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Characterization and Purification of Phenols from *Olea europaea* L. and Assay against *Xylella fastidiosa* subsp. *pauca*

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Abstract - The present study reports the characterization of main glucoside secoiridoid phenols from olive and of the respective aglycones as well as the bioassay of these compounds against *Xylella fastidiosa*, a pathogen microorganism responsible for a serious olive phytosanitary event occurred in Apulia (Italy) since 2013. The assayed secoiridoids were two native secondary metabolites, namely oleuropein and demethyloleuropein isolated and purified from leaves and from ripe drupes, respectively. Moreover, the corresponding aglycones were prepared in an easy, sustainable and bio-based way, by adding olive juice drops on buffered solutions of oleuropein and demethyloleuropein. The secoiridoids oleuropein and demethyloleuropein were obtained with 80% chromatographic purity. The aglycones obtained from native compounds were respectively a mixture of isomers derived

from oleuropein and oleacein derived from demethyloleuropein. These purified compounds were assayed against *Xylella fastidiosa* subsp. *pauca* at a starting concentration of 1 and 2 mM after 10, 100 and 1000-fold dilutions. Relevant activities were observed also towards the biofilm form of *Xylella fastidiosa*, especially for aglycones, after 100 (0.01 and 0.02 mM) and 1000 dilutions (0.001 and 0.002 mM). The data of the present study can pave the way for a sustainable method to face the relevant problem of *Xylella fastidiosa* infection.

Key Words: olive secoiridoids, aglycones, antibacterial activity, *X. fastidiosa* subsp. *pauca*

INTRODUCTION

Olive tissues from leaves and drupes are known to be relevant sources of phenolic compounds. The phenol compounds characteristic of olive belong to the class of secoiridoids, and their impact on the organoleptic and healthy properties of olive and of the processed products derived from them are well recognized[1]. The native secoiridoids from olive are known since decades[1] and have more recently attracted interest for their biological properties and for the very differentiated concentrations found in the wide range of existing olive cultivars: they are mainly represented by oleuropein **1** (Figure 1), found in leaves and immature drupes, and by its acidic derivative demethyloleuropein **2** (Figure 1), present in mature olive drupes[2]. These native glucosides can be hydrolyzed to the corresponding aglycones as a result of the cleavage of the glycosidic linkage catalyzed by beta-glucosidases. These hydrolytic enzymes are activated after tissue decompartimentation and, in the case of olive, also during the technological processes of olive oil production[3-4].

The olive secoiridoids have been studied in the past years and their significant repellent activity against the olive fly *Bactrocera oleae* was evidenced since several decades[5]. Moreover, it has to be noted that oleuropein has been object of studies regarding the interaction between host plant, such as privet (*Ligustrum* spp.), and adapted herbivore insects[6].

Aglycones from both oleuropein and demethyloleuropein can be obtained after olive fruit grinding and, for their relatively high lipophilicity, they can be found in the resulting oil[7]. The

aglycones from oleuropein are represented by a combination of different isomers **3-5** in equilibrium (Figure 1). On the other hand, the aglycone of demethyloleuropein (**6**) affords the stable dialdehyde oleacein **8** after the loss of a CO_2 molecule (Figure 1). Oleacein was firstly isolated from the juice of mature olives[8] and it is one of the main phenolic components in extra-virgin olive oil[7]. Moreover, it is to note that the aglycone forms often possess a relevant anti-microbial activity with respect to the corresponding glycosidic native derivatives[9].

In this context, the question faced in the present study is if extracts from olive, composed by the native secoiridoid glycosides and by the subsequently prepared aglycones, could have also a biological action against *Xylella fastidiosa* (*X. fastidiosa*). *X. fastidiosa* is a long-known pathogenic bacterium that represents an ancient problem in the Americas and it was recently introduced also in Europe.

The epidemic by *X. fastidiosa* subsp. *Pauca*, affecting olive groves since 2013 in Salento area, represents the most important phytosanitary event occurred in recent years in Italy, with very serious economic, landscape and social consequences[10-11]. In particular, the local cultivars Cellina di Nardò and Ogliarola salentina are threatened. These cultivars are traditionally grown in Salento since ancient times and are characterized by a relevant content of polyphenols with antioxidant properties, remarkable for nutrition and human health[12]. Among the curative strategies aimed at managing this disease, the use of bacteriophages, antagonistic bacteria, antibacterial microelements, nanoparticles, synthetic peptides, and naturally occurring organic compounds has been proposed[13]. Recently, the possibility of applying zinc-based compounds to manage *X. fastidiosa* has gained particular attention. Indeed, a nanoformulation of zinc oxide distributed on the soil significantly reduced the *in-planta* multiplication of *X. fastidiosa* in greenhouse[14]. In addition, zinc-based formulations have shown antibacterial activity also against the subsp. *pauca*, either in *in vitro* tests or with potted olive plants. Zinc and copper ions complexed by citric acid have also shown a relevant efficacy to mitigate the field symptoms caused by *X. fastidiosa* subsp. *pauca* in the olive groves of Salento[15-17]. Interestingly, these findings were confirmed and supported by the EFSA panel regarding the EU official position on the phytopathological problem suffered by olive crops in Italy since 2013[18]. Antibacterial activity has also been reported for several phenolic compounds including flavonoids, stilbenes and coumarins. In particular, catechol, caffeic acid and resveratrol were found to be the most effective against *X. fastidiosa* subsp. *fastidiosa*[19], whereas 4-methylcatechol, catechol, veratric acid, caffeic acid and oleuropein

showed in vitro bacteriostatic activity[20], as well as also raw phenolic extracts from olive leaves[21].

The present study is aimed to characterize and prepare in a sustainable way extracts of the main characteristic olive phenols secoiridoids, considering for the first time the possibility to obtain purified oleacein **8** from demethyloleuropein **2**, as previously hypothesized by Lo Scalzo and Scarpati[8]. It is worthy to mention that these compounds can be obtained and purified by means of a simple extraction method starting from easily available plant material, i.e. olive leaves and drupes. Hence, the present study reports on the isolation and characterization of the main secoiridoid compounds from olive, both as native glycosides (oleuropein **1** and demethyloleuropein **2**) and the corresponding aglycones (oleuropein aglycones **3-5** and oleacein **8**), as well as their *in vitro* bactericidal activity towards *X. fastidiosa* subsp. *pauca*.

RESULTS

Extraction and characterization of purified assayed compounds. The first steps of this investigation was the isolation and purification of four compounds from olive, namely oleuropein **1** from leaves and demethyloleuropein **2** from ripe drupes and the preparation of the corresponding aglycones (oleuropein aglycones **3-5** and oleacein **8**, respectively). Purification was performed by preparative chromatography, monitoring the composition of the extracts and of collected fractions by analytical HPLC (Figure 2). In accordance with previous studies, the average content of oleuropein in sampled olive leaves ranged from 0.8 to 2.3 % on dry weight[22], averaging at 1.2 % dw. Analogously, demethyloleuropein in fresh ripe black olive drupes resulted in a content of 0.2 to 0.4 % averaging at 0.3 % on fresh weight[23]. However, these metabolites can present a high natural variability in the detected levels, as they are synthesized by the plant in response to unfavorable environmental conditions[22, 24]. In addition, also genotypic features given by the existing agrobiodiversity of the crops in some districts of Southern Italy may be invoked as a cause of these variations [25-26].

The spectral features and chromatographic behaviour of the two main native olive phenol iridoid glucosides oleuropein **1** and its demethoxylated derivative demethyloleuropein **2** have been known for many years. The chemical structure of oleuropein has been firstly established in 1960 by Panizzi et al.[27], while robust chromatographic method for its quantitative measurement in olive tissues was primarily described by Savournin *et al* in 2001[28]. In the

present study, oleuropein revealed to be the main metabolite present in the samples of aqueous extracts from leaves, in accordance with previous literature data[25]. Thus, it was identified through the direct comparison with a commercial standard as the compound corresponding to the main signal in the chromatograms from leaves extracts at around 70-80% initial concentration on the total extractable phenols (Figure S1).

One of the main glucosidic metabolite of oleuropein, which significantly accumulated in relevant amount, resulted demethyloleuropein, naturally present in ripe black olives. This compound was identified for the first time in olive tissues by Ragazzi *et al* in 1973[29] and its isolation was made possible due to its acidic nature by extraction after solubilization of the salified demethyloleuropein in an alkaline system. Its identification and a preliminary check of purity were made by HPLC and then confirmed by LC-MS experiments. The MS spectrum confirmed the molecular weight of demethyloleuropein given in literature at 526.5 (PubChem release 2021.10.14) and confirmed at 525.26 in negative mode (Figure S2).

It is evident from Figure 2 that the HPLC signal shapes attributable to the aglycones derived from native phenol compounds are characteristic, i.e. in both cases peaks are broader than those of the parent glycosides. This may be due to their peculiar chemical composition. Indeed, these compounds exist as equilibrium mixtures of different isomeric forms **3**, **4, 5** for oleuropein and **6** and **7** for demethyloleuropein aglycones, with the final formation of the oleacein **8** after an irreversible decarboxylation (Figure 1). However, the presence and the purity of the compounds in the dried and weighed extracts have been evaluated by HPLC in comparison with reference standards and, where necessary, were definitively confirmed by LC-MS and NMR, indicating a level of purity of around 80%. The enzymatic degradation of the native glucosides to obtain aglycones was performed by adding few drops of raw black olive juice with glucosidase activity to a 1 mM solutions of oleuropein **1** and demethyloleuropein **2**, following the findings from previous literature data[30]. These Authors monitored the enzymatic hydrolysis of oleuropein **1** and the present study confirmed previous data, obtaining a mixture of isomers representing the oleuropein aglycone (see chromatogram in Figure 2, and structures **3**, **4**, **5** in Figure 1). In this case, the type and the presence of different aglycones in reciprocal dynamic equilibrium did not allow the isolation of a single compound, hence the extract remained purified from other non-phenolic impurities and was characterized by the clear absence of oleuropein, completely consumed during the hydrolitic reaction, as monitored by HPLC.

It has to be noted that in the present work the same procedure of enzymatic hydrolysis was performed for the first time on the purified demethyloleuropein **2**, following the preliminary method, by exploiting the endogenous β-glucosidase activity in freshly obtained black olive juice. This method was used in the first study[8] for the identification of oleacein **8** in juice from ripe olives: it was suspected that, in these conditions, the cleavage of the β-glycosidic linkage of demethyloleuropein should give oleacein **8** as the final stable compound following the scheme proposed in Figure 1. This suspect was revealed exact, and oleacein **8** was finally obtained in yields ranging from 25 to 45%, depending on the used sample of olive juice, and in a level of purity of around 90 % (Figure 2). Its chromatographic profile resulted coincident with that of a commercial standard (Figure S3).

In addition to a peak at 320.8 *m/z* in the MS spectrum, 1D- and 2D NMR spectra fully confirmed the structure of oleacein **8.** In the ^{1}H-NMR spectrum the signals attributable to two aldehydic groups were identified at 9.21 and 9.67 ppm (Figure 3 and Figure S4). An oxygenated 1,3,4-trisubstituted phenyl ring was recognizable by chemical shifts and the typical pattern of three signals: a doublet with an *ortho* coupling constant (6.81 ppm, J = 8.1 Hz), a doublet with a *meta* coupling constant (6.74 ppm, J = 2.0 Hz) and a doublet of doublet showing the same coupling constants (6.63 ppm, J = 8.1 and 2.0 Hz). This latter signal is very close to a quartet (6.67 ppm, J = 7.1 Hz, 1 hydrogen), attributable to a vinylic proton coupling with a methyl group (doublet at 2.08 ppm, J = 7.1 Hz, 3 hydrogens). An ethylidene moiety can thus be present. Inspection of the APT ^{13}C and HSQC spectra (Figures S5 and S6) confirmed the above-mentioned attributions and revealed the presence of four methylene and one aliphatic methyne groups (signals in the ^{13}C-NMR spectrum at 34.2, 36.9, 46.26, 65.17 and 27.17 ppm, respectively, Figure 3). H-H correlations in the COSY spectrum (Figure S7) suggested the presence of a two-carbon ethyl chain carrying an esterified oxygen atom and a phenyl ring at the two ends (δ_H 4.22, δ_C 65.1 and δ_H 2.79, δ_C 34.2, respectively) and a methyne flanked by two methylene groups (δ_H 3.65, δ_C 27.2; δ_H 2.96 and 2.80, δ_C 46.2; δ_H 2.63 and 2.75, δ_C 36.9, respectively); all the chemical shifts were consistent with the presence of neighbor double bonds. Analysis of the HMBC spectrum (Figure S8) allowed the determination of the connectivity of the recognized fragments and the attribution of quaternary carbon atoms in the APT spectrum (Figure S5). On the basis of NMR characterization, the structure of oleacein was thus attributed to compound **8**. [7, 8, 31]

Antibacterial activity. The four purified extracts from olive leaves and drupes were evaluated for their antibacterial activity. The efficacy of all extracts was assessed on *X. fastidiosa* subsp. *pauca* 15 days after the inoculation using DMSO 30%, the extracts diluent, as the negative control. As shown in Figure 4 (black bars) all the dilutions of DMSO 30% didn't influence the bacterial growth (both planktonic and biofilm state). The planktonic growth was inhibited by all olive extracts at the dilution 1:10 v/v (Figure 4 A, C, E, G) at a statistically relevant level. Furthermore, oleacein (Figure 4 G) inhibited the planktonic growth also at dilution 1:100 v/v. Conversely, the growth of the biofilm was inhibited by all extract dilutions tested, as shown in Figure 4 B, D, F, H. Differently from what observed on planktonic growth, all the assayed solutions resulted very active on the biofilm at 1:10 dilutions and the activity was maintained even at higher dilutions, although at lower levels. It's interesting to note that, while oleuropein aglycones are able to completely inhibit both planktonic and biofilm growth at 1:10 v/v dilution (Fig. 4C), they are on the contrary able to significantly induce planktonic growth when tested at 1:100 dilution (Fig. 4D).

The dilutions 1:100 and 1:1000 revealed differences between planktonic and biofilm growth. Planktonic growth of *X. fastidiosa* subsp. *pauca* is generally unaffected by the presence of extracts, except for two contrasting cases: oleacein significantly inhibits *X. fastidiosa* subsp. *pauca* growth (Fig. 4G), in contrast to aglycone oleuropein, which induces *X. fastidiosa* subsp. *pauca* growth (Fig. 4C). The biofilm growth is significantly influenced by the presence of extracts, all of which remain effective up to the dilution of 1:1000 v/v. The activities of oleuropein aglycones (Figure 4 D), as well as of oleacein (Figure 4 H) show the same trends, meaning that a slight significant inhibitory effect has been evidenced in 1:1000 dilutions, implying a concentrations of active compounds of 2 and 1 μM, respectively.

DISCUSSION

The characterization of the secoiridoid phenolic components of olive is in a full accordance with previous studies and the compounds involved in the transition from native secoiridoids glycosides **1** and **2** are well noted since previous studies[7-8, 30]. The present study clearly underlines the relationship between the presence of demethyloleuropein **2** and oleacein **8**, its

stable aglycone, obtained after a β-glucosidase-like transformation of the native parent compound (Figure 1, compounds **6**, **7** and **8**).

Oleacein **8** is considered as a "strategic" final point of bio-mimetic synthesis of natural compounds from olive, such as oleuropein, of great interest for nutraceutical purposes[32]. Oleacein can be easily obtained and purified after the action of endogenous enzymes present in the juice of fresh olive.

The significant bacteriostatic activity of the secoiridoids aglycones of olive in is due to the so-called "glutaraldehyde effect"[9], as it can be assessed by the structures of compounds **5** and **8** (Figure 1). This different bioactivity between glycosides and aglycone derivatives appears to be evident when they were tested at 1:10 dilutions (Figure 4), also if not always supported by statistical significance. The here evaluated effect of glutaraldehyde-like compounds is in accordance with previous findings, where the different bioactivity of oleuropein *vs* its dialdehydic derivatives has been assayed on bacterial strain of human interest, such as *P. fluorescens*, *S. aureus* and *E. coli*[33]. In previous studies it has been highlighted that the presence of these dialdehydic derivatives is due to the specific transformation of native compounds following tissue laceration. For example, cross-linking reagents towards proteins are released as natural defensive allelochemicals following herbivores action. This was confirmed also by the presence of high levels of free amino compounds, e.g. glycine, in the digestive system of specialist insects, able to counteract the action of dialdehydic defensive compounds produced by the plant[9, 34]. Previous studies highlighted the effectiveness against microrganisms of aqueous solutions used for the storage of table olives[35]. The storage solutions of black olives resulted more active than that of green ones and chemical analysis of these solutions confirmed the relevant presence of dialdehyde oleacein **8** in the aqueous solution from black olives, accompanied by anti-bacterial activity against *E. amylovora* and *P. syringae* at the same initial concentrations used in the present study[35]. This is in a full accordance with our hypothesis that the presence of oleacein **8** is closely related to the presence of demethyloleuropein **2** (Figure 1), found in significant amounts only in black ripe olives.

In general, previous works demonstrated the activities of some low molecular weight phenols against different strains of *X. fastidiosa*. The screening performed by Maddox *et al*. in 2010[19] revealed that some acidic phenols, such as caffeic acid and gallic acid, as well as catechol and resveratrol, have effective activities against *X. fastidiosa*. Vizzarri *et al*. in a recent

experiment[21] assayed a crude olive leaves aqueous extract for the activity against *X. fastidiosa.* subsp. *fastidiosa*. The extract was prepared by ultrasonic treatment at room temperature and its composition showed minor amounts of oleuropein, considerable amounts of catechol, hydroxytyrosol and luteolin both free and conjugated, but no report on olive secoiridoid aglycones content. The inhibitory effect on 10^7 cells suspensions was evident at 1:10 and 1:100 dilutions, where theinitial content of total phenols in the leaf extract was of 2.3 %. The here presented data start from 2 mM (around 0.1%) or 1 mM (around 0.05-0.03%) of isolated secoiridoids, in fact the most important inhibition was obtained at 1:10 dilutions, in accordance with the previously reported data[21].

Moreover, Bleve *et al* in 2018[20] explored the possibility to counteract the infection of *X. fastisiosa*, strain Salento-1, isolated in the infected zone of Salento, Puglia (Italy), by phenols derived from olive mill waste-waters and by single phenols. The olive mill waste waters containing oleuropein as the major phenol compound at 2-4 mM was added to culture media at 10% (ten-fold dilution) and gave a complete depletion of bacteria presence in the growth medium in accordance with the data of the present study. On the other hand, pure oleuropein, assayed at 1 mM on agar plate, gave no significant inhibition of bacterial growth while at a concentration of 10 mM a slight inhibition activity was evidenced. The present work has differently shown that oleuropein resulted significantly active at a concentration of 0.2 mM in both planktonic and biofilm forms (Figures 4A and 4B) of *X. fastidiosa*, further showing activities at higher dilutions in biofilm forms (Figure 4B). Finally, a recent work[36] demonstrated the effectiveness of extract from peel of pomegranate against *Xylella fastidiosa* subsp *fastidiosa* at 10 and 100-fold dilutions from the initial extract at a concentration of around 26 mM of both ellagic acid and punicalagin. Resuming, the effectiveness of olive native secoiridoids and their aglycones, in this situation, seems very efficient, having effective concentrations in a range from 0.2 mM to 0.01 mM, with interesting potential practical applications.

CONCLUSION

These assayed phenolics from olive resulted very active and, in a perspective of a possible application for in field trials against *X. fastidiosa* or other olive parasites, they are easily available. High amounts of oleuropein can be extracted from olive leaves coming from pruning residuals, considered a product able only to obtain low-cost firewoods. The aglycones

can be obtained from oleuropein by enzymatic de-glycosilation, simplyadding to a solution of oleuropein few drops of olive juice at room temperature. This is a simple, effective and less costly procedure of hydrolysis with respect to other chemical approaches[32, 37].

Finally, the action against *Xylella fastidiosa* of phenolic olive metabolites can contribute to better understanding the complex relationship and co-evolution between plant metabolites and their potential parasites. Within this context, it is well known that some olive cultivars are better resistant than others to *Xylella*, and the possible reason could be in the composition of phenolics in plant and their transformation in more biologically active compounds upon infection. To confirm this, further research is needed, first of all by exploring the actual biodiversity of the olive cultivars for their phenol content.

EXPERIMENTAL SECTION

Extraction and isolation of oleuropein from olive leaves. Olive leaves of "Nocellara messinese", popularly named as "Nostrale", or attributed as "Brandofino" after a careful survey with traditional growers of the territory, is a typical olive cultivar from East Sicily (Taormina-Giardini Naxos, province of Messina, Italy, 37° 55′ 58″ N, 15° 20′ 14″ E) and were harvested in summer 2023, on 16 - 21 July. This olive cultivar was chosen for its known resistance to biotic stresses and for its typical presence in this area[38, 39]. Leaves were sampled from ten olive trees, differentiated for their age (50 – 150 years), and sun expositions. Just after sampling, leaves were delivered to the laboratory within 24 hours, manually reduced in small pieces (20 – 30 mm) and subjected to a quick freezing in an air blast tunnel at -50°C, freeze-dried and stored at - 20°C until extractions and analyses. Oleuropein **1** was extracted from olive leaves by decoction with boiling water 1:10 w/v (typically, 100g of freeze-dried material treated with 1 liter of solvent) for 1h, following the method established in early studies[27]. This method avoided the endogenous hydrolytic activities on native glucosides, ensuring a better stability of the obtained oleuropein. The liquid fraction was decanted and filtered under cheesecloth and evaporated under reduced pressure at 60°C to around a 30% of the initial volume. After concentration, the insoluble material was separated by centrifugation at 4000*g*, 10 minutes. The supernatant was diluted with 10% NaCl and extracted with an equal volume of ethyl acetate saturated with water for three times. The organic layers were combined and taken to dryness under reduced pressure at 50°C. The dry residue was re-dissolved in EtOH 80% and stored at -20°C until use. The purification of oleuropein was

performed by solid-phase-extraction (SPE) on a column (15 cm height, 1.8 cm internal diameter) filled with 15 g of ICN Adsorbentien Biomedicals, 32–63 μm, 60 Å C_{18} resin, conditioned with 2 × 30 mL of 10% EtOH. The raw extract (5 mL) was 5-fold diluted with water and eluted at a flow rate of 1 mL/min under a pressure of around 2 bars. After washing with 30 mL of EtOH 10%, the column was eluted with 30 mL solutions at increasing EtOH concentration, 20, 30, 40 and 80% in water. All the resulting fractions were individually diluted three-folds with deionized H_2O and extracted with ethyl acetate (two times, equal volume). The separated organic layers were concentrated to small volume to eliminate ethyl acetate and re-dissolved with EtOH 80% to a final volume of 10 mL. The fractions were analysed by HPLC and the presence of semi-pure oleuropein **1** (at least 80% by HPLC analysis) resulted present in the fraction formerly eluted with EtOH 40%. The identity of oleuropein was confirmed by the comparison with an authentic standard, used also for the HPLC calibration prior to the quantification of this metabolite (Figure S1).

Extraction and isolation of demethyloleuropein from ripe olive drupes. Samples of black ripe fresh, not processed olives were purchased in September-November 2023 from a popular market in Milano. They were represented by olives of unknown variety, and were chosen at an uniform level of drupe dimensions and maturity, i.e. full ripe black, and for a complete absence of defects by a visual inspection. These olives have been also used for the production of the juice used for enzymatic hydrolysis (see after).

The previously established[23] method for the extraction and purification of demethyloleuropein was used, with some modifications: fresh, free of visible defects black olives were treated in a ratio 1:1 w/v (typically, 100 g and 100 mL solvent) with boiling EtOH 80% for 60 minutes, to avoid endogenous enzymatic hydrolysis of native olive glucosides. The extract was decanted and filtered through cheesecloth, then evaporated under reduced pressure at around 30% of the initial volume. The insoluble material, was eliminated by centrifugation at 4000g, 10 minutes. After addition of 10% NaCl (around 3g) the supernatant was extracted four times with ethyl acetate saturated with water (50 mL each). The organic layer was then separated and cooled at 4°C overnight and then extracted with a cold solution of 2% $NaHCO_3$ (pH 9.5), in a ratio 10:1. The aqueous phase, containing salified demethyloleuropein, was acidified to pH 2 with 6N HCl and re-extracted two times by a five-fold volume of ethyl acetate. The organic layer was separated, washed twice with small volumes of water and concentrated to

dryness under reduced pressure. The dried extract, containing demethyloleuropein **2** at a degree of purity of minimum 80% monitored by HPLC, was redissolved in EtOH 80% and stored at -20°C for further experiments. The identity of demethyloleuropein was confirmed by its chromatographic behaviour and by LC-MS (Figure S2).

Preparation and purification of aglycones. The aglycones of oleuropein **1** and demethyloleuropein **2** were prepared following the methodologies of previous studies[30], with some modifications. The initial water-alcoholic solution of purified oleuropein or demethyloleuropein was diluted to a final concentration of about 1 mM (500 mg/L) with a 0.1 M acetate buffer, pH 4.5 in a total volume of 100 mL. An equal volume of $CHCl_3$ was added to this solution and, under an energic magnetic stirring, five drops of a fresh ripe black olive juice obtained by a puncture with a needle, were added to this mixture. The reaction was carried out for 60 minutes at room temperature (20 ± 2°C), monitoring by HPLC the disappearing of the peak attributable to the starting reagents in the upper phase. The organic layer was separated and the solvent was evaporated under reduced pressure. The residue was dissolved in EtOH 80% and stored at -20°C before further analysis. The extracts obtained from enzymatic hydrolysis have been subjected to purification on SPE, using the same system reported before for oleuropein. The sequence of eluting solvents was slightly different with respect to previous separations, using EtOH concentrations of 20, 30, 40, 60 and 80%. The main purified fraction obtained starting from oleuropein, and represented by a mixture of isomers **3**, **4** and **5** (see Figure 2), was recovered in the elution performed with EtOH 60%. Oleacein **8** resulted the only product from the hydrolysis of demethyloleuropein, and was eluted with EtOH 40% (Figure 2). Its structure was confirmed both by HPLC comparison with an authentic standard (Figure 3 and Figure S3) and by NMR spectroscopy (Figures S4-S8). The yield for the hydrolytic reaction of oleuropein was difficult to establish due to the presence of more than one stable isomer (Figure 2), while the yield of the reaction of demethyloleuropein was calculated by comparing its initial amounts *vs* the finally obtained purified oleacein. Each assay was performed in triplicate.

HPLC analysis. HPLC analyses were performed with an Agilent 1200 series HPLC system equipped with a single wavelenght detector (Agilent VWD G1314B). The pump was coupled with a quaternary gradient unit (Agilent G1311A). The analytical data were collected and

evaluated using Agilent LC ChemStation, rev. A.01.01 software. The separation was performed using a reverse-phase C_{18} Phenomenex Synergi MAX RP 4 μm 150 × 4.6 mm column. The flow rate was 0.45 mL/min, the injection volume 20 μL, the column temperature was kept at 40°C and the detection was performed at 280 nm. The mobile phase consisted of water with 2 mM ortho-phosphoric acid with 7% acetonitrile (solvent A) and acetonitrile 93% in water acidified with 2 mM ortho-phosphoric acid (solvent B). The gradient was as follows: (A/B): 92/8 0–5 min, from 92/8 to 65/35 in 10 min, 65/35 for 10 min, from 65/35 to 92/8 in 10 min, 92/8 for 10 min. Total analysis time was 45 min.

The samples were diluted before the HPLC injection with MeOH 75% and subsequently filtered on 0.45 μm filters. The identity of the samples and their purity were based on their retention times and their correspondence with the available commercial standards oleuropein and oleacein (Sigma-Aldrich). Moreover, where necessary, their identity and purity have been confirmed by the evaluation of the weighed dried extracts in comparison with pure standards by HPLC, mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR).

LC-MS analyses were carried out on a Thermo Scientific Dionex UltiMate 3000 HPLC instrument equipped with a single VWD (Variable Wavelength Detector) detector interfaced with a Thermo LCQ Fleet Ion-Trap mass spectrometer with an Electrospray ionization (ESI) system. Acquisition was in positive and in negative mode. Samples were dissolved in 75% methanol at 0.3-0.4 mg/mL concentration. The used column was a reverse-phase C18 Phenomenex Synergi MAX RP 4 μm 150 × 4.6 mm. Solvents were a mixture of water 93% and acetonitrile 7% containing 5 mM formic acid (solvent A) and acetonitrile 93% and water 7%, containing 5 mM formic acid (solvent B). Gradients was as follows: 0-5 min: isocratic elution 90% solvent A : 10% solvent B; from 5 to 15 min: linear gradient to 50% solvent A : 50% solvent B; 15-25 min: isocratic elution at solvent A : solvent B 50%; 25-35 min: linear gradient to the initial conditions 90% solvent A : 10% solvent B; 35-45 min: equilibration at the initial conditions.

^{1}H-NMR and ^{13}C-NMR spectra were acquired at 400.13 MHz and 100.61 MHz, respectively, on a Bruker Advance 400 spectrometer (Bruker, Karlsruhe, Germany) equipped with a TOPSPIN software package. ^{13}C signal multiplicities were based on attached proton test experiments (APT) and attributions were based on COSY (Correlated Spectroscopy), HSQC (Hetero Single Quantum Correlation) and HMBC (Hetero Multiple Bond Correlation) experiments. Chemical shifts are given in ppm (δ) and are referenced to solvent signal (δ_H

412 $CDCl_3$ 7.26 ppm, δ_C $CDCl_3$ 77.16 ppm). Spectra analyses were carried out with *inmr Reader*
413 software (ww.inmr.net).

414

415 *Antibacterial activity*. After purification, samples were prepared for the assays of antibacterial
416 activity. An aliquot of extract was dried under reduced pressure by a centrifugal evaporator at
417 50°C (Jouan, mod. RC 10.10) to eliminate EtOH and water, condensed at -90°C (Jouan, mod.
418 RCT 90). Then, the residues were redissolved in a 30% DMSO aqueous solution to reach a
419 final concentration of 2 mM for oleuropein and 1 mM for other compounds. In addition, a
420 sample constituted by 30% DMSO in water was treated in the same way as the olive extracts,
421 constituting the negative control. After this, samples have been filtered on 0.45 μm sterilizing
422 membranes and delivered to the laboratory of microbiology of CREA-DC for the *X. fastidiosa*
423 antibacterial assays.

424 *In Vitro Growth Assay in 96-well plates*. The assay for antibacterial activity on *Xylella*
425 followed validated methodologies and approaches by previous assays performed by the same
426 group of research whose data are available in previously published works[40-41]. The
427 antibacterial activity was assessed on *X. fastidiosa.* subsp. *pauca* (De Donno_CFBP8402),
428 isolated in Apulia (Italy) from olive trees. *X. fastidiosa* subsp. *pauca* was grown on PD2 agar
429 medium for 15 days at 28 °C. Stock of *X. fastidiosa* subsp. *pauca* were stored in PD2 broth
430 plus 20% glycerol at -80°C[42]. *X. fastidiosa* subsp. *pauca* was scraped from the plates and
431 resuspended in PD2 broth at about 10^7 colony forming units (CFU) mL^{-1} concentration
432 (optical density at 600nm of 0.1). Sterile polystyrene 96-well plates containing 200 μL of PD2
433 broth per well were used. Bacterial inoculum (20 μL) was transferred in 180 μL of PD2 broth
434 (final concentration 10^6 CFU/mL) alone, as positive control, or in PD2 broth supplemented or
435 not with 30% DMSO (as negative control) or oleuropein (native), demethyloleuropein
436 (native), aglycones (derived from oleuropein), oleacein (derived from demethyloleuropein).
437 All extracts were tested at three dilutions (1:10, 1.100 and 1:1000 v/v PD2/extract). After 15
438 days the cells in suspension were transferred into a new plate for the planktonic growth
439 quantification, measuring the OD_{600nm}. The biofilm growth was estimated on the original 96-
440 well plate with the crystal violet assay according to a previously validated method[43], with
441 some modifications. The wells were first rinsed and gently washed with sterile distilled water
442 and then the biofilm was stained with 200 μL of 0.01% crystal violet for 20 min. The crystal
443 violet was removed and wells were washed with sterile distilled water and subsequently with

200 µL of absolute ethanol. The absorbance at 620 nm of the resulting ethanol solution was measured with Multiskan FC (Thermo Fisher Scientific, Cleveland, OH, USA). The experiment was repeated three times with different inoculum, and each experiment contained twelve replicates.

Statistical Analysis. Statistical significance of the *in vitro* analyses was determined by the student t-test for pairwise comparison of means. All statistical analyses were carried out by using GraphPad Prism 10 Software (GraphPad Software, San Diego, CA, USA). The *p*-values less than 0.05 were considered significant. Data are the mean ± standard deviation (SD) of three independent experiments.

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Figure Captions

Figure 1. *Chemical structures of native glucosides from olive (1 and 2) and aglycones from oleuropein (3, 4, 5) and from demethyloleuropein (6, 7, 8).*

Figure 2. *Chromatograms of purified samples used for antibacterial assays. Compounds of interest are indicated by arrows.*

Figure 3. *^{1}H and ^{13}C chemical shifts of oleacein, desumed from 1D- and 2D-NMR spectra.*

Figure 4. *Graphical representation of X. f.* subsp. *pauca planktonic A), C), E) G) and biofilm B), D), F) H) growth at different olive extracts dilutions. Data are expressed as means ± S.D. (n = 36, experiment repeated 3 times with different inoculum); *p<0.05, **p≤0.005, ***p=0.0006 vs. Ctr ****p=0.0001vs. Ctr).*

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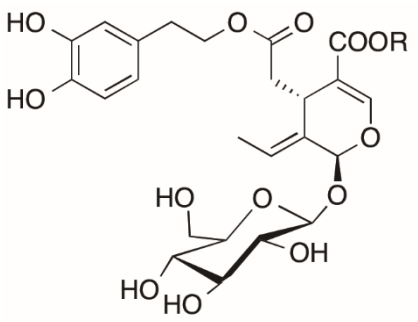
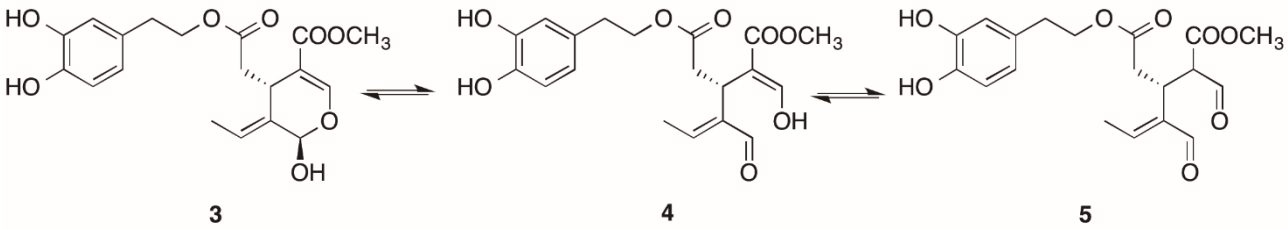
Figure 1



1 R = Me - oleuropein
2 R = H - demethyloleuropein



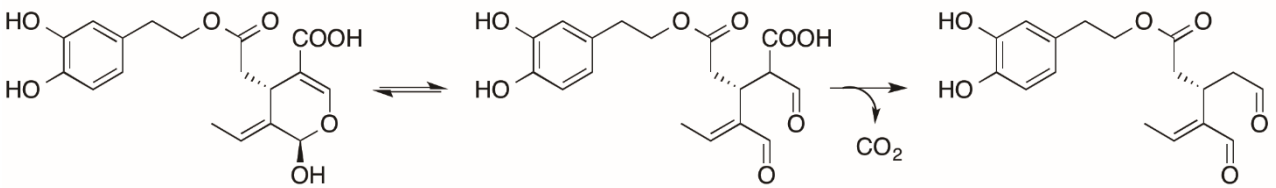


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Figure 2

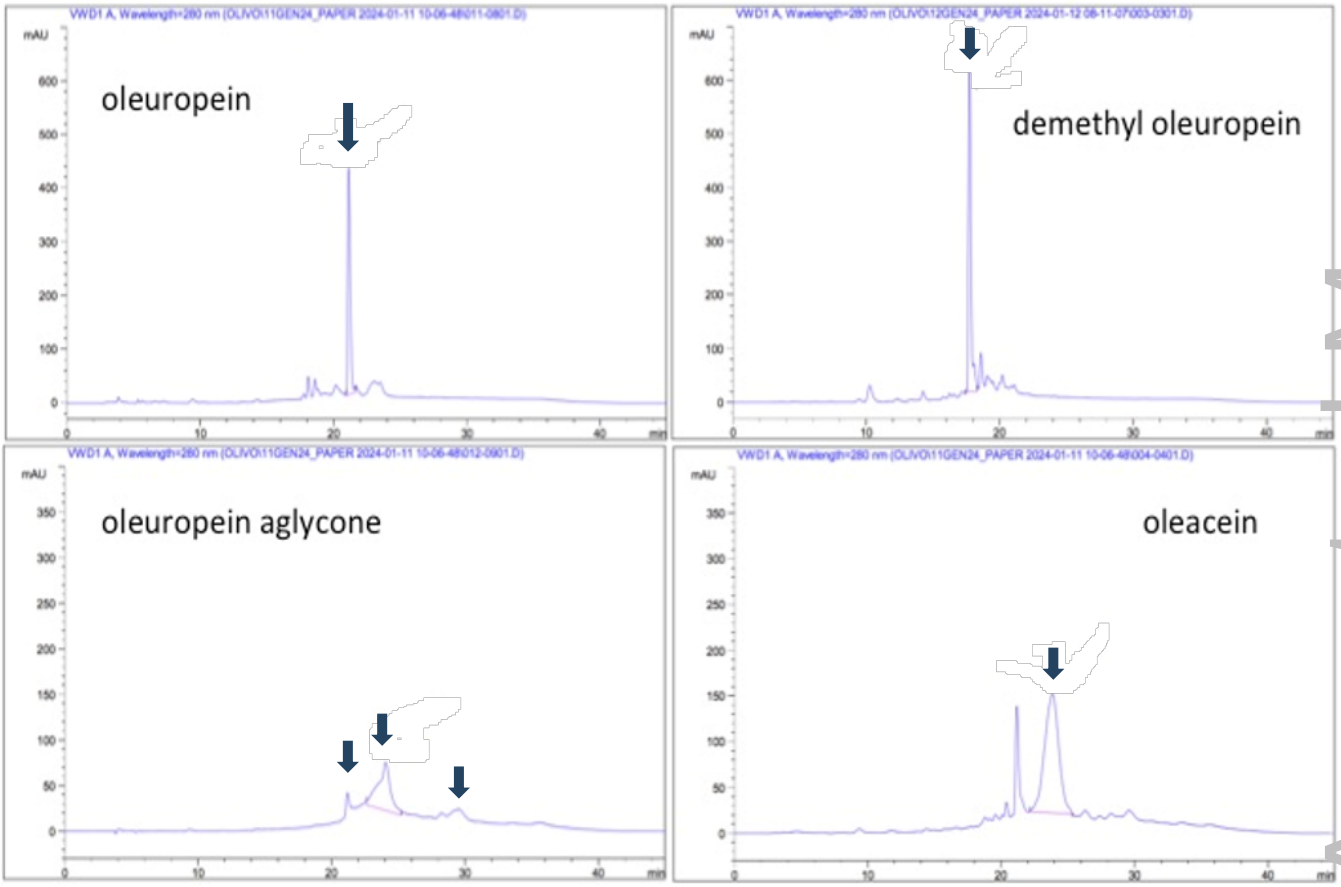

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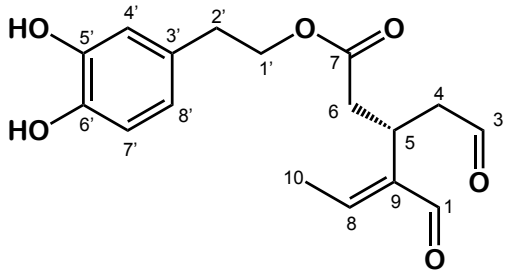
Figure 3



Carbon	^{13}C	^{1}H
1	195.73	9.21(d, *J* = 1.9 Hz, 1H)
3	200.83	9.67 (s, 1H)
4	46.26	2.78-2.84 (overlapping m, 1H) 2.96 (ddd, *J* = 18.4, 8.1, 1.1 Hz, 1H)
5	27.17	3.65 (ttd, *J* = 8.4, 6.3, 1.9 Hz, 1H)
6	36.96	2.63 (dd, *J* = 14.9, 6.5 Hz, 1H) 2.75 (dd, *J* = 14.9, 7.2 Hz, 1H)
7	171.83	-
8	155.15	6.67 (q, *J* = 7.1 Hz, 1H)
9	143.18	-
10	15.28	2.07 (d, *J* = 7.1 Hz, 3H)
1’	65.17	4.16 (dt, *J* = 10.8, 6.3 Hz, 1H) 4.23 (dt, *J* = 10.8, 6.6 Hz, 1H)
2’	34.22	2.78-2.84 (overlapping m, 2 H)
3’	130.58	-
4’	116.28	6.73 (d, *J* = 2.0 Hz, 1H)
5’	143.34	-
6’	142.70	-
7’	115.23	6.80 (d, *J* = 8.0 Hz, 1H)
8’	121.30	6.62 (dd, *J* = 8.0, 2.0 Hz, 1H)

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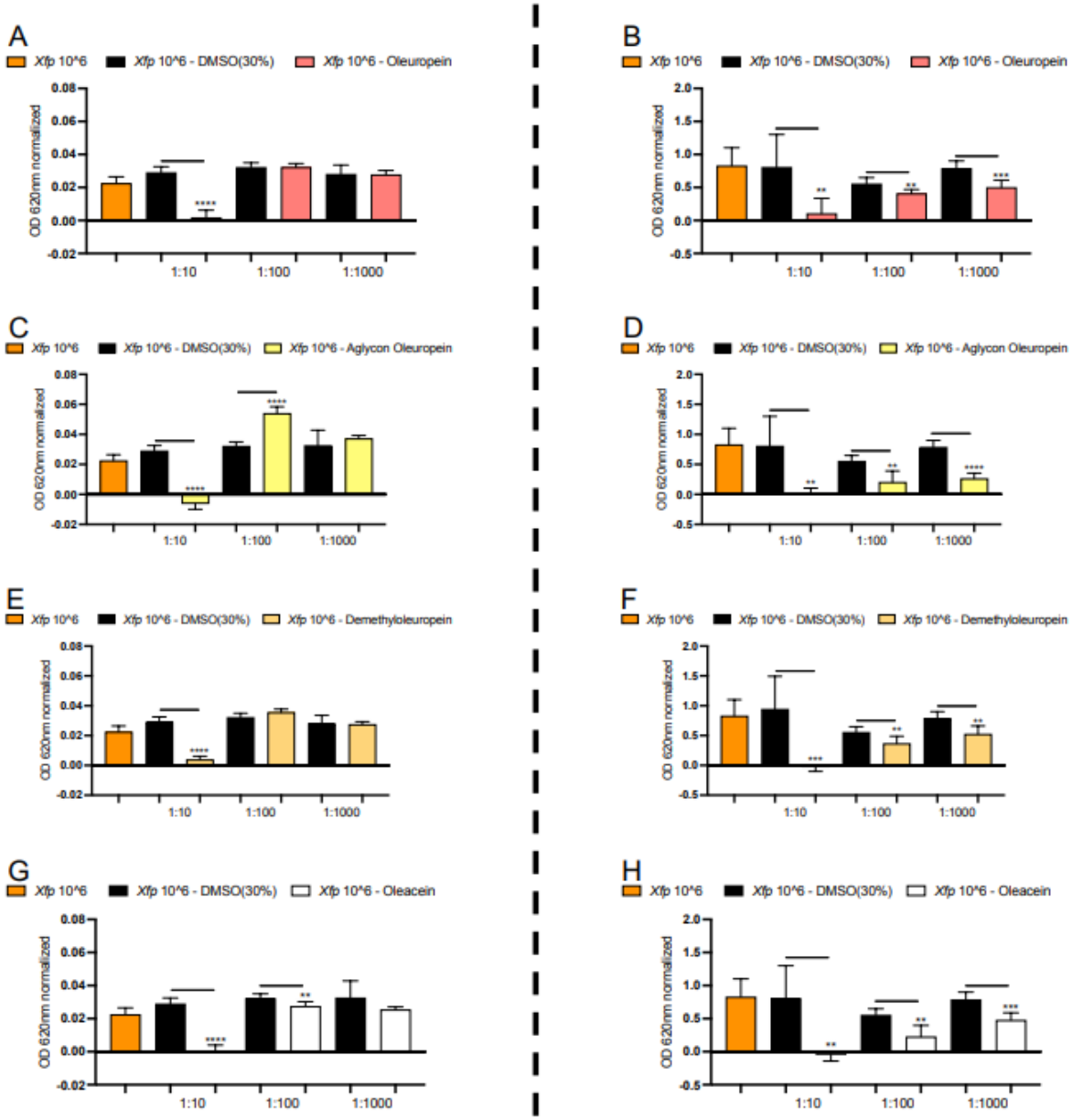
Figure 4



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Graphical Abstract