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SESQUITERPENE CONTENT ANALYSIS OF AGARWOOD INOCULATED USING THE SIMPORI TECHNIQUE

ANALISIS KANDUNGAN SESKUITERPEN PADA GAHARU HASIL INOKULASI DENGAN TEKNIK SIMPORI

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Abstract

Agarwood is a resin formed in plants that belong to the *Thymelaeaceae* family, including *Gyrinops Versteeg*, as a result of damage or microbial infection. The content of sesquiterpenes in both the wild and cultivated agarwood indicates its quality. Nevertheless, information regarding the comparison between the content has not been provided. This study aimed at identifying the sesquiterpenes in *G. versteegii* inoculated using the simpori technique with various dosages of inoculants (3; 9; and 18 mL/porous nail) and harvested at 18 months after inoculation. The harvested agarwood was extracted using n-hexane solvent. The chemical compositions were subsequently analyzed using the GC-MS. Compounds with a similarity index above 80% were analyzed, including their presence and percentage in the agarwood. The statistical significance test was carried out with a 5% level of significance. The Kruskal-Wallis test showed a P-value <0.05, indicating the significant differences between the inoculant dosages on the percentage of sesquiterpenes. Agarwood with F. solani at a dosage of 9 mL/porous nail shows the best result with a Mean Rank of 16 and 23.69% of sesquiterpenes. It contains aromadendrene; α-selinene; (1aS,4aS,8aR)-4a,8,8-trimethyl-2-methylene-1,1a,2,4a,5,6,7,8-ctahydrocyclopropa[d]naphthalene; 6-Methyl-2-(4-methylcyclohex-3-en-1-yl)hepta-1,5-dien-4-ol; alloaromadendrene; and eremophilene. Therefore, this dosage is recommended for optimizing agarwood production.

Keywords: Agarwood; Aromadendrene; Eremophilene; Sesquiterpene; Simpori

Abstrak

Gaharu merupakan resin yang terbentuk pada spesies anggota famili Thymelaeaceae, termasuk Gyrinops versteegii yang mengalami perlukaan atau terinfeksi oleh mikroorganisme. Kualitas gaharu baik alami maupun budi daya dapat dilihat dari kandungan sesquiterpene. Penelitian ini bertujuan mendeteksi kandungan sesquiterpene pada G. versteegii hasil inokulasi dengan teknik simpori pada 3 dosis inokulan berbeda yaitu 3; 9; dan 18 mL/paku simpori dan dipanen saat 18 bulan setelah inokulasi. Gaharu yang telah dipanen kemudian diekstrak menggunakan pelarut n-heksana. Ekstrak n-heksana selanjutnya dianalisis kandungan kimianya menggunakan GC-MS. Data yang digunakan pada hasil adalah deteksi senyawa dengan persentase Similarity Index (SI) di atas 80%. Untuk menguji signifikansi perlakuan maka selanjutnya dilakukan uji statistik dengan taraf signifikansi 5%. Berdasarkan uji Kruskal Wallis, Sig. hitung <0,05 artinya terdapat perbedaan yang signifikan dari tiga perlakuan dosis inokulan terhadap persentase senyawa seskuiterpen yang dihasilkan. Dosis 9 mL/paku simpori merupakan dosis terbaik dengan Mean Rank sebesar 16 dan memiliki kandungan seskuiterpen tertinggi yaitu 23,69%. Senyawa seskuiterpen yang terdeteksi pada gaharu hasil inokulasi dengan dosis 9 mL/paku simpori adalah aromadendrene; α-selinene; (1aS,4aS,8aR)-4a,8,8-trimethyl-2-methylene-1,1a,2,4a,5,6,7,8-ctahydrocyclopropa[d]naphthalene; 6-methyl-2-(4-methylcyclohex-3-en-1-yl)hepta-1,5-dien-4-ol; alloaromadendrene; dan eremophilene. Penggunaan dosis inokulan 9 mL/paku simpori dapat direkomendasikan untuk optimalisasi produksi gaharu yang lebih efisien.

***Kata Kunci**: Aromadendrene; Eremophilene; Gaharu; Seskuiterpen; Simpori*

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INTRODUCTION

Agarwood is one of the most sought non-timber forest products due to its high economic and aesthetic values, including for health, religious ceremonies, and aromatic purposes (Batubara et al., 2022; Tamyiz et al., 2022; Saputra et al., 2024). Agarwood is a resin formed in plants that belong to the *Thymelaeaceae* family as a result of damage or microbial infection (Wahyuni et al., 2020). In Indonesia, the major sources of agarwood are 26 species of seven genera, namely *Aquilaria*, *Gonystylus*, *Gyrinops*, *Aetoxylon*, *Enkelia*, *Phaleria*, and *Wikstroemia*, which grow naturally in the wild (Lukman et al., 2022). The first three genera are the most frequently used (Sutomo et al., 2021). *Gyrinops* dominated the Eastern, while *Aquilaria* dominated the Western Indonesia (Roemantyo & Partomihardjo, 2010).

Gyrinops versteegii is a major source of agarwood, particularly obtained from West Nusa Tenggara, East Nusa Tenggara, and Papua (Wahyuni et al., 2020). It is offered in various forms, from large-sized raw materials to powder, as well as finished products such as perfume and incense (Ceniza et al., 2021). Nevertheless, harvesting in nature has caused its amount to decrease drastically. Consequently, it is listed in the CITES Appendix II to specify that the trade of this species and its derivatives is restricted (Sutomo et al., 2021). Moreover, species belonging to *Aquilaria* and *Gyrinops* are also categorized “Critically endangered”, “Data deficient”, “Endangered”, and “Vulnerable” by the International Union for Conservation of Nature (IUCN) (Syameera et al., 2024).

The natural formation of agarwood requires a slow process and occurs only on 10% of wild trees due to fungal infection or physical damage (Gogoi et al., 2023). Therefore, cultivation is an alternative to obtain agarwood and to reduce the wild harvest at once. This effort comprises cultivation and inoculation technology to trigger the natural formation of agarwood. A modified inoculation technique called simpori has been conducted on *G. versteegii* in West Nusa Tenggara (Wahyuni et al., 2020). The technique combines nailing the tree trunk using porous nails and filling it with *Fusarium solani* in a liquid medium at a certain concentration. A previous study showed that the visual quality of agarwood using the technique with 3, 6, and 9 mL of *F. solani* inoculant per porous nail, and harvested months after inoculation was similar, namely the quality of agarwood with mastic content and weaker aroma (Grade C-medium) (Indonesian National Standard (SNI) 7631:2011, 2011). Sesquiterpene and chromone compounds were also found in the agarwood with slight variations (Wahyuni et al., 2020).

Sesquiterpene is a substantial chemical component for determining the quality of agarwood (Yu et al., 2023). The higher the sesquiterpene content in agarwood, the higher the quality of the agarwood. In addition, it is relatively stable against environmental changes, including temperatures (Syameera et al., 2024). A study on agarwood in wild *Gyrinops salicifolia* identified that it contained 12 sesquiterpenoids (Shao et al., 2016). Nevertheless, the information about the sesquiterpene compound of agarwood in *G.* versteegii, both in the wild and cultivated, is inadequate. After 7 months, *G. versteegii* inoculated using the Simpori technique yielded agarwood with alloaromadendrene and valerenol contents (Wahyuni et al., 2020). Information on sesquiterpenes of agarwood in the cultivated *G. versteegii* is required to provide a comparative overview of quality with the wild ones. This study aims to identify the sesquiterpene content of agarwood in the cultivated *G. versteegii* inoculated using the simpori technique at various inoculant concentrations (*Fusarium solani)*, namely 3; 9; and 18 mL/porous nail, and harvested 18 months after inoculation.

MATERIALS AND METHODS

Study Site/Location and/or Materials

This study was carried out in 2019. The extraction of sapwood was performed at the NTFPs processing laboratory, Research, and Development Institute of Technology for Non-timber Forest Products (*Balai Litbang Teknologi HHBK*) Mataram, West Nusa Tenggara, Indonesia. The GC-MS analysis was done at the Research Center for Chemistry-Indonesian Institute of Sciences, Serpong, Indonesia. The characteristics of sapwood agarwood were observed from the cultivated *G. versteegii* that was inoculated using the simpori technique at various inoculant concentrations (*Fusarium*

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solani), namely 3; 9; and 18 mL/porous nail. The agarwood was harvested 18 months after inoculation.

Methods

Extraction of Sapwood Agarwood

The sapwood was harvested, air-dried at room temperature, and ground using a blender. The powder was extracted using n-hexane repeatedly until the extracted liquid was clear. The agarwood powder to solvent ratio was 1:5. Subsequently, the yield was filtered and concentrated using a rotary vacuum evaporator.

GC-MS Analysis

A sample of 1 µL was injected into the GC-MS instrument with a glass column of 2.5 m long, 0.25 m diameter, 0.25 µm thickness, and CP-Sil 5 CB stationary phase. The carrier gas was helium. The device's starting temperature was 40 °C held for 1 minute and increased at 10 °C per minute until 250 °C. The temperature was held for 6 min since the compounds might have different retention times. Each compound's chromatogram and similarity index (SI) were analyzed using NIST MS Search version 2.0.

Statistical Analysis

The compounds with a similarity index (SI) above 80% were identified for further analysis. The presence and percentage of each compound on the agarwood were determined. A test of the statistical significance was carried out with the 5% level of significance.

RESULTS

The sesquiterpenes in agarwood from *G. versteegii* inoculated using the simpori technique are illustrated in Table 1. While the percentage of sesquiterpenes in *G. versteegii* agarwood inoculated using simpori technique is shown in Figure 1. The total percentage of sesquiterpenes in agarwood for each treatment is presented in Figure 2. The main structure of sesquiterpenes in *G. versteegii* agarwood inoculated using simpori technique is illustrated in Figure 3.

Table 1. Sesquiterpenes in the agarwood formed in *Gyrinops versteegii* inoculated using simpori technique with various dosages of inoculant

Compound	Presence in the agarwood		
	P0	P1	P2
Aromadendrene	+	+	+
γ-gurjunene	-	-	+
α-selinene	-	+	+
2-isopropylidene-3-methylhexa-3,5-dienal	-	-	+
(1aS,4aS,8aR)-4a,8,8-trimethyl-2-methylene-1,1a,2,4a,5,6,7,8-octahydrocyclopropa[d]naphthalene	-	+	-
6-methyl-2-(4-methylcyclohex-3-en-1-yl)hepta-1,5-dien-4-ol	-	+	-
Alloaromadendrene	-	+	-
Eremophilene	-	+	-

Note: *P0= *Fusarium solani* at a dosage of 3 mL/porous nail; P1= *Fusarium solani* at a dosage of 9 mL/porous nail; P2= *Fusarium solani* at a dosage of 18 mL/porous nail; += detected, -= not detected

Subsequently, based on the data in Figure 1, statistical analysis was done to test the effect of inoculant dosages on the amount of sesquiterpenes. The result of the normality test shows P-value is less than 0.05, implying that the data is not normally distributed and the Kruskal-Wallis test is required. Meanwhile, the result of the Kruskal-Wallis test shows P-value is less than 0.05, indicating the differences between the inoculant dosages on the percentage of sesquiterpenes in the agarwood are statistically significant. *F. solani* at a dosage of 9 mL/porous nail shows the best yield with a Mean Rank of 16.

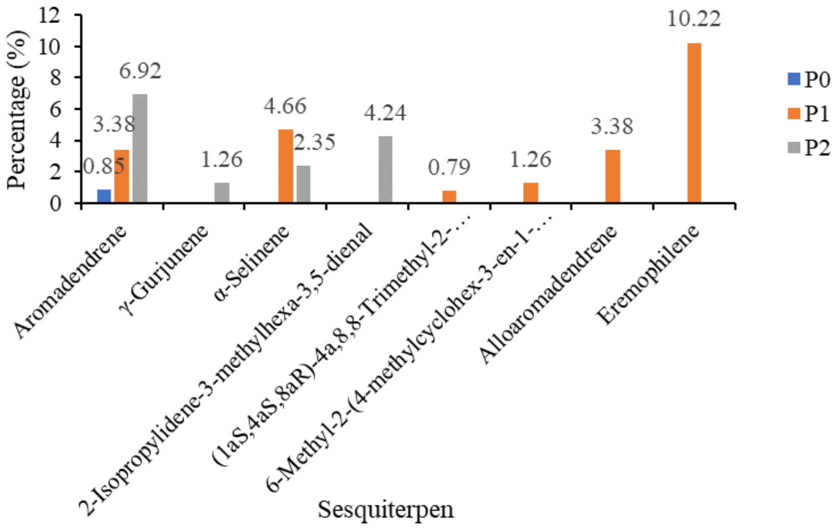

Figure 1. Percentage of sesquiterpenes in *Gyrinops versteegii* agarwood inoculated using simpori technique with various dosages of inoculant

Note: *P0= *Fusarium solani* at a dosage of 3 mL/porous nail; P1= *Fusarium solani* at a dosage of 9 mL/porous nail; P2= *Fusarium solani* at a dosage of 18 mL/porous nail

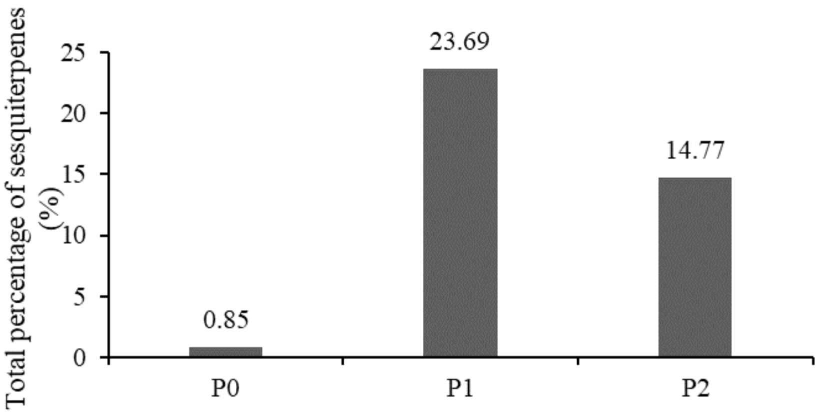

Figure 2. Total percentage of sesquiterpenes in *Gyrinops versteegii* agarwood inoculated using simpori technique with various dosages of inoculant

Note: *P0= *Fusarium solani* at a dosage of 3 mL/porous nail; P1= *Fusarium solani* at a dosage of 9 mL/porous nail; P2= *Fusarium solani* at a dosage of 18 mL/porous nail

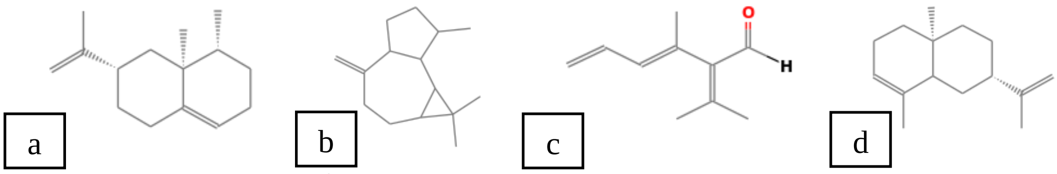

Figure 3. The main structure of sesquiterpenes in *Gyrinops versteegii* agarwood inoculated using simpori technique (Source: National Institute of Standards and Technology (NIST), 2023); eremophilene (a), aromadendrene (b), 2-isopropylidene-3-methylhexa-3,5-dienal (c), and α-selinene (d)

DISCUSSION

Based on the analysis, the types of sesquiterpene in n-hexane extracts of *G. versteegii* agarwood are diverse as illustrated in Table 1. The use of n-hexane as a solvent has been done previously, showing that sesquiterpenes can be obtained from extraction using nonpolar compounds (Ahmaed & Kulkarni, 2017). Most types of sesquiterpene were found in agarwood inoculated using simpori technique at an inoculant dosage of 9 mL/porous nail, namely six types. Subsequently, four types and one type of sesquiterpene were identified in those inoculated with inoculant dosage of 18 mL/porous nail and 3 mL/porous nail, respectively. Sesquiterpenes indicate the quality of agarwood. The more types of sesquiterpene identified in the agarwood, the higher the quality of the agarwood.

In this study, inoculation using simpori technique with an inoculant dosage of 9 mL/porous nail was able to produce aromadendrene; α-selinene; (1aS,4aS,8aR)-4a,8,8-trimethyl-2-methylene-1,1a,2,4a,5,6,7,8-octahydrocyclopropa[d]naphthalene; 6-methyl-2-(4-methylcyclohex-3-en-1-yl)hepta-1,5-dien-4-ol; alloaromadendrene; and eremophilene. These types of sesquiterpene also affect the aroma of the produced agarwood (Yang et al., 2021). Eremophilene, guaiane, and eudesmane are sesquiterpenes reported to be the main contributors to the aroma of agarwood (Hou et al., 2024). Eudesmane is mainly characterized by fresh, honey, sweet floral, and mint fragrance. The eremophilene is characterized by woody, camphor, amber, and other persistent strong aromas. Meanwhile, guaiane is characterized by a slight woody camphor aroma and a powerful honey aroma. Another type of sesquiterpenes, agarospirane, is found in *Aquilaria agallocha* and characterized by a spicy, peppery, and woody aroma (Yang et al., 2021).

The percentage of sesquiterpenes is presented in Figure 1. It shows that the eremophilene-type has the highest percentage (10.22%) and is only identified in agarwood inoculated using the simpori technique with an inoculant dose of 9 mL/porous nail. This type is found in natural agarwood from *Gyrinops salicifolia* from Papua New Guinea (Shao et al., 2016). The second highest percentage is the aromadendrene type. It is a member of the *Guaianes* group frequently found in *Aquilaria crassna* agarwood (Gao et al., 2019). Moreover, it is proposed to be a chemical compound that marks high-quality agarwood because its presence is always detected in high-quality agarwood (Sundaraj et al., 2023). In the present study, aromadendrene was identified in agarwood from all treatments, and the highest percentage (6.92%) was obtained in agarwood inoculated using simpori technique with an inoculant dose of 18 mL/porous nail.

The total percentage of sesquiterpenes in *G. versteegii* agarwood inoculated using the simpori technique with various inoculant dosages is presented in Figure 2. Based on the figure, the highest total percentage (23.69%) of sesquiterpenes was obtained from that inoculated with an inoculant dosage of 9 mL/porous nail. It confirms the findings shown in Table 1 that simpori technique with an inoculant dose of 9 mL/porous nail produces agarwood with more types of sesquiterpene than other dosages.

Several factors that potentially affect the amount of sesquiterpenes in agarwood are the degree of environmental exposure to sapwood, and volatilization and natural decomposition in trees, and the duration of injury/exposure to microorganisms (Hou et al., 2024). In the present study, all treatments underwent exposure to microorganisms with the same duration (18 months) and under the same environment (one layer). In the present study, the difference in the content of sesquiterpenes was mainly caused by the difference in the inoculant (microorganism) dosage that stimulated the formation of agarwood. A dosage of 9 mL/porous nail produced agarwood with the highest number of types and percentage of sesquiterpene. This phenomenon was reinforced through statistical tests using the Kruskal-Wallis test, P-value of less than <0.05 (P-value= 0.047), indicating that the administration of different dosages of inoculant produced a significantly different percentage of sesquiterpene. The dosage of 9 mL/porous nail produced the highest sesquiterpene with a Mean Rank of 16. In this research, a dosage of 9 mL/porous nail reaches an optimal dosage to produce sesquiterpene. Inoculant doses of more than 9 mL/porous nail are thought to produce negative effects on sesquiterpene production, but the mechanism related to secondary metabolite biosynthesis could not be explained in detail.

The chemical structures of aromadendrene, 2-Isopropylidene-3-methylhexa-3,5-dienal, eremophilene, and α-selinene are shown in Figure 3. Aromadendrene is structurally characterized by a dimethyl cyclopropane ring fused to a hydroazulene skeleton (Lamers, 2003). Aromadendrene has several functionalities that can be used as a handle for synthetic transformations (Lamers, 2003). Eremophilene is a $C_{15}H_{24}$, hydrocarbon first isolated from *Petasites officinalis* and *P. albus* (Piers & Keziere, 1969). The structure of eremophilene comes from 1,2,3,4,4a,5,6,7-octahydronaphthalene which is substituted by an isopropenyl group at position 3 and by methyl groups at positions 4a and 5 (the 3R,4aR,5S-diastereoisomer). Subsequently, α-selinene with a double bond in the octahydronaphthalene ring system is endocyclic (2R,4aR,8aR)-configuration while 2-isopropylidene-3-methylhexa-3,5-dienal has a molecular formula of $C_{10}H_{14}O$.

CONCLUSION

The administration of different dosages of inoculants, namely 3; 9; and 18 mL/porous nail has been investigated, showing a significant difference to the results of sesquiterpene content with a P-value of 0.047. *F. solani* inoculant at a dosage of 9 mL/porous nail obtains the best result with a Mean Rank of 16 and also the highest amount of sesquiterpene (23.69%). The sesquiterpenes identified in agarwood inoculated with a dosage of 9 mL/porous nail include aromadendrene; α-selinene; (1aS,4aS,8aR)-4a,8,8-trimethyl-2-methylene-1,1a,2,4a,5,6,7,8 octahydrocyclopropa[d]naphthalene; 6-methyl-2-(4-methylcyclohex-3-en-1-yl)hepta-1,5-dien-4-ol; alloaromadendrene; and eremophilene. The treatment of an inoculant dosage of 9 mL/porous nail can be recommended for optimizing agarwood production.

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