Antifungal Activity of Essential Oil Extract of Lemon Cui (*Citrus microcarpa*) Skin against *Trichophyton rubrum* Growth

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***Abstract*—Objective: The aim of this study was to test the antifungal activity of essential oil extract of lemon cui skin on *Trichophyton rubrum* growth.**
Method: This research is true experimental laboratory to see the existence of clear zone on SDA test media after giving the extract. The research was held 9 weeks in the laboratory of Health Analyst of Poltekkes Kemenkes Manado. Essential oil extract of Lemon Cui (*Citrus microcarpa*) Skin was taken using Steam Distillation method. Antifungal activity test do by inserting 1 ml of *Trichophyton rubrum* suspension into sterile petri dish then poured ± 20 ml SDA medium and made homogeneous. The diameter of well is about 6 mm Then into different wells put 20 µl extract and 20 µl positive control (ketoconazole 2%). After the whole process has been completed, the petri dishes are put into an incubator at 37°C. The clear zones was observed after 48 hours.
Result: Extraction with steam destilation method resulted a yellow gold oil with weighing 1.8 g and the yield is 0,75%. Antifungal activity test showed that essential oil extract of Lemon Cui skin had inhibitory effect on *Trichophyton rubrum* growth. The diameter average of clear zone around the well is 8,6 mm.
Conclusion: Essential oil extract of Lemon Cui Skin has inhibitory power to inhibit *Trichophyton rubrum* growth, marked by the clear zone around the well.

Index Terms*— anti fungal activity, Lemon Cui (*Citrus microcarpa*) skin, essential oil extract, *Trichophyton rubrum

I. Introduction

Indonesia is one of the countries who still familiar with skin disease problem. Indonesia Health Profile Data shows that the number of outpatient visits with medical diagnoses of "skin diseases and subcutaneous tissue" at hospitals throughout Indonesia in 2008 was 15,100 visits, ranking 12 of 21 diseases. In 2010, the number of patient visits in hospitals throughout Indonesia was 192,414 visits, of which 122,076 were new cases and ranked 3 out of 10 major outpatient diseases in 2010 [1].

The skin can be infected by microorganisms, bacteria, viruses and fungi. Dermatophytes are a group of filamentous fungi that cause infections and are among the most common causes of human diseases. Examples of dermatophyte-caused infections are tinea pedis and tinea capitis. Furthermore, deep dermatophytosis caused by dermatophyte infections has also been reported, in which the infection pen- etrates the skin barrier and reaches internal tissues and organs. Although dermatophyte caused infections rarely cause death, their prevalence, high incidence, difficulty to treat and contribution to morbidity represent a significant unsolved global public health problem [2]. Superficial fungal infections have been found in the last few decades to affect 20–25% of the world's population, making them one of the most frequent forms of infection [3].

Tinea pedis called Athlete's foot or "Ring worm of the foot" is the most common dermatophyte infection or fungal infection in humans [4]. The most common cause is *Trichophyton rubrum* [5]. Trichophyton rubrum is keratinophilic that can digest skin and anthropophilic keratin which selects humans as their permanent hosts. This fungus can live in air, land, water, clothing and even the human body itself. This fungus group can cause chronic and residual disease due to the body's very mild rejection reaction, on the human body of this fungus concerning the skin of ankles, soles of the feet and the sidelines of the toes [2]. Singapore's National Skin Care Hospital in 1999-2003 showed a percentage of Tinea pedis reaching 27.3% [6] while Chumitshu Chuo Hospital Tokyo Japan showed a percentage of Tinea Pedis reaching 64.2% [7]. Aproximately 15% of the population have a podiatric fungal infection at any given time and it is estimated that over 70% of the population have suffered at some point in their lives from tinea pedis [8].

Utilization of natural ingredients from plants as a cooking spices and preservatives has been done from a long time ago by the people of North Sulawesi, this is profitable enough because the raw material is easy to obtain or can be planted in the yard itself and relatively cheap. One of the plants that have been utilized by the community is Lemon Cui (*Citrus microcarpa*). Lemon cui is a local Indonesian fruit that is commonly found in Sulawesi, especially in Manado. This is why lemon cui is also called orange manado. This lemon is useful as a preservative, eliminating fishy smell and as a mixture of

Manuscript received September 14, 2018; revised February 21, 2019.

doi: 10.18178/ijpmbs.8.2.40-44

original chilli sauce with a distinctive aroma that is very appetizing. Reference [9] reported that orange peel contains essential oils used by the perfume chemical industry, adds citrus scents to beverages and foods, and is used in the field of health as an anti-oxidant and anti-cancer. Nevertheless the antifungal activity of orange peel essential oil has been scarcely studied. "Reference [10]", reported that volattile compounds of orange and lemon peel are capable to inhibit *Penicillium spp*..

We conducted this study to test the essential oil extract of lemon cui (*Citrus microcarpa*) skin against *Trichophyton rubrum* growth.

II. Method

This research is true experimental laboratory to see the existence of clear zone on SDA test media after giving the extract. The population in this study is Lemon cui that plucked directly in the yard of the house of population. The sample is Lemon cui skin with green colour, smooth skin not shrink.

The tools used in this study are biosafety cabinet, laminar air flow, autoclave, rotary evaporator, shaker, glass beaker, erlenmeyer, petri dish, ose needle, micropipette, measuring glass, test tube, analytical balance, spoon, hot plate, magnetic stirrer, extractor flask, distillation flask, steam generator, condenser, aluminum foil, plastic, tissue, mask, gloves, cap and camera. The material used in this study were lemon cui skin, *Trichophyton rubrum* isolated, Sabouraud Dextrose Agar (SDA), alcohol, ketoconazole, chloramphenicol, aquades and spiritus.

A. Essential Oil Extract of Lemon Cui Skin Collection

Lemon skin was obtained from fresh lemon cui (*Citrus microcarpa*). Lemon were washed and peeled. Essential oil extract of Lemon Cui Skin collected by using Steam Destilation Method [9]. Amount of 500 g Lemon Cui Skin were dry using an oven for 12 hours at 40^{o}C. Weigh lemon cui skin after drying then introduced into the distillation flask which was conntected to the steam generator via a glass tube and to a condenser to retrieve the oil. Water is put in a steam generator and then heated. The extractor flask is heated when water vapor from the steam generator starts to form. After the steam distillation process, the product was collected and separated by separatory funnel, then stored in the freezer to obtain oil free of water.

B. The Yield of Essential Oil Extract of Lemon Cui Skin

Calculation of the yield of lemon cui peel is done by weighing lemon cui skin before extracting and after extracting (1). The amount of yield is calculated by the formula [11]

$$R = \frac{A}{B} \times 100\% \quad (1)$$

R : Yield of Essential Oil Extract of Lemon Cui Skin

A : Weight of the essential oil of lemon cui skin from extraction

B : Weight of lemon cui skin before extracting

C. Making Sabouraud Dextrose Agar (SDA) Media

Suspended 65 g of the powder and 0,05 g of Chloramphenicol in 1 liter of distilled or deionized water. Mixed well and heated to boil until completely dissolved. Sterilize in autoclave at 121°C for 15 minutes. Final pH is 5.6 – 5.8.

D. Regeneration of Trichophyton rubrum

Culture of *Trichophyton rubrum* was cultivated in the slope agar medium of Sabouraud Dextrose Agar (SDA). The entire rim of the tube mouth is passed on the flame, the tube plug is opened slowly. Trichophyton rubrum culture is taken by ose and scratched on a sloping surface. The tube is closed slowly and passed again on a flame. Incubation at 25˚C for 2 weeks. Macroscopic of fungal growth can be seen by presence or absence of colonies. *Trichophyton rubrum* colonies exhibits a spectrum of overlapping characters; for example culture surface texture may vary from downy to suede-like; culture surface pigmentation may vary from white to cream to deep red; culture reverse pigmentation may vary from colourless to yellowish to yellow-brown to wine red [12].

E. Test for Antifungal Activity

1 ml suspension of *Trichophyton rubrum* is inserted into sterile petri dishes and poured ± 20 ml SDA media. Petri dishes are shaken until homogeneous and the media is allowed to solidify. After that, make two wells with diameter is about 6 mm. Then into different wells put 20 µl extract and 20 µl positive control. The positive control using ketoconazole 2% was prepared by weighing 0.02 grams ketoconazole then dissolved with 1 ml sterile aquades. After the whole process has been completed, incubation at 25˚C for 2 weeks. Repeated triplo in the same way. The presence or absence of clear zones was observed after 48 hours.

III. Result

A. Essential Oil Extract of Lemon Cui Skin by Steam Destilation Method

Extraction with steam destilation method resulted a yellow gold oil as shown in Figure 1, with weighing 1.8 g and the yield is 0,75%.

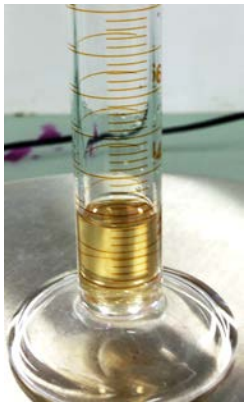

Figure 1. Essential oil extract of lemon cui skin.

B. *Antifungal Activity Test*

The results of the antifungal activity test of extract to *Trichophyton rubrum* showed the presence of clear zones around the well. The diameter of the clear zone of Essential Oil Extract of Lemon Cui Skin is 8,6 mm and the diameter of positive control is 11,8 mm as shown in Figure 2.

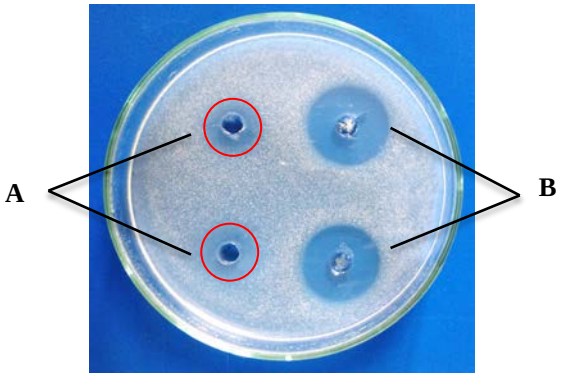

Figure 2. Clear zone of essential oil extract of lemon cui SKIN. A: Essential oil extract of lemon cui skin, B: Positive control.

IV. Discussion

Sample of this study is lemon cui skin because a large amount of skin is produce every year. Global production of citrus fruit has significantly increased during the past few years and has reached 82 million tons in the years 2009–2010, of which oranges commercially the most important citrus fruit accounts for about 50 million tons [13] and 34% of which was used for juice production, yielding about 44% peel as by product [14]. Citrus skin is the primary waste, is a good source of molasses, pectin and limonene and is usually dried, mixed with dried pulps and sold as cattle feed [15]. Due to the low cost and easy availability of fruit residues which other wise would be discarded as waste in the environment should be regarded as potential nutraceutic resources, capable of offering significant low-cost, nutritional dietary supplements [16].

Lemon cui skin is separated from the flesh and dried in an oven for 12 hours, temperature 40^{o}C. The drying of lemon cui skin aims to reduce the amount of water, so the weight of lemon cui is reduced more than 250 g after drying as shown in Table 1. “Reference [9]”, proved an increase in the yield of citrus peel oil on orange peel drying with a time of 12 hours compared to 24 hours drying or drying. This is supported by SEM (Scanning Electron Microscope) test results with 85X magnification from the surface of orange skin. Orange skin pores without drying have pores ranging from 140 to 165 µm. While with drying has larger pores around 288 - 363 µm. The bigger the pores open, the easier the oil stored under the surface of the orange skin evaporates. This is in accordance with research of “reference [17]” that the drying process along with the existence of this diffuse steam can damage the orange peel so it can open the pores. This is why the steam distillation method with the drying of raw materials can extract more oil yields than the same method without drying.

TABLE I. Lemon Cui Skin Weight Before and After Drying

Before Drying	After Drying (12 hours, 40^{o}C)
500.00 g	239.84 g

The yield of essential oil extract of lemon cui skin is 0,75 %. The yield of oil depends upon several factors-primarily the method of extraction. There is another way of extraction techniques to obtain essential oils beside steam distillation, namely hydro distillation. The yield obtained by hydro distillation process is around 0.35-0.37%, sometimes the hydrolysis process of the ester occurs, and the oil product mixes with another products [18].

Medium that have been used to test the activity of antifungal and regeneration of *Trichophyton rubrum* are Sabouraud Dextrose Agar (SDA) with the addition of Chloramphenicol. This medium is a selective medium that can be used for the cultivation of yeast, molds and aciduric microorganisms. It is used for cultivating pathogenic fungi, particularly those associated with skin infections. This medium is also used for determining the microbial and fungal content of cosmetics and for the mycological evaluation of food. Chloramphenicol is an antibiotic which aids in isolating pathogenic fungi from heavily contaminated material, as it inhibits most contaminating bacteria. It is a antibiotic that can use with media due to its heat stability and wide bacterial spectrum that use to inhibits the great majority of bacterial contaminants. Dextrose is the fermentable carbohydrate providing carbon and energy. Peptone mixture provides nitrogen, vitamins, minerals and amino acids essential for growth. Bacteriological agar is the solidifying agent. The high dextrose concentration and acidic pH make this medium selective for fungi [19].

Positive control that used in this study is ketoconazole, because ketoconazole is antifungal drug with wide spectrum of antifungal activity, effective against several of these superficial infections, such as candidiasis, pitiriasis versicolor, tinea curis and dermatophytosis [20]. This antifungal mechanism is inhibits the synthesis of ergosterol in fungi and cholesterol in mammalian cells. In addition it is an inhibitor of cortisol synthesis resulting from its ability to inhibit several cytochrome P450 enzymes in the adrenal glands [21]. Ketoconazole inhibits primarily the activity of 17α-hydroxylase, but it also inhibits 11-hydroxylation steps, and at higher doses the cholesterol side-chain cleavage enzyme. Therefore, ketoconazole is an inhibitor of cortisol and aldosterone synthesis. Ketoconazole is also an inhibitor of androgens synthesis, inhibiting the activity of C17-20 lyase in the adrenals and also in Leydig cells [22].

Agar well diffusion is the method that used to test the antifungal activity. This method is the most widely technique for assaying plant extract for their antimicrobial activity. In this technique, a well or reservoir containing the test compound at a known concentration is brought into contact with an inoculated medium and the diameter of the clear zone around the reservoir (zone inhibition diameter) is measured at the

end of the incubation period. Small sample requirements and the possibility of testing extract per plate against a single microorganism are specific advantages. The zone of inhibition of test organism growth around each well can be measured in mm. in order to enchance the detection limit, the inoculated plates can be kept at a lower temperature for several hours before incubation. In this way, compound diffusion can be enhanced over microbial growth, and better inhibition diameter is expected [23].

The results of the antifungal activity test showed an inhibitory activity against the growth of *Trichophyton rubrum*. This is evidenced by the formation of clear zones around the well of sample. Several previous studies have informed that essential oils may cause cellular damage as well as changes in the morphology of hyphae. This inhibition of fungal growth is thought to occur due to the presence of chemical compounds contained in the orange skin that can inhibit the growth of fungi. Essential oils are composed of lipophilic and highly volatile secondary plant metabolites, reaching a mass below a molecular weight of 300. Essential oils, were strongly identified with terpenes, principally mono and sesquiterpenes [24]. "Reference [4]" suggest that sesquiterpene compounds, especially sesquiterpen alcohol from essential oils, determine the antimicrobial and larvacid activity. Reference [25] gather information about herbal plants, including plants that are efficacious as an anti-fungal, it turns out that generally the component content consists of essential oils namely glucoside, saponin, flavonoid, tannin, polyphenol, eugenol, estragol, terpenena, sesquiterpena, phenylpropane, limonene, formic acid and peroxide. The antifungal activity of terpenoids is generally related to their functional groups, i.e., to the hydroxyl group of phenolic terpenoids. Carvacrol and thymol, produced from *p*-cymene, exhibit an important antifungal effect; they cause damage in the cell membrane by interacting with sterols and in particular with ergosterol. When tested alone, carvacrol, present in the essential oils of some oregano and thyme chemotypes, shows strong antifungal activity [26], [27]. The essential oil from *Citrus sinensis* epicarp (composed on limonene at 84.2%) is capable of inhibiting the growth of *Aspergillus niger*; it also leads to irreversible deleterious morphological alterations (in particular the loss of cytoplasm in fungal hyphae, and budding of hyphal tip [28] According to "reference [29]", the antifungal agents can deactivate the fungus by disrupting the structure and function of membranes or organelles of fungal cell and/or inhibiting the nuclear material or protein synthesis.

Chemically citrus skin contains different compounds, depending on the variety, so the aroma is different. However, the dominant compound of orange skin is limonene, this is based on previous studies that have analyzed the composition of the oil extracted constituents by GC-MS method, were detected which limonene was found as a dominant contributor [30]. It is the major component of the orange skin oil and probably the antibacterial and antifungal property of the oils.

V. Conclusion

Essential oil extract of Lemon Cui (*Citrus microcarpa)* Skin has inhibitory power to inhibit *Trichophyton rubrum* growth, marked by the clear zone around the well.

Acknowledgment

The author would like to thank the Health Polytechnic of the Health Ministry of Manado, Indonesia by RISBINAKES funds that have provided research funding, and also to Health Analysts Department of Manado Health Polytechnic that have supported through laboratory facilities until the research is completed, and to all relevant parties who have helped this research.

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