BUKTI KORESPONDENSI

ARTIKEL JURNAL INTERNASIONAL BEREPUTASI

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1.	Register pada European Journal of Dentistry	Mei 2021
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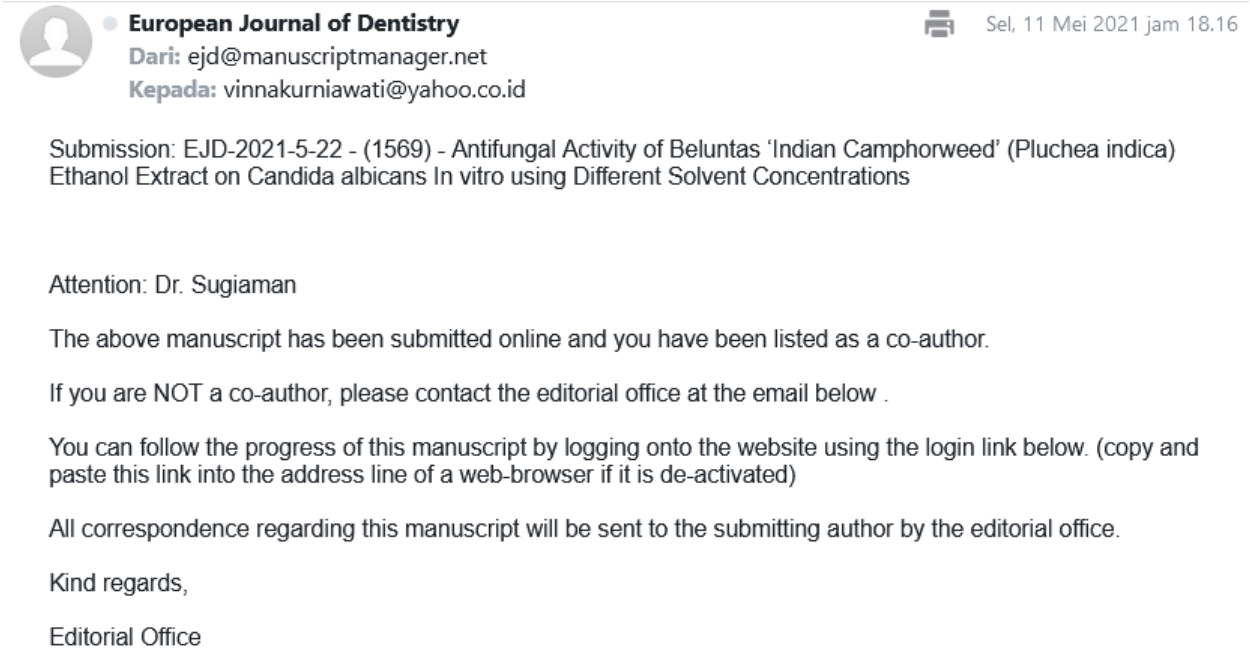

European Journal of Dentistry Sel, 11 Mei 2021 jam 18.16
Dari: ejd@manuscriptmanager.net
Kepada: vinnakurniawati@yahoo.co.id

Submission: EJD-2021-5-22 - (1569) - Antifungal Activity of Beluntas 'Indian Camphorweed' (Pluchea indica) Ethanol Extract on Candida albicans In vitro using Different Solvent Concentrations

Attention: Dr. Sugiaman

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Antifungal Activity of Indian Camphorweed 'Beluntas' (*Pluchea indica*) Ethanolic Extract on *Candida albicans In vitro* using Different Solvent Concentrations

Article types: Original Article

Conflict of interest: -

ABSTRACT

Background and Objective: Oral candidiasis is an infection caused by pathogenic fungi *C. albicans*, with a considerably high prevalence of 20% – 72%. Indian camphorweed (*Pluchea Indica*) or known as "beluntas" in local name has been known as traditional medicine in Indonesia. The objective of this study is to research the minimal inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of Beluntas ethanolic extract against the growth of *C. albicans*. **Methods:** The MIC and MFC were measured by microdilution assay and total plate count respectively with a variation of solvents DMSO 1%,

10%, and 4% and Beluntas extract with concentrations between 0.3125 mg/mL to 200 mg/mL. Amphotericin and nystatin were used as a comparison. **Statistical Analysis**: *One Way Anova* and *Post Hoc Tukey* were used to determine the significant difference between treatments. **Results:** It was found that the MIC ranged from 50 mg/mL – 200 mg/mL in the test with DMSO 10% solvent and MFC was found to be at a concentration of 200 mg/mL. However, there is a significant inhibitory effect and killing effect from DMSO 10% against *C. albicans* (p=0.000). MIC was also found within concentrations of 100 mg/mL of Beluntas extract in DMSO 4%. In this study, the DMSO 4% concentration neither showed significant inhibitory effects nor killing effects therefore the result was acceptable (p=0.357). **Conclusion:** Ethanol extract of Beluntas (*Pluchea Indica*) has the potential of being an antifungal with inhibitory activity in concentration ≥100 mg/mL which was equal to nystatin (p=0.278). The MFC for the extract was above 100 mg/mL, which cannot be measured with this method as a higher concentration of DMSO is needed which had a toxic effect on the tested fungi.

Keywords: *Candida albicans*, Nystatin, Amphotericin B, Indian camphorweed, *Pluchea Indica*, Beluntas

INTRODUCTION

Candida albicans and other *Candida* species colonized up to 75% in a healthy person's oral cavity. *C.albicans* is an opportunistic pathogen which can cause a wide range of disease manifestation from mild oral disease to disseminated candidiasis. This event can be triggered by several conditions including immunosuppression, endocrine imbalance, prolonged antibiotic therapy, smoking, and chemotherapy.[1,2]

Nystatin and amphotericin from the polyene class are the first-line therapy for candidiasis which work by affecting the fungi membrane permeability and thereby causing cells' death. However, several side effects had been reported regarding polyene formulation including delayed hypersensitivity attributed to cinnamic aldehyde and increase risk of carries due to sugar in the oral suspension. Rare cross-reactivity between nystatin and other macrolides and resistance to polyene antifungals has also been reported.[3] The most common adverse effects were poor taste and gastrointestinal adverse reactions.[4] Moreover, although rare, the resistance of fungi to polyene antifungal had been reported.[5] Thereby, novel drugs that are safer and less inducing resistance, especially coming from natural resources becomes the wide interest of research.

Indian camphorweed (*Pluchea indica*) or known as "beluntas" in the local name is a native plant of Indonesia that has been used as traditional medicine. The plant's leaf has a unique aroma and bitter taste. This part is used as a gastrointestinal agent, diuretic, antipyretic traditionally. It also has antiseptic properties and is used as a deodorant and vaginal leukorrhea medicine, which showed its potential as an antifungal.[6,7] This research is purposed to find the antifungal activity of Indian camphorweed (*Pluchea indica*)/beluntas against *C. albicans in vitro* as a novel natural-resource-based therapeutic for oral candidiasis.

METHOD

Beluntas Leaves Ethanol Extract

Beluntas leaves were obtained from Balai Penelitian Tanaman Rempah dan Obat (BALITTRO) (*Spices and Medicinal Plants Research Center*), Bogor, West Java, Indonesia. Leaves extract were obtained using maceration method. Maceration is used for the extraction as this method is the simplest method of extraction, also can be used to extract polar and non-polar fraction of the active compound and the thermolabile active compounds.[8] Firstly, the

leaves were dried using a food dehydrator and grounded with a food processor. The leaves powder was later soaked in ethanol 70% for 3 days. The active compound will be dissolved in the solvent during soaking. Ethanol 70% was used as solvent as this solvent is able to extract polar and non-polar active compounds of the leaves and also less cytotoxic. After 24 hours, the filtrate was taken and filtered with filter paper. This process is necessary to eliminate leaves grounds which doesn't contain any active compounds anymore. Later the extract was concentrated using rotary evaporator.[9] For experiment, Beluntas ethanol extract was diluted in DMSO 1%, 4%, and 10% with concentrations ranging from 10mg/mL to 0.3125mg/mL, 100mg/mL to 3.125 mg/mL, and 200mg/mL to 3.125mg/mL respectively. DMSO 1% was used in the initial experiment by refering to the Clinical Laboratory Standard Institute (2008) M-27 protocol of standard solvent used for microdilution assay.[10] However, at this concentration we cannot find the MIC and MBC value as the maximum diluted extract is 20mg/mL (see **Result**), hence we increase the DMSO concentration. Amphotericin and nystatin 0.25mg/mL in 1%, 4%, and 10% DMSO was used as comparison.

Preparation of *C. albicans* inoculum

C. albicans ATCC 10231 was used for this study and prepared in accordance to the clinical Laboratory Standard Institute (2008) M-27 protocol.[10] Fungi were a subculture in Potato Dextrose Agar (Himedia M096) for 24 hours before used. The 24-h old culture was used in the experiment as in this stage the yeast is in log phase (actively budding).[11] Later about 1 loop of *C.albicans* was taken and dissolved in PBS (Sigma Aldrich 11666789001). The turbidity of the suspension was adjusted to 0.5 McFarland turbidity standard (equals to 1 x 10^6 cfu/mL of yeast), which is defined as 0.08-0.1 absorbance value measured in 600nm wavelength, and further 3-times 10-fold diluted in Potato Dextrose Broth (Himedia, M403) so the final inoculum concentration was about $1x10^3$ cfu/mL.

Minimum Inhibitory Concentration (MIC)

The 96-well plate microdilution assay was used for this purpose according to the Clinical Laboratory Standard Institute (2008) M-27 protocol of antifungal susceptibility of yeast with slight modifications.[10] A hundred microliter of inoculum suspension was loaded unto the wells, except for the blank wells and positive control well. As much as 100µL PDB was added to the blank wells and positive control well. Thereby the positive control well consists of *C.albicans* suspension and 100µL medium without treatment. Later 100µL of diluted extract and nystatin and amphotericin in DMSO was added to each well, including the blank wells. A well filled with PDB only was used as the negative control. To measure the inhibitory effect of the solvent, a "solvent control" well was added which consists of 100µL *C.albicans* suspension and 100µL DMSO 1%, 4%, and 10%. Each treatment was plated 3 times. The plate was later incubated in 37^{o}C incubator for 24 hours, and later the absorbance was read at 530nm wavelength. Until now, there is no precise definition for MIC measured with absorbance. In its original definition, according to CLSI, the MIC value is the lowest concentration where there is no visual growth of microorganisms.[11] In one experiment, the MIC value was defined as the lowest concentration where there is a sharp decline in absorbance value.[12] In this experiment, MIC was defined as the lowest concentration which gives 95-100% inhibition. Growth inhibitory activity was calculated as follow:

$$\%\ Inhibition = \frac{(OD530\ C.albicans - OD\ medium) - (OD530\ treated\ wells - OD\ blanks - OD\ medium)}{(OD530\ C.albicans - OD\ medium)} \times 100\%$$

Minimum Fungicidal Concentration (MFC)

Minimum Fungicidal concentration (MFC) was determined through Total Plate Count assay according to CLSI (2008) standard.[10] A hundred microliter of suspension from wells with 100% inhibition, including the medium only well itself, *C.albicans* untreated, and *C.albicans*

in DMSO solvent were platted to Potato Dextrose Agar and incubated for 24 hours. Serial dilution was carried out if necessary to make colony counting easier. Each treatment was platted 3 times. The colony was counted and converted to *colony-forming unit* per mL, and %killing activity was calculated as follows:

$$\%Killing = \frac{CFU\ C.albicans\ untreated - CFU\ treatment}{CFU\ C.albicans\ untreated} \times 100\%$$

MFC is the lowest concentration where there is 99.9% killing activity according to CLSI (2008) definition.[10]

RESULT

Minimum Inhibitory Concentration

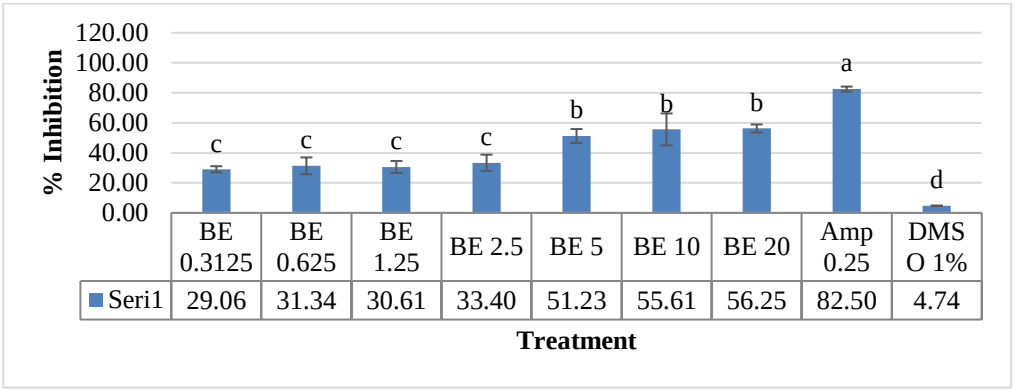
Different concentration of solvent is used for this experiment. The first experiment used 1% DMSO as suggested by CLSI (2008) guidelines.[10] The maximum concentration of Beluntas Extract (BE) that can be dissolved in this solvent is 20mg/mL. Amphotericin B was used as a comparison control for this setting. Maximum inhibitory activity that can be achieved by 20mg/mL BE is 56.25%. The inhibitory activity is very low in this concentration. Amphotericin 0.25mg/mL only inhibits 82.5% of *C.albicans* growth, where it is expected to give 100% inhibition. DMSO 1% didn't have a toxic effect as it only inhibits 4.74% growth (**Figure 1A**).

As the first set didn't give MIC value, a second experiment was conducted where 200mg/mL BE was used as the highest concentration diluted in DMSO 10%. The inhibitory activity was very high for this setting, ranging from 82% in 3.125mg/mL to 100% in 50mg/mL - 200mg/mL concentration. The control was changed to nystatin 0.25mg/mL which gave 100% inhibition. Inhibitory activity of BE 100mg/mL and BE 200mg/mL gave equal result with nystatin ($p = 0.960$), thereby it will be plated for MFC analysis. However,

DMSO10% it self showed high inhibitory activity (74.1%) (**Figure 1B**). This result may lead to interpretation bias whether the inhibitory activity is the result of the DMSO or the extract.

As there was confounding effect from inhibitory activity of DMSO, we conducted the next experiment using a lower DMSO concentration (4% DMSO). The maximum extract concentration that can be soluble is 100mg/mL. Nystatin was still used as a comparison for this setting. BE 100mg/mL showed 100% inhibition, comparable with nystatin (p =0.995). DMSO 4% only gave slight inhibitory activity of 11.88% (**Figure 1C**).

A



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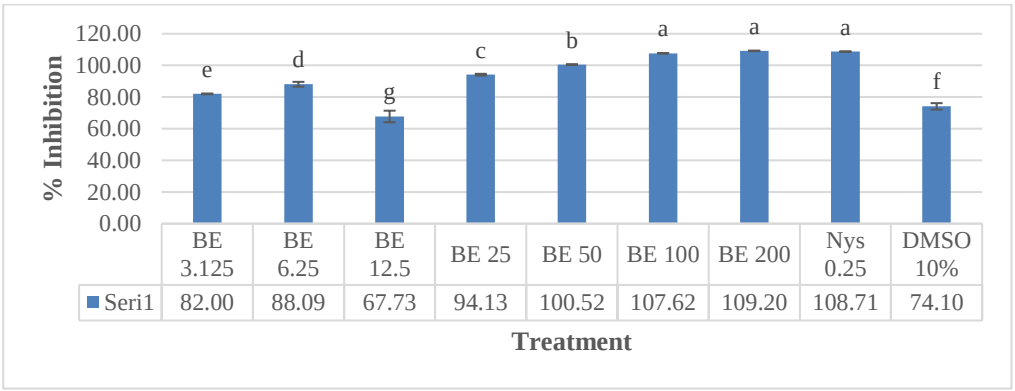

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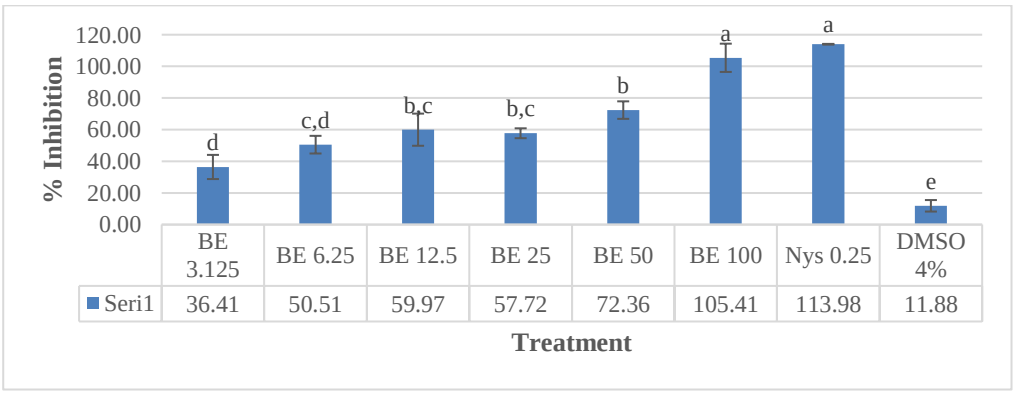
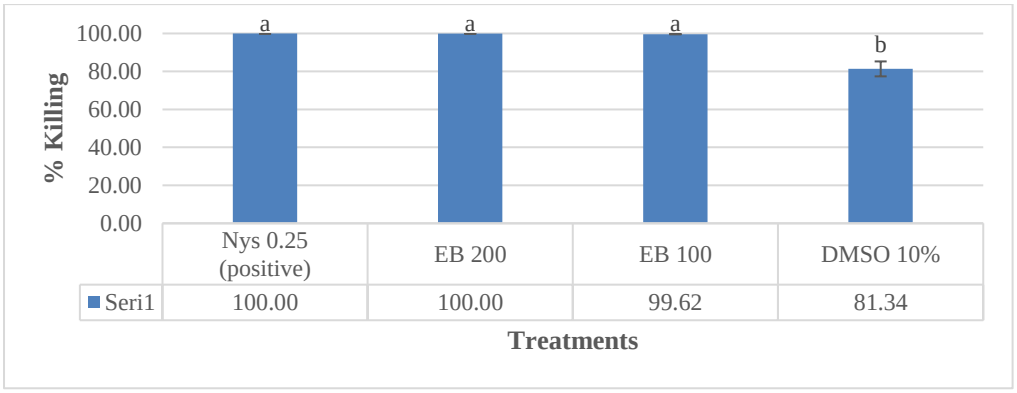


Figure 1. Percent of *C. albicans* growth inhibition after Beluntas Extract (BE) challenge in microdilution assay. Different solvents were used: (A) 1% DMSO, (B) 10% DMSO, (C) 4% DMSO which resulted in different maximum diluted extract and different inhibitory activities. Different alphabets located on top of the bar show statistical difference between groups ($p>0.05$) as calculated with ANOVA and Post-Hoc Tukey Test.

Minimum Fungicidal Concentration

Nystatin 0.25mg/mL in DMSO10% and BE 200mg/mL in DMSO10% showed fungicidal activity. Meanwhile BE 100mg/mL showed fungistatic activity both shown in DMSO 10% solvent and DMSO 4% as the killing activity didn't reach 99.9%. DMSO10% showed a quite high killing activity (81.34%). Whereas, DMSO4% showed slight killing activity (13.25%). It can be safely concluded that DMSO 4% is a suitable concentration to carry out MIC and MFC experiment, which doesn't give significant inhibitory and killing activity.

A



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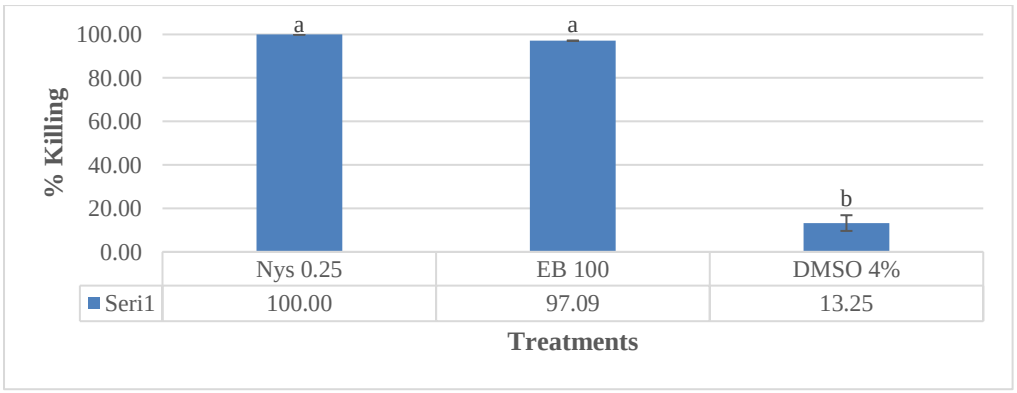

Figure 2. Percent of *C. albicans* killing as assessed with total plate count in (A) 10% DMSO and (B) 4% DMSO setting. Different alphabets located on top of the bar show statistical difference between groups ($p>0.05$) as calculated with ANOVA and Post-Hoc Tukey Test.

DISCUSSION

This study showed beluntas ethanol extract activity towards growth inhibition of *C. albicans.* In the first setting, 20mg/mL BE is not sufficient to induce a fungistatic effect, however, it still showed an inhibitory effect. This effect is still can be seen in the DMSO 10% setting where the inhibitory effect of each concentration significantly higher in contrast with DMSO10% alone. From the series of experiments, the MIC value of beluntas extract is 100mg/mL, which didn't differ from nystatin positive control. Our result is in line with previous research by Samalo (2014) where the MIC of Beluntas ethanol extract is at 16% concentration, while the MFC is at 20% using the macro dilution method. However, in this experiment, we cannot ascertain the type of solvent used and also the exact mg/mL concentration of the extract, as it is not fully accessible.[13] To our knowledge, this is the first research on Beluntas ethanol extract activity towards *C. albicans* inhibition using microdilution assay with different concentration of solvents used.

The antifungal activity of the ethanol extract of beluntas leaves is thought to be due to the synergistic effect of each secondary metabolite contained in the ethanol extract of beluntas leaves. Beluntas leaves contain flavonoids, phenols, saponins, tanins, steroids/triterpenoids, terpenoids, and alkaloids as reported in our previous study.[14] Based on the research of Widyawati et al., The ethanol extract of beluntas has levels of total flavonoids equivalent to 18,555 $\pm$ 1,792 mg CE / 100g dry weight and total phenolic equivalent to 16,958 $\pm$ 897 mg GAE / 100g dry weight.[15] In line with those research, where we found flavonoid content of 19.44 mg/g dry beluntas leaves.[16]

Flavonoids are known to be able to inhibit fungal growth through several mechanisms, namely efflux pump inhibition, cell division inhibition, inhibition of RNA / DNA synthesis or fungal protein, inhibition of fungal cell wall formation, mitochondrial dysfunction, and disruption of the fungal plasma membrane.[17]

Ergosterol is an important component in the formation of cell membranes. Phenols can inhibit ergosterol biosynthesis, and disrupt the cell membrane, which causes leakage of intracellular components and cause changes in the permeability of the fungal membrane. Deformation of the cell wall causes a significant reduction in cell size. Besides, phenols can also interfere with cell metabolism by inhibiting cell transports resulting in inhibition of fungal cell growth which resulted in apoptosis.[18] In more detail, phenol inhibits CYP51 enzyme activites and fungal suqlene epoxidase (SE), the first enzymes involve in ergosterol biosynthesis pathway.[19]

Saponin significantly induced the production of H2O2 and resulted in membrane lipid peroxidation, thus leading to an increase in cell membrane permeability and the leakage of K(+), soluble protein and soluble sugar.[20] Steroidal saponins are known to increase mitochondrial membrane potential, thus causing mitochondrial and reticulum endoplasma stress which leads to internal apoptotic pathway.[21,22] Meanwhile, triterpenoid saponin induced accumulatin of intracellular reactive oxygen species (ROS, resulting in mitochondrial dysfunction. It also breaks down the membrane barrier of *C. albicans* causing leakage of intracellular trehalose, entrance of extracellular imperbeable substance and ecrease of ergosterol content.[23]

In this experiment, two approved drugs from the polyene class were used as comparison control, nystatin and amphotericin B. Nystatin affects *C. albicans* by inhibiting the stages of glucose metabolism and influencing cell permeability, as a result, Candida cells will lack energy so they experience atrophy and over time their growth and multiplication are

inhibited.[14] Amphotericin B works by binding to ergosterol which is the main component of fungal cell membranes which causes depolarization of fungal cells thus causing the fungal cells to die.[24] In this experiment, 0.25mg/mL Amphotericin didn't give fungistatic effects. According to a previous study, MIC of amphotericin B in *C.albicans* ATCC10231 is 0.25 microgram/mL.[25] Probably amphotericin that was used in the experiment had degraded.

MFC value cannot be found in this experiment because a higher concentration of beluntas is needed while it can be met using DMSO4%. Higher DMSO concentration will result in toxic effects to *C.albicans* which may affect the interpretation of the result. We found that DMSO10% is toxic. This result is in line with previous research from Randhawa (2008) where DMSO10% gave significant inhibitory effects towards *C. albicans* as DMSO can dissolve fungal membrane, thereby higher concentration will result in cells' death.[26] Meanwhile, DMSO4% is considered safe and can be used for experiment.[27] However, a lower concentration of DMSO cannot dissolve beluntas ethanol extract which probably is hydrophobic.

Altough our study shows potential antifungal activity of Beluntas (*Pluchea indica*) leaves, several limitations had been met during the experiment. Firstly, is the only *Candida albicans* strain used in the experiment is ATCC 10231. Secondly, higher concentration of the extract is needed to carry out fungicidal activity. While higher concentration of DMSO is needed to dissolve the extract (as the extract is not solvable in water) might be one issue, and resulted in DMSO cytotoxicity, the high concentration of extract itself may have cytotoxic effect. Our previous study showed that ethanol extract of beluntas leaves has IC_{50} value at 311.77 μg/mL against 3T3/Balb-C mice fibroblast cells.[16]

Further research of beluntas extract activity against various *C. albicans* strains is needed to confirm its antifungal property. HPLC study might also be done to characterize the active compounds contained in the leaves and *in-silico* study might be performed to find optimum

solvents for the extraction. Another kind of assay or solvent is needed to find the exact value of Beluntas extract MFC.

CONCLUSION

From this study, we found that Beluntas (*Pluchea indica*) ethanol extract had inhibitory effects on *C. albicans* in vitro. The MIC value is 100mg/ml. MFC value cannot be determined exactly because a higher concentration of DMSO is needed to dissolve extract which may have toxic effects on the fungi.

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Bukti melakukan review yang pertama (02 Agustus 2021)

Reviewer 1 report:

1. Add the Phytochemistry information of Beluntas leaves.
2. Further discussion about the mechanism of inhibition activity of the leaves against fungal
3. Add previous data about the cytotoxicity of the leaves, that in this study showed the property of antifungal
4. Revise the result systematically
5. Table difficult to read (organize the data)
5. What is the limitation of the study
6. What is the future implication of the study

Reviewer 2 report:

1. Sometimes you write Candida albicans and sometimes you write C. albicans: try to unify
2. Beluntas Leaves Ethanol Extract: Try to add details about the protocol you used (why this protocol? references? why and the reason behind every step in the protocol)
3. Preparation of Candida albicans inoculum: the same like in comment "2": Try to add details about the protocol you used (references? why and the reason behind every step in the protocol)

Editor-in-Chief comments:

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Submission: EJD-2021-5-22/R1 RESUBMISSION - (1569) - Antifungal Activity of Beluntas 'Indian Camphorweed' (Pluchea indica) Ethanol Extract on Candida albicans In vitro using Different Solvent Concentrations

Attention: Dr. Sugiaman

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