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Immunomodulatory activity of ether insoluble phenolic components of *n*-butanol fraction (EPC–BF) of flaxseed in rat

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ABSTRACT

Objective: To investigate the immunomodulatory activity of ether insoluble phenolic components of *n*-butanol fraction (EPC–BF) of flaxseed in rat. **Methods:** Immunomodulatory activity of EPC–BF was assessed in rat at oral doses of 150 and 300 mg/kg using neutrophil adhesion test, hemagglutination test and measuring delayed type hypersensitivity response. **Results:** EPC–BF modulates immune response by significantly reducing cell-mediated immune response at oral dose 150 mg/kg but not at dose 300 mg/kg. However, EPC–BF did not affect on the neutrophil adhesion and humoral immunity at both the studied doses. **Conclusion:** EPC–PC modulates immune response by reducing cell-mediated immune response at lower dose but not at higher dose, which could be due to the presence of both immunostimulant and immunosuppressant phenols together in EPC–BF. These studies suggest that EPC–BF could be a promising source for new immune modifier.

1. Introduction

Searching of substances with immunostimulative or immunorestorative effects could contribute to the maintenance of the immune system[1]. Many plants have been evaluated for immunostimulant and immunosuppressive properties using simple techniques[2]. There are several plants such as *Tanacetum vulgare* (*T. vulgare*) L, *Actinidia macrocarpa* (*A. macrocarpa*), *Tinospora cordifolia* (*T. cordifolia*), *Curcuma longa* (*C. longa*), *Vernonia amygdalina* (*V. amygdalina*) etc. reported to have immunomodulatory properties[3–7]. Immune modulation helps to maintain disease-free state. Stimulation and suppression of immune response are the two ways of immunomodulation. Stimulation of immune response can be active, in which an immune response is induced through exposure to an antigen, or passive, in which preformed antibodies are administered directly. Most of the immune suppressive drugs, suppress immune response by inhibiting T cell activation, are used to prevent acute rejection after organ transplantation and to treat certain autoimmune diseases[8].

Linum usitatissimum (*L. usitatissimum*) L. belongs to family linaceae, commonly known as flaxseed or linseed. Flaxseed has been playing a major role in the field of diet and disease research due to its high content of omega–3 fatty acid, α -linolenic acid and a major lignan secoisolariciresinol diglucoside (SDG)[9]. Flaxseed or flaxseed oil is a well known immunomodulator. It has been reported that supplementation of flaxseed diet to human tend to suppress the cell-mediated immunity without affecting the humoral immunity in human[10]. Similarly, feeding of α -linolenic acid from flaxseed to rat also did not affect on antibody production[11]. However, role of flaxseed phenolic components in immunomodulation is still remains indistinct.

In our previous study, we have selectively isolated ether insoluble phenolic components (EPC–BF) such as caffeic acid, tannins and phenolic glycosides free from earlier reported ether soluble phenolics flavonoids and lignan, SDG from *n*-butanol fraction of defatted flaxseed meal and studied their antioxidant potential in various models[12,13]. The present study was undertaken to evaluate the effect of EPC–BF on immunomodulation in rat.

2. Materials and methods

2.1. Chemicals

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Hexane, methanol, *n*-butanol, solvent ether, sodium chloride, gum acacia were purchased from Molychem (Mumbai, India). Anesthetic ether (I.P.) was purchased from Narsons pharma, India. Alsever's solution was purchased from Sigma (St. Louis, MO, USA). All chemical and reagents were of analytical grade.

2.2. Preparation of extract

EPC–BF was prepared by our earlier described method^[13]. Briefly, a double pressed flaxseed cake powder obtained from 'Omega–3 oil unit, established under project NAIP–ICAR–Component–3 at Sangamner, M.S., India' was used for extraction. Residual oil from flaxseed meal was removed by *n*-hexane (1:4, w/v). Dried defatted flaxseed powder was extracted with methanol for 3 h at 60 °C in a soxhlet apparatus. Methanol extract was concentrated in rotavapour at 60 °C under vacuum. Then the dried residue was further partitioned with *n*-butanol: water (1:1, v/v). *n*-Butanol fraction was separated from aqueous fraction and dried in a rotavapour at 80 °C under vacuum. Dried *n*-butanol residue was dissolved in minimum quantity of methanol and precipitated with solvent ether (1:5, v/v), finally brown colored sticky precipitate of EPC–BF was obtained. Finally, it was dissolved in 2% gum acacia and used for the evaluation of immunomodulatory activity.

2.3. Animals

Female Wistar rats 120–200 g and 12 mice 25–35 g of either sex were used for the study. They were housed in animal house under standard condition of temperature (25 ± 2) °C, 12/12 h light/dark cycle and fed with standard pellet diet (Amrut, Sangali, M.S., India) and tap water ad libitum. Institutional Animal Ethical committee, Amrutvahini College of Pharmacy, Sangamner approved the study protocol. The animals were divided into three groups consisting six animals each. Group I was of normal control received only vehicle i.e. 2% gum acacia. Animals of Group II and III received orally 150 and 300 mg/kg of EPC–BF (dissolved in 2% gum acacia) respectively.

2.4. Acute oral toxicity study

Adult Swiss albino female mice 18–22 g were subjected to acute oral toxicity studies as per guideline (AOT No. 425) suggested by Organization for Economic Co-operation and Development (OECD) (2001). Dose of 175, 550, 1 750, 2 000 and 5 000 mg/kg of EPC–BF were administered orally to mice. The mice were observed by housing them individually in the polypropylene metabolic cages continuously for 2 h for behavioral, neurological, autonomic profiles and for any lethality during next 48 h.

2.5. Preparation of antigen

Antigen was prepared by collecting sheep blood in sterile Alsever's solution in 1:1 proportion and kept in the refrigerator. Sheep Red Blood Cells (SRBCs) for immunization were prepared by centrifuging at 2 000 rpm for 10 min and washed 4–5 times with physiological saline and then suspended into buffered saline for desired cell

concentration^[14].

2.6. Immunomodulatory activity

2.6.1. Neutrophil adhesion test

Neutrophil adhesion test was carried out according to method described by Dashputre and Naikwade^[15] with some modification. Rats were divided into three groups and treated orally with vehicle or EPC–BF for 14 d. On the 14th day of drug treatment, blood samples were collected from retro-orbital plexus into heparinized vials and analyzed for total leukocyte count (TLC) and differential leukocyte count (DLC) by the using hematology analyzer (Abacus Junior, Diatron, Australia). After initial counts, blood samples were incubated with 80 mg/mL of nylon fibers for 15 min at 37 °C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and percentage of neutrophil gives the neutrophil index (NI) of blood sample. Percentage of neutrophil adherence was calculated by equation:

$$\text{Percent neutrophil adhesion} = \frac{N_{iu} - N_{it}}{N_{iu}}$$

Where, N_{iu} was neutrophil index of untreated blood sample and N_{it} neutrophil index of treated blood sample.

2.6.2. Delayed type hypersensitivity response (DTH response)

C cell mediated immune response was measured by measuring delayed type hypersensitivity response^[16]. All rats were immunized on day 0 by i.p. administration of 5×10^9 SRBC/rat. The animals were treated with EPC–BF for 14 more days and challenged by subcutaneous administration of 1.25×10^9 SRBC/mL in to right hind foot pad on day 14. DTH responses were measured by using vernier caliper at 24 h after SRBCs challenged on day 14. The difference between pre and post challenge foot thickness expressed in cm was taken as a measure of delayed type hypersensitivity.

2.6.3. Determination of humoral immune response

Indirect haemagglutination test with some modification was used to measure humoral immune response^[17]. Rats were pretreated with the EPC–BF for 14 d and each rat was immunized with 0.4 mL of 5×10^9 SRBC/rat by i.p. route, including control rats. The day of immunization was referred to as day 0. The treatment of EPC–BF was continued for another 14 d and blood samples were collected from individual rats from retro-orbital plexuses on 15th day for determination of Haemagglutinating Antibody (HA) titer. The titer value was determined by titrating serum dilution with SRBC (1.25×10^9 cells) in microtiter plate. The plate were incubated at room temperature for 2 h and examined visually for agglutination. The highest number of serum dilution showing haemagglutination was noted and expressed as HA titer.

2.7. Statistical analysis

Data were expressed as the mean ± S.E.M. Statistical analysis was carried out by one-way ANOVA followed by Dunnett comparison test using graphpad prism 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. $P < 0.05$ was considered significant.

3. Results

3.1. Acute oral toxicity study

Acute oral toxicity study of EPC–BF in mice did not show any changes in the behavior, autonomic profiles and no mortality were observed in all treated and control groups of the mice up to the dose of 5 000 mg/kg.

3.2. Immunomodulatory activity

Table 1 shows the result of neutrophil adhesion to nylon fiber. EPC–BF at doses, 150 and 300 mg/kg non–significantly

Table 1.

Effect of EPC–BF on neutrophil adhesion in rats ($n=6$).

Groups	Neutrophil index		Percent neutrophil Adhesion
	UB	FTB	
I (Vehicle, p.o.)	149 379 \pm 11 336	116 209 \pm 1 309	19.5 \pm 15.6
II (150 mg/kg of EPC–BF, p.o.)	208 348 \pm 22 361	179 792 \pm 24 526	14.2 \pm 6.8 ^{ns}
III (300 mg/kg of EPC–BF, p.o.)	157 848 \pm 14 531	118 880 \pm 2 741	22.1 \pm 12.1 ^{ns}

All values are mean \pm S.E.M; ns= non–significant; UB= Untreated Blood; FTB= Fiber Treated Blood.

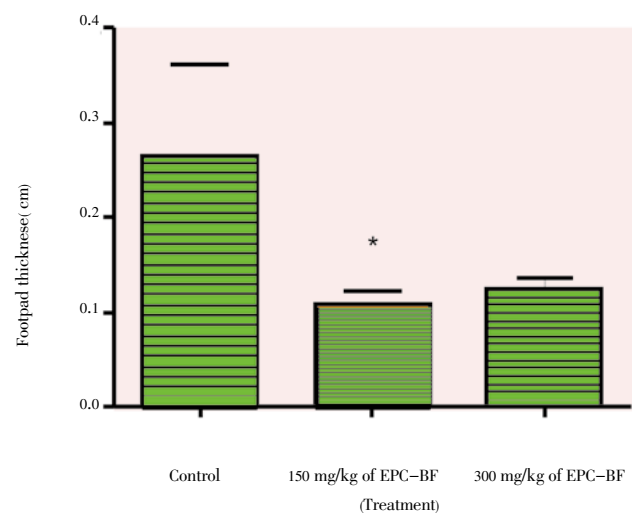


Figure 1. Effect of EPC–BF on Delayed Type Hypersensitivity (DHT) [Control: group received only vehicle i.e. 2% gum acacia; 150 mg/kg of EPC–BF: group treated with 150 mg/kg p.o., of EPC–BF (dissolved in 2% gum acacia); 300 mg/kg of EPC–BF: group treated with 350 mg/kg of EPC–BF (dissolved in 2% gum acacia)]. All values are mean \pm SEM, $n=6$, significantly different from control * $P<0.05$.

4. Discussion

Plant or plant products such as its powder, extract or fraction appears to modulates immune response by affecting on humoral immunity and /or act on cellular immunity. Whereas some time they modulate the activity of the innate branch of the immune system or may effect on specific cells or subsets of cells of acquired immunity^[18]. In the present study immunomodulatory activity of EPC–BF was first time investigated by evaluating its effect on neutrophil adhesion, cell–mediated and humoral immune responses.

Neutrophils represent a multi–functional cell type in innate immunity that contributes to bacterial clearance

affects on percent neutrophil adhesion, when compared with control group.

The data summarized in Figure 1, exhibits effect of EPC–BF at doses, 150 and 300 mg/kg on cell mediated immunity. The results of this study indicated that EPC–BF at dose 150 mg/kg significantly reduced delayed–type hypersensitivity, compared to control. However, EPC–BF at dose 300 mg/kg reveals further non–significant increase in delayed–type hypersensitivity response.

The haemagglutination antibody titer was used to assess humoral response. Figure 2 shows the effect of EPC–BF on haemagglutination antibody titer. The results of antibody titer reveals that EPC–BF at both studied doses did not significantly affect on antibody production i.e. humoral immune response in rats.

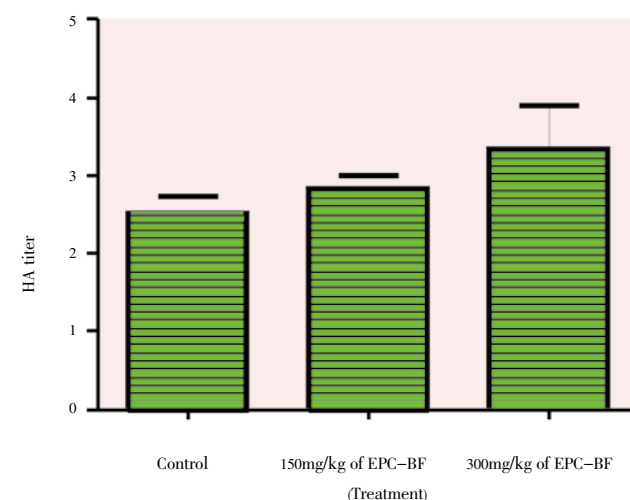


Figure 2. Effect of EPC–BF on haemagglutination antibody titer (HA titer) [Control: group received only vehicle i.e. 2% gum acacia; 150 mg/kg of EPC–BF: group treated with 150 mg/kg b.w, p.o., of EPC–BF (dissolved in 2% gum acacia); 300 mg/kg of EPC–BF: group treated with 350 mg/kg b.w, p.o., of EPC–BF (dissolved in 2% gum acacia)]. All values are mean \pm SEM, $n=6$.

by recognition, phagocytosis and killing^[19]. In neutrophil adhesion test, the adhesion of neutrophil to nylon fibers indicates the migration of cells in the blood vessels and the number of neutrophils reaching at the site of inflammation^[20,21]. The result of present study indicated that EPC–BF does not significantly affect on neutrophil adhesion. Delayed type hypersensitivity reaction is characterized by large influxes of non–specific inflammatory cells, in which the macrophage is a major participant^[22]. SRBC–induced delayed– type hypersensitivity was used to assess the effect of EPC–BF on cell mediated immunity. In cell mediated immune response, sensitized T–lymphocytes, when challenged by the antigen, are converted into lymphoblast and secrete lymphokines, attracting more scavenger

cells to the site of reaction. The infiltrating cells are thus, immobilized to promote defensive (inflammatory) reaction^[23]. Result shows EPC–BF suppressed cell – mediated immune response at dose 150 mg/kg. However, at dose 300 mg/kg EPC–BF exhibited further non-significant increase in cell mediated immune response. Suppressed delayed-type hypersensitivity by EPC–BF could be, due to their inhibitory effect on lymphocytes and accessory cell types function. The observed paradoxical immunomodulation activity by EPC–BF at different doses could be due to the presence of both immunostimulant and immunosuppressant phenols together in EPC–BF. However, to gain further insight into suppression of cell mediated immune response by EPC–BF at different doses, future experiments combining cytokine assays and gene expression analysis with disease models are warranted. Result of the hemagglutination test confirmed that EPC–BF does not modulates humoral immune response i.e. antibody production. Thus, EPC–BF shows immunomodulatory activity by suppressing cell– mediated immune response at dose 150 mg/kg, but not at dose 300 mg/kg just as activity shown by most of the immunosuppressants at lower dilution^[24].

In conclusion, EPC–BF modulates immune response in rat by affecting components of cell–mediated immunity and shows no effect on the humoral immunity. These studies suggest that EPC–BF could be a promising source for new immune modifier.

Conflict of interest statement

We declare that we have no conflict of interest.

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