Antifungal Activity and Identification of Active Compounds From Wood *Tristaniopsis whiteana* (Griff) Against Wood Rot Fungus

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Abstract

The aim of the study was to analyze the content of pelawan wood extractive substances (*Tristaniopsis whiteana* (Griff)) and to test it with the fungi *Schizophyllum commune* Fr and *Pleurotus ostreatus*. Pelawan heartwood powder was macerated with methanol. Then fractionated in stages with n-hexane, chloroform, ethyl acetate, and butanol. The resulting extract was then tested with *S. commune* and *P. ostreatus* fungi. The most active fraction was isolated using column chromatography with a gradient system, the eluent was methanol:chloroform. Sub-fractions were then tested for fungi to determine the most active sub-fractions, and the most active sub-fractions were then analyzed by 1H NMR. The results showed that the extractive content of pelawan wood was most soluble in chloroform. All extract fractions contain potential as anti-fungal. .The chloroform fraction was very active compared to the other fractions. Isolation of the chloroform fraction by column chromatography obtained 8 sub-fractions. All of these sub-fractions were able to inhibit the growth of *S.commune* and *P.ostreatus* with $IC_{(50)}$ = 54.55 - 64.69 mg/L and IC(50) = 54.17 - 64.44 mg/L respectively. PL.3 sub-fraction was the most active among the 8 sub-fractions. The results of ^{1}H NMR analysis on the PL.3 subfraction were shown to be Heptanoic Acid compounds.

Keywords: Chloroform, Heptanoic acid, NMR, *Schzophyllum commune* Fr, *Pleurotus ostreatus*.

Introduction

Fungi are organisms that can cause damage to agricultural plants, forest trees, wood and processed products made from wood. Some fungi are also useful in agricultural processes, pharmaceutical industry and biotechnology such as biopulping, bioremediation, biofuel (Zabed *et al*. 2019; Ghosh, 2020, Singh *et al*. 2020, Moya *et al*. 2023). Fungi attack wood because wood is a substrate that contains lignocellulosic materials.

The lignocellulosic material in wood can be damaged by wood rotting fungi with a hydrolytic enzyme system. Wood with a low durability class is very easily attacked by wood rot fungi. The impact of wood decay fungus attack is that it can reduce the quality and aesthetics of the wood. Rotting fungi are more capable of reducing the weight of wood than termite attacks (Goodell & Nielsen, 2023). The *P. ostreatus* fungus is able to remove 27.4% of lignin and 1.58% of cellulose in Beech wood (Bari *et al*. 2021). To overcome attacks by wood rot fungi, the wood needs to be preserved before use. However, the synthetic wood preservatives used can have a negative impact on the environment and human health and these materials cannot be renewed (Meena, 2022; Changotra *et al*., 2024). Hence, it is necessary to study biodegradable natural preservatives which the ingredients are easy to obtain.

Antifungal ingredients can be obtained from wood which has natural durability, because it contains toxic extractive substances. Extractive substances are secondary metabolic compounds that have ecological functions (Vek *et al*. 2022; Mai & Zhang, 2023).

Tropical forests in Indonesia are rich in wood species that are naturally durable against wood-destroying organisms attacks. One of them is pelawan wood (*Tristaniopsis whiteana*). *T. whiteana* wood belongs to the *Myrtaceae* family. There are 40 species of *Tristaniopsis*, from shrubs to trees. This wood is used as building material, making boats, bridges and floors (Denny *et al*. 2019; Hartanto, 2019; Aldi *et al*. 2021). Pelawan wood is resistant to fungus, termite and sea worms attacks and is classified as durable class I and II, and strong class I (Muslich & Sumarni, 2005; Jasni. 2016). Several species of pelawan wood have been studied for their wood, bark and leaves, such as *T. laurina, T. obovate, T. mergu*ensi which are anti-inflammatory, anti-diabetic, antioxidant, antibacterial and anti-toxic (Enggiwanto *et al*. 2018; Al Kadri *et al*. 2019; Pratiwi, 2019; Roanisca *et al*. 2019; Mahardika *et al*. 2020; Kusuma *et al*. 2022; Mathew *et al.* 2022).

Based on the description above, it is known that the *T. whiteana* wood species has bioactive potential. However, the antifungal activity of rotted wood on *T. whiteana* wooden terraces has never been reported. This research aims to analyze and determine the content of extractive substances, antifungal activity and to identify active compounds from *T. whiteana* wood.

Materials and Methods

Making Wood Powder

Pelawan wood with a diameter of 25 cm was obtained from tropical natural forests in Hanua Ramang Village, Central Kalimantan Province. Before being used as research material, the wood was first identified at the BRIN Biological

Research Center, Cibinong, to determine the correct scientific name. The heartwood is made into powder with a size of 40 mesh (Santoso *et al*. 2020). The wood powder obtained is then air-dried naturally until it reaches air-dry conditions (moisture content 12-15%). The resulting sawdust is then put into a plastic bag and closed tightly.

Maceration of Wood Powder

The maceration process referred to a procedure by El Aziz *et al*. (2019). 2000 g of pelawan sawdust were macerated with methanol solvent at room temperature and 1 atm pressure for 48 hours. Stirring was applied during the maceration process. The ratio of sawdust to solvent was 1:3 (v/v). The maceration process was carried out repeatedly with stirring every three hours, for 15 minutes until a clear filtrate was obtained. The methanol extract of pelawan wood was evaporated using a rotary evaporator at a temperature of 40^{o}C and a pressure of 1 atm to obtain a crude extract. The dry weight of the methanol extract was determined in the following way: 10 ml was taken from the methanol extract, then concentrated at 40°C until the extract was crystallized. After cooling,the dry weight of the methanol extract was determined. The methanol extract content was calculated based on the percentage of the solid weight of the methanol extract to the dry weight of the sawdust kiln.

Extract Fractionation

The fractionation procedure refers to the procedure of Lezoul *et al*. (2020). 100 ml of methanol extract was fractionated step by step with solvents namely n-hexane, chloroform, ethyl acetate and butanol. The crude extract mixture was shaken in a separating funnel for 10-15 minutes until the dissolved n-hexane extract and the crude extract dissolved in methanol were separated. Mixing was carried out until the filtrate becomes clear. The remaining fraction was then fractionated sequentially using chloroform, ethyl acetate, and butanol solvents. The extract filtrate of each fraction was concentrated using a rotary vacuum evaporator.

Column Chromatography Fractionation

The gravity column chromotography procedure referred to the procedure of Elfirta *et al*. (2018). The column was 50 mm in diameter and 100 cm long, containing 20 g G 60 (230 mesh) Merck silica gel. 0.5 g of the active fraction was placed in a column containing silica gel and methanol: chloroform eluent using a gradient system. .The extract obtained was collected in a 10 mL test tube. Next, analyzed using thin layer chromotography (TLC), the eluted fraction with the same Rf value was collected and evaporated using a rotary vacuum evaporator.

Cultivation of Wood Decay Fungi

The wood rot fungi used were *S. commune and P. ostreatus*, which are classified as white rot fungi (Li *et al*. 2022). The fungus was obtained from the IPB Pathology Laboratory. The fungus was first cultured on potato dextrose agar (PDA) growth medium for seven days. The composition of the growth media referred to the composition of Kusuma *et al*. (2004) as follows: 1 liter of growth medium contains 50 g of glucose, 120 g of onion extract, 50 g of glucose, 0.3 g of K2HPO4, 0.2 g of MgSO4.7H2O, 5 g of polyptone, and 30 g of agar powder, pH 6.0.

Testing of Anti-fungal Activity

The testing of all extractive substances were obtained from maceration, fractionation and column chromotography results. The treatments were variations in the concentration of extractive substances as follows: 0 mg/L, 50 mg/L, 100 mg/L, 250 mg/L, 500 mg/L and 1000 mg/L. The positive control was CCB (copper-chrome-boron) wood preservative with a concentration of 100 mg/L. Firstly, the petri dish was autoclaved for 15 minutes at a temperature of ± 120°C with a pressure of 1 atm. Filled with media and extractive substances according to the treatment, each treatment was repeated 3 times and planted with *S. commune* and *P. ostreatus* fungi. Then, it was incubated at 25^{o}C for 7 days in a dark room. Fungal growth inhibitory activity by extractive substances was carried out at the end of the incubation period, by measuring the growth diameter and comparing it with the growth of control mycelium. The basis for determining anti-fungal activity uses the following formula Kamaruzzaman *et al*. (2021) :

$$P = \frac{C - T}{C} \times 100\%$$

Note, *T* is the area of mycelium in the treated petri dish; *C* is the area of mycelium in the control petri dish; P is the percentage of mycelium growth inhibition.

Characteristics of Compounds with a 1H NMR Spectrophotometer

The compound structure of isolated 0.5 mg of the most active extract was determined using 1H NMR. Analysis was conducted at Advanced Chemical II Characteristics Laboratory, BRIN Serpong, Tangerang.

Data Analysis

Data on the percentage of fungal growth inhibition was analyzed to determine the $IC_{(50)}$ value. Determination was based on the logarithmic equation between the concentration of pelawan wood extract (y-axis) and the percentage of inhibition of fungal growth (x-axis). Calculations using the SPSS 17.0 program.

Results and Discussion

Pelawan Wood Extract Content

Maceration with methanol solvent was able to dissolve 104.39 g of pelawan wood extract (equivalent to 6%). This happens because the solvent has polar covalent bonds which will be polarized to form a partial charge which is able to attract electrons from the compounds in the pelawan wood. The results of maceration and extractive fractionation of Pelawan heartwood are presented in Table 1. The difference in extract content in each fraction is caused by differences in the polarity of each solvent. In terms of quantity, pelawan wood extract contains more non-polar compounds. Asmah *et al.* (2020) reported that the extraction of orange peel, *Dillenia spp* wood with chloroform was able to dissolve D-Limone, fatty acids, and wax. The n-hexane solvent dissolves wax, essential oils, vegetable oils and fats (Zhuang *et al*. 2018; Naqvi *et al*. 2020), the ethyl acetate solvent is able to dissolve alkaloids, terpenoids, flavonoids and aglycones (Kamalarajan *et al*. 2020). Butanol solvent is able to dissolve phenolic compounds, flavonoids, and saponins (Cai *et al*. 2021; Nurcahyo *et al*. 2022).

Table 1**.** Content of Pelawan heartwood extract

Fraction	Solid weight (g)	Extractive (%)
Dissolved n-hexane	0.21	0.01
Dissolved chloroform	55.61	3.26
Dissolved ethyl acetate	28.45	1.64
Dissolved butanol	1.19	0.06
Not Dissolved	17.93	1.03
Methanol extract (Amount)	104.39	6.00

Antifungal Activity of Pelawan Wood Extract

To determine the percentage of growth inhibition of the test fungus, pelawan wood extract dissolved in several solvents was then tested for *S. commune* and *P. ostreatus* fungi. . The results of the fungal growth inhibition test are shown in Figure 1 and Figure 2. Figure 1 displays a graph of the tendency to inhibit the growth of the fungus *S. commune* which is almost the same from a concentration of 50-1000 mg/L in each pelawan wood extract dissolved in n-hexane, chloroform, butanol. These three extracts are able to inhibit fungal growth by more than 68-79%. It exceeds the capabilities of CCB synthetic preservatives. Meanwhile, in Figure 2, the graph shows the trend of inhibiting the growth of *P. ostreatus* fungus, which continues to increase as the extract concentration increases. Pelawan wood extract was more active in inhibiting the growth of the fungus *S. commune* than *P. ostreatus*. This difference is due to the differences in extractive compounds contained in each fraction. Apart from that, the two types of fungi produce different enzymes for food metabolism (Kumar *et al*. 2022; Ibarra-Islas, *et al*. 2023). The difference in inhibition of *S. commune* fungal growth at a concentration of 50 mg/L n-hexane fraction was 75%, higher than the positive control. Because n-hexane extract of pelawan wood is able to inhibit non-enzymes and hydrolytic enzymes released by the fungus *S. commune* (Suryadi *et al*. 2022). The growth inhibition of *P. ostreatus* at an extract concentration of 50 mg/L chloroform fraction was 53%, also higher than the positive control. Differences in fungal growth inhibitory activity in pelawan wood extract, because the fungus *S. coummune* degrades cellulose while *P. ostreatus* degrades lignin in wood. The 100 mg/L CCB control was not able to inhibit the growth of *S. commune* and *P. ostreatus* fungi optimally because CCB was able to bind to the oxalic acid produced by *S. commune* and *P. ostreatus* fungi, then CCB no longer works. (Palmieri *et al*. 2019).

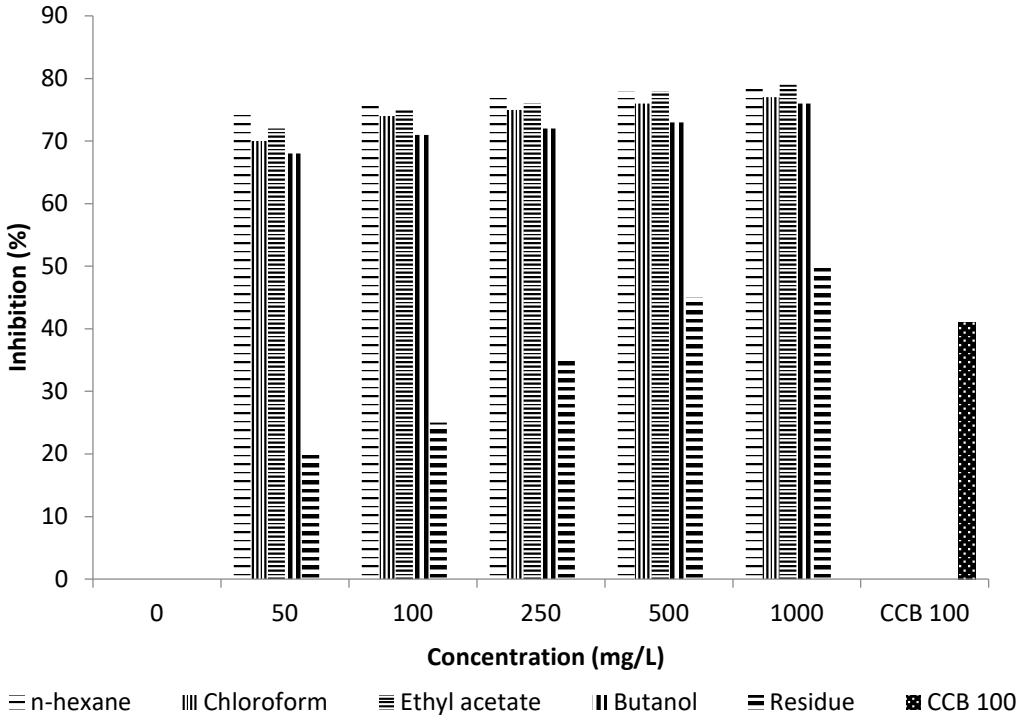

Figure 1. Inhibition of *S. Commune* mycelium growth at several concentrations of Pelawan wood extract.

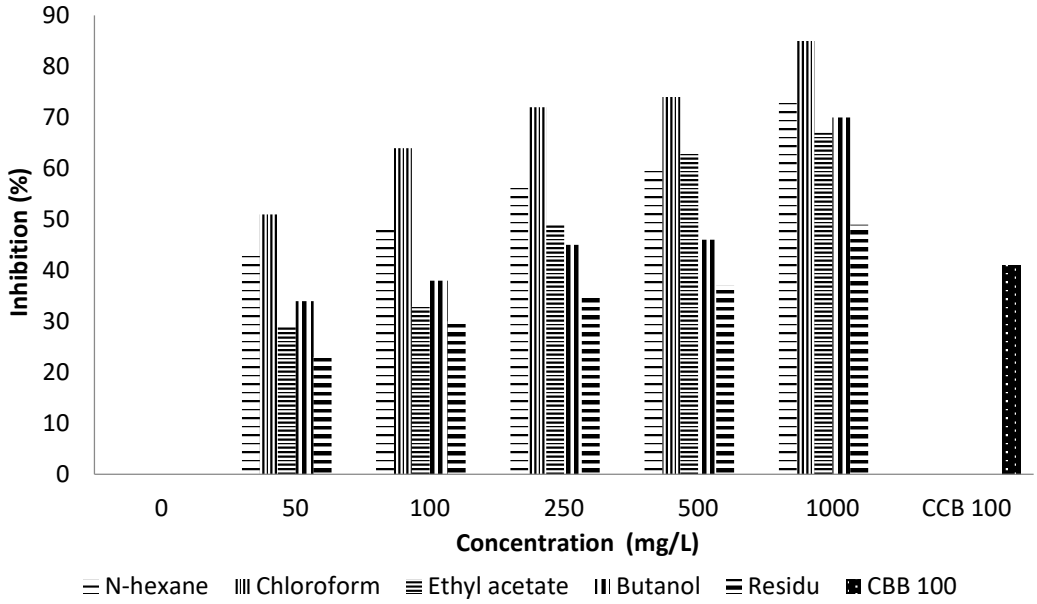

Figure 2. Inhibition of *P. ostreatus* mycelium growth at several concentrations of Pelawan wood extract

Isolation and Antifungal Activity of Chloroform Fraction from Pelawan Wood Extract

The selected pelawan heartwood chloroform fraction was isolated using column chromatography, because the results of testing the anti-fungal activity of the chloroform fraction extract were the most active of the 4 fractions. The chloroform fraction at a concentration of 50 mg/L was able to inhibit the growth of *S. commune* by 70% and *P. ostreatus* by 51%. In addition, the chloroform fraction produced a large yield of 55.61 g, making it is possible to isolate using column chromatography. A total of 0.61 g of the chloroform fraction was extracted again using column chromatography. The eluent is methanol: chloroform with a gradient system. The compounds obtained were combined based on thin layer chromatography (TLC) analysis, the results obtained for sub fraction 8 (PL.1-PL.8), in full are presented in Table 2.

Tabel 2. Eight sub-fractions of the chloroform fraction based on the results of TLC analysis

Sub faction	Weight (mg)	Rf	Value
PL.1	88.60	1.1	0.88
		1.2	0.98
PL.2	137.39	2.1	0.88
PL.3	14.50	3.1	0.73
PL.4	72.80	4.1	0.88
PL.5	10.10	5.1	0.65
		5.2	0.88
PL.6	15.10	6.1	0.85
PL.7	8.60	7.1	0.75
PL.8	18.70	8.1	0.69
		8.2	0.86

Rf = retention factor

The 8 (eight) subfractions (PL.1-PL.8) were tested for their antifungal properties to obtain the most active subfraction. The results of antifungal testing on compounds PL.1-PL.8 are shown in Figure 3. Tests on the fungi *S. commune* and *P. ostreatus* on all compounds PL.1-PL.8 showed that all subfractions were able to inhibit the growth of the fungus *S. commune* IC(50) = 54.53-64.69 mg/L and *P. ostreatus* IC(50)=54.17-64.44 mg/L. Compared to all fractions, CCB was only able to inhibit the growth of *S. commune* IC(50) = 81.49 mg/L and *P. ostreatus* IC(50) = 75.08 mg/L. This shows that the PL.1-PL.8 sub-fraction contains bioactive anti-wood rot fungal compounds whose ability exceeds that of CCB wood preservative. The PL.3 sub-fraction is the most active among the 8 sub-fractions (PL.1-PL.8), which is able to inhibit the growth of the fungus *S. commune* IC(50)= 55.09 mg/L and *P. ostreatus* IC(50)= 54.17 mg/L. Results of fungal testing on sun PL fraction. 3 is shown in full in Figure 3.

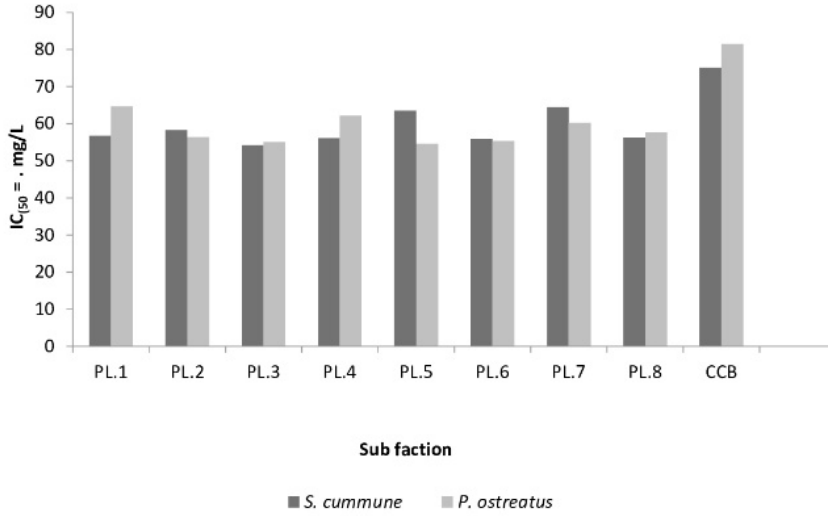

Figure 3. $IC_{(50)}$ growth of *S. commune* and *P. ostreatus* mycelium in the PL.1-PL.8 sub-fraction of Pelawan wood chloroform extract.

Identify anti-fungal compounds

The PL.3 subfraction is a thick green oil, has an unpleasant sour smell, with a boiling point: 222-245^{o}C. The identification results are in accordance with the reference for bioactive compounds in Buckingham (2006). The results of structural identification using 1H NMR are shown in Table 3. The structural forms of the compounds contained in the PL.3 subfraction are shown in Figure 4. As a comparison, the structure in Buckingham (2006) is used. The results of the identification of anti-fungal compounds from the PL.3 sub-fraction of the chloroform extract of Pelawan wood were heptanoic acid. This compound is a saturated fatty acid, with a molecular weight of 130,186 and the compound formula is C7H14O2. The 1H NMR spectrum displays signals of 7 (seven) hydrogen atoms δH 500 Mhz 1.26 (bs), 0.88 (s, J = 12.06 Hz, 3 H), the presence of a carboxylic acid group (-COOH) on C-1 and CH3 in position C-7.

Table 3. Position of 1H NMR signals for heptanoic acid

Position	δ_H 500 Mhz (ppm) (multiplicity *J* in Hz, amount H)
1	-
2 – 6	1.23 (bs)
7	0.88 (s, *J* = 12.06 Hz, 3 H)

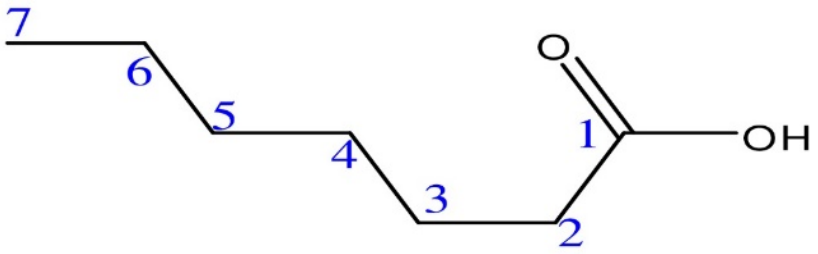

Figure 4. Heptanoic acid compound

The literature search for the mechanism of inhibition of heptanoic acid on fungal growth as follows: begins with the penetration of heptanoic acid into the cell membrane of wood rot fungi, especially at low sterol content, resulting in damage to myrysylated proteins, inhibition of the β-oxide reaction in triacylglycerol synthesis, and inhibition of topoisomerase activity (Guo *et al*. 2019; Castaño *et al.* 2022). It is because there is a carboxylic acid group in heptanoic acid which inhibits the pH regulation mechanism in cells. Finally, the function of the hydrolytic enzymes endo-1,4-β-glucoside, exo-1,4-β-glucoside and β-glucoside produced by eucalyptus rot fungal hyphae to hydrolyze cellulose into glucose is inhibited (Zhou *et al*. 2018; Sundararaj *et al.* 2020; Goodell 2020). Likewise, the enzymes O-acetyl-4-O-methyglucoroxylan and -aceylgalactoglucomannan hydrolyze Acetylarabinoglucuronomannan into glucose, mannose, galactose and acetic acid which are also inhibited by heptanoic acid (Ahuactzin-Pérez *et al*. 2018). If the penetration of heptanoic acid increases, it causes. wood rot fungi do not grow.

Conclusions

The results of multilevel fractionation showed that the chloroform fraction was the most active compared to the four n-hexane, ethyl acetate, butanol and residue fractions, was able to inhibit the growth of the fungi *S. commune* and *P. ostreatus*. The column chromatography resulted 8 subfractions (PL.1-PL8) from the chloroform fraction. The PL.3 subfraction was the most active compared to the other 7 subfractions (PL.1-PL.8) and the positive control (CCB). The PL.3 subfraction was identified as a heptanoic acid compound.

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