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Activity-Guided Isolation and GC-MS Analytical Identification of Potential Antibacterial Principle(s) of *Piper guineense* Leaf Extract

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ABSTRACT

Bacteria of medical importance have posed a tremendous challenge to global health care, hence, necessitating constant search for novel antibacterial agents. Plants have been documented as a rich source of drugs. *Piper guineense*, a flora of tropical Africa is commonly used in tradomedicine for treatment of broad range of bacterial infections. However, the isolation and identification of bioactive principles responsible for antibacterial activities are yet to be given the deserved attention. This study was designed to follow the activity-guided isolation andGC-MS analytical identification of potential antibacterial principle(s) of *Piper guineense* leaf extract. This was done by extracting the powdered leaves in solvents of different polarity, screening the crude extracts for antibacterial activities, fractionating the most bioactive crude extract by column chromatography that yielded seven pooled fractions (F_1–F_7) which were screened for antibacterial activities to obtain most bioactive fraction extract (F_2). The most bioactive fraction extract (F_2) was purified by fractionation, pooled together into three sub-fractions ($SF_{2:1}$–$SF_{2:3}$)and further screened for antibacterial activities. The most bioactive sub-fraction was subsequently analyzed by GC-MS technique. From the result, $F_{2:2}$extract was selected as the most bioactive pure sub-fraction, having exhibited the highest activity index of 0.361, 0.428, 0.683 and 0.582 against*E.coli, K. pneumoniae, B. subtilis and S. aureus* respectively, and also produced highest total antibacterial activityof 0.077 and 0.308respectively against gram negative and gram positive organisms used in this study. GC-MS analysis of $F_{2:2}$ reveals the presence of Elemecin, E-Nerolidol and Piperine. These principles are therefore responsible for antibacterial activity of *Piper guineese* leaf.

INTRODUCTION

Bacterial infections are on the increase and have remained the cause of significant morbidity and mortality world-wide, particularly in the developing countries.[1] The discovery and use of antibiotics which are substances produced by microorganisms that can kill or inhibit other microorganisms in low amounts, have contributed immenselyin combating bacterial infections until development of resistance and/or tolerance by most pathogenic microbes.[2] Microbial resistance/tolerance caused mainly by abuse and/or misuse of antibiotics, is a global problem that has continued to endanger human life because infections that were formerly treated with these substances (antibiotics) become much more difficult to control later.[3] In view of the above challenges, i.e., high rates of bacterial infections and microbial resistance, it has become necessary to discover and design new antibacterial therapies with better efficacy to enhance the health of mankind.[4,5]

Historically, plants have been documented as a rich source of drugs, as plant-derived compounds have made tremendous contributions to human health and well-being.[6 – 8] The undesirable effect associated with conventional antibiotics has stimulated research on medicinal and plant-derived compounds.[9,10] The use of plant-derived products in treatment of infections in humans have certain advantages besides being cheap to produce, are readily available, biodegradable, with little or no toxic side effects and environmentally friendly.[11]

Piper guineense Schaun & Thonn, a flora of tropical Africa is used in traditional medicine due to its numerous health benefits.[12] It is a spice plant commonly used in Nigeria in flavoring local dishes.[13] Studies have shown that extracts from *Piper guineense* are used in humans for a wide variety of purposes such as antibacterial[14-17], antifungal[18,19], anticancer.[20]*Piper guineens*e is of great ethnomedicinal importance, therefore, it became necessary to broaden knowledge of its antibacterial activity by pin-pointing the possible bioactive principle(s) which might be used as novel antibacterial agent(s) in for-seeable future. This study therefore, was designed to follow activity-guided isolation and GC-MS analytical identification of potential antibacterial principle(s) of *Piper guineense* leaf extract.

MATERIALS AND METHODS

Collection, Identification and Confirmation of Plant Material

Matured fresh leaves of the plant were harvested from a farm land in Naze, Owerri North LGA, Imo State, Nigeria. The leaves were identified and confirmed as *Piper guiueense* by a

taxonomist in the Department of Plant Science and Biotechnology, University of Port Harcourt where a sample was deposited in the herbarium with voucher number, UPH/P/251.

Drugs, Chemicals and Consumables

These include the following: Gentamicin (Surelife Pharm. Industry Ltd., Nigeria), MacConkey Agar (Highflow Biotech, UK), Nutrient Agar (Titan Biotech Ltd, India), Nutrient Broth (Titan Biotech Ltd, India), Ferric Chloride (Super Tek Chemical, Germany), Barium Chloride (Super Tek Chemical, Germany), Ethylacetate (Rankem, Mumbai, India), Sodium Hydroxide (Rankem Mumbai, India), Dimethylsulphoxide, Hydrochloric acid (Nice Laboratories Reagent, Kevala, India), Sodium tetraoxocarbonate IV (Sigma Aldrich Chemie, Germany), Tetraoxosulphate VI acid (Hi Media Laboratories Pvt Ltd, India), Methanol (Gungsdong Guandgua Chemical Factory, China), Glacial acetic acid (Sigma Aldrich Chemie, Germany).

Sources of Microorganisms

Microorganisms used in this study were obtained from Bioresources Development and Conservative Project Center, Nsukka. The organisms which included typed cultures of *Escherichia coli* (ATCC 11775), *Klebsiella pneumonia* (ATCC, 13883), *Staphylococcus aureus* (ATCC 25922) and *Bacillus subtilis* (ATCC 19659) were purified, identified and confirmed by repeated sub-culturing in appropriate media, gram stain reaction and biochemical tests respectively. The cultures were maintained on agar slant stored in refrigerator until when needed.

Preparation of MacFarland Standard

This was done as described in our previous work[21] by mixing and stirring 0.05ml 1.0% w/v $Bacl_2.2H_2O$ with 9.95ml concentrated H_2SO_4 to form a $BaSO_4$ precipitate that corresponded to 0.5 MacFarland turbidity standard of 1.5×10^8 cfu/ml of bacterial suspension.

Pre–extraction Preparation of Plant Material

The matured fresh leaves of *Piper guineense* were washed and rinsed with clean tap water, allowed for about 2 weeks to dry to a constant weight at room temperature. About 5kg of the dried leaves were pulverized into coarse powder and stored in a clean dry container for subsequent use.

Solvent Extraction of Plant Material

Various 1kg of powdered plant material was extracted by cold maceration using 1 liter of different solvents (water, methanol, isopropyl alcohol and n- hexane) of increasing polarity with intermittent agitation at 6 hours interval for 72 hours. The solutions were filtered through Whatman number 1 filter paper. The marc from each solvent was re-macerated (1 liter× 2) and filtered. Filtrates of various solvent were combined together in separate containers, concentrated in hot air oven (40°C) to obtain a solid residue. The entire process was repeated five times to generate enough quantities of various solvent crude extract for subsequent studies. The extracts were labeled appropriately as: aqueous extract (AE), methanol extract (ME), Isopropyl alcohol extract (IE) and n-hexane extract (HE). The labeled extracts were properly stored in refrigerator until when needed.

Phytochemical Screening

The crude extracts (AE, ME, IE and HE) were screened for presence or absence of phytochemical constituents using standard procedures described by:[22-25]

Antibacterial Studies (Determination)

The crude and fraction extracts were studied for antibacterial activities by determining growth zone inhibition diameter (mm) and minimum inhibitory concentration (MIC) using agar well diffusion and broth microdilution techniques respectively.

Determination of Mean Zone Inhibition (MZI) and Activity Index (AI) of Extracts

Using agar well diffusion method described by[21,26,27], preliminary antibacterial sensitivity screening was conducted on various crude extracts at different concentration (50, 100, 200, and 400 mg/ml) to determine the extract that would produce antibacterial activity and also to ascertain the concentration at which reasonable antibacterial activity would be produced. Subsequently, the extracts, at previously determined concentration in the preliminary screening, were deposited in triplicate agar wells (8mm diameter) bored on the inoculated agar plates. In separate triplicate agar wells, 50mcg/ml of Gentamicin was deposited as positive control while Dimethyl sulfoxide (DMSO) which was used in reconstituting the extract was introduced in a separate triplicate well as negative control. All the plates were kept for 2 hours for diffusion to take place and thereafter, incubated at 37° C for 24 hours. After incubation, zones of inhibition were measured and mean zone inhibition (MZI)

diameter was calculated. Activity index of each extract against different organisms was determined using the formula.[28,29]

$$\text{Activity Index (AI)} = \frac{\text{Inhibition Zone of Sample (Extract)}}{\text{Inhibition Zone of Standard (Gentamicin)}}$$

Determination of Minimum Inhibitory Concentration (MIC) and Total Antibacterial Activity (TAA) of Crude and Fraction Extracts

This was done by broth microdilution techniques as described by[30]with slight modifications. The extracts and the standard drug (Gentamicin) were diluted with DMSO to obtain 200mg/ml and 50mcg/ml stock solutions respectively from which two-fold serial dilution was done by mixing with broth medium in a 96-well microtiter plates. 100 microliter (0.1 ml) of bacterial suspension adjusted at 0.5MacFarland standard turbidity (1.5×10^8 cfu/ml) was added to each well. The 11th well served as negative control (without bacteria, with antibacterial) while the 12th well served as positive control (with bacteria, without antibacterial agent). The experiments were carried out in triplicate and the plates were incubated at 37° C for 24 hours. After incubation, assessment of bacterial growth was done by addition of resazurin to each well and the MIC was interpreted as the highest dilution that prevented a color change from blue to pink.[31] Total antibacterial activity (TAA) was determined using the formula:[32]

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$$\text{Total Antibacterial Activity (TAA)} = \frac{\text{Extraction yield (mg) per gram dried plant part}}{\text{MIC of the extract}}$$

Activity-guided Isolation and Fractionation/Purification of most Bio-active Crude Extract and Fractions

Following the preliminary sensitivity screening that eliminated aqueous crude extract, other solvent crude extracts (EE, IE and HE) were further studied for antibacterial activities. Judging from the values of activity index and total activity, methanol extract was found to produce highest antibacterial activities and was therefore selected for further fractionation/purification by series of column chromatographic technique as described by [33]where 10g of methanol extract (ME) was re-dissolved in 20ml methanol, introduced on top of the column previously wet packed with silica gel and then continuously eluted with 400ml chloroform/ethyl acetate/methanol (5:3:2) solvent system which gave the best resolution in the preliminary thin layer chromatography. The process was repeated in five cycles and one

hundred and ninety-five (195)-10ml fractions were obtained, pooled together based on their R_f values into seven fractions (F_1 - F_7) and screened for antibacterial activities. F_2extract having produced the highest antibacterial activities was further fractionated (2g ×5) to obtain three sub-fractions ($SF_{2:1}$ - $SF_{2:3}$) which were screened for antibacterial activities. Highest antibacterial activity was observed with $SF_{2;2}$ extract, and therefore, was subjected to GC-MS analysis for identification of bioactive compound(s).

STAITISTICAL ANALYSIS

Results of the experiments were expressed as mean +standard error of mean (SEM). One-way analysis of variance (ANOVA) was used to establish statistical difference at probability less than 0.05 ($p<0.05$), followed by Duncan's multiple comparison.

RESULTS

As shown in Table No.1, aqueous solvent produced the lowest extract yield (2.94g/ kg dry plant material) while methanol produced highest yield (13.62g/ kg dry plant material). Among the fraction and sub-fraction extracts, F_2 and $SF_{2:2}$ respectively showed yield of 256.48mg/100g and 19.24mg/10g residue.

Result in TableNo.2 indicates the presence of the tested phytochemicals in all the crude extracts in varying amounts with exception of saponins and carotenoids which are respectively absent in n-hexane and isopropyl alcohol extracts. The methanol extract contains alkaloids, tannins flavonoids, glycosides and carotenoids in large amounts.

From the result in Table No.3, the extracts produced greater zone of inhibition against gram positive organism than the gram-negative organisms. Methanol extract produced highest zone of inhibition than other extracts. Result in Tables No. 4 and 5 show that all the extracts generally exhibited higher activity index and total antibacterial activity against gram positive organisms than gram negative organisms.

Table No.1: Yield of Various Solvent Crude Extracts (g/kg dry powder), Fraction Extracts (mg/100gcrude residue) and Sub Fraction Extracts (mg/10g fraction residue)

Extract/Fraction	Yield
AE	2.94 + 1.47*
ME	13.62 + 1.19*
IE	9.83 + 2.07*
HE	7.66 + 1.95*
F_1	104.61+ 1.30*
F_2	256.48+1.11*
F_3	98.31+ 0.85*
F_4	36.25+ 1.52*
F_5	11.37 ± 2.38*
F_6	6.11 ± 2.17*
F_7	0.00
$SF_{2:1}$	2.22+ 1.66*
$SF_{2:2}$	19.24+ 1.03*
$SF_{2:3}$	2.16±1.50*

*Values represent + SEM of n = 5; AE = Aqueous Extract; ME = Methanol Extract; IE = Isopropyl alcohol Extract; HE = n – hexane Extract; SF = Sub-Fraction.

Table No.2: Qualitative Phytochemical Analysis of Various Solvent Crude Extracts of *Piper guineense* leaf

Solvent Extract	AE	ME	IE	HE
Alkaloids	++	+++	+++	+
Tannins	++	+++	++	++
Flavonoids	++	+++	+	++
Saponins	+++	++	+	_
Polyphenols	++	++	+++	+
Glycosides	+++	+++	++	++
Carotenoids	++	+++	_	++
Triterpens	+	++	++	+++

+++ =present in large amount; ++ = present in moderate amount; + = present in low amount, - = absent. AE = Aqueous Extract; ME = Methanol Extract; IE = Isopropyl alcohol Extract; HE = n – hexane Extract;

Table No.3: Preliminary Antibacterial Susceptibility Screening of Solvent Crude Extracts at different Concentrations against different Organisms

		Zone of Inhibition (mm)			
Extract	**Concentration (mg/ml)**	***E.coli***	***K.pneumoniae***	***B. subtilis***	***S.aureus***
AE	100	1.5	1.2	1.8	2.4
	200	1.9	1.7	2.5	2.8
	400	2.7	2.9	4.2	3.8
ME	100	8.2	9.4	13.0	14.6
	200	14.6	16	25.2	26.5
	400	16.2	15.8	26.9	28.2
IE	100	6.0	7.2	10.8	12.6
	200	11.8	12.4	15.2	16.5
	400	17.2	15.8	19.3	20.7
HE	100	4.8	5.6	7.4	6.6
	200	10.5	9.2	14.8	14.4
	400	12.8	13.4	17.8	16.8

AE = Aqueous Extract; ME = Methanol Extract; IE = Isopropyl alcohol Extract; HE = n – hexane Extract;

Table No.4: Mean Zone Inhibition (MZI) Diameter and Activity Index (AI) of Extracts at 200mg/ml and Gentamicin at 50mcg/ml.

Extract	*E.coli*		*K. pneumoniae*		*B. subtilis*		*S.aureus*	
	MZl(mm)	**AI**	**MZl (mm)**	**AI**	**MZI(mm)**	**AI**	**MZl(mm)**	**AI**
ME	13.34 + 0.78*	0.453	12.27 + 1.12*	0.470	23.14 + 1.20*	0.810	25.42 + 0.66*	0.839
IE	10.24 + 1.22*	0.348	10.52 + 1.46*	0.403	14.33 + 0.77	0.502	17.34 + 1.45	0.572
HE	11.62 + 0.95*	0.395	10.15+ 1.33*	0.389	14.04 + 1.41*	0.492	16.12 + 1.09*	0.531
F_1	5.62 + 1.44	0.191	6.12+ 0.63*	0.234	7.37+0.76*	0.258	8.25+ 0.52*	0.272
F_2	12.53 +1.02*	0.426	11.68 + 0.85*	0.447	21.78+ 1.01*	0.763	22.72 + 0.65*	0.749
F_3	4.02 + 2.77	0.137	2.84 + 1.62	0.109	3.72 + 1.27	0.130	6.31 + 1.42*	0.208
F_4	3.16 + 1.53	0.107	4.84 + 0.74	0.185	5.07±1.01	0.178	5.14 + 0.96	0.169
F_5	NIZ	ND	NIZ	ND	5.01±1.61	0.175	3.38±2.06	0.111
F_6	NIZ	ND	NIZ	ND	3.24±1.73	0.113	NIZ	ND
F_7	NIZ	ND	NIZ	ND	NIZ	ND	NIZ	ND
$SF_{2:1}$	3.61 + 0.92	0.123	2.53±1.90	0.098	6.28±0.32*	0.220	5.44 + 0.70	0.179
$SF_{2:2}$	10.62 + 1.31*	0.361	11.19+ 0.77*	0.428	19.51 + 1.06*	0.683	17.66 + 0.34*	0.582
$SF_{2:3}$	4.45 + 2.76	0.151	5.26 +1.65	0.201	5.73±1.31*	0.201	6.33±1.24*	0.209
Gentamicin	29.44 + 0.94*	ND	26.12 + 1.64*	ND	28.56 + 0.89*	ND	30.34 + 0.35*	ND

*Value represent + SEM of n = 3, p <0.05 is significant relative to negative control; NIZ = No Inhibition Zone, ND = Not Determined.AE = Aqueous Extract; ME = Methanol Extract; IE = Isopropyl alcohol Extract; HE = n – hexane Extract; SF = Sub-Fraction

Table No.5: Minimum Inhibitory Concentration (MIC) and Total Antibacterial Activity (TAA) of Extracts.

Extract	*E.coli*		*K.pneumoniae*		*B. subtilis*		*S.aureus*	
	MIC(mg/ml)	TAA	MIC(mg/ml)	TAA	MIC(mg/ml)	TAA	MIC(mg/ml)	TAA
ME	50	0.272	50	0.272	12.5	1.090	6.25	2.179
IE	50	0.197	100	0.098	25	0.393	12.5	0.786
HE	100	0.077	100	0.077	50	0.153	50	0.153
F_1	100	0.010	100	0.010	100	0.010	100	0.010
F_2	25	0.103	100	0.026	12.5	0.205	6.25	0.410
F_3	50	0.020	100	0.010	100	0.010	100	0.010
F_4	200	0.002	200	0.002	200	0.002	100	0.002
F_5	0.00	ND	0.00	ND	0.00	ND	0.00	ND
F_6	0.00	ND	0.00	ND	0.00	ND	0.00	ND
F_7	0.00	ND	0.00	ND	0.00	ND	0.00	ND
$SF_{2:1}$	100	0.002	50	0.004	50	0.004	50	0.004
$SF_{2:2}$	25	0.077	25	0.077	6.25	0.308	6.25	0.308
$SF_{2:3}$	100	0.002	100	0.002	100	0.002	100	0.002
Gentamicin	12.5mcg/ml	ND	12.5mcg/ml	ND	6.25mcg/ml	ND	3.12mcg/ml	ND

ND = Not Determined, AE = Aqueous Extract; ME = Methanol Extract; IE = Isopropyl alcohol Extract; HE = n – hexane Extract; SF = Sub-Fraction

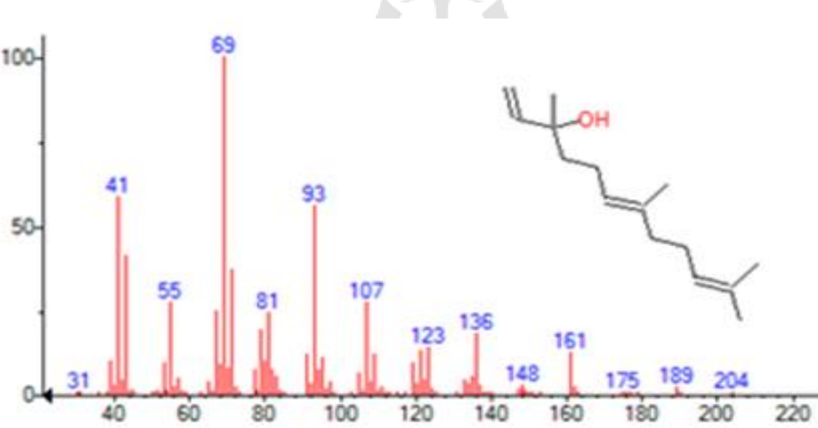

Figure No.1: National Institute of Standard and Technology

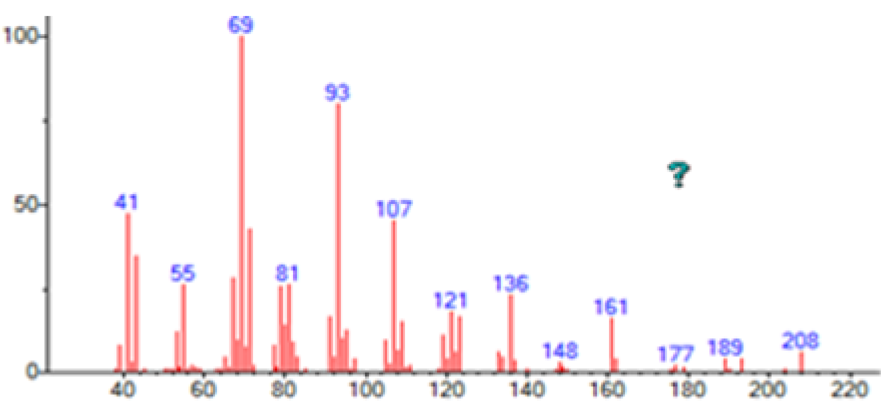

Figure No.2: Spectra of the Suspected Pure Compound (NIST) Reference Spectra of E-Nerolidol in *Piper guineense* leaves

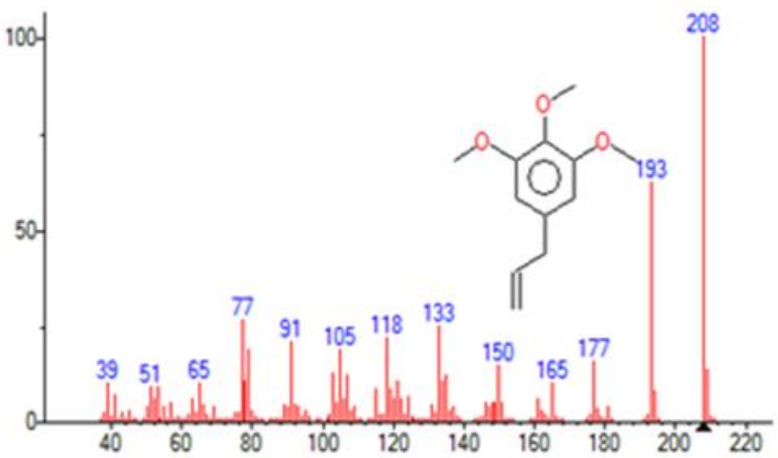

Figure No.3: National Institute of Standard and Technology

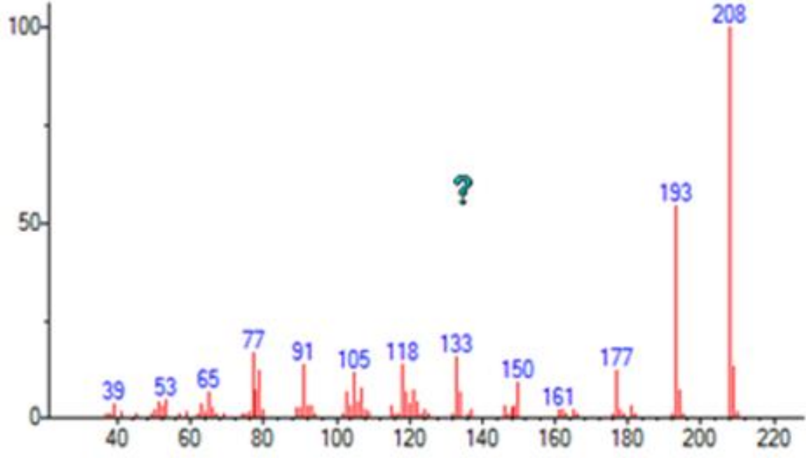

Figure No. 4: Spectra of the Suspected Pure Compound (NIST) Reference Spectra of Elemecin in *Piper guineense* leaves

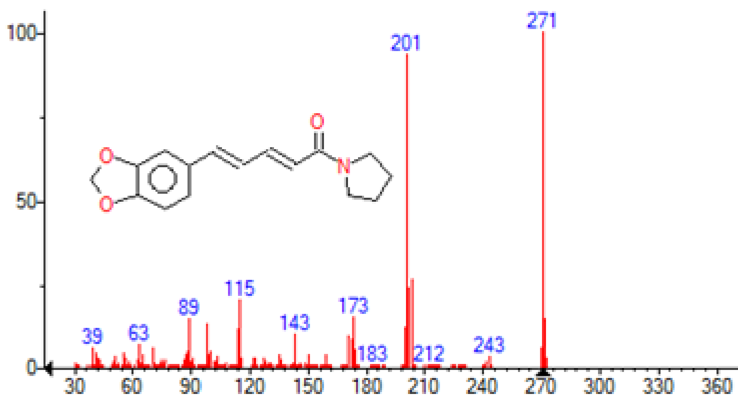

Figure No.5: National Institute of Standard and Technology

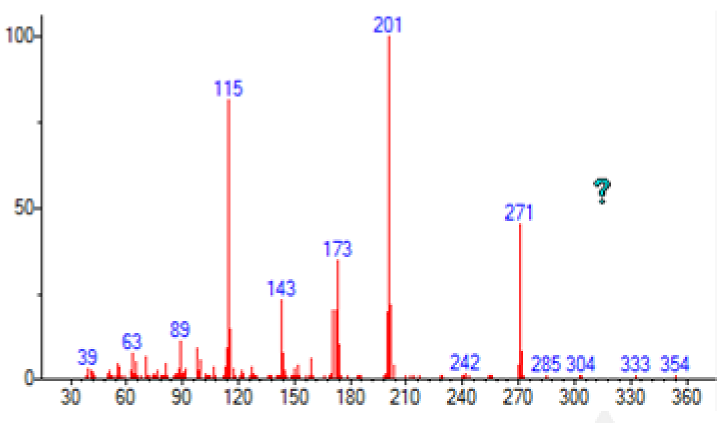

Figure No.6: Spectra of the Suspected Pure Compound (NIST) Reference Spectra of Piperine in *Piper guineense* leaves

DISCUSSION

Phytochemical analysis of different solvent extracts of *Piper guineense* leaf in this present study reveals the presence of flavonoids, triterpenes, polyphenols, alkaloids, tannins, saponins and glycosides which agrees with the findings of [34,35] that show that *Piper guineense* contains the phytochemicals mentioned above. This study also observed that these phytochemicals were extracted in varying amounts in different solvent which agrees with the report that plant constituents (phytochemicals) responsible for various biological activities exhibit varying degree of solubility in solvents of different polarities.[36] Studies have reported the antibacterial activities of flavonoids[37,38], tannins[39], glycosides[40], saponins[41], polyphenols and alkaloids.[42] Because these phytochemicals occur in the same plant or plants part (seeds, leaves, flowers stems, roots and fruits), they can work together to produce synergistic antibacterial activity.

Preliminary sensitivity screening was done to determine the solvent crude extracts with antibacterial activity and to also establish the concentration at which prominent antibacterial activity would occur. Agar well diffusion method was employed because it has been reported to be more sensitive than other methods.[43] The aqueous extract as indicated in Table No.3, having produced smaller zone of inhibition (i.e., less than 8mm) is assumed to have very little or no antibacterial activity. This finding conforms to the report that zone inhibition less than 8mm is considered not to have antibacterial activity.[44] Lack of antibacterial activity of aqueous extract may be due to poor water solubility of antibacterial principles of the plant.

Sensitivity of the test organisms to crude extracts was tested at three concentrations (100, 200 and 400 mg/ml) and the result showed a concentration-dependent antibacterial activity (Table No. 3). Similar observation was reported on increased antibacterial activity with increased concentration of *Millettia abuensis* extract against test organisms.[45]Methanol crude extract showed highest antibacterial activity and this may be due to ability of methanol to extract broad range of bioactive antibacterial compounds present in *Piper guineense* leaf which were made available in the agar medium during diffusion. This finding is supported by report that methanol can extract wide variety of non-polar and moderately polar bioactive compounds.[46]

Following preliminary sensitivity screening, other antibacterial activities were studied based on determining mean zone inhibition (MZI) diameter, activity index (AI), minimum inhibitory concentration (MIC) and total antibacterial activity (TAA). Mean zone inhibition diameter less than 8mmis considered no antibacterial activity.[44] Minimum inhibitory concentration is considered a "gold" standard for determining sensitivity of microorganisms to antibacterial agents.[47] It is the least concentration of antibacterial agent that can inhibit growth of microbes.[27] Activity index is the ratio MZI of the plant extract to MZI of standard antibiotics.[28,29] Activity index greater than 1 (AI >1) indicates that the plant extract exhibits greater antibacterial activity than the standard drug while activity index less than 1(AI <1) suggests that the standard drug exhibits greater antibacterial activity than the plant extract under consideration. The result in Table No.4 shows that the plant extracts have activity index less than 1. Total antibacterial activity(TAA), expressed as milliliter per gram, is the ratio extraction yield (milligram per gram) of pulverized plant material to minimum inhibitory concentration (milligram per milliliter).[32,48] Total antibacterial activity indicates the volume of water or solvent that can be added to 1 g of the plant extract that will enable it still inhibit microbial growth.[48] The MIC and TAA values are pharmacologically useful in

determining respectively the activity of the extracts in milligram per milliliter (potency) for isolation of bioactive principle and in milliliter per gram (efficacy) for selection of plant species.[48,49]

From the result in Table No.4 and 5, all the crude extracts showed significant ($p<0.05$) antibacterial activity, but most pronounced with methanol extract. This finding is in agreement with the report that methanol extract of medicinal plants have greater antibacterial activity than aqueous extract[50] on the account that methanol extracts a wide variety of non polar and moderately polar bioactive compounds.[46]In this study, antibacterial activities are greater with the gram-positive organisms than the gram-negative organisms. This observation is similar to report of greater antibacterial activity of plants extracts against gram positive organisms when compared to gram negative organisms. [51,52] The difference in antibacterial activities between the gram positive and gram-negative organisms is attributed to structural differences in their cell wall. While the gram-positive cell wall is simple and consists of peptidoglycan layer which is not an effective permeability barrier, the gram-negative cell wall is complex and consists of outer cell membrane that bears the lipopolysaccharide that constitutes permeability barrier to antibacterial agents.[53]

GC-MS analysis of purified $F_{2;2}$ extract of *Piper guineense* leaf pointed to the presence of Elemecin, E-Nerolidol and Piperine as antibacterial bioactive principles which were identified by matching and comparing the spectra produced by $F_{2;2}$ extract (Figure No. 2, 4 and 6) with the reference spectra of National Institute of Standard Technology (NIST) library source II (Figures 1, 3 and 5). Studies have reported antibacterial activities of Elemecin[54], E-Nerolidol [55] and Piperine.[56,57]

CONCLUSION

The results of this study have re-affirmed the antibacterial activities of *Piper guineense* leaf extract and also went further to establish that Elemecin, E-Nerolidol and Piperine are the antibacterial principles present in the plant.

CONFLICT OF INTEREST

The authors have no conflicting interest.

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