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Anticonvulsant activities of friedelan-3-one and n-dotriacontane both isolated from *Harungana madagascariensis* Lam (Hypericaceae) seeds extracts

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Chemical investigation of the crude methanol/methylene chloride (1:1) extract of the seeds of *Harungana madagascariensis* resulted in the isolation of two known compounds namely n-dotriacontane and friedelan-3-one. The structures of the isolated compounds were determined on the basis of 1D NMR spectroscopy and comparison with literature data. The crude extract and the two compounds were evaluated for their anticonvulsant effects on pentylenetetrazole or picrotoxin induced convulsions in mice. All the tested treatments showed anticonvulsant effects on experimental models of epileptic seizures chemically induced in mice. Friedelan-3-one and the crude extract showed up to 83% protection of animals against convulsions while n-dotriacontane produced a maximum of 33% protection.

Key words: *Harungana madagariensis* extracts, friedelan-3-one, n-dotriacontane, anticonvulsant activity.

INTRODUCTION

Plants are an important source of novel molecules for the development of new antiepileptic agents to solve the problems of toxicity, side effects, and ineffectiveness in the control of epilepsy (Hui-Ling et al., 2014). Though there is limited literature on pure components in herbal medicines that have been reported to treat neurological disorders, many kinds of herbs have been used to treat epilepsy.

In fact, this affects up to 50 million people (Wagner, 2016) of all age groups, sex and social class worldwide (Nirupam et al., 2012) with 2 million new cases every year including 75% from low- and medium-income countries (LMICs) (Wagner, 2016). In addition to this, with the emergence of numerous adverse outcomes and pharmaco-resistance to the available antiepileptic drugs (AEDs) (Wahab, 2010; Nirupam et al., 2012), there is an urgent need for the development of new AEDs with the ultimate aim of achieving complete freedom from seizures with improved tolerance in all patients. We therefore decided to investigate the anticonvulsant potentials of the crude extract of the seeds of *H. madagariensis* and compounds isolated from it. This with

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the hope of obtaining novel leads that could be further developed to obtain new, safe and efficacious antiepileptic agents.

MATERIALS AND METHODS

General

Extraction, purification and phytochemical analyses were carried out in the Natural Product Laboratory, Department of Chemistry, Faculty of Science, University of Buea. Samples were weighed on an ohaus electronic and scale balance. Column chromatography was carried out using Merck silica gel 60 (particle size 60-200 µm). For thin layer chromatographic (TLC) analysis, precoated aluminium sheets with silica gel (Alugramsil/UV $_{254}$) were used. Zones on these plates were visualized under UVGL-58 lamp at 254/365 nm. Alternatively, the plates were exposed to iodine vapour in an iodine chamber. Melting points were recorded on Meltemp II apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectroscopy was carried out in a Utah State University, USA. ^{13}C NMR (75 MHz-$CDCl_3$) and ^{1}H NMR (300 MHz-$CDCl_3$) spectra were recorded on variant mercury Vx-300 MHz machine. The chemical shift values were quoted in parts per million (ppm) on the scale, with tetramethylsilane (TMS) as internal standard. NMR spectra were obtained with deuteriated chloroform ($CDCl_3$).

Biological screening was carried out in the collaborating Zoology and Animal Physiology Laboratories of the Department of Zoology and Animal Physiology, Faculty of Science, University of Buea. Albino Swiss mice of either sex (18-22 g) were obtained from the Animal Centre, University of Buea, Cameroon and were kept in plastic cages at room temperature. They were fed with balanced rodent pellet diet and water ad libitum and were acclimatized for one week before using them for experiments. The procedures used in the study were in compliance with the ethical guidelines of the University of Buea and National Institutes of Health guide for the Care and Use of Laboratory Animals for experimental investigations. The following drugs were used: picrotoxin (Sigma-Aldrich, St. Louis, USA), pentylenetetrazole, PTZ (Sigma-Aldrich, St. Louis, USA), Clonazepam, CLZ (Roche, France).

Plant material

The seeds of *H. madagascariensis* were collected in Awing, Northwest region of Cameroon in February 2018. The plant was identified by Mr. Elias Ndive and a voucher specimen of it, N° SCA4408 is deposited at the Limbe Botanic Garden, Cameroon.

Extraction, isolation and identification of compounds

The seeds of *H. Madagascariensis* were air-dried then ground into coarse powder. The powder (1.9 kg) was macerated at room temperature with CH_2Cl_2/MeOH (1:1) (3 × 3 days each). The filtrate was then concentrated to dryness using a rotavapor to yield 227 g of crude extract. The extract was stored at 4°C. The crude extract was subjected to open column chromatography with silica gel as adsorbent. Elution was performed with hexane/ethyl acetate and ethyl acetate/methanol in increasing polarity. A total of 135 fractions (250 ml each) were collected, concentrated *in vacuo* in a rotator evaporator and their compound profiles monitored by using analytical TLC plates. Following the analytical TLC, the eluents were regrouped into 6 main fractions indexed A- F. AHM1 (n-dotriacontane, 60 mg) crystallized as a white solid (in acetone) from Fractions 1- 7 (Fraction A) that was obtained with pure hexane from the main column. It had a melting point of 68-70°C and an R_f of 0.5 (pure hexane). Repeated column chromatography of Fraction B (Fractions 8-20 obtained with 5% EtOAc/Hex from the main column) afforded a white solid (300 mg) in acetone coded AHM2; with a melting point of 248-250°C and an R_f of 0.4 (2% EtOAc/Hex).

Antiepileptic assay

Picrotoxin (PCT)-induced convulsion test

Mice were divided into fourteen groups of six mice each, and received the crude extracts: AHM (5, 10, 15 and 20 mg/kg; i.p.), AHM1 (5, 10, 15 and 20 mg/kg; i.p.), AHM2 (5, 10, 15 and 20 mg/kg; i.p.), clonazepam (1 mg/kg, i.p.) or vehicle (10 mL/kg p.o.).One hour later, tonic-clonic seizures were induced in mice by intraperitoneal (i.p) injection of 7.5 mg/kg PCT. The mice were observed for 15 min and the protective effects of the different treatments were recorded. Animals that did not convulse within the 15 min of observation were qualified as protected (Bum et al., 2001).

Pentylenetetrazole (PTZ)-induced convulsion test

Fourteen groups of six mice were treated as discussed previously. However, the positive control group received 0.1 mg/kg clonazepam i.p. Tonic-clonic seizures were induced in mice by the i.p. injection of 70 mg/kg PTZ. The protective effect of the different treatments given 1 h before PTZ injection was recorded. Animals that did not convulse within the 10 min of observation were qualified as protected (Bum et al., 2011).

Statistical analysis

The data were expressed as mean ± S.E.M. The data were analyzed with Graph Pad Prism Software version 4.03. Statistical analysis of data was done by One-way ANOVA, followed by Dennett's post-hoc test. A level of $p < 0.05$ was considered as statistically significant (Umukoro et al., 2013).

RESULTS

Identification of the AHM1 and AHM2

The structures of the compounds (Figure 1) were determined by analysis of their NMR data and comparison with those reported in literature. The ^{1}H NMR of AHM1 showed two signals; a triplet at about 0.88 ppm attributed to the six methyl protons (H-1 and H-32) and a broad signal at about 1.26 ppm attributed to the methylene protons (H-2 to H-31). Comparison of its ^{1}H NMR spectral data with the published data for n-dotriacontane, previously isolated from *Elaeocarpus lanceifolius* (Bordoloi et al., 2017) helped to identify compound AHM1 as n-dotriacontane; a long straight chain alkane with 32 carbons.

AHM2 showed a positive Lieberman Burchard test. Its ^{1}H NMR spectrum showed a quartet at 2.25 ppm (for proton H-4) and a doublet at 0.87 ppm (for the methyl protons H-23). Other methyl protons appeared at 0.72 ppm (H-24, singlet), 1.05 ppm (H-27, singlet), 1.18 ppm (H-28, singlet), 1.00 ppm (H-29, doublet), 0.93 ppm (H-30, doublet). The ^{13}C spectrum of AHM2 in addition showed a carbonyl signal at 213 ppm (C-3). Signals at

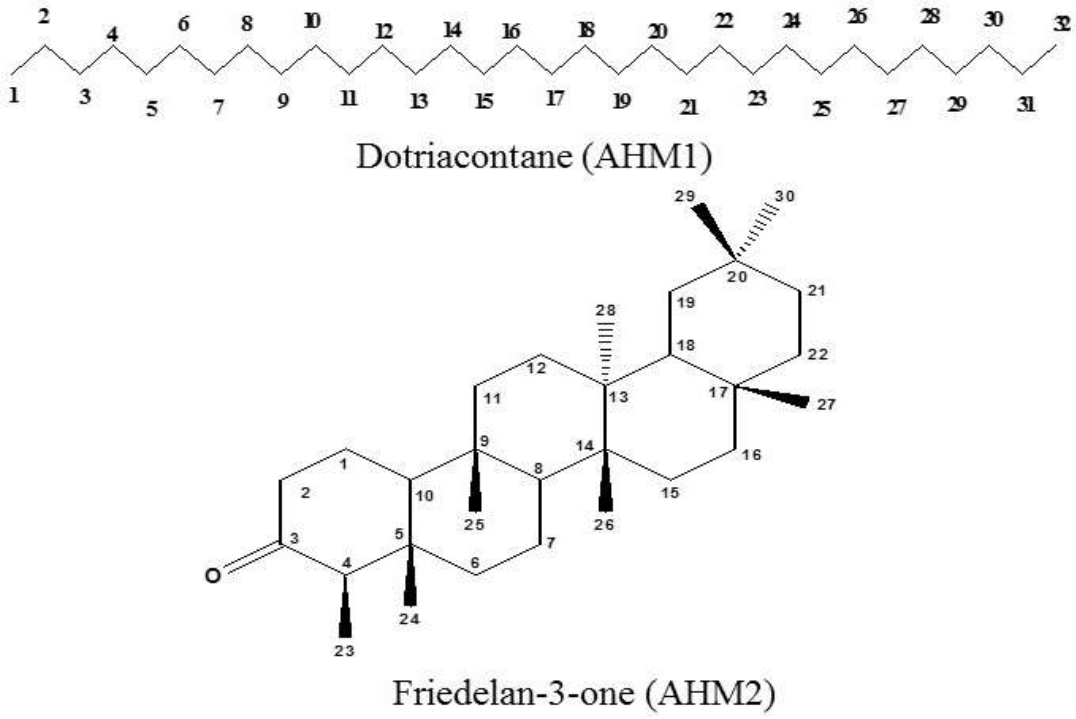

Figure 1. Structures of n-dotriacontane and friedelan-3-one.

6.80, 14.6, 17.9, 20.2, 18.6, 32.1, 35.0 and 31.8 ppm indicated the presence of methyl carbons C-23, C-24, C-25, C-26, C-27, C-28, C-29 and C-30 respectively. Those at 22.3, 41.5, 41.3, 18.2, 35.6, 30.5, 32.4, 36.0, 35.3, 32.8 and 39.2 ppm revealed the presence of methylene carbons C-1, C-2, C-6, C-7, C- 11, C-12, C-15, C-16, C-19, C-21 and C-22 respectively. The signals at 58.2, 53.1, 59.5 and 42.8 ppm indicated the presence of methyne carbons C-4, C-8, C-10 and C-18 respectively. Finally, the signals at 42.1, 37.4, 39.7, 38.5, 30.0 and 28.2 ppm indicated the presence of the quaternary carbons C-5, C-9, C-13, C-14, C-17 and C-20 respectively. Comparison of the NMR spectral features of AHM2 with existing literature for friedelan-3-one previously isolated from *Terminalia avicennioides* (Abdullahi et al., 2011) shows that AHM2 is friedelan-3-one; a pentacyclictriterpene.

Anticonvulsant activity

The anticonvulsant effects of the MeOH/CH_2Cl_2crude extract (AHM), n-dotriacontane (AHM1), and friedelan-3-one (AHM2) (5, 10, 15 and 20 mg/kg each) were assessed based on their abilities to prevent, or delay the onset of tonic-clonic seizures (convulsions) induced by pentylenetetrazole (PTZ) and picrotoxin (PCT), both chemoconvulsant agents (70 and 7.5 mg/kg respectively) in mice (Bum et al., 2001).

Effect of AHM on PTZ-induced convulsion

The effects of intraperitoneal (i.p) injection of AHM (5-20 mg/kg) on convulsions induced by PTZ (70 mg/kg, i.p) in mice are shown in Table 1 and Figure 2. AHM (5-20 mg/kg) protected 33.33-83.33% of mice against convulsions induced by PTZ while the reference drug, clonazepam (0.1 mg/kg) offered 100% protection against convulsions, as none of the animals exhibited tonic-clonic seizures induced by PTZ in mice. The effective dose needed for AHM to protect 50% of the animals (ED_{50}) was about 7.5 mg/kg (Figure 2). Statistical analysis showed that AHM (10-20 mg/kg) significantly increased the latency to the tonic-clonic components of PTZ-induced seizures in the unprotected mice (Table 1). At these doses, AHM significantly reduced the rate of seizures by 47 to 72% from the control, whereas the seizures rate was 100% with no protection against seizure, in the control group (Table 1).

Effect of AHM1 on PTZ-induced convulsion

The effects of AHM1 (5-20 mg/kg, i.p) on convulsions induced by PTZ (70 mg/kg, i.p) in mice are shown in Table 2 and Figure 3. AHM1 (5-20 mg/kg) protected 16.67-33.33% of mice against convulsions induced by PTZ (ED_{50} of about 33.3 mg/kg) while the reference drug, clonazepam (0.1 mg/kg) offered 100% protection against

Table 1. Effects of AHM on the latency to the seizure and the percentage of increase of the mean seizure onset time from the control.

Treatment	Dose (mg/kg)	Latency to the seizure (min) [mean±SEM]
Control (DW)	-	2.57 ± 1.08
AHM	5	3.29 ± 0.86
AHM	10	4.86 ± 0.27*
AHM	15	5.48 ± 0.00**
AHM	20	9.23 ± 0.00***
Clonaz	1	No convulsion

Values represent the mean ± S.E.M for 6 animals per group. *p < 0.05, **p < 0.01, ***p < 0.001compared to control group (ANOVA followed by Dunnett's post-hoc test).

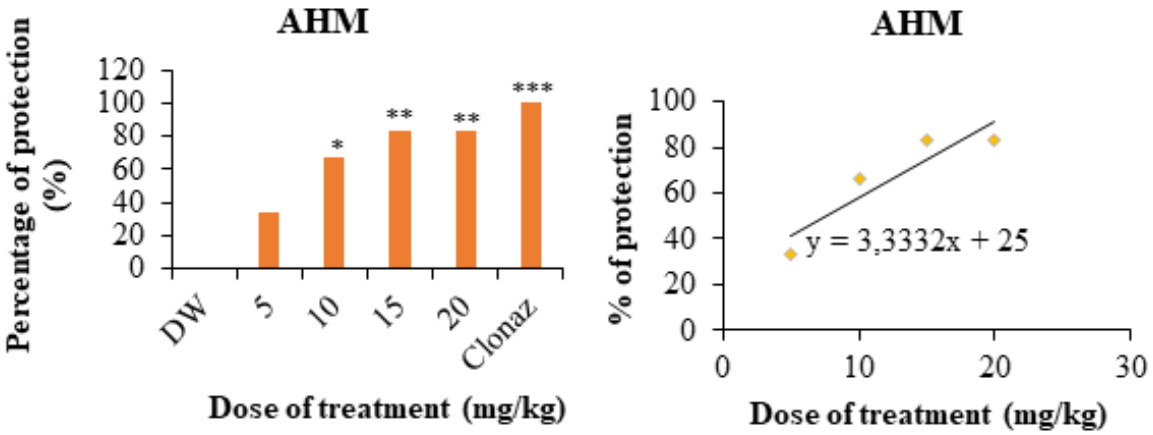

Figure 2. Effects of *H. madagascariensis* crude extract on the percentage of protected mice against seizures induced by PTZ.

Table 2. Effects of AHM1 on the latency to the seizure induced by PTZ.

Treatment	Dose (mg/kg)	Latency to the seizure (min) [mean±SEM]
Control (DW)	-	2.57±1.08
AHM1	5	3.01±1.37
AHM1	10	3.90±1.04
AHM1	15	4.61± 0.85*
AHM1	20	4.75±1.18*
Clonaz	1	No convulsion

Values represent the mean ± S.E.M for 6 animals per group. *p < 0.05 compared to control group (ANOVA followed by Dunnett's post-hoc test).

convulsions, as none of the animals exhibited tonic-clonic seizures induced by PTZ in mice (Figure 3).

Post-hoc test analysis by the Dunnett's multiple comparison test showed that AHM1 (10-20 mg/kg) significantly increased the latency to the tonic-clonic components of PTZ-induced seizures in the unprotected mice (Table 2). At these doses, AHM1 significantly reduced the rate of seizures by 34 to 46%, whereas the seizures rate was 100% with no protection, in the control group (Table 2).

Effect of AHM2 on PTZ-induced convulsion

The effects of AHM2 (5-20 mg/kg, i.p) on convulsions induced by PTZ (70 mg/kg, i.p) in mice are shown in Table 3 and Figure 4. AHM (5-20 mg/kg) protected 50-83.33% of mice against convulsions induced by PTZ (ED_{50}

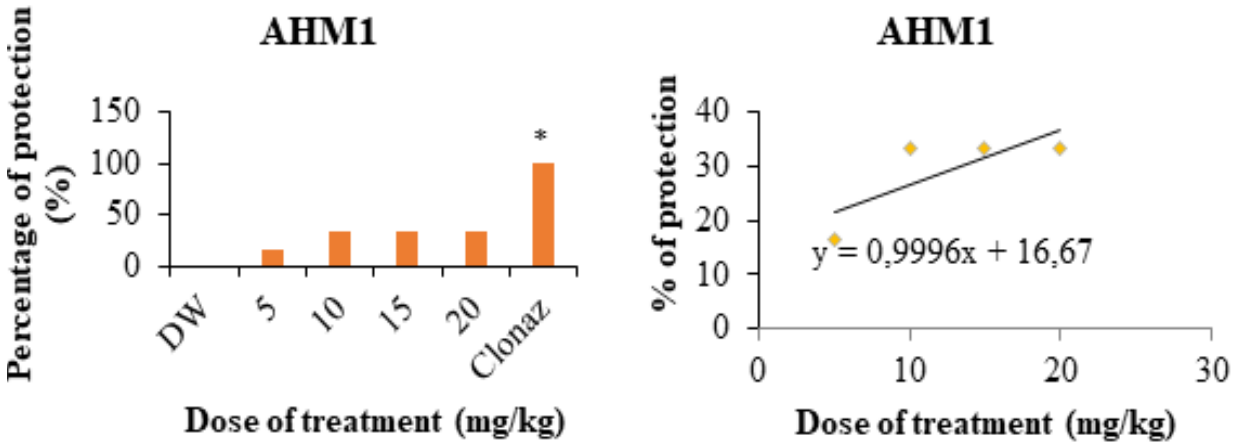

Figure 3. Effects of AHM1 on the percentage of protected mice against seizures induced by PTZ.

Table 3. Effect of AHM2 on the latency to the seizure induced by PTZ.

Treatment	Dose (mg/kg)	Latency to the seizure (min) [mean±S.E.M]
Control (DW)	-	2.57±1.08
AHM2	5	4.31±0.69*
AHM2	10	5.74±0.46**
AHM2	15	7.12± 0.00***
AHM2	20	8.53±0.00***
Clonaz	1	No convulsion

Values represent the mean ± S.E.M for 6 animals per group. *p < 0.05, **p < 0.01, ***p < 0.001 compared to control group (ANOVA followed by Dunnett's post-hoc test).

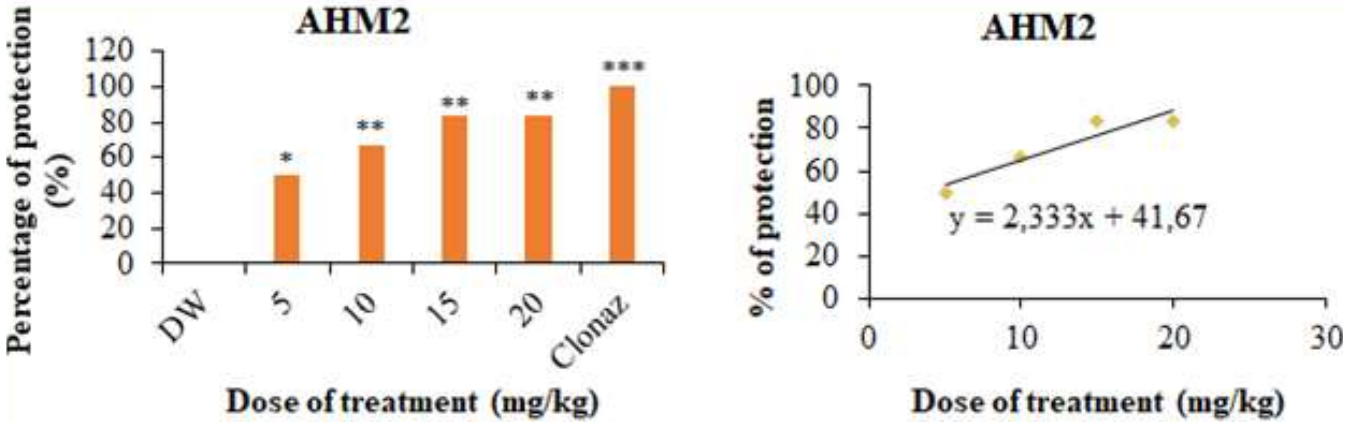

Figure 4. Effects of AHM2 on the percentage of protected mice against seizures induced by PTZ.

of about 5 mg/kg) while the reference drug, clonazepam (0.1 mg/kg) offered 100% protection against convulsions, as none of the animals exhibited tonic-clonic seizures induced by PTZ in mice (Figure 4).

Statistical analysis showed that AHM2 (5-20 mg/kg) significantly increased the latency to the tonic-clonic components of PTZ-induced seizures in the unprotected mice (Table 3). At these doses, AHM2 reduced the rate of seizures by 40 to 70%, whereas the seizures rate was 100% with no protection in the control group (Table 3).

Table 4. Effect of AHM on the latency to the seizure induced by PIC.

Treatment	Dose (mg/kg)	Latency to the seizure (min) [mean±S.E.M]
Control (DW)	-	5.97±1.35
AHM	5	7.84±9.52
AHM	10	9.99±21.31*
AHM	15	10.94±26.80*
AHM	20	13.52±28.98*
Clonaz	0.1	No convulsion

Values represent the mean ± S.E.M for 6 animals per group. *p < 0.05 compared to control group (ANOVA followed by Dunnett's post-hoc test).

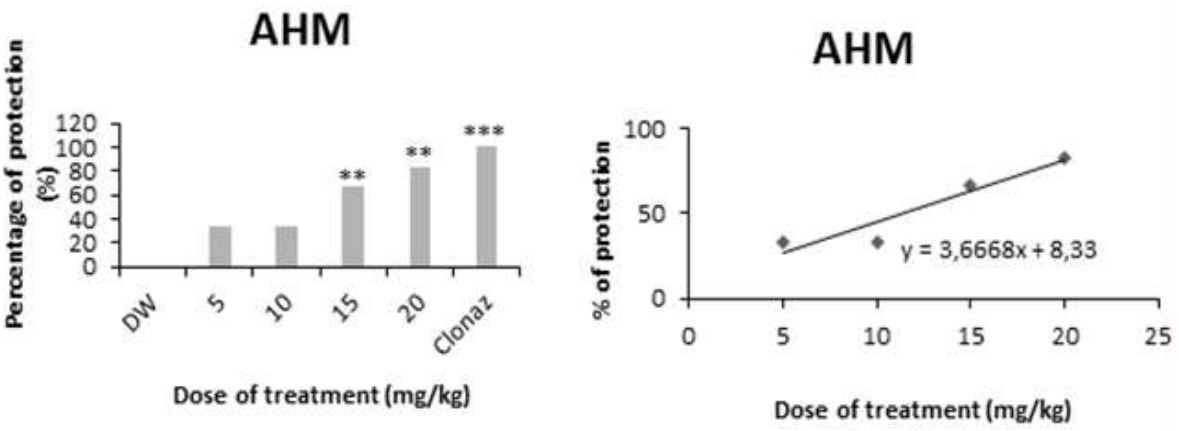

Figure 5. Effects of AHM on the percentage of protected mice against seizures induced by PCT.

Table 5. Effect of AHM1 on the latency to the seizure induced by PCT.

Treatment	Dose (mg/kg)	Latency to the seizure (min) [mean±S.E.M]
Control (DW)	-	5.97±1.35
AHM1	5	5.95±4.09
AHM1	10	6.96±8.79
AHM1	15	8.64±9.10*
AHM1	20	8.09±9.87*
Clonaz	0.1	No convulsion

Values represent the mean ± S.E.M for 6 animals per group. *p < 0.05, compared to control group (ANOVA followed by Dunnett's post-hoc test).

Effect of AHM on PCT-induced convulsion

The effects of AHM (5-20 mg/kg, i.p) on convulsions induced by PCT (7.5 mg/kg, i.p) in mice are shown in Table 4 and Figure 5. AHM (5-20 mg/kg) protected 33.33-83.33% of mice against convulsions induced by PCT (ED_{50} of about 11.4 mg/kg), while the reference drug, clonazepam (1 mg/kg) offered 100% protection against convulsions, as none of the animals exhibited tonic-clonic seizures induced by PCT in mice (Figure 5). Statistical analysis showed that AHM (10-20 mg/kg) significantly increased the latency to the tonic-clonic components of PCT-induced seizures in the unprotected mice. At these doses, AHM reduced the rate of seizures by 40 to 55%, whereas the seizures rate was 100% with no protection in the control group (Table 4).

Effect of AHM1 on PCT-induced convulsion

The effects of AHM1 (5-20 mg/kg, i.p) on convulsions induced by PCT (7.5 mg/kg, i.p) in mice are shown in Table 5 and Figure 6. AHM1 (5-20 mg/kg) protected

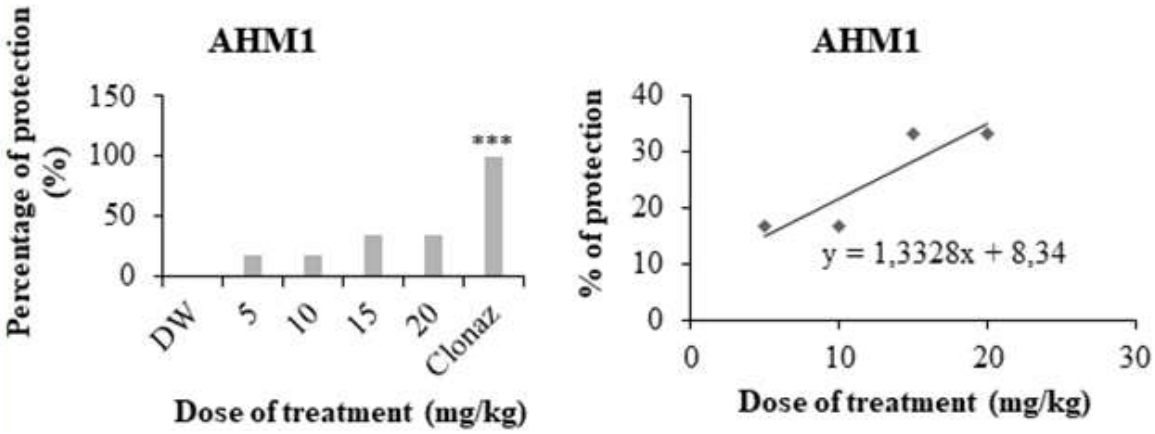

Figure 6. Effects of AHM1 on the percentage of protected miceagainst seizures induced by PCT.

Table 6. Effect of AHM2 on the latency to the seizure induced by PCT.

Treatment	Dose (mg/kg)	Latency to the seizure (min) [mean±S.E.M]
Control (DW)	-	5.97 ± 1.35
AHM2	5	7.91 ±14.44
AHM2	10	9.99 ± 20.08
AHM2	15	12.47 ± 26.36*
AHM2	20	13.59 ± 25.88*
Clonaz	0.1	No convulsion

Values represent the mean ± S.E.M for 6 animals per group. *p < 0.05, compared to control group (ANOVA followed by Dunnett's post-hoc test).

16.67-33.33% of mice against convulsions induced by PCT (ED_{50} of about 31.3 mg/kg) while the reference drug, clonazepam (1 mg/kg) offered 100% protection against convulsions, as none of the animals exhibited tonic-clonic seizures induced by PCT in mice (Figure 6).

Post-hoc test analysis by the Dunnett's multiple comparison test showed that AHM1 (10-20 mg/kg) increased the latency to the tonic-clonic components of PCT-induced seizures in the unprotected mice. At these doses, AHM1 reduced the rate of seizures by 14 to 30%, whereas the seizures rate was 100% with no protection in the control group (Table 5).

Effect of AHM2 on PCT-induced convulsion

The effects of AHM2 (5-20 mg/kg, i.p) on convulsions induced by PCT (7.5 mg/kg, i.p) in mice are shown in Table 6 and Figure 7. AHM2 (5-20 mg/kg) protected 33.33-83.33% of mice against convulsions induced by PCT (ED_{50} of about 10 mg/kg) while the reference drug, clonazepam (1 mg/kg) offered 100% protection against convulsions, as none of the animals exhibited tonic-clonic seizures induced by PCT in mice (Figure 7). Statistical analysis showed that AHM2 (10-20 mg/kg) significantly increased the latency to the tonic-clonic components of PCT-induced seizures in the unprotected mice (Table 6). At these doses, AHM2 reduced the rate of seizures by 40 to 56%, whereas the seizures rate was 100% with no protection in the control group (Table 6).

DISCUSSION

This is the first-time n-dotriacontane (AHM1) is isolated from this plant species. Friedelan-3-one (AHM2) was previously isolated from the stem bark of *H. madagascariensis* (Kouam et al., 2005). This is the first time it is isolated from the seeds of *H. madagascariensis*. However, Afieroho et al. (2012) used standard phytochemical screening reagents to reveal the presence of flavonoids aglycones, and terpenoids in the methanol extract of the seeds of *H. madagascariensis.*

The results of the antiepileptic assay show that AHM, AHM1 and AHM2 all provided protection against the development of seizure episodes and delayed instances of seizure induced by PTZ or PCT in mice in all their tested doses (5, 10, 15 and 20 mg/kg). At the dose of 20 mg/kg, AHM and AHM2 each considerably inhibited the action of PTZ and PCT, so much so that only 1/6 of the

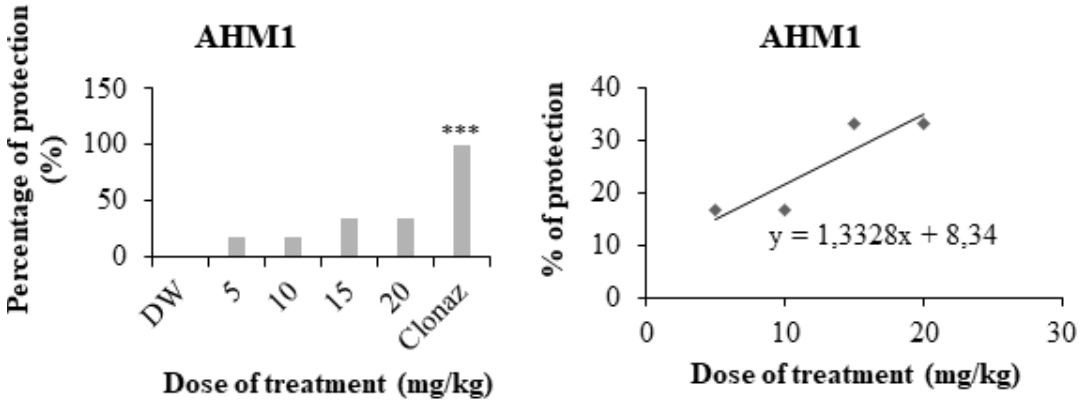

Figure 7. Effects of AHM2 on the percentage of protected mice against seizures induced by PCT.

animals exhibited tonic-clonic convulsions with a high latency suggesting that AHM and especially AHM2 might provide 100% like the reference drug clonazepam (1 and 0.1 mg/kg for PTZ and PCT respectively) if dose was increased to about 25 mg/kg. AHM2 could therefore possibly be the main compound responsible for the antiepileptic activity of the plant. To the best of our knowledge, this is the first report of the isolation of friedelan-3-one from the seeds of *H. madagascariensis* and also the first report of its anticonvulsant activity.

Pentylenetetrazole (PTZ) and picrotoxin (PCT) are chemoconvulsants widely used to induce convulsions in experimental animals (Umukoro et al., 2013). Seizures frequently result from an imbalance of excitation and inhibition due to a failure of inhibitory neurotransmission. The antagonism of the postsynaptic $GABA_A$ receptor leads to excessive neuronal excitation over neuronal inhibition that terminates in convulsions and death in laboratory animals (Greenfield, 2013; Annafi et al., 2014). PTZ induces convulsions by antagonizing $GABA_A$ in a competitive manner (Annafi et al., 2014). On the other hand, PCT is a non-competitive antagonist that acts through blockade of $GABA_A$ receptor chloride ion channel which is known to be resistant to most anticonvulsant agents (Umukoro et al., 2013).The fact that AHM, AHM1 and AHM2 significantly delayed the onset of seizures, and protected mice from seizures produced by both PTZ and PCT further suggests their beneficial role in curing epileptic patients or retarding the spread of seizures in epileptic brains (Annafi et al., 2014).

Conclusion

The anticonvulsant activity of the CH_2Cl_2/MeOH (1:1) crude extract and two compounds (n-dotriacontane and friedelan-3-one) isolated from the CH_2Cl_2/MeOH (1:1) extract of the seeds of *H. madagascariensis* (Hypericaceae) were assessed based on their abilities to prevent, or delay the onset of tonic-clonic seizures induced by PTZ and PCT in mice.The 3 treatments protected the mice at all their tested doses with the highest protection offered by friedelan-3-one followed by the crude extract and a moderate protection offered by n-dotriacontane. From these findings we suggest that n-dotriacontane and especially friedelan-3-one, could be important leads for the development of future antiepileptic drugs (AEDs). These compounds (n-dotriacontane and friedelan-3-one) have been isolated from the seeds of *H. madagarriensis* for the first time.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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