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Immunomodulatory effect of the hydroalcoholic extract of *Abrus precatorius* L. leaves against cyclophosphamide-induced immunosuppression in mice

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Abstract: This study presents the immunomodulatory potential of *Abrus precatorius* Linn. (Indian wild licorice) leaves. A hydroalcoholic extract of *A. precatorius* leaves (EAPL) was prepared by maceration. Thirty male mice were divided into five groups as follows: control group, model control group (cyclophosphamide-treated), and three treatment groups (treated with EAPL at doses of 100, 200 and 300 mg/kg, *per os*, daily for 14 days). Parameters, including hematological, biochemical, organ indices, hemagglutination test (HA titer), delayed-type hypersensitivity (DTH), interleukin-2 (IL-2) level, splenocyte proliferation and liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) analysis were evaluated. Histopathological examination was carried out for the spleen, kidney and liver. Cyclophosphamide (CPMD)-induced changes in white blood cells, lymphocytes and platelets were improved in the treatment groups. Total protein and albumin levels in the treatment groups were significantly higher. EAPL treatment significantly stimulated splenic T-lymphocyte-mediated proliferation. Neutrophil adhesion was insignificantly decreased in the model control group compared to the normal control group, which was slightly improved by EAPL treatment. EAPL treatment significantly improved the HA titer and cell-mediated immunity, which is an indication of antibody production. The IL-2 level was significantly higher in the treatment groups that received 200 and 300 mg/kg EAPL. LC-QTOF-MS analysis of EAPL showed the presence of flavonoids, lignans, iridoids and phenolic glycosides. These results suggest that *A. precatorius* leaves are a good candidate for a new immunomodulatory herbal formulation.

Keywords: *Abrus precatorius*; immunomodulation; cyclophosphamide; immunity; LC-QTOF-MS

Abbreviations: Concanavalin-A (ConA); Cyclophosphamide (CPMD); Delayed-type hypersensitivity (DTH); Enzyme-linked immunosorbent assay (ELISA); Extract of *Abrus precatorius* Leaves (EAPL); Hematoxylin and Eosin (H&E); Lipopolysaccharide (LPS); Liquid Chromatography Quadrupole Time of Flight Mass Spectrometry (LC-QTOF-MS)

INTRODUCTION

Research on alternate medicine, particularly the use of extracts of medicinal plants for immunomodulation, is increasing. Many plant-derived agents exhibit immunomodulating effects, and plant extracts also show such activity in drug-induced immunosuppression in laboratory animals [1]. However, little is known about the immunomodulatory properties of less frequently studied plants such as *Abrus precatorius* Linn.

Abrus precatorius Linn. (also known as *Chanothi* in Gujarati and *Gunja* in Sanskrit) is a leguminous plant of the Fabaceae family, which is a woody twining plant with characteristic toxic red seeds with a black mark at the base [2,3]. The roots, leaves, bark and stem of the plant are applied widely for treating various ailments; the leaves of the plant are used for fever, cough and cold, and the roots are used to treat jaundice and hemoglobinuria. The plant has been reported to produce different pharmacological actions, such as purgative, emetic, aphrodisiac and tonic effects [4]. It also possesses antibacterial [5], antiinflammatory [6], antimalarial [7], neuroprotective [8] and nephroprotective [9] properties. *A. precatorius* leaves have been reported to be a rich source of alkaloids, saponin, tannin and several polyphenols, acting as a good source of natural antioxidants that work mutually to support immunity [10].

A scientific study on the immunomodulatory effects of *A. precatorius L.* leaves has not been reported to date. Thus, the present study evaluated the *in vivo* ethnopharmacological effects of the hydroalcoholic extract of *A. precatorius* leaves against cyclophosphamide (CPMD)-induced immunosuppression. The major chemical constituents in the extract were tentatively identified by liquid chromatography-quadrupole time of flight mass spectrometric analysis (LC-QTOF-MS).

MATERIALS AND METHODS

Ethical statement

All procedures involving animal care and treatment were approved by the Institutional Animal Ethics Committee of the college (Approval No. JAU/JVC/IAEC/SA/35/18) as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Department of Animal Husbandry and Dairying, Government of India.

Chemicals and reagents

Alsever's solution (Lot No. RNBJ0060), RBC lysis buffer (Lot. No. RNBH9104), tetramethylbenzidine (TMB (3, 3', 5, 5')) solution (Lot. No. SLBV8513) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) (Lot No. MKCD4805) were purchased from Sigma-Aldrich, India. Cyclophosphamide monohydrate (Lot No. 3029855) was purchased from Merck, Darmstadt, Germany. RPMI-1640 without L-glutamine (Lot No. 29618006), fetal bovine serum (Lot. No. 42G6287K) and penicillin-streptomycin 100× solution (Lot. No. 4181012) were purchased from Genetix Biotech Asia Pvt. Ltd. Rabbit anti-mouse IL-2 (Lot No. 270814) was purchased from Bio-Rad, USA. Goat anti-rabbit HRP conjugate (Lot No. HRP-004-18-005) was purchased from Columbia Bioscience, USA.

Collection and authentication of the plant material

Leaves of the plant were collected in December 2018 from the herbal garden of the Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science and A.H., Junagadh Agricultural University, Junagadh (latitude 21°29'51.7" N, longitude 70°27'01.6" E). The plant material was verified by Botanist Dr. R. C. Viradia (Professor and Head, Department of Biology, Bahauddin Science College, Junagadh (Gujarat) India). A voucher specimen (JVC/VPT/SP/PS/01/18) of plant material was deposited in the department for future reference.

Preparation of the hydroalcoholic extract

Collected plant material was washed with tap water, shed dried, powdered and used to prepare the extract. The powder of *A. precatorius* leaves was extracted with 60% of methanol for 3 days and the extract was then filtered, and the filtrate evaporated to dryness [11]. The test extract was prepared in sufficient quantity and stored at -20°C until use. The yield of the hydroalcoholic extract from the crude dried powder of *A. precatorius* was found to be 15% (w/w).

Animals and experimental design

Thirty male BALB/c mice (7-8 weeks old) were obtained from Cadila Healthcare Ltd, Ahmedabad, India. The animals were housed at 22±3 °C at 50-60% relative humidity and maintained in a 12 h light/dark cycle. Drinking water and feed were provided *ad libitum* during the study. After 1 week of acclimatization, all mice were grouped randomly into 5 different groups, each comprising six mice. One group of healthy mice was kept as the control (C1) and provided with normal feed and water; the second group of mice was treated with CPMD alone at a dose rate of 100 mg/kg, i.p. on day 12 and considered as the model control (C2); the other three groups of mice were treated with EAPL at doses of 100, 200 and 300 mg/kg b.w., *per os*, daily for 14 days, along with a single dose of CPMD at a dose of 100 mg/kg, i.p. on day 12. At the end of treatment, spleen, kidney and liver were collected to assess the organ indices and the histological changes in all the treatment groups. A piece of spleen was collected to carry out a splenocyte MTT assay. Blood was collected to analyze hematological parameters and neutrophil adhesion, the serum was collected to analyze biochemical parameters, HA titer and ELISA. Footpad thickness was measured after injecting sheep

red blood cell (SRBC) to analyze the delayed-type hypersensitivity reaction.

Organ indices

The spleen, kidney and liver of each animal of all experimental groups were collected after the animals were killed and weighed. The spleen, kidney and liver indices were calculated based on spleen, kidney and liver weights and animal body weight (b.w.). The formula for calculating the organ index is the organ weight (in mg) divided by b.w. (in g).

Hematological and biochemical parameters

Hematological parameters such as hemoglobin (Hb; g/dL), packed cell volume (PCV; %), red blood cells (RBC $\times 10^6$/μL), platelets ($\times 10^3$/μL), total white blood cells (WBC $\times 10^3$/μL), neutrophils (%), lymphocytes (%) and monocytes (%) were measured using an automatic hematology analyzer (Abacus Junior Vet 7, Diatron, Hungary). Biochemical parameters like total protein (g/dL) and albumin (g/dL) were estimated using standard kits (Diatek Healthcare Pvt. Ltd., India) on a semiautomatic biochemistry analyzer (Dia-chem 240 plus, Diatek, China). The concentration of globulin was calculated by subtracting the value of albumin from the total protein.

MTT assay

The spleen from each animal was aseptically removed and collected in ice-cold phosphate buffered saline (PBS, pH 7.4), gently homogenized and passed through a 70-mesh filter (Genetix, India) to obtain a single-cell suspension of splenocytes. The erythrocytes were lysed with 1 mL of erythrocyte lysis buffer, while the remaining cells were centrifuged at 600 ×*g* for 8 min. After centrifugation, cells were resuspended in 1 mL of RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% antibiotic solution (100 U/mL penicillin and 100 μg/mL streptomycin). Spleen cells were counted using the trypan blue dye exclusion method. Cells were placed into 96-well plates at a density of 5×10^5 cells/mL (100 μL/well), then 100 μL/well of ConA (5 μg/mL) or LPS (10 μg/mL) was added to the wells. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 (LEEC, UK) for 48 h. After incubation, 20 μL of MTT solution (2 mg/mL) was added to each well. After a 4-h incubation period, the plates were centrifuged at 600 ×*g* for 5 min and the supernatant was discarded. Dimethyl sulfoxide (200 μL) was added to each well and the absorbance was measured in a microplate reader at 570 nm [12,13].

Neutrophil adhesion test

Each blood sample was analyzed for total and differential leukocyte counts. The samples were incubated with nylon fiber (80 mg/mL) at 37 °C for 15 min, followed by reanalysis for total and differential leukocyte count. The neutrophil index and neutrophil adhesion percentage were calculated according to the following formulas [14]:

$$\text{Neutrophil index} = \text{Total leukocyte count} \times \text{Neutrophil percentage}$$

$$\text{Neutrophil adhesion \%} = [(\text{NIus} - \text{NIts}) / \text{NIus}] \times 100,$$

where NIus is the neutrophil index of untreated samples and NIts is the neutrophil index of the treated sample.

Hemagglutination assay

An aliquot (25 μL) of a two-fold diluted serum in PBS (pH 7.4) was challenged with 25 μL of a 1% v/v SRBC suspension (prepared from a 10% SRBCs suspension) in V-bottom microtiter plates (Tarson, India). The plates were incubated at room temperature for 1 h and then examined for hemagglutination. The highest dilution giving hemagglutination was taken as the antibody titer. The antibody titers were expressed in a graded manner, and the minimum dilution was one-half [15].

Delayed-type hypersensitivity reaction

The mice of all groups were primed with 0.1 mL of SRBC suspension containing 5×10^9 cells/mL intraperitoneally on day 7 and challenged with 20 μL of SRBCs (5×10^9) in the left hind footpad on the 14th day. The thickness of the footpads of all the groups was measured at 0, 24 and 48 h after the challenge using a vernier caliper (Mitutoyo, Japan). The difference in

the thickness of the footpad in different groups served as the measure of the delayed-type hypersensitivity (DTH) reaction [16].

Determination of interleukin-2

Ninety µL of coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6) were added to each serum sample (10 µL) in a 96-well microtiter plate and incubated overnight at 4°C followed by incubation at 37°C for 30 min. After washing the plate twice with wash buffer PBS-T (phosphate buffer saline pH 7.4 containing 0.05% Tween-20), the unbound sites were blocked with 100 µL of blocking buffer (1% bovine serum albumin in PBS-T) and incubated at 37°C for 2 h. The plate was washed twice with 200 µL wash buffer, and 100 µL primary antibodies (rabbit anti-mouse IL-2) diluted in blocking buffer was added and incubated at 4°C overnight. Following the incubation, the plate was washed thrice with 200 µL of wash buffer and incubated at 37°C for 3 h with 100 µL/well of secondary antibody (goat anti-rabbit-HRP conjugate) specific to the primary antibody in blocking buffer and washed thrice with 200 µL wash buffer. Then, 100 µL of TMB solution was added to the wells and incubated at 37°C for 15 min. Then, 100 µL of 2N H_2SO_4 was added to stop the reaction and absorbance was measured at 450 nm in a microplate reader (MultiSkan Go, Thermo Scientific, USA) [17].

LC-QTOF-MS analysis of the hydroalcoholic extract of *A. precatorius* leaves

Chromatographic separation was achieved with a chromatographic system (Agilent Technologies, USA, Model 6540) with a C_{18} column ZORBAX 300SB (4.6×100 mm, 3.5 µm) at 25 °C. The method used a gradient at a constant flow rate of 0.6 mL min^{-1} with solvent A (0.1% formic acid/water) and solvent B (acetonitrile), programmed as follows: 0 min, linear change from A-B (95:5 v/v) to A-B (5:95 v/v); 12 min, isocratic A-B (5:95 v/v); 20 min, linear change to A-B (95:5 v/v) 22 min and 25 min, linear change to A-B (95:5 v/v). The extract was dissolved in the mobile phase and 10 µL was injected into the system. MS analysis was carried out using a 6540 Agilent Ultra-High-Definition Accurate-Mass QTOF-MS coupled to the LC, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in positive ionization mode as follows: drying gas flow (nitrogen): 10.0 L/min; nebulizer pressure: 45 PSIG; gas drying temperature: 325°C; gas vaporize temperature: 350°C; capillary voltage: 0.051 µA; chamber voltage: 4.23 µA. Fixed collision energies were set to 10, 20, 30, 40, 50 V; precursors per cycle were set to 5, and the precursor threshold was 400 counts; the mass scan range was 100-1700 m/z; scan speed was 25000 counts/spectrum. Integration and data elaboration were performed using Mass Hunter software (Agilent Technologies, Santa Clara, CA, USA). Agilent Technologies has provided the METLIN Compound Database available with an instrument having an accurate mass that was used as the reference.

Histopathological evaluation of organs

The isolated organs (spleen, kidney, liver) were transferred into 10% buffered formalin. The formalin-fixed tissues were subjected to paraffin wax embedding for tissue sectioning. Sections of each tissue were cut at 5-micron thickness with a semiautomated rotary microtome (Leica Biosystems, Germany) and were stained with hematoxylin and eosin (H&E) stain [18]. The stained slides were observed under a microscope and pathological lesions were recorded.

Statistical analysis

All the numerical data are expressed as the mean±standard error of mean (SEM). Data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test in GraphPad Prism 8. Statistical significance was considered if $P<0.05$.

RESULTS

Effect of EAPL on organ indices

The organ indices reflect the immune function of an animal. The data of organ to body weight index (mg/g) for liver, kidney and spleen are shown in Table 1. The organ index of the liver and spleen was unaltered and insignificantly increased in animals treated with the highest dose of EAPL when compared

to model control animals. However, the kidney index was significantly increased in the group of mice treated with 200 and 300 mg/kg of the extract along with CPMD as compared to the model control group (P<0.05).

Table 1. Organ indices (mg/g) of mice in treatment groups

Treatment groups	Spleen index	Kidney index	Liver index
C1	8.78±1.53	14.31±0.35	53.14±1.79
C2	6.10±0.99	12.38±0.62	49.90±2.76
T1	5.63±0.70	14.54±0.77	49.28±1.63
T2	6.13±0.89	15.04±0.80$^{\#}$	51.06±1.92
T3	7.33±1.35	15.39±0.37$^{\#}$	50.99±3.41

Data are expressed as the mean±SE (n=6). $^{\#}$ P <0.05 vs model control

Effect of EAPL on hematological parameters

Hematological parameters of different treatment groups are presented in Table 2. The platelets in animals of the model control group were insignificantly decreased compared to normal control animals. The treatment with EAPL significantly increased the number of platelets, which points to the capability of the extract to stimulate platelet production. The platelet-stimulating effect of the EAPL can be further explored and the potency of the EAPL in this regard can be useful in ailments where the platelet count is affected. The levels of Hb and PCV were significantly lowered, along with an insignificant decrease in total RBCs, WBCs and lymphocytes in the model control group compared to the normal control group.

Effect of EAPL on biochemical parameters

Serum biochemical parameters of different treatment groups at the end of the experiment are presented in Table 2. The concentrations of total protein and globulin were significantly decreased in the model control group as compared to the control group. However, total protein was significantly increased in the treatment groups when compared with the model control group. The concentration of albumin was significantly increased in the treatment group as compared to the model control group. Albumin was significantly increased in the group of mice treated with 100 and 300 mg/kg extract and CPMD when compared with the model control group. The treatment with EAPL improved the effect of CPMD on the concentration of total globulin (P>0.05).

Effect of EAPL on spleen cell count and the MTT assay

Splenocyte counts and splenocyte MTT assay were carried out to understand the immune protective action of EAPL against CPMD. Spleen cell counts ($\times 10^6$/mL) of different treatment groups are presented in Fig. 1A. Splenocyte counts were significantly reduced in the model control group (P<0.01) compared to the control group. However, EAPL treatment showed less reversal of the effect in the model control group than in other treatment groups. T and B splenic lymphocyte cell proliferation induced by ConA and LPS is shown in Fig. 1B and 1C, respectively. ConA and

Table 2. Hematological and biochemical parameters of animals in treatment groups

Parameters	C1	C2	T1	T2	T3
Hb (g/dL)	12.45±0.31	9.92±0.14***	10.42±0.33**	9.60±0.50***	10.26±0.45**
PCV (%)	38.48±1.16	32.50±0.98*	34.92±1.54	30.63±1.46**	31.12±1.80**
RBC ($\times 10^6$/µL)	8.22±0.23	6.97±0.05	7.42±0.23	6.89±0.49*	7.44±0.35
Total WBC ($\times 10^3$/µL)	11.20±1.94	7.62±0.42	7.76±0.83	8.58±0.94	9.49±0.79
Neutrophil (%)	25.67±2.78	48.33±1.09	33.50±8.18	50.33±8.88	46.50±11.14
Lymphocyte (%)	70.50±3.10	47.67±1.48	63.00±8.71	49.00±8.01	56.83±7.25
Monocyte (%)	3.83±1.83	3.83±0.40	3.50±0.85	4.00±0.86	5.17±2.15
Platelets ($\times 10^3$/µL)	877.17±28.38	672.33±150.85	941.83±48.76	1221.50±75.40$^{\#}$	1254.17±214.99$^{\#}$
Total protein (g/dL)	4.95±0.01	4.06±0.02***	4.87±0.08$^{\#\#\#}$	4.77±0.14$^{\#\#\#}$	5.14±0.16$^{\#\#\#}$
Albumin (g/dL)	2.18±0.04	1.54±0.05	1.81±0.14$^{\#}$	1.83±0.13	1.86±0.17$^{\#\#}$
Globulin (g/dL)	2.78±0.05	2.52±0.06**	3.06±0.09	2.93±0.21	3.28±0.14

Data are expressed as mean ±SE (n=6). * P<0.05 vs normal control; ** P <0.01 vs normal control; *** P<0.001 vs normal control; $^{\#}$ P<0.05 vs model control; $^{\#\#}$ P<0.01 vs model control; $^{\#\#\#}$ P<0.001 vs model control

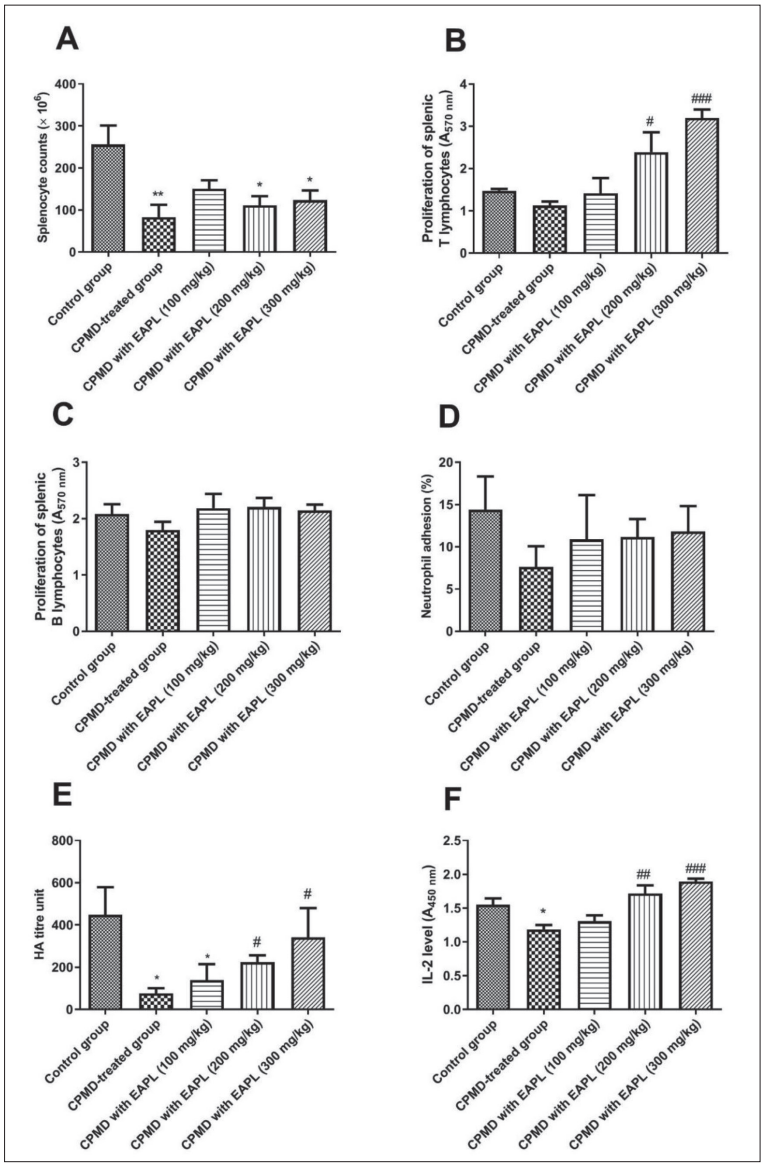

Fig. 1. **A** – Effect of EAPL on spleen cell counts (per mL). **B** – Effect of EAPL on splenocyte proliferation in the presence of ConA. **C** – Effect of EAPL on splenocyte proliferation in the presence of LPS. **D** – Effect of EAPL on neutrophil adhesion (%) in different treatment groups. **E** – Effect of EAPL on change in HA titer unit in different treatment groups. **F** – Effect of EAPL on IL-2 level in different treatment groups. * P<0.05 when compared with the control group; ** P<0.01 vs normal control; # P<0.05 vs model control; ## P<0.01 when compared with model control; ### P<0.001 vs model control.

LPS treatment resulted in insignificant decreases in T and B lymphocyte proliferation in the model control group when compared to the control group. T lymphocyte proliferation was significantly higher in the EAPL treatment groups (at the middle and higher doses), the response being dose-dependent. However, B lymphocyte proliferation was partially improved after EAPL treatment (P>0.05).

Effect of EAPL on neutrophil adhesion

The neutrophil adhesion (%) indices in different treatment groups are provided in Fig. 1D. Neutrophil adhesion (%) was insignificantly decreased in the model control group compared to the normal control group. The EAPL treatment resulted in partial improvement in neutrophil adhesion (P>0.05).

Effect of EAPL on antibody titer

The HA titer was used to assess the humoral immune response against SRBC in mice after various treatments, and the data are presented in Fig. 1E. The HA titer was significantly reduced in the model control group as compared to the normal control group. The EAPL treatment (200 and 300 mg/kg) significantly improved the HA titer, dose-dependently. The effect of EAPL on HA titer was important as improvement of HA titer points to the efficacy of the treatment on antibody production.

Effect of EAPL on the DTH reaction

The effect of EAPL treatment on the cell-mediated immune response in terms of an experimentally induced DTH reaction is shown in Table 3. The paw thickness in mice treated with CPMD at 0 and 48 h after the challenge significantly differed as compared to that observed before the challenge, which indicated an immunosuppressive effect of CPMD. The CPMD treatment reduced paw thickness in mice at 24 h (P>0.05) and 48 h (P<0.05) post-challenge as compared to that observed before the challenge (0 h), which pointed to immunosuppressive activity. The values of footpad thickness of the group of mice treated with 200 mg/kg (T2) and 300 mg/kg (T3) of extract

Table 3. Effects of treatments on delayed type hypersensitivity reaction

Hours/ Groups	C1	C2	T1	T2	T3
0 hour	2.60±0.09	2.53±0.05	2.52±0.08	2.61±0.0	2.47±0.10
24 hours	2.69±0.07	2.44±0.07	2.92±0.09#	2.78±0.05	2.84±0.15
48 hours	2.59±0.10	2.22±0.07*	2.59±0.16	2.60±0.07	2.53±0.17

Data are expressed as the mean ±SE (n=6). * P<0.05 when compared column-wise; # P<0.05 when compared row-wise

together with CPMD were insignificantly higher. However, the treatment with 100 mg/kg EAPL showed a significantly greater foot-pad thickness at 24 h when compared with the model control group, indicating a stimulatory effect of EAPL on cell-mediated immunity.

Effect of EAPL on IL-2 level

The effect of EAPL on the level of IL-2 in the different treatment groups is presented in Fig. 1F. The model control group showed a significant reduction in the IL-2 level when compared with the normal control group. The group of mice treated with 200 mg/kg and 300 mg/kg of extract and with CPMD displayed a significantly higher level of IL-2 when compared with the model control group, which indicated that EAPL stimulated the production of IL-2 in immunocompromised mice.

LC-QTOF-MS analysis of EAPL

LC-QTOF-MS analysis of the hydroalcoholic extract of *A. precatorius* leaves revealed the presence of various diverse primary and secondary metabolites. Out of them, six important phytochemicals were identified, which might be responsible for the observed immunomodulatory action (Table 4). The identified compounds were characterized according to the obtained mass spectra.

Effect of EAPL on histological changes in spleen, kidney and liver

Microscopic changes in spleen, kidney and liver of animals in the different treatment groups are shown in Figs. 2, 3 and 4, respectively. In the spleen, CPMD treatment caused atrophic changes with lymphoid depletion. The group treated with 100 mg/kg EAPL exhibited mild lymphoid depletion of white pulp along with reticuloendothelial hyperplasia when compared with the model control group. A slight reduction in splenic corpuscles near the capsule and increased total splenic thickness were observed in the spleen of mice treated with 200 mg/kg EAPL. Restoration of splenic white pulp lymphoid cells to almost normal architecture was observed in the spleen of mice treated with the highest dose of EAPL. After histological evaluation, the liver of the model control group displayed necrotic changes with infiltration of inflammatory cells, degeneration of hepatic cells and vacuolation. Slight degeneration of hepatic cells, fragmentation of nuclei and pyknotic nuclei were observed in the liver of mice treated with 100 mg/kg EAPL. The liver of mice treated with 200 mg/kg EAPL showed slight degeneration of hepatic cells, fragmentation of nuclei and pyknotic nuclei. Hypertrophy of hepatic cells with mild degeneration of the parenchyma and widening of the central vein were noted in the liver of mice treated with 200 mg/kg EAPL. The liver of mice treated with 300 mg/kg EAPL showed only hypertrophy of hepatic cells with mild degeneration of the parenchyma along with widening of the central vein. In the kidney, CPMD treatment resulted in severe degenerative changes in renal glomeruli and tubules, congestion and sloughing of

Table 4. Important phytochemicals observed in the extract of *A. precatorius* leaves by LC-QTOF-MS analysis.

Sr. No.	Name of phytochemical	Mol. formula	M. Wt.	t_R (min)	Peak height	Peak area	% area	CAS No.	METLIN ID
1	Dihydromikanolide	$C_{15}H_{16}O_6$	292.09	10.78	29252	211489	1.766	23758-04-5	67660
2	Abruquinone C	$C_{19}H_{20}O_8$	376.11	10.777	14103	102968	0.537	71593-11-8	48300
3	Allamandin	$C_{15}H_{16}O_7$	308.08	10.31	4122	19693	0.164	51820-82-7	67925
4	Corymbosin	$C_{19}H_{18}O_7$	358.10	10.76	7508	90744	0.758	18103-41-8	49496
5	Lappaol D	$C_{31}H_{36}O_{10}$	568.23	15.24	4680	34584	0.289	64855-01-2	71913
6	Methyl-picraquassioside A	$C_{19}H_{24}O_{10}$	412.13	9.81	11333	85318	0.712	169312-28-1	93067

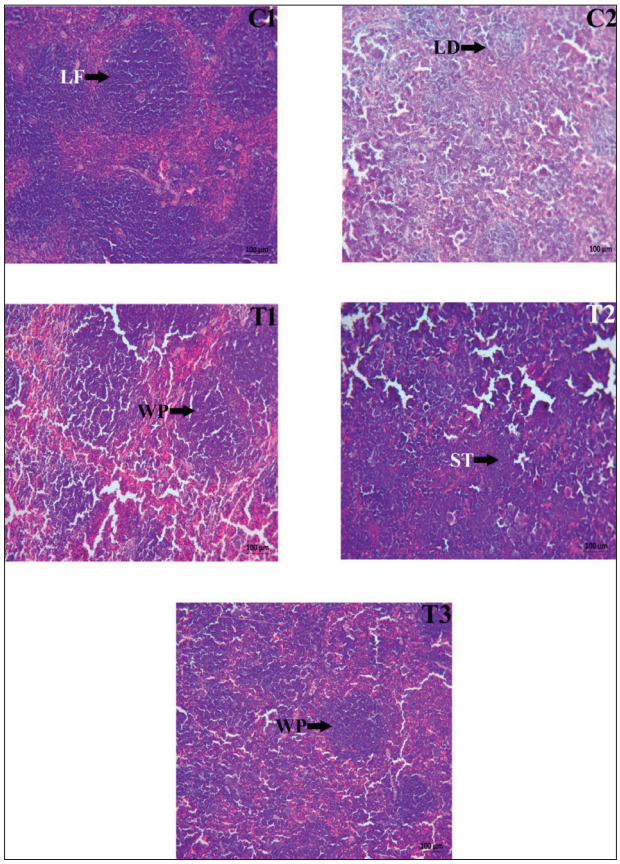

Fig. 2. Microscopic changes in spleen of animals in different treatment groups. **C1** – Control group; normal architecture of the lymphoid follicles (LF). **C2** – CPMD-treated group; note atrophic changes with lymphoid depletion (LD); **T1** – CPMD with EAPL (100 mg/kg); note mild lymphoid depletion of white pulp (WP) and reticuloendothelial hyperplasia. **T2** – CPMD with EAPL (200 mg/kg); note slight reduction in splenic corpuscles near the capsule and increased total splenic thickness (ST). **T3** – CPMD with EAPL (300 mg/kg); note restoration of splenic white pulp (WP) lymphoid cells almost normal architecture.

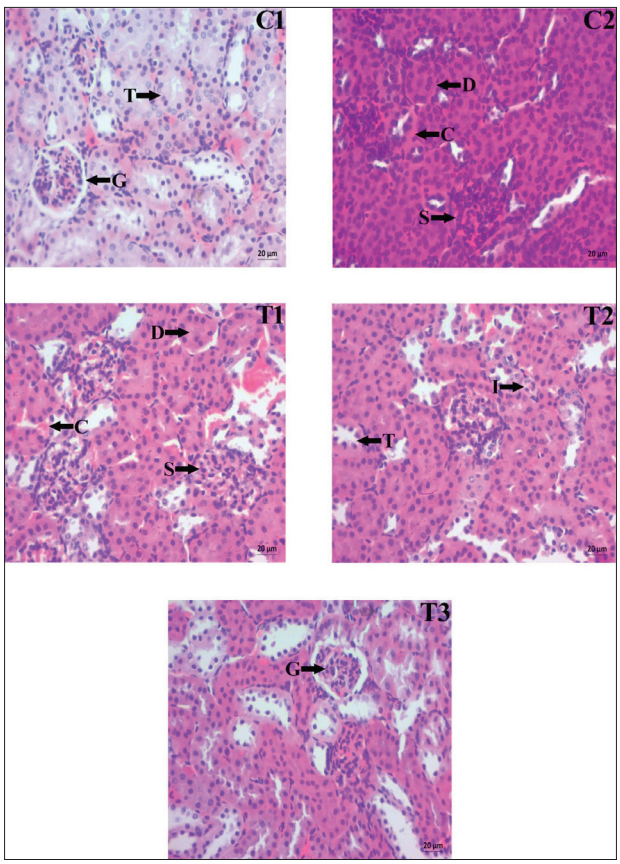

Fig. 3. Microscopic changes in kidney of animals in different treatment groups. **C1** – Control group; note normal architecture of glomeruli (G) and renal tubules (T). **C2** – CPMD-treated group; note severe degenerative changes (D) in renal glomeruli and tubules, congestion (C), and sloughing of glomeruli (S). **T1** – CPMD with EAPL (100 mg/kg); note mild degenerative changes (D) in renal glomeruli and tubules, congestion (C) and sloughing of glomeruli (S); **T2** – CPMD with EAPL (200 mg/kg); note mild interstitial infiltration (I) of inflammatory cells and almost normal architectural of tubules (T). **T3** – CPMD with EAPL (300 mg/kg); note improved degenerative changes of renal glomeruli (G) and tubules showing almost normal architecture.

glomeruli. Treatment with 300 mg/kg EAPL improved the degenerative changes in renal glomeruli and tubules and displayed near to normal architecture.

DISCUSSION

The status of immune organs directly affects immune function and the ability to resist disease; the immune organ index reflects immune organ development and immune function [19]. Cyclophosphamide damages the tissue sheath on organs such as the spleen, kidney, etc., and this may lead to atrophic changes in organ indices [20]. In the present study, the treatment with EAPL reversed the CPMD-induced alteration of the kidney index ($P<0.05$) and the spleen index ($P>0.05$) and it with no effect on the liver index, which points to an ameliorating effect of EAPL against CPMD-induced alteration in spleen.

CPMD is an oxazaphosphorine-alkylating agent commonly employed to treat cancer and also used to produce immunosuppression in autoimmune diseases. CPMD used in the study produced significant alterations in hematological parameters (Hb, PCV, RBCs, WBCs and platelets) that were similar to earlier

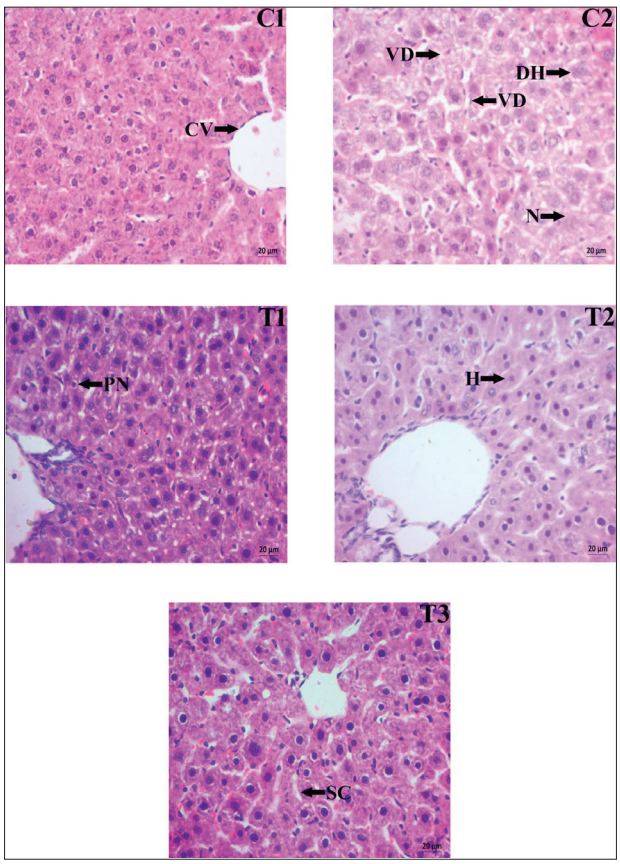

Fig. 4. Microscopic changes in the liver of animals in different treatment groups. **C1** – Control group; note normal architecture of hepatocytes (H) and central vein (CV). **C2** – CPMD-treated group; note necrotic changes (N) with infiltration of inflammatory cells, degeneration of hepatic cells (DH) and vacuolar degeneration (VD). **T1** – CPMD with EAPL (100 mg/kg); note slight degeneration of hepatic cells, fragmentation of nuclei and pyknotic nuclei (PN). **T2** – CPMD with EAPL (200 mg/kg); note hypertrophy of hepatic cells (H) with mild degeneration of the parenchyma and widening of the central vein; **T3** – CPMD with EAPL (300 mg/kg); note normal hepatic cells with mild sinusoidal congestion (SC).

reports of CPMD-induced changes in blood parameters [21,22]. In the present study, we observed a decrease in platelet count after CPMD treatment. CPMD administration in mice has also been reported to produce an observable decrease in the total number of platelets [23,24]. The treatment with EAPL prevented the reduction of platelets after exposure to CPMD. Treatment with CPMD is associated with bone marrow suppression and pancytopenia, which might be the reason for such an effect on platelet count. The previous report showed that the alterations in the levels of albumin and globulin caused by CPMD were due to a decrease in protein synthesis as a result of liver damage [25,26]. Herein, the EAPL treatment prevented liver damage and increased the levels of albumin and globulin.

Splenic T- and B-lymphocyte proliferation is the crucial indicator that mediates cell and humoral immunity, which are also crucial for the regulation of the immune response. Lymphocyte proliferation is a response to the stimulation induced by antigens or mitogens and is widely used to evaluate the cellular immune responses [27]. T lymphocyte division induced by ConA is commonly used to detect T lymphocyte immunity and LPS-induced activation of B cells, which as a lymphocyte-activating factor, plays a critical role in B lymphocyte immunity [28]. In activated T cells, ConA has been reported to activate nuclear activator protein 1 (AP-1) that is important in the development and functioning of the immune system including T-cell receptors; binding of ConA triggers cross-linking of the T-cell receptor complex leading to cell activation. Therefore, ConA- and LPS-induced splenocyte proliferation served as a parameter for cellular and humoral immunity, respectively [29]. In the present study, the spleen cell counts of EAPL-treated mice were improved when compared with the model control group ($P<0.05$). Splenic T lymphocytes were expressed dose-dependently compared to both the control and model control groups, while B-lymphocyte proliferation was found to be insignificant. Therefore, it can be concluded that EAPL enhances cellular immunity.

The neutrophil adhesion test is used for the assessment of immunomodulatory activities, particularly of cell-mediated immunity. CPMD reduced the percentage of neutrophil adhesion that was partially improved by the EAPL treatment, which indicates a positive effect of the extract on immunity. Cytokines are secreted by activated immune cells for margination and extravasations of phagocytes, mainly of polymorphonuclear neutrophils. An increase in the adhesion of neutrophils to nylon fibers reveals margination of cells in blood vessels and the increased number of neutrophils reaching the site of inflammation [30].

Enhanced antibody titer indicates an augmented humoral response mediated by T and B lymphocytes involved in the antibody synthesis [31]. The hemagglutination test was also performed to examine the effect

of EAPL on the humoral immune system. Humoral immunity involves the interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting cells. Antibodies function as the effectors of the humoral response by binding to the antigen and neutralizing it or facilitating its elimination by cross-linking to form a latex that is more readily ingested by phagocytic cells [32].

Cytokines play crucial roles in cell-cell communication in the immune system; they are involved in the preservation or restoration of homeostasis via coordination of lymphoid cells, inflammatory cells and hematopoietic cells [33]. IL-2 is a type of cytokine, a signaling molecule in the immune system. IL-2 is a 15.5-16 kDa protein [34] that regulates the activities of WBCs (often lymphocytes). CPMD has been reported to cause a reduction in IL-2 (Th1-type cytokine) in the sera of mice [35]. A similar result was obtained in the present study. CPMD suppresses the secretion of Th1-type cytokines and increases the secretion of Th2-type cytokines [36]. Moreover, IL-2 specifically promotes T-cell activation and proliferation, which creates an immunomodulatory effect [37]. Treatment with EAPL in mice increased IL-2, which might be responsible for the cell-mediated immunomodulatory effect.

Atrophic changes related to the depletion of lymphoid cells in the white pulp are one of the significant effects of CPMD on the spleen [38]. The recovery of pathological changes, such as restoration of the splenic white pulp and atrophic changes, support the immunomodulatory effect of EAPL.

CPMD causes degenerative changes in glomeruli, renal tubules, congestion and sloughing of glomeruli [39]. The treatment with EAPL improved the CPMD-induced microscopic changes in the kidney, which reveals an ameliorating effect of EAPL against CPMD at the cellular level.

In the case of the liver, CPMD induced necrosis at the cellular level, degeneration of hepatic cells and vacuoles [40]. This damage was restored if the treatment reduced the toxic effect of CPMD. In the present study, slight degeneration of hepatic cells, a decrease in the fragmentation of nuclei and pyknotic nuclei and the hypertrophy of hepatic cells with mild sinusoidal congestion in the liver revealed the protective effect of the treatment with EAPL. Different phytochemicals or metabolites (dihydromikanolide, abruquinone c, allamandin, corymbosin, lappaol D and methylpicraquassioside A) were identified in EAPL with the help of LC-QTOF-MS, which might be responsible for the immunomodulating effect. Dihydromikanolide, which is a principal active constituent of *Mikania micrantha*, has possesses antitumor and immunomodulatory activities [41]. Abruquinones are isoflavanquinone derivatives found in *A. precatorius*. Abruquinones have antiinflammatory activity observed as the inhibition of platelet aggregation induced by various inflammation-inducing chemicals [42]. The isoflavanquinones present in the extract have been reported to inhibit the respiratory burst in rat neutrophils, which is mediated partly by the blockade of phospholipase C (PLC) and phospholipase D (PLD) pathways, by suppressing the function of NADPH oxidase through the interruption of electron transport [43]. Allamandin, an iridoid lactone and active constituent of *Allamanda cathartica* and *Himatanthus sucuuba* (Spruce ex Müll.Arg.) Woodson (family Apocynaceae), was also detected in EAPL. An iridoid exhibits immunomodulating and antiinflammatory properties and also acts as an antileukemic, tumor-inhibiting and wound-healing agent [44]. Corymbosin, a chromone flavonoid glycoside, is commonly found in *Vitex negundo*, as well as in EAPL. Plants containing corymbosin can be used for treating inflammation, pain management and autoimmune diseases [45]. Lappaol D, a sesquilignan, was identified in EAPL and is also a main chemical constituent of *Arctium lappa* L., which has been claimed to strengthen the immune system, improve appetite, relieve pain and improve the overall quality of life. Lappaol, which was detected in the extract, has been reported to have potent anticancer and antiproliferative activity; in LNCaP cancer cells it arrests the cell cycle, and in mice it reduces tumors [46,47]. Methylpicraquassioside A is a phenolic glycoside generally found in *Ruta graveolens* (rue) and other spices and it is also found in the extract. Its *in vitro* immunomodulatory activity was reported [48].

CONCLUSIONS

Cyclophosphamide treatment caused hematological, biochemical changes and suppression of humoral and cell-mediated immunity in mice. Daily oral administration of 200 mg/kg of the hydroalcoholic extract of *Abrus precatorius* L. for 14 days in mice had an

immunomodulating effect against cyclophosphamide-induced immunosuppression. Further research, including gene expression studies, should be performed in order to examine the mechanism of the immunomodulatory effect in more depth.

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