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Evaluation of Total Phenolic and Flavonoid Contents, Antioxidant, and Antibacterial Activities of Crude Extracts from Roots of *Diospyros Gardneri*

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Abstract

This research aimed to investigate the total phenolic and flavonoid contents, along with the antioxidant and antibacterial activities present in roots of *Diospyros gardneri*, utilizing various solvents with different polarities. The samples underwent maceration using n-hexane, ethyl acetate, and methanol. Subsequently, the extracts obtained were subjected to phytochemical analysis to determine the levels of flavonoids and phenolics. The antioxidant activity was assessed using the DPPH method, while the antibacterial activity was tested using the disc diffusion method. The results revealed that the crude extracts contained phenolics at concentrations of 0.31, 0.60, and 0.82 mg GAE/mL sample, respectively. Additionally, they contained flavonoids at concentrations of 0.34, 0.65, and 1.38 mg QE/mL sample, respectively. The DPPH antioxidant activity percentages of the crude extracts stood at 47.08%, 79.95%, and 75.23%, respectively. The crude extracts exhibited antibacterial activity against *Bacillus cereus*, *Staphylococcus aureus*, and *Escherichia coli*. Furthermore, both the crude n-hexane and ethyl acetate extracts could inhibit *Vibrio harveyi*. Among these, the ethyl acetate extracts demonstrated significant inhibition against *S. aureus* (gram-positive bacteria), with the highest observed inhibition percentage recorded at 33.17±0.02%. These findings imply that the root of *D. gardneri* harbors bioactive compounds potentially beneficial for disease protection. Therefore, it stands as a valuable source of antioxidants and antibacterial elements, paving the way for the development of pharmaceutical applications and health products that capitalize on *D. gardneri*'s properties.

Keywords: *Diospyros gardneri*; Phenolic; Flavonoid; Antioxidant; Antibacterial

1. Introduction

The genus Diospyros is a diverse group of plants from the family Ebenaceae. It includes species that have economic and ethnomedicinal importance, yielding valuable timber and edible fruits. Diospyros plants have long been used in folk medicine and have been found to contain various bioactive compounds with therapeutic properties (Somat *et al*., 2020; Ojha *et al*., 2023; Wuttikit and Thanakijcharoenpath, 2023). Studies on the chemical composition of the genus Diospyros have revealed many important substances such as peptides, sterols, diterpenoids (Feusso *et al*., 2017), phenolics (Chang *et al*., 2009), flavonoids (Kwon *et al*., 2021; Somat *et al*., 2020), triterpenoids (Tameye *et al*., 2022), and naphthoquinones (Cai *et al*., 2000; Higa *et al*., 1998). These compounds have been associated with a range of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, and neuroprotective effects.

Phenolics and flavonoids can be extracted using a variety of solvents with varying degrees of polarity. Solvents commonly used for the extraction of phenolic and flavonoids include n-hexane (non-polar), ethyl acetate (semi-polar), and methanol (polar) (Islam *et al*., 2021). These compounds can neutralize free radicals in the human body. These compounds work by donating electrons to free radicals, preventing them from damaging cells and potentially indirectly preventing biological damage such as cancer, diabetes, and other diseases (Pádua *et al*., 2015; Cesari *et al*., 2013). Not only do these compounds enhance the antioxidant capacity of natural products, but they also exhibit promising antibacterial activities against a wide range of bacteria. Analytical techniques like spectrophotometry enable the quantification and characterization of these bioactive compounds, paving the way for their use in developing new therapies and improving the quality of consumer products.

In this research, we are interested in studying the roots of *Diospyros gardneri*, a medium-sized tree thriving in the moist, lowland tropics. Known as 'Paya-Fai' or 'Hung Hon' in Thai, this tree has been utilized in folk medicine. Despite its traditional use, there have been no reports regarding its phytochemical composition and bioactivities. Our aim is to evaluate the total phenolic and flavonoid contents, as well as the antioxidant and antibacterial activities of various crude extracts—hexane, ethyl acetate, and methanol—obtained from *D. gardneri* root.

2. Materials and Methods

2.1 Plant Materials

The roots of *Diospyros gardneri* were collected in June 2021 from Sri Mueang Mai District, Ubon Ratchathani Province, Thailand. The sample was certified by botanists at Faculty of Interdisciplinary Studies, Khon Kaen University, Nong Khai Campus, Nong Khai, Thailand.

2.2 Extraction Procedure

The fresh root of *D. gardneri* was thoroughly washed with water and then air-dried at room temperature for 24 h before being ground into powder using a hammer mill (Retsch type SK 100/C Gusseisen). Subsequently, it underwent extraction through the maceration method. The dried sample (3.1 kg) were sequential macerated extraction for with 15 L of solvent, initially using n-hexane, followed by ethyl acetate,

and finally methanol. The extraction time was two days for each solvent. Then, each extract was filtered using 150 mm diameter filter paper (Whatman No. 1) and subsequently evaporated using a rotary evaporator (Eyela type N-1200-BV) to yield a crude extract comprising n-hexane, ethyl acetate, and methanol components. The percentage extraction yield for the selected plants was calculated using the following equation (Felhi *et al*., 2017):

$$\text{Yield (\%)} = (X_1 * 100) / X_0$$

Where X_1 refers to the weight of extract after evaporation of solvent and X_0 refers to the dry weight of the plant before extraction.

2.3 Determination of Total phenolic content

The method for determining total phenolic content was adapted from the Folin-Ciocalteu reagent method as described by Lou *et al*. (2014). The crude extract was dissolved in methanol to achieve a concentration of 30 mg/mL. Subsequently, 0.2 mL of the extract was pipetted into a test tube, followed by the addition of 5 mL of distilled water. Then, 0.5 mL of Folin–Ciocalteu reagent was added, thoroughly mixed for 5 minutes, and thereafter, 1.5 mL of sodium carbonate solution (75 g/L) was added to the mixture. After thorough mixing, the solution was left at room temperature in the dark for 90 minutes, following which the absorbance was measured at 725 nm using a UV-vis spectrophotometer (Peak type E-1000V). The absorbance values were recorded and calculated relative to the gallic acid standard. The total phenolic content was expressed as mg gallic acid equivalents (GAE)/mL.

2.4 Determination of Total flavonoid content

The flavonoid content was determined following the method of Lasunon *et al*. (2022). The crude extract was dissolved in methanol to reach a concentration of 30 mg/mL. Subsequently, 0.3 mL of the extract was pipetted into a test tube. Then, 2 mL of distilled water and 0.15 mL of 5% sodium nitrite were added, mixed thoroughly, and left for 5 minutes. Following this, 0.15 mL of aluminum nitrate was added and left for another 5 minutes. Afterward, 1 mL of 1 molar sodium hydroxide was added, and the absorbance at 420 nm was measured using a UV-vis spectrophotometer. The absorbance value was recorded and calculated against the quercetin standard. The total flavonoid content was expressed as mg quercetin equivalents (GAE)/mL.

2.5 Antioxidant activity by DPPH Assay

Determination of antioxidant activity was conducted using the 2,2-Diphenyl-1-picrylhydrazyl radical scavenging capacity (DPPH assay), adapted from the method described by Chen *et al*. (2008). Initially, a solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) was prepared with a concentration of 0.1 mM in ethanol. The crude extract was dissolved in methanol to achieve a concentration of 30 mg/mL. Subsequently, 0.1 mL of the extract was taken and placed in a test tube, followed by the addition of 2.9 mL of methanol containing the DPPH solution at a concentration of 0.1 mM. The mixture was thoroughly shaken and left at room temperature in the dark for 30 minutes. Concurrently, a control sample was prepared by substituting the

extract with ethanol after the same 30-minute duration. Both the extract samples and control samples were collected, and their absorbance was measured at 517 nm using a UV-vis spectrophotometer. The percentage of antioxidant activity (percent inhibition) was calculated using the following formula:

$$\%\text{inhibition} = [(A_{control} - A_{sample}) / A_{control}] * 100$$

When $A_{control}$ was the absorbance of blank (95% ethanol) and A_{sample} was the absorbance of the extract

2.6 Antimicrobial activity

The antimicrobial activity of crude hexane, ethyl acetate, and methanol extracts from *D. gardneri* was evaluated using the disc diffusion method according to Gonelimali *et al*. (2018). The microorganisms used in this study included Gram-positive bacteria (*Bacillus cereus* 036 (BC), *Staphylococcus aureus* 746 (SA)) and Gram-negative bacteria (*Escherichia coli* 117 (EC), *Vibrio harveyi* 2088 (VH), *Pseudomonas aeruginosa* 357 (PA)), all obtained from the Thailand Institute of Scientific and Technological Research (TISTR). The bacteria were grown in nutrient agar (NA) at 37°C for 24 hours. Colonies were diluted with sterile water to an optical density (OD600) or compared for turbidity with McFarland No. 0.5 ($1.5x10^8$ CFU/mL). This solution was then spread on Mueller Hinton Agar (MHA). Subsequently, sterile paper discs with 6 mm diameters were impregnated with each type of crude extract at a volume of 250 µL or 2,000 mg/disc (8 mg/mL). The negative control was prepared using 5% DMSO (250 µL), while the positive control consisted of gentamicin (20 µL at 0.5 mg/mL). Incubation occurred at 37°C for 24 hours. The appearance of clear zones around the discs was observed, and the diameters of the three clear zones were measured. The percentage inhibition was calculated using the following equation:

$$\%\text{inhibition} = [(\text{clear zone } ØT - \text{clear zone } ØN) / \text{clear zone } ØP] * 100$$

When ØP is the clear zone Positive control (mm), ØT is the clear zone (mm) and ØN is the clear zone Negative control (mm)

2.7 Statistical analysis

All research results show repeated emphasis on the third point. ±Control section (SD) results were derived using a one-way analysis of variance (ANOVA) to test the significant control effort. Statistical significance, considered at $P<0.05$, was assessed solely through one-way distinctiveness analysis on SPSS (IBM Statistics SPSS 28).

3. Results and Discussion

3.1 Yields of extraction

The weight of the dried roots from *D. gardneri* before extraction was 3.1 kg. They were extracted using the solvents n-hexane, ethyl acetate, and methanol, respectively. This selection was based on each solvent's ability to dissolve polar compounds, as noted by Dlamini *et al*., 2023. The extraction yields of different fractions from *D. gardneri* are presented in Table 1. The crude methanol extract resulted in 150.10 g, constituting the highest fraction yield after extraction at 4.84%. This is attributed to methanol being a highly polar solvent, which enhances its efficacy in extraction. Additionally, the crude ethyl acetate extract weighed 37.60 g, equivalent to a yield after extraction of 1.21%. Similarly, the crude n-hexane extract weighed 5.39 g, corresponding to a yield after extraction of 0.17%.

Table 1. Fractions of extraction yield of *D. gardneri* root

Solvents extracted	Crude extracted (g)	Yield after extraction (%)
n-hexane	5.39	0.17
ethyl acetate	37.60	1.21
methanol	150.10	4.84

3.2 Total phenolic content

The quantification of total phenolics was conducted to establish the correlation between antioxidant activity and the total phenolic content present in the samples. This investigation stemmed from the well-documented strong antioxidant effects associated with phenolic compounds (Huyut *et al*., 2017). The results illustrating the total phenolic content of the *D. gardneri* extracts are presented in Figure 1. The highest level of total phenolic content was observed in the methanol extract, followed by the ethyl acetate and n-hexane extracts. This hierarchy can be attributed to the semi-polar and polar nature of phenolic compounds, resulting in a greater extraction of these compounds in semi-polar and polar solvents, specifically ethyl acetate and methanol. The higher total phenolic content in the methanol extract compared to the ethyl acetate extract is due to methanol being a more polar solvent than ethyl acetate, thereby facilitating the extraction of a larger quantity of phenolic compounds.

Our findings were aligned with research on the total phenolic content of *Diospyros* species like *Diospyros melanoxylon* Roxb (Jaiwal *et al.,* 2012). Methanol extracts exhibited higher total phenolic content (1.78±0.06 mg GAE/mL) compared to ethyl acetate (0.20±0.03 mg GAE/mL). In *Diospyros villosa* (Adu *et al.,* 2022), the highest total phenolic content in leaves was observed in the methanol extract (28.45±0.50 mg GAE/g) compared to n-hexane. Conversely, the highest total phenolic content in stem bark was found in n-hexane extract (14.40±0.58 mg GAE/g) compared to methanol. The studies indicated that the methanol was often gave the optimal extraction results for phenolic compounds in *Diospyros*. However, the choice of solvent was depended on the *Diospyros* species and plant part used for extraction.

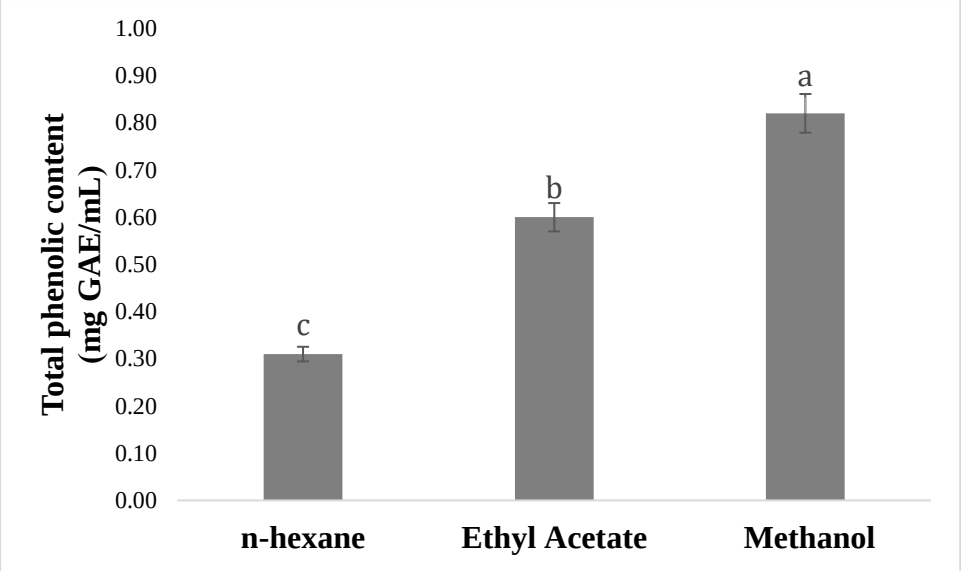

Figure 1. Total phenolic content of crude extracts from *D. gardneri* root

3.3 Total flavonoid content

The total flavonoid content was determined to establish the relationship between antioxidant activity and the concentration of flavonoid compounds in the samples. Flavonoids are widely recognized for their potent antioxidant effects (Huyut *et al*., 2017). The results of the determination of total flavonoid levels from *D. gardneri* extracts are presented in Figure 2. The highest total flavonoid content was observed in the methanol extract, followed by the ethyl acetate and then n-hexane extracts. This order is attributed to the semi-polar and polar nature of flavonoid compounds, which results in a greater extraction of these compounds in semi-polar and polar solvents, such as ethyl acetate and methanol. The higher total flavonoid content in the methanol extract compared to the ethyl acetate extract can be attributed to the higher polarity of methanol as a solvent, which facilitates the extraction of a greater number of flavonoid compounds.

Our results were in line with the research investigating the phytochemical screening of flavonoids in methanol, chloroform, and hexane extracts from the leaves and stem bark of *Diospyros villosa* (Adu *et al.,* 2022). Methanol extracts exhibited the highest flavonoid content in both parts compared to n-hexane, suggesting that methanol generally provides superior extraction results for flavonoid compounds in *Diospyros*.

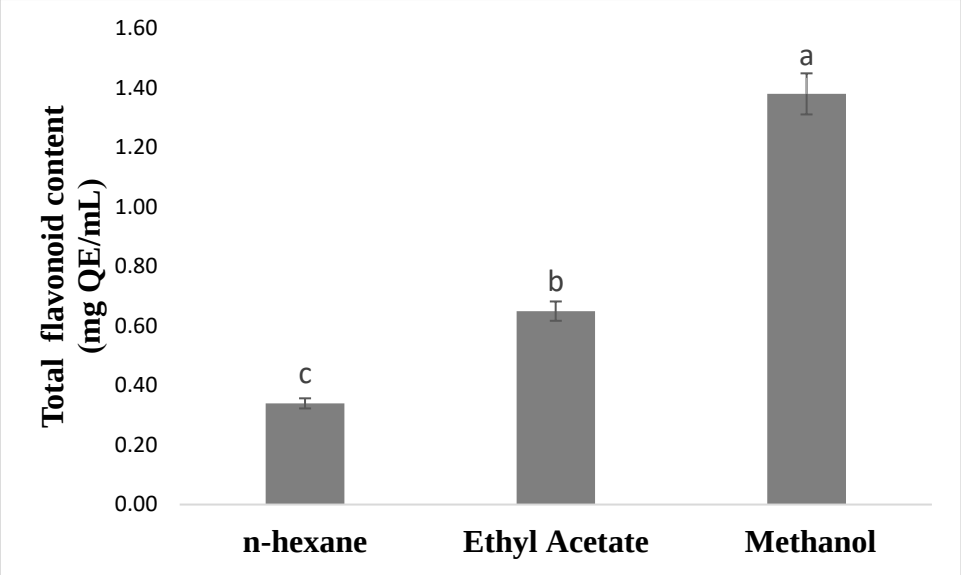

Figure 2. Total flavonoid content of crude extracts from *D. gardneri* root

3.4 Antioxidant activity

The antioxidant activity testing of *D. gardneri* extracts was conducted using the DPPH method. DPPH functions as a molecule that accepts hydrogen or free electrons from the precursor or antioxidant compounds present in the sample until it transforms into a stable diamagnetic molecule. When it receives hydrogen from an antioxidant and forms DPPH, its initial purple color changes to yellow (Moon and Shibamoto, 2009). The percentage value of antioxidant extracts from *D. gardneri* is depicted in Figure 3. This value indicates the extract's capability to inhibit the activity of free radicals. Extracts exhibiting a higher percentage of antioxidant activity are considered stronger. The DPPH antioxidant activity percentages of the crude extracts were 47.08%, 79.95%, and 75.23%, respectively. The higher antioxidant activity in the ethyl acetate extract compared to methanol suggests that the crude ethyl acetate might contain phenolic or flavonoid compounds that are more active than those in the crude methanol. Our findings were consisted with the research that examined the antioxidant activity of *Diospyros bipindensis* (Cesari *et al.,* 2013). The ethyl acetate extract showed the highest antioxidant activity (23.6%), followed by methanol (17.9%), and n-hexane (3.7%) at a concentration of 40 µg/mL. Consequently, further investigation into the secondary metabolites is warranted for confirmation.

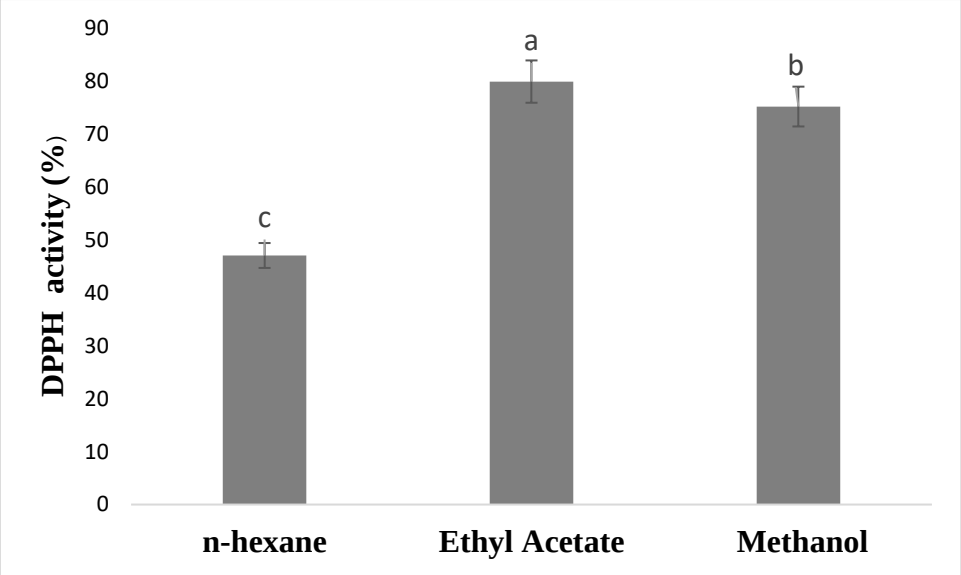

Figure 3. DPPH activity radicals of crude extracts from *D. gardneri* root

3.5 Antimicrobial activity

The research assessed the antimicrobial effects of n-hexane, ethyl acetate, and methanol extracts from *D. gardneri* against various bacterial species by observing the clear zones. Table 2 illustrates the antimicrobial activities of these extracts. Results indicated that all crude extracts demonstrated antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus*, and *Escherichia coli*. Moreover, the crude n-hexane and ethyl acetate extracts effectively inhibited *Vibrio harveyi*. However, none of the crude extracts showed a zone of inhibition against *Pseudomonas aeruginosa*. Figure 4 visually represents the zone of inhibition observed. The cell wall structure of gram-positive bacteria differs from that of gram-negative bacteria by lacking the outer membrane and possessing a thick layer of peptidoglycan that surrounds the plasma membrane, thereby providing protection to gram-positive bacteria (Jubeh *et al.*, 2020). However, this suggests the necessity for further investigation into the mechanism of bacterial inhibition to confirm these differences.

Our results were aligned with research investigating the antibacterial activity of *Diospyros villosa* (Adu *et al.*, 2022). The zone of inhibition of the n-hexane stem-bark extract against *Klebsiella pneumoniae* was higher than the methanol stem-bark extract at concentrations of 10, 5, 2.5, 1.25, and 0.625 mg/mL. Similarly, the zone of inhibition of the chloroform leaf and stem-bark extract against *K. pneumoniae* was higher compared to the methanol leaf and stem-bark extract and the n-hexane leaf extract at the same concentrations.

Besides these, despite having the highest yield, TPC, and TFC, the methanol extract exhibited the least antibacterial activity, likely due to the bioactive compounds present in the crude extracts. Therefore, further research is needed to isolate these active molecules and thoroughly evaluate their biological activities.

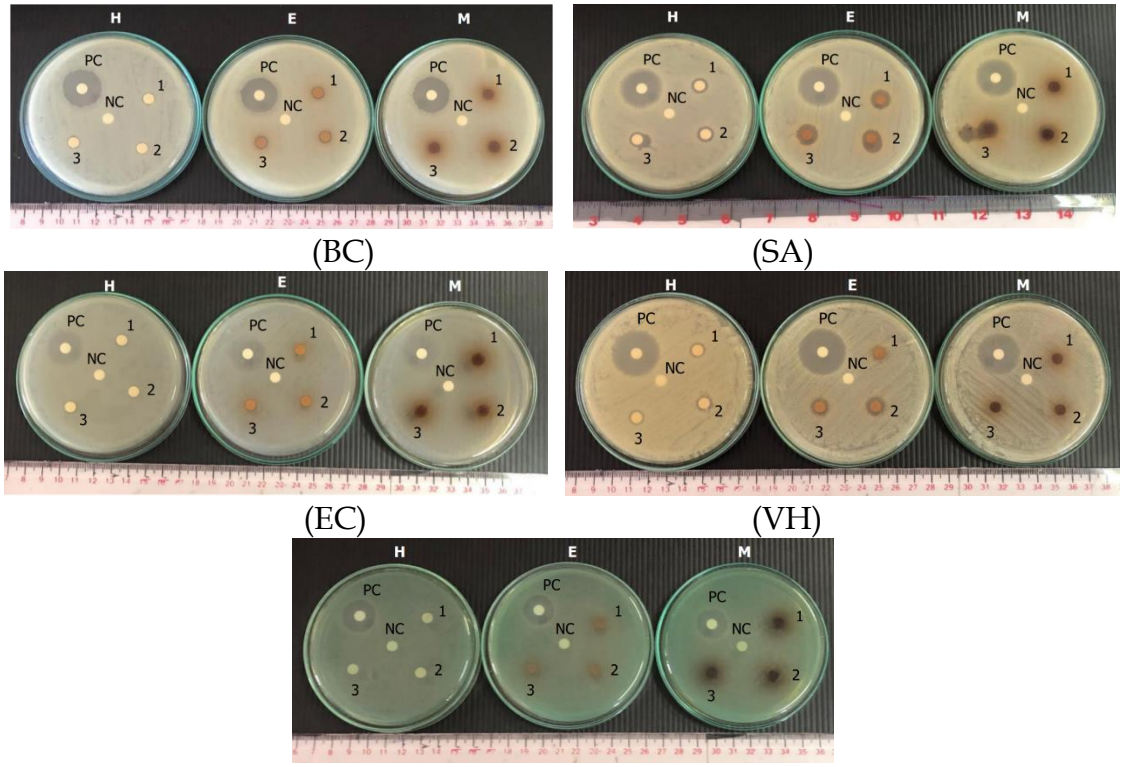

Figure 4. Disc diffusion inhibition zone of (BC) *Bacillus cereus*, (SA) *Staphylococcus aureus,* (EC) *Escherichia coli*, (VH) *Vibrio harveyi* and (PA) *Pseudomonas aeruginosa*. Tested with 250 µL of 2,000 µg/µL from *D. gardneri* extracts (H) n-hexane (E) ethyl acetate and (M) methanol. Disc (PC) is the positive control of 0.5 µg/µL (20 µL) from gentamicin. Disc (NC) is the negative control of 5% DMSO (250 µL).

Table 2. Antibacterial activity of crude extracts from *D. gardneri* root

Bacteria	**inhibition (%)**		
	n-hexane	**ethyl acetate**	**methanol**
Bacillus cereus	5.28±0.01^{e}	8.45±0.04^{d}	1.53±0.01^{e}
Staphylococcus aureus	21.18±0.03^{c}	33.17±0.02^{b}	11.22±0.21^{d}
Escherichia coli	18.86±0.02^{c}	6.60±0.02^{e}	4.31±0.02^{e}
Vibrio harveyi	15.19±0.02^{c}	17.79±0.14^{c}	ND
Pseudomonas aeruginosa	ND	ND	ND
Gentamicin	100±0.02^{a}	100±0.02^{a}	100±0.01^{a}

Values are means of with superscripts having the same letter is not significantly different (n=3), There was no inhibition discovered (ND).

4. Conclusion

The findings from this research reveal that crude extracts obtained from *D. gardneri*, namely n-hexane, ethyl acetate, and methanol, contain phenolics and flavonoids at various levels. The methanol extract of *D. gardneri* contained high levels of total phenolic and total flavonoid contents, which are well-known antioxidants. Among these extracts, the crude ethyl acetate extract exhibited the highest DPPH antioxidant activity. In terms of antimicrobial efficacy, the ethyl acetate extracts also

demonstrated significant inhibition against *S. aureus* (gram-positive bacteria), with the highest observed inhibition percentage. This study indicates that the root of *D. gardneri* contains bioactive compounds that potentially offer protection against diseases. Hence, it serves as a significant source of antioxidants and antibacterial agents, suggesting the possibility of developing pharmaceutical applications and health products to enhance the value of *D. gardneri*. Furthermore, exploring the chemical constituents of this plant in greater depth is of considerable interest.

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