Wiley
International Journal of Forestry Research
Volume 2026, Article ID 9492907, 13 pages
https://doi.org/10.1155/ijfr/9492907

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Research Article

Potential of Sumatran Frankincense (*Styrax benzoin*) *In Vitro* Technique for Producing Bioactive Alkaloids as a Strategy to Maintain the Preservation of Forest Plants

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Received 14 October 2025; Revised 18 December 2025; Accepted 23 December 2025

Academic Editor: Ahmad A. Omar

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Bioactive alkaloids that are naturally produced by plants need to be explored to identify chemical compounds with medicinal properties for pharmaceutical or industrial purposes. Sumatran Frankincense (*Styrax benzoin*) is known as a forest plant in North Sumatra, Indonesia, and produces bark sap containing bioactive compounds. The sap has been used as an ingredient in traditional and modern medicine. The problem is that producing incense resin is carried out by injuring the bark of the plant which has the potential to damage the plant. In vitro techniques are assumed to be an alternative for producing bioactive compounds to maintain plant sustainability. The research aims to obtain an in vitro technique for Sumatran Frankincense to produce bioactive alkaloids with medicinal properties as a strategy to maintain the sustainability of woody forest plants. The research stages include in vitro propagation of Sumatran Frankincense to produce callus raw materials, followed by bioactive isolation techniques, identification, and confirmation of the alkaloid compounds contained in the callus. The research succeeded in obtaining optimum conditions for the in vitro technique of producing callus, using MS media enriched with 3 mg/L IBA and 3 mg/L NAA to produce good quality callus in large quantities. The isolation from callus macerated using ethanol to produce a crude isolate that was positive for alkaloids. The crude isolate was purified by acid–base extraction using an organic solvent and fractionated by column chromatography to produce a pure alkaloid fraction. The alkaloid is in the form of a yellowish powder with a characteristic aroma, melting point of 129°C, boiling point of 307°C, and density of 1193 g·cm^{-1}, and it soluble in chloroform and alcohol solvents and insoluble in cold water. Identification of alkaloids using FT-IR and GC-MS confirmed the presence of the main compound piperine along with other chemical compounds. This study is a model for isolating alkaloids from callus to confirm the potential of the Sumatran Frankincense in vitro technique in producing compounds with medicinal properties.

Keywords: alkaloids; *In vitro* techniques; isolation; medicinal properties; Sumatran Frankincense

1. Introduction

The need for bioactive compounds from plants that have medicinal properties is increasing day by day in line with the increase in population because it is perceived that natural bioactives are safer for health and the environment than synthetic bioactives. Several forest plants are known to produce bioactive compounds such as alkaloids, flavonoids, terpenoids, and tannins, and some of them have been identified as having antioxidant, antibacterial, antiviral, anticancer, and anti-inflammatory properties [1–3].

Alkaloid compounds are produced naturally by plants, are characterized by containing nitrogen in their molecular structure, and have physiological effects on living creatures that function as plant guards against herbivores and pathogens. The broad therapeutic activity makes alkaloids able to act as medicinal raw materials to cure various diseases. Alkaloids have analgesic properties in relieving pain, and phytochemical functions reduce the risk of chronic diseases such as cardiovascular disease and other diseases [4]. Alkaloid compounds from plants are generally heterocyclic with nitrogen, which is basic, in the form of colorless crystals with a bitter taste. The alkaloid salts are soluble in water and acids, have pharmacological activity, and at high doses are mostly toxic [5].

Various studies on *Styrax benzoin* have been carried out to utilize incense resin, including as a raw material in the religious ceremony, beauty, perfumery, pharmaceutical, and industrial sectors [6]. The bioactivity of the chemical compounds contained in *Styrax benzoin* can be utilized for various purposes [7, 8]. Frankincense sap is also known to contain chemical compounds with medicinal properties such as cinnamic acid, benzoic acid, styracin, coniferyl benzoate, benziresinol, and resinotannol [9]. These compounds have been used as anti-inflammatory, antioxidant, antibacterial, and anticancer agents [10–12]. These facts will encourage the use of *Styrax benzoin* as a bioactive source and if exploited incorrectly will threaten the existence of this woody forest plant.

The use of alkaloid compounds encourages the production of bioactives from woody plants that grow in tropical forests. One of the forest plants known to be a potential source of alkaloids is Sumatran Frankincense (*Styrax benzoin*), which produces bark sap that contains bioactive medicinal properties, known as frankincense [13]. Frankincense has been widely used for various purposes, especially for religious and medicinal purposes. Many communities living around forests in North Sumatra, Indonesia, depend on these forest products. Frankincense is a commodity that is sold in the form of raw materials in traditional markets. Although frankincense is known to contain chemical compounds with medicinal properties, this product has not been isolated to separate the chemical content that is needed by pharmaceuticals and industry. This bark sap product is produced using a direct tapping technique from the Sumatran Frankincense plant, which has an impact on plant health.

The main problem faced in the production of bioactive compounds from Sumatran Frankincense plants is the potential for plant damage resulting from excessive plant exploitation. It has an impact on forest destruction. The sustainability of Sumatran Frankincense can be threatened if it is tapped carelessly to produce bark sap. The technique of tapping the sap will sacrifice the health and sustainability of the plant itself. The production of frankincense sap is carried out by making wounds on the plant stem to induce bark sap, and after harvest, it will leave a permanent gaping scar, which makes the plant vulnerable to attack by borer pests and the growth of pathogenic fungi [14]. Plant defenses will be reduced due to sap tapping, which will directly impact plant damage, which can result in plant death. Comprehensive efforts must be made to minimize the exploitation of plants in producing incense resin, and alternatives need to be considered that can keep plants safe and sustainable.

In vitro techniques for producing alkaloids are an alternative choice. It is believed that the production of the compounds that occur naturally in plants will also occur in plants at the callus level [15, 16]. In vitro techniques have been known as the best strategy for mass propagating plants, to produce seeds with the same genetic characteristics as the parent plant in a relatively short period of time. This advantage is appropriate to be adopted for producing natural alkaloids. Studies have reported that in vitro techniques can be relied upon to produce bioactive compounds [17, 18]. Thus, the in vitro technique of Sumatran Frankincense can also be used to produce alkaloids without having to sacrifice the parent plant [19].

Tropical forests are a good place to grow medicinal plants, and some plants have been used as a source of bioactive medicinal substances [20, 21]. Forest plants that produce medicinal substances are generally woody plants [22, 23]. Therefore, it must be managed properly as a source of medicinal compounds to maintain forest sustainability. The production of the chemical compounds with medicinal properties generally comes at the expense of the plant itself, as the compounds are extracted from productive plant parts such as leaves, stems, and roots [24–26]. Producing medicinal compounds from the roots and stems of plants must be avoided because it will damage plants, reduce plant populations, and if not properly conserved will result in plant extinction and damage of the forest environment.

In vitro techniques are a good alternative for producing alkaloids [27]. It can be carried out in a short time in the laboratory using young leaf explants, which will definitely not damage the parent plant. Several studies using in vitro techniques have been carried out to produce chemical compounds with medicinal properties [28]. In vitro techniques have been used to produce plant seeds and also naturally synthesize bioactive compounds [29, 30]. In vitro culture of Sumatran Frankincense has been proven to contain chemical compounds such as those contained in the parent plant [31, 32]. Plant tissue culture can be used to produce chemical compounds for medicinal raw materials. The technique will shorten the time to produce chemical compounds because it is carried out at the culture level [33]. This in vitro technique needs to be promoted for long-term goals, namely a high economic source from the production of the medicinal substances, saving forest plants from damage due to overexploitation, and keeping forests sustainable as a source of oxygen.

Scientific information regarding the potential of in vitro culture of Sumatran Frankincense in producing alkaloid compounds with medicinal properties needs to be disclosed in a comprehensive manner. In vitro techniques have the potential to be an economic source for the production of medicinal compounds and to preserve the environment because they do not damage the parent plant. The studies have been carried out on the advantages of the Sumatran Frankincense plant, including in vitro studies of plant propagation producing plant seeds that have the same superior genetic characteristics as the parent plant [34]. The studies regarding

the content of secondary metabolites in *in vitro* cultures have been carried out. It is reported that bioactive content at the callus level has a pattern like that of skin sap [35].

The success of Sumatra Frankincense's in vitro technique for producing chemical compounds will be able to provide a supply of the compounds as medicinal raw materials in a relatively short time without destroying forest plants. The compounds produced in vitro become an economic resource, preserve plants, and keep the forest environment sustainable [36]. This research aims to obtain an in vitro technique for Sumatran Frankincense as a protocol for producing alkaloids with medicinal properties as a strategy to preserve forest plants. The focus of the research is to obtain optimum in vitro techniques for producing Sumatran Frankincense callus as raw material for alkaloid isolation and techniques for isolating and identifying the chemical compounds with medicinal properties from callus for pharmaceutical and industrial purposes.

2. Methods

2.1. Materials and Chemicals. The raw material used in the *in vitro* technique is young leaf explants taken from the Sumatran Frankincense (*Styrax benzoin*) mother tree from a forest area in Aek Nauli II Village, Pollung District, Humbang Hasundutan Regency, North Sumatra, Indonesia. The chemical compounds used in this research include Murashige and Skoog (MS), Bacto Agar, the growth regulator indolebutyric acid (IBA), and naphthaleneacetic acid (NAA). Chemical substances include NaCl, HCl, NaOH, HCl, diethyl ether, ammonium hydroxide (NH_4OH), $FeCl_3$, sodium sulfate (Na_2SO_4), chloroform, methanol, ethyl acetate, *n*-hexane, and silica gel 60 (0.063–0.200 mm), all supplied from Merck Tbk. The culture medium used was MS enriched with IBA and NAA and prepared and sterilized following previous procedures [37].

2.2. Equipment. The equipment used is laminar air flow (LAF), UV–vis spectrophotometer, Fourier transform infrared spectroscopy (FT-IR) (ATR Nicolet, Spectrum one), gas chromatography mass spectroscopy (GC-MS) (7890B with MassHunter GC/MS Acquisition Agilent Technologies, Inc), chromatography column (CC) using silica gel $60F_{254}$, thin-layer chromatography (TLC) using UV 254 nm, rotary evaporator, UV lamp, and analytical balance. Chemical glass equipment includes beakers, volumetric pipettes, Erlenmeyer flasks, separating funnels, measuring flasks, sockets, pestles, and mortar.

The CC was a gravity-based, glass column (diameter 40 mm and length 40 cm) filled with 25 g of active silica gel (60 F254-activated by heating to 120°C) and chloroform–ethyl acetate eluent (8:2) with gradient elution, and separation of the alkaloid fraction was observed using UV λ254 nm.

2.3. Tissue Culture Experiment Design. Sumatran Frankincense plant tissue culture was carried out using a completely randomized design with varying concentrations of the growth regulators IBA and NAA supplemented into the MS medium, as shown in Table 1.

Table 1: Sumatran Frankincense tissue culture research design with a combination of indolebutyric acid (IBA) (I)/naphthaleneacetic acid and NAA (N) in MS media (seven replications).

		***Naphthaleneacetic acid* (NAA) (N), ppm**			
		N_0	N_1	N_2	N_3
Indolebutyric acid (IBA) (I), ppm	I_0	I_0N_0	I_0N_1	I_0N_2	I_0N_3
	I_1	I_1N_0	I_1N_1	I_1N_2	I_1N_3
	I_2	I_2N_0	I_2N_1	I_2N_2	I_2N_3
	I_3	I_3N_0	I_3N_1	I_3N_2	I_3N_3

Note: I_0 = IBA 0.0 ppm; I_1 = IBA 1000 ppm; I_2 = IBA 2000 ppm; I_3 = IBA 4000 ppm. N_0 = NAA 0.0 ppm; N_1 = NAA 500 ppm; N_2 = NAA 1000 ppm; N_3 = NAA 2000 ppm.

2.4. Research Procedures. The research procedure consists of two parts, namely in vitro culture and isolation of alkaloids. In vitro culture to produce callus is carried out through the stages of preparing young leaf samples for explants, providing media enriched with varying concentrations of growth regulators, sterilization, and planting explants following the procedures in previous research [6]. Briefly, young leaf explants from good quality mother plants were sterilized, cut into pieces (0.5–1.0 cm), and planted in MS medium enriched with IBA and NAA as in the research design in Table 1. Mass producing callus in this study was using 3 mg/L IBA and 3 mg/L NAA in the I_3N_3 treatment group. Next, the callus in the culture medium was incubated at 25 ± 2°C and irradiated using UV light for 16 h per day, and the sterile room was maintained by regularly spraying 70% alcohol. The growth and development of the callus are observed for 7 weeks before it is ready to be harvested. The wet callus is then cleaned, weighed, dried until it is free of water, crushed, sieved (mesh), stored in a desiccator to free up water content, and then becomes the raw material for isolating bioactive alkaloids [38, 39]. Data were analyzed using ANOVA followed by Duncan's multiple range analysis ($p = 0.05$) [40].

Isolation of alkaloids from callus cultures was carried out following previous research procedures [41–43]. The fine dry callus raw material was isolated through the stages of weighing, maceration, acid and base partition, Soxhlet extraction, chromatography (TLC and column), purification, and screening as summarized in Figure 1. A total of 6 g of dry callus was placed into a maceration bottle, soaked in ethanol (12 mL), stirred gently, and allowed to stand (3 × 24 h). Then, it was filtered to obtain a liquid extract, which was concentrated using a vacuum rotary evaporator at a temperature of (35°C). The thick extract was weighed again as a crude alkaloid isolate.

Qualitative alkaloid testing was carried out on the extract by placing 2 g of crude isolate into a beaker, adding 10 mL of chloroform, stirring, adding NH_4OH (5%), filtering and transferring the filtrate to a test tube, and adding H_2SO_4 (10%). It was then shaken and allowed to stand, and the acid layer solution was pipetted into a glass drop plate (three parts), and a drop of Dragendorff, Mayer, and Wagner reagents was sequentially added. The formation of a brownish orange/yellow to orange precipitate in the

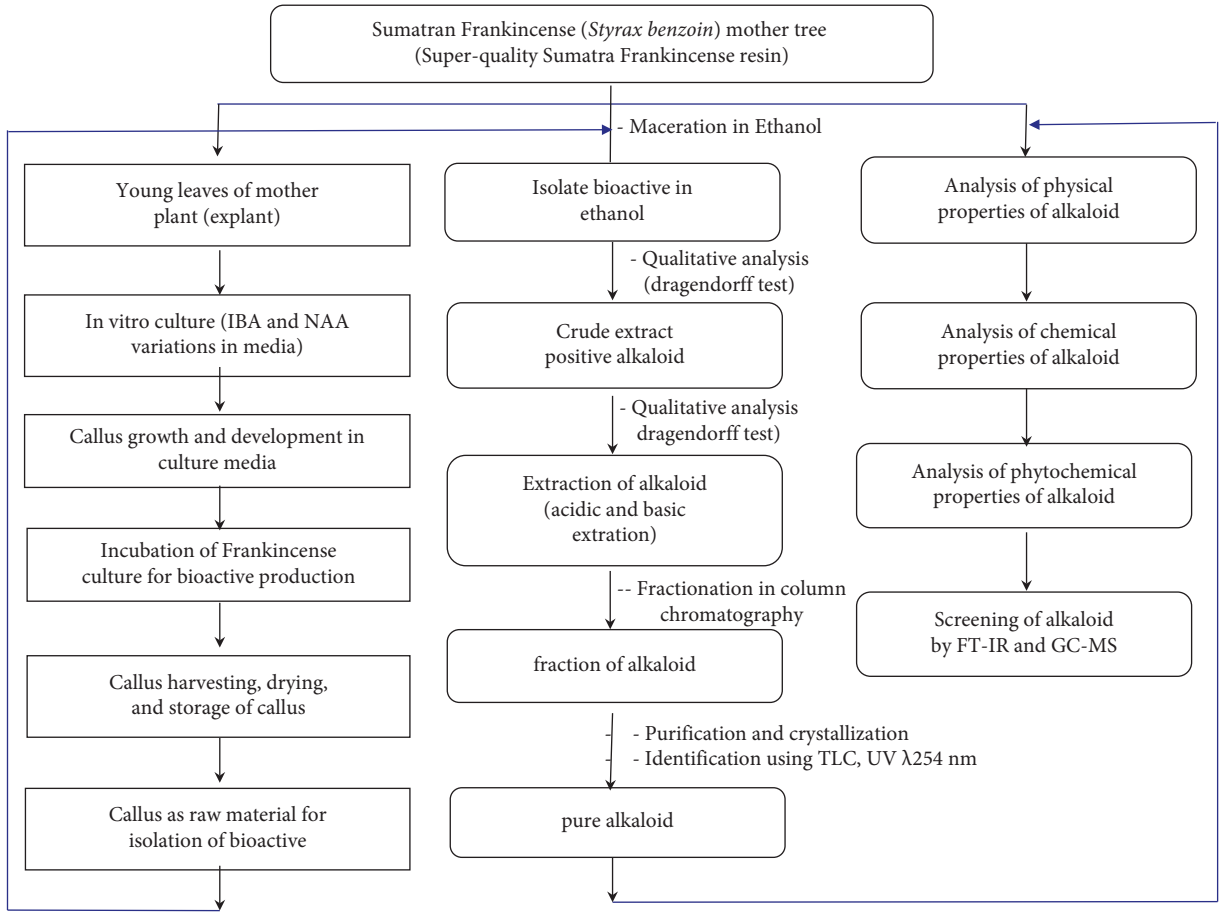


Figure 1: Isolation of bioactives from Sumatran Frankincense callus produces bioactive alkaloids.

Dragendorff reagent, a white precipitate/yellow precipitate in the Mayer reagent, and a brown/yellow precipitate in the Wagner reagent is considered a positive sign of alkaloids [44].

Isolates that were positive for alkaloids were extracted by weighing 70 mg of the extract and placing it in a separating funnel, and 2% citric acid (pH 3–4) was added while stirring gently, followed by chloroform (140 mL); then, the extract was shaken and allowed to settle, and the acid phase was separated and combined. Next, the acid phase was placed into a separating funnel, and then, 25% NH_4OH (pH 9–10) was added. It was then extracted using 100 mL of chloroform and shaken, and the chloroform phase was collected and concentrated using a vacuum rotary evaporator (35°C) to produce an alkaloid extract. The extract is then weighed and again tested qualitatively to ensure the presence of alkaloids.

Fractionation of the compounds in the alkaloid group was carried out chromatographically (silica 60 F_{254}, 63–200 μm) through step gradient polarity using the solvent chloroform–ethyl acetate (8:2). The separation was observed at UV λ254 nm (showing alkaloid fluorescence), and the eluate of the same fraction was collected, combined, and purified using preparative TLC to produce pure alkaloids. The pure alkaloid compounds are then ready to be analyzed to investigate the physical, chemical, and phytochemical properties and screening of chemical compounds.

As a comparison, alkaloids have been isolated using frankincense resin as a raw material following the same procedure as that used for Sumatran Frankincense callus culture. Isolation was carried out using raw materials of 6 g of frankincense resin in ethanol, stirred gently, and left to stand (1 × 24 h).

3. Results and Discussion

3.1. In Vitro Culture of Sumatran Frankincense. The in vitro technique for producing Sumatran Frankincense callus, which is obtained from very good quality young leaf explants of the parent plant, is shown in Figure 2. The growth and development of the callus vary in line with the composition of the growth regulator in the culture medium. A callus that is 7 weeks is ready to be harvested (Figure 2(d)), namely very good quality callus and a large quantity.

Table 2 data show that almost all explants grew in MS media, with the intensity of explant growth becoming callus varying according to the concentration of IBA and NAA supplemented. The pattern of callus growth and development is largely determined by the composition of growth regulators. Explants in cultures without IBA and NAA only enlarge and do not grow into a callus. The treatment group supplemented with low levels of growth regulators tended to have low callus growth intensity. Induction of explants into



Figure 2: In vitro production of Sumatran Frankincense callus for bioactive isolation raw materials: (a) super-quality mother plant, (b) explants from young leaf cuttings, (c) incubation of explants in culture media, and (d) callus ready for harvest.

Table 2: Growth patterns of callus Sumatran Frankincense in MS media with various combinations of IBA and NAA concentrations for up to 7 weeks.

Treatment group	Callus growth pattern (weeks)							
	1	2	3	4	5	6	7	Intensity of callus growth
I_0N_0	*	*	*	*	*	*	*	No callus
I_0N_1	*	*	+	+	+	+	+	Low
I_0N_2	*	*	+	+	++	++	++	Medium
I_0N_3	*	*	++	++	++	++	++	Medium
I_1N_0	*	*	+	+	+	+	+	Low
I_1N_1	*	*	+	+	++	++	++	Medium
I_1N_2	*	*	++	++	++	++	++	Medium
I_1N_3	*	*	+	+	+	++	++	Medium
I_2N_0	*	*	*	*	+	+	+	Low
I_2N_1	*	*	+	+	++	++	++	Medium
I_2N_2	*	*	+	+	+	+	+	Low
I_2N_3	*	*	+	+	++	++	++	Medium
I_3N_0	*	*	++	++	++	++	++	Medium
I_3N_1	*	*	+	++	+++	+++	+++	High
I_3N_2	*	*	+	++	+++	+++	+++	High
I_3N_3	*	*	++	+++	+++	++++	++++	Very high

Note: The experimental parameters are shown in Table 1.
* The explant enlarges.
+ The explant grows into a callus.

callus is very dependent on providing variations in the composition of IBA and NAA or a combination of both in the culture medium.

Increasing the concentration of growth regulators in the media has been proven to increase callus weight. The quantity of callus induced varied based on variations in

the composition of IBA and NAA or a combination of both in the culture medium, as summarized in Table 3. The trend of increasing callus weight was very significant with increasing growth regulators in the MS medium (Table 3). Callus weight with the greatest growth intensity was obtained with supplementation of 3 mg/L IBA and 3 mg/L NAA in the I_3N_3 treatment group (0.426 ± 0.117 g). To obtain callus in large quantities, in vitro culture of Sumatran Frankincense must be carried out under optimum conditions. A seven-week-old Sumatran Frankincense callus was harvested and then used as raw material for the isolation of bioactive alkaloids. Callus raw materials for alkaloid isolation have been obtained under optimum conditions of IBA and NAA in *in vitro* propagation of Sumatran Frankincense.

Table 3: Growth and development of callus in MS media at varying concentrations of IBA and NAA after 7 weeks (seven replications).

NAA (N)	Callus weight (grams) IBA (I) 0	1	2	3	Average (grams)
0	0.229^{a}	0.0443^{ab}	0.0543^{abc}	0.1814^{f}	0.076^{a}
1	0.0314^{a}	0.0557^{abc}	0.0857^{cde}	0.2029^{f}	0.094^{b}
2	0.0471^{ab}	0.0700^{bcd}	0.0971^{de}	0.2643^{g}	0.119^{c}
3	0.0471^{ab}	0.0729^{bcd}	0.1171^{e}	0.4257^{h}	0.166^{d}
Average	0.037^{a}	0.061^{b}	0.089^{c}	0.269^{d}	

Note: Values followed by different letters indicate a significantly different effect ($p < 0.05$) in the Duncan New Multiple Range Test (DNMRT).

3.2. Alkaloid Isolate. Isolation of alkaloids from frankincense culture was carried out by maceration using ethanol solvent, followed by solvent extraction and thin-column chromatography fractionation to produce alkaloid isolates as shown in Figure 3. Callus raw material (6.28 ± 0.04 g) after maceration and careful concentration (45°C) produced a crude alkaloid extract (4.56 ± 0.34 g) with a blackish brown color (yield 72.54%). It is then continued with qualitative identification to confirm the presence of alkaloids (Figure 3(a)). The acid and base solvent extraction process was effective in purifying the alkaloid extract and separating the alkaloids from other bioactive compounds (Figure 3(b)). Alkaloid fractionation on column chromatography produces pure alkaloid fractions, followed by TLC separation testing using UV 254 nm (Figure 3(c)). The pure alkaloid isolate is in the form of a crystalline powder, soft in texture, and reddish brown in color (Figure 3(d)). The quantity of pure alkaloids and their yields is summarized in Table 4. From 4.56 ± 0.34 g of crude alkaloid extract, 1.53 ± 0.22 g of pure alkaloid compounds were obtained, with a yield of 2.11%.

As a comparison, the alkaloid isolation has been carried out using the raw material of Sumatran Frankincense sap. The quantity of alkaloids obtained is summarized in Table 5. The raw material of Frankincense sap (7.19 ± 0.23 g), which was macerated, produced a crude alkaloid extract (5.59 ± 0.47 g) of blackish brown color (77.94% yield). The quantity of pure alkaloids was 1.64 ± 0.07 g, with a yield of 22.79%.

Testing the properties of alkaloids (physical, chemical, and phytochemical properties) is known to have a melting point of 129°C, boiling point of 307°C, and density of 1193 g·cm^{-1}. The alkaloid is in the form of a yellowish crystalline powder, has a characteristic aroma, is soluble in chloroform and alcohol, and is almost insoluble in water.

3.3. Alkaloid Screening From Sumatran Frankincense Callus. The screening of the isolates was performed, showing positive alkaloids. Characterization of chemical compounds was determined using FT-IR, and GC-MS confirmed the presence of alkaloid compounds in the Sumatran Frankincense callus culture. The FT-IR spectrum (Figure 4(a)) shows the main functional groups as alkaloid markers. The description of the marker absorption peaks has been compared against the absorption peaks of standard alkaloids, which are summarized in Table 6. The IR spectrum shows the presence of aromatic C=C bonds as well as aliphatic C–H stretches and amine N–H stretch absorption that is symmetric with O–H and amine C–N stretch bonds. Those are indications of the presence of N in the heterocyclic or aromatic molecular structure of the alkaloids.

Several chemical compounds contained in alkaloid isolates have been identified from the separation results as shown in the GC chromatogram (Figure 4(b) and Table 5) and fractionation in the MS spectrum (Figure 4(c)). Identification of the compounds based on comparison of the GC-MS library database confirmed that the main compound in the alkaloid group was piperine. It was identified in GC separation with a retention time of 25,929 with an identification quality of 91.62%. Identification based on the MS spectrum shows the presence of stable fragmentations at m/z 313.30, 285.16, 239.28, 201.09, 173.09, 157.12, 143.08, 129.08, 115.08 (base peak), 98.10, 84.11, 69.10, 55.09, and 41.08, as strengthening confirmation of piperine (m/z 313.30), which is a compound fragment that contains N atoms in its molecular structure. The results of the identification of pure alkaloid fractions have confirmed the presence of functional groups and chemical bonds as markers of aromatic alkaloid compounds. It was confirmed by the GC chromatogram separation analysis approach and the m/z molecular ion peak in the MS spectrum. After being compared to the library database, they have convincingly confirmed that the main alkaloid chemical compound is piperine (2E, 4E)-5-(benzo[d] [1, 3]dioxol-5-yl)1-(piperidin-1-yl)penta-2,4-dien-1-one ($C_{17}H_{19}NO_3$).

Identification results using GC-MS have confirmed the presence of several chemical compounds in the pure alkaloid fraction (Figures 4(b), 3(c)). A total of five chemical compounds have similar closeness identifications to the library database (quality > 89%) as summarized in Table 7. The piperine as the main chemical compound was confirmed as an alkaloid group that contains N in its molecular structure. The identification results also showed the presence of several other chemical compounds in the alkaloid isolates (with close quality < 89%). These results confirm that the in vitro technique of Sumatran Frankincense can be relied on to produce alkaloids.

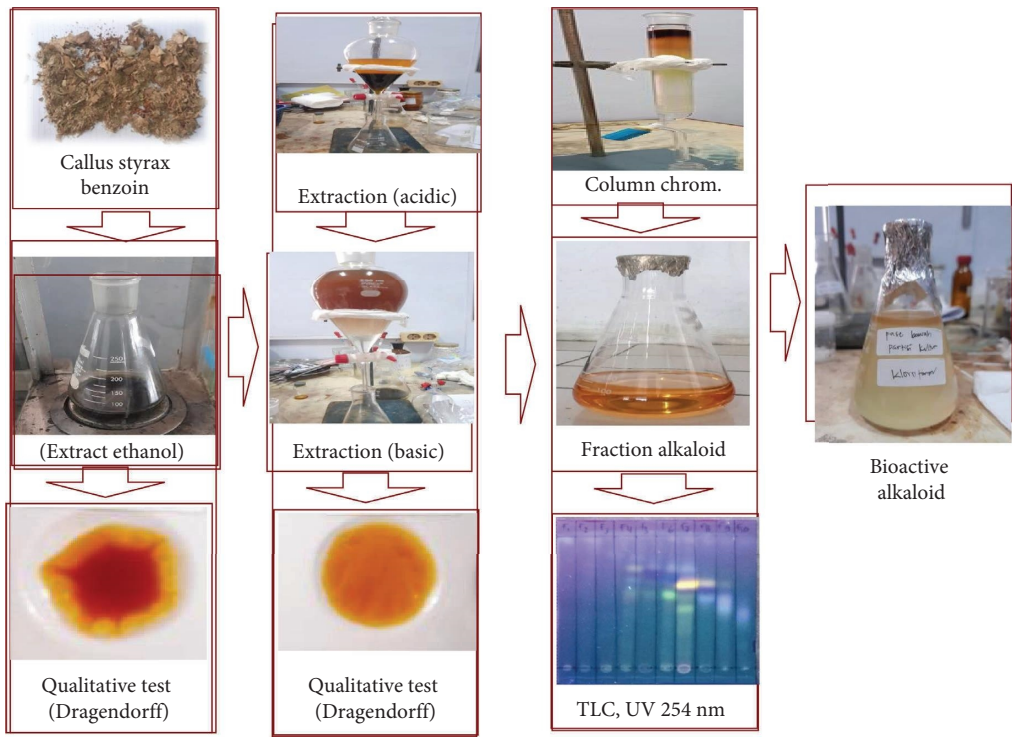


Figure 3: Isolation, extraction, fractionation, and purification of bioactive alkaloids from callus Sumatran Frankincense: (a) crude bioactive isolate from the results of culture maceration using ethanol, (b) extraction to purify the alkaloids, and (c)–(d) fractionation and purification to produce pure alkaloids.

Table 4: Quantity of crude extract of bioactive alkaloids isolated from the callus of Sumatran Frankincense.

Replication	Callus powder (grams)	Crude extract (grams)	Crude yield (%)*	Alkaloid (gr)	Alkaloid yield (%)**
1	6.25	4.18	66.85	1.29	1.93
2	6.33	4.82	76.15	1.72	2.26
3	6.27	4.68	74.64	1.58	2.11
Average	6.28 ± 0.04	4.56 ± 0.34	72.54	1.53 ± 0.22	2.11

*Compared to the dry callus weight.
**Compared to crude bioactive extract.

Table 5: Quantity of crude extract of bioactive alkaloids isolated from the sap of Sumatran Frankincense as a comparison.

Replication	Frankincense powder (grams)	Crude extract (grams)	Crude yield (%)*	Alkaloid (grams)	Alkaloid yield (%)**
1	7.09	5.21	73.44	1.59	22.41
2	7.01	6.12	87.25	1.72	24.52
3	7.45	5.45	73.11	1.60	21.43
Average	7.19 ± 0.23	5.59 ± 0.47	77.94	1.64 ± 0.07	22.79

*Compared to the weight of incense resin.
**Compared to crude bioactive extract.

4. Discussion

The tissue culture technique for producing callus is carried out using super-quality young leaf explants in an MS medium enriched with a combination of IBA and NAA [45]. The callus grew well in large quantities, fulfilling the requirements as raw material for the isolation of alkaloids. From the callus growth pattern, it can be seen that the callus develops rapidly over incubation time, and at the same time, secondary metabolites are formed, which are needed for the formation of plant buds, just like the natural processes that occur in plants. The growth and development of callus are greatly influenced by the composition of growth regulators. The higher the combined concentration of IBA and NAA,

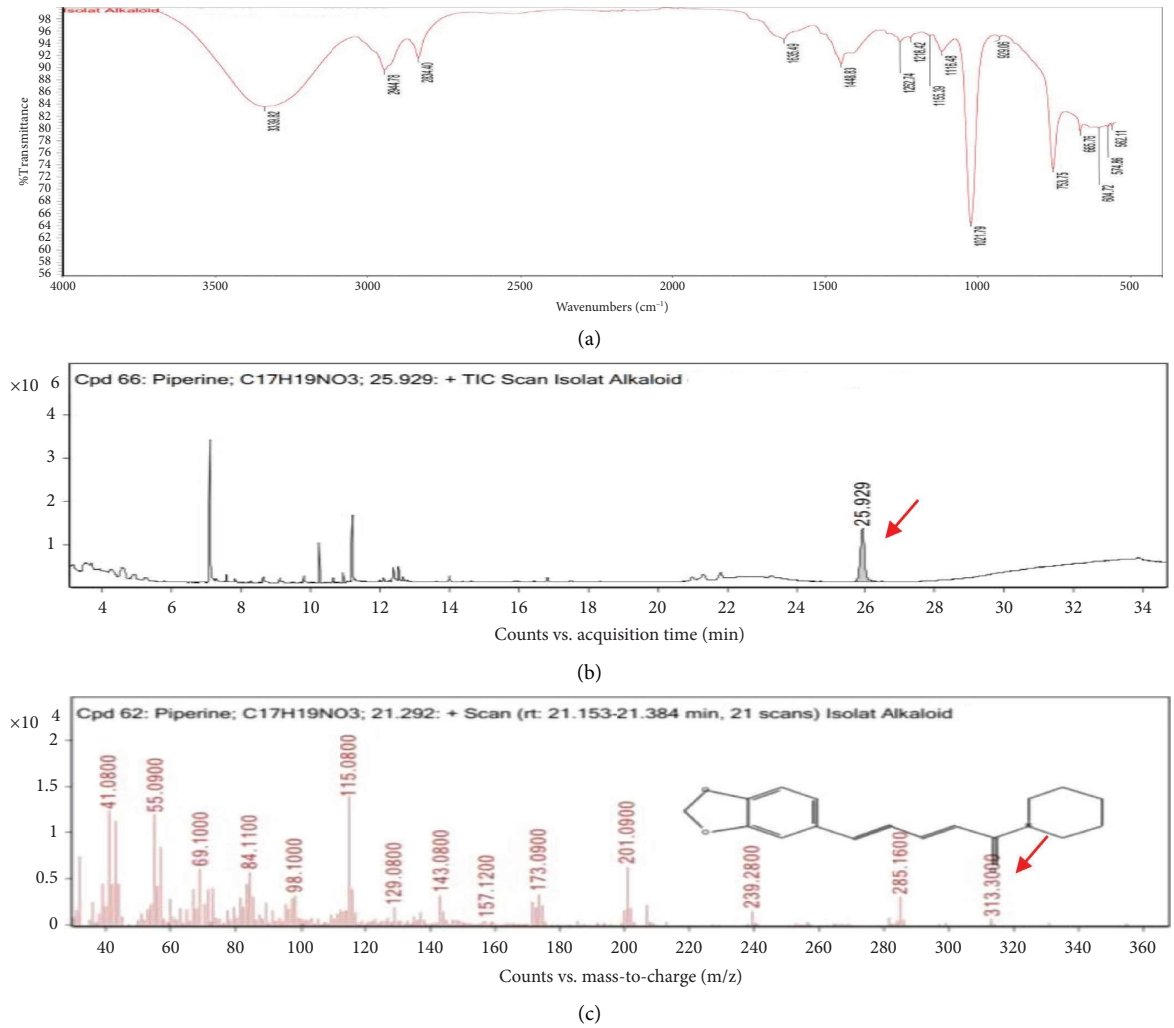


Figure 4: Identification of alkaloid compounds in bioactive isolates of callus Sumatran Frankincense: (a) FT-IR spectra, (b) GC chromatogram, and (c) MS spectra.

Table 6: Identification of alkaloid markers based on FT-IR analysis of isolates callus of Sumatran Frankincense.

Markers for functional groups and chemical bonds	Absorption (wavelength)		Confirmation indicators against predictors	Prediction (quality)
	Database	Alkaloid		
O–H and O–H	3350–3310	3339.82	Positive O–H	Alkaloid
C–N	1250–1020	1021.79–1218.42	Positive C–N amine	
C=C	1500–1400 and 1600–1585	1448.83–1635.49	C=C presence of aromatic	
C–H	2962–2853	2944.78	C–H presence of aliphatic	
C–O	1310–1250	1252.74	C–O presence of aromatic	

Table 7: List of bioactive compounds analyzed by GC–MS from isolates of callus of Sumatran Frankincense based on the proximity of the library database identification.

Peak	t_R	Bioactive compounds	Formula	Quality (%)*
1	7.079	Vanillin	$C_8H_8O_3$	95.47
2	10.228	Isopropyl myristate	$C_{17}H_{34}O_2$	92.57
3	10.9	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	90.96
4	12.363	9-Octadecenoic acid	$C_{18}H_{34}O_2$	89.26
5	25.929	Piperine (2E, 4E)-5-(benzo[d] [1,3]dioxol-5-yl)1-(piperidin-1-yl) penta-2,4-dien-1-one	$C_{17}H_{19}NO_3$	91.62

*The quality of compound similarity to the library database is > 90%.

the greater the quantity of callus [46]. The experimental design ensured that the best composition was obtained in I_3N_3 supplemented with 3 mg/L IBA and 3 mg/L NAA. In this condition, all plant callus grows perfectly and is used for callus production as raw material for isolating alkaloids.

The alkaloid isolation protocol from Sumatran Frankincense callus has been obtained through maceration using ethanol, producing a concentrated isolate that is reddish brown to black in color. Purification of isolates by solvent extraction, namely through salting and extraction using organic solvents, is effective in separating and recovering alkaloids from the mixture [47, 48]. The purity level is 73%. The alkaloid purification process using column chromatography fractionation with step gradient polarity has succeeded in obtaining pure alkaloids. The isolated product was identified as qualitatively positive for alkaloids [49]. The pure compound is in the form of a yellowish to brown crystalline powder, with an aromatic odor, soluble in chloroform and alcohol, and insoluble in water. Alkaloids have a density of 1193 g·cm^{-1}, a melting point of 129°C, and a boiling point of 307°C [50, 51].

The quantity of alkaloids contained in the Sumatran Frankincense callus was 2.11% and consistently gave relatively the same results for three replications. The quantity of alkaloids in Sumatran Frankincense callus is very low when compared with the alkaloid composition in Sumatran Frankincense sap of 22.79% using the same isolation protocol. However, these findings are very significant and promising for the potential of in vitro culture in producing alkaloids.

Several successes in producing bioactive compounds by in vitro plant culture have been reported, namely using cell culture, plant organ culture, and plant cell suspension [28, 29]. In vitro culture allows for engineering plants to produce chemical compounds for pharmaceutical and industrial purposes [52]. Tissue culture techniques are very advantageous because production is predictable, scalable, stable, and sustainable throughout the year. The process of extraction and purification of chemical compounds is easy and directed. Plant tissue culture can also guarantee the independence of bioactive production without being affected by geographical conditions and allows for the elimination of dependence on the quantity of parent plants.

Pure alkaloid compounds resulting from fractionation have been identified qualitatively (FT-IR and GC-MS) to screen the names of the main chemical compounds, namely based on identification using the closeness of compound quality compared to the library database [53]. Five compounds were found, which were separated by GC and m/z fragmentation on the MS spectrum and combined with functional group markers and chemical bonds on the FT-IR spectrum. It was confirmed that the main compound was identified as piperine.

A screening approach using FT-IR and GC-MS was used in this study because the chemical structure elucidation stage was not feasible. This qualitative identification is efficient, rapid, and reliable in predicting the presence of chemical compounds. The identification data support the results of phytochemical analysis, chemical properties, and physical properties of the target compounds. However, it must be acknowledged that relying on the proximity of library databases carries identification uncertainty, especially if the experimental parameters for sample and standard analysis are not exactly the same.

The findings of this research are very convincing that alkaloids can be produced by culturing plant tissue. The in vitro technique of Sumatran Frankincense can be relied on to produce alkaloids. In vitro production of medicinal compounds is very prospective because it produces alkaloids in a relatively short time, which corresponds to the incubation time for callus formation in the culture medium. Meanwhile, the Sumatran Frankincense plant takes years to mature to produce bark sap, which contains chemical compounds [54]. It is demonstrated that chemical compounds contained in the parent plant can be produced at the callus level. Therefore, this in vitro technique can also be applied to other forest plants, especially those with high economic value, as long as they can be cultured to produce callus.

The findings of this study indicate that the content of chemical compounds in callus has the same pattern as in the bark sap of Sumatran Frankincense. These findings imply that the in vitro technique of Sumatran Frankincense has a dual function, as plant propagation to produce good quality seeds and medicinal substances at the callus level in a relatively short time. These two roles will have an impact on producing chemical compounds as medicinal raw materials that have high economic value and also save plants from damage due to overexploitation. In vitro techniques can replace the role of tapping in producing sap from Sumatran Frankincense plants as long as the plant's health is guaranteed and forest sustainability is also maintained. The Sumatran Frankincense in vitro technique protocol and the alkaloid isolation technique from callus raw materials are ways to produce medicinal substances from woody forest plants. This strategy prevented plants from being damaged due to bark sap tapping, and the forest plants can grow sustainably for forest conservation.

Further studies need to be carried out to increase the quantity of alkaloids in callus cultures and to reveal the process of bioactive compound formation in callus cells. To increase the quantity of alkaloids, it is necessary to consider the variety of explant types in *in vitro* techniques. Several plant materials such as leaves, stems, roots, and flowers can be used as explant sources [55]. At this stage, alkaloid production is carried out using young leaf explants with varying concentrations of growth regulators (IBA and NAA) to obtain the largest quantity of callus [31].

To produce alkaloids in large quantities, the strategy can be achieved using biotechnological methods such as tissue culture and gene transfer in genetic engineering [56], using biotic and abiotic elicitors, precursors, and light modulation [57–59]. Revealing the contribution of genes in the process of forming bioactive compounds is also needed. Transcriptomic and metabolomic studies have proven that the culture method will influence gene expression and the formation of secondary metabolites [60, 61]. To explain the process of formation of bioactive compounds in *in vitro*

culture, combining this technique will provide an overview of the biological processes in the alkaloid production process in *in vitro* propagation of Sumatran Frankincense [62, 63]. Comprehensive studies are still needed to reveal the role of RNA active in cells during the formation of secondary metabolites and to provide insight into the contribution of genes in alkaloid biosynthesis in callus cells during in vitro plant propagation [64].

5. Research Limitations

The research has limitations in two aspects, namely the technical capacity to implement in vitro techniques and the dependence on identification using GC-MS. The small capacity of the LAF in this study resulted in the in vitro technique being carried out repeatedly to obtain large quantities of callus raw material. The low quantity of alkaloids contained in callus is also a technical obstacle that causes the isolation of alkaloids from callus raw materials to be carried out repeatedly to obtain pure compounds in large quantities. The obstacle in identifying chemical compounds to confirm the names of chemical compounds contained in alkaloids still relies on the proximity of the identification quality to the GC-MS database library. The findings of in vitro protocols and isolation of alkaloids have confirmed the potential of in vitro techniques to produce medicinal substances in a relatively short time. Implementing the findings of this protocol will maintain plant health and ensure forests remain sustainable.

6. Conclusion

An in vitro technique to produce bioactives from Sumatran Frankincense callus was successfully carried out to produce pure alkaloids with medicinal properties. Good quality callus raw material was induced from Sumatran Frankincense leaf explants in MS media containing 3 mg/L IBA and 3 mg/L NAA (I_3N_3) for 7 weeks, resulting in the highest callus weight (0.426 ± 0.117 g). Isolation stages included maceration of 6.28 ± 0.04 g of dry callus in ethanol, resulting in a crude isolate of 4.56 ± 0.34 g (yield 73%), followed by acid–base solvent extraction to recover alkaloids to a purity of 95.84% and fractionation to produce 1.53 ± 0.22 g of alkaloids (yield 2.11%). The alkaloid yield in callus was relatively small compared to the alkaloid yield in frankincense resin of 22.79%. The compound in the form of yellow to brown powder has a distinctive aroma as piperine ($C_{17}H_{19}NO_3$). Phytochemical analysis showed that the alkaloid is soluble in chloroform and alcohol, is insoluble in water, and has a melting point of 129°C, boiling point of 307°C, and density of 1193 g·cm^{-1}. The results of FT-IR and GC-MS analysis, compared to the closeness of identification in the library database (quality 91.62%), confirmed the alkaloid as piperine, (2E, 4E)-5-(benzo[d] [1, 3]dioxol-5-yl)1-(piperidin-1-yl)penta-2,4-dien-1-one.

This study demonstrates the potential of Sumatran Frankincense tissue culture to produce alkaloid compounds. However, the alkaloid content in the callus is still very low compared to the alkaloid content in the bark sap. Studies of in vitro bioactive production protocols need to be improved due to their significant potential for producing alkaloids in a relatively short time. The findings of this study provide an appropriate strategy for saving the health of woody medicinal plants from overexploitation that compromises plant sustainability, while also preserving forests and the environment. Further studies are needed to uncover the potential for producing other medicinal compounds at the Sumatran Frankincense callus level. These chemical compounds are essential as raw materials for pharmaceuticals and industry.

7. Research Implications and Recommendations

The research findings have direct practical implications, including the use of in vitro techniques on woody medicinal plants to produce medicinal chemical compounds. This protocol will increase the production of natural alkaloid compounds without damaging forest plants. The economic feasibility of producing alkaloids through plant tissue culture is considered high because it allows for the production of medicinal chemical compounds in a short time. Sumatran Frankincense trees take 10 years from seedling to producing frankincense resin containing alkaloids. Producing alkaloids from Sumatran Frankincense cultures is considered environmentally friendly and reduces plant exploitation, thus preserving forests.

To save medicinal plants from potential extinction, in vitro techniques for producing bioactive compounds are recommended. This protocol will meet the need for medicinal raw materials, protect woody forest plants from overexploitation, and serve as a strategy for maintaining sustainable forests.

Data Availability Statement

Complete research data and FT-IR and GC-MS analysis results are available from the author.

Conflicts of Interest

The authors declare no conflicts of interest.

Funding

Funding for this research was obtained from the Directorate of Research and Community Service, Directorate General of Research and Development Strengthening, Ministry of Research, Technology, and Higher Education of the Republic of Indonesia, Fiscal Year 2025, based on Regular Basic Research (Contract No. 28/UN5.4.10.K/PT.01.03/KP-DPPM/2025).

Acknowledgments

Thanks are extended to Panal Situmorang, the owner of the Frankincense forest in Aek Nauli II Village, Pollung District, Humbang Hasundutan Regency, North Sumatra, Indonesia,

who has provided his community forest land as a place for this research.

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