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DIPLOMA THESIS

Biological Activity of Plant Metabolites 7. Alkaloids from *Corydalis yanhusuo* W.T. Wang and Their Inhibitory Activity on Acetylcholinesterase

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Declaration

I declare that this diploma thesis is my original copyrighted work that I have developed separately. All literature and other sources from which I retrieved information are properly cited in the bibliography. This work has not been used to gain another or same title.

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1. INTRODUCTION

For thousands of years plants have been used for healing by humans. Fossil records date human use of plants as medicines at least to the Middle Paleolithic age, 60,000 years ago [1]. Many traditional medical systems using plants as a means of healing have been developed since. According to the World Health Organization (WHO), 80% of the populations of Asia and Africa still depend on traditional medicine for primary health care, while in many developed countries, 70% to 80% of the population has used some form of complementary and alternative medicine [2]. Despite the current preoccupation with synthetic chemistry as a vehicle to discover and manufacture drugs, even at the beginning of the twenty-first century, out of the 252 drugs considered as basic and essential by WHO, 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors [3].

One of the world's oldest documented medical systems based on herbal medicines is Traditional Chinese Medicine (TCM). Concepts of TCM, which are difficult to translate into Western medical terms, are Yin, Yang and Qi. These are components of 'vital energy', which is a term for collectively describing both the mental and physical energy. It is believed that an imbalance of Yin and Yang breaks body's harmony predisposing to diseases and restoring this harmony is the basic approach of treating illnesses. In Chinese tradition, ageing is considered a progressive decline of vital energy in the body, and anti-ageing herbs are able to correct the imbalance of vital energy components [4].

As the elderly population is growing, age-related diseases are becoming increasingly common and the need for a relief to the burden of society is crucial. The most frequent cause of senile dementia is known to be Alzheimer's disease (AD), for which at the moment there is no cure. The primary goals of treatment are to maximize the patient's ability to function in daily life, maintain quality of life, slow the progression of symptoms, and treat depression or disruptive behavior [5].

Although AD, as a defined medical condition, has only existed for about 100 years, age-related loss of memory and cognitive decline has been documented for thousands of years in human history. In common with many other conditions, ancient writings which describe the symptoms also suggest remedies, based usually on plant

extracts. One of the herbs from TCM that is thought to increase vital energy is *Corydalis yanhusuo*. The purpose of this diploma thesis is to evaluate the inhibitory effect of alkaloids from *Corydalis yanhusuo* on two of the main enzymes involved in the pathophysiology of AD, namely acetylcholinesterase (AChE) and butyrylcholineesterase (BuChE). This evaluation is based on the fact that the main currently approved therapy of AD is cholinesterase (ChE) inhibition.

2. AIM OF THE WORK

1) Isolation of a minimum of one alkaloid in pure form from chromatographic fraction. The fraction is prepared from primary extract of the tubers from *Corydalis yanhusuo*. Column chromatography and thin-layer chromatography will be used for the isolation of compounds.

2) Exploring the physico-chemical properties of isolated compound(s) (optical rotation and Rf values in two chromatographic systems – TLC). Determination of MS and NMR spectra.

3) Determination of antioxidative activity (DPPH test, ABTSD test) and influence on human cholinesterases (AChE, BuChE).

4) Calculation of IC_{50}, EC_{50} (statistical program GraphPad Prism 5.02 software).

3. THEORETICAL PART

3.1 Alzheimer's disease

3.1.1 Epidemiology of Alzheimer's disease

AD is a neurodegenerative disease of the brain that causes changes in brain function. An estimated 5-10% of the population aged 65 years and over and 40% of the population greater than 85 years of age are likely to be affected by AD. It accounts for 50%-75% of the total of 18 million people living with dementia in the world today. This number is expected to increase to the value of 34 million people by 2025 [5].

3.1.2 Definition and pathogenesis of Alzheimer's disease

In 1906, during the 37th Conference of German psychiatrists in Tübingen, the neuropsychiatrist Alois Alzheimer described for the first time the symptoms of "a particular disease of the cerebral cortex", characterized by a gradual and irreversible degeneration of the intellectual functions such as memory, orientation, judgement, language and the capacity to acquire new knowledge [6]. Since then, considerable effort has been made towards elucidating the causes and mechanism of the disease, as well as searching for therapeutic possibilities. At present, two main hypotheses have been developed: the cholinergic hypothesis and the amyloid cascade hypothesis. Both hypotheses have received abundant verification and increasing number of data demonstrates the connections between them. In addition, theories of oxidative stress and neurotoxicity attempt to add to the knowledge that would explain the underlying causes of the disease.

3.1.2.1 Cholinergic hypothesis

Brain shrinkage and localized loss of neurons, mainly in the hippocampus and basal forebrain, which are the main source of cholinergic innervation, explain the cognitive deficit and loss of short-term memory that occur in AD. Measurements on post-mortem AD brain tissue have shown that changes are present in many transmitter systems.

However, a relatively selective loss of cholinergic neurons in the basal forebrain nuclei is characteristic. Choline acetyl transferase (ChAT), acetylcholine (ACh) content, AChE and choline transport in the cortex and hippocampus are all reduced considerably [7]. Reductions in the activities of ChAT and AChE in brain tissues from AD patients were first reported in 1976 and 1977 [8, 9]. Those observations, in combination with the emerging role of ACh in memory [10] led to the cholinergic hypothesis of the disease.

The 'cholinergic hypothesis' was first proposed by Davies and Maloney in 1976 [8]. At the time of this discovery, the primary enzyme involved in the regulation of ACh was thought to be AChE, with BuChE considered a 'pseudocholinesterase' due to its apparently minor effect on ACh regulation. However, recent studies have found that BuChE can also play a significant role in the regulation of ACh in the human brain [11]. In addition, a growing body of evidence suggests that both enzymes may have roles beyond 'classical' co-regulatory esterase functions in terminating ACh-mediated neurotransmission. 'Non-classical' roles in modulating the activity of other proteins, regional cerebral blood flow, tau phosphorylation, and the amyloid cascade may affect the pathogenesis of AD and therefore its progression [12].

AChE and BuChE are encoded by different genes on chromosome 7 and chromosome 3, respectively. At the molecular level, they share 65% amino-acid sequence. Structural features of the two enzymes confer differences in their substrate specificity. AChE is highly selective for ACh hydrolysis, while BuChE is less substrate specific, accommodating the metabolism of several different molecules including various neuroactive peptides [13].

The two ChEs also differ in enzyme kinetics. It has been proposed that the efficiency with which AChE and BuChE hydrolyse ACh is dependent on the substrate concentration. AChE has greater catalytic activity at low ACh concentrations, resulting in substrate inhibition at higher doses [13]. However, BuChE is more efficient at high substrate concentrations [12].

Both AChE and BuChE exist in several globular and asymmetrical forms. In the human brain, AChE is present in G1 and G4 forms, the proportions of which vary in different brain regions [14]. BuChE also exists in G1, G2, and G4 molecular forms but,

similar to AChE, G4 is the predominant isoform in the mature brain [15]. In the brains of patients with AD, the level of the membrane-bound G4 form of AChE is selectively reduced by 90% or more in certain regions, probably due to the loss of presynaptic terminals. However, levels of G1 AChE remain largely unchanged [16]. The G1 form of BuChE shows a 30–60% increase in the AD brain, probably due to an increase in the number of BuChE-positive glia, while the G4 form decreases or remains the same as in the normal brain [12].

The majority of cholinesterase in the human brain is AChE. However, it is now known that BuChE has more widespread distribution than previously thought. BuChE is mainly found in glial cells and in endothelial cells, whereas AChE is in neurons and axons [12, 17]. AChE-positive neurons project diffusely to the cortex, modulating cortical processing and responses to new and relevant stimuli. BuChE-positive neurons project specifically to the frontal cortex, and may have roles in attention, executive function, emotional memory and behavior. Furthermore, BuChE activity progressively increases as the severity of dementia advances, while AChE activity declines. Therefore, inhibition of BuChE may provide additional benefits [12].

The ‘cholinergic hypothesis’ was the basis for the development of presynaptic, synaptic and postsynaptic treatment approaches designed to maintain and facilitate the activity of the surviving cholinergic system. Synaptic ChE inhibition has proved preferable to direct receptor agonist therapy, as ChE inhibitors amplify the natural spatial and temporal pattern of ACh release, rather than tonically or globally stimulating either nicotinic or muscarinic ACh receptors [12].

3.1.2.2 Amyloid cascade hypothesis

Along with descriptions of progressive loss of memory and general cognitive decline, Alzheimer noted the presence of intraneuronal tangles and extracellular "amyloid" plaques in the disease-damaged brain, but he could not decipher whether the tangles or plaques were causative or merely markers of the disease. The discovery of a pathogenic mutation in the β-amyloid precursor protein (APP) gene on chromosome 21 suggested that APP mismetabolism and β-amyloid deposition are the primary events in the disease

process. The occurrence of AD in Down syndrome, where chromosome 21 is duplicated added further value to this suggestion and led to the proposal of the "amyloid cascade hypothesis" of Alzheimer's disease [18].

In addition to the loss of cholinergic neurons, two features are characteristic of Alzheimer disease on the microscopical level: extracellular amyloid plaques, consisting of amorphous extracellular deposits of β-amyloid protein (known as Aβ), and intraneuronal neurofibrillary tangles, comprising filaments of a phosphorylated form of a microtubule-associated protein (Tau). Both of these deposits are protein aggregates that result from the misfolding of native proteins. They are also present in normal brains, although in smaller numbers [7]. Consequently it is believed that targeting the underlying mechanisms leading to plaques and tangles will ultimately generate novel therapeutics with disease-modifying properties.

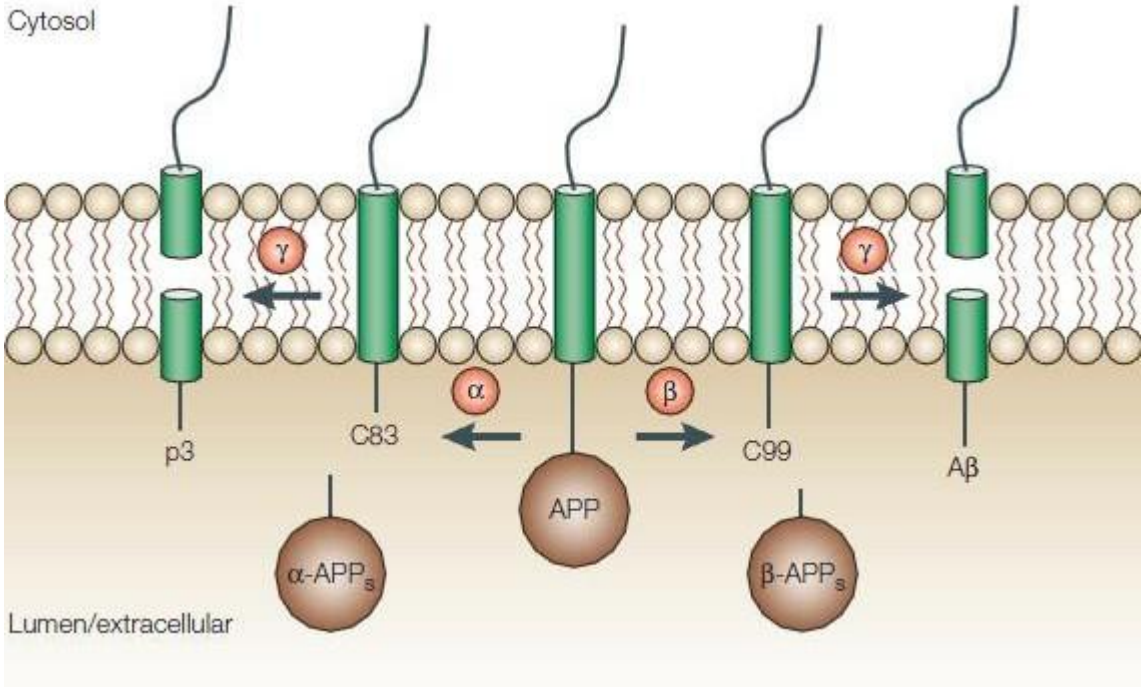

Fig. 1 APP processing [19]

The APP is a singlepass, integral transmembrane protein that is cleaved by several proteases called secretases [20]. APP is first cleaved by either α- or β-secretase (Fig. 1). α-Secretase cuts close to the transmembrane domain, in the middle of the Aβ region of

APP, to release a large ectodomain (α-APPs), leaving a carboxy-terminal fragment of 83 amino acids (C83) in the membrane. By contrast, β-secretase cleaves further from the membrane, producing a β-APPs ectodomain and a 99-residue carboxy-terminal fragment (C99) that contains the amino terminus of Aβ. Both C99 and C83 are substrates for a third protease, γ-secretase, which catalyses an unusual hydrolysis within the transmembrane region. Proteolysis of C99 by γ-secretase produces the 4-kDa Aβ, whereas C83 is cleaved to form p3, an amino-terminally truncated form of Aβ. Cleavage by γ-secretase produces primarily a 40-aminoacid Aβ-peptide (Aβ40). However, other shorter and longer forms of Aβ are also produced — in particular, a 42-amino-acid version (Aβ42) that has a high propensity to aggregate and is the principal Aβ species found in amyloid plaques [19].

3.1.2.3 Interactions between the amyloid and cholinergic hypotheses

In vivo and *in vitro* studies have consistently demonstrated a link between amyloid toxicity and the cholinergic hypofunction. Scientific efforts to restore the cholinergic function, by the administration of nicotinic or M1 muscarinic agonists or AChE inhibitors, have led to the observation that the effects of these drugs are not limited to the cholinergic system, but are capable of modulating the APP processing via different mechanisms. Therefore, a major challenge is to identify compounds that provide the optimal effect in respect of both the amyloid and the cholinergic hypothesis of AD [21].

3.1.2.4 Oxidative stress

Based on laboratory and clinical studies, it appears that reactive oxygen species (ROS) and reactive nitrogen species (RNS) that are generated extracellularly and intracellularly by various mechanisms are among the major intermediary risk factors that initiate and promote neurodegeneration in idiopathic AD [22]. The central nervous system is especially at risk from oxidative stress as a result of the brains high oxygen consumption, abundant lipid content, and relative lack of antioxidant compounds compared with other tissues [23].

3.2 Basic approaches of therapy in Alzheimer's disease

3.2.1 Inhibition of brain cholinesterases

The inhibition of AChE and BuChE increases and maintains the synaptic concentration of ACh and allows greater interaction with receptors. ACh is synthesized from choline and acetyl-coenzyme A (reaction catalyzed by ChAT), and then packaged in synaptic vesicles. Action potentials arriving at the presynaptic nerve terminal trigger the release of ACh into the synaptic cleft, where it interacts with muscarinic and nicotinic receptors located on the pre- and post-synaptic membrane. Muscarinic M2 receptors on the presynaptic membrane regulate ACh release via a negative feedback response. At the post-synaptic site, muscarinic M1 receptors transduce signals through pathways in the post-synaptic neuron. ACh is hydrolyzed in the synaptic cleft by membrane-bound tetrameric G4 AChE, or by soluble monomeric G1 AChE. A high affinity choline-uptake mechanism returns choline in the pre-synaptic neuron [12]. BuChE is also present in synapses as well as in the neuromuscular junction, and its role was proven to be more than a 'back-up' process. Thus, both enzymes, AChE and BuChE simultaneously participate in maintaining the functional integrity of cholinergic pathways [17].

At the intermolecular level of structure-activity relationships of AChE inhibitors, the important regions of an inhibitor appear to be a positively charged nitrogen, which binds to an aspartate residue, and a region, separated by a lipophilic area from the positive charge, which can form a hydrogen bond with a tyrosine or serine residue. A positively charged nitrogen is common in many alkaloids at body pH and it is not surprising that many of the most powerful AChE inhibitors are alkaloids. In the recent years, however, a number of non-nitrogenous natural compounds have been found to be AChE inhibitors although their interactions with AChE have generally not been investigated [24].

Four ChE inhibitors, tacrine, donepezil, rivastigmine, and galanthamine, are approved by the US Food and Drug Administration. In Europe, availability differs from country to country. Only rivastigmine has been approved centrally by the European Agency for the Evaluation of Medical Products (EMEA) [25]. Although donepezil, rivastigmine, and

galanthamine are part of the same therapeutic class, they differ in their pharmacology and pharmacokinetics. Donepezil is a piperidine derivative, which non-competitively and reversibly inhibits AChE. It is highly selective for AChE, and binds to plasma proteins (96%). It has a long half-life of elimination (70 h) and is given once daily. Rivastigmine is a slowly reversible (pseudoirreversible) inhibitor of both AChE and BuChE, has low plasma-protein binding (40%), and its short elimination half-life necessitates two doses daily. Galanthamine is a selective reversible AChE inhibitor and a positive allosteric modulator of ncicotinic receptors on pyramidal neurons that is poorly bound to plasma protein (18%) and with elimination of about 5 h, two doses are required daily [25].

Several randomized, double-blind, placebo-controlled studies reported positive effects of the ChE inhibitors on cognitive and functional symptoms, as well as on behavioral abnormalities in Alzheimer's dementia [26-29]. Clinical benefits from ChE inhibitors were also reported in two other meta-analyses published in 2004 [30, 31].

3.2.2 Glutamate-mediated neurotoxicity

Glutamate excitotoxicity, mediated through excessive activation of N-methyl-D-aspartate (NMDA) receptors, which is thought to increase intracellular Ca^{2+}, is believed to play a role in the neuronal death observed in AD and other neurodegenerative conditions [32]. Thus, NMDA-receptor antagonists may have a therapeutic potential for protecting neurons from glutamate-mediated neurotoxicity.

Memantine is a non-competitive NMDA-receptor antagonist with moderate affinity [33] that appears to be able to protect neurons while leaving physiological NMDA-receptor activation unaffected [34]. Memantine was approved in 2002 in Europe for the treatment of 'moderately severe to severe AD and in 2003 in the United States for the treatment of moderate to severe cases of AD [34].

3.2.3 Mechanism-based therapeutic approaches targeting β-amyloid and tau pathologies

The most effective treatments for complex, chronic diseases are usually those that stop an early step of the development of the disease, before irreversible damaging events have occurred. In this context, extensive research is being done to identify compounds that effect the β-amyloid and tau pathologies and they can be divided into four broad classes: (i) protease inhibitors that partially decrease the activities of the enzymes (β- and γ-secretase) that cleave Aβ from β APP; (ii) compounds that bind to extracellular Aβ and prevent its aggregation into cytotoxic amyloid fibrils; (iii) brain-specific anti-inflammatory drugs that block the microglial activation, cytokine release, and acute-phase response that occur in affected brain regions; and (iv) compounds such as antioxidants, neuronal calcium channel blockers, or antiapoptotic agents that interfere with the mechanisms of Aβ-triggered neurotoxicicity [35].

3.3 Current and potential therapy based on natural products

According to Newman and Cragg, 63% of the low molecular drugs developed from 1981 to 2006 are natural products or natural-derived compounds [36]. These reports suggest that there is great potential in developing biological active compounds from natural sources for AD. Natural compounds with most potential are those that inhibit ChEs and those that exhibit antioxidant activity. Natural ChE inhibitors can be classified into two broad groups, alkaloid and non-alkaloid, although the alkaloid inhibitors have proven to be stronger inhibitors. Antioxidant compounds are very abundant in nature, but here will be mentioned a few flavonoids and polyphenols, as well as plants containing them, that have potential to be developed successfully for use in AD.

3.3.1 Natural inhibitors of ChEs

3.3.1.1 Alkaloid inhibitors

3.3.1.1.1 Physostigmine and rivastigmine

Physostigmine (eserine), isolated from the seeds of *Physostigma venenosum* (Fabaceae) in the 19th century, has long been known as an AChE inhibitor. It is reported to have shown significant cognitive benefits in both normal and Alzheimer's patients [37]. Physostigmine is also a non-competitive nicotinic channel activator and BuChE inhibitor, both of which may have added value in AD. Despite positive results in cognition, the low half-life of physostigmine has prevented its clinical use [24].

The chemical structure of physostigmine provided a template for the development of rivastigmine, which was previously mentioned in the current treatment of AD. Moreover, it has brain-region selectivity and preferentially inhibits the G1 form of AChE, which predominates in the brains of patients with Alzheimer's disease [38].

3.3.1.1.2 Galanthamine and other compounds from Amaryllidaceae species

Galanthamine is another example of natural compound already in use for treatment of AD. It was first isolated in the 1950s from *Galanthus nivalis* (Amaryllidaceae) and occurs also in other genera of the Amaryllidaceae family [37]. This alkaloid is reported to be more selective for AChE than BuChE [39]. As mentioned earlier, galanthamine has a dual mode of action: an inhibitor of AChE and an allosteric potentiating ligand of nicotinic acetylcholine receptors [25]. A recent study also mentioned neuroprotective effects by induction of an antiapoptotic protein [37].

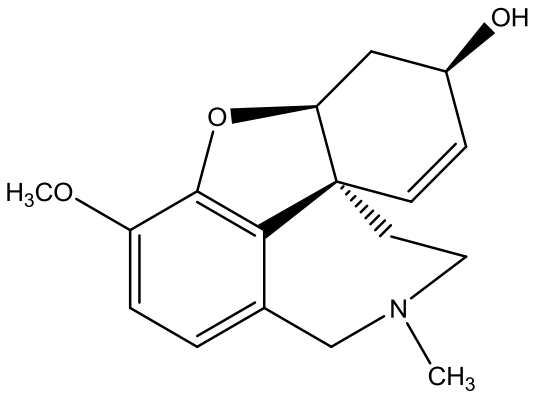

Fig. 2 Structure of galanthamine

In an attempt to find similar compounds to galanthamine, twenty-three alkaloids isolated from various Amaryllidaceae species were tested for their *in vitro* AChE inhibitory activity with galanthamine as a positive control (IC_{50} = 1.07 μM) [40]. Only the galanthamine and the lycorine types showed some activity. The most active compounds were sanguinine (IC_{50} = 0.10 μM) and 11-hydroxygalanthamine (IC_{50} = 1.61 μM). Among the lycorine-type, the highest activity was found for assoanine (IC_{50} = 3.8 μM) [40].

Two Crinum species, *Crinum glaucum* and *Crinum jagus*, have frequently been cited by traditional healers in southwest Nigeria for memory loss and other mental

symptoms associated with ageing. Four alkaloids were isolated, but only two, hamayne, (IC_{50} = 250 μM) and lycorine (IC_{50} = 450 μM), were active against AChE [41].

3.3.1.1.3 Huperzine A

Huperzia serrata (Lycopodiaceae) has been used in China for centuries in the treatment of such conditions as contusions, strains, swelling, and schizophrenia [42]. The active principle, huperzine A (a sesquiterpene alkaloid), has attracted attention because, relative to other well-known AChE inhibitors, it has greater potency, higher selectivity with respect to its AChE inhibitory effect, and marked memory-enhancing efficacy in a broad range of animal models of cognitive impairment. In addition, it has neuroprotection and APP metabolism modulation activities, indicating that it may interfere with the key factors of the disease [42].

Tacrine-huperzine A derivatives (huprines) have been synthesized and tested as AChE inhibitors. They proved to be very potent inhibitors and showed interesting properties for further studies in connection with their possible use in the treatment of AD. [43].

3.3.1.1.4 *Chelidonium majus* (Papaveraceae)

In a recent study a number of alkaloids from *Chelidonium majus* were extracted, crystallized and tested for human blood AChE (HuAChE) and human plasma BuChE (HuBuChE) inhibitory activity. The most active of the naturally-occurring alkaloids was chelidonine, which inhibited both HuAChE and HuBuChE in a dose-dependent manner with IC_{50} values of 26.8 ±1.2μM and 31.9 ±1.4μM respectively [44]. Other alkaloids from the order Papaverales that have inhibitory activity on ChEs will be discussed in the section 3.4.1

3.3.1.2 Non-alkaloid inhibitors of acetylcholine-esterase

3.3.1.2.1 Terpenoids

The majority of non-alkaloidal inhibitors discovered in plants are terpenoids. Many studies have been made on monoterpenes, but sesquiterpenes, diterpenes and triterpenes are also good candidates in the search for new potential treatments for AD [45].

One such group of plants was the various European species of Salvia, commonly known as sage. An ethanolic extract and oil of *Salvia officinalis* (Labiatae) and *Salvia lavandulaefolia* (Labiatae) were investigated for anti-ChE activity and it was found that all gave inhibition of AChE at quite low concentrations [46]. The ChE inhibition shown by the *Salvia lavandulaefolia* oil was shown to be partly due to the cyclic monoterpenes 1,8-cineole and α-pinene, which inhibit AChE *in vitro*, with some contribution from other constituents, perhaps by acting synergistically. However, the monoterpenes were considerably less active than the alkaloidal AChE inhibitors such as physostigmine [47].

Screening of 139 different Indian medicinal plants and spices for AchE inhibitory activity led to *Origanum majorana* (Labiatae) showing the highest activity. The active component was identified as the triterpene ursolic acid (IC_{50} = 7.5 μM), a fairly common compound [48].

3.3.1.2.2 Withanolides

Withania somnifera is a well-known ayuverdic medicine known as ‘ashwagandha’, and is classed among the rejuvenative tonics. Withanolides, C28-steroidal lactone triterpenoids, extracted from this plant, were shown to inhibit both AChE and BuChE [49]. *Withania somnifera* root and some constituents are also reported to have anti-oxidant properties [50].

3.3.2 Antioxidant therapy based on natural compounds

3.3.2.1 Flavonoids

The role of flavonoids in the diet as important antioxidant contributors has received much attention and their neuroprotective properties because of this effect have been demonstrated by several workers [24].

3.3.2.1.1 *Ginkgo biloba*

The standardized extract of *Ginkgo biloba* leaves (EGb 761) contains 24% of flavonoids (quercetin, keampferol and isorhamnetin) and 6% of terpenic lactones (diterpenic lactones, the ginkgolides A, B, C, J and M, and a sesquiterpenic trilactone, the bilobalide), which are suggested to be the pharmacologically active constituents [4, 51].Various *in vivo* and *in vitro* preclinical studies support the notion that EGb 761 may be effective in the treatment and prevention of AD and other age-related, neurodegenerative disorders. In addition to anti-oxidative effects, the following mechanisms of action have been proposed: anti-apoptosis, anti-inflammation, protection against mitochondrial dysfunction, amyloidogenesis and Aβ aggregation, ion homeostasis, modulation of phosphorylation of tau protein and even induction of growth factors. However, the clinical efficacy of EGb 761 still remains elusive [51].

3.3.2.2 Polyphenols

Brain penetrating property of polyphenols, as well as their antioxidant and iron-chelating properties make such compounds an important class of drugs to be developed for treatment of neurodegenerative disorders associated with oxidative stress [52].

3.3.2.2.1 Resveratrol

Resveratrol, a polyphenol that occurs in abundance in grapes and red wine, is suspected to afford antioxidant and neuroprotective properties and therefore contribute to the

beneficial effect of wine consumption in neurodegenerative process. It has also been found to have a potent anti-amyloidogenic activity by promoting the intracellular degradation of Aβ through a mechanism that implicates the proteasome [53].

3.3.2.2.2 Curcumin

Curcumin, the active ingredient in turmeric (dried ground rhizome of *Curcuma longa*, Zingiberaceae) has been used for thousands of years in Ayurvedic and Chinese medicine [4]. It has a surprisingly wide range of beneficial properties, including anti-inflammatory, antioxidant, chemo-preventative and chemotherapeutic acitivity [4, 54]. Its remarkable non-toxic nature makes it a promising therapeutic agent. It is currently in human clinical trials for a variety of conditions, including AD [54].

3.4 The therapeutic potential of *Corydalis yanhusuo*, its alkaloids and other Corydalis species

3.4.1 Inhibition of AChE by alkaloids of the order Papaverales

Isoquinoline alkaloids have been widely found in the order of Papaverales, and their structure-activity relationships have been studied. Alkyl chains of different lengths were added to the protoberberine skeleton and it was found that the activity decreased with the length of the chain (% inhibition at 0.5 μM: berberine 76 %, 13- methylberberine 64 %, 13-ethylberberine 56 %, 13- propylberberine 35 %), which could be connected with a steric restriction at the binding sites. An ionized hydroxyl group also decreased inhibition, which could be as a result of loss of cationic character [55]. It's also significant that compounds with a quaternary nitrogen (berberine 76 % of inhibition at 0.5 μM, escholamine 80 % of inhibition at 10 μM, californidine 61 % of inhibition at 10 μM) were better inhibitors than those with a tertiary nitrogen (papaverine 56 % of inhibition at 10 μM, protopine 38 % of inhibition at 10 μM) [55-57]. The inhibitory potency increases with the hydrophobicity of the alkaloids and the binding power of the alkaloid increases with the polarity of C7 substituent [56].

3.4.2 *Corydalis yanhusuo* and its alkaloids

Table 1. Botanical classification of *Corydalis yanhusuo* [58]

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Papaverales
Family	Fumariaceae
Genus	Corydalis
Botanical name	*Corydalis yanhusuo* W.T.Wang

Fig. 3 Dried plant of *Corydalis yanhusuo* [59]

Fig. 4 Dried tuber of *Corydalis yanhusuo* [59]

The genus Corydalis (Fumariaceae) of about 320 species is widely distributed in the Northern hemisphere and 70 species have been used in traditional herbal remedies in China, Japan, and Korea. *Corydalis yanhusuo* , a perennial herb up to 20 cm tall, is one of the medicinally important species of Corydalis [60]. The dried and pulverized tubers of *Corydalis yanhusuo*, also known as Yuanhu, Rhizoma Corydalis, are collected in early summer after the stems and leaves have withered [61]. It has been used in Traditional Chinese Medicine as an analgesic for the treatment of abdominalgia, menorrhalgia, menostasia, and traumatic pain [61], as well as for treatment of gastric, duodenal ulcer, cardiac arrhythmias [60] and memory dysfunction [62].

A number of alkaloids have been isolated and identified from the tuber of *Corydalis yanhusuo*. They are corydaline, tetrahydropalmatine, tetrahydro-coptisine, glaucine, protopine, allocryptopine, tetrahydrocolumbamine, corybulbine, dehydrocorydaline, tetrahydroberberine, palmatine, columbamine, lauroscholtzine, dehydroglaucine, yuanhunine, leonticine, and dihydrosanguinarine [61]. From the aerial part of *Corydalis yanhusuo*, 12 alkaloids and longchain fatty alcohol were isolated. They were identified as tetrahydrocoptisine, dehydroglaucine, protopine, glaucine, nantenine, allocryptopine, norglaucine, tetrahydrocolumbamine, thaliporphine, lirioferine, lauroscholtzine, isoboldine, and the fatty alcohol nonacosan-10-ol [61].

3.4.3 Pharmacology of *Corydalis yanhusuo* alkaloids

The abundant alkaloids contained in *Corydalis yanhusuo* can be divided into two groups: tertiary alkaloids (tetrahydropalmatine, tetrahydrocolumbamine, glaucine, corydaline), and quarternary alkaloids (columbamine, coptisine, palmatine, berberine and dehyrocorydaline) [63]. Below will be discussed few of these alkaloids, which have been more widely researched.

3.4.3.1 Tertiary alkaloids

3.4.3.1.1 Tetrahydropalmatine (THP)

Naturally occurring THP (Fig. 14) can be easily found in various plant species. It can also be directly synthesized from laudanosine, a benzyl-isoquinoline alkaloid, by a chemical

conversion [64]. This widespread availability of THP makes it a valuable compound worthy of study.

THP is the main active component of the tuber of *Corydalis yanhusuo*. Numerous studies aiming to characterize the mechanisms of THP actions have been carried out and the following pharmacological actions have been described:

i. THP depletes the levels of the neurotransmitters dopamine, noradrenaline and serotonin (5-HT) in the central nervous system [65];
ii. the two enantiomers of dl-THP act on different targets in the central nervous system, d-THP acts as a DA depletory while l-THP acts as a brain DA antagonist [66];
iii. l-THP was shown to have marked analgesic, sedative, and hypnotic effects, which were not observed with d-THP [67];
iv. THP decreases both arterial pressure and heart rate through a serotonergic release process in the hypothalamus [68];
v. inhibits picrotoxin-induced convulsion by blocking the amygdaloid release of DA [69];
vi. inhibits voltage-dependent Ca^{2+} channels [70], which has been shown to protect cardiomyocytes from apoptosis and therefore may explain some of the beneficial effects of *Corydalis yanhusuo* in heart failure [71];
vii. side effects of THP were rare and thus its therapeutic index appeared to be high [67];
viii. THP possesses anxiolytic effects avoiding sedation myorelaxation when administrated orally to mice at low dose regime, and this effect was found to be mediated, at least partly, through $GABA_A$ receptor BDS (benzodiazepine site) [72];

3.4.3.1.2 Corydaline

Corydaline (Fig. 5) isolated from *Corydalis yanhusuo,* was tested for its activity on AChE and showed weak inhibition (IC_{50} = 30.7 ± 1.5 μM) with mouse brain cortex as the source

of enzyme [73]. Corydaline was also shown to promote gastric emptying and small intestinal transit, and facilitate gastric accommodation, in an *in vivo* animal model [74].

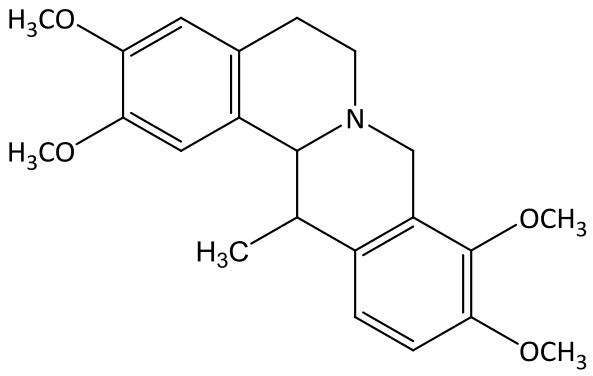

Fig. 5 Structure of corydaline

3.4.3.2 Quaternary alkaloids

3.4.3.2.1 Berberine

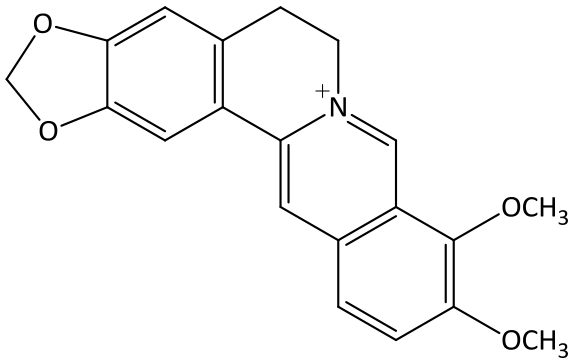

Fig. 6 Structure of berberine

In a recent study of anti-proliferative activities of several main constituents of *Corydalis yanhusuo*, berberine (Fig. 6) was identified as a powerful angiogenesis inhibitor. Both *Corydalis yanhusuo* extract and its active compound berberine suppressed the vascular

endothelial growth factor (VEGF)-induced upregulation of matrix metalloproteinase 2 (MMP2) at both mRNA and protein levels [75].

In addition, berberine demonstrated the highest inhibitory actions on uterine contraction induced by oxytocin in a study of Xiang-Fu-Si-Wu Decoction (XFSW). The decoction is a famous ancient prescription in Traditional Chinese Medicine which in addition to other plants also contains *Corydalis yanhusuo* and has been used to treat dysmenorrhea [76].

3.4.3.2.2 Pseudocoptisine

Pseudocoptisine (Fig. 7) was found to competitively inhibit AChE activity in a reversible, dose dependent manner (IC_{50} = 12.8μM) and also showed cognitive-enhancing activity in a series of experiments carried out in rat [73].

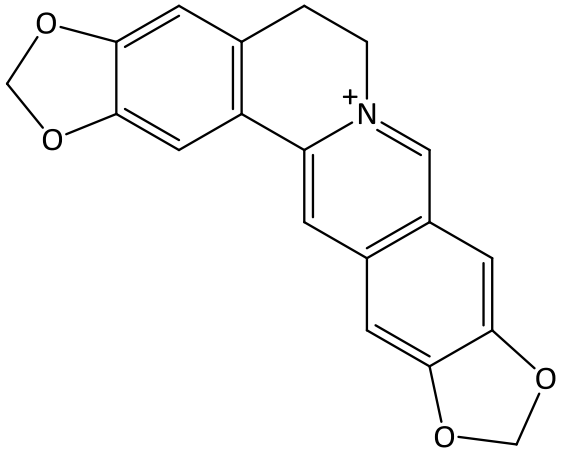

Fig. 7 Structure of pseudocoptisine

Another study showed that it reduces levels of the pro-inflammatory mediators, such as iNOS, COX-2, TNF-α, and IL-6 through the inhibition of NF-κB activation via the suppression of ERK and p38 phosphorylation in RAW 264.7 (mouse leukaemic monocyte macrophage cell line) cells [77].

3.4.4 *Corydalis bulbosa*

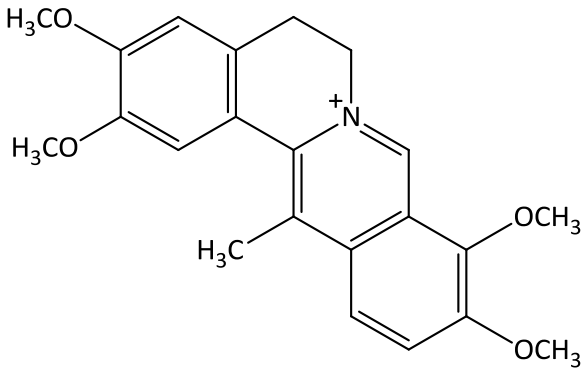

Fig. 8 Structure of dehydrocorydaline

Five protoberberine alkaloids, which inhibited AChE *in vitro*, were isolated from *Corydalis bulbosa*. The activity against larvae and adults of *Drosophila melanogaster* was also tested and it was partially related to the anti-AChE activity. (-)-Tetrahydroberberine (% inhibition at 1.0 mM = 78.7 %), (-)-tetrahydrocoptisine (% inhibition at 1.0 mM = 71.8 %) and (±)- dehydrocorydaline (Fig. 8) (% inhibition at 0.4 mM = 61.3 %) were the most active compounds for anti-AChE activity *in vitro* [78].

3.4.5 *Corydalis ternata*

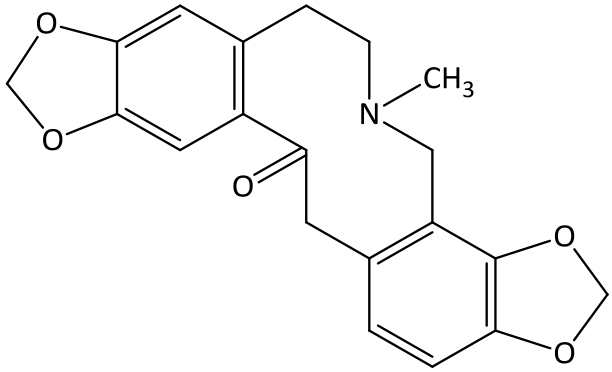

Fig. 9 Structure of protopine

A methanolic extract of *Corydalis ternata* showed a significant inhibitory effect on AChE activity which led to the isolation of a reversible and competitive inhibitor, protopine (Fig. 9) (IC_{50} = 50 μM). *In vivo*, mice treated with protopine exhibited a diminished scopolamine-induced dementia [79].

3.4.6 *Corydalis incisa*

In the course of screening Korean natural products for AChE inhibitory activity (AChE), it was found that a methanolic extract of the aerial parts of *Corydalis incisa* showed significant inhibitory effects on AChE (purchased Type V-S, from *Electophorus electricus*, compared with the enzyme from the mouse brain). Corynoline isolated from this plant inhibited AChE activity in a dose-dependent manner (IC_{50} = 30.6 μM). The AChE inhibitory activity of corynoline was reversible and noncompetitive [80].

3.4.7 *Corydalis speciosa*

In a study of *Corydalis speciosa* (Papaveraceae) four isoquinoline alkaloids with AChE (Type V-S) inhibitory activity were isolated; the activity of berberine (IC_{50} = 3.3 μM) and protopine (IC_{50} = 16.1 μM) has already been described [55, 79]; the inhibitory activities of palmitine (IC_{50} = 5.8 μM) and corynoxidine (IC_{50} = 89.0 μM) were described for the first time [81].

4. EXPERIMENTAL PART

4.1 General methods

4.1.1 Distillation and evaporation

Prior to use the solvents were distilled. First, the substances were applied (approx. 5%), and then the remaining solvent, about 90% in total, was distilled. Solvents were stored in brown glass containers. Evaporation of the chromatograph fractions were carried out on a vacuum evaporator under reduced pressure at 40℃.

4.1.2 Chromatography

4.1.2.1 Thin layer chromatography

Chromatography was carried out in a standard chamber system which was saturated with the mobile phase. The time of saturation was approx. 30 minutes and in case of preparative TLC, approx. 60 minutes. The chromatography was carried out in ascending order.

4.1.2.2 Column Chromatography

Column chromatography was carried out under gradient elution on a silica gel system, 0.1 - 0.25 mm, deactivated in 10% water. A suspension of the adsorbent in the solvent was then poured into the chromatographic column. The prepared column was coated with the sample diluted in a small amount of the solvent. The sample was dried in the exsiccator and then applied with a small amount of silica gel.

4.2 Plant material and equipment

4.2.1 Chemicals and solvents

Solvents:

- Cyclohexane
- Diethylamine
- Diethylether without stabilizer
- Ethanol 95%, denatured with methanol (EtOH)
- Chloroform ($CHCl_3$)
- Methanol (MeOH)
- Petrol (ČL 2006)
- Toluene

Chemicals:

- Acetic acid 99% p. a
- Bismuth subnitrate purum
- Hydrochloric acid 36% p. a. (HCl)
- Potassium iodide p. a. (KI)
- Sodium carbonate anhydrous purum
- Sodium hydroxide p. a. (NaOH)
- Sodium sulfate anhydrous purum
- Sulfuric acid 96% purum
- Tartaric acid purum

4.2.2 Chemicals and material for analysis of AChE and BuChE (IC_{50})

Chemicals:

- 0,1 M phosphate buffer pH 7.4
- 10 mM acetylcholine iodide (Sigma-Aldrich)
- 10 mM butyrylcholine iodide (Sigma-Aldrich)

- Dimethylsulphoxide p. a. (Sigma-Aldrich)
- 5,5′-Dithiobis(2-nitrobenzoic acid) (DTND) p. a. (Sigma-Aldrich)
- Eserine (Sigma-Aldrich)
- Galanthamine hydrobromide (Changsha Organic Herb Inc., China)
- Huperzin A (Tazhonghui Co., Ltd., China)
- Sodium dihydrogenphosphate dihydrate p. a. (Lachema)
- Sodium hydrogen dodecahydrate p. a. (Lachema)

Materials:

- Source of AChE: hemolysed human erythrocytes
  - Whole blood was centrifuged for 15 minutes at 10,000 rev / min., mass obtained from red blood cells was washed 3 times with 0.1 M phosphate buffer at pH 7.4 to remove residual plasma, 10% (v / v) lysate was prepared in water.
- Source of BuChE: human plasma
- Single semi-micro polystyrene cuvette 1.5 ml (PLASTIBRAND)

Equipment:

- Centrifuge type MPW–340 (Mechanika precyzyjna,Warsaw, Poland)
- Instrument for measurement of optical rotation: ADP 220 POLARIMETER B+S
- Micro-heated table Boetius
- pH meter Φ 72 METER (Beckmann, USA)
- UV-spectrophotometer UVIKON 942 (Kontron instruments, Switzerland)

4.2.3 Chemicals and material for analysis of antioxidant activity

- 2,2-diphenyl-1-picrylhydrazyl radical (Sigma-Adrich)
- Quercetine (Sigma-Adrich)
- Trolox p.a. (Sigma-Adrich)

4.2.4 Detection reagents

Dragendorf's reagent modified according to Munier:

- Solution A: prepared by dissolving 1.7 g alkaline bismuth nitrate and 20 g tartaric acid in 80 ml of water
- Solution B: prepared by dissolving 16 g potassium iodide in 40 ml water
- Stock solution: prepared by mixing solution A and B in ratio of 1:1. Stock solution may be stored for few months in the refrigerator.
- Solution for analysis: prepared by adding 5 ml stock solution to 5 ml tartaric acid dissolved in 50 ml of water

4.2.5 Chromatographic plates and adsorbents

Aluminum oxide neutral, 100-250 μm (fy Across)

Commercial chromatographic adsorbent was activated in a maximally 2 cm thick layer at 200 °C in dryer for 8 hours. After cooling to ~ 80 °C the adsorbent was poured into a flask, it was sealed and after cooling until at room temperature, 5% water (w/w) was added and was equilibrated upon periodic shaking.

Kieselgel 60 GF_{254}, plates for TLC (Merck)

For preparation of poured plates (90 x 150 mm) 3.9 g of commercial adsorbent were mixed with 13.5 ml water and mixture was homogenized for 30 seconds by using a micro-homogenizer. The suspension was poured on the plate, the surface layer was planed and plates were stored in horizontal position for 24 hours under room temperature. Solution of fraction was spotted in form of a line about 10 mm from upper edge of plate by using an application-tube.

4.3 Description and methods of alkaloid isolation

4.3.1 Origin of herbal drug

Ground tubers of *Corydalis yanhusuo* were supplied by the company Pragon s.r.o., Prague, and verification of the herbal drug was conducted by Assoc. Prof. L. Opletal.

4.3.2 Preparation of summary alkaloid extract

10.8 kg of dry tuber was percolated with 120 liters of 95% ethanol (< 1:11). Collected extract was evaporated to viscous residue, heated at 50°C and 2.5 liters of 2% hydrochloric acid was added. The brown solution was decanted, solid residue in flask was homogenized by using of 1 liter of 2% hydrochloric acid and sonified at 50 °C at level 10 (apparatus Sonorex 10HP) for 30 minutes. Then the suspension was filtered through viscous cellulose and the filtrate was diluted by water to 4.7 liters.

4.3.3 Preparation of extract A from primary extract

4.7 liters of acidic solution (pH < 1) was alkalized by 10% Na_2CO_3 to pH 9.7 (approx. 7 liters of solution were obtained) and suspension with alkaloids was operated by ether (5· 1.6 liters); organic layer was desiccated by sodium sulfate, filtered and evaporated to dryness.

4.3.4 Separation of extract A on particular groups of alkaloids

Dry residue of extract A was dissolved in 2% hydrochloric acid. The solution was filtered and shaken with chloroform. In this manner the mixture of alkaloids was divided on chlorides soluble and insoluble in chloroform. Each group of alkaloids was transferred into alkaloidal bases, which were dissolved in ether and divided into bases of phenolic and non-phenolic origin. Upon completion, 4 groups of alkaloids from extract A were obtained.

The mixture of non-phenolic alkaloids, which were obtained from the mixture of chlorides soluble in chloroform were examined in this diploma work. The alkaloid

fraction was obtained from the diploma supervisor (Assoc. Prof. L. Opletal). The first part of the separation was performed together with diploma co-worker Gabriella Cipra.

Fig. 10 TLC of alkaloidal bases from extract A

Non-phenolic bases obtained from chlorides soluble in chloroform. Silica gel plates for TLC $60F_{254}$ (Merck), 50 x 75 mm, toluene + chloroform + diethylamine (70:25:5), developing chamber was saturated by vapor of solvent system, developed 1x, detection UV λ = 254 nm, Dragendorf reagent (modified by Munier).

4.3.5 Separation of mixture of non-phenolic alkaloids from chlorides soluble in chloroform

9.73 g of yellow-orange oily residue was dissolved in a small volume of chloroform (minimal quantity only for dissolution). 30 g of aluminum oxide neutral was added and mixture was dried. After drying, 5 g of Celite 545 was added and the mixture was homogenized. This dry trituration was applied to the chromatographic column.

Table 2. Column chromatography of non-phenolic bases of alkaloids from chlorides soluble in chloroform

Adsorbent:	**Aluminium oxide neutral, 100-250 µm, grade 3 activity**
Quantity of adsorbent	350 g
Layer with extract	3 x 6 cm
Layer with adsorbent	3 x 39 cm
Dead volume	220 ml
Time of fraction collection	15-20 minutes
Fraction volume	100 ml

Each of the fractions was monitored by TLC (Silica gel for TLC $60F_{254}$ (Merck), plates 50 x 75 mm, solvent system: toluene + chloroform + diethylamine (70:25:5), developing tank was saturated by vapor of solvent system, developed 1·, detected UV λ = 254 nm, Dragendorf reagent (modif. Munier).

Fractions of the same quality were combined, evaporated under decreased pressure and temperature and desiccated in vacuum-desiccator over granulated silica gel.

Table 3. Results of column chromatography of non-phenolic alkaloids from chlorides soluble in chloroform

Fraction		Eluent	Weight	Description
1-42	1-12 13-22 23-42	Petrol+$CHCl_3$ 95:5 Petrol+$CHCl_3$ 92.5:7.5 Petrol+$CHCl_3$ 90:10	0.05 g	Yellow oil
43-56	43-56	Petrol+$CHCl_3$ 85:15	1.82 g	Bright brown, crystals
57-63	57-59	Petrol+$CHCl_3$ 85:15	0.57 g	Brown-red, crystals

	60-63	Petrol+$CHCl_3$ 80:20		
64-67	64-65 66-67	Petrol+$CHCl_3$ 80:20 Petrol+$CHCl_3$ 75:25	0.28 g	Brown, crystals
68-71	68-71	Petrol+$CHCl_3$ 75:25	0.86 g	Brown, very viscous
72-75	72-75	Petrol+$CHCl_3$ 75:25	0.86 g	Dark brown, viscous
76-97	**76-85** **86-95** **96-97**	**Petrol+$CHCl_3$ 75:25** **Petrol+$CHCl_3$ 70:30** **Petrol+$CHCl_3$ 25:75**	**3.20 g**	**Yellow-brown, crystals**
98-107	98-107	Petrol+$CHCl_3$ 25:75	0.19 g	Dark brown, very viscous
108-113	108-113	Petrol+$CHCl_3$ 25:75	0.16 g	Black, very viscous

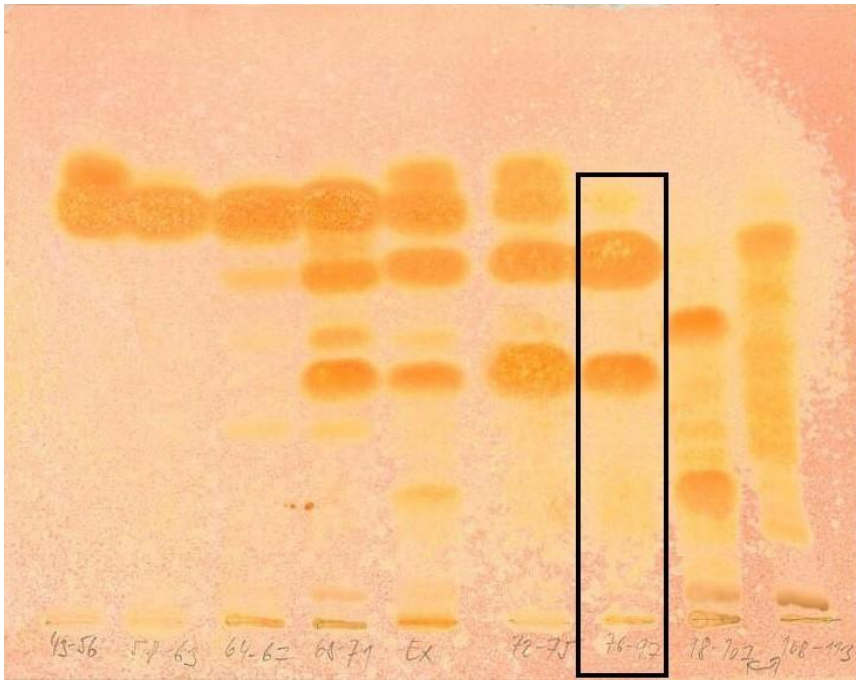

Fig. 11 Results of column chromatography on aluminum oxide –

Silica gel plates for TLC $60F_{254}$ (Merck), 50 x 75 mm, toluene + chloroform + diethylamine 70:25:5, developing chamber saturated by vapor of solvent system, developing 1x, detection UV $\lambda = 254$ nm, Dragendorf reagent (modif. Munier).

4.3.6 Isolation of alkaloid from combined fractions 76-97

3.20 g yellow-brownish crystalline mass was dissolved in 30 ml of chloroform. 60 ml of petrol were added and the solution was made slightly turbid. The solution was poured into chromatographic column with 48 g of aluminium oxide neutral (diameter x height 25 x 80 mm) and column has been eluted after soaking of solution by 180 ml of mixture chloroform + petrol 1 : 2. Yellowish eluent was then evaporated in rotation evaporator. Yellowish oily residue was obtained and was crystallized after short time in refrigerator. This crystalline mass was a mixture of 3 individual alkaloids.

2.65 g this yellowish residue was dissolved in 25 ml of mixture chloroform + ethanol 1:1. The solution was filtered ion Pasteur pipette (cellulose) and alkaloids were separated by using of preparative TLC.

Table 4. Preparative TLC of purified fractions 76-97

Adsorbent:	Kieselgel 60 GF_{254}
Number of plates:	70, format 15 x 9 cm
Separation line:	7,5 cm
Solvent system:	Toluen + Et_2NH = 95 : 5
Developing:	saturated by vapour of solvent system, 2x
Detection:	UV λ=254 nm

The zones of adsorbent containing the compounds (H; R_F = 0,90, D; R_F = 0,80) were separated from the plates. Portions of adsorbent were combined and elaborated under these conditions:

Table 5. Separation of alkaloids from adsorbent

Zone	Elution	Weight	Desription
H* $R_F = 0.90$	50 ml $CHCl_3$, 100 ml $CHCl_3$ + EtOH 1 : 1	1.425 g	Brownish, oily, non-crystallic
D $R_F = 0.80$	50 ml $CHCl_3$, 100 ml $CHCl_3$ + EtOH 1 : 1	0.642 g	Yellowish, crystallic, BK-1

* After measuring of mass spectra, it was found that zone H was composed of a mixture of minimally 2 compounds (non-crystallic).

4.3.7 Characterisation of the compound BK-1

0.642 g yellowish crystalline mass was crystallized two times from the mixture ethanol + toluen. 0.245 g colorless needles were obtained.

4.4 Method for determining MS spectra

The spectra were measured on the LC / MS Thermo Finningan LCQDuo, ion trap, electrospray ionization in positive mode (ESI +). MS / MS spectra were measured at a collision energy of 40 eV. The substance was dissolved in methanol.

4.5 Method for determining NMR spectra

The spectra were measured on a Varian VNMRS500 spectrometer with working frequency of 500 MHz for ^{1}H and 125 MHz for ^{13}C nuclei. 13C NMR spectra were measured in 5 mm broadband probe SW, ^{1}H and all 2D spectra in inverse 5 mm ID PFG probe using a modified version of standard pulse sequences. Experiments were measured in deuterochloroform at 25 ˚ C.

Values of chemical shifts are in ppm and are relative to internal standard (TMS, 0.04 ppm in ^{1}H spectra) or the solvent signal (76.99 ppm in ^{13}C spectra).

4.6 Method for determination of antioxidant activity

4.6.1 DPPH free-radical scavenging assay (EC_{50})

Radical scavenging activity of extracts and pure compounds were evaluated by means of the DPPH (2,2´-diphenyl-1-picrylhydrazyl radical) test using an SIA (PC-controlled Sequential Injection Analysis system) method developed in our laboratory [82]. The stock solution of extract/pure compound was prepared by dissolving 4 mg of the extract/pure compound in 4 ml of aqueous 50% w/w ethanol during 10 minutes of sonication; the same solvent was for appropriate dilution of the extract/pure compound stock solution (1, 0.5, 0.25, 0.01 mg/mL). DPPH solution (0.1 mM) was prepared by dissolving 3.9 mg DPPH in 100 mL 50 % w/w ethanol. The automated method is based on the known reaction of stable DPPH with antioxidants resulting in bleaching of DPPH due to its "quenching" by interaction with the analytes. The decrease in the absorbance of DPPH measured at 525 nm is related to the concentration of an antioxidant in the tested solution. The percentage of inhibition of DPPH is estimated by using the formula: % $Q_{DPPH} = (1-A_x/A_0) \times 100$, where A_0 is the height of the peak of the blank sample and A_x is the height of the peak after the extract/pure compound is added. All measurements are made in triplicate. The DPPH radical scavenging activity of samples is expressed as EC_{50} (mg/mL for extracts, μM for pure compounds), which is the amount of sample necessary to decrease by 50% the light absorbance.

4.7 Methods of determination of inhibitory activity against AChE and BuChE

4.7.1 Preparation of red blood cells ghost for AChE and BuChE

Ghosts were prepared from freshly drawn blood (taken from healthy volunteers), to which 1 mL of sodium citrate per 10 mL of blood is added, according to a slightly modified method of Steck and Kant [83]. Briefly plasma (HuBuChE) is removed from the whole blood by centrifugation at 4000 rpm in a Boeco U-32R centrifuge with a

Hettich 1611 rotor. Red blood cells were transferred to 50 mL tubes and washed 3 times with 5 mM phosphate buffer (pH 7.4) containing 150 mM sodium chloride (12000 rpm, Avanti J-30I, rotor JA-30.50). The washed erythrocytes were stirred with 5 mM phosphate buffer (pH 7.4) for 10 min to ensure lysis. The lysed cells were centrifuged at 20,000 rpm for 10 minutes and then the ghosts (HuAChE) were washed 3 times with phosphate buffer.

4.7.2 AChE and BuChE assay (IC_{50})

HuAChE and HuBuChE activities are determined with a modified method of Ellman et al. [84] using acetylthiocholine iodide (ATChI) and butyrylthiocholine iodide (BuTChI) as substrates, respectively. Briefly, 25-50 μL of either ghosts or plasma, 650 μL of DTNB and 25 μL of either the sample or appropriate solvent, as a blank sample, are added to the semi-micro cuvette. The reaction is initiated by addition of substrate (ATChI or BuTChI). The final proportion of DTNB to substrate is 1:1. The increase of absorbance at 436 nm (ΔA) is measured for 1 minute using a Shimadzu UV-1611 spectrophotometer. Each measurement is repeated 3 times. Galanthamine and huperzine A are used as positive controls. The IC_{50}, EC_{50} values are calculated with the use of GraphPad Prism 5.02 software. The inhibition (in %) is calculated according to the formula: $\%I = 100-(\Delta A_{BL}/\Delta A_{SA})*100$, where ΔA_{BL} is increase of absorbance of a blank sample and ΔA_{SA} is increase of absorbance of the measured sample.

4.8 Method of measurement of optical rotation

Optical rotation was measured on polarimeter ADP 220 BS in ethanol.

5. RESULTS

5.1 Structural analysis of tetrahydropalmatine

The structure of isolated compound has been determined by comparing spectral data with those reported in the literature as **(+)-tetrahydropalmatine**.

5.1.1 MS analysis of (+)-tetrahydropalmatine

ESI-MS *m/z* $[M+H]^+$ 356.19 (100), 192.20 (7).

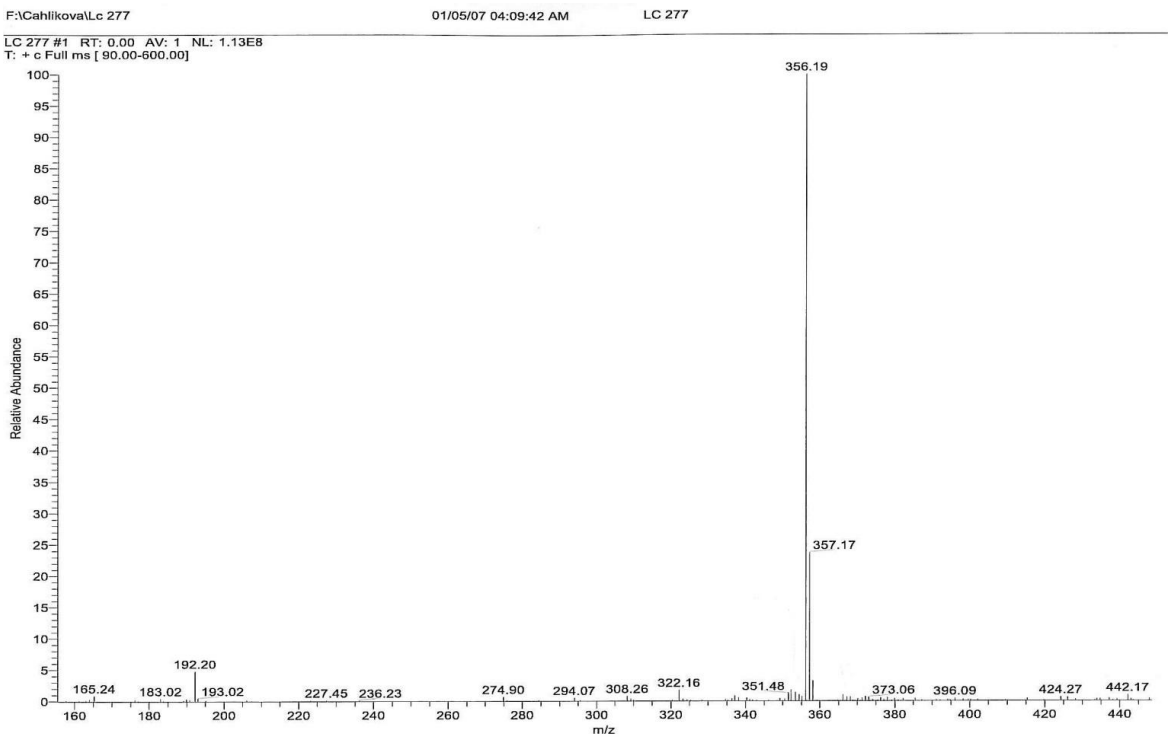

Fig. 12 MS spectrum of (+)-tetrahydropalmatine

5.1.2 MS/MS analysis of tetrahydropalmatine

MS/MS *m/z* 341.12 (5; $[M-CH3]^+$), 324.11 (7), 308.11 (12), 192.12 (100; $[C_{11}H_{14}O_2N]^+$), 165.07 (25; $[C_{10}H_{13}O_2]^+$).

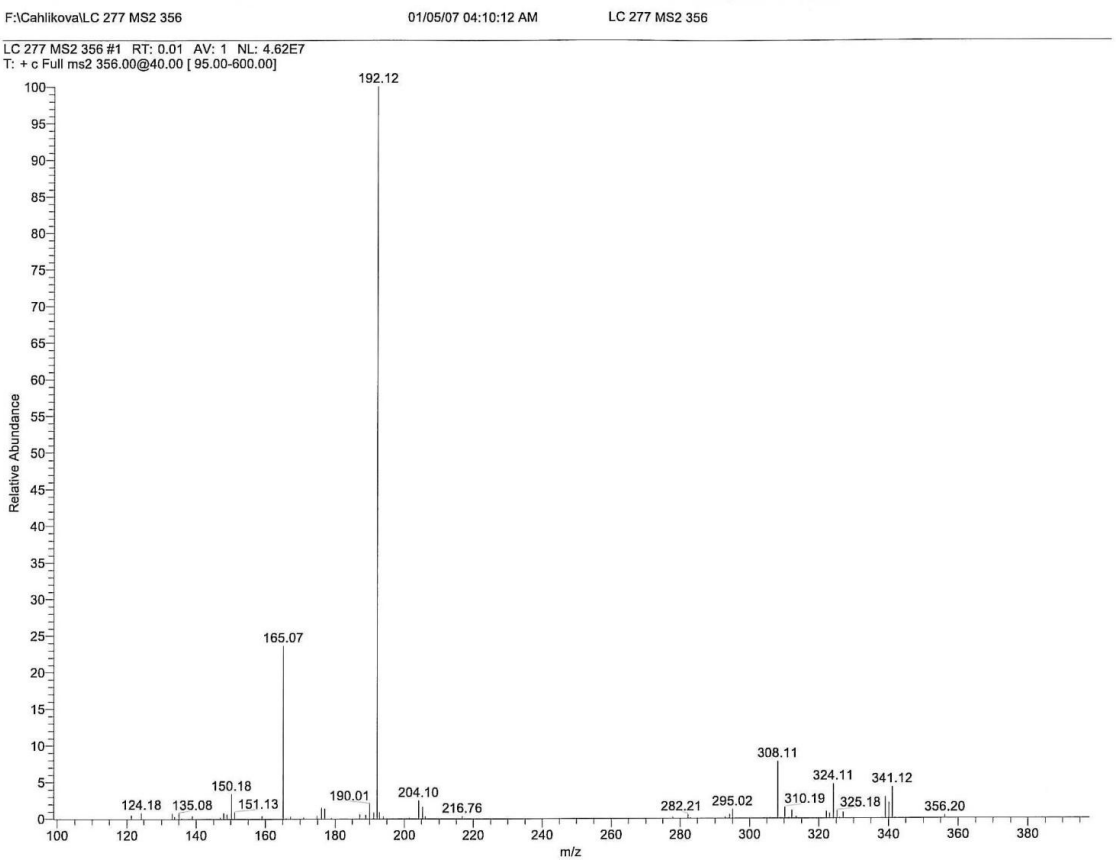

Fig. 13 MS/MS spectrum of (+)-tetrahydroapalmatine

5.1.3 Proposed MS/MS (ESI) fragmentation

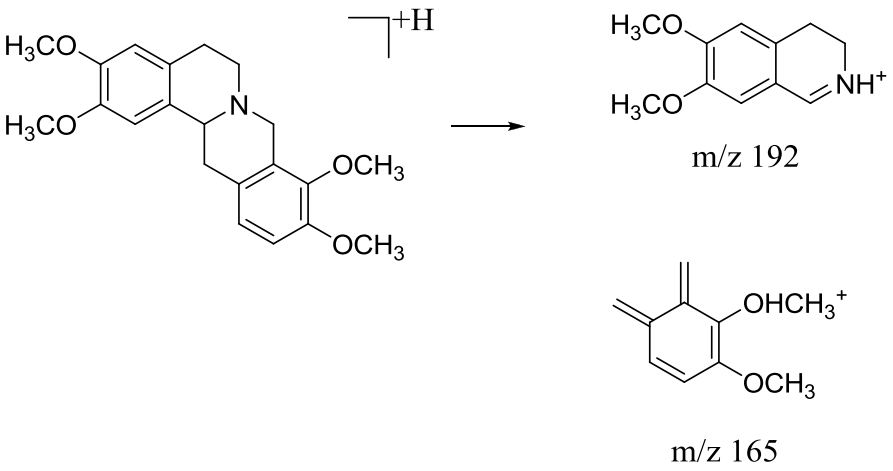

Fig. 12 Proposed retro-Diels-Alder (RDA) pathway

5.1.4 NMR analysis of tetrahydropalmatine

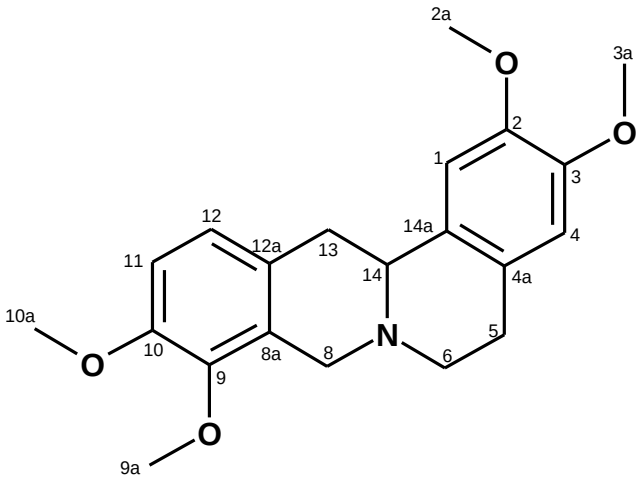

Fig. 14 Structure of tetrahydropalmatine

5.1.4.1 ^{1}H-NMR analysis of tetrahydropalmatine

^{1}H NMR ($CDCl_3$, 25°C):

2.66 br, 1H, H-5; 2.69 br, 1H, H-6; 2.86 br, 1H, H-13; 3.17 br, 1H, H-5'; 3.22 br, 1H, H-6'; 3.27 dd ($^{2}J_H$=15.8 Hz, $^{3}J_H$=3.6 Hz), 1H, H-13'; 3.56 br, 1H, H-8; 3.59 br ($^{3}J_H$=13.7 Hz), 1H, H-14; 3.85(4) s, 3H, H-10a; 3.86(0) s, 3H, H-9a; 3.87 s, 3H, H-3a; 3.89 s, 3H, H-2a; 4.26 d ($^{2}J_H$=15.6 Hz), 1H, H-8'; 6.63 s, 1H, H-4; 6.74 s, 1H, H-1; 6.81 d ($^{3}J_H$=8.4 Hz), 1H, H-11; 6.88 d ($^{3}J_H$=8.4 Hz), 1H, H-12;

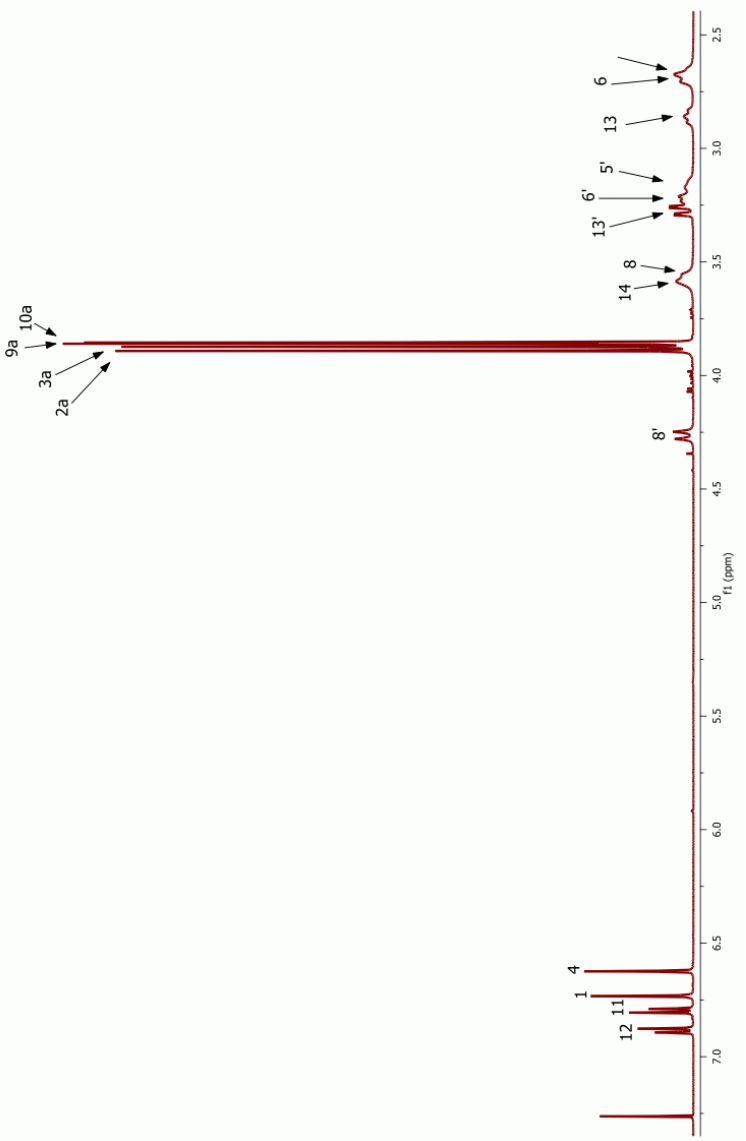

Fig. 15 ^{1}H-NMR analysis of tetrahydropalmatine

5.1.1.1 ^{13}C-NMR analysis of tetrahydropalmatine

^{13}C NMR ($CDCl_3$, 25°C):

29.23, C-5;36.45, C-13; 51.72, C-6; 54.18, C-8; 56.09, C-3a; 56.12, C-10a; 56.32, C-2a; 59.54, C-14; 60.42, C-9a; 108.83, C-1; 111.26, C-11; 111.60, C-4; 124.08, C-12; 126.93, C-4a; 127.88, C-12a; 128.75, C-8a; 129.75, C-14a; 145.34, C-9; 147.71, C-2; 147.80, C-3; 150.54, C-10;

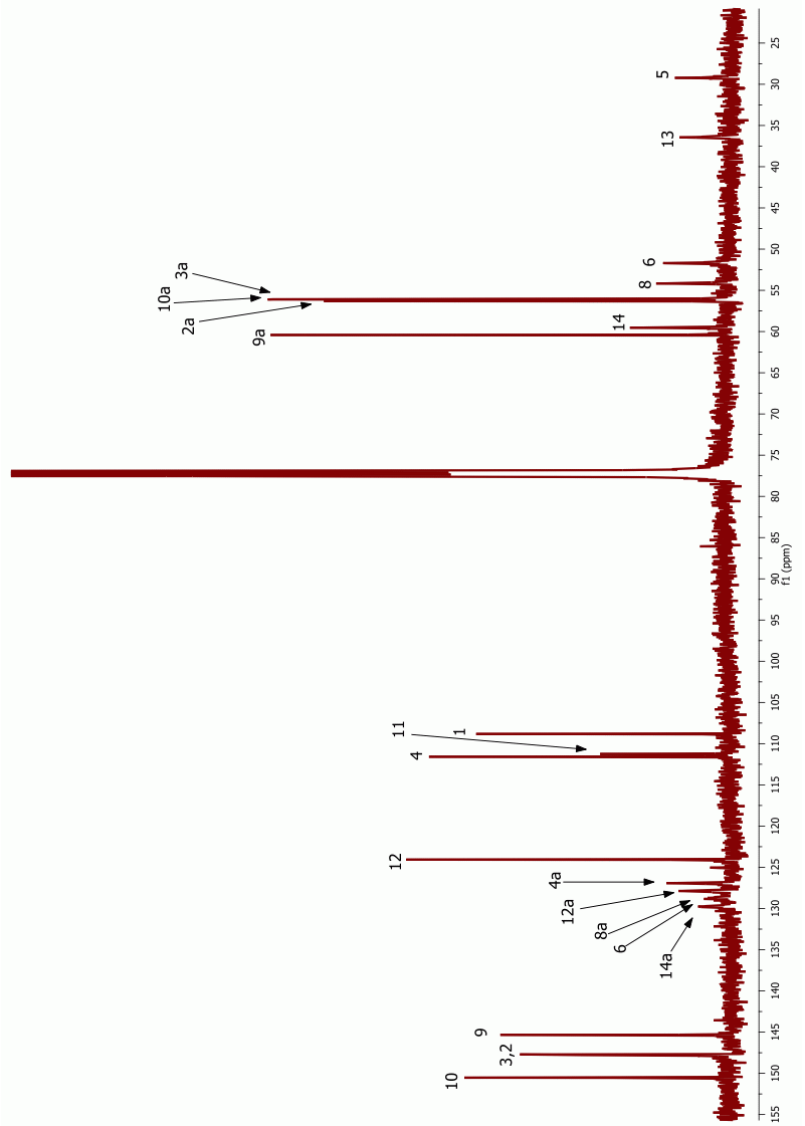

Fig. 16 ^{13}C-NMR analysis of tetrahydropalmatine

5.2 Inhibitory activity of tetrahydropalmatine against human AChE and BuChE

Table 6. *In vitro* HuAChE and HuBuChE inhibitory activity of isolated compound

Compound	IC_{50} (μM)[a]	
	AChE	BuChE
Tetrahydropalmatine	876 ± 15.3	>1000
Galanthamine[b]	6.9 ± 0.3	156 ± 6.9
Huperzine[b]	0.25 ± 0.01	>1000

[a]Results are the mean of three replications; [b]Reference compounds

5.3 Antioxidant activity of tetrahydropalmatine

Table 7. Antioxidant activity of isolated compound

Compound	EC_{50} (μM)[a]
Tetrahydropalmatine	> 1000
Quercetin [b]	25.3 ± 1.2
Trolox [b]	27.8± 0.8

[a]Results are the mean of free replications; [b]Reference compounds

5.4 Optical rotation

$[\alpha]^{20}_{D}$= + 291 (c 0.2, EtOH)

6. DISCUSSION

The aim of this diploma work was to isolate at least one pure compound (alkaloid) from the alkaloid extract of *Corydalis yanhusuo* and evaluate its inhibitory activity on human cholinesterases. The evaluation was done with the purpose of exploring natural alkaloidal compounds that could serve as a prototype in the search for novel structures to be used in the treatment of Alzheimer's disease.

Based on the methods described in the experimental part, one alkaloid was successfully extracted, isolated and crystallized. The alkaloid was named BK-1 and after MS and NMR spectral analysis, the results were compared with ones previously reported in literature. Consequently, the alkaloid was identified as tetrahydropalmatine (5,8,18,13a-tetrahydro-2,3,9,10-tetramethoxy-6H-dibenzo [a,q] quinolizine). Derived from the tetrahydroprotoberberine backbone structure, tetrahydroplamatine belongs to the isoquinoline alkaloid family [85, 86].

After evaluating the *in vitro* effect of tetrahydropalmatine on HuAChE and HuBuChE, the inhibitory activity was found to be negligible. IC_{50} values were 876 ± 15.3 μM for HuAChE and > 1000 μM for HuBuChE. In addition, the alkaloid was tested for antioxidant activity. The effective concentration was also insignificant (EC_{50} > 1000 μM).

The alkaloid tetrahydropalmatine has been previously isolated from various Corydalis species [85, 87] and also from other plants (i.e. *Stephania rotunda* (Menispermaceae) [86]). In comparison with literature data our results for AChE inhibition activity are surprising. Tetrahydropalmatine isolated from *Stephania rotunda* (2010) and *Corydalis turtschianovii* (2008) [85] has been found as a promising AChE inhibitor with IC_{50} values of 41.3 ± 3.1 μM and 41.3 ± 2.2μM, respectively. However, both studies used rat brain AChE and thus the rationale behind the differences in inhibitory activity could be due to the different enzyme source.

The same structure only with quarternary nitrogen, named palmatine, has also been isolated in our laboratory (by Martin Poruba, 2011) and tested for its HuAChE and HuBuChE inhibition activity. Values of IC_{50} from this study are significantly different (IC_{50} = 2,19 ± 0,11 μM (HuAChE) and IC_{50} = 68,31 ± 3,25μM (HuBuChE)). From these assays results that compounds with quarternary nitrogen are active AChE inhibitors and that the quarternary nitrogen plays an important role in binding with the enzyme;

however these compounds might have problems with crossing the blood-brain barrier [44].

BuChE inhibition activity by tetrahydropalmatine has been studied and tested for the first time within the framework of this diploma thesis. Thus, there are no data in the literature for comparing our results. However, when we compare the IC_{50} value of tetrahydropalmatine with IC_{50} value of palmatine, it seems that the quarternary nitrogen can also play an important role in the binding with BuChE. However, for these hypotheses further biological tests are needed.

From previous research which was discussed in the theoretical part, it can be said that tetrahydropalmatine has a potential for developing into a therapeutically active compound. However, the results of this diploma thesis suggest that tetrahydropalmatine is not a useful candidate in the development of compounds with dual inhibition activity (AChE/BuChE) for the treatment of Alzheimer's disease.

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8. ABBREVIATIONS KEY

Aβ - β-amyloid protein

ACh - acetyl choline

AChE - acetylcholinesterase

AD - Alzheimer's disease

APP - amyloid precursor protein

BDS - benzodiazepine site

BuChE - butyrylcholinesterase

ChAT - choline acetyl transferase

ChE - cholinesterase

CNS - central nervous system

COX-2 - cyclooxygenase 2

DA - dopamine

DPPH - 2,2´-diphenyl-1-picrylhydrazyl

EGb 761 - standardized extract of *Ginkgo biloba* leaves

EMEA - European Agency for the Evaluation of Medicinal Products

ERK - extracellular-signal-regulated kinase

$GABA_A$ - gamma amino butyric acid A subtype

HuAChE - human blood AChE (HuAChE)

HuBuChE - human plasma BuChE

IL-6 - interleukin

iNOS - inducible nitric oxide synthase

MMP2 - metalloproteinase 2

NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells

NGF - nerve growth factor

NMDA - N-methyl-D-aspartate

PGE_2 - prostaglandin E_2

RAW 264.7 - mouse leukaemic monocyte macrophage cell line

RNS - reactive nitrogen species

ROS - reactive oxygen species

TCM - Traditional Chinese Medicine

THP - tetrahydropalmatine

TNF-α - tumor necrosis factor-α

VEGF - vascular endothelial growth factor

WHO - World Health Organization

XFSW - Xiang-Fu-Si-Wu

ABSTRAKT

Bulëza Koci: Biologická aktivita obsahových látek rostlin 7. Alkaloidy *Corydalis yanhusuo* W.T.Wang. a jejich inhibiční aktivita vůči acetylcholinesteráze.

V rámci studia rostlin s obsahem alkaloidů, které vykazují inhibiční aktivitu vůči lidské erytrocytární acetylcholinesteráze a sérové butyrylcholinesteráze byl studován taxon *Corydalis yanhusuo.*

K izolaci alkaloidů bylo použito 10.8 kg suchých hlíz. Primární extrakt byl připraven perkolací 95% EtOH (120 l). V této diplomové práci byl zpracován výtřepek A-Et_2O. Alkaloidy tohoto výtřepku byly rozděleny na baze, jejichž chloridy jsou rozpustné a nerozpustné v chloroformu. Z každé uvedené frakce byly dále získány alkaloidy fenolické a nefenolické. Práce spočívala v dělení alkaloidů výtřepku s obsahem nefenolických alkaloidů, jejichž chloridy jsou rozpustné v $CHCl_3$. Z této směsi byl pomocí sloupcové chromatografie na Al_2O_3, preparativní TLC izolován (+)-tetrahydropalmatin. Látka byla identifikována na základě hmotnostního spektra, NMR spekter, teploty tání, optické otáčivosti a porovnáním získaných dat s literárními údaji. Při sledování inhibiční aktivity izolované látky vůči lidské AChE a BuChE a následném matematickém výpočtu byly pro tetrahydropalmatin zjištěny hodnoty IC_{50} 876 ± 15.3 μM pro HuAChE a IC_{50} > 1000 μM pro HuBuChE. Antioxidační aktivita (DPPH test) vykázala hodnotu EC_{50} > 1000 μM.

Na základě výsledků biologických studií lze konstatovat, že tetrahydropalmatin není atraktivní látkou potenciálně využitelnou v terapii Alzheimerovy choroby.

Klíčová slova: acetylcholinesterasa, butyrylcholinesterasa, alkaloidy, Alzheimerova choroba, *Corydalis yanhusuo*

ABSTRACT

Bulëza Koci: Biological Activity of Plant Metabolites 7. Alkaloids from *Corydalis yanhusuo* W.T. Wang and their inhibitory activity on acetylcholinesterase.

In the process of screening for plants containing alkaloids potentially inhibiting human erythrocytic AChE and human BuChE, *Corydalis yanhusuo* was studied.

10.8 kg of dry tuber was percolated with 120 liters of 95% ethanol. From the primary extract, extracts with individual types of alkaloids were prepared.

In this diploma thesis only one extract was processed (extract type A-ether, pH 9.7). Alkaloids from this extract were separated into bases whose chlorides were either soluble or insoluble in chloroform. From each of the above mentioned fractions phenolic and non-phenolic alkaloids were obtained. Alkaloids were separated from a fraction of alkaloids, whose chlorides are soluble in $CHCl_3$, non-phenolic (A- non-phenolic Cl^- S/$CHCl_3$). From this mixture tetrahydropalmatine was isolated by the use of column chromatography on alumina and preparative TLC on silica gel. This compound was preliminary identified according to data of MS spectra, NMR spectra and optical rotation and by comparison with literature data

. The biological activity of tetrahydropalmatine on human AChE and BuChE was found to be: IC_{50} 876 ± 15.3 μM for HuAChE and IC_{50} > 1000 μM for HuBuChE. Antioxidative activity (DPPH test) was EC_{50} > 1000 μM.

The results of this diploma thesis suggest that tetrahydropalmatine is not a useful candidate of compounds with dual inhibition activity (AChE/BuChE) for the treatment of Alzheimer's disease.

Key words: acetylcholinesterase, butyrylcholinesterase, alkaloids, Alzheimer's disease, *Corydalis yanhuuo*