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Antioxidant Activity of Bioproduced Endophytic *Fusarium sp.* HSFP-3 from Sijangkang Plant (*Hornstedtia scyphifera* Var. *fusiformis*)

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Abstract
Endophytic mold Fusarium sp. HSFP-3 is one of the endophytic molds isolated from Sijangkang plants (Hornstedtia scyphyfera var. fusiformis). The purpose of this study is to conduct bioproduction, and test the antioxidant activity of bioproduced extracts and identify active fractions that have antioxidant activity. The research method started from the cultivation of endophytic mold HSFP-3 on PDB media for 21 days, the bioproduction results were extracted using ethyl acetate and acetone solvents. Antioxidant activity of HSFP-3 extract was determined by IC_{50} and AAI values, using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method. Isolation of antioxidant active fraction was carried out by column chromatography method using sephadex LH-20 stationary phase and silica. Isolation of antioxidant active compounds of HSFP-3 extract using bioautography guided assay approach with TLC-DPPH Bioautography method. The antioxidant test results of HSFP-3 extract has moderate activity with IC_{50} value 74,64 and AAI 0,844. Fraction 2.4 is an active fraction that has spot compounds with higher yellow color intensity compared to other fractions. Spot with Rf 0.35 in fraction 2.4 has a dark zone under UV 254 light. has a sea blue color under UV 366 light and a dark purple color after with Vanillin-Sulfate reagent.

I. Introduction

Sijangkang (*Hornstedtia scyphifera* var *fusiformis*) is a plant of the genus *Hornstedtia* that grows in Sumatra, especially North Sumatra, West Sumatra, Riau and South Sumatra (Nurainas, Syamsurdi and Arbin, 2015). The ethyl acetate extract produced from Sijangkang plants has antioxidant activity that can reduce 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals with IC_{50} = 111.5 mg/L. Secondary metabolite compounds contained in sijangkang plants include phenolic groups, saponins, triterpenoids, and alkaloids. Antioxidants are compounds that function to inhibit the oxidation process. The oxidation process can produce free radicals, causing reactions that can damage organism cells. Antioxidant compounds will react with free radicals, forming inactive free radicals (Salehi *et al.*, 2018).

Endophytic molds are eukaryotic microorganisms found in all types of plant species, endophytic molds are capable of producing secondary metabolite compounds such as antioxidant compounds, which have biologic activity and are utilized in the health sector, especially in the pharmaceutical field. Medicinal plants are reported to be hosts for endophytic molds, endophytic fungi isolated from plants can produce compounds that have antioxidant activity (Pakaya *et al.*, 2023). Endophytic molds are known to have the ability to produce the same compounds as those produced by their host plants, this is because the bioactive compounds contained in plants are associated with endophytic molds contained in plants (Simarmata *et al.*, 2022). In sijangkang plants, several endophytic molds such as *Xylaria* sp, *Ganoderma sp,* and *Pyrrhoderma* sp were found, which have the ability to produce compounds with antioxidant activity (Munir *et al.*, 2022). *Fusarium sp* HSFP-3 endophytic mold is a collection of endophytic molds from the Bioprospection and Chemoprospection Laboratory Research Group, Center for Research on Raw Materials for Medicines and Traditional Medicines (PR BBO-OT) of the National Innovation Research Agency (BRIN).

Based on this, to obtain the latest information about endophytic molds that can be utilized as a source of raw materials for antioxidant compounds, it is necessary to conduct research related to the

antioxidant activity of the endophytic mold *Fusarium sp* HSFP-3.

II. Research Method

II.1 Rejuvenation and Scalle-Up of Endophytic Mold Bioproduction

HSFP-3 endophytic molds were rejuvenated into Potato Dextrose Agar (PDA) media and then incubated for 5 days at room temperature 25°C-30°C. Scalle-Up *of* HSFP-3 endophytic mold bioproduction was carried out by inoculating 5 pieces of HSFP-3 mold agar that had aged 5-7 days, into 200 ml of Potato Dextrose Broth (PDB) media in a 500mL Erlenmeyer container. Cultivation was carried out by the silent fermentation method, the fermentation medium containing the HSFP-3 mold was kept in a dark room at 25°-30° for 21 days (Praptiwi *et al.*, 2018).

II.2 Extraction of Endophytic Fungi

Endophytic molds HSFP-3 was blended with a Waring blender, the filtrate from fermentation was extracted with ethyl acetate while the biomass was extracted with acetone. Ethyl acetate and acetone solvents were evaporated with a Rotary evaporator, then the thick extract was dried by freeze drying so that the dry extract was obtained (Kumala, 2019). The extract samples were then analyzed using Thin Layer Chromatography (TLC) technique on silica plate GF_{254} (Merck). The ethyl acetate extract and acetone extract of endophytic mold HSFP-3 were then combined and the yield obtained was calculated.

II.3 Determination of IC_{50} Values for Antioxidant Activity and Antioxidant Activity Index (AAI) of HSFP-3 Extracts

Determination of IC_{50} value of antioxidant activity by free radical scavenging method (DPPH) was done by serial dilution using 96-well microplate. Each well in row B to the last row was filled with 100 µL of methanol pro analysis (p.a). Wells in row A were filled with 195 µL of methanol p.a, 5 µL of sample/extract with a concentration of 20480 ppm was added and homogenized. Serial dilution was done by pipetting 100 µL of row A mixture to row B in the same column. Dilution was carried out until the last row then 100 µL of the mixture in the last row was discarded. After the dilution is complete, each well is added with 100 µL of DPPH with a concentration of 61.5 ppm and incubated for 90 minutes in a dark container protected from light at room temperature 25°C-30°C. After incubation the absorbance of the extract/sample was measured using a microplate reader (Varioscan Flash, Thermo Scientific) at λ 517 nm. To calculate the inhibition concentration use Equation 1.

$$IC_{50}=\frac{(A_{DPPH100\%} - A_{Sample})}{A_{DPPH100\%}} \times 100\% \quad (1)$$

Description:

IC : Inhibitory Concentration

$A_{DPPH100\%}$: DPPH absorbance

A_{sample} : Sample Absorbance - Methanol p.a. Absorbance.

To calculate the percentage of inhibition of the IC_{50} value of the extract using a linear regression curve (Praptiwi, Sulistiarini, *et al.*, 2021). Antioxidant Activity Index (AAI) was calculated with the following equation 2 (Praptiwi *et al.*, 2018).

$$AAI = \text{Test DPPH Concentration} / IC_{50} \text{ Value} \quad (2)$$

II.4 Detection of Antioxidant Activity by TLC-DPPH Bioautography Method

Antioxidant activity screening was performed by Thin Layer Chromatography (TLC)-DPPH Bioautography (Dot-Blot and Elution). Dot-Blot: A total of 10 µL of sample at a concentration of 10000 ppm was blotted on a silica plate (Merck, F254) aerated until the blots were dry. Elution: A total of 10 µL of sample with a concentration of 10000 bpj was blotted on the silica plate, then eluted using the optimized eluent. The TLC plate was sprayed with DPPH solution in methanol. The TLC plate was kept in a container protected from light, after 30 minutes a yellow zone was seen around the sample. The yellowish-white zone indicates samples or compounds that have antioxidant activity. The intensity of the color indicates the capacity of antioxidant activity (Praptiwi, Ilyas, *et al.*, 2021).

II.5 Isolation of Antioxidant Active Fractions

A total of 200 grams of Sappan wood were macerated using 96% ethanol solvent for 3x24 hours and then re-macerated twice. The maceration process is assisted by occasional stirring so that the extraction process takes place optimally. The filtrate obtained from the maceration results was combined, then evaporated with a rotary evaporator.

II.3.4 Identification by Thin Layer Chromatography

Isolation of antioxidant active fraction using column chromatography method. Chromatography is based on the principle that the molecules in the mixture are interacted into the solid of the stationary phase and separated from each other while moving with the help of the mobile phase (Coskun, 2016). The column chromatography method used is column chromatography with normal phase, where the stationary phase used is sephadex LH-20. Sephadex LH-20 is used to separate compounds based on molecular weight and can also be used to purify and separate small organic molecules such as steroids, terpenoids, and lipids (Mottaghipisheh and Iriti, 2020). The samples were dissolved with methanol and filtered with whatman paper. The samples were column chromatographed with sephadex LH-20 stationary phase and methanol mobile phase with a flow rate of 5ml/min, then collected in a 25 mL test tube. The resulting fractions were column

chromatographed using silica stationary phase with a ratio of 50x sample weight, mobile phase used in stepwise separation with eluent *n*-hexan: ethyl acetate (10:1) to (1:1), ethyl acetate, dichloromethane: methanol (10:1) to (1:1) and methanol. Each eluent mixture used as much as 200 mL. The resulting fractions were collected in 20mL test tubes and identified by TLC.

III. Results and Discussion

Bioproduction of *Fusarium sp.* (HSFP-3) started from the rejuvenation of the mold isolate, inoculated as many as 3 pieces of mold mycelium along with the agar onto PDA media, then the mold was incubated at 25°C-30°C for 5 days. Cultivation of the mold was continued on PDB media. Scalle-Up bioproduction was carried out by inserting 5 pieces of HSFP-3 rejuvenated agar into 200 mL of PDB media with 500 mL fermentation containers, for a total of 22 fermentation containers. The total fermentation media used was 4400 mL. Cultivation and scale-up of molds in PDB media has the aim of increasing the number of molds so that more extracts and secondary metabolites are produced (Eevers *et al.*, 2015).

III.1 Sample Extraction

The cultivated mold was separated between the filtrate and biomass, and then the biomass was blended using a Waring blender. The filtrate was added with ethyl acetate solvent and then stirred using a magnetic stirrer to mix perfectly, then the filtrate was extracted using a liquid-liquid extraction method by partitioning, the ethyl acetate phase was separated and concentrated using a rotary evaporator until a thick extract was obtained. Biomass was extracted by maceration-sonication method with the help of a sonicator using acetone solvent, then filtered using gauze and Whatman filter paper, the filtrate was then concentrated using a rotary evaporator until a thick extract was obtained. Thick ethyl acetate and acetone extracts were dried using freeze-drying equipment until dry extracts were obtained. The use of freeze drying aims to reduce the water content in the extract by maintaining the quality of the chemical compound content in the extract. Weighed each dry weight of ethyl acetate extract and biomass. The weight of each dry extract can be seen in Table 1.

Table 1. Filtrate and biomass extract weights

No.	Sample Name	Extract Weight (mg)
1	Extract Filtrate	675.6
2	Biomass Extract	2173.9
3	Total	2849.5

III.2. Extract Chromatogram Profile

A total of 10 µL of filtrate and biomass extract samples with a concentration of 10,000 ppm were photographed on a silica plate and eluted with *n*-hexane - ethyl acetate (5:1) and dichloromethane - methanol (10:1) eluents shown in Figure 3. The silica plate was observed under UV 254 and 366 light and sprayed with vanillin-sulfate spotting agent. Vanillin-sulfate spotting agent is a general spotting agent and can display spots that are not visible under UV light 254 and 366, namely compounds that do not have chromophore groups (Fathoni *et al.*, 2021).

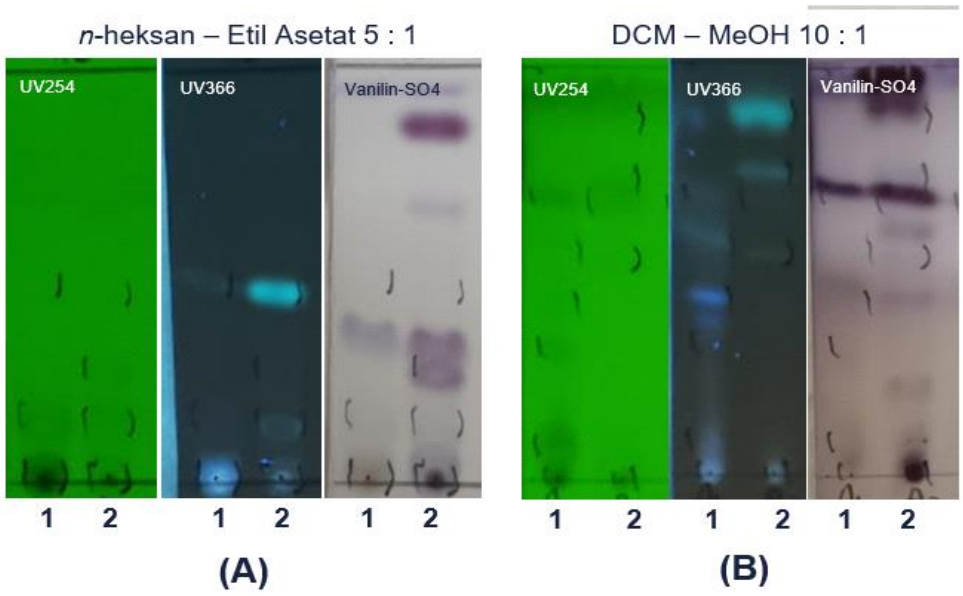

Figure 1. Chromatogram profile of filtrate extract (1) and biomass extract (2).

Selection of *n*-hexane : ethyl acetate (5:1) eluent to show the chromatogram profile of compounds contained in the extract with low solubility, while the selection of campurn dichloromethane : methanol (10:1) eluent to show the chromatogram profile of compounds with high solubility.

The results of spot identification at UV 254 light, UV 366 light and vanillin sulfate spotting, filtrate extract and biomass extract have similar spots in both chromatogram profiles with eluent *n*-hexane: ethyl acetate (5:1) and dichloromethane : methanol (10:1), so the filtrate extract and biomass extract were combined into one, for further antioxidant activity testing and isolation of antioxidant active fractions.

III.3. Determination of IC_{50} Values for Antioxidant Activity and Antioxidant Activity Index (AAI) of HSFP-3 Extracts

Determination of the IC_{50} value of antioxidant activity by free radical scavenging method (DPPH) was carried out by serial dilution using 96-well microplate. The smaller the IC_{50} value, the higher the activity of the sample in counteracting free radicals. IC_{50} value quantitative test of antioxidant activity of HSFP-3 extract was obtained from the linear regression curve of the relationship between ln concentration and % inhibition. The IC_{50} value indicates the concentration of the sample to reduce 50% of DPPH free radicals (Farida, Qodriah and Nilesh, 2022). The results of antioxidant activity test IC_{50} value and AAI are shown in Table 2. IC_{50} value antioxidant activity of HSFP-3 extract obtained 74.64 ± 1.09 µg/mL with 0.844 ± 0.012 which is classified as a moderate category as an antioxidant (Table 2). Antioxidant activity is said to be weak if the AAI value <0.5, moderate 0.5 -1, strong 1 - 2 and very strong >2 (Praptiwi, Ilyas, *et al.*, 2021). The potential antioxidant activity of HSFP-3 mold extract, needs to be further explored the content of compounds produced from the HSFP-3 mold bioproduction process, which plays a role in reducing DPPH free radicals.

Table 2. Antioxidant activity test results of HSFP-3 mold extracts

No.	Sample	IC_{50} Value (µg/mL)	Mean IC_{50} Value (µg/mL)	AAI Value	Average AAI Score
1	Repetition 1	75.86		0.83	
2	Repetition 2	74.32	74.64±1.09	0.848	0.844±0.012
3	Repetition 3	73.75		0.854	

Catechin was used as a comparator in this study, for antioxidant activity testing. The IC_{50} value of catechin obtained was 7.27 ppm with an AAI of 8.67 catechin can be seen in Table 3. Catechins include compounds that have very strong antioxidant activity seen from the AAI value that exceeds 2. Catechins are natural polyphenolic compounds flavan-3-ol (or flavanols), which belong to the flavonoid family. Catechins are single isolated from natural products. This compound is found in various natural products such as fruits and vegetables. The mechanisms of catechin antioxidant activity include chelating metal ions, inducing antioxidant enzymes, inhibiting pro-oxidant enzymes, and producing phase II detoxification enzymes and antioxidant enzymes. Catechins and their diastereoisomers all have phenolic hydroxyl groups that are able to stabilize free radicals (Bernatoniene and Kopustinskiene, 2018).

Table 3. Antioxidant activity test results of catechins

No.	Sample	IC_{50} Value (µg/mL)	Mean IC_{50} Value (µg/mL)	AAI Value	Average AAI Score
1	Repetition 1	7.27		8.662	
2	Repetition 2	7.37	7.24±0.15	8.550	8.706±0.812
3	Repetition 3	7.07		8.907	

A comparison of the antioxidant activity of HSFP-3 extracts and catechins is shown in Table 4. HSFP-3 mold has the potential to produce extracts and compounds that have antioxidant activity. HSFP-3 extract consists of various compound components, it is possible to have synergistic and antagonistic effects between compounds in providing antioxidant effects, so it is necessary to isolate and identify antioxidant active compounds in HSFP-3 extract.

Table 4. Comparison of antioxidant activity of HSFP-3 and catechin extracts

No.	Sample	IC_{50} (ppm)	AAI	AAI Value Criteria
1	HSFP-3 Extract	74.64±1.09	0.844±0.012	Medium
2	Catechins	7.24±0.15	8.706±0.812	Very Strong

III.4. Antioxidant Activity of Column Chromatography Fractions by TLC-Bioautography

Separation of the antioxidant active fraction of *Fusarium* endophytic mold HSFP-3 was performed by column chromatography. HSFP-3 extract as much as 1.94 g was column chromatographed using Sephadex LH-20 stationary phase, and 100% methanol mobile phase. Separation of active metabolites with this method produced 11 fractions, the weight of each Sephadex fraction and its antioxidant activity can be seen in Table 5. Separation using Sephadex LH-20 aims to classify compounds based on molecular weight, compounds with larger molecular weight will first go down through the Sephadex LH-20 membrane (Chen *et al.*, 2023).

Table 5. Weight of Sephadex Column Chromatography Fractions and TLC-Bioautography Results

No.	Sample	Weight (mg)	TLC-Bioautography Results
1	F1	25.1	+
2	F2	1655.3	+++
3	F3	131.6	++
4	F4	40.4	++
5	F5	46.6	+
6	F6	6.3	+
7	F7	7.4	+
8	F8	7.4	+
9	F9	3.8	+
10	F10	16.5	+

Note; Antioxidant activity of TLC-Bioautography results; Weak (+), Medium (++), Strong (+++)

All fractions of Sephadex column chromatography results were identified by TLC-Bioautography dot-blot, namely as much as 10 µL of sample with a concentration of 10000 ppm was bottled on a silica plate, dried then sprayed with 2% DPPH solution in methanol, silica plate incubated in a dark container for 30 minutes then observed the yellow spot that appears, indicating the presence of antioxidant activity (Figure 2). The change of purple color to yellow color is due to the presence of compounds that donate hydrogen atoms and donate electrons, so that DPPH which is radical will receive hydrogen atoms or electron pairs (Foti *et al.*, 2008).

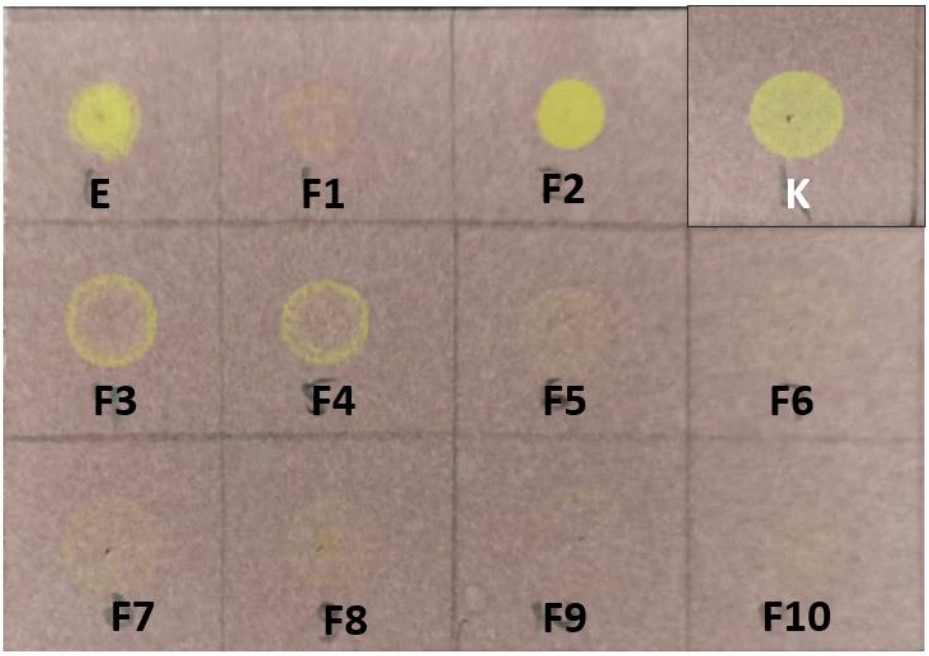

Figure 2. Bioautogram of antioxidant activity of HSFP-3 Extract (E), Fractions (1-10), Catechins (K).

HSFP-3 extract, fraction 2, and positive control of catechin showed good antioxidant activity by changing the target spot from purple to yellow color. Fraction 2 from column chromatography using Sephadex LH-20 has the largest fraction weight among the other ten fractions.

Fraction 2 was further separated using column chromatography with silica gel 60 stationary phase (0.2-0.5mm) (Merck®) and mobile phase *n*-

hexane: ethyl acetate, ethyl acetate, dichloromethane : methanol and methanol by Step Gradient Polarity (SGP). The working principle of column chromatography using silica stationary phase is based on the level of polarity of the compound will interact with the polar stationary phase and non-polar compounds will move first following the mobile phase. The chromatogram profile of the separation results of fraction 2 obtained 12 fractions shown in Figure 5. Identification of compounds from column chromatography fraction 2, using TLC with 3 different eluents, namely fraction 2.1 to fraction 2.6 using *n*-hexane - ethyl acetate eluent (5:1), fraction 2.6 to fraction 2.10 using dichloromethane - methanol eluent (10:1), and fraction 2.11 to fraction 2.12 using dichloromethane - methanol eluent (5:1). Eluent selection is adjusted to the polarity of the compounds in each fraction.

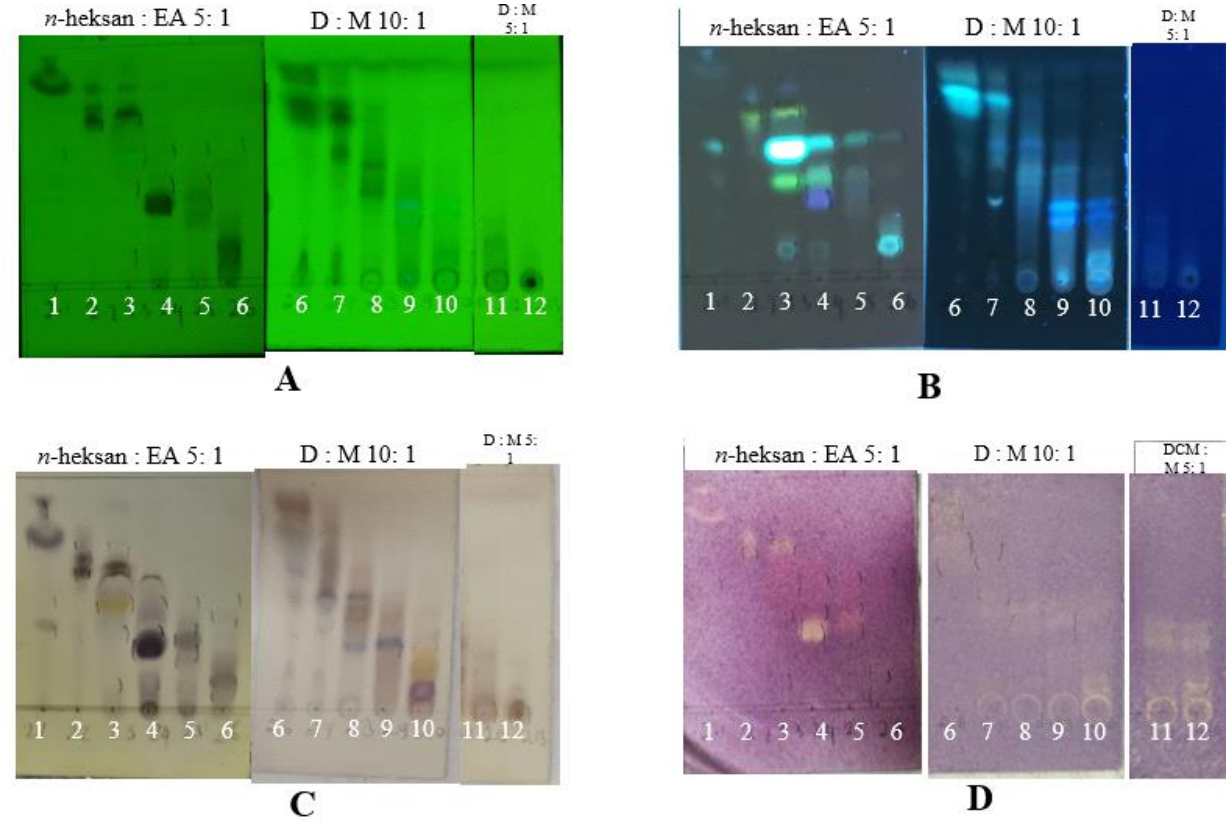

Figure 3. Chromatogram profile of silica column chromatography results of fraction 2 (no.1-12). Detection at UV 254 light (A), UV 366 light (B), after spraying Vanillin-sulfate color reagent (C), sprayed with 0.2% DPPH in methanol (D).

Fraction 2.1 to fraction 2.12 were identified by observing under UV light 254, UV light 366, sprayed with 0.2% DPPH in methanol, and sprayed with Vanillin-sulfate reagent. Observation under UV light 254 and UV light 366 to see spot chromophore compounds that have conjugated double bonds. Spraying with Vanillin-Sulfate reagent which is a general coloring reagent, aims to show the spot of compounds that are not visible by observation under UV light. Spraying with 0.2% DPPH in methanol to see the spots of fractions that have antioxidant activity. The weight of each fraction 2.1 to 2.12 and its antioxidant activity can be seen in Table 6. Identification of active compounds using TLC-DPPH Bioautography because it is one of the simple methods, requires a short time, and uses a small sample and reagents.

Table 6. Weight of Fraction 2.1 to Fraction 2.12 and TLC-Bioautography Results

No.	Sample	Weight (mg)	TLC-Bioautography Results
1	F2.1	105.6	+
2	F2.2	10.7	+
3	F2.3	198.2	+
4	F2.4	224.5	+
5	F2.5	94.9	+
6	F2.6	27	-
7	F2.7	35.5	+

8	F2.8	15.5	+
9	F2.9	132.8	+
10	F2.10	127.6	+
11	F2.11	421.6	+
12	F2.12	148.8	+

Description: (+) There are spots that have antioxidant activity, (-) there are no spots that have antioxidant activity.

Based on the TLC-Bioautography results of the twelve fractions, there is only one fraction, F2.6, which does not have a yellow spot with a purple background after being tested for antioxidant activity (Figure 3D) Fraction 2.4 has a compound spot with a higher intensity of yellow color compared to other fractions. Spot with Rf 0.35 in fraction 2.4 has a dark zone with a green background based on observation under UV 254 light. Terpenoid group compounds with conjugated double bonds show dark zones under UV254 (Fathoni *et al.*, 2021). Under UV 366 the compound with Rf 0.35 has a sea blue color and after being sprayed with Vanillin-Sulfate reagent has a dark purple color. Terpenoid compounds sprayed with Vanillin-Sulfate reagent give blue, or dark blue, or dark green, or brownish green, bluish green, light orange, purple, maroon, lavender, brown (Fathoni *et al.*, 2021). In Fraction 2.4 there are also 2 major spots observed under UV 366 light that have blue green and green fluorescence. Alkaloid group compounds when observed under UV 366 light have blue, green, or purple fluorescence (Fathoni *et al.*, 2021). Fraction 2.4 has a weight of 224.5 mg so that it can still be isolated compounds that have antioxidant activity using column chromatography. Isolation of active compounds is done to identify the compounds contained in the extract. The antioxidant activity of HSFP-3 extract is thought to be related to the many active antioxidant compounds that work synergistically.

IV. Conclusions

Endophytic molds HSFP-3 associated with sijangkang plants (*Hornstedtia scyphyfera* var. *fusiformis*) can produce extracts that have antioxidant activity. Isolation using column chromatography with sephadex LH-20 stationary phase followed by column chromatography using silica stationary phase, resulted in fractions with antioxidant activity identified by TLC-Bioautography.

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