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Isolation of anxiolytic principle from ethanolic root extract of *Cardiospermum halicacabum*

Rajesh Kumar[a], G. Murugananthan[a], K. Nandakumar[b,*], Sahil Talwar[b]

[a] *Department of Pharmacognosy, PES College of Pharmacy, 50 Feet Road, Hanumanthnagar, Bangalore, Karnataka, India*
[b] *Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal University, Manipal, Karnataka 576104, India*

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ABSTRACT

Cardiospermum halicacabum roots have been used traditionally for the treatment of epilepsy and anxiety disorders. The purpose of this study was to characterize the putative phytoconstituents present in the ethanolic root extract having anxiolytic activity using an elevated plus-maze (EPM) and light dark transition model. Control mice were orally treated with an equal volume of vehicle (4% gum acacia), and positive control mice were treated with diazepam (1 mg/kg). In the EPM test, out of pool of 19 master fractions (MF) only MF-14, 16 and 17 significantly (30 mg/kg, $p < 0.05$, $p < 0.01$ and $p < 0.001$) increased the number of entries in the open arm. MF-14, 16 and 17 (10, 20 and 30 mg/kg) had also increased the time spent by mice in illuminated part of the box significantly ($p < 0.05$, $p < 0.01$ and $p < 0.001$), as compared to control. However, significant changes ($p < 0.05$, $p < 0.01$ and $p < 0.001$) were recorded in other parameters, e.g., rearing, time spent in the closed arm and dark zone in both the models. These results suggested that *C. halicacabum* root is an effective anxiolytic agent. The phytoconstituent responsible for the observed central effects was isolated from MF-14 and identified as well-known compound, Cardiospermin, a cyanogenic glucoside.

Introduction

Anxiety disorders in a modern society have relatively high prevalence affecting between 10 and 30% of the general population with considerable financial resources (Rice and Miller 1998; Greenberg et al. 1999; Wittchen and Hoyer 2001). Excessive anxiety can debilitate and damage the quality of life (Seo et al. 2007). In the clinical treatment of anxiety benzodiazepines, $GABA_A$ receptor agonist and buspirone, $5\text{-}HT_{1A}$ receptor agonist, are mainly prescribed as first choice treatment. Chronic administration of benzodiazepines, however result in physical dependence such as sedation, myelorelaxation, ataxia, amnesia and pharmacological dependence (Lader and Morton 1991). Moreover buspirone also results in dizziness, headache, nervousness, paresthesia, diarrhea, excitation and sweating as adverse effects (Jordan et al. 1996; Rickels and Schweizer 1997). Therefore, research has been conducted to identify safer, more specific medications possessing anxiolytic effect without the complications. In past few years, several herbal medicines have been used for the management of anxiety in the world (Rex et al. 2002).

In India, *Cardiospermum halicacabum* (CH) has been used for several centuries in the treatment of rheumatism, stiffness of limbs and snake bite whereas the roots alone have been used for curing diseases related to the nervous system (Muthu et al. 2006). Similar uses of CH roots have been indicated in the folklore system for anxiety and epilepsy as well (Venkteshbabu and Krishnakumari 2006). Initially study was conducted with ethanolic root extract of CH and found to possess anxiolytic activity. The present study was designed to fractionate the ethanolic extract and to isolate the active constituent responsible for anxiolytic activity.

Materials and methods

Animals

Adult male Swiss albino mice (18–24 g) were procured from Bioneeds, Tumkur, India. They were housed in groups ($n = 6$) in polypropylene cages (11 cm × 17 cm × 28 cm) with wood shavings as bedding, under controlled conditions of light (12 h light dark cycle, light on at 7 a.m.) and temperature (25 ± 2 °C). The animals had free access to water and food except 1 h before and during the experiments. All protocols and experiments were conducted in strict compliance according to the ethical principles and guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

* Corresponding author. Tel.: +91 820 2922482x189; fax: +91 820 2571998.
E-mail address: nandakumar77@rediffmail.com (K. Nandakumar).

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Drugs

Diazepam was obtained from Ranbaxy Laboratories Limited, Thane, India. Other chemicals petroleum ether, methanol and ethyl acetate used for extraction, fractionation and phytochemical investigation were of analytical grade from SD Fine Chemicals, Mumbai, India.

Plant material

The roots used for this study were collected from wild area of Tiruchengodu, Tamil Nadu and was authenticated by Dr. K. Lakshman, Head of Department (Botany), Bangalore University, India. Roots collected were sun dried and powdered coarsely.

Isolation and identification of the compound

Dried powdered roots (sieved # 40–60) were extracted with absolute alcohol by soxhlation for 48 h. The extract was concentrated under vacuum. The major phytochemical constituents were identified by optimization of thin layer chromatography (TLC). The plates developed and scanned at 264 and 366 nm showed prominent band separation in TEF (toluene:ethylactate:formic acid 4.5:4.5:1). Based on the results of preliminary screening, 30 g of active crude ethanolic extract was chromatographed on silica gel column (Merk 60–120 mesh, 600 g) and successively eluted with stepwise gradient of petroleum, then ethyl acetate and finally methanol. A total of 384 master fractions were collected and each fraction was spotted on pre-coated silica gel (Merk-60 F254, 0.25 mm thick) plate and eluted in petroleum ether, ethyl acetate and methanol in varying proportions. Fractions with similar R_f values in TLC were pooled together to get 19 similar fractions. Fractions with sufficient yield were selected for further studies. Fraction 14 showed a single spot on the TLC. The structure of the isolated compound was elucidated on the basis of ^{1}H NMR, MS and FT-IR at the Indian Institute of Sciences, Bangalore, India (Fig. 1).

Pharmacological evaluation

Elevated plus-maze test (EPM)

According to Pellow and File (1986) elevated plus-maze apparatus comprised of two perpendicular open (16 cm × 5 cm) and two closed arms (16 cm × 5 cm × 12 cm) having an open roof, was elevated (25 cm) from the floor to observe anxiolytic behaviour in mice from the central platform (5 cm × 5 cm). The room was illuminated with four 25 W red bulbs giving a light intensity 12 lx on the arms. Mice (n = 6 per group) were randomly assigned to 13 experimental groups (vehicle control 4% gum acacia p.o., 1 mg/kg diazepam p.o. and 30 mg/kg of MF-2, 7, 10, 11, 13, 14, 15, 16, 17, 19). Drug administration was oral and 60 min prior to the test. The number of entries into and the time spent on each of the two types of arms and the latency to enter open arms were recorded during the 5 min trial. During the 5 min session the following parameters were noted.

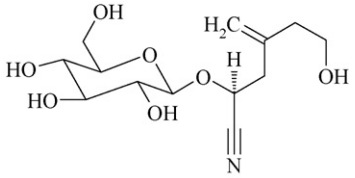

Fig. 1. Molecular formula of Cardiospermin with structure.

- Number of entries into open arm.
- Number of entries into closed arm.
- Time spent in the open arm.
- Time spent in the closed arm.

Every time before placing the animal, the arena was cleaned with 5% alcohol to eliminate the possible bias due to the odour left by the previous animal.

Light dark transition model

The method of Costall et al. (1989) was adopted and slightly modified. The apparatus used was a box made of wood with overall dimensions of 40 cm × 60 cm × 20 cm (length, width, height) and a grid floor composed by bars spaced 5 cm apart. The box was further divided by a barrier possessing a doorway (7 cm round hole), which mice could cross in two chambers of measures (40 cm × 20 cm): painted black, not illuminated, and (40 cm × 40 cm) painted white and illuminated with a 60 lx light source. On the test day, mice were administered orally diazepam (1 mg/kg, n = 6 for standard treated group) and three doses (10, 20 and 30 mg/kg) active fractions of extract (MF-14, 16 and 17) to other groups. MF-14, 16 and 17 were selected due their significant result in EPM as comparing to other MFs. Control group was administered vehicle (4% gum acacia, p.o.). One hour later, each animal was placed in the middle of the light compartment, facing the doorway separating the two compartments. The behaviour of animal was noted for 5 min and following parameters were recorded.

- Latency to the first crossing of the dark compartment.
- Number of crossings between light and dark area.
- Total time spent in the illuminated part of the cage.
- Total locomotion.
- Rearing.

Statistical analysis

Results are expressed as mean ± S.E.M. from (n = 6) animals per group. Statistical differences in the mean were collected were calculated/performed using One-way ANOVA followed by Dunnett's post hoc test for a significance level of $p < 0.05$.

Results

Identification of active compound

Fraction 14 of ethanolic extract of *Cardiospermum halicacabum* yielded a dark brown coloured active compound which was confirmed based on its FT-IR, MS and ^{1}H NMR values. These data led to the identification of a well-known compound, Cardiospermin, i.e. (2S)-6-hydroxy-4-methylene-2-[{3,4,5-trihydroxy-6-(hydroxymethyl)-tetrahydro-2*H*-pyran2yl}oxy] hexanenitrile (Fig. 1), with a molecular formula $C_{13}H_{21}NO_7$ and a molecular weight of 303. Molecular mass peak at 303 m/z and base peak at 141 m/z justifies that the base peak was formed by the aglycone part fragmented from the parent compound.

Assessment of anxiolytic activity

Elevated plus-maze test

Diazepam has long been reported for its anxiolytic activity in mice with EPM. In present study also, a pronounced anxiolytic affect was observed on mice after administration of diazepam (1 mg/kg) with significant increase in number of entries in the open arm ($p < 0.01$). The results are shown in Table 1. 3 out of 10 MFs (master fractions selected for activity), i.e. MF-14, 16 and 17 (30 mg/kg)