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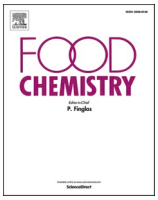

Isolation and structural determination of *cis*- and *trans-p*-coumaroyl-secologanoside (comselogoside) from olive oil waste (alperujo). Photoisomerization with ultraviolet irradiation and antioxidant activities

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ABSTRACT

p-Coumaroyl-ó-secologanoside (comselogoside) is a secoiridoid identified in large amounts in olive fruits, although no studies *in vitro* or *in vivo* of comselogoside have been reported. This work focuses on the recovery and purification of this compound from olive mill waste (alperujo). The successive isolation on Amberlite XAD-16 and Sephadex LH-20 resins, allowed a comselogoside extract with 80–85% of purity. A photoisomerization of the vinyl-double bond in the p-coumaroyl moiety occurred when the extract was exposed to ultraviolet radiation and a mixture of the *trans* and *cis*-isomers was obtained. Both isomers were characterized using NMR, mass spectroscopy, and UV spectrometry. The *J* (coupling constant) of the protons on the C7 and C8 on the unsaturated chain were found to be the difference between *cis* (12.8 Hz) and *trans*- (15.9 Hz) comselogoside. *Cis*-isomer exhibited lower radical-scavenging activity than *trans*, although a synergistic effect occurred when the *cis*-isomer was supplement by the *trans*-isomer.

1. Introduction

The olive oil sector is not only a very important part of the food industry in the Mediterranean area but also an essential element for improving our diet. The properties of olive oil are based on the composition of fatty acids which are rich in oleic acid, and above all, on the presence of minor components, among which phenolic compounds are the main ones. In the extraction of olive oil only 1–2% of phenols pass into the oil, with the rest (98–99%) remaining in the by-product which is generated, which is the so-called alperujo (olive pomace). The annual production of alperujo exceeds 5–7 million tons per year, and can be considered an important source of phenolic compounds which have been recognized for their therapeutic and nutritional properties as potent antioxidants and antimicrobials associated with the prevention of cardiovascular disease and cancer (Toledo et al., 2015). Among them, the comselogoside stands out. It is a type of secoiridoid, derivative of p-coumaric acid, identified as the p-coumaroyl ester of the secologanoside, which is present in large amounts in olive pulp (Fernández-Poyatos et al., 2021; D'Antuono et al., 2018; García et al., 2018) and has been identified in olive pomace (Obied et al., 2007; Innocenti, et al., 2006; Nunes et al., 2018). It is an iridoid glycoside with an exocyclic double bond between carbon atoms C^8 and C^{10} with a difference in oleuropein and ligstroside with a C═C bond between C^8 and C^9. Comselogoside has also been found in significant amounts in leaves from olive trees subjected to stress due to boron deficiency, which is the most frequent micronutrient disorder in olive orchards with oxidative damage accumulated in their cells (Karioti et al., 2006). However, in both cases, leaves and fruits of the olive tree, the amounts of phenolic compounds depend on various environmental factors such as ripening cycle, geographical origin, cultivation technique and olive tree variety (Malheiro et al., 2015). There is little or none information about the biological properties of this molecule, although some research has revealed that it has substantial antioxidant activity (Obied et al., 2007). A family of iridoid glycosides, including loganin and secoxyloganin, close to comsegoloside, has been found to have a number of health promoting properties, including neuroprotective, anti-inflammatory,

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hepatoprotective, and anticarcinogenic activities (Wang et al., 2020) (Przybylska et al., 2022). Furthermore, hydroxycinamic acid derivatives, especially p-coumaric acid and its conjugates with mono, oligo and polysaccharides, alkyl alcohols, organic acids, and lignin, exhibit interesting biological properties, including antioxidant, anti-cancer, antivirus, anti-inflammatory, antiplatelet aggregation, and anti-arthritis activities, and have been shown to be effective against diabetes, obesity, hyperlipaemia, and liver necrosis and cholestasis (Pei et al., 2016; Kaur & Kaur, 2022). Consequently, the recovery, isolation and purification of comselogoside have been studied in this work, and will facilitate the first studies on its possible biological activities.

Since comselogoside is a derivative of hydroxycinnamic acids, which contain a carbon-to-carbon double bond (1,2-disubsituted alkene moiety) and exist in both geometric molecular arrangements (geometric isomers): *trans* and *cis*- form (Salum & Erra-Balsells, 2013), it is logical to expect that the comselogoside exists in both *cis*-and *trans*- form in nature. However, although the presence of comselogoside isomers has been described (Fernández-Poyatos et al., 2021), their *trans- and cis*-configuration has never been verified. This may be important because the biological properties might change due to a variation in the stereo arrangement in the chemical structure. It is known that *cis*-cinnamic acid shows higher activities than the *trans*- isomer in physiological number or biological reaction. For example, *cis*-cinnamic acid exerts a greater antituberculosis effect than *trans-cinamic* acid (Chen et al., 2011) and *cis*-cinnamic acid also appears to exert a greater inhibitory effect against the invasion of human adenocarcinoma A549 cells than *trans*-cinnamic (Yen et al., 2011). In the case of hydroxycinnamic acids, it has been shown that *cis*-isomers could be obtained by photoisomerization (ultraviolet (UV)-irradiation) of *trans*-hydroxycinamic acids (Mitani et al., 2018).

The aim of the present work was the isolation and structure elucidation of two isoforms of a secoiridoid glucoside, 6́-*trans*-p-coumaroyl-secologanoside and 6́-*cis*-p-coumaroyl-secologanoside, based on their spectroscopic properties (UV spectra, ESI-MS/MS and NMR), as well as to investigate their radical scavenging properties using DPPH and ABTS assays. In addition, the effect of UV irradiation on these *trans*- and *cis*-isomers in racemic mixtures was also examined.

2. Materials and methods

2.1. Olive oil waste (alperujo)

An alperujo sample was obtained from yellow-green olive fruits of the Picual variety with an estimation of maturity index (MI) of 1.2 according to the method proposed buy Uceda and Frías (1975). The scale ranging from 0 (deep green colour) to 7 (black with colour throughout the pulp). The olive fruits were processed using a two-phase extraction system from an experimental oil mill in the Instituto de la Grasa (CSIC) (Seville, Spain). After crushing, the olives were malaxed and the paste was pumped into a horizontal centrifuge to obtain the olive oil. The alperujo sample was collected directly from the end of the horizontal centrifuge and stored at −20 °C until use.

2.2. Extraction and detection of phenolic compounds

For the recovery of phenolic compounds, 500 g of alperujo were extracted with 1000 mL of MeOH: H_2O (80: 20, v/v) twice at 80° C for 1 h. After filtration using Whatman n° 1 filter paper, the methanolic extract was concentrated under vacuum by rotary evaporation and brought to a final volume of 200 mL. The resulting extract was defatted using 200 mL of *n*-hexane twice to removal all possible traces of oil.

The defatted methanolic extract was injected into a HPLC-DAD-ESI-MS system consisting of a Dionex Ultimate 3000 RS U-HPLC liquid chromatography system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a photodiode array detector (DAD) and to a micrOTOF-QII High Resolution Time of Flight (UHR-TOF) mass spectrometer with a Qq-TOF geometry (Bruker Daltonics, Bremen, Germany). Chromatographic separation was carried out on a column Kinetex 5 μm EVO C18 (250 × 4.6 mm) (Phenomenex Inc, CA, USA). Water containing 0.01% trifluoracetic acid was used as eluent A and acetonitrile was used as eluent B. The flow rate was 1 mL/min with the following gradient: 95% A initially, 75% A at 30 min, 50% A at 45 min, 0% A at 47 min, 75% A at 50 min, and 95% A at 55 min until the run was completed. The wavelengths selected for DAD were 280 and 300 nm. A post-column split with 0.4 mL/min was inserted directly into the mass spectrometer electrospray (ESI) source. The instrument was operated in negative mode using a mass range of 50 to 1500 *m/z,* providing MS and MS/MS spectra simultaneously. Instrument control was performed using HyStar 3.2 software (Bruker Daltonics).

2.3. Isolation and purification of comselogoside

The defatted methanolic extract was purified by the Amberlite XAD-16 column (25 × 3 cm) using a step-wise gradient elution of EtOH: H_2O (0, 20, 30, 40, 50, 60 % v/v). The richest fractions containing comselogoside were collected and further separated on a Sephadex LH-20 column (18 × 2 cm) with a mixture of MeOH: H_2O (1:1, v/v) to give a fraction with a purity of 80–85 %. The isolation of comselogoside from this 'LH-20 extract' was performed by the HPLC system using a HP 1100 liquid chromatography (Agilent Technology, Santa Clara, CA, USA). The system was equipped with a diode array detector and Rheodyne injection valves (20 μL loop). Chromatographic separation was carried out on a reverse phase C18 analytical column Kinetex 5 μm EVO C18 (250 × 4.6 mm) (Phenomenex Inc, CA, USA), under the same conditions as described above for HPLC-DAD-MS. The comselogoside from the extract was detected at 280 nm and collected on the basis of retention time (28.1 min).

2.4. NMR spectroscopy

The sample for NMR analysis was prepared by dissolving the isolated sample in deuterated methanol-D4. The NMR spectra were recorded using a Bruker Advance III spectrometer operating at 500 MHz for protons. For ^{1}H NMR spectra, the acquisition parameters were: spectral width 10,000 Hz, relaxation delay 1 s, number of scans 16, acquisition time 3.277 s, and pulse width 90°, with a total acquisition time of 1 min 17 s. For ^{13}C spectra, the acquisition parameters were: spectral width 27,500 Hz, relaxation delay 2 s, acquisition time 1.188 s, and the number of pulses were dependent on the concentration of the sample. Chemical shifts were reported in δ units (ppm) relative to the solvent peak (MeOH-D4) and all coupling constants (J) were expressed in Hertz.

2.5. Quantification and UV–vis spectrophotometry of comselogoside

The quantification of comselogoside isomers was carried out by the integration of peaks at 280 and 300 nm with reference to the purified compound. The linearity of the curve was expressed in terms of the determination coefficient plots of the area of the integrated peaks versus the concentration of the isolated comselogoside. These equations were obtained over a concentration range of 0.15–1.0 mg/mL in accordance with the levels of the compound in the samples. The system was linear in all cases ($r > 0.99$). Three replicates were carried out on the same day.

The UV–visible spectra of isomers of comselogoside were recorded in an acidified H_2O: acetonitrile mixture when passed through a photodiode array detector (DAD) using a HPLC system (Agilent 1100 Series, Santa Clara, CA, USA).

2.6. UV-irradiation

A ethanolic solution of "XAD-extract" and a solution water: acetonitrile solution of a mixture of *trans*- and *cis*-comselogoside were placed in quartz cuvettes (3.5 mL volume, 12.5 × 12.5 × 45 mm outer dimension and 10 mm light path) under a photochemical chamber

(Desaga, Heildelberg, Germany) and exposed to ultraviolet light at 254 and 366 nm for a period of time in a dark room. The UV intensity average was of 0.0623 mW/m^2 (Välimaa et al., 2020).

2.7. Separation of the trans- and cis-comselogoside by semi-preparative HPLC

Trans and *cis*-comselogoside were isolated using a Jasco HPLC pump model PO2086 fitted with a Kinetex 5 μm EVO C18 semi-preparative reverse-phase column (250 × 21.2 mm) (Phenomenex, Torrance, CA, USA) and coupled with a Shimadzu SPD-10A Uv/Vis detector (Tokyo, Japan). The elution was carried out at a flow rate of 6 mL/min using the same solvent gradient as the water (0.01% trifluoracetic acid) and acetonitrile which were applied in the HPLC/DAD analysis. The injection volume of the sample was 500 μL, and the isomers were monitored at 280 nm, although in no instances did the collected isomers pass through the UV detection cell since after leaving the column, the flow path divides into a small amount in the detection section and the bulk of it into the collection section.

2.8. Assay of radical-scavenging of DPPH and ABTS radicals

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay was performed according to a previously reported protocol (Bermúdez-Oria et al., 2020). The DPPH assay was calibrated using a regression equation for the Trolox (6-hydroxy-2, 5, 7, 8-tetramethylpropionamidine) dihydro chloride) concentration (range between 0.08 and 4 mmol/L). The results are given in mmol of Trolox Equivalent per mol of comselogoside.

Radical scavenging activity against 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid radical (ABTS$^{\bullet+}$) was determined as described by Rubio-Senent, et al., (2012). This assay is based on the scavenging of ABTS$^{\bullet+}$ by antioxidants compared to the antioxidant potency of the Trolox which is used as standard. A calibration curve (in a range between 15 and 500 μmol/L) for Trolox was used. The results were expressed in terms of Trolox equivalent antioxidant capacity in mmol Trolox/mol of comselogoside.

2.9. Statistical analysis

Results were expressed as mean values ± standard deviation. STATGRAPHICS® plus software was used for statistical analysis. Comparisons among samples were made using one-way analysis of variance (ANOVA) and the LSD method. A p-value < 0.05 was considered significant.

3. Results and discussion

3.1. Isolation and purification of comselogoside

Fig. 1 shows the chromatogram of the methanolic extract obtained from an alperujo sample. The identification of phenolic compounds was carried out by HPLC-DAD-MS. Among the main detected phenols in this sample highlighted the comselogoside with a retention time (RT) of 28.1 min, which showed a pseudomolecular peak at *m/z* 535 [M−H]$^-$ and a UV absorbance spectra (λmax 225, 309 nm) similar to that detected for a p-coumaric acid derivative, which is in accordance with the reported by Romero et al., (2002) and Obied et al., (2007).

The reason for choosing this sample was to optimize the procedure in order to obtain an extract enriched in this compound of interest. The methanolic extract was fractionated by an Amberlite XAD-16 column eluted with aqueous ethanol (from 0 to 60% v/v). Comselogoside was eluted from the column in fractions from 40% to 50% EtOH (v/v), which were pooled and further fractionated using a Sephadex LH-20 column. The fractions eluted with aqueous methanol at 50% (v/v) containing comselogoside were combined to obtain an extract with 80–85% of purity in an adequate amount for further purification by semi-preparative reversed-phase RP-HPLC. Prior to this last step and for the isolation, quantification and structural elucidation of comselogoside, the extract was submitted to a purification by an analytical RP-C18

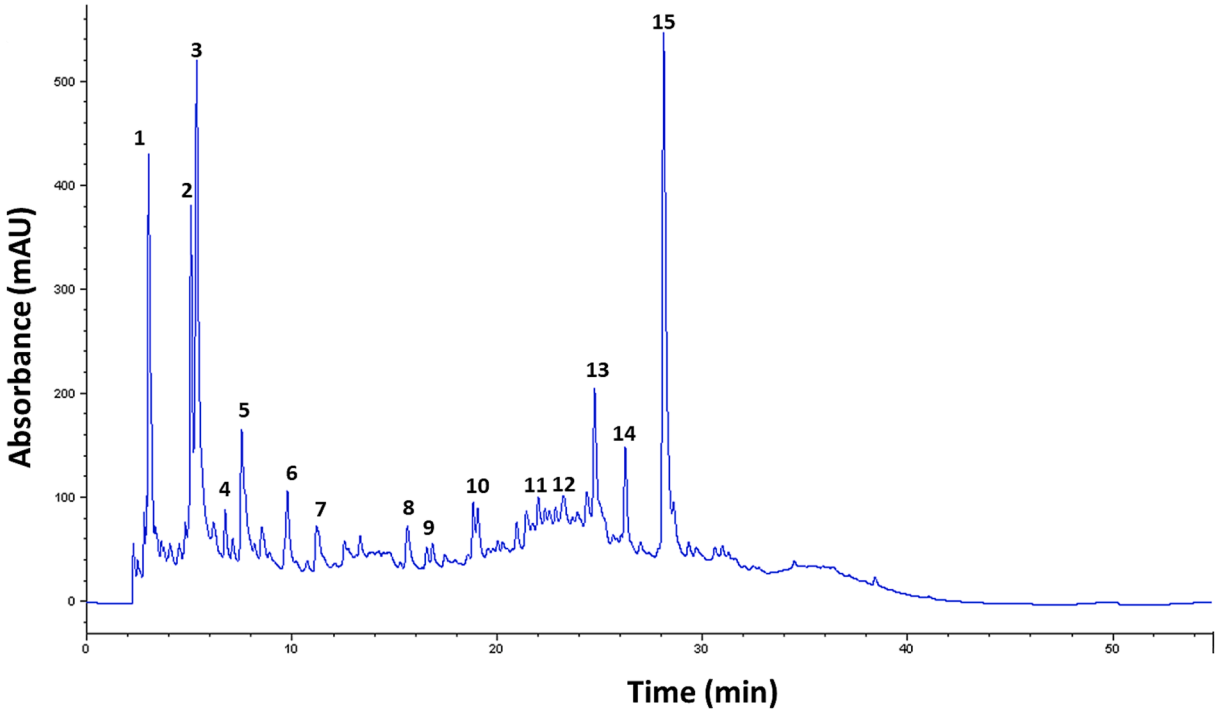

Fig. 1. HPLC-DAD profile of the alperujo methanolic extract at 280 nm. Peaks: 1. 3, 4-dihydroxyphenylglycol. 2. Hydroxytyrosol-glucoside. 3. Hydroxytyrosol 4. Salidroside. 5. Tyrosol. 6. Oleuropein derivative. 7. 11-mehyl ester diglucoside 8. p-coumaric acid. 9. Hydroxy-verbascoside. 10. Elenolic acid derivatives. 11. Verbascoside. 12. Luteolil-7-glucoside. 13. Cafeoyl-secologanoside. 14. Oleuropein. 15. Comselogoside.

column in HPLC-DAD. A compound with high purity was achieved from the peak at 28.1 min, although at the output of the HPLC column and the ultraviolet detector, the peak split into two, one at RT 28.1 min and the other at RT of 29.3 min (Fig. 2b), both with identical molecular mass (*m/z* 535 $[M-H]^-$). Direct exposure of this compound to ultraviolet light from the DAD detector suggested rapid photo-isomerization from *trans* to *cis*-isomers, resulting in a racemic mixture with 100% purity.

The peak at 28.1 min shows a compound with an absorbance spectrum (Fig. 2b) that seems to coincide with that described in the literature by Romero et al., 2006 and Obied et al., 2007, possibly the *trans-comselogoside.* The peak at 29.3 min, shows an absorbance spectrum, described for the first time, with a slightly different absorption maximum, possibly the *cis*-configuration. This is similar to the absorption spectrum of *cis*-p-coumaric acid, with a shift towards a shorter

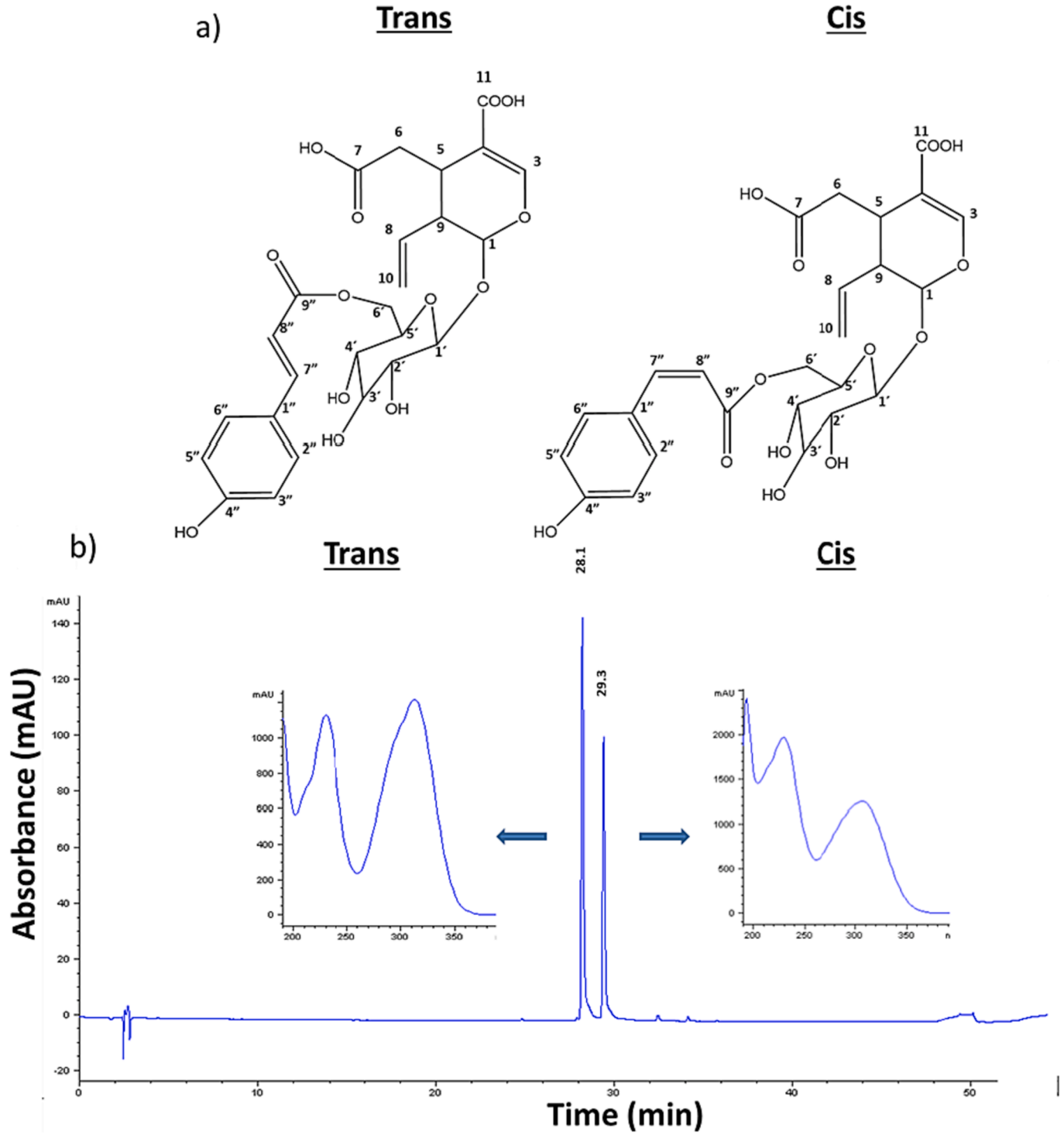

Fig. 2. a) molecular structure of *trans* and *cis*-p-coumaroyl-secologanoside (comselogoside). b) HPLC-DAD chromatographic profile of the two peaks at 280 nm with a retention time of 28.1 min and 29.3 min, obtained from comselogoside isolated from C_{18} analytical column and passed through the UV detector, and their corresponding absorbance spectrum profiles.

wavelength compared to the *trans*-isomer (Mitani et al., 2018). These results are in agreement with those reported for cinnamic acids, which indicate the presence of both isoforms in nature, with the *trans*-isomer being the predominant form because the *cis*-isomer tends to be less stable (La & Giusti, 2002; Salum & Erra-Balsells, 2013). Also, the *cis*-form could be a product of the photo-isomerization of *trans*-cinnamic acids (Mitani et al., 2018). The cinnamic acids (p-coumaric, ferulic, caffeic, and p-hydroxybenzoic acids) are present in all plant species and have in common the presence of a double bond in the acyl chain which can be in the *trans* and *cis* conformation, thus allowing a possible double-bond isomerization. Fig. 2a shows the structure of the molecule of the *trans*- and *cis*- comselogoside.

Also, to characterize these two peaks, the MS/MS fragmentation patterns of the ion $[M-H]^-$ at *m/z* 535 was made. The fragmentation patterns of the two isomers were identical to each other (Fig. 1S). Characteristic ions were detected at *m/z* 491, 389, 265, 205, 183, 177, 163, 145, and 117, which are in accordance with the literature (Obied et al., 2007; Innocenti et al., 2006; Fernández-Poyatos, et al. 2021).

3.2. Structural elucidation

An additional method of characterization was the ^{1}H and ^{13}C NMR spectral analysis of the possible mixture of *cis*- and *trans*-comselogoside. The results of the ^{1}H NMR and ^{13}C NMR spectra with the assignment of all the protons and carbons of secoiridoid, p-coumaroyl, and sugar moiety are shown in Table 1. The ^{13}C NMR spectrum exhibited the signals of all carbons of the molecule in duplicate form and with only a very close shift in value between them, except C1 and C3 of the secoiridoid group, with a single signal, showing only a slight difference in the p-coumaroyl moiety (Fig. 3S). This is indicative of the presence of two almost identical molecules. One of them completely agrees with the chemical shift reported by Karioti et al. (2006), although in our case at about 2 ppm lower in all the carbons, and with the only difference in carbon resonance of the p-coumaroyl moiety in one of the isoforms. Therefore, our data corresponds fairly well with the published data on *trans*-p-coumaroyl-6́-secologanoside (*trans*-comselogoside) (Karioti et al., 2006; Obied et al., 2007) and shows the presence of an isomer, possibly *cis*-comselogoside, which will be substantiated for the first time by ^{1}H NMR.

Table 1
^{13}C NMR chemical shift data for racemic mixture of comselogoside in methanol-d_4 at 500 MHz.

	TRANS Karioti et al., 2006	TRANS/CIS	TRANS	CIS
Secoiridoid Group				
C-1	98.2	96.27		
C-3	154.0	152.19		
C-4	110.7	108.78/ 108.74		
C-5	29.0	27.21/27.16		
C-6	35.5	33.64/33.61		
C-7	176.7	174.91/ 174.86		
C-8	134.9	133.05/ 132.99		
C-9	45.7	43.84/43.82		
C-10	121.0	119.21/ 119.18		
C-11	169.6	168.83/ 168.80		
Glucose				
C-1′	100.6	98.69/98.66		
C-2′	75.0	73.15/73.09		
C-3′	78.3	76.41/76.40		
C-4′	72.1	70.24/70.19		
C-5′	76.2	74.33/74.21		
C-6′	64.9	63.02/62.89		
p-coumaroyl group				
C-1′′	127.6		125.74	126.22
C-2′′/C-6′′	131.7		129.87	132.35
C-3′′/ C-5′′	117.3		115.43	114.51
C-4′′	150.2 (160.3 in Obied et al., 2007)		159.89	158.66
C-7′′	147.4		145.54	144.06
C-8′′	115.4		113.51	114.88
C-9′′	168.8		166.72	167.74

Table 2
^{1}H NMR spectral data for the racemic mixture of comselogoside in methanol-d_4 at 500 MHz.

	TRANS Karioti et al.,2006	TRANS	CIS
Secoiridoid group			
H-1	5.35 d (J = 4.0)	5.32 d (J = 4.0)	5.37 d (J = 4.0)
H-3	7.49 d (J = 1.8)	7.51 d (J = 1.65)	7.51 d (J = 1.65)
H5	3.23 m	3.25 m	3.25 m
H-6[a]	2.98 dd (J = 16.5, 4.8)	2.98 dd (J = 16.6, 3.05)	3.00 dd (J = 16.6, 3.05)
H-6B	2.21 dd (J = 16.5, 9.5)	2.23 dd (J = 16.6, 9.5)	2.23 dd (J = 16.6, 9.5)
H-8	5.60 td (J = 16.5, 9.5)	5.61 td (J = 16.9, 10.0)	5.61 m
H-9	2.98 ddd (J = 9.5, 5.5, 4.0)	2.81 m	2.81 m
H-10[a]	5.25 d (J = 17.2)	5.25 d (J = 16.9)	5.24 d (J = 16.9)
H-10B	5.22 dd (J = 10.0, 1.8)	5.19 dd (J = 10.2, 5.2)	5.18 dd (J = 10.2, 5.2)
Glucose			
H-1′	4.69 d (J = 8)	4.70 d (J = 8)	4.67 d (J = 8)
H-2′	3.25 dd (J = 8.0, 9.1)	3.22—3.42 m	3.22—3.42 m
H-3′	3.40—3.37 m	3.22—3.42 m	3.22—3.42 m
H-4′	3.40—3.37 m	3.22—3.42 m	3.22—3.42 m
H-5′	3.56 m	3.54 m	3.63 m
H-6A’	4.51 dd (J = 12.0, 2.2)	4.52 dd (J = 12.0, 2.2)	4.49 dd (J = 12.0, 2.2)
H-6B’	4.38 dd (J = 11.7, 5.8)	4.40 dd (J = 12.0, 6.0)	4.33 dd (J = 12.0, 6.0)
p-coumaroyl group			
H-2′′/H-6′′	7.46 d (J = 8.0)	7.48 d (J = 8.7)	7.66 d (J = 8.7)
H-3′′/ H5′′	6.80 d (J = 8.0)	6.82 d (J = 8.7)	6.78 d (J = 8.7)
H-7′′	7.64 d (J = 15.8)	7.66 d (J = 15.9)	6.90 d (J = 12.8)
H-8′′	6.37 d (J = 15.8)	6.39 d (J = 15.9)	5.82 d (J = 12.8)

The coupling constants (J values in Hz) are shown in parentheses.

The ^{1}H NMR spectrum signals (Fig. 2S) were also in good agreement with those obtained for *trans*- comselogoside (Karioti et al., 2006; Obied et al., 2007), with typical signals of secoiridoid and glucose moieties. However, the results (Table 2) show that the coupling constant (J) of *trans* protons 7 and 8 (δ 7.66, 6.39) with the vinyl double bond of the p-coumaroyl moiety was 15.9 Hz, while the coupling constant of 12.8 Hz between H-7 and H-8 (δ 6.90, 5.82) confirm the *cis* geometry outside of the plane of the double bond. This observation supported the presence of *cis*-comselogoside and agrees with the findings that the coupling constant between *cis*-proton is always lower than coupling between *trans*-protons in an oleifinic system (Kort et al., 1996).

3.3. Quantification

From this 100% pure racemic mixture, the quantification of comselogoside in its *trans*- and *cis*-isomers forms was carried out. Based on the observation of the HPLC-DAD chromatogram and the integration of both peak areas at 280 nm and 300 nm, the linearity was evaluated by plotting peak areas against known concentrations of the mixture (mg/mL) to construct the calibration curves. Regression equations of *trans*- and *cis*-isomers were linear in the investigated range, with a good correlation coefficient ($r^2 > 0.995$) (Table 1S). This was the first time that comselogoside in both isoforms was quantified from the isolated compound, because to the best of our knowledge comselogoside was always

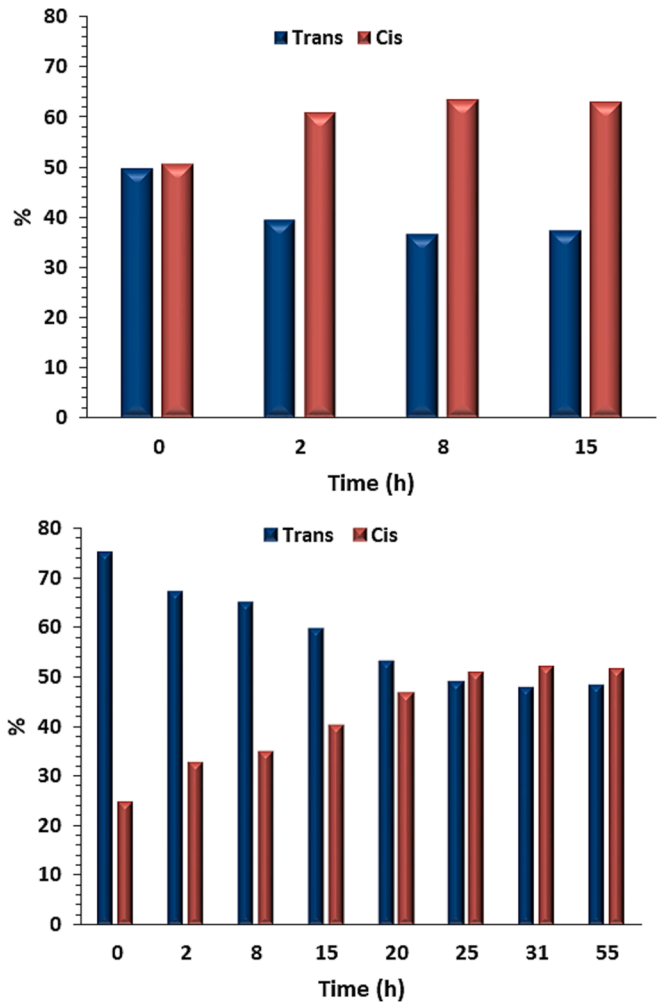

Fig. 3. Evolution of the ratio (%) the trans:cis isomers of comselogoside of a racemic mixture (a) and a "XAD-extract" (b) with the time of UV irradiation.

quantified using the response factor of p-coumaric acid (Romero et al., 2016; Nunes et al., 2018; D'Antuono et al., 2018; Fernández-Poyatos et al., 2019).

3.4. UV irradiation

Notably, *cis/trans* isomers of comselogoside have been detected. However, in the starting methanolic extract, the *cis*- form was present in very low amounts, which is similar to other cinnamic acids where the presence of a *trans*-isomer is predominant (Mitani et al., 2018). The *cis*-isomer of the comselogoside identified increased as the purification steps of the extract were conducted and could be a product of photo-isomerization from *trans*-comselogoside. This fact was clearly revealed previously, once the *trans*-isomer was isolated, when it passed through the diode array detector (DAD).

The ratio of *trans: cis* isomers after exposure to UV irradiation for several hours of the mixture *cis* and *trans* isolated by the analytical column of reversed phase in comparison to the extract obtained after the Amberilte XAD-16 column was investigated (Fig. 3). After 8 h of exposure, 63% of comselogoside of the purified mixture was in the *cis* conformation, which represents the steady state since no further changes in the ratio were observed during UV-exposure, while more hours (25 h) were necessary to achieve this steady state in the case of the XAD extract. The presence of other compounds seems to protect the structural transformation from the *trans* to its *cis*-isomer. Specifically, the presence of the other phenolic compounds can provide a stable photoprotective mechanism against UV radiation (Moreno et al., 2022). A significant difference of time of cis–trans isomerization is connected to formation the intermolecular H-bonds between phenolic compounds and solvent molecules (Kojima et al. 2005).

3.5. Antioxidant activity

DPPH and ABTS radical scavenging assays were used for the *in vitro* study of the antioxidant potential of comselogoside. The results of these experiments are shown in Fig. 4. Two methanolic extracts, containing 85% (Extract 1) and 80% (Extract 2) of comselogoside, mainly in its *trans* isoform, showed a moderate DPPH and ABTS radical scavenging activity, being the less pure extract, but the most active, which is explicable by the presence of a higher small amount of a probably active caffeic acid derivative in Extract 2 (Fig. 4S).

The photoisomerization allowed us to obtain the two isomers (*trans* and *cis*) of comselogoside in adequate amounts for a further purification step by a semipreparative reversed-phase (RP-HPLC). Under these experimental conditions assayed, the *trans* isomer was collected with high purity (100%) while the *cis* isomer was 80% together with 20% of *trans*. To study the influence of each of the isomers on antioxidant activity, a mixture of the isolated fractions was used, resulting in the *trans*: *cis* ratio: 100: 0; 70: 30; 55: 45 and 20: 80. The activity values in the DPPH and ABTS tests for *trans*: *cis* ratio (100:0) were 3 and 4 times higher than that for the 20: 80 ratio, although only slight significant differences ($p < 0.05$) were found when the mixture of cis and trans was close to 50: 50 (Fig. 4). The antioxidant property of the *trans* and *cis* mixture was more effective than of each isomer separately at certain proportions. Therefore, it can be concluded that the *trans*-comselogoside has higher antiradical capacity then *cis*-comselogoside, and the structural difference on the position of the double bond seems to influence this radical scavenger, although it seems that both isomers work in a synergistic fashion for radical scavenging, concretely a synergistic effect occurred when the *cis*-isomer was supplemented by the *trans*-isomer.

The phenylhydroxyl group of p-coumaryl moiety and the presence of the conjugated unsaturation which facilitates electron delocalization and should stabilize the resulting radical are more than likely responsible for the antioxidant activity of comselogoside. However, p-coumaric acid showed much lower DPPH and ABTS radical scavenging capacity. This fact indicates that the chemical structure of comselogoside also influences its *in vitro* radical scavenging effectiveness by impacting the process of hydrogen or electron donation. However, compared to recognized antioxidants such as hydroxytyrosol and caffeic acid, the antiradical activities of the *trans*-comselogoside found in the two tests were three times lower. This high radical scavenging ability of hydroxytyrosol and caffeic acid may be attributed to the presence of two hydroxyl groups adjacent to the aromatic ring (Platzer et al., 2022), unlike comselogoside and p-coumaric acid, which both have only one hydroxyl group, and achieved lower values and in the case of p-coumaric, the data found are consistent with the literature (Shen et al., 2019).

4. Conclusions

In this work, the isolation and purification of comselogoside from alperujo have led to a new isomer. The structures of these *trans*- and *cis-comselogosides* were established by means of NMR and MS spectroscopic analysis and compared with the data in the literature regarding *trans*-comselogoside. This is the first time that this *cis*-isomer was identified and there was induced isomerization of a vinyl double bound by UV irradiation. *Trans*-comselogoside exposed to UV irradiation underwent a structural transformation to their *cis*-isomer, resulting in a racemic mixture of comselogoside. A posterior separation of the *trans*- and *cis-isomers* was achieved by semi-preparative HPLC. Among the two isomers isolated, *cis*- exhibited lower DPPH and ABTS radical scavenging activity than *trans*, although a synergistic effect occurred when the *cis*-isomer

	DPPH		ABTS	
	µmol eq. trolox/g	mmol eq. trolox/mol	µmol eq. trolox/g	mmol eq. trolox/mol
Ext. 1	815 ± 87		1005 ± 146	
Ext. 2	543 ± 8		773 ± 132	
Trans 100%	616 ± 58	330 ± 31	895 ± 1	480 ± 1
Trans 70% Cis 30%	664 ± 37	356 ± 20	891 ± 30	477 ± 16
Trans 55% Cis 45%	597 ± 14	320 ± 7	1018 ± 14	546 ± 7
Trans 20% Cis 80%	217 ± 44	116 ± 24	244 ± 22	131 ± 12
p-Coumaric acid	11 ± 3	1.8 ± 0.4	319 ± 65	52 ± 11
Hydroxytyrosol	8784 ± 117	1353 ± 18	11322 ± 1712	1744 ± 264
Caffeic acid	7148 ± 486	1315 ± 90	10700 ± 607	1926 ± 112

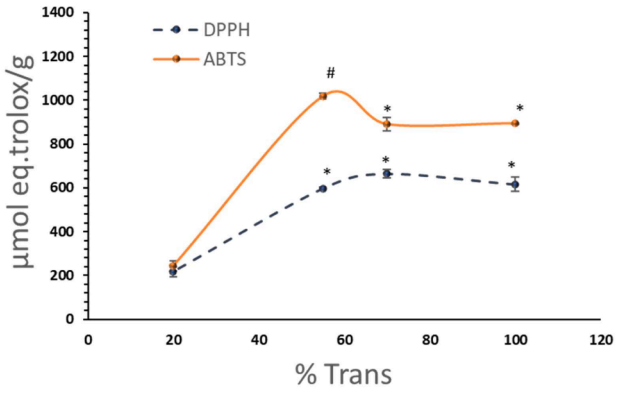

Fig. 4. DPPH and ABTS antioxidant scavenging activities of Extract 1 (85 % comselogoside) and Extract 2 (80 % comselogoside) and a mixture of trans and cis isomers in different proportions (trans: cis # 100: 0; 70: 30; 55: 45; 20: 80). Different symbols indicate a statistically significant difference between samples (ANOVA $p < 0.05$). Data are the average value of three replicates. The error bars represent standard deviations ($n = 3$).

was supplemented by *trans*-isomer. The results also showed a moderate DPPH and ABTS radical scavenging activity of *trans*-comselogoside in comparison with known *ortho*-diphenols antioxidants (hydroxytyrosol, caffeic acid), although with much higher activity than the p-coumaric acid from which it is derived.

It is the first time that comselogoside has been obtained from the main by-product of olive oil, showing an easily scalable chromatographic method at an industrial level. Further studies to determine the transformation kinetics, the stability and the non-covalent interactions with phenolic compounds on trans–cis conversion will be necessary through methodologies such as UV–Vis spectrophotometry. These new studies, together with the optimization of the purification process to avoid the use of organic solvents, could become the key to its future industrialization, following in the footsteps of other phenols that are allowing the use of a biorefinery for this by-product such as hydroxytyrosol or 3,4-dihydroxyphenylglycol.

Further studies are also needed to explore the biological properties of both isomers, together or separately, in order to determine their potential use in therapeutic fields or in food, which is currently underway in our laboratory.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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