

Investigation of Toxicity and Systemic Exposure of Bioactive Compounds of Aqueous Leaf Extract of *Azadirachta indica* A. Juss (*Meliaceae*) on Wistar Rats

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Abstract: Conventional medications used to treat ulcers are not easily accessible to remote areas, costly and not without side effects, thus causing many patients residing in rural areas to resort to herbal means of treatment. World Health Organization records that at least 80% of the world's population depends on herbal medicinal products. Herbal therapy is the belief that it will promote healthier living. *Azadirachta indica* is a tree extensively spread in the Northern parts and sparsely distributed in the Northwest Region of Cameroon, used as a remedy for several pathologies, amongst which we have gastric ulcers which is our area of interest. The objective of this study was to evaluate the acute oral toxicity (420 OECD guidelines), systemic exposure and biochemical parameters of toxicity of *Azadirachta indica* A. Juss on Wistar rats. Various biochemical parameters such as the: MDA, Catalase, Glutathione, Pepsin, SOD, ASAT, ALAT, Creatinine, XO, and total proteins, were quantified. The acute oral toxicity was of 2000 mg/Kg single dose and compared to a control group which was administered tap water. The administration of 2000 mg/Kg was well tolerated and no death was recorded throughout the fourteen days of observation. No toxic effects were recorded in the organs, implying that at the dose of 2000 mg/Kg, *Azadirachta indica* was safe. *Azadirachta indica* aqueous leaf extract contains active metabolites coumarins, catechic tannins, polyphenols, tannins, flavonoids and phlobotannins that were bioavailable in systemic circulation. Showed bioavailability at the tested doses (12.5, 25 and 50 mg/Kg), with the presence of phytochemicals being dose dependent. A clean toxicity profile, with just a slight increase in the level of creatinine.

Keywords: *Azadirachta Indica*, Systemic Exposure, Toxicity Bioactive Compounds Wistar Rats

1. Introduction

Traditional medicine has a long history and according to WHO, it is the sum total of the knowledge, skill, and

practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental

illness [1-5]. Large sections of the population in developing countries still rely on traditional practitioners and herbal medicines for their primary care; in Africa up to 90% and in India 70% of the population depend on traditional medicine to help meet their health care needs. In China, traditional medicine accounts for around 40% of all health care delivered [5-7].

The extensive use of traditional medicines could be backed up by several reasons, some of which include, they are more affordable, more closely correspond to the patient's ideology, allays concerns about the adverse effects of chemical (synthetic) medicines, satisfies a desire for more personalized health care, and allows greater public access to health information [2, 8-10]. In some parts of the world, traditional medicine is still considered as alternative medicine. Alternative medicine has been simply defined by WHO as a broad set of health care practices that are not part of that country's own tradition or conventional medicine and are not fully integrated into the dominant health-care system. They are used interchangeably with traditional medicine in some countries. [1, 11]

According to a survey by the National Center for Complementary and Alternative Medicine, herbal medicine (plant-derived materials or products with therapeutic or other human health benefits which contain either raw or processed ingredients from one or more plants or the usage of natural products other than vitamins and minerals was the most commonly used alternative medicine (18.9%) when all use of prayer was excluded [3, 12-15].

Herbs have been extensively used in different domains of life; nutritional relevance, recreation, disease remedies, ornamentation, fencing, etc. Herbal medicines have been widely utilized as effective remedies for the prevention and treatment of multiple health conditions for centuries by almost every known culture [16]. The first documented records of herbal medicine use date back 5,000 years in China. Similarly, India's Ayurvedic medicine tradition is thought to be more than 5,000 years old and herbal medicines remain an essential component of its practice. Today, vast populations of certain countries still depend on herbal medicines to address their healthcare needs [4, 17-20].

Medicinal plants (plants which have been used for medical purposes at one time or another, and which, although not necessarily a product available for marketing, is the original material of herbal medicines [21-23]) long played important roles in the treatment of diseases all over the world. Healing with medicinal plants is as old as mankind itself. The connection between man and his search for drugs in nature dates from the far past, of which there is ample evidence from various sources: written documents, preserved monuments, and even original plant medicines [5, 24-26].

Given the limitations associated to conventional medicines – poor access to medication in rural areas, informed choice to reduce incidence of side effects in urban areas, costly medications – many people tend to use herbal or traditional medicines. Regardless of why an individual uses it, traditional medicine provides an important health care service whether people have physical or financial access to allopathic medicine,

and it is a flourishing global commercial enterprise [27].

Currently, herbs are applied to the treatment of chronic and acute conditions and various ailments and problems such as cardiovascular disease, peptic ulcer disease, prostate problems, depression, inflammation, and to boost the immune system, to name but a few [21, 28]. The use of the Neem Tree dates as far back as mankind. Neem is an omnipotent tree and a sacred gift of nature. Neem tree is mainly cultivated in the Indian subcontinent. Neem is a member of the mahogany family, Meliaceae [3, 29]. Today it is known by the botanical name *Azadirachta indica* (*A. indica*) A. Juss. Neem has been used extensively by humankind to treat various ailments before the availability of written records which recorded the beginning of history. Very limited literature is available on the treatment of PUD by *A. indica* [11, 29-31]. It has a high tolerance of harsh environments, grows poorly in waterlogged and cold regions and grows best in altitudes of sea level to 800 above level in well drained soils.

2. Materials and Methods

2.1. Study Site, Research Design

This study was carried out in Laboratory for Preclinical Animal Studies and Toxicology Research of the Faculty of Medicine and Biomedical Sciences, FMBS, University of Yaoundé I.

The study was an experimental *in vitro* and *in vivo* design done in albino rats of *Wistar* strain conducted between the 6th November 2017 to May 2018.

2.2. Ethical Considerations

Ethical approval was demanded from the institutional review board of the Faculty of Medicine and Biomedical Sciences. Authorization was obtained from the administration of the FMBS, to work in the animal house of this faculty. The OECD Guidelines for the use of animals in preclinical studies was applied

2.3. Plant Material – *Azadirachta Indica*

2.3.1. Harvesting and Identification

The fresh leaves were harvested from a Neem plant growing in Ndop, capital of the Ngo Ketunjia Division of the Northwest Region, Cameroon, in the compound of Mr. Saki Madi Mahamat. These leaves were taxonomically identified and authenticated at the National Herbarium of Cameroon as *Azadirachta indica* leaves by Dr. Tchiengue Barthelemy. The sample was given the voucher code No 4447/SRFK.

We then proceeded to wash and dry the leaves in a shade, on mats placed on a flat surface. When dried, the leaves were ground, sieved to get fine powder and stored in airtight containers before extraction.

2.3.2. Extraction of Leaf Powder

There are several existing methods of extracting a plant but because this research is aimed at proving the presence of an activity claimed traditionally, we decided to use the

traditional method; maceration.

In this process, 150g of the coarsely powdered crude plant was put in a stoppered container with 1.5L of distilled water and allowed to stand at room temperature for a period of 48 hours with frequent agitation until the soluble matter has dissolved. The mixture was then strained, the marc (the damp solid material) was pressed, and the combined liquids were clarified by filtration using Whatman paper and collected the supernatant. This filtrate was evaporated and the extract collected. The percentage yield was then calculated:

$$\text{Percentage yield} = \frac{\text{Mass of extract obtained} \times 100}{\text{Mass of powder initially used}}$$

2.4. Animal Material

The experiments were done on adult albino rats of *Wistar* strain, gotten from the Animal House of FMBS, UY1.

2.4.1. Selection and Feeding of Rats

Wistar strain (*Rattus norvegicus*) albino rats were used. All animals used were bred in the FMBS animal house under favorable conditions of 12h of light and 12h of day. The rats were aged between 7 and 12 weeks, with average weight 203 ± 32 for the antiulcer activity. Also, for this activity, only male rats were used because literature demonstrates that the male gender is more prone to having ulcers. Both male and female rats were used for the toxicity studies, with average mass 118 ± 23 .

The animals were fed with a diet, consisting of corn meal (45 %), wheat flour (20 %), fish meal (20 %), soybean meal (10 %), palm kernel (5 %), bone flour for calcium intake (0.98 %), cooking salt (0.5 %) and vitamin complex (0.5 %). They were also allowed free access to regular tap water.

2.4.2. Accommodation of Rats

For each study, the animals were separated in different cages, with distinct and clear labels. The cages were made of plastic material with iron tops/doors and a space for food and water was made available. The floors were lined with saw dust to keep it dry. In conditions where the rats had to be starved, they were put in metabolic cages made of stainless steel material with spaced bars, allowing the feces to fall through, thus preventing them from eating their feces. In each cage, the tails of rats were marked with bold markers, with the number of lines denoting the rat number. These animals were then crosschecked to make sure that they were in good health and kept in natural environmental conditions (12h of light and 12h of darkness). Each day, the rats were fed with the above mentioned meal and given water ad lib.

2.5. Administration of Test Substances

The ulcerogenic agent, reference drugs or aqueous leaf extract were administered using an intubation needle, fitted into syringes of different volumes. Depending on the study, different volumes and doses of these substances were calculated and given to the rats according to their individual weights.

2.5.1. Acute Toxicity

The animals were randomly selected, marked to permit

individual identification and kept in their cages for acclimatization to laboratory conditions for at least five days before the experiment. Animals should be fasted prior to the administration of the substance; food were deleted, but not water, overnight. The extracts were administered in a single dose by gavage. The doses were prepared just prior to administration. After the period of fasting, the animals were weighed and then the extracts were administered. After administration of the substance, the animals were starved again, for 3 to 4 hours. Each step required three animals. For the initial dose, 2000 mg / kg body weight was chosen. The administration of the next lower dose was great if mortality was observed among animals. For 14 days, we observed the various manifestations of toxicity: salivation, diarrhea, lethargy, sleep and coma. The individual weight of each animal was determined shortly before administration of the test substance and then at least once a week. At the end of the test, the rats were euthanized for 14 days [32]. After a blood sample from the retro orbital sinus was collected for determination of blood biochemical parameters. Organs were immediately isolated, washed with cold saline and fixed in a neutral solution of 10% buffered formalin for histopathological evaluation

2.5.2. Zoo Technical Criteria

On day one, the animals were observed for one hour for abnormal behaviors like cleaning, abnormal postures, hyper/hypo activity, tremors, scratching and myoclonia. The animals' behavior were followed up with respect to feeding, water consumption and weight gain were observed and recorded throughout the fourteen days of studies. This was done every morning before 9:00 am

2.5.3. Preparation and Administration of the Extract

We prepared a 200 mg / ml concentration solution. 20 mL of solution was obtained from 4000 mg of extract and distilled water (sufficient for 20 mL). Then, the mixture was homogenized using a magnetic stirrer. From this solution, a dose of 2000 mg / kg of aqueous extract was administered to the male and female test groups according to their weight, while the control groups received distilled water. The animals were again deprived of food for four hours.

2.6. Quantification of Toxicity Biochemical Parameters

2.6.1. Evaluation of the Renal Activity

Creatinine quantification (CHRONOLAB KIT)

The assay is based on the reaction of creatinine with sodium picrate as described by JAFFÉ. Creatinine reacts with alkaline picrate forming a red complex. The time interval chosen for measurements avoids interferences from other serum constituents. The intensity of the color formed is proportional to the creatinine concentration in the sample.

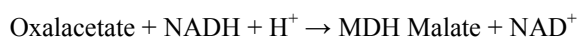
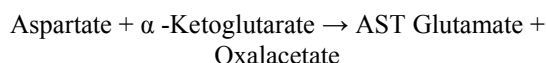
2.6.2. Quantification of ALAT (CHRONOLAB KIT)

Alanine aminotransferase (ALT) or Glutamate pyruvate transaminase (GPT) catalyzes the reversible transfer of an amino group from alanine to α -ketoglutarate forming glutamate and pyruvate. The pyruvate produced is reduced to lactate by lactate dehydrogenase (LDH) and NADH:

The rate of decrease in concentration of NADH, measured photo metrically, is proportional to the catalytic concentration of ALT present in the sample

2.6.3. Quantification of ASAT (CHRONOLAB KIT)

Aspartate aminotransferase (AST) formerly called glutamate oxaloacetate (GOT) catalyses the reversible transfer of an amino group from aspartate to α -ketoglutarate forming glutamate and oxalacetate. The oxalacetate produced was reduced to malate by malate dehydrogenase (MDH) and NADH:



The rate of decrease in concentration of NADH, measured photometrically, is proportional to the catalytic concentration of ASAT present in the sample

2.7. Evaluation of Systemic Drug Exposure

The systemic exposure of a drug determines to an extent, the bioavailability or absorption. Many *in vitro* studies indicated that neem possesses various bioactivities; however, a poor bioavailability may make them largely ineffective *in vivo*. Absorption levels are therefore of special interest. The same method used in phytochemical screening for secondary metabolites in the plant extract was used in evaluating the systemic exposure. After exposure of the rats to extract treatment, blood samples were collected and used phytochemical screening for metabolites.

From the curative activity, the least dose which offers the

best ulcer healing was used. The animals was administered this dose and at different time points, blood samples were collected from the tail vein and centrifuged. The plasma was extracted and screened for the presence of different secondary metabolites, referencing from what was found during the screening of the plant extract.

2.8. Statistical Analysis

The results were expressed in terms of mean \pm standard deviation. The comparisons between the groups were analyzed using one-way analysis of variance, the ANOVA test followed by Turkey's Kramer post hoc test using the GraphPad Instat version 5.0 software. A P-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Extraction Yield

The 1:1 ratio aqueous extraction of the *Azadirachta indica* leaf yielded 11.27%.

3.1.1. Acute Toxicity

Zootechnical parameters- observation of behaviour of rats after administration of plant extract.

Observation of test groups of male and female treated rats from 0-30 days showed no abnormal posture, vocalization, myoclonia and hypo activity. There were indications of scratching, hyperactivity, tremors and cleaning as shown in Table 1.

Table 1. Observation of behavior of rats after administration of NLEa.

Observation of test groups (male and female)		Day one, 0 – 30 minutes after administration of plant substance	Days two to fourteen
Appearance	Cleaning	++	-
	Abnormal posture	-	-
	Hyper activity	++	-
	Tremors	+	-
	Vocalization	-	-
	Scratching	++	-
	Myoclonia	-	-
	Hypo activity	-	-

3.1.2. Weights

The average weights of the control groups and animal groups receiving the plant substance increased from day one to day fourteen, with a slight decrease in the weight of the test males on day eight (Figure 1). These statistics showed no significant variation between the weights of the test and control groups, with a *p-value* > 0.05.

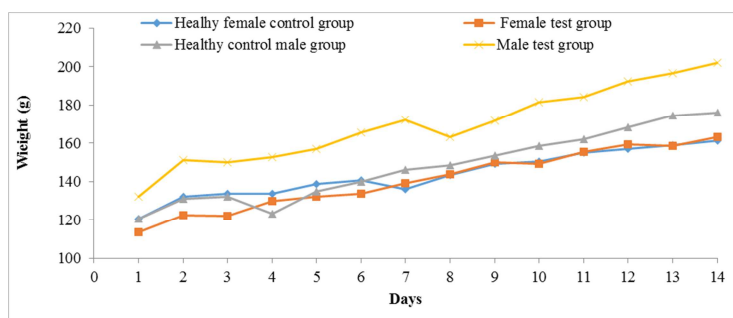


Figure 1. Kinetic evolution of weight during acute oral toxicity evaluation.

3.1.3. Food Intake

As concerns the food intake, a non-significant decrease was observed in the test females. Contrarily, an increase in the test males was observed, though not significant (Table 2).

3.1.4. Water Intake

A significant increase in the water intake was observed in both groups of animals that received the NLEa, with a *p-value* < 0.01, when compared to the control groups of animals (Table 2).

Table 2. Zootechnical parameters of acute oral toxicity of NLEa.

Parameters	Female control	Female test	Male control	Male test
Weight gain (g)	41,00 ± 6,56	50,00 ± 10,12	55,60 ± 6,54	81,40 ± 26,38
Food intake (g)	169,62 ± 11,41	155,31 ± 66,64	144,85 ± 46,33	207,69 ± 75,44
Water intake (ml)	124,15 ± 19,67	176,08 ± 31,08**	169,38 ± 44,78	232,08 ± 55,40**

The results are expressed in average ± standard deviation (n = 5).

3.2. Relative Weight of Organs

There was no significant difference in the weight of organs between the various test groups

Table 3. Relative weight of organs.

Organs	Control male	Test male	Control female	Test female
Liver	4,25 ± 0,20	4,03 ± 1,15	4,03 ± 1,13	3,98 ± 1,11
Brain	0,86 ± 0,07	0,76 ± 0,23	0,99 ± 0,25	0,90 ± 0,25
Lungs	0,91 ± 0,21	0,88 ± 0,26	0,69 ± 0,23	0,70 ± 0,22
Pancreas	0,62 ± 0,24	0,29 ± 0,23	0,43 ± 0,20	0,39 ± 0,17
Heart	0,30 ± 0,03	0,29 ± 0,08	0,32 ± 0,08	0,31 ± 0,08
Kidneys	Left	0,34 ± 0,04	0,34 ± 0,09	0,33 ± 0,09
	Right	0,35 ± 0,04	0,36 ± 0,09	0,35 ± 0,09
Adrenal glands	Left	0,01 ± 0,003	0,01 ± 0,006	0,02 ± 0,006
	Right	0,01 ± 0,005	0,02 ± 0,005	0,02 ± 0,006
Testicles /Ovaries	Left	0,54 ± 0,07	0,04 ± 0,002	0,03 ± 0,002
	Right	0,55 ± 0,07	0,03 ± 0,002	0,03 ± 0,002

The results are expressed in average ± standard deviation (n = 5).

3.3. Zootechnical Parameters of Acute oral Toxicity of NLEa

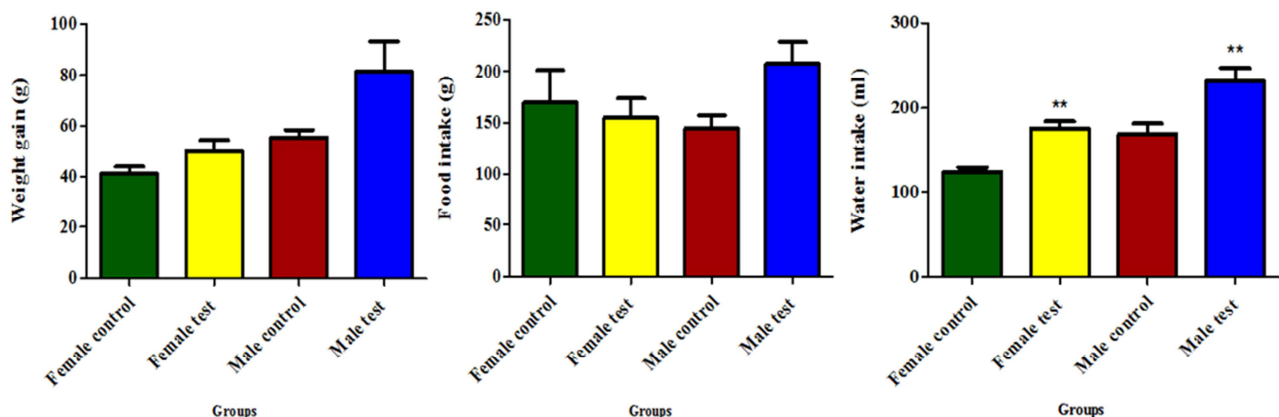


Figure 2. Zootechnical parameters of acute oral toxicity of NLEa.

3.4. Biochemical Parameters of Acute Oral Toxicity

Various enzymes, that served as organic markers were analyzed for further insight to the acute toxic effect of the aqueous Neem extract. Administration of the NLEa caused a significant increase in total proteins and creatinine in the test groups when compared to the control groups with a *p-value* <

0,001. On the other hand, the administration showed a non-significant decrease of ASAT and a non-significant increase of ALAT on male test groups (Table 4). There was no difference observed in the levels of ASAT and ALAT in the female test groups. The biochemical parameters are shown in the table (Table 4).

Table 4. Biochemical parameters of acute toxicity Parameters.

	Control female	Test female	Male control	Male test
Total proteins (mg/dl)	13,81 ± 3,20	61,13 ± 2,72***	13,74 ± 2,30	66,83 ± 4,45***
Creatinine (UI/L)	14,31 ± 1,46	5,59 ± 1,74***	9,26 ± 1,46	4,90 ± 1,04***
ASAT (UI/L)	23,28 ± 12,02	8,82 ± 1,21	23,51 ± 20,19	10,66 ± 0,77
ALAT (UI/L)	18,74 ± 9,64	18,70 ± 3,38	10,83 ± 1,05	21,67 ± 0,75

3.5. Evaluation of Systemic Exposure

The blood serum was tested for the presence of secondary metabolites which were originally present when the NLEa was screened. There was detected bioavailability of the following metabolites mucilage, catechic tannins, flavonoids, total polyphenols at 12.5 mg, 25 mg, 50 mg of NLE in the blood serum measured. Flavonoid was not detected at low dose of 12.5mg one hour after administration of the extract (Table 5).

Table 5. Systemic exposure of secondary metabolites of NLE in rat blood serum.

Metabolites	reagents	Blood serum containing different doses of NLEa		
		12.5 mg	25 mg	50 mg
Mucilage	Excess EtOH	+	++	++
Catechic tannins	STIASNY	+	+	+
Flavonoids	NaOH	-	+	+
Total polyphenols	Lead acetate	++	++	++
Tannins	Cu citrate + NH ₃	+++	+++	+++
Phlobotannins	HCl	-	-	-

Key: + = low concentration; ++ = moderate concentration; +++ = high concentration.

4. Discussion

Azadirachta indica, a tree stemming from the meliaceae family, is not a native tree of Cameroon. It was introduced in the drought prone North and Far North regions of Cameroon in the late 19th century [2, 32-35]. The acute toxicity studies done on the aqueous extract of *Azadirachta indica*, according to OECD 420 Guidelines was fixed at 2000 mg/Kg. the fixed dose procedure was used and shown to be better for the acute toxicity testing as it doesn't consider the death of animals as the endpoint of toxicity studies uses fewer animals to produce similar result to toxicity studies done using the classical method [32-35]. At this dose, no sign of toxicity or lethality was observed. These results obtained are similar to those obtained by Kanagasanthosh et al, who also didn't experience any animal death at the dose of 2000 mg/Kg. The LD50 was not determined in this study. Nonetheless, others have done studies beyond what was done in this case. At 2500 mg/Kg, Dorababu et al didn't observe any mortality either [36]. This suggests the safe nature of *A. indica*, as it correlates with the traditional use for centuries [2, 37]. However, our results were different from the overall results obtained by Ashafa et al, who found out that at all doses used, the ethanolic extract of *A. indica* demonstrated toxicity [38-40]. At a dose of 3200 mg/Kg, Yinusa et al obtained a 100% mortality in rats [12]. Contrarily, Adinortey et al found out that at a dose as high as 5000 mg/Kg, a combination of an aqueous extract of *A. indica* with an ethanolic extract of *Ficus racemose* in a ratio 1:1 caused no lethality. Probably, a combination of these two extracts dampens the toxic effect. His results suggest that the LD50 is > 5000 mg/Kg [13, 41].

Animals were observed on day one for thirty minutes for

any behavioral changes and slight agitation were noticed in some animals. This happened just for the first day and the animals returned to their normal calm state, like the animals of the control group for the rest of the study period. This is different from what Kanagasanthosh observed when he noticed no behavioral changes in his test animals [24, 42]. This slight agitation could have resulted from the fact that the animals were fasted before the administration of the leaf extract. Also, the process of gavage is never comfortable for the animals. We observed that there was a progressive increase in the body weight of all animals, whether test or control. This could be explained by the fact that the animals were fasted before the beginning of the experiment, but during the experiment, they were given free access to food. This could also mean that the extract enhances a sense of taste and appetite of the animals. These results correspond to what was observed by Ashafa et al. On the other hand, we observed a decrease in the water intake. This is similar to what Ashafa observed in rats taking 300 mg/Kg [43].

In this study, we also realized that there was no significant difference in the weight of organs, as compared to the control groups of animals. These results corroborate with those obtained by Dias et al [21, 44]. This is however different from what Dorababu and Ashafa obtained. This could be because he used a dose higher than what we used (2500 mg/Kg). This difference might have been because they used the barks and not the leaves. From the analyses of the histological cuttings of the livers and kidneys of all animal groups, there was no change observed in the cellular morphology. Biochemical parameters were used to measure the proper functioning of the metabolic and various endocrine systems and as earlier seen, nutritional functioning

or disorders. It is important to measure these parameters because they are useful in evaluating the functioning of various organs and in calculating damage percentages [28, 45]. The study recorded, upon the administration of the NLEa to test groups, a non-significant decrease of ASAT and a non-significant increase of ALAT on male test groups. No difference was observed in female groups, when compared to the control groups. This demonstrates the hepatoprotective abilities of the leaf extract. These results are similar to those obtained by Boadu *et al* [18]. He carried out this study in rabbits in 2011 and found no significant difference in the levels of ASAT and ALAT, when compared to the control group of animals. Determination of serum electrolytes, creatinine and urea are critical as they are the important markers of kidney function. In this study, there was a significant increase in the creatinine levels in both female and males groups with a *p-value* < 0,001 indicating that the renal function is negatively affected by this plant. The increase in total proteins without a damage in the kidneys or liver could mean that the plant has a high nutritional value.

5. Conclusion

According to this study, *Azadirachta indica* aqueous leaf extract contains active metabolites coumarins, catechic tannins, polyphenols, tannins, flavonoids and phlobotannins that were bioavailable in systemic circulation. Showed bioavailability at the tested doses (12.5, 25 and 50 mg/Kg), with the presence of phytochemicals being dose dependent. A clean toxicity profile, with just a slight increase in the level of creatinine.

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 Yaoundé I.

Competing Interests

Authors have declared that no competing interests exist.

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