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# Pytochemical Characterization, Evaluation of the Anti-diabetic Activity and Acute Toxicity of *Azadirachta indica* (Meliaceae) Seed Oil in Wistar Rat Models

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Authors BNA, FCN and TEF designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors NNB, NVN, TOY, NEM, NB and MG managed the analyses of the study, data mining and the literature searches. All authors read and approved the final manuscript*

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## ABSTRACT

**Aim:** *Azadirachta indica A. Juss* commonly called 'Indian Lilac' or 'Margosa' is used in the South West region of Cameroon to treat malaria, typhoid, intestinal worms and diabetes, and as mosquito repellent. Diabetes, a major risk factor for the development of cardiovascular diseases, is

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associated with long term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels. This study was designed to determine the phytochemical constituents and to investigate the anti-diabetic property of *Azadirachta indica A. Juss* seed oil obtained from the Far North region of Cameroon in alloxan induced diabetic Wistar rats.

**Method:** An *in vivo* experimental study was conducted in the laboratory for preclinical animal studies and pharmaco-toxicology research, of FMBS, UY1 Cameroon. A study population of 135 albino Wistar rats with average mass 100 ± 20g was used. The phytochemical screening of the seed oil used was done using the GC-MS technique. The antihyperglycemic property of the oil was evaluated after oral glucose hyperglycemia induction, using 2g/kg body mass of glucose. The anti-diabetic property of the oil was evaluated over a period of 28 days, and blood glucose concentration after diabetes induction using alloxan solution in citrate buffer. The oral acute toxicity profile of the oil was evaluated over a period of 14 days following oral single dose 3 mL/100g administration of neem oil.

**Results:** Physico-chemical results showed that the oil was composed mainly of five fatty acides (oleic acid (30-55%) being the most abundant and linoleic acid (11-26%) least abundant) and nineteen biochemicals with the three most abundant being: nonacosane (20.6575%), hentriacontane (14.1515%) and 2-methylbenzaldehyde (11.8674%). The oil was antihyperglycemic and maximum effect observed at 1mL/100g body mass, and at t=20minutes, compared to 0.5 mL and 1.5 mL.

**Conclusion:** This study showed that neem oil have a promising preventive diabetic properties. The oil also proved to be antidiabetic at the doses of 0.5 mL, 1 mL and 1.5 mL per 100g body mass, with maximum effect observed with neem oil at 1ml/100g. Acute toxicity results showed no lethality at the maximum standard toxicity range of 2000mg/kg body weight.

*Keywords: Azadirachta indica A. Juss; antidiabetic properties; antihyperglycemic properties; seed oil.*

## 1. INTRODUCTION

*Azadirachta indica A. Juss* commonly called 'Indian Lilac' or 'Margosa', belongs to the family Meliaceae, subfamily Meloideae and tribe Melieae. The most useful and valuable product of the tree are the seeds which yield 40% of a deep yellow oil, the well-known 'Margosa oil' [1-3].

In the Far North region of Cameroon, *Azadirachta indica A. Juss* seed oil is well known to possess nutritional, medicinal and cultural properties [1]. Kapok (*Ceiba pentandra Gaertn*.), mahogany (*Hhaya senegalensis*) and neem (*Azadirachta indica A. Juss*.) seed oils are used in this region as a mixture or individually in the treatment of various diseases like constipation, diarrhea, malaria, typhoid, worm infestation and hemorrhoids. Neem oil is equally applied as a pomade to treat "getti getti" (nappy rash)[2-5]. In the South West region of Cameroon, *Azadirachta indica A. Juss* seeds, leaves and bark are used to treat malaria, typhoid, intestinal worms and diabetes, and used as mosquito repellent [3,6].

Diabetes mellitus is a group of metabolic diseases characterized by elevated blood glucose levels resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels [4-7].

The world prevalence of diabetes in 2010 among adults (aged 20-79 years) was estimated to 6.4% affecting 285 million adults. By 2030 it is expected to increase to 7.7% and affecting 438 million adults. Between 2010 and 2030, there is an expected 69% increase in numbers of adults with diabetes in developing countries and a 20% increase in developed countries [5-9]. The age-standardized prevalence of diabetes in the rural and urban population ranged from 0.8% to 1.6% among adult Cameroonians. Over a 10-year period (1994-2003) there was an almost 10-fold increase in diabetes prevalence in Cameroonian adults. In 2010, the International Diabetes Federation (IDF) estimated the Nation prevalence of diabetes among adults aged 20 to 79 years at 4.4%. Prevalent undiagnosed diabetes is also very high - about 80% [6,10].

Treatment of diabetes involves the use of drugs that reduce glucose levels, including insulin and oral antihyperglycemic drugs. Although there are conventional drugs for the management of diabetes mellitus, most drugs in current use are

seriously constrained by both their side effects and cost of treatment. Due to these challenges, populations mainly in Sub-Saharan Africa have resorted to cheaper and readily available alternative sources of treatment, such as use of medicinal plants or traditional medicines [8]. The World Health Organization (WHO) estimates that 80% of the worlds' populations uses traditional medicine. The continued use of traditional medicines is linked to their low cost and a general belief that they have minimal side effects [9-12].

Medicinal plants have also served as valuable starting materials for drug development in both developing and developed countries [9-13]. It has been estimated that approximately one fourth of prescription medicines worldwide are derived from plants [14,15]. As a specific example, biguanide (metformin) is considered one of the first-line agents used for the treatment of type 2 diabetes, and its use can be traced to the traditional use of *Galega officinalis* to treat diabetes and the subsequent search to identify active compounds with reduced toxicity [16,17].

This study was designed to determine the phytochemical constituents and to investigate the antidiabetic property of *Azadirachta indica A. Juss* seed oil obtained from the Far North region of Cameroon in alloxan induced diabetic Wistar rats.

## 2. MATERIALS AND METHODS

This study was an *in vivo* preclinical experimental study, carried out in the laboratory for preclinical animal studies and pharmaco-toxicology research of the Faculty of Medicine and Biomedical sciences (FMBS), of the University of Yaounde I, Cameroon. For this study, 135 albino Wistar rats (*Rattus norvegicus*) were used, with an average mass of 100±20g. Plant material used was *A. indica* seed oil bought from a herbal medicine store in Marché B Dschang, cultivated and processed in the Far North Region of Cameroon. Animal feed composed of a mixture of corn flour (45 %), wheat flour (20 %), smoked fish (20 %), soybean flour (10 %), and palm kernel (5 %), bone flour for calcium intake (0.98 %), cooking salt (0.5 %) and vitamin B complex (0.5 %). Two to three times a month, a vitamin B complex (Olivitasol, Codex, France) was added in their water to drink and they had *ad libitum* access to food and water during the study.

### 2.1 Phytochemical Characterization and Phytochemical Analysis

This was done using gas chromatography coupled with mass spectrometer (GC-MS) for analysis of the seed essential oil. This test was done in Pretoria-South Africa, at the Council for Scientific and Industrial Research (CSIR), Department of science and technology. 50 ml of neem seed oil was used. 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 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.

### 2.2 Evaluation of the Antihyperglycemic Effect of *A. indica* Seed Oil in Nondiabetic Glucose-induced Hyperglycemic Albino Wistar Rats

To determine the dose of *A. indica* seed oil that could possess the antihyperglycemic effect, a pretest was performed with doses of 0.25 mL/100g, 0.5 mL/100g, 1 mL/100g and 2 mL/100g body mass, based on literature review.

For this study thirty healthy female albino Wistar rats, with average mass 100g were randomly selected and divided into six groups of five rats each. The rats were then put in rat cages and fasted for 18hours (no feed but provided water to drink *ad libitum*).Rats in each group were marked 1 to 5 using a bold marker.After the 18hours of fasting, FPG of rats was measured from the tail vein using the CERA-CHEK$^{TM}$ 1Code glucose meter and test strips and recorded at time t0.Based on the pretest results, the six groups consisted of:

**Group A**: Received *A. indica* seed oil at the dose of 1.5 mL/100g animal body mass;

**Group B**: Received *A. indica* seed oil at the dose of 1 mL/100g animal body mass;
**Group C**: Received *A. indica* seed oil at the dose of 0.5 mL/100g animal body mass;
**Group D**: Positive control (received glibenclamide solution at the dose of 10 mg/kg);

**Group E**: Negative control (received 1 mL/100g animal body mass of distilled water) **Group F**: Healthy control (SHAM) (received nothing).

Rats of each group were administered *per os*, using a gavage needle, the test and reference drugs as stated above, and 30 minutes later FPG was again measured and noted at time $T_{-30}$ and immediately glucose solution was administered per os at a dose of 2 g/kg to induce hyperglycemia. Blood glucose concentration was then measured at 10 minutes, 20 minutes, 30 minutes, 60 minutes and 120 minutes post hyperglycemia induction and noted at time T10, T20, T30, T60 and T120 respectively.

## 2.3 Evaluation of the Antidiabetic Property of *A. indica* Seed Oil in Alloxan-induced Diabetic Albino Wistar Rats

Twenty-five diabetic and five non-diabetic albino Wistar rats with average mass 100±20g were used for this study. To begin, 50 healthy female rats weighing averagely 100±20g were randomly selected and fasted for 18 hours. The FPG of each rat was measured and rats were injected freshly prepared alloxan (Sigma chemical co USA) in citric acid monohydrate ('AnalaR') solution, 80 mg/kg intraperitonealy to induce diabetes.

The volume of the solution to be administered was calculated with aid of the following formula:

$$\text{Volume} = \frac{\text{Dose}\left(\frac{\text{mg}}{\text{kg}}\right) \times \text{mass of animal (kg)}}{\text{concentration (mg/ml)}}$$

Immediately after alloxan injection, rats were provided 5% glucose solution to drink and feed to eat *ad libitum,* in order to prevent the hypoglycemic side effect of alloxan. Rats were observed and on the third day their blood glucose concentration was measured and rats with blood glucose concentration ≥200 mg/dl were selected for the study. Twenty-five diabetic rats were randomly selected and divided into five groups of five diabetic rats per group, and the day noted day 0. In another cage, five healthy female rats randomly selected were put to serve as the healthy control, SHAM. Each group of rats was put in a cage, marked 1 to 5 using a bold marker and provided feed of known mass and water of known volume, and drug as follows:

**Group A**: Received *A. indica* seed oil at the dose of 1.5 mL/100g animal body mass; **Group B**: Received *A. indica* seed oil at the dose of 1 mL/100g animal body mass; **Group C**: Received *A. indica* seed oil at the dose of 0.5 mL/100g animal body mass; **Group D**: Positive control (received glibenclamide solution at the dose of 10 mg/kg); **Group E**: Negative control (received 1 mL/100g animal body mass of distilled water); **Group F**: Healthy control (SHAM) (received nothing).

The feed and water intake and mass of rats per group were measured daily for 28 days, and based on their body mass, they were administered by gavage, test and reference drugs as stated for 28 days (period of the study). Blood glucose concentration of the rats was measured and recorded on days 0, 7, 14, 21 and 28, and cages were cleaned after every 2 days. On the 27$^{th}$ day rats were fasted for 18hours (overnight) and rats were sacrificed on the 28$^{th}$ day and the blood sample collected in test tubes and centrifuged at a velocity of 4000 rotations per 15minutes and sera collected in Eppendorf tubes for biochemical analysis. The liver, spleen, brain, lungs, kidneys, adrenal glands, stomach, testes or ovaries, heart and pancreas were collected and weighed, and the liver, pancreas and kidneys were preserved in 10% formol solution prior to anapathological analysis.

## 2.4 Acute Oral Toxicity Test of *A. indica* Seed Oil

This study was to identify, if present, $LD_{50}$ and adverse effects of *A. indica* seed oil that result from single dose oral administration of the seed oil in a short period of time (less than 24 hours) and within 14 days after administration of the oil. It was done following the modified OECD guidelines 420.

For this study the dose of 30 mL/kg body mass was used based on the absence of any death or visible adverse effect in rats that were administered 15 mL/kg test neem oil during oral neem oil antihyperglycemic pretest. Twenty healthy rats with average mass 100g (ten males and ten females) were randomly selected and divided into four groups of five rats each:

**Group A**: Five healthy female rats (female control); **Group B**: Five healthy female rats (female test); **Group C**: Five healthy male

rats (male control); **Group D**: Five healthy male rats (male test).

All rats were then fasted for 18 hours under normal laboratory conditions, after which rats of groups B and D were administered *A. indica* seed oil at 30 mL/kg body per os using the gavage needle. Rats of groups A and C were administered distilled water (vehicle), 30 mL/kg body mass per os. After the one hour of observation for death or any abnormal behavior: frequency that rats scratched their bodies, cleaned up, and agitation within one hour following drug administration. , rat feed of known mass and water of known volume were provided to the rats for them to eat and drink *ad libitum*. Animal masses, quantity of food and water intake of each group were determined daily for 14 days (toxicity study period) and cages were cleaned after every 2 days. On the fourteenth day, rats were sacrificed and the blood of each rat was put in a labelled Falcon™ conical tube prior to centrifugation. After centrifuging at a velocity of 4000 turns/15mins the blood sera were transferred to Eppendorf tubes ready for biochemical test. The liver, spleen, heart, brain, kidneys, adrenal glands, testes or ovaries, and lungs of each rat were isolated, then weighed, and the liver and kidneys for each group were conserved in 10 % formol solution prior to anapathological analysis.

Biochemical analyses of experimental rat sera.

Consisted of verifying renal activity by evaluating Creatinine quantification and total blood protein (CHRONOLAB KIT), hepatic activity by evaluating ALT and AST sera concentrations (CHRONOLAB KIT) and determination of lipid profile

## 2.5 Histopathological Analysis

Histopathological analysis of the conserved livers, kidneys and pancreas was done at the histology and anato-pathology laboratory of the faculty of medicine and biomedical sciences of the University of Yaounde 1.

## 2.6 Statistical Analyses

All data was registered using Excel 2013 (Microsoft office 2013, USA). Results were presented as average ± standard deviation. Comparison between different groups was done using analyses of variance (ANOVA) followed by the Post Hoc Test of Turkey's Kramer with the help of the logistic Graph pad instat version 5.0. The rate of significance was fixed at a probability value of 0.05 ($p<0.05$).

## 2.7 Ethical Considerations

Ethical approval was demanded from the institutional review board of the Faculty of Medicine and Biomedical Sciences. Authorization was obtained from the head of the laboratory of preclinical animal studies and toxicology research of the faculty of medicine and biomedical sciences (FMBS), of the University of Yaounde I.

# 3. RESULTS

## 3.1 Phytochemical Characterization of *A. indica* Seed Oil

The refractive index, specific gravity, iodine value and saponification value indicated in the Table 1 are parameters for neem oil identification. A brown coloured viscous liquid oil was obtained from the neem seed, with a garlic pungent repulsive ordour (Table 1). The saponification value ranged from 154-215 (mgKOH/g).

## 3.2 Fatty Acids Analysis

Table 2 shows the five fatty acids detected using Gas chromatography-mass spectrometer in the neem oil used in this study. Results showed that the most abundant fatty acid in the neem oil used

**Table 1. Physical characteristics of purchased *A. indica* seed oil**

| Colour | Brown colored viscous liquid |
|---|---|
| Odour | Garlic pungent repulsive. |
| Refractive Index @ 40ºC | 3 3217-1.2225 |
| Specific gravity @ 20ºC | 4 4510-0.7166 (g/ml) |
| Iodine Value | 5 8831 -93.0 (g/ml) |
| Saponification Value | 164-215 (mgKOH/g) |
| Azadirachtin by HPLC (A+B) | 110-258 PPM |

**Table 2. Fatty acids constituent of purchased *A. indica* seed oil**

| Lipid common name | Acid name | Composition range |
|---|---|---|
| Palmitic acid | Hexadecanoic acid | 21-42% |
| Palmitoleic acid | 9-hexadecanoic acid | 14% |
| Omega-6 | Linoleic acid | 11-26% |
| Omega-9 | Oleic acid | 30-55% |
| Stearic acid | Octadecanoic acid | 13-24% |

**Table 3. Chemical compounds found in *A. indica*, seed oil with retention time and percentage ratio in the oil**

| Sl No. | Name of compounds | Retention time | Seed oil (%) |
|---|---|---|---|
| 1 | Methyl petroselinate | 47.3077 | 11.2380 |
| 2 | Phytol | 46.2630 | 2.444 |
| 3 | Methyl isoheptadecanoate | 47.2573 | 2.5515 |
| 4 | Hexadecamethylcyclooctasiloxane | 48.8911 | 7.5111 |
| 5 | Butyl palmitate | 50.0237 | 6.2560 |
| 6 | 2,6,10,14-Tetramethylheptadecane | 51.3317 | 2.8601 |
| 7 | Heptacosane | 56.9823 | 8.3225 |
| 8 | Eicosane, 7-hexyl- | 63.1633 | 11.3732 |
| 9 | Heptacosane, 7-hexyl | 62.9192 | 6.7730 |
| 10 | (Z,E)-α-Farnesene | 30.0032 | 2.4999 |
| 11 | Hexahydrofarnesyl acetone | 37.1236 | 2.7055 |
| 12 | Methyl 14-methylpentadecanoate | 40.7132 | 6.01571 |
| 13 | Lineoleoyl chloride | 48.1990 | **12.43379** |
| 14 | Methyl isoheptadecanoate | 48.5614 | **11.6299** |
| 15 | Nonacosane | 67.3243 | **20.6575** |
| 16 | Levoglucosenone | 11.9058 | 7.1217 |
| 17 | Benzaldehyde, 2-methyl- | 15.2375 | **11.8674** |
| 18 | 2-Methyl-5-ethylfuran | 31.1565 | 4.8273 |
| 19 | Hentriacontane | 72.2455 | **14.1515** |

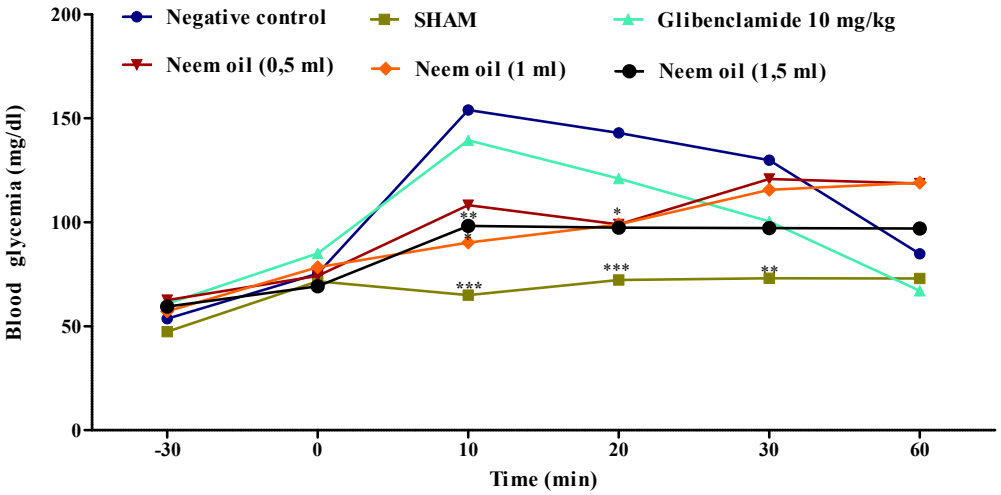


**Fig. 1. Antihyperglycemic effect of *A. indica* seed oil**

was oleic acid, then palmitic acid, palmitoleic acid, stearic acid and least abundant was linoleic acid.

### 3.3 Chemical Analysis

The chemical analysis results by Gas chromatography-mass spectrometer are given in Table 3 with their retention time and percentage ratio in the oil. Results showed that neem oil used in this study was made up mainly of 19 chemicals, with the five most abundant being nonacosane (20.6575%), hentriacontane (14.1515%), benzaldehyde, 2-methyl- (11.8674) and methyl isoheptadecanoate (11.6299%), and the three least most abundant being phytol (2.444%), (Z,E)-α-Farnesene (2.4999%) and Methyl isoheptadecanoate (2.5515%).

### 3.4 Biological Activities of *A. indica* Seed Oil Antihyperglycemic Test (Preventive test)

Blood glucose concentration of the rats of each group at t=0, that was, before administration of neem oil, glibenclamide and distilled water, showed no significant difference in the blood glucose concentration in the different test groups. 30 minutes later, after per os administration of *A. indica* seed oil at 0.5ml, 1ml, and 1.5ml per 100g, glibenclamide 10mg/kg and distilled water 1ml/100g animal mass, we observed no significant difference in blood glucose concentration in the different groups (Fig. 1).

At time t=10 minutes post hyperglycemia induction, we observed a significant decrease in glycemia in rats that received 1ml/100g (with p value <0.01) and 1.5ml/100g (p-value <0.05), and an insignificant decrease in glycemia in groups that received glibenclamide drug and neem oil at the dose of 0.5ml/100g, with p-value >0.05 compared to the negative control.

At time t=20 minutes after induction of hyperglycemia, we observed a significant decrease in blood glucose concentration with neem oil at 0.5ml, 1ml and 1.5 ml per 100g, with p-value <0.05, and insignificant decrease with glibenclamide, p value >0.05, compared to that of the negative control (Fig. 1).

At time t=30 minutes after hyperglycemia induction, we observed an insignificant decrease in blood glucose concentration in rats that receive glibenclamide and neem oil at all doses as compared to the negative control.

At time t=60 minutes, we observed an insignificant decrease in blood glucose concentration in group that received glibenclamide (p value >0.05). In contrast, neem oil at 05ml and 1ml per 100g animal mass showed an insignificant increase in blood glucose concentration compared to the negative control, and a significant increase compared to the reference drug glibenclamide (p value <0.01), meanwhile neem at 1.5ml/100g showed an insignificant increase in blood glucose concentration with p value >0.05 compared to the negative control and reference drug glibenclamide. These results are summarized in Fig. 1.

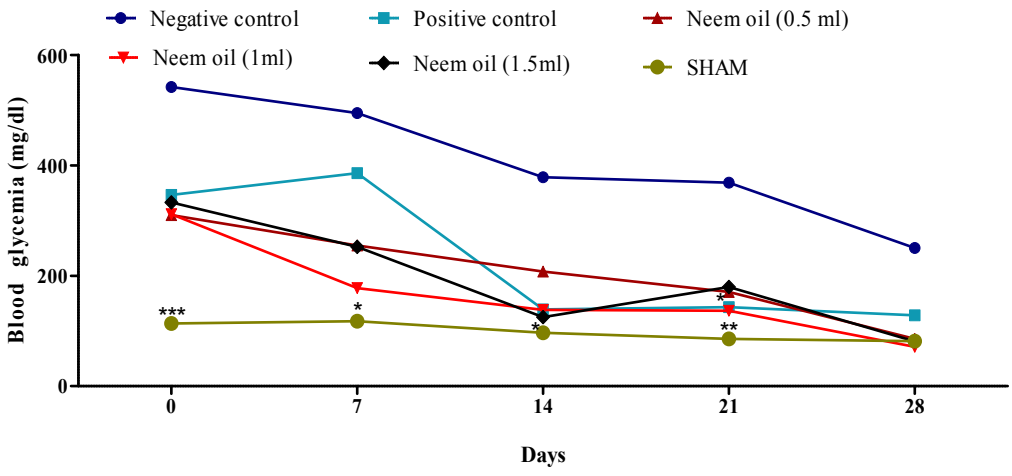


**Fig. 2. Effect of neem oil on diabetes induced by alloxan (80 mg/kg)**

### 3.5 Effect of *Azadirachta indica* Seed Oil on Plasma Glucose Concentration in Alloxan Induced Diabetic Rats

We observed a gradual decrease in blood glucose concentration in sera of test groups and positive control group during the 28 days of the study, compared to the healthy control (SHAM) which showed no significant change in blood glucose concentration (Fig. 2).

Neem oil at the dose of 1ml/100g body mass showed a marked decrease in glycemia on day 7 (p-value<0.01) as compared to neem oil at 0.5ml and 1.5ml and glibenclamide drug.

On day 14, we observed a significant decrease in blood glucose concentration with p-value <0.01, compared to the negative control (Fig. 2).

The antidiabetic effect of neem oil at 0.5ml, 1ml and 1.5ml, and glibenclamide was maintained throughout the study of 28 days as shown in Fig. 2. The blood glucose concentration of the negative control group remained high during the 28 days study, compared to the SHAM blood glucose concentration which showed no significant variation throughout the study, as shown in Fig. 2.

### 3.6 Effect of *Azadirachta indica* Seed Oil on the Liver and Kidney Functions in alloxan Induced Diabetic Rats

Fig. 3 shows a summary of the effect of *Azadirachta indica* seed oil on the liver and kidneys in alloxan induced diabetic rats. Serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) depicted liver integrity, total proteins depict the functional status of the liver and creatinine depicts the functional status of the kidneys. We observed a significant decrease in ASAT serum level (p<0.01) in healthy controls (SHAM), positive control, and all test groups, compared to the negative control group (Fig. 3).

### 3.7 Effect of *Azadirachta indica* Seed Oil on Lipid Profile in Alloxan Induced Diabetic Rats

The effect of distilled water, glibenclamide and Azadirachta indica seed oil on lipid profile in alloxan induced diabetic rats is summarized in Fig. 4. We observed an insignificant ($p > 0.05$) decrease in VLDL and triglycerides in the SHAM and positive control (glibenclamide) groups, and a significant decrease ($p < 0.05$) of total cholesterol in the positive control group, as compared to the negative control. Groups that received *A. indica* seed oil showed no influence of seed oil on lipid profile when compared to the results of the negative control.

### 3.8 Acute Toxicity of Neem Oil

After the oral administration of 3 mL/100g single dose neem oil to experimental rats the following results were obtained:

#### 3.8.1 Clinical observation for adverse effects

No animal died at the dose of 3 mL/100g indicating that $LD_{50}$ was more than 3 mL/100g. Rats of the test groups (males and females) were

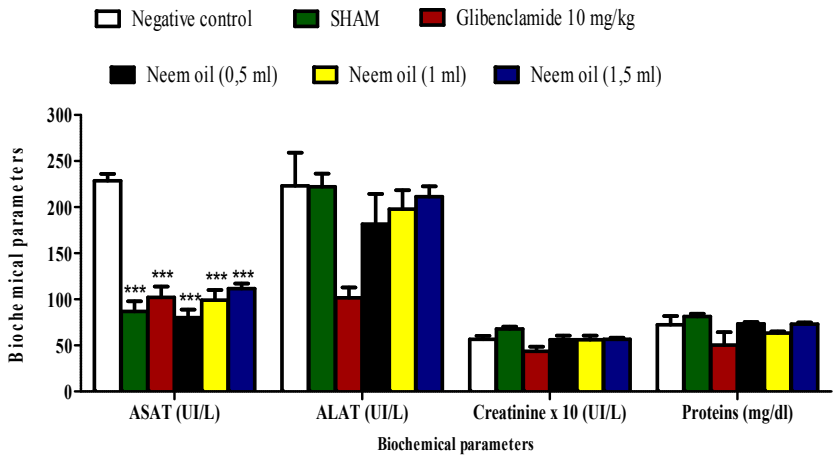


**Fig. 3. Antidiabetes serum biochemical parameters**

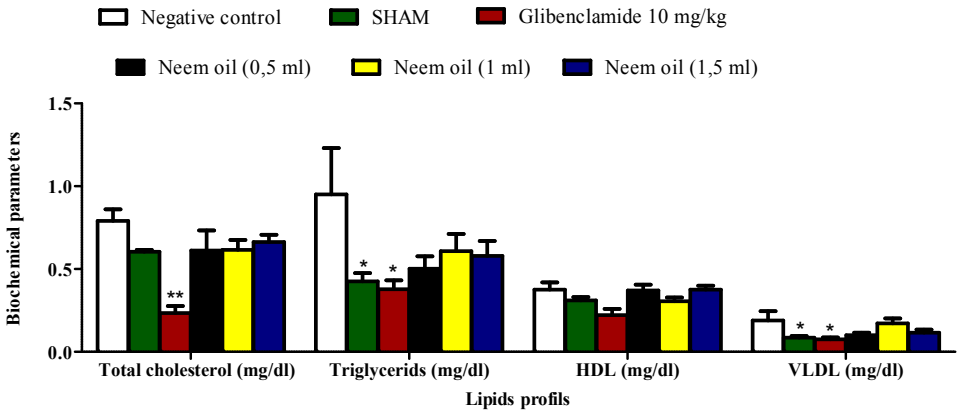


**Fig. 4. Antidiabetes sera lipid profile evaluation**

**Table 4. Summary of the clinical observation of acute toxicity experimental rats. (+++ (very frequent), ++ (frequent), +(less frequent))**

| Group\ clinical sign | Scratching | Cleaning | Agitation |
|---|---|---|---|
| Male test | + | +++ | + |
| Female test | +++ | +++ | + |
| Male control | + | + | - |
| Female control | + | + | - |

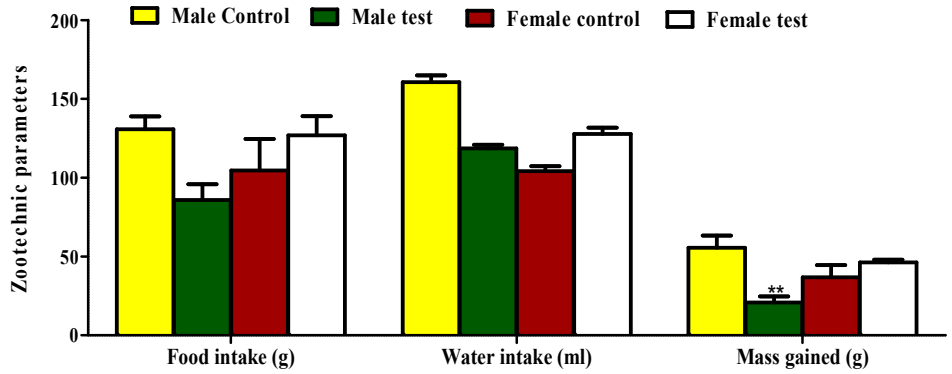


**Fig. 5. Illustration of the food and water intake, and mass of acute toxicity rats**

agitated compared to the control groups (males and females respectively) within the 10minutes following drug administration. Both test group rats frequently cleaned up, and female test frequently scratched their bodies indicating irritation, as compared to the control rats, as summarized in Table 4.

### 3.8.2 Water and food intake

All rats gradually gained weight from day one to day 14 of the study. We observed an insignificant increase in food and water intake and body mass (*p-value* > 0.05) in the male test group compared to the male control group. Female test and control groups showed significant increase in food intake, water intake and body mass (*p-value* < 0.01) as illustrated in Fig. 5.

### 3.8.3 Biochemical analysis of acute toxicity of rat sera

We observed a significant increase in serum ALAT concentration in the male test and female test groups, with *p-value* < 0.01, compared to the control male and female group sera respectively, as shown in Fig. 6.

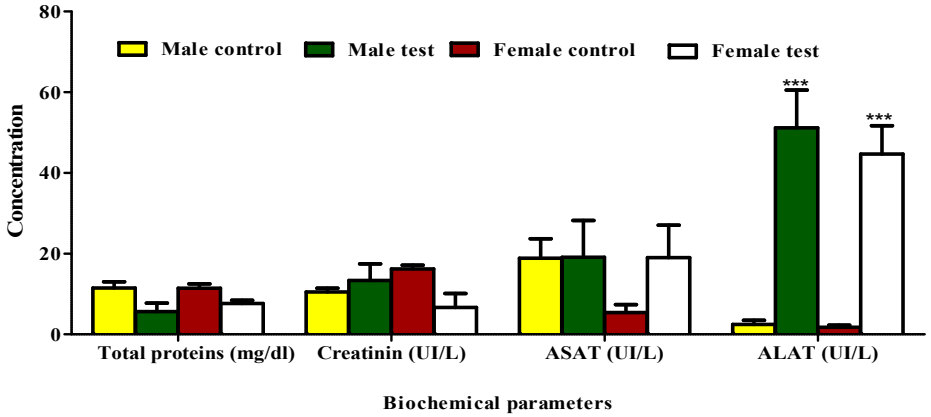


**Fig. 6. Acute toxicity biochemical serum parameters**

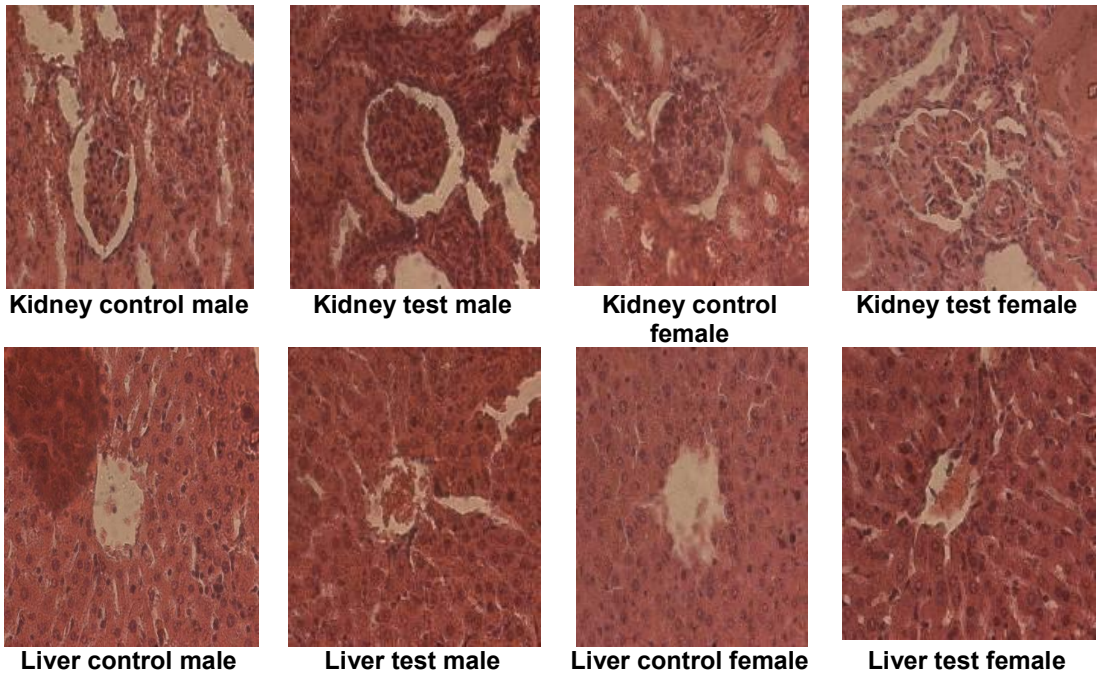


**Fig. 7. Histology of liver and kidney of neem oil acute toxicity experimental rats**

### 3.8.4 Weight of organs of acute toxicity rats

After weighing the liver, heart, pancreas, lungs, brain, kidneys, testes, ovaries and adrenal glands of the sacrificed acute toxicity rats, we observed an insignificant difference in the masses of the organs in the male test group compared to the male control group, as well as for the female test group compared to the female control group, with p-value >0.05. These results indicated that neem oil did not affect organ mass.

### 3.8.5 Acute toxicity histopathological test

The histopathological analysis of the livers and kidneys of the male test and female test groups compared to the male control and female groups respectively, showed no visible morphological changes and no evidence of liver and kidney damage (Fig. 7).

## 4. DISCUSSION

Results of the physicochemical test indicate that the oil is really *Azadirachta indica A. Juss* seed

oil. It's viscous nature, brown colour and pungent repulsive garlic odour, refractive index 1.2225 at 40°C, specific gravity of 0.7166g/ml at 20°C, iodine value of 93.0 g/ml, saponification value of 164-215mgKOH/g, and azadirachtin (A+B) amount by HPLC of 110-258ppm are very similar to the test specification results of *A. indica* seed oil established by NHR organic essential oils product specification of neem oil: viscous liquid, brown to greenish brown, refractive index of 1.450-1.490 at 40°C, specific gravity of 0.905-0.975 at 20°C, iodine value of 55-85g/ml, saponification value ≥180mgKOH/g, and azadirachtin content ≥1300 [52]. The oil used in this study is rich mainly in five fatty acids with the most abundant being oleic acid (omega-9) (30-55%), then hexadecanoic acid (palmitic acid) (21-42%), 9-hexadecanoic acid (palmitoleic acid) (14%), octadecanoic acid (stearic acid) (13-24%) and linoleic acid (omega-6). These results are slightly different from the results obtained by Sandanasamy et al. in 2013 [18,19], who showed that neem seed oil obtained from seeds cultivated and oil extracted through the heating technic in Malaysia, contained seven fatty acids with most abundant being linoleic acid (34.69%), then oleic acid (20.46%), stearic acid (20.42%), palmitic acid (18.66%), eicosanoic acid (arachidic acid)(3.59%), docosanoic acid (behenic acid)(0.80%), tetracosanoic acid (lignoceric acid)(0.55%) and palmitoleic acid (0.17%)[15]. These results are also slightly different from the results obtained by Diedhiou et al. in 2015 [19,31], who worked on neem seed oil of Senegal origin extracted through the heating technic. In his results, the number of fatty acids in neem seed oil were nine with most abundant being Oleic acid (41.91±0.69%), then Linoleic acid (19.59±0.44%), Stearic acid (18.71±0.46%), Palmitic acid (15.59±0.27%), Arachidic acid (1.33±0.01%), Behenic acid (0.86±0.38%), Gadoleic acid (0.08±0.00%), Linolenic acid (0.44±0.01%) and Palmitoleic acid (0.12± 0.00%). The difference in fatty acid composition and abundance from one country to the other could be as a result of difference in geographical features like nature of the soil and climate, oil extraction technic, as well as time of seed harvest and seed treatment.

Nineteen chemicals were identified in the neem oil used. The 8 most abondant compounds identified were: nonacosane as major (20.6575%), then hentriacontane (14.1515%), 2-methylbenzaldehyde (11.8674%), methylisoheptadecanoate (11.6299%), 7-hexyleicosane (11.3732%), methylpetroselinate (11.2380%), heptacosane (8.3225), and levoglucosenone (7.1217%). In the study to evaluate the antihyperglycemic effect of neem oil (preventive diabetes property of neem oil) in glucose-induced hyperglycemic non diabetic rats, the gradual decrease in blood glucose concentration in rats receiving *A. indica* seed oil at doses of 0.5ml, 1ml and 1.5ml, with an increase in the effect observed and appearing maximum at 20 minutes post hyperglycemia induction, compared to the positive and negative controls where glucose concentration remained high, are in agreement with the findings of P. Khosla et al in 2000 [11]. According to their findings, in diabetic rabbits where the seed oil was given 2 weeks prior to alloxan administration, neem seed oil prevented the rise in blood glucose levels compared to control diabetic rabbits [10,19]. Our findings are contrary to those of Bruna Dallaqua et al. in 2012 [20,21], who observed that treatment with neem seed oil and azadirachtin in pregnant rats had no hypoglycemic and anti-hyperglycemic effects on non-diabetic and diabetic rats, respectively [15].

The low rise in blood glucose levels observed in this study could be explained using the findings of Nagashayana G. et al in 2014 [22,30], who suggested that the preventive diabetic effect of neem oil could be due to its effect on glucose absorption, which is reduced in presence of neem. This mode of action still needs to be evaluated in this aspect. *A. Indica* may also act by increased release of insulin from beta cells of pancreas similar to sulfonylurea as suggested by khosla et al. [11,23]. Also, it may increase the uptake of glucose peripherally as suggested by Sonia Bajaj and Srinivasan [23], due to its blood glucose reducing action in type 1 diabetes model as well. Several other mechanisms have been suggested like decreased synthesis or release of glucose by liver, inhibition of proximal tubular absorption of glucose in kidney. In spite of all these explanations the exact mode of action still needs to be elicited and extensively studied in both human and animal models [24-26]. The effect of neem oil observed thirty minutes post hyperglycemia induction in the experimental rats; decrease antihyperglycemic effect and a rather hyperglycemic effect 60 minutes post hyperglycemia induction, as compared to the negative and positive controls observed in this study indicates that *A. indica* seed oil possesses antihyperglycemic properties but the effect does not last for long, approximately 30 minutes [6,27]. This is in corroboration with the findings of

Nagashayana et al. in 2014 who observed that neem oil has got the potential to reduce blood glucose levels within a short period of time [30]. The hyperglycemic effect observed with the neem oil 120 minutes after its administration in this study indicates that *A. indica* seed oil has the tendency of raising blood glucose concentration after sometime following drug administration. This finding is in agreement with that of Ngashayana G et al. in 2013 [30] who observed a rise in blood glucose levels in experimental rats on day 21 when working on the evaluation of the hypoglycemic activity of *A. indica* oil in Albino rats [31].

For the evaluation of the antidiabetic property of *A. indica* seed oil, a single intraperitoneal injection dose of freshly prepared alloxan monohydate in citrate buffer of 80 mg/kg body mass of the rats led to a persistent diabetic state characterised by severe hyperglycemia. Rats with nonfasted blood glucose concentration, 3 days after alloxan injection, of 200 mg/dl and above were used for the study. The study lasted for 28 days. In this study, *A. indica* seed oil and glibenclamide gradually decreased blood glucose concentrations from day 1 of administration to day 28 (study period), compared to the negative control of the study. The antidiabetic effect of *A. indica* seed oil in this study was maximum with the dose of 1 mL/100g body mass of rats and least with 0.5 mL/100g. The antidiabetic effect of the oil at 1 mL/100g was greater than that of glibenclamide (10mg/kg), indicating that *A. indica* seed oil possesses antidiabetic properties, and at a dose of 1ml/100g body mass of rats, the effect is greater compared to that of glibenclamide. This finding is in agreement with that obtained by Nagashayana G et al. in 2014 [30], who showed that oily extract of *Azadirachta Indica* produces a marked decrease in blood glucose levels in alloxan diabetic rats [30]. The results also corroborate with the findings of Saleem et al. in 2012 [9], who showed that with 0.5 mL/100g body mass of rats, *A. indica* seed oil causes a significant ($p<0.01$) reduction in blood glucose concentration in alloxan induced diabetic rats [57]. The results are also similar to the findings of Koshla et al. in 2000 who showed that *A. indica* leaf extract and seed oil produced a marked decrease in blood glucose in normal as well as alloxan diabetic rabbits. The antidiabetic action of *A. indica* as suggested by Sharma et al., may partly be due to extrapancreatic sites of action, that is by increased peripheral glucose utilization or by direct metabolic effect on tissues particularly on liver [10,28].

*Azadirachta Indica* seed oil showed no effect on serum lipid profile. These results are in corroboration with the findings of Bruna Dallaqua et al. in 2012 [29], who observed that the concentrations of total cholesterol, triglycerides, HDL-c and non-esterified fatty acids (NEFA) in non-diabetic rats treated with *A. indica* seed oil produced no statistically significant difference compared to non-diabetic rats treated with azadirachtin. The preventive and curative antidiabetic effect of neem oil observed in our study could be explained by the high amount of linoleic acid found in the neem oil. Studies by Belury and Vanden Heuvel [31] have shown that conjugated linoleic acid (CLA) exerts antidiabetogenic, anticarcinogenic, and antiatherosclerotic effects on the immune system as well as on fatty acid and lipid metabolism[55]. Also, the hydrocarbons found in *A. indica* seed essential oil could have an antioxidant activity, as demonstrated in other essential oils. The essential oil of *Rosa damascena*, rich in hexatriacontane (24.6%), 1-nonadecene (18.56%), and n-tricosane (16.68%), showed strong radical scavenging effect. A seed hexane extract of *Hypericum scabrum L.*, containing omega-3 fatty acids (37.6%), a bis (2-ethylhexyl)phthalate (35.7%), linoleic acid (6.9%) and nonacosane (3.9%) as major compounds, showed high radical scavenging activity [17,31].

In the oral acute toxicity we observed no mortality, indicating that the LD50 is greater than 30 mL/kg. This result does not corroborate with the findings of M Gandhi et al. in 1988, who obtained as 24-h LD50 of neem oil to be 14 mL/kg in rats. Animals of the tests groups frequently cleaned up, scratched themselves and were agitated compared to the control groups indicating irritation. In contrast to results obtained by Anofi Omotayo et al. in 2012 who obtained a significant increase in the body mass of male Wistar rats treated with 50, 100, 200, and 300 mg/kg ethanolic extract of *A. indica* stem bark [16], in this study rats of the male test group showed an insignificant variation in body mass. In this study, the significant elevation in ALT in test groups' sera compared to controls indicate that neem oil may be hepatotoxic at 30 mL/kg in experimental rats. These results are supported by Macdonald et al. in 2017 who, in a study revealed that neem oil was hepatoprotective, and that the hepatoprotective effect of the oil was dose-dependent [27]. They revealed that neem oil at 0.25 mL/kg, 0.5 mL/kg and 1.0 mL/kg was hepatoprotective, but might become toxic at a

comparatively higher dose [61]. In humans, particularly in children, there are several case reports of Neem oil poisoning causing vomiting, hepatic toxicity, metabolic acidosis, and encephalopathy [31]. Lai et al., reported 22 cases of neem oil poisoning in infants, who were given single doses of Neem oil (few drops to 5 mL), presented with features of toxic encephalopathy, metabolic acidosis, and hepatic toxicity [25]. The hepatotoxic effect of neem oil can be attributed to azadirachtin (C35H44O16) which manifests its toxicity possibly by interfering with mitochondrial bioenergetics, resulting in inhibition of the generation of the electrochemical proton gradient (primary form of energy generated in mitochondria) [31]. Acute poisoning with inhibitors of electron transporting complexes causes symptoms such as muscle weakness, easy fatigability, hypotension, headache, facial flushing, nausea, confusion, and aggravation of latent myocardial angina [5]. The inability to utilize oxygen is manifested as a cytotoxic hypoxia wherein the chemicals cause a metabolic acidosis and hyperpnea, despite normal pO2 [19,23]. However, inhibitors of the supply of reducing substrates for the respiratory chain cause a similar metabolic syndrome that is difficult to distinguish from inhibitors of the electron transport chain [30,31].

## 5. CONCLUSION

From this study, neem oil used in our study, precisely from the Far North region of Cameroon, consists mainly of five fatty acids: oleic acid, palmitic acid, palmetoleic acid, stearic acid and linoleic acid and nineteen biochemicals, with the eight most abundant being: nonacosane (20.6575%), hentriacontane (14.1515%), 2-methylbenzaldehyde (11.8674%), methylisoheptadecanoate (11.6299%), 7-hexyleicosane (11.3732%), methylpetroselinate (11.2380%), heptacosane (8.3225), and levoglucosenone (7.1217%). The neem oil has potential antihyperglycemic and antidiabetic properties, with maximum effect observed at the dose of 1ml/100g body mass. Theses biological activities could be attributed to the phytocostituents of the neem oil which have proven their antioxidative activities in other studies. Oral acute toxicity revealed no lethality indicating that LD50 may be greater than 3 mL/100g, even though hepatotoxicity was observed. *A. indica* seed oil thus holds the hope of a new generation of antidiabetic drugs with early onset of action, even though its preventive effect appears to be short lasting. There is therefore need for further studies on experimental animals to isolate the phytochemicals of the oil that possess the antidiabetic property, and to identify the mechanism of action of these chemicals.

## CONSENT

It is not applicable.

## ETHICAL CONSIDERATIONS

Ethical approval was taken from the institutional review board of the Faculty of Medicine and Biomedical Sciences. Authorization was obtained from the head of the laboratory of preclinical animal studies and toxicology research of the faculty of medicine and biomedical sciences (FMBS), of the University of Yaounde I.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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