Assessment of gastroprotective activity of aqueous leaf extract of *Ageratum conyzoides* L.: Role of mucous cells, anti-apoptotic (Bcl-2) and tumor suppressor (p53) proteins

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ABSTRACT

Introduction: Generally, factors that up-regulate gastric mucosal protective factors or down-regulate aggressive factors contribute to the maintenance of mucosal integrity. This study was done to assess the role of mucous cells, Bcl-2 and p53 proteins during the gastroprotective activity of aqueous extract of *Ageratum conyzoides*.
Methods: The phytochemistry of *A. conyzoides* extract was analyzed using a gas chromatography-mass spectrometer. Animals were subdivided into five groups, including non-treated normal control group A, non-treated test control group B, and treated groups C-E (Pre-treated with 100, 300, and 500 mg/kg *A. conyzoides*, respectively for 28 days). After the treatment period, pyloric-ligation was used to induce mucosal injury. Gastric tissues were harvested, grossly examined, and processed for histological, histochemical, and immunohistochemical studies. Stained sections were examined and quantified using image-J software. The data were analyzed using IBM-SPSS (version 23), and comparisons were checked via *t* test and analysis of variance.
Results: Mild mucosal erosion was observed in the treated groups, but intense erosion was prominent in the test control animals. There was an insignificant increase in mucous cells, a significant ($P < 0.05$) increase in Bcl-2 expression without a significant increase in p53 expression in gastric mucosa of pre-treated animals compared to normal control. Gastric mucosa of test control showed a significant ($P < 0.05$) decrease in mucous cell count and Bcl-2 expression with a significant concomitant increase in p53 expression.
Conclusion: Increased mucous cell population and reciprocal expressions of Bcl-2 and p53 proteins in the gastric mucosa of animals highlighted the sub-cellular mechanisms of gastroprotective activity of *A. conyzoides*.

Implication for health policy/practice/research/medical education:
Ageratum conyzoides L. extract exhibited gastroprotective effects by down-regulating the gastric mucosal aggressive factor and up-regulating the protective factor. Thus, phytochemicals derived from the plant extract might serve as a reliable source of therapeutic agents that can be used to manage gastric ulcers.
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Introduction

Gastric mucosal protection is often the result of a balance between the aggressive factors that disrupt the mucosal integrity leading to injury or ulceration and the protective factors that preserve the gastric mucosa against injury or ulceration (1). These gastric mucosal aggressive factors include endogenous factors (such as increased gastric acid secretion, ischemia and bile acids), exogenous factors (such as bacteria, ethanol, non-steroidal anti-inflammatory drugs) as well as oxidative stress (2,3). Conversely, the primary protective factors include mucus and bicarbonate ions produced by mucous and parietal cells in the gastric mucosa, respectively. The mucus and bicarbonate ions create a viscous layer of gel that helps ensure a near-neutral pH at the gastric mucosal surface protecting mucosal cells from the activity of digestive

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enzymes (such as pepsin) and the erosive effect of acidic gastric secretions (4). Other factors that play a vital role in gastric mucosal protection include mucosal microcirculation, nitric oxide, prostaglandins, epidermal growth factors, and cyclooxygenase (5-7). In general, exogenous factors that stimulate the protective factors of gastric mucosa and their protective effects and/or down-regulate the aggressive factors and their erosive effects contribute to gastric mucosal protection.

Several medicinal plants used in folklore medicine have been described to exhibit varying pharmacological or therapeutic properties, including anti-ulcerogenic or gastroprotective activity. *Aspilia africana, Ocimum gratissimum, Moringa oleifera,* and *Ageratum conyzoides* plants have been documented to have gastroprotective potentials against various gastric mucous (8-11). The *Ageratum conyzoides* Linnaeus is a tropical plant of the Asteraceae family, which has been in wide use for ages in different parts of the world, including Asia, South America, and Africa for varieties of ethnopharmacological applications (12,13). These include treatment of stomach ailments, gynaecological diseases, leprosy, high fever, rheumatism, infertility, and diabetes. Also, its usage as an anticoagulant, analgesic, anti-asthmatic, antispasmodic, haemostatic, anti-inflammatory, and antipyretic agent has been reported (14-17). However, in order to determine the bio-safety of medicinal plants especially those with wide-ranging therapeutic applications like *A. conyzoides*, assessing the subcellular mechanisms of their therapeutic activities remains a critical part. In the current study, we assessed the role of mucous cells and apoptotic markers such as Bcl-2 and p53 proteins during the gastroprotective activity of aqueous leaf extract of *A. conyzoides*.

Materials and Methods
Sources of chemical reagents
The reagents used for this study were procured from Bristol Scientific Company Limited Lagos, Nigeria, and Abcam, Cambridge, Massachusetts, USA.

Collection of study plant material
Fresh samples of the *A. conyzoides* plant were collected from the suburb of Isihor community, Benin City, Nigeria (6° 20′ 17.34″ N and 5° 37′ 32.70″ E) and authenticated at the Herbarium of the Department of Plant Biology and Biotechnology, University of Benin, Nigeria, and documented with an identification number – UBH$_A$344. Afterward, the plant material sufficient for use in the study was collected; leaves were detached, air-dried, pulverized, and used for subsequent extraction process.

Phytochemical profiling by gas chromatography-mass spectrometer (GC-MS)
The pulverized leaves were weighed and macerated in distilled water for 72 hours with intermittent agitation, filtered, and evaporated till dryness with the aid of a rotary evaporator to obtain aqueous extracts residue (18). The extract was stored in a refrigerator at 4°C before usage. For phytochemical analysis, 0.1 g of extract was dissolved in 1 mL methanol and filtered to obtain a clear, coloured filtrate used for GC-MS phytochemical analysis.

GC-MS phytochemical analysis
The GC-MS phytochemical analysis was performed using gas chromatography (7890A series) coupled to TSQ quantum XLS mass spectrometer with an injector device (7683B series). The pressure and temperature of column heater were set at 10.15 psi and 250°C, respectively. The average velocity was 66.45 cm/s and the carrier gas was Helium with 1 mL/min flow rate. The electron impact (EI) of the MS was set at 70 eV, scan interval set at 0.5 seconds; the injection volume was 1 μL, scan mass ranged from 50-650 amu, and polarity was positive. The phytochemical constituents of extract were separated on a column TG-5ms (with dimensions – 30 mL X 0.25 mmID X 0.25 μm film thickness) and flame ionization detector (at 250°C) was used to determine the percentage composition of phytochemicals. For identification, mass spectra of phytochemicals were compared with a database obtained from National Institute of Standard and Technology (NIST).

Experimental animals and grouping
Twenty-five adult Wistar rats used for this study weighed between 170 g-200 g. The animals were grouped into five groups (A-E) comprising five animals (n = 5) in each group. Group A represented the normal control (non-treated and non-induced) group administered with distilled water (5 mL/kg body weight). Group B represented the test control (non-treated but induced) group also administered with distilled water (5 mL/kg body weight). Groups C-E represented the pre-treated groups administered with 100, 300, and 500 mg/kg extract, respectively. The selected dosage levels of plant extract were considered safe without toxic effects (10). Administrations were done orally using orogastric gavage connected to a calibrated hypodermic syringe and lasted for 28 days.

Experimental gastric mucosal injury induction through pyloric ligation method
After the period of administration, experimental animals were subjected to 24-hour fasting but allowed access to drinking water. They were anesthetized with an injection of ketamine/xylazine (50 mg/kg at 1:1), intraperitoneally. Afterward, a midline incision was created on the abdominal wall of the experimental animals to gain access to the gastric pylorus. The pylorus was ligated and the abdominal incision closed. The experimental animals were subjected to a 5-hour observatory period to induce gastric mucosal injury (19). After the observatory period, they were sacrificed with their stomach tissues harvested for further study and processing (19).

Macroscopic examination of gastric tissues

A smooth cut was made along the greater curvature of the stomach tissues, the gastric secretion was emptied, and the internal aspect of the tissue was rinsed in distilled water. The gastric mucosal surface was photographed to document observable mucosal lesions following the prolonged exposure to the acidic gastric juice during the observatory period. The ulcer index was evaluated as the ratio of total lesion length per number of animals in a group (20).

Processing and sectioning of gastric tissues

The gastric tissues harvested from the experimental animals were transferred into 10% neutral buffered formalin and fixed for 24 hours. The tissues were dehydrated using ascending grades of alcohol (comprising of 70%, 90%, and absolute alcohol), cleared in xylene, and embedded in paraffin wax to form tissue blocks. The gastric tissue blocks were cut into sections at 3 μ and 5 μ thicknesses using a semi-automatic microtome, and the tissue sections were mounted on microscope slides.

Staining of tissues

The 3 μ tissue sections were used for the histological and histochemical staining using Haematoxylin and Eosin (H & E) and periodic acid-Schiff (PAS) techniques, respectively while the 5 μ tissue sections were used for immunohistochemical staining using horseradish-peroxidase-3,3-diaminobenzidine (HRP-DAB) technique.

H & E staining technique

The tissue sections were dewaxed in xylene, hydrated with descending grades of alcohol (100%, 90%, and 70% alcohol) and distilled water. They were stained in Haematoxylin for 10 minutes, washed in running water, differentiated in 1% acid alcohol, blued in Scott's tap water, and rinsed in water. The tissue sections were counterstained with Eosin for 3 minutes, rinsed in water, and dehydrated with ascending grades of alcohol (70%, 90%, and 100% alcohol), cleared in xylene, and mounted with distrene polystyrene xylene (DPX) (21).

PAS staining technique

The tissue sections were dewaxed in xylene and hydrated with descending grades of alcohol to distilled water. They were treated with 0.5% Periodic acid, rinsed in distilled water, treated with Schiff's reagent, and rinsed in distilled water. The tissue sections were counterstained in Haematoxylin, rinsed in water, dehydrated with ascending grades of alcohol (70%, 90%, and 100% alcohol), cleared in xylene, and mounted with DPX (22).

HRP-DAB staining technique

The tissue sections were hydrated, and antigen retrieval was done using citric acid solution (pH 6.0) in a microwave at 100 Watts for 15 minutes. They were equilibrated under running water to displace the hot citric acid. The tissue sections were exposed to peroxidase block and rinsed in phosphate buffer saline (PBS) mixed with Tween 20. Other proteins in sections were blocked with Nevocastra protein block and sections rinsed with PBS. The tissue sections were incubated in primary antibody (prepared with 1 in 100 dilution ratio), rinsed with PBS, treated with secondary antibody, and rinsed with PBS for 3 minutes. The polymer was added and allowed to polymerize for 15 minutes and tissue sections rinsed twice with PBS. The tissue sections were treated with 3,3-diaminobenzidine (DAB) substrate (prepared in 1/100 dilution ratio with the DAB substrate), rinsed with water, counterstained with haematoxylin, rinsed with water, dehydrated with ascending grades of alcohol (70%, 90%, and 100% alcohol), cleared in xylene, and mounted using DPX (23).

Photomicrography and analysis of photomicrographs

Microscopic examinations of stained tissue sections were done and photomicrographs were produced. Photomicrographs of H & E sections were examined to determine observable gastric mucosal surface erosion. Photomicrographs of PAS sections and HRP-DAB sections were quantitatively analyzed using an ImageJ software (National Institutes of Health, Bethesda, MA, USA) to quantify the distribution of mucous cells and Bcl-2 and p53 proteins in the gastric mucosa. The HRP-DAB sections were further examined to determine staining intensity of the Bcl-2 and p53 proteins. All data obtained were recorded and statistically analyzed.

Statistical Analysis

Study data were analyzed using IBM-SPSS (version 23, IBM Corp., NY, USA) and presented as mean ± standard error of the mean (SEM). Statistical values were compared using *t* test and one-way analysis of variance (ANOVA), wherein the level of significance was set at $P < 0.05$.

Results

Phytochemical analysis

The chromatogram of aqueous extract of *A. conyzoides* L. showed seven peak areas indicating seven phytochemical compounds (Figure 1), which included 1,2,4-Trizol 4-amineN-(2-thienylmethyl)-ester, 2-Pentadecenone 6,10,14-trimethyl ester, n-Hexadecanoic acid, 9,12,15-Octadecatrienoic acid (Z,Z,Z)-, 9,12-Octadecadienoic acid (Z,Z)-, Phytol and Methyl stearate. Their corresponding molecular structures are given in Figure 2.

Macroscopic examination

The gross appearance of the internal aspect of the gastric tissues of experimental animals showed varying degrees of gastric mucosal injury. These ranged from intense
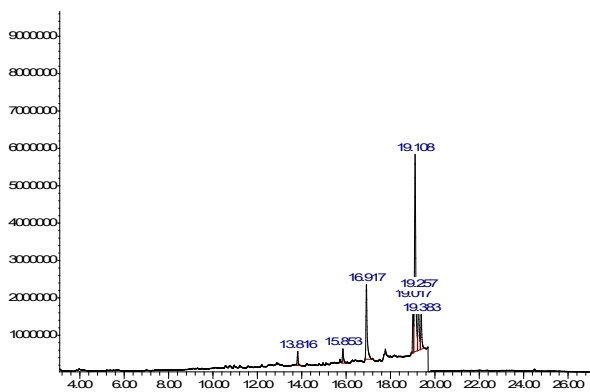
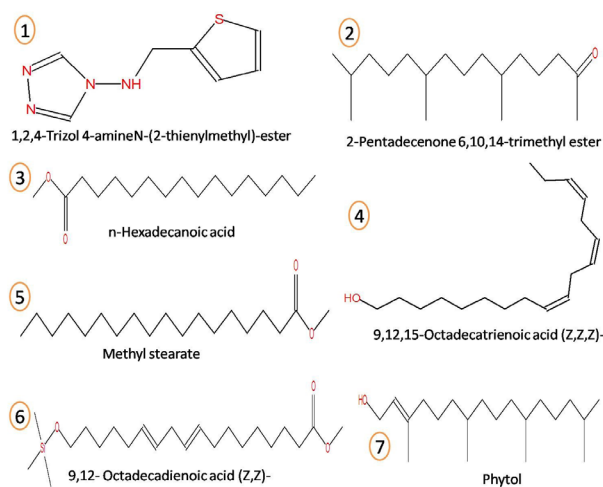**Figure 1.** Chromatogram of the aqueous extract of *Ageratum conyzoides* L. using GC-MS.

Figure 2. The structural formula of GC-MS-identified phytochemicals of *Ageratum conyzoides* L.

Figure 3. A-E: Gross macroscopic appearance of the gastric mucosa of experimental animals after the pyloric-ligation method of inducing gastric mucosal injury and showing different degrees of mucosal erosion with arrows pointing at mucosal lesion areas. F: Chart showing mean ulcer index of the gastric mucosa of groups A-E experimental animals (A = Normal control, B = Test control, C = 100 mg/kg extract, D = 300 mg/kg extract, E = 500 mg/kg extract; *Significant difference at $P < 0.05$ relative to group A; +Significant difference at $P < 0.05$ relative to group B).

erosion in test control group B to focal or mild erosion in treatment groups C-E compared to gastric mucosa of normal control group A (Figure 3).

Histopathological results

The result showed varying degrees of the gastric mucosal surface erosion relative to the gastric mucosal morphology of normal control group A. These range from intense mucosal surface erosion observed in test control group B to moderate or focal erosion observed in treatment groups C-E (Figure 4).

Histochemical results

This revealed the distribution of mucous cells within gastric mucosa of control groups A and B experimental animals as well as those in treatment groups C-E (Figure 5). The mucous cell count was insignificantly increased among treatment groups, but a significant reduction was observed in the test control group B when compared to normal control group A.
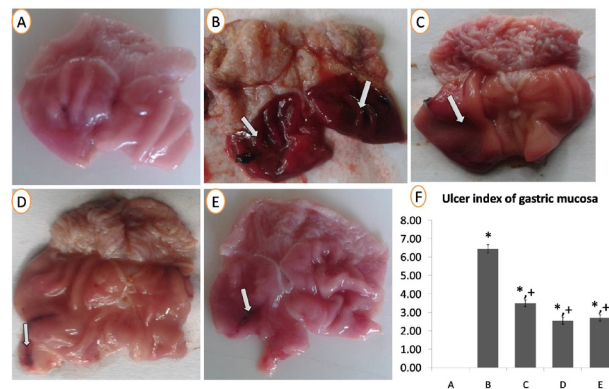
Immunohistochemical results

This showed the distribution of anti-apoptotic (Bcl-2) and tumor suppressor (p53) proteins in gastric mucosa of the experimental animals in control groups (A & B) and treatment groups (C-E) as indicated by the characteristic brownish coloration of immunostaining reaction (Figures 6 and 7). The staining intensity for Bcl-2 protein in gastric mucosa of experimental animals ranged from the moderate staining intensity in control groups A and B to deep intensity in treated groups C-E (Table 1). Accordingly, the quantification of Bcl-2 protein showed a significant ($P < 0.05$) up-regulation in its expression within gastric mucosa of treatment groups (C-E) compared to normal control group A (Figure 6). However, only test control group B showed deep staining intensity for p53 protein while treatment groups C-E showed mild staining intensity (Table 1). In addition, the p53 protein expression was significantly ($P < 0.05$) increased only in gastric mucosa of test control group B (Figure 7).

Discussion

Generally, the therapeutic properties of medicinal plants are dependent on their constituent phytochemicals (24,25). In other words, constituent phytochemicals confer on the medicinal plants their therapeutic potential, which can be harnessed in the treatment of various tissue pathologies, including those of gastrointestinal tissues. The results of this study (Figures 1 and 2) revealed the phytochemical constituents of the aqueous extract of *Ageratum conyzoides* These phytochemicals would account for the therapeutic activities of the plant extract including its gastroprotective activity. This activity was highlighted by the degree of gastric mucosal surface protection as grossly and microscopically observed in the
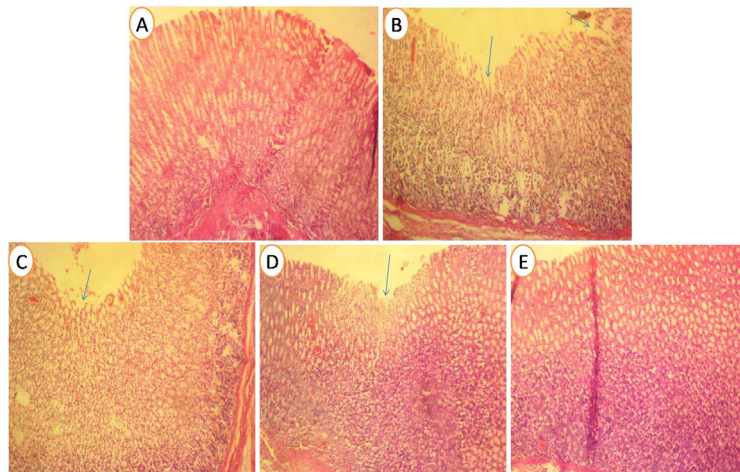
Figure 4. Photomicrographs showing gastric histomorphology of experimental animals in groups A-E (H & E X100). (A = Normal control, B = Test control, C = 100 mg/kg extract, D = 300 mg/kg extract, E = 500 mg/kg extract).
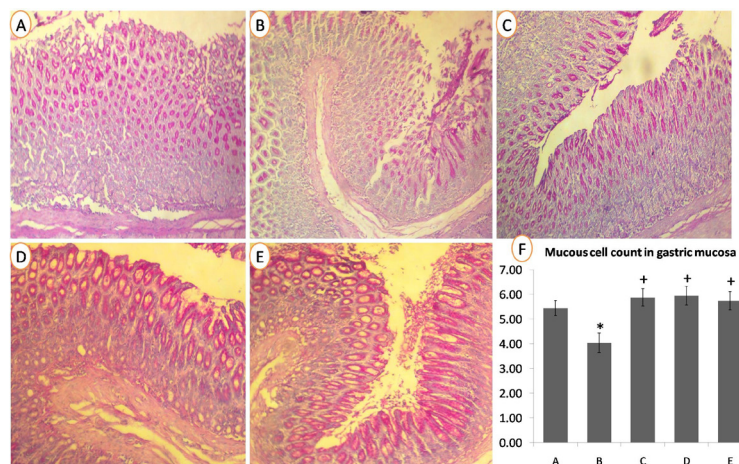

Figure 5. A-E: Gastric mucosa of experimental animals in groups A-E showing mucous cell distribution (PAS X100). F: Chart showing mucous cell distribution in gastric mucosa of experimental animals in groups A-E (A = Normal control, B = Test control, C = 100 mg/kg extract, D= 300 mg/kg extract, E = 500 mg/kg extract; *Significant difference at $P < 0.05$ relative to group A; +Significant difference at $P < 0.05$ relative to group B).

experimental animals (Figures 3 and 4). These ranged from intense mucosal erosion in the test control group B to moderate or focal erosion in the pre-treated groups C-E in comparison to the normal gastric histomorphology of normal control group A.

Based on the histochemical results of this study (Figure 5), the mucous cell count was insignificantly increased in the pre-treated groups C-E but a significant ($P < 0.05$) reduction was observed in the test control group B. The increased mucous cell population in the treatment groups, though insignificant, might contribute to the gastroprotective activity of the plant extract. This is because gastric mucous cells secrete alkaline mucus, which in turn forms a layer of gel on gastric mucosa that protects the gastric mucosal surface against the erosive effects of acidic gastric secretion (2,3). Studies have also shown that the impairment of the gastric mucus secretion

often leads to gastric mucosal injury or ulceration, while increased mucus secretion plays a vital role in gastric mucosal protection (10,26,27).

The integrity of gastric mucosa is a function of functional equilibrium between mucosal cells proliferation and death. Injury to gastric mucosa, therefore, results from a shift of the equilibrium towards mucous cell death due to stimulation of apoptosis and/or down-regulation of mucous cell proliferation rate. The regulation of these cellular events is usually brought about by the genes and protein products derived from anti-apoptotic members of Bcl-2 family as well as pro-apoptotic proteins such as p53 protein (28,29). The result obtained from this study (Table 1 and Figure 6) showed a significant ($P < 0.05$) up-regulation of Bcl-2 protein distribution in the gastric mucosa of the treatment groups C-E animals compared to the normal control group A. The Bcl-2 protein is an anti-
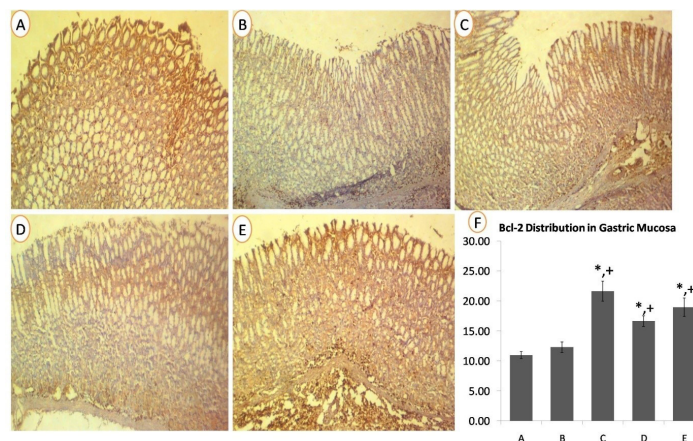
Figure 6. A-E: Gastric mucosa of experimental animals in groups A-E showing Bcl-2 protein expression indicated by the characteristic dark-brownish staining product of HRP-DAB reaction (HRP-DAB x100). F: Chart showing Bcl-2 expression in gastric mucosa of experimental animals in groups A-E (A= Normal control, B= Test control, C= 100 mg/kg extract, D= 300 mg/kg extract, E= 500 mg/kg extract; *Significant difference at $P < 0.05$ relative to group A; +Significant difference at $P < 0.05$ relative to group B).

Figure 7. A-E: Gastric mucosa of experimental animals in groups A-E showing p53 protein expression indicated by the characteristic dark-brownish staining product of HRP-DAB reaction (HRP-DAB x100). F: Chart showing p53 expression in gastric mucosa of experimental animals in groups A-E (A= Normal control, B = Test control, C = 100 mg/kg extract, D= 300 mg/kg extract, E = 500 mg/kg extract; *Significant difference at $P < 0.05$ relative to group A; +Significant difference at $P < 0.05$ relative to group B).
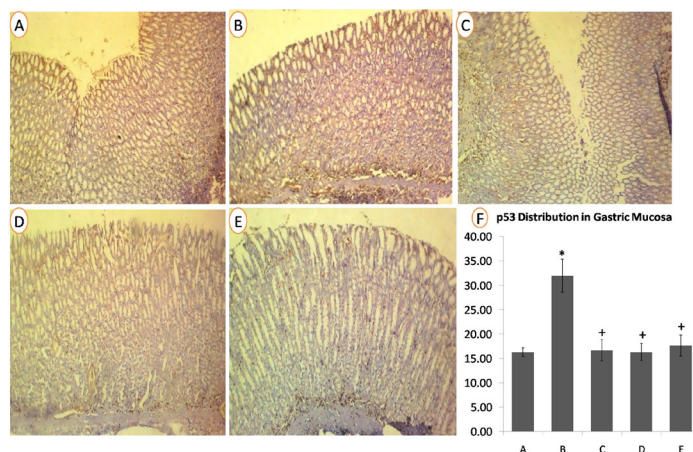
apoptotic protein, which functions to switch the fate of cells towards a survival pathway. The increased activity of Bcl-2 protein in gastric tissue suppresses pro-apoptotic (or death) signals that would be activated by exposure of the gastric tissue to aggressive factors (such as acidic gastric

Table 1. Staining intensity of Bcl-2 and p53 proteins in HRP-DAB sections of gastric tissue of experimental animals in groups A-E (A= Normal control, B= Test control, C= 100 mg/kg extract, D= 300 mg/kg extract, E= 500 mg/kg extract)

Groups	Bcl-2 staining intensity	P53 staining intensity
A	++	+
B	++	+++
C	+++	+
D	+++	+
E	+++	+

+ Mild intensity, ++ Moderate intensity, +++ Deep intensity.

secretion), thereby enhancing gastric mucosal protection. According to the study by Wang et al (30), the induction of gastric mucosal injury or ulceration is characterized by a significant loss of gastric mucosal cells due to stimulation of apoptosis and inhibition of proliferation by the aggressive factors. This usually results when the pro-apoptotic or aggressive factors overwhelm the anti-apoptotic or defensive factors, thereby committing the gastric mucosal cells to the death pathway leading to injury or ulceration. Hence, gastroprotective factors would function to inhibit apoptosis via up-regulation of the Bcl-2 protein distribution in the gastric mucosa leading to the prevention of mucosal injury.

Another study described the gastroprotective mechanisms to involve the blockage of mitochondrial and Fas apoptotic pathways via inhibition of pro-apoptotic proteins like Bax and Bak expressions while up-regulating

the expression of anti-apoptotic proteins like Bcl-2 and Bcl-X$_L$ (31). Gastric mucosal injury also results from an increased rate of apoptosis within the gastric mucosa. Hence, gastroprotective mechanisms may involve the down-regulation of pro-apoptotic signals and up-regulation of anti-apoptotic protein expression, especially Bcl-2 protein (32,33).

Furthermore, according to the results from this study (Table 1 and Figure 7), the expression of p53 protein was not significantly increased in the gastric mucosa of treatment groups but significantly ($P < 0.05$) increased in the test control group B compared to the normal control group A. The p53 protein is tumor suppressor factor which plays a key role during cell proliferation and apoptosis. It is regarded as the guardian of the genome, which functions as an inducer of apoptosis during an irreparable genomic alteration that may result from cellular exposure to toxic agents (34). P53 protein plays a critical role during the initiation of apoptosis such that mutation of its encoding gene or inactivation of wild-type protein results in unregulated epithelial plasticity of gastric tissues (35). Hence, mutated p53 gene, leading to aberrant p53 protein activity, has been closely linked with gastro-epithelial diseases, which range from gastritis to gastric carcinoma. The mechanisms of gastric mucosal protection include the down-regulation of apoptotic signals (such as p53 protein expression) so as to switch the fate of gastric mucosal cells toward the survival pathway (33).

Conclusion
The experimental animals pre-treated with aqueous extract of *A. conyzoides* showed an insignificant increase in the gastric mucous cell population, a significant increase in the Bcl-2 protein distribution, and a significant reduction in the p53 distribution in the gastric mucosa. Only the non-treated test control group showed a significant increase in the distribution of the p53 protein. Based on these findings, the plant extract potently exerts gastroprotective effects against gastric mucosal aggressive factor. Accordingly, the up-regulation of pro-cell survival factors and concomitant down-regulation of pro-cell death factors within the gastric mucosa can be described as key aspect of its gastroprotective mechanisms.

Authors' contributions
DRO and GIE conceptualized the research idea and created the study design. DRO performed the experiment work, data analysis and wrote the first draft of manuscript. DRO and GIE reviewed and approved the final manuscript for publication.

Conflict of interests
Authors confirm that there is no conflict of interest to disclose.

Ethical considerations
Animal handling and experimental procedures were in compliance with the guidelines for experimental animal care and use of the Faculty of Basic Medical Sciences, College of Medicine, University of Benin, Benin City, Nigeria. This study was approved by the Research and Ethics Committee of Faculty of Basic Medical Sciences, College of Medicine, University of Benin, Benin City, Nigeria (PG/BMS0616084).

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