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# Garcinexanthone G, a Selective Butyrylcholinesterase Inhibitor from the Stem Bark of *Garcinia atroviridis*

**Kooi-Yeong Khaw[1], Vikneswaran Murugaiyah[1], Melati Khairuddean[2], and Wen-Nee Tan[3,*]**

[1]*Discipline of Pharmacology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia*
[2]*School of Chemical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia*
[3]*School of Distance Education, Universiti Sains Malaysia, 11800 Penang, Malaysia*

**Abstract** – The present study was undertaken to investigate the isolated compounds from the stem bark of *Garcinia atroviridis* as potential cholinesterase inhibitors and the ligand-enzyme interactions of selected bioactive compounds *in silico*. The *in vitro* cholinesterase results showed that quercetin (**3**) was the most active AChE inhibitor (12.65 ± 1.57 μg/ml) while garcinexanthone G (**6**) was the most active BChE inhibitor (18.86 ± 2.41 μg/ml). It is noteworthy to note that compound **6** was a selective inhibitor with the selectivity index of 11.82. Molecular insight from docking interaction further substantiate that orientation of compound **6** in the catalytic site which enhanced its binding affinity as compared to other xanthones. The nature of protein-ligand interactions of compound **6** is mainly hydrogen bonding, and the hydroxyl group of compound **6** at C-10 is vital in BChE inhibition activity. Therefore, compound **6** is a notable lead for further drug design and development of BChE selective inhibitor.

**Keywords** – *Garcinia atroviridis*, Garcinexanthone G, Butyrylcholinesterase, Molecular docking

## Introduction

Alzheimer's disease (AD) is the common type of dementia among old people, characterized by gradual loss in memory, languages and cognitive function.[1] It is estimated that about 46.8 million people living with dementia and the number of cases will soar up to 131.5 million by 2050.[2] The cause of this disease still remained unknown and there are about 20 hypotheses referring to this disease,[3] where tau-protein aggregation, $\beta$-amyloid deposition, oxidative stress and cholinergic dysfunction are the common ones.[4,5] Drug discovery to alleviate this disease still remains as a challenge, whereby most of the drugs in clinical trials failed to demonstrate the disease-modifying ability to remove the accumulation of $\beta$-amyloid or tau-proteins in the brain.[4] To date, cholinergic system still remains as the plausible target to effectively manage the behaviour and cognitive symptoms in dementia, and most of the Food and Drug Administration (FDA) approved drugs for management of AD are acetylcholinesterase inhibitors.[6]

*Author for correspondence
Wen-Nee Tan, School of Distance Education, Universiti Sains Malaysia, 11800 Penang, Malaysia.
Tel: +604-6535906; E-mail: tanwn@usm.my

It is evidenced that a deficit of acetylcholine (ACh) neurotransmitter in the central cholinergic neurotransmission caused by the degeneration of the cholinergic neurons is an important neurochemical deficit of AD.[7] Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes are responsible for the hydrolysis of ACh and the regulation of cholinergic neurotransmission.[6] In the central nervous system, AChE is located mainly in the neuron while BChE is found in the glial cells.[8] AChE accounts for approximately 90% of cholinesterase activity in the temporal cortex of normal human brain.[9] Both AChE and BChE have a primary hydrophobic active-site gorge, approximately 20Å.[10,11] AChE has a narrower active gorge than BChE and it is substrate specific.[10,11] Although AChE plays an important role in the degradation of ACh, but the importance of BChE is postulated in patients affected with AD. The AChE activity decreases progressively from mild to severe stages with the AD disease, but the activity of BChE increases to as high as 120%.[7,9] Hence, a selective BChE inhibitor may produce a significant increase of ACh levels in the brain without triggering severe peripheral or central cholinergic adverse effects,[12] suggesting that BChE is a promising target in AD. In view of the existing treatment with AChE is less efficient, the drug discovery targeting BChE is of paramount.

Natural products play an important role in the management of various human diseases. Most of the drugs used in the treatment of AD including rivastigmine, galanthamine and huperzine A, were derived from natural products or their derivatives. *Garcinia atroviridis* is a tropical plant found in Peninsular Malaysia, Indonesia and Thailand. It possessed interesting pharmacology activities, including antimicrobial, antioxidant, anticancer and cholinesterase inhibition. Previous study from our group has demonstrated that the flavonoids from *G. atroviridis* possessed promising cholinesterase inhibitory activity.[13] In this study, 6 compounds from the stem bark of *G. atroviridis*, namely, 2,6-dimethoxy-p-benzoquinone (**1**), kaempferol (**2**), quercetin (**3**), 1,3,5-trihydroxy-2-methoxyxanthone (**4**), 1,3,7-trihydroxyxanthone (**5**) and garcinexanthone G (**6**) were evaluated for their cholinesterase enzymes inhibitory activity. The ligand-enzyme molecular interactions on the selected compounds using molecular docking were also reported for the first time.

## Experimental

**Chemicals and enzymes** – Acetylcholinesterase (AChE) from electric eel, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide,butyrylcholineterase (BChE) from equine serum, *S*-butyrylthiocholine chloride and physostigmine were purchased from Sigma (St Louis, MO, USA). Sodium dihydrogen phosphate anhydrous was purchased from R&M Chemicals (Essex, UK) while disodium hydrogen phosphate anhydrous was purchased from Merck (Darmstadt, Germany). All the other solvents and reagents used were of analytical grade.

**Plant materials** – The stem bark of *G. atroviridis* was collected in August 2009 from Kedah, Malaysia and was identified by Mr. Baharuddin Sulaiman from School of Biological Sciences, Universiti Sains Malaysia. A voucher specimen (USM 11201) has been deposited with the herbarium of Universiti Sains Malaysia, Penang, Malaysia.

**Extraction and isolation** – Air-dried stem bark (6 kg) of *G. atroviridis* was ground and sequentially extracted in a Soxhlet apparatus with *n*-hexane, dichloromethane, and methanol. The methanol extract (100.00 g) upon concentration was extracted with chloroform. The dichloromethane extract (17.00 g) was column chromatographed over silica gel to afford 58 fractions (D1-D58). The fraction D10 (0.30 g) was rechromatographed over a silica gel column and further purification to yield **1**. The fraction D22 (0.30 g) was subjected to column chromatography and further fractionation to yield **2**. The fraction D24 (0.40 g) was separated using silica gel column and subsequent purification to yield **3**. The fraction D17 (0.35 g) was rechromatographed using a 35 g silica gel column and further purification to yield **4**. The fraction D19 (0.30 g) was subjected to column chromatography and then purification to give **5**. The chloroform extract (17.00 g) obtained was subjected to silica gel column chromatography to afford 65 fractions (C1-C65). The fraction C15 (0.25 g) was rechromatographed and subsequent fractionation to yield **6**. The detailed isolation is described in Tan *et al*. (2016).[14]

***In vitro* cholinesterase inhibitory activity** – Compounds **1-6** were dissolved in methanol at an initial concentration of 1 mg/ml. The samples were filtered through a 0.22 μm sterile filter (JETBIOFIL) and stored at 4 ºC prior for experiments. Cholinesterase inhibitory activity of the isolated compounds was evaluated using Ellman's microplate assay.[15] For cholinesterase inhibitory assay, 140 μl of 0.1 M sodium phosphate buffer (pH 8) was first added into each well of a 96-well microplate followed by 20 μl of the test sample (in 10% methanol) and 20 μl of 0.09 unit/ml AChE/BChE. Subsequently, 10 μl of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) was added into each well followed by 10 μl of 14 mM acetylthiocholine iodide (ATCI) or *S*-butyrylthiocholine chloride. The absorbance of the coloured end-product was measured at 412 nm at designated intervals for 30 minutes after the initiation of enzymatic reaction using Tecan Infinite 200 ProMicroplate Spectrometer (Switzerland). Physostigmine and galanthamine were used as the reference standards. Each test sample was conducted in triplicate. Absorbance of the test sample was corrected by subtracting the absorbance of its respective blank. A set of five concentrations was used to estimate the 50% inhibitory concentration ($IC_{50}$) for the active compounds.

**Molecular docking** – Molecular docking of compounds **4 - 6** and galanthamine was performed using Autodock 3.0.5 along with AutoDockTools (ADT).[16] Initially, compounds **4 - 6** were drawn using Chemsketch; and Hyperchem 8 was used to build the compound and energy minimization was performed with a convergence criterion of 0.05 kcal/(molA). Crystal structures of BChE from Homo sapiens were obtained from Protein Data Bank with PDB ID: 2WIJ.[17] The protein was edited using ADT to remove all water molecules and the hydrogen atoms were added. Non-polar hydrogens and lone pairs were then merged and each atom was assigned with Gasteiger partial charges. A grid box of 50 × 50 × 50 points, with a spacing of 0.375Å was positioned at the centre of active site gorge. One hundred independent dockings were carried out and the most populated cluster was selected.

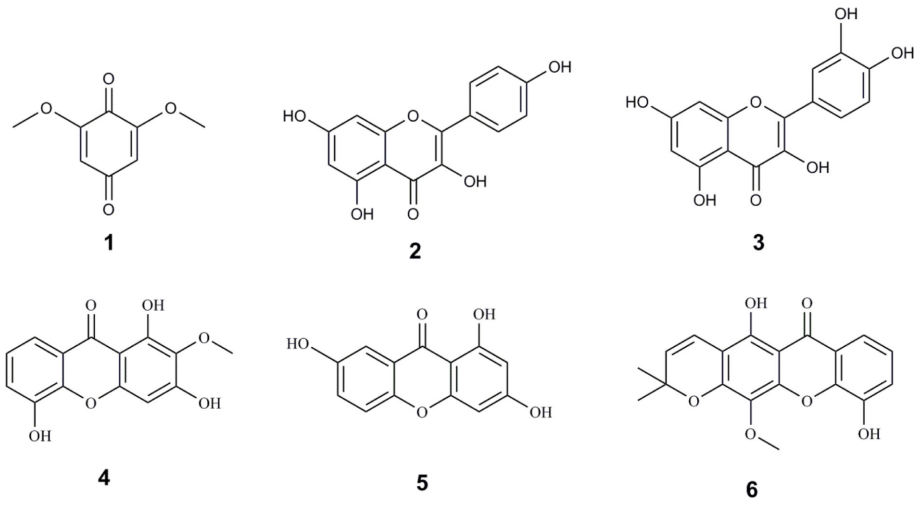

**Fig. 1.** The structures of compounds **1 - 6** from the stem bark of *G. atroviridis*.

**Table 1.** Cholinesterase inhibitory activities of compounds **1 - 6** from the stem bark of *G. atroviridis*

| Compounds | AChE | | BChE | | Selectivity Index | |
|---|---|---|---|---|---|---|
| | Inhibition at 100 μg/ml | $IC_{50}$ (μg/ml) | Inhibition at 100 μg/ml | $IC_{50}$ (μg/ml) | AChE | BChE |
| 1 | 61.35 ± 1.99 | 69.04 ± 6.15 | 55.00 ± 4.08 | 96.00 ± 0.25 | 1.39 | 0.72 |
| 2 | 68.93 ± 1.16 | 42.85 ± 3.60 | 40.02 ± 3.75 | 84.15 ± 5.23 | 1.96 | 0.51 |
| 3 | 54.75 ± 3.07 | 12.65 ± 1.57 | 25.25 ± 2.87 | ND | - | - |
| 4 | 5.10 ± 1.47 | ND | 16.04 ± 2.66 | ND | - | - |
| 5 | 9.49 ± 1.71 | ND | 64.06 ± 8.13 | 205.95 ± 10.15 | - | - |
| 6 | 42.28 ± 0.45 | 223.00 ± 18.27 | 93.30 ± 1.30 | 18.86 ± 2.41 | 0.08 | 11.82 |
| Physostigmine | - | 0.05 ± 0.007 | - | 0.14 ± 0.02 | 2.80 | 0.36 |
| Galanthamine | - | 0.27 ± 0.07 | - | 5.55 ± 0.02 | 20.56 | 0.05 |

Note: Data are presented as mean ± SD ($n$ = 3)
Selectivity for AChE is defined as $IC_{50}$ (BChE) / $IC_{50}$ (AChE)
Selectivity for BChE is defined as $IC_{50}$ (AChE) / $IC_{50}$(BChE)
ND = Not determined

Analysis and visualization of the docking results were conducted using VMD[18] and Acceryls Discovery Studio 2.5 (Accelrys Inc., San Diego, CA, USA).

## Result and Discussion

The isolated compounds (Fig. 1) from the stem bark of *G. atroviridis* were subjected to enzyme inhibitory test against AChE and BChE, using the Ellman's method at concentration of 100 μg/ml.[15] The compounds isolated belong to the chemical classes of flavonoid, xanthone and benzoquinone. The structures of compounds **1 - 5** were confirmed by comparison of their NMR, MS and UV data with those reported previously while compound **6** was identified as garcinexanthone G, a new compound isolated from the from the stem bark of *G. atroviridis*.[14] The cholinesterase inhibitory activities of the compounds are summarized in Table 1. Compounds showing good inhibitory activity were further investigated for their $IC_{50}$. Compounds **1 - 6** possessed a wide range of inhibitory activities with $IC_{50}$ values in the range of 12.65-223.00 μg/ml against AChE and 18.86-205.95 μg/ml for BChE, respectively. Compound **3** is the most potent AChE inhibitor, while compound **6** is the most potent BChE inhibitor in the present study. Both compounds are 46.6 and 3.4 times less potent than the standard drug, galanthamine. In addition, compound **3** is an AChE enzymes selective inhibitor while compound **6** is a BChE selective inhibitor. It is interesting to note that the presence of hydroxyl group at C-10 in compound **3** enhanced the

BChE inhibitory activity. Compounds **4** and **5** with the xanthone moieties showed weak to moderate BChE enzyme inhibition activities. Thus, suggesting that the cholinesterase activity might due to the interaction of hydroxyl group at C-10 and the well-fitting of the compound at the active sites of the enzymes.

Few studies have reported the cholinesterase inhibitory activities of flavonoids and xanthones *in vitro* and *in vivo*. Tan *et al*. (2014) reported garcineflavanone A is a selective cholinesterase inhibitor, while garcineflavonol A is a dual cholinesterase inhibitors, both isolated from the stem bark of *G. atroviridis*.[13] Khaw and colleagues reported cholinesterase inhibitory activities of nine prenylated xanthones analogues from the hull of mangosteen, in which garcinone C and γ-mangostin are the most potent BChE inhibitors with $IC_{50}$ values of 1.24 and 1.78 μM, respectively.[19] Louh and colleagues reported polyanxanthones and hydroxyxanthones from *Garcinia polyantha* potently inhibit the cholinesterase enzymes. From the study, polyanxanthone B was the most active AChE inhibitor ($IC_{50}$ 46.3 μM) while 1,5-dihydroxyxanthone was the most active BChE inhibitor ($IC_{50}$ 2.54 μM).[20] A few studies have reported cholinesterase inhibitory activities of compound **3** *in vitro* and *in vivo*. Khan and colleagues reported compound **3** inhibits AChE and BChE at 353.86 and 420.76 μM, respectively.[21] It is worthy to note that compound **3** is able to enhance the spatial memory of 6-hydroxydopamine-induced Parkinson disease animal model.[22]

The molecular docking study was performed for the most potent BChE inhibitor, compound **6**. In addition, compounds **4** and **5** were also chosen for the docking study even though they showed poor BChE inhibition despite having the same xanthone skeleton as compound **6**. This strategy was undertaken in order to reveal their binding orientations and to correlate with their *in vitro* cholinesterase inhibition results. Compounds **4 - 6** and the standard, galanthamine were docked into the active site of BChE (Fig. 2) in this study.

Table 2 shows the interaction sites, residues involved, bonding types and ligand-interacting moieties between BChE and compounds **4 - 6** together with standard drug, galanthamine. Compound **6** possessed the lowest free energy of binding (FEB; −8.93 kcal/mol), while the free energy of binding for compounds **4** (FEB; −8.37 kcal/mol) and **5** (FEB; −8.49 kcal/mol) are comparable. The FEB of galanthamine was greater than the three xanthones stood at −10.11 kcal/mol. The FEB values from the molecular docking of compounds **4 - 6** and galanthamine were in good agreement with the *in vitro* BChE inhibitory $IC_{50}$ values. On the other hand, by comparing the inhibition constant ($K_i$), compound **6** showed the lowest $K_i$ (indicating the most potent inhibitor) followed by galanthamine, compounds **5** and **4**. It is interesting to note that compound **6** possessed a different binding orientation as compared with compounds **4** and **5**. Compound **6** docked into the bottom of a narrow gorge of catalytic site by forming hydrogen bonding with two important residues of the catalytic domain represented by Ser 198 and His 438. Moreover, the hydroxyl group at C-10 formed a hydrogen bonding with Pro 285. On the other hand, compounds **4** and **5** with the same orientations, formed hydrophobic interactions and hydrogen bonding with amino acids at the choline binding site.

The molecular docking simulation for the xanthones completely corresponds with their *in vitro* cholinesterase

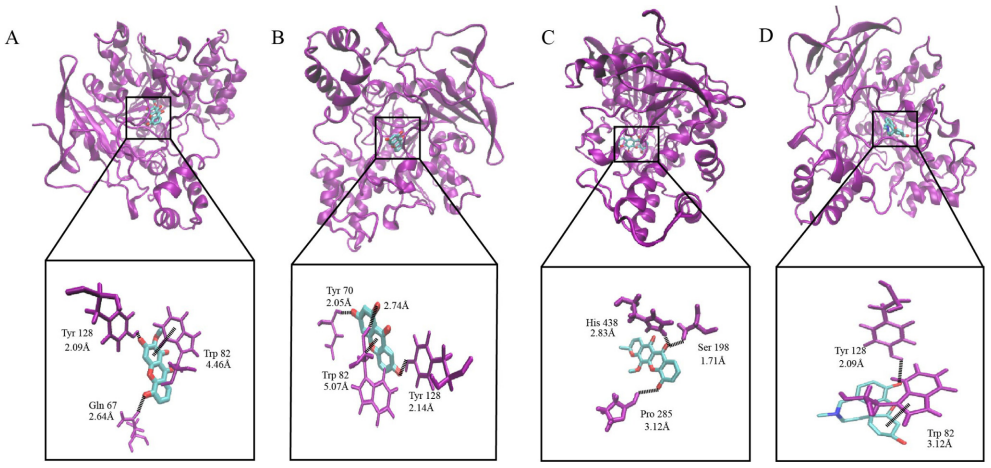


**Fig. 2.** Binding interactions of compounds **4 - 6** and galanthamine with the amino acids of butyrylcholinesterase. A-D represents compounds **4 - 6** and galanthamine, respectively.

**Table 2.** Binding interactions of compounds **4** - **6** with BChE

| Ligands | Binding energy (kcal) | Inhibition constant ($K_i$) | Residue | Type of interaction | Interacting site | Distance (Å) |
|---|---|---|---|---|---|---|
| 4 | -8.37 | 7.33 | Trp 82 | Hydrophobic | CBS | 4.46 |
| | | | Gln 67 | H-bond | | 2.64 |
| | | | Tyr 128 | H-bond | | 2.09 |
| 5 | -8.49 | 6.0 | Trp 82 | Hydrophobic | CBS | 5.07 |
| | | | | H-bond | | 2.74 |
| | | | Tyr 128 | H-bond | | 2.14 |
| | | | Tyr 70 | H-bond | | 2.05 |
| 6 | -8.93 | 2.85 | Ser 198 | H-bond | CT | 2.83 |
| | | | His 438 | H-bond | | 1.70 |
| | | | Pro 285 | H-bond | | 3.12 |
| Galanthamine | -10.11 | 3.85 | Trp 82 | Hydrophobic | CBS | 3.39 |
| | | | Tyr 128 | H-bond | | 2.97 |

Note: CBS = Choline binding site
CT = Catalytic site

inhibition results. Overall, compound **6** is an interesting selective BChE inhibitor. It could serves as a potential lead compound in drug design and the development for potent BChE inhibitor.

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