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THE QUALITATIVE COMPOSITION AND COMPARATIVE BIOLOGICAL POTENTIAL OF *Lunaria annua* L. (Brassicaceae) EXTRACTS

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ABSTRACT. *Lunaria annua* L. (Brassicaceae) in contrast to its name is a biennial plant native to the Balkans and southwest Asia. This research aimed to evaluate the composition of phenolic compounds (total phenolics, phenolic acids, flavonoids, flavonols, and gallotannins) of methanolic extracts of the aboveground parts (LAA) and roots (LAR) of *L. annua*, as well as antioxidant, antigenotoxic and anti-inflammatory properties of the extracts *in vitro*. LAA was richer in all groups of phenolics in comparison to LAR. LAA also had higher antioxidant potential except for the inhibition of lipid peroxidation. LAA and LAR showed inhibition of cyclooxygenase-1 and -2 (COX-1 and -2) enzymatic activities. The anti-inflammatory potential of *L. annua* extracts was outstanding, especially regarding COX-2 inhibition. Presented findings can lead to the isolation of compounds in *L. annua* responsible for this plant's remarkable anti-inflammatory properties.

Keywords: *Lunaria annua* L., annual honesty, Brassicaceae, phenolic compounds, antioxidants, anti-inflammatory activity, cyclooxygenase.

INTRODUCTION

Lunaria annua L., also known as Annual Honesty, Moonwort, Money Plant, Silver Dollar, or Satin Flower, is a biennial cruciferous plant from the genus *Lunaria*, family Brassicaceae. Genus *Lunaria* consists of five species, of which *L. annua* is the most abundant. *L. annua* is native to southeastern Europe and western Asia and it can be found in semi-shady places near roads. Due to its high ornamental value, it is also grown in flower gardens. *L. annua* (Fig. 1) grows up to 80–100 cm and in the late autumn, the plants shed the seeds and, after the germination in spring, they stay in vegetative mode until next year. In the spring of the second year, the plants enter the reproductive stage and flower during April and May. The flowers are mainly violet and purple, but *Lunaria* variations with white flowers can also be found. The formation of pale white, oval-shaped pods containing flat seeds, which have

ornamental value, resembling coins, follows the flowers (MASTEBROEK and MARVIN, 2000; MARTIN *et al*., 2005; DODOS *et al*., 2015). The seeds contain a high amount of oil (30–35%) that is consisted mainly of long-chain fatty acids of which erucic acid (C22:1, 44%) and nervonic acid (C24:1, 23%) are dominant. Both fatty acids are in use as industrial lubricants, but nervonic acid also has pharmaceutical value since it is used to produce a drug against multiple sclerosis (MASTEBROEK and MARVIN, 2000; MARTIN *et al*., 2005).

Figure 1. *Lunaria annua* L. (Brassicaceae) aerial part
(Photo: J. S. Katanić Stanković, April 2015).

Generally, the data regarding the phytochemical composition of *L. annua* extracts, oils, or other formulations are scarce. Some investigations showed that *L. annua*, like the other members of the Brassicaceae family, contains a group of characteristic chemical compounds called glucosinolates that give a specific pungent taste. Glucosinolates include in their structure sulfur and nitrogen and, under the action of the enzyme myrosinase which becomes active after the plant has been cut or crushed, glucosinolates are hydrolyzed and isothiocyanates are formed with potent, pungent, mustard-like aroma and taste (AVATO and ARGENTIERI, 2015). There is not much data on the glucosinolates present in *L. annua*. BLAŽEVIĆ *et al.* (2014) reported that twelve glucosinolates had been identified in *L. annua* of which glucohesperin, glucoalyssin, glucobrassicanapin, glucoputranjivin, and hex-5-enyl glucosinolate are the most abundant. BLAŽEVIĆ *et al*. (2020) also found that isopropyl isothiocyanate is the main volatile product of glucosinolate degradation in *L. annua* seeds was, with possible cytotoxic effects.

Another class of relevant bioactive compounds in *L. annua* is anthocyanins, purple flowers' characteristic pigments. TATSUZAWA *et al.* (2006) reported the isolation of three new anthocyanins with very complex structures, namely cyanidin 3-O-[2-O-(β-D-xylopyranosyl)-6-O-(trans-*p*-coumaroyl)-β-D-glucopyranoside]-5-O-[6-O-(malonyl)-β-D-glucopyranoside], cyanidin 3-O-[2-O-(β-D-xylopyranosyl)-6-O-(cis-*p*-coumaroyl)-β-D-glucopyranoside]-5-O-[6-O-(malonyl)-β-D-glucopyranoside], and cyanidin 3-O-[2-O-(β-D-xylopyranosyl)-6-O-(trans-feruloyl)-β-D-glucopyranoside]-5-O-[6-O-(malonyl)-β-D-glucopyranoside].

In this study, we aimed to investigate, for the first time, the amount of total phenolic compounds, phenolic acids, flavonoids, flavonols, gallotanins, and anthocyanins in methanolic extracts of *L. annua* aboveground parts (LAA) and roots (LAR). The biological potential

of those two extracts was also preliminarily analyzed using antioxidant, antigenotoxic, and anti-inflammatory assays.

MATERIALS AND METHODS

Chemicals and instruments

The chemicals and reagents used for spectrophotometric determination of total phenolic compounds and antioxidant activity were purchased from Merck KGaA (Darmstadt, Germany), Aldrich Chemical Co. (Steinheim, Germany), and Alfa Aesar (Karlsruhe, Germany). The broths used for microbial growth (nutrient agar – NA, Sabouraud dextrose agar – SDA, Müller–Hinton broth – MHB, and Sabouraud dextrose broth – SDB) were acquired from "Torlak" Institute of Virology, Vaccines and Sera (Belgrade, Serbia). The reagents for the COX-1 and -2 assays were purchased: purified prostaglandin H synthase (PGHS)-1 from ram seminal vesicles, human recombinant COX-2, NS-398, and arachidonic acid from Cayman Chemical Co. (Ann Arbor, MI, USA), Tris(hydroxymethyl)-aminomethanhydrochloride and Na_2EDTA (Titriplex III) from Merck KGaA, hematin (porcine) and indomethacin from ICN (Aurora, Ohio, USA), epinephrine hydrogen tartarate from Fluka (MO, USA), DMSO (> 99.98% purity) and formic acid from Sigma-Aldrich (MO, USA), competitive PGE_2 EIA kit from Enzo Life Sciences Inc. (Farmingdale, NY, USA). All spectrophotometric measurements were performed on a UV–Vis double beam spectrophotometer Halo DB-20S with temperature control (Dynamica GmbH, Switzerland). The anti-inflammatory activity was evaluated using a microplate reader (Tecan Rainbow, Tecan Group Ltd., Switzerland). Human leukemic monocytic cell line THP-1 (European Collection of Cell Culture; Item no. 88081201), RPMI1640, N-2-hydroxyethyl-piperazine-N-2-ethane sulfonic acid (HEPES), fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin, and strep-tomycin were purchased from Gibco® (NY, USA). Phorbol 12-myristate 13-acetate (PMA), GenElute™ Mammalian Total RNA Miniprep Kit, and lipopolysaccharide (LPS) were purchased from Sigma (MO, USA). High-Capacity cDNA Reverse Transcription Kit, Predeveloped TaqMan® Assay, COX-2 primers, and COX-2 probe were purchased from Applied Biosystems (NY, USA). Deoxyribonucleic acid from herring sperm was purchased from Carl Roth GmbH and 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH) from Acros Organics (New Jersey, USA).

Plant material and preparation of the extracts

The aboveground parts and roots (*herba et radix*) of *Lunaria annua* L. (Brassicaceae) were collected in April 2015, during the flowering season, in village Prijevor (43°55′19.3″N 20°15′14.5″E), near the Ovčar-Kablar Gorge (Western Serbia) by J. S. Katanić Stanković. Milan S. Stanković confirmed taxonomic and botanical identification and a voucher specimen (No. 124/015) was assigned and deposited in the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac (Kragujevac, Serbia).

The air-dried aboveground parts of *L. annua* (88.65 g) were powdered and macerated with methanol at room temperature for 24 h three times (250 mL each). The maceration of *L. annua* roots (26.8 g) was done in the same way, with methanol (100 mL, 24 h, for three times). Subsequently, the macerates were filtered and the solvent of the filtrate was completely removed using a rotary evaporator (RV 10 basic, IKA, Staufen, Germany) under low pressure to obtain dry extracts. The final yields of *L. annua* aboveground (LAA) and root (LAR) extracts were 10.5 and 2.1 g, respectively.

Phenolic compounds

Total phenolic content

The method of SINGLETON *et al.* (1999) was used for the determination of total phenolic content. In 0.5 mL of sample (LAA and LAR extracts, 2 mg/mL) were added 2.5 mL of Folin–Ciocalteu reagent (diluted 10-fold) and 2 mL of $NaHCO_3$ (7.5%). After 15 min of incubation at 45°C, the absorbance was measured at 765 nm. The results were expressed as gallic acid equivalents (mg GAE/g dry extract).

Total phenolic acids

The content of total phenolic acids was evaluated by the method of MATKOWSKI *et al.* (2008). In 1 mL of samples (LAA and LAR extracts, 2 mg/mL) was added 5 mL of distilled water, 1 mL of HCl (0.1 M), 1 mL Arnow reagent (10% w/v of sodium molybdate and 10% w/v sodium nitrite) and 1 mL NaOH (1 M), and the mixture was additionally filled up to 10.0 mL with distilled water. The absorbance was determined immediately at 490 nm. The results were expressed as caffeic acid equivalents (mg CAE/g extract).

Total flavonoids

The total flavonoid contents in LAA and LAR were evaluated by the aluminum trichloride method (BRIGHENTE *et al.*, 2007). To 0.5 mL methanol solution of samples (LAA and LAR extracts, 2 mg/mL) 0.5 mL 2% $AlCl_3$ was added, and the mixture was incubated at room temperature for 1 h. The absorbance was measured at 415 nm and the results were expressed as rutin equivalents (mg RUE/g extract).

Total flavonols

The flavonol contents in samples were determined according to a method published by YERMAKOV *et al.* (1987). To 1 mL of samples (LAA and LAR extracts, 2 mg/mL) 1 mL 2% $AlCl_3$ and 3 mL of sodium acetate (50 mg/mL) were added. After 90 min of incubation, the absorbance was measured at 440 nm and the results were expressed as rutin equivalents (mg RUE/g extract).

Total gallotannis

The method reported by HASLAM (1965) was used to evaluate gallotannin content. To 3.5 mL of a sample solution (LAA and LAR extracts, 2 mg/mL) 1.5 mL of a saturated potassium iodate solution was added. The mixtures were incubated at 45°C until the maximum absorbance was reached. The absorbance was measured at 550 nm and the results were expressed as gallic acid equivalents (mg GAE/g extract).

Total and monomeric anthocyanins

The monomeric and total (monomeric and polymerized) anthocyanins were evaluated in tested samples using a single pH and pH differential methods (GIUSTI and WROLSTAD, 2001). The methods are based on spectrophotometric measurements of samples and the procedure was reported in detail in a previous paper (KATANIĆ *et al.*, 2015). The results were expressed as cyanidin-3-glucoside (Cy-3-G) equivalents (mg Cy-3-G/g extract).

Antioxidant activity

Total antioxidant capacity

The method of PRIETO *et al.* (1999) was used to determine the total antioxidant capacity of *L. annua* extracts. To 0.3 mL of samples solution (LAA and LAR extracts, 2

mg/mL) 3 mL of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) were added. After the incubation at 95°C for 90 min and cooling mixtures to room temperature, the absorbances were measured at 695 nm. The results were expressed as ascorbic acid equivalents (mg AAE/g).

$DPPH^{\cdot}$ scavenging activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging activity was evaluated by the method of KUMARASAMY *et al.* (2007). To 1 mL of samples (LAA and LAR extracts, 2 mg/mL, eight double dilutions) in methanol 1 mL of DPPH solution (80 μg/mL) was added. The absorbance of samples was measured at 517 nm after 30 min incubation at room temperature in the dark. Gallic acid (GA) and butylated hydroxytoluene (BHT) were used as reference standards. A control was prepared using methanol instead of a sample. $DPPH^{\cdot}$ scavenging activity (%) was calculated using the following equation (1):

$$\% \text{ radical scavenging activity} = [(Ac - As) / Ac] \times 100 \quad (1)$$

where A*c* is the absorbance of the $DPPH^{\cdot}$ in methanol and A*s* is the absorbance of the samples. The IC_{50} value represents the concentration of the sample able to reduce 50% of the free-radical concentration and it was calculated as μg/mL using a sigmoidal dose-response curve in Origin 2019b statistical software.

$ABTS^{\cdot+}$ scavenging activity

The method reported by RE *et al.* (1999) was used for the evaluation of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium (ABTS) radical-cation ($ABTS^{\cdot+}$) scavenging activity. Briefly, $ABTS^{\cdot+}$ was prepared by mixing a 7 mM solution of ABTS with 2.45 mM potassium persulfate. It was left in the dark for 16 h at room temperature. Before the analysis, the generated $ABTS^{\cdot+}$ solution was diluted with 5 mM phosphate-buffered saline (PBS, pH 7.4) to reach the absorbance of 0.70 ± 0.02 at 734 nm. To 100 μL of the sample solutions (LAA and LAR extracts, 2 mg/mL, eight double dilutions) 900 μL of $ABTS^{\cdot+}$ solution was added and the mixture was incubated for 30 min. The absorbance was measured at 734 nm. GA and BHT were used as standard antioxidants. A control was prepared using methanol instead of a sample. The $ABTS^{\cdot+}$ scavenging activity was calculated using equation (1) and IC_{50} values were calculated accordingly.

Inhibition of lipid peroxidation

The thiocyanate method reported by HSU *et al.* (2008) was used for the determination of lipid peroxidation inhibition. The samples (0.5 mL, LAA and LAR extracts, 2 mg/mL, eight double dilutions) were mixed and homogenized with 2.5 mL of linoleic acid emulsion (0.2804 g linoleic acid, 0.2804 g Tween-80 as an emulsifier in 50 mL 40 mM phosphate buffer, pH 7.0). The phosphate buffer was added up to 5 mL and mixtures were placed in the incubator at 37°C for 72 h. Thereafter, 4.7 mL of 75% methanol, 100 μL of 20 mM $FeSO_4$, and 100 μL of 30% ammonium thiocyanate were added to 100 μL of the incubated mixture. After 3 min of shaking, the absorbance was measured at 500 nm. GA and BHT were used as standard antioxidants. Inhibition of linoleic acid peroxidation was calculated using the previous equation (1). The IC_{50} values were calculated as previously explained.

Antigenotoxicity

In vitro DNA protective activity of extracts in concentrations of 25, 50, 100, 200, and 400 μg/mL against hydroxyl and peroxyl radicals-induced DNA damage was assayed according to LIN *et al*. (2008) and Zhang *et al.* (2017), described in detail by KATANIĆ *et al.*

(2019) and SREĆKOVIĆ *et al.* (2020). Visualization of DNA from agarose gel electrophoresis was performed under UV light (UV transilluminator, Vilber Lourmat, France), photographed and analyzed using ImageJ software version 1.48 for Windows (Softonic International, Barcelona, Spain).

Anti-inflammatory activity

Cyclooxygenase-1 and -2 inhibition

The *in vitro* assays for inhibition of cyclooxygenases activity (COX-1 and COX-2) were used for the evaluation of the anti-inflammatory potential of *L. annua* extracts (FIEBICH *et al.*, 2005). A detailed description of both methods was reported by KATANIĆ *et al.* (2016). Briefly, 10 μL solutions of all tested compounds (LAA and LAR extracts in DMSO, indomethacin and NS-398 in ethanol p. a) were added to the incubation mixture (different in both assays) and preincubated for 5 min at room temperature. The used concentration of extracts was 50 μg/mL, the concentration of the positive controls indomethacin and NS-398 were 1.25 μM, and 5 μM, respectively. After adding arachidonic acid, the mixtures were incubated at 37ºC for 20 min, and the reaction was stopped by formic acid. Then, a competitive PGE_2 EIA kit was used to evaluate PGE_2 concentration by a microplate reader and calculated according to FIEBICH *et al.* (2005). Inhibition of COX-1/2 activities was expressed through the reduction of formation of PGE_2 compared to a blank sample, without an inhibitor.

Cyclooxygenase-2 gene expression

The reverse transcription-polymerase chain reaction and real-time PCR were used to evaluate the inhibition of COX-2 gene expression in human leukemic monocytic cell line THP-1. The procedure has been described in detail in a previous paper (KATANIĆ *et al.*, 2016). The extracts were applied at a concentration of 25 μg/mL, while dexamethasone was used as a positive control (2.5 nM). The $2^{-(\Delta\Delta CT)}$ method (LIVAK and SCHMITTGEN, 2001) was used to calculate the percent of inhibition of COX-2 gene expression.

Statistical analysis

The data were expressed as a mean of three independent experiments ± standard deviation (SD). One-way ANOVA was carried out to determine significant differences between the means by SPSS System (version 20 for Windows, IBM Corp., Armonk, New York, United States). Significance was determined at a level of $p < 0.05$.

RESULTS AND DISCUSSION

Total phenolic contents in Lunaria annua extracts

The results of the spectrophotometric analyses used to evaluate the total content of various classes of phenolic compounds in *L. annua* methanolic extracts are shown in Table 1. The aboveground part extract (LAA) had more than twice the higher content of total phenolic compounds compared to LAR. A similar trend was observed in the case of all tested groups of phenolic compounds. LAA had a much higher content of phenolic acids, flavonoids, flavonols, and gallotannins than LAR extract. Both extracts had a low content of monomeric anthocyanins, but LAA possessed a slightly larger quantity of total anthocyanins (sum of monomeric and polymeric). These results are consistent with TATSUZAWA *et al.* (2006) who reported a high content of various polymeric anthocyanins with particularly complex

structures, acylated cyanidin 3-sambubioside-5-glucosides with malonyl, feruloyl, and coumaroyl acyl residues.

Table 1. The total contents of various classes of phenolic compounds in *Lunaria annua* aboveground part and root extracts (LAA and LAR).

	Extracts	
Phenolics	**LAA**	**LAR**
Total phenolic compounds (mg GAE/g)	61.12 ± 0.86	27.04 ± 1.24
Total phenolic acids (mg CAE/g)	5.70 ± 0.29	0.88 ± 0.18
Total flavonoids (mg RUE/g)	56.65 ± 8.39	2.42 ± 0.25
Total flavonols (mg RUE/g)	16.95 ± 0.46	0.29 ± 0.04
Total gallotannins (mg GAE/g)	17.60 ± 0.64	0.83 ± 0.07
Total anthocyanins (mg Cy-3-G/g)	2.38 ± 0.01	0.72 ± 0.01
Monomeric anthocyanins (mg Cy-3-G/g)	tr.	0.35 ± 0.01

Data are represented as mean ± SD (n = 3). GAE – gallic acid equivalents; CAE – caffeic acid equivalents; RUE – rutin equivalents; Cy-3-G – cyaniding-3-glucoside equivalents; tr. – traces.

All other classes of phenolic compounds in *L. annua* extracts were reported for the first time. Since there are no further data in the literature regarding polyphenolic secondary metabolites of *L. annua*, a profound phytochemical characterization using HPLC and/or LC/MS is necessary to completely elucidate the phenolic composition of the analyzed extracts. There is only one previous study regarding the phytochemical composition of *L. annua* extracts, which was primarily aimed at GC-MS and UHPLC-DAD-MS/MS analyses for the identification of glucosinolates in seeds, a characteristic group of compounds for Brassicaceae family (BLAŽEVIĆ *et al*., 2020). Glucosinolates are mostly present in seeds of cruciferous plants, followed by leaves and roots, and have various biological properties (AVATO and ARGENTIERI, 2015). Phenolic compounds are well known for their diverse activities, from antioxidant, antimicrobial, and anti-inflammatory activities, all the way to cytotoxic and anticancer effects. Given the quantity of phenolic compounds in both *L. annua* extracts, they were expected to show a certain level of positive biological effects in various bioactivity assays applied.

Antioxidant potential of Lunaria annua extracts

Antioxidants can be defined as compounds that inhibit oxidation or suppress reactions initiated by oxygen, free radicals, or peroxides (AGUIRRE and BORNEO, 2013). To a smaller or larger extent, antioxidants can protect biomolecules from oxidative degradation by reducing or preventing oxidative stress (GUTTERIDGE and HALLIWELL, 2010). Medicinal plants and vegetable foods are often used as very good sources of antioxidants. Natural antioxidants have many benefits compared to many synthetic antioxidants that display some serious adverse effects (KATANIĆ STANKOVIĆ *et al*., 2020; MIHAILOVIĆ *et al*., 2021). In antioxidant action, phenolic compounds particularly stand out by acting with different mechanisms. Besides their protective role in a state of oxidative stress, they can serve as significant fighters in various diseases thanks to their multiple biological effects (ALBUQUERQUE *et al*., 2021).

The antioxidant activity of tested extracts was monitored using various spectrophotometric assays to demonstrate their potential to scavenge, reduce and neutralize free radicals leading to the prevention of oxidative stress in living organisms. The results are shown in Table 2.

Table 2. Antioxidant activity of *Lunaria annua* aboveground part and root extracts (LAA and LAR).

Samples and standards	Total antioxidant activity (mg AAE/g)	IC_{50} values (μg/mL) DPPH$^{\cdot}$ scavenging activity	ABTS$^{\cdot+}$ scavenging activity	Inhibition of lipid peroxidation
LAA	215.56 ± 11.75	578.9 ± 9.5^{b}	648.3 ± 7.1^{b}	558.3 ± 10.5^{b}
LAR	110.07 ± 11.02	1294.1 ± 17.2^{c}	1072.5 ± 16.6^{c}	342.7 ± 8.2^{b}
GA	-	0.98 ± 0.01^{a}	4.35 ± 0.28^{a}	> 200
BHT	-	12.27 ± 0.98^{a}	23.81 ± 2.15^{a}	2.42 ± 0.19^{a}

Data are represented as mean ± SD (n = 3). IC_{50} values were determined by nonlinear regression analysis; AAE – ascorbic acid equivalents; GA – gallic acid; BHT – butylated hydroxytoluene. Means in the same column with different letters as superscripts are significantly different at $p < 0.05$.

The aboveground part and root extracts of *L. annua* showed a wide range of antioxidant potential. In consistency with the content of total phenolics, LAA had more than two times higher total antioxidant activity than LAR extract. These results were expected because of the presence of a significantly higher concentration of phenolic compounds in the extract of the aerial parts of the plant, particularly flavonoids and flavonols, in comparison to roots. The results of free radical scavenging capacity towards DPPH and ABTS stable free radicals were consisted with the results of total antioxidant activity. In the DPPH assay, LAA was more than twice as effective in reaction with free radicals as LAR extract. Nevertheless, the capacity of tested *L. annua* extracts to neutralize free radicals was much lower and significantly different ($p < 0.05$) compared to the results of reference compounds, gallic acid and BHT. The only results that were not coherent with the obtained results of antioxidant activity was the potential of *L. annua* extracts to inhibit lipid peroxidation in the oil/water emulsion model system. In this case, the root extract LAR showed much better results than LAA (IC_{50} values 342.7 and 558.3 μg/mL, respectively), contrary to expectations. In this regard, it can be assumed that such activity depends on some additional groups of compounds or some single components that are not contained in the above-ground part extract or in a significantly smaller amount.

The DNA-protective potential of Lunaria annua extracts

The *in vitro* protective activity of *L. annua* extracts against hydroxyl radical-induced DNA damage was evaluated by agarose gel electrophoresis (Fig. 2). Lane 1 shows the band on agarose gel electrophoresis corresponding to the intact DNA, while line 2 represents the DNA damage caused by the hydroxyl radical. Statistically significant protection against DNA damage was observed at all applied doses (lanes 4-8: 25, 50, 100, 200, and 400 μg/mL) of both LAA and LAR extracts (Figs. 2A and 2B). The aerial part extract was slightly more effective in DNA protection against hydroxyl radicals than the root extract. The differences in the level of DNA protection of different doses of LAA extract were not statistically significant although it was observed to grow gradually. With respect to the LAR extract, the

highest DNA-protective potential was observed at the highest applied concentration (400 μg/mL), as expected.

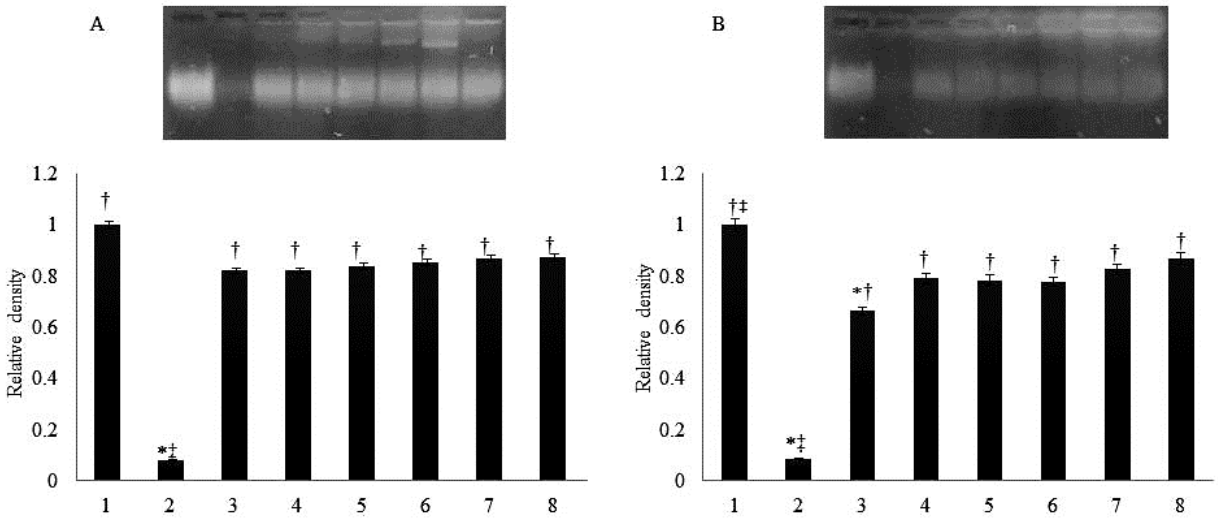


Figure 2. Protective potential of *Lunaria annua* aerial part (A) and root (B) extracts against hydroxyl radical-induced DNA damage. Untreated DNA (lane 1, negative control), damaged DNA (lane 2, positive control), quercetin (lane 3, 100 μg/mL, standard), *L. annua* extracts at the concentrations of 25, 50, 100, 200, and 400 μg/mL (lanes 4-8). $^{*}p < 0.05$ when compared with the negative control group; $^{\dagger}p < 0.05$ when compared with the positive control group, $^{\ddagger}p < 0.05$ when compared with the reference compound.

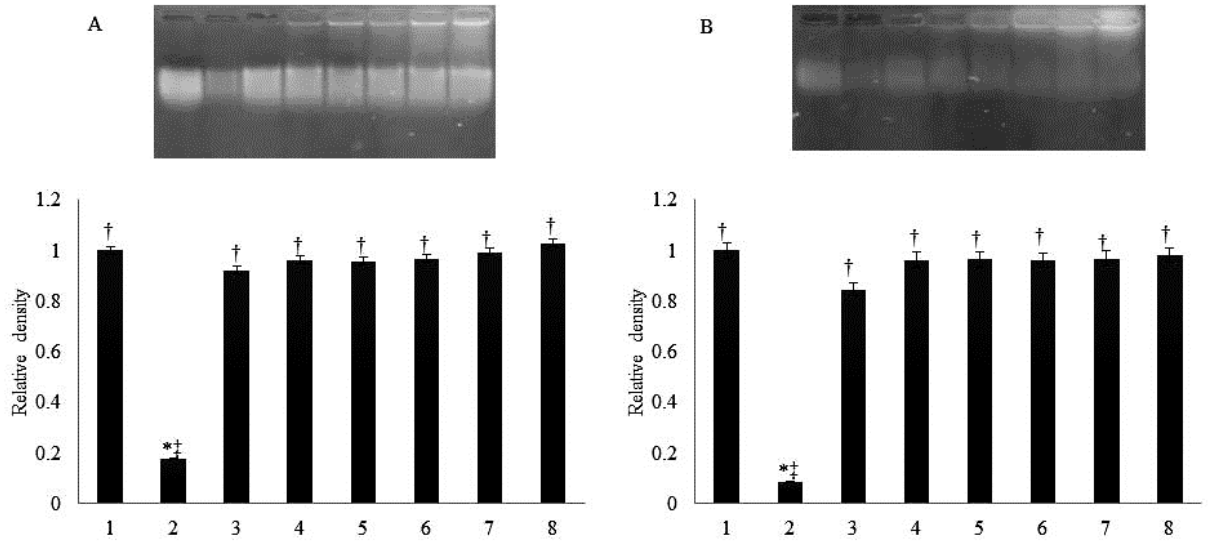


Figure 3. Protective potential of *Lunaria annua* aerial part (A) and root (B) extracts against peroxyl radical-induced DNA damage. Untreated DNA (lane 1, negative control), damaged DNA (lane 2, positive control), quercetin (lane 3, 100 μg/mL, standard), *L. annua* extracts at the concentrations of 25, 50, 100, 200, and 400 μg/mL (lanes 4-8). $^{*}p < 0.05$ when compared with the negative control group; $^{\dagger}p < 0.05$ when compared with the positive control group, $^{\ddagger}p < 0.05$ when compared with the reference compound.

To examine the protective effect of *L. annua* extracts on oxidative DNA damage induced by peroxyl radicals, DNA from herring sperm was incubated with 2,2'-azobis-(2-amidinopropane)-dihydrochloride (AAPH) and various concentrations of tested extracts. Bars 2 in Fig. 3 (A, B) depict a significant deterioration of DNA structure induced by produced

peroxyl radicals *in situ*. The results indicate similar protective effects of both *L. annua* extracts against peroxyl radical-induced DNA damage. Only a slight deviation can be observed for the highest concentration of LAA with the best DNA-protective properties, and the lowest concentration of LAR which appeared to be with the least capacity to protect DNA against free radicals.

These findings showed that the methanol extracts from *L. annua* aerial parts and roots have significant potential to prevent oxidative DNA damage, probably by AAPH-generated peroxyl radical scavenging activity and quenched hydroxyl radicals by the hydrogen atoms or electron-donating ability (SINGH *et al*., 2009). Interestingly, these results show the general pattern of antioxidant activity exerted by LAA compared to LAR extract. LAA showed the higher radical neutralizing potential that was reflected in the protection of DNA structure against free radical-induced breakdown. To the best of our knowledge, there are no studies regarding the ability of *L. annua* extracts to protect DNA from oxidative damage *in vitro*. The presented results show that the tested *L. annua* extracts are valuable natural agents with a significant ability to protect DNA from hydroxyl and peroxyl radicals and provide useful information for further *in vivo* studies.

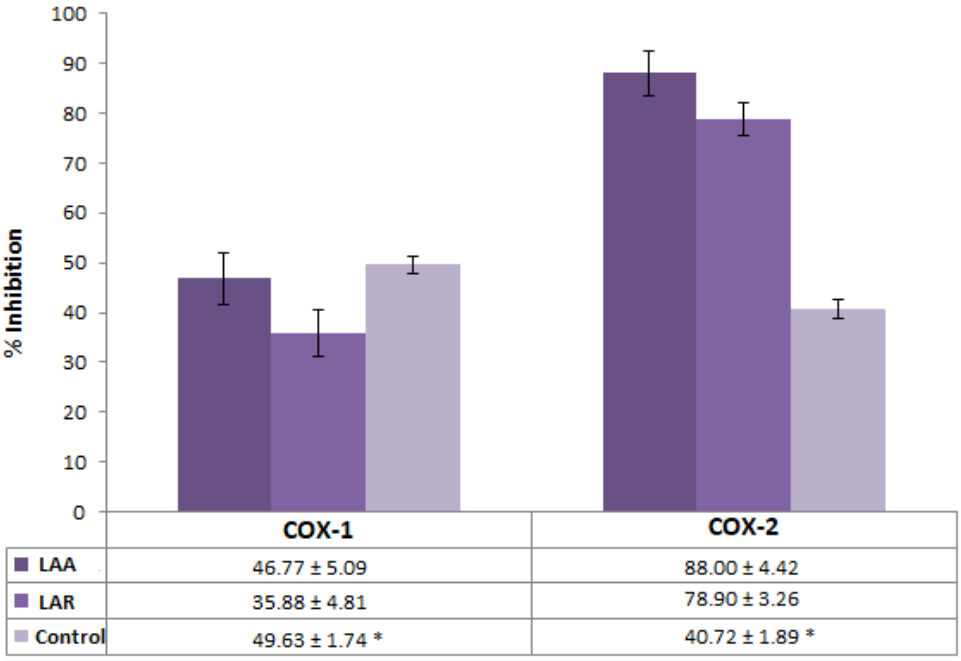
Lunaria annua extracts as anti-inflammatory agents

The anti-inflammatory potential of *Lunaria* extracts was monitored using assays based on the measurement of the inhibition of two cyclooxygenase isoforms (COX-1 and COX-2), and the inhibition level of COX-2 gene expression in macrophages. Inflammation is a protective response of the organism that ensures the removal of harmful stimulants (bacteria, parasites and viruses), but also a process that affects the healing and repairing of damaged tissue (TAKEUCHI and AKIRA, 2010). There is a wide range of inflammatory mediators that participate in many processes in the organism, above all in the immune response. The arachidonic acid pathway is one of the crucial sets of reactions that produces different classes of mediators (eicosanoids), such as prostaglandins (PG), thromboxanes (TX), and leukotrienes (LT). The cyclooxygenase pathway serves in the synthesis of PG and TX (FUNK, 2001), therefore, this feature was also used to evaluate the anti-inflammatory response of tested samples.

The results of COX-1/2 inhibitory activities of *L. annua* methanolic extracts, at a concentration of 50 μg/mL, are presented in Fig. 4.

Regarding COX-1 inhibition, LAA extract showed inhibition similar to the used positive control indomethacin at a concentration of 1.25 μM (46.8 and 49.6%, respectively). The root extracts showed somewhat lower COX-1 inhibition (35.9%) compared with LAA but the activity of both extracts was comparable to those of the reference compound. On the other hand, both extracts, at the same concentration, showed much higher inhibitory effects against COX-2 enzyme activity, with almost or more than twice the value of the positive control NS-398 at a concentration of 5 μM. What can be clearly seen from these results is the difference between COX-2 and COX-1 inhibition effects of the extracts. In fact, both *L. annua* extracts showed much higher inhibition towards COX-2 than the COX-1 isoform. The most common drugs used in the treatment of inflammation are nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, indomethacin, aspirin, diclofenac, etc. Their mechanism of action is mostly related to non-selective inhibition of both COX isoforms. Inhibition of COX-1 activity can led to various adverse effects in the long run, particularly related to inhibition of platelet function and the occurrence of gastrointestinal lesions and ulcers (DUGOWSON and GNANASHANMUGAM, 2006). Hence, there is a constant need for the synthesis of selective COX-2 inhibitors. However, some of them, like coxibs, also showed severe side effects (HAWKEY, 1999). Therefore, much research is turning to natural sources to find appropriate COX-2 inhibitors with less deleterious side effects. Various groups of phenolic compounds showed significant anti-inflammatory activity like numerous phenolic acids and flavonoids

(MALEKI *et al.*, 2019; TAOFIQ *et al.*, 2017) and some of them have even reached clinical trials (FÜRST and ZÜNDORF, 2014). In this respect, *L. annua* extracts stand out for their significantly stronger inhibition of COX-2 activity that can be applied to the definition and production of a new natural product that would mostly inhibit COX-2 activity by acting as an anti-inflammatory agent with significant antioxidant and genoprotective properties in addition.



	COX-1	COX-2
LAA	46.77 ± 5.09	88.00 ± 4.42
LAR	35.88 ± 4.81	78.90 ± 3.26
Control	49.63 ± 1.74 *	40.72 ± 1.89 *

Figure 4. *In vitro* COX-1/2 inhibitory activities of *Lunaria annua* methanolic extracts (LAA and LAR, 50 µg/mL). Positive controls: indomethacin (1.25 µM) for COX-1, NS-398 (5 µM) for COX-2. Data (% inhibition) presents the results of two independent experiments (mean ± SD). * the results for positive controls are as in KATANIĆ *et al.* (2016) since it was part of the same study.

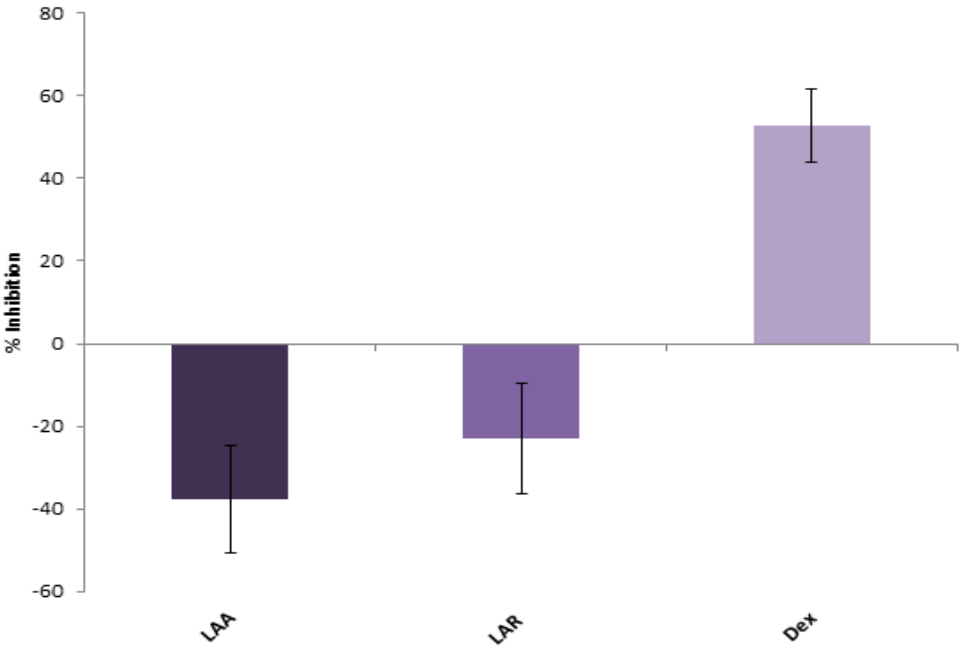


Figure 5. Inhibition (%) of COX-2 gene expression in macrophages of human leukemia cell line THP-1 by *Lunaria annua* methanolic extracts (LAA and LAR, 25 µg/mL). Dexamethasone (Dex; 2.5 nM) was used as a positive control. Data are expressed as mean ± SD of two independent experiments.

One of the possible mechanisms of COX-2 inhibition may be via the inhibition of COX-2 gene expression. The potential of *L. annua* extracts to inhibit COX-2 gene expression in THP-1 macrophages is presented in Fig. 5. As a positive control, dexamethasone was used at a concentration of 2.5 nM. However, both *L. annua* extracts showed stimulation of COX-2 gene expression at a concentration of 25 μg/mL. It is known that COX-2 mRNA expression is influenced by positive and negative feedback loops involving PGs produced by COX-downstream enzymes, and that inhibitors of COX-enzymes like aspirin, celecoxib or ibuprofen can attenuate COX-2 mRNA expression in return (DAVIES *et al.*, 1997; KIM *et al.*, 2018).

CONCLUSION

The preliminary phytochemical screening of methanolic extracts of *L. annua* aerial parts and roots showed the presence of various groups of phenolic compounds, with a higher content in the aerial part of the plant. The aerial part extract also showed somewhat better antioxidant and anti-genotoxic potential compared to root extract. *Lunaria annua* extracts exerted significant anti-inflammatory potential, especially towards the inhibition of COX-2 activity. Since this is the first study dealing with the bioactivity of *L. annua*, further work should be focused on the detailed evaluation of phytochemicals present in the extracts along with the determination of the exact mechanism of anti-inflammatory action. This would substantially contribute to the use of this plant species in the treatment of many inflammatory conditions.

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