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Partial Purification of Antimicrobial Compounds Isolated from Mycelia of Tropical *Lentinus cladopus* LC4

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***Lentinus cladopus* LC4 produced at least eight antimicrobial compounds (ACs) which are active against plant and human pathogens. Three ACs in its crude mycelial were extracted with methanol and partial purification was carried out with silicic acid column chromatography and by thin layer chromatography (PTLC). The antimicrobial activity was tested by paper disc method and antibiographic method. The chromatography purification eluted with dichloromethane containing 5% methanol gave one active fraction (FII). This fraction which was active against *X. campestris* pv. *glycines* and showing two inhibition zones against *Bacillus subtilis* on bioautographic plates with the R_fs 0.8 and 0.7. FI and FIII fractions eluted with dichloromethane containing 0 and 10% methanol performed one inhibition zone with R_fs 0.8 and 0.7 respectively. However, their activities were lower than that of FII fraction. The PLTC purification gave one separate fraction with R_f value of 0.73 and it was active against *X. campestris* pv. *glycines*. The compound of R_f 0.73 fraction should be further studied using TLC and HPLC to obtain the pure substance for molecule characterization.**

Key words: tropical *Lentinus cladopus* LC4, antimicrobial compounds, partial purification

INTRODUCTION

Mushrooms have been applied throughout the world as both food and medicine for thousands of years. Two thousand years have passed since the first century Greek physician Dioscorides included the polypore *Fomitopsis officinalis*, as a treatment for tuberculosis, in his *De Materia Medica* published approximately 65 C.E. The potential of mushrooms (macrofungi) as sources of antibiotics was reported by Anchel, Hervey, Wilkins in 1941 (Sandven 2000). They tested the extracts of fruiting bodies and mycelia culture from over 200 species. Several compounds that inhibit the growth of a large spectrum of saprophytic and phytopathogenic fungi and bacteria were isolated from basidiomycetes (Anke 1989; Coletto *et al*. 1994; Anke 1995; Bianco & Striano 2000; Smania *et al*. 2001; Badalyan *et al*. 2008; Minato 2008). The study on polypores, such as several species of *Ganoderma*, *Trametes versicolor*, *T. Marianna*, *T. cingulata,* and *Laetiporus sulphureus* and gilled mushrooms, such as *P. ostreatus*, *Lentinus connatus,* and *Lentinula edodes* showed either the antibacterial, anti-candida, antiviral or cytotoxic activities (Hirasawa *et al*. 1999; Gerasimenya *et al*. 2002; Babitskaya *et al*. 2003; Rukachaisirikul *et al*. 2005; Turkoglu *et al*. 2006; Ofodile *et al*. 2008). Despite their potential and enormous diversity in tropical ecosystems, few studies aiming at the discovery of bioactive compounds from mushrooms were conducted in Indonesia.

A number of species of *Lentinus* are edible and supposedly therapeutic properties and traditionally are used by local peoples, several among them are *L. sajor-caju*, *L. squarrosulus*, *L. badius* (Pegler 1983), and *L. tuberregium* (Isikhuemhen *et al*. 2000). Several other antimicrobial compounds have already been discovered from *L. crinitus* (Abraham & Abate 1995), *L. degener* (Anchel *et al*. 1948), and *L. adhaerens* (Lauer *et al*. 1991). In addition several metabolites were produced by *Lentinus* as the fragrance compounds from *L. lepideus* (Hanssen & Abraham 1987), the antiaggregant compound from *L. adhaerens* (Lauer *et al*. 1991), and the antitumor substances from *L. lepideus* and *L. trabeum* (Jong & Gantt 1987).

On silica gel thin-layer chromatograms, the crude extracts of tropical *Lentinus* were separated into several bioautographic spots; for the filtrate extracts of *L. squarrosulus* 55A into three spots (R_fs 0.75, 0.50, and 0.17), the mycelial extracts of *L. sajor-caju* LSC8 into two spots (R_fs 0.77 and 0.54), the mycelial extract of *L. torulosus* LU3 into two spots (R_fs 0.77and 0.48), the filtrate extracts of *L. cladopus* LC6 into one spot (R_f 0.76) but the mycelial extracts of this mushroom separated into two spots (R_fs 0.79 and 0.54), the filtrate and mycelial extracts of *L. cladopus* LC4 into three spots respectively (R_fs 0.75, 0.61, and 0.45 for the filtrate extract and R_fs 0.83, 0.73, and 0.60 for mycelial extract) (Sudirman 2005), while the fruiting body extract into two spots (R_fs 0.68 and 0.08) (Sudirman 2009, unpublished data).

This work is part of a screening program aiming to discover new bioactive metabolites from tropical mushrooms. We reported here the results of purification

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process of three antimicrobial compounds of *L. cladopus* LC4 by column chromatography and preparative thin layer chromatography.

MATERIALS AND METHODS

Fungal Isolate. *L. cladopus* LC4 isolate was obtained by mating of two compatible monokaryon mycelia come from spores which were isolated from a fruiting body of *L. cladopus* LC. Bacterial plant pathogens, *Xanthomonas campestris* pv. *glycines* and *Bacillus subtilis* were obtained from Bacteriology Laboratory, Research Center for Agriculture Biotechnology and Genetic Resources (BALITBIOGEN), Bogor, Indonesia and Laboratory of Industrial Microbiology, ENSAIA, Nancy, French respectively.

Mycelial Production. Mycelia were obtained from liquid cultures of *L. cladopus* LC4. One inoculant of 7 mm diameter mycelium of this isolate was inoculated on the surface of 100 ml of EMP media (malt extract 15 g, bacteriological peptone 5 g, glucose 20 g, distilled water 1 l) in 250 ml of Erlenmeyer flask. All cultures were incubated at 35 ^{o}C at static condition for 30 days.

Mycelial Extraction. Mycelia of the cultures were separated from the culture filtrate using filter paper. Mycelia were ground with a mortar and then extracted twice with 50 ml methanol and agitated overnight on a rotary shaker for each extraction. The extracts were separated from the mycelia with fritted glass filter no. 3 and then dried under vacuum on a 30 ^{o}C water bath and a rotary evaporator, then they were redissolved in methanol.

Activity Test of Extracts. The method used for extract activity test was paper disc method using *Xanthomonas campestris* pv. *glycines* as target microbe (Sudirman *et al*. 1994). The test media for this bacterium was Luria Bertani Agar (LBA) (tryptone 5 g, yeast extract 2.5 g, NaCl 5 g, agar 7.5 g, distilled water 1 l).

Cell suspensions of *X. campestris* pv. *glycines* obtained by inoculating one loop inoculator of bacterial colony to 20 ml Luria Bertani Broth. The cultures were agitated on a shaker at room temperature for two days. As much as 100 µl of the cell suspension were added into 100 ml of LBA media. Then the 10 ml of the media were poured on a Petri dish.

The extracts were prepared by placing 100 µl on a 13 mm paper disc. The treatment control was the discs with similar solvents without the extracts. The discs were dried on air in order to remove the solvents, sterilized with UV ray (254 nm) for 30 min and then placed on the surface of LBA media containing *X. campestris* pv. *glycines*. The media were then incubated at 10 ^{o}C for 3-4 hours in order to diffuse the extracts into agar media. After incubation at room temperature (28-30 ^{o}C) for two days, inhibition activities of extracts were estimated by measuring the diameters of inhibition zones.

Partial Purification with Column Chromatography. Three active compounds [R_f values of 0.83 (LC4-3 fraction), 0.73 (LC4-2 fraction), and 0.60 (LC4-1 fraction)] were purified by silicic acid column chromatography. The crude extract were applied to a column (2.1 x 65 cm) filled with silica gel silicic acid (Merck 60, Nr. 9385) mixed with celite (BDH 15284) (1:1, w:w) and eluted with steps of increasing concentrations of methanol in dichloromethane: 0, 5, 10, 20, 60, 100%. The fractions were tested against *X. campestris* pv. *glycines* by paper disc method, detected by bioautography against *B. subtilis* and by being viewed under ultraviolet radiation at 366 nm (Sudirman 2005).

Detection of Extract Separation by Bioautography Method using *Bacillus subtilis*. The media for *B. subtilis* was Tryptone Glucose Yeast (TGY) (tryptone 5 g, yeast extract 5 g, glucose 1 g, K_2HPO_4 1 g, agar 7.5 g, distilled water 1 l). The cell suspensions of *B. subtilis* were obtained by growing 100 ìl of stock suspension on 25 ml of TGY media in Petri dish and incubated at 28 ^{o}C for 15 days. Each Petri dish was then added with 3 ml of sterile distilled water, the suspension was then scrapped with glass rod, filtered with sterile cotton and pausterized three times at 60 ^{o}C for 30 min. One hundred microliter of this cell suspension were added to 100 ml of TGY media and then the media were poured on TLC plate explained below.

Thin-layer chromatography (TLC) analysis was carried out using silica gel plates (Merck 60 F 254, 0.1-mm thick, 20 x 5 cm). All fractions from column chromatography were deposited as spots and developed in a butanol-acetic acid-water mixture (3:1:1, vol:vol:vol). Dry chromatograms were covered with 15 ml TGY medium containing *B. subtilis* and then incubated at 10 ^{o}C for 3-4 hours. The location of inhibition zones were estimated by measuring the R_f values of inhibition spots (Sudirman *et al*. 1994).

Partial Purification with Preparative thin Layer Chromatography. Three active compounds [R_f values were 0.83 (LC4-3 fraction), 0.73 (LC4-2 fraction), and 0.60 (LC4-1 fraction)] were further purified as well by preparative thin layer chromatography by using larger and thicker silica gel plates (Merck, Kieselgel G Nr. 7731, 2 mm thick, 20 x 20 cm). Crude mycelial extracts were deposited as spots along plates and developed in a butanol-acetic acid-water mixture (3:1:1, vol:vol:vol). Based on LC4-3, LC4-2, and LC4-1 R_fs, silica gels containing each active fraction were removed, extracted with methanol, and then dried under vacuum in a 30 ^{o}C water bath and a rotary evaporator, then redissolved in methanol. Each fraction was tested against *X. campestris* pv. *glycines*, detected by bioautography against *B. subtilis* and by being viewed under ultraviolet radiation at 366 nm (Sudirman 2005).

RESULTS

Antimicrobial Activity of *Lentinus cladopus* LC4 Mycelial Extracts. On the basis of experimental evidence, it appears that mycelial extract of tropical *L. cladopus* LC4 was considered to be further investigated due to its strong antimicrobial activity against *X. campestris* pv. *glycines*, a pathogen of soybean bacterial pustule disease with inhibition zone diameter up to 39 mm.

Partial Purification with Column Chromatography. Three fractions separated by silicic acid column chromatography i.e. FI, FII, and FIII eluted with

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