

**An Anticonvulsant Diterpene Lactone Isolated From the Leaves of
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Chemical, physical and pharmacological methods were used to isolate, identify and evaluate an anticonvulsant diterpene lactone obtained from the leaves of *Leonotis leonorus*. Tonic seizures were chemically induced in mice using pentylenetetrazole (PTZ) at a dose of 95 mg/kg intraperitoneally. Extracts of the plant material obtained with hexane and methanol, and fractions obtained from the methanol extract were tested for anticonvulsant activity. The crude methanol extract at a dose of 100-400 mg/kg ip significantly delayed the onset of tonic seizures induced by pentylenetetrazole with 100 mg/kg ip of the extract protecting 50 % of the mice against seizures. Additionally, compound II (unknown) from the methanol extract at 200 mg/kg ip and 400 mg/kg ip protected 75 % and 87.5 % of mice respectively against the seizures while 100-400 mg/kg ip of 20-Acetoxy-9 α ,13 α -epoxylabda-14-en-6 β (19)-lactone (compound I) significantly delayed the onset of pentratetrazole-induced seizures and protected 50 % of the mice against seizures at 400 mg/kg. The active diterpene lactone was characterised using spectroscopic methods.

Key words: Anticonvulsant activity, isolation, diterpene lactone, *Leonotis leonorus*, mice.

INTRODUCTION

Natural products are used for pharmaceutical purposes, for preparing foodstuffs, insecticides, antioxidants, coloring matters, flavors and fragrances, extractions of enzymes and pheromones among other uses [1-4]. Many studies show that a vast array of these products occurs in the plant kingdom. They are found in the roots, stems, barks, leaves, flowers and fruits of the plant. The distribution of natural products in the plant kingdom is variable according to the species, parts of the plant and age. On the other hand several external factors also contribute to the nature and the concentration as well as the variability of natural products in the different sections of the plant [2, 5-9]. It is for this reason that one section of the plant viz. root can be used to treat disease while another section viz. the fruit can be used for a totally different purpose due to different compounds found in the different sections of a plant. Thus this variability

necessitates that the studies of natural products is to be done very carefully so as not to miss any potentially active agent, which might be found in a plant. In this way the isolated product is known to be active or not and the group and structure of this molecule determined using different physico-chemical methods. In this work, the anticonvulsant activity of *Leonotis leonorus* was undertaken.

Leonotis leonorus belongs in Angiospermae phylum, subphylum Dicotyledoneae in Labiatae (Lamiaceae) family [10-11]. During this study we combined chemical and pharmacological methods to isolate the anticonvulsant agents from the leaves of this plant. According to Watt *et al.* [12] and Van Wyk *et al.* [13], the dried leaves of *L. leonorus* were smoked as a treatment for epilepsy while the infusion or decoction has been used against other symptoms. Claims regarding the therapeutic successes of the plant have come only from oral communication. Thus, the need for

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scientific scrutiny of the claims becomes very necessary. Bienvenu *et al.* [14] verified the anticonvulsant activity of the crude aqueous extract of the leaves of *L. leonorus*. They also suggested a mechanism for antiepileptic activity of the plant extract. However, no attempt has been made to purify the compounds contained in the plant extract and to test for antiepileptic activity in the further purified fractions. This paper describes our investigation into the antiepileptic activity of the further purified compounds in the leaves of *L. leonorus*. The pharmacological and the phytochemical tests done on the active products showed that they are a diterpene lactone and a quinone, respectively.

MATERIALS AND METHODS

Plant material

The plant, *Leonotis leonorus* (L) R. BR., was collected between 9 am and 11 am during the summer months from Cape Flats Nature Reserve, Bellville, Republic of South Africa. It was identified at the Botany Department, University of the Western Cape and a voucher specimen (TRAD 10) was deposited in the University Herbarium.

Plant preparation and isolation of active compounds

The leaves mixed with young stems were washed with distilled water to remove dirt, placed on clean plates and dried in an oven at 30 °C for 72 h. The dried leaves were ground into powder with a warring commercial laboratory blender (KENWOOD CG100) and further milled (mesh size 850 µm). Sequential extraction of sample 890 g of the powder with hexane, then methanol was done using a Soxhlet extractor. The crude methanol extract was subjected to further analysis using both thin layer chromatography (TLC) (silica gel 60_{F254}, Merck) and column chromatography (CC). The stationary phase was silica gel (70-230 mesh), while the mobile phase was hexane: ethyl acetate (7:3). Five fractions were obtained from this column chromatography. The active fraction purified using a preparative thin layer chromatography (PTLC) and a compound obtained was re-crystallised in ethanol.

Pharmacological tests

Animals

Male albino mice, bought from the University of Cape Town, South Africa weighing 18-30 g, were used. The animals were kept in groups of eight per cage and had free access to food and water. Each animal was used for one seizure experiment only.

Drugs and chemicals

Pentylentetrazole (PTZ, Sigma Chemical Co) and phenobarbitone (Gardenal, Rhode-Poulenc Rorer, South Africa) were dissolved in physiological saline. Diazepam (Valium, Roche Products, South Africa) was dissolved in a minimum amount of polyethylene glycol 400 (Fluca, Buchs) and adjusted to the appropriate volume with physiological saline. The crude methanol extract was dissolved in a minimum amount of methanol (0.3 ml), while the hexane extract and further purified product isolated from the methanol extract by PTLC were dissolved in a minimum amount of Tween 80 (0.2 ml) and all adjusted to the appropriate volume with physiological saline. All drugs and extracts were injected intraperitoneally in a volume of 1 ml/100 g of mouse body weight. Control animals received equal volume injections of the appropriate vehicle. Fresh drug and plant material solutions were prepared on the days of the experiment.

Anticonvulsant activity assessment

The convulsant activity test of Velluci and Webster [15] was modified [14-17] and used to assess the anticonvulsant activities of crude methanol and hexane extracts as well as fractions of methanol extract. Mice were used in groups of eight per dose of drug or extracts. The animals were kept singly in transparent perplex cages (25 cm x 15 cm x 15 cm) for 30 min to acclimatize them to their new environment before drug or extract treatment. Seizures were elicited in mice with PTZ. Mice were observed for a period of 30 min following the administration of PTZ. The onset of tonic convulsion and the proportion of animals convulsing were recorded. Animals that did not convulse within 30 min of observation were regarded as not convulsing. To test for anticonvulsant activity the indices of measurement are either significant delay in onset

of tonic seizures or significant reduction in the incidence of seizures (proportion of animals convulsing) or both [16-17]. Experiments were repeated with mice pretreated with crude extracts, isolates or pure products (each for 15 min) and the standard antiepileptic drugs, phenobarbitone (10 min) and diazepam (20 min), prior to the administration of the convulsant agent, PTZ. The pretreatment times were obtained from preliminary studies in our laboratory. Control experiments involving the control vehicles (methanol, Tween 80 and polyethylene glycol 400) dissolved in physiological saline were done concurrently with the test experiments. Experiments were carried out between 9.00 am and 4.30 pm in a quiet laboratory with an ambient temperature of 22 ± 2 °C.

Phytochemical tests of active compounds

Phytochemical identification of active compounds was done using standard chemical methods for identification [18-20].

Spectroscopic characterisation of active compound

The IR absorption of the active compound was studied using a Perkin Elmer Paragon 1000 PC FT-IR Spectrophotometer. The mass spectra were obtained on a Finnigan Mat GCQ GC/Mass Spectrometer, while the NMR spectra were obtained using a Varian Gemini XR 200 NMR spectrometer.

Pharmacological results analysis

The results from the anticonvulsant assessments were analyzed using both paired student's t-test and chi-square test for the onset of seizures and the proportion of animals that exhibited tonic convulsion respectively.

RESULTS

Isolation and purification of active compounds

Sequential extraction of a sample of 890 g of the powder, by first hexane and then methanol, gave

64.5 g (7.24 %) and 220 g (24.72 %) for the hexane and methanol extracts respectively. The fractionation of methanol extract (CC, EtOAc:Hexane, 3:7; Silica gel) gave five fractions. These were characterised by their retardation factor and their colours on thin layer chromatography. Pharmacological testing of each fraction demonstrated that only two fractions, F₁ and F₂ with R_f values of 0.8 and 0.6 respectively, were active. These two fractions were further purified using CC and PTLC. Only the fraction with the R_f value of 0.8 was well purified and crystallized in methanol to give 2.55 g (0.28 % calculated from 890 g of fine powder) of the compound **I** (Figure 1). Fraction F₂ yielded compound **II** which was not pure enough for characterisation, but which was classified as a quinone by phytochemical tests.

Anticonvulsant activities assessment

Effects of hexane, methanol extracts and fractions F₁ and F₂

As preliminary anticonvulsant assessment, hexane and methanol extracts as well as all fractions of the crude methanol extract were tested on mice to assess their activity. Two fractions of methanol extract were found to be active against seizures. The crude hexane extract (100-400 mg/kg) did not affect PTZ-induced seizures to any significant extent. Crude methanol extract (100-400 mg/kg) significantly delayed the onset of PTZ-induced seizures; 100 mg/kg protected 50 % of mice against PTZ-induced seizures. Finally, 0.3 ml of 99.5 % methanol and 0.2 ml of Tween 80, both dissolved in physiological saline, did not affect the onset or incidence of seizures induced by PTZ. Fraction F₁ (100-400 mg/kg) significantly delayed the onset of PTZ-elicited seizures. Doses of 100 mg/kg, 200 mg/kg and 400 mg/kg protected 12.5 %, 50 % and 37.5 % of animals respectively against the seizures. Fraction F₂ (100-400 mg/kg) significantly delayed the onset of seizures induced by PTZ and significantly reduced the incidence of the seizures by protecting 50 %, 62.5 % and 87.5 % of mice at 100, 200 and 400 mg/kg respectively.

Effect of 20-Acetoxy-9 α ,13 α -epoxyabda-14-en-6 β (19)-lactone, compound I and compound II

The compound **I**, obtained from fraction F₁, significantly delayed the onset of tonic convulsion induced by PTZ. At all the doses (100-400 mg/kg, ip) used, Compound **I** reduced the incidence of PTZ-induced seizures. At doses of 200 mg/kg and 400 mg/kg Compound **II** significantly reduced the incidence of the seizures and protected 75 % ($p < 0.01$) and 87.5 % ($p < 0.005$) of mice, respectively (Table 1). 0.2 ml of Tween 80, dissolved in physiological saline, did not

affect either the onset or incidence of seizures induced by PTZ (Table 1).

Effect of phenobarbitone and diazepam on PTZ-induced seizures

Phenobarbitone (20 mg/kg) significantly delayed the onset of seizures induced by PTZ in only one animal and also significantly reduced the incidence of the seizures. Diazepam (0.5 mg/kg) effectively protected 100 % of the animals against the seizures (Table 2).

Table 1. Effect of 20-Acetoxy-9 α , 13 α -epoxyabda-14-en-6 β (19)-lactone, compound I and compound II (unknown) on PTZ-induced seizures in mice.

PTZ	Dose (mg/kg)		Number convulsed/ Number used	Protection (%)	Latency of tonic convulsion (min)	
	Compound I	Compound II			Mean	\pm S.E.M.
95	-	-	8/8	0	6.25	0.37
95	100	-	6/8	25	16.17 ^a	0.35
95	200	-	5/8	37.5	22.00 ^a	0.43
95	400	-	4/8	50	23.75 ^a	0.34
95	-	100	5/8	37.5	20.80 ^a	1.65
95	-	200	2/8 ^b	75	27.50 ^a	0.25
95	-	400	1/8 ^c	87.5	26.00 ^a	0.00
95	-	-	8/8	0	6.00	0.28

^a: $p < 0.001$ compared with PTZ (95 mg/kg) control, Student's *t*-test.

^b: $p < 0.01$.

^c: $p < 0.005$ compared with PTZ (95 mg/kg) control, Chi-square test.

Table 2. Effect of phenobarbitone and diazepam on PTZ- induced seizures in mice

PTZ	Dose (mg/kg)		No convulsed/ No used	Protection (%)	Latency of tonic convulsion (min)	
	Phenobarbitone	Diazepam			Mean	\pm S.E.M.
95	-	-	8/8	0	6.18	0.25
95	20	-	1/8 ^b	87.50	27.00 ^a	0.00
95	-	0.5	0/8 ^c	100	-	-

^a: $p < 0.001$ compared with PTZ (95 mg/kg) control, Student's *t*-test

^b: $p < 0.005$.

^c: $p < 0.001$ compared with PTZ (95 mg/kg) control, Chi-square test.

Physical and Spectroscopic characteristics of compound A

20-Acetoxy-9 α , 13 α -epoxylabda-14-en-6 β (19)-lactone (**I**), m.p. 105-106°C; ν_{\max} 1770 cm^{-1} and 1730 cm^{-1} (nujol); HRMS m/z $[M]^+$ 390.30236 calc. for $\text{C}_{22}\text{H}_{30}\text{O}_6$ 390.20424, 346.1775, 330.1832, 81; ^1H NMR δ_{H} 6.46 (1H, d, J 2.6, H-14), 5.15 (1H, d, J 2.6, H-15), 4.66 (1H, dd, J 6.6 and 4.4, H-6), 4.42 (1H, d, J 10.6, H-16), 4.35 (1H, dd, J 12.4 and 1.7, H-20 α), 4.20 (1H, d, J 12.4, H-20 β), 4.08 (1H, d, J 10.6, H-16), 2.42 (1H, d, J 4.4, H-5), 2.00- 2.22 (6H, m, H-8, H-7 ax, H-11 and H-12), 2.03 (3H, s, H-22), 1.40-1.80 (7H, m, H-7 eq, H-1, H-2 and H-3), 1.26 (3H, s, H-18) and 0.86 (3H, d, J 6.2, H-17); ^{13}C NMR δ_{C} 17.8 (C-17), 18.1 (C-2), 21.2 (C-18), 22.8 (C-1), 23.6 (C-22), 28.5 (C-3), 31.8 (C-11), 32.0 (C-7), 32.9 (C-8), 37.6 (C-12), 42.6 (C-10), 43.7 (C-4), 47.0 (C-5), 66.1 (C-20), 75.9 (C-6), 80.3 (C-16), 89.3 (C-13), 91.8 (C-9), 107.5 (C-14), 148.4 (C-15), 170.2 (C-21) and 182.7 (C-19).

DISCUSSIONS AND CONCLUSION

Pentylentetrazole is a convulsant drug [21], which is widely used to chemically induce convulsions in animals [16]. The mechanism of PTZ induced seizures in mice is still unknown [21]. According to Vida [22], PTZ does not block presynaptic or postsynaptic inhibition, but it activates all pathways. In the same way, the convulsant and respiratory stimulant drugs like PTZ (leptazol) act mainly on the brainstem and spinal cord, producing exaggerated reflex excitability, as well as an increase in activity of the respiratory and vasomotor centres [21]. However it was reported [23] that PTZ produces its convulsant activity by antagonising GABA activity in the brain [23]. In the present study, PTZ (95 mg/kg, ip) elicited seizures in 100 % of the animals used. This compares favourably with the finding of Vida [22], who used 85 mg/kg of PTZ to induce convulsion in more than 97 % of animals.

Phytochemical tests showed Compound **I** and **II** are terpenoid and quinone respectively. Strong anticonvulsant activity of terpenoids has already been shown in other types of medicinal plants like *Ginkgo biloba* [24]. Terpenoids could not only

cross the blood brain barrier (BBB), but could also act as a serotonin uptake inhibitor enhancing norepinephrine and dopamine activity and augmenting gamma amino butyric acid (GABA) concentration [25]. It is pertinent to note that the hypothesis that the enhancement of GABA activity in the brain attenuates convulsions is widely accepted [26-27].

It has been shown that *Leonotis leonorus* is rich in terpenoids essentially diterpene lactones [13, 28-30]. A detection of compound **I** made by placing a developed plate in a chamber containing iodine crystals gave a brown reddish colour while a test with concentrated H_2SO_4 gave a green colour with compound **I**. Both these characteristics may indicate the presence of a lactone ring in compound **I** [19]. Biological activity has been reported [29] in the sesquiterpene lactones. Furthermore, it was found that kava lactones possess anticonvulsant properties [31, 32-33]. Some synthetic lactones compounds have also found to have the anticonvulsant activity [34, 35-36]. In view of these anticonvulsant activities reported for the different groups of lactones, it is not surprising that the leaves of *Leonotis leonorus*, which have been found to be rich in diterpene lactones, could be used as an anticonvulsant drug in traditional medicine.

The results from phytochemical identification were confirmed by the structural elucidation of compound **I**. The structure of this new compound was established as 20-Acetoxy-9 α , 13 α -epoxylabda-14-en-6 β (19)-lactone (**I**) (Figure 1) based on the following spectroscopic evidence and a comparison with the rigorously analysed structure of leonotin [37] (Figure 2).

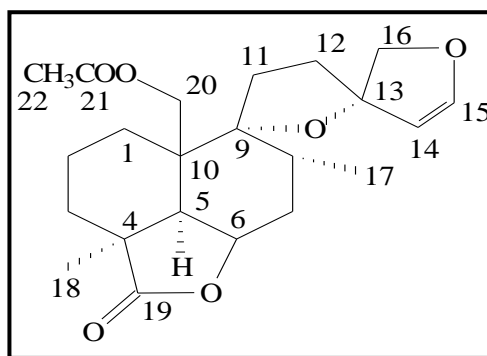


Figure 1. Compound **I**

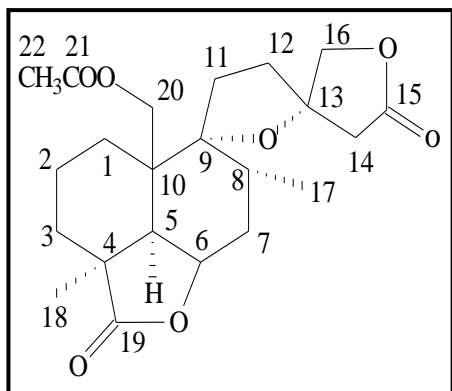


Figure 2. Leonotin

The infrared spectrum of **I** displayed two carbonyl frequencies, one at 1770 cm^{-1} for the lactone and the other at 1730 cm^{-1} for the ester group. The HRMS had a M^+ at 390.30236, which confirmed the molecular formula of $C_{22}H_{30}O_6$ (calc. 390.20424). Loss of the elements C_2H_4O , typical of the acetate group afforded the corresponding C-20 aldehyde as the base peak at m/z 346.17749 for the molecular formula of $C_{20}H_{26}O_5$ (calc. 346.17802). In addition the typical M^+-60 peak representing loss of CH_3COOH at m/z 330.18324 (calc. for $C_{20}H_{26}O_4$: 330.18311) further confirms the C-20 acetate. Finally a strong peak at m/z 81 for the fragment ion shown below (Figure 3) is also consistent with the proposed structure [28].

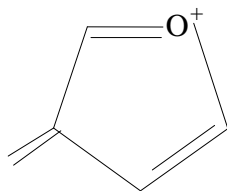


Figure 3. Fragment ion m/z 81

Assignment of structure **I** to the major isolate is based on the close similarities between its $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra to that published for leonotin [37] with the obvious exception of the H-14 and H-15 signals in the $^1\text{H-NMR}$ spectrum of the lactone ring being replaced by ortho-coupled olefinic proton signals at 6.46 and 5.15 ppm (J 2.6 Hz) for H-14 and H-15 respectively. A dd at 4.66 ppm (J 6.6 and 4.4 Hz) is assigned to H-6 (cf. 4.67 ppm and J 6.4 and 4.4 Hz for leonotin). The rest of the $^1\text{H-NMR}$ spectrum closely resembled

that of leonotin. Only two $\text{C}=\text{O}$ signals, viz., 170.2 and 182.7 ppm for C-21 and C-19 respectively were observed in the $^{13}\text{C-NMR}$ spectrum of **I** (cf. 170.1 and 182.3 in the case of leonotin for the same carbon atoms). Peaks at 41.5 and 174.6 ppm respectively for C-14 and C-15 observed in leonotin [37] have been replaced by two peaks at 107.5 and 148.4 ppm respectively for C-14 and C-15 in compound **I**. These indicated the presence of olefinic bond between C-14 and C-15 and the absence of carbonyl group at C-15 in **I**. The anticonvulsant activity of **I** could be in relation of the methyl group found at the α -position of lactone group. It was reported that lactone groups have either positive or negative modulatory activity, depending on the substitution incorporated in the lactone ring [36]. In addition, the presence of alkyl groups at the α -position generally results in anticonvulsant activity [34-36].

The structure of compound **II** is still unknown. However it has been classified as a quinonoid compound. This classification is supported by its yellow coloration on TLC and its positive test with the Bornträger reaction [19, 38]. The spectra obtained from this compound were not helpful in giving information about the structure of the compound since there were still impurities present that co-eluted. The potent anticonvulsant effect of quinones has been reported [39-40]. Herin *et al.* [39] have found that a quinone was able to alter the redox modulatory site of the NMDA receptor and was effective in limiting brain damage in rats. From the above results, it could be suggested that compounds **I** and **II** may be exerting their anticonvulsant activity by altering both NMDA and GABA receptor activities. This may be supported by the finding of Bienvenu *et al.* [14] who reported that the aqueous extract from *Leonotis leonorus* may be exerting its anticonvulsant activity by a non specific mechanism affecting both NMDA and GABA receptor activities. Furthermore, it was shown that PTZ may be exerting its convulsant effect by attenuating GABA_A receptor activity [23].

In conclusion, in the present study two anticonvulsant agents have been isolated. The major isolate compound was identified as 20-

Acetoxy-9 α , 13 α -epoxylabda-14-en-6 β (19)-lactone. The phytochemical tests done on the unknown second product show that it may be a quinone. It is possible from the characterization data that both compounds **I** and **II** may be exerting their anticonvulsant activity by modifying both NMDA and GABA receptor activities.

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