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Acetylcholinesterase and butyrylcholinesterase inhibitory activity of extracts from medicinal plants

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Abstract: Inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), enzymes which breakdown acetylcholine and butyrylcholine, are considered as a promising strategy for the treatment of Alzheimer's disease (AD). A potential source of AChE and BuChE inhibitors is provided by the abundance of plants in nature. In the present study, we selected five plants used in traditional medicine to treat different disorders of the central nervous system. Aqueous and methanolic extracts of sage (*Salvia officinalis* L.), arnica (*Arnica montana* L.), rue (*Ruta graveolens* L.), St. John's wort (*Hypericum perforatum* L.) and aronia (*Aronia melanocarpa* (Michx.) Elliot.) were tested for the AChE and BuChE inhibitory activity using Ellman's colorimetric method. Galanthamine hydrobromide was used as positive control. The results show that extracts from the aeiral parts of St John's wort and rue and flowers of arnica could inhibit the activity of AChE or BuChE or both. The best inhibition effect was observed using the water extract of rue, then methanolic extracts of arnica and St John's wort at concentration

INTRODUCTION

Alzheimer's disease (AD) is a progressive, neurodegenerative pathology that primarily affects the elderly population, and is estimated to account for 50-60% of dementia cases in persons over 65 years of age. The main symptoms associated with the later stages of AD involve cognitive dysfunction, primarily memory loss (Filho, Medeiros, Diniz *et al*., 2006). In mammalian brain, there are two major forms of cholinesterases, namely, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) (Giacobini, 2003). The most remarkable biochemical change in AD patients is a reduction of acetylcholine (ACh) levels in the hippocampus and cortex of the brain. Therefore, inhibition of AChE, the enzyme responsible for hydrolysis of ACh at the cholinergic synapse, is currently the most established approach to treating AD. While AChE is found in all excitable tissue, whether nerve or muscle, in most erythrocytes and in placental tissue, BuChE is present more commonly in the body including within the central and peripheral nervous system, liver and plasma. (Orhan, Kartal, Naz *et al*., 2007) The serious side effects caused by licensed drugs used to treat AD have forced researchers to investigate safer AChE or BuChE inhibitors from natural sources. Numerous plants and their constituents are reputed in traditional practices of medicine to enhance cognitive function and to alleviate other symptoms of AD, including depression (Politeo, Botica, Bilušić *et al*., 2011).

The aim of this study was to investigate a presence of possible AChE or BuChE inhibitors in few plants which traditionally used in European medicine. Selection of the

species screened in this study was based on their use as remedies for the enhance memory, central nervous system diseases, or as a source of well-known antioxidants. Aqueous and methanolic extracts of sage (*Salvia officinalis* L.), arnica (*Arnica montana* L.), rue (*Ruta graveolens* L.), St. John's wort (*Hypericum perforatum* L.) and aronia (*Aronia melanocarpa* (Michx.) Elliot.) were tested for the AChE and BuChE inhibitory activity.

EXPERIMENTAL

Plant material and chemicals

Plants were purchased from The Herbal Pharmacy-Vextra d.o.o. Mostar, Bosnia and Herzegovina. Each plant material was dried in shade at room temperature and then ground to a fine powder in a mechanic grinder. Plants and their parts used in this study are presented in Table 1.

AChE (EC 3.1.1.7) from electric eel (type VI-S), BuChE (EC 3.1.1.8) from horse-serum, acetylthiocholine iodide, butyrylthiocholine iodide, galanthamine hydrobromide, sodium dihydrogen phosphate monohydrate (NaH_2PO_4 x H_2O), disodium hydrogen phosphate (Na_2HPO_4) and methanol were purchased from Sigma-Aldrich (Germany). DTNB (5,5'-dithio-bis[2-nitrobenzoic acid]) was purchased from Zwijndrecht (Belgium). All reagents used in the study were of analytical grade.

Extract preparation

For extraction process 6 g of dried and grinded plant material was used. Water based extraction was done by using 150 ml of re-distilled water, while the temperature of the mixture was held constant at 70°C for 2 hours. Methanol based extraction was conducted by using 120 ml of methanol with the constant temperature of 60°C of the mixture for 2 hours. Both extracts were filtered and evaporated. To keep the extracts stable the evaporation temperature was not bigger than 60°C. After evaporation extracts were kept in the fridge at 4°C. Before using the extracts for the measurements they were diluted using phosphate buffer (pH=8.0).

Microplate assay

AChE and BuChE inhibitory activity were measured using a 96-well microplate reader (IRE 96, SFRI Medical Diagnostics) based on Ellman's method (Ellman, Courtney, Andres *et al.*, 1961). The enzyme hydrolyses the substrate acetylthiocholine or butyrylthiocholine resulting in the product thiocholine which reacts with Ellman's reagent (DTNB) to produce 2-nitrobenzoic-5-mercaptothiocholine and 5-thio-2-nitrobenzoate which can be detected at 412 nm. (Elman *et al.*, 1961, Rhee, Meent, Ingkaninan *et al.*, 2001)

In this method, total reaction volume of 220 µl consisted: 170 µl (0.1 mol/l) sodium phosphate buffer (pH 8.0), 20 µl of AChE/BuChE (0.45 U/ml), 10 µl test solution (plant extracts), 10 µl DTNB (0.03 mmol/l) and 10 µl of acetylthiocholine iodide/butyrylthiocholine iodide (final concentration of 0.68 mmol/l). The plant extracts were tested for AChE and BuChE inhibitory activity at concentrations from 100 µg/ml to 400 µg/ml. Different concentrations of dried plant extracts were prepared in phosphate buffer. Galanthamine hydrobromide was used as a AChE and BuChE positive control in a concentration range between 10 and 100 µg/ml. Appropriate amounts of buffer, extract and enzyme were incubated 15 min at 4˚C, the reaction was initiated by addition DTNB and substrate. Thereafter, the reaction mixture was incubated 30 min at 25˚C and absorbance read at 405 nm in a 96 well microtiter plate. A blank for each run consisted of 200 µl buffer, 10 µl substrate and 10 µl DTNB. Each sample was assayed in triplicate and it also included a control (C) in which buffer replaced the test solution.

Percentage of inhibition of AChE/BuChE was determined using the formula:

$$I = (C - T) / C \times 100 \qquad (1)$$

where C is the activity of enzyme without test sample and T is the activity of enzyme with test sample.

RESULTS AND DISCUSSION

In traditional practices, numerous plants have been used to treat cognitive disorders, including neurodegenerative diseases and different neuropharmacological disorders. The history of drug discovery has shown that plants contain active compounds that have become new sources to investigate for the pharmaceutical industry (Adewusi, Moodley and Steenkamp, 2010). In the present study, we selected five plants used in traditional medicine to treat different disorders of the central nervous system. The results on the effects of the tested herbal extracts on AChE and BuChE activity are summarized in Table 1., together with their family, plant part, solvent extract, percentage inhibition and concentration at which the enzyme is inhibited.

Data are expressed as mean with standard error. It was found out that galanthamine hydrobromide and plant extract had dose-dependent inhibitory activity. Galanthamine hydrobromide was used as a positive control, Figure 1. Galanthamine is an Amaryllidaceae alkaloid obtained from *Galanthus nivalis* L., and it is reported to be more selective for AChE than BuChE, and provides complete oral bioavailability. It is licensed in Europe for AD treatment, was well tolerated and significantly improved cognitive function when administered to AD patients (Mukherjee, Kumar, Mal *et. a*l., 2007).

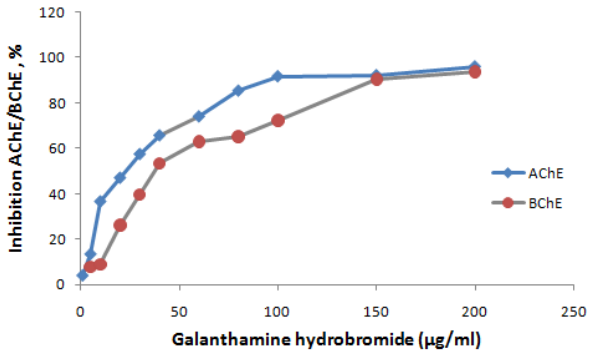

Figure1. AChE and BuChE inhibition efficiency of galanathamine hidrobromide.

Table 1. Anti-AChE and anti-BuChE acitivity of plant extracts.

Plant species	Family	Plant part analyzed	Solvent	BuChE inhibition (%)				AChE inhibition (%)			
				100 μg/ml	200 μg/ml	300 μg/ml	400 μg/ml	100 μg/ml	200 μg/ml	300 μg/ml	400 μg/ml
Arnica (*Arnica montana* L.)	Compositae	Flower	Methanol	25.4 ± 0.4	28.1 ± 0.1	32.3 ± 0.1	57.7 ± 0.1	50.7 ± 2.0	53.2 ± 2.7	60.2 ± 9.2	67.4 ± 9.3
			Water	6.4 ± 0.4	12.3 ± 0.3	18.9 ± 0.0	21,1 ± 0.5	40.7 ± 5.6	50.7 ± 3.1	53.9 ± 4.5	55.4 ± 4.1
Sage (*Salvia officinalis* L.)	Lamiaceae	Leaf	Methanol	1.4 ± 0.1	9.0 ± 0.5	34.8 ± 0.0	43,3 ± 5.4	3.8 ± 0.1	6.9 ± 0.1	9.9 ± 0.7	14.1 ± 0.4
			Water	8.0 ± 0.5	23.5 ± 0.4	27.2 ± 0.8	36.4 ± 0.6	4.4 ± 0.2	14.7 ± 0.2	17.2 ± 0.5	20.6 ± 0.6
Rue (*Ruta graveolens* L.)	Rutaceae	Herb	Methanol	9.4 ± 1.0	12.1 ± 2.3	17.0 ± 2.5	29.0 ± 2.8	30.8 ± 2.0	40.8 ± 6.4	53.8 ± 0.6	73.8 ± 5.7
			Water	19.7 ± 1.3	28.1 ± 1.1	30.9 ± 1.6	32.2 ± 1.5	55.7 ± 3.4	60.6 ± 8.2	65.7 ± 1.4	80.0 ± 0.8
St. John's wort (*Hypericum perforatum* L.)	Hypericaceae	Flower	Methanol	23.0± 0.1	37.1 ± 0.2	47.8 ± 0.7	50.5 ± 0.7	50.1 ± 0.9	58.7 ± 3.6	60.2 ± 4.2	73.5 ± 2.4
			Water	4,0± 0.1	15.4 ± 0.5	18.3 ± 0.6	19.2 ± 0.6	30.6 ± 6.9	38.5 ± 6.0	43.4 ± 8.5	52.7 ± 2.9
Aronia (*Aronia melanocarpa* (Michx.) Elliot.)	Rosacea	Fruit	Methanol	5.5 ± 0.4	13.0 ± 0.3	15.9 ± 2.2	18.9 ± 3.5	5.8 ± 2.7	8.9 ± 4.0	10.7 ± 7.5	18.3 ± 6.9
			Water	15.9 ± 1.9	18.1 ± 2.4	21.0 ± 3.4	22.6 ± 3.4	20.7 ± 4.7	55.9 ± 7.7	65.2 ± 6.1	70.2 ± 5.5

From ten investigated extracts seven of them have achieved 50% of inhibition activity for AChE and two for BuChE (Table 2). The strongest inhibition effect was detected with water extract of rue (IC_{50}=50 μg/ml) and methanolic extract of arnica (IC_{50}=75 μg/ml), following by methanolic extract of St. John's wort (IC_{50}=100 μg/ml) for AChE. Methanolic extracts of arnica (IC_{50}=389 μg/ml) and St. Jonh's wort (IC_{50}=353 μg/ml) have shown a significant inhibition effect towards BuChE.

Similar investigations were reported by Wszelaki, Kuciun and Kiss (2010). Significant inhibition effect in the fore mentioned research done by Wszelaki *et al*. was reported of AChE for hexane (IC_{50}=29 μg/ml) and methanolic (IC_{50}=43 μg/ml) extracts of the flowers of arnica (*Arnica chamissonis* Less. subs. *foliosa)*, and hexane extract (IC_{50}=34 μg/ml) of rue (*Ruta graveolens* L.) (IC_{50}=61 μg/ml) (Wszelaki *et. al*., 2010).

Table 2. The IC_{50} value of plant extracts.

Plant species	Solvent	IC_{50} (AChE) (μg/ml)	IC_{50} (BuChE) (μg/ml)
Arnica	Methanol	75	389
	Water	150	-
Sage	Methanol	-	-
	Water	-	-
Rue	Methanol	250	-
	Water	50	-
St. John's wort	Methanol	100	353
	Water	350	-
Aronia	Methanol	-	-
	Water	160	-
Galanthamine		22	24

- Not evaluated.

IC_{50} values were obtained from the dose-effect curves by linear regression.

Adsersen, Gauguin, Gudiksen, *et. al*., (2006) found AChE inhibitory activity water and methanolic extracts of rue, 0.1mg/ml caused 22 and 39%. Zheleva-Dimitrova and Balabanova (2012) reported the antioxidant and AChE inhibitory potential of methanol extract from *Arnica montana* cultivated in Bulgaria. The results demonstrated that arnica extract has strong antioxidant and AChE inhibitory activities (IC_{50}= 311 μg/ml). Many *Hypericum* species showed significant inhibition of AChE, with IC_{50} values between 0.62 and 1.79 mg dry extract/ml. The analysis indicated that chlorogenic acid, rutin, hyperoside, isoquercitrin, and quercitrin were the main compounds present in the water extracts. These compounds have strong anti- acetylcholinesterase activities, with IC_{50} values between 62 μg/ml and 196 μg/ml (Hernandez, Falé, Araújo *et al*., 2010).

In our research the lowest inhibition effect for the investigated enzymes was reported for sage exracts. Reserach done by Orhan *et al*. (2007) on *Salvia* plant family has indicated that only chloroform and petrol ether extracts have shown significant inhibitory activity on AChE. Essential oils of *Salvia lavandulaefolia,* and also some other isolated components, showed inhibition efficiency for AChE (Perry, Bollen, Perry *et. al*., 2003), while methanolic and water extracts did not show inhibition efficiency. It was also reported (Savelev, Okello and Perry, 2004) that the essential oils obtained from species of *Salvia* inhibit AChE and BuChE in a time dependent manner. The oils of *S. fruticosa* and *S. officinalis* var. *purpurea* had apparent dual cholinergic activity, namely they were active on both enzymes within the incubation time, while the duality of the oil of *S. officinalis* was less apparent.

CONCLUSION

Five medicinal plants were screened for inhibitory activity on AChE and BuChE. The results show that extracts from the aerial parts of St John's wort and rue and flowers of arnica could inhibit the activity of AChE or BuChE or both. The best inhibition effect was observed using the water extract of rue, methanolic extracts of St John's wort and arnica at concentration of 400 μg ml^{-1}. The results show that these plants could be very interesting for further isolation of acetylcholinesterase inhibitors, which are widely used in the treatment of Alzheimer's disease.

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Summary / Sažetak

Inhibicija enzima koji razgrađuju acetilkolin, acetilkolinesteraze (AChE) i butirilkolinesteraze (BuChE), smatra se obećavajućom strategijom za liječenje Alzheimerove bolesti (AD). Alzheimerova bolest je kronični neurološki poremećaj koji se očituje u smanjenju pamćenja, kognitivne disfunkcije i poremećajem ponašanja. Obilje biljaka u prirodi pruža potencijalni izvor inhibitora za AChE i BuChE. U ovom istraživanju odabrali smo pet biljaka koje se koriste u tradicionalnoj medicini za liječenje različitih poremećaja središnjeg živčanog sustava. Pomoću Ellmanove kolorimetrijske metode testiran je inhibicijski učinak vodenih i metanolnih ekstrakata kadulje (*Salvia officinalis* L.), arnike (*Arnica montana* L.), rute (*Ruta graveolens* L.), gospine trave (*Hypericum perforatum* L.) i aronije (*Aronia melanocarpa* (Michx.) Elliot.) na AChE i BuChE. Kao pozitivna kontrola korišten je galntamin hidrobromid. Rezultati ukazuju da ekstrakti nadzemnih dijelova gospine trave i rute i cvjetova arnike mogu inhibirati aktivnost AChE ili BuChE, ili oboje. Najjači inhibicijski učinak uočen je kod vodenog ekstrakta rute, potom metanolnih ekstrakata arnike i gospine trave pri koncentraciji od 400 μg ml^{-1}