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Anticonvulsant effect of kaurenoic acid isolated from the root bark of *Annona senegalensis*

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ABSTRACT

Context: The herbal preparations of *Annona senegalensis* Pers. (Annonaceae) root bark are used in Nigerian ethnomedicine for the treatment of epilepsy and febrile seizures. The scientific evidence for this effect has been reported.
Objective: The aim of this study was to identify and characterize the active constituent responsible for the anticonvulsant effect.
Materials and methods: Bioactive-guided fractionation of the methanol-methylene chloride root bark extract (MME) of *A. senegalensis* using pentylenetetrazole (PTZ)-induced seizures in mice, afforded a potent anticonvulsant ethyl-acetate fraction (EF). Further fractionation of the EF yielded eight sub-fractions (F_1–F_8) which were tested for anticonvulsant activity. The sub-fraction F_2 yielded white crystals that were purified to obtain *A. senegalensis* crystals, AS2. The AS2, which exhibited potent anticonvulsant effects, was characterized by 1D and 2D NMR spectroscopy, mass spectroscopy and X-ray crystallography.
Results: The AS2 was characterized as kaur-16-en-19-oic acid (KA), a diterpenoid. The AS2 indicated an oral LD_{50} of 3800 mg/kg. The results showed that the MME, EF and AS2 significantly ($P < 0.05$) and dose-dependently delayed the onset of myoclonic spasms and tonic–clonic phases of seizures induced by PTZ and maximal electroshock seizures (MES).
Discussion and conclusion: Kaurenoic acid was identified as the anticonvulsant principle in the root bark extract of *A. senegalensis*. The anticonvulsant effect of the MME, EF and AS2 is most likely being mediated through central inhibitory mechanisms.

1. Introduction

Epilepsy is one of the most common chronic neurological disorders in the world. According to the WHO report, it affects about 50 million people worldwide with almost 90% of these people found in the developing countries (WHO, 2001; Hirtz et al., 2007). The prevalence of active epilepsy is roughly in the range of 5–10 per 1000 people and 100–190 per 100,000 people in industrialized and developing countries respectively (Sander, 2003). In sub-Saharan Africa (SSA) there is a high prevalence of this disorder with Cameroon and Tanzania leading with prevalence rates of 58 and 35 per 1000 people, respectively, whereas Nigeria has 5.3 per 1000 (Osuntokun et al., 1987; Diop et al., 2003; Preux and Druet-Cabanac, 2005). Traditional healers in different parts of the world, use herbal preparations in the management of epilepsy due to their easy availability and affordability, when compared with standard antiepileptic drugs (AEDs), which are often associated with serious side effects. Teratogenic effects and long term toxicity are among such effects, together with high cost, inaccessibility and refractoriness to treatment (White, 1997). Despite the availability of AEDs, about one-third of individuals with epilepsy still experience seizures that do not respond to medication (Vezzani et al., 2011). Therefore, there is the need for new and potent AEDs. Medicinal plants serve as a reservoir for compounds with proven pharmacological effects. Preparations of root bark of *Annona senegalensis* Pers. (Annonaceae) are used in southeastern Nigeria in the folkloric treatment of epilepsy and febrile convulsions. The anticonvulsant effects of the root bark extract and fractions of *A. senegalensis* have been reported (Okoye et al., 2010; Okoye and Akah, 2010).

Some of the isolated pure compounds from medicinal plants with anticonvulsant effects include methysticin, a pyrone from the rhizomes of *Piper methysticum* G. Forst. (Piperaceae), linalool a monoterpene from *Aeolanthus suaveolens* G. Dom (Labiatae), a triterpenoid glycoside each from *Tetrapleura tetraptera* and *Spathodea campanulata* P. Beauv (Bignoniaceae) (Ilodigwe et al., 2010). Alkaloids with anticonvulsant effects have been isolated from *Capparis baduca* L. (Capparidaceae), *Picnomon acarna* L. (Compositae), *Pithecellobium saman* (Jacq.) Benth. (Leguminosae), while cannabinoids and flavonoids with same activity have been obtained from *Cannabis sativa* L. (Urticaceae) and *Galium*

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cruciata (L.) Scop. (Galiaceae), respectively (Chauhan et al., 1988). Saponins obtained from *Opuntia vulgaris* Mill. (Cactaceae) (Dilipkumar et al., 2005), embelin (2,5-dihydro-3-undecyl-1,4-benzoquinone) from *Embelia ribes* Burm. (Myrsinaceae) (Mahendran et al., 2011) and thymoquinone from *Nigella sativa* L. (Ranunculaceae) seeds (Hosseinzadeh and Parvardeh, 2004) have been shown to possess anticonvulsant effects. Terpenoids, such as acidic and neutral triterpene glycosides, isolated from *Patrinia intermedia* Roem. et Schult. (Leguminosae) (Chauhan et al., 1988), and a diterpene (kaurenoic acid) isolated from the aerial parts of *Espeletia semiglobulata* Cuatrec. (Compositae) (Dalo et al., 2007) have also exhibited anticonvulsant activities.

Further to the report of the anticonvulsant effects of the extract and fractions of *A. senegalensis* (Okoye and Akah, 2010), the study was designed to isolate, identify and characterize the active constituent(s) responsible for the anticonvulsant effects of root bark extract of *A. senegalensis*.

2. Methods

2.1. Animals

Adult albino mice (18–30 g) and rats (180–250 g) bred in the Laboratory Animal Facility of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka, were used in the studies. The animals were maintained under standard laboratory conditions and had free access to standard pellets (Guinea Feeds, Nigeria Plc) and water. On transfer to the work area, animals were allowed two weeks of acclimatization before the commencement of the experiments. All animal experiments were conducted in compliance with the National Institute of Health Guidelines for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985) and approved by the University Ethical Committee on the use of laboratory animals.

2.2. Plant material

Fresh roots of *A. senegalensis* were collected from Enugu-Ezike, Enugu State, Nigeria in the month of June 2007, and authenticated by Mr. A. O. Ozioko of the International Centre for Ethnomedicine and Drug Development (InterCEDD), Nsukka, Enugu State, Nigeria where a voucher specimen was deposited (specimen number: BDCP/INTERCEDD 64). The root-bark was peeled off, cut into pieces and allowed to dry. The dried root-bark was pulverized into coarse powder. The dried powdered root-bark (2.95 kg) was extracted with a mixture of methanol: methylene chloride (1:1) using Soxhlet extractor to obtain the *Annona senegalensis* root bark extract (MME). This was evaporated using a rotary evaporator at reduced pressure to obtain a yield of 375 g (12.71% w/w).

2.3. Solvent-guided fractionation of MME and bioactivity-guided studies

The MME (250 g) was subjected to solvent-guided fractionation in a silica gel (70–220 mesh, Merck Germany) column, successively eluted with *n*-hexane, ethyl acetate and methanol in order of increasing polarity. The fractions were concentrated under reduced pressure in a rotary evaporator (below 40 °C) to obtain the hexane fraction (HF; 115 g; 46.0% w/w), ethyl acetate fraction (EF; 61 g; 24.4% w/w) and methanol fraction (MF; 69.5 g; 27.8% w/w). Bioactivity-guided studies on the extract and fractions using pentylentetrazole (PTZ)-induced seizure showed that EF caused the highest delay in onset of myoclonic spasms and tonic–clonic phases of seizures and afforded 80% protection against seizure induced deaths. Subsequently, EF (50 g) was separated in a silica gel (70–220 mesh, Merck Germany) column eluted with gradient mixtures of *n*-hexane and ethyl acetate (1:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8) and the fractions collected in 500 ml volume. The collected fractions were subsequently pooled and concentrated into eight broad sub-fractions, F_1–F_8, based on the similarity of constituents visualized on silica gel pre-coated TLC plates developed with mixtures of n-hexane and ethyl acetate (8:2). Sub fraction F_2 (9–12; 2000 ml) gave a moderate yield of white crystals when concentrated. The crystals were harvested and purified by several washing with n-hexane and dried to obtain *A. senegalensis* crystals (AS2) (2.8 g; 5.6% w/w). Further activity-guided studies on the sub-fractions showed that the crystals yielded by F_2 exhibited the most potent anticonvulsant effects by causing the highest delay in the onset of myoclonic spasms and tonic and clonic phases of PTZ-induced seizures. MME, EF, and AS2 were also screened for further activities. Phytochemical tests of the extract and fraction were performed using standard procedures (Harbone, 1988) while the acute toxicity (LD_{50}) of MME, EF and AS2 were also determined using Lorke's method (1983).

2.4. Pentylenetetrazole-induced convulsion

Albino mice were randomly divided into five groups (n = five per group). Group I (control) received the vehicle (10 ml/kg, 20% Tween 80 + DMSO (1:1) solution, p.o.). Groups II–IV received the MME (200, 400 and 800 mg/kg, p.o.) while group V received diazepam (Hoffman-la Roche, 3 mg/kg, i.p.). Thirty minutes later, pentylenetetrazole (PTZ) (Sigma, 60 mg/kg, s.c.) was administered to all the animals. The animals were observed for the time of onset of myoclonic spasms and tonic–clonic phases of seizures. Percentage protection of mice against seizure-induced deaths was also recorded in each group. Animals devoid of seizure/convulsion without subsequent death during the 60 min observation period were considered protected (Akah and Nwaiwu, 1988; Nogueira and Vassilieff, 2000; Okoye et al., 2008). The same procedure was then repeated for EF (200, 400, 800 mg/kg, p.o.) and AS2 (100, 200 and 400 mg/kg, p.o.) using different group of animals.

2.5. Phenobarbitone induced sleeping time

Adult albino mice were randomly divided into five groups (n = five per group). Control (group I) animals were treated with the vehicle (10 ml/kg, 20% Tween 80 + DMSO (1:1) solution, p.o.). Mice in groups II–IV were treated with the MME (200, 400, 800 mg/kg, p.o.), while group V received diazepam (Hoffman-la Roche, 3 mg/kg, i.p.). These treatments were carried out 30 min before the administration of phenobarbitone sodium (Renaudin, France, 35 mg/kg, i.p.) to all the groups. Each mouse was observed for the onset (latency) of sleep and the duration of sleep using the loss of righting reflexes as the criterion for onset of sleep and the duration of sleep or hypnosis as the time the animal presented a loss of postural reflexes (Miya et al., 1973; Akah et al., 2007). The same procedure was then repeated for EF (200, 400, 800 mg/kg, p.o.) and AS2 (50, 100 and 200 mg/kg, p.o.) using different groups of animals.

2.6. Motor coordination (rota-rod performance)

This test was performed using Mouse Rota Rod (Ugo Basile, 47600). Adult albino mice were randomly divided into four groups (n = five per group). Group I (control) received the vehicle (10 ml/kg, 20% Tween 80 + DMSO (1:1) solution, p.o.). Groups II and III received the MME (200 and 400 mg/kg, p.o.) while group IV received diazepam (Hoffman-la Roche, 3 mg/kg, i.p.). The animals had already been subjected to the revolution speed (9 RPM) for acclimatization before treatment with the extracts and drugs. Five mice were simultaneously placed on the rotating rod 30 min post treatment and thereafter at interval of 30 min for the period of 90 min. The time lag before the animal fell off from the rotating rod during the 3 min run was recorded (Dunham and Miya, 1957; Mukherjee, 2007). The same procedure was then repeated for EF (200 and 400 mg/kg, p.o.) and AS2 (50 and 100 mg/kg, p.o.) using different groups of animals.

2.7. Maximal electroshock seizure (MES)

Swiss albino rats of both sexes (180–250 g) were divided into five groups with five animals per group. Groups I–III received MME 200, 400 and 800 mg/kg (p.o.) respectively. Group IV received phenobarbitone (30 mg/kg, i.p.) whereas group V, negative control, received the vehicle (10 ml/kg, 20% Tween 80 + DMSO (1:1) solution, p.o). All treatments were administered 30 min before the electroshock. The electroshock was induced in animals by passing a current of 100 mA for 0.2 s duration through electroconvulsiometer (ECT unit 7801, No. 18582, Ugobasile, Italy) using ear electrodes. A drop of electrolyte solution (0.9% Nacl) was used to moisten the ear prior to delivery of electroshock for good electrode contact (Swinyard and Kupferberg, 1985). Total duration of Hind limb Tonic Extension (HLTE) was recorded after the delivery of the electroshock to all the animals. The onset of stupor, death/recovery and the percentage of protection against mortality were also recorded (Mahendran et al., 2011).

2.8. Picrotoxin-induced convulsion

Albino mice of both sexes (18–30 g) were divided into five groups of five animals per group. Groups I–III received MME 200, 400 and 800 mg/kg (p.o.) respectively. Group IV received diazepam (Hoffman-la Roche, 3 mg/kg, i.p.) whereas group V, negative control, received the vehicle (10 ml/kg, 20% Tween 80 + DMSO (1:1) solution, p.o). All treatments were administered 30 min before picrotoxin (Sigma-Aldrich, 2 mg/kg, i.p.). Time for the onset and duration of first convulsive episode was recorded for each animal within the period of 30 min, while death and percentage protection of the animals also recorded at the end of the period (Ogbonnia et al., 2003).

2.9. Identification and characterization of AS2

The purity of AS2 was assessed by analytical HPLC using a Dionex P580 HPLC system coupled to a photodiode array detector (UVD340S, Dionex Softron GmbH, Germering, Germany). Detection was at 235, 254, 280 and 340 nm. The separation column (125 × 4 mm; length × internal diameter) was prefilled with Eurospher-10 C18 (Knauer, Germany), and a linear gradient of nanopure water (adjusted to pH 2 by addition of formic acid) and methanol was used as eluent. The molar mass was determined by liquid chromatography-electrospray ionization mass spectroscopy (LC-ESI-MS) using a ThermoFinningan LCQ-Deca mass spectrometer (Germany) connected to an UV detector. Complete structural characterization of the pure crystals of AS2 was achieved by 1D (HNMR, 13CNMR, DEPT) and 2D (HHCOSY, HMQC, HMBC) NMR spectroscopy using a Bruker ARX-500 and X-ray crystallography. Spot detection was done with ultra-violet (UV) light at 254 nm and spraying with vanillin sulfuric reagent. The melting point of AS2 was also determined using a melting point apparatus (Electrothermal®, Cat. No.: IA 6304, England). Measurements of HPLC and LC-ESI-MS were done at the Institut für Pharmazeutische Biologie, Heirich-Heine Universität Düsseldorf, Germany while NMR measurement was done at the Institut für Anaorganische und Structurechemie of the same University.

2.10. Statistical analysis

Data were analyzed using one way analysis of variance (ANOVA, SPSS Version 16) and expressed as mean ± SEM and multiple comparisons was done using Dunnet test as *post hoc*. Differences between means were regarded significant at $P < 0.05$.

3. Results

3.1. Phytochemical test of the extract

Phytochemical analysis of the extracts indicated the presence of the following constituents in the MME; alkaloids, glycosides, carbohydrates, resins, terpenoids, flavonoids, reducing sugars, steroids, fats and oils. The EF contains mainly alkaloids, resins, terpenoids, flavonoids, fats and oils while the F2 contains resins, terpenoids and alkaloids whereas the AS2 yielded a positive test for diterpenoids (Table 1).

3.2. Acute toxicity of AS2

The acute toxicity (LD_{50}) of AS2 was estimated to be 3800 mg/kg, while that of the MME and EF were 1296 and 2154 mg/kg, respectively, as previously reported (Okoye et al., 2012).

3.3. Pentylenetetrazole-induced convulsion

The results showed that the MME, EF and AS2 significantly ($P < 0.05$) delayed the onset of both myoclonic spasms (MS) and tonic–clonic phases of seizures (TCS) induced by PTZ. The EF and AS2 exhibited dose dependent prolongation of time of onset of both MS and TCS. The percentage protection against seizure induced deaths offered by MME, EF and AS2 was 60, 80 and 80%, respectively. However, the AS2 (400 mg/kg), significantly ($P < 0.01$) delayed the onset of TCS up to 54 min compared to that of diazepam which was 60 min. The order of their efficacy against PTZ induced MS and TCS was AS2 > MME > EF (Table 2).

3.4. Phenobarbitone induced sleeping time

The MME, EF and AS2 significantly ($P < 0.05$) reduced the latency for the onset of sleep and prolonged the duration of sleeping time, in a non-dose dependent manner when compared to the control. The order of both reduction in the latency and the prolongation of sleeping time was MME > EF > AS2 (Table 3).

Table 1
Phytochemical constituents of extracts.

Constituent	MME	EF	F2	AS2
Carbohydrates	+	−	−	−
Alkaloids	+	+	+	−
Reducing sugars	+	−	−	−
Glycosides	+	−	−	−
Saponins	+	−	−	−
Tannins	-	−	−	−
Flavonoids	+	+	−	−
Resins	+	+	+	−
Fats and oils	+	+	−	−
Steroids	+	−	−	−
Terpenoids	+	+	+	+
Acidic compounds	−	−	−	−

+ = present; − = absent.

Table 2
Effect of extracts and AS2 on pentylenetetrazole-induced convulsions.

Treatment	Dose (mg/kg)	Onset of seizure (min)		% protection
		Myoclonic spasms	Tonic–clonic phase	
Control	–	2.48 ± 0.27	6.74 ± 1.52	0
Diazepam	3	60.00 ± 0.00**	60.00 ± 0.00**	100
MME	200	7.88 ± 0.33*	44.80 ± 9.58**	60
	400	5.90 ± 1.00*	22.40 ± 2.11	60
	800	4.06 ± 0.46	16.20 ± 3.00	20
EF	200	4.54 ± 0.48	18.32 ± 10.47	80
	400	7.70 ± 1.81*	22.48 ± 4.62	60
	800	11.60 ± 4.50*	25.92 ± 9.13	60
AS2	100	4.06 ± 0.37	14.84 ± 1.68	0
	200	5.71 ± 0.79*	37.54 ± 9.70**	40
	400	5.92 ± 0.48*	54.00 ± 6.00**	80

Values are expressed as mean ± SEM; significance **P < 0.01, *P < 0.05 using ANOVA, *post hoc*-Dunnet's test compared with the control, n = 5.

Table 3
Effect of extracts and AS2 on phenobarbitone-induced sleeping time.

Treatment	Dose (mg/kg)	Sleep time (min)	
		Latency	Duration
Control	–	37.73 ± 0.95	37.96 ± 3.99
Diazepam	3	13.00 ± 1.53*	202.30 ± 21.07**
MME	200	28.00 ± 4.01	198.80 ± 14.00**
	400	34.40 ± 8.67	239.40 ± 5.41**
	800	16.40 ± 1.54*	135.80 ± 17.73**
EF	200	19.67 ± 1.46*	211.06 ± 2.42**
	400	13.81 ± 0.33*	180.83 ± 6.56**
	800	14.00 ± 1.83*	78.57 ± 9.59*
AS2	50	23.00 ± 4.32*	77.50 ± 6.90
	100	20.00 ± 2.04*	128.25 ± 24.40**
	200	47.00 ± 4.14	97.00 ± 3.72*

Values are expressed as mean ± SEM; significance **P < 0.01, *P < 0.05 using ANOVA, *post hoc*-Dunnet's test compared with the control, n = 5.

3.5. Motor coordination (rota-rod performance)

The extract and fraction progressively impaired the motor coordination as evidenced by the poor performance of the mice on the horizontal rotating rod or thread mill. The decrease in mean motor activity of MME and EF was significant ($P < 0.05$) up till the 60 min of treatment, while the AS2 exhibited non-significant motor in-coordination effects at the same duration of treatment. The motor coordination impairment or the decrease in the fatigue resistance exhibited by the MME was most potent and significantly ($P < 0.05$) dose dependent compared with EF and AS2 (Fig. 1).

3.6. Maximal electroshock seizure (MES)

The *A. senegalensis* root bark extract (MME) exhibited significant ($P < 0.05$) reduction in the duration of hind limb tonic extension (HLTE) caused by the maximal electroshock (ME)-induced seizure when compared with the control. MME at all the doses tested decreased significantly ($P < 0.05$) the onset of stupor in a dose dependent manner (Fig. 2).

3.7. Picrotoxin-induced convulsion

The MME at all doses tested delayed the onset of picrotoxin (PTX) induced convulsions when compared to the control. Similarly MME decreased in duration of seizures induced by PTX. The percentage protection against mortality was 60, 100 and 60% at 200, 400 and 800 mg/kg doses, respectively, compared to 0% and 100% protection offered by the control (vehicle) and diazepam, respectively, against PTX induced seizures (Table 4).

3.8. Identification and characterization of AS2

AS2 was isolated as a white crystalline compound. The melting point was estimated at 170–172 °C and in methanol it exhibited a UV maximum at 214 nm, which is typical of an unconjugated compound. It had strong peaks at 303.2 (M + H), 650.2 (2 M + 2Na) in the positive mode of LC-ESIMS and a corresponding peak at 301.6 (M − H) in the negative mode, which are consistent with the molar mass of 302. Based on this, and the analysis of ^{1}H and ^{13}C NMR, the molecular formula of AS2 was deduced as $C_{20}H_{30}O_2$. The analyses of the HNMR, HHCOSY, C-13 NMR, DEPT, HMQC and HMBC (Table 5) and comparison of data with literature reports (Pacheco et al., 2009; Brassy et al., 1988; Lee et al., 2008) established the structure of AS2 to be kaur-16-en-19-oic acid (Fig. 3). The absolute configuration as shown was based on the observed H NMR coupling constants, HMBC and X-ray crystallography and comparison with literature report (Bruno-Colmenarez et al., 2011).

4. Discussion

The extraction and bioactivity-guided fractionation of the root bark extract of *A. senegalensis* have led to the isolation of AS2 identified as kaurenoic acid, a diterpenoid, which exhibited potent anticonvulsant and sedative effects. Acute toxicity study of the AS2 gave an estimated oral LD_{50} of 3800 mg/kg while that of MME and EF were estimated to be 1296 and 2154 mg/kg, respectively, indicating high level of safety of the extracts and AS2 (Lorke, 1983; Okoye et al., 2012). The phytochemical constituents of the MME showed the presence of alkaloids, glycosides, carbohydrates, resins, terpenoids, flavonoids, reducing sugars, steroids, fats and oils while that of EF were mainly alkaloids, resins, terpenoids, and flavonoids. The fraction, F_2, which yielded the crystals revealed the presence of alkaloids, resins, and terpenoids. The extracts (MME and EF) and pure compound (AS2) exhibited significant ($P < 0.05$) dose dependent delay in the onset of both myoclonic spasms (MS) and tonic–clonic phases of seizures (TCS) induced by PTZ in mice. The AS2 (400 mg/kg) and diazepam offered 80% and 100% protection respectively against PTZ-induced deaths. Among the unprotected animals in the PTZ experiment the extracts and AS2 (100 mg/kg) significantly ($P < 0.05$) increased seizure latency in both the onset of MS and TCS compared with the control. The anticonvulsant effects of MME, EF and AS2 against PTZ-induced seizures indicated their possible effectiveness against absence seizures as drugs that inhibit PTZ-induced convulsions are generally effective against absence seizures (White, 1997; Rang et al., 2007a).

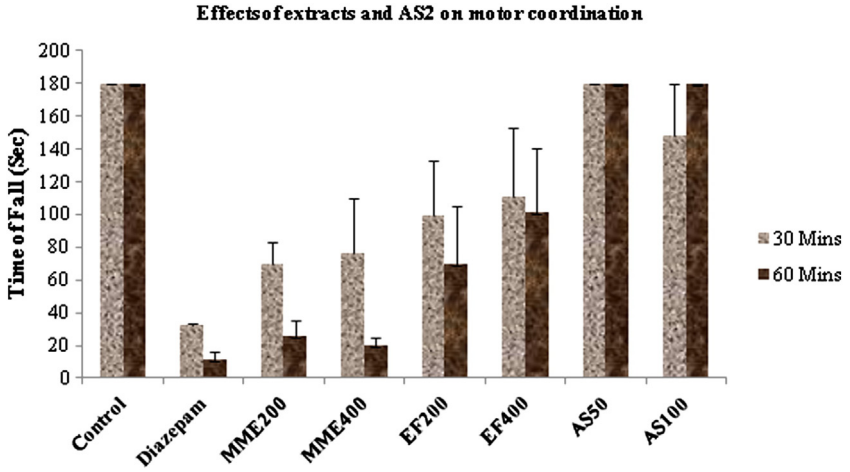

Fig. 1. Effects of extracts and AS2 on motor coordination.

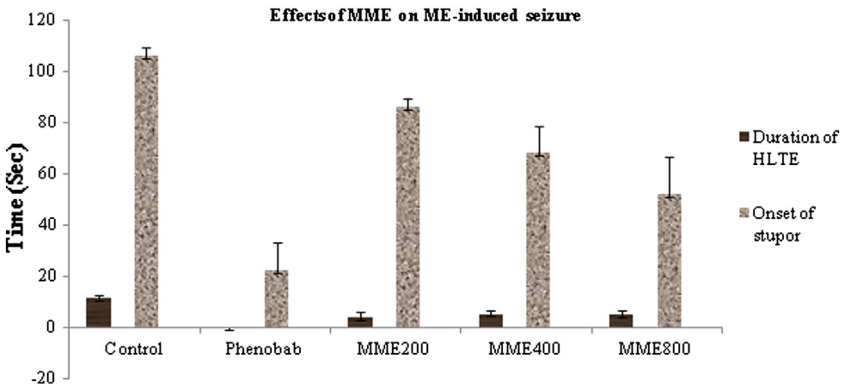

Fig. 2. Effects of MME on MES-induced convulsions.

PTZ induces convulsions by inhibiting the γ-aminobutyric acid (GABA) pathway in the CNS via the inhibition of $GABA_A$ receptor-chloride channel complex (Corda et al., 1990; Kasture et al., 2000). However, the effects of MME and EF against PTZ-induced seizures and prolongation of phenobarbitone sleeping time have been reported (Okoye and Akah, 2010).

Additionally, MME showed significant ($P < 0.05$) reduction in the duration of HLTE and delayed the onset of stupor after maximal electroshock (ME) induced seizures in rats compared with the control. MME and phenobarbitone exhibited 100% protection against mortality from maximal electroshock (ME) induced seizures. Protection against HLTE in ME-induced seizures predicts the ability of an agent to prevent the spread of seizure discharge from the epileptic focus in the brain and suppressing generalized tonic–clonic and partial seizures (White, 1997; Hosseinzadeh and Parvardeh, 2004). Therefore, the dose dependent blockade of HLTE and fast onsets of stupor exhibited by the MME, are indicative of its potent anticonvulsant effects against generalized or grand mal seizures. The extracts and AS2 significantly ($P < 0.05$) reduced the latency and prolonged the duration of phenobarbitone induced sleeping time. Decrease in the latency and the prolongation of duration of sleep by the MME, EF and AS2 is an indication of sedative and central inhibitory effects through the stimulation of the CNS inhibitory pathways (Akah et al., 2007). Also in the rota rod coordination test, MME and EF exhibited significant ($P < 0.05$) motor impairment activity up till 60 min of treatment compared with the control, while AS2 did not show an appreciable decrease in motor coordination at the doses tested. This could be due to lack of motor impairment by the AS2 (Dalo et al., 2007), a desired therapeutic advantage. The muscle relaxant effects of the extracts (MME and EF) could be likened to that of the standard sedatives such as benzodiazepines (Rang et al., 2007b), since rota rod coordination test is a means of assessing CNS depressant effects of pharmacologically active agents (Mukherjee, 2007). The anticonvulsant, sedative and motor impairment effects of extracts and AS2 tend to suggest central inhibitory effects as their possible mechanism of action. Benzodiazepines as well as certain anticonvulsants exhibit pharmacological actions by antagonizing GABA receptor/chloride channel complex (Rang et al., 2007b). Furthermore, MME significantly ($P < 0.05$) delayed both the onset and duration of picrotoxin (PTX) induced seizures and offered 100% (400 mg/kg) protection against mortality compared with the control. PTX is a CNS stimulant and chemical convulsant which acts as a $GABA_A$-receptor antagonist that produces seizures by blocking the chloride ion channels linked to $GABA_A$-receptors thus blocking the postsynaptic inhibitory effect of GABA (Nicoll, 2001). Therefore, the overall central inhibitory effects could be through GABA mediated synaptic inhibition (via GABA receptor chloride channel complex).

The results of the ^{1}H NMR, ^{13}C NMR and X-ray crystallography identified AS2 as kaur-16-en-19-oic acid (KA), a known diterpenoid compound. Forskolin, also diterpenoid, isolated from *Coleus forskohlii* (Willd.) Briq. (Labiatae) has been reported to prevent PTZ-induced seizures in mice (Sano et al., 1984). Interestingly, kaurenoic acid isolated from the aerial parts of *Espeletia semigloburata* has been reported to possess potent anticonvulsant and sedative activities (Dalo et al., 2007) as

Table 4
Effects of MME on picrotoxin induced convulsions.

Treatment (mg/kg)	Time (min)		Protection against mortality (%)
	Onset	Duration	
Control	11.02 ± 1.48	1.52 ± 0.58	0
MME 200	17.58 ± 3.50	1.51 ± 0.51	60
MME 400	18.69 ± 3.80	0.95 ± 0.42	100
MME 800	14.32 ± 1.76	0.90 ± 0.18	60
Diazepam	30.00 ± 0.00*	0.00 ± 0.00*	100

Values are expressed as mean ± SEM; Significance **P < 0.01, *P < 0.05 using ANOVA, *post hoc*-Dunnet's test compared with the control, n = 5.

Table 5
NMR spectroscopic data of AS2[a].

Position	δ_H	δ_C	HMBC
1	Ha 0.82 m Hb 1.54 m	41.48	Ha C-20 Hb C-2, C-3
2	1.42 m (2H)	19.29	1, 3, 4, 10
3	Ha 1.01 m Hb 2.17 m	37.97	1,2,4,5,19
4		43.95	
5	1.07 m	57.26	4, 6, 7, 10, 20
6	Ha 1.62 m Hb 1.84 m	22.03	4, 5, 7, 8, 10
7	Ha 1.44 m Hb 1.54 m	40.90	5, 6, 6, 8, 9, 15
8		44.05	
9	1.05 m	55.29	5,, 8, 10, 11, 14, 15, 20
10		39.85	
11	Ha 1.60 m Hb 1.88 m	18.64	9, 10, 12, 13
12	Ha 1.46 m Hb 1.62 m	33.97	11, 13, 16, 14
13	2.61 bs	43.95	C-8, C-12, C-14, C-16, C-17
14	Ha 1.16 Hb 2.02	39.9	
15	2.06	48.48	C-8, C-9, C-16, C-17
16		156.12	
17	Ha 4.72 s Hb 4.78 s	103.21	
18	1.22 s (3H)	29.18	C-2, C-3, C-4, C-5, C-19
19		184.94	
20	0.93 s (3H)	15.79	C-1, C-5, C-9, C-10

[a] Spectra were measured in $CDCl_3$ at 500 (^{1}H) and 150 (^{13}C) MHz. Assignments were made on the basis of DEPT, ^{1}H–^{1}H COSY, HMQC and HMBC experiments. The detailed NMR data are available with the author for correspondence and will be readily supplied on request.

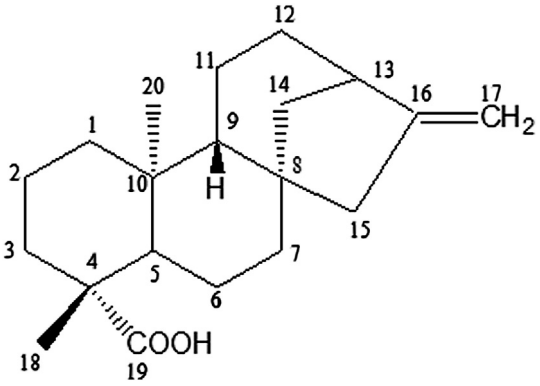

Fig. 3. Kaur-16-en-19-oic acid.

well as anti-inflammatory and anti-pyretic effects (Sosa-Sequera et al., 2010). Report has also revealed the isolation of kaurenoic acid from the leaves of *A. senegalensis* (Eshiet et al., 1971; Fatope et al., 1996) and root extract of *Viguiera arenaria* Baker (Asteraceae) (Porto et al., 2009) which exhibited anticancer and antibacterial activities, respectively. The specific mechanism of anticonvulsant actions of the kaurenoic acid from the root bark extracts of *A. senegalensis* could not be established at this stage of the work. However, mediation of the anticonvulsant and sedative effects of AS2 may likely be through central inhibitory mechanisms via GABA-receptor chloride channel complex. The evaluation of anticonvulsant effects of AS2 on isolated neuronal cultures for the determination of the specific mechanism of action is a point for further research.

4.1. Conclusions

In conclusion, the results of this study suggest that the isolated compound, AS2, from the root bark extracts of *A. senegalensis*, identified as kaurenoic acid, possesses potent anticonvulsant effects against PTZ-induced seizures and may be exhibiting these actions through enhancement of central inhibitory mechanisms mediated by $GABA_A$-receptor chloride channel complex. Kaurenoic acid has therefore, been identified as the possible phytoconstituents responsible for the anticonvulsant properties of root bark of *A. senegalensis*.

Declaration of interest

No conflict of interest declared.

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