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The antibiofilm potential of *Clidemia hirta* against *Candida albicans*: preliminary study

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Abstract

Objective This study aims to screen the activity of ethanol extract of *Clidemia hirta* fruits and leaves against 24 h old *Candida albicans* biofilm by measuring cell viability with 3-(4,5-dimethylthiazol- 2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) and planktonic cells of *C. albicans* using the broth microdilution method.

Results The ethanol extract of *C. hirta* fruits inhibited (50%) the *C. albicans* biofilm at a concentration fourfold higher than those required to inhibit planktonic cells (31.25 µg/mL vs. 7.8 µg/mL). This finding is supported by the morphological changes of *C. albicans* biofilm under scanning electron microscopy (SEM) visualization. A total of 55 compounds were detected using UHPLC coupled with Thermo Scientific™ Q Exactive™ High-Resolution Mass Spectrometer (HRMS). The top three compounds of the ethanol extract: dodecyl sulfate, bis(2-ethylhexyl) phthalate, and erucamide) might be responsible compounds for the antibiofilm activity of ethanol extract of *C. hirta*.

Keywords *Clidemia hirta*, *Candida albicans*, Biofilm

Introduction

Healthcare-associated infections (HCAIs) refer to infections that are acquired during obtaining medical care, typically in a hospital or other healthcare facility. These infections commonly occur due to invasive procedures, including inserting temporary indwelling devices, such as catheters (central venous or urinary catheters), and they also arise as a complication following surgical interventions involving implants [1].

The planktonic form of microorganisms and those organized in biofilms are responsible for contaminating the organisms in indwelling devices. *Candida albicans* is one of the critical priority groups in the WHO fungal priority pathogens [2]. Although the prevalence of biofilm-forming capability of *C. albicans* is lower than non-albicans *Candida* [3], infections produced by this pathogen remain a concern. The production of robust and structured biofilms on implanted medical devices such as urinary and central venous catheters allows this germ to colonize and cause systemic and invasive candidiasis with mortality rates ranging from 20 to 50% [2, 4].

Many strategies have been implemented to prevent and eradicate *Candida* biofilm. However, controlling the production of *C. albicans* biofilms remains a difficult task. The recommendation to remove the catheter is limited to the patient's medical condition. Indeed, some patients choose to use extended-stay urinary catheters at home for economic reasons, including not having enough money to pay for surgery or no national insurance [5]. Prevention and eradication using conventional antifungals also remain challenging due to biofilm-expressed resistance/tolerance to available therapeutics. This can be explained, for one example, by the presence of an extracellular matrix containing β− 1,3 glucan, which protects the yeast cells by absorbing conventional antifungals such as amphotericin B. Thus, the drug fails to kill the yeast cells [6]. A similar concern about the resistance of a newer antifungal, echinocandins, has been observed [7]. Thus, all the challenges to combating *Candida* biofilm

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involve exploring and evaluating natural products such as invasive plants.

Invasive plants are non-native plants that grow and proliferate outside their native region and compete aggressively with native plants for water and nutrients, damaging biodiversity. According to the Ministry of Environment and Forestry Republic of Indonesia, more than 300 invasive plants are spread in Indonesia. *Clidemia hirta*, locally known as *Harendong bulu*/*Senduduk bulu*/*Senggani*, is one of the invasive plants in Indonesia that belongs to the *Melastomataceae* family [8]. It originated in South America and has spread to Sri Lanka, India, Australia, East Africa, and South Asia. This plant is a serious weed that harms tea plantations and impacts the reduction of tea yields. However, most Indonesian people in West Java acknowledged it for healing wounds and carbuncles. In modern scientific research, the extract of *C. hirta* showed antioxidant and hepatoprotective activity against CCl_4-induced injuries and oxidative damage in mice [9]. The extract of *C. hirta* inhibits the growth of microorganisms such as *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* [10, 11]. The phytochemical screening study has shown that the ethanol extract of *C. hirta* contains flavonoids, saponins, tannins, and triterpenoids [10]. Flavonoids are well known to have the potency to inhibit the growth of *C. albicans*. Thus, based on those findings, it is essential to explore further the potency of *C. hirta* against *C. albicans* biofilm and to profile qualitative components of *C. hirta*.

Materials and methods

Collection and extraction of *Clidemia hirta*

This invasive plant, *Clidemia hirta* fruits and leaves, has been collected from West Java, Indonesia (GPS coordinate: 6.76132130611412, 107.42728367256899). No special permits were needed for the *C. hirta* collection for the present study. No special authorization is required for the location or activities of this invasive plant because this invasive plant is not protected in any way and is not a type of protected plant. The identification and determination process of the plant was conducted by Dr. Djoko Santosa (The Pharmacy Faculty of Gadjah Mada University) with the number identification 12.29.11. The voucher specimen of *C. hirta* was 98 CH-A and was deposited at the Pharmacy Faculty of Gadjah Mada University. The first step before the extraction process is cleaning the leaves and fruits of *C. hirta* under slow-running water. Both plant parts were shade-dried over several days (7 and 10 days, respectively), powdered using a grinder, and extracted in ethanol (Merck KGaA, Darmstadt, Germany) by the maceration process, according to the published method [12]. Briefly, 100 g of plant powder was soaked in 500 mL of ethanol for three consecutive nights with regular shaking and filtered (Whatman™ filter paper no 1) in the Buchner funnel. Then, the residuals were re-macerated for three days with fresh ethanol solvent. All obtained filtrates were pooled, filtered, and dried with a Heidolph™ rotary evaporator (Schwabach, Germany). Samples were kept in a refrigerator at − 20 °C until further testing.

High-Resolution mass spectrometer (HRMS)

Phytochemical screening of *C. hirta* sample was conducted using Thermo Scientific™ Dionex™ Ultimate 3000 RSLCnano UHPLC coupled with Thermo Scientific™ Q Exactive™ High-Resolution Mass Spectrometer. Water + 0.1% formic acid (solvent A) and acetonitrile + 0.1% formic acid (solvent B) were used as mobile phase. The analytical column was ACQUITY UPLC CSH Phenyl-Hexyl Column, 130 Å, 1.7 µm, 2.1 mm X 100 mm, 1/pk. The flow rate was 50 µL/min with two µL injection volume. The gradient mode has been configured as follows: at t = 0–2 min, B 5%; at t = 30–35 min, B 60%; at t = 35–45 min, B 90%; and at t = 45–50 min, B 5%. Full scan MS was measured with a full width at half maximum (FWHM) of 70,000, while MS2 was measured with an FWHM of 17,500. This experiment employed Heated Electrospray Ionization (H-ESI) in positive polarities. The identification of chemicals was then performed using the Thermo Scientific ™ Compound Discoverer Software. The extract was dissolved (0,1 mg/mL in ethanol), vortexed for 2–3 min, and filtered through a 0.22 µm Millex® filter membrane (Merck KGaA, Darmstadt, Germany) before injection into the chromatographic system.

Fungal strains and culture condition

The strains used in the antifungal study were *C. albicans* ATCC 10231 and one clinical isolate from the Microbiology Department of Faculty Medicine Universitas Gadjah Mada. All strains were grown in Sabouraud dextrose agar (SDA) at 37 °C for 24 h.

Determination of antifungal activity

The samples'minimum inhibitory concentration (MIC) against *C. albicans* was evaluated using the broth microdilution method [13]. All extracts were prepared by making stock solution at 10 mg/mL in 20% DMSO. The final DMSO concentration for all working samples was always kept below 2% in each well of microplate during all biological tests. Working samples were then prepared in 96-well microtiter plates serially two-fold dilution in Roswell Park Memorial Institute (RPMI) 1640, which contained 0.165 M per liter of morpholinepropanesulfonic acid (MOPS) (Himedia, AT180) buffer medium to get concentrations ranging from 1000 to 15.625 µg/mL.

Some wells were preserved for non-treated yeasts (negative control), yeasts treated by positive control, Sigma-Aldrich® fluconazole (≥ 98% HPLC powder, Merck KGaA, Darmstadt, Germany) at a similar final concentration to the samples, yeasts treated by DMSO 2% (DMSO control), and medium control. Five colonies of ~ 1 mm in diameter from 24 h-old cultures of *C. albicans* were picked up and suspended in sterile saline solution (0.89% of NaCl) and adjusted to 0.5 McFarland standard (equivalent to 2×10^6 CFU/ml). The suspension was diluted at 1:50, followed by a 1:20 dilution using RPMI 1640-MOPS to obtain 2×10^3 CFU/mL of yeast suspension. Then, 100 µL of diluted cell suspension was added to each corresponding well, filled with 100 µL of working samples. Therefore, the final concentrations of extracts were 500 to 7.8 µg/mL. The plates were then incubated at 37 °C for 24 h. Determining the lowest concentration of samples that causes a prominent decrease (~ 50%) (MIC_{50}) in visible growth was estimated after 24 h incubation.

Determination of antibiofilm activity

The colorimetric assay (MTT) was performed to determine the metabolic activity of mature *C. albicans* biofilm [12]. Yeast was first cultivated for 48 h on the SDA agar plate. After that, 30 mL of Yeast Extract-Peptone-Dextrose (YPD) medium (Difco™, Detroit, US) was filled with four loopfuls of this culture, and it was cultured for the entire night at 37 °C without shaking. After centrifuging this culture for 10 min at 3000 g, it was thrice washed in 0.1 M phosphate-buffered (PBS, pH 7.2) (Gibco™, NY, US), standardized to 0.5OD600 (equivalent to 3×10^7 CFU/mL), then diluted to obtain a suspension of approximately 1×10^6 CFU/mL in YPD medium. The 100 µL yeast suspension was then transferred into a sterile Corning® 96-well Clear Flat Bottom Polystyrene Microplate, and incubated for 24 h at 37 °C. Following 24 h incubation period, the non-adherent yeasts were eliminated by washing them twice in 0.2 mL of sterile PBS; after that, 100 µL YPD medium was added to the corresponding wells. Then, each well-containing biofilm was treated with serial two-fold dilutions (ranging from 1000 to 15.625 µg/mL) of the extracts prepared in another 96-well microplate, so the final concentrations of extract per well-containing biofilm ranged from 500 µg/mL to 7.8 µg/mL. Some wells were reserved for non-treated biofilm (negative control) and biofilm treated by DMSO 2%. The microplates were then incubated at 37 °C for 24 h. The wells were further cleaned twice using 0.2 mL PBS following 24 h incubation period at 37 °C. Next, 100 µL of MTT solution (5 mg/mL in PBS) was added to each well and left at 37 °C for 90 min [12]. After incubation, the solution was removed, and the formazan was dissolved in 100 µL of isopropanol-HCl. Solubilized formazan colour was measured using an absorbance microplate reader (Biochrom—Asys Expert Plus, NY, US) at a λ of 550 nm.

Microscopic observation of biofilm

The effect of *C.hirta* fruit extract on the morphology of *C. albicans* biofilms employing a scanning electron microscope (SEM) (JEOL JSM- 6510LA, US). Briefly, biofilms of *C. albicans* ATCC 10231 (control and treatment cells) were grown on Nunc™ Thermanox™ polystyrene coverslips (Thermo Fisher Scientific, US), as previously reported in the antibiofilm assay section. After 24 h, coverslips from control and treatment biofilms were rinsed twice with PBS and fixed with glutaraldehyde and 0.1 N PBS for 1 h at room temperature. The coverslips were rinsed with PBS and gradually dehydrated in low temperatures (pre-chilled in the refrigerator at 4 °C) of 50%, 70%, and 90% ethanol solutions for 10 min. Then, the coverslips were air-dried overnight in a desiccator before gold sputter coating.

Results

Clidemia hirta extract yields

After all the dry extract was weighed, the percentage yields of the ethanol extract of *C. hirta* fruits and leaves were determined by the following formula:

$$\text{Yield}(\%) = \text{weight of dry extract} \,/\text{weight of dry powdered plant part} \times 100$$

By calculation, the yield of the ethanol extract of *C. hirta* fruits was 15.33%, and the leaf extracts were 20.54%.

Antifungal activity of *C. hirta* against planktonic cells of *Candida albicans*

The ethanol extracts of *C. hirta* fruits and leaves showed activity against all strains of planktonic cells of *C. albicans*. The highest activity was shown by *C. hirta* fruits against all strains of *C. albicans*, with MIC_{50} at 7.8 µg/mL and 3.9 µg/mL against reference and clinical isolates of *C. albicans*, respectively. The activity was comparable to the fluconazole activity (Table 1).

Antibiofilm activity on reference and clinical *C. albicans* isolates

The antibiofilm activity of each extract in the treated and control groups was compared to determine whether both extracts inhibited the biofilm formed by *C. albicans* ATCC 10231 on the microplate. Both extracts inhibited biofilm with different potency (Fig. 1).

The fruit extract showed significantly more potent than the leaf extract, with an inhibitory concentration >50% at 31.25 µg/mL ($p < 0.05$). The leaf extract significantly

Table 1 Antifungal activity of *C. hirta* fruits and leaves against *C. albicans* planktonic cells

Strain	Ethanol extract of *C. hirta* fruits		Ethanol extract of *C. hirta* leaves		Fluconazole	
	MIC_{50} (µg/mL)					
	24 h	48 h	24 h	48 h	24 h	48 h
C.albicans ATCC 10231	7.8	15.625	15.625	31.25	3.96	7.8
IC *C.albicans*	3.96	7.8	7.8	15.625	7.8	15.625

*IC = Clinical Isolate. The test was run in triplicate and repeated at least twice

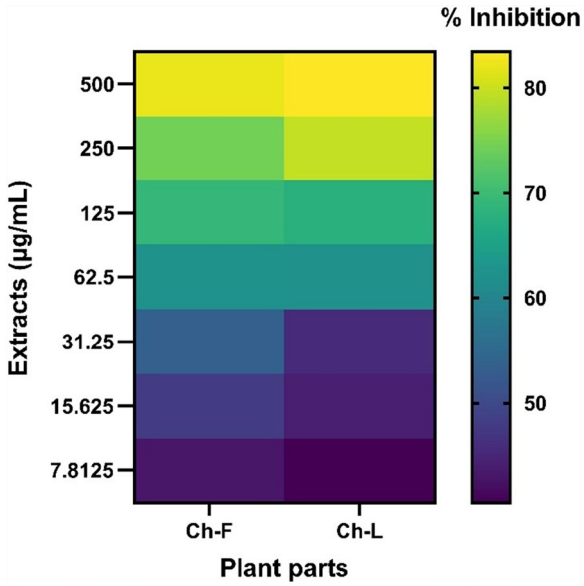

Fig. 1 The 2-dimensional data visualization of the inhibition percentages of the ethanol extract of *C. hirta* against 24 h old biofilm of *C. albicans* ATCC 1023. (Ch-F = *C. hirta* fruits; Ch-L = *C. hirta* leaves)

inhibited biofilm growth >50% at 62.5 µg/mL ($p < 0,05$). In addition, it was shown that the fruit extract of *C. hirta* was more active against clinical isolate biofilm than the leaves with the same inhibitory concentration as those that inhibit reference *Candida* (Fig. 2). While leaf extracts need a higher concentration to inhibit clinical isolate biofilm, at 125 µg/mL.

Scanning Electron Microscopy of reference *C. albicans* biofilm cells

The treated biofilm cells'features differed from the control cells (Fig. 3). In the magnificence 1000x, the control cells appear much more in number than the treated biofilm cells. In the magnificence 5000 ×and 10000 ×, the treated yeast cells appear rough and smaller in volume (shrinkage) starting at 250 µg/mL concentrations (red arrow). Even at the lowest concentration, the cells appear less in number, swelling, and rougher than the non-treated cells.

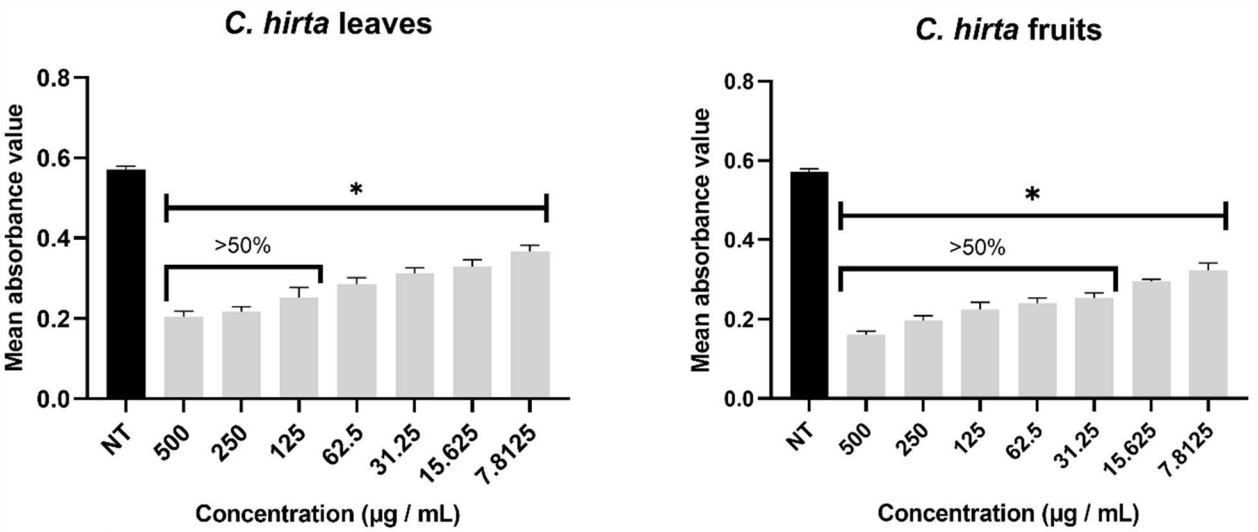

Fig. 2 Metabolic activity of ethanol extracts of *C. hirta* fruits and leaves against 24 h old clinical isolates biofilms. Asterisks denote statistically significant differences between treated and non-treated (NT) biofilm. *$p \leq 0.05$ was calculated by the Kruskal–Wallis test, followed by Dunn's multiple comparisons

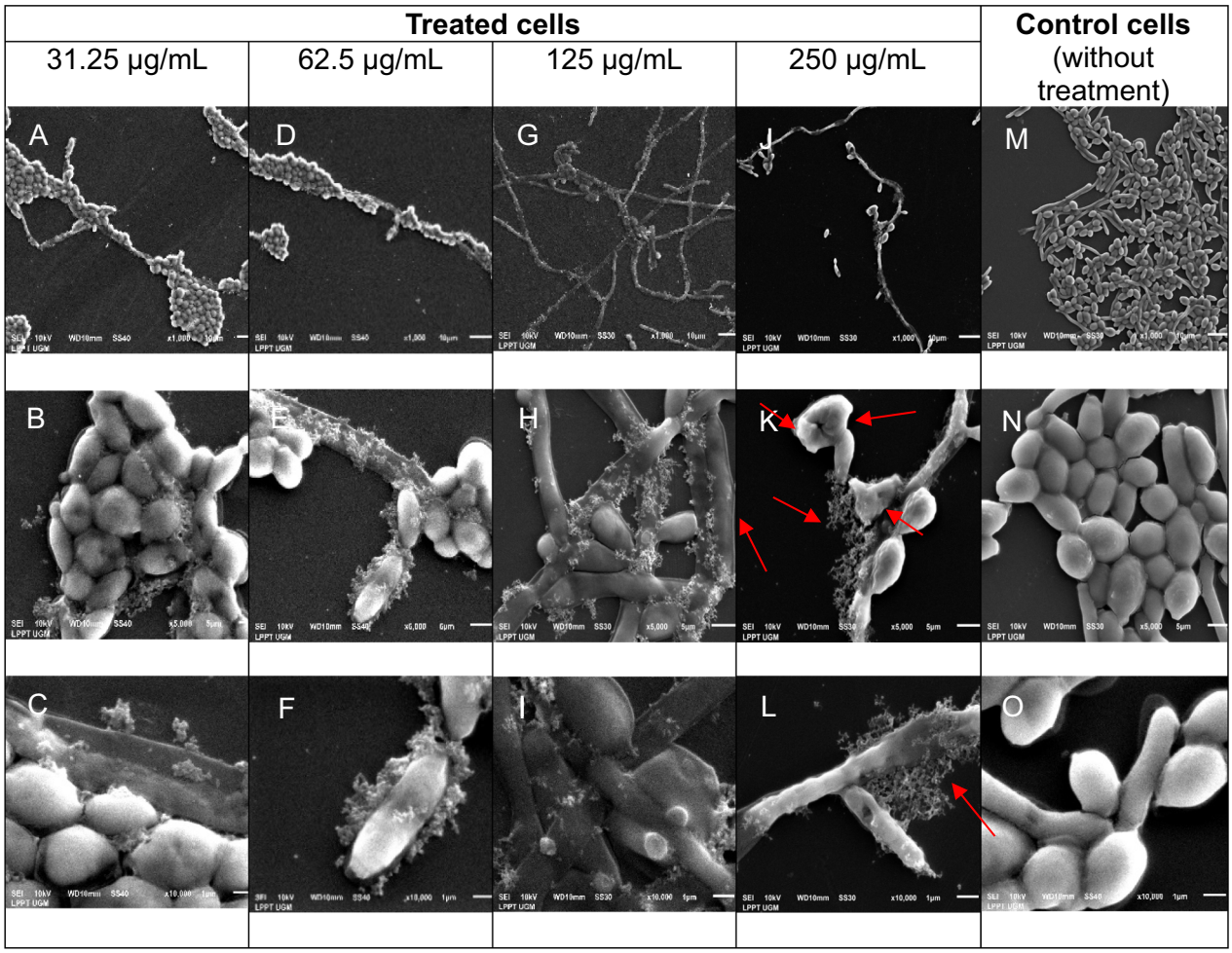

Fig. 3 Morphological structure of *C. albicans* biofilm observed by SEM (A–C = biofilm treated at 31.25 µg/mL; D–F = biofilm treated at 62.5 µg/mL; G–I = biofilm treated at 125 µg/mL; J-L = biofilm treated at 250 µg/mL; M–O = non-treated biofilm cells)

HRMS

Due to the fruit extract's best efficacy, its compounds have been screened qualitatively using HRMS. The number of compounds detected in the ethanol extract of *C. hirta* fruits was 55. To estimate the extent to which a compound can be found in an extract relative to a reference compound based on the largest peak area, the relative percentage abundance (% RA) was calculated by comparing the RA of the provided peak area to the RA of the *reference peak. Dodecyl sulfate, bis(2-ethylhexyl) phthalate, and erucamide were the top three components of the high RA (Table 2).

Discussion

Clidemia hirta is known to have a negative ecological impact, especially on the sustainability of the ecosystem and endemic flora in several areas of Indonesia [14, 15] Instead of throwing or burying the invasive plants, seeking out and exploiting their chemical resources would be profitable. In this case, turning the enemy into value is a rational solution that would eventually mitigate the cost of their removal.

The extraction yield of the active components from plant materials is well-known to be affected by many factors, such as solvents, extraction methods, plant parts, and different geolocations [16, 17]. In this study, even though the yield percentages of the ethanol extract of *C. hirta* fruit and leaves were different, both extracts had good yield results because the value of the yield was > 10% [17]. Compared to a previous study, the percentage yield of the ethanol extracts of leaves of *C. hirta* originated from Merapi Mount National Park area is higher than this study (37.98 vs. 20, 54%) [18], which might be affected by the difference in geolocation origin of the plant. This might indicate that in the Merapi Mount National Park area, the plant grows more worthwhile and generates more secondary metabolites than in West Java. However, we cannot conclude that the compound of C.

Table 2 Mass spectrometric analysis of ethanol extract of *C. hirta* fruits using HRMS in positive mode

No	Name	Formula	RT [min]	Area (Max.)	% RA*	mzCloud Best Match	Calculated Molecular Wight (MW)
1	Lidocaine	$C_{14}H_{22}N_2O$	9,873	80,365,087,95	0,76	99,7	234,17,266
2	PPG n6	$C_{18}H_{38}O_7$	11,589	69,296,658,93	0,66	99,1	366,26,057
3	3-tert-Butyladipic acid	$C_{10}H_{18}O_4$	11,608	14,801,730,97	0,14	85,8	202,11,949
4	DNH	$C_{14}H_{20}N_6O_7$	11,754	13,300,673,28	0,13	81,7	384,13,844
5	NP- 018716	$C_{11}H_{20}O_4$	12,182	14,527,657,17	0,14	94,7	198,12,502
6	Palmitic Acid	$C_{16}H_{32}O_2$	12,507	77,379,314,64	0,73	83,7	273,26,558
7	Dodecanedioic acid	$C_{12}H_{22}O_4$	12,694	144,494,009,3	1,37	85,2	230,15,077
8	NP- 004036	$C_{12}H_{22}O_4$	12,698	14,487,415,93	0,14	84,2	212,14,057
9	Perfluoroheptanoic acid	$C_7HF_{13}O_2$	12,755	131,155,204,1	1,24	94,5	363,97,594
10	Dibutyl phthalate	$C16H_{22}O_4$	12,785	40,294,685,93	0,38	88,2	278,14,867
11	(1R,4aS)– 7-(2-Hydroxypropan- 2-yl)– 1,4a-dimethyl- 9-oxo- 3,4,10,10a-tetrahydro- 2H-phenanthrene- 1-carboxylic acid	$C_{20}H_{26}O_4$	12,963	16,711,927,32	0,16	97,9	330,18,199
12	(2R,5R,6R)– 3-[(1E,3E)-hepta- 1,3-dien- 1-yl]– 5,6-dihydroxy- 2-(hydroxymethyl)cyclohexan- 1-one	$C_{14}H_{22}O_4$	13,23	8,529,640,833	0,08	91,5	276,13,298
13	NP- 021797	$C_{12}H_{22}O_3$	13,322	43,614,432,61	0,41	84,6	236,13,822
14	Perfluorooctanoic acid (PFOA)	$C_8HF_{15}O_2$	13,459	10,549,425,292	100**	99,5	413,97,234
15	DEET	$C_{12}H_{17}NO$	13,598	23,272,344,32	0,22	99,6	191,13,046
16	5-(6-hydroxy- 6-methyloctyl)– 2,5-dihydrofuran- 2-one	$C_{13}H_{22}O_3$	13,649	30,602,666,05	0,29	70,1	226,15,615
17	2,2,6,6-Tetramethyl- 1-piperidinol (TEMPO)	$C_9H_{19}NO$	13,728	55,428,487,18	0,53	92	157,14,626
18	NP- 019547	$C_{17}H_{26}O_5$	14,26	8,485,545,247	0,08	90,4	332,15,892
19	Dodecyl sulfate	$C_{12}H_{26}O_4S$	14,288	3,001,498,670	28,45	96,4	266,15,468
20	Ostruthin	$C_{19}H_{22}O_3$	14,546	409,649,674,4	3,88	95,1	298,15,962
21	Hexadecanedioic acid	$C_{16}H_{30}O_4$	14,593	12,517,105,77	0,12	88,3	286,21,397
22	NP- 019547	$C_{17}H_{26}O_5$	14,757	242,346,986,1	2,30	92,3	310,18,083
23	1-[(1S,4S)– 4-(1-Benzyl- 3,5-dimethyl- 1H-pyrazol- 4-yl)– 2-cyclopenten- 1-yl]– 3-isopropylurea	$C_{21}H_{28}N_4O$	15,04	58,183,158,51	0,55	76,8	398,23,311
24	Tetranor- 12(S)-HETE	$C_{16}H_{26}O_3$	15,208	12,549,938,23	0,12	78,6	248,17,446
25	4-Ethoxy ethylbenzoate	$C_{11}H_{14}O_3$	15,211	65,277,762,53	0,62	94,7	194,09386
26	Myristyl sulfate	$C_{14}H_{30}O_4S$	15,456	521,822,040,4	4,95	96	294,18,733
27	Resolvin E1	$C_{20}H_{30}O_5$	15,648	74,425,827,52	0,71	85	350,21,226
28	4-Dodecylbenzenesulfonic acid	$C_{18}H_{30}O_3S$	15,668	170,185,208,9	1,61	99,5	326,19,115
29	4-Dodecylbenzenesulfonic acid	$C_{18}H_{30}O_3S$	15,913	760,209,731,1	7,21	99,5	326,19,115
30	Tris(1,3-dichloro- 2-propyl) phosphate (TDCPP)	$C_9H_{15}Cl_6O_4P$	16,179	232,201,372,7	2,20	97,6	427,88,274
31	[(4S,6S)– 6-Isobutyl- 3,4,5,6-tetrahydro- 1H-azepino[5,4,3-cd]indol- 4-yl](4-morpholinyl)methanone	$C_{20}H_{27}N_3O_2$	16,793	94,376,609,06	0,89	79,3	341,20,964
32	3,5-di-tert-Butyl- 4-hydroxybenzaldehyde	$C_{15}H_{22}O_2$	17,045	28,647,683,97	0,27	96,4	234,16,144
33	Olomoucine	$C_{15}H_{18}N_6O$	17,377	26,493,251,05	0,25	79,4	298,1537
34	(2E)– 3-(Acetoxymethyl)– 5-[(1S,4aR,8aR)– 2-(hydroxymethyl)– 5,5,8a-trimethyl- 1,4,4a,5,6,7,8,8a-octahydro- 1-naphthalenyl]– 2-pentenoic acid	$C_{22}H_{34}O_5$	17,413	23,228,482,56	0,22	78,8	378,24,357
35	Docosahex	$C_{22}H_{32}O_2$	17,959	38,241,031,72	0,36	98,2	340,2582
36	NP- 020014	$C_{15}H_{26}O_3$	18,001	36,568,579,56	0,35	70,6	276,17,192
37	Galaxolidone	$C_{18}H_{24}O_2$	18,3	96,773,897,6	0,92	93,8	272,17,699
38	Dibutyl phthalate	$C_{16}H_{22}O_4$	18,348	203,479,713,3	1,93	99,7	278,15,102
39	Hexadecanamide	$C_{16}H_{33}NO$	19,375	34,927,049,54	0,33	94	255,25,557
40	NP- 001596	$C_{16}H_{30}O_4$	19,939	49,487,022,08	0,47	98,7	308,19,539
41	cis- 7-Hexadecenoic acid	$C_{16}H_{30}O_2$	20,138	136,058,574	1,29	95,8	254,22,392
42	Citroflex A- 4	$C_{20}H_{34}O_8$	20,145	43,364,356,39	0,41	98,7	402,22,425
43	Oleamide	$C_{18}H_{35}NO$	20,238	31,051,728,3	0,29	90,1	281,2712

Table 2 (continued)

No	Name	Formula	RT [min]	Area (Max.)	% RA*	mzCloud Best Match	Calculated Molecular Wight (MW)
44	NP- 013685	$C_{22}H_{22}O_{11}$	20,761	70,508,294,96	0,67	81,2	484,10,405
45	9(Z),11(E)-Conjugated linoleic acid	$C_{18}H_{32}O_2$	21,14	49,301,032,18	0,47	93,8	280,23,973
46	Palmitic acid	$C_{16}H_{32}O_2$	21,234	53,196,083,33	0,50	95,4	256,2397
47	1-Stearoylglycerol	$C_{21}H_{42}O_4$	21,538	51,082,538,54	0,48	96,1	358,30,752
48	Stearamide	$C_{18}H_{37}NO$	21,653	18,597,256,81	0,18	95,2	283,28,673
50	Oleic acid	$C_{18}H_{34}O_2$	21,957	124,322,409,1	1,18	95	282,25,529
51	Erucamide	$C_{22}H_{43}NO$	24,198	790,010,390,3	7,49	78,7	337,33,318
52	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	25,295	2,013,296,822	19,08	99,8	390,27,522
53	Octyl decyl phthalate	$C_{26}H_{42}O_4$	26,32	282,574,520,1	2,68	98,1	440,28,856
55	Vitamin E Acetate	$C_{31}H_{52}O_3$	25,939	40,755,828,76	0,39	98,4	489,41,646

*Relative percentage abundance (% RA) was measured by the ratio RA of the provided peak area to RA of the **reference peak

hirta growth in that area might be more active than *C. hirta* growth in West Java if we have never compared and proven it scientifically.

Commonly, the MIC of antifungals needs a higher concentration (15- to >1,000-fold higher) to inhibit biofilm than to inhibit planktonic cells [19]. Starting with this concept, an antifungal test was applied to determine the effective concentration of ethanol extract from *C. hirta* leaves and fruits to inhibit the 50% planktonic cells of *C. albicans*. Apart from the concept, the results showed that both extracts have the potency to be developed as fungistatics, and further studies can be conducted to determine whether they have fungicidal properties against the planktonic cells of *C. albicans*. Although the antifungal method and the geolocation origin of the plant are different, the principles of the present study are basically the same as the previous study, revealing that ethanol extract of *C.hirta* leaves had antifungal activity with the highest inhibition zone diameter was 28.73 mm [20].

To the present, there have been no established standards and guidelines for antibiofilm examination methods such as antifungals, which are generated by The Clinical & Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST). However, this assay is routinely used to evaluate antibiofilm activity. The assay is based on the ability of metabolically active cells to convert yellow, water-soluble tetrazolium salt into an insoluble purple formazan product using cellular oxidoreductase enzymes, specifically mitochondrial dehydrogenases. Only viable *C. albicans* cells with active metabolism will be able to convert MTT into formazan (purple color) with an absorbance of 550 nm [21]. In contrast, dead *C. albicans* cells lose this capability. Hence, purple colour formation is a unique marker for viable *C. albicans* cells only. Therefore, the effect of *C. hirta* extracts on the viability of *C. albicans* encapsulated in the biofilm can be checked by the MTT assay. By this assay, the ethanol extract of *C. hirta* fruits showed the best activity against *C. albicans* biofilm ($p \leq 0.05$). Thus, SEM was applied to confirm the effect of the ethanol extract of *C. hirta* fruits on *C. albicans* biofilm morphology. The result showed that the extract disrupted the structure of *C. albicans* biofilm. Thus, SEM was applied to confirm the effect of the ethanol extract of *C. hirta* fruits on biofilm morphology. The result showed that the extract disrupted the structure of *C. albicans* biofilm.

The HRMS results showed several compounds, particularly dodecyl sulfate, bis(2-ethylhexyl) phthalate, and erucamide, that may influence the effectiveness of *C. hirta* fruits against *C. albicans* biofilm and planktonic cells. Dodecyl sulfate, a surface-active agent that contains hydrophobic and hydrophilic groups, is used in hygiene products such as soaps, shampoos, toothpaste, foods, and pharmaceutical products. According to the previous study, dodecyl sulfate inhibited 50% of *C. albicans* biofilm formation at a concentration of 0.31% to 2.5% [22]. Although the mechanism by which these agents fight fungal remains elusive, a study reported that potential mechanisms of dodecyl sulfate against *C. albicans* are inducing mitochondrial membrane depolarization, inhibiting the morphogenesis of *C. albicans*, inhibiting the transport of Hyphal wall protein- 1 (Hwp1) to the tip and the cell wall, and affecting the expression of hyphae-related genes [23].

Interestingly, in this study, bis(2-ethylhexyl) phthalate, the most common member of the class of phthalates, was detected in the studied extract. Some microorganisms have also produced this compound [24–27]. However, this is the first study that showed that *C. hirta* fruit

contains bis(2-ethylhexyl) phthalate. Studies showed that bis(2-ethylhexyl) phthalate has antifungal activity against *C. albicans* [25–28]. Erucamide, a primary fatty amide, is to be found in this study plant, *C. hirta* fruit. A study reported that this compound, exuded from duckweed root exudates, was the most efficient biological compound stimulating bacterial nitrogen removal through enhancing denitrification [29]. Related to *C. albicans*, nitrogen is a critical nutrient for its development. During infection, this species must obtain nitrogen from various distinct and shifting sources within the host [30]. Thus, erucamide in the extract may function by affecting the nitrogen source of *C. albicans* biofilm and planktonic cells.

The three compounds, dodecyl sulfate, bis(2-ethylhexyl) phthalate, and erucamide, might interact synergistically to produce beneficial effects: antifungal and antibiofilm activity in this study. The synergistic impact always allows for lower doses of the drugs in the combination relative to their toxic effect, thus reducing the induction of drug resistance and overcoming side effects [31]. However, it does not rule out the possibility that other compounds in the studied extract have activity against sessile and/or planktonic cells of *C. albicans*. Thus, to prove which compound affects the activity, this study must proceed to the next step, bioassay-guided fractionation. Therefore, it can be established which chemicals have the most antibiofilm potential. This is a long journey, but it can still be attempted to manage the invasive plant, *Clidemia hirta*, as a plant that can positively impact the nation's health and economy.

Conclusion

This is the first study screening the potency of *C. hirta* against *C. albicans* sessile and planktonic cells. The ethanol extract of *C. hirta* fruit was shown to have the best efficacy in both cells compared to *C. hirta* leaves. The future directions must be applied to evaluate (1) the cytotoxicity and (2) the broader spectrum activity against other clinical isolates and species. Other essential directions are fractionation and isolation of the plants (bioassay-guided isolation) to find the active single compound.

Limitation

This study did not screen the activity of *Clidemia hirta* on the biofilm mass of *Candida albicans*. Thus, in the future, the crystal violet (CV) staining method could be applied to determine it.

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Author contribution
SD—conceived and designed the experiments; found materials tools; performed the experiments; wrote the articles; analyzed data.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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