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Antimicrobial and Antioxidant Potentials, and Chemical Constituents of the Leaf Extracts of the Nigerian *Piliostigma thonningii* (Caesalpiniaceae) Schum

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Authors' contributions

This work was carried out in collaboration between both authors. The bench work was done by author YR as a M.Sc. student under author OSK who designed and supervised the project. Author YR also drafted the report while author OSK did the final moderation of the manuscript for publication. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: In this work the 95% ethanol extract of the Nigerian *Piliostigma thonningii* known traditionally to possess a number of medicinal properties was screened for antimicrobial and antioxidant potentials as well as identified its chemical constituents. It was to confirm its traditional medicinal uses.
Study Design: The various local sources of the plant were identified for the collection of the leaves. The plant was authenticated by a taxonomist and a voucher specimen kept for future reference. The plant sample was extracted using 95% ethanol and the crude extract screened for phytochemicals and fractionated. The crude extract and fractions were screened for antimicrobial

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and antioxidant potentials. The antimicrobial hexane fraction was subjected to chromatographic separation to isolate the chemical constituents. Also, the fresh leaves were hydrodistilled to obtain the volatile components. The column isolates and volatile components were identified using GC-MS analysis.

Place and Duration of Study: The study was undertaken between October 2011 and April, 2013, in the Department of Chemistry, University of Abuja, Nigeria and the Advanced Chemistry Laboratory, Sheda Science and Technology Complex, Sheda, Abuja, Nigeria.

Methodology: The air-dried leaves powder of *P. thonningii* was extracted with 95% ethanol to obtain the crude extract. The fresh leaves were also hydrodistilled to obtain the volatile oils. The crude extract was fractionated into acidic, basic, non-polar neutral and polar neutral fractions. It was screened for phytochemicals using standard procedures. Both the crude extract and fractions were screened against some pathogens, including *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Streptococuss* spp. *and Salmonella* spp. The antioxidant potential of the crude extract was also determined using standard procedures. The antimicrobial hexane fraction was subjected to Flash column chromatography. The isolates and the volatile oil were analyzed for their constituents using GC-MS.

Results: The crude extract showed the presence of sterols, phenolics, alkaloids, flavonoids, glycosides, triterpenes, and tannins, but no carbohydrates. The crude extract and fractions and the hydrodistillate showed potential activity against the test organisms including, *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa, Streptococuss* spp. *and Salmonella spp.* The crude extract equally showed antioxidant potential in DPPH. Column chromatography of the hexane fraction followed by GC-MS analysis led to the identification of lupeol and lup-20(29)-en-3-one. GC-MS analysis of the volatile oil from hydrodistillation of the fresh leaf revealed the presence of 2,5-octadecadiynoic acid, cholestan-3-ol, 2-methylene, isoaromendrene, trans-Z-α-bisabolene epoxide,1-methyl-6-(3-methybuta-3-dienyl)-7-oxabicylo [4.1.0] heptane and aromadendrene oxide.

Conclusion: The crude 95% ethanol extract has demonstrated reasonable antioxidant potential. Also, the crude extract, fractions and the volatile oil have shown antimicrobial activity. Lupeol, lupenone and some volatile components have been identified from the leaves. The presence of these compounds may contribute to the bioactivities of the leaf extracts.

Keywords: Piliostigma thonningii; leaf extracts; antimicrobial; antioxidant; potentials; chemical constituents.

1. INTRODUCTION

Among the plants belonging to the genus Piliostigma the two African species, *P. reticulatum* (DC) Hochst and *P. thonningii* (Schum) Milne-Redhead, inhabit dry and moist savannahs, respectively [1-4]. Various parts of *P. thonningii* were found to be used in the management of ailments in phytomedicines. The bark is used in the management of cough, stomach infections, malaria, leprosy, sore throat and various forms of inflammation. The roots and twigs are used in treating fever, dysentery, snake bites, hookworms and skin infections, while the leaves decoctions are used as laxatives for children and for dressing wounds [5].

The results of a number of pharmacological investigations have revealed that *P. thonningii* has some bioactivities such as antimalarial [6,7],antibacterial [8,9], anthelmintic [10,11], antioxidant [12] and sub-acute toxicity [13,14]. Chemical studies by some researchers showed that the genus *Piliostigma* contains some flavonoids [15], polyphenols [16] and essential oils [17,18]. The presence of griffonilide and some unidentified sterols in the stem bark of the Nigerian *P. thonningii* has been previously reported [19]. Since then there have been several reports on the pharmacological properties, but reports on the chemical constituents have been scarce. In this paper we wish to report on the antimicrobial and antioxidant potentials and in particular on the chemical constituents of the leaf extracts of the Nigerian *P. thonningii.*

2. MATERIALS AND METHODS

2.1 General

All solvents and reagents used in this work were of standard grade and were purified by re-distillation before use.

For the antimicrobial screening, stock solutions of the extracts (crude, acidic, basic, non-polar and polar neutrals and hydrodistillate) were at concentration of 100 µg/ml; the petri-dish, micro-pipette, autoclave, incubator, bunsen flame, loop and dimethylsulphoxide as the solvent were used. The test organisms were *Escherichia coli, Staphylococcus aurues, Bacillus subtilis, Pseudomonas aeruginosa, Streptococcus* spp *and Salmonella* spp. from Gwagwalada Teaching Hospital in Mueller Hinton broth culture made by Beaker Company and kept in a refrigerator at 4°C.

Open flash column chromatography was performed using silica gel Merck 0.063-0.200 mm mesh, 60 G. Thin Layer Chromatography(TLC) analyses of extracts, fractions and isolates were carried out using silica 60 F_{254} precoated glass baked plates (0.25 mm, 20 X 20 cm; Merck Darmstadt, Germany). Spots were detected on TLC plates under short (λ=254 nm) and long (λ=366 nm) UV light as well as visualized by spraying with 2% (w/v) vanillin in sulphuric acid and in iodine vapour.

2.2 Plant Material

The fresh leaves of *Piliostigma thonningii* were collected from the Medicinal Plant Reserve Garden of Sheda Science and Technology Complex (SHESTCO), Abuja, Nigeria, in September 2011. It was authenticated at the National Institute for Pharmaceutical Research and Development (NIPRD), Idu, Abuja, Nigeria, and a voucher specimen was kept at the Ahmadu Bello University, Zaria, Herbarium (No.171). The leaves were air-dried and ground into fine powder and kept in a non-absorptive nylon bag for subsequent use.

2.3 Extraction and Fractionation Procedure

The ground plant material (1000 g) was extracted using a Soxhlet extractor with 50% aqueous ethanol for six hours. After filtering the extract the solvent was removed using a rotary evaporator to dryness on a water bath. The crude residue (32.0 g) was fractionated into acidic (1.9 g), basic (1.2 g), polar neutral (3.0 g), and non-polar neutral (11.0 g) extracts according to a standard procedure [20].

2.4 Hydrodistillation of fresh leaves

The freshly collected leaves of *P. thonningii* were subjected to hydrodistillation. The hydrodistillate was extracted with diethyl ether and the extract was evaporated to dryness to give an oily residue (2.6 g).

2.5 Phytochemical Screening

Phytochemical screening was carried out on the crude ethanol extract using standard methods [21].

2.6 Determination of Antioxidant Activity of Crude Extract

The antioxidant property of the crude extract was determined according to a standard method using the DPPH radical [22]. The crude extract (0.1 g) was weighed into a test tube and dissolved in 10 ml of methanol, then 5 ml, 2 ml, and 1 ml portions were taken into three separate test tubes labeled A, B, and C, respectively. Further, diluted concentrations were made from A, B, and C test tubes into five tubes D, E, F, G, and H (0.5, 0.1, 0.05, 0.02 and 0.01 mg/ml). From each of these test tubes 1ml was taken into another test tube and 3 ml of methanol and 0.5 ml of DPPH solution were added to prepare a standard test sample. This was allowed to stand for 15 mins. The same procedure was applied to the sample of Vitamin C. The samples were subjected to UV/Visible double beam spectrophotometer with the light set at 517 nm. A reference was also prepared by mixing 3ml of methanol with 0.5 ml of DPPH solution.

The percentage inhibition (I %) of free-radical DPPH was calculated using the standard equation[133]:

$$I\ \% = 100 \times (A_{Control} - A_{Sample}) / A_{Control}$$

Where $A_{Control}$ is the absorbance of the control reaction (containing all reagents except the test compound), and A_{Sample} is the absorbance of the test compound.

2.7 Antimicrobial Activity Screening of Crude Extract and Fractions

Antimicrobial sensitivity tests were carried out on the 95% ethanol crude extract, the acidic, basic, non polar and polar neutral fractions, and the steam distillate using the Disc Diffusion Method [23]. A stock solution of each extract at concentration of100 µg/ml was screened against the six test organisms. The test organisms were maintained on nutrient agar slants and kept at 4°C until required. Sterile paper discs of 6 mm in

diameter prepared from Whatman No.1 filter paper were impregnated with 100 µg/ml of the crude extract and fractions. The paper discs were kept in an incubator at 37°C for 24 hours to evaporate the solvent. 100 µl of the suspension of the organism (0.5 McFarland standard turbidity), containing 10^8 CFU/ml of bacteria was prepared from an overnight Mueller Hinton broth culture. The discs were arranged and firmly pressed on the agar surface of each seeded plate. The plates, after staying at 4°C for 2 hours, were incubated aerobically at 37°C for 24 hours for the organisms to grow. The negative control was also prepared using the same solvent employed to dissolve the plant extract. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism. All tests were carried out in triplicates and the average values were taken.

2.8 Column Chromatographic Separation of Hexane Fraction

The neutral non-polar hexane fraction (5 g) was chromatographed on a column of silica gel by eluting with mixtures of n-hexane and ethyl acetate in increasing polarity. Aliquots of 10 ml portion each were collected and the degree of purity of each was monitored by TLC using mixtures of hexane and ethyl acetate. Similar fractions were then combined, evaporated to dryness and weighed. Hexane eluted a fraction which on further purification gave the isolate, PT. H-d (0.4 g).

2.9 IR and GC-MS Analyses

The IR spectrum (neat) of the isolate PT.H-d was then obtained on FTIR 8400S Shimadzu Fourier Transform Spectrophotometer and the absorption values were recorded in wave numbers (cm^{-1}).

The isolate PT. H-d from the non-polar neutral hexane fraction of the crude 95% ethanol extract and the volatile oil (PT. STM-E) from the hydrodistillation of the fresh leaves were subjected to GC-MS analysis.

Thermo-Scientific Trace GC ULTRA system equipped with an AS 3000 auto sampler and a split/split-lessinjector was employed for the GC analysis. The column used was an DB-5 (optima-5), 30 m × 0.25 mm i. d., 0.25 µm d.f., coated with 5% diphenyl-95% polydimethylsiloxane, operated with the following oven temperature programme: 50°C, held for 1min, rising at 3°C/min to 250°C, held for 5 min, rising at 2°C/min to 280°C, held for 3 min; injection temperature and volume, 250°C and 1.0 µl, respectively ; injection mode, split ratio, 30:1; carrier gas was nitrogen at 30 cm/s linear velocity and inlet pressure 99.8 KPa; detector temperature, 280°C; hydrogen flow rate, 50 ml/min; air flow rate, 400 ml/min; make-up (H_2/air). Data were acquired by means of GC solution software (Thermo scientific). Thermo scientific Trace GC ULTRA AS 3000 auto sampler was interfaced with VG analytical 70-250, double- focusing mass spectrometer and the MS operating conditions were ionization: voltage 70eV with ion source of 250°C

3. RESULTS AND DISCUSSION

The results of qualitative phytochemical analysis of the crude extract of the leaf showed the presence of alkaloids, tannins, glycosides, saponins, flavonoids, phenols, triterpenes and sterols, but no carbohydrates. This is consistent with previous reports on some species of the genus [15,19,24], but only in few cases were the chemical constituents isolated and characterised [15,19].

The crude extract was subjected to antioxidant activity test and the results showed that the free radical scavenging activity of *P. thonningii* leaves crude extract using Vitamin C as standard was reasonably good, increasing with increasing concentration particularly at lower concentrations (Fig. 1). However, some workers have observed that DPPH scavenging activity may not be related to the concentration of the sample, but has a relationship with the time of reaction and that the composition of may have an impact on the antioxidant activity due to the presence of some constituents [25]. The antioxidant potential may be attributed to the presence of phenols and tannins. Previous workers had recorded the antioxidant activity of *P. thonningii* leaves using carbon tetrachloride-induced hepatic and oxidative damage in rat which showed that *P. thonningii* leaves protect liver against hepatic and oxidative damage by carbon tetrachloride possibly by acting as an *in-vivo* free radical scavenger through induction of antioxidant enzymes or drug detoxifying enzymes and by prevention of excessive stimulation of antioxidant enzyme and lipid peroxidation [12]. Thus, the results obtained in this work further support the antioxidant potential of *P. thonningii* leaves.

The results of antimicrobial activity screening of crude extract, fractions and hydrodistillate with

streptomycin as standard and measured as zones of inhibition (mm) are shown in Table 1. The crude extract and the non-polar neutral fraction when compared to the standard antibiotic, streptomycin, were found to be reasonably active against *E. coli, S. aureus, B. subtilis, P. aeruginosa, Streptococcus* spp *and Salmonella* spp at 100 µg/ml. The polar neutral fraction and steam distillate were active against all the test organisms except *B. subtilis*. The acidic fraction was not active against *S. aureus, P. aeruginosa, and Salmonella* spp at the same concentration (Table 1).

Thus, the antimicrobial and antioxidant activitivities of these extracts may be attributable to the phytochemicals found in the crude extract. Hence, it was very necessary to identify the chemical constituents of the fractions, particularly the hexane fraction, as well as the hydrodistillate through column chromatography and GC-MS analysis.

The biologically active n-hexane fraction was separated by column chromatography, eluting with mixtures of hexane and ethyl acetate. The hexane fractions 1-3 from the column were combined and purified to give an antimicrobial isolate PT.H-d (Table 2).

The bioactive column isolate, PT. H-d was subjected to IR analysis and gave the following absorption bands: 3347 supportive of OH group, 1732 which is suggestive of a ketone or ester carbonyl group and 1466 and 1375 for C-H bending vibrations. The GC of PT. H-d (Fig. 2) showed two important peaks at RT. 25.54 min for component PT. H-d-1 and at RT. 29.69 min for component PT.H-d-2. Direct comparison with standard computer library MS data and literature [25,26] were suggestive of lupeol, 1, and lup-20(29)-en-3-one 2. The presence of these components in PT.H-d is supported by the presence of OH and carbonyl CO absorptions in the IR spectrum, the molecular ion (M^+) m/z 426 for lupeol, the molecular ion (M^+) m/z 424 for lupenone and the characteristic MS fragment ions at m/z 218 (base peak), 207 and 189 for lupeol and lup-20(29)-en-3-one [26,27].

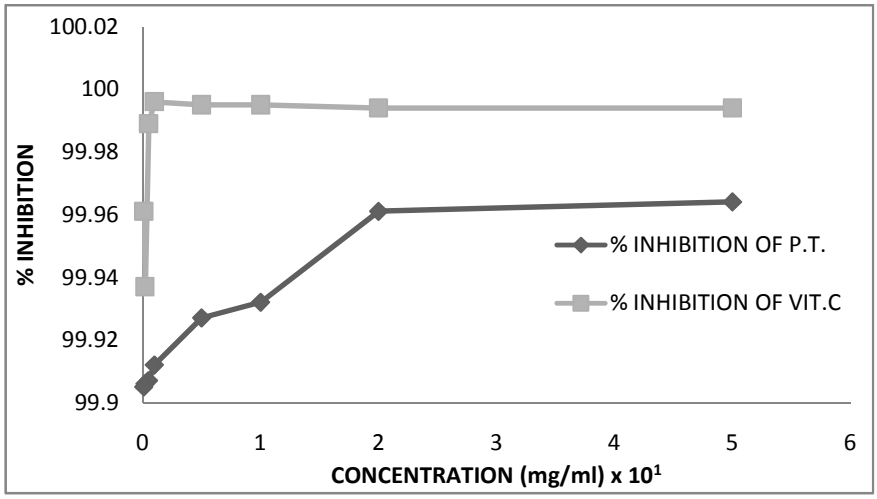


Fig. 1. Comparative percentage inhibitions of *Piliostigma thonningii* and vitamin C

Table 1. Screening of the antimicrobial potential of crude extract and fractions of leaves *P. thonningi* at 100 µg/mL (mm)

Sample	Sa	Ec	Bs	Ss	Pa	Sm
Crude extract	16.00±0.00	13.83±0.50	9.83±0.17	15.17±0.50	14.00±0.00	12.33±0.67
Non-polar	15.17±0.50	13.17±0.17	9.17±0.17	13.03± 0.03	13.00±0.67	10.07±0.07
Polar	14.17±0.50	10.10±0.57	-	12.23±0.10	12.10±0.57	12.00±0.00
Acidic	14.23±0.43	11.54±0.33	-	10.17±0.50	11.17±0.17	-
Basic	-	-	-	9.00±0.00	9.33±0.33	9.17±0.50
Hydrodistillate	10.10±0.67	10.17±0.50	-	11.00±0.67	12.07±0.07	11.83±0.50
Streptomycin	27.83±0.10	29.10±0.67	26.23±0.13	21.83±0.50	23.90±0.23	27.23±0.13

Values are means of triplicates, Sa= Staphylococcus aureus; Ec=Escherichia coli ; Bs = Bacillus substilis; Ss= Streptococcus spp; Pa= Pseudomonas aeruginosa; Sm=Salmonella spp

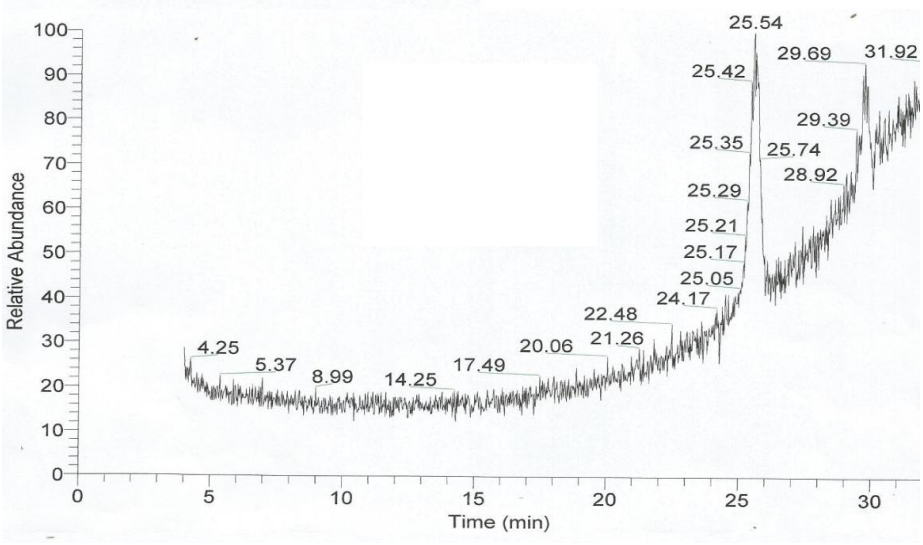
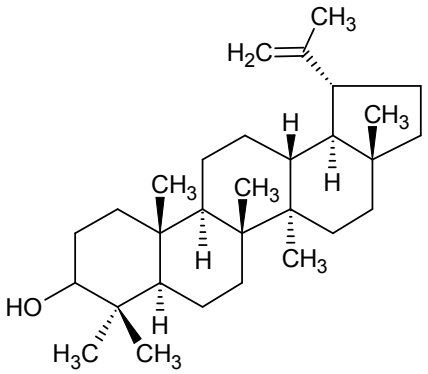
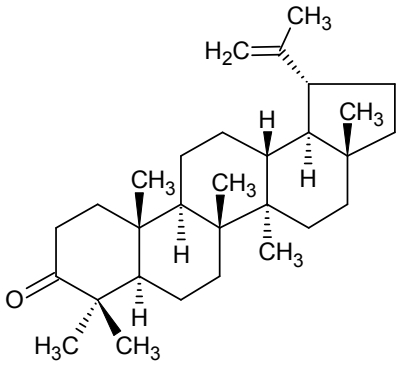


Fig. 2. Gas chromatogram of PT. H-d

The co-occurrence of lupeol, **1** and lup-20 (29)–en-3-one, **2** is not unusual as the ketone is actually a biogenetic oxidation product of the corresponding alcohol.



1. lupeol



2. lup- 20(29)-en-3-one

Table 2. Screening of antimicrobial potential of isolates from column chromatography of hexane fraction

Sample	Concentration/ Inhibition (mm)	
	5µg/ml	10µg/ml
PT. H-a	13.30±0.33	14.50±0.33
PT. H-b	14.67±0.33	18.50±0.33
PT. H-c	14.05±0.33	18.67±0.33
PT. H-d	15.00±0.00	19.50±0.33
PT. H-e	12.00±.00	14.00.±0.67

Values are means of triplicates, PT.H-a to PT. H-e = column chromatographic isolates from non –polar (hexane) fraction

The fresh leaves were hydrodistilled to give an antimicrobial oily residue, PT.STM-E (Table 1). The GC (Fig. 3) of PT.STM-E showed about six major components which on the basis of their Retention Times(RT), MS characteristics and by comparison with the NIST/EPA/NIH Mass Spectral Library data and literature were tentatively identified as a long chain acetylenic ester, 2,5–octadecadiynoic acid methyl ester [28], 1-methyl-6-3(methylbuta-1,3-dienyl)-7-oxa-bicyclo [4.1.0] heptane, trans-Z-a-bisabolene epoxide [29], cholestan-3-ol, 2-methylene-(3a,5a) [30], aromadendrene oxide-(2) [31] and isoaromadendrene epoxide [32]. The results of GC-MS analysis of PT.STM-E are summarized in Table 3.

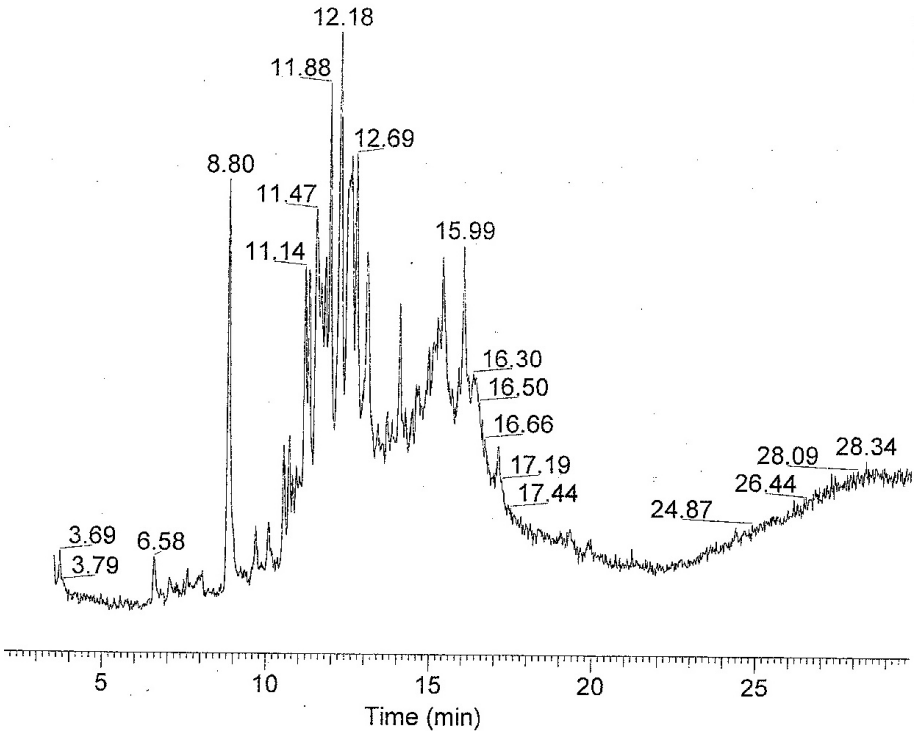


Fig. 3. Gas chromatogram of hydrodistillate PT.STM-E

Table 3. GC-MS of Volatile oil from hydrodistillation of fresh leaves of *P. thonningii*

S/N	Compounds	Retention time(mins)	Mol. Weight	Formula
1.	2, 5-octadiynoic acd methyl ester	8.82	290	$C_{19}H_{30}O_2$
2.	1-Methyl6-(3-methylbuta-1,3-dienyl)-7-oxabicyclo-[4.1.0]heptane	11.47	178	$C_{12}H_{18}O$
3.	Trans-Z- a-bisabolene epoxide	12.49	220	$C_{15}H_{24}O$
4.	Cholestan-3-ol, 2-methylene-(3a, 5a)	14.04	400	$C_{28}H_{48}O$
5.	Aromadendrene oxide-(2),	15.36	220	$C_{15}H_{24}O$
6.	Isoaromadendrene epoxide	15.95	220	$C_{15}H_{24}O$

4. CONCLUSION

The result of this study on the Nigerian *P. thonningii* has lent support to the antiinfective and antioxidant potentials of *P. thonningii* leaf irrespective of geographical location. Qualitative phytochemical screening results showed the presence of alkaloids, tannins, glycosides, triterpenes and phenolics in the 95% crude extract of the leaves. Two lupine triterpenoids known to possess antibacterial activity were identified from the bioactive n-hexane fraction of the *P. thonningii* leaf. The work also identified some volatile components in the leaf of *P. thonningii.* This is the first time these compounds have been identified from *P. thonningii* leaf. This work lends strong support to the traditional medicinal uses of the leaf of *P. thonningii* as an antiinfective agent in Nigeria.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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