The effect of ethanol extract of *Cosmos caudatus* leaves on the percentage of the cell cycle in *Candida albicans* culture

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Abstract

Candidiasis is a common fungal gynecological disease among humans. The use of antifungal agents, such as Fluconazole, has been reported to increase resistance to candidiasis by 7%. This study aimed to investigate the effect of antifungal flavonoids from *Cosmos caudatus* leaf extract on the cell cycle percentage in *C. albicans* culture. This research employed a true experimental post-test only with a control group design. The C. albicans isolate was obtained from the Microbiology Laboratory under the code *C. albicans* SV-1148. The isolates were cultured in Sabouraud Dextrose Agar (SDA) medium and Sabouraud Dextrose Broth (SDB). The sample group was divided into a negative control group, a positive control group with fluconazole (60 µg/ml), and a treatment group with various concentrations of ethanol extract from *Kenikir* leaves (*C. caudatus* Kunth.) - 5%, 10%, 20%, 40%, and combinations (20% ethanol extract from *Kenikir* leaves + 30 µg/ml Fluconazole). This study revealed a reduction in the percentage of cell cycles in the S phase (DNA synthesis) in the treatment group receiving ethanol extract from *Kenikir* leaves (C. *caudatus* Kunth.) and the combination treatment group compared to the negative control group. The study suggests that this decrease in the percentage of cell cycles results from DNA damage caused by the presence of flavonoids in *Kenikir* leaves (C. *caudatus* Kunth.). *Kenikir* leaves (C. *caudatus* Kunth.) have the potential to decrease the percentage of S-phase cell cycles (DNA synthesis) in the culture of C. *albicans*. This research demonstrates that *Kenikir* leaves (C. *caudatus* Kunth.) contain natural flavonoids with antifungal properties and have the potential to be used as an alternative medicine for candidiasis in humans. It is hoped that incorporating Kenikir as a food ingredient can serve as an alternative prevention and treatment approach for candidiasis.

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Key word: herbs; antifungal activity; *C. caudatus*; *C. albicans*; cell cycle.

Contributions: LL Conceptualization, Data Curation, Formal Analysis, Methodology, Validation, Visualization, Writing – Original Draft, Review & Editing; HS Methodology, Visualization, Writing – Review & Editing.

Conflict of interest: the authors declare no conflict of interest.

Ethics approval and consent to participate: the research has received ethical approval from the Ethics Committee, Medical Faculty, Universitas Brawijaya, Indonesia based on ethical certificate 166/EC/KEPK/05/2019. During the research, the researcher pays attention to the ethical principles of information to consent, respect for human rights, beneficence and non-maleficence.

Patient consent for publication: written informed consent was obtained for anonymized patient information to be published in this article.

Funding: this research did not receive external funding.

Availability of data and materials: all data generated or analyzed during this study are included in this published article.

Received: 11 September 2023.
Accepted: 2 October 2023.
Early access: 12 October 2023.

Healthcare in Low-resource Settings 2023; 11:11754
doi:10.4081/hls.2023.11754

Introduction

Fungal infections claim the lives of over 1.5 million people and afflict more than one billion individuals.[1] Serious yeast infections arise as a consequence of other health conditions, including asthma, AIDS, cancer, organ transplantation, and corticosteroid therapy.[2] Early and accurate diagnosis permits the immediate initiation of antifungal therapy.[3] Nonetheless, the administration of this therapy is frequently delayed, and the unavailability of treatment can result in severe chronic illnesses. Recent global estimates have identified 700,000 cases of invasive candidiasis.[4] The prevalence of candidiasis is reported to be twenty-two percent (22%), and this percentage remains consistent among adult women and female adolescents.[5,6] Furthermore, it is estimated that approximately 75% of women will experience at least one candidiasis episode in their lifetime.[7]

C. albicans is the species most commonly implicated in cases of candidiasis.[8] Candidiasis is often associated with the production of a thick, white, cream, or yellow discharge from the vaginal canal.[9] The primary and preferred treatment for Candidiasis is an antifungal drug, specifically Fluconazole.[8,10] The mechanism of action of the azole class of antifungal drugs involves the disruption of cell membranes by inhibiting the activity of lanosterol

14-α-demethylase, an enzyme essential for ergosterol biosynthesis.[11] Prolonged use of this drug has led to resistance against Fluconazole in C. albicans, despite its high cure rate.[12] According to a 2017 survey conducted by the Centers for Disease Control (CDC), resistance to *Candida* spp., specifically at a rate of 6.5%, was observed.[13]

Research on plants used in traditional medicine aims to identify alternative treatments, and numerous antimicrobial properties have been identified in natural ingredients used as herbal remedies.[14] According to Hayat *et al.* (2017), flavonoids exhibit antimicrobial activity against various strains of microorganisms, including Staphylococcus aureus.[15] Flavonoids have the ability to form non-specific bonds such as hydrophobic, hydrogen, and covalent bonds with proteins. They have also been studied for their lipophilic properties, which can disrupt microbial membranes.[16]

Research conducted by Han *et al.* (2016) revealed that flavonoids inhibit the cell cycle of C. albicans in the S phase when induced by Rubus chingii.[17] The synergistic activity is attributed to alterations in membrane fluidity, increased Fluconazole influx, interference with membrane-bound signaling proteins, and cell cycle arrest. Inhibition of the cell cycle in the S phase, specifically during DNA synthesis, is a response to DNA damage. Changes in the cell cycle are closely tied to DNA damage. The presence of chemical compounds in herbal extracts can induce DNA damage, leading to cell cycle arrest and apoptosis. During the S phase checkpoint, two key aspects are examined: the cell's adequate size for division into two individual cells and the accurate duplication of DNA replication.[18,19]

The content of flavonoids in *Kenikir* leaves measures 52.2±4.06 mg per 100 grams.[20] *Kenikir* (*C. caudatus* Kunth) is among the most commonly found plants in Indonesia. Research conducted by Rasdi *et al.* (2010) revealed that *Kenikir* (C. *caudatus* Kunth) can function as an antimicrobial agent against bacteria such as Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas aeruginosa, and C. albicans.[21] It is imperative to conduct in vitro testing for anti-Candida activity before experimenting on animals to assess the potential toxic effects of natural ingredient extracts directly on specific cell types or tissues within a controlled environment and a short timeframe.[22] Furthermore, in vitro tests, as highlighted by Visvesvara and Garcia (2002), are employed to identify potential therapeutic agents. This study demonstrates the impact of antifungal flavonoids from C. caudatus leaf extract on the cell cycle percentage in C. *albicans* culture.[23]

Materials and Methods

Design of the study

This research employs a true experimental research design, specifically a posttest-only control group design.

Manufacture of Kenikir leaf ethanol extract

The process of obtaining *Kenikir* leaf powder extract involves two stages: maceration and evaporation. In the maceration stage, finely ground *Kenikir* leaves, totaling 500 grams, are placed in a glass jar. Subsequently, 1 liter of 90% ethanol is added, and the mixture is stirred for 30 minutes to ensure thorough mixing. It is then left to settle for one hour, as recommended by Aswanida (2015) and Asworo (2017).[24,25] The maceration process is conducted at a temperature of 55°C. Afterward, the top portion of the ethanol-solvent mixture, containing the active substance, is separated using Whatman number 2 filter paper. The remaining active substance-solvent mixture proceeds to the evaporation stage.

In the evaporation stage, the filtered mixture is transferred into an evaporating flask, connected to a rotary evaporator and a water bath heater. Water is added to the water bath, and electricity is supplied with a temperature setting of 78.4°C. The separation of the ethanol solvent from the active substance in the evaporating flask continues until no more ethanol drips into the holding flask. Typically, this results in one flask containing approximately 900 ml of solution. The filtered solution is then further evaporated until the *Kenikir* leaf ethanol extract solidifies. The final yield obtained is 70 mL of *Kenikir* leaf ethanol extract paste.

The culture of C. *albicans* and treatment

C. *albicans* isolate (SV-1148) was procured from the Microbiology Laboratory of Brawijaya University in Malang, Indonesia. A smear of C. albicans (SV-1148) was obtained from a 36-year-old patient at Saiful Anwar RSSA Hospital in Malang. The C. *albicans* isolates were cultured on Sabouraud Dextrose Agar (SDA) medium and incubated for 48 hours at 37°C. The sample groups were divided into a negative control group, a positive control group treated with fluconazole (60 µg/mL),[26] and a treatment group subjected to ethanol extraction from C. caudatus leaves at concentrations of 5%, 10%, 20%, and 40%.[27]

Cell cycle assay

C. *albicans* isolates (1×10^3 cells), cultured in Sabouraud Dextrose Broth media, were harvested. Following 24 hours of incubation, the cells were washed with PBS three times and centrifuged at 3500 rpm for 3 minutes. Subsequently, they were fixed with 70% ethanol for 24 hours at 4°C [28]. Following the fixation, 50 µl of RNase A at a concentration of 200 µg/mL was added to the cells, and the mixture was allowed to react for 2 hours at 37°C. For DNA staining, 50 µg/mL of propidium iodide was introduced, and the mixture was incubated for 30 minutes at 4°C in the dark. The cells were then analyzed using a flow cytometer. The flow cytometers utilized in this research included the BD CellQuest flow cytometry machine, BD CellQuest Pro software (version 5.1 or higher), and BD™ Inits software (version 4.1 or higher).

Statistical analysis

All experiments were performed with four replicates as per the replication formula and sample size calculation. The data were assessed for homogeneity of variance using the Levene index and expressed as the mean ± standard deviation. The results of the normality test using the Shapiro-Wilk test yielded a $p>0.05$, indicating that the data is normally distributed. Furthermore, the data homogeneity test produced a p-value of 0.208 ($p>0.05$), indicating that the data possesses uniform variance. Based on these findings, statistical analysis proceeded with a one-way ANOVA test. Significant differences were analyzed through one-way analysis of variance (ANOVA). All statistical analyses were conducted using SPSS 25.0 (SPSS Inc., Chicago, IL, USA), with a significance level of $p<0.05$ considered statistically significant, and $p<0.01$ considered highly statistically significant. Ethical approval for this research was obtained from the Ethics Committee of the Medical Faculty at Universitas Brawijaya, Indonesia, under ethical certificate 166/EC/KEPK/05/2019. Throughout the research, the researcher adhered to ethical principles, including informed consent, respect for human rights, beneficence, and non-maleficence.

Results

The administration of *Kenikir* leaves (C. *caudatus* Kunth.), ethanol extract, fluconazole, and combination dosages resulted in a decrease in the percentage of cell cycles in the C. albicans culture. Specifically, the ethanol extract of *Kenikir* leaves (C. *caudatus* Kunth.) was effective in inhibiting the cell cycle in the S phase (DNA Synthesis). This inhibition was assessed using PI and RNAse A to detect changes in the cell cycle of C. *albicans* following the administration of the ethanol extract of Kenikir leaves (C. *caudatus* Kunth.).

The results, as depicted in Figure 1, indicate that ethanol extracts of Kenikir leaves (C. *caudatus* Kunth.) at various concentrations (5%, 10%, 20%, 40%), as well as in the combination group (C. *caudatus* Kunth. 20% + Fluconazole 30 µg/mL), effectively inhibited the cell cycle in the S phase (DNA synthesis) noted as "M2." This inhibition is demonstrated by a reduction in the percentage of the cell cycle in the S phase (DNA synthesis) in the C. *albicans* culture when compared to both the negative control and positive control groups.

In Figure 2, the average percentage of inhibition of the S-phase cell cycle (DNA synthesis) in the C. *albicans* culture is shown to decrease in the treatment group compared to the negative control and positive control groups. The lowest percentage of inhibition of the S phase cell cycle, measuring 1.16±0.38, was observed in the treatment group with a concentration of ethanol extract of Kenikir leaves (C. *caudatus* Kunth.) at 20%. In contrast, the highest percentage of S phase cell cycle inhibition was found in the negative control group, with a value of 25.4±1.61. Based on Figure 2 and the Tukey HSD test, it can be concluded that the concentration of ethanol extract of *Kenikir* leaves (C. *caudatus* Kunth.) significantly responsible for decreasing the percentage of the S phase cell cycle (DNA synthesis) in the C. *albicans* culture was a concentration of 10%. This is supported by the significant difference ($p<0.05$) when compared to both negative controls and positive controls.

Discussion

The objective of this study was to assess the impact of ethanol extracts from *Kenikir* leaves (C. *caudatus* Kunth.) as antifungals on the reduction of the cell cycle percentage in C. *albicans* culture. *Kenikir* leaves (C. *caudatus* Kunth.) are explored as potential natural alternatives for the safe and effective treatment of candidiasis. The antifungal activity of these extracts was examined at concentrations of 5%, 10%, 20%, and 40%, as well as in combination with fluconazole (20% ethanol extract + 30 µg/mL fluconazole). Based on the study results and subsequent statistical analysis, it was observed that the administration of ethanol extracts from Kenikir leaves (*C. caudatus* Kunth.) at different concentrations (5%, 10%, 20%, and 40%), along with the combination treatment group (20% ethanol extract of Kenikir leaves + 30 µg/mL fluconazole), significantly inhibited the cell cycle in the S phase. Notably, Kenikir leaves (*C. caudatus* Kunth.) were found to contain approximately 52.2 ± 4.06 mg of flavonoids per 100 grams, as documented by Andarwulan *et al.* (2010).[29] When comparing the positive control group to the *Kenikir* leaves ethanol extract treatment group at a 5% concentration, no significant difference was observed in reducing the percentage of the S phase in the cell cycle. However, significant differences were noted when compared to the negative control group and the treatment groups with ethanol extract concentrations of 10%, 20%, and the combination treatment group (20% ethanol extract of *Kenikir* leaves + 30 µg/ml fluconazole). Among the various concentrations tested, the concentration of 10% ethanol extract of *Kenikir* leaves (C. *caudatus* Kunth.) demonstrated significant effectiveness in reducing the percentage of the S phase cell cycle (DNA synthesis) in C. *albicans* cultures. This was supported by statistically significant differences ($p<0.05$) when compared to both the negative and positive control groups. Notably, the combination treatment group (20% ethanol extract of *Kenikir* leaves + 30 µg/ml fluconazole) exhibited the lowest decline in the percentage of the cell cycle in the S phase.

These findings align with prior research conducted by Jung *et al.* (2007) and Han *et al.* (2016).[30,31] Han *et al.* (2016) demonstrated inhibition of the S-phase cell cycle (DNA synthesis) in C. *albicans* when treated with a combination of Fluconazole and Rubus chingii extract, which contains flavonoids as antimicrobials. This is consistent with the biochemical compounds found in *Kenikir* leaves (C. *caudatus* Kunth.). Similarly, Jung *et al.* (2007) isolated resveratrol from ethyl acetate extract from grape skins, showing that resveratrol, a type of flavonoid, inhibits the S phase of the C. *albicans* cell cycle.[32]

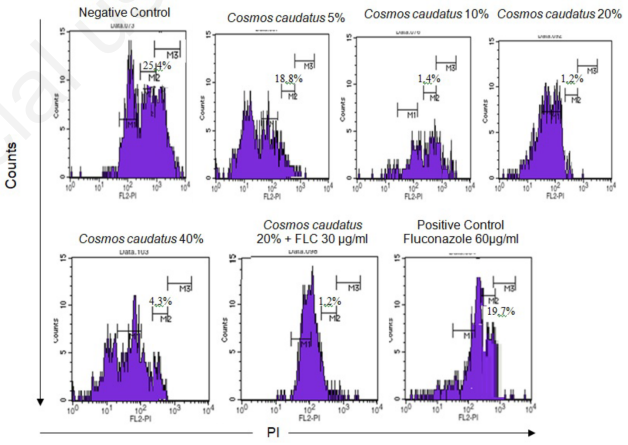

Figure 1. Percentage results of *C. albicans* cell cycle with flowcytometry analysis.

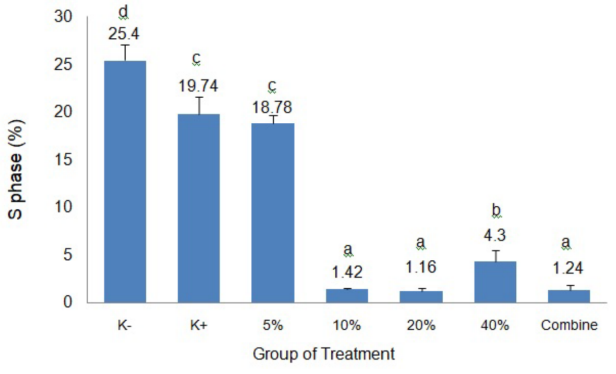

Figure 2. Histogram of the average percentage of *C. albicans* cell cycle in S. Phase.

This anti-Candida activity is mediated by cell entry via a transport system that requires the consumption of ATP. The action of resveratrol as a toxic agent in fungal cells induces several intracellular physiological changes, which are indicated by the inhibition of the cell cycle and the accumulation of trehalose (a class of carbohydrates) which is synthesized as an energy source, to survive in freezing conditions and lack of water. It can be concluded that resveratrol inhibits the process of cell division and affects the growth of fungal cells.[32,33]

According to Iyer and Rhind (2017),[34] cell cycle inhibition in the S phase (DNA synthesis) has been found in some organisms as a response to DNA damage. DNA can be damaged by extrinsic and intrinsic factors. Extrinsic factors that can damage DNA include ultraviolet light (UV, ionizing radiation (IR), and chemicals such as methyl-methane sulfonate (MMS), mitomycin C, cisplatin, psoralen, camptothecin (CPT), and etoposide.[34] Intrinsic factor wrong one of them is reactive oxygen species (ROS) which is produced as a product of cell metabolism, which can cause oxidative damage to DNA.[34] Changes in the cell cycle are closely related to DNA damage. The administration of extracts containing flavonoids can cause DNA damage in the presence of excessive accumulation of ROS, leading to cell cycle arrest and apoptosis.[31] Checkpoint activation is in response to faulty replication in mitosis, thus being one of the important reasons why cells lacking S phase checkpoints die. The S phase checkpoint is very important for chromosome replication in yeast.[35] In the process of the cell cycle, different checkpoints ensure that cellular events occur in the correct order and time. The first checkpoint is located at the end of the G1 phase, just before entering the S phase. There is an important checkpoint before the cell cycle enters the mitotic phase (checkpoint G2 / M), similar to the G1/S transition. In the S phase (DNA synthesis), there are two aspects examined, namely the cell is sufficiently sized to divide into two individual cells, and the DNA has been duplicated correctly.[36] Cell size is known to influence cell cycle regulation as a cell cycle checkpoint. Dysfunction of cell volume regulation leads to cell cycle arrest, leading to apoptosis.[37] Inhibition of the cell cycle in *C. albicans* can cause morphological changes, namely cells that are inhibited in the G1 phase tend to be more like hyphae, while inhibition in the S, G2, and M phases leads to cells like pseudohyphae.[38]

In this study, whole extract from *Kenikir* leaves *(C. caudatus* Kunth.) was used, which contains flavonoids, but the total flavonoid content in the ethanol extract of *Kenikir* leaves (*C. caudatus* Kunth.) has not been measured.

Conclusions

In this study, the mechanism underlying the antifungal activity of ethanol extracts from *Kenikir* leaves (C. *caudatus* Kunth.) is elucidated through the observed reduction in the cell cycle percentage. This research underscores that *Kenikir* leaves (C. *caudatus* Kunth.) are rich in natural compounds known as flavonoids. Flavonoids are believed to be the primary constituents responsible for the antifungal properties exhibited by *Kenikir* leaves, thus holding promise as a potential alternative medicine for the treatment of candidiasis in humans.

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