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In vitro Anti-inflammatory Activity of Carvacrol: Inhibitory Effect on COX-2 Catalyzed Prostaglandin E_2 Biosynthesis

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Possible anti-inflammatory effect of carvacrol was evaluated by *in vitro* cyclooxygenase-2 (COX-2) assay. Carvacrol inhibited production of prostaglandin E_2 catalysed by COX-2 with an IC_{50} value of 0.8 μM what is practically the same concentration as the IC_{50} obtained for the standard inhibitors indomethacin and NS-398 with values of 0.7 μM and 0.8 μM, respectively. The COX-1 was inhibited approximately at the same rate (IC_{50} of 0.7 μM for carvacrol), which suggests non-selective inhibition of both enzyme isoforms. The results of the study demonstrate possible anti-inflammatory potential of this compound due to the inhibition of inducible COX-2 isoform.

Key words: Carvacrol, Cyclooxygenase, Prostaglandin, Inhibition, Anti-inflammatory.

INTRODUCTION

Carvacrol (2-methyl-5-[1-methylethyl]phenol) (Fig. 1) is monoterpene phenolic constituent of essential oil produced by numerous aromatic plants and spices such as black cumin (*Nigella sativa* L.), marjoram (*Origanum majorana* L.), oregano (*Origanum vulgare* L.) summer savory (*Satureja hortensis* L.) and thyme (*Thymus vulgaris* L.) (Enomoto et al., 2001; Skocibusic and Besic, 2004; De Vicenzi et al., 2004). The above mentioned plants are traditionally used as natural remedies for pain, arthritis, asthma, bacterial infections and headache (Sosa *et al*., 2005; Enomoto et al., 2001) whereas their medicinal properties notably relate to occurrence of high content of biologically active monoterpenes and structurally related compounds (Sosa *et al*., 2005) such as carvacrol, for which a number of significant biological effects e.g. antimicrobial (Ben Arfa et al., 2006), anti-inflammatory (Sosa et al., 2005), antithrombotic (Enomoto et al., 2001) and acetylcholinesterase inhibitory properties (Jukic et al., 2007) have previously been described.

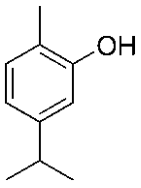

Fig. 1. Structure of carvacrol

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Most of antiphlogistic agens such as nonsteroidal anti-inflammatory drugs (NSAIDs) affect production of prostaglandins due to inhibition of cyclooxygenases (COXs), the enzymes which catalyze the first steps of prostaglandin biosynthesis from arachidonic acid (AA). The role of two main isoforms of COX in human organism can be simplified that first of them (COX-1) is responsible mainly for physiological production of prostanoids, whereas the second inducible isoform (COX-2) elevates their production in sites of inflammation (Warner and Mitchell, 2004). It has been shown that some monoterpene phenolic derivatives (e.g. thymol) inhibit both COX isoforms *in vitro* at nearly the same concentrations as commonly used anti-inflammatory drugs (Marsik et al., 2005). Therefore, some of biological activities of carvacrol, in particular its anti-inflammatory, analgesic and partially also antithrombotic effect might be caused by inhibition

of one or both COX enzymes, which is suggested by previous results concerning inhibition of COX-1 with carvacrol (Wagner et al., 1986). Nevertheless, no evidence concerning the interactions of COX-2 isoform with carvacrol *in vitro* has been published.

Hence the main goal of our work was testing its inhibitory potential on the biosynthesis of prostaglandin E_2 (PGE_2) from AA catalyzed by COX-2, which is the key enzyme of inflammatory processes in mammalian organisms.

MATERIAL AND METHODS

COX assay

Ovine COX-2 (specific activity 5,416 units/mg) and ovine COX-1 (specific activity 56,652 units/mg) purchased from Cayman Chemicals (Ann Arbor, MI, USA); [1-^{14}C]-AA (specific activity 48 mCi/mmol) obtained from PerkinElmer Life Sciences, Inc. (Boston, MA, USA); AA and prostaglandin E_2 (PGE_2) bought from Sigma (St. Louis, MO, USA) and finally, scintillation solution Monoflow^{TM} 2 was gained from National Diagnostics (Atlanta, GA, USA). Carvacrol (purity: 98%) was purchased from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade provided by either Sigma (St. Louis, MO, USA) or Pliva-Lachema (Neratovice, CR).

The slightly modified assay designed according to method previously published by Noreen et al. (1998) was used. In brief, COX-2 (0.88 μg protein) enzyme solution (10 μL) was activated with Tris–HCl (pH = 8.0) reaction mixture (60 μL) containing L-epinephrine (1 mM), reduced glutathione (1 nM), and hematin (1 μM) on ice for 5 min. Ten microlitres of tested solution of carvacrol dissolved in 10 μL of ethanol and [1-^{14}C] AA (50 nCi) were added into enzyme mixtures. After incubation at 37°C for 15 min, the reaction was terminated by adding 40 μL of 4 M formic acid. Resulting AA metabolic products were extracted by chloroform (200 μL), evaporated under a N_2 stream to dryness, dissolved in 10 μL of ethanol, and applied to HPLC analysis. The positive control was represented by indomethacin and N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulphonamide (NS-398); boiled enzymes were used as the negative control for all experiments. The corresponding volume of ethanol was used as a solvent control.

RP-HPLC analysis

The metabolic products were monitored according to method previously described by Marsik et al. (2005). Briefly, AA metabolites were separated onto a stainless steel column (4×250 mm) packed with C_{18} (Biospher PSI 120, 7 μm) stationary phase using a CostaMetric I pump (LDC Analytical, Riviera Beach, FL, USA), gradient keypad controller SCU-450 (LabAliance, PA, USA), and Rheodyne injector with 25 μL sample loop (Rheodyne, Cotati, CA, USA). Mobile phases A (0.02% HOAc in water) and B (0.02% HOAc in acetonitrile) were selected for gradient elution that started at 70/30 followed up to 0/100 (A:B; v/v) within 30 min. The injection volume 10 μL, flow rate 1 mL/min, and column temperature maintained at 25°C was set up. Product quantification was

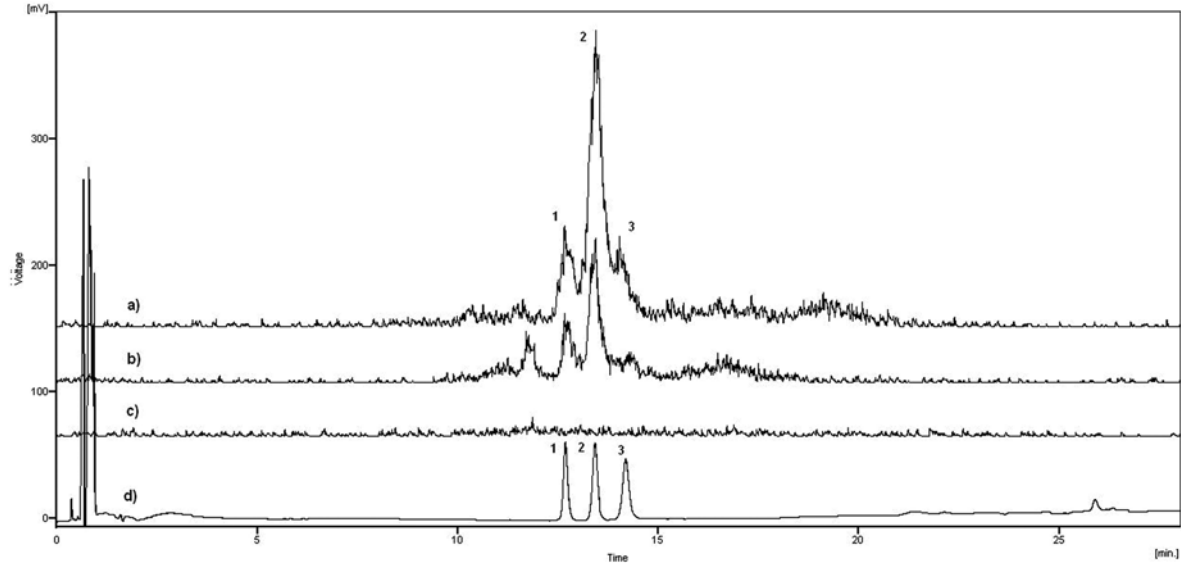

Fig. 2. Radiochromatogram of the COX-2 catalyzed reaction products in presence of 0 μM (a), 1 μM (b), 100 μM (c) concentration of carvacrol. Main prostaglandins (1 - $PGF_{2\alpha}$; 2 - PGE_2; 3 - PGD_2) analyzed under the same conditions are shown as UV chromatogram at λ=200 nm (d).

established using on-line liquid scintillation analysis equipped with flow-through β-RAM Detector (Model 3B, IN/US Systems, Tampa, FL, USA). The identification of PGE_2 was based on comparison of their retention times with those obtained from non-labelled authentic samples using a diode array detector (SpectraSystem UV6000LP, Thermo Finnigan, San Jose, CA, USA) at 198 nm and 200 nm (Fig. 2). The results were expressed as a percentage inhibition of PGE_2 formation against untreated samples (solvent blanks).

The IC_{50} values were determined by linear regression, where the independent variable was represented by decimal logarithm of applied concentration of inhibitor and dependent variable was inhibition of PGE_2 production. The relation between both variables is described by linear regression model: $y = a + \log(x) * b$. Therefore, the $\log(IC_{50})$ can be computed from formula: $\log(IC_{50}) = (y - a)/b$, where $y = 0.5$ and a and b are coefficients of regression equation. The IC_{50} value is antilogarithm of acquired figure.

Statistical analysis

The results were expressed as mean ± standard deviation of two independent experiments. Each experiment was performed in triplicates as minimum, statistical significance was calculated by Student's two tailed t-test. All the statistical analyses were carried out by STATISTICA 7.0 (StatSoft, Inc., OK, USA) software.

RESULTS AND DISCUSSION

Carvacrol exhibited a strong inhibitory activity against COX-2 *in vitro*. In comparison to untreated control, the production of PGE_2 was significantly reduced ($p<0.01$) at all tested concentrations. Observed inhibition was almost the same as the effect of reference inhibitors indomethacin and NS-398 (Table I) and the variability between tested substance and both positive controls had no statistical significance ($p<0.01$). Differences in IC_{50} values of all tested substances were negligible as well; they varied from 0.7 to 0.8 μM. In previous studies inhibitory potential of carvacrol against COX-1 isoform have been documented on enzyme isolated from ram vesicles (Wagner et al., 1986). This type of activity was also confirmed by our supplementary *in vitro* tests (Table II), where COX-1 was almost equally inhibited with carvacrol ($IC_{50} = 0.7$ μM) and indomethacin ($IC_{50} = 0.6$ μM). Aforesaid fact could be possible explanation of its biological effects linked to COX-1 such as suppression of AA-induced platelet aggregation (Enomoto et al., 2001). Moreover, our results showed that carvacrol is able effectively inhibit also inducible COX-2 isoform, which plays crucial role in process of inflammation (Warner and Mitchell, 2004), whereas its effect is comparable with selective and non-selective inhibitors used as positive controls in this study. Ability to inhibit both enzyme isoforms approximately at the same rate suggests it as a nonselective COX inhibitor such as classical non-steroidal anti-inflammatory drugs e.g. indomethacin or ibuprofen. A resembling effect of carvacrol has been reported *in vivo* in mice ear oedema model (Sosa et al., 2005), where the observed anti-inflammatory activity of carvacrol was comparable to that of the reference NSAIDs at the same level. Since COX-2 belongs to crucial factors involved in regulation of inflammatory process and presents target for the majority of antiphlogistic drugs, the observed ability of carvacrol to inhibit COX-2 *in vitro* could indicates one of possible mechanisms of its action *in vivo* (Sosa *et al.,* 2005; Botelho *et al.*, 2008).

In conclusion, this is the first work demonstrating inhibitory action of carvacrol against COX-2 isoform *in vitro*. Obtained results indicate that the inhibition of prostaglandin production mediated via AA pathway could be responsible for biological effects of this natural compound and suggest its possible contribution to anti-inflammatory activity of traditionally used carvacrol-rich plant drugs.

Table I. Inhibitory activity of carvacrol on production of PGE_2 catalyzed by COX-2 *in vitro*

Compound	Percentage of inhibition at different concentrations (μM)					IC_{50} (μM)
	0.1	1	10	50	100	
Carvacrol	27.7 ± 2.3	57.4 ± 4.2	72.6 ± 11.6	90.4 ± 8.1	100	0.8
Idomethacin	32.0 ± 3.5	50.7 ± 12.0	78.5 ± 3.4	83.5 ± 3.8	100	0.7
NS-398	22.2 ± 4.7	59.7 ± 7.3	77.9 ± 2.6	87.5 ± 1.6	100	0.8

Table II. Inhibitory activity of carvacrol on production of PGE_2 catalyzed by COX-1 *in vitro*

Compound	Percentage of inhibition at different concentrations (μM)					IC_{50} (μM)
	0.1	1	10	50	100	
Carvacrol	18.7 ± 1.4	60.9 ± 46.1	88.6 ± 2.3	94.9 ± 1.0	100	0.7
Idomethacin	27.0 ± 3.0	55.3 ± 1.2	85.6 ± 1.0	98.0 ± 2.0	100	0.6

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