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Antioxidant Activity of Endophytic Bacteria isolated from (*Pyrrosia piloselloides*) (L) M.G. Price

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Abstract:

Endophytic bacteria produced analogous secondary metabolites of their hosts. Similarly, the ability to generate antioxidants is not an exception. Dragon scales (*Pyrrosia piloselloides*), an epiphytic plant of the Polypodiaceae family, are frequently overlooked. This research aims to isolate antioxidant-producing bacteria from dragon-scale fern leaves. The antioxidant activities were tested after the extraction procedure using ethanolic extract. Bacteria were characterized and selected as candidates for antioxidant production by screening for the production of total phenolic compounds. Antioxidant levels were determined utilizing the ABTS, FRAP, and DPPH techniques. The preliminary findings of the entire phenolic compound test revealed that isolates B2, B3, C, and F produced the most phenolic compounds. The highest antioxidant activity was created by bacterial isolates B3. Bacterial isolates D1 have similar characteristics to B3 and it has been identified as *Bacillus subtilis* isolates D1AS. The assessment technique and extract dosages have the greatest effect on antioxidant concentration. In comparison to other approaches, the DPPH method yields considerable results when screening prospective isolates to manufacture antioxidants. Testing the ability of the results of bacterial extracts on other therapeutic effects needs to be done to ensure that the antioxidant abilities obtained in this study continue to be developed.

Keywords: Antioxidants, *Bacillus*, Dragon scales, Endophytic-bacteria, Phenols

Introduction:

Microbes often grow and associate with plants, so it is not surprising that this interaction is mutually beneficial. Not only are they mutually beneficial, but endophytic microbes from medicinal plants can produce the same active metabolite compounds as their host plants[1]. Endophytes are symbionts microbes that infiltrate and multiply in plant tissues without producing illness or injury. Endophytic fungi and bacteria are widely found in the healthy tissues of living plants and are essential components of plant micro-ecosystems. They influence plant metabolite development, which affects the quality and amount of chemicals produced from medicinal plants [2]. For example, *Methylobacterium radiotolerans* MAMP 4754 was isolated from the therapeutic herb's seed *Combretum erythrophyllum*, according to one investigation. This bacteria produced antioxidant compounds and antibacterial activity against various pathogens [3].

The dragon scales fern (*Pyrrosia piloselloides*) (L) M.G. Price is well-known in Indonesia as a medicinal plant [4]. Exploration of dragon scale endophytic fungus shows the ability as an active ingredient against bacteria [5]. The types of fungi obtained were *Aureobasidium melanogenum*, *Penicillium allii-sativi*, and *Aspergillus flocculosus*. Dragon scales contain flavonoids, tannins, steroids or triterpenoids, essential oils, and glycosides that have the potential as anticancer ingredients. Methanol and water extracts from dragon scales fern did not show inhibitory results on breast cancer cells [6]. Meanwhile, leukemia cells are toxic [7]. Extraction using dichloromethane by fractionation using a n-hexane-ethyl acetate and ethyl acetate-MetOH combination has increased its antioxidant capacity and toxicity to breast cancer cells [8].

The antioxidant activity of the endophytic microbe of dragon scales has the potential to be developed as an anticancer. Unfortunately,

information on endophytic microbes from these plants is still limited to the types of fungi. There is no available information regarding endophytic bacteria and their antioxidant abilities compared to dragon-scale fern leaf extract. Based on this description, the researcher intends to isolate antioxidant-producing endophytic bacteria from the leaves of the dragon scale fern (*Pyrrosia piloselloides*) (L) M.G. Price obtained from the Lampung University campus environment. This antioxidant potential plays a vital role as a modulator of the immune system and inhibits the development of cancer cells through its toxic power [9,10]. This will be able to complete the added value of information about dragon scale ferns that are not yet available. Therefore, this research was proposed to identify antioxidant-producing bacteria from the leaves of dragon-scale ferns and evaluate the scavenging capacity using various methods.

Materials and Methods:

Plant material

Fresh *Pyrrosia piloselloides* (L) M.G. Price. leaves were collected during the rainy season (September to November 2020) in the region between Southern Lampung and Bandarlampung, near the University of Lampung campus forest, Lampung, Indonesia. In addition, The creeping mature leaves were collected randomly from tree bark species such as *Thuja occidentalis*, *Dalbergia latifolia*, *Filicium decipiens*, *Hyophorbe lagenicaulis*, *Cycas rumphii* Miq., and *Mangifera indica*.

Endophytic bacteria isolation

First, dragon scale leaves (DSL) had picked from several locations across the Universitas Lampung campus forest. Leaf samples were placed in sterile plastic and labelled. DSL was then cleansed with sterilized distilled water and immersed in 70% ethanol for 20 seconds. Before hardening, the leaves were sliced symmetrically and put into an agar substrate. Bacteria were isolated using a medium consisting of 4 g Yeast extract powder, 10 g Malt extract powder, 4 g Dextrose, 20 g Agar, and 1 L of distilled water (dH2O). The isolates were cultured at 37°C for 5 days before being purified and identified using morphological, physiological, biochemical, and molecular approaches.

Molecular identification

The purified inoculum was sub-cultured on nutrient agar media for 24 hours before being utilized to isolate DNA for molecular identification. Bacterial endophyte and Gram-negative pathogens were identified by PCR amplification and sequencing of 16S rDNA. The bacterial primers used were 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [11,12]. Successfully amplified PCR results from bacterial strains were forwarded to IPB Bogor-Genetic Science in Jakarta for sequencing. The Basic Local Alignment Search Tool (BLAST) was used to assess the sequencing findings. Meanwhile, the sequencing data in FASTA format is displayed in a phylogenetic tree analysis of neighbour-joining relationships compiled using the bootstrap method using Mega11 software.

Extract preparation

A bacterial starter is made up by taking a loop of inoculum from a purified isolate in a Petri dish and incubating it for 24 hours in 10 mL of NB medium. The starter was placed into 240 mL of NB media and incubated overnight at 180 rpm in a shaking state at room temperature. Cells separated from media using a centrifugation technique at 1800 rpm to obtain free cells supernatant. For the extraction, the supernatant was extracted liquidly by adding the equivalent volume of ethyl acetate solvent. The mixture was macerated in a shaker at room temperature for 24 hours. After filtering using Whatman paper number 1, extracts are obtained using a rotary evaporator. The extracted samples were preserved for further analysis [13,14].

Total phenolic content (TPC)

TPC was determined using the Folin-Ciocalteu reagent test (Merck, Germany), as reported in [15]. First, 0.5 mL of Folin-Ciocalteu and 6 mL of dH_2O were mixed into aliquots of 1 mL of each MetOH- diluted extract (Each sample is repeated in triplicate). Then, after 5 minutes of agitation, 1.5 mL of 20 % sodium carbonate was supplemented into 1.9 mL of dH2O while trying to shake. A UV-Vis spectrophotometer (Shimadzu UV-1800, Japan) was utilized to quantify absorbance at 760 nm following 2 hours in the dark.

The blank was made by replacing the same quantity of MetOH-diluted extract. The data were represented as gallic acid equivalent (GAE) per dry weight (dw) in milligrams and quercetin equivalents per milligram of dry weight (dw) [16]. The standard curves were generated using a gallic acid dosages of 0.001, 0.005, 0.01, and 0.02 mg/mL, respectively.

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

Using the technique modified from [17], plant extracts' antioxidant efficacy against DPPH was tested. First, a DPPH 1×10^{-4} M methanolic dilution was made. Then, 1 mL pipetted of each sample in the MetOH extract was taken (at three distinct densities: 0.5, 1, and 2 mg/mL; two repetitions per dosage and sample) and was mixed with 2 mililiter of DPPH MetOH dilution.

For 16 minutes, the mixture was stored in the dark at 37° C before measuring absorbance at 517 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Japan). The blank was created by diluting DPPH with MetOH. The findings were represented in milligrams of quercetin equivalents per milligram of dry weight. The calibration line was created using the following quercetin densities: 0.001, 0.002, 0.005, 0.01, 0.02, and 0.04 mg/mL. Inhibition ability was measured using a formula that refers to [18]

$$Chelating\ effect\ (\%) = 1 - \frac{AS}{AC} X\ 100 \ldots\ldots\ldots 1$$

AS: absorbance of the sample
AC: absorbance of control

ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) inhibition

The antioxidant activity of the bacterial extracts in the research against ABTS was measured using the technique described in [19]. Oxidizing ABTS created the radical ABTS•+ with potassium persulfate ($K_2S_2O_8$). A 1:1 (v/v) combination of ABTS (7 mM) and potassium persulfate (4.95 mM) was produced and stored in the black at room temperature for 16 hours.

The mixture was then diluted with MetOH till transmittance values of 1-1.5 at 734 nm were obtained. Next, 3.9 mL of the $ABTS^{\bullet+}$ solution was increased to aliquots of 0.1 mL of each sample's MetOH extract (at 3 different compositions: 0.5, 1, and 2 mg/mL; with per concentration and sample duplicated). Next, a UV-1800 spectrophotometer was utilized to quantify the absorbance variation at 734 nm. Finally, the blank was prepared using $ABTS^{\bullet+}$.

The outcomes were stated in milligrams of quercetin equivalents per milligram of dry weight. The standard line was created using the following quercetin densities: 0.00062, 0.00125, 0.0025, 0.005, 0.01, and 0.032 mg/mL.

Ferric Reducing Antioxidant Power (FRAP)

The FRAP test was adjusted following approach [20]. An amount of 3.8 mL of FRAP catalyst was used to aliquots of 0.2 mL of MetOH extract (at three different densities: 0.5, 1, and 2 mg/mL; 2 duplicates per sample and densities). Previously, this reagent was made by combining ten parts 300 mM sodium acetate buffer dilution at pH 3.6, 1 part 10 mM $T_{PZ}T$, and 1 part 20 mM Iron (III) Chloride hexahydrate (Merck, Germany).

The resultant solution was hatched for 30 minutes at 37° Celsius. A UV-Vis spectrophotometer was used to detect the absorbance increase at 593 nm (Shimadzu UV-1800, Japan). The blank was prepared by replacing the same quantity of MetOH-diluted extract. The findings were reported in milligram equivalents of $FeSO_4$ per milligram dry weight. The adjustment curve was created using $FeSO_4$ values of 0.0025, 0.005, 0.01, and 0.02 mg/mL.

Data analysis

Bacterial extract, dose treatment, antioxidant test method, and bacterial isolate data were processed using SPSS 25 software and the Pearson correlation analysis method.

Result and Discussion:

Result

The dragon scales were collected from various locations around the University of Lampung (Unila) campus, including the Unila roundabout, the Hajimena roundabout, the dean counter building, and the chemical warehouse (Fig 1). Endophytic bacteria were obtained and cultured using the Yeast Starch Agar medium. After three days of incubation, several isolates occurred and were purified in Nutrient Agar as described follows (Fig 2 and Table 1). Most of the bacteria isolated were Gram-positive bacteria, rod-shaped.

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Table 1. Endophytic bacteria isolates collected from a variety of tree species topped with dragon scale fern on the Lampung University site

Isolates	Tree bark- Host	Local name	Gram	Shape
A1	*Cycas rumphii* Miq.	Pakis haji	+	Rod
A2	*Cycas rumphii* Miq.	Pakis haji	+	Rod
A3	*Cycas rumphii* Miq.	Pakis haji	+	Rod
A4	*Cycas rumphii* Miq.	Pakis haji	+	Rod
B1	*Hyophorbe lagenicaulis*	Palm botol	+	Cocci
B2	*Hyophorbe lagenicaulis*	Palm botol	+	Rod
B3	*Hyophorbe lagenicaulis*	Palm botol	+	Rod
C	*Thuja occidentalis*	Cemara	+	Rod
D1	*Filicium decipiens*	Kerai paying	+	Rod
D2	*Filicium decipiens*	Kerai paying	-	Rod
E	*Dalbergia latifolia*	Sono keling	+	Rod
F	*Mangifera indica*	Mangga	+	Rod

Figure 1. Location for sampling around the Unila campus

Figure 2. Bacterial culture and isolation on YS Agar media

The ethanolic extract from the endophytic bacteria of the dragon scale fern leaf found that Strain B3 has the largest TPC, followed by strains B2, C, and F in units of GAE per milligram of dry weight (dw) (Fig 3).

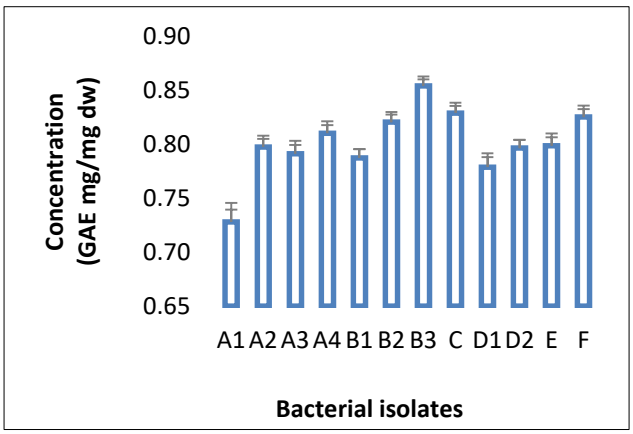

Figure 3. Total phenolic activity from the endophytic bacterial extract of dragon scale fern leaves.

Characterization was carried out on the DSL strain D1, referring to the data on the ability to produce total phenolic compounds (Fig 3). Almost all Gram positive isolates had the same characteristics, especially B3 and D1 (Table 2). These bacteria belong to the cellulolytic group, capable of growing on glucose, sucrose, and fructose substrates. Molecular identification was obtained that strain D1 was Bacillus subtilis strain D1AS with accession number OP108573 (Fig 4). Although strain D1 produced more TPC than the other strains, its capacity to block free radicals was not superior. The highest antioxidant ability was produced by strain B3 (Fig 5).

Table 2. DSL endophyte bacterium isolates B3 and D1 characteristics

Test		Result
Hydrolase	Cellulase	+
	Amylase	-
Antibiotic susceptibility	Streptomycin	Not resistance
	Chloramphenicol	Not resistance
	Clindamycin	Not resistance
	Griseofulvin	Not resistance
	Ketoconazole	Not resistance
Carbon Sources	Glucose	+
	Lactose	-
	Fructose	+
	Galactose	-
	Sucrose	+
	Starch	-
	Cellulose	+
Indole		-
Motile		-
Gram stain		+

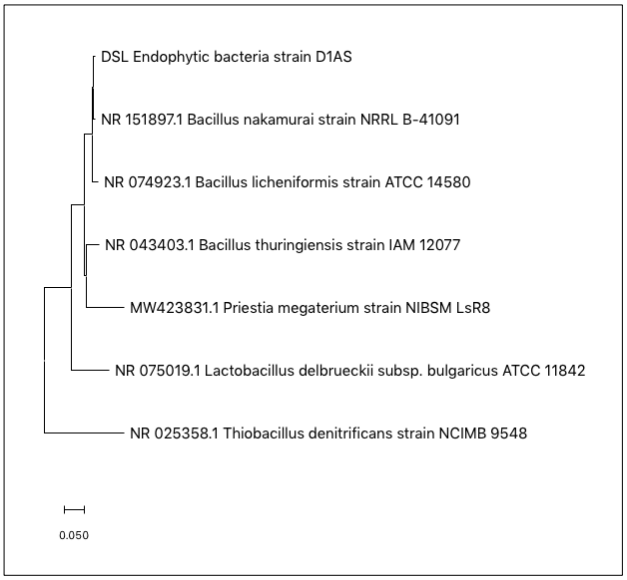

Figure 4. The DSL Endophytic bacterium strain D1 was built utilizing the neighbor-joining approach. Characterization data of isolates B3 and D1 have similarities in morphology, biochemistry, and molecular terms.

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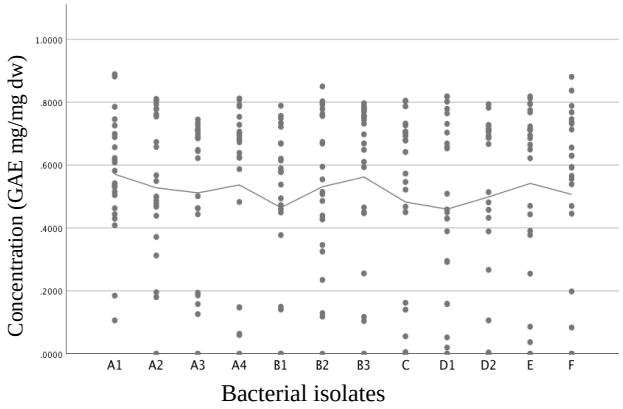

Figure 5. Simple scatter with fit line of concentration by bacterial isolates

Related to the types of methods for measuring antioxidants produced by the endophytic bacteria group in DSL, it turns out that there is an effect of differences in use. Based on different tests on non-parametric data plots using Pearson correlation, the DPPH method had significant differences compared to other methods in measuring antioxidant ability P<0.01 (Fig 6). The measured antioxidant concentration will increase depending on the types of accurate measurement method and the accuracy of dosing P<0.01 (Fig 7, Table 3). Thus, the doses treatment of ethanol extract from endophytic bacteria of DSL was higher at 2 mg/ml compared to other concentrations in antioxidant activity (Fig 8).

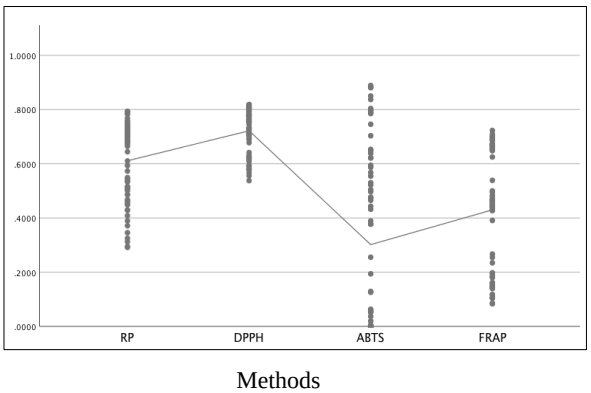

Figure 6. Simple scatter with fit line of concentration by methods. QE: quercetin equivalent per milligram of dry weight for RP, DPPH, and ABTS. $FeSO_4$ equivalents per milligram of dry weight for FRAP method.

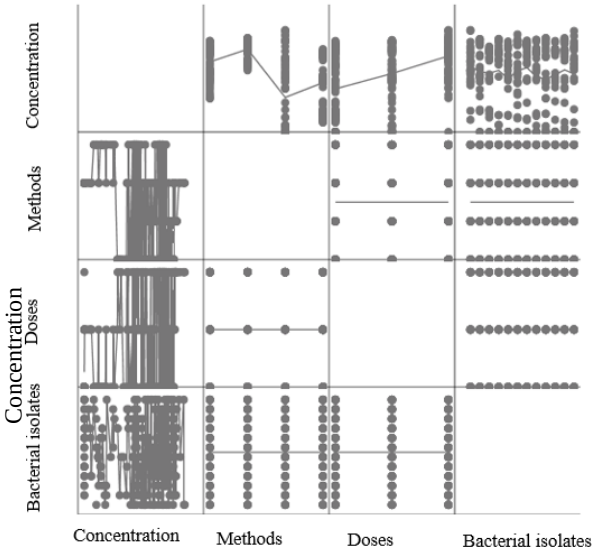

Figure 7. The relationship of a number of parameters is visualized in the Scattermatrix using the Pearson Correlation calculation method

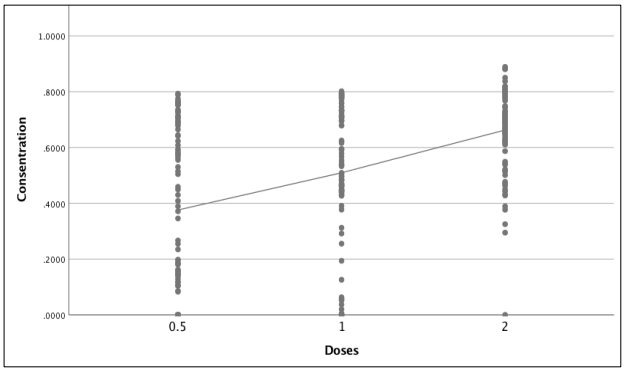

Figure 8. The effect of doses treatment of ethanol extract from endophytic bacteria of DSL to antioxidant activity using FRAP methods. Concentration calculated in FeSO4 equivalents per milligram of dry weight, while doses treatment given in $mg.mL^{-1}$

Table 3. Pearson correlations between bacterial isolate, doses and antioxidant methods

		Bacterial isolates	Doses	Methods	Concentration
Bacterial isolates	Pearson Correlation	1	.000	.000	-.044
	Sig. (2-tailed)		1.000	1.000	.455
	N	288	288	288	288
Doses	Pearson Correlation	.000	1	.000	.441**
	Sig. (2-tailed)	1.000		1.000	.000
	N	288	288	288	288
Methods	Pearson Correlation	.000	.000	1	-.406**
	Sig. (2-tailed)	1.000	1.000		.000
	N	288	288	288	288
Concentration	Pearson Correlation	-.044	.441**	-.406**	1
	Sig. (2-tailed)	.455	.000	.000	
	N	288	288	288	288

**. Correlation is significant at the 0.01 level (2-tailed).
If it returns a negative value, it indicates that the connection is not linear.

The Pearson value of correlation among concentration and bacterial isolates was -.044. Because this statistic was negative, this indicates that these two parameters own a negative relationship. The p-value/ Sig. (2-tailed) related to the coefficient of correlation for concentration and bacterial isolates. 455. Since this value is not less than 0.01, the two variables don't have a statistically significant association. To determine the Pearson correlation analysis, 288 pairings (N) were employed. Concentration gave a significant relationship with doses (.441) and methods (-.406). Positive correlation occurs when antioxidant concentration is measured according to doses treatment, in the opposite not only bacterial isolate show a negative correlation to antioxidant concentration but also happened to antioxidant methods utilized.

Discussion:

Unpaired electrons in atoms or molecules constitute free radicals. Because electrons require a partner to balance their spin values, the radical molecule becomes unstable and quickly interacts with other molecules, resulting in a chain reaction [21]. If the free radicals are neutralized by an antioxidant, the chain reaction will come to an end [22]. Most illnesses are originated and induced by an overabundance of free radicals in the body. Due to the effect of free radicals that are harmful to the body's health, the body requires a crucial component that counteracts free radical assaults [23]. Antioxidants are an important component that can protect human body cells from the risks of free radicals [24].

The ability of DSL to produce antioxidants has been widely reported. Physiological activities between hosts and their symbionts are often recorded to have similarities. Medicinal plant hosts, for example, have anticancer abilities, followed by the same abilities by endophytic microbes [25]. This study aims to determine the ability of the antioxidant activity of DSL endophytic bacteria.

In this study, DSL was collected from different tree species, namely *Cycas rumphii* Miq., *Hyophorbe lagenicaulis*, *Thuja occidentalis*, *Filicium decipiens*, *Dalbergia latifolia*, and *Mangifera indica*. Endophytic bacteria which obtained dominated by Gram-positive bacteria, with the exception found in *Filicium decipiens* whereas both Gram-positive and negative are presented to live. DSL was a fern group that crept to the host, *Pyrrosia piloselloides* was also a facultative mild hemiparasite rather than a typical parasite. DSL was a fern group that crept to the host, *Pyrrosia piloselloides* was also a facultative mild hemiparasite rather than a typical parasite. To live with the host, DSL anchored the root hairs and inserted two or three cell layers deep inside the cortex of the host branches [26]. It is no wonder why endophytic bacteria from DSL were able to produce the cellulolytic enzyme. Cellulase was utilized by bacteria to degrade cellulose compounds that were commonly found in wood tissue [27].

TPC determination seeks to determine the relationship between antioxidant activity and total phenolic content. The Folin-Ciocalteu solution and a gallic acid sample were used to calculate TPC. The presence of hydroxyl groups in phenolic compounds contributes to their antioxidant capacity. When interacting with radical chemicals, the hydroxyl group acts as a donor of hydrogen atoms via an electron transfer mechanism. Therefore, inhibiting the oxidation process [28]. According to the result, most of the bacterial strains were able to produce phenolic compounds (Fig 3) and chelate free radicals that were measured in an antioxidant assay. High

TPC levels have been shown to affect increasing antioxidant abilities. This concept was linear with the findings of [29]. It can be seen when B3 isolates were placed at the top of TPC producers, they also ranked in a similar position at an antioxidant concentration (Fig 5).

The DPPH method has the best sensitivity in this study to measure the concentration of antioxidant activity produced by endophytic bacteria of DSL. The DPPH technique was discovered to be the most often used method for evaluating in vitro antioxidant activity, with ethanol being the most frequently used solvent for extraction [30,31]. Antioxidant concentration is mostly influenced by the evaluation method and doses of extract in our findings (Fig 7 and Table 3). Although the principles of each category of antioxidant test techniques are similar, their sensitivity is affected by a variety of parameters, including medium pH, the presence of lipophilic and/or hydrophilic chemicals, and others. As a result, it is strongly advised to use more than one approach to evaluate antioxidant qualities, especially when studying such phytochemically complex matrices and products [32].

Antioxidant activity does not only play a role concerning free radicals but the capacity to scavenge free radicals can also lower cancer risk [33,34]. Besides cancer, heavy metal stress also can be alleviated by producing antioxidants [13,35]. Administration of *Bacillus subtilis* is reported to be able to induce antioxidant mechanisms and reduce the impact of injury, which often thwarts herbaceous plant grafting technology in tomatoes. This is because *B subtillis* is able to control the production of antioxidant immune systems such as superoxide dismutase (SOD), catalase (CAT), and phenylalanine ammonia-lyase (PAL) [36]. It should be suspected that this role also helps DSL in penetrating its host tissue without being hindered by the host's immune system. On another role for humans, antioxidants can also help to prevent cell damage by stimulating cell regeneration. Reduced free radicals will eventually result in fewer metabolic abnormalities, allowing blood circulation to flow freely and the enzymatic process to function normally [37]. Exploration of compounds that produce antioxidants also needs to be identified, which means that outstanding opportunities are still available. Further research development is essential to ensure that the metabolite capabilities obtained from the current results are more comprehensive.

Conclusion:

This study discovered 12 bacteria strains retrieved from the leaves of the dragon scales fern, with Gram-positive bacteria showing greater than Gram-negative. *Cycas rumphii* Miq plant contains much more isolates compared to other host plants. Using the TPC, DPPH, ABTS, and FRAP techniques to identify bacteria that produce more antioxidant activity, isolate B3 outperforms the others. Statistical analysis revealed that the DPPH method has significant findings in evaluating the potential isolates to generate antioxidants rather than other techniques.

Authors' Declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in Universitas Lampung.

Authors' Contributions Statement:

A.A. conceived of the presented idea, developed the theory and performed the computations. S.F. verified the analytical methods, encouraged to investigate bacteria and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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نشاط مضادات الأكسدة للبكتيريا الباطنة المعزولة من *(Pyrrosia piloselloides) (L) M.G.*

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الخلاصة:

أنتجت البكتيريا الباطنة مستقلبات ثانوية مماثلة لمضيفيها. وبالمثل، فإن القدرة على توليد مضادات الأكسدة ليست استثناءً. Dragon scales(Pyrrosia piloselloides)، هو نبات هوائي من عائلة Polypodiaceae كثيرا مايتم تجاهلها. يهدف هذا البحث إلى عزل البكتيريا المنتجة لمضادات الأكسدة من أوراق السرخس على Dragon scales. تم اختبار الأنشطة المضادة للأكسدة بعد إجراء الاستخراج باستخدام المستخلص الإيثانولي. تم وصف البكتيريا واختيارها كمرشحة لإنتاج مضادات الأكسدة عن طريق الفحص لإنتاج المركبات الفينولية الإجمالية. تم تحديد مستويات مضادات الأكسدة باستخدام تقنيات ABTS و FRAP و DPPH. كشفت النتائج الأولية لاختبار المركب الفينولي بأكمله أن عزل B2 و B3 و C و F ينتج معظم المركبات الفينولية. تم إنشاء أعلى نشاط مضاد للأكسدة بواسطة العزلات البكتيرية B3. تتمتع العزلات البكتيرية D1 بخصائص مماثلة لـ B3 وقد تم تحديدها على أنها عصوية فرعية تعزل D1AS. تقنية التقييم والجرعات المستخلصة لها أكبر تأثير على تركيز مضادات الأكسدة. بالمقارنة مع الأساليب الأخرى، تحقق طريقة DPPH نتائج كبيرة عند فحص العزلات المحتملة لتصنيع مضادات الأكسدة. يجب إجراء اختبار لقدرة نتائج المستخلصات البكتيرية على التأثيرات العلاجية الأخرى لضمان استمرار تطوير القدرات المضادة للأكسدة التي تم الحصول عليها في هذه الدراسة.

الكلمات المفتاحية: مضادات الأكسدة ، العصيات ، قشور التنين ، بكتيريا داخل النبات، الداخلي ، الفينولات