MICROBIOLOGY
INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol.13, No.3, September 2019, p 103-108

Available online at
http://jurnal.permi.or.id/index.php/mioline
DOI: 10.5454/mi.13.3.5

The Antifungal Activity of Artesunate toward *Candida albicans*: Two Opposite Activities

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The artemisinin and its derivatives antifungal activity continue to be an interesting research object, with the potential shown to be developed as an antifungal compound. Artesunate, one of the artemisinin derivatives known to have antifungal activity against various pathogenic fungi, including *Candida albicans*. This study aims to determine the effect of artesunate on antifungal activity toward *C. albicans in vitro* at concentrations below 1 mg mL^{-1}. The method used is yeast-plate count, with a parameter of observation were the number of *C. albicans* colonies viable after exposure with artesunate for five days. The concentration of artesunate used was divided into six groups, which were 10; 1; 10^{-1}; 10^{-2}; 10^{-3}; and 10^{-4}mg mL^{-1}. Compared to control, a significant decrease in colony counts was only shown at the highest concentration of 10 mg mL^{-1}. Interestingly, at the lowest concentration of 10^{-4}mgmL^{-1}, it showed an increase in a number of colonies almost twice of the blank. These results suggest that while at higher concentration of artesunate may inhibit the growth of *C. albicans*, a lower concentration of artesunate may stimulate their growth.

Key words: antifungal, artesunate, *Candida albicans*

Aktivitas antifungi artemisinin dan turunannya terus menjadi objek penelitian yang menarik, dengan potensi yang ditunjukkan untuk dikembangkan sebagai senyawa antifungi. Artesunat, salah satu turunan artemisinin diketahui memiliki aktivitas antifungi terhadap berbagai fungi patogen, termasuk *Candida albicans*. Penelitian ini bertujuan untuk mengetahui pengaruh artesunate terhadap aktivitas antifungi terhadap *C. albicans* secara *in vitro* pada konsentrasi dibawah 1 mg mL^{-1}. Metode yang digunakan adalah perhitungan cawan-ragi dengan parameter pengamatan berupa jumlah koloni *C. albicans* yang tampak setelah terpapar dengan artesunat selama lima hari. Konsentrasi artesunat yang digunakan dibagi menjadi enam kelompok, yaitu 10; 1; 10^{-1}; 10^{-2}; 10^{-3}; dan 10^{-4} mg mL^{-1}. Dibandingkan dengan kontrol, penurunan jumlah koloni yang signifikan hanya ditunjukkan pada konsentrasi tertinggi 10 mg mL^{-1}. Menariknya, pada konsentrasi terendah 10^{-4}mg mL^{-1} menunjukkan peningkatan jumlah koloni hampir dua kali lipat dari blanko. Hasil ini menunjukkan bahwa disaat artesunat dengan konsentrasi tinggi dapat menghambat pertumbuhan *C. albicans*, artesunat dengan konsentrasi yang lebih rendah justru dapat merangsang pertumbuhannya.

Kata kunci: antifungi, artesunat, *Candida albicans*

In addition to its antimalarial activity, artemisinin and its derivatives are known to have various other activities (Pan *et al*. 2018). Artemisinin is known to have activity against various types of parasitic infections such as Leishmania, Schistosoma, and Toxoplasma (Li and Zhou 2010). Artemisinin and its derivatives are also known to have several other activities such as antiviral (Efferth *et al*. 2008; Pratama and Gusdinar 2017), antimicrobial (Appalasamy *et al*. 2014), and even anticancer properties (Willoughby *et al*. 2009; Li *et al*. 2008). Artesunate, one of artemisinin derivative with potent antimalarial activity, is currently being developed for the treatment of another disease, including those caused by infection (Loo *et al.* 2017; Zuo *et al*. 2016). One of the artesunate properties currently being further investigated is as antifungal, where artesunate is known to have antifungal activity against various types of opportunistic pathogenic fungi such as *Candida albicans* and *Cryptococcus neoformans* (De Cremer *et al*. 2015; Galal *et al*. 2005). The exact mode of action of artesunate as antifungal itself remains elusive, but several studies have shown that artesunate and other artemisinin derivatives show antiproliferative activity on eukaryotic cells including fungi (Wang *et al*. 2017; O'Neill *et al*. 2010). One of the causes is that artemisinin derivatives including artesunate are known to inhibit various cyclin-dependent kinases (CDKs) enzymes induce growth arrest of cell cycle division that are the key factors in

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the proliferation of fungi cells (Zhang *et al.* 2018; Tran *et al*. 2014; Ho *et al*. 2014; Kundu *et al*. 2015). However, studies conducted generally carried out in doses above 1 mg mL^{-1}, while testing under these doses has never been done before. Thus, the antifungal activity of artesunate in this dose range is still unknown.

This study aims to determine the effect of different concentration of artesunate on the antifungal activity toward *C. albicans* as well as proving the potency of artesunate as the antifungal compound. Testing was performed with antifungal assay by counting the number of *C. albicans* viable colonies after administration of exposure to artesunate with multiple levels of dosage compared to controls. A decrease in the number of viable colonies showed that *C. albicans* cells divide at a lower rate and produce fewer colonies (Kwolek-Mirek and Zadrag-Tecza 2014). Before the test, Growth Promotion Test (GPT), and Sterility Test (ST) are conducted to determine the ability of the medium used to grow *C. albicans* and its asepticity, respectively (Sutton 2011). The results of this study will reveal how exactly the effect of artesunate exposure on the antifungal activity toward *C. albicans*.

MATERIALS AND METHODS

Drug and Test Solution. Artesunate was provided by Guilin Pharmaceutical Co. Ltd, China in the form of sodium artesunate reconstitution and dissolved in sterilized sodium bicarbonate. The test compound is further diluted with physiological saline (sodium chloride 0.9%) sterile solution to obtain 6 concentrations of 10 mg mL^{-1}; 1 mg mL^{-1}; 10^{-1} mg mL^{-1}; 10^{-2} mg mL^{-1}; 10^{-3} mg mL^{-1}; and 10^{-4} mg mL^{-1}, respectively. Each test solution was homogenized with vortex mixer then immediately used within half an hour.

Fungal Strains. The *C. albicans* strains ATCC 10231 used were obtained from Laboratory Microbiology, School of Pharmacy, Bandung Institute of Technology. The strain was grown in Sabouraud Dextrose Broth (SDB) with a pH range of 5.6 ± 0.2 then diluted with SDB until a number of colonies less than 100 CFU $mL^{-1}$are obtained. The inoculum suspension of *C. albicans* then stored in the cold room with temperature -20 °C and can be stored up to 1 week.

Growth Promotion Test and Sterility Test. The GPT was conducted to determine the ability of the medium used in the observation process to grow the test colony. The test medium used was Sabouraud Dextrose Agar (SDA), which has been known to grow colonies of *C. albicans*. As much as 1 mL of *C. albicans* inoculum suspension was added into SDA until a mixture of 15 mL was obtained. The mixture was then homogenized with a vortex mixer for a minute, then poured into a sterile petri dish and made in triplicate. The dishes then incubated at 25°C for five days. Observations were made on the fifth day by counting the number of colonies growing on each dish. All dishes should be able to grow between 30 and 300 colonies.

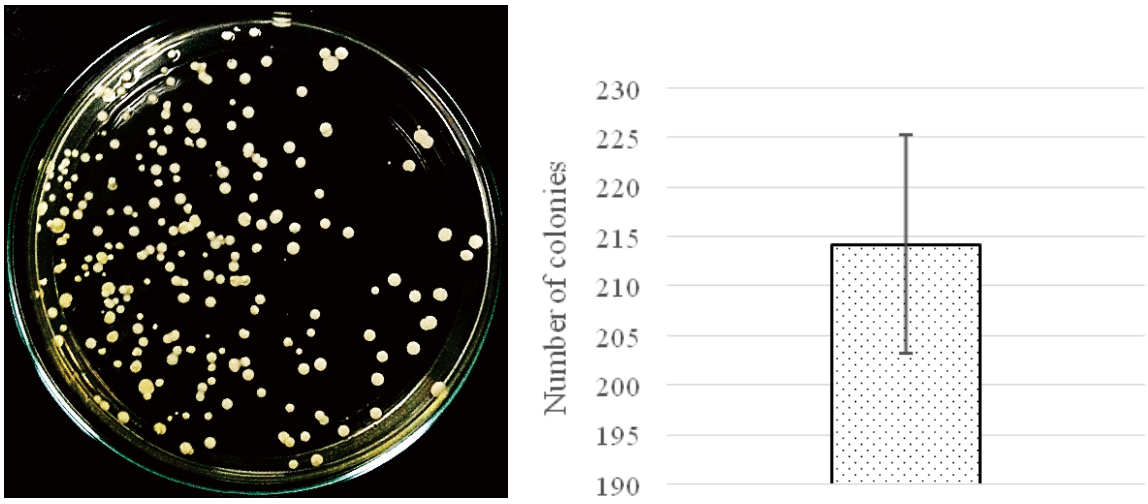
The ST was performed to ensure that the sterilization process is done successfully so that no contaminants grow in the petri dish due to non-aseptic process. As much as 1 mL of physiological saline sterile solution was added into SDA until a mixture of 15 mL was obtained. The mixture was then homogenized with a vortex mixer for a minute, then poured into a sterile petri dish and made in triplicate. The dishes then incubated at 25 °C for five days. Observations were made on the fifth day by counting the number of colonies growing on each dish. All dishes should not show the growth of the colony.

Antifungal Assay. As much as 1 mL of C. albicans inoculum suspension was added to 1 mL of test solution for each concentration. The mixture was then added with SDA until a final mixture of 15 mL was obtained. The mixture was then homogenized with a vortex mixer for a minute, then poured into sterile Petri dishes. Each series of test solution concentration was made in triplicate. The physiological saline sterile solution was used as a blank. The entire process was carried out in the Laminar Air Flow in aseptic conditions.

All Petri dishes were incubated at 25 °C for five days. Observations were made on the fifth day by counting the number of colonies growing on each dish then calculated the mean value of each test solution concentrations.

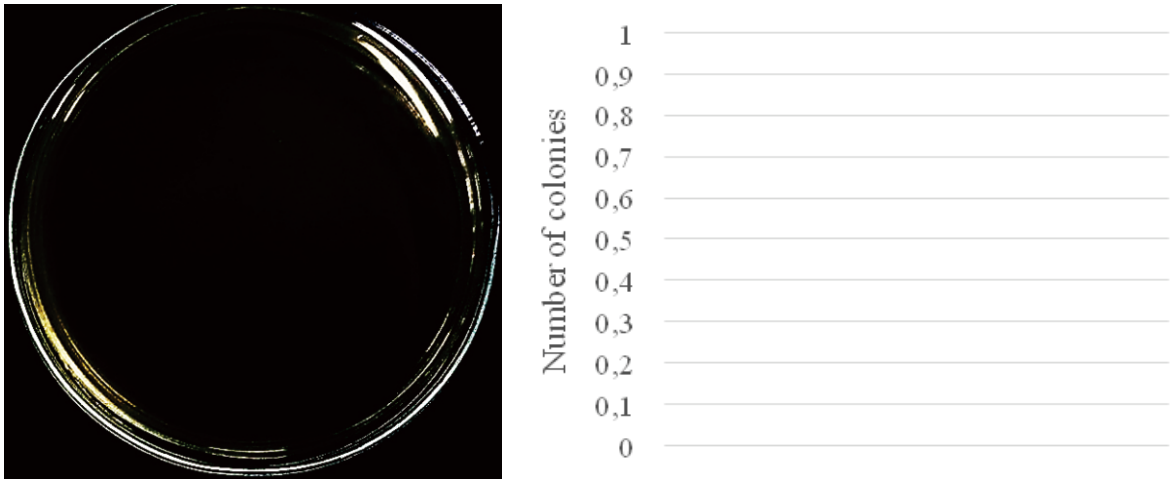
RESULTS

Growth Promotion and Sterility. The number of colonies that grow on each petri dish was calculated entirely using a colony counter. All visible colonies were counted regardless of the size of each colony. All dishes on GPT show considerable colony growth with a colony range between 203 to 226 colonies. In contrast to GTP results, all dishes on ST show no colony growth of all Petri dishes (Fig 1, 2). The GPT and ST itself is a mandatory requirement before conducting yeast-plate



(**A**) (**B**)

Fig 1 The result of growth promotion test. (**A**) The growth of *C. albicans* colonies in one of the petri dish; (**B**) Number of *C. albicans* colonies from all petri dishes with the average of 214.3 colonies.



(**A**) (**B**)

Fig 2 The result of sterility test. (**A**) No growth of colonies in one of the petri dish; (**B**) Number of *C. albicans* colonies from all Petri dishes.

count testing, where the test medium must be able to grow the test microbes and on the other hand the working process must be guaranteed aseptic to prevent any contaminants from outside.

Antifungal Assay. All petri dishes except on blanks covered by *C. albicans* colonies. The calculations of the number of colonies were performed on all parts of the petri dish. The colony growth in the antifungal assay is shown in figure 3 below.

DISCUSSION

Surprisingly, the decrease in the number of *C. albicans* colonies was significantly demonstrated only by the test solutions with the highest artesunate concentrations of 10 mg mL^{-1}. Besides the number of colonies in the concentration is still not reached half of the number of colonies on the blank, indicating that the IC_{50} of artesunate against *C. albicans* is in the range greater than 10 mg mL^{-1} or equivalent to 26 µM, which is very large value. The results clearly show that the potency of artesunate as an antifungal is relatively weak, especially compared to other derivative compounds of natural products such as those derived from *Origanum vulgare*, *Eucalyptus* and *Thymus sp* like carvacrol, thymol, and α-terpineol (Nazzaro *et al*. 2017; Gucwa *et al*. 2018).

More interesting results are at lower concentrations

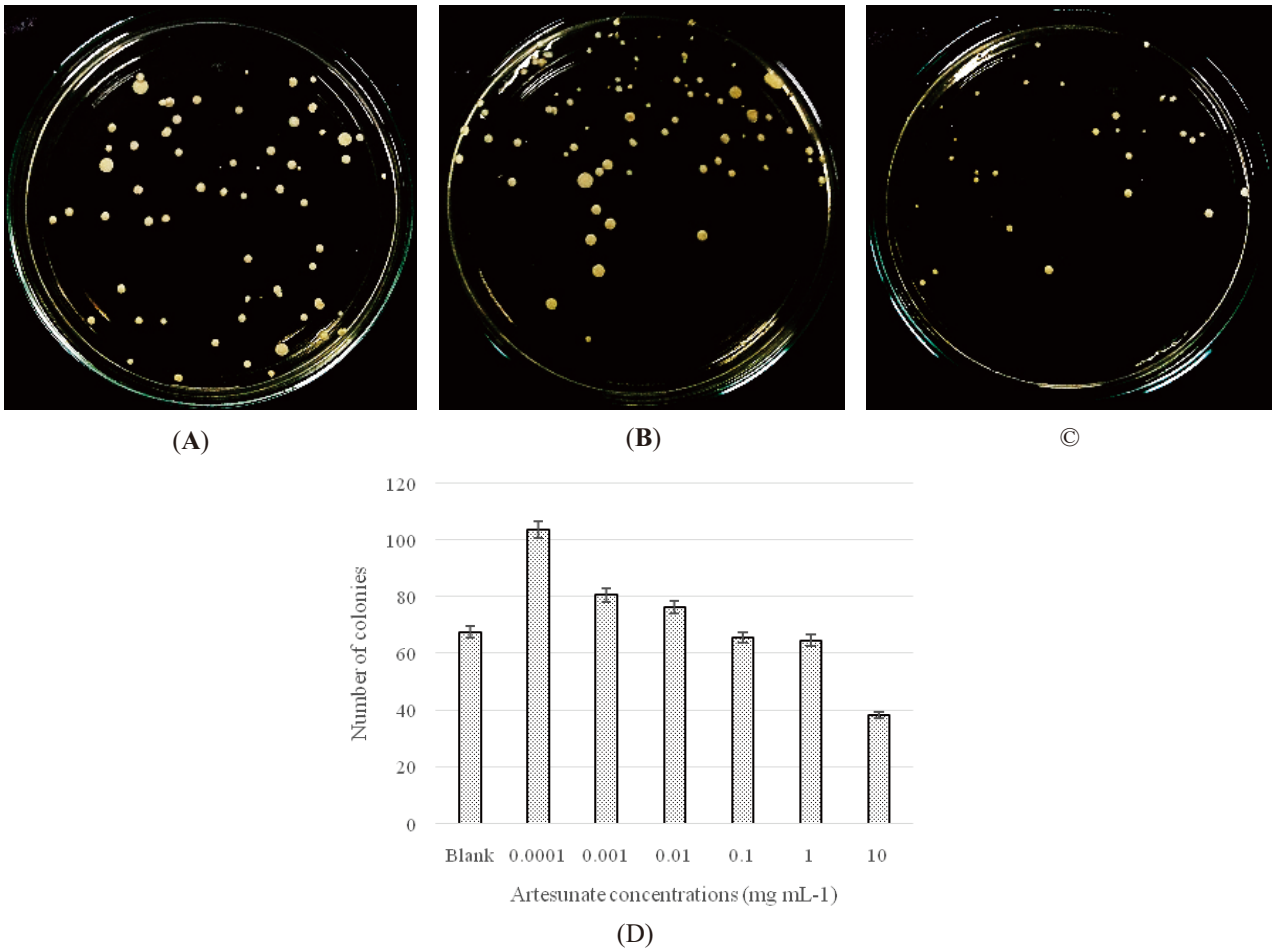


Fig 3 The result of the antifungal assay. Growth of colonies on one of the petri dish from (**A**) negative control (blank); (**B**) artesunate 10^{-4}mg mL^{-1}; (**C**) 10 mg mL^{-1}; (**D**) Number of *C. albicans* colonies from all petri dishes from each group of test solutions, with the average number for blank; 10^{-4}mg mL^{-1}; 10^{-3}mg mL^{-1}; 10^{-2}mg mL^{-1}; 10^{-1}mg mL^{-1}; 1mg mL^{-1}; and 10 mg mL^{-1} were 67.7; 103.7; 80.7; 76.3; 65.7; 64.7; and 38.3 colonies, respectively. The dashed line indicates the average colonies number of the blank.

that are below 10^{-1}mg mL^{-1}, artesunate actually triggered the growth of the number of *C. albicans* colonies. At the lowest concentration of 10^{-4}mg mL^{-1} even the number of colonies that grow almost 50% more than the blank (103.7 to 67.7 colonies). The cause of the increasing number of *C. albicans* colonies is still unknown, but it is probably related to transcription regulator Pdr1p and its target genes PDR5 and TPO1 (Alenquer *et al*. 2006; Fardeau *et al*. 2007). Another possibility is that artesunate interacts with apoptosis protein regulators in eukaryotic cells as shown in plantaricin E and F (Nurhayati *et al*. 2015).

Another interesting feature is that the increase in the number of colonies appears to occur linearly to the concentration of artesunate, where smaller artesunate concentrations lead to more *C. albicans* colonies growth. Unfortunately, the following study is only done at the lowest artesunate concentration of 10^{-4}mg mL^{-1}. It is interesting to observe how the change in the number of *C. albicans* colonies at artesunate concentrations smaller than 10^{-4}mg mL^{-1}, whether the number of colonies is still increasing, as well as the lowest artesunate concentration which still gives an increase in the number of *C. albicans*. Based on the author's search to date, no studies have reported this. In summary, we show for the first time an actual antifungal activity of artesunate against colonies of *C. albicans*, which shows that the antifungal activity of artesunate will only appear at high doses. On the contrary at low doses, artesunate actually increases the number of *C. albicans* colonies. The results of this study open new possibilities that the use of artesunate at low doses can actually increase the potential for candidiasis due to overgrowth of *C. albicans*.

ACKNOWLEDGEMENT

This work was supported by the internal grant from Institut Teknologi Bandung. In addition, financial support was also provided by the Institute for Research

and Community Services Universitas Muhammadiyah Palangkaraya. We thank Marlia Singgih Wibowo (Institut Teknologi Bandung) for the help with results interpretation.

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