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Anti-inflammatory effects and mechanisms of the ethanol extract of *Evodia rutaecarpa* and its bioactive components on neutrophils and microglial cells

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Abstract

Evodia rutaecarpa is commonly used as an anti-inflammatory drug in traditional Chinese medicine. We previously identified four bioactive compounds (dehydroevodiamine (I), evodiamine (II), rutaecarpine (III), and synephrine (IV)) from the ethanol extract of *E. rutaecarpa*, but their effects and mechanism(s) of action remain unclear. To study the anti-inflammatory potential and the possible underlying mechanism(s), their effects on phorbol-12-myristate-13-acetate (PMA)- and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced reactive oxygen species production in neutrophils was studied, as well as lipopolysaccharide (LPS)-induced nitric oxide (NO) production and inducible NO synthetase (iNOS) expression in microglial cells. The ethanol extract of *E. rutaecarpa* displayed potent antioxidative effects against both PMA- and fMLP-induced reactive oxygen species production in neutrophils (with IC_{50} values of around 2.7–3.3 μg/ml). Although less potent than the ethanol extract of *E. rutaecarpa*, compounds I–IV all concentration-dependently inhibited PMA- and fMLP-induced reactive oxygen species production, with compound IV consistently being the most potent agent among these active components. The antioxidative effects of the ethanol extract of *E. rutaecarpa* and these compounds were partially due to inhibition (10%–33%) of NADPH oxidase activity, a predominant reactive oxygen species-producing enzyme in neutrophils, and to a minor extent to their direct radical-scavenging properties. The ethanol extract of *E. rutaecarpa* also inhibited LPS-induced NO production (with an IC_{50} of around 0.8 μg/ml) and iNOS upregulation in microglial cells that was partially mimicked by compounds I, II, and III, but not compound IV. Our results suggest that the ethanol extract of *E. rutaecarpa* and its four bioactive components all exhibited anti-inflammatory activities which could be partially explained by their different potentials for inhibiting NADPH oxidase-dependent reactive oxygen species and/or iNOS-dependent NO production in activated inflammatory cells.

Keywords: Dehydroevodiamine; *Evodia rutaecarpa*; Evodiamine; Inflammation; Microglial cell; Neutrophil; Reactive oxygen species; Nitric oxide (NO); Inducible NO synthetase (iNOS)

1. Introduction

Evodiae Fructus, (known in Chinese as *wu-chu-yu*), is the dried, nearly ripe fruit of *Evodia rutaecarpa* (Juss.) Benth. (Rutaceae). It is officially listed in the *Chinese Pharmacopoeia* and is used as an analgesic, antiemetic, anti-inflammatory, and

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astringent agent, and for the treatment of hypertension (Tang and Eisenbrand, 1992). It is also used as a remedy for gastrointestinal disorders (abdominal pain and dysentery), headaches, amenorrhea, and postpartum hemorrhage (Liao et al., 1981); and for the treatment of bacterial infections (e.g., pneumonia bacteria) and inflammation-related disorders such as eczema and ulcerative stomatitis (Chang and But, 1987). A number of alkaloids with biological activity have been identified in *E. rutaecarpa* including dehydroevodiamine (I), evodiamine (II), and rutaecarpine (III) (Tang and Eisenbrand, 1992) (Fig. 1). Our previous study also revealed that synephrine (IV), in addition to compounds I–III, is another bioactive compound found in the ethanol extract of *E. rutaecarpa* (Ko et al., 2002, 2003).

Pharmacological investigations have revealed that different extracts of *E. rutaecarpa* and its chemical constituents display a number of biological activities related to inflammation, e.g., antinociception (Matsuda et al., 1998), anti-inflammation (Chang and But, 1987), immune modulation (Chang et al., 1995), nitric oxide (NO) inhibition (Chiou et al., 1997), protection against endotoxin shock in rats (Chiou et al., 2002), and anti-inflammatory activity in human skin (Yarosh et al., 2006). Since modulation of inflammation-related disorders by *E. rutaecarpa* predominates its biological activity, it is reasonable to hypothesize that the contents of these bioactive components (e.g., compounds I–IV) may correlate with the anti-inflammatory activity of extracts of *E. rutaecarpa*, and may be used as biological markers for further elucidation.

In this study, we attempted to set up two acute inflammation-related cellular models including (1) activation of human neutrophils by receptor-mediated (e.g., *N*-formyl-methionyl-leucyl-phenylalanine; fMLP) and non-receptor-mediated (e.g., phorbol-12-myristate-13-acetate; PMA) induction of reactive oxygen species production, and (2) lipopolysaccharide (LPS)-induced nitric oxide (NO) production and inducible NO synthetase (iNOS) expression in murine microglial cells as two parameters for evaluating the anti-inflammatory potential of the ethanol extract of *E. rutaecarpa* and the possible mechanisms of its action, while simultaneously correlating its anti-inflammatory activities with its bioactive components (compounds I–IV).

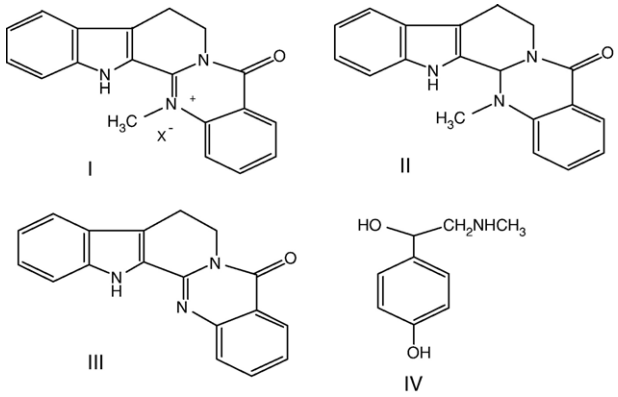

Fig. 1. Chemical structures of four bioactive compounds from *Evodia rutaecarpa*: dehydroevodiamine (I), evodiamine (II), rutaecarpine (III), and synephrine (IV).

2. Materials and methods

2.1. Preparation of the ethanol extract of *E. rutaecarpa* and drugs

The ethanol extract of *E. rutaecarpa* was prepared as in our previous reports (Ko et al., 2002, 2003). After being vacuum-dried, the ethanol extract of *E. rutaecarpa* was re-dissolved in dimethyl sulfoxide (DMSO) as a 10 mg/ml stock solution. Three bioactive components, dehydroevodiamine (I), evodiamine (II), and rutaecarpine (III), were purified from *E. rutaecarpa*. Their identities were confirmed by comparing their NMR and IR spectra with those reported in the literature (Lin et al., 1991). Synephrine (IV) was purchased from Sigma-Aldrich (USA). These drugs were all dissolved in DMSO as stock solutions of 10 mM. The final concentration of DMSO in the reaction buffer was less than 0.25%, and at that concentration, it showed no significant cytotoxicity or biological activity as compared with drug-free samples as reported in our previous report (Wang et al., 2006).

2.2. Measurement of PMA- and fMLP-induced reactive oxygen species production by human neutrophils

Reactive oxygen species production was measured as described in our previous study (Shen et al., 2003). Briefly, after incubation for 10 min with the test drugs, a luminol-preloaded neutrophil suspension was triggered by adding 50 μl PMA (0.5 μM) or fMLP (1 μM), the chemiluminescence was monitored during a 30-min observation period using a microplate luminometer reader (Orion,® Germany), and results are presented as relative light units (RLU). Peak levels were recorded in order to calculate the 50% inhibitory concentration (IC_{50}) in response to PMA- or fMLP-triggered chemiluminescence.

2.3. Measurement of NADPH oxidase activity

NADPH oxidase activity was measured as described previously (Wang et al., 2006). Test drugs were added to the wells of a bioluminescence plate and incubated with 50 μg of cell homogenate for 20 min at 37 °C in the dark. $O_2.^-$ production was stimulated with 200 μM NADPH, and the chemiluminescence was monitored for 30 min, after which the AUC (area under the curve) was calculated to represent reactive oxygen species production (NADPH oxidase activity).

2.4. Measurement of 1,1-diphenyl-2-picrylhydrazyl (DPPH)'s radical-scavenging capacity

The DPPH· radical-scavenging capacity assay was performed as described in our previous report (Lin et al., 2006). The DPPH solution (200 μl, at a final concentration of 200 μM in methanol) was added to 10 μl of diluted drugs in each well of a 96-well microplate, and the resulting solution was allowed to react for 30 min in the dark at room temperature. The absorbance (A517 OD units) is defined as the optical density (OD) measured at 517 nm caused by the DPP. radical as determined using a PowerWave™ XS (BioTek) microplate-spectrophotometer. The