Effect of aqueous extracts of *Enicostema axillare* and *Toddalia asiatica* on complement system alternative pathway activity

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Complement system, precisely, the Alternative complement pathway (AP) is the first line of defence against microbial infection. Herbal extracts are known to suppress as well as boost these pathways and have applications accordingly. Here, we explored the effect of aqueous extracts of two commonly used medicinal plants *Enicostema axillare* (Poir. ex Lam.) A.Raynal (AEEA) and *Toddalia asiatica* (L.) Lam. (AETA), on *in vitro* and *in vivo* AP activity and also compared factor B activity with that of the known immunostimulant levamisole. The *in vitro* results revealed a concentration dependent increase in the AP for levamisole, AEEA and AETA. The *in vivo* AP and factor B were studied using rat animal model. The studies were carried out independently using Veronal Buffer Saline (VBS)-AP buffer and Triethanolamine (TEA)-AP buffer to compare the suitability of TEA buffer. The AP activity in terms of concentration of the sample required to cause 50 % hemolysis (APC50) and factor B activity of AEEA and AETA treated animals were significantly (*P* <0.05) higher than control. However, AP 50 was significantly (*P* <0.05) higher for levamisole when compared with that of AEEA or AETA. Significant increase in Factor B activity was observed for AETA, AEEA and levamisole when compared with that of control. Factor B activity of AETA was comparable with that of levamisole (*P* <0.05) where as factor B activity of AEEA treated animal was significantly less than levamisole (*P* <0.05). The results suggest VBS buffer to be more sensitive than TEA buffer.

**Keywords:** Alternative pathway, Complement system, Factor B, Haemolytic activity

Microbial infection is a major health hazard, particularly in developing countries. Living beings protect themselves from infectious diseases by various defence mechanisms collectively known as immune system. Complement system (CS) is a part of immune system, consisting nine different proteins denoted by C1-C9 which are present in human beings and animals in inactive form1-4. These proteins can be activated by three routes: classical pathway (CP), alternative pathway (AP) and lectin pathway (LP). The alternative pathway of the complement system is innate immune system, non-specific and considered as the first line defence against microbial infection5-7. Excessive complement activation is observed in inflammatory condition as well as in autoimmune disease8-10.

Many synthetic agents and herbal extracts have been reported to activate the complement system CP11-13. There are only few reports available on agents that enhance AP activity14,15. Many plants are reported to decrease alternate pathway activity, thereby finding application as immunosuppressant in organ transplantation16,17. There are many reports on use of herbal drugs to increase the AP activity in fish and in fish hatchery to prevent infection18,19.

In ayurvedic system, the rasayanas are used to strengthen the body defence mechanism and many plants have been recommended in alternate system of medicine for the treatment of fever, cough, cold, etc. Herbal preparations exhibiting protective effect against infection may not necessarily have bactericidal/bacteriostatic effect as has been demonstrated earlier using *Caenorhabditis elegans* infection model20. Hence, the herbal drugs used in the treatment of infection may, in fact, strengthen or increase the first line defence mechanism, the alternative complement pathway (AP).

In this study, we evaluated the effect of two medicinal plants, the Indian white head *Enicostema axillare* and the Orange climber *Toddalia asiatica*, commonly used in traditional medicine, on alternative complement pathway activity in search of a potential herbal extract to improve body defence mechanism against infection.
Materials and Methods

Leaves of Enicostema axillare (Poir. ex Lam.) A.Raynal and root bark of Toddalia asiatica (L.) Lam. were collected from Tirunelveli district, Tamilnadu, India and authenticated by the CCRAS-Clinical Research Unit (Sidha), Palayamkottai, Tamilnadu, India. Voucher specimens of the plants have been deposited in the Department of Pharmacy, Annamalai University.

Levamisole, the standard drug was a gift sample from MMC healthcare Limited, Chennai. Other chemicals/drugs and their respective sources were as follows: Cyclophosphamide (Endoxan Injection), German Remedies (Mumbai, India); Triethanolamine (TEA), Qualigens Fine Chemicals, India; and EGTA [ethyleneglycol-bis-(2-aminoethyl ether) N,N,N',N'-tetraacetic acid], Sigma Aldrich, USA. Veronal (VBS-AP) buffer with Mg²⁺ and EGTA containing 10 mM barbital, 145 mM NaCl, 0.5 mM MgCl₂ and 5 mM EGTA, pH 7.4 ± 0.2 was from Boston Bioproducts (Ashland, USA). Anticoagulant, sodium citrate 3.8% w/v solution and Phosphate Buffer saline (PBS, pH 7.2) was from Himedia Laboratories Pvt. Ltd. India. Normal saline was from Baxter (India) Pvt. Ltd. India. All other chemicals used were of GR/AR grade. Human serum (HS) samples were obtained from healthy volunteers.

Experimental animals

Healthy male Wistar rats weighing between 150-200 g (from animal house, Rajah Muthiah Medical College, Annamalai University, Tamilnadu. Reg. No.160/1999/CPCSEA – Proposal No:1026), were kept in polyacrylic cages (@ 6/cage) and maintained under standard housing conditions of temperature (24-27°C) and humidity (60-65%) with 12:12 light:dark cycles. They were fed with standard pellet diet (D.S. Trading Company, Mumbai, India) and water ad libitum. The animals were acclimatized to laboratory conditions for 48 h prior to the experiment to minimize non specific stress.

Preparation of extract

Aqueous extracts of dried leaves of E. axillare and dried root bark of T. asiatica were prepared as described earlier²¹. Briefly, repeated extraction of the powdered plant material with boiling distilled water (1:20, w/v) till colourless extracts was obtained. The combined extract was concentrated in a rotary evaporator and Lyophilised. The yield was 28.48% w/w for aqueous extract of E. axillare (AEEA), 19.04% w/w for aqueous extract of T. asiatica (AETA).

Preparation of 1% Rabbit erythrocytes (RbE)

Fresh Rabbit blood was collected in a sterile bottle with anticoagulant (blood: anticoagulant, 9:1 v/v), mixed and centrifuged at 2000 rpm for 10 min. Supernatant was removed by decantation, cells were washed twice with PBS and twice with VBS-AP buffer (VBS/Mg₂⁺/EGTA). About 1% v/v suspension of RbE in VBS-AP buffer was prepared by suspending washed erythrocytes in VBS-AP buffer.

Similarly, RbE suspension in TEA-AP buffer was prepared by suspending washed erythrocytes in TEA-AP buffer containing 20 mM TEA, 0.15 M sodium chloride (NaCl), 0.8 mM Sodium azide, 8 mM ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid and 2 mM magnesium chloride²².

In vitro alternate pathway haemolytic activity

The assay was performed in flat-bottom 96-well microtitre plates (Tarsons – 941196, Kolkata, India), 5% w/v solutions of Levamisole (Standard drug), AEEA and AETA, in triplicate, were prepared separately in VBS-AP buffer. Further dilutions were made in the micro-centrifuge tubes (1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256) with the VBS-AP buffer resulting in a final volume of 100 µL in each tube and 25 µL of HS was added to each tube. After incubating for 30 min at 37°C, 25 µL RbE suspension was added to each tube and the tubes were incubated at 37°C for 60 min. Subsequently, the tubes were centrifuged at 1000rpm for 6 min. About 50 µL of the supernatant was transferred to a flat-bottom microtiter plate (Tarsons – 941196, Kolkata, India), mixed with 200 µL water and the absorbance was measured at 412 nm in an ELISA automatic plate reader (Multiscan EX, Thermo Fisher scientific, India). Controls in this assay consisted of RBC incubated in distilled water (Total lysis), RBC incubated in buffer (Blank) and the colour of HS-dilution (complement blank). The absorbance of complement blank was subtracted from absorbance values of test sample to get the corrected absorbance of test serum¹⁶,¹⁸,²²-²⁵

Percentage haemolysis for each dilution was calculated by the following formula:

\[
\text{Percentage haemolysis} = \frac{(\text{Corrected absorbance of test sample} - \text{Absorbance of blank})}{(\text{Absorbance of Total lysis} - \text{Absorbance of blank})} \times 100
\]

The percentage hemolysis was plotted against concentration of the drug/extract. The concentration of drug/extract required for producing 50% haemolysis (APC50) was calculated from the graph.
A similar study was carried out using TEA-AP buffer in place of VBS-AP buffer (APC50).

**In vivo complement system AP50**

**Grouping of animals**

Wistar male rats were randomized into four groups of six animals each and treated with drug/extract for 14 days as given in Table 1. At the end of 14 days, blood samples were collected from all animals by puncturing the retro orbital plexus. Serum was separated from blood by centrifugation and 1:25 dilution was made in VBS-AP buffer. Series of dilutions, in VBS-AP buffer, of each 1:25 diluted serum sample (1:10; 1:5; 1:4; 1:3; 1:2; 1:1; 2:1; 3:1; 4:1; 5:1; 10:1; 10:0) was made resulting in final volume of 50 µL in each tube. 50 µL RbE suspensions in VBS-AP buffer was added to each tube. Control tubes consisted of 50 µL RbE for total lysis, 50 µL RbE and 50 µL VBS-AP buffer for blank. Incubated at 37°C for 30 min with intermittent agitation. To all serum tubes and blank tube, 150 µL ice-cold normal saline was added and to total lysis tubes 200 µL distilled water was added. All the tubes were centrifuged at 1000 rpm for 6 min. 200 µL supernatants were transferred to flat-bottomed 96-well plate. Absorbance of the supernatants was measured to 412 nm in ELISA automatic plate reader (Multiscan EX, Thermo Fisher scientific, India). The absorbance of cell supernatants lysed in water was noted as 100% lysis and incubated with VBS-AP buffer was noted as blank (0% lysis). Factor B activity was calculated by subtracting % lysis of test serum from % lysis of test serum with RB. A similar study was carried out using TEA-AP buffer in place of VBS-AP buffer. The data was analysed using one-way analysis of variance (ANOVA), followed by Dunnett’s t – test. P <0.05 was considered significant.

**Results**

The in vitro studies revealed that there is a concentration dependent increase in the AP activity for the standard drug levamisole, AEEA and AETA. The APC50 value was found to be 313.54 ± 7.47, 435.50 ± 7.5 and 404.13 ± 1.67 µg/mL for levamisole, AEEA and AETA, respectively with VBS-AP buffer (Fig. 1A). Similarly with TEA-AP buffer, the APC50 was 343.49 ± 2.17, 493.46 ± 2.85 and 454.40 ± 1.93 µg/mL for levamisole, AEEA and AETA, respectively (Fig. 1B).

In vivo study also exhibited similar results with APD50 value of 0.03 ± 0.01, 7.52 ± 0.05, 2.20 ± 0.08 and 3.15 ± 0.02 for control, levamisole, AEEA and AETA treated groups, respectively with VBS-AP buffer (Fig. 2A) and 0.02 ± 0.01, 5.81 ± 0.37, 1.93 ± 0.18 and 3.04 ± 0.05 for control, levamisole, AEEA and AETA treated groups, respectively with TEA-AP buffer (Fig. 2B).

The percentage lysis was plotted against heating time. Baseline hemolytic activity was obtained when heated up to 3.3 min (210 s). Hence, sera heated at 56°C for 3.3 min was considered as rat serum

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**Table 1** — Grouping and treatment of animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Received normal saline 10 mL/kg body wt. for 14 days</td>
</tr>
<tr>
<td>LEV</td>
<td>Received 50 mg/kg body wt., p.o. for 14 days</td>
</tr>
<tr>
<td>AEEA</td>
<td>Received 200 mg/kg body wt., p.o. for 14 days</td>
</tr>
<tr>
<td>AETA</td>
<td>Received 100 mg/kg body wt. p.o for 14 days</td>
</tr>
</tbody>
</table>
depleted factor B (RB) and used for further studies to evaluate factor B activity (Fig. 3).

The factor B enhancement activity of AETA is comparable with that of levamisole ($P < 0.05$). AEEA factor B enhancement activity is significantly less when compared with levamisole ($P < 0.05$), although it is having significant activity when compared with control ($P < 0.05$) indicating AEEA also has AP enhancement activity and factor B enhancement activity though less than that of levamisole (Table 2).

**Discussion**

Our previous finding revealed that AEEA and AETA have immunostimulant activity$^{21}$ and the work was extended to evaluate their effect on AP activity in terms of haemolytic activity as well as factor B activity, since it is the first line defence against infections. Both *in vivo*$^{11}$ and *in vitro*$^{15,16,26}$

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Fig. 1 — *In vitro* AP activity expressed as a percentage haemolysis using (A) VBS-AP buffer; and (B) TEA-AP buffer. [Values are expressed as mean ± S.E.M, n=6 in each group, *$^a$* $P <0.01$ AEEA and AETA when compared with Levamisole]
Table 2 — Factor B activity

<table>
<thead>
<tr>
<th>Groups</th>
<th>Without factor B depleted serum</th>
<th>With factor B depleted serum</th>
<th>Mean difference in factor B activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VBS</td>
<td>TEA</td>
<td>VBS</td>
</tr>
<tr>
<td>Control</td>
<td>33.55±0.29</td>
<td>27.50±0.20</td>
<td>36.04±0.29</td>
</tr>
<tr>
<td>LEV</td>
<td>78.65±0.45</td>
<td>76.55±0.28</td>
<td>97.73±0.16</td>
</tr>
<tr>
<td>AEEA 200 mg/kg</td>
<td>68.36±0.22</td>
<td>67.66±0.21</td>
<td>83.40±0.45</td>
</tr>
<tr>
<td>AETA 100 mg/kg</td>
<td>73.60±0.27</td>
<td>70.18±0.19</td>
<td>91.51±0.19</td>
</tr>
</tbody>
</table>

Values are expressed as means ± S.E.M, n=6 in each group. *P <0.05 Levamisole, AEEA 200 mg/kg and AETA 100 mg/kg when compared with control.

experimental models have been used to evaluate AP activity. VBS-AP buffer is commonly used for evaluation of complement activity and there are few reports on the use of TEA-AP buffer. However, after inclusion under banned drugs category, procuring VBS-AP buffer has become a difficult task and also became more expensive. Hence, we explored the utility of TEA-AP buffer as a better alternative of VBS-AP buffer.

In agreement with earlier findings, the present study also revealed that levamisole increased the AP activity significantly (P <0.05) when compared to control. Similarly, AEEA and AETA also significantly increased the AP activity but their effects were significantly less than that of levamisole. Both in vitro and in vivo studies exhibited similar effect.

Interestingly, in vitro and in vivo AP enhancement activity of AETA was significantly less than that of levamisole (P <0.05) but factor B enhancement activity of AETA was comparable with that of levamisole (P <0.05). This may be due to the fact that AETA acts specifically only on factor B, whereas levamisole, a well known immunostimulant, may act on many other sites apart from factor B. AETA potentially activating only factor B which leads to cleavage of factor B to Ba and Bb. Bb is involved in the proliferation of pre-activated B lymphocytes, while Ba inhibits their proliferation thereby causing homeostasis of B lymphocyte activity. It is possible that uncontrolled stimulation of AP by levamisole may cause unwanted side effects like inflammatory reactions and autoimmune type of reactions. Thus, AETA might be a better agent to defend against infections without producing inflammatory disorders and autoimmune type of reactions when compared with levamisole.

AEEA factor B enhancement activity was significantly less when compared with levamisole (P <0.05), although it was having significant activity when compared with control (P <0.05) indicating AEEA also has AP enhancement activity and factor B enhancement activity though less than that of levamisole.

The results also revealed that the haemolysis was significantly higher (P <0.05) when VBS-AP buffer was used than with TEA-AP buffer although the final result of AP activity was similar.

From the present study, it can be inferred that the simple in vitro procedure using cost effective TEA-AP buffer can be used for preliminary screening of large number of samples and further confirmation can be done by in vivo studies using sensitive but costlier VBS-AP buffer.

In accordance with widely believed concept that whole plant extract gives a balanced biological effect than a pure synthetic or pure drug from plant, the present study revealed that the root bark extract of *Toddiella asiatica* (AETA) increased Alternative complement pathway (AP) activity compared with the common drug levamisole.

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References


