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Research Article

ANTIFUNGAL POTENTIAL AGAINST *Aspergillus flavus*: SECONDARY METABOLITE COMPOUND FROM UNIQUE ORGANISM OF LICHEN *Teloschistes flavicans*

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ABSTRACT

Lichen is a unique symbiotic organism formed from the mutualism relationship between fungus and algae. The aims of this research are to isolate and to examine antifungal activity of secondary metabolite compound from lichen *Teloschistes flavicans*. This research was conducted by extracting lichen dried powder using acetone solvent for 3×24 hours. The extract was isolated by Column Chromatography (CC) and Thin Layer chromatography (TLC) with G60 silica gel as stationary phase. Then it was eluted by n-hexane and ethyl acetate solvents in a gradient elution. The isolate was obtained and purified by recrystallization process then determined by using UV-VIS, FTIR, LC-MS, and 1H-NMR. The isolated compound is an orange needle "3-[1'-(2",3"-dihydroxy-phenyl)-propyl]-7hydroxy-chroman-4-one" with the formula of $C_{18}H_{18}O_5$. The antifungal activity test against *Aspergillus flavus* using disc diffusion method showed that the inhibitory zone was 11 mm in the concentration of 1000 mg mL^{-1}.

Keywords: Lichen, *Teloschistes flavicans*, antifungal, *Aspergillus flavus*, compound

INTRODUCTION

Trends "back to nature" is a trend in the last decade to produce natural drugs for human health. Indonesia is one of the mega-biodiversity countries in the world because it has a very abundant biodiversity and potential as a raw material for new drugs. The increase of public awareness about natural medicines is substantial to reduce the lack of side effects compared with the synthetic drugs[1]. In addition, it is rapidly to identify the raw material medicines from natural drugs based on various plant/organism types. A lot of natural plants/organisms were exploited by researchers to obtain the metabolite secondary for antimicrobial activity[2]. Due to the high activity, it is applicable for production the human drugs. The unique organism of lichen has not widely explored by researchers in Indonesia[3]. It is a organism which a mutual symbiosis with fungus and algae. In addition, it was a symbiotic organism between fungus (mycobiont) from the *Ascomycetes* and *Basidiomycetes* groups, with algae (phycobiont) from the *Cyanobacteria* or *Chlorophyceae* groups[4]. Lichen is known as an organism that contain a lot of potentials, especially in the chemical sciences, pharmaceutical sciences, and medicine. Lichen had been used in traditional medicine such as wounds, skin diseases, respiratory, and digestive disorders[2,5].

The high-content of lichen compounds were amino acid derivatives, pulvinic acid, peptides, sugar alcohols, terpenoids, steroids, carotenoids, aliphatic acids, monocyclic phenols, depsidone, depsidone dibenzofuran, anthraquinones, xanthone and other terpene derivatives[6]. The compound groups provide antimicrobial activities which are rarely found in other organisms. Based on the literature, the secondary metabolite from lichen showed the high-biological activity for antimicrobial and anticancer. Generally, the active compounds were usnic acid, eumitrin A1, and atranorin. The chemical structures content of identified lichen were approximately 350 compounds[7].

In recent decades, lichen has advantages as antioxidants[4], antibacterials[8], antifungals[9,10], antivirus[11], antimalarial[12], antiproliferative[13], cytotoxic activity[14] and anti HIV[15]. This condition leads to pharmaceutical activity so it has not been utilized as food security. The high problem of food poisoning among the public was caused by *Aspergillus flavus* fungus that endangers the health and human deadly. It is most likely to attack the pulmonary or cerebral arteries in Kenya. A hundred people were died due to fungal infection[16]. Aflatoxin have been produced by *A. flavus* and A. parasiticus as a group of toxic compounds. Aflatoxicosis in humans or livestock is due to consuming food or feed contaminated with high levels of aflatoxin.

In this work, we have explored the secondary metabolite from lichen *Teloschistes flavicans* to inhibit *A. flavus* fungal. This species has not been widely reported as antifungal activity. This is attributed to lichen as an anti-poison drugs on pharmaceutical science.

MATERIALS AND METHODS

Materials

The materials used were lichen *T. flavicans* organism taken from mountainous areas of Enrekang, South Sulawesi Province, Acetone (Merck, Germany), Ethyl Acetate (Merck, Germany), n-hexane (Merck, Germany), methanol (Merck, Germany), distilled water (IPHA, Indonesia), G.60 silica gel (0.063-0.200 mm), 10% sulfuric acid (Sigma-Aldrich), Potato Dextrose Agar (PDA) (Sigma-Aldrich), *Aspergillus flavus* fungal, physiological NaCl solution, ketoconazole, and aluminum foil.

Apparatus

The apparatus used in this research were rotary vacuum evaporator, analytic scale, column, UV lamp (λ 254 nm and 365 nm), Erlenmeyer, measuring cup, beaker, chamber, Thin Layer Chromatography (TLC) plate, porcelain, micropipette, drop pipette, vial bottle, capillary pipe, stative & clamp, spatula, stirring rod, scissors, tweezers, cutter, ruler, petri dish, autoclave, vortex, Bunsen, test tube, UV-VIS spectrophotometer (Jasco, Japan), LC-MS (Thermo Scientific), FTIR (Shimadzu: prestige 21), and NMR spectrometer (JEOL JNM ECA 500).

Sample Preparation and Purification

Lichen *T. flavicans* was cleaned from impurities and dried at ambient temperature. It was cut into the small size and mashed using a blender. Furthermore, 560 g lichen powder was extracted by maceration process using acetone solvent for 3 × 24 hours then filtered and separated by a rotary vacuum evaporator at temperature of 30-40°C. The extract was separated by gravity column chromatography (GCC) with G.60 silica gel as stationary phase using gradient eluent of n-hexane: ethyl acetate to obtain the extract fractions. It was analyzed using Thin Layer Chromatography (TLC) to obtain the spots color and purity compound. The final step, it was identified using UV-Vis, FTIR, LC-MS, and 1H-NMR spectrometers.

Antifungal Activity Test against *A. Flavus*

The antifungal activity test was carried out using disc diffusion method. At first, the materials and apparatus like petri dishes, test tubes, and Potato Dextrose Agar (PDA) media were sterilized using autoclaves at 121°C for 30 minutes. *A. flavus* fungus is rejuvenated using PDA media for 5-7 days. Subsequently, it was taken using an ose wire and put in a test tube containing 0.9% NaCl solution. Then, it was exported to obtain the same turbidity as the Mc-Farland standard was stated to be equal to 109 CFU mL^{-1}.

Extract and isolated compound were prepared in several concentrations of 1000 g mL^{-1}, 750 g mL^{-1}, 500 g mL^{-1}, and 100 g mL^{-1}. A positive and negative control were used for ketoconazole (+ control) and acetone (- control). *A. flavus* suspension was taken 1000 µL then mixed with PDA media and poured in a petri dish. After solidly, disc papers (6 mm) were dipped into each extract and isolated compound. It was placed on the surface media and incubated for 5-7 days at 37°C. The diameter clear zone formed in media then calculated using the calipers.

RESULTS AND DISCUSSION

Lichen Powder Extract

The *T. flavicans* lichen was extracted by maceration method and separated using vacuum rotary evaporator to obtain the concentrated extract. Based on the results, we have obtained as much as 57.27 grams. Then, it is separated using column chromatography to get separated extract as 135 eluents and analyzed using TLC to show the spot identity. The same Rf value was mixed and obtained the 12 fractions. Based on observations, the F3 fraction is needle crystal with orange color then recrystallization process to get the pure isolated compound. The compound was reanalysis using two dimensions TLC to explain the pure isolated compound. The analysis result can be seen in Figure 1.

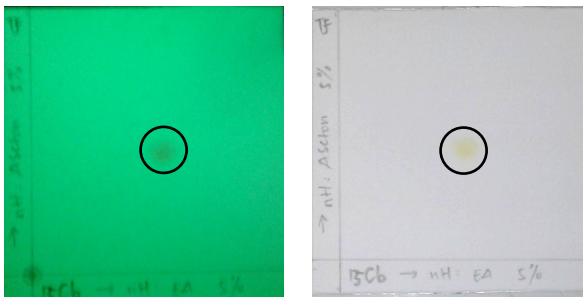


Figure 1: The 2 dimension TLC under UV light irradiation at wavelength of 254 and 365 nm

Figure 1 shows that the 2 dimension analysis using UV light was not appear other impurities. This was assumed that it is the pure isolated compound. It was characterized by UV-Vis spectrophotometer to obtain the maximum wavelength. Figure 2 explains that the isolated compound has a wavelength spectra of 270 nm. This phenomenon indicated that the sample has adsorb in UV light irradiation with high energy due to the low wavelength spectra.

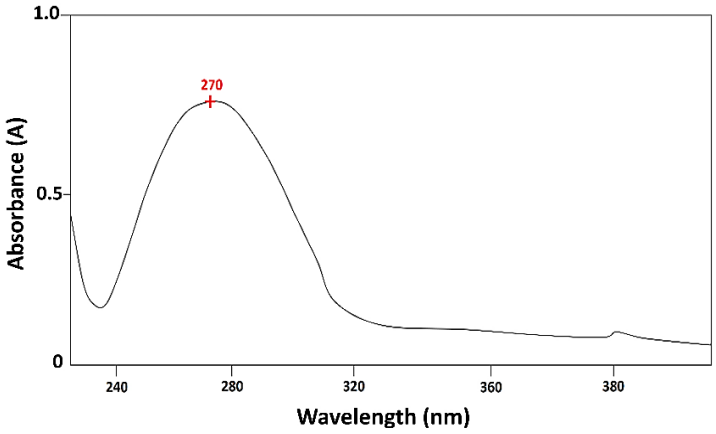


Figure 2: UV-Vis spectrophotometer spectra of isolated compound

Based on the data (Figure 2), the range wavelength determination on 200-400 nm explain that the carbonyl group absorbance was in wavelength range on 270 to 300 nm. This signifies the extracted compound containing the carbonyl group. This condition was confirmed by using Mass Spectrometer in order to identify the mass molecule of the isolated compound and we have found that it was contained a mass molecule [M+] is 315.21 m/z.

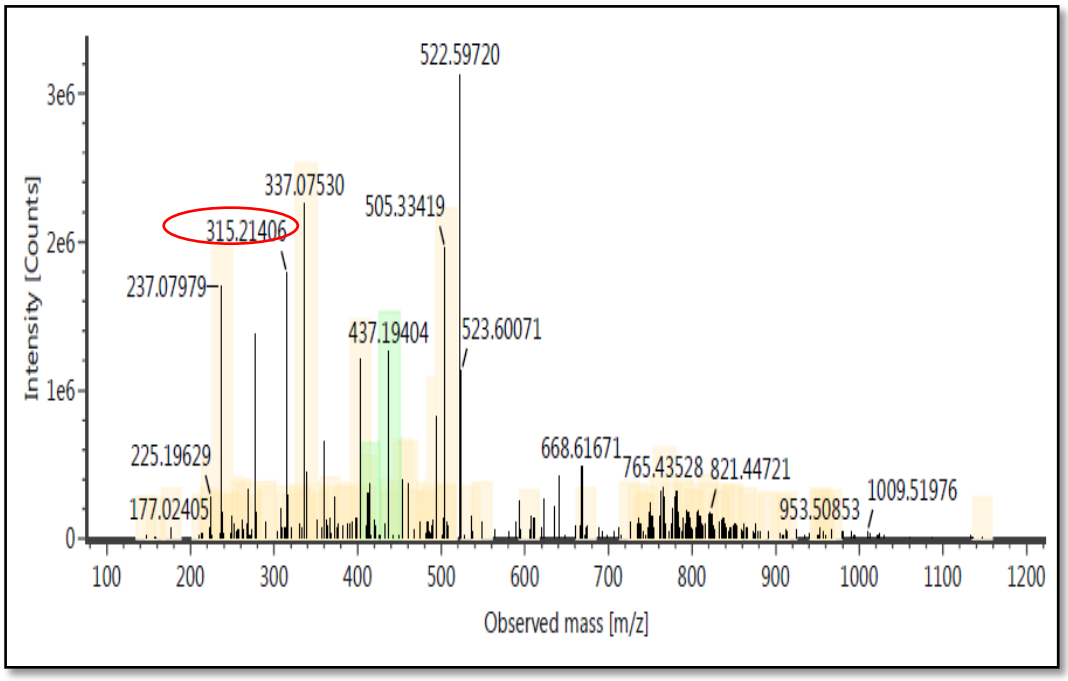


Figure 3: The mass spectrometer spectra of isolated compound

Figure 4 shows that the several characteristics of the functional group absorption have appeared. Absorption bands was identified in the area of 3321 cm^{-1}, 3209 cm^{-1}, and 3170 cm^{-1} indicate the presence of hydroxyl groups (-OH), the absorption area of 2932 cm^{-1} and 2860 cm^{-1}. Subsequently, the vibration indicates the Csp_3-H bonding and CH bending in the area of 1453 cm^{-1} and 1381 cm^{-1}. The area of 1326 cm^{-1} and 1239 cm^{-1} were a presence of C-C bond. The presence of C=C bond was shown in the area of 1625 cm^{-1} and 1566 cm^{-1} and the C-O stretch in the absorption area of 1037 cm^{-1} and 978 cm^{-1}. Based on FTIR spectrum data can be concluded that the isolates have hydroxyl (-OH) functional groups, aliphatic carbon (CH_3 and CH_2), carbonyl (C=O), alkene (C=C), and ether (C-O), respectively. The existence of functional groups from FTIR spectrum analysis was supported by ^{1}H-NMR spectroscopic analysis which can be seen in Figure 5.

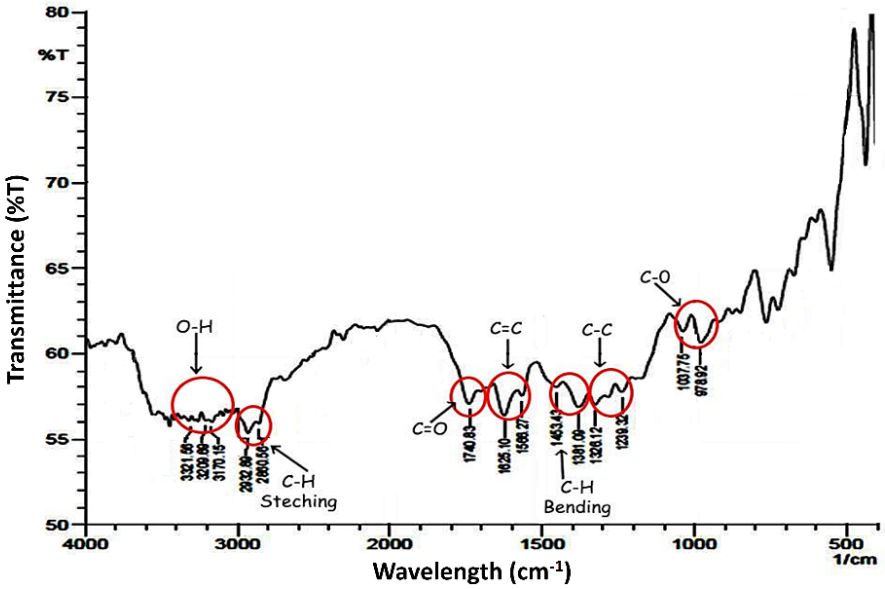


Figure 4: FTIR spectra from isolated compound

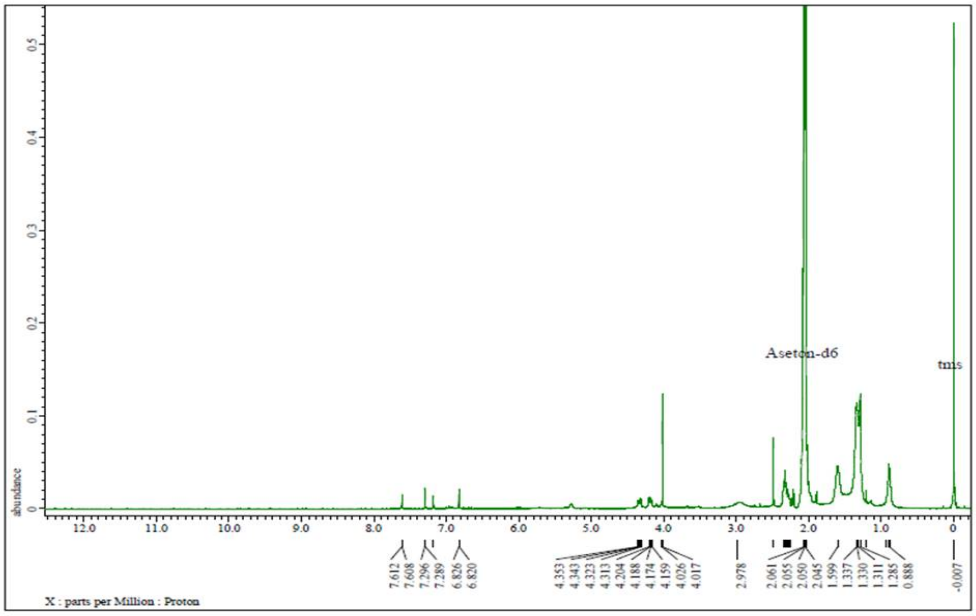


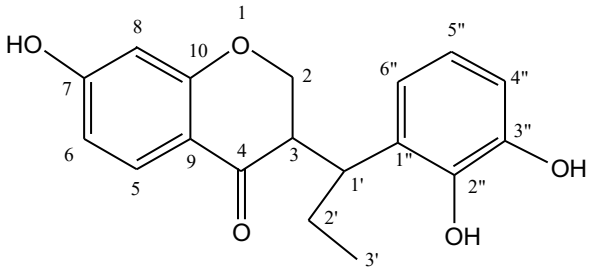
Figure 5: H-NMR spectra of isolated compound

The ^{1}H-NMR signal isolates in Figure 5 shows 13 types of proton signals representing 18 protons. There is 1 methyl proton signal at δH 0.833. The 4 types of protons have a chemical shift above 6 ppm which is 6.82; 7.182; 7.289 and 7.612 ppm which indicates that the proton has a low electron density or is bounding electron-attracting group like alkene group.

Table 1: ^{1}H-NMR data of isolated compound

δH (ppm)	Σ**H**, ***m***, **J (H)z**
0.088	1H, *t*, 3,5
1.330	1H, *t*, 3,5
1.599	1H, *s*
2.300	1H, *q*
2.484	1H, *s*
2.978	1H, *s*
4.017	1H, *s*
4.174	1H, *dd*, 7,5, 8
4.343	2H, *q*, 5, 5
6.820	1H, *d*, 3
7.182	1H, *d*, 1
7.289	1H, *d*, 3,5
7.612	1H, *d*, 2

Based on the spectrum data interpretation of UV-Vis spectrophotometer, LC-MS, FTIR, ^{1}H-NMR and compared with references, we have obtained the isolated compound with molecular formula $C_{18}H_{18}O_5$ and a molecular weight of 315.21 g mol^{-1}. The structure propose of isolated compound can be seen in Figure 6.



3-[1'-(2",3"-dihydroxy-phenyl)-propyl]-7-hydroxy-chroman-4-one

Figure 6: The structure purpose of isolated compound

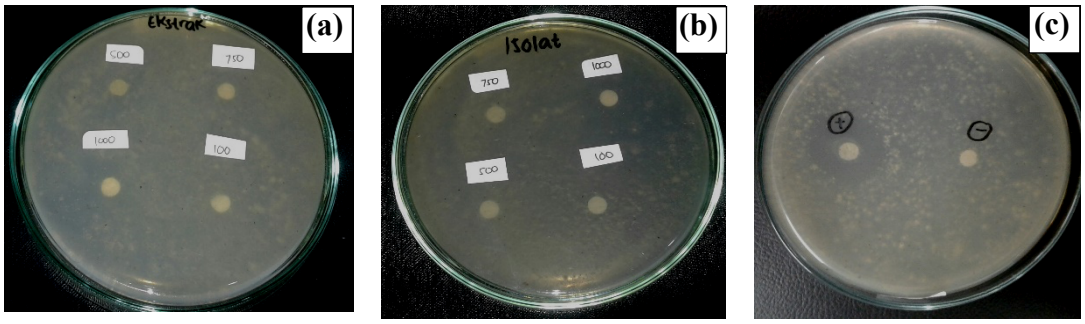


Figure 7: The antifungal activity test (a) lichen *T. Flavicans* extract, (b) isolated compound (c) positive-negative controls

Table 2: The results of diameter clear zone of antifungal test

Concentration (mg mL^{-1})	Diameter clear zone (mm)	
	Extract	Isolated compound
1000	10	11
750	7	8
500	4	6
100	0	0

Table 3: **The results of positive-negative controls**

Controls	Diameter clear zone (mm)
Ketoconazole (C+)	24
Acetone (C-)	0

Table 4: **Category of inhibitory power to inhibit of *A. flavus* fungus colonies**

Inhibit zone (mm)	Activity
≤5	Weak
6-10	Medium
11-20	Strong
>20	Very Strong

The result of antifungal activity test has carried out by using concentrated extract and isolate compound of lichen *T. flavicans*. It can be seen in Figure 7. Meanwhile, the results of diameter clear zone measurements in the antifungal activity test can be seen in Table 2 and Table 3. Based on the clear zone measurement, the acetone extract of lichen *T. flavicans* at 1000 and 750 mg mL^{-1} concentrations had medium antifungal activity, while at 500 mg mL^{-1} had weak antifungal activity. The isolated compound of lichen at a concentration on 1000 mg mL^{-1} has strong antifungal activity test against *A. flavus*. Subsequently, The concentrations of 750 and 500 mg mL^{-1} with medium activity. According to Maulidiyah et al. the category of inhibitory against antifungal activity test14 can be seen in Table 4. The positive control produces a clear diameter zone of 24 mm indicates that ketoconazole is a commercial fungal drug is very strong in inhibiting the growth of *A. flavus* fungus. According to Pizzolitto et al. reported the ability of the organic compounds to perturb the cell membrane integrity. Thus, the mechanism depends on the organic ability to affect the function of cellular lipoprotein membranes[17]. Moreover, refractivity index and molar volume are related to the antifungal effect, indicating that specific interactions between the phenolic compounds and the target receptor could be implicated in the inhibitory activity. Based on results, the lichen extract and isolated compound have the potential to inhibit *A. flavus* fungus.

CONCLUSION

Based on the spectrum data and elucidation measurement of isolated compound from lichen *T. flavicans* showed that the compound has a molecular formula $C_{18}H_{18}O_5$ with a molecular weight of 315.21 g mol^{-1}. The compound was proposed namely "3-[1'-(2",3"-dihydroxy-phenyl)-propyl]-7hydroxy-chroman-4-one". Antifungal test result of lichen extract and isolated

compound were obtained that the highest inhibition zone data at a concentration of 1000 mg mL^{-1} with diameter clear zone was 11 mm. This shows that the secondary metabolite compound was successfully isolated from lichen *T. flavicans* and as antifungal activity against *A. flavus*.

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