ANTIFUNGAL ACTIVITY OF PHENOLIC COMPOUNDS EXTRACTED FROM DRIED OLIVE POMACE

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The antifungal activity of phenolic compounds extracted from olive pomace against Alternaria solani, Botrytis cinerea and Fusarium culmorum was investigated. Olive pomace, a by-product of olive oil production, was used as a source of phenolic compounds. The extracted phenolics were added to a medium to reach phenol concentration of 0.1 and 0.2 % (w/v) and tested for their antifungal activity against the three fungi. In addition, the fungi were cultivated in a medium containing a commercial fungicide in the concentration of 0.2 % (w/v). Both of the phenolic concentrations inhibited the growth of all three fungi with the higher being more effective. It resulted in a four-fold reduction of the mycelial growth rate of A. solani and F. culmorum. The present study demonstrated that the phenolic compounds derived from olive pomace hold a good promise as a natural fungicide against common pathogens to crops.

Key words: antifungal activity; olive pomace; Alternaria solani; Botrytis cinerea; Fusarium culmorum

INTRODUCTION

The actual consumer concern for better human health increased the interest for olive oil. Olive oil has a high content of oleic acid and is rich in phenolic compounds, which act as natural antioxidants contributing to the prevention of human diseases. Epidemiological studies have shown that consumption of foods and beverages rich in phenolics is correlated with reduced incidence of heart disease [1].

In unripe olive fruits, Olea europaea, one of the main phenolic compounds is the secoiridoid oleuropein, responsible for the bitterness of the olives that must be eliminated before they can be made palatable. It is a heterosidic ester of beta-glucosilated elenolic acid and 3,4-dihydroxyphenylethanol (hydroxytyrosol) (Fig. 1). Other phenolic compounds isolated from the olive fruits are demethyloleuropein, rutin, luteolin 7-glucoside, verbascoside, ligstroside and elenolic acid glucoside [2]. Some of them are thought to have originated from oleuropein [3].

Fig. 1. The structural formulae of oleuropein

Apart from their antioxidant properties [4], these compounds possess antimutagenic, anticarcinogenic, antiglycemic [5] and antimicrobial characteristics [6, 7]. They appear to be involved
in the defence of plants against invading pathogens, including bacteria, fungi and viruses [8]. Currently, crop plant protection agents play an essential role in the protection of plants or harvested fruits against micro-organisms. These agents are effective and economically advantageous but, by modern standards, they lack selectivity and reliability. Being applied at high rates they are threat to human health and environment [9]. Complying with the growing public awareness of these hazards, increasing emphasis is placed upon the search for novel, natural products with pesticidal activity, competitive with already existing agrochemicals.

Olive oil is extracted mechanically by pressure and by a three-phase centrifugation system, which results in the production of two by-products: black olive mill wastewater and olive pomace. The disposal of both of these by-products creates a major environmental problem in the main olive-producing countries [10,11]. Therefore, a suitable use of these olive oil residues could not only improve the economic status of olive oil production but could also reduce this environmental problem.

Bearing these in mind, the objective of this study was to investigate the antifungal activity of phenolic compounds extracted from dried olive oil pomace against Alternaria solani, Botrytis cinerea and Fusarium culmorum. Although there have been studies on the antimicrobial activity of phenolic compounds derived from olive fruits, this is, to our knowledge, the first time that the extract from olive pomace has been used against these three fungi known to be capable of causing plant diseases.

EXPERIMENTAL

**Fungal species and media**

Alternaria solani, Botrytis cinerea and Fusarium culmorum belonging to the Collection of the Institute of Food Technology in Bonn were used as test microorganisms. They were maintained on agar plates at 4 °C on medium containing per litre: 30 g malt extract, 3 g meat peptone extract and 20 g agar. For inoculum preparation and submerged growth, the same medium, without agar was used.

**Extraction of phenolic compounds**

Olive pomace, collected during 2002/2003-harvest season from organic cultivars in the Kalamata region in Greece was supplied by Friedrich Bläuel Organics. The olive residue was dried at 60 °C and packed in vacuumed plastic bags, which were stored at 4 °C until used.

The extraction of the olive pomace was performed in a man-made extractor (a stirred-tank batch extractor) agitated at 750 min⁻¹ at room temperature. In the first step, the dry olive pomace was extracted, three times successively, with hexane in ratio 1:4 (w/v) to remove the residual oil and pigments. In the second step, the polyphenols were extracted from the pomace using a mixture of water and ethanol (1:1, v/v) adjusted to pH 9 with NaOH. The ratio of olive pomace and extraction mixture was 1:6 (w/v). After two successive extractions, the total ethanol extract was filtered (0.45 μm) and then concentrated by a rotary-evaporator at 30 °C until more than a half of the volume has evaporated. This extract was stored in dark at 4 °C. It was analysed for phenolic and dry matter content.

**Assay of antifungal activity**

Agar plates (10-cm Petri dishes) were inoculated at the centre with a mycelial disc which was taken at the periphery of the fungal colony. The plates were incubated for 5 days at 25 °C and served for inoculum preparation. 10 ml sterile distilled water was added to each plate and the resulting spore suspension was transferred to a 250-ml Erlenmeyer flask which contained 100 ml medium. The inoculated medium was incubated on a rotary shaker (200 min⁻¹) at 25 °C for 2 days and then used to inoculate the media for testing the antifungal activity in concentration of 5 % (v/v). The media for assaying the antifungal activity of the phenolic compounds were prepared by supplementing the growth medium with appropriate quantities of the extract to reach phenolic concentration of 0.1 and 0.2 % (w/v). The fungi were also grown on a medium containing the commercial agrochemical Euparen MW G (Bayer) in concentration of 0.2 % (w/v) as recommended by the producer. The initial pH of the media, before sterilisation, was adjusted to 7, apart from the medium with Euparen, when it was 8. All fermentations were carried out in 125 ml Er-
Antifungal activity of phenolic compounds extracted from dried olive pomace

lenmeyer flasks containing 50 ml medium, on a rotary shaker (125 min–1) at 25 °C for eight days.

At defined time intervals, the content of the entire flask was vacuum filtered through previously dried and weighed Millipore filter (0.45 μm) and the filtrate was used for pH and redox potential measurements. The cell residue was twice washed with distilled water and dried for cell mass determination.

Analytical methods

The total phenolic content was determined spectrophotometrically at 720 nm using Folin-Ciocalteu reagent. In a cuvette, the extract (10 μl) was mixed with distilled water (840 μl) and Folin-Ciocalteu reagent (50 μl). After three minutes 100 μl saturated NaCO₃ solution was added. The mixture has been equilibrated for one hour at room temperature before reading the absorbency. A calibration curve was calculated using pure oleuropein (Roth). The total phenols were expressed as mg oleuropein per ml extract.

The dry matter content of the extract was estimated by drying the samples (50 ml) at 105 °C to a constant mass. The cell mass concentration was determined by drying the fungal biomass at 105°C to a constant cell mass. The morphology of the fungal mycelium was observed on a light microscope (Leitz, Diaplan).

RESULTS AND DISCUSSION

Preparation of crude extracts

Representing 40 % of the original olive weight used for oil production, olive pomace, with 0.3 % phenolic content [10] presents an abundant and cheap source of natural antimicrobial compounds. Using a mixture of ethanol and water, after removal of pigments and lipids with hexane, we extracted the phenolic compounds from the dry olive pomace applying a multistep extraction procedure. The crude extract had 23.6 g/l dry matters that equals to 2.43 %(w/w). The total phenol content of the extract, estimated as oleuropein equivalents, was 0.24 % (w/v). Calculated on a dry matter basis, the total phenols represented 10.25 % (w/w).

Effect of the phenolic compounds on the growth of the fungi

The antimicrobial activity of oleuropein and other phenolic compounds found in olives have been studied mostly against various bacteria involved in lactic acid fermentation, Lactobacillus plantarum, L. brevis [12], Leuconostoc mesenteroides, Geotrichum candidum, Rhizopus sp. [13] and against bacteria connected with infections of the human intestinal or the respiratory tract [14]. In our current study we have tested these phenolic compounds for their potential usage as pest agents against three fungi, which are known to cause reduction in the yield and quality of crops. As all other fungi, they are extremely responsive to environmental pressures and exhibit a capacity to adapt to and to colonise a variety of ecological niches [9]. Alternaria solani is an attendant to tomatoes, potatoes, paprikas, Botrytis cinerea attacks grapes, berry-fruits, some vegetables too, while Fusarium culmorum causes a degradation of cereal grains. The latter is also known for its production of mycotoxins [15].

The antifungal activity of the phenolic compounds present in the extract derived from the olive pomace was assayed by cultivating three selected fungi in medium supplemented with appropriate quantities of the extract to reach phenolic concentration of 0.1 and 0.2 % (w/v). Additionally, the fungi were cultivated on a medium without phenols, and on a medium with a conventional fungicide, Euparen MW G, both serving as controls. The levels of phenols in the medium were selected on the basis of our previous investigations carried out on agar plates. The recommended concentration of the commercial fungicide (0.2 % w/v) was also considered. The time courses of biomass production and pH changes of A. solani, B. cinerea and F. culmorum, are shown in Figs. 2, 3 and 4.

The phenolic compounds in concentration of both 0.1 and 0.2 % (w/v), inhibited the growth of all three fungi with a higher phenol concentration being more effective. A. solani prolonged its lag phase and the exponential phase of growth (Fig. 2). The onset of growth was delayed for 72 hours and up to 140 and 160 h, respectively, the fungus

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grew slower than on medium with Euparen MW G. The antifungal activity of the phenol extract on the growth of A. solani is illustrated the best in Figure 5.

Compared to the other two fungi, B. cinerea, in general, grew much slower (Fig. 3, Table 1). Its maximal biomass concentration of 2.78 g/l (control) was considerably smaller than those of F. culmorum, 6.30 g/l and A. solani, 8.65 g/l. Although the mycelial growth rate on the medium with commercial fungicide (0.061 g/l·d) was lower than the rates produced in the medium with phenolic compounds (0.098 and 0.076 g/l·d), the final biomass concentration in all three media was almost the same, around 1.6 g/l (Table 1, Fig. 3).
The higher concentration of phenols in the medium, 0.2 % (w/v), decreased the final cell mass of *F. culmorum* more than the commercial fungicide (Fig. 4). However, in terms of reducing the growth rate of the organism, Euparen MW G was more effective with 0.112 versus 0.152 g/l-d (Table 1).

**Table 1**

Influence of the phenolic compounds extracted from the dried olive pomace and Euparen MW G on the mycelial growth rate of the fungi

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Phenolic compounds (% w/v)</th>
<th>Euparen MW G (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mycelial growth rate (g/l-d)</td>
<td></td>
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<tr>
<td><em>A. solani</em></td>
<td>1.022 0.370 0.242 0.156</td>
<td></td>
</tr>
<tr>
<td><em>B. cinerea</em></td>
<td>0.256 0.098 0.076 0.061</td>
<td></td>
</tr>
<tr>
<td><em>F. culmorum</em></td>
<td>0.633 0.301 0.152 0.112</td>
<td></td>
</tr>
</tbody>
</table>

* Time of cultivation, 180 h

The medium with Euparen MW G influenced the growth of all three fungi in the same fashion (Figures 2–4). Although with a reduced rate, they grew till the fifth day when their growth slightly declined. In media containing phenolic compounds, the growth was delayed and slow but did not decline. The changes in the growth of all cultures were followed by corresponding changes in pH values. As the fungi enhanced the growth, pH dropped.

Investigating the inhibitory effect of the commercial oleuropein on the growth of *S. enteritidis*, Tassou and Nychas [16] found that the growth was inhibited by 0.6 % (w/v) oleuropein, especially at pH 5.5 and 6 and with a low inoculum size. Oleuropein in concentration of 0.1 and 0.2 % (w/v) delayed the onset of the growth. However, a concentration of 0.2 % (w/v) oleuropein was sufficient to reduce the growth of *S. aureus* to half while inhibiting, at the same time, toxin production by the factor of 320 [17]. In contrast, Aziz et al. [7], working with pure phenolic compounds isolated from olive cake, reported very low concentrations of phenols in medium (0.02 to 0.04 % w/v) to completely inhibit growth of bacteria (*Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumoniae*) and fungi (*Aspergillus flavus*, *A. parasiticus*).

**CONCLUSION**

The presence of phenolic extract in a medium, which normally supports the growth of the fungi, inhibited the growth of all three fungi, with a higher phenol concentration (0.2 % w/v) being more effective. This makes the application of the phenolic extract as natural antifungal agent practically possible. Olive residue can be successfully used as an inexpensive source of phenolic compounds. Further studies should focus on optimizing the inhibitory concentration of the phenolics and the conditions of application. In the long run, attention should be paid to the mechanism of the antifungal action.

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**REFERENCES**


Резюме

Антифунгальная активность на фенолии соединения из маслина песчаника

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Ключевые слова: антифунгальная активность; маслина песчаника; *Alternaria solani; Botrytis cinerea; Fusarium culmorum*

Фенольные соединения добиваем из экстракции песчаника маслинового песчаника через испытание, в одно из низкого антифунгального действия в гребничные *Alternaria solani, Botrytis cinerea* и *Fusarium culmorum*. Какое быть изворо на всех фенол беши, а иногда и производство, что останавливает презиуа на маслинки при производстве масло. Гребницы без культивирования на медикум со 0,1 и 0,2 % (w/v) фенол и со 0,2 % (w/v) коммерции фунгицида. И двете концентрации на фенол беши ингибаторни для сите испитани габи, со тоа што поголемата концентрация беши поефикасна и доведе до четирикратно намалување на растот на *A. solani* и *F. culmorum*. Ова испитување покажа дека фенолни соединения добивани от маслинан песчаник би можеле да се користат како природен фунгицид против науочно присутните патогени организами кај посевите.