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Screening of immunomodulatory activity of Sphaeranthus indicus Linn. whole plant

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Abstract: *The aim of the present investigation is to evaluate the immunomodulatory activity for methanolic extract of Sphaeranthus indicus Linn. (MESI) at different doses as 100, 200 and 400 mg/kg bd.wt. in healthy wistar albino rats. Immunomodulatory activity of MESI was evaluated for humoral immunity (antibody titre, plaque forming cell assay and quantitative haemolysis of SRBC) and cellular immunity (delayed type hypersensitivity, T- cell population and drug induced myelosuppression) with antigen challenge by sheep RBCs. MESI along with the antigen (sheep red blood cells) showed significant increase in the circulating antibody titer and the number of plaque forming cells (PFC) in the spleen at the dose of 100 mg/kg bd.wt. as compare to 200 and 400 mg/kg bd.wt. MESI also showed significant ($p < 0.01$) increase in the DTH response, restoration of histological parameters, increase in lymphocytes and rosettes formation in T-cell population at dose of 400 mg/kg bd.wt. as compare to 100 and 200 mg/kg bd.wt. The present investigation reveals that Sphaeranthus indicus can be used as immunomodulator for activation of specific and non- specific immune responses.*

Keywords: *Immunomodulators, Sphaeranthus indicus, humoral mediated immunity and cell mediated immunity.*

I. Introduction

Immune system is a remarkably sophisticated defense system within vertebrates, to protect them from invading agents. It is able to generate varieties of cells and molecules capable of recognizing and eliminating limitless varieties of foreign and undesirable agents. Modulation of the immune system denotes to any change in the immune response that can involve induction, expression, amplification or inhibition of any part or phase of the immune response. Thus, immunomodulator is a substance used for its effect on the immune system. There are generally two types of immunomodulators based on their effects: immunosuppressants and immunostimulators. Immunopharmacology is a comparatively new and developing branch of pharmacology aims at searching for immunomodulators. The potential uses of immunodulators in clinical medicine include the reconstitution of immune deficiency (e.g. the treatment of AIDS) and the suppression of normal or excessive immune function (e.g. the treatment of graft rejection or autoimmune disease). Specific immunomodulators administered together with antigens known as immunological adjuvants to boost the immune response to the vaccine constituents[1].

Sphaeranthus indicus Linn. (Asteraceae) is a branched herb with purple flowers that grows abundantly in rice field and distributed throughout India. It is used indigenously in the Indian system of medicine as an anthelmintic[2]. The plant has a wide range of medicinal value and has been used in hemicranias, jaundice, leprosy, diabetes, fever, pectoralgia, cough, gastropathy, hernia, hemorrhoids, helminthiasis, dyspepsia, skin diseases and nerve tonic[3,4].

Sphaeranthus indicus showed immunomodulatory[5], antimicrobial[6,7], antibacterial[8,9], anxiolytic[4], wound healing action[10] activities. Phytoconstituents isolated from Sphaeranthus indicus were eudesmanolides[11], isoflavonoids[12], 7-hydroxy eudesmanolides[13], sterol glycoside[14], essential oil (cadiene, ocimene, citral, p-methoxycinnamaldehyde, geraniol, eugenol and geranyl acetate[15], and eudesmanolides[16].

II. Materials And Methods

Plant material collection and extract preparation

Sphaeranthus indicus whole plant was collected from Kamalapur of Karimnagar district, Telangana (India) in the month of January, 2015 and was identified and authenticated by Dr. Venkatesh. The plant material was cleaned, made into small pieces, dried under sun and coarsely powdered and stored. The coarsely powdered plant material (500g) was subjected to extraction with methanol using simple distillation. The extract was concentrated to semisolid mass and stored in air tight containers.

Animals used

Wistar albino rats (Approx 180 to 250 g) were procured from Albino Labs, Hyderabad. Present study was carried out in CPCSEA approved animal house of Gokaraju Rangaraju College of Pharmacy, Bachupally, Hyderabad, India (Reg. No.1175/ac/08/CPCSEA)**.**

Antigens

Sheep Red Blood Cells (SRBC) were collected in Alsever's solution from NIN slaughter house Hyderabad, India. SRBC were washed 3-4 times with large quantity of sterile and pyrogen free saline.

Acute toxicity studies

Methanolic extract of Sphaeranthus indicus whole plant was tested for acute toxicity studies as per procedure given in OECD guidelines 425 and limit test method was followed. Mice were starved for 4h and fed orally with methanolic extract of Sphaeranthus indicus at doses 2000 and 5000 mg/kg bd.wt. animals were observed for 14 days for mortality.

Preliminary phytochemical screening

MESI was subjected to preliminary phytochemical investigations.

Estimation of total phenolic content

For the estimation of total phenolic content 2 mL of MESI was prepared, 10 mL of the water and 2 mL of Folin-phenol reagent were added and volume of solution was made up to 25 mL by adding sodium carbonate solution. Resultant solution was kept for 30 minutes for incubation in dark. Then, the absorbance of solutions was measured using UV spectrophotometer at 760 nm [17].

Estimation total flavonoid content

Total flavonoid of MESI was determined using the method of Liu et al.

In brief, MESI was diluted with 80% aqueous ethanol (0.9 mL). 0.5 mL of extract was added to test tube containing 0.1 mL of 10 % aluminum nitrate, 0.1 mL 1M aqueous potassium acetate and 4.3 mL of 80 % alcohol. The reaction tubes were set aside for 40 minutes at room temperature. At the end, optical density of each sample was determined at 415 nm using a UV spectrophotometer. Total flavonoids content was calculated by interpolation on a standard curve established with a reference standard, quercetin. Quercetin and Folin-Ciocalteu reagent were obtained from Sigma-Aldrich, Germany[17].

In vitro antioxidant assay

Reducing Power Assay

One mL of MESI (20 µg/mL) was mixed with phosphate buffer (2.5 mL) and potassium ferricyanide (2.5 mL). The mixture was incubated at 500°C for 20 min. Aliquot of trichloroacetic acid (2.5 mL) was added to the mixture, and centrifuged at 3000 rpm for 10 min. The upper layer of resultant solution (2.5 mL) was mixed with distilled water (2.5 mL) and ferric chloride solution (0.5 mL). The absorbance was measured at 700 nm. Ascorbic acid (20 µg/mL) was used as standard. Increased absorbance of the reaction mixture indicates increase in reducing power[18].

Hydrogen Peroxide Assay

The ability of MESI to scavenge hydrogen peroxide was determined according to the method given by Ruch et al. A solution of hydrogen peroxide (2mmol/L) was prepared in phosphate buffer (pH 7.4). MESI (1–10 µg/mL) were added to hydrogen peroxide solution (0.6 mL). Absorbance of resultant solution was determined after 10 min at 230 nm against a blank solution, and ascorbic acid was used as reference compound[19].

In vitro immunomodulatory assay

The experiments was done according to the CPCSEA guidelines and approved by the Institutional Animal Ethical Committee. In the present study MESI was dissolved acacia and in distilled water. Doses selected of MESI were 100, 200 and 400 mg/kg body weight. Albino rats were divided into groups comprising of six animals each.

Plaque forming cell assay

On 0 day, all groups were sensitized with 0.1 mL of SRBC containing 1×10^8 cells, i.p.
Group I served as control and was administered 1% Gum acacia suspension in saline
Group II received 100 mg/ kg bd.wt. of MESI p.o. respectively (1 to 5 days)
Group III received 200 mg/ kg bd.wt. of MESI p.o. respectively (1 to 5 days)

Group IV received 400 mg/kg bd,wt. of MESI p.o. respectively (1 to 5 days)
Group V received standard drug 50 mg/kg bd.wt. Levamisole. p.o. respectively (1 to 5 days) (Immunostimulant)

The PFC assay was performed using the method of Raisuddin et al. The animals were humanized on the fifth day of immunization with SRBC. The spleen was removed, cleaned free of extraneous tissues, and a single cell suspension of 10^6 cells/mL was prepared from it in RPMI-1640 medium. For PFC assay, the SRBC were prepared at a density of 5 x 10^8 cells/ mL in PBS. One milliliter of SRBC in medium along with 0.5 mL of diluted rabbit serum complement (1:10 diluted with normal saline) was added to 1 mL of spleen cell suspension. Cuningham chambers were prepared using glass slide, coverslips and double- sided tape (Scotch Brand, St. Paul, MN). The chambers were loaded with a known volume of assay mixture, sealed with petroleum jelly and incubated at 37˚C for 1 h. The plaques were counted under a light microscope (Olympus BX50) and expressed as PFC per 10^6 spleen cells[20].

Quantitative haemolysis of SRBC (QHS) assay
On 0 day, all groups were sensitized with 0.1 mL of SRBC containing 1×10^8 cells, i.p.
Group I served as control and was administered 1% Gum acacia suspension in saline
Group II received 100 mg/ kg bd.wt. of MESI p.o. respectively (1 to 5 days)
Group III received 200 mg/kg bd.wt. of MESI p.o. respectively (1 to 5 days)
Group IV received 400 mg/kg bd,wt. of MESI p.o. respectively (1 to 5 days)
Group V received standard drug 50 mg/kg bd.wt. Levamisole. p.o. respectively (1 to 5 days)
(Immunostimulant)

QHS assay was performed using the methods of Simpson and Gozo and with some modifications. Spleens were removed and a cell suspension of 1 $X10^6$ cells /mL was prepared in PBS. One mL of 0.2% SRBC and 1 mL of 10% rabbit serum were mixed with cell suspension and incubated for 1hr at 37˚C. After centrifugation at 3000 rpm for 3 min, optical density of the supernatant was measured at 413 nm using a spectrophotometer (Shimadzu UV- 1201)[20].

In vivo immunomodulatory activities
Antibody (HA) titre response to SRBC
On 0 day, all groups were sensitized with 0.1 mL of SRBC containing 1×10^8 cells, i.p.
Group I served as control and was administered 1% Gum acacia suspension in saline
Group II received 100 mg/kg bd.wt. of MESI p.o. respectively (1 to 7 days)
Group III received 200 mg/kg bd.wt. of MESI p.o. respectively (1 to 7 days)
Group IV received 400 mg/kg bd.wt. of MESI p.o. respectively (1 to 7 days)
Group V received 50 mg/kg bd.wt. of standard, Levamisole, p.o. respectively (1 to 7 days)

On 7th day before challenge, blood was withdrawn from retro-orbital plexus of each animal. Blood was centrifuged, and serum was separated. Serial two fold dilutions were made i.e. 50 µL of serum was added to 1st well of 96-well micro titer plate containing 50 µL normal saline. To this 1% SRBC (50 µL) dissolved in normal saline was mixed. From 1st well 50 µl of diluted serum was added to 2nd well containing 50 µl normal saline and 50µl 1% SRBC. Such dilutions were done till 24th well. Plates were incubated at 37ºC for 1h highest dilution that has shown visible agglutination was considered as haemagglutination antibody[21].

Delayed type hypersensitivity
On 0 day, all groups were sensitized with 0.1 mL of SRBC containing 1×10^8 cells, i.p.
Group I - Control, 1% Gum acacia suspension in saline
Group II received 100 mg/kg bd.wt. of MESI p.o. respectively (1 to 7 days)
Group III received 200 mg/kg bd.wt of MESI p.o. respectively (1 to 7 days)
Group IV received 400 mg/kg bd.wt of MESI p.o. respectively (1 to 7 days)
Group V received 50 mg/kg bd.wt. of standard, Levamisole, p.o. respectively (1 to 7 days)

On 7th day prior to injection, right hind footpad thickness was measured with digital vernier callipers (Mitutoyo digimatic). Then animals were challenged by injecting 1% SRBC (20 µL) into the right hind footpad. On 8th and 9th day footpad thickness was again measured. Difference between prior and post challenge footpad thickness was reported as DTH response[22].

T-cell population
On 0 day, all groups were sensitized with 0.1 mL of SRBC containing 1×10^8 cells, i.p.
Group I - Control, 1% Gum acacia suspension in saline
Group II- received 100 mg/kg bd.wt. of MESI p.o. respectively (1 to 10 days)
Group III- received 200 mg/kg bd.wt. of MESI p.o. respectively (1 to 10 days)

Group IV- received 400 mg/kg bd.wt. of MESI p.o. respectively (1 to 10 days)
Group V- received 50 mg/kg bd.wt. of standard, Levamisole, p.o. respectively (1 to 10 days).

On 11th day, blood was collected from the retro-orbital plexus and anticoagulated with Alsever's solution in separate test tubes. Test tubes containing blood were kept in sloping position (45˚) at 37˚C for 1 h. RBCs were allowed to settle at bottom and supernatant was collected from each test tube by using micropipette which contains lymphocytes. An amount of 50 µl of this lymphocyte suspension & 50 µl SRBC were mixed in test tube and incubated. Resultant suspension was centrifuged at 200 rpm for 5 min and kept in a refrigerator at 40˚C for 2 h. The supernatant fluid was removed and one drop of cell suspension was placed on a glass slide. Total lymphocytes were counted and a lymphocyte binding with three or more erythrocytes was considered as rosette[23].

Drug induced myelosuppression

On 0 day, all groups were sensitized with 0.1 mL of SRBC containing 1×10^8 cells, i.p.
Group I – (Control) received, 1% gum acacia suspension in saline
Group II - Negative control, received, 2 mg/kg bd.wt Azathioprine. p.o. respectively (on 11th, 12th and 13th day).
Group III- received 100 mg/kg bd.wt of MESI p.o. respectively (1 to 13 days)
Group IV- received 200 mg/kg bd.wt. of MESI p.o. respectively (1 to 13 days)
Group V- received 400 mg/kg bd.wt. of MESI p.o. respectively (1 to 13 days)
Group VI received 50 mg/kg bd.wt. of standard, Levamisole, p.o. respectively (1 to 13 days)

On 0 day, blood was withdrawn from retro-orbital plexus of animals of each group and subjected to haematological parameter determination. MEFB was administered to Group-III, IV, V and VI from 1 to 13 days. Azathioprine (2 mg/kg, bd.wt) is given to all animals on 11th, 12th and 13th day, 1h after MESI administration except control and standard group. On day 14th blood was withdrawn from retro-orbital plexus and hematological parameters were estimated[24].

Histopathology of spleen and thymus

For histopathology the rats were sacrificed by cervical dislocation and their spleen and thymus gland were dissected out. Tissues (spleen and thymus) obtained from all the experimental groups were washed immediately with saline and then fixed in 10% buffered neutral formalin solution. After fixation, the tissues were processed by embedding in paraffin. The tissues were sectioned and stained with haematoxylin and examined under high power microscope (200 & 400X) and photo micrographs were taken.

Statistical analysis:

Graph Pad prism 3 software and MS excel was used for statistical analysis of data. All the results were expressed as mean±standard error of mean (SEM), analyzed for ANOVA and Dunnet's t-test (Multiple). Differences between groups were considered significant at $p<0.05$, $p<0.01$ levels.

III. Results And Discussion

Acute toxicity testing:

The acute toxicity testing was performed in female animals. The animals treated with different doses of Sphaeranthus indicus whole plant showed no signs of toxicity. No animal was found to be in moribund state and no animal died even after 14 days. So, it was confirmed that the Sphaeranthus indicus whole plant was safe up to 2000 mg/kg bd.wt.

The preliminary phytochemical investigation for MESI showed the presence of carbohydrates, terpenes, glycosides, flavonoids, phenolics, proteins, amino acids, steroids and tannins. Saponins are present in minor quantities. The total phenolic and flavonoid content of MESI was found to be 2.95 µg/mg of gallic acid and 2.4 µg/mg of quercetin equivalent.

MESI showed good antioxidant activity by reducing power assay and hydrogen peroxide assay with IC_{50} value 35 and 31 µg/mL respectively. Ascorbic acid was used as reference standard showed the IC_{50} value 27 and 24 µg/mL with reducing power assay and hydrogen peroxide assay respectively (Table. 1).

MESI was evaluated for in vitro immunomodulatory assay where MESI showed good immunomodulatory activity with plaque forming cell (PFC) and QHS assay (Table. 2).

Number of antibody secreting cells from spleen was determined using plaque forming cell assay. The effect of methanolic extract of Sphaeranthus indicus on antibody secreting cells of mouse spleen have indicated that immunostimulation was achieved through humoral immunity. The humoral mediated immunity involves the interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody-secreting plasma cells. Antibody functions as the effector of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross- linking to form clusters that are more readily ingested by phagocytic cells. The effect was significant ($P<0.01$) compared to control.

The humoral mediated immunity involves the interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody secreting plasma cells. Antibody functions as the effector of the humoral immune response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells. The effect of MESI has indicated that immunostimulation achieved through humoral immunity.

MESI was evaluated for in vivo immunomodulatory acitivity with antigen-antibody titer response i.e. haemagglutination titer. MESI showed significant antibody titer, when compared to control ($p<0.01$) and standard ($p<0.01$) (Table. 3). The reaction of an antibody and antigen can be easily detected by agglutination (clumping) of the antigen. If the antigen is an erythrocyte the term haemagglutination is used. Agglutination tests can also be used to measure the level of antibodies to particulate antigens. Humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody screening plasma cells. Antibody functions as the effectors of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells. The relative strength of an antibody titre is defined as the reciprocal of the highest dilution which is still capable of causing visible agglutination. The significant value of agglutination was observed at the dose (100 mg/kg bd.wt) for methanolic extract of Sphaeranthus indicus. The haemagglutination antibody titer obtained in case of MESI has indicated that immunostimulation was achieved through humoral immunity.

MESI showed significant inhibition of hypersensitivity reaction at the dose of 400 mg/kg bd.wt. compared with control ($p<0.01$) and standard ($p<0.01$) (Table. 4).

DTH is an antigen specific and mediated by T cells rather than antibody. T cells are required to initiate the reaction. Activation of T cells releases lymphokines, which lead to activation and accumulation of macrophages, increases vascular permeability, induce vasodilatation, and produce inflammation. It also boosts phagocytic activity and increases concentration of lytic enzymes for more effective killing, ultimately results in increase footpad thickness in immunized animals. General characteristics of DTH are an invasion of immune cells at site of injection and induction became apparent within 24 to 72 hrs. Increase in paw edema after 24 hrs of challenge was observed in all extract treated groups when compared to control. Increase in DTH response of animals revealed the stimulatory effect of MESI on T lymphocytes i.e. cell mediated immunity or specific immunity. The result indicates that there was significant difference in the foot paw thickness at doses of 100 mg/kg bd.wt ($p<0.01$), 200 mg/kg bd.wt ($p<0.01$) and 400 mg/kg bd.wt ($p<0.01$) methanolic extract of treated group when compared against normal control and standard.

MESI showed a significant increase in lymphocytes and rosettes when compared with control ($p<0.01$) and standard ($p<0.01$). 400 mg/kg bd. wt. was found to be potent dose amongst other doses as 100 and 200 mg/kg bd.wt. (Table. 5).

T lymphocytes are involved in both the cellular and humoral immune response and T cell formation is a very important factor. These cells do not secrete the antibody but attack the tissue cells that have been transplanted from one host to other. Therefore, only T cells come into close contact with foreign or infected cell in order to destroy them and to provide cell mediated immunity. Attachment of lymphocytes to foreign or infected cell is called as rosette. MESI showed dose dependant immunomodulatroy activity. In present study increasing doses had shown the significant increase in lymphocytes and rossettes count when compared with control.

MESI showed a significant effect in restoration of haematological parameters when compared with control ($p<0.01$, $p<0.05$), and standard ($p<0.01$) (Table. 6).

Myelosuppression is a decrease in the production of blood cells. Azathioprine is a most potent cytotoxic and immunosuppressive agent which act at various levels on cells involved in defense mechanism against various invaders by inhibiting both cell mediated and humoral immunity. It also causes dose dependant bone marrow suppression means it significantly decreases the Hb, RBC, and WBC counts. Azathioprine treatment for the period of 3 days showed significant reduction in Hb, RBC count and WBC count and thereby exerted immunosuppressant effect when compared to control animals. Combined treatment of extracts and myelosuppressive drug at all doses showed restoration of all haematological parameters when compared to azathioprine treated groups. Levamisole, a standard immunomodulatory drug, has shown marked difference in Hb count, RBC count and WBC count.

The histopathology studies of spleen (Fig. 1 a,b,c,d,e) and thymus gland (Fig. 2 a,b,c,d,e) further conformed the immunostimulatory activity of the methanolic extract of Sphaeranthus indicus. The methanolic extract of Sphaeranthus indicus had shown the significant protective effect from SRBC indicated by mild atrophy and lymphoid depletion in the spleen and thymus gland. Even there was moderate follicular atrophy in the cortical region of the thymus gland. Its effect was comparable to that of standard levamisole. Earlier studies reported that saponins, flavonoids and glycosides are most likely candidates eliciting the immunostimulatory activity. Hence the immunostimulant activity of the methanolic extract of Sphaeranthus indicus can be attributed to the phenols, flavonoids, glycosides and saponins present in it.

IV. Conclusion

Extensive literature survey revealed the presence of flavonoids, glycosides, steroids and saponins in Sphaeranthus indicus, are well established for their antioxidant, anti-inflammatory, analgesic properties. Methanol extract of Sphaeranthus indicus also showed potential effect on haemopoetic system. Immunomodulatory potential of Sphaeranthus indicus could be attributed for the presence of flavonoids, polyphenols and terpenoids which may modulate and potentiate humoral as well as cellular immunity. This emphasizes the future scope of this study.

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Table 1: Effect of methanolic extract of Sphaeranthus indicus for in vitro antioxidant assays

Test extract	Reducing power assay IC_{50} value (µg/mL)	H_2O_2 scavening assay IC_{50} value (µg/mL)
MESI	35	27
Ascorbic acid (standard)	31	24

Values are expressed as mean ± SEM

Table 2: Effect of methanolic extract of Sphaeranthus indicus for in vitro immunomodulatory assays

Groups	Treatment	PFC×10^6 cells	O.D ×10×10^6
I	Control	540 ±3.395	0.661±0.0054
II	MESI 100 mg/kg	655.33±4.957 ^{**,b}	0.769±0.01004 ^{**,b}
III	MESI 200 mg/kg	620.83±3.70 ^{**,a}	0.745±0.0017 ^{**,a}
IV	MESI 400 mg/kg	563±1.366 ^{**,a}	0.707±0.0127 ^{**,a}
V	Standard	678.833±1.701 **	0.88±0.1124 **

Values are expressed as mean± SEM, (n=6). All the groups were compared with control and standard groups using Dunnett's t- test. Significant values are expressed as control (**=p<0.01), standard (a=p<0.01, b=p<0.05).

In vivo immunomodulatory models

Table 3: Haemagglutination titer test for methanolic extract of Sphaeranthus indicus whole plant

Groups	Treatment	Antibody titre mean±SEM
I	Control	1.33±0.21
II	MESI 100 mg/kg	6.166±0.307^{**,a}
III	MESI 200 mg/kg	4.16.±0.307^{**,a}
IV	MESI 400 mg/kg	3.16±0.307^{**,a}
V	Standard	7.56±0.333**

Values are expressed as mean± SEM, (n=6). All the groups were compared with control group and standard group. (Dunnett's t- test). Significant values are expressed as control (**=p<0.01) and standard (a=p<0.01).

Table 4: Delayed type hypersensitivity of methanolic extract of Sphaeranthus indicus whole plant

Groups	Treatment	DTH Response (mm) 24 h mean±SEM	DTH Response (mm) 48 h mean±SEM
I	Control	0.2453±0.0053	0.243± 0.0053
II	MESI 100 mg/kg	0.564±0.0317^{**,a}	0.507±0.0236^{**,a}
III	MESI 200 mg/kg	0.730±0.0253^{**,a}	0.661±0.0335^{**,a}
IV	MESI 400 mg/kg	1.302±0.0185^{**,a}	1.166±0.0592^{**,a}
V	Standard	1.661±0.0435**	1.533± 0.0269**

Values are expressed as mean± SEM, (n=6). All the groups were compared with control and standard groups using Dunnett's t- test. Significant values are expressed as control (**=p<0.01), standard (a=p<0.01).

Table 5: Lymphocytes and rosettes count for methanolic extract of Sphaeranthus indicus whole plant

Groups	Treatment	Lymphocyte count mean± SEM	Rosettes count mean± SEM
I	Control	131±1.932	10.00±0.365
II	MESI 100 mg/kg	150.833±1.641^{**,a}	15.616±0.477^{**,a}
III	MESI 200 mg/kg	159.166±0.090^{**,a}	17.833±0.654^{**,a}
IV	MESI 400 mg/kg	169±1.033^{**,a}	20±0.577^{**,a}
V	Standard	171±1.713**	22±0.856**

Values are expressed as mean± SEM, (n=6). All the groups were compared with control and standard groups using Dunnett's t- test. Significant values are expressed as control (**=p<0.01), standard (a=p<0.01).

Table 6: Haematological parameters for methanolic extract of Sphaeranthus indicus whole plant (Drug induced myelosuppression)

Groups	Treatment	WBC count ($\times 10^3$ /mm^3) mean±SEM		RBC count ($\times 10^6$ /mm^3) mean±SEM		Hb count (g/dL) mean±SEM	
		0 day	14th day	0 day	14th day	0 day	14th day
I	Control	9.82±0.214	9.68±0.428	12.1±0.181	12.0±0.23	10.23±0.2	10.08±0.23
II	Negative control	9.01±0.311	4.7±0.39^{**,a}	11.2±0.248	5.6±0.24^{**a}	9.39±0.34	6.1±0.19^{**,a}
III	MESI 100 mg/kg	9.80±0.301	7.5±0.36^{**,a}	11.3±0.158	9.4±0.46^{**,a}	9.91±0.31	8.8±0.27^{**,a}
IV	MESI 200 mg/kg	10.24±0.21	8.3±0.20^{**,a}	12.8±0.124	9.6±0.31^{**,a}	10.5±0.34	8.9±0.18^{*,a}
V	MESI 400 mg/kg	10.47±0.19	9.6±0.207^{*,a}	14.1±0.255	12.6±0.2^{*,a}	11.23±0.2	9.6±0.32^{*,a}
VI	Standard	11.285±0.2	11.9±0.28**	14.81±0.34	15.1±0.3**	11.4±0.18	11.9±0.2**

Values are expressed as mean±SEM, (n=6). All the groups were compared with control and standard groups using Dunnett's t- test. Significant values are expressed as control (**=p<0.01, *=p<0.05) standard (a=p<0.01).

Histopathological studies of rat spleen for methanolic extract of Sphaeranthus indicus whole plant

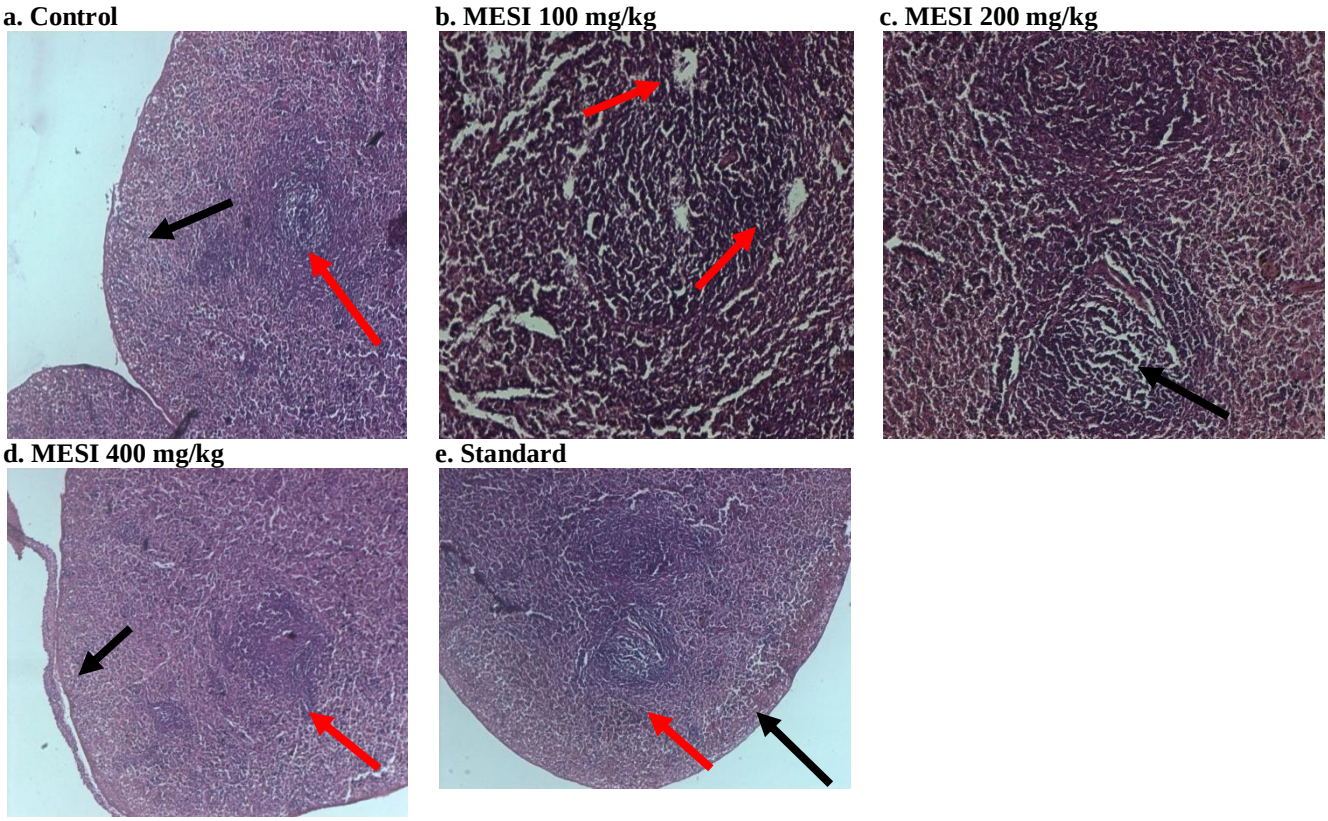

Figure 1. (a-e) rat spleen: In control spleenic cortex (black arrow) and lymphatic follicles (red arrow) appeared normal. In MESI 100 and 200 mg/kg treated group mild to moderate lymphoid depletion noticed in the lymphatic follicles located in the medullary region of spleen. In 400 mg/kg treated group spleenic cortex appeared normal (black arrow) and mild to moderate lymphoid follicular atrophy noticed in few follicles (Red arrow). In standard treated group spleenic cortex appeared normal and it contain large amount of RBCs (black arrow) and medullary region containing lymphatic follicles showed mild atrophy and lymphoid depletion- red arrow.

Histopathological studies of rat thymus for methanolic extract of Sphaeranthus indicus whole plant

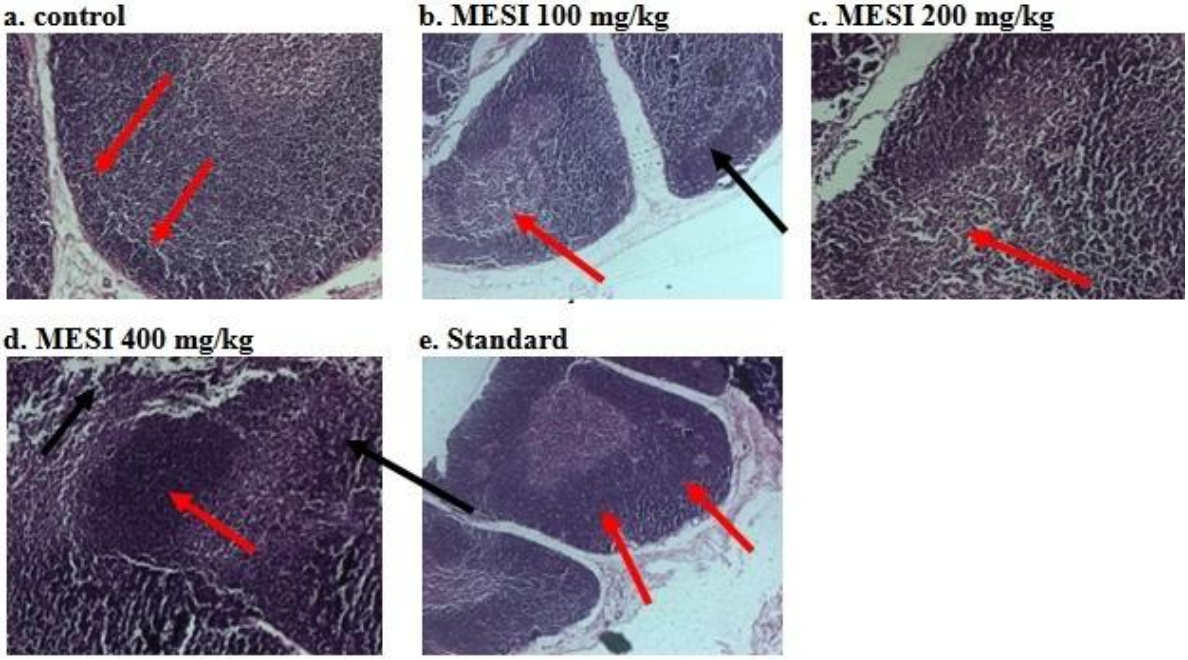

Figure (a-e) rat thymus: In control cortex region of thymus appeared normal (red arrow] it contained pockets/spread of lymphocytes appeared normal. In MESI 100 mg/kg treated group thymic atrophy / fibrosis noticed in the medullary region of thymus (Red arrow) and cortex region containing lymphoid pockets with follicles appeared normal (black arrow). In MESI 200 mg/kg treated group thymic atrophy / fibrosis noticed in the medullary region of thymus (red arrow). In 400 mg/kg treated group medullary region appeared normal (red arrow) and mild to moderate lymphoid cells proliferation or follicular hypertrophy noticed in the cortex region of thymus (black arrow). In standard treated group cortex region appeared normal it contain large amount of lymphocytes appeared as follicles (red arrow).