

Phytochemical characterization and antioxidant potential of rustyback fern (*Asplenium ceterach* L.)

SUZANA ŽIVKOVIĆ^{1,*}, MARIJANA SKORIĆ², BRANISLAV ŠILER¹, SLAVICA DMITROVIĆ¹, BILJANA FILIPOVIĆ¹, TIJANA NIKOLIĆ², AND DANIJELA MIŠIĆ¹

¹Institute for Biological Research "Siniša Stanković", University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia

²Faculty of Biology, University of Belgrade, Takovska 43, 11060 Belgrade, Serbia

*Corresponding author: suzy@ibiss.bg.ac.rs

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Asplenium ceterach L. (syn. *Ceterach officinarum* Willd.) or rustyback fern is a plant species traditionally used in Mediterranean countries as an expectorant, diuretic, against spleen complaints, kidney stones and hemorrhoids. Phytochemical analysis of gametophytes and sporophytes of *A. ceterach* was performed, followed by comparative analysis of phenolic composition and the antioxidant properties of the extracts (scavenging capacities against ABTS^{•+} and DPPH[•]). Totally 16 phenolic compounds belonging to the classes of phenolic acids (hydroxybenzoic and hydroxycinnamic acids), flavonoids (flavan-3-ols, flavonols), and xanthenes were identified using UHPLC/DAD/HESI-MS/MS analysis. Different phenolics' composition of the two phases of the life cycle of this fern was observed which significantly determined their radical scavenging activities. In sporophytes of *A. ceterach* considerably high amounts of chlorogenic acid were found (~21 µg 100 mg⁻¹ fresh weight), while xanthenes were the most abundant in gametophytes (mangiferin glucoside, reaching 2.54 µg 100 mg⁻¹ fresh weight), recommending this fern species as a valuable source of bioactive compounds.

Key words: *Asplenium ceterach*, fern, sporophyte, gametophyte, antioxidant activity, phenolics

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1. INTRODUCTION

Ferns (Class Polypodiopsida) include over 12,000 species, many of which being of ornamental, medicinal, and ethnobotanical importance, or with a specific role in habitat conservation (Soare, 2008). Very few works have been done on the phytochemical composition of ferns, even though their ethnobotanical importance has been investigated and studied by numerous authors (De Britto et al., 2012). Ferns are peculiar in having the life cycle which alternates between two distinct phases: a diploid sporophyte and a haploid gametophyte, the former representing the asexual, spore-producing phase and the later the sexual, gamete-producing generation. This feature provides an opportunity for monitoring bioactivity and changes in the content of bioactive compounds in both sporophytic and gametophytic plant bodies Pauline Vincent et al. (2012). However, phytochemical investigations of fern gametophytes are rarely performed, since they are characterized by fragile structure, microscopic dimensions, and difficult collection procedure. *Asplenium ceterach* L. (syn. *Ceterach officinarum* Willd., family Aspleniaceae) or rustyback is widely spread fern species in Europe, North Africa, and the Near East. It is being used medicinally as a diuretic, expectorant, against spleen complaints, kidney stones and hemorrhoids (Vokou

et al., 1993; Guarrera and Lucia, 2007). Its aqueous extracts were reported to show antioxidant, antimicrobial and DNA damage protection potentials (Berk et al., 2011). Bioactive components of ferns mainly belong to the phenolic, flavonoid, alkaloid, and terpenoid families Ho et al. (2011), and *Asplenium* species, mainly sporophytes, have been only partially phytochemically characterized (Durdevic et al., 2007; Mir et al., 2013).

To the best of our knowledge, this study is the first report of comparative phytochemical analysis of gametophytes and sporophytes of *Asplenium ceterach*, aiming to further explore the relationship between their phenolic profiles and antioxidant activity.

2. MATERIALS AND METHODS

1. Plant material

Mature sporophytes of rustyback (*Asplenium ceterach* L.) were collected during September 2013 in Krepoljin (Eastern Serbia) and further grown in a greenhouse of the Institute for Biological Research "Siniša Stanković", Belgrade (Serbia), at the temperature of 25±2°C and a relative humidity of 60-90%. Spores for in vitro gametophyte establishment were taken

from single sporophytes and kept at -20°C until use. Sterilized spores were germinated on half-strength MS medium (Murashige and Skoog, 1962) with 20 g L^{-1} sucrose, 7 g L^{-1} agar (Torlak, Serbia) and 0.1 g L^{-1} myo-inositol (Merck, Germany). Gametophyte cultures were grown aseptically in 9 cm Petri-dishes under white fluorescent tubes (8 h light / 16 h dark cycle) with a photon flux density of $50\text{ mmol}^{-2}\text{s}^{-1}$, at $25\pm 2^{\circ}\text{C}$ and a relative humidity of 60-70%.

2. Plant methanol extracts preparation

Plant material was powdered in mortars using liquid nitrogen and extracted with 80% methanol (AppliChem, USA). After centrifugation for 10 min at $10000 \times g$, the supernatants were collected and filtered (pore size $0.45\text{ }\mu\text{m}$, Econofilter, Agilent Technologies, USA) prior to analyses or further assays. All extractions were performed in triplicate.

2.1. Determination of total phenolic content

Total phenolic concentration (TPC) was quantified using Folin-Ciocalteu assay (Singleton and Rossi, 1965) with minor modifications (Šiler et al., 2014). The TPC was calculated from a standard calibration curve based on gallic acid and results were expressed as mg of gallic acid equivalents (GAE) per g of fresh weight ($\text{mg GAE g}^{-1}\text{ FW}$).

2.2. UHPLC/DAD/QQQ MS conditions for targeted metabolomics analysis

Separation, identification, and quantification of components in methanol extracts of gametophytes and sporophytes was performed using Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Germany) equipped with a diode array detector (DAD) and connected to a triple-quadrupole mass spectrometer. Elution was performed at 40°C on Synchronis C18 column ($100 \times 2.1\text{ mm}$) with $1.7\text{ }\mu\text{m}$ particle size (Thermo Fisher Scientific, USA). The mobile phase consisted of (A) water + 0.1% formic acid, and (B) acetonitrile (MS grade, Fisher Scientific, UK), which were applied in the following gradient elution: 5% B in the first 2.0 min, 2.0-12.0 min 5–95% B, 12.0-12.2 min from 95% to 5% B, and 5% B until the 15th min. The flow rate was set to 0.4 mL min^{-1} and the detection wavelengths to 260 and 320 nm. The injection volume was $5\text{ }\mu\text{L}$. All analyses were performed in triplicate.

A TSQ Quantum Access Max triple-quadrupole mass spectrometer (Thermo Fisher Scientific, Switzerland) equipped with a heated electrospray ionization (HESI) source was used. The vaporizer temperature was set to 200°C , while ion source settings were as follows: spray voltage 5000 V, sheath gas (N_2) pressure 40 AU, ion sweep gas pressure 1 AU and auxiliary gas (N_2) pressure 8 AU, capillary temperature 300°C , skimmer offset 0 V. Collision-induced fragmentation experiments were performed using argon as the collision gas, and the collision energy (eV) varied depending on a compound. Time selected reaction monitoring (tSRM) experiment for quantitative analysis of 16 phenolic compounds was performed by using two MS2 fragments for each compound. Xcalibur software (version 2.2) was used for the instrument control, data acquisition, and analysis.

Phenolics were identified by the direct comparison with the commercial standards (Sigma Aldrich, Germany) and by the comparison with the literature data. The total amount of each targeted compound in sporophyte (S) or gametophyte (G) samples was calculated based on the calibration curve of pure compound and expressed as $\mu\text{g } 100\text{ mg}^{-1}\text{ FW}$. Mangiferin glucoside was quantified using calibration curve of mangiferin.

2.3. DPPH and ABTS radical scavenging activity

DPPH assay was performed as previously described by Brand-Williams et al. (1995), with slight modifications (Šiler et al., 2014). The 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic

acid (ABTS) radical cation decolorization assay was determined spectrophotometrically using the method of Re et al. (1999), modified for this assay. All the analyses were run in triplicate and mean values were calculated. The percentage of scavenging activity was plotted against the sample concentration to obtain the IC_{50} , defined as the concentration of sample necessary to cause 50% inhibition.

2.4. Statistical analysis

Statistical analyses were performed using STATGRAPHICS software, v. 4.2 (STSC Inc. and Statistical Graphics Corporation, USA). The quantification data were subjected to Student's t-test to compare the mean values of samples (sporophytes and gametophytes). The data obtained in antioxidant assays were subjected to the analysis of variance (ANOVA), and the comparisons between the mean values of treatments were made by the least significant difference (LSD) test calculated at the confidence level of $P < 0.05$.

3. RESULTS AND DISCUSSION

A total of 16 phenolic compounds were identified in samples of *A. ceterach* belonging to phenolic acids (hydroxybenzoic and hydroxycinnamic acids), flavonoids (flavan-3-ols and flavonols), and xanthenes. The analysis included a comparison of the retention times, UV and MS spectra with the available standards in UHPLC/DAD/–HESI-MS/MS analysis. The UHPLC/–HESI-MS/MS data and the major –MS² product ions of the compounds detected, as well as DAD data (λ_{max}), are presented in Table 1. Corresponding UHPLC-MS/MS and UHPLC-DAD chromatograms are shown in Figure ??.

The following hydroxybenzoic acids were identified in the samples: protocatechuic acid and gentisic acid both showing $m/z[\text{M-H}]^{-}$ of 153, and *p*-hydroxybenzoic acid with $m/z[\text{M-H}]^{-}$ at 137. Protocatechuic acid was the most abundant compound, especially in sporophytes ($\sim 0.47\text{ }\mu\text{g } 100\text{ mg}^{-1}\text{ FW}$). Aesculin, chlorogenic, caffeic, *p*-coumaric, and rosmarinic acids belonging to the hydroxycinnamic acids and showing molecular ions $m/z[\text{M-H}]^{-}$ at 339, 353, 179, 163, and 359, respectively, were also identified. Hydroxycinnamic acids were shown to be the dominant phenolic compounds in sporophytes of *A. ceterach*, with chlorogenic acid being by far the most abundant (Table 1), reaching concentration of $21\text{ }\mu\text{g } 100\text{ mg}^{-1}\text{ FW}$. Among the phenolic acids, *p*-coumaric, caffeic, ferulic, *p*-hydroxybenzoic, and chlorogenic acids have been previously reported for *A. unilateralis* (Bohm, 1968; Glass and Bohm, 1969), while catechol derivatives have been identified in *A. adiantum-nigrum* (Bohm, 1968). To the best of our knowledge, there are no literature data on phenolic acids' content for *A. ceterach*. Flavonoids identified in sporophytes and gametophytes of rustyback belong to either flavan-3-ols (gallocatechin, epigallocatechin, catechin, epicatechin, and epigallocatechin gallate) or flavonols (rutin) (Table 1). Flavan-3-ols were more abundant in *A. ceterach* gametophytes than in sporophytes, where gallocatechin ($0.52\text{ }\mu\text{g } 100\text{ mg}^{-1}\text{ FW}$) and epigallocatechin gallate ($0.41\text{ }\mu\text{g } 100\text{ mg}^{-1}\text{ FW}$) were the major compounds from this group. Interestingly, sporophytes preferably accumulated epicatechin ($\sim 0.12\text{ }\mu\text{g } 100\text{ mg}^{-1}\text{ FW}$), while catechin ($\sim 0.15\text{ }\mu\text{g } 100\text{ mg}^{-1}\text{ FW}$) was present only in gametophytes. Both compounds showed molecular ions $m/z[\text{M-H}]^{-}$ at 289. Flavan-3-ols have not been previously reported for *A. ceterach*, and are only rarely identified in species from this genus (Ondo et al., 2013). Interestingly, rutin ($m/z[\text{M-H}]^{-}$ at 609) was the only flavonol identified in *A. ceterach*, and has been previously reported for ferns belonging to other genera (Ansari and Ekhlas Kazaj, 2012). Rutin ($\sim 0.06\text{ }\mu\text{g } 100\text{ mg}^{-1}\text{ FW}$) was present only in gametophytes of *A. ceterach*. Further analysis of phenolics in sporophytes and gametophytes of *A. ceterach* revealed the presence of xanthenes: mangiferin glucoside with molecular ion $m/z[\text{M-H}]^{-}$ of

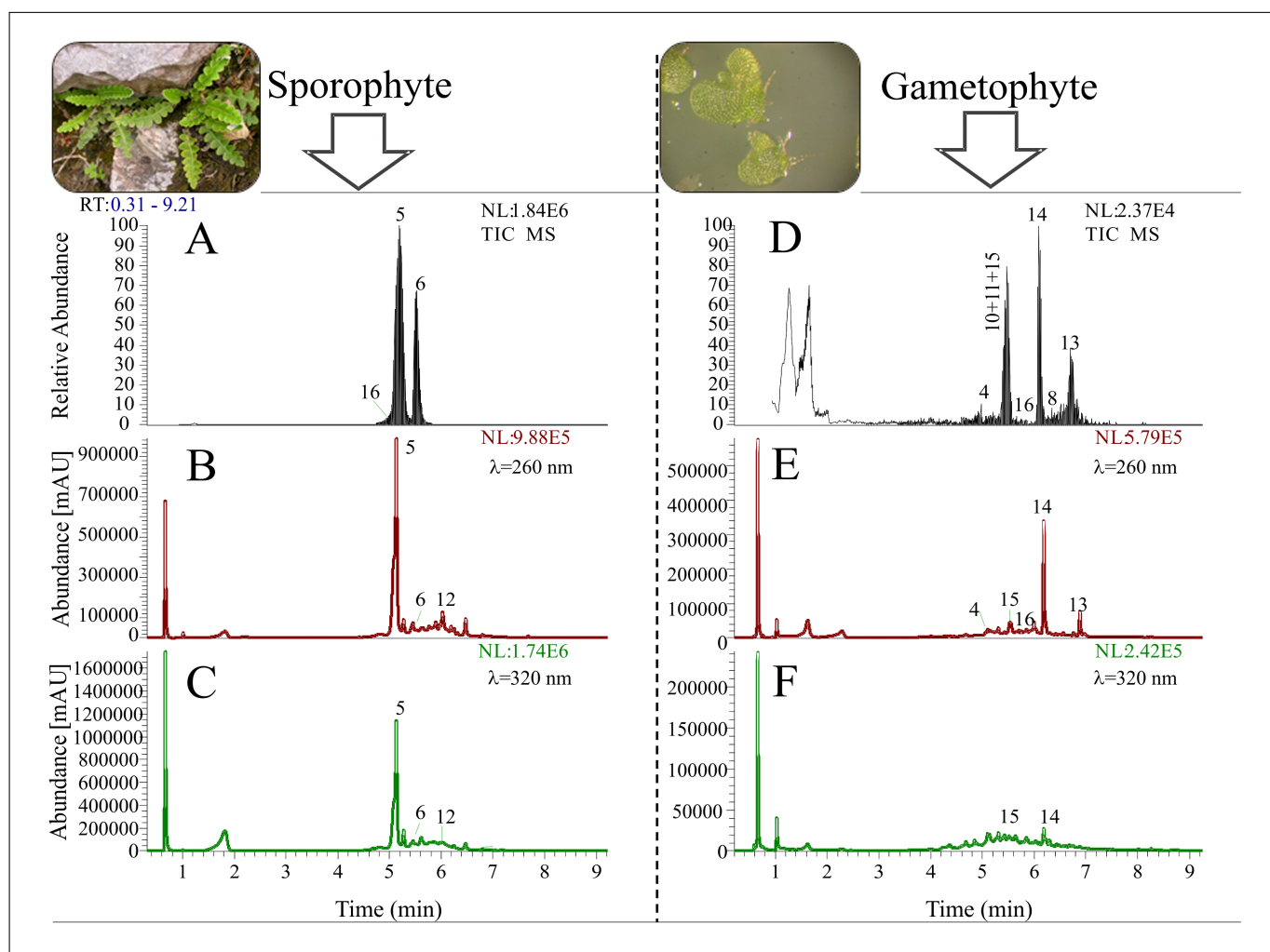


Fig. 1. Methanol extracts of *Asplenium ceterach* gametophytes and sporophytes-fronds were analyzed using UHPLC/DAD/-HESI-MS/MS method, and first 9 min of the analysis is presented. UHPLC/-HESI-MS/MS Total Ion Chromatogram (TIC) of sporophyte (A) and gametophyte (D) show major peaks corresponding to: (4) aesculin; (5) chlorogenic acid; (6) caffeic acid; (8) rosmarinic acid; (10) epigallocatechin; (11) catechin; (13) epigallocatechin gallate; (14) rutin; (15) mangiferin glucoside; (16) mangiferin. Peak numbers correspond to the compounds listed in Table 1. Chromatograms of sporophytes (B and C) and gametophytes (E and F) represent UHPLC-DAD chromatograms at $\lambda=260$ nm and $\lambda=320$ nm, respectively.

583 and mangiferin showing $m/z[M-H]^-$ of 421. Gametophytes of *A. ceterach* were especially rich in mangiferin glucoside, with concentration reaching $2.54 \text{ g } 100 \text{ mg}^{-1} \text{ FW}$ (Table 1). Mangiferin was present in significantly lower amounts ($\sim 0.01 \text{ } \mu\text{g } 100 \text{ mg}^{-1} \text{ FW}$). The presence of xanthones was previously reported in sporophytes of *A. ceterach* (Imperato, 1983). To the best of our knowledge, this is the first record of mangiferin and mangiferin glucoside in gametophytes of ferns in general. In the recent years, much attention has been devoted to ferns as potential sources of natural antioxidants, and several *Asplenium* species have been evaluated for their antioxidant potential (Lai et al., 2009; Talukdar et al., 2011; Ondo et al., 2013; Andrade et al., 2014). High superoxide anion and nitric oxide radical scavenging activities have been determined for *A. ceterach*, as well as significant radical scavenging activity against DPPH radical (Berk et al., 2011; Karadeniz et al., 2015). Methanol extracts of sporophytes and gametophytes of *A. ceterach* exhibited scavenging capacities against both ABTS and DPPH radicals. Regarding radical scavenging activity, more efficient were the fronds of *A. ceterach* sporophyte (Table 2), showing IC_{50} values of 0.46 and 0.18 mg mL^{-1} for DPPH and ABTS assays, respectively. Antioxidant activity can be ascribed to high phenolics' content ($\sim 232 \text{ mg GAE g}^{-1} \text{ FW}$), especially to chlorogenic acid, the major compound found in the methanol extracts in sporophytes. Chlorogenic acid is well-known for its high antioxidant potential (Xu et al., 2012;

Kamiyama et al., 2015). Gametophytes displayed lower TPC ($\sim 51 \text{ mg GAE g}^{-1} \text{ FW}$) and thus lower antioxidant activity in DPPH and ABTS assays than sporophytes. areXanthone mangiferin glucoside is most likely mainly responsible for the antioxidant activity of *A. ceterach* gametophytes and is followed by the flavan-3-ols. Mangiferin has previously been reported to show very good antioxidant potential in both ABTS and DPPH assays (Siler et al., 2014), as well as galloylated and non-galloylated catechins (Rice-Evans et al., 1996; Guo et al., 1999). Methanol extracts of sporophytes displayed higher antioxidant potential than pure compounds (chlorogenic acid, mangiferin and ascorbic acid) used as the reference (Table 2). Among reference compounds, catechin was the most efficient antioxidant in both assays, followed by ascorbic acid and mangiferin.

Table 1. Phenolics identified in methanol extracts of *Asplenium ceterach* sporophytes (S) and gametophytes (G), using UHPLC/DAD/-HESI-MS/MS analysis, with peak numbers, expected ranges of retention times (*t*R), mass of parent ions in a negative ion mode [*m/z*], and masses of product ions [*m/z*] with specified collision energies (eV) used in tSRM experiment. UHPLC/DAD data, including *t*R, and λ_{\max} , are also presented. Concentrations of phenolic compounds in samples are presented as $\mu\text{g } 100 \text{ mg}^{-1} \text{ FW}$.

Peak #	Assignment	Start	Stop	[M-H] ⁻	(collision energy, eV)	<i>t</i> R	λ_{\max}	S	G
		[min]	[min]	[<i>m/z</i>]	[<i>m/z</i>]	[min]	[nm]	[mg 100 mg ⁻¹ FW]	
Phenolic acids and their derivatives									
<i>Hydroxybenzoic acids</i>									
1	Protocatechuic acid	3.48	4.48	153.01	108 (23); 109 (14)	3.77	220, 260	0.471±0.181 *	0.129±0.001
2	Gentisic acid	4.68	5.68	153.00	108 (5); 109 (15)	5.13	210, 330	0.007±0.004 ns	0.013±0.001
3	p-Hydroxybenzoic acid	4.58	5.58	137.06	93 (19); 108 (22)	5.03	210, 320	0.007±0.004 *	0.115±0.001
<i>Hydroxycinnamic acids</i>									
4	Aesculin	4.39	5.39	339.08	133 (44); 177 (25)	4.80	250, 350	0.082±0.012 *	0.032±0.001
5	Chlorogenic acid	4.73	5.73	353.10	191 (25)	5.20	230, 330	20.905±1.811 *	0.058±0.001
6	Caffeic acid	5.01	6.01	179.00	134 (13); 135 (16)	5.53	230, 330	0.022±0.001 *	0.017±0.001
7	p-Coumaric acid	5.65	6.65	163.03	93 (39); 119 (16)	6.04	230, 280	0.089±0.005	-
8	Rosmarinic acid	6.20	7.20	359.06	133 (43); 161 (21)	6.70	240, 330	0.009±0.003 *	0.064±0.001
Flavonoids and their derivatives									
<i>Flavan-3-ols</i>									
9	Gallocatechin	3.37	4.37	305.12	125 (27); 179 (17)	3.80	210, 270	0.114±0.066 *	0.516±0.193
10	Epigallocatechin	4.57	5.57	305.11	125 (27); 179 (17)	4.95	210, 270	-	0.206±0.017
11	Catechin	5.00	5.50	289.09	203 (23); 245 (31)	5.22	210, 270	-	0.153±0.001
12	Epicatechin	5.50	6.00	289.08	203 (23); 245 (31)	5.70	230, 280	0.120±0.041	-
13	Epigallocatechin gallate	6.41	7.21	457.16	161 (25); 359 (16)	6.85	230, 280	0.061±0.036 *	0.414±0.024
<i>Flavonols</i>									
14	Rutin	5.54	6.54	609.20	300 (42); 301 (32)	6.04	260, 350	-	0.056±0.006
Xanthones									
15	Mangiferin glucoside	4.50	5.50	583.00	301 (20); 331 (20)	4.95	260, 320, 360	0.011±0.002 *	2.541±0.284
16	Mangiferin	5.00	6.00	421.30	301 (20); 331 (20)	5.46	260, 320, 360	0.020±0.001 *	0.011±0.005

^a All components except Mangiferin glucoside (15) were confirmed by standard

^b Within each parameter (compound) independently, values with the same letter are not significantly different at the $P < 0.05$ level according to the LSD test.

Table 2. ABTS•+ and DPPH• scavenging activity (IC₅₀ [mg ml⁻¹]) of methanol extracts of *Asplenium ceterach* sporophytes (S) and gametophytes (G).

Samples	Radical scavenging activity IC ₅₀ ^a	
	DPPH [mg ml ⁻¹]	ABTS [mg ml ⁻¹]
A. ceterach sporophyte (S)	0.46±0.02 a	0.18±0.02 a
A. ceterach gametophyte (G)	30.47±3.26 b	2.22±0.11 b
Chlorogenic acid	8.64±0.38 c	5.44±0.12 b
Mangiferin	7.27±0.54 c	3.20±0.03 a
Ascorbic acid	1.37±0.49 a	2.08±0.33 a

^a Values are presented as the means of three independent measurements ± SE. Within each assay, and for reference compounds independently, values with the same letter are not significantly different at the P<0.05 level according to the LSD test.

CONCLUSION

Phytochemical analysis of rustyback fern provided for the first time data on phenolics' composition in both gametophytes and sporophytes and highlighted an interesting phenomenon. Not only that they represent different stages of the ferns ontogenetic cycle (vegetative and generative; diploid and haploid), but sporophytes and gametophytes are clearly diversified by their qualitative and quantitative phenolics' content. Sporophytes are characterized by the prevalence of phenolic acids (especially of chlorogenic acid), while xanthenes (mangiferin glucoside) are the major group of phenolics in gametophytes. These phytochemical differences further influence their antioxidant properties. Results recommend *Asplenium ceterach* as a significant and alternative source of biologically active compounds, which can bring this species into the spotlight of pharmacological industry and medicine.

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