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ACTIVATION OF COMPLEMENT SYSTEM ALTERNATE PATHWAY BY *ANDROGRAPHIS PANICULATA, OCIMUM SANCTUM* AND *AZADIRACHTA INDICA*

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ABSTRACT

The effect of aqueous extracts of *Andrographis paniculata* (AEAP), *Ocimum sanctum* (AEOS) and *Azadirachta indica* (AEAI) on *in vitro* complement system alternate pathway (AP) activity was evaluated and compared with that of the known immunostimulant levamisole. The results suggest that there is a concentration dependent increase in the AP activity for levamisole, AEOS and AEAI. The activity exhibited by *Andrographis paniculata* was less than that of other herbs studied.

KEYWORDS: Complement system, Alternate pathway, Haemolytic activity, *Andrographis paniculata, Ocimum sanctum and Azadirachta indica.*

INTRODUCTION

Microbial infection is a very serious problem and Living beings protect themselves from infectious organisms by various defense mechanisms called as immune system. Complement system (CS) is a part of innate immune system, consist of nine proteins denoted by C1-C9 which are present in human beings and animals in inactive form.[1, 2] These proteins can be activated by three pathways: classical pathway (CP), alternate pathway (AP) and lectin pathway (LP). The alternate pathway of the complement system is mainly innate immune system and considered as first line defense against microbial infection.[3-5] Excessive complement activation is observed in inflammatory reactions and autoimmune disease.[6,7] Many herbal extracts have been reported to activate the complement system Classical pathway such as *Picrorhiza scrophulariiflora, Tamarindus indica,* Asteraceae family.[8-10]

Whereas there are very few reports on agents that enhance Alternate pathway activity such as Levamisole, Trichosanthin, *Aloe vera, Agaricus blazei* Murill.[11 -14]

Fever is mostly due to microbial infection and large number of herbal drugs is being used for fever. These herbal drugs are might be a cure for fever not only due to antipyretic action but also for the infection. Hence any plant which is used for the treatment of fever can have potential immunostimulating effect including activation of alternate pathway because it is first line defense.

Andrographis paniculata (Kalmegh) is a herbaceous plant in the family Acanthaceae and it has been reported as antibacterial, antifungal, antiviral, choleretic, hypoglycemic, hypocholesterolemic, adaptogenic, anti-inflammatory, emollient, astringent, diuretic, carminative, anthelmintic, antipyretic (Dengue fever), immunosuppressant, gastric and liver tonic. [15, 16]

Ocimum sanctum L. (Tulsi) in the family Lamiaceae and all parts of the plant are known to possess therapeutic potentials and have been used, by traditional medical practitioners, as expectorant, analgesic, anticancer, antiasthmatic, antiemetic, diaphoretic, antidiabetic, antifertility, hepatoprotective, hypotensive, hypolipidmic immunomodulatory and antistress agents. It is also been used in treatment of fever, bronchitis, arthritis, convulsions etc. Aqueous decoction of Tulsi leaves is given to patients suffering from gastric and hepatic disorders. The juice of fresh leaves is also given to patients to treat chronic fever, dysentery, hemorrhage, popular remedy for cold and dyspepsia.[17, 18]

Azadirachta indica (Family: Meliaceae) commonly known as neem and all parts of the tree have been used medicinally for centuries. It has been used in Ayurvedic medicine for more than 4000 years due to its medicinal properties. Hot water extract of the entire plant is used for the treatment of fever, diabetes, insecticide and purgative. It also posses immunostimulant activity, hypoglycaemic activity, antiulcer effect Antifertility effect, Antimalarial activity, Antifungal activity, Antibacterial activity, Antiviral activity and Anticancer activity. [19,20]

Under this background the present work was carried out to evaluate the effect of AP, OS and AI extracts on complement system Alternative pathway activity.

MATERIALS

Leaves of AP, OS and AI were collected from Cuddalore district and the above plants were authenticated by Dr. V. Chelladurai, Research Scientist, Botany (Scientist – C), Centre for research of Ayurveda and Siddha, Palayamkottai, Tirunelveli District, India. Voucher specimens of the plants have been deposited in the Department of Pharmacy, Annamalai University for future reference.

Drugs and Chemicals

Levamisole the standard drug was a gift sample from MMC healthcare Limited, Chennai. Veronal buffer with Mg++ and EGTA containing 10mM barbital, 145 mM NaCl, 0.5 mM $MgCl_2$ and 5 mM EGTA, pH 7.4 ± 0.2] (VBS-AP buffer) was from Boston Bioproducts (Ashland, US). Anticoagulant, sodium citrate 3.8% w/v solution was from Himedia Laboratories Pvt. Ltd. Phosphate Buffer saline (PBS, pH 7.2) was from Himedia Laboratories Pvt. Ltd. India. Normal saline was from Baxter (India) Pvt. Ltd. All other chemicals used were of GR/AR grade. Human serum (HS) samples was collected from healthy volunteers.

EXPERIMENTS AND RESULTS

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 *Andrographis Paniculata*, *Ocimum sanctum*, *Azadirachta Indica* 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 25.2% w/w for aqueous extract of *Andrographis Paniculata* (AEAP), 18.3% w/w for aqueous extract of *Ocimum sanctum* (AEOS), 17.45% w/w for aqueous extract of *Azadirachta Indica* (AEAI).

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 minutes. Supernatant was removed by decantation, cells were washed twice with PBS and twice with VBS – AP buffer

(VBS/Mg_{2+}/EGTA). 1% v/v suspension of RbE in VBS – AP buffer was prepared by suspending washed erythrocytes in VBS – AP buffer.

In vitro alternate pathway haemolytic activity [21-25]

The assay was performed in flat-bottom 96-well microtitre plates (Tarsons - 941196). 5mg / ml solutions of Levamisole (Standard drug), AEAP, AEOS and AEAI, 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. Subsequently, 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 1000 g for 6 min. 50 µL of the supernatant was transferred to flat-bottom microtiter plate (Tarsons - 941196), mixed with 200 µL water and the absorbance was measured at 412 nm in an ELISA automatic plate reader (Multiscan EX). 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 serum to get the corrected absorbance of test serum. Percentage haemolysis for each dilution was calculated by using the following formula:

$$\text{Percentage haemolysis (y)} = \frac{(\text{Corrected absorbance of test serum} - \text{Absorbance of blank})}{(\text{Absorbance of Total lysis} - \text{Absorbance of blank})} \times 100$$

The concentration of drug / extract required for producing 50% haemolysis was calculated from the graph. Percentage lysis was plotted against the drug concentration and the results are given in Table 1 and Figure 1.

Table 1. *In vitro* AP activity expressed as a percentage haemolysis using VBS – AP buffer

NHS	Percentage lysis			
	7.93 ± 0.39			
Concentration (µg/ml)	**LEV**	**AEAP**	**AEOS**	**AEAI**
1000	60.58 ± 0.29	21.82 ± 0.11	53.81 ± 0.33	51.60 ± 0.25
500	53.55 ± 0.29	18.90 ± 0.17	42.40 ± 0.23	40.94 ± 0.23
250	47.37 ± 0.33	16.81 ± 0.12	37.22 ± 0.23	34.64 ± 0.37
125	40 ± 0.33	14.35 ± 0.18	32.72 ± 0.19	30.09 ± 0.19

62.5	31.82 ± 0.29	11.80 ± 0.13	24.33 ± 0.35	23.26 ± 0.37
31.25	23.77 ± 0.29	9.73 ± 0.15	20.72 ± 0.21	17.42 ± 0.29
15.625	16.25 ± 0.30	7.98 ± 0.24	14.33 ± 0.35	12.77 ± 0.35
50% hemolysis	**355.11 ± 9.72**	●	**834.5 ± 10.30***	**873.31 ± 11.32***

● **AEAP increases the activity of Alternate pathway to lesser extent and concentration required for 50% Hemolysis couldn't been found**

Values are expressed as mean ± S.E.M, n=6 in each group, * p<0.01 AEEA and AETA when compared with Levamisole.

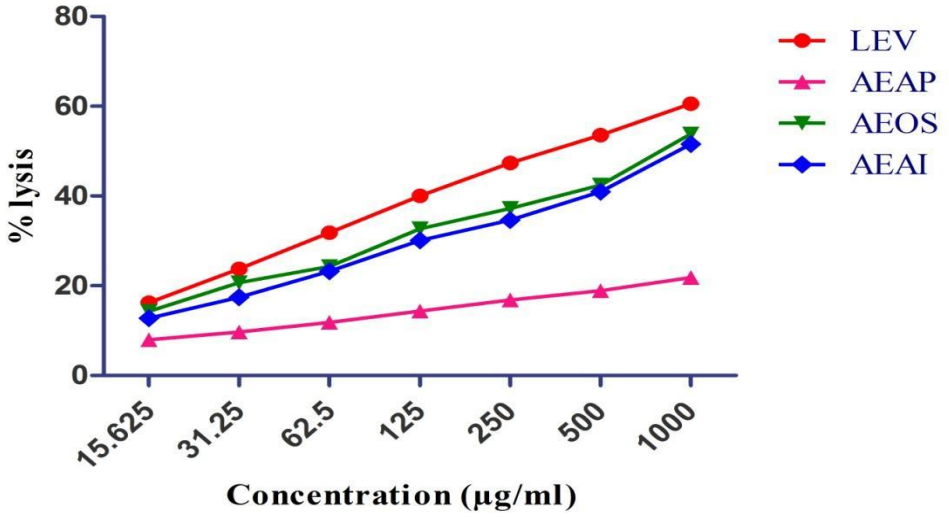

Figure 2. *In vitro* AP activity expressed as a percentage haemolysis using VBS – AP buffer

Statistical analysis

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

DISCUSSION

Ocimum sanctum ([18]) and *Azadirachta indica* ([19]) is traditionally used for the treatment of fever. In addition to this they are reported to have immunostimulant effect. Based on these reports, AEOS and AEAI were further evaluated for their effect of complement system alternate pathway activity and the results were compared with that of levamisole. Traditionally aqueous extracts of herbs in the form of decoction is used in folklore medicines. Hence in the present study the lyophilized aqueous extracts were used and the use of organic solvent was avoided. The *in vitro* studies revealed that there is a concentration dependent increase in the AP activity for the standard immunostimulant drug levamisole (LEV), AEOS

and AEAI. The concentration required for 50% haemolysis was found to be 355.11 ± 9.72 µg/ml, 834.5 ± 10.30 µg/ml and 873.31 ± 11.32 µg/ml for levamisole, AEOS and AEAI respectively with VBS – AP buffer.

In the case of *Andrographis paniculata* it is reported to immunosuppressant activity ([16]) at the same time there are reports that *Andrographis paniculata* is used as antibacterial, antifungal ([15]) and in the treatment of viral infections like dengue fever.

However, AEAP slightly increased the Alternate pathway activity and increase in the activity is dose dependent. It may be due to the fact that AEAP specifically suppress certain adaptive immune system which results in autoimmune disorder stimulated excessively and stimulate innate immune system like complement system alternate pathway activity.

CONCLUSION

In the present study attempt has been made to evaluate the effect of aqueous extracts of *Andrographis paniculata, Ocimum sanctum* and *Azadirachta indica* on *in vitro* complement system alternate pathway activity. There was significant rise in the AP activity for AEOS, AEAI as well as levamisole even at the least dose level 15.625 µg/ml when compared to NHS. However AEOS, AEAI exhibited less activity when compared with levamisole. AEAP exhibited dose dependent increase in the complement system alternate pathway activity though significantly less than that of the other plants studied as well as that of levamisole. There is possibility that AEAP specifically suppress certain adaptive immune system while stimulating innate immune system like complement system alternate pathway activity and the better choice for the treatment of infectious disease without causing autoimmune disorder.

Conflict of interest statement

The authors declare that there is no conflict of interest of any kind.

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