EMBRIO JURNAL KEBIDANAN
p-ISSN: 2089-8789
e-ISSN: 2714-7886

Acute Toxicity Test of Mulberry Leaf Extract (Morus rubra L) as a Basic Ingredient of Candida Albicans Antifungal on Rattus Norvecigus

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ARTICLE INFORMATION

Received: 29, May, 2024
Revised: 1, June, 2024
Accepted: 2, June, 2024

KEYWORD

Acute toxicity; Mulberry leaf extract; Candida albicans; LD50

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DOI

https://doi.org/10.36456/embrio.v16i1.9028

A B S T R A C T

Previous research on the effectiveness of mulberry leaves in the growth of candida albicans showed that C. albicans is inhibited in vitro. Mulberry leaf extract is used as a basic ingredient for treatment. In addition to being tested for effectiveness, testing is needed to ensure the level of acute toxicity in experimental animals in determining toxicity levels. The study aimed to test the acute toxicity of mulberry leaf extract on Rattus Norvecigus media in determining the level of toxicity before becoming the basic ingredient of VVC antifungal. The research method uses an experimental laboratory post-test-only control group design with a 9 Rattus Norvegicus media. The average dose is not lethal using LD50 values. Research analysis using ANOVA is One Way to determine the difference in the average weight change of experimental animals. The study's results did not show any deaths of experimental animals or toxic symptoms in administering mulberry leaf extract doses of 0 mg, 100mg, 1000 mg, 1600mg, 2900mg, 5000mg. LD50 value >8g/kg BW, included in the non-toxic category (5-15 g/kg BW). The ANOVA statistical test showed a P-value of 0.781 > a P-value of 0.05, that is, there was no difference in the average change in body weight in the experimental animal group given different treatment doses in each group. The research concludes that mulberry leaf extract does not show a lethal dose of acute toxicity, so it is safe to use as a basic ingredient in treatment sourced from natural ingredients.

Introduction

Vulvo Vaginal Candidiasis (VVC) is the most common condition in the presence or absence of symptoms (Swari et al., 2020), with a world prevalence of 1/3–3/4 of the population. Direct examination of 400 women to identify the cause of VVC was found to be 59.6% caused by Candida albicans, and the average occurred in women of childbearing age (29 years) (Djohan et al., 2019). Candida albicans in the form of yeast can survive well in the vaginal epithelium, but after morphotyping, switches to invasive hyphae forms and is regulated along with genes that code for virulence factors such as aspartyl proteases and candidalysin are secreted beyond tolerance limits, to trigger an intense inflammatory response and tissue damage (Marrazzo, 2002). Common predisposing factors for candidiasis are personal hygiene, pregnancy conditions, comorbidities such as endocrinopathy and autoimmune, consumption of antibiotics, and systemic corticosteroids (Marrazzo, 2002). Common symptoms of VVC are vaginal itching, vulvar edema, fissures, and excoriation accompanied by thick and thick vaginal discharge (Sobel et al., 1998). There are also non-physiological symptoms such as depression, helplessness, and decreased quality of life (Talapko et al., 2021). According to clinical practice

guidelines, VVC can be treated with topical or oral antifungals. Azol group is a treatment that is often given to cases of VVC, however, static activity of azole can make RVVC (Recurrent Vulvo Vaginal Candidiasis) or VVC recurrent (Willems et al., 2020). A marked increase was obtained in C. albican's resistance to antifungal agents (Moshfeghy et al., 2020). The degree of antifungal azole resistance varies widely and may be influenced by prescription patterns aimed at prophylaxis and treatment (Workowski et al., 2021). In particular, azole treatment can reduce clinical recurrence during administration in RVVC patients, however, there is usually no change in long-term use. In addition, the safety risks of fluconazole use are characterized by liver toxicity, drug interactions, and warnings during pregnancy (Bitew & Abebaw, 2018). Therefore, alternative tapes known as complementary therapies are needed to reduce VVC and RVVC effectively. Using natural materials in the current era is becoming a major concern in preventing or treating a disease. Plant-derived compounds are gaining widespread attention in identifying alternatives to microbial control (Lírio et al., 2019).

Mulberry plant (Morus alba) in Asian countries is used as an infusion and herbal treatment because of its content, such as flavonoids, amino acids, vitamins, and other nutritious nutritional elements (Farr et al., 2021). Berry compounds show antimicrobial activity that can protect against pathogenic bacteria in humans. The antimicrobial mechanism of berries is bacterial anti-adherence in epithelial cells, a prerequisite for colonization and infection of some pathogenic bacteria (Yang et al., 2020). Flavonoids are ingredients often found in plants that are useful as antimicrobials. The function of flavonoids is to keep cells from degradation, stress, anti-cancer, and antimicrobial. Flavonoids can actively help provide nutrients by trying to produce natural enzymes to fight disease (Latifah et al., 2022).

Identification of phytochemical components in Morus Rubra L ethanol extract showed the presence of triterpenoids, alkaloids, sugars, glycosides, tannins, resins, phenols, flavonoids, and saponins (Hidayatunnikmah et al., 2022). Previous research on the phytochemical identification of Morus Rubra L Extract showed the content of carbohydrates, monosaccharides, galactose, amino acids, fatty acids, phenol components, flavonoids, and tannins. The results of antibacterial screening at extract concentrations of 500 mg/dl, 250 mg/dl, and 125 mg/dl showed an obstacle in the development of Staphylococcus Aureus (Thiriloshani & Bharti, 2018), while other studies identified phenol 671.8 g-1 and anthocyanin 615.5 g-1, red mulberry extract was shown to suppress the growth of gram-positive (L. lactis, M. luteus and S. aureus) and gram-negative (S. typhimurium and E. coli) bacteria (Khan, 2021). In addition to antibacterial identification, mulberry leaf extract reduced the titer of pathogenic viruses such as human coronavirus (HCoV 229E) and cytopathogenic effects (Dimitrijevic et al., 2022). Previous research related to antifungals has shown that phenol components have antifungal properties. Most plants that exhibit antibacterial properties will also have antifungal properties (Hidayatunnikmah et al., 2022).

There are few studies on the incidence of VVC related to using red mulberry leaves as an antifungal candida albicans. Mulberry leaves are one of the plants that grow easily and are found in many areas in Indonesia, so more complex utilization needs to be done. Previous years of research have

shown the effectiveness of mulberry leaf extract on the development of C. albicans fungi in vitro (Memete et al., 2022) and compounds that work effectively in inhibiting the growth of C. albicans in this study, namely flavonoids (Miljković et al., 2018). Previous research showed that mulberry leaf extract was ineffective in inhibiting the growth of lactobacillus acidophilus at acidic or alkaline pH conditions. Lactobacillus acidophilus is a good bacteria found in the vaginal mucosa to stabilize physiological conditions. The use of mulberry leaf extract can inhibit the growth of C. albicans but does not inhibit the growth of lactobacillus acidophilus bacteria in the vagina. Mulberry leaf extract is the basic ingredient used to prevent and treat VVC. In addition to being tested for effectiveness, further testing is needed to ensure safety in experimental animals. The formulation of the problem is how the level of toxicity of Mulberry leaf extract as the basic ingredient of C. albicans antifungi, both in acute toxicity tests in experimental animals Rattus Norvecigus.

Method

This research method aims to analyze the acute toxicity of Mulberry leaf extract as the basic ingredient for making new product formulations that will be an alternative to the antifungal that causes VVC, namely the C. albicans strain. Experimental Laboratories research design using Rattus Norvegicus media with Post-Test Only Control Group Design approach.

The first step is the preparation of mulberry leaf extract. The leaves are picked from the tree, separated by branches, then dried and mashed using a blender. 500 g of powder was obtained from the Mulberry leaf simplisia, after which maceration was carried out using 99% PA ethanol for 72 hours. The resulting mixture will be filtered using filter paper, and the Filtrate obtained will be heated with a rotary evaporator to get a concentrated extract. Furthermore, the extract is stored in a clean, airtight place and a desiccator until the extract is used.

The second step is Acute Toxicity Studies, which will determine the median oral lethal dose of LD50 from the extract in experimental animals. Experimental animals were satisfied for one night, and the evaluation of LD50 administration was carried out in 2 phases. In the first phase, nine mice were randomly placed in 3 groups, each consisting of 3. Group I, II, and III will be given extracts at doses of 10, 100, and 1000 mg/kg body weight orally. The second phase is determined from the results obtained in the first phase. In the second stage, three mice are included in 3 groups, each consisting of 1 mouse. Groups I, II, and III were given extracts at 1600, 2900, and 5000 mg/kg body weight, respectively. Both phases were observed for 24 hours for signs of toxicity and death. The LD50 value is calculated as the average of the highest nonlethal dose (no death) and the lowest lethal dose (where death occurred).

Statistical Analysis The data is expressed as an average weight and analyzed using Way Analysis (ANOVA). P- value in this study is 0,05. The hypothesis in this study is Ho: There was no average change in body weight of experimental animals after being given mulberry leaf extract, and Ha: there was an average change in body weight of experimental animals after being given mulberry leaf extract.

Results

Mulberry leaves come from the family medicinal plant, Faculty of Science and Health, Universitas PGRI Adi Buana, Surabaya. Mulberry leaves that have been picked, cleaned, washed, aerated overnight, and then dried by drying them. After drying the leaves in the blender so that they become simplisia. Simplisia of mulberry leaves obtained as much as 500 grams. Furthermore, the extraction of mulberry leaf simplisia using the maceration method is carried out using 400 grams of mulberry leaves with 96% ethanol solvent as much as 1600 ml. The extraction process is allowed to stand for five days. Occasional stirring is carried out. The liquid that has been obtained is then filtered and allowed to stand for one day, then filtered again and taken to the filtrate. The filtrate obtained is evaporated so that a concentrated extract is obtained. Mulberry leaf extract is made with as much as one series of dilutions, which has been proven to inhibit the growth of Candida albicans by 100%.

The results of acute toxicity tests on nine experimental animals divided into three groups in Phase I and three in Phase II will be described in the table below.

Table 1. Number of Deaths of Experimental Animals on Observation for 24 Hours After Oral Administration of Mulberry Leaf Extract Preparations

Group	Phase	Mulberry Leaf Extract Dosage (mg/kg BW)	Number of experimental animals	Number of Dead Animals	Number of Live Animals	Percentage of dead animals (%)
A	I	10 mg/kg body weight	3	0	3	0
B		100 mg/kg body weight	3	0	3	0
C		1000 mg/kg body weight	3	0	3	0
D	II	1600 mg/kg body weight	1	0	1	0
E		2900 mg/kg body weight	1	0	1	0
F		5000 mg/kg body weight	1	0	1	0

Table 2. Observation of Toxic Symptoms for 24 Hours After Administration of Mulberry Leaf Extract

Group	Phase	Mulberry Leaf Extract Dosage (mg/kg BW)	Number of experimental animals	Toxic symptoms
A	I	10 mg/kg body weight	3	After oral administration of mulberry leaf extract, rats did not show toxic symptoms of a substance such as increased activity, convulsions, tremors, ataxia.
B		100 mg/kg body weight	3	After oral administration of mulberry leaf extract, rats did not show toxic symptoms of a substance such as increased activity, convulsions, tremors, ataxia.
C		1000 mg/kg body weight	3	After oral administration of mulberry leaf extract, rats did not show toxic symptoms of a substance such as increased activity, convulsions, tremors, ataxia.
D	II	1600 mg/kg body weight	1	After oral administration of mulberry leaf extract, rats did not show toxic symptoms of a substance such as increased activity, convulsions, tremors, ataxia.
E		2900 mg/kg body weight	1	After oral administration of mulberry leaf extract, rats did not show toxic symptoms of a substance such as increased activity, convulsions, tremors, ataxia.
F		5000 mg/kg body weight	1	After oral administration of mulberry leaf extract, rats did not show toxic symptoms of a substance such as increased activity, convulsions, tremors, ataxia.

Table 1 explains the results of observations of giving mulberry leaf extract in phase I for 24 hours in 3 groups, of which each group, there were three experimental animals with doses of mulberry leaf extract 10 mg/kg body weight, 100 mg/kg body weight, 1000 mg/kg bodyweight it was found that the number of deaths of experimental animals was 0 (0%) all experimental animals namely 9 remained alive. No deaths were obtained in these experimental animals. Meanwhile, observations of giving mulberry leaf extract in phase II for 24 hours in 3 groups, of which, in each group, there was one experimental animal from the results of phase 1 observations who experienced the most weight gain with doses of mulberry leaf extract 1600 mg/kg body weight, 2900 mg/kg body weight, 5000 mg/kg bodyweight it was found that the number of deaths of experimental animals was 0 (0%) all experimental animals namely three remained alive.

Based on table 2 shows the results of the observation of phase I toxicity symptoms after administration of mulberry leaf extract at a dose of 10 mg/kg body weight, 100 mg/kg body weight, and 1000 mg/kg body weight for 24 hours each in 3 groups with three experimental animals in each group were obtained rats did not show toxic symptoms of a substance such as increased activity, convulsions, tremors, ataxia. Meanwhile, observations of giving mulberry leaf extract were in phase II for 24 hours in 3 groups which each group, there was one experimental animal from the results of phase 1 observations who experienced the most weight gain with doses of mulberry leaf extract 1600 mg/kg body weight, 2900 mg/kg body weight, 5000 mg/kg bodyweight it was found rats did not show toxic symptoms of a substance such as increased activity, convulsions, tremors, ataxia.

Table 3. Observation of Changes in Body Weight of Experimental Animals During Mulberry Leaf Extract Administration

Group	Phase	Mulberry Leaf Extract Dosage (mg/kg BW)	Code of experimental animals	Initial Weight Loss	Final Weight Loss	Symptoms
A	I	10 mg/kg body weight	1	155	161	Healthy
			2	148	156	Healthy
			3	124	128	Healthy
B		100 mg/kg body weight	4	143	154	Healthy
			5	145	153	Healthy
			6	121	130	Healthy
C		1000 mg/kg body weight	7	131	136	Healthy
			8	144	143	Healthy
			9	152	164	Healthy
D	II	1600 mg/kg body weight	2	156	184	Healthy
E		2900 mg/kg body weight	4	154	187	Healthy
F		5000 mg/kg body weight	9	164	172	Healthy

Table 3 shows an increase in body weight in experimental animals after being given mulberry leaf extract for 24 hours at stage 1 and stage II with dose. The average weight gain of experimental animals in the phase I group was as much as 6 grams, and in phase I group A, the most weight gain was found in animals with code number no 2 with a weight gain of 8 grams. In phase I, group B, the average weight gain of 9.3 grams, the highest weight gain, was found in animals with code number 4. In Phase 1 group C, the average weight gain was 5.3 grams; the highest weight gain was found in animals with code number 9, as much as 12 grams. In the phase II group, the average weight gain was 17 grams. The highest weight gain was found in animal No. 9, as much as 18 grams,

Table 4. One Way Analysis (ANOVA)

	Sum of Squares	Number of experimental animals	Mean Square	F	Sig
Between Groups	830.250	5	166.050	.899	.537
Within Groups	1108.000	6	184.667		
Total	1938.250	11			

Based on table 3 shows the results that the results of the statistical analysis test obtained a significance value of P 0.537 > 0.05, which means that Ho's hypothesis is accepted that there was no average change in body weight of experimental animals after being given mulberry leaf extract.

Discussion

The main ingredient used in this study was Mulberry Leaf extract (Morus Rubra L). Morus Rubra L or Red Mulberry is a plant that belongs to the Moraceae family and has the characteristics of easy fall, fast-growing, and tree height from small to medium size to 15-20 m high. The growth of mulberry fruit in Indonesia is one of the plants that grows wildly. Its use is relatively small in Indonesia because of the lack of public ignorance of the pharmacological benefits of mulberry plants. The mulberry leaves used in this study are young mulberry leaves. Previous studies showed that the total polyphenol content found in young leaves is higher than in old mulberry leaves.

Mulberry leaves (Morus Rubra L) that have been identified will be sorted to remove dirt and rotten leaves. After sorting, washing the leaves with running water is carried out, then drying them, which aims to remove the moisture content still contained in the simplisia. The following process is drying under the sun but giving a net protector above it so that it is not directly exposed to sunlight, which can damage the content in mulberry leaves due to excessive heating. Dried mulberry leaves will be mashed until they form a fine powder. Then, the fine powder is sifted to obtain the same particle size, usually called simplisia

Mulberry leaf simplisia is then extracted to attract the chemical content in the simplisia. The extraction process, namely powder making, wetting, and watering, is carried out by maceration. Maceration is a process of making simpliasia extract using 96% ethanol solvent with a volume of 2000 ml. It is carried out several times by shaking or stirring at room temperature. The maceration process on mulberry leaf powder is carried out by soaking it with a solvent for 24 hours. After 24 hours, screening is carried out to obtain the filtrate. Then, the remaining residue is rewashed using the same solvent. This is done continuously and obtained filtrate, whose color is pale so that its chemical content can be extracted optimally. The maceration method is simple, so it is easy to do.

After the maceration process, the thickening or concentration process is continued using a rotary evaporator. The working principle of the tool is based on pressure drop so that the solvent can evaporate at temperatures below its boiling point. The purpose of using a rotary evaporator is to remove the solvent in the filtrate so that a thick extract is obtained from mulberry leaves (Morus Rubra L). Previous research by (Hidayatunnikmah et al., 2022) showed that the concentration of mulberry leaf extract that can inhibit the growth of Candida albicans is 80%, 95%, and 100%. The content contained in mulberry leaves are alkaloids, flavonoids, and polyphenols. Bioactive compounds can be found by extracting

these plants. Previous research has shown that bioactive compounds of alkaloids, flavonoids, and polyphenols can act as antimicrobials.

In this study, the experimental animals used were Rattus Norvegicus. The rats used were first acclimatized for two weeks, intended so that the experimental animals could adapt to the surrounding environment. During the acclimatization process and observation, mice are weighed daily to determine the weight changes that occur. The average body weight of experimental animals used was 1.5 grams. After the acclimatization process, in stage I, white rats will be grouped into three groups containing three white rats having different doses: group A with a dose of 10 mg/kg body weight, group B with a dose of 100mg/Kg body weight, group C with a dose of 1000 mg/kg body weight and each rat will be given a preparation of test materials that have been adjusted to their respective body weights. According to the dose, the extract is administered orally using a sonde. Observation will be conducted for 24 hours to find out the dead test animals and see toxic symptoms, which generally occur in tremors, ataxia, high heart rate, convulsions, and decreased activity, and observation of white rat body weight in each group. The results of phase I observations in the three groups of experimental animals, dosed 10 mg/Kg body weight, 100 mg/Kg body weight, and 1000 mg/Kg body weight, showed no dead animals were found and showed symptoms of toxicity. The results of weight observation in experimental animals obtained an increase. The increase in body weight was due to the immunostimulant content in mulberry leaf extract, previous studies showed that the effect of mulberry leaf extract on the immune system was evaluated using different experimental models such as carbon clearance assay, cyclophosphamide-induced neutropenia, neutrophil adhesion assay, effect on serum immunoglobulin, rat mortality test, and indirect hemagglutination test. Morus alba methanol extract was administered orally at low and high doses of 100 mg/kg and 1 g/kg, respectively. Ocimum sanctum (100 mg/kg, PO) was used as the standard drug. Morus alba extract at both doses increased serum immunoglobulin levels and prevented death in rats (Bharani et al., 2010).

After obtaining the results of observations in stage I, proceed with conducting toxicity tests in stage II. Observation of phase II with higher doses of 1600 mg/kg body weight, 2900 mg/kg body weight, and 5000 mg/kg body weight in each group of experimental animals. The phase II experimental animal group contains one experimental animal per group, based on the results of the phase 2 experiment, which has the highest weight change in each group. The extract is administered according to the dose given orally using a sonde. Observation will be carried out for 24 hours to find the dead test animals and see toxic symptoms and weight changes. The results of 24-hour observations in each group in phase II found that all experimental animals did not experience death toxic signs, and there was an increase in body weight in each experimental animal.

Observations made to determine the dose of LD50 in rats that had been given mulberry leaf extract showed no mortality and clinical symptoms of toxicity. With no deaths in experimental animals, the f factor, obtained in the Thomson and Weil formula tables, is not obtained, so the LD50 value cannot be calculated. The criteria for acute toxicity testing conducted to assess LD50 are based on expert agreement: if the maximum dose does not cause death in test animals, then LD50 is said to be a pseudo

LD50 or not a real LD50 (Loomis TA, 1987). If the dose reached up to 5000 mg/kg bodyweight does not cause death, then acute toxicity testing does not need to be continued using higher doses (BPOM RI, 2022). From the study results, the pseudo LD50 value > 8 g / kg body weight for mulberry leaf extract, where the pseudo dose is included in the non-toxic category (5-15 g / kg body weight). Based on body surface area, the dose converts to the maximum human dose in experimental animals. Experts agree that if there is no death in test animals at the maximum tidal dose, then it is clear that the compound falls under the criteria of "practically non-toxic" (Hafid & Rahayu, 2022).

Conclusions

Research on acute toxicity tests on mulberry leaf extract using rattus norvecigus experimental animal media conducted for 24 years showed no deaths in experimental animals and no symptoms of toxicity. There was an increase in body weight in experimental animals, which showed that mulberry leaf extract had a good impact on being used as an herbal treatment ingredient. A score of LD50 mulberry leaf extract from the test results in this study was >8g / kg BW, which showed that the value was included in the category of not causing toxicity (5-15 g / kg BW).

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