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Anticonvulsant Effect of *Annona diversifolia* Saff. and Palmitone on Penicillin-induced Convulsive Activity. A Behavioral and EEG Study in Rats

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Summary: *Purpose:* To evaluate hypnotic and anticonvulsant activities of *Annona diversifolia* Saff. and palmitone by using behavior and electroencephalographic (EEG) analysis in an experimental model of focal seizures in rats.

Methods: For hypnotic assessment, EEG analysis of polysomnographic slow-wave sleep (SWS) and rapid eye movement (REM) sleep for a 1 h period were performed after vehicle, *A. diversifolia* extract or palmitone, administration. For anticonvulsant effect, 60 minutes after treatments, EEG and behavior were analyzed during penicillin-induced seizures. Latency to the onset of the first paroxystic spike, first seizure and frequency, as well as seizure severity using Racine's scale, were determined.

Results: Palmitone, but not *A. diversifolia* extract, produced a delay in the latency to the SWS phase. In addition, both palmitone and extract decreased SWS duration and accumulated REM sleep phase. With regard to the seizures, both the extract and palmitone increased the latency to the onset of spikes and seizures, but also decreased the duration of penicillin-induced seizures. This reduction in the EEG recordings was associated with an attenuation in the severity of behavioral seizures.

Conclusions: A. diversifolia and palmitone did not produce a sedative-hypnotic effect although both of them were effective in reducing the severity of behavioral and EEG seizures induced by penicillin in rats, suggesting that the diminution in the paroxystic activity by *A. diversifolia* is likely produced by palmitone through GABAergic neurotransmission. This study justifies and reinforces the traditional use of this plant in epilepsy.
Key Words: *Annona diversifolia* Saff.—EEG—Palmitone—Penicillin—Seizures—Traditional medicine.

Epilepsy is one of the major neurological disorders of the brain, affecting approximately 0.5–1.0% of the world population. The goal of the epilepsy treatment is the complete abolition of seizures, and today this aim seems more attainable than before because of more accurate diagnosis of epilepsy syndromes, rational polypharmacy, surgical intervention (Wyllie, 1988; Engel, 1993) and a ketogenic diet (Swink et al., 1997). Although, 70% to 80% of patients with this affliction control their seizures with minimal side effects, there are still 20% to 30% suffering from intractable epilepsy (Leppik, 2001). Epilepsy is considered to be intractable when the seizures cannot be brought under full control within 1 year after the initiation of appropriate therapy documented with blood levels (Leppik, 1992). In addition to refractoriness, adverse effects or poor response to available antiepileptic drugs are compelling reasons to further research new treatment alternatives.

In Mexico as in all over the world, "herbolaria," healing with plants, is considered a very commonly therapeutic alternative. In the pre-Hispanic Badiano Codex, some plants such as "flor de corazon" and "tumbavaqueros" are mentioned as remedies for controlling seizures. On the other hand, it has been reported that plants belonging to the Annonaceae family are also used in traditional medicine for their anticonvulsant properties (Gill, 1948). Experimental models of epilepsy are essential for the development of new treatment alternatives, as well as for the discovery of

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new drugs to manage epileptic seizures. Previous studies in mice have demonstrated that the ethanol extract from *A. muricata* leaves not only delays the presence of myoclonus and clonic–tonic seizures induced by pentylenetetrazole (PTZ), but also reduces mortality in these animals (N´Gouemo et al., 1997). In 1998 we reported that *Annona diversifolia* leaves possessed the same anticonvulsant properties of *A. muricata*, but that anticonvulsant effect was concomitant to pronounced motor incoordination and to potentiation of the pentobarbital-induced hypnotic effect (González-Trujano et al., 1998).

Topical administration of sodium penicillin G is an experimental model commonly used to produce epileptic foci and interictal activity, both in the motor cortex (Gloor et al., 1977; Collins, 1978) and the amygdala (Fernández-Guardiola et al., 1995; Martínez et al., 2004), that resembles interictal spikes recorded in the human cortex (Gloor et al., 1977; Fisher, 1989). In the present study, we evaluated whether the anticonvulsant effect of *A. diversifolia* ethanol extract and its natural product palmitone was due to a reduction in the paroxystic cerebral activity and whether this effect was associated with hypnotic activity.

METHODS

Plant material

Leaves of *A. diversifolia* Saff. were collected in Tejupilco, Edo. de México, in September 2004. A voucher specimen of the plant was deposited for its confirmed identification in the herbarium Maximino Hernández in the Universidad Autónoma Chapingo, Edo. de México, México.

Preparation of the extract and palmitone

The air-dried powdered mature leaves (3 kg) were extracted through successive hexane (10 L × 3) and ethanol (10 L × 3) maceration at room temperature (22 ± 1°C). The extracts were concentrated in vacuum and the final hexane (130 g) and ethanol (110 g) crude extracts were obtained. Palmitone (16-hentriacontanone, purity 99.79%) was isolated from an hexane fraction (3.2 g) obtained from *A. diversifolia* leaf ethanol extract sample (10 g) as previously described (González-Trujano et al., 2001), to yield 32.76 mg; the IR, NMR, and mass spectral analysis of this compound matched the compound described in the extraction process (González-Trujano et al., 2001).

Animals

Experiments were carried out in compliance with the ethical committee guidelines laid down by the Instituto Nacional de Psiquiatría "Ramón de la Fuente Muñiz" regarding the care and use of animals for experimental procedures. Male Wistar rats weighing 280–300 g (at the beginning of the study) were used. Animals were individually housed under standard laboratory conditions (22 ± 1°C, 12:12 h light/dark cycle) and maintained on standard pellet diet (Lab diet) and water ad libitum.

Drugs

All substances were freshly prepared on the day of the experiments. Extract (30 mg/kg, $n = 12$) and palmitone (10 mg/kg, $n = 6$) were suspended in vehicle [0.5% tween 80 in saline solution 0.9% (s.s.), $n = 18$] and all treatments were injected by the intraperitoneal (i.p.) route in a volume of 1 mL/kg body weight. Preliminary acute experiments using the Rota-rod test demonstrated that 30 mg/kg of *A. diversifolia* extract (González-Trujano et al., 1998) and 10 mg/kg of palmitone are appropriate doses producing anticonvulsant effect without motor impairment (González-Trujano et al., 2001, 2006). Sodium penicillin G (Lakeside, S.A. de C.V. México), 100 IU dissolved in 1 μL deionized water, pH 7.4, was locally applied into the central amygdala nucleus. All rats were housed individually within a sound-insulated chamber and handled at intervals for 3 days prior to the beginning of the experiments to adapt them to manipulation.

Experimental procedure

Animals were deeply anesthetized with a combination of ketamine (Anesket^{TM}), 100 mg/kg, and xylazine (Rompun^{TM}), 20 mg/kg, i.p. Then, a bipolar stainless steel electrode bound to a cannula, directed to the central amygdala nucleus (–2.4 mm Bregma, 4.3 mm lateral, and 7.0 mm vertical) and a second to the dorsal hippocampus (–7.3 mm Bregma, 2.0 mm lateral, and 6.4 mm vertical) were stereotaxically implanted according to the Paxinos and Watson atlas (Paxinos and Watson, 2005). Two stainless steel nail electrodes were epidurally implanted into the prefrontal cortex. The electromyogram was recorded using flexible electrodes inserted into the nape muscles. Animals were left to recover for a 7-day period prior to the experimental session. During the experiments, a concurrent electroencephalography (EEG) of the prefrontal cortex, central amygdala nucleus, and dorsal hippocampus was recorded, as was an electromyogram. Each experiment was conducted on a pair of rats at the same time, one served as a control (vehicle) and the second underwent treatment (extract or palmitone); both were placed inside a sound-insulated box. EEG records were obtained using a Grass Model 78 D. Basal cerebral activity was traced for 30 minutes before the administration of vehicle and the extract or palmitone.

To determine whether the extract or palmitone administration produced a hypnotic response, the EEG was analyzed and the following parameters were evaluated: latency to the onset and duration of the first slow-wave sleep (SWS) and accumulated rapid eye movement (REM) sleep during 60 minutes.

To evaluate seizures, 60 minutes after vehicle and extract or palmitone administration, sodium penicillin G was injected in the two rats being simultaneously tested and

latency to the first spike and to first seizure, as well as frequency and duration of seizures, were analyzed in each experiment. Seizure behavior was assessed simultaneously with the EEG recording for a 240-min period. Seizure severity was scored according to Racine's scale (1972): (I) immobility, eye closure, ear twitching, twitching of vibrissae, sniffing, facial, clonus; (II) head nodding associated with more severe facial clonus; (III) clonus of one forelimb; (IV) bilateral clonus accompanied by rearing without falling; (V) generalized clonic seizures accompanied with rearing and falling. Status epilepticus was also recorded.

At the end of the experiments, animals were euthanatized by decapitation and brains were frozen on dry ice to be sliced into 40-μm-thick sections, in a coronal plane, on Cryostate Microm and stained with cresyl violet to establish the precise location of the cannula and electrodes (data not shown) (Paxinos and Watson, 2005).

Statistical analysis

Data are shown as mean ± SEM or median with 25 and 75 percentiles. Area under the curve (AUC) was calculated to measure the convulsive activity. The AUC is an expression relating the number of events in a 2 h recording period versus behavioral stage and calculated by using the trapezoidal rule (Rowland and Toser, 1989). Comparisons between control (vehicle), extract, and palmitone groups were done by analysis of variance (ANOVA) followed by Dunnett's test. When data were not normally distributed, the Kruskall–Wallis test (nonparametric one-way ANOVA) followed by Dunn's multiple comparison test was applied. A statistical difference was determined for values $p < 0.05$. SIGMA STAT® 2.3 software was used.

RESULTS

A 30-min baseline trace was obtained prior to the injection of the treatments (Fig. 1; panels A, C, and E). After administration of the vehicle (Fig. 1; panel B), extract (Fig. 1; panel D) or palmitone (Fig. 1; panel F), the recording was analyzed for an hour to evaluate the latency and duration to the first SWS phase. In the group receiving vehicle, the latency to and duration of the first SWS was 785 ± 159 s and 202 ± 68 s, respectively. After both extract and palmitone there was a delay in the latency (1209 ± 207 s and 1993 ± 557 s; $F_{2,33} = 9.76$, $p < 0.001$; Fig. 2A) and a nonsignificant diminution in the duration of the first SWS (76 ± 18 s and 73 ± 32 s; Fig. 2B). Accumulated REM sleep phase was detected in 100% of rats from vehicle group with a duration of 232 ± 59 s/1 h. Only 8% of rats administered with extract [12 ± 10 s/ 1 h ($H = 8.53$, $df = 2$, $p < 0.01$)] and 33% of rats receiving palmitone (134 ± 80 s/1 h) showed REM sleep (Fig. 2B).

After penicillin injection into the central amygdala nucleus, results were analyzed for cortex, amygdala, and hippocampus recordings as follows: the presence of the first spike was respectively delayed by 15-9-35% in the group receiving extract and, by 53-34-39% in the palmitone group, in comparison to the vehicle group. Whereas, the first seizure was retarded in the corresponding percentages, 165-172-161% in rats receiving extract and 8-11-7% for those administered palmitone, in comparison to the vehicle group. In addition, a decrease in the duration of the first seizure was also observed in all cerebral areas registered, mainly in the amygdala, where a 50% ($F_{2,33} = 8.07$, $p < 0.001$) and 36% reduction was found in rats receiving extract and palmitone, respectively, as compared with the vehicle group (Table 1).

The severity score was determined using Racine's scale (Racine, 1972). In general, rats receiving extract and palmitone administration showed fewer spikes and a decreased duration in the seizures in comparison with the vehicle group (Fig. 3). These EEG features were associated with a reduction in the convulsive behavior. All rats treated with extract exhibited seizure stages I to III, but only 40% reached IV or V (Fig. 4). Concerning palmitone, frequency of stages was decreased, but 50% of the rats displayed stages IV or V and one rat reached status epilepticus (Fig. 4). In contrast, almost 100% of the rats receiving vehicle demonstrated all seizure stages (I–V) (Fig. 4) and two of them reached status epilepticus. These effects were interpreted as AUC in relation to the number of events in 2 h versus the stage achieved. A significant ($F_{2,33} = 16.77$, $p < 0.001$) diminution was observed in the extract [AUC = 23.08 ± 6.89 area units (au)] and palmitone (AUC = 16.50 ± 2.91 au) groups in comparison to the vehicle group (AUC = 56.50 ± 3.98 au) (Fig. 5).

DISCUSSION

In this study, we investigated the hypnotic and anticonvulsant effect of an ethanol extract of *A. diversifolia* and its natural product palmitone by analyzing EEG recordings and the convulsive behavior induced by a topical administration of sodium penicillin G into the central amygdala nucleus.

The EEG activity of SWS and REM sleep represents a measure of sleep intensity (Tobler, 1995). Sleep has been considered as a use-dependent local phenomenon that stimulates synapses, which were insufficiently utilized during wakefulness to maintain neuronal connections (Krueger et al., 1995). In fact, diagnostically, it is commonly used to increase the yield of epileptiform activity (Malow et al., 1999). In previous studies, administration of the *A. diversifolia* extract increased the hypnosis induced by sodium pentobarbital, thus suggesting depressant properties (González-Trujano et al., 1998). Nevertheless, any synergism of sodium pentobarbital–induced sedative or hypnotic activity was observed in presence of

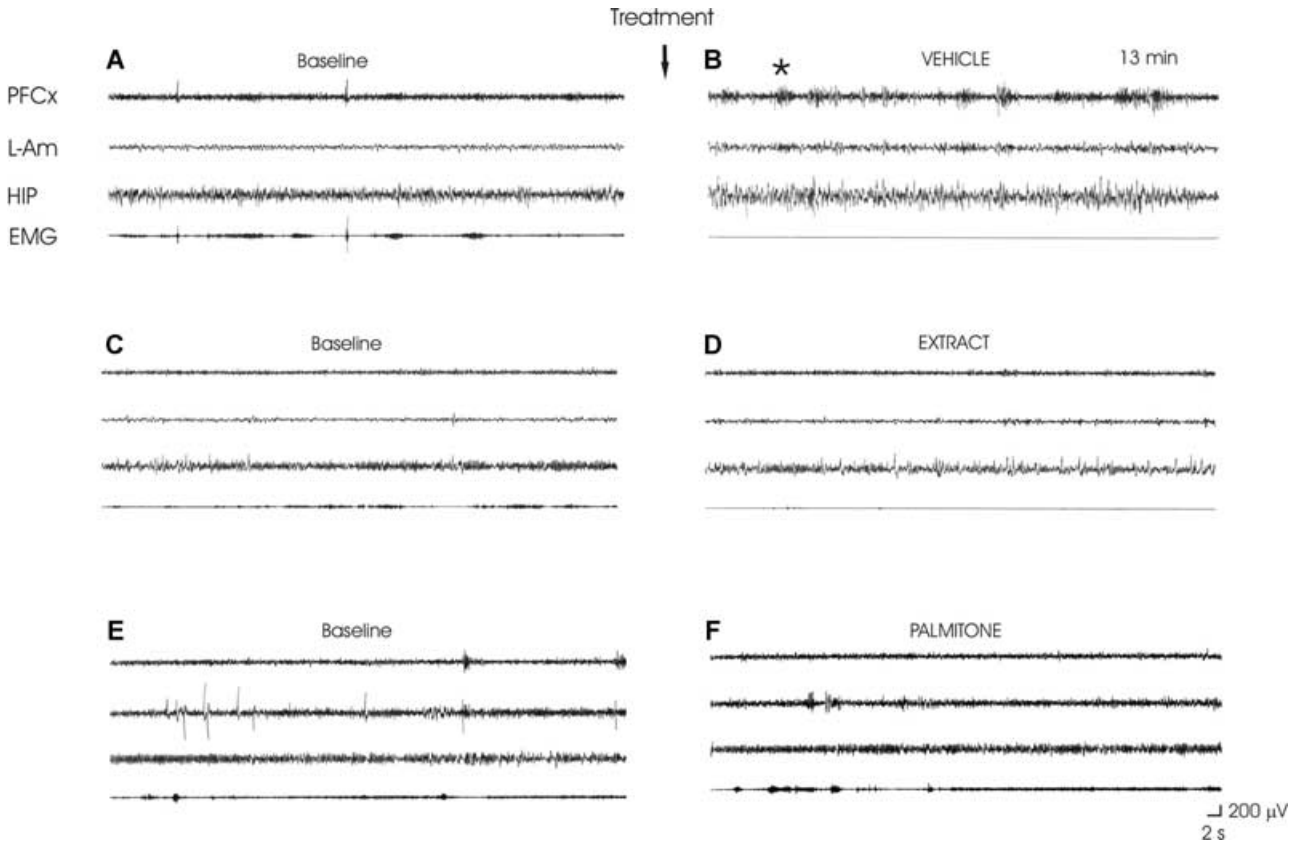

FIG. 1. Sampling traces showing baseline electrographic activity in prefrontal cortex (PFCx), left amygdala (L-Am); hippocampus (HIP) and electromyogram (EMG) in absence of treatment (panel **A**, **C**, and **E**) and 13 min after of intraperitoneal administration (arrow) of 0.5% tween 80 in s.s. as vehicle (panel **B**), 30 mg/kg extract (panel **D**), or 10 mg/kg palmitone (**F**). The asterisk on the trace indicates characteristic spindles of the slow wave sleep phase in a rat from vehicle group in comparison to rats from the extract and palmitone groups.

palmitone, the major anticonvulsant active principle isolated from *A. diversifolia* extract (González-Trujano et al., 2001). It has been described that some plant extracts enhance barbiturate-induced sleeping time by inhibition of the hepatic enzymatic metabolism (Gyamfi et al., 2000). The enzymatic metabolism has not been measured in presence of *A. diversifolia* extract, but since we neither observed in the EEG a facilitation in the SWS nor in the REM parameters, we cannot rule out that enzymatic inhibition may be involved in the potentiation produced by *A. diversifolia* extract on the sodium pentobarbital–induced hypnosis. Further studies are necessary to demonstrate this new hypothesis. However, this study demonstrates that effects of the *A. diversifolia* extract and palmitone during the first 60 min of administration are not related to sedative-hypnotic effects per se.

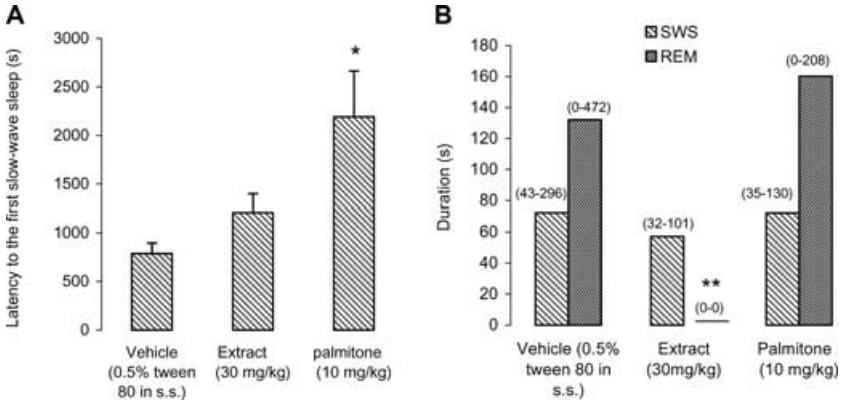

FIG. 2. Effect of vehicle (0.5% tween 80 in s.s., n = 18), palmitone (10 mg/kg, n = 6) and extract (30 mg/kg, n = 12) on the latency (s) to the onset of SWS spindles (**A**) and duration (s) of SWS (the left bar of each group) and REM (the right bar of each group) phases in rats (**B**). Each bar represents the mean ± S.E.M or the median with 25 and 75 percentiles, respectively. *$p < 0.01$, ANOVA followed Dunnett's test. **$p < 0.01$, Kruskall-Wallis test (ANOVA on ranks) followed Dunn's test.

TABLE 1. *Effect of the extract and palmitone administration on the penicillin-induced convulsive activity in rats*

Group	n	Latency to the first spike (s)			Latency to the first seizure (s)			Duration of the first seizure (s)		
		Cortex	Amygdala	Hippocampus	Cortex	Amygdala	Hippocampus	Cortex	Amygdala	Hippocampus
Vehicle (0.5% tween 80 in s.s.)	18	89 ± 31	86 ± 29	95 ± 36	162 ± 85	157 ± 80	165 ± 87	35 ± 9	49 ± 6	34 ± 5
Extract (30 mg/kg)	12	103 ± 37	95 ± 38	128 ± 45	431 ± 219	428 ± 220	431 ± 219	28 ± 6	24 ± 5*	27 ± 6
Palmitone (10 mg/kg)	6	136 ± 47	116 ± 47	132 ± 63	175 ± 125	175 ± 125	176 ± 127	30 ± 3	31 ± 2	27 ± 4

Data represent mean ± S.E.M. *$p < 0.001$, ANOVA followed by Dunnett's test.

On the other hand, both the extract and palmitone treatments produced a delay in the presence of sodium penicillin G–induced first spike, but only the extract delayed the occurrence of the first seizure in comparison to the vehicle group. It has been well established that penicillin epileptogenic focus is chloride dependent and exerts its antagonist effect on the $GABA_A$ receptor (McDonald and Barker, 1977; Hablitz, 1981). In previous studies in mice, *A. diversifolia* extract and palmitone produced a dose-dependent and significant increase in the latency to the onset of PTZ-induced seizures (González-Trujano et al., 1998, 2001), a selective GABAergic antagonist (McDonald and Barker, 1977; Rehavi et al., 1982). Also, palmitone retarded the presence of seizures produced by bicuculline (González-Trujano et al., 2001), a specific antagonist of the $GABA_A$ receptor site (Heyer et al., 1981), but not that of seizures produced by other kind of convulsant substances like strychnine or kainic acid (González-Trujano et al., 2001). In the present study, palmitone retarded the presence of the penicillin-induced first paroxystic spike indicating a delay in the onset of the convulsive action of sodium penicillin G. These results suggest that anticonvulsant effects of palmitone are likely to involve an inhibitory effect through GABAergic neurotransmission. However, it is important to mention that palmitone is a hydrophobic aliphatic ketone; consequently, possible changes in the cellular membrane may modify the coupling of convulsant agents, such as PTZ, bicuculline, and penicillin, with the $GABA_A$ receptor. In fact, after acute and repeated administration of palmitone, a diminution in flunitrazepam binding has been observed in brain mice (González-Trujano et al., 2000). Furthermore, although palmitone may be the main active principle involved in the anticonvulsant activity of the extract in penicillin-induced paroxystic activity, it may not be unique, since there is no participation of this compound in the delay to the penicillin-induced first seizure.

Regarding the delay in the onset of the first seizure, it may indicate that the extract is interfering with neuron recruitment. Besides, a decrease in the severity of behavioral seizures was observed in rats receiving extract or palmitone; these animals remained in stages I, II, and III and rarely reached stages IV and V, in contrast to the vehicle group. A correspondingly reduced cerebral activity was also observed in EEG activity. Some rats did not show convulsive behavior even when they showed paroxystic activity in the EEG record. These results indicate that the extract reduces the severity of paroxystic activity but mainly in reference to generalized seizures. It has been reported that neurons surrounding the penicillin exert substantial inhibitory activity in an attempt to limit the spread of the seizure (Prince and Wilder, 1967; Engel and Ackermann, 1980). According to Racine's scale (1972), stages I, II, and III are considered partial seizures, whereas stages IV and V indicate generalized convulsive activity. Furthermore, it has been described that during the onset and spreading of penicillin-induced paroxystic activity, several cerebral regions are directly involved with the amygdaloid complex, such as the prefrontal cortex (Fernández-Guardiola et al., 1991, 1995), thalamic regions (Miller, 1992), and the hippocampus (Bertram et al., 1998). Since extract and palmitone administration were systemically applied, this route reinforces their central effects and suggests that inhibitory mechanisms may be activated in more than one cerebral area.

On the other hand, it is well known that ictal and interictal activity is facilitated or inhibited during, respectively, synchronization and desynchronization associated with sleep–waking states (Fernández-Guardiola et al., 1995; Bazil and Walczak, 1997; Kumar and Raju, 2001). The generalization of epileptic discharges is attenuated during states of thalamocortical EEG desynchronization, such as alert waking and REM sleep, and favored during states of thalamocortical EEG synchrony, notably SWS (Shouse et al., 1989; Calvo and Fernández-Mas, 1991; Fernández-Guardiola et al., 1995; Martínez et al., 2004). Large populations of thalamocortical neurons discharge synchronously in high-frequency bursts during the SWS phase and may provide a natural mechanism for epileptic EEG discharges, whereas EEG desynchronization, with its asynchronous modes of neuronal discharge, does not (Gloor et al., 1977). Sleep studies have demonstrated that some anticonvulsant substances promote changes in SWS and REM sleep phases (Placidi et al., 2000a, 2000b). Also, specific increases have been demonstrated in interictal spikes and sharp during the SWS phase (Sammaritano

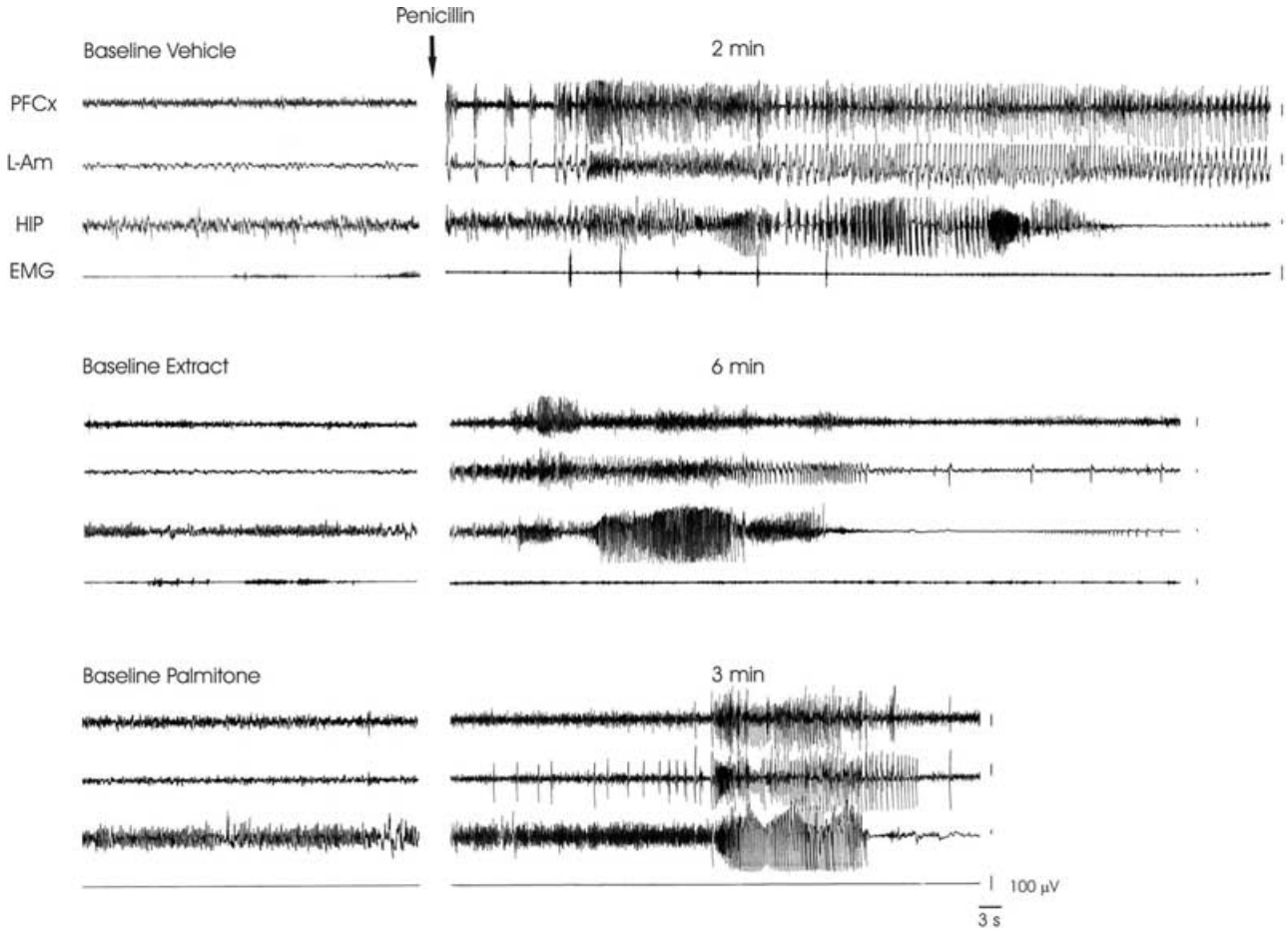

FIG. 3. Sampling traces showing electrographic activity in prefrontal cortex (PFCx), left amygdala (L-Am); hippocampus (HIP) and electromyogram (EMG). The arrow on the trace indicates the time of penicilllin (100 UI) injection into the central amygdala nucleus after 1 h of baseline recording in rats treated with extract (30 mg/kg) and palmitone (10 mg/kg) in comparison with a rat receiving vehicle (0.5% tween 80 in s.s.). Subsequent to penicillin injection representative traces of the duration of convulsive activity for each treatment are shown.

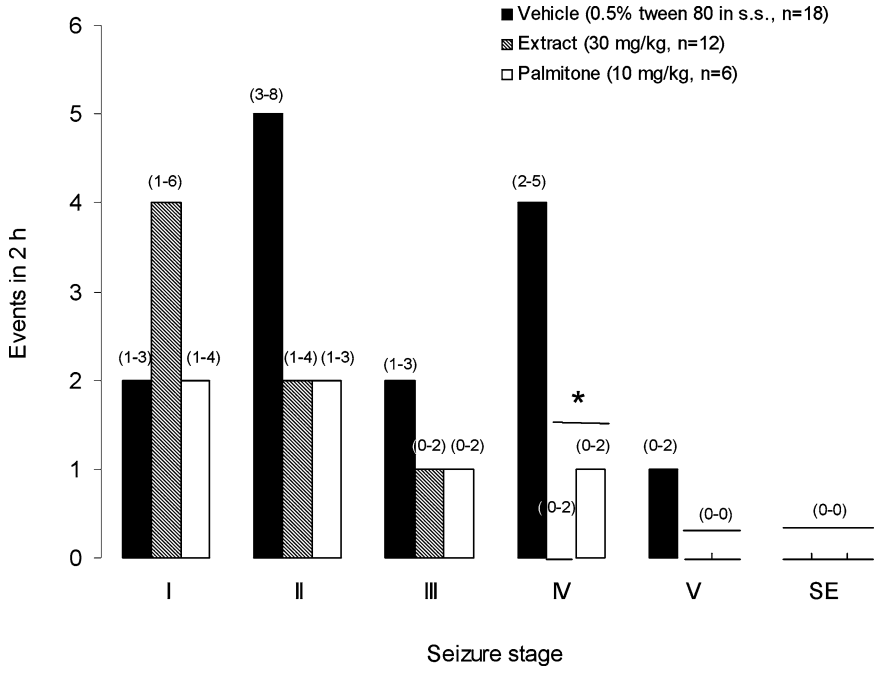

FIG. 4. Convulsive behavior represented as an enhancement in the frequency and severity of seizures induced by penicillin (100 UI) injection into the central amygdala nucleus in rats receiving vehicle (0.5% tween 80 in s.s., n = 18), extract (30 mg/kg, n = 12), and palmitone (10 mg/kg, n = 6). The severity score was determined by using Racine's scale considering stages I, II, and III as partial seizures, and stages IV and V as generalized seizures. Each bar represents the median with 25 and 75 percentiles per group. *Kruskall-Wallis (ANOVA on ranks) followed by Dunn's test, $p < 0.001$.

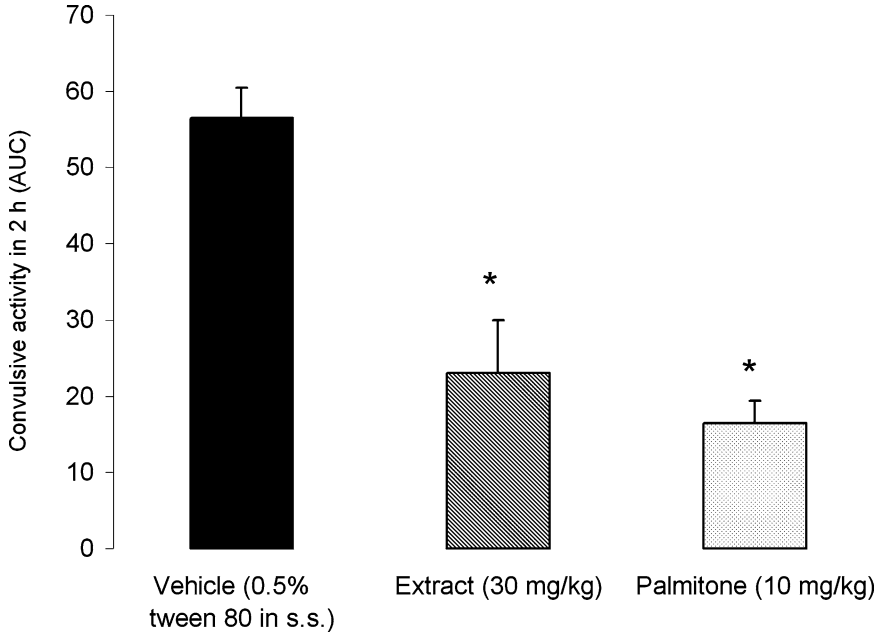

FIG. 5. Effect of intraperitoneal administration of vehicle (0.5% tween 80 in s.s., n = 18), extract (30 mg/kg, n = 12) and palmitone (10 mg/kg, n = 6) on the convulsive behavior induced by injection of penicillin (100 UI) into the central amygdala nucleus in rats. Data represent the mean of the area under curve (AUC) ± S.E.M. obtained from the number of seizures in 2 h recording period for each behavioral stage. *ANOVA followed by Dunnett's test, $p < 0.001$.

et al., 1991). In contrast, epileptiform discharges occurring during the REM phase are less frequent or they are severely decreased (Malow and Aldrich, 2000; Nobili et al., 2001), thus suggesting a relative protection of the REM phase against the occurrence of seizures (Bazil and Walczak, 1997). Besides, it has been described that increased inhibition of the paroxystic activity is also associated with facilitation of cortical desynchronization mechanisms by interaction of GABAergic and cholinergic processes (Nitz and Siegel, 1997; Martínez et al., 2004; Xi et al., 2004). Since we observed that extract and palmitone modify the polysomnographic analysis previous to the penicillin-induced seizures, it may be possible that the extract and palmitone promote a desynchronization effect, with consequent reduction in the convulsant activity produced by GABAergic antagonists like penicillin. However, in the future, it may be interesting to investigate the specific effect of *A. diversifolia* and palmitone on the mechanisms involved in the sleep–epilepsy relationship.

In conclusion, *A. diversifolia* and palmitone did not produce a sedative–hypnotic effect although both of them were effective in reducing the severity of behavioral and EEG seizures induced by penicillin. This reinforces the idea that the anticonvulsant effect of *A. diversifolia* extract is, at least in part, through the action of palmitone, which may also be mediated by GABAergic neurotransmission. However, further studies are necessary to elucidate the specific mechanisms of action in the anticonvulsant activity of *A. diversifolia* and palmitone. Since participation of other constituents in anticonvulsant activity of the extract cannot be discarded, further phytochemical and pharmacological studies need to be undertaken. Our experiments in the present study support the traditional use of *A. diversifolia* in folk Mexican medicine to control epilepsy.

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