CARVEOL AND RELATED MONOTERPENES INHIBIT THE FUNCTION OF THE HUMAN α7 NICOTINIC ACETYLCHOLINE RECEPTOR

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CARVEOL AND RELATED MONOTERPENES INHIBIT THE FUNCTION OF THE HUMAN α7 NICOTINIC ACETYLCHOLINE RECEPTOR

Yosra Adnan Lozon

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Medical Sciences (Pharmacology and Toxicology)

Under the Supervision of Professor Murat Oz

March 2015
Declaration of Original Work

I, Yosra Adnan Lozon, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled “Carveol and related monoterpenes inhibit the function of the human a7 nicotinic acetylcholine receptors”, hereby, solemnly declare that this thesis is an original research work that has been done and prepared by me under the supervision of Professor Murat Oz, in the College of Medicine and Health Sciences at UAEU. This work has not been previously formed as the basis for the award of any academic degree, diploma or a similar title at this or any other university. The materials borrowed from other sources and included in my thesis have been properly cited and acknowledged.

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Abstract

Plants and phytochemicals have been used for centuries for therapeutic purposes. Essential oils from these plants are complex mixtures and may possess a large spectrum of biological activities many of them of clinical interest. Among the active constituents of essential oils, monoterpenes, demonstrated valuable antioxidant, antiviral, antimicrobial, anticancer, analgesic and anti-inflammatory effects. In recent years, actions of monoterpenes on the function of ion channels have been investigated. In the present study, effects of different monoterpenes including carvacrol, carveol, d-carvone, eugenol, (+)-pulegone, thymol, thymoquinone, menthome and limonene, on the function of the cloned α7 subunit of human nicotinic acetylcholine (nACh) receptor expressed in Xenopus oocytes were investigated by using the two-electrode voltage-clamp technique. All monoterpenes caused a variable extent of reversible inhibition of ACh (100 μM)-induced currents except vanillin and d-carvone. Carveol showed maximum potency of inhibition with an IC50 value of 8.3 μM. The effect of carveol was further investigated and found to be independent of the membrane potential. Carveol (10 μM) did not affect the activity of endogenous Ca2+-dependent Cl− channels since the extent of inhibition by carveol was unaltered by the intracellularly injected Ca2+ chelator BAPTA and perfusion with Ca2+-free bathing solution containing 2 mM Ba2+. The effect of carveol was associated with decreased potency of the ACh, and the inhibition was fully reversed by increasing ACh concentrations, suggesting that this drug acts in a competitive manner. In conclusion, these results demonstrate for the first time that monoterpenes inhibit directly the function of human α7-nACh receptors expressed in Xenopus oocytes. It appears that the extent of inhibition by monoterpenes differs significantly depending on their chemical structures.

Keywords: Monoterpenes, human nicotinic α7 acetylcholine receptors, carveol, Xenopus oocytes.
كاريفرول و مركبات التربينات الأحادية المتعلقة به تثبط وظيفة مستقبلات الأسيتايل كولين ألفا 7 النيكوتينية

المنصوص

لقرن طويل، استُخدمت النباتات و المواد المستخلصة منها في أغراض علاجية متعددة. الزيوت العطرية عبارة عن خليط معقد من مواد كيميائية أظهرت عدة نشاطات حيوية ذات أهمية علاجية. أثبتت الدراسات أن من هذه المواد، التربينات الأحادية لها تأثيرات علاجية قيمة كمضادات للالتهاب و والأكسدة و الألم و مضادات ميكروبية و فيروسية و مضادات للسرطان. ضمن هذا السياق، التربينات الأحادية (monoterpenes) من المواد التي جذبت اهتمام الباحثين لدراسة تأثيرها على الدقات الأيونية. في هذه الدراسة، تم تقييم تأثير عدة تربينات أحادية و هي كارفرول و كاريفرول و كرفون و كريفون و كريفون و باتون و بوليجون و فانيلين و ميثون و ثيموكوينون و ليمونين على وظيفة مستقبلات الأسيتايل كولين النيكوتينية المكونة من وحدات ألفا 7 و المستنسخة في بويضات ضفادع (Xenopus) وذلك باستخدام تقنية تثبيت الجهد الكهربائي باستخدام قطبين كهربائيين. كل التربينات الأحادية - عدا الفانيلين و الكارفرول - قللت بدرجات مختلفة حجم التيار الناتج عن 100 ميكر مول من الأسيتايل كولين و ذلك بصورة قابلة للانعكاس. وقد ظهر أن كاريفرول هو المثبط الأكثر فعالية حيث أن تركيز 8.3 ميكر مول بثبت 50% من حجم التيار الأصلي. بعد إجراء تجارب إضافية تبين أن تأثير الكارفرول لا يعتمد على جهد الغشاء الخلوي. كما أن الكارفرول لم يتأثر على نشاط و عمل قنوات الكلوريد المعتمدة على الكالسيوم المتواجدة في غشاء البيوضات حيث أن درجة التثبيط التي أحدثها الكارفرول لم تتغير في البيوضات التي حققت بمادة BAPTA الحاسبة للكلسيوم و نضحت بمحلول خالي من الكالسيوم. تأثير الكارفرول قلل من فعالية الأسيتايل كولين و هذا التأثير انعكس تماما عند زيادة تركيز الأسيتايل كولين مما دل على أن التثبيط الناتج عن الكارفرول ذو طبيعة تنافسية. و كنتيجة للدراسة، فقد تبين أن العديد من التربينات الأحادية تثبط عمل مستقبلات الأسيتايل كولين النيكوتينية المكونة من وحدات ألفا 7 المستنسخة في بويضات ضفدع (Xenopus) بشكل مباشر. درجة التثبيط الناتجة عن التربينات الأحادية تعتمد بدرجة كبيرة على تركيبها الكيميائي.
Acknowledgements

It is a pleasant opportunity to express my gratitude to all those who have helped and inspired me during my research and thesis writing.

As a start, I would like to express my sincere and deepest gratitude to my supervisor, Murat Oz, Professor, Department of Pharmacology and Therapeutics, College of Medical and Health Sciences, UAE University. His expertise, understanding, and patience, continuous guidance and support added considerably to my graduate experience. In addition, he was always accessible and willing to help. My sincere thanks to Professor Frank Christopher Howarth, Chair of Physiology department in CMHS, for reviewing and correcting the thesis.

I would like to express my gratitude and sincere thanks for the contributions of Syed Muhammad Nurulain, Medical Research Specialist for his continuous support, guidance and understanding. Very special thanks to my dear colleagues and lab mates Abrar Ashoor, Lina Al Kury, Khawla Salem, Islam AlNebrisi, Hiba AlSamman, Iman Abdeen and all those who motivated and helped me regularly during the course of my study.

Words prove a meagre media to express my gratitude for my parents and family for providing me constant encouragement, faithful prayers, divine presence and supporting me along the way.
Dedication

To my beloved parents and family
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List of Abbreviations

5-HT 5-hydroxytryptamine
AcCoA acetylCoA
ACh Acetylcholine
AChE Acetylcholinesterase
ADHD Attention Deficit Hyperactivity Disorder
AITC Allyl Isothiocyanate
BAPTA 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
CAP Compound Action Potential
ChAT Choline Acetyltransferase
CHT Choline Transporter
COX-2 Cycloxygenase-2
DHP Dihydropyridine
DMPP 1,1-dimethyl-4-phenylpiperazinium iodide
DMSO Dimethyl Sulfoxide
DRG Dorsal Root Ganglion
EPSCs Excitatory Postsynaptic Currents
GABA γ-Amino Butyric Acid
Gly Glycine
HEK293 Human Embryonic Kidney 293
HEPES 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
IC₅₀ Half maximal Inhibitory Concentration
IPSCs Inhibitory Postsynaptic Currents
LPS Lipopolysaccharide
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>LTP</td>
<td>Long-term Potentiation</td>
</tr>
<tr>
<td>MBS</td>
<td>Modified Barths Solution</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimal Inhibitory Concentration</td>
</tr>
<tr>
<td>nAChRs</td>
<td>Nicotinic Acetylcholine Receptors</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D- Aspartate</td>
</tr>
<tr>
<td>PAG</td>
<td>Periaqueductal Grey</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>PPARs</td>
<td>Peroxisome Proliferator-Activated Receptors</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>SCG</td>
<td>Superior Cervical Ganglion</td>
</tr>
<tr>
<td>TBOB</td>
<td>t-butyl-bicyclo-ortho-benzoate</td>
</tr>
<tr>
<td>TEVC</td>
<td>Two-Electrode Voltage Clamp</td>
</tr>
<tr>
<td>TNBS</td>
<td>Trinitrobenzenesulfonic Acid</td>
</tr>
<tr>
<td>TRP</td>
<td>Transient Receptor Potential Channels</td>
</tr>
<tr>
<td>TrkA</td>
<td>Tropomyosin Receptor Kinase A</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>VAT</td>
<td>Vesicular Acetylcholine Transporter</td>
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<tr>
<td>α-BT</td>
<td>α-Bungarotoxin</td>
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Chapter 1: Introduction

1.1. Introduction to Monoterpenes

Plants and phytochemicals have been used for centuries for therapeutic purposes and constitute the backbone of pharmacology and medicine. In recent years, there has been an increased interest in unveiling the biological activities of specific phytochemicals and understanding the mechanisms of action of their molecular and cellular effects. One of the major, biologically active groups of phytochemicals are the terpenes. Terpenes are a large and diverse group of naturally occurring compounds that exist as the main constituents of essential oils of several plant families such as Apiaceae, Asteraceae, Lamiaceae (Labiatae), Pinaceae, Piperaceae, Rutaceae, and Zingiberaceae (Ramawat et al., 2013; Silvestre & Gandini, 2008).

Terpenes are mostly hydrocarbon in nature and their building block is a five-carbon isoprene unit (2-methyl-1, 3-butadiene) (Figure 1.1). Isoprene was discovered by Wallach in 1887, who defined the structure of most terpenes as the ‘head-to-tail’ condensation of isoprene units (Figure 1.1) which is known as ‘isoprene rule’ (Christmann, 2010). Therefore, terpenes can be classified according to the number of isoprene units with a molecular formula of \((\text{C}_5\text{H}_8)_n\) \((n\) is the number of isoprene units). Accordingly, there are monoterpenes, sesquiterpenes, diterpenes, triterpenes and tetraterpenes with two, three, four, six, and eight isoprene units respectively (Aldred et al., 2009).
Figure 1.1: Chemical structure of the Terpenes. Isoprene constitute the main building block of terpenes and can be found in either “head to tail” or “tail to tail” configurations (Blaber, 2001).

1.2 Physicochemical properties of Monoterpenes

Most monoterpenes are highly volatile and non-polar (lipophilic) in nature which enables them partition and dissolve in cellular membranes. They are colorless liquids that are chemically reactive and have characteristic aromatic odors (Clarke, 2008). Monoterpenes possess variable basic skeletons and also exhibit stereoisomerism (Ramawat et al, 2013). Monoterpenes can exist in acyclic structures such as myrcene, as well as cyclic structures that could be monocyclic as (+)-limonene and dicyclic as (-)-α pinene (see Figure 1.2). Moreover, there are a wide range of oxygenated derivatives (alcohols, aldehydes, ketones and carboxylic acids) that can be derived from native basic skeletons (Silvestre & Gandini, 2008). These oxygenated forms are known as terpenoids (Clarke, 2008)
Figure 1.2: Structure of some cyclic and acyclic monoterpenes (Heyen & Harder, 2000)

Figure 1.3: Structure of oxygenated terpenes (terpenoids)
1.3 Biological activity of Monoterpenes

Essential oils have been used for therapeutic and culinary purposes for centuries. Several ancient civilizations valued the therapeutic and medicinal effects of essential oils and utilized those natural oils for health promotion, well-being and mental relaxation. Essential oils are complex mixtures and may possess a large spectrum of biological activity many of them of clinical interest. Many recent investigations focused on studying the biological effects of essential oils as a whole or, more recently, on their major isolated active constituents i.e. monoterpenes. These studies have found that monoterpenes have valuable anti-oxidant, anti-viral, anti-microbial, anti-cancer, analgesic and anti-inflammatory effects (for reviews, see Acharya et al, 2010; Clifford & DiGiovanni, 2010; de Cassia da Silveira e Sa et al, 2013; Guimaraes et al, 2013; Kamatou et al, 2013; Nostro & Papalia, 2012; Slamenova & Horvathova, 2013; Wagner & Elmadfa, 2003). Some of these studies have been reviewed in the following sections.

1.3.1. Anti-oxidant activity

Excessive formation of reactive oxygen species (ROS), as a result of exposure to environmental factors like pollutants and UV radiation, constitutes a major initiation factor in the progression of many pathological conditions. When the formation of ROS reaches beyond the capacity of cellular antioxidant defenses, it leads to lipid peroxidation and eventually damages lipid membranes, DNA bases and proteins (Frankel & Neff, 1983). Several studies demonstrated the potential antioxidant properties of single or multiple monoterpenes (for reviews, see Koziol et al, 2014; Porres-Martinez et al, 2014; Raskovic et al, 2014; Slamenova &
Horváthová, 2013). In a study on the antioxidant effects of eucalyptus and clove oils, thymol and eugenol (400 μg/ml), it has been shown that pure isolated monoterpenoid that exists as a major constituent of these oils caused inhibition of the formation of malonaldehyde (product of lipid oxidation) by 43.3% and 57.2%, respectively (Lee & Shibamoto, 2001). In a later study, the antioxidant potential of carvone, α-terpineol and perillyl alcohol was investigated. Carvone showed maximum activity (IC<sub>50</sub> 32.1 g/L) that was 10 and 23 times greater than the activity of α-terpineol (IC<sub>50</sub> 332.8 g/mL) and perillylacohol (IC<sub>50</sub> 738.3 g/mL) (Bicas et al., 2011). Recent investigation of the antioxidant properties of isolated monoterpenes in Spanish sage, α-pinene and 1,8-cineole, demonstrated considerable protection of cells against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress. ROS production was significantly inhibited by 1,8 cineole (30-45% decrease at concentrations of 10 and 25 μM) (Porres-Martínez et al., 2014). Further evaluation of the antioxidant activity of the individual isolated monoterpenes is needed for better understanding of their actions.

1.3.2. Anti-inflammatory activity

Inflammation is associated with a cascade of biochemical events including the formation of cytokines and inflammatory mediators that leads to inflammatory damage of tissues (for review Kemp et al., 2008; Kovacs et al., 1994; Rosengren & Delves, 1998). In the context of finding new natural anti-inflammatory agents, essential oils and monoterpenes have been studied in several in vitro and in vivo models.

In an earlier study, 1,8-Cineole (cineole) demonstrated anti-inflammatory activity when used orally in doses ranging from 100 to 400 mg/kg in different
inflammation models in rats (Santos & Rao, 2000). Another study by the same group, a monoterpane oxide, 1, 8-cineole that exists in several essential oils was evaluated for its anti-inflammatory activity using the trinitrobenzenesulphonic acid (TNBS)-induced colitis model in rats (Santos et al., 2004). In this study, both 200 and 400 mg/kg doses of 1, 8-cineole caused a significant decrease in gross colonic damage scores and wet weights of colonic segments of treated rats when compared with vehicle treated rats. This effect was evident only in rats that were pre-treated with this compound but not the post-treated rats. 1, 8-cineole also reduced myeloperoxidase activity and increased glutathione levels (Santos et al., 2004), suggesting the potential value of this compound in preventing gastrointestinal inflammation and ulceration.

Several studies reported an anti-inflammatory effect of carvacrol, an aromatic cyclic monoterpane that exists as a major constituent in the volatile oil of thyme (Nabavi et al., 2015). In a recent study, (Hotta et al., 2010) it was found that carvacrol suppresses the activity of cyclooxygenase-2 (COX-2), the rate-limiting enzyme in prostaglandin and other inflammatory mediator synthesis, through a mechanism involving peroxisome proliferator-activated receptors α and γ (PPARs). They showed that carvacrol can activate PPAR α and γ which in turn causes concentration dependent suppression of COX-2 expression (40% and 60% suppression by 200 and 400 µM carvacrol incubated for 24 hours) when examined in cell-based transfection assays using bovine arterial endothelial cells. In this study (Hotta et al., 2010), it was also shown that carvacrol caused suppression of lipopolysacharide-induced COX-2 mRNA and protein expression in macrophage-
like U937 cells. Collectively, these results indicate that monoterpenes have significant anti-inflammatory activities that might be of clinical value.

1.3.3. Analgesic activity

Pain has been reported to be the most common reason for seeking medical attention (Ruoff, 2002). Different classes of analgesic medication play a central role in the management of painful conditions. However, in many chronic pain conditions such as cancer and neuropathic pain, currently available pain killers are clearly insufficient for proper control of pain (Okuse, 2007). As a result, the search for novel analgesic agents has been a major focus for discovery and development of new drugs. In this context, many monoterpenes have proven to be effective pain relieving molecules in various experimental pain models (reviewed de Sousa, 2011; Guimaraes et al, 2013; Guimaraes et al, 2014). Carvacrol is one of the monoterpenes that demonstrated clear antinociceptive effect using different pain models (Cavalcante Melo et al, 2012; de Santana et al, 2014). Carvacrol showed activating effects on PPAR α and γ which are known to regulate the expression of COX-2 enzyme responsible for synthesis of multiple pain and inflammatory mediators. In this study, carvacrol inhibited LPS-induced COX-2 mRNA and protein expression in human macrophage-like U937 cells (Hotta et al, 2010). Furthermore, carvacrol demonstrated anti-hypernociceptive and anti-inflammatory properties when examined in mice using mechanical hypernociception models. Systemic pretreatment with carvacrol (50 or 100 mg/kg; i.p.) inhibited the development of edema and mechanical hypernociception caused by carrageenan and TNF-α. Besides this, carvacrol significantly decreased tumor necrosis factor α (TNF-α) levels in pleural lavage and suppressed the recruitment of leukocytes without
affecting their morphology (Guimaraes et al, 2012b a). This monoterpene also caused a significant antinociceptive effect on formalin-, capsaicin-, and glutamate-induced orofacial nociception in mice (Guimaraes et al, 2012a). Carvone is a monoterpene ketone that was proved to have antinociceptive activity associated with decreased peripheral nerve excitability when investigated using writhing and formalin tests in mice (Goncalves et al, 2008). Linalool is one of the monoterpenes that was clearly reported to demonstrate antinociceptive properties using variable experimental models. Multiple reports showed that this compound reduced pain sensation through modulation of muscarinic, opioid, dopaminergic, adenosinergic and glutamatergic systems (Batista et al, 2011; Batista et al, 2010; Batista et al, 2008; Peana et al, 2003; Peana et al, 2004a; Peana et al, 2004b; Peana et al, 2006a; Peana et al, 2006b). Other studies suggested the involvement of nicotinic receptors at the neuromuscular junction (Re et al, 2000). Many other monoterpenes have also shown significant analgesic activities, in several in vitro and in vivo pain models (de Sousa, 2011), suggesting that monoterpenes might be good candidates for analgesic drug development.

1.3.4. Antimicrobial activity

It is well established that some essential oils have antimicrobial properties likely due to their function in most plants as a defensive mechanism (Mahmoud & Croteau, 2002). Recently, there has been an increased interest in essential oils and their constituents as preservatives to prevent food-borne diseases (reviewed Burt, 2004). The aromatic monoterpene carvacrol and various essential oils containing carvacrol have been studied. Carvacrol demonstrated a wide spectrum of activity
against many Gram-positive and Gram-negative bacteria, molds and yeasts (Nostro & Papalia, 2012). In a recent study by Rivero-Cruz et al. (2011), the antimicrobial properties of the essential oil of *Poliomintha longiflora* and *Lippia graveolens* and their major constituents were examined. Carvacrol showed better antimicrobial activity than other components such as thymol and p-cymene. Carvacrol had a minimal inhibitory concentration (MIC) of 128 µg/ml compared to 256 to 1024 µg/ml MIC values of thymol and p-cymene against various bacterial strains (Rivero-Cruz et al, 2011). Another study investigated the effects of essential oils and its constituents for prevention of oral infections (Botelho et al, 2007). Using the disk diffusion method, they showed that essential oil of *Lippia sidoides* and its major constituents, thymol and carvacrol, had significant antibacterial and anti-candidal properties. Thymol and carvacrol showed better inhibitory effect compared to the essential oil in specific bacterial strains and *Candida albicans*. MIC values for the constituents ranged from 2.5 to 5.0 mg/ml while MIC for the oil ranged from 5 to 10 mg/ml (Botelho et al, 2007). Minimum fungicidal concentration of carvacrol was 2.5 mg/ml while thymol showed similar activity to the essential oil. Minimum bactericidal concentration of the essential oil was 20-40 mg/ml while it was 5.0 mg/ml and 10 mg/ml for carvacrol and thymol respectively (Botelho et al, 2007). Collectively, these studies emphasize the potential for utilizing essential oils and monoterpenes as antimicrobial agents or adjuvant to conventional treatment.

**1.3.5. Anti-carcinogenic activity**

Cancer is a chronic process characterized by abnormal cell proliferation and differentiation. Chemoprevention is a pharmacological approach to arrest or reverse the process of carcinogenesis mainly by inhibiting enzymes or receptors of chemical
mediators that play an important role especially during the initiation and progression phases. Chemotherapy mainly involves inhibition of tumor cell proliferation and enhancing apoptosis (Sporn & Suh, 2000). There are many studies reporting that some monoterpenes show either chemopreventive or chemotherapeutic effects. For example, in a study investigating anticancer activity of thymol using peripheral blood mononuclear cells (PBMC) and promyelotic cancer cell line HL-60, it was shown that thymol has dose dependent cytotoxic effects on HL-60 cells after 24h of exposure starting from 25µM. However, thymol did not show any cytotoxic effect in normal human PBMC up to 48 hours of exposure (Deb et al, 2011). In a similar study exploring the apoptotic potential of d-limonene in two human leukemia cell lines (K562 and HL60 cell lines), it was shown that d-Limonene induced apoptosis in a dose-dependent manner starting from 0.1mM up to 0.8 mM with similar potency in both cell lines (Ji et al, 2006). The anticarcinogenic actions of various monoterpenes have been further discussed in recent reviews (Bhalla et al, 2013; Gautam et al, 2014; Sobral et al, 2014).
1.4. Effects on Monoterpenes on Different Ion Channels and Receptors

Ion channels are integral membrane proteins that control passage of charged ions (\(\text{Na}^+, \text{K}^+, \text{Ca}^{2+}, \text{or} \text{Cl}^-\)) through the cell’s lipid bilayer. Opening and closing (gating) of these channels can be controlled by membrane voltage, ligand binding, stretch, or other physical stimuli. Any of these events deforms the channel protein affecting its conformation leading to opening of the gate and ion permeation (for reviews see Cannon et al, 2014; Tien et al, 2014; Weir, 2013; Wu & Cui, 2014). Activity of ion channels underlies the fundamental basis of cellular excitability, muscle contraction, neurotransmitter release, excitation transcription coupling, hormone secretion, and other transmembrane signaling processes (Cannon et al, 2014; Tien et al, 2014). Many drugs exert their therapeutic effects by acting on the ion channels of various excitable and non-excitable cells. Many phytochemicals have provided opportunities for development of new medications. In this context the effects of monoterpenes have been recently investigated on the functional properties of various ion channels (de Araújo et al, 2011). The results of some of the recent studies will be reviewed in the following sections.

1.4.1. Sodium channels

Voltage-gated sodium channels are important for initiation of action potentials in neurons, muscle, and other excitable cells. Sodium (\(\text{Na}^+\)) channels are activated when membrane depolarization induces conformational changes in the channel structure leading to opening of the pore allowing sodium influx. Local anesthetic, antiepileptic, and antiarrhythmic drugs bind to a receptor site within the
pore of the Na\textsuperscript{+} channel and block it, whereas many classes of neurotoxins bind to
distinct receptor sites and alter sodium channel function (Catterall \textit{et al}, 2013).

In an earlier study (Haeseler \textit{et al}, 2002), the effects of thymol and menthol
were evaluated on whole cell sodium inward currents via heterologously (HEK293
cells) expressed rat neuronal and human skeletal muscle sodium channels. Sodium
currents were inhibited with half maximal concentration (IC\textsubscript{50}) values of 104 and 149
\(\mu\text{M}\) for thymol and 376 and 571 \(\mu\text{M}\) for menthol for the skeletal muscle and the
neuronal Na\textsuperscript{+} channel, respectively. The blocking potency of both compounds
increased at depolarized holding potentials (Haeseler \textit{et al}, 2002). The results of this
study suggested that the antinociceptive and local anesthetic effects of thymol and
menthol may be mediated via blockade of Na\textsuperscript{+} channels. In agreement with these
results, Gaudioso \textit{et al}., (2012) examined the effect of menthol on voltage-gated Na\textsuperscript{+}
channels in dorsal root ganglion (DRG) neurons. By use of a patch clamp, the effects
of menthol application were evaluated on tetrodotoxin (TTX)-resistant Nav1.8 and
Nav1.9 channel subtypes in DRG neurons, and on TTX-sensitive Na\textsuperscript{+} channels in
immortalized DRG neuron-derived F11 cells. The results indicated that menthol
inhibited voltage-gated Na\textsuperscript{+} channels in a concentration, voltage, and frequency-
dependent manner. Menthol promoted fast and slow inactivation states, causing use-
dependent depression of Na\textsuperscript{+} channel activity (Gaudioso \textit{et al}, 2012). In current
clamp recordings, menthol inhibited firing at high-frequency stimulation with
minimal effects on normal neuronal activity. Furthermore it was found that low
concentrations of menthol cause analgesia in mice, relieving pain produced by a Na\textsuperscript{+}
channel-targeting toxin. In this study, it was concluded that menthol is a state
selective blocker of Nav1.8, Nav1.9, and TTX-sensitive Na\textsuperscript{+} channels, indicating a
role for Na\(^+\) channel blockade in the efficacy of menthol as a topical analgesic compound (Gaudioso et al, 2012).

Recently, two groups, investigated the effect of carvacrol and related monoterpenes on Na\(^+\) channels of mammalian (Goncalves et al, 2010) and vertebrate (Kawasaki et al, 2013; Matsushita et al, 2013) neurons. Goncalves et al., (2010) investigated the effects of (+) and (-)-carvone, carvacrol, carveol, and limonene on compound action potential (CAP) characteristics using a modified single sucrose-gap method. It was demonstrated that (-)-carvone was less potent (IC\(_{50}\)=10.7 mM) in reducing nerve excitability than its enantiomer, (+)-carvone (IC\(_{50}\)=8.7 mM). In a structure-activity relationship study, it was demonstrated that hydroxyl groups in the carveol and carvacrol molecules enhanced the CAP blocking-effect, while the absence of the oxygen moiety in limonene resulted in the effect being almost abolished. In another study (Kawasaki et al, 2013), it was shown that (-)-menthol and (+)-menthol concentration-dependently reduced CAP peak amplitude with IC\(_{50}\) values of 1.1 and 0.93 mM, respectively. However, other monoterpenes such as p-menthane, limonene and menthyl chloride at 7-10 mM minimally affected CAPs. On the other hand, (-)-menthone, (+)-menthone, (-)-carvone, (+)-carvone and (-)-carveol and pulegone inhibited CAPs with potencies similar to that of menthol (Kawasaki et al, 2013). In another study by Joca and his co-researchers (Joca et al, 2012), carvacrol’s effect was tested on CAP in isolated rat sciatic nerve. In agreement with previous reports, carvacrol reversibly blocked the excitability of the rat sciatic nerve in a concentration-dependent manner with an IC\(_{50}\) value of 0.5 mM. Also, carvacrol blocked the generation of action potentials (IC\(_{50}\)= 0.36 mM) of the intact DRG neurons without altering the resting potential and input resistance (Joca et al, 2012).
Furthermore, carvacrol reduced the voltage-gated Na\(^+\) current of dissociated DRG neurons (IC\(_{50}\) = 0.37 mM). In conclusion these studies demonstrated that carvacrol blocks neuronal excitability by direct inhibition of the voltage-gated Na\(^+\) current suggesting that this compound can act as a local anesthetic.

Eugenol, a monoterpenoid widely used in dentistry for its anesthetic properties, was investigated for its effect on voltage-gated Na\(^+\) channels (Cho et al, 2008; Park et al, 2006). Cho et al demonstrated that an inhibitory effect of eugenol was evident for both TTX-sensitive and TTX-resistant Na\(^+\) channels in rat DRG neurons. The K\(_D\) values were 308 µM and 543 µM for TTX-sensitive and insensitive Na\(^+\) channels, respectively. Eugenol did not influence the activation voltage of either type of sodium current. However, eugenol moved the steady-state inactivation curves of both Na\(^+\) currents to a hyperpolarizing direction and reduced the maximal Na\(^+\) current (Cho et al, 2008) suggesting that eugenol inhibits sodium currents by interacting with resting and inactivated channels. The recovery from inactivation of both Na\(^+\) currents was slowed by eugenol. But, the eugenol inhibition of Na\(^+\) currents was not dependent on the stimulus frequency. In another study, using a whole-cell patch-clamp technique, the effect of eugenol was tested on voltage-gated sodium channels in rat dental primary afferent neurons (Park et al, 2006). Eugenol inhibited action potentials and two types of Na\(^+\) currents; TTX-resistant and TTX-sensitive. Another group (Moreira-Lobo et al, 2010) examined the effect of eugenol on action potentials recorded intracellularly from rat superior cervical ganglion (SCG). They showed that eugenol caused reversible, concentration-dependent blockage of action potentials with an IC\(_{50}\) of 0.31 mM (Moreira-Lobo et al, 2010). The results of these
studies suggested that inhibition of Na\(^+\) current can be considered as one of the mechanisms by which eugenol exerts analgesia.

In DRG neuron, another monoterpane, linalool reduced the amplitude of action potentials in a reversible and concentration dependent manner with an IC\(_{50}\) of 1.85 mM (Leal-Cardoso \textit{et al}, 2010). Linalool also inhibited excitability of the sciatic nerve with an IC\(_{50}\) of 0.78 mM (Leal-Cardoso \textit{et al}, 2010). In this study, it was also shown that linalool, in the concentration range of 0.1 to 6 mM, reversibly blocked the generation of action potentials in intact DRG neurons and inhibited the sodium current of dissociated DRG neurons (Leal-Cardoso \textit{et al}, 2010). In another study using newt olfactory receptor cells, linalool inhibited voltage-dependent Na\(^+\) currents with an IC\(_{50}\) of 0.56 mM (Narusuye \textit{et al}, 2005).

1.4.2 Calcium channels

Voltage-gated Ca\(^{2+}\) channels are responsible for controlling Ca\(^{2+}\) influx through the plasma membrane. Entrance of extracellular Ca\(^{2+}\) through these channels is crucial for many physiological functions of excitable cells such as neurons and various types of muscle. The structural diversity of Ca\(^{2+}\) channels is related to their function in different tissues, such as neurotransmitter release from nerve endings, cardiac and smooth muscle contraction, Ca\(^{2+}\)-dependent modulation of enzyme activity and gene transcription (Reuter \textit{et al}, 2013).

In early studies, several monoterpenes have been shown to modulate the functions of voltage-dependent Ca\(^{2+}\) channels. Among these monoterpenes, menthol has attracted much interest. High-potassium induced Ca\(^{2+}\) increases in Leech neurons (Dierkes \textit{et al}, 1997), synaptosomes and chick retinal neurons (Hawthorn \textit{et al}, 1988)
have been shown to be suppressed by menthol. Menthol inhibits K⁺ depolarization-induced and electrically stimulated responses in ileum, and atrial and papillary muscles. IC₅₀ values in the ileac preparation ranged from 7.7 to 28.1 µg/ml and in the cardiac preparations from 10.1 to 68.5 µg/ml. Furthermore, both menthol and peppermint oil competitively inhibited specific binding of [³H]nitrendipine and [³H]PN 200-110 to smooth and cardiac muscle and neuronal preparations with potencies comparable to those measured in pharmacological experiments (Hawthorn et al., 1988). In another study (Sidell et al., 1990), brief exposure of LA-N-5 cells to menthol decreased the depolarization-induced Ca²⁺ influx though both dihydropyridine (DHP)-sensitive and DHP-insensitive channels. The effect was concentration dependent with IC₅₀ value of 0.25 mM, rapid in onset and readily reversible. In a recent study, in agreement with earlier results, menthol has been shown to induce relaxation and inhibit contraction in rat aorta, mesenteric and coronary arteries, primarily through inhibiting Ca²⁺ influx via nifedipine-sensitive Ca²⁺ channels (Cheang et al., 2013). Similarly, the results of another study indicated that menthol (0.1-30 mM) induced spasmolytic effects in human colon circular muscle inhibiting contractility of the gastrointestinal smooth muscle, by blocking Ca²⁺ influx through L-type Ca²⁺ channels (Amato et al., 2014). Similar results have also been reported in bronchial smooth muscle (Wright et al., 1997) where menthol caused relaxation of KCl and ACh preconstricted bronchi in vitro.

In addition to functional studies, there have also been electrophysiological investigations. The effects of menthol (0.1-0.5 mM) on Ca²⁺ current inactivation was studied in Helix neurons (Swandulla et al., 1986). External, but not internal, application of menthol accelerated the Ca²⁺-dependent rapid phase of inactivation.
Later, the effect of menthol on voltage-dependent Ca\textsuperscript{2+} currents was investigated in cultured dorsal root ganglion cells from chick and rat embryos (Swandulla et al., 1987). Application of menthol (0.1-1 mM) had different effects on the various types of Ca\textsuperscript{2+} currents in these neurons. Below -20 mV, the low threshold Ca\textsuperscript{2+} currents were reduced in amplitude in a dose-dependent manner by menthol with little effect on their activation kinetics. In contrast, the time course of inactivation of the high-threshold Ca\textsuperscript{2+} currents, activated above -20 mV from a holding potential of -80 mV, was drastically accelerated by external menthol. The action of menthol was unchanged with more positive holding potentials (-50 mV). Importantly, menthol exerted its action only when applied from the outside. Collectively, the results of these studies indicated that menthol blocks Ca\textsuperscript{2+} currents through the low voltage-activated Ca\textsuperscript{2+} channel, and facilitates inactivation gating of the classical high voltage activated Ca\textsuperscript{2+} channel. Recently, Baylie and his group investigated the actions of menthol on L-type Ca\textsuperscript{2+} currents in rabbit ventricular myocytes at near-physiological temperature (~35°C) using whole-cell recording (Baylie et al., 2010). Menthol, concentration-dependently, inhibited peak Ca\textsuperscript{2+} currents (IC\textsubscript{50}=74.6). In addition, menthol blocked the late currents remaining at the end of depolarising pulses with greater efficacy (96.1% block at 1 mM) than peak Ca\textsuperscript{2+} currents (68.9% block at 1 mM) (Baylie et al., 2010).

In cardiac muscle, other monoterpenes have been shown to modulate the function of L-type Ca\textsuperscript{2+} channels. The negative inotropic effect of Pulegone was investigated in guinea pig atria (de Cerqueira et al., 2011). The effects of pulegone were compared with nifedipine, a well-known L-type Ca\textsuperscript{2+} channel blocker, and pulegone showed a similar pattern of concentration-dependent inhibition of atrial
contractility to that of nifedipine with much lower potency. Concentration-response curves demonstrated an IC$_{50}$ of pulegone of 777 µM compared to 1.1 µM for nifedipine. To explore the involvement of L-type Ca$^{2+}$ channel in the negative inotropic effects of pulegone, they used the selective L-type Ca$^{2+}$ channel agonist, BAY K8644. In these experiments, upon preincubation with 3.2 mM pulegone, the EC$_{50}$ of BAY K8644 increased significantly from 0.1 µM to 0.6 µM. To further confirm the role of L-type Ca$^{2+}$ channels, whole-cell patch clamp experiments were preferred in isolated mouse ventricular myocytes exposed to pulegone (de Cerqueira et al., 2011). Pulegone caused a reversible inhibitory effect on peak L-type Ca$^{2+}$ current. In another set of experiments, using isolated cardiomyocytes pre-loaded with the intracellular Ca$^{2+}$ indicator Fluo4-AM, the effect of pulegone was examined on global Ca$^{2+}$ transients. The cells were electrically stimulated to record intracellular Ca$^{2+}$ transients. The results, consistent with the previous findings, indicated that pulegone, concentration-dependently, reduced intracellular Ca$^{2+}$ transients (de Cerqueira et al., 2011).

Using the whole-cell configuration of the patch-clamp technique, thymol (1 µM-1 mM range), inhibited peak amplitude of L-type Ca$^{2+}$ current in NG108-15 cells (Huang et al., 2005) and rat skeletal muscle fibers (Szentandrassy et al., 2003) and ventricular cardiomyocytes (Magyar et al., 2002). Similarly, in isolated cardiac myocytes, both eugenol (Sensch et al., 2000) and carvacrol (Magyar et al., 2004) caused inhibition of peak L-type Ca$^{2+}$ currents with IC$_{50}$ of 226 µM and 98 µM, respectively. Eugenol was also examined on cloned T-type Ca$^{2+}$ channel isoforms expressed in HEK293 cells, using whole-cell patch clamp. In this study, eugenol caused inhibition of Cav3.1, Cav3.2, and Cav3.3 currents in a concentration-
dependent manner (Seo et al, 2013). Another recent study (Peixoto-Neves et al, 2014) demonstrated that eugenol dilates arteries in a concentration-dependent manner and inhibits voltage-dependent Ca\textsuperscript{2+} currents in isolated cerebral artery smooth muscle cells. Another monoterpene, linalool has also been shown to suppress voltage-gated Ca\textsuperscript{2+} currents in retinal horizontal cells and Purkinje cells (Narusuye et al, 2005). Collectively, these findings suggest a significant inhibitory action of some monoterpenes on L-type Ca\textsuperscript{2+} channels and may provide a structural template for the development of new L-type Ca\textsuperscript{2+} channel blockers.

1.4.3. Potassium channels

Voltage-dependent K\textsuperscript{+} channels are activated by changes in membrane potential and function to repolarize the membrane potential and function to repolarize the membrane potential to resting levels by selectively allowing passage of K\textsuperscript{+} ions down its electrochemical gradient across the cell membrane. Voltage-dependent K\textsuperscript{+} channels are widely distributed in mammalian tissues and play important physiological roles (for recent reviews, see Gonzalez et al, 2012; Jan & Jan, 2012; Nerbonne, 2014; Oudit et al, 2004). The functional roles of voltage-dependent K\textsuperscript{+} channels include setting the duration of action potentials and the interspike interval during repetitive firing in neurons and in the heart, secreting K\textsuperscript{+} in epithelia and adjusting the contractile tone of the smooth muscle. Voltage-dependent K\textsuperscript{+} channels consist of four subunits. The tremendous diversity of voltage-dependent K\textsuperscript{+} channels can be attributed to the large number of different genes present, auxiliary β-subunits and metabolic regulation (Latorre et al, 2013).
There are very few studies exploring the effect of monoterpenes on voltage-dependant $K^+$ channels. Thymol had an inhibitory effect on transient outward potassium currents in a concentration-dependant manner in canine and human ventricular myocytes (Magyar et al, 2002). Thymol caused inhibition of $K^+$ currents with an EC$_{50}$ of 60.6 µM, however, the kinetics of $K^+$ currents were not much altered by thymol. These effects developed rapidly (within 0.5 min) and were readily reversible (Magyar et al, 2002). Similarly inhibition of $K^+$ current by thymol has also been reported in skeletal muscle fibers (Szentandrassy et al, 2003). Another study using whole-cell patch clamp reported that eugenol also inhibits voltage-gated $K^+$ currents in rat trigeminal ganglion neurons and human Kv1.5 currents stably expressed in Ltk(-) cells in a concentration-dependent and reversible manner (Li et al, 2007). In addition to voltage-dependent $K^+$ currents, Ca$^{2+}$-activated $K^+$ currents have also been shown to be modulated by monoterpenes. For example, Ca$^{2+}$-activated $K^+$ channels were activated by menthol in human glioblastoma cells (Wondergem & Bartley, 2009) and by thymol in pituitary GH3 cells (Huang et al, 2005).

1.4.4. Non-selective cation channels

Transient Receptor Potential channels (TRP channels) are a group of non-selective cation channels. The founding member of this family was discovered in Drosophila and divided into six subfamilies, named C, V, M, A, P, and ML, on the basis of amino acid sequence homology: canonical, vanilloid, melastatin, ankyrin repeat, polycystins, and mucolipins, respectively (Clapham & Squire, 2009). TRP channels function as cation channels that mostly increase intracellular Ca$^{2+}$ levels. However, only a few of the TRPs are highly Ca$^{2+}$-selective, and exhibit a variety of gating
mechanisms. They are activated by variable stimuli like changes in temperature, pH, and osmolarity, and are modulated by growth factor and G-protein-coupled receptor pathways. These channels are involved in diverse physiological processes, such as photoreception, pain perception, mechanosensation, thermosensation, cell growth, secretion, and ion channel regulation (Freichel & Flockerzi, 2007).

A major difficulty in the study of TRP channels is the lack of specific pharmacological agents that modulate most members of the TRP superfamily. Nevertheless, exploring the effect of different monoterpenes has recently been a highly attractive area for researchers in this field. Several reports screened the activity of a wide range of structurally related monoterpenes on selected TRP channel subtypes. One of these studies focused on TRPV3, a thermosensitive ion channel expressed predominantly in the skin and neural tissues, and screened a selection of monoterpenoid compounds using whole cell patch clamp techniques. Monoterpene camphor is a known agonist of TRPV3 (Moqrich et al, 2005). Out of 33 screened compounds, six monoterpenes significantly more potent than camphor, were identified: 6-tert-butyl-m-cresol (290%), carvacrol (265%), dihydrocarveol (255%), thymol (245%), carveol (150%) and borneol (118%). Dose-response curves for these six compounds were determined using voltage clamp techniques in TRPV3 expressing *Xenopus leavis* oocytes. Their EC$_{50}$ values were found to be up to 16 times lower than that of camphor (Vogt-Eisele et al, 2007). Another study examined the effect of thymol and other related compounds on human TRPA1 channels, a TRP channel subtype that is known to respond to noxious cold (Story et al, 2003). Thymol (6.25 and 25 µM) caused depolarization of HEK293 cells expressing hTRPA1 determined by a strong increase in dye fluorescence using a Ca$^{2+}$ imaging
assay. Cinnamaldehyde, as a positive control, showed a similar profile and both compounds showed no activity on the parental HEK293 cells, indicating specific activity on hTRPA1 channels (Lee et al., 2008). In the same study, the direct effects of thymol on TRPA1 mediated currents were tested using whole-cell voltage clamp. Thymol activated TRPA1 currents with an EC\textsubscript{50} of 127 µM, but this activation was eliminated upon repeated application of thymol due to channel desensitization. Desensitization by thymol reduced the response to AITC (allyl isothiocyanate, a known agonist), indicating that both compounds act on the same channels (Lee et al., 2008).

In an attempt to identify the pharmacological mechanism of its antinociceptive effect, actions of carvone were examined using DRG neurons and TRPV1-expressing HEK293 cells in Ca\textsuperscript{2+} imaging experiments. In this study, carvone stimulated a significant increase in intracellular Ca\textsuperscript{2+} levels (Goncalves et al., 2013). This increase in cytosolic Ca\textsuperscript{2+} level was completely abolished when cells were preincubated with Ca\textsuperscript{2+}-free bath solution, 5 µM ruthernium red (non-specific antagonist of TRP channels) and 10 µM of capsazepine (specific TRPV1 antagonist), confirming that the effects of carvone were mediated by TRPV1 activation (Goncalves et al., 2013). Collectively, these results suggest that monoterpenes can be useful for the development of new drugs involving TRP channel-related pathological conditions.

1.4.5. GABA and Glycine receptors

Receptors for γ-aminobutyric acid (GABA) and glycine (Gly) are pentameric ligand-gated ion channels that respond to inhibitory neurotransmitters by opening a
chloride-selective central pore (Minier et al, 2013). GABA is the major inhibitory neurotransmitter in the central nervous system. GABA is synthesized in GABAergic neurons and released in the synaptic cleft in response to an action potential. Upon its release, GABA acts at two different types of receptors: the ionotropic GABA_A receptors (chloride-selective ion channels) and the metabotropic GABA_B receptors (G-protein coupled receptors). In case of GABA_A receptor, binding of GABA triggers a conformational change in the receptor leading to the opening of the pore. This causes a state of hyperpolarization and therefore neural suppression (Minier et al, 2013). Glycine receptors are found predominantly in the spinal cord and brain stem. Glycine released from nerve endings act by binding to the glycine receptor (GlyR) and cause opening of chloride channels leading to hyperpolarization and subsequent neural suppression (Ashcroft, 2000).

Several monoterpenes have been used in conventional medicine as anesthetic and anxiolytic agents (Bakkali et al, 2008; Denner, 2009). These effects of monoterpenes lead to the investigation of these compounds on GABA and glycine receptors. Among the monoterpenes, the effect of menthol on GABA current was extensively studied by different groups of researchers using various in vitro and in vivo models. In an earlier study screening the effects of monoterpenoid alcohols and ketones on recombinant human GABA_A (α1β2γ2s) and glycine (α1 homomers) receptors expressed in Xenopus oocytes, GABA-induced currents were enhanced by coapplication of 10–300 µM of monoterpenes in the following order, (+)-menthol > (-)-menthol > borneol > menthone = camphor = carvone (Hall et al, 2004). However, application of menthol alone (up to 1 mM) did not induce direct current indicating no agonistic activity of menthol. Menthol, 100 µM, reduced EC_{50} values for GABA and
glycine from 82 to 25 µM and from 98 to 75 µM respectively, indicating that menthol acts as a positive modulator of GABA and glycine inhibitory currents (Hall et al., 2004). In contrast to menthol, application of diastereomers of thujone inhibited GABA_A receptor currents. Similarly, another group of researchers investigated the effect of menthol and other monoterpenes in *Xenopus laevis* oocytes expressing GABA_A receptors (Watt et al., 2008). They found that upon co-application of sub-maximal (EC_{20}) GABA concentrations, the current sizes were enhanced concentration-dependently in the following order: (+)-menthol (e.g. by ~2-fold at 50 µM) > isopulegol > isomenthol > α-terpineol >> cyclohexanol (Watt et al., 2008). In this study, they also compared the effect of (+)-menthol to clinically used anesthetics such as propofol. At 2 µM propofol and 50 µM (+)-menthol, the level of GABA EC_{20} current enhancement was almost similar (119% and 96.2%, respectively). When co-applied, currents directly activated by 50 µM propofol were significantly inhibited (up to 26%) by 50 µM (+)-menthol suggesting that menthol and propofol compete for a common binding site on GABA_A receptors. In an *in vivo* tadpole assay (based on using gradually increasing concentration of the anesthetic agent and examining loss of righting reflex of the tadpole), addition of (+)-menthol resulted in an anesthetic effect with an EC_{50} of 23.5 µM (~10-fold less potent anesthesia than propofol) (Watt et al., 2008). In radioligand binding experiments, it was found that only (+)-menthol, among the five stereoisomers analyzed, was active, stimulating the binding of [³H]-flunitrazepam, an allosteric GABA_A receptor ligand, in a dose-dependent manner (Corvalan et al., 2009). In another radioligand binding study in housefly head membrane preparations, specific binding of [³H]-tbutylbicycloorthobenzoate (TBOB), a non-competitive inhibitor of picrotoxin, was
enhanced by 1,8-Cineole, carvacrol, citronellic acid, pulegone and thymol. On the other hand, four monoterpenoids, camphor, menthol, safrole and vanillin, significantly inhibited the [³H]-TBOB binding (Tong & Coats, 2012).

Another study showed that menthol reduced the excitation of cultured rat hippocampal neurons and suppressed the epileptic activity induced by pentylenetetrazole injection and electrical kindling in vivo (Zhang et al, 2008). It was found that menthol not only enhanced the currents induced by low concentrations of GABA but also directly activated GABAₐ receptors in hippocampal neurons in culture. Furthermore, in the CA1 region of rat hippocampal slices, menthol enhanced tonic GABAergic inhibition although phasic GABAergic inhibition was unaffected. Another study examined the effects of menthol on respiratory rhythm generation in brainstem–spinal cord preparations isolated from newborn rats (Tani et al, 2010). The results suggested that menthol has potent inhibitory action on burst generation of pre-inspiratory neurons due to direct activation of tonic GABAₐ channels by menthol. In another recent investigation, the actions of menthol on GABAₐ receptor-mediated currents in intact midbrain slices were examined (Lau et al, 2014). Whole-cell voltage-clamp recordings in periaqueductal grey (PAG) neurons in midbrain slices were used to determine the effects of menthol on GABAₐ receptor-mediated phasic inhibitory postsynaptic currents (IPSCs) and tonic currents (Lau et al, 2014). Menthol (150-750 μM) produced a concentration-dependent prolongation of spontaneous GABAₐ receptor-mediated IPSCs, but not non-N-Methyl-D-Aspartate (NMDA) receptor-mediated currents. Menthol actions were unaffected by TRPM8 and TRPA1 antagonists, tetrodotoxin and the benzodiazepine antagonist, flumazenil. Menthol also enhanced a tonic current, which was sensitive to the GABAₐ receptor
antagonists, picrotoxin and bicuculline. These results suggested that menthol positively modulates both synaptic and extrasynaptic populations of GABA\(_A\) receptors in native neurons of CNS.

Thymol is another monoterpane that has been tested on GABA\(_A\) receptors. One group used primary cultures of cortical neurons to examine the effect of thymol on native GABA\(_A\) receptors (Garcia et al., 2006). They showed that thymol enhanced GABA-induced chloride influx at concentrations lower than those exhibiting direct activity in the absence of GABA and that thymol is a positive allosteric modulator of GABA\(_A\) receptors as it enhanced \[^3\text{H}\]flunitrazepam binding with an EC\(_{50}\) = 131 µM. Interestingly, both bicuculline, a competitive GABA receptor antagonist, and picrotoxin, a non-competitive antagonist, were able to completely inhibit this increase in \[^3\text{H}\]flunitrazepam binding induced by thymol. The thymol structural analogues menthol and cymene, which lack an aromatic ring or a hydroxyl group, did not affect \[^3\text{H}\]flunitrazepam binding (Garcia et al, 2006).

In another investigation (Reiner et al, 2009), researchers determined and correlated several lipophilic parameters for GABAergic agents and three other related phenolic monoterpenes (eugenol, carvacrol and chlorothymol). The results of their correlation studies demonstrated the high capacity of these monoterpenes to interact with phospholipid membrane phases, which can be predicted by physicochemical parameters such as logP\(_{\text{o/w}}\). Finally, the fact that all monoterpenes studied were able to interact with membranes suggested that some alteration of the GABA\(_A\) receptor lipid environment can be exerted by thymol and other phenolic compounds (Reiner et al, 2009). In another study (Reiner et al, 2013b), researchers,
using Langmuir films, analyzed the effects of thymol on the molecular properties of the membranes. All the compounds studied were able to expand phospholipid films, by their incorporation into the monolayer membranes. Epifluorescence images revealed the presence of monoterpenes between phospholipid molecules, probably at the head-group region. The results of this study indicated that the thymol and other phenols studied were able to interact with membranes, suggesting that their biological activity could be the combined result of their interaction with specific receptor proteins and with their surrounding lipid molecules (Reiner et al, 2013b). In a recent study on the native GABA_{A} receptor using primary cultures of cortical neurons, Reiner et al., (2013b) investigated the effects of these compounds on the micro viscosity of membranes by means of fluorescence anisotropy. Phenolic monoterpenes such as carvacrol, chlorothymol, and eugenol were able to enhance the binding of [^{3}H]flunitrazepam with EC_{50} values in the μM range and to increase the GABA-evoked Cl\textsuperscript{-} influx in a concentration-dependent manner, both effects being inhibited by the competitive GABA_{A} antagonist bicuculline, strongly suggesting that the compounds studied are positive allosteric modulators of this receptor (Reiner et al, 2013a). Collectively, these results indicate that thymol and other monoterpenes can modulate the function of GABA_{A} receptors by directly acting on the receptors and also altering the biophysical characteristics of lipid membranes. Importantly, some studies suggest that thymol also acts on G-protein coupled receptors such as GABA_{B} (Parker et al, 2014) and α1, α2, and β-adrenergic receptors (Beer et al, 2007). (+)-Borneol, a bicyclic monoterpen used for analgesia and anaesthesia in traditional Chinese and Japanese medicine, was also tested alone and with GABA at recombinant human α_{1}β_{2}γ_{2L} GABA_{A} receptors expressed in Xenopus laevis oocytes.
using the two-electrode voltage-clamp technique. (+)-Borneol (EC50 248 µM) and (-)
-borneol (EC50 237 µM) were able to greatly enhance the effect of low concentrations of GABA. At high concentrations (>1.5 mM) both enantiomers directly activated GABA_A receptors indicating weak partial agonist activity (Granger
et al, 2005).

The effects of monoterpenes on the glycine receptors are less well understood. Menthol was examined for its effect on homomeric α1 glycine receptors expressed in Xenopus oocytes (Hall et al, 2004). The results of this study revealed that both (+) and (-) enantiomers of menthol could enhance glycine (EC20 = 50µM)-induced currents (Hall et al, 2004).

1.4.6 Serotonin type-3 receptors

Serotonin type-3 (5-HT3) receptors are another member of the ligand-gated ion channels that belong to the Cys-loop superfamily. The effects of menthol on these receptors have been described in several studies (Ashoor et al, 2013b; Heimes et al, 2011; Walstab et al, 2014). Heimes et al., (2011) tested the effect of menthol on 5-HT3 receptors using three different in vitro models: [14C]guanidinium influx into N1E-115 cells which express 5-HT3 receptors, isotonic contractions of the isolated rat ileum and equilibrium competition binding studies using a radioactively labelled 5-HT3 receptor antagonist ([3H]GR65630). Menthol inhibited [14C]guanidinium influx through 5-HT3 receptor channels as well as contractions of the ileum induced by 5-HT. Menthol, however, was not able to displace [3H] GR65630 from 5-HT3 binding sites. In this study, it was concluded that menthol exerts its well-known antiemetic effects at least partly by acting on the 5-HT3
receptor ion-channel complex by binding to a modulatory site distinct from the 5-HT binding site. In another study, Ashoor et al., (2013) investigated the effects of menthol on the function of human 5-HT$_3$ receptors expressed in *Xenopus laevis* oocytes. 5-HT-evoked currents were reversibly inhibited by menthol in a concentration-dependent (IC$_{50}$ = 163 μM) manner (Ashoor et al, 2013b). The effects of menthol developed gradually, reaching a steady-state level within 10-15 minutes and did not involve G-proteins, since GTPγS activity remained unaltered and the effect of menthol was not sensitive to pertussis toxin pretreatment. The actions of menthol were not stereoselective as (-), (+), and racemic menthol inhibited 5-HT$_3$ receptor-mediated currents to the same extent. The maximum inhibition observed for menthol was not reversed by increasing concentrations of 5-HT. Furthermore, specific binding of the 5-HT$_3$ antagonist $[^3]$HGR65630 was not altered in the presence of menthol, indicating that menthol acts as a non-competitive antagonist of the 5-HT3 receptor. Finally, 5-HT$_3$ receptor-mediated currents in acutely dissociated nodose ganglion neurons were also inhibited by menthol (Ashoor et al, 2013b). The results of this study demonstrated that menthol, at pharmacologically relevant concentrations, is an allosteric inhibitor of 5-HT$_3$ receptors. In another study the impact of menthol on human recombinant homeric 5-HT$_{3A}$ and heteromeric 5-HT$_{3AB}$ receptors in HEK293 cells was determined by radioligand binding, a luminescence-based Ca$^{2+}$ assay, and a membrane potential assay (Walstab et al, 2014). Menthol enantiomers inhibited 5-HT$_{3A}$ and 5-HT$_{3AB}$ receptor with IC$_{50}$ of 179 and 2041 μM for (-) menthol and 369 and 819 μM for (+) menthol suggesting enantiomer selectivity (Walstab et al, 2014). These studies indicate clearly that monoterpenene menthol is an allosteric inhibitor of 5-HT$_3$ receptors. In another study,
carvacrol and thymol showed allosteric agonist activity on human homomeric 5-HT$_3$ receptors expressed in *Xenopus* oocytes and also lower concentrations of these monoterpenes potentiated submaximal concentrations of 5-HT. Interestingly, both effects were species-specific as they were completely absent when using mouse 5-HT$_3$ receptors (Lansdell *et al.*, 2015). The effects of other monoterpenes on the functional properties of 5-HT$_3$ receptors are currently unknown and require further investigation.

1.5. Monoterpenes and the Cholinergic System

1.5.1. Cholinergic system

Acetylcholine (ACh) is the principal neurotransmitter in the parasympathetic autonomic nervous system as well as inside the central nervous system (CNS). The neurochemical events in the life cycle of ACh are shown in Figure 1.4. Synthesis of ACh is dependent on the availability of choline. There is very little de novo choline synthesis in cholinergic neurons; therefore, the pool of choline is built mainly from dietary sources. Choline is loaded inside the cholinergic neurons from the extracellular space by two transport systems (Westfall, 2009). One of them is a widely distributed, low-affinity, sodium independent transporter while the other transporter is a high-affinity, sodium and chloride dependent choline transporter system. The latter exists predominantly in cholinergic neurons and provides choline for ACh synthesis (Westfall, 2009). ACh is synthesized in the cytosol from choline and acetylCoA (AcCoA) by the choline acetyltransferase (ChAT) enzyme and then loaded into storage vesicles by the vesicular acetylcholine transporter (VAT) which can be inhibited by vesamicol. When depolarized by an action potential, voltage-
 gated Ca\textsuperscript{2+} channels are opened and the presynaptic neuron releases the stored ACh into the synaptic cleft, where it interacts with muscarinic (mAChRs) or nicotinic acetylcholine receptors (nAChRs). Once ACh dissociates from the receptors, it is rapidly hydrolyzed by acetylcholinesterase (AChE), which breaks down ACh into acetate and choline. Choline is then taken back into the presynaptic neuron by sodium-dependent choline transporter (CHT) to be recycled. CHT can be inhibited by hemicholinium drugs. Most cholinergic synapses are highly supplied with acetylcholinesterase therefore, the half-life of acetylcholine molecules in the synapse is extremely short (a fraction of a second) (Katzung, 2012). Reuptake and availability of choline is the rate-limiting step in ACh synthesis.
Inside the CNS, there are specialized cholinergic neurons. Early attempts to localize these neurons were based on immunohistochemical methods to localize (AChE) enzyme, but this was not specific for specialized cholinergic neurons as it revealed cholinoceptive neurons as well. In more recent studies, localization of ChAT-immunoreactive cell bodies was considered as more selective and specific for cholinergic neurons (Cuello, 2009). The main central cholinergic cell groups and pathways are shown in Figure 1.5. In the human CNS, the cholinergic system
Involves a highly complex network that has three main components, the first two components are diffuse, divergent projection systems that innervate wide areas of the brain. They include: (1) projections from nuclei of the basal forebrain; these include the medial septal nucleus, the nucleus basalis of Meynert (a site associated with profound degeneration in Alzheimer's disease), the vertical nucleus of the diagonal band and the horizontal limb of the diagonal band nucleus, which innervate the hippocampus, most cortical regions and some subcortical nuclei. Although this system is located in separate areas of the brain, they are usually referred to as the magnocellular basal forebrain cholinergic system (Mesulam et al, 1983). (2) The pedunculopontine-lateral dorsal tegmental projections from the brainstem to the thalamus, midbrain and other brainstem regions. The third central cholinergic component includes (3) interneurons in the neostriatum (most abundant) and correspond to the caudate nucleus-putamen complex. Also the ventral striatum has cholinergic interneurons in the nucleus accumbens and islands of Calleja (Everitt & Robbins, 1997; Perry et al, 1999).

Based on the complex nature of cholinergic neurotransmission in the human CNS, cholinergic neurons have been shown to play roles in diverse brain functions such as sleep, cognition, memory, motor control, and sensory processing (Scarr et al, 2013). For several decades, the CNS cholinergic system was suspected to play an important role in memory and learning.
One of the most important early studies demonstrating central cholinergic function was carried out by Drachman and collaborators in healthy young human subjects. They showed that subjects who were treated with low dose scopolamine, a muscarinic antagonist, showed similar memory and cognitive decline aged subjects (Drachman et al., 1980). There is experimental evidence indicating that the basal forebrain cholinergic neurons do participate in attention, learning, and memory functions (Gibbs & Johnson, 2007; Leanza et al., 1996; Wrenn & Wiley, 1998). Several earlier investigations have highlighted the role of the cholinergic system in conditioning and sensory learning (Deiana et al., 2011; Robinson et al., 2011). The
general observation is that blockade of both muscarinic and nicotinic receptors led to widespread impairments in either acquisition learning or long-term memory processing, and this can be reversed by boosting levels of ACh. Consistently, stimulation of either muscarinic (mainly M₁) or nicotinic (predominantly α7) receptors was beneficial for learning and memory and has been suggested to be valuable in the treatment of neurodegenerative or psychiatric disorders with cognitive impairment (Deiana et al, 2011; Robinson et al, 2011). Researchers have shown that variable performance of the central cholinergic system in the elderly, measured by assessing the AChE enzyme activity, is closely linked and can underlie the inter-individual variability in memory function (Richter et al, 2014). Therefore, cholinergic dysfunction can be considered an early hallmark even before onset of dementia.

The nicotinic α7 receptor subtype was found to be involved in working memory, whereas the α4β2 subtype has been linked to tests of attention. In addition to learning and memory, nicotinic receptors are also associated with the modulation of depression and anxiety (Graef et al, 2011).
1.5.2. Nicotinic receptors

Nicotinic acetylcholine receptors (nAChRs) belong to a large family of ligand-gated ion channels that include also GABA<sub>A</sub> receptors, serotonin 5-HT<sub>3</sub> receptors and glycine receptors. Several structural features are conserved in all members of this gene family, most notably a disulfide-linked sequence of fifteen amino acids that constitutes what is called the “signature Cys-loop” and the whole family is referred to as the “Cys-loop superfamily” of ligand-gated ion channels (Gotti et al, 2009). The nicotinic receptor was the first pharmacologic receptor to be purified and the cDNAs encoding its subunits cloned, therefore they are considered as prototype for the ligand-gated ion channel family (Itier & Bertrand, 2001).

Nicotinic receptors are widely distributed in the central and peripheral nervous systems. In skeletal muscle they are found in a high density at the motor end plate. In ganglia, the primary nicotinic receptor is found on the postsynaptic dendrite and nerve cell body. In the CNS, the majority of receptors are presynaptic, so they have regulatory function as they control the release of other transmitters or work as autoreceptors in cholinergic neurons (Gotti et al, 2006; Taylor, 2012).

Overall, nicotinic receptors are ionotropic receptors that exist as pentamers with intrinsic cation-permeable pores that are gated by the binding of ACh. The five subunits of the receptors can be identical (homomeric) or variable (heteromeric). Muscle-type ACh receptors were first isolated from electrical organs of fishes such as *Torpedo marmorata* (Karlin, 2002; Unwin, 1998). The receptors in *Torpedo* electric tissue and in fetal muscle are heteropentamers, with subunit composition of α<sub>2</sub>βγδ (Figure 1.6). In adult muscle, the receptor contains an ε subunit in place of the
γ subunit and has a greater single-channel conductance and a smaller mean single-channel-open time than fetal receptor (Hurst et al., 2013). There are nine other neuronal α subunits (α2–α10) and three known neuronal β subunits (β2–β4); the α subunit constitutes the principal component for binding site, while the β subunit is a complementary component of the receptor (Hurst et al., 2013). Some neuronal α subunits (e.g., α7) form functional homopentamers. Different regions of the peripheral and central nervous system express different combinations of the neuronal subunits (Karlin, 2010).
The structure of each subunit of nAChRs consists of β-strands, four transmembrane α-helices segments (M1-M4), a variable intracellular loop between M3 and M4, and an extracellular carboxy-terminus. The extracellular domain carries the acetylcholine–nicotine binding sites at the boundary between subunits. The number of binding sites per pentamer is variable, depending on its composition, from two (in muscle nAChRs or brain α4β2 nAChRs) to five (in the α7 homopentamer). Sites for allosteric modulators are located in the transmembrane domain. A schematic representation of the quaternary structure, showing the arrangement of the subunits in the muscle-type receptor, the location of the two acetylcholine-binding sites (between an alpha- and a gamma-subunit, and an alpha- and a delta-subunit), and the central cation-conducting channel. A cross-section through the 4.6-Å structure of the receptor determined by electron microscopy of tubular crystals of Torpedo membrane embedded in ice. Dashed line indicates proposed path to binding site (Adapted from Karlin, 2002).

1.5.3. Neuronal nicotinic receptors

The most abundant subtypes of nicotinic receptors in the brain are the low affinity α7 homomeric and high affinity α4β2* heteromeric nAChRs (Figure 1.7). Different subunit types are distributed in most central cholinergic areas as shown in table (1). Due to variable types of subunits, heteromeric nAChRs are rather complex
as they can contain two or three alpha subunits co-assembled with two or three beta subunits. For example, α4β2 nAChRs can be formed either as [(α4)2(β2)3] or as [(α4)3(β2)2]. This variability causes many differences between the various forms of nAChRs. In the previous example, [(α4)2(β2)3] nAChRs showed very high sensitivity to ACh that is almost 100 times greater than that of [(α4)3(β2)2] nAChRs (Moroni et al, 2006; Zwart & Vijverberg, 1998). In addition, some subtypes identified express more than one type of alpha and/or beta subunit. For example, the α4α6β2β3* nAChR was detected in midbrain dopaminergic neurons (Liu et al, 2012).

![Figure 1.7: Schematic presentation of homomer and heteromer nAChRs.](image)

(A) Homomorphic receptors consist of subunits only and usually have low affinity for agonist. To date, only mammalian α7, α9, and α10 (not shown) subunits may form functional homomers. (B) The majority of high affinity nAChRs are heteromeric and consist of a combination of α and β subunits. Importantly, multiple α subunits may coassemble with multiple β subunits in the pentameric nAChR complex (illustrated here by α4α6β2β3). ACh binding sites are depicted as red triangles (Adapted from Hendrickson et al, 2013).

The most abundant homomer α7 nAChRs has been extensively studied owing to its unique physiological and pharmacological properties. It is exclusively characterized by high permeability to Ca\(^{2+}\) (PCa:PNa of ≥ 10 compared to 3-4 for most other nAChRs) and rapid desensitization. The α7 subunit is expressed at high levels in the hippocampus and hypothalamus (Seguela et al, 1993). Significant proportion of α7 nAChRs are located on presynaptic terminals where they facilitate
Ca²⁺ dependent release of neurotransmitters. This may occur indirectly as a result of Na⁺ influx causing membrane depolarization and activation of voltage-gated Ca²⁺ channels or directly by Ca²⁺ influx through the nAChR itself (Albuquerque, 2009). Eventual increase of intracellular Ca²⁺ level upon nAChR activation makes these receptors relevant for the regulation of other Ca²⁺-mediated events such as cell excitability, activation of second messenger pathways, gene expression and cell differentiation (Berg & Conroy, 2002). The change in the pattern of α7 nAChR expression over time suggests that these receptors play a role during brain development. Their concentration is high during the stage of synapse formation (Gotti & Clementi, 2004). During development, α7 nAChRs are also expressed in non-neuronal cell types, including muscle, glia, epithelial cells, and chondrocytes (Berg & Conroy, 2002; Gotti & Clementi, 2004).

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<th>Table 1: Nicotinic receptor subunit protein expression in human brain (Adapted from Gotti &amp; Clementi, 2004)</th>
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1.5.4. Nicotinic receptors and monoterpenes

As mentioned earlier, being one of the most widely distributed nicotinic receptor subtypes in different higher brain areas, α7 nAChRs have been proven to be involved in many neural physiological and pathological conditions. There is considerable interest in finding selective ligands for nAChRs individual subtypes. In case of homomeric α7 nAChRs, many reports have indicated the beneficial effects of activation of these receptors in improvement of cognitive deficits of patients with schizophrenia and Alzheimer's disease (Bourin et al., 2003; D'Andrea & Nagele, 2006; Deutsch et al., 2014; Leiser et al., 2009; Levin, 2002; Ripoll et al., 2004; Thomsen et al., 2010; Wallace & Porter, 2011; Young & Geyer, 2013).

Fewer studies, mostly in mice and rats, have demonstrated that α7 nAChRs antagonism showed anti-depressant activity using several common tests of antidepressant efficacy including the forced swimming test and tail suspension test (Andreasen et al., 2009; Rabenstein et al., 2006). Clinical data elucidated that the non-selective nAChR antagonist, mecamylamine, reduces depression-like symptoms suggesting that targeting nAChRs could be a reasonable strategy for developing novel antidepressant medications (Shytle et al., 2000; Shytle et al., 2002). Interestingly, the α7 nAChRs have also been suggested to play an important role in both central and peripheral mechanisms involved in eating behavior and energy balance (reviewed in details by McFadden et al., 2014).

In the context of searching for selective novel agents that modulate the activity of nAChRs, natural phytochemicals have proven to be a good source at least for structural models (Daly, 2005; Romanelli et al., 2007). Regarding monoterpenes,
only few studies have been performed to screen the effects of the monoterpenes on nicotinic receptors. Park and co-workers tested the effect of camphor (Park et al., 2001) and borneol (Park et al., 2003) on catecholamine secretion in bovine adrenal chromaffin cells. Both compounds reduced [³H]norepinephrine secretion induced by a nAChR agonist, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), with similar IC₅₀ values (70 µM). To confirm that this effect is mediated by nAChRs, cytosolic Ca²⁺ and Na⁺ levels were measured upon treatment of the cell with DMPP alone and in the presence of monoterpenes. The rise of [Ca²⁺], and [Na⁺], an essential step for the release of catecholamines by nAChRs activation, was inhibited with an IC₅₀ of 88 and 19 µM for camphor and 56 and 49 µM for borneol, respectively. Binding assay of [³H]nicotine to nAChRs showed that these monoterpenes did not alter the binding of nicotine indicating that their binding site is distinct from nicotine's and ACh binding sites. However, another monoterpene, carvacrol was examined on nAChRs of housefly and showed non-competitive concentration dependent inhibition of [¹⁴C]-nicotine binding (Tong et al., 2013).

In earlier studies, it was shown that menthol is an antagonist of nAChRs as well. Hans and co-workers studied the effect of (-)-menthol on nicotine induced current through nAChRs recorded in rat trigeminal neurons (Hans et al., 2012). Menthol showed reversible and concentration-dependent inhibition of nAChRs. Single channel and whole-cell recordings from recombinant human α4β2 nAChR expressed in HEK tsA201 cells demonstrated that (-)-menthol is a negative allosteric modulator of this nicotinic subtype (Hans et al., 2012). The effect of menthol was also examined on another subtype, α7 nAChRs, expressed in Xenopus oocytes by recording ACh-induced currents using the two electrode voltage clamp technique.
Racemic, (-) and (+) forms of menthol caused concentration-dependent, non-competitive inhibition of α7 nAChRs. Consistent with these results, experiments done on neural cells (PC12) endogenously expressing α7 nACh receptors demonstrated that menthol attenuates α7 mediated Ca^{2+} transients in the cell body and neurite (Ashoor et al, 2013a).

Drug development efforts have recently focused on direct manipulation of α7 and α4β2 nAChRs. Monoterpenes could be helpful in better understanding of factors influencing these nicotinic subtypes and their function and may provide specific drug candidates or at least lead structures for further design of monoterpene-based compounds.
Chapter 2: Aims and Objectives

Our main objective was to investigate the effects of carveol and other related monoterpenoids on the functional properties of α7 nACh receptors. In all experiments, homomeric human α7 nACh receptors were expressed heterologously in a *Xenopus* oocyte expression system. This expression system is convenient for assaying the actions of tested compounds on cloned receptors for the following reasons. 1) Procedures for acquiring and maintaining oocytes are well established. Therefore, large number of efficiently expressing oocytes can be easily obtained on a routine basis for the intended experiments. 2) Two-electrode voltage clamp allows a relatively high throughput assay, so several cells can be screened on a daily basis. 3) Recordings using two-electrode voltage clamp can be stable over long periods of time, which makes it particularly useful for analyzing properties that require long protocols, such as slow time course of drug action.

In the present study, several monoterpenes were screened to test their effects on the activity of human α7 nACh receptor expressed in *Xenopus* oocytes. In order of fulfil this goal, the following experiments were done:

1. Functional expression of human α7 nACh receptor in *Xenopus* oocytes.
2. Exploring the effects of the selected monoterpenes on the function of human α7 nACh receptor.
3. Defining voltage-dependence of carveol's action on the α7 nACh receptor.
4. Investigating the involvement of cytosolic calcium in the effects of carveol on α7 nACh receptor.
5. Determination of the binding site of carveol in the α7-nACh receptor.
Chapter 3: Materials and Methods

3.1 Animals and materials

3.1.1 Animals: Female *Xenopus laevis*

Mature female African clawed frogs (*Xenopus laevis*) were purchased from Xenopus Express, Haute-Loire, France (Figure 3.1). They were kept in a container (32 cm width, 130 cm length and 66 cm height) filled to a height approximately 50 cm with chlorine-free water. The room temperature was maintained at 19-21°C with a 12 hour alternate light and dark cycle. Frogs were fed twice a week with food pellets, supplied by Xenopus Express, France. Tank-water was changed twice a week. Animal care and handling were consistent with institutional guidelines.

![Figure 3.1: An adult female *Xenopus laevis*.](image)
3.1.2 Chemicals

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Formula weight</th>
<th>Manufacturer / Catalogue number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine chloride</td>
<td>181.7</td>
<td>Sigma, USA / A6625</td>
</tr>
<tr>
<td>BAPTA</td>
<td>764.68</td>
<td>Sigma-Aldrich/ A-1076</td>
</tr>
<tr>
<td>Barium chloride (BaCl₂)</td>
<td>244.28</td>
<td>BDH, England/R-20-25 S:45</td>
</tr>
<tr>
<td>Benzocaine</td>
<td>165.2</td>
<td>Sigma, USA/ E-1501</td>
</tr>
<tr>
<td>Calcium chloride (CaCl₂)</td>
<td>110.9</td>
<td>Sigma-Aldrich, USA / C-4901</td>
</tr>
<tr>
<td>Collagenase-A (From Clostridium histolyticum EC.3.4.24.3)</td>
<td>~8500</td>
<td>Roche Diagnostic Corporations USA/ Lot number 93211521</td>
</tr>
<tr>
<td>Gentamicin reagent solution</td>
<td>Conc. 50mg/ml</td>
<td>Invitrogen Corporation/China Cat. number 15750-060</td>
</tr>
<tr>
<td>HEPES</td>
<td>238.3</td>
<td>Sigma, USA / H3375</td>
</tr>
<tr>
<td>Hydrogen chloride (HCl)</td>
<td>37%</td>
<td>Sigma-Aldrich, USA.25.814.B</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>95.22</td>
<td>Sigma, USA / M8266</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>246.5</td>
<td>Sigma, USA / M9397</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>356.4</td>
<td>Sigma, USA / P3032</td>
</tr>
<tr>
<td>Potassium chloride (KCl)</td>
<td>74.55</td>
<td>Mallinckrodt USA / 6858</td>
</tr>
<tr>
<td>Sodium bicarbonate (NaHCO₃)</td>
<td>84.01</td>
<td>Sigma, USA / S6014</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>58.4</td>
<td>Sigma, USA / S-3014</td>
</tr>
<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>40</td>
<td>Amresco, USA / Lot. # 214613010</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>110.04</td>
<td>Sigma-Aldrich, USA /P2256</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>1457.4</td>
<td>Sigma, USA / S9137</td>
</tr>
<tr>
<td>α-Bungarotoxins</td>
<td>~8500</td>
<td>Sigma / T-0195</td>
</tr>
</tbody>
</table>

Table 2: Chemicals required for experiments.

3.1.3 Experimental setup

The experimental setup for electrophysiological recordings using two electrode voltage clamp is shown in Figure 3.2 and 3.3. Two-electrode voltage clamp (TEVC) technique was applied using a GeneClamp-500B amplifier (Axon Instruments, Molecular Devices, Inc., Sunnyvale, CA, USA), as described previously (Ashoor et al, 2013a) recording setup included magnetic holding devices (Kanetec USA Corporation, Bensenville, IL, USA), two manual micromanipulators (M33;
Märzhäuser, Wetzlar, Germany) and head-stages for voltage (HS-2A Headstage, Gain 1 MG, Axon Instruments, Molecular Devices, Inc., Sunnyvale, CA, USA) and current (HS-2A Headstage, Gain 10MG) attached to manipulators. Electrodes were inserted in electrode holders and connected to the head-stages.

The perfusion apparatus consisted of perfusion tubes and bottles containing extracellular solutions connected to the recording bath by silicon tubing (Cole Parmer Instrument Company, I.D. 1/16 inch, O.D. 1/8 inch and WALL1/32, Vernon Hills, Illinois, USA). Flow rate of perfusion was set to 3 to 5 ml/minute. A multichannel perfusion system was used for drug applications included tubing (C-Flex tubing, Cole-Parmer Instrument Company, I.D. 1/32 inch, O.D. 3/32 inch and WALL1/32 inch, Vernon Hills, Illinois, USA), 50 mL glass syringes, plastic valves and coupling devices. The drug application system was based on gravity flow by means of a micropipette that was set at a distance of about 2-3 mm from the oocyte position in the perfusion chamber/recording chamber (Warner Instruments LLC, Hamden, CT, UK) designed for placing oocyte to be impaled with the microelectrodes (Figure 3.3). In initial control studies using high K+ (20 mM) containing solution, we determined that the response time of our recording system is 0.1 to 0.4 sec (0.32 ± 0.07 s; mean ± SEM, n=8).

An optic fiber light source was used for illumination of the recording chamber (Fiber Lite, High Intensity Illuminator Series 180, Dolan-Jenner Industries Inc. Boxborough, MA, USA). A Low-power stereo-dissection microscope (Olympus, Tokyo, Japan, SZ-STB1, 100 AL0.5 X, WD186) was used for visual observation of the recording chamber. The computer set up for recording consisted
of a Compaq computer, (Compaq Corporation, Product of UK, City of Wynyard) and analog-digital converter, BNC 2081 (National Instruments, Austin Texas, USA).

Figure 3.2: A photograph of the two-electrode voltage clamp used to study the alpha7 nicotinic receptor in *Xenopus* oocyte. ACh solution (4,7) and perfusion solution (3) lines are connected by tubes to the chamber (9). Micromanipulators (6) are used to control electrodes (8) to impale the oocyte visualized using microscope (5). An amplifier (2) is used to record current traces (1). Acquired data were stored on a computer (Prof. Murat Oz Lab).
Figure 3.3: Illustration of the plastic chamber. The oocyte was placed on the chamber and perfused continuously during the experiment. The suction line conveyed the solution to a waste flask.

3.1.4 Other materials

<table>
<thead>
<tr>
<th>Material</th>
<th>Specifications/company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Automatic nanoliter injector</td>
<td>Nanoject, Drummond Scientific Company, Broomall, PA, USA</td>
</tr>
<tr>
<td>Borosilicate Glass tubing for microelectrodes.</td>
<td>Glass Thin-walled w/filament 1.5 mm, Catalogue number: TW150F-4, World Precision Instrument, Sarasota, FL, USA</td>
</tr>
<tr>
<td>Electrode holder</td>
<td>World Precision Instrument, Sarasota, FL, USA</td>
</tr>
<tr>
<td>Magnetic stand and manipulators.</td>
<td>Catalogue number: 7739, Narishige, Tokyo, Japan</td>
</tr>
<tr>
<td>Micro-4 Microsyringe pump controller</td>
<td>Model UMC4-C, World Precision Instruments, Sarasota, FL, USA</td>
</tr>
<tr>
<td>Microfil filling syringe</td>
<td>World Precision Instruments, Sarasota, FL, USA</td>
</tr>
<tr>
<td>Petri dishes</td>
<td>Sterilin, Newport, UK, Catalog number: 127, 60 mm</td>
</tr>
<tr>
<td>pH meter</td>
<td>Corning pH meter model 450, Albany, NY, USA</td>
</tr>
<tr>
<td><strong>Picofuge</strong></td>
<td>Stratagene, Santa Clara, CA, USA, Catalog number: 400550</td>
</tr>
<tr>
<td><strong>RNase free water in 1.8 mL Eppendorf tubes</strong></td>
<td>Lot number: M25-80502, Epicentre Biotechnologies, Madison, Wisconsin, USA</td>
</tr>
<tr>
<td><strong>Silver wires</strong></td>
<td>World Precision Instruments, Sarasota, FL, USA</td>
</tr>
<tr>
<td><strong>Stirrer</strong></td>
<td>Rotomix, Type 50800, Barnstead/Thermolyne, Dubuque, IA, USA, Model number: M50825</td>
</tr>
<tr>
<td><strong>Surgical accessories</strong></td>
<td>Scissors, forceps, scalpels; World Precision Instrument, Sarasota, FL, USA</td>
</tr>
<tr>
<td><strong>Surgical sutures</strong></td>
<td>Catgut chrom, reverse cutting 3/8 circle, USP 4/0, SMI, DemeTech corporation, Miami, Florida, USA</td>
</tr>
<tr>
<td><strong>Vertical puller (Heater and solenoid settings were adjusted to 48 and 70, respectively, to get optimal resistance (1-2 MΩ) glass microelectrodes)</strong></td>
<td>Model 700D, David Kopf Instruments, Tujunga, CA, USA</td>
</tr>
</tbody>
</table>

**Table 3: Required materials**
3.2 Solutions required

3.2.1 Preparation of Modified Barth’s Solution (MBS)

The composition of Modified Barth Solution is shown in the table below.

a. Calcium-free Modified Bath Solution.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mM)</th>
<th>1x (weight in gram)</th>
<th>10x (weight in gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
<td>10</td>
<td>2.38</td>
<td>23.8</td>
</tr>
<tr>
<td>KCl</td>
<td>1</td>
<td>0.075</td>
<td>0.75</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.8</td>
<td>0.20</td>
<td>2.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>88</td>
<td>5.14</td>
<td>51.4</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.4</td>
<td>0.20</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 4: Calcium-free MBS solution composition.

The above substances were dissolved in 1 L of distilled water and the pH of the resulting solution was adjusted to 7.5 using NaOH.

b. Modified Barth’s Solution with Calcium.

Similar to previous composition but in addition of 2 mM CaCl₂ (0.22 g for 1x and 2.2 g for 10x stock solutions), and the final solution is made up to 1L with distilled water and pH adjusted to 7.5 with NaOH.

3.2.2 Preparation of frog's Ringer solution (ND96)

The composition of the ND96 bathing solution is given in the table below.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mM)</th>
<th>1x (weight in gram)</th>
<th>10x (weight in gram)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>96</td>
<td>5.61</td>
<td>56.1</td>
</tr>
<tr>
<td>HEPES</td>
<td>5</td>
<td>1.19</td>
<td>11.9</td>
</tr>
<tr>
<td>KCl</td>
<td>2</td>
<td>0.15</td>
<td>1.5</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1</td>
<td>0.10</td>
<td>1.0</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1.8</td>
<td>0.20</td>
<td>2.0</td>
</tr>
<tr>
<td>OR BaCl₂</td>
<td>1.8</td>
<td>0.439</td>
<td>4.39</td>
</tr>
</tbody>
</table>

Table 5: ND96 solution.
The above weighed substances were dissolved to make 1L solution with distilled water and the pH was adjusted to 7.5 using NaOH.

### 3.2.3 Preparation of oocytes storage solution

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mM)</th>
<th>Weight in gram</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>88</td>
<td>5.14</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td>2.38</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.4</td>
<td>0.20</td>
</tr>
<tr>
<td>KCl</td>
<td>1</td>
<td>0.075</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.8</td>
<td>0.20</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2</td>
<td>0.22</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>10000U/L</td>
<td>0.019</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>10 mg/L</td>
<td>0.01</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>50 mg/L</td>
<td>2mL</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>2</td>
<td>0.22</td>
</tr>
<tr>
<td>Theophylline</td>
<td>0.5</td>
<td>0.09</td>
</tr>
</tbody>
</table>

**Table 6:** Oocytes storage solution.
3.3 Methods

3.3.1 Isolation of oocytes from *Xenopus laevis*

*Xenopus laevis* female frog was anesthetized by keeping it in 1L of 0.03% w/v benzocaine solution, prepared by dissolving 300 mg of ethyl p-aminobenzoate in 15 mL of 70% ethanol and then adding that to 1L of cold tap water. The end point of anesthesia was determined by failure to respond to noxious stimuli like pinching of the lower limbs. On average it took 5-10 minutes to achieve anesthesia under the described conditions. Experimental procedures applied in this study have been approved by the Animal Ethics Committee of the CMHS, UAE University.

The anesthetized frog was then placed on crushed ice covered with a wet tissue paper to avoid skin from drying out during surgery and to maintain low core body temperature. Sterile surgical procedures were applied to remove oocytes. Sterilized surgical tools and regular use of 70% ethanol was employed to maintain overall sterility. A small incision was made through the epidermal layer in the lower abdominal area of about 1.5 cm length slightly to the left or right of the midline, and a similar cut in the inner muscular layer. Using sterilized forceps, one to two ovarian lobes (small clumps of oocytes) were removed and placed in a petri dish containing Ca$^{2+}$- free MBS (Figure 3.4). After removing the ovarian lobes, the muscular layer as well as the outer skin was sutured with absorbable Catgut sutures. After surgery, the frog was kept in a container filled with tap water and closely monitored for recovery. After 3-4 hours of recovery, the frog was returned to the main frog container. Each frog was utilized for three to four surgeries keeping a gap of two to three months between each procedure.
3.3.2 Oocyte preparation

The preparation of oocytes was performed according to procedures described earlier (Oz, Ravindran, Diaz-Ruiz, Zhang & Morales, 2003). Using fine forceps, inner ovarian epithelium, theca, and follicular layers were removed as much as possible to produce smaller clusters of oocytes. The resulting oocyte clusters were then treated for one hour with 12.5 mL of collagenase solution, prepared by dissolving 50 mg of Collagenase-A in 25 mL of Ca\(^{2+}\) free MBS, in a small conical flask with continuous stirring (60-80 RPM). This procedure was repeated again for another one hour using the remaining fresh collagenase solution (12.5 mL). Afterwards, oocytes were washed thoroughly with Ca\(^{2+}\)-free MBS six times, then with Ca\(^{2+}\) containing MBS another six times. Subsequently, oocytes were transferred to a petri dish containing MBS for selection using a dissecting microscope (Bunton Instruments Co Inc., Model GSZ, Rockville, MD, U.S.A.).

Morphologically intact, healthy looking and mature oocytes (stage V–VI) are selected. These oocytes are large in size, round in shape, about 1.1 to 1.2 mm in
diameter and have characteristic brown and white colored poles, a dark brown animal pole and a yellowish vegetal pole (Figure). The oocytes were subsequently maintained in MBS at 18 °C.

![Isolated stage V and VI oocyte of *Xenopus laevis* after collagenase treatment.](image)

**Figure 3.5:** Isolated stage V and VI oocyte of *Xenopus laevis* after collagenase treatment.

### 3.3.3 Synthesis of cRNA

The cDNA clone of human α7-nACh was kindly provided by Dr. J. Lindstorm (University of Pennsylvania, PA, U.S.A.). Capped cRNA transcripts were synthesized *in vitro* using a mMESSAGE mMESSAGE kit (Ambion, Austin, TX, U.S.A.) and analyzed on 1.2% formaldehyde agarose gel to check the size and quality of the transcripts (Figure 3.6).
Human α7 nACh receptor mRNA was prepared by *in vitro* transcription and confirmed by gel analysis. Restriction enzyme (Xbal) was used to digest the cDNA of human α7 nACh receptor. Linearized plasmid cDNA was transcribed *in vitro* by SP6 RNA polymerase to produce α7 nACh receptor RNA using a mMESSAGE mMACHINE kit. This RNA was cleaned and purified by phenol:chloroform extraction and ethanol precipitation. The quantity of RNA was estimated by OD260 measurement and quality was assessed by agarose gel. Capping enzyme and 2'-O-methyltransferase were used to add a cap and poly(A) tail to RNA, and the resulting mRNA was suspended in DEPC-treated water. Once more, mRNA was cleaned by
phenol:chloroform extraction and ethanol precipitation, then run against RNA in agarose gel to confirm the addition of 5’ cap and 3’ poly(A) tail (Figure 3.7).

3.3.4 Injection of cRNA into oocytes

The concentration of human α7 nACh receptor mRNA synthesized was 3.7 µg/µL. It was stored in 1 µL aliquots in -80 °C freezers. On the day of injection only one aliquot was transferred to the laboratory on ice. In order to control RNase contamination, gloves and face mask were used and the bench was wiped with 70% ethanol before handling cRNA. Eight µL of RNase and DNase-free water was added to the cRNA using a sterile pipette and RNase free and DNase free tips (Denville Scientific Inc., Metuchen, NJ, U.S.A.). Only 3 µl of diluted cRNA was used for one batch of oocytes and the remaining cRNA was returned to the freezer.

A vertical puller was used to pull an autoclaved glass capillary (World Precision Instruments, Sarasota, FL, USA) to make a needle with a long shaft. The tip of the needle was broken by applying pressure using fine forceps (Fine Science Tools Inc, Vancouver, BC, Canada) under the dissecting microscope (Bunton Instrument Co, Rockville, MD, USA). The needle was back filled with mineral oil (Sigma, St. Louis, MO, USA) using a glass 1 mL syringe. The needle was then placed in the micro-dispenser connected to a micromanipulator. Before starting the microinjection procedure, the surface of the microdispenser and the microscope was cleaned with 70% ethanol. To the centre of a drop of mineral oil kept on parafilm (American National Can, Greenwich, CT, USA), 3 µL of either diluted cRNA or distilled water was transferred. Using the microscope, the intended aqueous phase was carefully withdrawn into the needle using the microdispenser control panel.
Thereafter, denuded selected oocytes are placed in a petri dish containing MBS and having a mesh bottom to prevent movement of oocytes during the microinjection procedure. Each oocyte was injected with 25 nL of mRNA by the nanoliter injector, driven by a Micro-4, micro-syringe pump controller. Lastly, injected oocytes were kept at 18 °C in a petri dish containing oocyte storage solution for 48 hours for optimal expression of α7 nAChRs. The injected oocytes were used for electrophysiological experiments during a period of one week. Every day after the microinjection procedure, the storage solution was exchanged and dead or unhealthy oocytes were removed.

3.3.5 Drug preparation

Stock solutions of tested compounds were prepared in millimolar concentration in ND96 solution using the following formula:

\[
\text{Weight in mg} = (\text{MW}) \times (\text{volume in L}) \times (\text{mM concentration})
\]

Further dilutions were prepared by using Charles equation:

\[C_1 \times V_1 = C_2 \times V_2\]

Where,

\[C_1 = \text{concentration of stock solution}\]
\[V_1 = \text{volume of stock solution to be used}\]
\[C_2 = \text{desired concentration to be prepared}\]
\[V_2 = \text{desired volume to be prepared}\]

Fresh stock solution and further dilutions were prepared before starting experiments.
3.3.6 Two-electrode voltage clamp recording

Two-electrode voltage clamp (TEVC) is a conventional electrophysiological technique used to artificially control (or clamp) membrane potential using feedback amplifier in large cells to study the properties of electrogenic membrane proteins such as ion channels. The functional properties of ion channels exogenously expressed in *Xenopus* oocytes can be studied conveniently using this technique. In TEVC, two intracellular microelectrodes are employed, a voltage sensor electrode and a current injection electrode to control membrane voltage. In this way, membrane potential can be set at a desired value and current through the membrane can be recorded to analyze ion channel properties. The injected current to maintain command voltage is equal to total membrane current (Figure 3.7).

![Two-electrode voltage clamp](image)

**Figure 3.7:** Schematic illustration of two-electrode voltage clamp setup using *Xenopus* oocytes: The membrane of the oocyte is penetrated by two microelectrodes, one for voltage sensing (V1) and one for current injection (I2). The membrane potential as measured by V1 and a high input impedance amplifier (amp1) is compared with a command voltage, and the difference is brought to zero by a high gain feedback amplifier (amp 2). The injected current is monitored via a current-to-voltage converter to provide a measure of the total membrane current.
The experimental setup for TEVC is depicted in Figure 3.2 and 3.7. At the beginning of the experiments, perfusion containers and application systems were filled with the appropriate solutions and allowed to run through the connected tubes. The experiments were performed using the TEVC technique. For each experiment, a single oocyte was placed in the recording chamber and continuously perfused with ND96 solution at a rate of 3-5 ml/minute. The oocyte was impaled with two glass microelectrodes (resistance ≈ 1-2 MΩ) prepared using a vertical microelectrode puller (heater and solenoid values were adjusted to a setting of 50 and 70, respectively; David Kopf Instruments Tujunga, CA, USA) and filled with 3 M KCl solution. Drugs were applied by a gravity-based multichannel application system via a micropipette positioned about 2-3 mm from the impaled oocyte. TEVC was achieved using a Geneclamp 500 amplifier (Axon Instruments, Molecular Devices, Inc., Sunnyvale, CA, USA), connected to a PC computer loaded with electrophysiology software (Win WCP, University of Strathclyde, Glasgow, UK), for data acquisition.

3.3.7 Parameters tested by electrophysiological recordings

3.3.7.1 Concentrations response curve (IC₅₀ determination)

The oocytes were voltage-clamped at a holding potential of −70 mV using a GeneClamp-500 amplifier (Axon Instruments, Molecular Devices, Inc., Sunnyvale, CA, USA), and current response induced by application of 100 µM of acetylcholine chloride (ACh) was recorded digitally on an IBM\PC.

A typical experiment began with three to five control recordings of α7 nACh receptor ion currents induced by 100 µM of ACh with five minute intervals of
washing with ND96 solution. The average of three to four stable readings was calculated as the control value. Following the control recordings, the oocyte was perfused for a total of 15 minutes with the selected concentration of tested compound. The average of two to three responses at the end of the 15 min drug application were calculated to determine the effect of the compound. Subsequently, the application of drug was stopped and recovery recordings were taken for the 100 µM ACh again with 5 minutes intervals of ND96 perfusion.

The percent inhibition was calculated by dividing the average of currents induced in presence of the selected concentration of the tested compound by the average control values obtained before drug application. These values were normalized and displayed as percent of changes in current size (compared to controls). Monoterpenes such as carveol and carvacrol were used at varying concentrations to construct dose-response curves. The concentration of a compound which produced 50 % inhibition of ACh-induced currents (IC$_{50}$) was determined by nonlinear curve-fitting and regression fits (logistic equation) using computer software (Origin, OriginLab Corp., Northampton, MA, U.S.A.). Different concentrations of compounds were plotted on the X-axis and % inhibition of ion current of respective concentrations was plotted on the Y-axis. Concentrations of drugs close to their IC$_{50}$ values were employed for further studies. Also, 10 µM effect of monoterpenes such as L-carvone, eugenol, vanillin and (+)-pulegone was examined for comparison. With the exception of vanillin, all compounds were water insoluble. These compounds were first dissolved in 100% dimethyl sulfoxide (DMSO). DMSO, at the final concentration of 0.001 % (v/v) used in our studies, did
not induce a statistically significant effect on the maximal amplitudes of ACh-induced currents.

3.3.7.2 Experiments on the competitive and non-competitive nature of monoterpenene inhibition

Concentration-response curves for ACh were determined by using increasing ACh concentrations starting from 3 μM to 3 mM. The effects of carveol on different concentrations of ACh-induced currents were determined in the absence and presence of this compound. During these experiments, oocytes were voltage-clamped at a holding potential of −70 mV and three gradually increasing concentrations of ACh and maximal ACh (3 mM) were used on a single oocyte. For any set of concentrations of ACh, experiments were repeated in 5-6 different oocytes and percent inhibition was calculated as described earlier.

3.3.7.3 Experiments on the voltage-dependency of drug inhibition

Voltage-dependence of the compound's action was determined by holding the membrane potential at different values for 30 s. At each point, membrane potential was returned to -70 mV, and subsequent readings were taken every five minutes. During these experiments, ACh was used at a concentration of 100 μM. Current-voltage (I-V) relationships for ACh-induced currents were determined in the absence and presence of tested compound.
3.3.7.4 Experiments on the determination of Ca\textsuperscript{2+} contribution to observed drug action

In this series of experiments, 50 nL of BAPTA (1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) stock solution (100 mM) was injected into each oocyte (Sands, Costa & Patrick, 1993). Stock solution of BAPTA was prepared in distilled-water and the pH was adjusted to 7.4 by CsOH. Following BAPTA-injections, oocytes were kept in ND96 for 5 to 10 min, and placed in a ND96 solution containing Ba\textsuperscript{2+} instead of Ca\textsuperscript{2+} (prepared ND96 solution contained 1.8 mM BaCl\textsubscript{2} instead of 1.8 mM CaCl\textsubscript{2}). In order to determine the contribution of intracellular Ca\textsuperscript{2+} levels, the effect of the tested compound on the ACh-induced currents in BAPTA injected oocytes were investigated in either Ca\textsuperscript{2+} or Ba\textsuperscript{2+} containing ND96 solutions, and the extent of drug inhibition was compared.

3.3.7.5. Experiments for determination of the effect of exposure time on the compound's action

In this set of experiments, the compound's perfusion time was changed starting from zero exposure time (Ach is coapplied with the tested compound without prior incubation of that compound) to a maximum of 15 minutes of preincubation with the compound. Effect of increasing exposure time was analyzed by comparing the change in percent inhibition of the fixed concentration of the compound tested. These experiments were repeated on 5-6 different oocytes.
3.3.8. Data analysis

For the nonlinear curve-fitting, the computer software Origin (Origin Lab Corp., Northampton, MA, U.S.A.) was used. Average values were calculated as the mean ± standard error of the mean (SEM). 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,

\[ y = \frac{E_{\text{max}}}{1 + [x/EC_{50}]^n}, \]

Where x and y are drug concentration and response, respectively, \( E_{\text{max}} \) is the maximal response, \( EC_{50} \) is the half-maximal concentration, and ‘n’ is the slope factor (apparent Hill coefficient).
Chapter 4: Results

4.1 Functional expression of the human α7-nACh receptor in Xenopus oocytes

Application of 100 µM of acetylcholine (ACh) did not cause noticeable currents in uninjected oocytes (n=11) or in oocytes injected with distilled water (n=38). When oocytes injected with cRNA transcribed from cDNA encoding the α7 human nACh receptor were used, application of 100 µM ACh for 3 to 4 seconds caused fast inward currents that were rapidly desensitized (Figure 4.1 A, left panel). Co-application of 30 nM α-bungarotoxin, a potent nAChR antagonist obtained from snake venom, with 100 µM ACh caused almost complete inhibition of ACh-induced currents (n=14), indicating that the response was mediated by α-bungarotoxin-sensitive neuronal α7 nAChRs (4.1A, middle panel). After a 30-40 minutes washing period, incomplete or no recovery of the neurotoxin effect was observed (Figure 4.1A, right panel). Data from these experiments is presented in Figure 4.1B.
**Figure 4.1:** Functional expression of human α7 nACh receptors in *Xenopus* oocytes.

**A.** Records of currents induced by ACh (100 μM) in control conditions (left) during co-application of 30 nM α-bungarotoxin and ACh after 10 min preincubation with 30 nM α-bungarotoxin (middle), and 15 min following α-bungarotoxin washout (right).

**B.** Bar presentation of the means of the maximal amplitudes of ACh-induced currents in distilled water injected oocytes and human α7 nACh receptor cRNA injected oocytes are shown on the left-side of the Figure 1B. In oocytes injected with human α7 nACh receptor cRNA, the mean maximal amplitudes of ACh-induced currents in the presence and absence of α-bungarotoxin (30 nM) were presented on the right-side of the Figure 1B.
4.2 The effect of selected monoterpenoids on the function of human α7 nACh receptor

Initial experiments were done to screen the effects of various selected monoterpenes at 10 µM concentration, on the function of α7 nACh receptors. Eugenol, d-carvone and vanillin did not have a significant effect on α7 nACh function. Carvacrol, carveol, thymol and pulegone caused variable inhibitory effects of ACh (100 µM)-evoked currents. Carvacrol, thymol and carveol caused significant effects while pulegone caused weak inhibition (~20%). Carveol showed maximum inhibition (57%) of 100 µM ACh-induced current through α7 nAChR-expressing oocytes and therefore it was selected for further investigations. Summary of the effects of 15 minutes bath applications of monoterpenes on the ACh-induced ion currents are shown in Figure 4.2.

Traces of currents showing the inhibitory effect of carveol are presented in Figure 4.3. In these experiments, in the absence of the tested monoterpene, maximal amplitudes of currents elicited by the application of 100 µM ACh every 5 min remained unchanged in experiments lasting 50-60 min (control record in left panel of Figure 4.3 and open circles in Figure 4.4). However, bath application of 10 µM carveol caused a gradually developing strong inhibition of α7 nACh receptors (Figure 4.3, middle panel). Time-course of the effect of 10 µM carveol application for 15 min on the amplitudes of ACh-induced currents is presented in Figure 4.4. The inhibition by carveol was almost reversible within 10 to 15 min of washout period (Figure 4.3, right panel). In the presence of carveol, there was a statistically significant difference in the amplitudes ACh-induced currents (n=7, ANOVA, P < 0.05).
It was noteworthy that the inhibitory effect of carveol was dependent on the application or exposure period. As depicted in Figure 4.5, without preincubation, the coapplication of carveol (10 μM) and ACh (100 μM) caused minimal inhibition (0 time point in the Figure 4.5). However, incubation with carveol caused a significant inhibition which reached a maximal level with a half-time ($\tau_{1/2}$) of 3.4 min ($n=4$; Figure 4.5). As the magnitude of carveol effect was time-dependent, 10 to 15 min carveol application was routinely used in the rest of the experiments to ensure that equilibrium conditions for the monoterpene action were achieved.

Carveol's inhibitory effect of $\alpha_7$ nAChRs was concentration-dependent with IC$_{50}$ of 8.3± 0.9 μM (Figure 4.6). Based on these findings, 10 μM of carveol was employed routinely in the rest of the experiments.
Figure 4.2: Effects of selected monoterpenes on α7 nACh receptor-mediated ion currents. Bar-graph displaying the means of % of effect of 10μM of tested monoterpene on 100μM Ach elicited α7 nACh currents. Data represents the mean ± S.E.M of indicated number of oocytes.

Figure 4.3: Current traces of carveol’s effect on α7 nAChR currents. Records of currents activated by ACh (100 μM) in control conditions (left) during co-application of 10 μM carveol and ACh after 10 min pre-treatment with 10 μM carveol (middle), and 15 min following carveol washout (right).
Figure 4.4: Time-course of carveol inhibition of α7 nACh receptor-mediated ion currents. Inhibition of α7-nACh receptor is reversible after wash out of carveol. Data points represent the mean ± S.E.M of 4 to 6 oocytes.
Figure 4.5: Time-dependence of carveol inhibition of α7 nACh receptor-mediated ion currents. Inhibition of α7 nACh receptor increases with the prolongation of carveol application time. The half maximal inhibition was reached within a half-time ($\tau_{1/2}$) of 3.4 min. Data points represent the mean ± S.E.M of 6 oocytes.
Figure 4.6: Concentration-dependent inhibitory effects of carveol on α7 nACh receptor currents. Carveol inhibits the function of α7 nACh receptor in a concentration-dependent manner. Data points represent the mean ± S.E.M of 6 to 8 oocytes. The curve is the best fit of the data to the logistic equation described in the methods. The IC$_{50}$ is 8.3 ± 0.9 µM.

4.3 The role of intracellular calcium levels in carveol-inhibition of α7 nACh receptor

Some monoterprenoids were reported to alter intracellular Ca$^{2+}$ homeostasis in different cell types (Chang et al., 2011; de Cerqueira et al., 2011). In the oocyte expression system, the increased level of intracellular Ca$^{2+}$ can be detected by Ca$^{2+}$-activated Cl$^{-}$ channels and concomitant alterations in the holding currents. In control experiments, carveol alone (10 µM for 15 min) did not alter the magnitude of holding current in oocytes voltage-clamped at −70 mV (n=12), indicating that intracellular Ca$^{2+}$ levels were not altered by carveol.
As previously mentioned, high Ca\(^{2+}\) permeability is an important physiological characteristic of α7 nAChR (Albuquerque, 2009; Vernino et al., 1992). Ca\(^{2+}\)-activated Cl\(^{-}\) channels have been shown to be expressed endogenously in *Xenopus* oocytes (Miledi, 1982). Activation of α7 nAChRs allows influx of sufficient amounts of Ca\(^{2+}\) to activate endogenously expressed Ca\(^{2+}\)-dependent Cl\(^{-}\) channels in *Xenopus* oocytes (Sands et al., 1993; Seguela et al., 1993). Therefore, it was necessary to investigate whether the effect of carveol was mediated by its direct actions on α7-nAChRs or by the subsequent activation of Cl\(^{-}\) currents induced by Ca\(^{2+}\) entry through nACh receptors. In this set of experiments, extracellular Ca\(^{2+}\) was replaced with Ba\(^{2+}\) since Ba\(^{2+}\) can pass through α7-nAChRs but causes little, if any, activation of Ca\(^{2+}\)-dependent Cl\(^{-}\) channels (Barish, 1983; Sands et al., 1993).

In the presence of Ba\(^{2+}\), some activity of Ca\(^{2+}\)-dependent Cl\(^{-}\) channels has been reported. The contribution of the remaining Ca\(^{2+}\)-dependent Cl\(^{-}\) channel activity has been shown to be abolished by injection of the Ca\(^{2+}\) chelator BAPTA (Sands et al., 1993). For this reason, we recorded ACh-induced currents in presence and absence of 10µM carveol using BAPTA-injected oocytes bathed in a Ba\(^{2+}\) containing solution. Carveol produced the same level of inhibition (54 ± 6 % in controls versus 48 ± 7 % in BAPTA-injected oocytes n=5-8; ANOVA, P > 0.05) of ACh-induced currents when BAPTA-injected oocytes were recorded in Ca\(^{2+}\) free, 2 mM Ba\(^{2+}\) containing solutions (Figure 4.7).
4.4 The voltage-dependence of carveol action on the α7 nACh receptor

Voltage-dependence of carveol-inhibition was examined on α7 nACh receptors. Each tested membrane potential was held for 30 s and then returned to -70 mV. As shown in Figure 4.8A, the inhibition of 100 μM ACh-induced currents by carveol (10 μM) does not appear to be voltage-dependent. At all tested membrane potentials from -100 to -20 mV, the extent of carveol inhibition were similar. Evaluation of data from current-voltage relationship displayed that there is no significant difference in the extent of the inhibitory effect of carveol of α7 nAChR currents at different holding potentials (Figure 4.8B; P>0.05, n=6, ANOVA).
Figure 4.8: Carveol inhibition of ACh-induced currents is independent of membrane potential.

A. Current-voltage relationships of ACh-activated currents in the absence and presence of carveol (10 μM). Normalized currents activated by 100 μM ACh before (Control, •) and after 10 μM carveol treatment. B. Quantitative evaluation of the effects of carveol is presented as percent inhibition at different voltages indicated in panel B. Data points represent the mean ± SEM of 7 to 8 experiments.
4.5 Studies on the competitive or non-competitive nature of carveol's inhibitory effect of α7 nACh receptor

Carveol may decrease the binding of the agonist to the receptor by acting as either a competitive or non-competitive antagonist. For this reason, the effect of carveol was examined at different concentrations of ACh. Concentration-response curves for ACh in the absence and presence of 10 µM carveol are presented in Figure 4.9. Carveol caused a right-ward shift of the concentration-response curve, without causing a decrease on the the maximal ACh-induced current. In the absence and presence of carveol, the EC\textsubscript{50} values were 94 ± 8 µM and 248 ± 14 µM (n=7), and slope values were 1.6 ± 0.2 and 1.7 ± 0.3 (n=7), respectively, suggesting that carveol inhibits the ACh responses in a competitive manner.
Figure 4.9: Concentration-response curves for acetylcholine-induced currents in the presence and absence of 10µM carveol. Oocytes were voltage-clamped at -70 mV and currents were activated by applying acetylcholine (100 µM). Oocytes were exposed to 10 µM carveol for 15 min and ACh was reapplied. Paired concentration-response curves were constructed and responses normalized to maximal response under control conditions. EC$_{50}$ and slope values were determined by fitting the curves from 5 oocytes to the standard logistic equation as described in the methods section. Data points obtained before (Control) and after 15 min treatment with carveol (10 µM) were indicated by filled and open circles. Data points represent the normalized mean ± SEM of 6 to 8 experiments.
Chapter 5: Discussion

In this study, we provide electrophysiological evidence indicating that some monoterpenes inhibit the function of human α7 nACh receptor expressed in Xenopus oocytes. There were significant differences in the effects and the extent of activity among the monoterpenes tested in this study. At the same selected concentration, 10 µM, vanillin showed weak activation (<10% of control readings), d-carvone almost had no effect while all other monoterpenes demonstrated variable extent of inhibition. In the order of potency of inhibitory effect of the amplitude ACh-induced currents, carveol was most potent, then thymol, carvacrol, pulegone and least is eugenol (see Figure 4.2). Further experiments focused on carveol, being most potent among all tested monoterpenes. Our results demonstrated that preincubation time was a significant factor determining the extent of carveol inhibition. The time course required for carveol to show its effect on α7 nACh receptors was relatively slow (τ = 3.4 min). This might indicate possible interaction of carveol with the lipid membrane. As stated earlier, monoterpenes are lipophilic in nature and several in vitro studies on either artificial membrane systems, selected bacterial cells or mammalian cell lines demonstrated alteration of cell membrane properties to variable extents (Cristani et al, 2007; de Carvalho & da Fonseca, 2007; Di Pasqua et al, 2006; Reiner et al, 2013a; Reiner et al, 2009).

Ion channel blockers can have different mechanisms of actions. Depending on the type of ion channel and the blocker, the channel may be stabilized in the closed state leading to decreased rate and/or duration of the opened state. Many blockers inhibit channel activity by physically obstructing the channel pore near the
selectivity filter. This is known as open-channel blockade (Hille, 2001).

Open-channel blockade is widely accepted as a model to describe the block of ligand-gated ion channels (Hille, 2001; Hille & Dennis, 2010). However, this model cannot account for the results of the present study. Firstly, for an open channel blocker to act, the co-existence of the agonist and blocker is required to allow the blocker to enter the channel pore as a result of agonist-induced conformational change and channel opening. However, there was no inhibition of nACh receptors during coapplication of ACh and carveol. Increased preincubation period enhanced carveol-induced inhibition of channel activity (Figure 4.4), indicating that carveol can interact with the closed state of the α7 nACh receptor. Secondly, inhibition by carveol is not voltage sensitive (Figure 4.8), suggesting that the carveol-binding site is not influenced by the transmembrane electric field.

In electrophysiological studies, the EC$_{50}$ value of ACh, a natural ligand (agonist) for this receptor, increased significantly from 94 µM in controls to 248 µM in the presence of carveol. Efficacy of ACh remained unaltered in the presence of carveol. However, the potency was decreased significantly, indicating that carveol competes with the ACh binding site on the receptor.

Earlier studies in mammalian cells showed that monoterpenes such as thymol (Szentandrassy et al, 2004; Szentesi et al, 2004) and eugenol (Lofran-Alves et al, 2005) causes alteration of intracellular Ca$^{2+}$ handling and Ca$^{2+}$ mediated signal transduction pathways. As α7 nACh receptors are known to have high Ca$^{2+}$ permeability, activation of those receptors in Xenopus oocytes can permit sufficient Ca$^{2+}$ entry to possibly activate endogenous Ca$^{2+}$ dependent Cl$^{-}$ channels (Sands et al, 1993; Seguela et al, 1993). Carveol was still able to inhibit α7 nACh receptor-
mediated ion currents in oocytes injected with BAPTA and superfused with Ba\textsuperscript{2+} containing solution, suggesting that Ca\textsuperscript{2+} dependent Cl\textsuperscript{−} channels were not involved in carveol-induced inhibition of nicotinic responses (Figure 4.7). In addition, as the Ca\textsuperscript{2+} activated Cl\textsuperscript{−} channels are highly sensitive to intracellular levels of Ca\textsuperscript{2+} (for a review see Fraser & Djamgoz, 1992; Machaca et al., 2002; Weber, 1999), the alterations in intracellular Ca\textsuperscript{2+} levels would be reflected by changes in the holding current under voltage-clamp conditions. However, during our experiments, application of carveol, even at the highest concentrations (300 µM in n = 6), did not influence the baseline or holding currents, suggesting that carveol did not affect intracellular Ca\textsuperscript{2+} levels.

It appears therefore that the actions of carveol reported in our study, are not mediated by changes in intracellular Ca\textsuperscript{2+} levels, and independent of the entrance of extracellular Ca\textsuperscript{2+} through α7 nACh receptors.

5.1 Monoterpenes concentrations used in this study

Monoterpenes, in the concentration range used in the study have been shown to modulate the functions of several other receptors and ion channels. For example, menthol causes concentration-dependent inhibition of the α7 nACh receptors expressed in *Xenopus* oocytes with an IC\textsubscript{50} of 32.6 µM (Ashoor et al., 2013a). An earlier study showed that camphor inhibited nACh receptor function with an IC\textsubscript{50} of 70 µM in bovine adrenal chromaffin cells (Park et al., 2001). Similarly, borneol also showed an inhibitory effect on nACh receptors functions with a smaller IC\textsubscript{50} of 56 µM (Park et al., 2003). Also screening the effects of monoterpenes, thymol and carvacrol, on the functions of 5-HT type 3 receptors expressed in *Xenopus* oocytes, 10 µM concentration, when coapplied with small concentration of 5-HT (EC\textsubscript{25}),
caused significant potentiation of the response (Lansdell et al, 2015). Moreover, previous reports showed that coapplication of 50 µM menthol with GABA caused 2 fold potentiation of the response to GABAA receptors which are also expressed in *Xenopus* oocytes (Hall et al, 2004). Thymol showed similar effects on GABAA receptors concentration-dependently over the range 1-100 µM (Priestley et al, 2003). These results suggest that the concentrations of monoterpenes (Carveol IC50= 8.3 µM) are in a similar range to earlier reports.

5.2 Functional importance of monoterpenes actions on human α7 nACh receptor

It has been shown that a number of monoterpenes improve the cognitive performance in patients with cognitive disorders such as Alzheimer disease (Dalai et al, 2014; Khan et al, 2014; Perry et al, 2003; Perry et al, 2001; Videira et al, 2014). Most of these studies demonstrated the value of tested monoterpenes in Alzheimer disease and improvement of cognitive impairment through either anticholinesterase activity or inhibition of β-amyloid accumulation which is one of the key steps in the progression of the disease. Modulation of specific nicotinic receptors is another possible mechanism that might be of value. Since neuronal α4β2 and α7 nACh receptors have been strongly associated with cognitive function (Haydar & Dunlop, 2010; Levin, 2002; Okada et al, 2013; Picciotto et al, 2000) further investigations for the interaction of monoterpenes with these nACh receptor subtypes may be useful for drug development. It was evident from several earlier studies that agonistic effects on nicotinic receptors are associated with beneficial effects of various drugs. Interestingly, the monoterpenes tested in our study showed antagonistic actions on α7 nACh receptors.
Agonistic actions of drugs on nicotinic receptors can be mediated by activation of nACh receptors or indirectly by increasing the release of neurotransmitter (for review, see Dorostkar & Boehm, 2008). However, it has been shown in several earlier studies that both nicotine and nACh receptor antagonists have almost similar effects (Anderson & Brunzell, 2012; Picciotto et al, 2008). For example, high doses of nAChR antagonists such as α-bungarotoxin, d-tubocurarine and mecamylamine have been shown to cause excitatory responses (promoted population spikes) in rat CA1 hippocampal slices that were similar to those produced by nicotine (Ropert & Krnjevic, 1982). Another study using mouse hippocampal slices demonstrated an increase in amplitude of population spikes caused by bath-application of nicotine and the antagonists such as d-tubocurarine and α-bungarotoxin acted in a very similar manner (Freund et al, 1990). In another in vitro study, both nicotine and methyllycaconitine (selective α7 nACh receptor antagonist) reversed the muscimol induced block of long-term potentiation (LTP) after their application to CA1 region of the hippocampus (Fujii et al, 2000a; Fujii et al, 2000b).

Furthermore, application of nicotine as well as high concentration of the nicotinic antagonist mecamylamine increased serotonin release in rat dorsal hippocampal slices despite smaller concentration of the antagonist blocked nicotine-induced effects when both were co-applied (Kenny et al, 2000; Mihailescu et al, 1998). Another study has shown that mecamylamine and the high-affinity antagonist, dihydro-β-erythroidin, exerted effects similar to nicotine on dopamine release under conditions of phasic and tonic activity (Rice & Cragg, 2004).

It was reported that mecamylamine, in low doses, promotes the performance
in memory-related tasks to an extent comparable to nicotine. For instance, chronic administration of 3 mg/kg doses of mecamylamine was found to reverse the nicotine-induced improvement in spatial working memory in the radial-arm-maze test (Levin et al., 1993). Nevertheless, when mecamylamine was given alone to tested rats, it caused unexpected significant improvement in radial-arm-maze working memory performance during the first week of infusion (Levin et al., 1993). Using also radial-arm maze with repeated acquisition procedure, low dose range of mecamylamine showed significant improvement in learning relative to saline (Levin & Caldwell, 2006). It was also shown that chronic administration of mecamylamine had complex effects using T-maze alternation test. During the early phase of administration, there was a paradoxical improvement in working memory performance, but during the latter period of administration there was a deficit (Levin et al., 1997). Moran saw a similar paradoxical improvement in T-maze alternation with mecamylamine when given as an acute injection of 10 mg/kg (Moran, 1993). Corroborating evidence regarding the dose-specific effects of mecamylamine on memory was also gained using non-human primates as administration of mecamylamine caused improved performance of delayed matching-to-sample accuracy in monkeys (Terry et al., 1999). Similar improvements in cognition were also evident following low doses of methyllycaconitine, a selective α7 nAChR antagonist, in a rodent model of attention (Hahn et al., 2011). In the absence of nicotine, low doses of methyllycaconitine (0.4 and 1.3 mg/kg) improved response accuracy although concurrent administration with nicotine caused reversal of nicotine-induced attention-enhancing effects. In the same study, dihydro-β-erythroidine, a competitive antagonist at α4β2, α4β4, α3β2 and α2β2 nAChRs, almost showed almost no effect compared to control, indicating that
the α7 nAChR subtype may have a fundamental role in cognitive function (Hahn et al., 2011). The role of nACh receptor desensitization in mediating the effects of nicotine and nicotinic antagonist has been reviewed recently (Buccafusco et al., 2009; Picciotto et al., 2008). It has been concluded that an overall decrease in nicotinic stimulation could be clinically valuable for cognitive enhancement. In a recent study, group of researchers (Dobryakova et al., 2015) studied the effects of different nicotinic receptor antagonists on passive avoidance learning in rodents and demonstrated that mecamylamine, when given after a training phase, had positive effects on memory consolidation. In a pilot study (Potter et al., 2009) where human participants i.e. fifteen non-smoking young adults diagnosed with attention deficit hyperactivity disorder (ADHD), received, in a double-blind fashion, an acute, single dose of mecamylamine (0.2, 0.5 or 1 mg) orally and placebo. Mecamylamine showed beneficial effects on recognition memory with the 0.5 mg dose. Similar finding on recognition memory was demonstrated when acute nicotine dose was given for young adolescents with ADHD (Potter & Newhouse, 2004; Potter & Newhouse, 2008).

The similarities in effects of nicotine and nAChR antagonists were also seen at cellular level and on receptor regulation. Continuous treatment with mecamylamine in vivo or in vitro experiments was reported to cause brain region specific and/or subtype-specific upregulation of nACh receptors in a way similar to the effect of nicotine (Abdulla et al., 1996; Collins et al., 1994; Pauly et al., 1996; Peng et al., 1994). Some studies have demonstrated modulation of downstream signaling events as a result of nACh receptor antagonism. In vitro exposure of PC12 cells to nicotine (0.01-10 µM) for 24 hours produced an increase in the level of
expression of tropomyosin receptor kinase A (TrkA) receptors, high affinity nerve growth factor receptors, that was reversed upon concurrent exposure to mecamylamine (5 μM) (Jonnala et al., 2002). Interestingly, exposure to low concentration of mecamylamine (10-100 nM) in the absence of nicotine also induced a significant increase in expression of TrkA receptors with reference to control. Consistent with the in vitro findings, in vivo administration of mecamylamine (24 mg/kg/day) caused a moderate increase (20%) in expression of TrkA receptors (Jonnala et al., 2002). This suggests a possible neuroprotective effect of nACh receptor antagonist that is important for delaying or preventing neural degeneration associated with cognitive deficits. Collectively, these effects may contribute to the procognitive effects of nACh receptor antagonism.

As described earlier, α7 nACh receptors are characterized by fast desensitization, and this makes it difficult to discriminate whether the effects of α7 nACh receptor agonists are due to direct activation or subsequent activation-induced receptor desensitization (Banerjee et al., 2005). Binding of agonists stabilizes the desensitized state of the receptor. This state display high affinity for ACh and other ligands binding, nevertheless, receptor becomes unresponsive to subsequent stimulus. As a result, chronic exposure to nicotine was demonstrated in animal studies and in smoking human subjects to induce strong compensatory receptor upregulation (Benwell et al., 1988; Parker et al., 2014; Perry et al., 1999). In this context, it has been suggested that many of the nicotinic effects can be explained as a result of receptor desensitization rather than activation (Picciotto et al., 2008). It is still unclear whether agonists of nACh receptors are truly functioning in vivo as antagonists due to desensitization process.
Chapter 6: Conclusion

Monoterpenes display variable biological activities like analgesic, anti-inflammatory and anticancer effects. This is, to our knowledge, the first study reporting effects of several monoterpenes on the function of the human $\alpha_7$-nACh receptor, a ligand-gated ion channel. The results obtained in this study suggest an inhibitory effect of monoterpenes on the function of $\alpha_7$ nACh receptors. It appears that the extent of inhibitory action varies significantly among the different monoterpenes.

6.1 Limitations and future directions

In our study, we did not examine monoterpenes effects on mammalian cell lines and native neurons. It is possible that post-translational modification and interacting proteins in *Xenopus* oocytes are different from mammalian cells. In future studies, we intend to study the effect of monoterpenes on the other nicotinic ACh receptor subtypes to determine the specificity of monoterpenes action on these receptors. We are also interested in conducting experiments to further study the structure activity relationship of these phytochemicals on nicotinic receptors expressed in mammalian cell lines.
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