Increased mucous cell population and modulation of Bax/Bcl-2 factors characterize *in vivo* gastroprotective activity of *Cissampelos owariensis* in rats

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SUMMARY

The physiological integrity of the gastric mucosa is dependent on the balance between the mucosal protective and aggressive factors. Medicinal plants or their derivatives generally exhibit gastroprotective effect by promoting the protective factors against the aggressive factors of the gastric mucosa. The study was conducted to elucidate the gastroprotective mechanism of the methanol extract of *C. owariensis* (MECo) in rats. Twenty male Wistar rats were divided into four groups, which include control groups A and B – given distilled water – and treated groups C and D – animals given 100 and 300 mg/kg MECo respectively for 28 days. After the treatment period, gastric mucosal injury was induced for groups B-D by pyloric ligation method. The gastric tissue of animals was collected, processed for histology (haematoxylin and eosin technique), histochemistry (periodic acid-schiff technique) and immunohistochemical staining (for Bcl-2 & Bax proteins). The results of the gastric histomorphology showed prominent and widespread mucosal erosion in positive control group B compared to normal control group A, while treated groups C and D showed only mild or focal mucosal erosion. Furthermore, the histochemical results showed significant increase in mucous cell population in treated groups C and D compared to positive control group B. The immunostaining results showed significant up-regulation of anti-apoptotic Bcl-2 protein and down-regulation of pro-apoptotic Bax protein in the treated groups C and D compared to the control groups A and B. In conclusion, the findings of this study indicate that the increased mucous cell population and modulation of apoptotic signaling highlights the mechanism of gastroprotective activity of MECo.

Key words: *Cissampelos owariensis* – Gastroprotection – Mucous cell – Apoptosis – *In vivo*

INTRODUCTION

The gastrointestinal tract represents a collection of tissues which function to convey and digest ingested food substance, as well as absorb

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water, electrolytes, vitamins and other nutrients into the body (Barret et al., 2019). From the external aspect inward, the morphological composition of the tract includes serosa, muscular layers, submucosa and mucosa. Among the viscera of the tract, the stomach function as the major storage of ingested food substance, and as a site for churning or mixing the food substance with acidic gastric secretions to form a semi-fluid product known as chyme (Hall, 2016). Generally, the innermost mucosal layer of the gastrointestinal tract provides the protective covering against the digestive effect of the secretions or other toxic ingested substances. However, the gastric mucosal protection is functionally achieved by an equilibrium between the mucosal protective and aggressive factors (Omotoso and Eze, 2022).

Essentially, the gastric mucosa contains mucous and parietal cells which produce mucus and bicarbonate ions respectively. These in turn form a viscous layer of gel that constitutes the major protection for the mucosa against the acidic gastric secretions and digestive enzymes. Other contributing factors to the gastric mucosal protection include the microcirculation, prostaglandins, cyclooxygenase and epidermal growth factors (Bongu and Vijayakumar, 2012; De Lima et al., 2021). On the other hand, the mucosal aggressive factors include oxidative stress, ischaemia, exogenous factors such as bacteria, ethanol, non-steroidal anti-inflammatory drugs and endogenous factors such as elevated acidic gastric secretions, and bile acids (Sembulingam and Sembulingam, 2010). The mechanism of gastric mucosal injury or ulceration by most aggressive factors involves the induction of oxidation stress through the production of reactive oxygen species (ROS) and other free radicals (Ajeigbe et al., 2014). Therefore, there is a probable potency of antioxidants to mitigate the erosive effect of the aggressive factors on the gastric mucosa. These antioxidants include chemically synthesized compounds and naturally derived from medicinal plants.

Medicinal plants have been widely applied for diverse therapeutic purposes and their applications have been on a steady increase in recent years due to their wide accessibility, affordability and negligible adverse effects (Adelanwa and Tijani, 2013). Among the diverse medicinal plants is the *Cissampelos owariensis* (*C. owariensis*), which is a twiner plant commonly found in tropical regions especially sub-Saharan African countries. It belongs to the menispermaceae family, which comprises about 70 genera and 450 species (Akande et al., 2013). It has been reported to exhibit a wide range of therapeutic activities, which include the treatment of dysentery, diarrhoea, enteritis, colic and intestinal worm infections (Ekeanyanwu et al., 2012; Erhirhie et al, 2015). In our previous study, the gastroprotective potential of the methanol extract of the *C. owariensis* against the erosive effect of prolonged exposure to acidic gastric secretions (a potent gastric mucosal aggressive factor) has been reported (Omotoso et al., 2019b). However, the mechanism of its gastroprotective has not been fully elucidated in contrast to the anti-ulcer activity of another species of the same family – *Cissampelos mucronata* –which has been previously described and linked with its cytoprotective and antispasmodic mechanisms (Nwafor and Okoye, 2005).

The aim of this study was to elucidate the *in vivo* gastroprotective mechanism of the methanol extract of *C. owariensis* (MECo) through assessment of the role of gastric mucous cell and apoptotic signaling factors, which include the anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins using rat model.

MATERIALS AND METHODS

Study Reagents

The reagents used for the study were of analytical grade and procured from Sigma-Aldrich Chemical Company (St. Louis, MO, USA) except the primary and secondary antibodies that were procured from Abcam, Cambridge, Massachusets, USA.

Experimental animals

This study involved twenty (20) adult male Wistar rats with a weight range of 140-170 g. They were sourced from the Central Animal House facility of the School of Basic Medical Sciences, Igbinedion University, Okada, Edo State, Nigeria. The animals were housed at the facility throughout the study period and kept in standard animal cages under hygienic conditions, room temperature of 25 ± 2°C and twelve (12) hour light/dark cy-

cle. They were fed with standard animal feed and access to drinking water was ensured throughout the study period. The study was approved by the Research and Ethical Committee, School of Basic Medical Sciences, Igbinedion University, Okada, Edo Sate, Nigeria (11/013413/HSC). The experimental procedures were conducted in compliance with international guidelines for handling and care of experimental animals.

Plant material collection and extraction

The plant material was collected from the suburb of the Okada community in Edo State, Nigeria. Following the verification of the plant, the leaves were detached and air-dried for three weeks. With the aid of mechanical grinder, the dried leaves were pulverized into powder form. The powdered plant material was infused into methanol for seventy-two (72) hours, filtered and the filtrate was evaporated through a rotary evaporator. The alcohol-based extractio, adopted in the current study essentially exhibits the potency for extracting more phytochemicals, especially those with antioxidant properties than aqueous-based extraction (Safarzaei et al, 2020). The extract residue was cooled and kept in a refrigerated chamber until its use for the study.

Experimental design

The animals were randomly divided into four groups, which include control groups A and& B and treated groups C and D. Each group comprised five animals (n=5). The normal control group-A animals were given distilled water (5 ml/kg body weight) and not induced by pyloric ligation. The positive control group-B animals were given distilled water (as in group A) and induced by pyloric ligation. The treated groups C and D were given 100 and 300 mg/kg MECo respectively and induced by pyloric ligation. The treatment period was 28 days and the administration was carried out via oral route using an orogastric gavage coupled to a calibrated syringe.

Pyloric-ligation method of inducing gastric mucosal injury

At the end of the treatment period, the animals were fasted for 24 hours but allowed free access to water. Animals were anaesthetized by intraperitoneal injection of Ketamine/Xylazine (50 mg/kg at ratio 1:1). The pylorus of the stomach of the animals in groups B to D was accessed in the abdominal cavity via a small midline abdominal incision. The pylorus was gently pulled up, ligated and returned into the cavity. The abdomen was closed and a five (5)-hour observatory period followed (Shay et al., 1945). Thereafter, the animals were euthanized and stomach tissue harvested for subsequent examination

Tissue processing, histological and histochemical staining

The stomach tissue was fixed using 10% neutral buffered formalin, dehydrated using ascending grades of alcohol (70%, 90% and absolute alcohol), cleared using xylene and embedded in paraffin to form tissue blocks. The tissue blocks were sectioned using rotary microtome at 5 µ, and tissue sections were mounted on slides for histological and histochemical staining.

For histological staining using haematoxylin and eosin (H & E) technique, the tissue sections were dewaxed using xylene and hydrated using descending grades of alcohol (100%, 90%, and 70% alcohol) and distilled water. They were stained in haematoxylin, washed in running water, differentiated and blued using 1% acid alcohol and tap water respectively, and rinsed in water. The counter-staining of tissue sections was done using eosin. The sections were rinsed in water, dehydrated using alcohol, cleared using xylene and mounted using distrene polystyrene xylene (DPX) (Fischer et al., 2008).

For histochemical staining using the periodic acid-schiff (PAS) technique, the tissue sections were dewaxed using xylene and hydrated using alcohol to distilled water. Sections were treated with 0.5% periodic acid, rinsed in water, treated with schiff's reagent. The tissue sections were rinsed in water, counterstained in haematoxylin and rinsed in water. Dehydration of sections was done using alcohol, clearing using xylene and mounting using DPX (McManus, 1948). The quantification of the mucous cell population within the gastric tissue sections was conducted using the image-J software. This was achieved by creating

uniformly-defined count areas on the tissue section in each of the five replicates per group using grid-lines. The mucous cell count for three uniformly selected count areas was recorded in each replicate and the mean value of mucous cell count was determined for each group.

Immunohistochemical staining for Bax and Bcl-2 proteins

Tissue sections were hydrated using alcohol and water and antigen retrieval was done using citric acid solution (pH 6.0) in a microwave (at 100 Watts). Equilibration was done using running water to displace the hot citric acid; tissue sections were then exposed to peroxidase block, rinsed in phosphate buffer saline (PBS). Non-specific proteins in sections were blocked using Nevocastra protein block. Tissue sections were rinsed in PBS and incubated in primary antibody (prepared with 1 in 100 dilution ratio). They were rinsed with PBS, treated with secondary antibody and further rinsed with PBS. The polymer was added to the tissue sections and rinsed with PBS. Tissue sections were treated with 3,3-diaminobenzidine (DAB) substrate, rinsed with water, counterstained with haematoxylin and further rinsed with water. Dehydration was done using alcohol, clearing with xylene, and mounting with DPX (Omotoso and Eze, 2022). The quantification of the protein distribution within the gastric tissue sections was conducted using the image-J software.

Statistical analysis

The data derived during this study were analyzed using IBM-SPSS (version 22) and statistical results were presented as mean ± standard error of mean (SEM). Comparison of statistical mean values was done using one-way analysis of variance (ANOVA), with $p < 0.05$ and $p < 0.01$ considered as statistical significant levels.

RESULTS

Histological study

The gastric tissues of the study animals showed varying histomorphology. The normal control group (Fig. 1A) presented normal gastric histomorphology that shows the component parts of the gastric mucosal glands, which include gastric pits, isthmus, neck and base. The positive control group (Fig. 1B) showed intense mucosal erosion

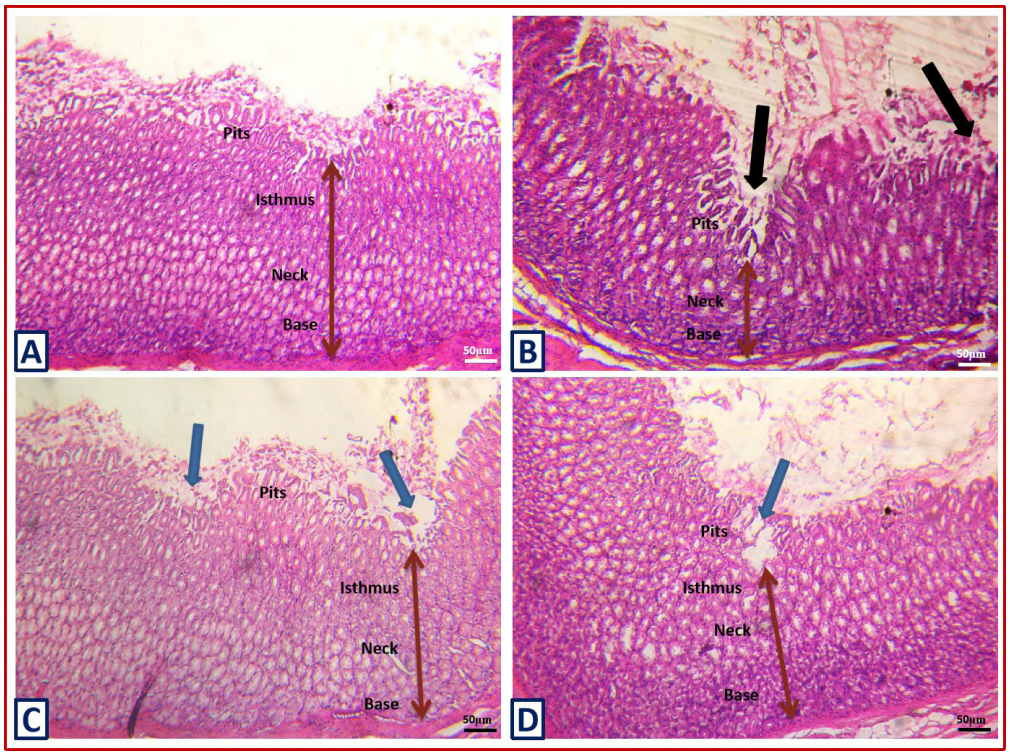

Fig. 1.- Photomicrograph of stomach tissue of experimental animals showing normal gastric histomorphology in control group A, intense mucosal erosion (black arrow) in positive control group B and mild focal mucosal erosion in treated groups C and D (H&E, X100). Double end red arrow indicates the minimum mucosal layer thickness. Scale bars: 50 µm.

with significant reduction of the mucosal isthmus due to the exposure to the gastric acid secretion. However, the pre-treatment with *C. owariensis* resulted into mild or focal mucosal erosion in the gastric histomorphology of groups C and D after exposure to the gastric acid secretion (Fig. 1C, 1D).

Histochemical study

The PAS staining of the gastric tissues revealed the distribution of the mucous cells of gastric mucosa of study animals. The normal control group presented normal distribution of the surface and neck mucous cell population (Fig. 2A). The positive control group (Fig. 2B) showed significant decline of the mucous cell population following the exposure to the gastric acid secretion. However, the pre-treatment with *C. owariensis* resulted into significant increase of the mucous cell population which include the surface and neck cells (Fig. 2C, D).

Immunohistochemical study

The immunostaining of the gastric tissues revealed the distribution of the anti-apoptotic Bcl-2 protein within the gastric tissues of the study animals. The Bcl-2 protein distribution of the positive control group-B animals showed significant decline compared to the normal control group A. However, the pre-treatment with *C. owariensis* resulted into significant increase of the Bcl-2 protein distribution compared to the control groups A and B (Fig. 3).

The immunostaining of the gastric tissues further revealed the distribution of the pro-apoptotic Bax protein within the gastric tissues of the study animals. The Bax protein distribution of the positive control group B animals showed significant increase compared to the normal control group A. However, the pre-treatment with *C. owariensis* resulted into significant decline of the Bax protein distribution compared to the positive control groups B (Fig. 4).

DISCUSSION

The physiological integrity of the gastric mucosa has been described to be dependent on the balance between the mucosal protective factors (which include increase mucous cell population or mucus secretion) and mucosal aggressive factors (which result into oxidative stress and apop-

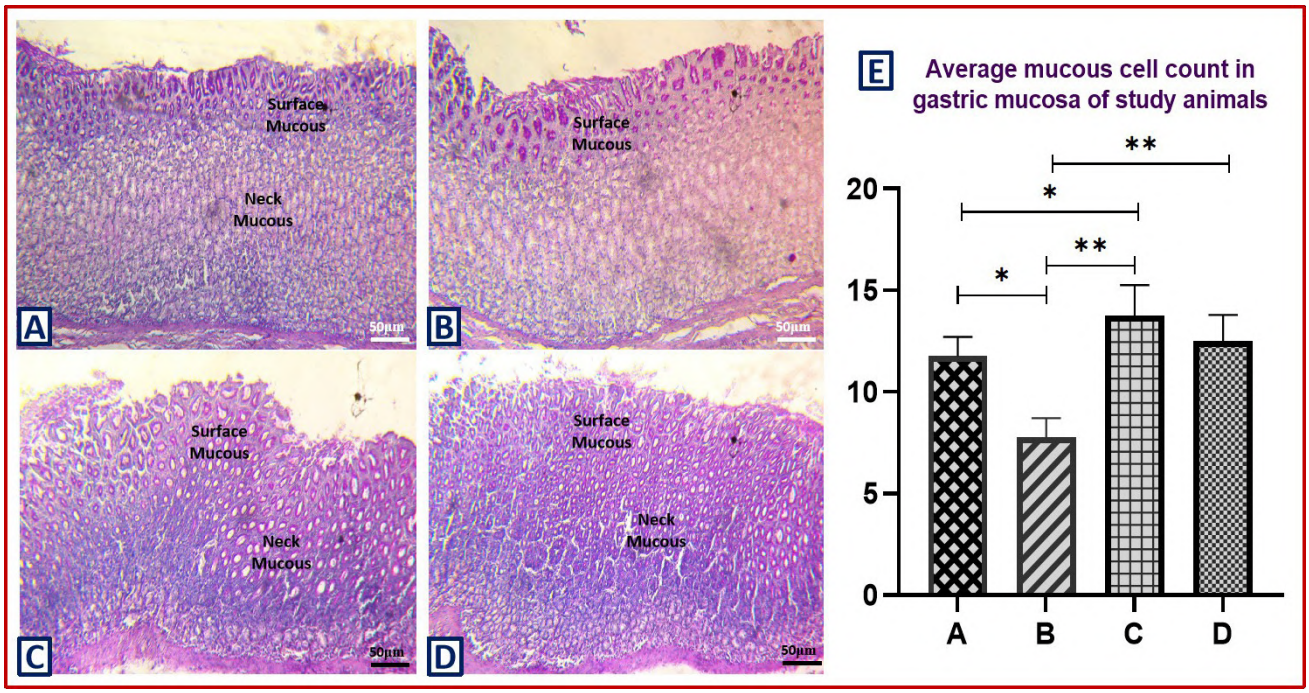

Fig. 2.- A-D: Histochemical staining of gastric tissue of groups A-D animals showing the mucous cell population (surface and neck mucous cells) within the gastric mucosa (PAS, X100). **E:** Evaluation of mucous cell distribution in the gastric mucosa of experimental animals in groups A–D using five replicates per group. (*,** indicate significant difference at $p < 0.05$ (0.0212) and $p < 0.01$ (0.0032) respectively). Scale bars: 50 µm.

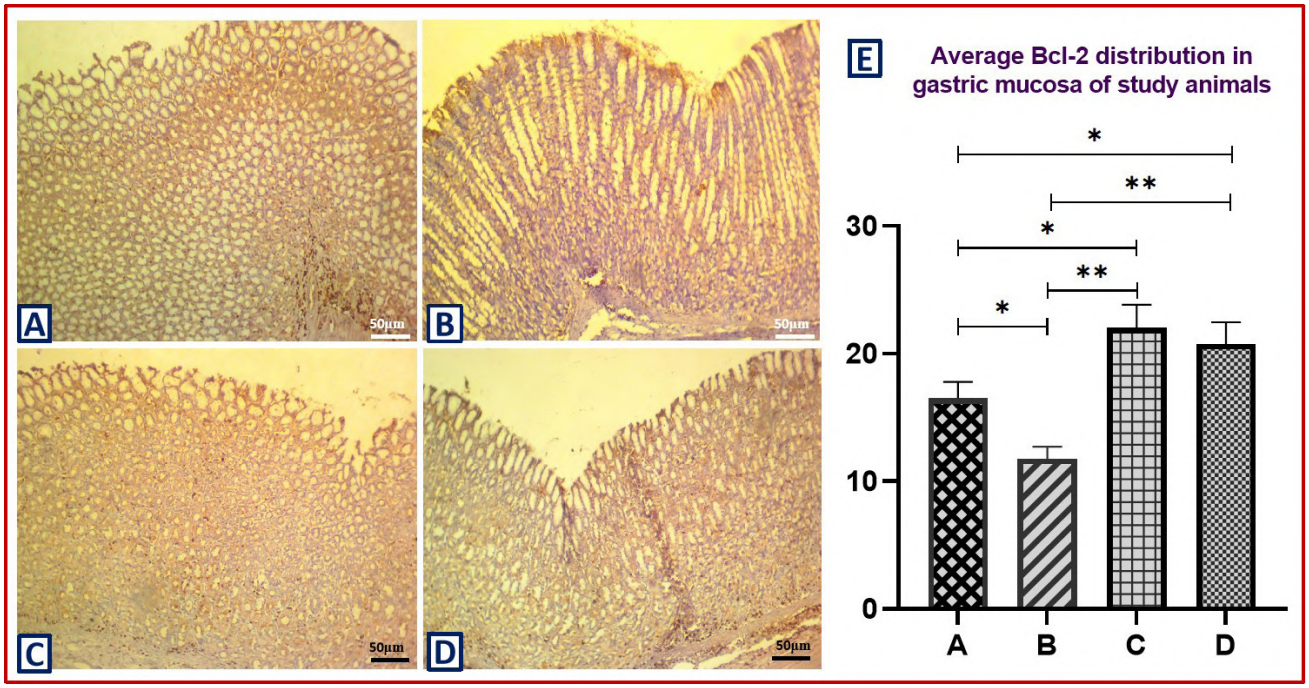

Fig. 3.- A-D: Immunostaining of gastric tissue of groups A-D animals showing Bcl-2 protein distribution indicated by dark-brown coloration. **E:** Evaluation of Bcl-2 protein expression in gastric mucosa of groups A-D animals using five replicates per group (*,** indicate significant difference at $p < 0.05$ (0.0344) and $p < 0.01$ (0.0048) respectively). Scale bars: 50 µm.

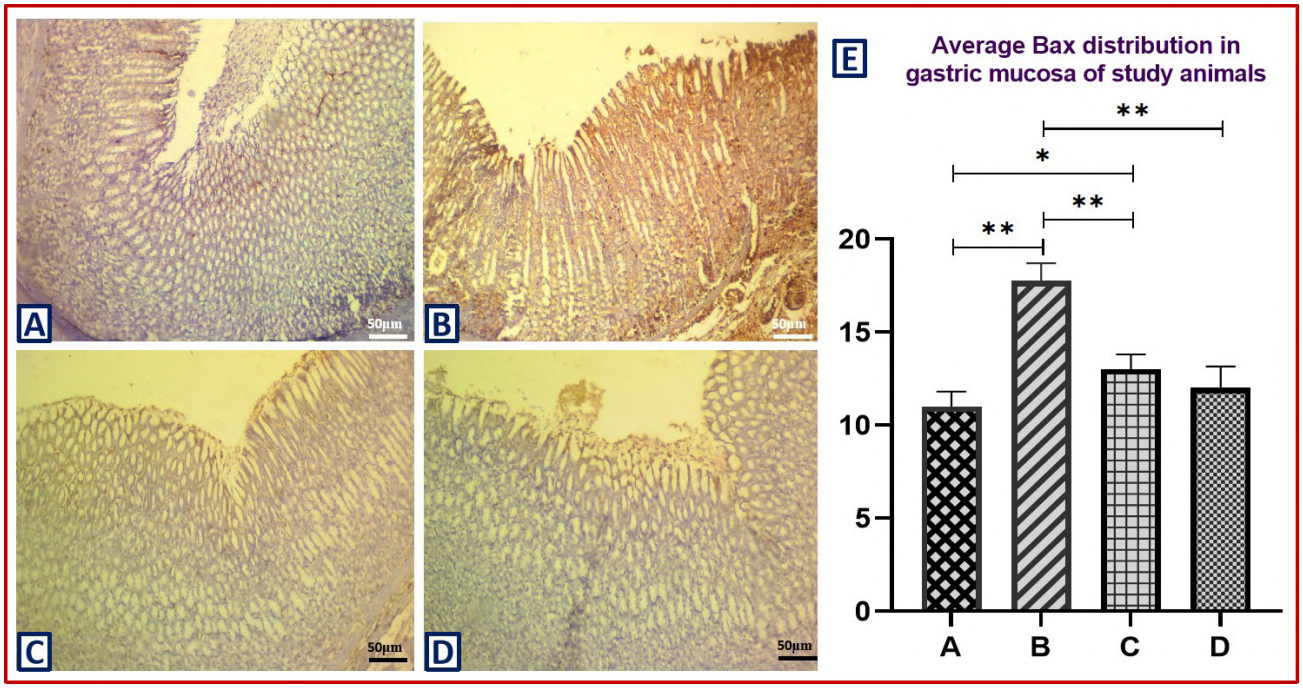

Fig. 4.- A-D: Immunostaining of gastric tissue of groups A-D animals showing Bax protein distribution indicated by dark-brown coloration. **E:** Evaluation of Bax protein expression in gastric mucosa of groups A-D animals using five replicates per group (*,** indicate significant difference at $p < 0.05$ (0.0134) and $p < 0.01$ (0.0037) respectively). Scale bars: 50 µm.

tosis) (Gryko et al., 2014; De Lima et al., 2021). Medicinal plants or their derivatives generally exhibit gastroprotective effect by promoting the protective factors against the aggressive factors of the gastric mucosa. This is indicated in the current study whereby MECo exhibited gastroprotective effect against the gastric mucosal aggressive factor (acidic gastric secretion). Based on the his-

tological results of this study (Fig. 1), the gastric histomorphology of the positive control group-B animals showed prominent and widespread mucosal erosion compared to the normal control group A. However, the gastric histomorphology of treated groups C and D revealed mild or focal mucosal erosion which indicates the gastroprotective effect of MECo. Previous studies on the phytochemical analysis of *C. owariensis* have reported the presence of bioactive compounds which include flavonoids, alkaloids, tannins and saponin. These are secondary metabolites that provide the therapeutic activity of the extract which include reparation of tissue histomorphology following exposure to toxicants (Ekeanyanwu et al., 2012; Omotoso et al., 2019a).

According to the histochemical results of this study (Fig. 2), the mucous cell population showed significant ($p < 0.05$) increase among the animals of groups C and D, which were pre-treated with MECo compared to the animals of control groups A and B. Essentially, the mucous cells of gastric mucosa produce alkaline mucus, which contributes to the formation of a layer of protective gel that protects the mucosal surface against the erosive effects of gastric mucosal aggressive factors (Sembulingam and Sembulingam, 2010). In previous studies, the functional impairment of mucus secretion had been associated with increased mucosal injury, while elevated gastric mucus secretion, usually due to increase in the mucosal cell population, enhanced the gastric mucosal protection (Zakaria et al., 2015; Ige et al., 2016). The increase in the mucous cell population among the treated groups C and D in the current study would enhance a physiological elevation of mucus secretion, which would in turn mitigate the erosive effect of the acidic gastric secretion. Previous studies have similarly reported the increase in gastric mucus production as one of the gastroprotective mechanism which helps to preserve the mucosal protective covering (Nwangwu et al., 2013; Chantharangsikul et al., 2016; Kim et al., 2019; Kim et al., 2020).

Furthermore, the exposure to aggressive factors results into gastric ulceration or injury through induction of oxidative stress and stimulation of apoptosis. Hence, the modulation of apoptotic signaling pathway plays an important role in determining the physiological integrity of the gastric mucosa. The B-cell lymphoma-2 (Bcl-2) protein family regulates apoptosis through interaction with mitochondrial function leading to mitochondrial membrane permeabilization and activation of the caspase signaling cascades. The members of the protein family include the anti-apoptotic Bcl-2 protein and pro-apoptotic Bax protein (Kale et al., 2018). According to the result of the current study (Fig. 3), there was a significant ($p < 0.05$) increase in the anti-apoptotic Bcl-2 protein in the gastric tissue of the treated groups C and D compared to the control groups A and B. Conversely, there was a significant ($p < 0.05$) increased level of the pro-apoptotic Bax protein in the gastric tissue of the positive control group B compared to the normal control group A and treated groups C and D (Fig. 4).

Therefore, the findings of this study concluded that the gastroprotective mechanisms of MECo include the upregulation of anti-apoptotic and down-regulation of pro-apoptotic signaling factors. According to the studies by Arab et al. (2015) and Liu et al. (2018), gastroprotective mechanisms involve the inhibition of pro-apoptotic signaling factors, which include Bax/Bak expressions and activation of anti-apoptotic signaling factors like Bcl-2/Bcl-X_L. This outcome was further corroborated by the findings of Zhou et al. (2020), Raish et al. (2021) and Fu et al. (2021), which showed that the inhibition of Bax and activation of Bcl-2 signaling as significant contributory mechanisms during the gastroprotective activity of gallic acid, sinapic acid and *Periplaneta americana* extract respectively. Moreover, previous studies have reported that the anti-apoptotic effects of therapeutic drug candidates such as paeonol, irbesartan and betaine-homocysteine significantly contribute to their gastroprotective activity in rat model (Hafez et al., 2018; Shahin et al., 2018; Gundogdu et al., 2022).

CONCLUSION

The findings of this study indicated that a significant increase in the mucous cell population, up-regulation of anti-apoptotic (Bcl-2) and down-regulation of pro-apoptotic (Bax) protein

expression and distribution within the gastric mucosa characterize *in vivo* gastroprotective activity of the methanol extract of *C. owariensis* in rat model.

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