Indian Journal of Pharmaceutical Education and Research | Association of Pharmaceutical Teachers of India

Immunomodulatory and Antioxidant Activity of Chloroform Extract of *Ficus racemosa* Linn. Stem Bark

Ravi Kumar P, Sama Venkatesh and Madhava Reddy B*

G. Pulla Reddy College of Pharmacy, Mehdipatnam, Hyderabad-28, India.

ABSTRACT

Submitted: 23/2/2012 Revised: 16/8/2012 Accepted: 13/11/2012

The immunomodulatory effect of chloroform extract of *Ficus racemosa* stem bark (CEFR) was evaluated in pyrogallol immunosuppressed rats by measuring humoral antibody response to SRBC (Sheep Red Blood Cells) and found that the primary response on 13th day was 7.67 (The level of rank on 0-12 scale indicating the highest dilution in which the antibodies were found) and secondary response on 20th day was 8.67 at a dose of 100 mg/kg body weight. The primary and secondary responses were found as 9.17 and 11.00 respectively at a dose of 200 mg/kg body weight. These results indicates the improvement in humoral antibody mediated immunity when compared to response produced by the standard, Vitamin-E, which has shown a primary response of 6.33 and a secondary response of 7.50 at 150mg/kg dose. The results are significant (P<0.01) and dose dependent. Phagocytic index and neutrophil index for CEFR were determined in normal mice to assess cellular immunity. The phagocytic index values were 0.0269 (natural logarithmic value of the phagocyte number) at 100 mg/kg dose and 0.0375 at 200 mg/kg dose. These values are greater than control value i.e. 0.0164, revealing the increase in the number of phagocytes and hence cellular immunity, significantly (p<0.05). Neutrophil index values of CEFR were found as 7400 and 7933 at 100 mg/kg and 200 mg/kg doses respectively, which are higher when compared to control value i.e. 7000, indicating the increase in the number of neutrophils and there by confirming improvement in cellular immunity. Oxidative stress also assessed in the blood of pyrogallol immunosuppressed rats on 22nd day of the experiment with reference to lipid peroxidation (LPO), reduced glutathione content (GSH) and catalase activity (CAT). The LPO level found to decrease significantly (P< 0.05) when compared to that of standard, Vitamin-E. GSH and CAT were increased significantly (P<0.05) when compared to that of standard, Vitamin-E. Thus, it was evident that CEFR enhanced the activity of endogenous antioxidant system. Finally the study concluded that CEFR stem bark possess promising immunomodulatory and antioxidant activities.

Keywords: *Ficus racemosa,* Immunomodulation, Humoral immune response, SRBC, Phagocytic index, Neutrophil index.

INTRODUCTION

It has been proved that immune system plays a key role in biological adaptation, contributing to homeostasis and establishment of integrity of the body. It is also known that immune system involves pathophysiology of many diseases. Immunomodulator offers important contribution in a variety of diseased conditions when host defense mechanism need to be activated i.e. in case of impaired immune responsiveness or when a selective immunosuppression has to be induced as in case of autoimmune disorders or organ transplantation[1]. According to ayurveda RASAYANA is the drug used for enhancement of body resistance against infections and acts as adaptogen, immunomodulator and antimutagenic[2,3]. Many plants used in traditional medicine were found to have immmunomodulatory properties and it could be because of synergistic effects of variety of phytoconstituents such as phenolics, terpenoids, steroids and flavonoids[4,5].

Ficus racemosa Linn. (Syn: *F. glomerata* Roxb,) belongs to family Moraceae was reported to possess ethno medicinal uses in treatment of asthma, diabetes, threatened abortions, menorrhagia, diarrhoea, haemorrhages, leucorrhoea etc. The bark was also reported to possess free radical scavenging, glucose lowering, antidiuretic, antibacterial, antiulcer, antitussive, larvicidal, anti-inflammatory and chemomodulatory activities[6]. In view of the medicinal importance and based on the literature, the stem bark of *Ficus racemosa* is investigated for immunomodulatory effect, which has not been studied sofar.

***Address for Correspondence:**

Madhava Reddy B, Professor and Principal, G. Pulla Reddy College of Pharmacy, Hyderabad- 500 028, India

E-mail: madhavareddyb@ymail.com

MATERIALS AND METHODS

Collection of plant material

The stem bark of *F. racemosa* was collected from G. Pulla Reddy Educational Campus, Mehdipatnam, Hyderabad in the month of December, 2010. The plant was identified and authenticated by Dr. Prabhakar Reddy, Taxonomist, Osmania University, Hyderabad. A Voucher specimen is deposited in laboratory of Phytochemistry and Pharmacognosy, G. Pulla Reddy College of Pharmacy, Hyderabad. The collected bark was made free from extraneous matter and shade dried. The dried bark was pulverized, packed and stored in well closed container.

Extraction and screening

The dried stem bark powder (1000 g) was successively extracted with chloroform, ethyl acetate and methanol in soxhlet extractor. The resultant extracts were concentrated

under reduced pressure and dried in desiccator. Preliminary phytochemical screening was carried out as per the standard test procedures.

Experimental protocol

Experimental procedures were carried out in strict accordance with guideline prescribed by the Committee for the Purpose of Control and Supervision on Experimentation on Animals (CPCSEA) and were approved by the Institutional Animal Ethics Committee. Wistar rats (175-225 g) and albino mice (25-30 g) of either sex were purchased from M/s. Mahaveer Enterprises, Hyderabad. The animals were maintained under standard environmental conditions and had free access to pellet diet and water *ad libitum*.

Acute toxicity test

According to Organization for Economic Co-operation and Development (OECD) draft guideline 423, the study was conducted in albino mice and rats of either sex. The animals were divided into two groups containing three animals each. They were fasted overnight and maintained with water *ad libitum.* CEFR was administered orally in the form of suspension in olive oil at a dose of 2000 mg/kg body weight to first group animals and observations were recorded after 4 hrs and upto 24 hrs and 48 hrs. Then the drug was administered to three animals of second group and observations were recorded same as that of first group i.e. test was carried out in a two step manner.

Humoral antibody response to SRBC in immunosupressed rats[4,7]

The method of pyrogallol induced immunosupression was employed to study the immunomodulatory potential of CEFR. The maximum tolerable dose of pyrogallol was determined in rats by administering 100, 75 and 50 mg/kg/day, for three groups (6 in each group) of rats, for 7 days and observed up to 30 days for mortality.

The experimental rats were randomly divided into 6 groups, consisting of 6 animals in each group. Group-I served as negative control and received equivalent volume of 0.1% sodium CMC suspension as vehicle, orally. All the groups except Group-I were administered pyrogallol 75mg/kg/day, i.p; for 7 days. Group –II served as positive control. Group- III received Vitamin -E 150mg/kg/day, p.o; from 1-22 days. Group- IV and V were considered as test groups and were administered the test extract 100 mg/kg/day & 200 mg/kg/day, p.o, respectively, from 1-22 days. On 7^{th} & 13^{th} days all the groups were received sheep red blood cells (SRBC $5X10^8$ cells /100 gm of body weight) i.p. in normal saline, as antigenic material to sensitize them for immunological studies. On 13^{th} and 20^{th} day, measurement of antibody titer by haemagglutination test was carried out as per Miller *et al*[8] method. Blood samples were collected from the retro orbital plexus under mild ether anesthesia and centrifuged to obtain serum. The antibody titer was determined using micro plates (U bottomed 96 well plate). Each well of micro titer plate was filled initially with 50µl of normal saline. 50µl of serum was added to 50 µl of normal saline in the first well of the micro titer plate. Subsequently the 50 µl diluted serum was removed from the first well and added to the next well to get two fold dilutions of the antibodies present in the serum. Same procedure was carried out till the last well of that row i.e.12^{th}well. Likewise equal volumes of individual serum samples of each group were pooled, so that the antibody concentration of any of the dilutions was half of the previous dilution. To each well, 25µl of 1% SRBC suspension in normal saline was added and the micro titer plates were incubated at 37^0C for one hour. Then, observed for haemagglutination. The antibody titer was expressed in a graded manner, the minimum dilution being ranked as 1 and the mean ranks of different groups were compared for statistical significance. The antibody titer obtained on 13^{th} day after sensitization with SRBC considered as primary humoral immune response and that on 20^{th} day as secondary humoral immune response.

Cellular Immune Response in Normal Mice

To study the cellular immune response, carbon clearance test (phagocytic index) and neutrophil index were performed in normal mice.

1) Carbon clearance test[3,9]

The mice were divided into 3 groups of 6 animals each. Group-I was control and received 0.1% sodium CMC suspension orally as vehicle. Groups II & III were test groups and administered CEFR in sodium CMC suspension at doses of 100 mg/kg/day & 200 mg/kg/day, p.o., respectively for 7 days. At the end of seven days the mice were injected with 0.1 ml of carbon ink (Camel fountain pen ink, black color) suspension (1.6%v/v in 1.0% gelatin, in normal saline) via the tail vein. At 0 min (t_1) and 15 min (t_2), after injecting the carbon ink, blood samples were withdrawn from the retro-orbital plexus, into tubes containing 5 µl of (20 mg/ml) EDTA solution. A 25 µl of blood sample was lysed in 2.0 ml of 0.1% sodium carbonate solution and the absorbance was measured at 660 nm using 0.1% sodium carbonate solution as blank. Then, the phagocytic index was calculated using the following formula.

$$K = \frac{\ln A_1 - \ln A_2}{(t_2 - t_1)}$$

Where A_1 and A_2 are optical densities at time t_1 and t_2 respectively.

2) Neutrophil index (NI)[3,10]

Three groups of mice were treated same as in case of carbon clearance test. On 7th day blood was withdrawn from all the groups and analysed for Total Leucocyte Count (TLC) and Differential Leucocyte Count (DLC).Then, neutrophil index was calculated by the following formulas.

$$\% \text{ Neutrophil} = \frac{\text{Neutrophil count} \times 100}{\text{TLC}}$$

NI = TLC X % Neutrophil

Oxidative stress parameters[7]

These were assessed in the blood of the immunosuppressed rats, withdrawn from the retro-orbital plexus on day 22 of the experiment.

1) Lipid peroxidation

To 2.0 ml of the 5% suspension of RBC in normal saline, 2.0ml of 28% trichloroacetic acid was added and centrifuged. To the supernatant, 1.0 ml of 1% thiobarbituric acid (TBA) was added, heated in boiling water for 60 min and then cooled. The absorbance was measured at 532 nm. Lipid peroxidation was calculated based on the molar extinction coefficient ($1.56X10^5$) of malondialdehyde (MDA) and expressed in terms of nanomoles of MDA/gHb[11].

2) Reduced glutathione (GSH)

Blood glutathione was measured by addition of 0.2 ml of whole blood to1.8 ml distilled water followed by 3.0 ml of precipitating mixture (1.67 g metaphosphoric acid, 0.2 g EDTA and 30g NaCl to make 100 ml solution). It was centrifuged at 5000 xg for 5 min and 1.0 ml of the filtrate was added to 1.5 ml of phosphate buffer solution (pH 7), followed by 0.5 ml of DTNB reagent. The absorbance was measured at 412 nm. GSH content was calculated and expressed as units per ml[12].

3) Catalase (CAT)

Catalase activity was determined in erythrocyte lysate using Aebi's method[13] with some modifications. 50 µl of the erythrocyte lysate was added to a cuvette containing 2.0 ml of phosphate buffer (pH 7) and 1.0 ml of 30 mM H_2O_2. Absorbance was measured immediately at 0 min and at 1 min at 240 nm. The molar extinction coefficient ($43.6Mcm^{-1}$) of H_2O_2 was used to determine the catalase activity. One unit of activity is equal to one millimole of H_2O_2 degraded per minute and is expressed as units per ml[13].

Statistical analysis

The data was statistically analyzed by student "t-test". All the values were expressed as Mean ± SEM and were considered as statistically significant when $p<0.05$.

RESULTS .

CEFR did not produce any signs of mortality in mice and rats at the dose 2000mg/kg, p.o. even after 24 hrs. Hence, the extract is considered as safe up to the dose level of 2000 mg/kg. Thus, LD_{50} value of the extract is 2000mg/kg. Hence 100 mg/kg and 200mg/kg doses were selected for the experiments.

The CEFR is investigated for its effect on humoral immune response, cellular immune response and antioxidant properties. In the humoral immune response to SRBC, the primary response on 13th day and secondary response on 20th day were measured in terms of the antibody titer, produced as a response to sheep red blood cells (which acts as an antigen) in pyrogallol induced immunosuppressed rats. The effectiveness of CEFR on humoral immune response is determined by the haemagglutination. The compound causing haemagglutination at highest dilution indicated by the higher well number (96 well plate) is considered as more effective because each successive well gets double diluted in terms of serum containing antibodies. Here in this test, in primary response, CEFR at 100 mg/kg dose has shown haemagglutination in 7.67th well (average of 6 animals) and at 200 mg/kg dose in 9.17th well as compared to Vit-E, in 6.33 well and control in 5.5 well. Similarly the secondary response also has shown in 8.67th well and 11th well at 100 mg and 200 mg doses respectively. So, this experiment clearly indicates that CEFR has caused considerable immunostimulation in rats at both the doses in primary response and secondary response and the immunostimulation is also dose dependent. The results are shown in Table-1.

The cellular immune response of CEFR was studied by carrying out carbon clearance test and neutrophil index. In carbon clearance test, the immune response is measured by the phagocytic index wherein the effectiveness of the macrophages in engulfing the ink particles (foreign body) introduced into the mice is determined. The greater the difference in the absorbance of the lysed blood sample withdrawn immediately after introduction of carbon and after

Table 1: Effect of CEFR on Humoral immune response to SRBC

Group	Treatment (mg/kg)	Primary response (13th day)	Secondary response (20th day)
I	Control	5.50± 0.22	6.17± 0.31
II	Pyrogallol- 75	3.50±0.22**	5.33± 0.21*
III	Pyrogallol-75 +Vitamin-E-150	6.33±0.21**	7.50± 0.22**
IV	Pyrogallol-75 + CEFR -100	7.67± .21**	8.67± 0.21**
V	Pyrogallol-75 + CEFR -200	9.17±0.31**	11.00± 0.37**

Values are mean ± SEM, N=6. *P<0.05; **P<0.01
CEFR : Chloroform extract of *Ficus racemosa* stem bark

15 min indicates the effectiveness of the phagocytosis. The animals treated with CEFR have shown a higher difference (0.0269 at 100mg/kg, 0.0375 at 200mg/kg) in the absorbance indicating increased phagocytic activity as compared to the control animals (0.0164) confirming the effect of the extract as immunomodulator. The results are shown in Table-2.

Neutrophils also one of the major immune responsive cells. CEFR treated animals have shown considerable increase in the neutrophil count (NI) as compared to control animals indicating the effectiveness of the extract as immunomodulator. The results are shown in Table-3.

CEFR also studied for its antioxidant properties by testing its effect on lipid peroxidation, reduced glutathione and catalase activities, simultaneously along with humoral immunity studies in pyrogallol induced immunosuppressed rats. In lipid peroxidation, malondialdehyde (MDA) is the end product of the oxidative stress, the measurement of which gives the effect of extract in preventing the oxidative stress. The extract has shown decreased levels of MDA (383.55 at 100 mg/kg dose and 264.96 at 200 mg/kg dose) as compared to untreated animals (618.59) and control animals (396.37). In reduced glutathione test CEFR has caused the increased levels of reduced glutathione units (0.130 at 100mg/kg dose and 0.195 at 200mg/kg dose). In catalase activity test CEFR has shown positive results by increasing the catalase activity as shown in the table-4.

Table 2: Effect of CEFR on Carbon clearance test.

Group	Treatment	Phagocytic Index
I	Control	0.0164 ± 0.002
II	CEFR-100 mg/kg	0.0269 ± 0.002*
III	CEFR-200 mg/kg	0.0375 ± 0.003*

Values are mean ± SEM, N=6. *P< 0.05,
CEFR : Chloroform extract of Ficus racemosa stem bark

Table 3: Effect of CEFR on Neutrophil index

Group	Treatment	Neutrophil Index
I	Control	7000.25 ± 0.10
II	CEFR-100 mg/kg	7400.13 ± 208.04
III	CEFR-200 mg/kg	7933.30 ± 66.67**

Values are mean ± SEM, N=6. **P< 0.01,
CEFR : Chloroform extract of Ficus racemosa stem bark

DISCUSSION

When rats were sensitized with SRBC, an antigen gets diffused in the extra vascular space and enters the lymph node via the lymphatics. Particulate antigens are taken up by macrophages, lining the sinuses or disperse in the lymphoid tissue and are processed. Small, highly antigenic peptides are combined with MHC class-II molecules. B cell with receptors for antigens binds and internalizes it into an endosomal compartment, process and presents it on MHC class-II molecules to the TH_2 cells. These cells are triggered to proliferate, giving rise to clones of large number of daughter cells. Some of the cells of these expanding clones serve as memory cells, others differentiate and become plasma cells that make and secrete large quantities of specific antibodies. During a primary response, IgM is secreted initially, often followed by a switch to an increasing proportion of IgG[14,15]. The magnitude of the secondary response to the same antigen is amplified in terms of antibody production. In the present study, the anti SRBC- antibody titer was raised in *F.racemosa* extract treated groups when compared to the control, pyrogallol and Vit-E treated groups, in both primary and secondary responses.

In Carbon Clearance test carbon ink was injected into the animals via tail vein, the carbon particles are engulfed by the macrophages. The phagocytic activity of reticuloendotheilial system was measured by the rate of removal of carbon particles from the blood stream and considered as cellular immune response[16]. There was a significant ($P< 0.05$) increase in the phagocytic activity of CEFR at both doses and it is dose dependant. The increase in the carbon clearance index in plant extract treated mice reflects the enhancement of the phagocytic function of macrophages and non-specific immunity. Activated immune cells, often called as neutrophils, secrete Cytokines. These constitute the majority of the blood leucocytes. Effect of CEFR on neutrophil activation is determined by calculating the neutrophil index. CEFR at a dose of 200 mg/kg significantly increased the number of neutrophils when compared with the control.

The cellular and humoral components of the immune system are adversely affected by free-radical induced oxidative

Table 4: Effect of CEFR on Oxidative stress

Group	Treatment (mg/kg)	Lipid Peroxidation (MDA/g Hb)	Reduced glutathione (Units/ml)	Catalase (Units/ml)
I	Control	396.37± 12.76	0.093± 0.007	2628.44± 89.82
II	Pyrogallol- 75	618.59 ± 10.03*	0.067± 0.007*	1041.28± 38.86*
III	Pyrogallol-75 +Vitamin-E-150	365.38± 22.87*	0.080± 0.002*	1059.17± 46.55*
IV	Pyrogallol-75 +CEFR-100	383.55± 12.1*	0.130± 0.002*	1424.30± 56.7*
V	Pyrogallol-75 + CEFR- 200	264.96± 11.18*	0.195± 0.005*	1798.15± 45.87*

Values are mean ± SEM, N=6. *P<0.05, CEFR : Chloroform extract of *Ficus racemosa* stem bark

stress, particularly sensitive to increased levels of reactive oxygen species, which may cause premature immunosenescence[17,18]. The endogenous antioxidant system prevents the deleterious influence of the free radicals on the immune cells and preserves their normal function. Impairment, in immune system leads to over utilization of endogenous antioxidants[19]. In view of this, it appears that pyrogallol, might impair the immune response through oxidative stress. This is evident by increase in LPO levels, decrease in GSH content and decrease in catalase activity, after pyrogallol treatment. The peroxidative toxicity of pyrogallol in vivo may also be through its effect on iron release from ferritin[20], which can induce lipid peroxidation via Fenton Haber Weiss reaction. CEFR, being a potent antioxidant counteracted the effects of pyrogallol. The antioxidant activity of CEFR can be attributed to the phenolic compounds, which are generally known as good antioxidants[21].

CONCLUSION

The results obtained in the present study have shown that CEFR significantly increased the humoral antibody mediated immunity as evident by showing the agglutination at a highest dilution as compared to the standard compound vitamin-E, at 100 mg/kg and 200 mg/kg doses. The cellular immunity levels were also increased as evident by an increase in the phagocytic index and neutrophil index at both the doses tested. The results of lipid peroxidation, reduced glutathione content and catalase activity of CEFR revealed the improvement in the activity of endogenous antioxidant system in immunosuppressed rats. These results clearly indicate that the stem bark of *Ficus racemosa* possess immune boosting properties and suggest its therapeutic usefulness in disorders of immunological origin and in diseases in which antioxidant system is adversely affected.

REFERENCES

1. Joshi Uttara P, Mishra SH. Evaluation of aqueous and methanolic extracts of *Pistacia integerrima* galls as potent immunomodulator. Phcog.Mag 2008; 4(14): 126-31.
2. Thatte UM, Dahanurkar SA. Rasayana concept: Clues from immunomodulatory therapy. In:, Upadhyay SN, editor. Immunomodulation, New Delhi: Narosa Publishing House;1997. 41-5.
3. Ghaisas MM, Shaikh SA, Deshpande AD. Evaluation of the immunomodulatory activity of ethanoloic extract of the stem bark of *Bauhinia variegata* Linn. Int J Green Pharm.2009; 70-4.
4. Mallurwar VR, Pathak AK. Studies on immunomodulatory activity of *Ficus religiosa.* Indian J Pharm Edu Res 2008; 42: 341-3.
5. Kirtikar KR, Basu BD. Indian Medicinal Plants. 2nd ed.Vol I, New Delhi: Periodical Book Agency; 1998. 686-9.
6. Padmaa Paarakh M. *Ficus racemosa* Linn.- An overview. Nat Prod Rad. 2009; 8(1): 84-90.
7. Joharapurkar AA, Wanjari MM, Dixit PV, Zambad SP, Umathe SN. Pyrogallol: A novel tool for screening immunomodulators. Indian J.Pharmacol 2004; 36(6): 355-9.
8. Miller LE, Ludke HR, Peacock TE, Torner RH. Manual of Immunology. 2nd ed. London: Lea and Febiger; 1991.1-57.
9. Bafna MR, Mishra SH. Immunomodulatory activity of methanol extract of flower-heads of *Sphaeranthus indicus* Linn. J Herb Pharmacother 2007; 25-37.
10. Mallurwar VR, Johrapurkar AJ, Durgakar NJ. Studies on immunomodulatory activity of *Mucuna pruriens.* Indian J Pharma Educ Res 2006; 40: 205-7.
11. Stocks J, Dormandy TL. The autooxidation of human red cell lipids induced by hydrogen peroxide. Br J Hematol 1971; 20: 95-111.
12. Ellman GL. Tissue sulphydryl groups. Arch Biochem Biophys 1959; 82:70-7.
13. Aebi H. Catalase *in vitro.* Methods Enzymol 1984; 105: 121-6.
14. Goldsby RA, Kindt TJ, Osborne BA, Kuby J. Immunology. 5th ed. New York: W.H.Freeman and Co; 2003; 1-25.
15. Dale MM, Formanj C. Text book of Immunopharmacology. 2nd ed. Oxford: Blackwell Scientific Publication; 1989; 14.
16. De P, Dasgupta SC, Gomes A. Immunopotentiating and Immunoprophylactic activities of Immune 21: A polyherbal product. Indian J Pharmacol 1998; 30: 163-8.
17. Gate L, Paul J, Ba GN, Tew KD, Tapiero H. Oxidative stress-induced pathologies: The role of antioxidants. Biomed Pharmacother 1999; 53: 169-80.
18. Knight JA. Review: Free radicals, antioxidants and the Immune system. Ann Clin Lab Sciss 2000; 30: 145-58.
19. Devasagayam TP, Sainis KB. Immune system and Antioxidants, especially those derived from Indian medicinal plants. Indian J Exp Biol 2002; 40: 639-55.
20. Agarwal R, Sharma PK, Rao GS. Release of iron from ferritin by metabolites of benzene and superoxide radical generating agents. Toxicology 2001; 168: 223-30.
21. Li AS, Bandy B, Tsang SS, Davison AJ. DNA- breaking versus DNA protecting activity of four phenolic compounds *in vitro*. Free Radic Res 2003; 33: 551-6.

****