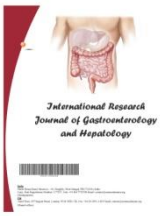

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Phytochemical Screening, Evaluation of Anti-Peptic Ulcer Activities of Aqueous Leaf Extract of Neem *Azadirachta indica* A. Juss (*Meliaceae*) in Wistar Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Authors TFE, FCN, NSA, designed the study, wrote the protocol, and wrote the first draft of the manuscript. Authors MGA, TMVE, BH, DJF managed the analyses of the study; and data mining. Authors NBN, RZ, KGY performed the statistical analysis, and the specified literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Introduction: Peptic ulcer disease (PUD) is a major public health concern, affecting up to 10% of the world's population. This disease results from an imbalance between the gastro protective and aggressive factors of the gastric mucosa. Prevalence of PUD is about 10% in Cameroon and conventional medications used to treat ulcers are not easily accessible to the population in the rural areas. The drugs are expensive to the poor population and comes with numerousside effects, thus causing many patients residing in rural areas to rely on herbal medicines. The herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products, that contain as active ingredients parts of plants, or other plant materials, or combinations). World Health Organization (WHO), records that at least 80% of the world's population depends on herbal medicine products. Herbal therapy is believed toto promote healthier living. *Azadirachta indica* is a treethat is common in the Northern parts and sparsely distributed in the Northwest Region of Cameroon. It is used as a remedy for several pathologies, amongst which we have gastric ulcers which is our area of interest.

Objectives: To qualitatively identify the secondary metabolites present in the aqueous leaf extract of *A. indica* and investigate its preventive and curative activity on gastric ulcers

Methods: The aqueous leaf extract was phytochemically screened following the method used by Prashanth and Krishnaiah. The extract was screened for the presence of sulphite ions which could be useful in ulcer prevention/healing. For *in vitro* investigations, the antacid properties (using the Food and Drug Administration (FDA) test) was tested, the acid neutralization capacity, acid neutralization speed (Rossett-Rice method) and buffering capacity (Holber's method). Ulcers were induced using the absolute ethanol and hydrochloric acid experimental model. Various biochemical parameters such as the: MDA, Catalase, Glutathione, Pepsin, SOD, ASAT, ALAT, Creatinine, XO, and total proteins, were quantified. Ulcer preventive and curative properties of three doses (12.5, 25 & 50 mg/Kg) of the extract were compared with a positive control (Sucralfate 25 mg/Kg) and a negative control Histological studies of the stomach were conducted, after samples were exposed to herbal products.

Results: Phytochemical screening of the *A. indica* aqueous leaf extract showed the presence of mucilage, tannins specifically catechin, flavonoids, total polyphenols, coumarins and phlobotannins. The leaf extract tested positive for ferric, iodide, carbonate, sulphite ions, as well as proteins. These bioactive molecules showed promising antiulcer and antioxidant properties. The neem leaf extract (NLE) fulfilled the FDA conditions for an antacid, had a capacity to buffer an acid milieu for about 40 minutes and had a neutralization capacity well within the designed pH range of 3.5 – 5. The preventive and curative studies showed significant reduction in the gastric juice and ulcer surface. A percentage inhibition of 71.27 in the preventive studies and percentage regeneration of 99.33 for curative studies was obtained from rats dosed at50 mg/Kg body weight.

Conclusion: This study showed that the aqueous extract of *Azadirachta indica* leaves had a promising gastro-protective and gastric healing activities in rats at 50 mg/Kg.

Keywords: Azadirachta indica; aqueous extract; antacid; gastric ulcer; phytochemicals; bioactive molecules.

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 [1-4]. 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 [2,5].

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 [5-7]. 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 [7-10].

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 [11] 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 [12-14].

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 [15]. 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 [16-18].

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 [7,19] 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 [20-23]. The neem plant (*Azadirachta indica* A. Juss) is a treethat is common in the Northern parts and sparsely distributed in the Northwest Region of Cameroon. It is used as a remedy for several pathologies, amongst which we have gastric ulcers which is our area of interest.

To phytochemically screened bioactive metabolites and investigate *in vitro* and *in vivo* the antiulcer & antacid activity of the aqueous leaf extract of *Azadirachta indica* in *Wistar* albino rats.

2. METHODOLOGY

2.1 Source of Plant Materials

Harvesting and identification of plant leaves: The fresh leaves were harvested from Ndop, capital of the Ngo Ketunjia, Division of the Northwest Region of Cameroon. Plant was identified during harvesting by an ethnobotanist and transported for taxonomic identification and authentication at the National Herbarium of Cameroon as *Azadirachta indica* A. Juss, with a sample voucher code 4447/SRFK.

2.2 Study Site and 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. This study was carried out from 6th November 2017 to May 2018.

2.2.1 Plant material drying of leaves

The leaves were cleaned and dried in a shade, on mats placed on a flat surface. The dried leaves were pulverized to get fine powder and stored in airtight containers before extraction.

2.2.2 Extraction of leaf powder

In this process, 1500g 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.

Percentage yield = (Mass of extract obtained /Mass of powder initially used) X100

2.2.3 Phytochemical screening

Several research papers have proven the presence of diverse functional groups in the

Neem plant. In the scope of our research, we carried out phytochemical tests on alkaloids, carbohydrates, steroids, terpenoids, cardiac glycosides, saponins, betacyanide, tannins, proteins, alkaloids, resins and flavonoids.

2.3 Molisch's Test for Carbohydrates

A few drops of Molisch's reagent were added to the extract dissolved in distilled water. Afterwards, 1 ml of conc. H_2SO_4 was added by the side of the test tube. The mixture was allowed to stand for two minutes and then diluted with 5 ml of distilled water. Formation of a red or dull violet color at the interphase of the two layers indicated a positive test [11].

2.3.1 Identification test for betacyanide

In a test tube, 2 mL of the extract was added with. 2 mL of 2N NaOH and heated the tube in a hot water bath at 100°C for 5 minutes. The appearance of a yellow coloration indicated the presence of betacyan [12].

2.3.2 Identification test for coumarins

In a test tube containing 1 mL of the plant extract and 1 mL of distilled water, we added a few drops of 10 % $FeCl_3$. Obtaining a green or blue coloration that turns yellow by addition of nitric acid (HNO_3) indicated the presence of coumarins [12].

2.3.3 Identification test for tannins

About 0.5g of the aqueous extract of *A. indica* was stirred with about 10ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate occurrence of a blue-black, green or blue-green precipitate indicated the presence of tannins [13].

2.3.4 Differentiation of catechic and gallic tannins

It is obtained by STIASNY reaction, which was carried out as such; to 30mL of infused solution, was added 15mL of STIASNY reagent (10mL of 40 % formalin and 5 mL of concentrated HCl) and heated for 15 minutes in a water bath at 90 °C. The formation of a precipitate indicate the presence of Catechin . After filtration, we saturated the filtrate with powdered sodium acetate, then 1 mL of a solution of 1 % ferric perchloride ($FeCl_3$). The presence of gallic tannins not previously precipitated by the STIASNY reagent was indicated by the development of a shade dark blue [14].

2.3.5 Borntrager's Test for anthraquinones

About 0.2 g of each portion to be tested was shaken with 10 ml of benzene and then filtered. Five milliliters of the 10% ammonia solution were added to the filtrate and thereafter homogenized. Appearance of a pink, red or violet color in the ammoniacal (lower) phase signified the presence of free anthraquinones [15].

2.3.6 Liebermann-burchard test for steroids

To 0.2g of the extract 2ml of acetic acid was added, the solution was cooled in ice followed by the careful addition of conc. H_2SO_4. A color appearance from violet to blue or bluish-green indicated the presence of a steroidal ring i.e. aglycone portion of cardiac glycoside [13].

2.3.7 Identification test for terpenoids

0.2 g of each extract was dissolved in ethanol. To it (the aqueous extract), 1 ml of acetic anhydride was added followed by the addition of concentrated H_2SO_4. A change in color from pink to violet showed the presence of terpenoids [16].

2.3.8 Identification test for saponins

5 mL of 1% of the plant extract was put in a test tube and violently shaken for about 30 seconds. The test tube was then allowed to stand. If the foam persisted for up to 15 minutes, it indicated the presence of saponins. If the foam was ≥ 1cm, it indicated an abundance of saponins [17].

2.4 Tests for Flavonoids

2.4.1 Shinoda's test for flavonoids

About 0.5g of each portion of plant extract is dissolved in ethanol, warmed and then filtered. Three pieces of magnesium chips will be added to the filtrate followed by few drops of conc. HCl. A pink, orange, or red to purple coloration indicates the presence of flavonoids [18].

2.4.2 Ferric chloride test for flavonoids

To a test tube containing 1 mL of the extract, a few drops of 10% ferric chloride solution were added. A green-blue or violet coloration indicates the presence of a phenolic hydroxyl group [13].

2.4.3 Lead ethanoate test for flavonoids

A small mass of the each portion is dissolved in water and filtered. To 5ml of each of the filtrate, 3ml of lead ethanoate solution is then added. Appearance of a buff-colored precipitate indicates the presence of flavonoids [19].

2.4.4 Sodium hydroxide test for flavonoids

A small quantity of the each portion is dissolved in water and filtered; to this 2 ml of the 10% aqueous sodium hydroxide is added to produce a yellow coloration. A change in color from yellow to colorless upon addition of dilute hydrochloric acid is an indication for the presence of flavonoids [13].

2.4.5 Identification test for alkaloids

A small quantity of the each portion was stirred with 5ml of 1% aqueous HCl on water bath and then filtered. From the filtrate, we put 1 mL into 2 test tubes. To the first portion, few drops of Dragendorff's reagent were added; occurrence of orange-red precipitate was taken as positive. To the second 1 mL, Mayer's reagent was added and appearance of buff-colored (brownish yellow) precipitate will be an indication for the presence of alkaloids [20].

2.4.6 Identification test for soluble starch

A small quantity of each portion was boiled with 1ml of 5% KOH, cooled and acidified with H_2SO_4. A yellow coloration was considered as the presence of soluble starch [21].

2.4.7 Identification test for resins

In a test tube containing 1 mL of the extract, we added a few drops of solution of anhydrous acetic acid and 1 mL of sulfuric acid (H_2SO_4) The appearance of a yellow color indicated the presence of resins [21].

2.5 Oxalate Identification Test

In a test tube, we put 1 mL of the 1 % extract then added a few drops of ethanoic acid. Obtaining a greenish-black color indicated the presence of oxalates [21].

2.5.1 Identification test for phlobotannins

To 1 mL of the plant extract in a test tube, we added a few drops of hydrochloric acid. This mixture was put in a water bath containing water at 100°C and heated for 10 minutes. The appearance of a red precipitate indicated the presence of phlobotannins [12].

2.5.2 Identification test for genins

1 mL of the plant extract was put in a test tube. To this extract, hydrochloric alcohol (HCl + H_2O + EtOH). The appearance of a white precipitate indicated that the extract contained genins [15].

2.5.3 Preparation of animal material

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

2.5.4 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.

2.5.5 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 libitum*.

2.5.6 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.

2.5.7 Study procedure

The *Wistar* rats were divided into six groups of 5 animals each:

- The first group consisted of rats that did not receive ulcerogenic substances (sham group)
- The second group was made of rats that received ulcerogenic substances without pretreatment with leaf extract
- The third, fourth and fifth groups received pretreatments of the NLE at progressive doses then an ulcerogenic substance
- The sixth received the ulcerogenic substance after pretreatment with an existing antiulcer drugs, as a comparative model

2.6 *In vitro* Antiulcer activity

2.6.1 Acid neutralizing capacity (ANC)

This method was adopted from the USP 29 guide for measurement of the ANC. The one mentioned here is unadulterated. The acid neutralizing capacity (ANC) of an antacid is the amount of acid that it can neutralize. This ANC was best measured in the laboratory by a process known as back titration. That consisted of dissolving the antacid in an excess of acid and then titrating the acidic solution against a known concentration of base until the endpoint is reached. The concentration of acid neutralized equals the difference between the concentration of acid added and the concentration of base required for the back titration [22].

For this investigation:

$$\boldsymbol{moles\ of\ acid\ neutralized} = \boldsymbol{moles\ of\ HCl\ added - moles\ of\ NaOH\ required}$$

$$*= (\boldsymbol{Volume_{HCl}\ x\ Molarity_{HCl}}) - (\boldsymbol{Volume_{NaOH}\ x\ Molarity_{NaOH}})$$

$$\boldsymbol{acid\ neutralizing\ capacity\ per\ gram\ of\ antacid} = \frac{\boldsymbol{moles\ of\ HCl\ neutralized}}{\boldsymbol{grams\ of\ antacid}}$$

After completing this laboratory investigation, the acid neutralizing capacity of an antacid was determined and the acid neutralizing capacity of various antacids were compared.

In the procedure 4 antacids were chosen for investigation. Their names and amount of the active ingredients in each antacid were recorded. The ring stand was used with the double burette clamp and two burettes to set up the titration apparatus. One burette was labeled as acid and the other as base. A 250 mL beaker was placed under each burette tip. Then 5-10 mL of 0.15 M HCl was added to the acid burette to rinse the burette and drain into the 250 mL beaker. The same process was completed with the base burette using the unknown concentration of NaOH, then the acid and base was discarded [23].

An antacid tablet was obtained and its mass measured and recorded in the data table. With a mortar and pestle, the antacid tablet was crushed and a clean 125 mL Erlenmeyer flask was placed on the electronic balance and zero or tare the balance. Approximately 0.25 grams of the antacid tablet was added to the 125 mL Erlenmeyer flask and the mass recorded and added to the flask in the data table.

The acid burette was filled with the 0.15 M HCl carefully until the acid was above the zero mark. The acid was dispensed from the burette into a discard beaker until all air was removed from the burette tip and the level of acid was within the graduated portion of the burette. The burette level was recorded as the initial burette reading of HCl. The bottom of the meniscus for the burette level reading was used. The same process was completed with the base burette using the 0.2 M NaOH.

Into the 125 mL Erlenmeyer flask containing the powdered antacid, was dispensed approximately 25 mL of the acid. Approximately 50 mL of deionized water was added and 3-5 drop of phenolphthalein solution to the flask and swirled to mix, then allowed to stand for 10 minutes. Titration was done slowly by adding the base into the flask containing the acid with stirring until a pink color started to persist in the beaker. The flow of base was decreased to a slow drop by drop process and continued until the pink colour persisted for more than a few seconds. In case the titration endpoint was overshot, acid drop was slowly added by drop until the pink colour disappeared. Then the base was added again

drop by drop until a faint pink colour persisted. The titration process was repeated until one drop of base caused the faint pink colour to remain. The final acid and base burette readings were recorded in the data Table 1. The same steps was repeated with three more different antacids.

2.7 Data Analysis

1. Calculate the volume of HCl dispensed and NaOH required from each trial and place in the calculations table.
2. Using the molarity formula, calculate the moles of HCl dispensed in each trial and place in the calculations table.
3. Calculate the moles of HCl neutralized by the antacid.
4. Calculate the neutralizing capacity of the antacid per gram of antacid.
5. Calculate the neutralizing capacity of the antacid per tablet of the antacid.

2.7.1 Determination of the buffer capacity

The buffer capacity was determined according to the recommended method of Holber et al [24]. A quantity of 0.5g of powder of each sample was put into 25ml of 0.1N HCl contained in a 50 ml beaker and subjected to constant stirring the magnetic stirrer. The pH of the mixture was determined at intervals of 0.5, 2, 4, 6.8 and 10 minutes. Then an amount of 5 ml of the mixture was removed using a pipette and replaced with 5ml of HCl 0.1N. This process was repeated at 10-minute intervals until a pH below 2.75 was attained, which showed that the buffering capacity of the antacid had been exhausted [25].

2.7.2 Food and drug administration (FDA) trials on antacids

The FDA defines antacids according to the minimum buffering capacity. To be considered an antacid, a molecule must contribute to 25% of the product's total neutralization. A 0.25g of a sample (plant extract and standard antiulcer) was weighed and added 2.5 ml of HCl 0.5N and 10 ml of distilled water. The mixture was homogenized for 10 minutes on a magnetic stirrer and the pH obtained. For each sample, the procedure was repeated five times. A final pH between 3 and 5 qualified the sample as an antacid, according to FDA.

2.8 *In vivo Antiulcer* Activity

2.8.1 Preparation of test solutions

2.8.1.1 Preparation of A. indica aqueous leaf solution

A solution of concentration 40 mg/mL was prepared for the preventive and curative studies. In total, of 70 mL for both studies, using 17.5 mL for preventive studies and the rest for curative studies. This means the mixture was obtained from 280 mg of extract and 70 mL of distilled water. The solution was administered the animals undergoing curative studies for three days consecutively. 0.5 mL, 1 mL and 2 mL of the solution were administered to groups taking 12.5 mg/kg, 25 mg/kg and 50 mg/kg respectively, according to their different weights [26].

2.8.1.2 Preparation of reference drug solution, Sucralfate

A solution of 10 mg/mL was prepared. This therefore implies that 15 mL of solution were obtained from 150 mg of Sucralfate tablets. For each study, preventive and curative studies, the positive control animals were administered 7.5mg/kg of the Sucralfate solution. Each study consumed equal amounts of standard drug solution.

Table 1. Titration process: Final acid and base Burette readings Molarity of HCl solution Molarity of NaOH solution

	Antacid 1	Antacid 2	Antacid 3	Antacid 4
Name of antacid				
Name and mass of active ingredients				
Mass of antacid tablet (g)				
Mass of antacid added to flask (g)				
Initial burette reading for HCl (mL)				
Final burette reading for HCl (mL)				
Initial burette reading for NaOH (mL)				
Final burette reading for NaOH (mL)				

2.8.1.3 Preventive and curative activity of Azadirachta indica aqueous leaf extract

30 Wistar rats were used for each of these experiments, weighing 100-180 g. The rats were purchased from the FMBS Animal House and left to acclimatize to the laboratory conditions for 5 days, and given free access to water and food. The rats were separated into 6 groups of five animals each, per study.

2.8.2 Induction of ulcers

Healthy animals were selected for this study and allowed to acclimatize with the laboratory conditions for 5 days. The animals were randomly grouped into 6 groups of 5 animals each animal was subjected to a fasting period of 48hrs. After this period, the first group, the negative control group, received only a vehicle (water). The second, third and fourth groups received specified doses of the NLEa, 12.5, 25 and 50 mg/kg respectively. The fifth group, the positive control group, received a standard or already approved drug, Sucralfate 25mg/kg. The sixth group, the sham, received nothing. One hour after oral administration, all animals except those of the sixth group were given EtOH/HCl, in order to induce gastric ulcers. One hour after the EtOH/HCl administration, all animals were sacrificed using an overdose of ether. The stomachs were extracted, opened along the greater curvature and rinsed with a 0.9% NaCl solution. The ulcer index was determined by the method described by Tan et al [27].

2.8.3 Determination of ulcer index

Three days after induction of ulcers, the animals were sacrificed using an overdose of ether. The stomachs were extracted and observed for ulcers in the glandular and non-glandular regions. The ulcer dimensions were measured in mm by tracing the wound boundaries on a transparent paper. We later calculated the surface areas of the ulcers. The surface area of each lesion was measured and scored as described by Tan et al [27]. The ulcer index for each rat was taken as the mean score. The percentage ulcerated surface was calculated as the total area covered by lesions and expressed as a percentage of the total corpus mucosal surface area as described by Nguelefack et al, [28] the percentage curative ratio (% CR) or percentage inhibition (%I) of ulcer will be calculated using the formula:

$$\%\,\mathrm{I} = \frac{(USc - USt).100}{USc}$$

Where, *USc* = ulcer surface area in control
USt = ulcer surface area in test/treated animals.

2.8.4 Quantification of biochemical parameters in gastric juice and homogenates

2.8.4.1 Pepsin

Pepsin is used as a biomarker for the integrity of the gastric mucosa. In an acid medium, it hydrolyzes the peptide bonds of the proteins which contain the aromatic amino acids to give the polypeptides which, in the presence of the Folin reagent, give a violet blue complex which exhibits a maximum absorption at 660 nm. The intensity of the staining is proportional to the amount of polypeptide present in the solution [29, 30].

2.8.4.2 Catalase

The catalase assay was performed according to the method described below.

Hydrogen peroxide is disrupted in the presence of catalase. This destroyed peroxide binds to potassium dichromate to form a green blue precipitate of unstable perchloric acid which will then be decomposed by heat and form a green complex that absorbs at 570 nm. The activity of the catalase which is proportional to the optical density will be determined by means of a calibration curve. A 0.9 ml of phosphate buffer (0.01M, pH 7) and 0.4 ml of H2O2 are introduced into each tube to initiate the reaction. The reaction is interrupted after 30 seconds by the introduction of 2 ml of dichromic acetic acid. The whole is heated at 100 °C for 10 minutes. After cooling, the optical density is read at 570 nm. The amount of hydrogen peroxide remaining in the solution after addition of the perchloric acid is evaluated using the calibration curve (represented in table IX) [1,15]. The specific activity of catalase was expressed in µM H_2O_2 /min/mg protein [31].

2.8.4.3 Reduced glutathione assay

The study was conducted according to the method described. here in. The 2, 2-dithio-5,5'-dibenzoic acid (DTNB) reacts with the SH groups of the glutathione to form a yellow colored complex which absorbs at 412 nm according to the following principle, 0.02 mL of the homogenate 10 % of the stomach and 3 mL of the Ellman reagent were introduced into a test

tube. The Ellman reagent was prepared by dissolving 4.96 mg of 5,5'-dithio-bisnitrobenzoic acid (DNTB) in 250 ml of 0.1 M phosphate buffer (pH 6.5). After vortexing, the coloration was allowed to develop for 60 minutes at ambient temperature. 0.02 mL of phosphate buffer, pH 0.1M and 3 mL of Ellman's reagent were placed in the control tube. The optical density was read at 412 nm against the white and the concentration of the reduced glutathione was calculated using a molar extinction coefficient ε = 13600 / mole.cm [31].

2.8.4.4 Malondialdehyde (MDA)

Carbonyl compounds such as malondialdehyde from the decomposition of fatty acid hydroperoxides react with thiobarbituric acid (TBA) to give pink chromophores whose concentration was determined by reading the absorbance at 532 nm.

We pipetted into test tubes, 100 µL of sample, 2 mL of reagent (TCA-TBA-HCL mixture) and sealed tightly. The mixture was heated in the water bath at 100 °C for 15 minutes. This was then cooled in a cold water bath for 30 minutes leaving the tubes open. Centrifugation was at 3000 rpm for 5 minutes and the absorbance of the supernatant read at 532 nm. The concentration of MDA was determined using its molecular extinction coefficient (ε = 1.56 105 M-1cm-1). The results were expressed in µmol/L.

2.8.4.5 Superoxide dismutase (SOD)

Principle Adrenaline (epinephrine), in the presence of the superoxide anion O2, is oxidized spontaneously to adrenochrome; A colored compound which absorbs at 490 nm. SODs, whose role is to reduce the O2 anion, inhibit this reaction. The procedure is described in Table 2.
Expression of results as:

$$\text{inhibition } \% = \frac{100 - \Delta A\ sample}{\Delta A\ White}\ x\ 100$$

The specific activity of SOD is evaluated in units of SOD/mg of protein. A unit of SOD is defined as the amount of SOD required to cause an inhibition of 50 % of the oxidation of adrenaline to adrenochrome for one minute.

2.8.5 Free mucous

The quantification of free mucous as described in Table 3.

2.8.6 Proteins quantification

This was done using sample solutions described in Table 4.

2.8.7 Xanthine oxidase

The quantification of xanthine oxidase has been described in the Table 5.

3. RESULTS

3.1 Extraction Yield

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

3.2 Phytochemical Screening

Phytochemical screening of the *A. indica* aqueous leaf extract showed the presence of mucilage, tannins specifically catechin, flavonoids, total polyphenols, coumarins and phlobotannins (Table 6). The leaf extract tested positive for ferric, iodide, carbonate, sulphite ions, as well as for proteins (Table 7).

3.3 *In vitro* Antacid Activity

3.3.1 Acid neutralization capacity and pH of samples

The pH of the different samples tested was greater or equal to 6.20. $NaHCO_3$ had the highest pH, 8.94, while Mg $(OH)_2$/Al $(OH)_3$ had the lowest pH (Table 8). At 0.25g, our leaf extract was shown to have a neutralization capacity averagely of 6.07 mEq. The extract showed a higher ANC than $CaCO_3$/$MgCO_3$ and Al $(OH)_3$, $Mg_2O_8Si_3$, Mg $(OH)_2$ Sim. Rennie® (the Al $(OH)_3$, $Mg_2O_8Si_3$, Mg $(OH)_2$ + Sim combination) presented the lowest ANC.

3.3.2 Buffer capacity

Our experiment showed that the Mg $(OH)_2$/Al $(OH)_3$ (Maalox) had the most durable buffer capacity, taking about 85 minutes for its pH to drop below 2.75. Our NLEa at 1g, took about 40 mins before it lost its buffering capacity (Table 9).

3.3.3 FDA test of antacids

At 0.25g, the leaf extract gave a pH less than the expected range (3≤pH≥5) but when a dose of 0.5g was used, the results fell well within the range.

Table 2. Description of the SOD quantification

Put in the cuvette	Sample	White
Sample	134 µL	
Carbonate buffer	1666 µL	1666
Adrenaline	0.2 mL	0.2 mL
Distilled water		134 µL

The mixture is homogenized by rapid inversion of the cuvette. Read the variation of the absorbance at the 20th and the 80th second at 480nm

Table 3. Description of the free mucus quantification

Test tube content	Sample volume	White/Blank
Gastric juice	250 µL	-
Phosphate citrate mac vains buffer	825 µL	825 µL
Alcian blue	50 µL	50 µL
Distilled water	125 µL	375 µL

The test tube was incubated for a period of 24 hours at room temperature, then centrifuged at 2000 turns per minute and after which the supernatant was withdrawn and the optical density read at 615nm against the blank.

Table 4. The quantification of proteins is described in the table

Put in the test tubes	Sample	White
Sodium hydroxide 0.1N	190 µL	200 µL
Gastric juice	10 µL	/
Solution C	1000 µL	1000 µL
Incubate for 10 minutes		
Solution D	100 µL	100 µL
The test tubes are vortexed		

Incubate the test tubes for a period of 30 minutes at room temperature under shade, then read the optical density at 600 nm against the White

Table 5. Description of the Xanthine Oxidase quantification

Test tube content	Sample	White/Blank
Tris HCl buffer 50mM pH 7.4	300 µL	300 µL
Copper II sulphate in 10mM	300 µL	300 µL
Xanthine solution 2.6 mM dissolved in glycine 0.05 pH 7.4	50 µL	50 µL
Distilled water	250 µL	300 µL
Stomach homogenate or gastric juice	50 µL	

The reaction was initiated by the addition of the homogenate and the increase in absorbance was observed and noted at 290 nm at the 15 seconds intervals for a period of 2 min. The activity was obtained from the molar extinction coefficient of 1.22.

Table 6. Identification of secondary metabolites

Secondary metabolites	Tests or reagents used	results
Mucilage	Absolute ethanol	+++
Saponins	Vigorously shake and allow to stand for 15 mins	-
Total polyphenols	$FeCl_3$ 10%	+++

Secondary metabolites	Tests or reagents used	results
	Lead acetate	
Tannins	Cu citrate	+++
Alkaloids	Wagner	-
	Mayer	-
	Hager	-
Catechic tannins	STIASNY	+++
Gallic tannins	FeCl3	-
	Sodium acetate	
Anthocyans	H2SO4	-
	NaOH	
Genins	Hydrochloric alcohol (H2O/HCl/EtOH)	-
Betacyans	NaOH and heat	-
Phlobotannins	HCl	++
Steroids	Acetic acid	-
	H_2SO_4	
Carbohydrates	H_2SO_4 + distilled H_2O	-
Resins	Acetic acid	-
	H_2SO_4	
Coumarins		++
Oxalates		-
Quinones		-
Cardiac glycosides	Acetic acid	-
	$FeCl_3$	
	H_2SO_4	

Table 7. Identification of ions

Ions	Reagents	Results
Al^{3+}	NaOH	-
Zn^{2+}		-
Fe^{3+}		+++
Fe^{2+}		-
I^-	HNO_3 + Lead acetate	+++
$(CO_3)^{2-}$	HNO_3	+++
SO_3^{2-}	Potassium dichromate	+++
Cl^-		
Br^-		
ClO^-		
N		
Lipids	Ethanol	-
Proteins	NaOH + Cu	+++

Key: *+ Mildly present; ++ fairly present; +++ abundant; - absent*

Table 8. pH and ANC of samples tested at 0.25g

Test samples	pH of samples	ANC
Neem leaf extract	6.78 ± 0.14	6.07 ± 0.7
$NaHCO_3$	8.94 ± 0.09	6.37 ± 0.18
$Al(OH)_3$, $Mg(Si)_3$, $Mg(OH)_2$ Sim	7.3 ± 0.18	5.58 ± 0.45
$CaCO_3/MgCO_3$	6.27 ± 0.19	5.85 ± 0.12
$Mg(OH)_2/Al(OH)_3$	6.20 ± 02	8.07 ± 0.38

3.3.4 FDA minimal neutralization capacity of the different test substance

The Table 9 is a demonstration of the capacity of different samples to maintain the pH of a milieu

at or above 3, in order to completely neutralize the acid solution. The plant at 0.25g, GESTID and RANITIDINE had pH < 3 while the rest (Maalox, Rennie, Sodium Bicarbonate and NLEa at 0.5g) had pH > 3 (Table 10).

3.4 *In vivo* Activity

3.4.1 Preliminary studies

Various doses of the NLEa were tried out for the preventive and curative studies, to identify the dose with optimal biological activity; we used 12.5, 25, 50, 100, 200, 400 mg/kg. The best results were gotten at the 25 mg/kg dose.

3.5 Preventive and Curative Activity

There was significant differences in ulcer surface, gastric juice and free mucous in preventive activity between the control and treatment group. Plant extract at 25mg/kg showed ulcer surface of 4.69mm^2 comparable to Sulcralfate 5.98mm^2 (Fig. 2).

In terms of ulcer surface, volume of gastric juice showed significant positive effect compared to the control as indicated in Fig 3.

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 [17,32]. In our search for the biological activity with respect to peptic ulcers, we examined the aqueous leaf extract of *A. indica* harvested from the Northwest region of Cameroon, specifically from Ndop. The ulcers were induced by using absolute ethanol. Ethanol readily penetrates the gastric mucosa due to its ability to solubilize the protective mucous and expose the mucosa to the proteolytic and hydrolytic actions of hydrochloric acid and pepsin causing damage to the membrane. Moreover, it stimulates acid secretion and reduces blood flow leading to microvascular injuries, through disruption of the vascular endothelium and facilitating vascular permeability [33-35].

According to this study, the phytochemical screen of aqueous leaf extract of *A. indica* showed the presence of several metabolites, commonly known as phytochemicals. We found mucilage, total polyphenols, tannins, catechic tannins, phlobotannins and coumarins. These results obtained are similar to those report in earlier studies[4,36] but different from that found by Dash *et al,* who found alkaloids, saponins, glycosides and reducing sugars [7,37]. This discrepancy could have resulted from the fact that in earlier studies different reagents were used from what we used. Research shows that mucilage possesses beneficial effects on burns, wounds, ulcers, external and internal inflammations and irritations [38,39,40,37], hence contributing to the preventive and curative properties of *A. indica*. Flavonoids as well as total polyphenols have been shown to possess antioxidant properties [12-15, 41]. Flavonoids equally increase the blood circulation and inhibit COX enzymes, thus inhibiting the inflammatory process of the body [42]. Phlobotannins have been reported to possess wound healing, anti-inflammatory, antioxidant and analgesic activities [21,43]. All these activities from the various phytochemicals may have contributed to the antiulcer properties of *A. indica* aqueous leaf extract.

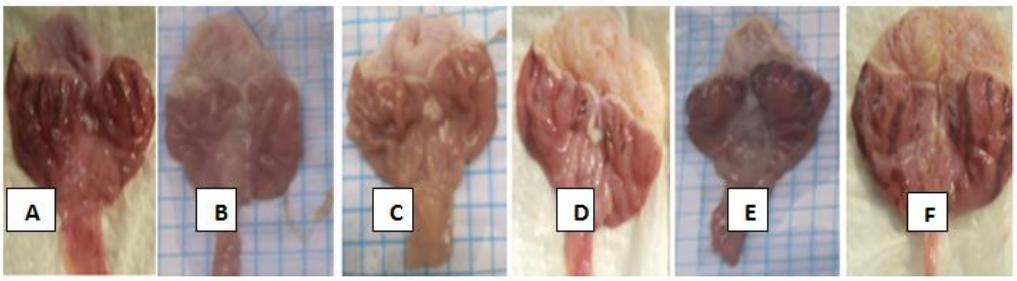

Fig. 1. Images of preliminary studies A = 12.5mg/kg; B = 25mg/kg; C = 50mg/kg; D = 100mg/kg; E = 200mg/kg; F = 400mg/kg

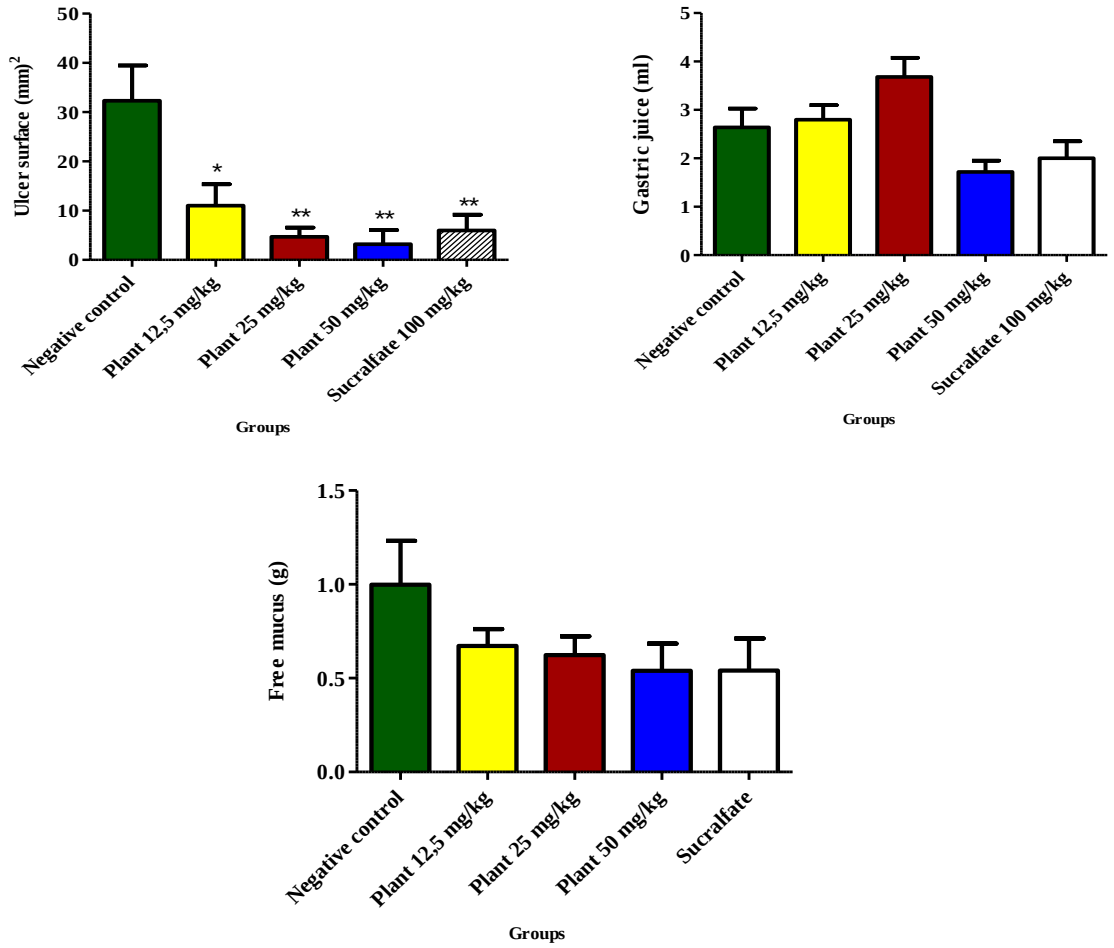

Fig. 2. Preventive activity of free mucus at different plant extract treatments Curative activity

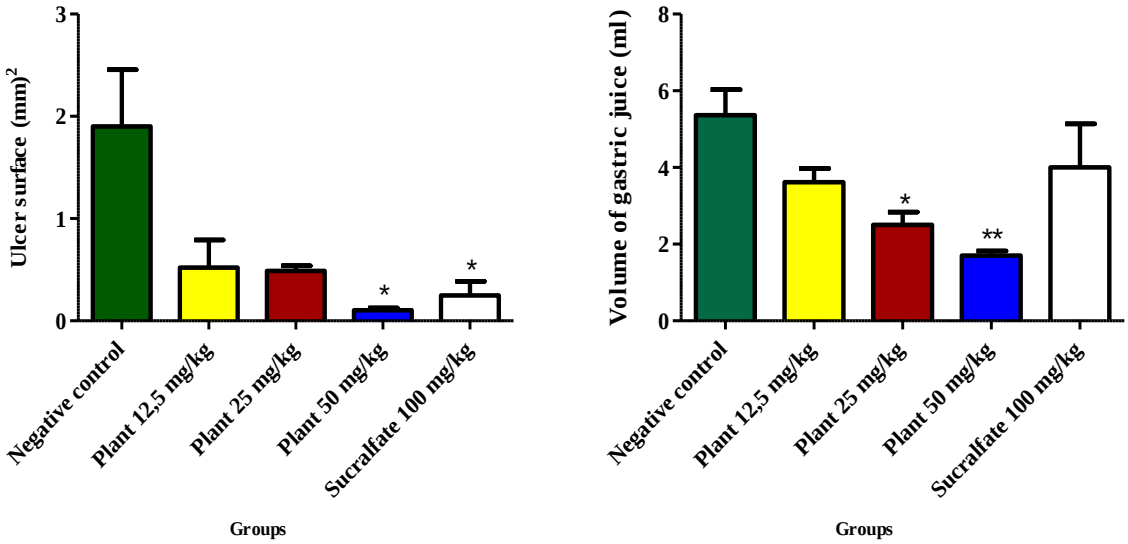

Fig. 3. Curative activity of ulcer surface at different extract treatment groups

Table 9. Time necessary for the pH of a sample to drop below 2.75

Times	NLEa	Buffer Capacity (Time Taken to Attain PH < 2.75)				
		$C_{13}H_{22}N_4O_3S$ (Ranitidine®)	$NaHCO_3$ (Sodium bicarbonate®)	$Al(OH)_3$, $Mg(Si)_3$, $Mg(OH)_2$ Sim (Gestid®)	$CaCO_3$/ $MgCO_3$ (Rennie®)	$Mg(OH)_2/Al(OH)_3$ (Maalox®)
1	30-40	<10	60-70	10	80-90	80-90
2	30-40	<10	50-60	40-50	60-70	70-80
3	30-40	<10	50-60	40-50	60-70	90-100
4	30-40	<10	50-60	30-40	60-70	70-80
5	30-40	<10	50-60	40-50	50-60	80-90

Table 10. The FDA minimal neutralization capacity of the different test substance

FDA test	pH after 10 minutes
GESTID®	2.75 ± 0.56
NLEa at 0.25g	2.54 ± 0.09
RANITIDINE®	1.77 ± 0.06
MAALOX®	4.61 ± 0.08
NLEa at 0.5g	4.57 ± 0.16
RENNIE®	7.04 ± 0.33
SODIUM BICARBONATE®	9.25 ± 0.1

There are several mechanisms of action involved in the reduction of acid in the stomach. These mechanisms involve stimulation of the body's natural defense processes like mucus production or prostaglandin, ionic neutralization of acid topically, inhibition of acid secretion or elimination of *H. pylori*, in the case of bacterial infection. According to this study, the NLEa was shown through the FDA antacid test to possess antacid properties at 0.5g, as opposed to the demanded 0.25g. This could be because we worked on an extract and not on fractions or pure compounds.

5. CONCLUSION

Results obtained from the phytochemical screening of the *A. indica* aqueous leaf extract showed the presence of mucilage, tannins specifically catechin, flavonoids, total polyphenols, coumarins and phlobotannins. Also, the leaf extract was shown to possess ferric, iodide, carbonate, sulphite ions, as well as proteins. This study showed that the aqueous extract of *Azadirachta indica* leaves had a promising gastro-protective and gastric healing activities in rats at 50 mg/Kg.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Ethical approval was obtained from the institutional review board of the Faculty of Medicine and Biomedical Sciences (Ref No: 008/UY1/FMSB/VDRC/CSD). Authorization was obtained from the administration of the FMBS, to work in the animal house of this faculty. The OECD Guidelines 423, for the use of animals in preclinical studies was applied for the acute toxicology studies.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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