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Select bioavailable metabolites from *Lippia citriodora* and *Olea europaea* extracts exhibit anticancer effects on pancreatic cancer cell lines

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Phenolic compounds are widely recognized for their anti-proliferative and chemopreventive properties, making them potential candidates for cancer therapy. *Lippia citriodora* (LC) and *Olea europaea* (OE) are two phenolic-rich plant extracts with established antitumor activity. Despite their distinct phytochemical compositions, a clinical intervention study identified nine common bioavailable metabolites in human plasma following ingestion of these extracts. This study aimed to evaluate the anticancer effects of selected shared bioavailable metabolites identified in human plasma after ingestion of LC and OE extracts, oleuropein (Oleu), vanillic acid sulfate (VA-Sul), and homovanillic acid sulfate (HVA-Sul), and compared them to the parent compounds, on pancreatic cancer cells *in vitro*. Using two human pancreatic cancer cell lines, the metabolites were assessed for their effects on cell viability, apoptosis, and cell cycle progression. Among the shared metabolites, Oleu exhibited the highest plasma bioavailability and significantly inhibited cancer cell growth by reducing the cell cycle progression. VA-Sul and HVA-Sul also suppressed tumor cell proliferation, likely through non-apoptotic mechanisms. In conclusion, these findings underscore the therapeutic relevance of bioavailable phenolic metabolites and highlight the importance of evaluating metabolite-specific bioactivity in the development of plant-based interventions for pancreatic cancer.

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1. Introduction

Phenolic compounds, particularly those derived from plant sources, have gathered significant attention for their diverse bioactivities, including antioxidant, anti-inflammatory, and antitumor properties.[1,2] Among these, phenolic compounds found in *Lippia citriodora* (LC) and *Olea europaea* (OE) have shown promising antiproliferative effects. Despite their differing phytochemical profiles—LC being rich in glycosylated phenylpropanoids and iridoids (*e.g.*, verbascoside), and oleuropein (OE) in secoiridoids[3,4]—both extracts exhibit similar bioactivities, suggesting a potential role for shared bioactive metabolites.

Upon ingestion, these phenolic compounds undergo extensive metabolic transformations. Hydrolysis in the stomach, enzymatic activity in the intestines, and phase I and II metabolism in the liver lead to the formation of bioavailable metabolites not originally present in the extracts.[5] In our previous human intervention study, we identified nine such common metabolites in plasma following acute consumption of LC and OE extracts,[6] including vanillic acid sulfate (VA-Sul), homovanillic acid sulfate (HVA-Sul), and hydroxytyrosol glucuronide (HT-G). These metabolites are formed through processes such as sulfation, glucuronidation, and methylation, and may be responsible for the observed bioactivities of the parent extracts.

While the metabolic fate of phenolic compounds is well-characterized, the biological activity of their metabolites, particularly their potential anticancer effects, remains underexplored. Some phase II metabolites, such as glucuronide and sulfate derivatives of resveratrol and quercetin, have demonstrated anticancer properties in other contexts,[7,8] suggesting that similar metabolites from LC and OE may also exert antiproliferative effects.

This is particularly relevant in the context of pancreatic cancer, a highly aggressive and treatment-resistant malignancy. With a five-year survival rate of just 13% and nearly as

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many annual deaths (611 720) as new cases (2 001 140), pancreatic cancer ranks as the 6th leading cause of cancer-related deaths worldwide.[9] Current chemotherapeutic options offer limited benefit and are often associated with severe toxicity,[10] highlighting the urgent need for novel therapeutic strategies.

Building on our previous findings, the current study evaluated the anticancer potential of selected common metabolites (VA-Sul, HVA-Sul, and HT-G) identified in human plasma following ingestion of LC and OE extracts. With the goal of investigating the role of bioavailable phenolic metabolites as potential modulators of cancer-related pathways, we specifically assessed their anti-cell growth properties in the context of pancreatic cancer, quantified their concentrations in plasma and urine, and compared their bioactivity to that of their precursor compounds.

2. Materials and methods

2.1 Chemicals and reagents

LC-MS grade methanol and formic acid for mobile phases were procured from Fluka, Sigma-Aldrich (Steinheim, Germany). Water was purified utilizing a Milli-Q system from Millipore (Bedford, MA, USA). Ethanol and methanol (Fisher Scientific Madrid, Spain) for plasma treatment were of LC-MS grade. Oleuropein (≥98%), hydroxytyrosol (≥98%) and homovanillic acid (≥97%) were purchased from Sigma-Aldrich (St Louis, MO, USA) and a stock solution (100 mM) were prepared in sterile DMSO. Vanillic acid (≥97%) was purchased from Merck KGaA (Darmstadt, Germany) and a stock solution (100 mM) was prepared in sterile DMSO. 3′-Hydroxytyrosol 3′-glucuronide (≥98%) was purchased from TRC-Canada (Toronto, Ontario, Canada) and a stock solution (100 mM) was prepared in sterile DMSO. Homovanillic acid sulfate sodium salt (≥96%) and vanillic acid 4-sulphate sodium salt (≥97%) were purchased from TRC-Canada (Toronto, Ontario, Canada) and a stock solution (100 mM) were prepared in sterile water. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (≥97.5%), RIPA lysis buffer, Halt Protease Inhibitor Cocktail, and Phosphatase Inhibitor Cocktail were purchased from MilliporeSigma (St Louis, MO). SuperSignal™ West Dura Extended Duration Substrate were purchased from ThermoFisher Scientific (Waltham, MA). Bradford protein assay reagent, 30% (w/v) Acrylamide/Bis Solution, 4× Laemmli sample buffer, Immun-Blot Polyvinylidene difluoride (PVDF) Membranes and were purchased from Bio-Rad (Hercules, CA).

2.2 Selection of common metabolites derived from ingestion of LC and OE leaf extract

Following the acute nutritional intervention study in humans described in the previously published study,[6] the identification of common metabolites derived from the ingestion of nutraceuticals made from LC leaf extract and OE leaf extract allowed the identification of common metabolites using an untargeted metabolomics approach. Ethical approval for the study was obtained from the Ethics Committee of Miguel Hernández University of Elche and the General University Hospital of Elche (Alicante, Spain; reference PI 57/2019). All procedures were conducted in accordance with the Declaration of Helsinki, and all participants provided written informed consent prior to enrolment.

In that study, volunteers were divided into three groups: 8 consumed 500 mg of encapsulated LC leaf extract, 8 consumed 500 mg of encapsulated OE leaf extract and 9 consumed encapsulated placebo. Before ingesting the capsule, a nurse inserted a cannula into the ulnar vein of each volunteer's non-dominant arm, and baseline blood samples were taken. Additional blood samples were collected at 0.5, 1, 2, 4, 6, 8, and 10 hours after consuming the 500 mg capsule. Plasma was separated through centrifugation (10 min at 3000 rpm and 4 °C) and stored at −80 °C until further analysis. Urine samples were collected in plastic containers at baseline (prior to capsule ingestion, $t = 0$) and at two subsequent time intervals following supplement intake: interval 1 (0–6 h) and interval 2 (6–12 h). Additionally, volunteers provided a 24-hour urine sample, marking the conclusion of the urine sample collection.

In relation to the results obtained by the previous work,[6] the common metabolites detected in plasma following the ingestion of the two plant matrices were selected to quantify the selected compounds. The selection of the metabolites for the cellular assays was based on the availability of their commercial standards. Thus, among the common metabolites identified in the previous work, HT-G, VA-Sul and HVA-Sul were selected. In addition to these three, the metabolites Oleu, HT, VA, and HVA were also selected and analyzed. These metabolites were chosen to compare their bioactivity, as they are generally present in the extract or are the result of the breakdown of compounds found in the extract, with their selected glucuronidated/sulfated derivatives.

2.3 Quantification of target metabolites in plasma and urine samples

Plasma samples were initially treated using a mixture of methanol and ethanol (50 : 50; v : v) to remove proteins, following previously reported protocols.[11] Urine samples were normalized to a standardized osmolality value of 100 mOsm kg^{-1} using Milli-Q water, measured with an OSMOMAT 3000 (Gonotec, Berlin, Germany). Samples were centrifuged for 10 min at 14 800 rpm and 4 °C and 60 µl of supernatant was taken in an HPLC vial and stored at −80 °C until the day of analysis.

The metabolites Oleu, HT, HT-G, VA, VA-Sul, HVA and HVA-Sul present in plasma and urine samples were quantified using an analytical method based on high performance liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS). Briefly, high performance liquid chromatography (Agilent 1290 HPLC, Agilent Technologies, Palo Alto, CA, USA) coupled to mass spectrometry with a quadrupole time-of-flight analyzer (Agilent 6545 QTOF Ultra High Definition, Agilent Technologies, Palo Alto, CA, USA) was used. Chromatographic

analysis was performed in reversed phase using a C18 ACQUITY UHPLC BEH column (1.7 μm, 2.1 mm, 150 mm, 130 Å, Waters Corporation, Milford, MA, USA). The mobile phases were (A) water acidified with 0.1% formic acid (v/v) and (B) acetonitrile. The following mobile phase gradient was used for optimal separation: 0.00 min [A : B 100/0], 5 min [A : B 90/10], 18 min [A : B 15/85], 24 min [A : B 0/100], 25.50 min [A : B 0/100], 26.50 min [A : B 95/5] and 32.50 min [A : B 95/5]. Finally, the acquired data were processed using MassHunter workstation software version B.10.0 (Agilent Technologies) and MZmine 3.9.0.

The compounds were quantified using calibration curves prepared with their corresponding analytical standards. Different dilutions (0.01–500 μM) of the analytical standards were prepared from a pooled mixture with a concentration of 100 mM per standard.

Based on the previous study in which common bioavailable metabolites were detected in biological samples using an untargeted metabolomic approach,[6] plasma and urine samples from volunteers with the highest and lowest relative content, were selected for quantification. This approach will enable the establishment of the maximum and minimum concentration values of the metabolites of interest within the recruited cohort.

Metabolite nutrikinetics (C_{max} and AUC) were studied using PKSolver, an add-in program for pharmacokinetic dana analysis in Microsoft Excel.[12]

2.4 Cell culture

Human pancreatic cancer cell lines (Panc-1 and MIA PaCa-2) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin. Cells were maintained in a 5% (v/v) CO_2, humidified atmosphere at 37 °C. These cells were grown as monolayers under conditions suggested by ATCC. Both cell lines were characterized by cell morphology and growth rate and passaged in our laboratory less than 6 months after being received.

2.5 Cell viability

After treating cells with the different compounds for 24, 48 and 72 h. The concentrations of the selected metabolites used in the *in vitro* assays (5–20 μM) were selected based on the maximum plasma concentrations (C_{max}) observed in the human intervention study following a single 500 mg dose of *Lippia citriodora* or *Olea europaea* extracts (see section 3.1, Table 1). Although some of the tested concentrations are higher than the measured plasma levels, these concentrations allow for the assessment of concentration–response relationships, mechanistic pathways, and scenarios reflecting potential tissue accumulation or repeated intake.

The reduction of MTT dye was determined according to the manufacturer's protocol (MilliporeSigma, St Louis, MO, USA).

2.6 Western blot

Following treatment with compounds for 24 hours, cells were lysed, and total cell fractions were obtained according to previously described methods.[13] Aliquots of total fractions containing 10–30 μg protein were separated using 10% (w/v) polyacrylamide gel electrophoresis and electroblotted onto PVDF membranes. After blocking with the "EveryBlot" Blocking Buffer (Bio-Rad, Hercules, CA) for 5 minutes, membranes were probed overnight with the following primary antibodies (1 : 1000 dilution) from Cell Signaling Technology (Danvers, MA): Cleaved Caspase-3 (Cat #9664), Caspase-3 (Cat #14220), Cleaved PARP (Cat #9541), PARP (Cat #9542), Cyclin E1 (Cat #20808), Bcl-xL (Cat #2802), XIAP (Cat #14334), Survivin (Cat #2808), p-ERK1/2 (Cat #4370), and ERK1/2 (Cat #9102). β-Actin (Cat #8457) was used at the same time as a loading control. After incubation for 60 min at room temperature in the presence of the secondary antibody (HRP-conjugated; 1 : 2500 dilution), the conjugates were developed and visualized using

Table 1 Nutrikinetic parameters of selected metabolites detected in human plasma after consumption of Lippia citriodora and Olea europaea extracts

			LC			OE		
RT (min)	$[M-H]^-$	Metabolite	N	C_{max} (μM) range	AUC (μM h^{-1}) range	n	C_{max} (μM) range	AUC (μM h^{-1}) range
2.92	329.0055	HT-G isomer 1	—	N.D.	N.D.	8	0.37–1.17	0.65–2.61
2.96	153.0553	HT isomer 1[a]	5	<LOQ–0.17	<LOQ–0.81	2	<LOQ–0.16	<LOQ–1.07
3.29	329.0050	HT-G isomer 2[a]	4	<LOQ–0.07	<LOQ–0.34	8	0.87–2.23	2.30–5.06
6.25	181.0508	HVA isomer 2	2	<LOQ–0.45	<LOQ–1.41	3	<LOQ–0.35	<LOQ–0.67
6.32	246.9921	VA-Sul isomer 1[a]	7	<LOQ–0.74	<LOQ–3.37	8	0.22–0.31	1.69–1.69
6.70	246.9910	VA-Sul isomer 2	5	<LOQ–0.07	<LOQ–0.63	—	N.D.	N.D.
8.37	261.0071	HVA-Sul isomer 1[a]	7	<LOQ–0.15	<LOQ–0.84	8	<LOQ–0.10	0.10–0.76
9.10	261.0075	HVA-Sul isomer 2	5	<LOQ–0.15	<LOQ–0.81	8	<LOQ–0.08	0.13–0.72
10.34	539.1795	Oleu isomer 1[a]	—	<LOQ	N.D.	3	<LOQ–6.90	<LOQ–28.39
10.53	539.1787	Oleu isomer 2	—	<LOQ	N.D.	2	<LOQ–1.40	<LOQ–4.09

RT: retention time; *n*: number of volunteers in whom the metabolite appears after ingestion of the *L. citriodora* (LC) or *O. europaea* (*OE*) extracts; C_{max}: relative maximum plasma level; AUC: area under the zero-moment curve; N.D.: not detected; LOQ: limit of quantification. [a] Isomer corresponding in retention time to that of the analytical standard used and selected for evaluation in the *in vitro* cell line assays; Oleu: oleuropein; HT: hydroxytyrosol; HT-G: hydroxytyrosol glucuronide; VA-Sul: vanillic acid sulfate; HVA: homovanillic acid; HVA-Sul: homovanillic acid sulfate.

a Molecular Imager FX™ System (BioRad; Hercules, CA) and analyzed using ImageJ software (V1.54, NIH, Bethesda, MD, USA).

2.7 Statistical analysis

The data, derived from a minimum of three independent experiments, were presented as mean ± standard deviation (SD). Statistical analysis was conducted using unpaired *t*-test or one-way ANOVA followed by Tukey's test for multiple comparisons with GraphPad Prism version 8.01 (GraphPad Software, San Diego, CA, USA). A p-value < 0.05 was considered statistically significant.

3. Results

3.1 Quantification of metabolites in plasma and urine samples

The analysis of results from our previous study, which employed an untargeted metabolomic approach based on HPLC-ESI-QTOF-MS, led to the detection of 66 circulating metabolites, including 9 common to both extracts, such as homovanillic acid sulfate and its glucuronide derivatives, hydroxytyrosol sulfate, among others. Among the 9 common metabolites, HT-G, VA-Sul and HVA-Sul were selected for analysis. Plasma and urine samples from volunteers, selected based on the highest and lowest relative abundance of metabolites detected in the non-targeted study,[6] were chosen in this study for quantification. Additionally, plasma concentrations of Oleu, HT, and HVA were also quantified. Although these compounds were not reported in the previous untargeted study, they were detected in plasma samples but in less than 50% of the recruited volunteers. For each analytical standard, the calibration range, the limits of detection (LOD) and quantification (LOQ) and the coefficient of determination (R^2) were calculated (Table S1), showing good linearity ($R^2 > 0.99$).

Table 1 shows the results of the nutrikinetics values of the metabolites present in plasma after ingestion of LC and OE extracts in an acute nutritional intervention study. This table includes the ranges of the AUC and C_{max} parameters, calculated from the study samples with the highest and lowest values. For those signals that were not previously detected in all eight volunteers of each group, the lower limit of the range was considered the LOQ value.

In the present study, the selected metabolites displayed T_{max} trends consistent with those reported in the previous study,[6] exhibiting significantly different T_{max} values depending on their source, suggesting distinct metabolization pathways for each extract.

In the volunteers who consumed LC, the metabolite with the highest plasma concentration was VA-Sul isomer 1, followed by HVA isomer 2. On the other hand, in the volunteers who ingested OE, Oleu, which is the main phenolic compound present in the extract in five different isomeric forms,[14] proved to be the most bioavailable metabolite, with the highest concentration detected in plasma. However, the two isomers of Oleu detected in plasma were present in only 2 and 3 volunteers, respectively. After Oleu, the two isomers of HT-G were the most abundant metabolites in plasma. Both isomers were detected in all eight volunteers who ingested the OE extract, indicating that their elevated plasma concentrations are consistently associated with extract intake.

Following a similar approach to that used for plasma samples, the selected metabolites were also quantified in urine samples to assess their excretion levels. Table 2 shows the range of concentrations between the subjects with the highest and lowest levels of the quantified metabolites in urine collected over 24 hours after ingestion of the LC and OE extracts.

Notably, although Oleu is the most abundant compound in the OE extract (258 ± 37 and 8.4 ± 0.7 μmol g^{-1} dry extract for isomer 1 and 2, respectively), its urinary excretion was very low, suggesting that Oleu undergoes significant metabolism before

Table 2 Selected metabolites excreted in urine (from 0 to 24 h) by healthy volunteers after consumption of Lippia citriodora and Olea europaea extracts

			LC	OE
RT (min)	$[M - H]^-$	Metabolite	Total 24 h (μmol) range	Total 24 h (μmol) range
2.92	329.0055	HT-G isomer 1	2.02–3.96	15.57–25.31
2.96	153.0553	HT isomer 1[a]	0.09–0.28	1.71–1.93
3.61	153.0541	HT isomer 2	1.34–1.38	0.47–2.02
3.29	329.0050	HT-G isomer 2[a]	4.67–10.50	27.20–63.87
5.41	181.0508	HVA isomer 1[a]	8.04–16.21	0.81–6.44
6.25	181.0508	HVA isomer 2	114.50–151.18	71.59–163.90
6.32	246.9921	VA-Sul isomer 1[a]	7.86–80.97	1.44–59.86
6.70	246.9910	VA-Sul isomer 2	75.60–133.15	0.43–41.48
6.92	167.0345	VA[a]	3.96–5.33	2.58–8.97
8.37	261.0071	HVA-Sul isomer 1[a]	16.22–24.55	11.07–26.62
9.10	261.0075	HVA-Sul isomer 2	2.57–5.74	2.71–6.65
10.34	539.1795	Oleu isomer 1[a]	<LOQ	0.06–0.44

RT: retention time; LC: *L. citriodora*; OE: *O. europaea*; LOQ: limit of quantification. [a] Isomer corresponding in retention time to that of the analytical standard used and selected for evaluation in the *in vitro* cell line assays; Oleu: oleuropein; HT: hydroxytyrosol; HT-G: hydroxytyrosol glucuronide; VA: vanillic acid; VA-Sul: vanillic acid sulfate; HVA: homovanillic acid; HVA-Sul: homovanillic acid sulfate.

excretion. In contrast, the metabolite with the highest excretion was HVA isomer 2 for both LC and OE extracts.

In the case of the two isomers of HT and the two isomers of its glucuronidated form, the excreted content was higher after consumption of the OE extract compared to the consumption of the LC extract, which may be due to differences in the concentration content of the original compounds in the extracts.

Furthermore, it was observed that VA is predominantly excreted in its sulfated form in both the LC and OE extracts, with particularly high levels associated with the consumption of the LC extract. Conversely, HVA was excreted in larger quantities in its non-sulfated form.

3.2 Effect of LC and OE metabolites on cell growth of pancreatic cancer lines

To test the growth-inhibitory effect of the metabolites compounds (Oleu, HT, HT-G, VA, VA-Sul, HVA and HVA-Sul) on cancer cell growth, we treated, two human pancreatic cancer cell lines (Panc-1 and MIA PaCa-2), with three concentrations of each of the metabolites (5, 10 and 20 μM) for 24, 48 and 72 h (Fig. 1).

In the case of the Oleu metabolite, the lowest percentage of cell growth was observed for the Panc-1 line at a concentration of 10 μM in the 24 h treatment. In addition, the cell growth inhibitory effect of the Oleu metabolite was the strongest when incubated with it for 24 h, compared to 48 and 72 h (Fig. 1A).

When comparing the effect on cell growth of the metabolites HT and HT-Glu, we observed that for HT-Glu, there were no significant differences in cell growth compared to the vehicle-treated controls at any of the concentrations or incubation times, tested, in either cell line. In contrast, HT exerted a significant reduction in cell growth in both cell lines for all three concentrations, especially showing a greater reduction at the 24-hour time point across the concentrations (Fig. 1B).

For the VA and VA-Sul metabolites, the former exerted no significant effect on cell growth compared to the control for any of the concentrations or incubation times in either cell line, except for the concentration of 10 μM at 24 h. While VA-Sul does exert a significant effect decreasing cell growth with respect to the control in general at the three concentrations tested in both cell lines, being the most significant for 20 μM at 72 h for both Panc-1 and MIA Paca-2 (Fig. 1C).

Finally, when considering the results obtained for HVA, it generally does not exert a significant effect compared to the control for both cell lines at the various concentrations tested and incubation times, except for 20 μM at 24 hours in Panc-1 and 10 μM at 48 hours in MIA Paca-2. In contrast, its sulfated form, HVA-Sul, does exhibit a significant overall effect in

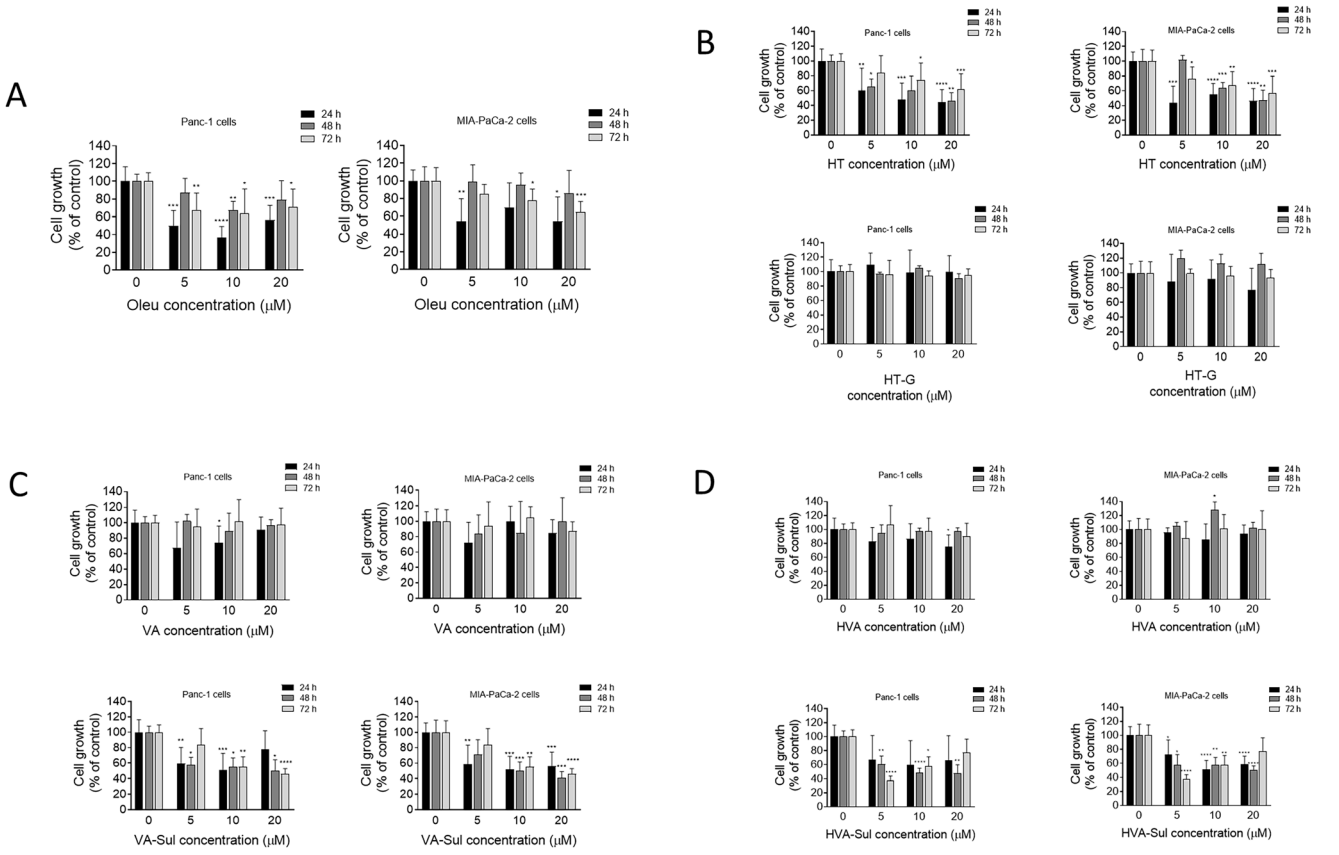

Fig. 1 Effect of the compounds oleuropein (Oleu) (A), hydroxytyrosol (HT) (B), hydroxytyrosol glucuronide (HT-G) (B), vanillic acid (VA) (C), vanillic acid sulfate (VA-Sul) (C), homovanillic acid (HVA) (D) and homovanillic acid sulfate (HVA-Sul) (D) on cell growth in Panc-1 and MIA PaCa-2 cells (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

decreasing cell growth compared to the control for both cell lines, with the most notable effect observed at 5 μM at 24 hours for both cell lines. Given the obtained results, we chose the concentration of 10 μM for 24 hours cell lines, for the subsequent studies (Fig. 1D).

3.3 Effect of LC and OE common metabolites on apoptosis-related protein expression

To explore the mechanism of metabolites-induced apoptosis, we determined, in Panc-1 and MIA PaCa-2 cells, the expression levels of multiple proteins that regulate apoptosis, including proteins of the inhibitor of apoptosis protein (XIAP) and Bcl-2 family.

As shown in Fig. 2, Oleu was the metabolite with the highest proapoptotic activity reducing Bcl-xl and survivin levels, but not XIAP, in both cell lines. Like Oleu, VA also exerted a proapoptotic effect, reducing Bcl-xl and survivin levels without affecting XIAP expression. However, unlike Oleu, this effect was observed only in the Panc-1 cell line. In the case of HT, it only exerted a significant effect on the reduction of Bcl-xl for the MIA PaCa-2 cell line, while its glucuronidated form exerted a negative regulatory effect on Bcl-xl for the Panc-1 cell line and on XIAP for the MIA PaCa-2 cell line.

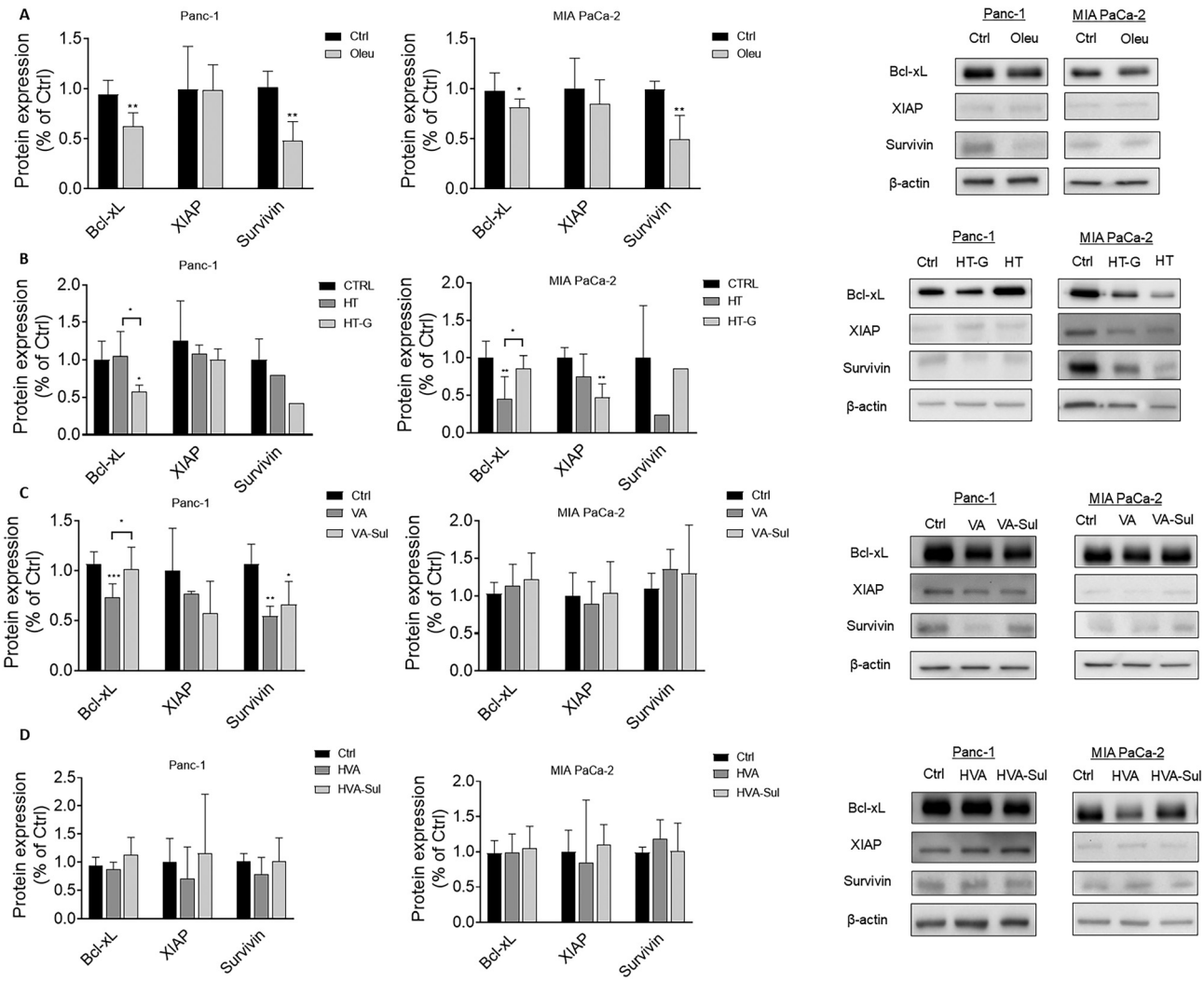

Fig. 2 Effect of the compounds oleuropein (Oleu), hydroxytyrosol (HT), hydroxytyrosol glucuronide (HT-G), vanillic acid (VA), vanillic acid sulfate (VA-Sul), homovanillic acid (HVA) and homovanillic acid sulfate (HVA-Sul) on apoptosis-related protein expression. (A) Immunoblots for Bcl-xL, XIAP, and survivin in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μM of Oleu for 24 h. Loading control: β-Actin. Bands were quantified and results are expressed as percentage of control. *$p < 0.05$, **$p < 0.01$ *vs.* control. (B) Immunoblots for Bcl-xL, XIAP, and survivin in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μM of HT and HT-G for 24 h. Loading control: β-Actin. Bands were quantified and results are expressed as percentage of control. *$p < 0.05$, **$p < 0.01$ *vs.* control. (C) Immunoblots for Bcl-xL, XIAP, and survivin in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μM of VA and VA-Sul for 24 h. Loading control: β-Actin. Bands were quantified and results are expressed as percentage of control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs.* control. (D) Immunoblots for Bcl-xL, XIAP, and survivin in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μM of HVA and HVA-Sul for 24 h. Loading control: β-Actin. Bands were quantified and results are expressed as percentage of control.

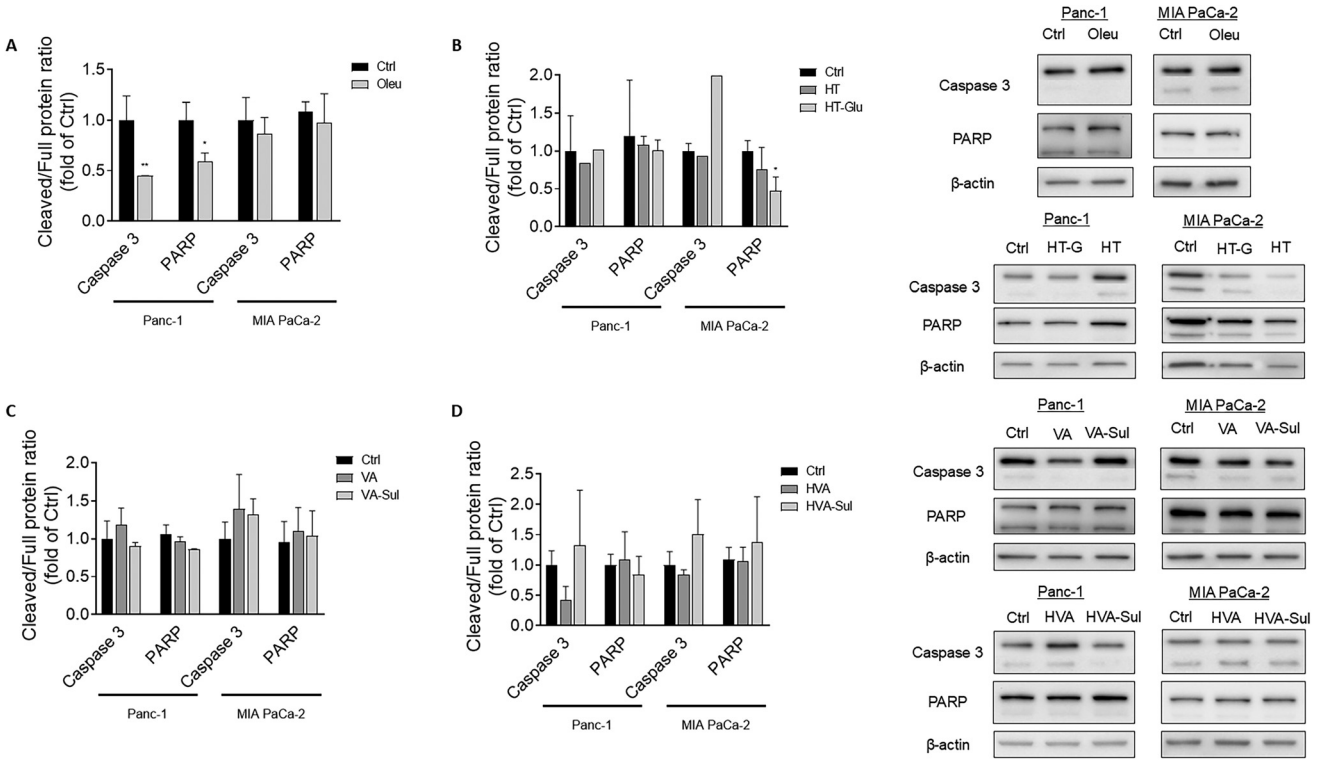

Fig. 3 Effect of the compounds oleuropein (Oleu), hydroxytyrosol (HT), hydroxytyrosol glucuronide (HT-G), vanillic acid (VA), vanillic acid sulfate (VA-Sul), homovanillic acid (HVA) and homovanillic acid sulfate (HVA-Sul) on apoptosis-related protein expression. (A) Immunoblots for full length and cleaved caspase 3 as well as full length and cleaved PARP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μM of Oleu for 24 h. Loading control: β-Actin. Bands were quantified and results are shown as the ratio between the cleaved/full length protein. $*p < 0.05$, $**p < 0.01$ *vs.* control. (B) Immunoblots for full length and cleaved caspase 3 as well as full length and cleaved PARP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μM of HT and HT-G for 24 h. Loading control: β-Actin. Bands were quantified and results are shown as the ratio between the cleaved/full length protein. $*p < 0.05$ *vs.* control. (C) Immunoblots for full length and cleaved caspase 3 as well as full length and cleaved PARP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μM of VA and VA-Sul for 24 h. Loading control: β-Actin. Bands were quantified and results are shown as the ratio between the cleaved/full length protein. (D) Immunoblots for full length and cleaved caspase 3 as well as full length and cleaved PARP in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μM of HVA and HVA-Sul for 24 h. Loading control: β-Actin. Bands were quantified and results are shown as the ratio between the cleaved/full length protein.

In order to test whether Oleu and VA mediated activation of apoptosis in pancreatic cancer cell lines was exerted through activation of caspases pathways, the level of apoptosis-related caspase 3 was assessed by western blot. As shown in Fig. 3, there was no activation of caspase 3 for both cell lines by either Oleu or VA, even in Panc-1 the activation was significantly decreased by the action of Oleu. Consequently, cleaved poly(ADP-ribose) polymerase (PARP) levels followed the same pattern as for caspase 3.

These results suggest that the Oleu-mediated decrease in cell growth in both cell lines and the VA-mediated decrease in cell growth in Panc-1 occur through activation of cell apoptosis, but that apoptosis is not mediated by activation of the caspases pathway.

3.4 Effect of LC and OE common metabolites on cell cycle progression

Next, we evaluated whether the metabolites could influence cell cycle progression by examining the expression of the cell cycle regulator Cyclin E1 using western blot analysis. Oleu treatment led to a significant reduction in Cyclin E1 levels in both Panc-1 and MIA Paca-2 cell lines, while VA treatment significantly decreased Cyclin E1 levels only in Panc-1 cells (Fig. 4).

It is noteworthy that both VA-Sul and HVA-Sul despite having an overall negative regulatory effect on cell growth for both cell lines do not affect cell growth through activation of apoptosis or cell cycle regulation.

4. Discussion

This study provides evidence that the biological activity of OE and LC extracts is largely mediated by their metabolites rather than the parent compounds themselves. The detection of key metabolites in plasma and urine—rather than the original phenolic compounds—highlights the critical role of metabolic

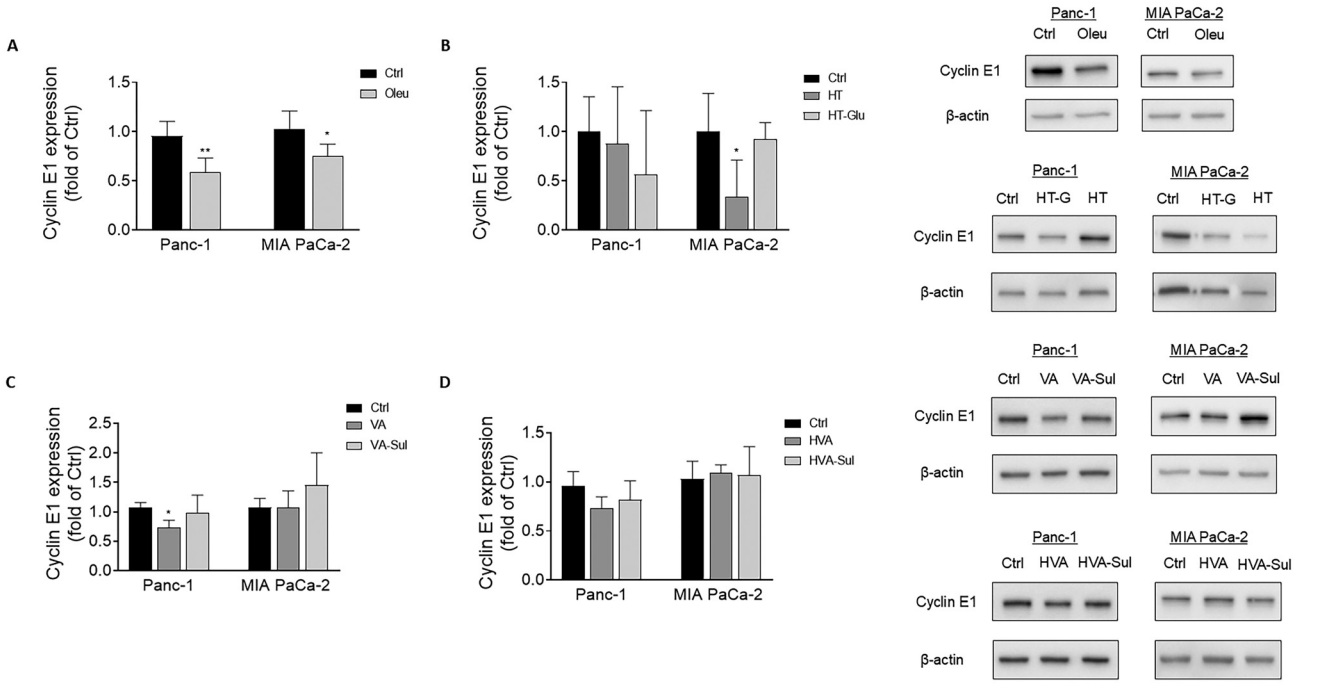

Fig. 4 Effect of the compounds oleuropein (Oleu), hydroxytyrosol (HT), hydroxytyrosol glucuronide (HT-G), vanillic acid (VA), vanillic acid sulfate (VA-Sul), homovanillic acid (HVA) and homovanillic acid sulfate (HVA-Sul) in cell cycle arrest in pancreatic cancer cells. (A) Immunoblots for cyclin E1 in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μM of Oleu for 24 h. Loading control: β-Actin. Bands were quantified and results are expressed as percentage of control. *$p < 0.05$, **$p < 0.01$ *vs.* control. (B) Immunoblots for cyclin E1 in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μM of HT and HT-G for 24 h. Loading control: β-Actin. Bands were quantified and results are expressed as percentage of control. *$p < 0.05$ *vs.* control. (C) Immunoblots for cyclin E1 in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μM of VA and VA-Sul for 24 h. Loading control: β-Actin. Bands were quantified and results are expressed as percentage of control. *$p < 0.05$ *vs.* control. (D) Immunoblots for cyclin E1 in total cell protein extracts from Panc-1 and MIA PaCa-2 cells treated with 10 μM of HVA and HVA-Sul for 24 h. Loading control: β-Actin. Bands were quantified and results are expressed as percentage of control.

transformation in shaping the bioactivity and systemic availability of these phytochemicals.

Particularly, major extract constituents such as verbascoside and Oleu were either undetectable or present at very low levels in biological samples. For instance, verbascoside, the predominant compound in LC extract, was absent in both plasma and urine, suggesting complete metabolic conversion. This aligns with the detection of downstream metabolites such as HVA and VA-Sul, which originate from the HT and caffeic acid moieties of verbascoside, respectively.[6]

Similarly, although Oleu was detected in plasma following OE extract consumption—particularly isomer 1—it was only present in a subset of volunteers and was minimally excreted in urine. This supports previous findings[15] indicating that Oleu undergoes extensive metabolism, with its aglycone derivatives appearing in conjugated forms in urine. These results reinforce the notion that the health effects of phenolic-rich extracts are mediated by their metabolites, not the parent compounds.

The higher concentrations of metabolites observed in both plasma and urine following OE extract consumption, compared to LC, can be attributed to the greater abundance of precursor compounds in OE. This is supported by prior quantification of HT equivalents, which were significantly higher in OE extract (328 ± 42 μmol g^{-1}) than in LC (219 ± 37 μmol g^{-1}).[6] Consequently, OE extract yielded higher levels of HT- and Oleu-derived metabolites, while LC extract favored the production of HVA and VA derivatives.

Importantly, individual variability in metabolite profiles was evident. For example, HVA isomer 2 was detected in only a few volunteers across both extract groups. This interindividual variability likely reflects differences in gut microbiota composition, genetic polymorphisms, sex, age, BMI, and other physiological factors.[16] Sex-specific differences in Oleu metabolism have also been reported, with women showing higher plasma Oleu levels and men exhibiting greater concentrations of HT conjugates.[17,18]

Interindividual variability in metabolite detection and plasma concentrations was evident among volunteers, which may influence, to some extent, systemic exposure and biological efficacy. This variability is a common feature in studies of dietary (poly)phenols and is largely attributable to differences in gut microbiota composition and metabolic capacity, which shape individual metabotypes.[19] Furthermore, genetic polymorphisms affecting metabolizing enzymes and transporters may also account for differential absorption and conjugation of phenolic compounds.[16] Lifestyle, diet, age, and sex-related factors may further contribute to the observed variability.

The intestinal microbiota is another factor that can shape the metabolic fate of olive-derived phenolics, including oleuropein. Oleuropein has been reported to modulate gut microbial composition, promoting beneficial bacterial taxa and metabolic activity.[20] In turn, gut microbial biotransformation, together with hepatic metabolism, generates bioactive metabolites such as hydroxytyrosol and vanillic acid derivatives, which are the focus of this study.[21] This bidirectional interaction between phenolic compounds and the gut microbiota further reinforces the translational relevance of investigating circulating metabolites, rather than parent compounds, as drivers of biological effects. Overall, these findings stress the need to account for metabolic and microbiota-related processes to properly interpret the bioactivity of phenolic metabolites, supporting the relevance of the *in vitro* assays conducted in this study.

A main finding of this study was that OE and LC extracts are valuable sources of phenolic compounds that, upon ingestion, are metabolized into bioavailable derivatives with significant anticancer potential. Among the metabolites evaluated, Oleu exhibited the strongest antiproliferative activity against pancreatic cancer cells. Its high plasma concentration following OE extract intake, despite minimal urinary excretion, suggests extensive metabolism and systemic availability. Mechanistically, Oleu induced apoptosis through a caspase-independent pathway, likely involving mitochondrial mediators such as AIF and endonuclease G—an important distinction from previously reported caspase-dependent mechanisms in other cancer types.[22] Additionally, Oleu inhibited the G1/S cell cycle transition by downregulating Cyclin E1, reinforcing its role in disrupting key proliferative checkpoints. However, it is important to note that Oleu's therapeutic potential is challenged by its metabolic instability. Encapsulation strategies, such as sodium alginate-based microencapsulation, have shown promise in protecting Oleu during digestion and enhancing its bioaccessibility and bioavailability, offering a viable approach to improve its clinical utility.[23,24] Beyond these approaches, recent studies have explored advanced delivery platforms to overcome Oleu's pharmacokinetic limitations. For instance, nanostructured lipid carriers (NLCs) have demonstrated high encapsulation efficiency (>99%), sustained release profiles, and improved antioxidant and anti-inflammatory efficacy in lung epithelial and colitis models.[25,26] Liposomal formulations have further improved Oleu's stability and solubility without compromising its biological activity.[27] Additionally, PEGylated nano-phytosomes combining Oleu and rutin achieved significantly enhanced anticancer efficacy (IC_{50} = 0.14 μM) in colon cancer cell models compared to free compounds.[28] More recently, intranasally administered NLCs have been shown to deliver Oleu effectively to the brain, offering sustained release and potential therapeutic benefits for central nervous system applications.[29] Collectively, these findings highlight the translational relevance of advanced encapsulation and delivery systems to enhance Oleu's stability, bioavailability, and therapeutic potential.

Although VA-Sul and HVA-Sul significantly reduced pancreatic cancer cell viability, they did not modulate apoptotic markers or Cyclin E1 expression, suggesting that their growth-inhibitory effects may occur through alternative mechanisms. Potential pathways include the induction of autophagy, modulation of oxidative stress, triggering of cellular senescence, or disruption of mitochondrial function. For instance, phenolic compounds such as curcumin and resveratrol have been reported to reduce cancer cell proliferation through autophagy-dependent mechanisms.[30,31] While further studies are required to delineate the precise pathways for VA-Sul and HVA-Sul, these observations suggest the potential involvement of non-apoptotic processes in their anticancer activity.

The observed antiproliferative effects of these metabolites can be partially contextualized by their systemic exposure. For instance, VA-Sul and HVA-Sul reached C_{max} values up to 0.74 μM and 0.15 μM, respectively, while Oleu isomers were detected at higher plasma concentrations (up to 6.90 μM, AUC up to 28.39 μM h^{-1}). These data suggest that metabolites with higher bioavailability may exert more pronounced growth-inhibitory effects, although compound-specific mechanisms, such as modulation of autophagy, oxidative stress, or mitochondrial function, are likely also involved.

Despite the fact that the metabolites studied are physiologically relevant and naturally present in human plasma after consumption of olive and lemon verbena extracts—ingredients with a long history of safe dietary use—the lack of a non-tumor pancreatic cell line limits the ability to directly assess selectivity and safety *in vitro*. Future studies should incorporate such models to further validate these findings.

These *in vitro* findings suggest potential antiproliferative effects; however, translation to clinical efficacy requires further investigation in appropriate *in vivo* models and human studies. While the current study provides valuable insights, several limitations should be acknowledged. First, the experiments were limited to two pancreatic cancer cell lines, which may not fully reflect the heterogeneity of the disease. Second, no *in vivo* validation was performed, so extrapolation of these findings to clinical settings remains uncertain. Finally, interindividual variability in metabolite bioavailability and cellular responses—potentially influenced by differences in metabolism, gut microbiota, or genetic polymorphisms—could affect the systemic efficacy of these compounds. Recognizing these limitations allows for a more balanced interpretation of the results and highlights key directions for future research.

In conclusion, we identified Oleu, VA-Sul, and HVA-Sul as bioavailable phenolic metabolites with distinct and potent anticancer effects in pancreatic cancer cells. These findings underscore the importance of evaluating circulating metabolites, rather than parent compounds, when assessing the bioactivity of plant-based extracts. The observed interindividual variability in metabolite profiles—driven by factors such as gut microbiota, genetics, and physiological status—further highlights the complexity of phenolic metabolism and its implications for therapeutic outcomes. Future research should focus on: (a) characterizing the mechanisms of action of these and other metabolites; (b) enhancing their stability and delivery through formulation technologies, and (c) exploring their

efficacy *in vivo* and across diverse cancer models. Such efforts are deemed essential for translating the health benefits of dietary polyphenols into effective, targeted strategies for cancer prevention and treatment.

Abbreviations

AIF	Apoptosis-inducing factor
ATCC	American type culture collection
AUC	Area under the zero-moment curve
Bcl-2	B-cell lymphoma-2
Bcl-xl	B-cell lymphoma-extra-large
BMI	Body mass index
Caspase	Cysteinyl aspartate specific proteinase
C_{max}	Maximum plasma level
DMEM	Dulbecco's modified Eagle's medium
FBS	Fetal bovine serum
HPLC-ESI-QTOF-MS	High performance liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry
HT	Hydroxytyrosol
HT-G	Hydroxytyrosol glucuronide
HVA	Homovanillic acid
HVA-Sul	Homovanillic acid sulfate
LC	Lippia citriodora
LOD	Limit of detection
LOQ	Limit of quantification
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OE	Olea europaea
Oleu	Oleuropein
PARP	Cleaved poly(ADP-ribose) polymerase
PVDF	Polyvinylidene difluoride
SD	Standard deviation
VA	Vanillic acid
VA-Sul	Vanillic acid sulfate
XIAP	X-linked inhibitor of apoptosis protein

Author contributions

María del Carmen Villegas-Aguilar: conceptualization, methodology, investigation, data curation, formal analysis, writing – original draft; María de la Luz Cádiz-Gurrea: conceptualization, methodology, formal analysis, writing – review & editing, supervision; Patricia Fernández-Moreno: methodology, investigation, data curation, formal analysis; Álvaro Fernández-Ochoa: conceptualization, methodology, investigation, software, formal analysis, writing – review & editing, supervision; David Arráez-Román: conceptualization, methodology, resources, supervision, project administration, funding acquisition, writing – review & editing; Antonio Segura-Carretero: resources, supervision, project administration, funding acquisition; Gerardo G. Mackenzie: conceptualization, methodology, resources, supervision, project administration, funding acquisition, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the SI.

Supplementary materials include the original, uncropped Western blot data. See DOI: **https://doi.org/10.1039/d5fo02688a**.

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