Investigation of antifungal activity mechanisms of alpha-pinene, eugenol, and limonene

Conference Paper

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Article info
Submission: 10-09-2022
Accepted: 29-12-2022
Online First: 30-12-2022
Publication: 31-12-2022

e-ISSN: 2548-1150
doi prefix: 10.31797/vetbio
- http://dergipark.org.tr/vetbio

ABSTRACT

Plant essential oils are preferred in cosmetics, medicine, food, and beverage industries for various purposes. α-Pinene is found mainly in eucalyptus oils, eugenol is the active ingredient in clove oil, and limonene is the major component in the oil of citrus fruit peels. In this study, we aimed to determine the antifungal activity of α-pinene, eugenol, and limonene against *Saccharomyces cerevisiae* yeast cells. Besides, we focused on revealing the target side of the compounds on the yeast cells. Firstly, the antifungal activity of compounds was tested via minimum inhibitory concentration (MIC) measurement. After that, we performed a sorbitol effect assay to understand whether it acts on the cell wall or not. With sorbitol, the MIC values were not changed. It means that they are not effective on the yeast cell wall. Then, we measured the extracellular conductivity increase upon treatment with the compounds to understand the effect on the cell membrane. Eugenol and limonene were not changed the extracellular conductivity, and there was no ion leakage from the cell membrane. On the other hand, α-pinene damaged the yeast cell membrane causing a sudden increase in conductivity due to ion leakage. An ergosterol effect assay with α-pinene was performed to detect cell membrane disruption via ergosterol or not. With ergosterol, the MIC value was not changed. α-Pinene must have another target than the ergosterol in the yeast cell membrane. Finally, revealing the mode of action of compounds against yeast cells will provide new insights into their usage in various fields.

Keywords: Antifungal activity, Eugenol, Limonene, Mode of action, α-Pinene, *Saccharomyces cerevisiae*.

INTRODUCTION

Natural compounds are vital sources in medicine for discovering drugs (Harvey et al., 2015)and for the food industry to improve food safety against microbial growth (Burt et al., 2013; Harvey et al., 2015). Several plant essential oils have been studied, and various studies have proven their biological activities, such as anti-inflammatory (Koudou et al., 2005), antiviral (Loizzo et al., 2008), cytotoxic (Zarai et al., 2011), and antimicrobial properties (Alviano & Alviano, 2009). Essential oils including pine, rosemary, lavender, and turpentine can contain pinenes and bicyclic terpenes. They have various chemical structures and biological properties. α-Pinene and β-pinene are two constitutional isomers (Silva et al., 2012). α-Pinene, ((1RS,5RS)-2,6,6-trimethylbi-cyclo [3.1.1] hept-2-ene) with a sum formula of C10H16 is an essential secondary metabolite in many conifer-derived essential oils (Allenspach et al., 2020), and it is an important biologically active natural monoterpene (Wei et al., 2020).

How to cite this article
Lüleci, HB., Ergüden B. (2022). Investigation of antifungal activity mechanisms of alpha-pinene, eugenol, and limonene. *Journal of Advances in VetBio Science and Techniques*, *7*(3), 385-390. https://doi.org/10.31797/vetbio.1173455

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Limonene is another cyclic monoterpene, the major component of citrus fruit peels. As a phenolic compound, eugenol is an important essential oil involved in clove oil (Cai et al., 2019). All three biologically active compounds have high industrial and commercial values (Allenspach & Steuer, 2021), and they are widely used in cosmetics, medicines, food industry and fine chemicals (Alonso et al., 2015; Wei et al., 2020) because of their antimicrobial activity against bacterial and fungal cells (Cahyari et al., 2018; Cai et al., 2019; Silva et al., 2012).

There have been many studies on the antibacterial (Allenspach & Steuer, 2021; Bevilacqua et al., 2010; Yang et al., 2015), antifungal (Bevilacqua et al., 2010; Cai et al., 2019; Nóbrega et al., 2021), and antiparasitic (da Franca Rodrigues et al., 2015) activities of α-pinene, limonene and eugenol in recent years. For antifungal activity, Candida species have been mostly used (Nóbrega et al., 2021). However, to our knowledge, studies concerning the antifungal activity of these compounds against *S. cerevisiae* are limited. *S. cerevisiae* is a significant spoilage yeast type for food and beverage industries (Stratford, 2006), and it is also frequently used in studies associated with diseases (Shen et al., 2004). Thus, determining the antifungal activity of the corresponding compounds against *S. cerevisiae* is crucial for various fields.

Natural compounds, especially essential oils, have a various modes of action to inhibit the yeast cells (Konuk & Ergüden, 2017). The cell wall, cell membrane, intracellular protein, mitochondria, DNA, and RNA are prominent and significant target sites of the natural compounds (Burt et al., 2013; Hyldgaard et al., 2012). In this study, we aimed to improve the understanding of the antifungal activity mechanism of the α-pinene, limonene and eugenol against yeast cells for usage in antifungal drugs or food/beverage industries.

MATERIAL and METHOD

Chemicals and yeast strain

D-Glucose, peptone, yeast extract powder, agar powder (HIMEDIA), dimethyl sulfoxide (DMSO), α-pinene, limonene, eugenol, sorbitol, and ergosterol (Sigma-Aldrich) were used in all the experiments. An ultrapure purification system produced distilled water (Thermo Scientific, Smart2Pure Water Purification System). YPH499 (MATa ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1; (Sikorski & Hieter, 1989) was used as the *S. cerevisiae* strain in all the experiments. Cells were grown in a yeast extract peptone glucose (YPD) medium at 28 °C and then precultured in a fresh YPD medium.

Minimum inhibitory concentration (MIC) measurement

The *S. cerevisiae* yeast cells were cultured overnight at 28 °C in a YPD medium and were diluted in the YPD to give a final density of 1×10^6 CFU/mL. Different concentrations of α-pinene, limonene and eugenol were prepared with DMSO, and put in a 24-well microtiter plate. After that, the *S. cerevisiae* cells were added to each well separately for three compounds. The yeast cells suspension without α-pinene, limonene and eugenol were used as a control groups. 24-well microtiter plates were incubated for 48 h at 28 °C. After the incubation, MIC values were detected to be the lowest concentration of compounds completely inhibiting the visual growth of the yeast cells.

Extracellular conductivity measurement

Extracellular conductivity measurement was performed like Konuk et al. (Konuk & Ergüden, 2017). All experiments were carried out in parallel with dH_2O and yeast cells as a negative control and dH_2O, yeast cells, and α-pinene, limonene and eugenol as the experimental groups. 20 mM, and 40 mM for α-pinene; 1 mM, 2 mM, and 4 mM for limonene / eugenol

were added to the cell suspensions at zero point, separately. Extracellular conductivity was recorded every 10 min in the first 60 min with an AD 31 Waterproof EC/TDS tester.

Sorbitol and ergosterol effect assay

Sorbitol and ergosterol effect assays were performed similarly to MIC. After adding α-pinene, limonene, and eugenol, 8 mM sorbitol was added to each well for the sorbitol effect assay (Pereira et al., 2015). Ergosterol effect assay was performed for α-pinene. 200 µg/mL ergosterol was added to each well after adding α-pinene for the ergosterol effect assay (Pereira et al., 2015). Sorbitol and ergosterol effect assays were performed separately, on different plates. 24-well microtiter plates were incubated for 48 h at 28 °C. The lowest concentration of compounds that completely inhibits visual growth of the yeast cells was detected visually for sorbitol and ergosterol effect assays.

Statistical analyses

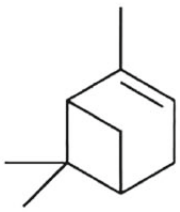
MIC, extracellular conductivity, sorbitol, and ergosterol effect assays were repeated at least three times to decrease the experimental errors. Averages and standard deviations of all groups were calculated by using Microsoft Excel. To understand whether treatment with α-pinene caused the statistically significant change, student's t-tests were performed to compare the plotted regression line slopes (Andrade & Estévez-Pérez, 2014)

RESULTS and DISCUSSION

MIC shows the antifungal activity of α-pinene, limonene and eugenol but not via yeast cell wall

The antifungal activity of the three biologically active compounds against *S. cerevisiae* cells was detected via MIC measurement. Here, we focused on the antifungal activity of the compounds and their modes of action against *S. cerevisiae* cells, because similar studies with *S. cerevisiae* are limited. Moreover, *S. cerevisiae* is a significant spoilage yeast for food, and beverage industries (Stratford, 2006), and medical areas (Shen et al., 2004). The MIC values of α-pinene, limonene and eugenol were demonstrated in Table 1. In addition to detecting antifungal activity, understanding the mode of action of these compounds is an indispensable necessity for medical or various industrial areas (Etebu & Arikekpar, 2016; Schäfer & Wenzel, 2020). After determining antifungal activity, the mechanism by which compounds exert an antifungal effect was determined. The cell wall of yeasts may be considered the first target for antifungal agents because of its structure involving chitin, glucan, and mannan (Nazzaro et al., 2017). Therefore, a sorbitol effect assay was performed to test the effectiveness of the compounds on the cell wall. The cell wall protects the yeast cells from environmental stresses (Pereira et al., 2015). With sorbitol, the MIC value of α-pinene, limonene and eugenol were not changed (Table 1). It means that the antifungal activity of the compounds does not interfere with the yeast cell wall. This result is in accord with the previously published work by Miron et. al. who investigated the damage of eugenol and α-pinene on the cell wall of another fungal cell type and observed no effect of sorbitol on MIC values (Miron et al., 2014).

Table 1. MICs (mM)value for α-pinene, limonene, and eugenol after MIC measurement, sorbitol and ergosterol effect assay



Compounds	MIC measurement (mM)	Sorbitol effect assay (mM)	Ergosterol effect assay (mM)
α-pinene	10 - 20	10 - 20	10 - 20
limonene	1 - 2	1 - 2	-
eugenol	1 - 2	1 - 2	-

α-Pinene triggered a higher amount of ion leakage but not via ergosterol biosynthesis

α- pinene is a well-known representative of the monoterpenes group and is found in various essential oils. It has a wide range of biological activities, including antibacterial effects against *E.coli* and *S. aureus*; antifungal effects against *C. albicans* (Salehi et al., 2019). In this study, we tried to elucidate the antifungal activity mechanism of the compound against the *S. cerevisiae* cells. The extracellular conductivity of the yeast cells treated with 20 mM and 40 mM α-pinene for 0–60 min are demonstrated in Fig. 1. Ion release was found to be statistically significantly different ($p < 0.05$) between control group (no compound), and 40 mM α-pinene (Fig. 1.). Ion release from the cell membrane with 40 mM α-pinene demonstrated that it disrupts cell membrane integrity with the corresponding concentration against *S. cerevisiae* cells.

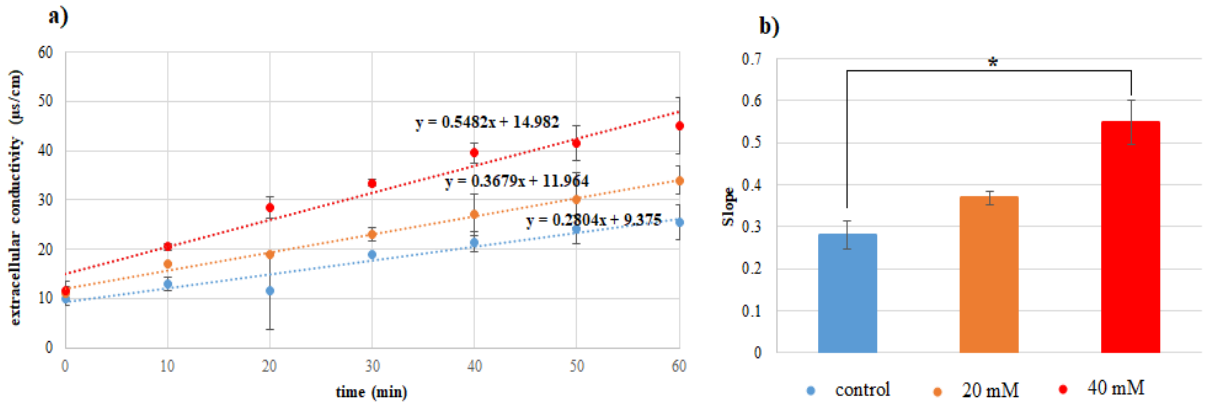


Figure 1. Extracellular conductivity measurement. a) Effect of α-pinene on the extracellular conductivity of *S. cerevisiae* yeast cells. b) The bars indicated standard deviation of the results from the mean values. Asterisk (*) indicates that values are statistically significantly different from each other ($p < 0.05$).

On the other hand, 1,2, and 4 mM limonene or eugenol did not cause efficient ion leakage from the yeast cell membrane. They must have another target side than the cell membrane in the yeast cell. Based on the conductivity measurement, 40 mM α-pinene caused efficient ion leakage from the yeast cell membrane. Ion leakage from the cytoplasm is associated with membrane disruption and cell death (Ergüden & Ünver, 2022).

After detecting ion leakage, we tested whether the membrane deformation was due to ergosterol or not. Ergosterol is a sterol included in the cell membrane of yeast cells, and it is an essential regulator of membrane fluidity. Since ergosterol is not present in animal and human cell membrane structure, it is the target site for many antifungal agents. Hence, disruption of ergosterol biosynthesis inhibits fungal growth (Minnebruggen et al., 2010). Nevertheless, MIC value of α-pinene after ergosterol effect assay did not change (Table 1). The findings indicate that α-pinene does not act by a mechanism that seems to involve inhibition of the ergosterol biosynthesis. In other words, the antifungal activity of α-pinene is not due to the disruption of ergosterol biosynthesis. It must have another target than the ergosterol in the yeast cell membrane.

CONCLUSION

The antifungal properties of α-pinene, limonene and eugenol against *S. cerevisiae* yeast cells were revealed, and the compounds' mode of action was studied. It has been proven that α-pinene, a biologically active natural monoterpene, has antifungal activity on the yeast cells. We have also shown that α-pinene disrupts cell membrane integrity and causes ion leakage resulting in cell death. These observations might be helpful for further

investigation for their medical applications or various industrial usage of monoterpenes.

ACKNOWLEDGMENT

This study was presented in ICABB 2022.

Ethical approval:

Ethics committee is not required

Conflict of interest: The authors declare that there is no conflict of interest

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