Menthol acts as a positive allosteric modulator on nematode levamisole sensitive nicotinic acetylcholine receptors

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Abstract
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Keywords
Menthol, allosteric modulation, nAChR, monoterpenoid, nematode

Disciplines
Entomology | Natural Products Chemistry and Pharmacognosy | Plant Sciences

Comments

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ACh + 0.1 µM Menthol + 10 µM Menthol

**pEC\textsubscript{50}**

- ACh
- + 0.1 µM menthol
- + 10 µM menthol

****
Menthol acts as a positive allosteric modulator on nematode levamisole sensitive nicotinic acetylcholine receptors

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Abstract

The ongoing and widespread emergence of resistance to the existing anti-nematodal pharmacopeia has made it imperative to develop new anthelmintic agents. Historically, plants have been important sources of therapeutic compounds and offer an alternative to synthetic drugs. Monoterpenoids are phytochemicals that have been shown to produce acute toxic effects in insects and nematodes. Previous studies have shown nicotinic acetylcholine receptors (nAChRs) to be possible targets for naturally occurring plant metabolites such as carvacrol and carveol. In this study we examined the effects of monoterpenoid compounds on a levamisole sensitive nAChR from \textit{Oesophagostomum dentatum} and a nicotine sensitive nAChR from \textit{Ascaris suum}. We expressed the receptors in \textit{Xenopus laevis} oocytes and used two-electrode voltage-clamp to characterize the effect of various compounds on these cys-loop receptors. At 100 µM the majority of these compounds acted as antagonists. Interestingly, further experiments revealed that both 0.1 µM and 10 µM menthol potentiated acetylcholine and levamisole responses in the levamisole sensitive receptor but not the nicotine sensitive receptor. We also investigated...
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**Keywords**

Menthol, allosteric modulation, nAChR, monoterpenoid, nematode

**Abbreviations**

*Asu*, *Ascaris suum*; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis (acetoxymethyl ester); cRNA, complementary RNA; DMSO, dimethyl sulfoxide; *Hco*, *Haemonchus contortus*; nAChR, nicotinic acetylcholine receptor; *Ode*, *Oesophagustomum dentatum*; PAM, positive allosteric modulator; STHs, soil transmitted helminths.
1. **Introduction:**

Parasitic nematode infections are a threat to health and have significant socio-economic consequences. Among the different classes of nematodes, soil transmitted helminths (STHs) are major contributors to the global parasite burden. According to WHO (2018), more than 1.5 billion people, or 24% of the world’s population, are infected with at least one species of soil transmitted helminth, which mainly include *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms (*Necator americanus* and *Ancylostoma duodenale*). These infections are most commonly found in tropical and sub-tropical countries due to suboptimal sanitation, lack of access to clean water and favorable climatic conditions for the parasites (Bethony et al., 2006; Brooker et al., 2006; Hotez et al., 2008; Savioli and Albonico, 2004). Although mortality is rare, high morbidity is a common feature of intestinal helminthiasis. The disease manifestations include weight loss, anemia, diarrhea, abdominal pain, malnutrition, general malaise, weakness, as well as impaired growth and physical development (Bethony et al., 2006). Children bear the highest burden of these infections which profoundly affect their physical growth, cognitive development and cause educational deficits (Alum et al., 2010; Brooker, 2010; Stephenson et al., 2000). STH infections such as *Ascariasis* have also been reported to exacerbate other diseases such as malaria, tuberculosis and HIV/AIDS (Fincham et al., 2003; Le Hesran et al., 2004). In addition, production losses caused by intestinal nematodes of livestock have adverse effects on nutritional status and the food economy especially in poverty-stricken regions (Morgan et al., 2013).

Effective vaccines are the most desirable approach but even after almost 30 years of dedicated research, a licensed vaccine against human parasites remains elusive. Current control strategies for treatment and prevention of helminth infections rely heavily on chemotherapy. Unfortunately, there are a limited number of classes of anthelmintic agents,
which include benzimidazoles, macrocyclic lactones and nicotinic agonists (Abongwa et al., 2017; Martin, 1997; Robertson and Martin, 2007). Moreover, widespread and indiscriminate administration combined with genetic factors has led to the development of resistance in animals (Albonico et al., 2003; Taman and Azab, 2014). There have also been reports of reduced therapeutic efficacy to anti-nematodal drugs in humans. De Clercq et al. (1997) and Flohr et al. (2007) reported poor efficacy of benzimidazoles against human hookworm infections. There is a need to focus research on drug discovery and development to overcome the issue of increasing resistance and reduced efficacy against existing drugs.

The use of phytotherapeutics for parasitic infections has emerged as an attractive alternative. Plants have dominated the human pharmacopoeia for thousands of years (Balick and Cox, 1996; Newman and Cragg, 2016; Raskin and Ripoll, 2004). Many therapeutic products, including codeine, quinine, artemisinin, digitoxin, morphine, paclitaxel, galantamine, currently used in modern pharmacotherapy are of plant origin (Butler, 2004; Heinrich and Lee Teoh, 2004; Newman and Cragg, 2016; Newman et al., 2000; Petrovska, 2012; Pirttila et al., 2004; Samuelsson, 2004). Plant based therapeutic compounds are environment-friendly and exhibit a wide array of medicinal properties (Balunas and Kinghorn, 2005; Coats, 1994; Dias et al., 2012; Harvey et al., 2015; Hu and Coats, 2008). Interestingly, an estimated four billion people living in the developing world rely on medicinal plants for their primary pharmaceutical care (WHO, 2005). Herbal remedies in the form of complementary and alternative medicine (CAM) are also being widely embraced in many developed countries (Anquez-Traxler, 2011; Calapai, 2008; Committee on the Use of Complementary, 2005). Among phytochemicals, essential oils and their active principles have been well documented for their broad range of functions including insecticidal and nematocidal activities (Camurca-Vasconcelos et al., 2007; Cetin
et al., 2009; Coskun et al., 2008; Enan, 2005; Macedo et al., 2009; Panella et al., 2005).

Historically, thymol (found in oil of thyme), santonin (derived from buds of the local
Artemisia spp.) and ascaridol (isolated from Chenopodium oil) were used in the late
nineteenth and early twentieth century for the treatment of ascarids and hookworms in
humans (Ferrell, 1914; Kaplan et al., 2014; Lamson and Ward, 1932). Terpene and
terpenoid biomolecules, particularly monoterpenes and sesquiterpenes, found in essential
oils have been identified as promising anthelmintic compounds and are of great
pharmaceutical interest (Jaradat et al., 2016; Katiki et al., 2011; Lei et al., 2010; Pessoa et
al., 2002; Squires et al., 2010). Lei et al. (2010) reported in vitro nematocidal activity of
thymol and carvacrol (major components of thyme and oregano essential oils)
against Caenorhabditis elegans and Ascaris suum. Trailović et al. (2015) also
demonstrated the potential anthelmintic effects of carvacrol in A. suum.

In the present study, we tested the activity of monoterpenoid compounds on two different
nematode nAChR types: levamisole sensitive (L-type) nAChRs from Oesophagostomum
dentatum and a nicotine sensitive (N-type) nAChR (ACR-16) from A. suum. The receptors
were expressed in Xenopus laevis oocytes and we used two-electrode voltage-clamp to
study the pharmacological effects of the compounds. We also investigated the effects of
menthol on the contractility of A. suum somatic muscle strips. The focus of this study was
to evaluate the effects produced by monoterpenoid compounds to identify potential
alternatives to current chemotherapeutic agents.

2. Materials and Methods

2.1 cRNA preparation and X. laevis oocyte expression

All nAChR subunit and ancillary factor cRNAs from O. dentatum (Ode-unc-29, Ode-unc-
38, Ode-unc-68 and Ode-acr-8), A. suum (Asu-acr-16 and Asu-ric-3) and Haemonchus
contortus (Hco-ric-3, Hco-unc-50 and Hco-unc-74) were prepared as previously described (Abongwa et al., 2016b; Buxton et al., 2014). The receptors were expressed in defolliculated X. laevis oocytes purchased from Ecocyte Bioscience (Austin, Texas, USA). N-type (nicotine sensitive) nAChRs from A. suum were expressed by co-injecting 25 ng of Asu-acr-16 with 5 ng of Asu-ric-3 in a total volume of 50 nL in nuclease-free water. Heteromeric (levamisole sensitive) nAChRs from O. dentatum were expressed by co-injecting 1.8 ng of each subunit cRNA (Ode-unc-29, Ode-unc-38, Ode-unc-68 and Ode-acr-8) with 1.8 ng of each H. contortus ancillary factor (Hco-ric-3, Hco-unc-50 and Hco-unc-74) in a total volume of 36 nL in nuclease-free water. The injections were made in the animal pole of the oocytes using a nanoject II microinjector (Drummond Scientific, Broomall, PA, USA). The injected oocytes were separated into 96-well plates containing 200 µL incubation solution (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂·2H₂O, 1 mM MgCl₂·6H₂O, 5 mM HEPES, 2.5 mM Na pyruvate, 100 U·mL⁻¹ penicillin and 100 µg·mL⁻¹ streptomycin, pH 7.5); each well contained one oocyte. The solution was changed daily to maintain oocytes in optimal conditions. The oocytes were incubated at 19 °C for at 5-7 days to allow functional expression of the receptor.

2.2 Two-electrode voltage-clamp (TEVC) electrophysiology

We used two-electrode voltage-clamp electrophysiology to record the inward current generated by the activation of expressed receptors in X. laevis oocytes. The oocytes were incubated with 100 µM BAPTA-AM (an intracellular calcium chelator) for ~3 hours prior to recordings to prevent activation of endogenous calcium-activated chloride currents. The oocytes were clamped at -60mV for all the experiments with an Axoclamp 2B amplifier; all data were acquired on a desktop computer with Clampex 10.2 (Molecular Devices Inc., CA, USA). The microelectrodes used to impale oocytes were pulled using a Flaming/Brown horizontal micropipette puller (model P-97, Sutter Instruments Co., USA)
and filled with 3 M KCl. The tips of the microelectrodes were carefully broken with a tissue paper to achieve a resistance of 2-5 MΩ in recording solution (100 mM NaCl, 2.5 mM KCl, 1 mM CaCl$_2$·2H$_2$O and 5 mM HEPES, pH 7.3). The low resistance allowed passage of large currents required to maintain adequate voltage-clamp of the oocyte. Oocytes were placed into a tiny groove of the narrow oocyte-recording chamber. The Digidata 1322A (Molecular Devices, CA, USA) was used to control the switches that controlled the perfusion of the chamber at a speed of ~6 mL/min. Un-injected oocytes served as the negative control.

2.3 A. suum muscle flap contraction measurements

The adult female A. suum worms were collected from the slaughterhouse at Surčin, Belgrade, Serbia and maintained at 32 °C in Locke’s solution (NaCl 155 mM, KCl 5 mM, CaCl$_2$ 2 mM, NaHCO$_3$ 1.5 mM and glucose 5 mM). The Locke’s solution was changed twice daily. Each batch of worms was used within 4 days of collection. For contraction studies, A. suum muscle flaps were prepared as described in Trailović et al. (2015). Briefly, an anterior part of the worm, 2-3 cm caudal to the head was dissected to prepare a 1 cm muscle flap and the lateral line was removed from the edge of the flaps. Each muscle flap was attached to a force transducer in an experimental bath maintained at 37 °C, containing 20 mL Ascaris Perienteric Fluid Ringer (23 mM NaCl, 110 mM Na acetate, 24 mM KCl, 6 mM CaCl$_2$, 5 mM MgCl$_2$, 11 mM glucose, 5 mM HEPES, pH 7.6) and bubbled with room air. The isometric contractions were monitored on a PC computer using a BioSmart interface with eLAB software (EIUnit, Belgrade). The preparations were allowed to equilibrate for 15 min under an initial tension of 0.5 g following which acetylcholine (1–100 µM) in the absence and presence of menthol (0.1 µM) was applied to the preparation. The responses for each concentration were expressed in grams (g).
2.4 Drug Applications

Acetylcholine chloride, L-menthol, (-)-menthone, eugenol, (S)-(--)β-Citronellol, (+)-limonene oxide, carvacrol, ((R)-(--))-carvone, menthyl acetate, phenethyl propionate, trans-cinnamaldehyde and BAPTA-AM were purchased from Sigma-Aldrich (St Louis, MO, USA). (+)-Pulegone was purchased from Kodak (Rochester, NY, USA) and geraniol from Berje (Carteret, NJ, USA). Acetylcholine was dissolved in recording solution while monoterpenoid compounds (Figure 1) were dissolved in DMSO such that the final working concentration did not exceed 0.1%.

Oocytes were perfused with drugs by an 8-channel system (VC-8 valve controller) (Warner Instruments, USA) at a flow rate of ~6 mL/min. For the agonist experiments, all the essential oils were used at a concentration of 100 µM. All the responses were normalized to the control (100 µM acetylcholine) response. Each drug was applied for 10s and the wash off time between each drug was 2 minutes. The application sequence of agonists for determining the potency series was random and not predetermined. The concentration-response relationship for acetylcholine was generated by applying increasing concentrations of acetylcholine (0.1-300 µM) for 10 s.

For positive allosteric modulator experiments, we tested the effects of menthol (0.1 and 10 µM) on acetylcholine and levamisole concentration-response relationship. Control concentration-responses were obtained by applying ascending concentrations of the agonist for 10 s. Concentration-response relationships in the presence of 0.1 µM and 10 µM menthol were generated by first applying a control 100 µM acetylcholine for 10 s, followed by a 2-min application of menthol, and 10 s applications of increasing concentrations of the agonist in the continued presence of menthol. The responses were
normalized to the control 100 µM acetylcholine current. Between each drug application a 2 min wash off time was allowed, and the responses were normalized to the control 100 µM acetylcholine current.

In order to characterize the antagonist properties of the phytocompounds, a control concentration of 100 µM ACh was first applied for 30 s followed by a 2 min wash with recording solution. Thereafter, 100 µM acetylcholine was applied for 10 s, immediately followed by 10 s application of the antagonist (100 µM) in the continued presence of 100 µM ACh and then a final 10 s application of 100 µM ACh. At least 2 min drug wash off interval was allowed between applications in order to minimize desensitization. Note that, the potency for each antagonist is likely to be slightly underestimated due to the short time of drug application with this approach (Abongwa et al., 2016a). Acetylcholine concentration-response relationships in the presence of 100 µM limonene oxide and 100 µM carvacrol were generated by first applying a control 100 µM acetylcholine for 10 s, followed by a 2-min application of the antagonist, and 10 s applications of 3, 10, 30, 100 and 300 µM acetylcholine in the continued presence of antagonist. The responses were normalized to the control 100 µM acetylcholine current.

2.5 Data and statistical analysis

Data acquired from electrophysiological recordings were analyzed with Clampfit 10.3 (Molecular Devices, Sunnyvale, CA, USA) and GraphPad Prism 7.0 (Graphpad Software Inc., La Jolla, CA, USA). The peak current responses in oocytes to applied drugs were normalized to the 100 µM ACh control current (unless otherwise indicated) and expressed as mean ± SEM. All completed drug application sequences on the oocytes were used for analysis without exclusion. If the recording became unstable, indicated by a change in the baseline holding current, all of that recording was rejected for analysis. The mean %
inhibition produced by monoterpenoid compounds on currents elicited by 100 μM acetylcholine were calculated as previously described (Zheng et al., 2016). For *A. suum* contraction experiments, mean contraction responses in grams (g) to each concentration of acetylcholine in the absence and presence of menthol was determined. The sigmoidal concentration-responses for the oocytes experiments and isometric contraction studies were described using the following equation:

\[
\text{% response} = \frac{1}{1 + [\text{EC}_{50}/X_a]^{nH}}
\]

where \( \text{EC}_{50} \) is the concentration of agonist (Xa) producing 50% of the maximum response and \( nH \) is the Hill coefficient (slope). Differences in \( p\text{EC}_{50} \) and \( I_{\text{max}} \) were assessed using extra sum of squares F-test. Two-way ANOVA and paired t-tests were used to test differences between control contractions and test contractions on the same muscle flap. The difference was considered significant if \( P<0.05 \).

3. RESULTS:

3.1 Pharmacology of monoterpenoids on *Ode* (29-38-63-8) receptor

3.1.1 Agonist pharmacology

The agonist properties of 12 monoterpenoid compounds were investigated on the levamisole sensitive *Ode-unc-29*:*Ode-unc-38*:*Ode-unc-63*:*Ode-acr-8* nAChR (Figure 2). 11 of the 12 monoterpenoids we tested showed no significant agonistic effect (<1.0% of control acetylcholine current; Figure 2A) on the expressed levamisole sensitive channel. Menthol was the only compound tested that produced an agonist effect, showing only \( \approx 6.5\% \) of the control acetylcholine currents.

3.1.2 Antagonist pharmacology

We tested potential antagonistic effects of the same 12 monoterpenoids on the levamisole sensitive receptor (Figure 3). Among all the phytocompounds tested limonene oxide,
citronellol, carvone, carvacrol, pulegone and eugenol reduced the acetylcholine response. Limonene oxide was the most potent inhibitor (mean inhibition% = 36.0 ± 3.2%). The rank order potency of inhibition was: limonene oxide > citronellol > carvone > carvacrol = pulegone = eugenol (Figure 3A). Interestingly, monoterpenoids including menthol, menthone, menthyl acetate and geraniol increased the amplitude of currents produced by acetylcholine instead of inhibiting them (data not shown). Menthol produced the most potent positive allosteric modulation and increased the response by 3.1 ± 0.7% (mean ± SEM) with a maximum potentiation of 6.3%.

3.1.3 Antagonistic effects of limonene oxide and carvacrol on acetylcholine concentration-response relationship

The concentration-response relationship for acetylcholine was examined by applying increasing concentrations of acetylcholine 0.3–300 µM to oocytes expressing the levamisole sensitive nAChR (Figure 3B & C). The sigmoidal concentration-response fit gave pEC50 = 5.3 ± 0.0 (EC50 = 5.3 µM), a hillslope (nH) of 2.0 ± 0.2 and I_max value of 104.6 ± 1.6%. We investigated the effects of limonene oxide (100 µM) and carvacrol (100 µM) on the acetylcholine concentration-response relationship. I_max for acetylcholine in the presence of limonene oxide and carvacrol were 62.8 ± 7.0% and 74.1 ± 3.2%, respectively. Both the monoterpenoid compounds produced a statistically significant reduction in the maximum response (P<0.001 for limonene oxide; P<0.0001 for carvacrol; Figure 3B & C). Neither carvacrol, pEC50 = 5.7 ± 0.1 (EC50 = 2.0 µM) nor limonene oxide, pEC50 = 5.4 ± 0.2 (EC50 = 4.0 µM) increased the EC50 values c.f. control. This indicates limonene oxide and carvacrol both produced non-competitive inhibition and are not binding at the agonist-binding site to produce their effect.

3.1.4 Menthol as positive allosteric modulator
Menthol was a weak agonist on the expressed levamisole sensitive receptor. However, it appeared to potentiate acetylcholine responses in our antagonist experiments (data not shown). To investigate positive allosteric modulation further, we analyzed the effect of menthol on acetylcholine and levamisole concentration-response relationships (Figure 4 and Figure 5). We observed a left shift in the sigmoidal concentration-response curves for both levamisole and acetylcholine in the presence of menthol. Sensitivity of the receptor was increased significantly for acetylcholine, $pEC_{50} = 5.3 \pm 0.0$ ($EC_{50} = 5.0 \mu M$); in the presence of 0.1 µM menthol, $pEC_{50} = 6.4 \pm 0.1$ ($EC_{50} = 0.4 \mu M$) and with 10 µM menthol, $pEC_{50} = 6.5 \pm 0.1$ ($EC_{50} = 0.3 \mu M$).

For levamisole alone, $pEC_{50}$ and $EC_{50}$ values were 6.3 ± 0.1 and 0.5 µM respectively. In the presence of 0.1 µM menthol $pEC_{50}$ and $EC_{50}$ values were 6.5 ± 0.1 and 0.3 µM respectively. In the presence of 10 µM menthol the $pEC_{50}$ and $EC_{50}$ values were 6.8 ± 0.2 and 0.2 µM. Only 10 µM menthol produced a significant left shift in the levamisole concentration-response curve ($P<0.05$; Figure 5C). The positive allosteric modulation produced by menthol was more pronounced with acetylcholine as an agonist in comparison to levamisole. In conclusion, menthol not only acts as an agonist on the levamisole sensitive receptor but also displays significant PAM activity on this nAChR.

### 3.2 Effects of menthol and carvacrol on A. suum ACR-16 nAChR

Figure 6 shows the concentration-response relationship for acetylcholine on the expressed Asu-ACR-16 nicotine sensitive nAChR. The sigmoidal concentration-response fit gave $pEC_{50} = 5.0 \pm 0.1$ ($EC_{50} = 11.1 \mu M$), $I_{max} = 109.1 \pm 6.6\%$ and a hillslope ($n_H$) of 1.6 ± 0.3.

We investigated the effects of menthol (10 µM) and carvacrol (10 µM and 100 µM) on the acetylcholine response. There was no significant change in the sensitivity or efficacy of acetylcholine on the N-type receptor in the presence of menthol ($pEC_{50} = 5.0 \pm 0.1$ and $I_{max} = 101.6 \pm 7.5\%$). The concentration-response fits (Figure 6C) show that 100 µM carvacrol
decreased the maximum response while 10 μM carvacrol failed to exhibit any significant antagonist properties. In the presence of 10 μM carvacrol \( pEC_{50}, EC_{50} \) and \( l_{max} \) values were 5.1 ± 0.1, 8.1 μM and 94.6 ± 5.9% respectively. Carvacrol produced a significant reduction in \( l_{max} \) (55.8 ± 4.4%; \( P<0.05 \)) when applied at 100 μM concentration but did not produce a significant right shift in the \( pEC_{50} \) (5.1 ± 0.1). This indicates carvacrol at 100 μM produced non-competitive inhibition.

3.3 Effect of menthol on A. suum muscle

We examined the effect of menthol on the A. suum muscle flap contractions induced by increasing concentrations of acetylcholine. Figure 7A shows the representative trace for acetylcholine, in the absence and presence of 0.1 μM menthol in the experimental bath. Acetylcholine produced concentration-dependent isometric contractions with a \( pEC_{50} \) of 5.2 ± 0.2 (\( EC_{50} = 7.0 \) μM) and \( R_{max} = 1.7 ± 0.2 \) g. We did not observe a significant left shift in \( pEC_{50} \) (5.2 ± 0.2) or change in \( R_{max} \) (2.0 ± 0.2 g) in the presence of menthol. As expected, based on two-way ANOVA, acetylcholine concentration had a significant effect on muscle contraction (\( P<0.0001 \)). Importantly the presence of menthol also had a significant effect on muscle contraction (\( P<0.0001 \)). Further analysis revealed a significant potentiation of contractions at each concentration of acetylcholine in the presence of menthol (paired \( t \)-tests, \( P<0.05 \), Figure 7B for examples). This modulatory effect of menthol is similar to our observed effects on the levamisole sensitive receptor.

4. DISCUSSION:
Monoterpenoids are a group of plant secondary metabolites that have been shown to modulate the function of nicotinic acetylcholine receptors of mammals and insects (Lozon et al., 2016; Park et al., 2003; Park et al., 2001; Tong et al., 2013). We were interested in finding possible agonists, antagonists or modulators for these cys loop receptors of
parasitic nematodes. In this study, we provide evidence of significant modulatory effects of several monoterpenoid compounds on the levamisole sensitive *O. dentatum* receptor and nicotine sensitive *A. suum* nAChR. We chose to use the levamisole sensitive nAChR from *O. dentatum* due to unreliable expression of the levamisole sensitive nAChR from *A. suum* in our hands.

The majority of the phytocompounds tested failed to exhibit agonist properties on the nAChRs but we were able to identify inhibitors of the levamisole sensitive nematode receptor with a rank order:

limonene oxide > citronellol > carvone > carvacrol = pulegone = eugenol.

Limonene oxide and carvacrol produced significant non-competitive inhibition suggesting neither compound acts at the ligand binding sites. Limonene oxide is a monoterpane found in essential oils of various citrus fruits and is an odorous component of some types of mushrooms (Breheret et al., 1997; Lemes et al., 2018; Lota et al., 2002; Rapior et al., 1997; Vieira et al., 2018); it has been reported to stimulate TRPA1 cation channels (Kaimoto et al., 2016). There are limited data available on the effects of limonene oxide on nAChRs and this is the first example of an antagonistic effect of the compound on a levamisole sensitive nematode nAChR. According to a study done by Kim et al. (2013) limonene oxide was reported to be a safe phytochemical in mammals and thus could be used in combination with other nicotinic inhibitors for anti-nematodal therapy. Carvacrol, found in many plants (thyme, oregano, Alaska yellow cedar; (Bouchra et al., 2003; De Vincenzi et al., 2004) acted as a non-competitive inhibitor on both levamisole sensitive and nicotine sensitive nematode receptors. Various studies have shown alpha-7 homomeric nAChRs, GABA (gamma amino-butyric acid) and tyramine receptors as target sites for carvacrol (Lozon et al., 2016; Tong and Coats, 2012; Tong et al., 2013). In addition, carvacrol was shown to produce significant inhibitory effects on acetylcholine induced
muscle contractions in *A. suum* (Trailovic et al., 2015). This polypharmacological effect of carvacrol might make it efficacious as an anthelmintic phytotherapeutic, although it may be best considered as an adjunct to other anti-nematodal compounds to increase efficacy due to toxicity considerations when used alone at high concentrations (Bimczok et al., 2008; Roselli et al., 2007; Stammati et al., 1999).

The most interesting finding was the positive allosteric modulatory activity of menthol on the levamisole sensitive nematode nAChR. Menthol is a monocyclic terpene alcohol extracted from peppermint plants, *Mentha* spp. and regulates somatosensory sensations such as warmth, pain and irritation (Cliff and Green, 1994; Eccles, 1994; Farco and Grundmann, 2013). Menthol showed very mild agonist activity but produced statistically significant potentiation of acetylcholine induced inward currents at concentrations as low as 0.1 µM and of levamisole induced currents at 10 µM on the levamisole sensitive cation channel. PAM compounds exhibit potentiation of the agonist-activation effects by binding to a site distinct from the ligand binding sites. This property is highly desired in drug discovery and development as PAMs can achieve subtype selectivity more easily in comparison to compounds binding to orthosteric sites (Chatzidaki and Millar, 2015; Williams et al., 2011) as all the nAChRs have highly conserved acetylcholine binding sites. In comparison, the conservation for amino acid composition for other sites is less which makes PAMs preferable in terms of selectivity and reduced potential off target effects (Chatzidaki and Millar, 2015; Williams et al., 2011). Menthol modulates the pharmacological properties and expression of vertebrate nicotinic acetylcholine receptors; it inhibits the effect of nicotine on α7-nACh receptors in neural cells in a non-competitive manner (Ashoor et al., 2013; Hans et al., 2012; Ton et al., 2015). However, it is worth noting that these negative allosteric modulatory and inhibitory effects of menthol on α7-nACh receptors were produced by much higher concentrations (30-300 µM) in comparison
to our study. In addition, menthol at 10 µM had no significant effect on the nematode nicotine sensitive nAChR (the closest ortholog to vertebrate α7-nAChRs in the nematodes). This makes menthol an interesting candidate that can potentiate the effects of cholinergic agonist anthelmintics acting on levamisole sensitive nAChRs. Importantly, 0.1 µM menthol produced significant potentiation of contractions produced by acetylcholine on *A. suum* muscle flaps. The presence of positive allosteric modulation in the somatic muscle flap preparation can be attributed to the presence of levamisole receptors and is important *in vivo* confirmation of our findings from the *in vitro* heterologous expression studies.

5. **Conclusion**

Natural products have formed the basis of sophisticated traditional therapies and they continue to play essential roles in modern healthcare. Phytocompounds, with their complex chemistry and structural diversity, offer the potential for discovery and development of new pharmaceuticals. Botanical anthelmintics may reduce the need for chemical drug treatment and alleviate the pressure on the limited group of anthelmintics available. Carvacrol produced significant antagonism on the acetylcholine induced inward currents on both levamisole sensitive and nicotine sensitive nematode ligand gated ion channels. This illustrates a multifaceted mode of action with involvement of multiple target subtypes that can help ameliorate the issue of drug resistance. It may be possible to combine these compounds with the nAChR antagonist derquantel or perhaps with a macrocyclic lactone as with Startect® (derquantel + abamectin).

Our results provide promising evidence of positive allosteric modulation by menthol which could be used in combination therapy with cholinomimetic drugs like pyrantel or levamisole to increase efficacy. For future investigations menthol could be used as the starting point
in structure activity relationship studies to find new potent PAM compounds suitable for stand-alone use as anthelmintics. In summary, the use of plant derived compounds, either alone or in combination, could facilitate sustainable control of parasitic nematodes. The results of the present study are encouraging and suggest monoterpenoids can be exploited as components of new anthelmintic formulations.

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References

Abongwa, M., Baber, K.E., Martin, R.J., Robertson, A.P., 2016a. The cholinomimetic morantel as an open channel blocker of the Ascaris suum ACR-16 nAChR. Invert Neurosci 16, 10.


Buxton, S.K., Charvet, C.L., Neveu, C., Cabaret, J., Cortet, J., Peineau, N., Abongwa, M., Courtot, E., Robertson, A.P., Martin, R.J., 2014. Investigation of acetylcholine receptor...
diversity in a nematode parasite leads to characterization of tribendimidine- and derquantel-sensitive nAChRs. PLoS Pathog 10, e1003870.


Tong, F., Gross, A.D., Dolan, M.C., Coats, J.R., 2013. The phenolic monoterpenoid carvacrol inhibits the binding of nicotine to the housefly nicotinic acetylcholine receptor. Pest Manag Sci 69, 775-780.


**Figure Legends:**

**Figure 1:** Chemical structures of monoterpenoid compounds used in the present study.

**Figure 2:** Agonist effects of monoterpenoid compounds on levamisole sensitive *O. dentatum* nAChR. **A.** Sample traces for the agonist experiment. **B.** Bar chart (expressed as mean ± SEM%) showing agonistic effects of the monoterpenoid compounds: menthol (6.5 ± 2.4%, n = 6), geraniol (0.9 ± 0.2%, n = 6), cinnamaldehyde (0.6 ± 0.2%, n = 6), citronellol (0.4 ± 0.2%, n = 6), menthy acetate (0.3 ± 0.1%, n = 6), eugenol (0.3 ± 0.2%, n = 6), phenethyl propionate (0.3 ± 0.1%, n = 6), limonene oxide (0.3 ± 0.2%, n = 6), carvacrol (0.2 ± 0.2%, n = 6), menthone (0.2 ± 0.1%, n = 6), pulegone (0.1 ± 0.1%, n = 6) and carvone (0.0 ± 0.0%, n = 6).

**Figure 3:** Effect of monoterpenoid compounds as antagonists on levamisole sensitive *O. dentatum* receptor acetylcholine mediated responses. **A.** Bar graph showing the rank order potency of monoterpenoid compounds as antagonists. Results were expressed as %
mean inhibition ± SEM of currents elicited by 100 µM ACh: limonene oxide (36.0 ± 3.2%, n = 6) > citronellol (18.0 ± 1.6%, n = 6) > carvone (14.0 ± 0.6%, n = 6) > carvacrol (11.0 ± 1.6%, n = 6) = pulegone (11.0 ± 2.6%, n = 6) = eugenol (11.0 ± 1.0 %, n = 6). Inset: image showing predicted acetylcholine response in the absence of limonene oxide (dotted line) and inhibition of acetylcholine mediated response in the presence of limonene oxide (highlighted in green).

**B.** Acetylcholine current responses, recorded from the *Xenopus oocyte* expressing the levamisole sensitive channel, alone and in the presence of 100µM carvacrol (n=6) & 100 µM limonene oxide (n = 5). **C.** Concentration-response plots for acetylcholine alone (n = 6, black) and acetylcholine in the presence of 100 µM carvacrol (n = 6, maroon) & 100 µM limonene oxide (n = 5, green). $\rho EC_{50}$ (mean ± SEM), $EC_{50}$ (mean, µM), Hill slope ($n_H$) (mean ± SEM) and $I_{max}$ (mean ± SEM%) values were respectively: 5.3 ± 0.0, 5.3 µM, 2.0 ± 0.2 and 104.6 ± 1.6 for acetylcholine alone; 5.4 ± 0.2, 4.0 µM, 2.2 ± 1.7 and 62.8 ± 7.0 in the presence of limonene oxide; 5.7 ± 0.1, 2.0 µM, 1.7 ± 1.1 and 74.1 ± 3.2 in the presence of carvacrol. Bottom was constrained to zero for curve fitting.

**Figure 4:** Effects of menthol as a PAM on levamisole sensitive *O. dentatum* nAChR acetylcholine responses. **A.** Sample traces (above) for two-electrode voltage-clamp recording showing inward currents in response to ascending concentrations of acetylcholine alone, acetylcholine in the presence of 0.1 µM and 10 µM menthol. **B.** Concentration-response relationships for acetylcholine alone (n = 7, black), acetylcholine in the presence of 0.1 µM (n = 6, blue) and 10 µM menthol (n=7, purple). $\rho EC_{50}$ (mean ± SEM), $EC_{50}$ (mean, µM), Hill slope ($n_H$) (mean ± SEM) and $I_{max}$ (mean ± SEM%) values were respectively: 5.3 ± 0.0, 5.0 µM, 1.4 ± 0.1 and 107.5 ± 2.6 for acetylcholine alone; 6.4 ± 0.1, 0.4 µM, 1.0 ± 0.1 and 109.0 ± 3.6 in the presence of 0.1 µM menthol; 6.5 ± 0.1, 0.3 µM, 1.0 ± 0.1 and 110.5 ± 3.7 in the presence of 10 µM menthol. Bottom was constrained to zero for curve fitting. **C.** Bar chart showing comparison of $\rho EC_{50}$ (expressed as mean ± SEM).
SEM) for acetylcholine in the presence and absence of menthol. ****P<0.0001; significantly different as indicated; extra sum of squares F-test.

Figure 5: Effects of menthol on levamisole sensitive nAChR levamisole responses. A. Representative current traces for two-electrode voltage-clamp recording showing inward currents in response to ascending application of levamisole alone, levamisole in the presence of 0.1 µM and 10 µM menthol. B. Concentration-response relationships for levamisole alone (n = 7, steel gray) and in the presence of 0.1 µM (n = 6, light green) and 10 µM menthol (n=7, dark green). pEC$_{50}$ (mean ± SEM), EC$_{50}$ (mean, µM), Hill slope (n$_H$) (mean ± SEM) and $I_{max}$ (mean ± SEM%) values were respectively: 6.3 ± 0.1, 0.5 µM, 1.0 ± 0.2 and 96.3 ± 5.2 for levamisole alone; 6.5 ± 0.1, 0.3 µM, 1.1 ± 0.2 and 94.6 ± 5.0 in the presence of 0.1 µM menthol; 6.8 ± 0.2, 0.2 µM, 1.0 ± 0.2 and 89.2 ± 6.0 in the presence of 10 µM menthol. Bottom was constrained to zero for curve fitting. C. Bar chart summarizing the results showing comparison of pEC$_{50}$ (expressed as mean ± SEM) for levamisole in the presence and absence of menthol. *P<0.05; significantly different as indicated; extra sum of squares F-test.

Figure 6: Effects of menthol and carvacrol on nicotine sensitive A. suum ACR-16 nAChR acetylcholine responses. A. Representative current traces for two-electrode voltage-clamp recording showing inward currents in response to increasing acetylcholine concentrations in the presence of menthol (10 µM) and carvacrol (10 µM and 100 µM) B. Concentration-response plot for acetylcholine alone (n = 7, black) and acetylcholine in the presence of menthol (n = 6, red). pEC$_{50}$ (mean ± SEM) and EC$_{50}$ (mean, µM) values were respectively: 5.0 ± 0.1 and 11.1 µM for acetylcholine alone, 5.0 ± 0.1 and 9.5 µM in the presence of 10 µM menthol. Hill slope (n$_H$) (mean ± SEM) values were: 1.6 ± 0.3 for acetylcholine alone; 2.5 ± 1.2 in the presence of 10 µM menthol. $I_{max}$ (mean ± SEM%) values were: 109.1 ± 6.6
for acetylcholine alone; 101.6 ± 7.5 in the presence of 10 µM menthol. Bottom was constrained to zero for curve fitting. **C.** Concentration-response plots for acetylcholine alone (n = 7, black) and in the presence of carvacrol (n=6, blue). pEC$_{50}$ (mean ± SEM), EC$_{50}$ (mean, µM), Hill slope (n$_H$) (mean ± SEM) and I$_{max}$ (mean ± SEM%) values were respectively: 5.1 ± 0.1, 8.1 µM, 2.5 ± 0.8 and 94.6 ± 5.9 in the presence of 10 µM carvacrol, 5.1 ± 0.1, 8.2 µM, 2.0 ± 0.7 and 55.8 ± 4.4 in the presence of 100 µM carvacrol. Bottom was constrained to zero for curve fitting.

**Figure 7: A.** Sample trace for the *A. suum* muscle flap contraction experiments. Inset: concentration-response plots for acetylcholine alone (n = 6, black) and in the presence of 0.1 µM menthol (n = 6, red). pEC$_{50}$ (mean ± SEM) and EC$_{50}$ (mean, µM) values were respectively: 5.2 ± 0.2 and 7.0 µM for acetylcholine alone; 5.2 ± 0.2 and 6.5 µM in the presence of 0.1 µM menthol. R$_{max}$ (mean± SEM, g) values were: 1.7 ± 0.2 g for acetylcholine alone and 2.0 ± 0.2 g in the presence of 0.1 µM menthol. Hill slope (n$_H$) (mean ± SEM) values were: 0.8 ± 0.2 for acetylcholine alone and 0.8 ± 0.1 in the presence of 0.1 µM menthol. Bottom was constrained to zero for curve fitting. **B.** Bar chart summarizing the results showing significant potentiation in amplitude of acetylcholine mediated contractions in the presence of 0.1 µM menthol. *P<0.05, ***P<0.001; significantly different as indicated; paired t-tests.
carvacrol

((R)-(−))-carvone

(+)-limonene oxide

L-menthol

(−)-menthone

(+)-pulegone

geraniol

(S)-(−)-β-citronellol

menthyl acetate

phenethyl propionate

eugenol

trans-cinnamaldehyde
Figure 2

A

B

Normalized current (%)

menthol

0 20 40 60 80 100

500 nA

2 min

Figure 2
Figure 3

A

![Graph showing percentage inhibition with different molecules.]

B

![Graphs showing current normalized to 100 µM ACh control with different molecules.]

C

![Graph showing % current normalized to 100 µM ACh control with log[ACh], M.]

- Control
- carvacrol
- limonene oxide
- citronellol
- carvone
- pulegone
- eugenol

Molecules: carvacrol, limonene oxide, citronellol, carvone, pulegone, eugenol

% Current normalized to 100 µM ACh control

log[ACh], M
Figure 4

A

B

C

**pEC\textsubscript{50}**

**ACh** + 0.1 μM menthol

**ACh** + 10 μM menthol
Figure 5

A

Ach 100 μM 0.3 1 3 10 30 100 200 nA 5 min

Ach 100 μM + 10 μM menthol 0.3 1 3 10 30

Ach 100 μM + 0.1 μM menthol 0.1 0.3 1 3 10

B

% Current normalized to 100 μM Ach control

% Current normalized to 100 μM Ach control

C

pEC50

lev + 0.1 μM menthol + 10 μM menthol
Figure 6

A

- menthol 10 µM
- carvacrol 10 µM
- carvacrol 100 µM

B

% Current normalized to 100 µM

log [ACh], M

C

% Current normalized to 100 µM

log [ACh], M
Figure 7

A

B
Highlights

• We screened 12 monoterpenoid compounds to search for a potential anthelmintic.

• Menthol acted as PAM on levamisole sensitive nematode nAChRs in vitro & in vivo.

• Carvacrol produced antagonism on both levamisole and nicotine sensitive nAChRs.

• Monoterpenoid compounds can be used as an adjunct with cholinergic anthelmintics.