Anti-oxidant and anti-diabetes activities of agarwood extracts from *Gyrinops versteegii* (Gilg.) Domke and their cytotoxicity

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Abstract. *Gyrinops versteegii* is an endemic plant in Lombok, West Nusa Tenggara, which produce agarwood. In vitro anti-oxidant and anti-diabetes activities from several extracts of agarwood produced by *G. versteegii* were investigated. The 1,1-diphenyl-2-picrylhydrazyl (DPPH)-radical scavenging activity assay was used to determine the anti-oxidant activity, whereas the α-glucosidase inhibitory activity assay was conducted to determine the anti-diabetes activity. The toxicity test using brine shrimp lethality test (BSLT) and phytochemical analysis were also conducted in this experiment. As the results, the acetone extracts of infected wood of *G. versteegii* had the highest anti-oxidant activity with the IC50 value of 65.62 μg/mL, whereas the acetone extracts of agarwood had the α-glucosidase inhibitory activity with the IC50 value of 53.46 μg/mL. The toxicity test to the both samples shows the high toxicity with the LC50 values of 1.98 and 15.44 μg/mL, respectively. The high anti-oxidant and anti-diabetes activities in both of the acetone extracts of infected wood and agarwood may be caused by their high content of total phenolic (12.75 and 9.17%, respectively) and total flavonoid (19.96 and 14.34%, respectively). The anti-diabetes activity found in agarwood extracts produced by *G. versteegii* from Lombok is firstly reported.

1. Introduction

Agarwood is an aromatic resin produced by several types of plants that are included in 5 genera, including: Aetoxylon, Gonystylus, Wikstroemia, Aquilaria and Gyrinops. The last two genera, namely Aquilaria and Gyrinops, which belong to the same family Thymelaeaceae, are very common genus known as agarwood plant producer. A dark resin and aromatic wood of agarwood is caused by its response to various forms of injury, including natural injuries, such as lightning strikes, animal grazing, insect attacks or microbial invasions, or artificial injuries, such as cutting, nailing, binding, fire, chemical wounds, and intentional fungal inoculation [1][2].

The Agarwood is claimed to possess aphrodisiac, flatulence and diuretic properties. It is one of the ingredients used in the treatment of smallpox, rheumatism, spasm of bronchus and respiration, abdominal cramp, diarrhea, nausea, vomiting, anxiety, elderly fatigue, pregnancy and post partum illness [3]. The presence of benzenoid derivatives in the form of balanophonin and (+)-Lariciresinol
from agarwood extract produced by *Aquilaria sinensis* was reported [4]. Both have cytotoxicity properties for gastric cancer with IC$_{50}$ of 34 and 75 μg/mL respectively. Agarwood oil can also be used as an anti-bacterium [5] and anti-fungi [6]. Mangiferin content was identified presence in *A. sinensis* and *A. Crassna* [7]. Mangiferin compounds are compounds that have the potential to be anti-diabetic, anti-HIV, anti-cancer, and anti-inflammatory [8]. Whereas the compound iriflophenone 2-O-α-L-rhamnopyranoside in *A. sinensis* is effective for anti-diabetes [9]. The Agarwood oil is used in perfumery and incense industries.

Agarwood leaves of *Gyrinops versteegii* type have also been used as burn medicine [10]. Previous study reported the presence of anti-oxidant activity in the leaves of *G. versteegii* methanol extract. In this study, we described the potent of agarwood extract from *G. versteegii* as anti-oxidant and anti-diabetes agents [11].

2. Materials and Methods

2.1. Materials

2.1.1. Plant materials

There are 4 types of wood extract in the form of agarwood, waste scraping, wood-inoculated fungi, and wood of *G. versteegii* were collected from Genggelang, Lombok Utara District, Nusa Tenggara Barat Province. All samples were dried and then mashed into powder before extraction. All specimens were deposited in Laboratory of Bioassay, Non-Timber Forest Product of Research and Development Agency, Mataram.

2.1.2. Chemicals

Quercetin dehydrate, dimethylsulfoxide (DMSO), p-nitrophenyl-D-glucopyranoside, α-glucosidase, 1,1–diphenyl-2-picrylhydrazl (DPPH), bovine serum albumin, and quercetin as a control were purchased from Wako Chemical Co. Ltd. (Osaka, Japan). All solvents were purchased from Wako Chemical Co. Ltd. (Osaka, Japan) and distilled before used.

2.2. Methods

2.2.1. Sample preparations

Plant material *G. versteegii* is mashed into a powder and dried by avoiding direct sunlight, then stored at room temperature before extracting. The dried wood was extracted for 48 hours at room temperature with methanol and acetone solvent. The extraction was repeated twice. The methanol and acetone extracts was concentrated with a rotary evaporator under reduced pressure. Both of methanol extract were a dark yellow semisolid. Activity-guided isolation was conducted using 1,1-diphenyl-1-picrylhydrazl (DPPH) radical-scavenging and α-glucosidase inhibitory activity assay. Brine shrimp lethality test (BSLT) was conducted to investigate the toxicity of extracts.

2.2.2. DPPH-radical Scavenging Assay

Scavenging activity of the DPPH radical was monitored according to the method described by [12]. A 0.1 ml methanolic solution containing from 0.4 to 2.0 mg of extracts was mixed with 2 ml of methanol, and a methanolic solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) (1mM, 0.5 ml) was then added. The mixture was stirred for 15 s, and then left to stand at room temperature for 30 minutes. The absorbance of this solution was then read at 517 nm. Antioxidant activity was calculated using the following equation: % Antioxidant activity (AA) = 100 x [(A$_{10}$ − A$_{xe}$)/(A$_{b0}$ − A$_{be}$)] where A$_{10}$ and A$_{xe}$ were absorbances of the sample at 0 and 120 min, and A$_{b0}$ and A$_{be}$ were absorbances of the control at 0 and 120 min. Assays were carried out in triplicate and the results were expressed as the mean ± standard deviation. Quercetin and ascorbic acid were used as standards.
2.2.3. α-Glucosidase inhibitory activity assay
Inhibition of methanol and acetone extract was monitored by α-glucosidase activity described by [13]. The reaction mixture consisted of 250 μL of 5 mM p-nitrophenyl-D-glucopyranoside and 1 mL of 100 mM phosphate buffer (pH 7.0) and was added to a flask containing 250 μL of the sample dissolved in DMSO at various concentrations. The reaction mixture was pre-incubated for 5 min at 37 °C and the reaction was then initiated by adding 250 μLα-glucosidase, and the incubation was continued for 15 min. The reaction was stopped by adding 1 mL of 200 mM Na2CO3. The activity of α-glucosidase was determined by measuring the release of p-nitrophenol at aUV max of 400 nm. The percentage inhibitory activity was calculated from \[(A_0 - A_1)/A_0\] ×100, where A0 is the absorbance of the control and A1 is the absorbance of the extract or standard. The 50% inhibition concentration (IC 50) of each compound was determined from the graph of percentage α-glucosidase inhibitory activity against sample concentration. Assays were performed in triplicate and the results were expressed as mean values ± standard deviations. Quercetin was used as the positive control.

2.2.4. Cytotoxicity test
Cytotoxicity test was conducted using Brine Shrimp Lethality Test (BSLT) method described by [14]. Toxicity of each sample is tested at various concentrations of 10, 100, 250, 500, 750 and 1000 μg/mL in seawater containing 1% DMSO (v/v). Three replications were used for each concentration. A parallel series of tests with the standard potassium dichromate solution (LC50=20–40µg/mL) the blank control was always included. After 24 hours, survivors were counted, by dissection microscope, and percentage of mortality (% M) of each dose was calculated as compared with control. Cytotoxicity was considered the significant if the LC50 value is less than 20-30 µg/mL.

2.2.5. Phytochemical analysis
Phytochemical analysis of the test sample was carried out according to standard methods described by [15] as below:

Test for saponin: About 5 ml of the extract was boiled in 20 ml of distilled water in a water bath and filtered. 10 ml of the filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion, which confirms a positive presence of saponin.

Test for alkaloid: Various methanol and acetone extracts of the G. verstegii wood were acidified by adding few drops of Dragendroff’s reagent (Potassium bismuth iodide). Appearance of orange red precipitate indicated presence of alkaloids.

Test for flavonoid: 3 ml of 1% Aluminum chloride solution were added to 5 mL of each extract. A yellow coloration was observed indicating the presence of flavonoids. 5 ml of dilute ammonia solution were added to the above mixture followed by addition of concentrated H2SO4. A yellow coloration disappeared on standing. The yellow coloration, which disappeared on standing, indicates a positive test for flavonoids.

3. Results and Discussion
3.1. Brine shrimp lethality test
Bioactive compounds are almost toxic at high doses. Therefore, resistivity in vivo (toxicity) containing bioactivity is used to filter plant extracts that have bioactivity and also act as bioactive fractions during fractionation and purification [16]. The toxicity test in this study used the BSLT method with shrimp larvae (Artemia salina Leach.) as a bio-indicator. This method is practical and inexpensive because shrimp eggs A. Salina can be easily obtained and can be stored for a long period [17].

According to Table 1, information is obtained that the highest yield is owned by acetone extract (AI) in inoculated wood with a yield value of 7.4549%. Then each extract was tested for BSLT and LC50 data were obtained as shown in Table 2.
Table 1. The yield of *G. versteegii* extract in the form of explants from scraping, waste scraping, healthy trees and inoculated trees with various types of solvents

| Sample | Weight of sample (g) | Weight of extracts (g) | Yields (%) |
|--------|----------------------|------------------------|------------|
| MS     | 800                  | 20.94                  | 2.62       |
| MAS    | 200                  | 3.13                   | 1.56       |
| AS     | 200                  | 9.11                   | 4.56       |
| NS     | 200                  | 1.71                   | 0.86       |
| MI     | 800                  | 28.97                  | 3.62       |
| MAI    | 200                  | 2.49                   | 1.24       |
| AI     | 200                  | 14.91                  | 7.45       |
| NI     | 200                  | 3.44                   | 1.72       |
| MK     | 800                  | 37.84                  | 4.73       |
| MAK    | 200                  | 6.42                   | 3.21       |
| AK     | 200                  | 14.38                  | 7.19       |
| NK     | 200                  | 1.93                   | 0.97       |
| ML     | 800                  | 34.80                  | 4.35       |
| MAL    | 400                  | 12.62                  | 3.15       |
| AL     | 200                  | 7.11                   | 3.55       |
| NL     | 125                  | 1.63                   | 1.30       |

The Brine Shrimp Lethality Test (BSLT) test on several agarwood extracts used concentrations of 10, 100, 500 and 1000 µg/mL which were carried out in duplicate from each test material based on testing using *A. salina* Leach biota larval phase. The results of the analysis using probit analysis methods for the data obtained can be seen in Table 2.

Table 2. Value LC50 for several agarwood samples in different solvents

| Sample | LC50 (µg/mL) |
|--------|--------------|
| MS     | 1.65         |
| MAS    | 15.43        |
| AS     | 23.54        |
| NS     | 794.33       |
| MI     | 1.00         |
| MAI    | 1.37         |
| AI     | 1.98         |
| NI     | 100.00       |
| MK     | 23.62        |
| MAK    | 12.33        |
| AK     | 15.44        |
| NK     | 51.49        |
| ML     | 1.08         |
| MAL    | 1.22         |
| AL     | 2.07         |
| NL     | 67.88        |

An active substance is toxic if it has LC50<1000 µg/mL for extracts, and <30 µg/mL for compounds [18]. According to Table 2, it can be seen that all extracts of agarwood samples show active toxicity.
because all test samples have LC50<1000 µg/mL. Nevertheless, the toxicity level of each sample varies based on the LC50 value. The highest toxicity level based on the data in Table 2 is in the MI sample which is part of the agarwood tree of the type *G. versteegii* which is inoculated and extracted using MeOH solvent (LC50 1.00 µg/mL) as a prospective extract, followed by several extract samples which have LC50<2.00 µg/mL, including methanol extract on agarwood waste (ML), methanol extract on healthy *G. versteegii* wood (MS), methanol-water extract on agarwood waste (MAL), methanol-water extract on agarwood inoculated *G. versteegii* (MAI), and acetone extract on inoculated *G. versteegii* wood (AI), with the LC50 values respectively being 1.08; 1.17; 1.22; 1.37; and 1.98 µg/mL. Therefore, it can be concluded that wood methanol extract from *G. versteegii* which is inoculated has better toxic properties than other extracts.

The high toxicity value of methanol, methanol-water, and acetone solvents in all test samples makes it possible that the sample has the criteria as an active compound for anti-cancer. Cytotoxicity criteria for crude extract as determined by the National Cancer Institute (NCI) is that the LC50 value has the potential as an anticancer if <30 µg/mL. Even so, of course this is still to be carried out anti-cancer testing further by using cancer cells to determine its bioactivity [19].

The n-hexane solvent in all test samples showed a low toxicity value indicated by a large LC50 value. The study also showed the same tendency, that the methanol extract of leaves of *G. versteegii* was more toxic (LC50 = 39.81 µg/mL) compared to n-hexane extract (53.70 µg/mL) [20].

### 3.2. Phytochemical analysis

Phytochemical screening results on *G. versteegii* plants in the form of explants from scraping, waste scraping, healthy trees and inoculated trees showed that methanol-water extract in the soil contained alkaloids, flavonoids, and saponins, as can be seen in Table 3.

**Table 3.** Phytochemical screening results on *G. versteegii* extract in the form of explants resulting from scraping, waste scraping, healthy trees and inoculated trees

| Sample | Alkaloid | Flavonoid | Saponin | Tanin | Kuinon |
|--------|----------|-----------|---------|-------|--------|
| MS     | +        | +         | -       | -     | -      |
| MAS    | +        | +         | -       | -     | -      |
| AS     | +        | +         | -       | -     | -      |
| NS     | +        | +         | -       | -     | -      |
| MI     | +        | +         | -       | -     | -      |
| MAI    | +        | +         | -       | -     | -      |
| AI     | +        | +         | -       | -     | -      |
| NI     | +        | +         | -       | -     | -      |
| MK     | +        | +         | -       | -     | -      |
| MAK    | +        | +         | +       | -     | -      |
| AK     | +        | +         | -       | -     | -      |
| NK     | +        | +         | -       | -     | -      |
| ML     | +        | +         | -       | -     | -      |
| MAL    | +        | +         | -       | -     | -      |
| AL     | +        | +         | -       | -     | -      |
| NL     | +        | +         | -       | -     | -      |

While extracts containing alkaloids and flavonoids alone are extracts of methanol, methanol-water, acetone and n-hexane in healthy wood; methanol, methanol-water, acetone and n-hexane extract in
inoculated wood; extract of methanol, acetone and n-hexane in kemedangan; and methanol, methanol-water, acetone and n-hexane extract in agarwood waste. Test results of tannins and quinones in all samples showed no content of tannins and quinones. Therefore, further quantitative phytochemical testing was carried out to determine the total phenol content (TFC), total flavonoid content (TFC), and total saponin/triterpenoid content (TSC) as shown in Table 4.

**Table 4.** Quantitative results of phytochemical testing on plant extracts of *G. versteegii* in the form of explants resulting from scraping, waste scraping, healthy trees and inoculated trees

| Sample | Content (% or mg/100 g) |
|--------|-------------------------|
|        | TFC  | TFIC  | TSC  |
| MS     | 11.97| 14.93 | 0.07 |
| MAS    | 8.55 | 6.65  | 0    |
| AS     | 18.00| 28.81 | 0.71 |
| NS     | 1.21 | 10.28 | 7.13 |
| MI     | 11.38| 14.18 | 0.39 |
| MAI    | 9.16 | 8.05  | 0    |
| AI     | 12.75| 19.96 | 1.72 |
| NI     | 0.82 | 9.42  | 0.72 |
| MK     | 10.91| 8.55  | 1.27 |
| MAK    | 8.28 | 5.20  | 0.92 |
| AK     | 9.17 | 14.34 | 1.06 |
| NK     | 4.18 | 11.17 | 7.38 |
| ML     | 8.63 | 8.10  | 3.79 |
| MAL    | 7.64 | 5.70  | 1.74 |
| AL     | 8.39 | 11.62 | 4.55 |
| NL     | 5.39 | 9.60  | 10.86 |

The total test results on phytochemical testing confirmed the presence of phenol content in all extract samples with the highest total phenol content found in acetone extract on healthy *G. versteegii* plant wood with a TFC value of 18.00% (fluroglucinol equivalents). The test results for total flavonoid levels also showed that acetone extract in healthy *G. versteegii* plant wood had higher levels of flavonoids with TFIC values of 28.81% (quercetin equivalents). The test results of total saponin/triterpenoid levels showed that n-hexane extract in agarwood waste had a higher TSC value of 10.86% (batulinol equivalents).

3.3. DPPH-radical scavenging
The results of the anti-oxidant test in all samples using DPPH radical scavenging can be seen in Figure 1 below. According to Figure 1, all extracts of methanol, methanol-water, and acetone showed high anti-oxidant activity in concentrations of 500 µg/mL. While extracts with n-hexane solvent showed low activity, there was not even any activity at all extract of agarwood (NL).
Figure 1. Antioxidant activity using DPPH radical scavenging test on *G. versteegii* plant extracts in the form of explants from scraping, waste scraping, healthy trees and inoculated trees at a concentration of 500 µg/mL.

To determine the IC$_{50}$ value of antioxidant activity for each extract, further testing was carried out at several concentrations and analyzed using a regression equation to obtain the results as shown in Table 5 below.

**Table 5.** IC$_{50}$ value of DPPH testing on crude extracts of *G. versteegii* in the form of explants resulting from scraping, waste scraping, healthy trees and inoculated trees using various types of solvents

| Sample | IC$_{50}$ (µg/mL) |
|--------|------------------|
| MS     | 132.70           |
| MAS    | 304.29           |
| AS     | 97.55            |
| NS     | >1000.00         |
| MI     | 139.69           |
| MAI    | 103.90           |
| AI     | 65.62            |
| NI     | >1000.00         |
| MK     | 183.19           |
| MAK    | 205.69           |
| AK     | 253.77           |
| NK     | >1000.00         |
| ML     | 170.15           |
| MAL    | 187.82           |
| AL     | 234.39           |
| NL     | >1000.00         |
| Quercetin | 8.59         |
According to Table 5, it can be seen that the best anti-oxidant value is possessed by acetone extract in inoculated wood with the IC50 value of 65.62 µg/mL. This is supported by the fact that acetone extract in inoculated wood also has very high toxicity levels, i.e. with LC50 of 1.98 ppm (while an active substance can be toxic if it has LC50 <1000 µg/mL for extract, and <30 µg/mL for compound). The high level of toxicity can occur because the acetone extract in inoculated wood has a high concentration of secondary metabolites, one of which is the presence of high antioxidant activity. The high level of toxicity does not rule out the possibility of also having active compounds for anti-diabetes, anti-cancer, and other activities, which of course this must be proven through further testing. Although acetone extract in inoculated wood has the best anti-oxidant activity, this activity has not been as good as quercetin which has an IC50 of 8.59 µg/mL. It is expected that the results of the isolation of the active compounds contained in the acetone extract will later have a better anti-oxidant activity.

Methanol, methanol-water and acetone extracts in inoculated wood have better anti-oxidant activity compared to healthy wood, kemedangan, or agarwood waste. Whereas the n-hexane solvent in all test samples showed inactive anti-oxidant activity. This might occur because the inoculated wood has high levels of total phenol and total flavonoids which influence anti-oxidant activity and is included in the group of moderate to very polar levels of compounds. The agarwood tree stem type *A. sinensis* has a high flavonoid content and 12 compounds were isolated which belonged to the flavonoid group, of which 7 were active as anti-oxidant compounds [21].

In this study, n-hexane extract of all test samples showed very low or inactive anti-oxidant activity. This is in line with the research of [22] who also stated that in the agarwood species of type *A. malaccensis*, extracts of n-hexane agarwood leaves had very low anti-oxidant activity with an IC50 value of 800 µg/mL.

### 3.4. α-Glucosidase inhibitory activity assay

The results of testing the inhibitory activity of α-glucosidase enzymes can be seen as shown in Figure 2 below. According to figure 2, it can be seen that acetone extract in the middle has the highest inhibitory activity value of 63.62% at a concentration of 100 µg/mL, followed by methanol extract in the dark with inhibitory activity value of 58.61%.

![Figure 2](image)

**Figure 2.** Inhibiting activity of the enzyme α-glucosidase extract of *G. versteegii* in several solvents at a concentration of 100 µg/mL.
In general, it can be seen that at a concentration of 100 µg/mL, methanol extract and acetone extract all samples have a fairly good inhibitory activity of the α-glucosidase enzyme. However, in mortality and agarwood waste it was seen that methanol extract and acetone extract had much higher activity compared to methanol extract and acetone extract in healthy wood and inoculated wood.

Table 6. IC₅₀ value of inhibitory activity of the enzyme α-glucosidase crude extract of *G. versteegii* in the form of explants resulting from scraping, waste scraping, healthy trees and inoculated trees using various types of solvents

| Sampel   | IC₅₀ (µg/mL) |
|----------|--------------|
| MS       | 216.58       |
| MAS      | 449.86       |
| AS       | >1000.00     |
| NS       | >1000.00     |
| MI       | 281.02       |
| MAI      | 675.74       |
| AI       | >1000.00     |
| NI       | >1000.00     |
| MK       | 56.73        |
| MAK      | >1000.00     |
| AK       | 53.46        |
| NK       | >1000.00     |
| ML       | 107.16       |
| MAL      | >1000.00     |
| AL       | 107.72       |
| NL       | 115.20       |
| Quercetin| 5.34         |

According Table 6 it can be seen that the lowest IC₅₀ value is found in acetone extract kemedangan with IC₅₀ value of 53.46 µg/mL. This shows that acetone extract has the highest inhibitory activity of the α-glucosidase enzyme. In addition, the methanol extract has a low IC₅₀ value of 56.73 µg/mL. This shows that methanol extract has the potential to be anti-diabetic. The interesting thing in this situation is the high IC₅₀ value of water-methanol extract, which is>1000 µg/mL which means that methanol-water extract has no inhibitory activity of the α-glucosidase enzyme. This condition indicates that the active compound in the medium has good solubility in medium to high polarity solvents, in this case acetone and methanol, but has very low solubility in solvents with polarity above methanol, in this case methanol-water.

4. Conclusions
Agarwood extract from *G. vesteegii* has potential as an antioxidant and antidiabetic agent due to their high bioactivity. The high antioxidant value of inoculated wood acetone extract (65.62 µg/mL) it can be determined that acetone is an appropriate solvent for extraction that is in accordance with the active antioxidant in inoculated wood. The high inhibitory activity of the α-glucosidase enzyme (53.46 µg/mL), acetone extract is an appropriate solvent for extracting an anti-diabetic compounds from agarwood of *G. versteegii*. 
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