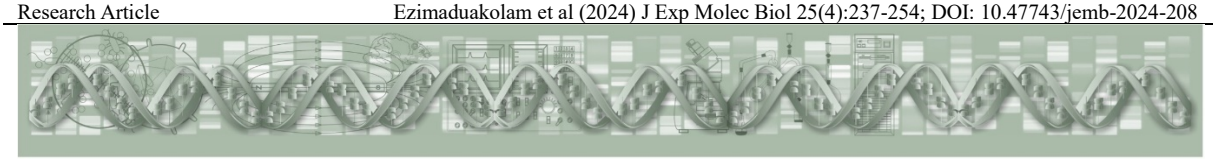

PHYTOCHEMICAL ANALYSIS AND TOXICITY STUDIES OF ETHANOL LEAVES EXTRACT OF *AZADIRACHTA INDICA*, *ANACARDIUM OCCIDENTALE* AND *MORINGA OLEIFERA* IN RATS

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Abstract

Medicinal plants are important natural products in drug discoveries due to their pharmacological activities. *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* have been used in traditional medicine practices for the treatment of many diseases. Various pharmacological activities of these plants have been reported and documented. Beside the medicinal value and pharmacological activities of these plants their phytoconstituents and safety for consumption should also be considered. This study aims at evaluating the phytochemicals composition and toxicity profile of ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* in rats. The phytochemicals analyses were performed using standard analytical methods. Acute and sub-acute toxicity tests were conducted using Lorke's method and OECD guidelines, respectively. This finding revealed that ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* demonstrated the presence of significant amount of many phytochemicals. In acute toxicity test, the LD_{50} value of ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* obtained was 3807.89 mg/kg, 4505.55 mg/kg, and above 5000 mg/kg, respectively. In sub-acute toxicity test, ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* demonstrated an increase in the levels of ALT, AST, total bilirubin, and globulin, while the *Moringa oleifera* exhibited a significantly ($p < 0.05$) decrease in the levels of ALT, AST, and total bilirubin coupled with increase in total protein and albumin levels. The concentrations of urea, creatinine, sodium, potassium, calcium, chlorides, and HCO_3^- were significantly ($p < 0.05$) increased in ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* while decreased in ethanol leaves extract of *Moringa oleifera* in rats. However, ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* demonstrated a significant ($p < 0.05$) decrease in RBC count and PCV while *Moringa oleifera* exhibited a significant ($p < 0.05$) increase in RBC count and PCV. Ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* contain significant amount of phytochemicals which might be responsible for their pharmacological activities. Ethanol leaves extract of *Moringa oleifera* at 5000 mg/kg dose is relatively non-toxic and safe for gavage administration while ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* are slightly toxic for oral administration.

Keywords: *Azadirachta indica*, *Anacardium occidentale*, *Moringa oleifera*, phytochemicals, toxicity

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Introduction

Medicinal plants have been recommended for the treatment of diseases and their safety measures have been documented (WHO 2000). Medicinal plants contain bioactive compounds known to demonstrate pharmacological activities and medicinal properties. Bioactive compounds include phytochemicals that have significant application in drug discovery and development (Abubakar et al. 2021). Phytochemicals from many plants have been reported to demonstrated various pharmacological activities including anti-diabetic (Ebong et al. 2008), anti-hypertensive (Mamta et al. 2013, ACS 2000), anti-cancer (Madhuri and Pandey 2009, Shaikh et al. 2016), anti-inflammatory (Patra et al. 2009), antioxidants (Lobo et al. 2009, Adamu et al. 2017, Ibrahim et al. 2024), anti-ulcer (Abubakar et al. 2020a, 2020b, Abubakar et al. 2021), antipyretic activities (Patra et al. 2009, Chandra et al. 2010), and anti-microbial (Mahesh and Satish 2008, Falowo et al. 2018).

Azadirachta indica commonly known as neem, is a medicinal plant belonging to the family Meliaceae, found abundantly in the tropical and subtropical countries of the world (Mohammad 2016). In Nigeria, *Azadirachta indica* is commonly called Atu yabasi in Igbo, Maina in Hausa, and Dongoyaro' in Yoruba. Neem plant has many therapeutic applications such as traditional medicine, modern medicine, nutraceuticals, food supplements, folk medicine, pharmaceutical intermediates and chemical entities for synthetic drugs (Mahima et al. 2013, Kumar et al. 2015). Different parts of *Azadirachta indica* have been used for the treatment of various diseases including inflammation, viral infections, hypertension, and fever and gastrointestinal diseases (Mossini and Kemmelmeier 2005, Shah et al. 2009, Talpur et al. 2013, Kaur et al. 2020). The parts of the plants have been reported to demonstrated antifungal, antibacterial, antiviral, and antioxidant activities (Adamu et al. 2017).

Anacardium occidentale L. is an evergreen perennial plant found abundantly in tropical countries and belongs to the family Anacardiaceae. The plant is popularly known as cashew and locally called kashu (Igbo), Fisa (Hausa), and Kaju (Yoruba) in Nigeria. Different parts of *Anacardium occidentale* have been used in treatment of many diseases including diarrhea, dysentery, pain, ulcer, diabetes, inflammation, dermatitis, headache, and infectious (Lizcano et al. 2010, Baptista et al. 2018, Gandji et al. 2018). It has been reported that cashew nuts demonstrated anti-diabetic activity due to its low sugar and high fiber contents (Bes-Rastrollo et al. 2007). Aqueous ethanol extract of leaves and nuts of *Anacardium occidentale* L. were reported to demonstrated anti-ulcerogenic activity in rats (Konan and Bacchi 2007, Behravan et al. 2012). Study showed that the acetone extract of cashew stem bark in rodents contains antibodies, and exhibited anti-inflammatory and antinociceptive activities (Vanderlinde et al. 2009). Onasanwo et al. (2012) reported that dichloromethane leaves extract of *Anacardium occidentale* L. demonstrated analgesic activity in rats. Aqueous extract leaves of *Anacardium occidentale* L. exhibited anti-diabetic activity in rats (Sokeng et al. 2001). Studies showed that petroleum ether and ethanolic extracts of cashew leaves demonstrated antimicrobial activities (Dahake et al. 2009, Doss and Thangavel 2011, Onasanwo et al. 2012).

Moringa oleifera Lam. (Family Moringaceae) is an arboreal plant found in many regions in the world mainly in tropical areas (Estrada-Hernández et al. 2016). The plant has been used daily as food vegetable and has nutritional and medicinal value (Ahmad et al. 2014, Santos 2014). *Moringa oleifera*, commonly called Moringa, is widely cultivated in various parts of Nigeria and is locally called Odudu oyibo in Igbo, Zogale in Hausa, and Aweigbale in Yoruba. *Moringa oleifera* has been used in the treatment of many diseases which include skin infections, anaemia, anxiety, asthma, blackheads, blood impurities, bronchitis, catarrh, chest congestion, and cholera (Khawaja et al. 2010, Hamza 2010, Singh and Sharma, 2012). Different parts of *Moringa oleifera* have been reported to demonstrate many pharmacological activities including anti-diabetic, anti-inflammatory, anti-spasmodic, anti-hypertensive, anti-tumor, anti-oxidant, anti-

pyretic, anti-ulcer, anti-epileptic, reported antibacterial, antifungal, diuretic, cholesterol lowering, renal, cardioprotective, and hepatoprotective activities (Lai et al. 2010, Paliwa et al. 2011, Huang et al. 2012, Sharma et al. 2012, Kou et al. 2018). The plant has been used in the manufacture of different pharmaceutical products (Ariana et al. 2022). Leaves, flowers, pods, and seeds of the plant have been used in production of drugs against bacteria, fungi, viruses, and other pathogens in humans (Falowo et al. 2018, Ariana et al. 2022).

Safety is one of the major criteria in the selection of medicinal plants of research interest. Efficacy of plant extracts should not only be considered but also their safety for consumption. Consumption of medicinal plants without knowledge of their toxic level could lead to damaging of organs especially liver and kidney due to their roles in metabolism and excretion of substances. Thus, screening of plant extracts for treatment of disease is couple with the knowledge of their toxicity profile. Research interest on pharmacological activities and medicinal properties of medicinal plants has been increase worldwide. High percentage of the people in the world relies on plants and herbs for remedies than the use of synthetic drugs. Conventional treatments produce side effects and are expensive for higher population of people in the world. Plants and herbs have fewer side effects and are less expensive than synthetic drugs. *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* are common medicinal plants familiar in the tropical and subtropical countries. These plants have been locally used for therapeutic purposes and they are availability, less cost and widely distributions in almost every community. Because of these the interest of researchers on these plants has drastically increased. This study aims at evaluating the phytochemicals composition and toxicity profile of ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* in rats.

Materials and Methods

Plant Materials

Fresh leaves of *Anacardium occidentale*, *Azadirachta indica*, and *Moringa oleifera* were obtained from the Umudike forest, Ikwuano Local Government Area, Abia state, Nigeria. The plants samples were collected in the last quarter of the rainy season in October, 2023. The samples were immediately transported to the Herbarium unit of Michael Okpara University of Agriculture, Umudike, Abia for identification. The *Anacardium occidentale* (MOUAU/CVM/VPP/HERB/16/008), *Moringa oleifera* (MOUAU/CVM/VPP/HERB/16/009) and *Azadirachta indica* (MOUAU/CVM/VPP/HERB/16/010) samples were identified and authenticated by a taxonomist at the Department of Forestry, College of Natural Resources and Environmental Management, Michael Okpara University of Agriculture, Umudike, Abia, Nigeria.

Preparation of Plant extracts

The leaves of the plants were air dried under a shade for 14 days. The dried leaves were pulverized to fine powders using fabricated grinding machine. Preparation of the plant extracts was carried out using the method described by Abubakar et al. (2022a) with little modification. The fine powder (500 g) of each plant sample was extracted with 1.5 L of ethanol for 48 hours with intermittent stirring at 2 hour interval period. Each of the plant extract was filtered using Whatman No. 1 filter paper and concentrated using rotary evaporator under reduced pressure at a temperature 40^0C for three hours. For all the three plant samples a solid dark-green extract was obtained with a weight 100.66 g, 140.18 g and 110.94 g and percentage yield 20.13%, 28.04% and 22.19% for *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* leaves, respectively. The extracts were stored in desiccators until further used.

Experimental Animals

A total of 32 Wistar rats (8 – 10 weeks) of either sex weighing between 180 – 200 g were used in this study. The animals were obtained from the laboratory animal production unit of the

Zoology and Environmental Biology, Michael Okpara University of Agriculture, Umudike. The animals were housed in aluminum cages (5 rats per cage) under standard conditions temperature 23±2°C, relative humidity 30-70%, and 12/12 hours light-dark cycle. The animals were fed with standard grower pellets (Chikkun Finisher Mash, Chikkun Feeds Ltd) and had access to water *ad libitum* for seven days of acclimatization before the commencement of the experiment.

Qualitative Determination of Phytochemicals

Test for the Presence of Alkaloids

The presence of alkaloids in the ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* were determined using Wagner's test method described by Trease and Evans (1989) and Abubakar et al. (2020a, 2022). The plant extracts (3 mL) was added into a test tube containing 3 ml of 1% HCl and then heated at 60°C for 20 minutes. The contents were allowed to cool and then Wagner's reagent (1 mL) was added drop by drop into the test tube. The presence of alkaloids was detected by the formation of reddish-brown precipitate.

Test for the Presence Flavonoids

Flavonoids in the plant extracts were identified by sodium hydroxide test described by Mosa et al. (2012) and Abubakar et al. (2022, 2020a). The plant extracts (3 ml) was added into a test tube containing 1 ml NaOH 10% solution. The deep yellow colour which became colourless following addition of dilute HCl solution indicated the presence of flavonoids.

Test for Tannins

Ferric chloride test was used for qualitative determination of tannins in the three plant extracts as described by Trease and Evans (1989). The plant extracts (1 mL) was added into a test tube containing two milliliters of 5% $FeCl_3$ solution. The presence of tannins was detected by the formation of black or blue-green colour.

Tests for Glycosides

Glycosides in the plant extracts were identified by Salkowski's test method described by Trease and Evans (1989) and Abubakar et al. (2022, 2020a). The plant extracts (5 mL) was added into a test tube containing 25 mL of 1% sulphuric acid and then boiled for 15 minutes. The contents were allowed to cool and then neutralized with 10% NaOH and followed by addition of 5 mL of Fehling's solution A and B. The presence of glycosides was observed by the formation of brick red precipitate of reducing sugars.

Test for Cardiac Glycosides

Keller-Killani test was employed for the screening of cardiac glycosides as described by Mosa et al. (2012) and Abubakar et al. (2020a, 2022). The glacial acetic acid (2 mL) containing one drop of ferric chloride solution was added into each test tube containing 5 mL of the respective plant extracts. One mile of concentrated sulphuric acid was added into the test tube. The formation of brown ring at the interface, violet colour below the brown ring, and greenish colour throughout the acetic acid layer indicated the presences of cardiac glycosides.

Test for Steroids

The presence of steroids in the plant extracts was determined using the method of Trease and Evans (1989). Chloroform (5 mL) and sulphuric acid (5 mL) were added into a test tube containing 500 µl of the plant extracts. The presence of steroids was observed by the formation of violet colour which changed to blue-green.

Test for Saponins

The plant extracts was analyzed for the presence of saponins using Froth test as described by Trease and Evans (1989) and Abubakar et al. (2020a, 2022). The plant extracts (3 mL) was added into a test tube containing 3 mL of distilled water. The contents were thoroughly mixed by vigorous shaken of test tube for about 30 seconds and allowed to stand for 30 minutes. The presence saponins was detected by the formation of stable persistent froth.

Test for Phenols

The qualitative determination of phenols in the plant extracts was carried out in accordance to method described by Trease and Evans (1989) with little modification. Ethanol (5 mL) and ferric chloride (5 mL) was added into each test tube containing 5 mL of plant extracts. Phenols in the plant extracts were identified by the formation of an ink blue color.

Test for Terpenoids

Terpenoids screening in the plant extracts was carried out using the method described by Trease and Evans (1989) and Abubakar et al. (2020a, 2022). The plant extract (1 mL) was added into a test tube containing ethanol (1 mL) and acetic anhydride (1 mL) and then thoroughly shaked. Concentrated sulphuric acid (10 mL) was added into the test tube. The presence of terpenoids was observed by the formation of pink color.

Quantitative Determination of Phytochemicals

Determination of Alkaloids Content

The quantity of alkaloids in each of the plant extracts was determined using the method of Harborne (1973) with little modification. Five grams of each of the plants dried extract was dissolved in 200 mL of 10% acetic acid and 50 mL of ethanol. The contents were allowed to stand for 2 hours, filtered using Whatman No. 1 filter paper and then concentrated using water bath. Concentrated NH_4OH solution was added to the extract in drops and the precipitate formed was collected, washed with dilute NH_4OH solution and then filtered using Whatman No. 1 filter paper. The final alkaloid residue was dried and weighed and the alkaloids content was obtained.

Determination of Flavonoids Content

The flavonoids content in each of the plant extracts was determined using the method described by Harborne (1973) with some modifications. The plants dried extract (5 mg) was boiled (100°C) in 50 ml of 2M HCl solution for 30 minutes under reflux. The content was allowed to cool and then filtered using Whatman No. 1 filter paper. The ethyl acetate (50 mL) was added into the mixture in drops and then to the volume. The mixture was filtered using filter paper and then dried in oven at 6ºC. The dried flavonoids residue was weighed and the quantity of flavonoids in the plant extracts was estimated.

Determination of Saponins Content

Quantitative determination of saponins in each of the plant extracts was carried out using the method of El-Olemylet al. (1994) with a little modification. Five grams of the plants dried extract was dissolved in 150 mL of 50 % ethanol. The contents were boiled for 30 minutes and then filtered using Whatman filter paper. One gram of charcoal was added to the filtrate and the content was heated at 100°C for 30 minutes. The content was filtered using Whatman filter paper and then allowed to cool at room temperature. Acetone (150 mL) solution was added into the filtrate and the content was filtered using Whatman filter paper. The filter paper was quickly introduced into desiccator containing anhydrous calcium chloride. The saponins residue was concentrated to dryness in oven at 6°C. The dried saponins residue was weighed and the saponins content in the plant extracts was determined.

Determination of Steroids Content

The quantitative determination of steroids content in the grapefruits aqueous extract was carried out according to the method described by Evans (1996) with some modifications. Each of the plant extracts (1 mL) was introduced into 10 mL volumetric flask followed by addition of sulphuric acid (2 mL) and iron chloride (2 mL). The contents were treated with 2 mL of potassium hexacyanoferrate (III) solution and then incubated at 70°C for 30 minutes with constant shaking. Absorbance of the sample against the blank was read at 780 nm wavelength using spectrophotometer. The amount of steroids in the plant extracts was determined.

Determination of Tannin Content

Tannin in each of the plant extracts was quantitatively determined using the method of AOAC (1999) with some modifications. The tannic acid standard solution was prepared by dissolving 10 mg of tannic acid in 100 mL of distilled water. Different concentrations of tannic acid standards (0 – 2.5 ml aliquots) were prepared in the respective 25 mL volumetric flasks followed by the addition of Folin-Denis reagent (2.5 ml) and sodium carbonate solution (1.25 mL) and then the content was made up to the volume. After 30 minutes absorbance was measured at 760 nm wavelength using spectrophotometer. The dried powder extract (1 g) of each of the plants was boiled in distilled water (80 mL) for half hour. The contents were allowed to cool and then diluted in 100 mL volumetric flask. The contents were filtered using Whatman filter paper No. 1 and the amount of tannin in the filtrate was determined. The tannin content in the plant extracts was obtained using tannic acid standard curve and expressed as mg/g of the extract.

Determination of Cardiac Glycosides

Quantitative determination of cardiac glycosides in the plant extracts was performed using the method of Harborne (1973). One gram of each of the plants dried powder extract was dissolved in 20 mL of distilled water and then 2.5 mL of 15% lead acetate was added. The mixture was filtered using Whatman filter paper. A volume of 2.5 mL chloroform was added into the filtrate and the content was vigorously shaken. The lower layer was collected and evaporated to dryness in oven at 6°C. The residue was dissolved in 3 mL of glacial acetic acid followed by addition of 0.1 mL of 5% ferric chloride and 0.25 mL of concentrated H_2SO_4. The contents were shaken and incubated for 2 hours in the dark. Absorbance of the sample against the blank was measured at 530 nm spectrophotometrically.

Determination of Terpenoids Determination

Terpenoids content in each of the plant extracts was analyzed using the method of Harborne (1973). The dried plant extracts (1 g) were dissolved in 50 mL of ethanol and then filtered using Whatman filter paper. The filtrate was treated with 2.5 ml of 5% aqueous phosphomolybdic acid and 0.25 mL of concentrated H_2SO_4 gradually. The contents were allowed to stand for 30 minutes. Absorbance of the sample against the blank was read at 700 nm using spectrophotometer.

Determination of Phenols

The quantitative analysis of phenols in each of the plant extracts was carried out using the method described by Santhi and Sengottuvel (2016). One gram of each of the plant extracts was dissolved 50 mL of ether and boiled for 15 minutes. The plant extracts was pipetted out into a 50 mL conical flask and then 10 mL of distilled water, 2 mL of NH_4OH, and 5 mL of conc. amyl alcohol was added into the flask. The contents were shaked and incubated for 30 minutes at room temperature. The absorbance of the sample against the blank was measured using spectrophotometer at 505 nm wavelength.

Determination of Glycosides

Glycosides in the each of the plant extracts were determined using the method of Nbaeyi-Nwaoha and Onwuka (2014). The plant extracts (1 g) was added into the respective test tubes containing 2.5 mL of 15% lead acetate. The contents were mixed thoroughly and then filtered using Whatman filter paper. The chloroform (2 mL) was added into the filtrate, mixed thoroughly and allowed to settle at room temperature. The lower portion was collected and evaporated to dryness in oven at 60°C. The dried lower portion was mixed with 3 mL of glacial acetic acid, 0.1 mL of 5% ferric chloride and 0.25 mL of conc. H_2SO_4 and then allowed to stand for 3 hours. Absorbance of the sample against the blank was read at 568 nm wavelength using spectrophotometer and the amount of glycosides was obtained.

Acute Toxicity (LD_{50}) Study

The acute toxicity test of each plant extracts was carried out in using the Lorke's method (1983). The method involves two phases. In the first phase, nine rats were divided into three groups; three rats per group. The animals in group 1, 2, and 3 were orally administered with 10, 100 and 1000 mg/kg doses of the plant extracts per body weight of the rats, respectively. In the second phase, three rats were used. The animals were treated with 1600, 2900 and 5000 mg/kg doses of the plant extracts per body weight, respectively. All the animals were observed for any sign of toxicity and lethality for 24 hours. The animals were initially observed for 24 hours and up to 7 days. The LD_{50} value of each of the plant extracts was calculated using the following formula:

$LD_{50} = \sqrt{D0 \text{ X } D100}$

Where; D_0= highest dose that produced no mortality, D_{100} = lowest dose produced mortality.

Sub-acute Toxicity Study

Sub-acute toxicity test was conducted using the Organization for Economic Co-operation and Development (OECD) guidelines 407 (2008) with some modifications. A high dose (800 mg/kg) of each of the three plant extracts was selected based on the results of the acute toxicity test. The Wistar rats (8 – 10 weeks, 180 – 200 g, n = 20) were divided into 4 groups each of 5 rats. Group 1 (normal control) was treated with 10 ml/kg/day b.w. of normal saline by gavage. Group 2, 3, and 4 were administered orally with the 800 mg/kg/day b.w. of the ethanol leaves extract of *Anacardium occidentale*, *Azadirachta indica*, and *Moringa oleifera*, respectively. The repeated dose **(**800 mg/kg) extract was administered daily for 28 days. Signs of toxicity and mortality were observed, and body weight of the animals was evaluated weekly. At the end of the administration period, the animals were fasted for 12 hours, anaesthetized using chloroform and then euthanized by cervical dislocation. Blood samples were collected by cardiac puncture into EDTA and plain tubes for haematological and biochemical analysis, respectively. For the biochemical analysis the blood samples were allowed to clot and retract within two hours and then centrifuged at 10,000 x g for 10 minutes. The sera were separated for the various biochemical analyses.

Biochemical analysis

The analysis of biochemical parameters total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT) alkaline phosphatase (ALP), total bilirubin, globulin, urea, creatinine, sodium, potassium, calcium, chlorides, and HCO_3^- was conducted spectrophotometric/colometric method using standard diagnostic kits (Sigma-Aldrich) according to the manufacturer's instructions.

Hematological analysis

The hematological counts parameters white blood cells (WBC), red blood cells (RBC), haemoglobin (Hb), platelet count (PLT), packed cellular volume (PCV) mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were analyzed using hematology analyzer (Sysmex).

Statistical Analysis

The results were expressed as mean ± SD. Statistical Package for Social Sciences (SPSS) Statistics version 22 software (IBM Corp., Armonk, NY, USA) was used for data analysis. Significant differences were determined using one way analysis of variance (ANOVA) at confidence level (95%) by Dunnett's test. Mean differences were considered statistically significant at $p < 0.05$.

Results

Qualitative Phytochemical Screening of the Plant extracts

Table 1 shows the result of qualitative phytochemical screening of the ethanol leaves extract of *Anacardium occidentale*, *Azadirachta indica*, and *Moringa oleifera*. The result showed that all the plant extracts contain various phytochemicals including alkaloids, flavonoids, saponins, glycosides, tannins, terpenoids, steroids, cardiac glycosides, and phenols (Table 1). However, the *Azadirachta indica* and *Anacardium occidentale* leaves extract demonstrated high amount of alkaloids and saponins while *Moringa oleifera* leaves extract exhibited moderate amount of alkaloids and saponins. The *Azadirachta indica* and *Moringa oleifera* leaves extract contained moderate amount of tannins, glycosides, and phenols which were fairly found in the *Anacardium occidentale* leaves extract. The moringa, *Anacardium occidentale*, and *Azadirachta indica* leaves extract exhibited high, moderate, and low amount of flavonoids, respectively. Moderate amount of terpenoids and steroids were found in the *Azadirachta indica* and *Anacardium occidentale* leaves extract while *Moringa oleifera* leaves extract demonstrated low amount of terpenoids and steroids. All the three plant extracts demonstrated low amount cardiac glycosides (Table 1).

Table 1. Qualitative Phytochemicals Screening of the Plant extracts

Phytochemical	*Ao* Extract	*Ai* Extract	*Mo* Extract
Alkaloids	+++	+++	++
Tannins	+	++	++
Flavonoids	++	+	+++
Phenols	+	++	++
Terpenoids	++	++	+
Saponins	+++	+++	++
Glycosides	+	++	++
Steroids	++	++	+
Cardiac glycosides	+	+	+

+++ = High amount, ++ = Moderate amount, + = Low amount. *Azadirachta indica* (Ai), *Anacardium occidentale* (Ao), *Moringa oleifera* (Mo)

Quantitative Phytochemicals Composition of the Plant extracts

The quantitative phytochemicals composition of the ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* is shown in Figure 1. The ethanol leaves extract of *Azadirachta indica* demonstrated high significant amount of alkaloids (60.49 mg/g), saponins (35.45 mg/g), and tannins (13.79 mg/g), than the *Anacardium occidentale* (35.11 mg/g, 28.55 mg/g, 1.99 mg/g), and *Moringa oleifera* (15.34 mg/g, 25.19 mg/g, 12.84 mg/g), respectively. High quantity of flavonoids (12.84 mg/g) and phenols (19.07 mg/g) was recorded in *Moringa oleifera* ethanol leaves extract compared to the amount demonstrated by the *Azadirachta indica* (5.44 mg/g, 9.93 mg/g) and *Anacardium occidentale* (10.32 mg/g, 7.12 mg/g), respectively. *Anacardium occidentale* ethanol leaves extract exhibited high value of glycosides (7.90 mg/g), steroids (11.14 mg/g), cardiac glycosides (5.62 mg/g), and terpenoids (10.60 mg/g) than the *Azadirachta indica* (4.82 mg/g, 3.92 mg/g, 3.32 mg/g, 7.72 mg/g) and *Moringa oleifera* ethanol leaves extract (6.62 mg/g, 5.66 mg/g, 2.69 mg/g, 6.33 mg/g), respectively (Figure 1).

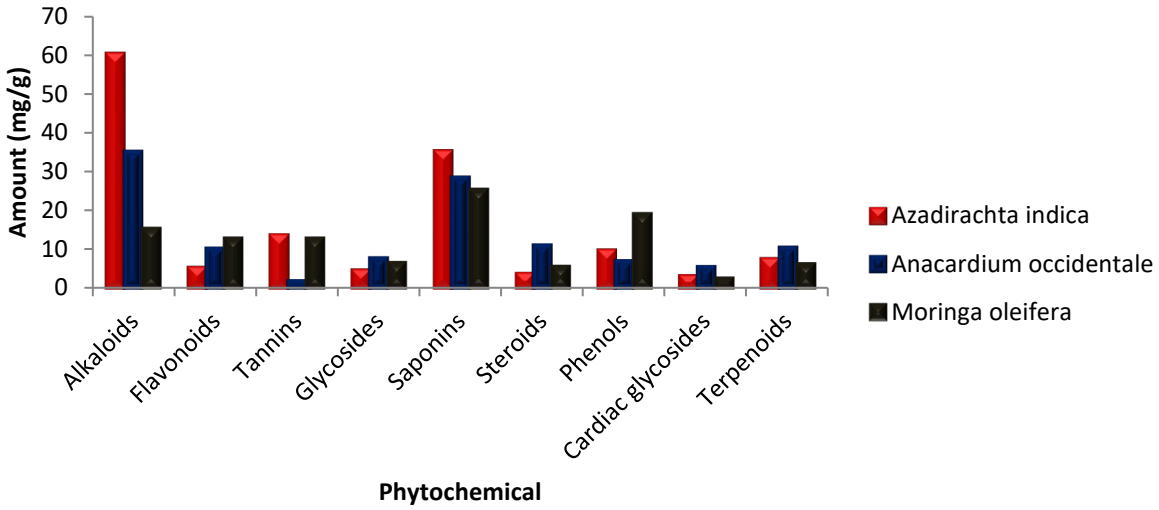

Figure 1. Quantitative Phytochemicals Composition of the Plant extracts
Values are expressed as mean ± SD (n = 3)

Acute Toxicity Test

For the ethanol leaves extract of *Azadirachta indica*, no sign of toxicity was observed and no mortality recorded at all the doses administered in the first phase (10, 100 and 1000 mg/kg). In the second phase, 1600 mg/kg and 2900 mg/kg doses of the extract the animals did not show any sign of toxicity and no mortality found. However, at 5000 mg/kg of the extract, rats were initially calm and depressed, and 100% mortality was recorded before the end of 24 hours period of administration. Thus, the LD_{50} value of the ethanol leaves extract of *Azadirachta indica* was 3807.89 mg/kg. In the first phase of acute toxicity test of ethanol leaves extract of *Anacardium occidentale* there was no sign of toxicity observed nor mortality recorded at all the administered doses (10, 100 and 1000 mg/kg). In the second phase, administration of 1600 mg/kg and 2900 mg/kg produced no mortality while at 5000 mg/kg, 33.33% mortality was recorded and 100% mortality rate was recorded at 7000 mg/kg of the extract. Thus, the LD_{50} value of ethanol leaves extract of *Anacardium occidentale* was 4505.55 mg/kg body weight. The ethanol leaves extract of *Moringa oleifera* at all the doses in phase 1 (10, 100 and 1000 mg/kg) and phase 2 (1600, 2900, and 5000 mg/kg) did not produce mortality or any sign of toxicity in rats during the observation period. Thus, the LD50 value of ethanol leaves extract of *Moringa oleifera* was greater than 5000 mg/kg.

Sub-acute Toxicity Studies

Effect of the Plant extracts on Body Weight of Rats

Figure 2 shows the effect of the plant extracts on body weight of rats. In the third week of the plant extracts administration, ethanol leaves extract of *Azadirachta indica* demonstrated significant ($p < 0.05$) decrease in the body weight of rats as compared with the control. In comparison with the control, administration of ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* significantly ($p < 0.05$) decrease the body weight of rats in week 4. However, the ethanol leaves extract of *Moringa oleifera* exhibited significant ($p < 0.05$) increase in the body weight of rats as compared with the control in week 4 (Figure 2).

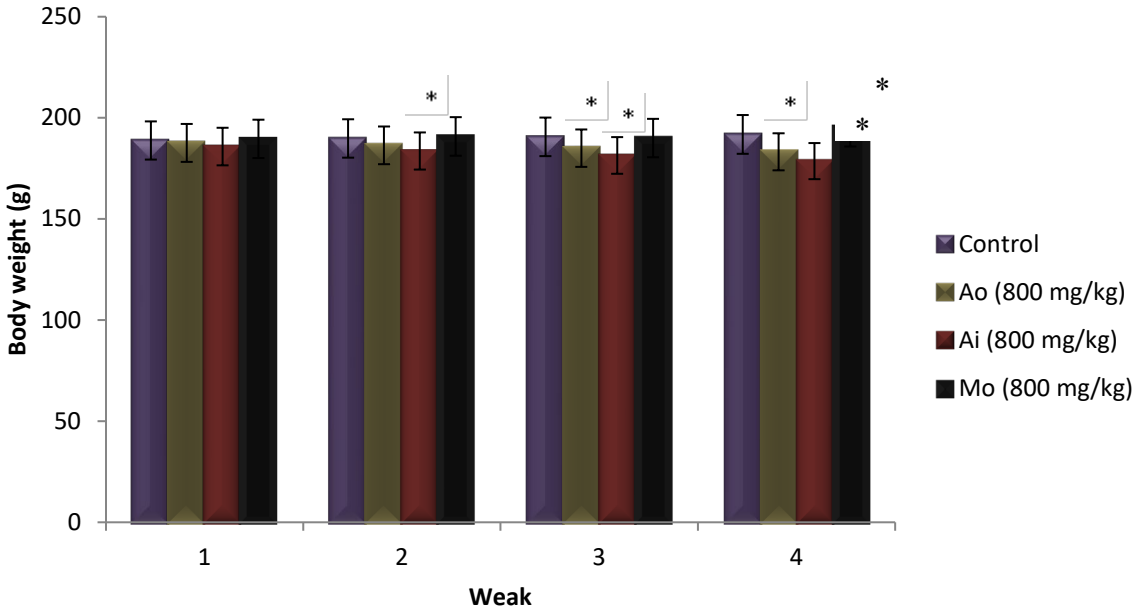

Figure 2. Effect of the plant extracts on the body weight of rats
Data are expressed as mean ± SD (n = 5).
*$p < 0.05$ statistically significant compared with control (One-way ANOVA) followed by Dunnett's multiple comparison test. *Azadirachta indica* (Ai), *Anacardium occidentale* (Ao), *Moringa oleifera* (Mo)

Effect of the Plant extracts on Biochemical Parameters in Rats

Table 2 shows the effect of ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale* and *Moringa oleifera* on liver function parameters in rats. The ethanol leaves extract of *Moringa oleifera* demonstrated significant ($p < 0.05$) increase in total protein levels in the rats compared with the control. The concentration of albumin in the rats was significantly ($p < 0.05$) decrease following administration of the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* when compared with the control. In comparison with the control, the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* exhibited significant ($p < 0.05$) increase in the level of ALT, AST and total bilirubin in the rats. The ethanol leaves extract of *Azadirachta indica* demonstrated a significant ($p < 0.05$) decrease in the level of ALP compared with control and the other treated groups. However, the result also showed that ethanol leaves extract of *Azadirachta indica* and *Moringa oleifera* demonstrated significant ($p < 0.05$) increase in globulin levels in the rats compared with the control (Table 2).

Table 2. Effect of Plant extracts on Liver Function Parameters in Rats

Group	Control	*Ao* (800 mg/kg)	*Ai* (800 mg/kg)	*Mo* (800 mg/kg)
Total protein (g/dl)	5.78±0.11^{a}	5.65±0.15^{a}	5.81±0.28^{a}	6.19±0.10^{b}
Albumin (g/dl)	3.29±0.04^{a}	3.18±0.09^{ab}	3.09±0.07^{b}	3.39±0.07^{b}
Globulin (g/dl)	2.49±0.13^{a}	2.48±0.08^{a}	2.72±0.23^{ab}	2.90±0.09^{b}
ALT (u/l)	35.00±1.00^{a}	39.33±1.16^{b}	43.00±3.61^{b}	34.14±3.00^{a}
AST (u/l)	42.33±2.52^{a}	45.00±4.58^{b}	44.67±2.52^{b}	41.00±1.73^{a}

ALP (u/l)	81.33±4.73[a]	79.33±4.51[a]	76.67±6.11[b]	80.00±9.17[a]
TB (mg/dl)	0.69±0.04[a]	0.83±0.09[b]	0.85±0.06[b]	0.66±0.04[a]

Data are expressed as mean ± SD (n = 5)
Values with different letters are statistically significant ($p < 0.05$) (One-way ANOVA) followed by Dunnett's multiple comparison test. *Azadirachta indica* (Ai), *Anacardium occidentale* (Ao), *Moringa oleifera* (Mo), standard deviation (SD), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB)

The effect of ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale* and *Moringa oleifera* on the renal function parameters in rats is shown in Table 3. All the three plant extracts exhibited non-significant ($p > 0.05$) changes in the levels of urea, creatinine, potassium, calcium, chlorides, and HCO_3^- when compared with the control. Although the no significant changes were observed, the levels of urea, creatinine, potassium, calcium, chlorides, and HCO_3^- were increased following administration of the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* and decreased by the administration of the ethanol leaves extract of *Moringa oleifera* in rats. However, in comparison with the control, the ethanol leaves extract of *Anacardium occidentale* demonstrated significant ($p < 0.05$) increase in concentration of sodium in the rats.

Table 3. Effect of the Plant extracts on Renal Function Parameters in Rats

Group	Control	*Ao* (800 mg/kg)	*Ai* (800 mg/kg)	*Mo* (800 mg/kg)
Urea (mg/dl)	20.25±0.47[a]	21.34±1.84[a]	20.93±0.79[a]	19.87±1.12[a]
Creatinine (mg/dl)	0.76±0.06[a]	0.81±0.09[a]	0.79±0.03[a]	0.74±0.06[a]
Na+ (mEq/L)	130.73±1.96[a]	135.57±2.31[b]	132.20±2.86[a,b]	129.67±2.93[a]
k+ (mEq/L)	4.36±0.15[a]	4.47±0.14[a]	4.50±0.11[a]	4.54±0.18[a]
Cl^- (mEq/L)	89.60±0.70[a]	90.93±0.94[a]	92.20±2.27[a]	89.83±1.01[a]
HCO_3^- (mmol/L)	19.87±0.31[a,b]	20.3±0.10[b]	20.23±0.15[b]	19.73±0.35[a]

Values are expressed as mean ± SD (n = 5)
Values with different letters are statistically significant ($p < 0.05$) (One-way ANOVA) followed by Dunnett's multiple comparison test. *Azadirachta indica* (Ai), *Anacardium occidentale* (Ao), *Moringa oleifera* (Mo), standard deviation (SD), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TB)

Effect of the Plant extracts on Haematological Parameters

Table 4 shows the effect of ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale* and *Moringa oleifera* on the haematological parameters in rats. The ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* demonstrated significant ($p < 0.05$) decrease in RBC count and PCV compared with the control. In comparison with the control, the ethanol leaves extract of *Moringa oleifera* exhibited non-significant ($p > 0.05$) increase in RBC count and PCV (Table 4). Hemoglobin content was significantly ($p < 0.05$) decrease in rats administered with the ethanol leaves extract of *Azadirachta indica*. All the three plant extracts demonstrated significant ($p < 0.05$) increase in WBC count (Table 4). In comparison with control, ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* exhibited significant ($p < 0.05$) decrease and increase in PLT and MCH content, respectively (Table 4).

Table 4. Effect of the Plant extracts on Haematological Parameters in Rats

Group	Control	*Ao* (800 mg/kg)	*Ai* (800 mg/kg)	*Mo* (800 mg/kg)
RBC (x10^6/mm^3)	7.07±0.38^a	6.57±0.14^b	6.41±0.21^b	7.16±0.11^a
PCV (%)	44.00±1.22^a	41.00±1.50^b	40.33±2.08^b	44.04±1.00^a
Hb (g/dl)	16.73±0.46^a	16.07±0.12^{a,b}	15.73±0.70^b	16.80±0.26^a
WBC (x10^3/mm^3)	8.95±0.45^a	9.73±0.08^{b,c}	10.16±0.28^c	9.56±0.15^b
PLT (x10^3/mm^3)	234.00±2.61^a	227.67±2.52^b	229.00±2.19^b	233.33±3.16^a
MCV (fl)	62.31±2.15^a	62.43±0.22^a	63.02±4.29^a	61.48±0.52^a
MCH (pg)	23.69±0.63^a	24.47±0.40^b	24.55±0.31^b	23.47±0.25^a
MCHC (g/dl)	38.03±0.53^a	39.20±0.73^a	39.10±0.30^a	38.19±0.35^a

Values are expressed as mean ± SD (n = 5)
Values with different letters are statistically significant ($p < 0.05$) (One-way ANOVA) followed by Dunnett's multiple comparison test. *Azadirachta indica* (Ai), *Anacardium occidentale* (Ao), *Moringa oleifera* (Mo), standard deviation (SD), packed cell volume (PCV), haemoglobin (Hb), white blood cell (WBC).

Discussions

Result of this study showed that ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* contained many phytochemicals including alkaloids, flavonoids, tannins, glycosides, saponins, steroids, cardiac glycosides, phenols, and terpenoids. Phytochemicals have been reported with many pharmacological activities and have medicinal importance (Oghenejobo 2017, Abubakar et al. 2022). It has been reported that flavonoids isolated from different plant extracts exhibited antioxidant activity scavenging activity, anti-cancer, anti-malarial, antihypertensive and anti-ulcer activity (Ballard and Marostica 2018). Brewer (2011) reported that alkaloids isolated from different plant extracts demonstrated analgesic activity. Saponins are source of steroidal hormones which have been shown to demonstrated anticholesterolmia activity (Kar 2007). Tannins isolated from plant extracts have been used as wound healing agents, astringents agents and in treatment of gastrointestinal diseases (De-Bruyne et al. 1999, Kar 2007). Denwick (2002) reported that cardiac glycosides isolated from different plant extracts have been used in management of cardiovascular diseases and including congestive heart failure and cardiac arrhythmia. Steroids are important precursors for biosynthesis of sex hormones and steroidal drugs such as corticosteroid (Majeed et al. 2004). Phenols from plant extracts demonstrated many pharmacological activities including anti-ulcer, anti- inflammatory, cytotoxic and antitumor, anti-spasmodic, and anti-depressant activities (Ghasemzadeh et al. 2010).

Acute toxicity test (LD_{50}) provides basic information for classification and hazard assessment of new chemical substances which can be used for safety statements on labels of potentially poisonous substances (OECD 2001). Lethal dose 50 % (LD_{50}) is an indicator of the potential of substance to produce harmful effect and can be used to obtained therapeutic dose and therapeutic index of test substance (Barile, 2010). In this study, the LD_{50} value of ethanol leaves extract of *Azadirachta indica* (3807.89 mg/kg) and *Anacardium occidentale* (4505.55 mg/kg) was less than 5000 mg/kg; while that of *Moringa oleifera* was greater than 5000 mg/kg. Substances with an LD_{50} value greater than 5000 mg/kg through gavage administration are regarded as being safe (Abubakar et al. 2020a; OECD, 2001). It has been reported that substance with $LD_{50} \leq 50$ mg/kg, $LD_{50} = 50 - 300$ mg/kg, $LD_{50} = 301 - 2000$ mg/kg, $LD_{50} =$

2001 - 5000 mg/kg, and LD_{50}> 5000 mg/kg is regarded as highly toxic, toxic, moderately toxic, slightly toxic, and non-toxic, respectively (Loomis and Hayes 1996, Erhirhie et al. 2018). This indicated that the ethanol leaves extract of *Moringa oleifera* at 5000 mg/kg dose is relatively non-toxic and safe for gavage administration. This also suggested that the ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* are slightly toxic for oral administration.

Change in body weight is important indicator of adverse effects of a substance (Abubakar et al. 2020a). In this study, ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* demonstrated significant decrease in the body weight of rats while *Moringa oleifera* exhibited significant increase in the body weight of rats. Decrease in body weight is associated with toxic effect of a chemical substance (Teo et al. 2002, Abubakar et al. 2020a). Liver and kidney are major organs affected by the toxic effect of substances (Auerbach et al. 2012, Awe and Banjoko 2013). Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are important markers of liver functions and used as biomarkers to evaluate toxic effect of a substance (Rahman et al. 2000). High levels of AST and ALT in the blood are attributed to destruction of liver parenchymal cells (Abubakar et al. 2020a). High level of AST and ALP is an indicator of bile duct, lungs, kidneys and liver damage (Awe and Banjoko 2013, Mujahid et al. 2017). In the present study, the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* demonstrated an increase in the levels of ALT, AST, total bilirubin, and globulin. This indicated that liver cell functions in the rats might be affected by the oral administration the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica*. However, the ethanol leaves extract of *Moringa oleifera* demonstrated a decrease in the levels of ALT, AST, and total bilirubin coupled with increase in total protein and albumin levels. This suggests that repeat-dose oral administration of the *Moringa oleifera* did not caused changes in the liver cell functions in the rats.

Urea and creatinine are waste products passed into the bloodstream and excreted by the kidney. Urea and creatinine levels are vital renal functions biomarkers (Rahman et al. 2000). High levels of urea and creatinine in the blood is an indicator of renal function impairment (Cameron and Greger 1998). In the present study, administration of the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* increased the levels of urea, creatinine, sodium, potassium, calcium, chlorides, and HCO_3^- while decreased by the administration of the ethanol leaves extract of *Moringa oleifera* in rats. This suggested that sub-acute administration of the *Moringa oleifera* extract does affected normal renal functions while *Anacardium occidentale* and *Azadirachta indica* extract could cause renal impairment.

In this study, ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* demonstrated significant decrease in RBC count and PCV while *Moringa oleifera* exhibited significant increase in RBC count and PCV. Hemoglobin content was significantly decrease by the ethanol leaves extract of *Azadirachta indica*. All the three plant extracts demonstrated significant increase in WBC count. High rate of hematopoiesis renewal has been considered as a sensitive target for toxicity (d'Yvoire et al. 2012). Increase in the rate of hematopoiesis is associated with increase in erythrocytes and leukocytes. In conditions such as dehydration and blood loss, more cells are produced in biological system to meet out the demand for more mature cells (d'Yvoire et al. 2012). Thus, increase in WBC counts by the plant extracts could be attributed to any such factors. Haemoglobin (Hb) is responsible for the transport of oxygen into the tissues and carbondioxide out of the tissues and absorbs nutrients used to release energy for normal body function (Enechi et al. 2019). Presence of phytochemicals is associated with elevated level of red blood cell and hemoglobin due to their potential to activate the production of erythropoietin that stimulates the stem cells of the haemopoietic tissue to produce more red blood cells (Arunsi et al. 2020). Phytochemicals demonstrated inhibitory effect on red blood cells hemolysis which elevates the serum levels of bilirubin resulting to harmful effects on the hematological system (Fevery 2008). White blood cells (WBC) play an important role in the

immune system by fighting infections and inflammation in the body. Thus, the non-significant changes in WBCs may indicate that the extracts did not increase or suppression the immune response of treated animals (Tousson et al. 2011). This suggested that the ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* could affect the erythropoiesis process.

Conclusions

The ethanol leaves extract of *Azadirachta indica*, *Anacardium occidentale*, and *Moringa oleifera* contain several phytochemicals. The presence of these bioactive metabolites implies that the plants have medicinal properties. The ethanol leaves extract of *Moringa oleifera* at 5000 mg/kg dose is relatively non-toxic and safe for gavage administration while ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* are slightly toxic for oral administration. The repeat-dose oral administration of the *Moringa oleifera* did not caused changes in the liver and kidney cell functions in the rats while repeat-dose oral administration of the ethanol leaves extract of *Anacardium occidentale* and *Azadirachta indica* might affect the normal liver and kidney cell functions. This study also suggests that ethanol leaves extract of *Azadirachta indica* and *Anacardium occidentale* could affect the erythropoiesis process. Further studies should be done to evaluate chronic toxicity profile of the plants and to investigate medicinal properties and pharmacological effects of the plants.

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Conflict of Interest
The authors declare no conflict of interest.

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