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Phytochemical characterization, *in vitro* antibacterial activity, *in vivo* acute toxicity studies of the seed oil of *Azadirachta indica* (neem oil) in Wistar rats

Abstract

The aim of this study was to phytochemically characterize, evaluate *in vitro* the effect of neem oil on some pathogenic bacteria and to investigate *in vivo* the acute oral toxic effects neem. The neem oil extracted from neem seeds was obtained from the University of Maroua Student Centre (commercial production). Extraction was done by the mechanical cold pressing method. The composition was analysed via Gas Chromatography/Mass spectrometry (GC/MS). The *in vitro* antibacterial activity was conducted through Agar disc diffusion and broth macro dilution methods. Tested microorganisms included both standard and clinical isolates: *Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC35218, *Enterococcus faecalis* ATCC51299, *Aerococcusviridans* ATCC11563, *Klebsiella. pneumoniae, Salmonella typhi, Shigellaspp, Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli* and *Staphylococcus aureus.* The acute oral toxicity test was performed according to the Organization for Economic Co-operation and Development (OECD) guidelines 420 in Wistar rats. Fatty acid analysis resulted in the detection of 5 fatty acids. GC/MS identified 19 bioactive compounds. Inhibition zone diameters varied from 9 (*P. mirabilis*) to 14 mm (*A. viridans* ATCC11563 and *E. faecalis* ATCC51299). *S. aureus* ATCC25923, *E. coli* ATCC35218, *K. pneumoniae, S. typhi, Shigellaspp,* and *S. aureus* were resistant. It was observed that, the ratio of the minimum inhibitory and bactericidal concentrations was equal to 2 (MBC/MIC=2), implying the bactericidal property of the oil. Oral administration of neem oil at a dose of 3mL/100g in rats caused no lethal effects

Keywords: *Azadirachta indica* A Juss, neem oil, antibacterial activity, acute toxicity

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Introduction

Plant-based antimicrobials have immense potential to combat bacterial, fungal, protozoal and viral diseases without any known side effects. The use of plants and its products has a long history that began with folk medicine and through the years has been incorporated in traditional and allopathic medicine as well as to extend the shelf life of foods, showing inhibition against bacteria, fungi and yeasts.[1] Most of their properties are due to essential oils produced by their secondary metabolism. Essential oils also known as volatile oils are products of secondary metabolism of aromatic plants.[2] Essential oils are derived from a variety of natural sources including plants or components of plants such as flowers, leaves, bark, roots, berries, seeds and/or fruit.[3] Essential oils and extracts from several plant species are able to control microorganisms related to skin, dental caries, and food spoilage, including Gram-negative and Gram-positive bacteria. Following the success of early antibiotic therapies such as penicillin, leaders in public health during the mid-20th century declared that the end of infectious diseases was approaching. However infectious diseases currently are the second leading cause of death worldwide, being responsible for approximately 15million deaths each year. This is because treatment by antibiotics faces major challenges of drug resistance. The active principles present in plants appear to be one of the important alternatives when compared to many synthetic medicines, because of their less or no side effects, cheap, readily available, and their better bioavailability.[4] Hence, researchers have recently paid attention to safer phytomedicines and biologically active compounds isolated from plant species used in herbal medicines with acceptable therapeutic index for the development of novel drugs.[4,5] As disease causing bacteria continue to exhibit resistance to antibiotic therapy, essential oils represent a potential weapon against emerging ''superbugs''. In the Northern region of Cameroon, neem oil is used to control constipation, rheumatism, skin infections and also as an appetite suppressant.

It is in this context that the seed oil of neem from the neem plant, one of the world's versatile plants will be screened for antibacterial activity against pathogenic bacteria as a contribution in a new horizon of combating bacterial antibiotic resistance. The objective of study was to phytochemically characterize, evaluate *in vitro* the antibacterial activity of the essential of *Azadirachta indica* A. Juss (neem seed oil) on some pathogenic strains of bacterial and determine *in vivo* the acute oral toxicity of this oil.

Materials and methods

Plant material

1.5L of the essential oil of neem was bought at the Maroua University Student Center. A follow up on the method of extraction for future extractions at the production site indicated the oil was extracted through the mechanical method by cold pressing.

Test microorganisms

Eleven bacterial strains were used. The microorganisms were obtained from the bacteriology laboratory of the Yaoundé University Teaching Hospital (CHUY). They included four standard strains and seven clinical strains. They are: *Staphylococcus aureus* ATCC25923, *Escherichiacoli* ATCC35218, *Enterococcus faecalis* ATCC51299,

Aerococcusviridans ATCC11563, *Klebsiella pneumoniae, Salmonella typhi, Shigella spp, Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli, Staphylococcus aureus.*

Experimental animals

A total of 20 Wistar albino rats of both sexes (10 males and 10 females), 6-8 weeks old and of average weight 100g were used for the study. All the animals were obtained from the animal house of the Faculty of Medicine and Biomedical Sciences (FMBS) -UY1.The rats were maintained at room temperature with a 12h light/dark cycle. The rats were randomized into experimental and control groups of 5 animals each and housed in clean plastic cages (25cm long, 18cm wide and 14cm tall). Animals were given free access to standard diet and water. All experimental procedures were performed according to the Organization for Economic Co-operation and Development (OECD) guidelines 420 in Wistar rats and were approved by the Faculty of Medicine and Biomedical Sciences Ethical Committee with an approval number 0226/UY1/FMSB.

Phytochemical characterisation

This was done in collaboration with an external laboratory-Council for Scientific and Industrial Research (CSIR), department of science and technology, Pretoria-South Africa. 50ml of neem seed oil was packaged and sent to the Centre for Science and Industrial Research(CSIR), Bio-analytical laboratory for chemical characterization of *Azadirachta indica* oil from neem seed. The method used was gas chromatography coupled with mass spectrometry. The essential oil obtained was dried over anhydrous sodium sulphate and kept at 4°C for analysis.

Analysis of the essential oils

GC/MS analysis of the essential oil was carried out on an Agilent 6890 equipped with amass spectrometric detector (MSD), model Agilent 5973, equipped with an HP-5MS column (30m×0.25mm, 0.25μm); programming from 80 (3 min) to 260°C at 8°C/min, 10 min hold; carrier gas, helium; flow rate, 1.0 ml/min; injection in split mode (60.1); injector and detector temperatures 225 and 300°C, respectively. The EIMS mode at 70 ev; electron multiplier,2500 V; ion source temperature, 250°C; mass spectra data were acquired in the scan mode in the *m/z* range 50 to 700.The essential oil components were identified by comparing their mass fragmentation patterns with those of the available reference. In addition, qualitative analysis was carried out by using internal normalization method (peak area measurement) and compound identification was confirmed by electronic Wiley and NIST mass spectral data base. The retention indices (RI) of the volatile oil components were determined relative to the retention times of series of hydrocarbons of the analytes.

Biological activity

The antimicrobial susceptibility tests were done using a slightly modified version of the agar disk diffusion method as described by Tendencia E; A, in the laboratory manual of standardized methods for antimicrobial sensitivity tests for bacteria isolated from aquatic animals and environment. 2004.[6]

Preparation of culture media

The culture media, MHA and MHB were prepared according to the manufacturer's instructions, sterilized in an autoclave at 121°C for 15minutes.

Preparation of inocula bacteria

Inoculum of test organism was prepared by the direct colony suspension method. From a pure bacterial culture not more than 24hours old, 1to 3 well isolated colonies are transferred in to 5ml of saline solution with the help of a platinum loop. The turbidity of the bacterial suspension is adjusted to match the 0.5McFarland standard corresponding to 1-2 x10^8cfu/ml and further diluted to contain 10^6 organisms by adding 100uL of saline solution to obtain bacteria density suitable for antimicrobial susceptibility testing.[7]

Inoculation of agar plates

To inoculate the agar plates, a sterile swab is dipped into the bacterial suspension and the agar inoculated by streaking with the swab containing the inoculum. This is repeated two times by rotating at 60° to ensure an even distribution of the inoculum.

Application of neem oil impregnated disks

Using a sterile forceps, an oil impregnated disk is placed on the surface of the inoculated plate. After the application of oil disks, the plates are incubated in an inverted position at 37°C for 18hours. Zones of inhibition were observed and measure using a sliding callipers after the 18hours of incubation.

Determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The broth macro dilution method was used. Only bacteria strains which showed susceptibility to our neem oil by the agar disk diffusion method with a diameter of zone of inhibition greater than or equal to 8mm were used to determine the MIC and MBC by the broth macro dilution method.

Preparation of stock solution

A stock solution concentration of 800mg/mL was prepared by weighing 8g of neem oil and dissolving in 10 mL of DMSO. This solution was homogenized in a vortex mixer.

Preparation of inocula bacteria

Inoculum of test organism was prepared by the direct colony suspension method. From a pure bacterial culture not more than 24hours old, 1to 3 well isolated colonies are transferred in to 5ml of saline solution with the help of a platinum loop. The turbidity of the bacterial suspension is adjusted to match the 0.5McFarland standard corresponding to 1-2 x10^8cfu/mL and further diluted to contain 10^6 organisms by adding 100uL of saline solution.[8]

Procedure

1mL of sterile MHB was transferred into a series of 8 test tubes numbered T1 to T8. Briefly, 1mL of stock solution at a concentration of 800mg/mL was added into the first tube T1. From T1, 1mL of this extract concentration was transferred to another test tube and this dilution continued until the 8th test tube was reached giving extract concentrations of400mg/mL, 200mg/mL, 100mg/mL, 50mg/mL, 25mg/mL, 12.5mg/mL, 6.25mg/mL and 3.13mg/mL in different test tubes. Then 1mL of inoculum of test organism containing 10^6 organisms was added into each of the 8 test tubes. This resulted in a bacterial suspension containing 10^5organisms and new concentrations of stock solution; 200mg/mL 100mg/mL, 50mg/mL, 25mg/mL, 12.5mg/mL, 6.25mg/mL, 3.13mg/mL and 1.56mg/mL. Three test

tubes T9 to T11 served as the control. T9(1mL of MHB+1mL of inoculum), T10(1mL of MHB+1mL of extract), T11(2mL of MHB) This procedure was repeated for each test organism and all the test tubes (T1 to T11) were incubated at 37°C for 18hours.[9] The MIC is expressed as the lowest dilution which inhibited growth judged by lack of turbidity in the tube[9] The main advantage of the broth dilution method for the MIC determination lies in the fact that it can readily be converted to determine the MBC as well. After recording the lowest concentration inhibiting growth as the MIC, all tubes not showing visible growth in the same manner as the control tube are subcultured and incubated at 37°C for 18hours.

The control tube T9 (1ml MHB+1ml inoculum) containing a bacterial concentration of 10^5organism is used to prepare dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} of start inoculum corresponding respectively to 10%, 1%, 0.1%, 0.01% of survivors. 50uL of each suspension of these dilutions was subcultured and incubated at 37°C for 18hours.

After incubation, the subculture in which bacterial colonies were less than or equal to the number of colonies present in the 1/10000 dilution of the start inoculum was identified. The MBC was expressed as the highest dilution showing at least 99% inhibition (0.01% of survivors). The MBC/MIC ratio was calculated and used to define the bacteriostatic, bactericidal and absolute bactericidal nature of our neem oil. When the MBC/MIC ratio is less than 4, the substance is bactericidal. If it is greater than or equal to 4, the substance is bacteriostatic and if equal to one, it is absolute bactericidal.[10]

Acute toxicity

Acute oral toxicity can be defined as adverse effect(s) that occur immediately or in a short period following the oral administration of a single dose of a test substance or multiple doses given within 24 hours.[11,12] Acute oral toxicity studies was performed according to the Organization for Economic Co-operation and Development guideline 420(OECD) for testing of chemicals.[13] A total of 20 Wistar albino rats of both sexes (10 males and 10 females), 6-8 weeks old and of average weight 100g were used for the study. All the animals were obtained from the animal house of the FMBS-UY1.

The animals were randomly selected and divided into 4 groups of five animals each; 2 tests groups consisting of 5 animals each (5 males, 5 females) and two control groups consisting of 5 animals each (5 males, 5 females). Rats were tail marked using a permanent marker to provide individual identifications and were kept in clean plastic cages (25cm long, 18cm wide and 14cm tall) in a 12hour light/dark cycle under standard conditions and room temperature (25±2°C). The first test group consisting of 5 males received a single dose of 3mL/100g of the essential oil extract by oral gavage. The second test group consisting of 5 females also received a single dose of 3mL/100g of the essential oil extract. The third and the fourth groups consisting of 5 males and 5 females respectively were not treated with the essential oil extract. They received 3ml of water only and therefore served as the control groups. During the first four hours after dosing, the tests groups were observed for behavioral changes and signs of toxicity (mortality, alertness, spontaneous activity, grooming, restlessness, wheezing, tremors and convulsions) and once daily further for a period of 14 days. Four hours after dosing the animals were allowed free access to water and food. Body weight and water and food intake were recorded daily for 14 days.

Daily body weight

The body weight of each rat was monitored carefully from the commencement of the study till the day of sacrifice.

Relative organ weight

On day 14, blood samples were collected via the jugular vein into non-heparinised tubes for biochemical analysis and the rats were humanely killed. Internal organs (heart, liver, spleen, kidney, lungs, brain, testes, and ovaries were excised and weighed using an electronic balance (Camry®). The relative organ weight of each animal was calculated as follows;

$$\text{Relative organ weight} = \frac{\text{Organ weight}}{\text{Body weight of animal on sacrifice day}} \times 100$$

Organs such as the liver and kidneys were collected, formalin fixed and labeled for histopathological studies.[14,15]

Biochemical estimations

Blood samples collected in non-heparinised tubes were centrifuged at 5000 r/min for 20mins. The sera separated were stored at -20°C until analysed for biochemical parameters. The sera were assayed for liver function (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)), kidney function (creatinine) and total proteins using standardized diagnostic kits (fortress®) and a mass spectrophotometer (Jenway6305®) measuring at 340nm.[16]

Histopathological study

Histopathological investigation of the organs was done according to the method described by Lamp.[17] The liver and kidneys previously preserved by fixing in formalin were dehydrated with increasing concentrations of ethanol and cleared with xylene and paraffin blocks were made. Then thin sections of about 5 to 7um were made and stained with hematoxylin and eosin stains. Histopathological examination of the slides was carried out by at the histology anatopathology laboratory of the Yaoundé University Teaching Hospital. (YUTH)

Results

Phytochemical characterization

Neem oil analysis: physical characteristics

The extracted oil has physical properties of brown colored viscous liquid with a garlic pungent repulsive smell. The saponification value was found to range from 170-172mg/g and the iodine value was between 570.0-74.0mg/g. The Azadirachtin content varied from 100-258ppm (Table 1).

Table 1 Physical characteristics of neem (*A. indica* A. Juss) seed oil

Colour	Brown coloured viscous liquid
Odour	Garlic pungent repulsive
Refractive Index@40°C	3 2.553-1.3715
Specific gravity@20°C	31.22-0.87
Iodine Value	5 70.0 -74.0
Sap. Value	170-172
Azadirachtin by HPLC (A+B)	100-258 PPM

Composition: Fatty acid analysis

The analysis of *A. indica* seed oil shows the presence of 5 different fatty acids that includes both saturated and unsaturated fatty acids (Table 2). In this study, oleic acid was found to be the major composition of fatty acid in *A. indica* seed oil at the percentage range of 32-60%. Other fatty acids that were hexadecanoic acid, octadecanoic acid, 9-hexadecanoic acid and linoleic acid were present

at the percentage of 32-60%, 22-35%, 11-22%, 11.8% and 10-25% respectively (Table 2).

Table 2 Fatty acid composition of Neem (*A. indica*) seed oil

Lipid common name	Acid name	Composition range
Palmitic acid	Hexadecanoic acid	22-35%
Palmitoleic acid	9-hexadecanoic acid	11.8%
Omega-6	Linoleic acid	10-25%
Omega-9	Oleic acid	32-60%
Stearic acid	Octadecanoic acid	11-22%

Mass spectrometer analysis of oil

The chemical composition of the oil was found to contain 19 bioactive molecules. The majority components of the oil were; m-Toluyladehyde (23.511%), Hentriacontane (13.453%), Benzaldehyde, 2-methyl- (11.867%), Eicosane, 7-hexyl- (11.613%), Methyl petroselinate (10.560%), Levoglucosenone (7.450%), Octacosane (7.138%), Hepatacosaane, 7-hexyl- (6.773%), Methyl 14-methylpentadecanoate (6.580%), Butyl palmitate (6.509%), Phytol (6.240%), 2-methyl-5ethylfuran (5.057%), Isobutyl stearate (4.252%), Gamma-elemene (4.0456%), and Nonadecane (3.915%). The high percentage of these major components can be attributed to their high retention time (Table 3).

Table 3 Chemical characterization of the essential oil of neem seed of *Azadirachta indica*

Sl No.	Name of compound	Retention time	Seed (%)
1	**Methyl petroselinate**	**46.885**	**10.560**
2	Methyl isoheptadecanoate	48.333	2.347
3	Butyl palmitate	50.226	6.509
4	2,6,10,14-Tetramethylheptadecane	48.801	2.520
5	Nonadecane	51.116	3.915
6	Isobutyl stearate	55.261	4.252
7	**Eicosane, 7-hexyl-**	**61.440**	**11.613**
8	Heptacosane, 7-hexyl	62.919	6.773
9	Octacosane	65.144	7.138
10	(Z,E)-α-Farnesene	27.201	2.340
11	Gamma.-elemene	29.259	4.0456
12	Methyl 14-methylpentadecanoate	43.111	6.580
13	Phytol	47.714	6.240
14	1-Tridecene	53.147	2.866
15	Levoglucosenone	11.857	7.450
16	**Benzaldehyde, 2-methyl-**	**16.375**	**11.867**
17	2-Methyl-5-ethylfuran	32.901	5.057
18	**Hentriacontane**	**72.199**	**13.453**
19	**m-Toluylaldehyde**	**17.223**	**23.511**

Antimicrobial susceptibility testing

Diameters of zone of inhibition

Five strains showed clear zones of diameters of inhibition ≥8mm, and were considered susceptible. Some strains showed lack of clear zones of inhibition >8mm or showed diameters of zone of inhibition with discrete individual colonies, and were considered resistant. The inhibitory zone diameter recorded with the agar diffusion of neem oil indicates that the diameters varied from 9 to 14 mm (Table 4). The largest diameter was observed with *Aerococcus viridans* ATCC11563 and *Enterococcus faecalis* ATCC51299. The values obtained from the test on *Pseudomonas aeruginosa and Escherichia coli* were similar. The smallest diameter was obtained with *Proteus mirabilis*.

Table 4 Diameter of zone of inhibition by neem oil against test bacteria

Name of bacteria with diameter of zone of inhibition		
Bacteria	Diameter of zone of inhibition (mm)	Interpretation
*Aerococcus viridans*ATCC11563	14	S
*Enterococcus faecalis*ATCC51299	14	S
*Staphylococcus aureus*ATCC25923	<8	R
*Escherichiacoli*ATCC35218	<8	R
Pseudomonas aeruginosa	12	S
Escherichia coli	11	S
Proteus mirabilis	9	S
Klebsiellapneumoniae	<8	R
Salmonella typhi	<8	R
Shigellaspp	<8	R
Staphylococcus aureus	<8	R

S=Susceptible; R=Resistant

Broth macrodilution

The two parameters evaluated during the broth macro dilution were the Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC). The highest MIC and MBC were obtained with *Enterococcus faecalis* ATCC51299 and *Escherichia coli*, 100 and 200 mg/mL respectively. For the others, the MIC and MBC values were equal to 50 and 100 mg/ml respectively. This ratio of the minimum inhibitory and bactericidal concentrations was equal to 2 for all microorganisms; (MBC/MIC = 2) (Table 5).

Table 5 MIC and MBC values against test organism

Bacteria	Name of bacteria with MIC,MBC,MBC/MIC values		
	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
Aerococcus viridansATCC11563	50	100	2
Enterococcus faecalisATCC51299	100	200	2
Pseudomonas aeruginosa	50	100	2
Escherichia coli	100	200	2
Proteus mirabilis	50	100	2

Acute toxicity studies

No lethal effects were observed 4 hours after the administration of a single dose of 3mL/100g of neem oil. About 20% of the animals were sleepy with decreased ambulation and 30% were grooming. No wheezing, tremors and convulsions were recorded.

Changes in body weight during 14days of study

There were no significant differences in body weight gain observed between the treated groups and the control groups, $p > 0.05$ (Figure 1). However the administration of a single dose of 3mL/100g of neem oil caused a significant decrease in water and food intake in the treated groups compared to the control groups (Table 6).

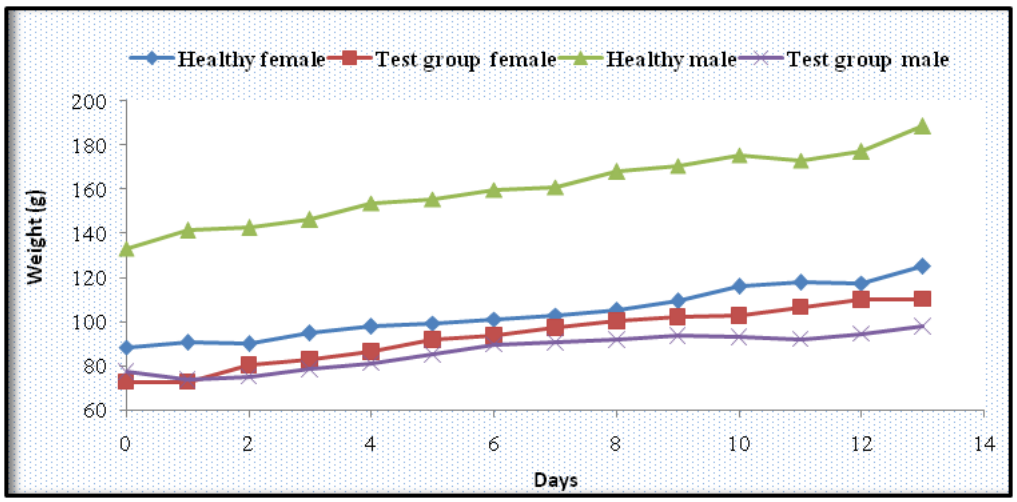

Figure 1 Changes in body weight of the control and treated rats in the acute toxicity studies.

Table 6 The effect of neem oil onfoodintake (g) and water intake (mL) of animals of control groups and treated during 14 days

Group	Healthyfemale (3 mL H_2O)	Test female 3 mL NO	Healthy male (3 mL H_2O)	Test male (3 mL NO)
Food intake (g)	124,15±65,43	80,23±30,83*	130,77±29,22	70,92±24,27**
Water intake (ml)	114,77±74,06	99,23±22,90	182,62±52,23	80,00±31,36***

Values are expressed as the mean ± SD (n = 5; for each group); One–way ANOVA followed by Turkey's multiple comparison test. $P > 0.05$. Not significant. NO = neem oil, H_2O = water.

Relative organ weight

There were generally no significant differences observed in the relative organ weight of the test groups compared to the control groups (Table 7).

Table 7 The relative organ weight of rats of test and control groups after 14days

Organ		Control male (3 mL H_2O)	Test male (3 mL NO)	Control female (3 mL H_2O)	Test female (3 mL NO)
Liver		3,25±1,67	4,58 ± 1,35	4,44 ± 1,36	4,60 ± 1,33
Brain		0,86 ± 0,07	1,40 ± 0,42	1,09 ± 0,30	1,23 ± 0,40
Lungs		0,81 ± 0,22	0,79 ± 0,29	0,95 ± 0,33	0,92 ± 0,29
Spleen		0,53 ± 0,19	0,68 ± 0,21	0,66 ± 0,22	0,38 ± 0,21
Heart		0,32 ± 0,05	0,35 ± 0,10	0,37 ± 0,10	0,34 ± 0,10
Kidney	Left	0,32 ± 0,03	0,47 ± 0,13	0,32 ± 0,09	0,37 ± 0,12
	Right	0,33 ± 0,04	0,45 ± 0,12	0,34 ± 0,09	0,40 ± 0,11
Testes / Ovaries	Left	0,55 ± 0,14	0,41 ± 0,25	0,03 ± 0,28	0,02 ± 0,24
	Right	0,56 ± 0,10	0,41 ± 0,25	0,04 ± 0,28	0,02 ± 0,24
Suprarenals	Left	0,01 ± 0,003	0,02 ± 0,006	0,02 ± 0,004	0,02 ± 0,006
	Right	0,01 ± 0,003	0,02 ± 0,006	0,02 ± 0,006	0,02 ± 0,003

Values are expressed as the mean ± SD (n = 5; for each group); One–way ANOVA followed by Turkey's multiple comparison test. $P > 0.05$. Not significant. Relative organ weight was calculated as (organ $weight_{(g)}$ / body weight of animal on sacrifice $day_{(g)}$) x 100.

Citation: Ngum WL, Hortense G, Barthélémy N, et al. Phytochemical characterization, *in vitro* antibacterial activity, *in vivo* acute toxicity studies of the seed oil of *Azadirachta indica* (neem oil) in Wistar rats. *MOJ Toxicol.* 2019;5(1):31–38. DOI: 10.15406/mojt.2019.05.00149

Biochemical analyses

Biochemical results show a significant increase in levels of plasma transaminases (AST, ALT) in the treated groups compared to the test groups. Also, there was a significant increase of level of creatinine of the test group with respect to the control groups. We also observed a significant decrease in total serum proteins (Table 8).

Table 8 Effect of neem oil on biochemical parameters in acute oral toxicity study

Parameter	Control male	Test male	Control female	Test female
ASAT (UI/L)	18,74±9,64	19,80±1,94	10,83±1,04	29,32±6,08*
ALAT (UI/L)	30,73±2,19	74,76±2,48***	36,67±2,85	128,80±8,81***
Creatinine (UI/L)	1,05±0,16	4,58±0,53***	1,62±0,16	4,93±0,46***
Proteins (mg/dl)	114,74±26,62	65,82±4,99**	114,21±19,10	72,38±6,18**

Values are expressed as the mean±SD (n = 5; for each group); One–way ANOVA followed by Turkey's multiple comparison test. $P > 0.05$. Not significant.*$P < 0,05$, **$P < 0,01$, ***$P < 0,001$.

Histopathological study

There were no gross abnormalities observed with the kidney and liver (Figure 2).

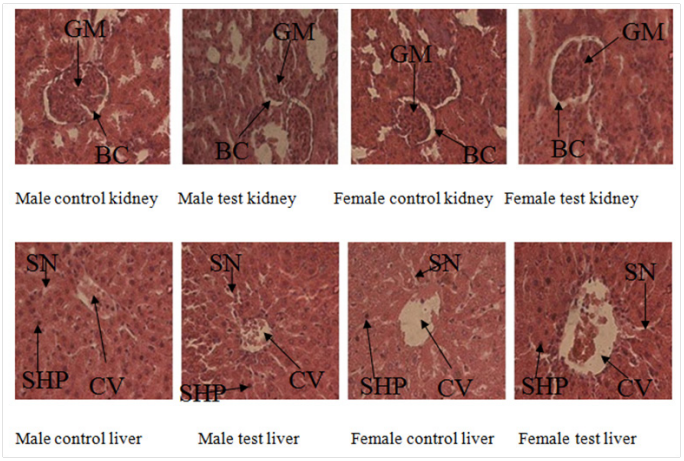

Figure 2 Histology of kidney and liver. GM= Glomerulus, BC= Bowman's capsule, SN= Sinusoid, SHP= Sheet of hepatocyte, CV= Central vein.

Discussion

The GC/MS fatty acid analysis resulted in the detection of 5 fatty acids. Oleic acid was found to be the major composition of fatty acid in *A. indica* seed oil at the percentage range of 32-60 % followed by hexadecanoic acid, octadecanoic acid, linoleic acid and palmitoleic acid at the percentage of 32-60 %, 22-35 %, 11-22 %, 10-25 % and 11.8 % respectively. These results obtained from the fatty acid analysis were supported by many past studies which have reported that the major fatty acid content of neem *(A. indica)* seed oil is oleic acid whereby the percentage lies between 25-61.9%.[4,14,16] The lowest content of fatty acid is represented by palmitoleic acid (11.8%) and it is agreeable with some of the identified studies whereby the percentage was 0.1%. Other studies report that the major content was linoleic acid at 38.26% and followed by oleic acid at 34.09%.[17] The difference in fatty acid and its composition may be attributed to the origin, plant species and their growth conditions.[7] Many studies so far in our literature review have been limited to the analysis of the fatty acid composition of neem seed oil. To our knowledge no studies have been conducted to determine the biological active compounds present in neem seed oil. However, the results obtained from the GC/MS analysis of our oil extract from neem seed were supported by other reported studies on biological activities of essential oils of *Azadirachta indica* leaves and flowers, in which the same chemical compounds were identified.

These reports identified the presence of major chemical compounds such as methyl petroselinate (11.24%), methylisoheptadecanoate (2.19%), butylpalmitate (6.69%), heptacosane (8.10%), eicosane, 7-hexyl- (10.01%) and heptacosane7-hexyl (6.77%), in the n-hexane crude leaf extract, -toluylaldehyde(22.76%),methyl14methylpentadecanoate(38.12%), and methylisoheptadecanoate(12.27%), in the methanol crude leaf extract, and levoglucosenone (7.12%),benzaldehyde,2-methyl-(11.86%),2-methyl-5-ethylfuran(4.82%),methyl14 methylpentadecanoate(13.44%),nonadecane(12.87%) and hentriacontane (13.98%), in the butanol crude leaf extract.[18–22]

This study aimed at assessing the antimicrobial potential of the essential oil extracted from seeds of *Azadirachta indca* revealed inhibition of growth for some tested organisms with inhibition zone of 14mm for *Aerococcus viridans* ATCC11563 and *Enterococcus faecalis* ATCC51299, 12 mm for *Pseudomonas aeruginosa*, 11 mm for *Escherichia coli* and 9 mm for *Proteus mirabilis*. It had been reported that the hexane seed oil extract of this plant shows inhibition zones of large diameters against bacteria strains of *E. coli* (19.5 mm), *E. faecalis*(24 mm), and *P. aeruginosa* (17 mm).[23] This difference can be due to the fact that our oil is not soluble and does not easily diffuse in the agar medium to allow greater contact with the tested microorganisms. To our knowledge, this was the first time the oil was tested against *A.viridans* ATCC11563 and *P. mirabilis*. Some bacterial strains (*Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC35218, *Shigella spp*, *Salmonella typhi* and *Staphylococcus aureus*) showed inhibition zones with individual growth bacterial colonies against our neem oil and were considered resistant, while *Klebsiella pneumoniae* showed no activity at all to this oil. These results do not concur with works on other studies which showed that neem oil has antibacterial activity on *Staphylococcus aureus*, *Salmonella typhi*, and *Shigella spp* with zones of inhibition of 19mm, 17.5mm and 15.4 mm respectively.[3,24] The bactericidal potential (MBC/MIC=2) of the oil was observed in this study. This may be associated with the presence of compounds like gamma elemene, nonadecane, eicosane, Phytol, and stearic acid.[13,25,26] Out of the bacterial strains tested in this study the highest activity recorded was obtained with *Aerococcus viridans* ATCC11563 and *Enterococcus faecalis* ATCC51299 (inhibition diameter equal to 14 mm), while the least susceptible strain was *Klebsiella pneumoniae* (paper disc diameter). *Escherichia coli* ATCC35218 was resistant compared to *Escherichia coli*. The two strains of *Staphylococcus aureus* were both resistant.

For centuries, herbal medicines and their formulations have been considered to be safe and effective due to their negligible side effects. This assumption may have influenced the indiscriminate use of these formulations to a large extent amongst the rural population. These formulations are usually administered over a long period of time without proper dosage monitoring by the experts and lack of awareness of the toxic effects that might result from such prolonged usage.[11,27] Therefore, scientific knowledge towards oral toxicity is much needed, which will not only help identify doses that could be used subsequently, but also to reveal the possible clinical signs elicited by agents under investigation. Regardless of the pharmacological benefits of *A. indica*, detailed knowledge about acute toxicity of this medicinal plant is lacking. Hence, the current study was undertaken to evaluate and focus on the acute toxicity of *A. indica* seed oil in an animal model. In screening natural products for pharmacological activity, the evaluation of the toxic characteristics of medicinal products (extract, isolated compounds, and formulation) is usually a preliminary step. During such evaluation, the determination of LD50 is usually an initial step to be conducted. The acute toxicity study may provide initial information on the mode of toxic action agent, acts as the basis for classification and labelling, and helps in deciding the dose of novel compounds in animal studies.[5,21] In this study, the essential oil of neem at a single dose of 3mL/100g had adverse effects on the treated rats in up to 14 days of observation. There were no significant changes in the weight and the relative organ weight of the rats of test and control groups. However following the administration of a single dose of 3mL/100g of neem oil, there was a significant decrease in water and food intake in the treated groups compared to the control groups. A possible explanation could be that, neem oil has activity on the central nervous system by partially inhibiting leptin thereby acting as an appetite suppressant. Most of the biochemical parameters of test groups were altered. There was a significant increase in levels of transaminases, and creatinine. High levels of transaminases could be indicative of hepatic cytolysis. Additionally, there was significant decrease in levels of total serum proteins, indicating possible malnutrition. These observations were not confirmed by the histological assessment of the organs as no histopathological abnormalities were observed. Therefore, this study indicates that the seed oil of neem causes acute toxicity effects at the dose tested and with LD50 value greater than 3mL/100g. This does not correlate past studies where the LD50 of neem oil in rats was found to be 1.4mL/100g.[28]

Conclusion

The fatty acid analysis resulted in the detection of 5 fatty acids whereby the dominant compound is oleic acid and followed by palmitic acid, Stearic acid, linoleic acid and palmitoleic acid. GC/MS analysis identified 19 bioactive compounds with major chemical compound being m-Toluylaldehyde and followed by hentriacontane, benzaldehyde, 2-methyl-, eicosane, 7-hexyl- and methyl petroselinate. The antibacterial activity of this oil could be attributed to the presence of these bioactive compounds. The seed oil of this study indicated antibacterial activity against *A. viridans* ATCC11563, *E. faecalis* ATCC51299, *P. aeruginosa E. coli* and *P. mirabilis* that were promising. The minimum inhibitory and bactericidal concentrations ratio was equal to 2 (MBC/MIC=2) implying the bactericidal property of the oil. The bactericidal potential of neem oil on Gram positive and Gram negative bacteria confirmed the oil is a potential candidate for new conventional antimicrobial drug production and infectious diseases prevention. Oral administration of neem oil at 3mL/100g in rats caused no lethal effects.

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Conflicts of interest

The author declares no conflicts of interest.

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