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Investigation of polysaccharide extracts from Iranian and French strains of *Agaricus subrufescens* against enzymes involved in Alzheimer's disease

[Investigación de extractos de polisacáridos de cepas iraníes y francesas de *Agaricus subrufescens* contra enzimas relevantes para la enfermedad de Alzheimer]

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Abstract: In this work, the inhibitory activity of a wide range of polysaccharide extracts from two Iranian and French strains of *Agaricus subrufescens* were evaluated toward acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Among them, two extracts S9 and S′7 obtained from Iranian and French strains under different extraction conditions showed selective AChE inhibitory activity with IC_{50} values of 154.63 and 145.43 µg/mL, respectively. It should be noted that all extracts from both strains demonstrated no BChE inhibitory activity. S9 and S′7 were also tested for their effect on amyloid beta (Aβ) aggregation, antioxidant activity, and neuroprotectivity. Their activity against Aβ aggregation was comparable to that of donepezil as the reference drug but they induced moderate antioxidant activity by DPPH radical scavenging activity and negligible neuroprotectivity against Aβ-induced damage.

Keywords: *A. subrufescens*; Alzheimer's disease; Amyloid beta (Aβ); Antioxidant activity; Polysaccharide; Neuroprotectivity.

Resumen: En este trabajo, se evaluó la actividad inhibitoria de acetilcolinesterasa (AChE) y butirilcolinesterasa (BChE) para varios extractos de polisacáridos de dos cepas iraníes y francesas de *Agaricus subrufescens*. Los extractos más potentes mostraron valores de IC50 de 154,63 y 145 µg/ml para las cepas iraní (S9) y francesa (S′7), respectivamente, las cuales se obtuvieron de diferentes condiciones de extracción; sin embargo, todos los extractos no mostraron actividad inhibitoria de BChE. Además, S9 y S′7 se probaron para determinar su efecto sobre la agregación de beta-amiloide (Aβ), así como su actividad antioxidante y neuroprotectora. Su actividad inhibitoria de la agregación de Aβ fue comparable con donepezil, fármaco de referencia, pero indujeron una actividad antioxidante moderada, medida mediante la captación de radicales DPPH, y una neuroprotectora insignificante contra el daño inducido por Aβ.

Palabras clave: *A. subrufescens*; Enfermedad de Alzheimer; Beta amiloide (Aβ); Actividad antioxidante; Polisacarido; Neuroprotección.

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INTRODUCTION

Alzheimer's disease (AD) is a serious neurodegenerative disorder which is mainly caused by multiple mechanisms such as accumulation of extracellular Aβ42 plaques (Sikanyika *et al*., 2019), intracellular hyper-phosphorylated tau neurofibrillary tangles (Naseri *et al*., 2019), mitochondrial dysfunction leading to oxidative stress (Bhat *et al*., 2015), and reduction of acetylcholine (ACh) (Chen & Mobley, 2019) in the brain. AD is usually characterized by the loss of cognitive functions and reduction in intelligence to perform daily activities and mostly is led to dementia. In this respect, published information on the population of patients with AD is worrying and it is estimated that 5.8 million Americans of all ages are living with AD dementia in 2019 (Hebert *et al*., 2013). The worldwide prevalence will be predicted to increase to 100 million by 2050 (Cai *et al*., 2016). The prevalence of AD on the one hand and the poor quality of life along with expensive cost on the other hand have brought lots of difficulties for patients, their families, and societies. However, as the nature of AD is complicated there is no definite approach to the treatment of AD and available drugs only help to improve cognitive and behavioral symptoms with no significant impact on the disease itself. It should be mentioned that they generally are cholinesterase (ChE) inhibitors such as donepezil, rivastigmine, and galantamine (Hansen *et al*., 2008). In search of efficient drugs for the treatment of AD, various drug candidates have been designed and synthesized based on the involved mechanisms in the creation of AD (Mangialasche *et al*., 2010; Sameem *et al*., 2017), however, the clinical trial of single-target drugs have failed (Cummings *et al*., 2018; Mullane & Williams, 2018). Consequently, looking for an efficient treatment is currently in high demand and at this juncture, natural resources (Koynova & Tenchov, 2018) have attracted lots of attention due to lower adverse effects and more diversity comparing with currently available drugs.

Polysaccharides, the most abundant group of biopolymers are versatile biologically-active macromolecules possessing antitumour, immunomodulating, antithrombotic, antiviral, antiinflammation, and antioxidative activity (Liun *et al*., 2015). Recent research on antioxidant polysaccharides have endorsed their efficacy for the treatment of neurodegenerative diseases as they have been known for the reduction of oxidative damages in cellular and animal models (Li *et al*., 2017). In addition, polysaccharide obtained from *Vitis vinifera* L. has depicted therapeutic effect on AD in a rat model *via* inhibition of inflammatory response (Ma *et al*., 2018) which has been known as an important factor in the pathogenesis of AD (Morales *et al*., 2014). The study reported by Pei *et al*. (2017), revealed that oral administration of the polysaccharides as well as aqueous extract of *G. lucidum* improved neural progenitor cell (NPC) proliferation to enhance neurogenesis and alleviated cognitive deficits in transgenic AD mice. Moreover, it was found that those polysaccharides activated fibroblast growth factor receptor 1 (FGFR1) and downstream extracellular signal-regulated kinase (ERK) and AKT cascades (Huang *et al*., 2017).

Intragastric administration of polysaccharides obtained from *C. pilosula* diminished hyperphosphorylation leading to improvement of cognitive defects in hTau infected mice (Zhang *et al*., 2018). Also, the cognitive-enhancing effect of polysaccharides extracted from *Flammulina velutipes* was investigated by compatibilizing with ginsenosides on D-galactose-induced Alzheimer's disease (AD) rats and beneficial effect were associated with promoting anti-oxidant and anti-apoptosis (Zhang *et al*., 2018). It is worth mentioning that recently the efficacy of polysaccharides from traditional Chinese medicine (TCM) on AD has absorbed lots of attention to develop carbohydrate-based drug discovery (Liu *et al*., 2017).

Mushrooms and their medicinal properties have drawn lots of attention as they are thought to demonstrate approximately 130 medicinal properties such as immunomodulating, antitumor, antioxidant, radical scavenging, antiviral, antibacterial, anti‑ parasitic, antifungal, antihypercholesterolemic, cardiovascular, detoxicating, hepatoprotec-tive, and antidiabetic activities (Wasser, 2014). Recently, they have been interpreted as a versatile therapeutic agent for AD according to *in vitro*, *ex vivo*, cell line and animal models (Rahman *et al*., 2016). In this respect, *Agaricus subrufescens* is an edible and medicinal mushroom which has become a valuable natural source in many countries and been cultivated throughout the world due to versatile pharmaceutical properties including anti-cancer and tumor suppressive, anti-genotoxicity, anti-allergy, antimicrobial activity activities (Kerrigan, 2005;

Wisitrassameewong *et al*., 2012). The alcoholic extraction of *A. subrufescens* obtained at 60°C, showed an antioxidant activity on mitochondrial membranes lacking cytotoxicity in both cells systems evaluated CFU-GM and V79 at concentrations lower than 2.5 mg/mL (Lavitschka *et al*., 2007). Also, it has shown important biological responses on the immune system though modifying cytokines (Guggenheim *et al*., 2014). Recently, the extract of *A. blazei* as the main synonym of *A. subrufescens* has been reported for its crucial role in the regulation of proteins expression such as DAT and VMAT2 and pro-apoptotic and anti-apoptotic in Parkinson's disease (PD) (Venkatesh *et al*., 2019). Also, the neuroprotectivity of *A. blazei* against rotenone-induced motor and monmotor symptoms has been investigated in mice which confirmed strengthening effect of mitochondrial dysfunction, oxidative stress, and apoptosis (Venkateshgobi *et al*., 2018).

In this study, focusing on the reported potential anti-AD activity of mushrooms and the neuroprotective effect of *A. subrufescens*, various polysaccharide extracts of two Iranian and French strains of *A. subrufescens* was evaluated for ChE and Aβ aggregation inhibitory activity as well as neuroprotectivity and antioxidant properties.

MATERIAL AND METHODS

Cultivation of mushrooms

Cultivation of two Iranian and French strains of *A. subrufescens* was carried out under standard conditions as described in the literature for *A. subrufescens* (Llarena-Hernandez *et al*., 2014; Mahdizadeh *et al*., 2017). They were deposited at TMU herbarium (collection of mushrooms at Tarbiat Modares University (TMU)), Tehran, Iran and CGAB herbarium (Collection of the Germ plasm of Agaricus at Bordeaux, Institute National de la Recherche Agronomique, INRA, MycSA, Villenave d'Ornon, France) and indicated by codes VM098 and CA487, respectively.

Preparation of extracts

Initially, the fruiting bodies of mushrooms were cleaned, dried and powdered for the preparation of different polysaccharide extracts. The powder (5 g) was soaked in desired solvent (100 mL, Table No. 1) either at reflux for 1 h or room temperature overnight. Then, the solution was filtered off and the extraction was repeated under the same conditions. Finally, the solvent was evaporated under vacuum and the residue was lyophilized.

In the case of entries 11, 12, and 13, the corresponding extracts were obtained from ethanol precipitation. For this purpose, the extraction was conducted using refluxing distilled water, the solvent was reduced to 50 mL under vacuum, then, EtOH 50%, 70%, or 90% (50 mL) was added and the solution was kept at 4°C for 2 h. Then, it was centrifuged at 2000 rpm for 5 min and the precipitates were separated. All extracts were obtained ranging from 95 to100 mg.

Polysaccharide determination

The total polysaccharide content of extracts was determined using the phenol-sulphuric acid method in term of α-D-glucose (Chow & Landhäusser, 2004).

Calibration curve of standard α-D-glucose

A stock solution of α-D-glucose was prepared by dissolving 10 mg of the corresponding extract in a 100 mL volumetric flask. A series of eleven 25-mL volumetric flasks respectively containing 1, 2, 3, 4, 5, 6, 7, 8, 10, 20 and 25 mL of stock solution were prepared and diluted to the related volume with distilled water. Then, 0.5 mL of 5% (w/v) phenol was added to 0.5 mL of each sample solution, followed by 2.5 mL of concentrated H_2SO_4 and mixed well on a vortex. After cooling to room temperature, the absorbance was measured using a spectrophotometer at 490 nm and the related calibration curve was plotted.

Estimation of polysaccharide in term of α-D-glucose

To determine the polysaccharide content of samples, at least 10 mg of each lyophilized extract was transferred to a 25-mL volumetric flask and made up to volume with distilled water. In some cases, sonication was used for rapid solution. Polysaccharide content in 0.5 mL of each extract stock solution was determined using the above validated spectrophotometric method.

Protein determination

For determination of total proteins in mushrooms, Bradford method was used (Bradford, 1976). Absorbance measurements were recorded on a UV-VIS spectrophotometer at 595 nm.

At first, Coomassie Brilliant Blue G-250 (100

mg) was dissolved in 95% ethanol (50 mL). To this solution, phosphoric acid 85% (w/v) (100 mL) was added and the resulting solution was diluted to a final volume of 1 L. Bovine serum albumin (BSA) was used as a standard protein at concentration of 100 µg/mL in distilled water.

Standard assay method

1, 2, 3, 4, 5, 6, 7, 8 and 9 mL of BSA stock solution (0.1 mg/mL) were pipetted into the volumetric flasks and made each up to 10 mL with distilled water. 1 mL of each solution following with 5 mL of protein reagent were transferred to the test tubes and the solutions were gently shaken. Then, the absorbance was measured within 2-60 min after addition of the reagent at 595 nm.

Determination of total proteins in mushrooms

At least 30 mg of each extract was transferred to a 5-mL volumetric flask, made up to volume with distilled water, and in some cases, sonication was used for rapid solution. Protein content of 1 mL of each solution was estimated using the above validated spectrophotometric method.

AChE and BChE inhibition assay

Acetylcholinesterase (AChE, E.C. 3.1.1.7, Type V-S, lyophilized powder, from electric eel, 1000 unit), butylcholinesterase (BChE, E.C. 3.1.1.8, from equine serum), all required reagents were obtained from Sigma-Aldrich. *In vitro* anti-AChE activity was performed according to the modified Ellman's method (Ellman *et al*., 1961; Mahdavi *et al*., 2019). For this purpose, the corresponding extracts were dissolved in the mixture of MeOH and water (50%, v/v). Each well contained 50 µL potassium phosphate buffer (KH_2PO_4/K_2HPO_4, 0.1 M, pH 8), 25 µL prepared sample solution as described above, 25 µL enzyme with final concentration of 0.22 U/mL in buffer.

They were preincubated for 15 min at rt, and then 125 µL DTNB (3 mM in buffer) was added to each well. Characterization of the hydrolysis of ATCI catalyzed by AChE was performed spectrometrically at 405 nm followed by the addition of substrate (ATCI, 3 mM in water). The absorbance measurements were recorded at 405 nm. The IC_{50} values were determined graphically from inhibition curves (log inhibitor concentration *vs.* percent of inhibition). A negative control was also performed under the same conditions without inhibitor and donepezil was used as the positive control. For all tests, four different concentrations were tested for each extract in triplicate. Similarly, BChE inhibitory assay was conducted for all extracts.

Kinetic studies of AChE inhibition

To gain an insight into the inhibition model and inhibition constant *Ki*, reciprocal plots of 1/V versus 1/[S] were obtained using different concentrations of the substrate acetylthiocholine (Mahdavi *et al*., 2019). For this purpose, experiments were completely conducted according to the method as mentioned above. The rate of enzymatic reaction was recorded using different concentrations of inhibitor (0, 80, 300, and 500 µg/mL) as well as in the absence of inhibitor. For each experiment, reaction was initiated by adding acetylthiocholine and the progress curves were recorded at 405 nm within 2 min. Next, double reciprocal plots (1/v vs. 1/[s]) were made using the slopes of progress curves to achieve the type of inhibition. Slopes of these reciprocal plots were then plotted against the concentration of extract in a weighted analysis, and *Ki* was determined as the intercept on the negative x-axis. All rate measurements were performed in triplicate and data analysis was performed with Microsoft Excel 2013.

Inhibition of self-induced Aβ (1-42) aggregation

Inhibition of self-induced Aβ (1-42) aggregation was measured using a Thioflavin T(ThT)-binding assay (Bartolini *et al*., 2007). HFIP pretreated Aβ (1-42) samples (Anaspec Inc) were resolubilized with a 50 mM phosphate buffer (pH 7.4) to afford a solution at concentration of 20 µM. To prepare a stock solution, each tested sample was firstly prepared in DMSO. A mixture of the peptide (10 µL, 10 µM, final concentration) with or without the tested sample (10 µL, 50 µg/mL, final concentration) was incubated at 30°C for 48 h. Blank sample containing 50 mM phosphate buffer (pH 7.4) instead of Aβ with or without inhibitors were also considered. After incubation, samples were diluted to a final volume of 200 µM with 50 Mm glycine-NaOH buffer (pH 8.0) containing thioflavin-T (5 µM). Each measurement was run in triplicate and fluorescence was measured on a Synergy HTX Multi-Mode reader from BioTek Instruments with excitation and emission wavelengths at 440 nm and 485 nm, respectively. The percent inhibition of aggregation was calculated

by the expression (1- IF_i/IF_O)×100% in which IF_i and IF_O are the fluorescence intensities obtained for Aβ in the presence and absence of inhibitors, respectively.

Neuroprotection effect against Aβ-induced damage
The ability of polysaccharide extracts in protecting neuronal PC12 cells against damage induced by $Aβ_{25-35}$ was examined by the MTT assay as previously described in the literature (Iraji *et al*., 2017).

DPPH radical scavenging activity (DPPH)
All compounds and reagents were obtained from Sigma and antioxidant activity of extracts were evaluated using DPPH (1,1-diphenyl-2-picrylhydrazyl) according to the literature (Kumarasamy *et al*., 2007). At first, the solutions of extracts were freshly prepared at concentration of 1 mg/mL. 1 mL of each solution was serially diluted using methanol to obtain concentrations ranging from 500 to 16 μg/mL. Then, solution of DPPH in metanol (1 mL, 70 μg/mL) was added to each diluted solution and they were kept at 25°C for 30 min in the dark. Finally, the absorbance measurements were recorded at 517 nm comparing with butylated hydroxyanisole (BHA) which was used as a positive control. All tests were achieved in triplicate and IC_{50} value was reported as means ± SD.

RESULTS AND DISCUSSION
Initially, various polysaccharide extracts of two Iranian and French strains (series **S** and **S′**, respectively) of *A. subrufescens* were prepared under various conditions as reported in Table No. 1 (entries 1-13). They were obtained using distilled water, absolute ethanol, ethanol 70%, ethanol 50% at different temperatures and pH conditions. Also, three extracts (entries 11-13) were obtained *via* ethanol precipitation. All of them were evaluated for their total polysaccharide and total protein contents using phenol-sulphuric acid (Chow & Landhäusser, 2004) and Bradford (Bradford, 1976) methods, respectively.

Polysaccharide content
As can be seen in Table No. 1, the highest content of polysaccharides generally was found in ethanol precipitated extracts (entries 11-13) and the lowest contents belonged to the absolute ethanol extracted samples (entries 3 and 4). Extracts **S11**, **S12**, and **S13** contained 71.3, 68.1, and 53.1% respectively comparing with their counterparts **S′11**, **S′12**, and **S′13** having 34.1, 12.1, and 20.0% of polysaccharides, respectively. Extracts **S4** and **S3** possessed 0.3 and 0.8%, and **S′4** and **S′3** contained 0.8 and 0.4% of polysaccharides, respectively. Other extracts from Iranian and French strains showed polysaccharide contents of 4.0-29.2% and 2.4-18.7%, respectively. Also, it should be noted that Iranian strain usually demonstrated higher content of polysaccharides than French strain.

Protein content
According to our results shown in Table No. 1, the highest content of proteins was afforded by ethanol precipitated extracts (entries 11-13). Extracts **S11**, **S12**, and **S13** contained 2.81, 2.75, and 2.12% of proteins, respectively and extracts **S′11**, **S′12**, and **S′13** possessed 5.44, 3.83,, and 3.23% of proteins, respectively. Among extracts obtained from Iranian strain, **S6**, **S3** and **S8** contained the lowest content of proteins 0.02, 0.06, and 0.10%. Also, **S′4**, **S′5**, and **S′6** obtained from French strain possessed 0.27, 0.76, and 0.56%, respectively. However, French strain usually demonstrated higher content of proteins than Iranian strain.

Cholinesterase inhibitory activity
Anti-cholinesterase activity of different polysaccharide extracts of two Iranian (**S**) and French (**S′**) strains of *A. subrufescens* reported in Table No. 2, was evaluated according to the modified Ellman's method (Ellman *et al*., 1961; Mahdavi *et al*., 2019). Our results clearly confirmed the effect of extraction conditions on the ChEI activity of polysaccharide extracts. Among different polysaccharide extracts from Iranian strain (**S1-13**), the best inhibitory activity was induced by the extract which was obtained by refluxing water at pH = 4 (**S9**, IC_{50} = 154.63 μg/mL). Interestingly, increasing the pH to 8 deleted anti-AChEI activity (**S10**, IC_{50}>500 μg/mL). It was founds that extraction using distilled water afforded different results. The extract obtained at room temperature (**S2**) (IC_{50} = 288.49 μg/mL) was much higher active than that of obtained under reflux condition (**S1**). Changing solvent from distilled water to absolute ethanol (**S3** and **S4**) led to lack of anti-AChE activity either at room temperature or at reflux (IC_{50} > 500 μg/mL). Some extracts were prepared from the mixture of ethanol and water (70% (v/v) and 50% (v/v)) at room temperature or reflux. It should be mentioned that extracts obtained from the

mixture of water and EtOH at room temperature (**S6** and **S8**) showed no activity (IC_{50}>500 µg/mL), however, their counterparts which was prepared at reflux (**S5** and **S7**) were found to be moderate AChE inhibitor with IC_{50} values of 226.66 and 382.18 µg/mL, respectively.

Table No.1
Polysaccharide and protein content of polysaccharide extracts

Entry	Extraction condition	% (w/w) of total polysaccharides		% (w/w) of total proteins	
		S	**S′**	**S**	**S′**
1	Distilled water, reflux	22.6	18.7	0.63	1.21
2	Distilled water, rt	4.0	3.0	0.91	1.69
3	Absolute ethanol, reflux	0.8	0.4	0.06	0.84
4	Absolute ethanol, rt	0.3	0.8	0.58	0.27
5	Ethanol 70%, reflux	16.0	6.5	0.59	0.76
6	Ethanol 70%, rt	9.0	3.6	0.02	0.56
7	Ethanol 50%, reflux	24.5	11.0	0.73	1.24
8	Ethanol 50%, rt	10.4	2.4	0.10	0.92
9	Distilled water, reflux, pH = 4	19.5	3.7	0.56	1.26
10	Distilled water, reflux, pH = 8	29.2	9.0	1.36	1.06
11	Ethanol precipitation (50%)	71.3	34.1	2.81	5.44
12	Ethanol precipitation (70%)	68.1	12.1	2.75	3.83
13	Ethanol precipitation (90%)	51.3	20.0	2.12	3.23

Table No. 2
The IC_{50} values of different extracts against AChE and BChE.[a]

Entry	**AChEI (S) [IC_{50} (µg)/mL]**	**BChEI (S) [IC_{50} (µg)/mL]**	**AChEI (S′) [IC_{50} (µg)/mL]**	**BChEI (S′) [IC_{50} (µg)/mL]**
1	>500	>500	>500	>500
2	288.49±6.39	>500	>500	>500
3	>500	>500	318.63±10.21	>500
4	>500	>500	>500	>500
5	226.66±4.76	>500	>500	>500
6	>500	>500	>500	>500
7	382.18±3.77	>500	145.43±0.48	>500
8	>500	>500	336.36±5.045	>500
9	154.63±0.62	>500	211.55±2.19	>500
10	>500	>500	>500	>500
11	>500	>500	>500	>500
12	>500	>500	>500	>500
13	>500	>500	>500	>500
Donepezil	0.020 ± 0.002		1.500 ± 0.268	

[a]Data are expressed as mean ± SD (three independent experiments).

It seems that the presence of higher amounts of ethanol in the mixture of solvent afforded extract having better AChEI activity (**S5** > **S7**), however, it should be remembered that the ethanolic extracts were not potent enough to induce AChEI activity and the presence of water was crucial for obtaining extracts possessing anti-AChE activity. In the case of ethanol precipitated extracts (**S11-13**), all of them were not active toward AChE.

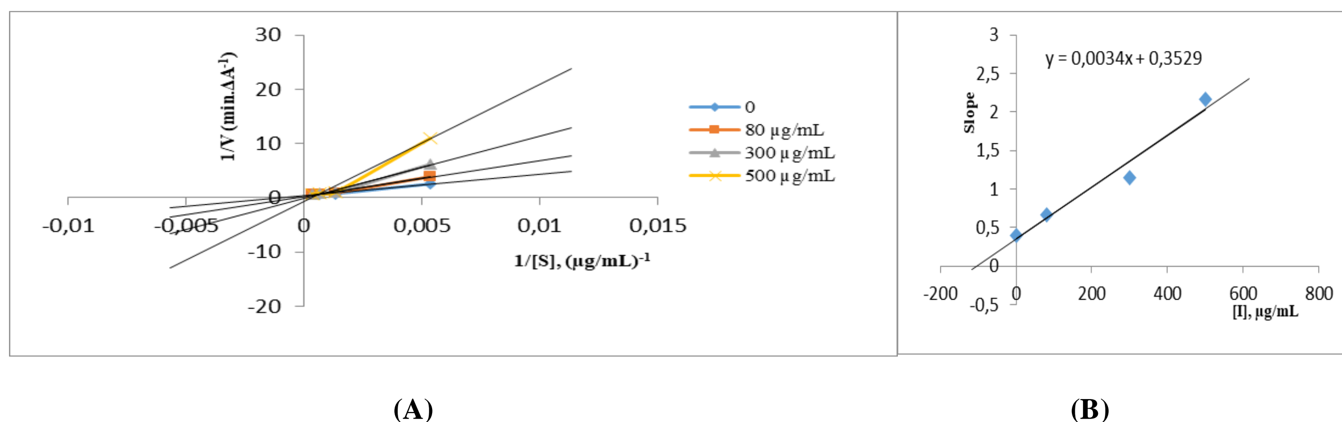
Among different polysaccharide extracts from French strain (**S′1-13**), the best AChEI activity was afforded by the extract **S′7** which was obtained from refluxing ethanol (50%) (IC_{50}=145.43 µg/mL). It should be noted that the extract **S′8** which was afforded from ethanol (50%) at room temperature induced lower activity than **S′7** (IC_{50}=336.36 µg/mL). Also, increasing the amounts of ethanol in the mixture of solvent led to the extracts which were inactive toward AChE either at room temperature or reflux condition (**S′5** and **S′6**, IC_{50}>500 µg/mL). Extracts **S′1** and **S′2** obtained from distilled water under different temperatures showed no activity (IC_{50}>500 µg/mL). Also, extract **S′3** obtained from refluxing absolute ethanol depicted moderate activity (IC_{50}=318.63 µg/mL), however, **S′4** obtained from absolute ethanol at room temperature did not display activity (IC_{50}>500 µg/mL). It should be noted that polysaccharides obtained from refluxing distilled water under acidic condition (**S′9**) depicted relatively good activity (IC_{50}=211.55 µg/mL) and increasing pH to 8 deleted activity. As observed in the case of ethanol precipitated extracts (**S′11-13**), no inhibitory activity was obtained.

Comparing AChEI activity of polysaccharide extracts from two Iranian **S** and French **S′** counterparts revealed some similarity and differences. Similarly, aqueous extract obtained at reflux, ethanolic extracts both 70% and absolute at room temperature, aqueous extract obtained at pH=8, and ethanol precipitated extracts showed no activity (entries 1, 4, 6, 10-13). The others, entries 2, 3, 5, 7, 8, and 9 depicted generally inverse activity. It is worth mentioning that the extracts obtained at pH=4 (**S9** and **S′9**) were found to be good inhibitor, however, Iranian stains showed better activity than French under the same conditions. It is clear that the extraction methods provided different polysaccharide extracts with different degrees of glucose polymerization (Júnior *et al*., 2015).

In the case of BChEI activity, all extracts from both Iranian and French strains showed no BChE inhibitory activity.

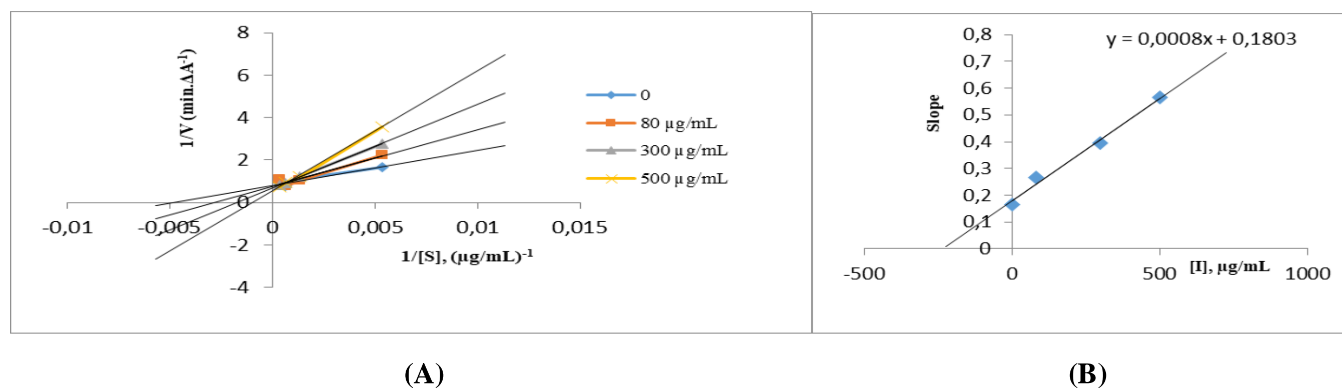
Kinetics study

In order to determine the mode of AChE inhibitory of the most potent polysaccharide extracts **S9** and **S′7**, a kinetic study was conducted based on the modified Ellman's method (Mahdavi *et al*., 2019). As it can be seen in Figure No. 1, graphical analysis of the Lineweaver-Burk reciprocal plots showed a competitive inhibition confirming the fact that the extract S9 can simultaneously bind to the catalytic anionic site (CAS) of enzyme. The inhibition constant (K_i=103.8 µg /mL) was calculated using secondary replots of the slope versus concentrations of extract.



(A) **(B)**

Figure No. 1
(A): Lineweavere-Burk plot for the inhibition of AChE by S9 at different concentrations of acetylthiocholine (ATCh). (B): Steady-state inhibition constant (*Ki*) of S9



(A) **(B)**

Figure No. 2
(A): Lineweavere-Burk plot for the inhibition of AChE by S′7 at different concentrations of acetylthiocholine (ATCh). (B): Steady-state inhibition constant (*Ki*) of compound

Also, the same mechanism of inhibition was obtained in the case of French strain **S′7** (Figure No. 2) and K_i=225.37 µg/mL was calculated.

Inhibition of self-induced Aβ (1-42) aggregation
The role of accumulation of the highly amyloidogenic self-associating Aβ is obviously known in the pathogenesis of AD (Sikanyika *et al*., 2019). Recently, carbohydrates have been found to prevent the formation of amyloid fibrils by Aβ peptides (Aβ (1-42), Aβ (1-40), and Aβ (25-35)) (Miura et al., 2007). In this respects, extracts **S9** and **S'7** were candidate for the evaluation of Aβ aggregation inhibitory activity. The self-induced Aβ aggregation inhibition of the most potent AChE inhibitors **S9** and **S′7** was investigated by Thioflavin T(ThT)-binding assay (Bartolini *et al*., 2007). Those extracts showed relatively good inhibitory activity of 13.88±1.45% and 11.70±2.6%, respectively at 50 µg/mL comparing with donepezil (14.70±2.35%) at 10 µg/mL.

Neuroprotectivity effect against Aβ-induced damage
Neuroprotectivity of the most potent AChE inhibitors **S9** and **S′7** against damage induced by $A\beta_{25-35}$ was investigated in PC12 cells by MTT assay (Iraji *et al*., 2017). They demonstrated 6.5% and 5.0% protection at concentration of 50 µg/mL, respectively.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity
Extracts **S9** and **S′7** were evaluated for their 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity (DPPH) comparing with to hydroxyanisole (BHA) as a standard antioxidant (Kumarasamy *et al*., 2007). They showed moderate antioxidant activity with IC_{50} values of 330.0±0.2 and 378.1±0.2 µg/mL comparing with BHA (IC_{50}=91.28± 0.13 µg/mL)

CONCLUSION
In conclusion, some biological properties related to anti-AD activity of polysaccharides from two Iranian and French strains of *A. subrufescens* were investigated. Those polysaccharides were obtained under various conditions which directly affected their anti-AChE activity, it which may be related to different degrees of glucose polymerization in the polysaccharide extracts of mushrooms. However, refluxing with water at pH=4 and refluxing with ethanol (50%) were found to be the best conditions to give polysaccharides possessing desirable activities. Also, it was perceived that candidate extracts from both Iranian and French strains induced good effect on Aβ aggregation comparing with donepezil. However, it should be noted that they showed moderate DPPH radical scavenging activity and insignificant neuroprotectivity against Aβ-induced damage.

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