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Acetylcholinesterase-inhibitory properties of dietary supplements derived from natural products marketed against neurodegenerative disorders

Diploma thesis

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Declaration

I declare that I have elaborated my diploma thesis "Acetylcholinesterase-inhibitory properties of dietary supplements derived from natural products marketed against neurodegenerative disorders" independely under the supervision of the thesis supervisor and consultant and using literature and other information sources cited in the thesis and listed in the bibliography at the end of this document. In addition, as the author of the thesis I declare that I have not infringed the copyrights of third parties.

In Prague, 2.4.2021 _____

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Acetylcholinesterase-inhibitory properties of dietary supplements derived from natural products marketed against neurodegenerative disorders

Summary

Every year, more and more people are diagnosed with neurodegenerative diseases. With increasing life expectancy, it is expected that the number of people diagnosed with the disease will increase. The drugs that are currently available to treat only address the symptoms, and many of them have side effects. That is why it is necessary to look for new drugs. The best option seems to be plants that show the ability to inhibit the enzyme acetylcholinesterase (AChE). This study is focused on determination of in vitro AChE-inhibitory effect of commonly available dietary supplements marketed for treatment of neurodegenerative disorders, including Alzheimer's disease (AD), namely curcumin, *Boswellia serrata*, *Mucuna pruriens, Centella asiatica, Withania somnifera, Bacopa monnieri, Ginkgo biloba*, glutathione, choline, and lecithine. Galathamine was used as a control.

Only in the case of a single *W. somnifera*, the IC_{50} was less than 512 μg/mL, namely 283.36 μg/mL. The remaining samples showed an IC_{50} higher than 512 μg/mL. W. somnifera extract was further fractionated with the use of a semi-preparative HPLC method and retested for AChE inhibition. . The 5th faction showed the most promising results, which are most likely due to withanolides. The results suggest that *W. somnifera* could be helpful for people suffering from neurodegenerative diseases, especially AD. However, further research is needed. The rest of the tested samples are either useless in treatment of neurodegenerative disorders or are exerting their therapeutic benefit via other mechanism not related to AChE inhibition.

Keywords: Alzheimer's disease; acetylcholine; Ashwagandha; dietary supplements; medicinal plants; plant extracts

Souhrn

Každý rok přibývá lidí, kterým je diagnostikováno neurodegenerativní onemocnění. S rostoucím věkem dožití se předpokládá, že počet lidí s neurodegenerativním onemocněním bude růst. Léky, které jsou momentálně dostupné pro léčbu tohoto onemocnění se zaměřují jenom na symptomy a mnoho z nich má nežádoucí účinky. To jsou důvody, proč je potřeba hledat nové léky. Jako nejlepší možnost se zdají být rostliny, které mají schopnost inhibovat enzym acetylcholinesterázy (AChE). Tato diplomová práce je zaměřena na stanovení in vitro inhibičního účinku AChE u běžně dostupných doplňků stravy prodávaných pro léčbu neurodegenerativních onemocnění včetně Alzheimerovy choroby. Konkrétně se jedná o kurkumin, *Boswellia serrata*, *Mucuna pruriens, Centella asiatica, Withania somnifera, Bacopa monnieri, Ginkgo biloba*, glutation, cholin, and lecitin. Galantamin byl použit jako kontrola.

Pouze v případě *W. somnifera* byla IC_{50} menší než 512 μg/mL, konkrétně 283.36 μg/mL. Ostatní vzorky, které byly zkoumány vykázaly IC_{50} vyšší než 512 μg/mL. Extrakt *W. somnifera* byl dále frakcionován s užitím semipreparativní metody HPLC a následně znovu testován na inhibici AChE. Frakce číslo 5 ukázala nejslibnější výsledky; za aktivitu této frakce jsou nejspíše zodpovědné withanolidy. *W. somnifera* by mohla být nápomocná při léčbě neurodegenerativní poruch, zejména Alzheimerovy choroby. Avšak je zapotřebí další výzkum. Zbytek testovaných vzorků jsou při léčbě neurodegenerativních poruch buď neúčinné nebo jejich účinek je způsoben jiným mechanismem, který nesouvisí s inhibicí AChE.

Klíčová slova: Alzheimerova choroba, acetylcholine, Ashwagandha, potravinové doplňky, léčivé rostliny, rostlinné extrakty

Content

1 Introduction

Neurodegeneration is the loss of neurons in the CNS (central nervous system), where neurons die out due to programmed cell death, specific proteins accumulate, and this leads to the triggering of diseases such as Alzheimer's, Parkinson's, Huntington's disease or multiple sclerosis (Forman, 2004; Amor, 2010). In recent years, these diseases have been increasingly discussed. Why? This is due to the fact that the population is aging, and people are living longer, with the prevalence of most of these diseases increasing with age. By 2050, the number of patients is expected to triple and neurodegenerative diseases will become the second most common cause of death. This growing trend is not limited to developed countries; on the contrary, it is expected that the growth of patients with neurodegenerative diseases in developing countries will be even higher (Forman, 2004; Skovronsky, 2006; Durães, 2018).

Great attention is paid to acetylcholinesterase inhibitors. Acetylcholinesterase is an enzyme that occurs in the brain, muscles and, to a lesser extent, in erythrocytes. Its main function is to terminate nerve excitation at cholinergic synapses by rapidly hydrolyzing the washed-out acetylcholine to choline and acetate. During Alzheimer's disease, the release of acetylcholine from presynaptic terminals is reduced. Treatment of the disease with AChE inhibitors is that the reduced amount of acetylcholine is compensated by its prolonged effect on the postsynaptic receptor. There are drugs that belong to the groups of AChE inhibitors, namely donepezil, galantamine and rivastigmine, in China then Huperzin A (Mukherjee, 2007; Martin, 2011; Mehta, 2012). The above-mentioned drugs, which are used only to treat and alleviate symptoms, can improve the quality of life, but do not cure the disease itself (Folch, 2018). In addition, there are adverse drug effects that complicate treatment. And so, there is a great demand for the development of new drugs that will have better effects than those that are currently available.

The sale of dietary supplements focused on brain health is in the limelight. These supplements are designed to improve memory, increase energy or focus on cognitive performance or protection against stress. Anyone can buy them, whether in pharmacies or on the Internet. Buyers are among the older people affected by aging, cognitive decline and their goal is to prevent it, as well as adults of productive age who are trying to improve their performance. Among the main reasons why dietary supplements are popular are the return to natural

remedies and herbs that have been used for centuries in traditional medicine. There is also the influence of the media, which promotes faith in the products. Many of these herbal preparations are still not well researched (Chang, 2000; Guillemin, 2017).

In view of what is written above, this study is focused on characterization of in vitro acetylcholinesterase-inhibitory effect of dietary supplements marketed for supportive management of neurodegenerative disorders, especially Alzheimer's disease. The sample with the most promising result was further separated using a semi-preparative high performance liquid chromatography (HPLC) and resubmitted to the acetylcholinesterase-inhibitory assay. Results of this study may be helpful in the better understanding of the mechanism of action behind the therapeutic benefit of these dietary supplements.

2 Scientific hypothesis and aims of the thesis

The diploma thesis is divided into two parts - theoretical and practical. The theoretical work will describe the problem of neurodegenerative disease (ND) and the potential of plants (and their active principles) known and used for centuries that could help with the development of new drugs. In the practical part, it will be about the potential of selected dietary supplements in the treatment of neurodegenerative diseases.

The aim of this work is to determine the in vitro acetylcholinesterase (AChE)-inhibitory activity of selected dietary supplements that are commonly available and marketed as agents with therapeutic benefit in neurodegenerative diseases, especially Alzheimer's disease. The most promising sample will be then fractionated with the use of semi-preparative HPLC and resubmitted to the AChE-inhibitory assay again, in order to trace the constituent with the strongest AChE-inhibitory effect Results of this study may be further helpful in the development of new drugs or supplements to be administered to patients with ND.

Hypothesis: Systematic screening of plant extracts and isolated natural compounds may lead to the discovery of an extract/compound that would have strong inhibition against AChE and thus might be useful in the development of new drugs or dietary supplements with therapeutic benefit in ND, especially Alzheimer's disease.

3 Literature review

3.1 Description of diseases

Nowadays we face a big problem with neurodegenerative diseases. Hundreds of millions of people worldwide are affected by neurological disorders such as Alzheimer disease (AD), Parkinson disease (PD), Huntington disease (HD). Understanding and finding a cure that would improve the quality of life is one of the major challenges of this century. And as the incidence of these diseases increases with age, there is expected to be a large increase in people with neurodegenerative diseases. One of the main reasons is that the lives of people in developing countries will be extended (Eckert, 2010).

3.1.1 Huntington disease

George Huntington wrote a description of known as Huntington disease these days. It's a neurodegenerative disease with a 4-10 to 100,000 prevalence (Ross, 2011). The first symptoms appear around 40 years of life. However, there is also a juvenile form where the first symptoms can come as early as 20 years. There is still no cure for the disease and is usually fatal 20 years after the outbreak (Walker, 2007).

The essence of the mutation is the expansion of a triplet containing cytosine adenine guanine (CAG) with a critical limit of 40 or more repeats on the short arm of the 4th chromosome. The product of the mutation is an altered protein called huntingtin (McColgan, 2018). However, in two individuals with the same length of recurrence, the onset of the disease may not be at the same time. A relatively large study was carried out to examine the Venezuelan relatives in the Maracaibo area. It was found that 59% of the variability of age of onset is familial and therefore there are important genetic and shared environmental factors. They also dealt with the correlation and found that for the sister correlation we are at 0.49, while for the brother and sister it is 0.40 and for the purely fraternal 0.18 (The U.S.-Venezuela Collaborative, 2004). The onset of psychic symptoms is uncharacteristic and is mostly nonspecific personality and behavioral changes, affective or cognitive disorders. Typical neurological manifestations are disorders of free movement, choreatic and dystonic dyskinesia, gait disturbance, dysphagia, dysarthria, less frequently cerebellar symptoms. The progression of HD cannot be affected by treatment, but a number of symptoms can be at least temporarily curtailed (Solanki, 2016).

3.1.2 Parkinson disease

Parkinson's disease is the second most common neurodegenerative disorder. PD is a chronic progressive disease of the nervous system that originates from the degenerative neuronal death in the pars compacta substantiae nigrae and other pigmented nuclei of the brain stem, resulting in a deficiency of dopamine and other neuromediators in the basal ganglia of the brain and the development of Lewy body (Růžička, 2006; Gazewood, 2013; Sarrafchi, 2016).

Some diseases are hereditary and cannot be influenced. They are transferred by genes from parents to their children. Heredity studies in PD show that it is low. Although research has focused on neurodegenerative diseases for many years, we still cannot say what is the cause of PD, what is the trigger. There are two forms of PD. The first is the familial form, which is genetically inherited. The familial form accounts for approximately 10-15% of all Parkinson diseases. Disease symptoms occur tens year earlier (Kollárová 2007; Chen, 2018; Ball 2019). Currently, 7 genes are identified that are related to the familial form of PD. These genes are: α-synuclein (SNCA), parkin (PARK2), DJ-1 (PARK7), putative PTEN-induced kinase I (PINK1), glucocerebrosidase (GBA), leucine-rich repeat kinase 2 (LRRK2), a protein associated with by vacuum polarization of protein 35 (VPS35) (Keller 2012). The rest is a sporadic form of the disease. Human genotypes are unique, so even if individuals are affected by the same environmental factors, they are affected differently and thus lead to different disease phenotypes (Ball 2019).

With age, the risk of disease outbreak increases, the older the age, the higher the prevalence and severity of symptoms. The average age for the onset of the disease is 68 years, but this is not always the case. The disease can break out much earlier, from adolescence to old age. The turning point is 55 years, when people who get PD before 55 years are classified as early onset and over 55 years as late onset (Keller 2012). In addition to age, which is the main factor, there are other factors. Examples include coffee drinking, ibuprofen use, pesticide exposure, etc. (Chen, 2018). In the early 1980s, synthetic neurotoxin MPTP (1-methyl, 4-phenyl, 1,2,5,6-tetrahydropiridine) induced symptoms in infected persons that were identical to those of Parkinson's disease (Kollárová 2007).

Symptoms of PD include movement disorders such as tremor, rigidity, postural instability and deformities, gait disturbances, speech disorder, swallowing disorder. Rest tremor is a

typical symptom of Parkinson's disease. The typical Parkinson's tremor is quiescent, with a frequency of 4-6Hz and does not shake the head. Shaking the head, tongue, lips is rare. However, these symptoms may be present (Růžička, 2006; Jankovic, 2008). Rigidity is associated with hypokinesia. These are muscle pain and stiffness of the limbs. Mostly occur with passive limb movement. The examination involves the so-called Froment's maneuver, where rigidity is enhanced by the movement of the second-sided limb. Postural instability is standing uncertainty, hesitant walking. So-called freezing is manifested by sudden movement blockages and is the most common reason for falls (Broussolle, 2007). In PD there are also non-motor symptoms such as automatic dysfunction, cognitive disorders, sleep abnormalities (Růžička, 2006; Jankovic, 2008). Růžička (2006) mentions the oxidative events that dopaminergic neurons are subject to and thus significantly contribute to the disease.

3.1.3 Relaps-remitting multiple sclerosis

Multiple sclerosis (MS) is a neurodegenerative, inflammatory, chronic central nervous system disease. RS starts in about 85% of patients, i.e. RR-RS, when relapses and remission alternate. Relapse is an acute development of neurological symptoms due to active autoimmune inflammation, these symptoms may include, for example, loss of coordination, change in vision, histological changes in bowel or bladder tissue. Attacks are then symptoms that last more than 24 hours and appear at least 30 days after the previous attack. There may be varying degrees of severity in an attack, but it is always an acute inflammation in the CNS (Krasulová, 2008; Weinstock-Guttman, 2013).

The genetic risk factor is the HLA-DRB1 * 1501 gene from the MHC class II gene. Thanks to this gene, the risk of infection increases up to threefold. Conversely, some of the HLA system may have a protective effect (Horáková 2011; Parnell, 2017). The disease affects young people, starting between the ages of 20 and 40 and affecting women 2-3 times more often than men. Furthermore, the risk of illness increases if someone in the family suffers from MS (Sawcer, 2014; Parnell, 2017). Disease also vary widely worldwide. There is an opinion that increasing the level of vitamin D_3 acts as a prevention against the outbreak of the disease. Vitamin D_3 helps activate T cells, when vitamin levels are low, it can lead to immunoregulatory deficits and thus increase the risk of MS (Oksenberg, 2010).

3.1.4 Alzheimer disease

Alzheimer's disease is the most common neurodegenerative disease in the world. It is a form of dementia, with memory loss, cognitive impairment and often accompanied by various behavioral disorders, such as depression or aggression (Sivaraman, 2019). There are approximately 35 million people in the world who suffer from AD. In recent years, there has been an increasing incidence of AD, which is due to the fact that people live to an increasingly older age, as well as a more demanding lifestyle that leads to brain oxidative stress, which, according to Guzior (2015) and Sivaraman (2019), may lead to a doubling of the incidence of AD.

The familial form of AD is relatively low, about 5-10% of cases, and the onset of the disease begins very early, between 30 and 60 years. The early onset of the disease is due to mutations in one of three genes: the amyloid precursor protein (APP gene, chromosomal locus 21q21), pro presenilin 1 (PSEN1 gene, chromosomal locus 14q24.3) or presenilin 2 (PSEN2 gene, chromosomal locus) 1q31–42) (Liu, 2013). PSEN1 is located on chromosome 14 and approximately 150 mutations in this gene are described. The onset of clinical symptoms is usually between 25 and 65 years. PSEN1 mutations are the cause of the most severe forms and cause complete penetration (Van Cauwenberghe 2016, Vyhnálek 2019). The APP is stored on chromosome 21. It is a transmembrane protein that is thought to play a role in cell movement and cell adhesion. Approximately 30 gene mutations are described. Pathogenic variants in this gene may account for 10-15% of the early onset of the disease (Schu 2012). The onset of clinical symptoms is a few years later than in PSEN1 - specifically around 49 years (Van Cauwenberghe 2016, Vyhnálek 2019). PSEN2 is located on chromosome 1 and about 20 mutations in the gene are currently described. Here is the onset of symptoms between 39 and 83 years (Van Cauwenberghe 2016, Vyhnálek 2019).

The remaining 90-95% of cases are the so-called sporadic form of AD. As with PD, AD is caused by a combination of genetic and risk factors. There are risk factors that may be associated with AD. For example, it may be increased blood pressure, which in the middle age increases the risk of cognitive disorders and dementia. High pressure increases the AD onset risk by reducing vascular integrity of the blood-brain barrier, causing white blood cells to pass through the vascular wall of the capillaries and enter adjacent tissues. Another risk factor may be type 2 diabetes, cerebrovascular diseases, and high body weight (Reitz, 2011).

In people suffering from AD, beta amyloid is stored, followed by accumulation of tau protein. Increasing acetylcholine levels by inhibiting its cholinesterase activity can improve memory and cognitive impairment in people suffering from AD, since cognitive impairment is caused by decreased acetylcholine (Solanki, 2016).

3.2 Available drugs in treatment of neurodegenerative disorders

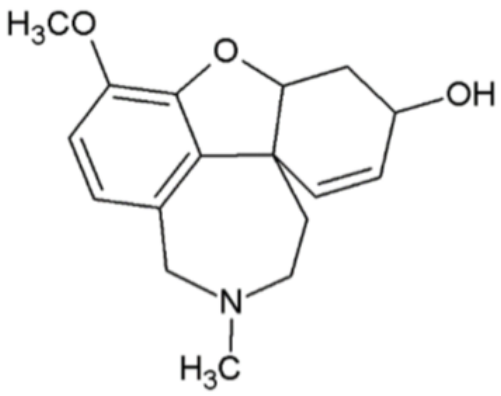
3.2.1 Natural compounds

3.2.1.1 Alkaloids

Alkaloids are nitrogen-containing compounds where the nitrogen may or not may be part of the heterocyclic ring. They usually have a strong bitter taste and are also very toxic, at relatively low doses. Plants use them mainly to defend themselves, for example, against invertebrate pests or herbivores (Mustafa, 2017).

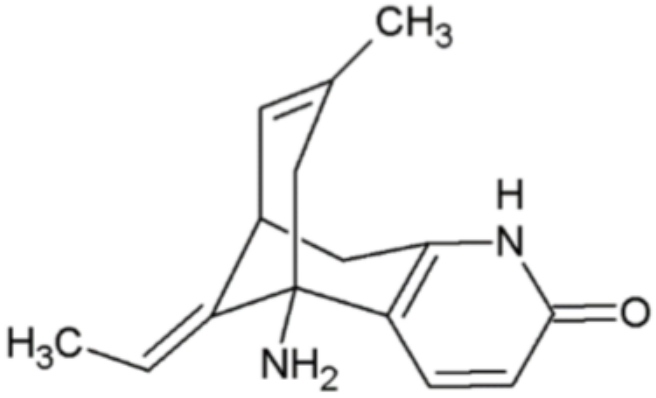
Plants of the genus Amaryllidaceae have been known since ancient Greece and have been used in traditional medicine as herbal remedies for thousands of years. Most alkaloids provided by these plants are not present in any other plant. The first alkaloid with AChE inhibitory which was isolated was lycorine. This occurred in 1877 from *Narcissus pseudonarocissus* (Amaryllidaceae). It has been found to have antitumor activity, to be able to inhibit ascorbic acid biosynthesis, and in addition to have acetylcholinesterase inhibitory activity (Ingrassia, 2008; Bastida, 2011).

Another important alkaloid already mentioned above is galantamine, which is one of the tertiary amines. Galantamine was first isolated from *Galanthus nivalis* (Amaryllidaceae) in the 1940s. The trade name is Reminyl. It is a long-acting, reversible, competitive and selective acetylcholinesterase inhibitor. In addition, it also acts as a modulator of nicotinic-type acetylcholine receptors, which increase the release of neurotransmitters and thereby stimulate neuronal function. Thus, galantamine also acts as a memory-improving drug. At the same time, it has the ability to bind to sites other than, for example, choline or acetylcholine and thus acts as their agonist (Orhan, 2006; Eckert, 2010; Bastida, 2011).



Picture 1: Galantamine

Huperzine A is isolated from the Chinese herb *Huperzia serrata* (Huperzinaceae). It is a reversible acetylcholinesterase inhibitor and increases the amount of acetylcholine in the synaptic cleft by inhibiting cholinesterases. It has antioxidant properties and is neuroprotective. As an herb found in China and Chinese traditional medicine, a number of clinical studies have been conducted here, looked at how huperzine A works in patients with AD. In most cases, huperzine A was given to one group of patients and placebo to another. Huperzine A was found to have a positive effect on memory, improving patients' memory quotients, cognitive and non-cognitive activities. The side effects were not significant. These include dizziness, diarrhoea, vomiting or nausea (Orhan, 2006; Girdhar, 2015).

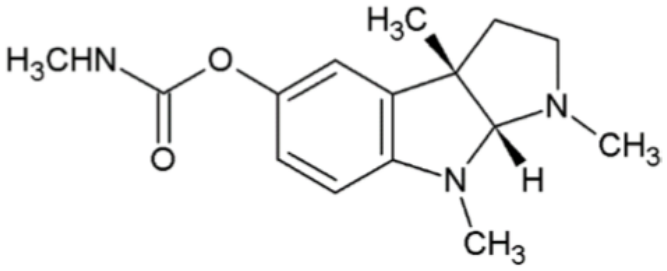


Picture 2: Huperzine A

3.2.1.2 Carbamate derivatives

The very first alkaloid to be investigated as a potential acetylcholinesterase inhibitor was physostigmine. It is one of the tertiary amines and was isolated from the West African perennial shrub *Physostigma venenosum* (Fabaceae), specifically from its seeds. It was originally used for ophthalmic purposes but was synthesized for the first time in 1935. Initial

experiments on mice and rats showed that their learning improved. In addition to inhibiting AChE, physostigmine is able to inhibit butyrylcholinesteraze (BChE). Today, it is not used to treat AD because it has a number of side effects and also a short half-life (Triggle, 1998; Arens, 2018).

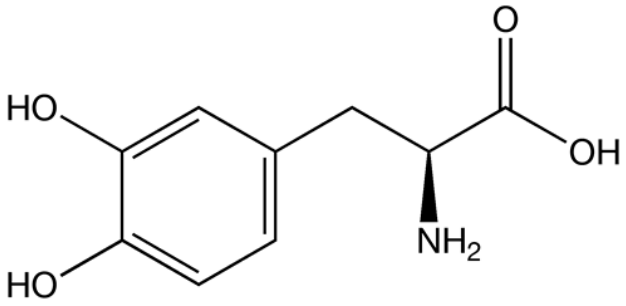


Picture 3: Physostigmine

3.2.1.3 Amino acid

Levodopa is a natural persecutor of dopamine. Dopamine is formed in the brain by the action of dopa-decarboxylase. Dopamine is exogenous and is also produced in the adrenal medulla in a small amount, so levodopa is given in combination with other peripheral dopa-decarboxylase inhibitors. Dopamine alone cannot be used in treatment because it is hydrophilic and therefore cannot cross the blood-brain barrier (Goole, 2009). Levodopa is a D_1 and D_2 receptor agonist and has a short half-life of about three hours. Synthetic levodopa was approved by the US Food and Drug Administration in 1970 for use in treatment of neurodegenerative disorders (Hauser, 2009).

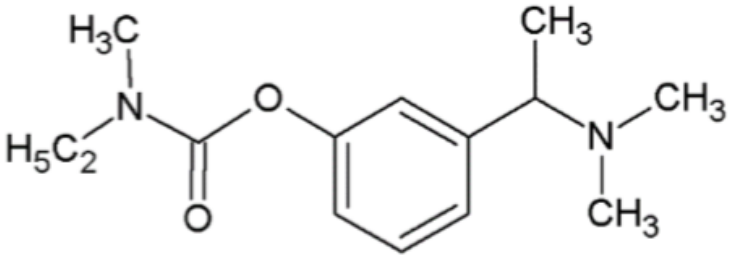
Here, too, we find side effects, namely uncontrollable movements, where these complications appear in 4-6 years of levodopa treatment in 40-75% of patients (Hauser, 2009). These motor complications have been shown to be a greater problem in young patients and those taking higher doses (Obeso, 2000; Stocchi, 2008). An "on-off" effect, which results in the patient stopping in the middle of a sentence when the effect of the drug stops (Abbott, 2010).



Picture 4: Levodopa

3.2.1 Semisynthetic derivatives

Rivastigmine is a semisynthetic derivative of physostigmine. Rivastigmine tartrate acts as a pseudo-reversible inhibitor of both AChE and BChE. This means that it has a long inhibition time of up to 10 hours, despite the fact that its elimination time is about two hours. Rivastigmine acts selectively in the CNS. It binds at the ester site of the enzyme and forms a carbamoylate complex with a serine residue. The carbamyl portion of rivastigmine cleaves more slowly than the acetyl portion of AChE. Rivastigmine has low protein binding properties, which means that there is no problem of interaction with other drugs. This is especially important for the elderly who are taking several medicines at the same time. Improvements in cognitive function have also been observed with rivastigmine (Annicchiarico, 2007; Onor, 2007; Russo 2013 Birks, 2015).



Picture 5: Rivastigmine

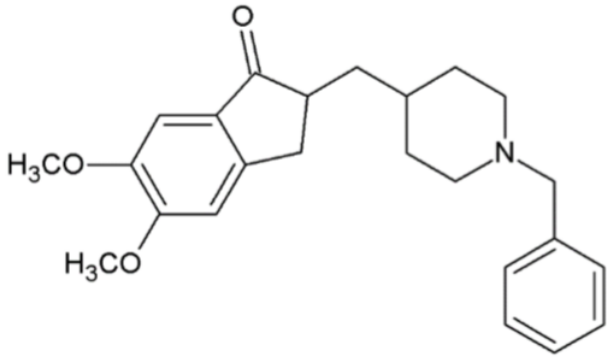
3.2.3 Synthetic derivatives

We live in a modern world where science is still shifting. Likewise, organic synthesis has evolved. Modern techniques and new reagents have developed that have advanced the possibilities of organic synthesis in a number of areas - natural substances, drugs or complicated structures for materials chemistry. (Svoboda 2000)

3.2.3.1 Piperidine derivatives

Donepezil is one of the other AChE inhibitors. It is a derivative of indanone benzylpiperidine with reversible AChE activity. It is highly selective and inhibits AChE in particular, it is 500-1000 times more selective for AChE than for BChE. It became the second inhibitor to be approved by the US FDA for the treatment of mild to moderate AD. It is one of the most widely used drugs for the treatment of AD and is available in more than 50 countries. It is known under the trade name Aricept. In addition to inhibiting AChE, it also indirectly stimulates nicotinic receptors in cortical neurons and thus acts to increase protective effects

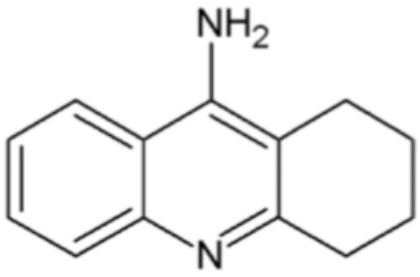
on the nervous system. Like the aforementioned rivastigmine, it has minimal interaction with other drugs. A major advantage of donepezil is its half-life, which is approximately 70 hours and therefore can only be administered once daily (Sugimoto, 2002; Jackson, 2004; Cacabelos, 2007; Adlimoghaddam, 2018).



Picture 6: Donepezil

3.2.3.2 Acridine derivatives

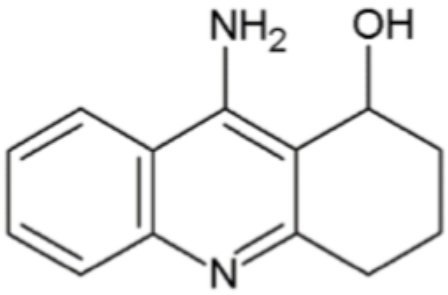
Tacrine is a reversible, non-competitive inhibitor that binds to the anionic part of the enzyme. It was the first drug to be FTA-approved in 1993 after treating mild to moderate AD. It was sold under the trade name Cognex. However, after a few years it was withdrawn from the market. The main reason was his hepatotoxic effect, which was manifested by an increase in alanine aminotransferase levels in serum. Tacrine has a low molecular weight, good inhibitory activity, and therefore scientists are trying to find hybrid or multi-target compounds that would take the good properties of tacrine and at the same time would not be hepatotoxic (Romero, 2013; Hamulakova, 2014; Sameem, 2017; Girek, 2019).



Picture 7: Tacrine

Velnacrine is a hydroxy metabolite of tacrine. It is also classified as an acridine derivative, but has lower toxicity than tacrine, which has been shown in experimental models. Although it is a potent AChE inhibitor, it has been excluded from clinical trials. The reason was adverse

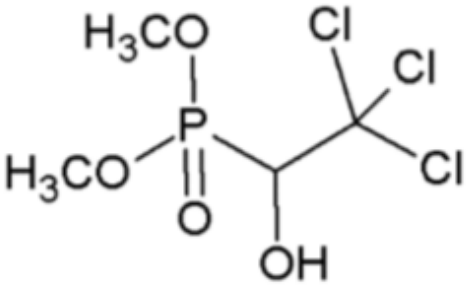
effects such as nausea, insomnia, restlessness, but mainly its high hepatotoxicity. Although velnacrine has a lower liver toxicity than tacrine, it is still significant (Bosch, 1993; Orhan, 2006).



Picture 8 Velnacrine

3.2.3.3 Organophosphates

Metrifonate was synthesized in the 1950's. It has found its use in the treatment of schistosomiasis, which occurs mainly in tropical and subtropical regions. It is an AChE inhibitor that is transformed non-enzymatically in the body into the active metabolite 2,2-dichlorovinyl dimethyl phosphate (DDVP). Despite the good clinical effect, metrifonate was withdrawn from clinical use due to its side effects such as leg cramps and reduced heart rate (Schneider, 2000; Orhan, 2006; E. Becker, 2010).



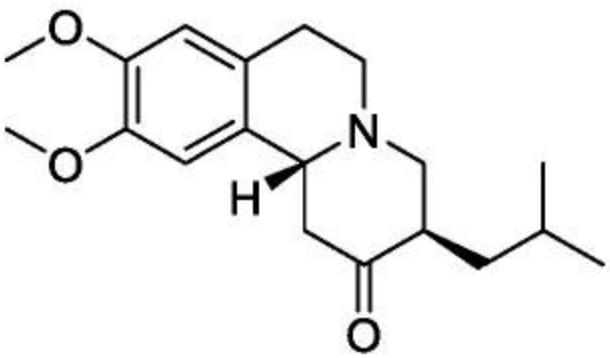
Picture 9: Metrifonate

3.2.3.4 Quinolizine derivatives

Tetrabenzine (TBZ) was originally developed for the treatment of schizophrenia, but over time it has been shown to have greater potential for the treatment of neurodegenerative diseases characterized by involuntary movements, such as chorea in HD (Paleacu, 2007; Tommaso, 2011; Klempíř, 2015; Stahl, 2020). Tetrabenazine is a quinolizine derivative. Reversible inhibition of the reuptake of all three monoamines - serotin, noradrenaline and especially dopamine into presynaptic vesicles. This increases the degradation of neurotransmitters in the cytoplasm. Vesicular monoamine transporters are special proteins

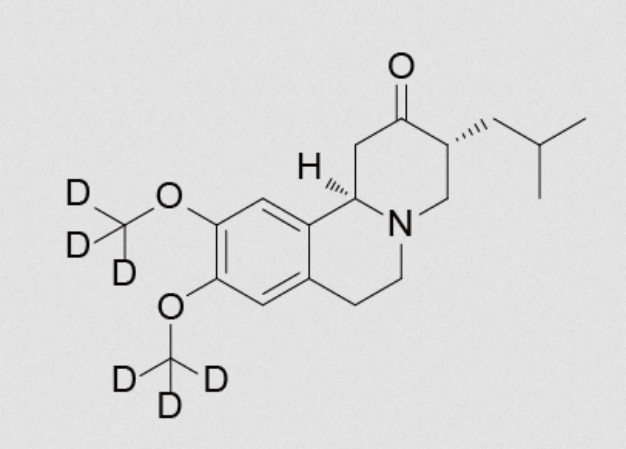
that are designed to transport neurotransmitters to vesicles. Tetrabenazine binds to these special proteins and thus prevents them from doing their work. It also acts as a blocker of dopamine D_2 receptors at high doses (Paleacu, 2007; Frank, 2009; Tommaso, 2011; Klempíř, 2015; Stahl, 2020).

TBZ is metabolized to two compounds, α-DTBZ and β-DTBZ. The first-mentioned compound being less bound to proteins. β-DTBZ has a lower half-life and therefore must be administered multiple times daily. TBZ is very well absorbed from the intestinal tract (Paleacu, 2007). Negative effects can be sedation, insomnia, or gastrointestinal problems. In this case, it is recommended to reduce the dose to a tolerated amount. However, with complete discontinuation, the chorea recurs, which returns to the same extent as before the drug was taken. In addition to the already mentioned negative effects, TBZ may also worsen the symptoms of Parkinsonism or akathisia, presumably via its effect on dopamine depletion (Frank, 2010; Videnovic, 2013; Klempíř, 2015).



Picture 10: Tetrabenazine

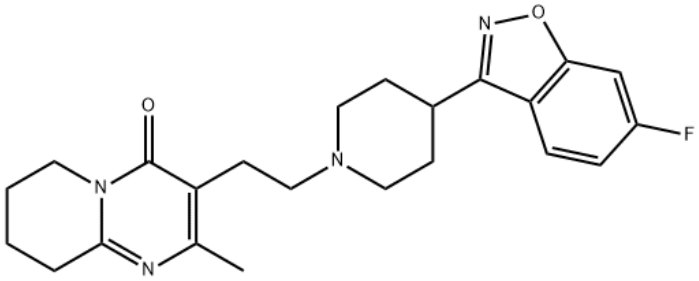
Deutetrabenazine is a deuterated form of tetrabenzine where selected hydrogen atoms are substituted with deuterium. Deuterium is a non-radioactive, naturally occurring, stable, non-toxic isotope of hydrogen and has the same size and shape as a hydrogen atom, differing only in that the chemical bonds are eight times stronger. Due to hydrogen substitution, deutetrabenazine has a longer half-life than TBZ, thus reducing daily dosing. The half-life is around 9-10 hours. Deutetrabenazine is a reversible inhibitor of the vesicular monoamine transporter 2 (VMAT2) (Dean, 2018; Richard, 2019). According to Claassen (2017), it has the potential to improve the benefit-risk profile for patients.



Picture 11: Deutetrabenazine

3.2.3.4 Benzisoxazol derivatives

Risperidone is an atypical antipsychotic that has been used mainly to treat schizophrenia and other bipolar disorders. It antagonizes dopamine - D_1, D_2, D_3, serotonin S_2, andrenergic - α_1, α_2 and to a lesser extent also histamine receptors (Klempíř, 2015). It is effective in psychomotor restlessness, irritability or aggressive behavior in HD (Duff, 2008). Blockade of α_1 adrenergic receptors may cause hypotension and tachycardia at the beginning of treatment. It can cause weight gain, which is an advantage with HD (Tinsmith, 2015). According to a study by Stahl (2020), risperidone was identified as the first choice of antipsychotic in 43% of all respondents to HD. Risperidone has a higher risk of drug-induced extrapyramidal syndromes, more sedentary effects and a higher incidence of apathy compared to tiapride (Klempíř, 2015).

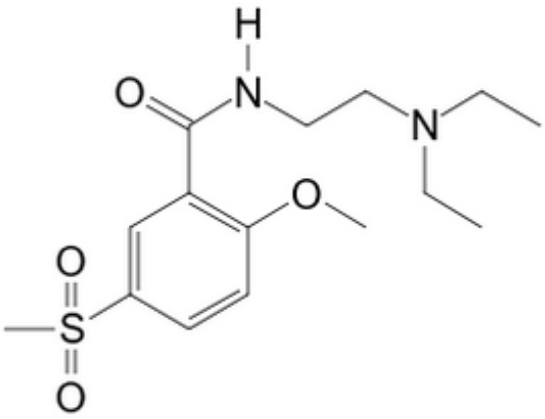


Picture 12: Risperidone

3.2.3.5 Benzamide derivatives

Tiapride was synthesized by L. Justin-Besancon together with a group of other benzamides. It is one of the atypical antipsychotics that block presynaptic and postsynaptic D_3 and especially D_2 receptors. It has no demonstrable affinity for other receptors (adrenergic, histamine, muscarinic). Like risperidone mentioned above, it is used in HD to alleviate irritability,

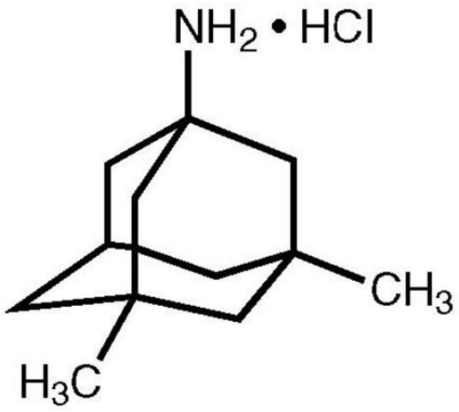
aggression or delirium. It has minimal interactions with other drugs. The side effects with tiapride are mild, most often fatigue, dizziness and restlessness (Dose, 2000; Klempíř, 2015). Triapide is relatively widely used in Europe but is not available in the United States (Klempíř, 2015; Stahl, 2020). Tiapride also has side effects such as weight gain, which is not completely detrimental. Then there is drowsiness, parkinsonism, akathisia, when a person is not able to stay calm and feels that he must constantly move or dyskinesia, which is an involuntary movement disorder (Adam, 2008; Stahl, 2020).



Picture 13: Tiapride

3.2.3.6 Aminoadamantane derivatives

Memantine (1-amino-3,5-dimethyladamantane) is one of the compounds that is unusual in three-dimensional tricyclic structures. Memantine was originally synthesized in the early 1960's by Eli Lilly as a potential antidiabetic but did not show any hypoglycemic activity. In 1982, it was registered in Germany and was used to treat PD. In the 1990s, studies showed that it had an effect in the treatment of AD and vascular dementia. It has been available in the European Union since 2002 and was registered in the US a year later (Rogawski, 2003; Robinson, 2006; Thomas, 2009). Memantine is a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, protecting neurons from the neurotoxic effects of glutamate by replacing Mg^{2+} in the ion channel and blocking the entry of Ca^{2+} ions into cells. Memantine is completely absorbed from the gastrointestinal tract and has almost 100% bioavailability (Rogawski, 2003; Robinson, 2006; Thomas, 2009).



Picture 14: Memantine

3.3 Ethnobothany and ethnopharmacology as a tool in discovery of novel drugs

From time immemorial, plants have been an important part of human life, without them life on Earth would not be possible. Whether it was the food or the medicine the plants gave us. It is also their ability to convert sunlight into food energy or their importance in regulating the concentration of gases in the air. They have also become the basis of traditional medicine around the world. In many developing countries, knowledge of plants and their effects have been passed down from generation to generation. Today, 80% of the world's population still lives in developing countries and is still dependent on traditional health care, whether in terms of poor infrastructure or money (Gómez-Estrada, 2011; Lopez, 2011; Mustafa, 2017; Aslam, 2016).

Nowadays, more and more scientist, focus on folklore medicine and more plants are studied as a protentional treatment. Such as *Salvia lavandulaefolia* (Lamiaceae), which has inhibitory activity on the acetylcholinesterase enzyme. *Ginkgo biloba* (Ginkgoaceae) leaves showed favorably effects in the treatment of neurodegenerative diseases and some other phytochemicals e.g. *Salvia lerrifolia* (Lamiaceae), *Epimedium koreanum* (Berberidaceae), peels of *Citrus medica* (Rutaceae), *Acorus calamus* (Acoraceae) or *Phagnalon saxatile* (Asteraceae) that have anticholinesterase activity (Sivaraman, 2019).

One of the oldest medicines is considered to be African, which is based on a holistic system that includes both body and soul. Cultural and spiritual aspects are also used in practice. Plants are seen not only as a tool for healing, but as a living organism that creates a kind of life force. Plants such as *Aloe ferox* (Xanthorrhoeaceae) are known from this medicine, which is used for skin problems because it has regenerating and soothing effects. Furthermore, it is rooibos tea, which is becoming increasingly popular in the Western world. This drink is prepared from *Aspalanthus linearis* (Fabaceae), which is showing medicinal effects as well – it is traditionally used in Africa to treat colic, allergy, asthma, and skin disorders (Joubert, 2008). Another very popular plant in African folklore medicine is *Prunus africana* (Rosaceae) (Lopez, 2011), also known as African cherry. Its bark is used in treatment of variety of conditions, including constipation, gonorrhea, fever, malaria, stomach-ache, kidney disease, insanity, and to heal wounds (Stewart, 2003).

Other traditional medicines include Chinese medicine, which is thought to be around 5,000 years old. In this medicine, the Earth is divided into wood, fire, earth, metal and water. And each of these elements is associated with important organs such as the liver, heart, spleen, lungs and kidneys. If an illness occurs, it is assumed that external influences such as heat or cold or emotions of fear, anxiety, anger are to blame. Plants that have become important to the world also come from Chinese medicine. These include, for example, *Ginkgo biloba* (Ginkgoaceae) or *Artemisia annua* (Asteraceae) and *Ephedra sinica* (Ephedraceae). *G. biloba* contains ginkgolides, a group of compounds thought to be helpful in neurodegenerative disorders by virtue of increasing cerebral blood flow. Today, the possible effects of ginkgolides on cancer are also being intensely investigated (Lopez, 2011). Artemisin from *A. annua* is showing strong anti-malarial effect and is currently used solely, or in combination with other drugs (e.g. quinine) in treatment of malaria. *E. sinica* is a source of ephedrine that is used in western medicine as a bronchodilator in treatment allergic conditions (e.g. asthma).

Another holistic system is Indian traditional medicine called Ayurveda, which is practiced not only in India but also in Sri Lanka and other countries. There is a great resemblance to the aforementioned Chinese traditional medicine. Even here there are five elements and once the disease breaks out, it means the imbalance between the elements. In addition to herbal remedies, treatment approach also includes application of other techniques, such as yoga. We are also indebted to Indian medicine for important plants. *Curcuma longa* (Zingiberaceae) gives us curcumin, which is used for inflammation and pain. Black pepper (*Piper nigrum*; Piperaceae) is used in Indian medicine as a digestive aid. Another plant gaining in popularity is the so-called Indian ginseng or *Withania somnifera* (Solanaceae). It contains several active ingredients such as alkaloids (isopelletierin, anaferin, anahygrin, etc.), steroid lactones (withanolides, withaferins) and saponins. These agents may then be useful in the treatment of neuropsychiatric or neurodegenerative disorders (Mukherjee, 2010; Lopez, 2011).

The pharmaceutical industry began to develop during the Industrial Revolution. At a time that meant the transition to factory production and also began the development of organic chemistry. For humans, medicines derived from plants became goods that were for people with lower incomes or with lower education. Thus, the economic power of pharmaceutical companies that were able to obtain pure compounds or make structural modifications that led to drug development grew (Rates, 2001; Heinrich, 2010).

Morphine from poppy seed (*Papaver somniferum,* Papaveraceae), which was first identified in 1804. In 1817, it was first chemically characterized as an alkaloid. Poppy seed is an annual plant that comes from Asia. It is grown in fields for food purposes – seeds and seed oil. In the countries of the golden crescent and triangle, it is then abused as a recreational drug, where opium is obtained from poppies. Already in ancient Greece it was used as a painkiller, suppressed cough or used as a sedative (Heinrich, 2010; Lopez, 2011).

Synthesis of galantamine was also important. It was firstly synthetized in 1990s. Initial research took place in Bulgaria and the USSR during the Cold War. The first work demonstrating the AChE-inhibiting properties of galanthamine was published in 1951 by M. D. Mashkovsky and R. P. Kruglikojva-Lvov. The original idea was that AChE inhibitors would be used for several years, but over the next few years, no new drugs was developed to replace the AChE inhibitors, and even after those years, they remain the main drugs for neurodegenerative diseases (Eckert 2010; Heinrich, 2010; Lopez, 2011).

Lastly, I will mention one of the best-selling medicinal products, and that is the extract obtained from the leaves of *Ginko biloba* L. (Ginkgoaceae). In many countries, it is not considered a medicinal product, only as a dietary supplement, in others, on the contrary. It comes from China, where it originally occurred mainly in monasteries and mountains. In Asia, it is considered a sacred tree. In 1969, the tree was transported to Europe, specifically to Utrecht by the German naturalist Engelbert Kaempfer. It is used to improve blood flow to the limbs, strengthens memory, spatial orientation and also improves overall brain function. It also contains antioxidants that help protect cells and tissues from oxidation (Heinrich, 2010). Flavonoids and terpene lactones have been identified as active ingredients in EGb 761, a standardized extract (Eckert 2010).

There are currently about 250,000 species of flowering plants in the world, half of which are found in tropical forests. So far, only a small percentage of world flora has been studied for pharmaceutical potential (Verpoorte, 2000). Consequently, the potential to find other, new compounds is still enormous. However, this requires a multidisciplinary approach, from botany, ethnobotany to phytochemical and biological techniques (Jachak, 2007; Rout, 2009).

The whole process begins with a person who identifies plants - botanist, ethnopharmacologist, ethnobotanist. Collection can take place in various ways. Plants where biological activity is

known or whole taxa that are used for a large screening program can be harvested. Throughout this process, it is necessary to respect the intellectual property of the country where this research and collection takes place. There are a number of methods that can be used to obtain compounds. Most often it is isolation from plants and natural sources, synthetic chemistry or combinatorial chemistry (Jachak, 2007).

Although it has been mentioned above that plant-derived medicines are of great importance and potential, pharmaceutical companies have nevertheless reduced their efforts to find new natural products. This attitude is most likely due to the fact that the collection of plants and samples can be difficult, for example the collection of marine organisms, which requires expensive equipment as well as qualified people. Another reason is the length of the process and also the finances. The discovery of the new drug takes about 10 years and the cost of the research is around $ 800 million (Jachak, 2001; Lopez, 2011).

On the other hand, the production of herbal medicines is cheaper than the production of synthetic ones. Furthermore, it is assumed that herbal medicines usually do not have adverse effects, as they have been used for thousands of years as medicines for many diseases around the world. Conversely, synthetic drugs usually warn of side effects and risks. However, they usually have faster or even greater effects than herbal medicines (Mustafa, 2017).

Natural substances have played an important role in the search for new drugs, even in the case of neurodegenerative diseases. The treatment we currently have available alleviates the symptoms of the disease, or it can also improve the quality of life a bit, but it is not able to cure the disease or slow it down significantly (Folch, 2018). In addition, some drugs have side effects and may not work as well in all patients. That is why it is necessary to develop new substances with better effectiveness, less side effects.

3.4 Dietary supplements marketed against neurodegenerative disorders

As it is noted above, drug development is a time-consuming and economically demanding activity. Dietary supplements may be one of the possible alternatives to pharmaceutical drugs, for which the approval process is not so strict and they can still be effective in the treatment of various human diseases (their introduction to the market may be significantly faster in comparison to medicinal drugs). The vast majority of dietary supplements intended for medicinal use (the so-called herbal remedies) are derived from plants that have a long history of use in folklore medicine, which points to their therapeutic usefulness (ethnomedicinal indications) as well as health safety for their user. Some of these dietary supplements are also made up of isolated compounds. Down below are summarized some of the natural products that are currently marketed for treatment of neurodegenerative disorders.

3.1 Curcumin

Curcumin is obtained from turmeric spices and is isolated from the rhizomes of *Curcuma longa* (Zingiberaceae). As an anti-inflammatory, neuroprotective component, it is used not only by traditional Chinese medicine, but also by Ayurveda (Pogačnik, 2020). The discovery of curcumin dates back about two centuries, when Vogel and Pelletier isolated the yellow dye from the rhizomes of the aforementioned *C. longa* and named it curcumin. However, the chemical structure of curcumin was not described until 1973 by Rough and Whiting (Ghosh, 2015).

C. longa is one of the perennial flowering plants. There are over 130 different species in the world (Bhat, 2019). *C. longa* grows to a height of up to 1 meter and has elongated root rhizomes. Long oval leaves sprout from the rhizomes. The rhizome is thick, fleshy and has potential healing properties. It is these rhizomes that are later dried and used as a spice. The plant reproduces only by rhizomes and produces no seeds. *C. longa* needs temperatures between 20-30 ° C with an annual total precipitation of 1500 mm to increase. *C.longa* occurs in tropical and subtropical regions of the world, especially in India and China (Kuete, 2017; Verma, 2018; Chanda, 2019).

Curcumin, 7-bis (4-hydroxy-3-methoxyphenol) -1,6-heptadiene-3,5-dione or diferuloylmethane, is a natural polyphenol compound that inhibits various factors, including NFkB, a group of factors that they thus affect important genes for immunity, cell growth and

cell death, the inflammatory response and other processes. NF-κB is thought to be of great importance in a number of chronic diseases (Goel, 2008). It also binds β-amyloid plaques, which are characteristic of neurodegenerative diseases, restores homeostasis of the inflammatory system and increases the level of defence systems. Curcumin is less well absorbed but is well soluble in fats (Hu, 2015; Rigacci, 2015; Winiarska-Mieczan, 2020). Pogačnik (2020) and Bhat (2019) mention that the Indian population, which makes extensive use of a diet rich in curcumin, has a lower prevalence of AD compared to the United States. Despite of what is written above, therapeutic efficacy of curcumin is still far from conclusive. More studies are required, as turmeric contains other components which may be more responsible for the observed biological activity than curcumin (Baker, 2017).

3.2 *Boswellia serrata*

Boswellia serrata (Burseraceae) became known for its use in incense and embalming in ancient times. Burning resin has become a ritual of everyday life. They believed that this would prevent the influence of evil spirits on their souls (Siddiqui, 2011; Bansal, 2013; Mundeep, 2020). The resin used to make incense is obtained from the trunk of a tree. However, it is also known from traditional Indian medicine, where it has been used to treat chronic inflammatory diseases, brain diseases, and to increase intelligence (Mundeep, 2020; Rajabian, 2020;).

B. serrata is a medium to large tree with deciduous leaves. It can grow to a height of up to 8 meters. Sometimes they grow into more trunks, so they resemble more a shrub than a tree. Boswellia grows in hilly, arid regions of India, North Africa and the Middle East. The Burseraceae family is represented by 17 genera and 600 species that are widespread in tropical areas (Siddiqui, 2011; Mundeep, 2020).

Boswellia Serrata contains monoterpenes consisting of two isoprene units, diterpenes are composed of four isoprene units, triterpenes of six isoprene units, tetracyclic triterpene acids and four major pentacyclic triterpene trispenic acids - β-boswellic acid, acetyl-β-boswellic acid, 11-keto -β-boswellic and acetyl-11-keto- β-boswellic acid. Boswell acids are the most important ingredient due to their strong anti-inflammatory effects (Bansal, 2013; Nemat, 2013).

3.3 *Mucuna pruriens*

Mucuna pruriens (Fabaceae) is used in traditional Indian medicine to treat more than 200 diseases. Patients with Parkinson's disease were given crushed Mucuna seeds, which were diluted and given as a drink. These patients showed improvement. It is also traditionally used to treat high cholesterol, diabetes, to induce vomiting or to treat snake bites (Madhyastha, 2011; Chandra Verma, 2014; Maldonado, 2018).

M. pruriens is an annual shrub that can grow to a height of 18 meters. It has purple flowers that form 3 petals. The fruit is a pod that resembles a bean in shape, which is why it is sometimes called velvet beans. This pod is coated with orange hairs that itch when in contact with the skin due to the presence of 5-hydroxytryptamine (5-HT). Over time, however, these hairs disappear, and contact is safe. It can be found in places where the climate is hot with an ideal temperature between 19-27 ° C, with precipitation up to 2,500 mm and high light intensity (Chandra Verma, 2014; Maldonado, 2018).

The main reason why Mucuna has become a sought-after crop in recent years is that beans contain natural levodopa, which is important in the fight against PD. Levodopa was first synthesized from seeds in 1937 (Madhyastha, 2011). According to Maldonado (2018), it is better tolerated than the synthetic levodopa given to patients. In addition to levodopa, methylated and unmethylated tetrahydroisoquinolines are found in beans. Then flavonoids, phenolic compounds and tannins (Chandra Verma, 2014).

3.4 *Centella asiatica* (Gotu kola)

The traditional use in Indian and Chinese medicine can also be found in *Centella asiatica* (Apiaceae), also known as Gotu kola. In the past, Gotu kola extract was used as a nerve tonic to delay brain aging, help regenerate nerve tissue, and improve memory, attention, and concentration. *C. asiatica* is also used to help with other ailments such as asthma, to treat wounds (wound healing), and liver disorders. Utilization as a dietary supplement is increasing every year (Hassim, 2011; Lokanathan, 2016).

C. asiatica is a small, perennial climbing plant that reproduces with the help of stems and stretches to a height of up to two meters. The leaves are long petiolate, and the small flowers are first green and then dark to purple to red. Gotu kola comes from Southeast Asia, where it

thrives in swampy mountain areas and is widespread throughout the humid zone of the tropics and subtropics almost all over the world. It thrives in soils rich in humus, sandy and clayey soils. *C. asiatica* grows to an altitude of 600 m. It often grows with other commercial crops, such as the coconut tree, which protects it from sunlight (Hassim, 2011; Lokanathan, 2016; Gray, 2018).

The main chemical component of *C. asiatica* is triterpene saponins, which consist of many compounds. In particular, it is asiatic, centelic, madecasic acid, then asiaticoside and brahmoside, which is responsible for the effects on the CNS (Hassim, 2011). Asiatic acid has a positive effect on collagen synthesis (Hashim, 2011). These triterpene saponins are responsible for the induction of wound healing. In addition to the triterpene saponins mentioned above, *C. asiatica* also contains tannins, essential acids, phytosterols, resins, free amino acids, flavonoids, alkaloids and fatty acids (Gohil, 2010).

3.5 *Withania somnifera* (Ashwagandha)

Withania somnifera (Solanaceae) is also known as Indian ginseng. In Ayurveda, traditional Indian medicine, Ashwagandha is used as a rasayana or herbal tonic to refresh a person's physical and mental condition, increase longevity, increase immunity or against infectious diseases. It is also used in CNS disorders such as neurodegenerative diseases, epilepsy. It has also been used to treat insomnia, anxiety and stress (Kulkarni, 2008; Kumar, 2015).

W. somnifera is a rather inconspicuous, green woody shrub that is up to 2 meters high. Fine grey hairs grow on the woody branches and the leaves, which are small due to their height, have a light green color. Their length is between 5 and 10 cm and their width is a maximum of 7 cm. The flowers are light yellow in color and the fruit is an orange-red berry (Mirjalili, 2009). Propagation takes place mainly by seeds. Ashwagandha is best suited to arid savannas, rocky semi-deserts and similar inhospitable areas that begin in sub-Saharan Africa and end in India (Kumar, 2015; Singh, 2015).

The main chemical component found in *W. somnifera* are steroidal alkaloids and lactones, which are known as withanolides. Withanolides are steroid lactones that have an ergostane backbone (Kulkarni, 2008; Kumar, 2015). Thanks to these substances, oxidative and inflammatory processes in cells are inhibited and nerve inflammation is alleviated (Sun,

2016). Withanamides, which are contained in the plant, are then able to cross the blood-brain barrier and thus have a neuroprotective effect (Vareed, 2014).

3.6 *Bacopa monnieri* (Brahmi)

Bacopa monnieri (Plantaginaceae) also known as Brahmi has its history in traditional Indian medicine. It is used as a tonic as a means to improve intellect, memory, against anxiety or lack of concentration. It is currently marketed as a nutritional supplement that protects the brain from oxidative damage, helps improve cognitive function, and as a regulator of blood sugar. It is also gaining research attraction due to its anti-inflammatory, antioxidant, anti-cancer and antimicrobial properties (Charoenphon, 2016; Chaudhari, 2017; Nemetchek, 2017).

B. monnieri is an undemanding perennial creeping herb that grows to a height of 90 cm. It is decorated with white to light purple flowers with four or five petals and small leaves. The fruit is a capsule that is pointed and ovoid (Aguiar, 2013; Charoenphon, 2016; Chaudhari, 2017). Brahmi can be found in the tropical and subtropical belt, especially in Asian countries such as India, Nepal, Sri Lanka, Japan or China. It roots in moist and swampy areas. It grows to an altitude of approximately 1500 m (Aguiar, 2013; Simpson, 2015; Charoenphon, 2016; Chaudhari, 2017; Nemetchek, 2017).

B. monnieri contains a large amount of substances. The main ones are triterpene saponins (bacosides A, B, C), steroids (stigmasterol), alkaloids (nicotine, brahmin, herpestin), and flavonoids (luteolin, apigenin). Bacosides in particular, which improve the transmission of nerve impulses, repair damaged neurons, have beneficial effects on learning and improve memory in the elderly, may be responsible for the beneficial effects on the CNS. Some of these components are able to cross the blood-brain barrier (Simpson, 2015; Charoenphon, 2016; Brimson, 2021).

3.7 *Ginkgo biloba*

Ginkgo biloba (Ginkgoceae) is a tree that has lived around the world for more than 200 million years and is also nicknamed the "living fossil". The use as a natural remedy has already been described in the Chinese Materia Medica and is used in traditional Chinese medicine as a remedy for asthma, eye diseases, heart activity, oxidative stress, brain support

related to concentration, depression, headaches, anxiety. It is one of the best-selling dietary supplements available on the market (Jacobs, 2000; Birks, 2009).

G. biloba is a slow-growing tree that can live up to 1000 years. It is a dioecious tree, which means that there are both male and female individuals, but their resolution is possible after 30 years. The leaves are bright green, fan-shaped, and bilobed (heart shape). Female plants produce a cherry-sized fruit that has a strong-smelling flesh and edible kernels. The tree grows to a height of about 35 meters and the trunk circumference can be up to 10 meters. It is characterized by exceptional resistance to insects and fungi. It grows mainly in China, Korea, Japan, Europe and the United States (Jacobs, 2000; Strømgaard, 2004; Singh, 2008; Birks, 2009).

In terms of chemical composition, *G. biloba* contains a large number of compounds. Mainly terpenoids (ginkgolides, bilobalides) and flavonovids (flavones, tannin, ginkgetin, quercetin, kaempferol). More than 30 flavonoids contained in *G. Biloba* are known. Terpene trilactones (TTL) are the collective name for ginkgolides and bilobalides, which are only available in *G. biloba*. Ginkgolides have a cage structure with six five-membered carbocyclic rings and bilobalides are sesquiterpenes (Singh, 2008). Currently, a standardized extract of EGb 761 is used, which contains 22-27% flavonoid glycosides, 5-7% terpene lactones and less than 5 ppm ginkgolic acid. Ginkgolic acids are allergenic constituents capable of inducing contact dermatitis, as well as being potentially cytotoxic and mutagenic (Jacobs, 2000). The EGb 761 extract is obtained from dried green leaves and subsequent extraction with acetone/water (Strømgaard, 2004).

3.8 Glutathione

Glutathione is the most important antioxidant in the human body. Chemically, it is a tripeptide (gamma-1-glutamyl-1-cysteinil-glycine), which is composed of three amino acids - glutamic, cysteine and glycine. It occurs in most cells at high levels - 5 millimolar, which is the same concentration as, for example, cholesterol or glucose (Pizzorno, 2012; Pizzorno, 2014; Minich, 2019). Glutathione exists in two forms - reduced (GSH) and oxidized (GSSG). Resting cells have a GSH/GSSG ratio higher than 100, but when exposed to oxidative stress, this ratio decreases to a ratio between 10 and 1 (Pizzorno, 2012). Glutathione is involved in a number of biological processes and is characterized by an important role in detoxification

reactions, in the protection of cells against the harmful effects of xenobiotics or environmental and intracellular oxidants. Glutathione plays an important role in reducing oxidative stress and short half-lives (Pizzorno, 2012; Pizzorno, 2014; Minich, 2019).

3.9 Choline

Choline is an essential micronutrient that is needed for the proper functioning of organs, the construction of cell membranes (Meschino, 2005). It is found in all living cells, forms part of lecithin, and is known for its broad field of activity, from central nervous system maintenance to multiple metabolic functions. Choline is a precursor of acetylcholine, a naturally occurring neurotransmitter in the brain that plays an important role in nerve and muscle function (Wójcicki, 1995; Meschino, 2005; Zeisel, 2018). When choline is deficient, the body begins to use the supply of nerve cells, which can lead to increased storage of lipids in the liver. The liver is not able to properly process and consume fat without choline. Acetylcholine levels increase after ingestion of choline, and so its ingestion is recommended for neurodegenerative diseases. Choline intake into the body takes place mainly from the diet, namely eggs, chicken and beef, milk, fish. It is also available in the form of dietary supplements (Wójcicki, 1995; Meschino, 2005; Zeisel, 2018).

3.10 Lecithin

Lecithin (phosphatidylcholine) is distributed among phospholipids, which are a major component of cell membranes (Sovová, 2009). Lecithin is sometimes called "brain food" because it provides choline, which is involved in the development of brain, nerve and liver functions as the basis of acetylcholine and carnitine replacement compounds and is a source of fatty acids (Mach, 2012; Ibrahim, 2013). The main source of commercial lecithin is soybean and sunflower oil. Natural resources are eggs, nuts, seeds, soybeans. Lecithin is an important by-product in the processing of edible oils, and in the 1930s, oil was produced using hexane as a solvent. Lecithin reduces the absorption of cholesterol in the intestine and regulates the homeostasis of cholesterol and fatty acids, stabilizes membranes and improves liver function (Shahidi, 2006; Sovová, 2009; Mach, 2012; Ibrahim, 2013).

4 Methodology

4.1 Samples

10 dietary supplements were selected based on their therapeutic claim to help prevent neurodegenerative diseases to be further submitted to the AChE-inhibitory assay. These dietary supplements were purchased through online e-shops, and information on them, including the manufacturer, is summarized in Table 1. All of these supplements are sold to help with memory problems.

Table 1. Information on the dietary supplements used in this study

Product name	**Manufacturer**
Boswellia serrata	India Minature, Slovakia
Ginkgo biloba	Warrior, the Czech Republic
Mucuna pruriens	Vito life, the Czech Republic
Centella asiatica	Swanson, the USA
Bacoba monnieri	Vieste group, the Czech Republic
Withania somnifera	Natu, the Czech Republic
lipophilic curcumin (liquid)	Adelle Davis, Slovakia
lipophilic glutathione (liquid)	Adelle Davis, Slovakia
Choline (powder)	Natural Nutrition, the Czech Republic
Lecithin (powder)	Dr. Max, the Czech Republic

4.2 Chemicals

Acetylthiocholine iodide (ATCI), acetylcholinesterase (AChE) type VI-S, from electric eel, 5,5′-dithiobis[2-nitroben-zoic acid] (DTNB), tris[hydroxymethyl]aminomethane (Tris buffer), , and galantamine were purchased from Sigma-Aldrich (Prague, Czech Republic). Dimethyl sulfoxide (DMSO; *per analysis* grade) was obtained from Lachner (Neratovice, Czech Republic). Analytical grade methanol (MeOH) was acquitted from VWR (Prague, Prague, Czech Republic). NaCl and $MgCl_2$ (hexahydrate) salts were purchased from PENTA Chemicals (Prague, Czech Republic).

4.3 Samples and extract preparation

Individual samples were weighed in 2 grams and 20 mL of 80% MeOH was added, except for the *Mucuna pruriens* sample, where water was added as a better solvent. The samples were then extracted for 24 hours using a shaker. The samples were then filtered and evaporated

using a rotary evaporator at a water bath temperature of 40 ° C. The dried samples were then dissolved in 100% DMSO to a final concentration of 51.2 mg/mL. The extracts were stored at -20 ° C.

4.4 Micro-plate assay for inhibition of acetylcholinesterase

Inhibition of acetylcholinesterase activity was determined using the Ellman colorimetric method modified by Eldeen et al. (2005). Each sample was pipetted in 25 µL portions into a 96-well microtiter plate, followed by the addition of 25 µL ATCl in water, 125 µL DTNB in Buffer C (50 mM Tris – HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl\ 2 \cdot 6H_2O$), 50 µL Buffer B (50 mM, pH 8, containing 0.1% bovine serum albumin). Subsequently, 25 µL of freshly prepared AChE (0.2 IU/mL) was pipetted into all wells and the plate was left in the dark for 5 minutes. Galantamine served as a positive control for the method. The concentration of the sample showing half maximal inhibitory concertation (IC_{50}) was obtained by plotting the percentage inhibition against the concentration of the extract/compound. The IC_{50} are expressed in µg/mL. Each sample was measured in three independent tests, each performed in duplicate; IC_{50} values of each sample replicate were expressed as mean ± standard deviation (SD).

4.5 Semi-preparative high performance liquid chromatography (HPLC) analysis

The extract, that showed the most promising result in AChE-inhibitory assay, was further fractionated with the use of Dionex UltiMate 3000 semi-preparative HPLC equipped with UV/Vis detector (Thermo Scientific, Waltham, USA). The fractions were separated on a Gemini-NX C18 column (5 µm, 250 × 4.6 mm; Phenomenex, Torrance, USA) using a gradient elution employing mobile phase A (water) and B (MeOH) under following conditions: 0 min, 95:5 (A:B); 5 min, 95:5; 15 min, 0:100; 20 min, 0:100; and 22 min, 95:5 (total run time: 28 min). The column temperature was maintained at 26 °C, injection volume was set at 100 µL, and flow rate was 1 mL/min. Five fractions were collected based on 280 second time windows (F1, 0.0-4.6 min; F2, 4.6-9,35 min; F3, 9.35-13.75 min; F4, 13.75-18.65min, and F5, 18.65-25.5 min). UV absorption was monitored at wavelengths between range of 190 and 400 nm. The analytical outputs were acquired and processed using Chromeleon 7.2.8 (Thermo Scientific). The fractions were evaporated to dryness using a

vacuum rotary evaporator (Büchi) at 40 °C, redissolved in buffer A to a concentration of 5.12 mg and resubmitted to the AChE-inhibitory assay.

5 Results

The results are presented in Table 2. It can be seen that only *Withania somnifera* shows a promising result of AChE inhibition. The mean IC_{50} of *W. somnifera* was 283.36 µg/mL with the % RSD 11.6 %. The rest of the extracts have final IC_{50} values at concentrations higher than 512 µg/mL. The galantamin used as a control showed IC_{50} at a concentration of 2 µg/mL.

Table 2.: AchE-inhibitory activites of tested samples

Sample	**Mean IC_{50} (µg/mL)**	**SD[a]**	**% RSD[b]**
Galantamin	2.00	0.59	29.40
Boswellia serrata	>512	-	-
Ginkgo biloba	>512	-	-
Mucuna pruriens	>512	-	-
Centella asiatica	>512	-	-
Bacopa monnieri	>512	-	-
Withania somnifera	283.36	32.86	11.60
Curcumin	>512	-	-
Glutathione	>512	-	-
Choline	>512	-	-
Lecithin	>512	-	-

[a] Standard deviation
[b] relative standard deviation

As can be seen from Table 2, of all tested extracts, *W. somnifera* showed the most promising results for AChE inhibition. This extract was further fractionated into a total of five fractions (Figure 1). It can be seen from Table 3 that the strongest AChE inhibition occurred in fraction 5, where the mean IC_{50} value was 538.96 µg/mL. The galantamine used as a control had a final concentration of 4.68 µg/mL.

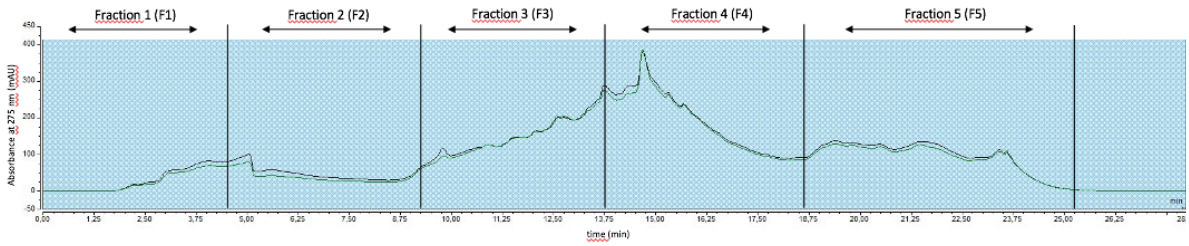


Figure 1. Semi-preparative HPLC-UV profile of Ashwaghanda (*Withania somnifera*; Solanaceae) crude extract

Table 3: AChE-inhibitory activity of fractions of *Withania somnifera* extract

Sample	Mean IC_{50} (µg/mL)	SD[a]	% RSD[b]
Galanthamin	4.68	1.28	27.38
Faction 1	>512	-	-
Faction 2	>512	-	-
Faction 3	>512	-	-
Faction 4	>512	-	-
Faction 5	538.96	127.17	23.60

[a] Standard deviation
[b] relative standard deviation

Figure 2 shows that the fraction 5 of *W. somnifera* extract is composed of 6 compounds. It appears that one of these compounds (or combination of these) are responsible for the observed AChE-inhibitory effect of *W. somnifera* extract. Corresponding UV/Vis spectra of these active constituents are shown in Figure 3. All 6 compounds strongly absorb UV light in the spectrum of 220 and ~288 nm, indicating that these compounds are of the similar chemical nature.

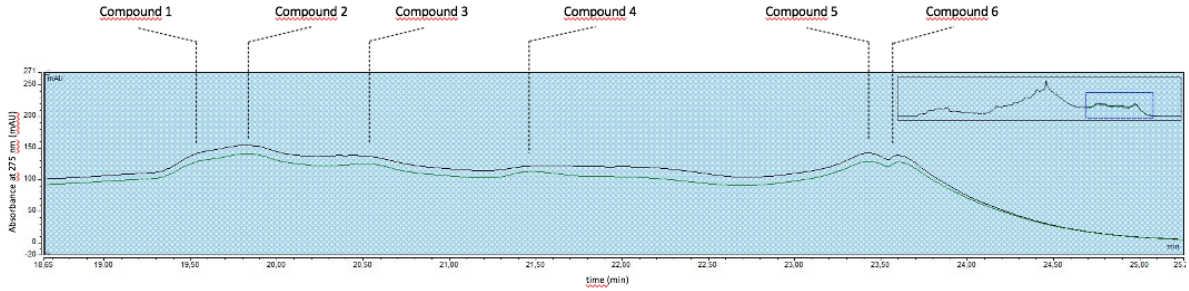


Figure 2. Detailed chromatogram of fraction 5 of Ashwaghanda extract (*Withania somnifera*; Solanaceae) crude extract and retention behaviour of present compounds (compounds 1-6)

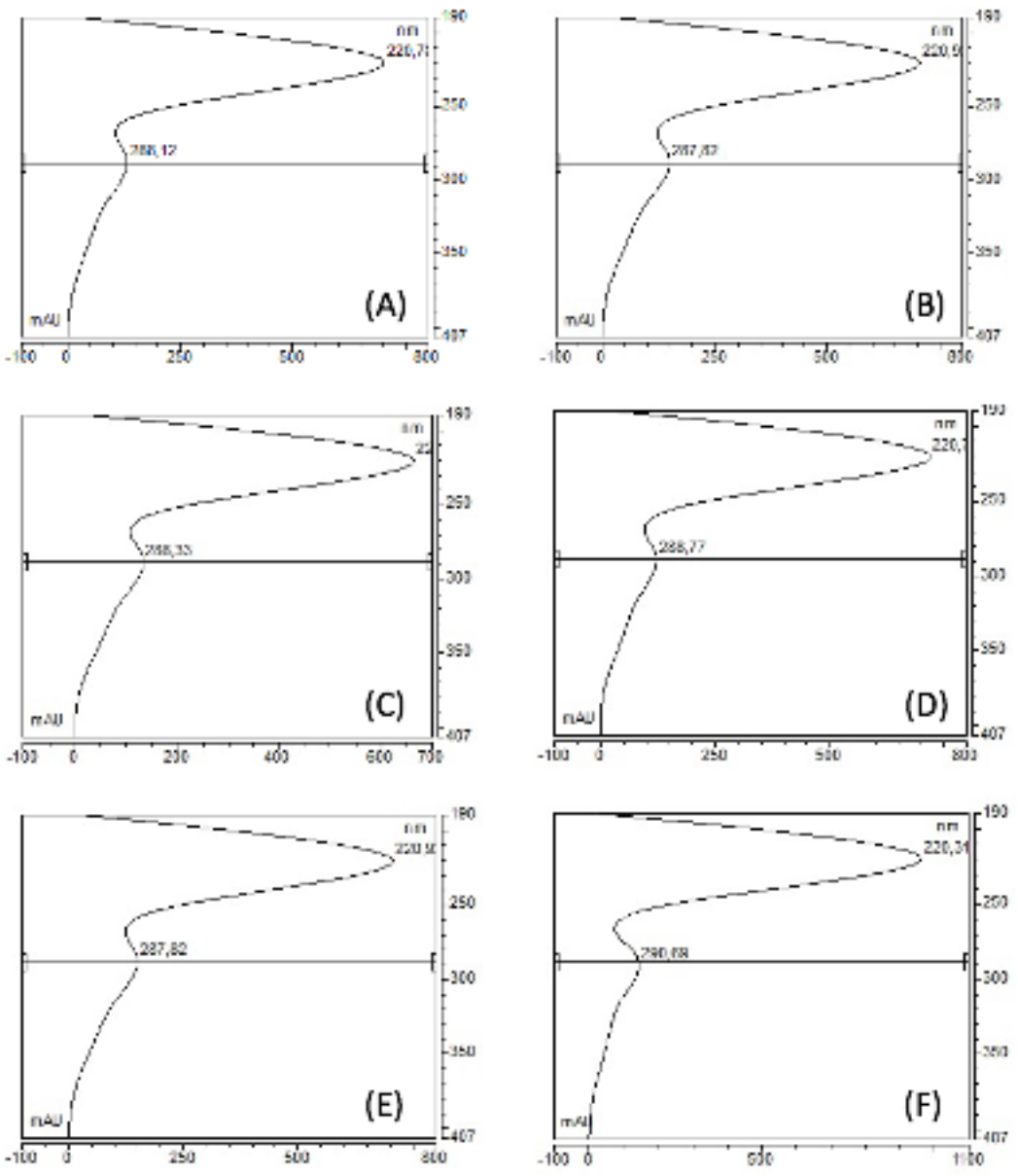


Figure 3. UV spectra of present compounds in fraction 5 of Ashwaghanda (*Withania somnifera*) crude extract: (A) compound 1, (B) compound 2, (C) compound 3, (D) compound 4, (E) compound 5, and (F) compound 6.

6 Discussion

We see more and more that there is a trend to return to traditional medicine, to the use of plants that have been known for centuries. Hectic life, little time, poor lifestyle lead to people increasingly reaching for dietary supplements to supplement daily nutrient requirements (Webb, 2006). This is also used by companies that provide dietary supplements. The use of these supplements has increased dramatically over the last 20 years, and this trend is expected to continue (Dwyer, 2018). Last year, the global dietary supplement sector was estimated at $ 140.3 billion and is projected to grow at an annual rate of 8.6% by 2028. But the key to success is research and development (ReportLinker, 2021). The discovery of natural cholinesterase inhibitors has been very challenging for drug development (Vinutha, 2007). AChE is a vital enzyme that increases acetylcholine levels in the brain and thereby improves cholinergic function in patients with AD (Krasowski, 1997; Murray, 2013). Drugs currently used to treat AD have limited efficacy as well as side effects, so there is still a demand for a new drug. In recent years, therefore, researchers have embarked on research and tried to find new AChE inhibitors, thanks to the development of colorimetric methods that are relatively fast, and Ellman's method has become the most widely used method for detecting AChE inhibitors and has also been used in this work (Murray, 2013).

In this diploma thesis, the inhibition of AChE on selected 10 dietary supplements, which are used against neurodegenerative diseases and are claimed to improve memory, was investigated. Of the 10 samples selected, only *W. somnifera* showed satisfactory results. The rest of the tested samples are ineffective or are showing its therapeutic benefit via different mechanism of action. Results of this study also shows, that withanolides are probably responsible for the AChE-inhibitory activity of *W. somnifera* extract. As it is shown in the Figure 3, the active constituents absorbed the UV in the spectrum of 220 and ~288 nm. Similar UV absorption ranges for withanolides were also observed in previous studies (Chaurasiya 2008; Nile, 2019).

Additionally, Choudhary (2004) investigated Ashwaganda withanolides and their potential to inhibit AChE. In his work, he found that 4 of 6 withanolides show activity to inhibit AChE in the amount of 50 to 161.5 μg/mL. Further research was focused on individual parts of the plant. All parts of the plant were found to show strong inhibition towards AChE. In another study, the best inhibition of AChE was shown by ripe fruit, which had an IC_{50} of 170 μg/mL

(Mahrous, 2017). Mathew (2014) focused on 20 plants used in Ajurveda and their potential to inhibit AChE, including *W. somnifera*, which demonstrated AChE inhibition at an IC_{50} of 124 μg/mL. In this diploma thesis, the IC_{50} was 283.36 μg/mL. The value is slightly higher than the values from the mentioned studies. There can be several reasons behind these differences, for example, manufacturers used different extraction procedures, as well as seasonal fluctuations that could affect specific concentrations of substances in the plant, or it may be due to different plant chemotypes (Ganzera, 2013).

Dhanani (2017) examined the total content of withanolides in *W. somnifera* by HPLC, finding that the total number depended on the solvent. The largest number was in the samples where methanol was used as the solvent, followed by water-ethanol, and the smallest number was in the aqueous solvent. The same conclusion was reached by Balkrishna (2019), who examined 3 fractions of Ashwagandha - water, water-methanol, methanol. The IC_{50} was greater than 512 μg/mL for the aqueous fraction, 306.72 μg/mL for hydro-methanol, and methanol was best with 203.79 μg/mL. In the same way, in this work, where the methanol fraction with a value of 538.96 μg/mL came out best. Different values can be given by the suitability of selected parameters such as temperature (Kumar 2018).

Although Ashwagandha is a plant that has been known and used for many centuries, its potential to treat neurodegenerative diseases has only recently been discovered. There are not many studies that look at its potential to inhibit acetylcholinesterase, but they are increasing every year. A study used in rats found that *W. somnifera* has nootropic effects, it perfuses the CNS (Bhattacharya, 1995). During another study, recovery of acetylcholine activity was found to be much more pronounced at higher doses of Ashwaganda (Pandey, 2018). In a study involving 50 people over the age of 35 who had similar problems with forgetfulness, one half was given a placebo and the other *W. somnifera* . In the group given to Ashwagandha, there was a significant improvement in logical memory, in pictures of family members after only eight weeks. Both placebo and *W. somnifera* were well tolerated and no adverse events were reported in either patient (Choudhary, 2017).

In view of the results of this study, it can be said that the most promising dietary supplement studied in this work is *W. somnifera*, which is suitable for further research, as there is also the potential for it to be better tolerated than currently used drugs. Moreover, only *W. somnifera* are marketed as the dietary supplement, and the active principles, the withanolides, still

remain commercially unavailable. Future studies should also be focused on them as they appear to be a promising leads for discovery of novel pharmacological agents with therapeutic benefit (apparently not only) in neurodegenerative disorders.

7 Conclusion

Current study provides information on in vitro AChE-inhibitory activity of commonly available dietary supplements marketed for neurodegenerative disorders. Extract of *W. somnifera* exhibited most promising result. It appears that the other tested materials either are possibly ineffective in the treatment of neurodegenerative disorders or provide their therapeutic benefit via different mechanism. Further analyses showed that withanolides (most probably the steroidal lactones withaferines) are very likely responsible for the observed AChE-inhibitory effect of *W. somnifera* extract. In conclusion, *W. somnifera*, and perhaps more importantly the present active principles (withanolides), may serve as a prospecting material for further development of plant-based AChE-inhibiting agents and should recieve considerable research attention in the future. Withanolides are currently not commercially available. It appears that these compounds (especially the steroidal lactones withaferines) have the potential of being employed into dietary supplements or pharmaceutical drugs that might be of value in treatment (not only) of neurodegenerative disorders, particularly Alzheimer's disease. However, more studies are required (in vivo tests on animals, clinical trials) in order to verify their potential practical use.

7 Literature

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8 List of abbreviation

AChE	Acetylcholinesterase
AD	Alzheimer disease
APP	Amyloid precursor protein
BChE	Butyrylcholinesterase
CAG	Cytosine adenine guanine
CNS	Central nervous system
HD	Huntington disease
MS	Multiple sclerosis
ND	Neurodegenerative disease
NMDA	*N*-methyl-D-aspartate receptor
Parkinson disease	PD
PSEN 1	Presenilin 1
PSEN 2	Presenilin 2
RS	Relapse sclerosis
TBZ	Tetrabenzine
VMAT2	Vesicular monoamine transporter 2