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IMMUNOMODULATORY ACTIVITY OF AQUEOUS EXTRACTS OF *ENICOSTEMA AXILLARE* LEAVES AND *TODDALIA ASIATICA* ROOT BARK

***BALAJI PANDIYAN, VENKATACHALAM VENKATACHALAM, SIVAKKUMAR THILLAINAYAGAM AND KANNAN KAMARAJAN.**

Department of Pharmacy, Faculty of Engineering & Technology, Annamalai University, Annamalainagar - 608 002, Tamil Nadu, India.

ABSTRACT

Aqueous extracts of leaves of *Enicostema axillare* (200 mg/kg body weight) and root bark of *Toddalia asiatica* (100 mg/kg body weight) were evaluated for cell mediated and humoral immunity immune response in rats. The parameters studied for cell mediated immune response were delayed type hypersensitivity response, neutrophil adhesion test, carbon clearance test and cyclophosphamide induced myelosuppression whereas, humoral mediated immunity was evaluated by its effect on humoral antibody titer and serum immunoglobulins. Aqueous extracts significantly increased the paw thickness, neutrophil adhesion to nylon fibers, phagocytic index and effective in restoring cyclophosphamide-induced myelosuppression, indicating its effect on overall cell mediated immunity. Similarly both extracts exhibited a significant increased level of circulating antibody titer and serum immunoglobulins suggesting an increase in humoral mediated immunity also. The effects exhibited are comparable to that of levamisole at 50 mg/kg dose level.

KEYWORDS: Immunomodulation, *Enicostema axillare*, *Toddalia asiatica*, Delayed type hypersensitivity response, Humoral antibody titer and Cyclophosphamide

***Corresponding author**

BALAJI PANDIYAN
Research scholar, UGC MRP, Department of Pharmacy, Annamalai University, Annamalai Nagar – 608 002.

1. INTRODUCTION

The immune system plays a vital role in protecting the host from invading pathogens. In recent years, many chemical substances both synthetic as well as from plants have been shown to stimulate or suppress the specific or non specific defense mechanism[1]. In Ayurveda a number of medicinal plants, as rasayanas, like *Withania somnifera, Tinospora cordifolia, Asparagus racemosus* and *Mangifera indica* are used as immunomodulatory agent[2]. The immunomodulatory activity may be cell mediated or humoral immunity[3-5]. *Enicostema axillare* (Lam.) A. Raynal (Gentianaceae), Vellarugu in tamil language, is a perennial glabrous herb found throughout India and more commonly in coastal areas. Traditionally *E.axillare* is used to treat diabetes, rheumatism, abdominal ulcers, hernia, hypotensive, swelling, itching and insect poisoning. Hot water extract is used in India for fever, diabetes, stomach ache, dyspepsia and malaria. Other biological activities reported for this plant extracts are anti-inflammatory, antidiabetic, *invitro* antioxidant and antimicrobial, hypolipidemic[6], anticancer[7], and in vitro antiplasmodial activity[8] and immunomodulatory activity[9] *Toddalia asiatica* (L.) Lam. (Rutaceae) Milakaranai in tamil language is an important medicinal plant used for the treatment of a range of diseases like malaria, cough, fever, bronchitis, stomach ache, snake bite, cholera, diarrhoea, rheumatism, indigestion, influenza and lung diseases. Other biological activities reported for this plant extracts are anti-inflammatory, antifeedant, antimicrobial and wound healing, spasmolytic, antimalarial**,** antiplasmodial**,** antidiabetic and diuretic activity. Root bark is used in traditional medicine as analgesic, antipyretic[10]. The plant is reported to be useful in the treatment of malaria, cough, fever, cholera and diarrhea, which are associated with microbial infection and the effect may be immunomodulatory activity.

2. MATERIALS AND METHODS

Leaves of *Enicostema axillare* & Root bark of *Toddalia asiatica* were collected from Tirunelveli district and the above plants were authenticated by Dr. V. Chelladurai, Research Scientist, Botany (Scientist – C), Centre for research on Ayurvedha and Siddha, Palayamkottai, Tirunelveli Dist, Govt. of India. Voucher specimens of all the plants were deposited in the Department of pharmacy, Annamalai University for future reference.

Drugs and Chemicals

Levamisole was procured as gift sample from MMC healthcare Limited, Chennai. Leishmann's stain (Merck, Mumbai India), Cyclophosphamide (Endoxan Injection) was from German Remedies (Mumbai, India), Colloidal carbon (Indian ink, camel India Pvt. Ltd.). All other chemicals used were of analytical grade and high purity.

Experimental animals

Healthy Wistar male rats weighing between 150 – 200 gm (from animal house, Rajah Muthiah Medical College, Annamalai University, Tamilnadu. Reg. No.160/1999/CPCSEA – Proposal No:1026), were kept in polyacrylic cages (Six animals / cage) and maintained under standard housing conditions of temperature (24-27 ^{o}C) and humidity (60%-65%) with 12:12 light: dark cycles. They were fed with standard pellet diet (D.S. Trading Company, Mumbai) and water ad libitum. The animals were habituated to laboratory conditions for 48 hr. prior to the experiment to minimize non specific stress.

Preparation of Extract

Fresh plant material was, washed under running tap water to remove adhering material, dried under shade, pulverized in a mechanical grinder and passed through sieve # 40. The 25g powder of each dried leaves of *Enicostema axillare* (EA), and dried root bark of *Toddalia asiatica* (TA) was extracted separately by boiling with distilled water (1:20, w/v) for 6 hrs and then filtered. Other portions of the distilled water were added to the marc and the extraction was repeated until the last extract was colorless. The combined extract was concentrated in a rotary evaporator at a temperature not exceeding 50ºC. The resulting concentrate was Lyophilised. The yield was 28.48% w/w for aqueous extract of *Enicostema*

axillare (AEEA), 19.04% w/w for aqueous extract of *Toddalia asiatica* (AETA).

Drug preparation

For the present study the oral dose of AEEA and AETA was fixed at 200 mg/kg and 100 mg/kg respectively for 14 days based on the acute toxicity study already reported[11,12].

Preparation of Sheep Red Blood Cells (SRBCs)

Fresh sheep blood was collected from local slaughter house, in a sterile bottle containing sodium citrate 3.8% w/v with a blood to anticoagulant ratio of 9:1 v/v, mixed and centrifuged at 2500-3000 rpm for 10 minutes. SRBCs were separated and washed three times in pyrogen free, sterile normal saline (0.9% Nacl, w/v), centrifuged, the supernatant was discarded and SRBCs cells are counted using Neubauer chamber. SRBC suspension in normal saline was prepared so as to number of cells in 0.1ml was 0.5 x 10^9 cells and 0.025 x 10^9 cells for immunization and challenge respectively.

Grouping of animals

Wistar male rats were randomized into eleven groups of six animals each.

Groups	Treatments
I.	Control (received normal saline – 10ml/kg body weight) for 14 days
II.	Levamisole (50 mg/kg body weight, p.o.) for 14 days – SRBC induced
III.	AEEA (200 mg/kg body weight, p.o.) for 14 days – SRBC induced
IV.	AETA (100 mg/kg body weight p.o) for 14 days – SRBC induced
V.	Levamisole (50 mg/kg body weight, p.o.) for 14 days
VI.	AEEA (200 mg/kg body weight, p.o.) for 14 days
VII.	AETA (100 mg/kg body weight p.o) for 14 days
VIII.	Cyclophosphamide (30 mg/kg, p.o. for 11,12 & 13 day)
IX.	Levamisole 50 mg/kg p.o. for 14 days + Cyclophosphamide (30 mg/kg, p.o. for 11,12 & 13 day)
X.	AEEA (200 mg/kg., p.o.) for 14 days + Cyclophosphamide (30 mg/kg, p.o. for 11,12 & 13 day)
XI.	AETA (100 mg/kg., p.o.) for 14 days + Cyclophosphamide (30 mg/kg, p.o. for 11,12 & 13 day)

Group I to Group IV animals blood samples were collected (0.3 ml) by puncturing the retro orbital plexus and analyzed for Humoral antibody titer, and then the animals were used for SRBC induced Delayed type hypersensitivity response. Group V to Group VII animals blood samples were collected (0.3 ml) by puncturing the retro orbital plexus and analyzed for Neutrophil adhesion test and estimation of serum immunoglobulin levels, and then the animals were used for the carbon clearance test.

SRBC – induced delayed–type hypersensitivity (DTH) Response

All the rats of Group II to group IV were immunized on day 0 by i.p. administration of 0.5 x 10^9 SRBC / rat and on 14^{th} day challenged with subcutaneous (subplantar region) administration of 0.025 x 10^9 SRBC / mL into right hind foot pad. The thickness of the right hind footpad was measured (before and 24 hrs after challenge) using vernier caliper. Footpad reaction was assessed after 24 hrs, (on 15^{th}day) in terms of increase of thickness in footpad due to oedema as a result of an hypersensitivity reaction. The difference in the thickness of the right and the left hind paw was used as a measure of delayed type hypersensitivity (DTH) reaction and expressed as the difference in the thickness (mm.) [13].

Humoral antibody titer

All the rats of Group II to group IV were immunized on day 0 by i.p. administration of 0.5 x 10^9 SRBC / rat. On 14^{th} day, blood was withdrawn from the retro-orbital plexus of all antigenically challenged rats. Twenty-five µl of serum was serially diluted with 25 µl of phosphate-buffered saline. SRBC (0.025x10^9 cells) were added to each of these dilutions in microtiter plates. The plates were incubated at room temperature for 2 hr and examined visually for agglutination. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titer[14].

Neutrophil Adhesion Test

At the end of 14 days Group V to group VII animals, blood samples were collected (before challenge) by puncturing the retro orbital plexus and analyzed for total leucocyte count (TLC) as well as differential leucocyte count (DLC) by fixing blood smears and staining with Leishman's stain. After initial counts, the blood samples were incubated with 80 mg/ml of nylon fibers at 37 °C for 15 min. The incubated blood samples were again analyzed for TLC and DLC. Percenatge of neutrophil adhered to nylon fibre is calculated[15]. The % neutrophil adhesion for each of the test groups was determines as follows:

Neutrophil adhesion (%): **$NI_u - NI_t / NI_u \times 100$** Where NI_u =Neutrophil index of untreated blood sample

NI_t =Neutrophil index of treated blood sample

Carbon clearance assay

At the end of 14 days Group V to group VII animals received an intravenous injection 0.1ml carbon ink (Camel fountain pen ink, black color) dispersion (warmed at 37ºC) via the tail vein. Blood samples were collected from retro orbital bleeding by using glass capillaries at an interval of 3 min and 15 min after the administration of ink dispersion. Every blood samples were added to 4ml of 0.1% sodium carbonate solution to lyse the erythrocytes. Absorbance of these samples was measured at 660 nm using UV-visible spectrophotometer[16].

Phagocytic index (K) was calculated by using following formula

$$\text{Phagocytic index (K)} : \frac{\text{Log OD 3} - \text{Log OD 15}}{\text{T2} - \text{T1}}$$

Effect on serum immunoglobulins

At the end of 14 days Group V to group VII animals blood samples were collected (before challenge) by puncturing the retro orbital plexus. A control tube containing 6 ml of distilled water and a test tube containing 6 ml of zinc sulphate solution was prepared. To each, 0.1 ml of serum was added, mixed and left to stand for 1 hr at room temperature. The first tube served as blank and the second tube was taken as sample. The turbidity developed was measured using a digital nepheloturbidity meter. The turbidity obtained (sample-blank) was compared with that obtained with standard barium sulphate ($BaSO_4$) solution. The standard BaSO4 solution was prepared by adding 3 ml of $BaCl_2$ solution (1.15% w/v) to 97 ml of 0.2N sulphuric acid. The turbidity obtained with this solution was expressed as 20 zinc sulphate turbidity (ZST) units[17].

Cyclophosphamide Induced Myelosuppression

On 11th, 12th, 13th day Group VIII, IX, X and XI were administered with cyclophosphamide (30mg/kg, i.p.) 1hr after administration of the extract or vehicle. On day 14th, blood was withdrawn from retro- orbital plexus of animals of each group and haematological parameter like red blood cells (RBC), haemoglobin (Hb%), platelets (PLT), total white blood cell counts (WBC) and differential leucocytes counts (DLC) were determined[18-20].

Statistical analysis

The data were analysed using one-way analysis of variance (ANOVA), followed by Dunnett's t – test. $P<0.05$ was considered significant.

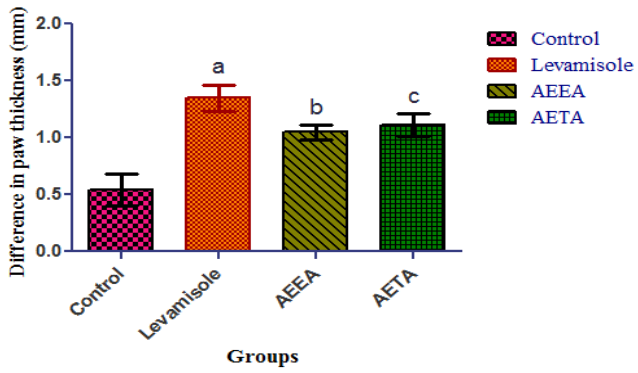
3. RESULTS

Effect of plant extracts on Delayed type Hypersensitivity response (DTH)

The impact of 200mg/kg AEEA and 100mg/kg AETA on T- cell mediated DTH reaction was shown in Figure 1. The difference in paw thickness was measured after 24 hours. In group II where levamisole, as a standard immunostimulant has shown significant increase ($p<0.001$) in the mean difference, in the foot pad thickness as compared to control group. AEEA and AETA administered group exhibited a significant increase ($p<0.05$,

p<0.01 respectively) in DTH response in rats at 24 h when compared with the control group.

Figure 1
Effect of AEEA and AETA on delayed type hypersensitivity in rats

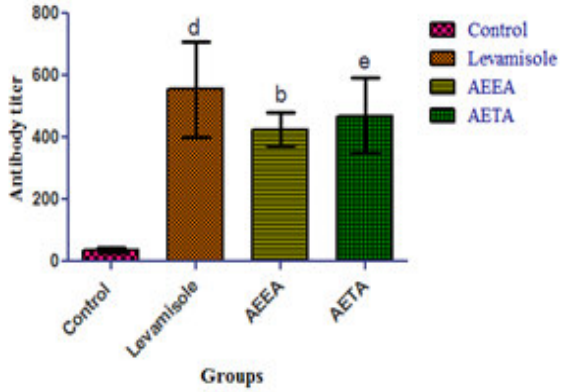


Values are expressed as mean± S.E.M, n=6 in each group,
[a]p<0.001 Levamisole, when compared with Control.
[b]p<0.05 AEEA 200 mg/kg, when compared with Control.
[c]p<0.01 AETA 100 mg/kg, when compared with Control.

Effect of plant extracts on Humoral antibody (HA) titer

From the results was showed in Figure 2, it was observed that there was a significant increase (p<0.01) in the HA titer values of group treated with Levamisole alone (group-II) as compared to control. Both AEEA (group-III) and AETA (group-IV) showed a significant increase (p<0.05) as compared to control group.

Figure 2
Effect of AEEA and AETA on humoral antibody titer in rats

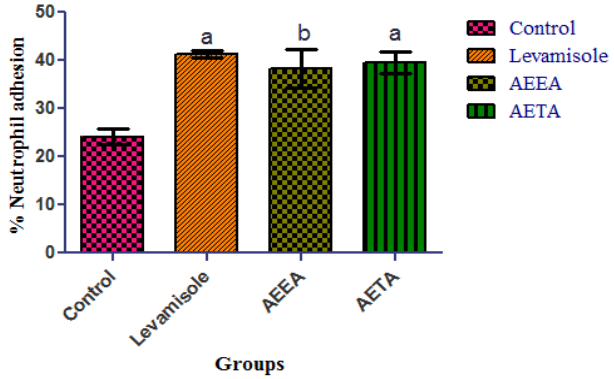


Values are expressed as mean± S.E.M, n=6 in each group,
[a]p<0.01 Levamisole, when compared with Control.
[b]p<0.05 AEEA 200 mg/kg and AETA 100 mg/kg, when compared with Control.

Effect of plant extracts on Neutrophil adhesion test

There was a significant increase in the percent neutrophil adhesion for AEEA 200 mg/kg, AETA 100 mg/kg and the standard Levamisole 50 mg/kg as compared to control group as increases percent neutrophil adhesion value to 38.24 ± 3.98, 39.58 ± 2.24 and 41.26 ± 0.73 respectively which was shown in Figure 3.

Figure 3
Effect of AEEA and AETA on Neutrophil adhesion in rats

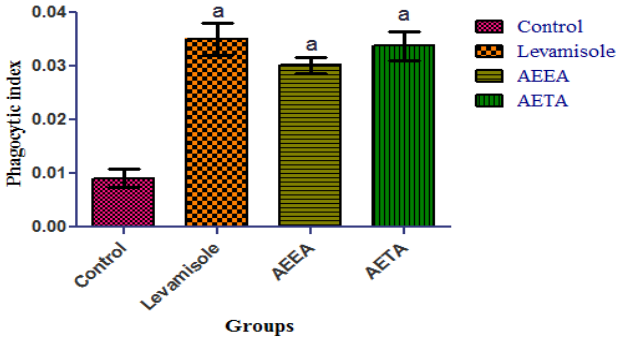


Values are expressed as mean± S.E.M, n=6 in each group,
[a]p<0.001 Levamisole and AETA 100 mg/kg when compared with Control.
[b]p<0.01 AEEA 200 mg/kg, when compared with Control.

Effect of plant extracts on carbon clearance test

The phagocytic activity of AEEA 200 mg/kg, AETA 100 mg/kg and Levamisole 50 mg/kg was showed in Figure 4. The results concluded the increase in the clearance of colloidal carbon from the blood after administration of AEEA, AETA and Levamisole compared to control group, indicating the influence of aqueous extracts and standard on circulating phagocytes.

Figure 4
Effect of AEEA and AETA on Phagocytic index in rats

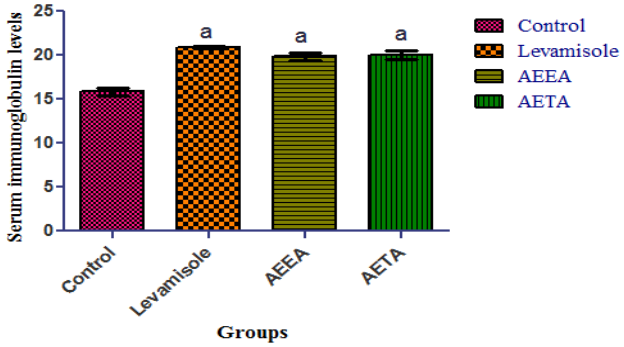


Values are expressed as mean± S.E.M, n=6 in each group,
[a]p<0.001 Levamisole, AEEA 200 mg/kg and AETA 100 mg/kg when compared with Control.

Effect of plant extracts on serum immunoglobulin level

Administration AEEA and AETA showed a significant (P< 0.001) increase in the serum immunoglobulin levels with a dose of 200 and 100 mg/kg/day, respectively in comparison to control group; whereas, levamisole also showed significant increase in the serum immunoglobulin levels (P< 0.001) when compared to control which was shown in Figure 5.

Figure 5
Effect of AEEA and AETA on Serum immunoglobulin levels in rats



Values are expressed as mean± S.E.M, n=6 in each group,
[a]p<0.001 Levamisole, AEEA 200 mg/kg and AETA 100 mg/kg when compared with Control.

Effect of plant extracts on Cyclophosphamide induced myelosuppression

Administration of Cyclophosphamide at the dose of 30 mg/kg (intraperitoneal) caused a significant (p<0.001) reduction in total WBC, RBC and platelets counts, Hb% and HCT% as compared to control group (Group I). A good recovery of total WBC along with Hb% in Levamisole, AEEA and AETA treated cyclophosphamide induced myelosuppressed rats (p<0.001, p<0.01) compared to the negative control (Group II) showed in table 1 and 2. Other hematological parameters RBC, Platelet count and HCT did not show any significant restoration.

Table 1
Effect of aqueous extract of Enicostema axillare and aqueous extract of Toddalia asiatica on cyclophosphamide induced myelosuppression in rats

Groups	WBC (10^3/L)	N (%)	L (%)	E (%)	M (%)	B (%)
Control	9.08 ± 0.26	24.83 ± 1.50	68.27 ± 1.91	1.85 ± 0.15	4.23 ± 0.33	1.012 ± 0.10
Cyclophosphamide (Negative control)	3.63 ± 0.53[a]	13.3 ± 0.36[a]	57.44 ± 0.77[a]	0.45 ± 0.02[a]	2.54 ± 0.22[a]	0.435 ± 0.01[a]
Levamisole + CP	7.58 ± 0.41[b]	21.39 ± 0.48[b]	68.64 ± 0.56[b]	1.37 ± 0.14[c]	4.47 ± 0.16[b]	0.591 ± 0.06[ns]
AEEA + CP	6.77 ± 0.71[b]	19.12 ± 0.79[b]	67.80 ± 1.64[b]	0.76 ± 0.17[ns]	4.42 ± 0.07[b]	0.575 ± 0.05[ns]
AETA + CP	6.03 ± 0.31[c]	17.83 ± 0.64[c]	66.62 ± 1.98[b]	0.77 ± 0.14[ns]	4.38 ± 0.29[b]	0.578 ± 0.03[ns]

Values are expressed as mean± S.E.M, n=6 in each group, [a]p<0.001 CP, when compared with Control.
[b]p<0.001 Lev + CP, AEEA 200 mg/kg and AETA 100 mg/kg, when compared with CP.
[c]p<0.01 Lev + CP, AETA 100 mg/kg, when compared with CP
[ns]- no significant AEEA 200 mg/kg, AETA 100 mg/kg when compared with CP.

Table 2
Effect of aqueous extract of Enicostema axillare and aqueous extract of Toddalia asiatica on cyclophosphamide induced myelosuppression in rats

Groups	RBC (10^6/L)	Hb (g/dL)	HCT (%)	PLT (10^3/L)
Control	7.36 ± 0.22	14.45 ± 0.21	32.86 ± 1.21	694.1 ± 13.06
Cyclophosphamide (Negative control)	6.44 ± 0.09[a]	12.24 ± 0.17[a]	29.62 ± 0.53[d]	671.98 ± 11.25[ns]
Levamisole + CP	7.56 ± 0.15[b]	13.43 ± 0.30[b]	31.50 ± 0.77[ns]	741.9 ± 8.85[ns]
AEEA + CP	6.94 ± 0.28[ns]	13.67 ± 0.17[c]	31.69 ± 0.67[ns]	736.08 ± 9.38[ns]
AETA + CP	7.14 ± 0.36[ns]	13.42 ± 0.14[b]	31.82 ± 0.38[ns]	742.61 ± 10.90[ns]

Values are expressed as mean± S.E.M, n=6 in each group,
[a]p<0.001 CP, when compared with Control.
[b]p<0.01 Lev + CP and AETA 100 mg/kg, when compared with CP.
[c]p<0.001 AEEA 200 mg/kg, Lev + CP and AETA 100 mg/kg when compared with CP
[d]p<0.05 CP, when compared with Control
[ns]- no significant CP vs control and Lev + CP, AEEA 200 mg/kg + CP, AETA 100 mg/kg + CP when compared with CP.

4. DISCUSSION

Enicostema axillare is traditionally used for the treatment of fever and malaria. Similarly *Toddalia asiatica* is used in the treatment of fever, malaria, cholera and influenza. It might be possible that the above activity may be by strengthening the body defense mechanism that is by immunostimulant activity. In addition to these, the methanolic extract of whole plant of *Enicostema axillare* was reported to have immunostimulant effect, whereas leaf extract found to reduce the fever[21] and hence leaf part may have better immunostimulant activity than the whole plant extract. Delayed Type Hypersensitivity is an antigen specific immunological response where the inflammation is produced in response to specific memory T cells[22]. During the inflammation process cytokines are released, chemotaxis is induced and there is increased recruitment of neutrophils & phagocytic cells. In the present study the AEEA and AETA significantly ($p<0.05$ & $p<0.01$) increased paw thickness of the SRBC treated animals compared to control group animals. The result of the AEEA is not in agreement with the findings of the methanolic extract of whole plant[9] Increase in the humoral response is evidenced by an increase in antibody secretion as a result of interaction between antigen and B cells followed by proliferation and differentiation into antibody secreting plasma cells[23]. The results of HA titer test showed that both AEEA and AETA significantly ($p<0.05$) increased the level of circulating antibodies as evidenced by increased antibody titer in albino rats challenged with SRBC, showing that the plant extracts increased humoral immune response. Neutrophils are a short lived immune cells involved in innate immunity. The neutrophilic phagocytic system has several benefits that contribute to clearance of bacteria by both intracellular and extracellular killing, chemotaxis, phagocytosis and thereby enhance immunity[24]. Therefore neutrophil adhesion test was carried out by TLC and DLC before and after treatment with nylon fibre under standardized conditions. In this test, a significant ($p<0.01$ and $p<0.001$ for AEEA and AETA respectively) increase in % neutrophil adhesion was observed when AEEA 200 mg/kg or AETA 100 mg/kg was administered orally to the rats. Carbon clearance test was carried out to evaluate the effect of aqueous extracts on the ability of reticulo-endothelial system (RES) in the removal of carbon particles from the blood stream by phagocytosis[25]. Both AEEA 200 mg/kg and AETA 100 mg/kg increased the phagocytic activity ($p<0.001$) when compared to control animals. The estimation of serum immunoglobulin level could be a direct measure to detect the humoral immunity. In the present study, serum immunoglobulin levels were estimated by using zinc sulphate turbidity test (ZST)[26], where the correlation between the concentration of specific immunoglobulin present in the serum and the intensity of the zinc sulphate reaction. The serum of AEEA 200 mg/kg and AETA 100 mg/kg treated rats showed significantly more level of turbidity ($p<0.001$) which indicates the increase in the serum immunoglobulin level as compared to control group proving that the AEEA and AETA can enhance humoral immunity. Cyclophosphamide is extensively used as immunosuppressant which suppresses humoral, cellular, non specific and specific cellular immune responses[27]. In the present study also the total WBC, RBC and Platelet counts, HCT, hemoglobin [Hb], all decreased significantly ($p<0.001$ and $p<0.05$) as compared to control when treated with the immunosuppressive agent cyclophosphamide. The suppressing effect of cyclophosphamide on WBC and Hb has been restored by the administration of AEEA 200 mg/kg or AETA 100 mg/kg whereas RBC, HCT and platelet counts were not restored. It may be due to the fact that the life span of WBC is 1 day, whereas it is 120 days for RBC and 9 days for platelets[28]. That means generation and degeneration of WBC are very fast when compared to RBC and platelets. Further, it takes 3 – 6 days for the development of fully matured RBC from reticulocytes and the measurement was taken less than 24 hours after administration of cyclophosphamide, there was sufficient time to overcome the effect of cyclophosphamide for regeneration of WBC back to the normal whereas sufficient time was not available for regeneration of

RBC and platelets to restore its normal level. Hemoglobin content will be more which shows the initial stage of proerythroblast production in the mitochondria which matures in to RBC[29]. In all the above studies AEEA and AETA exhibited statistically significant activity when compared to control. However, the activity is not significant when compared to levamisole.

5. CONCLUSION

The AEEA and AETA increase the T lymphocytes, the Phagocytosis activity of macrophages, neutrophil adhesion and also can restore the myelosupperessive condition induced by cyclophosphamide confirming their immune boosting properties through cell mediated immunity. The study also confirms that enhance the circulating antibodies level and B cell production by Humoral immunity. Thus the present study confirms the immunostimulant properties and their potential therapeutic applications of aqueous extracts from leaves of *Enicostema axillare* (AEEA) and root bark of *Toddalia asiatica* (AETA) in the treatment of infectious disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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