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

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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.
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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.

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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

$m/z = 319$, collision energy 7 V, product ion $m/z = 301$). In order to avoid the need for calibration curves for each compound, and to simultaneously compensate the matrix effects, the internal standard approach was chosen. Analyte-to-internal standard (PGB_2) peak area ratio was used for all calculations.

2.5. Statistical analysis

Percent of inhibition achieved by different concentrations of extracts was calculated by the following equation in performed COX-1 and 12-LOX assay: $I(\%) = 100 \times (R_0 - R)/R_0$, where R_0 and R were response ratios (metabolite peak area/internal standard peak area) in the control reaction and in the examined samples, respectively. Both R and R_0 were corrected for the value of blank probe. Corresponding inhibition-concentration curves were drawn using Origin software, version 8.0 and IC_{50} values (concentration of extract that inhibited COX-1 and 12-LOX by 50%) were determined. All of the results were expressed as mean ± SD of three different trials. A comparison of the group means and the significance between the groups were verified by one-way ANOVA. Statistical significance was set at $p < 0.05$.

3. Results and discussion

The application of several COX-1 and 12-LOX *in vitro* assays is known and they were applied for evaluation of anti-inflammatory potential of pure natural or synthetic compounds [6,8,9,12,13,25,26] as well as plant extracts [10]. In most of these assays COX-1 and 12-LOX activity is measured by mean of their metabolites concentration, 12-HHT and 12-HETE, respectively, even if 12-HHT formation does not entirely correlated with COX-1 inhibition because thromboxane synthase is also involved in 12-HHT formation. Moreover, most of these tests include usage of the isolated enzyme instead of intact cells, primarily platelets, where both COX-1 and 12-LOX are present [20,25,26]. Furthermore, it is also known that phenolic compounds from plant extracts may act as electron donors, being co-oxidized in the hydroperoxidase step of prostaglandin synthesis, and preferentially effect on the inactivation, instead on the inhibition of cyclooxygenase. This process can be avoided by using adrenalin as cofactor or intact cell system, since normal cell posses a high peroxidase potential [27]. So, platelets were the most suitable cell system for testing inhibitory activity of our plant extract, because they can provide physiological cell conditions and possibility to examine inhibition of both enzymes at the same time. Considering experimental procedure, we used human platelet concentrate, which was out of date for medicinal use. Namely, human platelet concentrate is used in treatment of patients with thrombocytopenia or an abnormality of platelet function, patients with bleeding, oncology patients, during open-heart surgeries, etc. This concentrate, older than five days, should not be used for therapies. Our tests showed that these platelets were viable up to eight days with no significant change in COX-1 and 12-LOX activity, and only decrease of platelet number was observed during that period. We also found that in some batches of platelet concentrate initial content of targeted metabolites was present, probably caused by some donor's unnoticed inflammation process. Thus, a blank probe was done for all probes, including control, and final content of metabolites was corrected for this value. Also, tests with plant extracts have some particular features, like formation of gel after the last incubation, mostly when higher concentrations of extracts were used. This can be explained by presence of polysaccharides in *Plantago* species [17,28], which can effect this process.

Considering determination of formed metabolites, our research was focused on LC–MS/MS technique in order to attain high sensitive and specific method with short analysis time. In previous papers [6,29,30], isocratic elution mode was mostly used to separate eicosanoids, leading to long run times (up to 60 min) and accumulation of matrix components onto column. Even some of methods with gradient elution [31] are still time-consuming, resulting in HPLC determination being the rate-limiting step of the assay. In this work, an attempt was made to replace the isocratic elution with more efficient gradient mode, thus enabling the high-throughput analysis of large batches of samples.

Mobile phase based on water and methanol or acetonitrile in various ratios, and acidified with 0.01–0.6% (v/v) of formic or acetic acid was used in previous investigations [6,29–31]. At early stage of method development, a mobile phase consisting of 0.6% (v/v) aqueous acetic acid (component A) and methanol (component B), volatile and ESI-compatible, was chosen [6]. In order to achieve satisfactory resolution while keeping the run time as short as possible, a number of rapid resolution reversed-phase columns, gradient profiles, temperatures and flows were tested on standards and representative samples. Zorbax SB-C18 100 mm × 2.1 mm × 1.8 μm (Agilent Technologies) at 45 °C, with mobile phase delivered at flow of 0.4 ml/min in gradient: 0 min 67% B, 10–15 min 80% B, gave acceptable resolution, but with long run time (PGB_2 eluting at 4.09 min, 12-HETE at 9.88 min, and total analysis time of about 20 min) and bad peak symmetry (unacceptable tailing). The application of Zorbax SB-C18 Plus 30 mm × 2.1 mm × 1.8 μm at 65 °C, and faster gradient (0 min 65% B, 4–10 min 95% B) resulted in significantly shorter analysis time (PGB_2 eluting at 2.27 min and 12-HETE at 5.26 min). Further attempts to shorten the analysis by gradient tuning (0 min 65% B, 2–7 min 100% B, and 0 min 75% B, 2–7 min 100% B) resulted in worsened peak shapes without significant time reduction. Finally, the Zorbax SB-C18 30 mm × 2.1 mm × 3.5 μm at 65 °C, with mobile phase delivered at flow of 1 ml/min in gradient: 0 min 65% B, 2–3.5 min 80% B, with post-time of 3 min, provided satisfactory resolution and peak shape within less than 1.5 min (retention times: 0.31 min PGB_2, 0.56 min 12-HHT, 0.95 min 12-HETE), while allowing all matrix components to elute. The injection volume was set to 5 μl, with higher volumes negatively affecting the peak shape (since samples were dissolved in stronger solvent than the starting mobile phase), and lower volumes decreasing the sensitivity while not improving the peak shape.

Tandem mass spectrometry was chosen as a detection technique, since it provides far better sensitivity and selectivity than traditional UV detectors and does not depend on presence of chromophores.

The standards were analyzed in both positive and negative mode, and the negative ionization is chosen based on the fact it resulted in $[M-H]^-$ (i.e. carboxylate anion) as only pseudomolecular ion (unlike the positive mode, which resulted in formation of significant amounts of sodium and potassium adducts, as well as dehydrated protonated molecule, and only a minute amount of $[M+H]^+$), thus providing better sensitivity and robustness. Also, it provided simpler fragmentation and lower noise level.

In order to gain maximum sensitivity for the detection of eicosanoids at trace levels, both fragmentor voltage and collision energy were optimized. In order to determine the fragmentor voltage that provides complete ion desolvation yet does not result in premature fragmentation, the standard mix was analyzed in MS2SIM mode (set to monitor $[M-H]^-$ ions) using fragmentor voltages of 60, 80, 100, 120 and 150 V, and peak areas were measured. The optimal value of voltage is determined for each analyte as the one giving the maximal signal, and is approximately 120 V for all compounds (Fig. 1). In the next phase, in order to obtain fragmentation spectra, all standards were analyzed in Product Ion Scan mode, with collision energies of 0, 10, and 20 V. Several abundant fragment ions were generated for each compound, of which the high-mass $[M-H-H_2O]^-$ ions were selected as target ions.

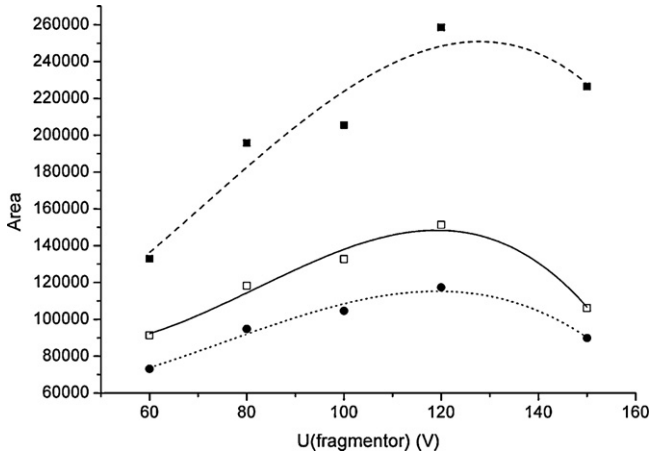

Fig. 1. Optimization of fragmentor voltage (peak area vs. voltage); solid line-HHT, dashed-PGB_2, dotted-HETE.

The final phase was optimization of collision cell voltage, in order to obtain the highest ion yield and thus the highest sensitivity. Standards were analyzed in MRM (multiple reactions monitoring) mode for $[M-H]^- \rightarrow [M-H-H_2O]^-$ transition with collision energies varied 0–20 V, in 5 V increments. The optimal voltages were determined to be 13 V for PGB_2, 5 V for 12-HHT, and 7 V for 12-HETE (Fig. 2). An example of typical chromatogram of test-mixture, containing both metabolites (12-HHT and 12-HETE) and internal standard PGB_2 is presented in Fig. 3.

To evaluate the method performance, several parameters were determined. Standard calibration curves SC (in methanol) and matrix-matched calibration curve MMC (matrix blank – extract of non-activated platelets – spiked with reference standards) were prepared for concentration ranges 67–1333 ng/ml (12-HETE) and 50–1000 ng/ml (12-HHT). Unweighted linear regression was chosen for all $A/A_{ISTD} = f(C_{analyte})$ curves since it provided the best random distribution of errors in residual plot. The regression equations and correlation coefficients were as follows: for 12-HETE/SC $y = 0.00414x - 0.1235$ ($r^2 = 0.987$), for 12-HETE/MSC $y = 0.00429x - 0.1648$ ($r^2 = 0.994$), for 12-HHT/SC $y = 0.00227x - 0.0189$ ($r^2 = 0.994$) and for 12-HHT/MMC $y = 0.00213x - 0.03742$ ($r^2 = 0.998$). Matrix effects were calculated as signal suppression/enhancement (SSE), i.e. slope ratio for MMC and SC, which equals 104% for 12-HETE and 94% for 12-HHT. For each level of MMC, signal-to-noise ratio (with signal defined as peak area) is calculated and plotted vs. concentration. Limit of quantitation (LoQ) is determined as concentration that results in peaks with S/N ratio of 10, and is estimated to be 0.15 μg/ml for 12-HETE and 0.10 μg/ml for 12-HHT, i.e. 0.75 and 0.5 ng injected on column, respectively. These values correspond to eicosanoid concentrations in highly inhibited probes ($I > 80$–85%) and do not hinder the determination of IC_{50}. Inter-assay precision is calculated as a pooled variance of IC_{50} values for 8 *Plantago* extract samples (for each sample, three 6-points inhibition curves were prepared and IC_{50} determined), and equals 8.6% and 9.3% for 12-HETE and 12-HHT, respectively.

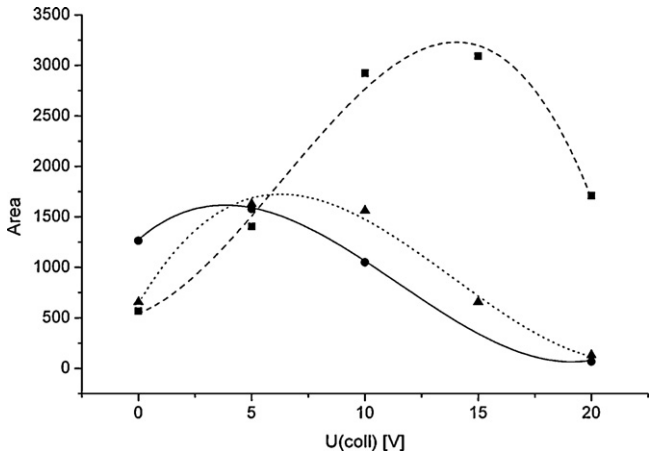

Fig. 2. Optimization of collision cell voltage (peak area vs. voltage); solid line 12-HHT, dashed-PGB_2, dotted 12-HETE.

Finally, COX-1 and 12-LOX inhibitory activities of *P. lanceolata* and *P. major* extracts, as well of aspirin and quercetin, well-known inhibitors of COX-1 [1] and 12-LOX [20], respectively, were determined (Table 1). These activities were concentration dependent, as it is shown in Figs. 4 and 5. Accordingly, both extracts showed inhibitory potential towards COX-1 ($IC_{50} = 2.0$ and 0.65 mg/ml for *P. lanceolata* and *P. major*, respectively), even it was significantly lower than activity of greatly potent inhibitor aspirin ($IC_{50} = 0.001$ mg/ml). Also, 12-LOX inhibition was expressed by both *P. lanceolata* and *P. major* extracts ($IC_{50} = 0.75$ and 1.73 mg/ml, respectively), but at higher IC_{50} than IC_{50} of quercetin (0.084 mg/ml).

In conclusion, an optimized *in vitro* test for anti-inflammatory activity by means of COX-1 and 12-LOX inhibition was presented in this paper. Using intact cell system (platelets) as 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 them, this test provides efficient method

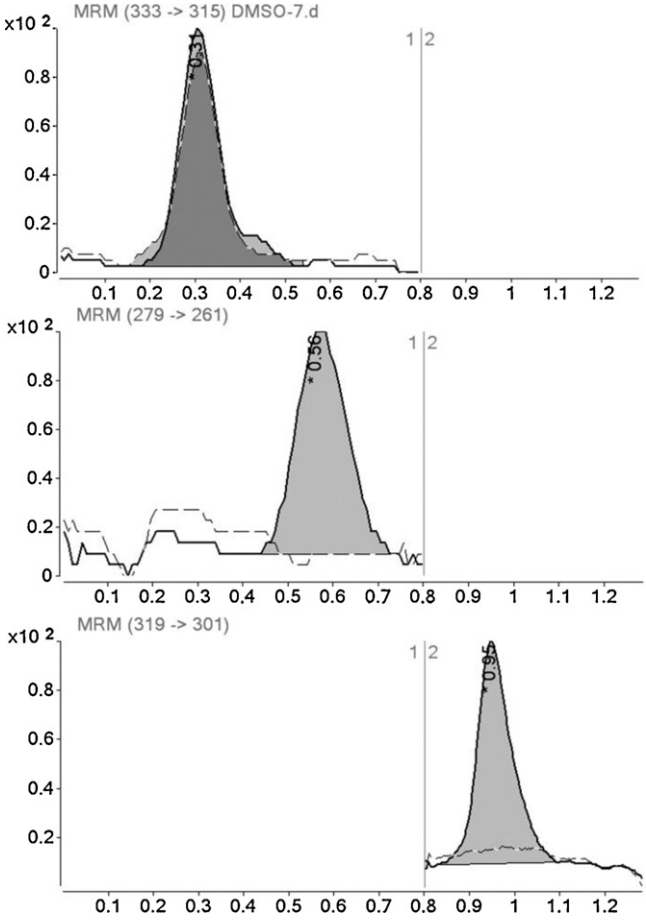

Fig. 3. Typical chromatogram of test sample (platelets treated with plant extract $C = 25$ mg/ml) and blank (solid and dashed lines, respectively), showing resolution of arachidonic acid metabolites 12-HHT and 12-HETE and internal standard PGB_2 (retention times: 0.31 min PGB_2, 0.56 min 12-HHT, 0.95 min 12-HETE).

Table 1
Determined IC_{50} values[a] for COX-1 and 12-LOX inhibitory activities of examined extracts and standards.

	IC_{50} values[a] (mg/ml)	
	COX-1 inhibition	12-LOX inhibition
Extract		
P. lanceolata	2.00 ± 0.34 c	0.75 ± 0.084 b
P. major	0.65 ± 0.04 b	1.73 ± 0.18 c
Standard		
Aspirin	0.001 ± 0.00008 a	na[b]
Quercetin	na[b]	0.084 ± 0.007 a

[a] Values are means ± SD of three measurements. Means within each column with different letters (a–c) differ significantly ($p < 0.05$).
[b] na—not active in applied concentration range.

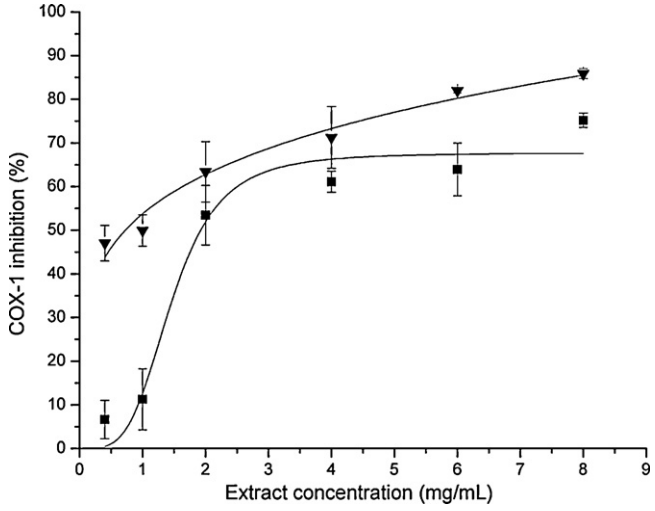

Fig. 4. Concentration dependent inhibition of COX-1 by *P. lanceolata* (■) and *P. major* (▼) extracts. Data are means ± standard deviation of three determination.

to evaluate anti-inflammatory potential of plant extracts and isolated compounds. Furthermore, methanol extracts of *P. major* and *P. lanceolata* showed COX-1, as well as 12-LOX inhibitory activity, which was reported for the first time. Overall, these results undoubtedly suggest need for further examination of other *Plantago* species as potential anti-inflammatory agents.

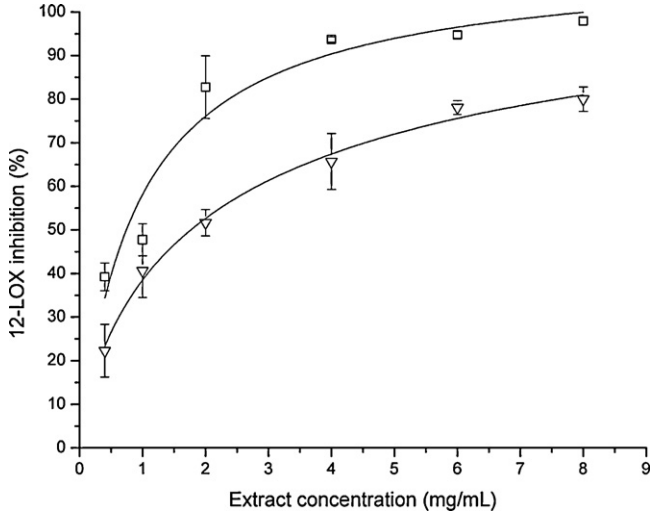

Fig. 5. Concentration dependent inhibition of 12-LOX by *P. lanceolata* (□) and *P. major* (▽) extracts. Data are means ± standard deviation of three determination.

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