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PHYTOCHEMICAL AND ANTICONVULSANT STUDIES ON THE METHANOL LEAF EXTRACT OF *COMBRETUM HYPOPILINUM* DIELS (COMBRETACEAE)

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ABSTRACT

Combretum hypopilinum is used by traditional medicine practitioners for the treatment of epilepsy, hepatic disorder, snake bites, relief of pains and headache. This study was aimed at investigating the anticonvulsant potentials in chicks and mice and to isolate compound(s) present in the methanol leaf extract (MLE) of *Combretum hypopilinum*. Preliminary phytochemical screening and oral median lethal dose (LD_{50}) were conducted using standard methods. Anticonvulsant studies were conducted using maximal electroshock test (MEST) in chicks and pentylenetetrazole induced-seizure (PTZ) in mice. The phytochemical screening revealed the presence of terpenoids, steroids, carbohydrates, cardiac glycoside, saponins, flavonoids, tannins and alkaloids. The column chromatographic analysis of the n-hexane fraction from the MLE led to the isolation of Lupeol which was characterized using chemical tests and spectroscopy analysis which include; Nuclear Magnetic Resonance (NMR), Infrared (IR) Spectroscopy and by comparison with data from literature. The LD_{50} of the extract was found to be greater than 5000 mg/kg. The extract at doses 125, 250 and 500 mg/kg did not exhibit significant effect on mean recovery time in MEST as none of the chicks were protected. However, the MLE at the dose of 500 mg/kg produced a significant ($p<0.05$) increase in the mean onset of seizures induced by PTZ, and dose independently protected the mice. In conclusion, the result of the study suggests that the MLE of *Combretum hypopilinum* contains phytochemical constituents with potential anticonvulsant activity and provides scientific rationale for the ethno-medicinal use of *Combretum hypopilinum* in the management of epilepsy.

Keywords: Anticonvulsants, Epilepsy, *Combretum hypopilinum*, Lupeol, seizure.

INTRODUCTION

The plant *Combretum hypopilinum* belongs to the family Combretaceae. The family has 600 species of trees, shrubs and tianans. *Combretum hypopilinum* is commonly called bush willow. In Nigeria, it is locally known as "*Jan Taramniya*" in Hausa, "*Katankara*" in Kanuri and "*Aro*" in Yoruba (Burkill, 1985). It is a small to medium size deciduous tree growing straight up to about 6-13 m tall (Burkill, 1985). It is used in Africa by the traditional medicine practitioners for the treatment of many ailments including epilepsy, snake bites, lung problems, jaundice, relief of pains and headaches, tooth aches or to plug a tooth with caries, hepatic disorder (Idoh *et al.,* 2018).

Epilepsy is a chronic disorder of the brain which affect people worldwide and it is characterized by recurring seizures which are brief episodes of involuntary movement

that may involve a part of the body or the entire body, and are also accompanied by loss of consciousness and control of bowel or bladder functions (WHO, 2018).According to the world health organization more than 50 million people are living with epilepsy globally, 80% of which live in low and middle-income countries and 75% do not receive treatment due to poor access to anti-epileptic medicines, societal misconceptions, poverty and low prioritization for the treatment of epilepsy (WHO, 2015). It was also estimated globally that in high income countries, 2.4 million people are diagnosed yearly with, new annual cases are between 30 and 50 per 100, 000 people in the general population, while, in low and middle income countries, this figures are two times higher, which is likely due to the increased risk of endemic conditions such as malaria, the higher incidence of road injuries, birth-related injuries, availability of medical infrastructure and preventive health care programmes which can be easily accessible and affordable (WHO, 2019). These scientific investigations of the anticonvulsant potential of the leaf part of *C. hypopilinum* will confirm or otherwise disprove its traditional use for the management of epilepsy.

MATERIALS AND METHODS

Collection and Identification of Plant Material

The leaf of *Combretum hypopilinum* was collected from Giwa local government, Kaduna State, Nigeria, in the month of April 2018. The plant sample was identified and authenticated by Mallam Namadi Sunusi of the Herbarium unit of the Botany Department, Faculty of Life Sciences, Ahmadu Bello University, Zaria, by comparing it with a standard specimen with voucher number 090143.

Experimental Animals

Locally breed adult Swiss Albino mice of either sex weighing between 19-40g were obtained from animal house of the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria were used for the study. In addition, chicks weighing between 24-34 g were obtained from Chi farms limited along Ibadan-Lagos express way, Ibadan, Oyo state. The animals were maintained and fed with standard animal feed and water *ad libitum*.

Extraction and Partitioning of Plant Material

The leaves of the plant were shade dried, pulverized maually using a clean wooden mortar and pestle to reduced the sizes. The powdered plant material (1500 g) was extracted with 80% methanol (10 L) using cold maceration for 72 hours with occasional shaking. The solvent was removed using a rotary evaporator at reduced pressure to yield a dark greenish residue (183.03g) subsequently referred toas methanol leaf extract (MLE). The MLE (170.03 g) was subjected to dry partitioning with n-hexane, chloroform, ethyl acetate and acetone which afforded n-hexane (nHF), chloroform (CF), ethyl acetate (EF) and Acetone fractions (AF) respectively.

Preliminary Phytochemical Screening

Various chemical tests were carried out on the MLE, nHF, CF, EF and AF according to standard chemical procedures to identify the presence or absence of phytochemical constituents (Trease and Evans, 1983).

Isolation of C_2

The column was packed using slurry method with silica gel (60-120 mesh size) suspended in n-hexane and allowed to settle. The n-hexane fraction (10g) was dissolved in n-hexane, and small amount of silica gel was added to it, which was then allowed to dry in

order to form a powdered mixture. The dried mixture was then packed on the cotton wool slightly above the silica gel bed. The column was eluted starting with n-hexane 100% followed by mixture of n-hexane and ethyl acetate 98:2 and increasing the polarity gradually by 2% up to 100% ethyl acetate, then finally washed with 100% methanol. A total of 130 collections of 100 ml each were obtained. Based on the TLC profile of the various collections, different column fractions were pooled together into eight (8) major column fractions depending on the similarities of their TLC profiles which were coded as A, B, C, DE, F, J, L and M. The column fraction coded C showed a major spot which was chromatographed over silica gel (60-120 mesh size) in a small capillary column using isocratic elution technique of a mixture of n-hexane and ethyl acetate 5:1. A total of 20 collections were obtained, and were pooled together based on their TLC profile and coded C_1. C_1 was further washed with 100% n-hexane to produce a white solid crystal coded as C_2. C_2 was then subjected to chemical test and spectral analysis using IR, 1D and 2D NMR in order to elucidate their structure.

Acute Toxicity Study

Nine (9) mice were divided into three groups of three mice each. In the first phase, varying doses of the MLE (10, 100 and 1000 mg/ kg body weight) were administered orally and the mice were observed for 24 hours for any signs of toxicity and mortality. In the second phase, based on the outcome of the first phase, three (3) groups of one mouse each were treated with the doses of the MLE (1600, 2900 and 5000 mg/ kg) and the mice were observed for any sign of toxicity and death for 24 hours. (Lorke, 1983). The median lethal dose was estimated as a geometric mean of the highest non-lethal dose (with no death) and the lowest lethal dose (where death occurred using formula).

$$LD_{50} = \sqrt{minimum\ lethal\ dose \times maximum\ tolerated\ dose}$$

Maximal Electroshock test (MEST) in Chicks

The Swinyard and Kupferberg (1989) method was employed. Day old Chicks weighing between 24-34 g were randomly divided into 5 groups of 10 each. Group 1 were treated with normal saline 10 ml/kg. Group 2, 3 and 4 were treated with 125, 250 and 500 mg/kg doses of the MLE respectively, while the group 5 were treated with phenytoin 20 mg/kg used as positive control. 60 minutes later, maximal electroshock was administered to induce seizure in all the groups using Ugo Basil electroconvulsive machine (Model 7801) with corneal electrodes placed on the upper eyelids of the chicks. The current, shock duration, frequency and pulse width was maintained at 80 mA, 0.8 s^{-1}, 100 pulse per second and 0.6 Ms respectively. The chicks were observed for hind limb tonic extension (HLTE). An episode of HLTE was regarded as full convulsion while lack of HLTE was regarded as protection. For the unprotected animals, their recovery time were recorded.

Subcutaneous Pentylenetetrazole (sc PTZ) induced-seizure in Mice

The Swinyard and Kupferberg, (1989) method was employed. Twenty-five (25) mice of either sex weighing between 18-32 g were randomly divided into five (5) groups of five (5) mice each. Mice in group 1 were treated with normal saline 10 ml/kg. Group 2, 3 and 4 were treated with 125, 250 and 500 mg/kg doses of the MLE respectively, while group five (5) were treated with 200 mg/kg sodium valproate. 60 minutes later, mice in all groups were treated with 90 mg/kg body weight (CD_{97}) of freshly prepared PTZ orally. The mice were then observed for the presence or absence of clonic spasm (seizure) for at least

five (5) seconds. The absence of seizure was considered as protection.

RESULTS AND DISCUSSION

The preliminary phytochemical screening of the MLE of *Combretum hypopilinum* revealed the presence of carbohydrates, saponins, cardiac glycosides, alkaloids, tannins, flavonoids, triterpenes/steroids, while the n-hexane fraction revealed the presence of triterpenes and steroids only. However, anthraquinones were not detected in both the MLE and the fractions (table 1).

Table 1: Phytochemical Constituents of Methanol Leaf Extract of *Combretum hypopilinum* and its Fractions

Constituents	Test	Inferences				
		Extract/fractions				
		MLE	HF	CF	EF	AF
Alkaloids	Meyers	+	-	-	+	+
	Dragendoff	+	-	-	+	+
	Wagners	-	-	-	-	-
Anthraquinones	Bontrager	+	-	-	-	-
Carbohydrates	Molisch	-	-	-	+	+
Cardiac Glycosides	Keller-kiliani	-	-	+	+	+
Flavonoids	Sodium hydoxide	+	-	-	+	+
Saponins	Frothing	+	-	+	+	+
Steroids	Salkwoski	+	+	+	-	-
Tannins	Ferric chloride	+	-	+	+	+
Triterpenoids	Liebermann Buchard	+	+	+	-	-

Key: + = present, - = absent

Column chromatographic separation of the n-hexane fraction followed by column chromatography of column fraction C led to the isolation 8 mg of a white solid crystals coded C_2. C_2 gave a single homogenous spot when subjected to thin layer chromatography using H: EA (5:1) and H: EA (9:1) as solvent system with Rf values of 0.50 and 0.31 respectively, this indicates the purity of the compound. C_2 was found to be completely soluble in chloroform and has a melting point of 150-152^{0}C. The appearance of red colour to Liebermann-Burchard's test and reddish-brown ring at the interphase to Salkowski's test suggests that compound C_2 to be a triterpenoid/steroid (Trease and Evans, 1996).

The IR spectrum of compound C_2 showed characteristic absorption frequencies at 3362.1 cm^{-1} (OH stretching), 2922.2 cm^{-1} (asymmetric C-H stretching) for CH_3 part, 2855.1 cm^{-1} (symmetric C-H stretching) for CH_2 part, a weakly intense band at 1640 cm^{-1} was observed due to the olefinic unsaturation (C=C stretching), medium intense bands at 1453.7 cm^{-1} (C-H bending) for the CH_3 part and 1379.1 cm^{-1} (C-H bending) for the CH_2 part and the corresponding C-O vibration was shown as weakly intense band at 1032.5 cm-^{1} (Haruna *et al*., 2017).

The ^{1}H NMR ($CDCl_3$) spectrum of compound C_2 varied between δ_H 0.79 – δ_H 4.68 revealed the presence of seven methyl proton resonances each appears as singlets at δ_H 1.01 (23), δ_H 0.79 (24), δ_H 0.83 (25), δ_H 1.07 (26), δ_H 0.97 (27), δ_H 0.76 (28) and δ_H 1.68 (30) ppm respectively (integrated for 3H-each). This proton signals are attributed to resonance of over lapping methyl and methylene protons, a characteristic framework of steroids (Yun-song *et al*., 2006; Jephtha *et al*., 2014). A methine signal at δ_H 2.34 was attributed to the proton at C-19 position. The H-3 proton resonance a multiplet at δ_H 3.20 while a pair of doublets at δ_H 4.68 and δ_H 4.57 ppm (IH, each) was indicative of olefinic proton signals δ at (H-29 a and b) respectively. These assignments are in good agreement for the structure of lupeol (Abdullahi *et al.,* 2013; Haruna *et al.,* 2017).

The ^{13}C NMR ($CDCl_3$) experiment revealed the presence of 30 signals for the triterpenoid of lupane skeleton. It also revealed the de-shielded quaternary carbon atom at δ_C 151.2 (C-20) due to the presence of the olefinic bond.

The DEPT-135 experiment of compound C_2 revealed thirteen (13) positive signals due to seven methyl (CH_3) δ_C 28.21 (C-23), δ_C 15.59 (C-24), δ_C 16.34 (C-25), δ_C 16.19 (C-26), δ_C 14.77(C-27), δ_C 18.22 (C-28), and δ_C 19.52 (C-30).
Six methine (CH) carbon atoms δ_C 79.22 (C-3), δ_C 55.51 (C-5), δ_C 50.66 (C-9), δ_C 38.27 (C-13), δ_C 48.52 (C18), δ_C 48.21 (C-19).
Eleven (11) negative signals due to the methylene (CH_2) carbon atoms δ_C 39.08 (C-1), δ_C 27.64 (C-2), δ_C 18.54 (C-6), δ_C 34.50 (C-7), δ_C 21.15 (C-11), δ_C 25.36 (C-12), δ_C 27.66 (C-15), δ_C 35.80 (C-16), δ_C 30.07 (C-21), δ_C 40.22 (C-22), δ_C 109.54 (C-29). This agrees with the ^{13}C NMR signals (Abdullahi *et al*., 2013).
The HSQC experiment of compound C_2, helped in determining the direct connectivity of the hydrogen and carbon atoms, among others are as follows; δ 4.68 (H-29a) and δ 4.57 (H-29b) and δ 109.54 (C-29), δ 2.41 (H-19) and δ 48.21 (C-19), δ 1.91 (H-21) and δ 30.07 (C-21), δ 1.37 (H-18) and δ 48.52 (C-18), δ 1.29 (H-9) and δ 50.66 (C-9), δ 3.20 (H-3) and δ 79.22 (C-3) and δ 0.69 (H-5) and δ 55.51 (C-5), δ 1.68 (H-30) and δ 19.52 (C-30).
The HMBC spectrum of compound C_2 showed the established two (2) to four (4) multiple bonds between the hydrogen and carbon resonances of the compound which

were also helpful in the assigning the quaternary carbon atoms, among others are as follows; δ_H4.68 (H-29a), δ_H4.57 (H-29b) correlate with δ_C19.52 (C-30) and δ_C48.21 (C-19), δ_H2.41 (H-19) correlate with δ_C19.52 (C-30), δ_C109.54 (C-29), δ_C151.20 (C-20) and δ_C48.52 (C-18), δ_H1.68 (H-30) correlate with δ_C48.21 (C-19), δ_C109.54 (C-29) and δ_C151.20 (C-20). These spectral data (as summarised in table 2) were in agreement with what those reported for Lupeol by (Abdullahi *et al*. 2013). The proposed structure of compound C_2 was found to be lupeol (Figure 1).

The oral median LD_{50} of the MLE of *Combretum hypopilinum* was found to be greater than 5000 mg/kg body weight in mice which is relatively safe (Lorke, 1983). The MLE was tested using MEST and PTZ induced-seizure for anticonvulsant activity. The results obtained were summarised on Table 3 and 4.

The MLE exhibit no significant effects on the mean recovery time in MEST and none of the chicks were protected against the hind limb tonic extension (HLTE) induced by the maximal electroshock test (MEST).

Table 2: Comparison of Proton (^{1}H), Carbon-13 (^{13}C) and DEPT Chemical Shifts of the Isolated Compound and Lupeol with literature data (Abdullahi *et al*., 2013)

S/No	δ^1H	δ^{13}C	DEPT	δ^1H Abdullahi *et al*, 2013	δ^{13}C Abdullahi *et al*, 2013	DEPT 135 Abdullahi *et al*, 2013
1	0.94	39.08	CH_2	0.93	38.80	CH_2
2	1.63	27.64	CH_2	1.60	27.20	CH_2
3	3.20	79.22	CH	3.19	79.30	CH
4	-	38.93	C	-	39.10	C
5	0.69	55.51	CH	0.68	55.30	CH
6	1.20	18.54	CH_2	1.40m 1.19m	18.30	CH_2
7	1.39	34.50	CH_2	1.38	34.20	CH_2
8	-	41.05	C	-	41.10	C
9	1.28	50.66	CH	1.28	50.60	CH
10	-	38.39	C	-	37.31	C
11	1.41	21.15	CH_2	1.40	21.10	CH_2
12	1.71	25.36	CH_2	1.68	25.50	CH_2
13	1.62	38.27	CH	1.63	38.30	CH
14	-	43.05	C	-	43.20	C
15	0.99	27.66	CH_2	0.97	28.10	CH_2
16	1.45	35.80	CH_2	1.45	35.20	CH_2
17	-	43.22	C	-	43.30	C
18	1.37	48.52	CH	1.38 (1H, dd)	48.30	CH
19	2.41	48.21	CH	2.36 (1H, m)	48.10	CH
20	-	151.20	C	-	150.9	C
21	1.91	30.07	CH_2	1.28 (2H, m)	30.00	CH_2
22	1.26	40.22	CH_2	1.17	40.50	CH_2
23	1.01	28.21	CH_3	0.99	28.00	CH_3
24	0.79	15.59	CH_3	0.75	15.60	CH_3
25	0.83	16.34	CH_3	0.80	16.20	CH_3
26	1.07	16.19	CH_3	1.02	16.00	CH_3
27	0.97	14.77	CH_3	0.91	14.80	CH_3
28	0.76	18.22	CH_3	0.75	18.20	CH_3
29	4.68 4.57	109.54	CH_2	4.67 (1H, dd) 4.56 (1H, d)	109.50	CH_2
30	1.68	19.52	CH_3	1.68 s	19.40	CH_3

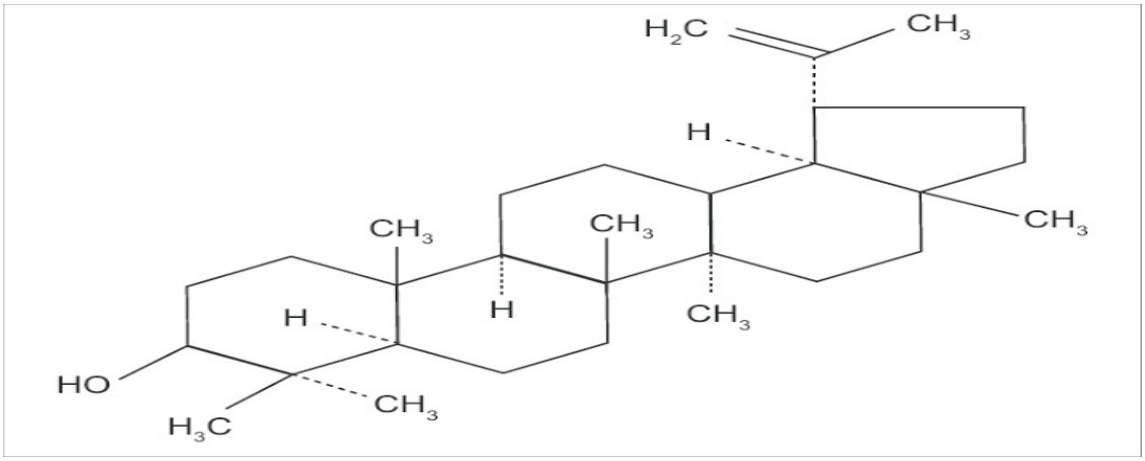

Figure 1: Proposed structure of C_2: Lupeol

Table 3: Effect of Methanol Leaf Extract of *Combretum hypopilinum* and Phenytoin (PHT) on Maximal Electroshock-induced seizure in Chicks

Treatments	Mean recovery Time (min)	Quantal protection of HLTE
NS 10 ml/kg	10.70 ± 1.21	0/10
MLE 125 mg/kg	8.10 ± 0.38	0/10
MLE 250 mg/kg	6.70 ± 0.58*	0/10
MLE 500 mg/kg	6.40± 0.87*	0/10
PHT 20 mg/kg	_	10/10

One-way ANOVA was used for the statistical analysis, mean recovery time expressed as mean ± SEM; n = 10 per group; at *$p < 0.05$ statistically significant difference when compared with normal saline (NS)

However, the MLE showed significant and dose-dependent increase in protections against clonic spasm (seizure) in all tested doses 125, 250 and 500mg/kg as 20%, 40% and 60% respectively, against the PTZ-induced clonic spasm (seizure) when compare to the control group (PHT 20 mg/kg), and also increase in the mean onset of seizures with a statistically significant difference (*p<0.05) at the highest dose 500 mg/kg.

Phenytoin (PHT) the standard drug used for the MEST gave 100% protection to the chicks against HLTE as shown in Table 4. The MEST is a non-mechanistic seizure model that are clinically effective in identification of anticonvulsant drugs such as; phenytoin, carbamazepine, primidone, phenobarbital, sodium valproate and lamotrigine which all prevent the HLTE in MEST for the management of generalized and partial seizures (Browning, 1992; Rho and Sankar, 1999). The ability of an agent to prevent against HLTE or to prolong the latency (delay) of the HLTE was considered as an indication of anticonvulsant activity (Swinyard, 1969; Sayyah *et al.,* 2002). Therefore, the inability of the MLE to prevent against HLTE suggest that, it may not be beneficial in the management of generalized and partial seizure.

Table 4: Effect of MLE of *Combretum hypopilinum* and Sodium Valproate on Pentylenetetrazole-induced Clonic Spasm (seizure) in Mice

Treatments	Mean onset of clonic spasm (min)	Quantal Protection against clonic spasm	% Protection against clonic spasm	Quantal Protection against mortality
NS 10 ml/kg	5.00 ± 0.55	0/5	0	0/5
MLE 125 mg/kg	6.80 ± 0.58	1/5	20	2/5
MLE 250 mg/kg	7.20 ± 0.58	2/5	40	4/5
MLE 500 mg/kg	9.80 ± 2.33*	3/5	60	4/5
VPA 20 mg/kg	_	5/5	100	5/5

One-way ANOVA was used for the statistical analysis, Mean onset of clonic spasm expressed as Mean ±SEM, n = 5, at *$p<0.05$* statistically significant differences when compared with normal saline (NS), VPA = Sodium valproate.

However, the MLE at all tested doses (125, 250 and 500 mg/kg) produced a dose dependent anticonvulsant activity against PTZ-induced clonic spasm (seizures) in mice. The MLE of the highest dose (500 mg/kg) gave 60% maximum protection against clonic spasm induced by the PTZ which was compared with the standard drug (sodium valproate) that gave 100% protection against clonic spasm (Table 5). It has been found empirically that drugs which inhibit PTZ-induced seizures and raise the threshold for production of electrically (mean onset of time) induced seizures are generally effective against absence (Petit mal) seizures (Rang *et al.,* 2003). The dose dependent anticonvulsant activity against PTZ-induced seizures suggest the presence of bioactive compounds effective in the therapy of absence (petit mal) or myoclonic seizures. Lupeol and its related compounds have also demonstrated to possess some activity in the nervous system. For example, lupeol significantly enhanced [3H]-glutamate uptake by astrocyte cultures and may play a role in treatment for neurodegenerative disorders (Martini *et al.,* 2007).

CONCLUSION

Based on the results presented above, the leaf of *Combretum hypopilinum* contains alkaloids, flavonoids, terpenoids, triterpenoids/steroids, tannins, carbohydrates and cardiac glycosides.

Lupeol has been isolated from the leaf of *Combretum hypopilinum*. The compounds were characterized on the basis of IR, NMR spectral data. To the best of our knowledge, this is the first report on the presence of this compounds in *Combretum hypopilinum*. The MLE demonstrated significant anticonvulsant activities by increasing the onset of seizure and giving protections against seizure in the PTZ-induced seizure in mice, the findings of this study suggested that the MLE contain bioactive principles that may be beneficial in the treatment/management of petit mal seizure and further provided scientific validity for the use of the leaf of *Combretum hypopilinum* in the management of epilepsy in traditional medicine. Further work will involve purification of the column fractions in order to isolate the compound responsible for observed anticonvulsant activity.

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