Potency of Massoia Bark in Combating Immunosuppressed-related Infection

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ABSTRACT

Background: As part of our search for new potential natural resources to eradicate infection, we have revealed the prominent potency of massoia bark (Massoia aromatica Becc, Lauraceae) in combating immunosuppressed-related infection. Materials and Methods: The extract was prepared by macerating the pulverized dried bark in ethanol 95%, followed by solvent evaporation. The oil was extracted from the dried bark by steam-hydrodistillation of which preparative thin-layer chromatography was performed on the oil to isolate the active constituent, C-10 massoia lactone (ML). Anti-biofilm assay against Candida albicans was conducted on polystyrene 96 wells microtiter plates, followed by a confocal laser scanning microscope observation to get three-dimensional profiles of the affected biofilms. Effects on the hyphae development inoculated on RPMI-1640 agar plates were observed for 7 days. Influences of samples on mice macrophage phagocytosis were examined by an in vitro technique. Samples concentration tested were in the range of 2.0-0.0625 mg/mL and done in triplicate. Results: Massoia bark extracts (oil and solid phase) and ML exhibited promising activities as anti-biofilm against C. albicans at IC50 0.074% v/v, 271 µg/mL and 0.026 µg/mL, respectively. The ML did not inhibit the hyphae development at the concentration tested; however, the extracts showed inhibition at 62.5 µg/mL. Macrophage phagocytosis stimulation was correlated to the ML content. Conclusion: Massoia bark is potential to be developed as an anti-infective in immunosuppressed condition of which the C10 ML (C10H16O2) plays a major role in exerting activity. Key words: Biofilm, Candida albicans, macrophage, massoia bark, massoia lactone

SUMMARY

• Massoia bark extracts (oily and solid phase) and C-10 Massoia lactone exhibited promising activities as antibiofilm against Candida albicans at IC50 are 0.074% v/v, 271 µg/mL and 0.026 µg/mL respectively. The major constituent, C-10 Massoia lactone (C10H16O2) plays major role in exerting anticaandida activity and potentially acts as an immunomodulator as well. However extracts showed inhibition of hyphae development of C. albicans which showed no correlation to the content of the Massoia lactone.

INTRODUCTION

Massoia aromatica Becc. (synonym Cryptocarya massoy Oken, Lauraceae) has been used commercially to produce essential oils. Its traditional usage by Javanese and Balinese women is to prepare a warming ointment having a pleasant smell, called bobory. Detailed chemical evaluation using gas chromatography/mass spectrometry (GC/MS) revealed the major components in the bark oil as C-10 massoia lactone (ML) or 5,6-dihydro-6-pentyl-2H-pyran-2-one, (65-68%) and the C-12 ML, or 5,6-dihydro-6-heptyl-2H-pyran-2-one, (17-28%). The MLs are rare essential oil components, but its production by chemical synthesis route has been reported. C. albicans is a normal flora in single cell phase which can convert to an opportunistic pathogen as multicellular fungi, mostly in an immunocompromised condition. The capacity of C. albicans to cause disease are closely associated with its ability to grow as biofilm communities by adhering both to cellular and inanimate surfaces of implanted devices such as catheters, prosthetic cardiac valves, contact lenses, and the new creations are licensed under the identical terms. This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms. For reprints contact: reprints@medknow.com

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lenses, artificial joints, and intrauterine devices. Microorganisms involved in biofilms are generally more resistant to antimicrobials and also against the host defense mechanism in comparison to the single cell planktonic state.[7] This fact makes them a progressive source of recurrent infections.[8] Although the drug resistance mechanisms to candida biofilms are still poorly understood, the action of antifungal may be limited by their poor penetration into the biofilm matrix.[9] Microbes in biofilm state normally have slower growth causing the antimicrobials targeting the microbial growth lose the efficacy. Moreover, biofilm heterogeneity is thought to be a contributing factor for the drug resistance phenomenon.[10] Biofilm communities develop differential gradients in nutrients and metabolic waste, so that groups of cells in the same biofilm react variably in response to environmental cues, leading to a variety of phenotypes.[11] Our previous study has suggested the prominent potency of the massoia oil in preventing and breakdown of C. albicans, Pseudomonas aeruginosa, and Staphylococcus aureus biofilms. In this research, we studied the potency of the massoia bark ethanolic extract as well as the oil major constituent, ML, toward the C. albicans biofilm and hypha development as well as toward mice macrophages.[12] No effect of ML on immune response has been reported, yet. However, other lactone substances, such as homoserine,[13] macrolides, lactone, and[14] sesquiterpene lactone, do exhibit immunomodulatory properties.[15,16] Ethanolic oil of other close related plant of Lauraceae family, Cinnamomum zeylanicum, has been reported to have immunomodulatory effect.

MATERIALS AND METHODS

Cell, chemical, and biochemical

Silica gel 60 F254 aluminum precoated plate, toluene, ethyl acetate, which are analytical grade (Merck, Germany) were used in thin-layer chromatography (TLC) densitometry. GC-2010 gas chromatography (Shimadzu, Japan) equipped with a GC-MS-QP2010 plus mass spectrometer (Shimadzu, Japan). An Agilent J & W DB-1 column (30 m length, 0.25 mm diameter, 0.25 mm film thickness, Agilent) was used for the component analysis. C-10 ML content in extract and oil were quantified by Camag TLC scanner slit dimensions 8.00 mm × 0.40 mm and a scanning speed of 80 mm/s.

C. albicans ATCC 10231, RPMI-1640 medium, L-glutamine (Sigma), sterile phosphate-buffered saline (PBS), crystal violet, fluconazole were used for biological assays. Macrophages were obtained from 3-month-old mice, taken from the peritoneal fluid with RPMI.

A Carl Zeiss LSM5 Exciter Laser Scanning Confocal Microscope (Leica Microsystems, Germany) was used to observe the architecture of the C. albicans biofilms, analog with the standardized cell suspension Candida biofilms (3 mL of a suspension containing 1.0 × 10⁹ cells/mL in LB medium, live/dead fluorescent staining (10 μL of 3.34 μM SYTO9 and 10 μL of 20 μM propidium iodide (PI) both in dimethyl sulfoxide (DMSO)) (Molecular Probes, USA). The image was subsequently analyzed using the freely available image processing software ImageJ Version 1.46 (Rasband, National Institutes of Health [NIH], Bethesda, Maryland, USA: http://rsb.info.nih.gov/ij/) including the LSM reader plug into open LSM5 formatted image stack created by the microscope software.

Plant material

Three kilograms of M. aromatica bark were obtained from a traditional market in Yogyakarta, Indonesia, in February 2010. A voucher specimen was deposited at Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Indonesia, and identified by Mr. Djoko Santosa, M. Sc., (plant taxonomist) under Nr. BF/14/Ident/Det/I/2015.

Sample preparation

The essential oil was obtained by steam-hydrodistillation process. Oil sample was stored in a sealed dark glass vial and kept at 4°C. Stock solutions of 50% (v/v) essential oils were prepared in methanol (MeOH). Ethanolic extract was prepared by macerating the pulverized bark in ethanol 95% for 24 h, repeated once. The filtrate was combined and evaporated to yield thick constituent.

Planar chromatographic separation and analysis

Chromatography was performed on 10 cm × 20 cm silica gel 60 F254 aluminium sheet TLC plates (Merck, Germany).[16,17] Before use, the plates were preconditioned by heating at 120°C for 3 h. The position of the starting line was 1.0 cm from the bottom and 1.0 cm from the left side. After sample application, the TLC plates were developed with the previously optimized mobile phase. For the separation of essential oils, toluene–ethyl acetate, 93:7 v/v, was used as a mobile phase. The development mode was ascendant in a saturated twin trough chamber (Camag, Switzerland). All TLC separations were performed at room temperature.

After chromatographic separation, the absorbent layers were dried in an oven at 90°C for 5 min to remove the solvent completely. An alcoholic vanillin–sulfuric acid reagent was used to visualize the separated spots. The developed layers were dipped into this reagent and heated for 5 min at 100°C. Detection of the separated compounds was performed according to retardation factor (Rf) value, i.e. the ratio of the distance traveled by the center of a spot to the distance traveled by the solvent front; and color of the standards.

A preparative TLC separation of M. aromatica oil was performed on a 10 cm × 20 cm silica gel 60 F254 aluminum sheet TLC plates (Merck, Germany) as described above by spotting the essential oil over the whole width of the starting line (streaking the plate). UV<sub>254</sub> instead of stain reagent was used for the visualization. The area (band) which has similar Rf value with the inhibition zone was marked and carefully rubbed out of using a spatula. The loosened silica then was transferred onto a glass funnel of which glass wool is present, and ethyl acetate was used as a solvent. The eluted fraction that has been collected and dissolved in ethanol for further analyses using TLC, of

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**Figure 1:** Thin-layer chromatography profile of purified massoia oil and extract by using silica gel F254 aluminum precoated plate; toluene: EtOAc = 93:7 v/v; observation under UV<sub>254</sub> (left) and under visible light after sprayed with annisaldehyde H<sub>2</sub>S<sub>4</sub>O<sub>6</sub> (right). (a) C-10 massoia lactone purified massoia oil, (b) C-10 Massoia lactone on extract (solid phase), (c) C-10 massoia lactone on extract (oil phase)
which observation was conducted under UV24. GC-MS analysis also
performed to obtain information about the chemical composition of
the eluted fraction.

Effect of massoia lactone and massoia extracts on
Candida albicans hyphal growth

Standard cell suspension of C. albicans was grown on RPMI-1640 agar
plates supplemented with different concentrations of sample tested. The
plates were incubated for 7 days at 37°C and the morphology of fungal
colony was photographed using a digital camera.

Effect of massoia lactone and massoia extracts on
Candida albicans planktonic growth

The antifungal activity of M. aromatica's essential oil and extracts
against C. albicans planktonic cells was evaluated using microdilution
method with Sabouraud Dextrose Broth (SDB) as the medium.18
The test compounds were prepared with different concentrations using
methanol. The inoculum of 5 × 105 CFU/mL in SDB was used for the
assay and the final volume in each well was 200 µL including SDB,
cells, and test compound. As positive control, amphotericin concentration
of 125 µg/mL in DMSO was used. Inhibition growth activity was monitored and calculated based on the optical density at
595 nm of the sample tested versus control after 48 h of incubation at
37°C.

Effect of massoia lactone and massoia extract on
Candidas biofilm inhibition

Biofilms were formed on polystyrene flat bottom 96-well microtiter plates
(Sarstedt, Inc., Newton, NC, USA).19,20 Briefly, 100 µL of a standardized
cell suspension (105 cells/mL) in the RPMI-1640 medium (without
sodium bicarbonate) supplemented with L-glutamine (Sigma) was
transferred into each well of a microtiter plate, and the plate was
incubated for 90 min at 37°C of adhesion phase. The cell suspensions
were then aspirated, and each well was washed twice with 150 µL of
PBS to remove loosely adhered cells. A total of 100 µL RPMI media
containing concentration of C-10 ML ranging from 100 µg/mL to
0.30 µg/mL, massoia solid extract in RPMI-1640 medium with concentrations ranged from
0.30 µg/mL to 0.03% v/v to 0.03% v/v; were then added to the washed wells and the plates were incubated at 37°C for another 24 and
48 h. The effects on established biofilms were estimated using crystal
violet stained and read by microtiter plates (Bio-Rad 680XR) at 595 nm.
Testing was performed in triplicate. Amphotericin B concentration
125 µg/mL was used as a positive control in this study.20 Compound found to reduce at least 50% biofilm formation were considered as biofilm preventive agents.

Anti-biofilm activity observed by confocal laser
scanning microscope

Preformed Candida biofilms were prepared by dispensing the
standardized cell suspension (3 ml of a suspension containing
1.0 × 105 cells/mL in LB medium) onto 25 mm diameter presterilized
plastic coverslips (Thermanox; Nulge Nunc International) placed in the
wells of presterilized flat bottomed six-well plates (Iwaki).20,21 The
plate was incubated at 37°C for 90 min (adhesion phase). Following the
incubation period, the supernatant was removed, and a total of 3 mL
RPMI medium containing different concentrations of tested samples
were added to the washed wells. The plate was then incubated for
24 h (intermediate phase) and 48 h (maturation phase) at 37°C. To
obtain information about the efficacy of oil on degrading the
established Candida biofilm, biofilms were performed for 24 and 48 h
at 37°C on 25 mm diameter presterilized plastic coverslips (Thermanox;
Nulge Nunc International) placed in the bottom of six wells microtiter
plates. Following incubation period, the supernatant was removed, and a total of 3 mL RPMI medium containing different concentrations of
plant oils were added to the washed wells. The plate was then incubated
for another 24 and 48 h at 37°C. Afterward, the coverslips were washed
twice with PBS and stained using the Bac light bacterial viability kit of
Molecular Probes (10 µL of 3.34 µM SYTO9 and 10 µL of 20 µM PI both
in DMSO) (Molecular Probes, USA) before examined under the Carl
Zeiss LSM5 to observe the architecture of C. albicans biofilms. A 40x oil
immersion objective was used with 488 nm Argon laser excitation and
500–640 nm band pass emission setting. The images were subsequently
analyzed using the freely available image processing software ImageJ
Version 1.46 (Rasband, NIH, Bethesda, Maryland, USA: http://rsb.info.nih.gov/ij/). The images’ scale bar used to calibrate the ImageJ area
measurement algorithm. The observations were made in triplicates, and representative images are presented here.

**Effect of massoia lactone, massoia extracts, and massoia oil on phagocytosis activity of macrophages**

Measurement of phagocytosis activity conducted by using 3 μm latex beads suspended in PBS to obtain a concentration of 2.5 × 10⁶ mL⁻¹. Culture of peritoneal macrophages was 24 h in wells equipped with coverslips, washed twice with RPMI-1640, and then added to the samples with various concentrations ranging from 1000 μg/mL to 30 μg/mL (200 μL/well). Incubation was performed in 5% CO₂ incubator at 37°C for 4 h. The cells were washed 3 times with PBS, and then added to a suspension of latex beads (200 μL/well). Incubation was performed in 5% CO₂ incubator at 37°C for 1 h. The cells were washed 3 times with PBS to remove the latex beads. Fixation with methanol took 30 s, the cover slip was allowed to dry, and 2% v/v Giemsa dye was left for 20 min. After being washed with distilled water and left to dry, the number of macrophages which engulfed the latex and total amount of enguled latex beads were counted under a light microscope to count the macrophage phagocytosis index. The phagocytic index was calculated according to the following formula:

\[
PI = \frac{\text{Total number of engulfed cells}}{\text{Number of macrophages engulfed cells}} \times \frac{\text{Number of macrophages engulfed cells}}{\text{Total number of counted macrophages}} \times 100
\]

The animal handling has been approved by the Ethical Clearance Commission for preclinical studies of the LPPT-UGM (The Integrated Research and Testing Laboratory, Universitas Gadjah Mada) under Nr. KEC-LPPT/II/2015.

**Data analyses**

One-way ANOVA was used at 95% confidence level for statistical data analyses.

**RESULTS AND DISCUSSION**

Yield of massoia oil was 0.4% v/w while the extract was 11.33% w/w (in total). The extract was separated into two phases, oil and solid. Phytochemical content comparison by TLC as shown in Figure 1 that describes the present of ML C-10 in the extract in much lower amount than in the oil. The oily part of the extract showed similar profile with the solid, but with different spots intensities. Content of C-10 ML in oil and extract was quantitatively determined by the TLC-densitometry at 211 nm [Figure 2]. Table 1 described the content of C-10 ML in massoia oils and extracts resulted from the TLC-densitometry measurement. Massoia oil has the highest content of C-10 ML than oil extracts and solid extracts, i.e., 46.00%; 33.68%; 10.25%, respectively. However, according to the GC-MS profiles of the massoia oil extract (oily), and solid phase extract, C-10 ML was detected at the percentage of 48.50%, 67.62%, and 59.54%, respectively. The results described the relative percentage of C-10 ML to other volatile substances in the samples detected by GC.

**Effect of massoia lactone and massoia extracts on Candida albicans hyphal growth**

The effect of different concentration of tested compounds on C. albicans hyphal growth on solid media was observed by cultivating fungal cells and observing colony morphology on Spider agar and RPMI agar at 37°C. It was found that until the lowest concentration tested (62.5 μg/mL for massoia solid extract and 62.5 μg/mL for massoia oily extract) growth of C. albicans hypha was inhibited by the extracts. On the contrary, up to highest concentration tested of C-10 ML (100 μg/mL), the compound was found failed to abrogate C. albicans filamentation. This finding can be interpreted that other constituents other than the ML were responsible for the hyphae development inhibition.

**Effect of massoia lactone and massoia extract on Candida albicans planktonic growth**

*Candida* planktonic growth was inhibited depending on the concentration of the sample [Figure 3]. C-10 ML inhibition on the planktonic growth was showed as IC₅₀ of 0.167 μg/mL. While the calculated IC₅₀ of massoia solid and oily extracts to inhibit *Candida* planktonic growth were determined at 6.40 μg/mL and 0.017% v/v, respectively.

**Effect of massoia lactone and massoia extract on Candida albicans biofilm inhibition**

It is interesting to note that despite the lower concentration of C-10 ML in the solid extract, the biofilm inhibition activity was comparable to the oily suggesting that there are other active constituents present in the solid extract contributing to the activity [Figure 4]. Concentration-dependent inhibitions were observed. A comparison effect between the C-10 ML and the oily extracts exhibited no significant different, except for the lowest concentration tested.

**Table 1: Content of C-10 massoia lactone in massoia oils and massoia extracts**

| Sampel          | Percentage b/v ML on sampel | Mean   | SD    | CV   |
|-----------------|-----------------------------|--------|-------|------|
| Massoia oil     | 45.42                       | 42.75  | 46.00 | 46.78 |
| Oily extract    | 34.54                       | 33.47  | 33.02 | 33.65 |
| Solid extract   | 10.50                       | 10.30  | 9.95  | 10.25 |

ML: Massoia lactone; SD: Standard deviation; CV: Coefficient of variation
C. _albicans_ biofilm formation is preceded in two distinct developmental phases: Intermediate (12–30 h) and mature (38–72 h).\textsuperscript{10} Massoia liquid (oily) extract at concentration of 1–0.03% v/v, C-10 ML at concentration of 100–0.31 µg/mL and massoia solid extract at concentration of 1,000–30 µg/mL were tested against _C. albicans_ adherent cells populations at different stages of biofilm development. The result of this experiment, as shown in Figure 4, demonstrated that all inhibition of _C. albicans_ intermediate and mature biofilms occurred in the presence of compound tested. Until the concentration of 12.5 µg/mL, C-10 ML exhibited up to 50% inhibition of _C. albicans_ biofilm intermediate phase formation, but higher concentration needed to exhibit up to 50% inhibition of the mature phase. The calculated IC\textsubscript{50} of C-10 ML application to inhibit formation _Candida_ biofilm was 1.63 µg/mL and 8.92 µg/mL toward intermediate and mature biofilm, respectively. Similar patterns were also showed by massoia oily and solid extracts, which partially inhibited _C. albicans_ intermediate biofilm (IC\textsubscript{50} = 0.006% v/v and 3.43 µg/mL, respectively), but needed a higher concentration to disturb the mature phase biofilm.

### Effect of massoia lactone and massoia extracts on fungal established biofilms

The phenomena in biofilm inhibition were also shown in the established biofilm but in less effect [Figure 5]. The extracts showed slightly less inhibitory effects in comparison to the oil. The calculated IC\textsubscript{50} values resulted from the C-10 ML application to degrade _Candida_ biofilm were 0.026 µg/mL and 0.43 µg/mL toward intermediate and mature biofilm, respectively. Similar patterns were also showed by the massoia oily and solid extract, which partially degraded the _C. albicans_ intermediate biofilm at concentration of 0.06% v/v and 250 µg/mL (calculated IC\textsubscript{50} = 0.074% v/v and 271 µg/mL, respectively), but a higher concentration (0.12% v/v and 500 µg/mL) was required to disrupt the mature phase biofilm (calculated IC\textsubscript{50} = 0.16% v/v and 427 µg/mL).

### Anti-biofilm activity observed by confocal laser scanning microscope

The anti-biofilm activity in the microtiter plate assay was confirmed by confocal laser scanning microscope (CLSM) analysis of _C. albicans_ biofilm. The biofilms were grown on the surface of coverslips and stained with SYTO9 and PI for live/dead cells monitoring. We observed a preventive activity on biofilm formation when we inoculated _C. albicans_ with different concentration of samples. Growth suppression of _C. albicans_ biofilm by essential oils was clearly seen by CLSM with the live/dead stain, compared with dense biofilm growth of the control.

The architecture of _C. albicans_ biofilm in the presence of tested oils in biofilm formation inhibition was shown in Figure 6. The estimated three-dimensional view of the biofilm refers to the total area in the x-y-z dimension, where x and y are the coordinates of the pixel positioning and z is the intensity collected using imageJ. Live cells are labeled in green (SYTO9), and dead cells are labeled in red (PI).

PI is a nonpermeant fluorescent nucleic acid stain that enters nonviable cells by binding to double-stranded nucleic acids through intercalation between base pairs with no preference to purine or pyrimidine base pairs.\textsuperscript{22} Figure 7 shows intense green fluorescence resulting from membrane permeant SYTO9 binding to DNA of _C. albicans_ intact cells while PI stains the cells red. Thus, areas of red fluorescence represent dead cells, and green fluorescent indicates healthy cells. The yellow color (formed as a result of overlapping of red and green images which were the representative of SYTO9 and PI colocalization due to the presence of dead cells or eDNA.

The result of this experiment, as assessed by colorimetric assay and microscopy, demonstrated that negative control (biofilm formed in the absence of samples) exhibited typical biofilm architecture. At intermediate phase of biofilm development, _C. albicans_ biofilm was found to be composed of yeast cells, elongated pseudohyphae and hyphae. At mature phase, _C. albicans_ biofilms composed mainly of intertwining mycelia structures, and a basal layer of blastospores [Figure 6].

Pretreatment of _C. albicans_ biofilm with various C-10 ML concentrations showed that there was a dose-dependent effect on biofilm formation. CLSM study shows that the number of viable cells (green) decreased when the concentration of extract exposure

**Figure 4:** (a) Biofilm formation inhibition after sample application (24 h). (b) Biofilm formation inhibition after sample application (48 h)

**Figure 5:** Inhibition of preformed _Candida albicans_ biofilm by C-10 massoia lactone and massoia extracts against: (a) intermediate (24 h) biofilm (b) mature (48 h) biofilm
Table 2: Phagocytic index of macrophage treated with massoia oil, C-10 massoia lactone and massoia extract

| Concentration | Massoia oil | C-10 massoia lactone | Extract (solid phase) | Extract (oily phase) |
|---------------|-------------|----------------------|-----------------------|----------------------|
| Control (0%)  | 0.99        | 0.99                 | 0.99                  | 0.99                 |
| 62.5 µg/mL    | 454.66      | 111.34               | 36.34                 | 357.01               |
| 125 µg/mL     | 594.37      | 881.01               | 42.34                 | 102.64               |
| 250 µg/mL     | 123.99      | 371.69               | 41.33                 | 56.65                |
| 500 µg/mL     | 35.02       | 31.00                | 66.65                 | 41.98                |
| 1,000 µg/mL   | 18.00       | 50.65                | 102.65                | 24.98                |

Effect of massoia lactone, massoia extracts, and massoia oil on phagocytosis activity of mice macrophages

Effect of C-10 ML, massoia extracts, and massoia oil on the immune system was observed through the influence on the phagocytosis activity of mice macrophages. Phagocytosis activity was evaluated by counting phagocytic index. As much as 300 macrophages were observed for each treatment, revealed that the highest phagocytic index were achieved at concentration 125 µg/ml for massoia oil and C-10 ML, 1000 µg/ml for oil extract and 62.5 µg/ml for solid extract. However, the greatest value of the phagocytic index was indicated in the presence of C-10 ML suggesting the main contribution of the compound to the activity [Table 2]. As described previously, massoia oil has the fewest content of C-10 ML which may explain the low value of phagocytic index. Nevertheless, increase massoia extract concentration could not increase the phagocytic index, which may happen due to the poor solubility in the media. This was also the case in the application of C-10 ML and massoia oil at concentrations of 500 and 1,000 µg/ml.

CONCLUSION

Massoa bark extracts (oily and solid phase) and C-10 ML exhibited promising activities as anti-biofilm against C. albicans at IC_{50} are
0.074% v/v, 271 µg/mL and 0.026 µg/mL, respectively. The major constituent, C-10 ML (C_{10}H_{16}O_{2}) plays major role in exerting anti-candida activity and potentially acts as an immunomodulator as well. Phagocytosis stimulation effect on mice macrophage was shown in a sequence from the highest to the lowest phagocytic index as follows: C-10 ML, massoia oil, massoia extract (solid phase), and massoia extract (oily phase). The C-10 ML did not inhibit the hyphal development at the concentration tested; however, the extracts showed inhibition at 62.5 µg/mL (oily extract) and 62.5 µg/mL (solid extract).

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Conflicts of interest
There are no conflicts of interest.

REFERENCES
1. Rali T, Wossa SW, Leach DN. Comparative chemical analysis of the essential oil constituents in the bark, heartwood and fruits of Cryptocarya massoy (Oken) Kosterm. (Lauraceae) from Papua New Guinea. Molecules 2007;12:149-54.
2. Barros ME, Freitas JC, Oliveira JM, da Cruz CH, da Silva PB, de Araújo LC, et al. Synthesis and evaluation of (-)-Massoialactone and analogues as potential anticancer and anti-inflammatory agents. Eur J Med Chem 2014;76:291-300.
3. Shapiro RS, Robbins N, Cowen LE. Regulatory circuitry governing fungal development, drug resistance, and disease. Microbiol Mol Biol Rev 2011;75:213-67.
4. Bandara A, Fraser S, Chambers PJ, Stanley GA. Trehalose promotes the survival of Saccharomyces cerevisiae during lethal ethanol stress, but does not influence growth under sublethal ethanol stress. FEMS Yeast Res 2009;9:1208-16.
5. Lal S, Sandaranaraj E, Jada SR, Kong MC, Lee LH, Goh BC, et al. Influence of APOE genotypes and VKORC1 haplotypes on warfarin dose requirements in Asian patients. Br J Clin Pharmacol 2008;65:260-4.
6. Kojic EM, Darouiche RO. Candida infections of medical devices. Clin Microbiol Rev 2004;17:255-67.
7. Donlan RM. Biofilms: Microbial life on surfaces. Emerg Infect Dis 2002;8:881-90.
8. Agarwal V, Tanwar C, Pruthi V. CLSM Studies of Candida albicans Biofilm on Biomaterials. ASM Conference-Biofilm. Quebec, Canada: American Society for Microbiology; 2007.
9. Lewis K. Riddle of biofilm resistance. Antimicrob Agents Chemother 2001;45:989-1007.
10. Jabri-Rizk MA, Fakhler WA, Meiller TF. Fungal biofilms and drug resistance. Emerg Infect Dis 2004;10:14-9.
11. Pratiwi SU, Lagendijk EL, de Weert S, Hertiani T, Idroes R, Hondel CA. Effect of Cinnamomum burmannii Nees ex Bl. and Massoia aromatica Becc. Essential oils on planktonic growth and biofilm formation of Pseudomonas aeruginosa and Staphylococcus aureus in vitro. Int J Appl Res Nat Prod 2015;8:1-13.
12. Khajanchi BK, Kirtley ML, Brackman SM, Chopra AK. Immunomodulatory and protective roles of quorum-sensing signaling molecules N-acyl homoserine lactones during infection of mice with Aeromonas hydrophila. Infect Immun 2011;79:2646-57.
13. Tauber SC, Nau R. Immunomodulatory properties of antibiotics. Curr Mol Pharmacol 2008;1:68-79.
14. Niphade SR, Asad M, Chandralekha GK, Toppo E, Sheshadri RR. Immunomodulatory activity of Cinnamomum zeylanicum bark. Pharm Biol 2009;47:1168-73.
15. Roshan N, Saviti P. Review on chemical constituents and parts of plants as immunomodulators. Res J Pharm Biol Chem Sci 2013;4:76-89.
16. Wagner H, Blättler S. Plant Drug Analysis: A Thin Layer Chromatography Atlas. London, New York: Springer Science and Business Media; 1996. p. 373.
17. Horváth G, Szabolcs LG, Lemberkovics É, Botz L, Kocsis B. Characterization and TLC-biautographic detection of essential oils from some thymus taxa. Determination of the Activity of the oils and their components against plant pathogenic bacteria. J Planar Chromatogr Mod TLC 2004;17:300-4.
18. Faria NC, Kim JH, Gonçalves LA, Martins Mde L, Chan KL, Campbell BC. Enhanced activity of antifungal drugs using natural phenolics against yeast strains of Candida and Cryptococcus. Lett Appl Microbiol 2011;52:506-13.
19. Pierce CG, Uppuluri R, Tristan AR, Wormald FL Jr., Mowat E, Ramage G, et al. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat Protoc 2008;3:1484-500.
20. Ramage G, Saville SP, Wickes BL, López-Ribot JL. Inhibition of Candida albicans biofilm formation by farnesol, a quorum-sensing molecule. Appl Environ Microbiol 2002;68:5459-63.
21. Jin Y, Zhang T, Samarayake YH, Fang HH, Ip HK, Samarayake LP. The use of new probes and stains for improved assessment of cell viability and extracellular polymeric substances in Candida albicans biofilms. Mycopathologia 2006;159:353-60.
22. Dusane DH, Dam S, Nanchanawe YV, Kumar AR, Venugopalan VP, Zinjarde SS. Disruption of Yarrowia lipolytica biofilms by rhamnolipid biosurfactant. Aquat Biosyst 2012;8:17.
23. Syamsudin, Darmoni, Kuswadi. The Effect of Garcinia parvifolia Miq (Active Fraction) on phagocytosis by peritoneal macrophages during Plasmodium berghei Infection in Mice. Res J Immunol 2008;1:16-20.
24. Tatarczuch L, Bischof RJ, Philip CJ, Lee CS. Phagocytic capacity of leucocytes in sheep mammary secretions following weaning. J Anat 2002;201:351-61.
25. Stocks SM. Mechanism and use of the commercially available viability stain, BacLight. Cytometry A 2004;61:189-95.

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