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Original research paper

Oleuropein in olive leaf, branch, and stem extracts: stability and biological activity in human cervical carcinoma and melanoma cells

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ABSTRACT

Olive leaves as a main byproduct of olive oil and fruit industry are a valuable source of phytochemicals such as polyphenols, with multiple biomedical effects. Apart from leaves, olive branches and stems make up a significant amount of olive waste. It is well known that the drying process and long-term storage affect the stability and concentration of polyphenols present in raw materials. For that matter, two different means of storing olive waste, at room temperature and +4 °C, were compared by determining the content of the polyphenol oleuropein (OLE) in olive leaf, branch, and stem extracts (LE, BE, and SE) by HPLC-DAD method. Total phenols (TPC), *o*-diphenols (*o*-DPC), and total flavonoids (TFC) content in extracts were assessed by UV-Vis measurements. LE prepared from leaves stored at +4 °C had the highest OLE content, 30.7 mg g^{-1} of dry extract (DE). SE from stems stored at +4 °C was the richest in TPC and TFC (193 mg GAE/g DE and 82.9 mg CE/g DE, respectively), due to the higher purity of the extract. The biological activity of extracts was determined on cervical cancer (HeLa), melanoma (A375), metastatic melanoma (A375M) tumor cell lines, and on spontaneously immortalized cell line of keratinocytes (HaCaT), using the MTT assay. The data show that all extracts had a similar dose-dependent effect on cell viability in HeLa cells, while the effect of LE on melanoma A375 and A375M, and HaCaT cells was cell-line dependent.

Keywords: oleuropein, olive leaf, olive branch, olive stem, long-term storage, biological activity

INTRODUCTION

Olea europaea L., also familiar as an olive tree, is one of the main cultures in the countries of the Mediterranean region, widely recognized for its fruit that is a source of valuable olive oil, rich in monounsaturated oleic acid and in minor, but very significant phenolic components

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endowed with many beneficial health effects (1, 2). In the olive fruit and oil industry, olive branches and leaves are regularly discarded as waste after the pruning or the harvesting process, although they can be further utilized as stock feed, fertilizers, novel materials, biofuels, food, and pharmaceutical products, contributing to environmentally sustainable waste management (3–5). Olive leaves and leaf extract (LE) preparations are already used by some industries to obtain natural products rich in bioactive compounds that could be adequate food additives, dietary supplements, or cosmetic ingredients, mostly owing to their antioxidant and antimicrobial properties (6–9). Those properties are mainly attributed to olive leaf phenolics, the composition of which heavily depends on the cultivar, harvesting season, drying method, and extract preparation (10–15). Olive leaf is particularly rich in oleuropein (OLE), the main polyphenol found in LE, renowned for its antioxidant, anti-inflammatory, antidiabetic, anti-atherogenic, antihypertensive, anti-obesity, neuroprotective, cardioprotective, anticancer and many other effects (2, 16–20). Other major bioactive compounds found in LE are sugar alcohol mannitol, and triterpene oleanolic acid, along with compounds ordinarily present in lower amounts as luteolin-7-*O*-glucoside, ligstroside, hydroxytyrosol, chlorogenic acid, verbascoside and maslinic acid (8, 21, 22). The stability of valuable polyphenolic compounds in olive leaves and other pruning waste highly depends on handling *e.g.,* proper drying and storing of raw material until extraction. These processes should ensure the removal of moisture from plant material to avoid bacterial infestation and preserve valuable nutrients (4). In the case of olive leaves, drying methods mostly include air-drying at different temperatures (usually from 25 °C to above 100 °C) and freeze-drying under vacuum pressure (10, 12). The effect of various drying methods, storage time, and temperatures on OLE stability in olive leaves was examined recently by Feng *et al.* (23). In this study, oven drying at high temperatures significantly reduced TPC, TFC, OLE, and hydroxytyrosol content in olive leaves extract when compared with air-drying at room temperature and freeze-drying method. Storage temperature had no significant effect on OLE and hydroxytyrosol content in powdered olive leaves, while the period of storage affected their content in a different manner. The greatest change was observed after the first week of storage when the concentration of OLE was slightly decreased and the concentration of hydroxytyrosol increased, probably due to the ability of OLE to be converted to hydroxytyrosol by β-glucosidase in the storage process. For the rest of the storage period (from the third to the ninth week) the contents of OLE and hydroxytyrosol were essentially unchanged.

Anticancer effects of LE and OLE have been investigated in many different cancer cell lines and in animal models as well (11, 17, 24, 25). Anticancer effects of OLE in human cervical carcinoma (HeLa) cells include reduction of cell viability and induction of apoptosis *via* the JNK signaling pathway (26, 27). Regarding LE effects, recent studies showed dose-dependent inhibition of cellular growth in HeLa cells, proapoptotic effects with molecular mechanism elucidated, and reduced cisplatin-chemoresistance in case of co-treatment (28, 29). Several studies conducted on A375 human melanoma cells showed that OLE and OLE-enriched LE could induce melanoma cell death and potentially act as an adjuvant in conventional anticancer therapies (16, 24, 30). Similar effects of LE were observed in earlier studies on mouse melanoma B16 cells *in vitro* and *in vivo* (31). Another study on mouse metastatic melanoma B16F10 cells showed apoptosis through ERK 1/2 and p53-mediated pathways when treated with aqueous LE, and this effect was mostly attributed to OLE (32).

Unlike LE, olive branch (BE) and olive stem extracts (SE) have been much less researched in terms of phenolic content and potential anticancer effects, even though they

present a major percentage of total biomass after the pruning process (3, 22). Despite this bias, scarce results obtained on BE and SE point out that this pruning waste could also be used as a source of valuable bioactive compounds (5, 33). In that perspective, this study aims to compare the content of OLE, total phenols content (TPC), *o*-diphenols content (*o*-DPC) and total flavonoids content (TFC) found in LE, BE, and SE prepared from air-dried olive leaves, branches with leaves, and stems (*O. europaea*) of the autochthonous Croatian varieties 'Rosinjola' and 'Istarska bjelica' (34, 35), stored at room temperature (RT) and +4 °C for a period of three months. The plant material used for analyses was a blend of these two varieties. The biological activity of LE, BE, and SE, and the main polyphenolic component OLE were tested on the HeLa cell line and compared. Additionally, LE was tested on human melanoma cell line A375, a metastatic variant of A375 (A375M), and spontaneously immortalized cell line of keratinocytes HaCaT.

EXPERIMENTAL

Raw material

Dry one-year-old olive branches with leaves (*O. europaea*) of the variety 'Rosinjola' (synonym 'Rošinjola', 'Rovinježa') and 'Istarska bjelica' (synonym 'Bianchera') (34, 35) in an ecological olive grove at the location of Southern Istria (Croatia) 44°58'23"N 13°51'03"E and 120 m altitude were collected in several time points during olive pruning from the end of the March to the middle of May 2022. When pruning olive trees, one-year-old saplings were left for two to three days in the olive grove at medium daily temperatures from 18 °C to a maximum of 29 °C. Then they were transferred to the canopy and kept until the beginning of July 2022 at ambient conditions with average daily temperatures of 24 to 26 °C in the shade protected by plastic nets. At the beginning of July, a certain part of samples of dry twigs with leaves were taken, divided, and stored at RT and at +4 °C, for three months, until analysis.

Reagents and standards

Methanol and acetonitrile (HPLC gradient grade) were supplied from J. T. Baker (Germany). Formic acid (LC-MS grade) was purchased from Merck (Germany). Ultra-pure water obtained by the WaterPro water system Labconco (USA) with a resistivity of 18.2 MΩ cm (25 °C) was used in all experiments. Sodium molybdate dihydrate, sodium nitrite, and aluminum chloride were purchased from Merck. Ethanol, dimethyl sulfoxide (DMSO), and oleuropein standard were obtained from Sigma-Aldrich Chemie GmbH (Germany). Folin-Ciocalteu reagent (FC) was purchased from Fluka Chemie GmbH (Switzerland) and sodium carbonate anhydrous and sodium hydroxide were from Kemika (Croatia).

Cell line

The human cervical carcinoma HeLa cell line was purchased from a cell culture bank (GIBCO BRL, USA). Human melanoma cell lines A375 (36) and A375M (37) were kindly given to us by Neda Slade, PhD, from the Division of Molecular Medicine, Ruđer Bošković

Institute, Zagreb, Croatia, whereas HaCaT (38), a spontaneously immortalized keratinocytes, were a gift from Prof. Jasmina Lovrić, University of Zagreb Faculty of Pharmacy and Biochemistry, Zagreb, Croatia. Originally, the melanoma cell lines were purchased from the American Type Culture Collection (USA) and HaCaT from Cell Line Services GmbH (Germany).

HeLa cell line was grown as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA), whereas A375, A375M and HaCaT cell lines were grown in Roswell Park Memorial Institute medium (RPMI; Sigma-Aldrich). Both media were supplemented with 10 % fetal bovine serum (FBS; Sigma-Aldrich) in a humidified atmosphere of 5 % CO_2 at 37 °C and were sub-cultured every three to four days. The cells were regularly tested for mycoplasma contamination by the Hoechst DNA staining method (Thermo Fisher Scientific, USA).

The dry extracts (DE) LE, BE, and SE were dissolved in DMSO (100 mg mL^{-1}) and stored at –20 °C. Just before use, these stock solutions were diluted with growth medium to the appropriate concentrations. Concentrations above 100 μg mL^{-1} were not used to avoid the solvent's toxicity. In that way, the highest solvent concentration in the well was under 0.5 %, which is not toxic for the experimental models used, as confirmed using the same MTT test (data not presented). All explored extracts diluted with growth media DMEM in concentrations 50 and 100 μg mL^{-1} developed crystals during 72 h incubation at 37 °C.

Extraction of phenolic compounds from olive leaf, branch, and stem

Polyphenolic compounds were extracted from previously air-dried olive leaves, olive branches with leaves, and olive stems following the procedure described in the literature with some modifications (39). A weighted amount of powdered (0.5 mm mesh size) plant material (2.50–4.00 g) was added to 50 mL of ethanol and the mixture was sonicated for 30 min using an ultrasonic bath (Elma Transonic T570 HF = 320 W, Germany), with temperature being held below 30 °C. After extraction, the samples were centrifuged at 4000 rpm for 30 min (Hettich centrifuge D-78532, Germany), and the supernatant was collected and concentrated using a rotary evaporator at a temperature below 40 °C (Büchi Heating Bath B-490, Büchi Labortechnik AG, Switzerland) till dryness. The weighted amount of DE was dissolved in methanol for spectrophotometric and HPLC-DAD analysis.

Spectrophotometric analysis of extracts (total phenolic, o-diphenolic and total flavonoid content)

The content of total phenols (TPC) in methanolic LE, BE, and SE was determined spectrophotometrically with Folin-Ciocalteu reagent at 725 nm according to Gutfinger (40), and the procedure and corresponding standard calibration curve are minutely described in Torić *et al.* (41). TPC in extracts was expressed as mg of gallic acid equivalent (GAE)/g of dry extract (mg GAE/g DE) and as mg of gallic acid equivalent (GAE)/g of dry weight (mg GAE/g DW).

The content of *o*-diphenols (*o*-DPC) in methanolic LE, BE, and SE was determined spectrophotometrically at 370 nm according to Mateos *et al.* (42) and the procedure along with the standard calibration curve could be found in Torić *et al.* (41) with the only difference

being in the volume of extract used (0.2 instead of 0.5 mL). The concentration of *o*-diphenols in extracts was expressed as mg GAE/g DE and as mg GAE/g DW.

The total flavonoid content (TFC) in the methanolic LE, BE, and SE was determined according to the spectrophotometric assay published by Kim *et al*. (43) with the procedure and standard calibration curve data already described in Torić *et al.* (41). TFC was expressed as mg of catechin equivalents (CE)/g of dry extract (mg CE/g DE) and as mg of catechin equivalents (CE)/g of dry weight (mg CE/g DW).

HPLC-DAD analysis

In the analysis of the samples, the Agilent 1260 series UHPLC system was used with a DAD detector by Agilent Technologies (USA). Data acquisition and processing were performed using OpenLab ChemStation also by Agilent Technologies. The HPLC analysis was performed using chromatographic column Ultra Aqueous C18 (4.6 × 250 mm, 5 μm particle size; Restek, USA) with an operating temperature of 30.0 ± 0.1 °C. The mobile phase consisted of two eluents, ultra-pure water/acetonitrile (99:1, *V*/*V*) (eluent A) and acetonitrile/ultra-pure water (99:1, *V*/*V*) (eluent B) both acidified with formic acid (0.1 %) and delivered at a flow rate of 1.0 mL min^{-1} with a gradient system (0–30 min 0–30 % B; 30–35 min 30 % B 35–40 min 30–100 % B). After each run, the column was equilibrated with 100 % of eluent A for 5 min. Both eluents were filtrated throughout a 0.45 μm membrane filter with a 47-mm diameter (Sartorius, Germany). The injection volume of each sample was 10 μL. To avoid carryover, the syringe and injection valve of the autosampler were set to be washed after each injection with wash solution (methanol). The DAD quantitation was performed at 280 nm with a slit of 4 nm. The absorbance of the analytes during a chromatographic run was collected in the spectral range of 200–400 nm. The stock solution of OLE (100 μg mL^{-1}) was prepared by dissolving the appropriate amount of analytical standard in methanol. Further dilutions of the stock solution with methanol produced a series of standard working solutions in the concentration of 20–100 μg mL^{-1}. A calibration curve of OLE was created using five concentration levels in the range from 20 to 100 μg mL^{-1} to assess the method's linearity. The calibration curve was found to be linear with a good regression coefficient value (r > 0.999). Samples were prepared by dissolving 10 mg of DE in 2 mL of methanol. Samples were filtrated through a PES Syringe Filter, with a diameter of 25 mm, and a pore size of 0.22 μm (FilterBio® Labex Ltd, Hungary). The identification of OLE in samples was made by comparison of retention times and UV spectrum with OLE standard.

Viability assay

HeLa cells were seeded (2.5 × 10^3 cells/0.18 mL medium/well) in 96 well plates and 24 hours later the cells were treated, in quadruplicate, with different concentrations of compounds. Due to the different doubling times, A375 and HaCaT cells were seeded at a concentration of 2 × 10^3 cells/well, whereas A375M cells were at 7 × 10^3 cells/well. The controls contained the test model cells and culture medium DMEM or RPMI (containing either the complete medium or the DMSO in the highest final concentration, 0.2 %) but no test compound. Following 72 h incubation (for HeLa cells) or 48 h incubation (for A375, A375M and HaCaT cells) at 37 °C, the medium was aspired and modified 3-(4,5-dimethylthiazol-2-yl)--2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the effect of tested

compounds on cell metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity (44). Three hours later, formazan crystals were dissolved in DMSO (0.17 mL/well), the plates were mechanically agitated for 5 min and the optical density at 545 nm was determined on a microtiter plate reader (Awareness Technology Inc., USA). The experiments were repeated at least three times.

Statistical analysis

The GraphPad Prism software version 9.0.0 for Windows (GraphPad Software, USA) (45), was used for the determination of inhibitory concentration as well as for statistical analysis. Analysis of variance (ANOVA) tests was used to assess significant differences among treatments. Results are shown as the mean values ± standard deviation (SD). All *p*-values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Characterization of olive leaf, branch, and stem phenolic extracts

Olive leaf phenolic extracts of numerous olive cultivars from and outside the Mediterranean region have been vastly investigated and characterized in the last decades, all of them confirming secoiridoids, especially OLE derivatives, as the most abundant polyphenols in olive leaves (14, 27, 46–48). As opposed to this, olive branch and stem extracts have been much less researched so far, with only one study reporting on OLE content and TPC in olive SE (33), to the best of our knowledge. In our study, LE, BE, and SE prepared from air-dried olive leaves, branches with leaves, and stems of *O. europaea*, the autochthonous Croatian varieties 'Rosinjola' and 'Istarska bjelica' (34, 35), were analyzed for their OLE content, TPC, *o*-DPC and TFC (Tables I and II). Results were evaluated in terms of dry extract weight (DE, Table I) to assess the quality of each extract, and in terms of dry plant material weight (DW, Table II) to allow the comparison of different olive plant parts regarding the phenolic content.

The concentration of OLE in LE, BE, and SE was analyzed by the HPLC-DAD method as previously described and the representative chromatogram is presented in Fig. 1. The results showed that OLE content was significantly higher in LE when compared with SE, 18.02 mg/g DE and 2.47 mg/g DE respectively, for samples stored at RT, and 30.66 mg/g DE and 4.41 mg/g DE respectively, for samples stored at +4 °C (Fig. 2). As opposed to this, in the single previous study on olive SE, some samples showed about 4 times higher OLE content in SE compared with LE (33). This discrepancy may be due to different experimental designs and remains to be clarified in future studies. OLE content in BE was 1.68 mg/g DE for samples stored at RT, and significantly greater 14.00 mg/g DE for samples stored at +4 °C (Fig. 2), which could be a result of a higher percentage of stem versus leaves in branch material, but this parameter was not strictly controlled in this study.

Generally, the content of OLE in LE spreads over a wide range of concentration values from 0.073 to 210.6 mg/g DW as was recently reviewed (13), and the reason for this wide range is numerous factors affecting the content of phenolics, as was reported throughout the literature (10–15). Our results for OLE in LE of 1.48 mg/g DW (RT) and 2.88 mg/g DW (+4 °C) fall within the range reported in the literature (13), but closer to lower values.

Table I. Oleuropein, total phenols, o-diphenols, and total flavonoids content in olive leaf, branch, and stem extract, stored at RT[a] and +4 °C[b]; expressed per dry extract weight (DE)

Extract	OLE mg/g DE ± SD	TPC mg GAE/g DE ± SD	*o*-DPC mg GAE/g DE ± SD	TFC mg CE/g DE ± SD
LE[a]	18.02 ± 0.02	132.1 ± 9.9	117.0 ± 0.0	58.2 ± 3.3
BE[a]	1.68 ± 0.01	122.0 ± 4.7	135.8 ± 21.8	54.1 ± 0.4
SE[a]	2.47 ± 0.02	182.7 ± 11.0	140.7 ± 5.9	74.3 ± 7.3
LE[b]	30.66 ± 0.21	150.5 ± 12.5	150.4 ± 21.6	63.7 ± 5.8
BE[b]	14.00 ± 0.40	156.9 ± 11.0	133.3 ± 30.5	65.1 ± 7.4
SE[b]	4.41 ± 0.04	192.7 ± 3.7	136.8 ± 13.6	82.9 ± 12.2

OLE – oleuropein; TPC – total phenols content; *o*-DPC – *o*-diphenols content; TFC – total flavonoids content; LE – leaf extract; BE – branch extract; SE – stem extract; GAE – gallic acid equivalent; CE – catechin equivalent; DE – dry extract weight; SD – standard deviation; RT – room temperature

Table II. Oleuropein, total phenols, o-diphenols, and total flavonoids content in olive leaf, branch, and stem extract, stored at RT[a] and +4 °C[b]; expressed per dry plant material weight (DW)

Extract	OLE mg/g DW ± SD	TPC mg GAE/g DW ± SD	o-DPC mg GAE/g DW ± SD	TFC mg CE/g DW ± SD
LE[a]	1.48 ± 0.00	10.8 ± 0.8	9.55 ± 0.00	4.77 ± 0.27
BE[a]	0.11 ± 0.00	8.20 ± 0.31	9.12 ± 1.46	3.64 ± 0.03
SE[a]	0.09 ± 0.00	6.84 ± 0.41	5.26 ± 0.22	2.78 ± 0.27
LE[b]	2.88 ± 0.03	14.15 ± 1.18	14.14 ± 2.03	5.99 ± 0.54
BE[b]	0.62 ± 0.02	6.92 ± 0.48	5.87 ± 1.34	2.87 ± 0.32
SE[b]	0.16 ± 0.00	6.89 ± 0.13	4.89 ± 0.49	2.96 ± 0.44

OLE – oleuropein; TPC – total phenols content; *o*-DPC – *o*-diphenols content; TFC – total flavonoids content; LE – leaf extract; BE – branch extract; SE – stem extract; GAE – gallic acid equivalent; CE – catechin equivalent; DW – dry plant material weight; SD – standard deviation; RT – room temperature

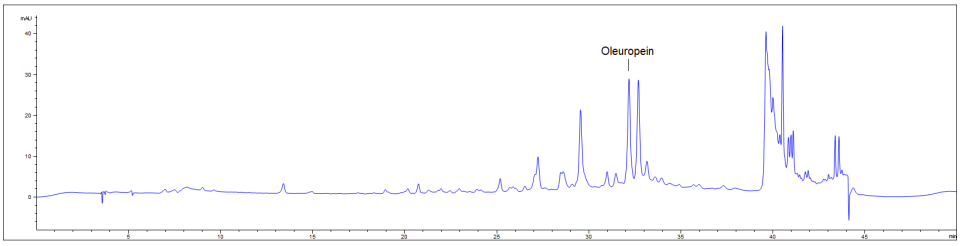

Fig. 1. HPLC-DAD chromatogram of olive LE prepared from leaves stored at +4 °C; recorded at 280 nm.

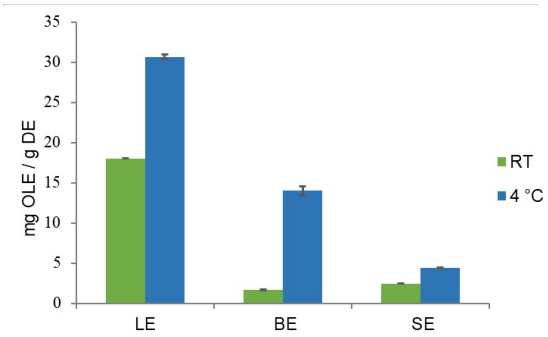

Fig. 2. The comparison of oleuropein (OLE) content in olive leaf (LE), branch (BE), and stem (SE) extract, stored at room temperature (RT) and +4 °C; expressed per dry extract weight (DE).

Indeed, in the very recent study on the same olive cultivars 'Rosinjola' and 'Istarska bjelica', with slightly different drying and extraction procedure, the OLE content was equal to 27.8 and 27.3 mg/g DW, respectively (48). Nevertheless, another study on 'Rosinjola' variety LE resulted in 6.46 mg/g DW of OLE, (49), and a study on 'Istarska bjelica' resulted in 2.23–6.83 mg/g DW of OLE (12), values much closer to our result.

Comparison of TPC values in LE, BE, and SE is somewhat different regarding the way results are expressed (per DE or DW). Results presented in Table I show the highest TPC for SE, followed by LE and BE, with values being 182.7, 132.1, and 122.0 mg GAE/g DE, respectively. This order of values points out that SE is the richest in phenolic compounds but doesn't tell much about phenol abundance in olive stem versus olive leaf or branch. TPC expressed per DW in Table II shows the highest value in LE, followed by BE and SE, and amounts 10.8, 8.20, and 6.84 mg GAE/g DW, respectively. The same phenomenon was reported earlier for olive SE and LE with TPC values very similar to ours (33). This difference that emerges may be explained by the effect of plant material and extraction method on the yield of extraction, purity, and richness of extract (14). The study of TPC in LE of 'Rosinjola' was 68.89 mg GAE/g DW, significantly higher compared with our result (49), while TPC values determined in LE of 15 Italian olive cultivars ranged between 11 and 49 mg GAE/g DW (46). LE prepared from Tunisian olive cultivars had a mean TPC value of 39.1 mg GAE/g DW or 161.5 mg GAE/g DE, reflecting the richness of the extract slightly greater than ours (14).

o-DPC and TFC determined in LE, BE, and SE are presented in Tables I and II. Similar trends as in the case of TPC could be observed regarding content expression (per DE or DW), with SE being the richest in *o*-diphenols and flavonoids (140.7 mg GAE/g DE and 74.3 mg CE/g DE, respectively), and olive leaf the most abundant in *o*-diphenols and flavonoids (9.55 mg GAE/g DW and 4.77 mg CE/g DW, respectively) compared with olive branch and stem. According to results, *o*-diphenols present a major part of total phenols in LE and SE, almost 89 and 77 %, respectively, and in the case of BE, the value for *o*-DPC exceeds TPC, probably because of minor experimental error connected to greater SD. A similar relation between *o*-DPC and TPC of approximately 73 % was published for 'Frantoio' olive LE (50). TFC is usually expressed as mg of rutin or quercetin equivalents (RE or QE) in literature, while in our study catechin equivalents (CE) were used, so the comparison of results is somewhat difficult and not straightforward, but the similarity of structures and molar

weights of catechin and quercetin, and presumption of their similar reactivity in the spectrophotometric assay (43) allows for some approximate comparison. In the study on the 'Oblica' cultivar LE, the TFC ranged from 11.80 to 26.52 mg QE/g DW, values significantly higher than ours, probably due to immediate processing and different extraction procedures (15). TFC in LE of two Portuguese cultivars 'Negrinha do Freixo' and 'Cornicabra' amounted to 14.52 and 7.95 mg QE/g DW, respectively (51), while for the LE of the 'Chemlali' cultivar (Algeria) the TFC was 1.05 mg QE/g DW (52). These comparisons place our values for TFC somewhere in the middle, confirming that even after long-term storage, the concentrations of flavonoids in olive leaf, branch, and stem could still be significant.

Effect of storage temperature on the stability of olive leaf, branch, and stem phenolic compounds

Olive leaf has been traditionally used in folk medicine as an herbal drug and preservative for centuries, and nowadays it is usually consumed as an olive tea infusion (8, 53). Nonetheless, studies on the production procedure of olive leaves for commercial purposes, including storage protocols are scarce (53, 54). In our study, olive branches with leaves were air-dried and subsequently stored for 3 months at RT and +4 °C. OLE content found in LE, BE and SE was approximately 2 times higher in samples stored at +4 °C pointing to the greater stability of this secoiridoid in plant material at lower temperatures. As opposed to this, the study on OLE content in olive leaves at three different storage temperatures (–20, 4, and 25 °C) showed no significant difference, but the olive leaf was powdered before the storing, and the grinding process possibly affected the OLE stability in the first place and diminished the effect of storage temperature (23). In the same study, the OLE content was followed for nine weeks of storage, and the major changes in OLE content were after the first week of storage (about 6 % of loss) but then generally became unchanged under those storage conditions (23). Another study on OLE in powdered olive leaf showed good stability of OLE at RT and relative humidity below 57 % for 6 months (54). Ahmad-Qasem *et al*. studied the stability of olive polyphenols in LE after different storing conditions (at 4, 25, and 35 °C for 4 weeks) and determined a non-significant influence of the temperature on the content of OLE and other polyphenolic compounds quantified in the extracts (55).

The effect of storage temperature on TPC, *o*-DPC, and TFC found in LE, BE, and SE could be seen as confusing at first sight because some values are higher in samples at RT than those at 4 °C. This is because the degradation of secoiridoids produces simple phenols, molecules such as hydroxytyrosol for example, that still give a positive reaction in total phenolic and *o*-diphenolic assays. A similar phenomenon could be seen in the case of flavonoids. Taking this into account, the results of TPC, *o*-DPC, and TFC for each extract are balanced when compared between two different storage temperatures, although a 2-fold difference in OLE content suggests the change in phenolic composition during the storage period.

Biological activity of LE, BE, SE, and OLE on human cervical carcinoma HeLa cell line

The results of studies on cervical cancer show that LE (28, 29, 56) and the main component of LE, the polyphenol OLE, inhibit HeLa cell proliferation in a dose- and time-dependent manner (17, 27). However, the biological impact of BE and SE on cervical cancer cells has not yet been studied.

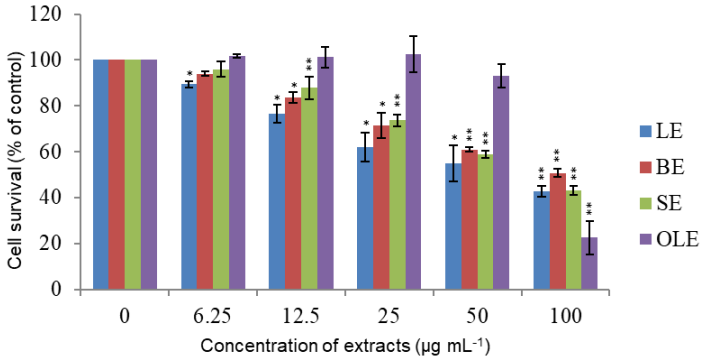

Fig. 3. Cancer cell viability after 72 h exposure to various concentrations of leaf extract (LE), branch extract (BE), stem extract (SE), and oleuropein (OLE) on the HeLa cell line. Metabolic activity was measured using an MTT assay. Values are the mean ± SD, $n = 4$. Statistically significant differences compared with non-treated cells are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

Here, the highest concentration of LE, BE, and SE tested was 100 μg mL^{-1}. In that way, the highest solvent concentration in the well was under 0.5 %, which is not toxic for the experimental models used, as confirmed using the same MTT test (data not presented). At concentrations of 50 and 100 μg mL^{-1}, all tested extracts crystallized limiting the calculation of IC_{50} but it seems that all extracts have a similar effect on cell viability (Fig. 3). At this point, we can only assume that other polyphenols present in extracts act synergistically with OLE, but this should be corroborated with further investigation. The strong impact of 100 μg mL^{-1} OLE and rather weaker effect of LE, BE, and SE on HeLa cells could be explained by the fact that at this concentration, the crystallization of all three extracts under the microscope is visible, and therefore we can assume that the biological effect is weaker. It is planned to determine whether inhibition of cell proliferation or cytotoxicity plays a role in the demonstrated biological effects of extracts LE, BE, and SE.

Biological activity of LE on two human melanoma cell lines A375 and A375M, and HaCaT keratinocytes

The LE was additionally tested on human melanoma cell lines, A375 and A375M, and a spontaneously immortalized cell line of keratinocytes HaCaT. The anti-melanoma activity of OLE, the main component of olive leaf extract, has been intensively studied (16, 17, 25, 57). LE was also studied, and the results showed that it suppressed human melanoma cell migration, invasion, and colony formation (16, 30–33, 58–60). In our study, neither A375 nor A375M tumour cells showed a statistically significant difference in metabolic activity at the applied concentrations of LE compared with cells treated with the highest concentration of DMSO. Similar results were already observed in A375 cells (31), human melanoma SK-MEL-5 cells (30), B16 melanoma cells (33), and B16F10 mouse melanoma cells (59) indicating the related general response of melanoma cells despite their diversity with regard to cell type origin, although some studies showed a significant reduction of cell viability

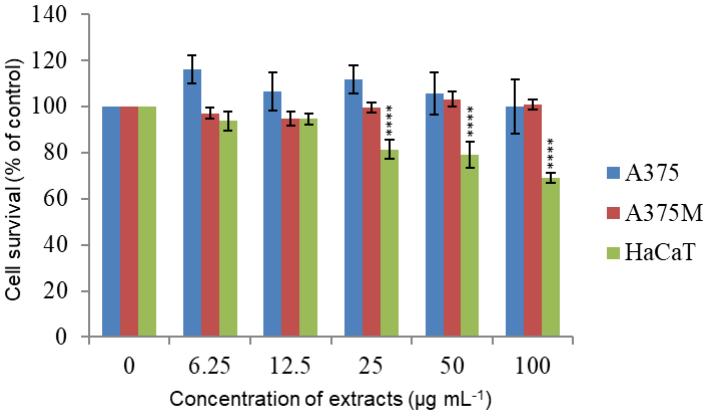

Fig. 4. Cancer cell viability after 48 h exposure to various concentrations of leaf extract (LE) on the A375 melanoma cell line, the A375M metastatic melanoma cell line, and HaCaT immortalized keratinocytes. Metabolic activity was measured using an MTT assay. Values are the mean ± SD, $n = 4$. Statistically significant differences compared with non-treated cells are marked with an asterisk (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

at 100 μg mL^{-1} of LE (32, 58). Nevertheless, the greater effect on cell viability of melanoma cells was shown at higher concentrations of LE (200 μg mL^{-1} and over) (30–32, 58), which unfortunately could not be tested in our experimental setup. In our case, only non-tumorous cells HaCaT were sensitive to LE in concentrations of 25 μg mL^{-1} or higher, inducing a slightly, dose-dependent biological effect (Fig. 4). The results of this study clearly show the importance of testing the toxicity of extracts/compounds simultaneously on tumor as well as non-tumor cells, which is often neglected in many studies. In the literature, one can very often find data on the toxicity of an individual extract/compound, but the data on the effect on healthy cells are missing. Without putting that information into the perspective of the overall impact on various cells, raw data collected on tumor cells can cause misleading conclusions about the beneficial effects of tested compounds. It is not surprising that at low concentrations, toxic effects are visible only on healthy cells, as shown by the results of this research on melanoma cells and keratinocytes. The reason for this lies probably in numerous genetic and biochemical changes that occur during the transformation of healthy cells into tumor cells, which make them more resistant to changes in environmental conditions, and increase their power of survival, proliferation, and recovery. Without additional investigation, it is hard to hypothesise what could be a reason for the detected effect.

CONCLUSIONS

Results presented in this study confirm that olive stems and branches together with olive leaves could be used as the source of valuable olive polyphenols, such as secoiridoid OLE. Even after long-term storage of this pruning waste, the concentrations of OLE remained in accordance with literature values, although affected by storage temperature. Future

studies on this issue are needed to optimize the conditions of storing olive waste and minimize the loss of bioactive olive polyphenols. The biological effect of SE and BE, assessed here for the first time, along with LE, had a weaker effect on HeLa cells' metabolic activity compared to 100 μg mL^{-1} OLE. Additional studies of LE on A375 and A375M melanoma cells did not show a significant biological effect in the investigated range of LE concentrations, though a weak toxic effect was observed on non-tumorous HaCaT cells. Moreover, it seems that the non-cancer HaCaT cell line is more sensitive to LE compared to two melanoma cells. The biological effects of olive leaf polyphenols seem to be cell-type dependent and remain to be clarified in future investigations.

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Conflicts of interest. – The authors declare no conflict of interest.

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