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The determination of antioxidant and lead content of hemiparasite *Dendrophthoe vitellina* (F. Muell) Tiegh on Nutmeg

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ABSTRACT

Mistletoe, a parasitic plant growing on nutmeg trees, is conventionally perceived as a parasitic entity with limited regard for its potential medicinal value. On the other hand, mistletoe has a remarkable potential source of valuable medicinal compounds, particularly in traditional healthcare, due to its secondary metabolites such as flavonoid, phenolic, ascorbic acid, and antioxidant activity. Nevertheless, both the host tree and the environment, like Pb, influence the adaptive responses of secondary metabolites. This study aimed to determine the secondary metabolites, such as flavonoids, phenolics, ascorbic acid, antioxidant activity, and Pb content in the leaves of *D. vitellina* and nutmeg (*M. fragrans*). The samples were obtained via maceration using ethanol. The spectrophotometric analysis method was used to measure several parameters, using particular reagents for phenolic compounds using Folin-Ciocalteu, flavonoids using $AlCl_3$, and ascorbic acid using sulfosalicylic acid), antioxidant activity using DPPH, and Pb using the AAS method. The results indicated that *D. vitellina* possesses a higher concentration of flavonoid and phenolic compounds, followed by 3.36 ± 1.92 % (w/w) and 18.45 ± 2.35 % (w/w) respectively. Conversely, nutmeg had a significant ascorbic acid concentration of 3.99 ± 4.38 % (w/w). The significant presence of phenolics and flavonoids had a crucial role in the antioxidant activity seen in *D. vitellina*, which exhibited exceptionally potent antioxidant properties. All samples contain Pb, ranging from 0.09-2.10 µg/g, within the maximum allowable limits. Therefore, mistletoe is notable for being a reliable and encouraging plant species that can provide a natural supply of antioxidants and be safely used in traditional medicine.

Keywords: Antioxidant, *Dendrophthoe vitellina*, Lead (Pb), Nutmeg (*Myristica fragrans*)

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INTRODUCTION

Mistletoe is recognized as medical plant due its rich contains a wide range of secondary metabolites that function as antioxidants, protecting against many stress-induced ailments such as diabetes, hypertension, epilepsy, arthritis, and cancer (Majeed et al., 2021). Mistletoe belongs to the category of hemi-parasitic species that attaches itself to the stems and branches of other plants using a specialized structure called a haustorium. The plant undergoes photosynthesis to make carbohydrates. Additionally, it extracts moisture and essential nutrients from the xylem of its host tree, indicating that its metabolic compounds rely on the host tree (Assanga et al., 2020; Mourão et al., 2016; Skrypnik et al., 2021). Host trees that are infected by mistletoe undergo changes in their metabolites, namely in phenolic compounds, and enhance their ability to absorb essential elements from the ground (Kolon et al., 2013; Lázaro-González et al., 2019).

D. vitellina species of mistletoe grows on nutmeg (*M. fragrans*) found in Ambon. Nutmeg, a superior plantation commodity crop, possesses a high development and economic value because it produces nutmeg oil, essential oils, medicinal ingredients, antioxidants and antimicrobial agents, and sunscreen (Ansory et al., 2019). Development products on nutmeg were carried out mostly in the seed and mace (Assa et al., 2014; Gupta et al., 2013). A few investigations showed the potential of nutmeg leaves. According to Ginting et al., (2016), as antioxidants and can be utilized in traditional medicinal plants for disease prevention and treatment (Fawwaz et al., 2017).

Environmental stressors like lead (Pb) stress also affect the mistletoe's ability to produce secondary metabolites (Karunaratne & Uduwela, 2020). Plants absorb Pb in two ways: from soil and air. Pb is derived from anthropogenic activities, notably through sources like traffic emissions (Kolon et al., 2013) and the application of fertilizers in agriculture (Rahayu et al., 2018). Pb is non-essential, non-biodegradable, and harms the environment, including plants and humans (Mahfoud et al., 2018). Plants produce secondary metabolites when they are exposed to Pb. This shows that these changes happen because plants must adapt to stressful conditions. High levels of Pb contamination in plants cause an increase in reactive oxygen species (ROS), which may cause an increase in the production of flavonoid, phenolic, and ascorbic acid (Kandziora-Ciupa et al., 2022; Njoya et al., 2018).

Monitoring secondary metabolite compounds and Pb stress is vital for optimizing the exploration of plants in conventional medicine. The determination of Pb in numerous plant species has been carried (Kamar et al., 2018; Karayil & Ch, 2014; Lee et al., 2017; Rufai et al., 2019). However, a scarcity of study references regarding Pb and secondary metabolite compounds in mistletoe grows on nutmeg; if any, it is mostly on nutmeg seed and mace. Consequently, this study aimed to determine the secondary metabolites, such as flavonoids, phenolics, ascorbic acid, antioxidant activity, and Pb content, in the leaves of *D. vitellina* on nutmeg (*Myristica fragrans*). Furthermore, in the future, information regarding the safety and potential uses of mistletoe for the local people of Ambon, Moluccas, could be explored.

MATERIALS AND METHOD

Materials

The main components utilized were the leaves of *D. vitellina* and the host nutmeg (*M. fragrans*). The samples were obtained from six areas in Ambon, Moluccas, such as Welmaho (1), Toisapu (2), Rutong (3), Ulutung (4), Soya (5) and Hatalai (6). The other materials used in this study were HNO_3 (Merck), HCl (Merck), Ethanol 96% (Merck), Quercetin (Sigma), $AlCl_3$ (Merck), Gallic acid (Merck), Na_2CO_3 (Merck), CH_3COOK (Merck), Na-molybdate (Merck), H_2SO_4 (Merck), $NaHPO_4$ (Merck) and DPPH (Sigma). The equipment utilized in this study is a rotary evaporator (B-One Horizontal), a spectrophotometer for UV-Vis analysis, specifically the Hitachi UV Mini 1240 model and an atomic absorption spectrophotometer (AAS) (Shimadzu AA-7000).

Sample preparation

All samples were desiccated for seven days without exposure to sunlight. Moreover, the samples were prepared by being cut and ready for extraction. The samples were obtained using the maceration procedure using ethanol with a concentration of 96% as the diluent for three cycles over 24 hours each. The filtered solution obtained after three sets of maceration was evaporated through a rotary evaporator (B-One Horizontal) until it reached a paste-like consistency to analyze flavonoid, phenolic, and antioxidant activity.

Measurement of Pb in leaves with AAS

The measurement of Pb in leaves was conducted employing the atomic absorption spectroscopy (AAS) technique. Sample preparation involved the dry destruction method, where leaves were oven-dried at 150°C for 3 hours. Subsequently, approximately 0.5 g of the dried leaves were transferred into a porcelain crucible positioned in a furnace until they became ash for 6 hours at 600°C. The porcelain crucible and the ash samples were carefully retrieved using tongs and allowed to cool in a desiccator for 30 minutes. Following the cooling phase, 5M HNO_3 (5 mL) and 2M HCl (5 mL) were introduced into the porcelain crucible. The samples underwent heating on a hot plate until the solution reached an approximate volume of 5 mL following this step; all samples underwent a cooling process and were subsequently diluted with deionized water in a 10 mL volumetric flask. The prepared samples were then analyzed using AAS, with a specific wavelength set at 283.3 nm (Istiaroh et al., 2014).

Measurement of flavonoid

The concentration of flavonoids in the extract was assessed through a colorimetric method, which involved the use of aluminium chloride. The calibration curve was constructed using quercetin as the standard. For this purpose, 0.01 g of quercetin was dissolved in 100 mL of ethanol and subsequently diluted to achieve concentrations ranging from 20 to 100 µg/mL. The prepared standard solutions and extract samples (0.5 mL) were blended with ethanol (1.5 mL), 10% $AlCl_3$ (0.1 mL), 1M CH_3COOK (0.1 mL), and distilled water (2.8 mL). After a 30-minute incubation at room temperature, the absorbance of the reaction blend was measured at 415 nm using a spectrophotometer (Thoa & Cuong, 2018). The calibration process involved establishing a linear regression equation expressed as y = ax + b. Following cooling and dilution with deionized water; the total flavonoid content (QE) was calculated using equation (1).

$$QE = C\frac{v}{m} \quad (1)$$

Where:
QE: The total flavonoid concentration in milligrams per gram (mg/g).
C: Flavonoid concentration obtained from the standard curve of quercetin, measured in milligrams per liter (mg/L).
v: The volume of the extract in liters (L).
m: The mass of the extract in grams (g).

Measurement of phenolic

The Folin-Ciocalteu as used in phenolic analysis. Gallic acid served as the standard phenolic compound, with a concentration range of 15–150 µg/mL. Extract samples (0.1 mL) were combined with Folin-Ciocalteu reagent (0.75 mL) and allowed to stand at room temperature for 5 minutes. Subsequently, 6% (w/v) Na_2CO_3 (0.75 mL) was carefully added to the mixture. After incubating at room temperature for 90 minutes, the absorbance of the reaction mixture was measured at 725 nm using a spectrophotometer (Azlim Almey et al., 2010). The concentration of the standard Gallic acid

was correlated with its absorbance to establish the linear regression equation, represented as y = ax + b. The calculation of total phenolic concentration was conducted using equation (2).

$$Total\ phenolic = C\frac{v}{m} \quad (2)$$

Where:
C: The total phenolic concentration in milligrams per gram (mg/g).
v: The volume of the extract in liters (L).
m: The mass of the extract in grams (g).

Measurement of ascorbic acid

The determination of ascorbic acid content was carried out using the sulfosalicylic acid reagent, following the method outlined by Maliya et al. (2019) that had been modified. The test solution was made by mixing 1 ml of sample extract plus 2% Na-molybdate solution (2 mL), 0.15M H_2SO_4 (2 mL), and 15mM $NaHPO_4$ (1 mL). The test solution was homogenized and incubated at 60°C for 40 minutes. Subsequently, the solution was cooled to room temperature. The absorbance was measured at a wavelength of 660 nm. The standard ascorbic acid curve served as a reference to determine the concentration of ascorbic acid. The computation of ascorbic acid content involves utilizing the linear regression equation derived from the standard ascorbic acid curve.

Antioxidant activity test

The antioxidant activity was quantified using the DPPH (1,1-diphenyl-2picrylhydrazyl) method. A DPPH solution was prepared by dissolving 6.0 mg of DPPH in 100 mL of methanol. Subsequently, 1 mL of extract from each dilution was added to a test tube containing 2 mL of DPPH solution and incubated in the absence of light for 30 minutes. The absorbance of the mixture was measured using a Shimadzu UV Mini 1240 ultraviolet-visible (UV-Vis) spectrophotometer at a wavelength of 517 nm (Azlim Almey et al., 2010). The calculation of inhibition is expressed as a percentage in equation (3).

$$Inhibition(\%) = \frac{Control\ abs - sampel\ abs}{Control\ abs}\ x\ 100\ \% \quad (3)$$

Data Analysis

Pb content, flavonoid, phenolic, ascorbic acid, and antioxidant activity in mistletoe and nutmeg leaves were tested using two-way ANOVA (Analysis of Variance) test analysis and further analysis using Duncan's test, performed with SAS Software (Statistical Analysis System) version 9.1.3.

RESULT AND DISCUSSION

D. vitellina belongs to Loranthaceae, the largest mistletoe family, and is found growing on nutmeg trees (*M. fragrans*) in the region of Ambon. It is characterized by haustorium that grows along the stem of its host. The young stems of the parasitic plant range in color from green to brown and have a rough texture. Leaves that are displaced-opposite or alternate are spear-or oval-shaped with a blunt apex. The flowers, generally green and later turn into shades of orange or red as they mature, are curved and shaped backwards. The fruit is ovoid and has a yellow-to-red color (see Figure 1).

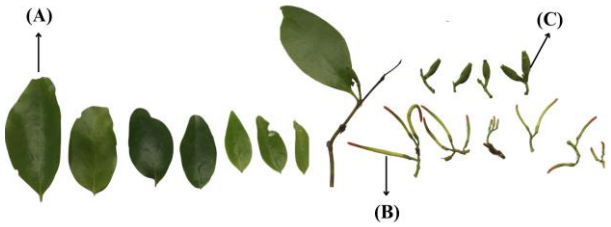

Figure1. Part of *D. vitellina* from host *M. bfragrans:* leaf (A), flower (B), fruit (C)

This study measured the concentrations of flavonoids, phenolic acids, and ascorbic acid in nutmeg and *D. vitellina* leaves (see Table 1). The highest flavonoids 3.36 % (w/w) ± 1.92 and phenolic 18.45 % (w/w) ± 2.35 were obtained from mistletoe, while ascorbic acid 3.99 % (w/w) ± 4.38 was obtained from nutmeg.

Table 1. Flavonoid, phenolic and ascorbic acid and Pb contents on nutmeg and *D.vitellina* Leaves

Samples	Locations	Flavonoid (% w/w)	Phenolic (% w/w)	Ascorbic acid (%w/w)	Pb (µg/g)
Nutmeg	1	2.25 ± 0.93^{e}	10.05 ± 1.35^{f}	2.48 ± 1.39^{c}	0.40 ± 0.002^{g}
	2	21.9 ± 2.30^{e}	9.43 ± 1.70^{g}	3.33 ± 1.76^{b}	0.44 ± 0.037^{fg}
	3	2.37 ± 1.23^{ed}	12.02 ± 0.01^{d}	3.99 ± 4.38^{a}	1.03 ± 0.112^{d}
	4	2.22 ± 0.27^{e}	7.04 ± 4.08^{j}	3.02 ± 3.36^{b}	2.10 ± 0.031^{a}
	5	2.42 ± 1.21^{ed}	12.25 ± 2.62^{d}	2.95 ± 3.67^{b}	1.43 ± 0.009^{b}
	6	1.52 ± 0.53^{f}	13.27 ± 3.28^{c}	2.33 ± 2.91^{c}	0.24 ± 0.003^{h}
D. vitellina	1	3.36 ± 1.92^{a}	7.90 ± 1.95^{i}	1.53 ± 1.15^{d}	0.17 ± 0.003^{i}
	2	2.57 ± 2.12^{cd}	18.45 ± 2.35^{a}	1.44 ± 2.14^{d}	0.48 ± 0.005^{f}
	3	2.82 ± 0.35^{b}	13.62 ± 3.53^{c}	1.64 ± 1.02^{d}	1.15 ± 0.006^{e}
	4	2.78 ± 1.41^{cd}	8.92 ± 1.12^{h}	1.48 ± 3.42^{d}	0.72 ± 0.014^{e}
	5	2.57 ± 0.68^{cd}	11.35 ± 3.05^{e}	2.37 ± 2.14^{c}	0.40 ± 0.004^{g}
	6	2.94 ± 0.81^{b}	16.60 ± 5.00^{b}	1.26 ± 1.76^{d}	0.09 ± 0.006^{j}

Note: Numbers with similar superscript letters indicate non-significant differences as determined by the DMRT test ($p<0.05$). 1 = Welmaho, 2 = Toisapu, 3 = Rutong, 4 = Ulutung, 5 = Soya, and 6 = Hatalai

D. vitellina contents higher flavonoid than nutmeg, but it depends on the plant species. Through a qualitative test, previous studies reported that nutmeg leaves contained flavonoids. However, the amount of flavonoids in nutmeg leaves has yet to be reported (Ginting et al., 2016). *V. album* L on the hosts *U. villosa* L and *J. regia* L were 2.61 ± 0.15 mg/g and 2.38 ± 0.05 mg/g (Majeed et al., 2021). This indicated that the levels of flavonoids found in mistletoe *D. vitellina* and its host nutmeg were higher compared to earlier research findings. Additionally, our results show that *D. vitellina* had the highest level of flavonoid compared to its host (see Table 1). Assanga et al. (2020), discovered a similar result, *Phoradendron californicum* showed a higher flavonoid content than its host, suggesting that the flavonoid content was influenced by its host. Flavonoids play an important role in abiotic stress as antioxidants, scavenging the singlet oxygen, scavenging ROS, inhibiting the activity of several enzymes, causing ROS production increase, and having the ability to detoxify free radicals and chelate heavy metals (Neggaz & Yssaad, 2019).

The highest phenolic content found in *D. vitellina* sample, at location 2 (Toisapu), was 18.45 % (w/w), while the lowest was in nutmeg at location 4 (Ulutung) with 7.04 % (w/w). Compared to the results of the phenolic on nutmeg and mistletoe leaves that have been carried out, *V. album* L on host *J. regia* L was 13.73 ± 0.83 mg/g (Majeed et al., 2021). Meanwhile, the phenolic in nutmeg leaves was

183.55 mg/g (Fawwaz et al., 2017). This indicated that there was a relatively higher phenolic content shown in the present study. The content of phenolic in *D. vitellina* and nutmeg showed significantly different values. The result shares similarity with flavonoid content. Phenolic contents are comparable to that of flavonoids. In this case, *D. vitellina* had the largest, while nutmeg had the lowest concentration of phenolic (see Table 1).

Ascorbic acid contents in all samples showed significantly different values (see Table 1). Mistletoe infestation affects metabolism in the host plant, including changes in the levels of ascorbic acid (Üstüner, 2019). This showed that mistletoe decreased the overall concentration of ascorbic acid. However, this study confirms that the ascorbic content is higher in the nutmeg than in the *D. vitellina*. Correspondingly, it indicates an increase in the ascorbic acid response to mistletoe. The specific conditions and interactions with *D. vitellina* can vary depending on the ascorbic acid content of host plants. Ascorbic acid is crucial in detoxifying reactive oxygen species (ROS) and is particularly important during environmental pressure (Singh et al., 2016). Likewise, a previous study also showed a higher ascorbic acid content in Loranthus sp, which was higher than in this study (Kristiani & Kasmiyati, 2022).

Figure 2 shows an increase in antioxidant activity with higher extract concentrations. As shown by DPPH tests, this study found that *D. vitellina*, which has a lot of flavonoids and phenolic compounds, had strong antioxidant activity at locations 2 (Toisapu) and 6 (Hatalai). However, at location 3 (Rutong), ascorbic acid was in high concentration, and nutmeg displayed significant antioxidant activity. The IC_{50} value, which comes from the results of the regression analysis, also evaluates the antioxidant capacity value. In this value, 50 is substituted for the Y-axis, and the coefficient x denotes the concentration of the standard or extracts that impedes free radical activity by 50%. A lower IC_{50} value represents stronger antioxidant activity in the extract (Yismairai et al., 2019). Based on the IC_{50} value, *D. vitellina* demonstrates much higher antioxidant activity than nutmeg (see Table 2). More highly, *D. vitellina* displayed highly potent antioxidants at locations 2 and 6, whereas nutmeg showed moderate antioxidant activity at locations 1, 2, and 4. The comparison indicates that *D. vitellina* leaves exhibit much higher antioxidant activity in comparison to nutmeg, ascribed to the existence of flavonoids and phenolics (Noreen et al., 2017). Multiple investigations have found that the claimed antioxidant activity in diverse samples can be attributed to flavonoids, phenolics, and ascorbic acid. The compounds possess anti-inflammatory, antiviral, anticancer, and antibacterial effects (Atun et al., 2018; Noman et al., 2019). Although certain species of mistletoe belonged to Loranthaceae family and found on clove and duku trees have been previously documented to possess antioxidant properties (Fitrilia et al., 2015; Salni et al., 2023), our study represents the initial investigation into the antioxidant potential of nutmeg and mistletoe, specifically in Ambon, Moluccas. Furthermore, Nurmilasari et al., (2017) showed that nutmeg leaves possess a higher concentration of potent antioxidants. The IC_{50} values of the methanol and chloroform extracts were 36.31 µg/g and 28.30 µg/g, respectively, showing the strongest activity of antioxidants.

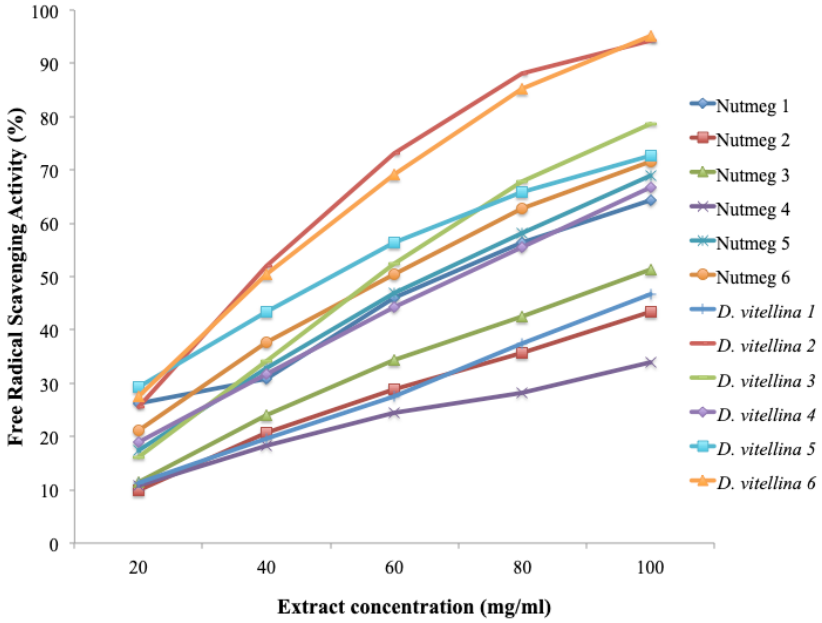

Figure 2. The graph of antioxidant activity (%) of nutmeg and *D. vitellina* leaves

Table 2. IC_{50} Values and Antioxidant Activity on Nutmeg and Mistletoe leaves.

Samples	Locations	IC_{50}	Strength of Antioxidant*
Nutmeg	1	118.92 ± 3.65^{b}	Medium
	2	114.67 ± 2.25^{c}	Medium
	3	95.03 ± 0.36^{e}	Strong
	4	155.2 ± 4.15^{a}	Medium
	5	60.12 ± 0.59^{f}	Strong
	6	56.68 ± 0.97^{g}	Strong
D. vitellina	1	110.97 ± 0.90^{d}	Medium
	2	41.52 ± 0.90^{d}	Very strong
	3	61.1 ± 0.63^{f}	Strong
	4	60.68 ± 1.95^{f}	Strong
	5	58.39 ± 2.15^{gf}	Strong
	6	41.13 ± 1.76^{h}	Very Strong

Note: Numbers with similar superscript letters indicate non-significant differences as determined by the DMRT test ($p<0.05$). 1 = Welmaho, 2 = Toisapu, 3 = Rutong, 4 = Ulutung, 5 = Soya, and 6 = Hatalai. Power classifications based on IC_{50} values (µg/g) are as follows: < 50: categorized as very strong; 50-100: categorized as strong; 100-250: categorized as medium; 250-500: categorized as low; and 500: categorized as not active

Multiple studies have demonstrated that locals in Sulawesi use mistletoe for medicinal purposes, specifically for treating many diseases. For instance, it has been employed in the treatment of mumps in Sulawesi (Dianto et al., 2015) and in the management of diabetes and tonsillitis in Central Kalimantan and Southeast Sulawesi (Arung et al., 2009). This study reports the first documented nutmeg and *D. vitellina* as medicinal plants in the forest region and roadside of Ambon, Moluccas. Both plants have been shown to contain phenolic acids, flavonoids, and ascorbic acid, which possess

strong antioxidant properties that could potentially improve human health. Flavonoids, phenolics, and ascorbic acids have been synthesized and utilized as agents with antibacterial properties (Anita et al., 2014), anti-diabetic effects (Fitrilia et al., 2015), anticancer activity (Lee et al., 2017), and antimicrobial capabilities (Fahmi et al., 2018).

The findings from this study indicate that nutmeg exhibited the highest Pb content at 2.10 ± 0.031 µg/g, while mistletoe demonstrated the lowest concentration at 0.09 ± 0.006 µg/g (see table 1). Samples were collected from locations 1-3 near the roadside and 4-5 within the forest area. The lead (Pb) concentration in all samples from the roadside and forest areas was within the acceptable limit of 10 µg/g, based on BPOM (2014). Afterward, nutmeg with a higher concentration of heavy metal at location 4 revealed lower antioxidant activity. These findings are relevant to the research carried out by Ukom et al., (2019), which showed that plants with higher antioxidant activity demonstrate adaptability to the accumulation of heavy metals in the ecosystem. Therefore, the results indicated that the samples are safe for consumption. The variations in Pb contamination levels between nutmeg and mistletoe may derive from the differences of the collected samples and the plant's ability to accumulate heavy metals (Vuong, 2020).

CONCLUSION

Based on the flavonoid, phenolic, ascorbic acid, and antioxidant activity assays, *D vitellina* and its host, nutmeg (*M. fragrans*), have demonstrated that *D. vitellina* possesses the highest amount of flavonoid and phenolic. In contrast, nutmeg shows higher levels of ascorbic acid. Furthermore, *D. vitellina* and nutmeg were found to contain detectable levels of Pb. However, these levels remain below the permissible limit, ensuring their safety for traditional medicinal use by the local people of Ambon.

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