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Huperzia saururus, activity on synaptic transmission in the hippocampus

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Abstract

Huperzia saururus (Lam.) Trevis. (Lycopodiaceae) known as cola de quirquincho is used in folk medicine to improve memory. The cholinergic neurons of the basal forebrain, including those in the medial septum, and in the vertical limbs of the diagonal band of Broca and the nucleus basalis of Meynert, provide a major source of cholinergic enervation of the cortex and hippocampus. These neurons have also been shown to play an important role in learning and memory processes. Thus, the effects of this traditional Argentinean species were studied in relation to its activity on synaptic transmission in the hippocampus. The alkaloid extract obtained first by decoction of the aerial parts and by subsequent alkaline extraction, was purified by using a Sephadex LH 20 packed column.

Electrophysiological experiments were developed with the purified extract (E_2) on rat hippocampus slices, thus eliciting long-term potentiation (LTP). Results show a marked increase in the hippocampal synaptic plasticity. The threshold value for generation of LTP was 22 ± 1.01 Hz on average for E_2, while for controls it was 86 ± 0.92 Hz. All of these factors could explain the use of *Huperzia saururus* as a memory improver as is reported in the ethnomedicine.

Keywords: *Huperzia saururus*; *Lycopodium* alkaloids; Memory and learning; Electrophysiological assays

1. Introduction

Aerial parts of *Huperzia saururus* (Lam.) Trevis. (syn. *Lycopodium saururus* Lam., syn. *Urostachys saururus* (Lam.) Herter) (Lycopodiaceae) have long been used in folk medicine mainly as an aphrodisiac (Amorin, 1974; de la Sota, 1977; Ratera and Ratera, 1980) and moreover it is believed to improve memory (Martinez Crovetto, 1981). It is consumed as an infusion and also as a decoction. Depending on the concentration, the latter can produce severe adverse effects such as vomiting, diarrhea, convulsions and even death (Amorin, 1974; Ratera and Ratera, 1980; Toursarkissian, 1980).

Results of previous studies on *Huperzia saururus* showed that its main constituents are alkaloids; sauroine, a novel alkaloid (Ortega et al., 2004a), sauroxine, 6-hydroxylycopodine, *N*-acetyllycodine, lycopodine, lycodine, *N*-methyllycodine, and clavolonine were isolated and identified (Ortega et al., 2004b) (Fig. 1). It has also been demonstrated that the alkaloid extract has a marked anticholinesterase activity with a selectivity on true acetylcholinesterase (Ortega et al., 2004b). All the alkaloids that occur in *Huperzia saururus* belong to Lycopodium alkaloids. This group forms a special kind of alkaloid found only in some restricted genera. For all the Lycopodium alkaloids, only a few of them have been demonstrated to be acetylcholinesterase inhibitors. Among them, Huperzine A, first isolated from *Huperzia serrata* has this effect (Liu et al., 1986) and in addition improves learning and memory (Vincent et al., 1987).

Abbreviations: E_2, purified alkaloid extract; LTP, Long-Term Potentiation; AD, Alzheimer's disease; NaOH, sodium hydroxide; $CHCl_3$, chloroform; MeOH, methanol; H_2O, water; E_1, crude total alkaloid extract; GLC-MS, gas liquid chromatography-mass spectrum; NaCl, sodium chloride; KCl, potassium chloride; $MgSO_4$, magnesium sulphate; $HKPO_4$, potassium phosphate monobasic; $HNaCO_3H$, sodium bicarbonate; $CaCl_2$, calcium chloride; Ctrol, control; fEPSP, field excitatory postsynaptical potential; GDS, global deterioration scale

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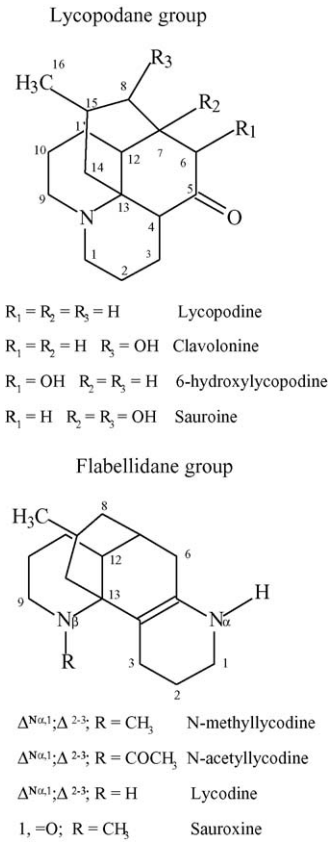

Fig. 1. *Lycopodium* alkaloids present in the *Huperzia saururus* purified extract (E_2).

Alzheimer's disease (AD) is characterized by loss of memory, functional decline and behavioral disturbance (American Psychiatry Association, 1987). AD is characterized by early cholinergic neuronal loss in a more consistent way than for other systems (Katzman, 1986). However, many neurotransmitter systems also being affected (Perry and Perry, 1985). As the cholinergic function is required for short-term memory processes, it is believed that the cholinergic deficit in AD is also responsible for much of the short-term memory deficit (Galizia, 1984; Jarvik et al., 1972).

At the present time, it is known that acetylcholinesterase inhibitors give temporary cognitive benefit to a percentage of AD patients (McGeer and McGeer, 2003). The fore brain cholinergic system is very important in memory functions. Furthermore, there is evidence that the septo-hippocampal pathway is involved in short-term learning and memory processes. The hippocampal long-term potentiation (LTP) has therefore been proposed as a potential neuronal mechanism for memory storage (Bliss and Lomo, 1973; Bliss and Colingridge, 1993). Considering all these antecedents, the aim of the present study was to determine if the purified alkaloid extract of *Huperzia saururus* elicits and maintains LTP in hippocampus rat slices measured by electrophysiological assays, and to attempt to correlate the results obtained to the supposed effects on memory claimed by ethnomedicine.

2. Materials and methods

2.1. Plant material

Plant material was collected in Pampa de Achala, Departamento San Alberto, province of Córdoba, in November 2001, and identified by Dr. Gloria Barboza, Instituto Multidisciplinario de Biología Vegetal, Universidad Nacional de Córdoba. A voucher specimen is deposited at CORD as Altamirano No. 684.

2.2. Extraction and purification

In order to obtain a similar extract to that which people consume, the following method was used to obtain the extract. The aerial parts of *Huperzia saururus* (500 g) were dried, ground and then decocted with boiling water (21) for 1 h. This process was repeated twice. Filtered and combined decoctions were concentrated under reduced pressure to reduce the volume, and then alkalinized to pH 12 by the addition of 0.1N NaOH. The alkaline aqueous extract was partitioned with $CHCl_3$ in a liquid-liquid extractor. The chloroformic extract was evaporated until dry in order to obtain 1.56 g of the crude total alkaloid extract E_1 (% w/w, 0.31; % w/w, calculated relative to dry starting material).

E_1 was chromatographed over Sephadex LH-20 in a glass column using $MeOH/H_2O$ (1:1) as the mobile phase. All the fractions resulting positive to Dragendorff's reagent were combined and evaporated under reduced pressure to yield the alkaloid purified extract E_2 (0.75 g; 0.15 % w/w).

2.3. Chemical composition

In order to separate and identify the components present in E_2 this extract was submitted to GLC-MS (Ortega et al., 2004a). By using a Perkin-Elmer Qmass-910 apparatus, and a capillary column SE 30, 30 m in length, E_2 analysis was performed. The injection volume was 0.5 μl with He as carrier, with the flow rate being 1 ml/min. Temperature program: 140 °C (3 min), 140–250 °C at 5 °C/min, 250 °C (5 min), 250–280 °C at 5 °C/min, 280 °C (5 min). The temperatures of injector, interface and ion source were 300, 280, and 280 °C respectively. The ionization energy was 70 eV and individual alkaloid identifications were made by comparing breakdown patterns with those found in the literature (MacLean, 1963; Alam et al., 1964; Ayer et al., 1965; Loyola et al., 1979; Sun et al., 1993; Ortega et al., 2004b).

Thus, the presence of sauroine, 6-hydroxylycopodine, lycopodine, and clavolonine (Lycopodane group) and sauroxine, lycodine, *N*-methyllycodine, and *N*-acetyllycodine (Flabellidane group), was confirmed, along with other alkaloid compounds whose structures have not yet been determined.

2.4. Animals

Male Wistar rats 65–70 days old and weighing 190–260 g were used. Animals were housed in groups of five in their home boxes and kept under a 12 h-light/12 h-dark cycle (lights on at 7 a.m.) and regular temperature conditions (22 ± 1 °C). Food and water were available ad libitum.

2.5. Krebs solution

NaCl 124.3 mM, KCl 4.9 mM, $MgSO_4 \cdot 7H_2O$ 1.3 mM, H_2KPO_4 1.25 mM, $HNaCO_3$ 25.6 mM, glucose 10.4 mM, and $CaCl_2 \cdot 2H_2O$ 2.3 mM.

2.6. Electrophysiological procedures

Electrophysiological experiments were carried out using the in vitro hippocampal slice preparation described elsewhere by Pérez et al., 2002. Briefly, rats were sacrificed by cervical dislocation between 11:00 and 12:00 a.m. to prevent variations caused by circadian rhythms or nonspecific stressors (Tayler and Discena, 1984, 1987). The brain was then removed for electrophysiological assays. The hippocampal formation was dissected and transverse slices, approximately 400 μm thick, were placed in a (BSC-BU Harvard Apparatus) recording chamber and perfused with standard, saturated Krebs solution with 95% O_2 and 5% CO_2. The rate of perfusion was 1.6 ml/min, while the bathing solution temperature was kept at 28 °C by the use of a Temperature Controller (TC-202A Harvard Apparatus). A stimulating electrode made of two 50 μm diameter insolated twisted wires (except for the cut ends) was placed in the perforant path, and then a recording microelectrode made with a micropipette (tip 10–20 μm) was inserted in the dentate granule cell body layer. Only slices showing a stable response were included in this electrophysiological study. Ten field potentials that responded to the stimuli were sampled at 0.2 Hz, averaged on line using a PC computer, and the data thus obtained were stored on diskettes for further analysis. Once no further changes were observed in the amplitude of the response, for 20 min, the intensity of the electrical stimulus to the perforant path was set at a value that would elicit spikes at approximately 30% of the maximum response.

Hippocampal slices were perfused with the E_2 extract (0.1 μg/ml E_2 group) during 20 min, or with Krebs solution (Control group). After this time, long-term potentiation eliciting frequency threshold was determined. Tetanus, consisting of a train of pulses (0.5 ms, each pulse) of 2 s duration and with increasing frequency, was delivered to the slice by a A310 Accupulser Pulse Generator (World Precision Instruments Inc.), at intervals that ranged from 20 min up to 45 min (starting with a 5 Hz tetanus). Frequency intensity was increased with each train to 10, 25, 50, 75, 100, 150 up to 400 Hz. Fifteen to twenty minutes after a tetanus, a new averaged response was recorded, and if long-term potentiation was not observed, another tetanus at the next higher frequency was applied.

Long-term potentiation was considered to have occurred when the amplitude of the evoked field potential recorded after the tetanus had risen by at least 30% and had persisted from 20 to 60 min. Once long-term potentiation was achieved no further tetanus were given.

2.7. Statistics

The experimental data were analyzed by one-way ANOVA, followed by Newman–Keuls pairwise comparisons of means. $P \leq 0.05$ represents a significant difference between groups.

3. Results and discussion

In Argentina *Huperzia saururus* is one of the most frequently used plants to obtain aphrodisiac effects but its use is also reported in folk medicine for memory improvement. In order to explain this latter use of the species, we selected for our research the LTP phenomena in hippocampus, because it is the neurological process that underlies to the learning and memory processes.

A purified alkaloid extract was used to perform the assays on hippocampus rat slices. After a single-pulse stimulation in the perforant path of the hippocampus, consisting of a gradual

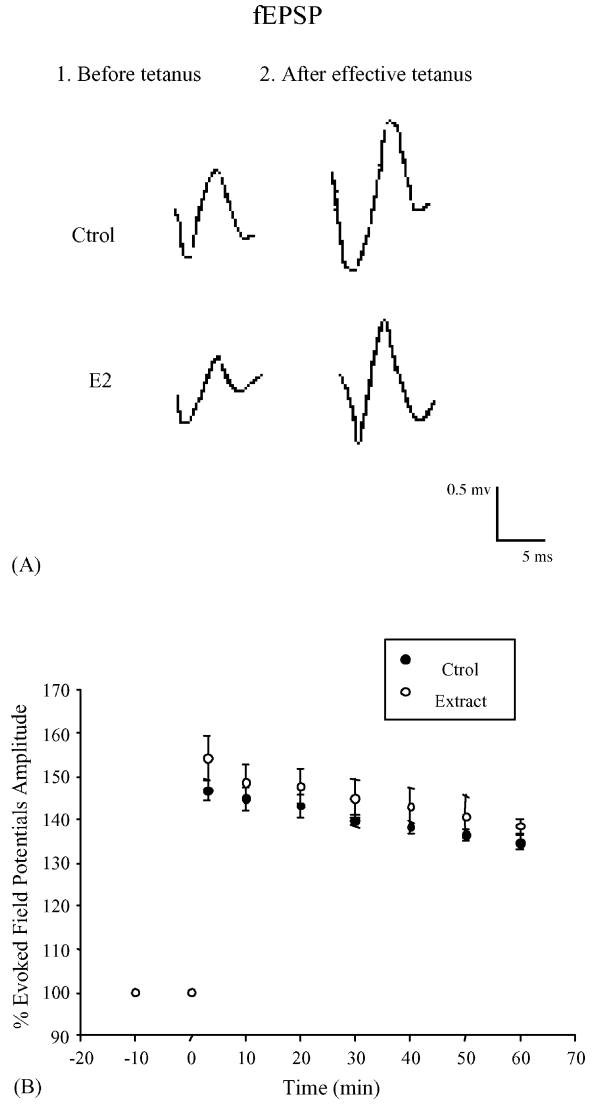

Fig. 2. (A) Oscilloscope modified photographs corresponding to typical averaged field potentials, recorded from the granule cell layer of the *dentate gyrus* following stimulation of the PP for each group: (1) before tetanus; and (2) after an effective tetanus. Calibration bars represent: 5 ms and 0.5 mV. (B) Plots of evoked amplitude of potentials recorded over time in granule cell layer of the dentate gyrus by stimulation of the PP in hippocampal slices in Control and E_2 groups. Values before time point zero show the baseline levels of each group. Evoked field potentials are expressed as percentage of baseline.

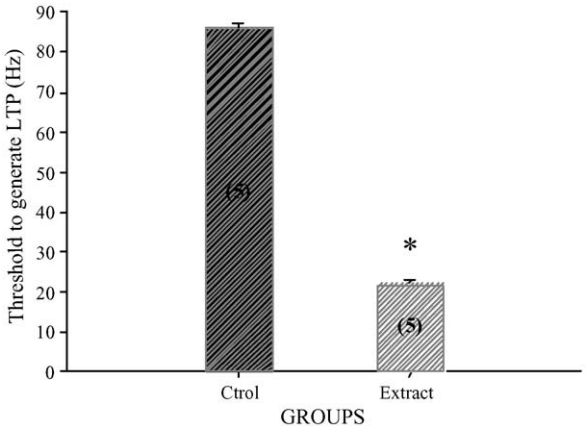

Fig. 3. Threshold to induce LTP in hippocampal *dentate gyrus* in Control and E_2 groups. Each bar represents the mean and vertical bars ± SME. The number of animals used in each group is indicated between parentheses. (*) $P < 0.0004$ compared to Control group.

positive-going field excitatory postsynaptic potential (fEPSP), a characteristic evoked field response is produced in the granule cell layer of the *dentate gyrus*. This can be seen as an increase in the amplitude after an effective tetanus for the control (Ctrol) as well as for E_2 groups, in Fig. 2A. The fEPSP reflects synaptic currents at perforant path-dentate granule cell synapses in *stratum moleculare*. In Fig. 2B the increased amplitude of fEPSP over time can be seen, after an effective tetanus for Ctrol and E_2 groups expressed as a percentage of basal fEPSP. The potentiation levels are comparable and there were no statistical differences between groups.

A one-way ANOVA was used to evaluate the differences in threshold in order to induce LTP for Ctrol and E_2 groups. A significant interaction was revealed ($F(1,225) = 45.5.$; $P < 0.0002$) among the effects in E_2 perfusion and Ctrol. Newman–Keuls pairwise comparisons of means test showed that when slices were perfused with E_2 an increment in the hippocampal synaptic plasticity was observed, measured as a diminution in the threshold necessary to generate LTP, when taking into consideration that $P < 0.5$ represented a significant difference between groups. Thus, the E_2 perfused slices ($n = 5$) showed an average threshold of 22 ± 1.01 Hz, while for Ctrol ($n = 5$) the average was 86 ± 0.92 Hz (Fig. 3).

It is important to point out that nowadays the treatment of AD is based on cholinesterase inhibitors, especially for the light to moderately-serious stage (4–6 of Global Deterioration Scale, GDS) of Reisberg et al. (1982). There are few inhibitors authorized to be commercialized for this purpose, so the discovery of a new source such as *Huperzia saururus* that besides being an acetilcholinesterase inhibitor (Ortega et al., 2004a) also shows a modification in the threshold for LTP generation, is important. All of this indicates that a new drug of natural origin could be a potential aid in AD treatment as well as in physiological ageing.

4. Conclusions

Long-term potentiation (LTP) of synaptic transmission is a relevant phenomenon and is seemingly linked to neural information storage (Douglas and Goddard, 1975). In the hippocampal formation, LTP can be produced by repetitive activation of afferent pathways (Harris et al., 1984; Lomo, 1971).

The results obtained so far show that the perfusion of hippocampal rat slices with the purified alkaloid extract of *Huperzia saururus* facilitates the LTP production. Thus, we have demonstrated for the first time that *Huperzia saururus* has a marked effect on the hippocampal synaptic plasticity. This effect, shown by an increase in plasticity that it is manifested as a diminution in the threshold, could explain the memory improvement effect claimed for *Huperzia saururus* by folk medicine. In view of the fact that the assayed extract contains Lycopodium alkaloids, and that there exists the antecedent of alkaloids such as Huperzine A improving memory as, it can be said that one or more of the alkaloids present is/are responsible for the action here demonstrated. More behavioral and biochemical studies will be necessary to clarify, which the active compounds are, and to identify the action mechanism through which they perform their action.

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