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Optimization of Bis-anthraquinones Production from Endophytic Fungi *Diaporthe* sp. GNBP-10

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Abstract

Bis-anthraquinones with a unique molecular backbone, (+)-2,2'-epicytoskyrin A (epi) and (+)-1,1′-bislunatin (bis), were produced by endophytic fungi *Diaporthe* sp. GNBP-10 associated with Gambir plant (*Uncaria gambier* Roxb. Rubiaceae). Epi and bis possess robust antimicrobial activity toward various pathogens. This study focus on knowing the optimum condition of epi and bis production from endophytic fungi *Diaporthe* sp. GNBP-10. A series of culture media with various nutrient compositions was investigated in epi and bis production. The content of epi and bis was determined by measuring the area under the curve from TLC-densitometric (scanner) experiment. The linear regression analysis was then applied to obtain the results. The optimized media to produce the highest extracted mass is media 7, while epi and bis were greatly produced in liquid media 3. The nutrient content of media 3 is potato starch and dextrose with the amount of epi component produced 0.484 mg and bis content is 0.163 mg. The presence of carbohydrates, whether simple sugar or carbohydrate complex, plays an essential role in the bis-anthraquinones production from *Diaphorthe* sp. GNBP-10 culture. The presence of minerals and excessive protein sources did not significantly affect bis-anthraquinones production.

Keywords: Bis-anthraquinone, Diaporthe, Endophyte media, Regression, TLC.

Introduction

Endophytic fungi are colonized and detected within the healthy host plant tissue [(1)]. Endophytes build a symbiotic relationship with the host plants, majorly defined as mutualism constitutive and inductive mutualism [(2,3)]. Endophytic fungi are believed to be a biological protector for the host plants from phytopathogens [(2)]. The endophytes could express secondary metabolites against the foreign intruders [(4)]**.** The released metabolites stimulate the activation of induced systemic resistance that is related to the host defense mechanism [(5)]. Furthermore, the exerted metabolites can promote plant growth via phytohormones [(6)]. The produced metabolites also help the host plant to degrade xenobiotics [(7)] and improve the resistance to soil contaminants [(8)].

Due to the unique relationship between endophytic fungi and host plants, endophytic fungi can produce similar secondary metabolites as their host plant [(9)].The interaction between endophytes and the host leads to the co-production of bioactive compounds[(10)]. That phenomenon is hypothesized due to horizontal gene transfer between endophytes and the host plant [(11)].

A considerable amount of invaluable therapeutic compounds were discovered from endophytic fungi such as Paclitaxel/taxol (anticancer), and Camptothecin (anticancer) [(12)], Ergoflavin (anti-inflammatory) [(13)], Cyclosporine (antiviral) [(14)], etc.

The endophytic fungi *Diaporthe* sp. GNBP-10, which is associated with the tea plant (*Camellia sinensis* (L.) O. Kuntze) and Gambir plant (*Uncaria gambier* Roxb.), generated two bis-anthraquinones (+)-2,2'-Epicytoskyrin A (epi) (Figure 1a) and (+)-1,1′-bislunatin (bis) (Figure 1b) [(15–17)]. The formed bis-anthraquinones, epi, were reported to have displayed strong *in vitro* antimicrobial activity against *S. aureus* (MIC: 0.06 µg mL^{-1}), *B. subtilis* (2 µg mL^{-1}), and *K. pneumonia* (4 µg mL^{-1}) [(16)]. Meanwhile, bis exhibited moderate activity against *B. subtilis, S. aureus, E. coli, M. luteus, P. vulgaris,* and *P. mirabilis* with a MIC value (64 µg mL^{-1}) [(17)]. Epi and bis retain a robust in-vitro antimycobacterial activity against *M. tuberculosis* h37rv, as indicated by MIC values 0.844 µM and 0.422 µM, respectively [(18)]. Additionally, the oral toxicity assays showed that both metabolites have low acute toxicity profiles [(19)].Due to their immense biological activities, a certain amount of pure epi and bis compounds is needed to meet sufficient criteria for further research. The cultivation method that optimized the production of epi and bis has not been yet investigated. The growth medium has a vital role in natural product production.

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A previous study demonstrated that various conditions and nutrients significantly contribute to the diversity and quantity of the expressed secondary metabolites [20]. Culture conditions variations are applied to optimize selected metabolites expressionby the endophytic fungi [12,21]. Potato Dextrose Broth (PDB) and Potato Dextrose Agar (PDA) were utilized in the cultivation of *Diaphorthe* sp. associated with the tea plant [15]. Ethyl acetate was used as a diluent to get the endophytic extracts. For the same amount of media culture volume, PDB (525 mg) was reported to have a higher extracted mass of endophytic fungi than PDA (80 mg). The production of epi and bis from PDB cultured-endophytic fungi were 47 mg and 10.3 mg, respectively. When the endophytic fungi cultured in the PDA generated 17.5 mg and 5.5 mg for both components [15].

The previous research showed a media influence in the production of bioactive compounds isolated from endophytic fungi. The purpose of this study is to investigate the effect of different media on the production of bis-anthraquinones from endophytic fungi *Diaphorte sp*. GNBP-10. The media selection will particularly be elaborated in optimizing the production of epi and bis, which are structurally depicted in Figures 1a and b.

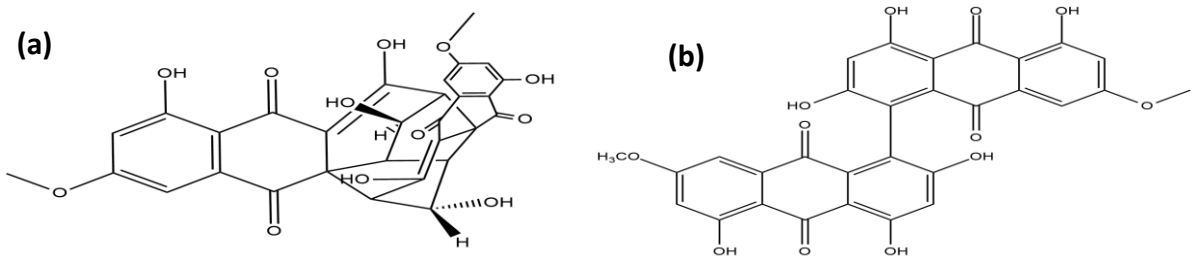


Figure 1.(a) (+)-2,2-Epicytoskirin -A (epi) (MW: 574.494 g/mol). (b) 1,1 Bislunatin (bis) (MW : 570.462 g/mol)

Materials and Methods

The optimized bioproduction of bis-anthraquinones was obtained via a selection of culture media from a series of culture medium variations representing a wide range of nutrient content. In general, this study contained three major workflows: cultivating endophytic fungi *Diaporthe* sp. GNBP-10 from various culture media, extraction of secondary metabolite, and determining bis-anthraquinones content using TLC scanner. The identification of endophytic fungal *Diaporthe* sp. GNBP-10 and the optimization of fungal growth were previously reported by Ilyas et al (2009) [22].

Endophytic fungi cultivation in various culture media

The culture media was prepared in Erlenmeyer flasks. The composition of each media is listed in Table 1. A volume of 200 mL culture media was used in this assay. The endophytic fungi, *Diaporthe* sp. GNBP-10 was isolated from the Gambir plant (*Uncaria gambier* Roxb.: Rubiaceae). The fungi
culture growth was obtained after 14 days of incubation at room temperature (25 – 28 ^{o}C) with shaking at 120 rpm in the orbital shaker (Newsbrunwich Scientific).

Table 1. The nutrient's content and compositions versus each media applied in the assay

Media ID	Nutrient content	Composition gL^{-1} H_2O	Media ID	Nutrient content	Composition gL^{-1} H_2O
1	Potato starch Table sugar (sucrose)	4.0 20.0	8	Peptone (Bacto™) Yeast extract (Bacto™) Malt extract (Bacto™) Dextrose (Merck Milipore)	2.0 2.0 2.0 20.0
2	Potato starch Glucose (Merck milipore)	4.0 20.0	9	PDB (Himedia®) Dextrose (Merck Milipore)	0.48 19.6
3	Potato starch Dextrose (Merck milipore)	4.0 20.0	10	PDB (Himedia®) Glucose (Merck Milipore)	0.48 19.6
4	Yeast extract Malt extract Glucose (Merck milipore)	1.0 1.0 20.0	11	Glucose (Merck Milipore) Yeast extract Peptone K_2HPO_4 $MgSO_4.7H_2O$ $FeSO_4.7H_2O$ $CaCO_3$	20.0 1.0 5.0 0.5 0.5 0.01 1.0

Continued table 1.

5	PDB (BD Difco™)	24	12	Dextrose (Merck Milipore) Yeast extract Peptone K_2HPO_4 $MgSO_4.7H_2O$ $FeSO_4.7H_2O$ $CaCO_3$	20.0 1.0 5.0 0.5 0.5 0.01 1.0
6	PDB (BD Difco™)	12	13	Potato infusion Dextrose (Merck Milipore) Agar	 4.0 20.0
7	Peptone (Bacto™) Yeast extract (Bacto™) Malt extract (Bacto™) Glucose (Merck)	5.0 3.0 3.0 200.0		-	-

Secondary metabolites extraction

The grown fungi mycelium was transferred from the media and crushed using a laboratory blender. A solvent mixture from ethyl acetate and acetone 7:1 ratio (v/v) (Merck Millipore) was added to the crushed media and left to extract the secondary metabolites. The crushed mycelium was macerated for 3x 6 hours at room temperature. The liquid-liquid solvent fractionations were implemented to separate the organic and aqueous fractions from culture media. The ethyl acetate fraction was vacuum dried at 35 ^oC using a rotary evaporator (IKA® RV 8), weighted, and reserved for further analysis.

Determination of bis-anthraquinones content by Thin Layer Chromatography (TLC) scanner

A calibration curve was constructed to determine the epi and bis content in the extracts. A stock solution (1 mg/mL) from epi and bis was prepared by dissolving the preserved sample with ethanol (Merck Millipore). A serial dilution was carried out to obtain100, 500, 1000, 2500, 5000, and 7500 ng/mL standard solutions. A spot of 7.5 µL aliquot from the sample extract was applied to thin-layer chromatography (TLC) silica plate (Merck Millipore, TLC Silica gel 60G F_{254} plate) by pinpointed capillary tube and subsequently dried before being put to the TLC chamber. The applied spot was developed on the chromatogram with dichloromethane-methanol-acetic acid (Merck Milllipore) (10:1:0.1) v/v/v in a TLC chamber saturated environment with the mobile phase vapor. The air-dried chromatogram TLC plate was then placed into a TLC scanner (Shimadzu) and the area under the curve was measured for both epi and bis components at 433 and 481 respectively. The calibration curve was established by plotting the measured area under the curve. The calibration curves are shown in Figures 2a and 2b.

Each endophytic extract obtained from different culture media was solvated with ethanol to form 1 mg/mL solutions. The volume of 7.5 µL sample specimens from the extracts was TLC assayed and scanned using the same conditions mentioned above. The calibration of the area under the curve was carried out for all the samples using the below equation and the anthraquinones contents in each extract were obtained. Linear regression analysis was used to determine the quantity of both epi and bis in the extract samples. The original extract epi and bis components were calculated by multiplying with the aliquote factor when the sample was deposited into the TLC plate as described in equation 1,

Equation 1:

$$OC: \left(\frac{a}{1000} \mu g \; x \; 100\%\right) xb$$

a: bis-anthraquinones (epi or bis) obtained from measurement (in μg)

b: mass of extract sample (μg)

OC: concentration of bis-anthraquinones on the initial extract

Results and Discussion

Gambir plant (*Uncaria gambier* Roxb) consisted of various types of endophytic fungi.. One of the endophytic fungi known for its biological activity is *Diaporthe* sp. GNBP-10 [(22)]. Taxonomic identification of *Diaporthe* sp. GNBP-10 was conducted through observation of macroscopic and microscopic characters based on several references as described comprehensively in Ilyas et al (2009) [(22)]. The macroscopic appearance of GNBP-10 is presented as dark-yellow thick colonies, with mycelium characterized as immersed, branched, septate, hyaline [(22)]. Anthraquinones produced by *Diaporthe* sp. GNBP-10 in various culture media linear regression analysis. The retention factor (rf) of epi after the elution was 0.4 demonstrated by was

measured with yellow spots on the chromatogram. The bis compound was moved by the mobile phase to 0.6 rf appearing as a red spot on the chromatogram. The TLC Scanner was used to evaluate the content targeted samples quantitively. This instrument combines the UV chamber equipped with a specific wavelength with a detector that can detect the area of the separated sample and convert it into a particular curve pattern [23]. The TLC scanner was chosen due to its quick analysis time, lack of complex sample preparation, low solvent consumption, which makes it more environmentally friendly, and ability to analyze multiple samples simultaneously.

According to the calibration curve, each standard of epi and bis concentration was linear over this concentration range. The correlation coefficient (r2) for epi and bis is 0.9944 and 0.9888, respectively, as shown in Figures 2a and 2b. The linear concentration versus absorbance of the standard anthraquinones relation was applied to determine the concentrations of the metabolites in the samples while the correlation coefficient (r2) for both authentic samples epi and bis were 0.9779 and 0.9893 respectively, Figure 2a and 2b.

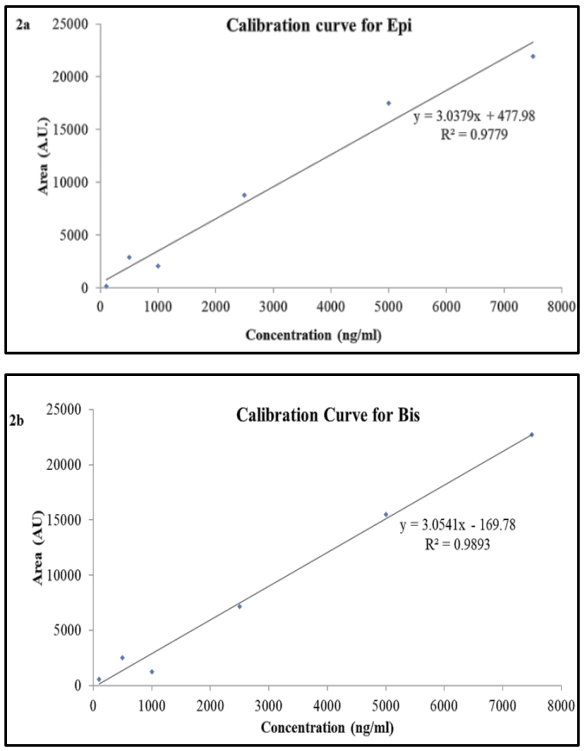


Figure 2. (a)The calibration curve for Epi. (b) calibration curve for bis.

The nutrient variation of the culture media greatly affects the obtained extract mass, as shown in table 2 and Figure 3a. The Malt extract induces higher cell mass production, indicating the highest extract mass (159.4 mg) obtained from the cultivation of media 7 (Figure 3a). The differences in the weight of the extracts could also be influenced by the extraction process such as the maceration step, liquid extraction step, and filtrations. The difference in composition and the diversity of secondary metabolites might contribute to the differences in extracted mass from various culture media [20]. As shown in Figures 3a and 3b, the optimized extract mass was obtained from media 7, while the targeted metabolites were found high in media 3. This finding indicates that the promoted robust fungal growth media and a high amount of the extract may not always produce high amount of the targeted compounds. The previous study discussed PD (Potato Dextrose) and YESD (soy peptone, dextrose, yeast extract, H_2O) media facilitated a large amount of G24 extract but failed to produce high content of the targeted compound [20].

The availability of simple sugar improves the production of bis-anthraquinones. Media 1 containing the disaccharides showed lower epi expression than media 2 and 3 containing monosaccharides (glucose and dextrose). Dextrose displayed a better nutrient than glucose for epi production in *Diaporthe* sp. GNBP-10, as shown by higher epi production in media 3 (0.484 mg) than in media 2 (0.3705 mg). Dextrose is D-glucose, while glucose contains both L-glucose and D-glucose. Carbohydrates, energy, minerals, and vitamins (especially thiamin) are key nutrients for fungal growth [24]. Dextrose, glucose, and potato starch are the source of carbohydrates [25]. Though simple sugars are vital for endophytic fungi growth, the ultimate glucose content in the culture media was reported to induce oxidative stress in yeast. It can negatively affect important cellular components like DNA, Lipid, and proteins [26].

Potato dextrose media containing potato infusion and dextrose have been considered the main media for fungi cultures [25]. Serving as a carbohydrate and energy source. Moreover, potato is a material full of nutrients such as nitrogen, enzyme, vitamins, and minerals needed for fungal growth [27]. Malt extract is a carbohydrate source that consists primarily of maltose. Both malt extract and potato dextrose are the carbohydrates and energy sources for the growing fungi. In this case, potato starch exhibited better performance to support the production of metabolites, as shown by all media containing potato (1,2, and 3) having higher bis-anthraquinones content than media with malt extract (4,7,8). The acidic environment provided by the malt extract [28] probably influences the biosynthetic pathway of both bis-anthraquinones in *Diaporthe* sp. GNBP-10. Figure 3a depicts the comparison of the extracted mass, while Figure 3b represents bis-anthraquinones production from endophytic fungi *Diaporthe* sp. GNBP-10.

Regarding the production of the bis metabolite, the highest production rate was attained from media 3, showing the number of bis content in the extract is 0.1621mg. The bis component production was undetected in *Diaporthe* sp. GNBP-

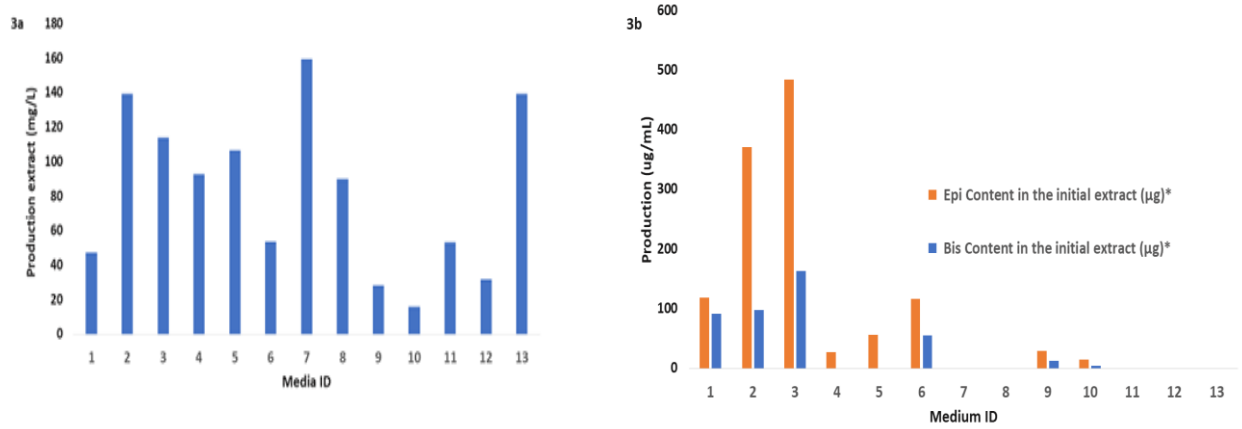
10 cultured in media no, 4,5,7,8, 11,12, and 13. There is no distinct content in the nutrient from the mentioned media. Media number 1 showed the optimized culture media to produce bis compound. Another study is needed to examine the induction capacity of the culture media composition on bis compound production in more regarding the effect of culture media composition to induce bis production in *Diaporthe* sp. GNBP-10.

The peptone and yeast incorporation as nitrogen sources showed an insignificant effect on the production of both anthraquinones from *Diaporthe* sp. GNBP-10. Media 11 (Table 1) demonstrated the presence of various minerals seemed not to substantially contribute to the improvement of bis anthraquinone production. Though the culture media contain relatively complete minerals, protein sources, and simple sugars, the absence of complex carbohydrates such as potato starch appeared to decrease the production of bis-anthraquinone in media 11. Table 2 and Figure 3b present the production of bis-anthraquinones was better in the manually prepared media compared to in the commercially available media (PDB Himedia and PDB Difco). Both PDB Difco and Himedia contain potato starch and dextrose.

Table 2. The measurement results of bis-anthraquinone from endophytic fungi cultured with various nutrient composition

Culture Media ID	Extract sample (mg)	Epi			Bis		
		Area under curve	measured content (µg/mL)	Content in the initial extract (µg)*	Area under curve	Measured content (µg/mL)	Content in the initial extract (µg)*
1	47.3	8117.37	2.51	118.723	5762.68	1.94	91.762
2	139.3	8571.91	2.66	370.538	1973.12	0.7	97.51
3	113.9	13405.05	4.25	484.075	4226.79	1.43	162.877
4	92.6	1367.9	0.29	26.854	ud	ud	ud
5	106.7	2108.52	0.53	56.551	ud	ud	ud
6	53.8	7069.69	2.17	116.746	2962.14	1.02	54.876
7	159.4	ud	ud	ud	ud	ud	ud
8	90.1	317.63	< 0.1	< 1.0	ud	ud	ud
9	28.2	3613.99	1.03	29.046	1185.16	0.44	12.408
10	16	3334.65	0.94	15.04	699.67	0.28	4.48
11	53.4	222.84	< 0.1	< 1.0	ud	ud	ud
12	31.7	ud	ud	ud	ud	ud	ud
13	139.3	307.7	< 0.1	< 1.0	ud	ud	ud

*Calculation refer to equation 1, ud: undetected



a **b**

Figure 3. The comparison of (a) Production of extract mass and (b). Bis-antraquinones produced from endophytic fungi *Diaporthe* sp. GNBP-10 is cultured in various media.

This study shows the optimized culture media composition for the production of bis-anthraquinones from endophytic fungi *Diaporthe* sp. GNBP-10. This finding can be used for optimum production of epi and bis components to meet a sufficient amount of epi and bis to further biological evaluation of both bis-anthraquinones.

Conclusion

This study is a cultured compositions optimization assay for the endophytic fungi *Diaporthe* sp. GNBP 10 (associated with Gambier plant) growth and metabolites expression. The optimized fungal extract was obtained from media no. 7 (159.4 mg), consisting of malt extract as a carbohydrate source. Meanwhile, the optimum epi and bis components production was observed in media no. 3 with the content of epi and bis in the respective extract are 0.484 mg and 0.1628 mg. The manually prepared culture media showed a better environment for producing the targeted anthraquinones than the commercially available PDB media. Potato starch provides a higher production level for bis-anthraquinones matched to the malt extract. The presence of glucose and dextrose is important to the production of bis-anthraquinones. Hopefully, this finding can be useful to produce the optimum amount of epi and bis compounds, especially from endophytic fungi *Diaphorte* sp GNBP-10.

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The authors' responsibilities are described as follows: **L.O:** concepting and analyzing. **R.F:** collecting and analyzing data **Evana:** analyzing and contributing to calculation, and **A.A:** designing, reviewing and supervising the project. All authors have equal contributions to this manuscript.

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