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In vitro and *in vivo* screening of *Clitoria ternatea* (Linn.) for Immunomodulatory activity

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Abstract : To evaluate immunomodulatory activity for methanolic extract of *Clitoria ternatea* Linn.**Method**: Methanolic extract of *Clitoria ternatea*(MECT) was evaluated for *in vitro* antioxidant assay by reducing power assay and hydrogen peroxide assay and immunomodulatory activity [specific and humoral immunity] at the dose of 100, 200 and 400 mg/kg bd. wt. by various models as plaque forming cell assay, quantitative haemolysis assay and antibody titre (Cell mediated) T cell population, delayed type hypersensitivity and drug induced mylosuppression with antigen challenge by sheep RBCs. **Results**:Methanolic extract of *Clitoria ternatea* at the dose of 100, 200 and 400 mg/kg bd. wt. along with the antigen (sheep red blood cells) showed significant increase in the production of circulating antibody titre and the number of plaque forming cells (PFC) in response to (SRBC's) in the spleen.MECT showed significant ($p<0.01$) increase in the delayed type hypersensitivity response facilitated by footpad thickness response, significantly ameliorated haematological parameters (WBC, RBC and Hb) and also restored the myelosuppressive effects induced by Azathioprine. MECT also showed increase in the levels of lymphocytes and rosettes formation when results were compared with standard as levamisole. MECT showed significant immunomodulatory activity at the dose of 100, 200 and 400mg/kg bd. wt. but amongst 100 mg/kg bd. wt. was found to be potent. **Conclusion**: MECT showed significant immunostimulating activity with specific and non-specific mechanism which may be due to the presence of prominent amount of flavonoids and phenols. So MECT can be used as immunostimulating agent in various disease conditions.
Key words: Immunomodulatory activity, *Clitoria ternatea,* Delayed type hypersensitivity, Antibody, Drug induced myelosuppression.

1. Introduction

Immune system is a remarkably sophisticated defense system within vertebrates, to protect them from invading agents.The basic role of the immune system is to distinguish self from non-self. This non-self could be an infectious organism, a transplanted organ or an endogenous cell that can be mistaken as a foreign[1].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 [2]. Immunomodulatormay be immunosuppressants and immunostimulators. They have the ability to increase an immune response or defend against pathogens or tumors. 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[3]. Immunomodulators are the biological or synthetic substances, which can stimulate, suppress or modulate any of the immune system including both adaptive (humoral and cell mediated) and innate arms of the immune

response[4]. Many proteins, amino acids, and natural compounds have shown a significant ability to regulate immune responses, including interferon-γ (IFN-γ), steroids[5]. The popularity of herbal medicine is increasing due to the perceived tremendous side effects of allopathic and synthetic medicines [6]. Plants secondary metabolites have been implicated for most plants therapeutic activities. Drug discovery from plants involves a multidisciplinary approach combining botanical, ethnobotanical, phytochemical and biological techniques[7].A variety of plant-derived compounds such as polysaccharides, Phenols, peptides, flavonoids, tannins, and sterols have been reported to modulate the immune system[8].The natural immunomodulators act to strengthen weak immune systems, about three quarters of the world population relies on the plants and plant extracts for healthcare. India has an extensive forest cover, enriched with plant diversity[9]. So in the present investigation *Clitoria ternatea* is screened for immunomodulatory activity with various models.

*Clitoria ternatea*is native to tropical equatorial Asia, but has been introduced to Africa, Australia and America. The plant has a wide range of medicinal value and has been used in diabetes, fever, pectoralgia, cough, gastropathy, hernia, hemorrhoids, helminthiasis, dyspepsia, skin diseases and nerve tonic[10]. *Clitoria ternatea* showed immunomodulatory, antidiabetic, antimicrobial, nootropic, anxiolytic, antidepressant, tranquilizer, wound healing action, diuretic and local anesthetic [11]. Phytoconstituents isolated from *Clitoria ternatea* were β-Sistosterol, Kaempferol-3-O-rhamnosyl-(1,6)-galactoside, Taraxerol, Clitorin, Delphidin, p-hydroxycinnamic acid, Taraxerone, arginine, ornithine, histidine,α-aminobutyric acid, ethyl α-D-galactopyranoside, delphinidin 3,3' ,5'α –triglucosides[12].Anti oxident is known that constituents of plants are associated in reducing the risk of many chronic diseases, in which antioxidants play a major role in their protective effects[13] .

2. Materials and Methods

2.1 Plant material collection, authentication and extraction

Clitoria ternatea Linn. (aerial parts) was collected from BHEL, Hyderabad, Telangana state in the month of December 2015, identified and authenticated by Dr. T. Srinivas, (Prof and HOD botany) Govt degree college, Karimnagar. The crude plant material was cleaned, made into small pieces, dried under sun and coarsely powdered. The powdered material was extracted by simple distillation process using methanol as solvent. Filtrate obtained was evaporated to dryness and extract obtained was stored in air tight containers for further use. Methanolic extract of *Clitoria ternatea* was subjected to preliminary phytochemical investingation[13].

2.2Animals used

Wistar albino rats (Approx 150 to 180 g) were procurd 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/PO/Ere/S/08/CPCSEA).

2.3 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.

2.4 Acute toxicity studies

The methanolic extract of *Clitoria ternate* 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*Clitoria ternatea* at doses 2000 and 5000 mg/kg bd.wt. Animals were observed for 14 days for mortality [14].

2.5 Estimation of total phenolic content and total flavonoid content

Total flavonoid content was determined by the aluminium calorimetric method[15], using quercetin as a standard. For the estimation of total phenolic content 2 mL of MECT 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.

Total flavonoid of MEFB was determined using the method of Liu *et al*. In brief, methanolic extract of *Clitoriaternatea*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 potassiumacetate 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[15].

2.6 In vitro antioxidant assay

Reducing Power Assay

1 mL of MECT (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.

Hydrogen Peroxide Assay

The ability of MECT 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). Methanolic extract of *Clitoriaternatea* (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[16].

2.7 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 methanolic extract of *Clitoria ternatea* was dissolved in acacia and distilled water. Doses selected of methanolic extract of *Clitoria ternatea* was 100, 200 and 400 mg/kg body weight. Albino rats were divided into groups containing 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 MECT *p.o.* respectively (1 to 5 days)
Group III received 200 mg/ kg bd.wt. of MECT*p.o.*respectively (1 to 5 days)
Group IV received 400 mg/kg bd,wt. of MECT*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 RPMI-1640 medium. For PFC assay, the SRBC were prepared at a density of 5 x 10^8 cells/ mL in PBS. 1mL 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[17].

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 MECT *p.o*. respectively (1 to 5 days)
Group III received 200 mg/kg bd.wt. of MECT*p.o*. respectively (1 to 5 days)
Group IV received 400 mg/kg bd,wt. of MECT*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 with some modifications. Spleens were removed and a cell suspension of 1 $X10^6$ cells /mL was prepared in PBS. 1mL of 0.2% SRBC and 1 mL of 10% rabbit serum were mixed with cell suspension and incubated for 1h 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)[18].

2.8 In vivo immunomodulatory activities

Antibody (HA) titre response to SRBC

On 0day, all groups were sensitized with 0.1 mL of SRBC containing 1×10^8cells, *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 MECT *p.o.* respectively (1 to 7 days)
Group III received 200 mg/kg bd.wt. of MECT *p.o.* respectively (1 to 7 days)
Group IV received 400 mg/kg bd.wt of MECT *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[19].

Delayed type hypersensitivity

On 0day, 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 MECT *p.o.* respectively (1 to 7 days)
Group III received 200 mg/kg bd.wt of MECT *p.o.*respectively (1 to 7 days)
Group IV received 400 mg/kg bd.wt of MECT *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 verniercallipers (Mitutoyodigimatic). Then animals were challenged by injecting 1% SRBC (20 µL) into the right hind footpad. On 8thand 9thday footpad thickness was again measured. Difference between prior and post challenge footpad thickness was reported as DTH response[19].

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
GroupII- received 100 mg/kg bd.wt. ofMECT *p.o*. respectively (1 to 10 days)
Group III- received 200 mg/kg bd.wt. of MECT *p.o*.respectively (1 to 10 days)
Group IV- received 400 mg/kg bd.wt. ofMECT *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 11thday, 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. 50 µl 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[20].

Drug induced myelosuppression

On 0 day, all groups were sensitized with 0.1 mL of SRBC containing 1×10^8cells, *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, 12thand 13thday).
Group III- received 100 mg/kg bd.wt. ofMECT *p.o*. respectively (1 to 13 days)
Group IV- received 200 mg/kg bd.wt. ofMECT *p.o*.respectively (1 to 13 days)
Group V- received 400 mg/kg bd.wt. ofMECT *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 0thday, blood was withdrawn from retro-orbital plexus of animals of each group and subjected to haematological parameter determination. MECT was administered to Group-III, IV, V and VI from 1 to 13 days. Azathioprine (2 mg/kg, bd.wt) was given to all animals on 11th, 12th and 13th day, 1h after MECT administration except control and standard group. On day 14th blood was withdrawn from retro-orbital plexus and hematological parameters were estimated[21].

2.9 Histopathological study:

Rats were sacrificed by cervical dislocation and their spleen and thymus gland were dissected out and used for histological study. 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.

2.10 Statistical analysis:

Graph Pad prism 3 software was used for statistical analysis of data. All the results were expressed as mean±standard error of mean (SEM), analyzed for ANOVA and Student t-test (Multiple). Differences between groups were considered significant at $p<0.05$, $p<0.01$ levels.

3.Results

Acute toxicity testing: The acute toxicity testing was performed in female animals. The animals treated with different doses of *Clitoria ternatea* 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 *Clitoria ternatea* whole plant was safe up to 2000 mg/kg bd.wt.

The preliminary phytochemical investigation for MECT showed the presence of, flavonoids, phenolics, steroids, tannins, terpenes, alkaloids, carbohydrates. The total phenolic and flavonoid content of methanolic extract of *Clitoria ternatea* was found to be 2.95 µg/mg of gallic acid equivalent and 2.4 µg/mg to quercetin equivalent.

Methanolic extract of *Clitoria ternatea* showed good antioxidant activity by reducing power assay and hydrogen peroxide assay with IC_{50} value 34 and 29 µg/mL respectively. Ascorbic acid was used as reference standard showed the IC_{50} value 27 and 22.5 µg/mL with reducing power assay and hydrogen peroxide assay respectively (Table 1).

Table 1: Effect of methanolic extract of *Clitoria ternatea* for *in vitro* antioxidant assays

Test drug	**Reducing power assay IC50 (µg/mL)**	**Hydrogen peroxide scavenging assay IC50 (µg/mL)**
MEFB	34	27
Ascorbic acid(Standard)	29	22.5

Assay was performed in triplicate, Values are expressed as mean ± SEM

Table2: Effect of methanolic extract of *Clitoria ternatea* for *in vitro* immunomodulatory assays

Groups	Treatment	PFC×106 mean±SEM	OD×10×106 mean±SEM
I	Control	565.83±3.725	0.564±0.0016
II	MECT 100 mg/kg	663.16±5.598**, b	0.767±0.0066^{**,b}
III	MECT 200 mg/kg	628.33±5.679** a	0.745±0.0014^{**,a}
IV	MECT 400 mg/kg	540±3.396^{**,a}	0.673±0.0104^{**,a}
V	Standard	681.66±7.853**	0.904±0.0132**

Values are expressed as mean± SEM, (n=6). All the groups were compared with control group and standard group (Student t- test). Significant values are expressed as control (**=*P* <0.01) and standard (a=*P* <0.05, b=*P* <0.01).

Table 3: Haemagglutination titer test for methanolic extract of *Clitoria ternatea*

Groups	Treatment	Antibody titer Mean ± SEM (n=6)
I	Control	1.33±0.210
II	MECT 100 mg/kg	6.166±0.307^{**,a}
III	MECT 200 mg/kg	4.166±0.307^{**,a}
IV	MECT 400 mg/kg	3.166±0.307^{**,a}
V	Standard	7.666±0.333**

Values are expressed as mean± SEM, (n=6). All the groups were compared with control group and standard group (Student t- test). Significant values are expressed as control (**=*P* <0.01), standard (a=*P* <0.01).

Table 4: Delayed type hypersensitivity of methanolic extract of *Clitoriat ernatea*.

Groups	Treatment	DTH Response (mm) 24 h mean±SEM	DTH Response (mm) 48 h mean±SEM
I	Control	0.247±0.0052	0.247±0.0052
II	MECT 100mg/kg	0.566±0.0338**, a	0.466±0.0265**, a
III	MECT 200mg/kg	0.744±0.0292**, a	0.660±0.0333**, a
IV	MECT 400 mg/kg	1.470±0.0139**, a	1.304±0.0172**, a
V	Standard	1.675±0.0474**	1.533±0.0264**

Values are expressed as mean± SEM, (n=6). All the groups were compared with control group and standard group (Student 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 *Clitoria ternatea*

Groups	Treatment	Lymphocytes count mean±SEM	Rosettes count mean±SEM
I	Control	128±0.210	10.00±0.365
II	MECT 100 mg/kg	134±5.483^{*,a}	15.616±0.477^{**,a}
III	MECT 200 mg/kg	159±0.909^{**,a}	17.833±0.654^{**,a}
IV	MECT 400 mg/kg	171±1.712^{**,a}	20±0.577^{**,a}
V	Standard	187±6.305**	22±0.856

Values are expressed as mean± SEM, (n=6). All the groups were compared with control group and standard group (Student t- test). Significant values are expressed as control (**P* <0.05), (**=*P* <0.01), standard (^{a} *P* <0.01).

Table 6: Haematological parameters for methanolic extract of *Clitoriat ernatea*(Drug induced myelosuppression)

Groups	Treatment	WBC count ($\times10^3$/mm^3) mean±SEM		RBC count ($\times10^6$/mm^3) mean±SEMs		Hb count (g/dL) mean±SEM	
		0th day	14th day	0th day	14th day	0th day	14th day
I	Control	9.82±0.21	9.68±0.42	12.19±0.18	12.03±0.23	10.23±0.27	10.08±0.23
II	Negative control	9.01±0.31	4.75±0.39$^{**, a}$	11.22±0.24	5.61±0.24$^{**, a}$	9.39±0.34	6.17±0.19^{**,a}
III	MECT 100 mg/kg	9.80±0.3	7.59±0.36$^{**, a}$	11.35±0.15	9.42±0.46$^{**, a}$	9.91±0.31	8.83±0.27^{**,a}
IV	MECT 200 mg/kg	10.24±0.21	8.33±0.20$^{**, a}$	12.80±0.12	9.60±0.31$^{*, a}$	10.51±0.34	8.93±0.18^{*,a}
V	MECT 400 mg/kg	10.47±0.19	9.67±0.207$^{ns, a}$	14.10±0.255	12.69±0.214$^{ns, a}$	11.23±0.231	9.65±0.327^{ns,a}
VI	Standard	11.285±0.22	11.97±0.28	14.81±0.340	15.14±0.3090	11.47±0.187	11.92±0.213

Values are expressed as mean± SEM, (n=6). All the groups were compared with control group and standard group (Student t- test). Significant values are expressed as control ($^{*}P$ <0.05, $^{**}P$ <0.01), (ns= non significant), standard (^{a}P <0.01).

Methanolic extract of *Clitoria ternatea* was evaluated for *in vitro* immunomodulatory assay where methanolic extract showed good immunomodulatory activity with plaque forming cell (PFC) and QHS assay (Table 2). MECT was evaluated for *in vivo* immunomodulatory activity with antigen-antibody titer response i.e. haemagglutination titer. MECT showed significant antibody titer, when compared to control ($^{**}P$ <0.01) and standard (a=*P* <0.01) (Table3). MECT showed significant inhibition of delayed type hypersensitivity reaction dose dependently compared with control (**P <0.01) and standard (P <0.01) (Table 4).Methanolic extract of *Clitoria ternatea* showed a significant increase in lymphocytes and rosettes when compared with control ($^{**}P$ <0.01) and standard (^{a}P <0.01). 100 mg/kg bd. wt. was found to be potent dose amongst other doses as 200 and 400 mg/kg bd. wt. (Table 5).MEFB showed a significant effect in restoration of haematological parameters when compared with control ($^{*}P$<0.05, $^{**}P$ <0.01) and standard (^{a}P <0.01)(Table. 6).

Discussion

Number of antibody secreting cells from spleen was determined using plaque forming cell assay. PFC is used to assess potential modulation of the humoral immune response, in which the number of B cells produces SRBC-specific IgM. Methanolic extract of *Clitoria ternatea* showed significant increase in plaque forming cells as compare to standard drug (Levamisole), which indicates immunostimulation 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. The effect for methanolic extract of *Clitoria ternatea* was significant when results were compared to control ($^{**}P$ <0.01) and standard (^{a}P <0.05, ^{b}P<0.01).

Particulate antigen combines with its antibody in the presence of normal saline at a suitable pH and temperature the antigen-antibody complexes forms. The complex is either large clumps or aggregates or agglutinate that are visible and can be seen with unaided eye. Such reactions are called agglutination reactions. If the antigen is an erythrocyte, the term haemagglutination is used. Antibody molecules which are secreted by plasma cells mediate the humoral immune response. This augmentation of the humoral response to SRBC indicated an enhanced responsiveness of the macrophages and T and B lymphocyte subsets involved in antibody synthesis. The maximum serum dilution that shows visible agglutination is considered as antibody titer.

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 humoral mediated immunity involves the interaction of B cells with the antigen and their subsequent proliferation and differentiation into antibody- secreting plasma cells. The effect of methanolic extract of *Clitoria ternatea* was significant when compared to control ($^{**}P$ <0.01) and standard (^{a}P <0.05, ^{b}P<0.01).

The delayed type hypersensitivity was evaluated in the present investigation by inflammation. DTH is an antigen specific and mediated by T cells rather than antibody. DTH reaction is characterized by large influxes of non-specific inflammatory cells, in which the macrophages are accumulated, increases vascular permeability, induce vasodilatation and causes inflammation. MECT showed dose dependant increase in DTH response. As DTH requiresthe specific recognition of a given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines. These in turn increase vascular permeability, induce vasodilatation, macrophage accumulation and activation, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective killing. The T-cell mediated DTH response to SRBC showed a dose-dependent increase due to the treatment with MECT. The significant differences in the foot paw thickness at doses of 100, 200 and 400 mg/kg bd. wt. in DTH response was statistically significant when results were compared to control group ($^{**}P < 0.01$) and standard group ($^{a}P < 0.01$). The drug influences cell mediated immune response in dose dependent manner.

T-Cells come in contact with infected or foreign cell to destroy them and provide cell mediated immunity. Attachment of lymphocytes to foreign or infected cell is called as rosette. As activation of CD4 and CD8 cells influence T-cell mechanism, results increase in T-cell immune response significantly. 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. Methanolic extract of *Clitoria ternatea* showed significant increase in number of lymphocytes and rosette when results were compared with control ($^{**}P < 0.01$) and standard ($^{a}P < 0.01$).

Myelosuppression is a decrease in the production of blood cells. Azathioprine is a immunosuppressive agent which act at various levels on cells which is involved in immune defense mechanism against various invaders by inhibiting both cell mediated and humoral immunity. Azathioprinesignificantly decreases the Hb, RBC, and WBC counts due to the dependent bone marrow suppression. Azathioprine treatment for the period of 3 days showed significant reduction in Hb, WBC count and RBC count and thereby exerted immunosuppressant effect when compared to control animals. Levamisole, a standard immunomodulatory drug, has not shown marked difference in Hb and RBC count, but WBC count was increased. Methanolic extract of *Clitorea ternata* showed significant increase in Hb, RBC and WBC count when results were compared with control($^{*}P<0.05$, $^{**}P < 0.01$), standard ($^{a}P < 0.01$).

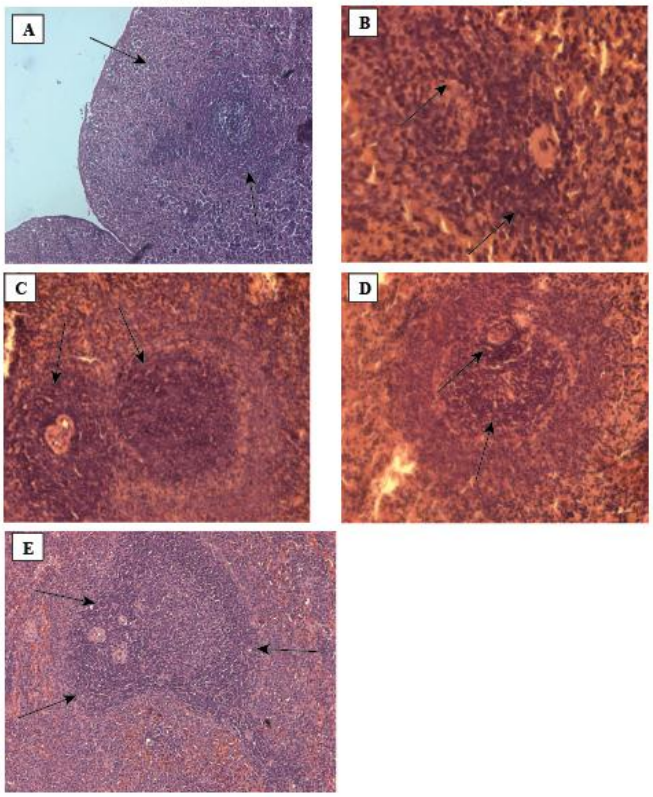

Figure 1: Histopathological studies of rat spleen for methanolic extract of *Clitoria ternatea*

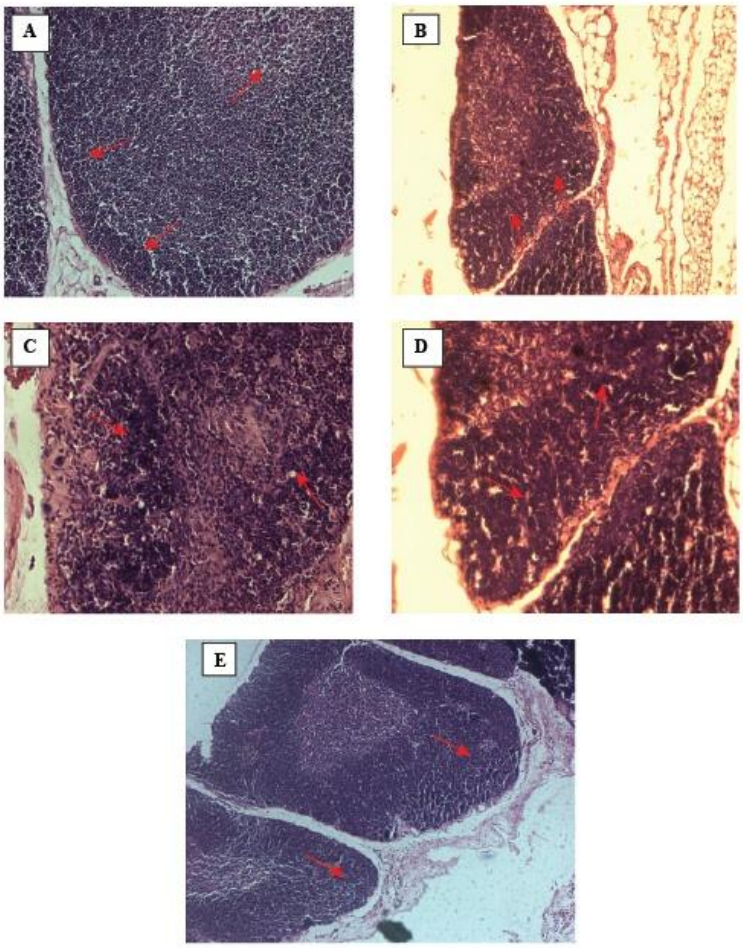

Figure 2: Histopathological studies of rat thymus for methanolic extract of *Clitoria ternatea*

The histopathology studies of spleen (Figure1 A, B, C, D, E) and thymus gland (Figure 2 A, B, C, D, E) further conformed the immunostimulatory activity of the methanolic extract of *Clitoriat ernatea* which had shown the significant protective effect from SRBC indicated by mild hyperplasia along with hypertrophy in the spleen and thymus gland. Even there was moderate follicular hypertrophy in the cortical region of the thymus gland. Its effect was comparable to that of standard levamisole. Earlier studies reported that phenols, flavonoids and glycosides are most likely candidates eliciting the immunostimulatory activity. Hence the immunostimulant activity of the methanolic extract of *Clitoria ternatea* can be attributed to the phenols, flavonoids, glycosides present in it.

References

1. Nagarathna PKM, Reena K, Reddy S, Wesley J. Review on Immunomodulation and Immunomodulatory Activity of Some Herbal Plants. *Int J Pharm PharmSci Res*2013; 22(1):223-30.
2. Patil US, Jaydeokar AV, Bandawane DD. Immunomodulators: A Pharmacological Review. *Int J Pharma PharmaSci*2012; 4(1): 30-36.
3. Manu KA, Kuttan G. Immunomodulatory activities of Punarnavine, an alkaloid from *Boerhaavia diffusa*. *Immunopharma Immunol T.* 2009; 31(3):377-387.
4. Chakraborthy G.S., P. Vikas, N. Kaushik Kailash,. Evaluation of immunomodulatory activity of *Aesculus indica*. *J Pharm Res.* 2009; 2(3): 385-387.
5. Benjamini E., Sunshine G., and Leskwitz S. Immunology a short course, Wiley LissInc New York; 3rd edition. 1996, p. 125.
6. Chang J. Scientific evaluation of traditional Chinese medicine under DSHEA: A conundrum.*J Alt Comple Med.* 1999; 5: 181–189.

7. M De La Fuente and VM Victor. Anti-oxidants and modulators of immune function. *Immunol Cell Biol* 2008; 15(16):754-774.
8. Bodhankar S., N. Makare, V. Rangari. Immunomodulatory activity of alcoholic extract of *Mangifera indica* L. in mice. *J Ethnopharmacol*2001, 78(2): 133-137.
9. Sharma K, Arora V, Rana AC, Bhatnagar M. Anxiolytic effect of *Convolvulus pluricaulis* petals on elevated plus maze model of anxiety in mice. *J Herb MedToxicol* 2009; 3:41-6.
10. Mukherjee, P.K., Wahile, A. Integrated approaches towards drug development from Ayurveda and other Indian system of medicines. *Exp J Chem.* 2006; 103, 25–35.
11. Nagarathna PKM, Reena K, Sriram Reddy, Johnson Wesley. Review on Immunomodulatory Activity of Some Herbal Plants. *Int J Pharm PharmSci Rec*. 2013; 22(1):223-230.
12. Morita, N., Arisawa, M., Nagase, M., Hsu, H., Chen, Y., 1977. Studies on the constituents of *Formosan* Leguminosae I. The constituents in the leaves of *Clitoria ternatea* L. *J Ethanopharmcol*1997, 649–653.
13. Mallick , Anindya Bose , SangeetaMukhi Comparative Evaluation of the Antioxidant Activity of Some commonly used Spices, International Journal of PharmTech Research 2016, 9 (1): 1-8.
14. Khandelwal K.R., Practical Pharmacognosy Techniques and Experiments, Nirali Prakashan, Pune, 2005; pp.30-149.
15. S. P. Pattanayak , P. Mitra Mazumder1 , P. Sunita, Total phenolic content, flavonoid content and in vitro antioxidant activities of Dendrophthoe falcata (L.f.) Ettingsh, International Journal of PharmTech, July-Sept 2011, 3(3) : 1392-1406
16. OECD guideline for testing of chemicals, Acute Oral Toxicity Up-and-Down Procedure., 425: 2001.
17. Singhal M, A Paul, H Pratap Singh, SK Dubey and G Kalpesh. Evaluation of reducing power assay of *Chalcone- semicarbazones.J Chem Pharm Res* 2011, 3(3): 639-645.
18. Nagulendran K, S Velavan, R Mahesh, VH Begum.In vitro Antioxidant activity and total poly phenolic content of *Cyperus rotandus* rhizomes. *Exp J chem*, 2007, 4(3): 440-449.
19. Bilal Bin-Hafeez, Rizwanul Haque, Suhel Parvez, SuwarnaPandey, Iqbal Sayeed, Raisuddin S. Immunomodulatory effects of fenugreek (*Trigonella foenumgraecum* L.) extract in mice. *Int J Immunopharmacol*2003; 3(2): 257–265.
20. Agrawal SS, CK Saurabh, S Gokul. Studies on Immunomodulatory Activity of *Capparis zeylanica* Leaf Extracts. *Int J PharmaSci Nano*. 2010, 3(1):887-892.
21. Verma S, Shaban KA, Purohit R, Chimata LM, RaiG and VermaO. Immunomodulatory activity of *Withania somnifera* (L.). *J Chem Pharm Res* 2012, 4(1): 559-561.
22. Damre AS.,Gokhale AB and Saraf MN. Investigations into the immunomodulatory activity of *Argyreia speciosa*. *J Ethnopharmacol* 2003; 84(1): 109-114.
23. Yadav AK, SinghS. Evaluation of immunomodulatory activity of *Grewia asiatica* in laboratory animals.*J Chem Pharm Res*2014; 6(7): 2820-2826.

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