Bioactivity-guided fractionation of the lipoxygenase and cyclooxygenase inhibiting constituents from *Chisocheton polyandrus* Merr

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1. Introduction

Inflammation is a protective response to cell injuries caused by physical damage or infection by pathogens. Persistent inflammatory conditions may lead to the development of pathophysiological states, including inflammatory bowel disease, atherosclerosis, arthritis, carcinogenesis and bronchial asthma. Under these conditions, the arachidonic acid pathway, which involves two metabolic pathways, is one of the major mechanisms of inflammation [1]. The cyclooxygenase (COX) pathway involves the production of prostanoids (prostaglandins, prostacyclins and thromboxanes) by cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), and the lipoxygenase (LOX) pathway involves the production of leukotrienes (LTs) and hydroperoxy fatty acids by 5-lipoxygenase (5-LOX), 12-lipoxygenase (12-LOX) and 15-lipoxygenase (15-LOX) [2–4].

Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used medication in the conventional treatment of inflammation. These drugs achieve their therapeutic effects by inhibiting the activity of COX-1 and COX-2 to suppress the production of inflammatory mediators. COX-1 inhibitors exhibit several side effects, including gastrointestinal irritation and renal toxicity. The newer, selective COX-2 inhibitors are more effective against inflammation and have reduced gastrointestinal irritation side effects. However, rofecoxib (Vioxx®), a selective COX-2 inhibitor, was withdrawn from the market due to increased risk of myocardial infarction [5,6].

The inhibition of COXs by NSAIDs is now believed to potentially increase the conversion of arachidonic acid to LTs through the 5-LOX pathway as a result of substrate diversion. The resulting LTs are pro-inflammatory mediators, and they could play an important role in amplifying the inflammatory response associated with the usage of COX inhibitors [7]. Thus, the development of compounds that inhibit both COX-2 and 5-LOX would be advantageous due to their ability to target both proteins, enhancing their individual anti-inflammatory effects and reducing their associated side effects [8]. In the search for potential dual COX/LOX inhibitors, natural products containing chemical entities with a wide structural diversity may serve as a useful source of potential compounds.

*Chisocheton* genus, a member of the Meliaceae family, contains approximately 50 species that are distributed mainly in India and Malaysia [9]. *Chisocheton* species are traditionally used to treat stomach complaints, backache, fever, kidney...
complaints, rheumatism and malaria [10,11]. Phytochemical and biological studies of Chisocheton species have led to the isolation of compounds, including protolimonoids and limonoids, which were reported to have anti-inflammatory, anti-feedant, insecticide, anti-tumor and anti-malarial activities [12–149]. Chisocheton polyandrus Merr. was first reported as a plant that exhibited strong soybean LOX inhibition (IC50 = 7.2 μg/mL) during the screening of 160 plant samples, which represent more than 30 plant families collected from the Malaysian forests, for anti-inflammatory properties [15].

Therefore, to continue the evaluation of anti-inflammatory properties of C. polyandrus, we isolated and identified two dammarane triterpenoids with LOX inhibitory activity using a bioactivity-guided fractionation technique coupled with a ferrous oxidation-xylgenol orange (FOX) soybean LOX inhibition assay. Soybean LOX was used in the bioactivity-guided fractionation step because it resembles human LOX in its substrate specificity and inhibition characteristics, displays potential selectivity in subtype inhibition and dual inhibition activities.

2. Materials and methods

2.1. Reagents

Soybean LOX (type V), linoleic acid, porcine arachidonic acid, xylgenol orange (XO), indomethacin, phenidone, dimethyl sulfoxide (DMSO), ferrous sulfate, calcium chloride, tris(hydroxymethyl)aminomethane, L-α-phosphatidylcholine (type II-S), adenosine 5′-triphosphate disodium salt (ATP), Tween-20 and vanillin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant 5-LOX, 15-LOX and COX-2, and on ovine COX-1 to evaluate their activity of the assays.

2.2. Plant materials

The leaves of C. polyandrus Merr. of the family Meliaceae were collected by L. Madani of the Forest Research Center, Sepilok, Sandakan, Sabah, Malaysia. The leaves (1 kg) were powdered and macerated with sufficient hexane in conical flasks for 7 days with stirring. The extracting hexane solvent was decanted and filtered, and the remaining plant powder was air-dried and further extracted by 7 days of maceration using dichloromethane. After the removal of dichloromethane, the plant powder was finally macerated with methanol for an additional 7 days. The solvent extracts were then evaporated in vacuo to produce hexane- methanol soluble (92.7 g; yield: 9.3%) extracts.

Therefore, to continue the evaluation of anti-inflammatory properties of C. polyandrus, we isolated and identified two dammarane triterpenoids with LOX inhibitory activity using a bioactivity-guided fractionation technique coupled with a ferrous oxidation-xylgenol orange (FOX) soybean LOX inhibition assay. Soybean LOX was used in the bioactivity-guided fractionation step because it resembles human LOX in its substrate specificity and inhibition characteristics, displays good stability and is readily available from commercial sources [16–18]. The isolated compounds were then tested on human 5-LOX, 15-LOX and COX-2 and on ovine COX-1: to evaluate their potential selectivity in subtype inhibition and dual inhibition activities.

The leaves of C. polyandrus Merr. of the family Meliaceae were collected by L. Madani of the Forest Research Center (Sandakan, Sabah) from the Sapagaya Forest Reserve between Silam and Danum Valley in the state of Sabah in Malaysia (GPS coordinates: N 4° 58′ 39″ and E 118° 06′ 22″) and were then air-dried. The collection was authorized by the Forest Research Center, Department of Forestry, Sandakan, Sabah, Malaysia. The plants were authenticated, and the voucher samples (SAN 143376) were retained at the herbarium of the Forest Research Center, Sepilok, Sandakan, Sabah, Malaysia.

2.3. Extraction, fractionation and isolation procedures

The leaves (1 kg) were powdered and macerated with sufficient hexane in conical flasks for 7 days with stirring. The
The control consisted of 50 μL of soybean LOX and 20 μL of 50 mM Tris–HCl buffer pH 7.4 containing DMSO (0.2% v/v). The background contained 50 μL of soybean LOX and 20 μL of 50 mM Tris–HCl buffer pH 7.4 containing DMSO (0.2% v/v) during the first incubation period, and 50 μL of linoleic acid (40 μM) was only added after the addition of 100 μL of FOX reagent to terminate the reaction. The percentage inhibition of the test sample was calculated as follows:

\[
\text{Percentage inhibition of test sample} = \frac{\left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{background}}}{\text{Abs}_{\text{background}}} - \left(\frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{background}}}{\text{Abs}_{\text{background}}}\right)\right)}{\times 100%}
\]

where \(\text{Abs}_{\text{control}}\) = absorbance of control well; \(\text{Abs}_{\text{background}}\) = absorbance of background well; \(\text{Abs}_{\text{sample}}\) = absorbance of sample well.

### 2.5. 5-LOX and 15-LOX inhibition assays

The isolated compounds (1 and 2) were also tested on human 5-LOX and 15-LOX activity. The 5-LOX assay was adopted from Cho et al. [19], with modifications. Briefly, 100 μL of human recombinant 5-LOX (0.5 units/mL, final) was pre-incubated with 50 μL of compound or standard inhibitor (various final concentrations), 100 μL CaCl₂ (0.4 mM), 100 μL L-α-phosphatidylcholine type II-S (24 μg/mL) and 100 μL ATP (0.2 mM) in 50 mM Tris–HCl buffer pH 7.4 containing DMSO (0.2% v/v) for 5 min at room temperature in the dark. The reaction was initiated by the addition of 100 μL of arachidonic acid (50 μM) to the mixture in a quartz microcuvette. The increase in absorbance associated with the formation of the conjugated diene product \((\varepsilon = 27,000 \text{ M}^{-1} \text{ cm}^{-1})\) was measured at 234 nm for 4 min using a Shimadzu UV-1601 spectrophotometer. The IC₅₀ value of dammaran-20,24-dien-3-one (1) was also determined (5–100 μg/mL).

In the 15-LOX assay, 200 μL of human 15-LOX (4 μg/mL, final) was incubated with 80 μL of compound or standard inhibitor (various final concentrations) in 50 mM Tris–HCl buffer pH 7.4 containing DMSO (0.2% v/v) for 5 min at room temperature in the dark. The premixture was then added to 200 μL of arachidonic acid (30 μM) and mixed. The increase in absorbance at 237 nm was measured for 10 min, and the initial rate of reaction of the formation of 15-LOX was determined. The control sample consisted of 200 μL of human 15-LOX and 80 μL of 50 mM Tris–HCl buffer pH 7.4 containing DMSO (0.2% v/v). The percentage inhibition of the test sample was calculated as follows:

\[
\text{Percentage inhibition of test sample} = \frac{\left(\frac{\text{ROR}_{\text{control}} - \text{ROR}_{\text{sample}}}{\text{ROR}_{\text{control}}}\right)}{\times 100%}
\]

where \(\text{ROR}_{\text{control}}\) = initial rate of reaction of control and \(\text{ROR}_{\text{sample}}\) = initial rate of reaction of sample.

### 2.6. COX-1 and COX-2 assays

The effects of the active compounds on COX-1 and COX-2 activities were further tested using the COX Inhibitor Screening Kit to measure the formation of prostaglandin E₂ (PGE₂). To test for COX-1 and COX-2 inhibition activities, 950 μL of 100 mM Tris–HCl buffer pH 8.0, 10 μL of heme (1% v/v) and 10 μL of COX-1 (ovine) or COX-2 (human recombinant) were added to the respective test tubes. Next, 20 μL of a test compound or standard inhibitor dissolved in DMSO (0.5% v/v) was added to the COX-1 and COX-2 inhibitor tubes. The tubes were incubated for 10 min at 37 °C. The reaction was initiated by the addition of 10 μL of arachidonic acid (100 μM), and the samples were incubated for 2 min at 37 °C. Fifty microliters of 1 M HCl was added to terminate the reaction. The formation of PGE₂ was quantitated using an ELISA method, and the reaction mixture was transferred to a 96-well plate coated with mouse anti-rabbit IgG. Tracer prostaglandin acetylcholine esterase and primary antibody (mouse anti-PGE₂) were added to the plate and incubated at room temperature overnight. The reaction mixtures were removed, and the wells were washed with 10 mM potassium phosphate buffer pH 7.4 containing 0.05% Tween-20. Two hundred microliters of Ellman’s reagent was added to each well, and the plate was incubated at room temperature in the dark for 60 min. The plate was then read at 412 nm using a microplate reader. A standard curve for PGE₂ was generated from the same plate to quantify the PGE₂ levels produced in the presence of test samples. Dose–response curves were generated to estimate the IC₅₀ values. The control consisted of 950 μL of 100 mM Tris–HCl buffer pH 8.0, 10 μL of heme (1% v/v), 10 μL of COX-1 or COX-2 and 20 μL of 100 mM Tris–HCl buffer pH 8.0 containing DMSO (0.5% v/v). The background sample was similar to the control, except the enzyme was heat inactivated. The results are expressed as a percentage relative to the control (solvent treated samples), according to the following equation:

\[
\text{Percentage inhibition of test sample} = \frac{\left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{background}}}{\text{Abs}_{\text{background}}} - \left(\frac{\text{Abs}_{\text{sample}} - \text{Abs}_{\text{background}}}{\text{Abs}_{\text{background}}}\right)\right)}{\times 100%}
\]

where \(\text{Abs}_{\text{control}}\) = absorbance of control well at 100% initial activity; \(\text{Abs}_{\text{sample}}\) = absorbance of sample well; and \(\text{Abs}_{\text{background}}\) = absorbance of background well.

### 2.7. Statistical analysis

The results are expressed as the mean ± SEM (n = 3). Statistical significance was determined by one-way ANOVA followed by Dunnett’s post hoc test (GraphPad Prism, version 5.00, San Diego, California, USA). Differences were considered significant when p < 0.05.

### 3. Results

#### 3.1. Bioactivity-guided fractionation

The percentage inhibition of soybean LOX by the hexane-soluble extract was 18.41 ± 1.04% (mean ± SEM; n = 3) at 5 μg/mL. Higher concentrations of the extract could not be tested, as its solubility in DMSO was low. Both the dichloromethane and methanol extracts showed inhibition of 90.92 ± 0.72% and 70.43 ± 2.23% on soybean LOX, respectively. As the dichloromethane extract showed higher percentage inhibition compared to methanol extract, it was subjected to further fractionation.
The first separation of the dichloromethane extract on silica gel yielded 15 fractions (F-1 to F-15). When tested at 10 μg/mL, two of the most active fractions, F-1 (908.5 mg; yield, 0.53%) and F-2 (739.6 mg; yield, 0.43%), produced 98.23 ± 3.29 and 97.86 ± 3.31% inhibition, respectively (Fig. 1B). Other active fractions such as F-3, F-5 and F-6 were not subjected to further fractionation as the yields were too low. Interestingly, some fractions, including F-4 and F-7 through F-15, appeared to potentiate the activity of LOX, but this observation was not further investigated.

Trituration of F-1 in hexane yielded white crystals of dammara-20,24-dien-3-one (1) (730.2 mg; yield, 0.43% of the dried leaves). Further separation of F-2 on silica gel yielded 11 fractions [F-2(1) to F-2(11)], and F-2(4) (163.9 mg) demonstrated 90.02 ± 5.74% inhibition at 10 μg/mL (Fig. 1C). F-2(4) was subjected to silica gel separation, which was followed by crystallization in hexane–acetone to give white crystals of 24-hydroxydammara-20,25-dien-3-one (2) (27.7 mg; yield, 0.04% of the dried leaves). Both isolated compounds, dammara-20,24-dien-3-one (1) and 24-hydroxydammara-20,25-dien-3-one (2), were characterized spectroscopically using 1H and 13C NMR, COSY, HMBC and HMQC, and the results were consistent with the published literature [20,21] (Fig. 2). Compounds (1) and (2) were also further evaluated to determine the IC50 of LOX inhibition, and the values were 0.69 ± 0.07 and 1.11 ± 0.38 μM, respectively (Table 1).

3.2. Inhibition of human LOX and COX

Compounds (1) and (2) were further tested on human 5-LOX, 15-LOX, COX-2 and ovine COX-1 activity. They were initially tested at 100 μg/mL to determine their percentage inhibition. For the 5-LOX assay, dammara-20,24-dien-3-one (1) and 24-hydroxydammara-20,25-dien-3-one (2) showed 51.85 ± 2.85 and 35.49 ± 0.50% inhibition, respectively. However, they did not inhibit 15-LOX at this concentration. Dammara-20,24-dien-3-one (1) inhibited COX-1 and COX-2 by 77.81 ± 0.01 and 68.83 ± 0.01%, respectively, and 24-hydroxydammara-20,25-dien-3-one (2) inhibited COX-1 and COX-2 by 24.92 ± 5.05 and 11.06 ± 1.10%, respectively (Fig. 1D). The IC50 values of dammara-20,24-dien-3-one (1) were also determined for 5-LOX, COX-1 and COX-2 and are shown in Table 1. The IC50 values of the standard inhibitors used for each assay were determined and are consistent with the reported values; these inhibitors included phenidone, as a soybean LOX inhibitor [16]; zileuton, as a 5-LOX inhibitor [22]; nordihydroguaiaretic acid, as a 15-LOX inhibitor [23], and indomethacin, as COX-1 and 2 inhibitors [24] (Table 1).

4. Discussion

Dammara-20,24-dien-3-one (1) and 24-hydroxydammara-20,25-dien-3-one (2) isolated from the dichloromethane...
extract showed potent inhibitory activities towards soybean LOX during bioactivity-guided fractionation, with IC50 values of 0.69 μM and 1.11 μM, respectively. These values are lower than that of phenidone (IC50 = 1.44 μM). The IC50 for phenidone was similar to the values reported by Chung et al. [15] (4.3 μM) and Hlasta et al. [25] (0.48 μM). Both the dammarane compounds

Table 1
Inhibitory activity of compounds (1) and (2) and of the standard inhibitors of LOXs and COXs. Each value represents the mean ± SEM (n = 3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Soybean LOX IC50</th>
<th>Human 5-LOX IC50</th>
<th>Human 15-LOX IC50</th>
<th>Ovine COX-1 IC50</th>
<th>Human COX-2 IC50</th>
<th>Ratio COX-1/COX-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dammara-20,24-dien-3-one (1)</td>
<td>0.69 ± 0.07 μM</td>
<td>24.27 ± 2.92 μM</td>
<td>NA</td>
<td>31.91 ± 16.19 μM</td>
<td>3.17 ± 0.90 μM</td>
<td>10.06</td>
</tr>
<tr>
<td>24-Hydroxydammara-20,25-dien-3-one (2)</td>
<td>1.11 ± 0.38 μM</td>
<td>-</td>
<td>NA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenidonea</td>
<td>1.44 ± 0.09 μM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zileutonb</td>
<td>-</td>
<td>1.15 ± 0.33 μM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NDGAc</td>
<td>-</td>
<td>-</td>
<td>9.63 ± 0.38 μM</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indomethacin d</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.90 ± 0.01 nM</td>
<td>8.90 ± 0.01 nM</td>
<td>0.55</td>
</tr>
</tbody>
</table>

NA: not active at the tested concentrations. NDGA: nordihydroguaiaretic acid.

a Soybean LOX inhibitor
b 5-LOX inhibitor.
c 15-LOX inhibitor.
d COX-1 and -2 inhibitor.
did not show inhibition of subtype human 15-LOX; however, dammara-20,24-dien-3-one (1) selectively inhibited subtype human 5-LOX with an IC50 value (24.27 μM) that is twenty-fold less potent than that of zileuton (IC50 = 1.15 μM), the only available 5-LOX inhibitor on the market for the treatment of asthma. Despite its modest potency on 5-LOX inhibition, dammara-20,24-dien-3-one (1) could still be useful as an anti-inflammatory agent if it also affects other therapeutic targets in the anti-inflammatory pathways.

Due to substrate diversion of arachidonic acid metabolism as described in the introduction section, the development of dual inhibitors of COX-2 and 5-LOX can improve the efficacy of and reduce the side effects associated with NSAIDs. Therefore, both of the identified compounds were further tested for COX-1 and COX-2 inhibitions. Dammara-20,24-dien-3-one (1) inhibited COX-1 (IC50 = 31.91 μM) and COX-2 (IC50 = 3.17 μM), with an IC50 COX-1/COX-2 ratio of 10.06, whereas indomethacin showed stronger inhibitions on both COX-1 and COX-2. However, indomethacin lacked selectivity towards COX-2 with an IC50 COX-1/COX-2 ratio of 0.55.

Similar to dammara-20,24-dien-3-one (1), the natural flavonoid, myrcitrin, that has been recognized to possess anti-cancer and anti-inflammatory activities [26,27], inhibited COX-1 (IC50 = more than 100 μM) and COX-2 (IC50 = 5.10 μM) with higher selectivity on COX-2. Furthermore, the COX-1/2 selectivity of dammara-20,24-dien-3-one (1) was also comparable to that of celecoxib, a selective COX-2 inhibitor [28].

24-Hydroxydammara-20,25-dien-3-one (2) showed insignificant 5-LOX, COX-1 and COX-2 inhibitions, compared to dammara-20,24-dien-3-one (1). This result may be explained by the possibility that only one of the (R) or (S) stereoisomers is biologically active. Further investigation such as computational modeling of enzyme protein–ligand interactions may provide a better understanding of the inhibition of these dammarane compounds on LOX’s and COX’s.

Triterpenoids have been implicated as the active constituents responsible for the biological activity in the many medicinal plants that are used for their anti-inflammatory properties. Recently, dammarane triterpenoids isolated from the flour of the palmry palm Borassus flabellifer Linn. [29,30] and the dried fruit of Forsythia suspensa Vahl. [31–33] have been reported to have anti-inflammatory effects. Both dammara-20,24-dien-3-one (1) and 24-hydroxydammara-20,25-dien-3-one (2), as isolated in this study, display structural similarities to the isolated B. flabellifer dammarane triterpenoid (17α)-23-(E)-dammara-20,23-diene-38,25-diol, which showed potent immunosuppression activity in both in vitro and in vivo assays [29]. Structure–activity relationships (SARs) also suggest that a methylene substituent at C-20 is essential for immunosuppression and anti-inflammatory activity [30].

In summary, dammara-20,24-dien-3-one (1) isolated from C. polyanthus displays dual inhibition on LOX and COX particularly 5-LOX and COX-2, while 24-hydroxydammara-20,25-dien-3-one (2) produced weak or minimal LOX and COX inhibitions. Although dammara-20,24-dien-3-one (1) is less potent than indomethacin on COX inhibition, it exhibits higher selectivity towards COX-2 inhibition and its potency on COX-2 inhibition is similar to that of myrcitin. Through the dual activity, its anti-inflammatory activity may be enhanced and gastrointestinal side effects normally associated with NSAIDs reduced. Further studies including in vivo efficacy and pharmacokinetics studies need to be carried out to further evaluate compound (1) as a potential anti-inflammatory agent.

Acknowledgments

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References


