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Research Article

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Correlation of total phenolic and flavonoid contents on the antioxidant activity of *Psychotria gitingensis* and *Psychotria pilosella*

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ABSTRACT

The genus *Psychotria* (Rubiaceae) possesses various biological properties, ranging from phytochemical and pharmacological properties of their chemical constituents to traditional medical applications. Most *Psychotria* species remain unstudied despite high diversity and endemism in the Philippines. Hence, this study investigates the total phenolic and flavonoid contents of *Psychotria gitingensis* and *Psychotria pilosella* methanolic crude extracts, evaluates their antioxidant properties, and assesses their antibacterial properties. Results revealed that *P. gitingensis* fruit extract exhibited the highest phenolic content (254.45 ± 6.63 mg GAE/g extract) and flavonoid content (9.85 ± 0.49 mg QE/g). In addition, it also displayed the highest antioxidant activity (0.993 ± 0.041 mg/mg) in the ABTS assay. *P. pilosella* leaf extract exhibited the highest antioxidant activity in DPPH (70.53% ± 1.50), and *P. gitingensis* leaf extract showed the highest iron-reducing antioxidant power (86.06% ± 0.73) in FRAP. Paper disk diffusion tests, however, did not exhibit activity against selected nosocomial pathogens. The results of this study contribute to expanding the field of knowledge on alternative treatments and paving the way for the development of new medicinal products.

KEYWORDS: Antioxidant, DPPH, ABTS, FRAP, *Psychotria*, Rubiaceae

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INTRODUCTION

Free radicals are unstable unpaired electrons and highly reactive, which are derived from normal metabolic processes (phagocytosis, aerobic metabolism, and synthesis of prostaglandins), external factors (ionizing radiation, xenobiotics, and pollutants), and invasion of pathogens (National Institute of Health, 2017). Unpaired electrons of the free radicals can bind to biomacromolecules and cause protein and DNA damage, which is a significant cause of mutation and progression of several illnesses such as cancer, asthma, and diabetes. These free radicals can be eliminated from the body through antioxidant enzymes and a proper diet. Antioxidants can scavenge free radicals by donating electrons to neutralize free radicals and prevent the denaturation of biomacromolecules (Patel & Preedy, 2021). Phenolic compounds, such as flavonoids, can strengthen the antioxidant power of the body, and are considered to be the most effective class of phytochemicals or plant-derived metabolites. These phytochemicals also serve as antimicrobial agents depending on their functional groups, concentration, structure, and composition (Babu *et al.*, 2017).

Over 250 medicinal plants have been a great source of alkaloid and phenolic compounds, which are known for exhibiting remarkable pharmacological activities (Petrovska, 2012). Many studies have proven the therapeutic and pharmaceutical potential of various plant species worldwide. Plant biomolecules have been used as alternatives to antibiotics, due to their effectiveness in fighting microbial pathogens and their accessibility (Wintola & Afolayan, 2015). These natural remedies are essential to provide medicines for geographically isolated areas with poor primary healthcare systems. In the Philippines, approximately 16,690 medicinal plants have been recorded, but only ten are approved as Herbal Medicinal Plants by the Department of Health (Cordero *et al.*, 2020). Medicinal plants require intensive research through bioprospecting, and proper protocols which have to be followed to consider plant prospects as a potential source of cures for different ailments.

The genus *Psychotria* of the Rubiaceae family is one of the most speciose angiosperm genera, with over 1600 species. It is widely distributed in tropical and pantropical regions and consists mainly of shrubs, herbaceous, and epiphytes. With its diversity and high population density, numerous phytochemical, ethnobotany, pharmacological, chemotaxonomic, and molecular phylogenetic studies of *Psychotria* species have been conducted over the last decade. There are 112 identified *Psychotria* species in the Philippines, with the majority identified as endemic (Biag & Alejandro, 2021). However, with the rapid loss of habitat and anthropogenic activities, almost half of the identified endemic species are assumed to be extinct (Batuyong *et al.*, 2020). Despite this group's diversity, only a few phytochemical and antimicrobial studies have been conducted regarding the Philippine *Psychotria*. In a study conducted by Castro *et al.* (2016), four compounds were identified by GC-MS in the hexane extract of *Psychotria luzoniensis* while its hexane, $CHCl_3$, and n-BuOH extracts showed moderate antibacterial activities against *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Compounds isolated from *P. luzoniensis* showed active cytotoxicity against the human colon adenocarcinoma cell line (Ramil *et al.*, 2021). The isolation of vomifoliol as a false-positive alkaloid based on Dragendorff's reagent was reported from *P. gitingensis* (Tan *et al.*, 2012). Despite the country's high diversity and endemism of *Psychotria* species, only a few ethnobotanical, phytochemical, and pharmacological studies have been tackled, despite their promising phytochemical properties. Hence, this study investigates the total phenolic and flavonoid content, evaluates the potential antioxidants using mechanism-based antioxidant assays, and assess the antibacterial activities of *P. gitingensis* and *P. pilosella* Elmer.

MATERIALS AND METHODS

Plant Materials

Fresh leaves of *Psychotria pilosella* (USTH-016846) were collected from Mt. Huraw, San Jose De Buan, Samar, on July 2022. Fresh leaves and fruits of *Psychotria gitingensis* (USTH-017170) were collected from Barangay Del Carmen, Siargao Island on October 2022. Species identification was performed using descriptions and keys from Sohmer (2007) and using online databases such as philippineplants.org (Pelser *et al.*, 2011) and Plants of the World Online (POWO, 2023). Voucher specimens were deposited in the University of Santo Tomas Herbarium (USTH).

Extraction of the Crude Extracts

All leaf samples were washed, air-dried and ground using a mill, while fruit samples were washed, freeze-dried and crushed. *P. gitingensis* fruit (30 g) were soaked in 950 mL of MeOH for five consecutive days. *P. gitingensis* leaves (512 g) and *P. pilosella* leaves (100 g) were soaked in a total volume of 6.4 L and 2.4 L of MeOH, respectively. Methanol extraction was done for 5 consecutive days, followed by filtration and concentration under reduced pressure. A total of 82 g of leaf extract and 4.65 g of fruit extract were collected from *P. gitingensis*, while 4.46 g of crude extract were obtained from the extraction of *P. pilosella leaves*.

Determination of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The modified Folin-Ciocalteu colorimetric method (Gajula *et al.*, 2009) was used to determine the total phenolic content (TPC) of the crude extracts. Briefly, 12.5 µL of the prepared extract samples and gallic acid standard were separately added to distilled water (50 µL). Folin-Ciocalteu's phenol reagent was added to the mixture, followed by the addition of 7% Na_2CO_3 (125 µL). Samples were incubated for 90 min at 25 °C and were subjected to spectrometric analysis. The diluted solution's absorbance was measured at 750 nm against a blank sample. A standard curve for the total phenolics was established using the gallic acid standard solution. Results of the TPC were expressed as milligram of gallic acid equivalents per gram of dry extract (mg GAE/g).

The modified Dowd method (Arvouet-Grand *et al.*, 1994) was used to determine the total flavonoid content (TFC) of crude extracts. A 50 µL extract solution and quercetin standard were separately mixed with 10% (w/v) $AlCl_3$ solution in MeOH (10 µL), 1M potassium acetate (10 µL), and distilled water (200 µL). Subsequently, the mixture was incubated for 30 min at room temperature and subjected to spectrometric analysis. The solution's absorbance was measured at 415 nm against the blank. The results of TFC were expressed as milligram of quercetin equivalents by gram of dry extract (mg QE/g).

DPPH Free Radical Scavenging Assay

The DPPH radical scavenging assay (Clarke *et al.*, 2013) was used to screen the antioxidant activity of *Psychotria* fruit and leaf extracts. A concentration of 40 µg/mL DPPH in methanol (180 µL) was added to 20 µL of the extract diluted appropriately in DMSO in the wells of a 96-well plate. The plate was incubated for 15 min at room temperature, followed by spectrometric analysis. The absorbance was measured at 540 nm against appropriate blanks (DMSO) and standards (ascorbic acid in DMSO). The % scavenging of DPPH is calculated using the equation:

$$\% \text{ scavenging of DPPH} = [(Ac - As)/Ac] \times 100$$

where Ac and As is the absorbance of the control and of the sample extract, respectively.

ABTS Radical Scavenging Activity

The antioxidant activities of the *Psychotria* fruit and leaf extracts were evaluated according to the method described by Moreira (2019). The concentrations of the plant extracts used in the assay were 125, 250, 500, and 1000 ppm, respectively. The decolorization due to the plant extracts or standards was computed using the equation:

$$\% \text{ Decolorization} = [(Ac - As)/Ac] \times 100$$

Where Ac is the absorbance of the control and As is the absorbance of the sample/standard. The Trolox equivalents was computed based on linear regression following the equation:

Trolox equivalents (mg/mg) = [(% decolorization – b)/a]/sample concentration (mg/mL)

Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power (FRAP) assay method described by Yu *et al.* (2020) was used to measure the reducing power of the plant extracts, with slight modifications. The concentration of the plant extracts used in the assay was 500 ppm. Seventy (70) µL of methanol (negative control), positive controls (butylated hydroxytoluene (BHT), quercetin, and L-ascorbic acid) at 565.6 ppm, and the plant extracts were mixed with 0.2 M sodium phosphate buffer (pH 7.4) and 1% [$K_3Fe(CN)_6$]. Incubation of the mixture was done at 50°C for 20 min. The reaction mixtures were then acidified with 10% trichloroacetic acid (176.5 µL), followed by centrifugation at 650 rpm for 10 min. An aliquot of the supernatant was added to 100 µL of deionized water. Lastly, 0.1% of FeCl3 (20 µL) was added to the solution, and the absorbance was measured at 700 nm. The percentage reduction of plant extract and standard compounds was calculated using the following formula: % Reduction = [(As – Ab)/As] x 100

where As is the absorbance of the control, and Ab is the absorbance of the sample extract.

Antibacterial Assay

Isolates of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterobacter* spp., *Proteus mirabilis*, *Staphylococcus aureus* and *Enterococcus faecalis* were obtained from the University of Santo Tomas Collection of Microbial Strains (UST-CMS). These were inoculated in a 9 mL sterile saline solution, each adjusted according to the 0.5 McFarland standard (Abebe & Mekonnen, 2016). The Kirby-Bauer disk diffusion method was used to preliminarily assess and screen the antibacterial activity of the plant extracts following the method of Gutierrez *et al.* (2013). Bacterial suspension was inoculated on Mueller-Hinton (MH) agar. Paper discs were immersed in 15 µl of 2000 ppm and 5000 ppm of each plant extract (n = 3). For the positive control, 25 µg/disc Streptomycin (Himedia Laboratories) and 5 µg/disc Ciprofloxacin (Cypress Diagnostics) were utilized, and methanol for the negative control. Zones of inhibition were measured after incubation for 24 h at 35 ± 2 °C.

Statistical Analysis

The Shapiro-Wilk test and Levene homogeneity test were used to determine the normality and homogeneity of variance. One-way ANOVA ($\alpha = 0.05$) was used for TPC, TFC, DPPH, ABTS, and FRAP to determine if there was a significant difference between the plant samples and the standard/s. Tukey test ($\alpha = 0.05$) was used as a post hoc test. Pearson's correlation coefficient test ($\alpha = 0.05$) was used to determine the statistical relationship or the magnitude of the correlation between the parameters used in the study. All analyses were performed in Microsoft Excel ver. 2022.

RESULTS AND DISCUSSION

Estimation of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The TPC of *gitingensis* fruit and leaf extracts and *P. pilosella* leaf extract was computed based on the calibration curve of standard gallic acid ($y = 0.0019x + 0.1037$, $R^2 = 0.9979$). *P. gitingensis* fruit extract exhibited the highest TPC (254.45 ± 6.63 mg GAE/g extract). *P. gitingensis* leaf extract showed 114.24 ± 5.06 mg GAE/g extract, while *P. pilosella* leaf extract gave the lowest TPC (38.47 ± 1.51 mg GAE/g extract) (Figure 1a).

The TFCs of the leaf and fruit extracts, as shown in Figure 1b, were computed based on the standard quercetin calibration curve ($y = 0.0034x + 0.0374$, $R^2 = 0.9971$). Among plant extracts, *P. gitingensis* fruit extract gave the highest activity (9.85 ± 0.49 mg QE/g extract), followed by *P. gitingensis* leaf and *P. pilosella* leaf extract at 6.99 ± 0.15 and 6.64 ± 0.08 mg QE/g, respectively.

Phenolic compounds are known to act as natural antioxidants due to their redox characteristics linking them to have a wide range of biomedical and pharmaceutical applications. Antioxidants are also linked to illness risk reduction and human health enhancement by preventing cell damage caused by free radical oxidation. Flavonoids are phenolic structures found in plants, fruits, vegetables, and grains that are beneficial for overall human health. Flavonoids are antimutagenic, anticarcinogenic, antioxidant, anti-inflammatory, and antiviral, essential components of medicines and supplements of pharmaceutical and nutraceutical companies (Panche *et al.*, 2016; Ullah *et al.*, 2020). Fruit extracts generally have higher TPC and TFC values compared to leaf extracts, thus, exhibited better antioxidant activity. In a study by Praptiwi *et al.* (2021), the TFC found in *Psychotria celebica* fruit extract is higher compared to its bark and leaf extracts. The same trend is also shown by *P. gitingensis* and *P. pilosella*.

DPPH Free Radical Scavenging Assay

The DPPH free radical scavenging activities were compared to standard ascorbic acid (Figure 2). At 1000 ppm, *P. pilosella* leaf extract showed the highest free radical scavenging activity of 70.53% ± 1.50. Surprisingly, a significant difference was observed in the DPPH radical scavenging activity of *P. gitingensis* fruit (61.67% ± 2.61) and leaf (19.08% ± 0.83) extracts. *P. gitingensis* fruit and *P. pilosella* had comparable DPPH radical scavenging activities at $p < 0.05$. None of the plant extracts were comparable to the free radical scavenging activity of the standard ascorbic acid of 98.64% ± 0.42.

Scavenging Activity of ABTS Radical

Table 1 shows the result of ABTS radical scavenging activity expressed as mg Trolox equivalent/mL. Among extracts, *P. gitingensis*

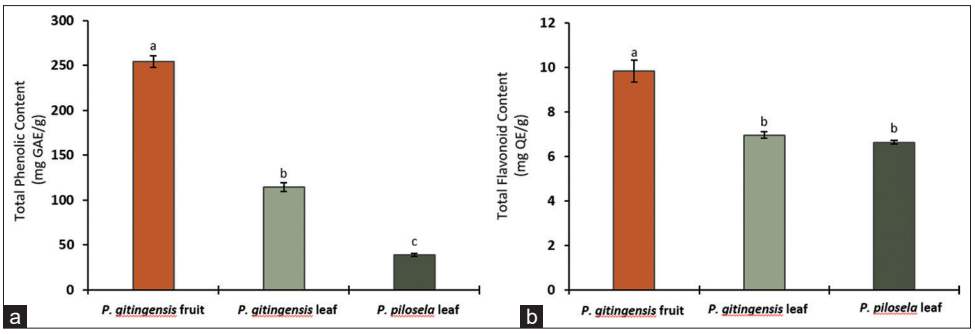

Figure 1: Phytochemical content of *Psychotria* methanolic extracts: a) total phenolic content (in mg GAE/g), b) total flavonoid content (in mg QE/g). Values are expressed as mean ± standard deviation (n = 3). (a – c) denote significant difference at $p < 0.05$

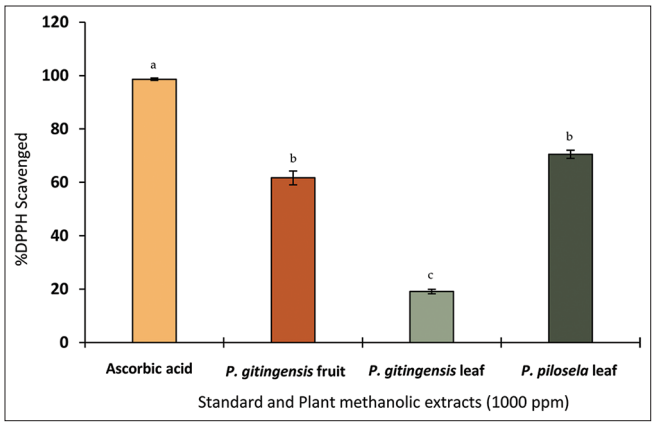

Figure 2: DPPH free radical scavenging assay of the *Psychotria* methanolic fruit, leaf crude extracts, and standard ascorbic acid; values expressed as mean ± standard deviation (n=3). (a – c) denote significant difference at $p < 0.05$

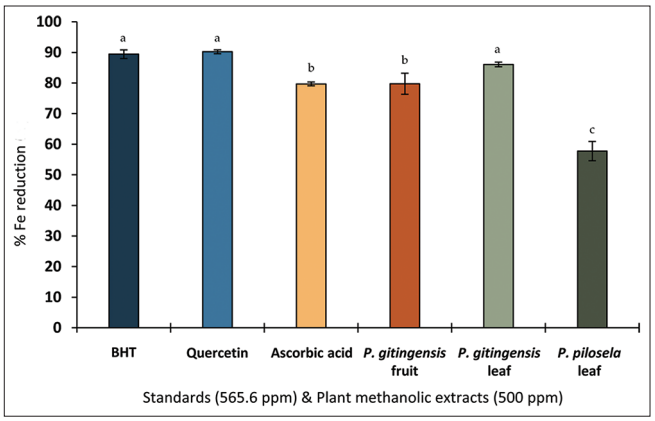

Figure 3: FRAP assay of *Psychotria* fruit and leaf methanolic crude extracts, and standard references, namely BHT, quercetin, and ascorbic acid. Values are expressed as mean ± standard deviation (n=3). (a – c) denote significant difference at $p < 0.05$

fruit exhibited the highest Trolox-Equivalent Antioxidant Capacity (TEAC) (0.993 ± 0.041 mg/mL) with an IC_{50} of 0.698 ± 0.039 mg/mL. This was significantly comparable with *P. gitingensis* leaf extract with TEAC of 0.981 ± 0.036 mg/mL and and IC_{50} of 1.338 ± 0.044 mg/mL. *P. pilosella* leaf extract showed the lowest TEAC and highest IC_{50} at 0.789 ± 0.011 mg/mL and 7.91 ± 1.11 mg/mL, respectively.

Table 1: Trolox-Equivalent Antioxidant Capacity (TEAC) and IC_{50} values of *Psychotria* plant extracts

Sample	TEAC (mg Trolox equivalent/mL)	IC_{50} (mg/mL)
Psychotria gitingensis fruit	0.993 ± 0.041[a]	0.698 ± 0.039[a]
Psychotria gitingensis leaf	0.981 ± 0.036[a]	1.338 ± 0.044[b]
Psychotria pilosella leaf	0.789 ± 0.011[b]	8.76 ± 1.11c

Values are expressed as mean ± standard deviation (n = 3). (a – c) denote significant difference at $p < 0.05$

Ferric Reducing Antioxidant Power (FRAP)

FRAP is measured using percentage of iron ion reduction. As shown in Figure 3, *P. gitingensis* leaf extract at 500 ppm (86.06% ± 0.73) is statistically comparable ($p < 0.05$) with the standards BHT (89.43% ± 1.42) and quercetin (90.21% ± 0.65). *P. gitingensis* fruit extract at 500 ppm (79.77% ± 3.45) is statistically comparable ($p < 0.05$) with the standard ascorbic acid (79.68% ± 0.62). *P. pilosella* leaf extract (57.73% ± 3.14) showed the lowest FRAP activity at 500 ppm.

Correlation between parameters used for *Psychotria* plant extracts

Correlations between parameters used for analyzing *Psychotria* plant extracts are shown in Table 2. FRAP showed a high positive correlation with TPC and ABTS (r = 0.60779 and r = 0.89829, respectively). In contrast, it showed a high negative correlation with DPPH (r = -0.9196) and a low positive degree of correlation with TFC (r = 0.39059). ABTS has a high positive correlation with TPC and TFC (r = 0.74636 and r = 0.59283, respectively) and a high negative degree of correlation with DPPH (r = -0.78347). DPPH showed a low negative correlation with TPC and TFC (r = -0.30646 and r = -0.04779, respectively). TFC and TPC showed a high positive correlation with a correlation value 0.95209.

On the effects of the free radical scavenging activity, ascorbic acid, vitamin E, dehydroascorbic acid, and reducing sugars are only a few examples of the many interfering substances that the Folin-Ciocalteu assay may contain because it is not specific for phenolic compounds (Gonzales *et al.*, 2021). The types of phenolic compounds present in the plant extracts and their

Table 2: Pearson coefficients of correlation between *Psychotria* methanolic plant extracts parameters

	TPC	TFC	DPPH	ABTS
TFC	0.95209			
DPPH	-0.30646	-0.04779		
ABTS	0.75922	0.59388	-0.78347	
FRAP	0.60779	0.39059	-0.91960	0.89829

corresponding chemical characteristics, such as their hydrophilic nature and number of hydroxyl groups may also affect their free radical scavenging activity, with the low correlation to the total phenolic contents (r = -0.31). Other possible interferences are interactions that are either synergistic or antagonistic between phenolic chemicals or between phenolic and non-phenolic compounds. On the other hand, a low negative correlation with total flavonoid content can be explained as not all flavonoids have strong antioxidant properties (r = -0.048) (Indradi *et al.*, 2017).

In the ABTS assay, the Trolox Equivalent Antioxidant Capacity (TAEC) of each plant extract increases in the following order: *P. gitingensis* fruit > *P. pilosella* leaf > *P. gitingensis* leaf (0.993 ± 0.041 mg/mg, 0.981 ± 0.036 mg/mg, 0.789 ± 0.011 mg/mg, respectively). Moreover, a similar trend was observed in the IC_{50} value in which *P. gitingensis* fruit extract showed the lowest concentration to produce a 50% ABTS reduction. TPC plays a part in the plants' antioxidant activity (Muflihah *et al.*, 2021). Thus, the strong correlation between antioxidant activity and polyphenol content states that phenolic compounds, like flavonols, are thought to have a significant role in the fruits' ability to function as antioxidants. Phenolic content may contribute to the antioxidant activity exhibited in the scavenging activity of ABTS radical, considering ABTS showed a high positive correlation with TPC (r = 0.759) and TFC (r = 0.594).

Iron is an important trace element in many organisms, as it plays an essential role in various metabolic processes, such as DNA synthesis and oxygen and electron transport (Patel & Preedy 2021). However, it is only controlled by absorption through the reduction of insoluble ferric (III) ions to absorbable ferrous (II) ions (Rodgers & Gilreath, 2019). Thus, FRAP is used to determine the ability of antioxidants present in the plant extract to reduce Fe^{3+} to green-colored Fe^{2+}. Its reducing power is associated with the compounds that exert action by breaking free radical chains by donating an electron. BHT, quercetin, and ascorbic acid are good antioxidants that protect cellular components from free radicals and are used as a standard for comparison with plant extracts. One-way ANOVA shows a significant difference between the methanolic extracts of the plant samples and the standard references ($p < 0.05$). Based on Tukey's HSD post hoc test, *P. gitingensis* leaf extract was not significantly different with BHT and quercetin ($p < 0.05$), and *P. gitingensis* fruit extract was not significantly different with ascorbic acid ($p < 0.05$) indicating that both extracts of *P. gitingensis* showed comparable % Fe reducing power with the standards. The notable electron donation ability of the said extracts could be attributed to their high phenolic content, which can transfer electrons to neutralize free radicals and chelate metal catalysts (El-Shiekh *et al.*, 2019) considering TPC and FRAP showed a high positive correlation (r = 0.60808). *P. pilosella* leaf extract exhibited 57.85% ± 0.84 at 500 ppm, with the lowest reducing ability to convert Fe^{3+} to Fe^{2+}.

Antibacterial Activity of *Psychotria* Leaves and Fruit Extracts

Antibacterial activity of 2000 ppm and 5000 ppm methanolic extracts of *P. gitingensis* fruit and leaf extracts and *P. pilosella* leaf extract against bacteria species were evaluated using the Kirby-Bauer disk diffusion assya. The antibacterial activities of the three *Psychotria* methanolic extracts were compared with Streptomycin (25 µg/disc) and Ciprofloxacin (5 µg/disc) and methanol as the negative control. Results (data not shown) showed that the plant extracts exhibited no inhibition as evidenced by the absence of clearance in the growth of microorganisms in the plate. Thus, this study reports *P. gitingensis and P. pilosella* MeOH extracts as having no antibacterial activity at 2000 and 5000 ppm concentrations.

CONCLUSION

This study reveals the presence of phenolic compounds and flavonoids in the methanolic extracts of two Philippine endemic *Psychotria* species, with notable antioxidant properties despite the absence of antibacterial activities. The varying results of the antioxidant assays among the methanolic leaf and fruit extracts may suggest that polyphenols could not be solely responsible for antioxidant action. Still, other compounds may be responsible for their antioxidant activity. We recommend that bioactive compounds from these extracts should be isolated and purified for further studies. Moreover, further pharmacological studies and investigations on *Psychotria* species and other Philippine endemic plants are vital for potential drug development and formulation that can help improve healthcare in treating existing and novel diseases in the future.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Conceptualization and Design – JAO and MAT; Plant Collection and Experimentation – JAO, MYA, JDD, SRF, KMP, MAV; Data

analysis and Interpretation - JAO, MYA, JDD, SRF, KMP, MAV, MAT; Wrting – initial draft - MYA, JDD, SRF, KMP, MAV; Writing – revision of the manuscript – JAO and MAT. All authors approved the final version of the manuscript.

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