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Anti-Inflammatory Principles from the Fruits of *Evodia rutaecarpa* and Their Cellular Action Mechanisms

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The fruits of *Evodia rutaecarpa* Benth (Rutaceae) has long been used for inflammatory disorders and some anti-inflammatory actions of its constituents such as dehydroevodiamine, evodiamine and rutaecarpine were previously reported. Since the pharmacological data is not sufficient to clearly establish the scientific rationale of anti-inflammatory medicinal use of this plant material and the search for its active principles is limited so far, three major constituents (evodiamine, rutaecarpine, goshuyuamide II) were evaluated for their anti-inflammatory cellular action mechanisms in the present study. From the results, evodiamine and rutaecarpine were found to strongly inhibit prostaglandin E_2 synthesis from lipopolysaccharide-treated RAW 264.7 cells at 1-10 μM. Evodiamine inhibited cyclooxygenase-2 induction and NF-κB activation, while rutaecarpine did not. On the other hand, goshuyuamide II inhibited 5-lipoxygenase from RBL-1 cells (IC_{50} = 6.6 μM), resulting in the reduced synthesis of leukotrienes. However, these three compounds were not inhibitory against inducible nitric oxide synthase-mediated nitric oxide production from RAW cells up to 50 μM. These pharmacological properties may provide the additional scientific rationale for anti-inflammatory use of the fruits of *E. rutaecarpa*.

Key words: *Evodia rutaecarpa* Benth (Rutaceae), Evodiamine, Rutaecarpine, Goshuyuamide, Anti-inflammation, Cyclooxygenase, Lipoxygenase

INTRODUCTION

Inflammation is a multifaceted event of pathological process. Numerous molecules are involved in this process. Among them, eicosanoids including prostaglandins (PG) and leukotrienes (LT) synthesized from arachidonic acid (AA) as well as nitric oxide (NO) are important players (Gallin and Snyderman, 1999). The inducible enzymes such as cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) are mainly responsible for the massive synthesis of PGs and NO, respectively. In addition, 5-lipoxygenase (5-LOX) is an enzyme to synthesize LTs deeply involved in allergic disorders. Therefore, an inhibition of these enzymes and/or a down-regulation of COX-2/iNOS may give a beneficial effect for exerting anti-inflammatory action.

The fruits of *Evodia rutaecarpa* Benth (Rutaceae) have been used in Chinese medicine for several disease conditions including inflammatory disorders (Bae, 2000). From this plant materials, various types of alkaloids, flavonoids, and limonoids were previously isolated (Tang and Eisenbrand, 1992). And some of their anti-inflammatory activities have been investigated *in vitro* and *in vivo*. For instance, dehydroevodiamine and evodiamine were shown to inhibit iNOS-mediated NO production when added before priming on RAW 264.7 cells (Chiou *et al.*, 1997). Rutaecarpine was found to strongly inhibit COX-2 and show in vivo anti-inflammatory activity (Moon *et al.*, 1999). Several quinolones from this plant material have been shown to inhibit LT biosynthesis from human polymorphonuclear granulocytes (Adams *et al.*, 2004) and most of them also inhibited Nfat-1 and nuclear transcription factor-κB (NF-κB) activation observed by transfection assay (Jin *et al.*, 2004). However, the pharmacological data is not sufficient to clearly establish the scientific rationale of anti-inflammatory medicinal use of this plant material, and the search for its active principles is limited so far. Therefore, it may be necessary to explore anti-inflammatory principles

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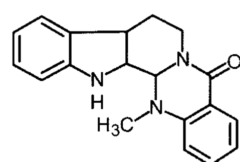
further and their anti-inflammatory action mechanism(s). Moreover, there is a need to define major active constituents having anti-inflammatory action for standardizing this plant material in the crude form as well as in the form of extracts. For these purposes, in this investigation, anti-inflammatory activity has been established with three major ingredients from this plant material, evodiamine, rutaecarpine and goshuyuamide II using the cellular assay system of COX-2-mediated PG production, iNOS-mediated NO production and 5-LOX-mediated LT production. And some of their cellular effects on a transcription factor, NF-κB, were also investigated.

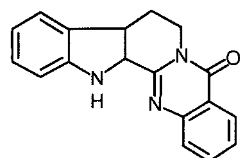
MATERIALS AND METHODS

Chemicals

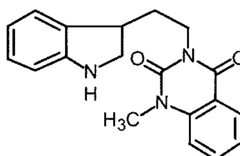
N-[2-cyclohexyloxy-4-nitrophenyl]methane sulfonamide (NS-398) was obtained from Biomol (Plymouth Meeting, PA). 2-Amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine hydrochloride (AMT) was purchased from Tocris Cookson Ltd. (UK). Arachidonic acid (AA, 99%), nordihydroguaiaretic acid (NDGA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), L-tosylamido-2-phenyl ethyl chloromethyl ketone (TPCK) and lipopolysaccharide (LPS, *Escherichia coli* 0127:B8) were purchased from Sigma Chem. (St. Louis, MO). Prednisolone was from Upjohn Co. (Kalamazoo, MI). LipofecAMINE PLUS, DMEM and other cell culture reagents including FBS were products of Gibco BRL (Grand Island, NY). Protein assay kit was purchased from Bio-Rad Lab. (Hercules, CA). Evodiamine, rutaecarpine and goshuyuamide II (Fig. 1) were isolated from the fruits of *E. rutaecarpa* and structurally identified according to the previously described method (Bergman and Bergman, 1985; Hwang *et al.*, 2001; Noboru *et al.*, 1989).



evodiamine



rutaecarpine



goshuyuamide II

Fig. 1. The chemical structures of the isolated compounds used in this study

RAW 264.7 cell culture and measurement of NO and PGE_2 concentrations

RAW 264.7 cells obtained from American Type Culture Collection (ATCC) were cultured with DMEM supplemented with 10% FBS and 1% antibiotics (penicillin, 100 U/mL and streptomycin, 100 μg/mL) under 5% CO_2 at 37°C and activated with LPS based on the previously described procedures (Chi *et al.*, 2001). Briefly, cells were plated in 96-well plates (2×10^5 cells/well). After pre-incubation for 2 h, test compounds and LPS (1 μg/mL) were added and incubated for 24 h, unless otherwise specified. Test compounds dissolved in DMSO were diluted with serum-free DMEM into appropriate concentrations. Final concentration of DMSO was adjusted to 0.1% (v/v). Cell viability was assessed with MTT assay as described previously (Mossman, 1983). For determination of NO concentration, the stable conversion product of NO, nitrite (NO_2^-), was measured using Griess reagent and optical density was checked at 550 nm. PGE_2 concentration in the medium was measured using ELISA kit for PGE_2 (Cayman Chem. Co.) according to the manufacturer's recommendation.

Effects on 5-lipoxygenase (5-LOX)

In order to evaluate the inhibitory activity against 5-LOX, RBL-1 cells purchased from ATCC were cultured. The cells were cultured in RPMI 1640 with 10% FBS, 2 mM glutamine and 1% antibiotics (penicillin, 100 U/mL and streptomycin, 100 μg/mL) under 5% CO_2 at 37°C, and plated in 96-well plate for 2 h. The tested compounds were added and preincubated for 10 min. For 5-LOX activation, A-23187 (ionophore) was added and incubated further for 15 min. Media was collected and the concentration of 5-LOX products, cysteinyl leukotrienes ($LTC_4/D_4/E_4$), was measured using ELISA kit (Cayman Chem. Co.) according to the manufacturer's recommended procedures.

Western blot analysis

For measuring the protein level of iNOS and COX-2, Western blotting technique was used (Chi *et al.*, 2001). RAW cells were cultured in 6-well plates (5×10^6 cells/well) in the presence or absence of LPS (1 μg/mL) with/without test compounds for 20 h. After preparing cell homogenate, the supernatant was obtained by centrifugation at 15,000 g for 30 min. Using Tris-glycine gel (8%), electrophoresis was carried out and bands were blotted to PVDF membranes. iNOS antibody (N32030, Transduction Lab.) and COX-2 antibody (No-160116, Cayman Chem.) were incubated and bands were visualized by the

treatment of secondary antibody and DAB reagent (Vector Lab.).

Effects on the activation of NF-κB by reporter gene assay

The transfectant RAW 264.7 cells were plated on a 12-well plate (1.5 × 10^5 cells/well). This cell line releases the secretory alkaline phosphatase (SEAP) as a transcription reporter to NF-κB activity and contains the neomycin phosphotransferase (NPT) gene for a dominant selection marker for geneticin resistance. The cells were incubated for 24 h, changing a new media and pretreated with the compounds for 2 h, and then were treated with LPS (1 μg /mL) for 5 h. Incubation mixture, containing dilution buffer (25 μL), assay buffer (97 μL), culture media (enzyme source, 25 μL), and 4-methylumbelliferyl phosphate (MUP, 1 mM, 3 μL) in a 96-well plate were left for 60 min in the dark at room temperature. Fluorescence from the product of the SEAP/MUP was measured using a 96 well plate fluorometer (Molecular Devices, Gemini XS, Sunnyvale, CA, U.S.A.) by excitation at 360 nm and measuring light emission at 449 nm (Ahn *et al.*, 2003; Ahn *et al.*, 2005).

Statistical analysis

Experimental values were represented as arithmetic mean ± SD. The one-way ANOVA test was used to determine the statistical significance. All experiments were performed at least twice and they gave the similar results.

RESULTS

By LPS (1 μg/mL) treatment, PGE_2 and NO production by the induced COX-2 and iNOS were dramatically elevated from RAW 264.7 cells for 24 h incubation. PGE_2 concentration increased to 22.9 ± 0.9 nM from the basal level of 3.3 ± 1.7 nM (n = 3). NO concentration also increased to 42.1 ± 3.1 μM from the control level (1.7 ± 0.1 μM) (n = 3). On this experimental condition, evodiamine and rutaecarpine concentration-dependently inhibited PGE_2 production whereas goshuyuamide II did not (Fig. 2A). The IC_{50} values for evodiamine and rutaecarpine were found to be 3.7 and 1.4 μM, respectively. The reference compound, NS-398 (COX-2 selective inhibitor), showed potent inhibition (IC_{50} < 0.01 μM). It is noteworthy that the concentrations of higher than 50 μM of evodiamine and rutaecarpine showed significant cytotoxic effects on RAW cells for 24 h incubation by MTT assay (data not shown). But, the inhibitory activity of PGE_2 production by evodiamine and rutaecarpine may not be associated with their cytotoxic effect since the concentration ranges of evodiamine and rutaecarpine potently inhibiting PGE_2 production were 1-25 μM, which did not affect cell viability.

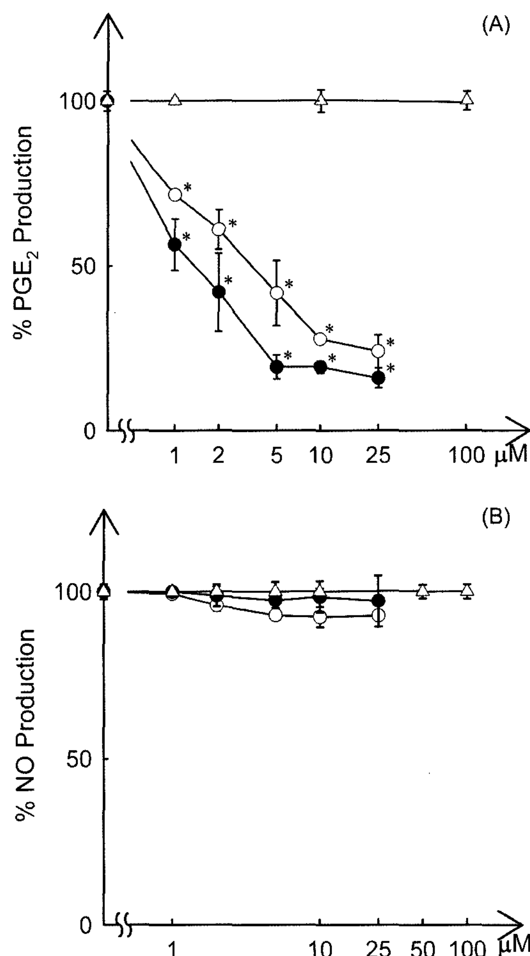


Fig. 2. Effects of the isolated compounds from *E. rutaecarpa* on PGE_2 and NO production from LPS-treated RAW cells. (A) Effects on PGE_2 production. (B) Effects on NO production. RAW cells were incubated with LPS and test compounds for 24 h, and PGE_2 and NO concentrations in the medium were measured as described in the experimental section. Percent production was calculated after subtracting basal level concentrations of PGE_2 and NO. Evodiamine (○), rutaecarpine (●), goshuyuamide II (△). The data points and bars represented arithmetic mean ± SD (n = 3). *, $P < 0.001$, Significantly different from the LPS-treated control group.

On the contrary, these three compounds did not show meaningful inhibition of NO production (Fig. 2B). AMT, a selective iNOS inhibitor, showed potent inhibition of NO production as expected (IC_{50} = 0.02 μM). Collectively, evodiamine and rutaecarpine inhibited PGE_2 production, but not NO production from LPS-treated RAW 264.7 cells.

Next, cellular action mechanism of PGE_2 inhibition by evodiamine and rutaecarpine was investigated. In order to check COX-2 expression level, Western blotting technique was employed. LPS treatment induced COX-2 expression (Fig. 3A). Under this condition, evodiamine (25 μM) potently down-regulated COX-2 expression, while rutaecarpine did not. And this down-regulation by evodiamine is strongly

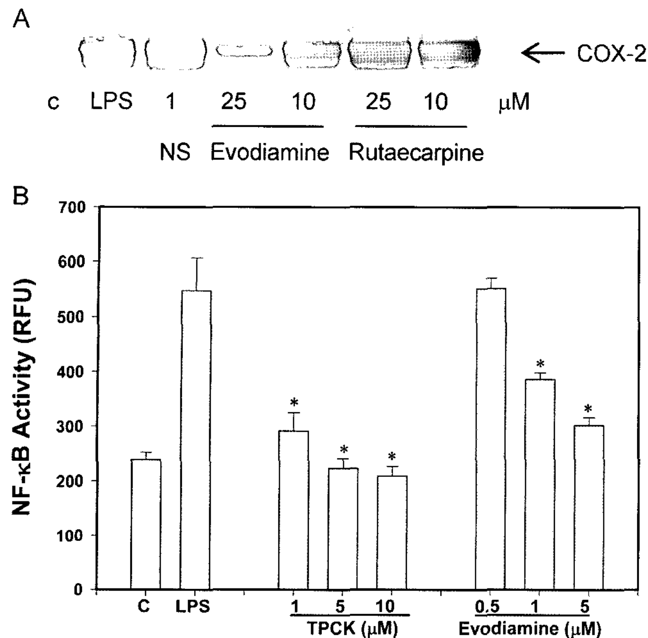


Fig. 3. Effects of the isolated compounds from *E. rutaecarpa* on COX-2 expression from LPS-treated RAW cells. (A) Western blotting analysis. (B) Inhibition of evodiamine on NF-κB activation by reporter gene assay. C, control without LPS; NS, NS-398; RFU, relative fluorescence unit. The data points and bars represented arithmetic mean ± SD (n = 3). *, $P < 0.001$, Significantly different from the LPS-treated control group.

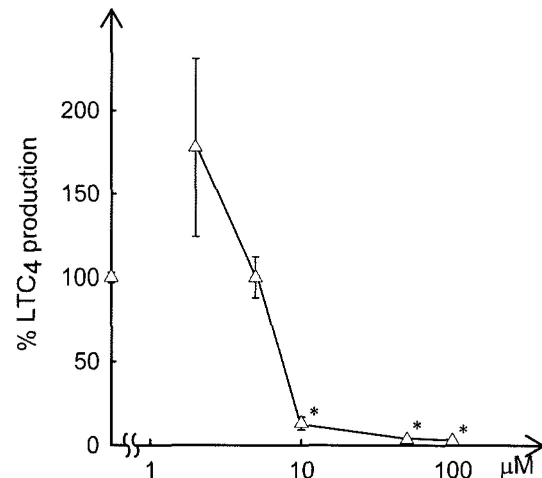


Fig. 4. Effects of goshuyuamide II on 5-LOX-catalyzed LT generation from RBL-1 cells. For 5-LOX activation, RBL-1 cells were incubated with A-23187 in the presence or absence of test compounds. LTs were measured with ELISA as described in Method section. Percent production was calculated after subtracting basal level concentrations of $LTC_4/D_4/E_4$. The data points and bars represented arithmetic mean ± SD (n = 3). *, $P < 0.001$, Significantly different from the LPS-treated control group.

related to an inhibition of transcription factor activation, NF-κB with an IC_{50} of 1.6 μM measured by reporter gene assay (Fig. 3B). In this experiment, the reference compound (TPCK) also strongly inhibited NF-κB activation. However, rutaecarpine did not affect NF-κB activation as expected (data not shown). Thus, it is revealed that an inhibitory activity of PGE_2 production by evodiamine is at least in part due to a down-regulation of COX-2 expression. And COX-2 down-regulation by evodiamine is related with an inhibition of NF-κB activation.

For elucidating the effects on 5-LOX activity, RBL-1 cells were used. Upon activation with an ionophore, A-23187, this cell line produced massive amounts of 5-LOX products, cysteinyl-leukotrienes ($LTC_4/D_4/E_4$) for 15 min incubation (489.7 ± 142.8 pg/mL from the basal level of 4.2 ± 1.2 pg/mL, n = 3). Under this experimental condition, the test compounds were added and the concentrations of LTs in the media were monitored by ELISA. While evodiamine and rutaecarpine did not show an inhibition up to 50 μM (data not shown), goshuyuamide II potently and concentration-dependently inhibited 5-LOX (IC_{50} = 6.6 μM) (Fig. 4). A reference compound, NDGA (nonspecific lipoxygenase inhibitor), also showed a potent inhibition (IC_{50} = 0.2 μM), as expected.

DISCUSSION

This investigation has clearly proven that three major ingredients from the fruits of *E. rutaecarpa* possess anti-inflammatory activity. The present study also provides an additional scientific rationale of the medicinal use of this plant material on some inflammatory disease conditions.

Dehydroevodiamine and evodiamine were previously found to inhibit NO production from RAW 264.7 cells stimulated by IFN-γ/LPS treatment and the latter was reported to only affect priming signal, but not iNOS protein synthesis (Chiou *et al.*, 1997). This previous finding may be matched with our results that evodiamine did not show the meaningful inhibition of NO production from LPS-treated RAW cells when simultaneously added with LPS. Recently, evodiamine was found to inhibit NF-κB activation from several cancer cell lines, leading to down-regulation of the expression of some proinflammatory molecules such as COX-2 and intercellular adhesion molecule-1, while rutaecarpine did not (Takara *et al.*, 2005). These results were also correlated with our finding that evodiamine down-regulated COX-2 expression by inhibition of NF-κB activation from LPS-treated RAW 264.7 cells, but rutaecarpine did not. The present study has also clearly shown that rutaecarpine did not affect COX-2 expression level, supporting the previous finding that this compound directly inhibited COX-2 as a COX-2 inhibitor (Moon *et al.*, 1999), although there were some controversial results showing phospholipase A_2 inhibitory activity of the same compound

(Woo *et al.*, 2001). On the other hand, there has been no report describing the biological activity of goshuyuamide II. Some of the effects of goshuyuamide II on eicosanoid and NO synthesis were unveiled here, but further pharmacological characterization is needed.

From our results, it is reasonably suggested that evodiamine inhibits COX-2-mediated prostanoid synthesis by COX-2 down-regulation, while rutaecarpine reduces prostanoid synthesis by COX-2 inhibition. In addition, goshuyuamide II inhibits LT synthesis by 5-LOX inhibition. On the contrary, all these compounds did not significantly affect iNOS-mediated NO production. Evodiamine was repeatedly proved to down-regulate COX-2 induction by inhibiting NF-κB activation, whereas rutaecarpine neither inhibited COX-2 induction nor NF-κB activation. It is surprising that evodiamine, rutaecarpine and goshuyuamide II possess very different action mechanisms despite the similarity of their chemical structures. This point should be studied further in detail. To our knowledge, this is the first report to demonstrate an inhibition of COX-2-mediated prostanoid synthesis from macrophages by evodiamine and 5-LOX inhibition of goshuyuamide II.

Based on the traditional Chinese medicine, the fruits of *E. rutaecarpa* have been utilized for treating the inflammatory disorders such as gastrointestinal disorders, headache, post-partum hemorrhage and amenorrhea. The present investigation has shown that anti-inflammatory activity of *E. rutaecarpa* could be attributed at least in part by inhibition of COX-2 and 5-LOX by three major ingredients studied here. All results from the present study have shown that these constituents may block eicosanoid synthesis in the inflamed sites, leading to anti-inflammation. Our results could also serve as an additional rationale for using *E. rutaecarpa* in the inflammatory-related disorders.

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