ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Appraisal of *In-Vitro* and *In-Vivo* Antioxidant Activity Potential of the Ethanolic Leaf and Bark Extracts of *Ficus dalhousiae* Miq.

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ABSTRACT

The present study was aimed to investigate the antioxidant activity of ethanolic extract of the leaf and stem bark (EELFD, EEBFD) of *Ficus dalhousiae* Miq. Fam. Moraceae. The antioxidant activity of the extracts has been evaluated by using a variety of *in vitro* assays and an *in vivo* hepatoprotective model. The test extracts exhibited potential scavenging effects on DPPH, hydrogen peroxide and nitric oxide free radicals. In the *in vivo* hepatoprotective model the EELFD and EEBFD significantly increased the hepatic levels of reduced glutathione, antioxidant enzymes and decreased the lipid peroxidation. The free radical scavenging and antioxidant activities may be due to the presence of phenolic and flavonoid compounds of the EELFD and EEBFD. The result obtained in the present study justifies that *Ficus dalhousiae* is a potential source of natural antioxidant activity.

Keywords: *Ficus dalhousiae*, Flavonoid, Total Phenolic, Antioxidant, Free radical scavenging.

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ISSN: 0975-8585

INTRODUCTION

Most of the living organisms have well-organized defense system to protect themselves against oxidative stress induced by reactive oxygen species (ROS)[1]. Oxidative stress is an imbalance between the production of reactive oxygen species and their cellular detoxification by antioxidants. Oxidative stress or free radicals are involved in many diseases including atherosclerosis, Parkinson's disease, heart failure, myocardial infarction, Alzheimer's disease and fragile X syndrome [2]. Antioxidants are agents that scavenge the free radicals and prevent the damage caused by ROS and reactive nitrogen species (RNS) and are essential for maintaining optimal cellular activity, systemic health and well being [3]. The ROS is composed of super oxide anion (O_2^-), hydroxy (OH^-), hydroperoxyl (OOH^-), peroxyl (ROO^-), alkoxyl (RO^-) radicals and non free radicals like hydrogen peroxide (H_2O_2) hypochlorus acid (HOCl), ozone (O_3), singlet oxygen (O^1) and RNS contains nitric oxide (NO) peroxy nitrite (ONOO), nitrogen dioxide (NO_2) which cause oxidation of membrane phospholipids, proteins and DNA. Antioxidants significantly reduce the damage caused by the oxidants by neutralizing the free radicals before they attack the cells and prevent the damage to lipids, proteins, enzymes, carbohydrates and DNA. There are two classes of antioxidants viz. enzymatic and non-enzymatic. The enzymatic antioxidants produced endogenously and they include superoxide dismutase (SOD), catalase (CAT) and glutathione, peroxidase and non-enzymatic antioxidants include ascorbic acid, carotenoids, flavonoids, tocoperols and tannins, which are obtained from the plant species. Many plants from *Ficus* genus possess antioxidant constituents that provide efficacy by additive or synergistic activities. In traditional Indian medicine, *Ficus dalhousiae* (Moraceae), a rare and endemic species of Andhra Pradesh was used for liver damage and skin diseases [4-7].

The objective of this present study was to investigate the antioxidant activity of the ethanolic extract of leaf and stembark of the *Ficus dalhousiae* by using different *invitro*methods and an *in vivo* CCl_4 induced hepatoprotective model as well as determination of total phenolic, tannin and flavonoid content to evaluate the relationship between the antioxidant activity and the chemical constituents.

MATERIALS AND METHODS

Plant Material

Fresh leaves and bark of *Ficus dalhousiae* Miq were collected from Tirupati Hills, Andhra Pradesh, India during the month of May 2013. The plant material was taxonomically identified and authenticated by Dr. P. Jayaraman at the Plant Anatomical Research Centre (PARC), Tambaram, Tamil Nadu. A voucher specimen and herbarium have been preserved in the Department of Pharmacognosy, Ratnam Institute of Pharmacy, Nellore, Andhra Pradesh for future reference. The collected plant materials were then dried under shade and then powdered coarsely with a mechanical grinder and were extracted using petroleum ether and ethanol by cold maceration method. All the extracts of leaves and bark of *Ficus dalhousiae* Miq (PELFD, PEBFD, EELFD and EEBFD) were concentrated by using rotary evaporator and were stored in refrigerator till further use. The percentage yields of the extracts were 2.52%, 1.80%,18.45 % and 10.22% respectively.

Chemicals

All the chemicals used were of analytical grade.

Animals

Adult male Wistar albino rats weighing 170-200 g and female Swiss albino mice weighing 20-30gm were obtained from the animal house of Ratnam Institute of Pharmacy, Nellore and used for the studies. The animals were housed in polypropylene cages in a temperature-controlled room (22ºC±2ºC) with 45-65% relative humidity and were maintained in a 12 h light/ dark cycle, fed with standard rat and mice food and water *ad libitum.* Food pellets were with held overnight prior to dosing. The study was duly approved by the Institutional Animal Ethical Committee.

Preliminary phytochemical screening

Preliminary phytochemical screening was carried out with the extracts of leaf and stembark of *Ficus dalhousiae* (EELFD and EEBFD) for the detection of various phytoconstituents as per the standard methods [8,9].

Quantitative analysis of antioxidative components

Determination of Total Phenolic Content

Total phenols were determined by Folin-Ciocalteu reagent [10]. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram of sample, using a standard curve generated with gallic acid.

Determination of Total Flavonoid Content

Aluminium chloride colorimetric method was used for the determination of total flavonoidcontent [11]. The absorbance of a total flavonoid content expressed as quercetin equivalents (QE) in mg per gram dry weight of extracts was also determined using the standard curve of quercetin.

Determination of Tannin Content

The tannins were determined by Folin–Ciocalteu method. The tannin content was expressed in terms of mgof GAE/g of extract [12].

***In vitro* antioxidant activity**

1, 1-Diphenyl-2-pycrylhydrazyl (DPPH) free radical scavenging activity

The free radical scavenging activity of the ethanolic extract of the leaf and stem bark of *Ficus dalhousiae* (EELFD and EEBFD) was based on the scavenging activity of the stable 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical, by the method described by Blois. The IC_{50}values (IC_{50} value is the concentration of the sample required to inhibit 50% of radical) were then calculated[13].

Nitric oxide radical scavenging Activity

This method is based on the inhibition of nitric oxide radical generation from sodium nitroprusside, which were measured by the Gries- Illosvoy reaction with some modifications. The IC_{50} values were calculated [14,15].

Hydrogen peroxide scavenging activity

The hydrogen scavenging activity was determined according to method of Ruch with some modifications [16].

Acute toxicity test

Acute toxicity tests were performed according to OECD 423 guidelines [17].

***In vivo* antioxidant activity**

Male Wistar rats were divided in to seven groups comprising of six rats in each. Group I served as normal, Group II served as CCl_4 treated control, both received 1 ml of 0.5% CMC. Group III & IV received EELFD of 200 & 400 mg/kg body wt and group V and VI received EEBFD Group VII received the standard Vitamin E; at 50 mg/kg body wt. On the fifth day except for Group I, all other animals received 0.5ml/kg body wt of CCl_4, intraperitoneally. On the seventh day, all the animals were anaesthetized using diethyl ether and the liver was removed, weighed and homogenates were prepared and used for the following estimations. Catalase (CAT)

was estimated by following the breakdown of hydrogen peroxide [3,18]. Superoxide dismutase (SOD) assay based on inhibition of epinephrine auto-oxidation by the enzyme [19]. Lipid peroxidation was measured in terms of malondialdehyde (MDA) content was measured following the TBARS method [20]. Glutathione was measured according to the method given by Ellman [3,21] and the values were expressed as µmoles GSH mg/protein.

Statistical analysis

The data are expressed as Mean values ± S.E.M and tested with one-way ANOVA followed by Tukey's multiple comparison tests for the control and treatment groups using Graph Pad prism5.0. The results were expressed as the Mean ± S.E.M. $P< 0.05$ was considered as statistically significant.

RESULTS

The results of the phytochemical screening revealed that, the EELFD and EEBFD contain flavonoids, alkaloids, tannins, phenolic compounds, triterpenoids and carbohydrates.

For acute toxicity studies, the extract does not show any, change in behavior, toxicity signs and mortality up to 2000mg/kg body weight in the female albino mice.

Total phenol, tannin and flavonoid contents were determined in terms of mg gallic acid equivalents/ g and mg quercetin equivalent / g for the EELFD and EEBFD using the calibration curve and it was found to be 10±0.4, 12±4.5, 9.18±0.33, 24±2.3, 32±2.8, 10.12±0.12 respectively.The result reveals that the EEBFD have more phenol, flavonoids and tannin content than EELFD. There is excellent supported evidence that the Phenolic compounds in the plant materials possess free radical scavenging properties.

***In-vitro* Assay**

Among the ethanolic extract of leaf and stembark and the standard drug tested for antioxidant activity using DPPH, nitric oxide and hydrogen peroxide scavenging assay, the ethanolic extract of stem bark showed the maximum antioxidant activity with IC_{50} value of 101.52µg/ml, 95.80µg/ml and 105.00µg/ml respectively (Table. 1). However, the ethanolic extract of leaf showed the antioxidant value of 159.60µg/ml, 161.21 µg/ml and 158.12µg/ml respectively, which indicates the less antioxidant effect of the leaf than the stem bark. But these values are found to be lower than the standard ascorbic acid. Ascorbic acid, the known antioxidant exhibited IC_{50} value of 37.93µg/ml, 45.90µg/ml and 89.62 µg/ml, respectively.

Table 1: Antioxidant IC $_{50}$ values of EELFD and EEBFD in different *In vitro* models.

S.No	Method of Assay	EELFD (µg/ml)	EEBFD (µg/ml)	STD (µg/ml)
1.	**DPPH**	159.60	101.52	37.93
2.	**Nitric Oxide**	161.21	95.80	45.90
3.	**Hydrogen peroxide**	158.12	105.00	89.62

***In-vivo* Assay**

Table 2: *In-vivo* antioxidant activity of EELFD and EEBFD

Treatment	Dose (kg^{-1}body weight)	Catalase (µM /min /mg of tissue)	SOD (Unit/min//mg of tissue)	MDA (nM/mg of tissue)	Glutathione (µg/mg of tissue)
Normal (0.5% CMC)	1ml	9.913±0.3080	3.847±0.1120	5.150±0.1080	15.077±0.7510
Control CCl_4)	0.5ml	4.700±0.0870†††	2.1470±0.1050†††	11.7830±0.2800†††	5.467±0.262†††
EELFD	200mg/kg	5.470±0.1990^{ns}	2.1650±0.0290^{ns}	10.5300±0.1780*	6.3330±0.268^{ns}
EELFD	400mg/kg	6.110±0.3530*	2.865±0.1120**	9.983±0.3500**	10.9930±0.5190*
EEBFD	200mg/kg	6.273±0.2100**	2.669±0.0990*	6.560±0.1500***	8.200±0.1970^{ns}
EEBFD	400mg/kg	9.060±0.2250***	4.823±0.0640***	3.680±0.2020***	12.620±0.5440**
Vitamin E + CCl_4	50mg	9.420±0.1283***	4.734±0.1210***	4.980±0.2770***	13.217±0.7270**

Results are mean ± S.E.M. (n=6); ns- non significant, *p <0.05,** p <0.01 and ***p<0.001, when compared with control;†††p <0.001, when compared normal.

Administration of the ethanolic extract of leaf and stem bark at 200 and 400 mg/kg for 5 days prior to CCl_4 treatment caused a significant decrease in the level of MDA and increase in the levels of SOD, glutathione and catalase in liver ($p<0.001$), when compared to the CCl_4 treated control (Table. 2). A significant reversal of these changes towards normal group was also observed. The increase in the level of SOD and decrease in the level of MDA by EEBFD 400mg/kg was found to be higher than the standard drug VitaminE.

DISCUSSION

Many studies have investigated the role of antioxidant drugs and plant derived compounds in the prevention of oxidative stress. In the present study, the antioxidant capacity of the ethanolic extracts of leaf and stem bark of the plant *Ficus dalhousiae* (EELFD, EEBFD) were evaluated by standard *in vitro* and *in vivo* models. The *in vitro* studies using DPPH, nitric oxide and hydrogen peroxide scavenging assays showed strong antioxidant nature of the EEBFD compared to EELFD. In *in vivo* method, administration of the EEBFD at 400mg/kg body wt to treated groups increase the level of catalase, SOD and glutathione in liver of the CCl_4 intoxicated rats more significantly than EELFD of both doses (200 & 400 mg/kg body wt) when compared to diseased group. The present study also shows that EEBFD 400 mg/kg body wt depletes lipid peroxidation more significantly than EELFD 200 & 400 mg as observed by the significant decrease in MDA content of liver. Based on the obtained results it was revealed that EEBFD of 400 mg/kg body wt showed good free radical scavenging activity equivalent to that of a natural antioxidant Vitamin E, which is used as a standard.

CONCLUSION

In conclusion, this study was planned to investigate the *in vitro* and *in vivo* antioxidant activities of ethanolic extract of leaves and stem bark of *Ficus dalhousiae* and it evidently reveals that the stem bark extract has potent free radical scavenging effects in *in vitro* models, and exhibits a dose dependent antioxidant activity by inhibiting lipid peroxidation and enhancing antioxidant enzymes such as catalase, glutathione and SOD levels in the *in vivo* CCl_4 intoxicated rat model. These activities may possibly due to the presence of the phytoconstituents viz., flavonoids, phenolic compounds which may indirectly help to decrease the level of MDA and increases the antioxidant activity in rats. Further work is under progress to identify and isolate the active constituent responsible for the preventive effects of *Ficus dalhousiae* against various diseases caused by oxidative damage.

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