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Stimulatory effects of *Caesalpinia volkensii* Harm on the innate and Cell mediated immunity in Balb/c mice

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ABSTRACT

Many studies have demonstrated that plant extracts possess various biological and pharmacological properties including immunomodulatory activity. *Caesalpinia volkensii* Harm (Caesalpiniaceae), a medicinal herb native to Kenya was investigated for its immunomodulatory effects. Ethanolic, methanolic and aqueous extracts of the plant was prepared and administered orally in normal albino rats at different concentrations. The effect of C. volkensii on various immunological parameters such as neutrophil adhesion, phagocytosis, nitroblue tetrazolium reduction (NBT) test and macrophage phagocytosis was assessed. The effect of C. volkensii on in vivo cell mediated immunity was also measured by delayed type hypersensitivity (DTH) reaction.The percentage neutrophil adhesion for extract treated animals with 125 to 1000mg/kg of the extracts ranged from 13.3% to 42% showing a significant stimulatory activity. Phagocytic index (PI) for animal treated group was significantly enhanced($P<0.001$). NBT reduction test as well as macrophage phagocytosis revealed that in animal treated groups, these were significantly enhanced. In DTH skin test, the mean footpad thickness of all rat groups treated with 125, 250, 500 and 1000mg/kg of the extract at 24 hours after immunization with antigen was 3.5+0.6mm compared to 2.5+0.5mm for the non treated group. Results of this study showed the ability of C. volkensii extract to strengthen and stimulate the innate immune system and enhance cellular immune response.

INTRODUCTION

The genus Caesalpinia (Caesalpinaceae) comprises many species with a broad distribution in both temperate and tropical regions. In Kenya, many species have been reported [1]. Some of the plants belonging to this family are used in folk medicine to cure malaria, pneumonia, coughs, bronchitis, gonorrhoea and other venereal diseases [1,2]. Examples of such plants are *Bauhinia acuminata*, *B. esculenta, B. rufescens, Brachstegia eurycoma, Cassia hirsuta* and *C. occidentalis* from the family Caesalpinia whose stem, bark, roots, seeds and leaves have been used in the treatment of malaria, coughs, extrusion of guinea worms and as antihelminthic [1,3]. *C. volkensii* is found in five provinces in Kenya namely Rift Valley, Central, Nairobi, Western and Coast [4]. Many medicinal uses of the various parts of this plant has been reported in traditional folklore medicine. The most frequently cited uses are antibacterial, antimalarial and antihelminthic [5]. However, its therapeutic mechanism remain largely unclear. Several possible mechanisms of actions of plant extracts used in ethno medicine have been postulated. It is believed that extracts and compounds from plants could be acting directly on pathogens thereby destroying them. It has also been reported that extracts of plants could be acting by modulating the immune system which would in turn clear these pathogens. It is also possible that plant extracts and compounds could be employing both mode of actions in eliminating pathogens, controlling and preventing disease establishment [6,7,8]. In a study conducted by Chhabra and Uiso [7], crude methanolic extracts of some members of the genus *Caesalpinia* were investigated and demonstrated to lack antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa, Neisseria gonorrhea* as well as *Klebsiella pneumoniae in vitro*. Studies by Ogila *et al* [9] showed that organic and aqueous extracts of *C. volkensii* had negligible or were devoid of antibacterial and antifungal activity. This lack of

antimicrobial activities in the Caesalpinia genus, encouraged us to investigate the possible immunomodulatory activity of *C. volkensii* Harm, a native plant of this genus to Kenya. *C. volkensii* is used for the treatment of infectious diseases in Kenyan traditional medicine. In the present study, in order to explore the possible immunomodulatory activities of *C. volkensii*, the effect of its extracts on innate immune activity and *in vivo* cell mediated immune response in rats was investigated.

MATERIALS AND METHODS

Experimental Animals

Male and female albino rats weighing between 140-210g used in the study were obtained from the animal house in the Department of Zoology, JKUAT. They were housed five per cage, were maintained in animal room under a 12:12-h light-dark cycle at a temperature of 25 °C and fed on rat pellet and tap water *ad libitum*.

Plant materials and their collection

Plant materials were colleted from Gatundu [1°3'0"S: 36°54'0"E] located in Central province of Kenya. Gatundu is approximately 40km north of Nairobi. The leaves, stem and root of *Caesalpinia volkensii* were collected. The plant was identified in the herbarium, Department of Botany JKUAT, where voucher specimen is deposited. The plant materials were dried under shade at temperature below 30°C and pulverized in a hammer mill fitted with a sieve of 0.5mm pore.

Assessment of immunomodulatory activity of the plant extracts

The ability of crude extracts of *C. volkensii* to affect a number of immune functions was assessed in Balb/c mice. This included looking at the ability of extracts to affect, neutrophils, macrophage functions and cellular immunity.

Preparation of extracts for administration in rats

The methanol, ethanol and aqueous extracts of *C. volkensii* leaf, stem and root, were prepared by dissolving in dimethylsulfoxide. All the plant extracts were dissolved so that the final volume of the solutions did not exceed 1ml.

Experimental Groups and Treatment of the rats

Rats were divided into 11 groups of 5 individuals each with two groups serving as controls. The groups were categorized as follow: - Group 1 to 3 rats were treated with methanolic extracts and coded as MCVL, MCVS, MCVR, while groups 4 to 6 were treated with ethanolic extracts of the plant parts and coded as follow, ECVL, ECVS, ECVR. Groups 7 to 9 were treated with aqueous extracts of C. *volkensii* and coded as ACVL, ACVS, ACVR

The 11 groups of experimental animals used in the study were first screened for immunomodulatory activity. Group 10 categorized as the dry control was not manipulated in any way while Group 11 which was taken as treated control was administered with the solvent used to dissolve the extracts, dimethyl sulfoxide. All the other remaining groups were treated with the various crude extracts of *C. volkensii*. The treated groups were dosed orally with the extracts at a dosage of 500mg/kg body weight for three consecutive days. The plant crude extracts were administered through intra gastric route using the stomach tube to ensure the safe ingestion of the extracts and the vehicle.

Evaluation of the effect of crude extracts and fraction isolated from the plants on neutrophils and macrophages activity

Neutrophil Adherence

This was carried out as described by Thakur *et al.* [10]. Briefly, neutrophil adherence was analyzed by the initial count of TLC and DLC from the blood sample. After initial count, blood sample was incubated in sterile nylon fiber column (80mg/ml) packed in a silicanazed Pasteur pipette (column length 15 mm). After a few minutes of incubation, blood sample was again analyzed for TLC and DLC. The product of TLC and percentage of neutrophil gave the neutrophil index (NI) of blood sample.

Percentage of neutrophil adherence was calculated according to the formula described by Thakur *et al.* [10]

(Neutrophil index of untreated blood samples) (neutrophil index of treated blood sample) / (neutrophil index of untreated blood samples) x100 gave the percentage of neutrophil adherence.

Candida Phagocytosis

This was carried out as described by Srikumar *et al.* [11]. Briefly, the phagocytic ability of neutrophil was assessed by separating the buffy coat from the blood sample. To this, the incubating medium, 0.1 ml of inactivated fetal calf serum and 0.1ml of heat killed *Candida albicans* (2×10^8 cell/ml) was added and incubated briefly, followed by centrifugation. From the sediment, thin smears were made and stained with Leishman's stain. The number of neutrophils positive for *Candida* in 100 cells gave phagocytic index (PI).

Nitroblue Tetrazolium (NBT) reduction Test

The killing ability of the neutrophils was assessed by nitroblue tetrazolium reduction test (NBT). Briefly, the blood sample was incubated for a few minutes in a clean glass slide. After incubation, the slide was gently washed with cold saline to remove other cell populations. To this, NBT medium and 0.2ml of inactivated fetal calf serum was added and incubated for 30 minutes at 37° C. After incubation, slide was washed with cold saline and stained with safranin. When NBT was phagosomed by the cells, intracellular dye converted it into an insoluble blue crystalline form (formazon crystals). One hundred cells were observed and the positive cells with the formazon granules counted.

Dose dependent relationship of the most active plant extracts of *C. volkensii* with immunomodulatory activity

Different concentrations of the extracts (125, 250, 500 and 1000mg/kg) were administered orally in rats and the various immunomodulatory tests evaluated as previously described. This involved evaluating the effect of various doses of the plant extracts on nitroblue tetrazolium reduction test, phagocytic index, neutrophil adherence test following oral administration in white albino rats. In addition, phagocytic activity of elicited peritoneal macrophages and delayed type hypersensitivity tests were evaluated in rats to determine how the extracts affected functions of the macrophage as well as cellular immunity.

Determination of phagocytic activity of peritoneal macrophages *in vitro*

Animals were put into various groups and given various doses (125mg/kg, 250mg/kg, 500mg/kg, and 1000mg/kg) of the aqueous, ethanol and methanol plant extracts of the leaf, stem and

root of *C. volkensii*. The phagocytic activity was measured as described by Ottendorfer *et al.* (24). Elicited peritoneal macrophages ($1x10^6$ml) were prepared by injecting the animals with 1ml sterile paraffin oil, intraperitoneally and leaving them for several hours. After harvesting, cells were allowed to adhere for a few hours in fibronectin-coated flat bottomed microwell plates to minimize cell loss during washing procedure. They were then washed and incubated overnight with the plant extracts. Control cells received only complete cell culture medium. After 16 hours, the cells were washed and 10^7 sheep red blood cells (opsonized with anti-forsman-antibody, final dilution 1:500) were added for a short period of time. After vigorously washing, the residual sheep red blood cells were eliminated by short treatment (3 to 5 seconds) with erythrocyte lysis buffer. The number of ingested erythrocytes was determined by spectrophotometric determination of hemoglobin and readings were taken at 550nm.

Dose-dependent relationship of the plant extracts in the delayed type hypersensitivity using SRBC as an antigen

This was done using the method of Thakur *et al*. [31]. Treatment of all groups with extracts began 3 days before challenge. Animals were put into various groups and given various doses (125mg/kg, 250mg/kg, 500mg/kg, and 1000mg/kg) of the aqueous, ethanol and methanol plant extracts of the leaf, stem and root of *C. volkensii*. All the groups were immunized by injecting 20µl of 5×10^9 SRBC per ml subcutaneously into the right foot pad. After 7 days of treatment the thickness of left foot pad was measured using calipers. The mice were then challenged by injecting 20µl of 5×10^9 SRBC per ml intradermally on the left hind foot pad (time 0). Foot thickness was measured before and after 48 h of challenge. The difference between the thickness of left foot just before and after challenge in mm was taken as a measure of immunogen- elicited delayed type hypersensitivity.

Statistical analysis: Data were presented as mean and the differences between groups were assessed using SPSS software and the ANOVA test. P values less than 0.05 were considered significant.

RESULT

The effect of varying the different concentrations of extracts on percent neutrophil adhesion

The results of the effects of various concentrations of the aqueous, ethanol and methanolic extracts of *C. volkensii* leaf, stem and root on percent neutrophil adhesion are presented in table 1. The results are expressed as mean values of % neutrophil adhesion together with their standard errors. As can be seen from the table, the mean values for percent neutrophil adhesion ranged from 13.3% to 22.8% for group of animals treated with 125mg/kg of aqueous, ethanol and methanol extracts. The mean values ranged from 18.7 to 33.4 and 32.4 to 42.8 for animals treated with 250mg/kg and 500mg/kg respectively. The mean values were observed to range from 35.6 to 42.8 for group of animals treated with 1000mg/kg of the aqueous, ethanol and methanol extracts.

In enhancement of percent neutrophil adhesion, the leaf extracts of *C. volkensii* was found to be very effective at all the concentrations followed by the root extracts which was active as from concentration of 250mg/kg. The stem extract was only active at higher doses of 500mg/kg and above. This dose-dependent enhancement was found to be significant for ACVL (ANOVA, F4, 20(1) = 54.2811, P = 0.0000), ACVS (ANOVA, F4, 20(1) = 119.7087, P = 0.0000) and ACVR (ANOVA, F4, 20(1) = 264.9691, P = 0.0000).

An increase in enhancement of percent neutrophil adhesion

Table 1: The effects of increasing concentrations of the various extracts of *C. volkensii* on neutrophil adhesion

Neutrophil Adhesion Test						
	Concentrations					
Plant Extracts	125mg/kg	250mg/kg	500mg/kg	1000mg/kg	Control	P-Value
ACVL	21.82±1.93	33.44±0.57	42.8±0.83	42.8±0.85	14.02±0.57	1.85E-10
ACVS	14.4±0.77	18.78±1.29	32.46±0.7	35.6±1.15	14.02±0.57	1.14E-13
ACVR	16.71±0.61	29.36±1.02	41.12±1.03	41.36±0.61	14.02±0.57	5.14E-17
ECVL	22.28±0.83	34.08±1.14	41.6±0.76	42±0.91	14.02±0.57	6.27E-16
ECVS	13.92±0.87	21.2±1.00	37.38±0.67	39.42±0.39	14.02±0.57	2.23E-17
ECVR	22.88±1.16	33.58±1.18	41.46±0.94	41.44±0.61	14.02±0.57	4.44E-15
MCVL	14.38±0.49	26.34±0.73	36.82±1.16	38.66±0.98	14.02±0.57	6.64E-16
MCVS	13.32±1.34	22.12±0.75	36.28±0.88	38.44±0.73	14.02±0.57	6.05E-16
MCVR	18.62±0.90	23.4±0.96	33.2±085.	36.94±0.57	14.02±0.57	3.58E-15

with increasing doses was observed with ethanolic extracts of *C. volkensii* and this was found to be significant for ECVL (ANOVA, F4, 20(1) = 205.1109, P = 0.0000), ECVS (ANOVA, F4, 20(1) = 288.4429, P = 0.0000) and ECVR (ANOVA, F4, 20(1) = 167.6529, P = 0.0000). With the ethanol extracts of *C. volkensii* leaf, stem and root, the leaf and the root were observed to be more active and an increase in activity of the two extracts that was dose dependent was seen. The stem was only active at higher doses of 500 and 1000mg/kg.

A dose dependent increase in enhancement of percent neutrophil adhesion that was significant was observed with MCVL (ANOVA, F4, 20(1) = 203.9075, P = 0.0000), MCVS (ANOVA, F4, 20(1) = 205.8716, P = 0.0000) and MCVR (ANOVA, F4, 20(1) = 171.6529, P = 0.0000). With the ethanol extracts of *C. volkensii* leaves, stem and roots, the ethanolic extracts from the three parts of the plant were more active starting from a concentration of 250mg/kg and increasing with increasing concentrations though activity was same for the two highest concentrations.

The effect of varying the different concentrations of extracts on neutrophil phagocytic index

The result of the effect of various concentrations of the aqueous, ethanol and methanolic extracts of *C. volkensii* on *Candida* phagocytosis is presented in table 2 below. The results were expressed as mean of phagocytic index together with the standard error of the mean. As depicted in the table, the mean values for phagocytic index ranged from 70.8 % to 74 % for group of animals treated with 125mg/kg of aqueous, ethanol and methanol extracts. The mean phagocytic index values ranged from 73% to 79% and 82.4% to 90.6% for animals treated with 250mg/kg and 500mg/kg respectively. The mean phagocytic index values were observed to range from 85.8% to 91% for group of animals treated with 1000mg/kg of the aqueous, ethanol and methanol extracts. The extracts were not effective at lower concentrations (125 and 250mg/kg) but very effective at higher concentrations of 500mg/kg and 1000mg/kg when the mean phagocytic index values of the extracted treated groups were compared to the control groups.

The aqueous extracts of *C. volkensii* leaf, stem and root were largely active at higher concentrations of 500mg/kg and 1000mg/kg. An increase in enhancement of phagocytic index with increasing doses was observed and this was significant for ACVL (ANOVA, F4, 20(1) = 130.950, P = 0.0000), ACVS (ANOVA, F4, 20(1) = 104. 4538, P = 0.0000) and ACVR (ANOVA, F4, 20(1) = 132.1633, P = 0.0000).

An enhancement of phagocytic index with increasing doses was observed and this was significant for ECVL (ANOVA, F4, 20(1) = 99.1264, P = 0.0000), ECVS (ANOVA, F4, 20(1) = 120.7313, P = 0.0000) and ECVR (ANOVA, F4, 20(1) = 107.2378, P = 0.0000). The ethanolic extracts of *C. volkensii* leaf, stem and root were largely active at higher concentrations of 500mg/kg and 1000mg/kg.

An enhancement in phagocytic index with increasing doses was observed and this was significant for MCVL (ANOVA, F4, 20(1) = 61.3115, P = 0.0000), MCVS (ANOVA, F4, 20(1) = 144.8, P = 0.0000) and MCVR (ANOVA, F4, 20(1) = 49.2582, P = 0.0000). The methanolic extracts of *C. volkensii* leaf, stem and root were found to be active at higher concentrations of 500mg/kg and 1000mg/kg

Table 2: The effect of increasing concentrations of the various extracts of *C. volkensii* on *Candida* phagocytosis

Phagocytic Index						
	Concentrations					
Plant Extracts	125mg/kg	250mg/kg	500mg/kg	1000mg/kg	Control	P-Value
ACVL	72.8±0.73	74.6±0.51	89.6±1.21	91±0.71	73.6±1.08	4.81E-14
ACVS	74±0.55	77.2±0.86	85.2±0.66	89.2±0.66	73.6±1.08	4.17E-13
ACVR	73±0.95	78.8±0.86	90.2±0.58	91±0.71	73.6±1.08	4.41E-14
ECVL	72.2±0.66	77.8±0.58	88.2±1.32	90±0.71	73.6±1.08	6.86E-13
ECVS	72.6±0.60	76.6±0.93	87±0.71	90±0.71	73.6±1.08	1.05E-13
ECVR	73.6±0.60	74.8±0.86	86.8±1.11	89.6±0.40	73.6±1.08	3.25E-13
MCVL	72.4±1.63	74.6±1.17	87.8±0.86	89±0.55	73.6±1.08	6.09E-11
MCVS	70.8±0.66	72.6±0.60	86.2±0.86	89±0.71	73.6±1.08	1.83E-14
MCVR	74.4±1.17	77.2±0.66	84.2±1.02	86.8±0.58	73.6±1.08	4.45E-10

The effects of varying different concentrations of extracts of *A. setaceous* and *C. volkensii* on NBT reduction by neutrophils

The results of the effects of various concentrations of the aqueous, ethanol and methanolic extracts of *A. setaceous* aerial part, root and *C. volkensii* leaf, stem and root on NBT reduction is presented in table 3. The results are presented as mean NBT values together with their standard error. The mean NBT reduction at 125mg/kg to 1000mg/kg of the extract treated group of animals was from 21.2±0.58 to 42±0.55 demonstrating a strong enhancement compared to that of the control with a mean value of 25± 0.71. The enhancement was observed to be dose dependent increasing with increase in the concentration of the extracts although the two higher doses used had almost similar activity. The extracts were not effective at lower concentrations (125 and 250mg/kg) but very effective at higher concentrations of 500mg/kg and 1000mg/kg when compared with controls.

An increase in NBT reduction in a dose dependent fashion was observed in groups of animals treated with aqueous extracts of *C. volkensii* leaf, stem and root. This increment was found to be significant for ACVL (ANOVA, F4, 20(1) = 80.3395, P = 0.0000), ACVS (ANOVA, F4, 20(1) = 28.2272, P = 0.0000) and ACVR (ANOVA, F4, 20(1) = 101.0944, P = 0.0000). Although the lower doses of aqueous extracts of *C. volkensii* leaf, stem and root had slight activity at a concentration of 250mg/kg, they were very active at the higher concentrations used.

An increase in NBT reduction in a dose dependent fashion was observed and this was found to be significant for ECVL (ANOVA, F4, 20(1) = 104.4406, P = 0.0000), ECVS (ANOVA, F4, 20(1) = 104.4846, P = 0.0000) and ECVR (ANOVA, F4, 20(1) = 148.422, P = 0.0000). The ethanolic extracts of *C. volkensii* leaf, stem and root were active at higher concentrations of 500mg/kg and 1000mg/kg with minimal activity observed at a concentration of 250mg/kg while the lowest dose of 125mg/kg used proved to be very ineffective.

An increase in NBT reduction in a dose dependent fashion was observed in group of animals treated with methanolic extracts of *C. volkensii* leaf, stem and root and this was found to be significant for MCVL (ANOVA, F4, 20(1) = 122.7248, P = 0.0000), MCVS (ANOVA, F4, 20(1) = 119.2754, P = 0.0000) and MCVR (ANOVA, F4, 20(1) = 45.1532, P = 0.0000). The methanolic extracts of *C. volkensii* leaf, stem and root were largely active at higher concentrations of 500mg/kg and 1000mg/kg but also showed activity at a dose of 250mg/kg but were ineffective at the lowest dose of 125mg/kg. The leaf extract was the most active, followed by the stem and finally the root extracts.

The effect of extracts of *C. volkensii* and *A. setaceous* on macrophage phagocytic activity

The results of the effects of administration of the aqueous, ethanol and methanolic extracts of *A. setaceous* and *C. volkensii* at various doses on macrophage phagocytic activity are presented in **Table 4**. These values given are the means of the optical density together with their standard errors. The mean values of the extract treated groups were observed to range from 0.19±0.01 to 0.54 ±0.01 for the different doses used. The mean optical value observed for the control group was 0.2±0.01. Comparison analysis indicated that 500mg/kg and 1000mg/kg of all the extracts showed significant difference compared to the control group with their mean values ranging from 0.31±0.01 to 0.54±0.01. The extracts were not effective at lower concentrations (125 and 250mg/kg).

Table 3: The effect of increasing concentrations of the various extracts of A. setaceous and *C. volkensii* on NBT reduction

T Reduction Test						
	Concentration					
Plant Extracts	**125mg/kg**	**250mg/kg**	**500mg/kg**	**1000mg/kg**	**Control**	**P-value**
ACVL	24.2±1.07	30±0.71	38.2±0.58	39.6±0.87	25±0.71	4.96E-12
ACVS	23.4±0.51	26.4±1.08	32±0.71	33.2±0.86	25±0.71	5.65E-08
ACVR	23.6±0.75	31.8±0.58	40.4±1.08	40.4±0.81	25±0.71	5.69E-13
ECVL	24.2±0.58	30.6±0.93	39.4±0.75	40.4±0.81	25±0.71	4.18E-13
ECVS	24.4±0.68	31±0.71	39.2±0.66	40±0.84	25±0.71	3.18E-13
ECVR	24.2±0.37	32.8±0.80	41.2±0.66	41.6±0.81	25±0.71	1.44E-14
MCVL	24.8±0.66	33.2±0.66	38.2±0.58	40.8±0.66	25±0.71	8.97E-14
MCVS	22.6±0.87	29.8±0.86	39±0.71	40.6±0.51	25±0.71	1.18E-13
MCVR	22.2±0.58	23.8±0.58	31±0.71	32.2±0.73	25±0.71	9.70E-10

Table 4: The effects of increasing concentrations of the various extracts of *A. setaceous* and *C. volkensii* on macrophage phagocytic activity

Macrophage Phagocytic Activity						
	Concentration					
Plant Extracts	125mg/kg	250mg/kg	500mg/kg	1000mg/kg	Control	P-value
ACVL	0.20±0.01	0.27±0.01	0.47±0.01	0.51±0.01	0.2±0.01	5.62E-20
ACVS	0.19±0.01	0.23±0.01	0.31±0.01	0.37±0.01	0.2±0.01	5.04E-14
ACVR	0.22±0.01	0.32±0.01	0.43±0.01	0.48±0.01	0.2±0.01	1.44E-16
ECVL	0.21±0.01	0.28±0.01	0.48±0.01	0.53±0.01	0.21±0.01	9.74E-18
ECVS	0.21±0.01	0.23±0.01	0.32±0.01	0.41±0.01	0.21±0.01	9.33E-15
ECVR	0.23±0.01	0.33±0.01	0.47±0.01	0.54±0.01	0.21±0.01	7.43E-18
MCVL	0.19±0.01	0.26±0.01	0.41±0.01	0.5±0.01	0.21±0.01	1.06E-11
MCVS	0.2±0.01	0.23±0.01	0.31±0.01	0.35±0.01	0.21±0.01	1.06E-11
MCVR	0.21±0.01	0.29±0.01	0.42±0.01	0.52±0.01	0.21±0.01	1.09E-17

Table 5: The effects of increasing concentrations of the various extracts of *C. volkensii* on delayed type hypersensitivity

DTH						
	Concentration					
Plant Extracts	125mg/kg	250mg/kg	500mg/kg	1000mg/kg	Control	P-value
ACVL	2.58±0.06	3.08±0.07	3.92±0.06	4.46±0.05	2.42±0.07	8.04E-16
ACVS	2.28±0.08	2.32±0.07	2.66±0.08	3.18±0.01	2.42±0.07	4.33E-07
ACVR	3.06±0.09	3.34±0.07	4.14±0.05	4.34±0.08	2.42±0.07	1.50E-13
ECVL	2.42±0.07	2.78±0.09	4.1±0.11	4.42±0.07	2.42±0.07	3.08E-14
ECVS	2.26±0.05	2.52±0.07	2.86±0.05	0.62±0.02	2.42±0.07	0.00017
ECVR	2.58±0.04	2.84±0.04	3.6±0.04	4.28±0.04	2.42±0.07	2.83E-17
MCVL	2.46±0.05	2.72±0.04	3.64±0.07	4.34±0.05	2.42±0.07	4.11E-16
MCVS	2.34±0.06	2.48±0.05	2.82±0.06	3.26±0.09	2.42±0.07	1.83E-08
MCVR	2.52±0.04	3.06±0.07	3.98±0.06	4.52±0.04	2.42±0.07	3.11E-17

An increase in macrophage phagocytosis that was dose dependent fashion was observed and this was found to be significant for ACVL (ANOVA, F4, 20(1) = 529.2593, P = 0.0000), ACVS (ANOVA, F4, 20(1) = 130.3248, P = 0.0000) and ACVR (ANOVA, F4, 20(1) = 238.4524, P = 0.0000). Aqueous extracts of *C. volkensii* leaf, stem and root were very effective at higher doses of 500mg/kg and above but not at 250mg/kg and below.

For group of animals orally dosed with ethanolic extracts of *C. volkensii* leaf, stem and root, an increase in macrophage phagocytosis that was dose dependent fashion was observed and this was found to be significant for ECVL (ANOVA, F4, 20(1) = 313.8789, P = 0.0000), ECVS (ANOVA, F4, 20(1) = 155.2823, P = 0.0000) and ECVR (ANOVA, F4, 20(1) = 322.6607, P = 0.0000). Ethanolic extracts of *C. volkensii* leaf, stem and root were also only active at higher concentrations with the root and leaves being the most active.

An increase in macrophage phagocytosis that was dose dependent fashion was observed in group of animals treated with methanolic extracts *C. volkensii* leaf, stem and root. This increment was found to be statistically significant for MCVL (ANOVA, F4, 20(1) = 74.0680, P = 0.0000), MCVS (ANOVA, F4, 20(1) = 74.0680, P = 0.0000) and MCVR (ANOVA, F4, 20(1) = 310.3072, P = 0.0000). Methanol extracts of *C. volkensii* leaf, stem and root showed higher activity at higher concentrations of 500 and 1000mg/kg in enhancing macrophage phagocytosis

The effects of extracts of *C. volkensii* and *A. setaceous* on delayed type hypersensitivity (DTH) response in treated rats

The results of the effect of various concentrations of the aqueous, ethanol and methanolic extracts of *A. setaceous* and *C. volkensii* on delayed type hypersensitivity are presented in **Table 5.** The mean footpad thickness of all rat groups treated with 125, 250, 500 and 1000mg/kg of the extracts ranged from 2.28±0.08mm to 4.46±0.05mm compared to 2.42±0.07mm for the control group. The mean footpad thickness of the rats treated with 125mg/kg of the extracts of C. volkensii and A. setaceous ranged from 2.26±0.05mm to 3.06± 0.09mm. The range was 2.34± 0.06mm to 3.34±0.07mm for animals given 250mg/kg and 2.66 ±0.08mm to 4.14± 0.05 mm for animal dosed with 500mg/kg. Foot pad thickness for animals treated with 1000mg/kg aqueous, ethanol and methanol extracts ranged from 0.62± 0.02mm to 4.52± 0.04mm. This indicated that the exposure to higher concentrations of the extracts increased hypersensitivity reaction.

The aqueous extracts of *C. volkensii* leaf and root were very effective in enhancing the delayed type hypersensitivity especially at higher concentration while the stem caused a depression in delayed type hypersensitivity response and this was more depressed at the highest concentration used which was statistically significant (ANOVA, F4, 20(1) = 9.6194, P = 0.0001). Enhancement in DTH by extracts of *C. volkensii* leaf and root observed was dose dependent and this was found to be significant for ECVL (ANOVA, F4, 20(1) = 137.1905, P = 0.0000), and ECVR (ANOVA, F4, 20(1) = 281.611, P = 0.0000).

Methanolic extracts of *C. volkensii* leaf and root were active in enhancing DTH at higher concentrations tested while the stem extract did not have an effect . Enhancement in DTH was dose dependent was observed and this was found to be significant for MCVL (ANOVA, F4, 20(1) = 214.1807, P = 0.0000), MCVS (ANOVA, F4, 20(1) = 32.25, P = 0.0001) and MCVR (ANOVA, F4, 20(1) = 278.8616, P = 0.0000).

Enhancement in DTH that was dose dependent was observed and this was found to be significant for ACVL (ANOVA, F4, 20(1) = 199.9271, P = 0.0000), ACVS (ANOVA, F4, 20(1) = 22.0064, P = 0.0001) and ACVR (ANOVA, F4, 20(1) = 116.2687, P = 0.0000). Aqueous extracts of *C. volkensii* leaf and root enhanced DTH response in treated rats when applied at higher doses. Lower doses were not effective in enhancing DTH response. The aqueous stem extract also was largely ineffective at all the tested concentrations.

DISCUSSION

Immune activation is an effective as well as protective approach against emerging infectious diseases. Basic research on natural substances with immunomodulating properties are performed by stimulating cell of the immune system including neutrophils, macrophages, T and B cell, NK cell [11].Neutrophils are short lived phagocytic cells and are the most abundant circulating granulocytes. Their granules contain powerful microbicidal molecules [13]. A number of functions carried out by neutrophils and macrophages were examined. To carry out their activity effectively, neutrophils need to move from the bloodstream to areas of infections in the tissue. Margination of neutrophils from the bloodstream requires a firm adhesion, which is mediated through the interactions of the β2 integrins present on the neutrophils. The β2 integrin is stored in the cell granules and are up regulated for a firm adherence. Integrins also regulate the vascular development and angiogenesis by promoting proliferation, migration and adhesion of endothelial cells [14]. The adhesion of neutrophils to nylon fibers correlates to the process of migration of cells in blood vessels [15]. Adherence to nylon column was increased in groups treated with MCVL, MCVS, MCVR, ECVL, ECVS and ECVR. All the aqueous extracts were also observed to be effective in increasing percent neutrophil adhesion. This may have been due to the up regulation of β2 integrins [11]. It has also been suggested that the main attractive substances for neutrophil are pro-inflammatory cytokines, such as IL-Iβ, that regulate endothelial molecule expression on vascular endothelial cells and promote neutrophil adherence to these cells [16]. The plant extracts were also found to be more active at higher doses tested.

A polyherbal formulation Guard Sansar was demonstrated to have significant immunomodulatory activity by increasing the rate of carbon clearance and the percent neutrophil adherence to nylon fibers [12]. A polyherbal formulation NRZTCD which contain extracts of *Withania somnifera*, *Emblica officinalis* and *Ocimum sanctum*, was demonstrated to increase the number of peritoneal macrophages, rate of carbon clearance, and the percentage neutrophil adhesion to nylon fibers [15]. Oral administration of *Haridadi ghrita* was demonstrated to significantly increase neutrophil adhesion and delayed type hypersensitivity following antigenic challenge by SRBCs. Thus *Haridadi ghrita* potentiated the CMI by facilitating the footpad thickness response by SRBCs in sensitized rats. It showed no effect on the humoral immunity [18]

Phagocytosis by neutrophils constitutes an essential arm of the host defense against foreign antigens. Neutrophils have receptors for fragment crystallizable (Fc) and complement component (C36) which are involved in uptake of foreign antigen. Phagocytic index was enhanced in groups treated with MCVL, MCVS, MCVR, ECVL, ECVS, ECVR, ACVL, ACVS andACVR. The ability of the extracts to modulate phagocyte

functions might offer obvious therapeutic benefits for bacterial infections since phagocytes play an essential role in the host's defense against infection by ingesting invading microorganisms and by mediating inflammatory process [5].

NBT reduction was found to be significantly enhanced in groups treated with MCVL, MCVS, ECVL, ECVS, ECVR, ACVL, ACVS and ACVR. This enhancement was seen with higher concentrations of the extracts. NBT reduction was found to be depressed in animals treated with MCVR. The capacity to reduce NBT to formazon indicated that the neutrophils are producing oxygen radicals, which are important microbicidal mechanism of these cells that could cause pathogen death [19]. During the process of phagocytosis by the polymorphonuclear leucocytes, there occur significant increases in oxygen consumption, hexose monophosphate shunt activity and hydrogen peroxide formation. Nitroblue tetrazolium is yellow or white water soluble nitro substituted aromatic tetrazolium compound that reacts with cellular superoxide ions to form formazon derivative [20]. Superoxide is generated as a by product in aerobic organisms from a number of physiological reactions such as the electron flow in the chloroplasts and mitochondria and from some redox reactions in cells. It can react with hydrogen peroxide to produce hydroxyl radical ($0H^-$), one of the most reactive molecules in the living cells. Hydroxyl radical can cause the peroxidation of membrane lipids, breakage of DNA strands and inactivation of enzymes in cells. The cytoplasmic NADPH which is produced by oxidation of glucose through the hexose monophosphate shunt, serve as an electron donor. The oxidase system, available in the cytoplasm, helps transfer electrons from NADPH to NBT and reduces NBT into formazon [20].

The higher reduction in NBT dye reduction assay, represent higher activity of oxidase enzyme reflecting phagocytosis stimulation in proportion to intracellular killing [21]. NBT reduction test relies on the generation of bacterial enzymes like NADPH-oxidase in neutrophils during intracellular killing. These enzymes are necessary for normal intracellular killing against foreign antigens [11]. NBT reaction also indirectly reflects the ROS generating activity in the cytoplasm of cells as ROS are formed during phagocytosis by neutrophils [20]. It is known that neutrophils are capable of producing 100- fold higher amounts of ROS than non activated neutrophils, and activated neutrophils increases NADPH production via the hexose monophosphate shunt. The Myeloperoxidase system of both PMNL and macrophages also is activated, which leads to respiratory burst and production of high levels of ROS [20,22]. Such an oxidative burst is an early and effective defense mechanism in cases of infection for killing microbes. It would seem that these plant extracts have the ability to enhance the activity of neutrophils which means they should be able to respond to and destroy pathogenic agent more quickly. The extracts could possibly be useful for treating patients suffering from neutrophil function deficiency.

The seeds of seed and leaves of *Urtica dioica* were found to increase NBT reduction [23]. Dhillon and Bhatia [24] demonstrated that aqueous extracts of leaves and seeds of oat possessed *in vivo* immunomodulatory activity in Swiss albino mice by enhancing NBT reduction and phagocytosis. Aqueous extracts of *Nerium oleander* produced a significant increase in the percentage of NBT positive cells in extract treated group in comparison to control.

Macrophage phagocytosis was also found to be enhanced in groups treated with methanol, ethanol and aqueous extracts of *C. volkensii* (leaf, stem and root). This enhancement was seen with higher concentrations of the extracts. Macrophages constitute a major part of the host defense system against infection and cancer. Macrophages carry out their bactericidal and tumoricidal activities by the oxygen-dependent killing via products of oxidative metabolism such as H_20_2, superoxide anion, and hydroxyl radical, and the oxygen-independent killing via cytokines and hydrolytic enzymes [25]. Macrophages are stimulated by foreign agents to produce cytokines and reactive oxygen and nitrogen species [26].

The present study showed that cell mediated immunity as evaluated by DTH to sheep red blood cells, can be potentiated by the injection of the methanol, ethanol and aqueous extracts of *C. volkensii* (leaf, stem and root) and *A. setaceous* (aerial part and root). DTH reactions observed on the skin of the experimental animals treated with extracts 48h post inoculation expressed the existence of cellular mediated immunity response. The cutaneous reaction is attributed to liberation of lymphokines, skin reactive factor and monocytes chemotactic factor from sensitized T-cells. The thickening and reddening of skin in immunized animals are attributed to vasodilatations that causes increase capillary permeability and local influx of mononuclear cells at the site of inoculation [27]. The immunopotentiating action of the extracts on the DTH could be due to its effect on the number of specifically committed lymphocytes and the availability of blood monocytes that could be recruited locally at the site of interaction [28]. The mechanism(s) whereby the plant extracts can enhance the activity of these components is unclear, but it may be due to the increased secretion of cytokines or the increased chemotactic response of the immune cells [12,28]. It is known that DTH is the result of macrophage-mediated tissue inflammatory response driven by Th1 cells [29]. In general, DTH reactions are mediated by $CD4^+$ cells and down regulated by $CD8^+$ cells. Cells involved in DTH include langerhans and other cutaneous dendritic cells that transport inducers from the skin to regional lymph nodes where it is presented to T-lymphocytes. Before this can happen, however, protein antigens must be broken down into small peptides by the antigen presenting cells (APCs) such as dendritic cells, macrophages and monocytes. The peptide fragments are presented on the surface of the APCs and bound to major histocompatibility complex (MHC) molecules [30]. Once the skin is exposed to a new contact either in the same location or elsewhere an immune response causes a considerable reaction at the point of contact and this reaction is characterize by the infiltration of T-lymphocytes into challenged skin sites and the development of a cutaneous inflammation [30]. The DTH response which is a direct correlate of CMI was found to be statistically increased at doses of 500 and 1000mg/kg body weight of the extracts. During CMI responses, sensitized T-lymphocytes when challenged are converted to lymphoblasts and secrete lymphokines, attracting more scavenger cells to the site of reaction. The infiltrating cells are thus immobilized to promote defensive (inflammatory) reaction. In this study, the footpad thickness was enhanced after extract treatment suggesting CMI enhancement. Increase in the DTH responses indicates that the extracts had a stimulatory effect on lymphocytes and accessory cell types required for the expression of the reaction [18].

Sagrawat and Khan [31] in their review reported that an alcoholic extract of *A. racemosus* was found to enhance both humoral and cell mediated immunity of albino mice injected with sheep red blood cells as particulate agents. Lower concentration

of thyme essential oil was found to enhance murine DTH reaction and this was correlated with the known immunomodulatory effects of carvacrol which is one of the main substances in thyme oil. It is reported that carvacrol selectively activates the extracellularly- responsive kinase subgroup in Jurkat T- cells and stimulates the JNK (c- Jun N- terminal kinase) subgroup in THP-1 monocytic cells (Human acute monocytic leukemia cell line) and so may act as an effective agent to modulate the functions of immuno- responsive cell via intracellular pathways [32]. Aqueous extracts of *Nerium oleander* had immunopotentiating activity on cellular mediated immunity by enhancing DTH response in extract treated group in comparison to control (11). n-butanol soluble and ethyl acetate soluble fractions of methanolic extract of *Lagenaria siceraria* fruits significantly reduced SRBC induced DTH reaction in rats but significantly increased the WBC and lymphocyte count but had insignificant changes in monocytes, eosinophil and basophil counts. Both fractions significantly increased antibody titers in a dose dependent fashion showing augmentation of humoral response but not CMI [33].

CONCLUSION

The studies indicate that the aqueous, methanol and ethanol extracts of the two plants possess a potential of significant immunomodulatory and adaptogenic activity. The methanol, ethanol and aqueous extracts of *C. volkensii* leaves, stem and roots have the ability to enhance some function of the immune cells. The extracts were effective in activating and enhancing neutrophil adhesion, phagocytosis and formation of reactive oxygen species by neutrophils. It also enhanced phagocytosis by macrophages. It would seem that these plant extracts have the ability to enhance the activity of neutrophils and macrophage which means they should be able to respond to and destroy pathogenic agent more quickly. These findings demonstrate the *A. setaceous* and *C. volkensii* can be added to the ever growing list of herbal medicines that have the potential to enhance immune responses.

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REFERENCES

1. Kokwaro, J.O. Medicinal plants of East Africa. 1976 African Literature Bureau, Nairobi, Kenya.
2. Njoroge G.N. and Bussmann R.W. Diversity and utilization of antimalarial ethnophytotherapeutic remedies among the Kikuyus (central Kenya). Journal of Ethnobiology and Ethnomedicine 2006 2:8-14.
3. Iwu, M.M. Handbook of African medicinal plants. 2000 CRC Press Inc, USA.
4. Beenje H, J Kenyan trees, shrubs and lianas. Majestic printing works Ltd Nairobi, Kenya.
5. Lin, W.C. and Hsieh . Commercial application of *Anoectochilus formosanus*: Immunomodulating activities. International Journal of Applied Science and Engineering 2005 3(3):175-178.
6. Bray, D.H., Warhurst, D.C., Connolly, J.D., O'Neill, M.J. and Phillipson, J.D. Plants as sources of Antimalarial drugs.Part 7. Activity of some species of Meliaceae plants and their constituent limonoids. Phytotherapy Research 1990 41(1):29-35.
7. Chhabra, S.C. and Uiso, F.C. Antibacterial activity of herbs used in traditional medicine in Eastern Tanzania for treating gastrointestinal diseases. International Journal of BioChemiPhysics 1995 4 (1 and 2):24-28.
8. Valigra, L. Engineering the future of antibiotics. New Scientist 1994 Pp 25-27.
9. Ogila, Kenneth, Zipporah Ng'ang'a, Josephat Shililu, and Kimani Gichuhi Antibacterial and antifungal activity of the organic extracts of *Asparagus setaceous* Kunth and *Caesalpinia volkensii* Harm. Journal of Biomedical and Health Development 2009
10. Thakur, M., Bhargava, S. and Dixit, V.K. Immunomodulatory Activity of *Chlorophytum borivilianum* Sant. F. eCAM, 2006 doi:1093.
11. Srikumar, R., Parthasarathy N.J.and Devi,R.S. Immunomodulatory Activity of *Triphala* on Neutrophil functions. Biol. Pharm. Bull 2005 28(8):1398-1403.
12. Ottendorfer, D., Frevert, J., Kaufmann, R., Beuscher, N., Bodinet, C., Msonthi, D.J., Marston, A. and Hostettmann, K. Enhancement of *in vitro* non specific immune functions by African plant extracts. Phytotherapy Research 1994 8: 383-390.
13. Oladunmoye, M.K., and Osho, I.B. Anti inflammatory activity of Ethanolic leaf extract from *Carica papaya* in Rats orogastrically dosed with S*almonella typhi* and S*taphylococcus aureus*. Journal of Plant Sciences 2007 2(4): 447-452
14. Bang, J.I.Y., Kim, E.Y., Seong, N.K., Shin, Y.S. and Kang, I.C. Proteochip based library screening of integrin α5β1 antagonists from Korean medicinal plant extracts. Arch Pharm Res 2007 30 (12): 1584-1589.
15. Radzan, R., and Roy, S. Study of the immunomodulatory activity of a polyherbal drug. Pharmacology online 2008 3: 336- 345.
16. Reis, S.B., Oliveira, C. C., Acedo, S.C., da Conceicao, D. D., Ribeiro, M.L., Pedrazoli, J.J. and Gambero, A. Attenuation of colitis injury in rats using Garcinia cambogia extract. Phytotherapy Research 2009 23: 324-329.
17. Meera, S., Atyam, V.S.S.S., and Kumar, N.S. Immunomodulatory and antioxidant activity of a polyherbal formulation. International Journal of Pharmacology 2008 4(4): 287- 291.
18. Fulzele, S.V., Satturwar, P.M., Joshi, S.B. and Dorle, A.K. Study of the immunomodulatory activity of *Haridadi ghrita* in rats. Indian Journal of Pharmacology 2003 35: 51-54.
19. Muniz- Junqueira, M.I., Mota, M.I., Aires, R.B. and Junior, L.F.J.Differing phagocytic function of monocytes and neutrophils in Chaga's cardiopathy according to the presence or absence of congestive heart failure. Revista da Sociedade Brasileira de Medicina Tropical 2004 37(6): 447-453.
20. Esfandiari, N. Sharma, R.K., Saleh, R.A., Thomas, A.J.

and Agarwal A. Utility of the Nitroblue Tetrazolium Reduction Test for Assessment of Reactive oxygen species production by seminal leucocytes and spermatozoa. Journal of Andrology 2003 24(6): 862-870.

21. Saraphanchotiwitthaya, A., Ingkaninan K., and Sripalakit, P. Immunomodulating activity of Thai rejuvenating plants. Naresuan University Journal 2007 15(3):149-157.

22. Blake, D. R., Allen, R. E., Lunec, J. (1987). Free radical in biological system- a review oriented to inflammatory processess. Br Med Bulletin 1994 43: 371-385. 5

23. Basaran, A.A., Ceritoglu, I, Undeger, U. and Basaran, N. Immunomodulatory Activities of some Turkish Medicine Plants. Phytotherapy Research 1997 11: 609-611.3

24. Dhillon, P. and Bhatia, A. *In vivo* studies on the immunomodulatory potential of aqueous oat extract. Journal of Clinical and Diagnostic Research 2008 3: 887-891.

25. Okokon, J.E., Iyadi, K.C., Effiong, C.O. Effect of sub chronic administration of ethanolic leaf extract of *Croton zambesicus* on haematological parameters of rats. Nigerian Journal of Physiological sciences 2004 19(1-2):10-13.

26. Toshkova, R., Stefanova, T., Nikolova, N., and Serkedjieva, J. A plant extract ameliorates the dysfunctions of alveolar macrophages in influenza virus-infected mice. Pharmacologyonline 2006 3:778-84

27. Farwachi, M.I. *In vivo* and *in vitro* immunomodulatory activities of *Nerium oleander* aqueous leaf extract in rabbits. Journal of Animal and Veterinary Advances 2007 6 (9): 1047- 1050.

28. Amirghofran, Z., Azadmehr, A., Bahmani M. and Javidnia, K. Stimulatory Effects of *Euphorbia cheiradenia* on Cell Mediated Immunity and Humoral Antibody Synthesis. Iran. J. Immunol 2008:5 (2):11-123.

29. Chan, Y., Chang, T., Chan, C.H., Yeh, Y.C., Chen, C.W., Shieh, B. and Li, C. Immunomodulatory effects of *Agaricus blazei* murill in Balb/cBy J mice. J Microbiol Immunol Infect 2007 40:201-208.

30. Rios, J.L., Bas, E. and Recio, M.C. Effects of natural products on contact dermatitis. Curr. Med. Chem-Anti-inflammatory and Anti- Allergy Agents 2005 4: 65-80.

31. Sagrawat, H., and Khan, M.Y. Immunomodulatory plants: A phytopharmacological review. Pharmacognosy Review 2007 1(2):248-260.

32. Juhas, S., Bujnakova, D., Rehak, P., Cikos, S., Czikkova, J., Vesela, J. and Koppel, J.

a. Anti- inflammatory effects of the thyme essential oil in mice. Acta Vet Brno 2008 77: 327- 334.

33. Gangwal, A., Parmar, S.K., Gupta, G.L., Rana, A.C. and Sheth, N.R. (2008) Immunomodulating effects of *Lagenaria siceraria* fruits. Pharmacognosy Magazine 4(16): 234-238.