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First Evidence for an Anxiolytic Effect of a Diterpenoid from *Salvia cinnabarina*

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The potential anxiolytic and anti-depressive activity of CMP1 was studied in the elevated plus-maze test and in the forced swimming test. Furthermore, CMP1 sedative activity was evaluated in pentobarbital treated animals; the effect of CMP1 on spontaneous motor activity (total locomotion) was also evaluated. Our data show that CMP1, at doses that did not affect locomotion, was able to induce anxiolytic and sedative, but not anti-depressive effects. In conclusion, our results represent first evidence for an anxiolytic activity of this diterpenoid from *Salvia cinnabarina.*

Keywords: *Salvia cinnabarina*, diterpenoids, anxiolytic activity.

In a screening programme on *Salvia* medicinal plants (Fam. Lamiacae), a crude ethanolic extract from the aerial part of the plant *Salvia cinnabarina* demonstrated *in vitro* antispasmodic activity. This led to the isolation of a new diterpenoid of the pimarane skeleton, the compound 3,4-secoisopimar-4(18),7,15-triene-3-oic acid (CMP1) [1], the complete relative stereochemistry of which was determined by an X-ray diffraction analysis of a derivative [2] (Figure 1). Further studies have shown that CMP1 reduces intestinal motility *in vivo* [3], the urinary bladder contractility *in vitro* both with a mechanism involving calcium channel [4] and possess a weak hypotensive activity [5]. Diterpenoids are widely distributed throughout plant species, including some of the genus *Salvia* [6] displaying a spectrum of biological activities including those on the CNS [7]. Salvinorin A (*Salvia divinorum)*, miltirone, tanshinones II A and II B, carnosol and carnosic acid (*Salvia officinalis*) are examples of isolated diterpenes with hallucinogenic, neuroprotective, sedative and hypnotic properties (for review see [7]). This study investigates CMP1, for its CNS activity in the hope that, as with other *Salvia* species, it may generate potential leads for novel CNS drugs.

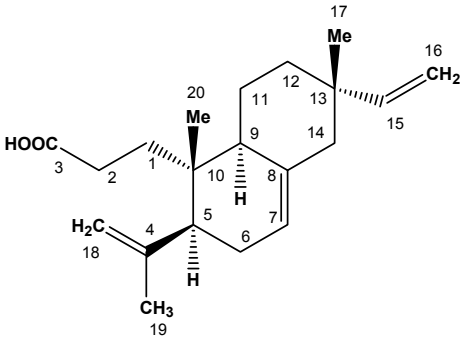

Figure 1: CMP1, chemical structure.

Our study shows that CMP1 (10 mg/kg ip.) has a potential anxiolytic effect evaluated either as the time spent in, or as the number of entries to, the open arms of the plus maze. Both CMP1 and diazepam (2 mg/kg) were able to increase both the time spent and the number of entries into the open arms.

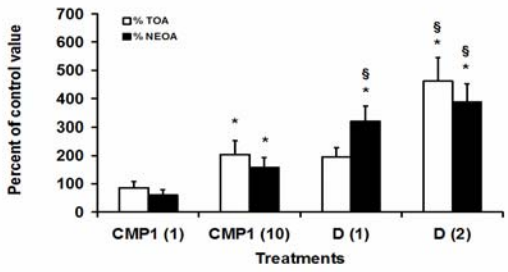

Figure 2: CMP1 effects in the plus maze test TOA, time spent in the open arms; NEOA, numbers of entry in the open arms. Animals were treated ip. 10 min before the test with CMP1 (1 and 10 mg/ kg) or 30 min before the test with diazepam (D, 1 and 2 mg/kg). Data are shown as percentage of the vehicle-induced effects (CMP1 vehicle: TOA, 22.4 ± 8.5 sec; NEOA, 3.1 ± 1.1; D vehicle: TOA, 21.1 ± 6.9 sec; NEOA, 2.9 ± 0.6).
* $p<0.05$ *vs* vehicle-treated animals; § $p<0.05$ *vs* CMP1 (10). N = 12-15.

Diazepam administered at the dose of 1 mg/kg was able to increase the number of entries into the open arms only, while CMP1 at the lowest dose (1 mg/kg) had no effect (Figure 2). Conversely, CMP1 does not display depressant activity, as it was ineffective in the swimming test (data not shown).

Furthermore, our findings demonstrate sedative properties for CMP1 as its ability to potentiate sleeping time of a pentobarbital injection, as diazepam. Indeed, CMP1 (10 mg/kg) significantly increased the sleeping time induced by pentobarbital (218.8 ± 25.5%, t-test: $t_{18} = 3.968$, $p < 0.001$, N = 10). Similarly, treatment with diazepam (2 mg/kg) significantly increased the sleeping time induced by pentobarbital (164.4 ± 20.9%, t-test: $t_{26} = 3.968$, $p < 0.01$, N = 15). The increase in sleeping time observed after CMP1 or diazepam administration were in the same order of magnitude and were independent of the dosage; for simplicity we report only the results obtained after highest dose of administration. Whether this effect of CMP1 on pentobarbital induced sleeping time is due to a pharmacodynamic or a pharmacokinetic interaction needs further investigation.

It is well known that a diazepam-induced sedative effect is paralleled by reduction in the spontaneous motor activity representing a possible limitation to its use [8]. This work showed CMP1 to have an anxiolytic effect without modifying total spontaneous motor activity and this may represent a therapeutically useful feature. Indeed, CMP1 (10 mg/kg ip.) did not change the spontaneous motor

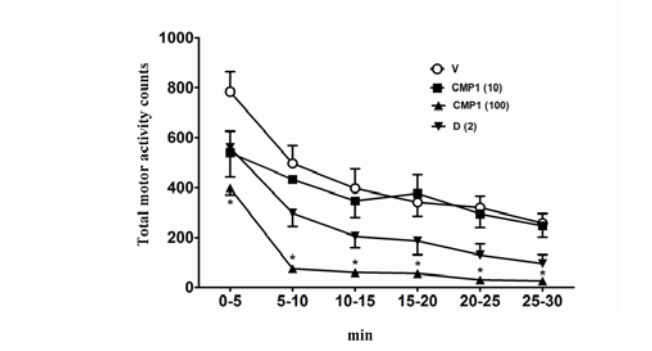

Figure 3: CMP1 effects on total locomotor activity.Animals were treated with CMP1 (10 and 100 mg/kg), diazepam (D, 2 mg/kg) i.p or vehicle (V) immediately before the test. * $p<0.05$ *vs* vehicle-treated animals. N = 6-8.

activity of mice; while a dose of 100 mg/kg significantly reduced spontaneous motor activity, in contrast to Diazepam (2 mg/kg) which reduced spontaneous motor activity (Figure 3).

In conclusion, our main finding is that CMP1, the major diterpenoid constituent of *Salvia cinnabarina*, has anxiolytic activity with fewer side effects in comparison to diazepam. Our findings further help delineate the pharmacological profile of diterpenoids and suggest that CMP1 could represent a lead compound for the development a new class of anxiolytics.

Experimental

Animals: Male CD-1 mice (Charles River, Italy) weighing 25–30 g were used for all experiments on CNS. Mice were housed in colony cages (7 mice in each cage) for at least 1 week before experimental sessions with the following conditions: exposed to normal light (from 7.00 a.m. to 7.00 p.m.); temperature 22 ± 1°C; relative humidity 60 ± 10%; and food and water available ad libitum. All experiments complied with the Italian D.L. N° 116 of 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609/ECC).

Drugs and treatment procedure: Extraction of CMP1 from leaf surface constituents of fresh aerial parts of *Salvia cinnabarina* was performed as previously described [1] to a HPLC purity of 96%. The sodium salt (prepared by reaction with an equivalent quantity of NaOH in MeOH solution and evaporation to dryness) was dissolved (5 mg) in

distilled water. On each test day, an aliquot of drug solution was diluted with phosphate buffered saline (PBS) and then ip. injected in a volume of 35 mL/kg. Diazepam solutions were prepared in distilled water added with Tween 80 (2 drops every 10 mL), dispersed by ultrasound and given ip. in a volume of 10 mL/kg. Drug dosage and time-courses were chosen on the basis of results obtained in preliminary experiments.

Elevated plus-maze test: This test has been widely validated to measure anxiety in rodents [9,10]. The apparatus, constructed from black Plexiglas, consisted of two open arms (50 cm × 10 cm x 40 cm each), and two enclosed arms (50 cm × 10 cm × 40 cm each) and a central platform (10 cm × 10 cm), arranged so that the two arms of a type were just opposite to the two arms of the other type. The maze was elevated 60 cm above the floor. Ten minutes after ip. treatment with CMP1 (1 and 10 mg/kg) or thirty minutes after ip. treatment with diazepam (1 and 2 mg/kg), or with the respective vehicle, each animal was placed at the centre of the maze, facing one of the closed arms. During 10 min period test, the number of open arms entries and the time (sec) spent in there was recorded. Animal behavior was monitored by using a video camera located above the maze.

Pentobarbital-sleeping time: This test was performed according to Mora *et al*. [11]. Ten minutes after ip. treatment with CMP1 (10 mg/kg) or thirty minutes after ip. treatment with diazepam (1 and 2 mg/kg) or vehicles, each animal was ip. injected with pentobarbital (50 mg/kg). The time required to the mouse to lay on its back was recorded as the sleep latency. Sleeping time was taken as the period between the loss of the righting reflex and its return. Experiments were carried out in a quiet room in which temperature was maintained at 22 ± 2°C.

Forced swimming test: The test was performed as described by Lucki [12] and Porsolt *et al*. [13]. Briefly, the apparatus consisted of a transparent plexiglas cylinder (50 cm × 20 cm diameter) filled up to 30 cm with water at room temperature. CMP1 (1 and 10 mg/kg) or vehicle were administered 10 min prior to the test. During 10 min test, floating, i.e. when mice made no further attempts to escape except the movements necessary to keep its head above the water, was recorded by a trained observer. Reduction in floating was considered an antidepressant-like action [14].

Spontaneous motor activity: Locomotor activity was recorded with an infrared photocell activity monitor (Ugo Basile, Italy), provided with one array of 15 infrared photocells spaced 2.5 cm apart. Total spontaneous motor activity was monitored during 30 min by evaluating all interruptions of photo beams, as described in literature [15]. All measurements were performed between 10:00 am and 1:00 pm. Animals were treated with CMP1 (10 and 100 mg/kg ip.), diazepam (1 and 2 mg/kg ip.) or the vehicles immediately before the test starting.

Data analysis and statistics: All data are expressed as mean ± ES and analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons or by t-test when the comparison was between two means. A value of $p<0.05$ was considered statistically significant.

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