Charles University in Prague, Faculty of Pharmacy

In Hradec Kralove

Department of Pharmaceutical Botany and Ecology

DIPLOMA THESIS

Biological Activity of Plant Metabolites XVII.

Alkaloids of Corydalis yanhusuo W.T. Wang

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Hradec Králové April, 2011 Gabriella Cipra

Declaration

I declare that this thesis is my original copyrighted work. All literature and other sources from which I extracted my research in the process are listed in the bibliography and all work is properly cited. This work has not been used to gain another or same title.

Acknowledgements

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1. INTRODUCTION

Plants as well as other natural sources have been used since ancient times as a means of medicinal therapy. Previously the root of a plants healing power was not known, however due to advances in technology and laboratory techniques, it can now be traced at least in part, to the various plant metabolites and active substances occurring uniquely within each herbal component. Among the main active constituents isolated are alkaloids, which consist of a large and diverse group of nearly 10,000 important secondary metabolites found abundantly in practically all plants, as well as in various species of animals, microorganisms, marine life and insects.

Alkaloids can be divided into various categories, depending on their chemical structures. An important family of alkaloids is isoquinoline alkaloids, which can further be divided into several sub-classes including benzylisoquinolines, phthalideisoquinolines, protopines, morphine, ipecac, and protoberberine type. Isoquinoline alkaloids are derived from amino acids tyrosine and phenylalanine and plants containing these alkaloids posses a wide array of pharmacological activities, many of which affect the nervous system. Notably plants from family's Papaveraceae, Amaryllidaceae, Ranunculaceae, and Fumariaceae are rich in these constituents.

Recently, attention has been drawn to isoquinoline alkaloids due to their potential as being potent inhibitors of acetylcholinesterase (AChE). For this reason, studies aimed at new or alternative therapies for neurodegenerative diseases connected to cholinergic depletion, such as Alzheimer's disease (AD), have shown increasing interest in further investigation of these compounds.

AD is a progressive form of dementia characterized by widespread loss of central cholinergic function affecting mainly the elderly population. In 2005, it was estimated that 24 million people suffer from dementia and that this amount will double every 20 years to 42 million by 2020 and 81 million by 2040. Within the spectrum of dementias, Alzheimer's dementia is the most prevalent subtype, accounting for about 60% of all

dementias [1]. Since there still remains no cure to prevent or treat AD, current therapy is based on the symptomatic treatment by use of AChE inhibitors.

One genus known to contain several species with AChE inhibitory activity is the genus *Corydalis*. Classified within this genus is *Corydalis yanhusuo,* a plant that has been used in traditional Chinese medicine for hundreds of years owing to its vast array of therapeutic indications. The tuber of *C. yanhusuo* is known to encompass various biologically active constituents, including isoquinoline alkaloids. Thus, it will be the topic of this diploma thesis to further investigate the extracts isolated from the tubers of *C. yanhusuo* and evaluate their inhibitory activity on AChE for potential use as natural alternatives in AD therapy.

2. AIM OF WORK

1) Isolation of one alkaloid in pure form from chromatographic fraction. The fraction was prepared from primary extract of tubers from *Corydalis yanhusuo*. Methods of isolation were carried out on column chromatography and thin-layer chromatography.

2) Determination of physico-chemical properties of isolated compounds (optical rotation and R_f values in two chromatographic systems – TLC). Determination of MS and NMR spectra.

3) Determination of antioxidant activity (DPPH test) and influence on human cholinesterases – acetylcholinesterase and butyrylcholinesterase.

4) Calculation of IC_{50}, EC_{50} (statistical program GraphPad from faculty web pages).

3. THEORETICAL PART

3.1 *Corydalis yanhusuo*

Kingdom: Plantae

Phyllum: Tracheophyta

Class: Magnoliopsida

Order: Papaverales

Family: *Fumariaceae*

Genus: Corydalis

Botanical name: *Corydalis yanhusuo* W.T.Wang

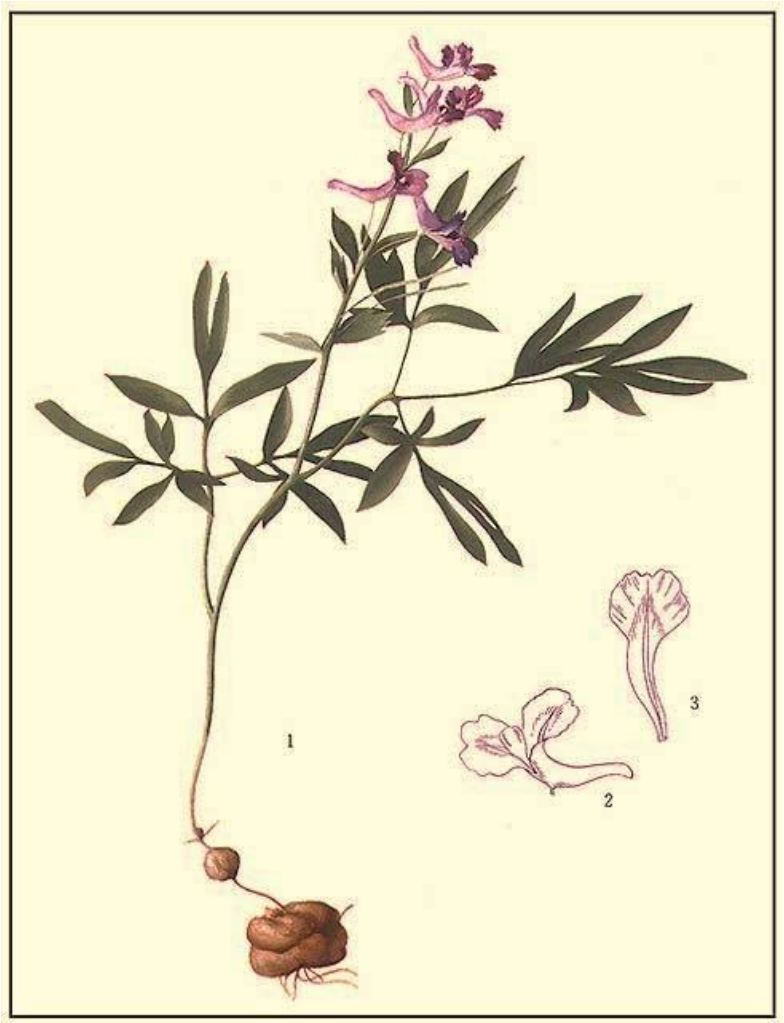

Fig. 1 ***Corydalis yanhusuo*** [2]

3.1.1 History and origin

The genus *Corydalis (*Fumariaceae) of roughly 320 species is widely distributed in the northern hemisphere and about 70 species are known to be used in traditional herbal remedies [3]. *Corydalis yanhusuo* W.T.Wang, a perennial herb belonging to the Fumariaceae family and important species of genus *Corydalis*, has been used in traditional herbal remedies in China, Japan, and Korea. *C. yanhusuo* grows wild in Siberia and Northern China and is cultivated principally in the Zhejiang province, where it is collected in the early summer season after the stems and leaves have wilted. The dried and pulverized tuber is also referred to as *Rhizoma Corydalis*. It is officially listed in the Chinese Pharmacopoeia, and in traditional Chinese medicine it has been used for hundreds of years in the treatment of gastric and duodenal ulcers, cardiac arrhythmia, rheumatism and dysmenorrhea [4]. *C. yanhusuo* has also been used to promote blood circulation, reinforce vital energy, move *qi*, and alleviate pain such as headache, chest pain, hypochondriac pain, epigastric pain, abdominal pain, backache, arthralgia, or trauma [5, 6].

3.1.2. Morphological description

The herbs of *C. yanhusuo* are perennial. The tuber is yellow, rounded, and about 1-2.5 cm in diameter. Stems are erect, 10-30 cm, with one or sometimes two scale leaves. Leaves are biternate or nearly triternate with leaflets measuring approximately 2-2.5 cm × 5-8 mm. Flowers are usually between 5-15 and bloom between April and June. Bracts are lanceolate or narrowly ovate, measuring 5-12 × 2-5 mm and sometimes lower bracts are slightly divided. The pedicel measures about 10 mm at flowering and in fruit up to 20mm. Outer petals are broad with dentate limbs and the spur of the upper petal is up curved, cylindrical, and measures about 11-13 mm. Lower petals have a short claw and inner petals measure 8-9 mm with claw longer than petal lobes. The stigma is nearly

orbicular while papillae are longer than in preceding species. Capsule are linear and measure between 20-28 mm and seeds are found in one row [7].

3.1.3. Chemical Constituents

The tuber of *C. yanhusuo* contains several tertiary and quaternary alkaloids that form the main bioactive components. However, there are still many alkaloids in the tubers that remain un-investigated, especially those in the micro, or even trace concentrations, which cannot be easily separated and identified by traditional phytochemical methods [8]. Among those identified, nearly 20 alkaloids of the tertiary and quaternary types have been isolated from *C. yanhusuo* thus far, which may be responsible for the biological activities of the drug. Their chemical structures belong to the isoquinoline family of alkaloids and can be divided into various skeletal structures [9]. They include protopine type: protopine and allocryptopine, and protoberberine/aporphine type: tetrahydropalmatine, palmatine, corydaline, dehydrocorydaline, berberine, pseudoberberine, canadine, columbamine, tetrahydrocolumbamine, glaucine, dehydroglaucine, corybulbine, dehydrocorybulbine, tetrahydrocoptisine, pseudocoptisine, and fumaricine [10].

3.1.4. Pharmacological Activity of Main Alkaloid Constituents

Numerous alkaloids displaying a wide range of pharmacological actions including analgesic [11, 12], anxiolytic [5], hypnotic [8], antiamnestic [9, 13] anti-inflammatory [14-16], antiplatelet [17] and cardioprotective [3, 18, 19] have been isolated form the tuber of *Corydalis.*

3.1.4.1 Tetrahydropalmatine

One of the main active constituents isolated from *C. yanhusuo* is *dl*- tetrahydropalmatine (*dl*-THP). Derived from the tetrahydroprotoberberine backbone structure, *dl*-THP belongs to the isoquinoline alkaloid family [5]. It can also be directly synthesized from laudanosine, a benzylisoquinoline alkaloid via chemical conversion [20].

Pharmacologically, it has been shown that *dl*-THP exerts marked analgesic, sedative-tranquilizing and hypnotic action and it has been listed in the Chinese Pharmacopoeia since 1977 for these indications [8, 11]. It was found that *dl*-THP could display such effect, probably due to its antagonistic action on the D_1/D_2 receptor in the brain [21]. However, in a study by Leung *et al.*, 2003, it was proposed that *dl*-THP could also act on the benzodiazepine site (BDS) of the $GABA_A$ receptor in the mouse brain. The main findings from the animal behavioral tests concluded that *dl*-THP manifests anxiolysis at defined low dosages at least in part, and this effect is mediated through the BDS, thus concluding that the anxiolytic effects of *dl*-THP could be produced by a combination of effects from several receptors in the CNS including D_1/D_2 receptor and $GABA_A$ receptor [5].

Studies aiming to distinguish between the various mechanisms of action by which *dl*-THP functions in the CNS have been carried out and in one such study, it was shown that *dl*-THP depletes levels of the neurotransmitters dopamine, noradrenaline and serotonin in the central nervous system [22]. In addition, it has been reported that the two enantiomers of *dl*-THP act on different targets – *d*-THP depletes dopamine while *l*-THP functions as a dopamine antagonist [23]. Interestingly, it was also found that *dl*-THP decreases both arterial pressure and heart rate through a serotonergic release process in the hypothalamus [24].

In a recent study by Oh *et al.*, 2010, on the inhibition of pro-inflammatory mediators, THP inhibited lipopolysaccharide (LPS)-induced interleukin (IL)-8 production

in a dose-dependent manner. Furthermore, THP inhibited extracellular signal-regulated kinase and p38 mitogen-activated protein kinase (MAPK) phosphorylation, which suggests that THP inhibits IL-8 secretion by blocking MAPK phosphorylation [14].

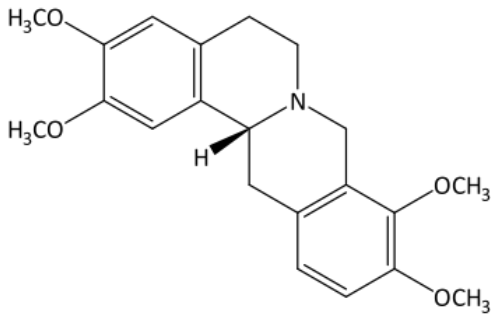

Fig. 2 Tetrahydropalmatine

3.1.4.2 Dehydrocorydaline

In a study by Kubo *et al.*, 1993, on the potential anti-inflammatory activities of methanolic extract from *Corydalis* tuber, it was found that among the tested alkaloidal components, dehydrocorydaline showed a stronger inhibitory effect than that of the standard drug, in this case disodium cromoglycate [15]. The methanolic extract of *Corydalis* tuber showed an inhibitory effect against histamine release from mast cells but also inhibitory effect on the released histamine when administered to isolated guinea pig ileum. It was therefore suggested as having a potentially important future implication in the therapeutic field of inflammatory disease [15].

Studies also show that dehydrocorydaline not only inhibits antibody-mediated allergic reactions but also influences cell-mediated allergic reactions [25].

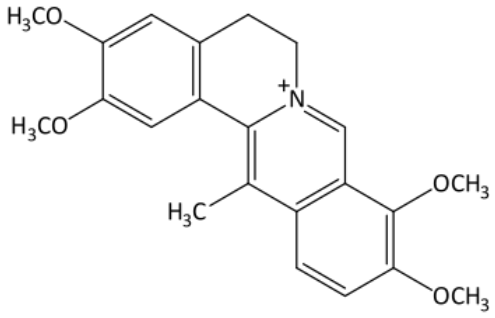

Fig. 3 Dehydrocorydaline

3.1.4.3 Protopine

Protopine was reported to exhibit an inhibitory activity on platelet aggregation [17]. Protopine was also found to possess potent anti-nociceptive effects due to its ability to function as an inhibitor of both serotonin and noradrenaline transporters [12]. It has also been described that following treatment with protopine, a significant decrease in glutamate level and an increase in glutamate dehydrogenase activity was observed in rat brains [26]. Since glutamate plays a significant role in nociceptive processing in central and peripheral nervous systems [27, 28], the decrease in glutamate level might also be associated with the anti-nociceptive effects of protopine [10].

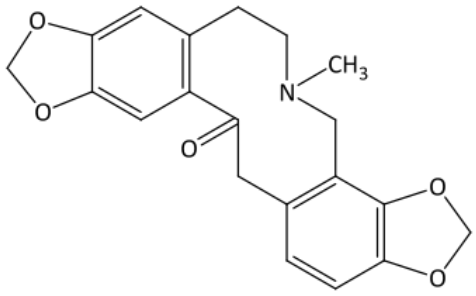

Fig. 4 Protopine

3.1.4.4 Pseudocoptisine

Based on a study conducted by Hung *et al.*, 2008, it was found that psuedocoptisine displayed remarkable cognitive-enhancing activity mediated in part by its ability to inhibit adult male rat AChE activity in a dose dependent manner (IC_{50} = 12.8 μM) [13]. Pseudocoptisine treatment (2.0, 5.0 mg/kg) also reversed the deficits produced by scopolamine treatment in the comparison with the vehicle-treated group on passive avoidance task. At a concentration of 2.0 mg/kg, pseudocoptisine significantly shortened the escape latency and improved swimming time within the zone of platform on water maze task [13]. The passive avoidance test is generally accepted as an indicator of long-term memory in animals [29], and the water maze-learning task was used to asses hippocampal-dependent spatial learning ability [30, 31].

Pseudocoptisine caused dose-dependent reductions in the levels of inducible nitric-oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) at both protein and mRNA levels and concomitant decrease in PGE_2 and NO production. In addition, it was found that pseudocoptisine suppressed the production and mRNA expressions of pro-inflammatory cytokines, such as, TNF-α and IL-6 [16].

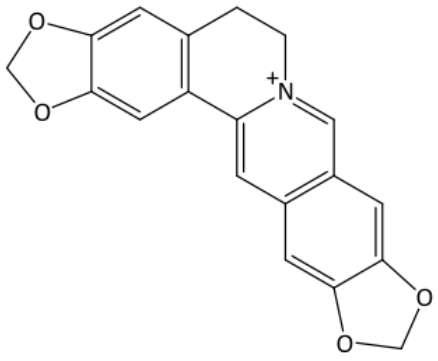

Fig. 5 Pseudocoptisine

3.1.4.5 Pseudoberberine

In a recent in vivo experiment, Hung *et al.*, 2008, found that pseudoberberine inhibited mouse brain cortex AChE activity in a dose-dependent manner with an IC_{50} value of 4.5 μM. Also, treatment in mice with 5.0mg/kg could reverse the deficits produced by scopolamine in comparison with vehicle-treated group on passive avoidance task, and significantly shortened the escape latency and improved the swimming time within the zone of platform on water maze task when evaluating spatial learning [9].

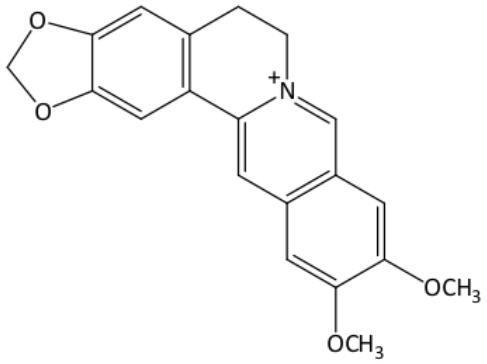

Fig. 6 Pseudoberberine

3.2 Other *Corydalis* species

In addition to the officially listed *C. yanhusuo*, there are many species of the genus *Corydalis* known to be of use in traditional Chinese medicine or folk medicine.

3.2.1 *Corydalis speciosa*

Corydalis speciosa has been used in Korea and China as a folk medicine for its antipyretic, analgesic and diuretic properties. In a study by Kim *et al.*, 2004, the methanolic extracts of the aerial parts of *C. speciosa* were found to exhibit significant AChE inhibitory activity. Four compounds were separated as active constituents and identified as corynoxidine, protopine, palmatine and berberine [32]. All four compounds inhibited male mouse AChE in a dose-dependent manner with IC_{50} values of 89.0, 16.1, 5.8, and 3.3 μM respectively [32].

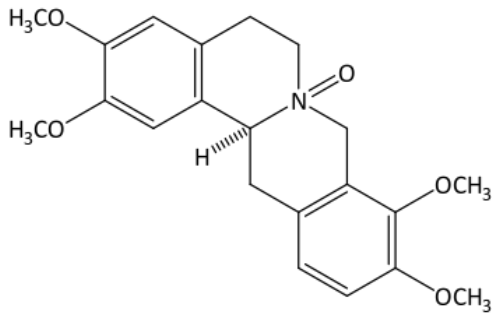

Fig. 7 Corynoxidine

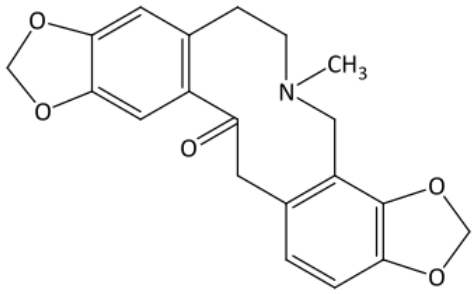

Fig. 8 Protopine

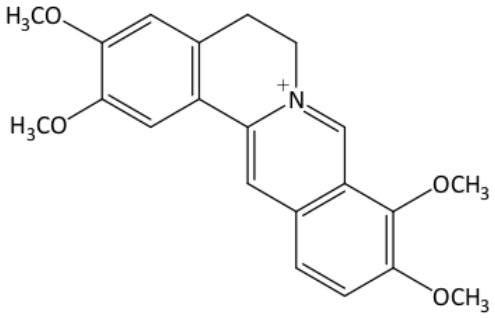

Fig. 9 Palmitine

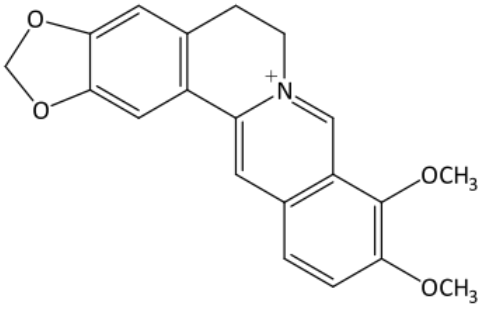

Fig. 10 Berberine

3.2.2 *Corydalis cava*

In a study using plants from Danish folk medicine described as memory enhancers, a crude methanolic extract of tubers from *Corydalis cava* demonstrated that corydaline, a tetrahydroberberine skeletal type alkaloid, inhibited AChE in a dose-dependent manner. The heads of Drosophila melanogaster were used as the enzyme source and an IC_{50} value of 15μM± 3μM was obtained along with bulbocapnine with an IC_{50} value of 40 ± 2 μM from [33].

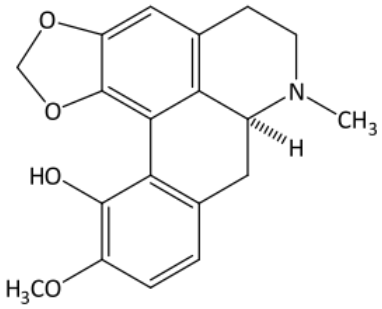

Fig. 11 Bulbocapine

3.2.3 *Corydalis saxicola*

Corydalis saxicola is a perennial herb native to China, and in traditional Chinese medicine it has been noted for its use in the treatment of inflammation, pain, and hepatic diseases. In a recent study by Cheng *et al.*, 2008, it was determined through a DNA cleavage assay that the alkaloids specifically inhibited topoisomerase through

stabilization of the enzyme–DNA complex. Among the isolated alkaloids, pallidine and scoulerine showed strong inhibitory activities toward topoisomerase I that were comparable to camptothecin, an atypical topoisomerase I inhibitor [34]. Interestingly, in another study on *C. saxicola,* it was found that alkaloidal constituents within this plant exhibited potential anti-hepatitis B activity [35].

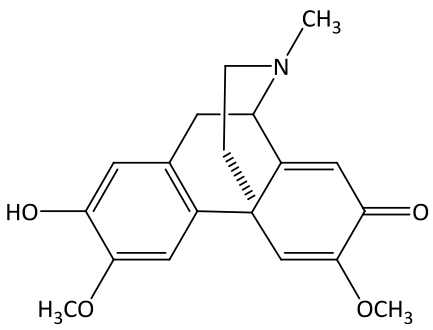

Fig. 13 Pallidine

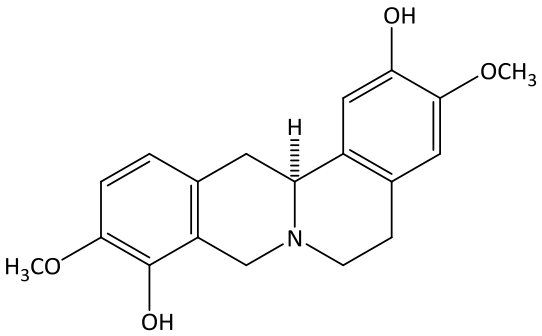

Fig. 14 Scoulerine

Another of the main active constituents of *C. saxicola,* dehydrocavidine, was found to exhibit a potent hepatoprotective effect on CCl_4-induced liver injury in rats owing to its antioxidant activity. In a recent study by Wang *et al.,* 2008, both pre- and post-treatment with dehydrocavidine prior to CCl_4 administration significantly prevented increases in serum enzymatic activities of alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, alkaline phosphatase and total bilirubin. Thus it was concluded that dehydrocavidine displays a potent hepatoprotective effect on CCl_4-induced liver injury in rats mediated through its antioxidant activity [36].

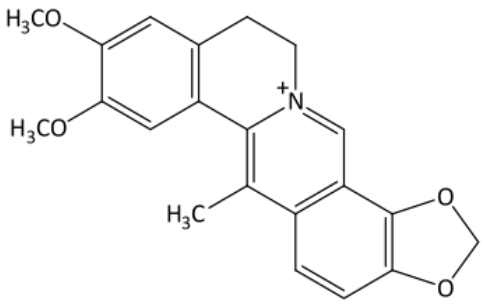

Fig. 12 Dehydrocavidin

3.3. Alzheimer's disease

Although age-related loss of memory and cognitive decline have been documented for thousands of years in human history, AD has only existed as a defined medical condition for roughly 100 years. As with many other conditions, ancient writings suggest remedies based on natural compounds and plant extracts. An example of such historical indications is *Withania somnifera*, or Ashwagandha in ancient Sanskrit, which was renowned in Ayurvedic medicine as 'medharasayan' or promoter of learning and memory retrieval in ancient India almost 4,000 years ago [37].

AD is a progressive and fatal neurodegenerative disorder manifested by cognitive and memory deterioration, progressive impairment of activities of daily living, and a variety of neuropsychiatric symptoms and behavioral disturbances [38]. In AD, the progressive nature of neurodegeneration suggests an age-dependent process that ultimately leads to degeneration of the synaptic afferent system, dendritic and neuronal damage, and formation of abnormal protein aggregates throughout the brain [39]. The main neuropathological changes associated with AD are β-amyloid (βA) plaques, neurofibrillary tangles (NFT's), and neuronal loss or dysfunction. The NFTs accumulate as abnormal components of the neuronal cytoskeleton aggregated into paired helical

filaments, whereas the plaques are comprised of dystrophic neurites and glial elements and have a core of amyloid peptide, which is derived from a larger amyloid precursor protein (APP).

Although the senile plaques and NFTs are considered to be the pathological hallmarks, there is strong evidence that multiple neurotransmitter systems are affected in the AD brain. Since the most prominent abnormalities are seen to arise in the cholinergic system, the cholinergic hypothesis of AD was suggested, and further became the leading strategy for the development of AD medication.

3.3.1 Pathological hallmarks of Alzheimer's disease

3.3.1.1 β-Amyloid plaques

The amyloid cascade is a sequence of events typically seen in AD which leads to the abnormal processing of the APP causing production, aggregation, deposition and toxicity of its Aβ derivative [40]. The production of Aβ from APP is dependent upon the activities of two enzymes, β-secretases and γ-secretases. The APP molecule is cleaved at different positions by two individual proteases, α- and β-secretase, further leading to the release of the large soluble N-terminal fragments, α-APPs and β-APPs, respectively. Cleavage by α-secretase occurs within the region containing Aβ, consequently preventing the formation of Aβ. However, since β-secretase cleavage generates the free N-terminus of Aβ, it is considered the first critical step in amyloid formation [41].

3.3.1.2 Presenilins and tau-phosphorylation

Presenilins are two proteins, presenilin 1 (PS1) and presenilin 2 (PS2), located in intracellular membranes, which are primarily expressed in neurons and universally expressed in the brain [42]. PS1 is required for proper formation of the axial skeleton and

is involved in normal neurogenesis and survival of progenitor cells and neurons on specific brain regions. PS1 also takes part in γ-secretase activity and binding of PS proteins to APP may play an important role in inducing intercellular signaling. Two conserved transmembrane aspartate residues in PS1 are critical for Aβ production, suggesting that PS1 either functions as an essential cofactor for γ-secretase, or is itself γ-secretase [43].

Mutations in genes of APP and presenilins have been shown to modify the processing of APP, through alteration of secretase activities. This process leads to an increase in Aβ and may trigger aggregation and induce the path to neurodegeneration [44]. The majority of early onset familial AD cases are caused by mutations within the PS genes [45].

Tau is a phosphoprotein containing multiple phosphorylation sites belonging to the family of microtubule associated proteins and widely expressed in the brain [45]. The primary function of tau is to maintain microtubule stability [46]. It is also found as the major component of NFTs. According to the tau and tangle theory, in AD the natural role of tau in stabilizing microtubules is impaired. Aggregated tau becomes hyperphosphorylated, where by reducing its ability to bind microtubules, and consequently diseased neurons microtubules are gradually replaced by tangles [45, 47].

3.3.2 The Cholinergic hypothesis

The cholinergic hypothesis of AD evolved from original observations by Davies and Maloney in 1976, which first reported decreased numbers of cholinergic neurons in autopsy brain tissue from patients with AD [48, 49]. The cholinergic hypothesis was supported by observations in loss of cholinergic markers, such as choline acetyltransferase and AChE, in patients with AD at post-mortem [50, 51], along with the

correlation of mental test scores and severity of dementia with cholinergic abnormalities in late-stage AD [48, 52].

Acetylcholine (ACh) is produced in cholinergic neurons from acetyl coenzyme A (CoA) and choline by the action of the enzyme choline acetyltransferase. ACh is concentrated in vesicles by the action of the vesicular ACh transporter and released from presynaptic cells following depolarization. The activation of postsynaptic muscarinic ACh and nicotinic receptors leads to the activation of biochemical pathways or depolarization of the target cell and thus, the propagation of the nerve impulse.

In the synaptic cleft, ACh is quickly inactivated by ChEs, breaking it down to acetate and choline. Two ChEs are present in mammals: AChE, which selectively hydrolyses ACh, and BuChE, which is capable of hydrolyzing ACh as well as other choline esters [53].

Both AChE and BuChE exist in several globular and asymmetrical forms. A G4 tetrameric form comprised of four globular protein subunits, and a G1 monomeric form with a single globular protein moiety are known to coexist [54]. The proportions of G1 and G4 forms vary in different human brain regions [55], but for both enzymes, G4 is found as the predominant isoform in the mature healthy brain [56].

At the molecular level, the structure of BuChE is similar to that of AChE, displaying only a slight difference in its amino acid sequence [57]. Both enzymes have a primarily hydrophobic active gorge, shown by X-ray crystallography to be 20 Å deep for AChE, into which ACh diffuses and is cleaved [58]. Once ACh enters this active site, it binds at two locations, a catalytic region near the base of the gorge and a choline-binding site midway up. Structural features of the two ChE enzymes explain the differences in their substrate specificity.

It has been proposed that the efficiency with which AChE and BuChE hydrolyze ACh is dependent on the substrate concentration. AChE shows greater catalytic activity

at low ACh concentrations, resulting in substrate inhibition at higher doses, whereas BuChE is more efficient at high substrate concentration [59]. These differences in the enzymatic kinetic properties and locations of brain AChE and BuChE propose that in the normal brain, AChE is the main enzyme responsible for ACh hydrolysis while BuChE maintains a supportive function [60]. However, it is interesting to note that in patients with AD, BuChE levels in the brain and CSF are found to increase whereas those of AChE decrease [61].

BuChE is widely distributed in the brain regions affected in AD, such as the temporal cortex, hippocampus and amygdala. Nuclei expressing high proportions of BuChE are implicated in working memory, attention, executive function and behavior, all of which are universal deficits in AD [62]. What is more, ACh metabolism may become increasingly dependent on BuChE activity as AD progresses [63, 64] and thus the inhibition of BuChE in addition to AChE would be expected as a valuable therapeutic approach [65].

It is proposed that both AChE and BuChE may also have a role in the aggregation of Aβ that occurs in the early stages of senile plaque formation [66]. As AD progresses, there is evidence indicating that both G1 forms AChE and BuChE become increasingly accumulated within the amyloid plaques and NFT's [66, 67]. Since levels of the G1 form of both enzymes are found to be positively correlated with plaque density and pathogenicity, inhibiting these enzymes could potentially augment cholinergic function in AD [68]. Thus, classical cholinergic signal transduction pathways may protect against neuronal degeneration by various routes including modifications in the formation of amyloidogenic compounds and reductions in tau-phosphorylation [69], as well as reductions in neuronal vulnerability to Aβ toxicity [70].

3.3.3 Current therapy in Alzheimer's disease

Currently, cholinesterase inhibitors are classified pharmacologically into three groups on the basis of their duration of inhibition- short acting, intermediate acting, and long acting. Donepezil and galanthamine are relatively selective for AChE and are fully reversible inhibitors that bind briefly to AChE and then dissociate to restore enzyme activity. Tacrine and rivastigmine co-inhibit both AChE and BuChE. Rivastigmine is a very slowly reversible ('pseudo-irreversible') inhibitor of both AChE and BuChE and tacrine produces reversible inhibition [65]. The pattern and types of symptomatic benefits differ between ChE´s and suggestions have attributed these differences in pharmacological effect to the various ChE forms within the CNS [71, 72].

In 1993, tacrine (Cognex®), an aminoacridine, was the first FDA approved AChE inhibitor for the treatment of cognitive decline in patients with AD. Since then, several other AChE inhibitors have appeared on the market including the piperidine derivative donepezil (Aricept®) in 1996, rivastigmine (Exelon®) in 2000, and the naturally based galanthamine (Reminyl®) in 2001 [73].

Recently in 2004, memantine (Namenda, Axura®), an N-methyl-D-aspartate (NMDA) receptor antagonist was approved by the FDA on the basis of glutamate-mediated neurotoxicity in AD [74]. Memantine functions as a neuroprotective at least in part through the inhibition of excitoxicity, which if not halted, leads to neuronal injury or death through over-stimulation of the NMDA receptors by excess exposure to the neurotransmitter glutamate [75].

Controlled clinical trials still prove the use of ChE inhibitors as being the most consistently successful method for treating the cognitive, functional and behavioral symptoms associated with AD [53]. In comparison to untreated AD patients, whose cognitive functions were reported to decline, those treated with ChE inhibitors were reported to display cognitive improvements from baseline [76, 77]. ChE inhibitor

treatment has also been proven to enhance quantitative electroencephalogram coherence with decreased slow-wave activity and increased faster frequencies, reflecting increased cortical arousal, improvements in concentration, sensory processing, learning, and memory [78, 79]. A recent meta-analysis by Trinh *et al.,* 2003, involving six randomized, double-blind, placebo-control trials of ChE inhibitors, concluded that as a class, these agents display a modest, beneficial impact on neuropsychiatric symptoms in patients with mild-to-moderate probable AD [80]. Although further investigations into evaluating the effectiveness of AChE and/or BuChE inhibitors are still needed, they currently remain and hold a promising future as the drugs of choice in treating the symptoms associated with mild-to-moderately severe forms of AD.

3.4 Natural compounds influencing the metabolism of AChE and BuChE

From 1981 to 2006, 63% of all low molecular weight drugs developed where from natural products or natural product-derived compounds [81]. In the quest for additional AChE inhibitors, various medicinal plants and natural resources have been screened in the hope of finding substances with comparative IC_{50} values to those of currently approved drugs on the market. The search for plant derived inhibitors of AChE has accelerated inlight of the benefits of these drugs not only in the treatment of AD but in other forms of dementia [82]. There are currently only a few synthetic medicines for the treatment of cognitive dysfunction and memory loss associated with mild-to-moderate AD [83]. Many of these compounds have been reported to present adverse effects including GIT disturbances, hepatotoxicity and problems associated with bioavailability [84-86], which further promotes the interest in finding more effective AChE inhibitors

from natural resources. Supplementary details beyond this text of natural product inhibitors of AChE can be found in reviews by Hostettmann *et al.,* 2006, [87] and Mukherjee *et al.,* 2007, [83].

3.4.1 Alkaloids

3.4.1.1 Physostigmine, *Physostigma venenosum,* Fabaceae

Physostigma venenosum was traditionally used in Africa as a ritual poison. Treatment with the indole alkaloid physostigmine, a short-acting reversible AChE inhibitor, isolated from *P. venenosum*, has shown cognitive benefits in both normal and AD patients [83]. However, due to its short half-life, physostigmine was found clinically impractical since a multiple dosing scheme would be required. Instead, the chemical structure of physostigmine was used as a prototype for the development of rivastigmine, a carbamate based AChE inhibitor now approved under the trade name Exelon® for symptomatic treatment of mild-to-moderately severe AD.

3.4.1.2 Galanthamine, *Galanthus nivalis,* Amaryllidaceae

Galanthus nivalis was used traditionally in Bulgaria and Turkey for neurological conditions. Initially derived from the extracts of snowdrop and daffodil bulbs, galanthamine is now a synthetically produced AChE inhibitor. In a randomized, 6-month, multicenter clinical trial, galanthamine showed improvements in activities of daily living and behavioral symptom when compared to placebo in patients with probable AD or vascular dementia [82]. Galanthamine can be taken as a novel representative for successful natural product substitution in place of synthetic drug treatment in AD.

3.4.1.3 Huperzine A, *Huperzia serrata,* Lycopodiaceae

Huperzia serrata is a moss used to treat contusions, strains, hematuria and swelling in traditional Chinese medicine [88]. The sesquiterpene alkaloid huperzine A is a potent, yet reversible inhibitor of AChE. In a study by Raves *et al.*, 1997, huperzine A improved memory retention process in cognitively impaired aged and adult rats [89]. In China, studies conducted by Wang *et al.*, 2006, showed enhancement in memory, cognitive skills, and improvements in daily activities, after administration of huperzine A to patients with AD [90].

3.4.1.4 *Chelidonium majus, Papaveraceae*

Chelidonium majus has traditionally been used as an herbal medicine in the treatment of gastric ulcer, gastric cancer, oral infections and general pain in Asian and European countries. In a recent study, Cahlikova *et al.*, 2010, demonstrated that the most active of the natural occurring alkaloids was chelidonine, which inhibited both human AChE and BuChE in a dose-dependent manner with IC_{50} values of 26.8 ±1.2μM and 31.9 ±1.4μM respectively [91].

3.4.2 Terpenoids

3.4.2.1 *Salvia lavandulaefolia,* Lamiaceae

The ChE inhibition produced by *Salvia lavandulaefolia* oil was shown to be partly due to the cyclic monoterpenes 1,8-cineole and α-pinene, which were found to inhibit AChE *in vitro*. Upon oral administration of *S. lavandulaefolia* essential oil to rat's, a decreased striatal AChE activity in both the striatum and the hippocampus was observed and therefore it was postulated that the *in vitro* and also *in vivo* inhibition of AChE in select brain regions was connected to the activity of either constituents or their metabolites [92].

3.4.2.2 *Melissa officinalis*, Lamiaceae

Melissa officinalis has been used for more than 2,000 years owing in part to its reputation for restoring memory and promoting long life. Although the constituents have not been thoroughly investigated, the plant is known to possess monoterpenes in its essential oil, including citral (a mixture of isomers geraniol and nerol), and it is known from previous studies that these compounds possess a weak inhibitory effect on AChE [93].

3.4.2.3 *Origanum majorana,* Lamiaceae

Origanum majorana is a plant found in Indian medicine and also more commonly known as a spice. In a study testing its inhibitory effect on AChE, the main active component, identified as the triterpene ursolic acid, exhibited an IC_{50} value of 7.5 nM [94].

3.4.3 Withanolides

3.4.3.1 *Withania somnifera,* Solanaceae

The root of this plant, also known as Indian ginger, is one of the most highly regarded herbs in Ayurvedic medicine where it is classified among the rejuvenating tonics known as 'Rasayanas'. Compounds present in *W. somnifera* are structurally related to steroids and more commonly referred to as withanolides. In a study aimed at the cholinesterase inhibitory effect of withanolides, Choudhary *et al.*,2004, isolated and identified six compounds, of which four dislayed inhibitory effect against electric eel AChE, while the remaining two inhibited horse-serum BuChE [95].

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 was 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. Chambers were saturated with the mobile phase. The time of saturation was approx. 30 minutes and in the 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, 0.1–0.25mm, 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)

Material:

- Source of AChE: hemolysed human erythrocytes

  Whole blood was centrifuged for 15 minutes at 10,000 rev / min. The 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,Warszaw, Poland)
- Instrument for measurement of optical rotation: ADP 220 POLARIMETER B+S
- Micro-heated apparatus 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 purum (Sigma-Adrich)
- Quercetin (Sigma-Aldrich)
- Trolox p.a. (Sigma-Aldrich)

4.2.4 Detection reagents

Dragendorf's reagent modified according to Munier:

- Solution A: prepared by dissolving 1.7 g bismuth subnitrate 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 some months in a refrigerator (4 °C).
- 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 layer maximally 2 cm thick at 200 °C in the dryer for 8 hours. After cooling to ~ 80 °C, the adsorbent was poured into a flask and sealed. After cooling at room temperature, 5% water (w/w) was added and equilibrated upon periodic shaking.

- *Kieselgel 60 GF_{254}, plates for TLC*

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 at room temperature.

Solution of fraction was spotted in the form of a line about 10 mm from the upper edge of the plate by using an application-tube.

4.3 Description of alkaloid and its isolation

4.3.1 Origin of herbal drug

Ground tubers *of C. 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 extract

10.8 kg of dry tuber were percolated with 120 liters of 95% ethanol (~ 1:11). Collected extract was evaporated to a viscous residue, heated at 50 °C, and 2.5 liters of 2% hydrochloric acid was added. The brown solution was decanted and the solid residue in the flask was homogenized with 1 liter of 2% hydrochloric acid and sonified at 50 °C on level 10 (apparatus Sonorex 10HP) for 30 minutes. The suspension was then filtered through viscous cellulose and the filtrate was diluted with 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). The suspension with alkaloids was operated by ether (5× 1.6 liters). The organic layer was then 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. This 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, four 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 Buleza Koci.

Fig. 15 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 $\lambda = 254$ 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 the mixture was dried. After drying, 5 g of Celite 545 was added and the mixture was homogenized. This dry trituration was then applied to the chromatographic column.

Table 1. 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 × 6 cm
Layer with adsorbent	3 × 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 $\lambda = 254$ nm, Dragendorf reagent (modif. according to Munier).

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

Table 2. Results of column chromatography of non-phenolic alkaloids from chlorides soluble in chloroform

Fraction		Eluent	Weight	Description
1-42	1-12	Petrol+$CHCl_3$ 95:5	0.050 g	Yellow oil
	13-22	Petrol+$CHCl_3$ 92.5:7.5		
	23-42	Petrol+$CHCl_3$ 90:10		
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	Petrol+$CHCl_3$ 80:20	0.28 g	Brown, crystals
	66-67	Petrol+$CHCl_3$ 75:25		
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	Petrol+$CHCl_3$ 75:25	3.20 g	Yellow-brown, crystals
	86-95	Petrol+$CHCl_3$ 70:30		
	96-97	Petrol+$CHCl_3$ 25:75		
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

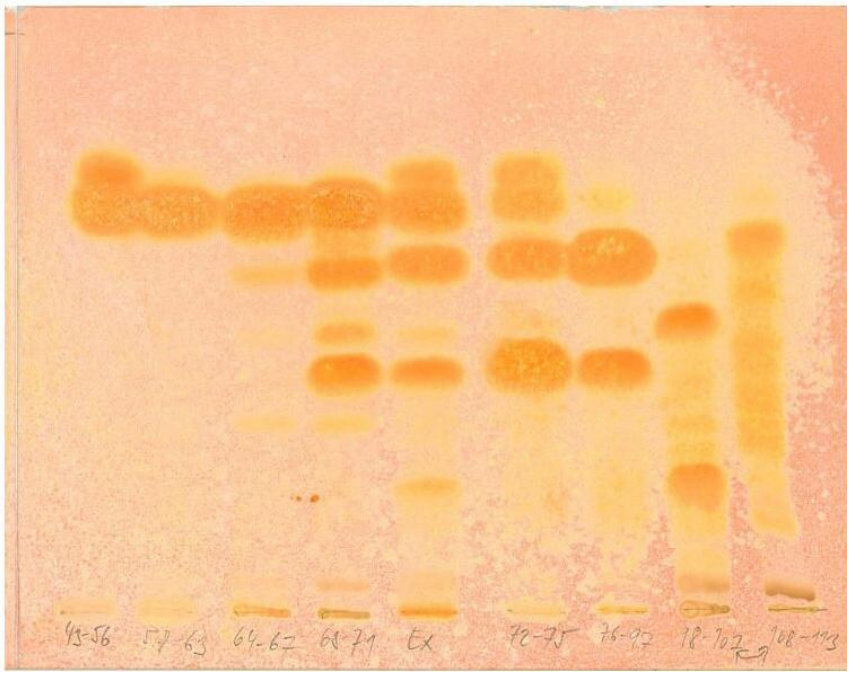

Fig. 16 Results of column chromatography on aluminum oxide

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

4.3.6 Isolation of alkaloid from combined fractions 57-63

0.57 g of brown-reddish crystalline mass was dissolved in a 15 ml of mixture chloroform + toluene 95 : 5 (w/w). The solution was filtered through 6.0 g of Aluminum oxide neutral in a micro-chromatographic column (diameter × height 8 : 12 mm) and from the compound, 20 ml of the mentioned mixture of solvents was eluted. After evaporation of filtrate, 0.50 g of yellowish crystalline mass was obtained and designated GC-1.

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 collision energy of 40 eV and the substance was dissolved in methanol.

4.5 Method for determining NMR spectra

The spectra were measured on a Varian Inova 500 spectrometer with a working frequency of 499.9 MHz for 1H and 125.7 MHz for 13C 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 (hexamethyldisilane, 0.04 ppm in ^{1}H spectra) or the solvent signal (76.99 ppm in 13C spectra)(Dr. M. Kurfürst, Ph.D., Institute of Chemical Process Fundamentals, ASCR, Prague).

4.6 Method for determining antioxidant activity- 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 [96]. 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 used 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 was 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 was related to the concentration of antioxidant in the tested solution. The percentage of inhibition of DPPH was estimated by using the formula: % $Q_{DPPH} = (1-A_x/A_0) \times 100$, where A_0 was the height of the peak of the blank sample and A_x was the height of the peak after the extract/pure compound was added. All measurements were made in triplicate. The DPPH radical scavenging activity of samples was expressed as EC_{50} (mg/mL for extracts, μM for pure compounds), which was the amount of sample necessary to decrease by 50% the light absorbance.

4.7 Methods for determinating 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 was added, according to a slightly modified method of Steck and Kant [97]. Briefly plasma (HuBuChE) was 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 were determined with a modified method of Ellman *et al.*,[98] at concentrations 500, 250, 125, 50, 25, 12.5, 5, 2.5, 0.5, and 0.25 μg/mL using acetylthiocholine iodide (ATChI) and butyrylthiocholine iodide (BTChI) as substrates, respectively. Briefly, 25-50 μL of ghosts or plasma, 650 μL of DTNB and 25 μL of either the sample or appropriate solvent, as a blank sample, were added to the semi-micro cuvette. The reaction was initiated by addition of substrate (ATChI or BTChI). The final proportion of DTNB to substrate was 1 : 1. The increase of absorbance at 436 nm (ΔA) was measured for one minute using a Shimadzu UV-1611 spectrophotometer. Each measurement was repeated three times. Galanthamine and huperzine A were used as positive controls. The IC_{50} and EC_{50} values were calculated with the use of GraphPad Prism 5.02 software. The inhibition (in %) was calculated according to the formula: $\%I = 100-(\Delta A_{BL}/\Delta A_{SA})*100$, where ΔA_{BL}= increase of absorbance of a blank sample and ΔA_{SA}= increase of absorbance of the measured sample.

4.8 Method for determining optical rotation

The optical rotation was measured on polarimeter ADP 220 BS ethanol.

5. RESULTS

5.1 Structural analysis of compound GC-1

The structure of the isolated compound was determined by comparing spectral data with those reported in the literature as (+)-corydaline (CAS: 3907-48-0).

5.1.1 MS analysis of (+)-corydaline

ESI-MS m/z $[M+H]^+$ 370.24 (100).

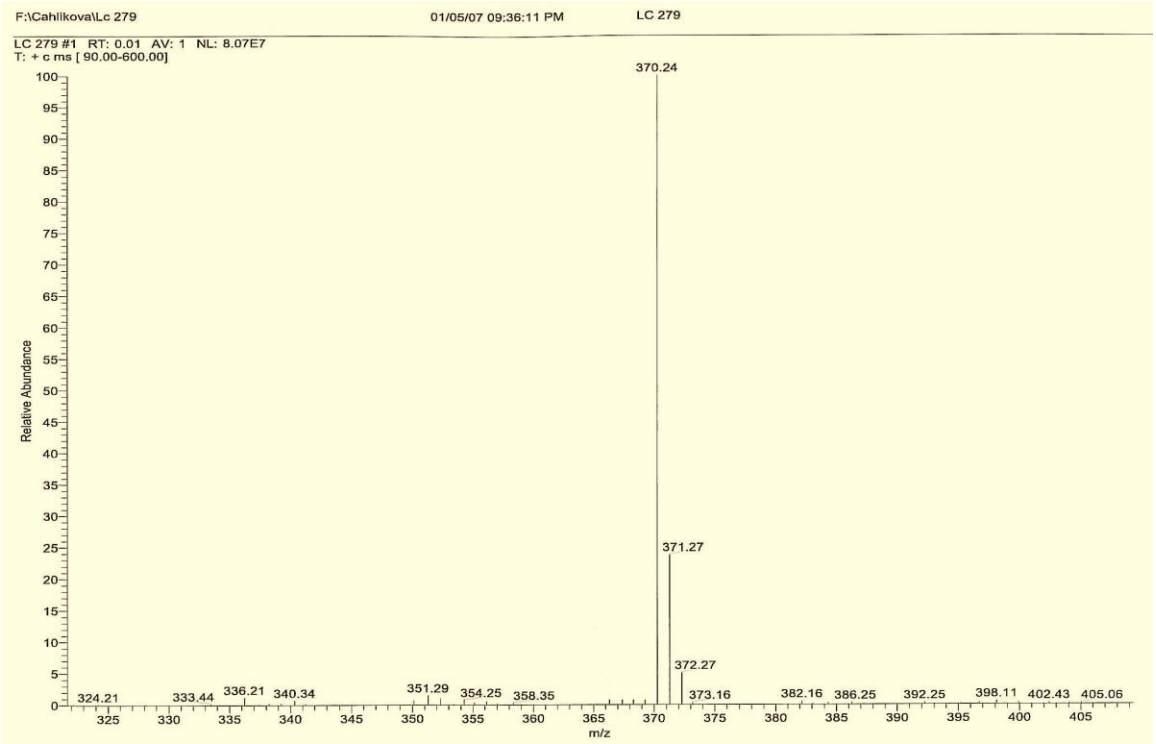

Fig.17 MS Spectrum of (+)-corydaline

5.1.2 MS/MS analysis of (+)-corydaline

MS/MS m/z 355.16 (20; $[M-CH3]^+$), 338.14 (15), 308.11 (12), 218.14 (22), 192.14 (100; $[C_{11}H_{14}O_2N]^+$), 165.07 (25; $[C_{10}H_{13}O_2]^+$).

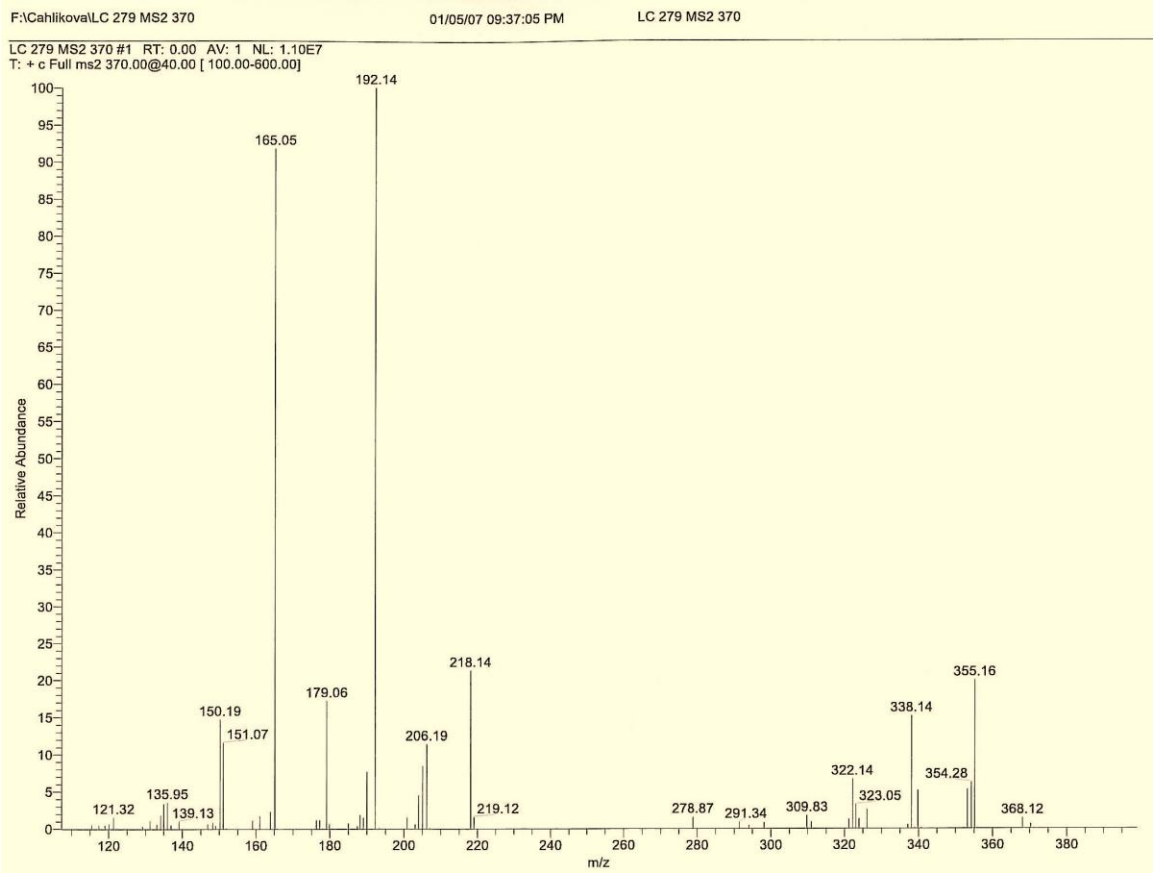

Fig. 18 MS/MS spectrum of (+)-corydaline

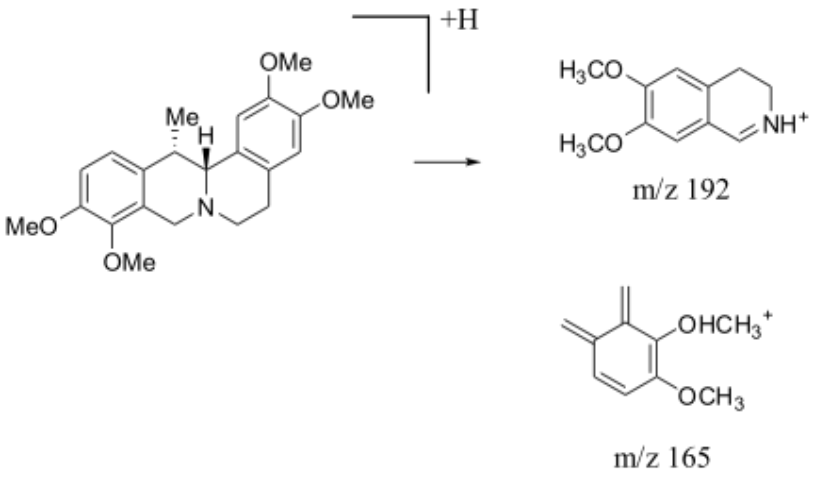

Fig.19 MS/MS spectrum of corydaline and its proposed retro-Diels-Alder (RDA) pathway

5.1.3 NMR analysis of (+)-corydaline

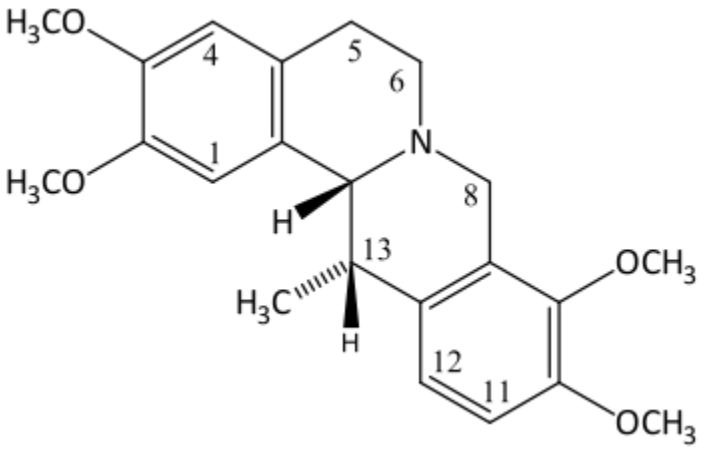

Fig. 20 Structure of isolated compound (+)-corydaline

5.1.3.1 ^{1}H-NMR analysis of (+)-corydaline

^{1}H NMR ($CDCl_3$, 25°C):

0.95 (3H, d, J = 7.0 Hz, R-CH_3); 2.59 - 3.17 (4H, m, H-5, H-6); 3.20 (1H, dd, J = 7.0 Hz, H-13); 3.50 (1H, d, J = 15.9 Hz, H-8α), 3.68 (1H, br s, H-13a), 3.85 (6H, s, 2 R-OCH_3), 3.87 (6H, s, 2 R-OCH_3), 4.20 (1H, d, J =15.9 Hz, H-8ß), 6.60 (1H, s, H-4), 6.68 (1H, s, H-1), 6.82 (1H, d, J = 8.4 Hz Hz, H-11), 6.90 (1H, d, J = 8.4 Hz, H-12)

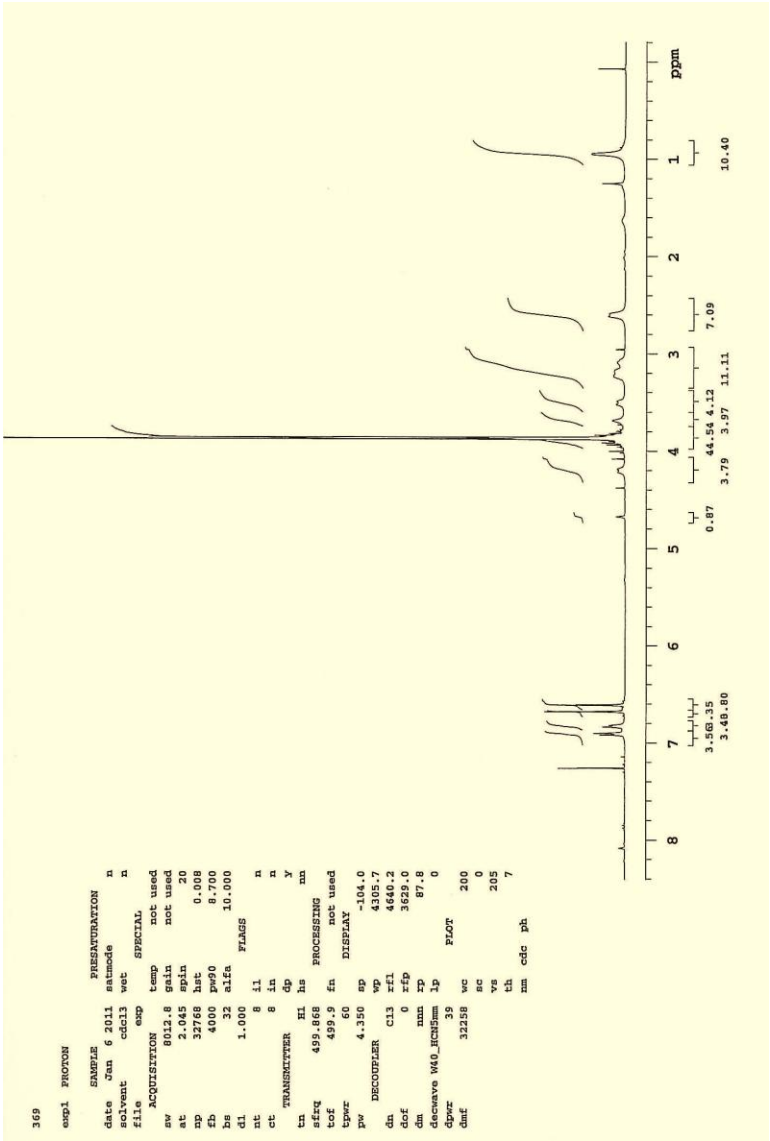

Fig.21 ^{1}H-NMR spectrum of (+)-corydaline

5.1.3.2 ^{13}C-NMR analysis of (+)-corydaline

^{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-1

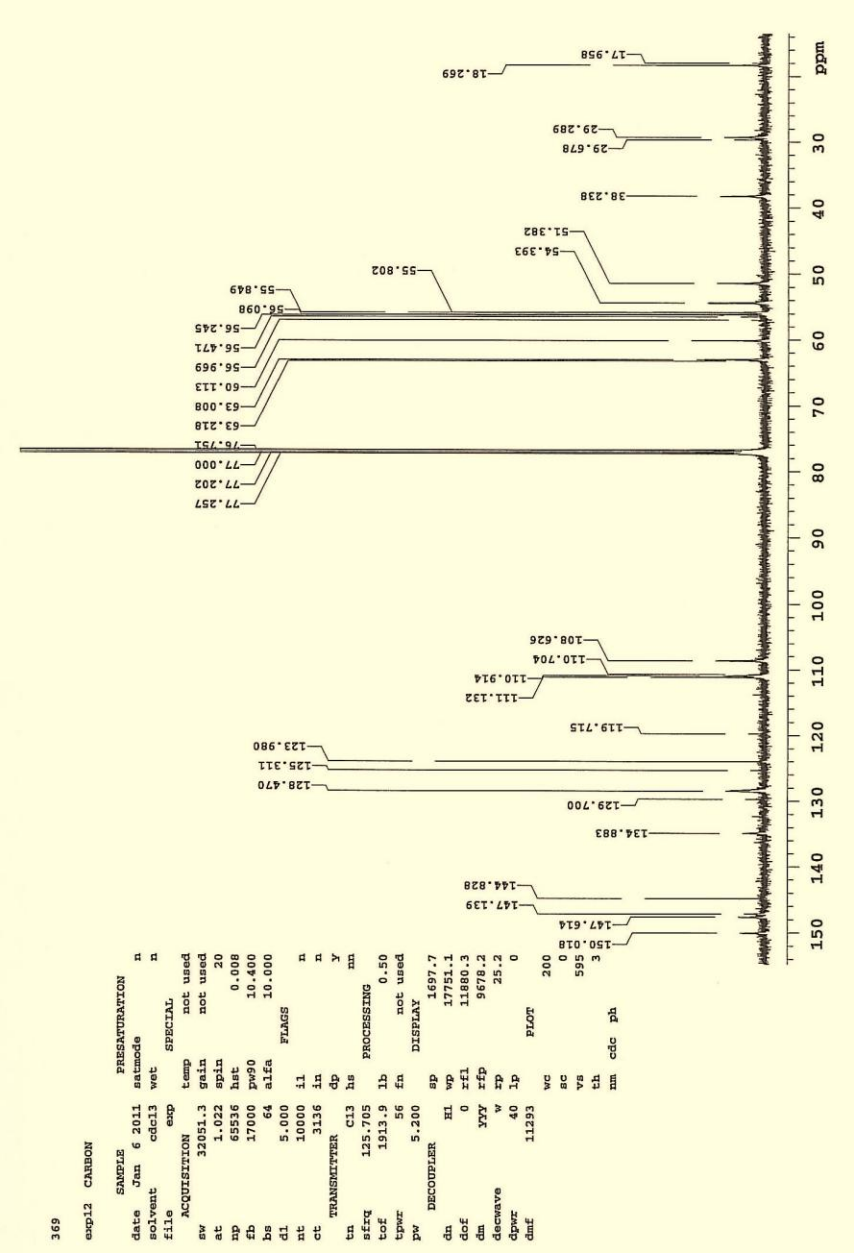

Fig. 22 ^{13}C NMR spectrum of (+)-corydaline

5.1.3.3 Optical rotation

$[\alpha]_D^{23}$ + 312 ° (EtOH; c = 0.2)

5.2 Inhibitory activity of (+)- corydaline against AChE and BuChE

Table 3 In vitro HuAChE and HuBuChE inhibitory activity of isolated compound

Compound	IC_{50} (μM)[a]	
	AChE	BuChE
(+)-Corydaline	40.5 ± 1.9	>1000
Galanthamin[b]	6.9 ± 0.3	156 ± 6.9
Huperzin[b]	0.25 ± 0.01	>1000

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

5.3 Antioxidant activity of (+)-corydaline

Table 4 Antioxidant activity of isolated compound

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

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

6. DISCUSSION

On the department of pharmaceutical botany and ecology, Faculty of Pharmacy in Hradec Králové, of Charles University, focus has been aimed at testing the biological activity of various alkaloids, notably isoquinoline type, with regard to their inhibitory activity on ChEs. This particular interest stemmed from the ever growing knowledge supporting the theory that these natural compounds could be employed as potential structural prototypes in the future development of more active ChE inhibitors. This class of agents is renowned for their use as the treatment of choice in mild-to-moderate symptomatic therapy of AD.

The aim of this work was to isolate alkaloids from the crude extract A of *C. yanhusuo* (from the part of chlorides non-phenolic alkaloids soluble in chloroform) by column chromatography and preparative thin-layer chromatography, and testing the biological activities related to ChE inhibition and antioxidant effect.

After chromatographic separation and fractionation of the extract A, one pure alkaloid was isolated. The alkaloid was termed GC-1 and further identified as (+)-corydaline on the basis of MS and NMR spectral studies. (+)-Corydaline is a tertiary alkaloid belonging to the structural group of isoquinoline alkaloids and can be further classified as having a tetrahydroberberine skeletal backbone (see Fig. 20).

The isolated alkaloid (+)-corydaline was further investigated on its inhibitory effect against human erythrocytic AChE and BuChE using the spectrophotometric method devised by Ellman [98], as well as antioxidant activity by use of the DPPH test. (+)-Corydaline was found to inhibit AChE in a dose-dependent manner with an IC_{50} value of 40.5 ± 1.9 μM, with huperzine A and galanthamine as positive controls. However, (+)-corydaline was found inactive against BuChE due to its IC_{50} value >1000 μM. The antioxidant activity was also considered negligible due to a value of $EC_{50.}$ >1000 μM.

Corydaline was isolated from *C. yanhusuo* in previous investigations [6, 9, 15, 96-98]. In a study by Hung *et al.*, 2008, corydaline was found to weakly inhibit AChE with an IC_{50} value of 30.7 ± 1.5 μM when mouse brain cortex was used as the source of

enzyme [9]. Interestingly, in another study on corydaline isolated from *Corydalis cava*, it was found to be the most active compound, inhibiting electric eel AChE in a dose-dependent manner with an IC_{50} value of 15 ± 3 μM [101]. Moreover, in the same study by Adsersen *et al*., 2007, corydaline was also found inactive against BuChE due to IC_{50} > 100 μM [101]. When comparing the spectrum of results obtained in this work with that of previous studies, it is important to note that differences in IC_{50} values could be due, at least in part, to the choice in enzyme origin.

In addition to possessing inhibitory activity against AChE, previous studies have demonstrated other unique pharmacological properties of corydaline. In one such study, corydaline was found to inhibit the enzyme GABA-transaminase, thus dispaling the basic mechanism of drug action used in the treatment of convulsive disorders [102]. Further more, corydaline derived from *C. yanhusuo* was found to exhibit significant antinociceptive effects with particularly high concentrations in the striatum [10].

The potential use of isoquinoline alkaloids against neurodegenerative processes in the human brain are concerned not only with their activity against AChE and BuChE, but another working theory in this area is on the inhibition of β- secretase (BACE1), as well as inhibition of NMDA receptors. From these reasons, it was also necessary to isolate 56 alkaloids that have previously been reported in literature for activity against human ChEs.

Based on the results obtained from this work along with comparisons from previous studies mentioned above, it can be said that (+)-corydaline does not prove sufficiently effective in the inhibition of AChE due to its weak IC_{50} values, and therefore may not be used as a direct means of alternative therapy in AD. However, the knowledge obtained with regard to the biological activity of (+)-corydaline can be considered as an important base in future production of more active synthetic analogues, where novel therapeutic strategies for dementia treatment may benefit from the combination of conventional Western approach and traditional Oriental medical.

7. LITERATURE

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8. ABBREVIATIONS KEY

β-A - β-amyloid

AChE - acetylcholinesterase

AD - Alzheimer's disease

APP - amyloid precursor protein

BACE1 - β-secretase

BuChE - butyrylcholinesterase

ChE - cholinesterase

CNS - central nervous system

COX-2 - cyclooxygenase 2

D_1/D_2 - dopamine 1/ dopamine 2 receptor

DA - dopamine

DPPH - 2,2´-diphenyl-1-picrylhydrazyl

EC_{50} - effective concentration

$GABA_A$ - gamma amino butyric acid A subtype

HuAChE -human acetylcholinesterase

HuBuChE -human butyrylcholinesterase

IC_{50} -inhibitory concentration

IL-6/ IL-8 - interleukin

iNOS - inducible nitric oxide synthase

MAPK - mitogen activated protein kinase

NMDA - N-methyl-D-aspartate (receptor)

NFT(s) - neurofibrillary tangle(s)

PGE_2 - prostaglandin E_2

PS1, PS2 - presenilin 1, presenilin 2

THP - tetrahydropalmatine

TNF-α - tumor necrosis factor-α

Abstrakt

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Farmaceutická fakulta v Hradci Králové

Katedra farmaceutické botaniky a ekologie

Kandidát: Gabriella Cipra

Konzultant: Doc. RNDr. Lubomir Opletal, CSc.

Název diplomové práce: Biologická aktivita obsahových látek rostlin XVII. Alkaloidy *Corydalis yanhusuo* W.T.Wang.

V rámci studia rostlin s obsahem alkaloidů, které inhibují aktivitu lidské erytrocytární acetylcholinesterasy a sérové butyrylcholinesterasy byl studován taxon *Corydalis yanhusuo.*

K izolaci bylo použito 10.8 kg suchých hlíz. Primární extrakt byl připraven perkolací 95% EtOH. V této diplomové práci byl zpracován výtřepek A-Et_2O (pH 9,7). 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 (+)-korydalin. Látka byla identifikována na základě hmotnostního spektra, NMR spekter, optické otáčivosti a srovnáním údajů s literárními daty. Při sledování vlivu (+)-korydalinu na lidskou AChE a BuChE a následném matematickém výpočtu byla pro (+)-korydalin zjištěno: IC_{50} 40,5 ± 1,9 μM. Antioxidační aktivita (DPPH test) vykázala hodnotu EC_{50} > 1000 μM.

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

Abstract

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Title of Thesis: Biological activity of plant metabolites XVII. Alkaloids of Corydalis yanhusuo W.T. Wang

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 dried 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 which 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 extract of non-phenolic chlorides soluble in chloroform. From this mixture (+)-corydaline (labeled GC-1) 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 (+)-corydaline on human AChE and BuChE was found to be: IC_{50} 40.5 ± 1.9 μM and IC_{50} >1000 μM, respectively, and antioxidative activity (DPPH test) was EC_{50} >1000 μM.

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