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ISOLATION OF STIGMASTEROL AND GASTROPROTECTIVE ACTIVITY OF THE ETHYL ACETATE PORTION FROM *ACACIA NILOTICA* LINN (FABACEAE) SEEDS

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ABSTRACT

Acacia nilotica Linn is used traditionally in the treatment of various diseases, such as diarrhea, ulcer, dysentery, asthma, inflammation and cancer. The present study aimed at isolation and characterization of a steroidal compound and evaluating the gastroprotective activity of the ethyl acetate portion of *A. nilotica* seeds in indomethacin-induced ulcer model. Fractionation of the ethyl acetate portion using standard chromatographic techniques led to the isolation of a compound (B2-I) which on further purification and characterisation using physical, chemical and spectral analysis, and by comparison with literature values was identified as stigmasterol. The effect of ethyl acetate portion of *A. nilotica* on experimentally induced ulcer was dose-dependent with curative ratios of 40%, 60% and 75% at concentrations of 100, 200 and 300 mg/kg body weights respectively. It significantly ($P<0.05$) decreased free and total acidity and increased the pH of gastric juice with respect to the indomethacin treated group. This justifies the ethnomedicinal use of *A. nilotica* seed as an antiulcer agent. The study recommends that *A. nilotica* seeds be evaluated against other ulcer models such as ethanol and stress induced ulcers.

Keywords: *Acacia nilotica*, phytochemical, ulcer, gastroprotective activity.

INTRODUCTION

Gastric ulcer occurs as a result of an imbalance between aggressive and defensive factors of the gastric mucosa (the mucous membrane layer of the stomach). Pepsin activity and hydrochloric acid secretion, decreased blood flow, infection caused by *Helicobacter pylori*, alcohol and tobacco consumption, and excessive use of non-steroidal anti-inflammatory drugs (NSAIDs) are the main aggressive contributors [1]. Blood flow, mucus and bicarbonate secretion, cytoprotective prostaglandins (PGs), nitric oxide, the endogenous

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antioxidant system, and sufficient blood flow are all mucosal defense components [2].

Even in this modern era, 80% of the global population especially from developing countries still use herbal medicine for primary health care because of better cultural acceptability, better compatibility with the human body, and lesser side effects, hence, herbal medicine is being promoted as an alternative treatment for the management of ulcer ailments [3, 4, 5]. Several Nigerian plants have been reported with antiulcerogenic potentials in the prevention and management of ulcers in Nigeria [6]. Phytochemical substances such as flavonoids and tannins are identified as active principles from medicinal plants that can be therapeutically used as antiulcer agents in modern medicine.

Acacia nilotica (Fabaceae) tree is found in well-watered Sahelian and Sudanian savannas, the southern Arabian Peninsula, Gambia, Sudan, Togo, Ghana, and Nigeria [7]. It is also found on lateritic soil in the Himalayan foothills in India [8]. Different parts of *A. nilotica* are widely used in traditional medicine for the treatment of various ailments. In different countries of West Africa, *A. nilotica* pods, bark, gum, root, flowers and leaves are implored for the treatment of several diseases, such as gastrointestinal disorders (diarrhea, dysentery, hemorrhoid, abdominal aches, toothaches, and sore throat), diabetes, asthma and hypertension [9]. In addition, the roots are used against cancers and tumors of the ear, eye, or testicles and tuberculosis [10]; the stem bark is used against diarrhea, dysentery, hemorrhoids, abdominal aches, toothaches, sore throat, diabetes, asthma and hypertension [11]. The ancient Egyptians used the fruits for the treatment of diarrhea, skin problems and internal bleeding [12]. Phenolic compounds, such as apigenin-6, 8-*bis*-C-*β*-D-glucopyranoside and ethyl gallate have been isolated from the leaves of *A. nilotica* [13, 14]. m-digallic acid, gallic acid, protocatechuic acid, ellagic acids, leucocyanidin, oligomer 3,4,7-trihydroxyflavan 3,4-diol and 3,4,5,7-tetrahydroxy flavan-3-ol have been isolated from the seeds [15]. (+) catechin, (-) epigallocatechin-7-gallate, quercetin, and umbelliferone were isolated from the stem bark [16]. Kaempferol-3-glucoside and isoquercitrin were isolated from the flowers of the plant [17].

The use of conventional drugs such as antacids, H2 receptor blockers (ranitidine, cimetidine), proton pump inhibitors (omeprazole), prostaglandin analogues and antibiotics used in the treatment of gastric ulcers is associated with resistance, relapses and side effects which makes their efficacy arguable [18]. However, the plant's seedless pods have been reported to possess significant gastroprotective activity [19]. In addition, medicinal plants possessing steroidal compounds as their active constituents have been found to possess gastroprotective activity [20]. Hence, the present study is aimed at isolating a steroidal compound and evaluating the gastroprotective activity of the ethyl acetate portion of the seed extract of *Acacia nilotica* Linn.

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MATERIALS AND METHODS

Materials

Different liquid chromatography columns (I.D X L:30 mmx 500 mm, 20 mmx 400 mm, and 10 mmx 150 mm) purchased from Kemtech America Inc. Pleasant Prairie, WI53158, USA with silica gel (70–230 mesh) were used for the purification of the active constituents, preparative TLC was carried out on pre-coated silica gel (60F254) (Merck®, Darmstadt, Germany), ^{1}H-,^{13}C- NMR spectra were measured using FT-NMR cryoprobeBrukerAvance III spectrometer (600 and 150 MHz, respectively). Cimetidine, 250 mg (Medico Remedies Ltd. Maharashtra, India), Indomethacin, 25 mg (Shanghai Himed Pharmaceutical Co., Ltd. Shanghai, China). All other chemicals and solvents used were of analytical standard.

METHODS

Plant material

The dried fruits (seed-containing pods) of *Acacia nilotica* were purchased from Kasuwan Gwari market, Minna, Niger State in January 2021 and authenticated at the Herbarium and Ethno Botany Unit, National Institute for Pharmaceutical and Research Development (NIPRD) Idu, Abuja, Nigeria (Voucher number: NIPRD/H/7228). The dried seeds were then powered and preserved in a dry plastic bag until extraction.

Extraction of plant material

The air-dried powdered seeds (540 g) were subjected to successive cold maceration in 2L of 70% methanol for 72 h with manual agitation at intervals. The resulting mixture was filtered, concentrated under reduced pressure using a rotary evaporator and finally dried over a water bath at 40^{o}C to yield a dark brown, gummy mass (48.6 g).

Partitioning of crude methanol extract

Partitioning of the crude methanol extract of *A. nilotica* was carried out using the liquid-liquid extraction method. The crude methanol extract (38 g) was solubilised in 400 cm^3 distilled water and then filtered. The filtrate was partitioned exhaustively with ethyl acetate (100 cm^3 x 5) in a separating funnel to afford an ethyl acetate soluble portion and the mixture was filtered and concentrated under reduced pressure with a rotary evaporator and then finally over a water bath at 40°C to obtain a dried portion which was stored until required for further processing.

Isolation

The ethyl-acetate portion (5.0 g) was subjected to silica gel (70–230 mesh) column chromatography and eluted with a gradient system of *n*-hexane/EtOAc (95:5 →5:95) to afford eight pooled fractions, A-G. Fraction B (0.8 g) was further re-fractionated with a gradient system of *n*-hexane/EtOAc (80:20 →20:80) to afford three pooled sub-fractions, B1-B3. Sub-fraction B2 (0.3 g), having two well-resolved spots was then subjected to further purification using preparative thin-layerchromatography (PTLC) using *n*-

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hexane/EtOAc (75:25) to give a pure compound, B2-I which tested positive for Liebermann-Burchard's and Salkwoski's tests.

Gastroprotective Activity

Study design and induction of ulcer was carried out as described by [21] with slight modification.

Study animals

A total of 24 Albino Wistar rats (150 – 200 g) of both sexes were used in the study, the animals were randomly divided into 6groups (three animals each). Experimental rats were kept for 2 weeks in the animal house to acclimatize before the study commenced. The animals had animal feed and water *ad libitum*. The animal feed was withdrawn 24 h before the inducement of ulcers on the rats while water was maintained. The National and Institutional Guidelines for the Protection of Animal were followed in handling the rats.

Induction of gastric ulceration

Following a 24 h of fasting, 30 mg/kg of indomethacin in 1 cm^3 of 1% Tween 80 was administered orally to all groups using gavage, except Group A which served as the negative control group.

Study design

Group A (negative control group): consisted of rats given 1 cm^3 of vehicle (1% Tween 80), Group B (indomethacin group): were rats given 30 mg/kg of indomethacin. The rats (Groups A and B) were sacrificed after 6 h. Groups C, D and E consisted of rats given 30 mg/kg of indomethacin and treated with ethyl acetate portion doses (100, 200 and 300 mg/kg body weights). Group F (cimetidine group), consisted of rats given 30 mg/kg of indomethacin and treated with cimetidine (50 mg/kg body weight). Test groups (C to F) received treatment doses twice daily for 8 days. At the end of the experiment, the animals were sacrificed following mild anesthesia using chloroform inhalation and the stomach opened along the greater curvature. The gastric lesions induced by indomethacin served as ulcer index (U.I).

Determination of gastric ulcer index

The dissected stomachs were opened along the lesser curvature; the inner surface was rinsed in saline water and examined for ulcerations. The magnifying lens was used to measure the length of lesions within 1 mm. The ulcer index (U.I) was determined using the modified scoring system of [22], where 0 = no lesion, 1 = hemorrhagic suffusions, 2 = 1–5 small ulcers that are up to 3 mm in length, 3 = many small ulcers more than 5 or 1 ulcer of more than 3 mm, 4 = many ulcers of more than 3 mm, and 5 = perforated ulcers. The curative ratio (CR) was calculated using the formula below:

$$CR = \frac{LC - LT \times 100\%}{LC}$$

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Where LC is the length of gastric ulcer in indomethacin group and LT is the length of gastric ulcer in treated group.

Determination of pH, free and total acidity of gastric juice

The gastric juice pH, free and total acidity was determined using the method described by [23]. Gastric juice was collected in centrifuge tubes, centrifuged at 3000 rpm and the supernatant titrated with 0.01 N NaOH using methyl orange (2 drops) as an indicator until yellowish-orange colour appears, and the result indicates free acidity. Then phenolphthalein (2 drops) was added as an indicator and the titration continued until a permanent pink color was observed. The total volume of alkali added indicates total acidity. The hydrogen ion concentration (pH) of gastric contents for each rat was determined using a precalibrated pH meter.

$$Acidity = \frac{\text{Vol.of NaOH x Normality of NaOH x 100 mEq/L}}{0.1}$$

Data Analysis

Analyses were carried out using Statistical Package for the Social Sciences 20 for windows (SPSS 20). The results were expressed as means and Standard Error of Means (SEM). Analysis of variance was obtained and the means were separated using Tukey's Kramer post hoc test at $p \leq 0.05$.

RESULTS AND DISCUSSION

Characterisation of the isolated compound

Appearance: white powder (16 mg); Melting point: 173-176 ^{0}C.

^{1}H NMR ($CDCl_3$, 500 MHz)spectrum of compound B2-Irevealed the presence of six methyl signals atδ_H0.70 (s, 3H, C-18), andδ_H1.03 (s, 3H, C-19), corresponding to the angular methyl protons; three methyl doublets (d) appeared at δ_H0.98 (d, 3H,C-21), δ_H0.82 (d, 3H, C-26) andδ_H0.85 (d, 3H, C-27), and a methyl doublet of doublets atδ_H0.87(dd, 3H, C-29), corresponding to the methyl protons. The spectrum showed one olefinic proton atδ_H 5.35 (t, 1H, H-6) and a proton corresponding to the hydroxyl group which appeared as a multiplet (m) at δ_H 3.54(m, 1H,OH-3). Also, vinyl protons appeared at δ_H 5.04 (dd, 1H,H-22) and δ_H5.18(dd, 1H, H-23). Vinylic protons resonate at around δ_H5.10 and δ_H5.5 ppm [24]. ^{13}C NMR ($CDCl_3$, 126 MHz) spectrum revealed twenty-nine carbon signals in the ^{13}C-NMR of compound B2-I, including six methyl viz. at δ_C11.9 (C-18), δ_C18.8 (C-19), δ_C19.1(C-21), δ_C19.4(C-26), δ_C19.8 (C-27) and δ_C11.9 (C-29), nine methylene at δ_C37.3(C-1), δ_C31.9 (C-2), δ_C42.3(C-4), δ_C31.7(C-7), δ_C21.1(C-11), δ_C39.8(C-12), δ_C24.3(C-15), δ_C28.2(C-16) and δ_C26.2(C-28), eleven methane viz. at δ_C71.8 (C-3), δ_C121.7 (C-6), δ_C31.9 (C-8), δ_C50.2 (C-9), δ_C56.8 (C-14), δ_C56.1(C-17), δ_C40.4(C-20), δ_C138.3 (C-22), δ_C129.3(C-23), δ_C51.2 (C-24) and δ_C29.2 (C-25) and three quaternary carbons at δ_C140.8 (C-5), δ_C36.5 (C-10) and δ_C42.2(C-13). The signals at δ_C140.8 and δ_C121.7 (C-5 and C-6), and at

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δ_C138.27 and δ_C129.31 (C-22 and C-23) are typical of alkene double bonds. The signals at δ_C19.80 and δ_C11.86 ppm correspond to angular methyl carbon atoms; the signal at δ_C71.79 ppm is attributable to the beta hydroxyl group attached to the carbon at position C-3. Based on these results and by comparison with existing literature, the chemical shifts are typical of stigmasterol [25].

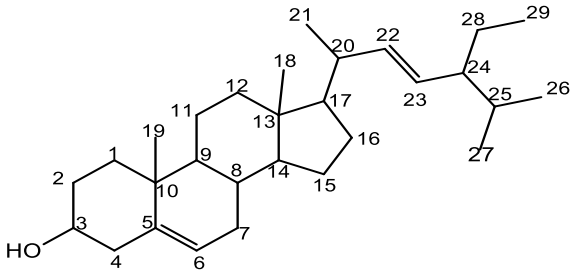

Figure 1: Structure of compound B2-I

Gastroprotective Activity

The animals treated with 30 mg/kg indomethacin only, had the lowest pH of 1.94±0.61 and highest free and total acidity values (72.15±0.78 and 92.94±0.87), indicating the ability of indomethacin to induce ulcer in rats. Table 1 shows the effects of oral administration of varying doses of the ethyl acetate fraction to the rats. The ethyl acetate portionsignificantly ($P<0.05$) reduced free and total acidity and increased gastric pH in a dose-dependent manner when compared to the indomethacin treated group.

Table 1: Biochemical Parameters of Rats Treated Orally with Ethyl acetate Portion

Group	pH	Gastric volume (cm^3)	Free acidity (mEq/L)	Total acidity (mEq/L)	Curative ratio (%)
Negative control (1% Tween 80)	3.21±0.12^{b}	5.12±0.02^{ab}	44.84±0.98^{a}	53.34±1.07^{a}	-
Indomethacin (30 mg/kg)	1.94±0.08^{a}	5.49±0.05^{c}	72.15±1.35^{c}	92.94±1.44^{d}	-
Cimetidine (50 mg/kg)	3.24±0.01^{b}	5.10±0.02^{a}	47.08±0.25^{a}	58.87±0.44^{ab}	91
Ethyl acetate portion					
100 mg/kg	2.20±0.15^{a}	5.15±0.04^{ab}	55.00±2.39^{b}	70.00±1.83^{c}	40
200 mg/kg	2.41±0.17^{a}	5.23±0.03^{ab}	48.15±2.62^{ab}	65.71±1.80^{bc}	60
300 mg/kg	2.35±0.24^{a}	5.31±0.08^{bc}	48.20±1.23^{ab}	63.45±2.01^{bc}	75

Values are Mean± SEM, values with different letters superscripts on the same column are significantly different ($p<0.05$, Tukey's post hoc comparisons).

Non-steroidal anti-inflammatory drugs (NSAIDs) induce gastric damage ranging from mere irritation and perforation to severe ulceration and perforation [26]. Indomethacin is

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a known ulcerogene which causes ulcers mostly on the glandular (mucosal) part of the stomach, especially when the stomach is empty by inhibiting prostaglandin synthase through the cyclooxygenase pathway [27]. In the present study, it was observed that indomethacin significantly ($P<0.05$) increased gastric juice volume, free and total acidity, and decreased gastric pH of rats when compared to the negative control group. In a previous study, indomethacin was found to cause ulcers by increasing gastric juice volume, free and total acidity, and decreasing gastric pH [27]. The ethyl acetate portion exhibited activity in a dose-dependent manner by significantly ($P<0.05$) decreasing free and total acidity and increasing the pH of gastric juice in comparison to the indomethacin treated group.There was, however, no significant difference ($P>0.05$) between the pH and that of indomethacin treated group. The portion had curative ratios of 40%, 60% and 75% at 100, 200 and 300 mg/kg body weights respectively. Previous study has shown that sterols exhibit gastroprotective activity [29] and this activity is attributed to the hydroxyl group at position C-3 [30]. Oxidative stress is believed to initiate and aggravate many diseases including gastric ulcers [31]. It has also been reported in the literature that phytosterols and their derivatives may act as potent radical scavengers [32]. The two main approaches in the treatment and management of peptic ulcer disease include reduction of stomach acid secretion or increasing stomach mucous production [33]. It has been suggested that the most common gastroprotective activity mechanism for sterols seems to be the activation of mucous membrane protective factors rather than the inhibition of gastric acid secretion [34]. Furthermore, other secondary metabolites including saponins, tannins, alkaloids, phenols, flavonoids, and terpenoids detected in the ethyl acetate portion have also been reported to exhibit gastroprotective efficacy through radical scavenging, anti-secretory, anti-*H. pylori*, prostaglandin synthesis, antioxidant, anti-inflammatory, and wound healing properties respectively [35, 36, 37].

Generally, the activity of the ethyl acetate portion may be attributed to the phytochemicals present in them. These phytochemicals exhibit bioactivity by acting either singly or in synergism with one another [38]. Hence the isolated compound contributed at least partly to the observed gastroprotective activity.

CONCLUSION

Fractionation of the ethyl acetate portion of *A. nilotica* by column chromatography led to the isolation of compound B2-I. Based on the obtained NMR spectroscopic data and by comparison with literature, the isolated compound was identified as stigmasterol. The ethyl acetate portion, in a dose-dependent manner, significantly offered gastroprotection to the ulcerated rats. The isolated compound may have contributed at least partly to the observed

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gastroprotective activity of the ethyl acetate portion.

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