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Comparative analysis of phenolic profile, antioxidant, anti-inflammatory and cytotoxic activity of two closely-related Plantain species: *Plantago altissima* L. and *Plantago lanceolata* L.

Ivana N. Beara [a,*], Marija M. Lesjak [a], Dejan Z. Orčić [a], Nataša Đ. Simin [a], Dragana D. Četojević-Simin [b], Biljana N. Božin [c], Neda M. Mimica-Dukić [a]

[a] Department of Chemistry, Biochemistry and Environmental Protection, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovića 3, 21000 Novi Sad, Serbia
[b] Oncology Institute of Vojvodina, Institutski put 4, 21204 Sremska Kamenica, Serbia
[c] Department of Pharmacy, Faculty of Medicine, Hajduk Veljkova 3, 21000 Novi Sad, Serbia

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ABSTRACT

The present study was designed to define the phenolic profile, antioxidant, anti-inflammatory and cytotoxic activity of *Plantago altissima* L., which has never been studied before and to compare it with closely-related, renowned, well-studied *Plantago lanceolata* L. The presence and content of 44 phenolics in methanol extracts were studied using LC–MS/MS. A similar qualitative composition including dominant compounds as *p*-hydroxybenzoic, vanillic, gallic and chlorogenic acid, besides apigenin, luteolin and luteolin-7-*O*-glucoside was found between both extracts. Antioxidant activity of extracts was determined using several assays. All results of these tests were comparable to butylated hydroxytoluene, a well-known synthetic antioxidant. Anti-inflammatory potential was studied by means of cyclooxygenase-1 (COX-1) and 12-lipoxygenase (12-LOX) inhibitory activity. Activity of *P. altissima* towards COX-1/12-LOX inhibition (IC_{50} = 4.4 and 3.6 mg/mL, respectively) was inferior to activity of *P. lanceolata* (IC_{50} = 2.0 and 0.8 mg/mL, respectively). Treatment of four cell lines resulted in a considerable dose-dependent inhibition of cell growth, where *P. lanceolata* exerted a stronger effect (IC_{50} = 172.3, 142.8, 405.5 and 551.7 μg/mL for HeLa, MCF7, HT-29 and MRC-5 cell lines, respectively). To conclude, *P. altissima* showed certain bio-potential, but was clearly inferior to *P. lanceolata*.

1. Introduction

The largest genus of the cosmopolitan family, Plantaginaceae is the genus *Plantago* L. and it comprises about 275 species. Extensive traditional use and modern medicinal application of several *Plantago* species is a consequence of their remarkable variety of curative properties: astringent, styptic, antimicrobial, expectorant, diuretic and demulcent (Huang et al., 2009; Jančić, 2002; Samuelsen, 2000; Tucakov, 1997; Wichtl, 1994; World Health Organisation, 1999). In addition, some studies confirm that certain *Plantago* species reveal considerable bioactivity, such as cytotoxic effects on cancer cell lines (Gálvez, Martín-Cordero, López-Lázaro, Cortés, & Ayuso, 2003), anti-inflammatory (Samuelsen, 2000), immunoregulatory (Huang et al., 2009), antioxidant (Gálvez et al., 2003; Heimler, Isolani, Vignolini, Tombelli, & Romani, 2007) and antispasmodic effects (Fleer & Verspohl, 2007). Furthermore, some *Plantago* species are also included in the diet. Usually, they are consumed as fresh salads, soups, side dish or they can be used as herbal tea. The seeds of some species can be cooked and used as starch, or can be ground into a powder and added to flour when making bread and cakes (Heimler et al., 2007; Jančić, 2002; Tucakov, 1997; Tull, 2003). Although previous studies of some plantains indicate the high potential of these species as a source of biologically active compounds and healing agents, the majority of *Plantago* representatives have not been described thus far in terms of phytochemical composition and biological activity. Therefore, in this study, we assessed phenolic profile, antioxidant, anti-inflammatory and anti-cancer activity of the methanolic extracts of *Plantago altissima* L. and *Plantago lanceolata* L. To the best of our knowledge, *P. altissima* has not been examined with respect to any biological activity, but several phenolic compounds, such as flavonoids (Grubešić & Vladimir-Knežević, 2004) have been described previously, but other data is

Abbreviations: COX-1, cyclooxygenase-1; 12-LOX, 12-lipoxygenase; 12-HHT, 12(*S*)-hydroxy-(5*Z*,8*E*,10*E*)-heptadecatrienoic acid; 12-HETE, 12(*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid; SRB, sulforhodamine B; dw, dry weight.

* Corresponding author. Tel.: +381 21 4852755; fax: +381 21 454065.
E-mail address: ivana.beara@dh.uns.ac.rs (I.N. Beara).

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limited. *P. altissima* and *P. lanceolata*, a well-known remedy and food, belong to the same subgenus, *Albicans* (Rønsted, Franzyk, Mølgaard, Jaroszewski, & Jensen, 2003) and yet, they can be easily mistaken for each other due to their minor morphological differences, mostly in the plants' height (Tutin et al., 1976). Consequently, the aim of this study was not only to examine the unexplored *P. altissima*, but also to compare its phenolic profile and biological activities with renowned *P. lanceolata*. Therefore, an LC–MS/MS technique was applied to evaluate quantitative content of numerous phenolics, including 14 phenolic acids, 25 flavonoids, 3 coumarins and 2 lignans. The antioxidant potential of extracts was determined using various assays related to free radical (DPPH$^{\bullet}$), reactive oxygen (HO$^{\bullet}$, $O_2^{\bullet-}$) and reactive nitrogen species (NO$^{\bullet}$) scavenging ability, in addition to the potential of lipid peroxidation (LP) inhibition and reducing power (FRAP assay). Anti-inflammatory activity was determined by means of inhibition of cyclooxygenase-1 (COX-1) and 12-lipoxygenase (12-LOX) enzymes, which are involved in the metabolism of arachidonic acid. To establish the anti-inflammatory activity of extracts, LC–MS/MS technique was used for quantification of COX-1 and 12-LOX metabolites 12(*S*)-hydroxy-(5*Z*,8*E*,10*E*)-heptadecatrienoic acid (12-HHT) and 12(*S*)-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-eicosatetraenoic acid (12-HETE), respectively. Finally, cell growth activity of extracts was evaluated *in vitro* in a panel of four human cell lines: HeLa (cervix epitheloid carcinoma), MCF7 (breast adenocarcinoma), HT-29 (colon adenocarinoma) and MRC-5 (human fetal lung). Presented results were obtained using SRB (Sulforhodamine B) assay (Skehan et al., 1990).

2. Material and methods

2.1. Chemicals and reagents

All standards of phenolic compounds were purchased from Sigma–Aldrich Chem (Steinheim, Germany), Fluka Chemie GmbH (Buchs, Switzerland) or from ChromaDex (Santa Ana, USA). Reagents used for antioxidant and anti-inflammatory assays were purchased from suppliers listed in Beara et al. (2009), Beara et al. (2010) and Lesjak et al. (2011). DMEM (Dulbecco's modified Eagle's medium) and FCS (fetal calf serum) were obtained from PAA Laboratories GmbH (Pashing, Austria). Penicillin and streptomycin were purchased from Galenika (Belgrade, Serbia). All 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. altissima* were collected in June 2009 in Novi Sad, Serbia and *P. lanceolata* in June 2009 from the mountain of Fruška Gora, Serbia. The voucher specimens (*P. altissima*, No. 2-1804; *P. lanceolata*, No. 2-1829) were prepared and identified by Goran Anačkov, PhD, and deposited at the Herbarium of Department of Biology and Ecology (BUNS Herbarium), Faculty of Sciences, University of Novi Sad, Serbia.

Air-dried and smoothly grounded herbal samples weighing 30 g were extracted by maceration with 300 mL methanol/water (4:1) 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 of removing non-polar compounds, the extracts were washed exhaustively with petroleum ether (fraction 40–60 °C) and concentrated to dryness under vacuum, yielding 4.71 g and 3.03 g for *P. altissima* and *P. lanceolata* extracts, respectively. Dried extracts were dissolved in methanol/water (4:1) to obtain 200 mg/mL or in dimethyl sulfoxide (DMSO) to obtain 200 and 100 mg/mL stock solutions for evaluation of the antioxidant, anti-inflammatory and cytotoxic activity, respectively. Also, dried extracts were dissolved in a mixture of formic acid/water (1:199) and methanol (in ratio of 7:3) for HPLC–MS analysis to obtain 0.2 mg/mL stock solutions.

2.3. LC–MS/MS analysis of the selected phenolics

The Agilent 1200 series liquid chromatograph, consisting of vacuum degasser, binary pump, autosampler and thermostated column compartment was used for separation of all analytes, whose detection was carried out by means of Agilent series 6410B triple-quadrupole mass spectrometer with electrospray ionization (ESI). MassHunter ver. B.03.01. software (Agilent Technologies) was used for instruments control and data analysis.

The injection volume for all samples was 5 μL. The binary mobile phase consisted of formic acid/water (1:1999; A) and methanol (B) and was delivered at a flow rate of 1 mL/min. Gradient elution was performed using the following solvent gradient: starting with 70% A/30% B, reaching 30% A/70% B in 6.00 min, then 100% B at 9.00 min, holding until 12.00 min, with post-time of 3 min. The separation was achieved using a Zorbax Eclipse XDB-C18 RR 4.6 mm × 50 mm × 1.8 μm (Agilent Technologies) reversed-phase column held at 45 °C. The eluate was forwarded, without flow splitting, into an ESI ion source with following settings: drying gas (N_2) temperature, 350 °C; flow, 9 L/min; nebulizer gas pressure, 40 psi; capillary voltage, 4 kV, negative polarity. All compounds were quantified in dynamic MRM mode (multiple reaction monitoring mode). Compound-specific, optimized MS/MS parameters are given in Table 1.

Extracts used for LC–MS/MS quantification were dissolved in starting mobile phase solvent to the concentration of 0.2 mg/mL. All used standards were dissolved in DMSO to prepare stock solutions of 10 mg/mL. The mix of stock solutions was prepared, with concentration of each compound being 100 μg/mL. The mix was subsequently serially diluted, giving working standard solutions with concentration ranging from 25.0 to 0.0015 μg/mL, which were used for construction of the calibration curves. Concentrations of standard compounds in extracts were determined from the peak areas by using the equation for linear regression obtained from the calibration curves (R^2 gt; 0.995).

2.4. Antioxidant activity

Assays considering reduction of DPPH$^{\bullet}$ radical, hydroxyl-radical, superoxide anion and NO scavenger capacity, lipid peroxidation and reducing power (FRAP assay) of plant extracts were performed according to the earlier reported procedures (Beara et al., 2009; Lesjak et al., 2011).

2.5. COX-1 and 12-LOX assay

Ex vivo COX-1 and 12-LOX assay was undertaken according to prior reported method (Beara et al., 2010).

2.6. Cell growth activity

2.6.1. Grow and culture of the cell lines

For the estimation of cell growth effects, human cell lines HeLa (cervix epitheloid carcinoma; ECACC No. 93021013), MCF7 (breast adenocarcinoma; ECACC No. 86012803), HT-29 (colon adenocarcinoma; ECACC No. 91072201) and MRC-5 (human fetal lung; ECACC No. 84101801) were used. Cell lines were grown in DMEM with 45 mg/mL glucose, supplemented with 100 μL/mL heat inactivated FCS, 100 IU/mL of penicillin and 100 μg/mL of streptomycin. All investigated cell lines grow attached to the surface. They were cultured in 25 cm^2 flasks at 37 °C in atmosphere of 5% CO_2 and 100%