β-Amyrin from *Ardisia elliptica* Thunb. is more potent than aspirin in inhibiting collagen-induced platelet aggregation

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*Ardisia elliptica* Thunberg (Myrsinaceae) is a medicinal plant traditionally used for alleviating chest pains, treatment of fever, diarrhoea, liver poisoning and parturition complications. The objectives of the study were to investigate the effect of *A. elliptica* on collagen induced platelet aggregation and to isolate and purify potential antiplatelet components. Fresh *A. elliptica* leaves were extracted using methanol (70\% v/v) by Soxhlet extraction and the extract was analysed for its inhibition of collagen-induced platelet aggregation. Inhibition of platelet aggregation was assessed by incubating the extracts with rabbit blood and collagen in a whole blood aggregometer and measuring the impedance. The leaf extract was found to inhibit platelet aggregation with an IC\textsubscript{50} value of 167 \(\mu\)g/ml. Using bioassay guided fractionation, \(\beta\)-amyrin was isolated and purified. The IC\textsubscript{50} value of \(\beta\)-amyrin was found to be 4.5 \(\mu\)g/ml (10.5 \(\mu\)M) while that of aspirin was found to be 11 \(\mu\)g/ml (62.7 \(\mu\)M), indicating that \(\beta\)-amyrin was six times as active as aspirin in inhibiting platelet aggregation. This paper is the first report that \(\beta\)-amyrin isolated from *A. elliptica* is more potent than aspirin in inhibiting collagen-induced platelet aggregation. In conclusion, *A. elliptica* leaves were found to inhibit collagen-induced platelet aggregation and one of the bioactive components responsible for the observed effect was determined to be \(\beta\)-amyrin.

Keywords: \(\beta\)-Amyrin; *Ardisia elliptica*; Collagen; Platelet aggregation

Aspirin, clopidogrel, abciximab and ticlopidine are highly efficacious in the treatments of thrombotic diseases, but they are associated with numerous adverse effects such as increased risk of bleeding, aggravating mucosal lesions\textsuperscript{2} and thrombotic thrombocytopenic purpura\textsuperscript{2}. Aspirin and clopidogrel resistance had also been reported in patients\textsuperscript{3,4}. To increase the efficacy of currently used drugs, combinative therapies involving the use of multiple antiplatelet agents have been studied\textsuperscript{5}. In addition, the discovery of novel antiplatelet drugs is actively being pursued.

Medicinal plants with antiplatelet and anticoagulant activities had been reviewed previously\textsuperscript{6}. Aspirin had its origins from salicin obtained from the willow plant. Willow leaves were used to treat pain since the times of ancient Assyrians and Egyptians\textsuperscript{7}. Other medicinal plants studied for their antiplatelet activities include garlic\textsuperscript{8}, ginkgo\textsuperscript{9}, green tea\textsuperscript{6} and the Korean red ginseng\textsuperscript{10}.

*Ardisia elliptica* Thunberg (family Myrsinaceae), also known as *Mata Ayam*, is a native medicinal plant commonly found in the Malay Peninsula\textsuperscript{12,13}. It is a small branched shrub up to 4 m tall with ob lanceolate or obovate leaves and small black drupes\textsuperscript{12,14}. A decoction of the leaves of the plant is used by the Malays for treatment of pain in the region of the heart (that is, to alleviate chest pains)\textsuperscript{14-18}. The roots may be used as a substitute for the leaves\textsuperscript{16,18}. The plant is also used to address complications attributed to parturition (child birth), and to treat fever, diarrhoea and liver poisoning\textsuperscript{14,16}. *A. elliptica* has been reported to inhibit platelet aggregating factor receptor binding\textsuperscript{17,19,20}. The bioactivity was attributed to 5-(Z-heptadec-4´-enyl)resorcinol\textsuperscript{17}. However, this component was not detected in our methanol extract. Hence, the objectives of the study were to investigate the effects of *A. elliptica* on collagen-induced platelet aggregation and to identify its potential antiplatelet components.
Materials and Methods

Plant material—Fresh leaves of A. elliptica were collected from the Medicinal Plant Garden, National University of Singapore between August 2006 and June 2007. Authentication was carried out by a botanist from the Singapore Botanic Gardens. A voucher specimen of the plant (KHL0060/CJH/AE/29062006) is stored at the Department of Pharmacy, National University of Singapore.

Reagents and standards—Methanol was purchased from Tedia (Fairfield, OH). Dimethyl sulfoxide (DMSO) was obtained from MP Biomedicals (Illkirch, France) while phosphate buffer saline (PBS, 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l NaHPO₄, 0.24 g/l KH₂PO₄) was bought from 1st Base (Singapore). Collagen (1 mg/ml) was purchased from Chronolog (Havertown, PA). α-amyrin and β-amyrin were obtained from Extrasynthase (Genay, France). Aspirin was obtained from Sigma Aldrich (USA).

Extraction and preparation of plant extracts—Fresh leaves of A. elliptica (327 g) were collected, washed and air dried, blended and extracted with 70% v/v methanol by Soxhlet extraction for 6 h. The 70% v/v methanol extract was evaporated to dryness and dissolved in DMSO to make up to concentrations of between 4 mg/ml and 40 mg/ml to determine its IC₅₀ value for the inhibition of platelet aggregation. α- and β-amyrin standards of concentrations between 0.1 mg/ml and 2 mg/ml were also dissolved in DMSO for the assay.

Isolation of β-amyrin using High Performance Liquid Chromatography—50 ml of HPLC grade methanol was added to 13.3 g of the dried 70% v/v methanol extract. After centrifuging and filtering to remove insoluble particles, the supernatant was fractionated using a preparative HPLC system (Agilent 1100 series, CA, USA) and a preparative Zorbax Eclipse XDB-C18 column (250 mm × 21.2 mm I.D., particle size: 7 µm; Agilent, CA, USA) using methanol as the mobile phase. The injection volume was 2 ml, flow rate 20 ml/min and run time was 40 min. The desired fraction was collected and further purified using a semi-preparative Zorbax SB-C18 column (250 mm × 9.4 mm I.D., particle size: 5 µm; Agilent, CA, USA) with methanol as the mobile phase. The injection volume was 2 ml, flow rate 8 ml/min and run time was 35 min. The UV detection for both preparative and semi-preparative HPLC was set at 210 nm. The identity of the β-amyrin was confirmed using gas chromatography as described in the next section.

Identification of β-amyrin using Gas chromatography-Mass spectrometry—Dried methanol extracts (70% v/v) were weighed, reconstituted in methanol, filtered with 0.45 µm nylon membrane syringe filter and analysed with gas chromatography-mass spectrometry (GC-MS) (Shimadzu, gc2010 and qp2010 MS, Japan). A DB-5MS column of film thickness 0.25 µm, length 30.0 m and diameter 0.25 mm was used. The oven temperature of the GC was held at 60°C for 5 min, and increased to 200°C in 7 min. The temperature was held for 10 min and increased to 280°C within 5 min. This temperature was held for another 30 min. Phytoconstituents were preliminarily identified by the Wiley Mass Spectral Library Registry 7 (NJ, USA) and confirmed by comparing the retention times and mass spectra of peak of interest and that of β-amyrin standard.

Platelet aggregation assay—Whole blood aggregation assay was carried out by the measurement of impedance using a whole blood aggregometer (Chronolog Corporation, Havertown, PA). Male New Zealand Whites, 2.5 ± 0.5 kg, were anaesthetised with ketamine/xylazine mixture at 0.2 ml/kg body weight of each and blood from the central ear artery of the rabbits was collected into a citrated tube (Tapval, 4 ml blood collection tubes, 0.106 M citrate, Deltalab S.A., Barcelona, Spain). The animals were kept in accordance with the internationally accepted principles for laboratory animal use and care as found in the US guidelines (NIH publication #85-23, revised in 1985). The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the National University of Singapore. 450 µl of the blood prewarmed to 37°C was then diluted with pre-warmed PBS (1:1) and allowed to equilibrate for two minutes. 4.6 µl of the sample (extract, β-amyrin and α-amyrin) in DMSO or control (DMSO) was added and the solution was allowed to equilibrate for two minutes. 2 µl of collagen was then added to initiate platelet aggregation. The final concentration of DMSO in the test solutions was kept constant at 0.5% v/v. The test was allowed to run for 5 min. All tests were carried out at least three times. Aspirin and the vehicle (DMSO) were used as the positive and negative control respectively. Inhibition of platelet aggregation (Z) is calculated as follows:

\[ \% \text{ inhibition of platelet aggregation, } Z = \frac{X - Y}{X} \times 100 \]

where X is the impedance value for the
control and Y is the impedance value of the sample. IC<sub>50</sub> values were calculated using Microsoft Excel.

**Statistical analysis—** All results are expressed as mean ± standard deviation. Statistical analyses of the groups were performed using one-way ANOVA with Tukey’s test. Significant differences were concluded for P < 0.05.

**Results and Discussion**

Dried methanol extract (23.5 g, 7.2% w/w crude yield) was obtained from soxhlet extraction of 327 g of *A. elliptica* leaves using 70% v/v methanol.

Analysis of the methanol (70% v/v) extract by GC-MS and using the autolibrary (Wiley Mass Spectral Library) search function showed the presence of two triterpenes, α- and β- amyrin. α- and β- amyrin are isomers, differing only in the position of a methyl substituent. The identities of the amyrins were determined by comparing the gas chromatogram and mass spectrum of the extract with that of the authentic standards. From the gas chromatogram of the extract (not shown), it was observed that both amyrins were present in relatively high amounts. The percentage areas of α- and β- amyrin in the gas chromatograms were around 13% and 27% respectively.

The results of the platelet aggregation assays are shown in Table 1. The IC<sub>50</sub> value of the 70% v/v methanol extract for inhibition of collagen-induced platelet aggregation was found to be 167 µg/ml. Using bioassay guided fractionation, a bioactive component was subsequently isolated and purified using preparative and semi-preparative HPLC and its identity was determined by GC-MS to be β-amyrin. About 24 mg of β- amyrin was isolated and purified from 13 g of dried 70% v/v methanol extract of *A. elliptica*. The IC<sub>50</sub> values of the isolated β-amyрин and a commercially available standard of β-amyрин for the inhibition of collagen-induced platelet aggregation were both found to be about 5 µg/ml. The β-amyрин standard was found to have an IC<sub>50</sub> value of 4.5 µg/ml (10.5 µM). This indicated that β-amyrin was six times more active than aspirin (IC<sub>50</sub> 62.7 µM); which was used as the positive control (P < 0.05); (Table 1). This paper is to first report that β-amyрин isolated from *A. elliptica* is more potent than aspirin in inhibiting collagen-induced platelet aggregation. Attempts to isolate α-amyрин were not successful as it co-eluted with other components. IC<sub>50</sub> value of the α-amyрин standard on platelet aggregation inhibition was nevertheless determined to be 9.1 µg/ml (21.3 µM). This indicated that α-amyрин was about three times more active than aspirin.

A mixture of α- and β-amyрин isolated from another plant, *Protium heptaphyllum*, in a ratio of 2:1 respectively was shown to inhibit platelet aggregation induced by collagen, adenosine diphosphate and arachidonic acid with IC<sub>50</sub> values of 38.4 µg/ml, 50.3 µg/ml and 77.4 µg/ml respectively<sup>21</sup>. The IC<sub>50</sub> value of α-amyрин for the inhibition of collagen-induced platelet aggregation at 9.1 µg/ml as determined in this study is 4 fold less. This discrepancy may be due to the different samples and experimental conditions (concentration of collagen, incubation time, vehicle etc) used in the two studies. For example, pure amyrins and whole rabbit blood were used in the current study while a mixture of α- and β-amyринs and platelet rich plasma from human blood were used in the previous study. α-amyрин was also reported to inhibit the platelet activating factor (PAF) receptor with an IC<sub>50</sub> value of 8.5 µg/ml (19.9 µM)<sup>22</sup>. Other activities had been reported for α-amyрин<sup>23-26</sup>, β-amyрин<sup>24,26</sup> and their mixtures<sup>27-37</sup>

Previously, 18.2 µg/ml of a methanol extract of *A. elliptica* was found to have an inhibitory effect of 53.9 % on the PAF of rabbit platelets<sup>19,20</sup> and 5-(Z-heptadec-4'-enyl)resorcinol from the leaf extract of *A. elliptica* was reported to have an IC<sub>50</sub> value of 2.4 µg/ml for the inhibition of PAF receptor binding on rabbit platelet using <sup>3</sup>H-PAF as a ligand<sup>17</sup>. However, in this work, 5-(Z-heptadec-4'-enyl)resorcinol was not detected by the current GC-MS method developed and used.

In conclusion, the methanol extract (70% v/v) from *A. elliptica* leaves was found to inhibit collagen-induced platelet aggregation and β-amyрин isolated and purified from *A. elliptica* was found to be more potent than aspirin in inhibiting platelet aggregation. Further work on the elucidation of the mechanism of action of β-amyрин is warranted.

### Table 1—IC<sub>50</sub> values for inhibition of collagen-induced platelet aggregation

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; values (µg/ml)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; values (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% methanol extract of <em>A. elliptica</em></td>
<td>166.9 ± 46.7</td>
<td>-</td>
</tr>
<tr>
<td>α-amyрин</td>
<td>9.1 ± 1.0</td>
<td>21.3 ± 2.3</td>
</tr>
<tr>
<td>β-amyрин</td>
<td>4.5 ± 0.6</td>
<td>10.5 ± 1.0</td>
</tr>
<tr>
<td>Aspirin</td>
<td>11.3 ± 2.0</td>
<td>62.7 ± 11.1</td>
</tr>
</tbody>
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All extracts and standards are significantly different from each other (P < 0.05).
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