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Induction of *In Vitro* Shoots in Liverwort Gametophyte Explant *Acrolejeunea fertilis* (Reinw., Blume & Nees) Schiffn. and its Comparative Metabolite and Bioactivity Analysis

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ABSTRACT

Plant-derived natural products, including those from bryophytes, are particularly advantageous in several aspects, as for antifungal, antibacterial, and anticancer properties. However, the bioactivity potential of secondary metabolites found in the liverwort species *Acrolejeunea fertilis* (*A. fertilis*) has not been extensively investigated; moreover, it has limited biomass in nature. An *in vitro* culture can be a solution for the propagation of *A. fertilis*. The application of plant growth regulatory substances with various concentrations is expected to increase the growth of *A. fertilis*. This study aimed to determine the optimum concentration of 2,4-dichlorophenoxyacetic acid and kinetin applied to the gametophyte phase of *A. fertilis in vitro* culture. This study also aimed to compare the metabolite profile between *A. fertilis* from nature and *in vitro* culture condition, using methanol and n-hexane solvent. The compounds in the extracts were identified using gas chromatography-mass spectrometry (GC-MS) also their antioxidant and antibacterial activities. The IC₅₀ values for nature and *in vitro* extracts were in the range of 58.41–63.10 µg/ml and 111.73–112.10 µg/ml. The total value of phenolic compounds in *A. fertilis* extracts ranged from 58.51±0.02 µgGAE/g and 272.53±0.02 µgQE/g, respectively. The antibacterial activity revealed inhibition on *S. aureus* using an n-hexane *A. fertilis* from nature. Thus, this study highlights the diverse secondary metabolite profiles and bioactivity potential of *A. fertilis* extracts. These findings have important implications for the development of natural products for medicinal and mass propagation in terms of the potential of *A. fertilis* as a source of bioactive compounds.

Keywords: antioxidants, *Acrolejeunea fertilis*, *in vitro* culture, phenolics, shoot induction

Introduction

Based on its classification, bryophyte consists of mosses, liverworts, and hornworts. *Acrolejeunea fertilis* (*A. fertilis*) (Reinw., Blume & Nees) Schiffn. is a leafy liverwort that belongs to the *Lejeuneaceae* family and the *Ptychantoideae* sub-family (Gradstein, 1991; Wang *et al.*, 2014). These liverworts can be found in several countries, such as Indonesia, Malaysia, the Philippines, Thailand, India, and Sri Lanka. In Indonesia, *A. fertilis* grows on the islands of Sumatra, Java, Kalimantan, Maluku, and Papua (Kornochalart *et al.*, 2012; Haerida *et al.*, 2015). According to Ah-Peng *et al.* (2007), areas with average to high humidity, exposure to sufficient amounts of sunlight, and only moderate rainfall intensity are ideal habitats for the growth of *A. fertilis* liverworts, with one such location being the area around the campus of the University of Indonesia in Depok, West Java. *A. fertilis* grows epiphytically with no attachment to the ground or any other obvious nutrient source. As an air plant, this liverwort can grow on another plant or object merely for physical support, like on the substrate of the ketapang (*Terminalia catappa*) and *Diospyros* sp. trees. According to Sidhu *et al.* (2020), the common environmental conditions needed for its growth are light intensity ranging from 375 µmol.m⁻².s⁻¹–555 µmol.m⁻².s⁻¹, an average temperature of 18°C–22°C, and a humidity percentage of 80 ± 5% (Vujicic *et al.*, 2010), which is suitable for its growth.

Liverworts have various ecological and biochemical benefits. Ecologically, epiphytic liverworts play the role of air bioindicators through their ability to indicate nitrogen levels in the air and air pollution due to their sensitivity to environmental conditions. Biochemically, liverworts also have the potential to produce secondary metabolites as a natural product that can be used in antifungal, antibacterial, anticancer, and antitumor agents (Nurhaeni *et al.*, 2019). According to Gradstein *et al.* (2013), *A. fertilis* contains terpenoid compounds and flavonoid bioactive compounds. However, research related to the compounds contained in *A. fertilis* and the potential for the activity of its compounds has not been further conducted due to the constraints related to obtaining sufficient biomass in nature. Therefore, an *in vitro* culture could be a solution for the propagation of *A. fertilis* biomass. An *in vitro*

culture is a technique of plant propagation based on the principle of totipotency, which refers to the ability of somatic cells to grow and develop into new individuals (Feher, 2019). Numerous *in vitro* cultures have been developed by utilizing various types of plants as explants, particularly mosses (Chen *et al.*, 2009). Previous studies used the sporophyte phase of mosses, which leads to explant growth in culture media. The growth of an explant involves the growth of a protonema, callus, and shoot. The use of liverwort and its gametophyte phase as an explant has not been explored much in extant literature. The application of *in vitro* culture techniques to liverworts can be useful in the study of their morphological and physiological characteristics.

More recently, such studies on liverwort *in vitro* culture were also conducted by Chen *et al.* (2009), who used Murashige & Skoog (MS), Knop, and White media; Makinde *et al.* (2014) who used MS media; and Tan *et al.* (2020) who used MS and Gamborg B5 for culture media. In the previous studies by Chen *et al.* (2009), Makinde *et al.* (2014), and Tan *et al.* (2020), all explants used were in the form of the sporophyte phase for moss and liverworts, with the addition of plant growth regulators (PGRs) in the culture media. Previous studies used the sporophyte phase for *in vitro* moss culture with an established method for the sterilization process and already achieved axenic condition for the explants. However, it is rather uncommon to obtain the sporophyte phase of a liverwort since it is not found in nature and its emergence lasts only for three or four days. Therefore, other types of explants, such as the gametophyte phase, can be an alternative explant source for the *in vitro* culture of liverworts. The use of the gametophyte phase as an explant as well as its sterilization method has not been widely observed. The acquisition of the gametophyte phase under axenic conditions is very difficult to accomplish; in comparison, since the sporophyte phase is protected by the capsule, the sterilization process for achieving an axenic condition is much easier. Meanwhile, the gametophyte phase is exposed to nature and the intense interaction with microorganisms requires more effort for the sterilization process to achieve an axenic condition.

PGRs can affect developmental or metabolic processes in plant tissues cultured *in vitro*. The application of PGRs on liverworts *in vitro* culture, such as auxin and cytokinin, at optimum concentrations could induce explant elongation as well as the formation of shoot, roots, or calluses. A PGR known to have an optimum growth result is a combination of 2,4-D and kinetin. Kinetin is the most active and effective type of cytokinin in inducing shoots in explants (Abu-Rohman *et al.*, 2016). The addition of 2,4-D in small amounts is capable of increasing growth responses, while large amounts have the potential to function as herbicides (Peterson *et al.* 2016). Based on research by Chen *et al.* (2009), the 2,4-D concentration range of 0–1 mg/L in moss *in vitro* culture will result in the growth of shoots. According to previous studies, a kinetin concentration of 1 mg/L can induce callus and protonema (Chen *et al.*, 2009; Makinde *et al.*, 2014). These results were obtained using the sporophyte explants and, thus, the effect on gametophyte explants has not been much studied. Research on *in vitro* culture using gametophyte explants of *A. fertilis* liverworts has been previously conducted by Putri *et al.* (2021), who reported 20% induced shoots from explants in ½ MS culture media without any addition of PGRs.

In this research, we modified the culture media by adding 2,4-D 0–1 mg/L and kinetin 0–2 mg/L as the PGRs, which has never been done before. The purpose of this study was to determine the response of *A. fertilis* liverwort explants to 2,4-D and kinetin as well as their optimum concentration to support the induction of shoots in the gametophyte explant. The addition of 2,4-D and kinetin with the combination given in culture media tend to increase the growth of shoots. Furthermore, we also analyze the secondary metabolites of the *Acrolejeunea fertilis* and evaluate their potential bioactivities, including their antioxidant and antimicrobial effects.

Materials and Methods

1. Explant collection and identification

The leafy liverwort *A. fertilis* for *in vitro* culture explant was collected from the *Diospyros discolor* tree (Figure 1) in the area around Universitas Indonesia in Depok, Jawa Barat, Indonesia, and confirmed through identification in Herbarium Depokensis (DEP) at Universitas Indonesia. Its identification was based on the presence and amount of a teeth-like structure on its lobule. The presence of its Massula-type oil body is also crucial for the identification of *A. fertilis*. These structures were observed under a light microscope [Leica DM500].

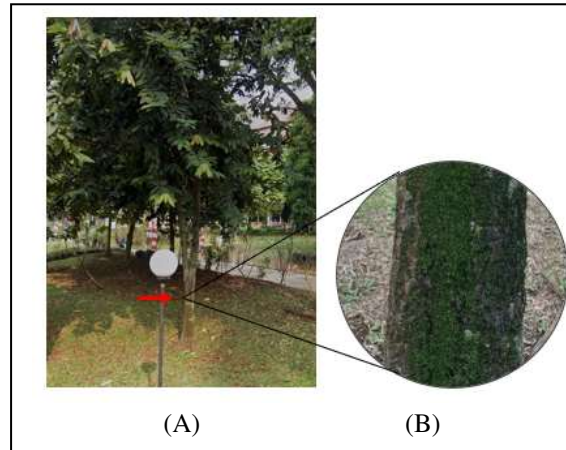


Fig. 1 (A) Sampling location of *Acrolejeunea fertilis* as explants, (B) *A. fertilis* grows epiphytically on *Diospyros discolor* tree barks

2. Explant sterilization

The sterilant used in this study consisted of detergent 0.4% (v/v), disinfectant (alcohol 35% (v/v), and commercial bleach [Natrium Chloride (NaOCl)] 1.25% (v/v)), fungicide (dithane) 2% (w/v), and antibiotics (Tetracycline) 0.5% (w/v). The anatomy of the explant was observed after sampling, washing using detergent 0.4%, and after completion of the entire sterilization process to evaluate the effect of each sterilization process to the cell of the explant, since its structure is small and fragile. The sterilization process was performed in the Laminar air flow cabinet (LAFC) [Type HS087], and only sterilization using detergent was performed outside the LAFC. In the first step of the sample sterilization process, whole explants were washed under tap water for three minutes to remove tree bark and dirt. Before washing with detergent, the explants needed to be cut to a size of ± 1 cm using a scalpel [Renz No. 3]. The explants were then washed using detergent and then sonicated [Cole-Parmer Instrument Company PC620E-1] for five minutes. Then, the explants were placed in the LAFC and sterilized in alcohol for 30 seconds, in disinfectant for 3 minutes, in fungicide for 5 minutes, and antibiotics for 5 minutes, respectively. After each step of the sterilization process, samples were rinsed using sterile water three times for a duration of five minutes each. This was done to ensure that there were no chemical compounds on or inside the samples. After the entire sterilization process was complete, samples were placed on a sterile petri dish and cut to precisely 1 mm using the millimeter block as a measurement tool. Thereafter, samples were placed in sterile water to be planted in culture media.

3. *A. fertilis* in vitro culture

All explants were then planted in $\frac{1}{2}$ MS culture medium with macro and micronutrients, amino acids, vitamins, and mineral salts [PhytoTechnology Laboratories], and various concentrations of 2,4-D and Kinetin were added, as shown in Table 1. The culture media was mixed with agar [Swallow Globe] to make the texture compact. Explants were then cultured for 50 days. There were 14 bottles for each treatment, with each bottle comprising three gametophyte explants of *A. fertilis*. The placement of the explant in the culture media is depicted in Figure 2. Every seven days, observations were made for each bottle and each treatment. The observations included the explant's color, growth indicator (i.e., the formation of shoots), number of shoots formed, shoot length, and whether any contaminants had appeared on the culture media.

Table 1 Treatments of PGRs used for explants (each treatment included $n = 14$).

2,4-D (mg/L)	Kinetin (mg/L)			
	0	0.1	1.0	2.0
0	P0	P1	P2	P3
0.1	P4	P5	P6	P7
1.0	P8	P9	P10	P11



Fig 2 Position of the *A. fertilis* explant in ½ MS culture media

4. Morphological observations

Explant colors were observed using a smartphone application (Color Grab on iPhone 11) and its color gradient categories were based on the Sherwin–Williams color grading[. Then, several explants were taken for further observation under a stereo microscope [Olympus LS], with magnification varying from 0.5x to 4.0x. This step was necessary to confirm whether the color analyzed by the application was accurate. Thereafter, several lobes were cut and taken to be observed under a light microscope [Leica DM500]. This step was important for observing the cell structure to determine the presence of an oil body, chloroplast condition, and trigon structure. The magnifications used were 1000x.

5. Sample Extraction and Metabolite Profiling

The extraction process for *A. fertilis* liverwort begins with cleaning the sample from remaining impurities, which can be tree bark (for origin sample) or in the form of culture media (for *in vitro* sample). The liverwort is washed under running water and then rinsed with sterile distilled water. The *A. fertilis* samples were then dried using the air-dry method for ± 3 days until its dry weight attained a constant value. The sample was then weighed at 0.1 gram and put into a falcon tube into which 100 ml of methanol and n-hexane was added, thereby yielding a concentration of 1 mg/ml, which is equivalent to 1000 ppm. The *A. fertilis* samples were then crushed using a TissueRuptor (Qiagen). The sample was then sonicated for 2×10 minutes to ensure that the moss sample had been completely destroyed. Then, the sample was centrifuged at 3000 rpm for 20 minutes and the supernatant was evaporated. Thereafter, 0.01 grams of methanol extract was diluted in 10 ml methanol to obtain the final concentration of 100 ppm. The supernatant was then transferred to a 25 ml vial tube and stored at 4°C for further use.

6. Gas Chromatography-Mass Spectrometry (GC-MS) Assay

The method for GC-MS is that given by Sukharak *et al.* (2011), which is specific to the *Lejeuneaceae* family. The stages for analysis using GC-MS begin with the filtering process of 2 ml of n-hexane extract that was diluted with methanol using a syringe and placing it into a specific vial. The GC-MS machine was tuned before inserting the sample for the analysis process, which includes an oven temperature of 50°C, an initial hold time of three minutes, and then setting the temperature at 250°C, with an increase of 5°C per minute and a hold process of 15 minutes after reaching a temperature of 250°C. The sample was injected at a temperature of 280°C and a helium flow rate of 1 ml per minute. The electron impact mode was 70eV with three scans. The temperature set is 230°C, with a mass range (m/z) of 40–500. The machine running process is ± 2 hours and the results obtained will be analyzed based on retention time and spectrum data using the latest version of the Wiley Library.

7. Total Phenolic Content

The method used to calculate total phenolic compounds (TPC) is the Folin-Ciocalteu method, and the number of repetitions was five. First, 200 μ l of liverwort extract *A. fertilis* methanol and n-hexane extract (concentration 12.5 ppm, 25 ppm, 50 ppm, 100 ppm) mixed with 120 μ l of Folin-Ciocalteu reagent and left at room temperature for 6 minutes. Thereafter, 80 μ l of Na_2CO_3 was added to the mixture. The mixture was then incubated in the dark for 120 minutes, and then the absorbance was measured using a spectrophotometer at a wavelength of 750 nm.

Thereafter, the total phenolic compounds were calculated and expressed as milligrams of gallic acid equivalent per gram of dry weight (Wang *et al.*, 2017). The following formula was used to calculate total phenolic compounds:

$$TPC = \frac{C.V.df}{gr}.$$

C = Phenolic concentration (µg/ml)

V = Extract's volume (ml)

df = Dilution factor

gr = Weight of the extract

8. Total flavonoid content

The total flavonoid compounds (TFC) were measured by mixing 200 µl of liverwort *A. fertilis* methanol and n-hexane extract, and the concentrations varied from 12.5 ppm, 25 ppm, 50 ppm, to 100 ppm with 215 µl of 80% methanol, 5 µl of 1 M CH₃COOK, and 5 µl AlCl₃.6H₂O 10% in each well. The mixture was then incubated at room temperature for 40 minutes. Thereafter, the wavelength was measured using spectrophotometry at a wavelength of 415 nm, with five repetitions. The total flavonoid compounds are expressed as milligrams of quercetin equivalent per gram of dry weight (Wang *et al.*, 2017). The following formula was used to calculate TFC:

$$TFC = \frac{C.V.df}{gr}.$$

C = Flavonoid concentration (µg/ml)

V = Extract's volume (ml)

df = Dilution factor

gr = Weight of the extract

9. Antioxidant activity

The method used to determine the antioxidant activity of *A. liverwort* extract is the DPPH method, and the number of repetitions was five. The process began by mixing each sample extract with 2,2-Diphenyl-1-picrylhydrazyl (DPPH), varying concentrations ranging from 125 ppm, 250 ppm, 500 ppm, to 1000 ppm with 0.5 ml of 0.1 mM DPPH. The mixture was then incubated in a dark room for 60 minutes. Then, absorbance was measured at a wavelength of 517 nm using spectrophotometry. The blank used was methanol and for control a mixture of 0.5 ml methanol and 0.5 ml 0.1 mM DPPH was used. Finally, the percentage of antioxidant activity and IC₅₀ value was calculated. The IC₅₀ value was determined by analyzing the linear regression curve. The expected R² coefficient value was above 0.99 and its categorization is in accordance with Reviana *et al.* (2021).

10. Antibacterial assay

The antibacterial assay was performed using the diffusion well method, in which the inhibition zone on the agar media was observed. *Staphylococcus aureus* (Gram-positive) and *Escherichia coli* (Gram-negative) were used as preculture for one night using nutrient broth (NB) [Himedia] media. An antibacterial test was performed using the Mueller Hinton Agar (MHA) [Difco] medium. As a positive control, tetracycline was used in a concentration of 7 µg/ml, while as a negative control, dimethylsulfoxide (DMSO) was used; for the treatment, each extract was used in a concentration of 100 ppm. The bacterial inoculum was adjusted to 0.5 McFarland standard as a reference to adjust the turbidity of the bacterial suspension, equivalent to 1.5 x 10⁸ CFU (Kralik *et al.* 2012). Thereafter, 10 µl of the inoculum was added to 10 ml of the medium and mixed gently. Then, the medium with the inoculum was poured over the previously hardened medium and allowed to dry before use. In all three treatments, the medium was perforated using a 6-mm diameter of cylinder to form a well and then 100 µl of the extract was loaded to each well. The culture was then incubated at 32°C for 16 h in the dark. Thereafter, the clear zone was observed and measured using a caliper. The measurement results were then categorized referring to Sarker *et al.* (2014).

11. Data analysis

Qualitative data—such as anatomy structure right after sample collection, after washing using detergent, and after sterilization—and the presence of contamination were then descriptively analyzed. The quantitative data consisted of the number of responses of the explant to forming shoots, the variation in the number of shoots from each explant, the average shoot length for each treatment, IC_{50} value, and the total phenolic and flavonoid compounds; moreover, the diameter of the inhibition zone was measured. All quantitative data were then analyzed using one-way analysis of variance (ANOVA), followed by a Duncan multiple range test (DMRT). The data analysis was conducted using SPSS (version 24).

Results and Discussion

1. The effect of sterilization on *A. fertilis*' explants

The process of sterilization is known to have various effects on the structure of explants, including the color and anatomy condition of explants. The color of the explants was observed at three different stages: immediately after the explants were taken from the substrate, after being washed using detergent, and after sterilization using several chemical compounds. As depicted in Figure 3, the color of the explants observed right after sampling was paradise. After washing using detergent, the same color was observed, thereby indicating that 0.4% detergent had no effect whatsoever on explant pigmentation. This finding is in accordance with the results reported by Ramadhani *et al.* (2023). The observations of explants sterilized using various chemical compounds revealed that the explants could be divided into two dominant color groups, which are paradise and verdant, immediately before planting (D0—day 0 of planting). Verdant indicates a darker green-to-brown color compared to Paradise, which is a bright green color.

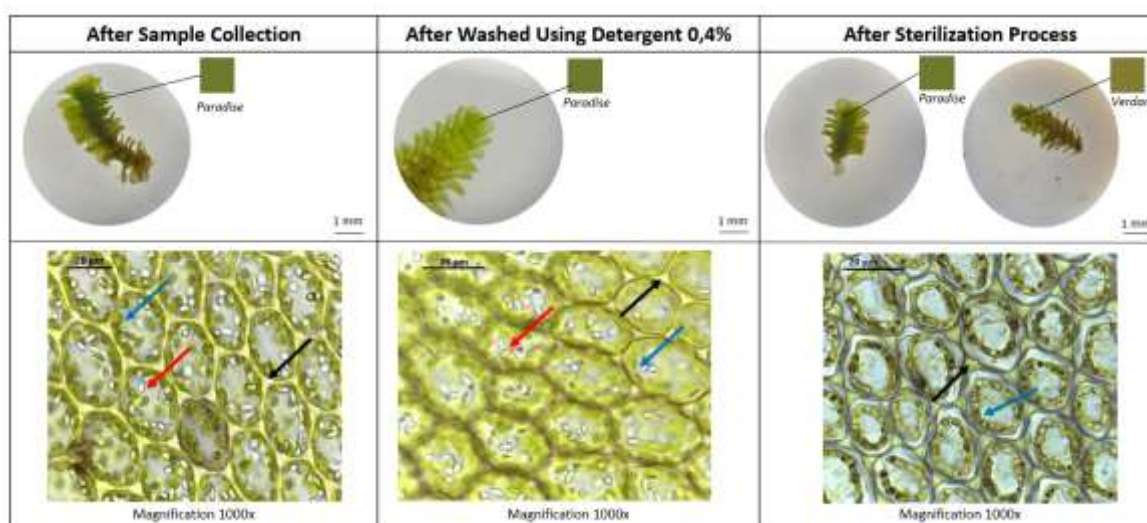


Fig 3 The anatomical-morphological structure of *A. fertilis* explants during the sterilization process.

The red arrow indicates the oil body, the blue arrow indicates chlorophyll, and the black arrow indicates the trigon observed on cells; magnification of 1000x was used.

According to Figure 3, the color of the explants after the entire sterilization process tends to be brown, which is indicated by verdant. The browning of the explants after the sterilization process may be caused by the exposure to alcohol and disinfectant. The gametophyte explant of *A. fertilis* has a rather fragile tissue, which are comprised only of a single layer of cell; hence, its cells are rather sensitive to the exposure to the disinfectant. Alcohol applied as a sterilant tends to cause browning of explants and damages the structure of cell membranes (Patra *et al.* 2006), as alcohol is known to be a hypertonic and phytotoxic solution (Mahmoud & Al-Ani, 2016). Based on our observations, an exposure to 35% alcohol for 30 seconds causes a semi-plasmolysis condition in the cell (Ramadhani *et al.*, 2023). Furthermore, a high concentration of alcohol can cause cell dehydration, which causes browning of the explants. The thinner and more fragile the tissue of the explant structure, the more easily it will be damaged by exposure to alcohol. Based on observations of cells through a microscope after sampling and after

washing with 0.4% detergent, the visible structures were hexagonal cells, sturdy trigons, chloroplasts, and oil bodies scattered in the cell compartment. After the explants were exposed to 35% alcohol for 30 seconds, changes were observed in the cell structure of the *A. fertilis* explants. Based on this observation, the chloroplasts that were previously scattered in cell compartments had become clustered around the cell membrane. This could have occurred due to the extraction of water contained in the cell, because the concentration of fluid outside the cell was higher than that inside the cell. While the cells in the liverwort explants of *A. fertilis* had not yet entered the plasmolysis stage, they did experience contraction in their cytoplasmic fluid (Lang *et al.*, 2014; Mahmoud & Al-Ani, 2016).

According to Putri *et al.* (2021), a commercial bleach concentration of 1.50% can cause severe browning of explants on D0 and bleaching of explants on D30 (30 days after planting). Browning conditions in explants may result in low growth rate and even cause death (Wang *et al.*, 2016). Further, explants sterilized using higher concentrations of disinfectant can be vulnerable to stress and produce further phenolic compounds. Phenolic compounds, generally polyphenols, cause the explants to become brown. They can activate polyphenol oxidase enzymes and become precursors to quinone through an oxidation process. Furthermore, quinone will gradually penetrate the tissue in the explants into the culture media, thereby suppressing enzyme activity. Compounds contained in the media become toxic and result in a lower survival rate of explants (Sukanto, 2011; Taghizadeh & Dastjerdi, 2021). The addition of antioxidant ingredients, such as polyvinyl pyrrolidone (PVP) and activated charcoal, can suppress the oxidation of phenolic compounds and inhibit the browning of explants (Taghizadeh & Dastjerdi, 2021). In addition, reducing the concentration of disinfectant from 1.50% to 1.25% resulted in less damage to the tissue of the explant and also reduced the contamination.

As depicted in Figure 4, no contamination was observed during the first seven days of the culture period. Fungi and bacteria contaminants were observed on the 14th day, while algae contaminants were observed on the 28th day. Generally, contamination in explants is located externally and does not affect explant growth. The most common type of contamination is fungi. As depicted in Figure 4A, the percentage of fungi contamination reached 18.45% up to 50 days after planting. The bacterial contamination rate was 11.90% and algae contamination rate was 3.57%. According to Figure 4B, the cultures that were not contaminated constituted 66.08%. The percentage of contamination in this study decreased significantly when compared to the previous research conducted by Putri *et al.* (2021), who reported a rate of contamination of approximately 90%. This reveals that the sterilization procedure conducted in this study was better than the previous study.

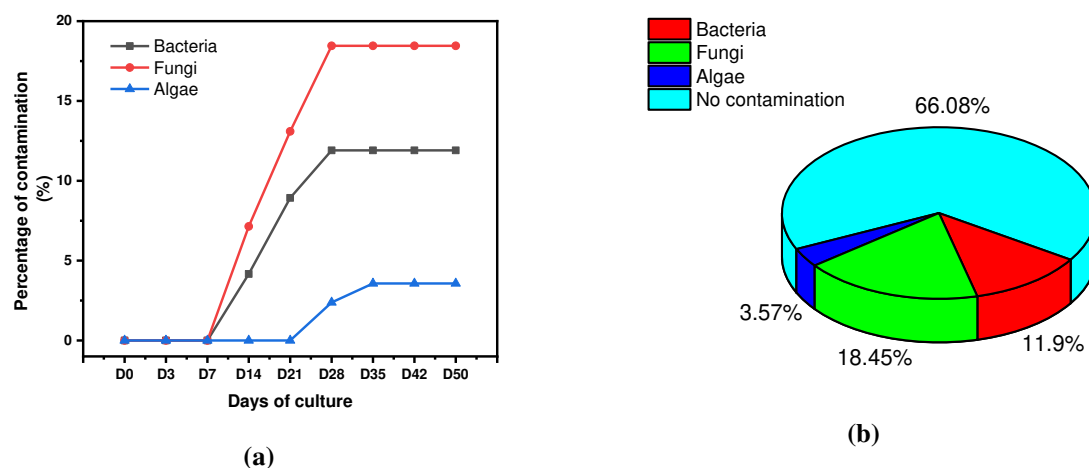


Fig 4 (a) Types and amounts of contamination that occurred each week from all treatments on *A. fertilis* liverwort explants; (b) percentage of explant contamination over the 50 days of culture (n = 168; n is the total number of culture bottles).

2. Shoot induction from *A. fertilis* gametophyte explant

The observation of *A. fertilis* *in vitro* culture lasted for 50 days, which indicated that the gametophyte explant was forming shoots (Figure 5). Each treatment resulted in various number of shoots. According to Figure 6, controls (0 mg/L 2,4-D and 0 mg/L kinetin) began to respond on the twenty-first day after the culture. Explants with treatments responded on the seventh day after the culture. The fastest growth rate was observed in explants treated with P1 (0 mg/L 2,4-D and 0.1 mg/L kinetin)—on day 50, the growth rate was 35.71%; this was followed by P9 treatment (1 mg/L 2,4-D and 0.1 mg/L kinetin), in which the growth rate was 30.95%. The lowest average response growth rate was observed in explants treated with P10 (1 mg/L 2,4-D and 1 mg/L kinetin) and P11 (1 mg/L 2,4-D and 2 mg/L kinetin), which was 4.76%. When compared to the control, it was observed that the application of 2,4-D and kinetin in ½ MS culture media triggered a better response in shoot formation. Referring to the result of this study, it is evident that the combination of 0–1 mg/L 2,4-D and 0.1 mg/L kinetin yields a better result in terms of shoot formation. In contrast, auxin acts synergistically with cytokinin to control the shoot stem-cell niche, while both hormones act antagonistically to maintain the root meristem. The higher amount of 2,4-D given in this combination should have led to root formation, but instead it led to the formation of a shoot. According to studies by Ashton *et al.* (1985) and von Schwartzenberg *et al.* (2007), the amount of endogenous auxin and cytokinin in *Physcomitrella patens* is 0.075 µg/g dry weight and 19.8 µg/g—426.36 µg/g dry weight, respectively. This might explain that the formation of the shoot in the *A. fertilis* culture is due to the higher endogenous cytokinin hormones in *A. fertilis*

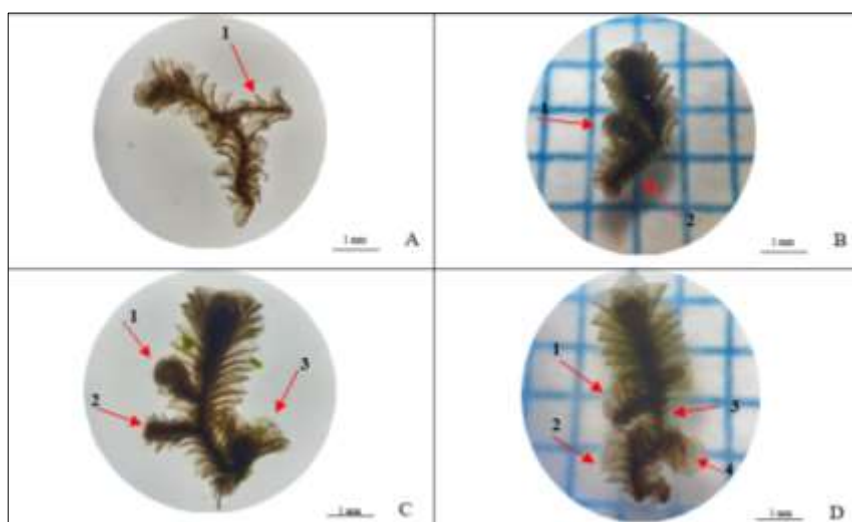


Fig 5 Variation in the number of shoots growing on explants from each treatment

(A): one shoot induced (n = 48; (B): two shoots induced (n = 14); (C): three shoots induced (n = 5); (D): four shoots induced (1)

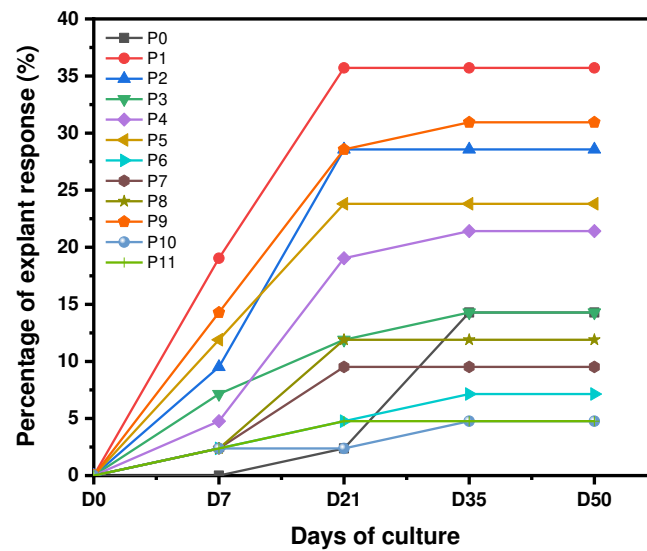


Fig 6 Percentage of explants that responded to PGRs used by a forming shoot in each treatment (n = 42 explant for each treatment).

Based on a one-way ANOVA statistical analysis (sig. 0.000 < α 0.05) there was a significant difference in the percentage of explants that formed shoots between control and treatments. Referring to the results of the post-hoc test used in this study in Figure 7, which is the Duncan multiple range test (DMRT), it was statistically proven that P1 (0.1 mg/L kinetin) and P9 (1.0 mg/L kinetin and 1.0 mg/L 2,4-D) were considered the optimum concentrations to enhance *A. fertilis*, while P10 (2,4-D 1 mg/L and 1.0 mg/L kinetin) and P11 (2,4-D 1 mg/L and 2 mg/L kinetin) were not recommended in accordance with their lowest growth rate until day 50.

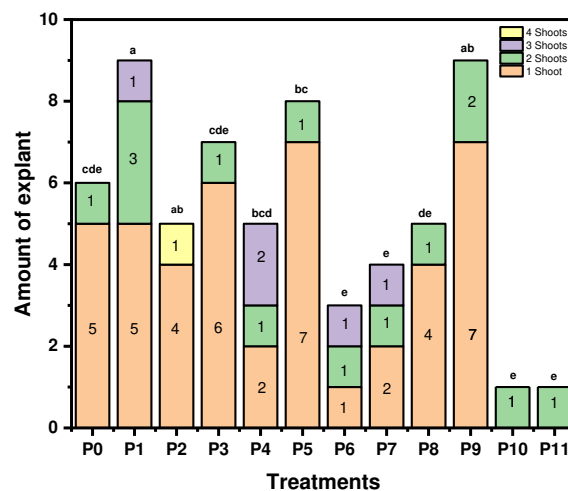


Fig 7 Number of explants that formed shoots in each treatment 50 days after culturing. N represents the total replications for each treatment (N—total explants cultured = 504; n—explants for each treatment = 42).

There was also a variation in the shoot length of each explant. As depicted in Figure 8, the longest average shoot length was observed in explants treated with P2 (1.307 ± 0.38 mm). When compared with the control results, there were significant differences between the explants in the P1, P2, P5, and P9 treatments. Explants treated with P3, P4, P6, P7, and P8 had shoot lengths that were more or less the same as the controls. However,

the explants treated with P10 and P11 had significantly smaller shoot length compared to the controls. The different average shoot lengths are a result of the application of 2,4-D and kinetin at various concentrations.

Based on the one-way ANOVA statistical analysis ($\text{sig. } 0.000 < \alpha 0.05$), there was a significant difference in the number of explants that formed shoots between the control and treatment media. Referring to the results of the DMRT post-hoc test, it was statistically proven that P1 (0.1 mg/L kinetin) and P2 (1.0 mg/L kinetin) were the optimum concentrations to enhance the growth of *A. fertilis*, and P10 (2,4-D 1 mg/L and kinetin 1 mg/L) and P11 (2,4-D 1 mg/L and kinetin 2 mg/L) were not recommended according to their lowest average shoot lengths of only 0.136 mm and 0.071 mm, respectively. The addition of kinetin 2 mg/L yielded the lowest shoot length. Moreover, it was revealed that the optimum concentration of kinetin to be applied to the culture media is under 2 mg/L.

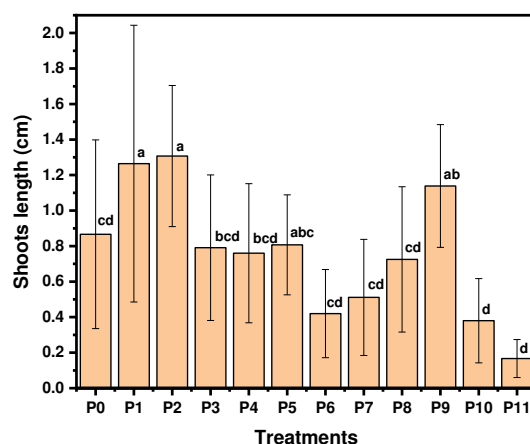


Fig 8 Average shoot length of *A. fertilis* explants from each treatment. (N—total explants planted = 504; n—explants per treatment = 42).

Based on the results of this study, the maximum concentration limit for the 2,4-D was 1 mg/L and that for kinetin was 2 mg/L. If any PGRs with a higher concentration were applied to the *in vitro* culture of *A. fertilis*, the possibility of the explants dying due to toxicity would have been much higher and the percentage of growth would also have been much lower. Toxicity can be triggered because endogenous hormone levels are available in large quantities and accumulate with the further addition of exogenous hormones. This finding is in accordance with research by Khan *et al.* (2015), thereby revealing that the application of PGRs such as BAP and 2,4-D with concentrations that are too high can cause death in explants. According to Kunikowska *et al.* (2013), PGRs from the group of cytokinin hormones—such as 6-benzylaminopurine (BAP), benzyl adenine (BA), and kinetin at high concentrations—can cause programmed cell death (PCD) in plants. PCD is defined as the genetically determined suicide of individual cells in response to pathogens and environmental stress during normal development and may be also exogenously induced. It is observed that cytokinin receptors (CK receptors) are involved in many physiological processes—such as seed germination, starch, and chlorophyll production, bud differentiation, and branching—but also play roles in CK-dependent PCD. These receptors could function as a multi-step phosphorelay system that functions like histidine kinase (HK) (Kunikowska *et al.*, 2013).

Cytokinins that regulate cyclin-dependent kinase activities control cell proliferation of cells, which arrests this proliferation at G1/S and/or G2/M transition points and, thus, triggers apoptosis. It is discovered that kinetin is converted with phosphoribosyl transferase to corresponding monophosphates, which are purine ligands specific to histidine kinase receptors (AHK, AHK2, and AHK3). Then, monophosphates that bind to receptors induce an efflux of calcium ions from the endoplasmic reticulum (ER) and directly or indirectly arrest cells in the G1 and G2 phases and direct them onto the PCD with DNA condensation, nuclei segmentation, and DNA degradation by nuclease (van Doorn 2011). According to Chen *et al.* (2009), the maximum concentration for 2,4-D is 1 mg/L, while Makinde *et al.* (2015) reported 5 mg/L as the maximum concentration for moss *in vitro* culture. According to Kowalczyk *et al.* (1997), the maximum concentration for kinetin is 1 mg/L, while the present study revealed that 2 mg/L is the maximum concentration for kinetin. However, the limits for every plant growth regulator differ

and are dependent on the physiological aspects of each species, such as growth hormone requirements and its response to environmental conditions.

3. Total phenolic content (TPC) and total flavonoid content (TFC)

The methanol extract of *A. fertilis* Depok extract had the highest value of phenol content, with 272.5 ± 0.02 $\mu\text{gGAE/gr}$ than from the *in vitro* culture with 58.51 ± 0.02 $\mu\text{gGAE/gr}$. Meanwhile, the n-hexane extract had a lower value of total phenolic content— 36.65 ± 0.02 $\mu\text{gGAE/gr}$ and 13.81 ± 0.02 $\mu\text{gGAE/gr}$ for original samples and *in vitro*, respectively. Meanwhile, the total yield of flavonoid compounds obtained for the methanol fractions of *A. fertilis* extract is consistent with the total phenolic compounds. The total flavonoid compounds identified for the methanol extract of *A. fertilis* Depok in the original sample extract was 8.90 ± 0.01 $\mu\text{gQE/gr}$ and 4.48 ± 0.01 $\mu\text{gQE/gr}$ in the *in vitro* culture. The total yield of flavonoid content for *A. fertilis* n-hexane extract was 5.32 ± 0.01 $\mu\text{gGAE/gr}$ and 2.94 ± 0.01 $\mu\text{gGAE/gr}$ for nature and *in vitro*, respectively. The results are summarized in Table 2.

Table 2 Total phenolic and flavonoid content of *A. fertilis* extract

Extract	Total Phenolic Content ($\mu\text{gGAE/g}$)	Total Flavonoid Content ($\mu\text{gQE/g}$)
Methanol from nature	272.53 ± 0.02	8.90 ± 0.01
Methanol from <i>in vitro</i> culture explant	58.51 ± 0.02	4.48 ± 0.01
n-hexane from nature	36.65 ± 0.02	5.32 ± 0.01
n-hexane from <i>in vitro</i> culture explant	13.81 ± 0.02	2.94 ± 0.01

These results support the assumption that the exposure of light intensity and UV index of *A. fertilis* in the habitat affects the phenolic content, with higher levels of flavonoids produced in response (Yildirim *et al.*, 2020; Martinez-Silvestre *et al.*, 2022). Furthermore, the low levels of total flavonoid compounds from original samples are the same with the study by Siregar *et al.* (2020), in which the detection of flavonoid compounds is difficult in the *Lejeuneaceae* family. These results support the assumption that the UV index of *A. fertilis* habitat affects the secondary metabolite content, with higher levels of flavonoids produced in response to the adaptation to a high UV environment (Yildirim *et al.*, 2020; Martinez-Silvestre *et al.*, 2022). The UV index recorded is in the range of 9–10. According to World Health Organization (WHO), a UV index in the range of 9–11 is categorized as rather high, which leads to higher accumulation of the phenolic compound.

Phenolic compounds are produced by plants mainly for their own growth, development, and protection. These aromatic benzene ring compounds are rather essential during the plant's biotic and abiotic stress interactions. The production of the phenolic compound in bryophytes is mostly caused by both biotic and abiotic stress, such as high light, low temperature, and high UV intensity. According to this study, *A. fertilis in vitro* extract reveals a lower value of both TPC and TFC. Lunic *et al.* (2020) identified *Hynum cupressiforme* Hedw. Phenolic and flavonoid content from *in vitro* extracts. They observed that there was a low yield of phenol and flavonoid content identified from *in vitro plant*. This might be because of the unfavorable conditions in the culture shelf leads to lower content of phenols and flavonoids.

4. Antioxidant activity

In this study, we compared the IC_{50} value to determine the antioxidant activity from all the *A. fertilis* samples. As presented in Tables 3 and 4, the n-hexane extract of *A. fertilis* collected from nature was categorized as strong, which is slightly better than the methanol extract. Meanwhile, all *A. fertilis in vitro* for both n-hexane and methanol extracts were categorized as moderate. From these results, it can be observed that the of *A. fertilis* from nature require a concentration of at least 58.41 mg/L to inhibit 50% of DPPH compounds, while *in vitro* extracts require a higher concentration of 111.73 mg/L to achieve the same level of inhibition. The study focused on liverwort habitats located in Universitas Indonesia, Depok, West Java, which are known for their relatively high and dry environmental temperatures. The recorded temperatures in Depok range between 33°C – 35°C , with low rain intensity. Drought conditions, which are prevalent in such habitats, can trigger the accumulation of ROS in plants by disrupting the electron transport system. Elevated ROS levels can cause peroxidation of membrane

lipids, thereby leading to damage to membrane integrity and the loss of electrolytes through damaged membranes (Mujeeb *et al.*, 2014). Plant antioxidant activity is mostly due to the role of polyphenol compounds, which function through multiple mechanisms and may, thus, be evaluated using a variety of assays.

Table 3 Percentage inhibition of *A. fertilis* extract

Concentration (ppm)	Percentage of Inhibition (%)			
	Collected from nature		<i>in vitro</i> culture	
	Methanol	n-hexane	Methanol	n-hexane
25	37.26 ± 2.48	39.13 ± 1.17	8.69 ± 1.83	10.55 ± 1.06
50	42.85 ± 3.01	47.82 ± 2.73	17.54 ± 2.76	21.42 ± 3.42
75	54.34 ± 1.95	60.24 ± 2.05	36.64 ± 2.43	32.60 ± 1.64
100	60.25 ± 4.51	66.15 ± 2.66	41.61 ± 1.33	42.85 ± 1.76

Table 4 IC₅₀ Value and Antioxidant Categorization

Extract		IC ₅₀ Value (ppm)	Categorization
Collected from nature	Methanol	63.10	Strong
	n-hexane	58.41	Strong
<i>in vitro</i> culture	Methanol	112.10	Moderate
	n-hexane	111.73	Moderate

Plants have various defense strategies to cope with drought stress, including the activation of the ROS system, increasing levels of osmoprotectants, and protecting membrane structures. The temperature recorded in Depok area is 32 ± 3°C, while the humidity ranges from 60%–75%. According to the Meteorology Climatology and Geophysics Council (BMKG), it is determined as dry season in Depok. Furthermore, the stimulation of the antioxidant potential in plants is particularly important in coping with ROS toxicity under drought stress. The first line of enzymatic defence against ROS is SOD, which actively converts O₂ to OH, followed by the conversion of H₂O₂ to H₂O with the help of other antioxidant enzymes, such as catalase (CAT) and ascorbate peroxidase (APX). The coordination among antioxidant components is critical to ensure better cell protection against oxidative damage caused by excessive ROS. These findings are consistent with research conducted by Ghosh *et al.* (2021) on the liverwort *Marchantia polymorpha*, which is found in dry habitats; this liverwort produces antioxidant enzymes as self-defense (Khaleghi *et al.*, 2019; Laxa *et al.*, 2019). In accordance with this data, it is stated that there is a high correlation between the phenolic content and the antioxidant activity (Platzer *et al.*, 2022).

5. Metabolomic profiling through GC-MS Analysis

This study investigated the methanol and n-hexane extracts of *A. fertilis* using GC-MS. The n-hexane extract of *A. fertilis* collected from nature from Depok contained the major compound of Drim-7-en-11-ol, as presented in Table 5, which was detected at retention times of 29.551 minutes. The compound had an abundance of 45.83%. Moreover, the extract from natural collected *A. fertilis* contained 27 types of secondary metabolites compounds, and all compounds are summarized in Appendix 1.

The results of *A. fertilis* extracts compound identification differed significantly between the *in vitro* and collected from nature. The chromatograms of the *in vitro* culture extracts revealed a larger number of secondary metabolites compared to the natural origin samples. Specifically, 59 secondary metabolites compounds were identified in the *A. fertilis in vitro* extract, as mentioned in Appendix 2. Additionally, the type of major compound identified varied between the two extracts. The major compound in the *A. fertilis in vitro* extract was n-hexadecanoic acid, which was detected at 34.373 minutes with an abundance of 14.89%, as presented in Table 4. However, the compound Drim-7-en-11-ol (further called as Drimenol) is also found in Caledonian liverworts, the *Bazzanioideae* subfamily, which was also detected in a relatively moderate percentage (Metoyer *et al.*, 2022).

Table 5 Major compound characterized using GC-MS from *A. fertilis* extracts from Depok, Jawa Barat

Sample	Solvent	Number of Compounds Identified	Major Compound	Major Compound Abundance
Nature	Methanol	2	Bis(2-ethylhexyl) methylphosphonate	35.6%
	n-hexane	27	Drim-7-en-11-ol	45.8%
<i>In vitro</i> culture	Methanol	0	-	-
	n-hexane	59	n-hexadecanoic acid	14.9%

The GC-MS analysis from methanol fractions of *A. fertilis*' extracts both from nature and *in vitro* culture also revealed significant differences. All compounds identified in the GC-MS machine is mentioned in Appendices 3 and 4. Moreover, as presented in Table 3, the methanol fractions of *A. fertilis* extracts from Depok contained two major compounds. The first compound identified was bis(2-ethylhexyl) methylphosphonate, with an abundance of 35.6% of the extract, followed by cyclononasiloxane and octadecamethyl, with an abundance of 28.74%. These compounds were detected at a retention time of five minutes. In this study, the use of GC-MS supports the theory that most of the compounds detected in liverworts are in the terpenoid group. Hence, GC-MS is most suited for detecting volatile compounds produced by oil bodies in *z* liverwort. However, since there might be non-volatile or polar compounds produce by *z* liverwort, the use of LC-MS may be required to complete this analysis for further study.

6. Antibacterial assay

As depicted in Figure 9, the antibacterial activity test using preculture of *Staphylococcus aureus* bacteria (Gram positive) and *Escherichia coli* bacteria (Gram negative) produced varying results. Table 6 presents the diameter of the observed inhibition zones, which vary in category. The categorization of the diameter of the inhibition zone was determined based on guidelines by Sarker *et al.* (2014), using tetracycline (7 µg/ml) as a positive control. If the diameter of the inhibition zone is ≤ 11 mm, the isolate is categorized as resistant to the extract. If the diameter of the inhibition zone is 12 mm–15 mm, the isolate is categorized as moderately sensitive to the extract. However, if the observed inhibition zone has a diameter of ≥ 15 mm, the isolate is categorized as sensitive to the tested extract.

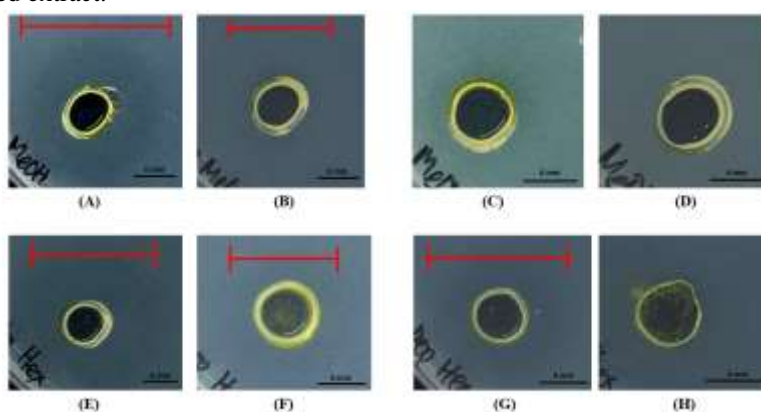


Fig 9 The antibacterial assay inhibition zone from each extract, (A) *A. fertilis* methanol extract on *S. aureus*; (B) *A. fertilis* from *in vitro* methanol extract on *S. aureus*; (C) *A. fertilis* methanol extract on *E. coli*; (D) *A. fertilis* from *in vitro* methanol extract on *E. coli*; (E) *A. fertilis* n-hexane extract on *S. aureus*; (F) *A. fertilis* from *in vitro* n-hexane extract on *S. aureus*; (G) *A. fertilis* n-hexane extract on *E. coli*; (H) *A. fertilis* from *in vitro* n-hexane extract on *E. coli*

Table 6 Zone inhibition diameter from *A. fertilis*' methanol and n-hexane extract collected from nature and from *in vitro* culture

Treatments	<i>S. aureus</i> (mm)	Zone Inhibition Category	<i>E. coli</i> (mm)	Zone Inhibition Category
Tetracycline	24.95	Sensitive	15.93	Sensitive
<i>A. fertilis</i> Methanol extract	19.63 ± 0.56	Sensitive	12.30 ± 0.18	Moderately Sensitive
<i>A. fertilis</i> , n-hexane extract	24.18 ± 1.41	Sensitive	11.45 ± 0.79	Moderately Sensitive
<i>A. fertilis</i> from <i>in vitro</i> culture, methanol extract	0	No Inhibition	0	No Inhibition
<i>A. fertilis</i> from <i>in vitro</i> culture, n-hexane extract	14.82 ± 0.83	Moderately Sensitive	0	No Inhibition

The antibacterial bioactivity tests conducted in this research reveal that the n-hexane extract of the *A. fertilis* extracts have larger inhibition zones than the methanol fraction when tested against the precultures of *S. aureus* bacteria. Further, secondary metabolite profile analysis reveals that the n-hexane extract contains Drim-7-en-11-ol, which is known for its antibacterial properties and is found in vascular plants. This compound belongs to the terpenoid group, in a subgroup of sesquiterpene alcohols. The results of the antibacterial bioactivity test for the n-hexane extract from the *A. fertilis in vitro* culture also reveal an inhibition zone in the two precultures tested, as mentioned in Figure 8. Rather than this, other compounds detected—such as 2,2-Dimethoxybutane and Cyclononasiloxane, octadecamethyl—also play a role as antibacterial agents (Wang *et al.*, 2019; Patil *et al.*, 2014).

The compound identified in the n-hexane extract of *A. fertilis* from *in vitro* culture was n-hexadecanoic acid, which has previously been identified in higher plants, such as *Ipomoea eriocarpa* (Ganesan *et al.*, 2022). This compound belongs to the fatty acid group. The results of *A. fertilis* methanolic extract, which was harvested from nature, had a lower inhibition zone value than the n-hexane extract. The test results of *A. fertilis in vitro* methanol extract showed no inhibition zone on bacteria growth. These results are in accordance with the test using the GC-MS machine that there are no compounds identified. Therefore, there is a possibility that very few polar compounds can dissolve in methanol compared to the more non-polar solvent n-hexane. This indicates a tendency for the compounds present in *A. fertilis* to be dominated by non-polar compounds, such as terpenoid. Based on previous research (Nogueira *et al.*, 2021) on the mechanism of bacterial growth inhibition using terpenoid compounds, it was suggested that these compounds could damage the bacterial cell membrane and affect its permeability. Moreover, terpenoid compounds could alter the morphological structure of the cell membrane in both *S. aureus* and *E. coli* bacteria, without causing cell lysis in both Gram negative and positive bacteria (Mahizan *et al.*, 2019). The activity of terpenoid compounds is believed to be linked to the inhibition of the synthesis of crucial components necessary for the bacterial cell membrane function. This hypothesis is supported by the electron micrograph results obtained by Nogueira *et al.* (2021).

Phenolic compounds possess antibacterial properties by interacting with various compounds synthesized in biological organisms, thereby making them molecular targets for the biological activity of phenolics. According to the current database, several phenolic compounds—including rutin, quercetin, diosmin, hesperitin, and genistein—can interact with a large number of compounds involved in bacterial metabolism. Polyphenols can act against bacteria at different levels and metabolic pathways by affecting the structure and formation of nucleic acids, cell walls, and various enzymes (Shamsuddin *et al.*, 2022; Lobiuc *et al.*, 2023). The synthesis of nucleic acid can be inhibited by polyphenols through topoisomerase inhibition, and it plays an important role in their antimycobacterial activity. Docking studies have proved that quercetin effectively binds to the subunit B of DNA gyrase through interaction with residues that are in the Toprim domain of the protein. The mechanism of action for affecting the cell wall is the hyperacidification at the plasma membrane interphase, which is a consequence of dissociation of phenolic acids, as one of the possible mechanisms of the antimicrobial action of phenolic acids. This hyperacidification alters cell membrane potential, thereby making it more permeable and causing irreversible alterations in the sodium-potassium ATPase pump, thereby leading to cell death (Makarewicz *et al.*, 2021).

Gram-negative bacteria were reported to be resistant toward numerous antibacterial substances due to the hydrophilic surface of their outer membrane and associated enzymes in the periplasmic space, which can break down numerous molecules introduced externally. Moreover, the negatively charged lipopolysaccharide (LPS) of the outer membrane protects the bacterial cell against catechins. Gram-positive bacteria appear to be more susceptible to the action of phenolic acids than Gram-negative bacteria. One of the explanations is that the Gram-positive bacterium lacks an outer membrane, which would facilitate diffusion of the phenolic acids through the cell wall and intracellular acidification (Makarewicz *et al.*, 2021). Despite all this, the strong and moderate categories of *A. fertilis* extracted from nature have the potential to be utilized as effective antioxidant and antibacterial agents.

Conclusion

The *A. fertilis* gametophyte explant produced shoots after being cultured on ½ MS media with the addition of 2,4-D and kinetin. Based on the statistical analysis, it was proven that 1.0 mg/L 2,4-D and 0.1–1.0 mg/L kinetin were the optimum combination for *A. fertilis in vitro* culture shoot induction. One main explant was able to form a single new shoot, but the others showed the potential to grow multiple shoots. Despite the growth response, there was still contamination from the sterilization process of *A. fertilis*; hence, this part of the process needs to be improved in further *in vitro* mass propagation using the gametophyte phase as an explant. The possibility of the browning of the *A. fertilis* explant should be considered when using alcohol as a sterilant. Furthermore, the study also highlights the variation in secondary metabolite profiles and bioactivity of *A. fertilis* extracts obtained from different sources. The study suggests that natural conditions, such as temperature and light intensity, may contribute to the differences observed in the composition and bioactivity of the extracts. These findings have important implications for the development of natural products for medicinal and industrial applications, followed by further research on its possibility application as anticancer and antiaging for humans.

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Author contributions NTR, WH, Y, and AP designed the study. NTR conducted the experiments under the supervision of WH and Y. NTR prepared the first draft of the manuscript. WH, Y, and AP finalized the manuscript. All authors read and approved the final manuscript.

Declaration of interest

The authors have no conflict of interest to declare

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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