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# Anthocyanins in Mulberry Leaves (*Morus rubra L.*) Ethanol Extract as the Inhibitor for the Growth of *Candida albicans*

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## ABSTRACT

Infections caused by *Candida albicans* are generally common in the vaginal mucosa or called *Vulvovaginal Candidiasis*. Herbal medicine is proven to be an alternative to treat vaginal candidiasis. Mulberry leaves have many chemical compounds, one of which is anthocyanins. Anthocyanins have pharmacological benefits and biological activity that can protect against human pathogenic bacteria. This study aims to observe the effect of anthocyanin compounds extracted from mulberry leaves (*Morus Rubra L*) on the growth of *Candida albicans*. Identification of compounds on mulberry leaves used the TLC spectrophotodensitometry on silica gel 60 F254. TLC plates were washed with methanol and activated at 110ºC for 30 minutes. The plates were eluted in a chamber that had been saturated with the mobile phase of n-butanol:glacial acetic acid:water (4:1:2) and transferred using a CMAG TLC densitometer with a spectrum in the wavelength range of 200-700 nm. Design study is experimental study with a short Post-Test Only Control Group. This study was conducted at the Pharmaceutical Biology Laboratory of PGRI Adi Buana University and the Laboratory of Professor Nidhom Foundation. The results showed that there was a content of anthocyanin compounds in mulberry leaves with antifungal function against *Candida albicans*.

*Infeksi yang disebabkan oleh Candida albicans umum terjadi pada mukosa vagina atau disebut Vulvo Vaginal Candidiasi (VVC). Pengobatan secara herbal dibuktikan bahwa dapat menjadi salah satu alternatif untuk mengatasi kondisi kandidiasis vagina. Daun mulberry memiliki banyak senyawa kimia salah satunya adalan antosianin. Anthocyanin dapat melindungi dari bakteri pathogen manusia yang memiliki manfaat farmakologis dan aktivitas biologi. Tujuan penelitian melihat senyawa antosianin dari daun mulberry (Morus Rubra L) terhadap antifungi Candida albicans. Identifikasi senyawa pada daun mulberry (Morus Rubra L) menggunakan alat KLT Spektrofotodensitometri pada plat silika gel 60 F254 yamh dicuci dengan methanol dan diaktifasi dengan suhu 110oC selama 30 menit. Plat akan dielusi pada chamber yang telah jenuh dengan fase gerak n-butanol:asam asetat glasial:air (4:1:2) dan dipindahi dengan menggunakan alat densitometer CMAG TLC spektrum dalam rentang gelombang 200-700 nm. Pengamatan senyawa anthocyanin daun mulberry terhadap antifungi Candida albicans menggunakan desain penelitian eksperimental laboratories dengan pendekatan Post-Test Only Control Group Design. Penelitian dilakukan di Laboratorium Biologi Farmasi Universitas PGRI Adi Buana Surabaya dan Laboratorium Profesor Nidhom Foundation Surabaya. Hasil menunjukan bahwa terdapatnya kandungan senyawa anthocyanin pada daun mulberry (Morus Rubra L) serta memiliki daya antifungi pada biakan Candida albicans.*

**Introduction**

*Candida albicans* is an opportunistic pathogenic fungus that exists as a harmless commensal (a small single-celled living thing that lives with other organisms). About 75% of women experience vaginal infections caused by *Candida albicans* at least once in a lifetime (Sobel, 2007). Infection caused by *Candida albicans* generally affects gastrointestinal epithelial cells, vaginal mucosa, and oropharyngeal mucosa (Schulze & Sonnenborn, 2009). Furthermore, such infection commonly occurs in the vaginal mucosa or so called Vulvovaginal Candidiasis (VVC), and this condition usually may also recur in the vaginal mucosa or so called Recurrent Vulvovaginal Candidiasis (RVVC). Candida species are the most common cause of infections caused by fungi (Meiller et al., 2009)

Treatment and prevention of a disease using natural ingredients called herbs are increasingly preferred by the community (Sharma et al., 2018). The value of herbal medicine lies in the number of chemical substances that produce certain physiological actions in the human body. There are many plants that have been used due to their antimicrobial properties, which are caused by the presence of compounds synthesized in secondary metabolism of the body. Plant extracts and phytochemicals which have antimicrobial properties are known to be very effective in therapeutic treatment (Niratker et al., 2015).

*Morus rubra L* or Red Mulberry is a plant that belongs to the *Moraceae* family which has the characteristics of easy to fall, fast growing, tree height from small to medium to 15-20 m high. *Morus rubra L* or Red Mulberry has been reported to have benefits in the world of health for the antimicrobial, astringent, hypoglycemic, anti-atherosclerotic, ophthalmic, and diuretic activities (Sharma et al., 2018). Antimicrobial activity and compounds in *Morus rubra L* or Red Mulberry are interesting to study and recent studies revealed that anthocyanins can protect against human pathogenic bacteria (Suriyaprom et al., 2021).

Anthocyanin compounds are the glycoside form of anthocyanin compounds and are part of secondary metabolites of Flavonoids. In plants, anthocyanin compounds are responsible for giving red, blue, purple, and other colour (Sharma et al., 2018). There are numerous scientific publications related to the benefits of anthocyanins in the world of health (Djohan et al., 2019). Anthocyanins have been shown to have antioxidant, anti-inflammatory, anticancer, lipid peroxidation and antimicrobial activities (Memete et al., 2022).

The treatment of the pathogenic fungus *Candida albicans* can be performed by chemical compounds that have antimicrobial activity, especially antifungal. Mulberry leaves (*Morus rubra L*) have been analyzed for their phytochemical and anthocyanin compounds. Anthocyanins have been shown to inhibit bacterial growth but have not been proven to inhibit fungal growth in pathogenic condition (Kim & Jang, 2011)

This study aims to determine the content of anthocyanin compounds identified in mulberry leaves extract on the growth of *Candida albicans*. The benefit of the study is the addition of herbal product as an alternative to treat the incidence of flour albus caused by *Candida albicans*.

Based on some of the theoretical studies above, there is a need to conduct studies regarding the anthocyanin compounds in mulberry leaves (*Morus rubra L*) to protect against *Candida albicans*.

## Method

### Extraction of Mulberry Leaves (*Morus rubra L*)

Identification of the inhibitory power of flavonoid compounds in preventing the growth of *Candida albicans* was performed. The agar media was placed in 4 petri dishes as much as 25 ml. Furthermore, 0.5 ml of *Candida albicans* suspension was inoculated into the media and spread evenly in a circular motion so that *Candida albicans* was evenly distributed on the petri dish and waited until the media was solid. Each petri dish that had been inoculated with *Candida albicans* were divided into 8 locations on the agar media to be occupied by paper disks that had been dripped with mulberry leaf extract solution. After the paper disks had been placed in 4 petri dishes and labelled according to the dosage, each paper disk was given drops of mulberry leaf extract using a micropipette according to the dosage label that had been made. The 4 petri dishes were incubated in an incubator. Incubation was carried out at 37oC for 24 hours. After 24 hours, the petri dishes were taken out, observed and measured to find out diameter of the inhibition zone in the form of the bright zone using a ruler (millimeter). The diameter of the inhibition zone showes the antifungal power of each test material.

The next stage was identification of anthocyanin compound using TLC Spectrophotodensitometry. A 60 F254 silica gel TLC plates with a size of 2x10 cm were prepared. The plates were washed with methanol and activated at 110oC for 30 minutes. The type of ethanolic viscous extract obtained was then spotted as much as 10µL on the activated plates using a linomat V spotter. Then the plates were eluted in a chamber that was saturated with the mobile phase of n-butanol:glacial acetic acid:water (4:1:2). The plates were transferred using a CMAG TLC scanner 3 densitometer with a wavelength of 210nm and a spectrum in the wave range of 200-700 nm. The maximum anthocyanin wavelength was determined by taking into account the spectrum of the scanned absorption results in the wavelength range of 200-700 nm. The maximum wavelength appeared from the spectra, namely the wavelength that showed the maximum absorbance value. The plates were transferred back at the maximum anthocyanin wavelength. Identification of anthocyanins was carried out by looking at the Rf value and the spectrum resulting from measurements of the Rf value and anthocyanin spectrum in the literature.

The plates were then re-identified using a spot sighter and color reagents in the form of ammonia (NH3), AICI3 5% and FeCI3 2%. Each plate was identified by a different spotting marker. The first plate was steamed with ammonia, the second plate was sprayed with 5% AICI3 color reagent, while the third plate was sprayed with 2% FeCI3 color reagent. The color changes produced were observed visually under UV light with a wave of 254 nm and under a wave of UV light 366 nm. The plates that had been sprayed were scanned again with a CAMAG TLC scanner densitometer with 3 maximum anthocyanin wavelengths and a wavelength range of 200-700 nm. The chromatogram as well as spectrum change or shift were observed to determined the suspected anthocyanin spot.

**Results**

Mulberry leaves were obtained from the Family Medicinal Plants of the Faculty of Science and Health, Universitas PGRI Adi Buana, Surabaya. Mulberry leaves that had been picked were cleaned, washed, dried in the wind overnight, then dried in the sun. The leaves were further blended to became 100 grams of mulberry leaves simplicia. Furthermore, simplicia extraction of mulberry leaves was carried out using the maceration method. Extraction used 100 grams of mulberry leaves and 400 ml of 70% ethanol solvent. The extraction result was left for 5 days with occasional stirring. The liquid obtained was then filtered and left for 1 day, then filtered again and the filtrate was collected. The filtrate obtained was evaporated to obtain a concentrated extract. Mulberry leaves extract was made in 8 series of dilution, namely 15%, 30%, 45%, 60%, 75%, 80%, 95%, 100%.

The results of identification of mulberry leaves showed that mulberry leaf simplicia contained anthocyanin compounds which were carried out by TLC spectrophotodensitometry on silica gel 60 $F_{254}$. TLC plates were washed with methanol and activated at 110$^{o}$C for 30 minutes. The plates were eluted in a chamber that had been saturated with the mobile phase of n-butanol:glacial acetic acid:water (4:1:2) and transferred using a CMAG TLC densitometer with a spectrum in the wavelength range of 200-700 nm. The maximum wavelength showed the maximum absorbance value. The resulting waves were recorded in the table below:

**Table 1.** Peak Wavelength on Band I and Band II of Mulberry Leaves Extract

| Isolate | Wavelength |
|---|---|
| Band I | 425 - 620 |
| Band II | 275 – 295 |

Based on the results of the table above, it was known that the peak values in band I and II were in the range of 425 – 620 nm and 275 – 295 nm, respectively. Absorption in the wavelength region of 425–620 nm was the absorption of anthocyanin compounds. According to Haeria et al (2016), the maximum wavelength to measure the anthocyanin activity of mulberry leaves extract was 490-535 nm. The extract spectrum data obtained showed the presence of anthocyanin compounds with native groups.

Identification of *Candida albicans* in this study used four isolates derived from vaginal swabs. These *Candida albicans* isolates were obtained from the Microbiology Laboratory, Faculty of Medicine, Brawijaya University, Malang. Prior to being used in this study, the fungus was identified by means of colony culture on Sabouraud Dextrose Agar (SDA) plates, gram staining, and the Germinating Tube Test.

This study aims to determine the inhibition of mulberry leaves (Morus Rubra L) extract on the growth of *Candida albicans* in vitro. The test samples were taken as many as 8 dilution series in 4 petri dishes based on the calculations in the preliminary study. Petri dishes were incubated for 24 hours at 37$^{0}$C in an incubator. Furthermore, calculations were performed to determine the inhibition zones of mulberry leaves (Morus Rubra L) extract using caliper in millimeters. The results are presented in the table below:

**Table 2.** Results of Assessment of Flavonoid Inhibitory Power against the Growth of *Candida albicans*

| Sample | Dilution Series (%) | Mulberry Leaves (*Morus Rubra L*) Extract |
|---|---|---|
| 1 | 15% | 0 mm |
| | 30% | 0 mm |
| | 45% | 0 mm |
| | 60% | 0 mm |
| | 75% | 0 mm |
| | 80% | 11.9 mm |
| | 95% | 12.3 mm |
| | 100% | 14.1 mm |
| 2 | 15% | 0 mm |
| | 30% | 0 mm |
| | 45% | 0 mm |
| | 60% | 0 mm |
| | 75% | 0 mm |
| | 80% | 14.7 mm |
| | 95% | 16,1 mm |
| | 100% | 17.6 mm |
| 3 | 15% | 0 mm |
| | 30% | 0 mm |
| | 45% | 0 mm |
| | 60% | 0 mm |
| | 75% | 0 mm |
| | 80% | 12.3 mm |
| | 95% | 13.2 mm |
| | 100% | 16.5 mm |
| 4 | 15% | 0 mm |
| | 30% | 0 mm |
| | 45% | 0 mm |
| | 60% | 0 mm |
| | 75% | 0 mm |
| | 80% | 12.8 mm |
| | 95% | 14.0 mm |
| | 100% | 15.1 mm |

## Discussion

*Morus rubra L* or Red Mulberry is a plant that belongs to the *Moraceae* family which has the characteristics of easy to fall, fast growing, tree height from small to medium to 15-20 m high. In Indonesia, mulberry in Indonesia is one of the plants that grows wild and the Indonesian people use it quite a bit due to the lack of public awareness of the pharmacological benefits of mulberry plants. The mulberry leaves used in this study were young mulberry leaves. According to previous study, it was shown that the total polyphenol content found in young leaves was higher than that of old mulberry leaves (Jurian et al., 2016; Maharani, 2012).

This study used extract made of mulberry leaves (*Morus rubra L*). The weight of the mulberry leaves used in this study was 500 grams, which were then dried to obtain a powder with a weight of 100 grams, and macerated with 70% ethanol with a volume of 400 ml and filtered three times to obtain a thick extract with a concentration100%. After that, the concentration of the extract was determined by serial thinning to obtain several concentrations, namely 15%, 30%, 45%, 60%, 75%, 80%, 95%, 100%. Of the 8 concentrations, it was found that at concentrations of 80%, 95%, 100%, there was no growth of *Candida albicans*. In contrast, at concentrations of 15%, 30%, 45%, 60%, 75%, there was still growth of *Candida albicans*. The ingredients contained in mulberry leaves were anthocyanins, alkaloids,

flavonoids, and polyphenols. Previous studies revealed that bioactive compounds can be found by extracting these plants. Anthocyanins, alkaloids, flavonoids, and polyphenols can act as antimicrobials Anthocyanins are compounds with antimicrobial activity that can inhibit the enzymes esterase sera DNA and RNA polymerase. In addition, anthocyanins are able to inhibit cell respiration and play a role in DNA intercalation (Siregar, 2004). Anthocyanin compounds work by inhibiting the biosynthesis of fungal nucleic acids, so that the fungus cannot develop and eventually dies (Swari et al., 2020). Furthermore, anthocyanins have genestein compounds that function to inhibit cell division or proliferation. This compound binds to microtubule proteins in cells and causes inhibition of fungal growth (Sharma et al., 2018). ROS (Reactive Oxygen Species) frequently find membrane lipids in *C. albicans* and generate lipid hydroperoxides, lipid peroxidation has been shown to disrupt the lipid bilayer and alter membrane potential, resulting in reduced fluidity, increased permeability, and disruption of phospholipids, which can form and induce membrane disruption, resulting in reduced cell size and leakage of intracellular components of *c.albicans* (Aboody & Mickymaray, 2020).

To find out whether or not there was a significant difference between concentrations, a statistical analysis was performed using the One-Way ANOVA test which showed that there were significant differences between concentrations in the inhibitory power against the growth of *C. albicans*. The test results regarding the antifungal activity of mulberry leaves extract against *C. albicans* showed that 80%, 95%, 100% concentrations of mulberry leaves extract were able to inhibit the growth of *C. albicans* fungus. Quercetin and anthocyanin compounds were contained in ethanol extract of mulberry leaves. Anthocyanins have an original group of phenolic compounds that act as protein coagulators. Phenol groups can bind to bacterial cell membranes in their hydrogen bonds, causing changes in protein structure. Changes in the structure of cell membrane proteins can result in disrupted cell membrane semi-permeability, so that cellular metabolism is disrupted and results in cell death

The results of the study showed that the inhibition was higher at the percentage of 100% mulberry leaves extract. The person correlation test result showed that there was a strong relationship between the variables. The higher the concentration of the extract, the lower the number of *Candida albicans*. The study finding also indicated that the increasing concentration of the extract led to the greater inhibition diameter. Such finding is in line by previous studies which revealed that the effectiveness of an antibacterial substance in inhibiting growth depended on the nature of the test bacteria, the concentration and the length of contact time (Pappas et al., 2004). Based on the results obtained, it was found that mulberry leaves extract at a concentration of 80% was the minimum concentration to inhibit the fungus *C. albicans* (Pappas et al., 2004).

## Conclusions

The results showed that mulberry leaves extract contained anthocyanin compounds as antifungals. In the inhibition test, it was found that administration of mulberry leaves (*Morus Rubra L*) extract significantly reduced the growth of *Candida albicans* with a p value of $0.000<\alpha$. Furthermore, the results showed that the inhibition zone ranged from 11.9 to 17.6 mm. Such finding indicated that the

anthocyanin compounds in mulberry leaves (*Morus rubra L*) extract had strong antifungal property to inhibit the growth of *Candida albicans*.

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