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Identification of active compounds and antifungal activity of *Toona sinensis* leaves fractions against wood rot fungi

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Abstract. *Elfirta RR, Falah S, Andrianto D, Lastini T. 2018. Identification of active compounds and antifungal activity of Toona sinensis leaves fractions against wood rot fungi. Biodiversitas 19: 1313-1318.* Inhibitory activities of surian or toon (*Toona sinensis*) leaves fraction origin Sumedang, West Java, Indonesia against wood rot fungi (*Ganoderma boninense* IPBCC 10.658*, Trametes versicolor* InaCC F200, and *Phanerochaete chrysosporium* IPBCC 93.259) were studied. The samples were macerated using methanol and acetone. The results of brine shrimp lethality test (BSLT) showed that the methanol extract had the highest cytotoxicity of LC_{50} at 29.76 µg/mL and inhibit the growth of *G. boninense* (27.78%), *T. versicolor* (79.26%) and *P. chrysosporium* (81.11%). The methanol extract was then subsequently fractionated using n-hexane, diethyl ether, and ethyl acetate respectively. The diethyl ether fraction was found to have the highest inhibitory activity against *T. versicolor* (46.30%) and *P. chrysosporium* (81.11%). This fraction was further separated using column chromatography and analyzed using thin layer chromatography (TLC), which gave six fractions. Antifungal test exhibited that fraction 5 had the highest antifungal properties against *T. versicolor* (74.07%) and *P. chrysosporium* (80.37%). Inhibition of *P. chrysosporium* resulted in abnormal growth of hyphae morphology as indicated by changes in its growth direction and excessive hyphae branching. Additionally, the results of LC-MS/MS experiment indicated toon leaves fraction 5 which contains N-[2- (D-Glucopyranosyloxy)ethyl]-2-hydroxy-N-[2hydroxy3 (octadecyloxy) propyl] butanamide dan Brucine compounds that were regarded as the antifungal compounds.

Keywords: Antifungal, surian, *Toona sinensis*, wood rot fungi

INTRODUCTION

Fungal attack is one of the biological factors of wood decays that must be controlled. Naturally, there are three types of wood rot fungi, i.e., white rot fungi, brown rot fungi and soft rot fungi. These fungi are capable of degrading the lignin component of the wood. The white rot fungi can degrade lignin, while brown rot fungi can break down cellulose and leave the lignin. The soft rot fungi accounted for making microscopic cavities inside the wood which make it softer; it is less aggressive decomposers on wet wood. White rot fungi, a group of wood rot fungi is reported to cause severe wood decays on industrial plant forests. *Ganoderma* sp. fungus was responsible for root decay of *A. mangium* in Sumatera, *P. falcataria* in Central Java and *A.chinensis* in West Java (Herliyana et al. 2012). Penetration of Ganoderma in host begins by degrading cell wall of root or basal stem enzymatically followed by tissue colonization. Microscopically, the success of penetration was evidenced by its swelling in epidermis (Suryantini and Wulandari 2018). Although the use of synthetic pesticide was useful to prevent the wood decay, it could potentially pollute environment due to its non-biodegradable properties. It contains chemicals derived from mineral and crude oil. Development of natural pesticide containing antifungal compounds must be carried out. Natural compounds are generally environmental friendly.

Surian or toon (*Toona sinensis*) is highly potent in comparison with other plants in West Java, but toon leaves only become the waste products of wood processing and has not been utilized properly. Empirically, almost all parts of the toon tree include leaves, seeds, bark, root bark, and sapwood has been used as traditional medicine in various countries (Shu et al. 2008). The toon leaves were reported to contain several bioactive compounds such as flavonoid, polyphenol, tannin, alkaloid, steroid, and terpenoid (Falah et al. 2015). Monisa (2016) reported that the ethanol extract of toon leaves contained 9.35 mg/g tannin that is associated with some biological activities including antifungal, antibacterial, antidiabetes, antitumor, antivirus, antioxidant, antihypertension, and antinematode (Hiyasih et al. 2002). Meanwhile, 0.3 mL methanol extract of toon leaves at dose of 10% (b/v) caused mortality of *X. festiva* larvae by 85% (Hidayati et al. 2013). This research was expected to provide scientific information about the utilization of bioactive compounds from toon leaf fraction as natural pesticide against wood rot fungi. The objective of this research was to determine the inhibitory activity, and identification of active compounds from toon leaves fractions as antifungal agent against wood rot fungi (*Ganoderma boninense, Phanerochaete chrysosporium,* and *Trametes versicolor)*.

MATERIALS AND METHODS

Study area

This study was conducted from July 2017 to March 2018 at the Laboratory of Department Biochemistry and Department Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Bogor, Indonesia.

Materials

The leaves (moisture content of 9.31%) were obtained from 30-year-old toon (*Toona sinensis*) tree in Regency of Sumedang, West Java, Indonesia. Samples were macerated using methanol and acetone solvents. The leaves extract was successively fractioned using n-hexane, diethyl ether, ethyl acetate, and water. The wood rot fungi *Ganoderma boninense* IPBCC 10.658, *Trametes versicolor* InaCC F200 and *Phanerochaete chrysosporium* IPBCC 93.259 were used. Other experimental materials included chromatography Potato Dextrose Agar (PDA), silica gel 60 G (230 mesh) Merck. All materials were sigma grade or equivalent. The laboratory glasswares were sterilized using autoclave at 121ºC (Tomy Model Es 315). The morphology of hyphae was observed under binocular microscope (Olympus CX3L). LC-MS/MS experiment using LC System Ultra Performance Liquid Chromatography dan Mass Spectrophotometry Electrospray Ionization.

Procedures

Extraction of toon leaves

The sample was macerated using methanol or acetone (1:10) and shook in a 150 rpm shaker for 24 h. The filtrate was then evaporated using rotary vacuum evaporator at 50°C.

Cytotoxicity test using Brine Shrimp Lethality Test (BSLT)

The cysts of *Artemia salina* were hatched in 100 mL of seawater for 48 h. The larvae obtained were then placed into a test tube, followed by addition of 1000 μL of sample solution at concentration of 5-1000 µg/ml. Each sample solution was carried out at triplicates. The dead larvae were counted after 24 h of sample exposure. The probit analysis was conducted to determined LC_{50} values at confidence level of 95% (Meyer et al. 1982).

Extract fractionation

A total of 2 g toon leaves extract was dissolved in 100 mL of water at 50°C. The solution was then transferred into a separating funnel and subsequently fractioned by addition of n-hexane (100 mL) to obtain n-hexane fraction and water fraction. The water fraction was then re-fractioned using diethyl ether and ethyl acetate. All resulting fractions (n-hexane, diethyl ether, ethyl acetate, and water) were evaporated using rotary vacuum evaporator (Kim et al. 2008).

Fractionation by column chromatography

The 21.5 cm diameter column was packed with 20 g silica gel G 60 (230 mesh) Merck. 1 g extracts were then introduced with the packed column. Three types of mobile phase were used, i.e., n-hexane, diethyl ether, and methanol. Eluent was collected in a 10-mL test tube and analyzed by thin layer chromatography (TLC). The eluted fraction with similar Rf value was collected and evaporated using rotary vacuum evaporator.

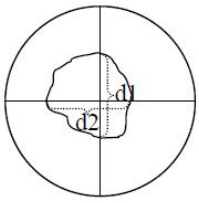

Figure 1. Determination of radial diameter of rot fungi colony (Winara 2014)

Determination of antifungal activity

The PDA (4 mL) sterile and leaves extract (1 mL) were mixed in 6-cm petri dish. The 7-day-old experimental fungus (at diameter of 5 mm) were inoculated at the center of petri dish, followed by incubation for 3-7 days at room temperature (Jemi et al. 2010). The hexaconazole (100 ppm) and water were used as positive control and negative control, respectively. The inhibitory activity was determined when mycelium in the control treatment fully covered the medium surface in petri dish (Figure 1).

The colony diameter and antifungal activity were determined as follow:

$$\text{Colony diameter} = \frac{d1 + d2}{2}$$

$$\text{Antifungal activity (AFA)} = \frac{GC - GT}{GC - A} \times 100\%$$

Where:
AFA = antifungal activity (%)
GC = mycelium growth in control treatment (mm)
A = initial mycelium growth (mm)
GT = mycelium growth in leaf extract treatments (mm)

Microscopic observation of fungal hyphae

The fungal mycelium (taken from inhibitory zone) was placed on the object glass. The preparation was set on wet tissue and incubated for 3 days at room temperature and observed under binocular microscope at magnificent of 400×.

Identification of active compounds using Liquid Chromatography-Mass Spectrometry and Tandem Mass (LC-MS/MS)

LC-MS/MS analysis was performed by gradient elution with a flow rate of 0.2 mL/min. The injected volume of samples was 5 µL, and analysis was performed for 23 min at 50°C.

Data analysis

Determination of LC_{50} in BSLT using probit analysis at confidence level of 95%. The quantitative data were analyzed using Analysis of Variance (ANOVA). When the result is significantly then Tukey Test was applied. The analysis was conducted using Minitab 16.

RESULTS AND DISCUSSION

Cytotoxicity test

The results of probit analysis at confidence level of 95% showed that LC_{50} values of methanol and acetone extract of toon leaves were 29.76 ± 1.20 µg/mL, 102.37 ± 3.41 µg/mL, respectively.

Antifungal activity of crude surian leaf extract

The result of antifungal activity showed that the effect of methanol extract was greater than that of hexaconazole as well as the positive control. The inhibitory activity of the extract against *P. chrysosporium*, *T. versicolor* and *G. boninens*e was 81.11 ± 0.00%, 79.26 ± 2.31%, and 27.78 ± 1.92%, respectively, while hexaconazole seemed to have lower antifungal activity against the experimental fungi, namely 80.07 ± 0.95%, 76.09 ± 0.48%, and 29.89 ± 0.10%. The results showed that the antifungal activity is affected by the crude extracts significantly (ρ <0.05). However, the crude extracts did not significantly affect (ρ > 0.05) *G. boninens*e (Table 1).

Liquid-liquid fractionation and antifungal activity

The resulting fraction was then tested for antifungal activity against *P. chrysosporium* and *T. versicolor* (Table 2). The results indicated that diethyl ether fraction of toon surian leaves showed the highest inhibitory activity against *P. chrysosporium* (80.74 ± 0.64 %) and *T.versicolor (*46.30 ± 1.28 %). The results showed that the antifungal activity were significantly (ρ <0.05) effected by the various fraction extract.

Column chromatography

Fractionation by column chromatography was performed to the fraction that had the highest antifungal activity, i.e., diethyl ether extract. Our experiment yielded 39 vial tubes of eluate and was further tested using TLC (observed under 254 UV light to appear the spots). Retardation factor (Rf) value of each spot was determined and pooled, which gave 6 fractions. They were then tested for antifungal activity against *P. chrysosporium* and *T. versicolor.* The results indicated that there was significant difference (ρ <0.05) in the antifungal activity of the chromatography fraction extracts.

Table 1. Inhibitory activity of crude methanol extract, acetone extract, and hexaconazole against *Phanerochaete chrysosporium Trametes versicolor,* and *Ganoderma boninense*

Crude extracts	Antifungal activity (%)		
	P. chrysosporium	*T. versicolor*	*G. boninense*
Methanol	81.11 ± 0.00[a]	79.26 ± 2.31[a]	27.78 ± 1.92[a]
Acetone	44.41 ± 1.06[b]	36.30 ± 0.32[b]	16.30 ± 0.64[a]
Hexaconazole (+ control)	80.07 ± 0.95[a]	76.09 ± 0.48[a]	29.89 ± 0.10[a]

Note: The values of antifungal activity represented mean ± standard error for 3 replicates; Antifungal activity with different superscript alphabetic letters was significantly different at < 0.05 by Tukey test

Table 2. Antifungal activity of liquid-liquid fractionation against *Phanerochaete chrysosporium* and *Trametes versicolor*

Fraction	Antifungal activity (%)	
	P. chrysosporium	*T. versicolor*
n-Hexane	66.67 ± 1.92[b]	35.56 ± 0[b]
Diethyl ether	80.74 ± 0.64[a]	46.30 ± 1.28[a]
Ethyl acetate	55.19 ± 3.57[c]	16.67 ± 1.11[c]
Water	50.19 ± 0.85[c]	4.26 ± 0.32[d]

Note: The values of antifungal activity represented mean ± standard error for 3 replicates; Antifungal activity with different superscript alphabetic letters was significantly different at < 0.05 by Tukey test

Table 3. Antifungal activity of toon leaves fraction after column chromatography fractionation against *Phanerochaete chrysosporium* and *Trametes versicolor*

Fractions	Antifungal activity (%)	
	P. chrysosporium	*T. versicolor*
1	1.85 ± 1.28[d]	32.59 ± 0.64[c]
2	58.15 ± 4.21[b]	21.85 ± 0.64[d]
3	50.00 ± 1.11[c]	39.63 ± 2.31[b]
4	77.04 ± 0.64[a]	42.59 ± 0.64[b]
5	80.37 ± 0.64[a]	74.07 ± 0.64[a]
6	1.85 ± 0.64[d]	6.30 ± 2.31[e]

Note: The values of antifungal activity represented mean ± standard error for 3 replicates; Antifungal activity with different superscript alphabetic letters was significantly different at < 0.05 by Tukey test

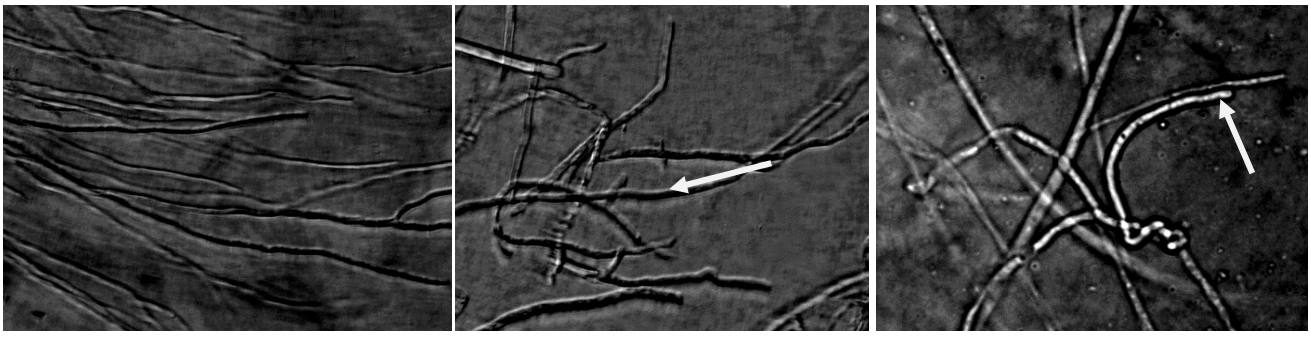

Figure 2. Microscopic figure of *Phanerochaete chrysosporium* hyphae: Left: wild culture, middle and right: fungus hyphae after exposed to toon leaves fraction

Microscopic morphology of hyphae

The fungal hyphae of *P. chrysosporium* were observed since it was most inhibited by the fraction 5. The results showed that addition of toon leaves fraction could affect hypha morphology, including changes in its growth direction and excessive hypha branching.

Identification of active compounds using LC-MS /MS

Analysis of LC-MS /MS chromatogram showed that the fraction 5 was comprised of some compounds (Figure 3). LC-MS/MS chromatogram displayed 21 peaks with different molecular weights. Our experiment successfully identified about 11 metabolite compounds in the fraction 5 (Table 4). The highest peak in chromatogram was found at retention time of 12.431 min with molecular weight of 634.4530 *m/z*. This compound presumably represents N-[2-(D-Glucopyranosyloxy) ethyl]-2-hydroxy-N-[2-hydroxy3 (octadecyloxy) propyl] butanamide. Furthermore, a peak at retention time of 3.032 min was attributed to secondary metabolite of indole alkaloid, Ajmalicine. Another alkaloid compound, Brucine was also detected at retention time of 3.650 min with molecular weight of 395.1986 *m/ z*.

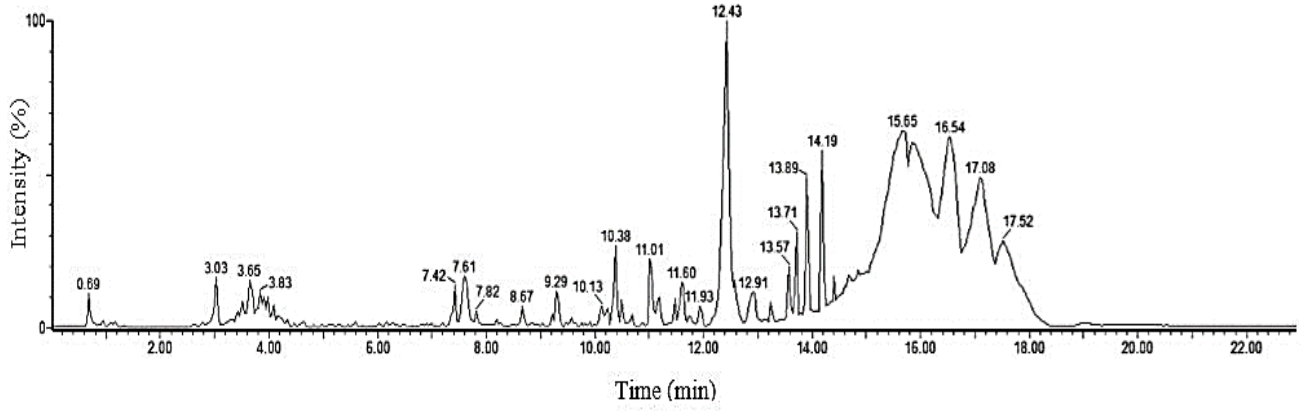

Figure 3. Chromatogram of the fraction 5

Table 4. Identified compounds of LC-MS / MS chromatogram from toon leaves fraction 5

No.	Retardation time (Rt)	Molecular weights (m/z)	Regarded compounds	Intensity (%)
1	3.032	353.1855	Ajmalicine	0.96
2	3.650	395.1986	Brucine	3.70
3	7.423	530.2987	1,3,4,6-Tetra-O-acetyl-2-deoxy-2- (dodecanoylamino) hexopyranose	1.93
4	9.286	397.1719	4-[(Z)-{[4,6-Di (1-pyrrolidinyl)-1,3,5-triazin-2yl] hydrazono}methyl]-2-nitrophenolate	0.63
5	10.384	270.1827	Orphenadrine	1.82
6	11.013	304.3010	2,4-Dimethyl-6-tetradecylpyrylium	1.08
7	11.596	496.3403	1- (β-D-Arabinofuranosyl)-4- (heptadecanoylamino)-2 (1H)-pyrimidinone	0.72
8	11.928	482.3585	1-{(2R,3S,5S)-3-Amino-2-hydroxy-5-[4-methoxy-3- (3-methoxypropoxy) benzyl] -6-methylheptyl}-3-pentylurea	0.02
9	12.431	634.4530	N-[2- (D-Glucopyranosyloxy)ethyl]-2-hydroxy-N-[2-hydroxy-3(octadecyloxy) propyl]butanamide	7.98
10	13.574	609.2687	3,8,13,17-tetramethyl-12-vinyl-2,7,18-Porphinetripropionic acid	0.01
11	14.191	593.2799	1-(1-{4-[6-(1-Methyl-1H-tetrazol-5-yl)-3-phenyl-2-quinolinyl]bezyl}-4-piperidinyl)-1,3-dihydro-2H-benzimidazol-2-one	6.49

Discussion

Clarkson et al. (2004) reported that the extract is considered as non-cytotoxic at LC_{50} > 1000 µg/mL, low cytotoxicity at LC_{50} 500-1000 µg/mL, moderate cytotoxicity at LC_{50} 100-500 µg/mL, and high cytotoxicity at LC_{50} < 100 µg/mL. Based on these criteria, crude methanol extract of toon leaves was highly cytotoxicity ($LC_{50} \leq 100$ µg/mL), while acetone extract of toon leaves was attributed to moderate cytotoxicity (LC_{50} 100 > 500 µg/mL). Sari et al. (2011) reported that the ethanol extract of toon leaves classified as toxic at LC_{50} of 35.76 µg/mL, while the ethanol extract of toon inner bark was highly toxic at LC_{50} of 34.35 µg/mL and the ethanol extract of toon sapwood exhibited higher toxicity at 12.37 LC_{50} of µg/mL. These findings suggest that toon leaves extracts obtained using organic solvent extraction was highly cytotoxicity. Brine shrimp lethality test (BSLT) was performed to evaluate bioactivity of an extract (Colegate and Molyneux 2007), including anti-microorganism, anticancer, antitumor, and antimalaria. In our study, BSLT was applied to determine concentration that showed the lowest toxicity for evaluation of antifungal activity.

In this present work, two solvents were used in maceration, i.e. methanol and acetone. With its high polarity, methanol was expected capable of extracting antifungal compounds. Presence of hydroxyl and methyl in methanol contributes to binding of polar, semi-polar, and non-polar components. Furthermore, acetone is a polar aprotic solvent with moderate polarity. This solvent enables to dissolve bioactive compounds such as phenolic, triterpenoid, steroid, flavonoid, and saponin. The methanol and acetone extracts of toon leaves were tested for antifungal activity against *P. chrysosporium*, *T.versicolor,* and *G. boninense.* Both extracts were applied at concentration of 30 µg/mL as obtained from LC_{50} values of methanol extract of toon leaves. Antifungal activity was determined according to mycelium growth compared to control. The positive control was hexaconazole, which is a commercial pesticide, and prepared at concentration of 100 µg/mL as recommended dose for rot fungi. Methanol is capable of dissolving secondary metabolites such as tannin. Tannin has hydroxyl groups and double bonds α-β that are responsible for its antimicrobial properties. These hydroxyl groups could alter biosynthesis of cell wall and membrane in fungal cells. Monisa (2016) reported that tannin found in toon leaves extract was regarded as hydrolyzed tannin. Saeida et al. (2015) reported that pyrogallol, a form of hydrolyzed tannin, exhibited biological activities of fungicidal. The antifungal activity of acetone extract against *P.chrysosporium*, *T. versicolor,* and *G. boninens*e was 44.41 ± 1.06 %, 36.30 ± 0.32%, and 16.30 ± 0.64 %, respectively. Valima et al. (2007) found that acetone could dissolve lignan and stilbene in genus Pinus that could serve as antifungal agent. The antifungal properties of the genus Pinus closely related to content of stilbene and marginal activity of lignan against fungi. Therefore, antifungal activity of acetone extract may be linked with presence of lignan and stilbene extracted by acetone.

The methanol extract of toon leaves with the highest inhibitory activity was subsequently fractioned using 4 solvents having gradual polarity, i.e. n-hexane (0.1), diethyl ether (2.8), ethyl acetate (4.4), and water (10) (Markom et al. 2007; Brown et al. 2015). Non-polar solvents enable to bind wax, lipid, and essential oils. Semi-polar solvents are capable of extracting phenol, terpenoid, alkaloid, aglycon, and glycoside, while polar solvents could extract alkaloid, phenolic, carotenoid, tannin, amino acid, and glycoside. The resulting fraction was then tested for antifungal activity against *P. chrysosporium* and *T. versicolor* (Table 2). The results indicated that diethyl ether fraction of toon leaves showed the highest inhibitory activity against *P. chrysosporium* and *T.versicolor.* Diethyl ether can dissolve terpenoid compounds. The antifungal mechanism of terpenoid is through alteration of membrane permeability. Terpenoid plays a role in penetrating other secondary metabolites into fungal membrane. Bioactivity of a compound could be affected by presence of other compounds, resulting in some interactions such as synergistic, anti-synergistic, or complementary. The antifungal activity of n-hexane fraction of toon leaves against *P.chrysosporium* and *T.versicolor* reached 66.67 ± 1.92% and 35.56 ± 0%, respectively. Jemi et al. (2010) found that n-hexane extract of Kupa *S. polycephalum (Mig)* wood had the highest antifungal activity against *S. commune* Fr, while its ethyl acetate fraction could retard growth of *Pleurotus* sp. Duraipandiyan and Ignacimuthu (2011) reported that n-hexane extract of S. cumini leaves was able to inhibit the growth of T. simil, E. floccosum, T. rubrum and M. grisea fungus. The n-hexane extract from *S. samarangense* contain coumarins, saponins, steroids compounds that can inhibit the growth of fungi (Kuo et al. 2004). Antifungal activity of ethyl acetate fraction of toon leaves against *P. chrysosporium* and *T. versicolor* was 55.19 ± 3.57% and 6.67 ± 1.11%, respectively. Yuh-Chi et al. (2004) found that ethyl acetate extract of *Syzygium samarangense* contained flavonoid and quinone that had antifungal properties. Phenolic compound was reported capable of denaturating cellular protein and inducing cell wall shrinkage, thus causing lysis. Presence of hydroxyl group in phenolic compound could also form linkage with sulphydryl group in fungal protein, altering protein conformation of the cell wall in fungi.

Fractionation by column chromatography was performed to the fraction that had the highest antifungal activity. The selected sample was diethyl ether fraction with antifungal activity against *P. chrysosporium* and *T. versicolor*. The 1.5 cm diameter and 30 cm height column are packed with 20 g silica gel 60 (230 mesh) Merck, which was first suspended in n-hexane for 3 h. The solvent system for mobile phase was prepared in gradient, aiming to induce faster component movement. The aluminum plat of silica gel was polar which could bind polar compounds. When the compound is non-polar, it is then faster moved at presence of non-polar solvent, vice versa. During elution, the first flushed component was non-polar compound (eluted with n-hexane), then semi-polar compound (eluted with mixture of n-hexane and ethyl acetate) and lastly followed by polar compound (eluted with methanol). The resultant of separating process was collected in vial tube (final volume of 10 mL for each eluate). Our experiment

yielded 39 vial tubes of eluate and was further tested using TLC (observed under 254 UV light to appear the spots). Retardation factor (Rf) value of each spot was determined and pooled. The spots with similar Rf was pooled, which gave 6 fractions. The results showed that fraction 5 had the highest inhibitory activity against *P. chrysosporium* and *T. versicolor* compared to other fractions. The fraction 5 was then used for microscopic morphology observation of hyphae and analysis of bioactive marker compounds using LC-MS /MS.

The results of microscopic morphology observation showed that addition of toon leaves fraction could affect hypha morphology, including changes in its growth direction and excessive hypha branching (Figure 2). This is caused by detrimental effects of secondary metabolite on culture medium. Other morphological changes included swelling hyphae, shrinkage, circular and excessive growth of hyphae (Hu et al. 2003). The mechanism of antifungal compounds may vary, including the neutralization of enzyme related to fungal invasion, destruction of fungal cell membrane, reduction of enzymatic activity regulating growth of hyphae, and altering nucleic acid and protein synthesis (Djunaidi 2008). The results of LC-MS/MS experiment indicated toon leaves fraction 5 contains N-[2-(D-Glucopyranosyloxy)ethyl]-2-hydroxy-N-[2hydroxy3 (octadecyloxy) propyl]butanamide and Brucine which may relate to the antifungal properties of the fraction 5 (Figure 3). Mallikharjuna and Seetharam (2009) reported that Brucine compound had antifungal activity against *A. niger, A. fumigatus, C. albicans* and *M. gypseum* fungi.

In conclusion, the research showed that the toon leaves fraction has inhibitory activity against wood rot fungi. Addition of toon leaves extracts altered the direction of hypha growth and induced excessive hypha branching of *P.chrysosporium*. The LC-MS/MS experiment showed that the fraction 5 of toon leaves extract contained N-[2- (D-Glucopyranosyloxy) ethyl]-2-hydroxy-N-[2-hydroxy3 (octadecyloxy) propyl]butanamide and Brucine compounds that were regarded as the antifungal compounds, which have not yet been reported.

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