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Low-molecular-weight Components of Olive Oil Mill Waste-waters

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A new lignan 1-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-6-(3-acetyl-4-hydroxy-5-methoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane, the secoiridoid 2H-pyran-4-acetic acid,3-hydroxymethyl-2,3-dihydro-5-(methoxycarbonyl)-2-methyl-, methyl ester, the phenylglycoside 4-[β-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-1,4-dihydroxy-2-methoxybenzene and the lactone 3-[1-(hydroxymethyl)-1-propenyl] δ-glutarolactone were isolated and identified on the basis of spectroscopic data including two-dimensional NMR, as components of olive oil mill waste-waters. The known aromatic compounds catechol, 4-hydroxybenzoic acid, protocatechuic acid, vanillic acid, 4-hydroxy-3,5-dimethoxybenzoic acid, 4-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, tyrosol, hydroxytyrosol, 2-(4-hydroxy-3-methoxy)phenylethanol, 2-(3,4-dihydroxy)phenyl-1,2-ethandiol, *p*-coumaric acid, caffeic acid, ferulic acid, sinapic acid, 1-*O*-[2-(3,4-dihydroxy)phenylethyl]-(3,4-dihydroxy)phenyl-1,2-ethandiol, 1-*O*-[2-(4-hydroxy)phenylethyl]-(3,4-dihydroxy)phenyl-1,2-ethandiol, D(+)-*erythro*-1-(4-hydroxy-3-methoxy)-phenyl-1,2,3-propantriol, *p*-hydroxyphenethyl-β-D-glucopyranoside, 2(3,4-dihydroxyphenyl)ethanol 3β-D-glucopyranoside, and 2(3,4-dihydroxyphenyl)ethanol 4β-D-glucopyranoside were also confirmed as constituents of the waste-waters.

Keywords: Phenols; lignan, secoiridoid; phenylglycoside; olive oil mill waste-waters.

INTRODUCTION

From an economical point of view, olive is the most important fruit tree in the Mediterranean basin, and Italy, Spain, Greece, Tunisia and Morocco are the leading olive oil producers in that area. The olive oil manufacturing process generates an aqueous effluent, referred to as olive oil mill waste-waters (OMW), which has significant polluting properties as indicated by high biological oxygen demand (BOD) (100 kg/m^3) and chemical oxygen demand (COD) (200 kg/m^3), marked acidity, high concentrations of mineral salts and a high content of toxic aromatic compounds (Borja *et al.*, 1992; Capasso, 1997; Arienzo and Capasso, 2000).

The annual disposal of several million m^3/year of OMW (800,000 m^3/year in Italy alone; Mulinacci *et al.*, 2001) poses a major environmental problem for agriculture in the Mediterranean area. OMW are usually released in the soil, but several studies have reported that this practice may be the cause of environmental pollution in the soil (Paredes *et al.*, 1986; Rodriguez *et al.*, 1988; Capasso *et al.*, 1992) as well as in ground and surface waters (DellaGreca *et al.*, 2001). All previous studies indicate that the major toxicity is due to phenolic components. Whilst catechol (**1**), tyrosol (**2**) and hydroxytyrosol (**3**) have been repeatedly found as components of OMW, the presence of other phenolic derivatives apparently depends on the ripeness of the olives, the environment of the olive cultivars, storage time and extraction procedures. In a recent study (DellaGreca *et al.*, 2001), the waste-waters from a Ligurian mill were fractionated by ultrafiltration, nanofiltration and reverse osmosis. Along with **1**–**3**, aromatic compounds **4**–**17** from the reverse osmosis fraction were isolated and identified (Fig. 1). The present paper reports on investigations performed on the nanofiltered fraction of the same effluent in order to isolate and characterize further low-molecular weight phenols from OMW.

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EXPERIMENTAL

General experimental procedures. All reagents were of HPLC grade (Romil, Cambridge, UK) except for ethyl acetate and chloroform, which were of analytical grade. NMR spectra were recorded in 99.8% deutero-methanol, 99.9% deutero-chloroform or 99.5% deuterium oxide solutions and were obtained using a Varian (Palo Alto, CA, USA) Inova 500 instrument (^{1}H-NMR at 500.13 MHz; ^{13}C-NMR at 125.75 MHz) with TMS as internal standard. Homonuclear ^{1}H connectivities were determined using two-dimensional (2D) COSY. One-bond heteronuclear ^{1}H-^{13}C connectivities were determined by a two-dimensional proton-detected HMQC experiment (optimised for $^1J_{HC}$ = 140 Hz); two- and three-bond ^{1}H-^{13}C connectivities were determined by a two-dimensional proton-detected HMBC experiment (optimised for $^1J_{HC}$ = 7 Hz). Low-resolution MS (LREIMS) spectra were determined with a Hewlett Packard (Palo Alto, CA, USA) HP 6890 spectrometer equipped with an MS 5973 N detector.

Analytical TLC was performed on Merck (Darmstadt, Germany) Kieselgel 60 F_{254} plates (0.2 mm layer thickness). Spots were visualised by UV light or by spraying with sulphuric acid:acetic acid:water (1:20:4) followed by heating for 5 min at 110°C. Preparative HPLC were

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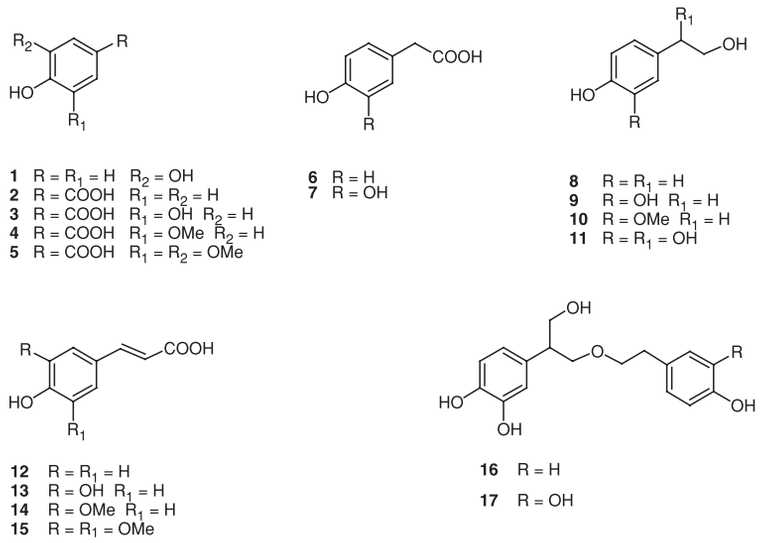

Figure 1. Structures of catechol (**1**), 4-hydroxybenzoic acid (**2**), protocatechuic acid (**3**), vanillic acid (**4**), 4-hydroxy-3,5-dimethoxybenzoic acid (**5**), 4-hydroxyphenylacetic acid (**6**), 3,4-dihydroxyphenylacetic acid (**7**), tyrosol (**8**), hydroxytyrosol (**9**), 2-(4-hydroxy-3-methoxy)phenylethanol (**10**), 2-(3,4-dihydroxy)phenyl-1,2-ethandiol (**11**) *p*-coumaric acid (**12**), caffeic acid (**13**), ferulic acid (**14**), sinapic acid (**15**), 1-*O*-[2-(3,4-dihydroxy)phenylethyl]-(3,4-dihydroxy)phenyl-1,2-ethandiol (**16**) and 1-*O*-[2-(4-hydroxy)phenylethyl]-(3,4-dihydroxy)phenyl-1,2-ethandiol (**17**).

performed on a Varian (Walnut Creek, CA, USA) Vista 5500 chromatograph using a Merck LiChrosper NH_2 column (250 × 10 mm i.d.; 10 μm). Flash column chromatography was performed on Merck Kiesegel 60 (230–400 mesh) at medium pressure. Sephadex LH-20 was from Sigma (St. Louis, MO, USA) and Amberlite XAD-4 was from Fluka (Buchs, Switzerland).

GC-MS analysis was performed on a Hewlett-Packard model 5890 instrument using a Supelco (Belefonte, PA, USA) SP-2330 capillary column (30 m × 0.25 mm i.d.) with helium as carrier gas (flow rate 0.8 mL/min). The oven temperature was maintained at 80°C for 2 min, increased to 170°C at 30°C/min, then increased to 240°C at 4°C/min and held at this temperature for 10 min.

OMW samples. Waste-water samples (10 L) were collected in November 2001 in Bitonto (Apulia, Italy) and filtered using a polypropylene net in order to eliminate the total suspended solids (consisting mainly of cellulose and pectins) derived from the olive pulp. The sludge-free fraction was microfiltered, ultrafiltered twice and finally nanofiltered in order to obtain four fractions on the basis of molecular weight of the constituents, i.e. MF (>120 kDa), UF1 (120–20 kDa), UF2 (20–1 kDa) and the nanofiltered fraction NF (<1 kDa).

Isolation of phenols. An aliquot (300 mL) of NF was extracted with ethyl acetate (3 × 300 mL), yielding an oily residue (7.3 ***g***; NFO) and an aqueous phase (NFW). An aliquot of NFO was chromatographed on Sephadex LH-20 eluted with different mixtures of methanol:water. Following elution with water and with methanol:water (2:3), fraction A was obtained and was made up of compounds **1**–**17** as described previously (DellaGreca *et al.*, 2001). Elution with methanol:water (3:2) gave fraction B containing compounds **18**–**22** (Fig. 2). Of these, compounds **18**, **19**, **20a** and **20b** were purified by HPLC using an NH_2 column eluted with acetonitrile:water (9:1), whilst compounds **21** and **22** were isolated by fractionation on a flash-silica gel column eluted with chloroform:acetone (8:2). The NFW fraction, after extraction with ethyl acetate, was concentrated under reduced pressure and chromatographed on Amberlite XAD-4, eluted first with water (300 mL) and subsequently with methanol (300 mL). The methanol eluate was submitted to flash chromatography, eluting with chloroform:methanol:water (13:7:2), to yield compounds **23** and **24** (Fig. 2) together with several phenols which had been previously detected in the organic phase.

Sugar analysis. A sample of compound **23** (1 mg) was methylated with methyl iodide and dimethylsulphinyl anion as described by Hakomori (1964). The crude reaction product was filtered on a C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA) that had been previously washed with ethanol (20 mL), acetonitrile (2 mL) and water (10 mL). Fractions were sequentially eluted with water (50 mL), water:acetonitrile (4:1) (8 mL), acetonitrile (2 mL) and ethanol (4 mL). The last two fractions were pooled and evaporated to give the methylated disaccharide, which was hydrolysed with 2 M trifluoroacetic acid. The partially methylated products were reduced with deuterated sodium borohydride, acetylated and analysed by GC-MS.

RESULTS AND DISCUSSION

Various low-molecular weight phenols were isolated and identified from the NF fraction obtained by fractionation of olive oil mill waste-waters. The known natural products (**1**–**20b**) were identified by the analysis of their spectral data and by comparison with literature values, mainly ^{1}H-NMR and ^{13}C-NMR chemical shifts, reported for **1**–**15** (DellaGreca *et al.*, 2001), **16** and **17** (DellaGreca

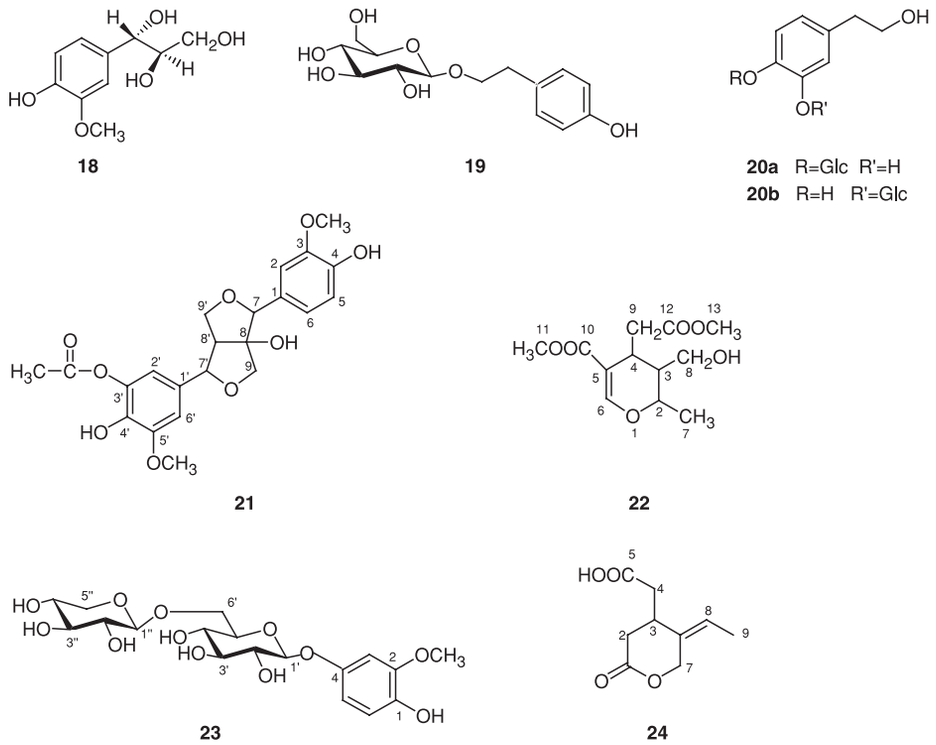

Figure 2. Structures of D(+)-*erythro*-1-(4-hydroxy-3-methoxy)-phenyl-1,2,3-propantriol (**18**), *p*-hydroxyphenethyl-β-D-glucopyranoside (**19**), 2(3,4-dihydroxyphenyl)ethanol 3β-D-glucopyranoside (**20a**), 2(3,4-dihydroxyphenyl)ethanol 4β-D-glucopyranoside (**20b**), 1-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-6-(3-acetyl-4-hydroxy-5-methoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane (**21**), 2H-pyran-4-acetic acid,3-hydroxymethyl-2,3-dihydro-5-(methoxycarbonyl)-2-methyl-, methyl ester (**22**), 4-[β-D-xylopyranosyl-(1→6)]-β-D-glucopyranosyl-1,4-dihydroxy-2-methoxybenzene (**23**), and 3-[1-(hydroxymethyl)-1-propenyl] δ-glutarolactone (**24**).

et al., 2000), **18** (DellaGreca *et al.*, 1998), **19** (Limiroli *et al.*, 1996), and **20a** and **20b** (Bianco *et al.*, 1998).

1-Hydroxy-2-(4-hydroxy-3-methoxyphenyl)-6-(3-acetyl-4-hydroxy-5-methoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane (21)

EIMS (*m*/*z* 432 $[M]^+$; $C_{22}H_{24}O_9$): ^{1}H-NMR ($CDCl_3$) δ_H 1.73 (3H, *s*, Me), 3.33 (1H, *m*, H-5), 3.82 (1H, *dd*, *J* = 3.2 and 8.1 Hz, H-4), 3.92 (3H, *s*, OMe), 3.95 (3H, *s*, OMe), 4.28 (1H, *d*, *J* = 7.9 Hz, H-8), 4.43 (1H, *dd*, *J* = 3.2 and 8.1 Hz, H-4′), 4.45 (1H, *d*, *J* = 7.9 Hz, H-8′), 4.77 (1H, *d*, *J* = 3.6 Hz, H-6), 5.10 (1H, *s*, H-2), 6.8–7.1 (5H, *m*, aromatic Hs): ^{13}C-NMR δ_C 132.1 (C-1), 114.4 (C-2), 145.7 (C-3), 145.8 (C-4), 146.8 (C-5), 114.0 (C-6), 86.0 (C-7), 59.0 (C-8), 69.9 (C-9), 119.5 (C-1′), 109.0 (C-2′), 146.3 (C-3′), 145.8 (C-4′), 111.7 (C-5′), 121.6 (C-6′), 87.2 (C-7′), 97.4 (C-8′), 75.4 (C-9′), 169.6 (CO), 56.1 (C-OMe), 56.1 (C-OMe), 21.0 (C-Me).

The LREIMS showed a molecular ion at *m*/*z* 432 and the inverse-gated ^{13}C-NMR spectrum showed twenty carbon signals that integrated for 22 carbons. These data were consistent with the molecular formula $C_{22}H_{24}O_9$. The ^{1}H-NMR spectrum, measured in deutero-chloroform, showed the aromatic H-2, H-5, H-6, H-2′ and H-6′ protons as overlapped signals in the 6.84–6.95 ppm range. Further, the spectrum showed the H-7 proton as a singlet at δ_H 5.10, the H-9 protons as two doublets at δ_H 4.25 and 4.45, the H-7′ proton as a doublet at δ_H 4.77, the H-8′ as a multiplet at δ_H 3.33, and the H-9′ protons as two double doublets at δ_H 4.43 and 3.82, together with two methoxy methyls at δ_H 3.89 and 3.92 and one methyl at δ_H 1.73. In the ^{1}H spectrum measured in deutero-methanol, the aromatic *meta* coupled H-2′ and H-6′ protons (*J* = 1.8 Hz) were split at δ_H 7.01 and 6.95 indicating the presence of an asymmetrical 1,3,4,5 tetrasubsituted benzene, whereas the signals in the 6.8–6.95 ppm range were typical of a 1,3,4 aromatic ring (Deyama *et al.*, 1987). In the ^{1}H–^{1}H COSY experiment, H-8′ was correlated with the methylene H-9′ and the methine H-7′. These data indicated that compound **21** was a typical furo-furanoid lignan (Deyama *et al.*, 1987), in which one of the two aromatic rings carried an acetyl group as confirmed by the values in the ^{13}C-NMR spectrum at δ_C 169.6 and 21.0. On the basis of the HMQC spectrum, the protons have been correlated to the corresponding carbons, while the entire structure has been assigned on the basis of an HMBC spectrum. In accordance with the assigned structure, the methyl protons at δ_H 1.73 yielded an NOE with the aromatic proton H-2′.

2H-Pyran-4-acetic acid,3- hydroxymethyl-2,3-dihydro-5-(methoxycarbonyl)-2-methyl-, methyl ester (22)

EIMS (*m*/*z* 258 $[M]^+$; $C_{12}H_{18}O_6$): ^{1}H-NMR ($CDCl_3$) δ_H 7.56 (1H, *s*, H-6), 4.26 (1H, *m*, H-2), 3.57 (1H, *dd*, *J* = 6.4 and 11.6 Hz, H-8), 3.49 (1H, *dd*, *J* = 4.8 and 11.6 Hz, H-8), 2.83 (1H, *m*, H-4), 2.70 (2H, *m*, H-9), 1.74 (1H, *m*, H-3), 1.38 (3H, *d*, *J* = 6.8 Hz, CH_3), 3.68 (3H, *s*, 11-OMe), 3.65 (3H, *s*, 2′-OMe): ^{13}C-NMR δ_C 75.8 (C-2), 46.7 (C-3),

31.3 (C-4), 110.2 (C-5), 157.9 (C-6), 21.1 (C-7), 63.9 (C-8), 39.9 (C-9), 171.0 (C-10), 176.0 (C-1′), 53.5 (C-11-OMe), 53.1 (C-2′-OMe).

The LREIMS and the ^{13}C-NMR data defined the molecular formula $C_{12}H_{18}O_6$ for compound **22**. The ^{1}H-NMR spectrum showed the H-6 olefinic proton as a singlet at δ_H 7.56, the methine H-3, H-4 and H-2 protons as multiplets at δ_H 1.74, 2.83 and 4.26, the methylene H-8 protons as two double doublets at δ_H 3.49 and 3.57, the methylene H-9 protons as a multiplet at δ_H 2.70, the H-7 methyl protons as a doublet at δ_H 1.38, and the two methoxyl methyls H-11 and H-13 at δ_H 3.68 and 3.65. Taking into account that in the COSY experiment the correlations between H-2/H-7, H-2/H-3, H-3/H-8, H-3/H-4, H-4/H-9 were evident, all these data suggested the presence of a dihydropyrane structure. The HMQC spectrum allowed the assignment of the protons to the corresponding carbons. Selected HMBC correlations between H-8/C-2, H-8/C-4, H-4/C-3, H-4/C-2, H-4/C-9, H-6/C-5, H-6/C-10, H-11/C-10, H-13/C-12, H-9/C12, H-9/C4 supported the assignment of the hydroxymethyl group at C-3, the methoxycarbonyl group at C-5 and the acetic acid methyl ester at C-4.

4-[β-D-Xylopyranosyl-(1 → 6)]-β-D-glucopyranosyl-1,4-dihydroxy-2-methoxy benzene (23)

EIMS (*m/z* 434 [M]$^+$; $C_{18}H_{25}O_{12}$): ^{1}H-NMR (D_2O) δ_H 6.75 (1H,*d*, *J* = 2.5 Hz, H-3), 6.70 (1H, *d*, *J* = 8.0 Hz, H-6), 6.60 (1H, *dd*, *J* = 2.5 and 8.0 Hz, H-5), 3.68 (3H, *s*, 2-OMe), 4.25 (1H, *d*, *J* = 7.6 Hz, H-1′), 3.07 (1H, *t*, *J* = 7.6 Hz, H-2′), 3.17 (1H, *t*, *J* = 7.6 Hz, H-3′), 3.70 (1H, *m*, H-4′), 3.41 (1H, *m*, H-5′), 3.94 (2H, *dd*, *J* = 10.6, H-6′), 4.90 (1H, *d*, *J* = 7.6 Hz, H-1″), 3.29-3.33 (2H, H-2″ and H-5″), 3.60 (1H, *m*, H-3″), 3.65-3.70 (1H, *m*, H-4″), 3.00 (1H, *t*, *J* = 7.6 Hz, H-5″): ^{13}C-NMR δ_C 150.1 (C-4), 147.7 (C-2), 140.4 (C-1), 115.4 (C-6), 108.6 (C-5), 102.9 (C-3), 55.8 (2-OMe), 103.1 (C-1′), 75.0 (C-2′), 75.2 (C-3′), 72.6 (C-4′), 75.2 (C-5′), 69.0 (C-6′), 100.7 (C-1″), 75.0 (C-2″), 68.8 (C-3″), 67.9 (C-4″), 64.7 (C-5″).

The positive FAB MS of compound **23** showed the parent peak at *m/z* 457 [M + Na]$^+$ which, together with the elemental analysis, defined the molecular formula as $C_{18}H_{26}O_{12}$. The ^{1}H-NMR spectrum in the aromatic region showed three protons as a doublet at δ 6.75 (*J* = 2.5 Hz), a doublet at δ 6.70 (*J* = 8.0 Hz), and a double doublet at δ 6.60 (*J* = 2.5 and 8.0 Hz), assigned to positions 2, 6 and 5 of a trisubstituted benzene. Also detectable were other protons which overlapped in the 3.8–3.0 ppm range, and two anomeric doublets at δ 4.25 and 4.90. Acid hydrolysis of **23** gave two sugars which were isolated by HPLC and identified as D-glucose and D-xylose by GC analysis (Leontein *et al.*, 1978). The GC-MS identification of 1,5-di-***O***-acetyl-2,3,4-tri-***O***-methylxylitol and of 1,5,6-tri-***O***-acetyl-2,3,4-tri-***O***-methylglucitol following the methylation analysis confirmed the 1 → 6 linkage between glucose and xylose and the couplings of 7.6 Hz for both the anomeric protons defined the β configuration of the two sugars.

3-[1-(Hydroxymethyl)-1-propenyl] δ–glutarolactone (24)

EIMS (*m/z* 184 [M]$^+$; $C_9H_{12}O_4$): ^{1}H-NMR δ_H 5.62 (1H, *q*, *J* = 6.0 Hz, H-8), 4.82 (1H, *d*, *J* = 12.5 Hz, H-7), 4.53 (1H, *d*, *J* = 12.5 Hz, H-7′), 3.30 (1H, *m*, H-3), 2.69 (1H, *dd*, *J* = 7.5 and 15.5 Hz, H-2), 2.75 (1H, *dd*, *J* = 7.5 and 15.5 Hz, H-2′), 2.47 (1H, *dd*, *J* = 9.0 and 15.0 Hz, H-4), 2.19 (1H, *dd*, *J* = 10.5 and 15.0 Hz, H-4′),1.70 (3H, *d*, *J* = 6.0 Hz, H-9): ^{13}C-NMR δ_C 179.2 (C-5), 176.6 (C-1), 136.0 (C-6), 125.6 (C-8), 74.0 (C-7), 44.0 (C-4), 36.7 (C-2), 32.3 (C-3), 14.2 (C-9).

The molecular formula of $C_9H_{12}O_4$ for compound **24** was based on the MS and ^{13}C-NMR data. The ^{1}H-NMR spectrum showed the H-8 olefinic proton as a quartet at δ 5.65 coupled with the H-9 doublet methyl at δ 1.74, the H-3 multiplet at δ 3.33 coupled with the H-4 methylene at δ 2.47 and 2.19 and with the H-2 methylene at δ 2.69 and 2.75; the H-7 methylene as two doublets at δ 4.50 and 4.80 was also detected. In the HMBC spectrum the H-3, H-7, and H-8 protons were correlated with the quaternary C-6 carbon. The H-8 had a cross peak with the C-9 carbon, the H-2 proton showed correlation with the C-1 carbonyl carbon at δ_C 176.6, and the H-4 protons were hetero-correlated with the C-5 carbon at δ_C 179.2. This molecule derives from breaking of the aglycone part of oleuropeine (Gil *et al.*, 1998).

In the present study the new lignan **21**, the seco-iridoid **22**, the phenylglycoside **23** and the lactone **24** have been isolated and identified as components of olive oil mill waste-waters along with the previously described aromatic compounds **1**–**20b**. D(+)-*Erythro*-1-(4-hydroxy-3-methoxy)-phenyl-1,2,3-propantriol (**18**), 2(3,4-dihydroxyphenyl)ethanol 3β-D-glucopyranoside (**20a**) and 2(3,4-dihydroxyphenyl)ethanol 4β-D-glucopyranoside (**20b**) have been isolated for the first time in olive oil mill waste-waters. The structures were established on the basis of spectroscopic data, including two-dimensional NMR analyses. It is reasonable to conclude, in agreement with the most recent literature in the field, that a detailed knowledge of all compounds to be found in OMW, as well as further studies on their phytotoxicity, is an important step for future developments in solving environmental problems related to the disposal of OMW.

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