Original article

EFFECTS OF AQUEOUS STEM BARK EXTRACT OF VITELLARIA PARADOXA ON HUMAN NEUTROPHIL FUNCTION AND VIABILITY


1. Introduction

Medicinal plants have been used in the treatment of both communicable and non-communicable diseases; they also play a vital role in the development of conventional drugs in modern age

[1]. Mankind has been using natural products from plant and animal sources for thousands of years either in pure forms or crude extracts for medical treatment [2]. More than 150,000 plant species have been studied, and several of them contain therapeutic substances [3]. However, the main drawback in the use of ethno botanical medicine is the fact that the dosage is non-standardized and most of the plants have not been evaluated for toxicity [4]. Nigeria has an extensive forest enriched with different variety of traditional and ethnic plants [5]. Here as elsewhere plants were used since ancient times for the treatment of many ailments and diseases, the compounds have properties of wound healing, enhancement of phagocytosis, and anti-cancer activity

[6]. A study showed the therapeutic activity of V. paradoxa against some selected member of the Enterobacteriaceae, Fungal and Viral agents [7]. Indeed, a study have indicated, the use of plant extracts in highdose could lead to toxic injury to the kidneys, liver, intestine and other immune cells, which interfere with normal biological functions [4]. People consume extract of V. paradoxa for treatment of many bacterial and fungal infections. But actually, they do not know the exact concentration they are taking. Considering the established anti-bacterial and anti-fungal activities of this plant, the present study focused on the potential effect of the bark of V. paradoxa on Neutrophils viability, phagocytic and Microbicidal indexes.
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MATERIALS AND METHODS

Study Area
The study was carried out in the Department of Immunology, School of Medical Laboratory Sciences, Usman Danfodiyo University, Sokoto (UDUS), Nigeria.

Study Subjects
A total of thirty (30) healthy volunteers comprising of 15 males and 15 females were recruited for this study. Their age ranges from 20-30 years and mean (Standard Deviation SD) age of 25.37 (2.67) years. The participants signed an informed consent and had no signs and symptoms of active disease at the time of phlebotomy.

Plant collection and Identification
The plant was collected locally. The stem bark, fruits and flower were identified at the Herbarium section of Botany Unit, Department of Biological Sciences, Faculty of Sciences, UDUS, and the voucher identification number issued was UDUS/ANS/0248.

Preparation of the plant extract
The stem bark of V. paradoxa was air-dried under shade for 14 days and the dried materials were chopped into small pieces and then pulverized into powdery form using a pestle and mortar. With the aid of 1.5 L sterile conical flasks, one hundred grams of the powdered plant material was soaked in 500 mL of distilled water, these were subjected to uniform agitation in a shaker water bath for 48 h. The content was then filtered with a piece of muslin cloth and then Whatman filter paper (No. 1). The filtrates were evaporated to dryness and the resultant extracts were packed in separate clean dry bottles and stored in a refrigerator at 4°C until required for use [13].

Peripheral Blood collection and Neutrophil Isolation
Three milliliter (3ml) of venous blood was aseptically collected from each participant and dispensed into Heparinized tube. The sample was mixed thoroughly and carefully layered onto Histopaque-1119 (Sigma Aldrich, UK) without getting mixed up, it was spun at 400 g for 30 minutes. After centrifugation, the upper layer was aspirated and discarded. With the aid of clean Pasteur pipette the opaque interface was carefully transferred into a conical centrifuge tube. The cells were washed by adding 10 ml of isotonic phosphate buffered saline solution and mixed by gently drawing in and out using Pasteur pipette and then centrifuged at 250 g for 10 minutes. It was repeated twice, the supernatant was discarded. Cells pellets were re-suspended in 2 ml of RPMI-1640 (Sigma Aldrich, UK).

Treatment of Neutrophils with stem bark Extract
Fifty microliter (50 µl) of neutrophils suspension were dispensed into falcon tubes, 50 µl of each of the following concentration 25 µg/ml, 50 µg/ml, 100 µg/ml and 1000 µg/ml of the stem bark extract were added into the tubes respectively. The tubes were labelled appropriately and incubated at 37°C for 30 minutes. A tube containing the cells suspension without the stem bark extract was considered as the control. All the treatments were carried out in duplicate.

Trypan Blue Exclusion Test of cell Viability
For pre-treatment cell count each 10 µl of 0.4% trypan blue solution (Loba chemie Mumbai, India) was transferred into two Eppendorf tubes and 10 µl of the neutrophils suspension (dilution factor = 2) were added respectively, mixed thoroughly and allowed to stand for 5-10 minutes at room temperature. Ten microliter (10µl) of trypan blue treated cell solutions was transferred onto haemocytometer, and observed under microscope for cell viability count [14]. For post treatment cell count, the trypan blue assay was carried out as described above, after incubating cells for 30 minutes with different concentrations of the stem bark extracts.

Preparation of Candida albicans suspension
The procedure was described by Mali and Hatapakki, [15] with some modification as follows; Candida albicans culture was incubated in Sabouraud broth for 24 h at 37°C then centrifuge to form a cell pellet the supernatant was discarded. The pellet was washed with sterile distill water and centrifuge again. The final pellet was re-suspended in a mixture of RPMI and human serum in a proportion of 4:1. Cell count of C albicans was carried out to get 2 x 106 cells/ml.

Neutrophil Phagocytic index
The reaction mixture was prepared by adding together, 0.5 ml of neutrophil suspension (2 x 106 cells/ml), 1 ml of C albicans suspension (2 x 106 cells/ml) and 0.2 ml of pooled serum in 5ml effendorf tubes. Stem bark extract dilutions were added in series of tubes and the mixture was incubated at 37°C for 30 minutes. Thick smears were prepared, fixed with methanol and stained with 3% Giemsa stain for 30 minute. Positive control was prepared without adding the plant extract. Slides were observed and immunostimulation was calculated by using the following equation [16].

\[
\text{Immunostimulation (\%)} = \frac{\text{PI (test)} - \text{PI (control)}}{\text{PI (Control)}} \times 100
\]
**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°C, for 60 minutes [17]. 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 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 hemocytometer under a phase contrast microscope. The number of dead C. albicans of the total phagocytosed by 100 neutrophils was determined (Microbicidal index). Results are expressed as percentage by giving 100% values to control [18].

**Statistical analysis**

The statistical analysis was carried out using SPSS version 21 (IBM, USA). Continuous variable were expressed in mean (SD). While categorical variables were expressed in percentage. Test for normality was performed to ascertain normal distribution of the variables. Data for viability test was not normally distributed whereas that of phagocytic and Microbicidal index were normally distributed. Analysis of variance (ANOVA) or Kruskal Wallis test was carried out to explore differences on variables across the concentrations of the stem bark extract. Bonferroni or Mann Whitney test was carried out as post hoc test to compare between different concentrations of the stem bark extract. Wilcoxon Signed Ranks Test was carried out to compare between pre- and post-treatment effect of the stem bark extract. A p-value ≤ 0.05 was considered as statistically significant.

**RESULT**

**Total Neutrophil Count and Percentage Viability**

The mean (SD) of total viable cell count (TVCC) before treatment was 2.35 x 10^6 cells/ml (SD = 1.55). Among the treatment, the Neutrophils treated with 1000 µg/ml of the stem bark extract shows the lowest mean score of viable cells count of 1.34 x 10^6 cells/ml (SD = 0.21). The total non-viable cells count (TNVCC) result shows that human Neutrophils treated with 25µg of the stem bark extract have the lowest mean score of non-viable cells 0.17 x 10^6 cells/ml (SD = 1.36). Mean percentage viability of Neutrophils before treatment was 95.8%. Human Neutrophils treated with highest concentration (1000 µg/ml) of the extract recorded a lowest mean percentage score of viability 82.2 % (Table 1).

As depicted from Table 2, a Kruskal Wallis Test showed that there was a statistically significant difference in human Neutrophils median percentage scores viability across the different concentration of the extract including control (Control: M = 95.36%, 25 µg: M = 92.38%, 50 µg: M = 86.33%, 100 µg: M = 84.59%, 1000 µg: M = 80.87%, F =38.08, p < 0.0001). Post-hoc comparisons using Mann-Whitney Test indicated that the median percentage viability score for treatment control was significantly different from human Neutrophils treated with 50 µg, 100 µg and 1000 µg (p< 0.0001).

As shown in Table 3, a Wilcoxon Signed Ranks Test revealed that there was no statistically significant decrease in the median percentage viability scores from pre-treatment Neutrophils (M = 96.00 %) to treatment control (M = 95.36 %), p = 0.230. There was statistically significant decrease in the mean percentage viability scores from pre-treatment Neutrophils to treatment with different concentration of the stem bark extract (25 µg, 50 µg, 100 µg, and 1000 µg; p< 0.05).

**Phagocytic and Microbicidal Index**

Neutrophils treated with 25 µg of the stem bark extract shows the highest mean percentage PI score of 6.33% (SD = 1.58) whereas those neutrophils treated with 1000µg concentration recorded lowest mean percentage PI score (0.27%, SD = 2.13). As depicted from Table 4, a one-way between-groups ANOVA shows that there was a statistically significant difference in human Neutrophils, mean
percentage Neutrophils phagocytic index (PI) scores across the different concentration of the plant extract (25 µg, 50 µg, 100 µg, 1000 µg) including control (p = 0.0001). Post-hoc comparisons using the Bonferroni tests indicated that the mean percentage PI score for treatment control was significantly different from human Neutrophils treated with different concentration of the stem bark extract (p < 0.001).

Neutrophils treated with 1000 µg/ml of the plant extract shows the highest mean percentage score of MI 99.7% (SD = 2.51) whereas human Neutrophils treated with 25 µg of the plant extract have the lowest mean percentage score of MI 89.3% (SD = 1.77). As shown in Table 5, there was no statistically significant difference in human Neutrophils mean percentage MI scores across the different concentration of the stem bark extract (25 µg, 50 µg, 100 µg, 1000 µg) as well as control (p = 0.48).

Table 4: Effect of different concentrations of V. paradoxa stem bark extract on neutrophils PI

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Mean PI (%)</th>
<th>SD</th>
<th>ANOVA</th>
<th>p-value</th>
<th>Compared mean</th>
<th>Mean difference</th>
<th>p-value</th>
<th>95% CI Lower</th>
<th>95% CI Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>92.3 (1.61)</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
<td>Control vs. 25µg</td>
<td>85.9</td>
<td>&lt;0.0001</td>
<td>84.9</td>
<td>112.3</td>
</tr>
<tr>
<td>25</td>
<td>6.33 (1.58)</td>
<td></td>
<td></td>
<td></td>
<td>Control vs. 50µg</td>
<td>89.2</td>
<td>&lt;0.0001</td>
<td>78.5</td>
<td>105.9</td>
</tr>
<tr>
<td>50</td>
<td>3.01 (1.99)</td>
<td></td>
<td></td>
<td></td>
<td>Control vs. 100µg</td>
<td>91.1</td>
<td>&lt;0.0001</td>
<td>87.4</td>
<td>104.8</td>
</tr>
<tr>
<td>100</td>
<td>1.14 (2.01)</td>
<td></td>
<td></td>
<td></td>
<td>Control vs. 1000µg</td>
<td>92.0</td>
<td>&lt;0.0001</td>
<td>89.8</td>
<td>113.2</td>
</tr>
<tr>
<td>1000</td>
<td>0.27 (2.13)</td>
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</tr>
</tbody>
</table>

PI = Phagocytic index

Table 5: Effect of different concentrations of V. paradoxa stem bark extract on neutrophils MI.

<table>
<thead>
<tr>
<th>Conc. (µg/ml)</th>
<th>Mean MI (%)</th>
<th>SD</th>
<th>ANOVA</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
<td>0.00</td>
<td></td>
<td>0.48</td>
</tr>
<tr>
<td>25</td>
<td>89.3</td>
<td>1.77</td>
<td></td>
<td></td>
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<td>50</td>
<td>91.2</td>
<td>1.93</td>
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<td>100</td>
<td>98.4</td>
<td>2.33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>99.7</td>
<td>2.51</td>
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DISCUSSION

In some African countries V. paradoxa aqueous stem bark extract is commonly used for treatment of some conditions such as diarrhea and dysentery therefore this raised the concern to evaluate its cytotoxicity on human neutrophils as well as other immune cells [19]. In this study we found that the percentage viability of the neutrophils was 95.8%, and this was considered to be within the reported range as the normal cell viability was 90–95% colorless cells [18]. This study shows that, the viability of neutrophil treated with different concentrations of the stem bark extract was found to be decreasing as the concentration of the extract increases. This suggests high concentration of the extract has more effect to the neutrophil survival. Plant material may contain some phytoconstituent that are harmful to the cells. Therefore, compounds that kill live cells are cytotoxic compounds [14]. Exposure of normal cells to these compounds may lead to apoptosis or incidental cell death [20]. A study reported that prolong administration of stem bark extract of V. paradoxa causes a decrease in white blood cells count [13]. However, plants like V. paradoxa possess antioxidant properties and it is believed to enhance the viability and normal function of cells in the body due to their phenolic components [21]. Phytochemical screening of the root, stem bark and leaves of V. paradoxa revealed the presence of Carbohydrates, simple reducing sugars, soluble starch, Saponins, Alkaloids and Tannins [22, 23] others are Flavonoids, Steroid, Terpene, Philoban, Cardiac glycoside, Phlobatannin and Anthraquinone [23]. Furthermore, Triterpenoids from the stem bark of V. paradoxa (Sapotaceae) and derived esters exhibit cytotoxicity against a breast cancer cell line [24].

The study further justify the effect of V. paradoxa on human neutrophils by pairing pre-treated neutrophils with those exposed to different concentration of the stem bark extract of the plant and control was included. Our findings shows that pre-treated neutrophils and control did not differ however the differences arise when pre-treated neutrophils were paired with treated ones. This affirm the effect and rule out confounding factors that may influence the result. However, viability of neutrophil depends on the conditions of their manipulation in vitro rather than their actual lifespan in vivo, which remain controversial [25]. This study revealed that phagocytic index of the neutrophils was adversely affected by the different concentrations of stem bark extract because as concentration increases so also the PI decreases drastically. This suggests the extract at the specific concentrations has effect on the immunostimulation of neutrophils. This may be related to phytochemical constituent of the stem bark extract. For instance polyphenols and flavonoids were reported to may have decrease the activity of neutrophils [18]. Flavonoids were suggested to affect neutrophil function by inhibiting important enzymes such as protein kinase C and phosphoinositide 3-kinase which actually involved in Neutrophils signaling [26]. In phagocytosis process different receptors and several activation pathways were involved, for instance, activation of protein kinase C (PKC) and regulation of DAG and DAG kinase were identified as the most important beside others [27]. On Microbicidal index of neutrophil we found that the V. paradoxa stem bark extract has considerably increase the ability of the neutrophils to intracellularly kill the ingested C albicans. Increase in MI is directly proportional to the stem bark extract concentrations. Ganachari et al., [28] reported increase in intracellular killing property and overall metabolic integrity of neutrophils after treatment with a plant extract. Whereas Chahra et al., [18] reported contrary after treatment with different concentration of plant extract. 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 [27]. Non-oxidative killing is mediated by various lysosomal enzymes, peptides and proteins, including lysozyme, bactericidal/permeability increasing proteins, cationic proteins, defensins and lactoferrin [27]. 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 [29].

Conclusion

The ability of the extract to decrease neutrophils viability with increase concentration of the extract was confirmed by trypan blue exclusion method. Phagocytic index adversely decreases with increase in the extract concentration. Therefore it decreases neutrophil immunostimulation. Microbicidal index increase in a specific manner with increase in extract concentration. Thus it increases the killing ability of the neutrophils. The stem bark extract of V. paradoxa have potential immunomodulatory effect on human neutrophils viability and function.

Conflict of interest

We declared that we have no conflict of interest.

Acknowledgement

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References:


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