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AAŚ and AP: research designing; AAŚ, KSB, AKo, KM, SG, AKa, and PP: conducting the experiments; AAŚ, KSB, and AKo: analysis of the data; AAŚ, KSB, AKo, GN, JT, and AP: drafting the manuscript

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Competing interests

No competing interests have been declared.

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ORIGINAL RESEARCH PAPER

Stimulation of phenolic compounds production in the in vitro cultivated *Polyscias filicifolia* Bailey shoots and evaluation of the antioxidant and cytotoxic potential of plant extracts

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In this study, an efficient method to enhance phenolic compound production in the in vitro cultured shoots of *Polyscias filicifolia* was developed. The phenolic compound content in *P. filicifolia* has not yet been reported. Shoots were treated with methyl jasmonate (JM) or salicylic acid (SA) at doses of 50, 100, or 200 µM. HPLC-UV-VIS and LC-MS techniques were used for the determination of chlorogenic, caffeic, and ferulic acids. The total phenolics and flavonoids were quantified, and the antioxidant capacity of plant extracts was determined using DPPH and ABTS methods. Finally, the cytotoxic activity of *P. filicifolia* extracts in normal (HaCaT) and cancer (A549) cells was investigated. Further, the effect of the extracts on cisplatin cytotoxicity was assessed.

The elicitors significantly enhanced phenolic production compared to that in untreated shoots and leaves of intact plants. Chlorogenic acid was the most abundant compound with the highest yield of 5.03 ± 0.25 mg/g DW after treatment with 50 µM SA. The total flavonoid and phenolic content was significantly and dose-dependently influenced by JM. The highest antioxidant capacity was noted in extracts derived from shoots grown on media supplemented with 50 µM SA and 200 µM JM; these doses were used for further cytotoxic activity investigations. The extracts from JM or SA treatments reduced cancer cell viability and increased their mortality, whereas the extract from JM treatment exhibited protective effect on normal cells. Moreover, the comparison of cytotoxic properties of plant extracts and cisplatin indicated that plant phenolic compounds in combination with anticancer drugs could reduce the detrimental effect of the latter on human cells.

Keywords*Polyscias filicifolia*; polyphenols; antioxidant capacity; cytotoxic activity; HaCaT; A549 cells

Introduction

Polyscias filicifolia (C. Moore ex E. Fourn.) L. H. Bailey, commonly known as fern leaf Panax [1], is a member of Araliaceae family that includes approximately 159 species of evergreen shrubs and large trees. Several taxa of *Polyscias* genus, including *P. filicifolia*, are widely cultivated in the tropical regions of South Asia; however, their native ranges are not yet known [2]. *Polyscias filicifolia* can be cultivated in Southeast Asia and the tropical islands, but harvesting from field-grown plants is associated with some disadvantages such as slow growth and variations in targeted compound content [3].

In traditional Southeast Asian medicine, *P. filicifolia* has long been used as an adaptogenic agent and was included in the national Vietnamese pharmacopoeia [4]. Other *Polyscias* species, such as *P. fulva*, have also been used in traditional Cameroonian medicine for the treatment of various cancers [5]. The available data indicate that the ethanolic extracts from *P. filicifolia* callus tissue exhibit many pharmacological properties such as antimicrobial [4], anti-inflammatory [1], and protein biosynthesis inhibitory activity during hypoxia and myocardial ischemia [6,7].

The phytochemical constituents of *P. filicifolia* are poorly recognized; however, other species of the genus *Polyscias*, such as *P. fruticosa* [8], *P. amplifolia* [9], *P. fulva* [5], *P. guilfoylei* [10], and *P. duplicata* [11], have been investigated in more detail and are known to contain oleanolic acid, hederagenin, and dammarane-type glycosides as well as polyacetylenic compounds [4]. However, phenolic compounds have been reported only in the leaves of *P. pinnata* and *P. scutellaria*, which represent the natural flora of Indonesia [12]. Polyphenolic compounds represent an abundant group and include flavonoids, phenolic acids, stilbenes, and lignans; they exhibit a wide range of physiological properties, including antioxidant, anti-inflammatory, antimicrobial, and cardioprotective [13]. Phenolics have also been shown to have anticancer activity, which is attributed to the induction of apoptosis, regulation of carcinogen metabolism, inhibition of DNA binding, and blocking of signaling pathways [14–17]. Moreover, the results of in vitro and in vivo studies showed that some phenolics might be useful as potential anticancer drugs or adjuvants in complex anticancer therapy [14–16,18,19]. However, polyphenolic compounds have been shown to be cytotoxic since they can exert prooxidant activity under certain conditions, e.g., in the presence of transition metals, leading to the generation of highly reactive oxygen radicals [19–23].

In the present study, we developed an efficient method for producing phenolic compounds from *P. filicifolia* shoots cultivated in vitro by applying selected concentrations of two elicitors: salicylic acid (SA) and methyl jasmonate (JM). To our knowledge, this is the first report on phenolic compound production from *P. filicifolia* shoots cultivated in vitro. In addition, we evaluated the antioxidant properties of plant extracts from the in vitro cultures by using DPPH and ABTS methods. The effect of *P. filicifolia* extract on cisplatin cytotoxicity against normal and cancer cells was also investigated.

Material and methods

Plant in vitro cultures and elicitor treatment

A voucher specimen of *P. filicifolia* is deposited at the Department of Pharmaceutical Biology and Medicinal Plant Biotechnology, Medical University of Warsaw, Poland (accession No. FW21/026/1999).

All reagents, if not indicated otherwise, were purchased from Sigma-Aldrich, Poland.

Polyscias filicifolia shoots were obtained by micropropagation of plants regenerated from somatic embryos, as described by Śliwińska et al. [24]. These shoots were cultivated on Linsmaier and Skoog (LS) medium [25] supplemented with 2.0 mg/L 6-benzylaminopurine (BAP) and 0.5 mg/L kinetin (KIN), 30 g/L sucrose, and 8.0 g/L Plant Propagation Agar (Biocorp, Poland) and passaged every 6 weeks onto fresh media, and then used as a plant material [24]. The shoots of *P. filicifolia* cultured on LS medium for 5 weeks were transferred onto LS medium supplemented with elicitors: JM and SA at concentrations of 50, 100, or 200 μ M. The shoots were cultured with the

elicitors for the next 7 days of culture. Eight 300-mL Erlenmeyer flasks containing 60 mL of medium and four 1–1.5-cm-long shoots with two or three leaves were used for each experiment. All plantlets from control and elicitor-treated cultures were harvested and separated into individual shoots. The multiplication rate of shoots, fresh and dry biomass, and shoot length were recorded after lyophilization (lyophilizer Christ Ralph 1-4 LSC, Germany). The increase in biomass was expressed as the ratio of final weight to initial weight. All experiments were conducted in three replicates, each including 12 explants.

Phenolic compound extraction and determination

The content of phenolic acids, chlorogenic (CGA), caffeic (CA), and ferulic (FA) in the leaves of parental intact plants and shoots cultivated under the six different elicitor treatments was determined. The powdered lyophilized shoots (0.1 ± 0.002 g) were sonicated (Sonorex Bandelin, Germany) with 100% methanol (3×1 mL; Avantor Performance Materials, Poland) for 15 min at 40°C. The extracts were centrifuged at 25,402 g (EBA 12 R Hettich Zentrifugen, Germany), and the supernatants were combined in 2-mL Eppendorf tubes and evaporated. The dry residue was dissolved in 10 mL distilled water and extracted three times with 15 mL petroleum ether (Avantor Performance Materials) for 5 min. Next, the water phase was transferred to 10-mL Falcon tubes and evaporated. The dry residue was dissolved in 100% methanol and subjected to HPLC-DAD-UV-Vis analysis on a DIONEX HPLC system (Sunnyvale, USA), equipped with an automated sample injector (ASI-100) and UVD 340S detector. The extracts were analyzed using gradient elution: solvent A, acetonitrile; solvent B, 0.04 M orthophosphoric acid. The gradient program was as follows: 0 min, B10%; 5 min, B45%; 15 min, B55%. The flow rate was 1 mL/min, and C18 reversed phase column (EC 250/4.6 Nucleosil 120–127 mm; Macherey-Nagel, Germany) was used; the data were recorded at 327 nm. Peaks were assigned by spiking the samples with the standards and comparing the retention times and UV spectra.

The HPLC method was validated by defining linearity, the limits of detection (LOD) and quantification (LOQ), interday and intraday precisions, and recovery. An external standard method was used for quantification, and the standard solutions were diluted to a series of nine different levels in duplicate. The LOD and LOQ values were determined based on the standard deviation of the response and the slope obtained from the linearity plot of each standard compound. The LOD and LOQ were calculated as $3.3 \alpha/S$ and $10 \alpha/S$, respectively, where α is the standard deviation of the y -intercept, and S is the slope of regression. Precision was determined by conducting intra- and interday variability test. One of the standard mixed solutions (25 $\mu\text{g/mL}$ of each standard compound) was analyzed eight times per day; for the intraday variability, the solution was assessed eight times per day on three consecutive days. The relative standard deviations (RSDs) for the peak areas were calculated as the measurements of precisions. The recovery was assessed by calculating the mean recoveries of the samples by using the standard addition method.

UHPLC-DAD-MS/MS analysis

The UHPLC analyses were performed using a Dionex Ultimate 3000RS series system (Germany). The instrument was coupled with an Amazon SL (Bruker, Germany) ion trap mass spectrometer. The eluate was introduced into the electrospray ionization (ESI) interface of mass spectrometer without splitting. Separation was performed using a Kinetex XB-C18 column (150 mm \times 2.1 mm; particle size 1.7 μm). Column temperature was maintained at 25°C. Mobile phase A was water:acetonitrile:formic acid (95:5:0.1, v/v/v; Sigma-Aldrich), and mobile phase B consisted of acetonitrile:formic acid (100:0.1, v/v). The flow rate was 0.2 mL/min. The gradient elution program was used as follows: 0–60 min, 5–26% B; 60–70 min, 26–95% B; injection volume, 3.00 μL . UV-Vis spectra were recorded in a range between 200 and 450 nm. Chromatograms were acquired at 254 nm. The analysis was performed at a scan range from $m/z = 70$ to 1,100. The MS spectra were recorded in a negative ion mode. Polyphenols detected in

the analyzed plant material were identified based on MS spectra and UV-Vis spectra according to the previously described methodology [26–28].

Determination of total flavonoid content

The flavonoid content was determined using spectrophotometric methods based on the formation of aluminum–flavonoid complexes, according to previously described procedures [29] with minor modifications. (+)-Catechin was used as a standard. All analyses were performed in three replicates.

Folin–Ciocalteu assay

Total phenolic content in the extracts was determined using the Folin–Ciocalteu method. All analyses were performed in five replicates according to a previously described protocol [29].

Estimation of antioxidant properties

For the determination of the total antioxidant capacity of extracts, DPPH and ABTS methods were used [30]. In both the methods, TROLOX (TE; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a standard. Results are expressed as mg TE/g dry weight (DW).

Assessment of cytotoxic activity

Human lung carcinoma (A549) and immortal keratinocyte from adult human skin (HaCaT) cell lines were purchased from American Type Culture Collection (USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) according to previously described procedures [31].

Cells were incubated with the extracts for 72 h, and cytotoxicity was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays. The cell viability was assessed by determining the conversion of MTT salt by mitochondrial dehydrogenase according to a previously described procedure [32]. The release of LDH from the cytosol to culture medium is a marker of cell death; this was determined according to previously developed methodology [31–33].

The reagents DMEM without phenol red and sodium pyruvate, heat-inactivated FBS, penicillin and streptomycin, 0.05% Trypsin-EDTA, Ca²⁺ and Mg²⁺ free phosphate-buffered saline (PBS), and PBS were purchased from Gibco-Invitrogen (USA). Dimethyl sulfoxide (DMSO) and MTT were purchased from Sigma-Aldrich. Absolute isopropanol and hydrochloric acid were purchased from Avantor Performance Materials. Cytotoxicity Detection Kit (LDH) and Triton X-100 were purchased from Roche Applied Science (Germany), fluid cisplatin (1 mg/mL CDDP) was purchased from EBEWE.

Statistical analysis

All results are presented as experimental mean values (\pm SD); they were compared using one-way analysis of variance with Tukey's post hoc test (Statistica ver. 12; StatSoft, Poland). Asterisk (*) indicates significant difference at $p < 0.05$, and double asterisks (**) indicate significant difference at $p < 0.001$.

Results

Effects of elicitor treatment on *P. filicifolia* shoot growth

An efficient approach for enhancing phenolic compound production in cultivated in vitro shoots of *P. filicifolia* was developed. The two elicitors used in the present study, salicylic acid (SA) and methyl jasmonate (JM), affected the rates of shoot multiplication and resulted in an increase in the fresh and dry biomass, as well as in the length of *P. filicifolia* shoots. The significantly ($p < 0.05$) highest shoot multiplication rate, nearly three newly formed shoots from the initial one, was achieved on LS medium with 50 μM JM, whereas the lowest rate of shoots multiplication was observed on LS medium with 50 μM SA (Tab. 1). The increase in shoot multiplication rate was significantly correlated with increasing concentration of SA ($2.0 < 2.28 < 2.41$). In contrast, the shoot multiplication rate decreased after treatment with JM in a dose-dependent manner ($2.97 > 2.56 > 2.34$), although the differences were not statistically significant.

The most detrimental impact on shoot fresh and dry biomass accumulation was noted after supplementation with SA, where the fresh and dry weight increases were 29.67% and 18.11% lower than in the control, respectively. The culture conditions and medium composition also influenced the length of cultivated *P. filicifolia* shoots. The longest shoots were obtained after treatment with 100 μM SA (Tab. 1). Furthermore, no significant morphological changes in shoots appearance were noted after SA treatment in comparison to the control. Shoots were characterized by dark green leaf color for the entirety of the experimental period (Fig. 1).

Supplementation of the medium with JM induced adverse effects on shoot length irrespective of its concentration. When shoots were grown in medium containing 200 μM JM, they were 31.33% shorter than those from the control (Tab. 1). Furthermore, the yellowing and death rate of shoots increased as the concentration of JM was increased (Fig. 1).

Tab. 1 The influence of methyl jasmonate (JM) or salicylic acid (SA) on the growth parameters of *Polyscias filicifolia* shoots cultured on various modifications of LS medium.

Type of culture	Fresh biomass increase	Dry biomass increase	Dry weight (%)	Multiplication rate	Length of shoots (mm)
Control	1.82 \pm 0.07	1.36 \pm 0.09	10.89 \pm 1.47	2.47 \pm 0.42	23.30 \pm 0.13
LS _{SA50}	1.24 \pm 0.18*	0.98 \pm 0.03**	10.55 \pm 1.06	2.00 \pm 0.20*	24.50 \pm 0.13
LS _{SA100}	1.67 \pm 0.01	1.24 \pm 0.04	10.69 \pm 0.71	2.28 \pm 0.66	25.70 \pm 0.22
LS _{SA200}	1.56 \pm 0.04**	1.04 \pm 0.04*	10.37 \pm 0.66	2.41 \pm 0.33	21.80 \pm 0.12
LS _{JM50}	2.15 \pm 0.10	1.34 \pm 0.07	10.01 \pm 0.60	2.97 \pm 0.82*	20.10 \pm 0.35
LS _{JM100}	2.16 \pm 0.11	1.37 \pm 0.04	9.86 \pm 0.62	2.56 \pm 0.47	21.40 \pm 0.44
LS _{JM200}	1.70 \pm 0.05	1.22 \pm 0.04	9.05 \pm 1.37	2.34 \pm 0.46	16.00 \pm 0.23

Control – shoots cultured without elicitor on LS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L KIN; LS_{SA50} – LS_{SA200} – shoots cultured on LS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L KIN and different doses of SA (50 μM , 100 μM , and 200 μM); LS_{JM50} – LS_{JM200} – shoots cultured on LS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L KIN and various doses of JM (50 μM , 100 μM , and 200 μM). Asterisks indicate a significant difference at $p < 0.05$ (*) and $p < 0.001$ (**) in the control shoots from in vitro cultures.

Phytochemical analysis

One of the aims of our study was to assess the potential of SA and JM to enhance the production of CGA, CA, and FA in in vitro cultivated shoots of *P. filicifolia*. The parameters of the HPLC-DAD-UV-Vis method developed for the determination of phenolic acids revealed good linear correlation ($r^2 > 0.9952$) for all of the compounds used in the investigated concentration range (Tab. 2). The LOD and LOQ values of all compounds, as well their relative standard deviations (RSD), used as a measure of

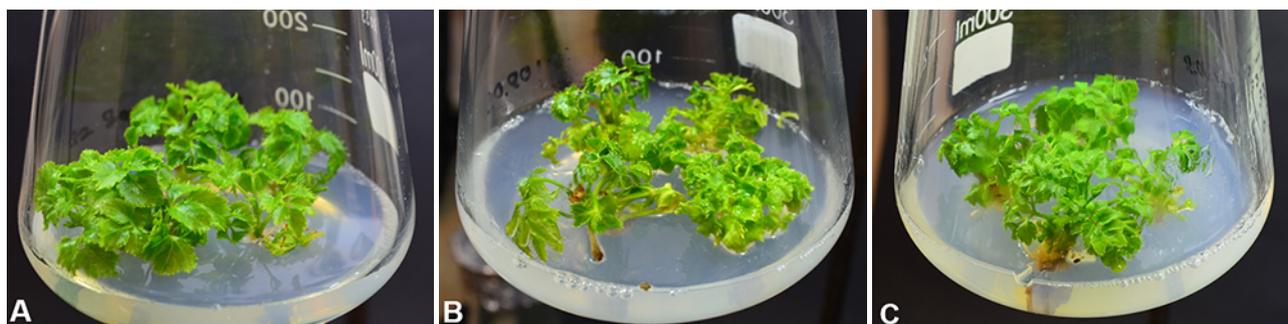


Fig. 1 Influence of elicitors on *Polyscias filicifolia* shoots growth: (A) cultivated on LS medium with 2 mg/L BAP and 0.5 mg/L KIN without elicitors (control); (B) cultivated on LS medium with 2 mg/L BAP, 0.5 mg/L KIN, and 200 µM JM; (C) cultivated on LS medium with 2 mg/L BAP, 0.5 mg/L KIN, and 50 µM SA.

Tab. 2 The results of regression equations, correlations coefficients, LODs, LOQs, precision tests, and recovery.

Standard compound	Regression equations	Correlation coefficient	LOD (µg/mL)	LOQ (µg/mL)	Precision (RSD; %)		Recovery (%)
					Intraday (n = 8)	Interday (n = 8)	
Chlorogenic acid	$y = 47.274x + 0.6654$	0.9997	0.10	0.29	0.029	0.043	95
Caffeic acid	$y = 59.978x - 3.8739$	0.9952	0.07	0.19	0.030	0.040	94
Ferulic acid	$y = 84.773x + 3.4695$	0.9968	0.27	4.02	0.020	0.043	102

precision (Tab. 2), indicated that the analytical method was sufficiently sensitive. The component recovery rates were in the range of 94–102%, indicating that the method had good reliability and accuracy.

According to the HPLC-DAD-UV-Vis analysis, CGA was the most abundant compound detected, with the highest amounts being 5.03 ± 0.25 and 3.17 ± 0.27 mg/g DW after treatment with 50 µM SA and 200 µM JM, respectively, three- or twofold higher than in the shoots of the control plants and the leaves of intact plants (1.48 ± 0.003 mg/g DW; Fig. 2).

The UHPLC-DAD-MS/MS analysis of methanol extracts of *P. filicifolia* shoots cultured with the tested elicitors resulted in the identification of 11 compounds comprising

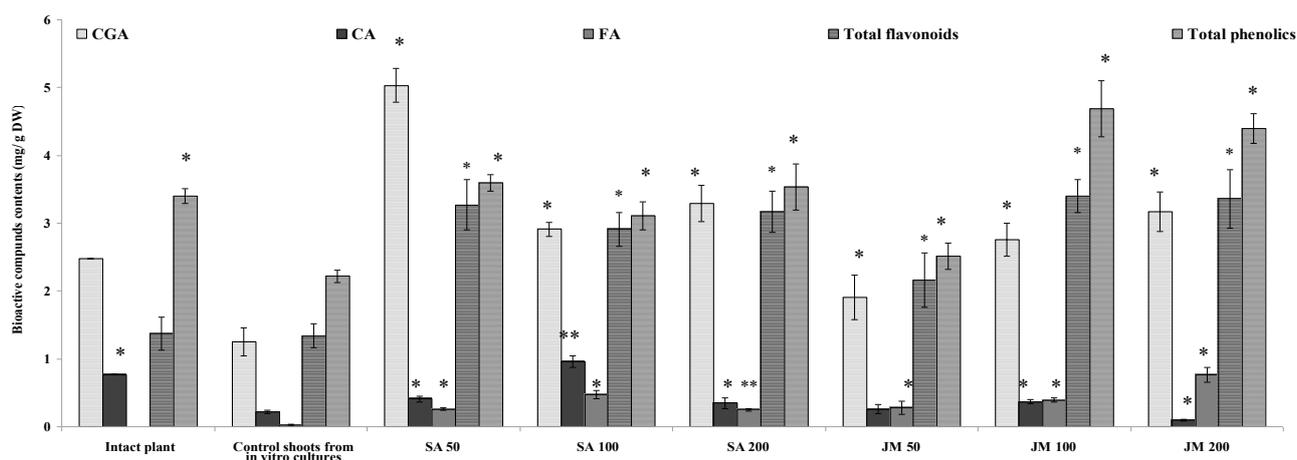


Fig. 2 The content (mg/g DW) of phenolic acids: chlorogenic (CGA), caffeic (CA), ferulic acid (FA), total phenolics (gallic acid mg/g DW), and total flavonoids [(+)-catechin mg/g DW] in the shoots of *Polyscias filicifolia* cultivated under various elicitor treatments: salicylic acid (SA; µM) or methyl jasmonate (JM; µM). Data are expressed as means \pm SD from two independent experiments performed in triplicate. Asterisks indicate a significant difference at $p < 0.05$ (*) and $p < 0.001$ (**) in the control shoots from in vitro cultures (without elicitors).

caffeoylquinic acids and their derivatives, as well as flavonoids (Tab. 3, Fig. 3). Caffeoylquinic acids and their derivatives constitute the major phenolic class found in the shoots cultivated after elicitation with SA and JM (in intact plants and shoots without elicitor treatment, we observed low amounts of these compounds; data not shown).

The compounds (Peaks 2 and 8), based on their parent ions in the negative mode ($[M-H]^-$ at m/z 353, 515), were classified into two groups: mono- and dicaffeoyl esters of quinic acid. Compound 2 exhibited MS/MS base ions at m/z 191 and secondary ions at m/z 179 and was identified as 5-*O*-caffeoylquinic acid. Compounds 3 and 4, located on the chromatograms by their parent deprotonated ions ($[M-H]^-$ at m/z 367), were classified as esters of quinic acid. Compound 3, exhibiting MS/MS base ions at m/z 191 and secondary ions at m/z 173, was identified as 5-*O*-feruloylquinic acid. Compound

Tab. 3 UHPLC-DAD-MS/MS analysis results of plant extracts from 50 μ M SA or 200 μ M JM treatments.

	Compound name	Retention time (min)	UV (nm)	$[M-H]^-$ m/z	MS/MS ions
1	Undefined compound	7.6	217, 279	203	-
2	5- <i>O</i> -Caffeoylquinic acid (chlorogenic acid) ^s	18.1	240, 301 ^{sh} , 325	353	214, 191 ^b , 179
3	5- <i>O</i> -Feruloylquinic acid	28.9	240, 300 ^{sh} , 324	367	191 ^b , 173
4	Feruloylquinic acid isomer	33.5	239, 302 ^{sh} , 324	367	301, 191 ^b , 173, 134
5	Quercetin 3- <i>O</i> -rhamnoglucoside (rutin) ^s	40.1	254, 262 ^{sh} , 353	609	563, 463, 301 ^b , 271
6	Methylquercetin derivative	46.9	252, 261 ^{sh} , 351	623	447, 315 ^b
7	Feruloylquinic acid derivative	47.5	238, 301 ^{sh} , 327	561	543, 367 ^b , 325, 191
8	3,5- <i>O</i> -Dicaffeoylquinic acid	47.6	239, 301 ^{sh} , 325	515	353 ^b , 335, 252, 191
9	Unidentified flavonoid	52.5	267, 332	917	741 ^b , 609, 475, 300, 271
10	Unidentified phenolic acid	68.6	222, 297	582	463 ^b , 437, 342
11	Unidentified phenolic acid	69.0	230, 321	577	533, 505, 433, 383 ^b , 355, 313

^s Comparisons with chemical standard have been made. ^b Base peak (the most abundant ion in the recorded spectrum). ^{sh} – shoulder.

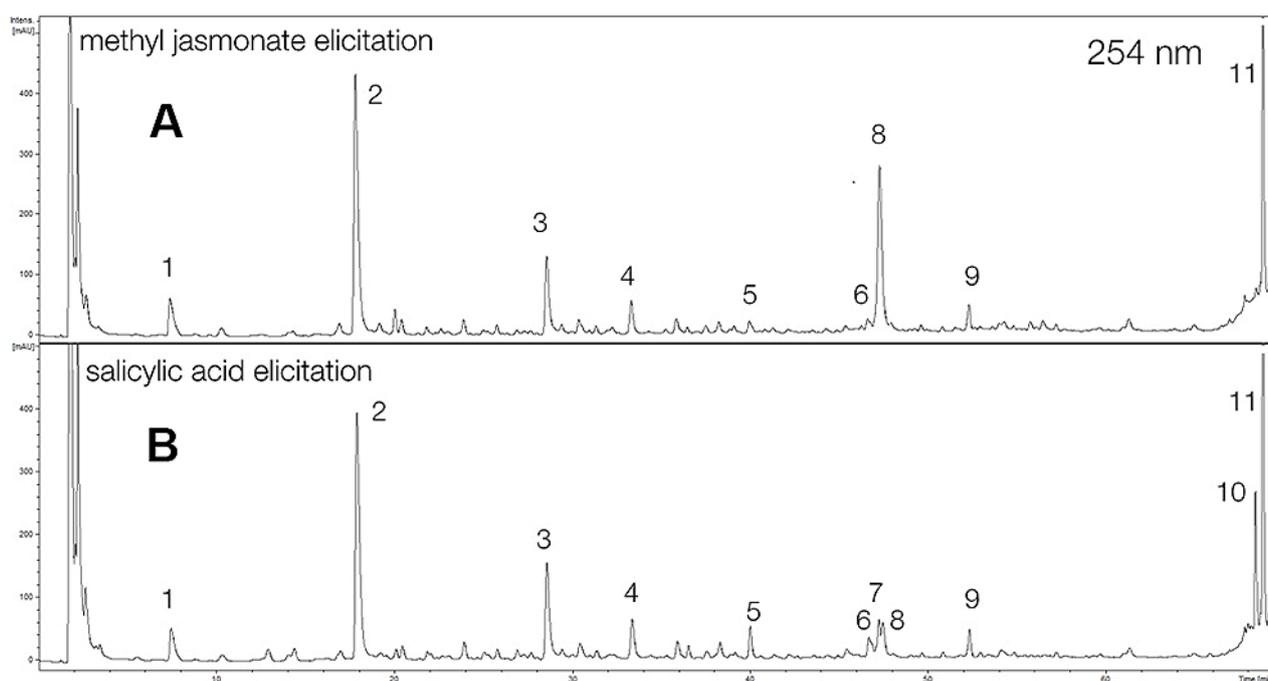


Fig. 3 UHPLC-DAD-MS/MS chromatograms of shoot extracts from 200 μ M JM (A) or 50 μ M SA (B) treatments.

4, exhibiting MS/MS base ions at m/z 191 and secondary ions at m/z 173, m/z 301, and m/z 134, was identified as an isomer of feruloylquinic acid (Tab. 3, Fig. 3).

Compound 8 (Fig. 3), eluting after CGA, was classified as dicaffeoylquinic acid, and exhibited UV-Vis absorption at 325 nm. Deprotonated molecular ions were found at m/z 515. In the MS/MS spectra, the compound that yielded a base peak at m/z 353 ($[M-H]$ -caffeoyl) and secondary ions at m/z 335 or 191 was identified as 3,5-di-*O*-caffeoylquinic acid. Our investigation revealed that, in the shoots cultivated in the presence of JM, CGA was further esterified with CA to produce 3,5-di-*O*-caffeoylquinic acid (Compound 8). In shoots elicited with SA, Compound 8 was found in significantly lower amounts than in the JM treated shoots (Tab. 3, Fig. 3).

The total content of flavonoids and phenolic compounds was the highest in extracts from shoots treated with 100 and 200 μ M JM and was twofold higher than in the control and 1.5-fold higher than that in the leaves of intact plants (Fig. 2). The UHPLC-DAD-MS/MS analysis revealed that Compounds 5 and 6 exhibited UV-Vis spectra characteristic of flavonoids with two absorption maxima, the first at 250–260 nm and the second at 350–370 nm. Flavonoids 5 and 6 were assigned as quercetin 3-*O*-rhamnoglucoside and as a methylquercetin derivative, respectively, based on their parent ions in the negative mode ($[M-H]^-$ at m/z 609 and 623).

Antioxidant activity of *P. filicifolia* extracts

The extracts from the shoots cultivated in the presence of SA or JM elicitors exhibited a significant increase in their free radical scavenging capacity in comparison to the control (Fig. 4). The highest antioxidant properties were observed in extracts derived from shoots growing on media supplemented with 50 μ M SA or 200 μ M JM (45.57 and 70.03 mg TE/g DW for DPPH and 44.07 and 64.84 mg TE/g DW for ABTS methods, respectively). In general, SA and JM treatments significantly enhanced the antioxidant activity of plant extracts compared to those of intact plants, whose antioxidant capacity was also significantly lower than that of extracts prepared from shoots cultivated without elicitation treatment.

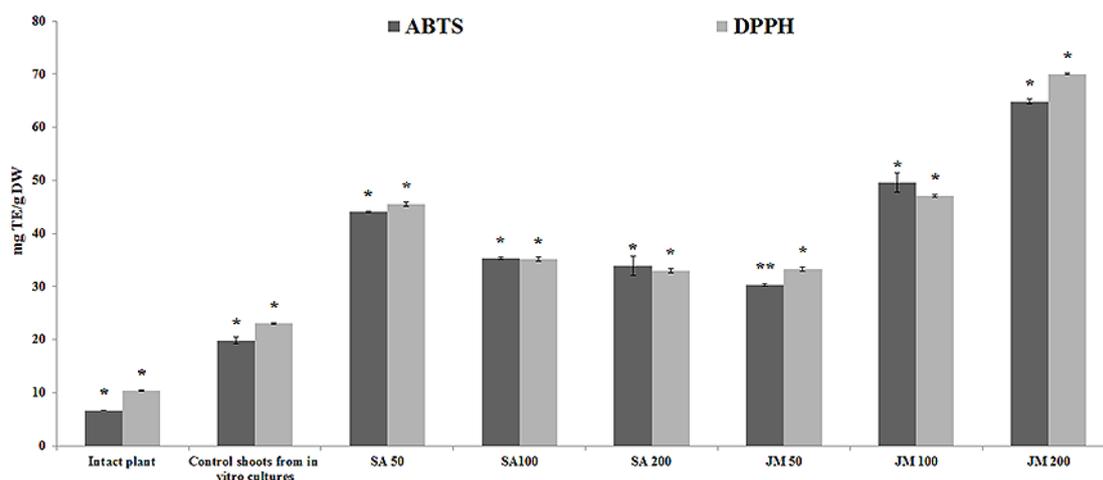


Fig. 4 Antioxidant properties assessed using ABTS and DPPH methods of plant extracts resulted from in vitro cultures of *Polyscias filicifolia* shoots cultivated under various elicitor treatments: salicylic acid (SA; μ M) or methyl jasmonate (JM; μ M). Asterisks indicate a significant difference at $p < 0.05$ (*) and $p < 0.001$ (**) in the control shoots from in vitro cultures.

Effect of *P. filicifolia* extracts on CDDP cytotoxicity against normal and cancer cells

For the cytotoxic assays, the extracts prepared from shoots growing on LS medium with 50 μ M SA or 200 μ M JM were selected. Incubation of HaCaT cells with JM extract resulted in an increase in cell viability compared to the control cells (Fig. 5A). The SA extract was poorly tolerated by these cells, and at a concentration of 60 μ g/mL a 50%

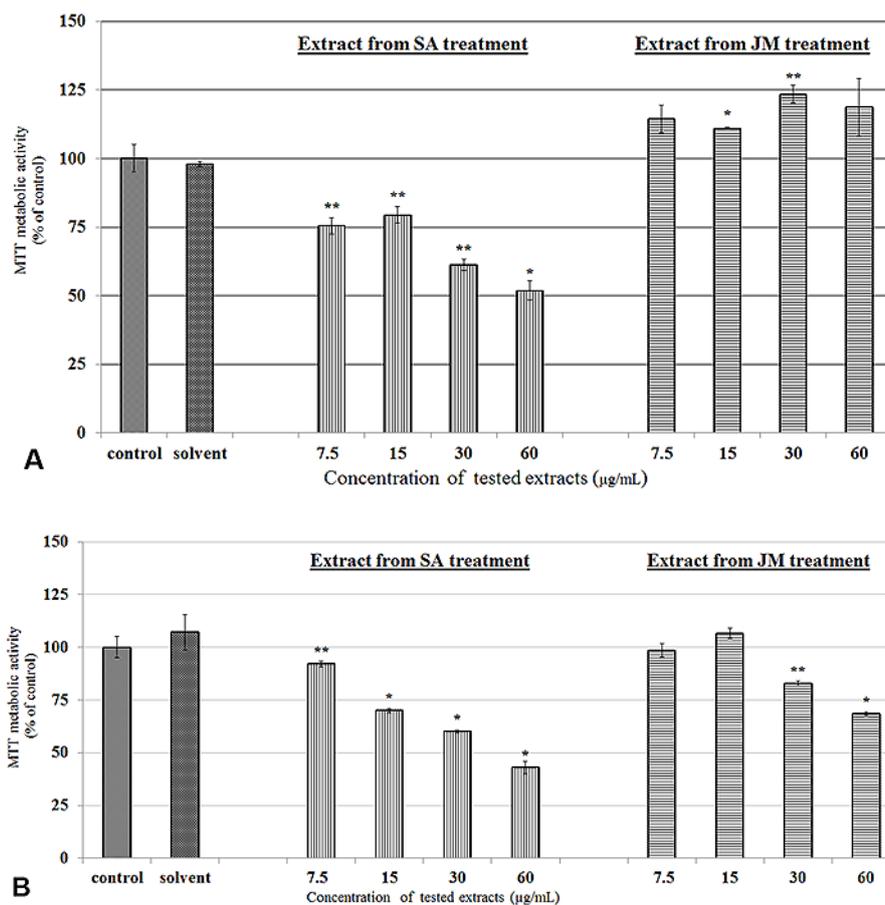


Fig. 5 Cell viability assessed by measuring MTT mitochondrial conversion in HaCaT (A) or A549 cells (B). Cells were treated with SA or JM extracts for 72 h. Statistically significant differences: * $p < 0.05$ and ** $p < 0.001$ refer to the control (untreated cells).

reduction in cell viability was observed. Incubation of HaCaT cells with both extracts resulted in a small (about 2%) release of LDH (Fig. 6A).

SA extract reduced A549 cancer cells viability (Fig. 5B) and induced LDH release in a concentration-dependent manner (Fig. 6B). After 72-h incubation, the IC_{50} of cells treated with 60 µg/mL SA showed LDH release of 70%, indicating a high level of cell death. Incubation of A549 cells with JM extract at a concentration of 60 µg/mL caused a 30% reduction in cell viability and observable morphological changes (Fig. 5B) and an increase in LDH release of more than 20% (Fig. 6B) compared to control cells.

Because of the recognized cytotoxicity of the studied extracts against cancer cells, the impact of these extracts on the cytotoxic activity of CDDP, a commonly used chemotherapeutic, against A549 and HaCaT cells was investigated. The cells were incubated (24 h) with the studied extracts and then incubated further (48 h) with the extract and CDDP, and their effects were compared to those of CDDP. Incubation of HaCaT cells with SA extract (at concentrations of ≤ 15 µg/mL) and CDDP slightly (about 10%) decreased CDDP cytotoxicity against these cells (assessed using MTT and LDH assays), while incubation with 60 µg/mL SA extract resulted in a 25–30% increase in LDH release, indicating increased cell death. HaCaT cells treated with 60 µg/mL JM extract and CDDP showed a decreased CDDP cytotoxicity of about 20% in the LDH assay (Fig. 7A, Fig. 8A). Incubation of A549 cancer cells with JM extract and CDDP caused cell mortality to be lowered by about 40%, assessed on LDH assay, in comparison to incubation with CDDP alone. When SA extract concentrations of ≤ 30 µg/mL were used, a decrease in CDDP cytotoxicity of approximately 40% (in both MTT and LDH assays) was noted (Fig. 7B, Fig. 8B).

The data indicate that JM extract in the studied concentration range (7.5–60 µg/mL) and SA extract at concentrations of ≤ 30 µg/mL (7.5–30 µg/mL) decreased CDDP cytotoxicity against human A549 cancer cells.

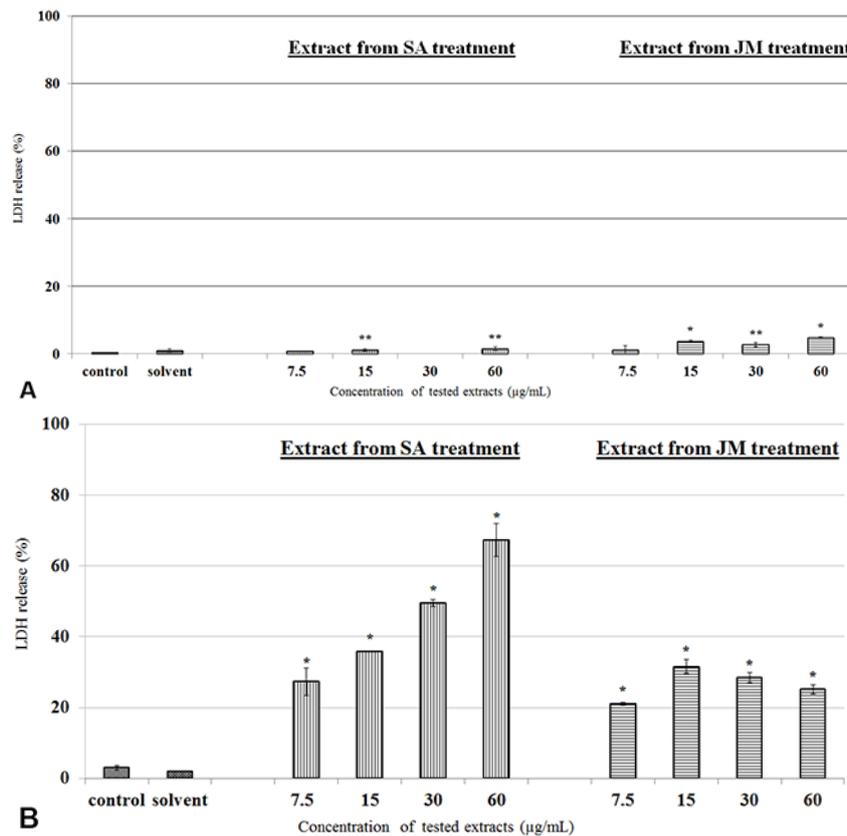


Fig. 6 LDH release as a marker of cell death: (A) HaCaT cells, (B) A549 cells. Cells were treated with SA or JM extracts for 72 h. Statistically significant differences: * $p < 0.05$ and ** $p < 0.001$ refer to the control (untreated cells).

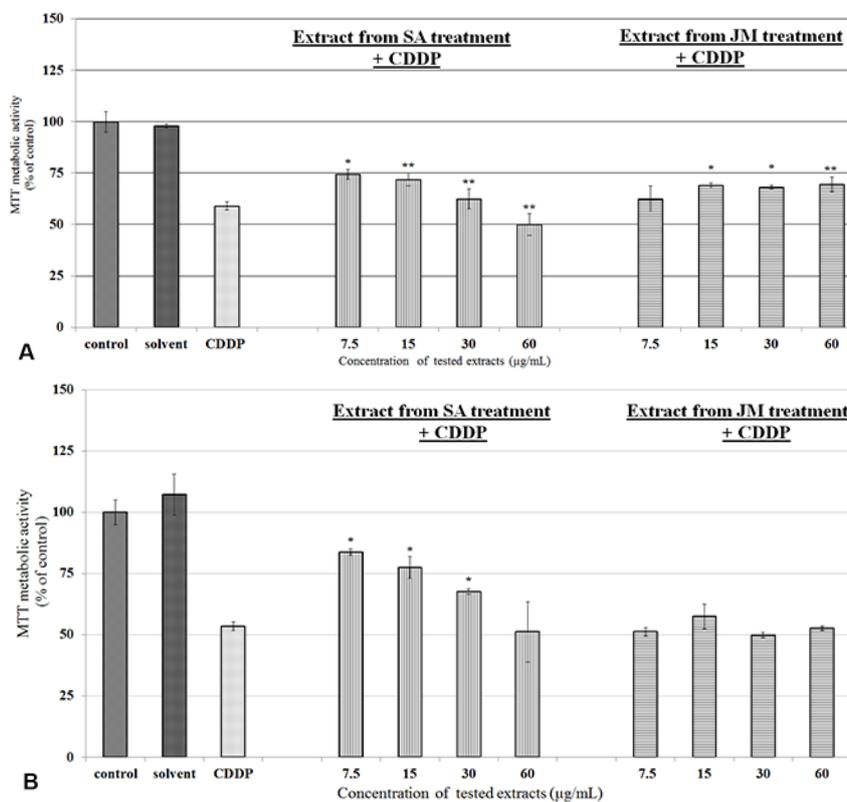


Fig. 7 Effect of the studied extracts on cisplatin (CDDP) cytotoxicity against HaCaT (A) and A549 cells (B) assessed using MTT assay (effect on cell viability). Cells were treated with SA or JM extracts for 72 h and for the last 48 h CDDP (7.5 µM) was added to the medium. Data are expressed as means \pm SD from three independent experiments performed in triplicate. Statistically significant differences: * $p < 0.05$ and ** $p < 0.001$ refer to the CDDP treatment.

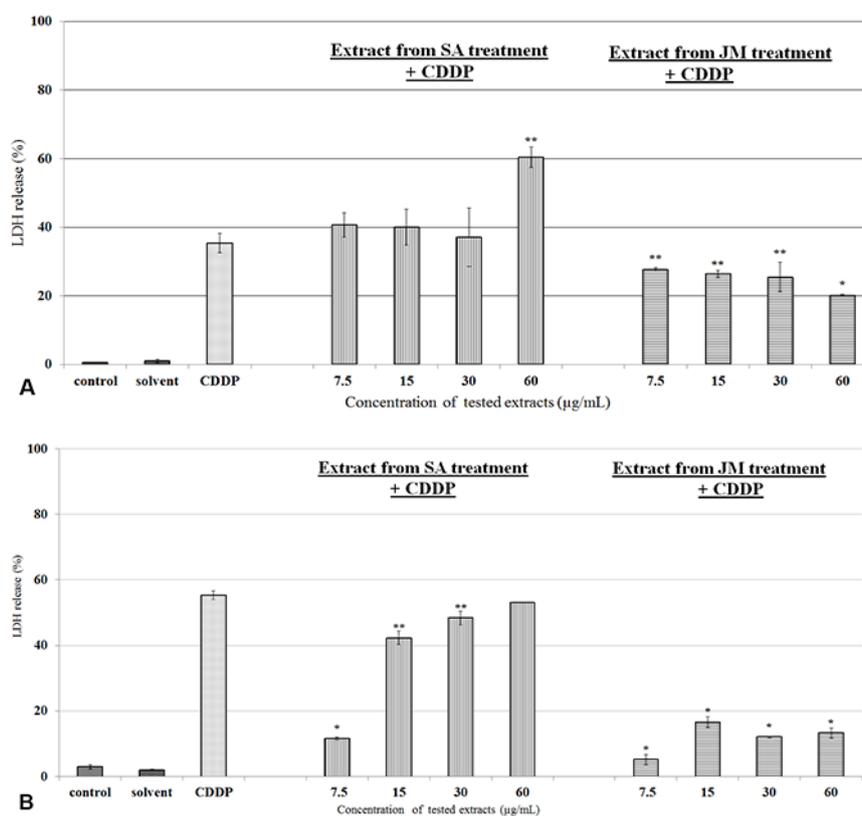


Fig. 8 Effects of studied extracts on cisplatin (CDDP) cytotoxicity against HaCaT (A) and A549 cells (B) assessed using LDH assay (effect on cell mortality). Statistically significant differences: * $p < 0.05$ and ** $p < 0.001$ refer to the CDDP-treated cells.

Discussion

Methyl jasmonate (JM) and salicylic acid (SA) are the most used elicitors in a wide range of concentrations and seem to be involved in the regulation of different biosynthetic pathways leading to the production of plant secondary metabolites in response to environmental stress [34–36]. The response to the application of these elicitors is postulated to depend on the type of secondary metabolites elicited and on the plant species [37,38].

This susceptibility of plant tissues to elicitation was used in our study to enhance phenolic compound production in shoot cultures of *P. filicifolia*. Elicitors used in our experiments affected not only biosynthetic potential but also plant growth parameters. Of the two elicitors applied in the current study, SA caused decreases in *P. filicifolia* shoot biomass accumulation, with the most pronounced effect exerted at a concentration of 50 µM. While biomass decreased under JM treatment in comparison to control, this was observed only when this elicitor was applied at a concentration of 200 µM. Ali et al. [39] reported diverse effects on the growth of adventitious roots of *Panax ginseng* (Araliaceae) following SA or JM treatment at a concentration of 200 µM. The authors described decreased root growth in comparison to the control and reported that this effect was more pronounced with SA treatment. Additionally, in adventitious root cultures of *Eleutherococcus koreanum* elicited with JM or SA at doses 50, 100, 200, or 400 µM, the fresh and dry biomass decreased as the concentration of the elicitors increased, while the percentage of dry weight was unchanged [40]. Under the culture conditions used in our study, biomass accumulation decreased significantly only in the shoots cultivated on media supplemented with 50 or 200 µM SA (Tab. 1).

In the current study, the elicitor treatments with 50 µM SA and 200 µM JM resulted in the highest accumulation of CGA, up to 5.03 ± 0.25 and 3.17 ± 0.27 mg/g DW, respectively, three or twofold higher than was found in the control shoots or leaves of the intact plants. In leaves of *Polyscias pinnata* and *P. scutellaria* previously collected from the wild, the content of CGA was 47.02 ± 0.81 and 14.13 ± 0.41 mg/100 g FW, respectively

[12]. Moreover, the authors reported values for the CA and FA content, compounds not found in *P. filicifolia* shoots cultivated under the conditions of our study. However, our results demonstrated that the CGA content determined in elicited shoots of *P. filicifolia* is comparable to, or is even higher than, that in other plants considered to be rich sources of CGA, e.g., apple (2.10 mg/g DW) or pear (3.72 mg/g DW) fruits [14].

Stimulation of phenolic biosynthesis through JM and SA elicitation was observed in previous investigations of some Araliaceae species [39–41]. In adventitious root cultures of *P. ginseng*, treatment with 200 μM of JM or SA enhanced total phenolic and flavonoid content. However, SA proved to be the more efficient elicitor, resulting in 62% and 88% increases in total phenolic and flavonoid accumulation, respectively [39]. The production of eleutheroside B and E, and of phenolic compounds, was strongly affected by elicitation with 100 μM JM [40]; however, the patterns of compounds produced differed depending on the added elicitor. JM stimulated the production of all types of investigated compounds, whereas SA exerted a detrimental effect on the biosynthesis of most studied compounds, with the exception of eleutheroside B, whose content increased sharply to 1,221% that of the control. A chlorogenic acid yield 3.2-fold higher than in the control was achieved in *E. sessiliflorus* by stimulation of embryogenic suspension cultures with 200 μM JM [41].

By comparing the chemical composition of the tested extracts with their free radical scavenging capacities, we recognized that the antioxidant properties of the tested extracts were related to their phenolic compound contents, among which the most abundant were flavonoids and phenolic acids. Chlorogenic acids have different subgroups that include caffeoylquinic, *p*-coumaroylquinic, and feruloylquinic acids, and they were reported to act at concentrations as low as 1.0 $\mu\text{mol/L}$ [42,43]. In our study, feruloylquinic acid derivatives were only observed in the SA extract. These derivatives were reported to possess strong antioxidant activities, stronger than that of FA itself [44]. Another compound possessing significant antioxidant properties, 3,5-*O*-dicafeoylquinic acid, was found to be present in considerably higher quantities in the JM extract than in the SA one, based on the UHPLC-DAD-MS/MS results (Tab. 3, Fig. 3). Literature data suggests that 3,5-*O*-dicafeoylquinic acid and its derivatives possess strong antioxidant and cytoprotective activities in normal cells, and induce DNA damage repair [43,45].

The cytotoxicity investigations of *P. filicifolia* extracts indicated that the studied extracts did not express cytotoxicity against normal cells (HaCaT) but induced significant cytotoxic effects in cancer cells (A549) when used at concentrations ≥ 30 $\mu\text{g/mL}$. This was consistent with previous reports in which the strongest cytotoxic activities of *P. filicifolia* extracts from shoots and leaves were found at concentrations of 15 and 31 $\mu\text{g/mL}$, respectively, in L929 murine fibrosarcoma cells [46]. Marczevska et al. [47] showed that extracts of *P. filicifolia* obtained from dry shoots, leaves, and callus cultivated in vitro, as well as a saponin fraction isolated from shoots, could increase the survival of murine mouse thymocytes cultured in the presence of hydrocortisone and cause an increase of IgM and IgG antibodies. Moreover, evaluation of the cytotoxic properties of the above-mentioned extracts and saponin fractions revealed that none of the investigated exposures showed genotoxic and cytotoxic effects [47]. Marczevska et al. [46] suggested that not only the saponin fraction but also other groups of active compounds were responsible for the cytotoxic activity of extracts prepared from in vitro cultures of *P. filicifolia* plant material.

The effects of the investigated extracts on A549 cancer cell viability and mortality were dose-dependent, which corroborates the results of previous studies [48,49]. Our study supports the data showing that the cytotoxic potential of phenolics was associated with their pro-oxidant activity for cancer cells, which was strongly concentration-dependent [16,17]. The SA extract was not well tolerated by normal cells, which could be related to its enhanced content of quercetin derivatives and have been reported to be associated with anticancer activity as, being easily oxidized, they acquire prooxidant properties [16,20,21,50,51]. Moreover, the results of the current study indicated that JM extracts applied in the studied concentration range (7.5–60 $\mu\text{g/mL}$), and SA extract at concentrations ≤ 30 $\mu\text{g/mL}$ (7.5–30 $\mu\text{g/mL}$), decreased CDDP cytotoxicity against human A549 cancer cells.

Conclusions

Medicinal plant cultivated in vitro could serve as an alternative and abundant source of pharmaceutically valuable compounds. The shoots of *P. filicifolia* cultivated in vitro, and stimulated with elicitors showed increased production of phenolic compounds. The chromatographic analysis revealed that the most abundant phenolic acid was CGA, and its content was the most enhanced up to 5.03 ± 0.25 mg/g DW after treatment with 50 μ M SA. Moreover, our results indicate that elicitor treatment could change the profile of biosynthesized phenolic compounds and derivatives. The phenolic compound content and antioxidant properties of plant extracts derived from in vitro cultivated shoots were significantly higher than those of intact plants. The free radical scavenging capacity of the tested extracts corresponds with phenolic compound levels, and its highest potential was noted for extracts from shoots treated with 50 μ M SA and 200 μ M JM.

Polyscias filicifolia extracts from 200 μ M JM treatment were not cytotoxic for normal HaCaT cells. Further, the extract from 50 μ M SA treatment was more cytotoxic for cancer cells than the JM extract. Moreover, the comparison of cytotoxic properties of tested plant extracts in combination with CDDP decreased cancer cells viability more than that of the normal cells. Our results suggest that the in vitro plant cell cultures could serve as a reliable source of phenolic compounds with different cytotoxic activity towards normal and cancer cells. However, further studies are necessary to elucidate the molecular mechanism of the synergistic activity of *P. filicifolia* extracts for the modulation of anticancer drug effectiveness, which seems to be a very promising direction in adjuvant therapy.

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