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Mentol, składnik olejków eterycznych, czynnikiem podnoszącym efektywność bendiokarbu, insektycydu z grupy karbaminianów

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Menthol, essential oils component, as a factor increasing effectiveness of bendiocarb insecticide

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List of abbreviations:

ACh - acetylcholine

AChE - acetylcholinesterase

ATP - adenosine triphosphate

CaM kinase II - Ca2+/calmodulin-dependent protein kinase II

cAMP – cyclic adenosine monophosphate

DAG - diacylglycerol

DDT – dichlorodifenylotrichloroetan

DEET - N,N-dietylo-m-toluamid

DUM - dorsal unpaired median neurons

ED_{50} – effective dose

GABAr - gamma-aminobutyric acid receptors

GPCRs - G-protein coupled receptors

IF – impact factor

IP3 -1,4,5-trisphosphate

mGluRIII - metabotropic glutamate receptors

OAr – octopamine receptor

PKA - protein kinase A

PKC - protein kinase C

SCBI - sodium channel blocking insecticides

TRPM8 - transient receptor potential cation channel subfamily M member 8

1. AUTHOR'S COMMENTARY

1.1 Introduction

Insect pests destroy crops and forests, limit food supplies, transmit infectious diseases, and give rise to hygienic problems in places inhabited by man. In the last decades, excessive increase in insect pest population has been observed [1]. Globalization (transport and travels), global warming, and pest resistance to insecticides are some factors responsible for the increasing risk of insect pests for humans and biodiversity.

Insects (mosquitos and flies) are vectors of serious diseases (e.g. malaria, Zika, dengue, chikungunya, Leishmaniasis, and West Nil Fever) that represent extremely important social problems in tropical regions of Earth. They have already been introduced in Europe too (e.g. outbreaks of Chikungunya in 2007 and 2017 in Italy) [2,3]. Climate change favors the spread of dangerous insects that were previously not able to survive in harsh European climates. Increasing temperatures not only affect the survival of exotic insects, but also vector activity and survival of the pathogens — parasites and viruses. It is predicted that the presence of disease-carrying mosquitos will progressed to the northern parts of Europe, due to increasing temperatures in Northern Europe and the desertification of Southern Europe [4,5].

Increasing temperatures worldwide affect population growth rates, insects metabolic rate, and rate of food consumption, all of which are related to crop loses. It is estimated that arthropods currently destroy 18-20% of crops annually, which is equivalent to about 470 billion US dollars [6]. A mathematical model of crop losses assumes that with an increase in global temperature of 2 °C, the losses in wheat caused by insects will increase by 46 % and losses in maize will increase by 31% [1].

The infestation of insects in households and utility buildings is a serious problem. Wood boring beetles (e.g. *Anobium punctatum*), Dermastides (e.g. *Dermestes sp*.), moths (e.g. *Tineola bisselliella*), and silverfish (*Lepisma saccharina*) damage materials, objects or parts of buildings [7]. Cockroaches (*Periplaneta americana, Blatta orientalis or Blattella germanica*), houseflies (*Musca domestica*), and other “dirty” insects carry and transmit bacteria and fungi [8,9].

The search for effective methods of protection against the development of insect pest populations is one of the most important needs in the world. Classical pest management is based on the use of chemical insecticides that mainly act as neurotoxic agents. The

chemical insecticides can be divided into different groups by their targets of action [10,11]:

1. voltage-dependent sodium channels modulators, which include pyrethroids, veratrum alkaloids, SCBI (sodium channel blocking insecticides: oxadiazine, indoxacarb, and metaflumizone), and polychlorinated insecticides. They cause prolonged opening of the sodium channels, which either results in uncontrolled action potentials and depolarization of the membrane or they block the sodium channels and prevent action potentials generation [12,13].
2. calcium channels modulators, such as ryanodine, which prolongs the opening of the channel [14,15].
3. nicotinic acetylcholine receptors agonists, such as neonicotinoids, which stimulate post-synaptic nerves [16,17].
4. acetylcholinesterase (AChE) inhibitors, such as organophosphates and carbamates, which inhibit the enzyme responsible for the proper level of the neurotransmitter in synapses (i.e., acetylcholine - ACh), which in turn leads to over-excitations of the post-synaptic nerves [18,19].
5. gamma-aminobutyric acid receptors (GABAr) modulators, such as polychlorocycloalkanes, isoxazolines, and meta-diamides, which also cause excessive stimulation of the nervous system due to the blocking of the influx of chloride ions into neurons [20,21].

Chemical insecticides are basically efficient but insects develop resistance to them over time. The widespread use of insecticides causes susceptible insects to be eliminated, however, some resistant individuals remain and are able to restore the population. The most commonly observed case is resistance to pyrethroids in many insect species. According to Naqquash et al. 2016, the level of resistance to pyrethroids is equal to 18,400x in *Musca domestica*, 959x in *Culex sp*., and 468x in *Blatella germanica*. Carbamates are much less resistance-inducing insecticides. The highest detected level of resistance to carbamates is equal to 290x, 2.93x, and 62x for the above mentioned insects species, respectively [22]. Several mechanisms of insecticide resistance have been described [23] and in general they can be divided into four categories: 1) behavioral resistance [24], 2) penetration resistance [25], 3) target-site resistance [26,27], and 4) metabolic resistance [22,28].

Another issue connected to the use of insecticides is their impact on the environment and non-target organisms. It was estimated that only 1% of released insecticides achieve their targets and 99% of them end up in the environment as pollutants [29]. Most insecticides get into the environment due to agricultural and forestry practices. Insecticide residues are detected not only in water and soil but also in rain, fog, and the air. Residues of DDT were detected even at an altitude of 4,250 m on the Nanjiabawa peak in Tibet [29]. Insecticides get into the environment basically due to run-off from fields, airborne drift, and intentional dumping. As a result, ground waters are contaminated and residues of insecticides are bioaccumulated in living organisms [30]. It was evidenced that insecticides have negative impact on birds and all aquatic organism: invertebrates, fish, as well as plants and microorganism [29,31].

Significant negative impact of some insecticides on animal and human populations led to their withdrawal from use. However, development of new insecticides, bypassing the discussed problems, recently plateaued [26]. In such situations, intensive scientific work is done in the field of insect genomic and genetic modifications, which aim to decrease insect pest population. While laboratory-scale experiments give promising results [32], there is still no technique to introduce genomic methods on a large scale in field conditions [33–35]. Moreover, introducing genetic modifications in organisms may be met with non-acceptance in society and lastly, may bring unpredictable consequences.

Sensible strategy that can be used straight away is to increase the effectiveness of available insecticides without increasing their negative impact on ecosystems and people. The example of such strategy is based on the combined use of substances belonging to two or more groups of highly differentiated target sites of action. The strategy is based on the synergistic effect of these substances [36]. By definition, “synergism is more than an additive effect and antagonism is less than an additive effect” [37]. There are data explaining the mechanism of such synergistic effect between insecticides. Most commonly, insecticides are combined with substances like piperonyl butoxide that directly inhibit detoxification enzymes in insects [38]. It has been proved that a positive interaction between pyrethroid (permethrin) and carbamate (propoxur) is carried out *via* muscarinic receptors, which regulate the release of ACh in the synaptic connections in insects bodies [39]. Muscarinic receptors belong to G-protein coupled receptors (GPCRs). Recently, more and more attention is paid to GPCRs and enzymes (kinases, ATPases, synthases and carboxylesterases) as potential “supporting” agents for insecticides

affecting key neural proteins: acetylcholinesterase, nicotinic acetylcholine receptors, and voltage-gated and ligand-gated ion channels [26].

GPCRs are metabotropic receptors, widely expressed in all animal species. They play crucial roles in processes such as development, reproduction, metabolism, and regulation of the functions of insect nervous system. Their activation through ligand binding leads to the activation of phosphorylation cascade and alteration in many cellular processes [40]. Two kinds of cellular pathways can be activated through GPCRs:

1) activation of phospholipase C, which hydrolyses phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 activates its specific receptors in the Golgi and the endoplasmic reticulum, which release internal calcium stores. Calmodulins, which are activated by calcium ions, activates Ca^{2+}/calmodulin-dependent protein kinases (CaM kinases). DAG activates protein kinase C (PKC) [40].
2) Activation of adenylyl cyclase, which hydrolyses ATP to cAMP. cAMP is a specific activator of protein kinase A (PKA) [40].

Elevation of intracellular Ca^{2+} and activation of protein kinases lead to activation and modulation of numerous cellular processes: gene expression, activity of ion channels and membrane receptors, exocytosis, metabolism, and cytoskeleton dynamics [41–44].

Innovative approaches in insect pest control focus on using activators of GPCRs receptors as synergistic agents [23]. Recently, synergistic interaction between GPCRs activators and insecticides are the subject of extensive research. It was demonstrated that activation of metabotropic glutamate receptors (mGluRIII) induces phosphorylation of voltage-gated sodium channel by CaM kinase II and changes its sensitivity to oxadiazine insecticides by an order of magnitude [45]. Positive, synergistic interaction was observed between repellent DEET and propoxur, a carbamate insecticide. DEET in low concentrations activates muscarinic receptors and through the increase in Ca^{2+} concentration and phosphorylation of acetylcholinesterase enzyme, changes its sensitivity to carbamates [46]. Another innovative approach relies on using baculoviruses as the agent to increase intracellular calcium level [47]. Positive results were obtained using *Autographa californica multiple nucleopolyhedrovirus* together with chlorpyrifos-ethyl on Sf9 cells expressing the enzyme acetylcholinesterase. That combination increases sensitivity of the enzyme 1.5 fold [48].

In the presented doctoral dissertation, emphasis was placed on one of the GPCRs – octopamine receptors (OAr). These receptors are specific to invertebrates since their agonist — octopamine — is found only in trace amounts in mammals. It plays a role similar to noradrenaline/adrenaline in mammals [49,50]. The effects of OAr activation have been described in detail in the first article from the presented set [51].

Potential action on octopamine receptors was reported for some components of essential oils [52–54]. Full description of the mode of action and effectiveness of essential oils against insects has been described in the first article from the presented set [51].

Preliminary tests revealed that menthol, the main compound of *Mentha* essential oil, has some insecticidal properties. Until now, there were no reports that menthol could act as an octopamine receptor agonist although there were known evidences that menthol is a ligand of TRPM8 receptor (cold receptor in mammals) [55] and is a positive modulator of GABA receptors [56,57]. Other preliminary tests indicated that menthol acts on insect (cockroach) nervous system octopamine receptors because its effect was abolished by phentolamine – inhibitor of OAr receptors (those results are included in Article II - [58]).

Following the latest approaches and results of my own preliminary tests, I decided to examine interactions between menthol and chemical insecticide. Bendiocarb, a carbamate insecticide was chosen. Carbamates are chemical insecticides whose mode of action is the inhibition of the enzyme, acetylcholinesterase (AChE). The choice was based on the following facts:

- Carbamates are classified as the temporary inhibitors of the AChE enzyme. Their effect is similar to that of organophosphates, but contrary to them carbamates are reversible and safer for humans [59].
- Resistance of insects to carbamates is relatively low, compared to other classes of insecticides (pyrethroids, DDT) [22].
- Carbamates are widely used as insecticides in agriculture and other industries, homes, offices, and gardens [60]. For example, the most used insecticide (over 95% of treatments) in dealing with wasp nest in Great Britain is Ficam D (commercial form of bendiocarb).
- Bendiocarb is approved for use: the European Union Commission Directive from 9 February 2012 [61] approved its use up to the year 2024. In Great Britain Ficam D can be used indoors until 2028 .

- The World Health Organization recommends using bendiocarb indoors [62].
- Bendiocarb is estimated as the most effective insecticide in practice for insect elimination from homes (information obtained during cooperation with local insecticidal company, INSE-TOX Jan Stromidło, Toruń).
- It was already evidenced that activation of metabotropic receptors can change the sensitivity of AChE to carbamate insecticides [46].

1.2 The aim of the study

Based on available knowledge and preliminary experiments carried out in our laboratory, the research goal was formulated – it was to verify the hypothesis that **the presence of essential oils component, menthol, will increase the efficiency of the carbamate insecticide, bendiocarb, on insects**.

For a deeper understanding of the problem, we put forward further alternative hypotheses:

- **the potentiating effect of menthol on bendiocarb occurs through the activation of octopamine receptors and then activation of protein kinase A, which lead to phosphorylation of AChE enzyme and alteration in its sensitivity to the inhibitor**
- **the potentiating effect of menthol on bendiocarb occurs through the activation of octopamine receptors and then activation of protein kinase C, which lead to phosphorylation of AChE enzyme and alteration in its sensitivity to inhibitor**

In order to verify these hypotheses, the following predictions were tested:

1. There will be more paralyzed insects after exposure to bendiocarb and menthol than after exposing them to bendiocarb only.
2. Menthol will modify (depending on the concentration) the bioelectric activity of insect`s central nervous system.
3. The effect of menthol on the electric activity of the insect nervous system will be abolished by an octopamine receptor antagonist – phentolamine.

4. Disruption of the electric activity of the central nervous system of the insect will be greater after the application of bendiocarb with menthol compared to using bendiocarb alone.
5. Menthol will inhibit the AChE activity.
6. Menthol will increase the inhibition of AChE caused by bendiocarb.
7. Inhibitors of protein kinase A will abolish the effect of menthol.
8. Inhibitors of protein kinase C will abolish the effect of menthol.
9. Effect of menthol will be mediated *via* the increase in intraneuronal Ca^{2+} level.

Results of performed experiments are presented in two original articles [58,63].

1.3 Justification for the consistency of the series of articles

The presented PhD thesis consists of three papers. The first article is the review article entitled: **"Molecular Targets for Components of Essential Oils in the Insect Nervous System-A Review"** and was published in Molecules (IF = 3.060), which is focused on substances of natural origin, such as essential oils. The paper discusses three major modes of action of essential oils: inhibition of acetylcholinesterase enzyme, positive modulation of GABA receptors, and activation of octopamine receptors. In the article, authors thoroughly examined current knowledge concerning impact of essential oils components on insects, with focus on their nervous system. The review paper preludes the next two original articles.

The second published paper is the original article entitled: **"The unusual action of essential oil component, menthol, in potentiating the effect of the carbamate insecticide, bendiocarb"** published in Pesticide Biochemistry and Physiology (IF = 2.87). Authors examined the impact of menthol on bendiocarb efficacy in several tests: 1) Toxicity tests, which included mortality tests on insects, paralysis tests, and ED_{50} value evaluation; 2) Electrophysiological experiments using extracellular recordings from connective nerves, where spontaneous activity and the response for stimulation were evaluated. In all tests, male cockroach *Periplaneta americana* served as a model.

It was established that menthol, although by itself did not cause mortality or knock-down effect, increased the number of insects knocked down by bendiocarb. With low concentrations of bendiocarb, menthol also enhanced the level of paralysis of insects, decreasing their ability

to turn back from their dorsal to ventral sides. In electrophysiological experiments, menthol decreased the response to stimulation in cockroach nerves, which was comparable to the octopamine effect and was abolished by phentolamine, the octopamine receptor antagonist. Menthol also increased the excessive excitation in the nervous system (size of burst of spontaneous activity) caused by bendiocarb, which was also abolished by phentolamine. In the paper it has been shown that menthol potentiates the effect of bendiocarb on the level of entire insect and on its nervous system.

The third paper, entitled: **"Menthol Increases Bendiocarb Efficacy Through Activation of Octopamine Receptors and Protein Kinase A"** and published in Molecules (IF = 3.060), presents a deeper insight into the mode of action of menthol and its potentiation effect on bendiocarb. I performed: 1) electrophysiological experiments on dorsal unpaired median (DUM) neurons, using microelectrode technique; 2) calcium imaging in DUM neurons; and 3) biochemical analysis of acetylcholinesterase activity.

In the paper, I showed that menthol caused rapid hyperpolarization accompanied by a decline in spontaneous action potential generated in DUM neurons, which was quite similar to octopamine effect and was abolished by phentolamine. Menthol also caused the rise in intracellular calcium level similar to octopamine. I also examined whether the effect of menthol is mediated through protein kinase A or protein kinase C. It was observed that the effect of menthol on the rise of calcium was dependent on protein kinase A. Biochemical analysis demonstrated that menthol alone does not have properties of the acetylcholinesterase inhibitor. However, it enhances inhibition caused by bendiocarb, which was abolished by protein kinase A inhibitor, but not by inhibitor of protein kinase C.

The presented papers form a coherent story. The review paper describes the background of research on essential oils components. The first original article shows the effect of menthol and its strengthening effect on bendiocarb activity in insect model. The second original paper provides a deeper insight into the mode of action of menthol and explains how menthol increases bendiocarb efficacy.

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2. List of articles, included in the PhD thesis

1.	**Jankowska M**., Rogalska J. Wyszkowska J., Stankiewicz M., (2018) Molecular targets for components of essential oils in the insect nervous system: a review. Molecules. 23(1): 1-20 **Contribution: 80%**	**IF - 3.060** **MNiSW - 100**
2.	**Jankowska M**., Lapied B., Jankowski W., Stankiewicz M., (2019) The unusual action of essential oil component, menthol, in potentiating the effect of the carbamate insecticide, bendiocarb. Pestic Biochem Physiol. 158: 101-111 **Contribution: 75%**	**IF – 2.87** **MNiSW - 100**
3.	**Jankowska M**., Wiśniewska J., Faltynowicz L., Lapied B., Stankiewicz M., (2019) Menthol Increases Bendiocarb Efficacy Through Activation of Octopamine Receptors and Protein Kinase A. Molecules. 24(20), 3775 **Contribution: 70%**	**IF - 3.060** **MNiSW - 100**
	Summarized **IF – 9.01** Summarized **MNiSW points: 300**	

3. Article I

Molecular targets for components of essential oils in the insect nervous system: a review

molecules

MDPI

Review

Molecular Targets for Components of Essential Oils in the Insect Nervous System—A Review

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Abstract: Essential oils (EOs) are lipophilic secondary metabolites obtained from plants; terpenoids represent the main components of them. A lot of studies showed neurotoxic actions of EOs. In insects, they cause paralysis followed by death. This feature let us consider components of EOs as potential bioinsecticides. The inhibition of acetylcholinesterase (AChE) is the one of the most investigated mechanisms of action in EOs. However, EOs are rather weak inhibitors of AChE. Another proposed mechanism of EO action is a positive allosteric modulation of GABA receptors (GABArs). There are several papers that prove the potentiation of GABA effect on mammalian receptors induced by EOs. In contrast, there is lack of any data concerning the binding of EO components in insects GABArs. In insects, EOs act also via the octopaminergic system. Available data show that EOs can increase the level of both cAMP and calcium in nervous cells. Moreover, some EO components compete with octopamine in binding to its receptor. Electrophysiological experiments performed on *Periplaneta americana* have shown similarity in the action of EO components and octopamine. This suggests that EOs can modify neuron activity by octopamine receptors. A multitude of potential targets in the insect nervous system makes EO components interesting candidates for bio-insecticides.

Keywords: acetylcholinesterase; bioinsecticides; essential oils; GABA receptors; insect nervous system; octopamine receptor

1. Introduction

Essential oils (EOs) are natural, complex substances extracted from different plant organs, and terpenoids are the main components of them [1]. People have taken advantage of EOs as well as their particular components for many centuries. Recently, the historical aspects of use of EOs has been described in detail [2,3]. Nowadays, we know more than 3000 kinds of EOs, about 300 of which are currently used. In traditional agriculture, farmers apply EOs to protect stored grain. EOs are widely utilized as insect repellents, mainly against mosquitoes [3–6]. EOs obtained from lemon and eucalyptus are used as the active substances in non-toxic repellent products that are recommended for children. Moreover, several studies demonstrate that EOs do not only repel the insects but also act on them as neurotoxic compounds [6–18].

It has been proved that EOs from 1500 plant species have insecticidal properties and are efficacious regarding both forms of insects—adults and larvae. For example, eugenol is toxic to a number of insect orders: Coleoptera, Hymenoptera, Isoptera; citral to insect species: *Ceratitis capitata* and *Anastrepha fraterculus*; geraniol to *Aedes aegypti*, *Aedes albopictus*, *Anopheles quadrimaculatus*, thymol to *Culex tritaeniorhynchus*, *Aedes albopictus* and *Anopheles subpictus*. All of them are also toxic to the cockroach *Periplaneta americana* [19–28]. Additionally, extensive research has provided evidence that some EO

components, applied in binary mixture, can exhibit synergistic or antagonistic activity. Such effects suggest diverse mechanisms of action of EO components [29–31].

Eugenol, α-terpineol and L-carvol cause hyperactivity in insects at first. Stretching the legs and numbness precede the insect's death [19–28]. These effects demonstrate the neurotoxic activity of EOs and motivate the investigation of their molecular targets in insect organisms. Understanding the spectrum of action of EOs on insect targets could be crucial for the application of EOs in the development of new, natural insecticides. The aim of this article is to present some identified targets for EOs and shed some light on their mode of action.

2. Essential Oils—Inhibitors of Acetylcholinesterase

A lot of research demonstrates that EOs inhibit the activity of acetylcholinesterase (AChE) (Figure 1) (e.g., [32]), which is one of the most important enzymes in neuro-neuronal and neuromuscular junctions in both insects and mammals [33–35]. Since the insect AChE differs from the mammalian one by a single residue, known as the insect-specific cysteine residue, AChE can be an insect-selective target for the newly developed insecticides, safe for non-target vertebrates [36–40]. Essential oils are estimated to be a potential source of insecticides due to their ability to modifying the insect AChE activity [41–51].

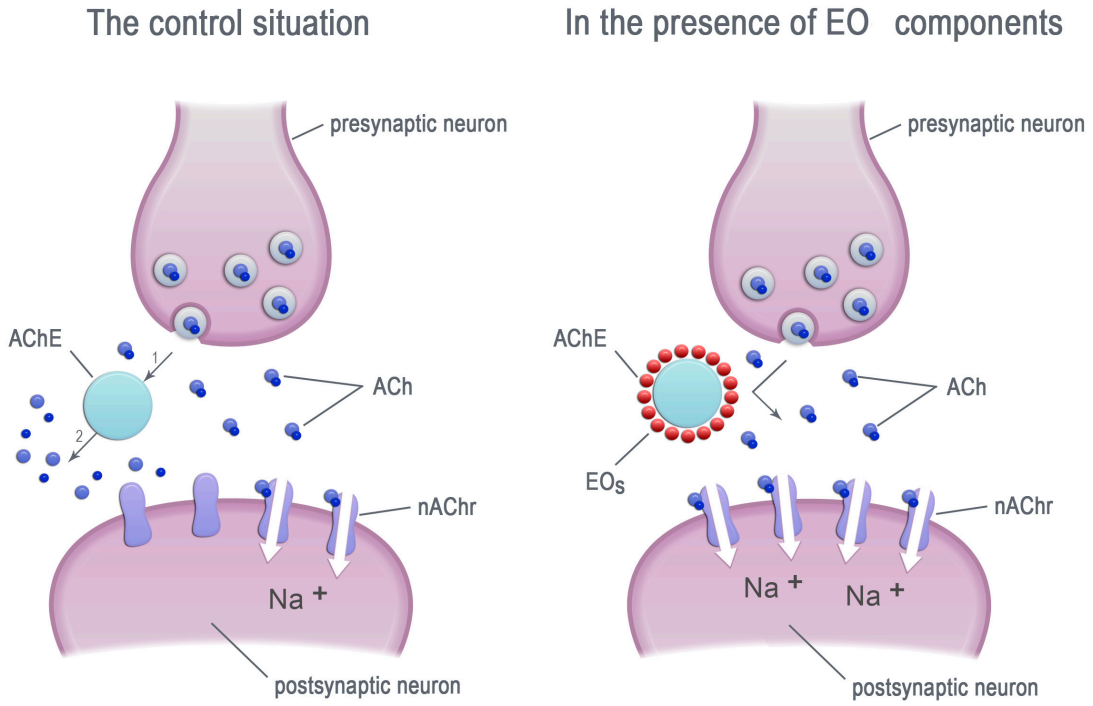

Figure 1. The EO components inhibit the acetylcholinesterase (AChE) activity. ACh—acetylcholinesterase, nAChr—nicotinic acetylcholine receptors, EOs—essential oil components.

It has been demonstrated that EOs from the following plants can inhibit AChE: *Chamaemelum nobile*, *Eriocephalus punctulatus*, *Ormenis multicaulis*, *Santolina chamaecyparissus*, *Cyclotrichium niveum*, *Thymus praecox* subsp. caucasicus var. caucasicus, *Echinacea purpurea*, *Echinacea pallida*, *Salvia chionantha*, *Anethum graveolens*, *Salvia lavendulaefolia* [41–45]. Moreover, the properties of the isolated components of EOs have been examined as well (Table 1, Table A1—Appendix A). Forty-eight of 73 examined substances exhibited an anti-AChE activity. However, the experiments were mainly conducted on the isolated AChE from the electric eel (*Electrophorus electricus*) and from some species of mammals. Only 28 components were tested on insect AChE and 23 of them inhibited the enzyme. The most efficacious of them were: α-pinene and β-pinene, β-phellandrene, carvacrol, limonene, menthol,

menthone, 1,8-cineole, *cis*-ocimene, niloticin [41,47–54]. Most of the EO components displayed anti-AChE activity in mM concentration. Only one study proved the inhibitory effect of EOs component (carvacrol) on AChE in μM concentration [48].

Table 1. The effects of the essential oil components on the acetylcholinesterase activity in insects.

No.	Essential Oils Components	AChE Source	IC_{50} (mM)	Ki (mM)	Reference
1	Anisaldehyde	BxACE-1 from *Bursaphelenchus xylophilus*	4.95		[46]
		BxACE-2 from *Bursaphelenchus xylophilus*	8.53		[46]
		BxACE-3 from *Bursaphelenchus xylophilus*	>50		[46]
2	Camphene	*Blatella germanica*	N.A.		[47]
3	Camphor	*Blatella germanica*	N.A.		[47]
4	3-Carene	BxACE-1 from *Bursaphelenchus xylophilus*	0.37		[46]
		BxACE-2 from *Bursaphelenchus xylophilus*	8.18		[46]
		BxACE-3 from *Bursaphelenchus xylophilus*	>50		[46]
5	Carvacrol	*Musca domestica*	0.0012		[48]
		Dermacentor variabilis	0.0018		[48]
		Periplaneta americana	0.0004		[48]
		Aedes aegypti	0.0012		[48]
		Drosophila suzukii	N.A.		[49]
		Sitophilus oryzae		0.05	[50]
6	Caryophyllene (humulene)	*Blatella germanica*	N.A.		[47]
7	1,8-Cineole	*Pediculus humanus capitis*	77		[51]
		Sitophilus oryzae		0.084	[50]
8	Coniferyl alcohol	BxACE-1 from *Bursaphelenchus xylophilus*	1.06		[46]
		BxACE-2 from *Bursaphelenchus xylophilus*	1.41		[46]
		BxACE-3 from *Bursaphelenchus xylophilus*	1.13		[46]
9	Cymene	*Sitophilus oryzae*		0.05	[50]
		Drosophila suzukii	N.A.		[49]
10	Estragole (Allylanisole)	*Blatella germanica*	N.A.		[47]
11	Eugenol	*Sitophilus oryzae*		0.096	[50]
12	Isoeugenol	*Sitophilus oryzae*		0.11	[50]
13	Isosafrole	*Sitophilus oryzae*		0.71	[50]
14	Limonene	*Sitophilus oryzae*		0.73	[50]
		Reticulitermes speratus Kolbe	0.95		[41]
15	Linalool	*Sitophilus oryzae*	N.A.		[50]
16	Methyleugenol	*Sitophilus oryzae*		0.051	[50]
17	Menthol	*Sitophilus oryzae*		0.048	[50]
		Drosophila suzukii	N.A.		[49]
18	Menthone	*Sitophilus oryzae*		0.39	[50]
		Drosophila suzukii	N.A.		[49]
19	Nerolidol	BxACE-1 from *Bursaphelenchus xylophilus*	9.98		[46]
		BxACE-2 from *Bursaphelenchus xylophilus*	15.28		[46]
		BxACE-3 from *Bursaphelenchus xylophilus*	19.06		[46]
20	Nootkatone	*Musca domestica*	>30		[48]
		Dermacentor variabilis	>30		[48]
		Periplaneta americana	>30		[48]
		Aedes aegypti	>30		[48]
21	Ocimene	Japanese termite	0.96		[52]
		Blatella germanica	N.A.		[47]
22	Perilla aldehyde	*Drosophila suzukii*	3.06		[49]

Table 1. *Cont.*

No.	Essential Oils Components	AChE Source	IC_{50} (mM)	Ki (mM)	Reference
23	Phellandrene	*Reticulitermes speratus* Kolbe	4.92		[41]
		Blatella germanica	2.2		[47]
24	α-Pinene	*Sitophilus oryzae*		0.44	[50]
		BxACE-1 from *Bursaphelenchus xylophilus*	0.24		[46]
		BxACE-2 from *Bursaphelenchus xylophilus*	0.64		[46]
		BxACE-3 from *Bursaphelenchus xylophilus*	0.68		[46]
		Reticulitermes speratus Kolbe	3		[41]
25	β-Pinene	BxACE-1 from *Bursaphelenchus xylophilus*	3.39		[46]
		BxACE-2 from *Bursaphelenchus xylophilus*	18.03		[46]
		BxACE-3 from *Bursaphelenchus xylophilus*	>50		[46]
		Reticulitermes speratus Kolbe	3.08		[41]
		Sitophilus oryzae		0.0028	[50]
26	α-Terpinene	*Sitophilus oryzae*		0.14	[50]
27	α-Terpineol	*Sitophilus oryzae*		3.94	[50]
28	β-Thujone	*Blatella germanica*	N.A.		[47]
29	Thymol	*Sitophilus oryzae*		0.57	[50]
		Drosophila suzukii	4.26		[49]

N.A.—the compound is not active or the inhibition is lower than 50%; IC_{50}—Concentration of component that cause 50% inhibition of enzyme; Ki—inhibitory constant. BxACE-1, BxACE-2 and BxACE-3 are three different acetylcholinesterases found in *Bursaphelenchus xylophilus*. Values in mg/mL were recalculated by the authors of this paper.

To understand the effectiveness of EOs in AChE inhibition we need to consider their exact mode of action and also to recognize the type of inhibition. First of all, knowledge concerning the modification of enzyme kinetics is necessary. In the majority of papers there is no data related to the changes in the AChE kinetics after the EOs' administration. The available research shows that some of the EO constituents function as competitive inhibitors and others as uncompetitive inhibitors (Table 2) [55–60]. It is also difficult to explain the EOs' mode of action because the activity of EOs as complex compounds differs from the activity of their single components. For example, EO from the tea tree (*Melaleuca alternifolia*) is an uncompetitive inhibitor, while its particular components are competitive inhibitors [57]. Such competitive inhibitors attach to the active sites in AChE and prevent the binding of ACh. It causes the decrease in the binding of the neurotransmitter but the maximal activity of the enzyme remains unchanged. On the other hand, the uncompetitive inhibitors bind to other sites of AChE and allosterically alter the action of the enzyme. They bind rather to the enzyme-substrate complex than to the enzyme itself and thus prevent product formation. As a result, the maximum activity of the enzyme decreases. Therefore, different inhibitory action of EOs on the AChE suggests the existence of diverse binding sites in the enzyme molecule.

Table 2. The EO components acting as the competitive and the noncompetitive inhibitors of AChE.

Competitive AChE Inhibitors	Reference	Noncompetitive AChE Inhibitors	Reference
Pulegon	[55]	Gossypol	[55]
Citral	[55]	Carvone	[60]
Linalool	[55]	Camphor	[60]
(−)-Bornyl acetate	[55]		
1,8-Cineol	[55,57,58]		
Terpinen-4-ol	[57]		
Fenchone	[60]		
γ-Terpinene	[60]		
Menthone	[50]		
Menthol	[50]		

The AChE enzyme has a deep "active-site gorge" with two target sites: "catalytic" at the bottom and "peripheral" at the entrance [61]. "Dual binding site" inhibitors interact with the AChE at both the catalytic and the peripheral site [62]. Thus, they can act both as competitive and uncompetitive inhibitors. EO components can act as dual inhibitors if they form a blend. López et al. [60] analyzed the kinetics of the inhibition and the spatial size of terpenoids on their binding capability. They conclude that two monoterpenoids can bind to one AChE molecule at a time. The binding of the first EO component favors the attachment of the second one. Moreover, by using a molecular docking, they demonstrated that some components (carvone and fenchone) can bind to several binding sites in the AChE. In contrast, they found only one binding site for terpinene and camphor.

The data described above may suggest a synergistic action of the EO components. In fact, the majority of essential oils exhibit greater activity than their single components. However, we have found only a few papers where the synergistic action of the EO components was estimated using statistical analysis of interaction. Savalev et al. [63] have proposed synergism between 1,8-cineole and α-pinene. They obtained similar results for 1,8-cineole and caryophyllene oxide. On the other hand, an antagonistic interaction was found between 1,8-cineole and camphor. Miyazawa et al. [64] also observed antagonism between some EO components. They compared the inhibitory effect of the natural EOs extracted from the plant with the sum of the inhibitory effectiveness of the major single components and with the "artificial" mixture of them. EOs exhibited the highest inhibition (46%), the sum of the inhibitory action of the EO components was lower (29.5%) and the "artificial" mixture of the EO components inhibited the AChE only by 19%. On the other hand, the study by Jukic et al. [65] showed that thyme EO exhibited less activity than its single components. Certainly, the positive or the negative interaction between the EO components depends on their relative quantity.

The structure-activity relationships for EOs are also unclear. It is difficult to define which chemical type of EO compounds is more active. Lee et al. [50] have suggested that monoterpenoid ketones are more active than alcohols or aldehydes. However, among 6 inactive compounds, two were ketones. In the same study, menthone (ketone) has a lower inhibitory activity than others. Moreover, among active compounds, two were phenolic alcohols. Certainly, it would be necessary to identify other features of chemical structure (e.g., double bond in phenolic ring) to determine the activity of the EO constituents. López et al. [60] have found a correlation between the size of the tested components and their inhibitory activity on the AChE. The substance with higher spatial size exhibited higher activity. Reegan et al. [53] performed a molecular docking of niloticin (large spatial size terpenoid) to the AChE of *Aedes aegypti*. They showed a high binding affinity of niloticin to the AChE and determined the binding residues as THR'58 and HIS'62. However, Dambolena et al. [66] provided mathematical analysis of factors affecting insecticidal activity of EO components and they discovered that compounds with lower molar volume and fewer rings are more active.

To sum up, the study on EOs as AChE inhibitors showed that monoterpenoids appeared to be weak AChE inhibitors. The inhibition of AChE requires mM concentrations of EOs [48] while usually neurotoxic symptoms of EOs are visible at their concentrations smaller by 3 orders of magnitude. Additionally, the inhibition of the AChE is always fast reversible [67,68]. Moreover, the chemicals (e.g., carvone) in one study caused the inhibition of AChE but in another study, using the same AChE, no activity was shown, so the results are not reproducible. Thus, AChE inhibition does not seem to be the primary neurotoxic action of EOs, however, some of the large sized EO chemicals can be consider as AChE inhibitors.

3. Essential Oils—Modifiers of GABA Receptors

3.1. Mammalian GABAA Receptors

Gamma-amminobutyric acid (GABA) is the major inhibitory neurotransmitter in the nervous system and the muscles in both mammals and insects (however in some cases it can play a role of excitatory neurotransmitter). It binds to specific receptors (GABArs) in synaptic or extrasynaptic

membranes [69–72]. In mammals there are two types of GABA receptors: ionotropic ($GABA_A$rs) and metabotropic ($GABA_B$rs) [73,74].

Many papers report essential oils action on the GABArs, primarily belonging to the ionotropic receptor group [75]. Studies that proved the influence of essential oils on the GABArs were conducted mainly on mammals. According to a great deal of data, EOs and their components are mostly positive modulators of the $GABA_A$ receptors (Figure 2). Menthol, thymol and other components increase the Cl^- current induced by the GABA neurotransmitter (Table 3) [76–82]. Such a situation occurs in low (μM) concentrations of EOs. Additionally, some of the EO constituents induce a weak Cl^- current themselves when applied at a concentration near 1 mM [75,76,78,80]. Higher concentrations of previously mentioned EOs do not exert any effects on $GABA_A$rs probably because of the desensitization of the receptors [82]. There are also EO components that do not induce any effect on the $GABA_A$rs Cl^- current, for example: camphor, carvone, menthon [76], linalool and α-terpineol [83].

The effect of the EO components on the GABA receptors depends on their chemical structure. Different EO stereoisomers vary in their potency to modulate the GABA receptors: (+)-menthol and (+)-borneol have higher activity than (−)-menthol and (−)-borneol [83,84]. The presence of a functional group is important as well. Alcohols have a stronger modulatory effect on the $GABA_A$rs (e.g., thymol, menthol, borneol)—than ketones—(linalool, α-terpineol) [76,83].

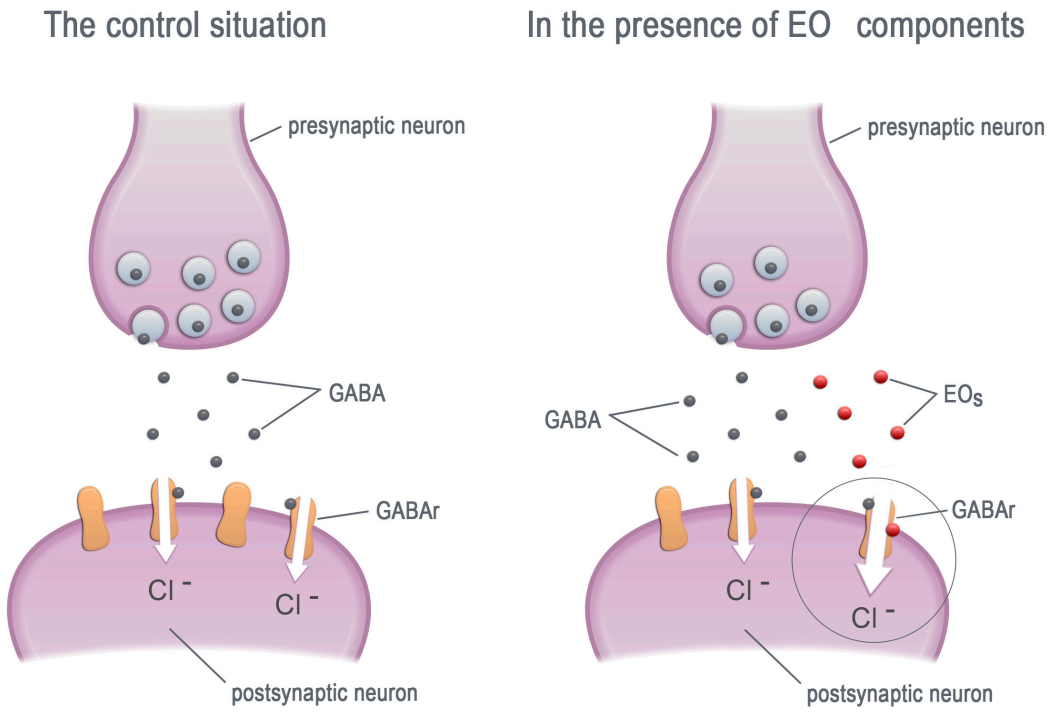

Figure 2. The EO components increase the chloride current by allosteric modulation of the GABA receptors. GABA—γ-aminobutyric acid, GABAr—GABA receptors, EOs—essential oil components.

Table 3. The intensification of the GABA-induced Cl^- current by EOs.

EO Components	Concentration of EOs Component (mM)	Change of GABA Current	Type of Receptor or Source of Receptor	Literature
(−)-Borneol	0.3	350%	α1β2γ2s $GABA_A$	[76]
Camphor	0.3	40% (inhibition)	α1β2γ2s $GABA_A$	[76]
Carvone	0.3	115%	α1β2γ2s $GABA_A$	[76]
cis-Jasmone	1	250%	Bovine $GABA_A$	[79]
Geraniol	1	500%	α1β1$GABA_A$	[79]
(+)-Isomenthol	1	327%	α1β2γ2s $GABA_A$	[78]
(+)-Isopulegol	0.3	380%	α1β2γ2s $GABA_A$	[78]
Linalool	1	350%	α1β1$GABA_A$	[79]
Nerolidol	1	150%	α1β1$GABA_A$	[79]
Menthol	0.32	200%	α1β2γ2s $GABA_A$	[78]
(+)-Menthol	0.1	596%	α1β2γ2s $GABA_A$	[76]
(−)-Menthol	0.3	600%	α1β2γ2s $GABA_A$	[76]
(−)-Menthone	0.3	150%	α1β2γ2s $GABA_A$	[76]
Methyleugenol	0.03	280%	hippocampal neurons	[80]
Methyl jasmonate	1	230%	Bovine $GABA_A$	[79]
α-Terpineol	1	299%	α1β2γ2s $GABA_A$	[78]
α/β-Thujone	0.3	40% (inhibition)	α1β2γ2s $GABA_A$	[76]
	0.1	715%	*Drosophila melanogaster* homomeric RDL_{ac} GABAr	[76]
α-Thujone	0.00066	208%	Rat $GABA_A$	[81]
	0.003	70% (inhibition)	rat dorsal root ganglion neurons	[82]
Thymol	0.1	416%	α1β3γ2s $GABA_A$	[75]
	0.01	150%	α1β1γ2s $GABA_A$	[75]

Values in mg/mL were recalculated by the authors of this paper.

Many studies have been performed to define the binding sites for the EO components in the GABArs [76,78,85]. However, such experiments are rather difficult to carry out in natural, neuronal membranes, because EOs are lipophilic substances and they can nonspecifically affect cellular membranes: they can increase the membrane permeability or cause damage [86]. The majority of data concerning EOs binding to the $GABA_A$rs was obtained using competitive studies with already known $GABA_A$rs ligands. Such experiments can only provide indirect evidences for the existence of binding sites for the EO components in the GABArs and should be complemented by more direct methods. Recent knowledge concerning the EO binding sites in the GABArs is presented below.

Although EOs do not compete with the GABA site antagonists [80], in mM concentrations EOs can cause weak Cl^- currents inhibited by bicuculline (a competitive antagonist of the $GABA_A$rs) [84].

The EO components do not bind to the benzodiazepine site despite the fact that the action of EOs is similar to the action of the benzodiazepines. Watt et al. [78] and Granger et al. [84] have shown that flumazenil (a benzodiazepine site antagonist) did not eliminate the potentiation of a Cl^- current induced by menthol and borneol. However, Sánchez-Borzone et al. [87] have observed that carvone can allosterically modify the flunitrazepam binding to the benzodiazepine site.

Moreover, the EO components do not bind to the picrotoxin site. If they did bind to the picrotoxin site, they would have induced the inhibition of the GABA-induced current—but such an effect was not observed. Additionally, picrotoxin completely inhibits the GABA-induced currents modulated by borneol [80,84]. EOs are also not competitive for the radioligand [^{3}H]-TBOB (non-competitive channel blocker), in contrast to all ligands of the picrotoxin site [83].

It was proposed that EOs bind to the $GABA_A$rs anesthetic site. The EO components (e.g., menthol, borneol or geraniol) are structurally similar to a known ligand of the anesthetic site—propofol. Both propofol and menthol are cyclic molecules containing the hydroxyl group. Borneol and geraniol have a similar structure to propofol as well. Moreover, the action of EOs and propofol is similar—they potentiate the GABA induced Cl^- current. Propofol itself (in μM concentration) can also cause currents via the $GABA_A$rs. In contrast, such a current was observed only after much higher (mM) concentrations of menthol. However, menthol competed with propofol and significantly decreased the propofol-induced current [78]. It was proposed that propofol binds to the $GABA_A$rs between β+-β−

and β+-α− subunits [88]. Additionally, propofol can bind to another β subunits combination but only at a 10× higher concentration [89]. Amino acid residues crucial for the propofol binding are located in positions: 265, 236, 296, 286, 444. These residues are also proposed as amino-acid residues participating in EOs binding [78,88–90].

To summarise, the EO components most probably share a binding site with propofol in the GABArs of mammals. A similar action of these compounds was proved in behavioral experiments. Both EOs and propofol cause sedation of a mouse (*Mus musculus*) [91] and a silver catfish (*Rhamdia quelen*) [92].

3.2. Insect GABA Receptors

The insect GABArs are related to vertebrate ionotropic GABArs. Similarly to vertebrates, in the insect nervous system GABArs mainly mediate the inhibitory effect on neurotransmission. However, there are several structural and pharmacological differences between the mammalian and the insect GABArs and thus the insect GABArs can be a very promising target for the development of new insecticides. The insect GABArs display features of both mammalian $GABA_A$ and $GABA_C$ receptors. The level of similarity between the insect and the mammalian GABArs is the same as between the insect GABArs and the insect nicotinic receptors. The insect GABArs are similar (85–99%) in different orders of insects. Three kinds of subunits were identified in the insect GABArs: RDL (resistant to dieldrin), GRD (GABA and glycine-like receptor of *Drosophila*) and LCCH3 (ligand gated chloride channel homologue 3). Among the insect GABArs subunits LCCH3 is the most similar to the mammalian GABArs—precisely to the β3 subunit of the $GABA_A$rs. The resemblance in the amino acid sequence between the LCCH3 and the $GABA_A$ β3 subunit amounts to 50% [93]. However, the presence of the LCCH3 subunit in insects is time- and tissue-limited. Experiments on *Drosophila melanogaster* showed that LCCH3 is located in cell bodies of the embryonic nerve cord and brain, in neuronal cell bodies surrounding the adult brain and in the olfactory system [94,95]. In contrast to the mammalian $GABA_A$rs, the majority of the insect GABArs is insensitive to bicuculline and, differently than the subclass $GABA_C$rs, they can be allostericaly modified by benzodiazepines and barbiturates [69].

Homomeric GABA receptors composed of the RDL subunits are accepted as a model to study the physiology and pharmacology of the insect GABArs because they are blocked by picrotoxin and they are insensitive to bicuculline [96,97]. The insect GABArs are targets for several chemical insecticides such as dieldrin, fipronil, insane, BIDN (bicyclic dinitrile convulsant). All of them act as antagonists of the GABArs and induce inhibition or overexcitation of the insect nervous system [96–100].

The efficacy of essential oils as insecticides was presented in many publications [101–103] although, the data concerning the effects of EOs on the insect GABArs are very limited. The research on RDL receptors has shown that thymol caused strong potentiation of the Cl^- current evoked by GABA. Moreover, thymol alone can induce a small current as well [75]. In addition, thymol, carvacrol and pulegone enhanced the binding of [^{3}H]-TBOB to membranes of the insect's neuronal cells. These monoterpenoids also increased the GABA-induced Cl^- uptake in the insect membrane preparations. It was proposed that these EO components are positive allosteric modulators of the insect GABA receptors [83]. It is supported by research by Waliwitiya et al. [104], who observed thymol induced reduction of flight muscle frequency at *Phaenicia sericata*, which was comparable to GABA effect. Anyway, EO action on the insect GABArs needs further studies.

4. Essential Oils—Ligands of Octopamine Receptors

Octopamine (OA) is an invertebrate multifunctional molecule, structurally and physiologically related to vertebrates noradrenaline. It has been found that it can act as a neurotransmitter, as a neurohormone and as a neuromodulator [105–107]. OA is present in the nervous system, neuroendocrine cells and hemolymph [108]. It is involved in the regulation of different forms of insect activity e.g., arousal level. It also plays an essential role in the insect stress response, aggressive behavior and social behavior [109–111]. Modern molecular biology techniques have made it possible to follow in detail the role of OA in the insect organism. OA binds to specific G protein-coupled

membrane receptors (OAr). The binding of OA to these receptors leads (via G protein) to the activation of the enzyme adenylyl cyclase. It transforms ATP to cAMP and causes an increase in the cAMP level, which is a signaling molecule, activating the protein kinase A (PKA). G protein also activates phospholipase C. It leads to the release of calcium from deposits in the endoplasmic reticulum and to the elevation of its intracellular level as well as to the activation of the calcium-dependent protein kinase C (PKC). Protein kinases phosphorylate a number of enzymes and receptors, which, lead to the modulation of their activity. This results in important changes in cell functions [112].

Three subclasses of OAr have been distinguished—depending on the kind of the G protein-coupled. Moreover, there are two kinds of receptors for which tyramine (TA—a precursor of OA) is a ligand [113]:

- α-adrenergic-like—the binding of OA to these receptors increases the level of the intracellular calcium; the secondary effect is an increase of the cAMP level;
- β-adrenergic-like—the binding of OA to these receptors increases the level of cAMP;
- octopamine/tyramine—the receptors are similar to α2—an adrenergic receptor in mammals. It is sensitive both to OA and TA. TA binding to this receptor causes a decrease in the cAMP level. In contrast, OA binding to the receptor causes an increase in the cAMP level;
- two classes of receptors for TA only: the activation of TyrR II causes an increase of the intracellular calcium level, the activation of TyrR III induces the increase of the calcium level and the decrease of the cAMP level [114–116].

In several papers the authors have demonstrated that EOs act in a similar way to OA (Figure 3). Eugenol, α-terpineol and their mixture with cinnamyl alcohol induced an increase in the cAMP level. However, at higher concentrations geraniol and citral decreased the cAMP level. The same EOs reduced the binding of [^{3}H]-OA to receptors [117]. Interestingly, cinnamic alcohol itself increased OA level over 20 times in *Blatella germanica* [118]. Price and Berry [28] have examined the effect of EOs on the bioelectrical activity of the cockroach (*Periplaneta americana*) ventral nerve cord and the functions of DUM neurons (dorsal unpaired median neurons) in the terminal abdominal ganglion. Geraniol and citral at low concentrations (μM) increased the spontaneous firing rate in the DUM neurons and in the nerve cord. Similar effects were observed after the OA application. However, in higher concentrations (mM) these compounds decreased the activity of the DUM neurons and the nerve cord as well. Eugenol reduced the activity of the DUM neurons and the nerve cord. The depressive effects of high concentrations of EOs may be explained by the destructive influence of EOs on neuronal membranes. A study performed by Enan [117] demonstrated that eugenol, cinnamyl alcohol, 2-phenethyl propionate and *trans*-anethole exert their toxic effects via OArs. EO components (eugenol, *trans*-anethole and 2-phenethyl propionate) increased Ca^{2+} concentrations in HEK-293 cells expressing OArs from cockroach *P. americana* and *D. melanogaster*. However, *trans*-anethole increased and eugenol decreased the cAMP level in these cells. All three of these EO components significantly decreased the binding of [^{3}H]-yohimbine (ligand of OArs). Kostyukovsky et al. [119] have shown that the EO component SEM-76 caused an increase in the cAMP level, in a similar way to OA. In addition, phentolamine (OArs antagonist) abolished SEM-76-induced changes in the concentration of cAMP.

The effect of EOs was also tested on *P. americana* tyramine receptors (TArs). Thymol, carvacrol and terpineol inhibited the binding of [^{3}H]-TA to membranes of S2 cells expressing TArs. Moreover, these EOs changed the cAMP level in S2 cells. The effects of EOs were observed in the μM concentrations corresponding to the physiological ligands activity [120].

All the presented data provides convincing arguments that the EO components interact with OA and TA receptors. They act mainly as agonists of these receptors. Importantly, EOs can be considered as agonists of all types of OArs and TArs. They cause an increase in both the cAMP level and in the intracellular Ca^{2+} level. Thus, they can induce the activation of kinases PKA and PKC and phosphorylation of many proteins (including ion channels, enzymes and receptors) [121]. The presence of OA in mammals is minor and no OArs was found in mammals (nevertheless, it should be taken into

account that OA is prohibited in sport owing to its stimulating properties) [112]. The effects of essential oils components on octopamine receptors specific to insects lead to the conclusion that essential oils represent a very interesting source of molecules for designing the insect pest control.

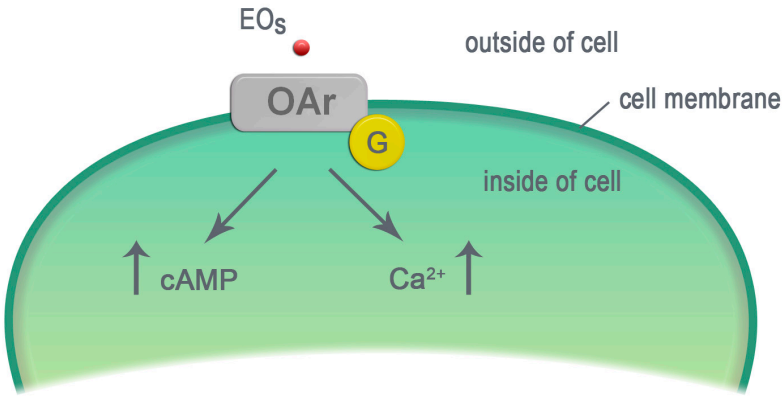

Figure 3. The EO components activate the octopamine receptors. EOs—essential oil components, OAr—octopamine receptor, G—protein G, cAMP—cyclic adenosine monophosphate, Ca^{2+}—calcium ions, ↑—increase in the molecule level.

5. Conclusions

Studies of neurotoxic effects of essential oils allowed their molecular targets to be determined: acetylcholinesterase enzymes, ionotropic GABA receptors and metabotropic octopamine receptors. The most evident proof concerns the effect of EOs on octopamine receptors, which are specific for invertebrates including insects. This fact strongly motivates future studies on EOs as bioinsecticides.

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Appendix A

Table A1. The effects of the essential oil components on the acetylcholinesterase activity in organisms other than insects.

No.	Essential Oil Components	AChE Source	IC_{50} (mM)	Ki (mM)	Reference
1	Anisaldehyde	Electric eel	N.A.		[122]
2	Anisole	Human erythrocyte	N.A.		[123]
3	Anethole	Electric eel	N.A.		[124]
		Electric eel			[67]
		Electric eel	0.88		[125]
		Electric eel	8.9		[126]
		Bovine erythrocyte	0.2		[127]
		Electric eel	N.A.		[122]
		Electric eel	N.A.		[128]
		Electric eel	0.87		[126]

Table A1. *Cont.*

No.	Essential Oil Components	AChE Source	IC_{50} (mM)	Ki (mM)	Reference
4	Borneol	Bovine erythrocyte	N.A.		[64]
		Human erythrocyte	N.A.		[68]
		Bovine erythrocyte	N.A.		[63]
		Electric eel	N.A.		[122]
		Human erythrocyte	N.A.		[123]
		Electric eel	N.A.		[124]
5	Bornyl acetate	Electric eel		21.3	[55]
		Human erythrocyte	N.A.		[68]
		Bovine erythrocyte	N.A.		[63]
6	Camphene	Electric eel	N.A.		[122]
7	Camphor	Electric eel	0.05		[67]
		Electric eel	N.A		[124]
		Electric eel	11.2		[60]
		Human erythrocyte	N.A.		[68]
		Bovine erythrocyte	N.A.		[63]
		Electric eel	N.A.		[122]
		Human erythrocyte	N.A.		[123]
8	2-Carene	Bovine erythrocyte	0.9		[58]
9	3-Carene	Human erythrocyte	0.2		[68]
		Bovine erythrocyte	0.2		[58]
		Electric eel	0.26		[126]
10	Carvacrol	Electric eel	0.41		[65]
		Electric eel	0.61		[126]
		Electric eel	0.21		[122]
		Electric eel	0.76		[128]
11	Carvone	Electric eel	0.3		[67]
		Bovine erythrocyte	N.A.		[64]
		Electric eel	N.A.		[122]
		Electric eel	5.5		[60]
12	Caryophyllene (humulene)	Human erythrocyte	N.A.		[68]
		Bovine erythrocyte	0.13		[68]
		Electric eel	N.A.		[124]
		Human erythrocyte	N.A.		[68]
		Electric eel	N.A.		[129]
13	Caryophyllene oxide	Human erythrocyte	N.A.		[68]
		Bovine erythrocyte	N.A.		[63]
14	1,8-Cineole	Electric eel		0.025	[55]
		Electric eel	0.1		[124]
		Electric eel	0.71		[126]
		Bovine erythrocyte	0.26		[64]
		Electric eel	0.6		[51]
		Electric eel	0.84		[122]
		Human erythrocyte	0.4		[68]
		Electric eel	0.04	0.03	[57]
		Bovine erythrocyte	0.29	0.1	[58]
		Bovine erythrocyte	0.39		[63]
		Human erythrocyte	0.67		[130]
15	Cinnamaldehyde	Electric eel	N.A.		[122]
16	Cinnamyl alcohol	Electric eel	N.A.		[122]
17	Citral	Electric eel		7	[55]
		Electric eel	N.A.		[124]
		Electric eel	N.A.		[122]

Table A1. *Cont.*

No.	Essential Oil Components	AChE Source	IC_{50} (mM)	Ki (mM)	Reference
18	Citronellal	Electric eel	N.A.		[122]
19	Citronellol	Electric eel	N.A.		[122]
20	Copaene	Human erythrocyte	N.A.		[68]
21	Cymene	Bovine erythrocyte	N.A.		[58]
22	Elemol	Bovine erythrocyte	0.16		[64]
23	Estragole (Allylanisole)	Electric eel	0.15		[67]
		Electric eel	12.6		[60]
		Electric eel	N.A.		[124]
		Electric eel	N.A.		[122]
24	Eugenol	Electric eel	2.9		[124]
		Electric eel	N.A.		[122]
		Human erythrocyte	N.A.		[123]
25	Fenchone	Electric eel	0.4		[67]
		Electric eel	7		[60]
26	Geraniol	Electric eel	0.1		[67]
		Electric eel	15		[60]
		Electric eel	N.A.		[122]
27	Globulol	Human erythrocyte	N.A.		[68]
28	Gossypol	Electric eel	1.5		[55]
29	Guaiol	Human erythrocyte	N.A.		[68]
30	Isoeugenol	Electric eel	N.A.		[122]
31	Limonene	Electric eel	N.A.		[124]
		Human erythrocyte	N.A.		[68]
		Electric eel	1.61		[125]
		Electric eel	4.33		[126]
		Electric eel	N.A.		[122]
		Bovine erythrocyte	N.A.		[64]
32	Linalool	Electric eel	0.3		[67]
		Electric eel		5.5	[55]
		Electric eel	N.A.		[124]
		Electric eel	15.6		[60]
		Electric eel	N.A.		[122]
		Human erythrocyte	N.A.		[68]
		Bovine erythrocyte	N.A.		[63]
		Bovine erythrocyte	N.A.		[64]
33	Linalyl acetate	Bovine erythrocyte	N.A.		[64]
		Electric eel	N.A.		[129]
34	Manool	Human erythrocyte	N.A.		[68]
35	Methylcinnamate	Electric eel	N.A.		[122]
36	Methyleugenol	Electric eel	N.A.		[122]
		Electric eel	N.A.		[124]
37	Menthofuran	Bovine erythrocyte	N.A.		[64]
38	Menthol	Bovine erythrocyte	N.A.		[64]
39	Menthone	Bovine erythrocyte	N.A.		[64]
		Electric eel	N.A.		[122]
40	Methol	Human erythrocyte	N.A.		[123]
41	Methoxycinnamaldehyde	Electric eel	N.A.		[124]

Table A1. *Cont.*

No.	Essential Oil Components	AChE Source	IC_{50} (mM)	Ki (mM)	Reference
42	Methyl acetate	Bovine erythrocyte	N.A.		[64]
43	Myrcene	Electric eel	N.A.		[122]
44	Myrtenal	Electric eel	0.17		[122]
45	Nerol	Electric eel	N.A.		[122]
46	Nerolidol	Electric eel	N.A.		[122]
47	Neryl acetate	Human erythrocyte	N.A.		[68]
48	Phellandrene	Electric eel	0.88		[129]
49	Phenylethanol	Electric eel	N.A.		[122]
50	α-Pinene	Electric eel	0.16		[124]
		Electric eel	10.5		[130]
		Human erythrocyte	0.7		[68]
		Human erythrocyte	0.63		[131]
		Bovine erythrocytes	0.66		[63]
		Electric eel	N.A.		[122]
		Bovine erythrocytes	0.4		[58]
51	β-Pinene	Electric eel	N.A.		[124]
		Human erythrocyte	1.5		[68]
		Bovine erythrocyte	1.5		[63]
		Electric eel	N.A.		[122]
52	Piperitenone oxide	Bovine erythrocyte	0.38		[64]
53	Piperitenone	Bovine erythrocyte	0.72		[64]
		Bovine erythrocyte	0.83		[64]
54	Pulegone	Electric eel		0.85	[55]
		Bovine erythrocyte	0.89		[64]
55	Sabinene	Human erythrocyte	N.A.		[68]
		Electric eel	1.25		[125]
56	Sclareol	Human erythrocyte	N.A.		[68]
57	α-Terpinene	Bovine erythrocyte	N.A.		[58]
58	γ-Terpinene	Electric eel	0.2		[67]
		Electric eel	N.A.		[124]
		Electric eel	5.8		[60]
		Bovine erythrocyte	N.A.		[58]
59	α-Terpineol	Electric eel	8.43		[124]
		Human erythrocyte	N.A.		[68]
60	Terpinen-4-ol	Electric eel	20.7		[124]
		Electric eel	10.30	4.7	[57]
		Bovine erythrocyte	N.A.	2	[58]
		Electric eel	N.A.		[129]
		Electric eel	N.A.		[122]
61	Terpinolene	Electric eel	1.1		[129]
62	α-Thujone	Human erythrocyte	N.A.		[68]
		Electric eel	N.A.		[122]
63	Thymohydroquinone	Electric eel	0.24		[65]
64	Thymol	Electric eel	4.9		[65]
		Electric eel	1.39		[126]
		Electric eel	N.A.		[122]

Table A1. *Cont.*

No.	Essential Oil Components	AChE Source	IC_{50} (mM)	Ki (mM)	Reference
65	Thymoquinone	Electric eel	0.85		[65]
66	Viridiflorol	Bovine erythrocyte	0.11		[64]
67	Verbenone	Electric eel Electric eel	2.66 0.73		[122] [128]

N.A.—the compound is not active or the inhibition is lower than 50%; IC_{50}—Concentration of component that cause 50% inhibition of enzyme; Ki—inhibitory constant. Values in mg/mL were recalculated by the authors of this paper.

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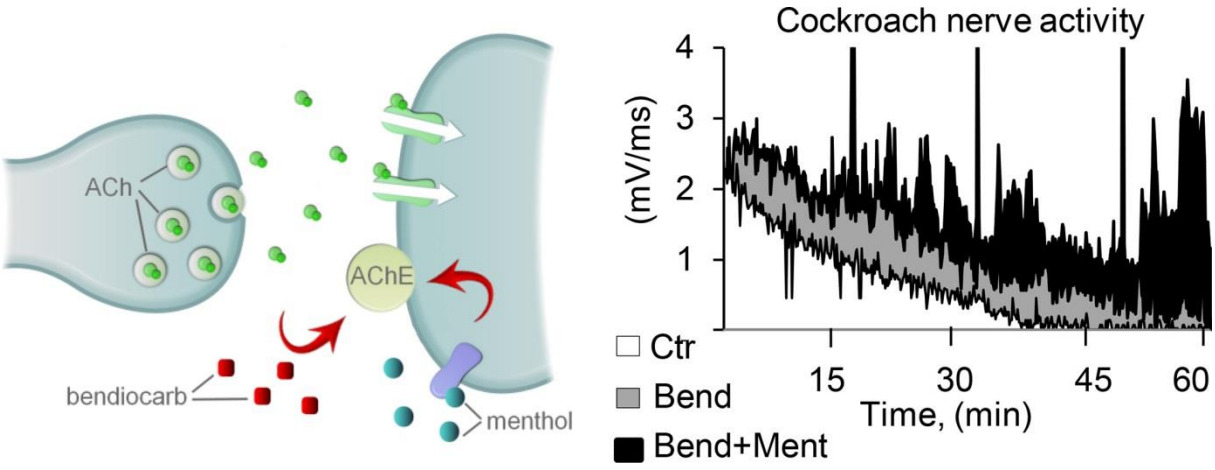
4. Article II

The unusual action of essential oil component, menthol, in potentiating the effect of the carbamate insecticide, bendiocarb



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The unusual action of essential oil component, menthol, in potentiating the effect of the carbamate insecticide, bendiocarb

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ABSTRACT

Standard chemical insecticides present mainly neurotoxic effects and are becoming less and less effective due to insects developing resistance to them. One of the innovative strategies to control insects pests is to find a way to increase the sensitivity of the target sites in the insect nervous system to the applied insecticides. In the presented research, we proposed menthol, a component of essential oils, as a factor increasing the effectiveness of bendiocarb, a carbamate insecticide. The aim of our study was to evaluate the potentiation of the bendiocarb effect by menthol. In toxicity tests performed on *Periplaneta americana*, menthol (0.1 μM) accelerated the lethal effect of bendiocarb, primarily in its low concentrations (lower than 0.05 mM). In the presence of menthol (1 and 0.1 μM), the ability of insects to turn back from its dorsal to the normal ventral side was significantly lower than with bendiocarb (1 μM) alone. We also evaluated the effectiveness of chemicals on the activity of the ventral nerve cord of the cockroach. In this preparation, bendiocarb (1 μM and higher concentrations) caused an irregular, spontaneous bursts of action potentials. The total nerve activity (including the response to stimulation and spontaneous firing) was much higher when bendiocarb was applied in the presence of menthol (1 μM). The effect of menthol was similar to the octopamine effect and was abolished by phentolamine, the octopamine receptor antagonist. Our results clearly indicated a strengthening effect of menthol on bendiocarb effectiveness; potentiation occurred through octopamine receptors activation.

1. Introduction

Insects are the most populous group in the animal kingdom and form a very important component of the ecosystem. However, some insects are harmful to human interest, transferring dangerous pathogens as well as causing damages to crops and stored food. Since the 1940's, people have fought against insect pests using chemical insecticides (Casida and Durkin, 2013). Although they are effective, they can have several undesirable consequences, such as negative impacts on human health (Ding et al., 2015; Schinasi et al., 2015) and toxicity to non-target animals (Antwi and Reddy, 2015; Pisa et al., 2015). Moreover, they have become less and less effective due to insects developing some resistance to insecticides (Buckingham et al., 2017; Dong, 2007; ffrench-Constant et al., 2004; Ihara et al., 2017; Perry et al., 2011).

One of the latest, innovative strategies to control insect pest is the co-application of chemical insecticides with activators of membrane metabotropic receptors such as cholinergic muscarinic receptors (Abd-Ella et al., 2015). The activation of intracellular pathways leads to an increase in intracellular Ca^{2+} concentration and the calcium-dependent phosphorylation/dephosphorylation of membrane functional proteins that modify their sensitivity to ligands (Apaire-Marchais et al., 2016; Lavialle-Defaix et al., 2010). These phenomena are considered important cellular mechanisms that are to be used to increase the sensitivity of insects to insecticides (Lapied et al., 2009; Raymond et al., 2017).

Octopamine is a vital regulatory biogenic amine in invertebrates and acts through metabotropic octopamine G-protein-coupled receptors (Beggs et al., 2011; Evans and Maqueira, 2005). It has been demonstrated that the activation of the octopamine receptors lead to the modification of proteins that can serve as targets for chemical insecticides (Leyton et al., 2014; Reale et al., 1986; Vehovszky et al., 2004, 2005). In experiments performed on mosquitoes, positive synergistic effects have been demonstrated between the activators of the octopamine receptors (formamidines) and different insecticide groups

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; cAMP, 3′,5′-cyclic adenosine monophosphate; ED, effective dose; EO, essential oil; GABA, gamma-aminobutyric acid; nAChR, acetylcholine nicotinic receptor; PKC, protein kinase C; TAG, terminal abdominal ganglion

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(pyrethroids, neonicotinoids, organophosphorus and insect growth regulators) (Ahmed and Matsumura, 2012; Ahmed and Vogel, 2015, 2016).

The octopamine receptors are one of the targets of the EO[1] components (Enan, 2001; Jankowska et al., 2017; Kostyukovsky et al., 2002). They cause a decrease in the binding of octopamine to its receptors (Enan, 2001). The EO components induce an increase in the cAMP[2] level in the epidermal tissue of the cotton bollworm which is inhibited by phentolamine (Kostyukovsky et al., 2002). These EO components mimic the octopamine effect on the firing rate in the nerve cord of the cockroach (Price and Berry, 2006). They also increase Ca^{2+} concentrations in HEK-293 cells expressing octopamine receptors from the cockroach and the fruit fly (Enan, 2005).

Essential oils and their components have become objects of increasing interest in integrative pest management. They exhibit an insecticidal and repellent activity and are increasingly being proposed as an alternative to synthetic insecticides (Pavela, 2016; Pavela and Benelli, 2016). However, they are usually less effective and thus cannot replace the chemicals alone in insect control. Different sites of action of essential oils in the insect nervous system are described: the inhibition of acetylcholinesterase, the positive modulation of the GABA[3] receptors, and as stated above, the activation of the octopamine receptors (Enan, 2001; Jankowska et al., 2017; Park et al., 2016; Tong and Coats, 2010). Menthol has been shown to act as a positive modulator of GABA receptors in mammals as well as an inhibitor of *Sitophilus oryzae* AChE[4] (Lee et al., 2001; Hall et al., 2004).

In our research, we hypothesise that menthol, acting through octopamine receptors, will enhance the efficiency of the carbamate insecticide - bendiocarb. Carbamates inhibit acetylcholinesterase enzyme in the insect nervous system (Casida and Durkin, 2013) and their efficiency has already been shown to be changed by the activation of the metabotropic muscarinic receptors (Abd-Ella et al., 2015).

In electrophysiological experiments we have shown the high similarity in the action of menthol and octopamine which indicates that menthol, like other active constituents of essential oils (Kostyukovsky et al., 2002), activates the octopaminergic receptors. In toxicity tests on cockroach, as well as in experiments on its nervous system, we demonstrated for the first time the potentiating effect of menthol on the efficacy of a carbamate insecticide.

2. Methods

2.1. Insects and reagents

2.1.1. Insects

Experiments were performed on adult male cockroaches (*Periplaneta americana*); obtained from own breeding. We always use only males because they have a more stable physiology than females (no changes related to reproduction). In addition, dissection of the nervous system of male cockroaches for electrophysiological experiments is easier. The animals were kept at 29 °C and fed oat flakes and cat chow with access to water *ad libitum*. The insects were moved from breeding cages in plastic boxes to the laboratory 24 h before the experiments to allow them to adapt to the new conditions.

2.1.2. Reagents

Bendiocarb insecticide was dissolved in ethanol to the concentration of 0.1 M since its solubility in water is low. Serial dilutions of 1 mM, and subsequently 500, 200, 100, 50, 10, 1, 0.1 μM were then made in water (for toxicity tests) or in physiological saline (for electrophysiological experiments). Physiological saline contained in mM: NaCl – 210, KCl – 3.1, $CaCl_2$–5, $MgCl_2$–5.4, and Hepes – 5. The pH = 7.2 was adjusted with NaOH. Menthol was dissolved in ethanol to the concentration of 0.1 M, then it was diluted in water or in physiological saline to the concentration of 1 μM and 0.1 μM. Bendiocarb (Pestanal, analytical standard), DL-octopamine hydrochloride, Phentolamine hydrochloride (≥98.0% (TLC), (±)-Menthol (racemic ≥98.0%), and Hepes were purchased from Sigma Aldrich. The physiological saline components — NaCl, KCl, $MgCl_2$, $CaCl_2$) — were obtained from Polskie Odczynniki Chemiczne. SA. Poland.

2.2. Toxicity tests

2.2.1. Assessment of motor skills

Bendiocarb as an acetylcholinesterase inhibitor causes hyperactivity and then paralysis of insects. In our toxicity tests, we estimated the level of cockroach paralysis induced by bendiocarb, menthol and bendiocarb and menthol applied together. Each cockroach was placed on the dorsal side (on cork arena) and its ability to turn back to its normal position was estimated by measuring the time necessary to return to the ventral side. We assumed that the more affected insects would need more time to turn back.

A group of five insects were placed in a 0.665 l non-absorbing, glass chamber (11 cm diameter, 7 cm height) and exposed to chemicals for 1 h. 1 ml of menthol (in the concentrations of 1 μM and 0.1 μM) was administered into a filter paper, then the paper was placed directly under the chamber lid. The volatile fraction of terpenoid is heavier than air so its vapor quickly reached the insects at the bottom of the chamber. 0.5 ml of bendiocarb – (in the concentrations of 0.1 mM and 1 μM) was dispensed in a form of spray with a glass atomizer directly into the insects in the glass chamber. The menthol and bendiocarb were applied at the same time. After 1 h of exposure, the insects were tested for motor skills. The tests were recorded with a video-camera which allowed for precise evaluation of the time necessary for the insects to turn back to their normal position with a degree of accuracy of 0.001 s. All experiments were repeated 3 times with $n = 15$ for all mean values.

2.2.2. Knockdown tests

To evaluate the efficiency of bendiocarb and the potentiation of its effect by menthol, we performed tests in which knockdown of the cockroaches was observed. Six adult male insects were placed together in a 1.7 l non-absorbing, cubic glass chamber (12 cm × 12 cm × 12 cm) for 12 h. 1 ml of menthol (in the concentration of 0.1 μM) was applied into a filter paper. Similar to previous test, the paper was placed directly under the chamber lid. 1 ml of bendiocarb (in the concentrations of 1, 0.5, 0.2, 0.1, 0.05, 0.01 mM) was dispensed in a spray form directly into the insects in the glass chamber using a glass atomizer. At intervals of one hour, the number of the paralyzed insects was noted repeatedly. An insect was considered knocked down when it laid upside down without moving. The experiment was replicated 5 times, hence the total number of insects in each variant and concentration was 30.

Statistical analysis was performed using R software (R Development Core Team, 2011). Since some data did not have a normal distribution, we used the Kruskal-Wallis test for equal median and Mann-Whitney post-hoc tests to compare groups of data using 'FSA' package (Ogle, 2018). Dose-response curves were computed using 'drc', 'sandwich' and 'lmtest' packages (Ritz et al., 2015; Zeileis, 2004; Zeileis and Hothorn, 2002). The knockdown doses were calculated using cumulative Behrens method (Wilbrandt, 1952).

2.3. Electrophysiological tests

The efficiency of bendiocarb, menthol and bendiocarb combined with menthol on the cockroach nervous system was tested *in vitro* at the first part of the insect escape system. The preparation contained the

[1] EO – essential oil.
[2] cAMP – 3′,5′-cyclic adenosine monophosphate.
[3] GABA - gamma-aminobutyric acid.
[4] AChE – acetylcholinesterase.

cerci, the cercal nerves, TAG[5] and the connective nerves with the ganglia from the abdomen. The cercal nerve is connected with the giant and other interneurons by the cholinergic synapses located in the TAG. Recordings of the bioelectrical activity were carried out from the postsynaptic part – the connective nerve close to the TAG.

The preparation was placed in a Petri dish and systematically irrigated with physiological saline to ensure the hydration of the cercal and connective nerves. The cercal appendages were not irrigated seeing that the mechanoreceptors that cover the cercus should be dry to react correctly to stimuli. An extracellular recording electrode (Alpha Omega Engineering LTD, Israel) was placed on the connective nerves while a non-polarized reference electrode was placed in the physiological saline nearby. The electrodes were connected with a preamplifier and then with a compensatory amplifier which allowed to record the spontaneous and induced activity of the nerve cord. The bioelectric signals were observed on an oscilloscope and intercepted by a modified software, Hameg (Germany).

One cercus was stimulated by delicate air puffs applied with 0.75 Hz frequency emitted by a mechanostimulator. A stimulus was evoked by a movement of a loudspeaker membrane, actuated by an impulse generator. The response to the stimulation (registered during 100 ms per one entry) and the total activity (registered during 10 s per one entry) of the connective nerve were recorded. Each entry consisted of one thousand bars, each one being a sum of compound action potentials (the height of the bar indicated the amount of the compound action potentials). As a result, in the case of recording the response to the stimulation one bar referred to the sum of the action potentials collected in 0.1 ms and in the case of the total activity, in 10 ms (Fig. 2A).

In all the experiments, the same patterns of the electrophysiological recordings were used. Each recording of the response to the stimulation consisted of (1) resting (spontaneous), usually very low activity, (2) response to a mechanical stimulation of the cercus, usually well-defined through time and (3) again resting activity mostly very low. The recordings of the total nerve activity were performed simultaneously. Each record (Fig. 2A) consisted of spontaneous nerve activity and reactions to stimulation visible in control as a single high peak of firing. The size of the total activity was determined mainly by the level of spontaneous activity (the participation of responses in the total activity did not exceed 10%). Two kinds of experiments were performed: (1) we logged 70 min of recordings in control conditions; (2) first we logged 10 min of control recordings, named “basic activity”, using only physiological saline. The preparations were then exposed to the tested compound(s) and observed for one hour (if it was variant with menthol applied with bendiocarb, the essential oil was administrated 5 min before carbamate).

The magnitude of the response to the stimulation was calculated, using the Hameg software as the “response surface”. This means that the specified response time was multiplied by all the bioelectric signals that appeared at that time. The duration of the response was stable in the given experiment and the time frame containing the response chosen at the beginning was applied to all the records in the experiment. In the case of the total connective nerve activity, the time of the entire record (10 s) was analyzed.

The collected data was further analyzed using R software (R Development Core Team, 2011). The results were expressed as mean values ± SE and the comparison of several data groups was made using Kruskal-Wallis test. The differences between groups were tested by Mann-Whitney post-hoc tests. The dose-response curves were established using ‘drc’, ‘sandwich’ and ‘lmtest’ (Ritz et al., 2015; Zeileis, 2004; Zeileis and Hothorn, 2002).

[5] TAG – terminal abdominal ganglion.

3. Results

3.1. Influence of menthol on the efficiency of bendiocarb in the toxicity tests

3.1.1. Assessment of motor skills

Motor skills were expressed as the time necessary for the insects to turn back from their dorsal (upside down) to their ventral (normal) position. In control conditions, all insects returned from the dorsal to the normal position within 0.7 ± 0.1 s. The application of menthol in the concentration of 1 μM did not change significantly the ability of the insects to turn back (Fig. 1A). However, a lower concentration of menthol (0.1 μM) extended the time needed for the insects to return to the normal position up to 10 ± 4.3 s ($p = .0002$) (Fig. 1A) surprisingly. Menthol in the concentration of 0.01 μM was less effective and caused an increase in time to 7.1 ± 3.3 s ($p = .03$). Bendiocarb tested alone at the concentration of 1 μM caused only a slight extension of the time necessary for the insects to return to the normal position At the concentration of 0.1 mM bendiocarb extended this time to 6.4 ± 3.1 s ($p = .02$) (Fig. 1A). Bendiocarb at concentrations lower than 1 μM did not cause any effect (Supplementary Fig. 1A). The lowest concentration of bendiocarb (1 μM) at which any effect was observed was chosen to assess the effect of menthol on the efficacy of bendiocarb. This was combined with two concentrations of menthol (0.1 and 1 μM).

The use of 1 μM bendiocarb in combination with 0.1 μM menthol caused an increase in the time necessary for the insects to return to the normal position to 18.9 ± 7.24 s ($p = .0037$) This represented a 14-fold increase in recovery time compared to when bendiocarb was applied alone (Fig. 1A). As a result, the effect of the mixed substances on the test insects was 56% higher than the sum of their individual effects. A corresponding effect was observed when 1 μM bendiocarb was applied together with 1 μM of menthol. The effect was 8 times higher than in the case of bendiocarb acting alone ($p = .0292$). This potentiating effect was 270% higher than the sum of the effects of bendiocarb and menthol applied in the same concentrations separately. Obtained results clearly indicated that menthol potentiated strongly the efficiency of bendiocarb.

3.1.2. Knockdown tests

In the previous series of experiments, menthol at the concentration of 0.1 μM produced a more significant effect on the motor skills of the insects. Knockdown tests were carried out using this concentration which alone never induced any visible signs of paralysis in the insects. The cockroaches were treated with bendiocarb separately and also in conjunction with menthol in concentrations previously indicated in the Methods section. The time from application to knockdown was then measured. Bendiocarb at the concentration of 1 mM when applied, almost immediately caused paralysis and within an hour produced paralysis in 100% of the insects. Lower concentrations of bendiocarb took longer to cause a knockdown—0.05 mM of bendiocarb took 5 h to cause the first signs of paralysis while it took 7 h to initiate knockdown at the concentration of 0.01 mM (Fig. 1B). At the concentration of 0.05 mM, bendiocarb applied in conjunction with menthol induced the first paralysis after 2 h. However, it took 6 h for 0.01 mM of bendiocarb applied with menthol to initiate paralysis. The development of the knockdown state was much faster when bendiocarb was used together with menthol. For example, while 7% of the insects were paralyzed after 5 h of exposure to 0.05 mM of bendiocarb alone, a treatment with a combination of bendiocarb and menthol caused 43% to be paralyzed in the same time frame and concentration. Likewise, 8 h of exposure to 0.01 mM bendiocarb alone and to a combined treatment of bendiocarb and menthol resulted in 12.5% and 46% paralyzed insects respectively (Fig. 1B). The co-application of menthol with bendiocarb at high concentrations (0.5 and 1 mM) did not result in any significant increase in the bendiocarb efficiency. Probably, the effect caused by bendiocarb alone was too fast and there was no time for the menthol to take effect. The cumulative dose-response curves for the knockdown effect for

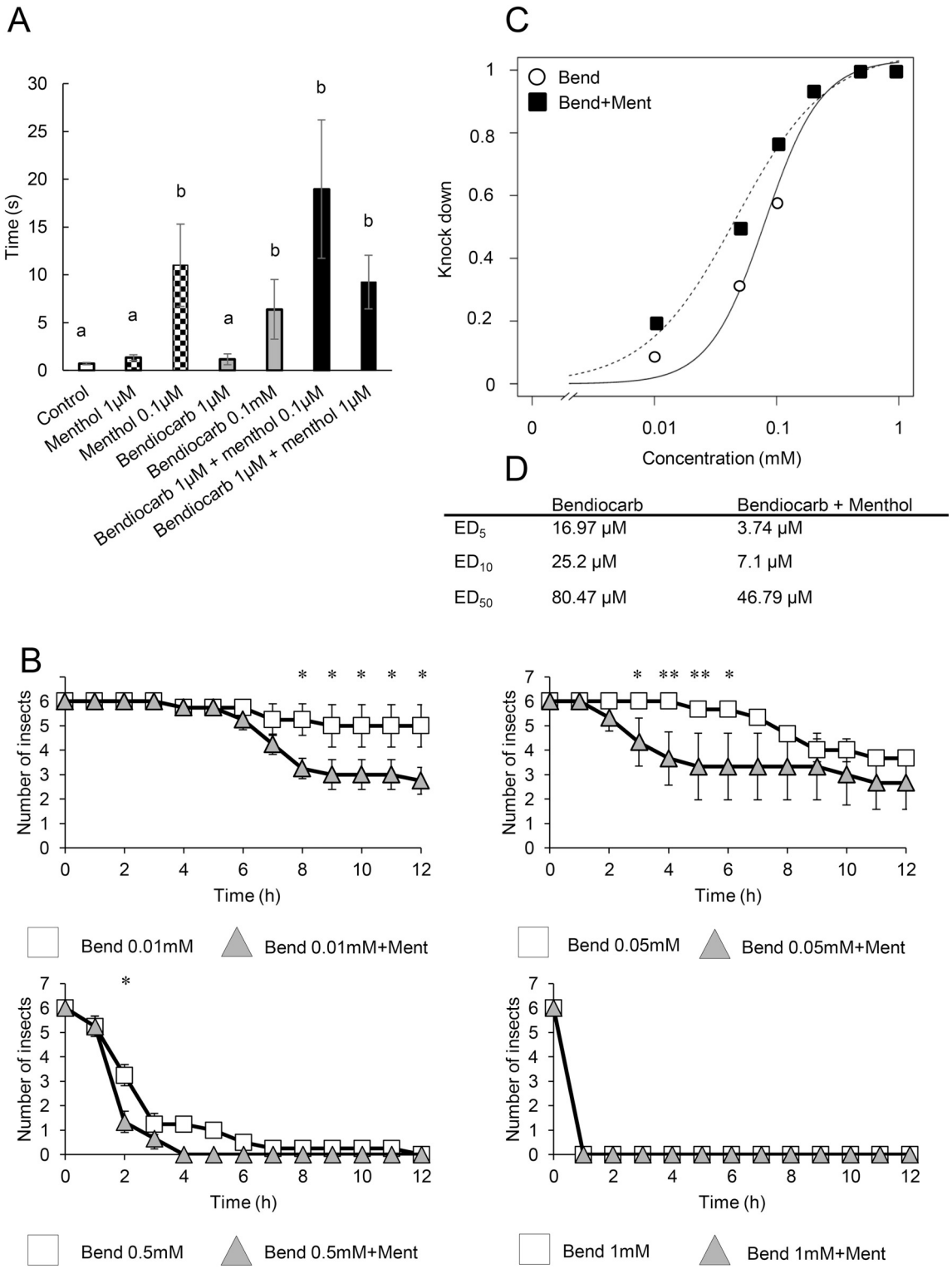


Fig. 1. Effects of bendiocarb and menthol on *in vivo Periplaneta americana* – toxicity tests. A). The assessment of motor skills: the insect motor abilities were estimated based on the time required to turn from the dorsal side to the normal position 1 h after the application of the indicated bendiocarb and menthol combinations. The data is expressed as mean values ± SE, $n = 15$ and the different letters above the bars refer to statistically significant differences between the data with $p < .05$. B). Knockdown tests—the reduction in the number of the mobile insects over time after the application of the indicated combinations of 0.1 μM menthol (Ment) and bendiocarb (Bend). Note the big difference in the menthol enhancing effect for low and high concentrations of bendiocarb. The data is expressed as mean values ± SE, $n = 5$ and the statistically significant differences between bendiocarb and bendiocarb combined with menthol are marked: * $p < .05$, ** $p < .01$. C). The dose-effect curves for bendiocarb (continuous line) and bendiocarb co-administered with 0.1 μM menthol (dashed line). The effect (normalized) is expressed as a cumulative number of the insects in the knockdown state 12 h after the administration of chemicals, $n = 30$. D). The effective dose values causing 5, 10 and 50% of effect in a knockdown tests.

different concentrations of bendiocarb alone and in the presence of menthol (0.1 μM) were shown in Fig. 1C. The curve plotted for data in the presence of menthol was shifted towards lower bendiocarb concentrations. This change was more pronounced for lower concentrations of bendiocarb. Menthol decreased ED^{6}_{5} value for bendiocarb 4.5 times and ED_{50} value 1.7 times (Fig. 1C,D).

3.2. Influence of menthol on the efficiency of bendiocarb in electrophysiological experiments

To evaluate the effect of bendiocarb and menthol on the cockroach nervous system, bioelectrical activity of the connective nerve close to the terminal abdominal ganglion was recorded. The magnitude of response to the cercus stimulation and the total activity (meaning the spontaneous activity together with the response to the stimuli) were observed.

3.2.1. Study of bendiocarb effect on the Periplaneta americana nerve cord

The examples of the total bioelectric activity of the connective nerve (meaning the responses to the stimulation and the spontaneous activity) were presented on Fig. 2A. In the control conditions, the reactions to the stimulation were well distinguished from the spontaneous activity. When bendiocarb was applied on the preparation the bursts of spontaneous activity were observed. Both parameters: the magnitude of the response to the stimulation and the spontaneous activity were analyzed and compared. In the experiments performed under control conditions, the size of the response slightly decreased at the beginning and usually reached a constant level (about 75% of initial value) within 15 min (Fig. 2B). During the experiment, the amount of total activity decreased as well (Fig. 2C).

Bendiocarb was tested at 4 concentrations (0.1, 0.2, 0.5, 1 μM) and at these concentrations it did not modify the magnitude of the response to the stimulation (Fig. 2B). However, we observed a new phenomenon where an increase in spontaneous activity appeared in a concentration-dependent manner, often in the form of bursts (Fig. 2A – Bend). When the spontaneous activity was low, the response to the stimulation was well-defined. Conversely, the higher and the irregular spontaneous activity caused by bendiocarb, made it impossible to distinguish the response from the spontaneous firing. In this situation, we decided to analyze the total activity of the preparation – this refers to the spontaneous activity and the response to the stimulation estimated together.

In control conditions, the initial total activity of the connective nerve varied between the preparations and for this reason the data was normalized. The average total activity from the first 40 recordings (10 min) was set to 1 and from then was referred to as the “basic activity”. In all control experiments, the spontaneous activity gradually decreased. After 60 min of recording only the response to the stimulation was visible and it constituted 14.3 ± 6.3% of the basic activity (Fig. 2C).

Bendiocarb in the highest electrophysiologically tested concentration (1 μM) induced two kinds of effects. (1) Bendiocarb increased the total activity of the connective nerve (Fig. 2A, C). 30 min after its application, the total activity was 1.8 times higher than in control conditions (66.5 ± 12.7% of the basic value after bendiocarb application compared to 36.1 ± 5.7% in control). 60 min after application, this activity was recorded as 3.2 times higher ($p < .05$) than in control (45.6 ± 11.4% *vs* 14.3 ± 6.4%, Fig. 2C). The observed increase in the total activity was related only to rise in the spontaneous firing because the size of the response to the stimulation remained almost on the control level (Fig. 2B). (2) Bendiocarb caused frequent, well detectable bursts of activity (Fig. 2A Bend). Such bursts were never observed in the control conditions on the connective nerve and after bendiocarb on the cercal nerve (Supplementary Fig. 1B). The use of bendiocarb in lower concentrations showed its concentration-dependent effect (Fig. 2C). An increase in the total activity was not observed after the application of bendiocarb in the concentration of 0.2 μM and lower (Fig. 2C). However, there were exceptions in some cases where the bursts of activity occurred. Calculated ED_{50} was estimated at 0.5 μM (Fig. 4D,E).

3.2.2. Study of the menthol effect on the Periplaneta americana nerve cord

Similar to bendiocarb, we began by testing the influence of menthol alone on the response to mechanostimulation. Menthol, in contrast to bendiocarb, changed the response size – Fig. 3A. After its application, the response decreased progressively; the effect was more visible for a lower concentration of the compound. After 30 min of menthol presence, the size of the response was 47.9 ± 8.9% of the basic value at the concentration of 1 μM ($p = .0037$) and only 33.9 ± 5.5% at the concentration of 0.1 μM ($p = .0009$). However, in a corresponding time in the control conditions, the response to the stimulation remained stable at the level of 74.2 ± 5.1%.

To test whether menthol can activate the octopamine receptors, we conducted a series of experiments with a receptor antagonist – phentolamine (1 μM), by which the preparation was pretreated. Interestingly, in the presence of phentolamine, 1 μM of menthol had no effect on the response to the stimulation and its evolution over time was exactly the same as in the control; after 30 min it reached 79.7 ± 5.0% of the initial value (Fig. 3A). Phentolamine applied separately did not cause any significant effect (Supplementary Fig. 1C).

In additional experiments, we demonstrated a high similarity between the action of menthol and octopamine. When octopamine (1 μM) was applied to the nerve cord, it reduced the size of the response to the stimulation recorded from the connective nerve. 30 min after this, it was at the level of 55.3 ± 2.0% of the initial value. In a corresponding time in the control conditions, the response to the stimulation remained stable on the level of 74.2 ± 5.1% (difference between octopamine and control in 30 min, $p = .0009$). A pretreatment of the nerve cord with phentolamine abolished completely the effect of octopamine and the changes in the magnitude of the response over time were similar to the changes in the control conditions (Fig. 3B).

We assumed that menthol and octopamine caused their effects by binding to the same receptor site. To verify this hypothesis, we studied the effect of menthol (1 μM) after pretreatment (10 min) with octopamine (1 μM). In 20 min of applying menthol together with octopamine, the size of the response was equal to 0.48 ± 0.08. This value was very similar to the one observed after 30 min of menthol presence alone at 0.47 ± 0.07 (Fig. 3C). This clearly suggests that these molecules bind to the same octopamine receptors.

Menthol (1 μM) induced a decrease in the total activity of the connective nerve. Total activity was now up to 15.7 ± 2.9% of the basic value compared to 36.1 ± 5.7% in the control over 30 min ($p = .00634$) and to 4.3 ± 1.7% compared to 14.3 ± 6.4% within 60 min (Fig. 3D). Quite similar results were obtained with 1 μM octopamine (Fig. 3D). When the nerve cord was pretreated with phentolamine, the difference between the control recordings and the recordings after the treatment with menthol was not statistically significant after 30 min. A small, insignificant increase in activity was observed after 60 min, compared to the control (Fig. 3D). The obtained results confirmed our assumption that the effects of menthol are carried out most likely through the octopamine receptors.

3.2.3. Effect of menthol on the efficiency of bendiocarb

After 10 min of the control recordings, the nerve cord was treated with 1 μM menthol for 5 min and then a mixture containing 1 μM menthol and 1 μM bendiocarb was applied. This did not change the magnitude of the response to the stimulation, just like with bendiocarb alone (Supplementary files, Fig. 1D). As a result, only the total activity of the connective nerve after bendiocarb application was considered in assessing the effect of menthol on bendiocarb efficiency. In 30 min, a slight increase in the total connective nerve activity was observed

[6] ED – effective dose.

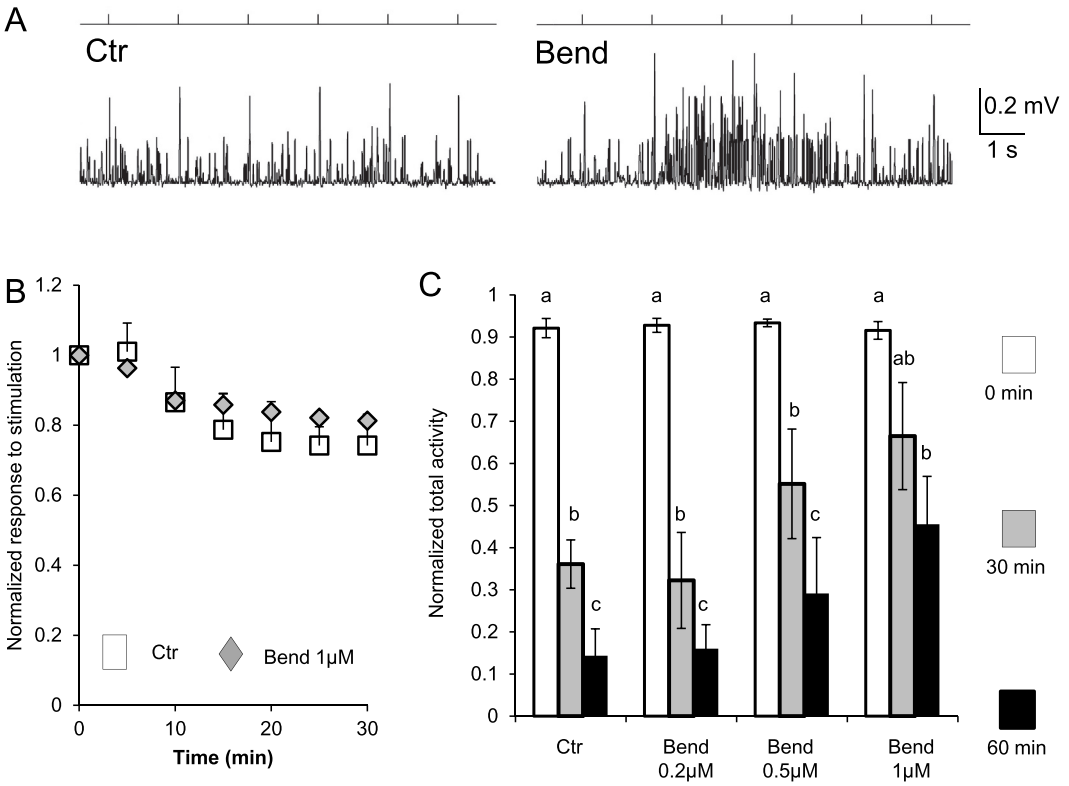


Fig. 2. Changes in the connective nerve bioelectric activity induced by bendiocarb. A). The original, representative extracellular recordings of the bioelectrical activity of the cockroach connective nerve in the control conditions (Ctr) and after 1 μM bendiocarb (Bend) application. In the upper part, the air puff stimuli are indicated while the lower parts show the activity of the connective nerve. Note: the highest peaks correspond to the response to the stimulation. B). The magnitude of the bioelectric response to the stimulation for the control (Ctr) and after applying 1 μM bendiocarb (Bend). The size of the response has been normalized (1 is the average value recorded in the first 10 min of each experiment). The data is presented as mean values ± SE, $n = 7$. C). The magnitude of the total activity at the beginning of the experiment (0 min) and within 30 and 60 min under the control conditions (Ctr) and after the application of bendiocarb (Bend) in the concentrations 0.2, 0.5, and 1 μM. The data was normalized where 1 is the average value of "the basic activity" recorded in the first 10 min of each experiment. The bars shown at time "0" indicate values 1 min before the given substance application. The data is presented as mean values ± SE, n = 7 and the different letters above the bars refer to statistically significant differences between values with $p < .05$.

compared to the effect of 1 μM bendiocarb applied alone (Fig. 4A). Within 60 min, 1 μM bendiocarb combined with menthol generated a total activity 2.1 times higher ($p = .0021$) than with bendiocarb applied alone – Fig. 4A. This value was 6.7 times higher ($p = .0015$) than in the control at a corresponding time of the experiment.

The strengthening effect of menthol was dependent on the concentration of bendiocarb. When the concentration of bendiocarb was higher, in the 60th minute of the experiment the increase in the total nerve activity after the mixture of compounds was bigger compared to the control (4 times for 0.2 μM, 5 times for 0.5 μM and 6.7 times for 1 μM; respectively p values were 0.0015, 0.026, 0.0015). However, the ratio of the activity in the presence of the mixture of the two substances and only bendiocarb was the highest (5 times) at the 0.2 μM bendiocarb concentration ($p = .019$) compared to 2.1 times for 1 μM ($p = .026$) – Fig. 4B. This indicates that the potentiation of bendiocarb effect by menthol was largest at the lowest bendiocarb concentration.

Our last goal was to check whether the potentiating effect of menthol on the efficacy of bendiocarb was mediated by the octopamine receptors. For this purpose, we used 1 μM phentolamine for 5 min before testing the effect of the mixture of menthol and bendiocarb on the total activity of the connective nerve. Our assumption was confirmed – the evolution of the level of the total activity over time was very similar to that under the control conditions (Fig. 4A). This confirmed unequivocally that menthol potentiates the effect of bendiocarb by activating the octopamine receptors.

In order to visualize the strengthening effect of menthol on the effectiveness of bendiocarb, we present Fig. 4C which convincingly illustrates the development of the potentiating effect of menthol on bendiocarb efficacy in time. On the figure, the high black bars correspond to the bioelectrical discharges with very high frequency. Such phenomena were never observed in the presence of bendiocarb alone.

To better assess the strengthening effect of menthol on bendiocarb activity, the dose-effect curves were calculated and plotted based on the increase in the total activity of the connective nerve in the presence of bendiocarb alone and bendiocarb with 1 μM menthol – Fig. 4D. Menthol shifted the dose-effect curve into the lower bendiocarb concentrations. Additionally, it induced the increase in the maximum possible effect of bendiocarb. For better comparison ED_{50}, ED_{10} and ED_5 were calculated (Fig. 4D,E). ED_{50} for menthol and bendiocarb together was 0.21 μM, this means it is over two times lower than for bendiocarb alone – 0.50 μM.

4. Discussion

The aim of our research was to check whether menthol – an essential oil component can be used as a substance to strengthen the effect of bendiocarb, a conventional, chemical insecticide. Essential oils have repellent and neurotoxic effects on insects, but their effectiveness is generally lower than that of the classic insecticides (Castillo et al., 2017). Neurotoxicity of essential oils results from the fact that their targets are the key proteins in the insect nervous system: AChE, the GABA and the octopamine receptors (Jankowska et al., 2017; Pavela and Benelli, 2016). One of the strategies to fight against insect pests is to use substances in pairs so that each of them acts on a different target.

Special attention is currently paid to the metabotropic receptor, whose activation may cause strengthening effects (sometimes

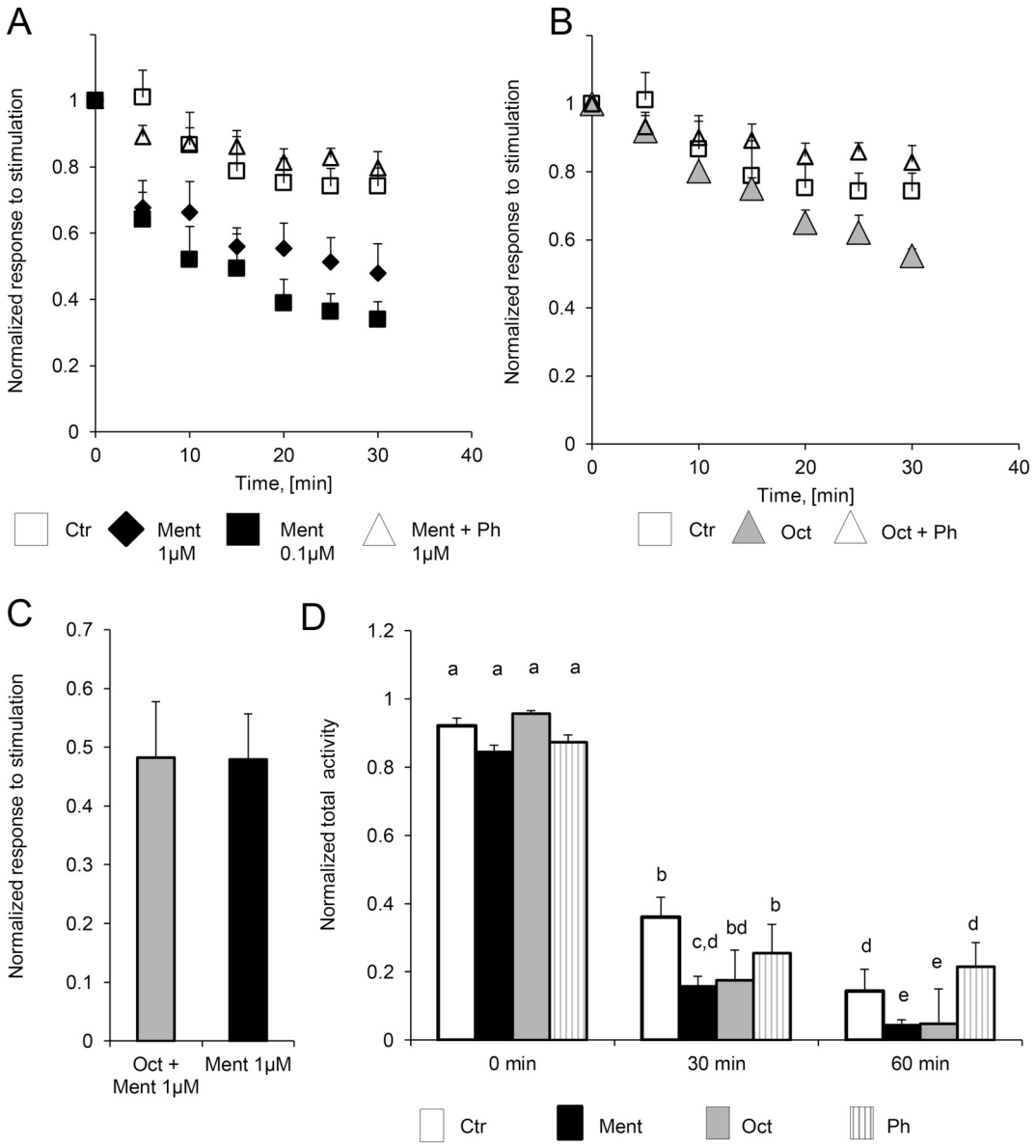
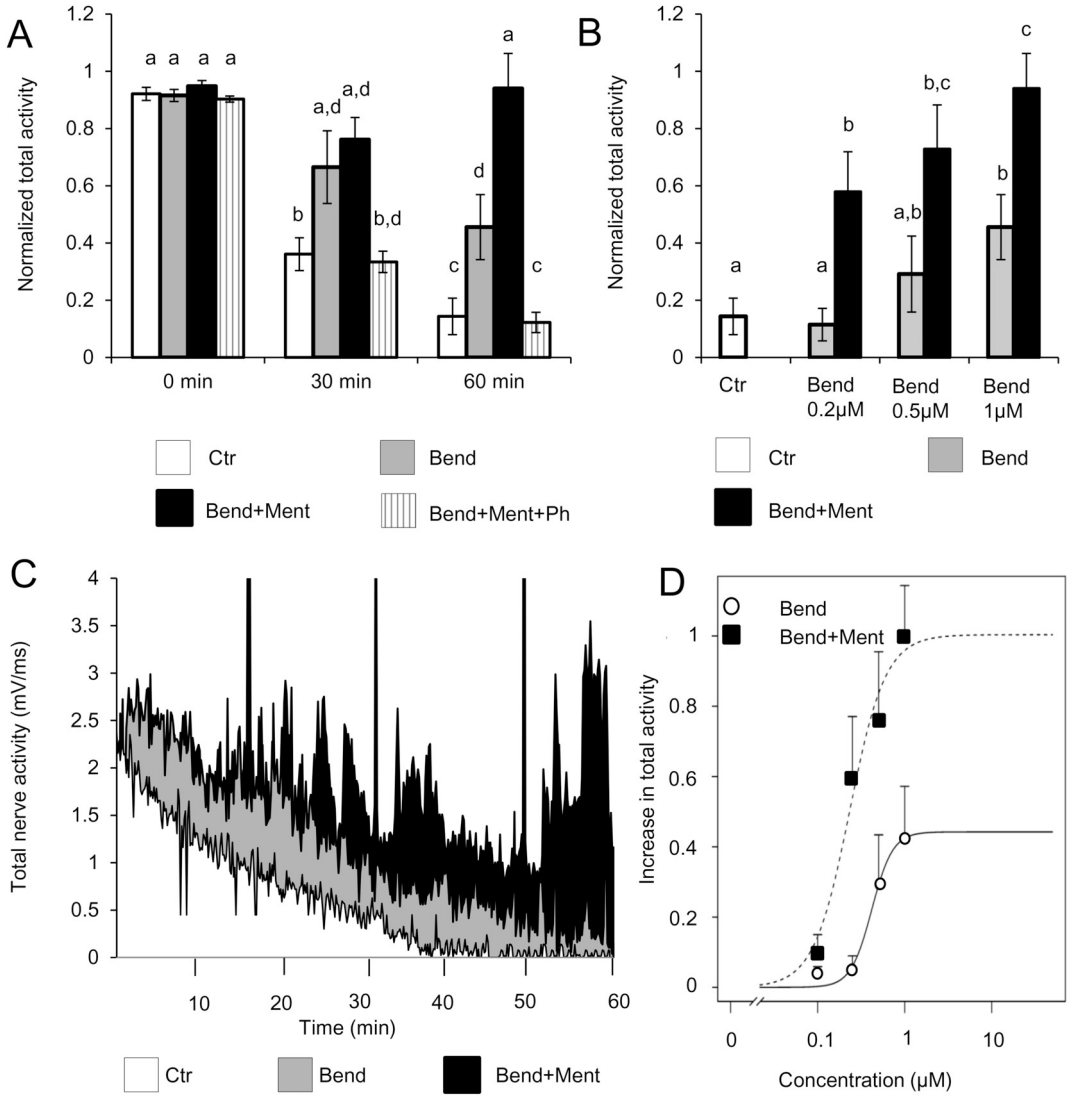


Fig. 3. Menthol changes the bioelectrical activity of the connective nerve. A). The magnitude of the bioelectric response to the stimulation for the control (Ctr), two indicated menthol concentrations (Ment), and menthol combined with 1 μM phentolamine (Ment + Ph). The size of the response has been normalized where 1 is the average value recorded in the first 10 min of each experiment. The data is presented as mean values ± SE, n = 7. For transparency, the statistical differences are not marked (for the data points in time 5–30 min, $p < .001$). B). The magnitude of the bioelectric response to the stimulation for the control (Ctr), octopamine (1 μM, Oct), and octopamine + phentolamine (1 μM Oct + 1 μM Ph). The size of the response has been normalized. 1 is the average value recorded in the first 10 min of each experiment and the data is presented as mean values ± SE, n = 7. For transparency, the statistical differences are not marked (for the data points in time 5–30 min, $p < .001$). C). The magnitude of the bioelectric response to the stimulation for 1 μM menthol (Ment) and 1 μM menthol after the pretreatment (10 min) with 1 μM octopamine (Oct + Menth) measured after 30 min of application of the compounds. The size of the response has been normalized where 1 is the average value recorded in the first 10 min of each experiment. The data is presented as mean values ± SE, n = 7. D). The size of the total nerve activity at the beginning of the experiment and after 30 and 60 min in the control (Ctr) and after the application of 1 μM menthol (Ment), 1 μM octopamine (Oct), and 1 μM phentolamine (pH). The data was normalized where 1 is the average value of "the basic activity" recorded in the first 10 min of each experiment. The bars shown at time "0" indicate values 1 min before the given substance application. The data is presented as mean values ± SE, n = 7 and the different letters above the bars refer to statistically significant differences between the data with $p < .05$.

synergistic) with the action of the chemical insecticides (Abd-Ella et al., 2015). We hypothesized that the activation of the octopamine receptors, which trigger the intracellular pathways associated with the increase in cAMP and Ca^{2+} concentrations, would modify the AChE activity and its sensitivity to chemical inhibitors (carbamates). Achieving a synergistic effect due to the stimulation of the octopamine receptors has already been described (Ahmed and Vogel, 2016). This is particularly valuable because the octopamine receptors play a key regulatory role in insects but occur only in negligible amounts in vertebrates. We assumed that the EO components will "mimic" the effect of octopamine at low concentration, since such an action had already been observed (Glanzman and Krasne, 1983).

In our studies we used menthol, which is considered one of the most active insecticide among the components of EOs (Isman and Machial, 2006). Menthol applied alone showed only insignificant effects in our toxicity tests. Nevertheless it proved to be a strengthening factor for bendiocarb activity. The toxicity tests adequately confirmed our hypothesis that bendiocarb in the presence of menthol is more efficient as an insecticide and this is the main conclusion of our research.

In addition, we wanted to know more about the mechanism of



E

	Bendiocarb	Bendiocarb + Menthol
ED_5	0.38 μM	0.049 μM
ED_{10}	0.41 μM	0.071 μM
ED_{50}	0.51 μM	0.21 μM

Fig. 4. Changes in the efficacy of bendiocarb induced by menthol. A). The size of the total activity of the connective nerve in 0, 30 and 60 min of the experiment under the conditions: Control (Ctr); after the application of 1 μM bendiocarb alone (Bend); 1 μM bendiocarb + 1 μM menthol, applied after a 5-min pretreatment with 1 μM menthol, (Bend + Ment); and 1 μM bendiocarb + 1 μM menthol after a 5-min pretreatment with 1 μM phentolamine (Bend + Ment + Ph). B). The size of the total nerve activity in 60 min of the experiment under the conditions: Control; application of 0.2, 0.5 and 1 μM of bendiocarb (Bend); and bendiocarb (the same concentrations) + 1 μM menthol (Bend + Ment) after a 5-min pretreatment with 1 μM menthol. The data was normalized where 1 is the average value of "the basic activity" recorded in the first 10 min of each experiment. The bars shown at time "0" indicate values 1 min before the given substance application. The data is presented as mean values ± SE, n = 7 and the different letters above the bars refer to statistically significant differences between the data with $p < .05$. C). The strengthening effect of menthol on bendiocarb activity. The comparison of the changes in the total bioelectrical activity of the connective nerve under the conditions: (Ctr) Control, (Bend) when induced by bendiocarb, and (Bend + Ment) when bendiocarb is applied together with menthol. The original size of the total activity throughout 60 min of the representative experiments (performed on the same day) is presented. The white area refers to the control experiment (Ctr), the grey area refers to the experiment with 1 μM bendiocarb (Bend), and the black area corresponds to the experiment with the mixture of 1 μM bendiocarb with 1 μM menthol (Bend + Ment) applied after a 5-min pretreatment with menthol. The figure is for reference only. D) Menthol shifts the dose-effect curve of bendiocarb. The dose-effect curve calculated for bendiocarb (Bend) and for bendiocarb together with menthol (Bend + Ment). The effect was calculated as the difference between the total activity of the connective nerves in the control experiments and the activity of the connective nerves in the presence of the indicated concentrations of bendiocarb and bendiocarb together with 1 μM menthol. The highest observed activity was set as 1. The data is presented as mean values ± SE, n = 7. E) Effective dose (ED) values causing 5, 10 and 50% of effect on the bioelectrical activity of the nerve cord treated with bendiocarb and with bendiocarb together with 1 μM menthol after a 5-min pretreatment with menthol. The effect was calculated as a difference between the total activity of the connective nerves in the control experiments and the activity of the connective nerves in the presence of the indicated concentrations of bendiocarb and bendiocarb together with 1 μM menthol.

interaction between bendiocarb and menthol at the level of the insect's nervous system. For this purpose, we performed electrophysiological experiments using extracellular recordings of the activity of the cockroach nerve cord.

The observed decrease (by approx. 25%) in the magnitude of the response to the stimulation under the control conditions resulted from the evolution of the preparation with time. Bendiocarb did not significantly modify the magnitude of the response to the mechanostimulation. This was a surprising phenomenon because the inhibition of AChE should have increased the size of the reaction. However, a release of the ACh[7] by the stimulus and its increased levels in a synaptic cleft in the presence of bendiocarb, could have triggered a negative feedback. This could possibly have been through the muscarinic receptors in the presynaptic part that limited the release of ACh in response to the stimulus. Such a phenomenon was observed in this preparation (Corbel et al., 2006). Moreover, in the insect central nervous system ACh binds to different types of nAChR.[8] These nAChR are composed from homomeric and heteromeric α 1–8 subunits as well as non α subunits and are found throughout the whole nervous system of insects. Among them homomeric nAChR (family 1) occurring in the giant fiber of *Drosophila melanogaster* is known to be crucial for the function of the escape system (Fayyazuddin et al., 2006). Their feature is that they are quickly desensitized with a high amount of ACh (Amar et al., 1995). Therefore, their function is mainly the detection of the stimulus and sending information to the muscles to start the escape. A high level of ACh after an inhibition of AChE may induce a desensitization of these receptors. However, non-desensitizing nAChR can be active and responsible for non-specific excitation of the post-synaptic part, which has been recorded as a high, irregular, bursting connective nerve activity after bendiocarb application. Such over-excitation prevents the detection of the stimuli and induces paralytic effects in the insects. Bendiocarb effects at the level of the insect nervous system corresponded well with the observations obtained *in vivo*. These were the reasons why mainly the spontaneous activity of nerves was accepted as a parameter to compare the action of bendiocarb alone and in the presence of menthol.

We also assumed that menthol would act as an octopamine receptor agonist so we compared the effects of menthol and octopamine. Octopamine (1 μM) caused a decrease in the size of the response to the stimulus compared to the control. A corresponding decrease was also observed in the total activity of the connective nerve. Similarly, octopamine inhibited the excitatory junctional currents in the glutamate neuromuscular junction in *Drosophila melanogaster* larvae (Nishikawa and Kidokoro, 1999). It also decreased the amplitude of electro-antennogram in *Periplaneta americana* (Zhukovskaya, 2012). Furthermore, in small concentrations, it decreased the firing rate in heart cells in *Drosophila melanogaster* (Papaefthimiou and Theophilidis, 2011). Very similar effects in terms of modifying the magnitude of the response to the stimulus and the total nerve activity were obtained after menthol (1 μM). The development of the effects with time was similar to that after octopamine.

All kinds of octopamine receptors (Oamb, Octß1R, Octß2R, Octß3R) cover the whole central nervous system of adult insects (El-Kholy et al., 2015). They are known to be present in the last abdominal ganglion and in the giant interneurons which make up the connective nerve of *Periplaneta americana* (Weisel-Eichler and Libersat, 1996). When menthol was applied after octopamine, no additional decrease in the magnitude of response to stimulation was observed. The pretreatment of the preparation with phentolamine - a blocker of the octopamine receptors - abolished completely the effect of octopamine and menthol. All these data strongly suggest that menthol acts through the octopamine receptors.

In the toxicity tests and the electrophysiological experiments we observed a surprising phenomenon: (1) cockroaches exposed to the lower concentration of menthol (0.1 μM) needed more time to return to the normal position than those exposed to 1 μM of menthol and (2) a decrease in the response to the stimuli after exposure to 0.1 μM of menthol was larger than after exposure to 1 μM of menthol. We suppose that with the higher menthol concentration the desensitization of the receptors could take place. The agonist-induced desensitization of the octopamine receptors has already been described (Orr and Hollingworth, 1990; Robb et al., 1994). Intracellular signaling pathway activated by the binding of menthol to the octopamine receptors can change the phosphorylation state of the receptors and their desensitization. A corresponding desensitization of the octopamine receptor by PKC activation has been described in the nervous system of *Drosophila* (Hoff et al., 2011).

In the nervous system of the cockroach, it has been found that the increase in the intracellular Ca^{2+} and the cAMP concentrations modify the affinity of AChE to carbamates (Abd-Ella et al., 2015). Thus, the effect of bendiocarb on AChE in our preparation was expected to change in the presence of menthol. However, the magnitude of the response under the influence of the menthol/bendiocarb mixture was similar to the values recorded under the control conditions. We propose an explanation for this phenomenon similar to that for the case of bendiocarb applied alone and in this situation we evaluated the changes in the total activity of the nerve cord. The effects of bendiocarb in the presence of menthol were more profound than that of bendiocarb only. The efficiency increased almost two fold, when comparing ED_{50} values (Fig, 4D,E). A very similar potentiation was observed in *in-vivo* tests. Moreover, the bursting activity was much more expressed, and it developed in time. This corresponded perfectly to the assumption that the strengthening effect of menthol developed during the time needed for the course of the intracellular processes, that end with the phosphorylation of the proteins engaged in the information transmission in the cockroach escape system. A detailed statistical analysis of the effects of bendiocarb alone and in the presence of menthol clearly showed that menthol potentiated the bendiocarb activity. All this interaction was abolished by phentolamine, which confirmed that the potentiation effect occurred through the octopamine receptors.

In our study, we demonstrated a high similarity between the effects of menthol and octopamine (both of which were inhibited by phentolamine). This may suggest that menthol activates octopaminergic receptors. We assumed that activation of octopaminergic receptors would result in higher efficacy of carbamate insecticide (bendiocarb) on insects. Our hypothesis has been confirmed: menthol strongly increases the efficiency of bendiocarb. This positive interaction between menthol and carbamate insecticide has been proven for the first time. The explanation for the mechanisms of this interaction requires further research.

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[7] ACh – acetylcholine.
[8] nAChR - acetylcholine nicotinic receptor.

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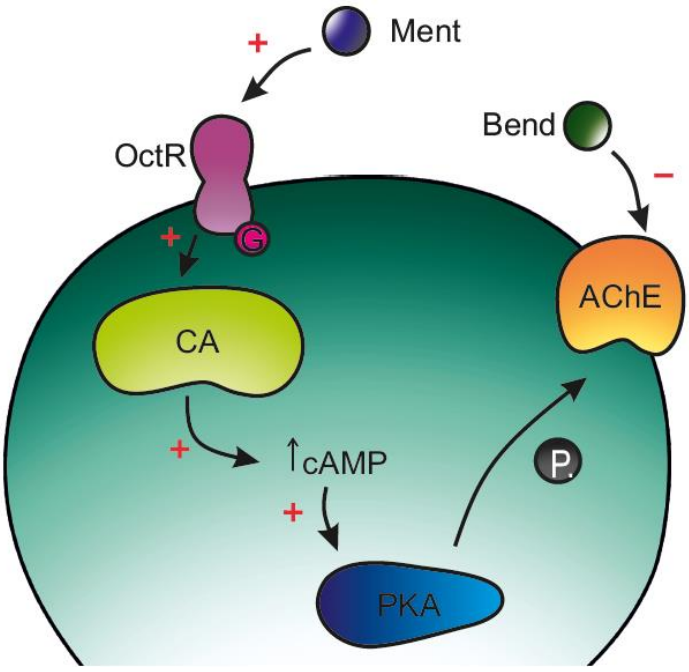
5. Article III

Menthol Increases Bendiocarb Efficacy Through Activation of Octopamine Receptors and Protein Kinase A



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Article

Menthol Increases Bendiocarb Efficacy Through Activation of Octopamine Receptors and Protein Kinase A

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Abstract: Great effort is put into seeking a new and effective strategies to control insect pests. One of them is to combine natural products with chemical insecticides to increase their effectiveness. In the study presented, menthol which is an essential oil component was evaluated on its ability to increase the efficiency of bendiocarb, carbamate insecticide. A multi-approach study was conducted using biochemical method (to measure acetylcholinesterase enzyme activity), electrophysiological technique (microelectrode recordings in DUM neurons in situ), and confocal microscopy (for calcium imaging). In the electrophysiological experiments, menthol caused hyperpolarization, which was blocked by an octopamine receptor antagonist (phentolamine) and an inhibitor of protein kinase A (H-89). It also raised the intracellular calcium level. The effect of bendiocarb was potentiated by menthol and this phenomenon was abolished by phentolamine and H-89 but not by protein kinase C inhibitor (bisindolylmaleimide IX). The results indicate that menthol increases carbamate insecticide efficiency by acting on octopamine receptors and triggering protein kinase A phosphorylation pathway.

Keywords: menthol; essential oils; bendiocarb; carbamates; octopamine receptor; protein kinase A; PKA; protein kinase C; PKC

1. Introduction

The impact of synanthropic insect species on human beings is highly significant and raises with global warming. The demand for food is increasing with a growing human population in the world, while insect pests destroy growing plants and stored crops. Therefore, new methods of protecting man and food against insect pests are constantly being developed [1,2].

Although conventional chemical insecticides are the most effective in controlling insect pest, they have many disadvantages. First, they are increasingly becoming less efficient. Long- lasting and excessive use have caused the development of resistance to different groups of insecticides (e.g., nicotinoids and pyrethroids) in various insect species [3–5]. Cross-resistance (i.e., the specific resistance for given insecticide can also cause resistance for other insecticidal substances) is also observed [5]. In consequence, it is necessary to use increasing doses of insecticides, which in turn leads to growing incidence of environmental pollution and danger to non-target organisms including humans [6–8].

Different strategies are used in Integrated Pest Management to increase the efficiency of insecticides and to reduce their dosages. One of them is the application of mixture of insecticides with different molecular targets [9–12]. Quite a new strategy has been proposed: increase the sensitivity of "classical insecticide targets" by synergistic agents which elevate intracellular calcium concentration and activate intracellular signaling pathways [13–16]. Potentiating effect of DEET repellent to carbamate insecticides by the activation of metabotropic receptors has been demonstrated [17] and a corresponding mechanism has been proposed for an essential oil component—menthol [18].

Essential oils derived from plants are proposed as alternative substances to chemical insecticides which are safer for animals and humans [19–21]. Even though they are much less efficient than classical insecticides and therefore cannot replace them [22]. However, it has been shown that some essential oil components activate the metabotropic octopamine receptors [23–25]. They can be especially interesting in potentiating the effects of insecticides because their target, octopamine receptors, play a key role in physiology of insects [26,27].

Octopamine receptors belong to a family of metabotropic G protein-coupled receptors. Their classification was made based on similarities between *Drosophila melanogaster* receptors and mammals' adrenergic receptors. In insects, the activation of receptors OCTβ-R triggers (*via* the increase of cAMP) protein kinase A (PKA) signaling pathway while the activation of OCTαR (*via* the increase of IP3) triggers protein kinase C (PKC) pathway [26,28]. In *Periplaneta americana,* one octopamine receptor has been characterized—$Paoa_1$—and its activation leads to increases in both intracellular calcium level and cAMP level [29].

In our previous studies, it was demonstrated that in the essential oils component, menthol, increases the efficiency of carbamate insecticide (bendiocarb), most probably by activating octopamine receptors [18]. Carbamates are inhibitors of acetylcholinesterase (AChE) enzyme, which hydrolyzes acetylcholine neurotransmitters. Acetylcholine activates synaptic and non-synaptic acetylcholine receptors in the central and peripheral nervous systems of many organisms including insects [30,31]. Increased sensitivity of AChE to carbamate insecticides via activation of metabotropic (muscarinic) receptors has already been shown [17]. The aim of our study was to verify the hypothesis that menthol potentiates the effect bendiocarb through the activation of octopamine receptors and thus the activation of phosphorylation cascade. Bendiocarb, the most often used carbamate insecticide, was approved by World Health Organization and European Union Commission [32,33].

Octopaminergic dorsal unpaired median (DUM) neurons of the cockroach (*Periplaneta americana*) nervous system was taken as a model. DUM neurons release octopamine from their axonal endings and express octopaminergic receptors on the cell body [34–36]; they have endogenous pacemaker activity that is precisely regulated by a large variety of ion channels and receptors. DUM neurons are often used as very sensitive models to study the intracellular signaling systems [13,17]. We conducted multi-approach analyses, including electrophysiological experiments on DUM neurons in situ, biochemical tests of AChE activity, and calcium imaging using fluorescent microscopy techniques. Presented study confirms our hypothesis that menthol increases the efficiency of bendiocarb by activation of octopamine receptors, activation of protein kinase A (PKA) signaling pathway, and increasing Ca^{2+} concentration.

2. Results

2.1. Electrophysiological Tests

Electrophysiological experiments were performed on DUM neurons in situ in ganglia, using the microelectrode technique. DUM neurons display spontaneous activity and generate rhythmical action potentials. Typical DUM neuron interspike "resting potential" is about −50 mV and the discharge frequency is about 2 Hz.

In control conditions, application of physiological saline directly on ganglion caused a short disturbance of DUM neurons activity, mostly manifested in small depolarization (no more than 2 mV) and increase in firing frequency (however with duration no longer than 3 s).

Tested substances changed spontaneous activity pattern in DUM neurons ($F_{5,45} = 5.65$, $p < 0.001$). Application of 0.1 µM menthol in all tests caused a rapid hyperpolarization of membrane potential which was accompanied with the switching off of the ability of neurons to generate action potentials (Figure 1Aa). This effect was short lasting and after some seconds (Av: 19.27 ± 4.5 s) the membrane potential returned to its control value. There was relatively large variation in the duration and size of hyperpolarization induced by menthol between preparations. To express quantitatively the changes in DUM neurons activity induced by tested substances, we decided to estimate the Relative Size of Hyperpolarization (RSH) value—this is the value of hyperpolarization (counted as each deviation from basal (control) membrane resting potential) multiplied by the duration of such hyperpolarization. In that manner, we obtained a "surface" of neuronal response for applied substances (Figure 1Ba). In the case of non-occurrence of hyperpolarization, we considered 15 s of activity after the application of tested substances or physiological saline. After application of menthol, RSH was equal to −793.39 ± 243.35 mV*s (Figure 1Bb) compare to −35.65 ± 30.55 mV*s in the control. The difference was statistically significant with $p = 0.0037$. The negative value of RSP for the control was due to the significant after-hyperpolarization (one of the phases) of each action potential.

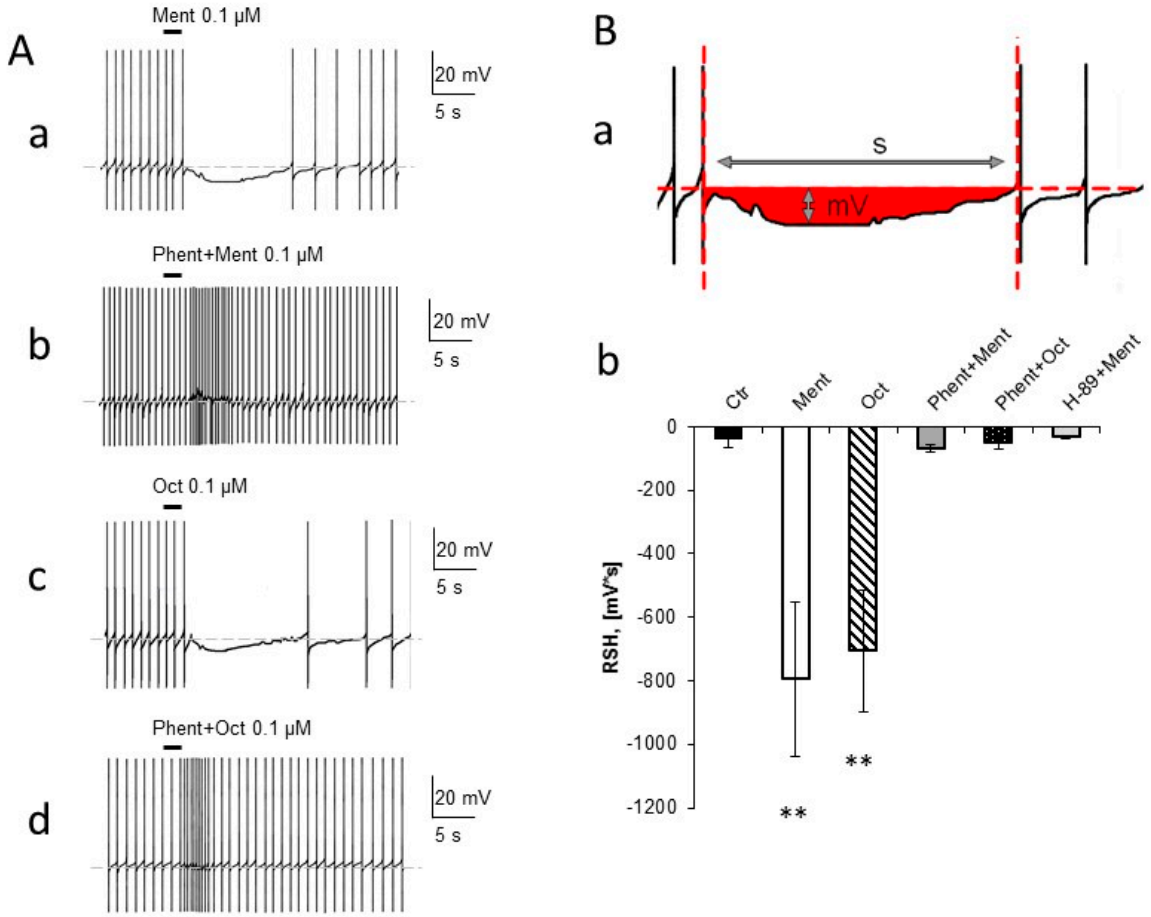

Figure 1. Menthol and octopamine change the electrophysiological properties of dorsal unpaired median (DUM) neurons. (**A**) Representative original recordings of spontaneous action potentials of DUM neurons: (**a**) deep hyperpolarization and switching off of the spontaneous potentials as a result of the application of menthol (0.1 µM); (**b**) phentolamine (10 µM) blocked the hyperpolarization caused by menthol; (**c**) octopamine (0.1 µM) caused the same effect as menthol; (**d**) phentolamine (10 µM) blocked the hyperpolarization caused by octopamine. (**B**) (**a**) The representation of RSH (Relative Size of Hyperpolarization) value. Size of DUM

neurons response (shown in red) to menthol and octopamine application was expressed quantitatively by RSH—value of hyperpolarization (mV) was multiplied by time (s) of its duration to obtain a response surface area. (**b**) Effect of menthol (0.1 µM, Ment) and octopamine (0.1 µM, Oct) which were reversed by phentolamine (10 µM, Phent + Ment; Phent + Oct). Inhibitor of PKA – H-89 (1 µM) abolished the hyperpolarization caused by menthol (H-89 + Menth). High negative values correspond to deep and long hyperpolarization. The data is presented as mean values ± SE, $n = 10$. The statistically significant differences between control and tested substances are marked: ** $p < 0.01$.

Very similar observations were made for 0.1 µM octopamine (Figure 1(Ac,Bb)), which caused hyperpolarization related to the lack of ability to generate action potentials. RSH value after its application was equal to −705.45 ± 190.47 mV*s and the difference with the control was significant with $p = 0.0032$. There was no difference with menthol trial.

We assumed that menthol acted through the octopamine receptors. To verify that hypothesis, we performed experiments by preincubating for 5 min ganglia with phentolamine which is an octopamine receptor antagonist. Phentolamine (10 µM) completely abolished the effect of menthol as well as octopamine (Figure 1(Ab,Ad,Bb)). RSH value for menthol in the presence of phentolamine was equal to −66.55 ± 12.30 mV*s and was statistically not different from the control.

One of the possible effects of activating octopamine receptor is the triggering off the PKA signaling pathway. In the next step, we performed an electrophysiological experiment by pretreating ganglia with H-89 (1 µM) which is an inhibitor of PKA. H-89 applied 5 min before menthol, completely blocked its effect and gave an RSH value of −34.46 ± 4.43 mV*s (Figure 1Bb).

2.2. Calcium Imaging

The activation of octopamine receptors can lead to an increase in intracellular calcium level, therefore we performed calcium imaging on dissociated DUM neurons, as seen in Figure 2. In control conditions, the signal resulting from binding free calcium ions with Oregon BAPTA-1 indicator was very low—it was equal to 6.65 ± 0.69 MGV and was observed mainly in the vicinity of the cell membrane (Figure 2A,C). Applied substances changed calcium signal ($F_{2,42} = 8.036$, $p = 0.001$). Application of 0.1 µM menthol in bath resulted in the increase in calcium signal to 13.92 ± 1.95 MGV, $p = 0.00013$. The highest signal intensity was observed near the cell membrane (Figure 2B,C). Since H-89 completely blocked the effect of menthol in electrophysiological studies, we decided to test its effect on menthol-induced increase of calcium level. After pre-treating cells with the PKA inhibitor, the intensity of fluorescence was equal to 6.93 ± 0.72 MGV and did not differ from control.

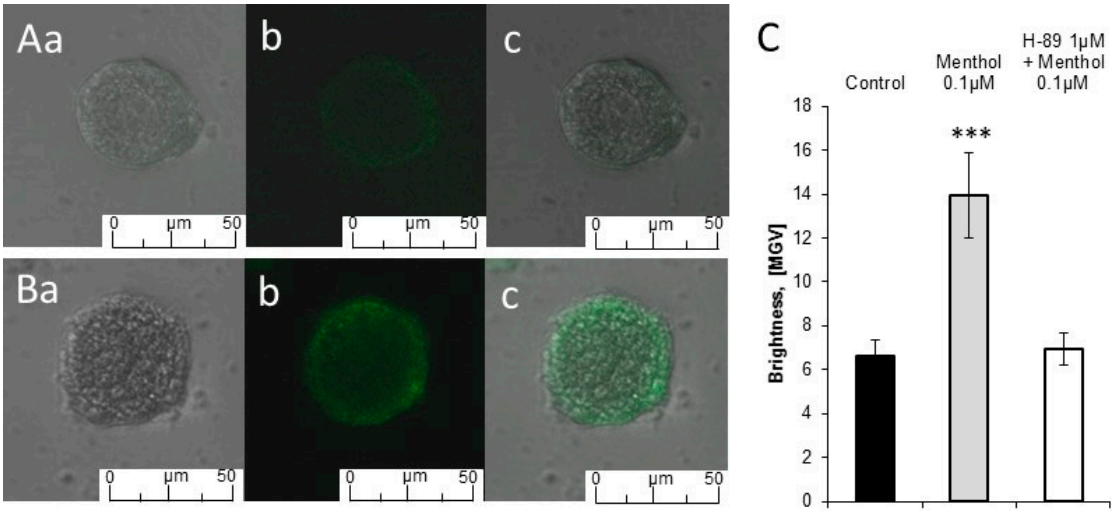

Figure 2. Menthol changed the level of free calcium ions in DUM cells. The free calcium ions were

labeled by Oregon Green BAPTA-1 and observation of fluorescence of the living cells was made using a confocal microscope Leica TCS SP8 (excitation at 308 nm, detection at 488 nm - green fluorescence). (**A**) Control confocal fluorescence images of DUM neuron and (**B**) after incubation with menthol 0.1 μM. (**a**) The bright-field transmission images; (**b**) the fluorescence transmission images; and (**c**) merged transmission images of both control and treated DUM neurons respectively. Note a high discrepancy between signal in control neurons and after application of menthol. Scale bars are 50 μm. (**C**) Numerical representation of free calcium level in DUM neurons in control, after application of menthol 0.1 μM alone, and preincubated with H-89. The data is presented as mean values ± SE. The statistically significant differences between control and tested substances are marked: *** $p < 0.001$.

2.3. Acetylcholinesterase Activity

In our previous work [18], in electrophysiological experiments on the cockroach escape system, we showed that menthol increased the efficacy of bendiocarb insecticide which is an AChE inhibitor. In this study, we analyzed bendiocarb efficiency in the presence of menthol on AChE enzymatic level. In control conditions, the activity of AChE obtained from dissociated terminal abdominal ganglia (TAG) of *P. americana* was equal to 1.86 ± 0.16 μmol/mg protein (Figure 3B). Menthol applied in concentration ranges 0.1 nM–100 μM did not significantly change AChE activity (Figure 3B) ($F_{8,16} = 1.26$, $p = 0.33$).

Tested substances changed activity of AChE enzyme ($F_{25,50} = 39.19$, $p < 0.001$). Bendiocarb inhibited AChE activity with $ED_{50} = 0.10$ μM and with a maximum effect of 88.76% of inhibition for 1 μM concentration. The lowest tested concentration of bendiocarb which caused enzyme inhibition (8.4%) was 0.05 μM (Figure 3A). Application of menthol (0.1 μM) shifted the dose-inhibition curve of bendiocarb toward lower concentrations and decreased by 36% ED_{50} value, which was equal to 0.074 μM. The maximum observed effect for 1 μM bendiocarb in the presence of menthol was 92.96% of inhibition and for the lowest tested bendiocarb concentration – 0.05 μM it was 35.7%. The increase in bendiocarb efficacy in the presence of menthol was statistically significant; activity of AChE in the presence of bendiocarb (0.5 μM) was equal to 18.87 ± 2.76% of native value and with addition of menthol (0.1 μM), it was equal to only 11.43 ± 2.62%; $p = 0.022$ (Figure 3(Aa,Ab),C).

The pre-incubation of ganglia with phentolamine (10 μM) and H-89 (1 μM) abolished the potentiating effect of menthol, leading to ED_{50} values of 0.11 and 0.12 μM for bendiocarb in the presence of menthol with the two respective inhibitors. The AChE activity in the presence of phentolamine (10 μM), menthol (0.1 μM) and bendiocarb (0.5 μM) was equal to 23.98 ± 0,99% of initial value and was higher than that of bendiocarb alone (18.87%) although not significantly. The AChE activity in the presence of H-89 (1 μM), menthol (0.1 μM), and bendiocarb (0.5 μM) was equal to 27.25 ± 2.47% of initial value and was higher than that of bendiocarb alone (18.87%) with $p = 0.01$ (Figure 3(Aa,Ab),C).

The pre-incubation of ganglia with bisindolylmaleimide IX (iPKC), an inhibitor of protein kinase C signaling pathway, did not have any effect on the action of menthol with the ED_{50} value being equal to 0.08 μM. The AChE activity in the presence of iPKC (1 μM), menthol (0.1 μM), and bendiocarb (0.5 μM) was equal to 12.89 ± 2.29% of initial value and was lower than that of bendiocarb alone (18.87%) with $p = 0.024$ (Figure 3Ab,C). The above values were similar to those of the menthol trial.

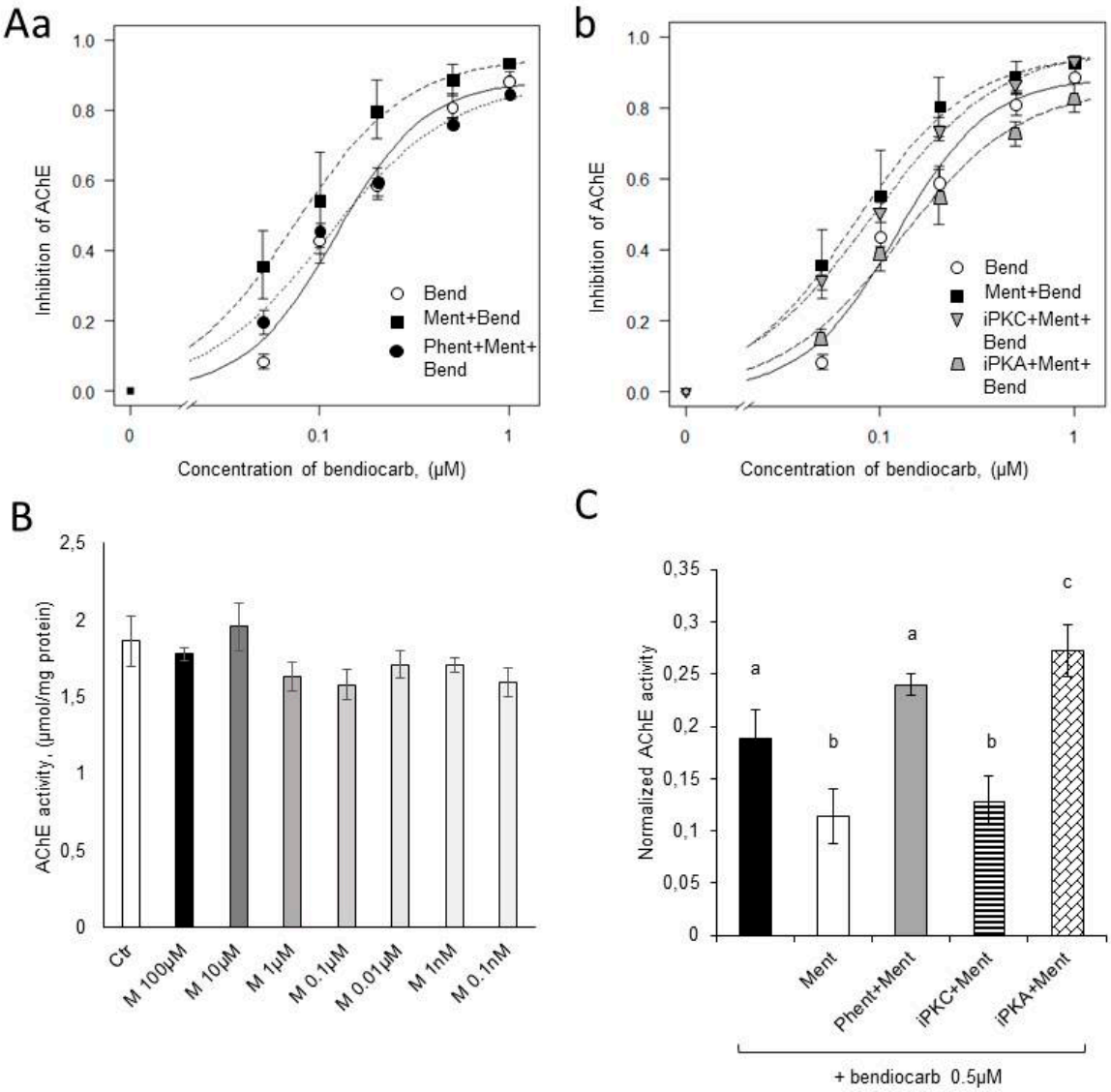

Figure 3. Biochemical analysis of acetylcholinesteraze (AChE) activity and its inhibition by bendiocarb insecticide. (**A**) Dose-inhibition curves representing inhibitory effect of bendiocarb on AChE activity: (**a**) Dose-dependency of AChE inhibition caused by bendiocarb (Bend) was shifted to its lower concentrations in the presence of menthol (0.1 μM, Ment + Bend). The effect of menthol was reversed by phentolamine (10 mM, Phent + Ment + Bend); (**b**) Dose-dependency of AChE inhibition caused by bendiocarb (Bend) was shifted to its lower concentrations in the presence of menthol (0.1 μM, Ment + Bend). The effect was reversed by protein kinase A inhibitor, H-89 (1 μM, iPKA + Ment + Bend) but not by protein kinase C inhibitor, bisindolylmaleimide IX (1 μM, iPKC + Ment + Bend). (**B**) Menthol applied in different concentrations (M) did not changed the AChE activity compared to control (Ctr). (**C**) Inhibition of AChE activity by bendiocarb (0.5 μM, black bar) and the changes caused by: menthol (0.1 μM, Ment); phentolamine (10 μM) with menthol (0.1 μM, Phent + Ment); inhibitor of protein kinase C, bisindolylmaleimide IX (1 μM) with menthol (0.1 μM, iPKC + Ment); and the inhibitor of protein kinase A, H-89 (1 μM) with menthol (0.1 μM, iPKA + Ment). The different letters above the bars refer to statistically significant differences between the data with $p < 0.05$.

3. Discussion

Cooperativity between essential oils and chemical insecticides in the control of pest insects becomes more and more interesting in modern pest management. Recent studies evaluated synergistic interaction between essential oils and chemical insecticides [37–39].

In our previous paper [18], it was demonstrated that menthol potentiates the effect of the carbamate insecticide, bendiocarb. As a possible factor increasing the efficiency of bendiocarb, the activation of octopamine receptors by menthol in the nervous system of the cockroach was proposed. However, the mechanism of this enhancement remained unclear. The aim of our new study was to obtain a deeper insight into this mechanism. Experiments were performed on cockroach DUM neurons, which are known to be octopaminergic.

Octopamine serves as a neurotransmitter, neurohormone, and neuromodulator in insects [40–43] although it is only a "trace amine" in vertebrates [28,43,44]. Octopamine receptors are G protein-coupled receptors. Their activation turns on cellular signal transduction pathways and changes various insect organism functions. Due to their key role being limited to invertebrates, they were tested as targets for insecticides: formamidine pesticides and plant essential oils [23,45].

Sensitivity of DUM neurons to octopamine was described previously. It has been observed that the electrical activity of isolated DUM neurons is regulated by octopamine in concentration dependent manner [46,47]. In our experiments on DUM neurons in situ, the ejection of octopamine induced rapid and short-lasting hyperpolarization of membrane potential and inhibition of spontaneous discharges. After several seconds, spontaneous action potentials were again generated. Highly similar effects were observed after the application of menthol and it was abolished by phentolamine, an octopamine receptor inhibitor. Identity in effects of octopamine and menthol on DUM neurons electrical activity confirmed the statement presented in our previous paper [18] that menthol activates octopaminergic receptors.

"Pacemaker potential"—depolarizing changes of membrane potential occurring during intervals between spikes—is "driven" by several calcium ion currents: low voltage activated (LVA) Ca^{2+} currents [48]; mid/low voltage (M-LVA) activated Ca^{2+} current [47]; and high voltage activated (HVA) Ca^{2+} current [36,47,48]. Between LVA Ca^{2+} currents, the maintained current permeable to Na^{+} and Ca^{2+} (mLVA Na^{+}/Ca^{2+}) has been described in DUM neurons [49]. It plays an essential role in regulating DUM neurons spontaneous discharge frequency [50]. Study of Lapied et al. [50] demonstrated that the application of octopamine on isolated DUM neurons decreased the amplitude of mLVA Na^{+}/Ca^{2+} via an increase in internal cAMP level and activation of protein kinase A (PKA). PKA activation induces a negative regulatory action on mLVA Na^{+}/Ca^{2+} by phosphorylation of DARPP-32 protein. The effect of octopamine on mLVA Na^{+}/Ca^{2+} was abolished by phentolamine.

We propose the mLVA Na^{+}/Ca^{2+} current to be one of the targets for the menthol action. The PKA inhibitor (H-89) has been shown to reverse the effects of octopamine on mLVA Na^{+}/Ca^{2+} current [50]. In our experiments, applying of H-89 in the bath eliminated the effect of menthol on the electrical activity of DUM neurons. The stimulation of octopamine receptors in *Periplaneta americana* causes the activation of adenylyl cyclase, increase of cAMP level, and activation of PKA [29]—factors which reduce the amplitude of mLVA Na^{+}/Ca^{2+} current [50]. When the depolarizing current was inhibited, hyperpolarization should occur—this was observed in our experiments. After blocking the PKA, its negative regulatory action was abolished and the effect of menthol was no longer observed.

The hyperpolarization state induced by octopamine and menthol can lead to activation of tLVA Ca^{2+}, which is normally activated at membrane potential of -70 mV and is involved in the initial part of the pre-depolarization phase of the activity of DUM neurons [48]. Depolarizing tLVA Ca^{2+} may cause a return of membrane potential from deep hyperpolarization to normal potential level after octopamine and menthol. Moreover, the hyperpolarization induced by octopamine and menthol can also increase the resting Ca^{2+} current that will participate in the return to the inter-spikes membrane potential level [51].

Activation of octopamine receptors, (Pa oa_1) from *Periplaneta americana*, OAMB from *Drosophila melanogaster*, and CsOA1 from hemocytes of *Chilo suppressalis*—induces an increase in both cAMP level and internal Ca^{2+} concentration [29,52–54]. In our experiments, we measured the intracellular level of Ca^{2+} in DUM neurons after applying menthol and observed a significant increase in its level. Intracellular Ca^{2+} concentration was indicated as a „key factor" in the regulation of ability to generate spontaneous action potentials in DUM neuron [36]. Elevated Ca^{2+} concentration after the activation

of octopamine receptors by menthol could induce modulatory effects on DUM neuron membrane ionic channels.

We consider the rise in Ca^{2+} level as secondary effect, not resulting from the direct activity of octopamine receptors. The increase in calcium level as a result of octopamine receptor has been evidenced [29,55,56], although we observed calcium rise which was blocked by protein kinase A inhibitor. We assume that menthol causes the activation of adenylyl cyclase and thus increase in cAMP level which activates PKA. The activities of PKA and adenylyl cyclase are highly regulated; cAMP can directly open the non-selective cation channels and cause Ca^{2+} to enter the cells [57,58]. The role of increasing level of Ca^{2+} ions is the regulation of adenylyl cyclase activity and thus the constitution of a negative feedback for PKA [59–62]. PKA is also responsible for up-regulation of M-LVA channel and voltage-independent Ca^{2+} resting current and thus increase the amount of calcium entering the cells which can be a reason for the rise in PKA-dependent calcium [63].

On the other hand, increase in Ca^{2+} level could indicate the involvement of PKC in the action of menthol. However, there is a lot of data indicating that PKA and PKC pathways exclude themselves and negatively regulates each other [64–67] and that cAMP inhibits PKC [68,69]. Our biochemical experiments (discussed later) confirm that PKC is not involved in the described mechanism. The inhibitor of PKC did not modify the effect of menthol on efficiency of bendiocarb.

In toxicity tests on the cockroach and experiments on its whole nervous system done during our previous study, we demonstrated that menthol increases the activity of bendiocarb—a carbamate—as an insecticide [18]. The experiments carried out on DUM neurons presented in this paper, allowed us to conclude that menthol activates the PKA signaling pathway through octopamine receptors and also helped demonstrate an increase in Ca^{2+} concentration. The modifications of the sensitivity of various molecular targets to insecticides by phosphorylation/dephosphorylation processes are already known [13,16,17,70–72]. The aim of the last part of our study was to determine the modification of bendiocarb efficiency by menthol at the level of enzymatic activity of AChE. In biochemical experiments, presence of menthol decreased ED_{50} of bendiocarb by 36% which closely correlates with results obtained in cockroach toxicity tests and in tests on its whole nerve cord [18]. Phentolamine eliminated the menthol-induced potentiation, which clearly indicates the involvement of octopamine receptors in its action. Moreover, bendiocarb was less potent in the presence of octopamine receptor antagonist (however, not significantly), which can indicate that some small level of octopamine is essential for normal activity of the enzyme. It is consistent with our previous results where phentolamine completely blocked the effect of bendiocarb on the whole nerve cord preparation [18]. The use of PKC inhibitor did not modify the effect of bendiocarb applied together with menthol. This suggests that the PLC signaling pathway is not involved in the straightening effect of menthol. However, the PKA inhibitor completely eliminated the effect of menthol and significantly reduced the efficiency of bendiocarb applied alone. These results clearly indicate that the potentiating effect of menthol is accomplished by the PKA signaling pathway and that phosphorylation of AChE is necessary to achieve the enhancement of bendiocarb effect by menthol. Some reports indicate that PKA, but not PKC, is responsible for phosphorylation of AChE [73].

The decrease in inhibitory activity of bendiocarb on AChE in the presence of H-89 compared to the action of bendiocarb alone confirms the conclusion from experiments with phentolamine. It can be explained by the fact, that some state of phosphorylation of AChE is required for its sensitivity for modulators, such as bendiocarb.

In this paper, we have confirmed the hypothesis that menthol increases bendiocarb efficacy and that its action is mediated through an octopamine receptor. We have shown first evidences that protein kinase A is involved in the menthol effect. We have also shown that menthol increases Ca^{2+} level and finally that protein kinase C does not participate in this action of menthol. Obtained results can be used in development of modern insecticide formulas, less harmful for human and non-target animals and more potent against insect-pests.

4. Materials and Methods

4.1. Insects

All experiments were performed on preparations from males of the cockroach *Periplaneta americana*. The insects were obtained from our breeding program. The animals were kept in temperature of 27–29 °C with access to water and food (cat food, oatmeal and apples) ad libitum. Insects were transferred to the laboratory 24 h before experiment for adaptation.

4.2. Reagents

Bendiocarb (Pestanal, analytical standard), DL-octopamine hydrochloride (≥95.0%), phentolamine hydrochloride (≥98.0%, TLC), and (±)-menthol (racemic ≥ 98.0%) were purchased from Sigma Aldrich (Saint Luis, MI, USA). They were dissolved in ethanol to a concentration of 10 mM and next serial dilutions were made in physiological saline. H-89 and bisindolylmaleimide IX were purchased from Abcam (Cambridge, United Kingdom). Chemicals were dissolved in DMSO to concentration 10 mM and then the serial dilutions were prepared in physiological saline.

Physiological saline contained in mM: NaCl – 210, KCl – 3.1, $CaCl_2$ – 5, $MgCl_2$ – 5.4, and Hepes – 5. The pH = 7.2 was adjusted with NaOH. Hepes were purchased from Sigma Aldrich. The physiological saline components—NaCl, KCl, $MgCl_2$, $CaCl_2$, ethanol 96%, and DMSO—were obtained from Polskie Odczynniki Chemiczne SA (Gliwice, Poland).

Acetyltiocholine chloride (99%, TLC, Sigma Aldrich) was dissolved in physiological saline to 0.1 mM. The solution stopping AChE reaction was composed of 1 mM 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) (Thermo Fisher, Waltham, MA, USA) and 2% sodium dodecyl sulfate (SDS) (Thermo Fisher) diluted in water. Collagenase (from *Clostridium histolyticum*, type XI) was purchased from Sigma Aldrich and was diluted to final concentration 2 mg/mL in physiological saline. Streptomycin/penicillin (Roche) (50,000 U/mL penicillin G and 50 mg/mL streptomycin (as sulfate) in 0.9% NaCl) were dissolved in physiological saline to working solutions of 100 IU/mL penicillin, 100 μg/mL streptomycin. Bovine serum (Sigma Aldrich) was diluted to 1% in physiological saline. Oregon Green BAPTA-1 (Thermo Fisher) was diluted in DMSO to 40 μM stock solution and then diluted in physiological saline to a working solution of 5 μM.

4.3. Electrophysiological Experiments

Electrophysiological recordings were performed on DUM neurons in situ in terminal abdominal ganglia (TAG) from the ventral nerve cord of the insect. The ganglia were dissected with micro-scissors. The protein-lipid sheets covering the ganglia were removed to allow chemicals to get to the neurons inside the ganglia and facilitate insertion of the microelectrodes. The TAG were then moved to Petri dishes filled with sylgard-polymer, to which the TAG were mounted with entomological needles. The ganglia were covered with physiological saline all the time. Recordings of bioelectric signals in DUM neurons were performed using glass microelectrodes with resistance equal to 30–40 MΩ. The electrophysiological set-up was consisted of: registration microelectrode filled with 3 M KCl, reference electrode placed in physiological saline near the preparation, headstage (HS-2A, Gain: 10 MGU; Axon Instrument's, Sunnyvale, CA, USA), amplifier Axoclamp 2A (Axon Instrument's) and oscilloscope (Hameg HM 507). Recordings were transferred to a computer and processed by modified Hameg software (version 6.0, Toruń, Poland).

In each experiment, the control recordings were made (for 5 min) after 10 min of preparation stabilization when spontaneously generated discharges had been stable in amplitude and frequency and the membrane potential had changed only in a range of 2–3 mV. Then tested substances (menthol and octopamine) were applied by fast ejection (Picoliter Microinjector PLI-100A, Warner Instruments, Holliston, MA, USA) in close vicinity of the ganglia to obtain the final 0.1 μM concentration of substances. In the case of preincubation, phentolamine (10 μM) H-89 (1 μM) were slowly applied by perfusion 5 min before introducing the tested substances.

The collected data was further analyzed using R software [74]. The results were expressed as mean values ± SE and the comparison of several data groups was made using one-way ANOVA. The differences between groups were tested by Tukey's post-hoc tests.

4.4. Acetylcholinesterase Activity—Biochemical Tests

AChE activity was determined using modified Ellman's method [75]. For biochemical experiments, 6 TAGs were dissected and placed on ice in tube containing 2 mL of physiological saline. The ganglia were then incubated with Collagenase IX in concentration of 2 mg/mL for 30 min in temperature of 30 °C. After incubation, the ganglia were transferred to cold (4 °C) physiological saline and rinsed twice to stop collagenase enzymatic digestion. Depending on the combination of tested substances, the different samples (shown in Table 1) of ganglia were prepared. In the next step, dissociation of already treated ganglia were made – they were passed through series of glass pasteur pipets with decreasing diameters since tissue fragments were no longer visible. Solution prepared in this way contained some whole nerve cells and fragments of cells with AChE enzyme activity. 90 µL of solution was placed in each well in 96-well plates with flat bottoms on ice. Solution containing AChE enzyme was incubated with the substrate, acetyltiocholine for 30 min in temperature of 30 °C. The reaction was stopped by 1 mM 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) with 2% sodium dodecyl sulfate (SDS). In variants of the experiments where efficiency of bendiocarb was estimated, tissue solution was incubated with insecticide 15 min before starting the reaction with acetyltiocholine. The amount of protein in solution was determined using Bradford method [76]. Calibration curve was always prepared at the same time as experiment. 10 min after stopping the reaction, the 96-well plate was read in microplate reader (BioTek, Epoch, Winooski, VT, USA) with wave lengths of 406 nm for Ellman's reaction and 595 for Bradford's reaction.

Table 1. Samples composition in biochemical tests.

Sample Name	Physiological Saline	Menthol (µM)	Bendiocarb (µM)	Phentolamine (µM)	iPKC (µM)	iPKA (µM)
Control	+					
Ment		0.0001, 0.001, 0.01, 0.1, 1, 10, 100				
Bend			0.05, 0.1, 0.2, 0.5, 1			
Ment + Bend		0.1	0.05, 0.1, 0.2, 0.5, 1			
Phent + Ment + Bend		0.1	0.05, 0.1, 0.2, 0.5, 1	10		
iPKC + Ment + Bend		0.1	0.05, 0.1, 0.2, 0.5, 1		1	
iPKA + Ment + Bend		0.1	0.05, 0.1, 0.2, 0.5, 1			1

Each replication was prepared using 6 ganglia. The experiment was replicated 3 times. The collected data was further analyzed using R software [74]. The results were expressed as mean values (from 3 replications) ± SE and the comparison of several data groups was made using one-way ANOVA. The differences between groups were tested by Tukey's post-hoc tests. The dose-response curves were established using 'drc', 'sandwich' and 'lmtest' [77–79].

4.5. Calcium Imaging

The determination of calcium level in living cells was performed on dissociated DUM neurons. 6 ganglia were dissected and placed in a tube containing 2 mL of physiological saline with streptomycin/penicillin in sterile conditions. The ganglia were then incubated with sterile Collagenase IX in concentration of 2 mg/mL for 30 min in temperature of 30 °C. The enzymatic activity was stopped by rinsing the ganglia with sterile physiological saline containing antibiotics. The ganglia were then moved to the physiological saline containing bovine serum and antibiotics. In the next step, the ganglia were dissociated as previous described, however, in sterile conditions all the time. Obtained medium

with DUM cells was divided into 2 Corning Petri dishes (covered earlier with poli-D-lysine (50 μg/mL)) and filled with sterile medium to 2 mL. The cells were incubated in temperature of 30 °C for 24 h to ensure good adhesion. After 24 h physiological saline with bovine serum and antibiotics was removed and the DUM cells were covered by sterile physiological saline.

Fluorescent marker of free calcium ions, Oregon Green BAPTA-1 at a concentration of 5 μM was applied for 1 h in the dark. Next, the liquid was removed, and cells were rinsed with fresh physiological saline. Firstly, the control observations were made. Menthol 0.1 μM was then applied in the bath and after 15 min the cells were observed. In case of pre-incubation with H-89 1 μM, the inhibitor was applied 10 min before menthol. The images were captured with a Leica TCS SP8 confocal microscope using an argon-ion laser emitting light at a wavelength of 488 nm (408 nm excitation wave). Optimized pinhole, long exposure time (400 kHz), and 20X (numerical aperture, 1.4) Plan Apochromat DIC H lens were used. For the quantitative measurements, each experiment was performed under condition of consistent temperature, incubation time, and concentrations of probes. The images were collected under consistent conditions of acquisition (low laser power at 3%, emission band, gain, and resolution) to ensure comparable results. For bleed-through analysis and control experiments, LASX program (Leica Application Suite X) software was used. Between 10 and 20 cells were analyzed for each experimental variant. The level of fluorescence was expressed in arbitrary units (as the mean intensity per μm^2). The statistical analysis was performed using SigmaPlot 11.0 software. The statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test ($p < 0.01$ or $p < 0.001$) to compare effects of different treatments.

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6. Conclusions

The presented research aimed to verify the main hypothesis that menthol increases efficacy of bendiocarb - carbamate insecticide and to clarify mechanism of its potentiating effect.

Using several methods: toxicity tests, electrophysiological and biochemical experiments, and fluorescent microscopic imaging, it was possible to define the following facts:

1. Menthol is moderately toxic for cockroach *Periplaneta americana*. It has no lethal effect and causes only minor paralysis, which can result from reduced activity of nervous system, observed in the study.
2. Menthol acts as an octopamine receptor agonist, and its effect is carried by activating protein kinase A and increasing intracellular calcium level.
3. Menthol potentiates bendiocarb effectiveness: it increases toxicity of the carbamate insecticide against *Periplaneta americana* on every level of organism organization – from entire insect to enzyme activity.

These findings allow the final conclusions to be established:

Essential oil component - menthol, potentiates carbamate insecticide - bendiocarb, effectiveness.

Observed potentiation is mediated by activating octopamine receptors, increasing of Ca^{2+} level and subsequently activating the protein kinase A, but not protein kinase C.

The findings of this thesis are important in the absence of effective and highly safe methods of insect-pest control. The search for new substances with an insecticidal effect requires a lot of work and financial resources, and here the widely known substance – menthol – showed moderate insecticidal properties, and more importantly, potentiates the effectiveness of an insecticide used and approved by world authorities.

Based on these conclusions, practitioners should consider using menthol in low concentrations before treatments with carbamates insecticides, which will allow to decrease the effective doses of insecticides.

To better understand the implications of these results, further studies could address the problem of other essential oil components mechanisms of action.

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Streszczenie pracy doktorskiej pt.: "Mentol, składnik olejków eterycznych, czynnikiem podnoszącym efektywność bendiokarbu, insektycydu z grupy karbaminianów"

Owady szkodniki powodują strat w żywności, niszczą lasy, przenoszą choroby zakaźne oraz zanieczyszczają miejsca zamieszkałe przez ludzi. Przez ostatnie dekady obserwuje się wzrost liczebność owadów szkodników, równocześnie zwiększa się ilość stosowanych insektycydów. Znaczący, negatywny wpływ insektycydów na człowieka oraz populacje zwierząt sprawił, że niezbędne stały się nowe metody kontroli liczebności owadów szkodników. Strategia, możliwa do wykorzystania natychmiast, polega na zwiększaniu efektywności dostępnych już insektycydów, nie podnosząc przy tym ich negatywnego wpływu na ekosystemy i człowieka. Innowacyjne podejście w projektowaniu nowych metod walki z owadami szkodnikami skupia się na wykorzystaniu substancji aktywujących receptory sprzężone z białkami G (GPCR) jako środków synergistycznych. W przedstawionej rozprawie doktorskiej badania zostały skoncentrowane na jednym z receptorów GPCR – receptorze oktopaminy. Potencjalnie niektóre składniki olejków eterycznych mogą mieć działanie oktopaminergiczne. Szczegółowy opis działania olejków eterycznych na receptory oktopaminy został przedstawiony w Artykule I, będącym częścią niniejszej rozprawy.

Celem prezentowanych badań była weryfikacja hipotezy, mówiącej, że ***obecność mentolu, składnika olejków eterycznych, zwiększa efektywność bendiokarbu, insektycydu z grupy karbaminianów u owadów***. Następnie kolejne dwie hipotezy alternatywne zostały postawione: 1) ***wzmacniający efekt mentolu na działanie bendiokarbu jest wynikiem aktywacji receptorów oktopaminy a następnie aktywacji białkowej kinazy A, co powoduje fosforylację enzymu AChE i zmiany w jego wrażliwości na inhibitor karbaminianowy*** lub 2) ***wzmacniający efekt mentolu na działanie bendiokarbu jest wynikiem aktywacji receptorów oktopaminy a następnie aktywacji białkowej kinazy C, co powoduje fosforylację enzymu AChE i zmiany w jego wrażliwości na inhibitor karbaminianowy***.

Eksperymenty prowadzące do weryfikacji hipotez zostały zaprezentowane w dwóch oryginalnych pracach badawczych: Artykuł II i III, które wraz z Artykułem I składają się na prezentowaną rozprawę doktorską. Przeprowadziłam wielowątkową analizę, na którą składały się: testy toksyczności (ocena możliwości motorycznych karaczana *Periplaneta americana* oraz ocena efektu letalnego/"knock-down" testowanych substancji); testy

elektrofizjologiczne (zewnątrzkomórkowa rejestracja z łańcuszka nerwowego *P. americana* oraz rejestracja mikroelektrodowa z neuronów DUM *in situ* w ostatnim zwoju odwłokowym); testy biochemiczne (ocena aktywności acetylocholinesterazy pozyskanej z *P. americana*) oraz obrazowanie poziomu wapnia w neuronach DUM.

Doświadczenia potwierdziły główną hipotezę, mówiącą, że: **mentol zwiększa efektywność bendiokarbu na owady**. Została ona potwierdzona na poziomie całego owada, gdzie mentol redukował czas pojawienia się paraliżu oraz zwiększał ilość porażonych owadów. Testowany składnik olejków eterycznych potęgował nienaturalne wybuchy potencjałów czynnościowych wywołane bendiokarbem, obserwowane w nerwach owada. Mentol wzmacniał hamowanie enzymu acetylocholinesterazy, wywołane bendiokarbem. Stwierdzono, że działa on za pośrednictwem receptorów oktopaminy. Mentol hiperpolaryzował neurony DUM oraz hamował typową dla tych neuronów aktywność spontaniczną. Efekty te były porównywalne do efektu oktopaminy i były znoszone przez fentolaminę. Co więcej, mentol powodował wzrost wewnątrzkomórkowego poziomu wapnia w neuronach DUM. Wszystkie efekty mentolu były znoszone przez inhibitor białkowej kinazy A.

Przeprowadzone badania pozwoliły na sformułowanie drugiego wniosku: **mentol podnosi efektywność bendiokarbu poprzez aktywację receptorów oktopaminy oraz szlaku białkowej kinazy A**. Stwierdzone i opisane zależności mogą być punktem wyjścia do projektowania nowych, efektywnych mieszanin insektycydów jak również mogą mieć wpływ na przyszłość zwalczania owadów szkodników.

Abstract of PhD thesis, entitled: "Menthol, essential oils component, as a factor increasing effectiveness of bendiocarb insecticide"

Insect pests destroy crops and forests, limit food supplies, transmit infectious diseases, and give rise to hygienic problems in places inhabited by man. Excessive rise in insect pest population and the application of insecticides have been observed in the last decades. Significant negative impact of some insecticides on non-target animal populations and humans has created the need to seek new methods to control insect pests. A sensible strategy that can be used straight away is to increase the effectiveness of available insecticides without increasing their negative impact on ecosystems and people. Innovative approaches in insect pest control focus on using activators of GPCRs receptors as synergistic agents. In the presented doctoral dissertation, emphasis was placed on one of the GPCRs – octopamine receptors. Potential action on octopamine receptors was reported for some components of essential oils. Detailed description of essential effects action on octopamine receptors was presented in Article I, which is part of the presented dissertation.

The aim of the presented study was to verify the hypothesis that *the presence of essential oils component, menthol, increases the efficiency of the carbamate insecticide, bendiocarb (inhibitor of acetylcholinesterase enzyme - AChE) on insects*. Further, the next two alternative hypothesis were put forward: 1) *the potentiating effect of menthol on bendiocarb activity occurs through the activation of octopamine receptors and then activation of protein kinase A, which leads to phosphorylation of AChE enzyme and alteration in its sensitivity to the carbamate inhibitor*; or 2) *the potentiating effect of menthol on bendiocarb occurs through the activation of octopamine receptors and then activation of protein kinase C, which lead to phosphorylation of AChE enzyme and alteration in its sensitivity to the carbamate inhibitor*.

Experiments leading to the verification of these hypotheses were presented in two original Articles, II and III, which together with Article I make up the presented dissertation. The author conducted multi approach analysis, which consisted of: toxicity tests (evaluation of motor abilities of cockroach *Periplaneta americana* and assessment of lethal/knock down effect of substances); electrophysiological tests (extracellular recordings from *P. americana* nerve cord and microelectrode recordings from the dorsal unpaired median (DUM) neurons *in-situ* in the last abdominal ganglion); biochemical tests (evaluation of acetylcholinesterase activity); and calcium imaging.

The experiments confirmed the main hypothesis **that menthol increases bendiocarb efficacy in insects**. It was proven on the level of the entire insect, where menthol reduced the time paralysis appeared and increased the number of knocked-down insects. Menthol also increased the abnormal burst of action potentials evoked by bendiocarb observed in cockroach nerves. Menthol enhanced the inhibition of acetylcholinesterase enzyme induced by bendiocarb. It was also possible to confirm that menthol effect depends on octopamine receptors. Menthol hyperpolarized DUM neurons and inhibited the spontaneous activity typical for these neurons - these effects were comparable to octopamine activity and they were abolished by phentolamine. Moreover, menthol induced a rise in intracellular calcium level in DUM neurons. All effects of menthol were abolished, when the inhibitor of protein kinase A was applied.

Thus, the second conclusion was made: **menthol increases bendiocarb efficacy through the activation of octopamine receptors and protein kinase A pathway**. These findings could be the starting point to design new and effective insecticide mixtures and may have impact on the future of insect pest management.

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Oświadczenie

Oświadczam, że mój udział w realizacji pracy: Jankowska M., Wisniewska J., Fałtynowicz Ł., Lapied B., Stankiewicz M., (2019) Menthol increases bendiocarb efficacy through activation of octopamine receptors and protein kinase A. Molecules, 24(20), 3775 polegał na przygotowaniu protokołów doświadczeń, wykonaniu eksperymentów, przeprowadzeniu analizy danych, pisaniu manuskryptu oraz uzyskaniu finansowania badań . Udział swój oceniam na 70%.

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I hereby declare that my contribution to the article « The unusual action of essential oil component, menthol, in potentiating the effect of the carbamate insecticide, bendiocarb » - Jankowska et al., Pestic. Biochem. Physiol. 2019, 158, 101 was 10% (conceptualization, writing-review and editing) and to the article « Menthol Increases Bendiocarb Efficacy Through Activation of Octopamine Receptors and Protein Kinase A » - Jankowska et al., Molecules 2019, 24, 3775 was 10% (conceptualization, writing-review and editing).

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