

ACUTE TOXICITY AND ANTIFUNGAL ACTIVITY OF THE OINTMENT *Murraya koenigii* ETHANOL EXTRACT

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ABSTRACT

Murraya koenigii (MK) plants are a type of plant whose bioactive compounds have been widely used in various countries as traditional medicines with metabolites containing alkaloids, flavonoids, tannins, steroids/triterpenoids and anthraquinones. The purpose of this study was to determine of acute toxicity effect and to test the antifungal effects of ointment MK ethanol extract. Acute toxicity testing is carried out with the initial stage of testing at a dose of <2000 mg/kgBW. The antifungal test was carried out by the diffusion disc method against *Candida albicans* by formulating the MK leaves extract into an ointment preparation. The results of testing the secondary metabolites of MK were alkaloids, flavonoids, tannins, steroid/triterpenoid, and anthraquinone. Toxicity testing was indicated by a decrease in body weight, but it was not seen in the MK extract because the weight difference did not show a difference of $p > 0.05$. The formulated ointment was subjected to an organoleptic evaluation test, pH, spreadability, and homogeneity. MK leaves ethanol extract can be formulated into ointment because it is safe to use with an LD₅₀ value >2000mg/kgBW. The concentrations of 12.5% and 25% were the best concentration to inhibit the growth of *Candida albicans*.

Keyword: *Murraya koenigii*, Acute Toxicity, Antifungal, Ointment

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INTRODUCTION

Murraya koenigii (MK) is a plant that is scattered in tropical and subtropical areas. This plant is known to have many benefits and benefits, especially in the health sector. Leaves, roots, stems, and fruit are known to have pharmacological activity.¹ MK leaves extract is reported to have anticancer, hypoglycemic, analgesic, antibacterial, and antifungal properties.² The antifungal effect of ethanol extract of MK leaves was tested against *Trichophyton mentagrophytes* and *Microsporum gypseum* using imidazole as a comparison. It was reported that the samples were able to inhibit fungal growth at concentrations of 10-50 µL/mL.³ The activity of MK leaves is inseparable from the active compound content. The ethanol extract of MK leaves is reported to contain 1-Methyl-pyrrolidine-2-carboxylic acid (69.00%), Ethyl α-dglucopyranoside (13.36%), Isolongifolene, 4,5-dehydro- (3.68%), γ-HIMACHALENE (2.88%), 1,2-Ethenediol, monoacetate (2.79%), 1,2-Benzenedicarboxylic acid diisooctyl ester (2.55%) which has antimicrobial properties⁴.

To facilitate the use of MK leaves extract as an antifungal, it is necessary to formulate it. The ethanol extract of MK leaves is formulated in the form of an ointment so that the dosage and effectiveness will be guaranteed.⁵ In addition, the formulation of MK leaves extract will facilitate the use of the product in therapy.⁶ The ethanol extract ointment of MK leaves will be tested for its fungicide activity against *Candida albicans*. *Candida albicans* is a type of fungus that causes most infections in up to 90% of fungal cases. The cases that occur vary, from low cases to serious cases that cause unexpected things.⁷ The development of this MK leaves extract dosage preparation is a step towards overcoming the problem of resistance to

Candida albicans against conventional drugs. Therefore, an alternative route to the use of traditional medicine is put forward.^{8,9} The safety of traditional medicines must also be evaluated.¹⁰ The toxicity assessment has never been carried out on ethanol extract of MK leaves. Acute toxicity is an initial toxicity test that can be done to ensure the maximum dose that can be used by MK leaves in therapy.¹¹ With the acute toxicity test, the LD₅₀ value of the ethanol extract of MK leaves will be obtained.¹² Based on the description above, the formulation and evaluation of the ointment preparation from the ethanol extract of MK leaves will be carried out accompanied by a chromatogram profile and an acute toxicity test.

EXPERIMENTAL

Materials

MK leaves were collected from North Padanglawas district, Indonesia. For chemical materials used were distilled water, NaOH, Na-CMC, H₂SO₄, HCl, methanol, ethanol, glacial acetic acid, chloroform, NH₄OH, HgCl₂, KI, anhydrate acetic acid, magnesium, HNO₃, FeCl₃, Bi(NO₃)₃, Iodine, Pb(C₂H₃O₂)₂, n-butanol, ethyl acetate, AlCl₃, n-hexane, chromatography plats silica gel 60F₂₅₄, paraffin liquid, and vaseline album. All materials used are certified and are used for analytical purposes.

Candida albicans obtained pure culture results from the microbiology laboratory, Faculty of Pharmacy, University of North Sumatra, Indonesia. For testing the antifungal activity required Whatman paper No.1, sabouraud dextrose agar (SDA), McFarland standard 0.5, and 10% dimethyl sulfoxide (DMSO). While for acute toxicity test required *strain swiss* female mice, 5-6 weeks old with a bodyweight of 20-35 g.

General Procedure

Extraction Process and Phytochemicals Analysis

Fresh MK leaves were processed into simplex. Simplex was used as a raw material for the manufacture of ethanol extract of MK leaves. 1000 g of simplex were processed by maceration using 10 L of ethanol for 24 hours, the filtrate was collected after 3 repetitions. To get the crude extract, the filtrate was evaporated with a rotary evaporator at a temperature of 50 °C.¹³ The phytochemicals constituent from ethanol extract of MK leaves was analyzed using TLC (silica gel 60F₂₅₄) by using one way ascending technique.¹⁴ The use of the mobile phase will determine the chromatography process. The mobile phase used is glacial acetic acid : butanol: water (1: 4: 5), chloroform : methanol (9: 1), methanol : water (6: 4), n-hexane: ethyl acetate (3: 7).¹⁵

Acute Toxicity Test

Acute toxicity test of MK leaves ethanol extract was determined according to Organization for Economic Cooperation and Development (OECD) guideline 423 on female mice.¹⁶ The determination of LD₅₀ was assessed from the number of deaths of tested animals during the 14 days of observation, both those that died directly and those who died were sacrificed due to suffering. If in the preliminary test there were no deaths at the dose level of <2000 mg/kgBW and in the main test only 1 head or none of the animals died at the dose rate of 2000 mg/kg BW, it is not necessary to give a dose exceeding 2000 mg/kg BW.¹⁷

Dosage Formulation, Evaluation and Antifungal Activity

MK leaves ethanol extract ointment has been prepared following the following formula as in Table-1¹⁸:

Table-1: Ointment Formula

Materials	F1	F2	F3	F4
MK leaves ethanol extract	-	6.25 g	12.5 g	25 g
Ointment base	100 g	93.75 g	87.5 g	75 g

Note: F1 (as a control), F2 (Extract with concentration at 6.25%), F3 (Extract with concentration at 12.5%), F4 (Extract with concentration at 25%)

The resulting ointment must go through the evaluation stage. The physical evaluation was carried out following general procedures including organoleptic test, spreadability test, homogeneity test, and pH of the preparation.¹⁹ For antifungal activity, the sample was carried out by disc diffusion method. *Candida albicans* was inoculated on SDA by dipping a sterile cotton swab into the inoculum, conditioned 5-15

minutes. Then, filter paper discs (about 6 mm in diameter) containing the sample at desired concentration were placed on agar surface. Incubated the petri and after that made observations by measuring the clear zone on the agar.²⁰

Data Analysis

The data obtained were analyzed using Microsoft Excel and SPSS 23 (Tukey HSD test).

RESULTS AND DISCUSSION

This research was started by producing the ethanol extract of MK leaves. Extraction was carried out using the maceration method with ethanol solvent for 24 hours and repeated for 3 days. Then the phytochemical compounds were analyzed qualitatively using the TLC method. This detection was carried out on 6 compounds suspected to be contained in the sample, namely alkaloid, flavonoid, saponin, anthraquinone, steroids/triterpenoid, and tannins. The TLC results can be seen in Fig.-1.

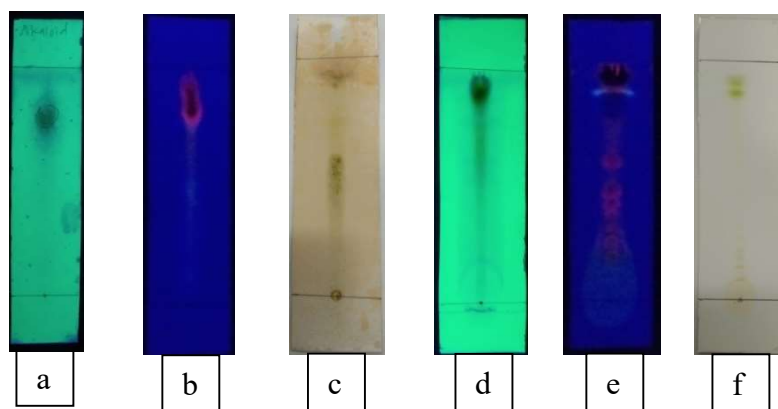


Fig.-1: The Chromatogram of Phytochemical Compounds in MK Leaves Ethanol Extract. (a) Alkaloid, (b) Flavonoid, (c) Tannin, (d) Saponin, (e) Steroid/triterpenoid, (f) Anthraquinone

The extract was prepared on a silica gel 60F₂₅₄ plate and eluted using the mobile phase. After the evaluated process, the sample was identified by the appearance of stains with the help of UV rays of 254 nm and 366 nm.²¹ TLC of MK leaves ethanol extract revealed the presence of alkaloid, flavonoid, tannin, steroid/triterpenoid, and anthraquinone. Alkaloids stain green under 254 nm UV light with the correct mobile phase.²² Alkaloid compounds can be seen on the silica plate with an R_f value of 0.647. Flavonoid stain purple under 366 nm UV light and have an R_f value of 0.705.²³ Tannin stain green to black with FeCl₃ 5% as an identification solution and have an R_f value of 0.752.²⁴ Steroid/triterpenoid stain purple under 254 nm UV and with Lieberman-Bouchard reagent, R_f value of 0.8.²⁵ Anthraquinone stain yellow with KOH 10% as identification solution under 366 nm UV light and the R_f value of 0.765.²⁶

The phytochemical content of the ethanol extract of MK leaves is the basis for its pharmacological activity. Therefore, to maintain its safety when consumed, an acute toxicity test was carried out.²⁷ The acute toxicity test was carried out for 14 days. During the observation, there were no animals that died as a result of giving the extract up to a dose of 2000 mg/KgBW, so it can be ignored that the extract is safe to give up to a dose of 2000 mg/KgBW and LD₅₀ of the extract was >2000 mg/KgBW.²⁸ The toxicity test was continued by looking at the effect of the extract on the weight loss of the tested animals. The results of weight loss for test animals can be seen in Table-2.

Table-2: Effect of Extracts on Weight Loss

Group	Weight Loss (gram)		p value
	Before Treatment	After Treatment	
Control	18.75	19.22	0.353
Dose 5 mg/kgBW	25.46	24.52	0.091
Dose 50 mg/kgBW	25.78	25.54	0.423

Dose 300 mg/kgBW	27.16	27.36	0.656
Dose 2000 mg/kgBW	30.42	30.38	0.932

Note: There was no significant difference in body weight between before giving the extract and after giving the extract with a p value >0.05

Observation of body weight of test animals was carried out for 14 days. Weight loss indicates toxic symptoms due to the administration of the test compound.²⁹ This was not seen in the ethanol extract of MK leaves, because the results of body weight before and after were obtained statistically, the paired-samples t test showed no significant difference with a p value >0.05 . After the extract safety test was fulfilled, the extract was formulated into an ointment for easy use as an antifungal. The ointment is evaluated for its quality. The results of the evaluation include organoleptic examinations, pH, spreadability, and homogeneity which can be seen in Table-3.

An ointment evaluation needs to be done to determine the quality of the ointment produced. For 14 days, organoleptic observations were made to see changes in the ointment.³⁰ For 14 days the ointment remained stable with white color for the control group and green color for the F1, F2, and F3 groups with a characteristic smell of MK leaves. The pH value of the ointment needs to be tested to make sure the ointment does not irritate. The F1, F2, and F3 test groups have $pH > 5$. This is as expected because a good ointment has a pH value of 5-7.³¹ The result of the spreadability test showed group tests have spreadability value >5 . The spreadability test aims to determine the softness level of the ointment mass so that it can be easily applied. Based on tests carried out on the three ointment preparations, where the three ointment preparations met the requirements for the diameter of the distribution of the ointment, namely ≥ 5 cm and ≤ 7 cm.³²

Table-3: Physical Evaluation of Ointments

Group	Physical Evaluation			
	Organoleptic	pH	Spreadability (cm)	Homogeneity
Control	White, specific ointment	5.8	5	Homogen
F1	Green, specific ointment	6.0	5	Homogen
F2	Green, specific ointment	6.2	5.1	Homogen
F3	Green, specific ointment	6.1	5.2	Homogen

Note: Control (ointment base), F1 (extract with concentration at 6.25%), F2 (extract with concentration at 12.5%), F3 (extract with concentration at 25%)

The activity of the ointment as an antifungal was tested against *Candida albicans* using the disc diffusion method. The test group used was divided into 3 with different extract concentrations. Nystatin was used in this test as a positive comparison. The results of the ointment activity test as an antifungal can be seen in Table-4.

Table- 4: Antifungal Activity of Ointments

Group	Inhibitor Zone Diameter (mm)
Control	0 ± 0.00^a
F1	9.582 ± 0.12^{ab}
F2	12.384 ± 0.22^{ac}
F3	15.111 ± 0.36^{ac}
Nystatin	16.116 ± 0.22^b

Note: a = $p < 0.05$, there is a significant difference with nystatin; b = $p < 0.05$, there is a significant difference with control; c = $p > 0.05$, there is not a significant difference with nystatin

The ointment with MK leaves ethanol extract formulas had the activity to inhibit the growth of *Candida albicans* through inhibition zone parameters. The F2 and F3 test groups had the best activity, able to inhibit growth with a value of 15.111 ± 0.36 and 15.111 ± 0.36 respectively which did not differ significantly from the nystatin group with a value of 16.116 ± 0.22 ($p > 0.05$).

The extract used in the ointment preparation is a crude extract. Not yet identified the main active compound which is useful for inhibiting the growth of *Candida albicans*.³³ The extract formulated into an ointment at a concentration of 12.5% and 25% effectively inhibits the growth of fungi such as nystatin. Ointments containing ethanol extract of MK leaves can be used as an alternative in the treatment of infections caused by *Candida albicans*.

CONCLUSION

MK leaves ethanol extract can be formulated into ointments because it is safe to use with an LD₅₀ value >2000mg/kgBW. The concentrations of 12.5% and 25% were the best concentrations to inhibit the growth of candida albicans

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