Journal of Pharmaceutical and Biomedical Analysis 52 (2010) 701–706

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Liquid chromatography/tandem mass spectrometry study of anti-inflammatory activity of Plantain (*Plantago* L.) species

Ivana N. Beara[a,*], Dejan Z. Orčić[a], Marija M. Lesjak[a], Neda M. Mimica-Dukić[a], Biljana A. Peković[b], Mira R. Popović[a]

[a] *Department of Chemistry, Biochemistry and Environmental Protection, University of Novi Sad, Faculty of Sciences, Trg Dositeja Obradovića 3, Novi Sad, Serbia*
[b] *The Institute for Blood Transfusion of Vojvodina, Hajduk Veljkova 9a, Novi Sad, Serbia*

ARTICLE INFO

Article history:
Received 13 October 2009
Received in revised form 9 February 2010
Accepted 10 February 2010
Available online 18 February 2010

Keywords:
Cyclooxygenase
Lipoxygenase
Anti-inflammatory activity
Plantago
LC–MS/MS
Platelets

ABSTRACT

To evaluate anti-inflammatory activity of selected *Plantago* species (*P. lanceolata* L. and *P. major* L.) an optimized *in vitro* test for determination of cyclooxygenase-1 (COX-1) and 12-lipoxygenase (12-LOX) inhibition potency was undertaken. By using intact cell system (platelets) as a source of COX-1 and 12-LOX enzymes and highly sensitive and specific LC–MS/MS technique for detection of main arachidonic acid metabolites formed by COX-1 and 12-LOX, this test provides efficient method for evaluation of anti-inflammatory potential of plant extracts and isolated compounds. Our results validated the well-known COX-1 inhibitory activity of *P. lanceolata* and *P. major* methanol extracts (concentration required for 50% inhibition (IC_{50}) was 2.00 and 0.65 mg/ml, respectively). Furthermore, 12-LOX inhibitory activity of examined extracts was reported for the first time (IC_{50} = 0.75 and 1.73 mg/ml for *P. lanceolata* and *P. major*, respectively). Although renowned inhibitors, such as acetylsalicylic acid and quercetin showed higher activity, this study verifies *P. lanceolata* and *P. major* as considerable anti-inflammatory agents.

1. Introduction

Arachidonic acid, usually derived from the second position of phospholipids in the plasma membrane by the action of phospholipase A_2, is precursor to the eicosanoids, physiologically and pharmacologically active compounds, which biochemical effect is expressed as local hormones act, functioning through G-protein-linked receptors. Arachidonic acid can be converted to these products by three different pathways: cyclooxygenase, leading to the formation of prostanoids (prostaglandins and thromboxanes), lipooxygenase, where leukotrienes and certain mono-, di- and tri-hydroxy acids are synthesized, and epoxygenase pathway, which includes cytochrome P-450 and epoxides as final products. Accordingly, cyclooxygenases, lipooxygenases and epoxygenases are enzymes involved in these pathways [1].

Cyclooxygenase (COX), implicated in cyclooxygenase pathway, exists in two forms, named COX-1 and COX-2. COX-1 is expressed constitutively in different tissues, blood monocytes and platelets, and transforms arachidonic acid to prostanoids, which are involved in normal cellular homeostasis. In contrast, COX-2 may be induced by a series of pro-inflammatory stimuli and its role in the progress of inflammation, fever and pain has been known [2]. Furthermore, three types of lipoxygenases, termed 5-, 12- and 15-lipoxygenase are engaged in lipoxygenase pathway. Some compounds, like 12(*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid (12-HETE), a product of 12-lipoxygenase (12-LOX), has influence on the regulation of platelet aggregation, but is also found to be involved in the progression of several human diseases like various cancers [3], psoriasis [4] and rheumatoid arthritis [5]. Aforementioned enzymes can be found in different cell types. Thus, in human platelets, COX-1 and 12-LOX are the initial enzymes responsible for arachidonic acid metabolism leading to the formation of thromboxane B_2, 12-HHT (12(*S*)-hydroxy-(5*Z*,8*E*,10*E*)-heptadecatrienoic acid) and 12-HETE. Other minor metabolites such as prostaglandins $F_{2\alpha}$, E_2 and D_2 are also formed [1]. Therefore, the relationship between these enzymes and their potential inhibitors can be established by quantifying COX-1 and 12-LOX metabolites 12-HHT and 12-HETE, respectively. Several assays [6–13] have been founded on this principle, but they differ significantly in source of platelets (intact human, rat or rabbit platelets), addition of exogenous arachidonic acid or inflammation induction agent (calcium ionophore A23187) and technique used for quantification of metabolites (high performance liquid chromatography (HPLC) with UV or radiochemical detection, preparative thin layer chromatography (TLC) with subsequent radioactive counting or enzyme immunoassay (EIA)).

Abbreviations: 12-HETE, 12(*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid; 12-HHT, 12(*S*)-hydroxy-(5*Z*,8*E*,10*E*)-heptadecatrienoic acid; 12-LOX, 12-lipoxygenase; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; MRM, multiple reaction monitoring; MS2SIM, single-stage mass spectrometry.

* Corresponding author. Tel.: +381 21 4852755; fax: +381 21 454065.
E-mail address: ivanak@eunet.rs (I.N. Beara).

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.02.014

Since our research is focused on biological activity of plant extracts, the aim of this study was to evaluate and optimize an *in vitro* assay for anti-inflammatory activity, that can be easily used to determine COX-1 and 12-LOX inhibitory potential of plant extracts or natural products, using LC–MS/MS technique for quantification of metabolites and human platelets as a source of enzymes. Furthermore, the advantage of applied experiment is avoidance of undesirable *in vivo* tests on experimental animals, since test commonly used to detect anti-inflammatory activity is carrageenan induced paw edema in rats [14,15]. Certainly, the exact anti-inflammatory activity can be validated only through *in vivo* tests, but creating *in vitro* assays in which physiological conditions similar to *in vivo* assays are used, can provide valuable information about inhibitory potential of tested compounds. However, the selectivity of COX-1/COX-2 inhibitors is one of the main targets of the researches concerning overall COX activity. Nevertheless, COX-2 inhibition can be determined in numerous assays, where various experimental conditions are applied. Thus, COX-2 can be of animal or human origin, native or recombinant, purified, in microsomal preparations or in different cell types where COX-2 is present, such as macrophages, monocytes, chondrocytes, synoviocytes or cell lines (i.e., osteosarcoma cell line 143.98.2, human endothelial cell line HUV-EC-C). Additionally, induction agents can also vary (bacterial lipopolysaccharide, various cytokines, such as interleukin-1 or tumor necrosis factor), as well as technique used for detection of COX-2 metabolites derived from endogenous or exogenous arachidonic acid [7]. Commonly, activities of COX-1 and COX-2 are determined in different cell type assays [7], even their activity can be measured in whole-blood or monocyte assays, where both isoenzymes are present [11].

Methanol extracts of *Plantago* species (*P. lanceolata* L. and *P. major* L.) were chosen to be examined towards anti-inflammatory potential by means of COX-1 and 12-LOX inhibition, due to their known remedial properties [16,17] and content of natural products such as phenolics and flavonoid compounds [17–19] which are highly potent 12-LOX inhibitors [20]. Also, some papers witness about anti-inflammatory potential of these two species [14,21,22] and their active compounds [23,24]. However, according to our knowledge, they have never been examined in assays similar to herein reported and there is no previous report on 12-LOX inhibitory potential of these two species.

2. Materials and methods

2.1. Chemicals

Following reagents were purchased from Sigma–Aldrich Chem, Steinheim, Germany: acetylsalicylic acid (aspirin), calcium ionophore A23187 (calcimycin), prostaglandin B_2 (PGB_2), quercetin, 12(*S*)-hydroxy-(5*Z*,8*E*,10*E*)-heptadecatrienoic acid (12-HHT), 12(*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid (12-HETE). Other reagents used in this study were of analytical grade.

Platelet concentrate was kindly provided by The Institute for Blood Transfusion of Vojvodina, Novi Sad, Serbia.

2.2. Plant material and extract preparation

The aerial parts of *P. lanceolata* L. and *P. major* L. were collected in June 2008 from the mountain of Fruška Gora, Serbia. The voucher specimens (*P. lanceolata*, No 2-1829; *P. major* No 2-1830) were prepared and identified by Goran Anačkov, PhD, and deposited at the Herbarium of Department of Biology and Ecology (BUNS Herbarium).

Air-dried and smoothly grounded herbal samples weighing 30 g were extracted by maceration with 80% aqueous methanol during 72 h at room temperature. After filtration, solvent was evaporated *in vacuo* at 45 °C and crude residue was dissolved in hot, distilled water (1 g/ml). With the aim to remove non-polar compounds, the extracts were washed exhaustively with petrol ether (fraction 40–60 °C) and concentrated to dryness under vacuum, yielding 10.1 and 11.3% for *P. lanceolata* and *P. major* extracts, respectively. Dried extracts were dissolved in DMSO to obtain 200 mg/ml stock solutions.

2.3. COX-1 and 12-LOX assay

In vitro COX-1 and 12-LOX assay was undertaken according to modified method of Safayhi et al. [6]. An aliquot of human platelet concentrate, viable, but outdated for medical treatment, which contains 4×10^8 cells was suspended in buffer (0.137 mol/l NaCl, 2.7 mmol/l KCl, 2.0 mmol/l KH_2PO_4, 5.0 mmol/l Na_2HPO_4 and 5.0 mmol/l glucose, pH 7.2) to obtain final volume of 2 ml. This mixture was slowly stirred at 37 °C for 5 min. Subsequently, 0.1 ml of extracts or standard compounds solutions in DMSO (concentration ranging from 10.0 to 200.0, 0.156 to 5.0 and 0.01 to 0.6 mg/ml for extracts, quercetin and aspirin, respectively) and 0.1 ml of calcimycin (Calcium Ionophore A23187, 125 μmol/l in DMSO) were added and incubated for 2 min at 37 °C, with moderate shaking. The exact amount of extract in control and calcimycin in blank probe were substituted with solvent (DMSO). Thereafter, 0.3 ml of $CaCl_2$ aqueous solution (16.7 mmol/l), substituted with water in blank probe, was added and the mixture was incubated for a further 5 min at 37 °C with shaking. Acidification with cold 1% aqueous formic acid (5.8 ml) to pH 3 terminated the reaction. If gel formation was occurred, vortexing was applied before mixing with the acid. Internal standard prostaglandin B_2 (50 μl of 6 μg/ml solution in DMSO) was added and extraction of products was done with mixture of chloroform and methanol (1:1, 8.0 ml) during vigorous vortexing for 15 min. After centrifugation at $7012 \times g$ for 15 min at 4 °C, organic layer was separated, evaporated to dryness, dissolved in methanol (0.5 ml), filtered and used for further LC–MS/MS analysis. All samples and control were made in triplicate.

2.4. LC–MS/MS analysis

The Agilent 1200 series liquid chromatograph, consisting of vacuum degasser, binary pump, autosampler and thermostated column compartment was used for separation of analytes, whose detection was carried out by means of Agilent series 6410B triple-quad mass spectrometer with electrospray ionization (ESI). MassHunter ver. B.01.03. software (Agilent Technologies) was used for instruments control and data analysis. The injection volume for all samples was 5 μl. The separation was achieved using a Zorbax SB-C18 30 mm × 2.1 mm × 3.5 μm (Agilent Technologies) reversed-phase column held at 65 °C. The binary mobile phase consisted of 0.6% aqueous acetic acid (A) and methanol (B) and was delivered at a flow rate of 1 ml/min. Components were eluted in gradient mode, starting with 65% B, reaching 100% B in 2 min and holding until 3.5 min, with post-time of 3 min, and the entire eluate was transferred to mass spectrometer, without flow splitting. ESI parameters were as follows: drying gas (N2) temperature 350 °C, flow 9 l/min, nebulizer gas pressure 40 psi, capillary voltage 4 kV. Compounds were quantified in negative ionization multiple reactions monitoring (MRM) mode, with time segments defined as follows: 0.0–0.9 min PGB_2 (fragmentor 120 V, precursor ion $m/z = 333$, collision energy 13 V, product ion $m/z = 315$) and 12-HHT (fragmentor 120 V, precursor ion $m/z = 279$, collision energy 5 V, product ion $m/z = 261$), 0.9–3.5 min 12-HETE (fragmentor 120 V, precursor ion