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EVALUATION, ISOLATION AND CHARACTERIZATION OF ANTIULCER PRINCIPLE(S) OF ETHANOL LEAF EXTRACT OF *PIPER GUINEENSE* ON INDOMETHACIN-INDUCED ULCER IN WISTAR RATS

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ABSTRACT
Peptic ulcer disease is a serious health challenge in the world. Synthetic drugs employed for treatment of the disease confer side effects and treatment relapse. Alternative therapies from plants are receiving a tremendous attention. *Piper guineense*, an African plant, is claimed by tradomedicine as a remedy for ulcer. However, the active principles responsible for bioactivities of many African plants have not been characterized, and this is the case for *Piper guineense*. The aim of this study was to evaluate, isolate and characterize the antiulcer principles of ethanol leaf extracts of *Piper guineense* on indomethacin-induced ulcer in wistar rats. This was done by extraction and fractionation of the plant material that yielded five pooled-fractions, labeled PF-1 to PF-5. Ethanol extract (EE) and fraction extracts were orally administered at 400mg/kg to six test groups of adult wistar rats. The positive and negative control groups were respectively administered with 100mg/kg cimetidine and 5ml/kg Tween 80 orally. Animals were sacrificed under anesthesia. Assessment of antiulcer activity identified PF-4 as the most bioactive extract, having produced the highest ulcer inhibition of 33.65%. Further purification of PF-4 by preparative thin-layer chromatography yielded three sub-pooled fractions (SPF-4:1, SPF-4:2, SPF-4:3), with SPF-4:2 identified as the most bioactive sub-pooled fraction, having produced the highest ulcer inhibition of 44.28%. Characterization of SPF-4:2 by GC-MS analysis confirmed the presence of 9,12,Octadecanoic acid methylester and Piperine. This study therefore concludes that these compounds are responsible for antiulcer activity of *Piper guineense* leaf.

KEYWORDS: Extraction, Fractionation, Bioactive principles, GC-MS analysis.

INTRODUCTION
Peptic ulceration is among the most prevalent gastrointestinal disorders characterized by pepsin and gastric acid mediated mucosal damage, as a result of imbalance between defensive and offensive processes.[1] Various synthetic drugs presently available for ulcer treatment have their characteristic side effects complications that hinder their frequent use, thereby limiting their clinical effectiveness[2], and prompting a search for non-toxic, accessible and affordable antiulcer medication. Natural products especially plant derived chemicals are considered as promising source for the development of new agent with safe therapeutic window.[3] Traditional medicine using plants have been shown to be successful in the treatment of gastrointestinal disorders including peptic ulcer disease.[4]

Piper guineense, is a spice plant from the family Piperaceae and from genus piper. It is commonly found in Nigeria, with local names "uziza" in Igbo, "monsoro" in Hausa and "iyeree" in Yoruba. It is widely used in traditional medicine for treatment of various forms of stomach disorders including peptic ulcer. It is a perennial plant that is characterized by heart-shaped leaves which are pale greenish in color when fresh and darker green when frozen or dried.[5] Various studies have been done on *Piper guineense* to determine its pharmacological and therapeutic properties such as antioxidant[6,7], larvicidal[8], hepatoprotective[9], hypolipidemic[10], fertility[11], aphrodisiac[12] and antibacterial.[13] However, there is still lack of comprehensive studies with regard to the active principles responsible for antiulcer activity of *Piper guineense* leaf. Hence, this study was carried out to identify bioactive principles of the ethanol leaf extract of

Piper guineense using Gas chromatography-Mass spectrometry technique with a view to justify its traditional claim as ulcer remedy.

MATERIALS AND METHODS

Collection and identification of plant material.

Piper guineense matured fresh leaves were collected from a farm land in Owerri, Imo State, Nigeria. The leaves were confirmed and deposited as voucher specimen in the herbarium of Department of Plant Science and Biotechnology, University of Port Harcourt with Herbarium Number designated as UPH/P/251

Study area

The animal study was conducted in the Animal Facility Centre, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Madonna University, Nigeria, Elele Campus

Animal ethics approval

Full animal ethics approval (Reference number: MAU/SREC/A/17) was obtained from University Senate Research and Ethics Committee of Madonna University, Nigeria, prior to the commencement of the study.

Experimental animals

The following experimental animals were used in the study.

- Healthy adult wistar rats of 12-15 weeks old that weighed between 170-190g.
- Healthy adult wistar mice of 12-15 weeks old that weighed between 20-22g.

The breeding of these animals was done at room temperature in Animal Facility Centre of Madonna University, Nigeria. Clean drinking tap water was supplied to the animals, and were fed freely with commercial poultry growers feed (Top feeds ^{R}, Nigeria). The handling of the animals was done in compliance to the highest standard for the humane and compassionate use of animals in biomedical research as published by.[14]

3.1.5 Drugs and Chemicals

Drugs and chemicals used in the study included: Indomethacin (Fine Chemicals, Mumbai India), Cimetidine (Cadila Pharmaceuticals Pvt Ltd, India), 96% Ethanol (Gungsdong Guandgua Chemical Factory ,China), Chloroform (Super Tek Chemicals, India), n-Hexane (Sigma Aldrich Chemie, Germany), Ethylacetate (Rankem, Mumbai, India), Dragendoff's reagent (Super Tek Chemicals, Germany), Tween 80 (3%v/v) (Super Tek Chemicals Germany),

Processing and extraction of plant material.

About three kilograms of matured fresh leaves of *Piper guineense* were properly washed and adequately air-dried for two weeks at room temperature. The dried leaves were ground into coarse powder and about a half of a kilogram (500g) of the powdered material was macerated in two litres of ethanol (80%) at room temperature. The macerated material was occasionally agitated six hourly for 72 hours, after which the filtration through Whatman filter paper was done to separate the filtrate from the marc. The marc was re-macerated twice and re-filtered twice. All the obtained filtrates were combined in a previously weighed clean empty beaker. The beaker and its content were placed in an oven at 40^{o}C to evaporate the ethanol

Determination of percent yield of ethanol-extracted residue of *Piper guineense* leaves.

The beaker and its dried content were re-weighed. Percent yield was determined using the formula proposed by[15] as the ratio of weight(gram) of extracted residue to weight(gram) of macerated powdered material multiply by hundred.

Chromatographic fractionation of ethanol-extracted residue

Eight solvent systems were prepared in different ratios, and using the procedure demonstrated by[16], preliminary thin-layer chromatography (TLC) was conducted on each of the solvent systems to determine the one that would give the best resolution. The solvent system that gave the best resolution was used in subsequent column chromatography and TLC analysis.

Fractionation of the extracted-residue was conducted using column chromatography in the solvent system that gave the best resolution. In a procedure demonstrated by[17], the column initially packed with sufficient quantity of wet silica gel (F_{254}), was allowed to for 24 hours to stabilize. A 10g amount of crude extract dissolved in 10ml ethanol, was placed on the column and then continuously eluted with the solvent system (Chloroform /Ethylacetate/Ethanol; 7:2:1) that gave the best resolution in the preliminary thin-layer chromatography.Seventeen-10ml fractions were collected and their TLC mobility (R_f values) was calculated as ratio of distance(cm) travelled by the spot from starting point in TLC to distance(cm) travelled by the solvent front in TLC

Pooling, Labeling and storage of the plant extracts

Fractions that showed similar R_f value and color reaction were pooled together in clean containers, evaporated to dryness in an oven at 40^{o}C and labeled appropriately as: ethanol extract (EE); pooled fraction-1 (PF-1); pooled fraction-2 (PF-2); pooled fraction-3 (PF-3); pooled fraction-4 (PF-4) and pooled fraction-5 (PF-5).The labeled containers were kept in the refrigerator until when needed.

Acute toxicity (LD_{50}) determination.

Both the ethanol and fraction extracts were used for acute toxicity test, to establish doses that could be safe in subsequent whole animal experiment. A method demonstrated by[18] was employed which involved two phases that used minimal number of thirteen (13) animals.

Phase I: In this phase, nine (9) healthy adult mice were used, divided into three groups of three animals per group. Doses of 10,100 and 1000mg/kg body weight of the extracts were orally administered to group one, two and three animals respectively. Then animals were monitored for signs of toxicity and death within 24 hours.

Phase II: This phase was conducted using the rest of the animals (i.e four healthy mice), which were divided into four groups of one animal per group. From the result obtained in phase I, the animal groups (one, two, three and four) were given 1000, 1600, 2900 and 5000mg/kg body weight of the extracts respectively and were monitored for 24 hours for signs of toxicity and death.

Experimental protocol.

Healthy adult wistar rats of 12-15 weeks old that weighed between 170-190g, were randomized into six test groups (labeled A to F) and two control (positive and negative) groups (labeled G and H respectively). Each group consisted of seven animals per group. The animals were fasted of food for 24 hours before commencement of experiment but had free access to water until two hours prior to experiment. Drug and extracts were orally administered through intragastric tube. Each group of animals was treated in following way.

Group A: Each rat was given 400mg/kg PF-1 orally as single dose.
Group B: Each rat was given 400mg/kg PF-2 orally as single dose.
Group C: Each rat was given 400mg/kg PF-3 orally as single dose.
Group D: Each rat was given 400mg/kg PF-4 orally as single dose.
Group E: Each rat was given 400mg/kg PF-5 orally as single dose.
Group F: Each rat was given 400mg/kg EE orally as single dose.
Group G (positive control): Each rat was given 100mg/kg cimetidine orally as single dose.
Group H (negative control): Each rat was given 5ml/kg 3% v/v Tween 80 orally as single dose.

The administered dose of extracts was safe, as established in acute toxicity study .Thirty minutes after respective treatment of animals in each group as outlined in the experimental protocol, ulcer was induced via intragastric administration of 100mg/kg indomethacin.[19] Six hours following the induction of ulcer, the animals were sacrificed under anesthesia and their stomachs cut open along greater curvature, rinsed under tap water, and pinned flat on a board.

Macroscopic assessment of stomach

The stomachs were assessed for ulcer formation using hand lens (magnification: x10). The number of ulcers were counted and scoring made as described by[20] using the following.

Normal colored stomach	0
Red coloration	0.5
Spot ulcer	1
Hemorrhagic streak	1.5
Deep ulcer	2
Perforation	3

Ulcer index and percent inhibition were calculated using equations proposed by.[21]

$$\text{Ulcer Index} = \frac{\text{total ulcer score}}{\text{number of animals ulcerated}}$$

$$\% \text{ Inhibition} = \frac{\text{ulcer index}_{(\text{control negative})} - \text{ulcer index}_{(\text{test group})}}{\text{ulcer index}_{\text{control negative}} \text{ group}} \times 100$$

Isolation and purification of pure compound from the most active fraction extract

The percentage inhibition of ulcer for each pooled fraction extracts was calculated and the pooled fraction with highest percentage of inhibition of ulcer was selected for purification.

Purification of the extract was done using preparative TLC as described by.[16] In this procedure, TLC plates were prepared and then activated in the oven at 110^0C for one hour before they were used. A five gram (5g) portion of the active fraction (ie the pooled fraction with highest percentage of ulcer inhibition) was dissolved in 10ml ethanol (80%), and 1ml syringe was used to streak the solution on the activated TLC plates from one side to the other in a straight band form, then the streak was allowed to dry on the plates. Chromatographic tank was saturated up to two centimeters (2cm) with solvent system that gave best resolution in the preliminary TLC. Two plates were placed into the tank and each was made to incline at an angle of 30^0 from the edge of the tank. The solvent front was allowed to run a distance of 18cm starting from the streaked end after which the plates were removed and air-dried. Each plate was placed under UV lamp at 254 and 365nm wavelength in a dark room to observe the fluoresced zones. The fluoresced zones were marked with a pin. The procedure was repeated several times on many plates. The separated zones/bands were scraped into different centrifuge tubes using a spatula, 5ml ethanol (80%) was added to dissolve the active principle and then centrifuged at 3,000 rpm for ten minutes to ensure proper separation of the eluents from the adsorbent (silica gel). Ten (10) ethanol solutions (sub-fractions) of the eluents were collected, labeled E_1 to E_{10}, subjected to TLC and pooled together into three sub-fraction based on their TLC mobility (*Rf value*) and color reaction. Each sub-pooled fractions was evaporated to dryness in a hot air oven (40^0C). The resulting material was weighed, put in sealed containers labeled as SPF-4:1, SPF-4:2 and SPF-4:3, which were stored in the refrigerator until when needed.

Determination of bioactivity of sub-pooled fractions on indomethacin-induced ulcer in wistar rats

Twenty-one (21) wistar rats of 12-15 weeks old that weighed between 170-190g were randomized into three

test groups (labeled: 1-3) of seven animals in a group. The animals in each group were treated as follows.
Group 1: Each animal received 400mg/kg SPF-4:1, orally as single dose
Group 2: Each animal received 400mg/kg SPF-4:2, orally as single dose
Group 3: Each animal received 400mg/kg SPF-4:3, orally as single dose
Thirty minutes after various treatment, ulcer was induced by oral administration of 100mg/kg indomethacin.[19] Six hours later, the animals were sacrificed under anesthesia and macroscopic assessment of the stomachs was done as explained above. The ulcer index and percent ulcer inhibition were calculated. The sub pooled fraction that gave highest percentage of ulcer inhibition was selected to have contained the active pure compound(s).

Removal (precipitation) of chlorophyll
This was done using the procedure by[22], to avoid interference chlorophyll with the GC-MS analysis and result.

GC-MS analysis of most active sub-pooled fraction (SPF 4:2)
Most bioactive sub-pooled fraction of *Piper guineense* leaf was analysed using Gas chromatography-Mass spectroscopy (GC-MS) as described by.[23] In this study, as much as one microliter of the bioactive fraction was used in GC-MS for identification of pure bioactive compound(s). Chromatographic instruments and conditions were carried out on the GC-MS system. Sample of one microliter was injected into GC-MS. The columns used were capillary model agilent number 19091s-433 HP-5MS 5% phenyl methyl siloxane with 30m length, 250um diameter and 0.25um thickness. The oven temperature used was between 100-220℃. The rate of increase in temperature was 15℃/minute, and the flow rate was 1.0ml/minute. The carrier gas was 10.5psi pressurized helium and total rate was 140ml/minute, and the split ratio was 1:50. Component evaluated was detected in the mass detector. The unknown organic compound in sample was identified by interpretation and by matching the produced spectra with reference spectra of National Institute of Standard and Technology (NIST) 11 Library Source.

STATISTICAL ANALYSIS
Results were expressed as ± standard error of mean (SEM). The values of test groups were compared to those of negative control group using one-way analysis of variance (ANOVA) followed by Post-Hoc (Duncan's) multiple comparison tests using SPSS software version 24. $P<0.05$ was considered as significant.

RESULTS
Yield of plant extract and fractions
21.08g was obtained as the quantitative yield of the ethanol extract, which is relatively low when compared to amount (500g) of macerated plant material. A total of seventeen (17) fractions were obtained from column chromatographic separation, which were pooled into five fractions based on their R_f value and color reaction

Acute toxicity test (LD_{50} determination)
In this study, the extracts of *Piper guineense* leaf at LD_{50} of 5000mg/kg , did not produce any obvious signs of toxicity nor death within 48 hours of observation.

Effect of plant extracts (ethanol and fractions) and standard drug (cimetidine) on indomethacin-induced ulcer in rats.
The effect of the standard drug (cimetidine) and extracts (ethanol and fraction) on indomethacin-induced ulcer in rats was studied by comparing the percent inhibition produced to that obtained with negative control (3% v/v Tween 80). From the result obtained, all extracts significantly ($P < 0.05$) produced inhibition of ulcer induced by 100mg/kg indomethacin on adult wistar rats, but the pooled fraction extract-4 (PF-4), produced highest ulcer inhibition of 33.65% as shown in table 1.

Effect of sub-pooled fractions on indomethacin-induced ulcer in rats
From the result in table 2 below, the sub-fractions produced significant ($P < 0.05$) ulcer inhibition when compare to the negative control. Sub-pooled fraction-4:2 (SPF-4:2) produced the highest ulcer inhibition (44.28%) and was therefore selected as the most bioactive sub fraction

GC–MS analysis of most active sub- fraction
GC-MS analysis was carried on the suspected pure compound of *Piper guineense* leaves Figure 1 below represents the National Institute of Standard and Technology (NIST) reference spectra of an unsaturated fatty acid (9,12,Octadecanoic acid methylester) which cross-matched and corresponded with the spectra produced by a compound contained in the most bioactive sub fraction, SPF-4:2, as shown in figure 2. Also, figures 3 and 4 represent respectively, the NIST reference spectra of a phenolic compound (Piperine) and spectra produced by another compound contained in the most bioactive sub-pooled fraction, SPF-4:2. Therefore, bioactive principles in SPF-4:2 are 9,12,Octadecanoic acid methylester (mol. weight: 280) and Piperine (mol. weight: 285) as shown in figures 5 and 6 respectively.

Structure-activity of the pure compounds
Figure 5 below shows the parent structure of a compound, 9,12,Octadecanoic acid metylester. The presence of conjugated diene (2 double bonds) and carboxylic functional group in a straight chain hydrocarbon suggest an unsaturated fatty acid compound. Figure 6 below shows the parent structure of another compound, Piperine, indicating structural features like: an aromatic ring with a methylenedioxy bridge, a conjugated dienone system and a piperidine ring constituting an amide bond. These features possess by these compounds have been considered important for

their exhibition of an array of bioactivities. Several modifications of above structural units have affected the biological properties of these compounds, either enhancing or in some cases completely abolishing their activities.

Table 1: Effect of extracts (ethanol and fractions) and standard drug (Cimetidine) on indomethacin-induced ulcer in wistar rats.

Treatment group	Dose(oral)	Ulcer index	% Inhibition
A (PF-1)	400mg/kg	6.14 ± 0.47	17.36*
B (PF-2)	400mg/kg	6.21 ± 0.24	16.42*
C (PF-3)	400mg/kg	6.00 ± 0.29	19.25*
D (PF-4)	400mg/kg	4.93 ± 0.17	33.65*
E (PF-5)	400mg/kg	6.36 ± 0.21	14.40*
F (EE)	400mg/kg	6.07 ± 0.28	18.30*
G (Positive control)	100mg/kg	1.71 ± 0.26	76.99*
H (Negative control)	5ml/kg	7.43 ± 0.20	—

Values represent mean ± SEM of seven animals in each group

****Significant relative to negative control (3% v/v Tween 80), P <0.05.***

Table 2: Effect of sub-pooled fraction on indomethacin-induced ulcer in wistar rats

Treatment Group	Dose (oral)	Ulcer index	% inhibition
Group 1 (SPF – 4:1	400mg/kg	5.71 ± 0.38	23.15*
Group 2 (SPF – 4:2	400mg/kg	4.14 ± 0.37	44.28*
Group 3 (SPF - 4:3	400mg/kg	6.14 ± 0.28	17.36*
G(Positive Control)	100mg/kg	1.71 ± 0.26	76.99
H(Negative Control)	5ml/kg	7.43±0.20	-

Values represent mean ± SEM of seven animals in each group

****Significant (P <0.05) relative to negative control.***

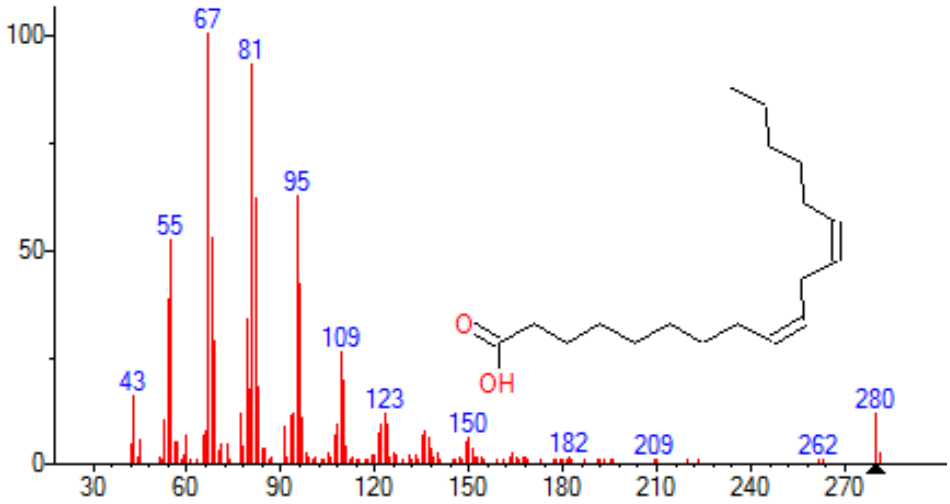

Figure 1: National Institute of Standard and Technology (NIST) Reference Spectra of 9,12 Octadecanoic acid methylester.

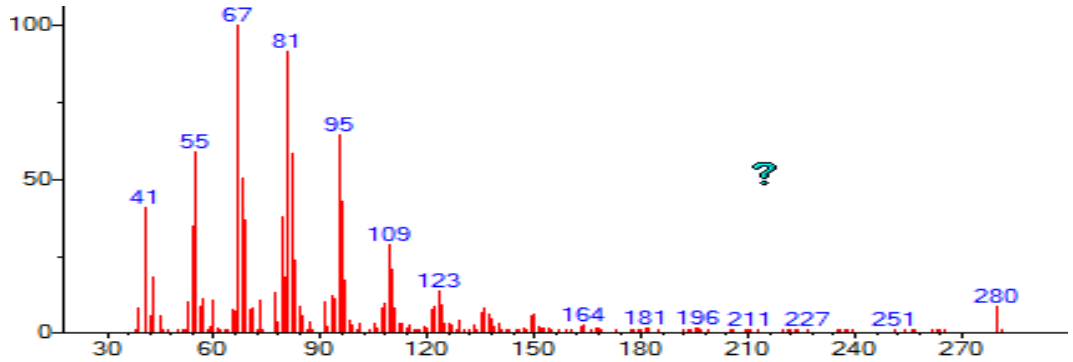

Figure 2: Spectra of the Suspected Pure compound in *Piper Guineense* Leaf.

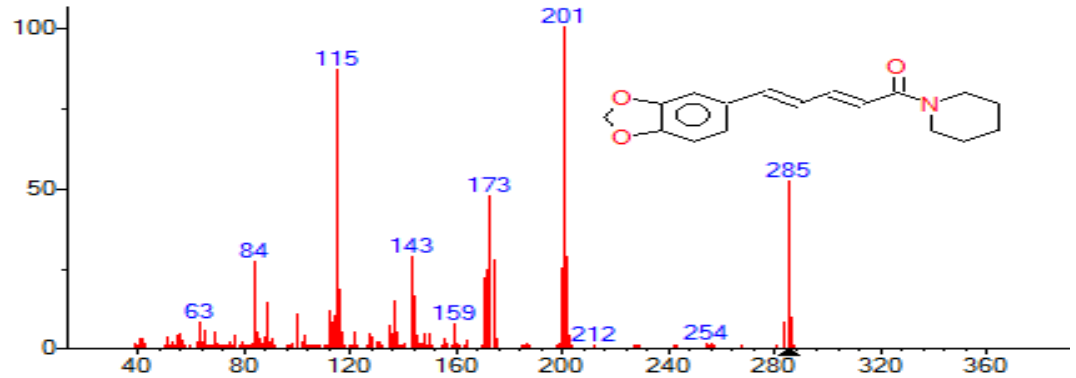

Figure 3: National Institute of Standard and Technology (NIST) Reference Spectra of Piperine.

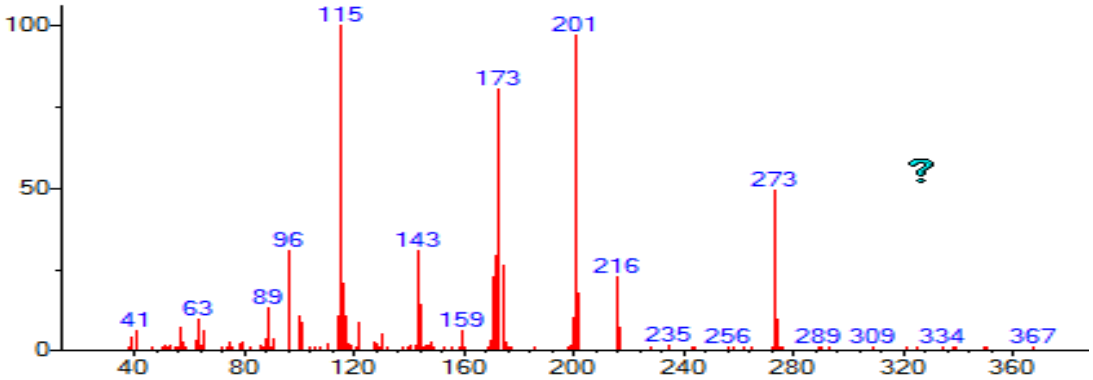

Figure 4: Spectra of the Suspected Pure Compound in *Piper Guineense* Leaf.

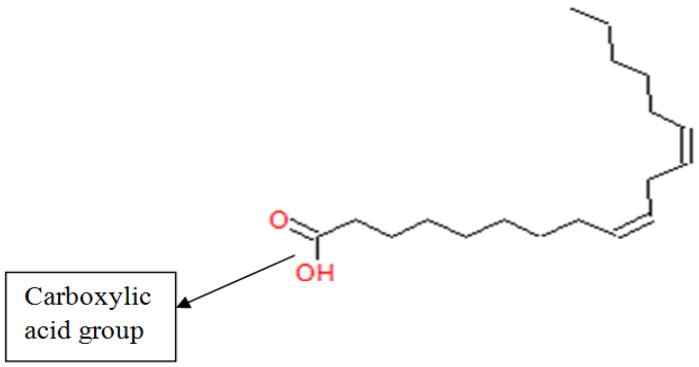

Figure 5: Name: 9,12-Octadecanoic acid methyl ester.

Formula: $C_{18}H_{32}O_2$
Molecular weight: 280; Exact Mass: 280.24023.24023.

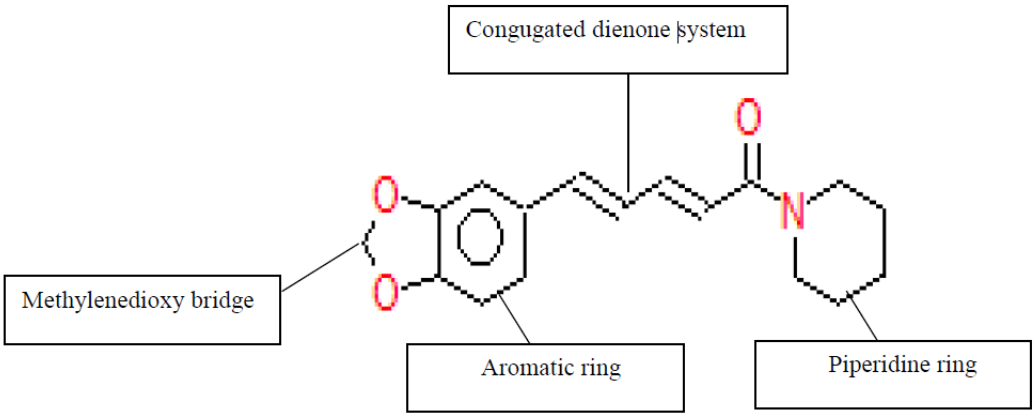

Figure 6: Name: Piperine
Formula: $C_{17}H_{19}NO_3$
Molecular weight: 285 Exact Mass: 285.136494

DISCUSSION

Yield: In this study, the yield of the ethanol extract was low (21.08g) when compared to the amount (500g) of macerated coarse powder. A study has shown that biologically active compounds usually occur in plants in low concentration.[24]

Acute toxicity test (LD_{50} determination)

The principal aim of evaluating safety on any medicinal plant is to identify the nature and significance adverse effect and to establish the exposure level at which the effect is observed.[25] Medicinal plants have been documented to have advantages of toxicity considerations based on their long term use by humans.[26,27] In this study, no toxicity nor death was observed with the extracts of Piper guineense leaves. This was substantiated in LD_{50} test which indicated that even at dose of 5000mg/kg, there was no signs of toxicity nor death during the period of observation. This finding correlated with the report that compounds that do not show adverse effects when given in doses of 3000mg to 5000mg per kilogram body weight are essentially non-toxic (National Academy of Science, 1975).

Antiulcer activity on indomethacin-induced ulcer

Currently, several ulcer models are available for evaluation of antiulcer properties of compounds from natural source.[21] Yet, selection of appropriate model is very difficult owing to associated advantages and disadvantages of those models. In this study, indomethacin ulcer model was chosen because it represents one of the most common cause of gastric ulceration in humans. Indomethacin induces gastrointestinal damage via inhibition of prostaglandin synthesis, production of free radicals and reduction gastric nitric oxide level.[28,29] Therefore, the ability of fraction and sub fraction extracts of *Piper guineense* leaves to significantly ($p<0.05$) inhibit indomethacin-induced ulcer suggests prostaglandin-mediated cytoprotective and free radical scavenging activities. This finding correlates with reports that plants with antiulcer activities inhibit indomethacin-induced gastric ulceration in rats through cytoprotective and antioxidant activities.[30,31] Although the ethanol extract (EE) and pooled fraction extracts showed significant ulcer inhibition, this study also observed increase in ulcer inhibition with the purified extract, particularly sub-pooled fraction-4:2 (SPF-4:2). This suggest that further fractionation/purification led to increased ulcer inhibition, which correlates with the report of.[32]

GC- MS analysis

The active components were identified by matching the spectra produced by SPF-4:2 with that of the reference spectra of National Institute of Standard and Technology (NIST) II library source, Gas chromatography-mass spectrometry analysis of SPF-4:2 confirmed the presence of two bioactive components: an unsaturated fatty acid, 9,12,Octadecanoic acid methylester (chemical formula $C_{18}H_{32}O_2$) and a phenolic compound, Piperine (chemical formula $C_{17}H_{19}NO_3$). This finding is substantiated by some reports:[33] identified 9,12,Octadecanoic acid methylester as one of the active constituents responsible for antioxidant and antimicrobial activities of *Buchholzia corkiacea.* Recently,[34] reported that unsaturated fatty acids accelerate nitric oxide (NO) synthesis and reduce oxidative damage, reduce inflammatory cells infiltration and TNF-alpha expression mice on skin injury induced by ischeamic-reperfusion model. Studies also identified Piperine as active constituent of *Piper nigrum* (Black pepper) and has been implicated to be responsible for antioxidant[35] and antidiarrheal[36] activities of the plant. These two active components identified in the *Piper guineense* leaf may be acting synergistically via antioxidant mechanisms to produced antiulcer activity, as evidenced by greater ulcer inhibition (44.28%) on indomethacin-induced ulcer in wistar rats.

CONCLUSION

The results of this study conclude that *Piper guneense* leaf extract contains among other constituents, two bioactive principles: 9,12,Octadecanoic acid methylester and Piperine, which are responsible for antiulcer activity of the plant

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