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Immunostimulatory activity of aqueous leaf extract of *Cassia occidentalis* on human neutrophils

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Abstract
The use of herbal extract in the treatment of infectious disease has been a regular practice in Nigeria and other developing countries. The study aim was to evaluate the immunostimulatory effect of aqueous leaf extract of *Cassia occidentalis* on human neutrophils. Thirty (30) healthy volunteers were recruited into this study, consisting of 15 male and 15 female. Blood sample collected was used to isolate the Neutrophils. Different concentrations (25 µg/ml 50 µg/ml, 100 µg/ml and 1mg/ml) of the plant extract were prepared. Giemsa stain was used to determine the neutrophils phagocytic function while methylene blue was used to carryout micro bicidal capacity analysis. There was significant increase in phagocytic index and micro bicidal capacity of neutrophils with increase in the plant extract concentration ($p<0.0001$). This study suggested that *Cassia occidentalis* leaf has potential immunostimulation of phagocytic activity and micro bicidal capacity on human neutrophils. As such this will enhance the phagocytic activity and micro bicidal capacity of the neutrophils.

Keywords: cassia occidentalis, human, immunostimulation, neutrophils, plant extract

Introduction
Previous studies highlight the importance of using medicinal plants for treatment of ailment which pre-exist thousand years ago [1, 2]. Thousands of plants were listed by the World Health Organization (WHO) as plants with medicinal importance [3]. Plant materials are still use widely in some part of the world as herbal medicine despite the advent of the modern medicines [4, 5]. These herbal medicine includes plant materials as formulation such as root, bark or leaves [6]. Some of these herbal medicine can be able to stimulate, suppress or modulate any part of the immune system [7], which comprise of innate and adaptive immune response. Immunomodulator can stimulate or suppress immune response. Immunostimulation increase immune reactions via stimulation of immune cells such as granulocytes, neutrophils, macrophages and lymphocytes [8, 9]. Regulation of immunostimulation and immunosuppression is important in order to control normal immune process [10]. Natural immunomodulators act to improve weak immune system and down regulate over reactive immune response [11, 12].

The use of herbal formulation is rampant globally especially when it comes to health issues [13]. Besides many of the herbal were claim to have immunomodulatory activities but were not fully researched only depends on local herbalists claim. The herbal medicine could be adulterated [14] or poorly regulated [15] as such can risk the life of an individual due to adverse effect and toxicity [14]. It is important to assess local herbal formulation on immunomodulatory activity, safety and toxicity as claimed [16].

Leaves of *Cassia occidentalis* has a wider application for treatment of diseases or conditions such as fever, ringworm, throat infection, cutaneous diseases etc [17]. It was also reported to have anti-inflammatory, immunosuppressive activities, anti-diabetic, etc [18]. Phytotoxins were also reported present in the plant [19]. However, the nature and amount of the phytochemicals in the plant depends on geographical location as well as season [20].

We understand there is paucity of data on the effect of the leaves extract of *C. occidentalis* on human neutrophils and there is no enough information on the rampant unprescribe consumption of the plant and desired effect which may be beneficial or harmful to its consumers. Therefore this study is set to determine the immunostimulatory activity of the aqueous leaf extract of *C. occidentalis* on human neutrophils this will provide an insight on immunodulatory effect of the plant on the cells. Neutrophils are the most predominant and indispensable immune cells which its function is to patrol and protect the body against infections [21].

2. Material and Methods

2.1 Study area

The study was carried out in the Department of Immunology, School of Medical Laboratory Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria.

2.2 Study subjects

The study subjects were thirty healthy volunteers with no sign or symptom of any disease at the time of phlebotomy. They comprise of 15 males and 15 females and age ranges from 20-30 years. The mean and Standard Deviation (SD) of their age was 25.37 (2.67) years. All subjects signed an informed consent. However, those individuals who did not gave consent and/or suffering from any kind of diseases were excluded in this study.

2.3 Plant collection and identification

Fresh leaves of *C. occendatalis* were collected locally. The plant taxonomic identification was carried out at the Herbarium section of Botany Unit, Department of Biological Sciences, Faculty of Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria. The voucher number issued was UDUS/ANS/0110.

2.4 Preparation of the leaves extract

Fresh leaves of *C. occidentalis* were washed thoroughly, shade-dried, coarsely powdered using mortar and pestle and sieved. After that 1300 g of the dried powder was dissolved in 7 L of distilled water. The preparation was left to soak for 24 hours in a water bath set at 40 ºC. The preparation was filtered using Whatman No. 1 filter paper. The resultant filtrate was concentrated to dryness at 40 ºC under reduced pressure [22]. About 0.1 g of the dried extract was dissolved in 1 ml of distilled water and this served as stock.

2.5 Sample Collection and Processing

Five millilitre (5 ml) of whole blood was collected using Monovette vacutainer system, out of which 3 ml was transferred into a lithium heparin tubes and mixed properly. The blood was used for isolation of Neutrophils while the remaining 2 ml was dispensed subsequently into a clean, plain Vacutainer tubes which was labeled and allowed to clot. The clotted blood was centrifuged at 3000 rpm for 10 minutes. The serum obtained was then transferred into sterile separation tubes, pooled and stored immediately at -20°C until used.

2.6 Isolation of human neutrophils

This procedure was followed according to the manufacturers' instruction. Three millilitres (3 ml) of Histopaque-1119 (Sigma-Aldrich, Co., UK) were added to a 15 ml conical centrifuge tube and bring it to room temperature then the 3 ml of whole blood were layered carefully onto the Histopaque-1119 and centrifuged (Biofuge 200, USA) at 400 × g for 30 minutes at room temperature. After centrifugation, the tubes were carefully removed from the centrifuge and upper layer was aspirated with a Pasteur pipette to within 0.5 cm of the opaque interface containing granulocytes. The upper layer was discarded. The opaque interface was carefully transferred with a Pasteur pipette onto a clean conical centrifuge tube. The cells were washed by adding 10 ml of isotonic phosphate buffered saline solution and mixed by gentle drawn in and out of a Pasteur pipette. The mixture was centrifuged at 250 × g for 10 minutes. The supernatant was aspirated and discarded. The cells pellets was resuspended with 5 ml of isotonic phosphate buffered saline solution and mixed by gentle drawn in and out of a Pasteur pipette and centrifuged at 250 × g for 10 minutes. The procedure was repeated again and the supernatant was discarded. The mixture was centrifuged at 250 × g, for 6 minutes at 5° C. The supernatant was discarded. The procedure was repeated again until the cell pellets appeared relatively free of RBC. The cell pellet was resuspended in 2 ml of cold Roswell Park Memorial Institute medium (RPMI 1640) (Sigma-Aldrich, Co., UK).

2.7 Treatment of neutrophils with plant extracts

Five set of 5 ml falcon tubes were placed in a test tube rack, onto each tube 50 µl of Neutrophil suspension was dispensed and then 50 µl of each of the following concentration 25 µg/ml, 50 µg/ml, 100 µg/ml and 1000 µg/ml of plant extract were added onto the tubes respectively. The tubes were labeled appropriately and incubated at 37°C for 30 minutes. Neutrophil suspension without plant extract was considered as control. All the treatments were carried out in duplicate.

2.8 Preparation of *Candida albicans* suspension

Candida albicans was grown on Sabouraud broth for 24 h at 37 °C then centrifuged at 1500 rpm for 5 minutes to form a pellet. The supernatant was discarded. The pellet was washed with sterile distil water and centrifuge again at 1500 for 5 minutes. The final pellet was resuspended in a mixture of RPMI 1640 (Sigma-Aldrich, Co., UK) and human serum in a proportion of 4:1. Cell count of *C. albicans* was carried out to get 2 x 10^6 cells/ml [23].

2.9 Neutrophil phagocytic index

The reaction mixture was prepared by adding together, 0.5 ml of neutrophil suspension (2 x 10^6 cells/ml), 1 ml of *C. albicans* suspension (2 x 10^6 cells/ml) and 0.2 ml of pooled serum in 5ml effendorf tubes. Leaf extract dilutions were added in the series of tubes and the mixture was incubated at 37ºC for 30 minutes. After the incubation, the set-up was centrifuged at 1500 rpm for 15 minutes. The supernatant obtain was used to prepare a thick smears then fixed with methanol and stained with 3% Giemsa stain for 30 minute. Positive control was prepared without adding the plant extract. Slides were observed under Light microscope and Immunostimulation was calculated by using the following equation [24].

$$\text{Phagocytic index (\%)} = \frac{\text{PI (test)} - \text{PI (control)}}{\text{PI (Control)}} \times 100$$

2.10 Microbicidal capacity of neutrophils

Mixture was prepared by adding together, 0.5 ml of neutrophil suspension (2 x 10^6 cells/ml), 1 ml of *C. albicans* suspension (2 x 10^6 cells/ml) and 0.2 ml of pooled serum. Extract dilutions were added in series of tubes and the mixture was incubated in a thermostatic bath at 37 ºC for 60 minutes [25]. After which, 2 ml of 0.01% methylene blue which stains the dead *C. albicans* was added but at 50 minutes. The samples were then centrifuged at 300 x g for 10 minutes, discarding two thirds of the supernatant. The remainder of the supernatant was shaken, and an aliquot taken for counting in a Neubauer haemocytometer under a phase contrast microscope. The number of dead *C. albicans* of the total phagocytosed by 100 neutrophils was determined (Microbicidal index). Results were expressed as percentage by giving 100% values to control [26].

2.11 Statistical analysis
The results were entered into SPSS version 21 (IBM, USA) for analysis. Continuous variables were expressed as mean percentage and standard deviation (SD). Test for normality was carried out to ascertain normal distribution of the variables. Data was normally distributed based on tests of normality results. One way between-groups analysis of variance (ANOVA) with Post-hoc Test (Bonferroni) was carried out to compare between the groups. The p value of ≤ 0.05 was used to determine the level of statistical significance.

3. Results
3.1 Phagocytic index of neutrophils after treatment with *Cassia occidentalis*
Neutrophils treated with 1000µg/ml of the plant extract shows the highest mean percentage PI score (80.2%, SD = 2.9) whereas Neutrophils treated with 25 µg concentration of the plant extract recorded lowest mean percentage PI score (21.9%, SD = 1.0) (Table 1). Depicted from Table 2, there was significant difference in human Neutrophils mean percentage PI scores across the different concentration of the plant extract (25 µg, 50 µg, 100 µg, 1000 µg) including control ($p < 0.0001$). The mean percentage PI score for treatment control was significantly different from human Neutrophils treated with 25 µg ($p < 0.0001$). There was also significance difference when control was compared with 50 µg concentration of the plant extract as well as 100 µg concentration ($p<0.0001$). But the control did not shows statistically significant difference with 1000 µg concentration of the plant extract ($p>0.05$). However, when Neutrophils treated with 100 µg concentration of the plant extract was compared with 1000 µg there was a statistically significant difference ($p<0.0001$).

Table 1: Phagocytic index of Neutrophils after treatment with plant extract

Concentrations (/ml) N=30	Phagocytic Index		Percentage Phagocytic Index	
	Test Mean	Control Mean	Mean	SD
25 µg	94.23	77.27	21.9	1.0
50 µg	105.5	77.27	36.5	1.3
100 µg	114.6	77.27	48.3	2.3
1000 µg	139.3	77.27	80.2	2.9

PI: Phagocytic index

Table 2: Effect of aqueous leaf extract of *C. occidentalis* on human Neutrophils phagocytic index

Conc. (/ml) N=30	Mean PI % (SD)	*p*-value	Post-hoc Test (Bonferroni)			95% CI	
			Compared Mean	Mean Difference	*p*-value	Lower Bound	Upper bound
Control	77.2 (9.6)	<0.0001	Control vs. 25 µg	55.3	<0.0001	37.1	69.8
25 µg	21.9 (1.0)		Control vs. 50 µg	40.7	<0.0001	22.9	55.6
50 µg	36.5 (1.3)		Control vs. 100 µg	28.9	<0.0001	19.5	75.2
100 µg	48.3 (2.3)		Control vs. 1000 µg	-2.93	>0.05	-19.3	13.3
1000 µg	80.2 (2.9)		100 µg vs. 1000 µg	-31.9	<0.0001	-78.3	-25.6

3.2 Microbicidal capacity of neutrophils after treatment with *Cassia occidentalis*
As shown in Table 3, the mean percentage score of microbicidal capacity of control was 100%. However Neutrophils treated with 1000 µg/ml of the plant extract shows the highest mean percentage score of microbicidal capacity 193.1% (SD = 4.0) whereas human Neutrophils treated with 25 µg of the plant extract have the lowest mean percentage score of microbicidal capacity 28.3% (SD = 1.2). There was a statistically significant difference in human Neutrophils mean percentage microbicidal capacity scores across the different concentration of the plant extract (25 µg, 50 µg, 100 µg, 1000 µg) including control ($p < 0.0001$). The mean percentage microbicidal capacity score for treatment control was compared with those human Neutrophils treated with 25 µg, 50 µg as well as 1000 µg, there was statistically significant difference ($p<0.0001$). But when the control was compared with 100 µg of the plant extract, there was no statistically significant difference ($p>0.05$). So also, when Neutrophils treated with 100 µg of the plant extract was compared with those treated with 1000 µg there was statistically significant difference ($p<0.0001$).

Table 3: Effect of aqueous leaf extract of *Cassia occidentalis* concentrations on human neutrophils microbicidal capacity

Conc. (/ml) N=30	Mean MI (%) (SD)	*p*-value	Post-hoc Test (Bonferroni)			95% CI	
			Compared Mean	Mean Difference	*p*-value	Lower Bound	Upper bound
Control	100 (0.0)	<0.0001	Control vs. 25 µg	71.7	<0.0001	53.4	89.9
25 µg	28.3 (1.2)		Control vs. 50 µg	32.2	<0.0001	13.8	50.3
50 µg	67.8 (0.4)		Control vs. 100 µg	8.10	>0.05	-10.2	26.2
100 µg	91.9 (1.3)		Control vs. 1000 µg	-93.1	<0.0001	-111.3	-74.8
1000 µg	193.1(4.0)		100 µg vs. 1000 µg	-101.2	<0.0001	-119.3	-82.8

4. Discussion
Plants materials were used for stimulation of host immune system to provide effective response against microorganisms thus lead to successful elimination of the pathogens [27, 28, 29]. For prevention and treatment of inflammatory conditions using immunomodulatory agents, neutrophils are the main target [30].

This study reported an increase of phagocytic activity of neutrophils up to 80.2% at 1000 µg/ml concentration of the plants. The findings suggest that there was significant increase stimulation of neutrophils at higher concentration of the plant extract. The finding is in line with the work of Ganachari and co-workers which reported *Ziziphus jujuba* leaves extract significantly increased the phagocytic function of human neutrophils [31]. It is also in consistent with the findings of Vikhe and co-workers where they reported a significant increase in phagocytic activity by neutrophils upon treatment with plants extracts [32]. *Rhodomyrtus tomentosa* extract was also reported to have immunostimulatory effect on human neutrophils, it can also alter the integrity of bacterial membrane, and this will possibly improve the killing activity of neutrophils against the bacteria [29]. However some

plants were reported to have decrease effect on neutrophil phagocytic functions. For instance, a recent study reported a decrease in neutrophils phagocytic activity by aqueous stem bark extract of *Vitellaria paradoxa* [33]. Similarly a decrease in neutrophils activity by *Santolina chamaecyparissus* polyphenolic leaf extract and *Santolina chamaecyparissus* aqueous leaf extract was also reported [34]. Immunostimulatory effect of the plants on neutrophils may be associated with its phytochemical constituents because some bioactive compound found in some plants extract have immunomodulatory activities, for instance Flavonoids, Terpenoids [35], Steroid and Tannins [36] as secondary metabolites were reported to have the immunomodulatory activity. Phytochemical screening of *C. occidentalis* revealed the presence of alkaloids, glycosides, proteins and amino acids, sterols, carbohydrates, phenolic compounds, flavonoids, saponins, and tannins [37]. Presence of these phytochemical constituents indicate potential wider application of the plant in phytomedicine [38]. Saponin is responsible for numerous pharmacological [39, 40] and anti-inflammation activities [41]. Flavonoids and resins are responsible for anti-inflammatory properties [38]. Flavonoids anti-inflammatory properties affect both acute and chronic inflammation [42, 43]. Ability of an extract to increase phagocytosis relies on the bioactive components of the plant and it varies between plants. Ability of neutrophils to fight against microorganisms depends on its phagocytic activities as well as intracellular killing by release of proteolytic enzymes and reactive oxygen species [34].

Our findings on Neutrophils Microbicidal capacity shows that there is high (193.1%) microbicidal killing at 1000 µg/ml concentration of the extract. This suggest microbicidal activity may be increase with increase in the concentration of the plant. There is tendency that the plant contains some constituents that can enhance the microbicidal ability of the neutrophils especially at high concentration. Ganachari and co-workers reported an increase in microbicidal ability of neutrophils by *Ziziphus jujube* leaves extract [31]. Similarly Kalgo and colleagues reported an increase in the ability of the neutrophils to intracellularly kill the ingested *C. albicans* when treated with stem bark extract of *V. paradoxa* [33]. In addition, a study also reported an increase in microbicidal ability and overall metabolic integrity of neutrophils after treatment with a plant extract [31]. In another study, iridoids fraction obtained from methanol extract of *Barleria prionitis* enhanced the intracellular killing activity of neutrophils against *Candida albicans* [27]. However, Chahra and co-workers reported contrary after treatment with different concentration of plant extract [26]. The final step of phagocytosis is the intracellular killing of microorganisms by the neutrophils, which is dependent on metabolic thrust generated through the hexose monophosphate shunt activation, an activation which is also necessary for normal microbicidal activity [43]. Neutrophil is one of the immune cells tasked to kill different kind of intracellular or extracellular pathogenic microorganisms in tissues by oxidative or non-oxidative means [44]. Non-oxidative killing is mediated by various lysosomal enzymes, peptides and proteins, including lysozyme, bactericidal/ permeability increasing proteins, cationic proteins, defensins and lactoferrin [44]. Whereas the oxidative killing of pathogenic microorganisms is due to generation of reactive oxygen species such as superoxide, hydrogen peroxide, hydroxyl radicals and hypochlorous acid and chloramines [45].

5. Conclusion

There was significant increase in immunostimulation of neutrophils at higher concentration of the aqueous leaf extract of *C. occidentalis* this will enhance the phagocytic function of the neutrophils. The microbicidal activity of the neutrophil increase with increase in concentration of the aqueous leaf extract of *C. occidentalis,* therefore, the plant tend to have positive effect on the microbicidal activity of the neutrophils. The leaves of *C. occidentalis* has immunostimulatory activity on human neutrophils.

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