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Research Article

IN VIVO EVALUATION OF IMMUNOMODULATORY POTENTIAL OF FERULIC ACID

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ABSTRACT

Immunology is a branch of biomedical science that involves all aspects of the immune system that recognize pathogens and initiate defense response that involve induction, expression, amplification or inhibition of phase of the immune response. Plant based medicines have potential immunomodulatory activity that can justify their use in past. Ferulic acid (FA) is a polyphenol abundant in several plants, possesses activity, such as antioxidant, antimicrobial, anti-inflammatory, anti-thrombosis, anti-cancer and also used in age-related diseases such as neurodegenerative disorders, cardiovascular diseases and diabetes. The study was proposed to determine immunomodulatory effects of FA. Assessment of immunomodulatory activity of FA was done by carbon clearance test, delayed type hypersensitivity (DTH) reaction, neutrophils adhesion test, effect on serum immunoglobulins and cyclophosphamide induced neutropenia. FA at doses 200 mg/kg produced significant increase in phagocytic index ($P<0.01$) which indicated stimulation of reticuloendothelial system, neutrophil count ($P<0.05$) in nylon fibers which causes the stimulation of neutrophils towards the site of inflammation, the serum immunoglobulin levels ($P<0.05$) shows direct measure of T and B cell activity, TLC count ($P<0.05$) and prevent neutropenia through activation of macrophages and increase in DTH response ($P<0.05$) which indicate stimulatory effect on lymphocyte. On the basis of present investigation, it can be concluded that FA has potential to stimulate overall immune system in the experimental animals.

Keywords: Ferulic acid, immunomodulatory, immunoglobulins, immunity

INTRODUCTION

Immunology is a branch of biomedical science that covers the study of all aspects of Immune system that comprises a complex defense mechanism to protect from invading agents and able to generate varieties of cells and molecules capable of induction, expression, amplification or inhibition of phase of the immune response [1]. Immunomodulation is a procedure which can alter the immune response of an organism by stimulating both nonspecific and specific immune responses; if immunomodulation results in an enhancement of immune response, termed as an immunostimulants while immunosuppressants reduce resistance against infections and stress [2]. Immunomodulatory drugs modify immune response by increasing or decreasing the production of antibodies and are valuable for prevention of immunodeficiency related disorders like AIDS. Immunostimulators enhance the immune response against infectious diseases, tumors and immunodeficiency. Immunosuppressives reduce the immune response against organs transplant and autoimmune diseases such as lupus, allergies [2,3].

Numerous plant-based products have been enlisted for immunomodulatory activity and many of these plant products are accessible to boost the immune system. There is great interest in the use of natural compound such as phenolics which have potential action against a wide range of diseases. Ferulic acid (FA) a phenolic compound has antioxidant and free radicals scavenger activity [4] and reported to have anticancer [5], antioxidant [6,7,8], anti-inflammatory [9], antidiabetic [10], hepatoprotective [11] , neuroprotective [12], anti-atherogenic [13], *in vitro* immunomodulatory [14] activity but no such evidences are available which support its activity on immune system. The current research work was aimed to *in vivo* investigation of immunomodulatory potential of FA in rats.

MATERIALS AND METHODS

Chemicals

FA was purchased from National Chemicals, Vadodara. Cyclophosphamide was purchased from Zydus Oncosciences. Levamisole tablet (Khandelwal laboratories) was purchased from local market. All the other chemicals used in study were of analytical grade.

Experimental Animal

Wistar albino rats of either sex, weighing about 150 to 200 gm were housed in group of six rats under controlled light and temperature environment. Food and water was provided *ad libitum*. The study designed was performed after prior approval by the institutional animal ethics committee of B. R. Nahata College of Pharmacy, Mandsaur bearing Registration number 918/AC/05/CPCSEA.

Preparation of Alsever's solution

All the solids (Citric acid -0.055gm, Sodium citrate -0.8gm, Glucose -2.05gm, Sodium chloride -0.42gm) was weighed and dissolved in distilled water, made the volume up to 100 ml and stored in refrigerator [15].

Antigen Preparation
Fresh sheep red blood cells will be collected from slaughter house in Alsever's solution (stock solution). Adequate amount of stock solution was taken and allow standing at room temperature. It was washed three times in large volumes (30 ml) of pyrogen free 0.9% normal saline and adjusted to a concentration of $0.5x10^9$ cells/ml for immunization and challenge [16].

Blood withdrawal
For withdrawing blood sample, the animal was lightly anaesthesized. A fine capillary was gently inserted into the lower angle of eye at 45º and blood was withdrawn from retro orbital plexus [17].

Grouping
The animals were divided in to four groups consisting of six animals each. A group of six untreated rats were taken as control (Group I). Group II were taken as standard and treated with Levamisole 50 mg/kg/day. FA was fed orally at a dose of 100 mg/kg/day (Group III) and 200 mg/kg/day (Group IV) for assessment of *in vivo* immunomodulatory effect.

EXPERIMENTAL PROTOCOL

Carbon clearance test
Weighed FA was dissolved in 5% CMC. The animal was treated with FA or vehicle for 5 days orally. After 48 h of the last dose of the drug, rats will have injected with 0.1 ml of Indian ink via the tail vein. Blood samples will with drawn at 0 min and 15 min. A 50 µl blood sample will be mixed with 4 ml of 0.1% sodium carbonate solution and the absorbance of this solution will be determined at 660 nm. The phagocytic index K will be calculated using the following equation:

$$K = (\text{Log OD1-Log OD2})/15$$

Where OD1 and OD2 are the optical densities at 0 and 15 min respectively [18].

Neutrophil Adhesion Test
Eighteen male rats were fasted overnight and randomly divided in to three groups and each group having equal animal (n=6). Control Group I treated with5% carboxy methyl cellulose (CMC), Group II (standard) were treated with levamisole 50 mg/kg and Group III (test) treated with FA 100 mg/kg body weight.
Albino Wistar rats were divided into different groups and were treated orally with drug or vehicle for 14 days. On day 14, blood samples were collected from the retro-orbital plexus into heparinized vials and the differential leukocyte count (DLC) was determined after fixing the blood smear and staining with the Leishmann's stain. After the initial counts, blood samples were incubated with 80 mg/mL of nylon fibers for 10 min at 37°C. The incubated blood samples were again analyzed for DLC. The difference in the neutrophil count before and after incubation of blood sample with nylon fibers was determined [19]. The percentage neutrophil adhesion was calculated by using the following formula:

$$\text{Neutrophil adhesion (\%)} = NI_u - NI_t \times 100 / NI_u$$

NI_u = neutrophil index of untreated blood
NI_t= neutrophil index of treated blood

Effect on serum immunoglobulins
Albino rats were treated with the drug orally for 21 days. Six hours after the last dose, blood samples were collected and the serum was separated by centrifugation, the collected serum was used for estimation of immunoglobulin levels. Briefly, for each serum sample to be analyzed, a control tube containing 6 mL of distilled water and a test tube containing 6 mL of zinc sulphate solution were prepared. To each, 0.1 mL of serum was added from a pipette. They were inverted to enable complete mixing of the reagents and left to stand for 1 h at room temperature in plugged tubes. The pH of the solution was monitored throughout the experimental period using pH meter. The first tube served as blank and the second tube was taken as sample. The turbidity developed was measured using a digital nepheloturbidity meter. The turbidity obtained (sample-blank) was compared with that obtained with standard barium sulphate (BaSO4) solution. The turbidity obtained with this solution was expressed as 20 zinc sulphate turbidity (ZST) units [19].

Cyclophosphamide induced neutropenia
Albino wistar rat received the drug orally for 10 days. On 10th day, a neutropenic dose of cyclophosphamide (200 mg/kg, s.c.) was administered and labeled as day zero. Blood samples were collected through retro-orbital vein. The total leukocyte count (TLC) and DLC were performed prior to and on day 3 after injection of cyclophosphamide. The TLC and DLC in treated groups were compared with the values of the control group [19].

Delayed type hypersensitivity (DTH) response
Six animals per group are immunized by *i.p.* administration of 1×10^9 SRBCs/rat after seven days pretreatment and challenged by *s.c.* administration of 0.25×10^9 SRBCs/rat into right hind foot pad after seven days of immunization. The volume for the inflammation was measured after challenge at 24 and 48 h by Plethysmometer [20].

Statistical Analysis

The data were analyzed by Graph pad Prism 5.0 software and results are expressed as Mean ± S.E.M. The significance of the difference in the response of treatment groups in comparison to the control was determined by one way of variance (ANOVA) followed by "Dunnet test". $P<0.05$ was considered significant, $p<0.01$ considered very significant and $p<0.001$ considered highly significant.

RESULTS

Effect of FA on Phagocytic Index
Administration of FA at doses 100mg/kg and 200 mg/kg produced increase in clearance of carbon particles from blood as indicated by a significant increase in phagocytic index ($P<0.01$) for 100 mg/kg and very significant increase in phagocytic index ($P<0.001$) for 200mg/kg (Figure 1).

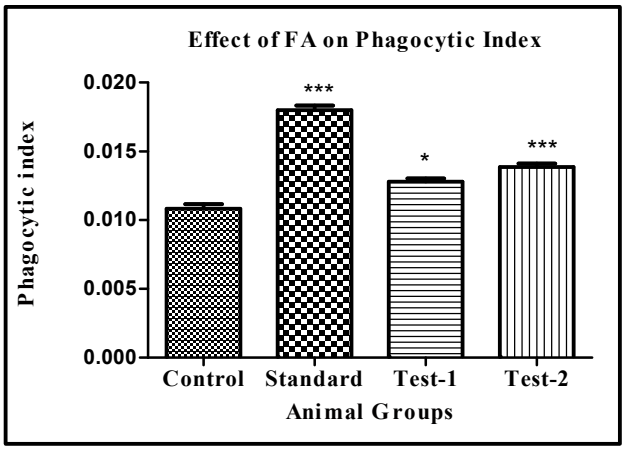

Figure 1: Effect of FA on Phagocytic Index in experimental animals. Values are expressed as Mean ± SEM (n=6).

Effect of FA on Neutrophil Adhesion

A reduction in neutrophil due to adhesion of neutrophils to the nylon fibers was observed. The percentage reduction in the neutrophil count in nylon fibers treated blood samples from the standard ($P<0.001$) and test group ($P<0.05$) was significantly more compared to the control group (Figure 2).

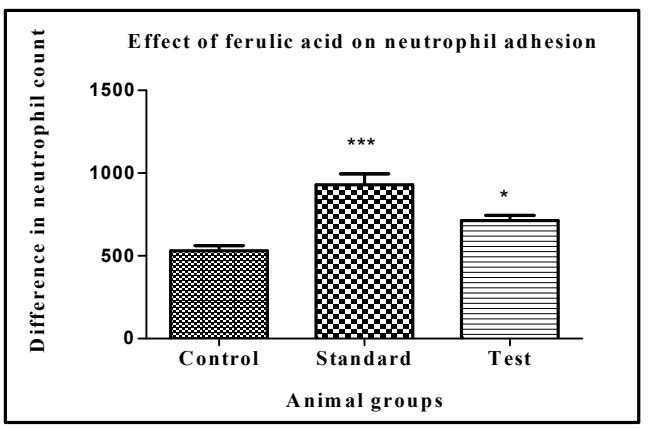

Figure 2: Effect of FA on Neutrophil Adhesion in experimental animals. Values are expressed as Mean ± SEM (n=6).

Effect of FA on Serum Immunoglobulin

The administration of FA significantly increased the serum immunoglobulin levels when compared to control (Figure 3).

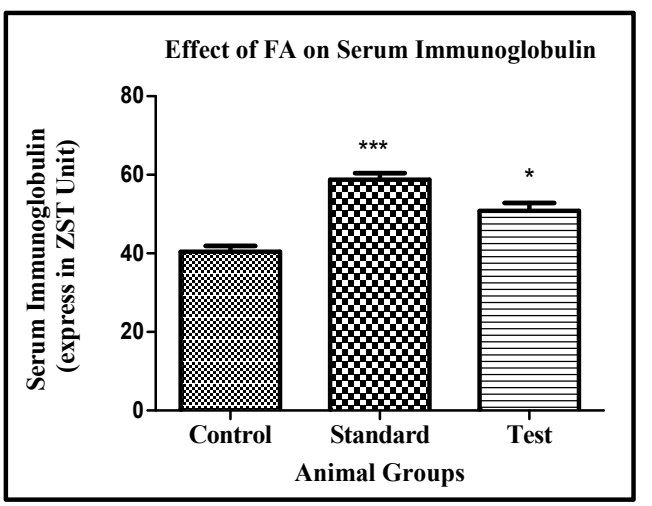

Figure 3: Effect of FA on Serum Immunoglobulin in experimental animals. Values are expressed as Mean ± SEM (n=6).

Effect of FA on Cyclophosphamide induced neutropenia

Administration of Cyclophosphamide (200 mg/kg, sc) produced a decrease in neutrophil count in all groups. However, the reduction in neutrophil count was less in levamisole and FA treated groups when compared to control. The levamisole administration produced a highly significant effect in TLC count and neutrophil reduction and FA produces significant effect in TLC count and neutrophil reduction when compared to Disease control (DC) (Figure 4,5).

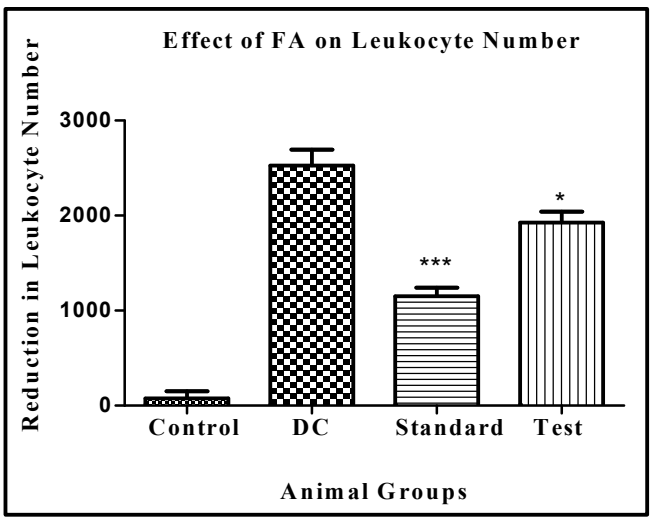

Figure 4: Effect of FA on Leukocyte number in experimental animals. Values are expressed as Mean ± SEM (n=6).

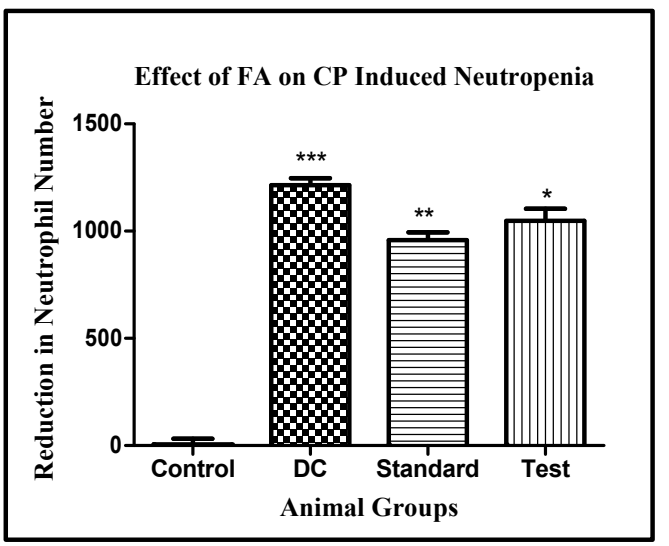

Figure 5: Effect of FA on CP induced Neutropenia in experimental animals. Values are expressed as Mean ± SEM (n=6).

Effect of FA on Delayed type hypersensitivity (DTH) response

Administration of FA 200 mg/kg increased the delayed type of hypersensitivity response significantly in terms of increase in paw thickness when compared to control group. Levamisole 50 mg/kg showed very significant effect in delayed type of hypersensitivity response (Figure 6).

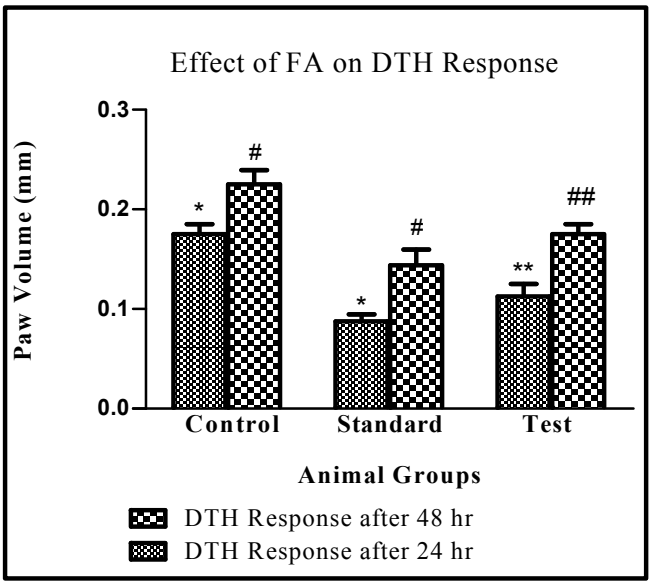

Figure 5: Effect of FA on DTH response in experimental animals. Values are expressed as Mean ± SEM (n=6).

DISCUSSION

Immunomodulatory agent obtained from the plant and animal origin enhance the immune responsiveness against a pathogen by activating the immune system [21]. The phagocytic index is a activity of reticulo-endothelial system (RES). The RES is a diffused system comprising of phagocytic cells, fixed tissue macrophages and mobile macrophages and participate in inflammation. The phagocytic cells comprise the mononuclear phagocyte system (MPS). Cells of the RES and MPS are known to be important in the clearance of particles from the bloodstream and contribute to non-specific immunity [18]. In the carbon clearance test, FA increase activity of reticulo-endothelial system observed phagocytic index. Active recruitment of neutrophils to sites of infection is fundamentally important to the innate immune system. The chemoattractants can enhance neutrophils function, thereby facilitating host defense. In neutrophil adhesion test, adhesion of neutrophil to the nylon fiber indicates the migration of cells in the blood vessels and the number of neutrophils reaching the site of inflammation [22]. FA acts as chemo attractants causes the stimulation of neutrophils towards the site of inflammation and plays role in innate host response. The estimation of serum immunoglobulin level is a direct measure of humoral immunity. Humoral immunity is mediated by T and B cell that produce specific protein which are called serum (antibody) and protect against extracellular microbes and their exotoxins.

In the present investigation, estimation of serum immunoglobulins determines the amount of immunoglobulins present in the serum [18]. The turbidity was expressed as ZST units, which indicate the amount of immunoglobulin present in the sample. FA increases the serum immunoglobulin level in this estimation.

Cyclophosphamide acts as an immunosuppressive agent by causing alkylation of DNA by interfering in DNA synthesis and function, used extensively as immunosuppressant. In this experiment, FA caused reduction in the cyclophosphamide induced neutropenia and leukocopenia suggesting that it may have an effect on the haemopoetic system. The prevention of neutropenia and decrease in leukocyte count induced by cyclophosphamide may be through activation of macrophages, which secrete a large number of substances including colony stimulating factor and interleukin 1 [23].

Delayed type of hypersensitivity reaction is antigenic specific and the characteristic are an influx of immune cell at the site of injection and induction become apparent within 24-72 h. T-cell required to initiate the reaction [24]. In this investigation, DTH response was increase by FA which has stimulatory effect on lymphocyte and necessary cell types required for the expression reaction. The present investigation therefore reveals that FA certainly process immunostimulant properties.

CONCLUSION

On the basis of the results obtained in the present investigation it can be concluded that ferulic acid has potential to stimulate cell mediated immunity as well as humoral immunity in the experimental animals. In the present investigation, ferulic acid showed a significant activity on immune system but the exact mechanism of immunomodulation on molecular basis have to explored further for the immune based therapy.

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