Antioxidant Activity of Fungus Comb Extracts Isolated from Indo-Malayan Termite *Macrotermes gilvus* Hagen (Isoptera: Termitidae)

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Abstract: *Indonesian termites are one of the wealthiest biological resources considering their very high species diversity (300 species) and extensive geographical distribution in the Indonesian mainland. However, its potential as a source of bioactive compounds has not been fully explored. One of the endemic termite sub-families of Indonesian, Macrotermitinae (Isoptera: Termitidae), has a food source in the form of fungal nodules that grow and spread in a particular structure in the termite nest that is shaped like a mammalian brain called a fungus comb, which likely serves as a source of bioactive compounds. This study aims to analyze the potential of fungus comb from an Indonesian Macrotermitinae sub-family species as a source of antioxidants. Antioxidant activity was tested on four different extracts of fungus comb (EFC), namely hexane extract, ethyl acetate extract, methanol extract and water extract, using DPPH• (1,1-diphenyl-2-picrylhydrazyl) and ABTS•+ (2,2'-azino-di-[3-ethylbenzthiazolinesulphonate]) radicals. The results showed that the highest antioxidant activity was found in methanol extract, followed by ethyl acetate extract with IC_{50} values of 0.6 mg/mL and 0.75 mg/mL, respectively. TEAC (Trolox equivalent antioxidant capacity) analysis confirmed the high antioxidant activity of methanol extract.*

Keywords: *Macrotermitinae; fungus comb extract; antioxidant; ABTS•+; DPPH•; IC_{50}; TEAC*

■ INTRODUCTION

Antioxidants are substances that, at low concentrations, inhibit or prevent the oxidation of a substrate through an oxidative chain reaction by radical oxidizing agents such as reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), superoxide anions ($O_2^{\bullet-}$) or hydroxyl radicals (OH^-). Antioxidants can detect and

prevent or terminate the cycle of oxidative proliferation reactions by stabilizing the radical molecules through several chemical mechanisms, such as single electron transfer (SET), hydrogen atom transfer (HAT), and the capability to chelate transition metals [1-2]. Studies on antioxidant compounds have been widely reported, both from natural sources, generally plants, such as ascorbic acid (vitamin C), phenolic compounds (e.g., tocopherols (vitamin E), gallic acid, resveratrol, etc.) and carotenoids (e.g., lycopene and β-carotene), as well as synthetic ones such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary-butylhydroquinone (TBHQ) and propyl gallate [1,3]. Antioxidant compounds have been extensively used: (i) as nutritional supplements or pharmaceuticals in the treatment of patients with diseases caused by the body's oxidative stress or excessive free radicals, such as cardiovascular disease, cancer, arthritis and asthma [4-5]; (ii) as an additive in food and fat based cosmetic products to protect fat or oil from oxidation during storage which triggers rancidity and changes the taste of food or integrity of natural cosmetic compounds [6-7]; and (iii) as stabilizers in fuels (e.g., jet fuel) and lubricants (e.g., turbine oils and hydraulic fluids) industry to prevent oxidation, and in the gasoline industry to prevent polymerization leading to the formation of engine fouling residues, as well as in polymers industry such as rubber, plastics and adhesives production to prevent degradation leading to a loss of strength and flexibility of materials [8-14]. The global antioxidants market is expected to grow from $ 4.13 billion in 2021 to $ 6.05 billion in 2028 at a CAGR (compound annual growth rate) of 5.61% in the forecast period 2021–2028 [15].

Termites (order Isoptera) are cellulose-eating insects that live in colonies with an organized social system, like ants and bees. Although sometimes referred to as white ants, termites are not closely related to ants but rather to cockroaches, based on phylogenetic studies [16-17]. Indonesian termites have a very high species diversity (300 species) and extensive geographical distribution. More than 80% of Indonesia's land area is suitable as a habitat for various types of termites [18]. One of Indonesia's endemic termite subfamilies, Macrotermitinae (Isoptera: Termitidae), has a unique nesting system and food source. Its nests are shaped like small hills or mounds reaching 3 m in height. In the interior of the mounds, termites build a unique structure shaped like a mammalian brain called a fungus comb.

Interestingly, within the fungus comb, a monoculture Termitomyces is growing, and its nodules serve as a nutrition source for the termite colony [19-20]. This condition is thought to be associated with a biologically active chemical content of fungus combs and Termitomyces nodules which can inhibit the growth of other microorganisms [21]. Thus, studies on the fungus comb as a source of bioactive compounds are intriguing and necessary. Nandika et al. [22] in 2021 have characterized the chemical composition of the fungus comb from the Indonesian termite mound *Macrotermes gilvus* Hagen (Isoptera: Termitidae) and have reported remarkable bioactivity in the extracts of fungus comb (EFC) in inhibiting the growth of the wood dye fungus *Aspergillus foetidus* [22]. The same EFC was also confirmed to show bioactivity as an anti-bacterial and an anti-fungal, as reported by Witasari et al. [23]. We conducted a study to look at the antioxidant capabilities of the bioactive components from fungus comb to investigate their potential further.

The antioxidant activity of a compound can be examined *in vitro* or *in vivo* through simple techniques. Results from *in vitro* tests are generally considered a direct indicator of antioxidant activity, and substances that perform poorly *in vitro* typically do not perform better *in vivo* [1,24]. Antioxidant activity cannot be quantified directly; instead, it is based on antioxidants' capability to control the level of oxidation. Antioxidant activity can be assessed using a variety of techniques. The mechanisms through which antioxidant activity reacts with various groups of chemicals rely on several variables. These variables include the chemical structure of the compounds, the type of solvent used, the temperature and pH, and the reactivity and chemical characteristics of free radicals. All of these variables have a significant influence on reaction rate. Hence, in antioxidant studies, it is crucial to apply more than one evaluation method [1,25]. Here we report the

antioxidant activity on the extracts of fungus comb (EFC) by the inhibition of 2,2-diphenyl-1-picrylhydracyl radical (DPPH•) and the inhibition of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) cation radical. IC_{50} values and Trolox equivalent antioxidant capacity (TEAC) was measured by the inhibition of DPPH• and $ABTS^{\bullet+}$, respectively.

■ EXPERIMENTAL SECTION

Materials

A sample of fungus comb was obtained from Indomalayan termite (*M. gilvus* Hagen) (Isoptera: Termitidae) mounds in Yanlappa Experimental Forest, Bogor, West Java Province, Indonesia [22]. The materials used in the antioxidant test include DPPH (2,2-diphenyl-1-picrylhydrazyl) (D9132**,** Sigma Aldrich), L(+)-ascorbic acid (EM 00468, Merck), methanol p.a. (EM 06009, Merck), acetone (EM 00014, Merck), the antioxidant Assay Kit (CS0790, Sigma-Aldrich), silica gel F_{254} (Merck), *n*-hexane (Merck), CH_2Cl_2 (Merck), ethyl acetate (Merck), and methanol (Merck).

Instrumentation

Instrumentation used include UV-Vis spectrophotometer (Thermo scientific Genesys 20), analytical balance (Ohaus), micropipette (Eppendorf), microplate reader (Biorad iMark), freeze dry (Christ), sonicator (Sonic Vibra Cell), microcentrifuge LM 17R (Thermo Scientific), TLC applicator (CAMAG Linomat 5), and UV detector (CAMAG Repsrostar 3 with WinCATS).

Procedure

Sample preparation

The fungus comb sample was grounded and extracted stepwise with successive solvents of *n*-hexane, ethyl acetate, methanol, and water. The yields of fungus comb extract (EFC) were 0.09, 1.73, 2.53, and 4.61%, respectively [22]. The results in the form of dry extracts were labeled as EFC_{hexane}, EFC_{EtOAc}, EFC_{MeOH}, and EFC_{water}, respectively. For the TEAC measurement, an extract of fungus comb in acetone was prepared by the maceration technique for 1 × 24 h at room temperature. The dried extract was then labeled as "crude $EFC_{acetone}$".

Antioxidant activity test using thin-layer chromatography (TLC)

Silica gel F_{254} was used as the stationary phase in an area of 20 × 10 cm with a thickness of 3 mm, while a 5 mL mixture of *n*-hexane:CH_2Cl_2 (1:9, v/v) was used as the mobile phase for elution of EFC samples. The antioxidant activity of EFC as a free radical scavenger was tested using the DPPH. After the elution, TLC was sprayed with a DPPH reagent of 5 mM (in ethanol). The chromatogram was checked 30 min after spraying. The stains formed were observed with visible light and a 366 nm UV lamp [26].

Kinetic test

The reaction kinetics were observed from the decrease in the concentration of DPPH• (% DPPH• remaining) after reacting with the EFC sample concerning time. Observations were conducted according to Brand-Williams et al. [27]. Two portions of sample solution having the same concentration in methanol were prepared. The concentration of the EFC_{hexane}, EFC_{EtOAc}, and EFC_{water} solutions was fixed at 2 mg/mL, while the EFC_{MeOH} solution was 1.5 mg/mL. One portion of the solution was used to measure the absorbance of the sample (A_{sample}), and the other was used as the correcting absorbance ($A_{corrector}$). A total of 800 µL of sample solution from the first portion was reacted with 800 µL of DPPH• solution 0.08 mM, while the same volume from the second portion was mixed with 800 µL of methanol. The absorbance of each mixture was measured every 2 min at a wavelength of 517 nm until a steady state (flat curve) was reached. The initial absorbance value when DPPH• has not reacted was determined using a mixture of 800 µL of 0.08 mM DPPH• solution with 800 µL of methanol. % DPPH• remaining was calculated by the following equation:

$$\%\text{DPPH}\bullet \text{ remaining} = \frac{A_t}{A_0} \times 100\% \quad (1)$$

whereas A_0 is absorbance of Initial DPPH• and A_t is $A_{sample} - A_{corrector}$.

IC_{50} measurement using radical DPPH•

The antioxidant activity and IC_{50} value of the EFC samples were measured with the DPPH• free radical as

previously reported by Bhuyar et al. [28] and Hu et al. [29] with slight modification.

Reagent preparation. A set of methanol solution for each EFC sample was prepared at different concentrations ranging from 1.0–8.0, 0.1–2.4, 0.2–1.5, and 0.2–4.0 mg/mL for EFC_{hexane}, EFC_{EtOAc}, EFC_{MeOH}, and EFC_{water}, respectively, as well as for the ascorbic acid solution with a concentration range of about 2.0–20 µg/mL. A DPPH• solution was prepared in methanol up to a concentration of 0.08 ± 0.005 mM or up to an absorbance value between 0.75–0.80 at 517 nm. DPPH• solution should be made fresh before each measurement and protected from light.

Stability test of DPPH• reagent. The stability test of the DPPH• reagent was carried out using the ascorbic acid solution and by the same method as the measurement of % inhibition, except the volume of the mixed reagents is 600 µL each instead of 800 µL and the reaction incubation was 18 min, instead of 30 min. Two types of ascorbic acid solution and DPPH• solution were prepared, namely a fresh solution and a not fresh solution. A fresh solution was a solution made just before the % inhibition measurement. In contrast, a not fresh solution was a solution that had been prepared 24 h before the measurement, which was stored in a refrigerator at 4 °C, tightly closed and protected from light.

Measurement of % Inhibition. As much as 800 µL of methanol solution of each EFC sample at different concentrations was added to 800 µL of 0.08 (± 0.005) mM DPPH• solution in methanol, and the reaction mixture was thoroughly shaken and stored for 30 min in the dark at room temperature. The solution's absorbance was measured at 517 nm using methanol as a blank. Correction solutions were prepared by adding 800 µL of methanol to 800 µL of sample solutions with different concentrations. The same procedure was used repetitively for positive control of ascorbic acid. The negative control was made by mixing 800 µL of DPPH•-methanol solution (~0.08 mM) with 800 µL of methanol. Antioxidant activity as the ability of the sample to inhibit DPPH• free radicals (% DPPH• inhibition) was calculated using the following equation:

$$\%\text{Inhibition} = \frac{A_{control} - \left(A_{sample} - A_{corrector}\right)}{A_{control}} \quad (2)$$

where A_{sample} is the absorbance of DPPH• and tested sample mixture, $A_{corrector}$ is the absorbance of the correction solution, and $A_{control}$ is the absorbance of the negative control.

TEAC measurement using ABTS•+ cation radical

This measurement was carried out using the Antioxidant Assay Kit (CS0790, Sigma-Aldrich) by following the procedure provided by the manufacturer. Trolox, a water-soluble vitamin E analog, served as a standard or positive control of antioxidants. The total antioxidant capacity (TAC) of each tested sample is represented or equivalent to the mM of the Trolox standard. Measurements were applied to (i) five different concentrations (0.000; 0.015; 0.105; 0.210; 0.420 mM) of trolox (provided in the Kit) for the standard curve construction; (ii) Solutions of 2% (w/v) of each EFC sample [22] in its respective extraction solvents, i.e., *n*-hexane, ethyl acetate, methanol and water; (iii) 2% (w/v) of ascorbic acid solution in 1× assay buffer (provided in the Kit); (iv) 2% (w/v) crude acetone extract of fungus comb (crude $EFC_{acetone}$) in 1× assay buffer; (v) turmeric extract which was prepared by maceration of 2% (w/v) of turmeric powder in Aqua DM for 2 × 24 h at room temperature, then filtered to get filtrate that ready to be tested; (vi) termite extract which was prepared by mixing 47 mg of powder of freeze-dried termites in 500 µL of 1× assay buffer, then sonicated for 1 h at 50 Hz frequency with a cycle of 30 sec on and 30 sec off for breaking down the cells/tissues, and centrifuged to obtain a supernatant that ready to be tested. Each sample was measured twice (Duplo) with a volume of 5 µL. The antioxidant concentration or TEAC of the tested samples were calculated following the instruction provided by the Kit manufacturer.

■ RESULTS AND DISCUSSION

A sample of fungus comb from a termite mound is shown in Fig. 1. Extracts of *n*-hexane, ethyl acetate, methanol, and water from the fungus comb (EFC) samples were prepared in a previous study and analyzed for their chemical content [22]. They have also been investigated for their antibacterial and antifungal activity [23]. In this study, the four EFC samples were

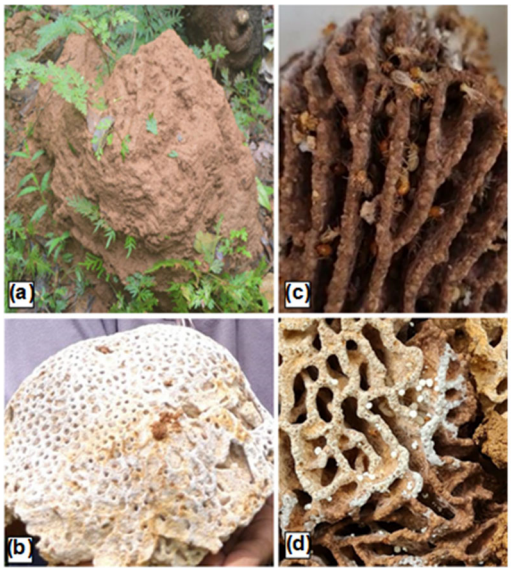

Fig 1. Appearances of the fungus comb
Nest mound found on the land of the Yan Lapa Nature Reserve, West Java, Indonesia (a); lumps of fungus comb from the interior of the nest (b); termites of various castes/age around the fungus comb (c); and fungal nodules appear as white granules (d)

analyzed for their antioxidant activity by including ascorbic acid and Trolox as a comparison or positive control of the reaction.

Antioxidant Activity Tested Using TLC

As a preliminary test, the antioxidant activity was analyzed on EFC samples using the TLC method. The tests were carried out specifically on the samples of EFC_{hexane} and EFC_{EtOAc}. Compounds suspected of having antioxidant activity were indicated by the formation of a pale-yellow color on a purple background after spraying the chromatogram with 5 mM DPPH• reagent. The formation of yellow spots was caused by the presence of compounds in the EFC that can donate hydrogen atoms or transfer single electrons, thus could reduce the radical DPPH• molecules (purple color) to neutral molecules (colorless) that appeared as a pale-yellow color on the chromatogram (Fig. 2). The test showed that the ethyl acetate and the *n-hexane* extracts of fungus comb contained compounds that could reduce DPPH• radicals. EFC_{EtOAc} may contain more free radical scavenging active compounds, as indicated by thicker pale-yellow spots on the chromatogram.

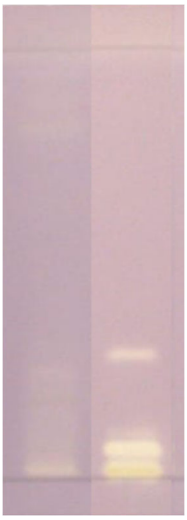

Fig 2. Chromatogram of EFC_{hexane} (left lane) and EFC_{EtOAc} (right lane) sprayed with DPPH• 5mM
The mixture of *n*-hexane:CH_2Cl_2 (1:9, v/v) was used as the mobile phase

Kinetic analysis

In this kinetic test, the antioxidant activity of tested samples was observed from a decline in the quantity of DPPH• free radicals (purple color) in the reaction mixture as indicated by decreasing intensity of purple color or decreasing absorbance value at 517 nm in the reaction mixture. This straightforward test is beneficial, especially for a preliminary qualitative analysis of the presence of antioxidants in the samples. Fig. 3 shows the results of the kinetic analysis of four EFC samples at the same concentration of 2 mg/mL, except for EFC_{MeOH} with 1.5 mg/mL. It was clearly shown that the highest antioxidant activity was found in EFC_{MeOH}, followed by EFC_{EtOAc} and EFC_{water}. EFC_{hexane}, on the other hand, has the lowest activity. Based on the position of the curve line at 30 min to the steady state, the IC_{50} value of each sample can be estimated, which is greater than 2 mg/mL for EFC_{hexane} and EFC_{water}, while for EFC_{EtOAc} and EFC_{MeOH} are less than 2 mg/mL and 1.5 mg/mL, respectively. This estimated value will be instrumental in determining the range of variations of sample concentration to measure IC_{50} with the DPPH• free radical inhibition method.

Stability of DPPH• reagent

The study was initiated by evaluating the stability of the DPPH• solution as a reagent in the antioxidant

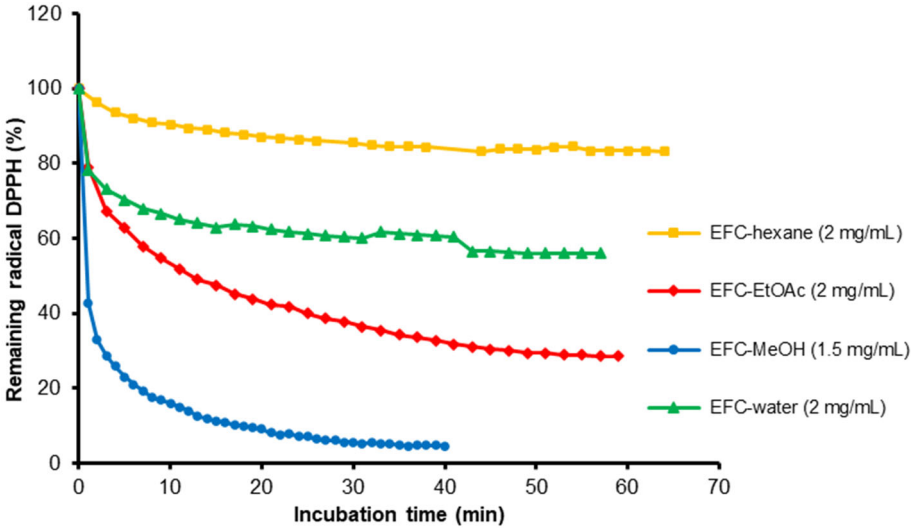

Fig 3. Four types of reaction kinetics observed in EFC samples

activity test of ascorbic acid positive control. The goal is to determine whether the storage of reagent solution over time will affect the test results and cause distortion in the measurement data. Methanol solutions of ascorbic acid at different concentrations were reacted with methanol solutions of DPPH• radicals. Two types of solutions tested here were a freshly prepared solution before the test (fresh solution) and the one that was prepared 24 h before the test, which has been stored in a cool, dark, and tightly closed container (not fresh solution). The evaluation results in Fig. 4 show that both fresh and not fresh ascorbic acid solutions which reacted with the fresh DPPH• solution gave a higher value of % inhibition compared to those that reacted with the not fresh DPPH• solution. These indicate that the ascorbic acid solutions were stable during storage. In contrast, the DPPH• solutions were unstable, i.e., their absorbance decreased after 24 h of storage, even in a cool, dark, and tightly closed condition. Therefore, to maintain accuracy and reproducibility, the DPPH• solution should be prepared freshly just before each %inhibition measurement.

IC_{50} of EFC samples

Antioxidant activity is the ability to inhibit or weaken the oxidation power of the DPPH• free radical molecules. The inhibitory level is expressed as the value of % inhibition. In the experiment, the inhibition is indicated by a decrease of the radical DPPH• quantity due to the reduction reaction or scavenging by any compound

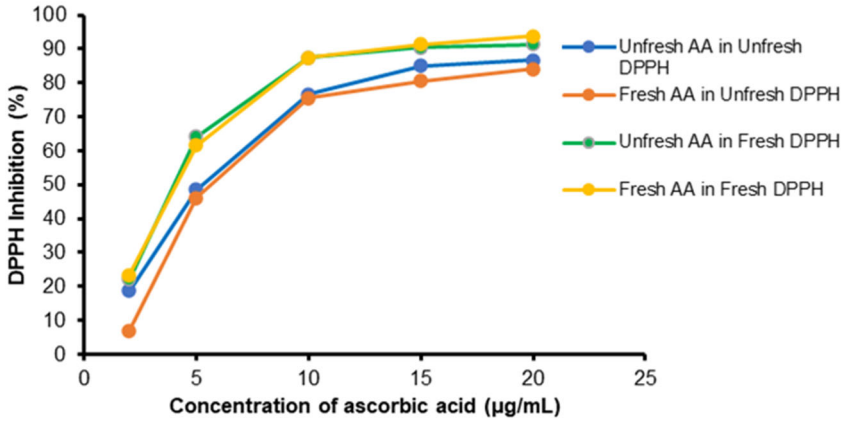

Fig 4. Result of stability test of DPPH• and ascorbic acid solution

For both DPPH• and ascorbic acid, two solutions with the same concentration were prepared and tested at different times, one immediately after preparation (fresh) and the other after being stored for 24 h (not fresh). AA = ascorbic acid

in the tested sample. DPPH• molecule having radical organic nitrogen is purple. After a reduction or scavenging reaction, it transforms into a non-radical diphenyl picrylhydrazine which is clear yellow in color (picryl group) or colorless. This color change (from purple to a clear yellow/colorless) observed in the reaction mixture can be measured spectrophotometrically as a change in the value of light absorbance at a wavelength of 517 nm. Thus, high antioxidant content in the tested sample will be depicted in a high % inhibition of DPPH• radical as shown by decreasing purple color intensity in the reaction mixture or the low A value. In this study, samples at various concentrations were reacted with a specific concentration of radical DPPH•, then the absorbances were measured, and the % inhibition was calculated using Eq. (2). A graph relating % inhibition to sample concentration was created to obtain the IC_{50} value.

Ascorbic acid, known as vitamin C, is an excellent antioxidant compound. In this study, ascorbic acid was used as a positive control of antioxidant activity. Ascorbic acid, a radical scavenger, can donate hydrogen atoms to reduce the radical DPPH• molecule to a non-radical or neutral one [1]. Fig. 5 shows a graph of the % inhibition resulting from ascorbic acid solution at different concentrations. Ascorbic acid exhibited a very high antioxidant activity, characterized by its ability to inhibit almost 90% of DPPH• radicals at low concentrations, i.e., 12 µg/mL.

Fig. 6 shows the graphs relating % inhibition to concentrations of the four EFC samples. The regression curve of EFC_{MeOH} has the steepest slope of the others, followed by EFC_{EtOAc}, having the second steepest curve. Meanwhile, EFC_{hexane} and EFC_{water} both have relatively sloping regression curves. The curve slope differences indicate that EFC_{MeOH} has the highest antioxidant activity (the most potent inhibitor), followed by EFC_{EtOAc} and EFC_{water}, while EFC_{hexane} has the lowest/weakest antioxidant activity.

IC_{50} is a parameter used to assess the strength of an antioxidant. IC_{50} specifies the required compound concentration to inhibit DPPH• radicals by 50%. Thus, the low IC_{50} value of a compound indicates a strong antioxidant activity. Ascorbic acid is a powerful antioxidant compound; thus, it exhibits a low IC_{50} value, i.e., 4.353 µg/mL (Fig. 5). The IC_{50} value of EFC_{MeOH} is 0.606 mg/mL, which is higher than that of the ascorbic acid but lower than that of the other EFC samples. The IC_{50} value of EFC_{EtOAc}, EFC_{water}, and EFC_{hexane} is 0.845, 2.777, and 2.836 mg/mL, respectively (Fig. 6, Table 1). Sukweenadhi et al. [30] reported that based on the DPPH method, the IC_{50} of turmeric extract (*Curcuma xanthorriza*) is 538 ppm (0.538 mg/mL) which is close to the IC_{50} of EFC_{MeOH}.

Trolox Equivalent Antioxidant Capacity (TEAC)

In the measurement of TEAC, Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], an analog of vitamin E, is used as a positive control due to its ability to reduce the ABTS•+ radical cation (green color) into an

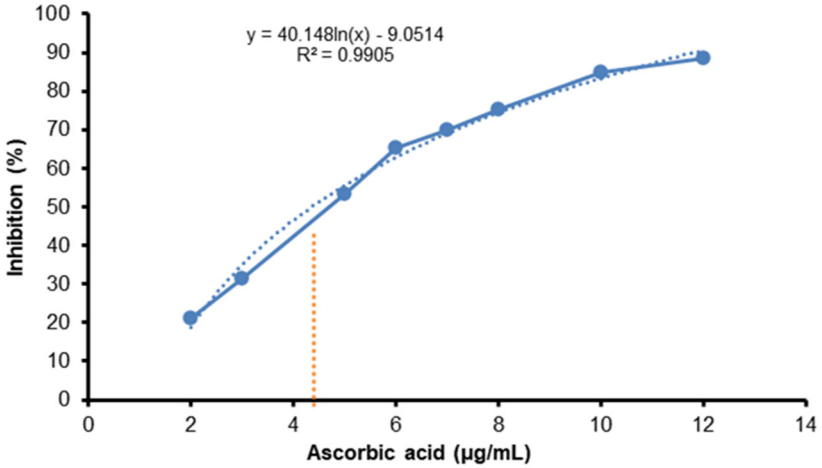

Fig 5. % Inhibition of DPPH• by ascorbic acid solution

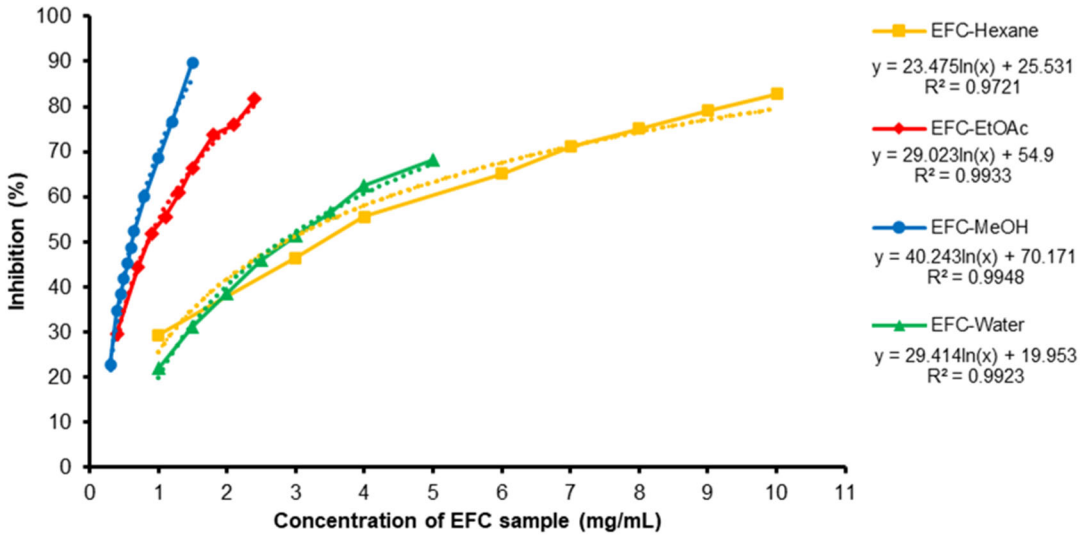

Fig 6. % Inhibition of DPPH• by the four EFC samples

Table 1. IC_{50} values of EFC samples

EFC sample	IC_{50} (mg/mL)
EFC_{MeOH}	0.606
EFC_{EtOAc}	0.845
EFC_{water}	2.777
EFC_{hexane}	2.836

$ABTS^{2-}$ anion (colorless). Its reducing ability is directly proportional to its used concentration. The standard curve that connects the Trolox concentration to the absorbance value, which is a value that represents the ABTS•+ radical cation remaining in the reaction mixture, is made as a comparison tool for determining the concentration of antioxidants in the tested sample. The antioxidant content in the tested sample is equivalent to the concentration of the Trolox standard. It is expressed as Trolox equivalent antioxidant capacity (TEAC), i.e., the capacity of the tested sample to scavenge the ABTS•+ radical cation. Fig. 7 shows the Trolox standard curve, which is linear with the equation of $y = -0.8462x + 0.6073$, where y is the absorbance value proportional to the remaining ABTS•+ radical cations in the reaction mixture, x is the Trolox concentration (mM), and the correlation coefficient $R^2 = 0.9904$.

The TEAC measurement on the three EFC samples [22] showed that the EFC_{MeOH} had the highest antioxidant capacity, i.e., Trolox equivalent (TE) of 0.565 mM, compared to the EFC_{hexane} (0.267 mM TE) and EFC_{water} (0.442 mM TE) (Table 2). Due to technical constraints, the measurement couldn't be performed on the EFC_{EtOAc} sample. Ascorbic acid as a well-known antioxidant compound and turmeric as a herbal product with reported high antioxidant activity [30] had relatively

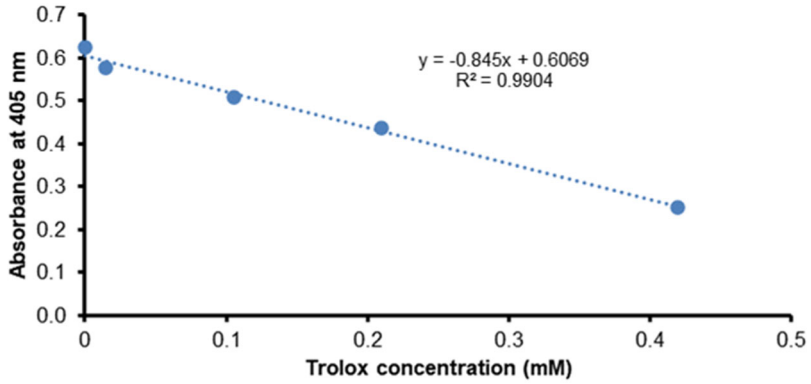

Fig 7. Standard Trolox curve

Table 2. Trolox equivalent antioxidant capacity (TEAC)
Samples were dissolved/incubated in their respective solvents as much as 2% (w/v) except termite 9.4% (w/v). The test volume was 5 μL

Sample, 2% (w/v)	TEAC (mM)
EFC_{MeOH}	0.565
EFC_{water}	0.442
EFC_{hexane}	0.267
Crude $EFC_{acetone}$	0.587
ground Termite*	0.330
ground turmeric	0.536
Ascorbic acid	0.670

*Test concentration was 9.4% (w/v)

high TEAC values, i.e., 0.670 and 0.536 mM, respectively. The TEAC value of EFC_{MeOH} resembled that of turmeric extract and was not significantly different from that of the ascorbic acid. Extract from the whole termite bodies provided a relatively low TEAC value, i.e., 0.33 mM. Extracts from the maceration of fungus comb by acetone (Crude $EFC_{acetone}$) showed relatively high TEAC values, i.e., 0.587.

According to the results of the GC-MS analysis in our previous study [22], three phenolic compounds were identified from *n*-hexane and ethyl acetate extracts of fungus comb from the *M. gilvus* Mound (Fig. 8). Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester (Fig. 8. compound **1**), is one of the dominant compounds found in the *n*-hexane extract. Nazaruddin et al. [31] reported that this compound has antifungal and antioxidant activities. In industry usage, as a plastic additive, this compound is known to act as an antioxidant or stabilizer [32]. The presence of antioxidant activity in the *n*-hexane extract (EFC_{hexane}) was observed in this study after testing using the TLC method, i.e., the formation of yellow spots after spraying the chromatogram with 5 mM DPPH• reagent (Fig. 2). Quantitatively, according to our previous study [22], nonpolar compounds (*n*-hexane extract) in the fungus comb were minimal. Its extraction yield was only 0.09% (the smallest one compared to other solvents), and the content of compound **1** in the *n*-hexane extract was relatively low, i.e., 1.16% [22]. The shallow content of this compound explains the lowest antioxidant activity observed quantitatively in EFC_{hexane} compared to in EFC of

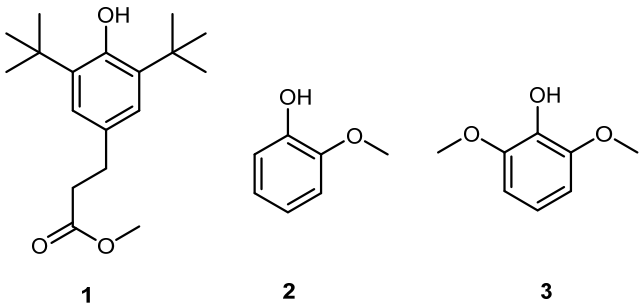

Fig 8. Phenolic derivatives reported from *n*-hexane and ethyl acetate extracts of fungus comb [22]

other solvents, where the highest IC_{50} value is in Table 1 and the lowest TEAC value is in Table 2. Also, in the TLC tests, the yellow spot chromatograms of EFC_{hexane} were found to be less bright (thinner) than those of EFC_{EtOAc} (Fig. 2).

Two phenolic compounds, i.e., 2-methoxyphenol (compound **2**) and 2,6-dimethoxyphenol (compound **3**), were identified from ethyl acetate extract (Fig. 8) [22]. These compounds are the product of lignin pyrolysis and a significant component of wood smoke [33-34]. Naturally, they were isolated from *Pinus densilora* and *Quercus serrata* with reported antifungal activity [35]. Compound **3** is widely used as a marker to measure the laccase activity (an enzyme that oxidizes phenolic compounds). This compound, along with an oxidative coupling product (dimer), is reported to have good antioxidant activity, EC_{50} DPPH 0.802 ± 0.005 and 0.415 ± 0.012 mM, respectively [34]. Our present study found a relatively high antioxidant activity in the ethyl acetate extract (ECF_{EtOAc}) (Table 1).

The highest antioxidant activity was found in methanol extract (EFC_{MeOH}) (Tables 1 and 2), which is more polar than ethyl acetate. According to our previous study [22], the more polar extracts were found to be more dominant in the fungus comb, as shown by the higher extraction yield of the methanol extracts (2.53%) compared to *n*-hexane and ethyl acetate [22]. Although the content of methanol extract could not be identified to the limitation of GC-MS analysis [22], we can assume that phenolic compounds would also be present in these more polar solvents, as proven by the qualitative test of the extracts [22]. Kähkönen et al. [36] and Wojdyło et al.

[37] reported their study on total phenolic contents (as GAE, gallic acid equivalents) in aqueous methanolic extracts of various plants (edible and non-edible) and its antioxidant activity. Among those extracts having high total phenolic content, as well as high antioxidant activity, were spruce needle (155.3 ± 6.1 mg of GAE/g dw), silver birch phloem (85.5 ± 2.1 mg of GAE/g dw), Scotch pine bark (76.0 ± 2.9 mg of GAE/g dw), and willow bark (75.5 ± 1.5 mg of GAE/g dw) extracts [36]. Therefore, the phenolic compounds that may compose the methanol extract (including compounds **2** and **3**) may contribute to the antioxidant activity of this extract (EFC_{MeOH}).

■ CONCLUSION

The results of the antioxidant activity testing using the DPPH• and ABTS•+ inhibition methods confirmed the presence of antioxidant compounds in the fungus comb extract from the Indo-Malayan termite *Macrotermes gilvus* Hagen (Isoptera: Termitidae). The highest antioxidant activity was found in methanol extract. The ethyl acetate extract had reasonably high activity, while *n*-hexane extracts possessed the lowest antioxidant activity. Aqueous extract showed low antioxidant activity in the test using the DPPH• reagent but had relatively high TEAC in the test by the ABTS•+. Based on this study, methanol extract from fungus comb has the highest antioxidant content, followed by ethyl acetate extract. The TEAC and IC_{50} of the methanol extract were comparable or close to that of the turmeric extract.

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■ AUTHOR CONTRIBUTIONS

Dodi Nandika conceptualized the study; Yanti Rachmayanti, Irmanida Batubara and Reza Ro'isatul Umma experimented; Dodi Nandika, Irmanida Batubara, Arinana and Lina Karlinasari collected samples and prepared EFC; Yanti Rachmayanti, Dikhi Firmansyah, and Reza Ro'isatul Umma calculated and analyzed the data; Yanti Rachmayanti wrote the first draft; Yanti Rachmayanti, Dodi Nandika, Irmanida Batubara, Lina Karlinasari, Arinana, Djoko Santoso, Dikhi Firmansyah, Lucia Dhiantika Witasari, I Ketut Sudiana and Decsa Medika Hertanto revised and edited the draft; All authors agreed to the final version of this manuscript.

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