

Gastroprotective Property of Stigmasterol In Ethanol-Induced Ulcer In Male Wistar Rats

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Abstract: Gastric ulcers have been treated over the years with proton pump inhibitors and histamine (H₂) receptor blockers. However, these medications have also been shown to cause side effects and relapse. Stigmasterol, a plant sterol, has been shown to possess various therapeutic properties. This study aimed to investigate the gastroprotective property of stigmasterol on ethanol-induced ulcer in Wistar rats. Thirty-five male rats were divided into seven groups. Groups one and two served as the normal and negative controls. Groups three and four were pretreated with 5 mg/kg and 10 mg/kg body weight of stigmasterol, respectively. Group five received 20 mg/kg rabeprazole, group six was pretreated with 50 mg/kg cimetidine, and group seven received 1% DMSO. Pretreatment was done for seven days. The ulcer was induced on the 8th day by administering 1 mL of ethanol. Stigmasterol 10 mg/kg body weight had the highest percentage inhibition (85.04 %) when compared to rabeprazole (52.22 %) and cimetidine (62.50 %). 5 mg/kg stigmasterol, 10 mg/kg stigmasterol, 20 mg/kg rabeprazole and 50 mg/kg cimetidine pretreated groups significantly ($p < 0.05$) reduced ulcer index, CRP, TNF- α , IL-6 concentrations and MDA level, but significantly ($p < 0.05$) increased SOD and catalase (CAT) activities when compared with the negative control.

Keywords: gastroprotective; cimetidine; ethanol; gastric ulcer; rabeprazole; stigmasterol, plant sterol.

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1. Introduction

Gastric ulcers are lesions in the mucosa of the stomach lining that extend through the muscularis mucosa, these breaks (lesions) are usually larger than 5 mm in diameter, and modifications in the gastric mucosa brought on by alterations in the stomach's defensive mechanisms may lead to erosion and, finally, ulceration of the stomach lining [1]. The two most frequent causes of stomach ulcers are gastric prostaglandin loss brought on by non-steroidal anti-inflammatory drugs and *Helicobacter pylori* (*H. pylori*) bacterium infection [2]. Gastric ulcer disease is recognized by discontinuity in the gastrointestinal (GI) tract's inner lining. It penetrates the stomach epithelium's *muscularis propria* layer. Usually, the stomach and proximal duodenum are affected. It might also affect the distal duodenum, jejunum, or lower oesophagus. Patients with gastric ulcers typically have epigastric pain 15 to 30 minutes

after eating; in contrast, patients with duodenal ulcers typically experience pain 2-3 hours after eating [3].

According to studies [4], the likelihood of developing stomach ulcers rises with age. Its prevalence is the same for both men and women. In the United States population, the frequency of *H. pylori* infection at age 60 is close to 50%. The peak incidence of ulcer disease occurs between the ages of 55 and 65, and it is now a disease that primarily affects older people [3]. 35% of patients who are diagnosed with stomach ulcers may experience serious side effects, it has been known from research that smoking increases the relative risk of getting stomach ulcers by two times, compared to non-smokers [3]. 5 to 10% of individuals will develop an ulcer in their lifetime, which is likely an underestimation of the disease's incidence because some people may experience no symptoms [5]. According to WHO (2020), ulcer contributes 5,846 or 0.39% of total deaths in Nigeria, and up to 10 % of the world's population may be impacted by gastric ulcers. Each year, 500,000 new cases are reported, affecting 5 million people in the United States (US) alone [3].

Proton pump inhibitors (PPIs) and histamine (H₂) receptor antagonists, the two common therapies for gastric ulcers, have been shown, however, to cause side effects, relapses, and a variety of pharmacological interactions; they might cause haematological abnormalities, hypersensitivity, arrhythmia, gynecomastia, impotence, and kidney diseases [6]. On the other hand, traditional medicine is a major contributor to primary healthcare globally. About 75–80% of people in underdeveloped nations use traditional medicine, since it is more palatable to local cultures and has fewer adverse effects [6,7]. Medicinal plants and the chemical compounds they produce can be used to cure and prevent a wide variety of illnesses [8].

Numerous studies from our laboratory and elsewhere have demonstrated that traditional medications can effectively prevent gastric ulcers in humans and various animal models [8-15]. There is a need to find an alternative therapy that can protect against gastric ulcers with better efficacy, affordability, accessibility, and fewer side effects.

Stigmasterol, also known as stigmasterin or the Wulzen anti-stiffness factor, is an unsaturated phytosterol found in diverse botanical species [16]. According to [17], stigmasterol has been found to play a role in both gravitropism and abiotic stress tolerance in plants. It is a plant sterol with numerous therapeutic properties and also serves as a precursor in the biosynthesis of diverse steroid hormones, such as androgens, a hormone called progesterone, corticoids, and estrogens, as well as in the synthesis of vitamin D₃ [16]. Stigmasterol has been known to possess diverse pharmacological potentials, encompassing anti-hypercholesterolemic, anticancer, hypoglycemic, antioxidant, and anti-inflammatory properties [18]. Therefore, this study aimed to investigate the gastroprotective property of stigmasterol on ethanol-induced ulcer in Wistar rats .

2. Materials and Methods

2.1. Chemicals.

Stigmasterol (Catalog Number - CFN97326) was procured from ChemFaces Biochemical Co., Ltd., Wuhan, China, with a chemical purity of 98% as determined by high-performance liquid chromatography (HPLC). Enzyme-linked Immunosorbent Assay (ELISA) kits were procured from Elabscience Biotechnology Inc., Beijing, China. All other chemicals and reagents used were of analytical grade.

2.2. *Animal grouping and treatment.*

A total of 35 Wistar rats weighing 140 ± 9 g on average were purchased from Babcock University, located in Ilishan-Remo, Ogun State. The rats were kept in plastic cages for two weeks, with a 12-hour light-dark cycle and a constant temperature of $25 \pm 2^\circ\text{C}$. They were given free access to water and food, which was to allow them to get acclimatized to their environment. After acclimatization, seven groups of five rats each were formed. For seven (7) days, pretreatment, as shown in Table 1, was administered once a day. For this research, 5 mg/kg and 10 mg/kg stigmasterol were administered, which were selected based on previous studies carried out by [19-21] on the effective doses of stigmasterol on inflammation and oxidation.

Table 1. Animal grouping and treatment.

Group (Where n = 5)	Pretreatment
1	Distilled water
2	No pretreatment + 1 mL absolute ethanol
3	Pretreatment with 5 mg/kg stigmasterol + 1 mL absolute ethanol
4	Pretreatment with 10 mg/kg stigmasterol + 1 mL absolute ethanol
5	Pretreatment with 20 mg/kg rabeprazole + 1 mL absolute ethanol
6	Pretreatment with 50 mg/kg cimetidine + 1 mL absolute ethanol
7	Pretreatment with 1% DMSO + 1 mL absolute ethanol

2.3. *Ulcer induction and quantification.*

The rats were fasted overnight on day eight, but they were given free access to water. After receiving an oral dose of 1 mL of 100% ethanol, the rats were euthanized after an hour. The rats' stomachs were cut along the larger curvature and rinsed with 1.15% KCl to determine the degree of ulceration, according to [22].

2.4. *Quantification of ulceration.*

The degree of ulceration in the rat and the percentage of inhibition were measured and computed using the techniques outlined by [23].

Ulcer scores were calculated using the method of [23]

- i) No lesion or bleeding = 0
- ii) Bleeding and slight lesions between 0.5 – 1.0 mm = 1
- iii) Moderate lesions between 1.0 and 1.5 mm in size = 2
- iv) Severe lesions between 1.5 - 2.5 mm in size = 3
- v) Perforated lesions between 2.5 – 3.5 mm in size = 4

$$\text{Ulcer Index (UI)} = \frac{\text{Sum of ulcer score}}{\text{Rats ulceration count}} \tag{1}$$

$$\text{Percentage Inhibition of Ulceration} = \frac{\text{UI untreated category} - \text{UI treated category}}{100} \times \text{UI untreated category} \tag{2}$$

2.5. *Collection of blood samples and preparation of serum.*

To obtain blood samples, the rats were anesthetized using diethyl ether and subsequently euthanized following completion of the treatment. The blood sample was collected from the rats' posterior vena cava using a needle and syringe. It was poured into a

standard sample bottle. Subsequently, it was left to clot at room temperature for approximately 15 to 30 minutes, followed by centrifugation at 3000 x g for 10 minutes using a refrigerated centrifuge. The serum was carefully decanted into a blood sample bottle. It was preserved at a temperature of -20°C before being subjected to various biochemical analyses.

2.6. Stomach homogenate preparation.

The rats were euthanized, their stomachs, removed, rinsed, and cut along the greater curvature, after which each stomach was divided into two. A portion of the stomachs was kept in phosphate buffer, after which they were homogenized according to the methods reported by [23]. The prepared stomach homogenates were stored at -20°C before analyses.

2.7. Calculation of total protein content.

The protein content of the different homogenates was assessed using the biuret technique, as outlined by [24], with some adjustments made. Specifically, potassium iodide was added to avoid the formation of cuprous oxide by the precipitation of Cu^{2+} ions. To enhance the stability of the complex, sodium potassium tartrate was included. Bovine Serum Albumin (BSA) was used as the standard protein. Different dilutions of the stock solution were prepared, and the Biuret reagent was added. The solution was left undisturbed at ambient temperature for 30 minutes. Subsequently, the optical densities (OD) of the consequent solutions were measured using a spectrophotometer at a wavelength of 540 nm. A blank solution was used as a reference. A graph depicting the relationship between absorbance and protein concentration was generated, and the proteins in the samples were determined by extrapolating from the standard curve.

2.8. The quantification of c-reactive protein (CRP) levels.

The determination of C-reactive protein concentration was done using the method described by [25].

A volume of 100 μL was extracted from each sample using a micropipette and thereafter introduced into the well. A blank well was prepared by pouring 100 μL of a 5 $\mu\text{g}/\text{mL}$ CRP standard into it. A volume of 100 μL of CRP enzyme reagent was introduced into each well, followed by gentle agitation and subsequent resting for 15 minutes. The wash buffer was diluted with 250 mL of distilled water and thereafter used to undergo three rounds of thorough washing. The wells were filled with 100 μL of the prepared working solution and left to incubate at room temperature for 15 minutes.

A volume of 50 μL of the prepared stop solution was introduced into the wells and gently agitated for 15 seconds. The absorbance was measured at a wavelength of 450 nm using a plate reader, with a reference wavelength of 630 nm. The mean and standard deviation of the absorbance were calculated using the absorbance values from the microplate reader.

2.9. The determination of tumor necrosis factor-alpha (TNF- α).

The concentration of serum TNF- α was determined using a commercially purchased rat TNF- α enzyme-linked immunosorbent assay (ELISA) kit according to the method reported by [26].

A volume of 100 μL was extracted from each sample using a micropipette and then transferred into individual wells. The blank well was filled with 100 μL of TNF-Alpha Standard

Working Solution at a concentration of 1000 pg/mL. The samples were incubated for 90 minutes at a temperature of 37°C. Following the termination of the procedure, the samples were subjected to decantation, followed by the addition of 100 µL of Biotinylated detection Ab working solution. The plates were covered and incubated for one hour at a temperature of 37°C. The solutions underwent decantation, followed by the addition of a small quantity of wash buffer. The resulting mixture was then patted dry by gently striking it against clean tissues. This procedure was done three times. Subsequently, a volume of 100 µL of HRP conjugate working solution was introduced into each well, followed by covering and incubation at a temperature of 37°C for 30 minutes. The dishes were retrieved and subjected to five rounds of washing before being patted dry. A volume of 90 µL of substrate reagent was introduced into the wells and shielded from sunlight by placing the plates within a film. The plates were then incubated at a temperature of 37°C for 15 minutes. The wells were filled with 50 µL of stop solution, and the optical density was measured at 450 nm. The mean and standard deviation of the mean were calculated using the optical density obtained from the microplate reader.

2.10. The determination of interleukin-6 (IL-6) concentration.

The concentration of serum IL-6 was determined using the ELISA technique as reported by [26].

A volume of 100 µL was extracted from each sample using a micropipette and then transferred into individual wells. The blank well was filled with 100 µL of IL-6 Standard Working Solution at a concentration of 800 pg/mL. The samples were incubated for 90 minutes at a temperature of 37°C. Following the termination of the procedure, the samples were subjected to decantation, followed by the addition of 100 µL of Biotinylated detection Ab working solution. The plates were covered and incubated for one hour at a temperature of 37°C. The solutions underwent decantation, followed by the addition of a small quantity of wash buffer. The resulting mixture was then patted dry by gently striking it against clean tissues. This procedure was done three times. Subsequently, a volume of 100 µL of HRP conjugate working solution was introduced into each well, followed by covering and incubation at a temperature of 37°C for 30 minutes. The dishes were retrieved and subjected to five rounds of washing before being patted dry. A volume of 90 µL of substrate reagent was introduced into the wells and shielded from sunlight by placing the plates within a film. The plates were then incubated at a temperature of 37°C for 15 minutes. The wells were filled with 50 µL of stop solution, and the optical density was measured at 450 nm. The mean and standard deviation of the mean were calculated using the optical density obtained from the microplate reader.

2.11. Concentration of malondialdehyde (MDA).

The concentration of MDA was assessed by quantifying the production of thiobarbituric acid reactive substances (TBARS) in the sample, using the methodology outlined by [27].

A 400 µL of the test sample was combined with 1600 µL of Tris-KCl buffer, followed by the addition of 500 µL of 30% TCA. Subsequently, a volume of 500 µL of 0.75% TBA was introduced and subjected to a 45-minute temperature of 80°C in a water bath. After being cooled to room temperature in ice, the sample was subjected to centrifugation at 3000 rpm for 10 minutes. The transparent liquid portion was collected, and the level of absorbance was determined by comparing it to a standard solution of distilled water at a wavelength of 532 nm.

The MDA level was determined by using an extinction coefficient of 0.156 $\mu\text{M}^{-1}\text{cm}^{-1}$, as described by [28].

$$\text{Lipid peroxidation (nmole MDA/mg protein)} = \frac{\text{Absorbance} \times \text{volume of mixture}}{E_{532\text{nm}} \times \text{volume of sample} \times \text{mg protein/mL}} \quad (3)$$

2.12. Determination of superoxide dismutase (SOD) activity.

The measurement of SOD activity was conducted using the [29] method.

A cuvette was used to combine 50 μL of the sample with 2.5 mL of the prepared carbonate buffer and 300 μL of epinephrine. The mixture was mixed using inversion, and the absorbance was measured at 30-second intervals for 2.5 minutes at a wavelength of 480 nm. The reference cuvette used in this study was identical to the one used for the samples, with water as a replacement.

$$\% \text{ Inhibition} = 100 - \frac{(100 \times \text{Increase in absorbance per minute for sample})}{\text{Increase in absorbance per minute for blank}} \quad (4)$$

1 unit of SOD activity was given as the amount of SOD necessary to cause 50% inhibition of the auto-oxidation of epinephrine.

2.13. Catalase (CAT) activity determination.

The determination of catalase activity was conducted using the [30] technique. This technique relies on the decrease in absorbance seen at 240 nm when catalase breaks down hydrogen peroxide. Although hydrogen peroxide does not exhibit a maximum absorbance at this particular wavelength, its absorbance demonstrates a satisfactory correlation with concentration, enabling its use in a quantitative experiment. The hydrogen peroxide solution was made, and a volume of 2,950 μL was transferred into a 1 cm quartz cuvette. Subsequently, 50 μL of the sample was added to the cuvette. The solution was rapidly transferred to a spectrophotometer after being inverted to ensure thorough mixing. The absorbance was measured at a wavelength of 240 nm at 5-minute intervals.

$$\text{Catalase activity} = \frac{\Delta A_{240} / \text{min} \times \text{reaction volume} \times \text{dilution factor}}{0.0436 \times \text{sample volume} \times \text{mg protein/mL}} = \mu\text{mole H}_2\text{O}_2 / \text{min/mg protein} \quad (5)$$

2.14. Histopathological examination of stomach samples.

A portion of each of the stomach samples was kept in 10% formalin. Samples were dehydrated by immersing them in ascending concentrations of alcohol solutions (70 -100%) and paraffin. Slides of stomach slices of 4-5 μm thickness were prepared and stained with hematoxylin and eosin (H and E) and then analyzed under a light microscope at x 40, x 100, and x 400. All slides were photographed with a Zeiss Axio photomicroscope.

2.15. Statistical analysis.

All data were expressed as mean \pm standard deviation of the mean and processed using GraphPad Prism (Version 8). The difference in variance was analyzed by the one-way analysis of variance (ANOVA). A post-hoc test was done using Bonferroni's multiple comparison test. Statistically significant results had $p < 0.05$.

2.16. Ethical approval.

Ethical approval was obtained from the Babcock University Health and Research Ethics Committee (BUHREC). Reference number, 915/23.

3. Results and Discussion

3.1. Images of stomachs after treatment with absolute ethanol.

Figure 1 shows representative images captured from each group. It was observed that the negative group had the most severe injury (ulcer induction), followed by the group treated with DMSO. Interestingly, treatment with varying doses of stigmasterol, rabeprazole, and cimetidine brought about a reduced ulcer induction.

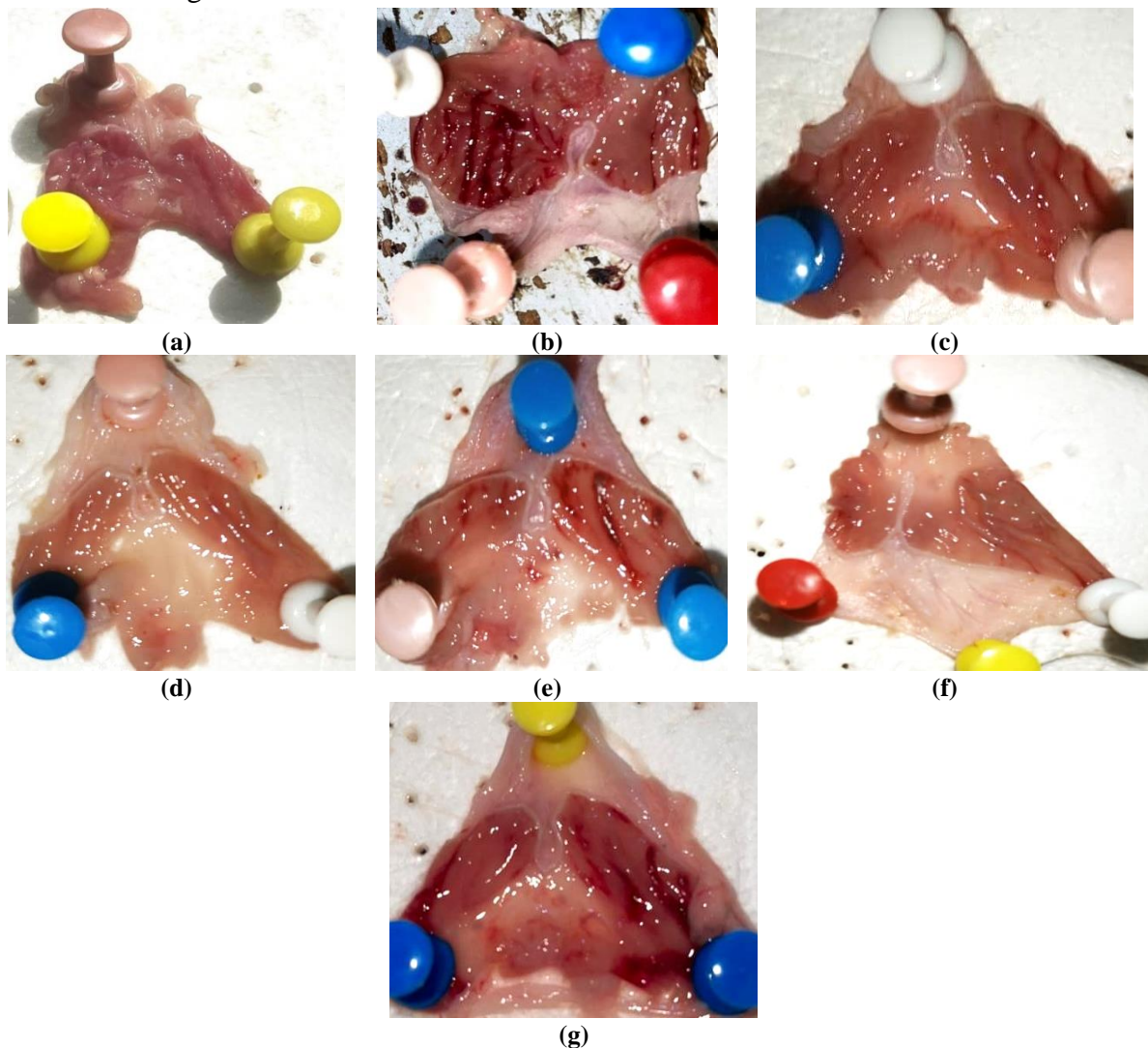


Figure 1. Effect of stigmasterol on ethanol-induced gastric ulcer. (a) Normal control group; (b) Negative control group; (c) 5 mg/kg stigmasterol; (d) 10 mg/kg stigmasterol; (e) 20 mg/kg rabeprazole; (f) 50 mg/kg cimetidine; (g) 1% DMSO.

This study used the method of ethanol induction, which involved administering 1 mL of absolute ethanol per kilogram of body weight to the rats. The use of this approach has been prevalent in various scientific research [9, 10, 12-15, 31]. This is similar to the harm induced by alcohol intake, and it is employed to investigate the impacts of different substances on the development and recovery of ulcers. The images displayed in Figure 1 showed the severity of ulceration caused by ethanol administration in the negative group and DMSO pretreated group;

this was evident by the bleeding observed. Moreover, this aligns with clinical manifestations of ulceration, such as stomach bleeding, epigastric pain, nausea, and early satiety [2]. Ulcer induction by ethanol may occur via the penetration and digestion of the gastric wall, facilitated by the proteolytic and hydrolytic effects of ethanol when it comes into contact with the stomach lining [15, 32].

3.2. Effect of stigmasterol on ethanol-induced mucosal injury (ulcer index).

Figure 2 shows that the stomach mucosa of the negative control suffered sufficient harm from ethanol. The gastric ulcer index of the negative control group (2.80 ± 0.45) was the highest, with severe lesions between 1.5 - 2.5 mm in size, and significantly different ($p < 0.05$) when compared with the pretreatment groups (reduction), aside from the DMSO group (2.4 ± 0.55). The pretreated groups at 5 mg/kg stigmasterol, 10 mg/kg stigmasterol, rabeprazole, and cimetidine showed reduced ulcer indices (1.40 ± 0.55), (0.80 ± 0.45), (1.60 ± 0.55), and (1.20 ± 0.45), respectively.

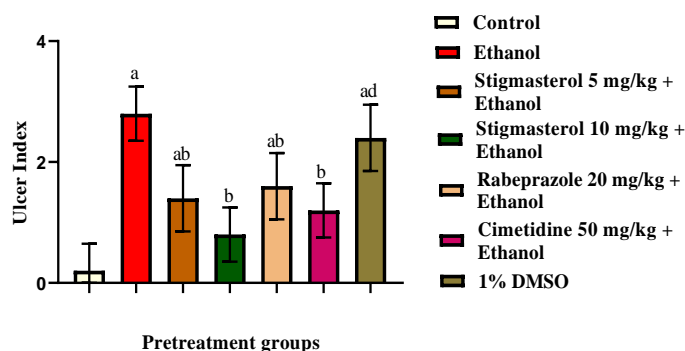


Figure 2. Effect of stigmasterol on gastric ulcer index. a, b, c, and d indicate statistically significant differences from the control, negative control (ethanol), 5 mg/kg stigmasterol, and 10 mg/kg stigmasterol, respectively ($p < 0.05$).

3.3. Effect of stigmasterol on percentage inhibition of ulceration.

Figure 3 shows that the stomach mucosa of the negative control group suffered severe harm from ulceration by absolute ethanol.

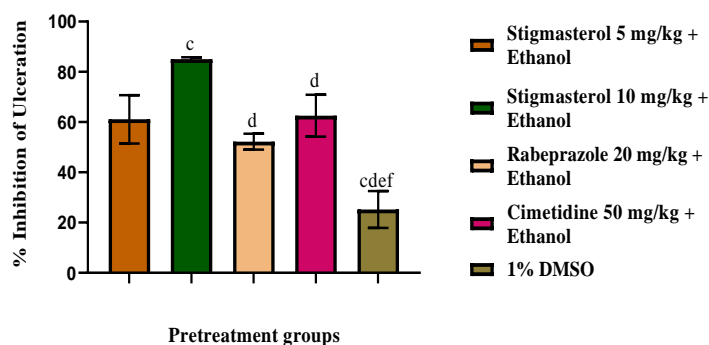


Figure 3. The percentage protection/inhibition by stigmasterol, rabeprazole, and cimetidine against ethanol-induced gastric ulcer. c: significantly different from 5 mg/kg stigmasterol ($p < 0.05$); d: significantly different from 10 mg/kg stigmasterol ($p < 0.05$); e: significantly different from 20 mg/kg rabeprazole ($p < 0.05$); f: significantly different from 50 mg/kg cimetidine ($p < 0.05$).

A significant percentage inhibition of ulceration was observed when the negative control group was compared with the 5 mg/kg stigmasterol, 10 mg/kg stigmasterol, rabeprazole, and cimetidine pretreated groups, having the following inhibition rates (61.11%),

(85.04%), (52.22%), (62.50%), respectively. This showed that the 5 mg/kg stigmasterol, 10 mg/kg stigmasterol, rabeprazole, and cimetidine provided a statistically significant increase in protection against the formation of ulceration by ethanol.

Based on the findings, 10 mg/kg stigmasterol gave the highest protection against ethanol, followed by cimetidine, 5 mg/kg stigmasterol, and rabeprazole. The groups pretreated with 10 mg/kg stigmasterol and 50 mg cimetidine had a higher percentage of ulceration inhibition, suggesting a more substantial decrease in ulcer development as a result of the therapy. Based on empirical investigations, it has been shown that phytosterols, such as beta-sitosterol and campesterol, have a gastroprotective quality and exhibit antiulcer effects against tissue necrosis. From this study, 20 mg/kg rabeprazole also gave a significant inhibition against the development of ulcers, as compared to the negative group. This may be attributed to the fact that rabeprazole can inhibit gastric acid secretion (as a proton pump inhibitor) by irreversibly inactivating the H^+/K^+ -ATPase pump, which plays a crucial role in maintaining gastric pH homeostasis [15, 33]. The 50 mg/kg cimetidine group had a notable reduction in ulceration, which may be attributed to its role as a histamine H2 receptor antagonist [15, 34]. Cimetidine has garnered significant support in scholarly literature as an agent with antiulcer qualities. It functions as a competitive inhibitor of histamine binding to histamine H2 receptors, hence eliciting several pharmacological effects that contribute to its antiulcer effect [15, 34].

3.4. Effect of stigmasterol on c-reactive protein (CRP) concentration.

Figure 4 demonstrated a statistically significant difference ($p < 0.05$) between the normal control (0.02 ± 0.01) and the negative control group (0.13 ± 0.02), the 5 mg/kg stigmasterol group (0.05 ± 0.01), and the group treated with DMSO (0.07 ± 0.01). However, no significant difference was observed when comparing the normal control to the cimetidine group (0.03 ± 0.01), the rabeprazole group (0.03 ± 0.01), and the 10 mg/kg stigmasterol group (0.03 ± 0.01). A statistically significant increase in the CRP level in the negative control group was seen in comparison to the pretreatment groups. There was a statistically significant difference seen between the group treated with 5 mg stigmasterol and the groups treated with 10 mg stigmasterol, rabeprazole, and cimetidine.

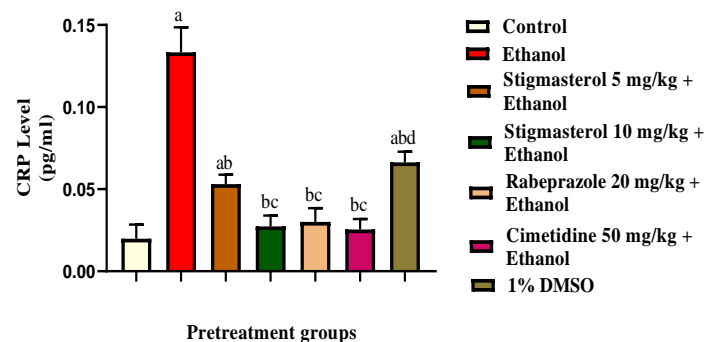


Figure 4. Effect of stigmasterol on CRP levels. a: $p < 0.05$ vs. control; b: $p < 0.05$ vs. negative control (ethanol); c: $p < 0.05$ vs. 5 mg/kg stigmasterol; d: $p < 0.05$ vs. 10 mg/kg stigmasterol.

3.5. Effect of stigmasterol on tumour necrosis factor-alpha (TNF- α).

Figure 5 demonstrated a statistically significant difference ($p < 0.05$) between the normal control group (0.01 ± 0.07) and the negative control group (0.02 ± 0.03), the 5 mg/kg stigmasterol group (0.01 ± 0.06), and the DMSO pretreated group (0.01 ± 0.01). However, no significant difference was observed when comparing the normal control group with the 10

mg/kg stigmasterol group (0.01 ± 0.06), the rabeprazole group (0.01 ± 0.01), and the cimetidine group (0.01 ± 0.07). A statistically significant increase in the negative control group was seen in comparison to the pretreatment groups. There was a significant difference between the group administered with 5 mg stigmasterol and the groups administered with rabeprazole and cimetidine.

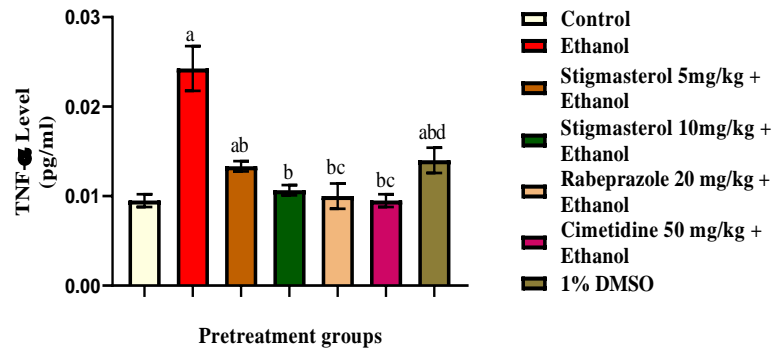


Figure 5. Effect of stigmasterol on TNF- α concentrations. a: statistically significant compared to the control; b: statistically significant compared to the negative control (ethanol); c: statistically significant compared to 5 mg/kg stigmasterol; d: statistically significant compared to 10 mg/kg stigmasterol.

3.6. Effect of stigmasterol on interleukin-6 (IL-6).

Figure 6 illustrates a significant difference ($p < 0.05$) when the normal control group (0.01 ± 0.01) was compared with the negative control group (0.05 ± 0.03). A statistically significant increase in the negative control group was seen in comparison to all the pretreatment groups. There was a statistically significant difference observed between the group treated with 5 mg stigmasterol (0.02 ± 0.07) and the group pretreated with cimetidine (0.01 ± 0.07) and DMSO pretreated groups (0.02 ± 0.07). However, no significant difference was found when comparing the groups pretreated with 10 mg stigmasterol and rabeprazole (0.01 ± 0.08). A statistically significant difference was seen between the group treated with 10 mg/kg stigmasterol (0.01 ± 0.01) and the group pretreated with DMSO.

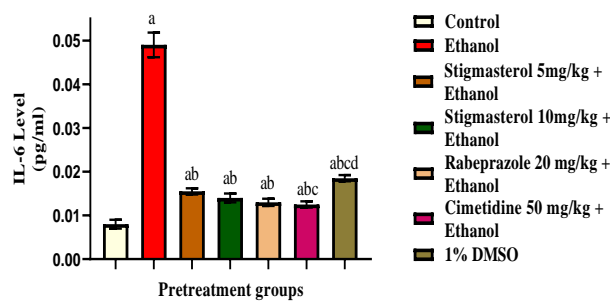


Figure 6. Effect of stigmasterol on IL-6 levels. a: $p < 0.05$ vs. control; b: $p < 0.05$ vs. negative control (ethanol); c: $p < 0.05$ vs. 5 mg/kg stigmasterol; d: $p < 0.05$ vs. 10 mg/kg stigmasterol.

The induction of ulcers with the use of ethanol has been shown in this study to have a significant impact on inflammatory cytokines like C-reactive protein (CRP), tumour necrosis factor-alpha (TNF- α), and interleukin-6 (IL-6), as seen in Figures 4, 5 and 6 respectively, since ethanol has been shown to induce oxidative stress, upregulate cytokine levels, and generate reactive oxygen species, hence causing tissue damage and inflammation inside the stomach region [15, 35]. From the results obtained, it was seen that ethanol induction increased the production of these inflammatory cytokines. Hence, it induced inflammatory responses in the rats. However, the rate of production of these inflammatory cytokines was reduced

significantly with stigmasterol pretreatment. Studies have shown that stigmasterol reduces the production of inflammatory cytokines. According to the literature [36], stigmasterol has demonstrated anti-inflammatory properties by alleviating allergic airway inflammation, attenuating inflammatory response via NF- κ B signalling [37], and suppressing phagocytosis while inhibiting inflammatory mediators like TNF- α . Stigmasterol's ability to reduce inflammatory cytokines like TNF- α is attributed to its impact on immune responses. Studies have also shown that it can, along with other phytosterols, scavenge cytokines like TNF- α , thereby reducing the inflammatory reaction induced by lipopolysaccharides (LPS) [38].

3.7. Effect of stigmasterol on malondialdehyde (MDA).

The results shown in Figure 7 indicated a significant difference ($p < 0.05$) between the normal control group (0.15 ± 0.08) and the negative control group (1.17 ± 0.07), 5 mg/kg stigmasterol group (0.57 ± 0.03), and DMSO pretreated group (0.68 ± 0.05). A significant increase in the negative control group was seen in comparison to all the pretreatment groups. There was a difference observed between the groups pretreated with 5 mg/kg stigmasterol and the groups pretreated with rabeprazole (0.26 ± 0.07) and cimetidine (0.21 ± 0.02). A statistically significant difference was observed between the group pretreated with 10 mg/kg stigmasterol (0.26 ± 0.06) and the group pretreated with DMSO, but no significant difference was observed when compared with groups pretreated with rabeprazole and cimetidine.

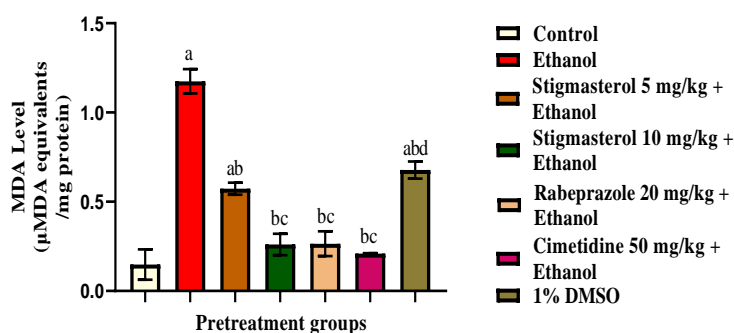


Figure 7. Effect of stigmasterol on MDA levels, a: statistically significant compared to the control; b: statistically significant compared to the negative control (ethanol); c: statistically significant compared to 5 mg/kg stigmasterol; d: statistically significant compared to 10 mg/kg stigmasterol.

The impact of ethanol and stigmasterol on malondialdehyde (MDA), a marker of oxidative stress, has been shown in this research, as seen in Figure 7. Ethanol increased the production of malondialdehyde, a product of lipid peroxidation and a marker of oxidative stress. Ethanol metabolism is known to generate reactive oxygen species (ROS) and free radicals [39]. However, pretreatment with stigmasterol showed reduced production of MDA. Stigmasterol has been shown in the literature to alleviate oxidative stress and reduce MDA levels in various studies. It acts as an antioxidant, preventing the deleterious effects of ethanol in conditions like alcoholic liver disease.

3.8. Effect of stigmasterol on superoxide dismutase (SOD).

Figure 8 presented statistical significance ($p < 0.05$) in the comparison between the normal control group (5.20 ± 0.33) and the negative control group (0.73 ± 0.12), the 10 mg/kg stigmasterol group (6.02 ± 0.36), and the DMSO pretreated group (2.65 ± 0.42). However, no significant difference was observed when comparing the normal control group to the 5 mg/kg

stigmasterol group (4.80 ± 0.47), the rabeprazole group (5.57 ± 0.45), and the cimetidine pretreated group (5.48 ± 0.41). The statistically significant decrease in the negative control group was seen in comparison to all of the pretreatment groups. The group administered 5 mg stigmasterol exhibited a statistically significant difference compared to the groups given 10 mg stigmasterol and DMSO. However, no significant difference was seen when comparing the groups treated with rabeprazole and cimetidine. A statistically significant difference was seen between the group treated with 10 mg/kg of stigmasterol and the group treated with DMSO.

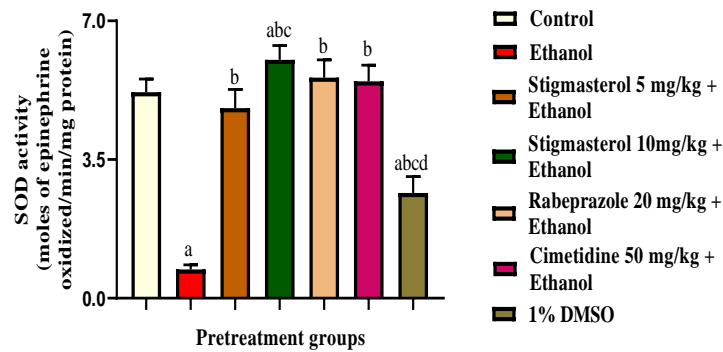


Figure 8. Effect of stigmasterol on SOD activities. a: statistically significant compared to the control; b: statistically significant compared to the negative control (ethanol); c: statistically significant compared to 5 mg/kg stigmasterol; d: statistically significant compared to 10 mg/kg stigmasterol.

3.9. Effect of stigmasterol on catalase (CAT).

Figure 9 demonstrated a statistically significant difference ($p < 0.05$) between the normal control group (2.99 ± 0.4) and the negative control group (0.40 ± 0.09), 5 mg/kg stigmasterol group (2.21 ± 1.14), rabeprazole (2.69 ± 0.03), cimetidine (2.76 ± 0.04), and DMSO groups (1.59 ± 0.05). However, no difference was observed when comparing the control to the 10 mg/kg stigmasterol group (2.85 ± 0.11). A significant decrease in the negative control group was observed in comparison to all of the pretreatment groups. The group that received 5 mg stigmasterol exhibited a statistically significant difference in comparison to 10 mg stigmasterol, rabeprazole, and cimetidine pretreatment groups. A statistically significant difference was seen between the group treated with 10 mg/kg of stigmasterol and the group pretreated with DMSO.

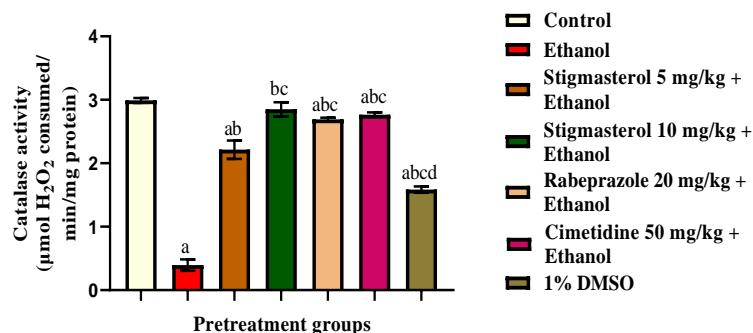


Figure 9. Effect of stigmasterol on the CAT activities of ethanol-induced gastric ulcer. a: $p < 0.05$ vs. control; b: $p < 0.05$ vs. negative control (ethanol); c: $p < 0.05$ vs. 5 mg/kg stigmasterol; d: $p < 0.05$ vs. 10 mg/kg stigmasterol.

The effects of stigmasterol on antioxidant enzymes like superoxide dismutase (SOD) and catalase, as seen in Figures 8 and 9, respectively, are crucial in understanding stigmasterol's impact on oxidative stress. According to the result obtained, induction with

ethanol brought about antioxidant damage, as well as oxidative stress, which led to an increased level of malondialdehyde (MDA) and a subsequent decrease in antioxidant enzymes (catalase and superoxide dismutase activity). Stigmasterol, however, did not lead to a decrease in catalase and SOD activity. It has been shown to possess antioxidant properties, which can impact antioxidant enzymes like catalase and SOD. Studies suggest that stigmasterol can alleviate oxidative stress and reduce MDA levels, indicating its potential to modulate antioxidant enzyme activity [40]. Also, the synergistic effect of stigmasterol on antioxidant activity has been reported, suggesting its ability to enhance the antioxidant defence system. This enhancement may contribute to the reduction of oxidative stress biomarkers like MDA and an increase or maintenance of the activity of Catalase and SOD.

3.10. Histopathology.

Figure 10 displays the photomicrograph of the groups taken at x100 H&E magnifications, respectively.

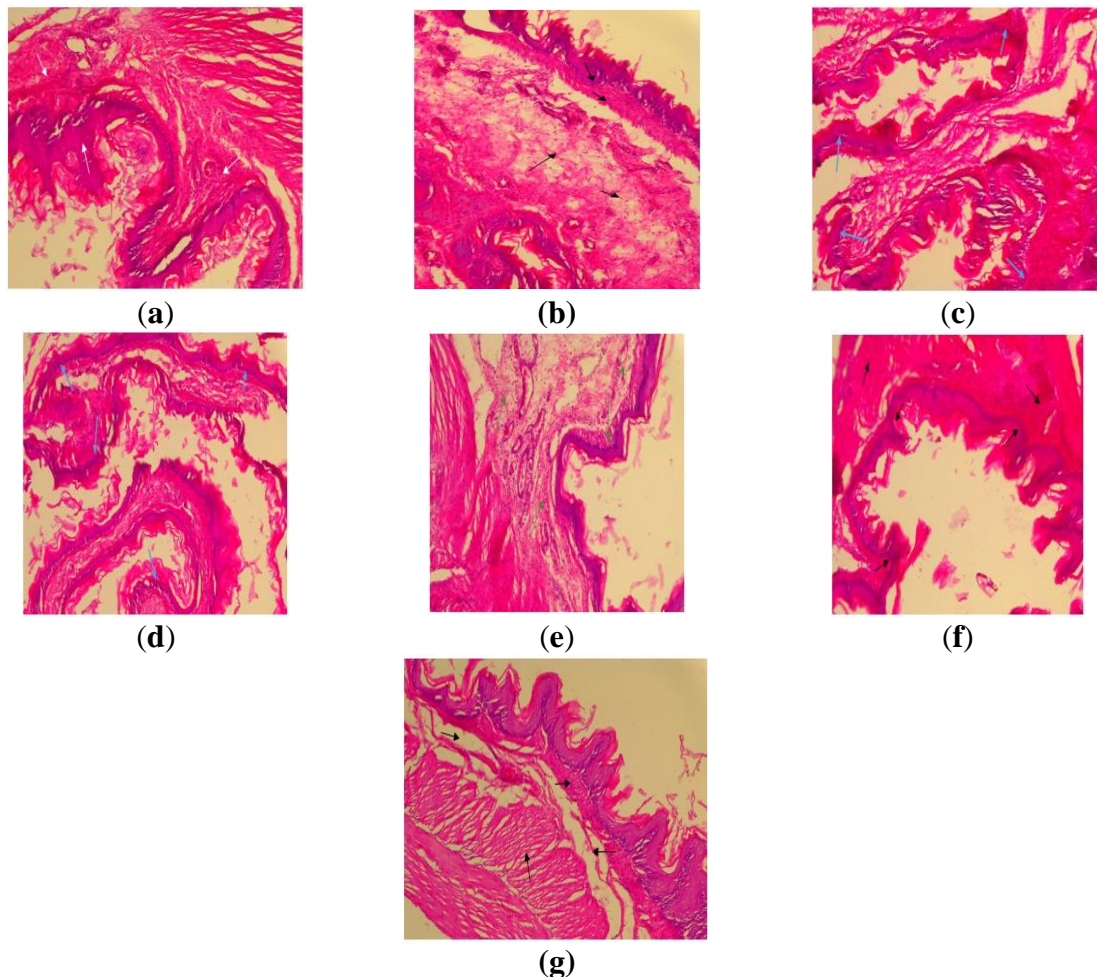


Figure 10. Microscopic effect of MEC on ethanol-induced gastric ulcer. (a) Normal x100 H&E section shows no disruption of surface epithelium with neither oedema nor leucocyte infiltration of the sub-mucosal layers; (b) ethanol x100 H&E section shows that there was severe disruption to the surface epithelium and oedema of the sub-mucosal layer with leucocytes infiltration; (c) and (d) stigmasterol 5 mg/kg and stigmasterol 10 mg/kg x100 H&E section show an improvement of mucus secretion disorders and also increased protein expression; (e) rabeprazole 20 mg/kg x100 H&E section shows that rabeprazole has no significant effect on lesions healing but promoted inhibition of gastric acid secretion and also an increase in the intragastric pH; (f) cimetidine 50 mg/kg x100 H&E section shows a suppressed increase in mucosal abrasion and promote lesion healing, inhibits gastric acid secretion; (g) 1% DMSO x100 H&E section shows an expression of superficial damage, noticed at the corpus mucosa.

As seen in Figure 10c and Figure 10d, administration of stigmasterol at a dose of 5 mg/kg and 10 mg/kg resulted in enhanced mucus production, alleviation of lesions, and increased protein expression. This indicated a favourable alteration in mucus production, a potential resolution of previous abnormalities, and an increase in protein expression within the investigated tissue. This result suggested that the pretreatment of stigmasterol at a dose of 5 mg/kg in the rats led to the restoration of normal mucus function and improved tissue health. As shown in Figure 10e, the administration of rabeprazole did not have any statistically significant impact on the healing of lesions. However, it demonstrated the ability to limit the release of gastric acid, which resulted in an elevation in the intragastric pH. This finding suggests that while rabeprazole did not have a significant effect on the repair of lesions, it did have a beneficial influence on decreased gastric acid output and increased pH levels in the stomach. This finding implied a possible therapeutic advantage in managing acid-related problems inside the stomach milieu. Based on the result from Figure 10f, it was observed that the administration of cimetidine resulted in a reduction in mucosal abrasion, facilitated the healing of lesions, and decreased the production of stomach acid. The study observed a strong protective impact of acute gastric mucosal damage, specifically in terms of the total area of erosions in the stomach. The findings indicated that cimetidine had a protective impact on acute gastric mucosal injury, perhaps leading to a decrease in tissue erosion and an improvement in the healing mechanism. Furthermore, the suppression of gastric acid production and the elevation of intragastric pH had a beneficial effect in regulating acid-related conditions inside the gastric region. According to Figure 10g, the application of DMSO resulted in the observation of superficial injury specifically at the corpus mucosa. The result suggested that there was observable damage to the outermost layer of cells at the corpus mucosa.

3.11. Histopathology.

Figure 11 illustrates the proposed mechanism of action of stigmasterol, as derived from the findings of the study

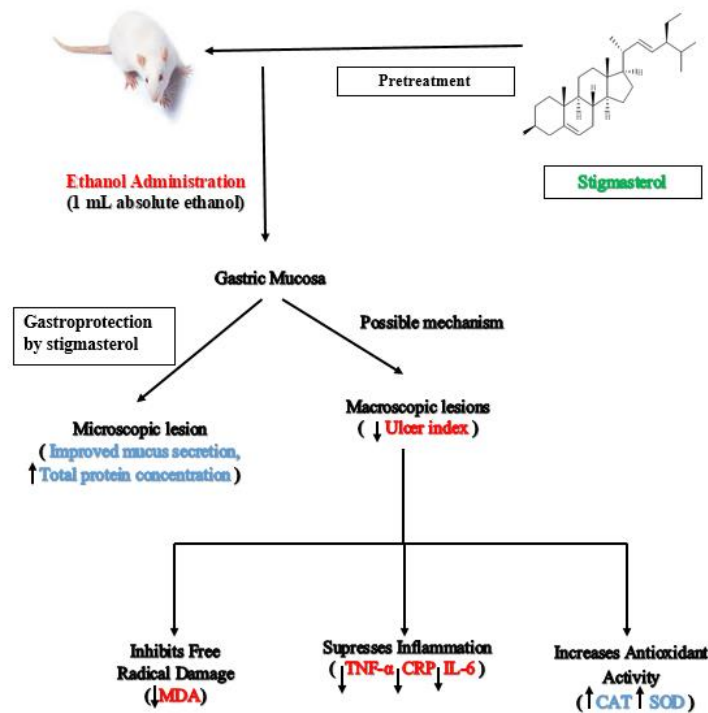


Figure 11. Proposed mechanism of action of stigmasterol.

4. Conclusions

The gastroprotective property of stigmasterol has been demonstrated through its efficient mitigation of ulceration, inflammatory reaction, and oxidation, induced by ethanol administration. Ethanol substantially impacted the ulcer index, levels of inflammatory cytokines (CRP, TNF- α , and IL-6), and MDA, and reduced the activities of antioxidant enzymes (SOD and CAT). Interestingly, stigmasterol at doses of 5 mg/kg and 10 mg/kg body weight demonstrated significant suppression of ulceration, inflammatory cytokine concentrations, and lipid peroxidation (MDA level). It also significantly increased protection against ulceration, TNF- α , and IL-6 activities in the treated rats. This study has successfully demonstrated that stigmasterol exhibited significant gastroprotective properties. Further research is recommended to look into the isolation of stigmasterol from plant sources in ulcer studies. This could help develop cheaper and more effective drugs in preventing and treating ulcers.

Author Contributions

Conceptualization, A.A.A.K.; methodology, A.A.A.K., and R.E.O.; software, R.E.O.; validation, A.A.A.K., and R.E.O.; formal analysis, R.E.O.; investigation, R.E.O., F.M.O., G.O.A., and B.B.O.; resources, A.A.A.K., R.E.O., F.M.O., G.O.A., and B.B.O.; data curation, A.A.A.K.; writing—original draft preparation, R.E.O., F.M.O., G.O.A., and B.B.O.; writing—review and editing, R.E.O.; visualization, R.E.O., and F.M.O.; supervision, A.A.A.K.; project administration, A.A.A.K.; funding acquisition, R.E.O., F.M.O., G.O.A., and B.B.O. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement

The animal study protocol was approved by the Ethics Committee of BABCOCK UNIVERSITY (BUHREC 915/23 approved on 12th March, 2023)

Informed Consent Statement

Not applicable.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

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