Anticonvulsant Activity of Essential Oils and Active Principles from Chemotypes of *Lippia alba* (MILL.) N.E. BROWN

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In the present work we studied the anticonvulsive effects of the essential oils (EOs) from three chemotypes of *Lippia alba* (MILL.) N.E. Brown (Verbenaceae). Animals (female Swiss mice, 25 g) were treated with the EO and, 30 or 60 min after intraperitoneal (i.p.) or oral (p.o.) administration, respectively, injected with pentylenetetrazole (80 mg/kg, i.p.) and observed for 30 min. The results showed that EO I (200 and 400 mg/kg), EO II (100, 200 and 400 mg/kg), and EO III (400 mg/kg), i.p., produced an increased latency time for the first convulsion as related to controls. Death latency was greater in the groups receiving EO I (50 and 100 mg/kg), EO II (100 and 200 mg/kg), and EO III (200 mg/kg), i.p. Orally, while no effect was demonstrated with EOs at doses of 200 or 400 mg/kg, significant increases in the latency of convulsion and latency of death were observed with EO I at the highest dose (800 mg/kg). Similarly, EO III at this dose was also effective as far as latency of convulsion is concerned. Animals treated with citral (100 mg/kg, i.p.), β -myrcene or limonene (200 mg/kg, i.p.), EOs chemical constituents, presented significant increases in the latency of convulsion and percentage of survival as compared to controls. After oral administration these effects were observed only with a higher dose (400 mg/kg). The association of EOs with diazepam significantly potentiated their effects, suggesting a similar mechanism of action and indicating that citral, β -myrcene, and limonene are probably the EOs active compounds.

Key words essential oil; *Lippia alba*; anticonvulsant activity

Lippia alba (MILL.) N. E. BROWN (fam. Verbenaceae) is a medicinal plant popularly known as *cidreira*, *erva-cidreira* or *falsa melissa*. In Brazil the tea from the plant leaves is largely used in the treatment of respiratory tract diseases, diseases of the central nervous system (CNS) such as insomnia and anxiety,^{1,2)} and also for gastrointestinal problems. We have previously shown that the plant hydroalcoholic extract (HAE) increased the latency time to thermic stimuli in the hot-plate test in mice, as well as decreased the number of abdominal contractions in mice, and significantly inhibited paw licking in the formalin test which is indicative of antinociceptive activity.^{3,4)} HAE also potentiated the barbiturate sleeping time and, in the model of pentylenetetrazole (PTZ)-induced convulsions, it increased the latency of convulsion and latency of death, but no effects were observed in the strychnine or pilocarpine-induced convulsions.⁵⁾

Recent results⁶⁾ showed that all three essential oils (EOs) significantly increased not only the number and percentage of entries but also time and percentage of permanence in the open arms, indicative of anxiolytic activity in the elevated plus-maze test. However, more intense effects were demonstrated with EO II. Psychopharmacologic properties have also been demonstrated in the aqueous extract of *L. multiflora*⁷⁾ which presented tranquilizer and analgesic activities similar to those observed with diazepam (DZP).

In Northeast Brazil there are three types of *L. alba* characterized by their EOs composition. Type I has a high content of myrcene and citral, and is the only one containing hexenile acetate. This is the commonest type in the North and South of Brazil. Type II is characterized by high contents of limonene and citral which are responsible for its nice aroma; and Type III by the presence of limonene and carvone and the absence of citral.⁸⁾ The objectives of the present work were to further explore the pharmacological effects of the

plant, studying in a comparative manner the essential oils from the three chemotypes of *L. alba* with respect to their anticonvulsant action, in an attempt to elucidate the mechanism of the observed effects.

MATERIALS AND METHODS

Plant and EO Extraction Leaves from the three chemotypes of *L. alba* cultivated at the Medicinal Plants Garden of the Federal University of Ceará were collected at the same day and hour for the extraction of the essential oils as well as for their chemical analyses. EOs were obtained from fresh leaves, extracted according to the method previously described,⁹⁾ and analyzed in equipment from Hewlett Packard, model 5971 GC/MS, under the following experimental conditions: fused capillary silica column with 5% phenylmethylsilicone and 20 m by 0.25 mm diameter, 1 ml flux/min and helium gas. Temperatures used were: injector 250 °C, detector 280 °C, heating of the column programmed to 50–180 °C at 4 °C/min and 180–250 °C at 20 °C/min, and electronic impact of 70 eV. EO constituents were identified by mass spectra followed by computerized comparison of data from two mass spectra libraries used to pre-select Kovacs indices. Confirmation was done by visual comparison of experimental data with that from the literature.¹⁰⁾

Animals Female Swiss mice from the Animal House of the Federal University of Ceará and weighing on average 25 g were used. The study was carried out in accordance with the "Guide and Use of Laboratory Animals" from the National Institutes of Health, U.S.A.

Experimental Protocol The EOs were emulsified in 0.5% cremophor before use and administered in doses ranging from 50 to 400 mg/kg. In the case of intraperitoneal or oral administration, respectively, animals were injected 30 or

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60 min before the experiment. The same procedure was followed for citral, limonene and myrcene. Controls were injected with vehicle. DZP was used as standard and PTZ as the convulsant agent.

PTZ-Induced Convulsion After drug treatment (30 and 60 min after i.p. and *p.o.* administration, respectively), animals were injected with PTZ (80 mg/kg, i.p.), placed in individual cages and observed for 30 min. Latency time of the first convulsion, percentage of animals showing convulsion, death latency, and percentage of death were the observed parameters. Severity of convulsions was evaluated by scores according to Czuczwar and Frey¹¹⁾ as: (0) no convulsive behavior, (1) myoclonic seizures, (2) clonic seizures without loss of postural reflexes, (3) clonic seizures with loss of postural reflexes, (4) tonic extension of hind paws, and (5) tonic extension of hind paws with death.

Statistical Analyses Results are expressed as mean $\pm$ S.E.M. and were analyzed by analysis of variance (ANOVA) followed by Dunnett as the post hoc test. Results with $p < 0.05$ were considered significant. For results expressed in percentages, the χ^2 test was used.

RESULTS

Animals intraperitoneally treated with EO I (200 and 400 mg/kg) as well as EO II (100, 200 and 400 mg/kg), and EO III (400 mg/kg) showed an increased latency time for the first convulsion compared to controls. The percentage of animals showing convulsions was 100% for all groups, except for those treated with EO II and EO III (400 mg/kg) which had 75.0 and 87.5% of convulsions, respectively. Death latency

was greater in the groups which received EO I (50 and 100 mg/kg), EO II (100 and 200 mg/kg), and EO III (200 mg/kg), i.p. Death latency for controls was 325.6 ± 52.5 s, while this parameter was not determined for those groups which received EOs at higher doses, due to the high percentage of animals survival. The percentage of survival ranged from 42 to 88% for EO I, 35 to 100% for EO II, and 25 to 100% for EO III for doses up to 400 mg/kg. This figure was 17% for the control group. The degree of severity of convulsions was 5 for controls and 3 for all groups treated with EOs I, II and III, at the dose of 400 mg/kg, i.p. (Table 1).

In the case of oral administration, while no effect was demonstrated with any EO at a dose of 200 or 400 mg/kg, significant increases in the latency of convulsion and latency of death were observed with EO I and III at the highest dose (800 mg/kg). At this dose an increased percentage of survival was detected not only with EO I but also with EOs II and III compared with controls (% of survival for controls was 16.7 and increased from 50 up to 85.7 for the EO treated groups). Similar to EO I, EO III was also effective at the dose of 800 mg/kg as compared to controls for latency of convulsion.

Animals treated with citral at 100 and 200 mg/kg, i.p., showed an increased latency of convulsions and percentage of survival in a dose-dependent way. Also, the degree of severity of convulsions was lower at the higher dose than in controls. With the dose of 400 mg/kg, *p.o.*, there was an increased latency of convulsion as well as an increased percentage of survival and a decreased degree of severity of convulsions in relation to controls. Myrcene (100 and 200 mg/kg, i.p.) did not interfere with latency of convulsion, percentage of survival, or degree of severity of convulsions.

Table 1. Effects of the EOs from Chemotypes of *L. alba* (MILL.) N. E. BROWN in the Model of Convulsions Induced by PTZ in Mice

Group	Latency of convulsion (s)	Animals with convulsion/No. animals	Latency of death	Animals alive/No. animals	Degree of sev. convulsion
Control	94.0 $\pm$ 6.3 (25)	25/25	325.6 $\pm$ 52.5	3/25	5 (3—5)
EO I (i.p.)					
50mg/kg	122.9 $\pm$ 9.9 (12)	12/12	780.3 $\pm$ 167.8*	5/12	5 (3—5)
100mg/kg	115.7 $\pm$ 9.0 (9)	9/9	1089.0 $\pm$ 221.5*	3/9	5 (3—5)
200mg/kg	134.3 $\pm$ 20.8 (9)*	9/9	ND	4/19	5 (3—5)
400mg/kg	241.1 $\pm$ 54.1 (8)*	8/8	ND	7/8 ^{a)}	3 (3—5)*
EO II (i.p.)					
100mg/kg	138.7 $\pm$ 24.9 (14)*	14/14	655.6 $\pm$ 157.2*	3/14	5 (3—5)
200mg/kg	166.6 $\pm$ 30.3 (12)*	12/12	955.0 $\pm$ 189.3*	6/12	4 (3—5)
400mg/kg	261.8 $\pm$ 39.8 (9)*	9/12	ND	9/9 ^{a)}	3 (0—3)*
EO III (i.p.)					
100mg/kg	102.6 $\pm$ 8.6 (12)	12/12	456.7 $\pm$ 99.9	3/12	5 (3—5)
200mg/kg	107.2 $\pm$ 7.9 (12)	12/12	983.2 $\pm$ 136.2*	3/12	5 (4—5)
400mg/kg	262.1 $\pm$ 70.0 (08)*	7/8	ND	8/8 ^{a)}	3 (0—3)*
Control	113.2 $\pm$ 7.1 (18)	18/18	324.6 $\pm$ 32.5	3/18	5 (3—5)
EO I (p.o.)					
200mg/kg	111.5 $\pm$ 10.7 (13)	13/13	539.5 $\pm$ 88.8	2/13	5 (3—5)
400mg/kg	113.3 $\pm$ 13.9 (16)	16/16	635.8 $\pm$ 120.9	3/16	5 (3—5)
800mg/kg	249.4 $\pm$ 68.4 (16)*	16/16	724.9 $\pm$ 176.7*	8/16 ^{a)}	5 (3—5)
EO II (p.o.)					
200mg/kg	146.3 $\pm$ 26.1 (12)	12/12	345.3 $\pm$ 39.0	2/12	5 (4—5)
400mg/kg	161.4 $\pm$ 10.9 (16)	16/16	580.9 $\pm$ 119.7	4/16	5 (3—5)
800mg/kg	199.7 $\pm$ 56.3 (11)	8/11	526.3 $\pm$ 92.2	6/11 ^{a)}	3 (1—5)
EO III (p.o.)					
200mg/kg	126.1 $\pm$ 10.9 (11)	11/11	473.3 $\pm$ 155.6	4/11	5 (3—5)
400mg/kg	135.9 $\pm$ 20.9 (17)	17/17	744.3 $\pm$ 147.2*	5/17	5 (3—5)
800mg/kg	233.4 $\pm$ 44.1 (12)*	10/12	ND	8/12 ^{a)}	3 (1—5)*

Female Swiss mice (20 g) were treated with EOs 30 min (i.p.) or 60 min (*p.o.*) before administration of PTZ (75 mg/kg, i.p.). Results are means $\pm$ S.E.M. of the latency of convulsion and latency of death. In parenthesis, number of animals per group. * $p < 0.05$ (ANOVA, Dunnett post hoc). ^{a)} $p < 0.05$ (Qui-square) as related to controls.

However, at the dose of 400 mg/kg, *p.o.*, it increased the latency of convulsions and percentage of survival with no alteration in convulsion severity. The latency of convulsions of animals treated with limonene (200 mg/kg, *i.p.*) was higher than that of controls while no difference was detected at the dose of 100 mg/kg, *i.p.* In this case the percentage of survival was 27.3 and 58.3% for the doses of 100 and 200 mg/kg, *i.p.*, respectively, while the degree of severity of convulsions was not different from that of controls. Orally, at a higher dose (400 mg/kg), limonene produced an increased latency of convulsion as well as an increased percentage of survival. In order to demonstrate a potentiation of the anticonvulsive activity of the EOs by a benzodiazepine drug, and also the possible involvement of the GABA-channel complex, the effects of EOs were studied in the absence and in the presence of DZP. EOs I, II or III at the dose of 200 mg/kg, *p.o.*, did not alter latency of convulsions, percentage of survival, or the degree of severity of convulsions. Similarly, DZP (0.05

mg/kg, *i.p.*) was not different from controls as far as those parameters are concerned. The association of EOs I, II or III at the dose of 200 mg/kg, *p.o.*, with DZP (0.05 mg/kg, *i.p.*) significantly increased the percentage of survival (75 to 87.5% increase) compared to controls. Also, the EO II (200 mg/kg, *p.o.*) plus DZP (0.05 mg/kg, *i.p.*) decreased the degree of severity of convulsions (Table 3).

DISCUSSION

L. alba (MILL.) N. E. BROWN is a medicinal plant popularly used in Brazil for its spasmolytic and central actions, and especially for its tranquilizing effects. In the present work we have demonstrated for the first time significant anticonvulsive effects in the EOs from leaves of the three chemotypes of *L. alba* with the pentylenetetrazole-induced convulsions model in mice. The anticonvulsive effects were also studied with the essential oils active constituents, such as citral, β -myrcene and limonene in an attempt to elucidate their mechanisms of action.

PTZ produces pro-conflict and convulsant effects in rodents,¹²⁾ but cognitive deficits have also been noted.¹³⁾ These pharmacological effects of PTZ appear to be mediated through a specific interaction with the γ -aminobutyric acid (GABA)-gated chloride ionophore. Disinhibition of inhibitory neurotransmitters such as GABA and/or activation of *N*-methyl-D-aspartate (NMDA) receptors appear to be factors involved in the initiation and generalization of the PTZ induced seizures. Glutamate seems to play a central role in epilepsy, and kindling is considered the most useful experimental model to reveal plastic changes associated with epileptic features.^{14,15)} On the other hand, the development of PTZ-induced kindling in mice is prevented by the co-administration of phenobarbital or diazepam.¹⁶⁾

In the present work we demonstrated that all three EO chemotypes significantly increased the latency of PTZ-induced convulsions at the dose of 400 mg/kg, *i.p.* Chemotype I was also active at a lower dose (50 mg/kg, *i.p.*). However, while the percentage of survival at the dose of 200 mg/kg, *i.p.*, was 44 and 50%, respectively for EO I and II, it was only 25% in the case of EO III and not statistically significant in relation to controls (17%). This parameter ranged from 88 to 100% after administration of the higher dose of EO (400 mg/kg, *i.p.*).

Previous studies¹⁷⁾ observed that kindling induced by repeated intraperitoneal injections of PTZ resulted in increased

Table 2. Effects of Active Constituents Present in the EOs from Chemotypes of *L. alba* (MILL.) N. E. BROWN in the Model of PTZ-Induced Convulsions in Mice

Group	Latency of convulsion (s)	Animals alive/ No. animals	Degree of severity of convulsion
Control (10)	91.4 $\pm$ 5.0	4/10	5 (4—5)
Citral (<i>i.p.</i>)			
100mg/kg (11)	130.7 $\pm$ 7.6*	7/11	3 (3—5)
200mg/kg (12)	247.1 $\pm$ 53.9**	11/12 ^{a)}	3 (1—5)*
Myrcene (<i>i.p.</i>)			
100mg/kg (11)	104.7 $\pm$ 8.6	4/11	5 (3—5)
200mg/kg (12)	110.3 $\pm$ 5.3	3/12	5 (3—5)
Limonene (<i>i.p.</i>)			
100mg/kg (11)	111.1 $\pm$ 7.5	3/11	5 (3—5)
200mg/kg (12)	143.8 $\pm$ 23.3*	7/12	4 (3—5)
Control (18)	113.2 $\pm$ 7.1	3/18	5 (3—5)
Citral (<i>p.o.</i>)			
200mg/kg (11)	124.5 $\pm$ 13.8	2/11	5 (3—5)
400mg/kg (12)	215.8 $\pm$ 34.3**	9/12 ^{a)}	3 (1—5)*
Myrcene (<i>p.o.</i>)			
200mg/kg (10)	93.6 $\pm$ 5.8	2/10	5 (4—5)
400mg/kg (11)	179.7 $\pm$ 28.5*	6/11 ^{a)}	3 (3—5)
Limonene (<i>p.o.</i>)			
200mg/kg (9)	121.6 $\pm$ 10.2	3/9	5 (3—5)
400mg/kg (10)	185.3 $\pm$ 15.8*	7/10 ^{a)}	3 (3—5)

Numbers are means $\pm$ S.E.M. of the latency of convulsion (1st tonic-clonic convulsion) or latency of death, and median of the degree of convulsion severity. Animals were treated with constituents 30 min (*i.p.*) or 60 min (*p.o.*) before PTZ administration. In parentheses, number of animals per group. * $p < 0.05$ and ** $p < 0.05$ (ANOVA and Dunnett, post hoc). ^{a)} $p < 0.05$ (Qui-square) as related to controls.

Table 3. Effect of the Association of EOs from Chemotypes of *L. alba* (MILL.) N. E. BROWN with DZP in the PTZ-Induced Convulsions in mice

Group	Latency of convulsion (s)	No. animals with convulsion/No. Animals	Animals alive/No. animals	Degree of severity of convulsion
Control (14)	129.6 $\pm$ 9.6	14/14	4/14	5 (3—5)
DZP (8)	97.6 $\pm$ 18.2	8/8	1/8	5 (4—5)
EO I 200 mg/kg (10)	157.0 $\pm$ 46.6	10/10	2/10	5 (4—5)
EO I 200+DZP (8)	124.0 $\pm$ 8.2	8/8	6/8 ^{a)}	5 (4—5)
EO II 200 mg/kg (8)	159.9 $\pm$ 37.1	8/8	2/8	3 (3—5)
EO II 200+DZP (8)	167.8 $\pm$ 25.0	8/8	7/8 ^{a)}	3 (3—5)*
EO III 200 mg/kg (11)	126.1 $\pm$ 10.9	11/11	4/11	5 (3—5)
EO III 200+DZP (8)	113.6 $\pm$ 8.8	8/8	6/8 ^{a)}	3 (3—5)

Numbers are means $\pm$ S.E.M. of the latency of convulsion (1st tonic-clonic convulsion), or median of the degree of severity of convulsions. The essential oil was administered orally, 60 min before the PTZ injection (75 mg/kg, *i.p.*). DZP (0.05 mg/kg, *i.p.*) was administered 20 min before PTZ. * $p < 0.05$ (ANOVA and Dunnett, post hoc). ^{a)} $p < 0.05$ (Qui-square) as related to controls.

density of [^3H]-glutamate binding sites in the hippocampus. More recently, Schroeder *et al.*¹⁸⁾ studied [^3H]-glutamate binding to hippocampal membranes and K^+ -stimulated [^3H]-D-aspartate release from hippocampal slices of rats after PTZ kindling. They concluded that aspartate is increased in the early phase of PTZ kindling development, whereas after its completion the density of excitatory amino acid binding sites is enhanced.

In the present work we showed a significant potentiation of the anticonvulsive effect of EOs by low doses of DZP. Thus, after the association of EO with DZP, percentages of survival increased from 2 to 3 times as compared to their effects in the absence of DZP. The antiseizure actions of benzodiazepines, as well as other effects that occur at non-sedating doses, result in large part from their ability to enhance GABA-induced increases in the conductance of Cl^- . Thus, benzodiazepine-induced behavioral responses are mediated by specific GABA_A receptors.^{19,20)} At therapeutically relevant concentrations, DZP and other active benzodiazepines augment the inhibitory effects by stimulating of various GABAergic pathways and enhancing GABA-induced changes in membrane potential. In animals, prevention of PTZ-induced seizures by benzodiazepines is much more prominent than is their modification of the maximal electroshock seizure pattern.²¹⁾

We also demonstrated in this work that citral produced an anticonvulsive effect at a lower dose as compared to those produced by β -myrcene and limonene, and was the most effective among the EO constituents studied. It has been shown that citral decreases locomotor activity in rodents and potentiates imipramine action in the forced swimming test.²²⁾ Previous work²³⁾ showed an analgesic effect of β -myrcene in the hot plate and writhing tests in mice. On the other hand, behavioral studies¹⁶⁾ did not demonstrate any anticonvulsant effect of β -myrcene and concluded that this compound has no action on the CNS. Although limonene has been identified as a tumor inhibitor²⁴⁾ and shown to decrease experimental breast tumor in rats,^{25–27)} no other pharmacological effect has been attributed to this compound.

The only described use for carvone, the other main constituent of EO III, is as a carminative and in the perfumery and soap industries. *l*-Carvone is also an oxidation product of *d*-limonene and probably responsible for allergic processes in humans.^{28,29)} Our results showed that β -myrcene, citral and limonene are responsible, at least in part, for the EOs anticonvulsant effects observed in the present work, and these constituents present a pharmacological profile similar to that shown by DZP-like drugs.

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