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Histochemical Studies of the Efficacy of an Anti-Ulcer Herbal Mixture on the Gastrointestinal Tract of Albino Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author OAA designed the study, wrote the protocol and the first draft of the manuscript, did the literature searches and performed the laboratory analysis. Author OGA supervised the study. Histology slides were read by author DEI while author AGFA assisted in the laboratory analysis. All authors read and approved the final manuscript.

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ABSTRACT

Objectives: The present study was performed to evaluate the efficacy and toxic effect of an anti-ulcer herbal mixture.
Method: Quantitative phytochemical analysis was carried out on the ethanol extract of the herbal mixture. The control group in the acute toxicity study received 2 ml of distilled water; other doses were 1 g/kg, 2 g/kg, 4 g/kg, 6 g/kg and 8 g/kg per body weight for 7 days. Rats were sacrificed 1hour after oral administration of 1 ml absolute ethanol and Gastrointestinal Tract taken for histological assessment. The anti-ulcerogenic activity was investigated by administering 2 ml of distilled water to control group of albino rats, omeprazole 20 mg/kg (reference group) and 100, 200, 400 and 800 mg/kg of the anti-ulcer herbal mixture to the test groups for 14 days and ulcers were induced in the rats by oral administration of 1 ml absolute ethanol.

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Results: secondary metabolites like flavonoids, saponins, tannins, alkaloids and anti-oxidant like phytic acid and oxalate were quantitatively present. There was no death up to an acute maximum dose of 8 g/kg of the herbal mixture. The Pre-treatment groups of animals both in gross examination and histopathological assessment exhibited significant protection from ethanol-induced gastric mucosal injury comparable with omeprazole group.
Conclusion: the study has shown that the extract from the herbal mixture could significantly protect the gastric mucosa against ethanol-induced ulcer.

Keywords: Anti-ulcer herbal mixture; phytochemicals; toxicity; ulcer; gastrointestinal tract.

1. INTRODUCTION

An herbal mixture involves mixing of two or more different herbal extracts together to achieve prevention or treatment of a specific health challenge. The Chinese herbal preparation named PC SPES is an example. It consists of extracts from eight herbs for the treatment of prostate cancer. Darynkiewkz et al. [1] reported that the mixture is an effective modality that alleviates some symptoms in advanced prostate cancer in a significant proportion of patients including the cases that failed conventional therapy. Peter et al. [2] evaluated a Chinese herbal mixture, Aden-1, a mixture of extracts from *Lonicera japonica* flower buds, *Forsythia suspensa* fruits with crude flavonoids from *Scutellaria baicalensis.* The mixture is for the treatment of respiratory disease. A comparable group was subjected to standard antibiotic therapy. The group treated with Aden-1 responded as well as the group on antibiotic therapy. It was concluded that Aden-1 has both antibacterial and antiviral effect.

The anti-ulcer herbal mixture in this current study contains; *Ageratum conyzoides, Vernonia amygdalina* and *Citrus aurantiifolia* and it is known to be a potent anti-ulcer herbal therapy.

Ageratum conyzoides is called goat weed in English, Imi-esu among Yorubas, Alkaura-tuturuwa in Hausa and Obiarakara among Igbo speaking people of Nigeria. It belongs to the family of Asteraceace. It is a pan-tropical herb. Its toxicity has not been well studied but the extracted oil has a powerful nauseating odour. It is an erect aural branched, slender, hairy and aromatic herb which grows to approximately 1m in height. It is used as fish feed with a long history of traditional medicinal uses in several countries of the world [3]. Its methanolic extracts were reported by Oladejo et al. [5] to have healing properties [4]. Excision was made on dorsolateral flank of rats. The wounds were packed with *Ageratum conyzoides* dressing. *Ageratum conyzoides* treated wounds showed fewer inflammatory cells histologically, more fibrosis, greater wound contraction and significantly fewer fibroblasts than honey treated wounds. Lorenzi described the fruit as an achene with an aristae pappus easily dispersed by wind while Nwachukwu et al. [6] referred to its seeds as positively photoblastic whose viability greatly diminish within 12 months. Durodola revealed range of chemical compounds analyzed from *Ageratum conyzoides* as alkaloids, flavonoids, chromenes, benzofurans and terpnoids [7]. He further reiterated its medicinal use to include treatment of pneumonia, wounds and burns. Borthakur and Baruah expatiated its use as a bacteriocide, anti-dysentric and antilithic herbal drug [8] while. Ekundayo reported the use of aqueous extract of the plant as a bacteriocide in Asia, South America and Africa [9]. Githen before 1948 highlighted the uses of *Ageratum conyzoides* to include the use as purgative, febrifuge, for opthalmia, colic treatment of ulcers and wound dressing.

Vernonia amygdalina, a member of the Asteraceae family, is a small shrub that grows in tropical Africa. It typically grows to a height of 2–5 m (6.6–16.4 ft). The leaves are elliptical and up to 20 cm (7.9 in) long. The leaves are green with a characteristic odour and a bitter taste. No seeds are produced and the tree has therefore to be propagated through cutting. Its bark is rough and it is commonly called bitter leaf in English because of its bitter taste, Ewuro in Yoruba, Shakwa shuwaka in Hausa and Onugbu among Igbo speaking people of Nigeria. Other African common names include grawa (Amharic), Etidot (Ibibio), Ityuna (Tiv), Oriwo (Edo), Mululuza (Uganda), Labwori (Acholi), Olusia (Luo) and Ndoleh (Cameroon). In Nigeria, *V. amygdalina* is used for food and medicinal purposes. The roots and twigs are used for ulcers, abdominal and other gastrointestinal problems in humans while the decoctions from the leaves are used as anti-malaria in Guinea and as cough remedy in Ghana [10-12]. It is widely described by livestock

farmers as a potent anti-helmitic [13]. The cooked leaves are a staple vegetable in soups and stews of various cultures throughout equatorial Africa. Anibijuwon assessed the physiologically active principles found in *Vernonia amygdalina* extract. Their results showed the presence of terpenoids, tannins, alkaloids, saponins and glycosides [14]. They confirmed that the aqueous extract of *Vernonia amygdalina* had very high value of minimum inhibitory concentration (MIC) on both *Staphylococcus aureus and Streptococcus mutans.*

Citrus aurantiifolia belongs to the family Rutaceae. It is used against nausea, indigestion and constipation. It exhibits activities for cold fevers, sore throats, sinusitis and bronchitis as well as helping asthma [15]. The Key lime (*Citrus aurantiifolia*) is a citrus hybrid (*C. micrantha* x *C. medica*) with a globose (spherical shaped) fruit, 2.5–5 cm in diameter (1–2 in), that is yellow when ripe but usually picked green commercially. It is smaller and seedier, with a higher acidity, a stronger aroma, and a thinner ring, than that of the Persian lime (*Citrus latifolia*). It is valued for its unique flavour compared to other limes. The name comes from its association with the Florida Keys, where it is best known as the flavouring ingredient in Key lime pie. It is also known as West Indian lime, bartender's lime, Omani lime, or Mexican lime, the last classified as a distinct race with a thicker skin and darker green colour. Philippine varieties have various names, including dayap and bilolo. Khanc evaluated the antimicrobial efficacy of *Citrus aurantifolia* leaves against some micro-organisms-bacteria and fungus were *Staphylococcus aureus, Esherichia coli, Klebsiella pneumonia, Pseudomonas* spp*, Aspergillus niger, Aspergillus fumigates, Mucor* spp *and Penicillium.* It was indicated by these researchers that the hydro-alcoholic extract of *Citrus aurantifolia* leaves possess good antibacterial and antifungal activity [15]. This confirms the presence of bioactive compounds and the great possibility of being used in primary health care.

2. MATERIALS AND METHODS

2.1 Collection and Extraction of the Anti-Ulcer Herbal Mixture

The anti-ulcer herbal mixture contains whole plant of *Ageratum conyzoides*, roots of *Vernonia amygdalina* and *Citrus aurantifolia*. These were collected from farms in Idogun and Owo, Ondo state. They were registered with the Department of Botany, Faculty of Life Sciences, University of Benin, Benin City with registration numbers UBH_A286, UBH_C288 and UBH_V287.

They were washed in running water to remove all dirt and air-dried for 4weeks [16]. They were separately crushed in a clean mortar with pestle and later reduced to fine powder by grinding using domestic electric blender. The granules were measured as specified by the herbal practitioner, 35 g of *Ageratum conyzoides*, 7.5 g of *Vernonia amygdalina* and 7.5 g of *Citrus aurantifolia*. They were thoroughly mixed together.

The powdered plant mixtures (1220 g) were weighed (*Ageratum conyzoides* 854 g, *Citrus aurantifolia* 183 g and *Vernonia amygdalina* 183 g) and dissolved in 15 litres of 70% alcohol. They were left at room temperature for 3 days, filtered using whatman filter paper (No 1). The filtrates were concentrated at 40°C in water bath for 2 weeks. The extracts were packed in brown colour sample bottles and stored in a refrigerator. The extract was reconstituted in distilled water for experimental protocol [16].

2.2 Extraction and Phytochemical Analysis of the Anti-Ulcer Herbal Mixture

The extraction was done in solvents; water, 70% alcohol, methanol, acetone and chloroform. The powdered plant mixtures (50 g) were soaked in each of the solvents (200 ml). The solutions were left at room temperature for three (3) days. The contents were filtered using whatman's filter paper (No. 1). The filtrates were subjected to quantitative analysis after vapourization.

2.2.1 Oxalate determination [17]

To about 1 g of the sample 75 ml of 1.5N H_2SO_4 was added and the solution was carefully stirred using a magnetic stirrer for 1 hour before being filtered using Whatman (No. II) filter paper. From the filterate, 25 ml was measured and titrated when hot against 0.1 N $KMnO_4$ solution to a faint pink colour end point.

2.2.2 Phytate determination [18]

About 4 g of the sample was soaked in 100 ml of 2% HCl for 3 hours. It was then filtered through Whatman filter paper (No II) out of which 25 ml was placed in 250 ml conical flask followed by

the addition of 5 ml of 0.3% Ammonium thiocyanate solution as indicator. Distilled water (53.5 ml) was added to give the desired acidity. This was then titrated with standard iron (III) chloride solution which contains about 0.00195 g of iron per ml until a brownish yellow persists for 5 minutes.

% Phytic Acid = (8.24T × 0.1 × wt of sample)

Where, T = titre value.

2.2.3 Determination of flavonoids

This was determined according to the method of Harborne, five grams of the sample was boiled in 50 ml of 2M HCl solution for 30 min under reflux. It was allowed to cool and then filtered through whatman (No 42) filter paper. A measured volume of the extract was treated with equal volume of ethyl acetate starting with drop. The flavonoid precipitated was recovered by filtration using weighed filter paper. The resulting weight difference gave the weight of flavonoid in the sample [19].

2.2.4 Saponin determination

Twenty grams of each sample were put into a conical flask and 100 cm^3 of 20% aqueous ethanol were added. The samples were heated over a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 ml 20% aqueous ethanol. The combined extracts were reduced to 40ml over water bath at about 90°C.The concentrate was transferred into a 250ml separatory funnel and 20 ml of diethylether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded, the purification process was repeated, n-butanol (60 ml) was added and the combined n-butanol extract was washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath, after evaporation the samples were dried in the oven to a constant weight and the saponin content was calculated as a percentage [20].

2.2.5 Tannin determination by van- burden and robinson method

500 mg of the samples was weighed into a 100 ml plastic bottle and 50 ml of distilled water was added and shaken for 1 hr in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtrate was pipetted out into a test tube and mixed with 2 ml of 0.1 M $FeCl_3$ in 0.1 N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm [21].

2.2.6 Alkaloid determination

Weigh 1 g of finely ground powered sample into a flask. Add 40 ml of 10% acetic acid in ethanol (10 ml of acetic acid into 90 ml of absolute ethanol). It was allowed to stand for 4hrs at 28°C. Filter and Heat the filtrate in a hot plate to one-quarter of its original volume by evaporation. Treat the remaining concentrate with drop wise addition of 5 ml Conc. Ammonium hydroxide (until all the alkaloid was precipitated). Weigh a dried filter paper (W_1). Filter the precipitate through the pre-weighed filter paper. Wash the filter paper while still inside the funnel with 2% ammonia solution. Dry in the oven at 55°C. Allow to cool and weigh the dried filter paper as (W_2). Alkaloid content was calculated [19].

In summary, phytochemicals and antioxidants like saponin, alkaloids, flavonoids, tannins, oxalate and phytic acids were recovered.

2.3 Acute Toxicity Assessment

Assessment for the oral median lethal dose (LD50) was carried out with six groups of six animals. The animals of both sexes were randomly distributed into each of the groups and were deprived of food overnight, the control group was given 2 ml of distilled water while other received doses as follows: 1 g/kg, 2 g/kg, 4 g/kg, 6 g/kg and 8 g/kg of the anti-ulcer herbal mixture. After the administration of the extracts, animals were observed for death, and symptoms of toxicity within three days initially and then for 30 minutes each day for another seven days. Signs and symptoms observed include; fast breathing and reduced locomotion. The LD_{50} result was then recorded Acute toxicity assessment was calculated using arithematic formula specified by Angalabiri-Owei and Isirima [22].

2.4 Preparation of Test Animals

The animals (both females and male rats) used were bred locally in the animal house of the Department of Anatomy, University of Benin, Benin City. They were also acclimatized for two weeks in the animal house. Animals were housed in standard plastic cages, fed with

standard pellet diet (Bendel Feeds and Flour Mill, Limited, Ewu, Nigeria) and allowed free access to water. All animals received humane care in accordance with international guidelines [23]. The animals were used for both acute toxicity tests as well as for ulcer efficacy test.

2.5 Anti-Ulcer Activities of the Herbal Mixture against Ethanol Induced Gastric Mucosa Injury in Albino Rats

The study was carried out to confirm the ulcerogenic efficacy of plants content against ethanol-induced gastric mucosal ulcers in rats. Six groups of locally bred albino rats were used. Each was orally treated respectively with distilled water (ulcer control group), omeprazole 20 mg/kg (reference group) and 100, 200, 400 and 800 mg/kg of the anti-ulcer herbal mixture (experimental group). Rats were sacrificed one hour after oral administration of 1ml absolute ethanol to induce injury in the gastric mucosa. Number of ulcers (UN) in the rats' stomach were counted using magnifying glass, percentage ulcer (UP) was determined by calculating the percentage of rats with ulcer. Ulcer severity (US) was scored as follows: Normal stomach…..(0), Red colouration….(0.5), Spot ulcer….(1) Heamorhagic stress…..(1.5), Deep ulcer…(2.0), Perforations….(3.0) Ulcer index=(UN+US+ UP) x 10 raise to power-1 [24].

2.6 Histological Assessment

The tissue specimens were fixed in neutral buffered formalin and processed in Thermo Scientific Spin Tissue Processors STP 120. The tissues were treated for half hour each in three baths of 50% alcohol and were transferred into three baths of 80/20 Ethanol/IPA for half hour each and later treated in three baths of neat Isopropyl alcohol (IPA) for 1 hour each. The samples were allowed to drain for 2 hours before they were immersed in two baths of cell path wax at 56°C for 1½ hours each [25]. The tissues were embedded in cell path paraffin wax, sectioned and stained using Haematoxylin and Eosin stains.

3. RESULTS

Results from this study are presented below:

Results of phytochemical analysis are shown in Table 1 shows various phytochemicals and their corresponding extracts, while Figs. 1 and 2 shows the gross appearances of stomach and ulcer count of rats treated with the anti-ulcer herbal mixture respectively. Plates 1 to 12 are photomicrographs showing the histopathological alterations in the stomach and small intestine of albino rats as a result of the anti-ulcer activities of the anti-ulcer herbal mixture under study. Control Plates 1, 7 showed various histopathological alterations while Omeprazole Plates 6, 12 and most test plates showed normal histology with few histological alterations seen.

4. DISCUSSION

Peptic ulcers are caused when the natural balances between the aggressive factors of acid, pepsin, defensive mechanism of mucus, bicarbonate, mucosal turnover and blood supply are disturbed [26]. A number of synthetic drugs are available to treat ulcer. However, they are expensive and produce some side effects like arrhythmias, impotence, gynaecomastia, arthralgia, hypergastronemia and haemopoetic changes [27]. In the present situation of economic decline, traditional medicine is enjoying an enviable patronage. It has maintained greater popularity all over developing countries and the use is rapidly on the increase. Plants are the basis of life on earth and are central to man's livelihood. Plants and phyto constituents are better choice to treat diseases than the allopathic drugs. This medicinal property is attributed mainly to the presence of secondary metabolites like, flavonoids, saponin, tannins, alkaloids, phytic acid and antioxidant micronutrients like copper, manganese and zinc [28]. Most of the drugs used in primitive medicine were originated from plants and are relatively free from toxic effects.

Table 1. Results of phytochemical analysis of plants mixture

Extracts	Mg/g oxalate	% phytic acid	% flavonoids	% saponin	% tannin	% alkaloid
Water	0.27	2.43	3.05	2.86	0.014	9.98
70% Alcohol	0.27	2.88	1.83	3.11	0.116	18.06
Methanol	0.18	3.17	2.54	1.09	0.024	17.73
Acetone	0.09	2.47	3.43	3.65	0.129	11.99
Chloroform	0.18	3.75	1.32	2.54	0.112	20.18

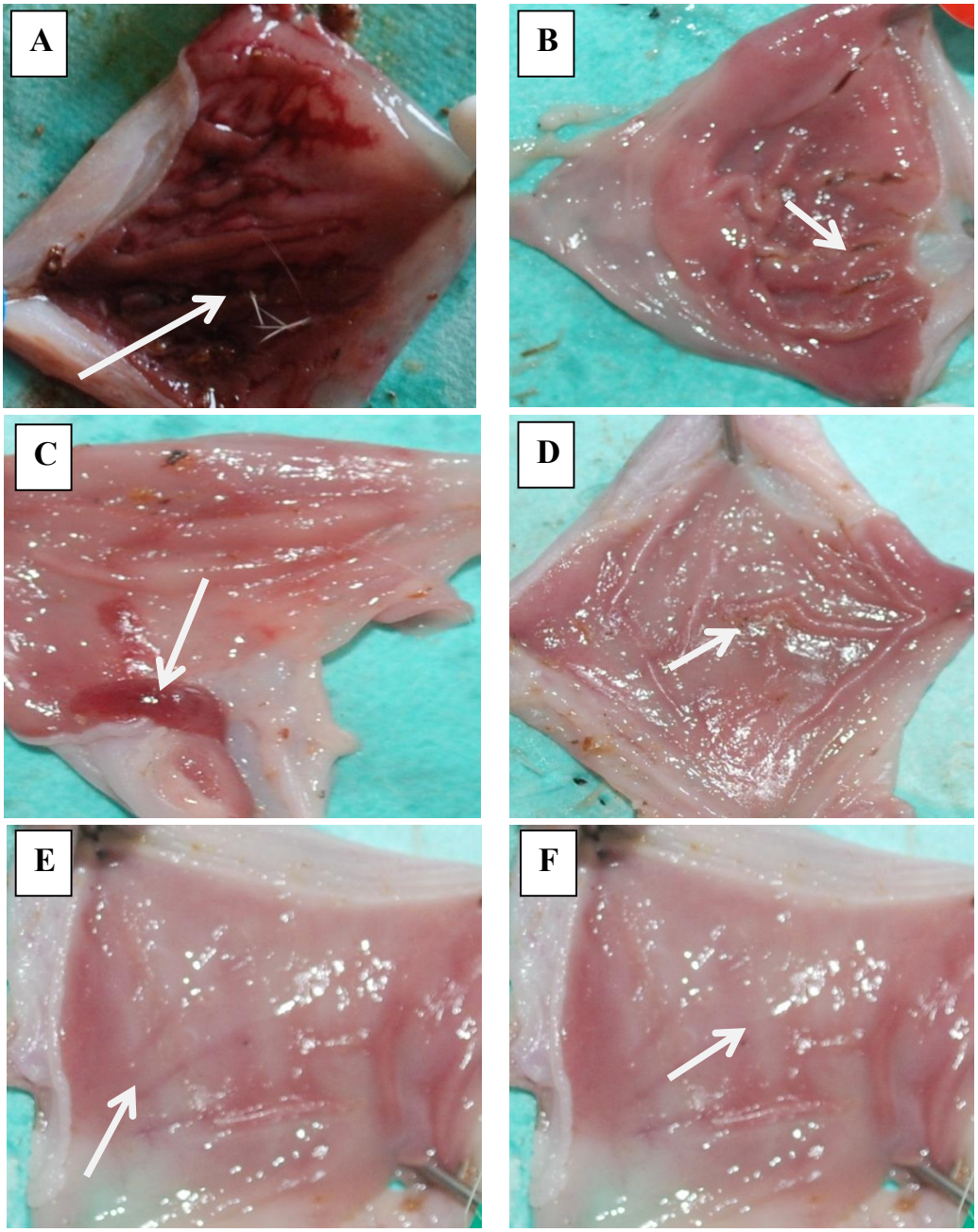


Fig. 1. Gross appearance of stomach of rats treated with pure plant mixture (Arrows (A) deep ulcers (B) spot ulcers (C) red coloration (D-F) normal coloration) A-F(Control, 100, 200, 400, 800 mg/kg and 20 mg/kg Omeprazole groups respectively)

The anti-ulcer herbal mixture used for this study contains 0.09 mg/g oxalate. This low concentration makes the herbal mixture safe for use since high level of oxalate in herbal extracts may increase the risk of developing kidney stones [29]. There is also the presence of phytic acid at concentration of 2.76%. Sudheer et al. [30] confirmed that phytic acid confers antioxidant activity and cytoprotection on gastric mucosa. Flavonoids have been reported to act in the gastrointestinal tract having anti-ulcer and anti-oxidant properties. They are among the cytoprotective material for which anti ulcerogenic efficacy has been extensively confirmed [31]. This protects the gastric mucosa against a variety of ulcerogenic agents via several

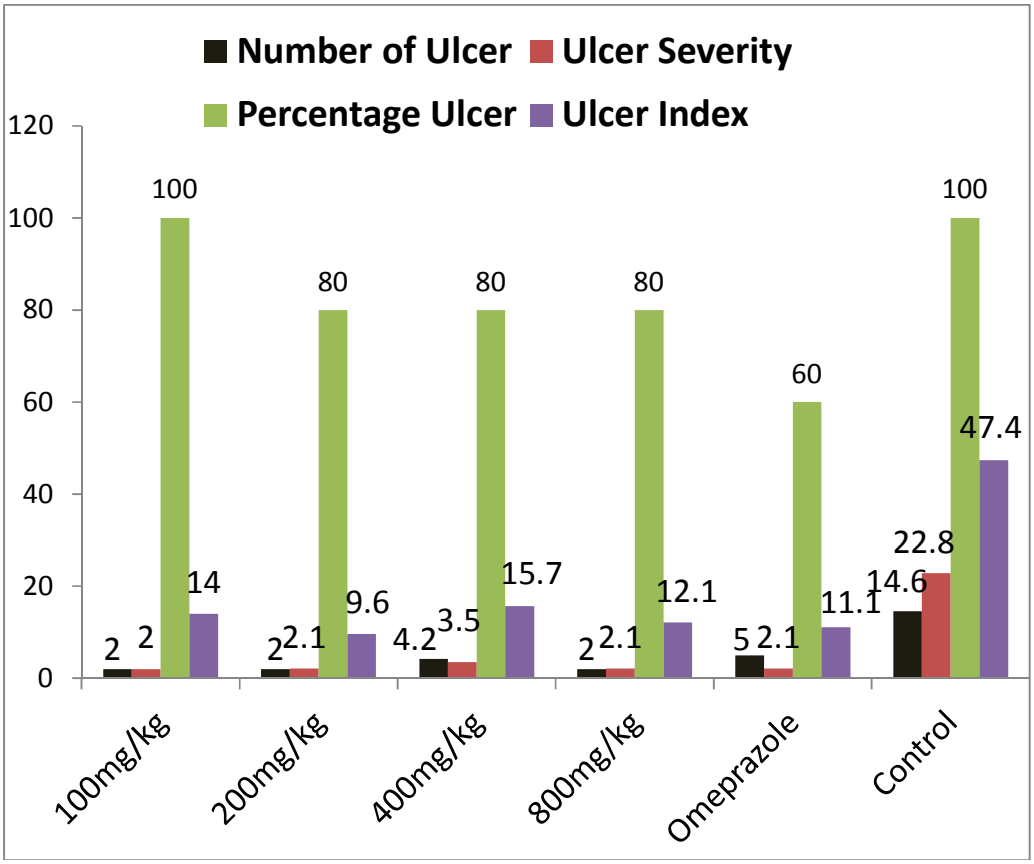
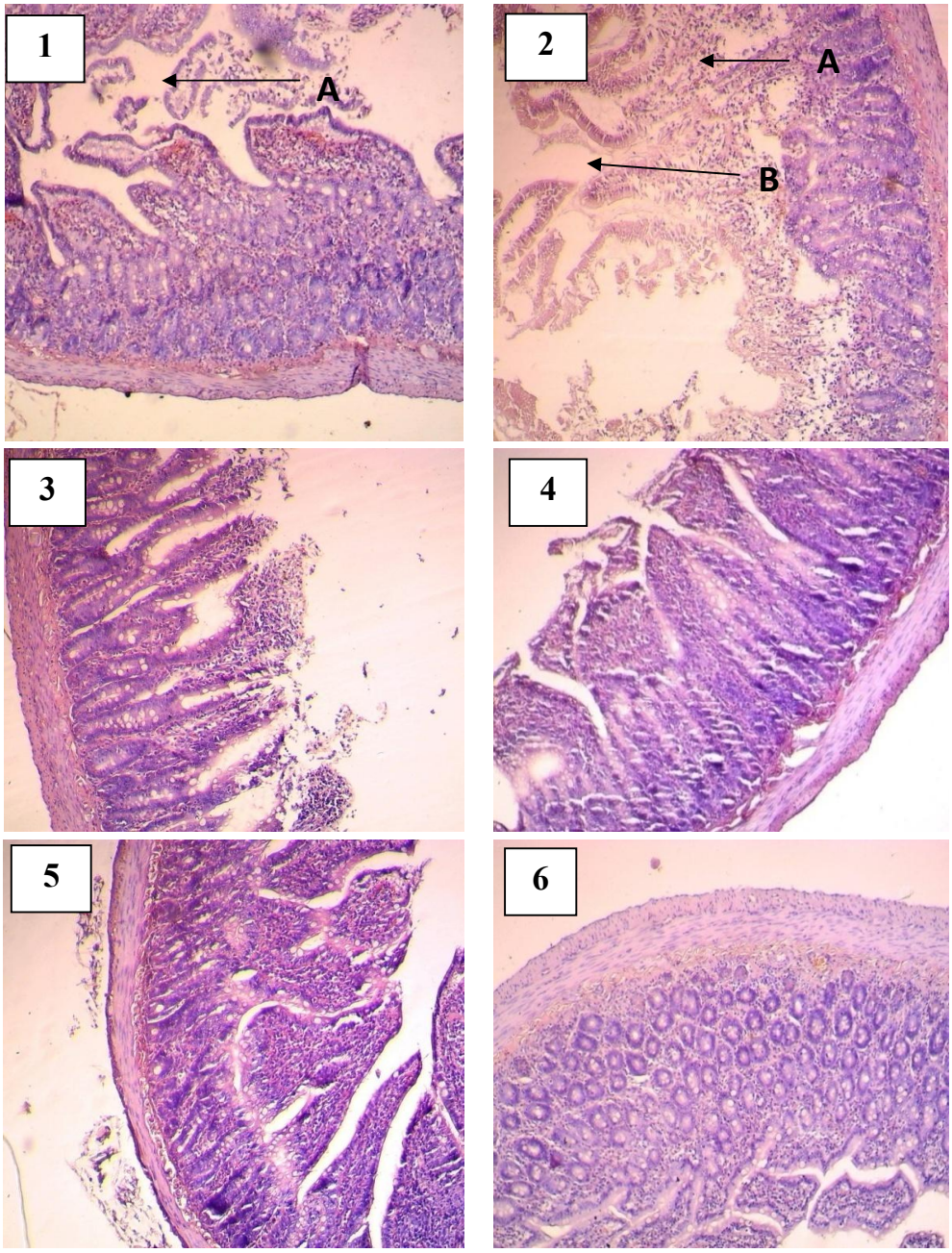


Fig. 2. Ulcer count pure plant mixture

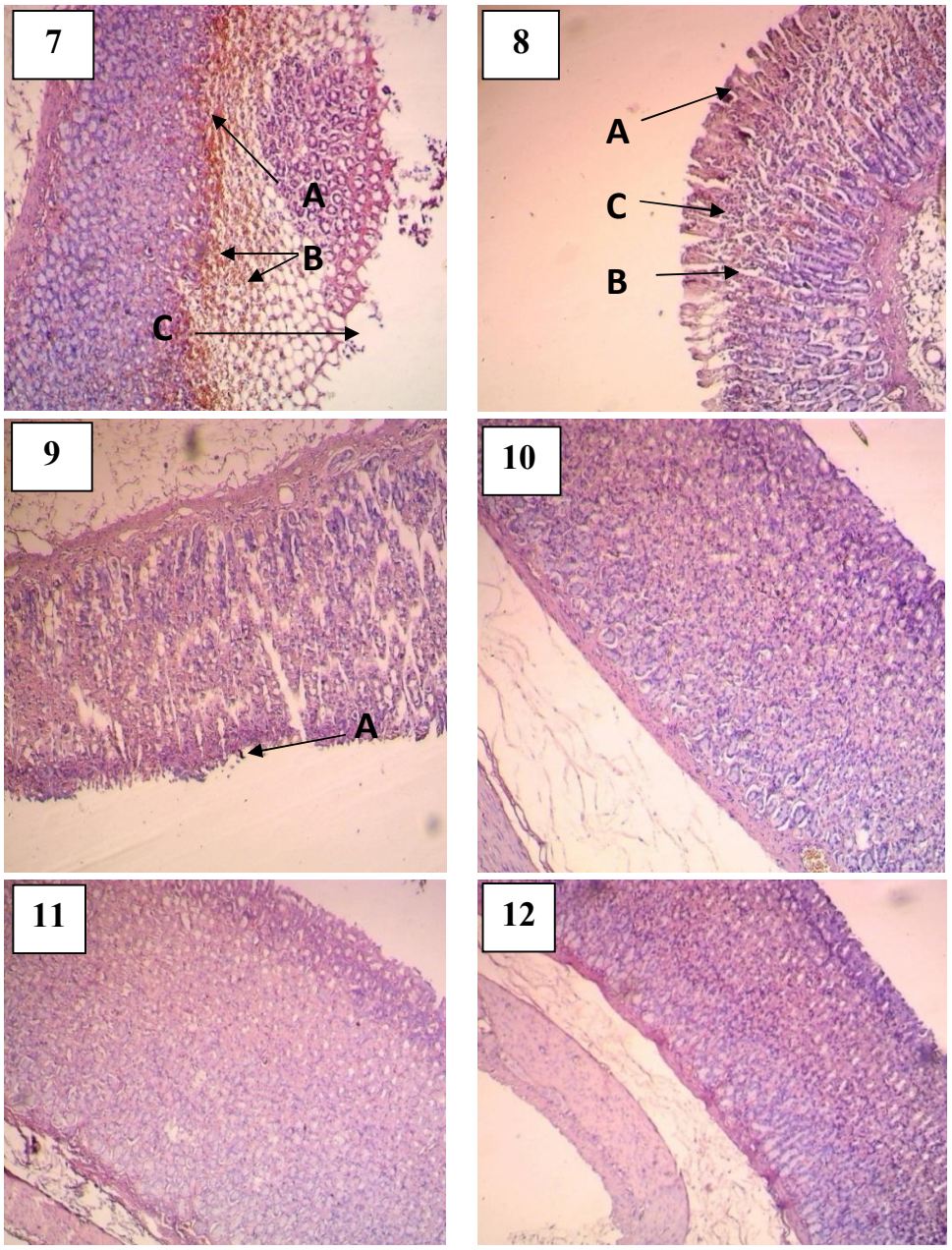
mechanism of action mainly; free radical scavenging, anti- oxidant properties, increased mucus production, anti-secretory action and the inhibition of the helicobacter pylori growth [32]. The anti-ulcer herbal mixture for this study contains flavonoids at 1.83%. Ma and Liu found out that *Conyza blinni* (CB) is a type of natural plant which contains triterpenoidal saponins and has gastric mucous protection activity [33]. At concentrations 5, 10, 20 mg/ml, CB was found to have a proportional protection activity against acute gastric ulcer induced by ethanol, the efficacy was compared with colloidal bismuth sub citrate. The concentration of saponin in the herbal mixture was 3.11%. Tannin was reported by Aguwa and Nwako to prevent ulcer development due to its protein precipitating and vasoconstriction effects [34]. Tannins precipitate mucoprotein on the ulcer site thereby forming an impervious layer over the lining which hinders induced gastric ulcers in rats as evidenced by the gut secretions and protects the underlining mucosa from reduction in the ulcer score [35]. This metabolite is present in the herbal mixture at concentrations of 0.0116%. Jia and Changping revealed that dried fruit *of Evodia ruteacarpia* is used for treating disorders of gastro intestinal tract in traditional oriental medicine [36] and that Asahna and Kash isolated alkaloids from the said fruit. In this s tudy, alkaloids were found at 18.06% in the herbal mixture. The presence of all these secondary metabolites (phytic acid, flavonoids, saponin, tannin and alkaloids) confirms the efficacy of the herbal mixture in protecting the gastric mucosa of albino rats from the deleterious effect of ethanol. This is consistent with the earlier findings by Sabiha that phenolic compounds that constitute the largest group of plants secondary metabolites have health – promoting characteristics [37]. The researchers noted that polyphenols have a role in the prevention of Peptic ulcer. [38] in 'Plants and Phytochemicals for peptic ulcer' observed that various plants like *Anogeisus latifolia, Alchornea castaneafolia, Ulteria salicifolia, Solalium nigrum, Ocimum sanctum, Asparajus racemosus, Scoparia dulcis Bysonima crassa* etc and their pytoconstituent proved active in anti-ulcer therapy.



Plates 1-6. Small Intestine (1) A, Tissue edema (2) A, Inflammation B, Tissue edema (3) Normal (4) Normal (5) Normal (6) Normal 1-6 (Control, 100, 200, 400, 800 mg/kg Plant mixture and 20 mg/kg Omeprazole groups respectively). H&E x400

The present study revealed the efficacy of the anti-ulcer herbal mixture under study. The gross evaluation of gastric lesions showed that rats pre-treated with it prior to administration of ethanol had significant gastric mucosal protection from deleterious effect of the absolute alcohol. The ulcer severity was greatly reduced when compared with the control group. At 800 mg/kg of the herbal mixture there was the flattening of the mucosa lobes of the rats treated with them suggesting protection of the site from the effect of absolute alcohol and suppression of formation of ulcers [39]. Histological sections of gastric mucosa of rats pretreated with 100 and 200 mg/kg of the herbal mixture, showed mild ulcers and no ulceration in rats in those

pretreated with 400 and 800 mg/kg as compared to those in the control groups which showed extensive ulceration of the gastric mucosa while rats in omeprazole group had their gastric mucosa mildly protected from ulceration. The intestines of rats dosed with 200-800 mg/kg of the anti-ulcer herbal mixture showed normal histological architecture when compared with the control, connoting protective effect of the mixture from the deleterious action of absolute alcohol. At the lowest concentration of 100 mg/kg of the anti-ulcer herbal mixture, the intestine showed mild inflammation and tissue edema.



Plates 7-12. Gastric Atrum (7) A, Hemorrhage B, Dense infiltration by mixed inflammatory cells C, Extensive ulceration (8) A, Ulceration B, Edema C, Infiltration by mixed inflammatory cells. (9) A, Ulceration (10) No ulceration, healing taking place (11) No ulceration, normal histology (12) Healing, No ulceration. 7-12 (Control, 100, 200, 400, 800 mg/kg Plant mixture and 20 mg/kg Omeprazole groups respectively) H&E x400

5. CONCLUSION

In conclusion, the study has shown that administration of the anti-ulcer herbal mixture under study could significantly protect the gastric mucosa against ethanol-induced injury. The phytochemical analysis showed presence of alkaloids, tannins, low oxalate content, flavonoids, saponin and phytic acid which might have contributed to the cytoprotection of gastric mucosal. The calculated lethal doses (LD_{50}) were high and therapeutically safe by oral route.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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