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Neuropharmacological Effects of Epinepetalactone from *Nepeta sibthorpii* Behavioral and Anticonvulsant Activity

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Abstract

Several cyclopentanoid monoterpenes related to the iridoid compounds (nepetalactones) have been isolated and characterized in many *Nepeta* species. We studied the neuropharmacological activity of *Nepeta sibthorpii* Bentham essential oil (1.5 mg/kg i.p.), methanol extract (50 mg/kg i.p. and p.o.), and fraction containing epinepetalactone (Fraction I) obtained from methanol extract (dose corresponding to 50 mg/kg of methanol extract i.p. and p.o.) in rodents. *N. sibthorpii* preparations produced definite alterations in general behavior pattern, potentiation of sodium pentobarbital–induced sleeping time, and protection against pentylenetetrazol (PTZ)-induced convulsions. The number of convulsions was significantly decreased after treatment with methanol extract (0.6 ± 0.5), fraction I (0.6 ± 0.9), and essential oil (0.7 ± 0.9) in comparison with the group treated only with PTZ (3.0 ± 0.5). The pretreatment of mice with flumazenil (3 mg/kg, i.p.), a benzodiazepine antagonist, blocked the anticonvulsant effect of all preparations. In our experimental conditions, methanol extract was the most active preparation. The depressant effect of *Nepeta sibthorpii* preparations on the CNS appears to involve $GABA_{ergic}$ mediation. This effect is certainly related to epinepetalactone, but in methanol extract other active principles are present.

Keywords: Anticonvulsant activity, epinepetalactone, flumazenil, GABA-receptor, *Nepeta sibthorpii* Bentham, sedative activity.

Introduction

Some lactones chemically related to a large family of methylcyclopentane monoterpenoids were isolated and characterized in various species belonging to the *Nepeta* genus (McElvain & Eisenbraun, 1955).

Nepetalactone (4aα,7α,7aα-nepetalactone), the major constituent of *Nepeta cataria* L. (Chalchat & Lamy, 1997), was the most studied compound. Other nepetalactones were isolated from *Nepeta* species: epinepetalactone (4aα,7α,7aβ-nepetalactone) (Eisenbraun et al., 1980), 4aβ,7α,7aα-nepetalactone (De Pooter et al., 1987), neonepetalactone (Sakan et al., 1965), dehydronepetalactone, dihydronepetalactone (Bicchi et al., 1984), and (4a*S*,7*S*,7a*R*)-nepetalactame (Eisenbraun et al., 1988). It has been shown that these compounds, classified as iridoids, are active insect repellents (Bicchi et al., 1984) and have behavioral activity in felines (Harney et al., 1978).

In rodents, the nepetalactone significantly increases hexobarbital sleeping time and alters Sidman avoidance behavior (Harney et al., 1978; Sherry et al., 1981); therefore, besides marked sedation, it shows significant analgesic activity (Aydin et al., 1998).

The epinepetalactone is a component of *Nepeta sibthorpii* Bentham (syn. *Nepeta argolica* Bory et Chaub.) Lamiaceae. In previous work, epinepetalactone was found to be the major component of the essential oil (82.5%) (Tzakou et al., 2000); we demonstrated that this compound is present also in the methanol extract of the aerial parts (Miceli, 2000).

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Therefore, it seemed interesting to study in rodents the neuropharmacological activity of *N. sibthorpii* essential oil, methanol extract, and fraction containing epinepetalactone obtained from methanol extract.

Materials and Methods

Plant material

Aerial parts of *N. sibthorpii* were collected in Greece (Mt. Parnes), at an altitude of 1200 m, from wild-growing plants during the flowering stage. Voucher specimens (OT-9E) were deposited in the herbarium of the Department of Pharmacognosy, University of Athens.

Preparation of extracts

Fifty grams of crushed aerial parts of *N. sibthorpii* were macerated with 500 ml of 95% methanol for 4 days. The extract obtained was filtrated, and the solvent was removed *in vacuo*. The yield was 11.41%.

The extract was cromatographed on a silica gel (230–240 mesh, 120 g) column (40-cm long, 2.5 i.d.) using chloroform as eluent. Fractions of 10 ml were collected and subjected to IR analysis in order to identify the fraction containing epinepetalactone. This fraction (Fraction I) was purified and dried; the yield was 5.12%.

Air-dried aerial parts of the plant were subjected to separate hydrodistillation for 3 h using a Clevenger-type apparatus. The distillate was saturated with NaCl, and the oil was extracted successively with hexane. The yield was 0.61%.

All the extractive preparations were suspended in propylene glycol for administration to animals.

Animals

Adult male Wistar rats (180–200 g) and Swiss mice (20–25 g) of both sexes were used in the experiments. They were kept in standardized conditions (temperature 22 °C ± 2 °C; humidity 60% ± 4%; natural lighting), fed with a standard diet, and water was provided *ad libitum*. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M. 116192), as well as with the European Economic Community (EEC) regulations (O.J. of E.C. L 358/1, 12/18/1986).

In all experiments, rats and mice were divided in groups of 10 animals each.

Data were expressed as mean ± SE of 10 determinations. The results were statistically analyzed by Student's *t*-test; $p < 0.01$ versus control was taken as significant.

LC_{50} and LD_{50} determinations

LC_{50} determination was carried out using the method of Meyer et al. (1982). This method, using brine shrimp (*Artemia salina* Leach), is a bioassay system largely employed in LC_{50} detection of active principles of plants (Meyer et al., 1982). Appropriate amounts of methanol extract and essential oil (10, 100, and 1000 µg/ml) were assayed. LC_{50} was determined from 24-h counts using the probit analysis method described by Finney (1971).

The acute toxicity was carried out on Swiss mice (5 females and 5 males) fasted for 18 h. The methanol extract of *N. sibthorpii* was given by gavage, in doses of 200, 400, 800, 1600, and 2000 mg/kg in a volume of 0.1 ml/10 g b.w. The essential oil was given i.p., in doses of 50 and 100 mg/kg in a volume of 0.1 ml/10 g b.w. The mice were kept under observation for 7 days to monitor mortality (Litchfield & Wilcoxon, 1949).

Irwin test

The Irwin test (Irwin, 1964) is a scanning procedure designed to assess and characterize the effects of a drug, in particular on the behavioral sphere in rat (Kyerematen & Ogunlana, 1987).

The methanol extract was administered in the morning, by gavage, to the animals, fasted for 18 h, at doses of 50 and 100 mg/kg, and i.p. at doses of 25 and 50 mg/kg.

Fraction I was given orally and i.p. at a dose corresponding to 50 mg/kg of methanol extract.

Essential oil was administered i.p. at a dose of 1.5 mg/kg.

The rats were monitored for 7 days and were also observed for their behavioral symptoms according to the Irwin scheme.

Sedative activity

N. sibthorpii methanol extract was administered at a dose of 50 mg/kg p.o. and i.p. Fraction I was administered p.o. and i.p. at a dose corresponding to 50 mg/kg of methanol extract.

The essential oil was administered i.p. at a dose of 1.5 mg/kg.

The control mice received vehicle (propylene glycol) only.

Thirty minutes later, all groups received sodium pentobarbital (Sigma, Milan, Italy) i.p. at a dose of 50 mg/kg in physiological saline.

The *sleeping induction* (time elapsed between injection and loss of the righting reflex) and the *sleeping time* (time interval between loss and recuperation of the righting reflex) were recorded, respectively, in seconds and minutes. The criterion for recuperation of the righting reflex was fixed so that the mice had to regain their normal posture three consecutive times (Carlini et al., 1983).

Determination of the hypothermic effect

The methanol extract was administered to rats at doses of 50 and 100 mg/kg p.o. and i.p.

Fraction I was given p.o. and i.p. at a dose corresponding to 50 mg/kg of methanol extract.

Essential oil was administered i.p. at a dose of 1.5 mg/kg.

Body temperature was measured by inserting the sensor probe of a digital thermometer (Termist II series, Ellab A/S, Roedovre, DK) about 5–6 cm through the rectum. The temperature was recorded before treatment (time 0) and 60, 120, and 240 min after drug administration.

Anticonvulsant activity

We tested the anticonvulsant activity of *N. sibthorpii* preparations on generalized tonic-clonic seizures produced by pentylenetetrazol (PTZ) in mice (Aguilar-Santamaria & Tortoriello, 1996) in comparison with diazepam (Valium, Roche, Milan, Italy). In order to study the involvement of benzodiazepine binding site on GABA receptor in PTZ-induced convulsions, we used flumazenil (Anexate, Roche, Milan, Italy), a specific antagonist of benzodiazepine receptor.

Seizures are manifested as wild running, followed by stunning or clonic convulsion and then tonic convulsion exhibited by tonic hindlimb extension accompanied in most cases by death.

Animals were divided into nine groups.

Groups I and II received *N. sibthorpii* methanol extract p.o. and i.p., respectively, at a dose of 50 mg/kg.

Fraction I was administered p.o. (group III) and i.p. (group IV) at a dose corresponding to 50 mg/kg of methanol extract.

Group V was treated with flumazenil (3 mg/kg) i.p., 15 min before the oral administration of methanol extract (50 mg/kg).

Group VI received flumazenil (3 mg/kg), i.p., 15 min after the oral administration of methanol extract (50 mg/kg).

The essential oil was administered i.p. at a dose of 1.5 mg/kg (group VII).

Diazepam was administered i.p. at a dose of 1 mg/kg (group VIII). The control mice (group IX) received vehicle (propylene glycol) only.

Thirty minutes later, all groups of mice received i.p. PTZ (Sigma) at a dose of 90 mg/kg in physiological saline. Animals were observed for 1 h to detect latency period of the convulsions (time elapsed between PTZ injection and convulsion arising), to monitor the number of convulsions, and to record mortality.

Results

LC_{50} and LD_{50} determinations

N. sibthorpii methanol extract does not contain any toxic compounds; in fact, the LC_{50} is more than 1000 µg/ml and the LD_{50} is more than 2 g/kg (Lu, 1990).

N. sibthorpii essential oil shows a moderate toxicity: the LC_{50} is 145.2075 µg/ml and the LD_{50} is more than 100 mg/kg (Lu, 1990).

Irwin test

The animals treated with *N. sibthorpii* preparations showed a significant alteration in behavior.

In our experimental conditions, we observed in rats a decrease in sensitivity to external stimuli; the animals became quiet and showed a decrease in locomotor activity, lasting for about 2 h.

At the highest dose, all phenomena were significant and the animals showed their maximal behavioral changes: higher passivity, loss of curiosity, and stereotypic movements. Twenty-four hours after treatment, the animals came back to normality.

Our results show that the responses are dose-dependent and that oral is more active than parenteral administration.

Sedative activity

The administration of *N. sibthorpii* preparations potentiates the hypnotic effects of sodium pentobarbital, decreasing the *induction time* and enhancing the *sleeping time*, versus control group treated with pentobarbital only.

Particularly, in animals orally treated with methanol extract, the *induction time* is about 4-times lower than controls, and the *sleeping time* is about 10-times as high as controls (Table 1).

Determination of the hypothermic effect

N. sibthorpii preparations produce a lowering of body temperature in mice. This decrement is maximum (about 1 °C) at the first hour and persists until the third hour after treatment.

Anticonvulsant activity

In our experimental conditions, *N. sibthorpii* preparations show anticonvulsant activity and depress mortality in mice. Particularly, methanol extract, orally administered, significantly increases the latency period, decreases the number of convulsions, and totally eliminates mortality.

N. sibthorpii methanol extract did not show any activity in animals that had previously received flumazenil; instead, the anticonvulsant effect persists when the flumazenil is administered after methanol extract (Table 2).

Discussion

Nepeta sibthorpii is an aromatic plant that contains monoterpenes, sesquiterpenes, and iridoid compounds such as epinepetalactone, which is chemically related to valepotriates (epoxy-iridoid esters present in *Valeriana officinalis* L. root that possess well-known mild sedative and tranquilizing properties).

The results of the current study indicate that the *N. sibthorpii* preparations depress the central nervous system.

Table 1. Effect of *Nepeta sibthorpii* preparations on pentobarbital induction time and sleeping time potentiation in mice.

Treatment	Dose (mg/kg)	Induction time (min and s) $\bar{\chi} \pm$ SE	Sleeping time (min) $\bar{\chi} \pm$ SE
Pentobarbital i.p.	50	5′10″ ± 1.8	20′ ± 2.3
N. sibthorpii MeOH p.o.	50	1′05″* ± 0.7	190′* ± 7.1
N. sibthorpii MeOH i.p.	50	3′50″* ± 1.2	121′* ± 5.4
Fraction I p.o.	[a]	1′50″* ± 1.4	150′* ± 6.0
Fraction I i.p.	[a]	2′30″* ± 0.9	110′* ± 4.1
N. sibthorpii essential oil i.p.	1.5	2′00″* ± 1.4	170′* ± 5.0

[a] Dose corresponding to 50 mg/kg of MeOH.
* $p < 0.01$.

Table 2. Effect of *Nepeta sibthorpii* preparations on pentylenetetrazol (PTZ)-induced seizures in mice.

Treatment	Dose (mg/kg)	Latency period (min and s) $\bar{\chi} \pm$ SE	No convulsions $\bar{\chi} \pm$ SE	Lethality $\bar{\chi} \pm$ SE
PTZ i.p.	90	40″ ± 2.1	3.0 ± 0.5	10/10
N. sibthorpii MeOH p.o.	50	11′05″* ± 31	0.6* ± 0.5	0/10
Flumazenil + *N. sibthorpii* MeOH p.o.	50	1′20″ ± 3.2	2.2 ± 1.5	10/10
N. sibthorpii MeOH p.o. + flumazenil	50	11′07″* ± 31	0.6* ± 0.6	0/10
N. sibthorpii MeOH i.p.	50	10′05″* ± 31	0.7* ± 0.5	2/10
Fraction I p.o.	[a]	6′05″* ± 15.1	0.5* ± 0.8	0/10
Fraction I i.p.	[a]	5′30″* ± 12.1	0.7* ± 0.9	3/10
N. sibthorpii essential oil i.p.	1.5	5′06″* ± 10.1	0.7* ± 0.9	3/10
Diazepam i.p.	1	28′36″* ± 41.7	0.5* ± 0.3	0/10

[a] Dose corresponding to 50 mg/kg of MeOH.
* $p < 0.01$.

Also, they provoke alteration of body temperature in normothermic mice. This result is not surprising, as it is known that psychoactive CNS-depressant drugs reduce temperature both in normal and pyretic conditions (Clark & Clark, 1980).

N. sibthorpii preparations potentiate the hypnotic effects of sodium pentobarbital and prevent PTZ-induced convulsions.

Several studies evidenced that the convulsing activity of PTZ is due to its ability to selectively antagonize GABA-mediated postsynaptic inhibition in the mammalian CNS (Macdonald & Barker, 1977). Other studies demonstrated that PTZ affects chloride permeability, which is also an indication of a direct effect upon the cellular membrane. This direct excitatory effect, together with its ability to block enhancing effects of GABA on chloride conductance, can account for the convulsant properties of PTZ (Pritchard et al., 1971).

N. sibthorpii preparations reduce the number and duration of PTZ-induced convulsions and depress mortality. The preventive administration of flumazenil, a specific antagonist of benzodiazepine binding site on GABA receptor, blocks the anticonvulsant effect of the *N. sibthorpii* preparations. The results permit the hypothesis that the *N. sibthorpii* active principles act as agonists on benzodiazepine binding site on $GABA_A$ receptor, increasing chloride conductance. It is evident that when flumazenil occupies benzodiazepine binding site on GABA receptor, *N. sibthorpii* active principles are not allowed to exert anticonvulsant activity.

On the other hand, the anticonvulsant effect persists when the flumazenil is administered after methanol extract; we can presume that flumazenil is not able to remove *N. sibthorpii* active principles from benzodiazepine binding sites.

Because it is known that iridoids provoke alterations in general behavioral pattern in several animal species (Cavill & Robertson, 1965; Cavill, 1969), the general depressant effects observed can be correlated to epinepetalactone.

In our experimental conditions, the methanol extract shows the greatest activity; the neuropharmacological effects could thus be related not only to epinepetalactone but even to other compounds present in the extract.

Moreover, the effects are dose-dependent and more marked after oral rather than after i.p. administration. We can

hypothesize that after oral administration, *N. sibthorpii* active principles are biotransformed into more active compounds in the gastroenteric tract. Further investigations are in progress in our laboratory in order to isolate and identify other active principles present in methanol extract that act in synergy with epinepetalactone, contributing to CNS depressant activity.

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