ORIGINAL ARTICLE

Biological evaluation of ferulic acid as potent immunomodulator: An *in vitro* study

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Objective: The development of immunity and suppression of undesired immune reaction are two of the strategies that are responsible to control the disease. Immunomodulators, which are devoid of any untoward effects, can be administered for a long period for prevention of variety of diseases. Ferulic acid, a hydroxylated cinnamic acid is an abundant phenolic phytochemical found in cell wall of plants. It is one of the important phyto-molecule with diverse therapeutic effects. The current work was proposed to determine *in vitro* immunomodulatory effects of ferulic acid. **Materials and Methods:** Nitroblue tetrazolium test, phagocytosis of killed *Candida albicans*, neutrophil locomotion and chemotaxis test and membrane stabilisation studies were performed to determine immunomodulatory effect of ferulic acid. **Results:** Ferulic acid caused stimulation of neutrophils causing phagocytotic activity to significant degree. Ferulic acid aroused the process of phagocytosis of killed *C. albicans* and demonstrated a significant ($P < 0.05$) chemotactic activity at all tested concentrations. Ferulic acid at concentrations of 50-300 µg/ml demonstrated protection to goat erythrocytes membrane against lysis induced by heat solution. **Conclusion:** The present study suggests that ferulic acid could be regarded as potential immunomodulatory compound. However, it would be interesting to understand *in vivo* behaviour of ferulic acid under varied experimental conditions.

Key words: Ferulic acid, immunomodulatory, membrane stabilisation

INTRODUCTION

Inflections of immune system snap to alleviate the generation of disease. The development of immunity and suppression of undesired immune reaction are two of the strategies, which are responsible to control the disease.[1] Immunomodulators are the substances that alter and regulate immune system. There are two classes of immunomodulators. Immunostimulants augments and boost up immune response, whereas immunosuppressants suppress the immune response. A number of immunomodulators are very often used in the management of autoimmune diseases; they are also used in organ transplantation to prevent rejection of the new organ.[2] Immunomodulators, which are devoid of any untoward effects, can be administered for a long period for prevention of variety of diseases.[3]

Ferulic acid, chemically hydroxylated cinnamic acid is an abundant phenolic phytochemical. It is an important component of plant cell wall. It is one of the important phyto-molecule with diverse therapeutic effects. Ferulic acid is potent antioxidant.[4-6] It offers protection against hydroxyl and peroxyl radical oxidation in biological system.[7] It ameliorates critical condition during alcohol and poly unsaturated fatty acids PUFA-induced toxicity.[8] Ferulic acid also protects cells against radiation[9] and ultraviolet light-induced photo-damage.[10] Ferulic acid is natural protector against carbon tetrachloride-induced toxicity in experimental animals.[11] It has also demonstrated protective effect on focal cerebral ischemic injury[12] and noise-induced hearing loss in the guinea-pig.[13] It also exhibit stimulatory effect on endurance exercise capacity in mice.[14] It improved depression-like behaviours in reserpine-treated mice.[15] It is known to show inhibition of tumour promotion and has tendency to block pro-carcinogen formation.[16,17] Plant polyphenols are known for immunomodulatory potential.[18-20]

The current work was proposed to determine *in vitro* immunomodulatory effects of ferulic acid.

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MATERIALS AND METHODS

Chemicals

Ferulic acid was purchased from Central Drug House, India. All the other chemicals used in the study were of analytical grade. Triple distilled water was used in the experiment.

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Study of Immunomodulatory Activity

Qualitative Nitroblue Terazolium Test

A suspension of leucocytes (5 × 10^6/ml) was prepared in 0.5 ml of phosphate buffered saline (PBS) solution in different tubes. A total of 0.1 ml of PBS solution (control) and 0.1 ml of endotoxin-activated plasma (standard) were added to first and second tubes, respectively, and to the other tubes were added 0.1 ml of different concentrations (10, 20, 40, 100 and 1000 µg/ml) of ferulic acid. A total of 0.2 ml of freshly prepared 0.15% nitroblue tetrazolium (NBT) solution was added to each tube and incubated at 37°C for 20 min. The tubes were centrifuged at 400 rpm for 3-4 min to discard the supernatant.

The cells were re-suspended in a small volume of PBS solution. A thin film was made with the drop on the slide, dried, fixed by heating, counterstained with dilute carbol-fuchsin for 15 s. The slide was washed under tap water, dried and focussed under 100 × oil immersion objective; 200 neutrophils were counted for the percent of NBT positive cells containing blue granules/lumps.[21]

Phagocytosis of Killed *Candida albicans*

Preparation of Candida albicans Suspension

Candida albicans culture was incubated in Sabouraud broth overnight and then centrifuged to form a cell button and the supernatant was discarded. The cell button was washed with sterile Hank's balanced salt solution (HBSS) and centrifuged again. This was done 3-4 times. The final cell button was mixed with a mixture of sterile HBSS and human serum in a proportion of 4:1. The final cell suspension of concentration 1 × 10^8 was used for the experiment.[22]

Slide Preparation

Goat blood (un-heparinised; 0.2 ml) freshly obtained from slaughter house was placed on a sterile glass slide and incubated at 37°C for 25 min to allow clotting. The blood clot was removed very gently and the slide was drained slowly with sterile normal saline, taking care not to wash the adhered neutrophils (invisible). The slide consisting of polymorphonuclear neutrophils (PMNs) was flooded with a concentration of ferulic acid and incubated at 37°C for 15 min. The PMNs were covered with *C. albicans* suspension and incubated at 37°C for 1 h. The slide was drained, fixed with methanol and stained with Giemsa stain.

Phagocytosis Evaluation

The mean number of *Candida* cells phagocytosed by PMNs on the slide was determined microscopically for 100 granulocytes using morphological criteria. This number was taken as Phagocytic Index (PI) and was compared with basal PI of control. This procedure was repeated for different concentrations (10, 20, 40, 100 and 1000 µg/ml) of the isolated compound.[21] Immunostimulation in percentage was calculated by using the following equation:

$$\text{Stimulation (\%)} = [PI_{test} - PI_{control}] \times 100/PI_{(control)}$$

Neutrophil Locomotion and Chemotaxis Test

Neutrophil cell suspension was prepared in PBS solution at about 10^6 cells/ml. The lower compartment of chemotactic chamber was filled with appropriate chemotactic reagents pre-adjusted to a pH of 7.2 (e.g. chamber 1 had PBS solution (control), chamber 2 had casein 1 mg/l (ferulic acid) and chambers 3, 4, 5, 6, 7 had different concentrations (10, 20, 40, 100 and 1000 µg/ml) of the isolated sample). The upper compartment (1 ml syringe) was filled with neutrophil cell suspension and the wet filter (Millipore) of 3 mm pore size was fixed at the bottom of the upper compartment. The upper compartment was placed into the lower compartment and incubated at 37°C for 180 min.

The upper compartment was removed and inverted to empty the fluid. The lower surface of the filter was fixed with 70% ethanol for 2 min and then stained with haematoxylin dye for 5 min. The fixed filters were observed under microscope using 100 × lenses and the number of neutrophil cells that reached the lower surface was counted.

Membrane Stabilisation Studies

In vitro heat-induced haemolysis of goat erythrocytes associated with membrane stabilising activity of a ferulic acid was evaluated.[23] Goat blood was collected from slaughter house and anticoagulant mixture was added to it. The blood was immediately transported to laboratory. Briefly, a vial containing 20 µl fresh goat blood in 2 ml of PBS was treated in triplicate with the ferulic acid or diclofenac (the standard drug) to obtain the final concentration of the ferulic acid or diclofenac in the vials became 50, 100, 150, 200, 250 and 300 µg/ml. Each control vial contained 20 µL of PBS. The vials were incubated for 15 min at 37°C followed by 54°C for 15 min then centrifuged it and absorbance of supernatant was measured spectrophotometrically at 540 nm. The percent inhibition of haemolysis was calculated as:

$$\text{Percent Inhibition} = [(A_{control} - A_{sample})/A_{control}] \, 100$$

The EC_{50} value of the ferulic acid and diclofenac for the plasma membrane stabilisation effect was calculated using the plot of concentration of the ferulic acid or diclofenac versus percent inhibition of haemolysis.

Statistical Analysis

The results are expressed as mean ± standard error of mean. Experiments were always performed in triplicates. Statistical comparison was performed using analysis of variance (ANOVA) followed by Bonferroni's test ($^*P < 0.05$).

RESULTS

Nitroblue Tetrazolium Test

Ferulic acid caused stimulation of neutrophils causing phagocytotic activity to significant degree ($P < 0.05$) of 67.27% and 61.99% at concentrations of 1000 and 100 µg/ml, respectively, as compared with endotoxin activated plasma [Figure 1].

Phagocytosis of Killed *C. albicans* Assay

Ferulic acid aroused the process of phagocytosis of killed *C. albicans*. The mean particle numbers (MPNs) were found to be 4-5, 4 and 4 for ferulic acid at concentrations of 1000, 100 and 40 µg/ml, respectively, when compared with positive control-pooled serum at the same concentrations [Table 1].

Neutrophil Locomotion and Chemotaxis

Ferulic acid demonstrated a significant ($P < 0.05$) chemotactic activity at all tested concentrations. Mean number of neutrophils per field for ferulic acid was found to be 138.02, 129.9, 122.3 at concentrations of 1000, 100 and 40 µg/ml, respectively, when compared with the standard-casein [Figure 2].

Membrane Stabilisation Studies

The result showed that ferulic acid at concentrations of 50-300 µg/ml demonstrated protection to goat erythrocytes membrane against lysis induced by heat solution. Significant results ($*P < 0.05$) were found at concentrations higher than 200 µg/ml. The standard drug diclofenac offered significant ($*P < 0.05$) protection against damaging effect by heat solution at a concentration of 150 µg/ml [Figure 3].

DISCUSSION

Phyto-immunomodulators alleviate the immune response of the body against pathogens by triggering non-specific immune system. A number of herbs and herbal preparations are traditionally used to modulate immune system. Yet, it is necessary to screen phytomedicine to evaluate therapeutic benefit.

PMNs have been related with competent innate defense mechanisms. PMNs activated by endotoxic/phagocytic stimuli generate a huge sum of 'microbicidal oxidant's, which play an important role in 'host defense against pathogen'.[24] In the current study, ferulic acid caused reduction in percentage of reduced neutrophils after treatment.

'Engulfment of microorganism by leucocyte' is among foremost 'defense mechanism' that is inherited in

Table 1: Mean particle number in phagocytosis of CANDIDA ALBICANS after treatment with ferulic acid

	Concentration (µg/ml)				
	1000	100	40	20	10
Phosphate buffered saline	0-1	1	0	0	0
Casein	5	3-4	3	3	3
Ferulic acid	4-5	4	4	4	4

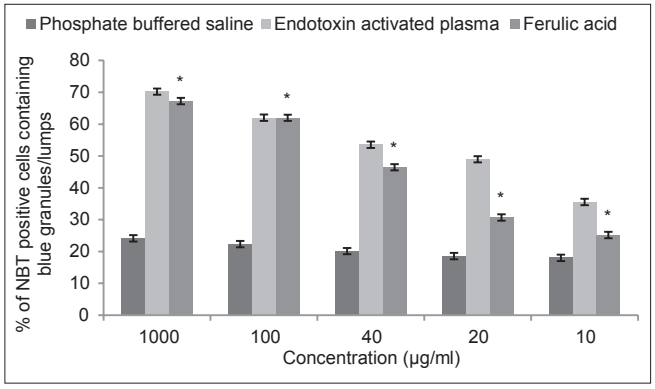

Figure 1: Percentage of reduced neutrophils after treatment with ferulic acid as determined by nitroblue tetrazolium test. The results are expressed as mean ± standard error of mean. Statistical comparison was performed using analysis of variance (ANOVA) followed by Bonferroni's test ($*P < 0.05$)

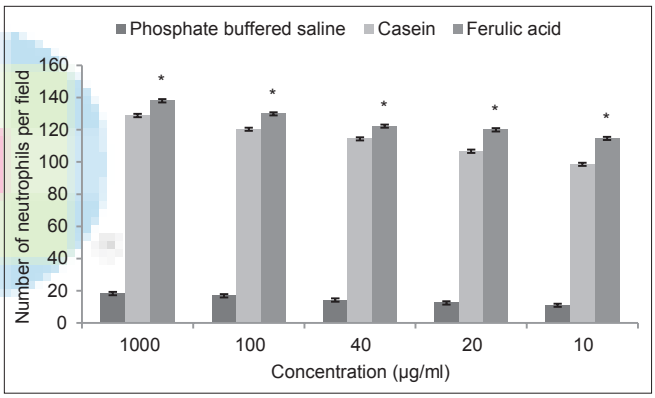

Figure 2: Number of neutrophils per field after treatment with ferulic acid determined by neutrophil locomotion and chemotactic activity. The results are expressed as mean ± standard error of mean. Statistical comparison was performed using analysis of variance (ANOVA) followed by Bonferroni's test ($*P < 0.05$)

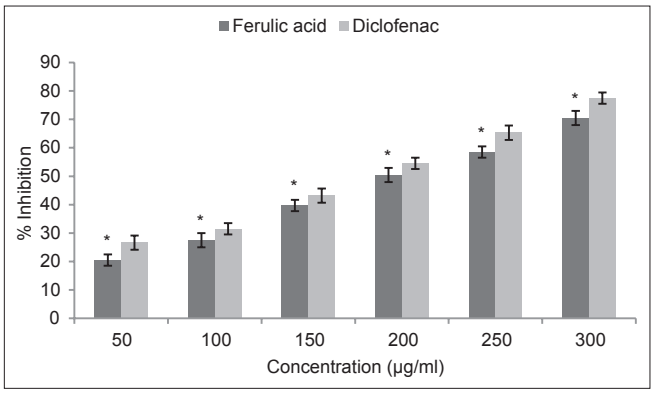

Figure 3: Membrane stabilising Activity of ferulic acid on goat RBC. The results are expressed as mean ± standard error of mean. Statistical comparison was performed using analysis of variance (ANOVA) followed by Bonferroni's test ($*P < 0.05$)

organisms.[25] In the present work, ferulic acid caused enhanced 'neutrophil chemotactic movement' and it establishes the fact that ferulic acid act as 'chemo-attractant'.

Chemo-attractants induce 'attraction of neutrophils toward certain chemicals'. Neutrophil locomotion and chemotaxis is course of action for chemoattarction.[20] In this procedure, ferulic acid was examined for chemo-attractant potential and mean number of neutrophils attracted per field was noted against positive control-casein. Ferulic acid caused increase in number of neutrophils attraction per field.

Red Blood Cell (RBC) membrane is similar to that of lysosomal membrane. RBC haemolysis inhibition provides an insight to the course of inflammation. Lysosomal injury causes liberation of phospholipase-A2, which aids in generation of phospholipids causing production of inflammatory mediators.[23] Stabilisation of membrane causes inhibition of arbitrator, which provokes inflammatory mediator's release. Drugs/compounds that endorse membrane stabilisation affords defence against injurious substances. The *in vitro* ability of ferulic acid to guard erythrocytes against heat-induced lysis in a dose-dependent manner reflects its ability to stabilise the lysosomal membrane.

Plant-derived polyphenolic compounds are reputed anti-cancer and immunomodulatory agents. These agents interfere with formation of reactive intermediates and thus, can terminate formation of reactive intermediates and thus avert instigation of process of carcinogenesis. A number of scientific evidences sustain immunomodulatory prospective of polyphenols.[2,3] The excellent radical scavenging capability of polyphenols makes them an effective weapon against cellular stress. Ferulic acid, in the present work, demonstrated ecstasy as immunomodulatory agent.

The present study suggests that ferulic acid could be regarded as potential immunomodulatory compound. Apart from therapeutic importance, it would be interesting to understand *in vivo* behaviour of ferulic acid under varied experimental conditions.

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