Toxicity Activity-Guided Characterization of Toxic Constituents in Azadirachta indica Seed

Edwin N. Okafor¹*, O. U. Njoku² and Okoli Charles³

¹Department of Medical Laboratory Sciences, University of Nigeria Enugu Campus, Nigeria.
²Department of Biochemistry, University of Nigeria, Nsukka, Nigeria.
³Department of Pharmacology and Therapeutics, University of Nigeria, Nsukka, Nigeria.

Authors’ contributions

This work was carried out in collaboration between all authors. Authors ENO and OUN designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors OUN and OC managed the analyses of the study. Authors ENO and OC managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJRB/2018/v2i4561

Editor(s):
(1) Asmaa Fathi Moustafa Hamouda, Assistant Professor, Faculty of Applied Health Sciences, Jazan University, Saudi Arabia.

Reviewers:
(1) K. Elumalai, University of Madras, India.
(2) Sobia Chohan, Bahauddin Zakariya University, Pakistan.
(3) Rupali Sengupta, SNDT Women’s University, India.

Complete Peer review History: http://www.sciencedomain.org/review-history/25769

Received 10th May 2018
Accepted 16th July 2018
Published 2nd August 2018

Original Research Article

ABSTRACT

Background: Azadiradita indica seed has been used in traditional system of medicine. It is known to be natural medicine with many benefits. Toxicological studies have reported that toxic effects may be related to the complex mixtures of active constituents and other chemicals which increase the risk of adverse reactions.

Objective: The purpose of the study was designed to isolate and characterize the toxic constituents with a view of recommending for clinical trials.

Materials and Methods: The study was conducted at the Department of Biochemistry, University of Nigeria, Nsukka. The seeds were collected, identified and extracted with conventional Soxhlet extraction technique. Chromatographic techniques were used for fractionation and isolation of the toxic constituents. A total of eighty- four (84) adult Albino rats of both sexes were randomly assigned into fourteen (14) groups containing 6 rats in each group. Each group I-VI received one of the 100, 500, or 1000 mg/kg of Methanol Extract (ME) or Hexane Extract (HE), respectively. Group VII was

*Corresponding author: E-mail: nkemyika.okafor@unn.edu.ng;
the control and received 0.5 ml/kg of the vehicle, 3% v/v Tween 80. Group VIII-XIV received 100 mg/kg of Pet-ether-ethylaceate fraction (PEF), Methanol fraction (MF), Methylene Chloride /Acetone fractions 9:1, 8:2 and 7:3 (MAC-1, MAC-2, MAC-3), Isolate 1 (TN-1), and Isolate 2 (TN-2), respectively. Extracts and fractions were administered orally once daily for 30 days for animals in group I-XII. Groups XIII-XIV were treated for only 10 days. On days 10, 20, 30, 3 ml of blood was withdrawn from each rat by an ocular puncture for liver function test (ALT and AST). Body/organ weights were equally used as a toxicity guide in the separation of toxic constituents.

**Results:** The ME and various fractions caused significant ($P<0.05$) increase in the activities of ALT and AST. The isolated compounds TN1 and TN2 also caused a significant effect on AST and ALT. The toxic effect of TN-2 was higher than that exhibited by TN-1. The methanol fractions caused significant ($P<0.05$) dose related increase in the body weight of treated animals. TN-1 and TN-2 caused significant ($P<0.05$) reduction in the organ weight of the treated animals.

**Conclusion:** Although there was no evidence of lethality for acute toxicity of the extract, chronic oral administration of the extract and solvent fractions caused hepatotoxicity. Structure elucidation revealed TN-1 and TN-2 to be 6-deacetylnimbin and Nimbolide, respectively.

**Keywords:** Toxicity; activity; Azadirachta indica; structural characterization; nimbolide.

### ABBREVIATIONS

- **ME:** Methanol extract.
- **HE:** Hexane extract.
- **PEF:** Pet-ether-ethylaceate fraction.
- **MF:** Methanol fraction.
- **(MCA):** Methylene chloride /Acetone.
- **ALT:** Alanine aminotransferase.
- **AST:** Aspartate aminotransferase.
- **TN:** Isolate.

### 1. INTRODUCTION

Plant products either as extracts or pure compound provide great opportunities for new drug discoveries because of the unlimited availability of chemical diversity [1]. Interest has grown in medicinal plants due to increasing demand for screening programs, seeking therapeutic drugs from natural remedies. The administration of plant extracts based on traditional practices represents a long history of human interactions with the environment. Approximately 20% of known plants have been used in pharmaceutical studies, impacting the healthcare system in a positive way such as treatment of cancer and harmful diseases [2].

Current available drugs for treating a variety of different diseases have a number of limitations, such as adverse effects and a high rate of secondary failure like microbial resistance. As there is a growing trend towards ethnopharmacognosy adjunct to conventional therapy, traditionally used plants might provide a useful source of new therapeutic compounds and phytochemicals. Recent studies reported that thousands of phytochemicals from plants are safe and act as effective alternatives with less adverse effect. Many beneficial biological activities such as anticancer, antioxidant, analgesic antidiarrheal and wound healing have been reported [16].

However, clinical applications are limited due to the absence of information on toxic constituents and lack of toxicological studies of these constituents. There is a need for pharmacokinetic and toxicological considerations to accelerate their development. In accordance with the drug development pipeline, critical studies must be performed at the preclinical stage before the drug can proceed to the clinical testing phase [3]. Clinical trials are necessary to know the effectiveness of the bioactive compound to determine the dosage range to support the safety and also to verify the traditional claim. Careful evaluations of the extracts and compounds are needed to safeguard the health of the population and answer specific research questions, which could be done by evaluating both immediate and long-term toxicity before the drug is applied to patients.

Medicinal plants play important role in the indigenous system of medicine. The phytochemicals derived from plants are an important source of antioxidants like glycosides, alkaloids, steroids, flavonoids and tannin. Studies have shown that many plants are rich in vitamins A, C, E and phenolic compounds [4,5,6]. The phenolic compounds play a dynamic role in delaying ageing, reducing inflammation and have been recommended by many agencies and healthcare system throughout the world [7]. The constituents such as alkaloids, triterpenoids and tannins of the medicinal plants play a major role.
in the process of wound healing in diabetes mainly due to their astringent and antimicrobial properties [8]. This research work aims to explore the toxic constituents in *Azadirachta indica* seed with the recent advancement in separation and characterization techniques.

Several studies reported that *A. indica* known as neem is native to the Indian subcontinent. Today, neem is well distributed across the world and can be found in at least 30 countries of Asia, Africa and America [9]. Natural compounds have been identified from various parts of *A. indica* including azadirachtin, Saianrin, nimbidol, nimbin and nimbic acid [10]. *A. indica* is accessible and affordable, owing to the abundance in tropical regions. More importantly, *A. indica* is used for its antibacterial [11], antimalarial [12], antioxidants [13], and antifeedant properties [14]. Parallel with recent increase in the use of *A. indica* seeds for the prevention and treatment of various illnesses, there is increasing concern about the safety. Reports of toxic effect due to the use of *A. indica*, [15,16] are scarce. It may be due to the fact that some of the active constituents have not been isolated and characterized as toxic constituents.

The study was designed to isolate and characterize toxic constituents of *A. indica* by studying the effect of chronic administration of the seed extract, fractions and isolates on liver function, body/organ weight of adult Albino rats.

2. MATERIALS AND METHODS

2.1 Plant Material

The neem seed extract was used as botanical sample for the treatment. The exact botanical name of neem is *Azadirachta indica* (A. Juss) which can be found under [http://www.theplantlist.org](http://www.theplantlist.org) as the accepted name. It belongs to the Meliaceae family. *Azadirachta indica* variety used in the study was collected in the month of June 2015 from the premises of College of Medicine, University of Nigeria, Enugu Campus. The collected neem seed was identified and authenticated at International Centre for Ethnomedicine and Drug Development (INTERCEDD), Nsukka, Nigeria by Dr. Chimene Mitch, Chief Botanist at the centre. A voucher specimen was deposited at the herbarium.

2.2 Preparation of Plant Extract

The seeds were dried under the sun for 2 weeks and reduced to coarse powder using Hammer mill (Gallen Kamp, U.S.A). The powdered plant material (12.5 kg) was successively extracted, first with 7.5 litres of n-hexane and then litres of methanol by continuous extraction in a Soxhlet apparatus. Both the extracts were filtered and concentrated using a rotary evaporator (Eyla N-1000, Japan) to obtain dried extract which was of 265 g (2.12% w/w) of methanol extract (ME) and 250 g (2.0%w/w) of the hexane (HE). It was stored in a refrigerator at 4°C until required.

2.3 Phytochemical Analysis of the Extracts

The preliminary qualitative analysis of the phytochemical was screened for the presence of carbohydrate (Molisch test) [17], alkaloids (Dragendorff test and Mayer’s test) [18], glycosides (Kellar-Kiliani test) [19], Reducing sugars (Fehling’s test) [20], Saponins (foam test) terpenoid, and steroid (Libermann-Burchar test) [21]. Quantitative analysis of various phytochemicals such as alkaloid [22], Saponins and Resin [17] was also done.

2.4 Animal

Adult albino rats (140-250 g) and mice (15-30 g) of both sexes were obtained from the laboratory animal facility of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka and kept at room temperature of (25±2) °C, relative humidity of (40-45%) and 12hr dark light cycle. The animals were housed in a steel cage within the laboratory animal facility of the College of Medicine, University of Nigeria, Enugu Campus and maintained on standard pellet (Vita Feed Ltd., Jos) water *ad libitum*. A period of four weeks was allowed for acclimatization before the commencement of the experiment.

2.5 Ethical Approval

The study protocol was approved by the Department of Biochemistry, under the institutional review board of the University of Nigeria, Nsukka. All animal experiments were in compliance with ethics for medical and scientific research and also existing internationally accepted principles for care and use of laboratory animal [23].

2.6 Toxicological Activity Guided Studies

2.6.1 Column chromatographic separation of ME

The toxicological effect of the extracts and successive fractions on liver enzymes was used
for activity guide in the separation of the bioactive seed extract (ME and HE). Based on the results of the preliminary toxicological studies on the extract, the methanol extract (ME) was separated in a silica gel (70-200 mesh size) column, successively eluted with petroleum ether: ethyl acetate (1:1) and methanol (100%). The silica gel column was packed dry, loaded on top with the extract (265 g) mixed with silica gel and eluted with the solvents. The solvent fractions were collected in 100 ml flasks and pooled together into 2 broad fractions on the basis of solvent of the elution. The concentration of these fractions in a rotary evaporator afforded 120 g of petroleum ether: ethyl acetate fraction (PEF) (PEF 45.28% W/W) and 80 g of methanol fraction (MF) (MF 30.19% w/w). Based on the result of toxicity studies, PEF was further subjected to toxicological separation because it was more toxic than MF.

2.6.2 Chromatographic separation of PEF

PEF (120 g) was separated in a silica gel (100-200 mesh size) column eluted with gradient mixtures of methylene chloride:acetone (9:1, 8:2, 7:3). Three fractions – MCA-1 (20 g; 16.67% w/w), MCA-2 (17 g; 14.17% w/w) and MCA-3 (15 g; 12.5% w/w) were obtained and subjected to further toxicological activity guided studies.

2.6.3 Isolation of toxic constituent

A 37 g mixture of MCA-1 and MCA-2 was separated in a silica gel column eluted with hexane:ethyl acetate (1:1). About 3 ml fractions were collected in 50 ml volumetric flasks. Based on the similarity of constituents visualized on silica gel, pre-coated thin layer chromatographic plates developed with acetone, the fractions were pooled into 2 broad fractions – TN-1 and TN-2. The broad fractions were concentrated to dryness in a rotary evaporator and recovered in acetone (100%). On standing at room temperature, TN-1 yielded brown coloured oil (200 mg) while TN-2 produced brown coloured crystals (150 mg). The purity of the two isolates was determined and confirmed using thin layer chromatography on the same grade of silica gel developed with acetone to obtain a single spot.

---

**Fig. 1. Chart for extraction and purification of bioactive compounds of neem**

(+) Biological active, (-) Inactive
2.7 Identification and Structural Characterization of TN-1 and TN-2

The identities and structures of TN-1 and TN-2 were established using 1-dimensional (¹D) nuclear magnetic resonance (NMR) spectroscopy, proton NMR (¹H) (NMR) AND 13 NMR (¹³C NMR) as well as 2-dimensional (²D) NMR involving DEPT, COSY, HMQC and HMBC. The obtained spectra were compared with the library of established compounds to arrive at the structures of both compounds.

2.8 Acute Toxicity and Lethality Test

The acute toxicity and Lethality (LD₅₀) of ME in mice (n = 12) was estimated using the method proposed by Lorke [24]. In the first stage, animals received oral administration of one 10, 100 and 1000 mg/kg (n=3) of ME and was observed for 24 h for the number of deaths. Since no death occurred in any of the groups in the first stage of the test 1600, 2900 and 5000 mg/kg doses of the extracts were administered to a fresh batch of animals (n=1) and no death was recorded within 24 hrs. Therefore the LD₅₀ of ME was estimated to be greater than 5000 mg/kg.

3. BIOCHEMICAL STUDIES

3.1 Experimental Design

Sixty-six adult Albino rats of either sex were used for the study. The animals were grouped into 11 groups of 6 animals each. Group I – III received one of 100, 500 and 1000 mg/kg of ME, respectively. Group IV was the control and received 0.5 ml/kg of the vehicle, 3% v/v Tween 80. Group V-XI received 100mg/kg of PEF, MF, MAC-1, MAC-2, MAC-3, TN-1 and TN-2, respectively. Extracts and fractions were administered orally once daily for 30 days. On 10th, 20th and 30th day, 3 ml of blood was withdrawn from each rat by an ocular puncture. The blood samples were put into centrifuge tubes, spun at 3000 rpm for 5 minutes and the serum were collected for biochemical analysis i.e., serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) determination [25].

4. RESULTS

4.1 Acute Toxicity and Lethality Studies

Oral administration of the methanol and hexane extracts to mice caused no deaths at doses ranging from 10-5000 mg/kg. Therefore, the oral LD₅₀ of the extracts in mice was estimated to be greater than 5000 mg/kg.

4.2 Phytochemical Constituents of the Extracts

Phytochemical tests on the methanol extract gave positive reactions for alkaloids, glycosides, resins, reducing sugars, terpenoids, steroids, and carbohydrates.

4.2.1 Characterization of TN-1 and TN-2

A comparison of the spectra obtained from the ¹D NMR, ¹HNMR, ¹³CNMR, ²D NMR involving DEPT, COSY, HMQC and HMBC of TN-1 and TN-2 with the published library of compounds established the identity of the isolates as 6-deacetyl-nimbin (Fig. 2a & 2b) and (Fig. 3a & 3b) nimbolide, respectively.

4.2.2 Effect of extract and the fractions on aspartate aminotransferase (AST) Activity

The methanol extract (ME) and the various fractions caused significant (P<0.05) increase in the activity of AST. The effect of ME was found

<table>
<thead>
<tr>
<th>Bioactive constituents</th>
<th>Relative presence methanol extract (ME)</th>
<th>Quantity in gram % (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloids</td>
<td>+</td>
<td>1.56±0.25</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>+</td>
<td>1.65±0.32</td>
</tr>
<tr>
<td>Glycosides</td>
<td>+</td>
<td>1.80±0.40</td>
</tr>
<tr>
<td>Resins</td>
<td>+</td>
<td>1.19±0.30</td>
</tr>
<tr>
<td>Reducing sugar</td>
<td>+</td>
<td>1.40±0.10</td>
</tr>
<tr>
<td>Saponins</td>
<td>+.</td>
<td>0.29±0.20</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
<td>1.59±0.20</td>
</tr>
<tr>
<td>Terpenoids</td>
<td>++</td>
<td>2.12±0.20</td>
</tr>
</tbody>
</table>

Results are mean ± SD of quadruplet determination on the basis of dry weight.
Fig 2a. Spectral data for TN-1

to be highest on day 20 at all dose levels (Table 2). The methanol (MF) and petroleum ether-ethyl acetate (PEF) fractions caused a consistent increase in AST activity (Table 3). Of the three solvent systems i.e., 9:1, 8:2 and 7:3 of methylene chloride/acetone, the fractions obtained with the 9:1 and 8:2 ratios caused a more pronounced increase in AST activity (Table 4). The isolated compounds TN-1 and TN-2 also produced marked increases in AST activity. However, TN-2 caused a greater effect than TN-1 (Table 5).

Fig 2b. Structure of TN1 = 6-deacetylminbin

Fig 3a. Spectral data for TN-2
4.2.3 Effect of extract and fractions on alanine aminotransferase (ALT) activity

The methanol extract (ME) and the fractions caused significant ($P<0.05$) increase in the activity of ALT. The methanol extract (ME) had a peak increase in activity at day 20 which subsequently decreased by day 30 (Table 6). In the methanol (MF) and petroleum ether/ethyl acetate (PEF) fractions range, PEF caused a more pronounced increase on day 20 than MF (Table 7). In the methylene chloride/acetone fractions range, fraction 9:1 caused a more consistent effect than fraction 8:2. Fraction 7:3 as well the control group also had an increase in ALT activity. The isolated compounds – TN-1 and TN-2 also increased ALT activity (Table 8).

TN-2 caused a greater increase than TN-1 (Table 9).

Fig 3b. Structure of TN2 = nimboide

Table 2. Effect of methanol extract on aspartate aminotransferase (AST) activity

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Doses (mg/kg)</th>
<th>AST activity (IU/L)</th>
<th>Baseline</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME</td>
<td>100</td>
<td>11.7 ± 7.96</td>
<td>15.5 ± 4.8</td>
<td>35.6 ± 7.1$^{a,b}$</td>
<td>15.6 ± 3.8$^c$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>14.0 ± 11.2</td>
<td>16.0 ± 4.5</td>
<td>34.8 ± 4.8$^{a,b}$</td>
<td>17.3 ± 2.0$^c$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>17.0 ± 8.98</td>
<td>20.0 ± 2.3$^a$</td>
<td>36.0 ± 3.8$^{a,b}$</td>
<td>17.0 ± 3.7$^c$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $P<0.05$ compared to baseline; $^b$ $P<0.05$ compared to day 10; $^c$ $P<0.05$ compared to day 20 (Student’s t-test); AST = aspartate aminotransferase; ME = Methanol extract

Table 3. Effect of methanol and pet ether-ethyl acetate fractions on aspartate aminotransferase (AST) activity

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Doses (mg/kg)</th>
<th>AST activity (IU/L)</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>100</td>
<td>28.7 ± 6.8$^a$</td>
<td>30.3 ± 4.8$^a$</td>
<td>31.8 ± 5.0$^a$</td>
<td></td>
</tr>
<tr>
<td>PEF</td>
<td>100</td>
<td>27.6 ± 5.1$^a$</td>
<td>33.0 ± 4.6$^a$</td>
<td>34.0 ± 6.0$^a$</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>18.0 ± 2.7</td>
<td>22.0 ± 5.2</td>
<td>24.0 ± 4.1</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $P<0.05$ compared to Control (Student’s t-test); MF = methanol fraction; PEF = petroleum ether-ethyl acetate fraction

Table 4. Effect of methylene chloride- acetone fractions on aspartate aminotransferase (AST) activity

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Doses (mg/kg)</th>
<th>AST activity (IU/L)</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCA 9:1</td>
<td>100</td>
<td>28.0 ± 2.5$^a$</td>
<td>30.0 ± 2.0$^a$</td>
<td>35.0 ± 2.6$^a$</td>
<td></td>
</tr>
<tr>
<td>MCA 8:2</td>
<td>100</td>
<td>27.0 ± 1.5$^a$</td>
<td>29.0 ± 2.1$^a$</td>
<td>33.0 ± 4.0$^a$</td>
<td></td>
</tr>
<tr>
<td>MCA 7:3</td>
<td>100</td>
<td>14.0 ± 1.4</td>
<td>15.3 ± 0.9</td>
<td>15.8 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>13.0 ± 1.5</td>
<td>13.9 ± 2.1</td>
<td>14.0 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $P<0.05$ compared to control (Student’s t-test); MCA= methylene Chloride – Acetone

Table 5. Effect of TN1 and TN2 on aspartate aminotransferase activity

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Doses (mg/kg)</th>
<th>AST activity (IU/L)</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN1</td>
<td>100</td>
<td>22.8 ± 4.5$^a$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TN2</td>
<td>100</td>
<td>37.5 ± 1.9$^a$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>20.0 ± 1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

$^a$ compared to Control (Student’s t-test)
Table 6. Effect of methanol extract on alanine aminotransferase activity

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Doses (mg/kg)</th>
<th>ALT activity (IU/L)</th>
<th>Baseline</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME</td>
<td>100</td>
<td>5.3 ± 4.08</td>
<td>10.66 ± 3.4</td>
<td>22.3 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.6 ± 2.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>6.6 ± 3.2</td>
<td>10.3 ± 4.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.0 ± 3.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>7.3 ± 3.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>4.7 ± 3.2</td>
<td>14.0 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.0 ± 2.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>7.0 ± 2.0&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

*<sup>a</sup> compared to baseline; <sup>b</sup> compared to 10 days; <sup>c</sup> compared to 20 days (Student’s t-test)

Table 7. Effect of methanol and pet ether-ethyl acetate fractions on alanine aminotransferase activity

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Doses (mg/kg)</th>
<th>ALT activity (IU/L)</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF</td>
<td>100</td>
<td>23.2 ± 2.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.3 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.8 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PEF</td>
<td>100</td>
<td>23.5 ± 4.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.0 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.5 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>14.0 ± 2.3</td>
<td>17.5 ± 3.8</td>
<td>20.0 ± 5.2</td>
<td></td>
</tr>
</tbody>
</table>

*<sup>a</sup> compared to Control (Student's t-test)

Table 8. Effect of methylene chloride-acetone fractions on alanine aminotransferase activity

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Doses (mg/kg)</th>
<th>ALT Activity (IU/L)</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCA 9:1</td>
<td>100</td>
<td>16.0 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.0 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.0 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>MCA 8:2</td>
<td>100</td>
<td>14.8 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.0 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.0 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>MCA 7:3</td>
<td>100</td>
<td>10.0 ± 0.9</td>
<td>13.0 ± 0.8</td>
<td>14.8 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>10.0 ± 1.0</td>
<td>13.0 ± 1.5</td>
<td>13.5 ± 0.9</td>
<td></td>
</tr>
</tbody>
</table>

*<sup>a</sup> compared to Control (Student's t-test)

Table 9. Effect of TN-1 and TN-2 on alanine aminotransferase activity

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Doses (mg/kg)</th>
<th>ALT Activity (IU/L)</th>
<th>Day 10</th>
<th>Day 20</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN-1</td>
<td>100</td>
<td>13.5 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TN-2</td>
<td>100</td>
<td>16.0 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>12.0 ± 1.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*<sup>a</sup> compared to Control (Student’s t-test)

4.3 The effect of Weight and Fraction on Body Weight and Organ Weight

The methanol extract and fractions caused a significant (*P*<0.05) dose related increase in the body and organ weight of treated rats (Table 10 Fig 4a). TN-1 and TN-2 caused a significant reduction in organ weight of the treated rats (Fig 4b).

5. DISCUSSION

These compounds are established constituents of neem plant and exhibited the greatest toxic...
Fig 4a. Effect of TN-1 and TN-2 on body weight of treated rats

effect in this study. 6 deacetylnimbin is a tetranortriterpenoid, related to nimbin, and nimbolide but less toxic. Nimbolide is a limonoid tetranortriterpenoid constituent of seed. It was isolated from fresh leaves of Nigerian sample of A. Indica for the first time and the study documented that nimbolide has molecular mass of 466, melting point 245-247°C and optical rotation of \([\alpha]_D +2060\). Its infrared radiation spectrum shows carbonyl absorptions at 1665 (C=C\(\text{CH}_3\)) and 1770 cm\(^{-1}\) (\(\gamma\)-lactone) [26]. An earlier study showed that it exerted anti-malarial effect by inhibiting the growth of Plasmodium falciparum [12]. Nimbolide has been shown to possess antibacterial activity against S. aureus and S. coagulase [11]. It has been shown to be more potent antioxidant in comparison to azadirachtin and ascorbic acid [13]. Nimbolide can induce apoptosis in cancer cells by modulation of apoptotic protein via both intrinsic and extrinsic pathway [27]. Nimbolide has potential to prevent procarcinogenic activation and oxidative DNA by inhibiting phase 1 carcinogen detoxification enzymes (glutathione-s-transferase (GST) and quinine reductase [28,29]. Although accumulating evidence from in-\textit{vivo} and in-\textit{vivo} animal studies indicated that nimbolide and 6 deacetylnimbin extract has multiple pharmacological effects. However, nimbolide was also shown to cause liver and kidney dysfunction and a sudden drop in arterial blood pressure [30]. The greatest toxic effect in this study was shown by nimbolide.
Fig. 4b. Effect of TN-1 and TN-2 on organ weight of treated rats

Preliminary assessment of acute toxicity of the extract in mice using the oral route revealed an LD_{50} value greater than 5000 mg/kg suggesting the extract may be generally regarded as safe [24]. This also implies a remote risk of acute intoxication. Despite this finding, evaluation of the effect of chronic administration of the extract and its solvent fractions on liver functions, body/organ weight showed serious deleterious effects.

Chronic oral administration of the extract and fractions increased the activities of ALT, and AST. This may be due to the hepatocellular damage since these enzymes are located in the periportal hepatocytes and thought to reflect their roles in oxidative phosphorylation and gluconeogenesis. Thus, an increase in their presence or activity in serum may be as a result of intracellular membrane damage and leakage as they are biochemical markers of hepatocellular necrosis [31].

A critical look at the time-course of events showed that enzyme activities peaked on day 20 in this study. The reasons for this are not clear. However, it may not be unrelated to further hepatocellular damage through bioactivation by toxic metabolites of the extract. These damaged hepatocytes have the ability to initiate immunological reactions (innate and adaptive). The necrotic cells also activate other cell types particularly Kuffer cells, natural killer cells, and natural killer T cells that contribute to the
progression of liver injury by producing proinflammatory cells and secreting chemokines to further recruit inflammatory cells to the liver. It has been shown that various inflammatory cytokines such as tumor necrosis factor (TNF) α, interferon (IFN) γ and interleukin (IL) β produced during liver damage are involved in promoting tissue damage [32]. Leukocytes (neutrophils and monocytes) as part of their defensive roles contribute to the injury in response to chemotactic factors during inflammation [33]. Activation of these inflammatory responses of hepatic origin may have led to the peak activity of AST and ALT on day 20. Conversely, at that point, adaptive immunity of the liver could also produce hepatoprotective mediators like interleukin (IL)-6 and 10, prostaglandins and some other anti-inflammatory mediators [34]. These hepatoprotective mediators counteract the effects of the proinflammatory mediators leading to the immunological tolerance, tissue regeneration and repair that may have reduced the activity of the enzymes on day 30. The decline in the activity of these enzymes may indicate recovery from fulminant injury, reflecting a major loss of functional hepatocytes. The liver, however, has a large functional reserve and favors the induction of immunological tolerance rather than immunity. It is the delicate balance of inflammatory and hepatoprotective mediators produced after activation that determines susceptibility and adaptation to liver injury.

Chronic administration of the extract and fractions increased body and organ weights in rats. This may suggest a direct stimulant effect on muscle and liver glycogen metabolism which prevents loss of muscle mass. This is possible because of the existence of functional reserve in a failing liver. It is, however, doubtful if this mechanism is associated with the toxic effect of the extract and fractions since the isolated compounds- 6-deacetylnimbin and nimbolide rather reduced organ weight even when they caused greater toxic effect than the fractions. Hepatocellular tissue damage increased with fractionation of the extract. The reduction in organ weight may directly correlate with the greater degree of tissue damage caused by these compounds and which manifested as toxicity. 6-deacetylnimbin and nimbolide were isolated. They were confirmed and identified as parts or members of the toxic constituents of the seed extract of the neem plant, A. indica. It was revealed that Nimbolide was more toxic than 6-deacetylnimbin.

6. CONCLUSION

Although there was no evidence of lethality from acute toxicity of the extract, chronic oral administration of the extract and solvent fractions caused harmful effects on the liver and kidney. 6-deacetylnimbin and nimbolide were isolated, identified and confirmed toxic constituents of neem seed plant. However, nimbolide was more toxic than 6-deacetylnimbin.

CONSENT

Written consent was not required in this study.

ETHICAL APPROVAL

Ethical approval for the study was obtained by the Central Animal Ethical Committee of University of Nigeria, Nsukka.

ACKNOWLEDGEMENT

The authors acknowledge the Department of Biochemistry University of Nigeria Nsukka.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES


[PubMed]

© 2018 Okafor et al.; This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history/25769