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Antifungal activity of olive leaf (*Olea Europaea* L.) extracts from the Trilye Region of Turkey

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Abstract - Antimicrobial properties of olive leaf extract on some yeast were examined in this study. Fresh olive leaf extracts were prepared using various solvents (water, ethanol, acetone, ethyl acetate) in Soxhlet apparatus. Antimicrobial effects of these extracts were tested against *Saccharomyces cerevisiae* ATCC 9763, *Schizosaccharomyces pombe*, *Saccharomyces uvarum*, *Candida oleophila*, *Metschnikowia fructicola* and *Kloeckera apiculata*. The antifungal activities of these extracts were tested by the disc diffusion assay, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC). All extracts showed various degrees of antifungal effects with 10-28 µg/ml MIC, 20-48 µg/ml MFC and 1.5-9.3 mm inhibitory zone values against yeasts utilised, except water. The results indicated that the tested yeasts were sensitive to acetone and ethyl acetate extracts. It was determined that *Saccharomyces cerevisiae* ATCC 9763 was the most resistant among the yeasts.

Key words: *Olea europaea* L., antifungal, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC).

INTRODUCTION

Historically, olive leaf extract has been widely used in folk medicine for combating fevers and other diseases, such as malaria (Benavente-Garcia *et al*., 2000). Olive leaf extract has the capacity to lower blood pressure in animals (Khayyal *et al.*, 2002) and increases blood flow in coronary arteries, relieves arrhythmia and prevents intestinal muscle spasms (Zarzuelo, 1991). In addition, olive leaf extract and its phenolic compounds such as oleuropein, tyrosol, hydroxytyrosol, caffeic acid, gallic acid and luteolin have antimicrobial activity against viruses, retroviruses, bacteria, yeasts, fungi, molds, and other parasites (Juven *et al*., 1968, Fleming *et al.,* 1973; Mahjoub and Bullerman, 1987; Gourama *et al.,* 1989; Tranter *et al*., 1993; Tassou and Nychas, 1995; Bisignano *et al.,* 1999; McDonald *et al*., 2001; Tuck and Hayball, 2002; Korukluoglu *et al*., 2004; Micol *et al*., 2005). Phenolic compounds isolated from olive fruit have been shown to inhibit the growth of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* (Aziz *et al*., 1998, Yigit *et al.,* 2001). Gourama and Bullerman (1987) tested the effect of oleuropein on growth and aflatoxin production by *Aspergillus parasiticus*, and found that oleuropein stimulated mold growth but inhibited the production of aflatoxins. Markin *et al*. (2003) tested water extract of olive leaf against some microorganisms and found that *Candida albicans* was killed within 24 h. Sousa *et al.* (2006) reported that the determination of phenolic compounds in 'alcaparra' table olives and the evaluation of their extract *in vitro* activity against Gram positive, Gram negative bacteria and fungi (*Candida albicans* and *Cryptococcus neoformans*). Some aldehydes obtained from olive fruit revealed antifungal activity against *Tricophyton mentagrophytes, Microsporum canis* and *Candida* spp. (Battinelli *et al*., 2006).

Many of the problems caused by yeasts results from the much higher attention paid to bacteria and moulds, which are more significant in terms of public health. Yeasts play a central role in the spoilage of foods and beverages, mainly those with high acidity and reduced water activity (a_w) (Loureiro and Queroly, 1999; Loureiro and Malfeito-Ferreira, 2003; Evans *et al.,* 2004). Ismail *et al.* (2000) reported that *Kloeckera apiculata, Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Candida* spp. are responsible for the spoilage of foods that have been processed and packaged according to the normal standards of good manufacturing practice. In addition, yeasts are used during biological control following harvest (*Candida oleophila*, *Metschnikowia fructicola,* etc.).

The objectives of this study were (i) to investigate antifungal activity of extracts from olive leaf, which has been utilised as traditional folk medicine, and (ii) to evaluate the potential usage of olive leaf extract as natural preservative.

MATERIALS AND METHODS

Microorganism strains. *Schizosaccharomyces pombe* and *Saccharomyces uvarum* were obtained from Department of Food Engineering; *Metschnikowia fructicola, Candida oleophila* and *Kloeckera apiculata* were obtained from Department

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of Plant Protection, Faculty of Agriculture of Uludag University, Bursa, Turkey; *Saccharomyces cerevisiae* was ATCC 9763 strain.

Preparation of test microorganisms. All strains were cultured twice at a 48 h interval before use on Potato dextrose agar (PDA, Difco, MI, Detroit, USA) slant at 35 °C for 24 h. A loopful of PDA culture was inoculated in 50 ml of Malt extract broth (Difco, MI, Detroit, USA) and then incubated at 30 °C for 24 h (Arora and Kaur, 1999).

Preparation of olive leaf extracts. Olive leaves collected from Trilye region, Mudanya, Turkey, were used as samples for our investigation. Leaves (30 g) were homogenised and extracted for 8 h in a Soxhlet apparatus with 150 ml of various solvents (ethanol, ethyl acetate, acetone and water). The crude extracts were concentrated in a rotary evaporator and then transferred into sterile vials with 10 ml extraction solvent. Extracts were kept under refrigerated conditions until further use (Rauha *et al*., 2000, Sagdic *et al.,* 2002). These experiments were repeated 5 times.

Antimicrobial assay (Disc diffusion assay). The crude extracts were dissolved in the same solvents (ethanol, ethyl acetate, acetone and water) to a final concentration of 30 mg/mL and sterilised through filtration by 0.45 µm Millipore filters. Antifungal tests were then carried out by the disc diffusion method (Yin and Tsao, 1999; Karaman *et al*., 2003) using 200 µl of suspension containing 10^6 CFU/ml of yeast, spread on Malt extract agar medium. The discs (6 mm in diameter) were impregnated with 10 µl of extracts placed on the inoculated agar. Negative controls were prepared using same solvents employed to dissolve the plant extracts. The inoculated plates were incubated at 30 °C for 48 h. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms. The results are expressed as net zone diameter 95% confidence interval (mm) of inhibition, which represents the subtraction of the diameter (6 mm) of the paper disk from the measured zone. Each assay in this experiment was repeated five times. The negative control with the same solvents did not inhibit the test microorganisms.

Determination of Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC). The MIC and MFC determinations were measured according to the methods described by Yin and Tsao, 1999; Rasooli and Abyaneh, 2004; Sahin *et al.,* 2004. Leaf extract was diluted with same solvents to produce two-fold serial dilutions ranging from 10 to 2000 µg/ml. From each prepared solutions 1 ml was added into test tubes containing 5 ml Malt extract broth. Yeasts' suspensions (1 ml) prepared from 24 h Malt extract broth cultures and adjusted to 0.5 McFarland turbidity, were added to the tubes containing olive leaf extracts or solvent used as control. Test tubes were then incubated at 30 ºC for 48 h. The MIC of the test extracts was the lowest concentration of extracts that did not permit any turbidity of the test tubes. The MFC was determined by culturing 0.2 ml of the mixed broth culture from all tubes that lacked visible turbidity in the MIC assay on Malt extract agar plates at 30 ºC for 48 h (Portillo *et al*., 2005). The MFC was defined as the lowest concentrations that completely inhibit the growth of yeasts.

Statistical analysis. Mean differences were analysed statistically by running a two-way analysis of variance test (ANOVA). Differences between means were determined by the least significance defined at $p < 0.05$.

RESULTS AND DISCUSSION

The antimicrobial activities of olive leaf extracts (ethanol, ethyl acetate, acetone and water) against tested yeasts and their potency were quantitatively assessed by the presence or absence of MIC (µg/ml) and MFC (µg/ml) and inhibition

TABLE 1 – The minimal inhibition concentration (MIC/MFC) and inhibitory zone of olive leaf extracts against some yeast

Yeast	Source	Solvent	MIC (µg/ml)*	MFC (µg/ml)*	Inhibitory zone diameter (mm)*
Saccharomyces cerevisiae	ATCC 9763	Ethyl acetate	24 ± 2.94	48 ± 1.81	3.0 ± 2.17
		Acetone	Nd	Nd	Nd
		Etyhl alcohol	Nd	Nd	Nd
Saccharomyces uvarum	Wine	Ethyl acetate	23 ± 1.41	46 ± 1.72	2.3 ± 0.23
		Acetone	12 ± 0.81	24 ± 1.06	9.3 ± 0.18
		Etyhl alcohol	13 ± 2.16	26 ± 1.47	8.8 ± 0.36
Metschnikowia fructicola	Decayed sweet cherries	Ethyl acetate	15 ± 4.08	30 ± 2.36	7.0 ± 0.75
		Acetone	16 ± 2.44	32 ± 2.75	5.8 ± 1.61
		Etyhl alcohol	15 ± 2.16	30 ± 2.01	7.5 ± 1.53
Kloeckera apiculata	Decayed apple	Ethyl acetate	16 ± 1.82	32 ± 1.80	5.3 ± 0.56
		Acetone	20 ± 2.44	20 ± 2.16	4.8 ± 0.77
		Etyhl alcohol	10 ± 1.41	20 ± 1.33	1.5 ± 0.46
Candida oleophila	Decayed sweet cherries	Ethyl acetate	23 ± 1.82	23 ± 1.70	2.3 ± 1.33
		Acetone	12 ± 2.44	24 ± 2.11	9.0 ± 2.29
		Etyhl alcohol	28 ± 1.82	28 ± 1.63	0.8 ± 0.39
Schizosaccharomyces pombe	Wine	Ethyl acetate	23 ± 1.41	23 ± 1.55	2.3 ± 1.42
		Acetone	16 ± 0.81	32 ± 1.01	5.3 ± 0.67
		Etyhl alcohol	23 ± 2.58	23 ± 2.37	2.0 ± 0.87

* The results are expressed as mean ± Standard Deviation of fourth detection time observations.
Nd: not determined.

zone diameters (mm). MIC, MFC values and inhibition zone diameters of olive leaf extracts against yeasts are given in Table 1. The range of MIC, MFC and inhibitory zone diameters was 10-28 µg/ml, 20-48 µg/ml and 1.5-9.3 mm, respectively.

Test yeasts showed various degree of antifungal sensitivity to acetone, ethyl alcohol, ethyl acetate extracts of olive leaves, except water extract that did not exhibit any inhibitory effects on tested yeasts. It might be explained that water weakly solved phenolic compounds, non-polar fraction, in olive leaf. On the other hand, Markin *et al.* (2003) reported that aqueous extract of olive leaf (15%) killed *C. albicans* within 24 h. These differences are likely to occur because of the different olive cultivars (Ranalli *et al.,* 2006), the content of the antimicrobial agents present in the extracts, or the sample preparation methods used.

Acetone extracts have been found most effective against *Saccharomyces uvarum* and *Candida oleophila* according to MIC values (12 µg/ml). The results have been supported by chemical characterisation of leaf extracts (Korukluoglu *et al.,* 2004). According to the chemical analysis, acetone extract was the most effective solvent for dissolving dicarboxylic phenolic content of olive leaf.

In our observation, none of the extracts have antifungal activity against *S. cerevisiae,* except ethyl acetate. *Metschnikowia fructicola* was found the most sensitive yeast to ethyl acetate extract (15 µg/ml) followed by *K. apiculata* (16 µg/ml) according to MIC values.

Ethanol extract generally indicated weak inhibitory action against yeast used ($p < 0.05$). However, this extract was found the most effective against *K. apiculata* with 10 µg/ml of MIC followed by *S. uvarum*, *M. fructicola*, *S. pombe* and *C. oleophila* with 13, 15, 23 and 28 µg/ml, respectively.

Saccharomyces cerevisiae demonstrated higher resistance than the other test yeasts while *C. oleophila* and *S. pombe* were the most sensitive microorganisms ($p < 0.05$).

Previous studies suggest that phenols in olive have antimicrobial properties (Le Tutour and Guedon, 1992, Soler-Rivas *et al.,* 2000). The effect of oleuropein, present in high amounts in olive leaves, was investigated on its antifungal activity against *Rhodotorula* ssp., *C. albicans* and *S. cerevisiae* by Juven and Henis (1970) and no inhibitory effect against these yeasts were observed. In the present study, similar results were obtained for *S. cerevisiae.*

Our results demonstrate that extracts obtained using various solvents have different effects on the growth of yeasts, which may cause deterioration in foods or are utilised in the food industry. The antifungal resistance may depend on the genus, species, strain and source of isolation, as well as on the active components in the leaf extracts. Solvents used in extracts, cultivars of olives, crop origin, harvesting time and climate may all influence the leaf composition and consequently affects the antifungal properties.

The results suggested that similar studies about olive leaf extract, as a natural preservative, could be an alternative to synthetic antimicrobial substances in various industries. Therefore, crude extracts obtained in future experiments should be chosen and evaluated according to their antimicrobial compounds.

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