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Gastroprotective Effects of β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside from *Bridelia ferruginea* Stem Bark

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Abstract: The methanol extract of *Bridelia ferruginea* Benth. (Euphorbiaceae) stem bark (BFME) was partitioned in chloroform-methanol-water (2:2:1) mixture to obtain the Chloroform (CF) and Aqueous Methanol (AMF) fractions. The BFME, CF and AMF were screened for antiulcer activity using indomethacin-induced ulcer as activity guide. The CF provided the highest gastroprotection and was subsequently fractionated in a silica gel (60-200 mesh) column eluted with different mixtures of *n*-hexane and ethyl acetate (100:0; 95:5; 90:10; 80:20) to obtain six fractions (I-VI). Fractions III and VI offered the highest protection against indomethacin-induced ulcer and were further purified in a sephadex LH-20 column eluted with methanol to yield two compounds, BF1 and BF2. Using Nuclear Magnetic Resonance (^{1}H-NMR, ^{13}C-NMR) and electron impact mass spectroscopies, BF1 and BF2 were confirmed to be β-sitosterol and β-sitosterol-3-*O*-βD glucopyranoside respectively. The BFME, fractions, β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside elicited dose-related and significant ($P<0.05$) protection against various ulcers in rats. β-sitosterol, 100 and 300 mg/kg, produced 79.70, 82.18, 42.31, 44.87, 65.97, 70.83, 80.22 and 87.91% gastroprotection; while β-sitosterol-3-*O*-β-D-glucopyranoside, 100 and 300 mg/kg, caused 69.80 and 74.26, 33.33, 35.26, 84.03, 95.83, 83.52 and 85.71% gastroprotection against indomethacin-, ethanol-, cold restraint stress- and pylorus ligation- induced ulcers, respectively. Results demonstrated gastroprotective effects of *B. ferruginea* stem bark, attributable to β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside.

Keywords: β-sitosterol, β-sitosterol-3-*O*-β-D-glucopyranoside, Cold Restraint Stress Ulcer, Indomethacin-Induced Gastric Ulcer, Pyloric Ligation

Introduction

Peptic ulcer is usually due to an imbalance between the injurious (acid, pepsin and *Helicobacter pylori*) and defensive (mucus, bicarbonate, prostaglandins, nitric oxide, some peptides) factors of the gastric mucosa (Wallace and Sharkey, 2011; Love and Thoma, 2014; Turner, 2015). To re-establish the balance, pharmacotherapeutic agents are used to inhibit gastric acid secretion, eliminate *H. pylori* infection, or enhance the mucosal defensive mechanisms.

Numerous plants are used in ethnomedicine to treat peptic ulcer disease. Various scientific studies have demonstrated the antiulcer efficacy of some of these medicinal plants (Falcão *et al*., 2008; Ezike *et al*., 2009; 2011; Vimala and Shoba, 2014). One of such plants with antiulcer activity is *Bridelia ferruginea* Benth (Euphorbiaceae).

Earlier studies in our Laboratory demonstrated the antiulcer (Ezike *et al*., 2011) and wound healing (Ezike *et al*., 2015) potentials of *B. ferruginea* stem bark. To further elucidate the antiulcer properties of the plant, this study sought to isolate and characterize the antiulcer compound(s) from extracts of *B. ferruginea* stem bark.

Materials and Methods

Chemicals, Reagents and Drugs

Solvents including those used for extraction, fractionation, separation and isolation of the pure compounds were purchased from Sigma-Aldrich (Darmstadt, Germany). Indomethacin was obtained from Sigma Aldrich (St. Louis, MO, USA), while cimetidine was procured from Jiangxi Xierkangtai Pharmaceutical Co. Ltd. (North Zone, High-New Technology Industrial Zone, Pingxiang, Jiangxi, China).

Animals: Adult Sprague Dawley rats (120-150 g) of either gender (the female rats were 12-13 weeks old, while the male rats were 11-12 weeks old) bred in the Laboratory Animal Facility of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka were used for the study. The animals were maintained *ad libitum* on standard pellets and water. All animal experiments were in compliance with National Institute of Health Guide for Care and Use of Laboratory Animals (Pub No. 85-23, revised 1985) and with prior permission from the National Health Research Ethics Committee (NHREC) of the University of Nigeria, with protocol clearance number NHREC/05/01/2012C.

Preparation of Extract

Fresh stem bark of *B. ferruginea* trees growing in Ezimo, Enugu State, Nigeria were collected in October. The plant was identified and authenticated by a taxonomist at the International Center for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Nigeria. A voucher specimen (specimen number: BFSB2011) of the plant was kept in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka. The bark was separated from the wood stem, cleared of the dead outer parts and thorns, cut into smaller pieces and dried under shade for seven days. The dried plant material was pulverized to powder using a milling machine.

The powdered plant material (8 kg) was extracted by cold maceration in methanol for 48 h at room temperature (28±1°C) and the mixture filtered. Subsequently, the plant material was repeatedly washed with methanol until the filtrate became clear. Removal of the solvent *in vacuo* using a rotary evaporator (40°C) afforded 2.01 kg of the methanol extract (BFME; 25.125% w/w).

Fractionation of the Extract

The BFME (2 kg) was partitioned, in eight separate batches, in a chloroform-methanol-water (2:2:1) mixture. The resulting chloroform layer was concentrated *in vacuo* using a rotary evaporator (40°C) to obtain 38.1 g of the Chloroform Fraction (CF; 1.905% w/w). While the aqueous-methanol portion was freeze-dried to afford 126.5 g of the Aqueous Methanol Fraction (AMF; 6.325% w/w).

Biological Activity Guided Studies and Isolation of Bioactive Constituents

The BFME and fractions were subjected to biological activity-guided studies using indomethacin-induced ulcer as activity guide. The CF offered higher protection than the AMF. Subsequently, CF (38 g) was further fractionated in a silica gel (60-200 mesh) column eluted with gradient mixtures of *n*-hexane and ethyl acetate (100: 0; 95: 5; 90:10; 80: 20). About 37 fractions of 100 ml each were collected. The fractions were pooled based on the similarity of constituents visualized on silica gel coated TLC plates developed with *n*-hexane-ethyl acetate (7:3) mixture to afford 6 broad fractions: fraction I (1.08 g; 2.84% w/w), fraction II (1.2 g; 3.16% w/w), fraction III (8.7 g; 22.89% w/w), fraction IV (1.5 g; 3.95% w/w), fraction V (1.65 g; 4.34% w/w) and fraction VI (11.1 g; 29.21% w/w). The fractions were also screened for antiulcer activity. Fractions III and VI gave comparably higher protection against indomethacin-induced ulcer. Further purification of fractions III (8.0 g) and VI (10.5 g), separately, by column chromatography using sephadex LH-20 with methanol as eluent yielded two white waxy amorphous powders; compound I (BF1; 2.60 g; 32.5% w/w) and compound II (BF2; 2.85 g; 27.14% w/w) respectively. The antiulcer activities of BF1 and BF2 were confirmed using the activity guide.

Characterization of Isolated Compounds

The molecular weights of the isolated compounds BF1 and BF2 were determined by Electron Impact Mass Spectroscopy (EI-MS). Electron impact mass spectra were measured on a Finnigan MAT 8430 mass spectrometer which uses energy for ionization (70 eV) achieved by accelerating the electrons through a potential drop of 70 V.

The molecular structures of the compounds were elucidated using ^{1}H-NMR and ^{13}C-NMR, as applicable. The spectra were recorded at 300° K on ARX 600 MHz NMR spectrometers. All the one-dimensional and two-dimensional spectra were obtained using the standard Bruker software with Tetramethylsilane (TMS) as internal standard reference signal. The observed chemical shifts (δ) were recorded in parts per million (ppm) and the coupling constants (*J*) were recorded in Hertz (Hz).

Proton signals of BF1 were captured and assigned using ^{1}H-^{1}H Correlation Spectroscopy (^{1}H-^{1}H COSY). Also, proton and carbon signals of BF2 were captured and assigned using ^{1}H-^{1}H COSY, Distortionless Enhancement by Polarization (DEPT), Heteronuclear

Single Quantum Coherence Spectroscopy/Heteronuclear Single Quantum Coherence Correlation (HSQC) and Heteronuclear Multiple Bond Correlation Spectroscopy (HMBC) as applicable.

Pharmacological Tests

Indomethacin-Induced Ulcer Test

This was carried out according to the method described by (Santin *et al*., 2010) with some modifications. Rats starved of food for 24 h prior to the experiment, but with free access to water, were randomly placed into 17 groups ($n = 5$). Groups 1-15 received oral administrations of one of BFME, CF, AMF, fractions I - VI, BF1 and BF2 at 100 and/or 300 mg/kg respectively. Groups 16 and 17 were the control groups and received cimetidine (100 mg/kg) and 1% Tween-80 (5 mL/kg), respectively. One hour after treatment, indomethacin (100 mg/kg) was given to the animals orally. Four hours later, the animals were sacrificed by cervical dislocation and their stomachs removed and opened along the greater curvature. The stomachs were rinsed carefully under running water, pinned on a cork board and examined with a hand lens (×10). Using a modification of the method described by (Main and Whittle, 1975), ulcers formed on the glandular portion of the stomach were observed and each graded on a 0-3 scale based on the length; 1 = <1 mm; 2 = 1-3 mm; and 3 = >3 mm. The Ulcer Index (UI) was calculated as (1× number of ulcers grade 1) + (2× number of ulcers grade 2) + (3× number of ulcers grade 3). The overall score was divided by a factor of 10 and the mean score for each group calculated. Ulcer protection (%) of the treated groups were calculated using the relation:

$$Ulcer\ protection(\%) = 100[1 - y / z]$$

Where:
y = Ulcer index of treated group
z = Ulcer index of control group (Ezike *et al*., 2014)

Absolute Ethanol-Induced Ulcer Test

This was carried out according to the method described by (Santin *et al*., 2010) with some modifications. Rats starved of food for 24 h prior to the experiment, but with free access to water, were randomly divided into 17 groups ($n = 5$). Groups (1-15) received oral administrations of one of BFME, CF, AMF, fractions I-VI, BF1 and BF2 at 100 and/or 300 mg/kg respectively. Groups 16 and 17 were the control groups and received cimetidine (100 mg/kg) and 1% Tween-80 (5 mL/kg), respectively. One hour after treatment, each animal received 1 mL of absolute ethanol orally. One hour after ethanol administration, the animals were sacrificed by cervical dislocation and their stomachs removed and opened along the greater curvature. The stomachs were rinsed carefully under running water, pinned on a cork board and examined with a hand lens (×10). Ulcers formed on the glandular portion of each stomach were observed and scored using a 0-7 scale, as described by (Ezike *et al*., 2014). Where 0 = No ulcer, 1 = One slight ulcer, 2 = More than one grade 1 ulcer, 3 = One ulcer of length ≤0.5 cm, 4 = One ulcer of length >0.5 cm, 5 = More than one grade 3 ulcer, 6 = More than one grade 4 ulcer, 7 = Complete hemorrhagic lesion of the mucosa. The overall score was divided by a factor of 10. Mean ulcer score for each group was calculated and expressed as the Ulcer Index (UI). Ulcer protection (%) of the treated groups were calculated as described for indomethacin-induced ulcer.

Cold Restraint Stress-Induced Gastric Ulcer Test

This was carried out according to the method described by (Viana *et al*., 2013) with some modifications. Rats starved of food for 24 h prior to the experiment, but with free access to water, were randomly placed into twelve groups ($n = 5$). Groups 1-10 received oral administrations of one of BFME, fraction III, fraction VI, BF1 and BF2 at 100 and/or 300 mg/kg respectively. Groups 11 and 12 were the control groups and received cimetidine (100 mg/kg) and 1% Tween-80 (5 mL/kg), respectively. One hour after treatment, each rat was restrained in a closed cylindrical cage maintained at 4-6°C. After 4 h, the animals were sacrificed by cervical dislocation and their stomachs removed and opened along the greater curvature. The stomachs were rinsed carefully under running water, pinned on a cork board and examined with a hand lens (×10). Ulcers were graded, also UI and ulcer protection (%) were determined as described for indomethacin-induced ulcer.

Pylorus Ligation-Induced Ulcer Test

The pylorus ligation-induced ulcer test was performed using the method described by (Shay, 1945) with some modifications. Rats starved of food for 24 h prior to the experiment, but with free access to water, were randomly placed into six groups ($n = 5$). The animals were anesthetized i.p. with phenobarbital sodium (35 mg/kg), the abdomen incised and the pylorus ligated. Immediately after pylorus ligation, groups 1-4 received one of BF1 and BF2 at 100 or 300 mg/kg administered intraduodenally respectively, while control animals (groups 5 and 6) received either 1% Tween-80 (5 mL/kg) or cimetidine (100 mg/kg). The stomachs were replaced carefully and the animals were allowed to recover. Four hours later, the animals were sacrificed by cervical dislocation, the abdomens opened and another ligature placed around the esophagus close to the diaphragm. The stomachs were removed, opened along the greater curvature, rinsed carefully under running water and pinned on a cork board for examination with a hand lens (×10). Ulcers were graded, also UI and ulcer protection (%) were determined as described for indomethacin-induced ulcer.

Statistical Analysis

Data were analyzed using One-Way ANOVA in GraphPad Prism 7.0. Results were expressed as Mean ± SEM. Differences between means were determined using Bonferroni's post hoc test for multiple comparison and regarded significant at $P<0.05$.

Results

Phytochemistry

Physical Characteristics, Spectral Data and Structure of BF1

β-sitosterol (BF1) was isolated as a white, waxy amorphous powder, soluble in chloroform, but insoluble in water. The molar mass was deduced as 414 g/mol based on the EI-MS m/z peaks at 414, 415 and 416.

The major fragment at m/z 396 occurred by a loss of 18 amu (loss of H_2O molecule). This suggested the presence of OH group in the compound. The BF1 was identified to be a steroid or triterpene with hydroxyl group at position 3. These observations suggested that BF1 was most likely a β-sitosterol.

The ^{1}H-NMR spectrum of BF1 showed a de-shielded proton peak at δ_H 5.33 (brs, 1H) assignable to the olefinic proton H-5 of the β-sitosterol. Another de-shielded proton at δ_H 3.49 was assignable to proton H-3. These two proton peaks showed correlation with the other protons of the steroid nucleus, when subjected to ^{1}H-^{1}HCOSY. The NMR spectrum also showed methyl signals at 0.66 (s, 3H), 0.99 (s, 3H), 0.96 (d, J = 6.5, 3H), 0.82 (d, J = 6.5, 3H), 0.81 (d, J = 6.5, 3H) and 0.80 (t, 3H) assignable to CH_3-18, CH_3-19, CH_3-21, CH_3-26, CH_3-27 and CH_3-29 respectively of the steroid nucleus. The proton signals of BF1 are shown in Table 1. The compound was accordingly identified as β-sitosterol based on the comparison of the NMR data with that reported in the literature. The molecular structure of BF1 is shown in Fig. 1.

Physical Characteristics, Spectral Data and Structure of BF2

β-sitosterol-3-*O*-β-D-glucopyranoside (BF2) was isolated as a white, waxy amorphous powder, soluble in chloroform, but insoluble in water.

The ^{1}H-NMR spectrum of BF2 was similar to that of BF1. The spectrum of BF2 also showed the presence of olefinic proton peak at δ_H 5.33 (brs, 1H) assignable to H-5 and the de-shielded proton signal at δ_H 3.45 (m, 1H) assignable to H-3. The spectrum also showed the presence of the methyl signals as previously described for BF1. The major difference, however, was in the presence of several other oxygenated proton peaks in the range of 2.80 to 4.50 ppm and carbon peaks in the range of 60 to 100 ppm. These additional signals suggested the presence of a sugar moiety. The signal at δ_H 4.22 (d, J = 7.8 1H) was assignable to the anomeric proton H-1'. This proton was found to show ^{1}H-^{1}H COSY correlations with the other oxygenated protons. Analysis of the ^{1}H-^{1}H COSY spectrum of BF2 showed that the signals at δ_H 2.89 (dd, J = 8.5, 13, 1H), 3.11 (dd, J = 4.8, 8.8, 1H), 3.02 (dd, J = 5.2, 8.9, 1H) and 3.06 (m, 1H) were assignable to H-2', H-3', H-4' and H-5' respectively, while the signals at δ_H 3.40 (dd, J = 5.9, 11.7, 1H) and 3.64 (dd, J = 54.2, 11.7, 1H) were assignable to the two diastropic methylene protons HA-6' and HB-6'.

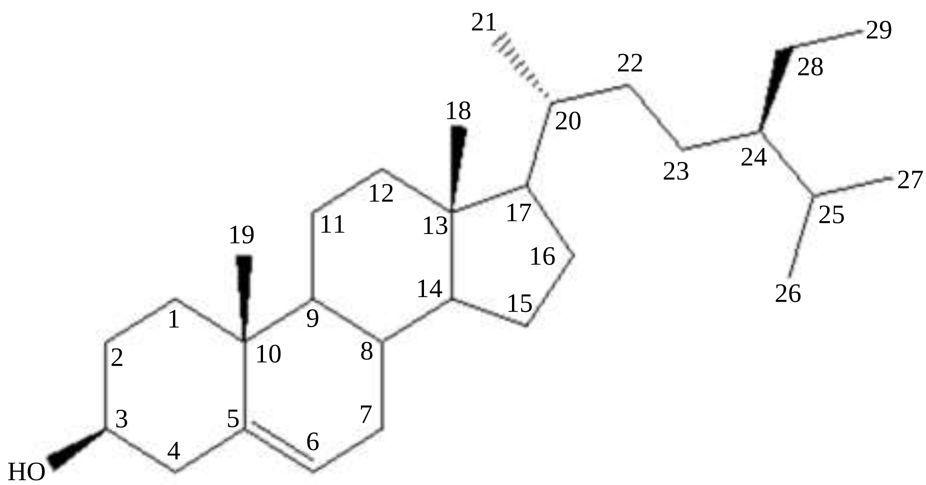

Fig. 1: Molecular structure of BF1 (β-sitosterol)

Table 1: NMR data of β-sitosterol (BF1) and β-sitosterol-3-*O*-β-D-glucopyranoside (BF2)

Position	β-Sitosterol (BF1)[a]	β-Sitosterol-3-*O*-β-D-glucopyranoside (BF2)[b]	
	δ_H	δ_H	δ_C
1	–	1.22 m (Ha) 0.93 m (Hb)	33.5
2	1.87 m (Ha) 1.47 m (Hb)	1.80 dd (Ha) 1.48 dd (Hb)	29.4
3	3.49 m	3.45 m	76.9
4	2.24 m	2.37 m (Ha) 2.11 m (Hb)	38.5
5	–	-	140.6
6	5.33 brs	5.33 brs	121.7
7	1.96 m (Ha) 1.47 m (Hb)	1.90 m (Ha) 1.51 m (Hb)	31.6
8	–	–	31.5
9	–	–	49.8
10	–	–	35.6
11	–	–	20.7
12	–	–	37.0
13	–	–	42.0
14	–	–	56.3
15	–	–	24.0
16	–	–	28.8
17	–	–	55.6
18	0.66 s	o.65 s	11.9
19	0.99 s	0.95 s	19.3
20	–	–	36.9
21	0.90 d (6.5)	0.90 d (6.5)	19.1
22	–	–	36.4
23	–	–	25.6
24	–	–	45.3
25	–	–	28.0
26	0.82 d (6.5)	0.82 d (6.5)	18.6
27	o.81 d (6.5)	0,82 d (6.5)	19.1
28	–	–	22.8
29	0.80 t	0,80 t	11.8
1'	–	4.22 d (7.8)	100.9
2'	–	2.89 dd (8.5, 13)	73.6
3'	–	3.11 dd (4.8, 8.8)	77.1
4'	–	3.02 dd (5.2, 8.9)	70.3
5'	–	3.06 m (5.9)	76.9
6'	–	3.64 dd (4.2, 11.7) 3.40 dd (5.9, 11.7)	61.3

[a]Spectra measured in Deuterated Chloroform ($CDCl_3$); [b]Spectra measured in Deuterated Dimethylsulfoxide (DMSO-d6)

The sugar moiety was confirmed as β-D-glucose based on the coupling constant of the anomeric proton and the chemical shifts of the proton and carbon signals. The proton and carbon signals of BF2 are shown in Table 1; they were assigned based on ^{1}H-^{1}H COSY, DEPT, HSQC and HMBC analyses. The attachment of the sugar moiety at position 3 of the steroid was confirmed by the correlation of the anomeric proton signals with C-3 (76.9) of the steroid nucleus in HMBC and that of the H-3 proton with C-1' (100.9) of the sugar moiety. This was also confirmed by the de-shielded position of the C-3 at δ_C 76.9 ppm. Based on the analysis of the NMR data and comparison with literature, BF2 was therefore confirmed to be β-sitosterol-3-*O*-β-D-glucopyranoside. The molecular structure of BF2 is shown in Fig. 2.

Effects of Extract, Fractions and Isolated Compounds on Indomethacin-Induced Ulcer

The BFME, CF, AMF, fractions III, IV, V and VI, β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside inhibited indomethacin-induced ulcer. Fraction I elicited slight inhibition, while fraction II elicited no ulcer protection. The BFME, CF, AMF, fractions III and VI, BF1 and BF2 caused significant (P<0.05) inhibition compared to control (Table 2). β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside elicited dose-related protection, with β-sitosterol causing greater protection (Table 2).

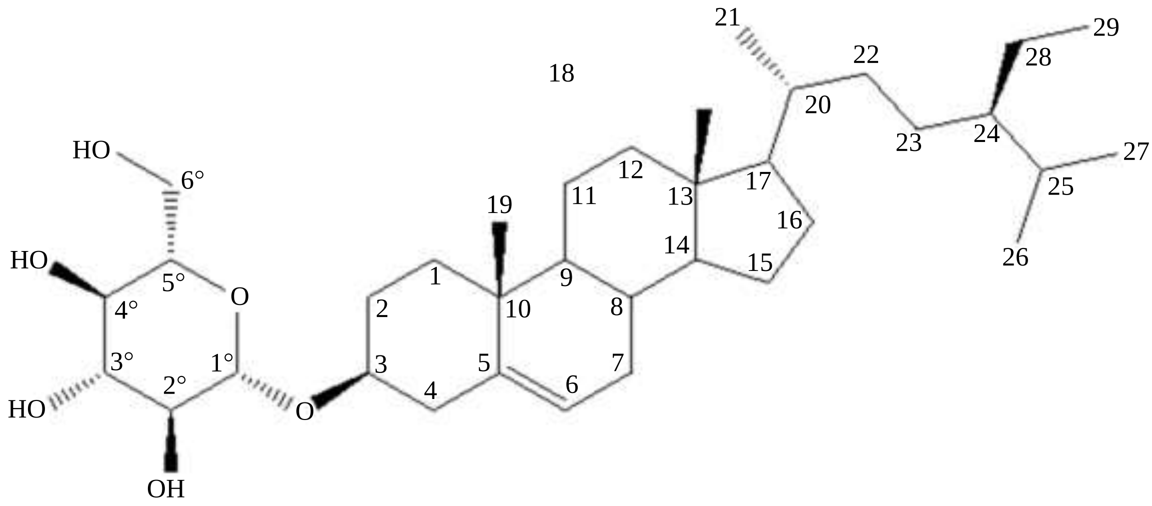

Fig. 2: Molecular structure of BF2 (β-sitosterol-3-*O*-β-D-glucopyranoside)

Table 2: Effects of phytoconstituents of *B. ferruginea* stem bark on indomethacin-induced ulcer

Treatment	Dose (mg/kg)	Ulcer index	Protection (%)
BFME	300	0.23±0.29*	88.61
CF	100	0.86±0.10*	57.43
	300	0.24±0.13	88.12
AMF	100	1.54±0.29*	23.76
	300	0.88±0.77	56.44
I	300	1.84±0.04	8.91
II	300	2.45±0.02	Nil
III	300	0.35±0.04*	82.67
IV	300	0.84±0.02	58.42
V	300	0.93±0.03	53.96
VI	300	0.78±0.02*	61.39
β-sitosterol	100	0.41±0.03*	79.70
	300	0.36±0.02	82.18
BST-G	100	0.61±0.04*	69.80
	300	0.52±0.07	74.26
Cimetidine	100	0.20± 0.11*	90.10
Control	-	2.02±0.15	—

*$P<0.05$ compared to the control (One Way ANOVA; Bonferroni's post hoc); BFME = Methanol Extract; CF = Chloroform Fraction; AMF = Aqueous Methanol Fraction; I-VI = fractions I-VI; BST-G = β-sitosterol-3-*O*- β-D-glucopyranoside

Effects of Extract, Fractions and Isolated Compounds on Absolute Ethanol-Induced Ulcer

The BFME, CF, AMF, fractions I, II, III, IV, V and VI, β-sitosterol and β-sitosterol-3-*O*-β- D-glucopyranoside inhibited the development of absolute ethanol-induced ulcers. The BFME and AMF produced higher protection than cimetidine. β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside elicited dose-related protection, with β-sitosterol causing greater protection (Table 3).

Effects of Extract, Fractions and Isolated Compounds on Cold Restraint Stress-Induced Ulcer

The BFME, fraction III, fraction VI, β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside caused significant ($P<0.05$) and dose-related reduction in ulcer lesion index in cold restraint stress-induced ulcers (Fig. 3). The ulcer protection (%) were 59.03 and 81.25, 47.92 and 61.81, 81.25 and 92.36, 65.97 and 70.83, 84.03 and 95.83 for 100 and 300 mg/kg of BFME, fraction III, fraction VI, β-sitosterol or β-sitosterol-3-O-β-D-glucopyranoside respectively. β-sitosterol-3-*O*-β-D-glucopyranoside (300 mg/kg) elicited the greatest reduction with ulcer protection of 95.83%, higher than cimetidine (93.06%).

Table 3: Effects of phytoconstituents of *B. ferruginea* stem bark on absolute ethanol-induced ulcer

Treatment	Dose (mg/kg)	Ulcer index	Protection (%)
BFME	300	0.46±0.32*	85.26
CF	100	1.51±0.04	51.60
	300	1.66±0.50	46.79
AMF	100	0.91±0.27*	70.83
	300	0.42±0.20	86.54
I	300	2.00±0.03	35.90
II	300	2.14±0.09	31.41
III	300	1.78±0.04	42.95
IV	300	2.04±0.14	34.62
V	300	2.60±0.06	16.67
VI	300	2.02±0.04*	35.26
β-sitosterol	100	1.80±0.13*	42.31
	300	1.72±0.10	44.87
BST-G	100	2.08±0.15*	33.33
	300	2.02±0.05	35.26
Cimetidine	100	1.04±0.05*	66.67
Control	-	3.12±0.32	—

*$P<0.05$ compared to the control (One way ANOVA; Bonferroni's post hoc); BFME = Methanol Extract; CF = Chloroform Fraction; AMF = Aqueous Methanol Fraction; I-VI = fractions I-VI; BST-G = β-sitosterol-3-*O*-β-D-glucopyranoside

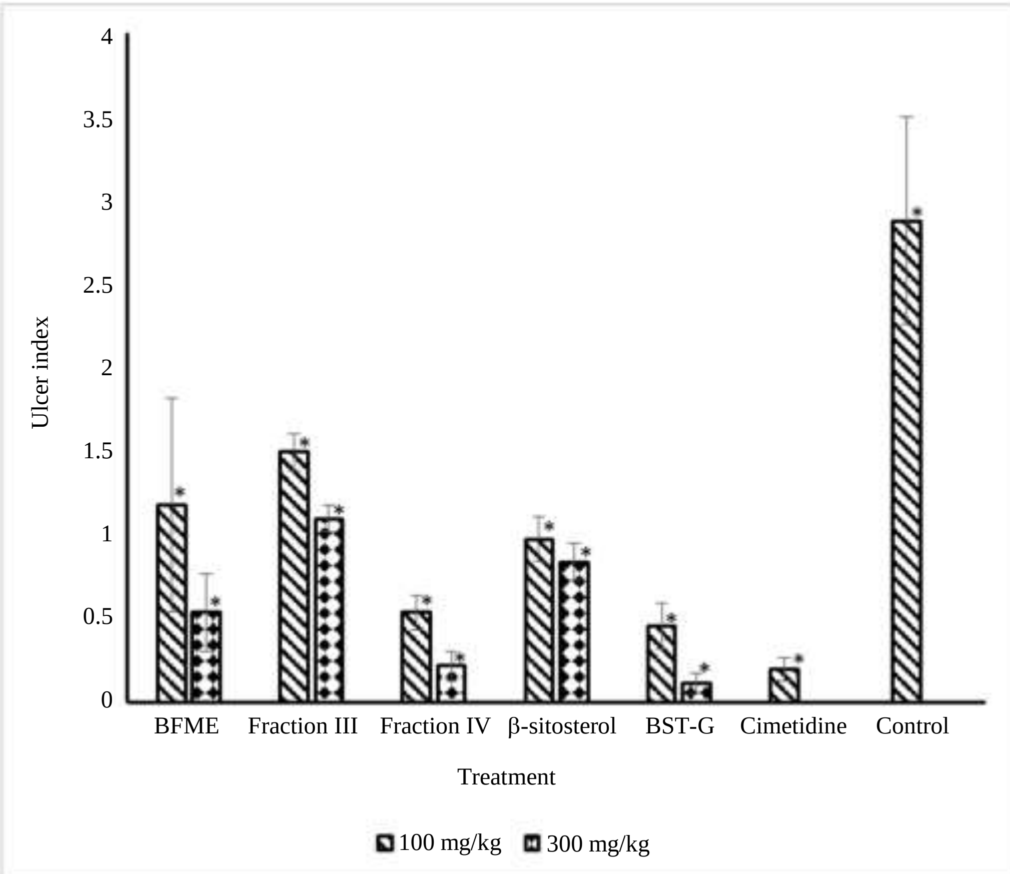

Fig. 3: Effects of bioactive constituents of *B. ferruginea* stem bark on cold restraint stress-induced ulcer; *$P<0.05$ compared to the control (one-way ANOVA; Bonferroni's post hoc); BFME = Methanol Extract, BST-G = β-sitosterol-3-*O*-β-D-glucopyranoside

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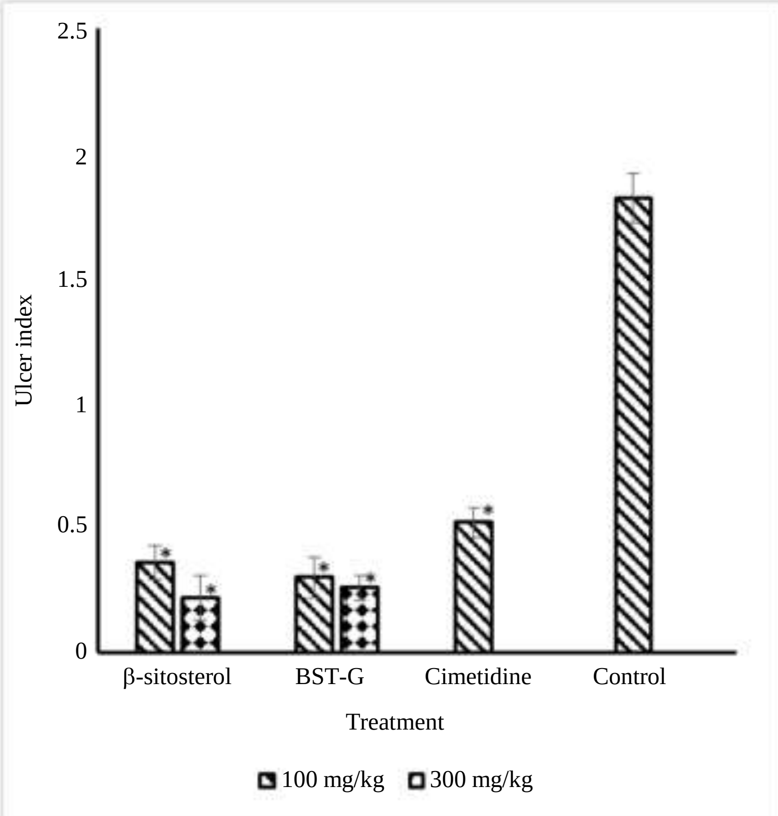

Fig. 4: Effects of β-sitosterol and β-sitosterol-3-*O*- β-D-glucopyranoside isolated from *B. ferruginea* stem bark on pylorus ligation-induced ulcer; *$P<0.05$ compared to the control (one-way ANOVA; Bonferroni's post hoc); BST-G = β-sitosterol-3-O- β-D-glucopyranoside

Effects of β-sitosterol and β-sitosterol-3-O-β-D-glucopyranoside on Pylorus Ligation-Induced Ulcer

The β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside produced significant ($P<0.05$) and dose-related reduction of ulcer lesion index in pylorus ligation-induced ulcers (Fig. 4). The ulcer protection (%) were 80.22 and 87.91, 83.52 and 85.71 for 100 and 300 mg/kg of β-sitosterol and β-sitosterol-3-*O*-β-D-glucopyranoside, respectively. The ulcer protective activity of β-sitosterol and β-sitosterol-3-O-β-D-glucopyranoside were greater than that of cimetidine (71.43%).

Discussion

Assessment of the antiulcer activity of *B. ferruginea* stem bark showed that the methanol extract, its fractions and isolated compounds (β-sitosterol and β-sitosterol-3-O-β-D-glucopyranoside), significantly protected the gastric mucosa against indomethacin-, absolute ethanol-, cold restraint stress- and pylorus ligation-induced ulcers.

The extract, fractions and isolated compounds significantly protected the rat stomach against indomethacin-induced lesions. Indomethacin, a Non-Steroidal Anti-Inflammatory Drug (NSAID), causes gastroduodenal ulceration by suppressing the production of prostaglandins (Wallace, 2001), increasing the secretion of gastric acid (Wallace, 2001) and generation of free radicals (Lichtenberger, 1995). Endogenous prostaglandins help to maintain mucosal integrity under injurious and adverse conditions by exerting negative feedback inhibition of acid secretion, mediating functional vasodilation in the gastric mucosa (Main and Whittle, 1973; 1975), maintaining mucosal blood flow and gastric microcirculation (Vane, 1971) and production of bicarbonate and mucus (Robert, 1982). Therefore, suppression of prostaglandins synthesis by indomethacin and other NSAIDs impedes the vasodilator, antisecretory and other protective mechanisms of prostaglandins, causing increased susceptibility to gastric mucosal lesions. Thus, indomethacin produces erosion and bleeding in the gastric mucosa by reduction in mucosal blood flow and increase in gastric acid production (Main and Whittle, 1975). Also, mucosal damage elicited by NSAIDs is characterized by changes in its permeability to ions, water and protein, which further erode the mucosal integrity. Hence the ability of the bioactive constituents of *B. ferruginea* stem bark to protect against indomethacin-induced ulcer suggests antisecretory and cytoprotective activity.

Ethanol produces severe hemorrhagic erosion on the glandular part of the stomach (Ezike *et al*., 2009) by reduction of bicarbonate and mucus, increase in leukotrienes and free radicals which cause lipid peroxidation and cell damage (Peskar *et al*., 1986; Glavin and Szabo, 1992; Ezike *et al*., 2009). It also reduces endogenous glutathione and prostaglandin levels and increases the release of histamine and influx of calcium ions (Glavin and Szabo, 1992). The observed effects of the bioactive constituents of *B. ferruginea* stem bark on ethanol-induced ulcer indicate gastroprotection through augmentation of the defensive factors.

The pathology of stress ulcer involves increased gastric acid secretion due to histamine release (Kitagawa *et al*., 1979), oxidative damages mainly due to hydroxyl radicals (Das and Banerjee, 1993; Liu *et al*., 1996; Das *et al*., 1997; Bandyopadhyay *et al*., 2002; Brzozowski *et al*., 2008), reduction of blood flow and mucus production in the gastric mucosa (Kitagawa *et al*., 1979), pancreatic juice reflux (Guth, 1972), inhibition of gastric mucosal prostaglandin synthesis (Brzozowski *et al*., 2008; Nur Azlina *et al*., 2013), inflammatory responses (Bregonzio *et al*., 2003) and increased vagal activity (Brodie and Hanson, 1960; Grijalva and Novin, 1990). Stress ulcers in humans and rats share similar pathophysiology (Konturek *et al*., 2003). Stress and distress decrease blood flow in the upper gastrointestinal tract (Kauffman Jr, 1997) and may render the human stomach and duodenum more susceptible to damage (Levenstein, 2002). Furthermore, human studies have shown that stress, anxiety and depression increase acid secretion (Feldman *et al*., 1992), impair ulcer healing and promote ulcer relapse (Levenstein, 2002). Stress also causes an increase in gastrointestinal motility resulting in folds in the stomach (Peters and Richardson, 1983) which increase susceptibility to damage by acid (Brodie and Hanson, 1960). Stress also decreases the quality and amount of mucus adhering to the gastric mucosa. Due to the significant role of mucus in protection and enhanced healing of the stomach membranes, stress-induced ulcer models are usually deployed to evaluate gastroprotective agents. The bioactive constituents of *B. ferruginea* stem bark protected the rat gastric mucosa against stress ulcers suggesting cytoprotective activity, potential to reduce acid secretion and shield against oxidative damage and enhancement of mucosal barrier and healing mechanisms.

Gastric acid accumulates in the stomach when the pylorus is ligated (Brodie, 1966), causing formation of gastric ulcers. Ulceration produced by pylorus ligation is due to auto-digestion and subsequent collapse of gastric mucosal barrier, secondary to high concentration of acid (Tovey, 2015). The bioactive constituents of *B. ferruginea* stem bark elicited remarkable and significant gastroprotective effects in rats subjected to pylorus ligation suggesting decrease in total acidity and volume of gastric secretion, hence antisecretory activity.

Bioactivity-guided fractionation technique was employed to aid the isolation of the antiulcer constituents of *B. ferruginea* stem bark. Successive separation and pharmacological screening using indomethacin-induced ulcer as bioactivity guide revealed that two steroids isolated from the plant, β-sitosterol and β-sitosterol-3-O-β-D- glucopyranoside possess gastroprotective activity. This is the first report of the isolation of these compounds from the stem bark extract of *B. ferruginea*. They are phytosterols which are steroid compounds composed of plant sterols and stanols related to cholesterol and differ in carbon chains and or presence or absence of double bond. β-sitosterol was more active against indomethacin- and ethanol-induced ulcers, while β-sitosterol-3-*O*-β-D- glucopyranoside elicited more protection against cold restraint stress-induced ulcer. However, there was hardly any difference in the degree of protection against pylorus ligation-induced ulcer elicited by both compounds. Earlier studies reported the gastroprotective effects of these and related phytosterols (Arrieta *et al*., 2003; Xiao *et al*., 1992; Navarrete *et al*., 2002). Phytosterols have been demonstrated to reduce the permeability of phosphatidyl-choline bilayers to water and enhance stability of phospholipid monolayers (Hąc-Wydro *et al*., 2007; Tovey, 2015), reduce the leakage of proton and sodium ions from cell membranes (Haines, 2001) and inhibit the release of histamine from peritoneal mast cells (Shoji *et al*., 1994); these may partly account for the observed cytoprotective effects of β-sitosterol and β-sitosterol-3-*O*-β-glucopyranoside isolated from *B. ferruginea* stem bark.

Conclusion

The results from this study reveal β-sitosterol and β-sitosterol-3-O-β-D- glucopyranoside as antiulcer constituents of *B. ferruginea* stem bark and account for antiulcer effects of the plant. These phytosterols may serve as lead compounds to develop novel antiulcer agent(s) with improved efficacy and safety profile.

Author's Contributions

Ejike Marcellus Nnamani: Participated in the study design, conducted the experiments and data analysis.

Peter Achunike Akah and Charles Ogbonnaya Okoli: Participated in the study design.

Adaobi Chioma Ezike: Participated in the study design, experiments and data analysis.

Michel Tchimene Kenne: Participated in phytochemistry and some other aspects of the experiments.

All the authors participated in the preparation of the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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