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RESEARCH ARTICLE

Cosmos caudatus Leaf Ethanol Extract Inhibit Growth and Viability through Membrane Permeability and Apoptosis in *Candida albicans*

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Abstract

Objective: Candidiasis is the most common fungal gynecological disease in humans. Essentially, the use of antifungal such as Fluconazole has reportedly increased resistance to candidiasis by 7%. The flavonoid has been shown to be used as an antifungal in *Candida albicans*. *Cosmos caudatus* contains 51% of Quercetin. To prove the influence of the use of flavonoid antifungal of quercetin group on the growth, viability, membrane permeability, and apoptosis of the *C. albicans*. Methods: The SV-1148 coded *C. albicans* isolate was obtained from the Microbiology Laboratory of Brawijaya University, Malang, Indonesia. Initially, the isolate was grown in Sabouraud Dextrose Agar (SDA) medium. The samples were divided into negative and positive control groups of fluconazole (60µg/ml) and difference concentrations (5%, 10%, 20%, and 40%) of leaves ethanol extract of *C. caudatus*. Results: Concentration of 40% ethanol extract *C. caudatus* has Minimum Inhibitory Concentration (MIC) of 30%. This result showed that 40% ethanol extract *C. caudatus* was able to provide the best growth resistance which was characterized by the absence of colonies from *C. albicans*. The data of Influx Assay Iodide increased membrane permeability of *C. albicans* to disrupt cell membranes and induce apoptosis. The percentage of apoptosis increased after treatment of 40% ethanol extract *C. caudatus* in the culture of *C. albicans*. Conclusion: *C. caudatus* has antifungal activity and can be used as an herbal drug in *C. albicans* infection.

Keywords: Antifungal activity, *Cosmos caudatus, Candida albicans*, Apoptosis.

Introduction

Candidiasis is a fungal infection caused by *Candida albicans (C. albicans)* [1]. The most common treatment for candidiasis is through the administration of fluconazole antibiotics [2]. Irregularities and length of time for the use of fluconazole leads to drug resistance and treatment failure [3]. About 7% of *Candida sp* were tested by the Central for Disease Control and Prevention (CDC) shown resistance to fluconazole [4]. This encourages the discovery of anti-fungal sources that can be used as anti-candida. *Cosmos caudatus* is one of the most widely used herbs as an antifungal. *C. caudatus* leaves contain 51% quercetin which is the main content of flavonoids [5]. Flavonoids have lipophilic properties that can disrupt microbial membranes, induce apoptosis, DNA fragmentation, damage mitochondria and accumulation of Reactive Oxygen Species (ROS) [6]. Quercetin and Catechin flavonoids also increase condensation and cell nucleus fragmentation [7]. The purpose of this study to determine the effect of *C. caudatus* leaf ethanol extract on growth and viability, membrane permeability and apoptosis of *C. albicans*, and find the effective dose of *C. caudatus* ethanol extract to suppress cell growth of *C. albicans* culture.

It is hoped that *C. caudatus* can be used as a more economical alternative medicine, and minimize resistance to antifungal antibiotics.

Materials and Methods

The Culture of *C. albicans* and Treatment

SV-1148 coded Candida albicans isolate was obtained from the Microbiology Laboratory of Brawijaya University, Malang, Indonesia. The *C. albicans* isolates were planted on Sabouraud Dextrose Agar (SDA) medium and incubated for 48 hours at 37°C. The sample groups were divided into negative and positive control groups of fluconazole (60µg/ml) and the treatment group with a different concentration of *C. caudatus* ethanol extract (5%, 10%, 20%, and 40%) [8]. For the hemolysis test, blood samples were taken from healthy volunteers.

Growth and Viability Assay

Various *C. caudatus* extract concentrations were prepared in the Sabouraud Dextrose Broth (SDB) medium for multiple repetitions in 96 well-plates. Each well contained an inoculum of C. albicans isolates 1×10^3 ml-1 and the final volume of SDB media in each well was 200 µl. The free-*C. caudatus* extract well was used as the control well. The microplates were incubated at 35°C for 48 hours and read spectrophotometrically at the 620 nm wave using a microplate reader (Multiskan EX, Thermo Electron Corp. USA). The lowest concentration caused a reduction in absorbance of 50% lower than control wells was considered as Minimum Inhibitory Concentration (MIC) [9].

The number of colonies were counted to know the effect of the viability. The *C. albicans* from each well were diluted to obtain 300 to 400 colonies and the samples were distributed to the SDA plate. The plate was incubated at 30°C for 24 hours and the number of the colony were counted then. The treated *C. albicans* viability percentage was calculated by comparing the number of colonies in the control well [10].

Propidium Iodide Influx Assay

Propidium Iodide (PI) staining was carried out to quantitatively analyze the loss of fungal cell membrane integrity. *C. albicans* (10^6) was grown in SDB media in Eppendorf. After that *C. albicans* was given treatment in accordance with the provisions of the sample. The sample were then incubated for 24 hours at 28°C. After the incubation process, the *C. albicans* isolates were centrifuged and suspended with Phosphate-buffered saline (PBS) and given PI 50 µg/ml for 25 minutes. After that, the isolates were analyzed by using flow cytometer [11].

Apoptosis Assay

C. albicans isolates (2 x 10^6/mL) were incubated in SDB for 24 hours at 30°C. The C. *albicans* isolates were harvested by centrifuging and washed in 0.1 M potassium phosphate buffer. Annexin V/propidium iodide (PI) tests were carried out according to the staining kit protocol, using 5 µg annexin V and 5 µg PI at 37°C for 20 minutes, then analyzed using by flow cytometer [12].

Statistic Analysis

All experiments were carried out in triplicate, and the results were presented as mean ± standard deviation (SD). Analysis of significant differences was carried out by one-way ANOVA analysis. Comparative tests were carried out using the Tukey HSD test and the Mann Withney test, to find out which treatment group had significant differences. The difference at $p < 0.05$ was considered significant. Statistical data analysis was performed with the Statistical Version of SPSS 25.0 software package (SPSS Inc, Chicago IL, USA).

Result

The Growth and Viability of *C. albicans*

The *Minimum Inhibitory Concentration* (MIC) concentration was measured by spectrophotometry to read the absorbance of each well. MIC determination was measured by calculating the fungal inhibition percentage which can be calculated by the following formula:

$$\% \text{ MIC } = \left(1 - \frac{OD\ MSJ - OD\ MS}{OD\ MPJ - OD\ MP}\right) \text{x } 100\%$$

MIC calculation results with inhibitory ability to *C. albicans* are presented in Figure 1. The 40% *C. caudatus* extract had 72.2% MIC, indicated the best growth inhibition compared to other concentration. The concentration of 5% *C. caudatus* extract shown no inhibition zone with 337.6% MIC value. 60 µg/ml Fluconazole shows 0% MIC value due to the fungistatic characteristic of fluconazole. The results of this data indicated that *C. caudatus* has an antifungal effect on *C. albicans*.

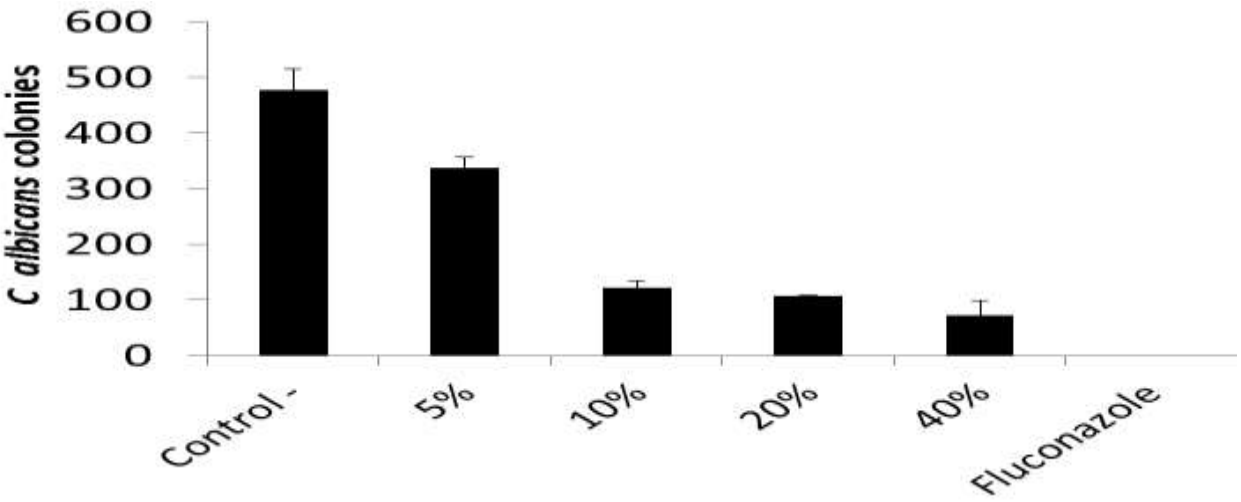


Fig. 1: The percentage of *Minimum Inhibitory Concentration* (MIC) of *C. albicans* after being given *C. caudatus* leaf extract with concentrations of 5%, 10%, 20% and 40%

The treatment of *C. albicans* samples after administration of *C. caudatus* leaf extract with various concentrations were incubated 24 hours at 30°C incubator.

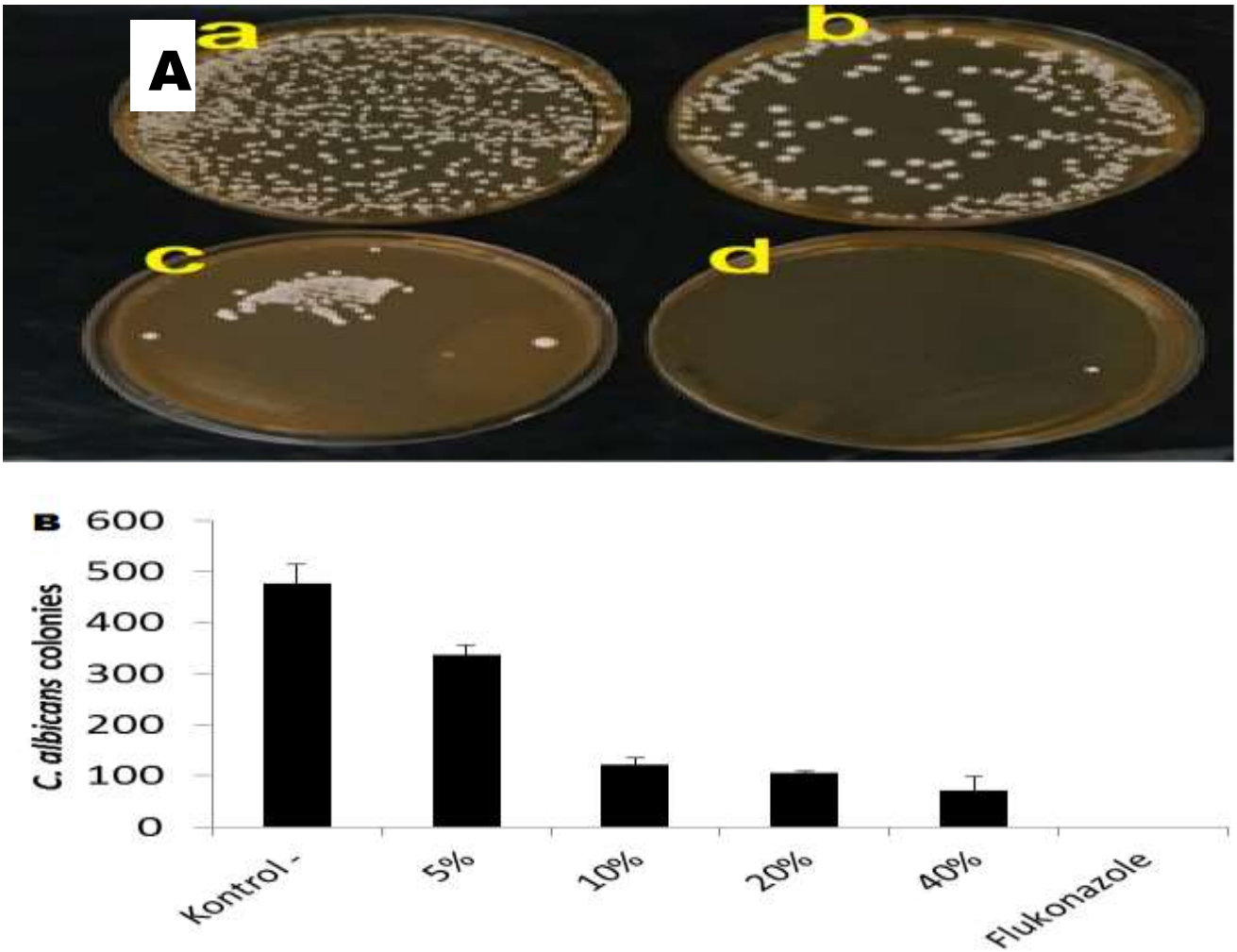


Fig. 2: A. The growth of C. *albicans* colonies in SDA post treated of (a) 5%, (b) 10%, (c) 20% and (d) 40% C. caudatus leaf extract. B. The total viability of C. albicans colonies post treated with various concentration of *C. caudatus* extract were calculated by using automatic colony counter

The Figure 2 shown the rapid growth of *C. albicans* colonies of 5% *C. Caudatus* extract, therefore, the colonies became uncountable. The concentration of 10% *C. caudatus* extracts produced *C. albicans* as much as 849.100,80 CFU/plate while 20% *C. Caudatus* extract resulted in 30.144,00 CFU/plate. These result indicated the viability of *C. Albicans* decreased in a dose-dependent *manner*. The concentration of 40% *C. caudatus* extract can inhibit the growth of *C. albicans* which was characterized by the absence of colony growth. This data suggest that *C. caudatus* extract has antifungal activity. The analysis of one-way ANOVA test p = 0,000 (p <0.05) showed a significant difference between the treatment and the control groups. Post Hoc test and Tukey HSD showed *C. albicans* colonies of 40% and 20% *C. caudatus* extract significantly decrease compare to control group. This data suggested that the inhibitory effect was progressively increased correlated with concentration of *C. caudatus* leaf extract. Our study showed that 40% *C. caudatus* extract has inhibitory capacity of the growth of *C.albicans* almost similar to fluconazole group.

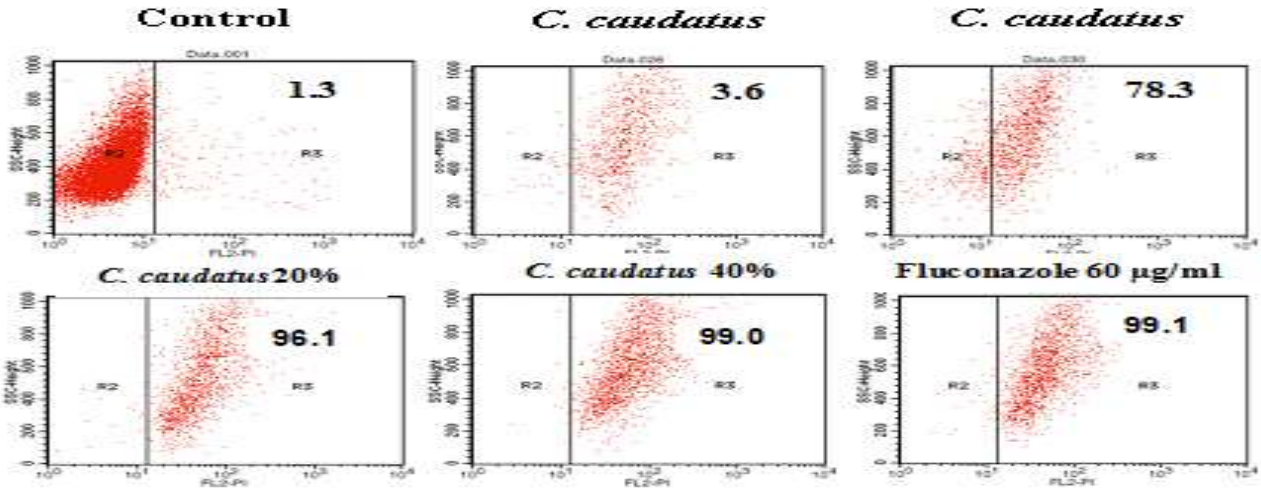
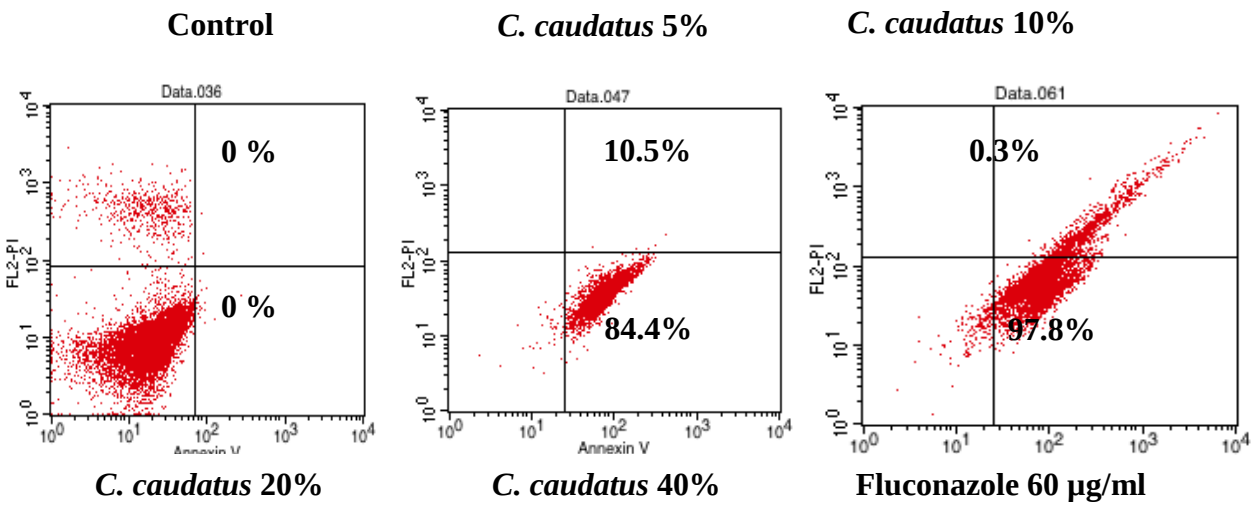


Fig. 3: The percentage of membrane permeability of *C. albicans* after being given *C. caudatus* leaf extract with concentrations of 5%, 10%, 20% and 40%

The difference concentrations of *C. albicans* lead to changes in membrane permeability. Furthermore, flow cytometry analyzed by using Propidium Iodide (PI) showed there were differences concentration of *C. caudatus* leaf extract could enhance membrane permeability of *C. albicans*. The One Way ANOVA test showed significant results with a value of p = 0.000 (p <0.05). The results showed the membrane permeability of *C. albicans*t treated with 40% *C. caudatus* leaf extract significantly increase compare to control group. This result suggested that membrane permeability also increased in a dose-dependent manner of *C. caudatus* leaf extract. Thus, 40% *C. caudatus* leaf extract was the most effective concentration to increase the membrane permeability of *C. albicans*.



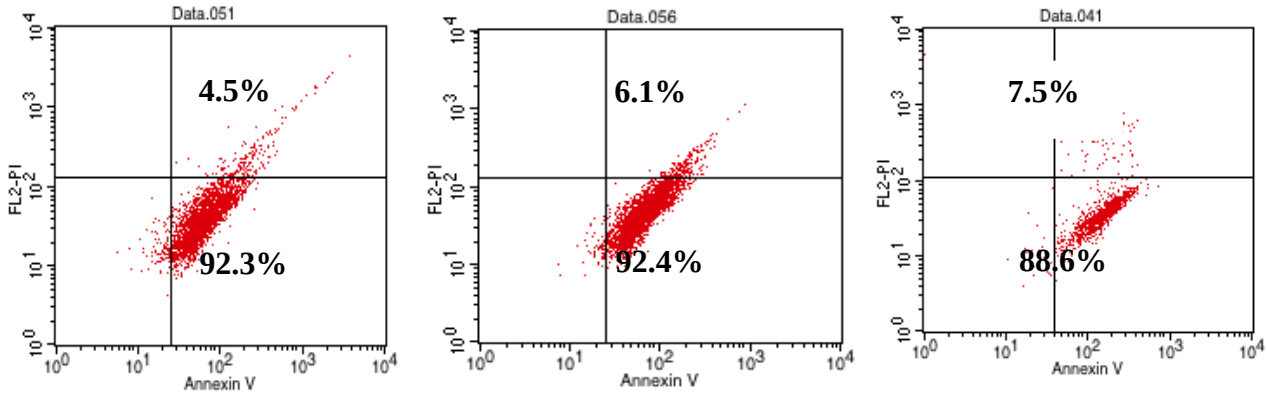


Fig. 4: The apoptosis of *C. albicans* after being given *C. caudatus* leaf extract with concentrations of 5%, 10%, 20% and 40%

The results of flowcytometry showed the percentage of early apoptosis and late apoptosis of different concentrations of *C. Caudatus* extract (84.4% and 10.48%; 97.83%, and 0.28%; 4.49%, 92.32%; 6.06% and 92.36%, and at the fluconazole concentration of 88.6% and 7.5%. One-way ANOVA statistical tests were carried out after the data normality test using the Shapiro-Wilk test and homogeneity test. The Shapiro-Wilk test showed the value of Asymp. Sig (p) 0.662 was higher than 0.05 (p> 0.05) which means normal distribution.

The One Way ANOVA test results showed significant results p = 0.000 (p <0.05), which means that there was a significant difference between C. caudatus leaf extract groups. The results of the comparison of the Post Hoc test showed a significant results between *C. Caudatus* extract concentration. This data indicated that the concentration of 40% of C. caudatus extract was as effective as the positive control group, so it could increase the percentage of apoptosis in *C. albicans*.

Discussion

This study was conducted to determine the value of Minimum Inhibitory Concentration (MIC) of C. caudatus leaf extract on the growth of *C. albicans*. C. *caudatus* leaves can be used as natural alternative ingredients that are appropriate, safe and effective to inhibit fungal growth, especially *C. albicans*. The antifungal activity of *C. caudatus* extracts was observed at concentrations of 5%, 10%, 20% and 40%, to determine the effectiveness of different dose intervals. The effectiveness of *C. caudatus* in inhibiting the growth of *C. albicans* caused by bioactive compounds including flavonoids, carbohydrates, phenolics, minerals, proteins and vitamins in C. *caudatus* [13]. Flavonoids contain quercetin has effect to eliminate cell viability. This is in line with in vitro study of flavonoid compounds in Scutellaria Baicaleins roots which showed that the MIC values for each strain and at 260 mg/ml resulted in loss of viability of fungal colonies compared to the untreated control group [14]. *C. caudatus* contains 52.2 $\pm$ 4.06 mg of flavonoids per 100 grams [5]. Its biological activity is closely related to hydroxyl groups or phenolic rings.

Phenolic compounds are able to associate with bacterial proteins and complexesmembranes form. The antibacterial mechanism of flavonoidsto inhibit nucleic acid synthesis, disrupt the function of the cytoplasmic membrane, inhibit metabolism, and peptidoglycan synthesis. Quercetin, a type of flavonoid, has been shown to be able to inhibit Gram-positive and Gram-negative bacteria through inactivation of extracellular proteins. The anti-bacterial mechanism of quercetin is affected by membrane disruption and inactivation of extracellular proteins by forming irreversible complexes [15].

Membrane permeability is a sign of cell death associated with membrane changes [16]. Isoquercitrin flavonoids can increase the permeability of *C. albicans* cell membranes caused by lipid peroxidation in cell membranes [11]. This results indicated membrane oxidation through the increased accumulation of Reactive Oxygen Species (ROS) which leads to increased permeability of the cytoplasmic membrane. The increasing of membrane permeability causes the cell membrane of *C. albicans* to be disrupted and initiate apoptosis. Quercetin modulate mitochondrial function effect mitochondrial membrane potential and the electron transport chain and ATP production, and ultimately inhibit or induce apoptosis [17].

Mitochondria not only participate in fungal apoptosis but also function as the main determinant of life and cell death [18]. Research conducted by Dai *et al*. (2009) monitored variations in mitochondrial membrane potential, which is a direct indicator of mitochondrial function. During apoptosis in yeast, the production of ROS triggers mitochondrial dysfunction.

This mitochondrial dysfunction causes depolarization of the mitochondrial membrane and translocation of pro-apoptotic factors from mitochondria to the cytoplasm (cytochrome c) or to the nucleus (Aif1p and Nuc1p) [19]. This research showed that quercetin caused mitochondrial dysfunction through the production of ROS, thus causing cell death by apoptosis. Therefore, mitochondria play an important role in apoptosis induced by *C. caudatus*.

Conclusion

In this study, the mechanism of antifungal activity of C. *caudatus* was indicated by growth inhibition and membrane permeability leading to apoptosis *C. albicans*. This study improve the understanding of quercetin flavonoids as antifungal and show that *C. caudatus* has the potential to be applied as an alternative drug for candidiasis in humans.

Ethical Clearence

All experimental procedures were approved by the Ethics Committee of the Medical Faculty of Brawijaya University, Malang, Indonesia number 166/EC/KEPK/05/2019.

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