

Cholinesterase and Glycation Inhibition Assay of Several Metabolites Obtained from Plant and Fungi

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ABSTRACT: Cholinesterase inhibition is one of the major strategies to develop better quality anti-Alzheimer's drug, while inhibition of glycation pathway is a useful therapeutic strategy to treat diabetic complications. This study was designed to evaluate the anti-acetylcholinesterase, anti-butyrylcholinesterase and anti-glycation activities of 8 secondary metabolites namely, RS-8, RS-22C (arborinine), CL-1 (betulinic acid), CL-2 (ursolic acid), CCL-1 (cajaninstilbene acid), CP-1 (beta-sitosterol), HS-1 (anhydrofusarubin) and HS-2 (methylether of fusarubin). It was found that RS-8 and arborinine showed promising activity in inhibiting acetylcholinesterase with IC₅₀ values of 24.40 ± 0.39 and 13.14 ± 0.07, respectively. Anhydrofusarubin and methyl ether of fusarubin exhibited moderate activities with IC₅₀ values of 47.82 ± 0.54 and 44.58 ± 0.8. Arborinine and cajaninstilbene acid potentially inhibited butyrylcholinesterase with IC₅₀ values of 26.34 ± 0.31 and 25.82 ± 0.34, respectively, but other metabolites did not show such inhibitory activities. Inhibition of glycation process was evaluated by bovine serum albumin assay but none of the metabolites significantly inhibited the glycation pathway.

Key words: Secondary metabolites, cholinesterase, acetylcholinesterase, butyrylcholinesterase, glycation

INTRODUCTION

Alzheimer's disease (AD) and diabetes mellitus (DM) are the two major health concerns globally. Alzheimer's disease is an irreversible, progressive brain disorder that slowly destroys memory and thinking skills and eventually the ability to carry out the simplest tasks. It occurs due to the cholinesterase induced declination of neurotransmitters. Cholinesterases are enzymes involved in breaking down neurotransmitters like acetylcholine (ACh) and butyrylcholine (BuCh) and are respectively called acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE).¹

Studies have shown that AD is defined by the rapid loss of AChE activity in the early stages of the disease, along with an increasing ratio of AChE as the disease progresses.^{2,3} Furthermore, BuChE has been found to be capable of compensating for the missing AChE catalytic functions in the synaptic cleft, and its activity increases by 30%-60% during AD.⁴⁻⁷ Due to the role of BuChE in the hydrolysis of ACh, the inhibition of both ChEs using a dual inhibitor should result in increased levels of ACh in the brain, which provide more successful clinical efficacy of AD.⁸

Diabetes mellitus (DM), commonly referred to as diabetes, is a group of metabolic diseases in which there are high blood sugar levels over a prolonged period. High blood sugar level occurs due to the increased glycation of proteins in the blood plasma. Glycation of proteins [advanced glycation end

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products (AGEs)] occurs through non-enzymatic condensation reaction between reducing sugars and amino groups of proteins that undergo rearrangements to stable ketoamines. These AGEs are thought to be the major causes of a number of diabetic complications.⁹

Although, synthetic drugs such donepezil, neostigmine and rivastigmine are available for the symptomatic treatment of AD, search for newer molecules from natural products has gained much attention by the researchers worldwide because of lower cost and less side effects. Majority of the secondary metabolites with potential AChE and BuChE inhibitory activity are alkaloids followed by terpenes, sterols, flavanoids, and glycosides. Examples of some secondary metabolites that have shown anticholinesterase activity include vasicinone, vasicine, harmine, deoxyvasicinone, deoxyvasicine, harmaline, harmol, and harmane.¹⁰ Most of the compounds are isolated from Buxaceae, Amaryllidaceae, Lycopodiaceae, Lamiaceae, Chenopodiaceae, Papaveraceae, Apocynaceae, and Labiatae species.^{11,12} Many indigenous plants have been used in folkloric medicine to manage diabetes from which some of them have received scientific attention.¹³ Several substances like glycosides, alkaloids, terpenoids, flavonoids, carotenoids etc. are reported to have antidiabetic effects such as diosmin, tangeritin, lycopene, syringin etc.¹⁴

In this present study eight (8) isolated secondary metabolites namely, RS-8, RS-22C (arborinine) isolated from *Ravenia spectabilis*, CL-1 (betulinic acid) and CL-2 (ursolic acid) obtained from *Chamaedorea linearis*, CCL-1 (cajaninstilbene acid) obtained from *Cajanus cajan*, CP-1 (beta-sitosterol) obtained from *Carica papaya*, HS-1 (anhydrofusarubin) and HS-2 (methylether of fusarubin) obtained from endophytic fungal strain, *Cladosporium* species, were assessed for the inhibition of AChE, BuChE and glycation pathway.

MATERIALS AND METHODS

Isolated secondary metabolites. Secondary metabolites that were used to assess the preliminary inhibitory effect on AChE, BuChE and glycation pathway are listed in figure 1. A brief of the sources of the isolated metabolites are: RS-8 and RS-22 were isolated from the plant *R. spectabilis* (Family: Rutaceae) through vacuum liquid chromatographic (VLC) separation technique. Later on RS-22C was confirmed as arborinine (1-hydroxy-2, 3-dimethoxy-10-methyl-9(10H)-acridinone) by nuclear magnetic resonance; while structure of RS-8 is yet to be confirmed.¹⁵ CL-1 (betulinic acid) and CL-2 (ursolic acid) were obtained from the plant *C. linearis* (Family: Myrtaceae);¹⁶ CCL-1 (cajaninstilbene acid) was obtained from *C. cajan* (Family: Fabaceae);¹⁷ CP-1 (beta-sitosterol) was obtained from *C. papaya* (Caricaceae).¹⁸ On the other hand, HS-1 (anhydro fusarubin) and HS-2 (methylether of fusarubin) were obtained from the endophytic fungal strain, *Cladosporium* species isolated from *R. serpentina* (Family Rutaceae).¹⁹

Materials and chemicals. 5, 5'-dithio-bis-(2-nitro) benzoic acid (DTNB), acetylcholine iodide, butyrylcholine iodide, Triton X-100, BCA (bicinchoninic acid) kit, trichloroacetic acid and rutin were purchased from (Sigma-Aldrich, Germany), Tris-HCl and bovine serum albumin were from Merck, Germany). Donepezil was a kind gift from Incepta Pharmaceuticals Ltd., Bangladesh. Other chemicals required were collected from local sources.

Acetylcholinesterase inhibition assay. The inhibition of AChE was assessed following Ellman method^{20,21} with minor modification using acetylthiocholine iodide as a substrate. As a source of AChE, rat brains were homogenized in a homogenizer with 5 times of a homogenation buffer [10 mM Tris-HCl (pH 7.2), which contained 1 M NaCl, 50 mM MgCl₂ and 1% Triton X-100],²² and then centrifuged at 10,000 rpm for 15 min. The supernatant so obtained was used as an enzyme source. The process of enzyme preparation was carried out at 4°C and the protein concentration was measured using the BCA (bicinchoninic acid kit)

using BSA as a protein standard. To carry out the reaction, each tested compound or standard (500 μ l) was mixed with an enzyme solution (500 μ l) and incubated at 37°C for 15 minutes, and then Ellman's reaction mixture [(3.5-ml 0.5 mM acetylthiocholine, 1 mM 5, 5'-dithio-bis-(2-nitro) benzoic acid) in a 50 mM sodium phosphate buffer (pH 8.0)] was added. Immediately after adding Ellman's reaction mixture, the rate of hydrolysis by acetylcholinesterase was

monitored spectrophotometrically taking absorbance at 405 nm. Recording of absorbance was repeated for 10 min at 2 min intervals to confirm the linearity of the reaction. Saline water in place of enzyme solution was used to carry out a blank reaction and donepezil was used as reference drug in the inhibitory assay. The inhibition of AChE activity was calculated in percentage using the following formula:

$$\% \text{ of inhibition of AChE activity} = \frac{(\text{Absorbance of control} - \text{Absorbance of test sample})}{\text{Absorbance of control}} \times 100$$

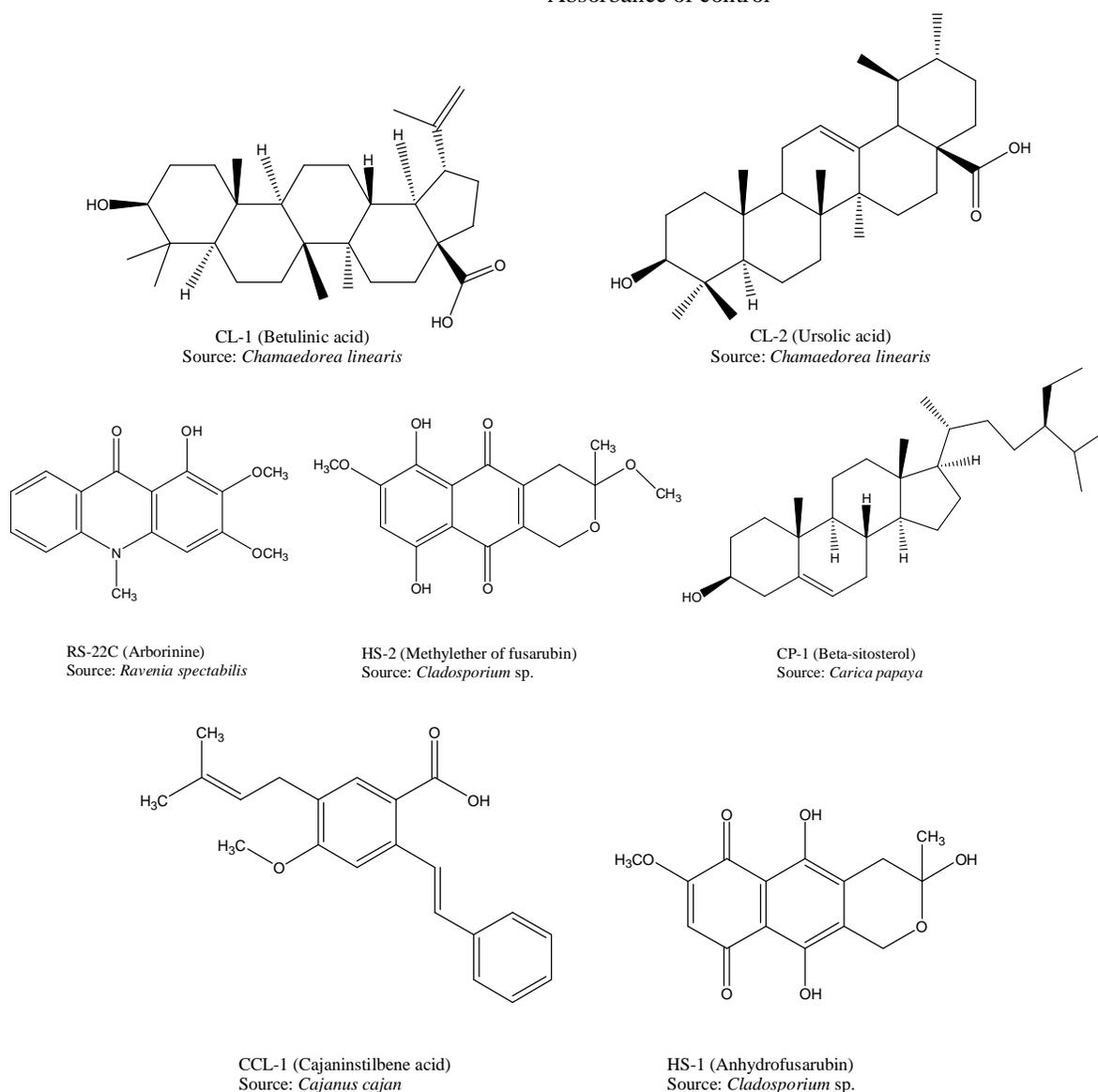


Figure 1. Secondary metabolites used for screening cholinesterase and glycation inhibition assay.

Butyrylcholinesterase inhibition assay. BuChE assay was performed according to the same method as described for AChE inhibition assay using butyrylthiocholine iodide as a substrate. However, as a source of enzyme, the human blood was homogenized in a homogenizer with 5 volumes of a homogenation buffer [10 mM Tris-HCl (pH 7.2), which contained 1 M NaCl, 50 mM MgCl₂ and 1% Triton X-100, and centrifuged at 10000 rpm for 15 min, and the resulting supernatant was used as an enzyme. The inhibition of BuChE activity was calculated in percentage using the same formula as mentioned above for AChE inhibition assay.

Glycation inhibition assay. Inhibition of glycation pathway was assessed using the bovine serum albumin (BSA) assay through Maillard reaction.²³ In all experiments, the final reaction volume was 1.0 ml and carried out in 1.5 ml Eppendorf tubes. In brief, 500 µl of BSA (1 mg/ml concentration) was incubated with glucose 400 µl (500 mM final concentration) and 100 µl sample; while 100 µl phosphate buffer saline was used as the sample control and 100 µl rutin as the reference standard. A negative control was carried out at the same time with BSA 500 µl (1 mg/ml concentration), 400 µl phosphate buffer saline and 100 µl sample incubated under same conditions. The reaction was allowed to proceed at 60°C for 24 hours and terminated by adding 10 µl of 100% (w/v) TCA. The TCA added mixture was kept at 4°C for 10 minutes and centrifuged 4 minutes at 13000 rpm. The precipitate was re-dissolved with alkaline phosphate buffer saline (pH 10) and was quantified for the relative amount of glycated BSA based on fluorescence intensity by Hitachi Fluorescence Spectrophotometer (f-7000). The excitation and emission wavelength used were at 370 nm and 440 nm, respectively. Each sample was analyzed in eight concentrations in triplicate manner. Inhibition of glycation was calculated in percentage (%) using the formula given below and the sample concentration required for the 50% of inhibition was calculated.

$$\% \text{ Inhibition} = \{(F_c - F_b) - (F_s - F_{sb}) / (F_c - F_b)\} \times 100$$

Where, F_c = fluorescence of incubated BSA, glucose and DMSO (control), F_b = fluorescence of incubated BSA alone (blank), F_s = fluorescence of incubated BSA, glucose and tested compound or rutin and F_{sb} = fluorescence of incubated BSA with the tested compound or rutin

Statistical analysis. The results are presented as mean ± SEM (standard errors) of triplicate experiment. The IC₅₀ values of the compounds were evaluated by non-linear regression analysis using GraphPad Prism Data Editor for Windows, Version 6.0 (GraphPad Software Inc., San Diego, CA). The data were analyzed by one-way ANOVA p < 0.05 and p < 0.01 were considered as statistically significant.

RESULTS AND DISCUSSION

***In vitro* AChE inhibitory activity.** Cholinesterase hydrolyses the choline esters such as acetylcholine, butyrylcholine, etc. These choline esters play an important role in the transmission of nerve impulse. Hence, inhibition of cholinesterases has become an important target for the treatment of neurodegenerative disorders, including Alzheimer's disease.²⁴ In this study, 8 isolated secondary metabolites were assessed for AChE and BuChE inhibitory activities and their results are shown in table 1. From the obtained data, it is observed that, RS-8 and RS-22C (arborinine) showed significant inhibition of AChE compared to the standard donepezil. Arborinine at a concentration of 25 µg/ml inhibited 56.34% AChE activities, while the standard drug donepezil inhibited 68.41% activities of cholinesterase at the same concentration. Activity was found to be increased at higher dose. RS-8, HS-1 and HS-2 also significantly inhibited AChE activities with values of 49.78, 44.40 and 42.45%, respectively. The inhibitory effects were found to be increased with increased dose and these activities were showed in table 1. The inhibition of cholinesterase was found promising with RS-22C (arborinine) which was compared to other tested compounds. This finding suggests that RS-22C might be a lead compound to

develop future drug in the treatment of AD. Previous study reported the effect of arborinine against AChE²⁶, malaria²⁷ and cancer cells.^{28,29} The present study further confirm the effect of arborinine in inhibiting AChE.

***In vitro* BuChE inhibitory activity.** In assessment of the butyrylcholinesterase inhibitory activities, as shown in table 1, the IC₅₀ (μg/ml ± SEM) values were found to be 49.88 ± 0.822 for RS-8, 26.34 ± 0.314 for RS-22C (arborinine) and 25.82 ± 0.3403 for CCL-1 (cajaninstilbene acid). Comparing the activity with standard donepezil, RS-22C (arborinine) and CCL-1 (cajaninstilbene acid) showed promising activities, whereas RS-8

(arborinine) exhibited moderate activity. Previous study reported the effect of cajaninstilbene acid activities against D-amino acid oxidase (DAAO), AChE and BuChE,³⁰ whereas arborinine exhibited BuChE inhibitory activities.³¹ The present study further confirm the effect of arborinine and cajaninstilbene acid in inhibiting BuChE. A comparison of the AChE and BuChE inhibition by the tested secondary metabolites are presented in figure 2. From the figure, it was found that arborinine, fusarubin and anhydrofusarubin more potentially inhibited AChE than BuChE; while cajaninstilbene acid more potentially inhibited BuChE than that of AChE.

Table 1. Anticholinesterase activity of secondary metabolites obtained from plants and endophytic fungi where donepezil was used as standard drug.

Tested compounds	AChE inhibition (IC ₅₀ (μg/ml) ± SEM)	BuChE inhibition (IC ₅₀ (μg/ml) ± SEM)
Donepezil	6.31 ± 0.09	11.93 ± 0.13
(RS-8)	24.40 ± 0.39	49.88 ± 0.82
Arborinine (RS-22C)	13.14 ± 0.07	26.34 ± 0.31
Betulinic acid (CL-1)	160 ± 2.31	108.58 ± 1.20
Ursolic acid (CL-2)	252.14 ± 5.27	309.80 ± 3.01
Cajaninstilbene acid (CCL-1)	117.12 ± 2.46	25.82 ± 0.34
Bitá-sitosterol (CP-1)	208.16 ± 2.11	288.52 ± 3.17
Anhydrofusarubin (HS-1)	47.82 ± 0.54	398.72 ± 18.57
Methylether of Fusarubin (HS-2)	44.58 ± 0.80	196.46 ± 4.30

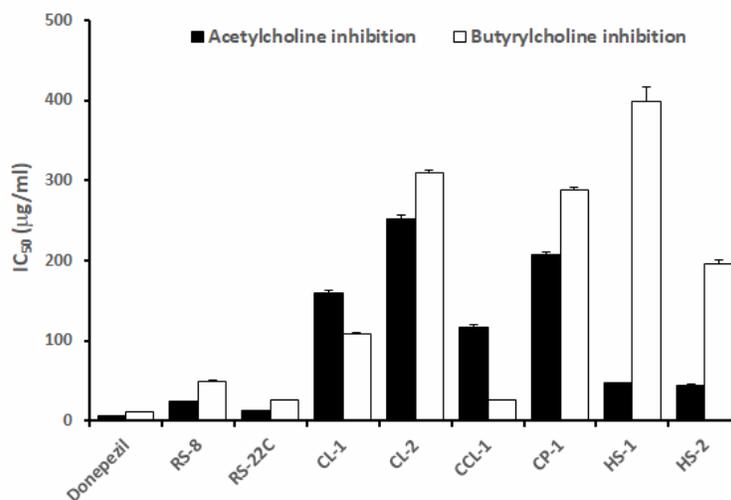


Figure 2. Comparison of acetylcholinesterase and butyrylcholinesterase inhibitory activities by secondary metabolites and donepezil as standard drug.

***In vitro* glycation pathway inhibition activity.**

These secondary metabolites were screened for antiglycation activities using the bovine serum albumin (BSA) assay. The antiglycation activities were presented as IC₅₀ (Inhibitory concentration 50%) ($\mu\text{g/ml} \pm \text{SEM}$) and the values were showed in table 2. Glycation process in human body is a key molecular pathway for diabetic complications like diabetes retinopathy, diabetic nephropathy etc. Rutin as standard drug potentially inhibit glycation process in our body. However, comparing the study data of the isolated secondary metabolites with that of rutin, it was found that none of the tested compounds showed any antiglycation activities.

Table 2. Antiglycation activity of secondary metabolites obtained from plants and endophytic fungi where rutin was used as standard.

Tested compounds	(IC ₅₀ ($\mu\text{g/ml}$) \pm SEM)
Rutin	46.77 \pm 0.42
RS-8	282.25 \pm 2.14
Arborinine (RS-22C)	234.36 \pm 1.91
Betulinic acid (CL-1)	320.31 \pm 5.22
Ursolic acid (CL-2)	308.26 \pm 1.24
Cajaniinstilbene acid (CCL-1)	187.83 \pm 2.74
Bitasitosterol (CP-1)	470.37 \pm 12.90
Anhydrofusarubin (HS-1)	220.60 \pm 1.26
Methylether of Fusarubin (HS-2)	212.54 \pm 0.16

CONCLUSION

Only three cholinesterase inhibitors such as donepezil, galantamine, rivastigmine and an NMDA receptor antagonist, memantine, currently approved drugs by Food and Drug Administration (FDA) are used to treat AD. The purpose of cholinesterase inhibitors is to inhibit the degradation of acetylcholine within synapses, resulting in increased amount of acetylcholine. Memantine, which is a partial NMDA receptor antagonist, protects neurons from glutamate-induced excitatory damages.³² The cholinesterase inhibitors employed in current treatments are limited and they show a wide variety of toxic effect including insomnia, anorexia, diarrhea, fatigue, nausea, gastrointestinal disorders and

cardiovascular disorders.^{33,34} Therefore, researchers have focused their attention towards the discovery of new drugs from natural sources such as plants, which have enormous potential for the treatment of AD. Here RS-22C (arborinine) showed significant anticholinesterase activity which has not been seen before, and in case of butyrylcholinesterase inhibitory activities, CCL-1 (cajaniinstilbene acid) showed the moderate activity. Our preliminary result suggest that arborinine and cajaniinstilbene acid can be studied further in other *in vitro* and *in vivo* model to confirm the activities.

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