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Research Article

Anti-ulcerogenic Activity of Methanol Fraction of *Hibiscus asper* Leaves in Albino Rats

Njoku U.O[1a], Umeh C.G.[1*] and Ogugofor M.O.[1b]

[1]Pharmacological Unit, Department of Biochemistry, University of Nigeria, Nsukka, Enugu State Nigeria;

ABSTRACT

Hibiscus asper, a herb used in folklore medicine has demonstrated strong and diverse therapeutic potentials, such as, anti-inflammatory, anti-oxidant, anti-depressant, and widely used in the management of diseases. Despite the ethno-pharmacological value of this plant, no study has been conducted to evaluate its anti-ulcerogenic activity. Methanol fraction of *Hibiscus asper* leaves (100, 200 and 400 mg/kg) was used to investigate anti-ulcerogenic activity in acidified ethanol-induced ulceration model, whereas, Omeprazole was used as the standard drug; both were administered orally. Pre-treatment of rats with the plant fraction significantly inhibited development of gastric ulcers by oral administration of acidified-ethanol in a dose-dependent manner, presenting protection to ulceration of 46.6 % to 81 % as doses increased from 100 to 400 mg/kg. No significant difference was observed in gastric protection presented by Omeprazole pre-treated control group (87.6 %) and the fraction, at doses of 200 (76.3 %) and 400 mg/kg (81 %). More so, histological examination performed revealed that the gastric mucosa of rats pre-treated with fraction, exhibited reduced gastric lesions and sub-mucosal oedema relative to the positive control, which comparatively showed gastric mucosal protection. Therefore, the study suggests that *Hibiscus asper* might possess some protective and healing potentials in rats which might be due to the stimulation of prostaglandin synthesis.

***Keywords**: Hibiscus asper,* acidified-ethanol, antiulcerogenic, gastric mucosa

*Author for correspondence: Email: *chinenyenwagoodness@gmail.com*; Tel: +234-8064778021

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INTRODUCTION

Peptic ulcer is a public health condition with high rate of morbidity and substantial mortality and has become the main focus of experimental and clinical investigations, mainly because of its high prevalence within the global population (Brito *et al*., 2018). Peptic ulcers are sores or lesions within the gastrointestinal mucosa extending throughout the muscularis mucosae, usually characterized by different stages of necrosis, neutrophil infiltration, blood flow reduction, increased oxidative stress and inflammation (Sharifi-rad *et al*., 2018). It is used in managing common disorder of the stomach and duodenum (Sharath *et al*., 2015; Araujo *et al*., 2011). The fundamental physiopathology of gastric ulcer results from an imbalance between some endogenous and exogenous aggressive factors like increased secretion of hydrochloric acid, pepsin, refluxed bile, leukotrienes, reactive oxygen species (ROS), the prolonged consumption of non-steroidal anti-inflammatory drugs, alcohol, Helicobacter pylori infection, oxidative stress conditions; and protective factors, which include the function of the mucus-bicarbonate barrier, surface active phospholipids, prostaglandins (PGs), mucosal blood flow, cell renewal and migration, nonenzymatic and enzymatic antioxidants, and a few growth factors (Brito *et al*., 2018; Sreeja *et al*., 2018; Alrashdi *et al*., 2012). The multifactorial pathogenesis of peptic ulcers is secretion of gastric acid. The disease has high prevalence and is related to significantly high annual mortality rates (Ayaz *et al*., 2017). peptic ulcer is a common global problem with increasing incidence and prevalence. Worldwide, 14.5 million people have the ulcers with a mortality of 4.08 million (Sharath *et al*., 2015).

The treatment of peptic ulcer is usually based on the inhibition of gastric acid secretion as well as acid-independent therapy such as using antacids, H_2 receptor blockers (ranitidine, famotidine) or proton pump blockers (omeprazole and lansoprazole) (Bighetti *et al*., 2005; AlRashdi *et al*., 2012). However, most of these drugs produce several adverse reactions including toxicities and even may alter biochemical mechanisms of the body upon chronic usage (Sharath *et al*., 2015; Luiz-Ferreira *et al*., 2010). Natural products, especially

plants derived chemicals are considered as promising source for the development of new agents with safe therapeutic window (Atanasov *et al*., 2015). Traditional medicine using plants have been shown to be successful in the treatment of gastrointestinal disorders, including peptic ulcer disease (Gadekar *et al*., 2010). Consequently, plant extracts stand out as the most promising substances in the search for new therapies for the treatment of gastric ulcer (Ayaz *et al*., 2017). *Hibiscus asper* Hook.f. (Malvaceae) is an important medicinal plant widely distributed throughout tropical Africa and in Madagascar. This species belongs to the genus Hibiscus represented by 250 species and characterizes by the presence of biological active compounds like flavonoids, phenolic acids, and polysaccharides (Vasudeva and Sharma, 2008). In the Western region of Africa, this plant is widely used by the traditional practitioners for the treatment of inflammation, anaemia, jaundice, leucorrhoea, poison antidote, depression and dysmenorrheal (Schippers and Bosch, 2004). In the Western region of Cameroun, the leaves are used as a potent sedative, tonic and restorative. It is also use to treat male infertility and skin infection (Foyet *et al*., 2011). Studies has shown that *H. asper* leaves have anti-oxidative property (Lucian *et al*., 2014); also some other species of hibiscus possess antiulcerogenic effect (Nyam *et al*., 2016; Srivastava *et al*., 2013). This research was aimed at determining the anti-ulcer activity of methanol fraction of *Hibiscus asper* leaves on acidified ethanol-induced gastric ulcer in rats.

MATERIALS AND METHODS

Animals: A total number of twenty-four (24) Wistar albino rats were used in the study, while eighteen (18) albino mice were used for acute toxicity test on the plant extract. All the animals used were obtained from the animal house of the Department of Zoology, University of Nigeria Nsukka. The animals were acclimatized for one week in the Department of Biochemistry animal house, University of Nigeria, Nsukka under 12-hour light and dark cycle, and at room temperature 25$_o$C. They were fed with finisher pelletized feeds and water prior to experiment.

Plant Collection and Identification: Fresh leaves of *Hibiscus asper* were collected from Isuofia in Aguata Local Government Area of Anambra State. The leaves were identified and authenticated by Mr. Felix Nwafor of Pharmacognosy and Environmental Medicine Department, University of Nigeria Nsukka, Enugu State, Nigeria

Preparation of Plant Material: *Hibiscus asper* leaves were air-dried at room temperature and pulverized into powder for extraction. The powder (1300 g) was macerated in 80 % methanol and allowed to stand for 48 hours at room temperature. The mixture was filtered with Whatman No. 1 filter paper and the filtrate was concentrated using rotary evaporator to get a brownish black semi solid extract.

Fractionation of Plant Extract: Solvent partition of crude methanol extract was done by using the protocol designed by Kupchan and Tsou (1973) and modified version of Wagenen *et al.* (1993). Fractionation was carried out using n-hexane, ethylacetate and 20 % methanol. Crude extract of 20 g was weighed and dissolved with 250 ml of 20 % methanol to form the methanol fraction. Then, 250 ml of n- hexane was added to the mixture and poured into a separating funnel. It was allowed to stand for 20 minutes for proper separation, the upper part was collected in a beaker while the lower part was further mixed with 250 ml of n- hexane, and it was also allowed to stand for 20 minutes for proper separation. The upper part was collected in another beaker; the process was repeated for the third time. The procedure above was continued with the same 20 g of crude extract, and 250 ml of ethyl acetate was added, the process was repeated three times. At the end, all the n- hexane fraction, ethylacetate fraction and methanol fraction were pulled together respectively and concentrated (Suganga and Thangaraj, 2014). Pilot study was conducted using the three fractions and it was observed that methanol fraction gave the greatest gastro protective effect after inducing ulcer with HCl-ethanol in the albino rats. Therefore, the methanol fraction was used throughout the experiment.

Acute Toxicity Studies: Acute Toxicity Study of crude ethanol extract of *Hibiscus asper* leaves was carried out using Lorke's method (1983).

Treatment Protocol: A total number of twenty-four (24) Wistar albino rats of both sexes were used. The animals were randomly divided into six (6) experimental groups (1-6), of four (4) rats each. After seven days of acclimatization, rats of the respective groups were pretreated Omeprazole, as standard control group and graded doses of methanol fraction of *Hibiscus asper* leaves (MFHAL) for fourteen days before induction on the fifteenth day. The treatment protocol is summarized below:

- *Group 1 received Distilled water (Normal control)*
- *Group 2 received HCl-ethanol induced with no treatment (negative control)*
- *Group 3 received 20 mg/kg b.w Omeprazole + HCl-ethanol (Standard control):*
- *Group 4 received 100 mg/kg b.w of MFHAL*
- *Group 5 received 200 mg/kg b.w of MFHAL*
- *Group 6 received 400 mg/kg b.w of MFHAL*

HCl-Ethanol-induced Ulceration: The ulcer induction was carried out an hour after pretreatment according to a modified method of Mota *et al.* (2008) using 0.8 ml of 5 % 0.3 M HCl /60 % absolute ethanol in Groups 2- 6. Rats were monitored for one hour and sacrificed while the stomachs were removed and opened along the greater curvature to remove gastric content.

Determination of Ulcer Index in the Stomach: The stomachs were opened along the greater curvature and rinsed with water to remove gastric contents and blood clots and examined by a 10× magnifier lens to assess the formation of ulcers. The ulcerative lesion index was calculated based on the following keys: No ulceration = 0, mildly ulcerated = 1, moderately ulcerated = 2, severely ulcerated = 3. The ulcer preventive/ inhibition index was calculated using the formula as described by Onwukwe *et al.* (2016):

$$\text{Ulcer index (UI)} = \frac{Total\ ulcer\ score}{Number\ of\ animals\ ulcerated}$$

Percentage inhibition of ulceration was calculated as below:
% Inhibition of ulceration/Ulcer protective index (%) =

$$\frac{\text{Ulcer index of untreated} - \text{Ulcer index of treated}}{\text{Ulcer index of untreated}} \times 100$$

Determination of Total Acidity in Gastric Fluid: A volume 1 ml of gastric juice was pipetted into 100 ml conical flask and diluted with 10 ml of distilled water. About 2 to 3 drops of Topfer's reagent was added and titrated with 0.01 N NaOH until the red color changed to yellowish orange. The volume of the alkali added was noted. Consumed volume corresponds to free acidity. Then 2 to 3 drops of phenolphthalein solution were added and titration was continued until red tinge color reappeared. The total volume of alkali added was noted. This volume corresponds to total acidity (Dashputre and Naikwade, 2011).

$$\text{Acidity} = \frac{\text{Volume of NaOH} \times \text{Normality} \times 100}{0.1}\ \text{mEq/L}$$

Determination of pH: An aliquot of 1 ml of gastric juice was diluted with 1 ml of distilled water, and pH of the solution was measured using pH meter (Dashputre and Naikwade, 2011).

Histological Examination: The histopathological evaluation was carried out using the method described by Drury *et al*. (1967). The stomachs of the sacrificed rats from all the experimental groups were collected for histopathological examination. The tissue samples were immersed in 10 % formalin for a minimum of 48 hours. The tissues were subsequently trimmed, dehydrated in 4 grades of alcohol (70 %, 80 %, 90 % and absolute alcohol), cleared in 3 grades of xylene and embedded in molten wax. On solidifying, the blocks were sectioned, 5 µm thick with a rotary microtome, floated in water bathe and incubated at 60 ˚C for 30 minutes. The 5 µm thick sectioned tissues were subsequently cleared in 3 grades of xylene and rehydrated in 3 grades of alcohol (90 %, 80 % and 70 %). The sections were then stained with hematoxylin for 15 minutes. Blueing was done with ammonium chloride. Differentiation was done with 1 % acid alcohol before counterstaining with eosin. Permanent mounts were made on degreased glass slides using a mountant; DPX.

Statistical Analysis

Results were analyzed using one-way analysis of variance (ANOVA) in statistical product and service solution (SPPS), version 21.0 and presented as mean ± S.D. value with $p < 0.05$ considered significant.

RESULTS

In the acute toxicity test, administration of 10, 100 and 1000 mg/kg of the crude methanol extract of *H. asper* to the animals caused no death in the first stage of the test. In the second stage of the test, no death was recorded even at the dose level of 5000 mg/kg. The LD_{50} of the crude extract of *H. asper* leaves was thus estimated to be greater than 5000 mg/kg as shown in Table 1.

Table 1:
Acute Toxicity of Crude Extract of *Hibiscus asper*

Group (n = 3)	Phases	Dosage (mg/kg b.w)	Mortality
I	1	10	0/3
II	1	100	0/3
III	1	1000	0/3
IV	2	1600	0/3
V	2	2900	0/3
VI	2	5000	0/3

The gastroprotective activity of methanol fraction of *Hibiscus asper* leaves in HCl/ ethanol-induced gastric lesion model is shown in Table 2. Treatment of rats with acidified ethanol solution produced extensive gastric ulcers in all the control animals. In the negative control group, the mean ulcer index was found to be 3.63 ± 0.13.

Pretreatment of rats with MFHAL at the dose of 100 mg/kg (MUI, 1.94 ± 0.11), 200 mg/kg (MUI, 0.86 ± 0.38) and 400 mg/kg (MUI, 0.69 ± 0.24) significantly ($p<0.05$) inhibited the formation of gastric lesions in a dose-dependent manner when compared with the negative control group. MFHAL exhibited 46.6 %, 76.3 %, and 81 % protection to ulceration at doses of 100, 200 and 400 mg/kg respectively. There was no significant difference ($p<0.05$) in ulcer inhibition between Omeprazole pre-treated control group (87.6 %), and the fraction at doses of 200 mg/kg (76.3 %) and 400 mg/kg (81 %). Compared to the ulcer control rats (negative control group), rats pretreated with graded doses of MFHAL (100, 200 and 400 mg/kg) showed significant ($p<0.05$) decreases in gastric volume (1.80 ± 0.08, 1.64 ± 0.05, 1.54 ± 0.08), total acidity (73.50 ± 5.20, 56.75 ± 2.99, 52.50 ± 3.42) and significant ($p<0.05$) increases in gastric pH (6.64 ± 0.37, 7.29 ± 0.56, 7.57 ± 0.16) respectively.

Table 2:
Effect of MFHAL Pretreatment in Acidified-Ethanol –induced Gastric Ulcer

Group	Ulcer Index	% Ulcer Inhibition	PH	Total Acidity	Gastric Juice Volume
Normal control	$0.00 \pm 0.00_a$	100	$7.47 \pm 0.16_c$	$48.50 \pm 7.23_a$	$1.39 \pm 0.41_a$
Positive control	$3.63 \pm 0.13_c$	—	$3.87 \pm 0.17_a$	$123.25 \pm 3.59_d$	$2.81 \pm 0.23_c$
20 mg/kg b.w Omeprazole	$0.45 \pm 0.26_{ab}$	87.6	$7.58 \pm 0.33_c$	$48.75 \pm 2.99_a$	$1.46 \pm 0.07_a$
100 mg/kg b.w of MFHAL	$1.94 \pm 0.11_b$	46.6	$6.64 \pm 0.37_b$	$73.50 \pm 5.20_c$	$1.80 \pm 0.08_b$
200 mg/kg b.w of MFHAL	$0.86 \pm 0.38_{ab}$	76.3	$7.29 \pm 0.56_c$	$56.75 \pm 2.99_b$	$1.64 \pm 0.05_{ab}$
400 mg/kg b.w of MFHAL	$0.69 \pm 0.24_{ab}$	81	$7.57 \pm 0.16_c$	$52.50 \pm 3.42_{ab}$	$1.54 \pm 0.08_{ab}$

Values are presented as mean ± SD (n = 4). Values with different superscripts differ significantly at p < 0.05

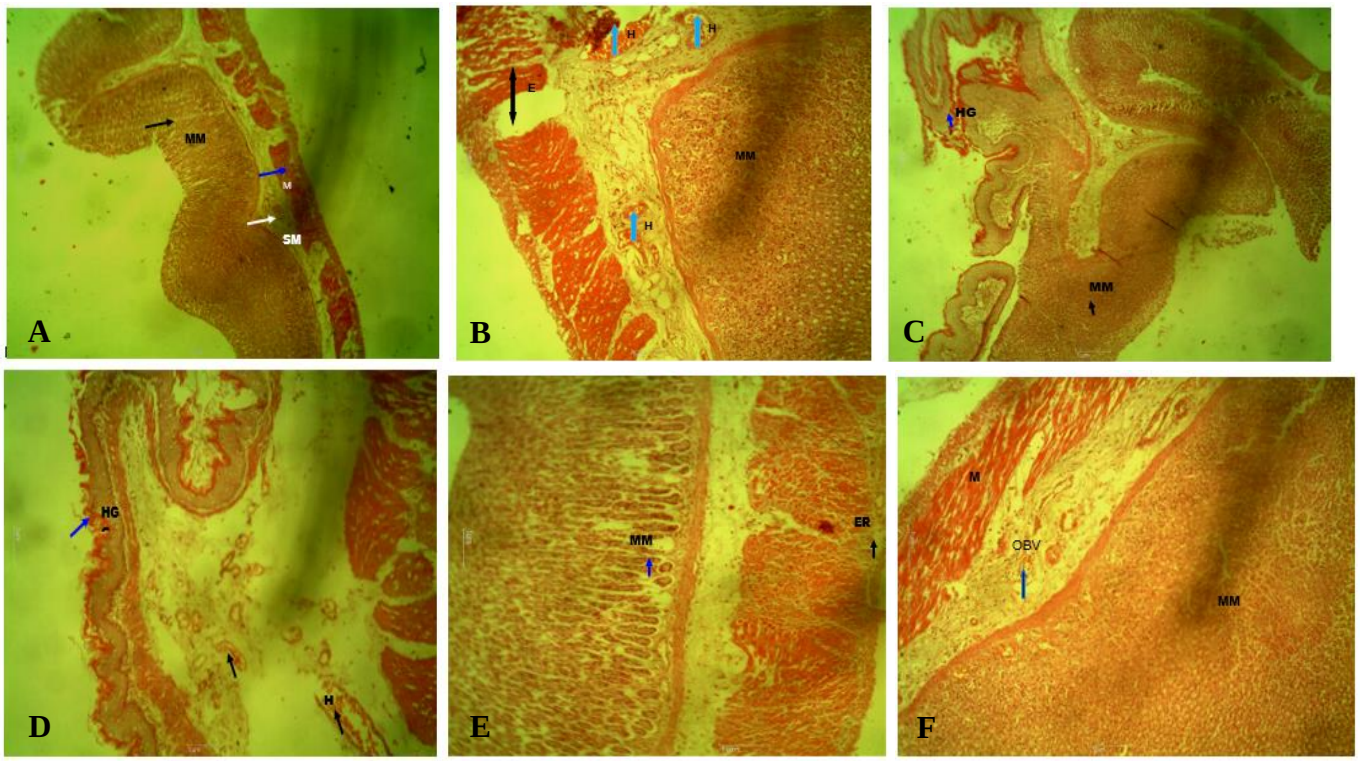

Plate 1:
A. Normal control (Group 1): Photomicrograph of stomach showed normal glandular stomach with intact mucosa (M) (blue arrow), submucosa (white arrow) and muscularis mucosa(MM) (black arrow) H&E. mag. 100X.
B. Positive Control (Group 2): Photomicrograph of stomach showed erosion (E) of the mucosa and deep multiple hemorrhage (H) (blue arrow) in the submucosa of the stomach, secondary to treatment related inflammation. The muscularis mucosa (MM) is intact
C: Standard Control (Group 3): Photomicrograph of the glandular stomach showed a wound healing process hypergranulation (HG) characterized by the appearance of light red or dark pink flesh that can be bumpy or granular (blue arrow). The mucosa is intact.
D: 100 mg/kg b.w of MFHA (Group 4): Photomicrograph of stomach showed deep multiple vascular hemorrhages (H) (black arrow) in the submucosa of the stomach, secondary to treatment related inflammation. The muscularis mucosa (MM) is intact. Meanwhile, the presence of hypergranulation (blue arrow) is due to wound healing
E: 200 mg/kg b.w of MFHA (Group 5): Photomicrograph of stomach showed normal appearance of the muscularis mucosa (MM) (blue arrow), minor erosion (ER) of the stomach epithelium was observed (black arrow). H&E. mag. 100X
F: 400 mg/kg b.w of MFHA (Group 6): Photomicrograph of stomach showed normal appearance of the muscularis mucosa (MM) and mucosa (M). The submucosa is filled with minor occluded blood vessels (OBV) (blue arrow) but no pathological lesion was observed

There were no significant differences ($p<0.05$) in gastric volume, total acidity and gastric pH between Omeprazole pre-treated group (1.46 ± 0.07, 48.75 ± 2.99, 7.58 ± 0.33) and 400 mg/kg MFHAL (1.54 ± 0.08, 52.50 ± 3.42, 7.57 ± 16) respectively.

Rats pretreated with Omeprazole and graded doses of MFHAL showed comparatively better protection of gastric mucosa as seen by reduction of ulcer area, reduced submucosal oedema, and leucocytes infiltration compared to the negative control group, which presented extensive gastric mucosa damage and deep multiple hemorrhages (Plate 1).

DISCUSSION

The plant showed to have high safety profile from the acute toxicity studies which relates to the report of Quattrochi (2012) for insignificant toxicity of *H. asper* leaves in humans and can be used as vegetable.

Peptic ulcers are caused by an imbalance between the protective and the aggressive mechanisms of the mucosa (Sharifi-Rad *et al*., 2018). HCl causes severe gastric mucosal damage in HCl/ethanol-induced gastric ulceration model (AIRashdi*et al*., 2012; Gopinathan and Naveenraj, 2013) whereas ethanol produces necrotic lesions by direct necrotizing action by impairing gastric defensive factors (Brito *et al*., 2018). Upon oral administration, absolute ethanol disrupts gastric mucosal barrier, provokes pronounced microvascular changes and other alcohol induced injuries in a matter of minutes. (Moleiro *et al*., 2009).

Oral administration of acidified ethanol was characterized by maximum hemorrhagic mucosal lesions as observed in negative control group of animals with ulcer index, 3.63 ± 0.25, while MHFAL significantly ($p < 0.05$) inhibited ulcer formation in the stomach in contrast to ulcerated group in a dose dependent manner. The dose dependent significant ($p<0.05$) decrease in ulcer index observed or caused by MFHAL, optimal at the highest tested dose of 400 mg/kg, when compared with the untreated group, indicates anti-ulcer property of the plant. This anti-ulcer effect of *H. asper* leaves at the highest dose of 400 mg/kg is comparable with the

commercially available antiulcer drug, omeprazole (20 mg/kg). Possibly, MFHAL suppressed the necrotizing action of ethanol, evident by the decreased gastric lesion. Hence, enhancing defensive factors, increasing the secretion of bicarbonates and enhancing gastric mucus production in the test group. Therefore, the experimental results of the study showed that methanol fraction of *Hibiscus asper* leaves (MFHAL) possibly has an effective antiulcer activity against HCl-ethanol-induced gastric mucosal injury.

Omeprazole, as a proton pump inhibitor (PPI), offered a fairly protected gastric mucosa and has been widely used as an acid inhibitor agent for the treatment of disorders related to gastric acid secretion (AIRashidi *et al*., 2012). PPIs are capable of producing almost complete suppression of acid secretion. The mechanism of action of omeprazole is such that it binds very specifically to a single subunit of the H_+, K_+-ATpase at the secretory surface of parietal cell and inactivates it, and it reduces acid secretion regardless of the source of secretory stimulation (Karen, 2015). Omeprazole exhibits an antisecretory and protective effect, they are effective in treating peptic ulcer disease and gastroesophageal reflux with both short- and long-term use (Ode and Asuzu, 2011). In the present study, different doses of the MFHAL (100, 200, and 400 mg/kg) were evaluated for their effect on volume of gastric secretion, pH, and total acidity, along with the standard drug omeprazole (20 mg/kg). The MFHAL decreased total acidity and gastric juice volume, invariably increasing the gastric wall mucus which is in consistent with results reported by Al-Attar (2011) in the study, "Protective effect of *Avicennia alba* leaves extract on gastric mucosal damage induced by ethanol". Similarly, Abebaw *et al*., (2017) in the evaluation of anti-ulcer activity of the leaf extract of *Osyris quadripartita* Decne (Santalaceae) in rats discovered a reduction in gastric acidity in treated animals. In negative control group, there was increased acid secretion resulting in increased in ulcer lesion. This model showed that at the highest tested dose of 400 mg/kg, MFHAL has got better antisecretory activity as evidenced by reduction in the mean volume of gastric secretion, rise in pH, and reduction in total acidity ($P< 0.05$) compared to the negative control.

Histopathology results revealed protection of gastric mucosa and inhibition of inflammatory markers infiltration into the gastric wall in rats pretreated with MFHAL as indicated in table 2. This is consistence with reports by Abdulla *et al*. (2010), Wasman *et al*. (2010), AIRashidi *et al*., (2012), Foyet *et al*., (2011) and Swarnakar *et al*. (2007)

The mechanism of anti-ulceration activity of *Hibiscus asper* may be antioxidant free radical scavenging, cytoprotection, gastric acid anti-secretory activity, increasing luminal prostaglandin secretion, increasing mucus secretory activity and increased gastric alkaline secretion.

In conclusion, the results from this study suggests that the methanol leaf fraction of *H. asper* possibly possesses anti-ulcerogenic property

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