

## Article

# Action of carvacrol on *Parascaris* sp. and antagonistic effect on nicotinic acetylcholine receptors

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**Abstract:** *Parascaris* sp. is the only ascarid parasitic nematode in equids and one of the most threatening infectious organisms in horses. Only a limited number of compounds are available for treatment of horse helminthiasis and *Parascaris* sp. worms have developed resistance to the three major anthelmintic families. In order to overcome the appearance of resistance, there is an urgent need for new therapeutic strategies. The active ingredients of herbal essential oils are potentially effective antiparasitic drugs. Carvacrol is one of the principal chemicals of essential oil from Origanum, Thymus, Coridothymus, Thymbra, Satureja and Lippia herbs. However, the antiparasitic mode of action of carvacrol is poorly understood so far. Here, the objective of the work was to characterize the activity of carvacrol on *Parascaris* sp. nicotinic acetylcholine receptors (nAChRs) function both *in vivo* with the use of worm neuro-muscular flap preparations and *in vitro* with two-electrode voltage-clamp electrophysiology on nAChRs expressed in *Xenopus* oocytes. We have developed a neuro-muscular contraction assay on *Parascaris* body flaps and obtained acetylcholine concentration-dependent contraction responses. Strikingly, we observed that 300 µM carvacrol fully and irreversibly abolished *Parascaris* sp. muscle contractions elicited by acetylcholine. Conversely, carvacrol antagonized acetylcholine-induced currents from both the nicotine-sensitive AChR and the morantel-sensitive AChR subtypes. Thus, we show for the first time that the body muscle flap preparation is a tractable approach to investigate the pharmacology of *Parascaris* sp. neuro-muscular system. Our results suggest an intriguing mode of action for carvacrol being a potent antagonist of muscle nAChRs of *Parascaris* sp. worms which may account for its antiparasitic potency.

**Keywords:** *Parascaris*, carvacrol, nicotinic acetylcholine receptors, muscle contraction, electrophysiology, *Xenopus* oocytes, mode of action

## 1. Introduction

Helminth infections of livestock are of considerable importance and cause major financial losses in the world [1]. *Parascaris* sp. is the only ascarid parasitic nematode in equids and one of the most threatening infectious organisms in horses. *Parascaris* sp. worms have a very high prevalence especially in foals with important impact in terms of morbidity and mortality [2,3]. The worms leave in the intestine of the equids and are targets for anthelmintic drugs. Only a limited number of compounds are available for treatment of horse helminthiasis with the macrocyclic lactones being the last drug class of veterinary anthelmintics marketed since the eighties [1,4]. Anthelmintic resistance is a major problem in veterinary medicine and *Parascaris* sp. worms have recently developed resistance to the three major anthelmintic families [5-9]. In order to overcome the appearance of resistance, there is an urgent need for new therapeutic strategies, especially new chemical entities [1,10]. Based on their pharmacological properties, the active ingredients of herbal essential oils are potentially effective antiparasitic drugs [11-13]. Carvacrol and

thymol are monoterpenic phenol isomers and the principal chemical of essential oil from Origanum, Thymus, Coridothymus, Thymbra, Satureja and Lippia herbs [14]. Carvacrol has been known for long time for its wide use in traditional pharmacopeia due to antimicrobial and disinfectant properties [15-17]. In addition, some studies previously indicated that carvacrol has anti-nematodal properties against pathogenic helminths such as the pig roundworm, *Ascaris suum* [18], the sheep parasite, *Haemonchus contortus* [19,20], the fish parasite *Anisakis simplex* [14,21], plant parasitic nematodes [22] and can kill the free-living model nematode *Caenorhabditis elegans* [23-25]. However, the antiparasitic mode of action of monoterpenoid compounds is poorly understood and their potential on horse parasites has not been investigated so far. Previous studies evidenced that the anthelmintic effect of carvacrol might be mediated through different ligand-gated ion channel subtypes including tyramine, acetylcholine and GABA receptors of nematodes [18,25-28] as well as acetylcholinesterase [14].

Acetylcholine is a major excitatory neurotransmitter in both vertebrates and invertebrates. The nicotinic acetylcholine receptors (nAChRs) are major targets for anti-nematodal drugs such as pyrantel and levamisole [29,30]. They are members of the cys-loop ligand-gated ion channel superfamily and consist of five subunits arranged around a central pore [31]. Despite the large diversity of nAChR subunit genes present in nematodes, only few receptor subtypes have been characterized so far. Two nAChR subtypes have been described to mediate fast neurotransmission at the neuromuscular junction in the free-living model nematode *Caenorhabditis elegans* [32]. The levamisole-sensitive nAChR (L-AChR) which is a heteropentameric ion channel made of five different subunits and the prototypical nicotine-sensitive nAChR (N-AChR) which is composed of five identical subunits encoded by the *Cel-acr-16* gene [33,34]. In parasites, the ACR-16 receptor subunit was recently isolated and characterized from *Parascaris* sp. as well as the pig parasite, *Ascaris suum*, which are nematodes species from clade 3 distantly related to *C. elegans* [35,36]. When expressed in *Xenopus laevis* oocytes, ACR-16 formed a functional homomeric N-AChR which is activated by nicotine. Furthermore, a new subtype of nematode AChR preferentially activated by morantel was reported in *Parascaris* sp. (M-AChR) and the small ruminant parasite *Haemonchus contortus* [29]. Interestingly, parasitic nematodes affecting human or animals possess two closely related AChR subunit genes that are essentially absent in free-living or plant parasitic species: *acr-26* encodes an alpha subunit and *acr-27* encodes a non-alpha subunit. Hence, ACR-26 and ACR-27 subunits from *Parascaris* sp. were found to form a functional AChR when co-expressed in *Xenopus* oocytes, with higher affinities for pyrantel and morantel than for acetylcholine. Importantly, the heterologous expression of *Parascaris-acr-26* and *acr-27* as transgenes in the model nematode *Caenorhabditis elegans* also drastically increased morantel and pyrantel sensitivity *in-vivo* [29].

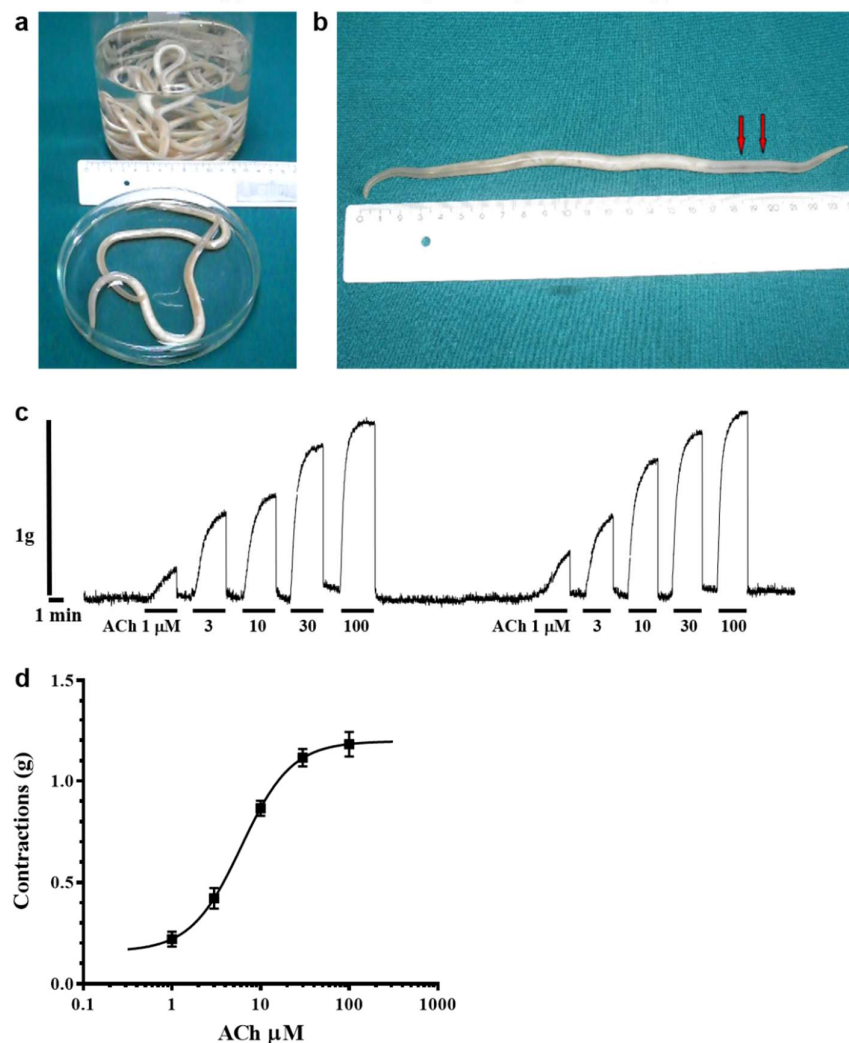
Here, the objective of the work was to characterize and investigate the activity of carvacrol at different concentrations on *Parascaris* sp. nicotinic acetylcholine receptors both on nAChR function *in vivo* with the use of worm neuro-muscular flap preparations and *in vitro* on nAChRs expressed in *Xenopus* oocytes. Strikingly, we observed that carvacrol abolished *Parascaris* sp. muscle contraction elicited by acetylcholine. Conversely, carvacrol inhibited acetylcholine-induced currents on both N-AChR and M-AChR subtypes. Thus we show carvacrol is a potent antagonist of muscle AChRs which may account for its antiparasitic potency against *Parascaris* sp. worms.

## 2. Results

### 2.1. Acetylcholine-induced contraction of *Parascaris* sp. body muscle flap preparation

*Parascaris* sp. and *A. suum* worms are closely related ascarid species with similar anatomy and morphology (Fig. 1A). The presence of acetylcholine receptors on *Parascaris* sp. muscles is anticipated as every nematode is supposed to synthesize acetylcholine and receptors although they have never been functionally evidenced so far. Therefore, as for *A.*

*suum*, it is expected that the application of acetylcholine on *Parascaris* sp. muscle strips would produce muscle contractions. As a first step, we adapted the muscle isometric contractions approach, which previously was used in *A. suum* studies [26,37]. Due to worm size differences, we had to modify the method of dissection. Unlike *A. suum*, the part of worm that we dissected for contractions was 4 to 5 cm behind the head instead of 2-3 cm in *A. suum* (Fig. 1B). In addition, in order to get contractions after acetylcholine application, we had to use a larger initial tension (1.5 g). On the other hand, the maximal contractions were no higher than the contractions previously obtained in *A. suum* experiments [26,37]. As a result, we were able to measure contractions of nerve-muscle strip preparations induced by ACh. The figure 1C shows representative recording of the *Parascaris* sp. muscle flap contractions produced by increasing concentrations of acetylcholine, while in the Figure 1D we present concentration-response plot for ACh fitted with non-linear regression. Increasing concentrations of ACh caused dose-dependent contractions of *Parascaris* sp. The control mean effective concentration ( $EC_{50}$ ) of ACh was  $6.08 \mu\text{M}$  ( $\log EC_{50} = 0.78 \pm 0.079$ ,  $n=5$ ), while maximal effect ( $R_{\text{max}}$ ) was  $1.19 \pm 0.051 \text{ g}$  obtained with  $100 \mu\text{M}$  ACh. Overall, these results indicate that *Parascaris* sp. body muscle flaps preparation is an amenable approach to investigate the pharmacology of its neuro-muscular system.

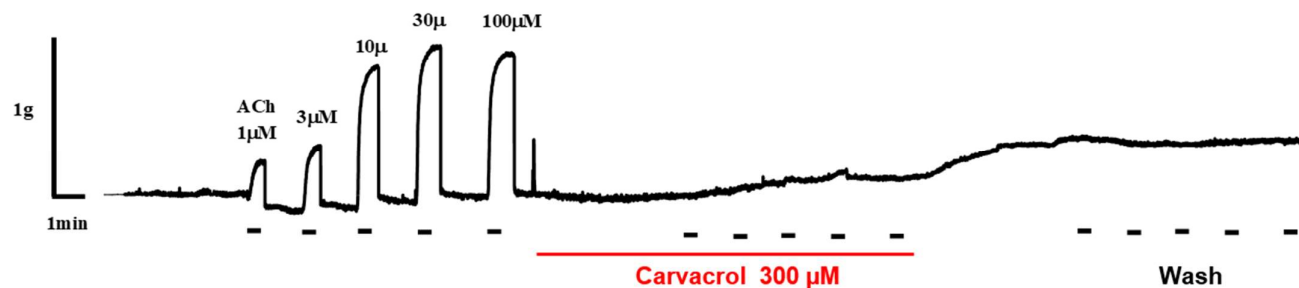


**Figure 1.** Contraction of *Parascaris* sp. muscle strips produced by acetylcholine. (a) Adult female *Parascaris* sp. collected from horses and used in this study; (b) Photograph of a single worm indicating the location of the body muscle flap (1 cm length between the two red arrows), within the anterior part of the worm (3-4 cm caudal to the head), to be dissected for isometric contraction measurements; (c) Isometric contractions of *Parascaris* sp. muscle flap produced by increasing

concentrations of acetylcholine (ACh) from 1 to 100  $\mu\text{M}$  (short bars); (d) Concentration-response plot for ACh fitted with non-linear regression, with mean contraction in  $\text{g} \pm \text{SE}$ .

### 2.2. Carvacrol abolishes acetylcholine-induced contractions of *Parascaris sp.* muscle strips

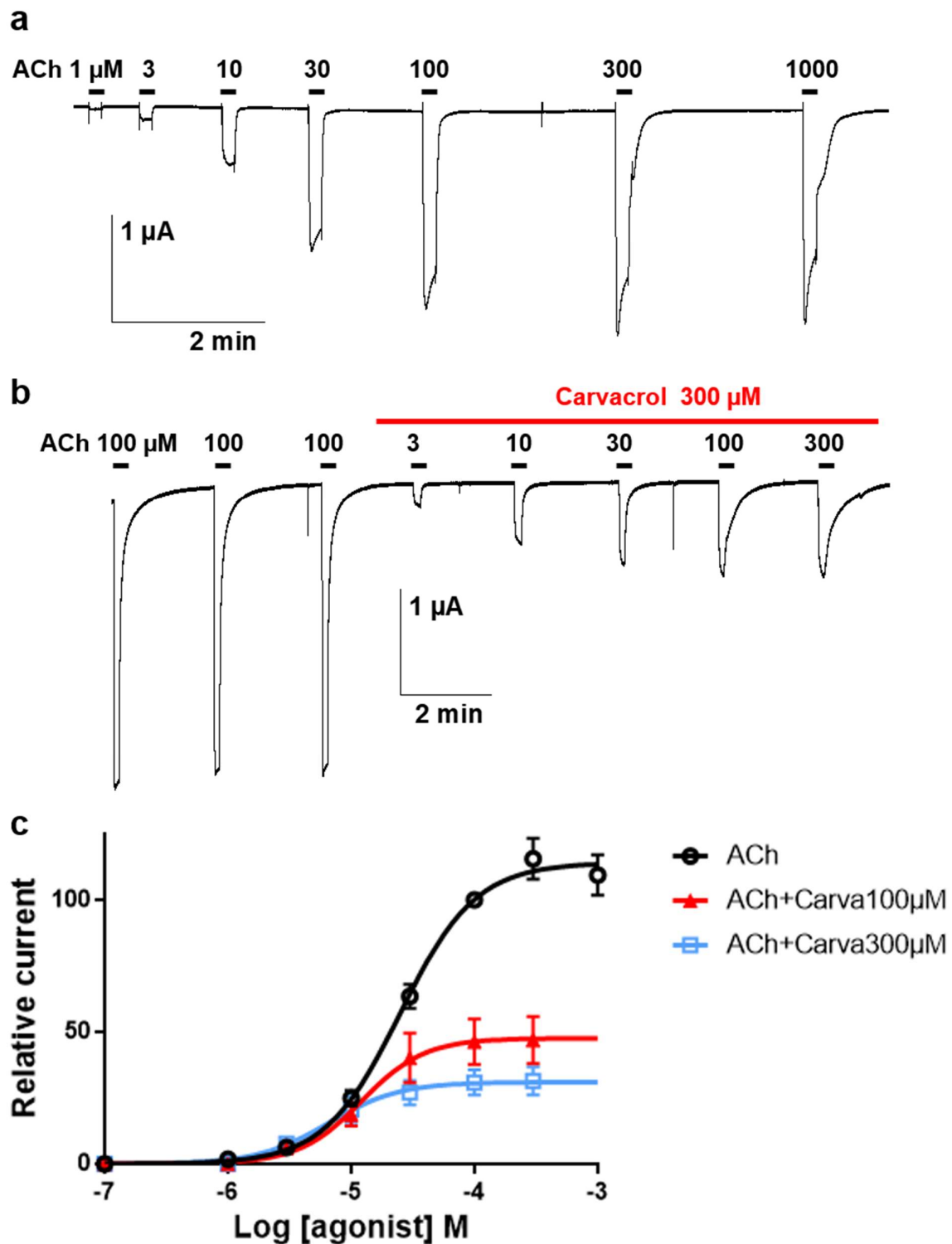
Previous studies highlighted the inhibitory effect of carvacrol on *A. suum* isolated muscle flap contractions caused by ACh [26,28]. In order to get first insights about the mode of action of carvacrol on *Parascaris sp.* worms, we determined the effect of carvacrol in isometric contractions of isolated segments of *Parascaris sp.* The figure 2 shows an inhibitory effect of carvacrol (300  $\mu\text{M}$ ) on the contractions of nerve-muscle preparation of *Parascaris sp.* induced by ACh. Strikingly, carvacrol completely abolished the contraction induced by ACh even at 100  $\mu\text{M}$  which is the highest concentration assessed that is used to give the maximal contraction effect. Interestingly, the inhibitory effect of 300  $\mu\text{M}$  carvacrol remained even after removal of carvacrol from experimental baths. Altogether, our results show isometric contractions of *Parascaris sp.* muscle strips produced by increasing concentrations of ACh and full inhibition of contractions following application of carvacrol.



**Figure 2.** Effect of carvacrol on contractions of *Parascaris sp.* muscle strips produced by acetylcholine. Isometric contractions of *Parascaris sp.* muscle flap produced by increasing concentrations of acetylcholine (ACh) from 1 to 100  $\mu\text{M}$  (left panel, short bars) and inhibition of contractions mediated by 300  $\mu\text{M}$  carvacrol (middle panel, full line). Absence of ACh-induced response recovery after washing the preparation (right panel).

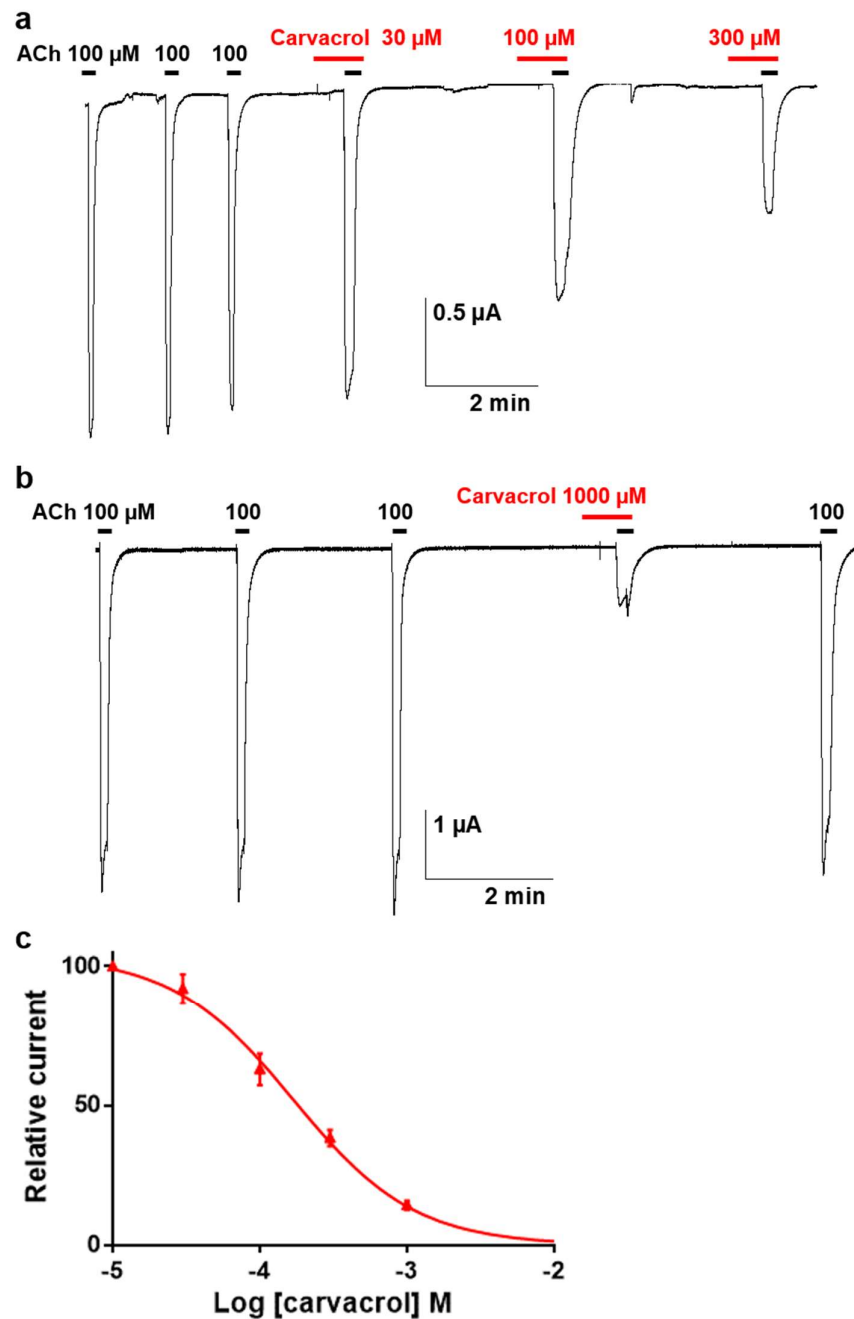
### 2.3. Effect of carvacrol on the *Parascaris sp.* morantel-AChR expressed in *Xenopus oocytes*

It was recently described that the co-expression of the *Parascaris sp.* ACR-26 and ACR-27 subunits in *Xenopus laevis* oocytes resulted in a functional morantel-sensitive AChR (M-AChR) (Courtot *et al.*, 2014). The expression of the *Parascaris* 26/27 M-AChR resulted in robust currents in the  $\mu\text{A}$  range when challenged with 100  $\mu\text{M}$  acetylcholine (Fig. 3A). The ACh  $\text{EC}_{50}$  value of 25.0  $\mu\text{M}$  ( $\log \text{EC}_{50} = 1.398 \pm 0.022$ ,  $n=6$ ) was estimated from the concentration response curve with current amplitudes normalized to the maximal response to 100  $\mu\text{M}$  (Fig. 3C). When carvacrol was perfused in the recording chamber, we observed no agonist action on the M-AChR (Fig. 3B). Strikingly, the continued perfusion of 100  $\mu\text{M}$  and 300  $\mu\text{M}$  carvacrol during the ACh concentration-response relationships significantly decreased the ACh  $\text{EC}_{50}$  values to 12.2  $\mu\text{M}$  ( $\log \text{EC}_{50} = 1.085 \pm 0.064$ ,  $n=5$ ), and 6.6  $\mu\text{M}$  ( $\log \text{EC}_{50} = 0.817 \pm 0.060$ ,  $n=6$ ), respectively ( $p < 0.0001$ ). The Hill coefficients were determined and remained stable in the presence of either 100  $\mu\text{M}$  ( $1.7 \pm 0.4$ ) or 300  $\mu\text{M}$  carvacrol ( $1.5 \pm 0.3$ ), compared to the absence of carvacrol ( $1.4 \pm 0.1$ ). In the same experiment, we observed that the perfusion of carvacrol significantly reduced the efficacy of ACh activation ( $I_{\text{max}}$ ) of this receptor ( $p < 0.0001$ ) (Fig. 3B).



**Figure 3.** Concentration-response relationships of acetylcholine on the *Parascaris* sp. ACR-26/27 M-AChR expressed in *Xenopus laevis* oocytes in absence of carvacrol (a) or in presence of carvacrol (b). Representative current traces for single oocytes. The concentrations of ACh and carvacrol ( $\mu$ M) are indicated above each trace; (c) Concentration-response curves. All responses are normalized to 100  $\mu$ M ACh. Results are shown as the mean  $\pm$  SE.

To characterize this effect, carvacrol antagonist concentration-response relationship was obtained by perfusing oocytes with increasing concentrations carvacrol for 10 seconds prior to the co-application with 100  $\mu$ M ACh (Fig. 4A-B). Hence, increasing concentrations of carvacrol (10  $\mu$ M – 1 mM) resulted in a dose-dependent reduction of the maximal ACh-elicited current amplitude. The  $IC_{50}$  value of carvacrol for the *Parascaris* M-AChR was  $169.3 \pm 1.0$   $\mu$ M (n=7) (Fig. 4C). Thus, carvacrol slightly increased the ACh affinity for the *Parascaris* M-AChR while acting as a non-competitive antagonist.

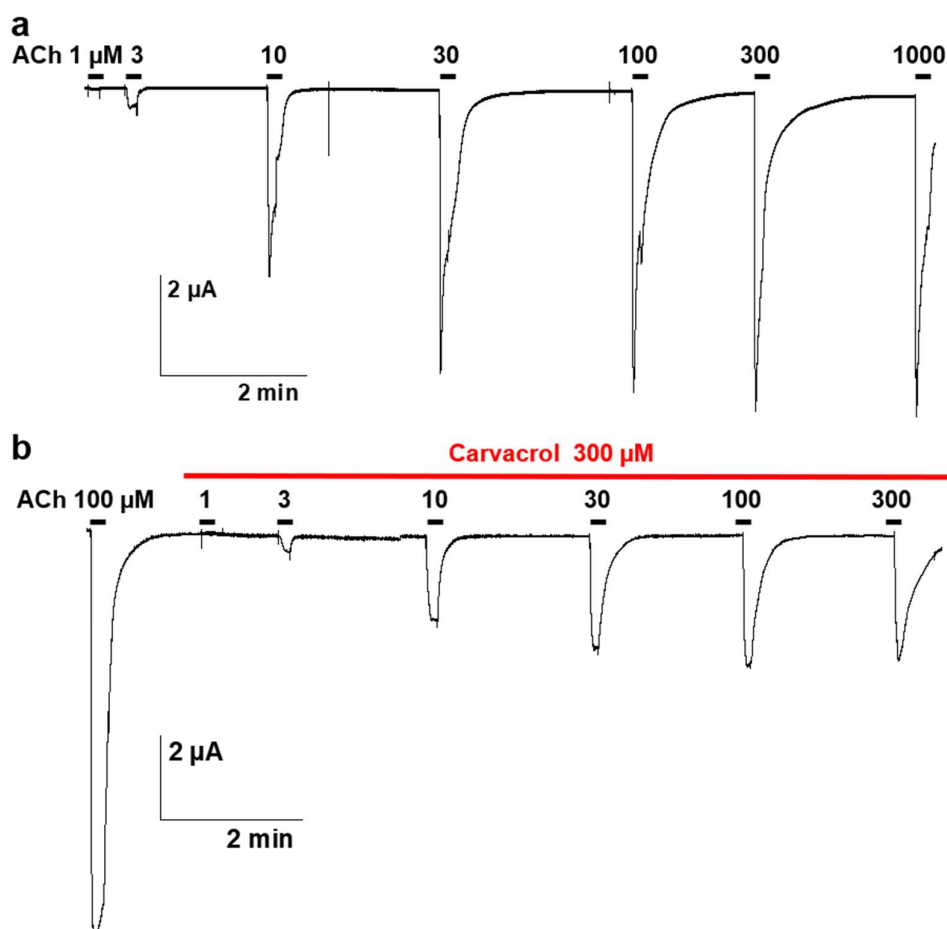


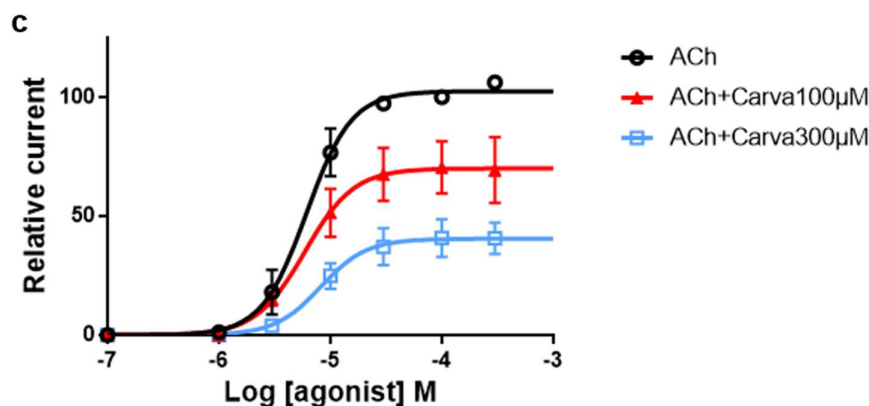
**Figure 4.** Concentration-inhibition relationship of carvacrol on the *Parascaris* sp. ACR-26/27 M-AChR expressed in *Xenopus* oocytes. Representative current traces for single oocytes challenged with acetylcholine (ACh) in the presence of increasing concentration of carvacrol from 30 to 300  $\mu$ M (a) and 1000  $\mu$ M (b). The concentrations of ACh and carvacrol ( $\mu$ M) are indicated above each trace; (c) Concentration-inhibition response curve of carvacrol. All responses are normalized to 100  $\mu$ M ACh. Results are shown as the mean  $\pm$  SE.



#### 2.4. Effect of carvacrol on *Parascaris* sp. and *Ascaris suum* nicotine-sensitive AChRs expressed in *Xenopus* oocytes

It was previously reported that the ACR-16 AChR subunit from *Parascaris* sp. and from the closely related species *A. suum* were able to form homomeric functional N-AChRs when expressed in *Xenopus* oocytes [35,36]. Recently, carvacrol proved to be a non-competitive inhibitor of the Asu-N-AChR [27]. In order to investigate the mode of action of carvacrol in *Parascaris* sp., we applied carvacrol on oocytes expressing the *Parascaris* sp. N-AChR (Fig. 5B). Perfusion of 100  $\mu$ M acetylcholine elicited large currents with maximum amplitude in the  $\mu$ A range (Fig. 5A) and the ACh concentration-response curve was characterized by an  $EC_{50}$  of 6.5  $\mu$ M ( $\log EC_{50} = 0.811 \pm 0.028$ ,  $n=6$ ) (Fig. 5C). As expected, a high concentration of carvacrol (300  $\mu$ M) had no agonist effect (Fig. 5B). In the presence of 100  $\mu$ M and 300  $\mu$ M of carvacrol, the  $EC_{50}$  values of ACh were 5.9  $\mu$ M ( $\log EC_{50} = 0.772 \pm 0.052$ ,  $n=6$ ) and 8.2  $\mu$ M ( $\log EC_{50} = 0.913 \pm 0.038$ ,  $n=10$ ), respectively, and not significantly different from the ACh  $EC_{50}$  obtained without carvacrol (Fig. 5C). As for the M-AChR, the Hill coefficients were similar with values of  $2.0 \pm 0.3$ ,  $2.1 \pm 0.4$  and  $2.0 \pm 0.2$  for 100  $\mu$ M, 300  $\mu$ M carvacrol and without carvacrol, respectively. However, the ACh maximal response amplitude was significantly reduced by 2 folds and 3 folds in the presence of 100  $\mu$ M and 300  $\mu$ M of carvacrol ( $p < 0.05$ ), respectively. Thus, ACh had a lower efficacy as an agonist of the *Parascaris* N-AChR in the presence of carvacrol.

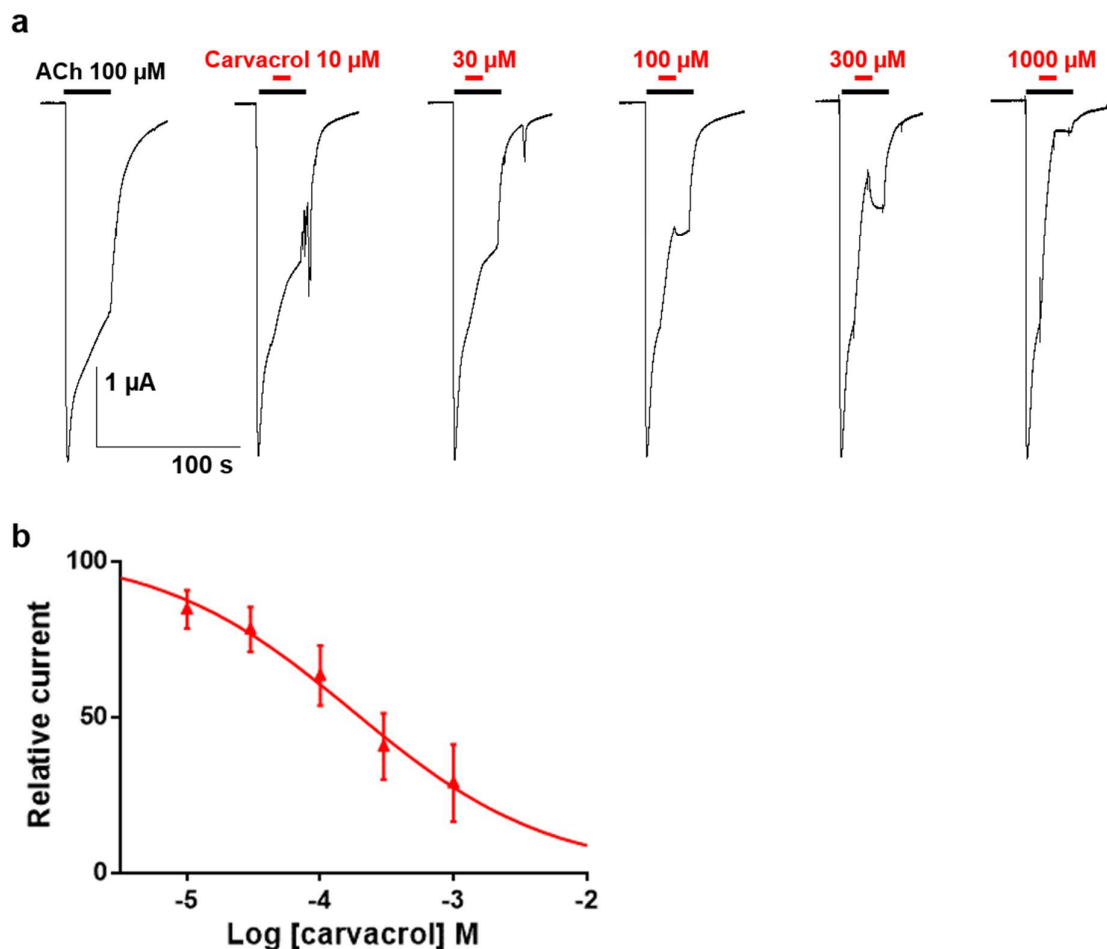




**Figure 5.** Carvacrol effect on the acetylcholine concentration-response relationships for the *Parascaris* sp. ACR-16 N-AChR expressed in *Xenopus* oocytes in absence of carvacrol (a) or in presence of carvacrol (b). Representative current traces for single oocytes. The concentrations of ACh and carvacrol ( $\mu\text{M}$ ) are indicated above each trace; (c) Concentration-response curves. All responses are normalized to 100  $\mu\text{M}$  ACh. Results are shown as the mean  $\pm$  SE.

To characterize this inhibition, carvacrol was perfused during the application of ACh as described elsewhere for different synthetic compounds (Fig. 6A) [38]. The carvacrol antagonist concentration-response relationship (10  $\mu\text{M}$  – 1 mM) resulted in a dose-dependent inhibition of the currents with an  $\text{IC}_{50}$  value of  $177.8 \pm 1.1$   $\mu\text{M}$  ( $n=6$ ) (Fig. 6B). Similarly, we confirmed that 100  $\mu\text{M}$  carvacrol had no impact on the ACh  $\text{EC}_{50}$  value for the *A. suum* N-AChR ( $6.0 \pm 1.0$   $\mu\text{M}$  ( $n=6$ ) versus  $4.9 \pm 1.1$   $\mu\text{M}$  ( $n=11$ )) without carvacrol), although the ACh  $\text{EC}_{50}$  value slightly increased ( $8.9 \pm 1.1$   $\mu\text{M}$  ( $n=5$ )) in the presence of 300  $\mu\text{M}$  carvacrol without being significantly different ( $p > 0.05$ ) (Fig. S1). As previously described [27], 100  $\mu\text{M}$  carvacrol led to a significant decrease in the ACh maximum response ( $73.6 \pm 1.7$  %,  $p < 0.05$ ,  $n=6$ ). Increasing the carvacrol concentration to 300  $\mu\text{M}$  further drastically reduced the effect of ACh ( $19.6 \pm 1.5$  %,  $p < 0.05$ ,  $n=5$ ). In addition, we performed a carvacrol antagonist dose-response relationship for the *Asu*-N-AChR and obtained an  $\text{IC}_{50}$  value of  $36.4 \pm 1.3$   $\mu\text{M}$  ( $n=6$ ) (Fig. S2). Altogether, these results indicate that carvacrol acted as a non-competitive antagonist on *Parascaris* sp. and *A. suum* N-AChRs.





**Figure 6.** Concentration-inhibition relationship of carvacrol on the *Parascaris* sp. ACR-16 N-AChR expressed in *Xenopus* oocytes. (a) Representative current traces for single oocytes challenged with acetylcholine (ACh) in the presence of increasing concentration of carvacrol from 10 to 1000 µM. The concentrations of ACh and carvacrol (µM) are indicated above each trace; (b) Concentration-inhibition response curve of carvacrol. All responses are normalized to 100 µM ACh. Results are shown as the mean ± SE.

### 3. Discussion

There has been a limited amount of published data reporting the contraction force transduction in adult parasite worms. In the present study, we carried out for the first time the investigation of *Parascaris* sp. worm pharmacology using contraction assays performed on nerve-muscle preparations. The contractions are not different from the contractions that were obtained in nerve-muscle preparation prepared from *A. suum*, except that the maximal effect is somewhat lower. Indeed, the  $EC_{50}$  of ACh from 6.08 µM is similar to the values ranging from 8.87 to 10.88 µM observed in *A. suum* innervated muscle strips [26,37]. These first results indicate that the body muscle flap preparation is a tractable approach to study the pharmacology of *Parascaris* sp. neuro-muscular system. In addition to *A. suum* [39], the measurements of force transduction were described in the sheep barber pole worm *Haemonchus contortus* [40] and in the canine hookworm *Ancylostoma caninum*, [41]. Interestingly, these studies helped to understand the diversity of body wall muscle nAChR subtypes that are preferentially activated or antagonized by different cholinergic anthelmintics [42]. In that context, it would be reasonable to expect that the muscle isometric contractions approach could be further adapted for pharmacological investigations in other nematode parasite species of interest such as the ascarids *Taxocara canis*,

*Ascaridia galli* and *Anisakis simplex*. Furthermore, when anthelmintic-resistant parasites were available, the comparison of muscle contraction assays with drug-susceptible nematode parasites have revealed new insights into the mechanisms underpinning resistance to anthelmintics [40,41]. As little is known on the effect of cholinergic anthelmintics on *Parascaris* sp. muscles the muscle contraction approach will be useful for to assess the nAChRs present in *Parascaris* sp. and the changes that could be associated with resistance. In *C. elegans*, *A. suum* and the pig nodule worm *Oesophagostomum dentatum*, single channel recordings revealed at least three main nAChR subtypes characterized by their conductances [43-45]. Likewise, single channel experiments in somatic muscle cells of *Parascaris* sp. could be helpful to investigate the muscle nAChR subtypes targeted by anthelmintics and carvacrol *in vivo*.

Given the limited number of anthelmintic drugs available for the control of *Parascaris* sp. infestations (benzimidazole, pyrantel, ivermectin and moxidectin), and the growing issue of anthelmintic resistance worldwide, there is an urgent need to develop new alternative control strategies [7,10]. Hence, more and more attention is given to the nematicidal potential of plant-based natural products, including essential oils, [46] which could replace or potentiate the effects of classical anthelmintic drugs [12]. The advantage of this approach is the possibility of continuous application of functional feeds, and thus prevent reinfection after deworming, which does not provide long-term protection against infection. Among the active ingredients from essential oils, carvacrol was shown to be active against animal parasitic nematodes, plant parasitic nematodes and the free-living nematode *C. elegans* [18,22,46]. There is a strong interest to explore essential oils and elucidate the mechanism of action of carvacrol remain high. Therefore, we took advantage of the adapted neuro-muscular contraction approach to assess the effect of carvacrol in *Parascaris* sp. Surprisingly, we found that carvacrol completely abolished the contractions induced by ACh, and this effect remained even after removal of carvacrol from experimental bath. Based on this result, we hypothesized that carvacrol may interact directly with nAChRs. Our electrophysiological investigations demonstrated the non-competitive inhibition of carvacrol on both the nicotine-sensitive ACR-16 and the morantel-sensitive ACR-26/27 AChRs from *Parascaris* sp expressed in *Xenopus* oocytes. In addition, this effect was further confirmed on the ACR-16 N-AChR from *A. suum* which is phylogenetically closely related to *Parascaris* sp. This not the first time that carvacrol has been assayed on *A. suum* nAChRs. It was previously observed that carvacrol produced significant inhibition of *A. suum* muscle contractions induced by ACh, inhibited depolarizations caused by acetylcholine and reduced membrane conduction of muscle cells [26]. Unlike menthol, carvacrol has further been reported to produce non-competitive inhibition on the *A. suum* ACR-16 N-AChR [27]. More recent contraction experiments revealed the antagonistic interaction of carvacrol with anthelmintic drugs at different muscle nicotinic receptors *in vivo* [28]. Interestingly, the full inhibition of the ACh contractile effect with 300  $\mu$ M of carvacrol is a major difference with the effect in *A. suum* did not exceed 49% [26,28]. This result suggests that *Parascaris* worms may be more sensitive to carvacrol than *Ascaris* worms. On the other hand, our data is consistent with the results on *A. suum* ACR-16 N-AChR, in which carvacrol acted as a non-specific antagonist [27]. In addition, we further confirmed this effect on *Parascaris* sp ACR-16 N-AChR and extended to the ACR-26/27 M-AChR. However, according to our concentration-inhibition data, carvacrol showed approximately 5-fold higher affinity for the *A. suum* N-AChR over the *Parascaris* sp N- and M-AChRs. Therefore, it is not possible to rule out that additional mechanisms may be involved in the activity of carvacrol in *Parascaris* sp.

Noticeably, carvacrol and cinnamaldehyde showed a better potency in multi-drug resistant *H. contortus* egg hatch assay when combined together and this result highlights the anthelmintic value of bioactive compounds from plant sources [20]. However, the literature is scarce on the clinical efficacy of herbal essential oils either alone or in combination with synthetic drugs *in vivo* whereas numerous studies have shown interesting effect *in vitro*. Some recent investigations on plant product combination with anthelmintic drugs

have reported potentially interesting synergistic effects against gastro-intestinal parasites [47-49]. The potential of carvacrol and essential oils either alone or in association with anthelmintic drugs in treating *Parascaris* sp. infections in equids remains to be evaluated. In summary, we report for the first time *in vivo* contraction assays from *Parascaris* sp. neuromuscular preparation. Our results indicate that the antimicrobial agent carvacrol preferentially acted by inhibiting nAChR function *in vivo* on *Parascaris* sp. muscle contractions and *in vitro* on both morantel- and nicotine-sensitive nAChRs. The present study opens the way for new prospects regarding the promising therapeutic properties of plant essential oils representing useful ingredients for deworming in veterinary medicine.

#### 4. Materials and Methods

##### 4.1. *Parascaris* sp. muscle flap contraction

For the contraction assay, adult female *Parascaris* sp. were collected weekly from the slaughterhouse at Vrčin, Belgrade, Serbia. Worms were maintained in Locke's solution, composition (mM): NaCl 155, KCl 5, CaCl<sub>2</sub> 2, NaHCO<sub>3</sub> 1.5 and glucose 5, at a temperature of 32 °C. The Locke's solution was changed twice daily, and each batch of worms was used within 2 days of collection. *Parascaris* muscle flaps for the contractions were prepared by dissecting the anterior part of the worm, 3-4 cm caudal to the head (Fig. 1B). Each flap (always the same length of 1 cm) was monitored isometrically by attaching a force transducer in an experimental bath maintained at 37 °C, containing 20 ml Ascaris Perienteric Fluid Ringer/APF Ringer (mM): NaCl, 23; Na-acetate, 110; KCl, 24; CaCl<sub>2</sub>, 6; MgCl<sub>2</sub>, 5; glucose, 11; HEPES, 5; pH 7.6, and bubbled with room air. After dissection, the preparations were allowed to equilibrate for 15 min. under an initial tension of 1.5 g. Different concentrations of ACh were then added to the preparation (1, 3, 10, 30, and 100 μM), and the maximum contraction was observed before washing and subsequent application of the next concentration of acetylcholine. Responses for each concentration were expressed in grams (g), produced by each individual flap preparation. The effect of Carvacrol (300 μM) on the acetylcholine dose-response plots was determined. Contractions were monitored on a PC computer, using a BioSmart interface, and eLAB software (EIUnit, Belgrade). The system allows real time recording, displaying, and analysis of experimental data. Sigmoid dose response curves for each individual flap preparation at each concentration of the antagonist were described by the Hill equation.

##### 4.2. Two-electrode voltage-clamp electrophysiology in *Xenopus laevis* oocytes

*Parascaris* sp. ACR-26/27 M-AChR as well as *Parascaris* sp. and *A. suum* ACR-16 N-AChRs were expressed in *Xenopus laevis* oocytes as previously described [29,35,36]. Briefly, *Xenopus laevis* defolliculated oocytes were obtained from Ecocyte Bioscience (Germany) and micro-injected with cRNAs mixes containing 50 ng/μL of each cRNA encoding subunits of interest and ancillary factors. After 3-4 days of incubation, the oocytes were assayed under voltage-clamp at -60 mV and electrophysiological recordings were performed as described previously. The carvacrol concentration-dependent inhibition of acetylcholine current response was performed on *Parascaris*- and *Asu*-ACR-16 channels with the protocol described by Zheng et al. [38].

##### 4.3. Drugs

Acetylcholine chloride (ACh) and carvacrol were purchased from Sigma-Aldrich.

##### 4.4. Statistical analyses

The results of contraction assay are expressed as means ± S.E. in grams (g) of contractions. Sigmoid concentration dose-responses were described by the equation as follows: % response =  $1/(1+[EC_{50}/X_a]^{nH})$ , where the median effective concentration (EC<sub>50</sub>) is the concentration of the agonist (X<sub>a</sub>) producing 50 % of the maximum response and nH is

the Hill coefficient (slope). GraphPad Prism® Software, (San Diego, CA.) was used to estimate the constants EC<sub>50</sub> and nH, by non-linear regression for each preparation. We determined the mean contraction responses to each concentration of acetylcholine. Whole cell current electrophysiology responses were analyzed using the pCLAMP 10.4 package (Molecular Devices). EC<sub>50</sub> and IC<sub>50</sub> values were determined using non-linear regression on normalized data (100 µM ACh as maximal response) using GraphPad Prism® software. One-way analysis of variance (ANOVA) was applied for the comparison of the differences between the EC<sub>50</sub> value and the maximal effect (R<sub>max</sub>). Differences were considered significant when the p value was <0.05. The statistical analysis was done using GraphPad Prism® software (San Diego, CA.), while all values are expressed as mean ± standard error (S.E.).

**Supplementary Materials:** Figure S1: Carvacrol effect on the acetylcholine concentration-response relationships for the *Ascaris suum* ACR-16 N-AChR expressed in *Xenopus* oocytes, Figure S2: Concentration-inhibition relationship of carvacrol on the *A. suum* ACR-16 N-AChR expressed in *Xenopus* oocytes.

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