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Anticonvulsant effects of a glycoside isolated from the leaf of *Spathodea campanulata* P. Beauv

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Herbal preparations of *Spathodea campanulata* leaves are used in southeastern Nigeria for the treatment of convulsions. A preliminary study of the ethanol leaf extract of *S. campanulata* has confirmed the anticonvulsant potency of the plant. This study was aimed to isolate the constituent(s) responsible for this claimed activity. The ethanol leaf extract was subjected to bioactivity guided fractionation and isolation of the active compound. Anticonvulsant effect of the isolated compound was performed using pentylenetetrazole (PTZ) and electrically –induced seizures. Activities related to anti-convulsion such as effect on rota rod performance and phenobarbitone induced-sleeping time were investigated. Also the acute toxicity studies, as well as the structural elucidation of the isolated compound were carried out using Nuclear Magnetic Resonance (NMR) and mass spectrometry. Results indicated that the new active compound (SCI) isolated from *S. campanulata* exhibited significant ($p < 0.05$) abolition of seizures induced by PTZ and maximal electro shock (MES) seizures. Acute toxicity studies of SCI estimated an oral and intraperitoneal LD_{50} of 323.59 and 158 mg/kg respectively. Structural elucidation of SCI provided a glycoside: urs-12-en-27α, 30 di-oic acid 3-0-α-L-rhamnopyranosyl (1→2) α-L- arabinopyranoside.

Key words: *Spathodea campanulata*, glycoside, anticonvulsant, pentylenetetrazole, electro shock.

INTRODUCTION

The huge diversity of medicinal plants species are endowed with a rich source of potentially therapeutic compounds with novel structures. Approximately 119 pure chemical substances isolated from higher plants are used in medicine throughout the world (Farnsworth et al., 1985). Therefore, study of medicinal plants used in different ancient cultures can be a valuable tool towards the discovery of new molecular compounds or isolates which could serve as a lead compound in the discovery of new therapeutic molecules. *Spathodea campanulata* P. Beauv (Bignoniaceae) is one of such medicinal plants commonly used in folkloric medicine in Nigeria. The plant is commonly known as the 'African Tulip' tree as the shape of the flower is like that of tulip, and is variously known as 'Imiewu' among the Ibo tribe of eastern Nigeria

and 'Oruru' among the Yoruba tribe of Southern Nigeria (Ilodigwe and Akah, 2009). *S. campanulata* leaf extract is used among the people of South Eastern Nigerian for its anticonvulsant, analgesic and anti inflammatory effects (Oliver, 1960; Ilodigwe et al., 2010a) and antiplasmodial effect (Markinde et al., 1987). The bark of *S. campanulata* is used in the treatment of fungal infections, impetigo, herpes, scabies as well as other skin infections (Ainslie, 1937; Oliver, 1960). Various plants isolates or pure compounds with anticonvulsant activity have been isolated from certain medicinal plants. These include methysticin, a pyrone from the rhizomes of *Piper methysticum* (Backhaub and Krieglstein, 1992), linalool, a monoterpene from *Aeolanthus snaveolens* (Elisabetsky et al., 1995), tetrahydrocannabinols (Wada et al., 1975), barcalein (Hamada et al., 1993) and a glycoside from *Tetrapleura tetraptera* (Adesina and Sofowora, 1979). Having established the anticonvulsant effects of *S. campanulata* extract (Ilodigwe et al., 2010a), the aim of this study therefore, was to isolate and characterize the

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active phytochemical(s) responsible for the anti-convulsant effects of *S. campanulata* leaf extract.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats (150 - 170 g) and Swiss albino mice (20 - 25 g) of either sex were obtained from the Laboratory Animal Center of University of Lagos, Lagos state, Nigeria. The animals were maintained under standard laboratory conditions and had free access to standard pellets (Guinea Feeds, Plc, Nigeria) 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 (Pub No. 85 – 23, revised 1985) and approval of the University Ethical Committee on the use of laboratory animals.

Plant material

The fresh leaves of *S. campanulata* were collected from Nawfia, Anambra State, Nigeria. The collections were authenticated by Mr. P. O. Ugwuozor of the Department of Botany, University of Nigeria, Nsukka (UNN), and the voucher specimen No. PCL0567/10 is deposited in the herbarium of the Department of Pharmacognosy UNN. The leaves were air-dried under shade for 5 days, and air-dried samples were pulverized using a mortar and pestle and extracted with ethanol (70%) using cold maceration. The mixture was filtered and the filtrate concentrated under vacuum using rotary evaporator to obtain the Ethanol Extract Residue (EER).

Solvent-guided fractionation of ethanol extract and bioactivity-guided studies

About 100 g of EER was subjected to solvent-guided fractionation in a silica gel (70 - 220 mesh, Merck Germany) column, successively eluted with 1 L of n-hexane, ethyl acetate and methanol in order of increasing polarity respectively. Bioactivity-guided studies on the fractions using pentylentetrazole (PTZ)-induced seizure showed that ethylacetate fraction (EF) exhibited the most potent activity. Subsequently, EF (50 g) was separated in a silica gel (60 – 200 mesh, J.T. Baker, USA) column eluted with gradient mixtures of n-hexane: ethyl acetate. The collected fractions were subsequently pooled and concentrated into six broad fractions, F1 – F6, based on the similarity of constituents visualized on silica gel pre-coated, Thin Layer Chromatography (TLC) plates developed with mixtures of chloroform: ethanol: ammonia (50: 50: 0.5). Crystals instantaneously precipitated out from the F1. The crystals were concentrated and purified by washing successively in solvents to obtain *S. campanulata* isolate (SCI) and stored for activity testing. SCI exhibited potent anticonvulsant activity against PTZ- induced seizure and further subjected to activity testing using maximal electro shock (MES) seizure, rota rod performance test and pentobarbitone induced sleep time test.

Acute toxicity studies of SCI

Seventy mice were divided into seven groups of ten per group after six- hour fasting period. Group 1 - 6 received oral doses of SCI (200, 250, 300, 350, 400 and 450 mg/kg) respectively, while mice in the seventh group received normal saline (10 ml/kg, p.o.), after 6 h fasting period. Mortality in each group was determined twenty four hours after administration. The animals were also observed for toxic symptoms within the stated period. Similarly five groups of mice (10 per group) received SCI (100, 125, 150, 175 and 200 mg/kg) intra peritoneal. Mortality and toxic symptoms were also determined. The median lethal doses (LD_{50}) for the two routes of administration were estimated using probit analysis (Miller and Tainter, 1944).

PTZ- induced seizure test

Thirty six mice were divided into six groups of six mice per group. Groups 1- 4 were treated with SCI (25, 50, 75 and 100 mg/kg, p.o.) respectively. Group 5 received normal saline (10 ml/kg) whereas group 6 received diazepam (Hoffman-la Roche, 2 mg/kg, i.p.), thirty minutes before the administration of PTZ (70 mg/kg, i.p.). The mice were then observed for onsets and durations of convulsions for 30 min post administration of PTZ (Ymitan and Adeyemi, 2005; Ogbonnia et al., 2003).

Maximum electric shock seizure test

Thirty six mice were fasted and divided into six groups of six mice per group. Animals in each group were stimulated through corneal electrodes by a 60 cycle (60 Hz) alternating current until MES indicated by hind limb tonic- extensor spasm was elicited, before and 30 min post treatment of animals in groups 1 - 4 with SCI (25, 50, 75 and 100 mg/kg), group 5 mice were treated with distilled water (10 ml/kg) while group 6 received diazepam (2 mg/kg, i.p.). The duration of electrically induced convulsion was noted for each mouse of the mice (Ymitan and Adeyemi, 2005).

Rota rod performance test

Rota rod performance test was done using the Rota-rod Treadmill (7600 model, Ugo Basile, Italy) as described by Dunham and Miya (1957) and Ozturk et al. (1996). The mice were initially trained by placing them on the rotating rod (18 rev. /minutes) twice daily for three consecutive days. Those mice that were able to maintain a stay on the rotating rod for 3 min or longer for three trials were selected. The mice were divided into five groups of six animals per group. Group 1- 4 received SCI (25, 50, 75 and 100 mg/kg, p.o.) respectively whereas group 5 received normal saline (10 ml/kg). The mice were placed on the rotating rod 30 minutes after administration of SCI and monitored for the time of fall form the rotating rod for the period of 3 min.

Phenobarbitone induced sleeping time test

Thirty mice were fasted for 6 h and divided into 5 groups of six mice per group. Groups 1 - 4 received SCI (25, 50, 75 and 100 mg/kg, p.o.), whereas group 5 received normal saline (10 ml/kg) 30 min before i.p phenobarbitone sodium (45 mg/kg; Renauding, France). The onset and duration of sleep were recorded using loss of righting reflex as onset of sleep (Wambebe, 1985; Akah et al., 2007).

Characterization of SCI

The purified crystals (SCI) were characterized using ^{1}H and ^{13}CNMR: Pyridine-ds with TMS as internal standard, Bruker AM 300. The multiplicities of ^{13}C were determined by DEPT PULSE sequence using Electron impact mass spectrometry (EI- MS) (70ev), Uvikon kotron 930 instruments. Spot detection was done

Table 1. Effects of SCI on PTZ-induced seizures.

Treatment	Dose (mg/kg p.o.)	No. of mice that convulsed/ used	Onset of convulsion (min)	Duration of convulsion (sec)	Percentage death (%)	Percentage protection (%)
Control	10 ml/kg	5/5	9.8 ± 1.6	21.8 ± 1.9	100	-
SCI	25	5/5	11.7 ± 1.1*	16.5 ± 2.6*	100	-
	50	3/5	18.2 ± 2.1*	7.2 ± 3.5*	40	40
	75	2/5	23.5 ± 1.6*	4.6 ± 1.2*	20	60
	100	0/5				100
Diazepam	2	0/5				100

Values are mean ± SEM, *$P < 0.05$ compared with the control, N/gp = 6.

Table 2. Effects of SCI on MES-induced seizures.

Treatments	Dose (mg/kg, p.o)	Duration of convulsion (sec.)	Protection
Control	10 ml/kg	22.6 ± 3.1	0/5
SCI	25	20.2 ± 1.8*	0/5
	50	14.9 ± 7.0*	1/5
	75	8.8 ± 8.2*	3/5
	100	6.2 ± 7.9	3/5
Diazepam	2	2.0 ± 4.0	4/5

Values are means ± SEM, *$p < 0.05$, compared with the control, N/gp = 6.

Table 3. Effects of SCI on time on the rota-rod.

Treatment	Dose (mg/kg)	Time (s)
Control	10	170.1 ± 2.5
SCI	25	169.5 ± 3.0
	50	168.2 ± 2.6
	75	166.3 ± 3.5
	100	164.7 ± 5.2

Values are means ± SEM, N/gp = 6.

with ultra-violet (UV) light at 254 nm and spraying with vanillin sulphuric reagent. The melting point of SCI was determined using the Gallenkamp melting point apparatus.

Statistical analysis

Experimental data were analyzed using one way analysis of variance (ANOVA) and LSD multiple range test to determine significant differences between means. Difference between means were regarded as significant at $p < 0.05$,

RESULTS

Acute toxicity studies

The SCI exhibited an oral and intraperitoneal LD_{50} of 323.59 and 158 mg/kg, respectively.

PTZ- induced seizure test

SCI significantly ($p < 0.05$) and dose dependently increase the onset and decrease the duration of convulsion in the PTZ- induced seizures. It offered 100% protection at the dose of 100 mg/kg and above (Table 1).

Maximum electric shock seizure test

The SCI reduced the mean duration of MES-induced seizures, and offered a significant ($p < 0.05$) degree of protection in a dose dependent manner (Table 2).

Rota rod performance test

The time lag for the animals to fall off the rota rod was dose-dependently but not significantly ($p > 0.05$) reduced by SCI (Table 3).

Table 4. Effects of SCI on phenobarbitone-induced sleeping time.

Treatment	Dose (mg/kg)	Onset of sleep (min)	Time (sec)
Control	10	5.1 ± 0.2	58.3 ± 2.0
SCI	25	5.0 ± 0.4	59.7 ± 3.5
	50	4.7 ± 0.6	62.2 ± 4.0
	75	4.4 ± 0.5	63.0 ± 5.4
	100	4.3 ± 0.4	64.0 ± 5.7

Values are means ± SEM, N/gp = 6.

Phenobarbitone induced sleep time test

Compared with the control, SCI did not significantly ($p < 0.05$) prolong phenobarbitone-induced sleeping time in mice (Table 4).

DISCUSSION

The bioassay-guided fractionation of the extract using the accelerated gradient column chromatography yielded the compound SCI, a pentacyclic triterpenoid (Figure 1). This compound was purified and subjected to GLC, mass spectrophotometer, nuclear magnetic resonance and UV for identification and structural analysis. The result showed that SCI was a triterpenoid saponin in which two sugar moieties were attached. The pentacyclic triterpenoid was isolated from the ethanol extract of *S. campanulata.* It was obtained as an off white powder, which crystallized from ethylacetate: methanol mixture of give off white plates, melting point 201 - 203°C. It gave purple colouration with vanillic sulphuric acid test. The structure of SCI was elucidated by a combination of ^{1}H and ^{13}C-Nuclear Magnetic Resonance (NMR), and mass spectrometry (Table 5). The aglycone of SCI had a peak due to M^{+} ion at *m/z* 486 in the mass spectrum. The ^{1}H-NMR signals at δ:5.22 (bs,H-12) and δ:2.83 (d, J = 12.0 Hz, H-18) in combination with ^{13}C-NMR signals at δ:128.4 (c-12) and δ:133.9 (c-13), indicated that SCI aglycone is a member of the Urs-12-en series. ^{1}H-NMR signals of six methyl protons, four as singlets (δ:0.99, 1.02, 1.08 and 1.12) and two doublets (δ:0.90, J = 6.4; δ:1.21 J = 6.6Hz) confirmed the ursolane skeleton of SCI. The peak of M^{+} ion at *m/z* 206 ($C_{14}H_{22}O_6$) and M^{+} at m/z 278 ($C_{16}H_{22}O_4$) due to retro Diels-Alder fragmentation c 233, 206 (100), 190, 179 and 129 characteristic of steroidal compounds with C_{12}-C_{13} double bond confirmed that SCI has pentacyclic triterpenoid skeleton. ^{13}C-NMR broad band data of the aglycone exhibited signals due to a total of 30 carbon atoms. This fact in combination with EIMS of molecular mass of 486 is consistent with the formula $C_{30}H_{46}O_5$. The C-13 NMR showed two quaternary carbonyl absorption at δ: 178.3 (C-30) and the olefinic quaternary carbon at δ:138.1 (C-13) and were confirmed by DEPT analysis. There were 6CH, 10CH$_2$ and 6CH$_3$

signals typical of pentacyclic compounds. ^{1}H-NMR and ^{13}C-NMR showed OH signal (δ:6.04) at position C-3 and ^{13}C-NMR absorbed at δ:77.5 due to OH signal at position C-3. ^{13}C-NMR absorption at δ:28.1, 22.9, 16.0, 18.4, 18.5 and 19.6 indicated the presence of six methyl groups. Mass spectra of SCI showed peaks due to M^{+} ion at 765, and 633. These were due to fragments from subsequent losses of rhamnose and arabinose. The ^{13}C-NMR broad band data of the glycoside has signals corresponding to 41 carbon atoms and the absence of CH$_2$ signal at 68.52 showed the absence of CH$_2$ linking rhamnose and arabinose. It is therefore proposed that the linkage between rhamnose and arabinose was 1 → 2. Together with the spectral analysis (Table 4) the structural elucidation was then found to be glycoside, urs-12-en-27α, 30 di-oic acid 3-0-α-L-rhamnopyranosyl (1→2)-α-L-arabinopyranoside, (Figure 1). SCI, being a triterpenoid is thought to be a secondary metabolite biosynthesized via the acetate-mevalonate pathway (Trease and Evans, 1994). It has been demonstrated that triterpenoid saponins exert various pharmacological activities including anti-seizure effects (Mahato and Garai, 1998; Melzig, 2001). The isolate (SCI) significantly ($p < 0.05$) attenuated the chemically (PTZ)- induced and electrically(MES)- induced seizures in mice. Decrease in the duration of convulsion by MES and abolition of PTZ seizures by the SCI are indications of central inhibition through the stimulation of the CNS inhibitory pathway. Earlier results had indicated the anticonvulsant effects of the ethanol leaf extract of *S. campanulata* (Ilodigwe et al., 2010a) These suggest a central inhibitory activity as a possible mechanism of action. Anticonvulsant drugs such as barbiturates and benzodiazepines exhibit their effects through enhancement of gamma amino butyric acid (GABA) receptor chloride channel complex which is a GABA/ benzodiazepine mediated inhibition pathway in the central nervous system (Nogueira and Vassilieff, 2000, McNamara, 2001). PTZ induces convulsion by inhibiting the GABA$_A$ receptor- chloride channel complex (Kasture et al., 2000; Corda et al., 1990) and therefore agents that abolish or tend to reduce the effects of PTZ possibly acts through the stimulation of such receptors. Benzodiazepines as well as certain anticonvulsants exhibit pharmacological actions through the reduction of muscle tone, sedation and induction of sleep by antagonizing the
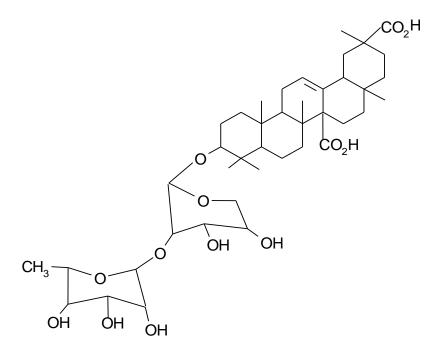
Figure 1. Proposed structure of the glycoside (SCI). Urs-12-en-27α, 30di-oic acid 3-0-α-L-rhamnopyranosyl (1 → 2) -α-L arabinopyranoside.

Table 5. Spectral results of SCI.

C	δ	CH	δ	CH$_2$	δ	CH$_3$	δ
C$_4$	(54.4)	C$_3$	(75.5)				
		C$_5$	(54.5)	C$_1$	(37.2)	C$_{23}$	28.1
C$_8$	(39.5)	C$_9$	(48.1)	C$_2$	(28.8)		
C$_{13}$	(133.7)	C$_{12}$	(128.6)	C$_6$	18.8	C$_{24}$	22.9
C$_{14}$	(48.3)	C$_{18}$	(56.2)	C$_7$	(34.4)	C$_{25}$	16.0
C$_{17}$	(37.0)			C$_{10}$	(36.5)	C$_{26}$	18.4
C$_{20}$	(28.2)					C$_{28}$	19.6
C$_{27}$	(177.5)			C$_{11}$	(27.6)	C$_{29}$	18.5
C$_{30}$	(178.4)			C$_{15}$	(25.5)		
				C$_{16}$	(24.0)		
				C$_{21}$	(30.0)		
				C$_{22}$	(39.6)		

EIMS 70ev: m/z: 486, 464, 453, 278(80), 233(100), 206 (90), 190 (64), 175 (39), 765, 633, ^{1}H-NMR (400mHZ, C$_5$D$_5$N), δ: 1.45(2H,m, H-11), 2.81(2H,d,H-18), 3.84 (1H,b,s,H-3), 5.25 (1H,b,s,H-12), ^{13}C-NMR (100MB$_2$, C$_5$D$_5$N).

GABA receptor/ chloride channel complex (Rang and Dale, 2007). The electroshock seizures are characterized by tonic limb flexion followed by tonic limb extension and finally generalized clonic movements (Swinyard and Woodhead, 1982). Only the abolition of the hind-limb tonic extensor spasm is recorded as the measure of anti-convulsant potency and the ability of the anticonvulsant to prevent seizure spread (McNamara, 2001). The abolition or suppression of the tonic-extensor component of the electroshock seizures as indicated by increase in onset and decrease in duration of MES by the SCI was an indication of its ability to prevent seizure spread (White et al., 1995) and suggest its effectiveness against partial and generalized seizures. Therefore SCI being potent against PTZ and MES seizures would be generally effective against absence seizures and generalized tonic-clonic seizures. Since the MES test identifies agents with activity against generalized tonic-clonic seizures,

seizures, whereas the PTZ test identifies compounds that are efficacious against generalized absence and myoclonic seizures (White, 1997). In this study SCI did not prolong the duration of phenobarbitone-induced sleeping time. Sedative effect of drugs can be evaluated by measurement of phenobarbitone sleeping time in laboratory animals (Ming-Chin, 1998; Amos et al., 2001). Endogenous neurotransmitters in the brain particularly dopamine and GABA have been implicated in sleep mechanisms (Osuide and Wambebe, 1980). It is, therefore, likely that the SCI do not have effect on the dopaminergic pathways or other mechanisms that may be remotely involved in the mechanism of sleep. Glycosides have been implicated in the anticonvulsant constituents of some medicinal plants (Adesina and Sofowora, 1979). Although, SCI appeared to have low oral and i.p LD_{50} values, earlier results on the LD_{50} of crude ethanol leaf extract (Ilodigwe et al., 2010b) revealed that it was generally tolerated and safe.

Conclusion

The results of these studies indicated that SCI is the anti-convulsant principle in *S. campanulata* leaf. Structurally it is a pentacyclic triterpenoid compound- urs-12-en-27α, 30 di-oic acid 3-0-α-L-rhamnopyranosyl (1→2)-α-L-arabinopyranoside, which has anticonvulsant properties with central inhibition as the probable mechanism of action.

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