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Evaluation of the immunomodulatory properties and microbial bioburden of three commercial herbal mixtures sold in Awka, Anambra State Nigeria

Christopher E. Enemor[1*], Malachy Ugwu and Chukwuemeka Nworu[2]

[1]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences. Nnamdi Azikiwe University, Awka, Nigeria.
[2]Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria Nsuka, Nigeria.

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The consumption of herbal medicine and herbal medicinal products has been on the rise lately. This has led to an increase in research on herbal medicine and its different formulations to gain more knowledge in their constituents, therapeutic effects, its mechanism, and their undesired or toxic effects. The purpose of this study was to evaluate toxicity profile, phytochemical constituents, microbial quality and immunomodulatory properties of Goko cleanser®, Beta cleanser® and Weifa body defense mixtures®. Acute oral toxicity, phytochemical screening and the microbial quality was evaluated. The immunomodulatory activities of the herbal mixtures were studied using the Carbon Clearance Test, Cyclophosphamide Induced Neutropenia, Delayed-type Hypersensitivity Test and Humoral Antibody Assay with the Sheep Erythrocytes as antigen. The result indicated that the herbal mixtures showed no toxic effect on the test animals. The phytochemical analysis showed an adequate presence of immunomodulatory phytochemicals. The result also revealed that Beta cleanser® was contaminated with *S. aureus* and *E. coli*. The three test herbal mixtures, at doses tested, increased the phagocytic index by stimulating the reticuloendothelial cells and increasing their phagocytosis ability. The test herbal mixtures also showed significant protection against cyclophosphamide-induced neutropenia by increasing the depleted levels of leucocytes. The herbal mixtures aided the mobilization of macrophages and memory T cells as seen in the result of the Delayed-Type Hypersensitivity Test. The result of the humoral antibody test showed that the herbal mixtures exhibited a dose-dependent stimulatory effect on B cell maturation and differentiation into antibody-secreting plasma cells.

Key words: Immunomodulatory, Phytochemistry, Microbial Quality, Toxicity profile, Herbal Formulation.

INTRODUCTION

Herbal medicines are in great demand in the developed world for primary health care because of their efficacy, safety and lesser side effects. They offer therapeutics in age-related disorders like memory loss, osteoporosis,

*Corresponding author. E-mail: enemoremmanuel@gmail.com.

immune disorders, etc. for which no modern medicine is available (Tyler, 2000). Medicinal plants play significant roles in the prevention and treatment of various diseases. In nature, there are various medicinal plants which are used as immunomodulator agents (Singh et al., 2011). *Vernonia amygdalina* has been proven to strengthen the immune system through many cytokines regulation and increase in mean absolute CD4 count (Erasto et al., 2007; Momoh et al., 2012.) *Moringa oleifera* antibacterial activity, immunomodulatory activity (Anwar and Bhanger, 2003); *Morinda citrifolia* stimulates the release of several mediators from murine effector cells, including TNF-α, interleukin-1beta (IL-Iβ), ILfl0, IL-12, interferon gamma (IFN-y) and nitric oxide (Hirazumi and Furusawa, 1999). There appears to be an overwhelming increase in the public awareness and usage of herbal medical products in the treatments and or prevention of diseases in Nigeria. With this increased usage, the safety, efficacy and quality of these medicines have been an important concern for health authorities and health professionals (Okunola et al., 2007; Oreagba et al., 2011). Microbiological assessment of non-sterile products, such as herbal mixtures, is particularly pertinent in view of the fact that microbial contamination can reduce or even eliminate the therapeutic effect of drugs or cause drug-induced infections (Adesanya et al., 2007). Microbes presented in drugs not only make them hazardous from the infectious standpoint, but may also change the chemical, physical and organoleptic properties of the drugs or change the contents of active ingredients. Microorganisms and their toxic metabolites, which persist even after the death of the primary contaminants, can convert drugs to toxic products (Esimone et al., 2007). The presence of low level of pathogenic microorganisms, higher levels of opportunistic pathogens or bacterial toxic metabolites, which persist even after the death of the primary contaminants, can render the medicinal product ineffective (Ratajczak et al., 2014). In Nigeria, studies have shown that many patients rely on the use of herbal remedies in managing infectious diseases and immune boosting (Falodun and Imieje, 2013; Ekeanyawu, 2011). A questionnaire-based study by Oreagba et al. (2011) showed that 267 (66.8%) out of the 388 individuals recruited for the study had used herbal medicine at one point in their lives. These remedies however, have not been properly studied to confirm their label claims. This implies that these patients would be on drugs which may have no direct effect on both disease progression and quality of the patients' life. On the other hand, these herbal remedies contain bioactive constituents which may have either a positive or negative effect on therapeutic outcome. There is the need to confirm the label claims and safety profile of commercially available herbal medicines and mixtures. This study is geared towards the evaluation of the microbial bioburden and the immunologic claims of herbal mixtures popularly sold and consumed in Anambra State, Nigeria.

MATERIALS AND METHODS

Test herbal mixtures

Goko cleanser®, Beta cleanser® and Weifa Body defense mixtures® (five bottle each) were purchased in Eke Awka market in Anambra state Nigeria. Table 1 shows the composition and other information about the products.

Equipment and instrument

These include incubator (Genlab UK), Autoclave (EQUITRON Medica, Instrument India), UV-Vis Spectrophotometer (JENWAY 6505, Bibby Scientific Ltd., UK).

Culture media and other reagents

Muller Hinghton Agar (Titan; Rajasthan India), Nutrient Broth (LabM; United Kingdom), Mannitol Salt Agar (HiMedia; Mumbia India), MacConkey Agar (Biotech United Kingdom), Salmonella Shigella Agar (Titan Biotech; Rajasthan India), Distilled Water, Normal Saline (Table 1).

Experimental animals used

Albino rats of both sexes, with weights range of 80-120 g, were used. They were housed under the standard condition of temperature (25±10°C) and relative humidity (60±10%) and fed with standard pellets diet and water. They were housed in the animal house in the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka. The animals were kept to acclimatize for about a week before they were randomly divided into the different experimental groups. The use of animals in this research was in accordance with the guidelines approved by the Animal Ethical Committee, Nnamdi Azikiwe University Awka Nigeria.

Microbial assay

Determination of the microbial load of the herbal sample was carried by the technique outlined by Oluyege and Adelabu (2010). Exactly 10 ml of each sample (Goko herbal mixture®, Beta herbal mixture®, Weifa body defense mixture®) was aseptically transferred into a corresponding sterile tube containing 90 ml of sterile distilled water and ten-fold serial dilution was carried out into three containers (1/10, 1/100 and 1/1000 respectively). One milliliter of each dilution of the test herbal mixtures was mixed with 15 ml of sterile molten standard plate count agar (Muller Hington Agar) and 15 ml molten Saburoud dextrose agar for bacteria and fungi respectively, and then poured into petri dishes. This was done in triplicate. The plates were allowed to set and incubated at 37°C and 24 h for bacterial counts and at 27°C for four days for fungal counts. Isolation and identification of potential pathogens in the herbal samples was also carried out using MacConkey agar, Manitol salt agar and Salmonella-Shigella agar. Potato dextrose agar was used to isolate fungi. For each herbal sample, 1.0 ml of the mixture was aseptically transferred onto each medium and spread on the surface with glass spreader. The plates were incubated at 37°C for 72 h. Pure cultures were obtained from the plates and stored

Table 1. Herbal mixtures with their composition.

Name of product	Composition	Producers	Batch Number	Date manufactured	Expiring Date	Nafdac Reg. number
Goko cleanser®	*Vernoniaamygdalina*-12% *Saccharum officinarum*-11.5% *Allium sativum*-13% *Cajanus cajan*-11.5% Caramel -1.5% *Zingiber officinale*-0.5% Treated Water-QS	Goko Herbs West Africa Ltd	0005	08/2014	07/2017	A7-0804L
Beta Cleanser®	*Aloe vera*-25ml M. oleifera-20ml *C. aurantifolia*-15ml *C. officinarum*-20ml *S. offocarium* -15ml *Allium sativa*-5ml Treated water-100ml	SJOESYLY CONSERNS LTD	008	09/2014	08/2017	A7-0738L
WEIFA BODY DEFENSE®	Roots and herbs Treated water *Dacryodes edulis* *Garcina spp* *Allium satium*	Benbela Tradomedicals Ltd	WBDAL/05	01/2014	01/2017	

onagar slants and kept in the refrigerator until used for biochemical identification. The limits presented in the European Pharmacopoeia (Microbial Quality of Pharmaceutical preparations; category 3B), for each test, was used to assess the result.

Phytochemical screening

The herbal mixtures were screened for the presence of various phytochemical constituents using standard methods as earlier described (Trease and Evans, 2009; Akinjogunla et al., 2010).

Determination of acute toxicity

The acute oral toxicity study was conducted on each of the products as described by Lorke (1983). The study was conducted in two phases using a total of 39 rats. In the first phase, 27 rats were

divided into three groups of nine per group and the nine of each group was further divided three groups of three rats per group. Groups one to three were given 10, 100 and 1000 mg/kg body and 1000 mg/kg body weight of Beta herbal mixtures®, and the third group received 10, 100 and 1000 mg/kg body weight of Weifa body defense mixtures® respectively, to possibly establish the range of doses producing any toxic effect and the rats were watched for 24 h for mortality rate. The death pattern in the first phase determined the dose for the second phase. Depending on the death pattern, further specific doses (1600, 2900, 3600, and 5000 mg/kg) of Goko herbal mixture®, Beta herbal mixture® and Weifa body defense® were administered to three rats (one per dose) to further determine the LD_{50} value. The herbal mixtures were serially diluted with sterile water and administered orally and the animals observed for 24 h. The LD50 was calculated as the geometric mean of the maximum dose that did not result in lethality and the least toxic dose that produce death in the albino rats.

Processing of sheep red blood cells for use as an antigen

Blood samples were obtained from the jugular vein of a healthy sheep maintained in the animal house of Faculty of Pharmaceutical sciences, Nnamdi Azikiwe University Awka, Nigeria and into a 5 ml EDTA bottle. Then red blood cells were washed thrice with copious volume of sterile normal saline by centrifugation at 3000× g for 10 min. The final cell volume was adjusted to a concentration of 1×10^9 cells/ml and used for immunization and challenge.

Selection of doses

The doses for the study were selected based on the outcome of the oral toxicity studies done up to dose level of 5000 mg/kg body weight. On the account of no death, doses of 100, 200 and 400 mg/kg body weight was used.

Experimental protocols for determination of immunological parameters

The animals were numbered, weighed and divided into eleven different groups of six animals per group as follows:

Group 1: positive control to receive pellet and distilled water
Group 2: negative control to receive 100 mg/kg body weight of Noni® Group 3: received 100 mg/kg body weight of Goko herbal mixtures® Group 4: received 200 mg/kg body weight of Goko herbal mixtures® Group 5: received 400 mg/kg body weight of Goko herbal mixtures®
Group 6: Received 100 mg/kg body weight of Beta herbal mixtures® Group 7: received 200 mg/kg body weight of Beta herbal mixtures® Group 8: received 400 mg/kg body weight of Beta herbal mixtures® Group 9: received 100 mg/kg body weight of Weifa body defense® Group 10: received 200 mg/kg body weight of Weifa body defense® Group 11: received 400 mg/kg body weight of Weifa body defense®

Carbon clearance test

This test assesses the phagocytic activity of reticuloendothelial system (RES). It was conducted as described by Tripathi et al. (2012). Briefly, eleven groups of animals were used. Group 1 and 2 are positive (100 mg/kg body weight of Noni®) and negative control respectively; Group 3 to 5 received Goko herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively; Group 6 to 8 received Beta herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively while group 9 to 11 received Weifa body defense mixture® at increasing doses of 100, 200 and 400 mg/kg body weight respectively. The treatment was done daily for 10 days. Carbon ink suspension was injected via the tail vein to each rat 48 h after the tenth day treatment. Blood samples (25 µl) was withdrawn from the retro-orbital plexus under mild ether anesthesia at 0 and 15 min after injection of colloidal carbon ink and lysed in 0.1% sodium carbonate solution (3 ml). The optical density was measured spectrophotometrically at 660 nm. The phagocytic index was calculated using the following formula:

Where OD_1 and OD_2 are the optical densities at time t_1 and t_2, respectively (Barbuddhe et al., 1998).

Cyclophosphamide induced nutropenia studies

The albino rats were divided into 11 groups with each group having six animals each. Group 1 and 2 are positive (100 mg/kg body weight of Noni®) and negative control respectively; Group 3 to 5 received Goko herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively; Group 6 to 8 received Beta herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively while group 9 to 11 received Weifa body defense mixture® at increasing doses of 100, 200 and 400 mg/kg body weight respectively, daily for 10 days. On the 11th day, blood samples were withdrawn from the animals via retro orbital puncture into an EDTA container. A neutropenic dose of cyclophosphamide (30 mg/kg body weight) was administered on the 11th, 12th, and 13th days one hour after the administration of the treatment intra-peritonially (i.p). Blood samples were withdrawn on the 14th day of the experiment by retro orbital puncture. Haematological parameters were studied (total white blood cell (WBC) counts and differential leucocyte count (DLC)) prior to and on the 3rd day after injection of cyclophosphamide. Data collected were expressed in mean and standard error of mean (S.E.M).

Delayed type hypersensitivity reaction

Animals were divided into eleven groups. Group 1 and 2 are positive (100 mg/kg body weight of Noni®) and negative control respectively. Group 3 to 5 received Goko herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively; Group 6 to 8 received Beta herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively while Group 9 to 11 received Weifa body defense mixture® at increasing doses of 100, 200 and 400 mg/kg body weight respectively for 5 days. On the fifth day, the animals were immunized with 0.1 ml of SRBCs suspension containing 1.0×10^9 cells/ml inter- peritonuosly (i.p). For 14 days, the animals in Group 3 to 11 were fed with their respective herbal mixtures and on the 19th day, they were sensitized again and then were fed with their respective herbal mixtures for another seven days. On the 8th day after immunization, the thickness of the right hind footpad was measured using a venier calliper. The animals were again challenged by the injection of 1.0×10^9 SRBCs footpad in the left leg. The thickness of the footpad was measured again after 24 h. The difference between the pre and post challenge footpad thickness was estimated and represents an index of the delayed

Table 2. Total Fungal and Bacteria population in the test herbal products.

Herbal product	Mean fungal count in CFU/ml ± SEM	Inference	Mean aerobic bacteria Count in CFU/ml ± SEM	Inference
Goko herbal cleanser®	0.23×10^{2} ± 0.083	Pass	3.2×10^{2} ± 0.780	Pass
Beta cleanser®	0.82×10^{2} ± 0.101	Pass	8.7×10^{2} ± 0.891	Pass
Weifa body defense®	0.20×10^{1} ± 0.022	Pass	1.1×10^{1} ± 0.060	Pass

type hypersensitity (DTH) response. The DTH response was obtained from this formula (Corrier and DeLoach, 1990):

$$\frac{Left\ foot\ pad\ challenged\ with\ antigen - right\ foot\ pad\ control}{Left\ foot\ pad\ challenged\ with\ antigen} \times \frac{100}{1}$$

Humoral antibody determination

This was done using sheep erythrocyte agglutination test (SEAT) as described by Kumar et al. (1996) and Ray et al. (1991). Briefly, animals were divided into eleven groups, each having six rats. Group 1 and 2 are positive (100 mg/kg body weight of Noni®) and negative control respectively; Group 3 to 5 received Goko herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively; Group 6 to 8 received Beta herbal mixtures® at increasing doses of 100, 200 and 400 mg/kg body weight respectively while Group 9 to 11 received Weifa body defense mixture® at increasing doses of 100, 200 and 400 mg/kg body weight respectively, daily for 10 days. All the animals were injected with 0.25 ml of 1×10^{9} SRBC/ml on 6th, 8th, and 10th days to achieve maximum titer of antibody. On day 11 blood was collected and serum separated by centrifuging at 3000×g for 15 min. The serum was diluted serially with normal saline in separate test tubes. Serial dilutions that were made are 20, 40, 80 up to 1280th. To these serial dilutions, 50 µl of SRBC was added and incubated at 37°C for 18 h. All the tubes were then subjected to physical examination visually for agglutination and compared with control. The highest dilution (lowest concentration of serum) giving hemagglutination was taken as the antibody titer for that group. The antibody titer was expressed in the graded manner, the minimum dilution being ranked as 1, and mean ranks of different groups was compared for statistical significance.

Statistical analysis

Results obtained were analysed using one-way analysis of variance (ANOVA) expressed as mean and standard error of mean to test for variations of the different parameters observed in the study. Test of significance was at $P<0.05$. The Microsoft excel 2010 was used.

RESULTS

Microbial assay

The results show that the total fungal count ranges from 0.2×10^{1} to 0.9×10^{2} cfu/ml (Table 5). The result also shows that the total aerobic bacteria count is less than 0.9×10^{3} cfu/ml with Weifa body defense been the least contaminated herbal product (Table 5). The result for the total *Escherichia coli* count in the herbal mixtures showed that Beta herbal cleanser®, after repeating the microbial quality assay, is contaminated with *E. coli* with the value of 0.3×10^{1} CFU/ml (Table 6). The same herbal product (Beta cleanser®), from the result of the total *Staphylococcus aureus* count, has a *S. aureus* count of 0.8×10^{1} CFU/ml and *Salmonella* spp. count of 0.4×10^{1} CFU/ml. From the reference (Microbial Quality of Pharmaceutical preparations; category 3B; European Pharmacopoeia), the herbal preparations all passed the limit test for fungi and aerobic bacteria (not more than 10^{2} CFU/ml and not more than 10^{4} CFU/ml respectively). Beta cleanser® failed the limit test for *E. coli* and *S. aureus* (absence of *E. coli* and *S. aureus* according to the reference material) as these organisms were isolated from the herbal product after inoculating it in their selective media. On the surface of the Salmonella shigella agar, few black colonies of *Salmonella* spp. were observed but not much enough to conclude that Beta cleanser® also failed the limit test for the organism. Two biochemical tests were conducted for the isolated microorganisms. *S. aureus* was positive for catalase test, *E. coli* was positive for indole, and *Salmonella* spp was negative for both indole and catalase test (Tables 2 to 4).

Phytochemical screening of the herbal mixtures

The three herbal mixtures contain high level of tannin (Table 7). Weifa body defense mixture® also has excess of alkaloid and flavonoids, while Goko herbal mixture® has normal alkaloids and flavonoids. The herbal mixtures lacked reducing sugar in the exception of Weifa body defense mixture® which has traces of it.

Acute oral toxicity testing

The results of the first stage of oral acute toxicity test for the herbal mixtures are shown subsequently. From Tables 8 to 10 (for the first phase of the test), it was observed that after the administration of the specific doses of the herbal product, no animal died. The result of

Table 3. *E. coli, S. aureus and Salmonella* spp population in the test herbal products.

Herbal product	Mean *E. coli* Count in CFU/ml ± SEM	Inference	Mean *S. aureus* count in CFU/ml ± SEM	Inference	Mean *Salmonella spp*. count in CFU/ml ± SEM	Inference
Goko herbal cleanser®	Nil	Pass	Nil	Pass	Nil	Pass
Beta cleanser®	0.3×10^{1} ±0.006	Fail	0.8×10^{1}±0.004	Fail	0.4×10^{1} ±0.020	Pass
Weifa body defense®	Nil	Pass	Nil	Pass	Nil	Pass

Table 4. Phytochemical screening of the herbal mixtures.

Bioactive compound	Goko herbal mixture®	Beta herbal mixture®	Weifa body defense®
Reducing sugar	_	_	+
Saponins	++	++	++
Flavonoids	+	++	++
Proteins	+	+	+
Tanins	+++	+++	+++
Alkaloids	++	+	+++
KEY: - = absence			
+ = trace			
++ = present			
+++ = excess			

Table 5. Acute oral toxicity test of Goko Herbal Mixture®.

First stage of acute toxicity		
Number of animals used	**Dose administered (mg/kg)**	**Results**
	Group one	
3.0	10.0	No death
3.0	10.0	No death
3.0	10.0	No death
Group two		
3.0	100.0	No death
3.0	100.0	No death
3.0	100.0	No death
Group three		
3.0	1000.0	No death
3.0	1000.0	No death
3.0	1000.0	No death

the first phase of the test where no death was recorded led to the second phase of the test which was conducted with the animals given higher doses of the herbal products. The result of the second stage acute oral toxicity for the herbal mixtures is shown in Table 11. From Table 11, it was also observed that after the administration of the mixtures at higher doses, no death was recorded.

Carbon clearance test

The carbon clearance assay result of this work is shown in Figure 1. Goko cleanser® shows an enhanced carbon clearance activity in the test animal with the dose of 100 mg/kg been significant when compared to the control result. Beta cleanser®, at doses of 100 and 400 mg/kg, indicated a significant increase in the phagocytic index

Table 6. Acute oral toxicity test of Bata herbal mixtures®.

First stage of acute toxicity		
Number of animals used	**Dose administered (mg/kg)**	**Results**
Group one		
3.0	10.0	No death
3.0	10.0	No death
3.0	10.0	No death
Group two		
3.0	100.0	No death
3.0	100.0	No death
3.0	100.0	No death
Group three		
3.0	1000.0	No death
3.0	1000.0	No death
3.0	1000.0	No death

Table 7. Acute Oral Toxicity Test of Weifa Body Defense Mixtures®.

First stage of acute toxicity		
Number of animals used	**Dose administered (mg/kg)**	**Results**
Group one		
3.0	10.0	No death
3.0	10.0	No death
3.0	10.0	No death
Group two		
3.0	100.0	No death
3.0	100.0	No death
3.0	100.0	No death
Group three		
3.0	1000.0	No death
3.0	1000.0	No death
3.0	1000.0	No death

when compared to the negative control and to other two herbal mixtures. At doses of 100 and 200 mg/kg, Weifa body defense mixture® significantly enhanced the phagocytic index when compared to the control group and to Goko cleanser® and Beta cleanser® mixtures.

Cyclophosphamide induced immunosupression

In this study, Goko cleanser® at dose of 400 mg/kg body weight, Beta cleanser® at doses of 100 and 200 mg/kg body weight and Weifa body defense mixture® at doses of 100 and 400 mg/kg body weight showed a significant percentage reduction of the inhibition effect of cyclophosphamide on total white blood cell count in treated rats when compared to the control group. In the evaluation of the percentage reduction of the lymphocyte count, Goko cleanser® at dose of 100 mg/kg body weight showed a significant percentage reduction in lymphocyte count (50.08%) when compared to the control group (73.10%). Beta cleanser® and Weifa body defense mixture® at doses of 200 and 400 mg/kg body weight showed significant reduction in the percentage lymphocyte count when compared to the control group. Goko cleanser® at doses of 100 and 400 mg/kg, Beta cleanser® at doses of 100 and 200 mg/kg and Weifa body defense mixture® at dose of 200 mg/kg body weight all showed a significant percentage reduction in neutrophil

Table 8. Second stage of acute oral toxicity test.

Acute oral toxicity of Goko herbal mixture®		
Number of animals used	**Doses administered (mg/kg)**	**Result**
1.0	1600.0	No death
1.0	2900.0	No death
1.0	3600.0	No death
1.0	5000.0	No death
Acute oral toxicity of Beta herbal mixtures®		
Number of animals used	**Doses administered (mg/kg)**	**Result**
1.0	1600.0	No death
1.0	2900.0	No death
1.0	3600.0	No death
1.0	5000.0	No death
Acute oral toxicity of Weifa body defense mixture®		
Number of animals used	**Doses administered (mg/kg)**	**Result**
1.0	1600.0	No death
1.0	2900.0	No death
1.0	3600.0	No death
1.0	5000.0	No death

Table 9. Effect of Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® on Phagocytic Index Values.

Treatment groups		**Mean absorbance ± SEM**		**Phargocytic index**
		0 min	**15 min**	**±SEM**
Positive control		0.1066±0.00101	0.0098±0.00038	0.0691*
Negative control		0.0592±0.00055	0.0212±0.00079	0.0025
Goko 100 mg/ml	cleanser®	0.0978±0.00083	0.0198±0.00085	0.0462*
Goko cleanser® 200 mg/ml		0.1020±0.00098	0.0101±0.00122	0.0669NS
Goko cleanser® 400 mg/ml		0.1064±0.00144	0.0095±0.00036	0.0699NS
Beta cleanser® 100 mg/ml		0.0732±0.00104	0.0292±0.00127	0.0266**
Beta cleanser®200 mg/ml		0.0964±0.00151	0.0108±0.00210	0.0633NS
Beta cleanser® 400 mg/ml		0.0980±0.00087	0.0090±0.00066	0.0691*
Weifa defense® 100 mg/ml	body	0.0988±0.00800	0.0213±0.00102	0.0444*
Weifa defense® 200 mg/ml	body	0.1045±0.00137	0.0158±0.00221	0.0547*, **
Weifa defense® 400 mg/ml	body	0.1184±0.00107	0.0102±0.00109	0.0720NS

Values are expressed in mean ± S.E.M., n=6
*= P<0.05 as compared with the control group
**=P<0.05 as compared with the three herbal mixtures
NS= P>0.05 not significant as compared to control (Negative) group.

count when compared to the control group (Table 12 and Figures 2 to 4).

Delayed type hypersensitivity reaction

Goko herbal cleanser®, Beta cleanser® and Weifa body defense® mixtures produced some significant percentage increase in the paw volume of the immunized animals. From Figure 5, Goko cleanser® showed a significant effect in the increase in the paw volume of the immunized rats. Doses of 200 and 400 mg/kg body weight showed a significant percentage increase in the paw volume when compared to the control group. Beta cleanser® at dose of

Table 10. Effect of Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® on the Cyclophosphamide induced leucopenia (Total WBC Counts).

Treatment group		Total white blood cell count (WBC/mm^3)		
		Before	After	%Reduction
Positive control		12.02 ± 3.26	7.15 ± 2.13	41.50*
Negative control		16.72 ± 2.91	2.92 ± 1.72	82.63
Goko 100mg/ml	cleanser®	18.04 ± 4.12	3.63 ± 0.96	80.05NS
Goko cleanser® 200 mg/ml		16.71 ± 4.44	3.90 ± 1.11	76.70**
Goko cleanser® 400 mg/ml		10.98 ± 3.02	4.41 ± 1.38	59.92*
Beta cleanser® 100 mg/ml		22.54 ± 4.81	4.48 ± 1.53	80.44*, **
Beta cleanser® 200 mg/ml		18.92 ± 4.03	3.95 ± 0.87	79.36*
Beta cleanser® 400 mg/ml		12.78 ± 3.88	5.65 ± 1.37	55.94NS
Weifa defense® 100 mg/ml	body	15.43 ± 3.11	4.31 ± 1.13	72.19**
Weifa defense® 200mg/ml	body	8.40 ± 2.94	3.47 ± 1.20	59.51*
Weifa body defense 400 mg/ml®		15.62 ± 2.72	9.04 ± 3.02	42.30*, **

Values are expressed in mean ± S.E.M., n=6
*= P<0.05 as compared with the control group
**=P<0.05 as compared with the three herbal mixtures
NS= P>0.05 not significant as compared to control (Negative) group.

Table 11. Effect of Goko cleanser®, Beta cleanser® and Weifa body defense® on Cyclophosphamide induced neutropenia (Lymphocyte counts).

Treatment group		Total lymphocyte count		
		Before	After	%Reduction
Positive control		32.82 ± 4.95	29.40 ± 6.04	10.42*
Negative control		41.62 ± 2.6	11.20 ± 2.98	73.10
Goko 100 mg/ml	cleanser®	40.30 ± 4.69	16.11 ± 2.0	50.08*
Goko cleanser® 200 mg/ml		50.71 ± 1.89	33.52 ± 4.11	34.28**
Goko cleanser® 400 mg/ml		43.50 ± 5.21	37.21 ± 3.56	13.90**
Beta cleanser® 100 mg/ml		35.41 ± 3.10	12.23 ± 2.21	65.54NS
Beta cleanser® 200 mg/ml		31.92 ± 2.55	24.40 ± 3.65	23.51*
Beta cleanser® 400 mg/ml		42.21 ± 5.17	34.85 ± 1.69	17.55*
Weifa defense® 100 mg/ml	body	44.60 ± 2.98	18.11 ± 2.89	59.42**
Weifa defense® 200 mg/ml	body	38.76 ± 3.84	26.23 ± 2.91	32.40*
Weifa defense® 400 mg/ml	body	46.04 ± 4.81	39.61 ± 4.02	13.98*, **

Values are expressed in mean ± S.E.M., n=6
*= P<0.05 as compared with the control group
**=P<0.05 as compared with the three herbal mixtures
NS= P>0.05 not significant as compared to control (Negative) group.

400 mg/kg body weight showed a significant percentage increase in paw volume of the immunized rats when compared to the control group, while at doses of 200 and 400 mg/kg there was a significant increase in the paw volume when the result is compared among the three test herbal mixtures. Weifa body defense mixture, at doses of 100, 200 and 400 mg/kg body weight showed significant percentage increase in paw volume of the immunized animal when the result was compared to control group (Table 13).

Humoral antibody determination

To evaluate the effect of the test herbal mixtures on humoral response, its influence was tested on sheep erythrocyte specific humoral antibody titre in experimental animals. The antibody titre was interpreted as the highest

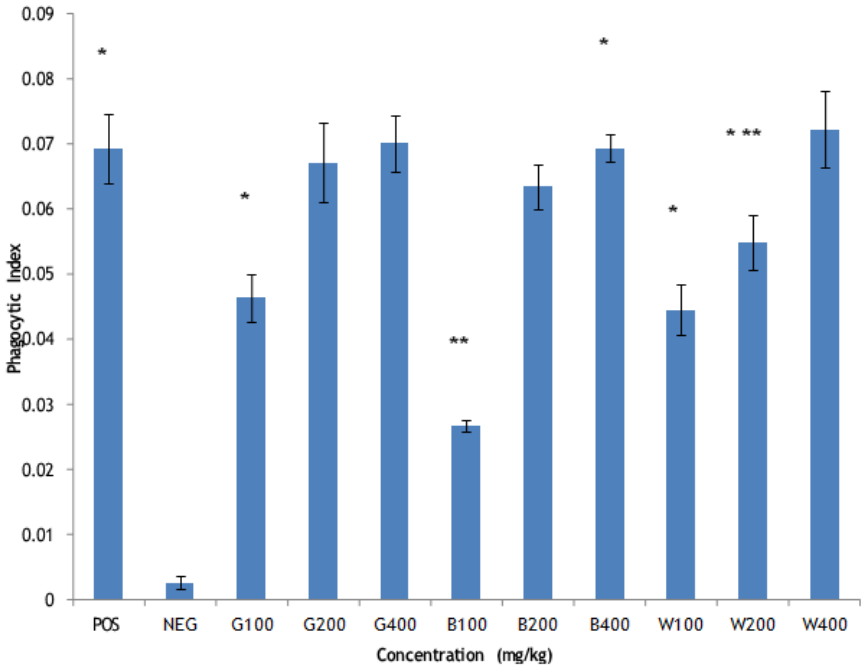

Figure 1. Phargocytic index of rats fed with Goko cleanser®, Beta cleanser® and Weifa body defense mixtures®.
Where G= Goko cleanser®, B= Beta cleanser® and W= Weifa body defense®.
*= P<0.05 as compared with the control group
**=P<0.05 as compared with the three herbal mixtures.

Table 12. Effect of Goko cleanser®, Beta cleanser® and Weifa body defense® on Cyclophosphamide induced neutropenia (Neutrophil counts).

Treatment group		Total neutrophil count		
		Before	After	%Reduction
Positive control		50.20 ± 6.90	38.40 ± 3.27	23.50*
Negative control		62.89 ± 3.21	19.02 ± 2.56	69.75
Goko 100 mg/ml	cleanser®	56.21 ± 5.13	21.55 ± 2.18	61.66*
Goko cleanser® 200 mg/ml		61.00 ± 9.52	32.30 ± 3.39	47.05**
Goko cleanser® 400 mg/ml		45.34 ± 6.12	31.82 ± 7.40	29.81*
Beta cleanser® 100 mg/ml		52.65 ± 8.22	18.23 ± 2.12	65.37*
Beta cleanser® 200 mg/ml		38.45 ± 4.99	22.61 ± 4.11	41.23*
Beta cleanser® 400 mg/ml		47.44 ± 6.34	32.12 ± 4.78	32.30NS
Weifa defense® 100 mg/ml	body	39.22 ± 2.82	17.32 ± 3.72	55.89**
Weifa defense® 200 mg/ml	body	58.32 ± 6.91	39.81 ± 3.31	31.73*, **
Weifa defense® 400 mg/ml	body	51.90 ± 3.83	40.61 ± 6.65	21.77NS

Values are expressed in mean ± S.E.M., n=6
*= P<0.05 as compared with the control group
**=P<0.05 as compared with the three herbal mixtures
NS= P>0.05 not significant as compared to control (Negative) group.

dilution that shows agglutination. In this study, Goko cleanser® at dose of 100 mg/kg body weight, Beta cleanser® at 400 mg/kg and Weifa body defense® at doses of 100, 200 and 400 mg/kg body weight showed a

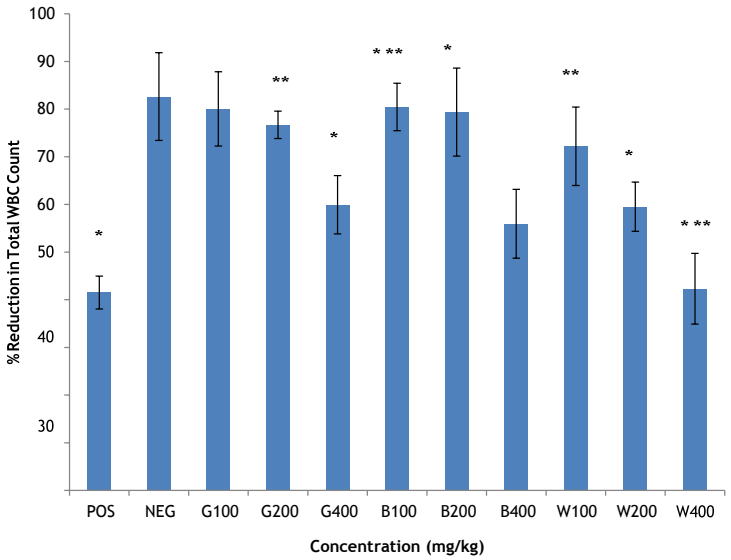

Figure 2. Cyclophosphamide induced leucopenia in rats fed with Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® (Total WBC Counts).
Where G= Goko cleanser®, B= Beta cleanser® and W= Weifa body defense®.
*= P<0.05 as compared with the control group
**=P<0.05 as compared with the three herbal mixtures.

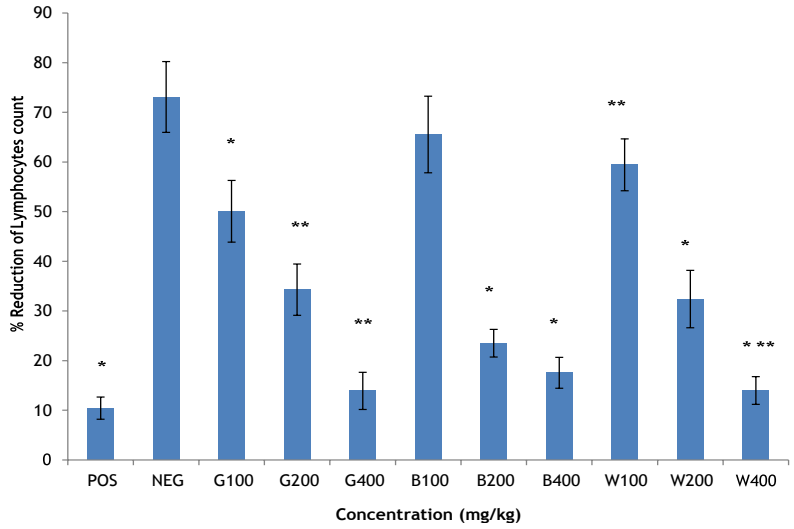

Figure 3. Cyclophosphamide induced nutropenia of rats fed with Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® (Lymphocyte Counts).
Where G= Goko cleanser®, B= Beta cleanser® and W= Weifa body defense®.
*= P<0.05 as compared with the control group
**=P<0.05 as compared with the three herbal mixtures.

significant increase in augmenting antibody production. When the three herbal mixtures were compared using statistical analysis (one way ANOVA), Goko cleanser® showed a significant effect at dose of 400 mg/kg body

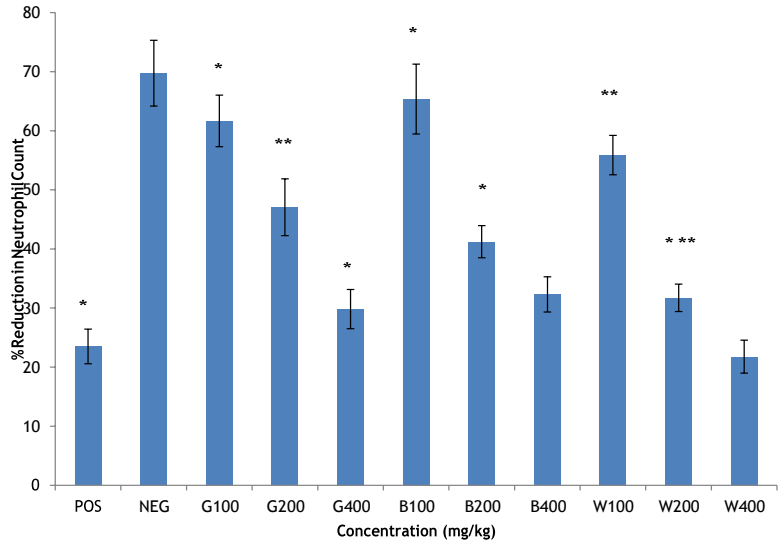

Figure 4. Cyclophosphamide induced neutropenia of rats fed with Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® (Neutrophil Counts).
Where G= Goko cleanser®, B= Beta cleanser® and W= Weifa body defense®.
*= *P<0.05* as compared with the control group
**=*P<0.05* as compared with the three herbal mixtures.

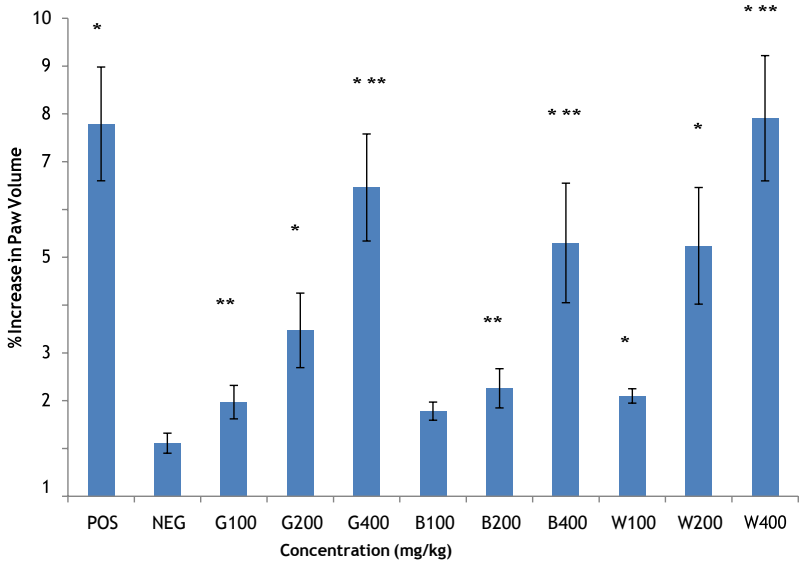

Figure 5. Delayed type hypersensitivity reaction of rats fed with Goko cleanser®, Beta cleanser® and Weifa body defense mixtures®.
Where G= Goko cleanser®, B= Beta cleanser® and W= Weifa body defense.
*= P<0.05 as compared with the control group
**=P<0.05 as compared with the three herbal mixtures.

weight, Beta cleanser® showed a significant effect at doses of 200 and 400 mg/kg body weight, while Weifa body defense®, at dose of 400 mg/kg showed a significant effect as shown in Figure 6 and Table 14.

Table 13. Effects of Goko cleanser®, Beta cleanser®, and Weifa body defense mixtures® on the delayed type hypersensitivity reaction in rats.

Treatment groups	DTH response % increase
Positive control	7.79 ± 0.041*
Negative control	1.11 ± 0.013
Goko cleanser® 100 mg/ml	1.97 ± 0.016**
Goko cleanser® 200 mg/ml	3.47 ± 0.032*
Goko cleanser® 400 mg/ml	6.46 ± 0.029*, **
Beta cleanser® 100 mg/ml	1.78 ± 0.020NS
Beta cleanser® 200 mg/ml	2.26 ± 0.025**
Beta cleanser® 400 mg/ml	5.30 ± 0.027*, **
Weifa body defense® 100 mg/ml	2.10 ± 0.017*
Weifa body defense® 200 mg/ml	5.24 ± 0.031*
Weifa body defense® 400 mg/ml	7.91 ± 0.037*, **

Values are expressed in mean ± S.E.M., n=6
*= P<0.05 as compared with the control group
**=P<0.05 as compared with the three herbal mixtures
NS= P>0.05 not significant as compared to control (Negative) group.

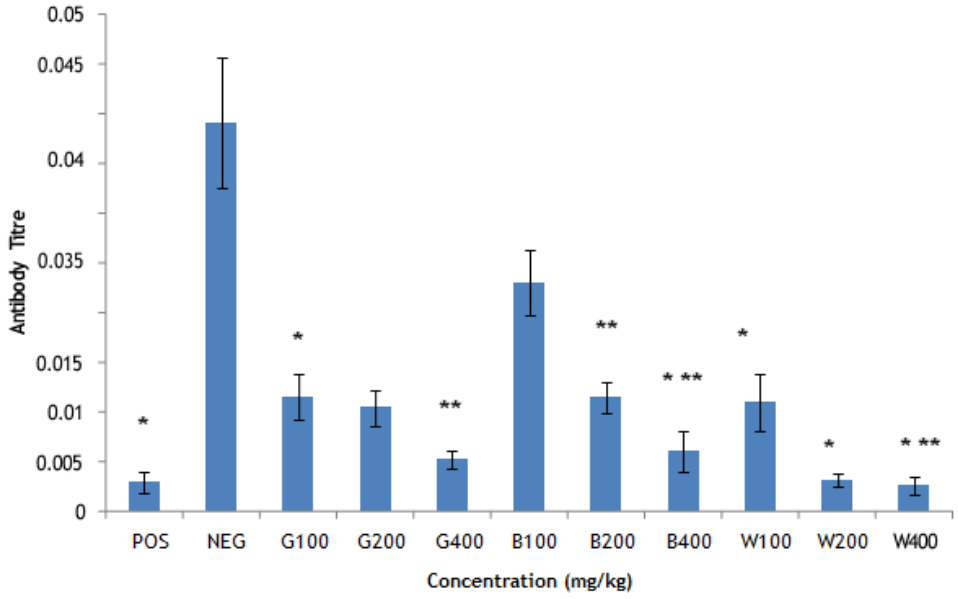

Figure 6. Humoral antibody determination in immunised rats fed with Goko cleanser®, Beta cleanser® and Weifa body defense mixtures®.
Where G= Goko cleanser®, B= Beta cleanser® and W= Weifa body defense.
*= P<0.05 as compared with the control group
**=P<0.05 as compared with the three herbal mixtures.

DISCUSSION

The presence of microbial contaminant in non-sterile pharmaceutical products can reduce or even inactivate the therapeutic activity of the product and has potential to adversely affect patients taking the medicines (Nakajima et al., 2005). Some infectious disease outbreaks have been associated with the use of heavily contaminated raw materials of natural origin. Since the microbial quality of the herbal medicinal products were influenced by the environments and quality of the raw materials used during formulation, the manufacturers should ensure that

Table 14. Effect of Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® on Humoral Antibody Determination Test.

Treatment	Mean Antibody Titre value±S.E.M.
Positive control	0.0029 ± 0.0011*
Negative control	0.0441 ± 0.0065
Goko cleanser® 100 mg/ml	0.0115 ± 0.0023*
Goko cleanser® 200 mg/ml	0.0104 ± 0.0018NS
Goko cleanser® 400 mg/ml	0.0052 ± 0.0009**
Beta cleanser® 100 mg/ml	0.0229 ± 0.0033NS
Beta cleanser®200 mg/ml	0.0114 ± 0.0015**
Beta cleanser® 400 mg/ml	0.0060 ± 0.0021* **
Weifa body defense® 100 mg/ml	0.0109 ± 0.0028*
Weifa body defense® 200 mg/ml	0.0031 ± 0.0007*
Weifa body defense® 400 mg/ml	0.0026 ± 0.0009* **

The antibody titre was interpreted as the highest dilution that shows agglutination Values are expressed in mean ± S.E.M., n=6.
*= P<0.05 as compared with the control group
**=P<0.05 as compared with the three herbal mixtures
NS= P>0.05 not significant as compared to control (Negative) group.

the microbial load is brought to a minimal safety level in the raw materials, finished dosage forms, and the packaging components, to maintain appropriate quality, safety, and efficacy of the products. Studies conducted on numerous herbal products sold and consumed in south east Nigeria showed that they were contaminated with bacteria and fungal isolates (Ujam et al., 2013). Govender et al. (2006) also reported contamination of herbal products with *Bacillus* spp., *Enterobacteriaceae* spp., *Salmonella* spp., *S. aureus*, *Penicillium* spp and *Aspergillus* spp. Moreover, elevated levels of bacterial and fungal contaminants, such as *Penicillium* spp., *Aspergillus spp* and *Fusarium* spp, have been observed in herbs and spices (Kneifel et al., 2002). In this study, *Staphylococcus aureus* and *E. coli* were isolated from Beta cleanser mixtures® and these contaminations can alter the physical, chemical and, to some extent, the pharmacological activity of the herbal product, and hence is said to be detrimental to consumers.

Studies have shown that different alkaloid extracted from numerous medicinal plants possesses a lot pharmacological activity including immunomodulatory activity (Manu and Kuttan, 2009). Kolodziej and Kiderlen (2005) attributed the immune modulatory effect of tanins extracted from different medicinal plant to their ability to cause macrophage activation. Pods of *Acacia concinna* (Leguminosae) contain several saponins which studies have shown to possess immunological adjuvant property (Ratiya et al., 2006). The result of this study was in concordance with the research work of other investigators on immunomodulatory effects of phytochemicals and suggests the origin of the immunological activity test of the herbal mixtures. At 5000 mg/kg body weight, Goko herbal cleanser®, Beta cleanser® and Weifa body defense mixtures® were safe and non-lethal as revealed by the study, hence the oral acute toxicity of these herbal mixtures is greater than 5000mg/kg body weight.

Reticuloendothelial systems are class of cells that occur in widely separated parts of the human body and that have in common the property of phagocytosis, whereby the cells engulf and destroy bacteria, viruses, and other foreign substances and ingest worn-out or abnormal body cells. German pathologist Karl Albert Ludwig Aschoff introduced the term reticuloendothelial system in 1924, collating the cells based on their phagocytic activity. The carbon clearance assay was used to evaluate the effect on reticuloendothelial cell mediated phagocytosis (Jayathirtha and Mishra, 2004). When ink containing colloidal carbon is injected intravenously, the macrophages engulf the carbon particles of the ink. Rate of clearance of (carbon particles) ink from blood is known as phagocytic index. When colloidal ink containing carbon particles are injected directly into the systemic circulation, the rate of clearance of carbon from the blood by macrophage is governed by an exponential equation (Gokhale et al., 2003). Several researches have proven that some medicinal plants have the potential of stimulating the reticuloendothelial cells and increase their phagocytosis ability. Study on the immunomodulatory property of methanolic extract of *Swietenia mahagoni* seeds shows that the extract stimulated the reticuloendotheial system and hence increased the phagocytic index significantly (Hajra et al., 2012; Yadev et al., 2011) administered extracts of *Quisqualis indica* to albino rats and the extract appeared to enhance the phagocytic function by exhibiting a clearance rate of

carbon from the blood stream of the animal by the cells of the reticulo-endothelium system. Ethanolic extract of *Trigonella Foenum-Graeceum* were administered to albino mice and the result indicted an enhanced phagocytic function when compared to the control (Smriti et al., 2012). Methanolic Extract of *Swietenia mahagoni* seeds also enhanced phagocytic function on test animals (Subhadip et al., 2012). From the results obtained, it can be concluded that the test herbal mixtures possessed immunostimulatory property.

Cyclophosphamide is a chemotherapeutic agent used in many experimental protocols such as induced myelo-supression in experimental animals. It is an alkylating agent of the nitrogen mustard type (Takimoto and Calvo, 2005). An alkylating agent adds an alkyl group to DNA. It attaches the alkyl group to the guanine base of DNA, at the number 7 nitrogen atom of the imidazole ring. This interferes with DNA replication by forming intrastrand and interstrand DNA crosslinks (Giraud, 2010). This was said to be the mechanism behind its myelo- supression. White blood cells (WBCs), also called leukocytes or leucocytes, are the cells of the immune system that are involved in protecting the body against both infectious disease and foreign invaders. There are five main types: neutrophils, eosinophils, basophils, lymphocytes, and monocytes. Neutrophils are the most abundant white blood cell, constituting 60-70% of the circulating leukocytes (Alberts et al., 2002). They are important components in the surveillance and protection systems for a broad spectrum of host defenses. They play the main role as an effectors or killer cell for many types of antigenic challenges especially for infections. The primary functions of the neutrophils in host resistance are the migration towards the challenge, which is called 'chemotaxis' and the intracellular killing of microorganisms by the formation of oxygen radicals (Badway and Karnovski, 1980). Lymphocytes include natural killer cells (NK cells) (which function in cell-mediated, cytotoxic innate immunity), T cells (for cell-mediated, cytotoxic adaptive immunity), and B cells (for humoral, antibody-driven adaptive immunity). They are the main type of cell found in lymph, which prompted the name lymphocyte. Prevention of neutropenia induced by cyclophosphamide is suggested to be through the induction of the maturation and activation of macrophages which secretes substances such as colony stimulating factor and interleukin 1 (Heppner and Calabresi, 1976). At a standard dose of 30 mg/kg body weight, cyclophosphamide have been shown to decreases total white blood cell, neutrophil and lymphocyte counts in all groups of the experimental animals. Different studies have demonstrated that some medicinal plant can increase blood parameters of experimental animals administered with cyclo-phosphamide (Stalin and Sampath, 2013) showed that aqueous extract of *Leucas aspera* used to treat immune-suppressed mice gave a result that indicated an increase in neutrophil and total leucocyte counts when compared to cyclophosphamide treated groups. Methanol extracts of fruits of *Solanum xanthocarpum* showed pronounced immunoprotective activity by increasing the depleted levels of total WBC count and RBC, percentage Hb, and percentage neutrophils adhesion in mice treated with cyclophosphamide at a dose of 30 mg/kg body weight (Rokeya et al., 2011; Gupta *et al*., 2010), in their study, demonstrated the immunomodulatory property of ethanolic (50%) extract of *Moringa oleifera* leaves on immune-supressed rats. Eze et al. (2013), while studying the immunologic effects of *M. oleifera* methanolic leaf extract in chickens infected with Newcastle disease virus discovered that the extract stimulated the production of white blood cells. The result showed a dose dependent increase in total WBC and percentage neutrophil counts by an action on both the cellular and humoral immunity when the result is compared to the control group, Hence, the test herbal products are said to be immunostimulatory in action.

Delayed type hypersensitivity (DTH) reaction as the reaction takes two to three days to develop. It is not antibody mediated but a type of cell-mediated response. CD4+ helper T cells recognize antigen in a complex with MHC II major histocompatibility complex on the surface of antigen-presenting cells. These can be macrophages that secrete IL-12, which stimulates the proliferation of further CD4+ Th1 cells. CD4+ T cells secrete IL-2 and interferon gamma, inducing the further release of other Th1 cytokines, thus mediating the immune response. Activated CD8+ T cells destroy target cells on contact, whereas activated macrophages produce hydrolytic enzymes and, on presentation with certain intracellular pathogens, transform into multinucleated giant cells. The DTH response directly correlated with T-lymphocytes especially T-DTH-lymphocytes, therefore increased the effect on cell mediated immunity. When antigens are challenged T-cells, sensitized T-lymphocytes to convert lymphoblasts and secrete lymphokines, attracting more scavenger cells such as macrophages and basophils and induction becomes apparent within 24-72 h in test animals such as rats (Poulter et al., 1982). There are two different types of reactions capable of causing tissue injury in this way. The first, known as delayed type hypersensitivity, (DTH for short) is mediated by CD4+ helper T cells (Th-1 and Th-17 cells). The second, known as cell mediated cytotoxicity, is mediated by CD8+ T cells. The increased response indicates that ethanol extract of *Spilanthus acmella* leaves has a stimulating effect on B-lymphocytes and macrophages killing activity through NO release by stimulating T cell for the hypersensitivity reaction (Yadev et al. 2011). Studies by (Stalin and Sampath, 2013 ; Lu et al. (2007) indicated an increase in DTH reaction in mice in response to T cell dependent antigen; this revealed the stimulatory effect of aqueous extract of *L. aspera* and of *Actinidia*

macrosperma on T cells. In this research, sheep red blood cells (SRBC) are used as the antigen to induce delayed type hypersensitivity reaction in rats and this was used to evaluate the immunomodulatory effect of the herbal mixtures. In DTH reaction, T cells initiate the reaction which leads to activation and accumulation of macrophages, induce vasodilation, increase bascular permeability and as an end result, produces inflammation. This ultimately leads to the increase in the foot pad volume of the immunized animals (Dashputre and Niakwade, 2010). The result stated above shows that the three test herbal mixtures have immunostimulatory property.

Humoral immunity involves interaction of B cells with the antigen and their subsequent proliferation and differentiation to antibody secreting plasma cells (Ose and Muenster, 1968). Antibody functions as the effector of the humoral response by binding to antigen and neutralizing it or facilitating its elimination by cross-linking to form clusters that are more readily ingested by phagocytic cells. Study by Hajra et al. (2012) showed that *Swietenia mahagoni* seeds extract significantly increased circulating antibody titre. This he suggested is as a result of an enhanced responsiveness of macrophages, T and B lymphocyte subsets involved in antibody synthesis. The result showed that high values of hemagglutinating antibody titre obtained in the case of methanolic extract of *Swietenia mahagoni* seeds indicated that immunostimulation was achieved through humoral immunity. Studies on the ethanolic extract of *Trigonella Foenum-Graeceum* leaves at a dose of 200 mg/kg body weight showed a significant agglutination and hence Antibody titre value when compared to the control group. Ethanol extract of *Spilanthus acmella* leaves, at a dose of 250mg/kg body weight showed an augumentation of the humoral response as evidenced by an enhancement of antibody responsiveness to sheep red blood cell antigen in rats as consequence of both pre and post-immunization drug treatment and this indicates the enhanced responsiveness of macrophages and B-lymphocyte subsets involved in antibody synthesis (Yadev et al., 2011). The result obtained shows that the test herbal mixtures have immunostimulatory property.

Conclusion

In conclusion, the three test herbal mixtures passed the microbial bioburden limit assay except for Beta cleanser® that failed the *S. aureus* and *E. coli* limit test, the presence of these microorganisms poses a danger to human upon consumption. The herbal mixtures contain some major plant phytochemicals such as flavonoids, saponins, tannins and alkaloids with established immunostimulatory/immunomodulatory activity. The toxicity profile test showed that the herbal mixtures were relatively safe and post no acute toxic event upon consumption. This study has recognized the immunomostimulatory properties of Goko cleanser®, Beta cleanser® and Weifa body defense mixtures® according to the outcome of the immunological assays done. Comparatively, Weifa body defense mixture exhibited the best immune potentiating activity than the other two herbal mixtures.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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