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Phytochemical screening, evaluation of anti-salmonella activity and acute toxicity of *Azadirachta indica* (A. Juss) oil seeds

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Abstract
Bacterial infection by *Salmonella* remains a real public health threat causing each year, more than 1.2 million deaths worldwide. The causal pathogen is *Salmonella spp*, whose treatment by antibiotic therapy is usually compromised due to the emergence of multi-resistant strains, supply chain problems and the high cost of drugs in less developed countries where the infection is usually endemic. Faced with these difficulties, the local populations with limited resources resort to medicinal plants and essential oils, whose anarchic use poses effectiveness and safety challenges. With the aid of microdilution, the antibacterial activity of *Azadirachta indica* seed oil was evaluated on three *Salmonella* strains. Subsequently, an acute toxicity study of the extract was performed on Wistar rats by dosing the test groups with 2 ml/100kg of body weight, according to the slightly modified Organisation for Economic Co-operation and Development (OECD) guideline 420. The extracted oil gave a yield of 18.6%. Two of the three identified bacterial strains were susceptible with diameters of 17 mm for *Salmonella typhi* and 18.5mm for *Salmonella paratyphi A*. Their minimum inhibitory and bactericidal concentration were 37.5mg/ml and 150 mg/ml respectively with a CMB/CMI ratio equals to 4 which demonstrated that, oil from the *Azadirachta indica* seeds used had anti-salmonella bacteriostatic properties. Acute toxicity analyses showed that the lethal dose 50 (LD 50) of this oil was greater than 2ml/100g. We concluded that *Azadirachta indica* seed oil contained important bioactive metabolites with promising anti-*Salmonella* bacteriostatic activity on two bacterial strains. It was toxicologically safe at the dose of 2 ml/100g of body weight.

Keywords: Phytochemical screening, anti-salmonella, toxicity *Azadirachta indica*

Introduction
Bacterial infections caused by *Salmonella* are responsible for more than 1.2 million deaths in the world annually. They continue to be a public health threat in developing countries in general and in Sub-Saharan Africa in particular, where they are endemic [1, 2]. About 11.9 to 26.9 million cases of typhoid fevers, and 129 to 270,000 typhoid-related deaths have been estimated to occur worldwide each year [3]. Their transmission is mainly via the faecal-oral route [4, 5]. In Cameroon, typhoid fever is endemic with episodes limited in time and space. Recent data shows that in December 2016, 262,149 cases were recorded nationally compared to 220,337 cases in 2015 [6, 7]. *Salmonella* can cause typhoid and paratyphoid fevers, gastroenterics, individual and community food poisoning, lethargy and other extra-intestinal complications in humans [8, 9]. In some developing countries, phenicoles, aminopenicillins and cotrimoxazole remain the reference treatment option for this infection. However, the emergence of multidrug-resistant *Salmonella* strains has led to new treatment regimens favouring parenteral cephalosporins, fluoroquinolones and sometimes azithromycin [10]. These multi-drug resistant serotypes pose a serious threat to the treatment of salmonellosis. Hence, there is a need to develop new active molecules for better preventive and curative management [11].
Although modern medicine has made great improvements in recent decades, about 80% of the population in developing countries still rely on medicinal plants, which occupies an important place in therapy [12].

Azadirachta indica A. Juss, a member of the *Meliaceae* family commonly known as Neem, is a tree mainly cultivated in the Indian subcontinent. Various parts of this plant have been used for centuries in traditional medicine as a household remedy for the treatment of several disease in several localities around the world, especially in India and South Asia [13, 14].

In Cameroon, *Azadirachta indica* is mostly found in the Far North region where it is mainly used as a bio-pesticide in the protection of vegetable and seed crops [15, 16]. It is a powerful disinfectant and in the Far North Region of Cameroon as in other Sahelian regions of Africa, *Azadirachta indica* leaves are used to treat malaria, leprosy and gastroenteritis. The fruit and bark are also part of the therapeutic arsenal. Rich in azadirachtin, the oil extracted from the seeds is used as a dewormer, antihypertensive, anti-hyperglycaemic and antibacterial; but given its use as a biopesticide [15], it exhibits some cytotoxic effects in humans [7, 16]. Thus, the extensive use of Neem oil raises many questions about its safety, quality, efficacy and long-term availability. So far, there are few studies in Cameroon demonstrating its safety to the organism. It is in this perspective that we evaluated the anti-Salmonella activity and acute toxicity of *Azadirachta indica A. Juss*

Materials and Methods

Preparation of the plant material

Oil from *Azadirachta indica* seeds was collected from the fruits of a mature tree in the Far North of Cameroon in the town of Yagoua. The fruits were taxonomically identified and authenticated by the National Herbarium of Cameroon as fruits of *Azadirachta indica* and the identification code assigned to the sample was 4447/SRFK. Traditionally, *Azadirachta indica* oil was obtained after crushing the kernels by cold pressing. The yield was calculated according to the following formula:

R=mb/mi
MB: mass of crude oil
MI: initial seed mass

Evaluation of the antibacterial activity

It was done by determining the inhibition diameters in solid media by the disc diffusion method and by determining the minimum inhibitory and bactericidal concentrations in liquid media by macro dilution according to the recommendations of the CA-SFM 2019.

Preparation of the inoculum

Using a sterile platinum loop, we collected 2 to 3 colonies aged 18 to 24 hours and made a suspension with sterile physiological water (normal saline) similar to a 0.5 McFarland standard, corresponding to the concentration of 108 Colony Forming Units/ml (CFU/ml). Inoculation was done within 15 minutes after preparation of the inoculum.

Preparation of the oil solution from *Azadirachta indica* seeds to be tested

The extracted oil solution of concentration 100 mg/ml was prepared each time it was to be used. This was done by dissolving 1 gram of the extract (obtained by weighing 1g of *Azadirachta indica* seed oil) in 10 ml of dimethylsulphoxide (DMSO).

Susceptibility testing: Agar disc diffusion method

To test for the susceptibility of the bacteria, the principle of aromatogram was used. 20 µL of *Azadirachta indica* oil supplemented with 10% dimethyl sulphoxide (DMSO) was introduced into a tube and homogenised with a vortex. Sterile discs of 6 mm in diameter cut in a Whatman n°. 2 paper were then added into the tube. Once the discs were impregnated with *Azadirachta indica* oil, they were removed and gently placed on the surface of a Mueller Hinton agar plate previously swabbed with the bacterial inoculum.

Simultaneously, Ciprofloxacin 500 mg antibiotic discs, were also deposited on the same agar as a positive control. The plates were then incubated for 24 hours under strict anaerobic conditions. Following incubation, the discs were surrounded by circular zones of inhibition corresponding to an absence of culture, which allowed us to measure the diameters of inhibition using a calliper. This experiment was performed trice for each bacterial strain.

The determination of the diameter of the zone of inhibition enabled a clinical categorisation of the strain, based on the correlation between the results obtained by the diffusion method and the reference method. The susceptibility to the oil is classified according to the diameter of the zones of inhibition as follows: Not susceptible (-) for diameter less than 8 mm; susceptible (+) for diameter between 8-13.9 mm; Very susceptible (+ +) for diameter between 14-19 mm; extremely susceptible (+++) for diameter over 19 mm. This susceptibility test allows access to qualitative results. The experiment was repeated three times.

Determination of Minimum Inhibitory Concentrations (MIC) and Bactericidal Concentrations (MB C)

The minimum inhibitory concentration (MIC) was expressed as the lowest dilution that inhibits growth due to the lack of turbidity in the tube. After recording the lowest MIC, all tubes not showing visible growth, as well as the control tube, were sub-cultured and incubated at 37°C for 18 hours. The minimum bactericidal concentration (MBC) is the lowest concentration at which a substance is capable of killing more than 99.9% of the initial bacterial inoculum after 24 hours of incubation at 37 °C. Thus, the determination of the MBC is based on subculture from the MIC on agar. The solution from each of the tubes without bacterial pellets and form the positive control with the concentration range performed for the determination of MIC, was inoculated by streaking on Mueller Hinton agar. The plates were then incubated for 24 hours at 37 °C. The MBC of the neem oil is deduced from the lowest concentration at which no culture is observed on Mueller Hinton agar. This procedure was repeated trice for each bacterium.

MBC/MIC ratios

The MBC/MIC ratio made it possible to determine the bacteriostatic or bactericidal character of our *Azadirachta indica* oil. An essential oil is said to be bacteriostatic when this ratio is greater than or equal to 4 and bactericidal when this ratio is less than 4.

Acute toxicity assessment according to OECD Guideline 420: We used the slightly modified OECD guideline 420 which recommends the administration of a single dose of extract and observation of the animals for 14 days.

Preparation of the animals

Twenty rats consisting of ten males and ten females with a mean weight of (106.4±15.05) g and (114.6±17.71) g respectively were used for this study. For each gender, these animals were divided into two groups of five; that is; the test group and the control group. All animals were weighed before being allocated to the different groups and then fasted for 24 hours. After 24 hours of fasting, the test groups received a concentrated extract solution of 2 ml/100g via a stomach tube and the control groups were administered distilled water only. Again, the animals were deprived of food for four hours during which their behaviour was observed, and during the 14 days of the experiment their water and food consumption, weight variation and behaviour were monitored and recorded. At the end of the 14 days, the animals were weighed just before their sacrifice, which took place after anaesthesia with excess of ether. A blood sample was taken from the carotid artery to determine the biochemical parameters of toxicity. The organs were isolated and weighed immediately. The livers and kidneys were fixed in 10% formalin for histological sectioning.

Analysis of biochemical parameters

The determination of the effect of *Azadirachta indica* seed oil on biochemical parameters was done by evaluating the serum level of the following parameters: creatinine, urea, total protein, Alanine Amino transferase, Aspartate Amino transferase, total bilirubin, alkaline phosphatase and Gamma GT through colorimetric assay using BIOLABO assay kits and a semi-automatic BIOBASE-Silver Plus Spectrophotometer.

Histological analysis

The histological techniques used in this work were basic techniques described by Cannet and consisted of; fixation, macroscopy, dehydration, inclusion, sectioning, staining and mounting. Microscopic analysis was performed with the aid of an AxiosKop 40 microscope connected to a computer where images were transferred, edited and analysed with MRGrab 1.0 and AxioVision 3.1 software's, all supplied by ZEISS (Hallbermoos, Germany).

Statistical analysis

The results were expressed in terms of mean +/- standard deviation. Comparison between groups was performed using the analysis of variance test (ANOVA) followed by Turkey's Kramer's post hoc test using the Graph Pad instat version 5.0 software.

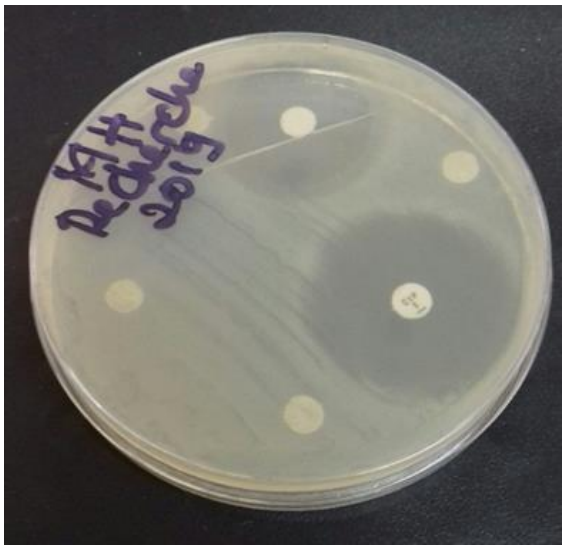
Results

Antibacterial activity of *Azadirachta indica* oil

The disc diffusion method used helped determine the susceptibility of Salmonella bacterial strains to *Azadirachta indica* oil.

Diameters of the zones of inhibition of *Azadirachta indica* seed oil

The three bacterial strains identified were tested for susceptibility to the control antibiotic and to *Azadirachta indica* oil. Only two of the three bacterial strains tested were susceptible to the *Azadirachta indica* oil (Figure 1).



A: *Salmonella typhi*,

B: *Salmonella paratyphi A*

Fig 1: Antibacterial activity of *Azadirachta indica* seed oil (Photograph by Yede, 2019)

Diameters of 6 mm, 17 mm and 18.5 mm were obtained. All these bacterial strains were susceptible to ciprofloxacin (Table I).

Table 1: Diameter of inhibition of *Azadirachta indica* seed oil and ciprofloxacin on isolated bacteria

Bacteria	Ciprofloxacin 500 mg	*Azadirachta indica oil*
Salmonella typhimurium	30.00±2.52	6.00±0,33
Salmonella typhi	32.00±2.52	17.00±0.26
Salmonella paratyphi A	34.00±2.65	18.50 ± 0.51

Minimum inhibitory and bactericidal concentrations of *Azadirachta indica* seed oil

The minimum inhibitory and bactericidal concentrations as well as the MBC/MIC ratio of *Azadirachta indica* oil are shown in Table 2. The MIC for all three strains was 37.5 mg/ml and the MBC was 150 mg/ml. The MIC/BMC ratio gave a value of 4. It was deduced that *Azadirachta indica* oil had a bacteriostatic action on the tested bacterial strains.

Table 2: MIC, MBC and MBC/MIC ratio values for strains tested

Bacteria	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC
Salmonella typhi	37.5	150	4
Salmonella paratyphi A	37.5	150	4
Ciprofloxacin 500 mg	/	/	/

MIC: Minimum inhibitory concentration
MBC: Minimum bactericidal concentration.

Acute toxicity assessment

Extraction efficiency

A yield of 8.567% was obtained from the extraction

Zootechnical criteria

Analysis of Figure 2 shows a weight gain among groups which received the extracted oil compared to those which did not with a p-value > 0.05.

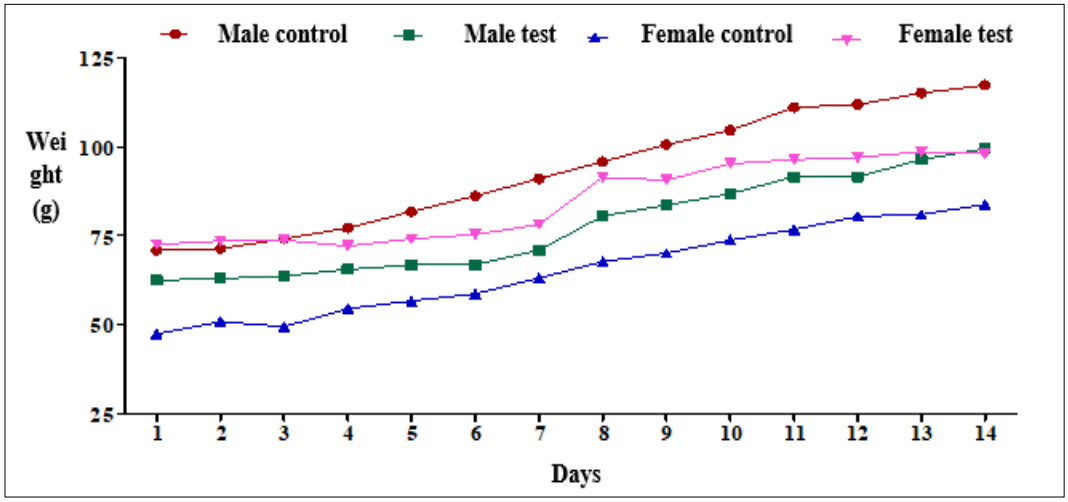


Fig 2: Kinetics of weight change in rats from different study groups

The results in Table 3 show a non-significant decrease in food intake. The same is true for water intake but with a significant decrease and a *p-value* < 0.05, in the female test group compared to the female control group.

Table 3: Assessment of zootechnical parameters

Parameters	Male Control	Test male	Female Control	Test female
Food intake (g)	88.31± 22.50	62.31±32.83	76.31±22.35	75.69±32.54
Water intake (ml)	90.31±18.54	75.69±14.31	77.54±18.73	56.31±19.57
Weight gain (g)	46.20±12.56	36.80±10.47	36.20±16.80	31.00±16.79

Comparative assessment of organs weight: Analysis of the results in Table 4 did not reveal any significant difference in the relative weight of the organs between the different study groups.

Table 4: Comparative assessment of the relative weight of organs

Organs		Male control	Male test	Female Control	Female test
Heart		0.39±0.05	0.42±0.05	0.40±0.06	0.45±0.08
Liver		3.67±0.28	4.32±0.58	4.07±0.30	3.70±0.32
Lungs		0.92±0.25	0.79±0.17	0.78±0.07	0.94±0.24
Brain		1.28±0.22	1.56±0.18	1.70±0.50	1.58±0.56
Spleen		0.90±0.22	0.90±0.10	0.66±0.29	0.72±0.21
Kidneys	Left	0.36±0.04	0.39±0.03	0.43±0.05	0.40±0.03
	right	0.38±0.04	0.41±0.03	0.46±0.03	0.45±0.06
Testis/Ovaries	Left	0.61±0.15	0.64±0.08	0.06±0.04	0.06±0.03
	Right	0.62±0.15	0.66±0.10	0.06±0.03	0.06±0.02
Adrenals	Left	0.02±0.01	0.02±0.005	0.03±0.01	0.02±0.008
	Droit	0.02±0.003	0.02±0.004	0.03±0.02	0.03 0.02

Assessment of biochemical parameters

Analysis of transaminase results showed a non-significant increase in ALT levels in the male test group (90.70±17.41) IU/L compared to the healthy control group (73.36±18.61) IU/L as shown in Figure 3. On the other hand, a non-significant decrease in activity was observed in the female test group (70.02±7.17) IU/L compared to the female control group (88.32±15.38) IU/L with a p-value > 0.05. The same results were observed for ASAT where we noticed a non-significant increase in enzyme activity in the male test group (206.80±33.37) IU/L compared to the control group (201.00±48.76) IU/L. In the female control group (278.75±56.62) IU/L, we observed a decrease in enzyme activity (231.80±49.25) IU/L with a *p-value* > 0.05. These results suggested the absence of hepatic cytolysis.

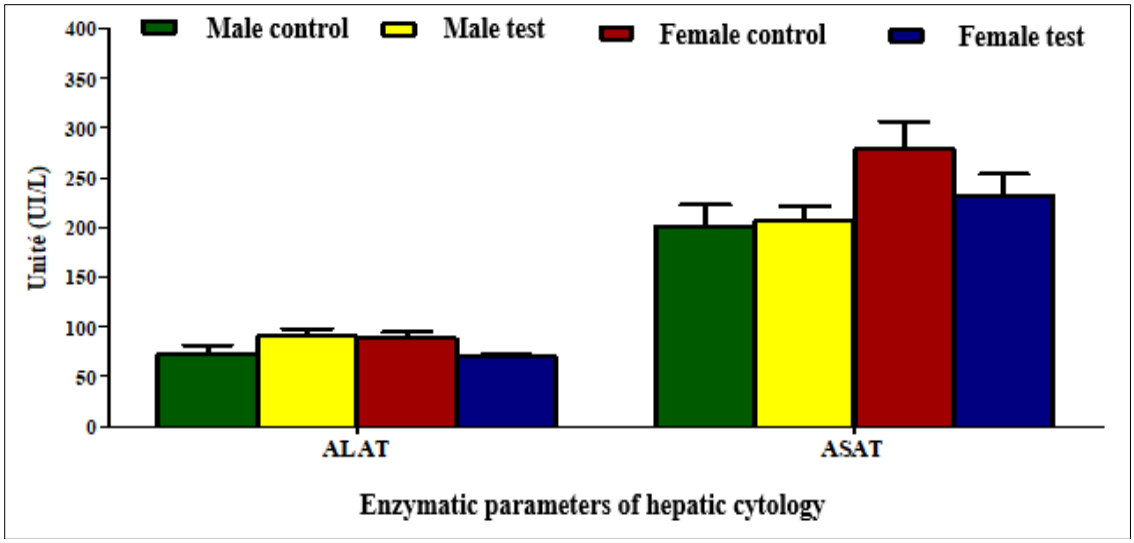


Fig 3: Effect of *Azadirachta indica* oil on serum transaminases

Hepatic cholestasis parameters showed a non-significant decrease in alkaline phosphatase activity in the male test group (569.80±192.51) IU/L compared to the male control group (607.75±32.25) IU/L with a *p-value* > *0.05*. In females, we observed a significant decrease in the test group (485.60±115.17) IU/L compared to the control group (777.20±206.03) IU/L with a *p-value* < *0.05*. Gamma GT analysis showed a significant elevation in the male test group (3.43±2.01) g/l compared to the male control group (3.08±1.22) g/l and the same result was observed in the females or in the female test group (Figure 4). A value of (4.59±1.72) g/l was obtained compared to the control group (2.86±1.46) g/l with a *p-value* > *0.05*. Observation of
Total bilirubin level gave a non-significant increase in activity in the male test group (7.16±1.72) mg/L compared to the male control group (5.97±1.34) mg/L. Compared to the female control group (8.10±0.71) mg/L we recorded a non-significant decrease in activity (6.66±2.23) mg/L in the female test group with a *p-value* > *0.05*. These results suggested an absence of hepatic cholestasis following oil administration.

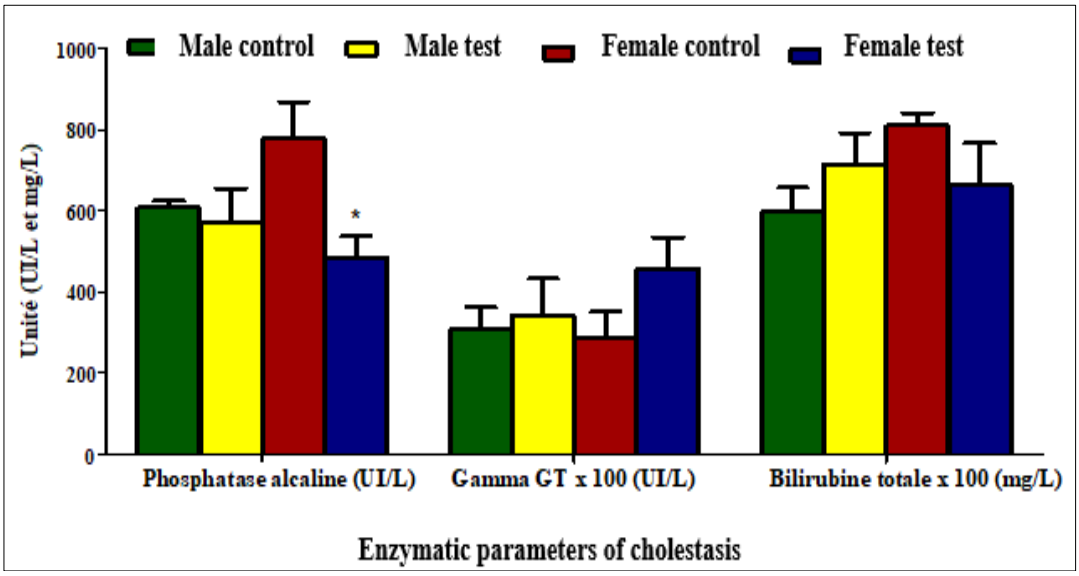


Fig 4: Effect of *Azadirachta indica* oil on liver cholestasis

Analysis of renal damage parameters showed for creatinine, a non-significant increase with a *p-value* > *0.05* in the male test group (4.38±0.58) mg/L compared to the male control group (4.32±0.32) mg/L. However, a difference was noted in females with a non-significant decrease in the female test group (4.54±0.15) mg/L compared to the female control group (4.84±0.31) mg/L (Figure 5).
A non-significant increase in urea level with a *p-value* > *0.05* was observed in the male test group (0.64±0.02) g/L compared to the male control group (0.49±0.06) g/L. Also, similar results were recorded for the female test group (0.61±0.08) g/L compared to the female control group (0.59±0.10) g/L. The observed creatinine and urea activity suggested no renal involvement. Moreover, a non-significant decrease in protein level with a p-value > 0.05 was observed in the male test group (53.54±5.56) g/L compared to the male control group (56.34±2.59) g/L, and in the female test group (52.68±3.73) g/L compared to the female control group (59.10±3.34) g/L (Figure 5). This result suggested a possibility of oil-induced malnutrition.

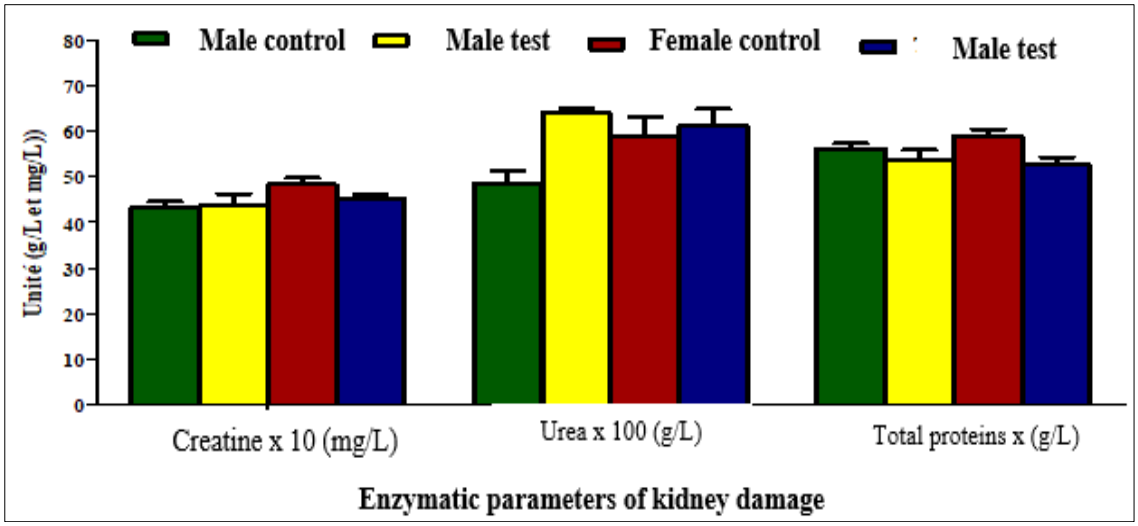


Fig 5: Enzymatic parameters of kidney damage

Assessment of histological parameters

Results from histological sections showed no major changes in internal organ structures between the study groups (Figure 6). However, there was a slight obstruction of the middle hepatic vein in the male test group, which may suggest mild hepatic damage (Figure 7).

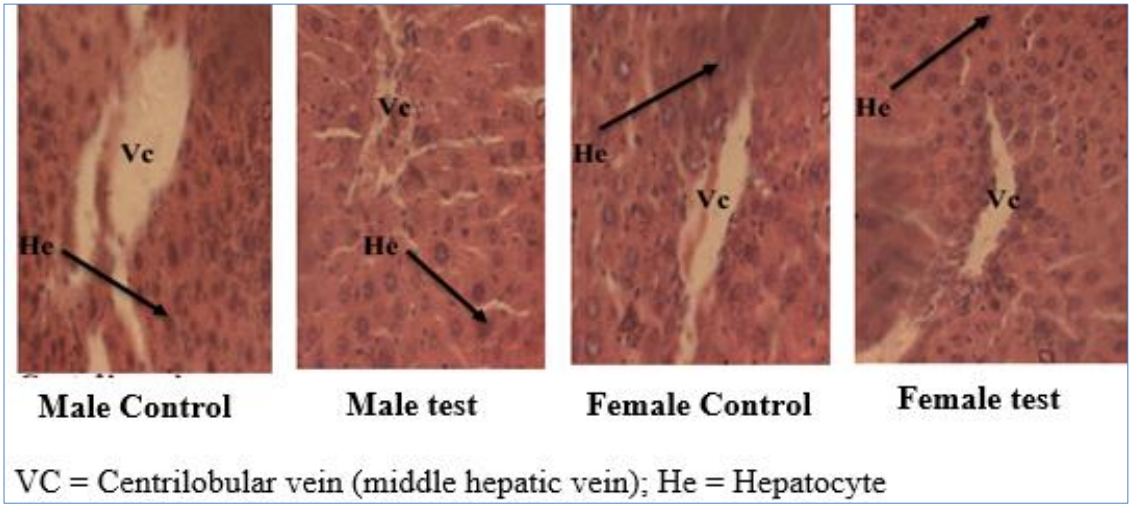


Fig 6: Liver histology for animals in the different study groups

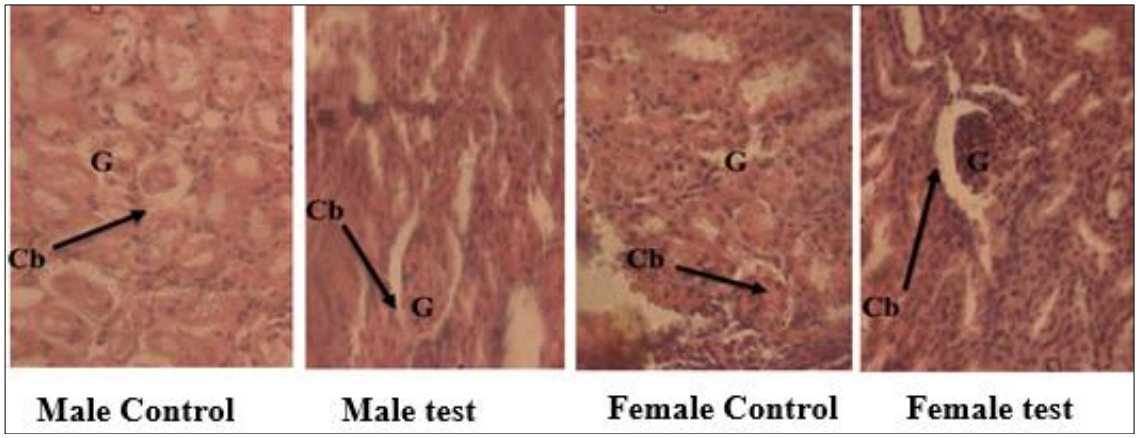


Fig 7: Kidney histology for animals in the different study groups

Discussion

Susceptibility of Salmonella strains to *Azadirachta indica* seed oil in this study showed that two out of the three Salmonella strains were susceptible to *Azadirachta indica* seed oil with varying diameters. Thus, determination of the inhibition diameter gave a result of 17 mm for *Salmonella typhi*. This diameter is higher than those found in previous studies by Ngum *et al*. 2019 who obtained a diameter of 8 mm [17, 17] and Zhong-hui *et al*., (2010) [23], who obtained a diameter of 10 mm. This difference could be explained by the fact that both oils had different origins and extraction methods. However, the diameter obtained shows that

Salmonella typhi is susceptible to our oil. *Salmonella paratyphi* A showed an inhibition diameter of 18.50 mm. We did not find any studies similar to ours reporting the inhibition diameters of *Salmonella paratyphi* A strain by *Azadirachta indica* oil. However, according to Moreira's classification, both bacterial strains are susceptible to *Azadirachta indica* seed oil.

The minimum inhibitory and bactericidal concentration of *Azadirachta indica* seed oil on bacterial strains showed values of the minimum inhibitory and bactericidal concentrations for the two bacterial strains studied were 37.5 mg/ml and 150mg/ml respectively. The ratio of the minimum bactericidal concentration to the minimum inhibitory concentration was 4. These values are different from those found by Zhong-hui *et al*., 2010 [23] in China who obtained a ratio of 1. This difference can be explained by the fact that they used the broth microdilution method [23]. Therefore, our oil has a bacteriostatic antibacterial activity. This could be related to the presence of some compounds such as: Lineoleoyl chloride 10.24%, methyl petroselinate 10.23%, phytol 9.6%, hentriacontane 8.8%, 7-hexyl eicosane 8.1% and many others revealed by gas chromatography.

Acute toxicity

At a single dose of 2 ml/100g, *Azadirachta indica* seed oil had no adverse effects on treated rats during 14 days of observation. A non-significant decrease in food intake was observed in the test groups and in water intake in the male test group. However, a significant decrease in water intake was observed in the female test group. There was a non-significant weight gain in both test groups but the relative weight of the internal organs of the rats showed no difference between the test and control groups. These results correlated with other studies carried out in Cameroon, Nigeria and China which also reported this decrease during the second week [22-25]. These studies suggest that the changes in water intake and in weight of the rats during the observation period may have occurred because the *Azadirachta indica* seed oil interfered with the absorption of nutrients making them unavailable or the oil intake may have made them feel satisfied for a while thus reducing their food intake [6, 12, 17].

The biochemical results of ALT, ASAT, alkaline phosphatase, total bilirubin, creatinine, urea and total protein were not significantly altered between animals in the test and control groups after 14 days of extract administration. Other studies found in the literature presented similar results to ours [2, 18]. Significant changes were found in Gamma GT levels but these do not necessarily indicate cholestasis or liver damage. However, a study conducted in Cameroon by Ngum *et al*. found a significantly elevated serum transaminase level and a statistically significantly decreased in total protein level [11]. This result could be explained by the difference in the dose administered in each study. Ngum *et al* administered a dose of 3 ml/100g. These observations confirmed the macroscopic and histological evaluation of the internal organs which showed no apparent damage or lesions. However, a slight obstruction of the central vein of the liver observed in the male test group could suggest a slight hepatic injury. These results are consistent with those reported by other authors [8, 25]. Therefore, the *Azadirachta indica* seed oil used does not cause acute toxicity effects at the dose tested and with LD50 values below 2 ml/100g. Nevertheless, these findings do not correlate with earlier studies by Ngum *et al.*, 2019 [11], where the LD50 of *Azadirachta indica* seed oil in rats was 3 ml/100 g [11, 26]. *Azadirachta indica* seed oil is reported to be hepatoprotective, and its hepatoprotective effect is dose-dependent. Some studies revealed that *Azadirachta indica* seed oil at 0.25 ml/kg, 0.5 ml/kg and 1.0 ml/kg was hepatoprotective, but could become toxic at a comparatively higher dose [5].

Conclusion

This study whose aim was an *in vitro* evaluation of the anti-Salmonella activity and acute toxicity of *Azadirachta indica* A.juss seed oil helped determine the bacteriostatic activity of our oil with a MIC equal to 37.5 mg/ml and a MBC of 150 mg/ml for the two strains that were susceptible. Acute toxicity study concluded that the LD50 of this extract is greater than 2 ml/100g as no animal deaths were recorded during the experiment. Also, according to the results of the analyses obtained, taken at low doses over a short period of time, this oil would have no notable toxic effects on the liver and kidneys. *Azadirachta indica* seed oil could therefore be a good alternative for modern medicine in the treatment of certain salmonellosis in order to reduce bacterial resistance to antibiotics.

Authors Contribution

Yede Marie Paule[1], Estella Achick Tembe[2], Mbong Grace Anih[3], Bayaga Herve[4], Njinkio Nono Borgia[2], Gonsu Kamga Hortense[5], Charles Fokunang*[2].

YMP, EAT, FCN conceived and designed the study and drafted manuscript. MGA, BH, GH and NBMO coordinated laboratory analysis and data assembly. YMP, BH did the data mining. All authors read and reviewed the final draft of manuscript for publication.

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Conflicts of interest

The authors declare no conflict of interest.

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