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Research Article

Gas Chromatography-Mass Spectrometry Analysis and Inhibitory Activity Against *Candida albicans* ATCC 10231 of the Leave Ethanolic Extract From *Citrus aurantifolia* (Christm.) Swingle

Monica Puspasari[1], Gloria Stefanie[1], Kris Herawan Timotius[1,2*]

[1]Faculty of Medicine and Health Sciences, Krida Wacana Christian University, Jakarta, Indonesia
[2]Research Center for Jamu and Herbal Medicine, Jakarta, Indonesia

*Corresponding author:
Kris Herawan Timotius, Faculty of Medicine and Health Sciences, Krida Wacana Christian University, Research Center for Jamu and Herbal Medicine, Jakarta, Indonesia
Email: kh_timotius@ukrida.ac.id

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Abstract

Background: *Candida albicans* is one of the important infectious yeasts that is associated with candidiasis, including oral candidiasis. The extracts of various herbal materials are potential for treating candidiasis.

Objectives: The objectives of this study were to determine phytochemical constituents of the leaf ethanolic extract of *Citrus aurantifolia* with gas chromatography-*mass spectrometry* (GC-MS) and to investigate the inhibiting effect of this extract on the planktonic growth of *C. albicans*.

Methods: The fresh leaves of *C. aurantifolia* were macerated overnight with ethanol. The extract was analysed with GC-MS. *C. albicans* ATCC 10231 was used in this study. The well-diffusion procedure was applied to detect the anti-candida activity qualitatively. Finally, real-time planktonic growth was employed for detecting the anti-candida activity quantitatively.

Results: GC-MS analysis revealed four dominant components in the ethanolic leaf extract of *C. aurantifolia*, namely, limonene, geraniol, phytol, and caryophyllene. The extract inhibited the growth of *C. albicans* either under the agar diffusion test or real-time planktonic growth. The specific growth rate of *C. albicans* was slower in the liquid culture with the extract. The specific growth rates of the 0 (control), 13.3, and 26.6 µg/mL were 0.582, 0.384, and 0.272, respectively. Eventually, the yields of the treated growth with 0 (control), 13.3, and 26.6 µg/mL were OD_{850} of 4.5, 3.0, and 3.7, respectively.

Conclusion: The leaf ethanolic extract of *C. aurantifolia* contains bioactive compounds which have anti-candida activity. Thus, it is a good material for new anti-candida ingredients in the future.

Keywords: Anti-candida, Caryophyllene, Geraniol, Limonene, Phytol

Background

Candida albicans belongs to the opportunistic/pathogenic yeast that is generally treated with antifungal agents. The current antifungal agents can induce side effects, resistance, and recurrence. Therefore, there is a need for screening new antifungal ingredients. Herbal materials can have a role as additional or alternative therapeutic materials (1). Herbal therapy is good and cheap for the treatment of various health disorders. The choice to use the leaf of *Citrus aurantifolia* as a source for new anti-candida is an appropriate approach (2,3).

Citrus is one of the essential plants economically, but previous evidence indicates attention to its fruits rather than leaves. Accordingly, this study is the first to analyse the leaf ethanolic extract and investigate its inhibitory activity against *C. albicans*. The leaves of *C. aurantifolia* (Family: Rutaceae) are available in the market for various cooking purposes. Particular bioactive compounds from *Citrus* species have antimicrobial, antioxidant, anticancer, antifungal, and antidepressant bioactivity (4), including anti-candida potential (5). The ethanolic extract from the leaf of *C. aurantifolia* may also exhibit particular bioactivities. The essential oils of *C. aurantifolia* are usually the product of hydrodistillation, either from the fruit or leaf (6,7). In this study, ethanol was used to obtain the leaf extract rich in essential oils.

This study aimed to analyse the constituents of the leaf ethanolic extract of *C. aurantifolia* with gas chromatography-*mass spectrometry* (GC-MS) and evaluate the influence of the extract on the *C. albicans* growth under planktonic or liquid broth media.

Materials and Methods

Extraction Techniques

Citrus aurantifolia leaves were coarsely powdered, and then, the powders were macerated with ethanol overnight at room temperature. The macerates were filtered through filter paper (Whatman No. 1). The obtained filtrate was dried by rotary evaporation at 80°C and stored until further analysis. Further, active charcoal was applied to absorb the chlorophyll content. Two types of extract were obtained, including the green and yellow ones that were

extracted before and after absorption with active charcoal, respectively. Both extracts were further analysed with GC-MS and well-diffusion tests. However, only the yellow extract was investigated for inhibitory activity with real-time growth inhibition experiments.

GC-MS Analysis

The leaf ethanolic extract (1 µL) was injected and subjected to GC-MS analysis on a Shimadzu GCMS-QP2010S, which was attached to an Abdel 5MS column (length: 30 m × 0.25 nm, film thickness: 0.25 µm) interfaced to a mass spectrophotometer (EI mode 70 Ev. in m/z range 28-600 employing the following condition: Helium was used as the carrier gas at a flow rate of 1.5 mL/min and the split ratio of 1:49. The column temperature was constant at 60°C for 5 minutes and then heated at 10 °C/min to 300 °C with a holding time of 41 minutes. The injection mode split less, and the MS spectra were identified using Library NIST62.LIB.

Candida applied in the Experiment

Citrus albicans ATCC 10231 was obtained from the Research Laboratory of the Faculty of Medicine and Health Sciences, Krida Wacana Christian University, Jakarta. The culture was maintained in the growth medium Sabouroud Dextrose agar.

Well-diffusion Agar Inhibition Test

The test aimed to check the presence of the inhibition zone caused by the extract. The Sabouroud Dextrose Agar and the liquid culture of *Candida* were used as growth agar and inoculum, respectively. Next, 0.5 mL of Mc Farland 0.5. was spread on the surface of the agar, and the diameter of the well was 1 cm. The agar was then incubated at 37°C for 24 hours.

Real-time Planktonic Growth Inhibition Experiment

This experiment was conducted with the RTS-1 Personal Bioreactor (Biosan) at 37°C. Three growth tubes were considered, including one control and two extract-added tubes. A control (without extract addition) growth tube and the growth tubes with extract, 13.3 and 26.6. µg sample/mL were carried out. The culture volume was 15 mL of Mueller-Hinton broth.

Statistical Analysis

The specific growth rate was calculated by following the instruction of the RTS-1C BioSan personal bioreactor. Then, the data of the exponential phase were extracted from the database of the BioSan instrument. The obtained data were compared with the two-way classification of ANOVA.

Results

GC-MS revealed the presence of four main constituents in the leaf ethanolic extract of *C. aurantifolia*, including limonene, geraniol, phytol, and caryophyllene

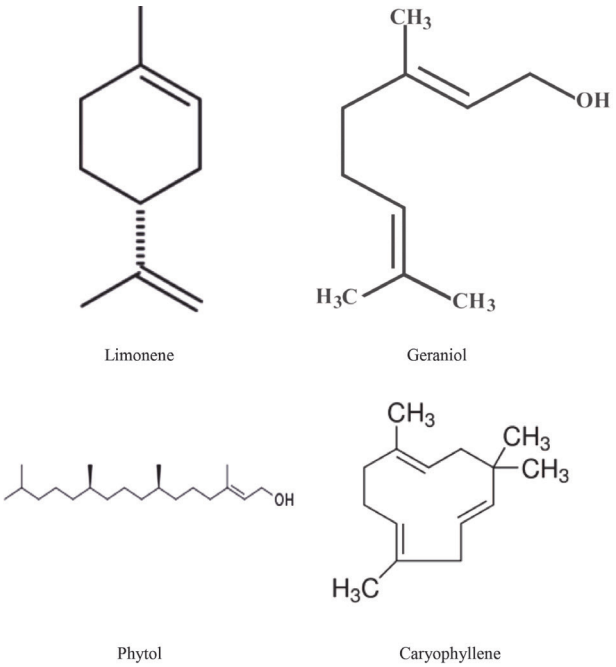


Figure 1. Chemical Structure of Limonene, Geraniol, Phytol, and Caryophyllene.

(Figure 1 and Table 1). The chromatograms and MS spectrums are illustrated in Figures 2 and 3.

Leaf ethanolic extracts could significantly inhibit the growth of *C. albicans*. The well-diffusion test of the extract showed a clear zone of inhibition (Figure 3), either the green or the yellow extract. The diameter of the inhibition zone was greater when more extracts were added to the well. The diameters of the inhibition zone from several repetitions were not stable (Figure 4). Therefore, the real-time planktonic growth of *C. albicans* was conducted to measure the inhibition activity of the leaf ethanolic extract (Figure 5). The lag phase was not inhibited, and inhibition was observed during the logarithmic phase or the active growth of the *C. albicans*. The specific growth rates of the 13.3 µg/mL and 26.6 µg/mL extract/medium and the control were 0.582, 0.384 and 0.272, respectively. The yields (the stationary phase) 13.3 µg/mL and 26.6 µg/mL extract/medium were OD_{850} of 3 and 3.7, respectively. These yields were lower compared to the control (OD_{850} 4.5). Statistically, the addition of the leaf ethanolic extract significantly decreased the exponential growth of *C. albicans* ($P \leq 0.01$).

Discussion

Except for phytol, the three constituents were already reported as the main constituents of Citrus leaf essential oils (8). They were occasionally reported as the primary or predominant constituents depending on the growth condition and the origin of the plant (9).

Limonene is the main component of the leaf ethanolic extract of *C. aurantifolia* and can inhibit the growth of *C. albicans*. In addition, it exhibits excellent anti-Candida activity, either against biofilm-producing or planktonic growth of *C. albicans* (10,11). Moreover, limonene as the

Table 1. Main Components (%) of the Leave Ethanolic Extracts of *Citrus aurantifolia* (GC-MS Analysis)

RT	Selected Compound	Formula	Green Extract	Clear Extract
10.895	Limonene	$C_{10}H_{16}$	9.35	1.87
15.420	Citral	$C_{10}H_{16}O$	2.09	1.46
16.839	Geraniol	$C_{10}H_{18}O$	3.75	10.96
17.117	Geranyl acetate	$C_{12}H_{20}O_2$	0.98	3.65
17.475	2,4-disopropenyl-1-methyl-1-vinyl-cyclohexane	$C_{15}H_{24}$	1.25	1.85
17.972	Trans Caryophyllene	$C_{15}H_{24}$	4.88	10.28
20.259	Carryophellene oxide	$C_{15}H_{24}O$	1.32	2.95
20.757	Spathulenol	$C_{15}H_{24}O$	1.05	1.20
22.967	1-Octadecyne	$C_{18}H_{34}$	4.59	0.75
23.042	2-Undecanone, 6,10-dimethyl-	$C_{13}H_{26}O$	2.59	1.64
23.889	Methyl stearate	$C_{19}H_{38}O_2$	1.98	2.05
24.606	Ethyl palmitate	$C_{18}H_{36}O_2$	6.00	6.81
25.730	Oleic acid, methyl ester	$C_{19}H_{36}O_2$	4.50	3.48
25.966	Phytol	$C_{20}H_{40}O$	16.45	16.70
26.376	9-hexadecenoic acid	$C_{12}H_{22}O_2$	7.34	8.14
26.585	Ethyl palmitate	$C_{18}H_{36}O_2$	2.42	2.53
	Total of selected compounds		70.54	76.32
	Minor/trace components		29.46	23.68
	Total		100.00	100.00
	No. peaks		71	40
	Total fatty acids		38.69	39.71

Note. RT : ; GC-MS: Gas chromatography-*mass spectrometry*.

natural insecticide is a good ingredient for a cleaning solvent (12) and in cosmetic products. It is also used in the food and pharmaceutical industries due to its smell (13).

Geraniol is an antifungal against *C. albicans* (14). As a monoterpene and alcohol, it is the primary component of the leaf ethanolic extract of *C. aurentifolia*. Furthermore, it is reported as a component of lemon essential oils that has an inhibitory effect on the growth of Candida strains (13,15).

Phytol is a terpene with a branched unsaturated chain and a product of chlorophyll metabolism. Additionally, it can inhibit microbes, including *C. albicans* (5,16,17). Although it is not yet clear whether phytol has an anti-candida potential, it is reported to have an anti-biofilm potential (18).

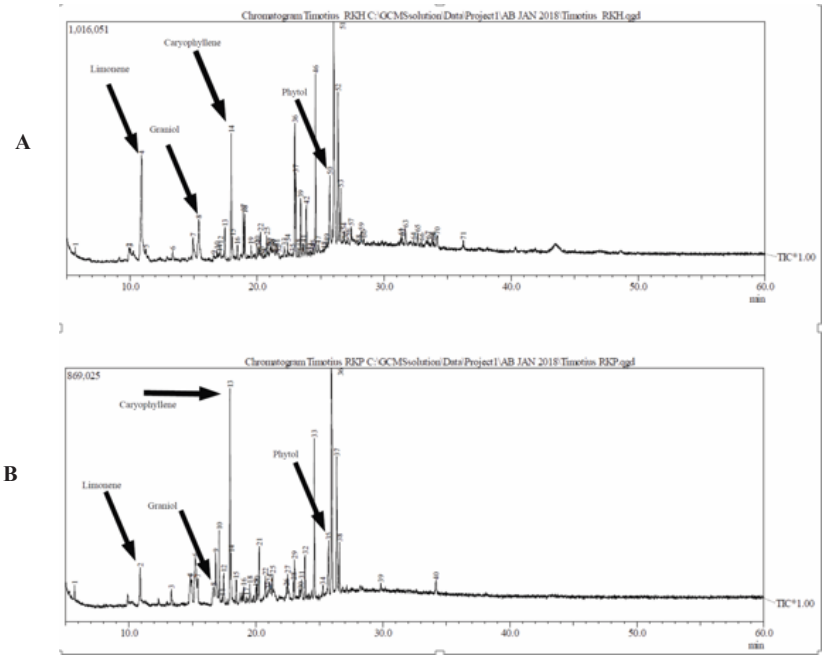
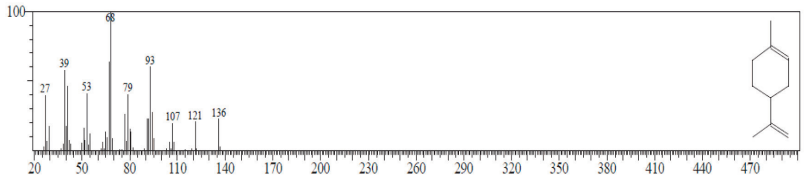
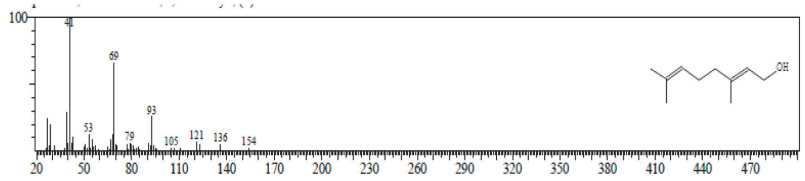


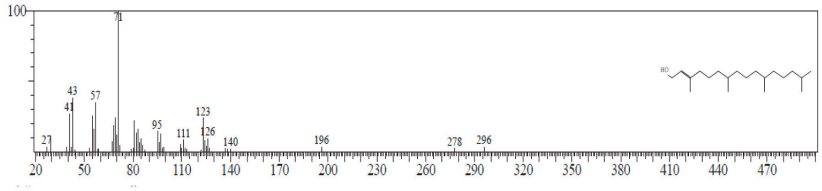
Figure 2. Chromatogram of the Leave Ethanolic Extract of *Citrus citrifolia*: (**A**) Green extract and (**B**) Yellow Extract .



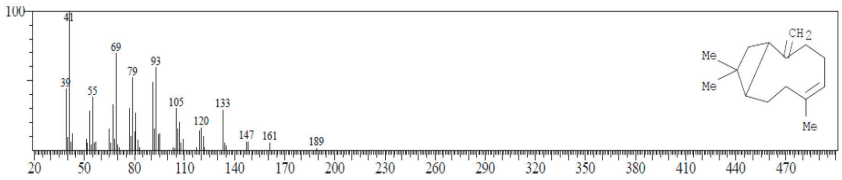
Mass spectra of limonene



Mass spectra of geraniol



Mass spectra of phytol



Mass spectra of caryophyllene

Figure 3. Mass Spectra of Limonene, Geraniol, Phytol, and Caryophyllene.

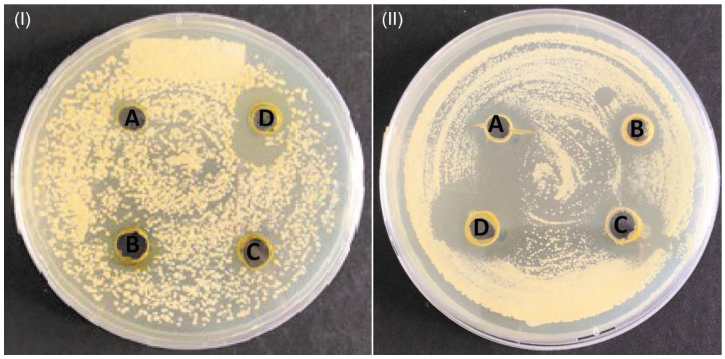


Figure 4. Inhibition Zones of *C. albicans* by the Leave Ethanolic Extract of *C. aurentifolia*: (**I**) Green Extract and (**II**) Yellow Extract. *Note*. *C. albicans*: *Candida albicans*; *C. aurantifolia*: *Citrus aurantifolia*. The four wells contained the extract amounts of (A) 40 ug, (B) 60 ug, (C) 80 ug, and (D) 100 gl.

Caryophyllene derivates are the constituents of various extracts with anti-candida potentials, including trans-caryophyllene (β-caryophyllene) and caryophyllene oxide (13,19). Trans-caryophyllene is a component of the essential oil of various plant species and is known for its anti-inflammatory activity (20). A combination of limonene, geraniol, phytol, and caryophyllene is good for inhibiting the growth of *C. albicans*.

Conclusion

The findings of our study demonstrated that the compounds present in the leaf ethanolic extract of *C. aurantifolia* have the potential to exert anti-candida effects and contain four main constituents, namely, limonene, geraniol, phytol, and caryophyllene. With these constituents, the leaf ethanolic extract inhibits the growth of *C. albicans*, which is often found as a pathogen in clinical cases of invasive oral

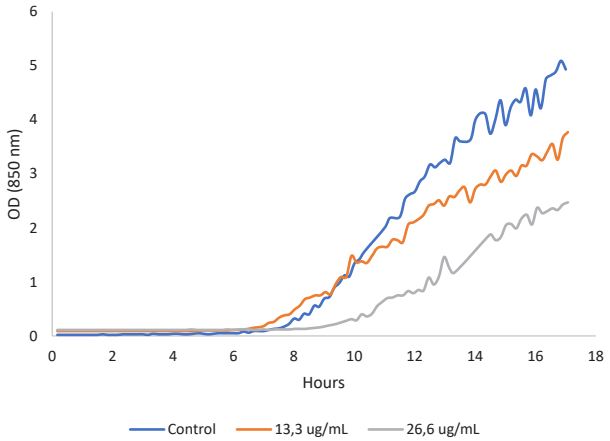


Figure 5. Plantonic Growth Curve of *Candida albicans*.*Note*. OD: Optical density.

candidiasis. Therefore, it is recommended that herbal toothpaste with the leaf ethanolic extract of *C. Aurantifolia* be developed for this purpose (2).

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Authors' Contributions
MP and GS are responsible for the study design and experimentation. KHT was responsible for the manuscript preparation.

Conflict of Interest Disclosures
None.

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