

## 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 *Oesophagostomum dentatum* and a nicotine sensitive nAChR from *Ascaris suum*. We expressed the receptors in *Xenopus laevis* oocytes and used two-electrode voltage-clamp to characterize the effect of various compounds on these cys-loop receptors. At 100  $\mu$ M the majority of these compounds acted as antagonists. Interestingly, further experiments revealed that both 0.1  $\mu$ M and 10  $\mu$ M menthol potentiated acetylcholine and levamisole responses in the levamisole sensitive receptor but not the nicotine sensitive receptor. We also investigated the effects of 0.1  $\mu$ M menthol on the contractility of *A. suum* somatic muscle strips. Menthol produced significant potentiation of peak contractions at each concentration of acetylcholine. The positive allosteric modulatory effects of menthol in both *in vivo* and *in vitro* experiments suggests menthol as a promising candidate for combination therapy with cholinergic anthelmintics.

### 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).

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**Abbreviations**

Asu	<i>Ascaris suum</i>
BAPTA-AM	1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)
cRNA	complementary RNA

DMSO	dimethyl sulfoxide
Hco	<i>Haemonchus contortus</i>
nAChR	nicotinic acetylcholine receptor
Ode	<i>Oesophagostomum dentatum</i>
PAM	positive allosteric modulator
STHs	soil transmitted helminths

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; Pirtila 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 and Alternative Medicine by the American Public, 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-63* 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-63* 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  $\mu$ L incubation solution (100 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 1 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 5 mM HEPES, 2.5 mM Na pyruvate, 100 U·mL<sup>-1</sup> penicillin and 100  $\mu$ g mL<sup>-1</sup> 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 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  $\mu$ M BAPTA-AM (an intracellular calcium chelator) for ~3 h prior to recordings to prevent activation of endogenous calcium-activated chloride currents. The oocytes were clamped at -60 mV 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 M $\Omega$  in recording solution (100 mM NaCl, 2.5 mM KCl, 1 mM CaCl<sub>2</sub>·2H<sub>2</sub>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<sub>2</sub> 2 mM, NaHCO<sub>3</sub> 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<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 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 (EUnit, Belgrade). The preparations were allowed to equilibrate for 15 min under an initial tension of 0.5 g following which acetylcholine (1–100  $\mu$ M) in the absence and presence of menthol (0.1  $\mu$ M) 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)-(-)- $\beta$ -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 (Fig. 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  $\sim$ 6 mL/min. For the agonist experiments, all the essential oils were used at a concentration of 100  $\mu$ M. All the responses were normalized to the

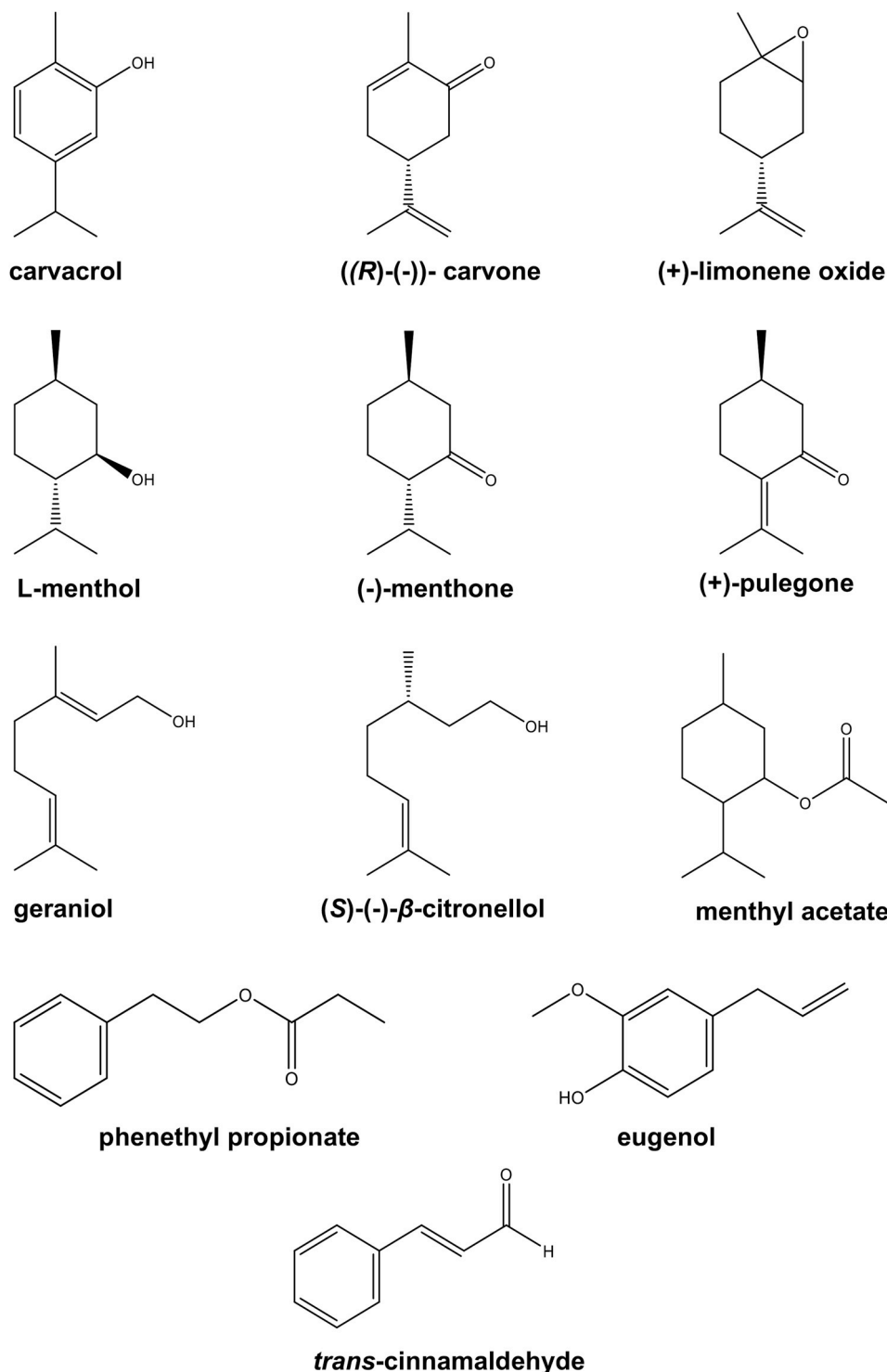
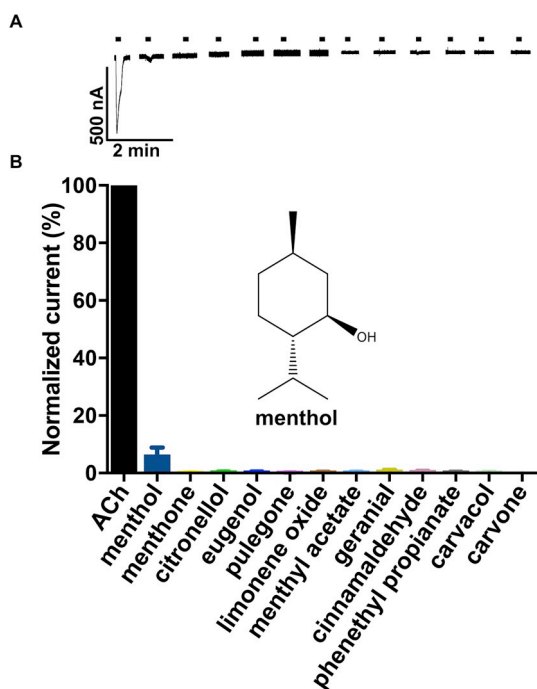


Fig. 1. Chemical structures of monoterpenoid compounds used in the present study.



**Fig. 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  $\pm$  SEM%) showing agonistic effects of the monoterpenoid compounds: menthol ( $6.5 \pm 2.4\%$ ,  $n = 6$ ), geraniol ( $0.9 \pm 0.2\%$ ,  $n = 6$ ), cinnamaldehyde ( $0.6 \pm 0.2\%$ ,  $n = 6$ ), citronellol ( $0.4 \pm 0.2\%$ ,  $n = 6$ ), menthyl acetate ( $0.3 \pm 0.1\%$ ,  $n = 6$ ), eugenol ( $0.3 \pm 0.2\%$ ,  $n = 6$ ), phenethyl propionate ( $0.3 \pm 0.1\%$ ,  $n = 6$ ), limonene oxide ( $0.3 \pm 0.2\%$ ,  $n = 6$ ), carvacrol ( $0.2 \pm 0.2\%$ ,  $n = 6$ ), menthone ( $0.2 \pm 0.1\%$ ,  $n = 6$ ), pulegone ( $0.1 \pm 0.1\%$ ,  $n = 6$ ) and carvone ( $0.0 \pm 0.0\%$ ,  $n = 6$ ).

control (100  $\mu$ M acetylcholine) response. Each drug was applied for 10 s and the wash off time between each drug was 2 min. 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  $\mu$ M) for 10 s.

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

In order to characterize the antagonist properties of the phyto-compounds, a control concentration of 100  $\mu$ M ACh was first applied for 30 s followed by a 2 min wash with recording solution. Thereafter, 100  $\mu$ M acetylcholine was applied for 10 s, immediately followed by 10 s application of the antagonist (100  $\mu$ M) in the continued presence of 100  $\mu$ M ACh and then a final 10 s application of 100  $\mu$ M 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  $\mu$ M limonene oxide and 100  $\mu$ M carvacrol were generated by first applying a

control 100  $\mu$ M acetylcholine for 10 s, followed by a 2-min application of the antagonist, and 10 s applications of 3, 10, 30, 100 and 300  $\mu$ M acetylcholine in the continued presence of antagonist. The responses were normalized to the control 100  $\mu$ M 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  $\mu$ M ACh control current (unless otherwise indicated) and expressed as mean  $\pm$  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  $\mu$ M 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 oocyte experiments and isometric contraction studies were described using the following equation:

$$\% \text{ response} = 1 / 1 + [EC_{50} / X_a]^{nH}$$

where  $EC_{50}$  is the concentration of agonist ( $X_a$ ) producing 50% of the maximum response and  $nH$  is the Hill coefficient (slope). Differences in  $pEC_{50}$  and  $I_{max}$  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-unc-8* nAChR (Fig. 2). 11 of the 12 monoterpenoids we tested showed no significant agonistic effect ( $< 1.0\%$  of control acetylcholine current; Fig. 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.

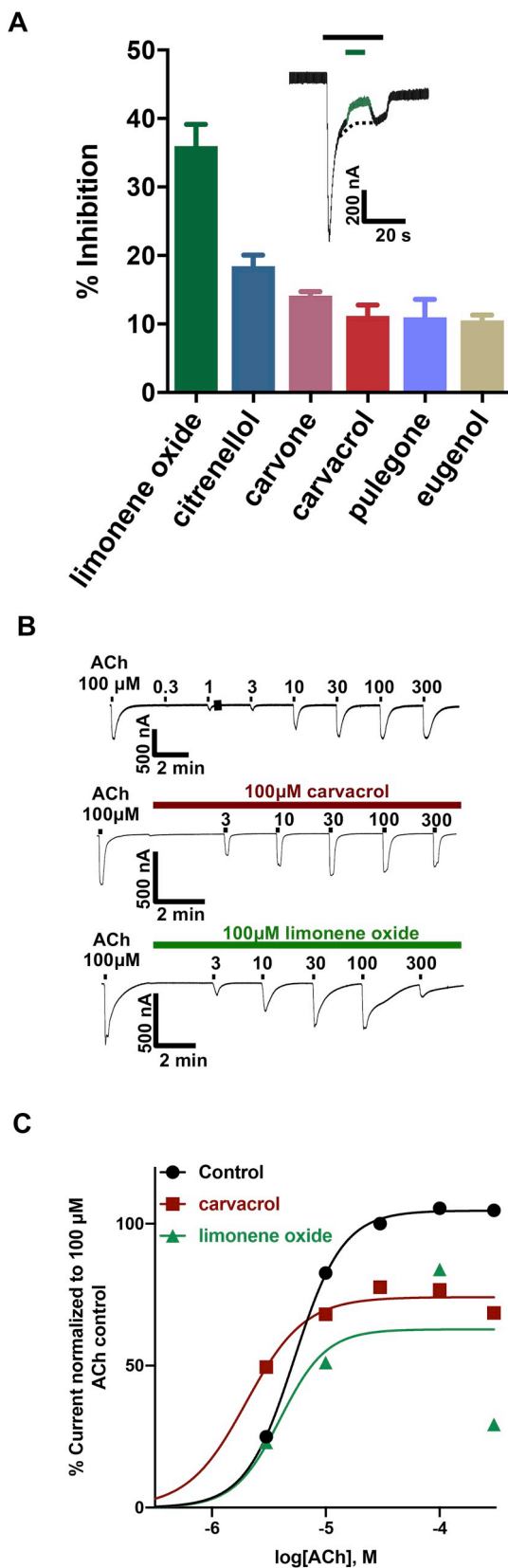
#### 3.1.2. Antagonist pharmacology

We tested potential antagonistic effects of the same 12 monoterpenoids on the levamisole sensitive receptor (Fig. 3). Among all the phyto-compounds tested limonene oxide, citronellol, carvone, carvacrol, pulegone and eugenol reduced the acetylcholine response. Limonene oxide was the most potent inhibitor (mean inhibition% =  $36.0 \pm 3.2\%$ ). The rank order potency of inhibition was: limonene oxide  $>$  citronellol  $>$  carvone  $>$  carvacrol = pulegone = eugenol (Fig. 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  $3.1 \pm 0.7\%$  (mean  $\pm$  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





**Fig. 3.** Effect of monoterpene compounds as antagonists on levamisole sensitive *O. dentatum* receptor acetylcholine mediated responses. **A.** Bar graph showing the rank order potency of monoterpene compounds as antagonists. Results were expressed as % mean inhibition  $\pm$  SEM of currents elicited by 100  $\mu$ M ACh: limonene oxide (36.0  $\pm$  3.2%, n = 6) > citrenellol (18.0  $\pm$  1.6%, n = 6) > carvone (14.0  $\pm$  0.6%, n = 6) > carvacrol (11.0  $\pm$  1.6%, n = 6) = pulegone (11.0  $\pm$  2.6%, n = 6) = eugenol (11.0  $\pm$  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 100  $\mu$ M carvacrol (n = 6) & 100  $\mu$ M limonene oxide (n = 5). **C.** Concentration-response plots for acetylcholine alone (n = 6, black) and acetylcholine in the presence of 100  $\mu$ M carvacrol (n = 6, maroon) & 100  $\mu$ M limonene oxide (n = 5, green).  $pEC_{50}$  (mean  $\pm$  SEM),  $EC_{50}$  (mean,  $\mu$ M), Hill slope ( $n_H$ ) (mean  $\pm$  SEM) and  $I_{max}$  (mean  $\pm$  SEM%) values were respectively: 5.3  $\pm$  0.0, 5.3  $\mu$ M, 2.0  $\pm$  0.2 and 104.6  $\pm$  1.6 for acetylcholine alone; 5.4  $\pm$  0.2, 4.0  $\mu$ M, 2.2  $\pm$  1.7 and 62.8  $\pm$  7.0 in the presence of limonene oxide; 5.7  $\pm$  0.1, 2.0  $\mu$ M, 1.7  $\pm$  1.1 and 74.1  $\pm$  3.2 in the presence of carvacrol. Bottom was constrained to zero for curve fitting. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

$I_{max}$  value of 104.6  $\pm$  1.6%. We investigated the effects of limonene oxide (100  $\mu$ M) and carvacrol (100  $\mu$ M) on the acetylcholine concentration-response relationship.  $I_{max}$  for acetylcholine in the presence of limonene oxide and carvacrol were 62.8  $\pm$  7.0% and 74.1  $\pm$  3.2%, respectively. Both the monoterpene compounds produced a statistically significant reduction in the maximum response ( $P < 0.001$  for limonene oxide;  $P < 0.0001$  for carvacrol; Fig. 3B and C). Neither carvacrol,  $pEC_{50} = 5.7 \pm 0.1$  ( $EC_{50} = 2.0 \mu$ M) nor limonene oxide,  $pEC_{50} = 5.4 \pm 0.2$  ( $EC_{50} = 4.0 \mu$ M) increased the  $EC_{50}$  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

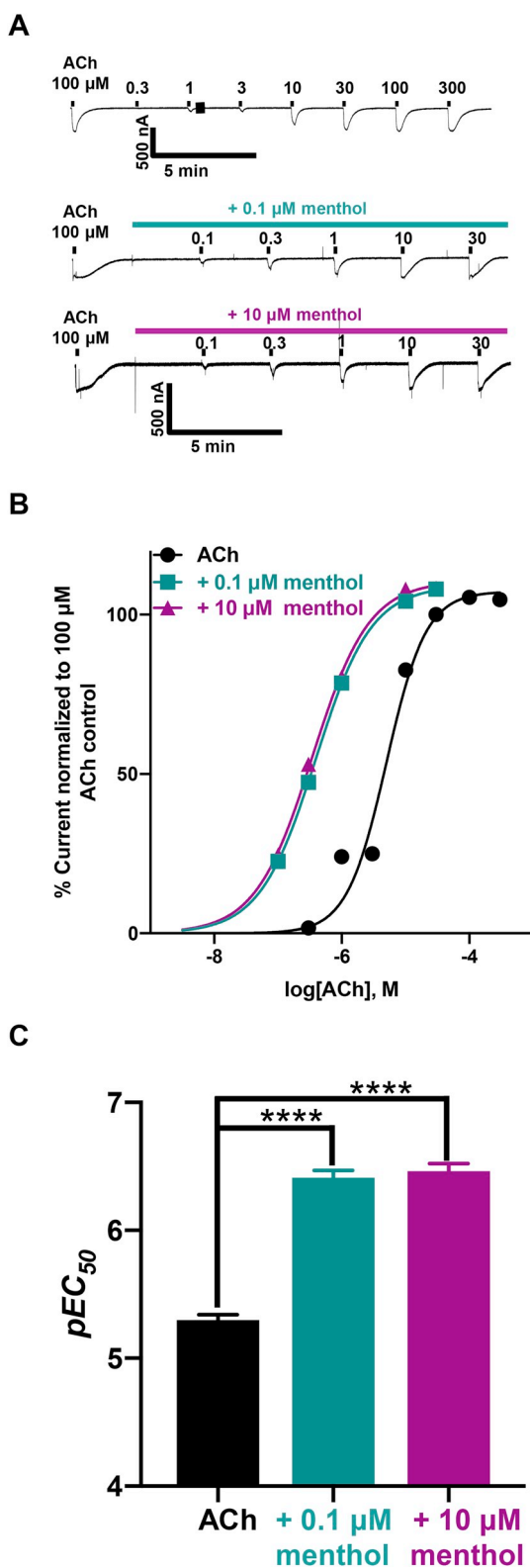
Menthol was a weak agonist on the expressed levamisole sensitive receptor. However, it appeared to potentiate acetylcholine responses in our antagonist experiments (data not shown). To investigate positive allosteric modulation further, we analyzed the effect of menthol on acetylcholine and levamisole concentration-response relationships (Fig. 4 and Fig. 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  $\mu$ M menthol,  $pEC_{50} = 6.4 \pm 0.1$  ( $EC_{50} = 0.4 \mu$ M) and with 10  $\mu$ M 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  $\pm$  0.1 and 0.5  $\mu$ M respectively. In the presence of 0.1  $\mu$ M menthol  $pEC_{50}$  and  $EC_{50}$  values were 6.5  $\pm$  0.1 and 0.3  $\mu$ M respectively. In the presence of 10  $\mu$ M menthol the  $pEC_{50}$  and  $EC_{50}$  values were 6.8  $\pm$  0.2 and 0.2  $\mu$ M. Only 10  $\mu$ M menthol produced a significant left shift in the levamisole concentration-response curve ( $P < 0.05$ ; Fig. 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

Fig. 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  $\pm$  0.3. We investigated the effects of menthol (10  $\mu$ M) and carvacrol (10  $\mu$ M and 100  $\mu$ M) on the acetylcholine response. There was no

examined by applying increasing concentrations of acetylcholine 0.3–300  $\mu$ M to oocytes expressing the levamisole sensitive nAChR (Fig. 3B and C). The sigmoidal concentration-response fit gave  $pEC_{50} = 5.3 \pm 0.0$  ( $EC_{50} = 5.3 \mu$ M), a hillslope ( $n_H$ ) of 2.0  $\pm$  0.2 and



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 (Fig. 6C) show that 100  $\mu\text{M}$  carvacrol decreased the maximum response while 10  $\mu\text{M}$

carvacrol failed to exhibit any significant antagonistic properties. In the presence of 10  $\mu\text{M}$  carvacrol  $pEC_{50}$ ,  $EC_{50}$  and  $I_{\max}$  values were  $5.1 \pm 0.1$ ,  $8.1 \mu\text{M}$  and  $94.6 \pm 5.9\%$  respectively. Carvacrol produced a significant reduction in  $I_{\max}$  ( $55.8 \pm 4.4\%$ ;  $P < 0.05$ ) when applied at 100  $\mu\text{M}$  concentration but did not produce a significant right shift in the  $pEC_{50}$  ( $5.1 \pm 0.1$ ). This indicates carvacrol at 100  $\mu\text{M}$  produced non-competitive inhibition.

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

carvacrol failed to exhibit any significant antagonistic properties. In the presence of 10  $\mu\text{M}$  carvacrol  $pEC_{50}$ ,  $EC_{50}$  and  $I_{\max}$  values were  $5.1 \pm 0.1$ ,  $8.1 \mu\text{M}$  and  $94.6 \pm 5.9\%$  respectively. Carvacrol produced a significant reduction in  $I_{\max}$  ( $55.8 \pm 4.4\%$ ;  $P < 0.05$ ) when applied at 100  $\mu\text{M}$  concentration but did not produce a significant right shift in the  $pEC_{50}$  ( $5.1 \pm 0.1$ ). This indicates carvacrol at 100  $\mu\text{M}$  produced non-competitive inhibition.

### 3.3. Effect of menthol on *A. suum* muscle

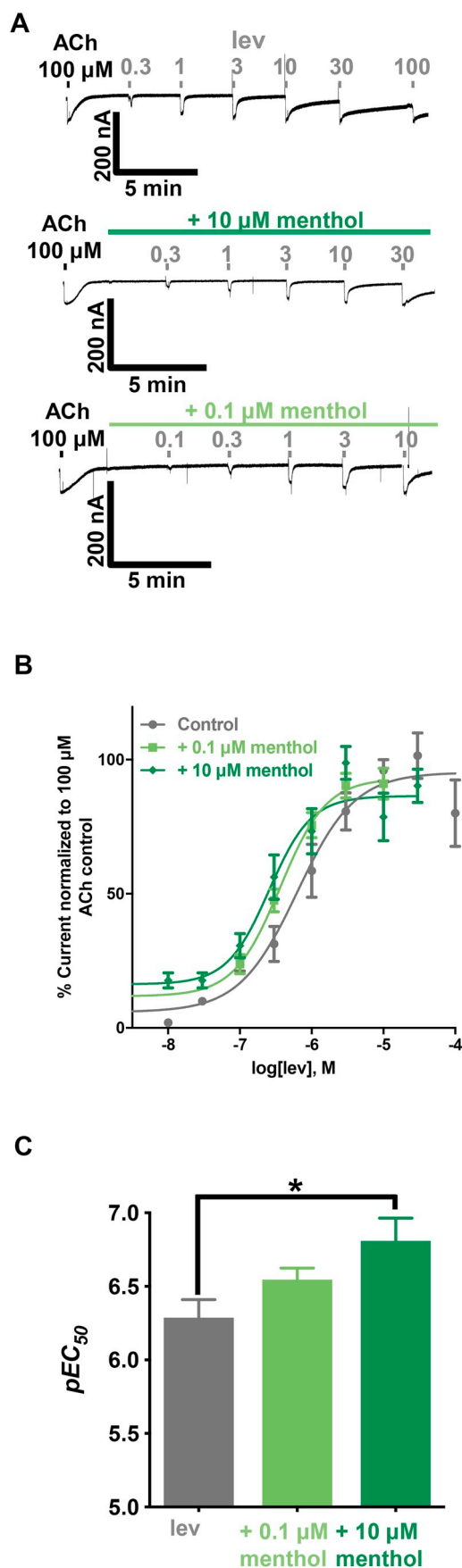
We examined the effect of menthol on the *A. suum* muscle flap contractions induced by increasing concentrations of acetylcholine. Fig. 7A shows the representative trace for acetylcholine, in the absence and presence of 0.1  $\mu\text{M}$  menthol in the experimental bath. Acetylcholine produced concentration-dependent isometric contractions with a  $pEC_{50}$  of  $5.2 \pm 0.2$  ( $EC_{50} = 7.0 \mu\text{M}$ ) and  $R_{\max} = 1.7 \pm 0.2$  g. We did not observe a significant left shift in  $pEC_{50}$  ( $5.2 \pm 0.2$ ) or change in  $R_{\max}$  ( $2.0 \pm 0.2$  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,  $P < 0.05$ , Fig. 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., 2001, 2003; 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 phytochemicals 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:

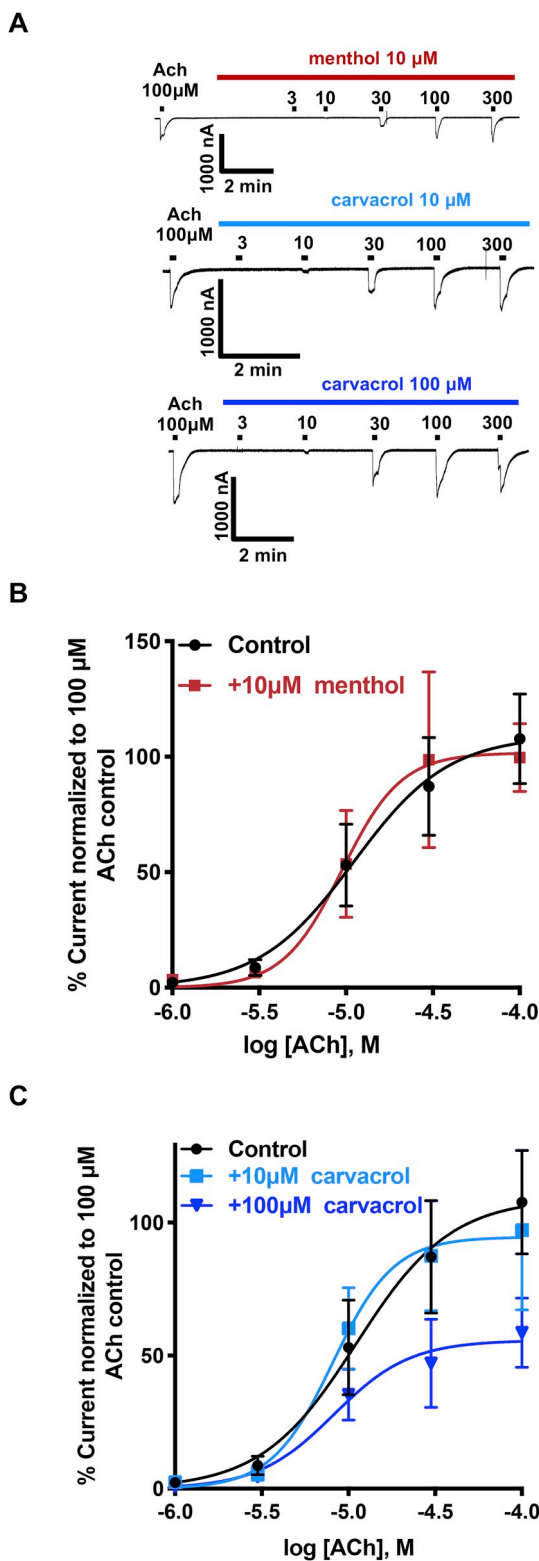
limonene oxide > citronellol > carvone > carvacrol = pulegone = eugenol.



**Fig. 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  $\mu\text{M}$  and 10  $\mu\text{M}$  menthol. **B.** Concentration-response relationships for levamisole alone ( $n = 7$ , steel gray) and in the presence of 0.1  $\mu\text{M}$  ( $n = 6$ , light green) and 10  $\mu\text{M}$  menthol ( $n = 7$ , dark green).  $pEC_{50}$  (mean  $\pm$  SEM),  $EC_{50}$  (mean,  $\mu\text{M}$ ), Hill slope ( $n_H$ ) (mean  $\pm$  SEM) and  $I_{max}$  (mean  $\pm$  SEM%) values were respectively:  $6.3 \pm 0.1$ , 0.5  $\mu\text{M}$ ,  $1.0 \pm 0.2$  and  $96.3 \pm 5.2$  for levamisole alone;  $6.5 \pm 0.1$ , 0.3  $\mu\text{M}$ ,  $1.1 \pm 0.2$  and  $94.6 \pm 5.0$  in the presence of 0.1  $\mu\text{M}$  menthol;  $6.8 \pm 0.2$ , 0.2  $\mu\text{M}$ ,  $1.0 \pm 0.2$  and  $89.2 \pm 6.0$  in the presence of 10  $\mu\text{M}$  menthol. Bottom was constrained to zero for curve fitting. **C.** Bar chart summarizing the results showing comparison of  $pEC_{50}$  (expressed as mean  $\pm$  SEM) for levamisole in the presence and absence of menthol. \* $P < 0.05$ ; significantly different as indicated; extra sum of squares  $F$ -test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Limonene oxide and carvacrol produced significant non-competitive inhibition suggesting neither compound acts at the ligand binding sites. Limonene oxide is a monoterpene 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 including thyme, oregano and 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 aminobutyric 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* (Trailović 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  $\mu\text{M}$  and of levamisole induced currents at 10  $\mu\text{M}$  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



**Fig. 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:  $5.0 \pm 0.1$  and  $11.1 \mu\text{M}$  for acetylcholine alone,  $5.0 \pm 0.1$  and  $9.5 \mu\text{M}$  in the presence of 10 μM menthol. Hill slope ( $n_H$ ) (mean ± SEM) values were:  $1.6 \pm 0.3$  for acetylcholine alone;  $2.5 \pm 1.2$  in the presence of 10 μM menthol.  $I_{max}$  (mean ± SEM%) values were:  $109.1 \pm 6.6$  for acetylcholine alone;  $101.6 \pm 7.5$  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:  $5.1 \pm 0.1$ ,  $8.1 \mu\text{M}$ ,  $2.5 \pm 0.8$  and  $94.6 \pm 5.9$  in the presence of 10 μM carvacrol;  $5.1 \pm 0.1$ ,  $8.2 \mu\text{M}$ ,  $2.0 \pm 0.7$  and  $55.8 \pm 4.4$  in the presence of 100 μM carvacrol. Bottom was constrained to zero for curve fitting. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

that these negative allosteric modulatory and inhibitory effects of menthol on  $\alpha 7$ -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  $\alpha 7$ -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.

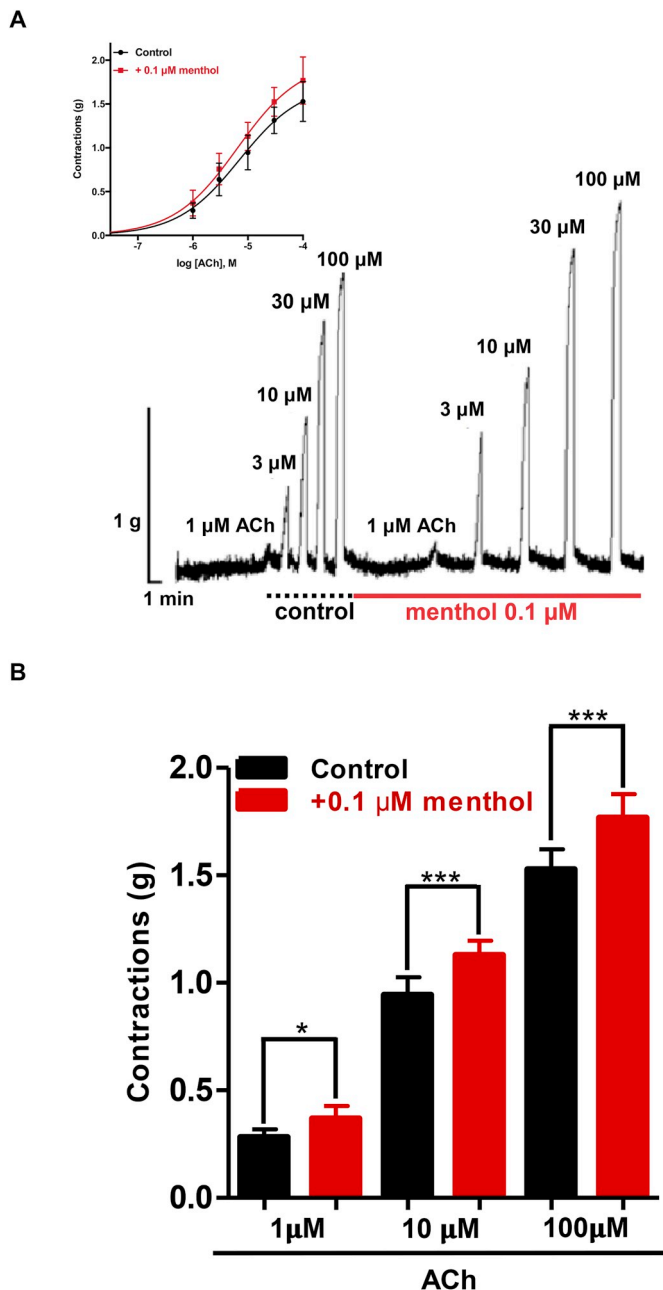
## 5. Conclusion

Natural products have formed the basis of sophisticated traditional therapies and they continue to play essential roles in modern health-care. Phytochemicals, 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.

pharmacological properties and expression of vertebrate nicotinic acetylcholine receptors; it inhibits the effect of nicotine on  $\alpha 7$ -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





**Fig. 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  $0.1 \mu\text{M}$  menthol ( $n = 6$ , red).  $pEC_{50}$  (mean  $\pm$  SEM) and  $EC_{50}$  (mean,  $\mu\text{M}$ ) values were respectively:  $5.2 \pm 0.2$  and  $7.0 \mu\text{M}$  for acetylcholine alone;  $5.2 \pm 0.2$  and  $6.5 \mu\text{M}$  in the presence of  $0.1 \mu\text{M}$  menthol.  $R_{\text{max}}$  (mean  $\pm$  SEM, g) values were:  $1.7 \pm 0.2$  g for acetylcholine alone and  $2.0 \pm 0.2$  g in the presence of  $0.1 \mu\text{M}$  menthol. Hill slope ( $n_H$ ) (mean  $\pm$  SEM) values were:  $0.8 \pm 0.2$  for acetylcholine alone and  $0.8 \pm 0.1$  in the presence of  $0.1 \mu\text{M}$  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  $0.1 \mu\text{M}$  menthol. \* $P < 0.05$ , \*\*\* $P < 0.001$ ; significantly different as indicated; paired  $t$ -tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpddr.2018.12.005>.

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