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Research Article
Chemical Constituents and Anti-ulcer Activity of Propolis from the North-West Region of Cameroon

[1]Tamfu Alfred Ngenge, [2]Domgnim Mokam Elisabeth Carol, [3]Talla Emmanuel, [2]Tan Paul Vernyuy, [1]Mbafor Tanyi Joseph, [4]Milena Popova and [4]Vassya Bankova

[1]Department of Organic Chemistry, Faculty of Science, University of Yaoundé I, P.O. Box 812, Yaoundé, Cameroon
[2]Gastroenterology Research Unit, Laboratory of Animal Physiology, Department of Animal Biology and Physiology, Faculty of Science, University of Yaoundé I, P.O. Box 812, Yaoundé, Cameroon
[3]Department of Chemistry, Faculty of Science, University of Ngaoundére, Ngaoundéré, Cameroon
[4]Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria

Abstract

Three extracts of propolis harvested from Nkambe, North-West region of Cameroon were characterized by GC-MS analysis and their gastric cytoprotective, antisecretory and antioxidant properties evaluated using experimentally-induced gastric ulcers in rats. The propolis extracts were rich in phytoconstituents occurring as sugars, triterpenes and a mixture (fatty acids+triterpenes+alkenyl resorcinols) in the methanol, hexane and acetone extracts, respectively with *Mangifera indica* as major plant source. Three triterpenes, lupeol, lupenone, 27-hydroxymangiferonic acid and an ester of fatty acid heptadecyl butanoate were isolated and characterized. The methanol, acetone and hexane extracts (200-600 mg kg^{-1}) dose-dependently prevented the formation of ethanol-induced gastric lesions (percentage of inhibition, 61, 54 and 55%, respectively, at the dose of 600 mg kg^{-1}). Increasing doses of the extracts inhibited pylorus ligation-induced lesions by 64.5, 73.1 and 16.2%, respectively for the highest dose but none of them showed antisecretory activity compared with controls. The most further significantly ($p<0.01$) reduced HCl/ethanol-induced ulcer indices from 4.33+0.32 in cytoprotective (acetone) extract (56.6-73.1% inhibition under highly acidic gastric environments), the controls to 1.25+0.53 and 0.6+0.04 at the dose of 400 and 600 mg kg^{-1}, respectively (percentage of inhibition: 71-86%). Furthermore, upon pretreatment of the rats with indomethacin prior to HCl/ethanol, the acetone extract significantly ($p<0.001$) decreased ulcer index from 5.55+0.73 in the controls to 1.89+0.15 at the dose of 600 mg kg^{-1}. Although pretreatment with indomethacin reduced the protective effect of the acetone extract by 23-27% and cytoprotection remained high (62-66% inhibition). The cytoprotective action of the most active (acetone) extract may involve the mediation of endogenous prostaglandins.

Key words: Propolis, GC-MS analysis, triterpenes, cytoprotection, antioxidant activity

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Corresponding Author: Tan Paul Vernyuy, Gastroenterology Research Unit, Laboratory of Animal Physiology, Department of Animal Biology and Physiology, Faculty of Science, University of Yaoundé I, P.O. Box 812, Yaoundé, Cameroon Tel: +237674921819

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Peptic ulcers are a deep gastrointestinal erosion disorder that involves the entire mucosal thickness, penetrating the muscular mucosa. An estimated 15.000 deaths occur each year as a consequence of peptic ulcer diseases[1] and as the prevalence of this disease increases over time, one would expect peptic ulcers to continue to have a significant global impact in the basic health and economic systems and in patient's life quality[2]. For decades it was believed that gastrointestinal ulcerations were caused by the excessive secretion of gastric acid, but many patients presenting such ulcerations had normal acid secretion rates[3]. Then some researchers reported that peptic ulcers are caused when the balance between aggressive factors (such as acid and pepsin) and defense mechanisms (such as mucus, bicarbonate, blood flow and mucosal turnover) are shifted in favour of the former[4]. Exogenous aggressive factors such as cigarette smoke, non-steroidal anti-inflammatory drugs (NSAIDs), alcohol, stress, fatty foods and *Helicobacter pylori* infections trigger tissue necrosis through mucosal ischemia, free radical generation and cessation of nutrient delivery. Hydrochloric acid together with pepsin, pancreatic enzymes and bile decrease the defense mechanisms of gastrointestinal mucosa, such as the intercellular junctions, local blood flow, mucus/bicarbonate secretion and cellular growth[5-7]. Although, histamine H_2-receptor blockers (for example ranitidine and famotidine), proton-pump inhibitors (for example omeprazole and lansoprazole), antibiotics (for example metronidazole, amoxicillin, clarithromycin and tetracycline) and other drugs are extensively used in the management of peptic ulcers, there are reports of adverse effects and relapse within one year[8] and also a number of side effects. For example, proton pump inhibitors (omeprazole and lansoprazole) may cause nausea, abdominal pain, constipation, diarrhea and H_2-receptor antagonists (cimetidine) may cause gynaecomastia and loss of libido. Due to the occurrence of many side effects triggered by use of synthetic drugs for many diseases, medicinal plants are considered as the main source of new drugs as they are believed to have less or no side effects. Herbal medicines are considered as safe for the treatment of ulcers with less adverse effects. Drugs for the treatment of gastric ulcers might be very expensive and unaffordable by many. Also in poor countries not everyone can have access to conventional and modern drugs and so they tend to recourse to medicinal plants and other natural products for treatment of various ailments. In addition to being economical, plant sources are effective and relatively less toxic and extensive study is presently being carried out in the study for potent antiulcer agents of plant origin[9-11]. Traditionally plants have not only provided food and shelter for mankind, but have also been used to cure many different ailments[12].

Propolis is an apicultural product that has been used for its various biological properties, particularly as a source of alternative medicines for disease treatment and prevention in different parts of the world. Bees use propolis to narrow the nest entrances, seal cracks and embalm dead organisms inside the hive and the antibiotic properties of propolis provide a healthy hive environment for the honeybee colony[13]. Recently, it has been reported to possess various biological activities such as antinociceptive[14], antimicrobial[15,16], antiviral[17,18], anti-inflammatory[19,20], anticancerous[21,22], antifungal[17,23], antitumoral[21,24], antioxidant[14,25,26], hepatoprotective[27], antiulcer[28,29], antiaging[30], antidiabetes[31,32], immunemodulating[33] and antibacterial[13] properties. The action of propolis against microorganisms represents the most essential pharmaceutical characteristic, for which it has been used by human beings since ancient times[34]. A number of chemical constituents are responsible for these pharmacological activities of propolis. Some of these compounds belong to flavonoids, prenylated p-coumaric acids and acetophenones, lignans, phenolic compounds, di- and triterpenes, caffeoylquinic acids, sugars, sugar alcohols, hydrocarbons and mineral elements[34] and these chemical compositions of propolis depend on the collection site, available plant sources and bee species.

In recent years, a remarkable number of studies have reported many advances made in the chemical and pharmacological studies of medicinal plants and other natural products as well as therapeutically active compounds obtained from propolis. Since incorrect use of the natural products offers can be dangerous to society, it is important to identify the active compounds, linking its structure with the biological activity and to report the correct manner, in which to use them with regard to dose, route of administration and frequency of use. In general, the metabolic profile of an extract gives an insight into its plant origin and allows the identification of its major constituents and also of a number of minor constituents, depending on the technique. It reveals the types of compounds present and gives an idea about the possible activities to be expected.

In the present study, three extracts (methanol, acetone and hexane extracts) were prepared from propolis harvested from Nkambe in the North-West region of Cameroon and the

chemical profiles were characterized by GC-MS analysis. The gastric cytoprotective activity, antisecretory and antioxidant properties of the extracts were then evaluated using experimentally-induced gastric ulcers in rats.

MATERIALS AND METHODS

Collection and significance of propolis in the locality of collection: The propolis was harvested from bee hives of an apiary located within the same area in Njap village, Nkambe town, North-West region of Cameroon during the months of February-March, 2013. A voucher specimen of this sample was deposited in the laboratory of natural products number III, Department of Organic Chemistry, University of Yaoundé 1. Propolis is called 'Nlaa-nfuu' or 'Mbihdong' in Limbum language and 'Dhatche-Nyaki' by the 'Bororo' inhabitants of this locality. Other popular local names include 'Kilei' in Oku, Bui division of the North-West region and 'Ndaki-goro' by the 'Gbayas' of the Adamawa region amongst others, where the use of propolis is becoming very popular. In Nkambe as well as many other localities in Cameroon, propolis is used by local sculptors for ornamental works and mending of calabashes. It is also exploited for its medicinal uses to treat tooth ache, stomach disorders, gastritis and sore throat by chewing directly. Its aqueous extract is used in treating wounds, skin rashes, boils and burns.

Extraction: One gram of raw propolis sample was dried and cooled (20°C) and ground in a mortar using a pestle to obtain a powder. The propolis powder was extracted successively by maceration with 10 V fold of hexane, acetone and methanol in a tightly closed glass jar kept in a dark cupboard at ambient temperature for 48 h with intermittent stirring. The supernatant was carefully decanted and filtered through a Whatmann No. 1 filter paper. The final filtrates were evaporated to near dryness on a rotary evaporator under reduced pressure to remove the solvent and the extract was collected in a clean vial. The maceration, filtration and evaporation process was repeated three times for each solvent after which the residual powder was dried before introduction of a new solvent. This yielded the hexane extract (PHEN), the acetone extract (PAEN) and the methanol extract of propolis (PMEN). All the extracts were well conserved for GC-MS analysis and antiulcer tests.

GC-MS analysis

Preparation of the analyte sample: About 5 mg of each extract were mixed with 50 L of dry (water-free) pyridine and 75 L of bis (trimethylsilyl)-trifluoroacetamide (BSTFA) and heated at 80°C for 20 min. The silylated extracts were analyzed by GC-MS.

GC-MS analysis: 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 60-300°C at a rate of 5°C min^{-1} and a 10 min hold at 300°C. Helium was used as a carrier gas at a flow rate of 0.8 mL min^{-1}. The split ratio was 1:10, the injector temperature 280°C, the interface temperature 300°C and the ionization voltage 70 eV. Every extract was analyzed in duplicate.

Identification and quantification of compounds: The identification of individual compounds were performed using computer searches on commercial libraries, comparison with spectra of authentic samples and literature data. If no reference spectra were available, identification was performed based on the mass-spectral fragmentation and in such cases for some compounds only tentative structures were proposed. Some constituents remained unidentified because of the lack of relevant references and information (none of them major constituents). The quantification of individual constituents is based on internal normalization. The percentage figures in the tables refer to percent of the Total Ion Current (TIC) and are semi-quantitative.

Isolation and characterization of pure compounds: Seventy five grams of the acetone extract were subjected to column chromatography with silica gel on a gradient of hexane-EtOAc (0-100%) then EtOAc-MeOH (0-40%) with increasing polarity to yield 352 fractions indexed ANT 1-352. Based on their TLC profiles, some of the fractions were regrouped into 12 pooled major fractions A-L, while others (ANT10, ANT37, ANT39, ANT46, ANT55, ANT103, ANT252 and ANT315) were left alone. Fraction D (ANT40-ANT45, 205 mg) was purified by column chromatography on silica gel using hexane-CH_2Cl_2 gradient (5-20%) to yield TA1 (48 mg) and TA2 (68 mg). Fraction G (ANT56-ANT84, 600 mg) was purified similarly by column chromatography on silica gel with mobile phase hexane-EtOAc (Hex/AcOEt 40%) to afford 30 mg of TA5. Fraction H (ANT85-ANT100, 2 g) was

purified by column chromatography on silica gel on a hexane-EtOAc40-50% to yield 8 mg of TA20 (hexane-EtOAc, 40% eluate) and 3 mg of TA33 (hexane-EtOAc, 45% eluate). Lastly, fraction I (ANT105-ANT170, 4.5 g) was purified on column chromatography on silica gel on a hexane-EtOAc (40-80%) to yield 24 mg of TA68 (hexane-EtOAc 65% eluate).

The ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC and HMBC were recorded on a Bruker AV500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). The ESI-MS spectra (ionization voltage 3.8 kV) were measured on a LTQ-FT Thermo scientific spectrometer. The structures of the compounds indexed, TA1, TA2, TA5 and ANT252 were elucidated based on their respective spectroscopic data and by comparison with some data reported in literature.

Anti-ulcer tests

HCl/ethanol-induced gastric lesions in rats: The rats were deprived of food for 36 h prior to experimentation but all the animals had free access to tap water. The HCl/ethanol solution was used to induce ulcers in the gastric mucosa according to the method of Hara and Okabe[35]. The animals received the plant extract by oral route, 1 h before they were given the necrotizing solution. Positive control rats received sucralfate in place of the extract. They were killed 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[36] and the Ulcer Index (UI), percentage of Inhibition (I%) and percentage of Ulcerated Surface (US%) were calculated.

HCl/ethanol-induced lesions in rats pre-treated with indomethacin: Indomethacin (Allphamed PHARBIL Arzneimittel GmbH Hildebrandstrasse 12 D-37081 Gottingen, Germany) was given to the rats (20 mg kg^{-1}) by intra peritoneal route at the end of the 24 h fast. This was followed 1 h later by the HCl/ethanol ulcer procedure as described above. Blood and gastric tissue samples were taken and prepared for the measurement of oxidative stress parameters.

Absolute ethanol-induced gastric lesions: The method described previously for the HCl/ethanol method was used, the only difference being that 1 mL of absolute ethanol was used as the necrotizing solution.

Pylorus ligated gastric secretion and ulceration in rats: The method of Shay *et al.*[37] was used to study the ability of the extract to reduce gastric acid secretion as well as prevent gastric ulceration resulting from auto digestion by stomach secretions. The test rats received the extract, while the controls received distilled water (1 mL) or cimetidine. One hour later, laparotomy was performed under ether anesthesia, the pylorus of each rat was ligatured and the abdominal incisions stitched up. The gastric juice produced during six subsequent hours was collected from each rat, the volume measured and 1 mL aliquots kept for gastric acid measurement. The ulcers produced in the glandular region of the stomachs were measured and ulcer index, percentage of inhibition, percentage of ulcerated surface were determined.

Measurement of mucus production: The mucus covering of each stomach was gently scraped using a glass slide and the mucus weighed carefully using a sensitive digital electronic balance.

Measurement of gastric acidity: One milliliter of centrifuged gastric contents from each rat was assayed for hydrogen ion concentration by pH-metric titration against 0.1 N NaOH using a digital pH meter. Gastric acidity was expressed as meq L^{-1}.

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 absorbence[38] was read at 412 nm. The glutathione concentration was calculated using the molar extinction coefficient $\varepsilon = 1.36\ 104\ M^{-1}\ cm^{-1}$. Lipid peroxidation was assessed by measuring the levels of malondialdehyde[39]. Quantification of MDA was done using an extinction coefficient of $\varepsilon = 1.56\ 105\ M^{-1}\ cm^{-1}$.

Statistical analysis: Pharmacological data were subjected to the one way analysis of variance (ANOVA) followed by the Turkey-Kramer post test. The p-values less than 0.05 were considered significant. Values in tables are given as arithmetic Mean±Standard Error of the mean (SEM).

RESULTS AND DISCUSSION

Different techniques are appropriate for the purpose of chemical profiling of propolis as demonstrated by numerous

Table 1: GC-MS profile of the methanol (PMEN) extract (silylated sample)

Compounds	TIC (%)
Glycerol	4.0
Glucose	10.2
Fructose	8.2
Pinitol	7.0
Quinic acid	6.3
Pentose	4.0
Manose	4.9
Glucitol	2.3
Inositol	1.2
Hexose	4.2
Pallatinose	1.2
Sucrose	2.8

Table 2: GC-MS profile of the acetone (PAEN) extract (silylated sample)

Compounds	TIC (%)	Compounds	TIC (%)
Glycerol	0.4	Anacardic acid (C17:2)	0.2
Hexadecanoic acid	0.6	Anacardic acid (C17:1)	1.5
Octadecenoic acid	0.8	Nonadecenyl resorcinol	0.4
Octadecanoic acid	0.2	Anacardic acid (C19:1)	0.3
Pentadecyl phenol	0.2	α-amyrenone	7.1
Eicosanoic acid	0.2	Cycloartenol	8.2
Pentadeceny resorcinol	0.9	α-amyrine	12.3
Heptadecyl resorcinol	0.8	Lupenone	10.4
Tetracosanoic acid	0.6	24-methylenecycloartenol	2.7
Anacardic acid (C15:1)	1.0	α-amyrine acetate	1.6
Heptadecadienyl resorcinol	0.6	Lupeol	1.2
Heptadecatrienyl resorcinol	0.6	β-amyrine acetate	5.6
Heptadecenyl resorcinol	2.4	3-hydroxydammarene	1.9

Table 3: GC-MS profile of the hexane (PHEN) extract (silylated sample)

Compound	TIC (%)
α-amyrenone	16.0
Germanicone	2.0
β-amyrine	3.4
Lupenone	24.7
Lupeol	7.1
α-amyrine	3.2
β-amyrine acetate	1.9
α-amyrine acetate	7.3

papers dealing with propolis analysis and hyphenated techniques are the most appropriate ones: HPLC-DAD, LC-MS, LC-MS-MS and GC-MS, etc[32]. The major compounds present in the different extracts PMEN, PAEN and PHEN identified by GC-MS analysis are listed in Table 1-3, respectively. Their percentages are given in the tables and refer to percent of the Total Ion Current (TIC), which are semi-quantitative since the ion current generated depends on the characteristics of the compound concerned and it is not a true quantitation.

The GC-MS techniques have been proven to be suitable for chemical profiling of propolis[32]. Even though these techniques provide a sufficient profile and identification of the compounds analyzed[40], the propolis has to be derivatised in order to increase the concentration of volatile compounds for detection. However, not all compounds comprising propolis are able to be derivatised or become volatile after derivatisation[40]. The GC-MS analysis of the silylated samples of the three extracts of propolis (PHEN, PAEN and PMEN) led to the identification of over 40 compounds belonging to various classes of natural products such as triterpenoids, alkenyl phenols and alkenyl resorcinols, fatty acids, sugars and anarcadic acids. The most abundant compounds in the acetone and hexane extracts are triterpenes and triterpene derivatives. The α-amyrenone, α-amyrine, 24-methylenecycloartenol, cycloartenol, α-amyrine acetate and lupenone were the most abundant in the PAEN, while lupenone, α-amyrenone, α-amyrine acetate and lupeol were the most abundant in the PHEN, based on the TIC% values. The hexane and acetone extracts (PHEN and PAEN) are similar in that they all contain α-amyrenone, β-amyrine, lupenone, lupeol, α-amyrine, α-amyrine acetate and β-amyrine acetate, but the PHEN contains germanicone and β-amyrine exclusively. Propolis samples from tropical and subtropical regions such as Cameroon have been proven to be rich in triterpenes and almost deprived of or contain only traces of other constituents[41].

Many studies with African propolis from different regions, like Kenya, Cameroon, Congo and Ethiopia showed that triterpenoids are major chemical components[42-44] and phytochemical studies of Cameroonian and some African propolis samples led to the isolation of or identification of a significant number of triterpenes[14,16,43,45-47]. Triterpenoids including β-amyrin, β-amyrone, lupeol and lupenone and polyprenyl benzophenones such as 7-epi-nemorosone, 7-epi-clusianone, xanthochymol and gambogenone have been detected in propolis samples from the Brazilian Amazon[48] and triterpenoids with major diterpenoids together with caffeate esters were reported in the propolis samples from Egypt but no aromatic acids and flavonoids[49,50]. The major constituents of the PHEN and PAEN are triterpenoids and triterpenes were found to be predominant in the hexanic and EtOAc phases of some Cameroonian propolis samples[45]. Therefore, the major source of triterpenoids is terrestrial vegetation[51]. Generally, the main constituents of propolis are resins and volatiles, which are substances obtained from a variety of botanical processes in different parts of plants found in the site of collection of the propolis samples and beeswax secreted by the bees. Besides triterpenes, a number of fatty acids, hexadecanoic acid, octadecenoic acid, octadecanoic acid, eicosanoic acid and tetracosanoic acid were also identified in the PAEN. A good number of fatty acids and organic acids have been identified in Turkish propolis[52,53] and also fatty acids have been revealed in Omani propolis[54]. To

the best of this knowledge, this is the first time that fatty acids are reported in important amounts in Cameroonian propolis and tropical propolis although methyl esters of these acids have been reported as major constituents of Ethiopian propolis[42]. An alkenyl phenol, pentadecyl phenol together with alkenyl resorcinols, pentadecenyl resorcinol, heptadecyl resorcinol, heptadecadienyl resorcinol, heptadecatrienyl resorcinol, heptadecenyl resorcinol and nonadecenyl resorcinol were also identified in the PAEN.

These compounds were reported previously in Cameroonian propolis[45] with the exception of heptadecadienyl resorcinol identified in Brazilian geopropolis[55] and heptadecatrienyl resorcinol. An inseparable mixture of four alk(en)ylresorcinols, (5-pentadecyl resorcinol, 5-(8′Z,11′Z-heptadecadienyl)-resorcinol, 5-(11′Z-heptadecenyl)-resorcinol and 5-heptadecyl resorcinol) were isolated and characterized from Indonesian propolis together with three cycloartane type triterpenes, mangiferolic acid, isomangiferolic acid and 27-hydroxy isomangiferolic acid[56]. Kardar *et al.*[45] attributed some characterized triterpenes with cycloartenol inclusive, mangiferonic acid, mangiferolic acid and isomangiferolic acid inclusive and some alk(en)ylresorcinols, 5-pentadecylresorcinol, 5-heptadecylresorcinol, 5-(11′Z-heptadecenyl)-resorcinol and 5-(12′Z-heptadecenyl)-resorcinol in Cameroonian propolis as known constituents of mango (*Mangifera indica*, Anacardiaceae) a resin-producing plant widely used in honey production in Cameroon and throughout tropical Africa[57,58]. Therefore mango could be a possible plant source of resin used by bees for the manufacture of propolis from the site of collection in Njap-Nkambe, a hypothesis that might require further verification. This fact is supported by the presence of anacardic acids, anacardic acid, (C15:1), anacardic acid (C17:2), anacardic acid (C17:1) and anacardic acid (C19:1) in the PAEN. Popova *et al.*[54] identified alkylphenol(cardanol), alk(en)ylresorcinols(cardols) and anacardic acids in Omani propolis[54] and documented that these three related compound types, which have been found in propolis samples from Brazil and cardols, which have been detected in propolis from Thailand and Indonesia[56,59,60], most probably originate from *Mangifera indica* fruit bark and are known antifungal substances[56,54]. Glycerol was identified in both PAEN and PMEN. Glycerol has been detected in a good number of propolis samples from different regions around the world for example in Turkish propolis, Canadian propolis and Brazilian geopropolis[52,55,61]. The PMEN is exclusively rich in sugars with glucose TIC% = 10.2, fructose TIC% = 8.2, pinitol TIC% = 7.0 and quinic acid TIC% = 6.3 as predominant constituents. Monosaccharides such as glucose, fructose, ribose, rhamnose, talose, gulose and saccharose are commonly present in propolis[62]. Sugars were also found in Turkish propolis and Omani propolis[52,54] and geopropolis from Northeast Brazil[55]. Some of the polyols alcohols identified in the PMEN such as pinitol, glucitol, inositol and quinic acid are known to possess good biological activities.

The structures of the compounds TA1, TA2, ANT252 and TA5 isolated were elucidated as (1) Lupenone, (2) Lupeol, (3) 27-hydroxymangiferonic and (4) Heptadecyl butanoate, respectively (Fig. 1). For the triterpenes, lupeol and lupenone have been described previously in Cameroonian propolis[14], while 27-hydroxymangiferonic acid was isolated from propolis of Myanmar[63]. Although, as a minor constituent, the presence of mangiferonic acid in the propolis substantiates the fact that *Mangifera indica* could be a major plant source of propolis in Cameroon.

The possible therapeutic usefulness of the rich chemical profiles of the three propolis extracts were tested using well known experimental methods of gastric ulcer, namely, absolute ethanol, HCl/ethanol, HCl/ethanol pretreated with indomethacin-and pylorus ligation-induced gastric ulcer. When the extracts were screened for cytoprotective activity against the highly corrosive absolute ethanol solution, control rats developed hemorrhagic lesions in the glandular portions of their stomachs 1 h after induction of the lesions. The methanol, acetone and hexane extracts (200-600 mg kg^{-1}) dose-dependently prevented the formation of gastric lesions, percentage inhibition attaining 61, 54 and 55%, respectively, at the dose of 600 mg kg^{-1}. Sucralfate (100 mg kg^{-1}) prevented lesion formation by 30.5%. Mucus production increased from 74.6 mg in the controls to 288.8, 375.8 and 375.2 mg, respectively, for the methanol, acetone and hexane extracts compared with 77.4 for sucralfate (Table 4). The highly corrosive nature of absolute ethanol to the gastric mucosa is well known. Absolute ethanol causes gastric mucosal lesions through the release of tissue-derived mediators, such as histamine and leucotriene C_4 as well as by superficial aggressive cellular necrosis. The action of these mediators on gastric microvasculature results in both mucosal and sub mucosal gastric tissue destruction[64]. The significant cytoprotection offered by the propolis extracts against absolute ethanol (54-61% inhibition) was accompanied by highly significant increases in mucus production, suggesting important inhibitory effects by extracts on the generation of the destructive tissue-derived mediators or inhibition of their action on the gastric microvasculature[65,66].

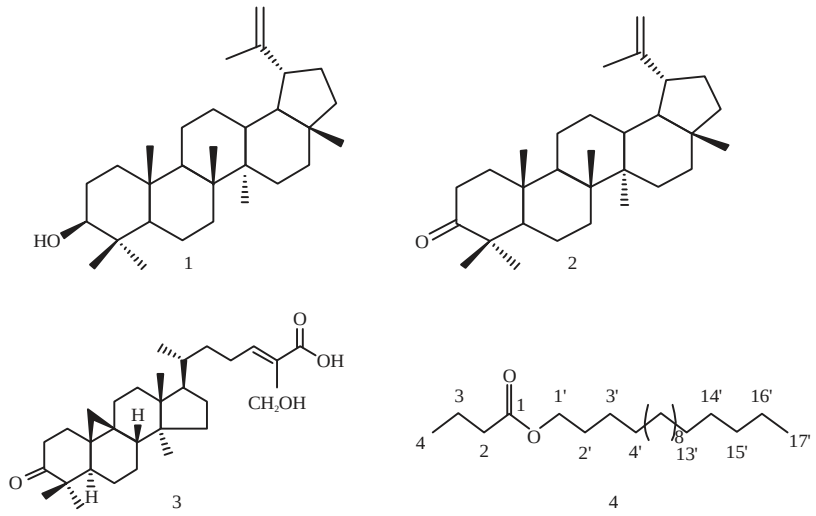

Fig. 1: Structures of the compounds TA1, TA2, ANT252 and TA5 isolated were elucidated as (1) Lupeol, (2) Lupenone, (3) 27-hydroxymangiferonic acid and (4) Heptadecyl butanoate

Table 4: Effects of propolis extracts on gastric ulcers induced by absolute alcohol in rats

Treatment	Dose (mg kg^{-1})	No. of rats	Ulcerated surface (%)	Ulcer index	Mucus production (mg)	Inhibition (%)
Control		5	14.4	5.22±0.40	74.63±6.31	-
Methanol extract	200	5	11.1	3.37±1.40	138.46±19.65*	35.4
	400	5	1 .9	2.32±0.60*	228.77±11.01***	54.9
	600	5	0.4	2.00±0.55*	230.34±17.11***	61.7
Acetone extract	200	5	11.3	4.16±1.20	128.13±19.53*	20.1
	400	5	3.9	3.02±0.18	371.81±52.13***	40.6
	600	5	0.7	2.36±0.64*	375.84±17.56***	54.8
Hexane extract	200	5	15.9	4.76±0.70*	215.60±32.64***	32.6
	400	5	4.9	2.66±0.44*	271.49±50.86***	47.8
	600	5	2.7	2.34±0.62*	275.16±8.95***	55.2
Sucralfate	100	5	1.4	2.04±0.47*	77.44±10.32	60.9

Statistically different relative to control, *p<0.05, ***p<0.001. The values are expressed as Mean±SEM

Since gastric acid and pepsin secretion are very important precursors for the creation of ulcers, the extracts were further screened for their ability to prevent gastric acid secretion using the pylorus ligation technique. In the control rats subjected to pyloric ligature alone, gastric lesion indices were 3.46+0.34. Increasing doses of the methanol, acetone and hexane propolis extracts inhibited lesion formation by 64.5, 73.1 and 16.2%, respectively for the highest dose of extracts compared with 61.8% for cimetidine. Cytoprotection was highest for the acetone extract and lowest for the hexane extract. Although, the cytoprotection was accompanied by significant increases in mucus production (Table 5), none of the extracts showed antisecretory activity compared with the controls (Table 6). Even though gastric acidity for the methanol and acetone extracts (55.8 and 65.6 meq L^{-1}, respectively) were statistically low compared with the controls, previous studies show that gastric acid levels of this magnitude are known to be highly ulcerogenic[36,67,68]. Unlike the methanol and acetone extracts which had slight tendencies to reduce gastric acidity at 400 mg kg^{-1} (45.6 and 34.0% reduction), the hexane extract (400-600 mg kg^{-1}), increased gastric acid levels by 8.1 and 16% compared with the controls (Table 6). Acid substances like hydrochloric acid, acetyl salicylic acid (aspirin) and glacial acetic acid are well known for their ulcerogenic effects on the gastric mucosa. The chemical profiles of the propolis extracts revealed the presence of 11 sugars and 1 acid in the methanol extract, 10 acids, 6 alkenyl resorcinols and phenols, 9 triterpenes and 1 sugar in the acetone extract and 8 triterpenes in the hexane extract. The presence of organic acids in the extracts (especially the methanol and acetone extracts) would have been expected to provide an additive ulcerogenic effect to the pylorus ligation-induced hyperacidity.

Table 5: Effects of propolis extracts on gastric ulcers induced by pylorus ligation in rats

Treatment	Dose (mg kg^{-1})	No. of rats	Ulcerated surface (%)	Ulcer index	Mucus production (mg)	Inhibition (%)
Control	-	5	7.48	3.46±0.34	38.00±5.19	-
Methanol extract	400	5	3.44	2.33±0.66	63.00±5.20*	32.66
	600	5	2.86	1.23±0.53*	63.20±4.39*	64.45
Acetone extract	400	5	1.73	1.50±0.38*	63.80±4.20*	56.65
	600	5	0.57	0.93±0.41**	71.00±8.73**	73.12
Hexane extract	400	5	5.26	3.20±0.33	15.60±1.40**	7.51
	600	5	5.60	2.90±0.60	81.00±6.81***	16.18
Cimetidine	50	5	0.31	1.32±0.61*	88.81±0.13***	61.80

Statistically different relative to control, *p<0.05, **p<0.01, ***p<0.001. The values are expressed as Mean±SEM

Table 6: Effect propolis extracts on gastric secretion in pylorus-ligated rats

Treatment	Dose (mg kg^{-1})	No. of rats	Gastric contents (mL)	Gastric pH	Gastricacidity (meq L^{-1})	Reduction of gastricacidity (%)
Control		5	6.40±0.70	1.93±0.09	84.20±7.07	-
Methanol extract	400	5	4.28±1.32	2.11±0.05	55.80±12.09*	45.6
	600	5	5.54±0.73	2.23±0.07	78.00±4.05	7.4
Acetone extract	400	5	4.28±0.92	2.07±0.10	65.60±12.16*	34.0
	600	5	2.88±0.83**	3.14±0.44**	75.80±8.90	10.0
Hexane extract	400	5	5.96±0.68	2.31±0.15	91.00±4.49	-8.1
	600	5	4.80±0.38	2.24±0.11	97.80±2.15	-16.2
Cimetidine	50	5		4.30±0.34	35.75±0.58**	57.5

Statistically different relative to control, *p<0.05, **p<0.01. The values are expressed as Mean±SEM

This was apparently not the case since the methanol and acetone extracts significantly (p< 0.01) prevented the formation of gastric lesions (64.5 and 73.1% inhibition, respectively) in spite of the highly acidic gastric environments (78.0 and 75.8 meq L^{-1}, respectively). The quantification of individual organic constituents of the extracts was done based on internal normalization and gave percentage values of Total Ion Current (TIC%) for each compound. Although, TIC% values are semi-quantitative, they may be useful in explaining the cytoprotective actions observed. The cocktail of 11 sugar molecules in the methanol extract represent 43.2% TIC compared with 6.3% TIC for quinic acid. Intragastric administration of a mannitol, glucose-fructose-sucrose-maltose mixture to pylorus ligated rats prevented the formation of mucosal lesions in an osmolality-dependent manner. The effect occurs by luminal dilution of the necrotising agent and acid without affecting acid content[69]. When Gharzouli *et al.*[70] obtained significant cytoprotection (87-100%) by a glucose-fructose-sucrose-maltose mixture against ethanol, indomethacin-and acidified aspirin-induced lesions in the rat, they concluded that the sugar-rich solutions may prevent gastric damage by a mechanism involving the release of some protective agents. Carbohydrates at high concentrations behave as mild irritants that can induce adaptive cytoprotection[71]. Hexoses, which are present in the methanol extract are major structural components of mucins, which in turn are the major components of the protective gastric mucus.

Six alkenyl resorcinol and alkenyl phenol compounds and 9 triterpenes in the acetone extract together account for 56.9% TIC compared with 5.6% TIC for the 10 acids. The possible ulcerogenic actions of the acid compounds may therefore be masked by the quantitative superiority of the triterpenes, sugars and phenolic compounds. Moreover, the quinic acid present in the methanol extract may well have cytoprotective effects since caffeoylquinic acids from Ligularia species possess peroynitrite-scavenging activity and showed antiulcer activity against HCl/ethanol-and indomethacin/bethanechol and reduced the volume of gastric juice[72]. In addition, the cytoprotective actions of phenolic compounds and triterpenes are well known. A study of antiulcer drugs of plant origin shows that triterpenes because of their ability to strengthen defencive factors such as stimulation of mucous synthesis or maintenance of the prostaglandins content of gastric mucosa at high levels are potentially the compounds with antiulcer activity[73]. These compounds exert cytoprotective actions through increased mucosal blood flow; increased mucus, bicarbonate and prostaglandin secretion and enhancement of the *in vivo* antioxidant status[74-77]. Polyphenolic compounds possess antioxidant activity often attributed to their redox properties which enable them to act like reducing agents and metals chelators and they scavenge free radicals[78]. Most effective medicinal plants are rich in polyphenols and possess high antioxidant potentials[79].

Although, all the three propolis extracts had no antisecretory activity, the acetone extract had the most

Table 7: Effects of propolis extracts on gastric ulcers induced by HCl/ethanol solution in rats

Treatment	Dose (mg kg^{-1})	No. of rats	Ulcerated surface (%)	Ulcer index	Mucus production (mg)	Inhibition (%)
Control	-	5	5.61	4.33±0.32	104.00±8.38	-
Acetone extract	400	5	0.49	1.25±0.53***	109.20±3.01	71.1
Acetone extract	600	5	0.07	0.60±0.40***	152.00±11.85*	86.1
Sucralfate	100	5	1.13	2.80±0.97*	105.90±12.17*	35.3

Statistically different relative to control, *p<0.05, ***p<0.001. The values are expressed as Mean±SEM

Table 8: Effects of propolis extracts on HCl/ethanol-induced gastric lesions in rats pre-treated with indomethacin

Treatment	Dose (mg kg^{-1})	No. of rats	Ulcerated surface (%)	Ulcer Index	Mucus production (mg)	Inhibition (%)
Control	-	5	25.5	5.55±0.73	50.60±4.63	-
Acetone extract	400	5	3.2	2.15±0.20***	120.80±9.46**	62.3
Acetone extract	600	5	2.2	1.89±0.15***	153.60±17.93***	65.9
Sucralfate	100	5	3.1	2.80±0.97*	59.40±6.81	49.5

Statistically different relative to control, **p<0.05, ***p<0.001. The values are expressed as Mean±SEM

Table 9: Effect of propolis extract on oxidative stress parameters in stomach tissues of rats subjected to HCL/Ethanol/Indomethacin-induced gastric lesions

Treatment	Dose (mg kg^{-1})	No. of rats	GSH (μmol g^{-1} of tissue)	MDA (μmol g^{-1} of tissue)
Normal rats	-	5	6.99±0.12	2.26±0.19
Control	-	5	4.09±0.45	4.70±0.49
Acetone extract	400	5	3.70±0.06	6.11±0.95
Acetone extract	600	5	3.65±0.05	6.35±1.07

significant cytoprotection (56.6-73.1% inhibition under highly acidic gastric environments). In addition, 5 out of the 8 triterpenes (α-amyrenone, α-amyrine, lupenone, lupeol and α-amyrine acetate) were present in both the hexane and acetone extracts. The acetone extract was therefore judged to be the most active and further tests were carried out to elucidate its possible mode of action. Table 7 shows the antiulcer actions of the acetone extract against HCl/ethanol solution. The extract significantly ($p<0.01$) reduced ulcer index scores from 4.33+0.32 in the controls to 1.25+0.53 and 0.6+0.04 at the dose of 400 and 600 mg kg^{-1}, respectively (%inhibition: 71-86%). Furthermore, pretreatment of the rats with indomethacin prior to HCl/ethanol raised ulcer index scores to 5.55+0.73 compared with 4.33+0.32 for the HCl/ethanol controls. In response, the acetone extract significantly ($p<0.001$) decreased ulcer index from 5.55+0.73 to 1.89+0.15 at the dose of 600 mg kg^{-1} (Table 8). Inhibition of ulcer formation was accompanied by highly significant ($p<0.001$) increase in mucus production. Although, pretreatment with indomethacin reduced protective effect of the acetone extract by 23-27%, cytoprotection remained high (62-66% inhibition) (Table 8). Indomethacin is a prostaglandin inhibitor, which suppresses gastro-duodenal bicarbonate secretion, disrupts the mucosal barrier, reduces endogenous prostaglandin synthesis as well as gastric mucosa blood flow in animals[80-83]. On the other hand, prostaglandins synthesized in large quantities by the gastrointestinal mucosa are known to prevent experimentally-induced ulcers caused by various ulcerogens. The role of prostaglandins in cytoprotection has been well discussed by Robert[84], Konturek *et al.*[85] and Robert *et al.*[86]. 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[87]. This may well be the case for the acetone extract of propolis used in our experiment. Table 9 shows that subjection of the rats to the HCl/ethanol/indomethacin treatment significantly decreased antioxidant enzyme concentration (GSH) and increased the MDA concentration compared with controls. Treatment with acetone extract did not prevent the drop in the concentration of GSH. The high MDA concentrations (4.70±0.49 mmol g^{-1}) created by the ulceration procedure were not reversed in the extract-treated groups. These results suggest that antioxidant effects may not be involved the mode of antiulcer activity of the propolis extract.

CONCLUSION

Herbal medicines are considered as safe for the treatment of ulcers and propolis, an apicultural by-product of bee farming (often with heterogeneous location-specific chemical composition) has been used for its various biological properties, particularly as a source of alternative medicines for disease treatment and prevention in different parts of the world. In conclusion, the results show that propolis from the Nkambe area of the North West region of Cameroon is rich in phytoconstituents occurring mainly as sugars in the methanol extract, as triterpenes in the hexane extract and as a mixture of acids, triterpenes and alkenyl resorcinols compounds in the

acetone extract. The propolis extracts do not possess antisecretory activity, but show cytoprotective actions that are linked to their phytochemical compositions. The cytoprotective action of the most active (acetone) extract may involve the mediation of endogenous prostaglandins. The propolis showed significant antiulcer activity and provide a justification for the therapeutic use of propolis extracts in the treatment of ulcers and other infectious diseases. These pharmacological activities of Cameroonian propolis are attributable to the presence of diverse chemical compounds including alkenyl resorcinols, fatty acids and triterpenes revealed by the GC-MS profiles of the extracts. *Mangifera indica* could be a major plant source of propolis in Cameroon. In particular, the discovery of propolis plant sources in different geographic regions could be of great importance in addition to the chemistry and biological action of propolis. Hopefully, this study could attract the attention of beekeepers and scientists to further study on propolis and explore its numerous therapeutic effects.

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