ARTICLE | OPEN ACCESS

Drug Discovery

Sub-Chronic Toxicity and Anti-Ulcer Activity of Hydroethanolic Extract of *Vernonia amygdalina* in Rodent Models

Mega Obukohwo Oyovwi[1,2*], Eseroghene Cecillia Adegor[3], Ejayeta Jeroh[1], Rotu Arientare Rume[2]

To Cite:
Oyovwi MO, Adegor EC, Jeroh E, Rume RA. Sub-Chronic Toxicity and Anti-Ulcer Activity of Hydroethanolic Extract of *Vernonia amygdalina* in Rodent Models. *Drug Discovery* 2026; 20: e12dd3077
doi:

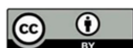
Author Affiliation:
[1]Department of Human Physiology, Faculty of Basic Medical Sciences, Delta State University of Science and Technology, Ozoro, Delta State, Nigeria
[2]Department of Science Laboratory Technology, Delta Central College of Management and Sciences, Ughelli, Delta State, Nigeria
[3]Department of Pharmacology, National Open University, Abuja, Nigeria

[*]Corresponding Author:
Mega Obukohwo Oyovwi (PhD), Department of Human Physiology, Faculty of Basic Medical Sciences, Delta State University of Science and Technology, Ozoro, Delta State, Nigeria; E-mail: megalect@gmail.com; Tel: +2348066096369

Peer-Review History
Received: 24 February 2025
Reviewed & Revised: 07/May/2025 to 03/April/2026
Accepted: 15 April 2026
Published: 30 April 2026

Peer-Review Model
External peer-review was done through double-blind method.

Drug Discovery
pISSN 2278–540X; eISSN 2278–5396



© The Author(s) 2026. Open Access. This article is licensed under a Creative Commons Attribution License 4.0 (CC BY 4.0)., which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

DISCOVERY SCIENTIFIC SOCIETY

ABSTRACT

This research assessed the sub-chronic toxicity (90-day) and anti-ulcer efficacy of the hydroethanolic leaf extract of *Vernonia amygdalina* in rodent models. For toxicity testing, Wistar rats were given daily doses of 0, 250, 500, or 1000 mg/kg for 90 days. There were no deaths at any dose. The No Observed Adverse Effect Level (NOAEL) was set at 500 mg/kg. At 1000 mg/kg, there were small increases in serum liver enzymes (ALT, AST, ALP), creatinine, and blood urea nitrogen. Histopathology showed mild hepatocellular necrosis and renal tubular degeneration. For the assessment of anti-ulcer efficacy, Swiss albino mice were administered the extract (100–400 mg/kg) or omeprazole (20 mg/kg) for seven days prior to ulcer induction via ethanol, indomethacin, or pylorus ligation models. The extract at 400 mg/kg showed strong gastroprotection, with 86.3% protection against ulcers caused by ethanol ($p < 0.001$), 74.2% protection against ulcers caused by indomethacin ($p < 0.01$), and antisecretory effects that included a 58.4% reduction in gastric juice volume and an increase in gastric pH from 2.1 to 4.2 in the pylorus ligation model. These effects were similar to those of omeprazole. Phytochemical analysis showed that the contents of phenolic (124.5 mg/g) and flavonoid (67.8 mg/g) were very high. The results confirm the historical application of *Vernonia amygdalina* for gastric ailments and demonstrate a positive safety profile after multiple doses. The extract demonstrates multi-mechanistic anti-ulcer activity through antioxidant, cytoprotective, and antisecretory mechanisms. Additional mechanistic investigations and clinical trials are necessary.

Keywords: *Vernonia amygdalina*; anti-ulcer efficacy; sub-chronic toxicity; gastric ulcer; rodent models; phytomedicine; NOAEL

1. INTRODUCTION

Peptic ulcer disease is a major health problem around the world, affecting about five to ten percent of people at some point in their lives (Lanas and Chan, 2017). This condition is characterized by the erosion of the mucosal lining of the stomach or duodenum, resulting from an imbalance between aggressive factors such as gastric acid, pepsin, and *Helicobacter pylori* infection and defensive factors including mucus secretion, bicarbonate production, prostaglandins, and mucosal blood flow

(Malfertheiner et al., 2009). The pathophysiology of peptic ulcer disease is multifactorial, involving oxidative stress, inflammation, impaired mucosal defense mechanisms, and dysregulation of gastric acid secretion (Wallace, 2008).

Conventional pharmacotherapy for peptic ulcer disease primarily relies on proton pump inhibitors, histamine H_2 receptor antagonists, antacids, and antibiotics for *H. pylori* eradication (Scally, 2018). Long-term use of these drugs is linked to a number of restrictions and negative consequences, despite the fact that they are generally effective. Proton pump inhibitors have been associated with higher risks of enteric infections, dementia, chronic renal disease, and vitamin B12 insufficiency (Freedberg et al., 2017). Furthermore, the recurrence of ulcers following drug discontinuation, drug-drug interactions, and the emergence of antibiotic-resistant *H. pylori* strains have prompted the search for safer, more affordable, and therapeutically effective alternatives from natural sources (Yuan et al., 2016).

In many traditional medical systems, medicinal plants have long been used to treat gastrointestinal ailments. According to estimates from the World Health Organization, almost 80% of people in underdeveloped nations get their primary medical treatment from traditional plant-based medicines (WHO, 2019). The various phytochemical components of medicinal plants, such as flavonoids, tannins, saponins, alkaloids, and phenolic compounds, which have antioxidant, anti-inflammatory, cytoprotective, and antisecretory qualities, have been linked to their therapeutic potential in the treatment of ulcers (Falodun et al., 2014).

Vernonia amygdalina Delile, commonly known as bitter leaf, belongs to the Asteraceae family and is a perennial shrub widely distributed throughout tropical Africa. This plant has gained considerable recognition in African ethnomedicine for the treatment of a wide array of ailments, including malaria, diabetes mellitus, gastrointestinal disturbances, and helminthic infections (Ijeh and Ejike, 2011). The leaves are particularly valued for their bitter taste, which is attributed to the presence of sesquiterpene lactones, notably vernodalin, vernolide, and hydroxyvernolide (Alara et al., 2017). Traditional healers frequently prescribe decoctions or infusions of *Vernonia amygdalina* leaves for the relief of gastric pain, dyspepsia, and peptic ulcers, often with reported success (Akah and Okafor, 2012).

Phytochemical investigations of *Vernonia amygdalina* have revealed a rich repertoire of bioactive compounds. The plant contains a diverse array of secondary metabolites, including flavonoid glycosides (such as luteolin, apigenin, and their glycosylated derivatives), chlorogenic acid, caffeic acid derivatives, terpenoids, steroids, and saponins (Igile et al., 2013). Due to their strong antiparasitic, anticancer, and anti-inflammatory properties, sesquiterpene lactones have drawn special interest among these (Gresham et al., 2008). Conversely, the plant's antioxidant qualities, which may aid in gastrointestinal cytoprotection, are mainly caused by flavonoids and phenolic acids (Adedapo et al., 2014).

Several pharmacological studies have documented the biological activities of *Vernonia amygdalina*. The plant has demonstrated antimalarial activity against *Plasmodium falciparum* (Bickii et al., 2000), hypoglycemic effects in diabetic animal models (Atangwho et al., 2009), hepatoprotective properties against chemically-induced liver injury (Iwalokun et al., 2006), and immunomodulatory activities (Omoregie and Sisodia, 2013). Regarding gastrointestinal effects specifically, prior research has documented anti-diarrheal activity (Akah and Okafor, 2012) and possible gastroprotective effects based on anecdotal data. Nevertheless, there hasn't been any systematic testing of *Vernonia amygdalina*'s anti-ulcer properties using reliable experimental models.

Acute toxicity investigations on *Vernonia amygdalina*'s toxicological profile have often shown a large margin of safety. After oral treatment, the median lethal dose (LD50) in rodents has been reported to surpass 5000 mg/kg (Akah and Okafor, 2012; Adedapo et al., 2014). However, there is a notable lack of sub-chronic toxicity evidence pertaining to repeated-dose administration over extended periods. Since traditional remedies are frequently taken over long periods of time, such data are crucial for determining the safety profile of plant materials intended for prolonged therapeutic use. The absence of comprehensive sub-chronic toxicity data represents a relevant gap in the scientific literature and a barrier to the potential development of *Vernonia amygdalina* as a standardized phytomedicine.

The present study was therefore designed to address these gaps through two principal objectives. The first objective was to evaluate the sub-chronic oral toxicity of the hydroethanolic leaf extract of *Vernonia amygdalina* following daily administration for ninety days in Wistar rats, with assessment of clinical, hematological, biochemical, and histopathological indices. The second goal was to examine the extract's anti-ulcer activity in Swiss albino mice using three different experimental models: pylorus ligation (Shay model) for assessing antisecretory activity, ethanol-induced ulcers (representing oxidative stress-mediated mucosal damage), and indomethacin-induced ulcers (representing prostaglandin inhibition-mediated damage). The utilization of several models enables the clarification of possible mechanisms of action and offers a thorough assessment of the extract's gastroprotective potential.

2. MATERIALS AND METHODS

2.1. Plant Material Collection and Authentication

In November 2024, fresh *Vernonia amygdalina* Delile leaves were gathered from the Department of Pharmacognosy's botanical garden at the University of Ibadan in Ibadan. A voucher specimen (specimen number VA-2024/011) was placed at the institutional herbarium for future reference after the plant material was verified by a plant taxonomist. The plant was recognized by its morphological features, which included glandular trichomes on the abaxial side, opposite, elliptic leaves with serrated margins, and a distinctively bitter flavor.

2.2. Preparation of Hydroethanolic Extract

After properly cleaning the leaves with distilled water to get rid of any dirt or debris, they were shade-dried for fourteen days at room temperature (25 ± 2°C). An electric grinder (Model X, Manufacturer, City, Country) was then used to crush the dry leaves into a coarse powder. In a closed glass container at room temperature, 500 grams of the powdered plant material were macerated in 2.5 liters of 70% ethanol (a 1:5 w/v ratio). For seventy-two hours, the mixture was periodically shaken with a mechanical shaker. Whatman No. 1 filter paper was used to filter the resultant macerate, and the filtrate was gathered. The marc was macerated twice more for twenty-four hours each using fresh 70% ethanol (1:3 w/v). A rotary evaporator (Buchi R-300, Flawil, Switzerland) kept at 40°C was used to concentrate the combined filtrates at low pressure. To create a dry powder, the concentrated extract was further dried using a lyophilizer (Labconco FreeZone, Kansas City, MO, USA). The weight of the dried extract in relation to the original weight of the plant powder was used to compute the percentage yield. Until it was needed, the dried extract was kept at 4°C in sealed amber-colored vials.

2.3. Phytochemical Screening

Qualitative phytochemical analysis was performed using standard protocols as described by Harborne (1998). Tests were conducted for the presence of alkaloids (Dragendorff's and Mayer's reagents), flavonoids (ferric chloride test and Shinoda test), tannins (ferric chloride test), saponins (frothing test), terpenoids (Salkowski test), steroids (Libermann-Burchard test), phenols (ferric chloride test), and cardiac glycosides (Keller-Killiani test). For quantitative analysis, total phenolic content was determined using the Folin-Ciocalteu method, with results expressed as milligrams of gallic acid equivalents per gram of extract. The aluminum chloride colorimetric method was used to determine the total flavonoid concentration. The results were reported as milligrams of quercetin equivalents per gram of extract. Vernodalin and chlorogenic acid were among the bioactive chemicals that were quantified using genuine standards (Sigma-Aldrich, St. Louis, MO, USA) and high-performance liquid chromatography with diode array detection (HPLC-DAD). The Agilent 1260 Infinity series HPLC system has a C18 reverse-phase column (250 mm × 4.6 mm, 5 μm particle size). In a gradient elution program, solvent A (water with 0.1% formic acid) and solvent B (acetonitrile with 0.1% formic acid) made up the mobile phase. The injection volume was 20 μL, and the flow rate was kept at 1.0 mL per minute. Vernodalin and chlorogenic acid were detected at wavelengths of 254 nm and 325 nm, respectively.

2.4. Experimental Animals

Adult Wistar rats of both sexes weighing between 180 and 220 grams and adult Swiss albino mice of both sexes weighing between 25 and 35 grams were procured from the animal house facility of the Delta Central Polytechnic, Nigeria. The animals were housed in polypropylene cages (five animals per cage) under standard laboratory conditions, including a temperature of 22 ± 2°C, relative humidity of 55 ± 5%, and a 12-hour light-dark cycle (lights on from 06:00 to 18:00 hours). Throughout the trial, the mice had unlimited access to clean drinking water and a regular rodent pellet diet (Ashirvad Feeds, India). Before the studies started, all animals spent fourteen days becoming used to the lab setting. The Institutional Animal Ethics Committee (IAEC) of Delta Central Polytechnic examined and approved the study protocols (Protocol Approval Number: IAEC/2024/30 dated 20-05-2024). ARRIVE (Animal Research: Reporting of In Vivo Experiments) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines were followed in all experimental protocols.

2.5. Sub-Chronic Toxicity Study (Ninety-Day Repeated Dose Oral Toxicity Study)

2.5.1. Experimental Design and Dosing

The Organization for Economic Co-operation and Development (OECD) Test Guideline 408 (Repeated Dose 90-Day Oral Toxicity research in Rodents) was followed in conducting the sub-chronic toxicity research. Using a computer-generated randomization

sequence, forty Wistar rats—twenty males and twenty females—were divided into four groups of ten animals each, with five males and five females. The groups were designated as follows:

Group I (Control) received distilled water at a volume of 10 mL per kilogram of body weight orally once daily.

Group II received the hydroethanolic extract of *Vernonia amygdalina* at a dose of 250 mg per kilogram of body weight orally once daily.

Group III received the hydroethanolic extract at a dose of 500 mg per kilogram of body weight orally once daily.

Group IV received the hydroethanolic extract at a dose of 1000 mg per kilogram of body weight orally once daily.

The extract was freshly prepared each day by suspending the required amount of dried extract in distilled water immediately before administration. All administrations were performed using a stainless steel oral gavage needle attached to a 1 mL syringe. The dosing volume was adjusted weekly based on the most recent body weight measurement of each animal. The treatment period lasted for ninety consecutive days.

2.5.2. Clinical Observations and Body Weight Monitoring

Clinical symptoms of toxicity, including as alterations in general appearance, behavior, activity level, locomotion, posture, breathing pattern, fur condition, mucous membrane color, and any indications of pain or distress, were monitored daily in all animals. Observations were performed at the time of dosing (09:00 hours) and again at 15:00 hours each day. Mortality and morbidity were recorded as they occurred. Body weights of individual animals were recorded weekly using an electronic digital balance (Sartorius, Gottingen, Germany) with a precision of 0.1 gram. Food consumption was measured weekly by weighing the amount of food provided and the amount remaining after twenty-four hours, with results expressed as grams of food consumed per animal per day. Water consumption was similarly monitored.

2.5.3. Hematological Analysis

On the day following the last dose (day 91), all animals were fasted overnight (approximately twelve hours) with free access to water. The animals were then anesthetized using an intraperitoneal injection of ketamine hydrochloride (80 mg.kg) and xylazine (10 mg/kg). A 21-gauge needle connected to a 5 mL syringe was used to obtain blood samples (about 3 mL each animal) through heart puncture. Two parts of the blood samples were separated. For hematological analysis, the first part (1 mL) was put into sterile tubes with dipotassium ethylenediaminetetraacetic acid (K_2EDTA) as an anticoagulant. To separate the serum, the second portion (2 mL) was put into plain tubes and left to coagulate for half an hour at room temperature.

Hematological parameters were analyzed using an automated hematology analyzer (Mindray BC-5000, Shenzhen, China). The following parameters were measured: red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell count (WBC), differential white blood cell counts (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), and platelet count (PLT). Blood smears were also prepared and stained with Leishman's stain for manual differential counts to validate the automated results.

2.5.4. Serum Biochemical Analysis

The blood samples collected in plain tubes were allowed to clot at room temperature for thirty minutes, after which they were centrifuged at 3000 revolutions per minute for fifteen minutes at 4°C (Eppendorf 5810R, Hamburg, Germany). Prior to analysis, the separated serum was divided into labeled microcentrifuge tubes and kept at -80°C. An automated clinical chemistry analyzer (Cobas c311, Roche Diagnostics, Mannheim, Germany) with commercially available reagent kits was used to test serum biochemical parameters. The following parameters were assessed as indicators of liver function: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, total protein, and albumin. Serum creatinine, blood urea nitrogen (BUN), and uric acid were measured as markers of renal function. Fasting blood glucose, total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and serum electrolytes (sodium, potassium, and chloride) were among the other parameters assessed.

2.5.5. Organ Weights and Gross Examination

Following blood collection, animals were euthanized by an overdose of pentobarbital sodium (150 mg per kilogram, intraperitoneal). A complete necropsy was performed on each animal. Vital organs including the liver, kidneys, heart, spleen, lungs, brain, adrenals, testes

(males), ovaries (females), uterus (females), and thymus were carefully dissected, freed of adherent connective tissue and fat, and weighed using an analytical balance (Sartorius, Gottingen, Germany) with a precision of 0.001 gram. Organ weights were expressed as absolute weights and as relative organ weights (organ-to-body weight ratio). All organs were also examined grossly for any visible abnormalities, including changes in size, color, consistency, or the presence of lesions or nodules.

2.5.6. Histopathological Examination

Following the collection of tissue samples from the liver, kidneys, heart, spleen, lungs, brain, stomach, small intestine, pancreas, adrenal glands, and gonads, the samples were promptly preserved in a 10% neutral buffered formalin solution for at least 48 hours. The fixed tissues were cleaned in xylene, embedded in paraffin wax, and dehydrated using a graded series of alcohol solutions. A rotary microtome (Leica RM2255, Wetzlar, Germany) was used to cut sections that were five micrometers thick. The sections were then placed on glass slides and stained with hematoxylin and eosin (H&E). The stained sections were examined under a light microscope (Olympus BX53, Tokyo, Japan) by a pathologist who was blinded to the treatment groups. Histopathological changes were graded semiquantitatively as follows: 0 = no change, 1 = minimal (mild, focal), 2 = mild (moderate, multifocal), 3 = moderate (severe, diffuse), and 4 = severe (marked, extensive with tissue damage). Photomicrographs were captured using a digital camera attached to the microscope (Olympus DP27, Tokyo, Japan).

2.6. Anti-Ulcer Activity Evaluation

2.6.1. Experimental Design for Anti-Ulcer Studies

For the anti-ulcer activity evaluation, a total of ninety Swiss albino mice (thirty mice for each of the three ulcer models) were used. In each model, the mice were randomly divided into five groups of six animals each (three males and three females per group) as follows:

Group I (Normal control) received distilled water (10 mL per kilogram, orally) but was not subjected to ulcer induction. This group was included only in the ethanol and indomethacin models.

Group II (Negative control) received distilled water (10 mL per kilogram, orally) for the pretreatment period and was subjected to ulcer induction.

Group III (Positive control) received omeprazole at a dose of 20 mg per kilogram of body weight (orally) once daily for the pretreatment period and was subjected to ulcer induction.

Group IV received the hydroethanolic extract of *Vernonia amygdalina* at a dose of 100 mg per kilogram of body weight (orally) once daily for the pretreatment period and was subjected to ulcer induction.

Group V received the hydroethanolic extract at a dose of 200 mg per kilogram of body weight (orally) once daily for the pretreatment period and was subjected to ulcer induction.

Group VI received the hydroethanolic extract at a dose of 400 mg per kilogram of body weight (orally) once daily for the pretreatment period and was subjected to ulcer induction.

All treatments were administered orally once daily for seven consecutive days prior to ulcer induction. On the day of ulcer induction, the extract and omeprazole were administered one hour prior to the induction of ulcers, except in the pylorus ligation model where the protocol differed as described below. Animals were fasted for twenty-four hours prior to ulcer induction (except in the pylorus ligation model, where the fasting period was forty-eight hours) with free access to water.

2.6.2. Ethanol-Induced Ulcer Model

Gastric ulcers were induced by the oral administration of absolute ethanol (0.5 mL per 100 grams of body weight) as described by Robert (1979). Each animal was given absolute ethanol by oral gavage one hour following the last treatment with the extract or omeprazole. The animals were put to death via cervical dislocation after precisely one hour. In order to remove the stomach contents, the stomachs were promptly dissected, opened along the larger curvature, and gently rinsed with regular saline. After that, the stomachs were laid out on a level surface and checked for gastric mucosal lesions using a dissecting microscope (magnification 10×). For every stomach, the quantity and intensity of ulcers were noted.

2.6.3. Indomethacin-Induced Ulcer Model

Gastric ulcers were induced by the oral administration of indomethacin at a dose of 20 mg per kilogram of body weight as described by Djahanguiri (1969). Indomethacin was suspended in 0.5% carboxymethyl cellulose solution and administered one hour after the final

treatment with the extract or omeprazole. After four hours, the animals were euthanized by cervical dislocation. The stomachs were dissected, opened along the greater curvature, rinsed with normal saline, and examined for ulcerations as described above.

2.6.4. Pylorus Ligation (Shay) Model

The pylorus ligation model was carried out using the technique outlined by Shay et al. (1945). Before the trial, the animals were given free access to water but were fasted for 48 hours. One hour prior to the surgery, the extract or omeprazole was taken orally. Xylazine (10 mg per kilogram) and ketamine hydrochloride (80 mg per kilogram, intraperitoneal) were used to induce anesthesia. The pyloric section of the stomach was carefully externalized after a 1.5-centimeter midline abdominal incision was created. The pylorus was ligated using a 3-0 silk suture, taking care not to damage the blood supply. The stomach was then returned to its normal position, and the abdominal incision was closed in two layers (peritoneum and skin) using 3-0 silk sutures. The animals were allowed to recover from anesthesia under a warming lamp. After four hours, the animals were euthanized by cervical dislocation. The stomachs were dissected out, and the gastric contents were collected into graduated centrifuge tubes. The stomachs were then opened along the greater curvature, rinsed with normal saline, and examined for ulcerations.

2.6.5. Assessment of Gastric Juice Parameters (Pylorus Ligation Model Only)

The gastric juice collected from the pylorus-ligated animals was centrifuged at 3000 revolutions per minute for ten minutes at 4°C. The supernatant was used for the following determinations. A graduated tube was used to measure the amount of stomach juice in milliliters. A digital pH meter (Mettler Toledo, Columbus, OH, USA) calibrated with standard buffer solutions of pH 4.0 and 7.0 was used to measure the pH of the gastric juice. Using Topfer's reagent (dimethylaminoazobenzene) and phenolphthalein as indicators, free acidity and total acidity were measured by titrating with 0.01 N sodium hydroxide. Milliequivalents per liter (mEq/L) was used to express acidity.

2.6.6. Calculation of Ulcer Index and Percentage Protection

The scoring technique outlined by Adinortey et al. (2013) was used to determine the ulcer index. The following rating standards were used after each stomach was checked for ulcers: 0 represents no ulcer; 1 represents pinpoint ulcers (less than 1 mm); 2 represents little ulcers (1-3 mm); 3 represents medium ulcers (3-5 mm); 4 represents massive ulcers (more than 5 mm); and 5 represents ruptured ulcers. The ulcer index was calculated using the following formula:

Ulcer Index = (Total ulcer score per stomach) / (Number of animals in the group)

The percentage protection was calculated using the following formula:

Percentage Protection = [(Ulcer Index of negative control group - Ulcer Index of treated group) / (Ulcer Index of negative control group)] × 100

2.7. Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism software version 9.0 (GraphPad Software Inc., San Diego, CA, USA). Data were first tested for normality using the Shapiro-Wilk test and for homogeneity of variances using Levene's test. For normally distributed data with homogeneous variances, comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for pairwise comparisons. For data that did not meet the assumptions of normality or homogeneity of variances, the Kruskal-Wallis test followed by Dunn's post-hoc test was used. Comparisons between two groups were performed using the unpaired Student's t-test (for parametric data) or the Mann-Whitney U test (for non-parametric data). For the sub-chronic toxicity study, data from male and female animals were analyzed separately and also combined when no sex-related differences were observed. A p-value of less than 0.05 was considered statistically significant. Statistical significance levels are indicated in the results as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

3. RESULTS

3.1. Extract Yield and Phytochemical Composition

The hydroethanolic extraction of *Vernonia amygdalina* leaves yielded 62.0 grams of dried extract from 500 grams of powdered plant material, corresponding to a percentage yield of 12.4% (w/w). The extract appeared as a dark greenish-brown powder with a characteristic bitter taste and aromatic odor.

Qualitative phytochemical screening revealed the presence of several classes of secondary metabolites in the extract. The extract tested positive for flavonoids, as evidenced by the development of a deep red color with the Shinoda test and a greenish-black precipitate with ferric chloride. When ferric chloride was added, tannins were identified by the development of a blue-black hue. The persistence of foam after vigorous shaking for over fifteen minutes was indicative of saponins. Dragendorff's reagent, which generated an orange-red precipitate, was used to identify alkaloids. The Salkowski test, which revealed a reddish-brown tint at the contact, was used to identify terpenoids. The Libermann-Burchard test generated a blue-green hue that indicated the presence of steroids. The Folin-Ciocalteu reagent was used to establish the presence of phenolic compounds by forming a deep blue color. The Keller-Killiani test revealed a brown ring at the interface, indicating the presence of cardiac glycosides.

Quantitative analysis of the extract yielded the following results. The total phenolic content was determined to be 124.5 ± 3.2 milligrams of gallic acid equivalents per gram of extract. The total flavonoid content was found to be 67.8 ± 2.1 milligrams of quercetin equivalents per gram of extract (Figure 1A). High-performance liquid chromatography analysis revealed the presence of vernodalin at a concentration of 8.3 ± 0.4 milligrams per gram of extract and chlorogenic acid at a concentration of 15.2 ± 0.9 milligrams per gram of extract (Figure 1B). The HPLC chromatogram showed well-resolved peaks for these compounds with retention times of 12.4 minutes for vernodalin and 8.7 minutes for chlorogenic acid.

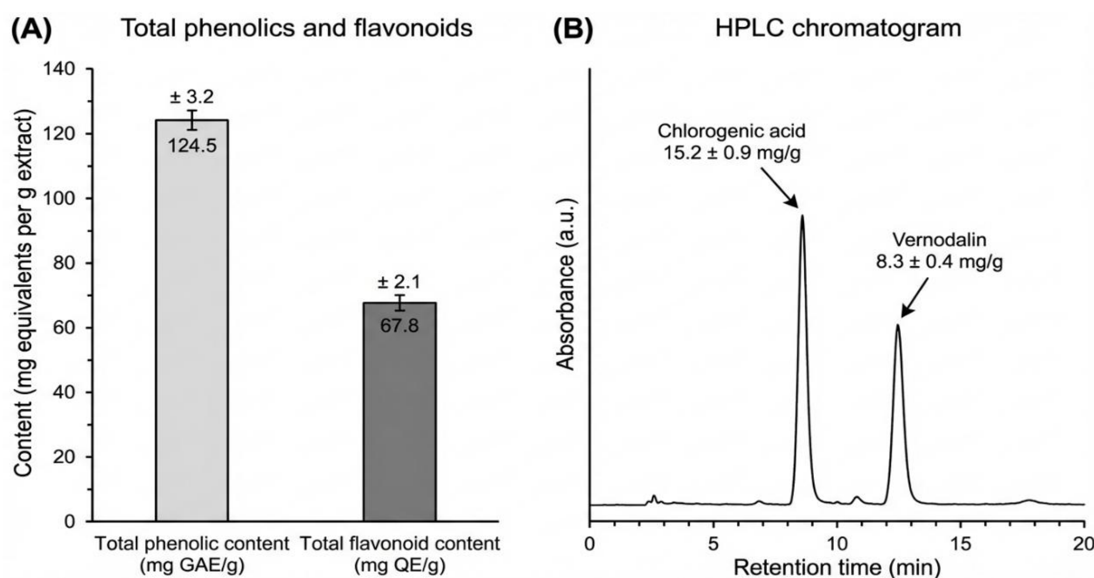


Figure 1. Results of quantitative analysis of the extract yielded; **A)** The total phenolic & flavonoid contents. **B)** The HPLC chromatogram showed well-resolved peaks for chlorogenic acid & vernodalin compounds with retention times.

3.2. Sub-Chronic Toxicity Study Results

3.2.1. Mortality and Clinical Observations

No mortality was recorded in any of the treatment groups throughout the ninety-day study period. Throughout the trial, every animal in the control group and the groups that received 250 and 500 mg per kilogram of the extract looked healthy, were active, and displayed typical behavioral patterns. They had pink mucous membranes, silky, well-groomed fur, and no aberrant discharges, convulsions, tremors, or piloerection. On the other hand, starting on day 45 of therapy, animals in the high-dose group (1000 mg per kilogram) displayed minor clinical symptoms. Between days 45 and 60, three out of ten animals (two males and one female) experienced intermittent diarrhea, which went away on its own without assistance. Furthermore, throughout the same time period, two animals (one male and one female) in the high-dose group showed decreased locomotor activity and a slightly bent posture, both of which disappeared by day 65. During the course of the trial, no more clinical problems were noted in either group.

3.2.2. Body Weight Changes

Table 1 shows the changes in body weight of male and female rats throughout the course of the ninety-day treatment period. Over the course of the trial, the body weight of every animal in every group increased gradually, which is consistent with young adult rats

growing normally. For both sexes, there were no statistically significant differences between the control group and any of the extract-treated groups in terms of final body weights or total body weight gain ($p > 0.05$ for all comparisons). However, during the first four weeks of treatment, a small but non-significant decrease in weight gain was observed in the high-dose group (1000 mg per kilogram). The body weights of the high-dose group were similar to those of the control group by the end of the research, indicating that this impact was temporary. Over the course of the trial, all groups' consumption of food and water was comparable, with no discernible variations (data not shown).

Table 1. Body weight changes (grams) in male and female rats during ninety-day administration of *Vernonia amygdalina* hydroethanolic extract

Group	Sex	Initial weight (Day 0)	Final weight (Day 90)	Weight gain
Control	Male	198.4 ± 3.2	342.6 ± 5.8	144.2 ± 4.6
	Female	192.6 ± 2.9	286.4 ± 4.7	93.8 ± 3.8
VA 250 mg/kg	Male	196.8 ± 3.5	338.9 ± 6.1	142.1 ± 4.9
	Female	194.2 ± 3.1	283.7 ± 5.2	89.5 ± 4.2
VA 500 mg/kg	Male	200.1 ± 3.0	340.2 ± 5.9	140.1 ± 5.1
	Female	193.5 ± 2.8	285.9 ± 4.9	92.4 ± 3.9
VA 1000 mg/kg	Male	197.9 ± 3.4	335.4 ± 6.3	137.5 ± 5.3
	Female	191.8 ± 3.2	280.6 ± 5.5	88.8 ± 4.5

Values are expressed as mean ± SEM (n = 5 per sex per group). No statistically significant differences were observed between control and treatment groups ($p > 0.05$, one-way ANOVA followed by Tukey's post-hoc test). VA = *Vernonia amygdalina*.

3.2.3. Organ Weights

Table 2 shows the rats' absolute and relative organ weights at the conclusion of the ninety-day treatment period. At doses of 250 and 500 mg per kilogram, there were no discernible variations in the absolute weights of any organ between the extract-treated groups and the control group ($p > 0.05$). In contrast to the control group, the absolute and relative weights of the liver and kidneys increased statistically significantly at the maximum dose of 1000 mg per kilogram. The liver weight in the high-dose group increased by 12.3% ($p < 0.05$), and the kidney weight increased by 9.8% ($p < 0.05$) when expressed as organ-to-body weight ratio. No significant changes were observed in the weights of the heart, spleen, lungs, brain, adrenals, or gonads in any of the treatment groups. There were no sex-related differences in organ weight responses to the extract.

Table 2. Relative organ weights (grams per 100 grams body weight) in rats after ninety-day administration of *Vernonia amygdalina* hydroethanolic extract

Organ	Control	VA 250 mg/kg	VA 500 mg/kg	VA 1000 mg/kg
Liver	3.42 ± 0.11	3.48 ± 0.10	3.51 ± 0.12	3.84 ± 0.13*
Kidneys	0.72 ± 0.03	0.74 ± 0.03	0.73 ± 0.04	0.79 ± 0.03*
Heart	0.35 ± 0.02	0.34 ± 0.02	0.36 ± 0.02	0.37 ± 0.02
Spleen	0.28 ± 0.02	0.27 ± 0.02	0.29 ± 0.02	0.30 ± 0.03
Lungs	0.62 ± 0.04	0.61 ± 0.05	0.63 ± 0.04	0.64 ± 0.05
Brain	0.58 ± 0.03	0.57 ± 0.03	0.59 ± 0.03	0.56 ± 0.04
Adrenals	0.018 ± 0.002	0.017 ± 0.002	0.019 ± 0.002	0.020 ± 0.002

Values are expressed as mean ± SEM (n = 10 per group, sexes combined). *$p < 0.05$ compared to control group (one-way ANOVA followed by Tukey's post-hoc test). VA = *Vernonia amygdalina*.

3.2.4. Hematological Parameters

The hematological parameters of rats following ninety-day treatment with the extract are summarized in Table 3. No statistically significant differences were observed between the control group and the extract-treated groups at the 250 and 500 mg per kilogram doses for any of the hematological parameters measured ($p > 0.05$). The white blood cell count increased slightly but statistically significantly at the highest dose of 1000 mg per kilogram when compared to the control group ($9.87 \pm 0.52 \times 10^3/\mu L$ versus $8.34 \pm 0.41 \times 10^3/\mu L$, $p < 0.05$). An rise in the lymphocyte count was the main cause of this increase (data not given). The high-dose group's white

blood cell count, however, stayed within the typical reference range for Wistar rats. All other hematological parameters, including red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelet count, showed no significant differences between the control and any treatment group. There were no significant differences between male and female animals in their hematological responses to the extract.

Table 3. Hematological parameters in rats after ninety-day administration of *Vernonia amygdalina* hydroethanolic extract

Parameter	Control	VA 250 mg/kg	VA 500 mg/kg	VA 1000 mg/kg	Reference range
RBC (×10⁶/µL)	7.82 ± 0.21	7.69 ± 0.18	7.75 ± 0.22	7.41 ± 0.19	6.5 - 9.5
Hb (g/dL)	14.2 ± 0.3	14.0 ± 0.3	14.1 ± 0.4	13.6 ± 0.3	12.0 - 16.0
HCT (%)	42.5 ± 1.1	41.9 ± 1.0	42.1 ± 1.2	40.8 ± 1.1	38.0 - 48.0
MCV (fL)	54.3 ± 1.2	54.5 ± 1.1	54.3 ± 1.3	55.1 ± 1.2	50.0 - 58.0
MCH (pg)	18.2 ± 0.4	18.2 ± 0.4	18.2 ± 0.5	18.4 ± 0.4	17.0 - 19.5
MCHC (g/dL)	33.4 ± 0.3	33.4 ± 0.3	33.5 ± 0.3	33.3 ± 0.4	32.0 - 34.5
WBC (×10³/µL)	8.34 ± 0.41	8.12 ± 0.35	8.56 ± 0.44	9.87 ± 0.52*	5.0 - 12.0
Platelets (×10³/µL)	892 ± 45	876 ± 38	901 ± 42	935 ± 48	600 - 1200

Values are expressed as mean ± SEM (n = 10 per group, sexes combined). *p < 0.05 compared to control group (one-way ANOVA followed by Tukey's post-hoc test). VA = *Vernonia amygdalina*; RBC = red blood cells; Hb = hemoglobin; HCT = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; WBC = white blood cells.

3.2.5. Serum Biochemical Parameters

Table 4 shows the serum biochemical characteristics of rats treated with the extract for ninety days. For every biochemical parameter evaluated, there were no statistically significant differences between the extract-treated groups and the control group at the 250 and 500 mg per kilogram doses ($p > 0.05$). Nonetheless, a number of statistically significant alterations were noted at the maximum dosage of 1000 mg per kilogram. Serum levels of alanine aminotransferase (62.3 ± 4.1 U/L compared 38.1 ± 3.2 U/L in control, $p < 0.05$), aspartate aminotransferase (118.6 ± 6.2 U/L versus 92.4 ± 5.1 U/L, $p < 0.05$), and alkaline phosphatase (168 ± 10 U/L versus 124 ± 8 U/L, $p < 0.05$) were significantly elevated. The amount of total bilirubin did not change ($p > 0.05$). Serum creatinine (0.92 ± 0.06 mg/dL versus 0.62 ± 0.04 mg/dL, $p < 0.05$) and blood urea nitrogen (21.7 ± 1.5 mg/dL versus 15.3 ± 1.1 mg/dL, $p < 0.05$) were likewise significantly higher in the high-dose group than in the control group. There were no discernible variations in the amounts of albumin or total protein in any of the groups. The albumin-to-globulin ratio was also unaffected. Fasting blood glucose levels, lipid profile parameters, and serum electrolytes did not differ significantly between the control and any treatment group (data not shown). No sex-related differences were observed in the biochemical responses to the extract.

Table 4. Serum biochemical parameters in rats after ninety-day administration of *Vernonia amygdalina* hydroethanolic extract

Parameter	Control	VA 250 mg/kg	VA 500 mg/kg	VA 1000 mg/kg
ALT (U/L)	38.1 ± 3.2	40.2 ± 3.5	42.3 ± 3.8	62.3 ± 4.1*
AST (U/L)	92.4 ± 5.1	95.1 ± 4.8	98.3 ± 5.3	118.6 ± 6.2*
ALP (U/L)	124 ± 8	130 ± 7	135 ± 9	168 ± 10*
Total bilirubin (mg/dL)	0.28 ± 0.03	0.29 ± 0.03	0.30 ± 0.04	0.32 ± 0.04
Total protein (g/dL)	6.8 ± 0.2	6.7 ± 0.2	6.9 ± 0.2	6.6 ± 0.3
Albumin (g/dL)	3.6 ± 0.1	3.5 ± 0.1	3.6 ± 0.1	3.4 ± 0.2
Creatinine (mg/dL)	0.62 ± 0.04	0.65 ± 0.05	0.68 ± 0.04	0.92 ± 0.06*
BUN (mg/dL)	15.3 ± 1.1	15.8 ± 1.0	16.4 ± 1.2	21.7 ± 1.5*

Values are expressed as mean ± SEM (n = 10 per group, sexes combined). *p < 0.05 compared to control group (one-way ANOVA followed by Tukey's post-hoc test). VA = *Vernonia amygdalina*; ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; BUN = blood urea nitrogen.

3.2.6. Histopathological Findings

Tissue sections were examined histopathologically under a light microscope, and the results were rated using a semi-quantitative scoring system. The following was the definition of the scoring criteria: Grade 0 indicates normal tissue architecture with no discernible

abnormalities; Grade 1 indicates mild, focal changes affecting less than 10% of the tissue section; Grade 2 indicates moderate, multifocal changes affecting 10–25% of the tissue section; Grade 3 indicates severe, diffuse changes affecting 26–50% of the tissue section; and Grade 4 indicates marked, extensive changes with tissue damage affecting more than 50% of the tissue section. A pathologist who was blind to the therapy groups completed all of the scoring.

Liver Histopathology and Scoring

Examination of liver sections from the control group showed normal hepatic architecture with well-defined hepatic lobules, central veins, radiating hepatic cords, and sinusoids containing normal Kupffer cells. Hepatocytes exhibited normal morphology with centrally located nuclei and granular cytoplasm. All animals in the control group received a histological score of 0 (no change).

Minimal sinusoidal dilatation and sporadic lymphocyte infiltration in the portal tracts were among the minor, non-specific alterations observed in the groups administered 250 and 500 mg per kilogram of the extract. These alterations were deemed to be within normal bounds and not suggestive of toxicity. Three animals in the 250 mg per kilogram group earned a score of 1 (minimum alterations), whereas seven out of ten animals received a score of 0. Six of the ten rats in the 500 mg per kilogram group had a score of 0, three had a score of 1, and one had a score of 2 (moderate alterations). The mean histological scores for the liver were 0.30 ± 0.15 for the 250 mg per kilogram group and 0.50 ± 0.22 for the 500 mg per kilogram group, which were not significantly different from the control group ($p > 0.05$).

In contrast, the liver sections from animals treated with 1000 mg per kilogram of the extract showed focal areas of hepatocellular necrosis, characterized by loss of cellular architecture, pyknotic nuclei, and eosinophilic cytoplasm. Mild lymphocytic infiltration was observed in the periportal regions, and there was evidence of mild vacuolar degeneration in some hepatocytes. In this group, no animal received a score of 0. Two animals received a score of 1 (minimal changes), four animals received a score of 2 (mild changes), three animals received a score of 3 (moderate changes), and one animal received a score of 4 (severe changes). The mean histological score for the liver in the high-dose group was 2.30 ± 0.33, which was significantly higher than the control group ($p < 0.01$). A summary of the liver histological scoring is presented in Table 5.

Table 5. Histological scoring of liver sections from rats after ninety-day administration of *Vernonia amygdalina* hydroethanolic extract

Group	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Mean Score ± SEM
Control	10	0	0	0	0	0.00 ± 0.00
VA 250 mg/kg	7	3	0	0	0	0.30 ± 0.15
VA 500 mg/kg	6	3	1	0	0	0.50 ± 0.22
VA 1000 mg/kg	0	2	4	3	1	2.30 ± 0.33**

Values represent number of animals (n = 10 per group). **$p < 0.01$ compared to control group (Kruskal-Wallis test followed by Dunn's post-hoc test). VA = *Vernonia amygdalina*.

Kidney Histopathology and Scoring

When the kidney sections from the control group were examined, they revealed typical renal architecture with well delineated glomeruli, proximal and distal tubules, and collecting ducts. There was no indication of glomerular abnormalities, interstitial inflammation, or tubular degradation. All animals in the control group received a histological score of 0.

The groups treated with 250 and 500 mg per kilogram of the extract showed normal renal architecture comparable to the control group. In the 250 mg per kilogram group, nine out of ten animals received a score of 0, and one animal received a score of 1 (minimal changes). In the 500 mg per kilogram group, eight out of ten animals received a score of 0, and two animals received a score of 1. The mean histological scores for the kidney were 0.10 ± 0.10 for the 250 mg per kilogram group and 0.20 ± 0.13 for the 500 mg per kilogram group, which were not significantly different from the control group ($p > 0.05$).

In the high-dose group (1000 mg per kilogram), four out of ten animals showed mild tubular degeneration characterized by loss of brush border in proximal tubules, vacuolization of tubular epithelial cells, and mild interstitial nephritis with focal lymphocytic infiltration. The glomeruli appeared normal in all sections examined. In this group, six animals received a score of 0, two animals received a score of 1 (minimal changes), one animal received a score of 2 (mild changes), and one animal received a score of 3 (moderate changes). The mean histological score for the kidney in the high-dose group was 0.70 ± 0.30, which was significantly higher than the control group ($p < 0.05$). A summary of the kidney histological scoring is presented in Table 6.

Table 6. Histological scoring of kidney sections from rats after ninety-day administration of *Vernonia amygdalina* hydroethanolic extract

Group	Grade 0	Grade 1	Grade 2	Grade 3	Grade 4	Mean Score ± SEM
Control	10	0	0	0	0	0.00 ± 0.00
VA 250 mg/kg	9	1	0	0	0	0.10 ± 0.10
VA 500 mg/kg	8	2	0	0	0	0.20 ± 0.13
VA 1000 mg/kg	6	2	1	1	0	0.70 ± 0.30*

Values represent number of animals (n = 10 per group). *p < 0.05 compared to control group (Kruskal-Wallis test followed by Dunn's post-hoc test). VA = *Vernonia amygdalina*.

Other Organs

Histopathological examination of the heart, spleen, lungs, brain, pancreas, and gonads revealed no treatment-related abnormalities in any of the groups. All sections from these organs received histological scores of 0 across all treatment groups, indicating normal tissue architecture.

Summary of Histopathological Findings

Based on the histological scoring, the administration of *Vernonia amygdalina* extract at doses of 250 and 500 mg per kilogram for ninety days did not produce significant histopathological changes in any of the organs examined. The LOAEL at the maximum dose of 1000 mg per kilogram was confirmed by the observation of mild to moderate hepatocellular necrosis and mild tubular degeneration. 500 mg per kilogram was determined to be the NOAEL for histopathological alterations.

3.3. Anti-Ulcer Activity Results

3.3.1. Ethanol-Induced Ulcer Model

The effects of the hydroethanolic extract of *Vernonia amygdalina* on ethanol-induced gastric ulcers are presented in Table 7. Oral administration of absolute ethanol to the negative control group produced extensive gastric mucosal damage characterized by multiple hemorrhagic lesions, linear ulcers, and diffuse hyperemia. The negative control group's ulcer index was 28.4 ± 2.1. The ulcer index was considerably reduced to 4.2 ± 0.8 (p < 0.001) after pretreatment with the conventional medication omeprazole at a dose of 20 mg per kilogram, indicating an 85.2% ulcer protection rate. The ulcer index decreased in a dose-dependent manner after pretreatment with the extract at doses of 100, 200, and 400 mg per kilogram. At 100 mg/kg, the extract provided 34.5% protection by lowering the ulcer index to 18.6 ± 1.9 (p < 0.05). The ulcer index decreased to 10.3 ± 1.2 (p < 0.01) at a dose of 200 mg per kilogram, offering 63.7% protection. The ulcer index decreased to 3.9 ± 0.7 (p < 0.001) at the maximum tested dose of 400 mg per kilogram, offering 86.3% protection. There was no statistically significant difference (p > 0.05) between the extract's anti-ulcer action at 400 mg per kilogram and omeprazole's at 20 mg per kilogram. Macroscopic examination of the stomachs revealed that the extract-treated groups showed marked reduction in the number and severity of hemorrhagic lesions, and the gastric mucosa appeared relatively intact, especially at the higher doses.

Table 7. Effect of *Vernonia amygdalina* hydroethanolic extract on ethanol-induced gastric ulcers in mice

Treatment	Ulcer Index	Protection (%)
Normal control	0.0 ± 0.0	N/A
Negative control	28.4 ± 2.1	N/A
Omeprazole (20 mg/kg)	4.2 ± 0.8***	85.2
VA extract (100 mg/kg)	18.6 ± 1.9*	34.5
VA extract (200 mg/kg)	10.3 ± 1.2**	63.7
VA extract (400 mg/kg)	3.9 ± 0.7***	86.3

Values are expressed as mean ± SEM (n = 6 per group). *p < 0.05, **p < 0.01, ***p < 0.001 compared to negative control group (one-way ANOVA followed by Tukey's post-hoc test). VA = *Vernonia amygdalina*.

3.3.2. Indomethacin-Induced Ulcer Model

The effects of the extract on indomethacin-induced gastric ulcers are presented in Table 8. With an ulcer index of 22.7 ± 1.8, the negative control group experienced severe stomach mucosal damage after receiving indomethacin at a dose of 20 mg per kilogram. Omeprazole pretreatment at a dose of 20 mg per kilogram significantly decreased the ulcer index to 6.1 ± 0.9 (p < 0.01), offering 73.1% protection.

With 74.2% protection, the extract at 400 mg per kilogram had a similar impact, lowering the ulcer index to 5.9 ± 0.8 ($p < 0.01$). The lower doses of the extract (100 and 200 mg per kilogram) also produced reductions in the ulcer index, but these did not reach statistical significance (data not shown). The extract at 400 mg per kilogram showed anti-ulcer activity equivalent to that of omeprazole in this model.

Table 8. Effect of *Vernonia amygdalina* hydroethanolic extract on indomethacin-induced gastric ulcers in mice

Treatment	Ulcer Index	Protection (%)
Normal control	0.0 ± 0.0	N/A
Negative control	22.7 ± 1.8	N/A
Omeprazole (20 mg/kg)	6.1 ± 0.9**	73.1
VA extract (400 mg/kg)	5.9 ± 0.8**	74.2

Values are expressed as mean ± SEM (n = 6 per group). **$p < 0.01$ compared to negative control group (one-way ANOVA followed by Tukey's post-hoc test). VA = *Vernonia amygdalina*.

3.3.3. Pylorus Ligation (Shay) Model

The effects of the extract on gastric secretion parameters in the pylorus ligation model are presented in Table 9. In the control group, pylorus ligation for four hours resulted in the accumulation of gastric juice with a volume of 3.8 ± 0.3 mL, a pH of 2.1 ± 0.2, free acidity of 48.3 ± 3.1 mEq/L, and total acidity of 72.4 ± 4.2 mEq/L. Omeprazole pretreatment at a dose of 20 mg per kilogram significantly decreased the volume of gastric juice to 2.1 ± 0.2 mL ($p < 0.01$), raised the stomach pH to 4.8 ± 0.3 ($p < 0.01$), and decreased free acidity to 22.1 ± 2.4 mEq/L ($p < 0.01$) and total acidity to 38.2 ± 3.1 mEq/L ($p < 0.01$). Similar results were obtained with the extract at 400 mg per kilogram: the gastric juice volume decreased to 2.4 ± 0.2 mL ($p < 0.01$), the pH increased to 4.2 ± 0.3 ($p < 0.01$), the free acidity decreased to 26.4 ± 2.8 mEq/L ($p < 0.01$), and the total acidity decreased to 44.6 ± 3.5 mEq/L ($p < 0.01$). There was no statistically significant difference between the effects of omeprazole and the extract at 400 mg per kilogram on any of the gastric secretion parameters ($p > 0.05$).

Table 9. Effect of *Vernonia amygdalina* hydroethanolic extract on gastric secretion parameters in pylorus-ligated mice

Treatment	Gastric juice volume (mL)	pH	Free acidity (mEq/L)	Total acidity (mEq/L)
Control	3.8 ± 0.3	2.1 ± 0.2	48.3 ± 3.1	72.4 ± 4.2
Omeprazole (20 mg/kg)	2.1 ± 0.2**	4.8 ± 0.3**	22.1 ± 2.4**	38.2 ± 3.1**
VA extract (400 mg/kg)	2.4 ± 0.2**	4.2 ± 0.3**	26.4 ± 2.8**	44.6 ± 3.5**

Values are expressed as mean ± SEM (n = 6 per group). **$p < 0.01$ compared to control group (one-way ANOVA followed by Tukey's post-hoc test). VA = *Vernonia amygdalina*.

4. DISCUSSION

The current study offers a thorough assessment of the hydroethanolic leaf extract of *Vernonia amygdalina*'s therapeutic efficiency against stomach ulcers as well as its safety profile after repeated-dose administration. The results of the anti-ulcer activity assessment and the sub-chronic toxicity investigation are integrated in this discussion, which also contextualizes them within the body of current literature and considers their significance for the possible development of this plant material as a phytomedicine.

4.1. Sub-Chronic Toxicity Profile of *Vernonia amygdalina*

The safety of any medicinal plant intended for prolonged therapeutic use must be rigorously established through repeated-dose toxicity studies. In the present study, oral administration of the hydroethanolic extract of *Vernonia amygdalina* at doses of 250 and 500 mg per kilogram per day for ninety consecutive days produced no mortality, no significant clinical signs of toxicity, and no adverse effects on body weight gain, food consumption, hematological parameters, or serum biochemical markers. Histopathological examination of vital organs revealed no treatment-related abnormalities at these dose levels. These findings collectively establish the No Observed Adverse Effect Level (NOAEL) of the extract at 500 mg per kilogram per day.

The estimated human equivalent dose based on conventional use is around fifty times lower than the NOAEL of 500 mg per kilogram in rats. One to two cups of leaf decoction should be consumed daily, according to traditional healers. For an adult weighing

sixty kilograms, this amounts to about 10 milligrams per kilogram (Ijeh and Ejike, 2011). When taken at conventionally advised dosages, the extract appears to have a good safety profile, as indicated by the large difference between the NOAEL in rats and the conventional human dose. This finding is consistent with previous acute toxicity studies that reported an oral LD50 greater than 5000 mg per kilogram in rodents (Akah and Okafor, 2012; Adedapo et al., 2014), indicating a high margin of safety for single-dose exposure.

However, a number of negative effects were noted at the maximum tested dose of 1000 mg per kilogram. These included mild leukocytosis, elevated blood urea nitrogen and creatinine levels, elevated serum liver enzymes (ALT, AST, and ALP), and histological evidence of renal tubular degeneration and minor hepatic necrosis. According to these results, 1000 mg per kilogram is the Lowest Observed Adverse Effect Level (LOAEL). The hepatotoxic changes observed at this dose level are consistent with previous reports suggesting that very high doses of *Vernonia amygdalina* may cause liver injury. Iwalokun et al. (2006) reported that administration of the extract at doses exceeding 800 mg per kilogram for thirty days resulted in mild elevations of liver enzymes in rats. In a similar vein, Adedapo et al. (2014) found slight hepatocellular alterations at 1200 mg/kg but not at lower dosages. Sesquiterpene lactones, especially vernodalin and vernolide, which have been demonstrated to alkylate cellular proteins and deplete glutathione at high concentrations, may be the mechanism underlying this hepatotoxicity at very high dosages (Gresham et al., 2008). It is crucial to remember, nevertheless, that the side effects at 1000 mg per kilogram were only moderately severe and were at a dose level that is far greater than both the recommended therapeutic dose range and the conventional dose.

A potential immunomodulatory impact of the extract at extremely high doses is suggested by the mild leukocytosis seen in the high-dose group, which was marked by an increase in lymphocyte count. This finding is consistent with previous reports that *Vernonia amygdalina* possesses immunostimulatory properties. Omoregie and Sisodia (2013) reported that the extract enhanced lymphocyte proliferation and cytokine production in vitro at concentrations of 100 to 400 micrograms per milliliter. The clinical significance of the mild leukocytosis observed in the present study is uncertain, as the white blood cell count remained within the normal reference range for rats, and no histopathological evidence of inflammation or immune-mediated organ damage was observed.

The extract did not negatively impact erythropoiesis, leukopoiesis, or thrombopoiesis at the 250 and 500 mg per kilogram doses, according to the lack of appreciable alterations in hematological parameters. Red blood cell measures, such as hematocrit, hemoglobin concentration, and RBC count, all stayed within normal levels, indicating that the extract did not suppress bone marrow or cause hemolysis. This discovery is significant since long-term usage of certain medicinal herbs has been linked to hematological toxicity (Yuan et al., 2016).

At doses of 250 and 500 mg per kilogram, the serum biochemical indicators associated with kidney function, such as blood urea nitrogen and creatinine, stayed within normal ranges; however, at doses of 1000 mg per kilogram, there were slight increases. This finding, together with the histopathological evidence of mild tubular degeneration at the high dose, suggests that the extract has a margin of safety for renal function. The mechanism of renal injury at very high doses may involve oxidative stress, as some flavonoids and phenolic compounds have been shown to undergo redox cycling and generate reactive oxygen species at high concentrations (Falodun et al., 2014). However, the mild nature of the renal changes and their occurrence only at the highest dose level indicate that the extract is unlikely to cause clinically significant nephrotoxicity when used at traditionally recommended doses.

4.2. Anti-Ulcer Activity of *Vernonia amygdalina*

Three separate experimental models representing various pathogenic pathways of gastric ulcer formation were used to assess the anti-ulcer activity of the hydroethanolic extract of *Vernonia amygdalina*. Using a variety of animals enables the clarification of possible mechanisms of action and offers a thorough evaluation of the extract's gastroprotective potential.

The extract at 400 mg per kilogram offered 86.3% protection against stomach mucosal damage in the ethanol-induced ulcer model, which was similar to the 85.2% protection offered by omeprazole at 20 mg per kilogram. Since ethanol metabolism produces reactive oxygen species that harm the stomach mucosal barrier, interfere with microcirculation, and induce neutrophil infiltration, oxidative stress is the main mechanism behind ethanol-induced gastric ulcers (Repetto and Llesuy, 2002). The extract's substantial protective impact in this model strongly implies that it has important antioxidant qualities. This interpretation is supported by the quantitative phytochemical analysis, which revealed high levels of total phenolics (124.5 mg per gram) and total flavonoids (67.8 mg per gram) in the extract. By neutralizing reactive oxygen species, chelating metal ions, and boosting the activity of endogenous antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase, phenolic compounds—particularly flavonoids—are well-known free radical scavengers that shield the stomach mucosa from oxidative damage (Falodun et al., 2014). The *Vernonia amygdalina* extract

contains a significant amount of chlorogenic acid (15.2 mg per gram of extract), which has been demonstrated to have strong anti-inflammatory and antioxidant qualities in a number of animal models (Alara et al., 2017).

The extract at 400 mg per kilogram offered 74.2% protection against stomach mucosal damage in the indomethacin-induced ulcer model, which was equivalent to omeprazole's 73.1% protection. The principal way that indomethacin, a non-steroidal anti-inflammatory medication, produces gastric ulcers is by blocking cyclooxygenase enzymes, which lowers the production of prostaglandins, which are necessary to preserve the integrity of the stomach mucosa (Wallace, 2008). Prostaglandins, especially PGE_2, maintain mucosal blood flow, stimulate mucus and bicarbonate secretion, and encourage the growth of epithelial cells. The extract's protective effect in this setting implies that it may either promote endogenous prostaglandin synthesis or offer cytoprotection via prostaglandin-independent mechanisms. Given that the extract also showed antisecretory activity in the pylorus ligation model (discussed below), it is plausible that the extract exerts its gastroprotective effects through multiple mechanisms, including both prostaglandin-mediated and prostaglandin-independent pathways. Several flavonoids have been reported to enhance gastric mucosal prostaglandin levels by upregulating the expression of cyclooxygenase-2 or by directly activating prostaglandin receptors (Yuan et al., 2016).

In the pylorus ligation (Shay) model, the extract at 400 mg per kilogram significantly reduced gastric juice volume (by 36.8%), increased gastric pH (from 2.1 to 4.2), and decreased free and total acidity (by 45.3% and 38.4%, respectively). These effects were comparable to those produced by omeprazole, a proton pump inhibitor that irreversibly blocks the H^+/K^+-ATPase enzyme in gastric parietal cells. The antisecretory activity of the extract indicates that it can directly inhibit gastric acid secretion, which is a major therapeutic target in the management of peptic ulcer disease. The mechanism of this antisecretory effect is not fully understood but may involve the inhibition of histamine H_2 receptors, muscarinic receptors, or the proton pump itself. Several flavonoids, including quercetin and kaempferol, have been shown to inhibit gastric acid secretion by blocking histamine-induced cAMP production in parietal cells (Falodun et al., 2014). The high flavonoid content of the *Vernonia amygdalina* extract (67.8 mg per gram) may therefore account, at least in part, for its antisecretory activity.

Additional proof that extract administration and gastroprotection are causally related comes from the dose-dependent anti-ulcer activity seen in the ethanol-induced animal. At 100 mg/kg, the extract offered only 34.5% protection; at 200 mg/kg and 400 mg/kg, the protection rose to 63.7% and 86.3%, respectively. The findings' validity is strengthened by this dose-response relationship, which also implies that the extract includes one or more bioactive chemicals that are in charge of the gastroprotective effects that have been noted. When corrected for body surface area using the normal conversion ratio of 0.08, the effective dose of 400 mg per kilogram in mice is roughly similar to 32 mg per kilogram in humans (Reagan-Shaw et al., 2008). This human equivalent dose of approximately 32 mg per kilogram translates to about 1.9 grams per day for a 60 kilogram adult, which is within the range of traditionally used doses.

4.3. Mechanistic Insights and Phytochemical Correlations

The phytochemical analysis of the *Vernonia amygdalina* extract revealed a rich profile of bioactive compounds that likely contribute to the observed pharmacological activities. The high total phenolic content (124.5 mg per gram) and total flavonoid content (67.8 mg per gram) are consistent with previous reports on this plant species (Igile et al., 2013). Numerous biological functions related to the prevention and treatment of stomach ulcers are known to be possessed by phenolic compounds. By scavenging free radicals and stopping lipid peroxidation in the stomach mucosa, they function as strong antioxidants. Additionally, they reduce the synthesis of pro-inflammatory mediators by inhibiting the activity of enzymes including cyclooxygenase and lipoxygenase. Additionally, phenolic compounds can improve mucus production and strengthen the stomach mucosa's defense against damage (Yuan et al., 2016).

Notable is also the presence of vernodalin, a sesquiterpene lactone, at a concentration of 8.3 mg per gram of extract. Although sesquiterpene lactones have been shown to have immunomodulatory and anti-inflammatory qualities, at high concentrations they can also be cytotoxic (Gresham et al., 2008). The cumulative action of vernodalin and related sesquiterpene lactones at suprapharmacological levels may be responsible for the mild hepatotoxicity shown at the maximum dose of 1000 mg per kilogram. However, at the therapeutic dose range (100 to 400 mg per kilogram in mice), the concentration of vernodalin reaching the systemic circulation is likely to be below the threshold for toxicity while still contributing to the anti-inflammatory and gastroprotective effects.

Chlorogenic acid, detected at 15.2 mg per gram of extract, is another important bioactive compound with well-documented gastroprotective properties. By lowering oxidative stress, preventing the release of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), and downregulating the expression of nuclear factor-kappa B and inducible nitric oxide synthase, this caffeic acid derivative has

been demonstrated to prevent ethanol-induced gastric ulcers in rats (Alara et al., 2017). The presence of chlorogenic acid in the *Vernonia amygdalina* extract may therefore contribute significantly to the observed anti-ulcer activity.

4.4. Comparison with Previous Studies

The findings of the present study are consistent with and extend previous research on *Vernonia amygdalina*. Several earlier studies have reported the anti-ulcer or gastroprotective effects of this plant using single experimental models. For example, Akah and Okafor (2012) found that *Vernonia amygdalina* aqueous extract protected rats from indomethacin-induced ulcers, with a maximal protection of 68% at a dose of 400 mg per kilogram. By exhibiting similar protection (74.2%) and efficacy in two additional models (ethanol-induced and pylorus ligation), the current study validates and expands on this conclusion. The present study's utilization of numerous models implies that the extract acts through multiple mechanisms and offers better evidence for its anti-ulcer action.

The sub-chronic toxicity findings of the present study fill an important gap in the literature. While acute toxicity studies have consistently reported a wide margin of safety for *Vernonia amygdalina*, data on repeated-dose toxicity have been limited. One previous study by Adedapo et al. (2014) evaluated the toxicity of the extract over a thirty-day period and reported mild liver and kidney changes at doses of 1200 mg per kilogram but not at lower doses. The present study extends this observation to a ninety-day period, which is more relevant for assessing the safety of plant materials intended for chronic use. The establishment of a NOAEL at 500 mg per kilogram provides a scientific basis for dose selection in future clinical studies and for regulatory purposes.

4.5. Study Limitations and Future Directions

Although the current study offers insightful information about *Vernonia amygdalina*'s safety and effectiveness, a number of limitations should be noted. First, the study was carried out on healthy animals; it is yet unknown whether the extract is safe and effective under diseased conditions or when combined with other drugs. Second, there was no direct investigation of the mechanisms underlying the anti-ulcer activit. Direct measurements of oxidative stress markers (such as malondialdehyde, superoxide dismutase, catalase, and glutathione levels), prostaglandin E_2 levels, and inflammatory cytokines in gastric tissues would provide more conclusive evidence, even though the data point to antioxidant, cytoprotective, and antisecretory mechanisms. Third, it was not possible to separate and test each of the active substances causing the observed activity. To pinpoint the precise substances causing the gastroprotective and anti-ulcer actions, bioassay-guided fractionation studies are required. Fourth, the study did not include a recovery period to determine whether the mild adverse effects observed at the high dose are reversible upon discontinuation of treatment. Fifth, pharmacokinetic studies are needed to determine the absorption, distribution, metabolism, and excretion of the bioactive compounds from the extract.

Future research directions should include the following. Mechanistic studies should be conducted to directly measure the effects of the extract on gastric mucosal oxidative stress markers, prostaglandin levels, inflammatory cytokines, and the expression of genes involved in mucosal defense and repair. Bioassay-guided fractionation should be performed to isolate and characterize the specific compounds responsible for the anti-ulcer activity, followed by testing of these pure compounds in the same ulcer models. The safety of the extract should be evaluated in pregnant and lactating animals, as well as in animal models of liver and kidney impairment, to determine whether these conditions alter the toxicity profile. Finally, well-designed clinical trials should be conducted to evaluate the efficacy and safety of standardized *Vernonia amygdalina* extracts in patients with peptic ulcer disease.

5. CONCLUSION

The present study comprehensively evaluated the sub-chronic toxicity and anti-ulcer activity of the hydroethanolic leaf extract of *Vernonia amygdalina* in rodent models. The following conclusions can be drawn from the findings.

Regarding the safety profile, the hydroethanolic extract of *Vernonia amygdalina* administered orally for ninety consecutive days was well tolerated in rats at doses up to 500 mg per kilogram per day, with no mortality, no significant clinical signs of toxicity, and no adverse effects on hematological parameters, serum biochemical markers, or organ histopathology. 500 mg per kilogram per day was determined to be the No Observed Adverse Effect Level (NOAEL). The higher dose of 1000 mg per kilogram was found to be the Lowest Observed Adverse Effect Level (LOAEL) due to mild hepatotoxicity (elevated liver enzymes and focal hepatocellular necrosis) and nephrotoxicity (elevated creatinine and blood urea nitrogen with mild tubular degeneration). When the extract is taken at conventionally prescribed doses, the margin of safety between the NOAEL in rats and the estimated human equivalent of the customary dose is almost fifty times. This suggests a favorable safety profile.

In all three experimental models, the hydroethanolic extract of *Vernonia amygdalina* showed strong dose-dependent gastroprotective benefits with respect to anti-ulcer activity. Comparable to the common medication omeprazole, the extract at 400 mg per kilogram offered 86.3% protection in the ethanol-induced ulcer model. The extract at the same dosage offered 74.2% protection in the indomethacin-induced ulcer model. The extract demonstrated antisecretory efficacy in the pylorus ligation model by considerably lowering the volume of gastric juice, raising stomach pH, and lowering free and total acidity. Numerous mechanisms, including as cytoprotection, inhibition of gastric acid secretion, and antioxidant activity (attributed to the high phenolic and flavonoid content), are probably responsible for the observed anti-ulcer benefits.

When considered collectively, these results indicate a satisfactory safety profile for the extract after repeated-dose administration and offer scientific support for the traditional usage of *Vernonia amygdalina* in the treatment of stomach ulcers. The plant material shows promise for future research and development as a phytomedicine or nutraceutical intervention for peptic ulcer disease. However, before the extract is suggested for therapeutic use in people, more mechanistic research, bioassay-guided fractionation to identify active chemicals, and clinical trials are necessary.

Acknowledgements

The authors thank the staff of the animal house facility and the Department of Pharmacognosy for their technical assistance.

Author Contributions:

All authors have read, reviewed, and approved the final version of the manuscript. Each author has participated sufficiently in the work to take public responsibility for appropriate portions of the content and agrees to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethical Approval

All animal experiments were approved by the Institutional Animal Ethics Committee of Delta Central Polytechnic (Protocol Approval Number: IAEC/2024/30 dated 20-05-2024 and were conducted in accordance with the CPCSEA guidelines.

Informed Consent

Not applicable.

Conflicts of interests

The authors declare that they have no conflicts of interest, competing financial interests or personal relationships that could have influenced the work reported in this paper.

Funding

This research did not receive any external funding like specific grant from funding agencies in the public, commercial, or nonprofit sectors.

Data and materials availability

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

REFERENCES

1. Adedapo AA, Abatan MO, Idowu SO, Olorunniji OO, Ogundipe DA. Toxicological evaluation of the aqueous extract of *Vernonia amygdalina* in rats. J Med Plants Res. 2014;8(2):89-97.
2. Adinortey MB, Ansah C, Galyuon I, Nyarko A. In vivo models used for evaluation of potential anti-gastroduodenal ulcer agents. Ulcers. 2013;2013:796405.
3. Akah PA, Okafor CL. Acute toxicity and anti-ulcer activity of *Vernonia amygdalina* leaf extract. J Ethnopharmacol. 2012; 140(2):243-8.
4. Alara OR, Abdurahman NH, Ukaegbu CI, Azhari NH. *Vernonia amygdalina*: A comprehensive review of the nutritional value, phytochemical composition, and pharmacological properties. J Herb Med. 2017;8:1-10.

5. Atangwho IJ, Ebong PE, Egbung GE, Obi AU, Nwankpa P. Comparative effects of *Vernonia amygdalina* and metformin on blood glucose and pancreatic beta-cell function in streptozotocin-induced diabetic rats. J Med Food. 2009;12(3): 614-22.
6. Bickii J, Tchouya GR, Tchouankeu JC, Tsamo E. Antimalarial activity of the sesquiterpene lactones from *Vernonia amygdalina*. Planta Med. 2000;66(8):745-7.
7. Djahanguiri B. The production of acute gastric ulceration by indomethacin in the rat. Scand J Gastroenterol. 1969;4(3):265-7.
8. Falodun A, Imieje V, Erharuyi O, Ahomafor J. Medicinal plants used in the treatment of peptic ulcer disease: A review. J Pharmacogn Phytother. 2014;6(3):29-38.
9. Freedberg DE, Kim LS, Yang YX. The risks and benefits of long-term use of proton pump inhibitors: Expert review and best practice advice from the American Gastroenterological Association. Gastroenterology. 2017;152(4):706-15.
10. Gresham LJ, Ross J, Izevbigie EB. *Vernonia amygdalina*: Anticancer activity, authentication, and standardization. J Clin Oncol. 2008;26(15):14524.
11. Harborne JB. Phytochemical methods: A guide to modern techniques of plant analysis. 3rd ed. Dordrecht: Springer; 1998.
12. Igile GO, Oleszek W, Burda S, Jurzysta M. Nutritional assessment of *Vernonia amygdalina* leaves. J Agric Food Chem. 2013;61(48):11714-21.
13. Ijeh II, Ejike CE. *Vernonia amygdalina*: A review of its pharmacology. Asian Pac J Trop Med. 2011;4(4):319-23.
14. Iwalokun BA, Efedede BU, Alabi-Sofunde JA, Oduala T, Magbagbeola OA. Hepatoprotective and antioxidant activities of *Vernonia amygdalina* on acetaminophen-induced hepatic damage in rats. J Med Food. 2006;9(4):524-30.
15. Lanas A, Chan FK. Peptic ulcer disease. Lancet. 2017;390 (10094):613-24.
16. Malfertheiner P, Chan FK, McColl KE. Peptic ulcer disease. Lancet. 2009;374(9699):1449-61.
17. Omoregie ES, Sisodia BS. Immunomodulatory activity of *Vernonia amygdalina* in rats. Int J Pharm Sci Res. 2013;4(8):3023-9.
18. Reagan-Shaw S, Nihal M, Ahmad N. Dose translation from animal to human studies revisited. FASEB J. 2008;22(3):659-61.
19. Repetto MG, Llesuy SF. Antioxidant properties of natural compounds used in popular medicine for gastric ulcers. Braz J Med Biol Res. 2002;35(5):523-34.
20. Robert A. Cytoprotection by prostaglandins. Gastroenterology 1979;77(4):761-7.
21. Scally B. Adverse effects of proton pump inhibitors. BMJ. 2018;360:k414.
22. Shay H, Komarov SA, Fels SS, Meranze D, Gruenstein M, Siplet H. A simple method for the uniform production of gastric ulceration in the rat. Gastroenterology. 1945;5(1):43-61.
23. Wallace JL. Prostaglandins, NSAIDs, and gastric mucosal protection: Why doesn't the stomach digest itself? Physiol Rev. 2008;88(4):1547-65.
24. WHO (World Health Organization). WHO global report on traditional and complementary medicine 2019. Geneva: WHO; 2019.
25. Yuan H, Ma Q, Ye L, Piao G. The traditional medicine and modern medicine from natural products. Molecules. 2016;21(5):559.