Original article

Cancer chemoprevention activity of labdane diterpenes from rhizomes of *Hedychium coronarium*

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\textbf{Introduction}

*Hedychium coronarium* J. Koenig, Zingiberaceae, is a medicinal plant popularly used to treat inflammatory conditions in different countries. Three labdane diterpenes [isocoronarin D (1), methoxycoronarin D (2), ethoxycoronarin D (3)] and benzoyl eugenol (4) were isolated from rhizomes and their chemopreventive potential was evaluated using in vitro assays, namely the inhibition of NF-κB, COX-1 and -2, the induction of antioxidant response element (ARE), and the inhibition of cell proliferation. Diterpene 1 activated ARE (EC\textsubscript{50} 57.6 ± 2.4 μM), while 2, 3 and 4 significantly inhibited NF-κB (IC\textsubscript{50} of 7.3 ± 0.3, 3.2 ± 0.3 and 32.5 ± 4.9 μM, respectively). In addition, 2 and 3 selectively inhibited COX-1 (IC\textsubscript{50} values of 0.9 ± 0.0 and 3.8 ± 0.0 μM, respectively). These data support the potential chemopreventive activity of constituents from *H. coronarium* rhizomes.

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One of the most important steps in the inflammatory response is the activation of the nuclear transcription factor-κB (NF-κB) (Ghosh and Karin, 2002; Kunnumakkara et al., 2008). NF-κB activation is involved in inflammation, cell proliferation and oncogenic processes (Skehan et al., 1990; Matsuda et al., 2002; Pizzuto et al., 2005; Maiti et al., 2007; Kang et al., 2009; Kiem et al., 2012). Thus, agents that suppress NF-κB activation can abrogate carcinogenesis. This finding has encouraged the search for specific inhibitors of NF-κB activation from natural sources, which might lead to new candidates for cancer chemoprevention (Skehan et al., 1990; Pizzuto et al., 2005). In addition, COX-2 is induced during the inflammatory response and has been implicated in the etiology of cancer (Ghosh and Karin, 2002; Maiti et al., 2007; Kunnumakkara et al., 2008). Coronarin D has been shown to inhibit the NF-κB activation pathway induced by different carcinogens and pro-inflammatory molecules, and also to impair the expression of constitutive NF-κB (Girón et al., 2008; Kunnumakkara et al., 2008). It also inhibits NF-κB by down regulating IKK activation (Girón et al., 2008; Kunnumakkara et al., 2008).

As part of our continuing search for bioactive molecules from medicinal plants, herein we report the potential cancer chemopreventive activity of labdane diterpenes isolated from rhizomes of *H. coronarium*. Chemopreventive activity was assessed by examining in vitro inhibition of NF-κB, COX-1 and -2, ARE induction, and cell proliferation inhibition.

### Isolation and identification of constituents of *H. coronarium*

After drying at 40°C, for 72 h, the rhizomes of *H. coronarium* were powdered (706.3 g) and percolated with 96% EtOH at room temperature. The solvent was removed under reduced pressure yielding 81.9 g of a dark residue that was successively extracted by partition with *n*-hexane, dichloromethane and methanol yielding three main fractions *n*-hexane (6.5 g), dichloromethane (26.0 g) and methanol (15.0 g). The dichloromethane fraction was re-chromatographed on a silica gel column (70-230 mesh, Merck), eluting with *n*-hexane-ethyl acetate (95:5-1:1) gradient. The sub-fraction eluted with *n*-hexane-ethyl acetate (9:1) afforded benzyoleugenol (48.8 mg, 4). The sub-fraction eluted with *n*-hexane-ethyl acetate (85:15) was re-chromatographed on a silica gel column eluting with dichloromethane-ethyl acetate (100:0-0:100) gradient. The sub-fraction eluted with 100% of dichloromethane yielded isocoronarin D (1, 311.2 mg) and the one eluted with DCM:EtOAc (98:2) yielded ethoxy coronarin D (epimers, 3a/3b, 44.0 mg). The methanol fraction was re-chromatographed on a silica gel column eluting with dichloromethane-ethyl acetate (100:0-0:100) gradient. The sub-fraction eluted with 100% of dichloromethane yielded coronarin D (1, 312.2 mg) and the one eluted with DCM:EtOAc (98:2) yielded ethoxy coronarin D (epimers, 3a/3b, 44.0 mg). Isocoronarin D was isolated as a pure compound. Structure identification was accomplished by spectroscopic methods (Taveira et al., 2005).

### Materials and methods

#### Plant material

The rhizomes of *Hedychium coronarium* J. Koenig, Zingiberaceae, were collected in June 2002, at UFMG campus, in Belo Horizonte, Minas Gerais state, Brazil. The species was identified by Dr. J. A. Lombardi, from the Departamento de Botânica, Instituto de Ciências Biológicas, UFMG, Belo Horizonte, Brazil, where a voucher specimen was deposited (BHC 68447).

#### Reagents and apparatus

All chemicals, unless specified otherwise, were purchased from Sigma-Aldrich Co. (St. Louis, MO).

#### Cell lines

Cell lines were obtained as follows: Wild-type HepG2 and HepG2 transfected with ARE-luciferase plasmid, human hepatoma cells, were supplied by Dr. Hong-Jie Zhang (University of Illinois at Chicago, Chicago, IL); 293/NF-κB, human embryonic kidney cells, transfected with NF-κB-luciferase plasmid and Hygromycin (RC0014) were purchased from Panomics (Fremont, CA); LU1, human lung carcinoma cells were supplied by the Department of Surgical Oncology, University of Illinois at Chicago, Chicago, IL; MCF-7, human breast carcinoma (ATCC HTB-22) and hormone-dependent human prostate carcinoma LNCaP (ATCC-CRL-1740) were purchased from American Type Culture Collection (Rockville, MD). All media were supplemented with 10% fetal bovine serum (FBS) (HyClone™Thermo Scientific, VWR, Atlanta, GA) containing 100 U/ml penicillin and 0.1 mg/ml streptomycins. Cells were incubated at 37°C with 5% CO₂. All cells were kept under twenty passages and used in experiments during the linear phase of growth. COX-1 enzyme was purchased from Animal Technologies Tyler (Texas). For the assays, the compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted to their final concentrations. An equivalent volume of DMSO was added to control wells and showed no measurable effect on the assayed culture cells or enzymes.
Luciferase assays

Luciferase assays were conducted as previously described (Pezzuto et al., 2005). In brief, transfected cells were incubated at an initial density of 1.5 x 10^5 cells per well for 48 h in 96-well plates. After a 6 h incubation with 12-O-tetradecanoylphorbol 13-acetate (TPA) (100 nM) and test compounds, cells were analyzed for luciferase activity. Cells were washed with PBS and lysed using 50 µl 1X Reporter Lysis Buffer for 10 min, and the luciferase determination was performed according to the manufacturer’s protocol (Promega, Madison, WI). Data for ARE induction (EC50 values) were expressed as the concentration of compound that provoked activation halfway between baseline (DMSO control) and maximum response at a concentration of 20 µg/ml. NF-kB constructs data were analyzed for luciferase activity inhibitor (IC50 25 mM). IC 50 values were generated by measuring PGE2 production as described previously (Kang et al., 2009). The potential cancer chemopreventive activity elicited by compounds isolated from the ethanol extract of Hedychium coronarium J. Koenig, Zingiberaceae, rhizomes (EHC) was assayed using a battery of in vitro tests (inhibition of NF-kB, COX-1 and -2, ARE induction, and cell proliferation). Compounds showing anti-proliferative effects against LNCaP and HepG2 cell lines. No signs of overt cellular toxicity were observed under the employed experimental conditions.

Assay for inhibition of COX-1 and -2 activities

The effect of test compounds on COX activity was determined by measuring PGE2 production as described previously (Kang et al., 2009).

Cell proliferation assay

Cell density determinations were performed as described by Skehan et al. (1990). This assay is based on the selective binding of sulforhodamine B with cellular proteins (Ahmad et al., 2006). The human cancer cell panel was comprised of LU-1, LNCaP, HepG2, and MCF-7 cells. The cells were seeded (1 x 10^4 cells/well) in 96-well plates; test samples were added at an initial density of 1.5 x 10^5 cells per well for 48 h in 96-well plates. After a 6 h incubation with 12-O-tetradecanoylphorbol 13-acetate (TPA) (100 nM) and test compounds, cells were fixed with 10% (w/v) trichloroacetic acid and stained for 30 min. Excess dye was removed by repeated washing with 1% acetic acid. Protein-bound dye was dissolved in 10 mM Tris base solution and measurements were performed at 510 nm using a microplate reader. For each cell line, four concentrations were tested in triplicate.

Statistical analyses

Results are presented as means ± standard error. Statistical comparisons were performed using the Student’s t-test, and the level of p < 0.05 was considered as significantly different.

Results and discussion

The potential cancer chemopreventive activity elicited by compounds isolated from the ethanol extract of Hedychium coronarium J. Koenig, Zingiberaceae, rhizomes (EHC) was assayed using a battery of in vitro tests (inhibition of NF-kB, COX-1 and -2, ARE induction, and cell proliferation). Compounds showing at least 50% inhibition at a concentration of 10 µg/ml (COX-1 and -2) or 20 µg/ml (NF-kB, ARE and cell proliferation) were tested in triplicate and dose-response curves were used to determine IC50 values. Inhibition of NF-kB and induction of ARE were carried out using a luciferase reporter gene assay (Kang et al., 2009). The results are shown in Table 1.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>EC50 (µM)</th>
<th>IC50 (µM)</th>
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<tbody>
<tr>
<td></td>
<td>ARE</td>
<td>NF-κB</td>
</tr>
<tr>
<td>Isocoronarin D (1)</td>
<td>51.2 ± 5.5</td>
<td>&gt; 62.9</td>
</tr>
<tr>
<td>Methoxy coronarin D (2)</td>
<td>&gt; 60.2</td>
<td>7.2 ± 0.3</td>
</tr>
<tr>
<td>Ethoxy coronarin D (3)</td>
<td>&gt; 57.8</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>Benzoyl-eugenol (4)</td>
<td>&gt; 74.6</td>
<td>32.5 ± 4.9</td>
</tr>
</tbody>
</table>

ARE, induction of antioxidant response element; NF-κB, TPA-induced NF-κB inhibition; COX-1 and -2, inhibition of cyclooxygenase-1 and -2; anti-proliferative effects against LNCaP and HepG2 cell lines.
in the inhibition against this target when the methoxy group at C-15 is replaced by an ethoxy moiety. These changes possibly interfere with the Michael acceptor character of the α, β-unsaturated carbonyl groups of these derivatives. The active Michael acceptor groups have been shown to react with critical cysteine residues of target proteins, and could interact with the cysteine residue 179 (C179) of IKK-β, a IkB kinase-β which releases NF-κB by phosphorylation of IkB (Ahmad et al., 2006; Hee-Juhn et al., 2007; Girón et al., 2008; Kunnunakkara et al., 2008). This assumption is supported by the work of Girón et al. (2008), which demonstrated the potential anti-inflammatory activity of two compounds from a library of hispanolone labdane-type diterpenes. According to these authors, the effects are mediated by the inhibition of IKK activity, which results in stabilization of the NF-κB/IκB complex and consequently impairs the nuclear translocation of NF-κB (Girón et al., 2008; Kunnunakkara et al., 2008). The active compounds described by Girón et al. (2008) bear one or two Michael acceptors as functional groups, whereas this feature is absent in the inactive structurally-related diterpenes. Recently, other labdane diterpenes isolated from H. coronarium, hedyconeron A and hedyconeron B, showed moderate inhibitory activity on TNF-α production (IC50 46.0 ± 1.3 and 12.7 ± 0.3 μM, respectively), whereas the corresponding deoxymethyl derivatives were inactive (Kiem et al., 2012). This finding suggests that the inhibitory effect against some nuclear factors increases by introducing a methoxy or ethoxy group at the C-15/16 positions.

Regarding COX-1 and -2 inhibition, the presence of a methoxy group at C-15 (compound 2; IC50 0.9 ± 0.0 μM) led to an increase of potency against COX-1, in a selective manner, in comparison to derivative 3 (IC50 3.8 ± 0.1 μM), which bears an ethoxy group. These compounds did not inhibit COX-2 under these assay conditions.

In addition to labdane diterpenes, fractionation of H. coronarium rhizomes afforded benzoyl eugenol (4), a derivative of eugenol. This compound showed weak activity (IC50 32.5 ± 4.9 μM) in the NF-κB inhibition assay and was considered inactive in the ARE induction assay as well as in the COX-1 and -2 inhibition assays. Resveratrol is chemically related to 4, since both compounds have a phenylpropanoid group, but with different location of the second aryl ring. Recently, two libraries of resveratrol derivatives were designed to evaluate structure features that could improve selective inhibition of NF-κB, COX-1 or COX-2 (Nakatani et al., 1994; Pezzuto et al., 2005). Among the COX-1 and -2 inhibitors, the absence of electron-donating atoms between the two aryl rings seems to be an important structure feature for activity (Skehan et al., 1990; Handler et al., 2007). This requisite is violated by compound 4, which possess an ester moiety that may account for its inactivity on the COX assay. Additionally, derivatives with a withdrawing substituent were either inactive or weakly active in the NF-κB inhibition assay (Pezzuto et al., 2005). The ester carbonyl group present in compound 4 fulfills this feature, and this is probably related with its low NF-κB inhibitory activity.

To further assess the potential of diterpenes 1-3 as cancer chemopreventive agents, their cytotoxicity was determined against four cell lines (HepG2, LU-1, LNCaP, and MCF-7), at concentrations up to 20 μg/ml. Proliferation suppression with LU-1 and MCF-7 cells was not significant, whereas weak antiproliferative effects against HepG2 (1 and 2) and LNCaP (1 and 3) was observed (Table 1). The HepG2 antiproliferative activity exhibited by compound 1 could be related to ARE induction activity, since the ARE-luciferase plasmids transfected cells are constructed from the HepG2 cell line. The low cytotoxicity of compound 2 in cancer cells MCF-7, A549 and SK-N-SH (Suresh et al., 2010), and Hela and HUMEC has been also reported (Zhan et al., 2012).

Altogether, these results indicate that the cancer chemoprevention effect of the labdane diterpenes from H. coronarium most probably involve the inhibition of NF-κB, which may also account for the alleged anti-inflammatory activity of the plant. An increase in the liposolubility of these labdanes increased NF-κB inhibition and decreased cytotoxicity. These data suggest the potential cancer chemopreventive activity of methoxy and ethoxy labdanes from H. coronarium.

Authors’ contributions

DCE designed the study, contributed in running the laboratory work, analysis of the data and drafted the paper and did a critical reading of the manuscript. FSNT contributed in plant samples collection and identification, confection of herbarium, running the laboratory work. TPK contributed to biological studies. GH contributed in plant identification and herbarium voucher specimen confection. JMP and FCB designed the study, supervised the laboratory work and contributed to critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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R E F E R E N C E S


Manish, M., 2013. Current status of endangered medicinal plant

Girón, N., Través, P.G., Rodríguez, B., López-Fontal, R., Boscá, L., Hortelano, S., las Heras, B., 2008. Suppression of


Hwang, D., a labdane diterpene, inhibits both constitutive and inducible nuclear factor-kappa B pathway activation, leading to potentiation of apoptosis, inhibition of invasion, and suppression of osteoclastogenesis. Mol. Cancer Ther. 7, 3306-3317.


