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Antimicrobial Activity of Olive Solutions from Stored Alpeorujo against Plant Pathogenic Microorganisms

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ABSTRACT: The aim of this work was to assess the in vitro antimicrobial effects that wastewaters from alpeorujo oil extraction have against phytopathogenic bacteria and fungi. Alpeorujo was stored for 6 months and then processed to extract its oil, pomace, and a new liquid waste (OWSA), which was characterized by its content in phenolic compounds. OWSA at 20% decreased bu >4 log the population of *Erwinia* spp., *Pseudomonas* spp., and *Clavibacter* spp. viable cells in test tubes, whereas OWSA at 50% in agar medium was necessary to inhibit mycelial growth of most fungi. It was found that the bactericidal effect was due to the joint action of low molecular mass phenolic compounds, although neither hydroxytyrosol, its glucosides, hydroxytyrosol glycol, nor a glutaraldehyde-like compound individually explained this bioactivity. Hence, OWSA constitutes a promising natural solution to fight plant phytopathogenic bacteria and fungi.

KEYWORDS: alpeorujo, antibacterial, antifungal, hydroxytyrosol, olive oil

INTRODUCTION

The two-phase centrifugation process employed to extract olive oil is widespread in Spain and many other olive oil producing countries. The only waste produced from the two-phase system is the semisolid olive residue called "alpeorujo", which can account for $>4 \times 10^6$ tons per year in Spain. This paste can be composted or stored for months in large ponds before its residual oil is extracted using a three-phase centrifugation system. This new centrifugation process gives rise to (i) alpeorujo oil, (ii) olive pomace, and (iii) a dark liquid.[1] At present, this new wastewater obtained from the extraction process of stored alpeorujo (OWSA) is discharged in large ponds for evaporation or concentrated under vacuum, but it constitutes a new environmental problem for the extraction plants. The chemical characteristics of this liquid waste differ from that of the traditional olive mill wastewater (OMW) because it is produced from stored alpeorujo, and during this storage period chemical and microbial changes occur that make this sludge unique.[2,3] To the best of our knowledge, the characteristics of OWSA have never been studied.

Phytopathogenic bacteria and particularly fungi can cause severe economically damaging diseases, ranging from spots, mosaic patterns, or pustules on leaves and fruits, or smelly tuber rots, to plant death. Among bacteria, *Erwinia uredovora*, *Pseudomonas savastanoi*, and *Clavibacter michiganensis* are the most phytopathogenic species for plants cultivated in the Mediterranean basin. Likewise, the fungi *Alternaria*, *Botrytis*, *Pestaloptiopsis*, *Phytophthora* and *Colletotrichum* contribute to many diseases in strawberry, carrot, potato, grape, and others, and huge amounts of fungicides are used by farmers to fight these pathogens.[4−6] However, concerns over synthetic pesticides have stimulated researchers and companies to discover and develop natural products for the management of plant disease in agriculture.[7,8] Precisely, olive products and byproducts possess antibacterial and antifungal activity[9,10] that could be exploited for the control of plant microbial diseases. In particular, researchers have demonstrated in vitro and in vivo antimicrobial activity of OMW against several phytopathogenic bacteria and fungi.[11−13] Most of these works have associated the antimicrobial activity of OMW with its content in phenolic compounds[14−16] and in some cases with specific phenolic substances such as hydroxytyrosol, methylcatechol, and others.[17−19]

Raw dry olive residue from alpeorujo has also been tested in vitro against phytopathogenic fungi, but no effect was observed.[20] In fact, large numbers of saprophytic fungi and earthworms are able to grow on this material and reduce its phytotoxicity.[21,22]

In this work, we investigated the efficacy of OWSA against phytopathogenic bacteria and fungi. Thus, we analyzed the direct effect of OWSA on mycelial growth of these fungi in Petri dishes and the in vitro bactericidal effect of bioactive phenolic compounds. To our knowledge, there are no data on the antimicrobial activity of the new liquid waste originated from the stored alpeorujo.

MATERIALS AND METHODS

Olive Materials. The semisolid pomace from the olive oil extraction (alpeorujo) was supplied by Oleícola el Tejar, SCA, from two different factories located in southern Spain (Pedro Abad and Palenciana). These two factories receive large amounts of alpeorujo paste during the months of December and January, and three representative batches of this material were transported within 24−48 h to the Instituto de la Grasa premises. Samples of 70 kg were put in 100 L open containers and stored in duplicates for 6 months. OWSA was obtained from alpeorujo at 0 and after 6 months of storage. Using an Abencor analyzer (Comercial Abengoa, Spain), 600 g of Alpeorujo was mixed with 100 mL of water, and the paste was kneaded at 40 °C for 40 min. The resulting paste was centrifuged, and the liquid was collected. After 30 min of decanting, the OWSA was passed through a paper filter and then preserved at −30 °C until analysis.

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Microorganisms. All bacterial strains were obtained from the Spanish Type Culture Collection (CECT) at Burjassot, Valencia (Spain), except *Erwinia uredovora* LMG 2676^T (Belgian Coordinated Collections of Microorganisms). *Pseudomonas savastanoi* CECT 5019, *Pseudomonas savastanoi* CECT 5023, *Pseudomonas syringae* CECT 4429^T, *Clavibacter michiganensis* subsp. *michiganensis* CECT 790, *Clavibacter michiganensis* subsp. *insidiosus* 5042^T, *Clavibacter michiganensis* subsp. *sepedonicus* CECT 4886^T, *Erwinia toletana* CECT 5263^T, and *Erwinia amylovora* CECT 222 were used. Bacterial strains were maintained at −80 °C in nutrient broth with 20% glycerol. Before each experiment, strains were cultured twice from the frozen stock.

The fungal strains tested were *Alternaria* sp., *Pestalotiopsis dyospiri*, *Botrytis cinerea*, *Phytophthora cactorum*, and *Colletotrichum acutatum* CECT 20240. They were isolated from persimmon and strawberry and identified by the Las Torres Tomejil IFAPA center.[4,5]

Chemical and Microbiological Analyses of Alpeorujo Paste during Storage. Sugars (glucose, fructose, sucrose, and mannitol), organic acids, and ethanol were analyzed by HPLC, as described elsewhere.[23]

The microbial population in alpeorujo before the OWSA extraction was monitored by spreading alpeorujo samples and appropriate decimal dilutions (in sterile 1 g/L peptone water) onto different culture media. Alpeorujo samples were aseptically taken from the 5−10 cm depth after removal of the dry surface with sterile tools. Alpeorujo samples were spread onto the different plates with Digralsky spreaders and their dilutions with a Spiral Plater (Don Whitley Sci. Ltd., model Wasp 2, Shirpley, U.K.). Enterobacteriaceae were counted on crystal-violet neutral-red bile dextrose agar (Merck, Darmstadt, Germany) incubated at 37 °C, lactic acid bacteria on MRS agar (Oxoid), and yeasts and molds on OGYE agar (Oxoid). The latter two were incubated at 32 °C. After 24−72 h of incubation, colony-forming units (CFU) per gram was counted (Counter-mat, IUL Instruments, Barcelona, Spain).

Analysis of Phenolic and Oleosidic Compounds. A mixture of 250 μL of OWSA, 250 μL of internal standard (2 mmol/L syringic acid), and 500 μL of deionized water was filtered through a 0.45 μm pore size nylon filter. An aliquot (20 μL) was injected into the chromatograph. The chromatographic system consisted of a Waters 717 plus autosampler, a Waters 600E pump, and a Waters 996 diode array detector (Waters Inc., Milford, MA). A Spherisorb ODS-2 (5 μm, 25 cm × 4.6 mm i.d., Waters Inc.) column was used. Separation was achieved using an elution gradient with an initial composition of 90% water (pH adjusted to 2.3 with phosphoric acid) and 10% methanol. The concentration of the latter solvent was increased to 30% over 10 min and maintained for 20 min. Subsequently, the methanol percentage was raised to 40% over 10 min, maintained for 5 min, and then increased to 50%. Finally, the methanol percentage was increased to 60%, 70%, and 100% in 5 min periods. A flow of 1 mL/min and a temperature of 35 °C were used. Phenolic and oleosidic compounds were monitored at 280 and 240 nm, respectively. The mixtures were analyzed in duplicate.

Phenolic and oleosidic compounds were also analyzed by LC-MS using a quadrupole mass analyzer (ZMD4, Waters Inc.) equipped with an electrospray ionization (ESI) probe, working in the negative-ion mode.[2]

Compounds Tested. 3,4-Dihydroxyphenyl glycol (hydroxytyrosol glycol) and glutaraldehyde were purchased from Sigma (St. Louis, MO). The dialdehydic form of decarboxymethyl elenolic acid (EDA) was isolated from a brine of black olives. Hydroxytyrosol, an EDA-like compound, and a mixture of hydroxytyrosol with hydroxytyrosol 1-glucoside were isolated from an OWSA solution using analytical high-pressure chromatography with the same analytical column, mobile phases, gradient, and equipment used for the polyphenol analysis except for the aqueous mobile phase, which was acidified with 2 equiv/L HCl (522 μL/L). Fractions were collected peak-by-peak with a Waters Fraction Collector II. The pooled extract for each peak was evaporated under vacuum close to dryness, and the residue was dissolved in 1 mL of distilled water. Finally, the purity and concentration of each compound were measured by HPLC-MS.

Table 1. Concentrations of Sugars, Acetic Acid, and Ethanol in the Solutions (OWSA) Obtained during Processing of Alpeorujo by the Abencor Methodology[a]

	concn (mmol/L) after alpeorujo storage time of	
compound	0 months	6 months
glucose	152.3 ± 14.2 a	1.7 ± 0.3 b
fructose	58.4 ± 2.8 a	7.8 ± 0.6 b
mannitol	132.5 ± 9.1 a	130.2 ± 22.6 a
acetic acid	7.8 ± 1.8 a	15.1 ± 2.1 b
ethanol	185.8 ± 38.8 a	208.8 ± 35.5 a

[a] Data are expressed as the mean ± standard error. Values for each compound not sharing a common letter were significantly different according to a Student's t test at $P < 0.05$. Lactic acid was never detected.

Extracts Tested. OWSA solutions were filtered through cellulose membranes (Amicon Corp.) with different cutoffs (0.22 μm, 100,000, 30,000, and 3,000 Da), and the filtrate was diluted with autoclaved tap water to reach 2.5, 5, 10, and 20% of their original concentrations.

An ethyl acetate extract was also obtained as follows: 800 μL of OWSA was extracted six times with 2 mL of ethyl acetate; extracts were collected, and the solvent was eliminated by vacuum evaporation. Finally, the residue was dissolved in 800 μL of distilled water and diluted with water to reach 5, 10, and 20% of their original concentrations. Eight hundred microliters of distilled water was also extracted with ethyl acetate and used as control.

Bactericidal Activity. One hundred and fifty microliters of the sample (pure compounds, extracts, or tap water as control) was inoculated with 10 μL of an overnight broth culture of the target microorganism diluted with saline to get an initial population ca. 10^6 CFU/mL. The mixture was incubated at room temperature for 5 min with occasional shaking and then plated onto nutrient agar to count survivors after up to 5 days of incubation at 32 °C. Experiments were run at pH 5.5. The pH (4.8−5.0) of the OWSA extracts was adjusted to 5.5 with 3 N KOH.

Fungistatic Activity of OWSA. The level of growth inhibition induced by olive solutions was tested by the poisoned medium technique.[4] Fungi were grown on potato dextrose agar (PDA) from Difco Laboratories (Detroit, MI) at 25 °C for 7 days. PDA (20 mL) prepared with different percentages of OWSA (pH adjusted to 5.5) to achieve different concentrations (5, 20, 50%) was poured into Petri dishes. A mycelial disk (5 mm diameter) was taken from the periphery of an actively growing PDA culture and placed at the center of an 85 mm × 13 mm Petri dish. The dishes were incubated at 25 °C. Controls consisted of Petri dishes with the mycelial disk, but PDA was prepared with sterile distilled water (pH 5.6). After 3−6 days of incubation, the diameter of the colonies was recorded. Fungitoxicity was expressed in terms of percentage of mycelial growth inhibition (%MGI) and calculated following the formula of Pandey et al.:[24] $\%MGI = d_c - d_t/d_c$, where dc is the average diameter of the fungal colony in the control and dt is the average diameter of the fungal colony in the treatment. Three replicate Petri dishes were used for each treatment, for each fungus, and for each of the doses tested.

Statistical Analysis. The different statistical techniques used in this work were implemented using Software Statistica version 7.0 (Statsoft Inc., Tulsa, OK). Significant differences were determined by using the Student's t test and the one-way factor analysis of variance (ANOVA, Duncan's test).

RESULTS AND DISCUSSION

The new liquid waste obtained from stored alpeorujo (OWSA) has never been characterized. Table 1 shows the carbohydrate, acetic acid, and ethanol composition of the liquid

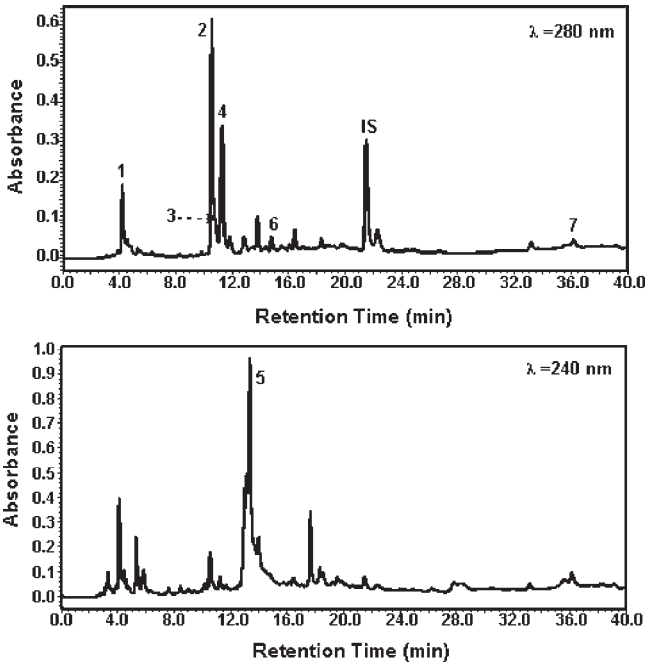

Figure 1. HPLC analysis of liquid waste obtained from fresh alpeorujo. Peaks: 1, hydroxytyrosol glycol; 2, hydroxytyrosol; 3, hydroxytyrosol 1-glucoside; 4, hydroxytyrosol 4-glucoside; 5, EDA-like compound; 6, tyrosol; IS, internal standard (syringic acid); 7, dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA).

wastes obtained from "fresh" and stored alpeorujo. As expected, sugars (glucose and fructose) were almost completely consumed after 6 months of storage, whereas mannitol was not metabolized. This sugar alcohol is also a recalcitrant compound in olive fermentation brines.[25] Low concentrations of acetic acid and ethanol were formed during alpeorujo storage, although a high amount of ethanol was also detected in the liquid waste obtained from fresh alpeorujo. Lactic acid was not found in fresh or stored alpeorujo liquid. It must be said that 2−3 days passed between the production of alpeorujo in the olive oil factory and its delivery to the laboratory. Thus, fermentation of the olive paste could occur during this period of time and ethanol could have been formed.

The prevalent microbiota in alpeorujo paste during storage were yeasts (data not shown), which reached a population between 10^5 and 10^6 CFU/g. Occasionally, Enterobacteriaceae were also detected at the beginning of storage, and lactic acid bacteria were not found. Consequently, the changes in sugars and metabolic end products (ethanol and acetic acid) could be related to the presence of yeasts in these olive pastes, which have been associated with the formation during storage of the off-odor 4-ethylphenol.[3]

Besides microbial growth, chemical changes in the oily phase of alpeorujo also occur during its storage in open-air ponds, and alpeorujo oil exhibits increased quantities of triterpenic acids, short-chain alcohol esters, and the off-odor 4-ethylphenol.[2,3,26] However, there is a lack of data regarding phenolic compounds in the aqueous phase of this stored alpeorujo. Figure 1 and Table 2 show that hydroxytyrosol 4-glucoside was the main phenolic compound in the aqueous phase of fresh alpeorujo, followed by hydroxytyrosol and hydroxytyrosol 1-glucoside. By contrast, hydroxytyrosol 4-glucoside was not detected in OWSA after 6 months of storage, which could be due mainly to its hydrolysis

Table 2. Concentrations of Phenolic Compounds in the Solutions (OWSA) Obtained during Processing of Alpeorujo by the Abencor Methodology[a]

	concn (mmol/L) after alpeorujo storage time of	
compound	0 months	6 months
hydroxytyrosol glycol	1.56 ± 0.48 a	1.91 ± 0.51 a
hydroxytyrosol	9.87 ± 2.04 a	18.38 ± 4.90 b
hydroxytyrosol 1-glucoside	6.85 ± 3.09 a	12.26 ± 3.40 b
hydroxytyrosol 4-glucoside	16.95 ± 5.97	nd[b]
tyrosol	1.99 ± 0.23 a	2.25 ± 0.48 a
HyEDA[c]	0.65 ± 0.31	nd
EDA-like compound[d]	9.72 ± 4.22 a	5.14 ± 2.46 a

[a] Data are expressed as the mean ± standard error. Values for each compound not sharing a common letter were significantly different according to a Student's *t* test at $P < 0.05$. [b] nd, not detected. [c] Dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol. [d] Quantified as EDA, the dialdehydic form of decarboxymethyl elenolic acid.

and, consequently, formation of hydroxytyrosol, as it has also been reported during the fermentation of natural black olives[27] and storing of olive oil wastewaters from the three-phase centrifugation system.[28] Other minor compounds detected in fresh alpeorujo were hydroxytyrosol glycol, tyrosol, and the dialdehydic form of decarboxymethyl elenolic acid linked to hydroxytyrosol (HyEDA). The latter substance possesses a high antimicrobial activity,[9] but it disappeared during storage, probably as a consequence of its hydrolysis into EDA and hydroxytyrosol in a similar manner as reported during table olive fermentation.[29]

In principle, peak 5 eluting at 14 min (Figure 1) was identified as the dialdehydic form of decarboxymethyl elenolic acid (EDA) on the basis of its retention time and UV spectrum with a maximum absorbance at 230 nm. However, the MS spectrum of peak 5 recorded in the negative ion mode exhibited a signal at m/z 199 instead of at m/z 183, corresponding to EDA. Hence, peak 5 was named an EDA-like compound, and it was quantified using the response factor of EDA. Finally, it must be said that the off-odor 4-ethylphenol, which is found in stored alpeorujo oil,[3] was not detected in OWSA.

Studies in vitro on the antimicrobial activity of diluted OWSA indicated a clear bactericidal effect of these solutions against the phytopathogenic bacteria *Erwinia*, *Pseudomonas*, and *Clavibacter* (Figure 2). It can be seen that 10% OWSA reduced the initial population by ca. 1−3 log CFU/mL, being notable the effect with 20% for the three genera. A high number of papers address the antibacterial activity of olive oil mill wastewaters,[12−18] but this is the first time that this activity against phtytopathogenic bacteria has been reported for OWSA. Obviously, these in vitro results must be confirmed in vivo, particularly taking into consideration the studies on the toxicity exerted in plants by olive oil wastewaters[22] and dry alpeorujo.[30]

Because many plant diseases are caused by fungi, the action of OWSA against several phytopathogenic fungi was also tested (Figure 3). OWSA showed a dose-dependent antifungal activity. In fact, a complete inhibition of mycelium growth was only reached for *P. cactorum* with 50% OWSA in the culture medium. Less activity was detected against *C. acutatum*, *Alternaria* sp., *B. cinerea*, and *P. dyospiri*, but growth inhibition of 30−60% was

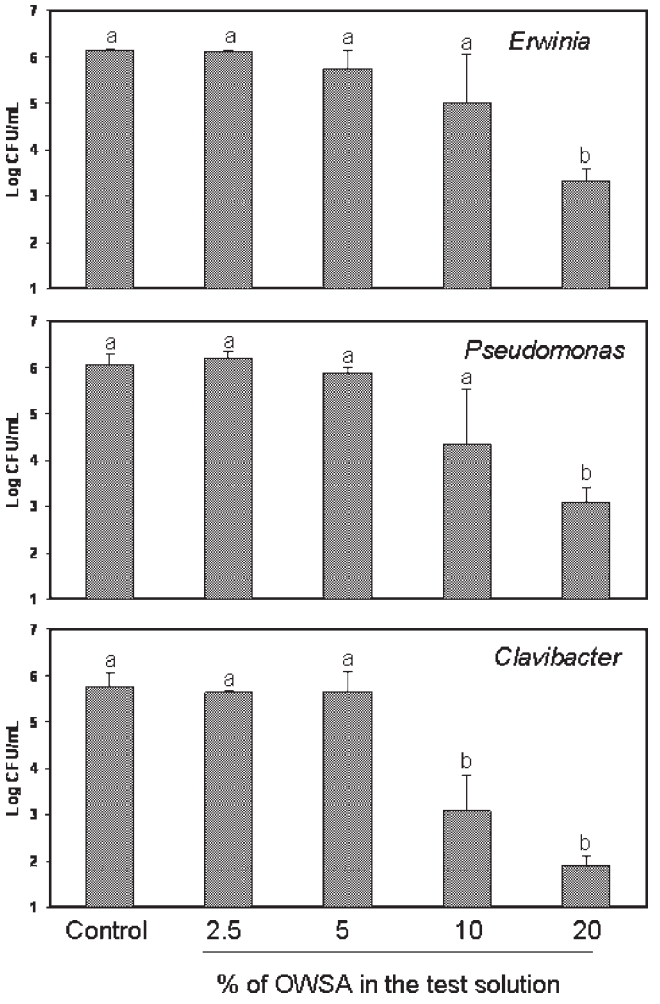

Figure 2. Bactericidal effect of OWSA against *Erwinia* (*E. uredovora* LMG 2676, *E. toletana* CECT 5263, and *E. amylovora* CECT 222), *Pseudomonas* (*P. savastanoi* CECT 5019 and 5023 and *P. syringae* CECT 4429), and *Clavibacter michiganensis* (subsp. *michiganensis* CECT 790, subsp. *insidiosus* CECT 5042, and subsp. *sepedonicus* CECT 4886). Microorganisms were in contact for 5 min with the OWSA solution or autoclaved tap water as control. Bars indicate the standard error of three strains of each genus, and those with different letters are significantly different according to a multiple Duncan tests ($P < 0.05$). The detection limit of enumeration was 1.3 log CFU/mL.

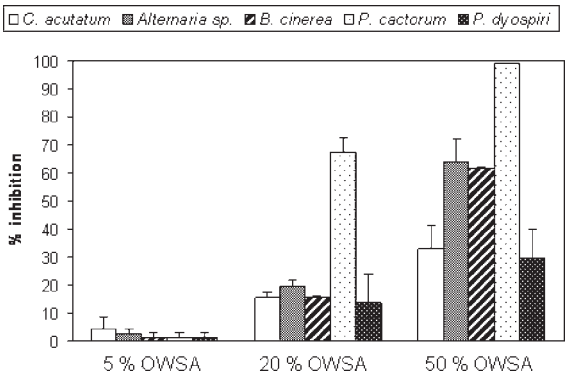

Figure 3. Mycelial growth inhibition of different concentration of OWSA in PDA on several phytopathogenic fungi. Bars indicate the standard error of triplicates.

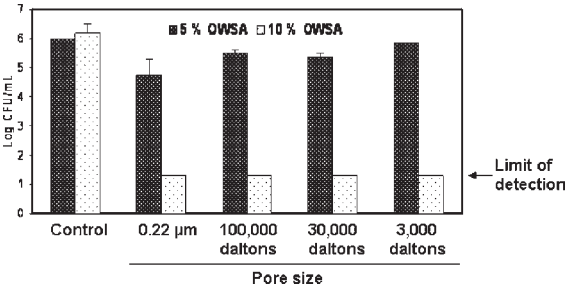

Figure 4. Bactericidal effect of ultrafiltered OWSA at 5 and 10% against *E. amylovora* CECT 222. Microbial cells were in contact for 5 min with the filtered OWSA or autoclaved tap water as control. Bars indicate the standard error of triplicates.

obtained in most cases with 50% OWSA in the culture medium. Our data provide first evidence that OWSA possesses antifungal activity against plant pathogens, although the concentration requested (50%) seems too high. Nevertheless, we have not tested an extract or a concentrated OWSA solution.

Some researchers have not found antifungal activity for OMW, but many others have reported mycelium growth inhibition by OMW or its phenolic extracts.[10,11,13] Indeed, hydroxytyrosol has mainly been associated with the antimicrobial activity of these solutions.[19] First, we tried to rule out the possible antibacterial activity of high polymers present in OWSA by ultrafiltering the solution through several pore size molecular weights and testing them. The results presented in Figure 4 demonstrated that the bactericidal activity of OWSA was likely due to low molecular mass compounds (<3000 Da) because 10% diluted OWSA in the culture medium induced the cell death of *E. amylovora* regardless of the ultrafiltration pore size used. Cell population was below the detection limit when polymers with molecular size higher than 3000 Da were removed from OWSA. Even though there are studies that report antimicrobial activity by high molecular mass compounds in olive-derived products,[31] it seems that in the case of OWSA the low molecular mass substances are responsible for this bioactivity. As commented above, lactic acid was not detected in OWSA and the concentration of acetic acid was very low. Ethanol was also ruled out because a concentration 3 times higher than present in the OWSA did not show any effect against the tested strains.

Moreover, many researchers have pointed out the phenolic compounds as the main antimicrobial substances in olives and OMW, although there is controversy over attributing this bioactivity to one specific compound. Capasso et al.[17] proposed methylcatechol as the main antimicrobial substance in OMW. Oleuropein,[32] tyrosol,[6] and the flavonoids luteolin and quercetin[33] have also been reported with high antimicrobial activity. Recently, an extract very rich in hydroxytyrosol from OMW showed a powerful antifungal and antibacterial activity against plant pathogens.[19] However, it has recently been demonstrated that the most powerful antibacterial substances in olive products are glutaraldehyde-like compounds such as HyEDA and EDA.[9] Thus, the antibacterial activity of peak 5, which was named EDA-like compound, was tested against *E. amylovora* and *P. savastanoi* at a concentration of up to 1 mM (Figure 5), which is double that estimated in a 10% OWSA solution (Table 2). The effect of the EDA-like compound was compared with that of pure EDA isolated from table olives (not present in

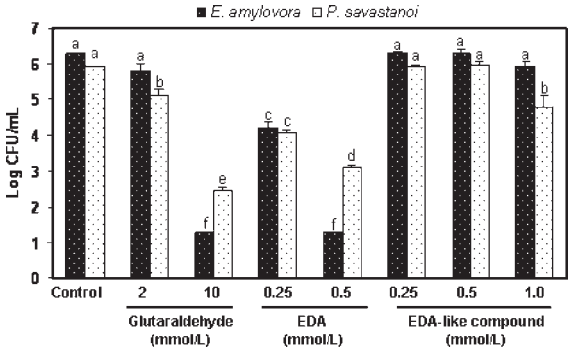

Figure 5. Bactericidal effect of EDA and isolated EDA-like compound against *E. amylovora* CECT 222 and *P. savastanoi* CECT 5019. Autoclaved tap water was used as control. Identical letters over error bars within the same microorganism tested indicate that there is no significant difference according to multiple Duncan tests ($P < 0.05$).

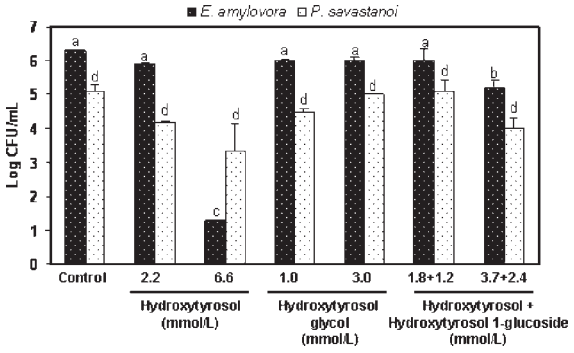

Figure 6. Bactericidal effect of hydroxytyrosol and hydroxytyrosol glycol against *E. amylovora* CECT 222 and *P. savastanoi* CECT 5019. Autoclaved tap water was used as control. Identical letters over error bars within the same microorganism tested indicate that there is no significant difference according to multiple Duncan tests ($P < 0.05$).

OWSA) and glutaraldehyde as positive controls. Unlike pure EDA isolated from table olives and glutaraldehyde, the EDA-like compound present in OWSA did not show bactericidal activity at a concentration of 0.5 mmol/L and only a slight decrease in *P. savastanoi* population when tested at double the concentration found in a 10% OWSA solution (Figure 5). We must note the high antimicrobial activity exerted by isolated EDA, although this substance was not detected in OWSA.

Hydroxytyrosol, its glucosides, and tyrosol have been assessed against lactic acid bacteria and did not show significant activity.[34] To our knowledge, there are no data on the antimicrobial activity of hydroxytyrosol glycol, which has been disclosed as an important phenolic compound in olive oil waste.[35] We tested hydroxytyrosol glycol against *E. amylovora* and *P. savastanoi* at a very much higher concentration (3 mM) than that found in a 10% OWSA solution (0.19 mM), and no antibacterial effect was observed ($P < 0.05$) (Figure 6). Reduction of cell viability by the action of hydroxytyrosol glycol was not significant ($P < 0.05$), and the contribution of this compound to the antimicrobial activity of OWSA was probably negligible. Hydroxytyrosol isolated from OWSA was also tested at a concentration of 2.2 mmol/L, which is a little higher than that found in the 10% OWSA solution (1.8 mM), and no significant activity ($P < 0.05$) was found. In contrast, 3 times the latter concentration (6.6 mmol/L) gave rise to a significant decrease ($P < 0.05$) in the

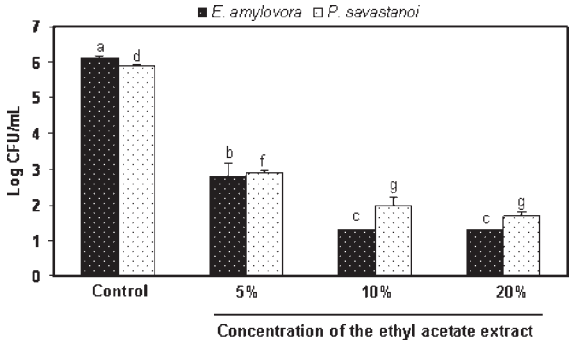

Figure 7. Bactericidal effect of an ethyl acetate extract of OWSA against *E. amylovora* CECT 222 and *P. savastanoi* CECT 5019. Control was an ethyl acetate extract of distilled water. Identical letters over error bars within the same microorganism tested indicate that there is no significant difference according to multiple Duncan tests ($P < 0.05$).

population of *E. amylovora*. In fact, hydroxytyrosol does not possess a strong bactericidal activity if it is compared with that of HyEDA and EDA, in particular against lactic acid bacteria.[9] Finally, a mixture of hydroxytyrosol and hydroxytyrosol 1-glucoside was assayed at a concentration similar to that found in 10% OWSA, and no significant effect ($P < 0.05$) was detected (Figure 6). Hydroxytyrosol 1-glucoside has also been tested against lactic acid bacteria, and only a weak activity was observed.[9] In consequence, the antibacterial activity of OWSA cannot be explained by that of hydroxytyrosol and its derivatives.

Because none of the individual phenolic compounds nor the polymer fraction of these liquids was responsible for the OWSA antimicrobial activity, we tested an ethyl acetate extract with a mixture of all the phenolic compounds (Figure 7). It was confirmed by HPLC analysis that all compounds reflected in Table 2 were present in the extract, and no new compounds were observed in the chromatogram. This mixture exerted a significant bactericidal activity at 5% ($P < 0.05$), this effect being higher with increasing concentration. It can be seen that the control of distilled water extracted with ethyl acetate allowed a survival of ca. 10^6 CFU/mL of both *E. amylovora* and *P. savastanoi*. On the contrary, the ethyl acetate extract of OWSA at a concentration of 5% decreased significantly ($P < 0.05$) the number of survivors of both species. A more outstanding effect was detected when 10 and 20% extracts were assayed, these results being similar for both concentrations.

In summary, the liquid phase originated after the processing of stored alpeorujo possesses antifungal and antibacterial activities against phytopathogenic microorganisms, but this activity cannot be assigned to a particular substance or to the polymeric fraction of this liquid. However, the antimicrobial activity exerted by the ethyl acetate extract of OWSA, which contained a mixture of monomeric phenolic compounds, was able to explain the bioactivity observed for OWSA.

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