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Phytochemical, Antimicrobial and GC-MS Analysis of the Bark Extracts and Fresh Leaves Hydro-distillate of *Piliostigma Thonningii* (Caesalpiniaceae)

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ABSTRACT: The plant *Piliostigma thonningii* (Caesalpiniaceae) is used in ethnomedicine for the treatment of a number of infections. In this work, the root and stem barks and the fresh leaves were investigated for antimicrobial activity and chemical constituents to confirm the traditional medicinal uses of the plant. The root and stem barks were separately subjected to extraction to obtain the crude methanol extracts, while the fresh leaves were hydro-distilled to obtain the volatile oils. The crude methanolic extract each was fractionated to obtain fractions; the hexane, chloroform, and 50% methanol-chloroform. Phytochemical screening of the two crude methanolic extracts showed that the stem bark extract contained most of the common phytochemicals such as saponins, terpenoids, glycosides, flavonoids, tannins, steroids, alkaloids, and carbohydrates, but the root-bark extract lacked terpenoids, flavonoids, steroids, and carbohydrates. The two crude extracts and their fractions were subjected to antimicrobial screening against some pathogens. The root-bark extracts generally did not exhibit reasonable activity (MICs=6.25mg/ml-50mg/ml) compared to the stem-bark extracts (MIC=500µg/ml-2000µg/ml). Also, for each of the extracts, the 50% chloroform/methanol fraction was the most active against the test organisms. The most active fraction, 50% chloroform/methanol of each extract, was subjected to GC-MS analysis which showed that the stem-bark extract had more volatile components (29) than the root-bark (17) at 0.5% and above. Also, for both extracts, the volatiles were mostly long-chain hydrocarbons, fatty acids, and derivatives (amides, esters, ketones, alcohols, and aldehydes) and phthalates. Of significance structurally, though in small quantity was 4-phenylbut-3-ene-1-yne in the root-bark extract. The fresh leaves of *Piliostigma thonningii* were subjected to hydro-distillation to obtain the volatile yellowish oil which was subjected to GC-MS to identify the chemical constituents. The gas-chromatogram showed 9 major components, including, 6-methyl-5-heptene-2-one, myrcene, linalool, carveol, trans-citral, cis-citral, cyclopropane methanol,2-methyl-2-(4-methyl-3-pentyl),6,10-dodecadien-1-yn-3-oland3,7,11-trimethyl-1,6,10-dodecatriene,7,11 dimethyl-3-methylene. Of great significance was the presence of citrals which constituted about 83% of the volatiles of the fresh leaves. Some of the above phytochemicals may be responsible for the traditional medicinal uses of the plant and therefore validate the ethnomedicinal uses of the plant in the management of microbial infections. Also, the high percentage of the citrals has shown that *Piliostigma* fresh leaves provide a new source of citrals which are important in the perfumery industry.

Keywords: Piliostigma *thonningii,* root bark, antimicrobial activity, chemical constituents, leaves, hydro-distillate, GC-MS, volatile components

INTRODUCTION

Members of the genus *Piliostigma* occur in tropical Africa and Indo-Malaya. *P. malabarium* (Roxb.) Benth. Var acidum de Wit, is however, confined to Asia and occurs in the drier monsoon belt of India, Malaysia and the

Philippines (Allen and Allen 1981) The two African species *P. reticulatum* (DC) Hochst and *P. thonningii* (Schum.) Milne-Redhead, inhabit dry and moist savannahs, respectively (Index kewensis, 1953; Supplementum XI 188:1941-1950). *Piliostigma thonningii* is a leguminous plant belonging to the family *Leguminosae -Caesalpinioideae*, It has *Synonym(s) like Bauhinia reticulata (non-DC) Broun and Massey, Bauhinia thonningii* (Schum), *Bauhinia abyssinica Rich, and Bauhinia pyrrhocarpa Hochst native to tropical Africa.* In Nigeria it bears such local names as *Abefe* (Yoruba), *Kalgo* (Hausa) and *Okpoatu* (Igbo). It is found growing abundantly as a wild uncultivated tree in many parts of Nigeria such as Zaria, Bauchi, Ilorin, Jos, Lagos, Abeokuta and some parts of Abuja. Ethno-medicinally, the bark, root, pod, young stem or leaves have been used for treating leprosy, smallpox, coughs, ulcer, heart pain, gingivitis, snake bite, dysentery, fever, wounds and a variety of closely related disease conditions (Irvine, 1961; Asuzu and Onu1994, Bombardelli, 1994, Dalziel, 1937, Watt and Breyer-Brandwijk, 1962; Bombardelli *et al*., 1973; Bombardelli *et al*., 1992; Okwute *et al.,* 1986).
Pharmacological investigations of some parts of *P. thonningii* such as the leaves (Okwute and Yakubu 2015; Asuzu, 1999) and stem bark (Asuzu and Onu 1994, Duniyan, 2011, Akinpelu and Obuotor 2000) had indicated that *P. thonningii* has some bioactivities. Also, chemical studies by some researchers had shown that the genus *Piliostigma* contains some flavonoids (Bombardelli *et al*., 1973), polyphenols (Bombardelli, 1994) and essential oils (Tira-Picos, 2010, Mustapha *et al.,* 2012). The presence of griffonilide in the stem bark (Okwute *et al*., 1986) and some other compounds in the leaves (Okwute and Yakubu 2015, Afolayan *et al*., 2018) of the Nigerian *P. thonningii* has been previously reported. This work reports on the comparative phytochemical, antimicrobial and GCMS analyses of the bark extracts and fresh leaves hydro-distillate of *Piliostigma thonningii.*

MATERIALS AND METHODS

The root and stem barks of *Piliostigma thonningii* were collected from Sheda, Abuja, Nigeria, and authenticated at Biological Sciences Department, Ahmadu Bello University, Zaria. A voucher specimen (Number: 171) was deposited in the Herbarium. The barks were cleaned by washing under a running water tap and air-dried under shade for three weeks. Each batch was then cut into small pieces and pulverized to a small particle size using a Hammer Mill machine. The fresh leaves of *Piliostigma thonningii* were collected from Angwan Lambu area in Keffi, Nasarawa State, Nigeria and identified at the Biological Sciences Department, Nasarawa State University, Keffi, Nigeria. The solvents used in the study were of standard grade and were re-distilled. Silica gel for TLC was mesh size 70-230. The TLC spots were viewed under UV lamp (254nm and 366nm) and observed with iodine vapour and Conc. H_2SO4-MeOH spray. The organisms for antimicrobial tests included *Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, Proteus mirabilis, and Streptococcus spp., Salmoneiia typhi, Bacillus subtillis, Candida albicans, and Aspergillus niger.* The test organisms were obtained from University of Abuja Teaching Hospital, Gwagwalada, Abuja, Nigeria and were maintained on nutrient agar slants. Aliquots of nutrient broth (20ml) were inoculated with the culture of test organisms using a loop and then incubated at 37 ^{0}C for 24 hours. Chloramphenicol was used as the reference antibiotic. GC-MS analysis was performed using GC-MS-QP2010 plus (Shimadzu, Japan) equipped with flame Ionization detector (FID). The injection was conducted in splitless mode at 250 ^{0}C for 3min by using an inlet of 0.75mm internal diameter (i.d) to minimize peak broadening. Chromatographic separations were performed by using DB-WAX analytical column 30 m x 0.25 mm (J&W scientific, Folsom C.A) with helium as carrier gas at a constant flow rate of 1.58ml/min. The oven temperature was programmed at 80 ºC for 2 mins, followed by an increase (held for 4 mins), and finally at 200^{0}C to 280ºC (held for 5 mins). The temperature of the FID was set to 250ºC. Mass Spectroscopy (MS) operating conditions (electron impact ionization mode) were an ion source temperature of 200ºC, ionization voltage of 70 eV and mass scan range of m/z 40-800.

Extraction and fractionation of the root bark

The dried powdered root and stem barks of *Piliostigma thonningii* (500g) were each extracted with methanol (1L) using a Soxhlet extraction apparatus. The extract was decanted and concentrated using a rotary evaporator to dryness. The crude extracts, 40.20g and 38.6g, respectively, were stored in the refrigerator until needed. The crude methanol extract, 20.0g each, was further partitioned into hexane, chloroform, and 50:50 chloroform/methanol to give approximately 0.34, 0.10 and 1.56%, respectively, of residues on evaporation to dryness.

Hydro-distillation of the fresh leaves

The fresh leaves (500g) were fully immersed in water in a flask and subjected to heat to boiling under a distillation set-up. A clean conical flask was placed to collect the distillate. After 4 hours of heating and boiling, 200ml of distillate was collected, followed by extraction of the aqueous medium at room temperature with diethyl ether in a 500ml separatory funnel to collect the oily mixture. Evaporation of the ether extract to dryness gave a pale

yellow oily residue (0.02g, 0.004%).

Phytochemical screening

Phytochemical tests on the crude methanol extracts were carried out according to standard methods (Sofowora, 2008).

Antimicrobial screening of root bark extractives

Antimicrobial activity tests were carried out on the methanol crude extracts and the hexane, chloroform and the 50 methanol/chloroform fractions using the Disc Diffusion method (Deeni and Sadiq 2002).

GC-MS analysis of extractives

The 50% chloroform-methanol soluble fractions from the root and stem bark extracts and the fresh leaves hydro-distillate were subjected to GC-MS analysis. The identification of the peaks was computer generated by comparing their mass spectra with those of the bibliography data of known compounds from the NIST library mass spectra database while the percentages were estimated according to the method of Wanakhachornkrai and Lertsiri (2003).

RESULTS AND DISCUSSION

The results of phytochemical tests for the stem and root bark crude extracts are presented in (Table 1). The two crude extracts and their fractions were subjected to antimicrobial tests. The results are shown in (Tables 2 and 3). The cleaned fresh leaves were steam-distilled and the hydro-distillate was subjected to GCMS analysis. The analysis data showing the major volatile components (0.50% and above) are shown in (Figure 1 and Table 6).
The stem and root barks were screened for phytochemicals and the results are reported in (Table 1). The stem bark extract is richer in phytochemicals than the root. While the stem bark contains terpenoids, steroids, tannins, glycosides, saponins, flavonoids, alkaloids and carbohydrates the root lacks terpenoids, steroids, flavonoids and carbohydrates. The presence of tannins, alkaloids, saponins flavonoids, cardiac glycosides in the bark has been earlier reported by Mustapha *et al.* (2012). Also, the rich tannins content of the root was validated by Tshisikhave *et al*. (2012). The absence of steroids and terpenoids in the root methanol extract is in agreement with the work of Bello *et al.* (2013). The antimicrobial tests results (Tables 2 and 3) on the extracts showed that the root bark extracts are generally more active against the test organisms, wit MIC values ranging from 500µg/ml to values>200µg/ml, while the stem bark extracts exhibited MIC values at 6.26mg/ml to 50mg/ml. Thus, the *Piliostigma* stem-bark extract was relatively inactive against the test organisms. However, for either stem or root bark the 50% chloroform/methanol was the most active among the fractions.

Consequently, the 50% chloroform/methanol fraction of each bark extract was subjected to GC-MS analysis for volatile chemical constituents (Tables 4 and 5). The tables contain organic volatiles present in 0.50% and above percent in each of the 50% chloroform/methanol fraction. From the tables, the stem bark fraction has about 29 major components while the root has about 17 components, consisting mainly of long-chain hydrocarbons, fatty acid and derivatives (amides, esters, alcohols, ketones and aldehydes) phthalates. Of some significance structurally and medicinally, though in small quantity (0.74%) is the presence of 4-phenylbut-3-ene-1-yne in the root extract. Among the volatiles, hexadecanoic acid, oleic acid and 4-phenylbut-3-ene-1-yne have been associated with spoilage metabolites (organic volatiles) from African horned cucumber fruits (Ibrahim *et al.,* 2011), and ripe tomato fruits inoculated with *Aspergillus flavus* (Karaye *et al.,* 2012). Hexadecanoic acid and octadecanoic acid (oleic acid) are also reported in literature among the fatty acids known to have antimicrobial and antifungal activity while oleamide (*cis*-9 10-octadecenoamide) is a brain lipid that has been isolated from the cerebral fluid of sleep-deprived cats (Cravatt *et al.,* 1996). It has also been identified as an antibacterial agent found in the seed of *Garcinea kola*. It showed activity against *Staphylococcus aureus*, *Plesiomonas shigelloides* and *Salmonella typhimurium* (Christinah and Roland, 2012).

Previously, we had investigated the biological activities and chemical constituents of the leaves of *Piliostigma thonningii* harvested in September, 2011, from the Medicinal Plant Reserve Garden of Sheda Science and Technology, Abuja, Nigeria. In that study the antimicrobial activity and chemical constituents of the hydro-distillate of the fresh leaves were reported (Okwute and Yakubu 2015). To investigate the effect of geographical location on the chemical constitutions of plants the hydro-distillate of fresh leaves collected from Keffi, Nasarawa State, Nigeria, in September, 2016, about 200 Km from the previous site in Abuja showed very striking differences in the chemical constitutions of the two hydro-distillates. In the new study, cis- and trans-citrals are the major constituents of the volatile oils, accounting for 83%. This is in addition to the presence of myrcene, linalool and carveol, all typical monoterpenoids in minor quantities (Figure 1 and Table 6). In contrast, the previous results did not reflect the presence of any monoterpenoids, but instead the presence of sesquiterpenoids such as aromadendrene and isoaromadendrene oxides and trans-Z-a-bisbolene epoxide. The citrals are known to occur naturally in a number of plants, including lemon grass from which the

Table 1. Phytochemical screening of stem and root bark crude extracts.

Phytochemicals	Stem Bark	Root Bark
Saponins	+	+
Terpenoids	+	-
Glycosides	+	+
Tannins	+	+
Flavonoids	+	-
Steroids	+	-
Alkaloids	+	+
Carbohydrates	+	-

Key: (+)=Present; (-)=Absent

Table 2. Antimicrobial screening of root bark extracts of *Piliostigma thonningii*

	MIC(µg/ml)/Microorganisms				
Extractive	**Ec**	**Kp**	**Pm**	**Sp**	**Sa**
Crude	>2000	>2000	>2000	>2000	>2000
Hexane	2000	>2000	1000	2000	2000
Chloroform	1000	>2000	**500**	2000	1000
50%Chloroform/methanol	500	1000	**500**	**1000**	**2000**
Ciprofloxacin	10	NT	NT	NT	10

Key: Ec=Escherica coli, Kp=Klebseilla pneumoniae, Pm=Proteus mirabilis, Sp=Streptococcus spp, Sa=Staphylococcus aureus, NT=Not tested

Table 3. Antimicrobial screening of the stem bark extracts of *Piliostigma thonningii.*

Parameters	**MIC(mg/ml) Microorganisms**						
Extracts	Sa	Bs	Ec	St	Kp	Ca	An
Crude methanol	12.5	25	12.5	12.5	-	-	-
Hexane	50	25	25	25	-	-	-
Chloroform	25	12.5	50	50	-	-	-
50%Chloroform/methanol	6.25	6.25	25	6.25	-	-	-
Ciprofloxacin (µg/ml)	10	-	10	-	-	-	-

Key: *Sa=Staphylococcus aureus, Bs=Bacillus subtillis, Ec=Escherichia coli, St=Salmonella typhi, Kp=Kleibsilla pneumonia, Ca=Candida albicans, An=Aspergillus niger***,** (-) =not tested.

Table 4. GCMS analysis data on 50% chloroform/methanol extract of root bark

Name of Compound	**RT**	**% Area**	**Mol. Formula**
4-Phenylbut-3-ene-1-yne	8.675	0.74	$C_{10}H_8$
Pelargonic acid	10.225	0.45	$C_9H_{12}O_2$
1-Dodecene	14.085	1.92	$C_{12}H_{24}$
Capric acid	14.404	1.79	$C_{10}H_{20}O_2$
2-Tridecene	16.739	4.67	$C_{13}H_{24}$
Palmitic acid	17.047	2.21	$C_{16}H_{32}O_2$
Isoprophyl myristate	17.300	0.44	$C_{17}H_{34}O_2$
Decanoic acid	19.289	3.75	$C_{14}H_{22}O_2$
Pentadecanoic acid	19.308	1.90	$C_{17}H_{34}O_2$
Decanoic acid	19.289	3.75	$C_{14}H_{22}O_2$
3-Tridecene	20.502	3.95	$C_{13}H_{26}$
n-Hexadecanoic acid	22.129	19.88	$C_{16}H_{32}O_2$
11-octadecanoic acid	22.530	3.61	$C_{19}H_{36}O_2$
Oleic acid	23.912	39.27	$C_{18}H_{34}O_2$
Adogen(oleamide)	26.207	4.33	$C_{18}H_{35}NO$
Di-n-octyl pthalate	27.714	22.40	$C_{24}H_{34}O_4$
9-octadecenyl aldehyde	29.409	3.83	$C_{18}H_{34}O_4$

Key: RT=Retention Time (mins.)

Table 5. GCMS Analyses data on 50% chloroform/methanol extract of stem bark.

Name of Compound	RT(Mins)	%Area	Mol. Formula
Tridecane	11.697	0.63	$C_{13}H_{28}$
Eicosanoic acid	13.394	0.69	$C_{20}H_{42}O_2$
Cetene	13.594	0.85	$C_{16}H_{32}$
Nonadecane	13.657	0.76	$C_{19}H_{40}$
Tritetracontane	14.525	1.12	$C_{43}H_{88}$
10-Heneicosene	15.279	1.58	$C_{21}H_{42}$
1-Nonadecene	15.280	3.32	$C_{19}H_{38}$
Heneicosane	15.325	2.08	$C_{21}H_{44}$
Vinyl 10-undecenoate	15.878	0.60	$C_{13}H_{22}O_2$
9-Heptadecanone	15.919	1.12	$C_{17}H_{34}O$
Oxiranedecyl	15.919	0.59	$C_{12}H_{24}O$
2-Methyl tetracosane	16.086	2.50	$C_{25}H_{52}$
Cyclopentane tridecanoic acid methyl ester	16.279	2.61	$C_{19}H_{36}O_2$
Hexadecanoic acid, methyl ester	16.282	4.58	$C_{16}H_{32}O_2$
Phthalic acid, butylundecyl ester	16.585	12.20	$C_{23}H_{36}O_4$
n-Hexadecanoic acid	16.589	11.67	$C_{17}H_{34}O_2$
1-Eicosanol	16.766	2.78	$C_{20}H_{42}O$
Tetratetracontane	16.807	2.71	$C_{44}H_{90}$
11-Octadecanoic acid, methyl ester	17.518	4.94	$C_{19}H_{36}O_2$
Methyl 9-methyltetradecanoate	17.674	0.95	$C_{16}H_{32}O_2$
9,12-Octadecadienoic acid(Z,Z)	17.786	5.93	$C_{18}H_{34}O_2$
Cis-Vaccenic acid	17.816	15.51	$C_{18}H_{34}O_2$
Oleic acid	17.951	5.91	$C_{18}H_{34}O_2$
1-Heneicosanal	18.109	7.83	$C_{21}H_{44}O$
Oxcyclododecan-2-one	18.779	2.88	$C_{11}H_{20}O_2$
Octacosanol	19.335	6.57	$C_{25}H_{55}O$
9-Octadecanal(Z)	19.880	6.00	$C_{18}H_{34}O$
7-Hexadecenoic acid, methyl ester	20.167	2.89	$C_{17}H_{32}O_2$
Bis(2-ethylhexyl)phthalate	20.262	10.49	$C_{24}H_{38}O_4$

Key: RT=Retention Time (mins.)

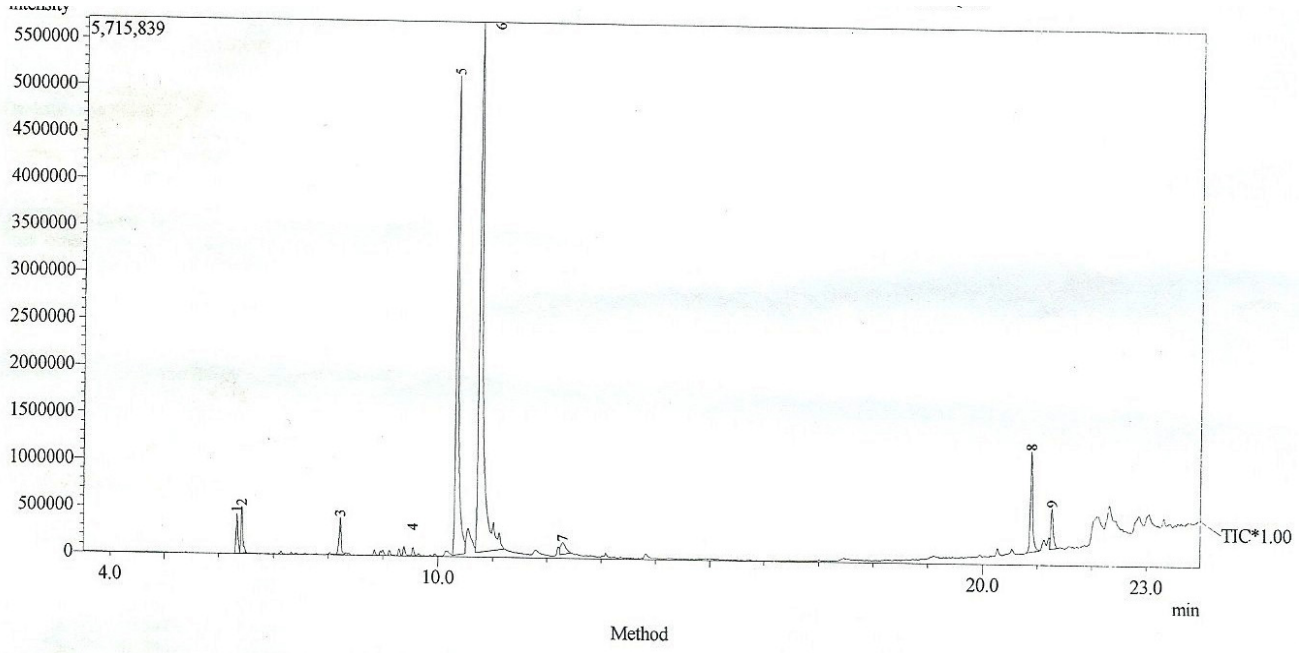

Figure 1. Gas chromatogram of fresh leaves hydro-distillate.

Table 6. GCMS analysis data on fresh leaves hydro-distillate.

Name of Compound	RT(Mins)	%Area	Mol. Formula
6-Methyl-5-heptene-2-one	6.330	1.95	$C_8H_{14}O$
Myrcene	6.420	2.23	$C_{10}H_{16}$
Linalool	8.214	2.02	$C_{10}H_{18}O$
Carveol	9.547	0.43	$C_{10}H_{16}O$
Trans-citral	10.334	31.78	$C_{10}H_{16}O$
Cis-citral	10.756	51.54	$C_{10}H_{16}O$
Cyclopropanemethanol, 2-methyl-2(4-methyl-3-pentyl)	12.294	1.50	$C_{11}H_{20}O$
6,10-Dodecadien-1-yn-3-ol,3,7,11-trimethyl	20.883	6.03	$C_{15}H_{24}O$
1,6,10-Dodecatriene, 7,11-dimethyl-methylene	21.268	2.54	$C_{15}H_{24}$

Key: RT=Retention Time (mins.)

mixture is extracted by hydro-distillation as a pale yellow liquid in 70-90%. It is about the most significant naturally occurring metabolite and is used as additives in diets, drinks and maquillages. It has antibacterial, antifungal and anticancer properties (San *et al*., 2015, Nashwa, 2017, Mohammad *et al*., 2019).Thus, apart from different geographical locations, home grown *Piliostigma thonningii* species has a different chemical constitution from the wild species.

Conclusion

This work has shown that the major volatile compounds of the bark extracts of Piliostigma are long-chain hydrocarbons and fatty acids and derivatives that have a wide range of therapeutic applications. In the work a rare aromatic terminal acetylenic compound, 4-phenylbut-3-ene-1-yne was also identified. In addition the fresh leaves have been demonstrated to contain a high content of citral and other monoterpenoids which are known to be antibacterial, antifungal and anticancer agents. The presence of these compounds may explain the ethno-medicinal use of the plant for the management of infections.

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