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Isolation and Characterization of an Anticonvulsant Principle from Leaf Extract of Pyrenacantha Staudtii

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Abstract:
Pyrenacantha staudtii was investigated as a potential source of novel anticonvulsant drug nucleus. Systematic isolation of compounds in the non – polar extracts (i.e n-hexane and dichloromethane) of the leaves of *Pyrenacantha staudtii* was carried out using column and thin layer chromatography. The isolated compounds were characterized using infra red (IR) and nuclear magnetic resonance (NMR) spectroscopic techniques to be bis(8-hydroxyl-2-methylnonyl) phthalate and bis(8-methylnonyl) phthalate. The two compounds were screened for anticonvulsant activity using mice. Bis(8-hydroxyl-2-methylnonyl) phthalate showed appreciable anticonvulsant activity but bis(8-methylnonyl) phthalate did not.
Key words: *Pyrenacantha staudtii*, Icacinacae, anticonvulsant

Introduction:
Pyrenacantha staudtii is an arborescent liana in the family of Icacinacae. It grows up to 6 m high and with a diameter of about 5-10 cm. It is found in secondary jungles in Southern part of Nigeria and West Cameroun (Burkill, 1994). The plant is widely used in traditional medicine for the treatment of hypertension, ulcer, inflammation, intestinal pain, blenorrhoea, hernia, and insomnia (Dalziel, 1995; Aguwa and Okunji, 1986). Phytochemical studies on the plant revealed the presence of Alkaloids, glycosides, saponins, tannins, flavonoids and acidic compounds (Falodun *et al*, 2005; Anosike *et al*, 2008). In a study to validate the traditional use of the plant in prevention of miscarriages, the leaf extract was found to exhibit significant inhibitory effect on oxytocin induced contractions on rat uterus at a dose dependent level (Falodun *et al*, 2005). Investigation of the methanol extract of the plant resulted in isolation of 3-carbomethoxypyridine (Falodun and Usifo, 2007) and 2-methoxylpyridine which was shown to have a relaxant effect on the smooth muscles of the uterus (Falodun *et al*, 2006). The crude aqueous extract of the plant has also been shown to possess analgesic, hypnotic and anticonvulsant activities (Awe *et al*, 2005). In this study, we report the isolation and characterization of two compounds from the leaf extracts of the plant one of which proved to be a potential anticonvulsant drug.

Experimental:
The infra red spectra data were obtained using KBr disc or nujol on Nicolet Avator FT – IR 330 by Thermo Electronic Corporation. The ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were obtained from a Varian Mercury spectrometer operating at 200 MHz for ^{1}H and 50 MHz for ^{13}C. The chemical shift values are reported in ppm relative to TMS as internal standard. All solvent used for extraction and column chromatography were General Purpose Reagent (GPR), redistilled before use. Gel filtration was performed using Sephadex LH – 20 previously swollen in specified solvent(s) prior to loading of extract onto the column (3.5 x 8.5 cm). Thin layer chromatography (tlc) were done with aluminium sheet pre coated with normal phase silica gel 60 F254 (Merck, 0.20 mm thickness). The tlc were run using suitable solvent systems. Spots were located on the developed tlc plates by visualization under ultraviolet light at 254 and 366 nm.

Sample collection:
The leaves of *Pyrenacantha staudtii* were collected from the premises of the University of Ibadan in the South-Western region of Nigeria, West Africa and identified at the herbarium of the Federal Institute of Forest Research, Ibadan, voucher specimen was deposited with number106886.

Extraction and Isolation:
A 660 g air-dried powdered leaves of *Pyrenacantha staudtii* was extracted at room temperature with 50% aqueous ethanol for 72 hrs. The resulting mixture was filtered and concentrated to dryness *in vacuo* on a rotatory evaporator to give the crude extract (75.0 g; 11.4%). The crude extract was redissolved in water and successively partitioned with n-hexane, dichloromethane and ethylacetate. The extracts thus obtained were concentrated to dryness on a rotatory evaporator yielding fractions as follows: n-Hexane (10.0 g; 1.52%); Dichloromethane (4.5 g; 0.68%) and Ethylacetate (2.3 g; 0.35%).
The n-Hexane extract (10.0 g) was subjected to silica gel open column chromatography eluting with different solvent combinations, starting with n-Hexane and a sequential increase in polarity by volume between n-Hexane, dichloromethane and ethylacetate. A total of ninety eight (98) fractions of 15 ml each were collected and

analyzed by tlc. Fractions with similar tlc profile were pooled together and concentrated to dryness *in vacuo* with a rotatory evaporator. Five fractions coded PH_A, PH_B, PH_C, PH_D & PH_E were obtained. Fraction PH_E was further purified on sephadex LH -20 (50% toluene-ethanol with gradient increase in polarity) to afford compound $1(P_1H_E)$.

The dichloromethane extract (4.5 g) was also subjected to silica gel open column chromatography with solvent combinations, starting with dichloromethane and then sequential increase in polarity by volume between dichloromethane, ethylacetate and methanol. A total of sixty seven (67) fractions of 15 ml each were collected and analyzed by tlc. Fractions showing similar tlc characteristics were pooled together and concentrated to dryness *in vacuo* with a rotatory evaporator. Eight fractions coded PD_A, PD_B, PD_C, PD_D, PD_E,PD_F,PD_G,PD_H were obtained and analyzed by tlc. Fraction PD_C was further purified through preparative thin layer chromatography with dichloromethane as the developing solvent. The band with R_f 0.54 was scraped, eluted with dichloromethane, filtered and concentrated to dryness to afford compound 2 (PD_1).

Animals:

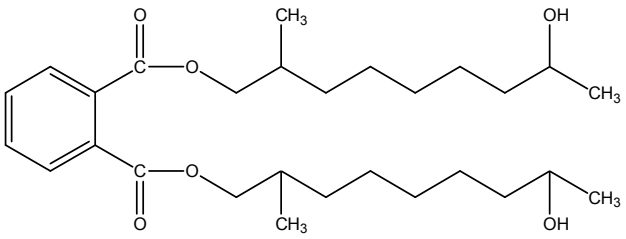
Mice (weighing 24-27 g) were used for the experiment. The animals were bred and housed under standard environmental conditions in the department of Pharmacognosy and Therapeutics, College of Medicine, University of Ibadan, Nigeria. They were fed with standard diet and water ad libitum. The animals were divided into four groups of five each.

DMSO – induced convulsion:

The method employed is as described by Elisha *et al* (1988). The first group of animals which serves as a control was administered with 2% DMSO intraperitoneally and the animals observed for latency minute of convulsion (i.e. time delay before the animal starts to show symptoms of convulsion which include: masticatory movement, head nodding and myoclonic jerking of limbs followed by wild running and subsequent tonic extension of the whole body) and mortality. In the other groups, 2%DMSO (10 mg/kg) was intraperitoneally administered into the mice thirty minutes after administration of different concentrations of the isolated compounds. The animals were then observed for tonic convulsions over a 24 hr period to note lethality.

Results and Discussion:

Compound 1 isolated from the n-Hexane extract was a yellow oil with R_f 0.5 in Dichloromethane : n-Hexane (3:1). The infrared spectrum of 1 showed signals at (υ cm^{-1}) 3377, 2927, 1728, 1580 and 744 corresponding to O – H stretching vibration of hydrogen bonded alcoholic group, C – H stretching vibration of alkane, carbonyl stretching of an ester, C = C stretching due to benzene ring and C – H out of plane bending of benzene C- H respectively. The signal at 744 cm^{-1} is also an indication of the 1,2-disubstitution of the benzene ring.



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The ^1H & ^{13}C NMR spectra data are presented in table 1. The ^{13}C-NMR spectrum of the compound showed it has a total of twenty eight carbon atoms comprising 4 methyl, 12 methylene, 8 methine and 4 quartenary carbons. However only half of each of the signals are observable in the spectrum because of the symmetrical nature of the molecule (having a plane of symmetry across the benzene ring and separating the two side chains). The ^1H NMR spectrum also showed the different proton environment as presented in table 1 and hence supports the structure.

Table1: ^{1}H NMR and ^{13}C NMR data for compound 1.

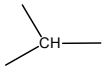
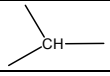
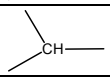
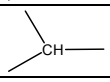
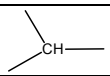
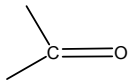
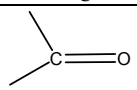
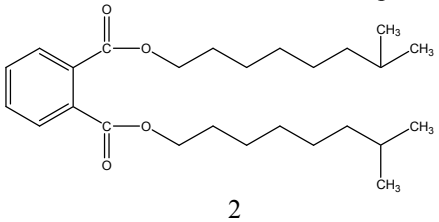
Position	δ_C (ppm)	C type	δ_H (ppm)	
2^1	11.182	CH_3	0.9	
9^1	14.285	CH_3	1.1	
3^1	23.215	CH_2	1.2	
4^1	23.959	CH_2	1.2	
5^1	29.140	CH_2	1.2	
6^1	29.949	CH_2	1.2	
7^1	30.574	CH_2	1.2	
2^1	38.943		2.3	
1^1	68.380	CH_2	3.5	
8^1	99.380		4.2	
4	129.022		7.5	
3	131.116		7.7	
1,2	132.664		1.2	
	168.004			

Table 2: ^{1}H & ^{13}C NMR data for compound 2

Position	δ_C (ppm)	C type	δ_H (ppm)	
8^1	11.204	CH_3	0.9	
9^1	14.314	CH_3	0.9	
2^1	23.236	CH_2	1.3	
3^1	23.964	CH_2	1.3	
4^1	29.153	CH_2	1.3	
5^1	29.949	CH_2	1.3	
6^1	30.579	CH_2	1.3	
7^1	38.939	CH_2	1.4	
11	68.396	CH_2	4.2	
4	129.040	CH	7.6	
3	131.164	CH	7.6	
2	132.626	C	-	
1	168.057		-	

A total of 26 carbon atoms were established in the ^{13}C - NMR, six of which are methine carbons at δ(ppm) 38.94, 129.04 and 131.16 ($C7^1$, C-4, C-3); two quartenary carbons at δ(ppm) 132.63 and 168.06 (C-2, C-1) and peak methylene carbons $C\text{-}2^1$, $C\text{-}3^1$, $C\text{-}4^1$ $C\text{-}5^1$, $C\text{-}6^1$, $C\text{-}1^1$ at δ(ppm) 23.24, 23.96, 29.15, 29.95, 30.58 and 68.40 respectively.The molecule is also symmetrical, hence only half of the signals are observed in the spectrum. This data is also consistent with the compound bis(8 - methylnonyl) phthalate.



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Tables 3 to 5 give the result of the anticonvulsant experiment. Table 3 shows that the average latency of minute

convulsion was three minutes on the average while the latency of minute death was four minutes. All the animals in this control group died given 100% mortality. In table 4, we have the result of the effect of compound 1 on the animals. At a dose of 1 mg/kg, the latency minute of tonic convulsion increased to five minutes when compared with the control and the percentage mortality decreased to 80%. Generally, as the concentration of compound 1 increases, the latency of minute tonic convulsion and latency of death minute increased while the percentage mortality decreased compared to control. At a dose of 4 mg/kg, the latent minute of convulsion increased to twenty minutes on the average and therefore the drug could be said to be anticonvulsant active at this concentration. Table 5, shows that all the rats treated with compound 2 died just as in the control even though there were marginal increases in latent minute of tonic convulsion and latent death minute compared to the control. This shows that compound 2 may not be effective as an anticonvulsant drug.

Table 3: Anticonvulsant activity on control group

2% DMSO

Animal	Dose mg/kg	Latency of minute tonic convulsion	Latency of death minute	% mortality	% protected
1	10	3	4	100	0
2	10	2	4	100	0
3	10	2	4	100	0
4	10	3	4	100	0
5	10	4	6	100	0
					All the animals died

Table 4: Effect of Compound 1 on DMSO – induced convulsion

Animal	Dose mg/kg	Minute tonic convulsion	Minute death	% mortality	%protected
1	1	5	7	80	20
2	1	6	8	80	20
3	1	5	6	80	20
4	1	5	7	80	20
5	1	5	7	80	20
					4 rats died

Animal	Dose mg/kg	Minute tonic convulsion	Minute death	% mortality	%protected
1	2	11	16	60	40
2	2	12	16	60	40
3	2	10	15	60	40
4	2	11	18	60	40
5	2	512	16	60	40
					3 rats died

Animal	Dose mg/kg	Minute tonic convulsion	Minute death	% mortality	%protected
1	4	16	20	20	80
2	4	20	25	20	80
3	4	18	22	20	80
4	4	21	26	20	80
5	4	25	29	20	80
					1 rat died

Table 5: Effect of compound 2 on DMSO - induced convulsion

Animal	Dose mg/kg	Minute tonic convulsion	Minute death	% mortality	%protected
1	1	5	8	100	0
2	1	6	9	100	0
3	1	7	10	100	0
4	1	5	7	100	0
5	1	5	6	100	0
					all rats died

Animal	Dose mg/kg	Minute tonic convulsion	Minute death	% mortality	%protected
1	2	5	9	100	0
2	2	6	12	100	0
3	2	7	11	100	0
4	2	7	13	100	0
5	2	8	15	100	0
					all rats died

Animal	Dose mg/kg	Minute tonic convulsion	Minute death	% mortality	%protected
1	4	6	13	100	0
2	4	7	14	100	0
3	4	9	16	100	0
4	4	10	18	100	0
5	4	12	19	100	0
					All rats died

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