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EVALUATION OF IN-VITRO AND IN-VIVO IMMUNOMODULATORY ACTIVITY OF AQUEOUS AND ETHANOLIC EXTRACT OF PROSOPIS CINERARIA (L.)

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ABSTRACT:

*Prosopis cineraria*Linn. is used for rheumatism, cough, common cold, bronchitis, dysentery, leucoderma, leprosy, asthma and scorpion stings. Present work was aimed to study immunomodulatory activity of *Prosopis cineraria*L. leaves extract. Both ethanol (PEE) and aqueous extract (PAE) was used for the in-vitro and in-vivo immunomodulatory activity of *Prosopis cineraria* L. After phytochemical evaluation and acute oral toxicity studies in-vitro immunomodulatory activity was studied by evaluating release of following immune mediators from murine peritoneal macrophages such as superoxide (NBT reduction), nitric oxide (NO), lysosomal and myeloperoxidase enzymes. In-vivo immunomodulatory effect was studied in test systems such as Carbon Clearance Assay, Delayed Type Hypersensitivity reaction, Antibody Titer Assay and cyclophosphamide induced myelosuppression in mice. Results showed that, the phagocytic index of PEE and PAE treated mice was found to be significantly higher than the vehicle control group in dose dependant manner. This in vivophagocytic response would be due to enhanced lysosomal enzyme activity of macrophages as observed during in-vitroassays. Study concluded that, both specific and non-specific immunostimulating properties of the PEE and PAE in in-vivo experimental methods suggest itstherapeutic effectiveness in immunocompromised conditions.

Keywords: *Prosopis cineraria*L., immunomodulatory activity, peritoneal macrophages, Carbon clearance assay, Delayed type hypersensitivity reaction, Antibody titer assay, etc**.**

INTRODUCTION:

Medicinal plants have been known for millennia and arehighly esteemed all over the world as a rich source of therapeutic agents for the prevention ofdiseases and ailments. Nature has bestowed our country with an enormous wealth of medicinalplants; therefore India has often been referred to as the Medicinal Garden of the world.[1, 2]*Prosopis cineraria* belongs to mimosaceae subfamily, commonly known as “khejri” or “shami tree”. Itis also known as king of Thar desert forest. It is anendemic tree which is majorly found in Rajasthan inIndia. *Prosopis cineraria* is a small tree, leaves arebipinnate, branches are thorned along the internodes.Flowers are small and yellow and seeds arepods. *Prosopis cineraria* indicates the presence of adeep-water table.[3]It holds an important place in the rural economy in the northwest region of Indian subcontinent.The genus Prosopis comprises about 44 species distributed mainly in dry regions of SouthwestAsia, Africa and, predominantly America from western North America to Patagonia.[2, 4]

The tree parts including leaves, pods, seeds and barks has been used in many ways as food, i.e., flour, drink, vegetable, and gum. Leaves and pods are used for ruminant and animal feed.[5]Whole plant of

*Prosopis cineraria*is useful for the treatment of many diseases such as ailments like leprosy, dysentery, asthma, leucoderma, dyspepsia and earache.The bark is used as a remedy for rheumatism, cough, common cold, bronchitis, dysentery, leucoderma, leprosy, asthma and scorpion stings.[6, 7] The whole plant is tikta, katu, sheeta (Sheetavearya), kashaya, laghu and purgative; beneficial in deranged kapha, vertigo, dyspnoea, piles and worms. Leaves also plays several vital roles in health benefits as its fresh juice can me mixed with lemon juice and used against dyspepsia and the crushed pods extract provides relief from the pain in fractured bones and is beneficial during pain in ear and tooth. Several skin diseases and wounds are disinfected and healed by externally applying the aqueous extract of bark and leaves.[8]

Literature survey reveals that the plant contains proteins, calcium, iron and vitamins. It has many chemical constituents as alkaloid, steroids, alcohol and alkane.Spicigerine and Prosapanol G are the alkaloids obtained from seeds. Flowers contain prosogerin A, B, C, D and E.[9, 10]Studies referred to the secondary metabolites compounds in plants that are considered bioactive compounds and have diverse antinutritional and nutraceutical features. Therefore, it can be potential as a source of bioactive products and used in functional products. In many literatures it is mentioned that Prosopis species tree generally contains various phytochemical compounds as tannins, 5-hydroxytryptamine, isorhamnetin-3-diglucoside, L-arabinose, quercetin, apigenin, and tryptamine. Studies conducted on phytochemical compounds of *Prosopis cineraria*showed that each part of the plant contains different types of these compounds.[5, 11] Leaves of plant contains –Alkaloid such as; spicigerine, Steroids like; campesterol, cholesterol, sitosterol, stigmasterol, actacosanol, Alcohols such as;octacosanal, triacontane-1-ol, Tricosan-1-ol and Alkanes likehentriacontane, Diisopropyl-10,11-dihydroxyicosane-1,20-dioate.[5, 12, 13, 14.]Leaf paste of *P. cineraria* is applied on boils and blisters, including mouth ulcers in livestock and leaf infusion on open sores on the skin. Smoke of the leaves is considered good for eye troubles and infections. [15, 16, 17]The objective of present study was extraction, phytochemical characterization and in-vitro and in-vivo immunomodulatory activity of leaves of *Prosopis cineraria (L.)*.

MATERIALS AND METHODS:

Plant source:

Leaves of *Prosopis cineraria* Linn were purchased from local Patil Nursery, Mulund, Mumbai in June 2019 and were authenticated by Dr. (Mrs.) BinduGopalkrishnan, Asst. Professor, Department of Botany at Mithibai College of Arts, Chauhan Institute of Science &AmruthbenJivanlal College of Commerce and Economics, Vile Parle (W), Mumbai- 400056 and the voucher specimen (No.MIT0156) were submitted to Pharmacology department, Oriental College of Pharmacy, Sanpada, Navi Mumbai- 400705 for future reference.

Chemicals:

Sulfanilamide and naphthylethylenediamine were obtained from Sisco Research Lab Ltd., Mumbai, India.RPMI (Roswell Park Memorial Institute)-1640 was obtained from Himedia Laboratories, Mumbai, India. Ethanol AR, phosphoric acid and Dimethyl Sulfoxide (DMSO) were obtained from S.D. Fine Chemicals, Mumbai, India. All other chemicals used were of analytical grade.

Methods:

Preparation of Extract:

The leaf was cut down into small pieces, shade dried and powdered to get moderately coarse powder, which is sieved under mesh. The powder was then subjected to successive extraction with petroleum ether, and water at (60°-80°C), by hot continuous percolation using soxhlet apparatus having 3 cycles each for 24 hours to give petroleum ether extract of leaves of Prosopis cineraria (PEE), similarly successive extraction with water to give aqueous extract of leaves of Prosopis cineraria (PAE). The extract were filtered and evaporated to dryness with a dryer.[10, 18]

Phytochemical Evaluation of Extracts:

Phytochemicals are the chemicals that naturally occur in plants. Phytochemical evaluation plays an significant role in the standardization of the crude extracts and was carried out for detecting the presence of various phytoconstituents in the plants under investigation.Various extracts of powdered plant materials were subjected to preliminary phytochemical evaluation using qualitative chemical tests for detecting the presence of the phytoconstituents like alkaloids, glycosides, tannins, phenolic compounds, phytosterols, carbohydrates, proteins and amino acids etc.[19, 20, 21]

Separation of compound using different solvent system by thin layer chromatography:

Thin Layer chromatography (TLC) was performed to isolate the principle compounds that were present in*Prosopis cineraria* leaves extract. The different solvent systems of different polarities were prepared and TLC studies were carried out to select the solvent system proficient of showing better resolution.The plant extracts were applied on TLC plates by using capillary tubes and developed on a TLC chamber using suitable mobile phase. The developed TLC plates were air dried and discover under ultraviolet light (UV) at both 254 nm and 316nm.[22, 23]

Approval from animal ethics committee

The study was performed after getting approval from Institutional Animals Ethics Committee (IAEC) of Oriental College of Pharmacy; Sanpada. Ref. No. OCP/IAEC/2019-2020/09.

Acute Oral Toxicity:

Acute oral toxicity studies were carried out for test extracts as per the OECD (Organisation for Economic Co-operation and Development) guideline 423. This guideline refers to those adverse effects occurring within a short time following oral administration of a single dose of a substance or multiple doses given within 24 hr. The acute toxic class method set out in this Guideline is a stepwise procedure with the use of 3 animals of a single sex per step. Depending on the mortality and/or the moribund status of the animals, on average 2-4 steps may be necessary to allow judgment on the acute toxicity of the test substance.[24, 25]

Animals: Female Swiss Albino mice of 20-25g body weight were used.

Administration of doses: The test extracts was administered in a single dose by gavage using a stomach tube. Mice were fasted for 3-4 hours (water was allowed) prior to dosing. Following the period of fasting, the animals were weighed and the test extracts administered. After the substance has been administered, food was withheld for a further 1-2 hours in mice.

Number of animals and dose levels: Three animals are used for each step. The dose level used as the starting dose was 2000 mg/kg body weight. Depending upon the effect (mortality/no mortality) next dose level (higher/lower) was selected.

Observations: Animals were observed individually after dosing at least once during the first 30 minutes, periodically during the first 24 hours, with special attention given during the first 4 hours, and daily thereafter, for a total of 14 days, except where they need to be removed from the study and humanely killed for animal welfare reasons or are found dead. All observations were systematically recorded. Observations included changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity and behavior pattern, if any. Attention was directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma, if any. In case of mortality or abnormal signs in no or one animal, the same study was repeated in a group of 6 animals of the other sex per group. In case of 2 or more mortality or abnormal signs in 2 or more animals per group, lower dose was tested.

In-vitro Immunomodulatory Activity of *Prosopis cineraria* leaves extract

In-vitro immunomodulatory activity studies for the test extracts were carried out by, screening for release of various immune mediators from isolated murine peritoneal macrophages.

Isolation of peritoneal macrophages and culture conditions

To collect macrophages, 2-ml syringe was filled with 2.0 ml 3% fluid thioglycollate medium (Himedia). Peritoneal cells were washed twice with PBS by discarding supernatant and resuspend the cell pellet by gently pepetting in and out the contents.Cell pellet resuspend in RPMI 1640 containing 10% fetal bovine serum (FBS), 20µm 2-mercaptoethanol, 100 u/ml penicillin, 100 µg/ml streptomycin and 25 mm HEPES buffer (complete RPMI-1640). Cell count and adjusting cell concentration: This was carried out as per the following procedure.To the cell sample (10µl) trypan blue dye (4%w/v) (10µl) was added on glass slide and mixed it well. Then cell count was determined using hemocytometer excluding dead cells. Cell count was adjusted by dilution in complete RPMI-1640 medium to appropriate cell concentration (105 or 5x105 or 1.5x105) as per the assay requirement.In sterile tissue culture plate, 100 µl of cell suspension was added to each well and plate was incubated at 37°C for minimum 2 hr under a humidified atmosphere of 95% air and 5% CO_2. Meanwhile, test extracts were prepared in RPMI media containing 10%DMSO maintaining sterility conditions using 0.22µ filter in syringe. Different concentrations were prepared in serially diluted manner in a separate 96-well plate as 832µg/ml, 416µg/ml, 208µg/ml, 104µg/ml, 52µg/ml, 26µg/ml, 13µg/ml and 6.5µg/ml.Two hours after the incubation, test extracts/drugs prepared as above were added (20 µl/well) to cultured macrophages and incubated for 24 hr in a humidified incubator containing 5% CO2 at 37ºC. Twenty four hours after incubation, following assays viz. Sulforhodamine B (SRB) assay, Nitric oxide assay, NBT dye reduction assay, cellular lysosomal enzyme assay and myeloperoxidase enzyme assay, were performed using (PHA) (Phytohemagglutinin-M) (10 µg/ml) as a positive control.[26, 27]

Measurement of NO production

Nitrite (Nitric oxide) assay on isolated murine peritoneal cells was carried out by following validated laboratory standard operating procedure. Nitric oxide (NO) production was determined by assaying culture supernatants for nitrite using Griess reagent. Isolated murine peritoneal macrophages (5×105 cells/ well) were cultured in complete RPMI 1640, incubated for 2 hr at 37ºC in a 5% CO2 atmosphere. The test extracts dissolved in complete RPMI-1640 containing 10% DMSO were added to cultured wells at various concentrations (832-6.5 µg/ml) in triplicate. The plates were incubated for 24 h at 37ºC in a

5% CO2 atmosphere in CO2 incubator.After 24 h, cell free supernatant (75 μl) was mixed with 75 μl of Griess reagent (sulfanilamide 1%, phosphoric acid 5%, naphthylethylenediamine 0.1%) in another 96 well tissue culture plate and incubated for 10 min at room temperature, protected from light. Cells incubated with PHA (10 μg/ml) were used as a positive control. The optical density (OD) was read at 540 nm using a microplate reader (BioTek).Nitrite concentrations were determined from standard curve of sodium nitrite in culture conditions. Stimulation index (SI) for NO release was calculated as the nitrite concentrations ratio of the treated and vehicle treated control cells (RPMI-1640 containing 10% DMSO).[28, 29]

Cellular lysosomal enzyme activity

Cellular Lysosomal enzyme activity assay on isolated murine peritoneal cells was carried out by following validated laboratory standard operating procedure. For lysosomal enzyme assay, isolated murine peritoneal macrophages (1.5×105 cells/ well) were incubated in CO2 incubator for 24 h with the test extracts (832-6.5 μg/ml) in triplicate. For determination of lysosomal enzyme activity, the cultured macrophage monolayers in a 96-well plate were solubilized by the addition of 25 μl of 0.1% Triton X-100. After 15 min incubation at room temperature, 100 μl of 10 mM p-nitrophenyl phosphate was added to a well as a substrate for acid phosphatase.Then 0.1 M citrate buffer (50 μl, pH 5.0) was added to each well and plate was incubated for 1 h at 37ºC.After incubation, 0.2 M borate buffer (150 μl, pH 9.8) was added to the mixture to stop the reaction. The optical density (OD) was measured at 405 nm by using a microplate reader. Stimulation index (SI) for lysosomal enzyme activity was calculated as the ratio of OD of test extract/ drug treated cells to vehicle treated control cells (RPMI-1640 containing 10% DMSO).[30, 31, 32]

Nitro blue tetrazolium (NBT) reduction

Nitro Blue Tetrazolium (NBT) reduction assay on isolated murine peritoneal cells was carried out by following validated laboratory standard operating procedure.For NBT dye reduction assay, isolated murine peritoneal macrophages (3×105 cells/well) were incubated in CO2 incubator at 370C for 24 h with the test extracts/ fractions (832-6.5 μg/ml) in triplicate.The reduction of NBT to insoluble blue formazan was used as a probe for superoxide generation.0.3% NBT (50 μl) solution in RPMI-1640 medium was added to the each well of 96 well tissue culture plate.The plate was incubated for 2 hr.After incubation, the supernatants were recovered and the macrophages were fixed by addition of 200 μl of absolute methanol.Then the plate was washed twice with 70% methanol and then air dried with the help of blow of hair dryer. The formazan deposits were solubilized in 120 μl, 2 M KOH and 140 μl DMSO. After homogenization of the contents in the wells for 10 min., the optical density (OD) was read at 630 nm using microplate reader (BioTek). The superoxide production was represented as the stimulation index (SI). The stimulation index (SI) of NBT for test extract was calculated as the ratio of OD of test extract to vehicle treated control cells (RPMI-1640 containing 10% DMSO).[33, 34]

Measurement of myeloperoxidase activity

Myeloperoxidase activity assay on isolated murine peritoneal cells was carried out by following validated laboratory standard operating procedure. For myeloperoxidase activity, macrophages (5 × 105 cells/well) were incubated in CO2 incubator at 370C for 24 h with the test extracts/ fractions (832-6.5 μg/ml) in triplicate.After incubation, cells from each well were washed three times with fresh RPMI medium.The mixture (100 μl) of o-phenylenediamine (0.4 g/ml) and 0.002% H2O2 in phosphate-citrate buffer (pH 5.0) was added. The reaction was stopped after 10 min using 0.1 N H2SO4.The optical density (OD) was measured at 490 nm using microplate reader.The myeloperoxidase (MPO) activity was

calculated as the stimulation index (SI). The myeloperoxidase stimulation index (SI) was calculated as the ratio of the OD of a test extract treated well to vehicle treated control cells (RPMI-1640 containing 10% DMSO.[32, 35]

Statistical Analysis for in vitro assays

Results are expressed as mean ± SEM (standard error of mean) for triplicate assays. Data were analyzed by one way ANOVA followed by Dunnet's multiple comparisons test using GraphPad Prism Ver 5 software against control samples. Values of $P<0.05$ were the criteria for statistical significance.

***In-vivo* Immunomodulatory Activity**

The test extracts of*Prosopis cineraria* Linn, dried leaves were studied for their in vivo effects in representative animal models like Phagocytic activity by Carbon Clearance Assay in mice, Ovalbumin-induced Delayed Type Hypersensitivity in mice, Antibody Titer against ovalbumin using ELISA method and Cyclophosphamide-induced myelosuppression in mice.

Treatment protocol for in vivo experiments

Animal species used: Swiss albino mice, Body weight: 18-24 g; Sex: both male and female.

Following are the different treatment groups of animals:

- Normal control (0.5% Na-CMC)
- Ethanol extracts of *Prosopis cineraria* (PEE) 50mg/kg
- Ethanol extract of *Prosopis cineraria* (PEE) 100mg/
- Ethanol extracts of *Prosopis cineraria* (PEE) 200mg/kg
- Aqueous extract of *Prosopis cineraria* (PAE) 50mg/kg
- Aqueous extract of *Prosopis cineraria* (PAE) 100mg/kg
- Aqueous extract of *Prosopis cineraria* (PAE) 200mg/kg

Treatment groups of standard drugs (positive/ negative control)

- Cyclophosphamide 25 mg/kg (Negative control)
- Immunosin 50 mg/kg (Positive control)

Phagocytic activity by Carbon Clearance Assay in mice

Mice were divided into different groups as mentioned above having six animals each. Treatment: The control group received 0.5% Sodium Carboxy Methyl Cellulose (Na-CMC) only as vehicle; while animals of the treatment groups were administered test extracts/drug at different doses, p.o. suspended in 0.5%Na-CMC daily for 20 days. Cyclophosphamide (25mg/kg) was used as a negative control and was administered for last 7 days of total 20 days treatment period.Colloidal carbon ink suspension (Rotring ink, Germany) in 1:8 proportions in normal saline was injected via tail vein to each mouse (10µl/1g, body weight) 48 hr after 20 days treatment of extracts and 7 days treatment for cyclophosphamide. Blood samples were withdrawn from retro-orbital plexus at 2 and 15 min after carbon injection on glass slide and 25 µl blood was added to 0.1% Sodium Carbonate (2 ml) in test tubes. The test tubes were then vortex mixed. The optical densities of these lysed solutions were determined at 660 nm keeping pre-injection blood samples as blank.The phagocytic index, K was calculated by using following equation:

$$K= (\ln OD1 - \ln OD2) / (T2 - T1)$$

Where OD1 and OD2 are the optical densities now and again T1 and T2individually.The phagocytic index of drug/extracts treated groups was statistically compared to that of vehicle control group.[36, 37]

Ovalbumin-induced Delayed Type Hypersensitivity in mice

Procedure for in vivo determination of delayed type hypersensitivity (DTH) response to ovalbumin. The control group received 0.5% Sodium Carboxy Methylcellulose (Na-CMC) only as vehicle; while animals in the treatment groups were given the test extracts/drug at different doses, p.o. suspended in 0.5% Na-CMC daily for 20 days. Immunosin was used as a positive control. The administration of immunosin (50mg/kg) was started 7 days before the first challenge on 21st day.4. On day 21 the animals were immunized subcutaneously with 3 mg of ovalbumin dissolved in normal saline emulsified with equal volume of Freund's complete adjuvant (Bangalore Genei). For determination of the delayed type hypersensitivity (DTH) reaction, all the mice were challenged subcutaneously (s.c.) with 50 µg ovalbumin in 25µl saline in the left hind footpad 14 days after the first immunization.The right hind footpad was injected with 25µl vehicle and this served as the control. The increase in footpad thickness was measured 24 hr after the challenge with the help of a Digimatic Caliper (Mitutoyo Corporation, Japan).The degree of DTH reaction was expressed as the difference in footpad thickness (L-R) over the control value.The increase/decrease in footpad thickness in drug/extracts treated group was statistically compared to that of vehicle control group.[38, 39]

Antibody Titer against ovalbumin using ELISA method

The administration of standard drugs was started seven days before the first challenge on 21st day. Cyclophosphamide (25mg/kg) was used as a negative control and Immunosin (25mg/kg) was used as a positive control. On day 21, the animals were immunized subcutaneously with 3 mg of ovalbumin dissolved in normal saline emulsified with equal volume of Freund's complete adjuvant (Bangalore Genei). Blood (400µl) was collected by retro orbital plexus under ether anesthesia after 7 days of immunization. The serum was separated by centrifugation. Quantification of serum IgG against ovalbumin i.e. serum antibody titer was estimated by Enzyme Linked Immunosorbent Assay (ELISA) as per the procedure described earlier. The procedure is as follows:

Flat bottom polystyrene plates were coated with 12.5 µg of ovalbumin dissolved in 100 µl of sodium carbonate buffer (pH 9.6) at 4ºC for 12 hr.The coated plates were washed three times with phosphate buffer saline (0.15M, pH 7.2) containing 0.05% Tween 20 (Tween-PBS).The wells were incubated with 100 µl of 1% BSA (Bovine serum albumin) prepared in sodium carbonate buffer at 37ºC for 1 hr.Serial dilutions of serum in Tween-PBS were prepared and 100 µl was incubated with coated wells for 1 hr at 37ºC. After washing, diluted (1:2000) Anti-mouse IgG conjugated with peroxidase (100 µl) was added and the plates were incubated at 37ºC for 1 hr.The enzyme activity was determined by addition of Tetra Methyl Benzidiene /$H2O_2$. The enzyme reaction was stopped by addition of 50 µl, 8 N Sulphuric Acid and the absorbance was measured at 450 nm.The antibody titer was expressed as the reciprocal of the highest dilution of the test serum showing three times more absorbance as compared to normal serum. The antibody titer of drugs/extracts treated groups was statistically compared to that of vehicle control group.[38, 40, 41]

Cyclophosphamide-induced myelosuppression in mice

Mice in treatment groups were treated with test extracts/drug at different doses, p.o. suspended in NaCMC daily for 16 days. Immunosin was used as a positive control. Cyclophosphamide treatment: On 17th, 18th and 19th day of study, all the animals except in the vehicle control group were injected with cyclophosphamide (25 mg/kg) intraperitoneally (i.p.) 1 hr after administration of the extracts or vehicle.

On 20th day of the study, blood samples (0.5ml) were collected from all the animals under ether anesthesia through retro-orbital plexus in micro centrifuge tube containing 4% disodium EDTA. Total leukocyte count of these blood samples was determined using hematology cell counter (Arcus, Diatron). Total leukocyte count of extracts treated groups were statistically compared to that of negative control group.[42, 43]

Statistical Analysis for in vivo assays

Results are communicated as mean ± SEM (n = 6). Data were analyzed by one way ANOVA followed by Dunnet's multiple comparisons test using GraphPadInstat software against control samples except in case of Cyclophosphamide-induced myelosuppression. In case of Cyclophosphamide-induced myelosuppression, extracts treated groups were controlled with Cyclophosphamide (negative control) group. Values of $P<0.05$ were the criteria for statistical significance.

RESULTS AND DISCUSSION:

RESULTS:

Phytochemical Evaluation of Extracts:

Phytochemical investigations revealed the presence of Alkaloids, Carbohydrates, Steroids, Glycosides, Phytosterols, fixed oils and fats, in ethanol extract of *Prosopis cineraria* dried leaves (PEE) and Carbohydrate, Steroids and Saponins in aqueous extract of *Prosopis cineraria* dried leaves (PAE). Results are shown in Table 1.

Table 1: Phytochemical Screening of Extracts of *Prosopis cineraria* Linn dried leaves

Phytochemical Test/Reagent	***Prosopis cineraria*Ethanol Extract (PEE)**	***Prosopis cineraria*Aqueous Extract (PAE)**
Alkaloids		
Mayer's Test	**+**	**–**
Dragendroff's Test	**+**	**–**
Wagner's Test	**+**	**–**
Carbohydrates		
Molisch's Test	+	+
Barford's Test	+	+
Benedicts Test	+	+
Glycosides		
Molisch's Test After Hydrolysis	+	**–**
Phytosterols		
Liebermann's Burchard's Test	+	+

Fixed Oils And Fats - Spot Test	+	–
Saponins - Foam Test	+	+
Phenolic Compounds And Tannins		
Ferric Chloride Test	+	+
Lead Acetate Test	+	+
Proteins And Amino Acids		
Biuret Test	+	+
Ninhydrin Test	–	+
Flavonoids - Shinoda Test	-	-
Yield (%W/W)	3.50	15.14

+ indicates present; - indicates absent

Separation of compound using different solvent system by thin layer chromatography:

TLC profiling of all extracts gives a magnificent result that directing towards the presence of number of phytochemicals. Various phytochemicals gives different Rf values in different solvent system.

TLC Alkaloid

TLC of leaves extract of *Prosopis cineraria* revealed the presence of 4 compound having Rf values of 0.4, 0.6, 0.7 and 0.92 when a solvent phase of hexane: ethyl acetate: petroleum ether (7:3:1) was used.

TLC Flavonoid

TLC of leaves extract of *Prosopis cineraria* revealed the presence of 2 compound having Rf values of 0.45, 0.89 when a solvent phase of Chloroform: methanol (18:2) was used.

TLC Saponin

TLC of leaves extract of *Prosopis cineraria* revealed the presence of 2 compound having Rf values of 0.83, 0.91 when a solvent phase of Chloroform: glacial acetic acid: methanol: water (6:2:1:1) was used.

TLC Terpenoid

TLC of leaves extract of *Prosopis cineraria* revealed the presence of 1 compound having Rf values of 0.88 when a solvent phase of Benzene: Ethyl acetate (1: 1) was used.

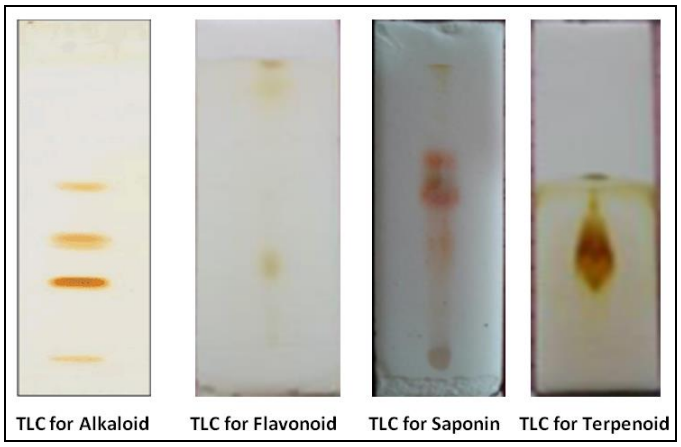

Figure 1: Separation of compound by using different solvent system for thin layer chromatography of *Prosopis cineraria* Linn

Table 2: Phytochemical Analysis of Different Parts of *Prosopis cineraria* Linn by Thin Layer Chromatography

Chemical Name	Solvent System	Plant Part	Solvent Run (Cms)	Peaks Obtained (Cms)	Rf Values	Spray Reagent
Alkaloid	Hexane:EthylAcetate:Petroleum Ether (7:3:1)	Leaves	5.0	2.0	0.4	Mayer's Reagent
				3.0	0.6	
				3.5	0.7	
				4.6	0.92	
Flavonoid	Chloroform: Methanol (18:2)		5.5	2.5	0.45	Uv Light
				4.9	0.89	
Saponin	Chloroform: Glacial Acetic Acid: Methanol: Water (6:2:1:1)		6.0	5.0	0.83	Iodine Vapours
				5.5	0.91	
Terpenoid	Benzene: Ethyl Acetate (1: 1)		4.5	4.0	0.88	10% H_2SO_4, UV Light

Acute Oral Toxicity:

PEE and PAE was found to be safe till a dose of 2000mg/kg since no mortality was observed till this dose level. At 2000 mg/kg dose levels, following signs of abnormal activity were observed: hypoactivity, sedation, disturbed somatomotor activity. Signs of intoxication were not observed 24hr post treatment.

***In-vitro* Immunomodulatory Activity of *Prosopis cineraria* leaves extract**

The ethanol and aqueous extracts of *Prosopis cineraria* leaves were evaluated for release of following immune mediators from murine peritoneal macrophages viz. superoxide (NBT reduction), nitric oxide (NO), lysosomal and myeloperoxidase enzymes.

Measurement of NO production

The nitrite level (nitric oxide) produced in cell culture supernatants was measured at 24 hr of treatment, PEE extract induced nitrite production in statistically significant higher ($p<0.05$) at), 832µg/ml (SI 1.884) 416µg/ml (SI 1.831), 208µg/ml (SI 1.809),104µg/ml (SI 1.671),52µg/ml (SI 1.545), 26µg/ml (SI 1.483), 13µg/ml (SI 1.332), 26µg/ml (SI 1.138) concentrations than controls in all three experiments performed. PHA (positive control) also showed significant increase ($p<0.05$) in nitrite release.

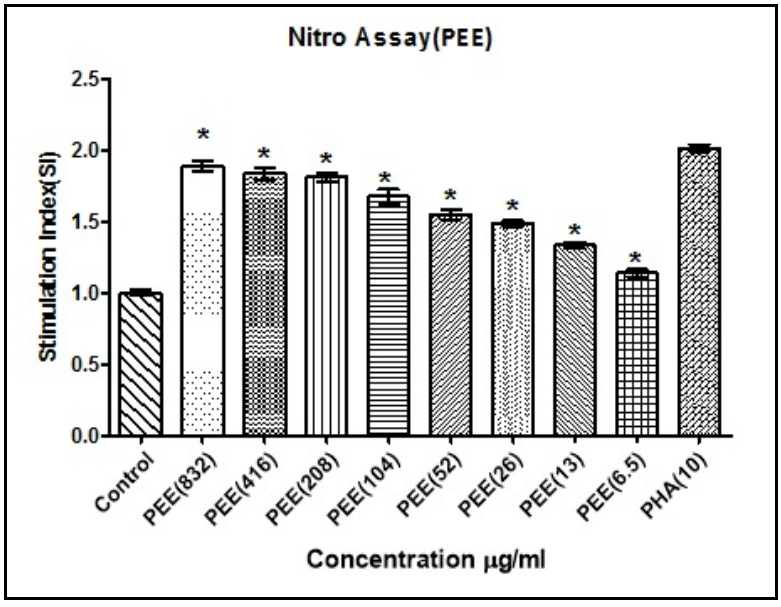

Figure 2: In-vitro effect of (PEE) on release of Nitric Oxide [*Significantly different from Control (P < 0.05)]

The nitrite level (Nitric Oxide) produced in cell culture supernatants was measured at 24 hr of treatment, showing that PAE extracts induced nitrite production in statistically SIgnificant higher ($p<0.05$) at 832µg/ml (SI 1.739), 416µg/ml (SI 1.551), 208µg/ml (SI 1.452), 104µg/ml (SI 1.331), 52µg/ml (SI 1.261), 26µg/ml (SI 1.205). PHA (positive control) also showed Significant increase ($p<0.05$) in nitrite release (SI 2.012)

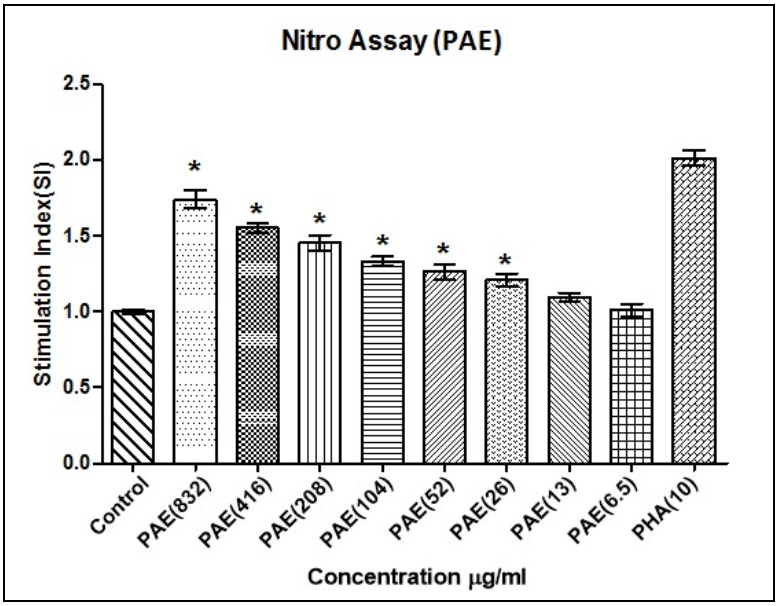

Figure 3: In-vitro effect of (PAE) on release of Nitric Oxide [*Significantly different from Control (P < 0.05)]

Cellular lysosomal enzyme activity

The effect of PEE extract showed significant stimulation p<0.05) on lysosomal enzyme release at 832µg/ml (SI 1.513), 416µg/ml (SI 1.397), 208µg/ml (SI 1.394) 104µg/ml (SI 1.577) 52µg/ml (SI 1.500), 26µg/ml (SI 1.394),13µg/ml (SI 1.462), 6.5µg/ml (SI 1.417), positive control, PHA showed significant stimulation (p<0.05) of (SI 1.971).

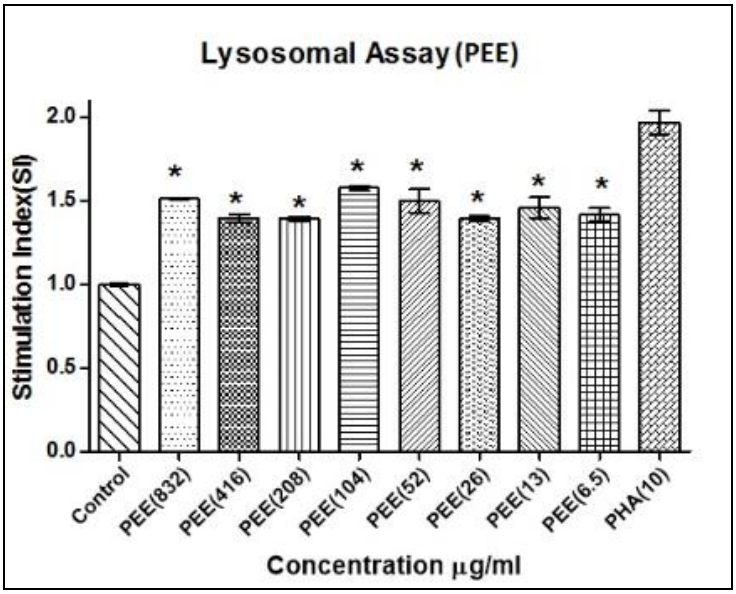

Figure 4: In-vitro effect of (PEE) on Cellular Lysosomal activity assay [*Significantly different from Control (P < 0.05)]

The effect of PAE extract showed significant stimulation (p<0.05) on lysosomal enzyme release at 832µg/ml (SI 1.404), 416µg/ml (SI 1.399), 208µg/ml (SI 1.388) 104µg/ml (SI 1.676) 52µg/ml (SI 1.391), 26µg/ml (SI 1.420),13µg/ml (SI 1.467), 6.5µg/ml (si 1.483), positive control, PHA showed significant stimulation (p<0.05) of (SI 1.971)

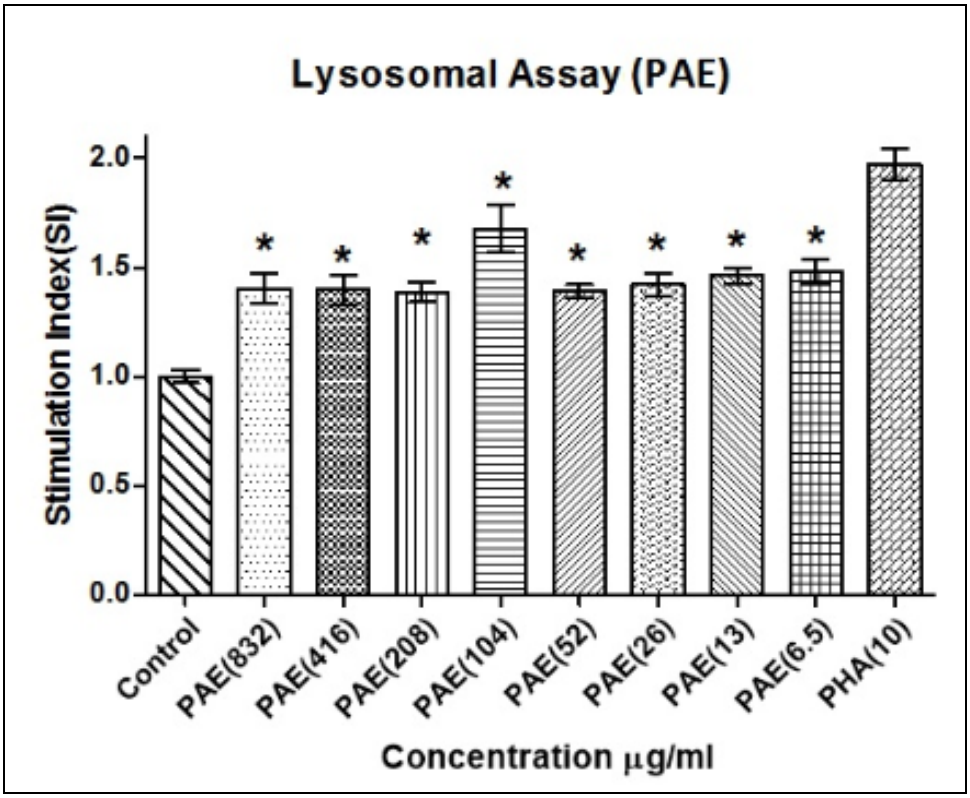

Figure 5: In-vitro effect of (PAE) on Cellular Lysosomal activity assay [*Significantly different from Control (P < 0.05)]

Nitro blue tetrazolium (NBT) reduction

The In vitro phagocytic effects of different concentrations of PEE extract on the reduction of NBT dye activity of macrophages are presented in table. PEE extract showed significant stimulation (p<0.05) at 832µg/ml (SI 1.662) 416µg/ml (SI 1.608), 208µg/ml (SI 1.447), 104µg/ml (SI 1.293), 52µg/ml (SI 1.183), 26µg/ml (SI 1.154) & 26µg/ml (SI 1.087). Positive control, PHA showed significant stimulation (p<0.05) of NBT reduction (SI 1.910).

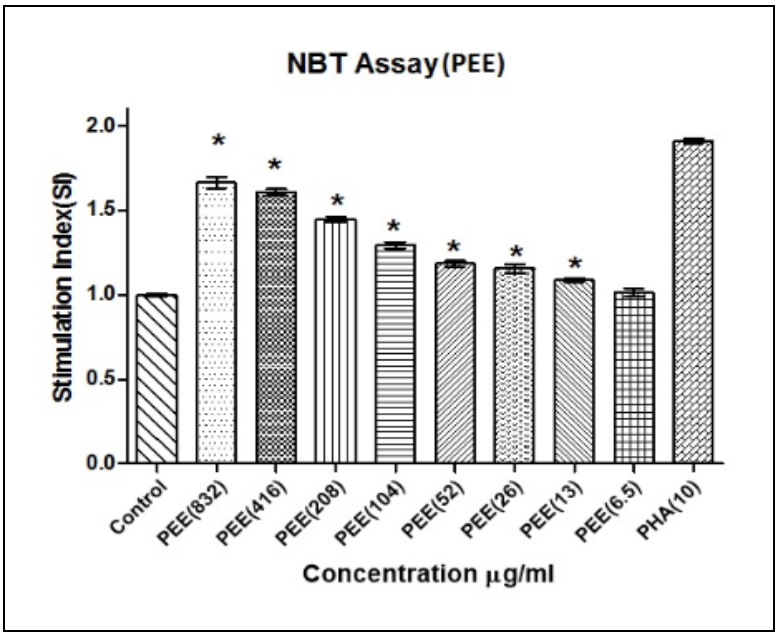

Figure 6: In-vitro effect of (PEE) on NBT dye reduction activity [*Significantly different from Control (P < 0.05)]

The In vitro phagocytic effects of different concentrations of PAE extract on the reduction of NBT dye activity of macrophages are presented in table. The effect of PAE extract showed significant stimulation p<0.05) on NBT reduction at 832µg/ml (SI 1.540), 416µg/ml (SI 1.379), 208µg/ml (SI 1.367) 104µg/ml (SI 1.315) 52µg/ml (SI 1.316), 26µg/ml (SI 1.215) 13µg/ml (SI 1.161), PHA showed significant stimulation (p<0.05) of NBT reduction (SI 1.910).

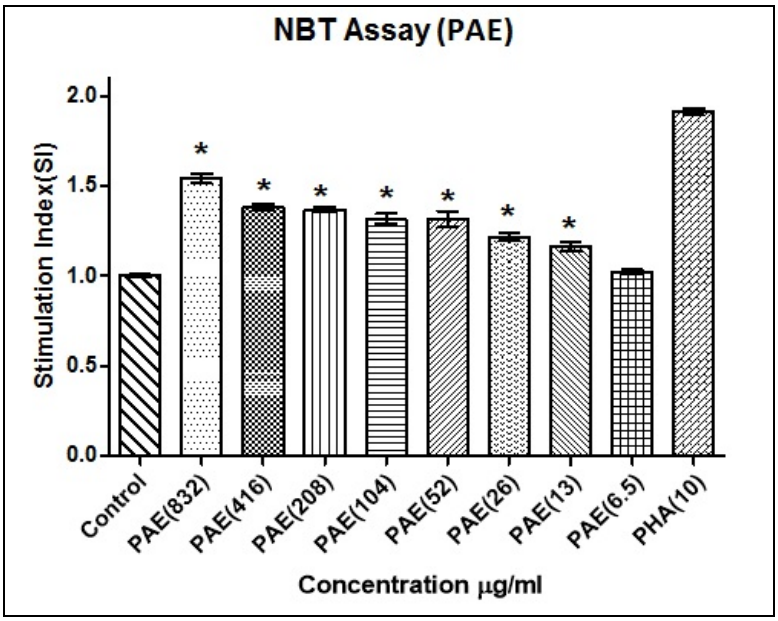

Figure 7: In-vitro effect of (PAE) on NBT dye reduction activity [*Significantly different from Control (P < 0.05)]

Measurement of myeloperoxidase activity

PEE extract at 832µg/ml (SI 1.772), 416µg/ml (SI 1.758), 208µg/ml (SI 1.738) 104µg/ml (SI 1.511) 52µg/ml (SI 1.573), 26µg/ml (SI 1.535),13µg/ml (SI 1.543), 6.5µg/ml (SI 1.405) as compared to control wells. Positive control, PHA showed significant stimulation with SI value 1.965.

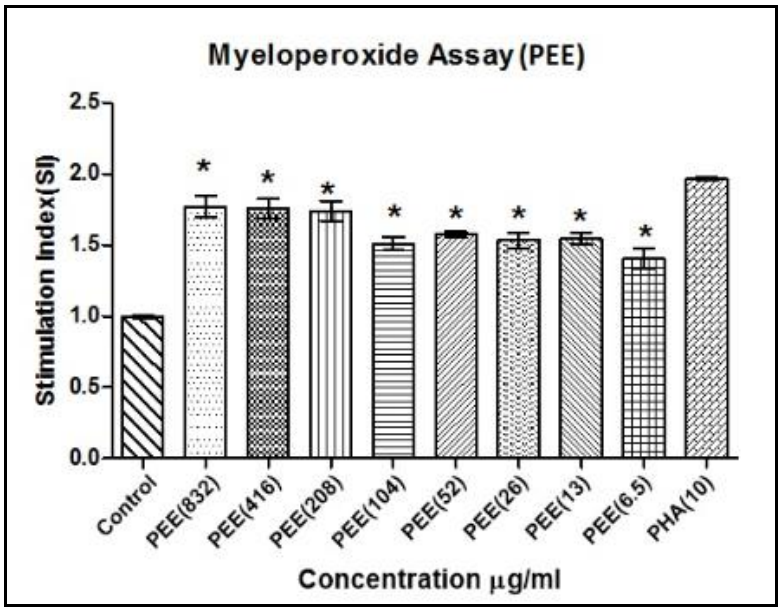

Figure 8: In-vitro effect of (PEE) on Myeloperoxidase activity assay [*Significantly different from Control (P < 0.05)]

The PAE extract showed significant (p<0.05) stimulation of myeloperoxidase activity of macrophages at 832µg/ml (SI 1.728), 416µg/ml (SI 1.695), 208µg/ml (SI 1.725) 104µg/ml (SI 1.730) 52µg/ml (SI 1.601), 26µg/ml (SI 1.541),13µg/ml (SI 1.427), 6.5µg/ml (SI 1.375), positive control, PHA showed significant stimulation (p<0.05) (SI 1.195) as compared to control wells.

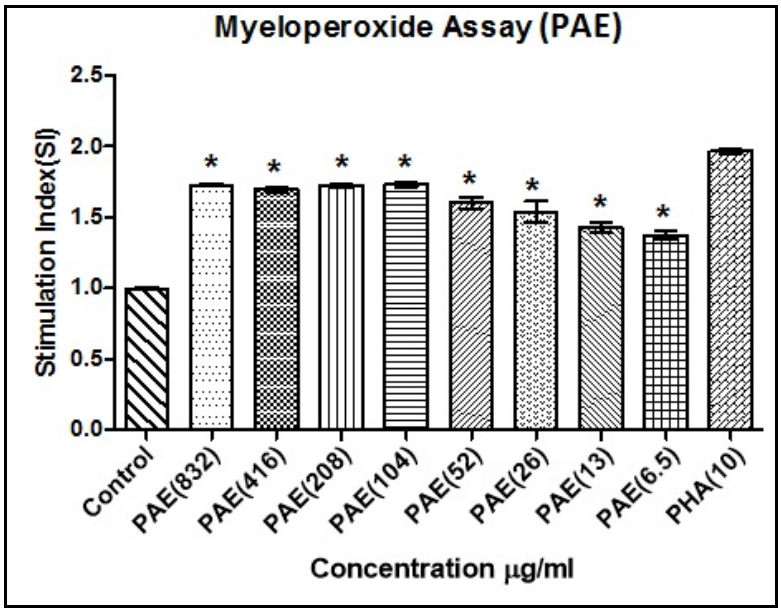

Figure 9: In-vitro effect of (PAE) on Myeloperoxidase activity assay [*Significantly different from Control (P < 0.05)]

Phytohemagglutinin

A positive control (10µg/ml) significantly stimulated nitric oxide (SI 2.013), NBT reduction (SI 1.910), lysosomal enzyme (SI 1.971) and myeloperoxidase activity (SI 1.965) as compared to control wells.

***In-vivo* Immunomodulatory Activity**

The ethanol extract (PEE), aqueous extract (PAE) and isolated (PEE) extract of *Prosopis cineraria* leaves were evaluated for their effects on in vivo immune system of experimental animals using validated animal models. The effect of the test extracts were evaluated in following test systems viz. Carbon Clearance Assay, Delayed Type Hypersensitivity reaction, Antibody Titre Assay and cyclophosphamide induced myelosuppression in mice.

Phagocytic activity by Carbon Clearance Assay in mice

The effect of the extracts (PEE and PAE) of *Prosopis cineraria* on phagocytic activity was studied through carbon clearance assay in mice. The Phagocytic Index (K) for PEE extract treated mice was significantly higher at 50 mg /kg (14.80%), 100mg/kg (31.91%), 200mg/kg (48.12%) dose levels as compared to control group. The Phagocytic Index (K) for PAE extract was significantly higher ($p<0.05$) at 50 mg /kg (15.86%), 100mg/kg (32.06%), 200mg/kg (35.47%). Isolated PEE extract was significantly higher ($p<0.05$) at 50 mg /kg (34.58%), 100mg/kg (51.51%), 200mg/kg (60.60%).

Positive control immunosin (50mg/kg) showed 71.12% higher K compared to control group. Cyclophosphamide (25mg/kg), a negative control group has produced significant decrease ($P<0.05$) in K value, a positive control group has produced significant increase in K ($P<0.05$) as compared to vehicle control group.

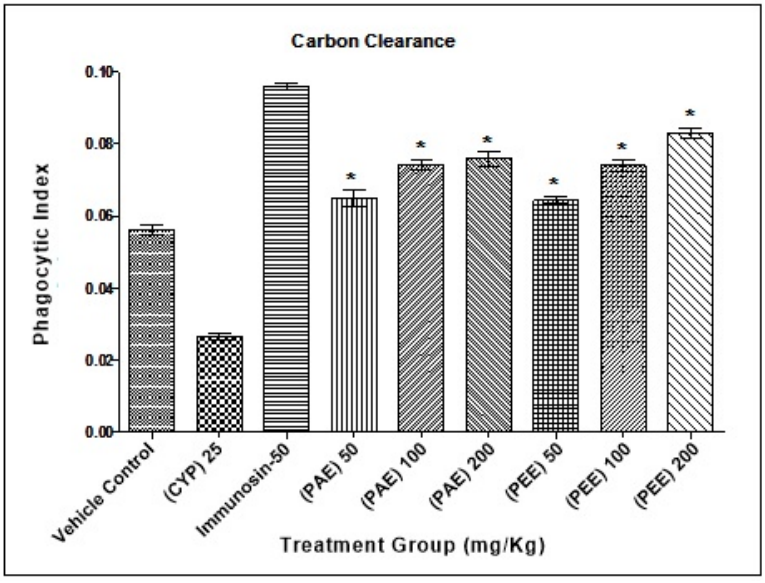

Figure 10: Effect of *Prosopis cineraria* ethanol and isolated (PEE) extract and aqueous extract (PAE) on phagocytic index [*Significant (P < 0.05)]

Ovalbumin-induced Delayed Type Hypersensitivity in mice

The DTH response of the extracts (PEE and PAE) of *Prosopis cineraria* were measured in mm(x 10-2) as the difference in the paw thickness 24hr after challenge with ovalbumin immunized mice. Ethanolic, aqueous and isolated extracts produced a significant increase the DTH response to ovalbumin at all the tested dose levels. Increase in DTH reaction in mice in response to t cell dependent antigen revealed the stimulatory effect of aqueous, ethanolic and isolated extract on t cells. Immunosin 50 mg/kg, PEE and PAE at 50, 100 and 200mg/kg ($p<0.05$).

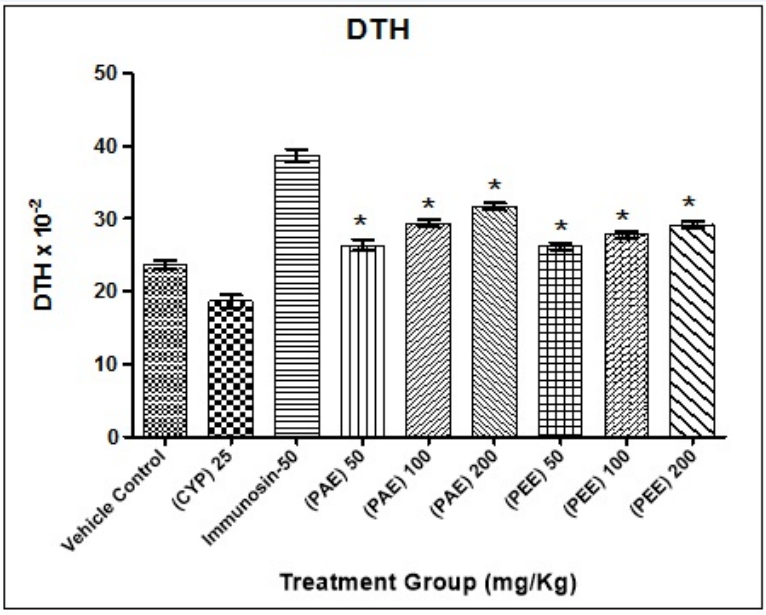

Figure 11: Effect of *Prosopis cineraria* ethanol and isolated (PEE) extract and aqueous extract (PAE) on DTH response [*Significant (P < 0.05)]

Antibody Titer against ovalbumin using ELISA method

Humoral response to ovalbumin was studied by elisa antibody titer assay. Mice treated with different doses of the PAE and PEE extract showed an increase in the antibody titer in a dose dependent manner. There was significant increase in serum antibody titer at 200mg/kg (768.01) ($p<0.05$) of PEE extract compared to control group (213.33). The PAE extract showed significant increase in serum antibody titer at 200mg/kg (768.33) ($p<0.05$) compared to control group (213.33) and isolated PEE extract showed significant increase in serum antibody titer at 200mg/kg (978.00) ($p<0.05$) compared to control group (213.33). Positive control immunosin (50mg/kg) showed (1195.00) ($p<0.05$) compared to control group.Cyclophosphamide, a negative control group showed decrease ($P<0.05$) in antibody titre (149.3).

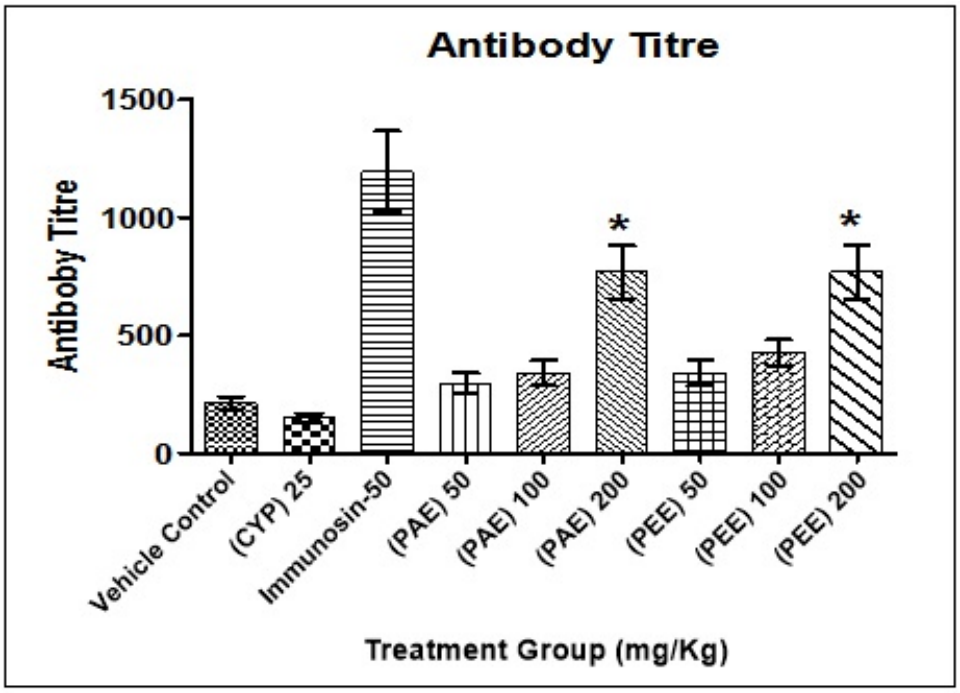

Figure 12: Effect of *Prosopis cineraria* ethanol and isolated (PEE) extract and aqueous extract (PAE) on Antibody Titer [*Significant (P < 0.05)]

Cyclophosphamide-induced myelosuppression in mice

The effect of ethanol extract (PEE), aqueous extract (PAE) of *Prosopis cineraria* leaves on total WBC count of CYP induced myelosuppression in mice is presented in Table 12. There was significant reduction ($p<0.05$) in total WBC count of Cyclophosphamide (25mg/kg) treated mice (8.792 x103 cells/cmm) as compared to Vehicle Control group (13.38 x103 cells/cmm). Cyclophosphamide dose of 25mg/kg caused significant reduction in the WBC, RBC, Haemoglobin and Platelet Count. Combined treatment of (PAE+CYP-25mg/kg), (PEE+CYP-25mg/kg) and isolated (PEE+CYP-25mg/kg) (50,100 and 200mg/kg) doses of PEE and PAE each with 25 mg/kg resulted in restoration of bone marrow activity as compared with negative control CYP treatment alone. Immunosin, a positive control group treated mice showed significant increase ($P<0.05$) in WBC, RBC, Haemoglobin and Platelet Count as compared to CYP alone treated group.

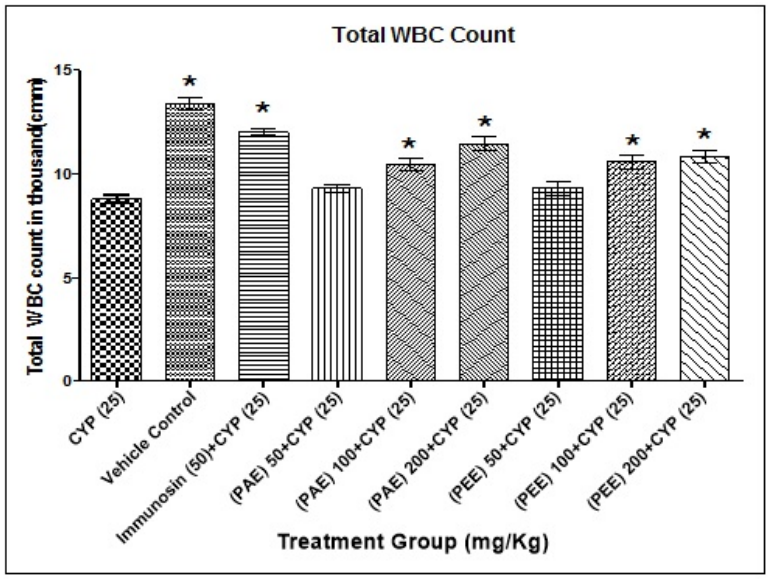

Figure 13: Effect of *Prosopis cineraria* ethanol and isolated (PEE) and aqueous extract (PAE) on Total WBC Count [*Significant (P < 0.05)]

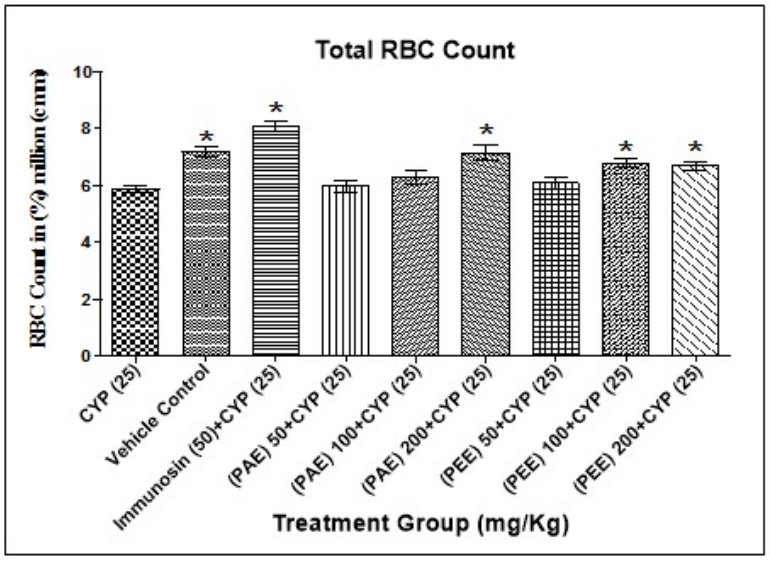

Figure 14: Effect of *Prosopis cineraria* ethanol and isolated (PEE) and aqueous extract (PAE) on Total RBC Count [*Significant (P < 0.05)]

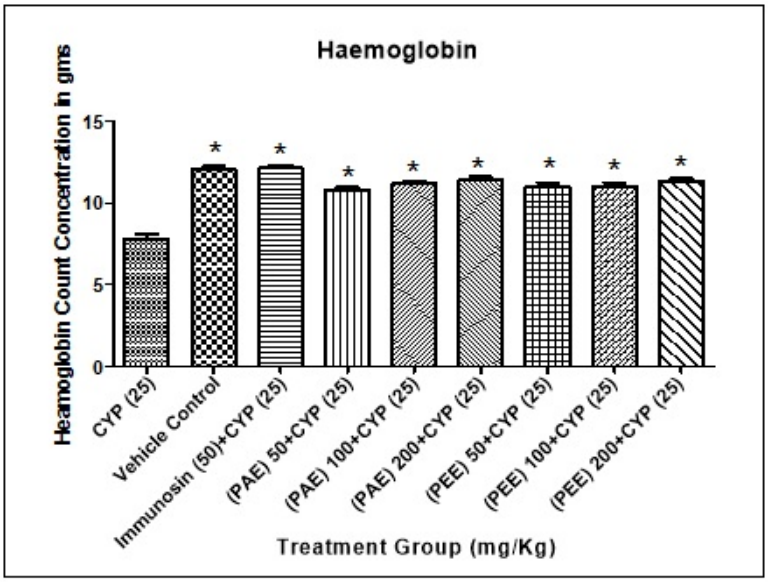

Figure 15: Effect of *Prosopis cineraria* ethanol and isolated (PEE) and aqueous extract (PAE) on Haemoglobin [*Significant (P < 0.05)]

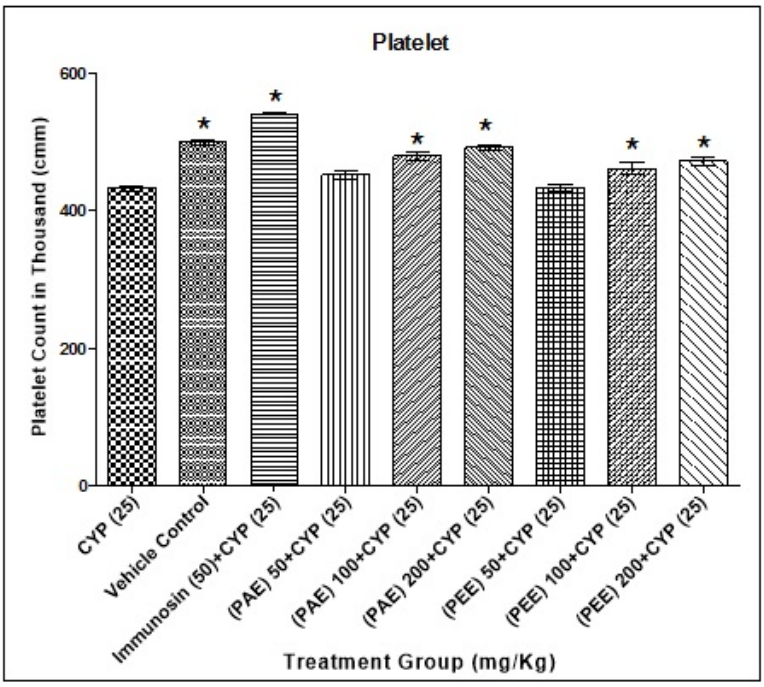

Figure 16: Effect of *Prosopis cineraria* ethanol and isolated (PEE) and aqueous extract (PAE) on Platelet count [*Significant (P < 0.05)]

DISCUSSION:

The ethanol and aqueous extract of *Prosopis cineraria* (PEE and PAE)has shown significant stimulation of nitric oxide, NBT reduction and lysosomal enzyme activity of murine macrophages with dose-dependant relationship. This release of the immune mediators from macrophages indicates enhanced in vitrophagocytic and cytotoxic activity of macrophages. This extract was further evaluated for in vivo

effects for confirmation of the results observed in vitro.The clearance rate of carbon was taken as a measure of phagocytic activity of the reticulo-endothelial system of mice according to the techniques of Biozzi et al. 1953 and Yang et al, 2007.[44, 45]PEE and PAE showed increase in phagocytic index (K) at 100 and 200mg/kg dose indicates stimulaton of reticulo-endothelial system. The mechanism for this effect seems to be stimulation of release of superoxides and lysosomal enzyme from macrophages.The DTH response of PEE and PAE was significant at 50,100 &200mg/kg indicates stimulation of T cell mediated immune response to ovalbumin.The antibody response to ovalbumin was significantly higher at 200mg/kg indicates stimulation of B cells to secrete antibodies.[46]The results of effect of the PEE and PAE extract in experimentally induced myelosuppression in mice are encouraging. In this assay, cyclophosphamide showed suppression of total WBC count i.e. leukocytopenia. The treatment of PEE and PAE extract in these animal groups produced increase in total WBC count of these mice. Statistically significant increase was observed at 100 &200mg/kg dose. No dose of PEE and PAE was able overcome the effect of cyclophosphamide completely and restore the total WBC levels to normal.

This release of the immune mediators from macrophages indicates enhanced in vitrophagocytic activity of macrophages. This extract was further evaluated for in vivoeffects for confirmation of the results observed in vitro. Carbon clearance assay in mice forphagocytosis showed significant increase in phagocytic index (K) at 50,100 &200mg/kg dose indicates stimulation of reticulo-endothelial system.[47]The mechanism for this effect seems to be stimulation of release lysosomal enzyme from macrophages. Mild stimulation of DTH response to ovalbumin was observed at 50, 100 and 200mg/kg indicates stimulation of T cell mediated immune response to ovalbumin.[48]The antibody response to ovalbumin was significantly higher at 100 &200mg/kg indicates stimulation of B cells to secrete more antibodies. PEE and PAE extract showed increase in total WBC count in cyclophosphamide induced myelosuppression in mice. Statistically significant increase was observed only with 200mg/kg dose. None of the tested dose of PEE and PAE was able to restore the suppressed WBC count to normal.Macrophages are the main pro-inflammatory cells that respond to invading pathogens by releasing many pro-inflammatory molecules, including short-living free radical nitric oxide (NO). NO is synthesized from L-arginine by NO synthase (NOS) in numerous types of cells. In mammals, 3 distinct isoforms of NOS have been cloned: endothelial, neuronal, and inducible NOS (iNOS). Among these isoforms, iNOS plays an important role in the regulation of cytotoxic responses.[44]Agents that modulate the activity of NO may be of considerable therapeutic value. NO mediates diverse functions, including vasodilatation, neurotransmission and inflammation.[49]Phytohemagglutinin (PHA), the positive control used in the experiments showed stimulation of immune parameters in all these assays indicates stimulation of immune system in vitro. The phagocytic index of PEE and PAE treated mice was found to be significantly higher than the vehicle control group in dose dependant manner. This in vivophagocytic response would be due to enhanced lysosomal enzyme activity of macrophages as observed during in-vitroassays. Cyclophosphamide, a negative control group has shown significant decrease whereasImunosin, a positive control group has shown significant increase in phagocytic index. This indicates that the test system model is well validated.

CONCLUSION:

The finding of study showed an overall stimulatory effect of *Prosopis cineraria* leaves extract on both humoral and cellular immunity of mice. The data obtained give justification for further research into the mechanisms of immunomodulatory activity of extracts from *Prosopis cineraria* leaves.The studies have demonstrated specific and non-specific immunostimulating properties of the PEE and PAE tubers in in vivo experimental methods. This suggests its therapeutic usefulness in immunocompromised conditions.

AUTHORS CONTRIBUTION STATEMENT:

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CONFLICT OF INTEREST:

Conflict of interest declared none.

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