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ORIGINAL ARTICLE

Effects of Insecticidal Ketones Present in Mint Plants on $GABA_A$ Receptor from Mammalian Neurons

Mariela Eugenia Sánchez-Borzone, Leticia Delgado Marin, Daniel Asmed García

Instituto de Investigaciones Biológicas y Tecnológicas (IIBYT), CONICET-Universidad Nacional de Córdoba, Av. Vélez Sarsfield 1611, Córdoba 5016, Argentina

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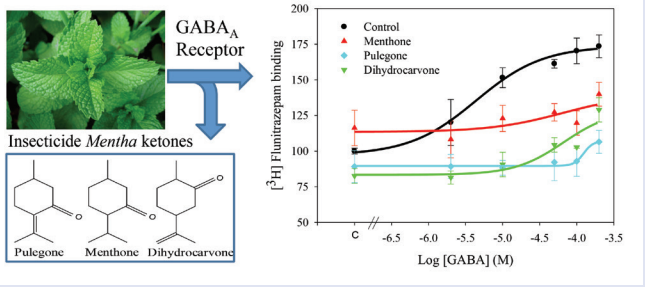
ABSTRACT

Background: The genus *Mentha*, an important member of the *Lamiaceae* family, is represented by many species commonly known as mint. The insecticidal activity of *Mentha* oil and its main components has been tested and established against various insects/pests. Among these, the ketone monoterpenes that are most common in different *Mentha* species demonstrated insect toxicity, with pulegone being the most active, followed by carvone and menthone. Considering that the $GABA_A$ receptor ($GABA_A$-R) is one of the main insecticide targets on neurons, and that pulegone would modulate the insect GABA system, it may be expected that the insecticidal properties of *Mentha* ketones are mediated by their interaction with this receptor. **Objective:** In order to discern the pharmacological actions of these products when used as insecticides on mammalian organisms, we evaluated the pharmacologic activity of ketones, commonly present in *Mentha* plants, on native $GABA_A$-R from rats. **Materials and Methods:** Determination of ketones effects on allosterically enhanced benzodiazepine binding, using primary cultures of cortical neurons, which express functional receptors and MTT assay to evaluate their cell toxicity. **Results:** Our results seem to indicate that ketone components of *Mentha*, with proven repellent or insecticide activity, were able to behave as $GABA_A$-R negative allosteric modulators in murine cells and consequently could exhibit convulsant activity in mammalians. Only pulegone at the highest assayed concentration (2 mM) showed a significant reduction in cell viability after exposure for 24 hr. **Conclusion:** The present results strongly suggest that the ketone components of *Mentha* are able to exhibit convulsant activity in mammalian organisms, but functional assays and *in vivo* experiments would be necessary to corroborate this proposed action.

Key words: Cell culture, $GABA_A$ receptor, insecticide, ketones, *Mentha*, toxicity

SUMMARY

- The pharmacological activity of insecticide ketones, commonly present in *Mentha* plants, was evaluated on native $GABA_A$ receptor from mammalian neurons.
- All studied compounds: pulegone, menthone and dihydrocarvone, were able to behave as negative allosteric modulators and could exhibit convulsant activity in mammalian organisms.
- Citotoxicity assays demonstrated that only pulegone affected the cell viability.



Abbreviations used: GABA: gamma aminobutyric acid, GABAA-R: GABAA receptor, MTT: 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazam, DMEM: Dulbecco's modified minimum essential médium, [3H]TBOB: [3H] t-Butylbicycloorthobenzoate

Correspondence:
Dr. Daniel Asmed García,
Instituto de Investigaciones Biológicas y Tecnológicas (IIBYT), CONICET-Universidad Nacional de Córdoba, Cátedra de Química Biológica, FCEFYN, Av. Vélez Sarsfield 1611, Córdoba 5016, Argentina.
E-mail: dagarcia@efn.uncor.edu

INTRODUCTION

The genus *Mentha*, one of the important members of the *Lamiaceae* family, is represented by many species commonly identified as mint, which has been known for its medicinal and aromatherapy properties. The insecticidal activity of *Mentha* oil and its main components has been tested and established against various insects/pests.[1] *Mentha*'s repellent properties against agricultural pests were investigated in a series of experiments by Odeyemi *et al.*[2] and Kumar *et al.*[3] Its repellent activity was also demonstrated in mosquito control, and thus for diseases of public health concern such as malaria, yellow fever, dengue, and viral encephalitis.[4–6] Many assays have reported insect mortality caused by *Mentha* toxicity,[7,8] and some have evaluated its antifeedant activity.[3,9] Species of the genus *Mentha* have been reported to contain a range of components.[10] The monocyclic ketones most commonly found in *Mentha* species are pulegone, menthone, carvone, and, to a lesser extent, dihydrocarvone.[11,12]

The $GABA_A$ receptor ($GABA_A$-R) is a major insecticide target along with the voltage-dependent sodium channel, the nicotinic receptor, and acetylcholinesterase.[13,14] Important insecticides acting at the $GABA_A$-R (e.g., lindane, α-endosulfan, dieldrin, and fipronil) recognize the picrotoxinin site, a noncompetitive antagonist site, to block GABA-induced chloride flux.[15] $GABA_A$-R in mammalian, and even in various insect species, differs a lot in their subunit combinations and sensitivities to different ligands.[16–18] The structure and nature of binding sites in housefly GABA receptors have been shown to be different from those in rat GABA receptors, and the differences may be related to the selectivity of antagonists for housefly versus rat receptor.[19]

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We described very recently the effects of carvone isomers on the mammalian $GABA_A$-R, demonstrating their inhibitory activity on this receptor. In this work, we evaluated the pharmacologic activity of the other monoterpene ketones commonly present in *Mentha* (pulegone, menthone, and dihydrocarvone; see structures in Figure 1) on native $GABA_A$-R from rats by determining their effects on allosterically enhanced benzodiazepine binding using primary cultures of cortical neurons, which express functional receptors,[20,21] in order to discern the pharmacologic activity of these products on mammalian organisms when used as insecticides. We also investigated the possible neurotoxic effects of *Mentha* components in the same cell culture system at concentrations relevant to their neuroactive ranges.

MATERIAL AND METHODS

Materials

(*R*)-(+)-Pulegone (purity 99%) (IUPAC name: (5*R*)-5-methyl-2-propan-2-ylidenecyclohexan-1-one), (−)-menthone (purity 99%) (IUPAC name: (2*S*,5*R*)-5-methyl-2-propan-2-ylcyclohexan-1-one), (+)-dihydrocarvone (mixture of isomers: ~77% *n*-(+)dihydrocarvone and ~20% iso-(+)dihydrocarvone) (IUPAC name: 2-methyl-5-prop-1-en-2-ylcyclohexan-1-one) , γ-aminobutyric acid (GABA), picrotoxin, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazam (MTT), Dulbecco's modified minimum essential medium (DMEM), trypsin, soybean trypsin inhibitor, DNase, amino acids, and poly-l-lysine were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Fetal calf serum was obtained from PAA (Pasching, Austria) and [^{3}H]flunitrazepam (84.9 Ci/mmol) from Perkin-Elmer (Boston, USA). All the other chemicals were of analytical grade.

Cell cultures

Primary cultures of cortical neurons were prepared from the cerebral cortices of 17-day-old rat fetuses, as previously described.[20] The cell suspension (1.6×10^6 cells/ml) was seeded in 24×- or 96× multiwell plates, according to the experiment, precoated with poly-l-lysine and finally incubated for 6-7 days in a humidified 5% CO_2/95% air atmosphere at 37°C. Twenty millimolar cytosine arabinoside was added after 48 h in culture to prevent glial proliferation.

[^{3}H]Flunitrazepam binding

The benzodiazepine binding to intact cultured cortical neurons was determined as previously described[20] using nearly 2.0 nM [^{3}H] flunitrazepam. Seven hundred fifty micromolar of ketones and variable concentrations of GABA, between 0 and 200 μM, were added to the incubation media for 30 min of incubation at 25 °C. Nonspecific binding was determined in the presence of 20 μM diazepam.

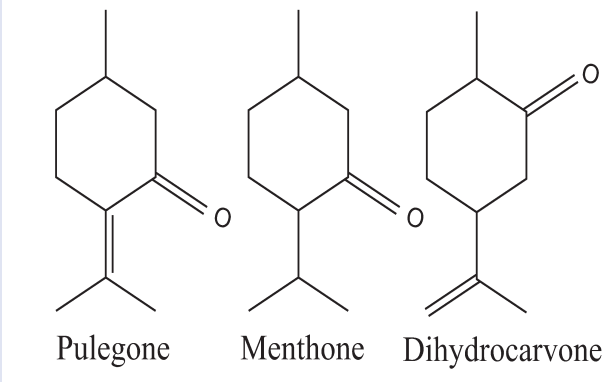

Figure 1: Chemical structures of the compounds analyzed

Cell viability

After 6-7 days *in vitro*, the cells were exposed to different concentrations of each compound for 30 min or 24 h. Ketones were added after solubilization in 0.2 ml of culture medium previously extracted from each well. Cell viability was determined by measuring the reduction of MTT to a colored formazan salt by mitochondrial reducing activity, as described previously.[20]

Data analysis

Data shown represent the mean ± standard error of mean (SEM). Sigmoid curves were fitted to concentration response data and statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). The two-tailed Student's *t*-test and one-way ANOVA were used to compare data. A *P* value less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Effects of *Mentha* ketones on the benzodiazepine binding enhanced by GABA

To evaluate the activity of *Mentha* ketones on native $GABA_A$-R from mammalians, their effects were observed on [^{3}H]flunitrazepam binding stimulated by the agonist GABA in primary cultures of rat cortical neurons. The results demonstrated that GABA was able to enhance radioligand binding in a dose-dependent manner as expected, showing an EC_{50} value of 4.4 μM [Figure 2] [Table 1]. This result is consistent with that reported previously.[22,23] All *Mentha* ketones studied in the present work were able to right shift the concentration–response curve of the effect of GABA on [^{3}H]flunitrazepam binding. At the beginning of each curve, pulegone and dihydrocarvone showed a negative effect in the absence of GABA (control samples), while menthone induced an increase; later, all ketones slowly enhanced the binding as the GABA concentration increased. Fitting the data to sigmoid curves revealed a rise in the EC_{50} value for the GABA-induced increase in [^{3}H] flunitrazepam binding to 123.7, 64.8, and 66.7 μM in the presence of pulegone, menthone, and dihydrocarvone, respectively. At the same time, the maximum response induced by GABA 200 μM (174% with respect to basal) was significantly reduced by all ketones [Figure 2 and Table 1] ($P < 0.05$, one-way ANOVA). Taking into account these effects, we can clearly consider all *Mentha* compounds as negative allosteric modulators, at least on mammalian neurons. It should be noted that the allosteric behavior of the receptor was tested by determining the improvement of [^{3}H]flunitrazepam binding exerted by GABA and its reduction by a noncompetitive GABA antagonist.

Pulegone is used as a flavoring agent, in perfumery and aromatherapy,[1] and an enantiomeric form ((*R*)-(+)-pulegone) was described as a psychoactive compound with the profile of an analgesic drug.[24] Tong and Coats[25] suggested that pulegone and other monoterpenoids act as positive allosteric modulators of the $GABA_A$-R in insects. However, this result supported by the $^{36}Cl^-$ uptake enhancement did not correlate very well with the increase found in [^{3}H]TBOB binding in the same work, since a reducing effect should be expected according to their suggested function as GABA allosteric agonist. The inhibitory effect of pulegone on GABA-stimulated [^{3}H]flunitrazepam binding described in the present work clearly indicates its activity as a negative allosteric modulator in murine cortical neurons. In another report, Bessette[26] described opposite effects of pulegone on [^{3}H]TBOB binding in house

Table 1: Effect of *Mentha* ketones on GABA-induced increase of [^{3}H]flunitrazepam binding

Compound	log EC_{50}	EC_{50} (μM)	Max. response (%)	*n*
GABA	-5.36 ± 0.40	4.4	174 ± 8.0	4
GABA/Pulegone	-3.91 ± 0.34	123.7	106 ± 8.1*	5
GABA/Menthone	-4.19 ± 0.60	64.8	140 ± 8.3*	6
GABA/Dihydrocarvone	-4.18 ± 0.16	66.7	129 ± 8.5*	4

EC_{50} values were calculated from data shown in [Fig. 2], as explained in the Material and Methods section. A minimum of 6 concentrations of GABA were used for each curve. Maximal response corresponds to the percentage of increase induced by GABA 200 μM with respect to the basal binding (without GABA or any compound). The values correspond to the mean ± S.E.M. *Different to GABA sample ($P < 0.05$, one-way ANOVA).

fly (enhancement) or in mouse (inhibition). These controversial results could be the consequence of the wide, complex subunit composition of $GABA_A$-R and its different expression in insect compared with mammalian organisms.

Menthone is extensively used in perfumery and as a flavoring agent.[1] The marked increase in the EC_{50} found in the present study induced by menthone strongly suggests an essentially negative effect on this receptor in mammalian cells. Therefore, considering dissimilar organisms, some differing responses would be expected,[27] as is discussed above with pulegone.

The last *Mentha* ketone assayed, dihydrocarvone, also showed inhibitory effects on the receptor and could accordingly also be considered an allosteric negative modulator. Even though it was described that hydroxydihydrocarvone, a similar compound, has significant psychopharmacologic activity with depressant effects,[28] this is the first report of the interaction of dihydrocarvone on $GABA_A$-R.

Effects of *Mentha* ketones on neuron viability

Considering that *Mentha* essential oils and their principal components have been described as toxic for many insects,[1,29-31] we analyzed the toxicity in mammalian cells of three *Mentha* ketones. *Mentha* ketones did not reduce cell viability after exposure for 30 min. After 24 h, just pulegone, at the highest tested concentration, showed significant reduction in viability (16%) compared with control (100%) in this cell assay system ($P < 0.05$, one-way ANOVA). It is interesting to note that the highest toxicity of pulegone, compared with menthone and dihydrocarvone, is in agreement with their reported insect toxicity.[29-31]

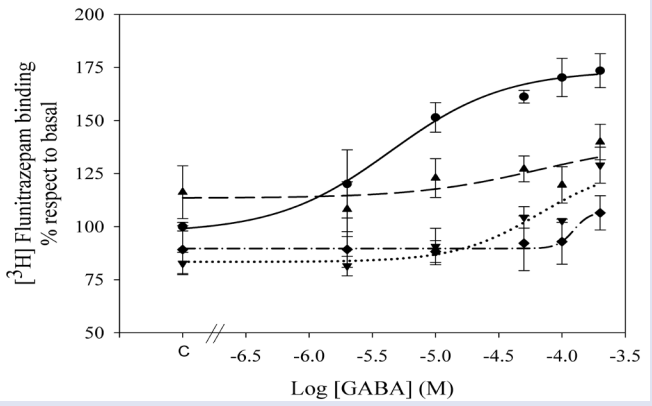

Figure 2: Concentration–response curves for the [^{3}H]flunitrazepam binding stimulated by GABA in primary cultures of cortical neurons. The points correspond to experiments made in the presence of GABA and in the absence (—•—) or presence of 750 μM pulegone (···♦···), menthone (—▲—), or dihydrocarvone (···▼···). Lines represent sigmoid curves fitted to the data. The values are expressed as the percentage of basal binding (without GABA or any compound). C: control values in the absence of GABA. Results are means ± SEM of four to six independent experiments done in triplicate

Mentha components as repellents or insecticide agents could expose them to other organisms, including mammalian, where their potential toxicity should be evaluated. Pulegone is recognized as potentially toxic to the liver and lung.[32,33] It has been suggested that its main action mechanism could involve metabolites of pulegone, such as menthofuran, which neutralize cytochrome P450s by modifying the prosthetic heme group or the apoprotein.[34] This mechanism could be occurring in the toxic effect of pulegone on rat neurons at high concentrations and over a long time, as described in the present work. Menthone showed moderate to low acute oral toxicity in rats.[35] In cell viability studies, using a cell line of human foreskin fibroblasts, menthone was found to be nontoxic, while pulegone showed adverse effects.[36] At present, there are no published reports about the cytotoxicity of dihydrocarvone. Its oxidized form, carvone, and its isomer had no effect on murine neuron viability, as we described previously.[37]

In conclusion, the possible use of natural repellents or insecticides based on extracts, essential oils, or their principal components should consider their potential effects on other organisms. The present results seem to indicate that ketone components of *Mentha*, with proven repellent or insecticide activity, are able to behave as $GABA_A$-R negative allosteric modulators in mammalian cells, although further assays, such as [^{35}S] TBPS and [^{3}H]muscimol binding, would be necessary to identify the exact binding site(s) of the ketones. Thus, the present results strongly suggest that the ketone components of *Mentha* are able to exhibit convulsant activity in mammalian organisms, but functional assays and *in vivo* experiments would be necessary to corroborate this proposed action.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Daniel A. García

ABOUT AUTHOR

Daniel A. García, is Professor at the Universidad Nacional de Córdoba and Independent Researcher of CONICET (Argentine). He works in the Instituto de Investigaciones Biológicas y Tecnológicas, (IIByT, CONICET- Universidad Nacional de Córdoba, Argentine). Dr. García has wide experience in the area of Medicinal Chemistry, Neuroscience and Membrane Biophysics. His research topic is focusing on the drug-membrane interaction with special interest in the non-specific modulation of Neuronal Receptors.