Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 7, Issue 9, 2015

Original Article

EVALUATION OF IMMUNOMODULATORY ACTIVITY OF PETROLEUM ETHER EXTRACT OF SEEDS OF *PITHECELLOBIUM DULCE* IN WISTAR RATS

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Received: 03 Jul 2015 Revised and Accepted: 30 Jul 2015

ABSTRACT

Objective: The present study was planned to assess the immunomodulatory action of petroleum ether extracts of seeds of *Pithecellobium dulce* (PEPD) in experimental models of immunity.

Methods: Metronidazole, pyrogallol and ethanol models were used for suppression of the immune system in Wistar rats. Humoral immunity was analyzed by haemmagglutination assay, whereas, cellular immunity was carried out by paw edema and carbon clearance assay. PEPD (400 mg/kg, p. o.) was selected by acute toxicity study. Levamisole (50 mg/kg, p. o.) was used as standard.

Results: Metronidazole, pyrogallol and ethanol significantly decreased the humoral and cellular immunity, whereas, PEPD and levamisole significantly increased the circulating antibody titer in the indirect haemagglunation test. PEPD and levamisole also produced significant increases in paw edema and increase in the phagocytic index in the carbon clearance assay. Animals treated with metronidazole, pyrogallol and ethanol decreased in peripheral blood RBCs and monocytes whereas, PEPD and levamisole treated groups significantly increased in peripheral blood RBCs and monocytes.

Conclusion: Our data showed that PEPD possesses potential for augmenting immune activity by cellular and humoral mediated mechanisms and also significantly restores the biochemical and histopathological parameters. The present study concluded that PEPD may have therapeutic and prophylactic value as immunostimulants.

Keywords: *Pithecellobium dulce*, Immunomodulation, Haemagglunation, Paw edema, Phagocytic response.

INTRODUCTION

The defensive action of the immune system is being involved in the etiology, as well as pathophysiologic mechanisms of many diseases. Modulation of the immune responses to diminish various diseases has been of much interest for many years [1]. Immunomodulation is a process which can modify the defensive organization of an organism by interfering with its purpose, if the results is an enhancement of immune reactions it is mentioned as an immunostimulative agent which primarily act through stimulation of nonspecific system, i.e. granulocytes, complement, macrophage, T-lymphocytes and different effector substances. Immunosuppression implies mainly to reduce resistance against infections, stress and may come about on account of environmental or chemotherapeutic agents [2].

Due to more side effects of conventional drugs, the use of natural plant product was rising in the last few decades as an alternative treatment in the healing and treatment of various diseases [3]. *Pithecellobium dulce* Benth. (Leguminosae) is a small to medium sized, evergreen, spiny tree up to 18 m height, native of tropical America and cultivated throughout the plains of India and in the Andamans [4]. The presence of steroids, saponins, lipids, phospholipids, glycosides, glycolipids and polysaccharides has been reported in the seeds [5]. The active constituents of plant derivatives such as polysaccharides, lectins, peptides, flavonoids and tannins have been reported to modulate the body's defense mechanism in different experimental models [6]. Aqueous and hydroalcoholic Pithecellobium dulce shows significant free radical scavenging action and also possess antacid secretory activity in several *in vitro* models. Pithecellobium Dulce Extract fraction of various extract show's differ activity such as Abortifacient, Anti-inflammatory, Antivenom, Protease inhibitor, Spermicidal, Antimicrobial, Hypolipidemic, Locomotor, Antioxidant properties and Anti tubercular activity and showed significantly equal activity when compared with standard drugs [7]. Nevertheless, there is no scientific data on the *in vivo* immunomodulatory activity of this flora. Hence the present work has been undertaken to explore the immunomodulatory action of petroleum ether extracts of Pithecellobium deals on several models of Wistar rats.

MATERIALS AND METHODS

Plant collection and preparation of extract

Pithecillobium dulce seeds were gathered locally from supermarket in Anantapuramu and was authentified by Dr. J. Raveendra Reddy, M. Pharm., Ph. D., Raghavendra Institute of Pharmaceutical Education and Research (RIPER), Anantapuramu. Seeds were dried at room temperature and shade dried seeds were made into powder. The powder was extracted with petroleum ether on a continuous reflex by using a Soxhlet apparatus for about 15 cycles. The extract was concentrated on rotary flash evaporator and air dried to get the semisolid extract (yield-62.50 g/kg).

Animals

Wistar rats of either sex, 150-200 g body weight were obtained from Raghavendra enterprises, Bangalore, and they were put up in small cages under standard husbandry conditions, (22±2ºC temperature, 12 h light/dark cycle) with standard rat feed (VRK Nutrition solution, Maharashtra Pvt Ltd. India) with water ad libitum. The experimental protocol was approved by the Institutional Animal Ethical Committee of Raghavendra Institute of Pharmaceutical Education and Research, Anantapuramu (878/ac/05/ CPCSEA/ 015/2014) and all the animals were conducted according to the guidance's of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Drugs and chemicals

Metronidazole (J. B chemicals and pharmaceuticals), Pyrogallol, Ethanol, DTNB, Tris-HCl, EDTA, Hydrogen peroxide, TCA, (Hi-Media Pvt Ltd.) IgG kit (Quanta Pvt Ltd.)

Instruments

Analytical UV-Visible Spectrophotometer (2200/Systronics), Electronic balance (LC/GC), centrifuge (13 REMI), Automated Analyser (Chem7/Erba), Homogenizer (Remi motor/Remi Electro Technik Ltd.)

Preliminary phytochemical screening

About 50 mg of the solvent free extract was subjected for each of the preliminary phytochemical screening of Alkaloids, Phenolic compounds, Tannins, Glycosides and Saponins and were found positive in all examinations as per Kokate *et al.* [8]

Acute toxicity study

Acute oral toxicity study of PEPD was performed as per OECD-423 guidelines (acute toxic class method). Wistar rats either sex was chosen randomly and divided into six groups (n=2). The animals were fasted overnight and extract in doses of 250, 500, 1000, 2000 and 4000 mg/kg body weight, were administered orally to II-IV groups. The group I which received vehicle (water) serves as controls. The animals observed continuously for 2 h, and then intermittently for 6 h and at the end of 24 h, the number of death was noted to determine the LD50 of the extract [9]. Animals were also observed for behavioral, neurological and autonomic profiles simultaneously [10].

Treatment protocol

The experimental rats were divided into ten groups of 6 animals each and treated as follows (table 1).

Immunological studies

Blood was withdrawn from the jugular vein of a sheep and RBCs were preserved in Elsevier solution. It was then suspended in phosphate buffered saline for further usage. All rats were antigenically challenged twice with sheep RBC (0.5 × 109 cells/100 g, i. p.).

For a humoral antibody response, a blood sample was collected from the retro-orbital plexus and the rat serum were used for determination of hemagglutination titer. The blood samples were centrifuged to collect serum and equal volume of individual serum samples of each group was seriously diluted (in doubling dilutions) in phosphate buffer saline (PBS) and placed in the well of a U-shape 96-Microtiter plates. Aliquots (25µl) of two folds diluted sera in PBS were challenged with 25 µl of 1% v/v SRBCs suspension and mixed. After mixing, the plates were incubated at 37 °C for 1 h and examined for hemagglutination. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titer [11].

Whereas the cellular immune response was assayed by the foot-pad reaction method in rats. The increase in paw volume by an injection of SRBCs (0.025 × 109 cells), in the sub plantar region of right hind paw, was considered as delayed type of hypersensitivity reaction and considered as an index of cell-mediated immunity. The volume of left hind paw, injected similarly with phosphate buffer saline, served as control [12] (table 2).

***In vivo* anti-oxidant parameters**

In vivo antioxidant parameters like SOD, Catalase, Reduced glutathione, and LPO were estimated on the last day of treatment, i.e. 15th day in case of metronidazole induced immunosuppression, 22nd day in case pyrogallol induced immunosuppression and 28th day in the case of ethanol induced immunosuppression respectively.

Preparation of erythrocyte lysate

The blood samples were withdrawn into EDTA containing eppendroff tubes on the last day of the treatment from the retro orbital venous plexus of rats. Then these eppendroff tubes were subjected to centrifugation at 8000 rpm for 15 min. The supernatant was discarded and erythrocyte lysate was prepared from the sediment.

Table 1: Treatment schedule

Groups	Metronidazole model	Pyrogallol model	Ethanol model
Normal	Distilled water	Distilled water	Distilled water
Negative Control	Metronidazole 114 mg/kg, i. p. For 14 days	Pyrogallol 100 mg/kg, i. p. For 7 days	Ethanol 2 g/kg, 20 % w/v *p. o*. For 28 days
Test	Metronidazole 114 mg/kg, i. p. For 14 days+test drug 400 mg/kg, *p. o*. For 14 days	Pyrogallol 100 mg/kg, i. p. For 7 days+test drug 400 mg/kg *p. o*. For 22 days.	Ethanol 2 g/kg, 20 % w/v *p. o.* For 28 days+test drug 400 mg/kg *p. o*. For 28 days.
Standard	Metronidazole 114 mg/kg, i. p. For 14 days+Levamisole 50 mg/kg, *p. o*. For 14 days	Pyrogallol 100 mg/kg, i. p. For 7 days+Levamisole 50 mg/kg, *p. o*. For 22 days.	Ethanol 2 g/kg, 20 % w/v *p. o*. For 28 days days+Levamisole 50 mg/kg, *p. o*. For 28 days.

Table 2: Humoral antibody response and cellular immune response

S. No.	Model	Antigen challenge for humoral immune response	Estimation day for humoral immune response	Antigen challenges for cellular immune response	Estimation day for cellular immune response
I	Metronidazole induced immunosuperssion	On the 9th day	On the 13th day	On the 13th day	On the 15th day
II	Pyrogallol induced immunosupression	On 7th and 13th day	On 13th and 20th day	On the 20th day	On the 22nd day
III	Ethanol induced immunosupression	On 14th and 20th day	On 20th and 27th day	On the 27th day	On the 29th day

Superoxide dismutase (SOD)–It was estimated in the erythrocyte lysate prepared from the 5% RBC suspension. To 50 µl of the lysate, 75 mM of Tris-HCl buffer (pH 8.2), 30 mM EDTA and 2 mM of Pyrogallol were added. An increase in absorbance was recorded at 420 nm for 3 min by spectrophotometer [14]. The activity of SOD is expressed as units/mg protein.

$$\text{SOD} = \frac{(0.025 - \text{Y})}{\text{Y} \times 50} \times 50$$

Where Y = final reading–initial reading

Catalase–50 µl of the lysate was added to a cuvette containing 2 ml of phosphate buffer (pH 7.4) and 1 ml of 30 mM H_2O_2 catalase activity was measured at 240 nm for 1 min using a spectrophotometer. The molar extinction coefficient of H_2O_2, 43.6 M cm^{-1} was used to determine the catalase activity. One unit of activity is equal to one mM of H_2O_2 degraded per minute and expressed as units per milligram of protein [15].

$$\text{Log}\frac{\text{A}}{\text{B}} \times 2297.3$$

Where, A: Initial absorbance

B: Final absorbance (after 30 min)

Units = μ moles of H_2O_2 consumed/min/mg

Reduced glutathione–To 1 ml of sample, 1 ml of Trichloroacetic acid (TCA) was added. The precipitated fraction was centrifuged and 0.5 ml of supernatant, 2 ml DTNB was added. The final volume was made up to 3 ml with phosphate buffer. The color developed and the optical density was measured at 412 nm using spectrophotometer [16].

$$X = \frac{Y - 0.0046}{0.0034}$$

Where Y= absorbance of test sample

Lipid peroxidation–2 ml of sample was mixed with 2 ml of 20% TCA and kept on ice for 15 min. The precipitate was separated by centrifugation and 2 ml of samples of clear supernatant solution were mixed with 2 ml aq. 0.67% TBA solution. This mixture was heated on a boiling water bath for 10 min. It was cooled in ice for 5 min and absorbance was measured spectrophotometrically at 535 nm. The values were expressed as nm of MDA formed/mg of protein values are normalized to protein content of tissues [17].

$$X = \frac{Y + 0.002}{0.0026056}$$

Where Y = absorbance differences of final (after 3 min) and initial reading of the test sample.

Hematological parameters

Rats were anesthetized and blood sample was collected from the retro-orbital plexus using EDTA coated tubes. Blood parameters like RBC, WBC, DLC and Hb were estimated using the kits on the last day of the treatment [18] i.e. 15th day in case of metronidazole induced immunosuppression, 22nd day in case of pyrogallol induced immunosuppression, and 28th in case of ethanol induced immunosuppression.

Serum immunoglobulins

Serum immunoglobulins, like IgG were estimated using the kit (Quantia) on the last day of the treatment, i.e. 15th day in case of metronidazole induced immunosuppression, 22nd day in case of pyrogallol induced immunosuppression, and 28th in case of ethanol induced immunosuppression.

Histopathological examinations

All groups of rats were sacrificed by cervical dislocation on the last day of treatment. Spleen of each rat was collected, fixed in 10% formalin and sectioned. Histopathological changes in the spleen were observed under light microscope.

Statistical analysis

All the data were expressed as mean±SEM. Statistical significance between more than two groups were tested using one-way ANOVA followed by 'Bonferroni: compares all pairs of columns' test using Graph Pad Prism-6 software. Differences were considered to be statistically significant when $P<0.05$.

RESULTS

Phytochemical screening

PEPD exhibits the presence of diterpinoids, steroids and essential fatty acids like linoleic, linolenic, palmitic, stearic, myristic and arachidic acid.

Acute toxicity studies

PEPD was found to be safe since no animal died at maximum dose of 4000 mg/kg when administered orally, and the animals didn't show any gross behavioral changes. Hence, 1/10 of maximum therapeutic dose (400 mg/kg) was selected for the present study.

Effect of PEPD on metronidazole induced immunosuppression

Effects on Immunological parameters

Animal treated with metronidazole alone for 14 days showed a significant ($P<0.001$) decrease in humoral immune response, cellular immune response, phagocytic response and IgG levels when compare to the normal group, whereas administration of PEPD or levamisole for 14 days significantly ($P<0.001$) prevent the influence of metronidazole on humoral immune response, cellular immune response, phagocytic response and IgG levels when compared to control group (table 3).

Table 3: Effect of PEPD on Metronidazole induced Immunological parameters

S. No.	Group	Hemagglutination (Antibody titer)	Cellular immune response (Paw edema) mm	Phagocytic response (% phagocytosis)	IgG
1	Normal	55.3±7.126	9.500±0.2646	56.93±1.841	0.3367±0.03930
2	Control	7.667±0.88#	7.433±0.2028#	27.23±1.855#	0.1833±0.01453#
3	Test	78.33±4.702*	11.60±0.6110*	66.67±1.507*	0.3933±0.008819*
4	Standard	77.33±2.906*	9.767±0.1453*	66.00±4.041*	0.3500±0.015*

All values are shown as mean±SEM and n = 6, # $P<0.001$ when compared to normal and * $P<0.001$ when compared to control group

Effects on *in vivo* anti-oxidant parameters

Animal treated with metronidazole alone for 14 days showed a significant (P<0.001) decreased in SOD, catalase, reduced glutathione and significantly (P<0.001) increased in LPO when compare to the normal group, whereas administration of PEPD or levamisole for 14 days significantly (P<0.001) preclude the influence of metronidazole on SOD, catalase, reduced glutathione and LPO when compared to control groups (table 4).

Effect on hematological parameters

Animal treated with metronidazole alone for 14 days showed a significant decrease in % Hb ($P<0.001$), RBC ($P<0.01$) and WBC ($P<0.01$) when compare to the normal group, whereas administration of PEPD for 14 days significantly prevent the influence of metronidazole on % Hb ($P<0.001$), RBC ($P<0.01$) and WBC ($P<0.01$) when compared to control group. Animals receiving levamisole for 14 days had shown significant influence on % Hb ($P<0.01$), RBC ($P<0.05$) and WBC ($P<0.05$) when compared to control group (fig. 1).

Table 4: Effect of PEPD on metronidazole induced *in vivo* anti-oxidant parameters

S. No.	Groups	SOD (Units/mg protein)	Catalase (Units/mg protein)	Reduced glutathione (μmol/gHb)	Lipid peroxidation (MMDA/gHb)
1	Normal	32.00±2.309	290.3±4.631	5.067±0.4096	85.67±2.404
2	Control	15.67±0.8819#	160±2.082#	1.633±0.2603#	122.0±2.309#
3	Test	33.67±1.453*	297.3±2.906*	5.667±0.2612*	72.33±2.028*
4	Standard	28.00±2.082*	293.0±2.517*	5.600±0.2517*	79.33±0.8819*

All values are shown as mean±SEM and n = 6, # $P<0.001$ when compared to normal and * $P<0.001$ when compared to control group

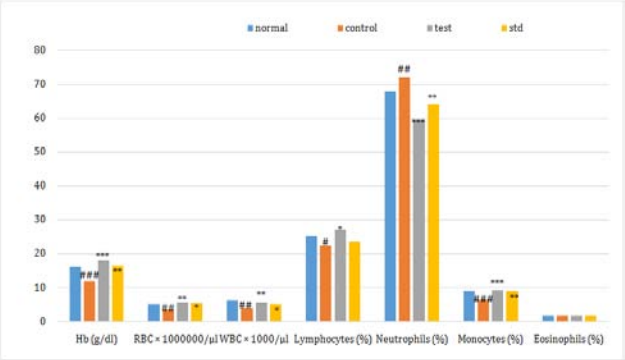


Fig. 1: Effect of PEPD on metronidazole induced hematological parameters

All values are shown as mean±SEM and n = 6, # *P*<0.05, ## *P*<0.01 and ### *P*<0.001 when compared to normal and * *P*<0.05, ** P<0.01 and *** *P*<0.001 when compared to control group

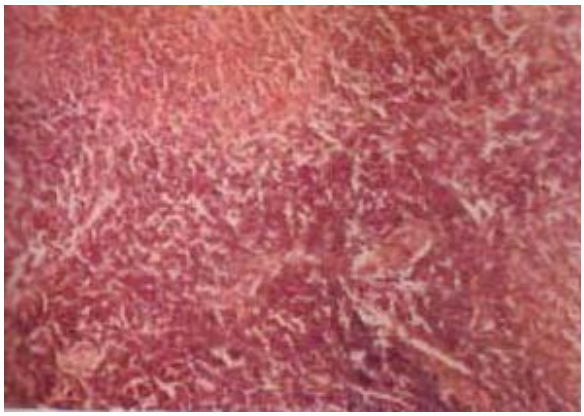
Effect on Histopathology of spleen

Light microscopic examinations of spleen did not show any significant differences between PEPD-treated and control groups, fig 2 (a) and fig 2 (c) whereas animals treated with metronidazole shows the congestion of red pulp, some eosinophil's and neutrophils, fig 2 (b).

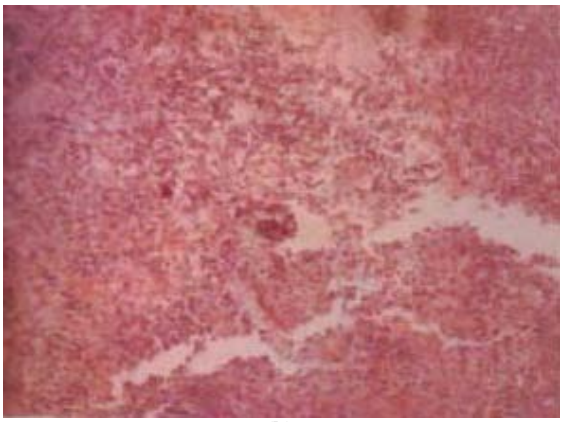
Effect of PEPD on pyrogallol induced immunosuppression

Effects on Immunological parameters

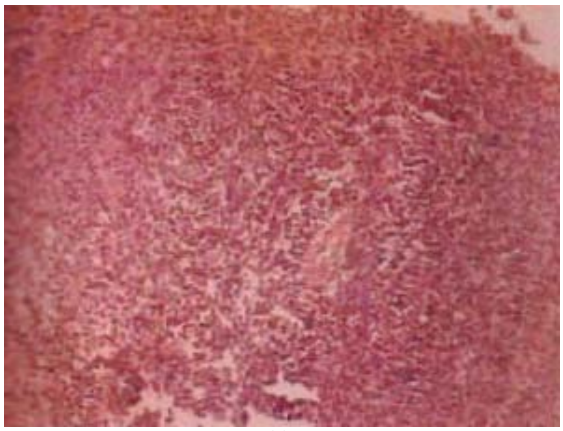
Animal treated with pyrogallol alone for 22 days showed a significant (*P*<0.001) decreased in humoral immune response, cellular immune response, phagocytic response, and IgG levels when compare to the normal group, whereas administration of PEPD or livamisole for 22 days significantly (*P*<0.001) prevent the influenced of pyrogallol on humoral immune response, cellular immune response, phagocytic response, and IgG levels when compared to control group (table 5).



(a)



(b)



(c)

Fig. 2: Histopathology of the spleens of metronidazole model; (a) normal, showing capsule and sub capsular spaces (b) metronidazole treated, congestion of red pulp, eosinophil's, and neutrophils and (c) PEPD treated, with congested red pulp

Table 5: Effect of PEPD on pyrogallol induced Immunological parameters

S. No.	Group	Hemagglutination (Antibody titer)		Cellular immune response (Paw edema) mm	Phagocytic response (% phagocytosis)	IgG
		Primary	Secondary			
1	Normal	57.33±1.856	68.67±1.764	10.07±0.8387	55.33±0.8387	6.383±0.01764
2	Control	13.67±1.453#	18.33±1.453#	7.533±0.1764#	22.03±1.146#	0.1533±0.03512#
3	Test	45.33±1.764*	61.0±1.732*	9.533±0.2333*	55.73±1.947*	0.3400±0.01732*
4	Standard	57.67±3.480*	71.33±1.453*	10.13±0.1453*	54.67±2.028*	0.4100±0.0152*

All values are shown as mean±SEM and n = 6, # *P*<0.001 when compared to normal and * *P*<0.001 when compared to control group.

Effects on *in vivo* anti-oxidant parameters

Animal treated with pyrogallol alone for 22 days showed a significant (P<0.001) decrease in SOD, catalase, reduced glutathione and increase in LPO when compare to the normal group,

Whereas administration of PEPD or levamisole for 22 days significantly (P<0.001) prevent the influence of pyrogallol on SOD, catalase, reduced glutathione and LPO when compared to control groups (table 6).

Effects on hematological parameters

Animal treated with pyrogallol alone for 22 days showed a significant decrease in % Hb (P<0.01), RBC (P<0.01) and WBC (P<0.01) when compare to the normal group, whereas administration of PEPD for 22 days significantly prevent the influence of pyrogallol on % Hb (P<0.01), RBC (P<0.01) and WBC (P<0.01) when compared to control group. Animals receiving levamisole for 22 days had shown significant influence on % Hb (P<0.01), RBC (P<0.01) and WBC (P<0.01) when compared to control group (fig. 3).

Table 6: Effect of PEPD on pyrogallol induced *in vivo* anti-oxidant parameters

S. No.	Groups	SOD (Units/mg protein)	Catalase (Units/mg protein)	Reduced glutathione (µmol/gHb)	Lipid peroxidation (MMDA/gHb)
1	Normal	39.33±1.453	298.3±1.325	5.5±0.3055	92.33±2.028
2	Control	15.33±1.764#	145.7±3.108#	1.53±0.08819#	133.7±3.282#
3	Test	36.00±2.082*	295.7±4.910*	4.8±0.1155*	96.67±2.603*
4	Standard	30.87±0.4055*	302±2.082*	5.467±0.1453*	88.33±0.8819*

All values are shown as mean±SEM and n = 6, # P<0.001 when compared to normal and * P<0.001 when compared to control group.

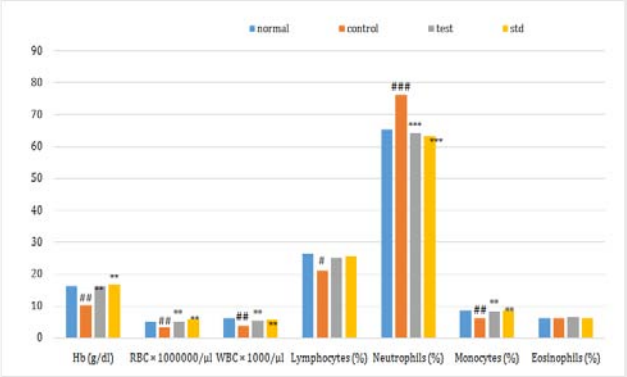


Fig. 3: Effect of PEPD on pyrogallol induced hematological parameters

All values are shown as mean±SEM and n = 6, # P<0.05, ## P<0.01 and ### P<0.001 when compared to normal and * P<0.05, ** P<0.01 and *** P<0.001 when compared to control group

Effect on histopathology of spleen

Complete loss of spleen architecture with undifferentiated red pulp and white pulp was observed in pyrogallol treated group, fig. 4 (ii) whereas Capsule and sub capsular spaces with red and white pulps were intact in both normal, fig. 4 (i) and PEPD, fig. 4 (iii) treated group.

Effect of PEPD on ethanol induced immunosuppression

Effects on Immunological parameters

Animal treated with ethanol alone for 28 days showed a significant (P<0.001) decrease in humoral immune response, cellular immune response, phagocytic response, and IgG levels when compare to the normal group,

Whereas administration of PEPD or levamisole for 28 days significantly (P<0.001) prevent the influence of ethanol on humoral immune response, cellular immune response, phagocytic response, and IgG levels when compared to control group (table 7).

Effects on *in vivo* anti-oxidant parameters

Animal treated with ethanol alone for 28 days showed a significant (P<0.001) decreased in SOD, catalase, reduced glutathione and significant (P<0.001) increase in LPO when compare to the normal group, whereas administration of PEPD for 28 days significantly (P<0.001) prevent the influenced of ethanol on SOD, catalase, reduced glutathione and LPO when compared to control group. Animals receiving levamisole for 28 days had shown significant (P<0.001) influenced on SOD, catalase, reduced glutathione and LPO when compared to control group (table 8).

Table 7: Effect of PEPD on ethanol induced immunological parameters

S. No.	Group	Hemagglutination (Antibody titer)		Cellular immune response (Paw edema) mm	Phagocytic response (% phagocytosis)	IgG
		Primary	Secondary			
1	Normal	70.33±2.963	77.33±3.283	10.63±0.1453	64.67±2.028	0.6267±0.04631
2	Control	11.67±1.453#	16.33±1.453#	6.567±0.2848#	14.33±2.028#	0.1333±0.02963#
3	Test	56.00±1.528*	66.77±2.603*	8.333±0.1453*	40.87±0.1764*	0.3533±0.01764*
4	Standard	71.33±3.180*	71.33±3.180*	9.933±0.2404*	66.00±2.082*	0.7200±0.02082*

All values are shown as mean±SEM and n = 6, # P<0.001 when compared to normal and *P<0.001 when compared to control group

Effects on hematological parameters

Animal treated with ethanol alone for 28 days showed a significant decrease in % Hb (P<0.01), RBC (P<0.01) and WBC (P<0.01) when compare to the normal group, whereas administration of PEPD for 28 days significantly prevent the influence of ethanol on % Hb (P<0.01), RBC (P<0.01) and WBC (P<0.05) when compared to control group. Animals receiving levamisole for 22 days had shown significant influence on % Hb (P<0.01), RBC (P<0.01) and WBC (P<0.01) when compared to control group (fig. 5).

Effect on histopathology of spleen

Complete distortion, atrophy of white pulp, congestion and hemorrhage of red pulp was observed in ethanol treated group, fig 6 (b), whereas intact capsule and sub capsular spaces and preservation of architecture was observed in both control, fig 6 (a) and PEPD, fig 6 (c) treated group.

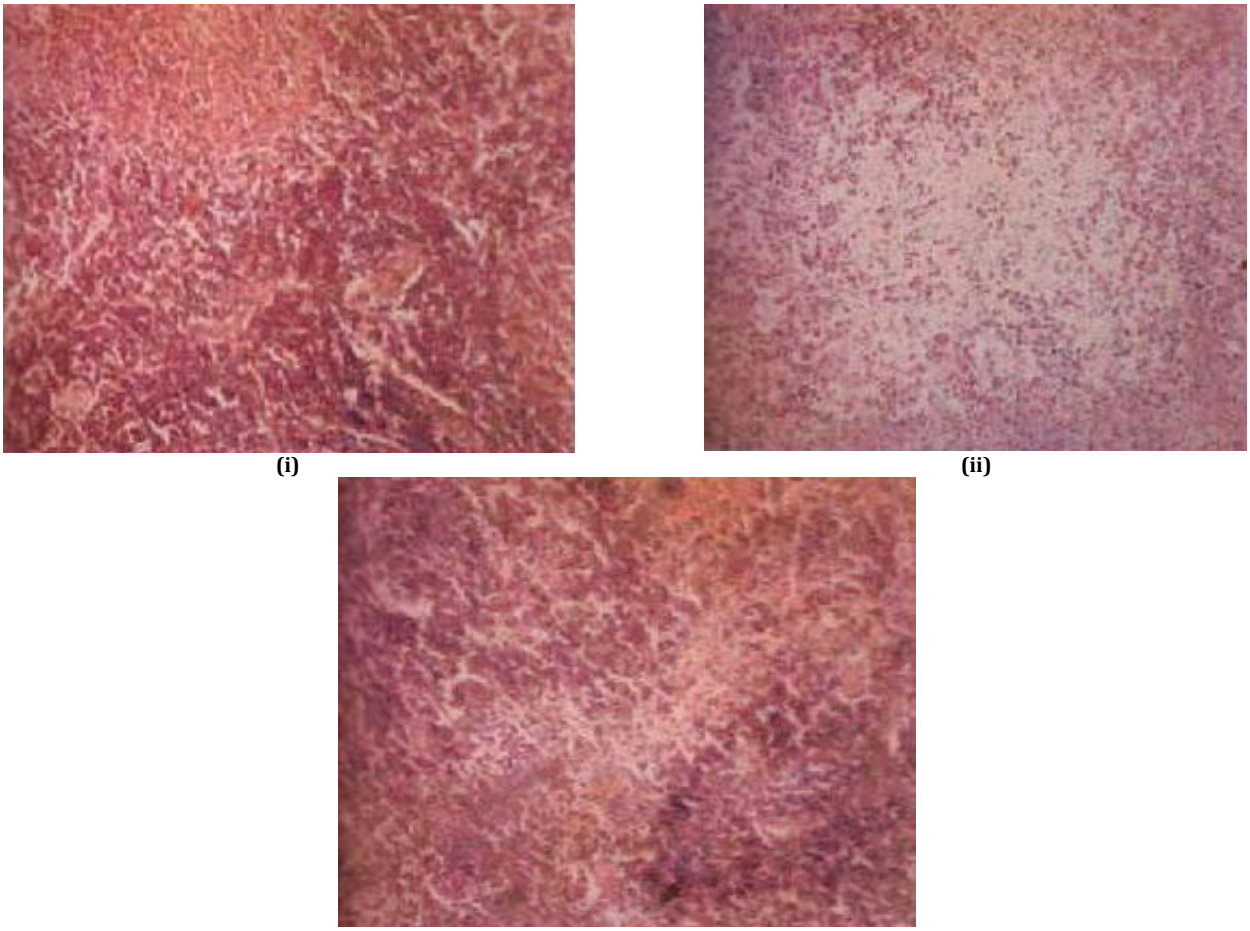


Fig. 4: Histopathology of the spleens of pyrogallol model; (i) normal, with capsule and sub capsular spaces (ii) pyrogallol treated, with complete loss of spleen architecture, red pulp and white pulp can't be differentiated and (iii) PEPD treated, with red pulp and white pulp showing normal cellularity

Table 8: Effect of PEPD on ethanol induced *in vivo* anti-oxidant parameters

S. No.	Groups	SOD (Units/mg protein)	Catalase (Units/mg protein)	Reduced glutathione (µmol/gHb)	Lipid peroxidation (MMDA/gHb)
1	Normal	41.67±2.333	299.0±4.041	5.833±0.1453	97.33±3.283
2	Control	11.67±1.764#	136±3.215#	1.167±0.1453#	157.33±1.856#
3	Test	21.67±1.453*	281±4.359*	3.867±0.1764*	127.5±1.160*
4	Standard	38.33±1.202*	303±3.606*	5.2±0.2082*	86.67±5.812*

All values are shown as mean±SEM and n = 6, # *P*<0.001 when compared to normal and * *P*<0.001 when compared to control group

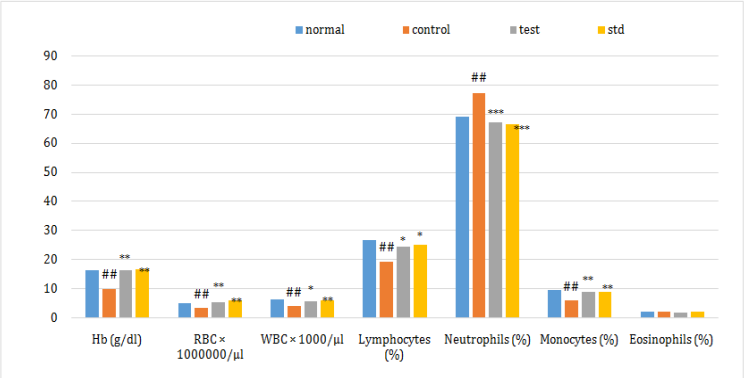


Fig. 5: Effect of PEPD on ethanol induced hematological parameters

All values are shown as mean±SEM and n = 6, # *P*<0.05, ## *P*<0.01 and ### *P*<0.001 when compare to normal and * *P*<0.05, ** *P*<0.01 and *** *P*<0.001 when compared to control group

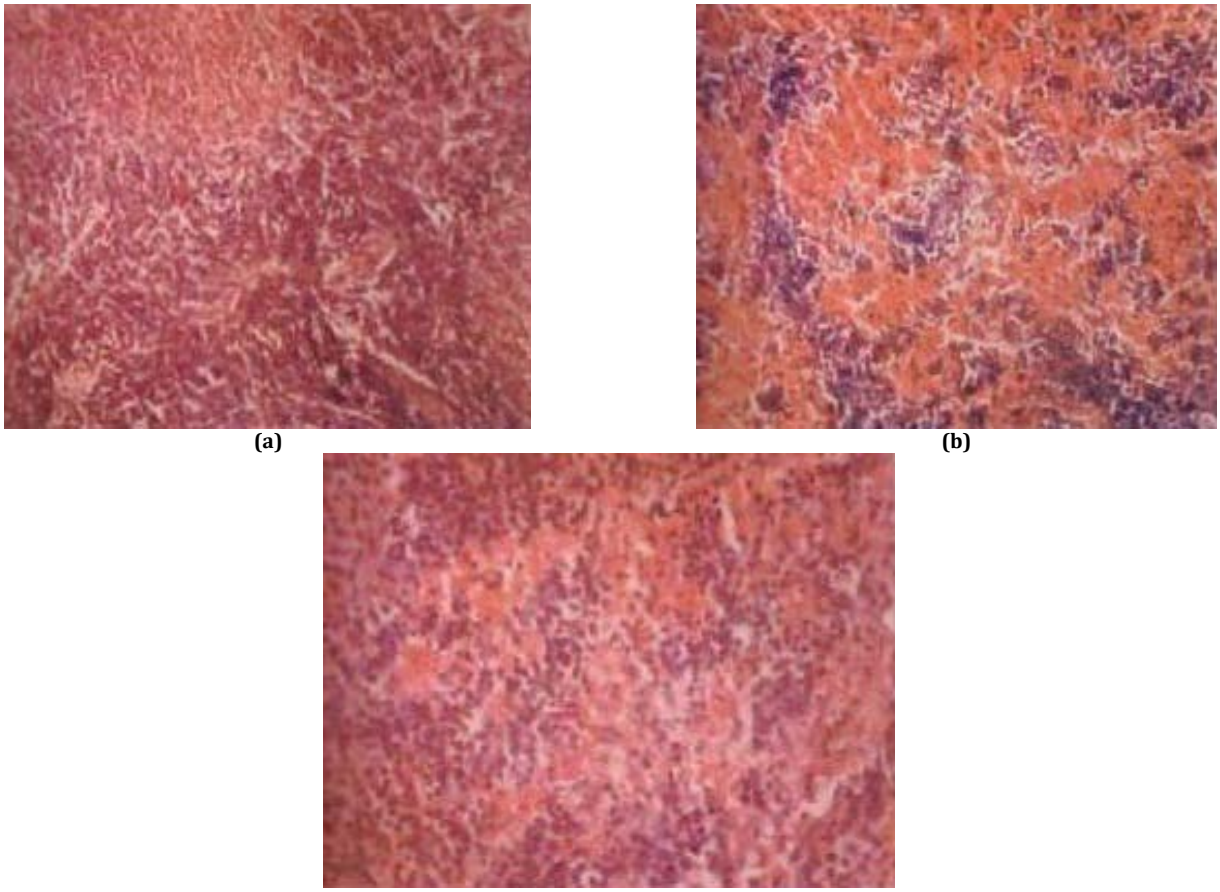


Fig. 6: Histopathology of the spleens of ethanol model; (a) normal, with showing capsule and sub capsular spaces (b) ethanol treated, with complete distortion, atrophy of white pulp, congestion and hemorrhage of red pulp and (c) PEPD treated, with preservation of architecture of spleen near to normal

DISCUSSION

In traditional medicine in different plant parts are believed to have specific medicinal properties including the ability to stimulate the body's defense mechanism [19]. An advance area of research is the development of immunomodulatory agents that is free from toxic side effects and can be utilized for a longer duration, hence resulting in continuous immune-activation [20]. While screening the immunomodulatory activity, most of the studies employed agents like Cisplatin, Cyclophosphamide, or Corticosteroids in order to bring on the immunosuppression in the experimental animals [21]. These factors are likewise experienced to generate free radicals in the biological system and thereby cause oxidative stress, but they are highly toxic and they will not produce reproducible results [22]. It is known pyrogallol is a potent generator of free radicals, less toxic agents and can curb the proliferation of mouse lymphocytes *in vitro* [23]. However, several works demonstrated that prooxidants suppress the immune response in experimental animals [24]. Ethanol exposure decreased SOD and reduced glutathione content which are an important defensive antioxidant enzyme found in almost all existing cells. The present investigation revealed that treatment with ethanol depleted glutathione, SOD and impairment of phagocytosis. In fact, free radical are signalling in T-cell activation. Nevertheless, the continued propagation of free radicals over a long period was shown to down regulate the activation of T-cell. Likewise, down regulation of T-cell activation has been observed when nitric oxide, a free radical generator, is constantly generated by macrophages, and grain alcohol is too recognized to generate nitric oxide [25]. Metronidazole has also been shown to induce suppression in the bone marrow, a primary lymphoid organ and metronidazole decreased the relative weight of the spleen and atrophy in the white pulp of the spleen. Metronidazole also decrease the percentage of neutrophils suggesting a suppression of the phagocytes defense mechanism. The reduction in the spleen relative weight, fall in the atrophy of spleen white pulp, decreased in peripheral blood RBCs and monocytes may be reason behind immunosuppression [26]. Levamisole is synthetic immunostimulant preparation, which enhance the immunological parameters by increasing the proliferation of macrophage and lymphocytes and by increasing the antibody production [27].

Pithecellobium dulce seeds contain mucilage and it consists of acylated triterpinoid saponin called pithecelloside and essential fatty acids like palmitic acid, stearic acid, linoleic acid, linolenic acid, myristic acid and arachidic acid (16%). Fatty acids are a sort of fat or lipid which have the power to act upon the immune system and the role of its various cellular components have been known for nearly 30 years [7]. Mice are sensitized with SRBC as antigen, and gets diffused in the extra vascular space and enters to the lymph node via the lymphatic system. Particulate antigens are taken up by macrophages lining the sinuses. Small highly antigenic peptides are combined with MHC class II molecules. B cells with receptors for antigens bind and internalize it into an endosomal compartment and process and present it the T-helper cells. These B cells are triggered to proliferate and gives rise to a large number of daughter cells. Some of the cells of these 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, frequently accompanied by a switch to an increasing proportion of IgG [28, 29]. In the present work, assessment of humoral immunity was carried out using the H. A. titer. The anti-SRBC antibody titer was raised in PEPD-treated groups with normal immune status, but was found statistically significant when compared to the control group in both primary and

secondary antibody titer responses. An increment in the carbon clearance index reflects the enhancement of the phagocytic function of macrophage and nonspecific immunity. Phagocytosis by macrophages is markedly enhanced by the opsonisation of parasites with antibodies and complementing C3b, leading to a more rapid clearance of parasites from the blood [30]. PEPD significantly increases the phagocytic activity when compared to the control. The phagocytic index and antibody titer increased significantly and the F ratios of the phagocytic index and WBC count were also significant in case of *Eclipta alba* and *Centella asiatica* [13].

In this study immunostimulant activity of PEPD may be ascribable to the presence of dietary fatty acids, they were reported to regulate immune responses through one or more of three major molecular mechanisms: altered membrane composition and function, modified eicosanoid production, and changed cytokine biosynthesis [31]. SOD is an enzyme that repairs cells and reduced the damage done by superoxide, the most common free radical in the consistency. Surveys hold shown that SOD acts as both an antioxidant and anti-inflammatory in the body, neutralizing the free radicals that can contribute to wrinkles and precancerous cell changes [32]. Metronidazole, pyrogallol and ethanol decreased the levels of SOD whereas PEPD and levamisole treated group exhibited increased levels of this enzyme. Catalase is a haem containing redox enzyme found in almost all living organisms that are exposed to oxygen, where it works to catalyze the decay of hydrogen peroxide to water and oxygen [33]. The present investigation has brought out that treatment with metronidazole, pyrogallol and ethanol depleted the reduced glutathione. It was further observed that PEPD and levamisole prevent the above influences of metronidazole, pyrogallol and ethanol on reducing glutathione levels. It is recognized that the peroxidative toxicity of metronidazole, pyrogallol and ethanol *in vivo* may also be through its result of iron release from ferritin, which can cause lipid peroxidation via Fenton or Haber-Weiss reaction [34]. Treatment with PEPD and levamisole prevented the above influence of metronidazole, pyrogallol and ethanol on lipid peroxidation.

Cassia auriculata (Caesalpiniaceae) showed a significant immunostimulant effect on cell mediated immunity and no force on the humoral immunity which may be due to petroleum ether extract-steroids alcoholic and aqueous extract-alkaloids, flavonoids, tannins, phenolics. *Bauhinia variegate (Caesalpiniaceae)* also possesses immunostimulant activity on both specific and non-specific immune system which might be ascribable to the presence of Tannins, steroids, alkaloids, flavonoids, β sitosterol, lupeol, vitamin C, kaempferol, flavones, quercetin & saponins. Likewise, other plants like *Couropita guianensis* (Lecythidaceae), *Withania somnifera*, *Baliospermum montanum* and *Trapa bispinosa* also showed a stimulatory effect on humoral immunity and stimulated phagocytosis furthermore, it stimulates the haemopoietic system and also enhances the differentiation of stem cells [35].

The PEPD not only attenuated the influence of metronidazole, pyrogallol and ethanol on the immune system but also found to prevent the changes in the oxidative stress parameters, which were induced by metronidazole, pyrogallol and ethanol. *Pithecellobium dulce* was reported to possess acylated triterpinoid, saponin, pithecelloside and essential fatty acids. Thus, the immunomodulatory activity of PEPD could be due to its saponins and fatty acid components, these components may exert its immunomodulatory activity by altered membrane composition and changed cytokine biosynthesis. Further studies are on course for establishing PEPD as a therapeutic intervention for several immunological disorders.

ACKNOWLEDGEMENT

We show our profound thanks and huge gratitude to Dr. Y. Padmanabha Reddy, M. Pharm., Ph. D., F. I. C Principle and Dr. S. Somasekhar Reddy, M. Pharm., Ph. D., Department of pharmacology, RIPER, Anantapuramu, for their cooperation, encouragement and for providing facilities, which enabled us to complete this work successfully. We are grateful to express our particular thanks to Mr P. Nagendra Kumar, Lab. Technician for his continuous help throughout our study.

CONFLICT OF INTERESTS

Declared None

REFERENCES

1. Sharififar F, Pournourmohammadi S, Rastegarianzadeh R, Ranjbaran O, Purhemmaty A. Immunomodulatory activity of aqueous extract of heracleum persicum. Desf in mice. Iran J Pharm Res 2010;8:287-92.
2. Prabhu VV, Guruvayoorappan C. Evaluation of immunostimulant activity and chemoprotective effect of mangrove Rhizophora apiculata against cyclophosphamide induced toxicity in BALB/c mice. Immunopharmacol Immunotoxicol 2012;34:608-15.
3. Fong HH. Integration of herbal medicine into modern medical practices: issues and prospects. Integr Cancer Ther 2002;1:287-93.
4. Anonymous. The Wealth of India: Raw materials. Vol. VIII. Publications and Information Directorate, CSIR, New Delhi; 1969.
5. Nigam SK, Gopal M, Uddin R, Yoshikawa K, Kawamoto M, Arihara S. Pithedulosides A-G, oleanane glycosides from Pithecellobium dulce. Phytochemistry 1997;44:1329-34.
6. Shivaprasad HN, Kharya MD, Rana AC, Mohan S. Preliminary immunomodulatory activities of the aqueous extract of terminalia chebula. Pharm Biol 2006;44:32-4.
7. Shweta S, Mehta BK. A review on pharmacological activities of Pithecellobium dulce extract, and there effective doses. J Med Pharm Allied Sci 2013;5:37-45.
8. Kokate CK, Purohit AP, Gokhale SB. A Text Book of Pharmacognosy. Nirali Prakashan Publisher, Pune, India; 2009.
9. Annie SSG, Lipton AP, Paul Raj R. Lethal concentration of methanol extract of sponges to the brine shrimp, Artemia salina. J Mar Biol Assoc India 2009;51:122–5.
10. Sheth JN. The Future of Buyer Behavior Theory. In: SV-Proceedings of the Third Annual Conference of the Association for Consumer Research. Eds. M Venkatesan, Chicago, IL: Association for Consumer Research; 1972. p. 562-75.
11. Sudha P, Asdaq SMB, Dhamingi SS, Chandrakala GK. Immunomodulatory activity of methanolic leaf extract of moringa oleifera in animals. Indian J Physiol Pharmacol 2010;54:133–40.
12. Fulzele SV, Satturwar PM, Joshi SB, Dorle AK. Study of the immunomodulatory activity of Haridradi ghrita in rats. Indian J Pharmacol 2003;35:51-4.
13. Jayathirtha MG, Mishra SH. Preliminary immunomodulatory activities of methanol extracts of Eclipta alba and Centella asiatica. Phytomedicine 2004;11:361-5.
14. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974;47:469–74.
15. Aebi H. [13] Catalase *in vitro*. Methods Enzymol 1984;105:121-6.
16. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70–7.
17. Stocks J, Dormandy TL. The autoxidation of human red cell lipids induced by hydrogen peroxide. Br J Haematol 1971;20:95–111.
18. Antai AB, Ofem OE, Ikpi DE, Ukafia SS, Agiang EA. Phytochemistry and some haematological changes following oral administration of ethanolic root extract of gonglonema latifolium in rats. Niger J Physiol Sci 2009;24:79-83.
19. Punturee K, Wild CP, Kasinrerk W, Vinitketkumnuen U. Immunomodulatory activities of centella asiatica and rhinacanthus nasutus extracts. Asian Pacific J Cancer Prevention 2005;6:396-6.
20. Su PF, Staniforth V, Li CJ, Wang CY, Chiao MT. Immunomodulatory effects of phytocompounds characterized by *in vivo* transgenic human GM-CSF promoter activity in skin tissues. J Biomed Sci 2008;15:813-22.
21. Hirokawa K, Utsuyama M. Animal models and possible human application of immunological restoration in the elderly. Mech Ageing Dev 2002;123:1055-63.

22. Knight JA. Review: free radicals, antioxidants, and the immune system. Ann Clin Lab Sci 2000;30:145-58.
23. Archer DL. Immunotoxicology of food borne substances: an overview. J Food Prot 1978;41:983-8.
24. Hafidh RR, Abdulamir AS, Bakar FA, Abas F, Jahanshiri F, Sekawi Z. Antioxidant research in asia in the period from 2000-2008. Am J Pharmacol Toxicol 2009;4:56.
25. Hafez MS. Histological and immunohistochemical study on the effect of noise stress on the spleen of adult albino rat and the possible role of Ginkgo biloba extract. Egyptian J Histol 2013;36:546-55.
26. Fararjeh M, Mohammad MK, Bustanji Y, Alkhatib H, Abdalla S. Evaluation of immunosuppression induced by metronidazole in Balb/c mice and human peripheral blood lymphocytes. Int Immunopharmacol 2008;8:341-50.
27. Salomi KR, Shafeen SS, Roopesh C, Reddy YCK, Sandya L, Nagarjuna S, *et al*. Evaluation of immunomodulatory activity of petroleum ether extract of seeds of Celastrus paniculatus. Pharm Lett 2011;3:87-93.
28. Shaikh S, Ghaisas M, Deshpande A, Karpe AS, Manikrao A. Immunomodulatory activity of ethanolic extract of the stem bark of bauhinia variegate Linn. Pharmacologyonline 2011;2:167-70.
29. Chahar MK, Kumar DSS, Lokesh T, Manohara K. *In-vivo* antioxidant and immunomodulatory activity of mesuol isolated from Mesua ferrea L. Seed oil. Int Immunopharmacol 2012;13:386-91.
30. Patil PR, Patil MR, Mane A, Patil S. Immunomodulatory effects of fruits of Barringtonia racemosa Linn. Int J Basic Clin Pharmacol 2013;2:216-9.
31. Fritsche K. Fatty acids as modulators of the immune response. Annu Rev Nutr 2006;26:45-73.
32. Lin CC, Shieh DE, Yen MN. Hepatoprotective effect of the fractions of Banzhilian on experimental liver injuries in rats. J Ethnopharmacol 1997;56:193-200.
33. Anbuselvam C, Vijayavel K, Balasubramanian M. Protective effect of Operculina turpethum against 7, 12-dimethyl benz (a) anthracene induced oxidative stress with reference to breast cancer in experimental rats. Chem Biol Interact 2007;168:229-36.
34. Li ASH, Bandy B, Tsang SS, Davison AJ. DNA-breaking versus DNA-protecting activity of four phenolic compounds *in vitro*. Free Radical Res 2000;33:551-66.
35. Naga Preethi N, Rajeshwari P. An overview on immuno-modulators. Int J Curr Pharm Res 2014;4:108-14.