Gastroprotective activity and acute toxicity of the aqueous extract of the trunk bark of *Cylicodiscus gabunensis* Harm. (Fabaceae)

Abstract

The aim of this work is the evaluation of 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, using spectral methods (MS, ^{1}H NMR, ^{13}C NMR, HSQC, and HMBC) we were able to obtain the structures of the isolated compounds.

At the end of this work, it appears that *C. gabunensis* does not present any acute toxicity at the doses tested. In general, 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. Eleven 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* is not toxic.

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

Introduction

In Africa, many plants are used by healers, medicinal plants sellers and people to relieve stomach pain (Karima et *al.*, 2016). In a context where 80% of the population uses traditional medicine to cure diseases, *Cylicodiscus gabunensis* (*Fabaceae*), whose bark is used in traditional medicine to treat various ailments such as malaria, measles, varicella, bacterial infections, rheumatism, migraine and stomach pain (Ngoule et *al.*, 2015). 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 has not been reported. To achieve this, we started with a gastroprotective activity test on stomachs pre-treated with different doses of aqueous extract of *C. gabunensis*, compared to an antacid sold in pharmacies. Thereafter, a phytochemical screening and an evaluation of the acute toxicity of this extract were carried out. Finally, the bioactive compounds were isolated and their chemical structures characterized.

Materials and methods

Plant material

The plant material 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.

Animal material

The animal material consisted of 57 male and female rats 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.

Extraction

The aqueous extract was obtained through a decoction of air-dried and powdered stem bark of *C. gabunensis* on distilled water (Ferreira et *al.*, 2020)

Gastroprotective activity

The test was conducted on 45 male and female 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 (Abebaw et *al.*, 2017)

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 (Robert et *al.*, 1983).

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° ethanol for 1 hour; 2 baths in 2 tanks of 95° 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°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.

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 other 3 were administered increasing concentration of *C. gabunensis* aqueous extract: 300, 2000 and 5000 mg/Kg, respectively [84]. 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, tremor, grooming, crowding, reaction to light and sound, appearance of secretion and excretion, mobility, coat, neurovegetative reactions. The animals were weighed every 5 days during the test period.

Phytochemical analysis

Phytochemical analysis of *C. gabunensis* aqueous extract was carried out using color reactions (Harborne, 1998) and the compounds were isolated using chromatography techniques (TLC, CC). The elucidation of the structures was made possible using mass spectroscopy and 1D (^{13}C and ^{1}H), 2D (HSQC, HMBC, COZY) NMR techniques.

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 (Nabavizadeh, 2011). The data obtained were entered by group (column) in an analysis table of the GraphPad prism 8 software. *P < 0,05 (Nabavizadeh, 2011).

Results and Discussion

Gastroprotective activity

Macroscopic evaluation

The figure 1 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).

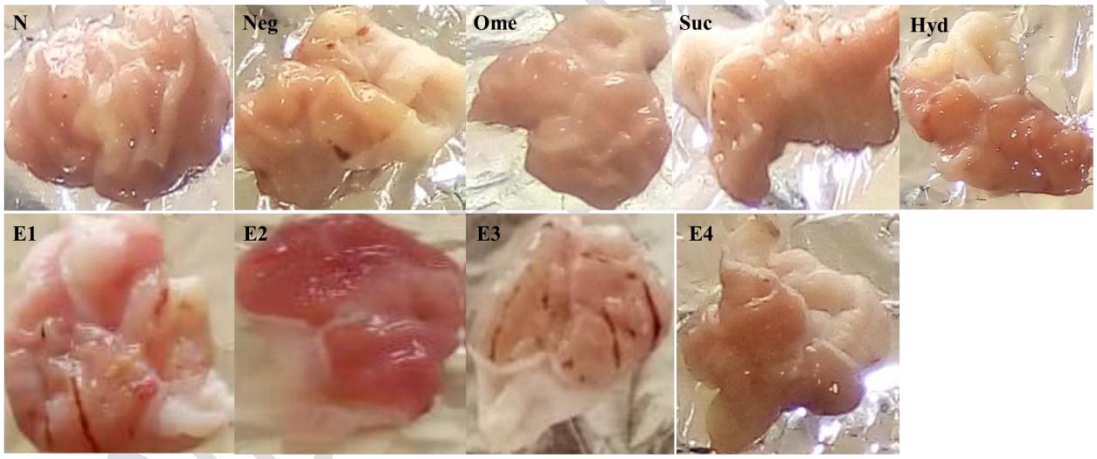


Figure 1: Macroscopic aspect of the stomachs of Wistar rats pre-treated with the aqueous extract of *C. gabunensis*. (N) Neutral Control, (Neg) Negative Control, (Ome) Omeprazole, (Suc) Sucralfate, (Hyd) Aluminum Magnesium Hydroxide, (E1) Extract 50mg/Kg, (E2) Extract 100mg/Kg, (E3) Extract 200mg/Kg, (E4) Extract 400mg/Kg.

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 1**).

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 2**).

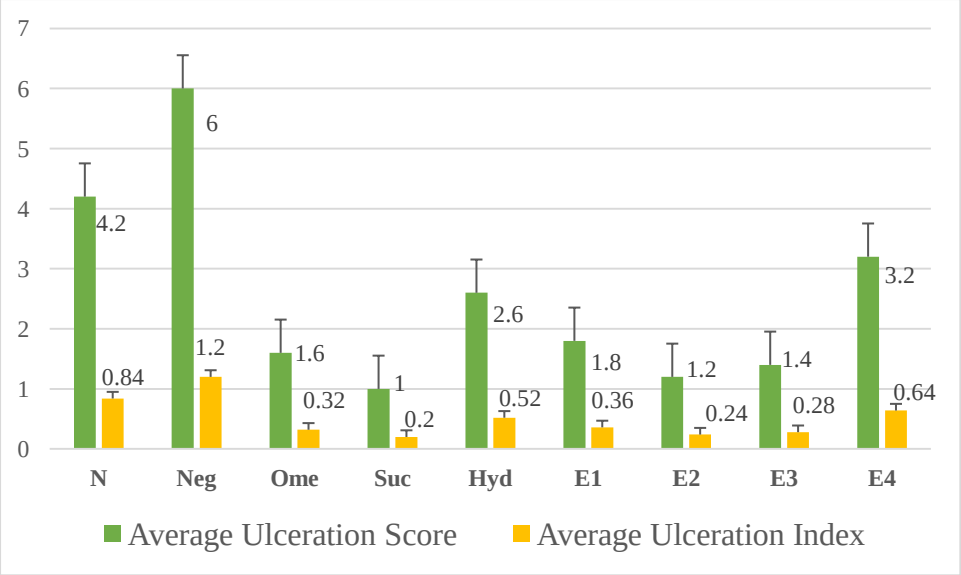


Figure 2: Average Ulceration Scores and Ulceration Index

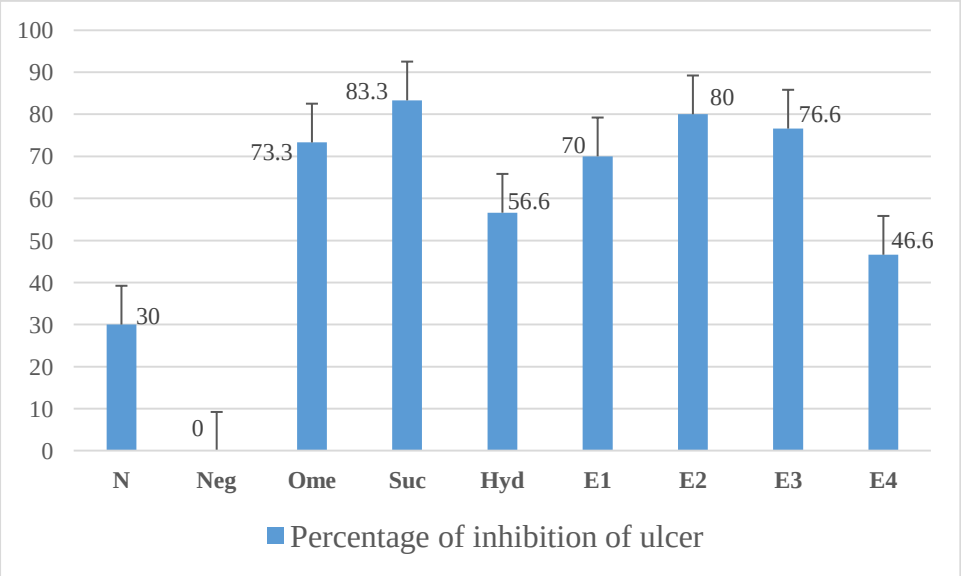


Figure 3: Percentage of inhibition of ulcer

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 2**). 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 3**).

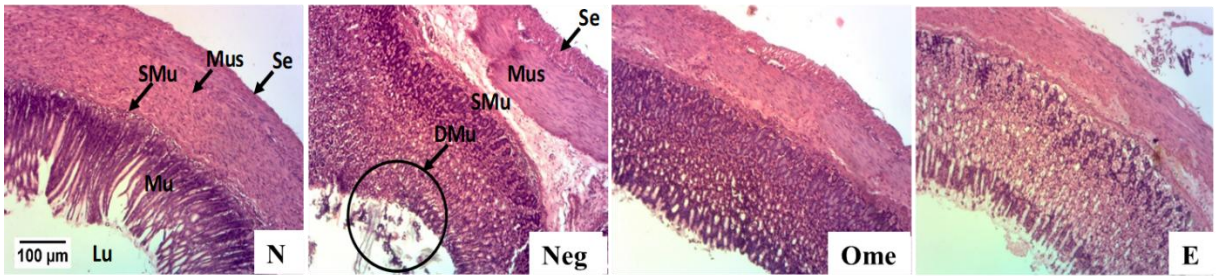


Figure 4: Micrographs of the stomach (X40) with hematoxylin-eosin staining. N = normal control; **Neg** = Negative control; **Ome** = Omeprazole; **E** = Extract at a dose of 100 mg/kg; Stomach: **Lu** = Light; **Mu** = Mucosa; **SMu** = Submucosa; **Mus** = muscular; **Se** = Serous; **DMu** = Destruction 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 (Szaingurten-Solodkin et *al.*, 2009).

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 (Driman et *al.* 1996). The E2 extract probably stimulated mucus secretion (Tovey et *al.*, 2011). 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 (Driman et *al.*, 1996).

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 (Kouitcheu Mabeku et *al.*, 2006). The presence of these secondary metabolites (sterols, flavonoids, phenols, saponins) explains the gastroprotective effect (Tovey et *al.*, 2011; Kouitcheu Mabeku et *al.*, 2006).

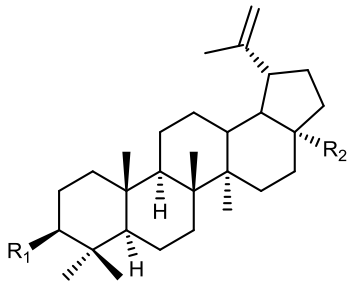
Identification of bioactive metabolites

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

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 is

soluble in chloroform. The Liebermann-Burchard test was positive, exhibiting a red color that turned purple, characteristic of triterpenes (Oluwatoyin et *al*., 2012).

The ^{1}H NMR spectrum of **2** (**Figure 5**) 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 (**Fig. 6**) 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 (Çulhaoglu et *al.*, 2015).



	R_1	R_2
2	OH	CH_3
3	OH	COOH
4	OH	OH

Figure 5 Chemical structures of compound 2-4

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 7**) 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 (Mahato and Kundu, 1994).

The ^{13}C-NMR spectrum of **3** (**Figure 8**) 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 (Tangmouo et *al.*, 2005).

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 8) 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 (Mahato and Kundu, 1994). The ^1H NMR spectrum (Figure 7) 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* (Prachayasittikul et *al*., 2010).

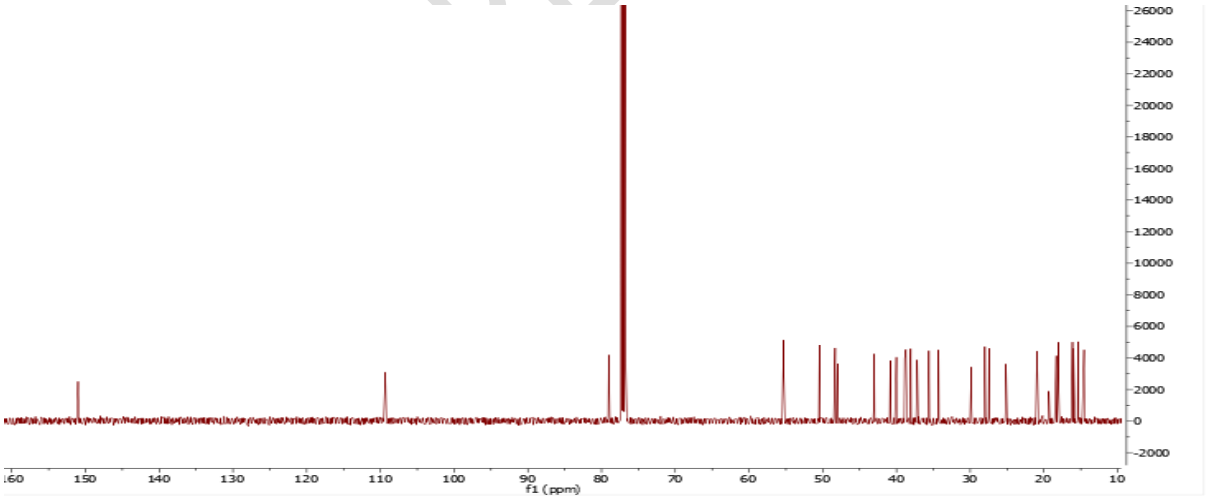


Figure 1: 13C NMR spectra ($CDCl_3$, 100 MHz) of compound 2

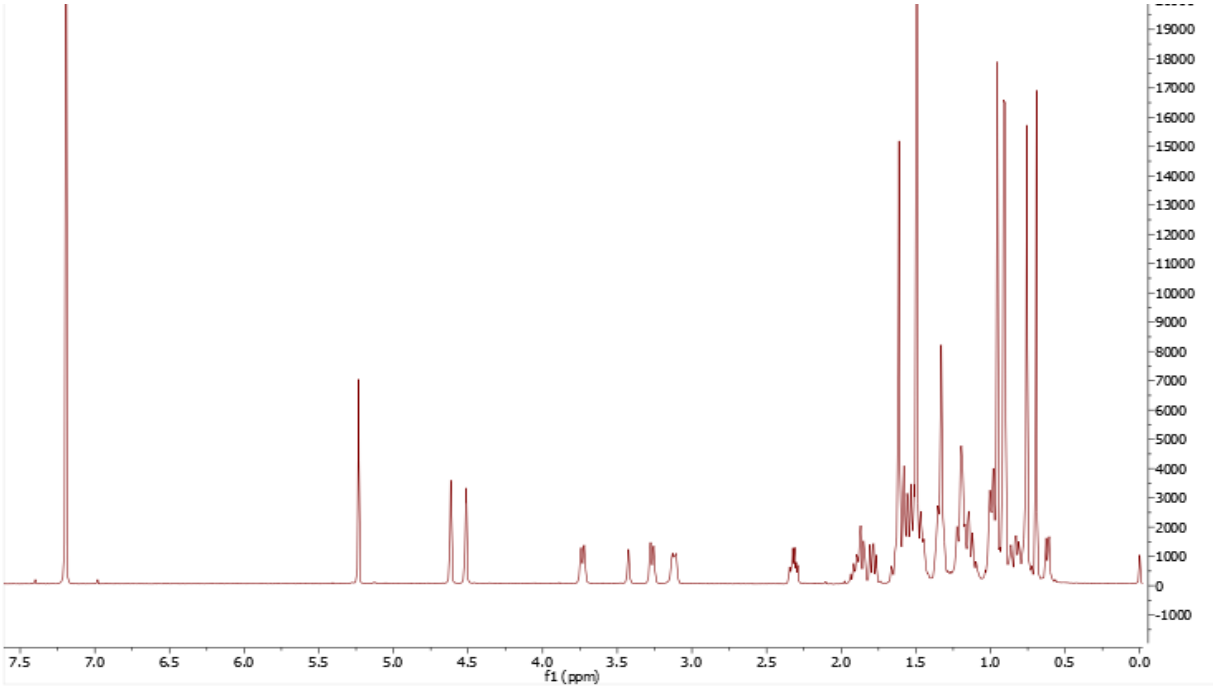


Figure 2: ^{1}H NMR spectra ($CDCl_3$, 500MHz) of compound 3

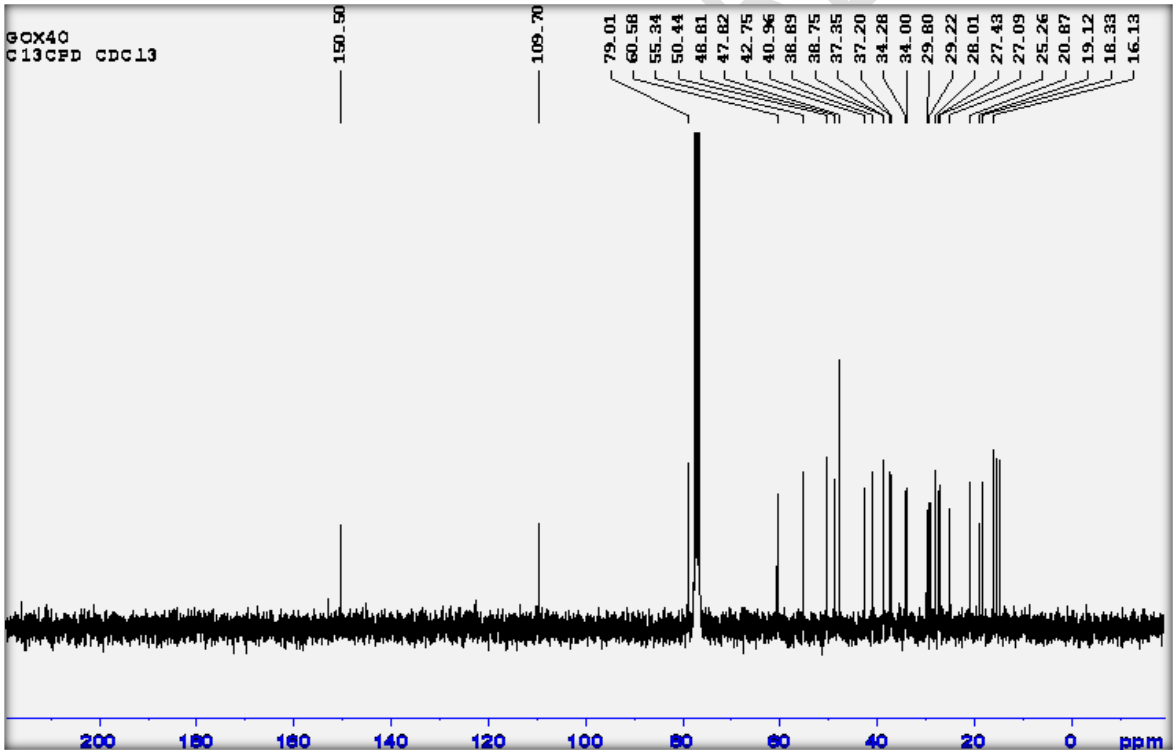


Figure 3: ^{13}C NMR spectra ($CDCl_3$, 125 MHz) of compound 3

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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