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Immunostimulatory and Antimicrobial Claims of Super-7 Herbal Mixture (SHM) and Odogwu Cleanser Herbal Mixture (OCHM) Commercially Available in Enugu State, Nigeria

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Abstract: The continuous increase in resistance associated with conventional drugs is gradually shifting focus to herbal remedies as an alternative globally. This study was designed to investigate the immunostimulatory and antimicrobial claims of Super-7 herbal mixture (SHM) and Odogwu cleanser herbal mixture (OCHM) commercially available in Enugu State, Nigeria. Immunomodulatory properties of the products on the Delayed Type Hypersensitivity reaction (DTHr), the Primary and Secondary humoral response and the *in vivo* leucocyte mobilization were evaluated using a total of one hundred and forty-six (146) Swiss albino rats. The spectrum of antimicrobial activity was evaluated by agar well diffusion method using eight (8) selected microorganisms. The LD_{50} of both products was greater than 100% ml/kg body weight. OCHM elicited significant ($p<0.05$) dose dependent increase in total leucocyte and neutrophil counts compared to Levamisole (2.5mg/kg) and the untreated group. A 100% ml/kg SHM (78.07%) also exhibited significantly ($p<0.05$) higher oedema inhibitory potentials compared to the standard drug (levamisole = 65.78%). Samples of SHM demonstrated dose dependent antibacterial activities against *B. subtilis* and *S. typhi*, while only exhibiting antifungal activity against *C. albicans* (IZD = 9.0±0.0 mm). OCHM was observed to inhibit *E. coli, S. typhi, S. aureus, B. subtilis, S. typhi* and the fungus *C. albicans*. An average bioload of 2.8 x 10^4±1.0 CFU/ml which was higher than the average count (2.8 x 10^4±1.0 CFU/ml) obtained for OCHM. Conclusively, this study showed that samples of SHM and OCHM exhibited significant immunostimulatory and antimicrobial properties, but also recorded the presence of microbial contaminants.

Keywords: Super-7 Herbal Mixture, Odogwu Cleanser Herbal Mixture, Antimicrobial Claims, Immunostimulatory, Enugu, Delayed Type Hypersensitivity Reaction (DTHr), The Primary, Secondary Humoral Response

1. Introduction

Research is gradually focusing on natural mechanisms for the management, treatment, and cure of human infections and diseases [1], due to the continuous increase in the development of drug resistance against conventional drugs. Traditional and folklore medicine remains an indispensable repertoire for tackling the health care needs of indigenous people across the globe, and particularly in developing countries. Genuine utility of herbal remedies for treating many ailments are recognized for both traditional and modern medicines [2, 3]. The wealth of natural reserve presented by medicinal plants is not only the bedrock from which newer medicinal agents are launched directly or indirectly, but have also been a therapeutic resort for hard-to-

cure illnesses due to reports of their potentials in cardioprotective, antimicrobial, immunological and neuroprotective therapies [4, 5], and to this ends, vast majority of indigenous people still rely on ethno-medicinal products to resolve their healthcare needs. There is also an increasing positive perception and wide acceptability of herbal medicines in developing countries, [6] which could be credited to both the therapeutic efficacies of herbal formulations in the treatment and management of human diseases, and the associated little or no adverse effects when compared to the alternative orthodox drugs. [7] The use of plants such as *Glycyrrhiza glabra, Panaxnoto ginseng, Zingiber officinale, Astragalus membranaceus*, etcetera for the treatment of various human ailments are well documented to have shown efficacy *in vitro* and *in vivo* studies. [8, 9]

Although, there remains a dearth of research evidence in Sub-Saharan Africa on the drivers and facilitators of traditional, complementary and alternative medicine use, factors associated with its use, and the impact it has on broader healthcare. [10] Several factors are known to contribute to the rise in the current global herbal medicinal products market size. The relative low side-effect profiles, local availability, accessibility, affordability and significant acceptability by patients perhaps, are the most glaring contributors to the popularity gained by herbal formulations when compared to the orthodox drugs. [7] Technically, the world health organization (WHO) through her policies and implementation programme had also encouraged the development and adoption for use of traditional medicines over the years. [3, 11-13] Scientific investigation to verify the therapeutic label claims of these commercially available herbal medicines is feasible and herbal research is even evolving towards herbogenomics. [14] Thus, this present work aims to evaluate the immunomodulatory and antimicrobial activities claims of Super 7 herbal mixture (SHM) and Odogwu cleanser herbal mixture (OCHM), and to justify their use in contemporary times.

2. Materials and Methods

2.1. Experimental Animals

Adult Swiss albino rats (150-200g) of both sexes were used for the experiments. The experimental animals were obtained from the Laboratory facilities of the Veterinary Parasitology and Entomology Department of the University of Nigeria, Nsukka. All the animals were housed and maintained under standard conditions (25 ± 2°C with 12 h of alternating light/dark cycle) in steel cages, and were fed with standard pellet diet and water *ad libitum.*

2.2. Antigen

The antigen used for the delayed type hypersensitivity assay was Sheep red blood cell (SRBC) which was obtained from the Veterinary Parasitology and Entomology department of University of Nigeria, Nsukka.

2.3. Test Microorganisms

Test microorganisms used include: Gram- negative bacteria (*Escherichia coli, Pseudomonas sp*, *Klebsiella pneumoniae, Salmonella typhi),* Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis),* and Fungi (*Candida albicans* and *Aspergillus niger).* All the microbial strains used in this study were clinical isolates deposited in the laboratory of Pharmaceutical Microbiology and Biotechnology Department, University of Nigeria, Nsukka.

2.4. Preliminary Phytochemical Screening of Selected Herbal Mixtures

The Preliminary qualitative phytochemical screening of steroids, alkaloids, glycosides, terpenoids, saponins, flavonoids, and tannins was conducted for the previously described herbal mixtures (SHM and OCHM) according to standard procedures. [15]

2.5. Acute Toxicity Study

In the acute toxicity study, a modified Lorke's method was adopted. The samples were administered in two phases. Phase one aimed to determine the toxic range which involved random placement of the mice into three groups (n = 3) to receive graded doses (6.26, 12.5, 25 (% ml/kg)) of each sample, orally administered every 12 hours. The treated mice were observed for 24 hours for number of deaths. The doses for the second phase were determined by death pattern in the first phase. A fresh batch of two mice each was administered 50% and 100% ml/kg of the sample respectively. The animals were observed for signs of acute intoxication for 24 hours. The LD_{50} were calculated as the median lethal dose of the test substance. [16]

2.6. Immunological Studies

Carrageenan Induced Leucocyte Mobilization in Rats

Swiss albino rats were randomly allotted into five groups (n = 5). Groups 1, 2, and 3 received the sample (25%, 50% or 100% ml/kg). Group 4 received Levamisole (2.5 mg/kg) as the standard control and group five was left as the untreated group. All samples were orally administered relative to the body weight of the rats. One hour later, each rat was given an intra-peritoneal injection containing 0.5 ml of 1% (w/v) carrageenan suspended in normal saline. The rats were sacrificed after the next four hours and the peritoneal cavity washed with 5 ml of 5% solution of Ethylenediaminetetraacetic acid (EDTA) in Phosphate buffer saline (PBS), thus recovering the peritoneal fluid. [17, 18] Total and differential leucocyte counts in the peritoneal fluid were carried out using Abacus analyzer (Buchi, Swtzerland).

Sheep Red Blood Cell (SRBC) Induced Delayed-Type Hypersensitivity Assay

Induction of Delayed type hypersensitivity was in the albino rats using the SRBC as antigen. Five groups (n =5) of rats were used for this test. Groups 1 to group 3 received 25%, 50% or 100% ml/kg of the sample respectively. Group

4 was treated with 2.5 mg/kg of Levamisole as standard control, while group 5 was received no treatment. Drug administration started after one hour when each animal received 0.1 ml of 40% sheep red blood cell (SRBC) suspension by means of parenteral route into the sub-plantar region of the right hind paw. The first day was considered as day zero. Daily oral administration of test sample (SHM or OCHM) was continued for seven days. On the 7th day, the animals were then challenged by injecting 0.1 ml of 40% SRBC into the left sub-plantar of the animal paw. The extent of delayed-type hypersensitivity (DTH) response in the rats was determined by measuring the footpad thickness after 4, 8, and 24 h of challenge using vernier calipers. The difference in the thickness of the right hind paw and the left hind paw was then used as a measure of DTH reaction and was expressed as a mean percent increment in thickness/edema. [19]

2.7. Hemagglutination Antibody Titre in Rats

The hemagglutination antibody titer assay was done using five groups (n=5) of rats. The first three groups received 25%, 50%, 100% ml/kg of the sample. Group 4 was treated with 2.5 mg/kg of Levamisole as standard drug while last group (Group 5) received no treatment. All samples and controls were orally administered. One hour later, each animal received 0.1 ml of 40% sheep red blood cell (SRBC) suspension carefully injected into the sub-plantar of the right hind paw. The day was considered as day zero. Daily administration of the drugs as described above was repeated for the next seven days. On the 7th day, the retro-orbital plexus was used as site for blood collection and then the serum samples were separated and used for estimation of primary hemagglutination antibody titre. Two-fold diluted serum in saline (25 µl) was challenged with 25 µl of 1% (% v/v) SRBC in U-shaped microtitre plates and incubated at 37°C for one hour and then hemagglutination for primary antibody titre was observed. The animals were challenged on day 7 by injecting 0.1 ml of 40% SRBC into the sub-plantar of the paw after a second blood sample collection via retro-orbital plexus. Drug administration was continued for the next seven days. On day fourteen, blood samples collection for secondary antibody titre assay was carried out. [20]

2.8. Antimicrobial Assay

The microbiological studies carried out involved the determination of the sensitivity of some microorganisms, both bacteria and fungi, to the herbal mixtures. These *in-vitro* antimicrobial studies were determined by the agar diffusion method as described in literatures. [21, 22] From the stock solution (100%%v/v) samples were prepared in distilled water by serial dilutions to arrive at five concentrations:(100%, 50%, 25%, 12.5% and 6.5%)%v/v. Agar plates were divided into four sections corresponding to the different concentrations of a particular sample. Each labeled medium plate was uniformly inoculated with overnight test organism adjusted to Mc Farlands standard, by using a sterile cotton swab. A sterile cork borer of 6 mm diameter was used to make wells on the medium. 0.1 ml of the various samples were dropped into each well in the increasing order of concentration. Dimethyl sulfuroxide (DMSO) served as negative control while Ciprofloxacin and Fluconazole served as positive control for bacteria and fungi, respectively. The inoculated plates were allowed to stand for one hour to allow the samples to diffuse into medium. The bacterial and fungal plates were incubated at 37°C for 24 hours and 27°C for 48 hours, respectively. The tests were carried out in triplicates, and inhibition zone diameters (IZD) produced by the samples against the test organisms were measured and recorded. The IZDs above 6mm was taken as significant susceptibility. [21, 23]

2.9. Determination of Bioburden of Herbal Products

The bioburden of the samples was evaluated using method as described by. [24] A precise volume (1 ml) of each herbal mixture sample (SHM and OCHM) was aseptically transferred into a sterile test tube containing 9 ml of sterile distilled water of which ten-fold serial dilutions were subsequently carried out. The following dilutions 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} of the herbal mixtures were aseptically transferred into clean sterilized Petri dishes and mixed with 20 ml of sterile nutrient agar for bacteria and Sabouraud Dextrose agar for fungi, allowed to cooled to 45°C. For bacteria counts, plated were incubated at 37°C for 24 h, while for fungal counts, plates were incubated at 27°C for 72 h. The procedures were done in triplicates for each sample and colony counts obtained expressed in CFU/ml.

2.10. Statistical Analysis

The results of the study are presented as Mean ± standard error of mean (SEM), where n= 5 in most cases. Data obtained were analyzed with SPSS (version 16.0) and thereafter subjected to least significant difference (LSD) post-hoc test. Differences between means of treated and control groups were accepted as significant at p-value less than 0.5 ($p<0.05$).

3. Results

3.1. Preliminary Phytochemical Screening

Result of preliminary phytochemical screening of Super 7 herbal mixture (SHM) and Odogwu cleanser herbal mixture (OCHM) revealed the presence of alkaloids, flavonoids, glycoside, carbohydrates, reducing sugar, tannins, steroids, saponins and terpenoids. However, samples of SHM and OCHM showed absence of steroids and reducing sugars respectively (Table 1).

Table 1. *Preliminary Phytochemical Screening.*

S/N	Phytochemical	Test/Reagents	SHM	OCHM
1	Alkaloids	Dragendorff	++	++
2	Flavonoids	Ethyl acetate in ammonia	++	++
3	Glycosides	dil. H_2SO_4 + Fehling Solution I & II	++	++
4	Carbohydrates	Molisch's test	++	++
5	Reducing sugars	Fehling's reagent	++	-
6	Tannins	Ferric chloride	++	++
7	Steroids	Ethanol + Chloroform + conc. H_2SO_4	-	++
8	Saponins	Frothing test	++	++
9	Terpenoids	Ethanol + Chloroform + conc. H_2SO_4	++	++

Key: - = absence, ++ = present

3.2. Acute Toxicity (LD_{50}) Test

Table 2. *Acute Toxicity (LD_{50}) Test.*

S/N		Dose of extract (% ml/ml)	Mortality	
			SHM	OCHM
1	Phase 1	6.25	0/3	0/3
2		12.5	0/3	0/3
3		25	0/3	0/3
4	Phase 2	50	0/2	0/2
5		100	0/2	0/2

The result of the acute toxicity tests presented in Table 2 showed that the LD_{50} for both SHM and OCHM is greater than 100% ml/kg. Both herbal mixtures did not cause any sign of acute intoxication or death within 24h post-treatment at all dose levels (6.25%, 12.5%, 25%, 50% and 100% ml/kg) tested in the two phases.

3.3. Results of Immunological Studies

3.3.1. In-Vivo Effect of Samples on Leucocyte Mobilization

In the result of i*n-vivo* effect of samples on leucocyte mobilization (Table 3), all the tested doses (SHM, OCHM and Levamisole) improved the total leucocyte counts compared to the control (untreated group). Samples of SHM and OCHM elicited a dose related increase in total leucocyte count (TLC) that was quite significant ($p<0.05$). The highest activity generated by SHM (1830.1±0.55 cell/mm^3) at 100% ml/kg was significantly lower than the standard control (2.5 mg/kg Levamisole = 6914±2.12 cell/mm^3). However, OCHM (7028±1.14 cell/mm^3) was observed to be significantly higher than the standard control. The differential leucocyte count of neutrophils was significantly different ($p<0.05$) between all treatments and the controls. SHM displayed non-dose related relationship, while OCHM demonstrated a dose related increase in neutrophil counts; at 50% ml/kg (neutrophil count = 15.10±0.04 cell/mm^3) and 100% ml/kg (neutrophil count = 15.50±0.04 cell/mm^3). SHM also elicited non-dose related decrease in lymphocyte count. 25% ml/kg of SHM elicited a significantly higher lymphocyte count (89.14±0.01 cell/mm^3) compared to Levamisole (14.36±0.01 cell/mm^3), while 100% ml/kg showed no significant difference ($p>0.05$) with Levamisole. On the other hand, OCHM demonstrated dose related decrease which was significantly different ($p<0.05$) for all treatment compared to the standard control. Only 25% ml/kg of OCHM elicited a significantly higher lymphocyte count (87.24±0.01 cell/mm^3) compared to Levamisole (14.36±0.01 cell/mm^3). At 100% ml/kg SHM, 100% ml/kg OCHM and 2.5 mg/kg Levamisole monocyte count of treatments were all significantly different with one another, but all showed increase in number when compared with the control group.

Table 3. *In-vivo effect of Samples on Leucocyte mobilization.*

S/N	Treatment	Dose	TLC	Differential Leucocyte Count (%)		
			(cell/mm^3)	Neutrophils	Lymphocytes	Monocytes
1	SHM	25% ml/kg	1366.0±1.4^a	8.48±0.01^a {30.8}	89.14±0.01^g (1.3)	0.0±0.00^a
		50% ml/kg	1726.5±0.63^b	14.24±0.01^e (16.2)	79.36±0.05^a {9.8}	0.0±0.00 ^{a}
		100% ml/kg	1830.1±0.55^c	9.28±0.01^b {24.3}	85.50±0.07^d {2.8}	0.1±0.02^b
2	OCHM	25% ml/kg	9544±1.07^h	11.28±0.01^c {8.0}	87.24±0.01^e {0.9}	0.0±0.00^a
		50% ml/kg	7959±1.33^g	15.10±0.04^g (23.2)	84.48±0.01^c {4.0}	0.0±0.00^a
		100% ml/kg	7028±1.14^f	15.50±0.04^h (26.4)	82.36±0.00^b {6.4}	0.2±0.10^c
3	Levamisole	2.5 (mg/kg)	6914±2.12^e	14.36±0.01^f (17.1)	85.42±0.03^d {2.9}	0.3±0.10^d
4	Control	2.5 ml/kg	5364±1.89^d	12.26±0.01^d	88.00±0.16^f	0.0±0.00^a

Key: TLC: Total leucocyte count, n=5 per group; Values are expressed as Mean ± SD. Values in the same column with different alphabets as superscripts differ significantly at p<0.05 when compared to control LSD post-hoc; Values in parenthesis represent increase IN percentages (%) of TLC, Neutrophil or Lymphocyte, respectively to control; Values in curly brackets {%} signifies percentage decrease in TLC, Neutrophil or Lymphocyte respectively compared to control.

The result of *In-vivo* effects of SHM, OCHM and Levamisole on delayed type hypersensitivity reactions are shown in Table 4. SHM significantly (p<0.05) inhibited SRBC induced oedema when compared with the control group. The peak inhibitory effect of the SHM was recorded with a dose of 100% ml/kg (78.07%) at 6 h. Comparatively, 100% ml/kg OCHM displayed a poor activity with 29.82% inhibition. Statistically, this was observed to be significantly lower compared to Levamisole (2.5 mg/kg) after 6 h treatment.

Table 4. *In-vivo effect of Samples on Delayed Type Hypersensitivity Reactions.*

S/N	Treatment	Dose	Oedema (ml)	Inhibition of oedema (%)
1	SHM	25% ml/kg	0.199±0.001^{e}	12.72
		50% ml/kg	0.104±0.001^{c}	54.39
		100% ml/kg	0.05±0.000^{a}	78.07
2	OCHM	25% ml/kg	0.219±0.001^{f}	3.95
		50% ml/kg	0.196±0.000^{e}	14.56
		100% ml/kg	0.16±0.001^{d}	29.82
3	Levamisole	2.5 mg/kg	0.078±0.000^{b}	65.78
4	Untreated	-	0.228±0.000^{g}	-

Key: n=5 per group. Values are expressed as Mean ± SD. Values in the same column with different alphabets as superscripts differ significantly at p<0.05 compared to control LSD post-hoc.

3.3.2. In-Vivo Eaffect of Samples on Hemagglutination Antibody Titre

The result of hemagglutination antibody titre is shown in table 5. There are observable dose dependent increases between the primary readings and secondary readings recorded. Maximal enhancement of secondary antibody response was found with rats administered with 100% ml/kg body weight of SHM. There was no significant (p>0.05) difference when the titre value of the group that received 100% ml/kg was compared to the standard drug. Groups that received 25% ml/kg (with titre value = 7.50±0.45) differed significantly (p<0.05) with groups that received 50% ml/kg and 100% ml/kg of SHM both of which had dose related increased titre values of 8.1±0.26 and 9.2±0.27 respectively. There was no significant (p>0.05) difference in the primary readings when the titre value of the group that received 50% ml/kg body weight was compared to the group that received 100% ml/kg OCHM and 2.5 mg/kg of levamisole.

Table 5. *In-vivo effect of Samples on Haemaglutination Antibiotic Titre.*

S/N	Treatment	Dose	Haemaglutination Antibody Titre	
			Primary	Secondary
1	SHM	25% ml/kg	4.1±0.07^{c}	7.5±0.34^{a}
		50% ml/kg	4.1±0.04^{c}	8.1±0.75^{b}
		100% ml/kg	3.7±0.06^{a}	9.2±0.01^{d}
2	OCHM	25% ml/kg	3.6±0.03^{ac}	7.5±0.00^{a}
		50% ml/kg	4.0±0.03^{b}	8.3±0.01^{c}
		100% ml/kg	4.1±0.03^{c}	8.4±0.01^{c}
3	Levamisole	2.5 mg/kg	4.7±0.03^{d}	9.7±0.04^{d}
4	Control	2.5 ml/kg	3.8±0.05^{ab}	7.4±0.01^{a}

Key: n=5 per group; Values are expressed as Mean ± SD. Values in the same column with different alphabets as superscripts differ significantly at p<0.05 compared to control LSD post-hoc.

3.4. Microbiological Studies

3.4.1. Result of Antimicrobial Assay

The results of antimicrobial activity of the herbal mixtures in this study are presented in table 6. The mixtures displayed a concentration dependent antimicrobial activity against the test isolates. At 100% concentration, OCHM demonstrated the best inhibitory activity with broad spectrum activity against *E. coli* (17.7±0.6), *S. typhi* (14.3±0.6), *S. aureus* (15.1±0.2), and *B. subtilis* (27.5±0.6). These obtained inhibition zones were observed to be higher than that of the positive control Ciprofloxacin. All other bacterial isolates used in the study were resistant to both herbal mixtures. Similarly, OCHM displayed the best antifungal activity against *C. albicans* (17.0±0.0). Both herbal mixtures were also observed to be resisted by *A. niger*.

Table 6. *Inhibitory Zone Diameter (IZD) of test organisms to SHM and OCHM.*

Organism	Sample	6.25%	12.5%	25%	50%	100%	CIP	FNZ
E. coli	SHM	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	11.0±0.0	-
	OCHM	0.0±0.0	9.2±0.3	12.0±0.0	14.0±0.0	17.7±0.6		
S. typhi	SHM	0.0±0.0	0.0±0.0	0.0±0.0	8.0±0.0	10.0±0.0	13.0±0.0	-
	OCHM	0.0±0.0	0.0±0.0	0.0±0.0	10.0±0.0	14.3±0.6		

Organism	Sample	6.25%	12.5%	25%	50%	100%	CIP	FNZ
K. pneumonia.	SHM	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	11.0±0.0	-
	OCHM	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0		
P. aeruginosa	SHM	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	13.1±0.0	-
	OCHM	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0		
S. aureus	SHM	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	14.1±0.0	-
	OCHM	0.0±0.0	8.0±0.0	8.9±0.2	12.0±0.3	15.1±0.2		
B. subtilis	SHM	0.0±0.0	13.0±0.0	20..0±0.0	25.0±0.0	31.0±0.0	18.9±0.0	-
	OCHM	6.3±0.6	13.0±0.0	17.0±0.2	22.3±0.6	27.5±0.6		
C. albicans	SHM	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	9.0±0.0	-	17.0±0.1
	OCHM	0.0±0.0	8.0±0.0	11.2±0.0	14.8±0.0	17.0±0.0		
A. niger	SHM	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	-	12.07±0.0
	OCHM	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0		

Key: SHM = Super 7 Herbal Mixture, OCHM = Odogwu Cleanser Herbal Mixture, CIP = Ciprofloxacin, FNZ = Fluconazole - = Not tested for

3.4.2. Result of Bioburden Test

The result of the bacterial count obtained from both herbal mixtures are presented in Table 7. The herbal mixture OCHM was observed to have a higher bacterial count of 4.0 x 10^5 ± 2.33 cfu/ml compared to the 2.8 x 10^4±1.0cfu/ml obtained for SHM.

Table 7. *Sterility test results.*

Product	Batch No.	Bacteria count (CFU/ml)	
		Number	Ẋ±SEM
SHM	Plate A	27	2.8 x 10^4±1.0
	Plate B	27	
	Plate C	30	
OCHM	Plate A	44	4.0 x 10^5±2.33
	Plate B	36	
	Plate C	41	

4. Discussion

This study investigated the immunomodulatory and antimicrobial activities of two herbal preparations, Super 7 herbal mixture (SHM) and Odogwu cleanser herbal mixture (OCHM) respectively. Samples of both products had evidence of NAFDAC registration and were within their viable shelf-life as at the time of this investigation. Modified acute toxicity study of SHM and OCHM on albino rats (Swiss) showed that the oral LD_{50} of each of the herbal mixture were greater than the maximum dose (100% ml/kg) and implies a remote risk of possible acute intoxication or death.

The result of the phytochemical analysis of SHM and OCHM respectively revealed the presence of secondary metabolites which include alkaloids, flavonoids, glycosides, saponins, tannins, reducing sugar, carbohydrates and terpenoids. These phytochemical compounds have been reported to play crucial roles in bioactivity of medicinal plants. For instance, the *Aloe vera* (5%) and *Azadirachita indica* (10%) content of SHM as well as *Mangifera indica* (10%) and *Zingiber officinale* (10%) content of OCHM are all reported to possess immunomodulatory activities as a result of their phytochemistry. *Aloe vera* (L.) Burm f. (Asphodelaceae) leaves increases phagocytosis and stimulates the production of superoxide, which implies possession of anti-oxidative potentials. [25, 26] The health-promoting effects of Neem plant (*Azadirachta indica*) are also attributable to its antioxidant constituents. *Azadirachta indica* A. Juss. (*Meliaceae*) leaves are known to increase IgM and IgG production and inhibits nitric oxide (NO) synthesis, as well as degranulation of neutrophils. [27, 28] Phytoconstituents of this plant are capable of modulating numerous molecular pathways like p53, pTEN, NF-κB, PI3K/Akt, Bcl-2 and VEGF without any adverse effect. It has played pivotal role not only in the management of cancer, but has also served in dentistry, antinephrotoxicity, growth promotion, neuroprotection and immunomodulatory management of disease cases. [29]

Leaves extract of *Mangifera indica* L. (Anacardiaceae) contain mangiferin, phenolic acid, benzophenones and other antioxidants such as flavonoids, carotinoids, ascorbic acid and tocopherols. The extracts from mango leaves (MLs) have been studied for their biological activities, including anticancer, antidiabetic, antioxidant, anti-microbial, antiobesity, lipid-lowering, hepatoprotection, and antidiarrheal. [30] *Mangifera indica* was also reported for its potential to increase in humoral antibody titre and DTH enhanced production of IgG1 and IgG2b. [31] The rhizome of *Zingiber officinale* (Zingiberaceae) contains gingerol which is a potent B cell stimulant, and posses pharmacologically verified benefits including antimicrobial, antiviral, antidiabetic, antihypertensive, gastroprotective, cardioprotective, chemopreventive, anticancer, and immunomodulatory activities. [32, 33] These culinary and medicinal properties of *Zingiber officinale* have been linked to the presence of specific phytochemicals likeβ-bisabolene, shogaols, cineole, gingerols, limonene, pardols, β-phellandrene, zingerone, curcumene, geranyl acetate, terphineol, terpenes, geraniol, β-elemene, zingiberol, borneol, linalool, α-zingiberene, β-sesquiphellandrene, zingiberenol and α-farmesene. [32, 34] However, recent study highlighted the possibility of extensive reactive lymphoid hyperplasia in the splenic cells associated with ethanol extract of *Zingerber officinale*, [35] hence, entails the application of cautions when administered in high doses.

The search for use of medicinal plants and identification of phytochemicals have recently witnessed a positive surge since some phytocompounds have shown antiviral potentials to disrupt various stages of viral life cycle and may alters

virus-specific host targets. [36] Fruits, beverages, leaves etcetera containing tannins has been recommended for inclusion daily diet to prevent and also combat various types of health ailments. Tannins have also been used for its customary protective effect on inflamed gastrointestinal surfaces and in the therapeutic management of wounds, hemorrhoids, diarrhea and catarrh. [36, 37] Phytochemicals like alkaloids, flavonoids, tannins, glycosides and sterols from *Adhatoda vasica* Linn; tannins of *Gymnema sylvestre origin*; and sterol, tannins, terpenoids, and proteins of *Azadirachta indica* have been shown to possess considerable antimicrobial and immunomodulatory properties. [38] The anti-allergic, antioxidant, analgesic and antimicrobial properties of bioflavonoids have been described as potentials which make them essential components of herbal medicines. [39] Bioflavonoids also possess protective pharmacological actions against platelets aggregation, allergies, inflammation, ulcers, tumours, hepatotoxins, and microbial pathogens. [40] Presently, pharmaceutical companies are developing innovative dosage forms to boost the market share of herbal products; some bioflavonoids with vasoprotective functions are readily obtainable in capsular forms in community pharmacies.

Findings on leucocyte mobilization test showed that both herbal mixtures significantly increased the total leucocyte counts, and also increased differential neutrophil and monocyte counts compared to control (untreated group). The differential count showed the granulocytes, neutrophils were significantly mobilized. These granulocytes are vital immune cells that play a significant role in the inflammatory process and have a profound effect on microbial infections and pathogens. [41] As subtype of the immune cells, polymorphonuclear leucocytes (PMNs) are well adapted to engulf and eliminate invading pathogens through phagocytosis. Accordingly, neutrophils represent the major phagocyte of the innate immunity that is pivotal in host defense even against staphylococcal bacterial infections. [42] This immune process is actualized through systematic opsonization of the bacteria with immunoglobulins and complement factors which enables efficient microbial recognition by the neutrophil and subsequent intracellular compartmentalization, killing and elimination of the immunogen. [43] Therefore, chemotactic movement of neutrophils towards antigens represents the early most crucial step in phagocytic elimination of the foreign body. The mechanisms that facilitate G- protein subunit $G\alpha_{13}$ facilitated motility and directionality of neutrophils to their chemoattractants have been described. [44] This process requires both temporal and spatial regulation of intracellular signaling pathway, which allows the β_2 integrins of neutrophils to detect, polarize, and migrate towards the area with highest concentration of the chemo-attractant with optimal speed.

The result also shows that higher titre of monocytes were mobilized at 100% ml/kg SHM (0.1±0.02), 100% ml/kg of OCHM (0.2±0.10) when compared to that of Levamisole treatment (0.3±0.10). At optimal dosage, all treatments were significantly higher than the monocyte count for the untreated group (0.0±0.00 cells/mm^3). Monocytes are precursors of tissue macrophages. When in the blood stream, macrophages float in the form of monocytes, but transform into tissue macrophages once they enter the interstitial space where they share the phagocytic function with neutrophils and also act as antigen presenting cells to T cells. Immunomodulatory role of flavonoid contents is vast, and could also be vital in this process whereby it activates pro-inflammatory to anti-inflammatory macrophages, leading to leukocytosis and improved neutrophil function. [45]

Again, sheep red blood cells (SRBC) induced delayed type herpersensitity reactions (DTHR) was significantly enhanced in all treatments compared to the untreated group. Both herbal mixtures showed dose dependent inhibitory activities. This increase in inhibition of oedema suggests clear potentiation of DTHR by both herbal mixtures with concurrent enhancement of leucocyte migration potentials under inflammatory stimulus. Similar mechanism of action is thought to be involved in the activation of DTHR by Levamisole, which is a synthetic drug that belongs to the class of imidazothiazole derivatives. Levamisole is mainly indicated for use in single-dose as an anthelmintic agent. As an antihelminthic, the drug exerts an agonistic action on the nicotinic acetylcholine receptors. This essential medicine has also demonstrated anti-parasitic, anti-viral and anti-bacterial agent, [46-48] hence, has been used in the treatment of various forms of microbial infections.

Levamisole has recently been deployed in myriad cases of inflammatory pathologies and as an adjunct in cancer therapy in association with 5-fluorouracil due to of its potent immunomodulatory properties. The mechanisms of immunomodulatory properties of levamisole are diverse, and yet to be clearly elucidated. It is thought that Levamisole acts principally on macrophages and T-lymphocytes to augment their phagocytosis, chemotaxis, adherence, intracellular killing, E-rosette formation, antigen-induced proliferation and delayed-type hypersensitivity functions. [49] Levamisole seems to efficiently recruit T helper-1 cells to which it have a preferential effect, subsequently up-regulate the serum titre of interleukin-2, interleukin-12 and interferon-γ. [49] Research has shown that it also has the potentials to inhibit the action of endogenous immunosuppressive factors such as the soluble immune response suppressor, and can also affect leucocyte counts by modulating B-cells lymphocytes, an effect which outcome may reflect as reduction in immunoglobulin G (IgG), immunoglobulin M (IgM) and circulating immune complex levels. [50] Herbal medicines that possess levamisole-like potentials to increase natural killer (NK) cells and activate T-cells, may exhibit anti-anergic properties significance enough to boost compromised immunity in humans.

Hemagglutination assay was performed to ascertain the effect of SHM and OCHM on enhancing the humoral immune response. Humoral-mediated immunity is practically orchestrated either by bursa equivalent lymphocytes or plasma cells following sensitization to specific antigen and

involves the synthesis of specific immunoglobulins. The results demonstrated that administration of SHM had a potential effect on stimulating the humoral immune response. This reflected as increase in the secondary hemagglutination antibody titer to SRBC antigens. The increase in antibody titre was only consistent and coincided with the increase in the lymphocyte counts by SHM at 50% ml/kg and 100% ml/kg. Research has also demonstrated that the primary responses consist mainly of humoral immunoglobulin G (IgG) while secondary hemagglutination response involves the interactions of B-cell lymphocytes. [51] Humoral immunity is a product of the body's lymphoid mechanism which is mediated by B-cell lymphocytes secreted antibodies. Antibody synthesis does not require B-cell lymphocytes working in isolation to produce antibodies. Instead it consists of the B-cells activation by the alpha (α-) globulin antibodies once in contact with antigenic substances such as proteins or polysaccharides foreign to the body, and cooperation of activated B-cells with macrophages and T lymphocytes to achieve the required outcome. [52, 53] B-cells are programmed, therefore, to produce antibodies when sufficiently stimulated, but they lack the capacity of self-stimulation.

In the antimicrobial screening of test microorganisms, the sample of SHM demonstrated dose dependent antibacterial activities against the clinical isolates *S. typhi*, *B. subtilis* and antifungal activity against *C. albicans* at 100%%v/v concentration. OCHM on the contrast was active against *E. coli, S. typhi, S. aureus, B. subtilis,* and *C. albicans*. This indicates that OCHM had a broader spectrum of activity when compared to SHM. Neither of the herbal mixtures demonstrated antimicrobial actions against *K. pneumoniae*, *P. aeruginosa* or *A. niger*. Thus, indicating that these organisms are resistant both herbal mixtures, even at 100% concentration. Again, the bioburden recorded for both herbal mixtures were high, albeit within permissible limit. Microbial contamination of herbal medicines can spoil such products and could contribute as source of infection if potentially virulent organisms are present. Microbial contamination of non-sterile pharmaceutical products not only have the potentials to minimize or even deactivate therapeutic properties of the medicines, but can also impact negatively on the health of the patients who patronize such products. [54, 55] Spoilage pattern of herbal medicines also involves initial microbial invasion which leads to biodegradation of wholesome products. [56] Once colonized, microbial activities on the complex nutrient media alters the surrounding pH and causes further deterioration of the product, and perhaps renders the product unwholesome for human consumption.

Apart from impact of potential microbial contamination, many herbal products are constituted with plants found to be toxic to specific organs of the body serious adverse effects. Disturbing reports of gastrointestinal side effects and more serious adverse effects such as liver and kidney toxicities have been cited in recent literatures, [57] thus, implicating some traditional medicinal plants like *Alstonia boonei* which may induce testicular and kidney damage while *Azadirachta indica, Morinda lucida,* and *Enantia chlorantha* were found to induce mutagenesis in modified Ames assays. [58] As a result of which the WHO developed guidelines to ensure safe monitoring and use of herbalproducts within the existing WHO pharmacovigilance frame-work. [59] The implementation of global public health policies on herbal medicinal products, therefore, entails that serious focus be channeled towards the thorough investigation of herbs used in traditional medicine formulation. Rigorous standardization measure of herbal products, as well as proper control and abatement of evident environmental contaminations must be checked, as these factors can potentially pose toxicity to patients, amidst other public health concerns.

5. Conclusion

This study showed that the herbal mixtures, SHM and OCHM, exhibited significant immunostimulatory properties. Both products also possess broad spectrum antimicrobial activity against some test microorganisms, which could be attributed to the presence of specific secondary metabolites present in these mixtures. Consequently, the products may be beneficial for the treatment of infectious and immune related diseases if quality control criteria of products are achieved through good manufacturing process (GMP).

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