GASTROPROTECTIVE ACTIVITY AND ACUTE TOXICITY OF THE AQUEOUS EXTRACT OF *CYLICODISCUS GABUNENSIS* HARM. (FABACEAE) TRUNK BARK

ABSTRACT

This study was aimed at evaluating gastroprotective activity and the acute oral toxicity of the aqueous extract of the stem bark of *Cylicodiscus gabunensis*. Gastritis was induced with 96% (*v/v*) ethanol after pre-treatment with different doses of the aqueous extract of *C. gabunensis*. The gastroprotective effect was determined with the help of macroscopic and microscopic analysis of injured stomachs and the percentages of ulcer inhibition were calculated. Subsequently, using colorimetric methods, a phytochemical screening was carried out on the dry extract of *C. gabunensis* in order to determine the families of compounds present therein. After the screening, the dry extract was fixed on silica gel and mounted on a chromatographic column in order to isolate the bioactive compounds. Finally, the structures of the isolated compounds were obtained using spectral methods (MS, ^{1}H NMR, ^{13}C NMR, HSQC, and HMBC).

The result of this study shows that *C. gabunensis* does not present any acute toxicity at the doses tested. However, the gastroprotective effect was observed for all doses, with a maximum inhibition of the ulcer at 63.94% at the dose of 400 mg/kg of extract; against 56.73% inhibition for aluminium and magnesium hydroxide at a dose of 50 mg/kg.

The phytochemical screening of the crude extract of *C. gabunensis* showed the presence of several secondary metabolites including sterols, triterpenes and saponins. Five compounds were isolated and characterized by spectroscopic methods as being: *β*-sitosterol (**1**), lupeol (**2**), betulinic acid (**3**), betulin (**4**) and 3-*O*-*β*-D- sitosterol glucopyranoside (**5**). LD_{50} has been found over 5000 mg/kg, indicating that *C. gabunensis* was not toxic.

Keywords: *Cylicodiscus gabunensis*, *Fabaceae*, Gastroprotective, Oral toxicity

1. INTRODUCTION

Digestion is a mechanical and chemical process by the way foods are transformed in necessary nutrients to maintain lives after pass through small intestine [1]. Chemical digestion of foods is carry out by stomach through acids secretions who are aggressive to secrete mucus necessary for its protection against auto digestion [2]. In Africa, a context where 80% of the population uses traditional medicine to cure diseases, many plants are used by traditional healers to relieve stomach pain [3]. Previous studies carried out shoes that they possess various secondary metabolites with grastroprotective potential such as sterols, polyphenols and flavonoids [4].

Cylicodiscus gabunensis (*Fabaceae*) bark is used in traditional medicine to treat various ailments such as malaria, measles, varicella, bacterial infections, rheumatism, migraine and stomach pain [5]. *Cylicodiscus gabunensis* (*Fabaceae*) appears to be an interesting alternative in the management of gastritis at a lower cost. Nevertheless, the gastroprotective activity and the acute oral toxicity of the trunk's bark have not been reported. Hence, this study, Gastroprotective activity and acute toxicity of aqueous extract of *Cylicodiscus gabunensis* Harm. (Fabaceae) trunk bark.

2. MATERIALS AND METHODS

2.1 Plant material

The plant material used in this study consisted of trunk bark of *C. gabunensis*. The bark was harvested in the Center Region, Cameroon, 4 km from the city of Makak, on the road to Ngouatè. The identification of the sample collected was confirmed at the National Herbarium of Cameroon by comparison with No. 43972/HNC.

2.2 Animal material

The animal material consisted of a total of 57 rats divided into 20 males and 37 females of the Wistar strain. All animals were 10 weeks old and weighed between 110 and 130 g. They were acclimatized and had unlimited access to water and food.

2.3 Extraction

The aqueous extract was obtained through a decoction of air-dried and powdered stem bark of *C. gabunensis* on distilled water: 500 g of the pulverized vegetable drug in 2,5 l of distilled water for a period of 48 hours. This operation was repeated once, in order to maximize the extraction. At the end of this operation, the filtrate obtained was brought to a water bath at a temperature of 60°C [6].

The extraction yield (EY) was calculated relative to the mass of dry plant matter according to the formula:

$$EY = \frac{\text{Mass of the extract}}{\text{Initial mass of powder}} \times 100$$

2.4 Phytochemical analysis

This analysis of *C. gabunensis* aqueous extract was carried out using phytochemical screening tests and the compounds were isolated using chromatography techniques (TLC, CC) [7]. The elucidation of the structures was made possible using mass spectroscopy and 1D (^{13}C and ^{1}H), 2D (HSQC, HMBC, COZY) NMR techniques.

Phytochemical screening

Phenol test: 2 ml of extract were placed in a test tube and 1 ml of 5% ferric chloride was added. The appearance of a thick blackish-blue color indicates the presence of phenols.

Shinoda test: the dry extract was dissolved into 3 ml of methanol, and a few shavings of magnesium were added, followed by 5 drops of hydrochloric acid. The appearance of a purple or red-orange color indicates the presence of flavonoids.

Liebermann Burchard test: a small amount of dry extract was dissolved in a few drops of chloroform. A few drops of acetic anhydride were added, followed by sulfuric acid. A purple color that turns green indicates the presence of a sterol. An initial brick-red coloration that turns purple indicates the presence of a triterpene.

Dragendorff test: the Dragendorff's reagent was prepared by mixing a concentrated solution of potassium iodide (8g of KI with 200 ml of distilled water) with an equal volume of a bismuth (III) nitrate solution (0.85g of Bi(NO3)3 in 100 ml of distilled water and 10 ml of acetic acid) and adding 100 ml of distilled water to 10 ml of this mixture, as well as 100 ml of acetic acid.
A small quantity of the extract was dissolved in methanol. The presence of alkaloids was materialized by the appearance of a precipitate when adding the Dragendorff's reagent or spraying a TLC plate. The color of the precipitate varied between yellow, orange, red and brown, depending on the nature of the alkaloid.

Bornstrager test: by adding 1 ml of 25% ammonia to an equal volume of methanolic extract. The appearance of a red coloration indicates the presence of anthraquinones.

Tannin test: the reagent used was 5% ferric chloride ($FeCl_3$). In a test tube, 2 ml of extract was introduced, then 1 ml of $FeCl_3$ solution. The appearance of a bluish to black color indicated the presence of tannins.

Saponin test: a small amount of dry extract was dissolved in 1 ml of distilled water. The aqueous solution obtained was subjected to energetic stirring for 3 seconds. The presence of saponins results in the formation of a thick foam which persists for about 30 minutes.

2.5 Gastroprotective activity

The test was conducted on a total of 45 rats divided into 20 males and 25 females Wistar rats divided into 9 batches of 5 animals each, including 01 neutral control, 01 negative control (distilled water), 03 positive controls treated with (omeprazole, sucralfate, hydroxide of aluminum and magnesium), 04 batches tested with the *C. gabunensis* aqueous extract at

increasing concentrations (50, 100, 200 and 400 mg/Kg). The rats were fasted without water for 48 hours before the experiment [8].

Macroscopic evaluation

The mass of extract to be sampled to prepare the doses to be administered was determined for each batch. The rats were individually treated by single intragastric gavage of a predetermined volume of the substance to be administered. One hour after treatment, gastritis was induced with a 1ml/200g intragastric administration of 96% (*v/v*) ethanol. After 1h, the rats were anesthetized with ether and sacrificed by sectioning the carotid artery. The stomachs were removed, opened along their greater curvature and then cleaned with sodium chloride. The stomachs were examined by measuring lesions or ulcerous plaques using the score method [9].

Microscopic evaluation

Following the macroscopic examination, the stomach of each animal was immersed in 10% formaldehyde in order to keep the cells in a state as close to life as possible. Subsequently, a thin slice of each fixed stomach was removed and placed in previously labeled plastic cassettes, then dehydration was carried out manually by passing the samples contained in the cassettes through 6 ethanol baths as follows: 1 bath in 1 tank of 70 °C ethanol for 1 hour; 2 baths in 2 tanks of 95 °C ethanol for 1 h and 1 h 30; and finally, 3 baths in 3 tanks of absolute ethanol (1h, 1h30 and 2h). Once out of the absolute alcohol bath, the cassettes were left to soak up in two baths of xylene for 1h and 1h30 respectively to achieve clarification, then in a series of 3 tanks of molten paraffin (60 °C) for 1h, 1h30 and 2h respectively.

After soaking, the organ portions were placed in a determined orientation in stainless steel molds. The surfaces of the molds were covered by the base of the cassette and the molds were filled with melting paraffin. The whole was put to solidify on a refrigerating plate. At the end of this process, 5 µm thick sections were made using a Reichert-Jung 2030 brand microtome. Gelatinized water (1%), then collected on clean, previously labeled slides. The slides were then kept for 24 hours in an oven at 45°C before undergoing hematoxylin-eosin (HE) staining. Deparaffinization was carried out in 3 baths of xylene (5 minutes per bath), then rehydration by successive baths of alcohol of decreasing degrees (3 baths of absolute ethanol, 1 bath of 95° ethanol, and 1 bath of 70 °C ethanol) was performed and the slides were rinsed with distilled water for 5 minutes.

Staining of the nuclear components and cytoplasm was carried out using a 10-minute bath in Mayer's hematoxylin, then a 5-minute bath in 0.5% alcoholic eosin.

After staining, the slides were dehydrated in 3 baths of absolute ethanol and then cleared in 3 baths of xylene (5 minutes per bath). Once out of the xylene, a few drops of resin were placed on the sections, which were then covered with glass coverslips for observation under a microscope. The microscope (Scientico STM-50) used was equipped with a Celestron 44421 digital camera connected to a Toshiba tecra A9 computer. The Digital Microscope Suit 2.0 software was used to take the photomicrographs.

2.6 Acute oral toxicity test

The acute oral toxicity test was carried out on 12 nulliparous, non-pregnant, 10 weeks-old female rats, divided into 4 batches. The animals were fasted 18 h before the experiment. The first batch was the control and was administered distilled water, while the others 3 were administered at increasing concentration of *C. gabunensis* aqueous extract: 300, 2000 and 5000 mg/Kg, respectively [10]. After gavage, the animals were observed individually at least for the first 30 minutes and regularly for the first 24 hours after treatment with particular attention during the first 4 hours. The observation focused on various manifestations such as tremor, grooming, eye color, reaction to light and sound, appearance of secretion and excretion, mobility, neurovegetative reactions, etc. The animals were weighed every 5 days during the test period.

2.7 Statistical analysis

The average weight of the animals and the gastric lesion scores of each batch were analyzed using a one-factor ANOVA test, coupled with a Turkey test, carried out on the GraphPad Prism 8 software [11]. The data obtained were entered by group (column) in an analysis table of the GraphPad prism 8 software. *P < 0,05 [11].

3. RESULTS AND DISCUSSION

3.1 Phytochemical analysis

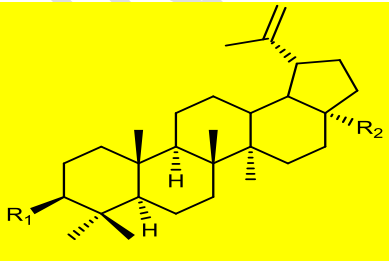
An aqueous extraction of *C. gabunensis* stem bark was performed, with a 4.1% yield.

The phytochemical screening of the extract revealed the presence of most of the secondary metabolites tested, namely alkaloids, flavonoids, phenols, coumarins, sterols, tannins, glycosides, terpenes and saponins. The test for anthraquinones was negative. These results are consistent with a previous study on the aqueous and methanolic extracts of this species [12]. The presence of these secondary metabolites (sterols, flavonoids, phenols, saponins) explains the gastroprotective effect [12, 13].

Identification of bioactive metabolites

Compounds **1** and **5** were identified by comparison with authentic samples available at our laboratory.

The chemical structures of compound 2 - 5 are:



	R_1	R_2
2	OH	CH_3
3	OH	COOH
4	OH	OH

Compound **2** was obtained in the form of white crystals in the mixture Hex-AcOEt (92.5:7.5). The melting temperature was determined to be between 215-216°C and the compound was soluble in chloroform. The Liebermann-Burchard test was positive, exhibiting a red color that turned purple, characteristic of triterpenes [14].

The ^{1}H NMR spectrum of **2** (**Figure 1**) showed the following: 2 singlets of one proton each at δ_H 4.62 and 4.73 characteristic of the terminal methylene protons at position C-29 of lup-20(29)-ene; a singlet of 3 protons at δ_H 1.73 attributable to vinyl methyl; a split doublet at δ_H 3.24 (1H, *J*=5.1; 10.8 Hz) attributable to the twin proton at the hydroxyl group in the C-3 position of a triterpene skeleton; 6 intense singlets of 3 protons each, at δH 0.81, 0.83, 0.87, 0.99, 1.01, 1.07, attributable to the 6 angular methyl groups.

The ^{13}C NMR spectrum (**Figure 2**) showed: a signal characteristic of the oxymethine carbon on triterpenes' C-3, at δ_C 79.0 in agreement with the biosynthesis; 2 characteristic signals of the C-20 and C-29 carbons at δ_C 151.0 and 109.4 lup-20(29)-enes, respectively. All of these spectroscopic data allowed the attribution of the structure of lupeol previously isolated from *Lonchocarpus sericeus* to Compound 2 [15].

Compound **3** was obtained in the form of a white powder in the hexane/ethyl acetate system (85:15). It was soluble in methanol, gave a purplish red color and responded positively to the Liebermann-Burchard test, characteristic of triterpenes.

It ^{1}H NMR spectrum (**Figure 3**) highlights a set of signals consisting, among all, of: two singlets of one proton each at δ_H 4.62 and 4.50 corresponding to olefinic protons; a terminal methylene and a singlet of three protons at δ_H 1.72 due to a methyl probably linked to a sp2 carbon, all characteristic of a propenyl group. A multiplet of one proton at δ_H 3.12 was also observed on this spectrum, attributable to the proton of a hydroxymethine, as well as five singlets of three protons each, between δ_H 0.97-0.75 corresponding to five angular methyl groups. These data are characteristic of the lupane series triterpenes [16].

The ^{13}C-NMR spectrum of **3** (**Figure 4**) shows thirty signals corresponding to the 30 carbon atoms in the molecular formula. These signals include: a signal at δ_C 177.6 characteristic of the carbonyl of carboxylic acids; 2 signals at δ_C 150.0 and 109.4 corresponding to the sp2 carbons of the terminal double bond C-20 and C-29; a signal at δ_C 79.2 characteristic of hydroxymethine at C-3 of triterpenes. The rest of the signals appearing in strong fields between δ_C 56.2 and 15.1 correspond to sp3 hybridized carbons. Comparing these results with the documented data enabled us to attribute to compound **3** the structure of betulinic acid [17].

Compound **4** was obtained in the Hex/AcOEt (80:20) system in the form of white crystals and is soluble in chloroform. The melting temperature is between 237-239°C and the compound reacts positively to the Liebermann-Burchard test, giving a purplish-red coloration, indicating the presence of a triterpene-like skeleton. The ^{13}C NMR spectrum (125 MHz; $CDCl_3$; Figure 4) showed the signals at δ_C 109.7 and 150.5, characteristic of the ethylenic carbons of the exocyclic double bond of a lup-20(29)-ene26-type skeleton [16]. The ^{1}H NMR spectrum (Figure 3) showed the following: a signal at δ_C 79.0 attributable to the hydroxylated carbon in position 3 of the triterpenes, in agreement with the biosynthesis; a signal at δ_C 60.5 attributable to the carbon of an oxymethylene. These results compared with

the data in the literature made it possible to attribute to compound 3 the structure of lup-20(29)-ene-3β,28-diol commonly called betulin and recently isolated from *Diospyros rubra* [18].

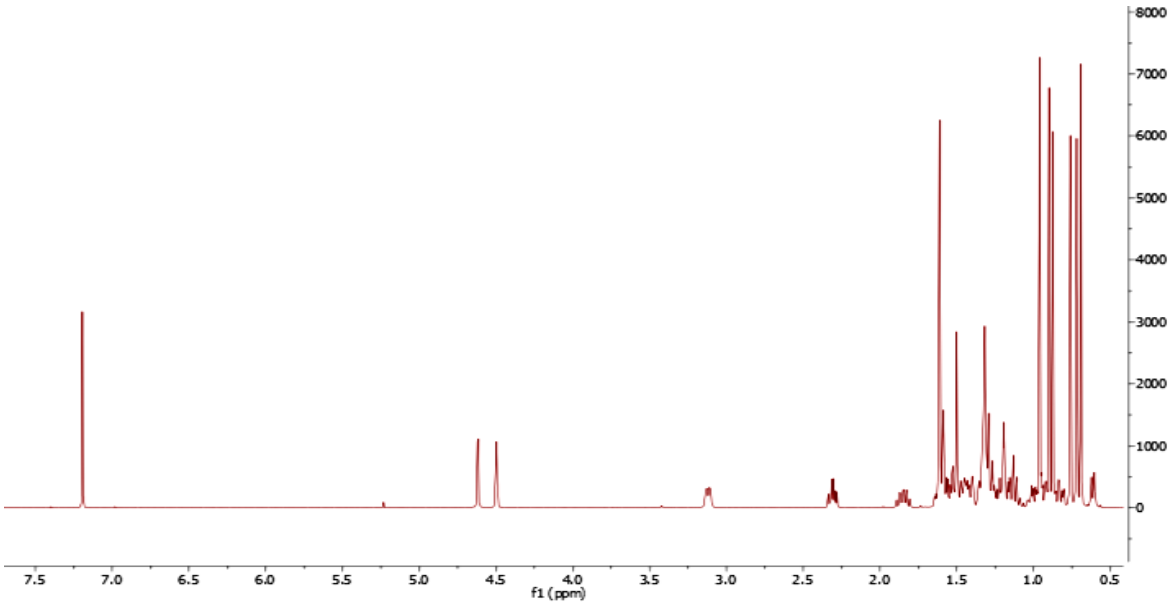


Figure 1: ^{1}H NMR spectra ($CDCl_3$, 400 MHz) of compound 2

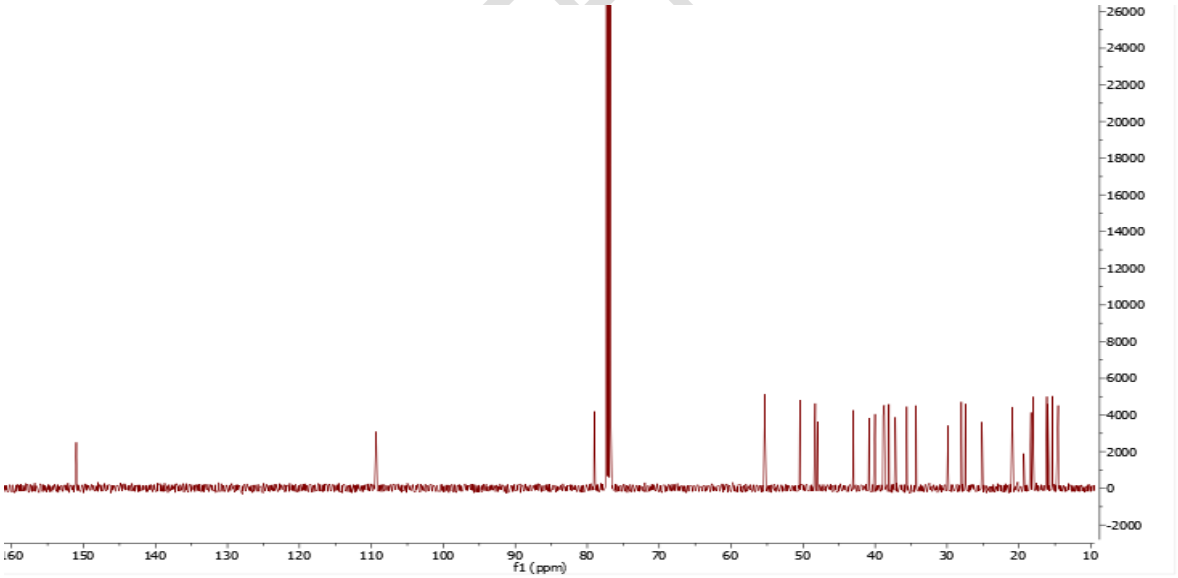


Figure 2: ^{13}C NMR spectra ($CDCl_3$, 100 MHz) of compound 2

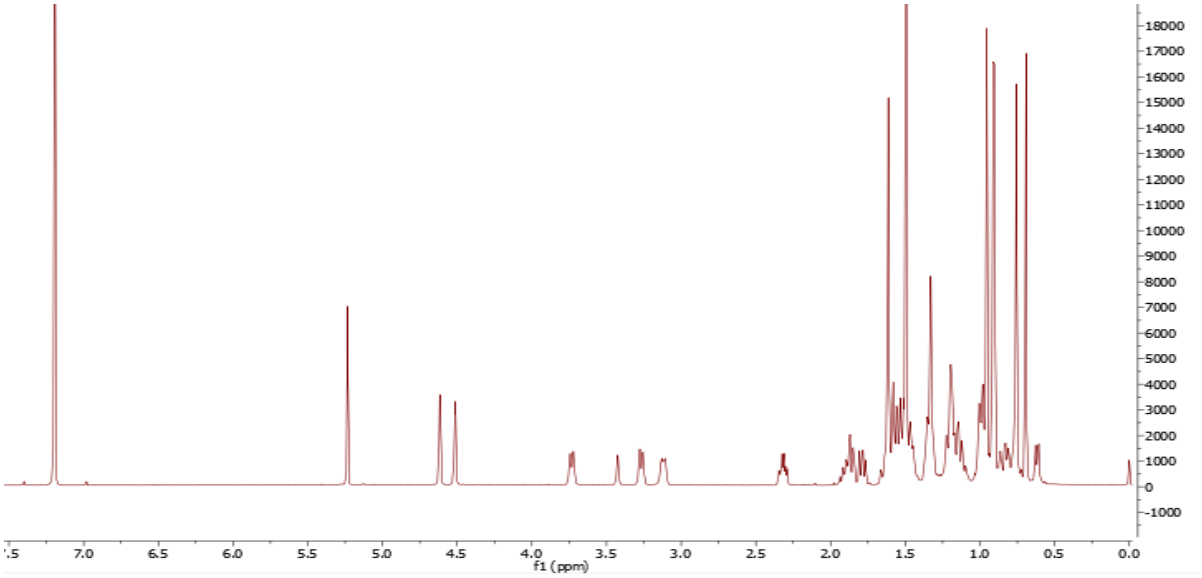


Figure 3: ^{1}H NMR spectra ($CDCl_3$, 500MHz) of compound 3

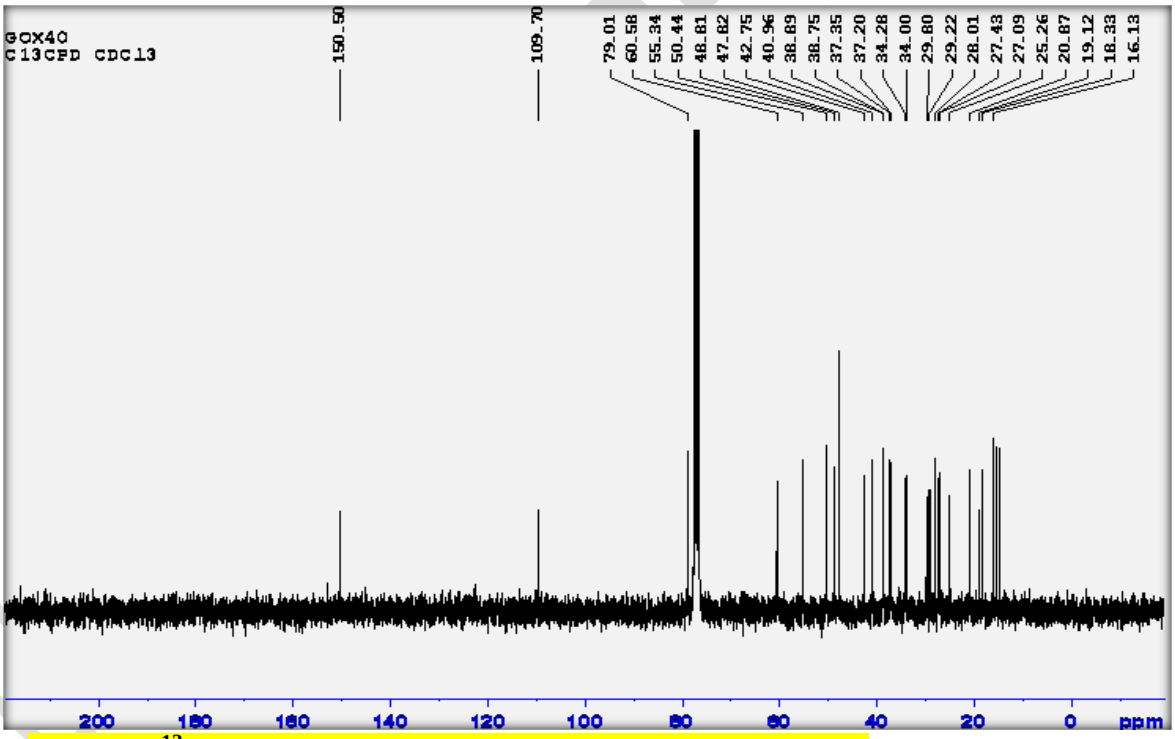


Figure 4: ^{13}C NMR spectra ($CDCl_3$, 125 MHz) of compound 3

3.2 Gastroprotective activity

Macroscopic evaluation

Figure 5 presents the macroscopic appearance of the stomachs of the different batches of Wistar rats and highlights the macroscopic alterations of the stomachs of the different batches in comparison with the neutral control batch (N).

Ethanol administered orally to rats caused lesions on the gastric mucosa in the negative control group (Neg) unlike the neutral control treated with distilled water (N), where gastric mucosa showed no lesions (**Figure 5**).

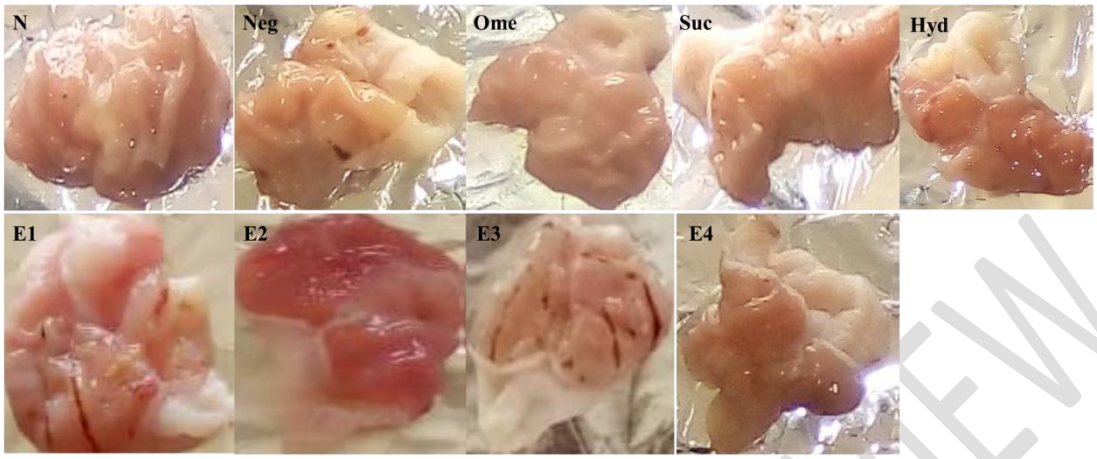


Figure 5: Macroscopic aspect of the stomachs of Wistar rats pre-treated with the aqueous extract of *C. gabunensis*. (N) Neutral Control, (Neg) Negative Control, (Ome) Omeprazole, (Suc) Sucralfate, (Hyd) Aluminum Magnesium Hydroxide, (E1) Extract 50mg/Kg, (E2) Extract 100 mg/Kg, (E3) Extract 200 mg/Kg, (E4) Extract 400 mg/Kg.

The pretreatment of rats with increasing doses of the extract (50, 100, 200, 400 mg/Kg) before the induction of gastritis with ethanol, significantly reduced (P <0.05) the average scores of gastric lesions to E1 (1.8); E2 (1.2); E3 (1.4) and E4 (3.2), compared to Neg (6) group (**Figure 6**).

For tested group, the greatest gastric lesions was obtained for the batch treated with E4 at the dose of 400 mg/Kg (score 3.2), while for the references drugs, aluminum and magnesium hydroxides (score 2.6) at a dose of 50 mg/Kg presented the highest lesion score (**Figure 6**).

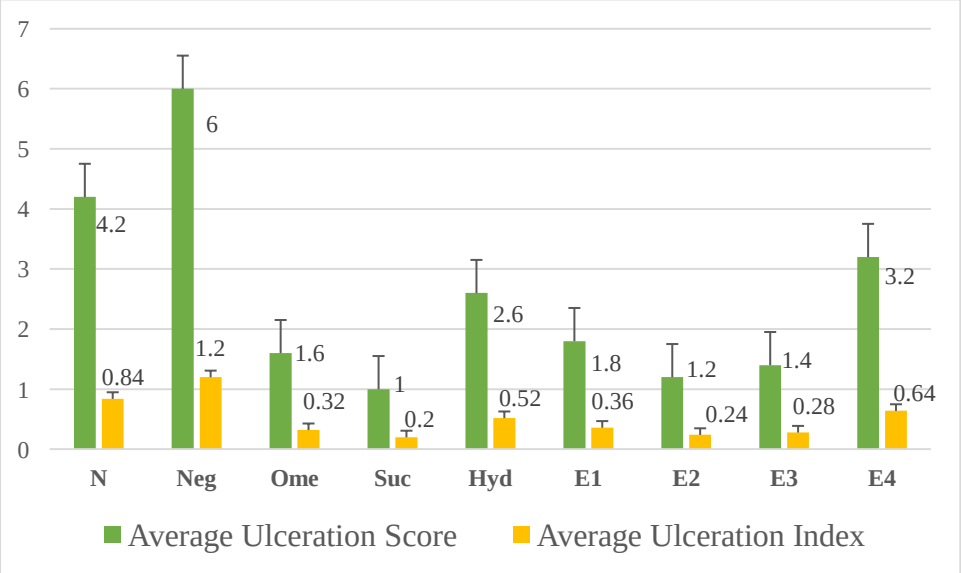


Figure 6: Average Ulceration Scores and Ulceration Index

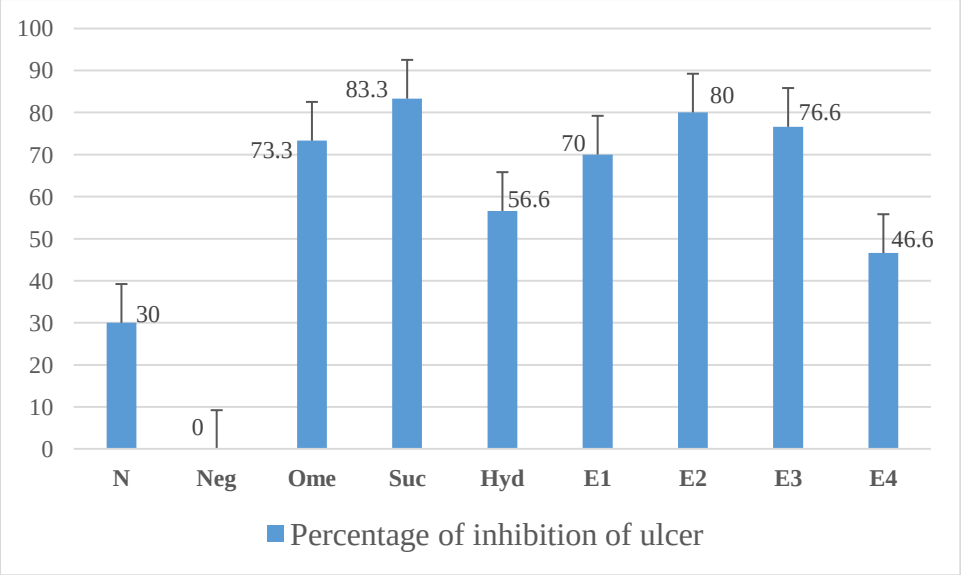


Figure 7: Percentage of inhibition of ulcer

All these scores were lower than Negative control group (score 6) and Neutral control group (score 4.2). The strongest gastroprotective activity was observed for E2 (80%), followed by E3 (76.6%) for tested groups, while sucralfate presented the highest gatroprotective activity for referential drugs (**Figure 7**).

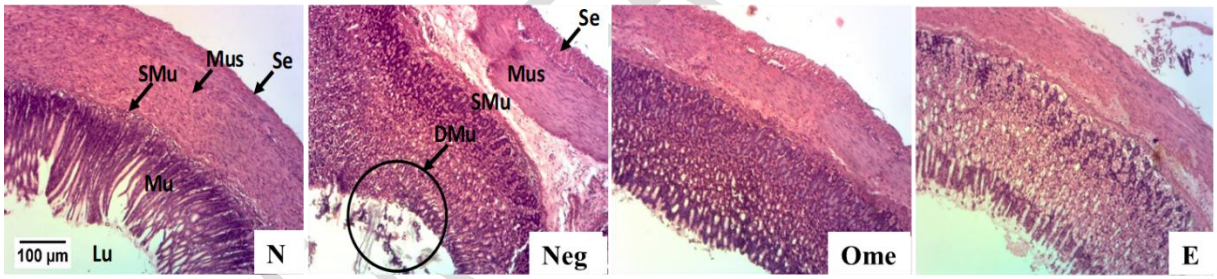


Figure 8: Micrographs of the stomach (X40) with hematoxylin-eosin staining. N = normal control; **Neg** = Negative control; **Ome** = Omeprazole; **E** = Extract at a dose of 100 mg/kg; Stomach: **Lu** = Light; **Mu** = Mucosa; **SMu** = Submucosa; **Mus** = muscular; **Se** = Serous; **DMu** = Destruction of the mucosa.

Microscopic evaluation

The ethanol negative control batch shows glandular loss of the gastric epithelium with cellular necrosis and the presence of an inflammatory infiltrate between the submucosa and the muscularis. These observations are explained by the fact that ethanol rapidly penetrates the gastric mucosa and leads to the formation of reactive oxygen species (ROS), the rupture of endogenous mucus and the reduction of endogenous prostaglandin production [19].

The positive control batch treated with omeprazole did not show any remarkable disturbance of the gastric epithelium. The presence of a weak layer of mucus in the gastric lumen and adaptive hyperplasia were observed, which resulted in a regeneration of the cells of the gastric mucosa [20]. The E2 extract probably stimulated mucus secretion [13]. Cellular

hyperplasia and hypertrophy were noted, suggesting that the aqueous extract of *C. gabunensis* prevented the back diffusion of H^+ ions by stimulating the secretion of protective mucus [20].

3.3 Acute oral toxicity test

The acute toxicity study looked at physiological changes in Wistar-strain albino rats. The aqueous extract of *Cylicodiscus gabunensis* trunk bark was administered into intra gastric of the rats at the doses of 300, 2000 et 5000 mg/Kg and observed during the first 2h, then at 4h, 8h and 15 days after administration. At the end of the fifteen days of observation, no anomalies were found in the parameters studied. In addition, no deaths were being recorded. The LD50 is therefore greater than 5000 mg/kg (**Table 1**).

Behavioral studies

The Table 1 bellow shows physical parameters observed 4 hours after intra gastric administration of the extract at increasing doses:

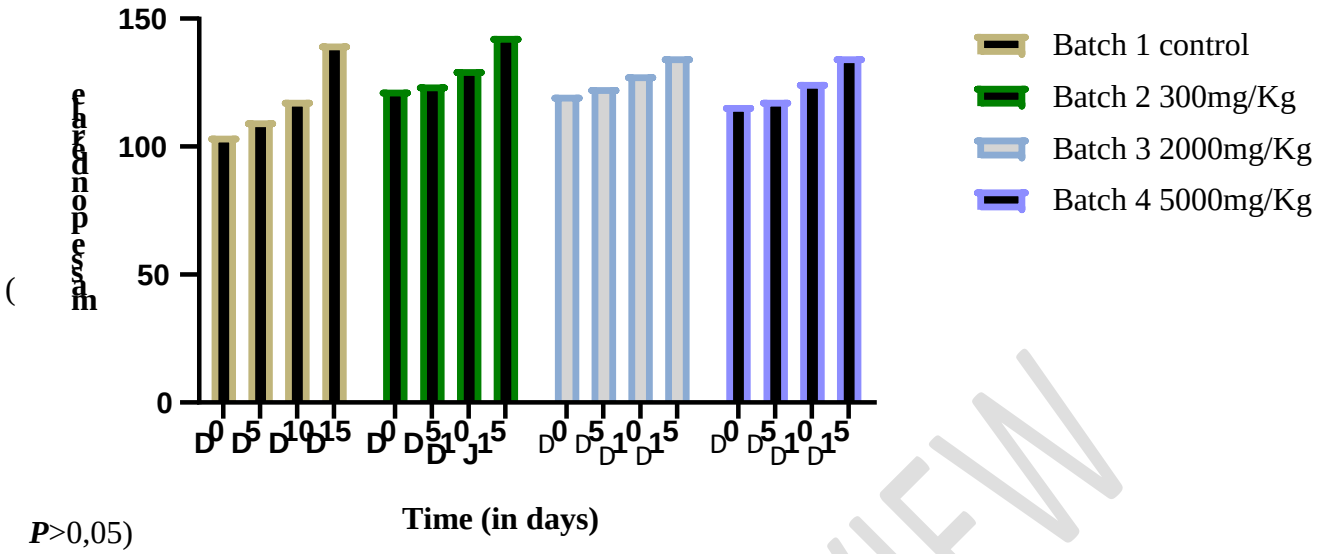
Table 1: Observation of physiological parameters of rats

Groups Criteria	Batch 1 control	Bath 2 300 mg/Kg	Batch 3 2000 mg/Kg	Batch 4 5000 mg/Kg
Toilet	-	+	+	+
Grooming	N	N	N	N
Tremor	-	+	-	-
Mobility	N	M	M	M
Sound and light reactions	N	N	M	M
Eye color	-	+	+	+
Secretion and excretion	N	N	N	N
Neurovegetative reactions	N	M	M	M
Gathering	-	+	+	+
Painful sensation	N	M	M	M

(-): Absent (+): Present N: Normal M: Modified

Effects of the aqueous extract *Cylicodiscus gabunensis* trunk bark on the evolution of average body masses of rats during the study of acute oral toxicity

The body masses of the animals were generally increasing during the acute oral toxicity study regardless of the batch chosen. Those of the control batch increased from 105 g on D0 to 140 g on D15, an increase of 35 g. Those of batch 2 increased from 125 g on D0 to 140 g on D15, an increase of 15 g. Rats in the third batch went from 120 g on D0 to 130 g on D15, an increase of 20 g. Finally, batch 4 went from 110 g on D0 to 130 g on D15, an increase of 20 g. Comparing the body masses of the same batch on D0 and D15 using the ANOVA test revealed very significant differences regardless of the batch chosen (**Figure 9**).



P>0,05)

Figure 9: Evolution of the body masses of batches of rats

TYPE OF ARTICLE: 1 Original Research Article

4. CONCLUSION

The main objective of this work was to study gastroprotective activity and acute toxicity of the aqueous extract of *Cylicodiscus gabunensis* Harm. (Fabaceae) trunk bark.

At the end of the study, the mass of the extract obtained after drying in an oven at 60 °C was 20,5 g with an extraction yield of 4.1%. Tests for alkaloids, flavonoids, phenols, coumarins, sterols, tannins, glycosides, terpenes and saponins were found to be positive with the extract, while that for anthraquinones was negative. *C. gabunensis* did not present any acute toxicity at the doses tested. The gastroprotective effect was observed for all doses, with a maximum inhibition of the ulcer at 63.94% at the dose of 400 mg/kg of extract. Five compounds were isolated and characterized: *β*-sitosterol (**1**), lupeol (**2**), betulinic acid (**3**), betulin (**4**) and 3-*O*-*β*-D- sitosterol glucopyranoside (**5**). *β*-sitosterol

The secondary metabolites named sterols and pentacyclic triterpenes present in this aqueous extract would justify this activity.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that they have no known competing financial interests OR non-financial interests OR personal relationships that could have appeared to influence the work reported in this paper.

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