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Isolation of Oleanolic Acid from *Parinari curatellifolia* (Planch Ex. Benth) Stem Bark and Evaluation of its Anticonvulsant and Sedative activities in Rodents

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ABSTRACT

Parinari curatellifolia is used by traditional medicine practitioners for the treatment of epilepsy. So far, no study has isolated the active principle that may be responsible for its anticonvulsant activity. The study aimed to isolate compound(s) present from *Parinari curatellifolia* that may be responsible for its anticonvulsant activity. The ethyl acetate fraction of the stem bark of *Parinari curatellifolia* was chromatographed over silica gel column chromatography which led to the isolation of compound **C**. The structure of the compound was elucidated using IR, ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectroscopy. Acute toxicity study of the isolated compound was evaluated in mice using OECD 425 guidelines (2000 mg/kg orally). The anticonvulsant study of the isolated compound (at 50, 75 and 100 mg/kg) was evaluated in mice using pentylenetetrazole (PTZ)-induced convulsion. The sedative properties of the compound (at 10, 50 and 100 mg/kg) were evaluated using the diazepam-induced sleep model in rats. Structure elucidation of the isolated compound confirmed the compound to be oleanolic acid. Acute toxicity study revealed no lethal effects at 2000 mg/kg. the compound (oleanolic acid) significantly ($p<0.05$) increased the onset of seizure at all doses and resulted in 25% protection against seizure at 100 mg/kg. It exerted sedative effect at all doses by significantly ($p<0.05$) reducing sleep latency and increasing total duration of sleep induced by diazepam. The results obtained from this study have revealed the presence of oleanolic acid in *P. curatellifolia* and have shown its anticonvulsant and sedative activities for the first time.

Keywords: Anticonvulsant, Acute toxicity, Oleanolic acid, *Parinari curatellifolia*, Sedative, Pentylenetetrazole.

Introduction

Epilepsy is a chronic neurological disorder which is estimated to affect 60 million people or more worldwide.[1] It is a heterogeneous syndrome often characterized by recurrent and spontaneous seizures.[2] Several attempts have been made to treat/manage this disorder using anti-epileptic drugs (AED). The contemporary treatment of epilepsy with modern AED is often associated with side effects.[3, 4] In search for safer alternatives, people have resulted to the use of traditional/herbal or complementary/alternative medicines in the treatment of this disorder. Among such herbal medicine is the use of the stem bark extract of *Parinari curatellifolia*.

Parinari curatellifolia Planch Ex. Benth (Chrysobalanaceae) is used in Africa by traditional medicine practitioners for the treatment of many ailments including cancer, malaria fever, epilepsy and bacterial infections.[5; 6] In Nigeria, oral communication has it that this plant is used for the treatment of malaria fever, epilepsy and convulsion.

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Previous scientific studies have shown the anticonvulsant properties of this plant.[7] Other pharmacological activities of this plant include antiplasmodial,[8] antioxidant,[9] anti-inflammatory[10] and hypoglycaemic effects.[11] Previous studies have reported the isolation and characterization of *β*-sitosterol, stigmast-4-en-3-one, stigmasterol, betulin and betulinic acid from the stem bark extract of *Parinari curatellifolia*.[9;12] This current study is therefore designed to isolate potential bioactive compound(s) from the ethyl acetate fraction of the stem bark of *Parinari curatellifolia* and to evaluate its anticonvulsant and sedative activities.

Materials and Methods

General Experimental Procedures

Thin layer chromatography was run on pre-coated aluminium-back plate (Silica gel 60 F_{254}; Merc Germany). The spots were visualized under UV light at 254 nm for detection of UV active compounds. The plate were stained with phosphomolybdic acid solution and heated in an oven for 2 minutes at 105ºC for identification of colourless compounds. Melting point was determined on Gallencamp USA melting point apparatus. ^{1}H-NMR, ^{13}C-NMR and DEPT-135 experiments were run on Top Spin 400 MHz NMR spectrometer (Bruker, Germany) using TMS as internal standard. IR analysis was done on KBr disc using the Perkin Elmer Spectrum RX FT-IR System.

Plant Material and Preparation of Extracts

The stem barks of *P. curatellifolia* were collected from Zaria, Kaduna State, Nigeria, in September 2011 and identified at the Herbarium Unit,

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Department of Biological Sciences, Faculty of Science, Ahmadu Bello University, Zaria-Nigeria by U.S. Gallah (Taxonomist). A voucher specimen (903) of the plant was deposited at the herbarium. The stem bark of the plant was shade dried for one week and was powdered using wooden pestle and mortar. The powdered plant material (3 kg) was extracted successively with 8 L each of n-hexane, ethyl acetate and methanol by cold maceration for 24 h. The extracts were filtered and concentrated using a rotary evaporator at reduced pressure. The yield of ethyl acetate extract was 8.2% (w/w) and was used for the study.

Column Chromatography
Silica-gel slurry (150 g) was packed in a glass column (65 cm x 3.0 cm) using 100% n-hexane and allowed to stabilize for 2 h. The powdered ethyl acetate extract (4 g) was adsorbed onto silica gel and loaded onto the column. The column was eluted with a mixture of organic solvents in a step-wise gradient of increasing polarity starting from hexane (100%), then hexane:ethyl acetate (99:1), the polarity was increased gradually up to hexane:ethyl acetate (80:20). Two hundred and five fractions (10 mL each) were collected and analyzed by TLC. Fractions with similar TLC profile were pooled together. Twenty-one major fractions were obtained and concentrated *in vacuo*. Fractions 90-116 gave single homogeneous spots with the same R_f and was designated as compound C which was subjected to further purification by re-chromatographing over silica gel column (35 cm x 2.0 cm) following the elution procedure described above.

Biological Activity
Experimental Animals
Albino mice (15-25 g) and rats (150-165 g) of either sex were obtained from the animal house of Faculty of Pharmaceutical Sciences, Ahmadu Bello University, Zaria, Nigeria. The animals were acclimatised for 2 weeks before the commencement of the study. Standard commercial chow and water were provided *ad libitum* for the animals. Housing conditions were maintained at 25 ± 2 ºC at 12 h day/night cycles. The study was approved by the Animal Research Ethical Committee, Usmanu Danfodiyo University, Sokoto (PTAC/PC(OA)/OT/007-18). The care and handling of the animals were done according to the established public health guidelines in Guide for Care and Use of Laboratory Animals (2011).

Acute toxicity Study
Oral acute toxicity study was carried out by 'Up-and- Down' method in nulliparous mice according to Organization for Economic Development (OECD) guideline no. 425.[13] A limit dose of oleanolic acid 2000 mg/kg was used for the study. Five female mice were used for the study. An animal was picked at a time, weighed and dosed with the equivalent volume of extract dissolved in 20% Tween 80 + DMSO (1:1) vehicle. The extract was administered orally using gastric feeding tube and monitored according to OECD guidelines.

Anticonvulsant Study (Pentylenetetrazole (PTZ)-induced convulsion)
Twenty-five mice (weight range 15 - 20 g) were divided into five groups of five animals each. Groups 1 and 2 received 5 mL/kg of 20% Tween 80 + DMSO (1:1) vehicle and diazepam 5 mg/kg, respectively. Groups 3-5 were treated with 50, 75 and 100 mg/kg of oleanolic acid, respectively. One hour before administration of the compound, 70 mg/kg of pentylenetetrazole (PTZ) was administered subcutaneously to each mouse.[14] Absence of an episode of clonic spasm of at least 5 seconds duration, hind limb extension or death indicated the compound's ability to abolish the effect of PTZ on seizure threshold (protected). The percentage seizure protection was calculated according to the formula below.

$$\% \text{ protection} = \frac{\text{Number of animals that convulsed in the group}}{\text{Total number of animals in the group}} \; x \; 100$$

Diazepam-induced Sleep in Rats
Rats were divided into five groups of 5 rats each and treated with 5 mL/kg of 20% Tween 80 + DMSO (1:1) solution, p.o. as the control group and oleanolic acid comprising 3 groups the dose of which was selected based on the LD_{50} (10, 50 or 100 mg/kg p.o). Thirty minutes later, all the groups received diazepam (5 mg/kg i.p.). The time from the injection up to the loss of the righting reflex is recorded as sleeping latency and the time between the loss and voluntary recovery of the righting reflex was recorded as the duration of sleep using a stop watch.[15]

Statistical Analysis
All data are presented as mean ± standard error of mean (SEM) of five replicate readings. They were analysed using graph pad prism version 6 software. One-way analysis of variance (ANOVA) was used to compare all groups followed by Dunnett post-hoc test. Differences were considered significant at $p < 0.05$.

Results and Discussion

Structure Elucidation
Compound C (45 mg) was isolated as white powder, soluble in DMSO, with melting point of 276 - 278ºC. The proton NMR spectrum showed characteristic signal at δH (ppm) 5.10 (1H, d) which is characteristic of olefinic hydrogen, δH (ppm) 4.03 (1H, br s) which is characteristic of hydrogen of an aliphatic hydroxyl group. Several multiplet signals occurring between the range of δH (ppm) 0.6 to 2.0 which are characteristic of methyl (CH_3), methylene (CH_2) and methine (CH) protons.
The ^{13}C-NMR spectrum of compound C showed 30 carbon signals. The most prominent signals include δc (ppm) 179.2 (C-28), 138.7 (C-13), 124.7 (C-12), 77.1 (C-3). Other signals were observed between the range of 17.1 to 54.9 ppm which are characteristic of methyl (CH_3), methylene (CH_2) and Methine (CH) carbon (Table 1).
The DEPT-135 spectrum of compound C showed twelve (12) positive signals due to methine (CH) and methyl (CH_3) at δc (ppm) 17.1, 17.2, 20.9, 23.4, 26.7, 27.7, 30.8, 47.2, 41.8, 54.9, 77.1 and 124.7, ten negative signals due to methylene (CH_2) were also seen at δc (ppm) 18.1, 23.4, 23.9, 27.7, 28.4, 30.3, 32.9, 36.5, 41.2 and 47.0. This agrees with the ^{13}C-NMR signals.
The IR spectrum of compound C showed characteristic signals at V_{max} (KBr): 3385 cm^{-1} (OH stretching), 2927 cm^{-1} (CH_2 stretching), 2868 cm^{-1} (CH_3 stretching) and 1686 cm^{-1} (C=O stretching) and 1453.58 cm^{-1} (C-C bending). The IR data is in agreement with the data of oleanolic acid reported by Narendra and Ameeta (2014).[17] The ^{1}H-NMR, ^{13}C-NMR and DEPT-135 data are in agreement with those reported in literatres.[16, 18, 19] The spectra data are characteristic and consistent with oleanolic acid. Therefore the proposed structure of compound C is oleanolic acid (Figure 1).

Acute Toxicity Study
The animals survived at the dose of 2000 mg/kg. There were no observed signs of toxicity. Therefore, the LD_{50} of the compound (oleanolic acid) is said to be greater than 2000 mg/kg. Previous studies have reported that the LD_{50} of oleanolic acid is greater than 2000 mg/kg.[12] This agrees with our study and shows that oleanolic acid is relatively safe when administered orally.

Anticonvulsant and Sedative Study
Oral administration of oleanolic acid resulted in a dose-dependent increase in the onset of PTZ-induced seizure (Figure 2). The compound provided 25% protection against seizure in the group administered 100 mg/kg of the extract and 100% protection in the group treated with diazepam (Figure 3). PTZ-induced seizure models are usually employed to test for possible activity of an agent against absence seizures, as drugs that inhibit PTZ-induced convulsions are generally used in the management of absence seizures.[20, 21] PTZ is a most frequently used model in the preliminary screening of potential anticonvulsant drugs as the model evaluates the activity of a drug against myoclonic or absence seizure.[22] The mechanism by which PTZ produces its action is not very well understood; it is believed to exert its action by acting as an antagonist at the gamma-aminobutyric acid $GABA_A$ receptor complex.[23] Therefore, agents that enhance $GABA_A$ receptor mediated inhibitory transmission such as benzodiazepines and barbiturates prevent PTZ-induced seizures. The activity of the compound against PTZ-induced seizure may also be due to its interference with GABA neurotransmission.
In order to further verify the mechanism of action of oleanolic acid, diazepam-induced sleep was conducted. In the diazepam-induced sleep test, oleanolic acid significantly ($p<0.0001$ at 10 and 50 mg/kg; $p<0.05$

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Table 1: ^{1}H-NMR and ^{13}C-NMR Data of Compound C7 (400 MHz at 300 K in DMSO).

Carbon No.	δ_H (ppm)	*δ_H (ppm)	δ_C (ppm)	*δ_C (ppm)	Carbon Type
1			32.9	39.0	CH_2
2			27.7	28.1	CH_2
3	4.03 s. br; 3.43	3.44	77.1	78.2	CH
4			39.5	39.4	C
5			54.9	55.9	CH
6			20.9	18.8	CH_2
7			36.5	33.4	CH_2
8			40.1	39.8	C
9			47.2	48.2	CH
10			37.4	37.4	C
11			23.9	23.8	CH_2
12	5.10	5.49	124.7	122.6	CH
13			138.7	144.8	C
14			41.8	42.2	C
15			28.4	28.4	CH_2
16			23.4	23.8	CH_2
17			47.0	46.7	C
18			41.8	42.1	CH
19			47.0	46.6	CH_2
20			30.8	31.0	C
21			41.2	34.3	CH_2
22			30.3	33.2	CH_2
23	1.23 s	1.24	27.7	28.8	CH_3
24	1.01 s	1.02	18.1	16.5	CH_3
25	0.87 s	0.88	17.1	15.6	CH_3
26	1.05 s	1.04	17.2	17.5	CH_3
27	1.29 s	1.30	26.7	26.2	CH_3
28	-	-	179.2	180.0	C=O
29	0.95 s	0.94	30.8	33.4	CH_3
30	1.01 s	1.02	23.4	23.8	CH_3

*δ_H ppm and *δ_C ppm = Ghias *et al*., 2011.[16]

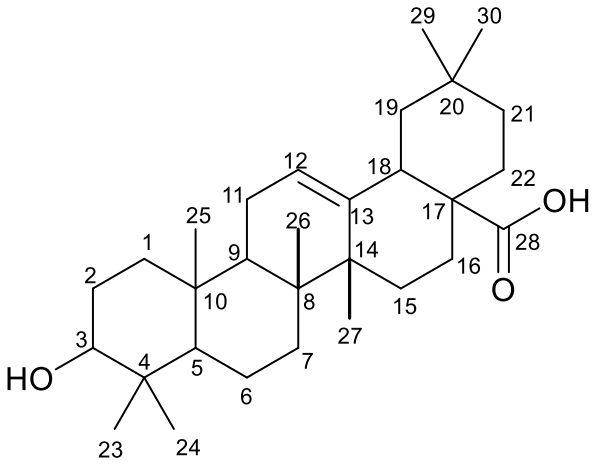

Figure 1: Structure of Oleanolic Acid.

at 100 mg/kg) decreased the onset of sleep (sleep latency) at all dose levels (Figure 4) and increased the duration of sleep (Figure 5). Diazepam acts by increasing GABA mediated synaptic inhibition either by directly activating GABA receptors or more usually by enhancing the action of GABA on $GABA_A$ receptors. Decrease in latency and prolongation of duration of sleep by oleanolic acid is an indication of a possible sedative and central inhibitory effects through the stimulation of the Central nervous system inhibitory pathways by acting through GABA pathway.[24]

Conclusion

Oleanolic acid have been isolated from the stem bark of *Parinari curatellifolia*. The compound was characterized on the basis of IR, ^{1}H-NMR, ^{13}C-NMR and DEPT-135 spectra data. To the best ofour knowledge, this is the first report of the presence of this compound in *Parinari curatellifolia*. The compound (oleanolic acid) demonstrated significant anticonvulsant and sedative properties by increasing the onset of seizure, providing protection against seizure similar to diazepam in the PTZ- induced seizure test and reducing sleep latency and increasing duration of sleep in experimental animals.

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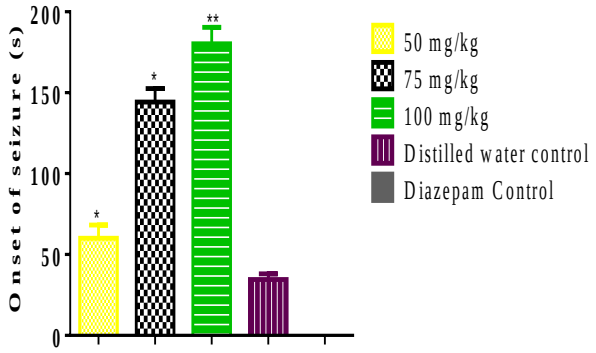

Figure 2: Onset of seizure.

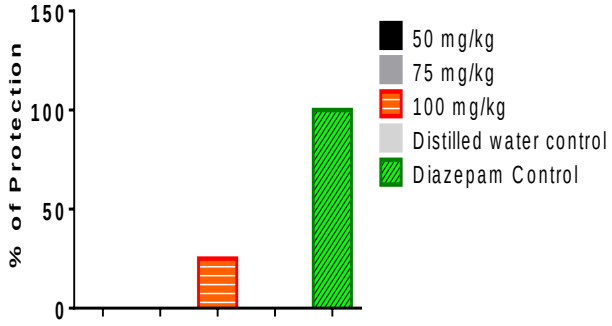

Figure 3: Effect of oleanolic acid on percentage protection.

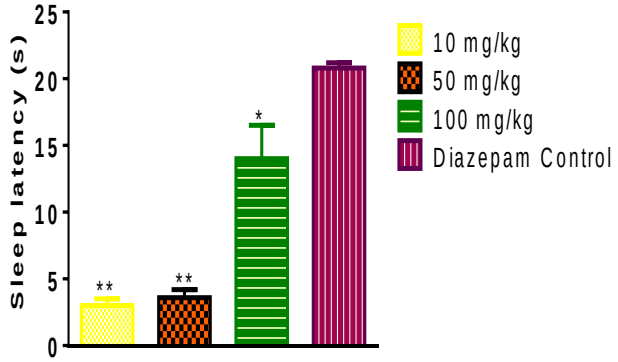

Figure 4: Effect of oleanolic acid on sleep latency time induced by diazepam (5 mg/kg).

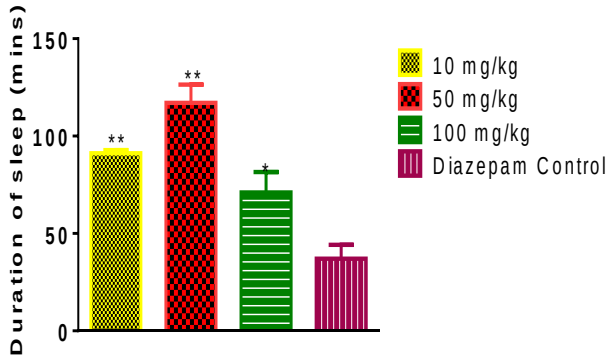

Figure 5: Effect of oleanolic acid on duration of sleep induced by diazepam (5 mg/kg).
Data represents mean ± S.E.M. n = 5, *$p < 0.05$; **$p < 0.001$ as compared to control.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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