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GC-MS Characterization and Antiulcer Properties of the Triterpenoid Fraction from Propolis of the North West Region of Cameroon

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TAN and DMEC did the field work and collected the propolis sample. Authors TPV, TAN and DMEC conceived the study and designed the experiments. Authors TAN, TE, DMEC and MTJ did extraction and column chromatography. Authors DMEC, TAN and TPV carried out antiulcer tests and analyzed the results. Authors MP and VB carried out GC-MS and interpreted the data. Authors TPV, MTJ, TE and VB supervised the work and provided materials. Authors DMEC, TAN and TPV wrote the first manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To identify the chemical components of the major bioactive fraction from a propolis extract and investigate the gastric cytoprotective effects of the said fraction.
Place and Duration of Study: Department of Animal Biology & Physiology (Animal Physiology

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Laboratory), Department organic chemistry (Laboratory of medicinal chemistry), Faculty of Science, University of Yaoundé I in collaboration with the IOCCP, Bulgarian academy of sciences between August 2014 and April 2017.

Methodology: The propolis extract previously shown to possess gastric cytoprotective activity was subjected to column chromatography to obtain fractions, the major one of which was used in this study. About 5 mg of this fraction was silylated using BSTFA and subjected to GC–MS analysis on a Hewlett–Packard gas chromatograph. Identification of individual compounds was performed based on mass-spectral fragmentation and by comparison with some literature data and authentic samples. Gastric cytoprotective activity and antioxidant properties of the fraction were then evaluated using experimentally-induced gastric ulcers in rat models including HCl/ethanol, HCl/ethanol pretreated with indomethacin, absolute ethanol and stress-induced gastric ulcers. In each experimental model percentage of ulcerated surfaces, ulcer indices, mucus production as well as the percentage inhibition of ulceration were evaluated. The effects of this fraction on the anti-oxidative parameters in stomach tissues of rats subjected to cold/restraint stress were equally evaluated.

Results: The GC-MS profile of the major fraction under investigation revealed that it was a mixture of pentacyclic triterpenes; lanosterol, α-amyrine, 28-norolean-12-en-3-ol, Cycloartenol, 3-epi-α-amyrine, lupeol and 24-methylenecycloartenol. In the HCl/ethanol-induced gastric lesions model the fraction significantly ($p < 0.01$) reduced ulcer index at dose 50 mg/kg with a percentage inhibition of 47.25%, a significant increase in mucus production from 70.60 mg ($p < 0.5$) to 88.00 mg ($p < 0.001$) for the 25 and 50 mg/kg doses, respectively, compared with 53.40 mg for the negative controls. HCl/ethanol-induced gastric lesions in rats pre-treated with indomethacin showed that ulcer index decreased significantly ($p < 0.001$) from 1.93 to 1.50 for the 25 and 50 mg/kg doses, respectively, compared with 5.77 for the control. This corresponded with 66.55 and 74 percentage inhibition for the same doses of triterpenes, and the quantity of mucus increased to 104.20, 116.20 and 139.40 mg for the 25, 50 and 100 mg/kg doses. With absolute ethanol the fraction significantly reduced ulcer indices to 3.63 and 2.28 for the 25 and 50 mg/kg doses, respectively, compared with 4.87 for controls. The highest dose (50 mg/kg) of the fraction provided 53.18% inhibition of ulceration and the increase in mucus production was significant ($p < 0.001$) at the two doses. In the cold/restraint stress model, the fraction showed significant reduction in ulcer index, and the highest dose (50 mg/kg) prevented lesion formation by 52.51% inhibition while mucus production increased significantly. Cold/restraint stress significantly increased tissue concentrations of NO and MDA but the positive control (Omeprazole) and the fraction (50 mg/kg) reduced these parameters back to normal levels. The fraction (50 mg/kg) also reverted the increased concentrations of SOD and GSH (but not of catalase) back to normal values.

Conclusion: Cameroonian propolis likewise tropical and subtropical propolis samples are rich in triterpenes. The triterpene fraction dose-dependently acts by different and complementary mechanisms to improve the mucosal defensive factors. The mode of its gastroprotective activity may be attributed to reduction in gastric mucosal lipid peroxidation (MDA), elevation of glutathione (GSH), superoxide dismutase (SOD) and nitric oxide (NO).

Keywords: Propolis; GC-MS analysis; triterpenes; antiulcer properties; antioxidant effect.

1. INTRODUCTION

Gastric and duodenal ulcers are illnesses that affect a considerable number of people in the world and some authors consider gastric ulcer as the new "plague" of the 21st century [1]. Gastric ulcer occurs when there is a disturbance of the normal equilibrium of the gastric mucosa caused by either enhanced aggression or diminished mucosal resistance [2]. Factors such as stress, smoking, presence of *Helicobacter pylori*, nutritional deficiencies and frequent ingestion of non-steroidal anti-inflammatory drugs (NSAIDs) increase the incidence of gastric ulcer [3-4]. In addition to models of mucosal damage induced by NSAIDs, drugs, ethanol, feeding restriction and stress, there is substantial evidence that oxygen-derived free radicals such as the superoxide anion, hydrogen peroxide, and hydroxyl radical are well-established factors implicated in the pathogenesis of ischemic injury of the gastrointestinal mucosa and play an important role in the pathogenesis of the injury of various tissues, including the digestive system

[5-6]. A number of drugs including proton pump inhibitors and H_2 receptor antagonists are available for the treatment of gastric ulcer, but clinical evaluation of these drugs have shown incidences of relapses, side effects and drug interactions [7-8]. Thus, there is an urgent need to identify more effective and safe antiulcer agents. Crude plant extracts or their pure compounds are good sources for the development of new drugs and have been shown to produce promising results in the treatment of gastric ulcers [9].

Propolis is a resinous hive product collected by worker bees from various parts of the plants [10]. The word propolis (from the Greek pro = in defense or for, and polis = city) reflects its importance to bees, since they use it to smooth out internal hive walls, as well as to protect the colony from diseases and to cover carcasses of intruders that die inside the hive, thereby avoiding their decomposition [11]. Propolis was used as a source of alternative medicines for disease treatment and prevention in different parts of the world. Recently, propolis has been extensively used in food and beverages to improve health and prevent disorders such as heart disease, diabetes, inflammation and cancer [12-13]. Scientists have been interested in the investigation of its constituents and biological properties in the last decades [14]. The medicinal uses of propolis in Cameroon are numerous and include the treatment of tooth ache, stomach disorders, gastritis and sore throat by chewing directly. Its aqueous extract is used in treating wounds, skin rashes, boils and burns [15]. Our previous studies have reported the anti-ulcer activity of propolis from the North West region of Cameroon. It was found that the acetone extract of propolis was rich in phyto-constituents (including triterpenes, fatty acids and alkenyl resorcinols) [15]. Some studies have mentioned the possible antiulcer activities of different classes or mixtures of triterpenoids [16-18].

In the present study, the GC-MS analysis of the triterpene fraction (isolated as the major fraction from the propolis) was carried out and the constituents identified and semiquantified. The gastroprotective effects of the triterpene fraction was then evaluated using experimental rats models including HCl/ethanol, absolute ethanol, HCl/ethanol in rats pretreated with indomethacin, and stress-induced gastric ulcers. The effects of this triterpene fraction on the *in vivo* anti-oxidative parameters were equally evaluated.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Male albino Wistar rats weighing between 180-200 g were used. Prior authorization for the use of laboratory animals in this study was obtained from the Cameroon National Ethics Committee (Reg. No. FWA-IRB00001954). The use, handling and care of animals were done in adherence to the European Convention (Strasbourg, 18.III.1986) for the protection of vertebrate animals used for experimental and other purposes (ETS-123), with particular attention to Part III, articles 7, 8, and 9. Animals were maintained under standard conditions in the animal house of the Animal Physiology Laboratory, Faculty of Science, University of Yaoundé I. The animals were fed a standard laboratory diet and given fresh water *ad libitum*.

2.2 Preparation of Triterpene Fraction

Previous anti-ulcer studies on three extracts (hexane, acetone and methanol extracts) of propolis from Nkambe (North West Cameroon) were evaluated and the acetone extract was found to be the most cytoprotective extract. Acetone extract (75 g) was subjected to column chromatography using silica gel on a gradient of Hexane-EtOAc (0-100%) then EtOAc-MeOH (0-40%) with increasing polarity to yield 352 fractions indexed ANT1-ANT352. Based on their TLC profiles, 344 fractions were regrouped and pooled into 12 major fractions D-L while the remaining 8 fractions (ANT10, ANT37, ANT39, ANT46, ANT55, ANT103, ANT252 and ANT315) were left. The major fraction in terms of quantity, ANT46 (combining ANT46-54), crystallized in the form of a whitish powder in the eluent Hexane-EtOAc (5-20%) on standing. It was then filtered and subjected to ^{1}H NMR on a Bruker AV500 spectrometer (500 MHz) to verify its degree of purity. GC-MS analysis was then used for the identification of its constituent compounds since it was found to be a mixture. Apart from fraction ANT46 which was obtained in significant quantity, the rest were obtained in minute quantities that could not be used in animal experimentation.

2.3 GC-MS Analysis

2.3.1 Preparation of the analyte sample

About 5 mg of fraction ANT46 were mixed with 50 μL of dry (water-free) pyridine and 75 μL of

bis (trimethylsilyl)-trifluoroacetamide (BSTFA) and heated at 80°C for 20 min. The silylated fraction was analyzed by GC-MS.

2.3.2 GC-MS analytical procedure

The GC–MS analysis was performed with a Hewlett–Packard gas chromatograph 5890 series II Plus linked to a Hewlett–Packard 5972 mass spectrometer system equipped with a 30 m long, 0.25 mm i.d., and 0.5 µm film thickness HP5-MS capillary column. The temperature was programmed from 60 to 300°C at a rate of 5°C/min, and a 10 min hold at 300°C. Helium was used as a carrier gas at a flow rate of 0.8 mL/min. The split ratio was 1:10, the injector temperature 280°C, the interface temperature 300°C, and the ionization voltage 70eV. The fraction was analyzed in duplicate.

2.3.3 Identification and quantification of compounds

The identification of individual compounds was performed using computer searches on commercial libraries, comparison with spectra of authentic samples and literature data. The quantification of individual constituents was based on internal normalization. The percentages in Table 1 refer to percent of the Total Ion Current (TIC), and are semi-quantitative [15,19]. The total ion chromatogram and the Mass spectra of the individual compounds are given in the appendix.

2.4 Anti-ulcer Tests

2.4.1 HCl/ethanol-induced gastric lesions

The HCl/ethanol solution was used to induce ulcers in gastric mucosa according to the method of Hara and Okabe [20]. Male rats were fasted for 48 h before administration of triterpenes. The animals received triterpenes (25 and 50 mg/kg) by oral route, while positive and negative controls received, respectively, sucrafalte (50 mg/kg) and distilled water (1 ml), 1 h before they were given the necrotizing solution. The animals were sacrificed using ether, the abdomen of each opened and the stomachs removed. The ulcers produced in the glandular region of each stomach were measured and scored as earlier described previously [21] and the ulcer index (UI), percentage of inhibition (%I) and percentage of ulcerated surface (%US) were calculated.

2.4.2 HCl/ethanol-induced gastric lesions in rats pre-treated with indomethacin

Indomethacin was given to the rats (20 mg/kg) by intra peritoneal route at the end of a 24 h fast. This was followed 1 h later by the HCl/ethanol ulcer procedure as described elsewhere [22]. Briefly, following indomethacin treatment, the animals received three doses of triterpenes (25, 50 and 100 mg/kg) by oral route, while positive and negative controls received, respectively, sucrafalte (50 mg/kg) and distilled water (1 ml), 1 h before they were given the HCl/ethanol solution. The animals were sacrificed using ether, the abdomen of each opened and the stomachs removed. The ulcers produced in the glandular region of each stomach were measured and scored.

2.4.3 Absolute ethanol-induced gastric lesions

Male rats were fasted for 48h before administration of triterpenes. The animals received triterpenes (25 and 50 mg/kg) by oral route, while positive and negative controls received, respectively, sucrafalte (50 mg/kg) and distilled water (1 ml), 1 h before they were given the necrotizing solution (1ml of absolute ethanol) by oral route. The animals were sacrificed using ether, the abdomen of each opened and the stomachs removed. The ulcers produced in the glandular region of each stomach were measured and scored as earlier described previously [21] and the ulcer index (UI), percentage of inhibition (%I) and percentage of ulcerated surface (%US) were calculated.

2.4.4 Cold/restraint stress-induced gastric lesions

Stress-induced gastric ulcers were provoked in rats using a slight modification of the method described elsewhere [23-24]. The animals were deprived of food for 24 hours. Rats in treatment groups were given triterpenes (25 and 50 mg/kg) by oral route while control rats received the vehicle or omeprazole (10 mg/kg). One hour later, the rats were placed in small individual wire cages and immersed in cold water (20 ± 2°C), up to the level of the xiphoid. 3 hours later the animals were sacrificed using ether and the stomachs removed. The lesion formation was assessed. Gastric tissue samples were also taken, prepared and preserved in a frozen state awaiting the measurement of different oxidative stress parameters.

2.4.5 Measurement of mucus production

The amount of mucus produced was measured by carefully scrapping the glandular mucosal surface with a glass slide and weighing the mucus obtained using a sensitive digital electronic balance.

2.4.6 Measurement of *in vivo* antioxidant capacity

Blood and gastric tissue samples were assayed for oxidative stress parameters as follows: Cellular glutathione (GSH) was measured based on the reaction between 2,2-dithio-5,5-dibenzoic acid and the thiol (SH) groups of glutathione to yield a complex whose absorbance was read at 412 nm. The glutathione concentration was calculated using the molar extinction coefficient ε = 13600 cm/mol and the results expressed in nmol/g of tissue. Lipid peroxidation was assessed by measuring the levels of malondialdehyde (MDA) in gastric tissue samples. Quantification of MDA was done using an extinction coefficient of 1.56 × 106 M-1/cm−1 and expressed as pmol of MDA per g of wet stomach tissue. Superoxide dismutase (SOD) activity was measured using a standard method and tissue protein was measured using the Biuret method of protein assay. Catalase (CAT) was determined and expressed as μmol of H_2O_2 per min per mg of protein.

3. RESULTS

3.1 Chemical Constituents of ANT46

Sample processing for GC-MS involves solubilization, concentration to dryness and consecutive derivatization often carried out in a two-step procedure. The first step is achieved by a reaction of sample components with diluted pyridine to stabilize thermolabile compounds. In the second step, extracted metabolites are derivatized with silylating reagents *N*,*O*-bis (trimethylsilyl) trifluoroacetamide (BSTFA) which substitute protons bound to heteroatoms in functional groups such as -OH, -COOH, -NH_2, -NH, -SH, and generate trimethylsilyl (TMS) derivatives. The latter step is crucial for the adequate derivatization of non-volatile compounds in order to capture a huge variety of metabolites with polar characteristics and high boiling points on a GC-MS system. The retention times (RT), %TIC and some key ion fragments of the triterpene constituents of ANT46 are given in Table 1.

Fraction ANT46 gave a violet coloration when reacted with Liebermann-Burchard reagent characteristic of triterpenes. The ^{1}H NMR of ANT46 revealed that it was a mixture of triterpenes. This fraction was then analyzed by GC-MS and its constituent compounds identified from their retention times, MS fragmentation

Table 1. GC-MS data of silylated constituents of ANT46 with their key ion fragments

RT (min.)	Compound	Key ion fragments of silylated sample (m/z and relative abundance)	% of TIC
67.2	lanosterol	498.6 (40.9%), 393.5 (100%), 69.2 (40.45%), 394.5 (31%), 109.2 (27.5%), 189.2 (17.6%)	2.4
69.2	α-amyrine	498.6 (4.5%), 218.3 (100%), 203.3 (36.2%), 189.3 (16.3%), 219.3 (18.5%), 135.2 (6.7%)	20.5
69.3	28-norolean-12-en-3-ol	498.6 (30.5%), 204.3 (100%), 177.2 (70.9%), 190.3 (43.2%), 109.2 (31.3%), 231.3 (30.2%)	0.9
71.8	Cycloartenol	498.6 (16.9%), 218.3 (100%), 408.5 (45.1%), 365.4 (35.2%), 189.3 (37.1%), 135.2 (26.9%), 175.2 (17.8%), 109.2 (23.7%)	36.3
72.3	3-epi-α-amyrine	498.6 (19.3%), 218.3 (100%), 189.3 (39.5%), 203.3 (25.3%), 191.3 (12.8%), 135.3 (19.1%), 109.2 (17.3%)	13.45
72.6	lupeol	498.6 (60.6%), 189.3 (100%), 218.3 (59.3%), 109.2 (55.9%), 135.2 (49.3%), 369.5 (40.9%), 107.2 (39.9%)	13.45
74.0	24-methylene cycloartenol	497.6 (9.7%), 422.5 (100%), 379.4 (89.9%), 353.4 (26.9%), 380.4 (27.9%), 203.4 (24.6%), 175.2 (34.8%), 107.2 (40.3%)	6.3

patterns and by comparison with internal standards and MS data bases and other data reported in literature. The qualitative and quantitative composition of triterpenes present in ANT46 were obtained from %TIC (percentage of total ion current) and fragmentation patterns from the MS fingerprint, respectively. These led to the identification of lanosterol, α-amyrine, 28-norolean-12-en-3-ol, Cycloartenol, 3-epi-α-amyrine, lupeol and 24-methylenecycloartenol as constituents of ANT46 (the triterpene fraction) (Fig. 1).

3.2 Anti-ulcer Effects of ANT46

The gastroprotective effects of triterpenes fraction against HCl/ethanol-induced gastric lesions are shown in Table 2. The fraction significantly ($p < 0.01$) reduced ulcer index at dose 50 mg/kg compared with the control, with 47.25% inhibition of ulceration. Mucus production increased significantly from 53.40 mg for the controls to 70.60 mg ($p < 0.5$) and 88.00 mg ($p < 0.001$) for the 25 and 50mg/kg doses, respectively.

Gastroprotective effects were also obtained when the triterpenes fraction was used to prevent HCl/ethanol-induced gastric lesions in rats pre-treated with indomethacin. Table 3 shows that ulcer index dose-dependently decreased ($p < 0.001$) from 5.77 in the controls to 1.93, 1.70 and 1.50 for the 25, 50 and 100 mg/kg doses, respectively. This corresponded with % inhibition of 66.55, 70.53 and 74 for the same doses of triterpenes. Mucus production increased dose-dependently to104.20, 116.20 and 139.40 mg for the 25, 50 and 100 mg/kg doses, compared with the controls.

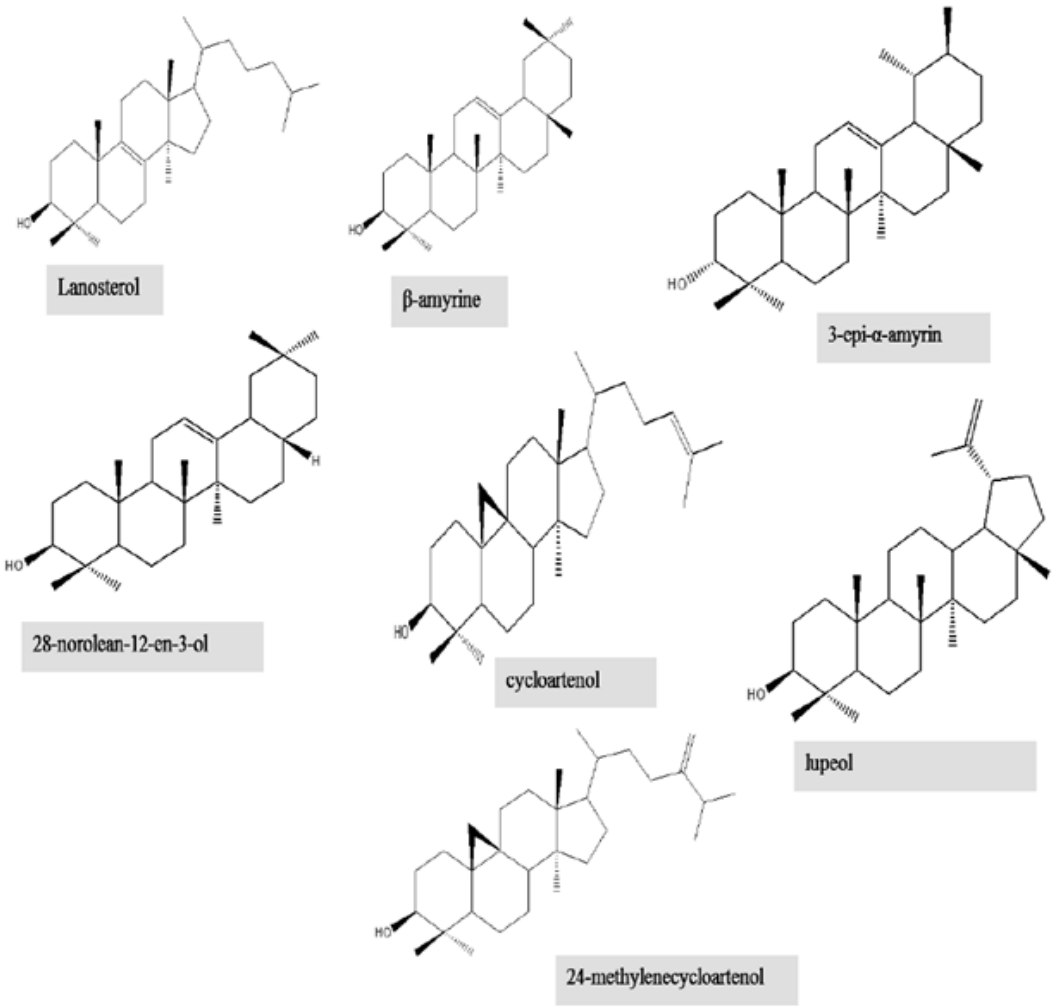

Fig. 1. Structures of the chemical constituents of triterpenoid fraction ANT 46

Table 2. Effect of triterpenes on HCl/ethanol-induced gastric lesions in rats

Treatment	Dose (mg/kg)	N	% ulcerated surface	Ulcer index	Mucus production (mg)	Inhibition (%)
Control	/	6	9.04	4.19 ± 0.60	53.40 ± 2.06	/
Sucralfate	50	6	0.75	2.21 ± 0.09**	105.00 ± 2.96***	47.25
Triterpenes	25	6	2.03	2.47 ± 0.26*	70.60 ± 4.51*	41.05
	50	6	0.84	2.21 ± 0.09**	88.00 ± 5.55***	47.25

*Statistically different relative to control; *: P < 0.5; **: P < 0.01; ***: P < 0.001; N: number of rats*

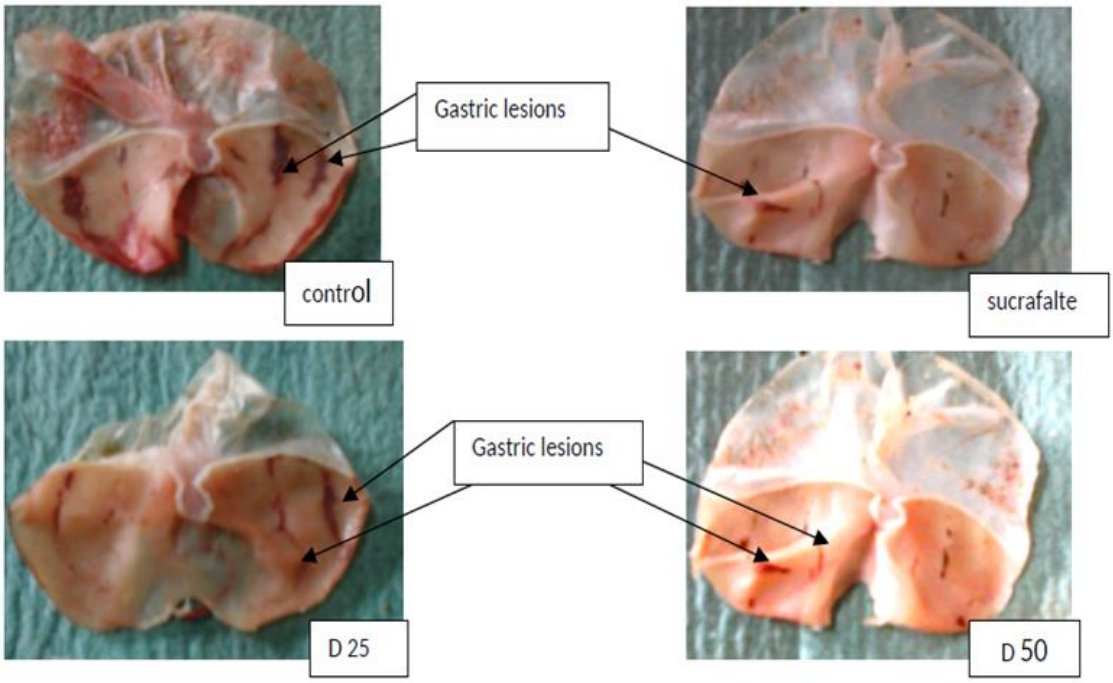

Fig. 2. Photograph of rat stomach subjected to HCl/EtOH-induced gastric lesions

Table 3. Effects of triterpenes fraction on HCl/ethanol-induced gastric lesions pre-treated with indomethacin

Treatment	Dose (mg/kg)	N	%ulcerated surface	Ulcer index	Mucus production (mg)	Inhibition (%)
Control	/	6	18.29	5.77 ± 0.48	49.49 ± 2.93	/
Sucralfate	50	6	1.94	2.74 ± 0.23***	72.00 ± 10.24	52.50
Triterpenes	25	6	0.58	1.93 ± 0.06***	104.20 ± 9.63**	66.55
	50	6	0.31	1.70 ± 0.20***	116.20 ± 9.32***	70.53
	100	6	0.27	1.50 ± 0.22***	139.40 ± 13.99***	74.00

*Statistically different relative to control; *: P < 0.5; **: P < 0.01; ***: P < 0.001; N: number of rats*

Table 4 shows the results obtained when the triterpenes fraction (25 and 50 mg/kg) was used to prevent the formation of gastric lesions induced using absolute ethanol. The fraction showed significantly reduced ulcer indices (3.63 and 2.28 for the 25 and 50 mg/kg doses respectively), compared with the controls (4.87). The highest dose of triterpenes fraction (50 mg/kg) provided 53.18% inhibition (Table 3) and the increase in mucus production was significant ($p < 0.001$) at the two doses.

In animals subjected to cold/restraint stress, all the two doses of triterpenes showed significant reduction in ulcer index compared with the controls. But only the highest dose (50 mg/kg)

prevented lesion formation by 52.51% inhibition. The mucus production increased significantly at both doses of triterpenes and Omeprazole (10 mg/kg) (Table 5).

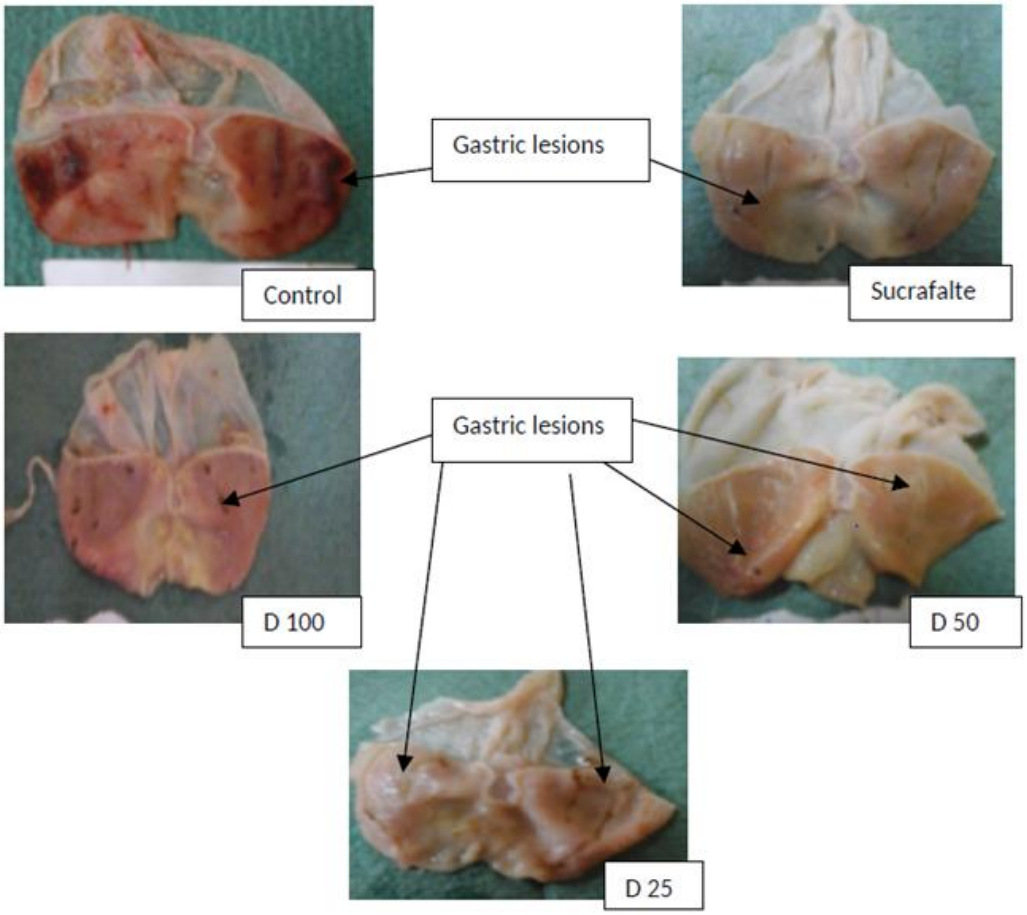

Fig. 3. Photograph of rat stomach subjected to HCl/ethanol-induced gastric lesions pre-treated with indomethacin

Table 4. Effect of triterpenes on absolute ethanol-induced gastric lesions in rats

Treatment	Dose (mg/kg)	N	% ulcerated surface	Ulcer index	Mucus production (mg)	Inhibition (%)
Control	/	6	17.88	4.87 ± 0.26	69.60 ± 2.64	/
Sucralfate	100	6	1.78	2.53 ± 0.15***	89.80 ± 4.15**	48.05
Triterpenes	25	6	4.99	3.63 ± 0.17**	96.40 ± 4.37***	25.46
	50	6	0.89	2.28 ± 0.17***	102.80 ± 3.40***	53.18

*Statistically different, relative to control; *: P < 0.5; **: P < 0.01; ***: P < 0.001; N: number of rats*

Table 5. Effect of triterpenes fraction on cold/restraint-induced gastric lesions in rats

Treatment	Dose (mg/kg)	N	%ulcerated surface	Ulcer index	Mucus production (mg)	Inhibition (%)
Control	/	6	1.89	2.19 ± 0.06	47.25 ± 2.78	/
Omeprazole	10	6	0.16	1.04 ± 0.04***	69.40 ± 3.82***	52.51
triterpenes	25	6	0.44	1.44 ± 0.07**	63.20 ± 2.20**	34.25
	50	6	0.18	1.04 ± 0.04***	89.40 ± 2.16***	52.51

*Statistically different relative to control; *: P < 0.5; **: P < 0.01; ***: P < 0.001; N: number of rats*

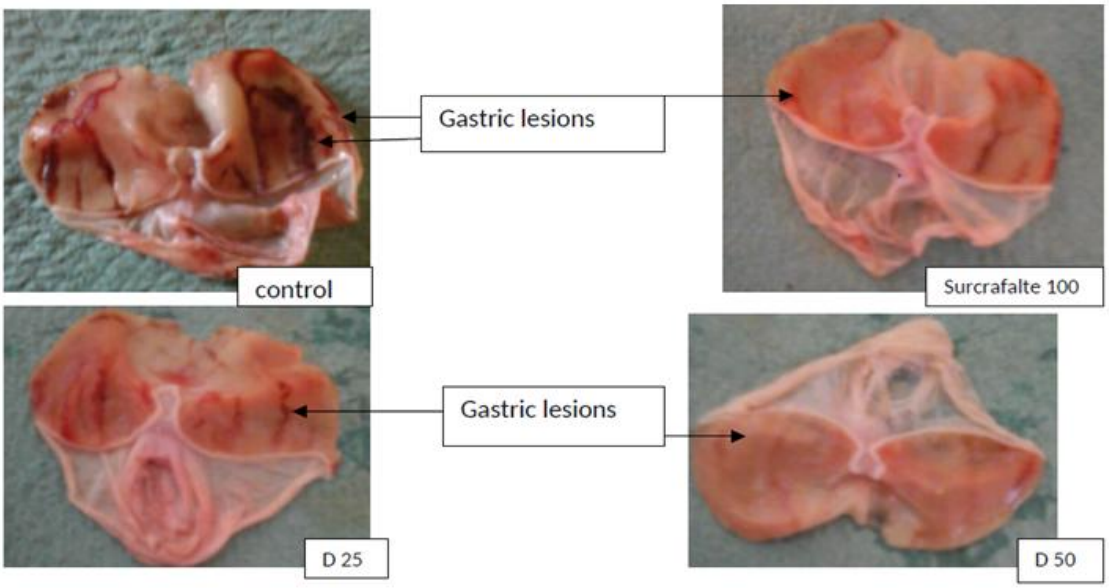

Fig. 4. Photograph of rat stomach subjected to absolute ethanol-induced gastric lesions

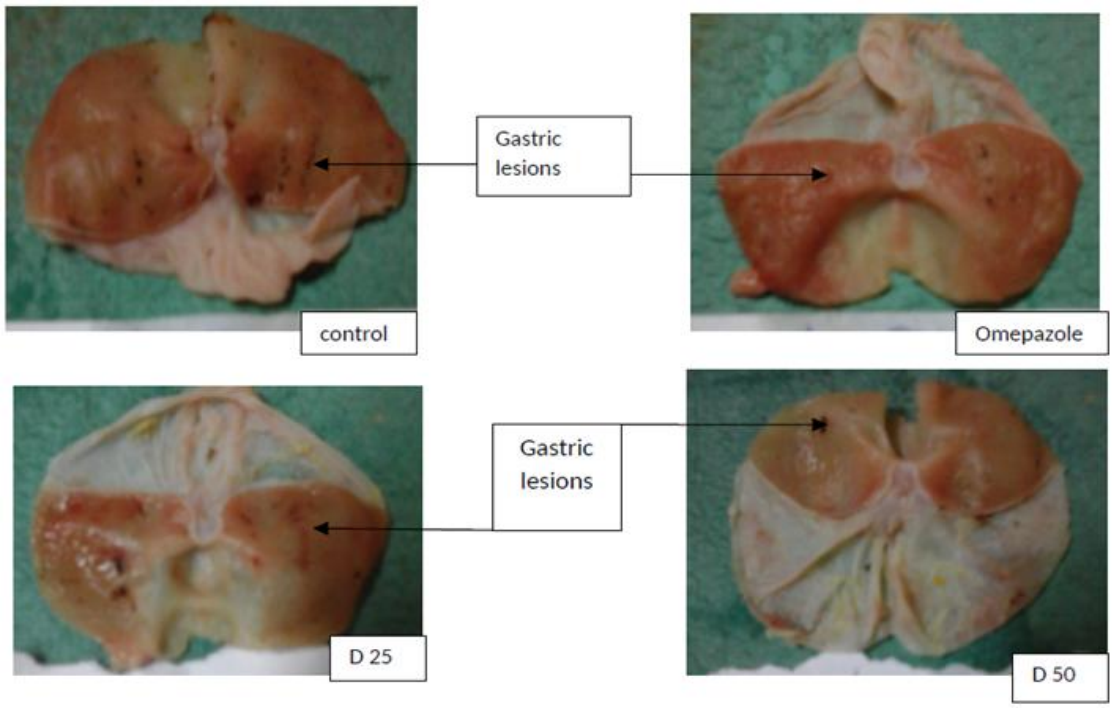

Fig. 5. Photograph of rat stomach subjected to cold/restraint stress

Cold/restraint stress significantly increased tissue concentrations of NO and MDA compared with normal values, but the positive control (Omeprazole) and triterpene fraction (50 mg/kg) reverted these back to normal levels. Cold/restraint also significantly reduced tissue levels of anti-oxidant enzymes, GSH, SOD and Catalase compared with normal values. Fraction ANT46 (50 mg/kg) also reverted the increased concentrations of SOD and GSH (but not of catalase) back to normal values. Fraction ANT46 at the dose of 25 mg/kg showed positive effects for tissue NO but not for GSH, SOD or MDA (Table 6).

Table 6. Effect of triterpenes on oxidative stress parameters in stomach tissues of rats subjected to cold/restraint stress

Treatment	Dose (mg/kg)	NO (µmol/g)	GSH (µmol/g)	SOD (µmol/g)	Catalase (µmol/g)	MDA (µmol/g)
Normal rat	/	$1.78\ 10^{-2} \pm 3.74\ 10^{-5***}$	$(5.63 \pm 0.17)\ 10^{-12***}$	$1.50 \pm 0.007^{***}$	$849.70 \pm 301.30^{*}$	$(1.18 \pm 0.04)\ 10^{-9***}$
Control	/	$5.22\ 10^{-3} \pm 2.41\ 10^{-4}$	$(3.46 \pm 0.13)\ 10^{-12}$	0.59 ± 0.07	144.80 ± 1.09	$(5.20 \pm 0.22)\ 10^{-8}$
Triterpenes	25	$1.25\ 10^{-2} \pm 4.11\ 10^{-4***}$	$(7.15 \pm 0.29)\ 10^{-12***}$	$1.40 \pm 0.079^{***}$	149.40 ± 2.94	$(1.35 \pm 0.04)\ 10^{-9***}$
	50	$7.52\ 10^{-3} \pm 2.69\ 10^{-4*}$	$(3.38 \pm 0.12)\ 10^{-12}$	0.46 ± 0.008	145.50 ± 1.59	$(5.59 \pm 0.34)\ 10^{-9}$
Omeprazole	10	$1.33\ 10^{-2} \pm 8.09\ 10^{-4***}$	$(5.86 \pm 0.01)\ 10^{-12***}$	$1.30 \pm 0.069^{***}$	140.40 ± 0.75	$(1.46 \pm 0.07)\ 10^{-9***}$

*Statistically different relative to control; *: P < 0.5; **: P < 0.01; ***: P < 0.001*

4. DISCUSSION

The structural identification of the compounds made use of fragmentation patterns, the Retro-Diels-Alder fragmentation of pentacyclic triterpenes giving the most characteristic diagnostic ion in each case. Hence ANT46 was characterized as a mixture of the pentacyclic triterpenes lanosterol, α-amyrine, 28-norolean-12-en-3-ol, Cycloartenol, 3-epi-α-amyrine, lupeol and 24-methylenecycloartenol.The peak corresponding to TMS (trimethylsilyl) group appears at 73.1 on all the spectra while molecular masses of the silylated compound corresponding to M+TMS appears at 498.55 for the triterpenes of molecular mass 426 g/mol. In several published papers, molecular weight or molecular ion and the base peaks were used as the criteria for the characterization of a compound. Analytical reviews on mass spectra of triterpenes present the use of the base peaks, main fragments and fragmentation mechanism/pattern of several skeleton pentacyclic and tetracyclictriterpenes for their identification [25]. A tentative quantitative composition of each of the triterpenes present in ANT46 were given from %TIC (percentage of total ion current) which depends on the nature of the compound and not its actual amount.

The integrity of the gastric mucosa requires continuous generation of prostacyclin and prostaglandin E_2. Thus, suppression of prostaglandin synthesis by non-steroidal anti-inflammatory drugs (NSAIDs) results in increased susceptibility of the mucosa to injury. When the gastric mucosal defense is compromised, exogenous noxious agents (e.g. ethanol, NSAIDs), together with HCl and pepsin, penetrate into the mucosa and damage the mucosal microvasculature. This damage reduces oxygen and nutrient delivery resulting in the release of pro-inflammatory and vasoactive mediators (serotonin, endothelin, leukotriene C_4, and platelet activating factor) that in turn exaggerate ischemic necrosis of the gastric tissue [26]. Some studies have reported that several triterpenoids possess useful cytoprotective activity [17,27]. In the present experiment, pre-treatment of the rats with indomethacin led to a significant increase in cytoprotection (66.55% 74.00% and 70.53% inhibition for the 25, 50 and 100 mg/kg doses of triterpenes, respectively) against the HCl/ethanol solution. When the cytoprotective action of an antiulcer agent is significantly decreased by pre-treatment with indomethacin, it can be interpreted that the cytoprotection is occurring through the mediation of endogenous prostaglandins [28]. The involvement of endogenous prostaglandins in cytoprotection by triterpene fraction is not therefore possible in this case since, in a separate experiment HCl/ethanol alone yielded comparatively lower percentage inhibition (47.35%) at the dose of 50 mg/kg. Various literature reports show that the lupane triterpenes, and specifically betulinic acid, possess anti-inflammatory properties but which are not of the NSAIDs type, but may be related to the inhibition of the non-neurogenic pathways and also due to interaction with glucocorticoid receptors [6]. The highly significant increase in gastric mucosal protection against HCl/ethanol in rats pre-treated with indomethacin can be due to the stereochemistry of triterpenes. It has been proposed that a hydroxyl group at position C-3 (free or derivative) is necessary for sterols and triterpenoids to exhibit antiulcer activity [29]. All the triterpenes present in the ANT46 fraction fulfill this stereochemical requirement (Fig. 1) and thus justify the observed cytoprotective activity of the fraction.

Ethanol is one of the ulcerogenic agents that induce intense damage in gastric mucosa by promoting disturbances of mucosal microcirculation, ischemia and appearance of free radicals, endothelin release, degranulation of mast cells, inhibition of prostaglandins synthesis and decrease of gastric mucus production [30]. In the ethanol-induced ulcer model which is used to screen drugs for cytoprotection, both doses of triterpenes (25 and 50 mg/kg) showed significant ulcer index reduction as well as Sucralfate (100 mg/kg). The major mechanism of gastroprotection by triterpenoids has been reported as involving the activation of mucous membrane secretion rather than the inhibition of gastric acid secretion. Triterpenes also stimulate gastric mucosal blood flow which is an important factor in gastroprotection [27]. This interpretation is supported by the significant increase in mucus production ((102.80 ± 3.40 mg) observed at the dose of 50 mg/kg of triterpenes as well as the corresponding high degree of ulcer inhibition (53.18%). In addition, the acetone fraction of propolis from which fraction ANT46 was obtained showed no antisecretory activity in our previous study (10% reduction of gastric acidity at 600 mg/kg) [15]. It is worth noting that the ANT46 fraction at the dose of 50 mg/kg was comparatively more cytoprotective than the 600 mg/kg dose of the mother acetone extract: 53.2%

inhibition versus 54.8% inhibition in the absolute ethanol model; 74% inhibition versus 65.9% inhibition in the HCL/ethanol/indomethacin model.

Body restraint, when combined with cold/water exposure, has been used as one of the methodologies because it can induce reliable gastric ulceration in a very short time [31,32]. Cold/ restraint stress is a frequently used and clinically relevant experimental model for induction of acute gastric ulcer [33]. Stress-induced gastric ulceration is probably mediated by the release of histamine, which not only enhances the acid secretion but also reduces mucous production. The major factors involved in the development of stress ulcers include an increase in gastric acid secretion and pepsin activity**,** and a decrease in mucosal protection due to the reduction in mucus secretion, mucosal blood flow, and NO production as well as prostaglandin levels [33]. Stress-induced gastric damage depletes the GSH levels which act as the first line of cellular defense against oxidative injury and this leads to aggravated tissue damage during stomach ulceration [34]. GSH and other antioxidant mechanisms (vitamins, melatonin, etc.) prevent tissue damage by keeping the reactive oxygen species at low levels and at certain cellular concentrations. In the present study, triterpenes treatment at the dose of 50 mg/kg significantly reverted GSH and SOD levels back to near normal values compared to control groups. Triterpenes such as lupeol suppress benzoyl peroxide (BPO)-induced skin toxicity by activating a series of antioxidant enzymes such as catalase and GSH that inactivate the BPO [35]. Oxidative stress in gastric tissue also causes damage to key biomolecules such as lipids. The stimulated lipid oxidation leads to increased accumulation of MDA in tissues. In the present study, triterpenes treatment significantly reverted the stress-induced changes in gastric tissue MDA concentrations back to normal levels, suggesting the high antioxidant activity of triterpenes. Nitric oxide (NO) is an endogenous defensive factor for gastric cells and exhibits gastro-protective properties against different types of aggressive agents [36]. It is involved in the maintenance of mucosal integrity through the regulation of mucus and alkaline secretion, gastric motility and microcirculation [37]. In the present study, cold/restraint stress significantly reduced gastric mucosal NO levels in the negative control group. The stress-induced decrease in NO biosynthesis is a result of decreased nitric oxide synthase (NOS) activity that is usually associated with an increase in the extent/severity of tissue damage [38]. Treatment with triterpenes significantly increased mucosal NO level when compared to the control group. This antioxidant activity is not proportionally dose-dependent in all cases and could be explained by the paradoxical effect of antioxidant compounds which can become pro-oxidant at high concentrations.

Triterpenes from propolis have been shown to possess DPPH scavenging activity [39] while triterpenes isolated from *Ganoderma lucidum* had antioxidant effects on ABTS(+) and superoxide radicals, significant ferric reducing activity, and highly effective reduction of *in vitro* lipid peroxidation [40]. These authors observed that the activities of antioxidant enzymes in blood and tissues were increased by the administration of total triterpenes to Swiss albino mice *in vivo,* and concluded that the ability of total triterpenes to scavenge the free radicals and to enhance the body's antioxidant defense systems indicates their potential use as antioxidants [40]. The anti-ulcer activity of Betulinic acid, a lupane type triterpene similar to the lupeol present in fraction ANT46, was reported to be due to its antioxidant effects and its ability to decrease malondialdehyde (MDA) production [6]. Dela Lastra and Motiva also attributed the anti-oxidant activity of Betulinic acid in gastric mucosa to its ability to reduce tissue MDA concentration thereby inhibiting lipid peroxidation [41].

5. CONCLUSION

Cameroonian propolis likewise propolis from subtropical and tropical regions has been shown to be rich in triterpenes. A triterpene mixture found to be the major fraction from a previously cytoprotective extract of propolis was prepared. GC-MS analysis of the triterpene mixture led to the identification of its chemical constituents as a mixture of the pentacyclic triterpenes lanosterol, α-amyrine, 28-norolean-12-en-3-ol, Cycloartenol, 3-epi-α-amyrine, lupeol and 24-methylenecycloartenol thanks to base peaks, main fragments andfragmentation mechanism/ pattern and by comparison with data reported in the literature. The gastroprotective activity of this triterpenes mixture from propolis was evaluated. The results showed that triterpenes seem to act by different and complementary mechanisms to improve the mucosal defensive factors. The mode of its gastroprotective activity may be attributed to reduction in gastric mucosal lipid

peroxidation (MDA), elevation of gastric reduced glutathione (GSH), superoxide dismutase (SOD) and nitric oxide (NO). Finally, the triterpene mixture at the dose of 50 mg/kg was more efficient than dose 25 mg/kg in antiulcer effects. These results support the ethno-medical use of propolis extracts in the treatment of gastric ulcer.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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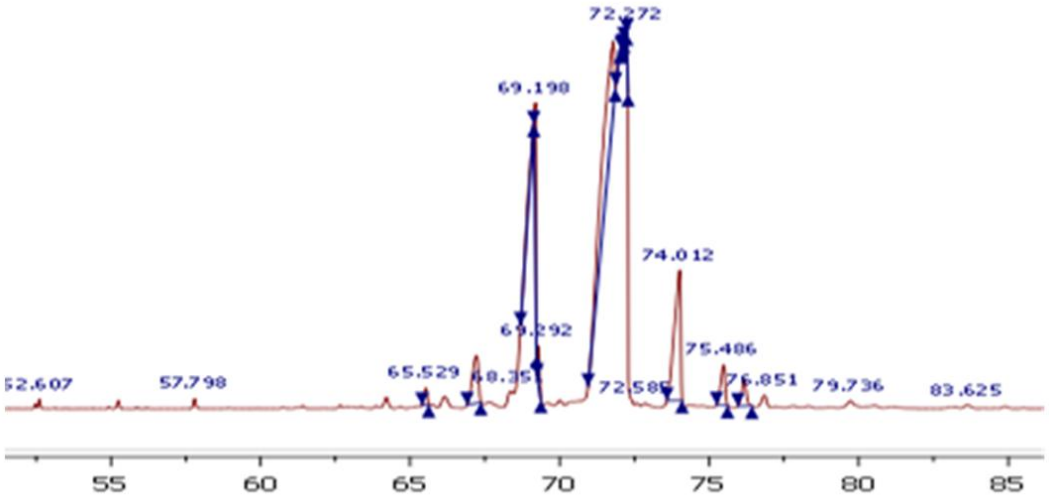
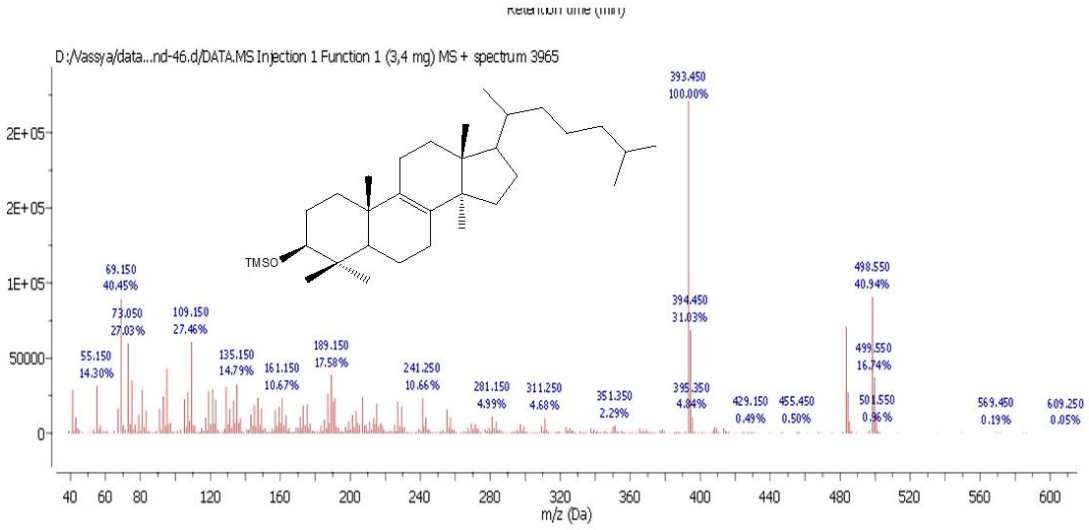
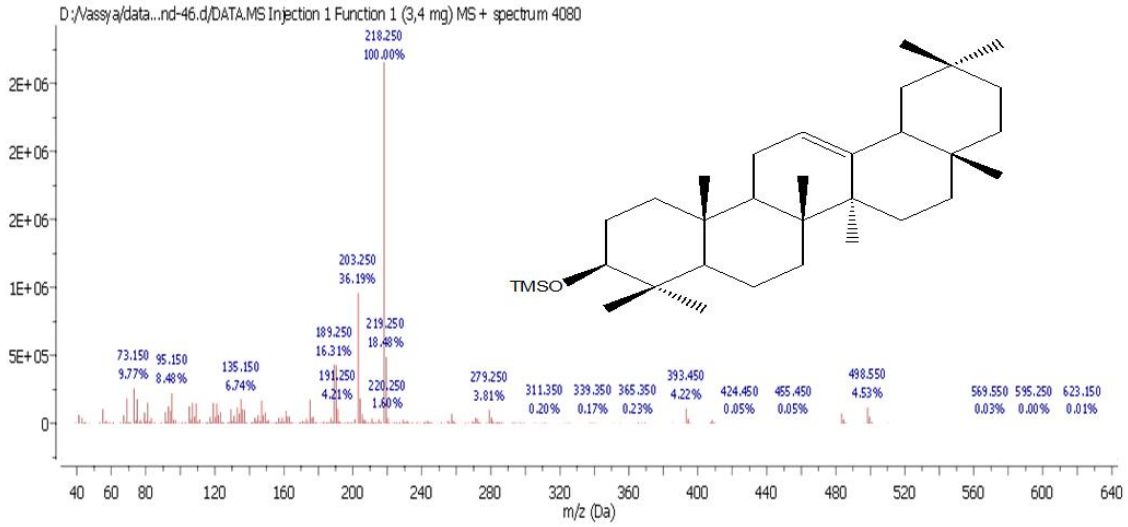
APPENDIX



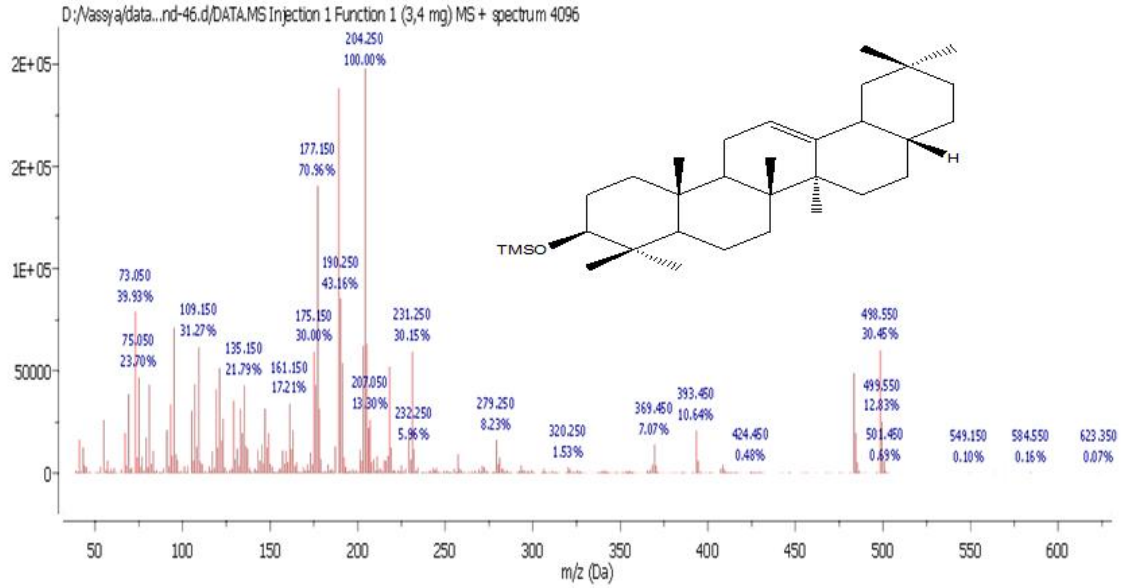
TIC of ANT46 silylated sample



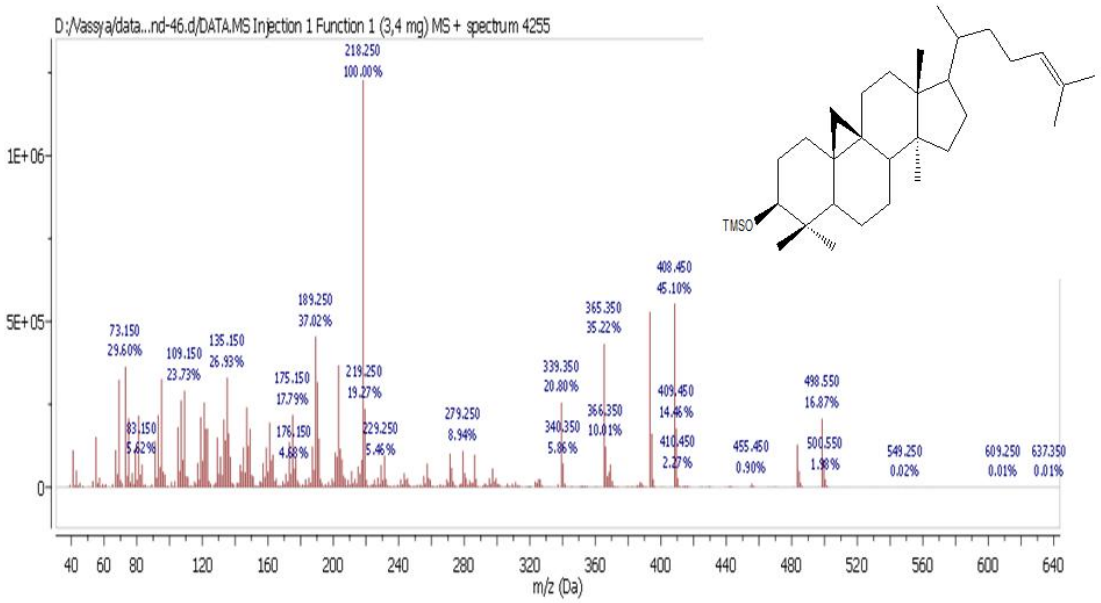
Mass spectrum of silylated Lanosterol



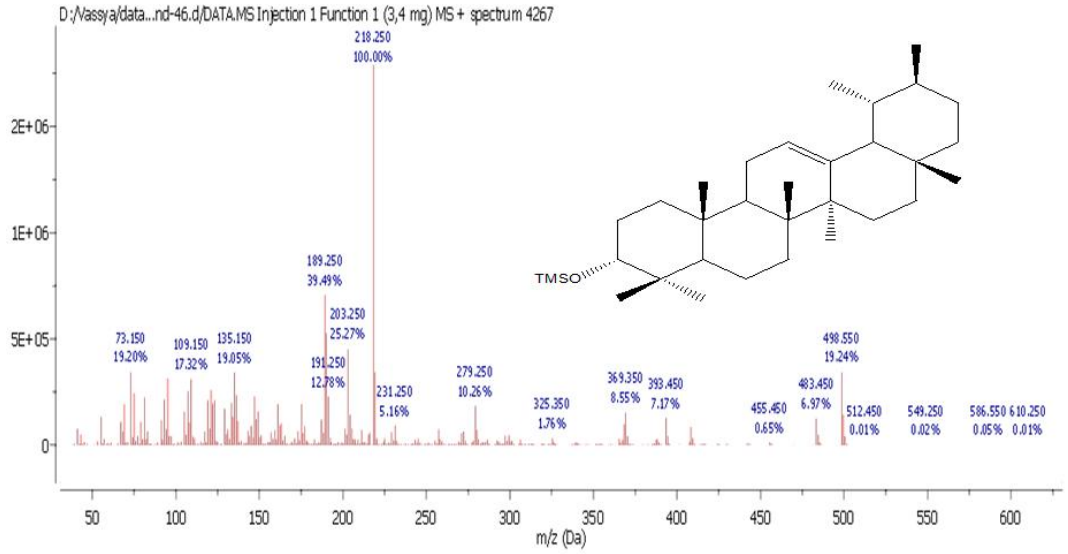
Mass spectrum of silylated β-amyrine



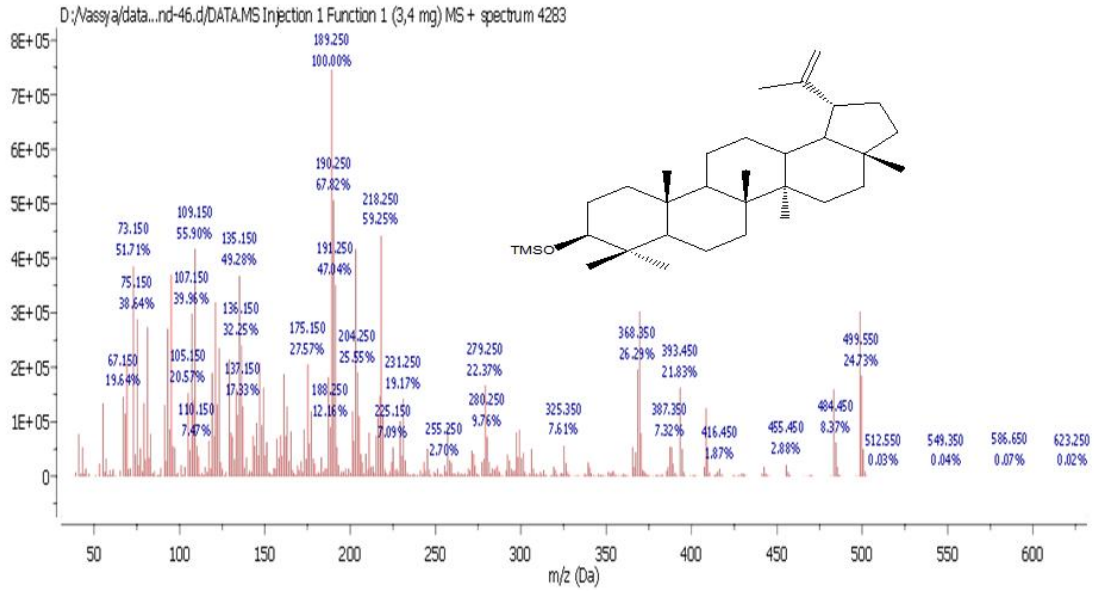
Mass spectrum of silylated 28-norolean-12-en-3-ol



Mass spectrum of silylated cycloartenol



Mass spectrum of silylated 3-epi-α-amyrin



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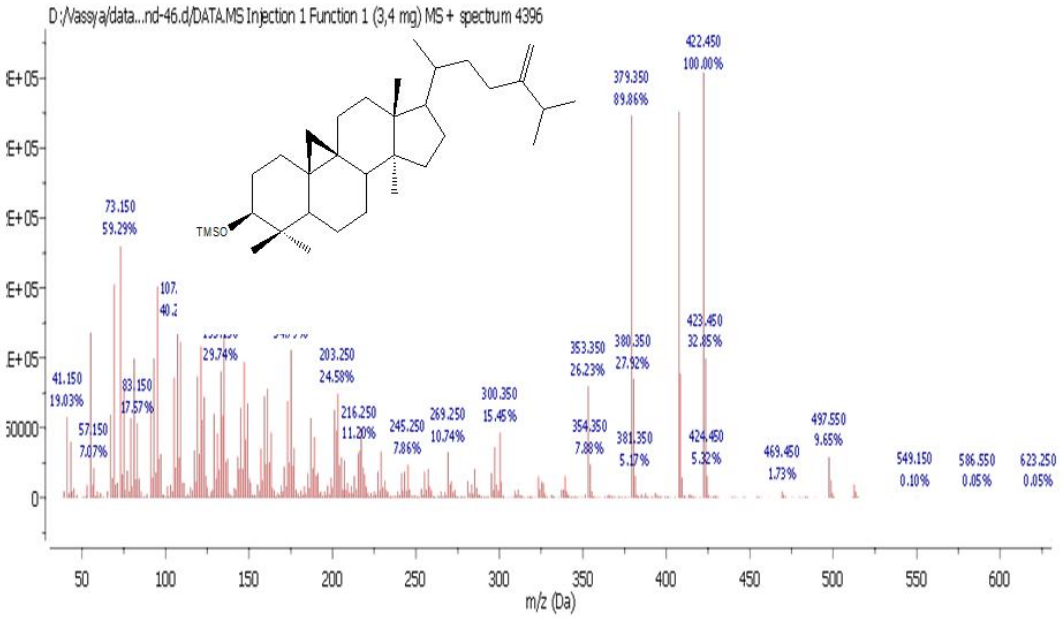

Mass spectrum of silylated 24-methylenecycloartenol

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