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Measuring the antimicrobial activity of natural extracts against food spoilage bacteria to enhance food hygiene: preliminary *in vitro* results

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ABSTRACT
Food products are prone to microbial contamination able to affect their safety and quality attributes and their nutritional value. The interest in the potential use of bioactive compounds deriving from natural matrices, especially agro-industrial wastes, as alternatives to classical food preservatives has rapidly increased. In the present study a food grade olive mill wastewater polyphenolic extract and a commercial mix were characterised and their antioxidant and antimicrobial capacity were assessed. The antimicrobial activity was preliminary assessed *in vitro* by agar well diffusion, subsequently by microdilution method to define the minimum inhibitory and bactericidal concentration. The olive mill wastewater polyphenolic extract registered a higher antioxidant capacity [(13.3 ± 1.0) 10^2 µgTE/(100 g)] and antimicrobial efficacy (max MBC value 0.2500 g/mL) compared to commercial mix with wide potential application in food industry.

Section: RESEARCH PAPER

Keywords: Olive mill wastewater; polyphenols; agro-industrial by-products; MIC; MBC; ORAC

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1. INTRODUCTION

Food products are characterised by high susceptibility to microbial contamination with several potential consequences on safety and quality attributes as well as on the reduction of the nutritional aspects [1]. To counteract this phenomenon, chemical additives with different activities such as antimicrobial and antioxidant, are commonly added to food stuff. Synthetic preservatives, indeed, are widely employed in the industry during food production to decrease or eradicate the unwanted presence of microorganisms and so extend the shelf life of food products [2], [3].Despite the fact that food additives are strictly regulated (Regulation (EC) No.1333/2008, s.m.i.) [4], consumers are sceptical of chemical substances that are purposefully added to foodstuff due to the possibility of long-term negative effects on human health[5].

Recently, however, the interest in bioactive substances derived from natural matrices, particularly agro-industrial wastes, as substitutes for conventional food preservatives has remarkably increased. These substances may represent a novel approach to prevent Foodborne illnesses and limiting food waste[6]-[8].

Fruit and vegetable processing does produce a high amount of waste, but reusing those wastes in the food business could help to solve the environmental, economic and social issues [6], [9]. Also, since these bioactive chemicals have the capability to limit the growth of microorganisms that cause food spoiling, their use in food manufacturing may have a favourable impact on food safety [7]. To effectively contain microbial contamination along the food production chain, for instance, the use of natural antibacterial agents defines a valuable and sustainable alternative method [10], [11]. For instance, olive oil by-products can be exploited as a source of bioactive molecules

that might be suitable for improving food hygiene [1], [12]. Olive oil by-products are characterised by several hydrophilic phenols, particularly represented by secoiridoids, whose presence has been revealed only for plants belonging to *Oleaceae* family, that seem to be able to inhibit the growth of several Gram-positive and Gram-negative bacteria and to express high antioxidant properties as well [12]. The recovery of the high-value bioactive compounds from the olive mill wastewater could enable the possible exploitation of this agro-industrial waste, enhancing the economic and environmental sustainability of the agro-industrial sector, especially considering its high generation rate (49 % of total mass) [1], [11]. In order to protect public health and reduce the significant economic and social effects of food waste, it is crucial that competent authorities and the scientific community continue to focus on ensuring food hygiene and safety for consumers [1].

The present study aimed to compare the antibacterial activity of a commercial mix used as an ingredient in meat product formulations with a food grade polyphenolic extract from olive mill wastewater. To determine the antibacterial activity the agar well diffusion was preliminarily applied. Than the microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The study targeted spoilage microorganisms strongly related to food hygiene.

2. MATERIAL AND METHODS

2.1. Extracts

The crude olive mill wastewater phenolic extract (PE) used was obtained by means of a membrane filtration process using fresh olive mill wastewaters. To obtain a stable powder formulation, the extract was subjected to spray drying after their combination with maltodextrins as carrier matrix (1:1dw) [13].

The beetroot commercial mix (CM) is represented by a fine powder intended to be added to hamburger meat batter functioning as stabilizing agent (MecImport GroupSrl, Perugia, Italy).

2.2. Polyphenols determination by LC-QTOF

Two aliquots of the extract were collected and diluted 50 and 500-fold, respectively, with a mixture of acetic acid 0.025 %/methanol 90/10 (v/v). After filtration, both aliquots were injected. Liquid-Chromatography Quadrupole Time-Of Flight spectrometry (LC-QTOF) was applied to determine twelve polyphenols. The equipment consisted of an ExionLC™ coupled to a 6600+TripleTOF™ (ABSciex, Foster, CA, USA) equipped with an electrospray ionization source operating in negative mode (ESI-). Chromatographic separation was carried out on an Acquity BEH C18 (150 mm × 2.1 mm, 1.7 μm, Waters, Milford, MA, USA).

Water with 0.025 % aceticacid (A) and methanol/ACN 90/10 v/v % (B) were used as mobile phases. The gradient started with 0 % of B (1min); the percentage of B was increased to 20 % in 10 min, followed by an increase to 50 % B in 4 min and another one to 100 % in 1 min. After 4 min, B percentage was reported to initial conditions (0 %) in 1 min. Finally, the system was re-equilibrated for 5 min (run time: 26 min). The column temperature was set at 40 °C and the autosampler temperature was kept at 25 °C. Flow rate and injection volume were 0.25 mL/min and 10 μL, respectively. Compressed air was used as GS1 (55 arbitrary units) and GS2 (55 arbitrary units), whereas nitrogen was the curtain gas (40 arbitrary units). The spray voltage was set at −4.5 kV and interface source temperature at 450°C. Single infusions of each analyte were carried out to optimize declustering potential (DP) and collision energy (CE). Theprecursor ([M-H$^+$]) and fragmentions acquired in MRM^{HR} mode are listed in Table 1. Mass error was ≤ 5 ppm.

Table 1. Analyte Retention Times and monitored ions.

Analyte	RT (min)	Molecular formula	Precursor (m/z)	Fragment (m/z)	DP (V)	CE (V)
Hydroxytyrosol	9.2	C8H10O3	153.0557	123.0455	-80	-14
Hydroxytyrosol-D4	9.2	C8H6D4O3	157.0808	125.0588	-80	-15
Tyrosol	11.9	C8H10O2	137.0608	119.0520	-90	-18
Vanillic acid	13.3	C8H8O4	167.0350	152.0111	-70	-15
Vanillin	14.9	C8H8O3	151.0401	136.0166	-60	-14
p-Coumaric acid	15.4	C9H8O3	163.0401	119.0500	-60	-14
Verbascoside	16.4	C29H36O15	623.1981	161.0251	-90	-38
Oleuropein	17.3	C25H32O13	539.1770	307.0824	-100	-27
Pinoresinol	17.4	C20H22O6	357.1344	151.0410	-80	-20
Luteolin	17.5	C15H10O6	285.0405	133.0293	-110	-36
Oleuropeinaglygone	17.6	C19H22O8	377.1242	307.0824	-80	-14
Apigenin	17.7	C15H10O5	269.0456	117.0343	-110	-35

2.3. Antioxidant capacity of extracts

The antioxidant capacity was evaluated using the Oxygen Radical Absorbance Capacity method ($ORAC_{FL}$). To do this, one gram of each extract was mixed separately with a buffer solution with a pH of 7.2, containing 13.19 g of K_2HPO_4 and 10.26 g of KH_2PO_4 dissolved in 900 mL of deionized water. This mixture was homogenised using an Ultra-Turrax homogenizer (Ultra Turrax T25 Basic, IKA Labortechnik Janke & Kunkel GmbH, Stavfen, Germany) for one minute, followed by two minutes of vortexing. After homogenization, the samples were centrifuged at 6000 rpm for 20 minutes at a temperature of 4°C. The resulting supernatant was then used to determine the antioxidant capacity through the $ORAC_{FL}$ method. The $ORAC_{FL}$ method measures antioxidant capacity by comparing it to a reference standard, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, obtained from Sigma-Aldrich, Steinheim, Germany). This comparison is based on the rate of fluorescence decay of a probe when exposed to a radical oxygen species (ROO). The $ORAC_{FL}$ assays were carried out through a FLUOstar OPTIMA microplate fluorescence reader (BMGLABTECH, Offenburg, Germany) with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The results are reported as micrograms of Trolox equivalents (TE) per 100 g of the sample.

2.4. *In vitro* measurement of antibacterial activity – Agar well Diffusion

Extracts' antibacterial activity was determined by the agar well-diffusion method against different food spoilage bacteria. In particular *Escherichia coli*, *Pseudomonas fluorescens, Pseudomonas aeruginosa, Lactobacillus plantarum subsp. plantarum*, *Lactobacillus sakei subsp. sakei, Lactococcus lactis* strains were bought from Microbiologics, St.Cloud, MN, USA, while *Shewanella putrefaciens* and *Brochothrix thermosphacta* derived from Istituto Zooprofilattico Sperimentale dell' Umbria and Marche "Togo Rosati" (IZSUM) collection isolated from meat samples.

For each organism, a suspension with a turbidity of 0.5 McFarland in a 0.9 % sterile saline solution was prepared. Subsequently, 100 μL of this suspension were evenly spread onto

each quadrant of Mueller-Hinton agar (MHA) or Mueller-Hinton agar supplemented with 5 % defibrinated sheep blood (MHAB) plates (manufactured by Oxoid Limited, Basingstoke, UK) using a swab [1]. Circular holes with a diameter of 7 mm were created in the agar plates by removing a portion of the medium with a sterilized cork borer. Subsequently, 50 μL of an extract solution in sterile demineralised water (with a concentration of 1000 mg/mL) were introduced into these holes. The plates were then incubated under conditions suitable for the growth of each specific bacterial strain. After the designated incubation period, the presence and size of the inhibition zone were assessed using a measuring gauge in millimeters [14], [15].

2.5. Minimum Inihibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination

The investigation into the antibacterial properties of the extracts was extended through the determination of MICs/MBCs for the specific microorganisms of interest. MICs/MBCs were assessed using the standard broth microdilution method, following the guidelines established by the Clinical Laboratory Standards Institute (CLSI) [16]. To carry out this procedure, bacterial suspensions were prepared, adjusting the bacterial count to 10^5 CFU/mL by diluting them in fresh Mueller-Hinton broth with 5 % blood (supplied by Biolife Italiana s.r.l., Milan, Italy). Aliquots of each suspension were added to 96-well microplates (manufactured by Starlab International GmbH, Hamburg, Germany) containing equal volumes of two-fold serial dilutions of the extract, ranging from concentrations of 0.5 to 0.0039 g/mL. Three control groups were established: an antibiotic control (using benzylpenicillin sodium salt from Sigma-Aldrich, St. Louis, MO, USA), an organism control (comprising culture medium and bacterial suspension), and a negative control. Subsequently, the plates were incubated under the best growth conditions of each bacterial strain. The MIC was defined as the lowest concentration of the extract that exhibited no bacterial growth when compared to the wells at the start of the incubation period [17]. The MBC was determined by transferring samples from the broths utilised for MIC determination onto culture media.

2.6. Statistical Analysis

The data obtained from the agar well-diffusion test underwent statistical analysis using an analysis of variance (ANOVA) model as implemented in SAS software (SAS Institute Inc., Cary, NC, USA, 2001). In order to elucidate any noteworthy differences in means (with a significance level of $p < 0.05$), Tukey's *post-hoc* analysis was applied.

3. RESULTS AND DISCUSSION

3.1. Polyphenols determination by LC-QTOF

Table 1 reports the Analyte Retention Times and monitored ions assessed by means of LC-QTOF. The chemical composition of this olive mill wastewater extract is in line with that of other extracts obtained from similar products already reported in the literature [17]-[19].

The most representative bioactive compounds belong to the class of phenols. Specifically, hydroxytyrosol (3,4-DHPEA) and tyrosol (p-HPEA) are phenolic alcohols, verbascoside is a hydroxycinnamic acid's derivative, the caffeic acid, the p-coumaric acid, and the vanillic acid are phenolic acids and derivatives. The luteolin is a flavone and the pinoresinol a lignans, verbascoside is a secoiridoid.

Due to their properties, these phenolic compounds can be adopted by the pharmaceutical sector as well as in cosmetics and medicine and as nutraceutical products and antioxidants in foods [20], [21]. The specific content of the major phenolic compounds in olive mill wastewater spray dry extract was 13.0 ±1.0, 2.2 ±0.3 and 0.59±0.01 mg/g for hydroxytyrosol, tyrosol, and vanillic acid, respectively. Notably, hydroxytyrosol and tyrosol are known to have numerous biological activities, proved both *in vitro* and *in vivo* [22].

3.2. Evaluation of antioxidant capacity of extracts

A higher antioxidant activity was found in the PE in comparison with CM containing ascorbic acid and beetroot (532 ± 4) and (13.3 ± 0.1) 10^2 μgTE/100g in CMandPE, respectively). The high antioxidant activity registered in the PE is in accordance with previous studies that refer to the powerful antioxidant activity of olive phenolic compounds [21], [23].

Oxidation has been demonstrated as the main non-microbial cause of food quality deterioration. For instance, oxidative deterioration is capable of limiting food acceptability and shortening its shelf-life by causing discoloration, the development of off-flavours and the formation of toxic compounds. Recently, great interest has been addressed to natural antioxidants that can be used as technological strategies applying antioxidants directly into food products or by coating packaging materials with natural extracts to improve the oxidative stability of the products, therefore avoiding or reducing the use of chemical compounds [23]. Another approach is represented by the dietary manipulations in which antioxidant compounds, or their metabolites, are introduced into the food (milk, muscle or egg) via feed [24].

3.3. Measurements of the in vitro Antibacterial Activity – Agar well diffusion

PE and CM were evaluated qualitatively and quantitatively for their in vitro antibacterial activity against the chosen bacteria based on the presence or absence of inhibition zones (Figure 1).

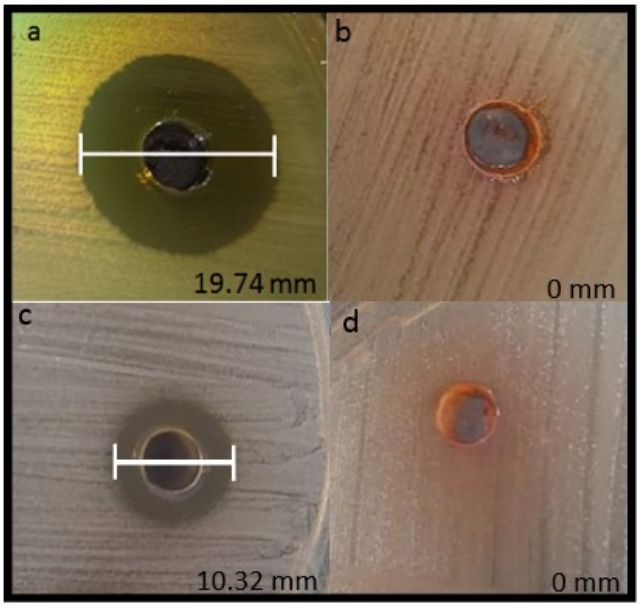

Figure 1. Example of inhibition halos obtained on Mueller-Hinton agar in the screening test for *B. Termosphacta* exposed to crude olive mill wastewater phenolic extract (a) and commercial beetroot mix (b) *L. Plantarum* exposed to crude olive mill waste-water phenolic extract (c) and commercial beetroot mix (d).

Table 2. Inhibition halos obtained for PE and CM for the different strains tested. Values are expressed as means ± standard deviation.

Olive mill wastewater extract (PE)					Positive Control
Concentration	**1 g/mL**	**0.5 g/mL**	**0.25g/mL**	**0.13g/mL**	**Tetracycline 30 µg/disc**
E. coli	17.7 ± 0.6	13.0 ± 0.7	9.0 ± 1	-	33.2 ± 0.5
S. putrefaciens	16 ± 1.0	8.7 ± 0.6	-	-	20.6 ± 0.6
P. fluorescens	24.0 ± 1.0	17.8 ± 0.5	14.0 ± 1	11.5 ± 0.2	34.7 ± 0.1
P. aeruginosa	16.5 ± 0.3	12.4 ± 0.5	8.2 ± 0.1	-	30.0 ± 1.0
B.termosphacta	19.7 ± 0.6	15.0 ± 0.0	11.6 ± 0.6	-	28.0 ± 1.0
L. plantarum	10.3 ± 0.6	-	-	-	20.2 ± 0.6
L. sakei	8.1 ± 0.5	-	-	-	19.0 ± 3.0
L. lactis	19.0 ± 1.0	10.0 ± 1.0	-	-	26.1 ± 0.4
Commercial mix (CM)					
E. coli	-	-	-	-	
S. putrefaciens	-	-	-	-	
P. fluorescens	-	-	-	-	
P. aeruginosa	-	-	-	-	
B.termosphacta	-	-	-	-	
L. plantarum	-	-	-	-	
L. sakei	-	-	-	-	
L. lactis	-	-	-	-	

A preliminary assessment utilized the agar well-diffusion technique to conduct a screening test. Table 2 displays the measurement of inhibition zones (in millimeters) for each microorganism. It demonstrates that the polyphenolic extract displays antimicrobial activity against all the tested microorganisms. As shown in Table 2 at the highest extract concentration (1 g/mL), the greater effect was registered for *P. fluorescens* (halo of 23.80 mm), while the lowest was registered for *L. Sakei* with a halo of 8.15 mm ($p < 0.05$).

Totally absent antibacterial activity was observed, instead, for beetroot commercial extract against Gram-negative and positive bacteria targeted in the study as no inhibition halos were measured (Figure 1 and Table 2).

3.4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericiadal Concentration (MBC) determination measured on PE and CM extract

The obtained results, reported in Table 3, highlight MIC/MBC values for PE ranging from 0.0156 g/ml to 0.2500 g/ml suggesting an antibacterial activity against the assayed strains, that seem to be exerted more efficaciously towards Gram-positive bacteria. These preliminary results confirm the outcome of previous studies testing the antimicrobial activity of different microorganisms relevant in food production where, on average, Gram-positive bacteria show lower MIC/MBC [25]. Furthermore, as expected, the CM extract didn't show relevant antimicrobial activity at the concentrations tested in the study.

Table 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the olive mill wastewater extract (PE) and of commercial mix (CM).

	PE (mg/mL)		CM (mg/mL)	
	MIC	**MBC**	**MIC**	**MBC**
E. coli	15.6	15.6	> 500	> 500
S. putrefaciens	15.6	15.6	> 500	> 500
P. fluorescens	15.6	31.3	> 500	> 500
P. aeruginosa	15.6	31.3	> 500	> 500
B.termosphacta	15.6	15.6	> 500	> 500
L. plantarum	125	250	> 500	> 500
L. sakei	62.5	62.5	> 500	> 500
L. lactis	62.5	125	> 500	> 500

In particular, attention has been oriented towards the microbial food spoiling process, in fact, despite chill chains, chemical preservatives, and a more in-depth understanding of microbial food spoilage, it has been estimated that 25 % of all foods produced globally is lost *post harvest* or *post slaughter* due to microbial spoilage [26].

Consequently, both consumers and producers of food products are looking for natural ingredients and efficient formulation strategies to improve the shelf life of final products [27]. It is important to consider, however, that the efficacy of a natural extract against microbial growth depends not only on its chemical composition and the extraction technique, but it is also strongly related to the specific sensitivity of microbial species and further studies are needed to better explore this aspect [1], [19].

4. CONCLUSIONS

The results demonstrate the *in vitro* efficacy of the tested polyphenolic extract against the growth of both Gram positive and negative undesired spoilage microorganisms, defining preliminary threshold values for future application on food models. Further studies are needed to address the main challenges in the use of natural antimicrobial such as its low stability, adverse effects on sensory properties, low solubility, high needed doses.

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