/ml of rutin equivalents flavonoids and FRL 68 µg/mg of rutin equivalents flavonoids.

***IN VITRO* ANTIOXIDANTASSAY**

The DPPH test provides information on the reactivity of the test compounds with a stable free radical. The free radical scavenging

activities of FRL and FRB extracts were assessed by DPPH. As shown in Figure. 1, both the FRL and FRB extract exhibited a significant inhibitory activity against the DPPH radical. Similar IC50 values for DPPH radicals in FRL and FRB extract are observed as 136.02µg/ml and 123.75µg/ml respectively. Standard was ascorbic acid with IC50 value 91.74 µg/ml (Figure. 1).

Reducing power assay measures the electron donating capacity of an antioxidant. The reducing power of FRL and FRB extracts are summarized in Figure2. From the figure, reducing power increased with an increased in extracts concentration.BHT was used as standard (Figure. 2).

Hydrogen peroxide non reactive, but sometimes it can be toxic to living cells, because in living cell it is converted into free radical called hydroxyl radicals (•OH), react with biomolecules, cause tissue damage and cell death. Figure 3 shows the IC50 of (•OH) scavenging effect by extracts FRL 229.75 µg/ml and FRB 138.42 along with α-tocopherol 91./28 µg/ml was standard (Figure. 3).

After ascertaining antioxidant potential and acute oral toxicity of FRL and FRB extract. Considerable knowledge about the *in vivo* antioxidant status has not been documented so far. Hence, it is aimed to study the enzymatic antioxidant profiles using various models: superoxide dismutase (SOD), glutathione (GSH) and Lipid peroxidation (LPO).

IN VIVO ANTIOXIDANTASSAY

Lipid peroxidation

MDA is a marker of LPO, the rate of MDA increased from 12.64 ±0.16 (control)for 6 days. A significant depletion in the lipid peroxide level was observed after post treatment of FRL and FRB extracts as compared to CYP group 14.52 ± 0.13. FRL attenuated LPO levels as 10.77 ± 0.14 whereas FRB 12.5 ± 0.08 in brain (P<0.05).

Reduced glutathione

GSH level decreased from 13.77 ± 0.22 (control) in brain after CYP treatment as compared to control (P<0.001). Post administration of FRL and FRB significantly increased the level of GSH as compared to inducer group. FRL recovered the GSH level 14.01±1.11 whereas FRB 6.88 ± 0.608 as in brain (P<0.05).

Superoxide dismutase

SOD activity inhibited from 71.64± 2.05(control) and 84.94 ± 1.93 (inducer)in brain by inducer (P<0.05). Post administration of FRL and FRB significantly protect the level of SOD in comparison to control group. FRL enhanced the level of SOD as 68.79± 2.34whereas FRB46.67 ± 0.652 in brain (P<0.05) (Table. 2).

CONCLUSION

Present study concluded that both of the plants FRL and FRB extracts administration were able to attenuate the stress-induced oxidative damage in brain. Therefore, it is clear by studying different biochemical parameters that both of these extracts are affected but in different manner. Thus the study indicates that FRL and FRB are potential candidates for further evaluation as an antioxidant to attenuate stress induced oxidative damage. Further studies will be needed to purify the bioactive compound(s) in the ethanolic extract, and use the purified compound(s) for bioassay directed experiments.

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Table 1: *Ficus religiosa* leaf extract (FRL) and *Ficus religosa* bark (FRB) fractions phytochemical screening

Test Metabolites	FRL				FRB			
	P.E.	C.M.	E. A.	M.L	P.E.	C.M.	E. A.	M.L
Tri terpenoids/ steroids	-ve	+ve	+ve	+ve	-ve	-ve	-ve	+ve
Glycosides	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve
Alkaloids	+ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve
Carbohydrate	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve
Flavonoids	-ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve
Tannin and Phenolic	-ve	-ve	-ve	+ve	-ve	-ve	-ve	+ve
Saponin	-ve	-ve	-ve	-ve	-ve	-ve	-ve	+ve
Protein and amino acid	-ve	-ve	-ve	-ve	+ve	-ve	-ve	-ve

FRL *Ficus religiosa* leaf extract and FRB *Ficus religiosa* bark extract in various solvent fractionate for phytochemical screening for various phytoconstituent.P. E.(Petroleum ether), C.M. (chloroform), E.A. (Ethyl acetate), M.L. (Methanol)

Table 2: *In vivo* antioxidative analysis of *Ficus religiosa* extract on brains of treatment groups

TREATMENT GROUP	SOD MEAN±SEM	GSH MEAN±SEM	MDA MEAN±SEM
Control group (vehicle)	71.64± 2.05	13.77±0.22	12.64±0.16
Inducer group (vehicle+ cyp(75mg/kg))	84.94 ± 1.93	2.76±0.4	14.52±0.13
Treatment group 1 (FRL(200mg/kg)+cyp(75mg/kg)	51.24± 1.4	10.81±1.1	12.64±0.16
Treatment group 2 (FRL (400mg/kg+cyp(75mg/kg)	68.79± 2.34	14.01±1.11	10.77±0.14
Treatment group 3 (FRB200mg/kg)+cyp(75mg/kg)	40.71 ± 1.171	5.75 ± 0.94	14.1 ± 0.04
Treatment group 4 (FRB 400mg/kg)+cyp(75mg/kg)	46.67 ± 0.652	6.88 ± 0.608	12.5 ± 0.08

Ficus religiosa extract where taken for *in vivo* antioxidative analysis of *Ficus religiosa* extract on brains of various treatment groups FRL (*Ficus religiosa* leaf extract) FRB (*Ficus religiosa* bark extract)

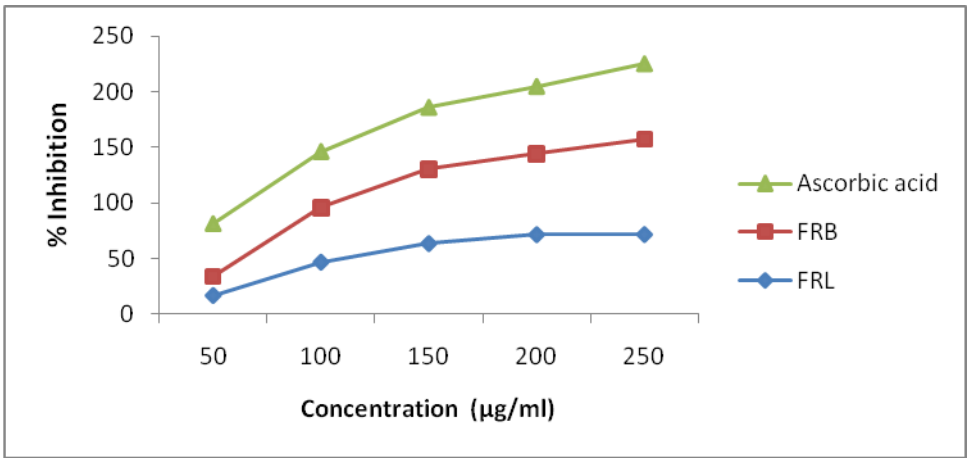

Fig. 1: showing DPPH scavenging activity of FRL, FRB and ascorbic acid

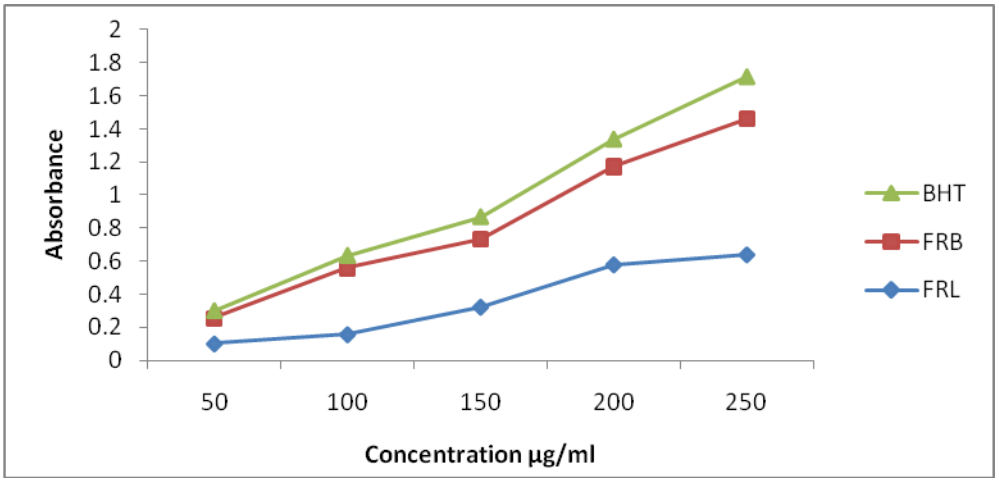

Fig. 2: Reducing power assay of *FRL*, *FRB* and BHT

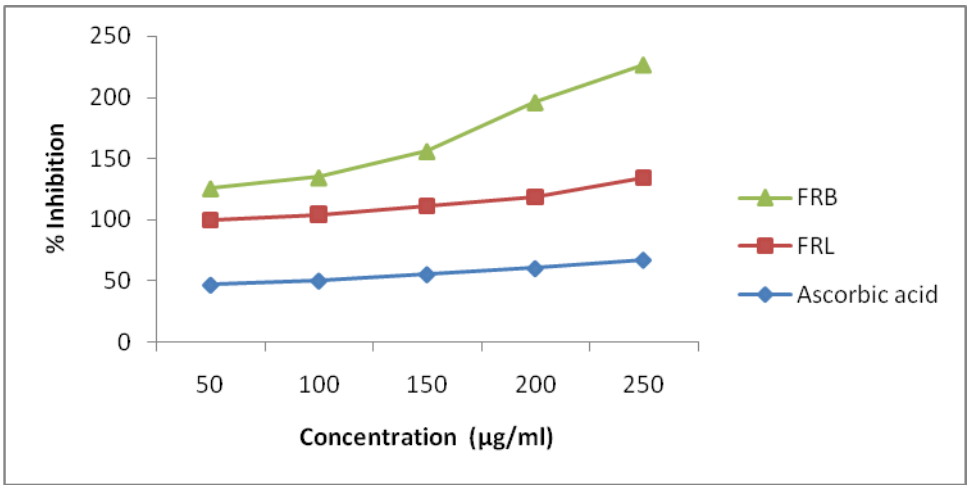

Fig. 3: Showing H_2O_2 scavenging activity of *FRL*, *FRB* and ascorbic acid

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