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Antifungal activity of various essential oils against *Saccharomyces cerevisiae* depends on disruption of cell membrane integrity

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Abstract: Antifungal activity and mode of action of nine essential oils (EOs) against *S. cerevisiae* cells were examined. Antifungal effects of commercial lemon peel, orange peel, tea tree, turpentine, rosemary, peppermint, thyme, oregano and clove oils were determined through Minimum Inhibitory Concentration (MIC), Minimum Fungicidal Concentration (MFC) and inhibition zone measurements. The most effective oil was turpentine oil. Orange peel, thyme and oregano oils were also effective, according to MIC and MFC. Inhibition zone measurements, also revealed oregano, orange peel, thyme, turpentine and clove oils as most efficient ones. Later, membrane damage of yeast cells was studied by measuring the extracellular pH and conductivity in a concentration dependent manner. Orange peel, turpentine, thyme and oregano oils caused a clear increase in extracellular pH of glucose-induced yeast cells and induced an increase in extracellular conductivity. These results point to the deterioration of yeast cell membrane integrity upon exposure to EOs. Since these oils can be used as food preservatives and pharmaceutical agents, it is very important to understand their mode of action and their main target sites in the cell. Thus this research not only opens new perspectives to understand antifungal activity mechanisms of EOs, but also help widen their use.

Introduction

Essential oils (EOs) are complex mixtures of volatile compounds produced by plants and fruits. They are known to have antioxidant (Yang *et al.*, 2010), antibacterial (Reichling *et al.*, 2009), antifungal (Hammer at al., 2004), antiplasmid (Schelz *et al.*, 2006), antiviral, antiparasitic and insecticidal properties (Unal *et al.*, 2009). It is clear from different studies on EOs which are defi ed 'generally regarded as safe' (GRAS) by the US' Food and Drug Administration that they have potential uses in medicine and applications in the cosmetic, pharmaceutical and food industries and also in cleaning products (Souza *et al.*, 2007; Van Vuuren *et al.*, 2009; Lima de Sousa *et al.*, 2013; Bialon *et al.*, 2014; Rajkowska *et al.*, 2014; Boire *et al.*, 2016).

There are many studies on the antimicrobial activity of essential oils in recent years. However, to our knowledge, studies concerning antifungal activity of plant essential oils against *Saccharomyces cerevisiae* are limited. Antifungal activity of lemon oil *(Citrus limon)* (Kunicka-Styczyńska, 2011), orange oil (*Citrus sinensis)* (Liu *et al.*, 2012), tea tree oil *(Melaleuca alternifoliae)* (Hammer *et al.*, 2004; Mantil *et al.*, 2015), turpentine oil *(Pinus* spp.*)* (Schelz *et al.*, 2006), rosemary oil (*Rosmarinus officinalis*) (Moreno *et al.*, 2006), peppermint oil *(Mentha piperita)* (Kunicka-Styczyńska, 2011), thyme oil (*Thymus vulgaris)* (Kunicka-Styczyńska, 2011), oregano oil *(Origanum vulgare)* and clove oil (*Eugenia caryophyllata)* (Chami *et al.*, 2005) against *S. cerevisiae* have been documented in various reports.

There are different antimicrobial mechanisms of EOs. Cell wall, cell membrane, intracellular proteins, enzymes and nucleic acids are signifi ant target sites for EO contents (Helander *et al.*, 1998; Burt *et al.*, 2004; Morten *et al.*, 2012). Cell membrane is the first line of defense against environmental stresses. It was suggested that the lipophilic nature of EOs allows them to easily pass through cell membranes to change biological responses of cells (Wang *et al.*, 2015). Especially phenolic compounds and terpenes may accumulate in the cell membrane and result in instant loss of membrane integrity, making it highly permeable to ions that might be responsible for the establishment of antimicrobial activity. It has also been shown that essential oil affects the membrane composition of *Yarrowia lipolytica* yeast and some bacteria (Di Pasqua *et al.*, 2006; Papanikolaou *et al.*, 2008). In other cases, changes in membrane fluidity and integrity of yeast cells were observed upon exposure to various stress conditions, by regulating the biosynthesis of fatty acids and

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sterols (Ding *et al.*, 2009; Ta *et al.*, 2010; Dupont *et al.*, 2011; Turk *et al.*, 2011).

TABLE 1

Antifungal activity of essential oils presented as MIC and MFC values. Essential oils were mixed with DMSO to increase solubility. DMSO level did not exceed 1% in all cases. MIC and MFC measurements were repeated three times.

#	Essential Oil	MIC (µL/mL)	MFC (µL/mL)
1	lemon peel	>10	>10
2	orange peel	0.04-0.08	0.02-0.08
3	tea tree	>10	>10
4	turpentine	0.01-0.04	0.01-0.04
5	rosemary	*ND*	*ND*
6	peppermint	*ND*	*ND*
7	thyme	0.2-0.3	0.2-0.3
8	oregano	0.2-0.3	>0.3
9	clove	*ND*	*ND*

ND: not determined

Although antifungal activity of EOs was examined before, their effects on cytoplasmic membrane of *S. cerevisiae* have not been extensively studied (Tao *et al.*, 2014a, 2014b). Due to our continuing interest on the mode of action of various chemicals on yeast membranes (Sezen, 2015), we set out to disclose the possible membrane dependent action of essential oils on yeast cells. In this study, we fi st examined the antifungal effects of lemon peel, orange peel, tea tree, turpentine, rosemary, peppermint, thyme, oregano and clove oil against *S. cerevisiae* by applying the measurement of Minimum Inhibitory Concentration (MIC), Minimum Fungicidal Concentration (MFC) and inhibition zone techniques. Then we evaluated the membrane damage by measuring the extracellular pH of glucose-induced cells and conductivity of yeast cells after exposure to different concentrations of above EOs.

Materials and methods

Essential oils

Lemon peel (*Citrus limon*), orange peel (*Citrus sinensis)*, tea tree (*Melaleuca alternifolia)*, pine turpentine (*Pinus* spp.), rosemary (*Rosmarinus officinalis)*, peppermint (*Mentha piperita)*, thyme (*Thymus vulgaris)*, oregano (*Origanum vulgare*) and clove (*Syzygium aromaticum*) essential oils were purchased from ÇİFTÇİZADE (Antalya, Turkey) and used for all experiments. Major components of the oils were determined by gas chromatography with electron-ionization mass-selective detector.

Yeast strain and growth conditions

YPH499 was used as the *S. cerevisiae* strain in all experiments. Typically, cells were grown in yeast extract peptone glucose medium (YPD) at 25°C.

TABLE 2

Antifungal activity of essential oils presented as Zones of Inhibition. Essential oils were mixed with DMSO to increase solubility. The values are mean of four replicates ± standard deviation.

#	Essential Oil	%	Zones of Inhibition (cm)
1	lemon peel	100	1,3±0,3
		20	<0,5
2	orange peel	20	3,3±0,9
3	tea tree	100	<1
		20	<0,5
4	turpentine	20	2,5±0,2
5	rosemary	100	<1
		20	<0,5
6	peppermint	100	1,7±0,3
		20	<1
7	thyme	20	3,0±0,5
8	oregano	20	3,6±0,1
9	clove	20	2,2±0,5

Minimum Inhibitory Concentration (MIC) measurement

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the essential oil at which the yeasts did not demonstrate visible growth (Tao *et al.*, 2009). S. *cerevisiae* strains were cultured overnight at 25°C in YPD broth. The essential oils dissolved in DMSO were diluted to the mentioned concentrations in 24-well microtiter plate and then *S. cerevisiae* strain was added to each well. As controls, suspensions of yeasts in the medium without essential oils and yeasts in the medium with only DMSO were tested. Plates were incubated for 48 h at 25°C. After incubation, viability of yeasts was controlled based on turbidity clearance (Talebi *et al.*, 2014).

Minimum fungicidal concentration (MFC) measurement

The minimum fungicidal concentration (MFC) was determined as the lowest oil concentration at which no growth was observed in the microplate (Martos *et al.*, 2010; Kunicka-Styczyńska, 2011). 5 µL of specimen was taken from all the wells of MIC plate after 24 hours and added on petri dishes. The petri dishes were incubated at 25°C for 48 hours.

Inhibition zone measurement

The agar diffusion method (Skocibusic *et al.*, 2006) was employed for the determination of antifungal activities of the essential oils. Five hundred µL of fresh culture of *S. cerevisiae* was spread on the YPD agar media plates and allowed to dry for 2 h. Later, 5 mm diameter wells were opened in solid media plates. EOs were diluted in DMSO at different concentrations and 55 µL of essential oils were filled into the each well. Plates were placed in the incubator at 25°C for 48 hours. After incubation, the diameters of the inhibition zones were measured to the nearest milimeter.

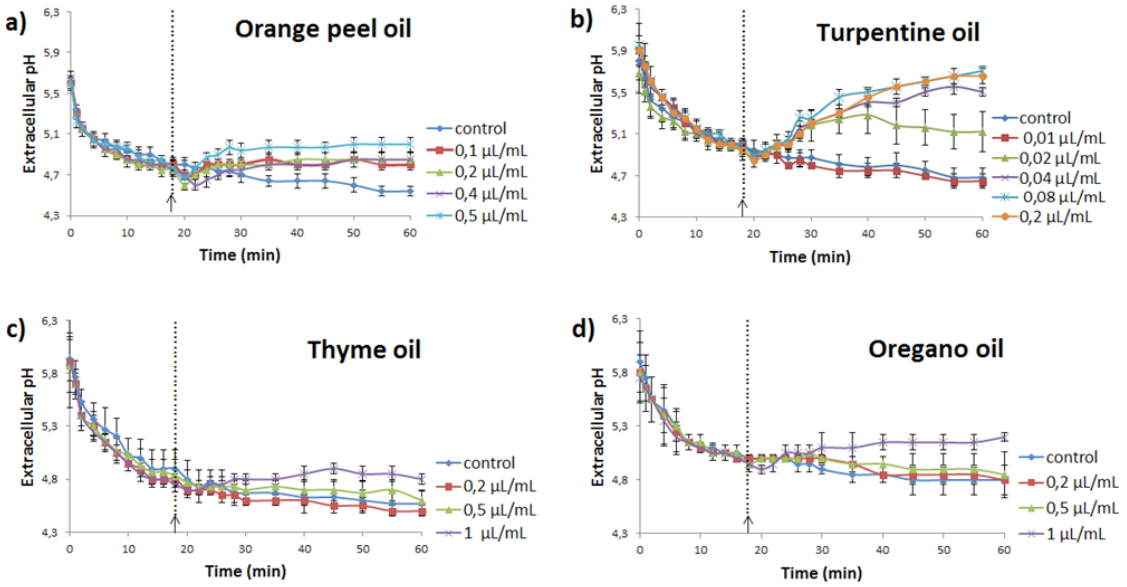

FIGURE 1. Effects of essential oils on the extracellular pH of *S. cerevisiae*. Concentration dependent effects of essential oils on yeast cells in glucose-induced medium are shown. The arrows indicate the time of addition of a) orange peel oil: 0.1; 0.2; 0.4; 0.5 µL/mL, b) turpentine oil: 0.01; 0.02; 0.04; 0.08; 0.2 µL/mL, c) thyme oil: 0.2; 0.5; 1 µL/mL, d) oregano oil: 0.2; 0.5; 1 µL/mL. The data represent the average of at least two independent experiments.

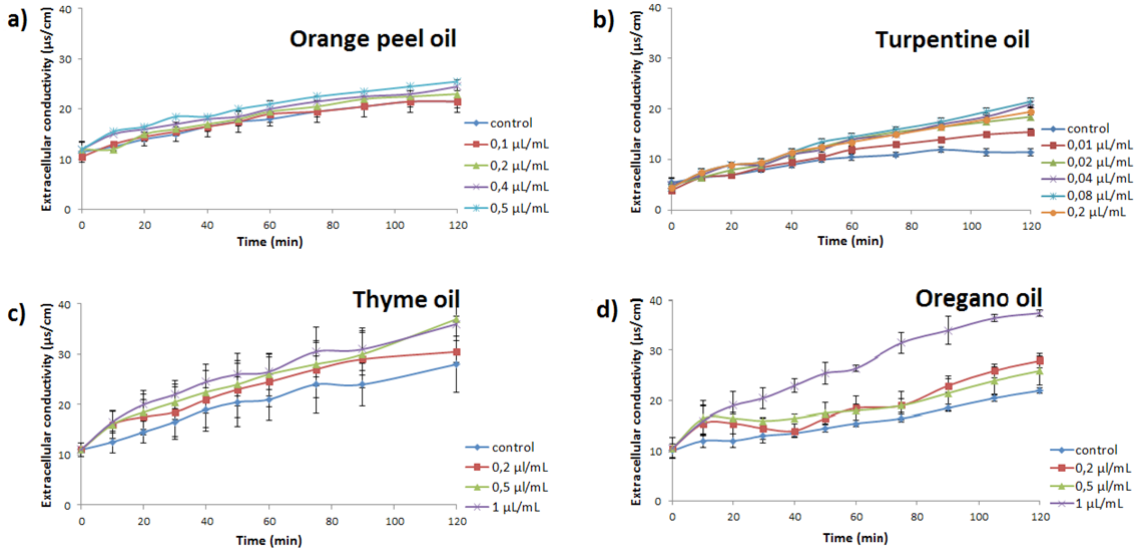

FIGURE 2. Effects of essential oils on the extracellular conductivity of *S. cerevisiae*. Concentration dependent effects of essential oils on yeast cells are shown. Point zero indicate the time of addition of a) orange peel oil: 0.1; 0.2; 0.4; 0.5 µL/mL, b) turpentine oil: 0.01; 0.02; 0.04; 0.08; 0.2 µL/mL, c) thyme oil: 0.2; 0.5; 1 µL/mL, d) oregano oil: 0.2; 0.5; 1 µL/mL. The data represent the average of at least two independent experiments.

Extracellular pH measurement

The permeability of *S. cerevisiae* cell membranes is expressed in terms of their electric conductivity and extracellular pH value (Gaskova *et al.*, 2013) and was determined by the following method: *S. cerevisiae* strain was cultured overnight at 25˚C in 20 mL of YPD broth. After incubation, the yeast cells were centrifuged at 3200 rpm for 5 min and pellet was washed twice with sterilized distilled water and resuspended. About 50 mg wet weight of yeast cells were used for each experiment. The essential oils dissolved in DMSO were diluted to the mentioned concentrations. Two% glucose (zero point) and essential oils were manually injected to the fi al concentrations. Extracellular pH was recorded with an HI 98127 water proof pH meter (HANNA, USA).

Extracellular conductivity measurement

S. cerevisiae cells were cultured overnight at 25°C in 50 mL of YPD broth. After incubation, the yeast cells were centrifuged at 3200 rpm for 5 min and the pellet was washed twice with sterilized distilled water and resuspended. About 200 mg wet weight of yeast cells were used for each experiment.

Essential oils at mentioned concentrations prepared in DMSO were manually injected at zero point. Extracellular conductivity was recorded with an AD 31 Waterproof EC/TDS tester (Adwa, Hungary).

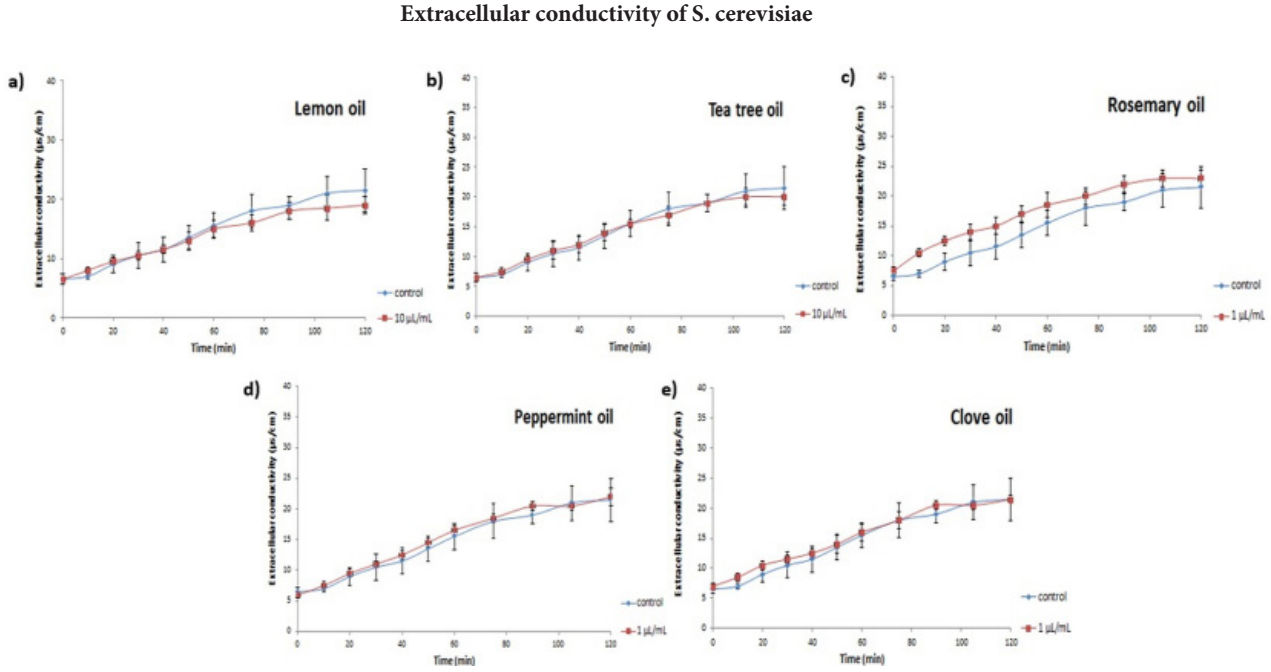

Figure 3. Effects of essential oils on the extracellular conductivity of S. cerevisiae. Point zero indicate the time of addition of a) Lemon peel oil: 10 µL/mL, b) Tea tree oil: 10 µL/mL, c) Rosemary oil: 1 uL/mL, d) peppermint oil: 1 uL/mL, e) clove oil: 1 uL/mL. The data represent the average of at least two independent experiments.

Results and discussion

The main purpose of this study was to disclose the dependence of antifungal activity of various essential oils on the integrity of cell membrane. After a thorough search of the literature we found that the information on the antifungal activity against *S. cerevisiae* was limited. More importantly, experimental data reported in the literature were based on studies with different experimental conditions preventing a simple comparison of the data with each other. Thus at the beginning of our studies we set out to determine the antifungal activity of lemon peel, orange peel, tea tree, turpentine, rosemary, peppermint, thyme, oregano and clove oils against *S. cerevisiae* via MIC, MFC and inhibition zone measurements.

The MIC and MFC values are shown in Tab. 1. The most effective oil against yeast cells was turpentine oil (MIC: 0.01-0.04 µL/mL and MFC: 0.01-0.04 µL/mL). Orange peel oil (MIC: 0.04-0.08 µL/mL and MFC: 0.02-0.08 µL/mL), thyme oil (MIC: 0.2-0.3 µL/mL and MFC: 0.2-0.3 µL/mL) and oregano oil (MIC: 0.2-0.3 µL/mL and MFC: >0.3 µL/mL) were also effective. Lemon peel and tea tree oil had slight antifungal activity (MIC: >10 and MFC: >10, for both). The MIC and MFC values of rosemary, peppermint, and clove oil could not be measured due to solubility problems.

The inhibition zones of essential oils are presented in Tab. 2. According to inhibitory zone measurements five oils with the highest efficacy were oregano oil (for 20% dilution, 3.6±0.1), orange peel oil (for 20% dilution 3.3±0.9), thyme oil (for 20% dilution 3.0±0,5), turpentine oil (for 20% dilution 2.5±0,2), and clove oil (for 20% dilution 2.2±0,5). The inhibition zone values generally confirmed the results obtained in the MIC and MFC data. Slight differences between the two data sets may be caused by different rates of diffusion of particular oil components into the agar medium or by evaporation of some of the components during the incubation time (Kunicka-Styczyńska, 2011). Recent studies highlight the role of water-soluble and vaporized components in the assessment of antimicrobial activity of essential oils (Inouye *et al.*, 2006; Fisher *et al.*, 2006).

Later, extracellular pH and conductivity measurements were performed to determine their effect on membrane integrity and membrane permeability of yeast cells (Gaskova *et al.*, 2013). It is well known that glucose-induced cells show a decrease in extracellular pH values (Souza *et al.*, 2007) (Supporting Information Fig. 1). In order to reach a maximal pH gradient across the cell membrane, yeast cells were glucose-induced before extracellular pH measurement experiments. Fig. 1 shows the changes in extracellular pH of orange peel, turpentine, thyme and oregano oil treated yeast cells for 0-60 min. Upon addition of essential oils, an increase in extracellular pH was observed. Especially extracellular pH of yeast cells treated with various concentrations of turpentine oil dramatically increased in a concentration dependent-manner. Higher concentrations than 0.2 µL/mL could not be tested due to solubility problems. Addition of

orange peel, thyme and oregano oils also caused an increase in the extracellular pH of yeast cells, possibly due to the neutralization of the glucose-induced pH gradient upon impairment of the cell membrane.

On the other hand, extracellular conductivity of *S. cerevisiae* cells treated with various concentrations of orange peel, turpentine, thyme and oregano oils for 0-120 min are demonstrated in Fig. 2. Conductivity values increased clearly in cells exposed to oils, which indicates rapid leakage of ions to the extracellular medium due to loss of integrity of cellular membrane. Higher concentrations than 0.2 μL/mL could not be examined as the pH measurements due to solubility problems of EOs. Conductivity change of other oils is shown in Supporting Information Fig. 3.

The interest in EOs has significantly grown in recent years and there has been an increase in the number of scientific publications of essential oils. Our results demonstrate that essential oils extracted from different plants show wide spectrum of antifungal activity against *S. cerevisiae* and that the cell membrane is the main target for the antifungal agents in the content of EOs, while disruption of yeast cell membrane integrity is the basic mode of action of these agents. These results will augment our knowledge about the mechanism of action of EOs against *S. cerevisiae* cells and help us widen their usage in food, cosmetic and pharmaceutical industries.

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