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RESEARCH ARTICLE

ACTIVATION OF COMPLEMENT SYSTEM, ALTERNATE PATHWAY ACTIVITY OF SELECTED HERBAL DRUGS

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ABSTRACT

Objective: To screen the selected herb / herbal preparations for their effect on complement system Alternative Pathway activity. **Methods:** The effect of aqueous extracts of selected eight plants (*Aegle marmelos, Withania somnifera, Centella asiatica, Curcuma longa, Cynodon dactylon, Phyllanthus amarus, Solanum trilobatum, Wedelia chinensis* an herbal preparation (Trikattu churanum) and an active constituent (curcumin) on *in vitro* complement system alternate pathway activity was evaluated and compared with that of known immunostimulant levamisole and negative standard cyclophosphamide. **Results:** The results suggest that there is a concentration dependent increase in the AP activity for levamisole, AETC, AEST, ACCD, AECA, AEPA, AEWS, AEWC and AEAM. Among the samples investigated for all the eight preparations exhibited increased AP activity though different extent when compared to positive control levamisole. Three preparations namely Cyclophosphamide (CYP-negative control), AECL and AECU did not exhibit any effect on Alternate pathway activity. It may be due to the fact that CYP, AECL and AECU suppress the immune system by different mechanisms and not through alternate pathway. Conclusion: In conclusion, the selected herb / herbal preparations stimulating innate immune system like complement system alternate pathway activity and also it is the better choice for the treatment of infectious disease without causing autoimmune disorder.

Keywords: Complement system, Alternate pathway, *in vitro* hemolytic assay, Levamisole, Cyclophosphamide.

INTRODUCTION

Infectious disease is a major concern for healthcare providing agencies, particularly in developing countries and it is mainly managed by using appropriate antibiotics apart from preventive measures including vaccination programmes. Antibiotics have many serious drawbacks like allergic reactions, tolerance, drug resistance, bone marrow suppressing effects, swipe out the good bacteria and tissue damage due to long course which produces reactive oxygen species [1, 2]. Above all, antibiotics can also interfere with the immune system and suppress antigen specific immune response [3, 4].

Hence long term use of antibiotic can cause irreversible damage to immune system and also there is possibility that the immune system may be shunted, in the generations to come, due to natural evolutionary process. Hence it is better to manage microbial infection by strengthening the immune system so that the infection can be taken care of by body defense mechanism itself instead of using antimicrobial agents. Many such preparations have been reported in ayurveda and siddha system of medicines to improve body immune system [5, 6].

Among various immune responses to microbial infection, complement system is a strong feature of innate immunity and alternate pathway is the first line defence against various infectious conditions. Complement system consists of circulating soluble plasma proteins with immediate pattern recognizing ability and instant fight capacity against pathogen, triggering specific cell functions, opsonisation, inflammation and secretion of immunoregulatory molecules [7 – 9].

Many herbal extracts have been reported to activate the complement system Classical pathway such as *Picrorhiza scrophulariiflora, Tamarindus indica, Asteraceae* family [10-12]. Whereas very few reports on agents that enhance Alternate pathway activity, such as Levamisole, Trichosanthin, Aloe vera, *Agaricus blazei* Murill [13-16], have been reported. Excessive complement activation is observed in inflammatory condition as well as in autoimmune disease [17,18].

Under this background the present work was carried out to screen the selected herb / herbal preparations for their effect on complement system Alternative Pathway activity. Those herb / herbal preparations reported to possess immunomodulatory activity, have been selected for the study.

S. No	Plant name / Family / Source	Folklore uses	Activity reported	Active constituents
1	*Aegle marmelos* [19-22]* Family: Rutaceae	Asthma, Anaemia, Fractures, Healing of Wounds, Swollen Joints, High Blood Pressure, Jaundice, Diarrhoea, dysentery, fever, hypoglycemia, febrifuge, hepatitis and analgesic	Contractile activity, Anti-microfilarial activity, analgesic, antipyretic, anti-inflammatory, Anti ulcer, anti convulsant, anti depressant, antifertility anti fungal, hepatoprotective, immunomodulatory ativity.	Skimmianine, Aegeline, Lupeol, Cineol, Citral, Citronella, Cuminaldehyde, Eugenol, Marmesinine
2	*Ashwagandha (Withania somnifera)* [23-25] Family: Solanaceae Purchased from local herbal medicine suppliers and used as such	Best rejuvenative that helps maintain proper nourishment of the tissues, particularly muscle and bones, while supporting the proper function of the adrenals and reproductive system and also it has been recommended for the treatment of various ailments which include Polyarthritis, rheumatoid arthritis, lumbago, painful swellings, spermatorrhoea, asthma,	Anti-inflammatory Activity, Antibiotic Activity, Antitumour Activity, Immunomodulatory Activity, Anti-stress/Adaptogenic Activity, Anticonvulsant Activity, Neuropharmacological Activity, Musculotropic Activity, Anti-oxidant Activity, Anti-ageing Effect, Anti-hyperglycaemic Effect, Macrophage-Activating Effect, Morphine Tolerance and Dependence-Inhibiting Effect,	Withanolides and several sitoindosides

		leucoderma, general debility, sexual debility, amnesia, anxiety neurosis, , scabies, ulcers, marasmus and leucorrhoea	Hepatoprotective Activity.	
3	*Centella asiatica* (L) [26-28]* Family: Apiaceae	CNS disorders like epilepsy, schizophrenia and cognitive dysfunction and it has been additionally used in the management of diarrhoea, cholera, measles, jaundice, leukorrhoea, haematemesis, hepatitis, urethritis, toothache, syphilis, smallpox, neuralgia, rheumatism, toothache and varices	Antipyretic, analgesic, antiinflammatory and immunomodulatory	Asiatcoside, centelloside, madecossoside, thankuniside, isothankunic acid, centellose, asiatic, centellic and madecassic acids and brahmoside, brahminoside, brahmicacid
4	*Curcuma longa* [29-33] Family: Zingiberaceae *Purchased from local herbal medicine suppliers and used as such*	Anti-inflammatory and for the treatment of flatulence, jaundice, menstrual difficulties, hematuria, hemorrhage, and colic	Anti- Inflammatory activity, Wound healing activity, Anti-tumour activity, Antimicrobial, Anticancer activity, Hepatoprotective, Repellent activity, Anti-platelet activity, Antitussive activity, Free radical scavenging and antioxidant activity, Antimelanogenic activity, Anti-nephrotoxic activity and immunosuppressant activity.	Curcumin
5	Curcumin [34] Purchased from Sigma Aldrich	variety of inflammatory conditions and other diseases,	anti-inflammatory, anti-cancer, anti-oxidant, wound healing and anti-microbial effects	-
6	*Cynodon dactylon* (L.) Pers. [35-38]* Family: Poaceae	cough, headache, diarrhea, cramps, epilepsy, dropsy, dysentery, hemorrhage, hypertension, hysteria, measles, snakebite, sores, stones urogenital disorders, tumors, and warts	Antibacterial, antipyretic, immunomodulatory, antiarrhythmic, anthelmintic, anticancer, antiepileptic, antioxidant, antidiabetic, antimicrobial, antiviral and wound healing properties.	β- sitosterol, β-carotene, vitamin C, palmitic acid, triterpenoids, arundoin, friedelin, apigenin, luteolin
7	*Phyllanthus amarus* [39-41]* Family: Euphorbiaceae	diarrhoea, dysentery, dropsy, jaundice, intermittent fevers, urinogenital disorders, scabies and wounds, kidney problems, urinary bladder disturbances, pain, gonorrhea, diabetes and chronic dysentery	Anti-tumour activity, Anti-amnesic, Antinociceptive Activity, Anti-oxidant, Antileptospiral Activity, Antimicrobial, Anticonvulsant, Antidiabetic, Antiinflammatory, antifertility, Nephroprotective and cardioprotective activity, Hepatoprotective effect, Antiviral activity, Immunostimulant activity	Phyllanthine, hypophyllanthine, quercertin, astralgin, quercertrin, isoquercitrin, rutin, phyllanthusiin D, amariin, amarulone, amarinic acid
8	*Solanum trilobatum* Linn [42-43]* Family: Solanaceae	Tuberculosis, respiratory problems and bronchial asthma.	Hepatoprotective activity, antimicrobial activity, antioxidant activity, cytotoxic activity, haemolytic activity, protective effect, immunomodulatory activity and antiinflammatory properties	Sobatum, β-solamarine, solasodine, solaine, glycoalkaloid and diosogenin
9	Trikkattu churanum traditional polyherbal preparations [44-47] containing Piper nigrum L (Piperaceae), Piper longum L. (Piperaceae) and Zingiber officinale Roscoe. (Zingiberaceae) *Purchased from local herbal medicine suppliers and used as such.*	Fever, asthama, cold, cough and other general health disorders.	Immunomodulatory, Anthelmintic, antimicrobial and analgesic activities	Piperine, piperlonguminine, zingerol
10	*Wedelia chinensis (W. chinensis)* (Osbeck) Merill [48-50]* Family: Asteraceae	Osteochondritis dissecans, multiple sclerosis, juvenile arthritis, gouty arthritis, Rheumatic fever, treatment of bites and stings, fever and infection. kidney dysfunction, cold, wound and amenorrhea	Hepatoprotective, wound healing, Anticancer, Immunostimulant, CNS depressant, antioxidant, Adaptogenic and antistress, Sedative, Anti-osteoporotic (post menopausal), Chemopreventive, Analgesic and anti-inflammatory, Androgen suppressing activity, Anthelmintic and febrifuge anticonvulsant, Anti ulcerogenic and mucosal protective agent, Antibacterial & antimicrobial, Insecticidal	Wedlolactone isoflavonoids, bisdesmoside, oleonelic acid and saponin

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 were obtained from healthy volunteers.

Extraction of plant materials

The selected plants were powdered, dried and extracted separately by boiling with distilled water (1:20, w/v) for 30 mts and filtered through muslin cloth. The marc was extracted with further quantities of purified water until the extract was colorless. The extracts were combined and concentrated in a rotary evaporator at a temperature not exceeding 50ºC. The resulting concentrate was Lyophilised and stored in the desiccators until use.

In *vitro* alternate pathway haemolytic activity [7]

Preparation of 1% Rabbit erythrocytes (RbE)

Fresh Rabbit blood was collected in 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.

The assay was performed in flat-bottom 96-well microtitre plates. 5mg / mL solution each of Levamisole (Standard drug), AEEA and AETA were prepared in triplicate in VBS-AP buffer and filtered through whatman filter paper. 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 1000 g for 6 min. 50 µL of the supernatant was transferred to flat-bottom microtiter plate, mixed with 200 µL water and the absorbance was measured at 412 nm in an ELISA automatic plate reader. 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 serum.

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

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

The percentage hemolysis was plotted against concentration of the drug / extract. Further log-log plot also made for the above data and the concentration of drug / extract required for producing 50% haemolysis (APC_{50}) was calculated from log-log plot.

RESULTS

Preparation of extract

25g each of selected powdered herb/herbal preparation was extracted separately by boiling with distilled water (1:20, w/v) for 30 mts and filtered through muslin cloth. The extraction was repeated with fresh distilled water until the extract was colourless. The combined extract was concentrated in a rotary evaporator at a temperature not exceeding 50ºC. The resulting concentrate was lyophilised. In case of curcumin the sample was dissolved in VBS – AP buffer and diluted to get a final concentration. The physical appearance and yield are given in table: 1.

Table 1: Properties of the extracts.

Name of the aqueous extract	Physical appearance	% yield
Aegle marmelos (AEAM)	Greenish yellow	25.12
Withania somnifera (AEWS)	Greenish	19.6
Centella asiatica (AECA)	Dark Greenish	18.2
Cynodon dactylon (AECD)	Yellowish green	20.2
Phyllanthus amarus (AEPA)	Greenish yellow	16.4
Solanum trilobatum (AEST)	Greenish white	16.9
Trikkattu churanum (AETC)	Brownish Red	26.45
Curcuma longa (AECL)	Yellowish	21.55
Wedelia chinensis (AEWC)	Greenish brown	16.4

***In vitro* alternate pathway haemolytic activity**

Preparation of 1% Rabbit erythrocytes (RbE)

Rabbit erythrocyte suspension was prepared in VBS buffer separately from 3ml of rabbit blood was collected in sterile bottle containing 0.33ml of anticoagulant. The *in vitro* alternate pathway hemolytic activity was evaluated and the results are given in table 2 and table 3. The % lysis vs concentration plots are given in figure 1 for VBS – AP buffer. The concentration of drug / extract (APC_{50}) required for producing 50% hemolysis was calculated from the log-log plot of % lysis vs concentration and it was shown in table 4.

Table .2: In vitro AP activity expressed as a percentage hemolysis using VBS – AP buffer.

NHS	Percentage lysis						
	7.93 ± 0.39						
Concentration (µg/mL)	LEV	AETC	AEST	AECD	AECA	AEPA	AEWC
1000	60.58 ± 0.29	52.94 ± 0.34	53.67 ± 0.37	52.14 ± 0.53	54.23 ± 0.36	54.81 ± 0.31	54.11 ± 0.29
500	53.55 ± 0.29	40.97 ± 0.20	41.75 ± 0.38	42.72 ± 0.47	45.30 ± 0.30	49.99 ± 0.26	47.81 ± 0.20
250	47.37 ± 0.33	37.39 ± 0.27	37.88 ± 0.46	37.68 ± 0.51	41.14 ± 0.34	46.22 ± 0.50	43.01 ± 0.19
125	40 ± 0.33	33.16 ± 0.24	33.06 ± 0.24	33.06 ± 0.24	37.56 ± 0.29	40.77 ± 0.30	40.04 ± 0.16
62.5	31.82 ± 0.29	23.86 ± 0.34	23.52 ± 0.40	24.76 ± 0.33	29.36 ± 0.36	31.04 ± 0.17	33.18 ± 0.20
31.25	23.77 ± 0.29	19.61 ± 0.16	19.85 ± 0.20	20.58 ± 0.28	24.93 ± 0.32	23.57 ± 0.38	26.37 ± 0.15
15.625	16.25 ± 0.30	13.45 ± 0.19	13.86 ± 0.36	14.67 ± 0.13	19.00 ± 0.23	15.49 ± 0.23	19.14 ± 0.61

Values are expressed as mean ± S.E.M, n=3 in each group

Table.3: *In vitro* AP activity expressed as a percentage hemolysis using VBS – AP buffer.

NHS	Percentage lysis				
	7.93 ± 0.39				
Concentration (µg/mL)	AEAM	AECL	CYP	AECU	AEWS
1000	54.20 ± 0.35	15.31 ± 0.14	16.90 ± 0.52	11.29 ± 0.15	52.67 ± 0.34
500	48.36 ± 0.15	13.53 ± 0.13	15.91 ± 0.13	9.63 ± 0.07	46.30 ± 0.30
250	43.50 ± 0.18	11.65 ± 0.15	14.28 ± 0.25	7.46 ± 0.09	42.50 ± 0.26
125	40.48 ± 0.24	9.20 ± 0.19	11.58 ± 0.13	5.13 ± 0.08	39.39 ± 0.24
62.5	33.67 ± 0.11	8.23 ± 0.05	10.09 ± 0.09	3.75 ± 0.08	29.65 ± 0.64
31.25	26.76 ± 0.15	6.64 ± 0.08	8.46 ± 0.17	2.43 ± 0.09	22.65 ± 0.20
15.625	19.61 ± 0.33	5.25 ± 0.12	7.95 ± 0.17	1.05 ± 0.10	14.79 ± 0.17

Values are expressed as mean ± S.E.M, n=6 in each group

Fig.1*: In vitro* AP activity expressed as a percentage hemolysis using VBS – AP buffer.

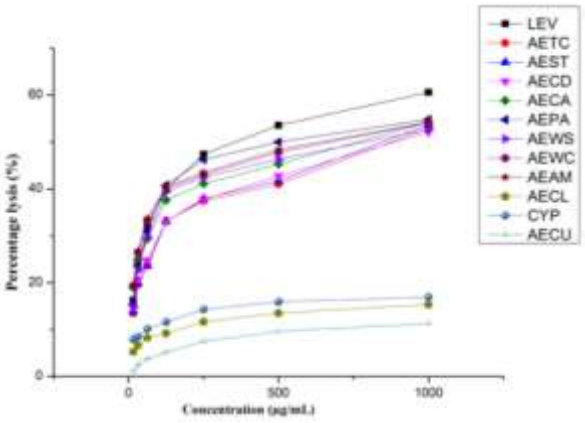

Table. 4: *In vitro* APC_{50} activity using VBS – AP buffer.

Test sample	APC_{50} activity
Levamisole	367.30 ± 4.2
AETC	702.57 ± 5.3 [a]
AEST	703.57 ± 27.49 [a]
AECD	713.69 ± 14.5 [a]
AECA	617.02 ± 16.4 [a]
AEPA	430.34 ± 14.4 [b]
AEWS	528.87 ± 4.07 [a]
AEWC	516.92 ± 7.8 [a]
AEAM	497.46 ± 7.6 [a]

Values are expressed as mean ± S.E.M, n=3 in each group, [a] p<0.01 AETC, AEST, AECD, AECA, AEWS, AEWC and AEAM when compared with Levamisole.[b] p<0.05 AEPA, when compared with Levamisole.

DISCUSSION

Antibiotics are life saving drugs for many clinical health conditions but the drawbacks of using of antibiotics is well documented. Apart from developing of drug resistant, cost and other side effects they also interfering with functioning of immunological system. Hence indiscriminate use of antibiotics may make the immune system less active is subsequent generations due to evolutionary process. Hence the better way of managing infectious diseases by strengthening of body defense mechanism so that the infection will be taken care of by them [1-4].

Immunological response not only neutralized toxic effect of antigens but also produce inflammatory reactions. Excessive stimulation of immune system may cause the unwanted inflammatory reactions and possibly autoimmune disorder. Hence a therapeutic use of immunomodulatory agent should have a balanced activity so that it helps eliminate the infection without inflammatory reactions [17, 18].

Among various immunological responses, complement system AP has distinct advantage of first line defense against invading microbes, being consist of soluble plasma proteins and the response is instantaneous and does not involved immunological memory. Hence agent capable of stimulating alternate pathway selectively could be a better choice to treat infectious disease or used as an adjuvant in the antibiotic therapy. Due to this fact, probably, complement system AP stimulants are stimulate are prevalently used in fish hatchery to prevent infection [51, 52].

Our previous study (under communication) revealed that certain herbs stimulate AP activity selectively by stimulate factor B. Those herbs may be 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. Hence it will not produce inflammatory disorder. These findings encouraged us to investigate the other medicinal herbs for their AP activity.

Among the eight plants, a herbal preparation (Trikattu churanum) and an active constituent (curcumin) investigated for AP activity. In which seven plants and the herbal preparation were reported as immunostimulant whereas *Curcuma longa* and its active constituent (Curcumin) reported to have immunosuppressant effect.

Traditionally aqueous extracts of herbs in the form of decoction is used in folklore medicines. Hence the present study the lyophilized aqueous extracts is used. Use of other organic solvents 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), AETC, AEST, ACCD, AECA, AEPA, AEWS, AEWC and AEAM. The concentration required for 50% haemolysis (APC_{50}) was found to be 367.30 ± 4.2 µg/ml, 702.57 ± 5.3 µg/ml, 703.57 ± 27.49 µg/ml, 713.69 ± 14.5 µg/ml, 617.02 ± 16.4 µg/ml, 430.34 ± 14.4 µg/ml, 528.87 ± 4.07 µg/ml, 516.92 ± 7.8 µg/ml and 497.46 ± 7.6 µg/ml for levamisole, AETC, AEST, ACCD, AECA, AEPA, AEWS, AEWC and AEAM respectively with VBS – AP buffer.

Among the samples investigated for all, the eight preparations exhibited increased AP activity though different extent when compared to positive control levamisole. Three preparations namely Cyclophosphamide (CYP-negative control), AECL and AECU did not exhibit any effect on Alternate pathway activity. It may be due to the fact that CYP, AECL and AECU suppress the immune system by different mechanisms and not through alternate pathway.

Interestingly *curcuma longa* and *curcumin* the well known herb commonly used in alternate system of medicine as antiseptic and antimicrobial agent as well as for wound healing [35], immunosuppressant [33]. It was expected that *curcuma longa* and its active constituent curcumin suppress immune system by various mechanism at the same time they might activate complement system AP activity thereby act as anti-infective agent. Hence this plant (*Curcuma longa*) and its active constituent (Curcumin) were included for the present study. However, contrary to the expectation this plant and its active constituent did not have any effect on complement system AP activity.

CONCLUSION

In the present study attempt has been made to evaluate the effect of aqueous extracts of selected eight plants, an herbal preparation (Trikattu churanum) and an active constituent (curcumin) on *in vitro* complement system alternate pathway activity and compared with that of levamisole and cyclophosphamide. There was significant rise in the AP activity for AETC, AEST, ACCD, AECA, AEPA, AEWS, AEWC and AEAM as well as levamisole even at the least dose level 15.625 µg/ml when compared to NHS. However AETC, AEST, ACCD, AECA, AEPA, AEWS, AEWC and AEAM exhibited less activity when compared with levamisole. AECL and AECU 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 AECL and AECU 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.

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