Furanocoumarins from Kaffir lime and their inhibitory effects on inflammatory mediator production

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Saisakun Kidarn1, Chalermpong Saenjum1,2, Darunee Hongwiset1 and Ampai Phrutivorapongkul1*

Abstract: Two known furanocoumarins, namely, 6,7-dihydroxybergamottin (1) and oxypeucedanin hydrate (2), were isolated from Kaffir lime (Citrus hystrix DC) fruit peel using an assay of nitric oxide scavenging effect-guided fractionation. The inhibitory activities of isolated coumarins on the production of nitric oxide (NO) and inducible nitric oxide synthase (iNOS) were studied in mouse macrophages RAW264.7 cells, whereas their inhibitory effect on cyclooxygenase-2 (COX-2) production was investigated using HT-29 and HCT116 cells. The results demonstrated that 1 inhibited lipopolysaccharide-interferon gamma-induced NO and iNOS production in RAW264.7 cells and COX-2 production in HT-29 and HCT116 cells with IC50 values of 16.16 ± 1.08, 18.63 ± 1.42, 18.19 ± 0.95 and 18.19 ± 0.95 µg/mL, respectively; 2 showed IC50 values of 18.23 ± 1.25, 22.54 ± 1.56, 22.27 ± 1.14 and 23.24 ± 1.05 µg/mL, respectively. Additionally, the positive control curcumin exhibited these activities with IC50 values of 12.52 ± 0.63, 15.55 ± 1.34, 14.29 ± 0.58 and 16.14 ± 0.65 µg/mL, respectively. The inhibitory effect of 1 on the production of iNOS and COX-2 was significantly stronger than that of 2.

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Saisakun Kidarn received her B.Sc. in Chemistry from Chiang Mai University (CMU), Thailand. Presently, she is studying M.S. in Pharmaceutical Sciences, Faculty of Pharmacy, CMU. Dr. Chalermpong Saenjum completed his B.Pharm., M.S. and Ph.D. from CMU. He is well versed in pharmaceutical chemistry research. He is now an assistant professor and a member of the Cluster of Excellence on Biodiversity-based Economy and Society (B.BES-CMU), CMU. Dr. Darunee Hongwiset completed her doctoral degree from Heinrich-Heine-Universität Düsseldorf, Germany, after receiving M.S.in Pharmaceutical Chemistry from Chulalongkorn University (CU) and B.Pharm. from CMU. Now she is a head of pharmacy service centre, Faculty of Pharmacy, CMU. Dr. Ampai Phrutivorapongkul received her B.Pharm. from CMU after that she finished doctoral degree under the Royal Golden Jubilee (RGJ) Ph.D. program from CU. She is interested in Pharmacognosy and Phytochemistry. She is now an assistant dean for graduate studies, Faculty of Pharmacy, CMU.

PUBLIC INTEREST STATEMENT

A sustained inflammation is a pathological condition. Non-steroidal anti-inflammatory drug is a drug of choice to treat inflammation, but many people suffer from their side effects. Nowadays, many researchers try to investigate the natural compounds to be developed as new drugs for treating inflammation. Curcumin is well-known natural compound used as therapeutic agents for inflammation-associated diseases. Citrus hystrix DC is an edible plant widely used in Thailand. Its leaf and fruit have been used as an ingredient in Thai foods. Its fruit is used as traditional medicine for headache, flu, fever, sore throats, bad breath, digestion, tonic and blood purifier. This fruit is rich in compounds showing various pharmacological effects. In this study, active furanocoumarins were isolated from C. hystrix fruit peel and showed a potential to develop as anti-inflammatory drug since these compounds presented similar inhibition on NO, iNOS and COX-2 productions to those of curcumin.
indicated that furanocoumarins from Kaffir lime fruit peels have potency for further development as an anti-inflammatory drug.

Subjects: Bioscience; General Science; Pharmaceutical Science

Keywords: Kaffir lime; 6', 7'-dihydroxybergamottin; oxypeucedanin hydrate; inflammatory mediators

1. Introduction

Inflammation, expressed in the form of swelling, heat, redness, fever and pain, is a normal reaction of living tissue to injury (Libby, 2007; Mequanint, Makonnen, & Urga, 2011). The inflammation process starts with the release of numerous chemical mediators from immune cells to the damage area. Two interesting chemical mediators, prostaglandins E$_2$ (PGE$_2$) and nitric oxide (NO), are produced to induce symptom-like pain, fever, redness and swelling (Vliet, Eiserich, & Cross, 2000). These two mediators are also associated with the pathogenesis of several diseases such as rheumatoid arthritis, acute gout and atherosclerosis (Vliet et al., 2000). The inhibition of NO, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) production are, therefore, an interesting option for reducing inflammation and associated diseases (Alderton et al., 2001; Surh et al., 2001).

Nowadays, it is desirable to use natural products as alternative or complementary medicines for the prevention and treatment of some diseases. Curcumin, for example, is a well-known natural compound used as a therapeutic agent for inflammatory-related diseases (Julie & Jurenka, 2009). In Thailand, some medicinal plants have been reported to be valuable sources of anti-inflammatory agents such as Terminalia chebula Retz. (Gall), which exhibited a potent inhibitory activity on lipopolysaccharide-induced NO production in RAW264.7 cells (Kakatum, Jaiarree, Makchucit, & Itharat, 2012). Various natural compounds with potential anti-inflammatory activity through the inhibition of NO production were isolated from plants in the Rutaceae family. Citrusin XI was isolated from the fruit of Citrus unshiu (Noh et al., 2015), essential oil from the seeds of Zanthoxylum myriacanthum wall.ex. Hook.f. (Li et al., 2014) and coumarins from the leaves of Z. avicennae (Cho et al., 2012). Interestingly, an ethyl acetate extract prepared from the leaves of Glycosmis parva inhibited COX-2 production (Buranabunwong, Ruangrungsi, Chansriniyom, & Limpanasithikul, 2015).

Previous reports led us to question the inhibitory effect of Kaffir lime (C. hystrix DC), an edible plant found throughout the Southeast Asia region, including Thailand. Its fruit has traditionally been used to treat abscesses, fever and coughs (Srisukha et al., 2012). Moreover, its fruit peel has been reported to be a component of a traditional Thai medicine called Prasapliai, which is active in the remedy of primary dysmenorrhea (Sriyakul, Kietinun, Pattaraarchachai, & Ruangrungsi, 2012). The chemical constituents found in this plant are alkaloids, flavonoids, phenolics and tannins (Sri Tunjung, Cinat, Michaelis, & Smales, 2015). Coumarins and glycosides were also isolated and identified (Seeka et al., 2016). The biological activity in the fruit peel of this plant is reported to have antimicrobial (Srisukha et al., 2012), anticholinesterase (Seeka et al., 2016) and antioxidant effects (Hutadilok, Chaiyamutti, Panthong, Mahabusarakam, & Rukachaisirikul, 2006). Furthermore, coumarins isolated from this fruit peel have demonstrated anti-inflammatory activity through their inhibitory effect on NO production (Murakami et al., 1999), but there are still no reports on their inhibitory effect on COX-2 production. In the present study, the fractionation of a hydroalcoholic extract of Kaffir lime fruit peel using a NO-scavenging effect-guided technique was performed. The isolated active compounds were accordingly investigated for a potential anti-inflammatory agent through their inhibitory effects on the production of NO and iNOS in macrophages RAW 264.7 cells and on COX-2 production in HT-29 and HCT116 cells.
2. Materials and methods

2.1. Plant material

The *C. hystrix DC* fruits were purchased from a local market in Chiang Mai, Thailand, in July 2017 and authenticated by Wannaree Charoensup, a botanist from the Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University. Voucher specimen No.023233 was deposited in the Herbarium at the Faculty of Pharmacy, Chiang Mai University, Thailand.

2.2. Extraction and isolation

The dried and powdered fruit peel from the *C. hystrix DC* was macerated with 70% ethanol for 3 days (Putri et al., 2013) and concentrated under reduced pressure. The crude extract was dissolved in 20% ethanol (Sreejayan & Rao, 1997) and then partitioned with *n*-hexane and ethyl acetate, respectively. Each portion was concentrated under reduced pressure to obtain three fractions, *n*-hexane fraction (CH), ethyl acetate fraction (CE) and aqueous fraction (CA). All three fractions were assayed for *in vitro* NO-scavenging activity. The CE fraction, which showed the most activity, was further separated for four consequential steps of column chromatography (CC) with NO-scavenging activity-guided fractionation technique. In the first step, the CE fraction was separated to become CEF1 to CEF13. In the second step, the highest potent fraction CEF10 was separated using a mixture of hexane: chloroform (5:95) as the eluent and sub-fractions CEF10-1 to CEF10-8 were obtained. In the third step, fraction 10–5 was selected for further separation by using a mixture of chloroform: methanol (99:1) to give 12 sub-fractions. In the fourth step, sub-fraction CEF10–5–5 was separated by using a mixture of *n*-hexane: ethyl acetate (1:1) as the eluent. Finally, sub-fraction CEF10–5–5 was purified by the preparative thin layer chromatography (TLC) to obtain compound 1. Another active fraction CEF 10–6 was also further separated by CC, using a mixture of chloroform: methanol (99:1), and sub-fraction CEF10–6–3 was finally purified by the preparative TLC, using hexane: ethyl acetate (2:3) as the developing solvent to give compound 2. The isolated compounds were identified by a nuclear magnetic resonance (NMR) spectroscopic technique and determined inhibitory activity on the inflammatory mediator production using a cell-based study.

2.3. General experimental procedure

The $^1$H NMR (400 MHz) and $^{13}$C NMR (100 MHz) spectra were recorded using a Bruker Biospin 400 MHz spectrometer with tetramethylsilane (TMS) as the internal standard and CDCl$_3$ as the solvent. The chemical shifts are expressed in δ values. The analytical and preparative TLC were performed on precoated silica gel 60F$_{254}$ (Merck, 0.25 or 0.50 mm thickness).

2.4. In vitro NO-scavenging activity

*In vitro* NO-scavenging activity using a *Griess reaction* was undertaken for every sample. The reaction mixture was composed of 800 μL of 6.25 mM sodium nitroprusside, dissolved in phosphate buffer saline (pH 7.4) and 200 μL of curcumin, used as a positive control or tested samples at concentrations of 10, 25, 50, 100 and 200 μg/mL. The reaction mixture was incubated at 37°C for 150 min. Then, the reaction mixture was transferred to the 96-well plate. The *Griess reagent*, a mixture of naphthylethylene diamine and sulphanilamide (Sreejayan & Rao, 1997), was added and incubated at room temperature for 5 min. The absorbance was measured at 540 nm.

2.5. Cell-based study for inhibitory activity on inflammatory mediator production

2.5.1. Cell culture and cell viability study

The cell viability of compounds 1 and 2 at concentrations of 10, 25, 50, 100 and 200 μg/mL and the positive control curcumin at concentrations of 2.5, 5, 10, 25, 50 and 100 μg/mL on RAW 264.7, HT-29, and HTC 116 cells were measured using cell proliferation regent WST-1 according to the described method (Saenjum, Chaiyasut, Chansakaow, Suttajit, & Sirithunyalug, 2012).
2.5.2. Inhibition of NO and iNOS production

To examine the effects of compounds 1 and 2 on NO and iNOS production in RAW 264.7 cells, a modified method (Hong et al., 2002; Hu et al., 2003; Kim et al., 2004; Sirithunyalug et al., 2018) was used. In brief, the RAW 264.7 cells were cultured with Dulbecco’s Modified Eagle medium (DMEM) in a 24-well plate for 24 h. Then, the cells were replaced with a new medium containing various concentrations of the tested samples (10–100 µg/mL) and incubated for 12 h. A mixture of 10 ng/mL lipopolysaccharide (LPS) and 250 pg/mL IFN-γ was added to the cell culture and then further incubated for 72 h. The supernatants of the culture media were collected to measure for NO production using a Griess reaction, and the cell lysates were measured for iNOS using a mouse total iNOS immunoassay kit. The results were expressed as 50% inhibitory concentrations (IC50) and curcumin was used as a positive control.

2.5.3. Inhibition of COX-2 production

To examine the effects of compounds 1 and 2 on COX-2 production in HT-29 and HCT 116 cells, the method using a human total COX-2 immunoassay kit was modified from previous studies (Colucci, Blandizzi, Ghisu, Florio, & Del Tacco, 2008; Hong et al., 2002; Sirithunyalug et al., 2018). The HT-29 and HCT 116 cells (1 × 105 cells/well) were cultured with DMEM in a black 96-well plate for 24 h and then the tested substances (1–50 µg/mL) were added. Then, the cells were incubated for 12 h. The combined LPS and IFN-γ was added and continued to incubate for 72 h. The cell supernatants, which were extracted with 10 mM Tris, pH 8.0, 1% NP-40, 0.15 M NaCl and 1 mM EDTA, were collected to measure for total COX-2 levels followed the manufacturer’s protocol. The amount of DNA was quantified by a Quant-iT PicoGreen Assay (Invitrogen, P11496) according to the manufacturer’s protocols, while that of the protein produced by RAW 264.7, HT-29 and HCT116 cells was analysed using a Bradford reagent (Bradford, 1976; Sirithunyalug et al., 2018).

3. Results

3.1. Extraction and isolation

The dried and ground fruit peel of the C. hystrix (1.2 kg) was extracted with 70% ethanol to obtain a 70% hydroalcoholic extract (185 g). This extract (48 g) was then partitioned with hexane and ethyl acetate together with the evaluated in vitro NO-scavenging activity using a Griess reaction to give a hexane extract (CH, 2.6 g; EC50 160 µg/mL), ethyl acetate extract (CE, 14.9 g; EC50 53.8 µg/mL) and aqueous extract (CA, 22.8 g; EC50 112.1 µg/mL). Next, the most active CE (10.1 g) was normal-phase flash chromatographed using a hexane–ethyl acetate (2:3) system to yield a white crystal of compound 1 (21.2 mg; EC50 14.0 µg/mL), which was identified as 6,7-dihydroxybergamottin. Sub-fraction CEF10-6 (364.3 mg; EC50 35.1 µg/mL) was normal-phase flash chromatographed using a chloroform–methanol (99:1) system to give six sub-fractions (CEF10-6-1 to CEF10-6-6). Next, CEF10-6-3 (47.9 mg), which contained more of the target compound, was purified by normal-phase preparative TLC with n-hexane–ethyl acetate (2:3) to yield a white crystal of compound 2 (oxypeucedanin hydrate, 42.8 mg; EC50 20.6 µg/mL). The in vitro NO-scavenging activity of major fractions and isolated compounds was shown in Figure 2.
3.2. Identification of the compounds

The chemical structures of the two purified compounds were successively elucidated by the $^1$H and $^{13}$C NMR spectroscopic techniques together with a comparison with the data from previous reports. Compound 1 was identified as a furanocoumarin namely 6′,7-dihydroxybergamottin (Figure 1) with a molecular formula of C$_{21}$H$_{24}$O$_6$ (Murakami et al., 1999; Ohta et al., 2002). The $^1$H and $^{13}$C NMR data of 1 were assigned as shown in Table 1. In the case of compound 2, it was identified as a furanocoumarin, namely, oxypeucedanin hydrate (Figure 1), with a molecular formula of C$_{16}$H$_{16}$O$_6$ (Gökay et al., 2010). The $^1$H and $^{13}$C NMR data of 2 are shown in Table 1.

3.3. Cell-based assay for inhibitory activity on inflammatory mediator production

Compounds 1 and 2 showed a significant ability to inhibit the induction of NO and iNOS production in RAW 264.7 cells. Both compounds also inhibit the induction of COX-2 production in HT-29 and HCT116 cells. Table 2 shows the IC$_{50}$ values obtained from both compounds.

4. Discussion

In this study, two known furanocoumarins, namely, 6′,7′-dihydroxybergamottin (1) and oxypeucedanin hydrate (2), were isolated from Kaffir lime (Citrus hystrix) peel. 6′,7′-Dihydroxybergamottin is found in grapefruit juice and presented cytochrome P3A4 inhibitory activity (Ohta et al., 2002), whereas oxypeucedanin hydrate was isolated from various plants such as Anethum graveolens, Radix imperatoria, Angelica dahurica and Ficus exasperata Vahl.

Figure 1. The structures of compounds 1 and 2.

Figure 2. The in vitro NO-scavenging activity of the fractions, sub-fractions and compounds 1 and 2.

The data were expressed as mean ± SD; n = 3. The different letters following the data show the significant content differences at $p \leq 0.05$. 
and its biological activities have been reported to be antimicrobial, anticholinesterase, antioxidant and anti-inflammatory (Amponsah, Fleischer, Dickson, Annan, & Thoss, 2013; Bai et al., 2016; Gökay et al., 2010; Seo et al., 2013; Stavri & Gibbons, 2005). The results of this study found that pharmacologically active compounds 1 and 2 were isolated from a 70% ethanol extract of *Citrus hystrix* DC fruit peel using in vitro NO-scavenging activity-guided fractionation. Both isolated compounds demonstrated potential anti-inflammatory activity through their inhibitory effect on NO, iNOS and COX-2 production in a cell-based assay without exerting cytotoxicity. Their activities were similar to those of curcumin, a naturally anti-inflammatory

<table>
<thead>
<tr>
<th>Position</th>
<th><em>δ</em>&lt;sub&gt;H&lt;/sub&gt;</th>
<th><em>δ</em>&lt;sub&gt;C&lt;/sub&gt;</th>
<th><em>δ</em>&lt;sub&gt;H&lt;/sub&gt;</th>
<th><em>δ</em>&lt;sub&gt;C&lt;/sub&gt;</th>
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<td>3</td>
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<td>8.18 (d, 10.0)</td>
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<td>7.18 (s)</td>
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<td>10’</td>
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**Table 1. The **<sup>1</sup>H and <sup>13</sup>C NMR spectral data of 1 and 2 in CDCl<sub>3</sub> (δ in ppm, J in Hz)**

**Table 2. The 50% inhibition concentrations of compounds 1 and 2 on NO, iNOS and COX-2 production**

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μg/mL)</th>
<th>NO</th>
<th>iNOS</th>
<th>COX-2</th>
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<tr>
<td></td>
<td></td>
<td>HCT 116</td>
<td>HT-29</td>
<td></td>
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<tr>
<td>Compound 1</td>
<td>16.16 ± 1.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.63 ± 1.42&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.53 ± 0.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.19 ± 0.95&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Compound 2</td>
<td>18.23 ± 1.25&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.54 ± 1.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23.24 ± 1.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.27 ± 1.14&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Curcumin</td>
<td>12.52 ± 0.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.55 ± 1.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.14 ± 0.65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.29 ± 0.58&lt;sup&gt;a&lt;/sup&gt;</td>
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</table>

The data were expressed as mean ± SD; n = 3. The mean values in a column superscripted by the same letter are not significantly different at p ≤ 0.05.
compound that is widely used to compare the anti-inflammatory capacity of plant-derived anti-inflammatory compounds and which clearly has revealed a signal transduction in NO, iNOS and COX-2 inhibition in cell-based studies (Aggarwal & Harikumar, 2009; Fürst & Zündorf, 2014) (Table 2). Interestingly, the inhibitory effect of 1 on the production of iNOS and COX-2 was significantly stronger than that of 2, whereas the inhibitory activity of 1 and curcumin on COX-2 production in HCT 116 cells was not significantly different. The inhibitory capacity of these compounds on NO production in RAW 264.7 cells corresponded to that of a previous report (Murakami et al., 1999)—that 6,7-’dihydroxybergamottin showed more inhibitory activity on NO production than oxypeucedanin hydrate with IC₅₀ values of 130 and 310 µM, respectively. Surprisingly, the inhibitory effect of both compounds on the induction of COX-2 production in HCT-116 and HT-29 cells was reported for the first time. However, some furanocoumarins were found to have drug interaction with dihydropyridine calcium channel blockers such as felodipine and nisoldipine, which are metabolized mainly via cytochrome P450, and could promote hepatotoxicity (Bailey, Arnold, Strong, Munoz, & Spence, 1999; Kakar, Paine, Stewart, & Watkins, 2004). For safety reasons, these isolated furanocoumarins should be further investigated for their effect on hepatocellular function and interference with the drugs’ metabolism.

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Competing Interests
The authors declare no competing interests.

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Disclosure statement
No potential conflicts of interest were reported by the authors.

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