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RESEARCH ARTICLE

Anti-Dandruff Activities of Ethanol Extract and Water, N-Hexan, Ethyl Acetate Fractions of Key Lime (*Citrus Aurantifolia*) Leaves Against *Malassezia Furfur*

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Abstract

The objective of this research was to determine the Anti-dandruff characteristic of ethanol extract and its fractions of key-lime leaves (*Citrus aurantifolia* L) against the fungus *Malassezia furfur*. *C. Aurifolia* leaves were collected from villagers' vegetable crops in Kendari, Southeast Sulawesi, Eastern Indonesia. It was macerated with ethanol 96%, rotary evaporated till viscous extract was obtained. The extract was analyzed its phytochemical substances and tested its Minimum Inhibitory Concentration (MIC). The extract was fractionated using ethyl acetate and n-hexane. Both ethanol extract and its fractions were tested their anti-dandruff activity using the fungus *Malassezia furfur*. DMSO was used as negative control and ketoconazole 1% as a positive control. All data were statistically analyzed. Phytochemical screening found that key-lime leaves contained flavonoids, polyphenols, saponins, steroids & triterpenoids, and tannin. The results showed that key-lime leaf extract was able to inhibit the activity of the Malassezia furfur fungus, the inhibition zones of the extract were 1% (6.0 mm), 4% (7.55mm), 8% (8.44 mm), 16% (9.9 mm), and 29% (16.7) mm) which was slightly greater than 1% ketoconazole as a positive control. The higher the concentration of lime leaf extract, the greater the inhibitory power against the Malassezia furfur fungus. Statistical data shows that the fraction of key-lime leaf had the best antifungal effect shown by the ethyl acetate fraction. The study concluded that either ethanol extract or its fractions of *c.aurantifolia* leaves has anti-dandruff activity against *M. furfur*. The ethyl acetate fraction was found to have a better activity compared to n-hexane and water fractions. This finding could be useful to elucidate the compound which is responsible for this action.

Keywords: *Citrus aurantifolia*, Anti-dandruff, MIC, *Malassezia furfur*, Ethanol extract.

Introduction

Hair is a part of the human body that functions as a protective head from the surrounding environment directly, besides physiologically, hair has an aesthetic function that supports one's appearance. Hair is also a hallmark of ethnicity and serves as a social and cultural symbol [1].These various functions of hair indicate that hair is a very important part of the body so it is only natural that severe hair loss can be annoying for people who experience it. The biggest impact that is felt due to hair loss is to reduce self-confidence which can interfere with the sufferer psychologically [2].The occurrence of hair loss is influenced by several factors both from within and outside the body. Internal factors that cause hair loss are systemic diseases, hormonal conditions, nutritional status, and genetic disorders, and external factors such as stimuli from the environment in the form of sunlight, pressure, and the use of hair cosmetics [3, 4].Heavy dandruff is said to cause hair loss, but dandruff doesn't directly cause hair loss. However, the itchiness it causes can lead to scratching. This can injure your hair follicles, leading to some hair loss, though not complete baldness [5]. The scalp commensal organism, *Malassezia*, has been recognized to be a source of oxidative damage and this oxidative stress appears to play a role in premature hair loss [6, 7]. The use of key-lime fruit to cure various diseases has been done for thousands of years.

Apart from its fruit, key-lime leaves are also often used as medicine by the community [8]. It is reported of having antioxidants [8], antimicrobial [9, 10], anti-cancer [11], and inhibiting the growth of *Aedes spp*. Larvae [12]. So far no publication on the dandruff activity of jeruk nipis (Indonesian for *C. aurantifolia*) leaf but there is an article on jeruk purut (Indonesian for *C. hystrix*) fruit [13].

This study reports the anti-dandruff activity of ethanol extract and water, n-hexane, ethyl acetate fractions of *C.aurantifolia* leaf against *M. furfur*. Several factors are thought to be the cause of dandruff such as increased sebum production, individual sensitivity to the activity of sebum, and microbiota on the scalp, namely seven distinct species within this genus, namely *M. furfur, M. pachydermatis, M. globosa, M.obtuse, M. restricta, M. slooffiae, and M. sympodialis.*

Furthermore, recently three new species were included in this genus, namely *M. dermatitis, M. yamatoensis, and M. nana* [14-18]. *M. furfur* was chosen in this study as it reported that *M. furfur* was most detected in human samples [19].

Materials and Methods

Materials

The daun jeruk nipis (Indonesian), *C. aurantifolia (Cristm.) Swingle)* leaves were obtained from the villagers' vegetable crops in Kendari, Southeast Sulawesi, Eastern Indonesia, and determined at the Laboratory of Taxonomy, Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran.

Fungus

The fungus used was *M.furfur* obtained from the Mycology Laboratory of Universitas Hasanudding, Makassar, South Sulawesi.

Mushroom Growth Media

The seed medium used was 65 grams of Sabouraud Dextrose Agar (SDA) plus 10 ccs of olive oil suspended in 1 liter of distilled water, boiled until completely dissolved, then sterilized in an autoclave at 1210C for 15 minutes.

Methods

Phytochemical screening was performed to detect the class of secondary metabolites in simplicia C. aurantifolia leaves. The screening was conducted using the modified Farnsworth method [20, 21].

Extraction

Extraction was done by the maceration method [22]. A total of 1 kg of crude drugs incorporated into the *C.auantifolia* leaves macerator, then added 3L of 70% ethanol and allowed to stand for 24 hours. The liquid extract obtained was then collected and added back 70% ethanol with the same amount. It performed three times a change of solvent. The entire liquid extract obtained was then concentrated by rotary evaporator at 60 °C with a speed of 60 rpm and evaporated over a water bath with a temperature of 40 ° C till constant viscous extract.

Testing Standards Parameter Extract

Testing parameters include organoleptic standardized extract and determination of moisture content and yield calculations based on existing textbook [23, 24]. The determination of the water content of the extract was done with the toluene distillation method. Condensed extract as much as 2 g inserted into the flask and add 200 ml of toluene, and then connected to a distillation apparatus. Pumpkin is heated until all the water was distilled and cooled at room temperature until the water and toluene separate perfectly**.**

Fractionation

C. aurantifolia leaf seed extract fractionation performed using Liquid Extraction - Liquid (ECC) [25]. A total of 30 g of extract dissolved in 500 mL of water and then put into a separating funnel. N-hexane entered with the same amount of water into the funnel and then shaken while the existing air occasionally released and allowed to stand until the two separate solvents. The fraction of n-hexane and water separated, this phase was repeated several times until a layer of n-hexane was no longer colored.

At the same separating funnel, ethyl acetate entered with the same amount of water, then shaken and separated using the same procedure. The result obtained three fractions, namely fraction of n-hexane, ethyl acetate, and water from the extract of *C. aurantifolia* leaves. The third fraction was evaporated and concentrated using a rotary

evaporator, and then calculates the yield of the fraction.

Standardization of Extracts

Standardization of wood extract was carried out to ascertain water content, drying losses, levels of soluble ethanol extracts, levels of .water soluble extracts, total ash content, acid insoluble ash content, and metal content of extracts guided by the Indonesian Pharmacopeia 4th edition [24].

Determination of Minimum Inhibitory Concentration (MIC)

Making Test Microbial Suspension

The test fungus was inoculated on Sabouraud Dextrose Agar (SDA) media and incubated for 3-5 days at 32°C. One ose of fungal colonies was suspended in 5mL of sterile physiological NaCl. The concentration of the fungus in the suspension was measured based on turbidity using a spectrophotometer. For *M. furfur* mushrooms the absorbance value was 0.3 - 0.5. The modification of Andrews method was applied [26].

Procedure for Determination of Minimum Inhibition Concentration (MIC of ethanol extract:

Provide an initial solution consisting of cabbage ethanol extract (100%), Provides 8 sterile test tubes for diluting key-lime extract, and 2 tubes for media control and growth control. Conducted the dilution of cabbage ethanol extract for tube 1 by 1mL cabbage extract the initial solution was added with 19mL of media to be solid (SDA), mixed until homogeneous, and poured into sterile Petri dishes allowed to condense (concentration of 5%).Conducted dilutions for tubes 2 to tube 8 using the same method and calculation, to obtain the concentration of *c. aurantifolia* ethanol extract in sterile Petri dishes 1%, 4%, 8%, and 16%. Each dilution concentration was made 3 times (triple). Apply test microbes to solidified agar in each petri dish (8 Petri dishes) and incubated for *M.furfur* at 32^{0}C for 3-5 days.

Setting up media controls and growing controls. Media control was agar media with *c. aurantifolia* extract without being planted with *M.furfur*. The growth control contained an agar medium that was planted with *M.furfur* without the ethanol extract. Ketoconazole 1 % and DMSO were applied respectively as a positive and negative control.

Procedure for Determination of Minimum Inhibition Concentration (MIC of water, ethyl acetate, and n-hexane fractions

To find out the anti-dandruff properties of fractions of *c. aurantifolia*, a similar same procedure was applied as for the determination of the anti-dandruff properties of the ethanol extract. The procedure of anti-fungal was based on the modification of Gozali method [27]

Data Analysis

Data analysis: carried out using the Newman-Keuls statistical test [28].

Results and Discussion

Extraction Results

The ethanol extracted results obtained a dry extract with a yield of 10.5 % w/w and the results of observations of organoleptic *c. aurantifolia* extract was in the form of dry powder, brownish red, smelly, and distinctive taste**.**

Phytochemical Screening: Phytochemical screening results can be seen in Table 1.

Table 1: Secondary metabolites of *C. aurantifolia*

No	Secondary metabolites	Leaf sample	Ethanol extract
1	Alkaloids	-	-
2	Flavonoids	+	+
3	Monoterpenoids & Sesquiterpenoids	-	-
4	Polyphenol	+	+
5	Saponins	+	+
6	Steroids & Triterpenoid	+	+
7	Tannin	+	+

Notes: + = detected; - = not detected

Abdallah [29] reported that their Sudan's *c. aurantifolia* sample showed the presence of saponin, phenolic compounds, and anthraquinone, and no other secondary

metabolites were reported. The results of phytochemical screening showed that the ethanol extract of 70% *C. amblycarpa* leaves contained flavonoids, polyphenols and tannins, glycosides, and essential oils [30]. The slight difference in phytochemical screening results was most likely due to the origin of the sample.

Standardization of Extracts

To find out the quality of the C. Aurantifolia leaves extract used more clearly, can be seen in Table 2.

Table 2: Results of Standardization of Extracts

Tests	Concentration (%)	Concentration based on Indonesian Herbal Pharmacopeia (%) [23]
Water-soluble extractive	19.5	-
Ethanol soluble extractive	40.5	-
Water content	0.5	<10
Drying shrinkage	0.5	-
Total ash content	3.22	<1.4
Acid-insoluble ash content	1.56	<0.6

Table 3 showed that nearly all parameters fulfilled the concentration described in Indonesian Herbal Pharmacopeia [23]. Provisions regarding the need for standardization of extracts from herbal ingredients are also required by Traditional Medicine Raw Materials and WHO [24, 31-33]. The water content obtained at 0.5% meaning it could reduce the risk of damage to the extract due to the growth of fungi and bacteria.

Besides, the value of water content was also related to the dosage on the preparation because the extract with a high value of the water content, the amount of extract needed to reach the desired dosage on more preparations. The results obtained from testing drying losses were 0.5%. It indicated that the compounds in the *C. aurantifolia* leaf were only lost or evaporated as much as 0.5% during the heating process. The results of total ash content determination were 3.22% and the results of the determination of acid-insoluble ash content were 1.56%. These results were not following the standards listed in Herbal Pharmacopoeia. This was possibly due to metal or mineral contamination during planting due to environmental factors.

Anti-dandruff Activities of Extract Ethanol Key-lime Leaf against *M. furfur*:

Fig. 1 and Table 3 were, respectively Inhibition Zone (MGIC) Ethanol Extract and statistical analysis of key-lime leaves as an anti-fungal against *M.furfur*.

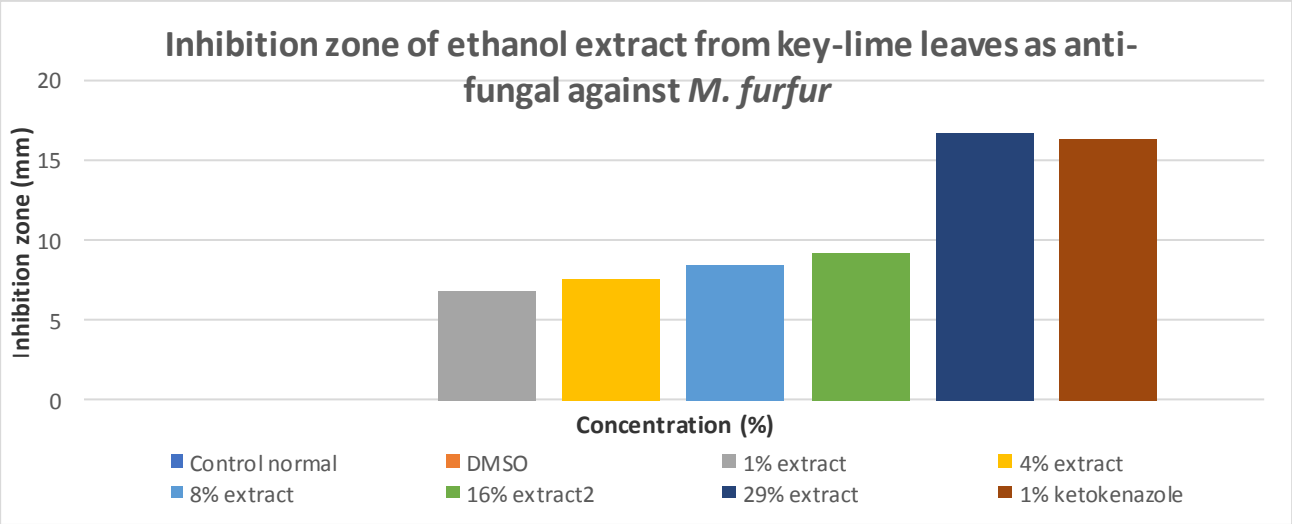

Fig. 1: Anti-dandruff activities of *C.aurantifolia* ethanol extract

All data from Fig. 1 was taken from three measurements. From statistical data, it was found that the Fcount value was greater than Ftable (Ho

was rejected), meaning that there was a significant difference in the effect of different shampoo formulas on the potential of the shampoo preparation. While from the results of the further analysis with the Newman Keuls test (Fig. 2), it might conclude that the greater the concentration of key-lime ethanol extract would increase antifungal activity in the shampoo preparation. This was because key-lime leaves contained flavonoids that would obtain a large diameter inhibiting antifungal activity.

Responds to fungal growth according to CLSI (Clinical and Laboratory Standards Institute), strong > 20 mm, moderate 15-19 mm, and weak if inhibition zones <14 mm [34]. In the library, it also stated if inhibition areas of more than 3 mm are fungal sensitive areas, whereas if the diameter of inhibitory regions is less than 2 mm, they were called fungal resistant or insensitive areas [35]. This meant key-lime extract ethanol had an inhibition anti-fungal activity higher at higher concentrations. Lime juice of *C.aurantifolia* Swingle) fruit was reported containing limonene and have a growth-inhibiting effect on Malassezia furfur [36]. The inhibiting effect on *Salmonella typhii* has been also reported [37]. So far, there was no report regarding the inhibiting effect on *M.furfur by C.auratifolia ethanol extract.*

Statistical Test

A. Normality test

Tests of Normality[b]

	Treatment group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Inhibition zone key-lime extract	1	.253	3	.	.964	3	.637
	2	.382	3	.	.757	3	.016
	3	.385	3	.	.750	3	.000
	4	.175	3	.	1.000	3	1.000
	5	.385	3	.	.750	3	.000

a. Lilliefors Significance Correction

b. inhibition zone of key-lime was constant when treatment group = 6. It had been omitted.

B. Homogencity test

Test of Homogeneity of Variances

Inhibition zones

Levene Statistic	df1	df2	Sig.
2.553	5	12	.085

C. Kruskal-Wallis Test

Ranks

	Treatment group	N	Mean Rank
Inhibition zone	1	3	5.50
	2	3	7.50
	3	3	11.83
	4	3	13.17
	5	3	17.00
	6	3	2.00
	Total	18	

Test Statistics[a,b]

	diameter zona hambat
Chi-Square	16.119
df	5
Asymp. Sig.	.007

a. Kruskal Wallis Test

b. Grouping Variable: treatment group

Fig. 2: Statistical analysis of key-lime ethanol extract

Anti-dandruff Activities of Fractions of Key-lime Leaves against M. furfur

Fig. 3 was the Inhibition Zone (MGIC) of fractions of key-lime leaves. Fig, 3 was obtained from 3 times measurement. The procedure and statistical following the ethanol extract method.

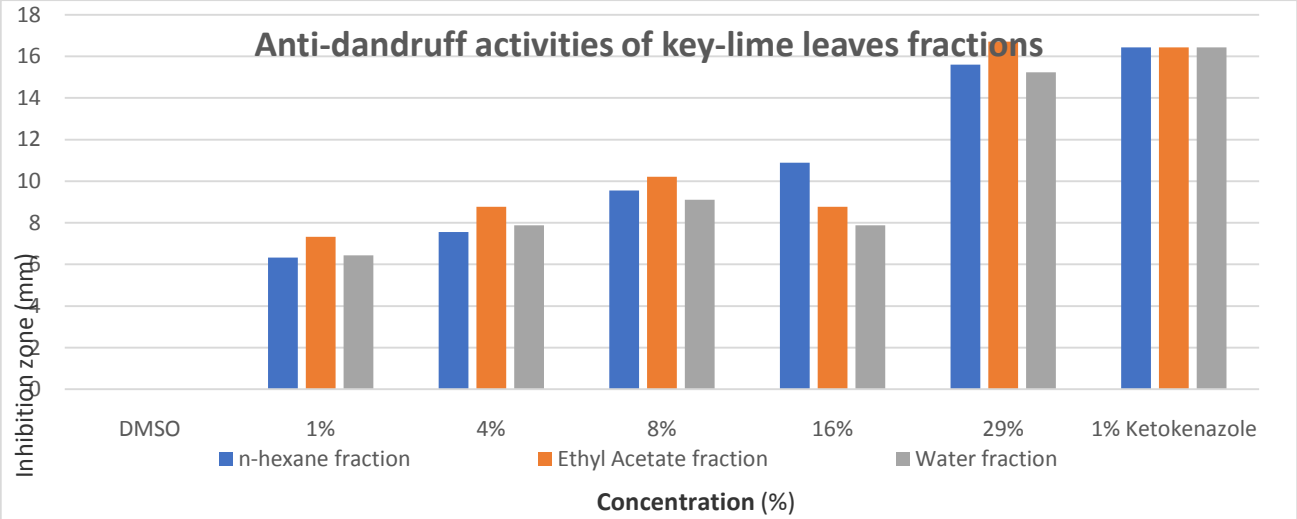

Fig. 3: Anti-dandruff activities of *C.aurantifolia* fractions

Fig. 3 was drawn based on Table 3 data.

Table 3: Anti-dandruff activities of *C.aurantifolia* fractions

Compounds	n-hexane fraction	Ethyl acetate fraction	Water fraction
DMSO	0±0.00	0±0.00	0±0.00
1%	6.33±0.50	7.33±0.71	6.44±4.16
4%	7.56±0.53	8.77±1.20	7.88±8.51
8%	9.56±0.73	10.22±0.97	9.11±9.83
16%	10.89±1.05	11.66±0.71	9.88±11.83
29%	15.60±1.05	11.71±0.71	15.23±7.52
1% Ketoconazole	16.44±0.53	16.44±0.53	16.44±5.26

Table 3 data was obtained from each of the three experiments as shown in Fig. 4.

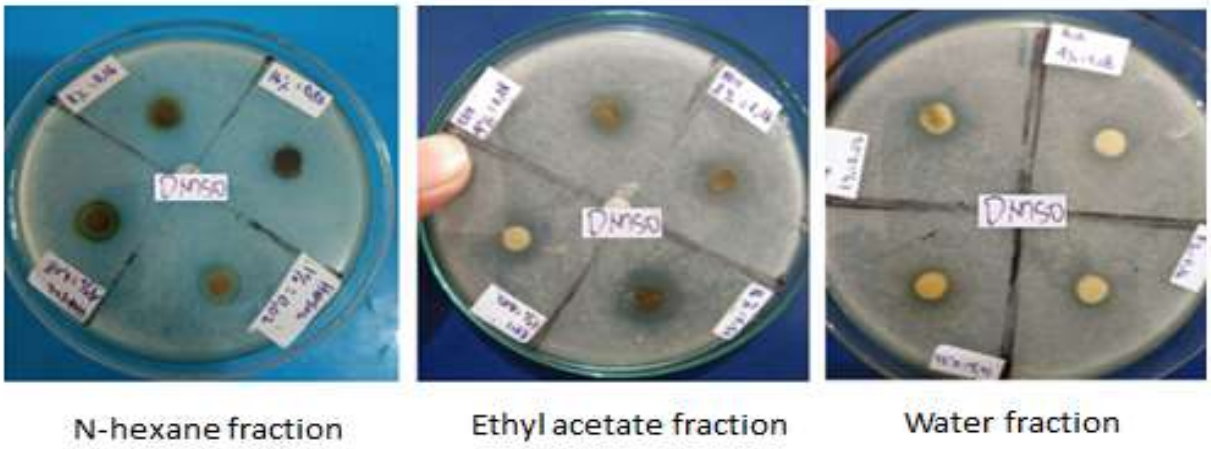

Fig.4: Inhibition zone in a petri-dish of key-lime fractions against *M.furfur*

From the data obtained, statistically, it was found that ethyl acetate fraction had a better anti-dandruff activity compared to other fractions. Unfortunately, no data from other researchers could be compared with.

Conclusions

Based on the results of this study, it was found that either ethanol extract or its fractions of *c.aurantifolia* leaves has anti-dandruff activity against *M. furfur*. Ethyl

acetate fraction was found to have a better activity compared to n-hexane and water fractions. This finding could be useful to elucidate the compound which is responsible for this action.

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