**CHOLINESTERASE INHIBITORS FROM HEARTWOOD OF ARTOCARPUS FULVICORTEX F. M. JARRET (MORACEAE)**

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**Abstract**

Cholinesterase inhibition activities were screened on crude extracts and isolated compounds of the heartwood of Artocarpus fulvicortex. Phytochemical studies on the heartwood of *A. fulvicortex* have resulted in the isolation of one flavonoid, one stilbenoid and two triterpenes. Their structures were determined by spectroscopic analysis as catechin (1), oxyresveratrol (2), lupeol-3-acetate (3) and friedelin (4). All isolates were evaluated for the first time for anti-cholinesterase activity. Inhibition of acetylcholinesterase (AChE), tested by Ellman’s method and modified TLC bioautographic assay showed catechin (1) and oxyresveratrol (2) were active agents with detection limit of 2 mM. The microplate assay was carried out using butyrylcholinesterase (BChE) to further confirmed that catechin (1) and oxyresveratrol (2) displayed positive cholinesterase activity with IC\textsubscript{50} values 50.0 mM and 6.25 mM respectively in dose dependent manner.

**Keywords**: Acetylcholinesterase (AChE), butyrylcholinesterase (BChE) *A. fulvicortex*, Ellman’s method, modified TLC bioautographic assay, microplate assay
1.0 INTRODUCTION

Alzheimer (AD) is the term referred to brain damaged disease which may result on the declining of the memory performance. AD often attacks aging people as the risk greater by the increasing age. However, AD is not a normal part of the aging, it might attacks the other age groups. US Food and Administration (FDA) has approved two types of medications to treat AD, cholinesterase inhibitor and memantine or drugs that used to treat the cognitive symptoms.

Acetylcholinesterase (AChE) enzyme has been proven to be the possible therapeutic target that caused cholinergic insufficiency which developed AD [1]. AChE is the primary enzyme that responsible to break down the neurotransmitter called, acetylcholine that used to interconnect the information throughout the body and brain whereas, butyrylcholinesterase (BChe) is the secondary enzyme. According to the previous reports on the cholinesterase inhibition, more compounds give better inhibition on BChe rather than AChE [2].

Inhibitor of AChE was considered to be a treatment strategy of AD. Natural-occurring AChE inhibitor, hyperzine A and galanthamine were previously isolated from the plant. This proves that the herbs are among the best source for AChE inhibitor and later be developed as potential drug for the treatment of AD [3].

Artocarpus species are very synonym with their rich composition of the bioactive compounds especially flavonoids [4]. This research continues the interest to explore more about the potential of Artocarpus species as example, A. fulvicortex F. M. Jarret; one of the Malaysia’s rare species usually found in East Coast of Peninsular Malaysia. Phytochemical study on the leaves of A. fulvicortex had led to isolation of remarkable compounds [5]. However phytochemical and bioactivity studies on the heartwood of this species has not been reported yet.

Therefore, this paper reports on the TLC bioautographic and microplate assay of AChE and BChe respectively of the heartwood. To the best of our knowledge, this is the first report on the phytochemicals and anti-cholinesterase activity from the heartwood of A. fulvicortex. In order to screen for cholinesterase inhibitory activity, an assay has been developed in which TLC is used in combination with bioactivity staining based on Ellman’s method [6].

In Ellman’s method, acetylcholine as substrate was hydrolysed into thiocholine and acetate catalysed by AChE. 5,5'-Dithiobis[2-nitrobenzoic acid](DTNB) was used as chromogen that produced yellow colour when it reacted with thiocholine. The inhibition of the enzymatic activity can be detected by reduction of yellow colour intensity that can be measured at 412 nm wavelength by microplate reader.

Scheme 1 explained the chemical reaction occurs during the procedure. Several simple methods had been developed to determine false-positive effects in the TLC assay including addition of thiocholine spray or fast blue B salt [3,7]. However, in this research the microplate assay was performed to eliminate the false-positive results in TLC bioautographic assay.

![Scheme 1 Ellman’s Method](image)

2.0 EXPERIMENTAL

2.1 Plant Material

The heartwoods of Artocarpus fulvicortex F. M. Jarret were collected from Sekayu Forest, Terengganu in August 2011 and identified by botanist from Universiti Putra Malaysia (UPM), Dr. Shamsul Khamis. The plant’s voucher specimen (SK1963/11) was deposited at Herbarium of UPM, Serdang, Selangor.

2.2 Extraction and Isolation

The air-dried heartwood of A. fulvicortex (2.0 kg) were macerated thrice (12.5 L for each extraction) with different polarity of solvent successively at room temperature for 3 days (72 hours). The filtrates were evaporated under reduce pressure to produce dichloromethane (CH₂Cl₂) and methanol (MeOH) crude extracts of heartwood. The MeOH extract (13.16 g) of heartwood were fractionated by increasing polarity of n-hexane and ethyl acetate (EtOAc) using Vacuum Liquid Chromatography (VLC) technique to
give six (AHM1-AHMH6) major fractions. Purification of AHM4 and AHM5 using Column Chromatography (CC) gave 1 [61.2 mg, 0.02%] and 2 [38.4 mg, 0.01%] correspondingly as brown powder. Compound 3 [43.7 mg, 0.5%] and 4 [33.9 mg, 0.41%] were isolated from repeated CC of CH2Cl2 extract. Compound 3 [43.7 mg, 0.5%] and 4 [33.9 mg, 0.41%] were observed as a white needle with m.p 214-216°C and 255-258°C, respectively.

Catechin (1)

\[ R_{s} = 0.4 \text{ [n-hexane: EIOAc = 1: 4;]}^{1} \text{H NMR (Acetone-d$_{6}$, 400 MHz):} \delta 2.51 (1H, dd, J = 16.4, 8.0 Hz, H-4b), 2.90 (1H, dd, J = 16.0, 5.2 Hz, H-4a), 3.97 (2H, m, OH, H-3, 4.56 (1H, d, J = 7.6 Hz, H-2), 5.89 (1H, s, H-8), 6.03 (1H, s, H-6), 6.76 (1H, d, J = 8.0 Hz, H-5), 6.79 (1H, dd, J = 8, 1.6 Hz, H-6'), 6.91 (1H, d, J = 1.6 Hz, H-2'), 7.93 (1H, s, OH), 7.98 (1H, s, OH), 8.09 (1H, s, OH), 8.28 (1H, s, OH). \] 

13C NMR (Acetone-d$_{6}$, 100 MHz): \[ \delta 27.1 (C-26), 58.3 (C-24), 50.4 (C-10), 38.4 (C-30), 31.8 (C-28), 32.1 (C-21), 32.4 (C-15), 32.8 (C-29), 35.0 (C-19), 35.4 (C-16), 35.6 (C-11), 36.0 (C-9), 37.5 (C-13), 38.3 (C-22), 39.3 (C-14), 39.7 (C-6), 41.3 (C-2), 41.5 (C-5), 42.1 (C-18), 53.1 (C-8), 58.3 (C-4), 59.5 (C-10), 213.1 (C=O). \]

2.3 Anti-cholinesterase Assays

2.3.1 Chemicals

Tris-hydrochloride (pH 8.0), acetylthiocholine iodide (ATCI), butyrylthiocholine chloride (BuCl), acetylcholinesterase from electric eel (type VI-s; lyophilised powder; 292 U/mg solid; 394 U/mg protein), butyrylcholinesterase, from horse 5,5′-dithiobis[2-nitrobenzoic acid] [DTNB] were purchased from Sigma-Aldrich and galanthamine was purchased from Calbiochem. TLC assay were conducted on silica gel F$_{254}$ on aluminum plates purchased from Merck. The microplate assay was carried out in 100 mM sodium phosphate buffer (pH 8.0) at 25°C. The buffer used was 50 mM Tris-hydrochloride (pH 8).

Acetylcholinesterase enzyme was dissolved in buffer to produce a stock solution containing 1000 units/mL, then further diluted with buffer to give 0.22 units/mL. Acetylthiocholine iodide (ATCI) was dissolved in buffer to prepare concentration of 1 mM. DTNB was used at a concentration of 1 mM in buffer.

2.3.2 TLC Bioautographic AChE Assays

The assay for AChE inhibitory activity was done according to Elman’s method and was referred to previous works of Rhee et al. [6]. First, all the crude extracts and compounds were dissolved in preferred solvent. The highest concentration tested for crude extracts and pure compounds were 10 mg/ml and 100 mM respectively. The series of solution of crude extracts 10, 8, 6, 4, and 2 mg/ml were prepared using serial-dilution method. Using the same method, the series of 100, 80, 40, 20, 10, 5 mM were prepared for compounds 1-4.

Each aliquot of all samples (5 μL) was spotted on a silica gel TLC plate. TLC plate was developed using selected solvent systems and was allowed to dry absolutely. Enzyme inhibitory activities of the samples on the TLC plate were started by spraying the substrate, and enzyme according to Elman's method. The plate was sprayed with DTNB/ATCI reagent (1 mM DTNB and 1 mM ATCI in buffer) until the layer was just saturated with the solvent, and the plate was then allowed to dry for 3–5 min after the enzyme solutions were sprayed on the TLC. The white spots visible after 5 min show the positive inhibition of AChE. These white spots were recorded within 15 min as they will disappear for AChE.
2.3.3 Microplate Assays

All the crude extracts and compounds were dissolved in MeOH, the same way as in the TLC bioautographic assays. The highest concentration, 100 mM of the sample (20 µL) was transferred into a flat-bottomed microplate and was diluted with MeOH by serial-dilution method. MeOH and galanthamine were used as the negative and positive control respectively. The samples were then mixed with 10 µL DTNB, 15 µL BChe and 140 µL sodium phosphate buffer and incubated for 15 min. After 15 minutes of incubation, 10 µL of BuCl was added to each sample. Then, the enzymatic inhibition was measured by microplate reader at 412 nm wavelength.

3.0 RESULTS AND DISCUSSION

The phytochemical investigation of 
A. fulvicortex heartwood by applying column chromatographic method yielded four known compounds, catechin (1), oxyresveratrol (2), lupeol-3-acetate (3) and friedelin (4). All the compounds were isolated for the first time from this species. The structures of compounds 1- 4 were confirmed by comparison of the 1H and 13C NMR spectroscopic data with those reported in the literature [8-12].

TLC bioautographic assay was performed to determine qualitatively the inhibitory effects towards the targeted enzyme, AChE. The cholinesterase inhibitory activities of the extracts and the compounds isolated from 
A. fulvicortex were firstly evaluated by TLC bioautographic assay and the results are shown in Table 1.

Table 1 Detection limit of crude extracts and isolated compounds measured by TLC assay

<table>
<thead>
<tr>
<th>Pure compounds</th>
<th>Detection limit (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechin (1)</td>
<td>5</td>
</tr>
<tr>
<td>Oxyresveratrol (2)</td>
<td>5</td>
</tr>
<tr>
<td>Lupeol-3-acetate (3)</td>
<td>ND</td>
</tr>
<tr>
<td>Friedelin (4)</td>
<td>ND</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Crude extracts</th>
<th>Detection limit (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFHD</td>
<td>2</td>
</tr>
<tr>
<td>AFHM</td>
<td>2</td>
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AFHD = A. fulvicortex heartwood dichloromethane, AFHM = A. fulvicortex heartwood methanol, ND = not detected

It was found that the CH2Cl2 and MeOH extracts of 
A. fulvicortex possessed AChE inhibitory activities. Catechin (1) and oxyresveratrol (2) those were isolated from MeOH extract gave positive indication toward constraining the enzyme action.

However, lupeol-3-acetate (3) and friedelin (4) isolated from the CH2Cl2 extract showed no activity in TLC assay. This result reveals that there are other compounds which present in the CH2Cl2 extract that could be the potent cholinesterase inhibitor. The negative results shown by lupeol-3-acetate (3) and friedelin (4) revealed that the non-nitrogenous triterpenes only inhibit the enzymatic activity. Previous report by Rahman et al. proved that nitrogen plays an essential role to inhibit cholinesterase activity [13]. All the isolates were tested using microplate assay.

![Figure 1](image-url)

Figure 1 Comparison Cholineasterease Inhibition of Catechin (1) and Oxyresveratrol (2) with Galanthamine

Microplate assay supported the results of TLC bioautographic assays by giving the same outcome. Catechin (1) and oxyresveratrol (2) revealed significant value of inhibition of BChe enzymatic activity in a dose dependent manner with the IC50 values 50.0 mM and 6.25 mM respectively. Figure 1 shows the trend of cholinesterase inhibition activity of catechin (1) and oxyresveratrol (2). Oxyresveratrol (2) showed almost similar inclination with the existing cholinesterase inhibitor, galanthamine or commercially known as Razadyne. Catechin (1) also gave the significant value toward inhibit the cholinesterase enzyme. The positive inhibition might due to the presence of phenolic hydroxyl groups that attached to both compounds which play an important role to form bonding with the enzyme [2].

4.0 CONCLUSION

Catechin (1) and oxyresveratrol (2) isolated from heartwood of 
A. fulvicortex showed cholinesterase inhibitory activity with detection limit up to 5 mM with the IC50 values 50.0 mM and 6.25 mM respectively. In light of these findings, it can be concluded that oxyresveratrol (2) could be the potential cholinesterase inhibitor in dose dependent manner and it could be considered for further studies in the treatment of AD. The results indicated that the screening may provide useful lead compounds in the discovery of new drugs for the treatment of AD.
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