2-2-2016

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Recommended Citation

Lozon Y, Sultan A, Lansdell SJ, Prytkova T, Sadek B, Yang KHS, Howarth FC, Millar NS, Oz M. Inhibition of human α7 nicotinic acetylcholine receptors by cyclic monoterpene carveol. *European Journal of Pharmacology*. 2016;776:44-51. doi: 10.1016/j.ejphar.2016.02.004

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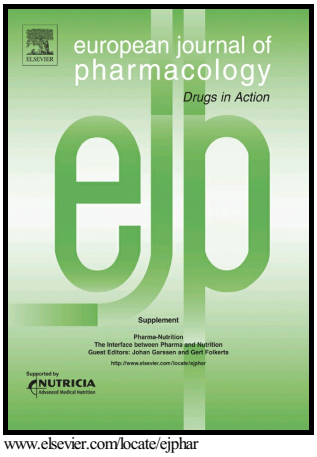

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PII: S0014-2999(16)30021-8
DOI: http://dx.doi.org/10.1016/j.ejphar.2016.02.004
Reference: EJP70438

To appear in: *European Journal of Pharmacology*

Received date: 1 October 2015
Revised date: 26 January 2016
Accepted date: 1 February 2016

Cite this article as: Yusra Lozon, Ahmed Sultan, Stuart J. Lansdell, Tatiana Prytkova, Bassem Sadek, Keun-Hang Susan Yang, Frank Christopher Howarth Neil S. Millar and Murat Oz, Inhibition of human α_7 nicotinic acetylcholin receptors by cyclic monoterpene carveol, *European Journal of Pharmacology* http://dx.doi.org/10.1016/j.ejphar.2016.02.004

Inhibition of human α_7 nicotinic acetylcholine receptors by cyclic monoterpene carveol

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Abstract

Cyclic monoterpenes are a group of phytochemicals with antinociceptive, local anesthetic, and anti-inflammatory actions. Effects of cyclic monoterpenes including vanilin, pulegone, eugenole, carvone, carvacrol, carveol, thymol, thymoquinone, menthone, and limonene were investigated on the functional properties of the cloned α_7 subunit of the human nicotinic acetylcholine receptor expressed in *Xenopus* oocytes. Monoterpenes inhibited the α_7 nicotinic acetylcholine receptor in

the order carveol > thymoquinone > carvacrol > menthone > thymol > limonene > eugenole > pulegone ≥ carvone ≥ vanilin. Among the monoterpenes, carveol showed the highest potency on acetylcholine-induced responses, with IC_{50} of 8.3 μM. Carveol-induced inhibition was independent of the membrane potential and could not be reversed by increasing the concentration of acetylcholine. In line with functional experiments, docking studies indicated that cyclic monoterpenes such as carveol may interact with an allosteric site located in the α_7 transmembrane domain. Our results indicate that cyclic monoterpenes inhibit the function of human α_7 nicotinic acetylcholine receptors, with varying potencies.

Keywords: Nicotinic acetylcholine receptor; Monoterpenes; Carveol; *Xenopus* oocyte

1. Introduction

Monoterpenes are a structurally diverse group of phytochemicals and are major constituents of essential oils. They are widely used for medicinal purposes and as food additives (Koziol et al., 2014). Monterpenes include acyclic compounds, such as myrcene and geraniol; cyclic compounds, such as thymol and carvacrol; and bicyclic compounds such as borneol and eucalyptol. In earlier studies, cyclic monoterpenes have been shown to exhibit significant anti-inflammatory, anti-oxidant, analgesic, and anxiolytic effects (for reviews, Guimarães et al., 2013; 2014; Koziol et al., 2014). However, molecular targets mediating these actions of cyclic monoterpenes are largely unknown. In earlier studies, cyclic monoterpenes have been shown to modulate the functional properties of various ion channels, including voltage-gated Na^+ channels (Haeseler et al., 2002; Gaudioso et al., 2012), K^+ (Sensch et al., 2000), voltage-gated Ca^{2+} channels (Seo et al., 2013), transient-receptor potential channels (Parnas et al., 2009), 5-HT3 receptors (Ashoor et al., 2013; Lansdell et al., 2015) and $GABA_A$ receptors (Priestley et al., 2003;

Kessler et al., 2013) (for reviews, de Araújo et al., 2011; Oz et al., 2015).

Nicotinic acetylcholine receptors (nAChRs) belong to the Cys-loop family of ligand-gated ion channels and are located throughout the central and peripheral nervous system. Neuronal nAChRs, such as the homomeric α_7 subtype, are located on presynaptic terminals or at extrasynaptic sites on soma and dendrites (for a review: Zoli et al., 2015). Moreover, these receptors have been shown to be expressed in non-neuronal structures (Egea et al., 2015), and in immune system cells (Matteoli and Boeckxstaens, 2013). The high Ca^{2+} permeability and presynaptic location of α_7 nAChRs suggest that these receptors exert an important modulatory influence in the neurotransmitter release and cellular excitability of the nervous system. These receptors have also been shown to play roles in inflammation, nociception, psychosis, and Alzheimer disease (for reviews Oz et al., 2013; Umana et al., 2013; Hurst et al., 2014). The objective of the present study was to examine the effects of cyclic monoterpenes such as carveol on the electrophysiological properties of human α_7 nAChRs expressed in *Xenopus* oocytes.

2. Material and methods

2.1. Recordings from oocytes

Female *Xenopus laevis* frogs were obtained from Xenopus Express (Haute-Loire, France) and housed in a container filled with dechlorinated water at 19-21 °C. Frogs were exposed to 12/12-h light/dark cycle and fed with dry food pellets obtained from Xenopus Express. The procedures used in the present study were in accordance with the 'Guide for the care and use of laboratory animals', National Institutes of Health (Bethesda, MD) and approved by the Institutional Animal Ethics Committee at the UAE University. Experimental procedures used in the present study were described in detail previously (Oz et al., 2004; Singhal et al., 2007). Briefly, oocytes were removed surgically under local tricaine (Sigma, St. Louis, MO) anesthesia (0.15%

w/V). Oocytes were dissected manually in a solution containing (in mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; $MgSO_4$, 0.8; HEPES, 10 (pH 7.5). Isolated oocytes were then stored for up to seven days in modified Barth's solution (MBS) containing (in mM): NaCl, 88; KCl, 1; $NaHCO_3$, 2.4; $Ca(NO_3)_2$, 0.3; $CaCl_2$, 0.9; $MgSO_4$, 0.8; HEPES, 10 (pH 7.5), supplemented with sodium pyruvate, 2 mM, penicillin 10,000 IU/l, streptomycin, 10 mg/l, gentamicin, 50 mg/l, and theophylline, 0.5 mM. During experiments, oocytes were positioned in a recording chamber with a volume of 0.2 ml and superfused at a rate of 2-3 ml/min. The bathing solution contained (in mM): NaCl, 95; KCl, 2; $CaCl_2$, 2; and HEPES 5 (pH 7.4). The cells were impaled with two glass microelectrodes (1-10 MΩ) filled with 3 M KCl. Throughout the experiments, the oocytes were voltage-clamped at a holding potential of -70 mV using a GeneClamp-500 amplifier (Axon Instruments Inc., Burlingame, CA), and current responses were recorded digitally at 2 kHz (Gould Inc., Cleveland, OH).

Drugs were applied by a glass pipette positioned about 2 mm from the oocyte. Bath applications of the compounds were attained by directly adding to the superfusate. All chemicals used in preparing the solutions were from Sigma-Aldrich (St. Louis, MO). All the monoterpenes and α-bungarotoxin were obtained from Sigma (St. Louis, MO). Stock solutions (10 mM) of monoterpenes used in this study were prepared in distilled water.

The cDNA clone of human α_7-nicotinic acetylcholine receptor was kindly provided by Dr. J. Lindstrom (University of Pennsylvania, PA). The cDNAs for human α_4, β_2, α_3, and β_4 subunits were kindly provided Dr. Subunit combinations, α and β subunits were injected at 1:1 ratio.

Capped cRNA transcripts were synthesized *in vitro* using an mMESSAGE mMACHINE kit from Ambion (Austin, TX) and analyzed on 1.2% formaldehyde agarose gel to check the size and the

quality of the transcripts.

2.2. Radioligand binding studies

Radioligand binding studies were performed with [^{3}H]-α-bungarotoxin (56.0 Ci/mmol; Quotient Bioresearch (Radiochemicals) Ltd, Cardiff, UK), essentially as described previously (Barker et al. 2004). Binding experiments were performed on intact transiently transfected human kidney (tsA201) cells in HBS buffer containing 0.5% BSA to reduce non-specific binding. Samples were incubated with radioligand (10 nM [^{3}H]-α-bungarotoxin) in a total volume of 150 µl for 2 hours at room temperature. Competition studies were carried out using [^{3}H]-α-bungarotoxin (10 nM) and varying concentrations (0.003 nM to 100 µM) of competing ligands. Samples were assayed by filtration onto Whatman GF/A filters pre-soaked in 0.5% polyethyleneimine, followed by rapid washing with phosphate buffered saline using a Brandel cell harvester and levels of bound radioligand were determined by scintillation counting, as described previously (Barker et al. 2004).

2.3. Docking studies:

An NMR structure of the human α_7 nAChR transmembrane domain (PDB code 2MAW) (Bondarenko et al., 2014) was used for docking calculations and analysis. Docking of vanillin (cid :01609418), pulegone (cid :442495), eugenole (cid : 3314), carvone (cid : 439570), carvacrol (cid : 10364), carveol (cid : 7438), thymol (cid : 6989), thymoquinone (cid : 10281), menthone (cid : 443159), limonene (cid :22311) to the structural model (Bondarenko et al., 2014) was made by the Autodock Vina program (Trott et al., 2010). Ligands and receptor files were prepared based on

the procedure described in Autodock Tools (ADT) (Morris et al., 2009) documentation. ADT assigned polar hydrogens, united atom's Kollman charges and solvation parameters. Prepared files were saved in PDBQT format. Affinity grid maps of 20x20x20Å with spacing 0.375Å were added. Grid center was designated x,y,z dimensions: -8.73,-11.56 and -20.68 which correspond to center of side chain on Phe453. These coordinates correspond to recently identified binding site for ketamine in the human α_7 nAChR transmembrane domain (Bondarenko et al., 2014). Docking calculations were performed using the Lamarckian genetic algorithm (LGA) (Morris et al., 1998) .The pose with the lowest binding free energy was aligned with receptor for further analysis of interactions.

2.4. Data analysis

Average values were calculated as the mean ± standard error of the mean (S.E.M.). Statistical significance was analyzed using Student's *t* test or ANOVA as indicated. Concentration-response curves were obtained by fitting the data to the logistic equation,

$y = E_{max}/(1+[x/EC_{50}]^{-n})$,

where x and y are concentration and response, respectively, E_{max} is the maximal response, EC_{50} is the half-maximal concentration, and n is the slope factor (apparent Hill coefficient).

3. Results

In uninjected oocytes (n=5), acetylcholine at the highest concentration used in this study (10 mM), did not cause detectable currents. On the other hand, application of 100 μM acetylcholine for 3 to 4 sec induced rapidly activated inward currents in oocytes injected with cRNA transcribed from cDNA encoding the human nAChR α7 subunit. These acetylcholine-induced inward currents were completely inhibited by 100 nM α-bungarotoxin (n=9, data not shown), indicating that currents recorded in our experiments were mediated by the activation of recombinant α_7 nAChRs.

Effects of 10 cyclic monoterpenes (vanilin, pulegone, eugenole, carvone, carvacrol, carveol, thymol, thymoquinone, menthone, and limonene) were investigated on the function of α_7 nAChRs (Fig. 1). In these experiments, ACh concentration of 100 μM was chosen since ACh (EC_{50} = 96 μM) at this concentration produces approximately 50 % of maximal response for α_7 nAChRs (see Fig. 6). A comparison of the effects of monoterpenes (10 μM for 10 min application) on ACh (100 μM)-induced currents is presented in Fig. 2. Vanilin, pulegone, eugenole, and carvone (at 10 μM) did not cause a significant change in the amplitude of currents evoked by acetylcholine (100 μM). However, all of the other monoterpenes compounds examined produced significant inhibitory effects on α_7 nAChRs (Fig. 2). The rank order of their inhibitory effects was: carveol > thymoquinone > carvacrol > menthone > thymol > limonene. Increasing the application time to 10 in to 20 min which is 6 fold more than τ1/2 for carveol (see Fig. 4A) did not cause any further change on the effects of the monoterpenes (see supplement Fig. 1). Dose-response curves for the compounds for monoterpenes that induced a significant inhibitory effect were shown in supplement Fig. 2. None of the monoterpenes tested alone at 10 μM induced an agonist action on

α_7 nAChRs (n=4-6, for each compound). Recordings of the acetylcholine-induced currents, in the absence and presence of 10 μM carveol and after 10 min recovery are shown in Fig. 3A, The time-course of effects of carveol on the amplitude of acetylcholine-activated currents are shown in Fig. 3B. Inhibition by carveol reached a steady-state level in less than 10 min and was fully reversible within a washout period of 10 to 15 min. In the absence of carveol (control condition), the maximal amplitude of acetylcholine-activated currents remained unaltered during the course of the experiments (Fig. 3B, controls).

It is noteworthy that the effect of carveol on α_7 nAChRs was dependent on the duration of pre-application. For example, without carveol preincubation, the coapplication of carveol (10 μM) and acetylcholine (100 μM) did not cause a significant alteration in the amplitude of maximal current (0 time point in Fig. 4A). However, increasing the preincubation time of carveol caused significantly enhanced inhibition, reaching a maximal level within 10 min (with a half-time ($\tau_{1/2}$) of 3.4 ± 0.4 min; Fig. 4A). Since the magnitude of the carveol effect was time-dependent, an application time of 10 min was used to ensure equilibrium conditions. Carveol inhibited the function of α_7 nAChRs in a concentration-dependent manner with IC_{50} and slope value of 8.3 ± 0.5 μM and 0.86, respectively (Fig. 4B).

We also investigated whether the inhibition of α_7 nAChRs by carveol was affected by changes in the membrane potential. Each tested potential was held for 30 sec and then returned to -70 mV. As shown in Fig. 5A, the inhibition by carveol (10 μM) of currents evoked by acetylcholine (100 μM) does not seem to be voltage-dependent. Examination of the current-voltage relationship (Fig. 5B) shows that the extent of inhibition by carveol was not altered significantly at different holding potentials (P>0.05, n=7, ANOVA).

Activation of α_7 nAChRs caused a significant increase in intracellular Ca^{2+} and activated endogenous Ca^{2+}-dependent Cl^- channels in *Xenopus* oocytes (Sands et al., 1993). It was possible that the effect of carveol was mediated by the inhibition of inward Cl^- currents induced by Ca^{2+} entry. Thus, we tested whether inward Cl^- currents contribute to the carveol-induced inhibition of α_7 nAChRs. For this reason, oocytes were injected with BAPTA (100 mM, 50 nL) to chelate intracellular Ca^{2+} increases. In addition, extracellular Ca^{2+} was replaced with Ba^{2+}, since Ba^{2+} can pass through α_7-nicotinic acetylcholine receptors but causes a significantly less activation of Ca^{2+}-dependent Cl^- channels (Sands et al., 1993). Carveol (10 μM) produced the same level of inhibition of α_7 nAChRs (54 ± 5 in controls versus 49 ± 6 in Ba^{2+} containing solutions, ANOVA, n=6-7, P>0.05; Fig. 5C). In agreement with these results, in control experiments, carveol at the highest concentration used in this study (300 μM for 10 min) did not cause any change on the magnitudes of holding-currents (43 ± 5 nA in control versus 46 ± 4 nA in carveol at -70 mV holding potential) or input resistance (1.8 ± 0.3 MΩ in control versus 1.7 ± 0.4 MΩ in carveol; n=9) of oocytes, indicating that intracellular Ca^{2+} levels were not altered by carveol.

Carveol may decrease the binding of acetylcholine to the α_7-nAChR and inhibit these receptors as a competitive antagonist. For this reason, the effect of carveol was examined at different concentrations of acetylcholine. In Fig. 6, concentration-response curves for acetylcholine were presented in the absence and presence of 30 μM carveol. In the presence of carveol, the potency of acetylcholine was not altered significantly. However the maximal response induced by acetylcholine decreased significantly (n=6-8). The respective EC_{50} values in the absence and presence of carveol were 96 ± 11 μM and 87 ± 9 μM (P>0.05, ANOVA, n=6-9) and slope values were 1.2 ± 0.3 and 0.9 ± 0.4, suggesting that carveol inhibits the nicotinic

acetylcholine receptors in a non-competitive manner with respect to acetylcholine.

If carveol is, indeed, binding at a site other than the orthosteric agonist binding site, it would not be expected to displace the binding of a competitive antagonist such as α-bungarotoxin. To examine this, ability of carveol to displace the specific binding of [^{3}H]-α-bungarotoxin was examined in a transiently transfected mammalian cell line (tsA201) expressing human recombinant α7 nAChRs. No significant displacement of [^{3}H]-α-bungarotoxin was observed with carveol, even at the highest concentrations tested (1 mM) (Fig. 7). In contrast, the competitive antagonist methyllycaconitine (MLA) caused complete displacement of [^{3}H]-α-bungarotoxin in a dose-dependent manner, with an IC_{50} value of 33 ± 3 nM (n=5).

The effect of carveol on the functional properties of other neuronal nAChR subtypes was also examined but did not show significant inhibitory or potentiating effects (Fig. 8).

Computer docking studies were conducted with a previously described NMR structure of the α7 transmembrane domain (Bondarenko et al., 2014). The aim of these studies being to test the hypothesis that monoterpines compounds might exert there inhibitory effect by binding to a site similar to that previously identified for the non-competitive antagonist ketamine (Bondarenko et al., 2014). The results of the docking studies (Fig. 9 and Table 1) support the conclusion that cyclic monoterpenes such as carveol are able to interact with a similar transmembrane site to that reported previously for ketamine (Bondarenko et al., 2014).

4. Discussion

In this study, we provide evidence that cyclic monoterpenes inhibit human α_7 nAChRs with varying potencies. While vanilin, pulegone, eugenole, and carvone did not alter functional properties of α_7-nAChR, other monoterpenes inhibited the activity of this receptor in the rank order: carveol > thymoquinone > carvacrol > menthone > thymol > limonene. The inhibitory effect of carveol was reversible and concentration-dependent. Increases in the concentration of acetylcholine did not significantly alter the inhibitory effect of carveol, suggesting that it acted through a non-competitive mechanism of action. This conclusion was supported by competition radioligand binding studies demonstrating that carveol was unable to displace the binding of the competitive antagonist [^{3}H]-α-bungarotoxin.

Many of the cyclic monoterpenes have been shown to release Ca^{2+} from intracellular stores (for a review Oz et al., 2015). In *Xenopus* oocytes, activation of Ca^{2+}-activated Cl^- channels by changes in intracellular Ca^{2+} levels (for reviews: Fraser and Djamgoz, 1992; Terhag et al., 2015) can interfere with α_7 nAChR-mediated currents. However, during our experiments, application of carveol, even at the highest concentration (300 μM) used in this study, did not cause any alteration on the holding currents, suggesting that carveol did not affect intracellular Ca^{2+} concentration. In *Xenopus* oocytes, activation of α_7 nAChRs, due to their high Ca^{2+} permeability, allows sufficient Ca^{2+} entry to activate endogenous Ca^{2+}-dependent Cl^- channels (Séguéla et al., 1993; Sands et al., 1993). However, in oocytes injected with BAPTA and recorded in solution containing 2 mM Ba^{2+}, carveol continued to inhibit α_7 nAChR-mediated currents, suggesting that Ca^{2+}-dependent Cl^- channels were not involved in carveol inhibition of nicotinic responses.

Open-channel blockade is a widely used model to describe the block of ligand-gated ion

channels (Hille, 2001). However, this model does not seem to be consistent with the results obtained in the present study. For an open channel blocker, the presence of the agonist is required for the entrance of the blocker to the channel after the receptor-ion channel complex has undergone an agonist-induced conformational change. In contrast to open channel blockers, preincubation of carveol caused further inhibition (Fig. 4) indicating that the channel does not need to be open in order to interact with carveol. Secondly, inhibition by carveol was not sensitive to changes in membrane potential, suggesting that the binding site for carveol is not within the transmembrane region.

In docking studies, our results show that pose of the ligand in the binding site is located in the cavity of lower part of transmembrane domain, and that hydrophobic interactions in the pocket between helices play an important role and can be correlated with % inhibition measured in the experiment (Table 1). As can be seen in Fig. 9, the binding position of carvacrol and thymoquinone is parallel to the ring of Phe453, and both ligands have favorable binding energies with -6.3 kcal.mol and -6.7 kcal/mol, respectively. Interestingly, these two compounds have demonstrated effective inhibition. In contrast, carvone, with binding energy of -6.2 kcal/mol, has a monoterpene ring is not parallel but rotated relative to ring of Phe453. Notably, all other ligands follow the same pattern and their behavior can be described similarly except limonene which has binding position similar to thymoquinone and relatively favorable binding energy -6.1 kcal/mol but demonstrates only 15 % of inhibition.

Moreover, factors other than effective binding position can also influence inhibition. For example differences in hydrophobicity of the ligands may play a role in the favorable interaction of ligands with the membrane and may prevent interaction with receptor. Monoterpenes are highly lipophilic compounds with Log p values ranging between 2 and 5 (Turina et al., 2006;

Zunino et al., 2011).

Analysis of binding interaction of ligands with residues of the binding was made . It was noticed that favorable interaction of the ligands with residues in the binding site such as Ser285, Leu248, Ile217 and Lys239 might be important in influencing inhibition. It is possible that the specific conformation of the ligand inside of the binding site may prevent gating movement of helix TM2. There is direct experimental evidence that the TM2 helix plays an important role in movements associated with the gating of nAChRs, with the pore-lining TM2 helix changing conformation during receptor activation (Unwin and Fujiyoshi, 2012). In closed conformation of the receptor, when helix TM2 has a kinked orientation, the hydrophobic cavity between helices is expected to have a larger volume, which may be favorable for the binding of receptor inhibitors. It is possible that the binding of ligans such as monoterpenes to the closed conformation will prevent the helix TM2 from adopting the straighter orientation that is characteristic of the open channel.

Monoterpenes such as menthol have been shown to activate Transient Receptor Channel Melastatin 8 (TRPM8) in the concentrations ranging from 10 μM to 1 mM (for reviews, de Araújo et al., 2011; Oz et al., 2015). In addition to menthol, in the concentration range of 100 μM to 10 mM, many other monoterpenes including menthone, geraniol, citronellal, eucalyptol, linalool, carvone, isopulegol, and euganol activate TRPM8 receptors (McKemy et al., 2002; Behrendt et al., 2004). However, these effects are not specific and actions of monoterpenes extend to other members of TRP channel family (for reviews, de Araújo et al., 2011; Oz et al., 2015).

In conclusion, our results demonstrate that cyclic monoterpenes directly inhibit the function of human α_7-nicotinic acetylcholine receptor, possibly by interacting with an allosteric

transmembrane site.

Acknowledgements

The experiments in this study were supported by the grants from CMHS, UAE University. Research in our laboratory was also supported by LABCO partner of Sigma-Aldrich. The authors cordially thank Dr. Syed M. Nurulain for his valuable help in oocyte injections and recordings. The authors gratefully acknowledge Dr. Jon Lindstrom for providing cDNA clones of the human α7 nicotinic acetylcholine receptor subunit and Dr. Isabel Bermudez for human α4, α3, β2, and β4 subunits.

Figure legends

Fig. 1. Chemical structures of cyclic monoterpenes used in the present study.

Fig. 2. Relative potencies of cyclic monoterpenes tested on human α_7 nicotinic acetylcholine receptor. The effects of 10 μM of monoterpenes; vanilin, pulegone, eugenole, carvone, carvacrol, carveol, thymol, thymoquinone, menthone, and limonene on acetylcholine (ACh; 100 μM)-induced currents. The effects of monoterpenes were tested after 10 min application of each drug. Each bar represents the normalized mean ± S.E.M. Numbers of experiments are shown on top of each bar. * indicate statistical significance at the level of $P<0.05$ using paired t-test (controls in the same oocytes).

Fig. 3. Effects of carveol on α_7-nicotinic acetylcholine receptor-mediated responses. **(A)** Records of currents activated by acetylcholine (ACh; 100 μM) in control conditions (*left*), during co-application of 10 μM carveol and acetylcholine after 10 min pretreatment with 10 μM carveol (*middle*), and 15 min following carveol washout (*right*). **(B)** Time-course of the effect of carveol (10 μM) on the maximal amplitude of the acetylcholine-activated currents. Each data point represents the normalized mean ± S.E.M. of 6 to 8 experiments. Application time for carveol is

shown by the horizontal bar in the figure.

Fig. 4. Time and concentration-dependent effects of carveol on α_7- nicotinic acetylcholine receptors. **(A)** Increasing the preapplication time of carveol (10 μM) enhances its inhibition of the α_7-receptor. Each data point represents the mean ± S.E.M. of 7 to 8 oocytes. **(B)** Inhibition of α_7-nicotinic acetylcholine receptor function by carveol is concentration-dependent. Each data point represents the mean ± S.E.M. of 6 to 8 oocytes.

Fig. 5. Inhibition of acetylcholine-induced responses by carveol is not dependent on changes in membrane potential. **(A)** Current-voltage relationships of acetylcholine-activated currents in the absence and presence of carveol (10 μM). Currents induced by 100 μM acetylcholine before (control,●) and after 15 min treatment with carveol (○) were normalized and plotted in the graph. Each data point presents the means ± S.E.M. of 6 to 8 experiments. **(B)** Inhibitory effect of carveol is presented at different voltages. **(C)** α_7-nicotinic acetylcholine receptor mediated currents in 2 mM Ca^{2+} containing MBS solution (control) or in 2 mM Ba^{2+} containing MBS solution. Bars represent the means ± S.E.M. of 5 to 7 experiments.

Fig. 6. Concentration-response curves for acetylcholine-induced currents in control and in the presence of carveol. Effects of carveol on the acetylcholine concentration-response relationship. In control conditions current was activated by acetylcholine (1 μM to 3 mM). Subsequently, 30 μM carveol was applied for 10 min and acetylcholine induced responses were measured in the presence of carveol. Currents activated by acetylcholine were normalized to the maximal response under control conditions. Concentration-response curves were plotted by fitting the curves from 6 to 7 oocytes as described in the methods section. Data points obtained before (control) and after 10 min treatment with carveol (30 μM) were indicated by *filled* and *open circles*, respectively.

Fig. 7. The effect of carveol on the specific binding of [^{3}H]-α-bungarotoxin. Human α_7-nicotinic acetylcholine receptors expressed in transiently transfected human kidney (tsA201) cells were incubated with the [^{3}H]-α-bungarotoxin (10 nM). The competitive antagonist methylycaconitine (MLA) displaced the binding of [^{3}H]-α-bungarotoxin in a dose-dependent manner IC_{50} = 33 ± 3 nM (n=5). In contrast, no significant displacement of [^{3}H]-α-bungarotoxin was observed with carveol (n=3), even at the highest concentrations tested (1 mM). All points are means ± S.E.M from 3-5 independent experiments.

Fig. 8. Comparison of the effect of 100 μM carveol on currents mediated by α_7, $\alpha_4\beta_2$, $\alpha_3\beta_4$, $\alpha_3\beta_2$, and $\alpha_4\beta_4$ subunits. Bars represent the means ± S.E.M. of 6 to 8 experiments.

Fig. 9. The results of docking calculations for cyclic monoterpenes obtained from their docking into the human α_7-nicotinic acetylcholine receptor transmembrane domain; A-C) position of carvacrol (red), thymoquinone (blue), carvone (green), vanillin (pink), and D) interaction of ligands with the residues of the binding site.

Supplementary Fig. 1. Inhibition of α_7-nicotinic acetylcholine receptor mediated currents at 15 and 20 minutes of perfusion with monoterpenes. The extent of inhibition at 10 and 20 min was shown in light and dark grey colors, respectively. Bars represent the means ± S.E.M. of 5 to 6 experiments.

Supplementary Fig. 2. Concentration-dependent inhibition of α_7-nicotinic acetylcholine receptor by monoterpenes. The IC_{50} values for carvacrol, thymol, thymoquinone, menthone, and limonene are 12.8, 20.1, 4.4, 18.3, and 29.7 μM, respectively. Each data points represent the means ± S.E.M.

of 5 to 7 experiments.

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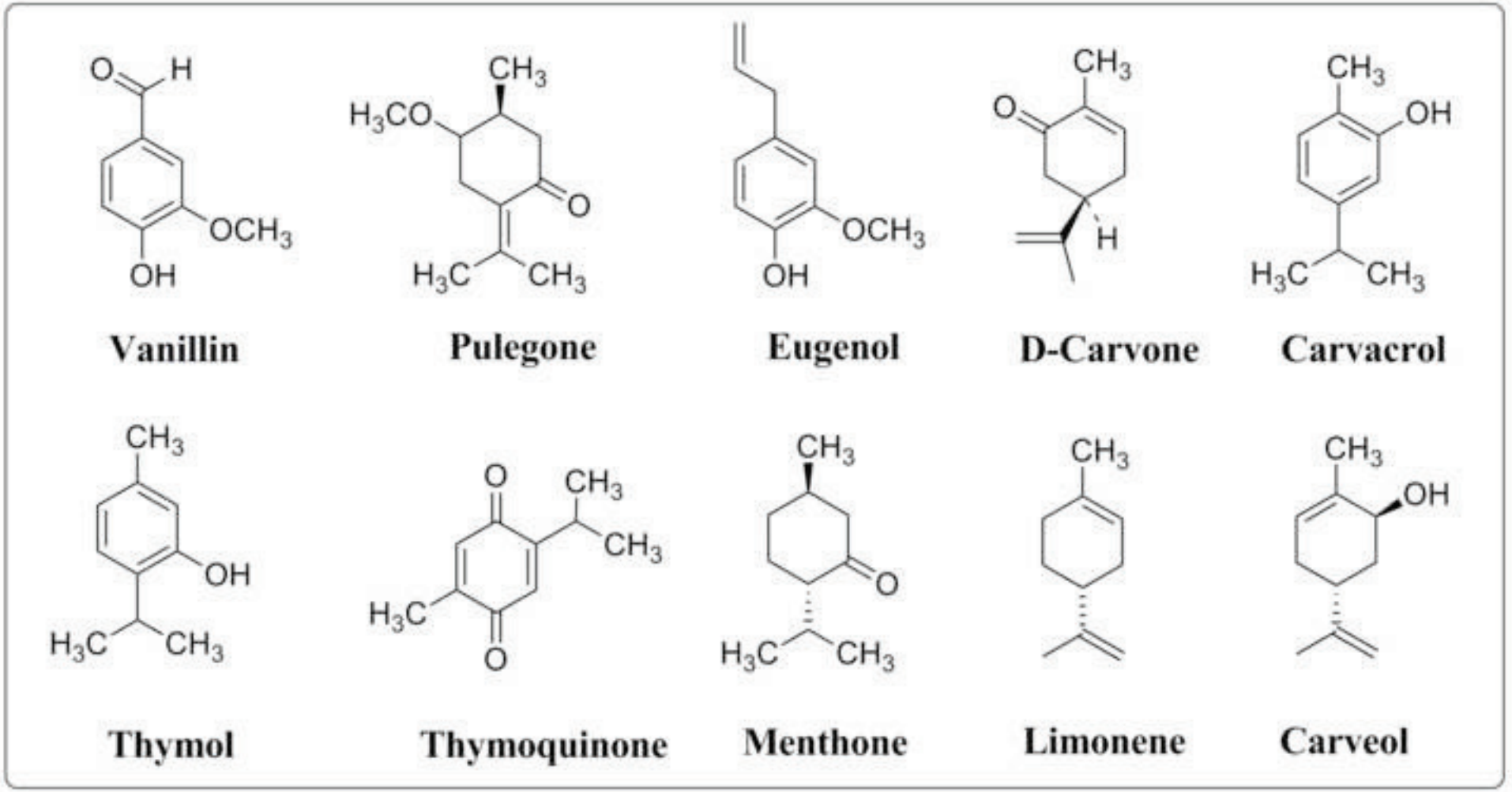
Table 1. Percent inhibition, calculated binding energies and location of lowest energy pose.

Compound	**Experimental % inhibition**	**Calculated binding energy, kcal/mol**	**Location lowest energy pose relative to binding site of Ketamine (Bondarenko et al., 2014)**
Vanillin	-14	-5.6	Not in a binding site
Pulegone	6	-6.0	Not in a binding site
Eugenol	9	-6.1	Not in a binding site
Carvone	1	-6.2	In a binding site
Carvacrol	39	-6.3	In a binding site
Carveol	57	-6.7	In a binding site
Thymol	26	-6.6	In a binding site
Thymoquinone	49	-6.8	In a binding site
Menthone	34	-6.7	In a binding site
Limonene	17	-6.1	In a binding site

Figure



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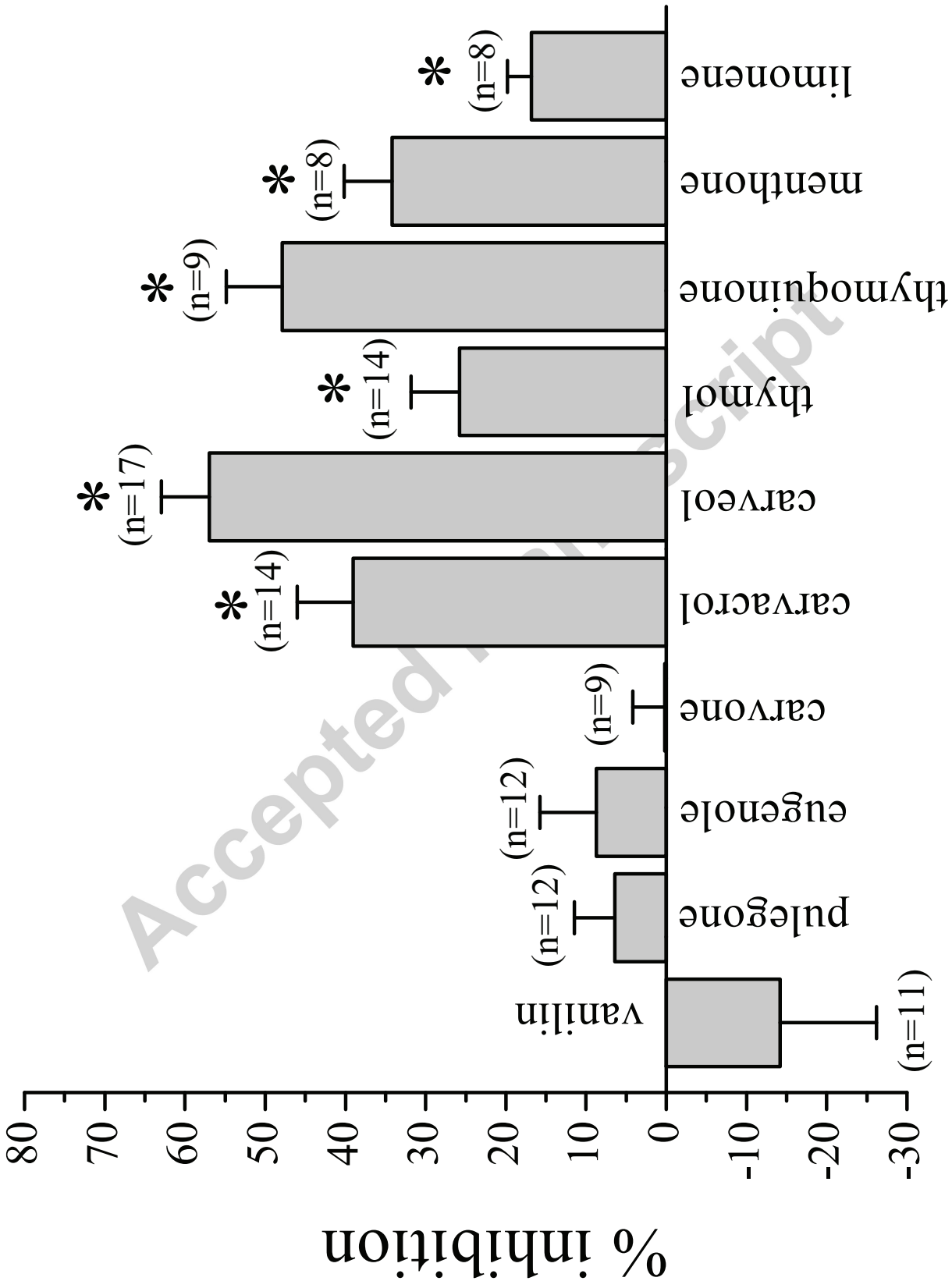

Figure

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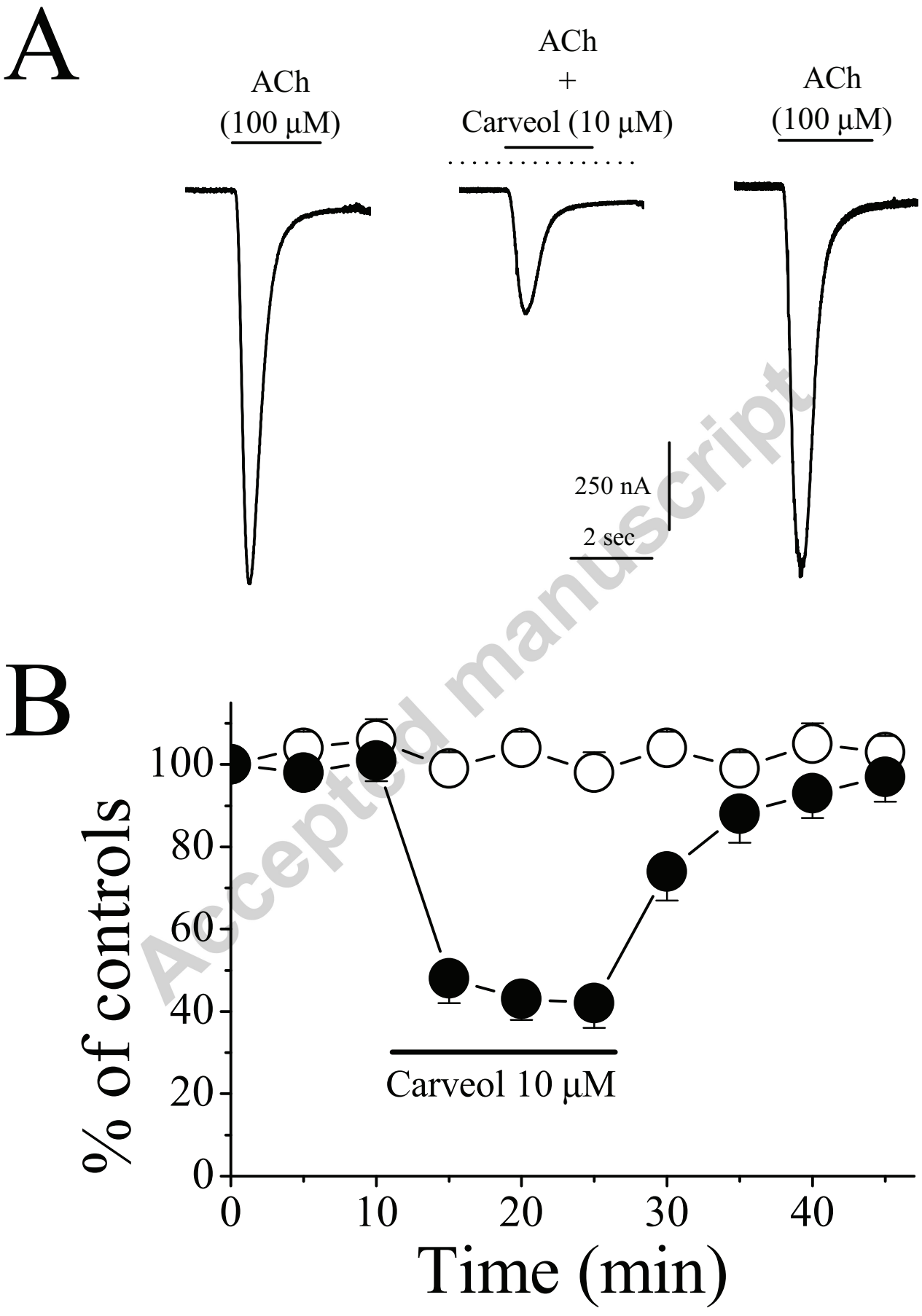
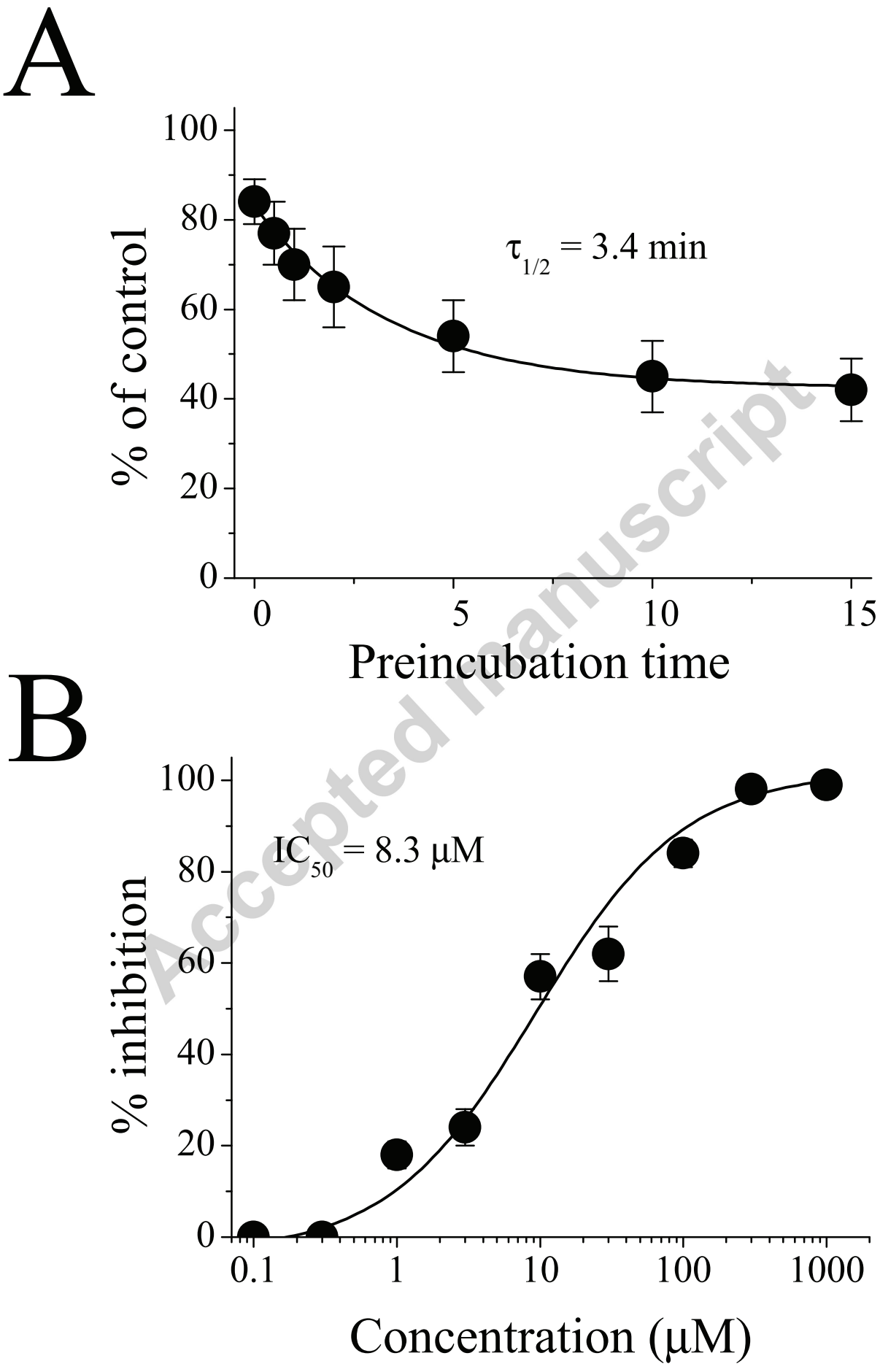
Figure 3



Figure

Figure 4



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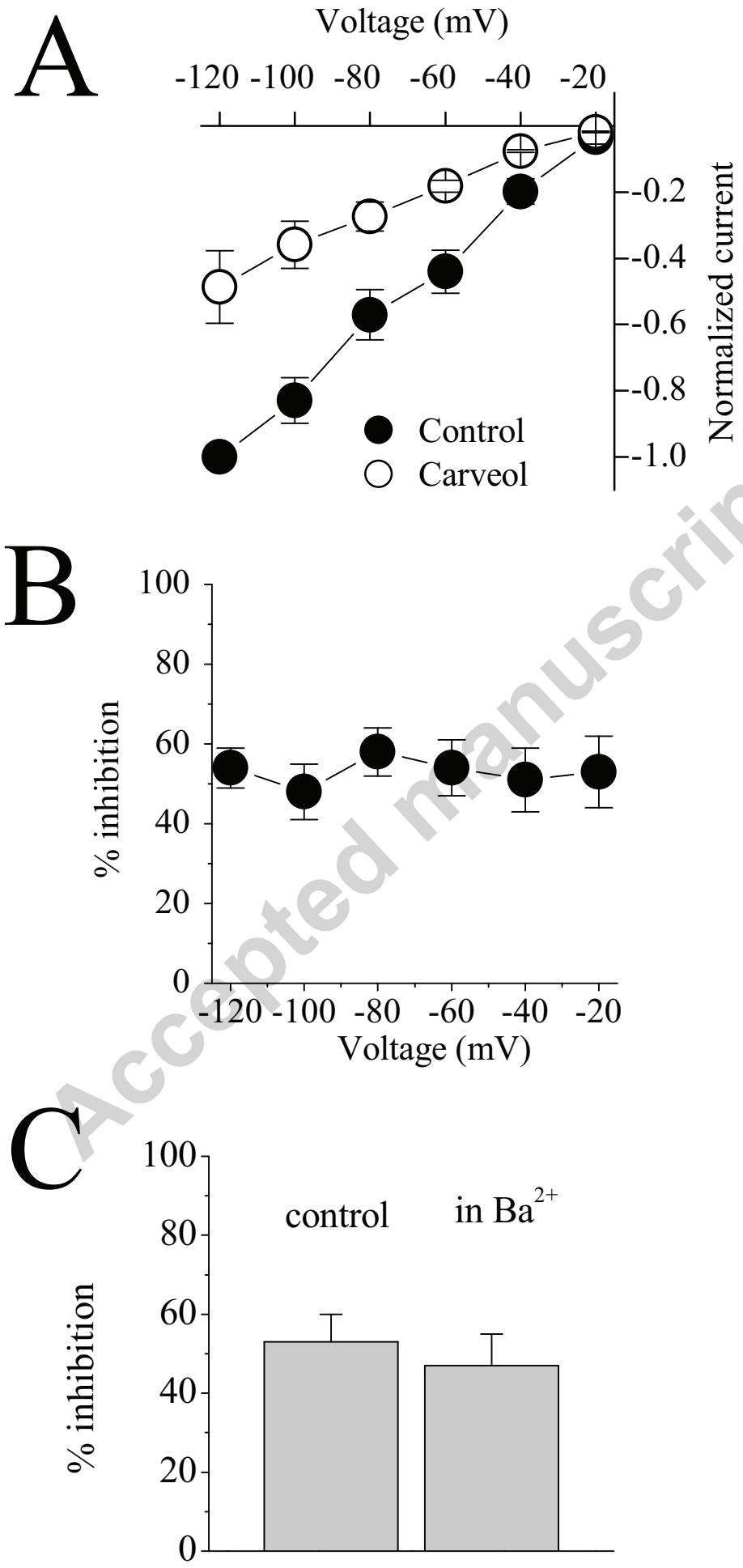
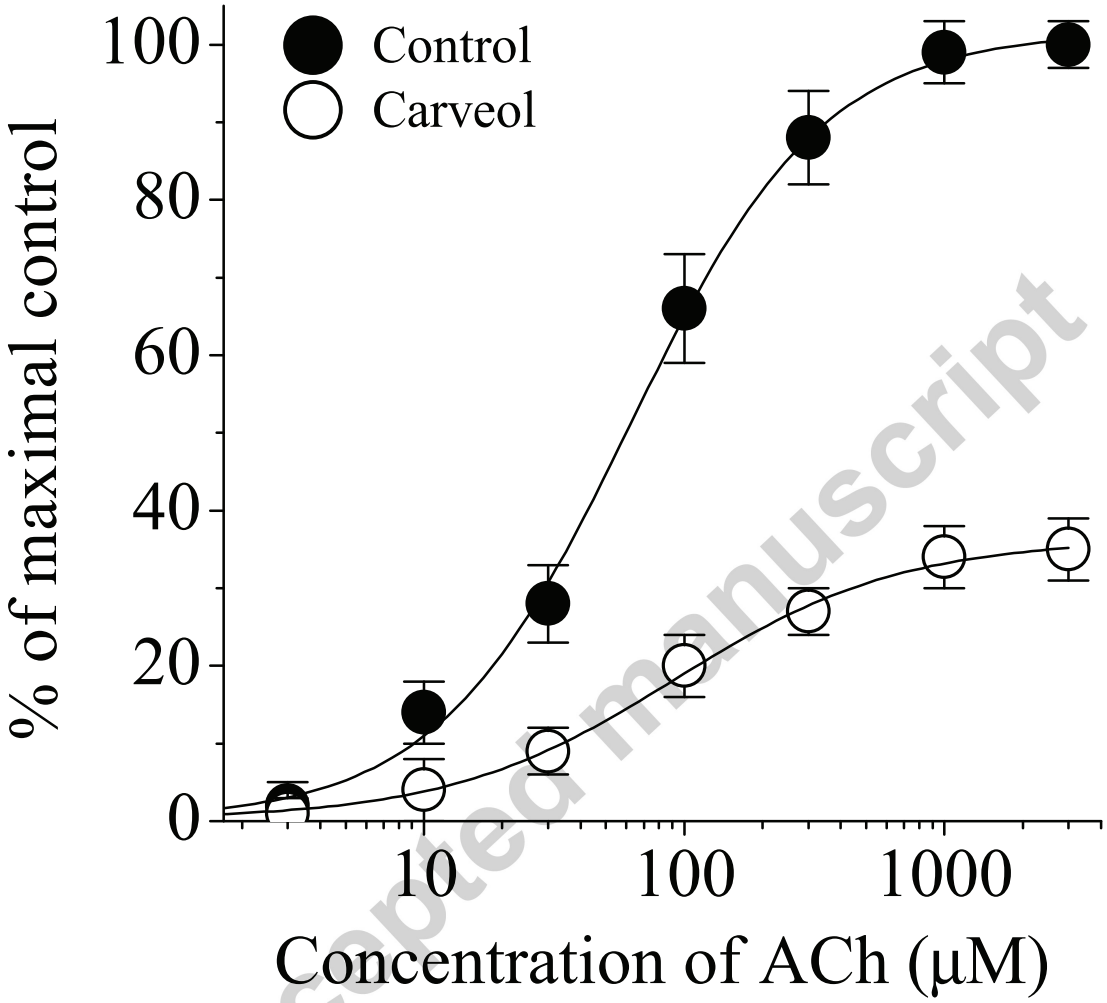


Figure

Figure 6



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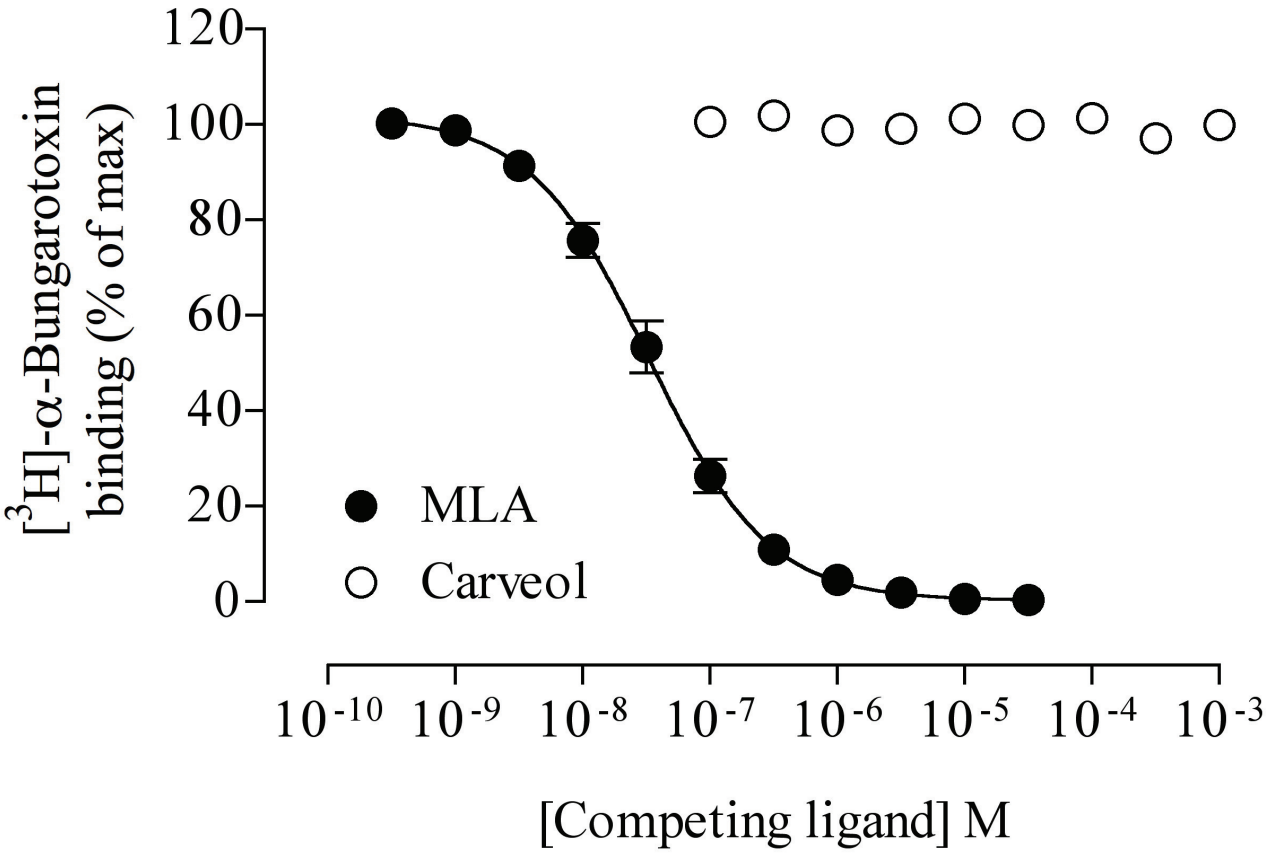

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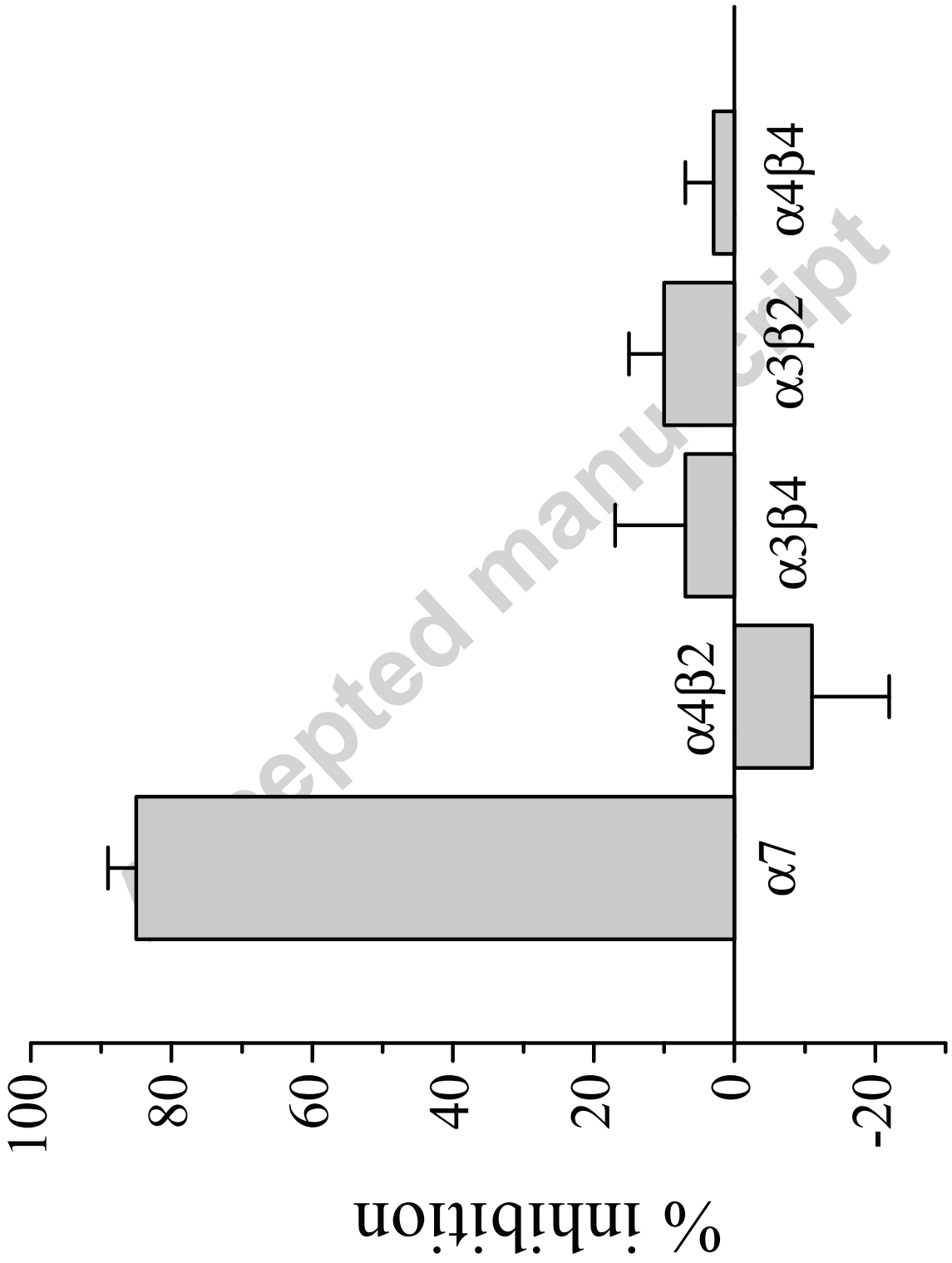

Figure

Figure 9