

**ETHYL ACETATE FRACTION OF GARCINIA MANGOSTANA-LINN PERICARP EXTRACT:
ANTI - *CANDIDA ALBICANS* AND EPITHELIAL CYTOTOXICITY****FEBRINA RAHMAYANTI^{1*}, DEWI FATMA SUNIARTI², ZAINAL ALIM MASÚD³, BOY M BACHTIAR²,
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ABSTRACT

Candida species are a commensal agent in oral, skin, and gastrointestinal tract environment, but when there is an underlying predisposing condition in the host, it causing candida infections. The increasing of antifungal resistance complicates patient management. Therefore, it is necessary to identify potential antifungal agents. Garcinia mangostana-Linn (GML) is a famous tropical fruit in Indonesia and other southeast Asia countries. This study was conducted to examine the antifungal activity of chromatographic column ethyl acetate fraction of GML pericarp against *Candida albicans*. GML was extracted with ethanol and fractionated with ethyl acetate. *C. albicans* ATCC 10231 was used in this study. Antifungal activities were expressed as the viability of *C. albicans* growth identification with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. There is no previous research that analyzes the antifungal effect of GML based on ethyl acetate fraction from the chromatographic fractionation technique, especially as anti-*C. albicans*. In this study, we also conducted an analysis of the cytotoxicity effect of ethyl acetate fraction on HaCaT cell line. The result of our study indicates that ethyl acetate fraction of GML pericarp extract has reduced the viability of *C. albicans*. There is a tendency of decrease in viability in line with the increase of the concentration of fraction. As anti-*C. albicans* ethyl acetate fraction of GML pericarp extract has no cytotoxicity to HaCaT cell line. This result showed the ability of ethyl acetate fraction of GML extract changes the viability of *C. albicans*, but not toxic to HaCaT cell line. It may consider ethyl acetate fraction of GML pericarp extract as potent anti-*C. albicans* and promising adjuncts in oral health product.

Keywords: Garcinia mangostana-Linn, Viability *Candida albicans*, Ethyl acetate fraction, HaCaT cytotoxicity.

INTRODUCTION

Fungal infections are common in immunocompromised patients. It increased morbidity and may be life threatening. 95% HIV patients have oral candidiasis, related to the decrease of their CD4 T cells counts [1]. *Candida* is the most common fungal pathogen in human, and the top four leading diseases in nosocomial bloodstream infection. Majority, the cause of infection is *Candida albicans*. *C. albicans* is dimorphic fungi usually found in an oral cavity in the non-pathogenic state as a commensal microorganism, its prevalence in healthy human ranges from 40% to 60% [2], but it can be transformed into the pathogenic state if there are substantial changes in the environment. The predisposing factors are salivary factors, temporary factor, smoking, oral topography, immune status, and oral microflora, play an important role in Candidiasis [3]. The overgrowth of *C. albicans* caused an opportunistic infection. Increasing incidence of candidiasis since the last few decade scan are due to increase immune compromised patients, and the use of antibiotics. In healthy patients, the treatment is relatively simple, but in immunocompromised patients, the therapy is more complicated. The widespread use of antifungal is potential cause antifungal resistant, especially in patients with long-term treatment. The development of new antifungal drugs or new treatment strategies is needed to combat *Candida* infections [4,5].

Current researches in formulated antifungal drugs from natural products, parallel with developing in synthetic drugs as an arsenal of fungal infection, interested us to search the potential antifungal effect, especially as anti-*C. albicans* from Indonesian indigenous plant. Previous studies have analyzed plant as medicinal compounds, since ancient time. Doddona *et al.* (2013) had tested the antimicrobial effect from plant extracts of tea leaves, onion leaves, onion bulb, mint leaves, *Aloe vera* and curry leaves, and it can inhibit the growth of *C. albicans*. However, using plant extract with antifungal effect may help to prevent candidiasis without side effects [6].

Garcinia mangostana-Linn (GML) known as mangosteen is a tropical fruit that growth in South East Asia, including in Indonesia [7]. Mangosteen is also known as the queen of fruit. Mangosteens are small round fruit with thick, brittle, and deep purple spherical outer pericarp.

Several previous phytochemical studies have shown active compounds of mangosteen pericarp, for examples α mangostin has been isolated from pericarps, whole fruit, stems, arils, and seeds. Its pericarp contains a variety of xanthenes, fifty xanthenes have been isolated from mangosteen pericarp, as secondary metabolites and have used as medicine purposes since ancient history [8]. People have used mangosteens as an antiseptic, anti-inflammation, antipyretic, antiparasitic, analgesic, antioxidant activity, and antibacterial agent. Previous studies have demonstrated that an ethanolic mangosteen extract was demonstrated as a broad-spectrum antibacterial [9-11]. Kaomongkolgit *et al.* (2009) have analyzed the antifungal effects of alpha mangosteen [12], but no studies have reported about ethyl acetate fraction of ethanol extracts from GML pericarp as antifungal and its effect to an epithelial cell. The purpose of this study was to determine the anti-*C. albicans* of ethyl acetate fraction of GML pericarp extract based on chromatographic fractionation technique and analyze the cytotoxicity effect to the HaCaT cell line. This research is very important for further research on the use of GML as a treatment of candidiasis in the oral cavity.

METHODS**Preparation of GML extraction and fractionation**

GML was collected from Puspahieng Village in West Java, Indonesia. We separated the pericarps and the arils. The pericarp were dried at the room temperature and blended to become powder. We extracted pericarp powder with ethanol 75% and macerated for 48 hrs. The solvent was evaporated with rotary vacuum evaporator at 50°C. The

crude extract was purified with chromatography column, in ethyl acetate. We made ethyl acetate fraction from ethanol extraction of GML pericarp [12,13].

C. albicans ATCC culture

Prior of the experiment, *C. albicans* ATCC 10231 was cultured at 37°C for 24 hrs on saboraud dextrose agar (SDA), and a loopful of growth was inoculated in saboraud dextrose broth (SDB) for 18 hrs, and serial dilution made for standardized the amount of *C. albicans* used in this experiment.

Antifungal activity test

Antifungal activity of GML pericarp extract and fractions were carried out by broth microdilution method. The testing condition we were analyzed ethyl acetate fractionation of ethanol extract GML pericarp with three different concentrations of GML pericarp fractions (500 ppm, 1000 ppm, and 2000 ppm), and in 60 minutes incubation periods. First, we made 900 µl for each concentration of GML pericarp extract and from chromatography fractions at 1 ml tube and added 100 µl *C. albicans*, incubated 37°C in three different times. Then, 100 µl of each tube plated onto SDA, incubated at 37°C for 24 hrs [14,15].

Preparation for C. albicans viability test

After culture in broth medium for 24 hrs, *C. albicans* optical density (OD) analyzed in a microplate reader. The level of OD has to be the equal or close to the value obtained by the standard.

Biofilm from saliva was made. Stimulated saliva were collected from volunteers and centrifuged in 4000 rpm for 20 minutes in 4°C. Take the supernatant and diluted with phosphate buffered saline (PBS) with ratio 1:9. Diluted saliva then filtered to eliminate bacteria and fungi. 200 µl of the saliva coated in the 96 well plates incubated in 37°C for 90 minutes, wash with PBS two times and 200 µl *C. albicans* coated the saliva biofilm, incubated at 37°C, 90 minutes. After coated with *C. albicans*, GML extract, and GML fractionation applied (except the control of test, only added with SDB) and incubated 37°C for 60 minutes. The concentrations of GML extracts and GML fractionated were 500 ppm (0.5 mg/ml), 1000 ppm (1 mg/ml), and 2000 ppm (2 mg/ml).

3-4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

MTT assay was determined for assessing the viability of *C. albicans* ATCC after GML fractionation applied. A parallel set of at least 3 replicate plates was set up for the MTT assay and exposed in an identical manner as described above in preparation for *C. albicans* viability test, after 60 minutes of GML exposure. 50 µl freshly MTT were added to each well plates. After 3 hrs incubation, 100 µl MTT fixation solutions (acidified isopropanol) were added to each well and shaken for 1 hr. The absorbance was measured at 490 nm using the microplate reader.

Cytotoxicity evaluation

The human keratinocytes cell line, HaCaT was given from Prof. Ikeda from Tokyo Medical and Dental University, routinely maintained in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum and antibiotics. HaCaT cell was cultured until confluence in 96 well plates. Then, HaCaT cells were exposed with chloroform, ethyl acetate, and ethyl acetate fraction from the ethanol extract of GML pericarp, and the concentrations were 500 ppm (0.5 mg/ml), 1000 ppm (1 mg/ml), and 2000 ppm (2 mg/ml). MTT analysis was used to evaluation *in vitro* cytotoxicity of HaCaT cell line. A parallel set of triplicate plate was set up for MTT assay of HaCaT cell after 24 hrs exposed to GML pericarp fractionation. After each time of exposure, washed the cells with PBS and added 100 µl of 5 g/ml MTT reagent, incubation for 3 hrs, and after that 150 µl acidified isopropanol added for 1 hr, shake and read the result at microplate reader at 490 nm.

Ethical clearance

This study was approved by the Ethical Committee Faculty of Dentistry Universitas Indonesia.

Statistical analysis

All statistic computation was performed by GraphPad Prism 6. Kruskal-Wallis analysis used to analyzes the different of *C. albicans* growth in the different concentration at 60 minutes time of exposure and to analysis the different of the viability of HaCaT cell line at 24 hrs. To compare between groups, we used unpaired Test analyzes. Statistical significance was defined as $P < 0.05$.

RESULT AND DISCUSSION

We tested the viability of *C. albicans* after 60 minutes exposure to ethyl acetate fraction, with 3 concentrations 500 ppm, 1000 ppm, and 2000 ppm (Table 1). There are significant differences of the viability of *C. albicans* between in three different concentration of 500 ppm, 1000 ppm, and 2000 ppm after 60 minutes exposure time ($p = 0.0001$) Kruskal-Wallis test.

In our study, *C. albicans* growth inhibition was seen at a concentration of 500 ppm, and the growth inhibition increase in line with the increase of concentration. The trend of viability showed decreased of *C. albicans* viability line to the increased of concentration. Unpaired test analysis showed the different between groups (Table 2).

This present study based on Table 1 above showed that mean different between the viability of *C. albicans* with a concentration of 500 ppm and 1000 ppm is about 9%, this little differences cause no statistically significant ($p = 0.0524$). However, there are statistically significant data when compare with other concentrations (Table 2).

Fig. 1 shows the IC_{50} , which is the ability of optimal concentration of ethyl acetate fraction from GML pericarp extract to inhibit 50% of *C. albicans* growth. Based on Fig. 1, the IC_{50} is around 1900 ppm. This is consistent with the results of descriptive analysis of the average viability in Table 1. In addition to investigating, the anti-*C. albicans* effect of ethyl acetate fraction of GML pericarp, we also analyze the cytotoxicity effect to HaCaT cell line.

There are significant differences of the viability of HaCaT cell line after 60 minutes exposure time ($p = 0.0035$) Kruskal-Wallis test. Based on concentration, the viability of HaCaT cell were higher parallel to the increase of concentration (Table 3). However, there were no significant different between concentrations of GML extract fractionation, except when it compare with control (Table 4).

There were no significant effects of ethyl acetate fraction of GML pericarp to HaCaT cell line viability. The HaCaT cell line viability is increasing parallel to the increase of the concentration of ethyl acetate fraction of

Table 1: Mean and SD of viability of C. albicans after exposure of ethyl acetate fraction GML in 60 minutes

Ethyl acetate	Mean±SD	p value
500 ppm	82.6667±5.85947	p=0.0001
1000 ppm	71.6667±3.78594	
2000 ppm	46.3333±4.16333	

Kruskal-Wallis test, SD: Standard deviation, *C. albicans*: *Candida albicans*, GML: *Garcinia mangostana*-Linn

Table 2: p value between groups concentration of ethyl acetate fraction concentrations with the viability of C. albicans

Ethyl acetate	p value
500 ppm versus control	p=0.0069*
1000 ppm versus control	p=0.0002*
2000 ppm versus control	p<0.0001*
500 ppm versus 1000 ppm	p=0.0524
500 ppm versus 2000 ppm	p=0.0009*
1000 ppm versus 2000 ppm	p=0.0015*

*Significant difference, *C. albicans*: *Candida albicans*

Table 3: Viability of HaCaT cell line after exposure of ethyl acetate fraction of GML pericarp extract in 24 hrs

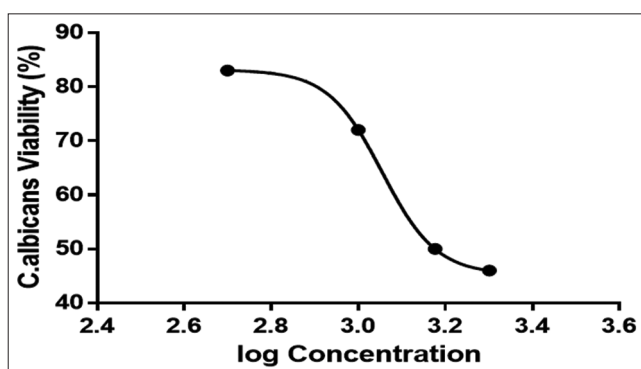
Ethyl acetate	Mean±SD	p value
500 ppm	74±6.6	p=0.0035
1000 ppm	81.5±5.9	
2000 ppm	82.25±6.8	

Kruskal-Wallis test, SD: Standard deviation, GML: Garcinia mangostana-Linn

Table 4: p value between group concentrations of ethyl acetate fraction concentrations and the viability of HaCaT cell line in 24 hrs

Ethyl acetate	p value
500 ppm versus control	p=0.0002*
1000 ppm versus control	p=0.0008*
2000 ppm versus control	p=0.0020*
500 ppm versus 1000 ppm	p=0.1438
500 ppm versus 2000 ppm	p=0.1343
1000 ppm versus 2000 ppm	p=0.8743

*Significant difference

**Fig. 1: IC₅₀ ethyl acetate fraction from ethanol extract of Garcinia mangostana-Linn pericarp to Candida albicans viability**

GML extract. It is an interesting condition and need more investigation. However, this present study shows although ethyl acetate fractionation can inhibit *C. albicans* growth, but is safe to HaCaT cell line.

C. albicans is a yeast-like fungus that can be found in our body, in skin, oral mucosa, gastrointestinal tract, and in vagina mucosa. *C. albicans* is commensal yeast in normal oral flora, but it is a primary agent of oral candidiasis, in immunocompromised individual. Nowadays, some cases of oral candidiasis are refractory to antifungal agents. Several studies have been conducted to analyze the use of active substances of the plant as antifungal. Zhao *et al.*, 2013 analyzed the used of tetradrine from stephaniatetrandra as antifungal [16]. Others previous research, of phytochemical studies, has shown GML pericarp extract have a potent antibacterial effect. Antifungal activity of several xanthenes isolated from mangosteen pericarp and some α -mangostin-derivates against three phytopathogenic fungi have been demonstrated by Gopalakrishnan *et al.*, 1997 [17].

In dentistry, ethanolic extract of GML and its purification, as α mangostin has been investigated as an anticaries [17]. How about the anti-*C. albicans* activity? Is it a potent agent as an anti-Candida activity? According to Kaomongkolgit *et al.*, α mangosteen from GML pericarp extract has the antifungal effect [12]. In this study, we evaluated the antifungal effect of ethyl acetate fractionation from the ethanol extract of GML pericarp, it showed interesting data (Table 1). The ethyl acetate fractionation of GML pericarp can inhibit the growth of *C. albicans* below 50% after 60 minutes exposure, in 2000 ppm concentration, and with IC₅₀ around 1900 ppm. Our study confirmed ethyl acetate fraction from

the ethanolic extract of GML has the potential effect to *C. albicans*. The previous study showed IC₅₀ of α mangostin isolated from ethyl acetate GML pericarp extract was > 200 μ M [7]. Our result IC₅₀ is higher than the previous study, because of the different research method. Besides, the secondary metabolites factors could be different, according to the origin of the mangosteen tree.

In our study, we also analyzed the toxicity to HaCaT cells because it is important as a basis data for treatment oral candidiasis in the oral mucosa. Ethyl acetate fraction from the ethanolic extract of GML pericarp was not toxic to HaCaT cell line for 24 hrs at concentration 500 ppm, 1000 ppm, and 2000 ppm [Table 3]. The previous study only analyzed of alpha mangostin on human gingival fibroblast cells demonstrated, it was not toxic and low dose used [12].

In our research, dose fraction of the extract used requires high doses, caused of the use of fraction extract, whereas when using isolated bio-component smaller doses required. As previous study by Aisha AFA, showed the lower dose of GML extract (81% α mangostin and 16% γ mangostin) has potent cytotoxicity as anticancer^[19] and also other studies with α mangostin and γ mangostin just required lower doses to be used as anti-inflammation, antioxidant [8], and antibacterial [20]. The used of dose extracts or fraction of medicinal plants is generally higher than the doses bio-component isolation. The use of dose extracts of medicinal plants is generally higher than the doses of the bio-component insulation because the extracts and fraction still contain other bio-components bound to the solvent used.

CONCLUSION

Our finding suggests ethyl acetate fraction from the ethanol extract of GML as a potential oral anti-*C. albicans*, and safe to HaCaT cell line. It is an alternative way to utilize antifungal effect from GML pericarp. However, further studies are needed, particularly on different time exposure of *C. albicans*, and acute toxicity test.

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