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Mechanism-based antioxidant activity of Rubiaceae species collected from Ilocos Norte, Philippines

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Abstract

The Rubiaceae family, comprising 550 species in the Philippines, is a significant source of bioactive components with ethnopharmacological uses. This study assessed the total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of six Rubiaceae species, collected in Ilocos Norte, Philippines: *Guettardella microphylla* (Bartl. ex DC.) Merr., *Timonius ternifolius* (Bartl. ex DC.) Fern.-Vill., *Kanapia monstrosa* (A. Rich.) Arriola & Alejandro, *Pyrostria triflora* Arriola, Calaramo & Alejandro, *Pyrostria subsessilifolia* (Merr.) Arriola & Alejandro, and *Psychotria luzoniensis* (Cham. & Schltdl.) Fern.-Vill. Identification of the plant species was done using morphological characterization. The TFC results ranged between 1.86-3.81 mg quercetin equivalent/g dry weight (GAE/g DW), while TPC indicated 5.47-17.17 mg gallic acid equivalent/g dry weight (QE/g DW). *G. microphylla* showed the highest TFC (3.81±0.20 mg QE/g DW) while *P. triflora* exhibited the highest TPC (17.17±0.83 mg GAE/g DW). Antioxidant profiling showed *P. luzoniensis* exhibiting the highest activity in the NOS, ABTS, DPPH, and FRAP assays. *G. microphylla* showed the highest hydrogen peroxide scavenging activity, while *T. ternifolius* demonstrated the highest hydroxyl radical scavenging activity. Findings suggest that the crude methanolic extracts of the Rubiaceae species have relatively high TPC and TFC values and exhibit promising antioxidant capacities.

Keywords: antioxidant; DPPH; *Guettarda*; *Psychotria*; Rubiaceae; total phenolic content; total flavonoid content

Introduction

Oxidants, particularly free radicals, are of key interest given their importance in various physiological processes and involvement in the pathogenesis of a wide range of diseases. Oxidants are generally categorized as

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reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS play biologically significant roles in several normal bodily functions, including adaptation to hypoxia, signal transduction, as well as control of autophagy, immunity, differentiation, and lifespan (Sena and Chandel, 2012; Di Meo *et al.,* 2016). However, when the body fails to counteract the overproduction of these species through antioxidant networks present in cells, the excessive levels of ROS, RNS, and associated free radicals can result in oxidative stress, which can subsequently lead to cellular dysfunction, excessive inflammation, and chronic conditions such as cancer, diabetes, cataracts, and many cardiovascular, inflammatory, and neurodegenerative diseases (Newsholme *et al.,* 2016; Hernández-Rodríguez *et al.,* 2019).

Antioxidants are substances that have become more significant in recent years because of their capacity to counteract some of the harmful effects of elevated ROS concentrations (Yu *et al.,* 2020). Although many synthetic antioxidants have been developed, certain studies have reported that these may induce adverse effects on human health (Lourenço *et al.,* 2019). Therefore, looking into natural products for safer and similarly effective antioxidants may offer a promising alternative. Plants that contain beneficial phytochemicals may serve as natural antioxidant sources, as exemplified in many studies (Altemimi *et al.,* 2017). Phenolic compounds contribute to the majority of the antioxidant activity in plants. Flavonoids, one of the largest groups of phenolics, have been discovered to have numerous biological effects such as antibacterial, antiviral, antiulcer, antiarthritic, anticancer, and hepatoprotective (Amarowicz and Pegg, 2019). However, its antioxidant properties are the most described due to its numerous mechanisms of action (Kumar and Pandey, 2013).

A key source of leads in pharmaceuticals is the family Rubiaceae. Numerous members of the Rubiaceae family were revealed to have the presence of cardenolides, iridoids, indole alkaloids, terpenoids, flavonoids, and anthraquinones, compounds that have been characterized as potential agents for various biological activities, most notably regarding their antioxidant properties (Suksungworn and Duangsrisai, 2021). Thus, many are widely used in traditional medicine, specifically for their antiplasmodial, antimicrobial, antioxidant, anti-inflammatory, immunostimulant, and analgesic effects (Heitzman *et al.,* 2005; Karou *et al.,* 2011; Martins and Nunez, 2015). Many medically significant compounds have been isolated from Rubiaceae plants. Examples include quinine from *Cinchona* spp., one of the first drugs used against malaria (Achan *et al*., 2011). Emetine, a drug mainly sourced from *Psychotria ipecacuanha,* has been used for treating amoebiasis (Bleasel and Peterson, 2020). It was also shown to have antiviral activity against HIV and Dengue (Yin Low *et al.*, 2009; Valadão *et al*., 2015). Similar antiviral effects were described in cephaeline, an analog of emetine derived from the same plant species. Both are implicated in drug design and discovery for Zika virus, Ebola virus, and SARS-CoV-2 (Yang *et al*., 2018; Wang *et al*., 2020; Ren *et al*., 2022). Their derivatives and other related compounds are being investigated for anticancer properties as well (Silva *et al.*, 2022; Peng *et al*., 2023). Rubiadin, first isolated from *Rubia cordifolia*, is currently being touted as a promising anti-cancer, anti-osteoporotic, hepatoprotective, and neuroprotective compound that can be a candidate for drug development (Watroly *et al.*, 2021). Currently, there are 550 Rubiaceae species in 80 genera that have been described within the Philippines (Davis *et al.,* 2009). Despite its rich biodiversity, the Philippine flora is still considered to be grossly understudied, not only taxonomically but also in terms of their potential medicinal and pharmacological properties.

Five Philippine endemic plant species from Ilocos Norte namely *Guettardella microphylla* (Bartl. ex DC.) Merr., *Timonius ternifolius* (Bartl. ex DC.) Fern.-Vill., *Pyrostria triflora* Arriola, Calaramo & Alejandro, *Pyrostria subsessilifolia* (Merr.) Arriola & Alejandro, and *Psychotria luzoniensis* (Cham. & Schltdl.) Fern.-Vill., as well as one indigenous plant species *Kanapia monstrosa* (A. Rich.) Arriola & Alejandro, were selected for antioxidant capacity testing. The four species *G. microphylla, T. ternifolius, P. triflora, and K. monstrosa* were selected based on the initial phytochemical screening (Batuyong *et al.,* 2021). The overall phytochemical composition of Rubiaceae plants lends to their potential as sources of bioactive compounds. In particular, a common target for collecting and extracting phytochemicals would be plant leaves as these provide a simple,

convenient, and reliable method for pharmaceutical and nutraceutical development (Vongsak *et al.*, 2013; Rafael *et al.*, 2023). Plant leaves are ideal prospects for ethnopharmacological use and phytochemical profiling as these are prolific sources of bioactive compounds, due to the protective role that secondary metabolites such as phenolics and flavonoids provide against biotic and abiotic stresses (Abdennacer *et al*., 2015; Dash *et al*., 2017; Chen, 2019; Laoué *et al*., 2022). Thus, this study aims to investigate the antioxidant capacities of crude leaf methanolic extracts derived from Philippine endemic and indigenous Rubiaceae species, namely, *G. microphylla*, *P. triflora*, *P. subsessilifolia*, *P. luzoniensis*, *T. ternifolius*, & *K. monstrosa* through assessment of their total phenolic and total flavonoid contents, and through antioxidant assays (DPPH, hydrogen peroxide, hydroxyl radical, nitric oxide, ABTS scavenging, and FRAP assays) that screen for various antioxidant mechanisms of action.

Materials and Methods

Plant sampling

Fresh leaves of *G. microphylla, P. triflora, P. subsessilifolia, T. ternifolius, K. monstrosa,* and *P. luzoniensis* were collected in Ilocos Norte Province in September 2022. Permits from respective local government units (LGU) and the Department of Environment and Natural Resources-Biodiversity Management Bureau (DENR-BMB) were secured (Wildlife Gratuitous Permit No. 2022-001). Samples of *G. microphylla, P. triflora,* and *P. luzoniensis* were acquired from the Metropolitan Ilocos Norte Watershed Forest Reserve (MINWFR). *P. subsessilifolia* was collected from Kalbario-Patapat Natural Park (KPNP), while *T. ternifolius* and *K. monstrosa* were from Mt. Pao Range. All species were identified based on morphological features, and voucher specimens (Table 1) were deposited at the Herbarium of Northwestern Luzon, located at the Northwestern University Ecotourism Park and Botanic Gardens (NUEBG). Figure 1 summarizes the workflow of experiments on the plant species.

Table 1. Collection information of the Rubiaceae species

Species	Herbarium – Accession number	Collection site
Guettardella microphylla	HNUL0021179	Brgy. Tadao, Pasuquin
Kanapia monstrosa	HNUL0021177	Mt. Pao Range, Adams
Psychotria luzoniensis	HNUL0021180	Brgy. Tadao, Pasuquin
Pyrostria subsessilifolia	HNUL0021182	KPNP, Pagudpud
Pyrostria triflora	HNUL0021181	Brgy. Tadao, Pasuquin
Timonius ternifolius	HNUL0021178	Mt. Pao Range, Adams

Extraction of the plant materials

Air-dried, ground leaves (approximately 100 g) were soaked in distilled methanol (MeOH) overnight and repeated thrice. The combined filtrates were concentrated under reduced pressure to obtain the crude MeOH extracts. The extracts were kept in the refrigerator in amber bottles until further use.

Determination of the total phenolic content (TPC)

TPC was determined based on Shackelford *et al.* (2009). One mg/mL of each extract and gallic acid as standard were dissolved in distilled water, followed by serial dilutions to obtain 1000, 500, 250, 125, 62.5, and 31.25 ppm sample solutions. For the reagents, 0.5 g of Folin-Ciocalteu (FC) reagent (Sigma-Aldrich®) was

dissolved in 5 mL distilled water, and 0.35 g of Na_2CO_3 was dissolved in 5 mL distilled water. In a 96-well plate, 50 µL distilled water and 12.5 µL FC reagent were added to 12.5 µL of sample solution, followed by incubation for 5 min at room temperature (RT). Then, 125.0 µL Na_2CO_3 was added to each well and incubated for another 90 min.

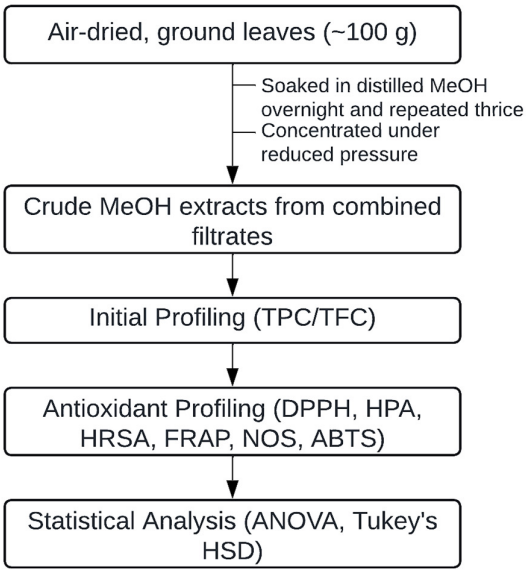

Figure 1. Workflow diagram of the experiments conducted starting from extraction, initial and antioxidant profiling, and statistical analysis of results

The absorbance was read at 740 nm with a microplate reader (Promega GloMax®). The TPC was measured against the gallic acid standard calibration curve and expressed as milligrams per gram of gallic acid equivalents (mg GAE/g) of the dry extract.

Determination of the total flavonoid content (TFC)

TFC was measured based on Aryal *et al.* (2019) utilizing quercetin as standard. One mg/mL of each extract and quercetin were prepared using MeOH, followed by serial dilution to yield 1000, 500, 250, 125, 62.5, and 31.25 ppm sample solutions. A 0.5 g $AlCl_3$ in 5 mL MeOH and 0.5 g potassium acetate in 5 mL distilled water were used as reagents. A 50 µL of the sample solution was measured in a 96-well plate and added subsequently with 10 µL of $AlCl_3$, 10 µL potassium acetate, and 200 µL distilled water. The microwell plate was then incubated at RT for 30 min. Then, the absorbance was measured against a blank solution at 415 nm using a microplate reader (Promega GloMax®). TFC was calculated from the standard curve and expressed as milligrams per gram of quercetin equivalents (mg QE/g) of the dry extract.

DPPH free radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was determined based on previous method (Clarke *et al.,* 2013). A 1 mg/mL in dimethyl sulfoxide (DMSO) solution of each plant extract and 0.6 mg of a 0.1 mM DPPH solution in MeOH were prepared. The diluted plant extracts (20 µL in 180 µL DPPH) were placed in a 96-well plate. The plate was incubated for 15 min in the dark at RT and the absorbance was read at 540 nm using a microplate reader (Promega GloMax®). Ascorbic acid, quercetin, and gallic acid (1 mg/mL) were used as standards. DPPH scavenging activity was calculated using the following equation: % DPPH RSA = $[(A_0 - A_1)/A_0]$ x 100, where A_0 is the absorbance of the blank (DMSO), and A_1 is the absorbance of the plant extracts and standards.

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity (HPA) was evaluated by the method described in Aryal *et al.* (2019). Plant solutions (1 mg/mL), phosphate-buffered saline (PBS) solution (blank, 1 mg/mL), and ascorbic acid (standard, 1 mg/mL) were prepared in MeOH. The H_2O_2 reagent was prepared by dissolving 453.5 µL H_2O_2 in 100 mL PBS (pH 7.4). Fifty microliters (50 µL) of sample solutions were mixed with 100 µL H_2O_2 solution in a 96-well plate. After 10 min of incubation, the absorbance was read at λ_{max} 230 nm. The % HPA was computed using the following equation: % scavenging of H_2O_2 = $[(A_0 - A_1)/A_0$ x 100, where A_0 is the absorbance of the blank, and A_1 is the absorbance of the plant solutions and standard.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay (HRSA) utilizing 1 mg/mL solution of plant extracts and ascorbic acid (standard) in MeOH, 0.75 mM PBS, 0.01% H_2O_2, 0.75 mM phenanthroline, and 0.75 mM $FeSO_4$ was described previously (Lei *et al.,* 2016). In a 96-well plate, 50 µL each of 0.2 mM PBS (pH 7.4) and $FeSO_4$ and 25 µL each of H_2O_2 and plant solutions were mixed with 50 µL phenanthroline. The resulting mixture was incubated at RT for 10 min followed by absorbance reading at 510 nm. The % hydroxyl radical scavenging activity was calculated using the following equation: % HRSA = A_1/A_2 x 100, where A_1 is the absorbance of the samples and standards and A_2 is the absorbance of the blank.

Nitric oxide scavenging assay

The nitric oxide scavenging (NOS) activity was assessed following a previous method (Boora *et al.,* 2014). Sodium nitroprusside (131 mg) and Griess reagent (2 g) dissolved in 50 mL PBS and 1 mg/mL in MeOH of the plant extracts and standards (ascorbic acid and gallic acid) were utilized in the assay. In a 96-well plate, 50 µL of the sample solutions were mixed with 70 µL sodium nitroprusside. The mixtures were incubated for 180 min at 25 °C before the addition of 120 µL Griess reagent. Then, the absorbance at 546 nm was read. The percentage inhibition was calculated following the equation: % NO Radical Inhibition = $[A_0 - A_1] / A_0$ x 100, where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample solutions.

ABTS radical cation assay

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) free radical scavenging of the plant extracts (125-1000 µg/mL) and Trolox standard (62.5-500 µg/mL) was measured as previously described (Re *et al.,* 1999). An equal volume of 0.7 mM ABTS and 2.45 mM potassium persulfate were mixed in the dark for 12 h at RT to generate the ABTS radicals. The sample solutions were mixed with the ABTS radical solution and incubated for 30 min in the dark at RT, followed by absorbance measurement at 734 nm. The % ABTS radical inhibition was calculated following the equation: % Inhibition of $ABTS^{+\cdot}$ = $[(A_0 - A_1)/A_0$ x 100, where A_0 is the absorbance of the control, and A_1 is the absorbance of the sample solutions.

Ferric reducing antioxidant potential (FRAP) assay

The FRAP assay of the plant extracts (at 500 and 1000 µg/mL in MeOH) and the butylated hydroxytoluene (BHT) standard (at 500 and 1000 µg/mL in MeOH) were measured based on a previously described method (Yu *et al.,* 2020). The working FRAP reagents were prepared by dissolving 0.2 M PBS (pH 7.4), 0.5 g $K_3Fe(CN)_6$, 5 g trichloroacetic acid (TCA), and 0.05 g $FeCl_3$ in 50 mL distilled H_2O. Sample solutions (70 µL) were mixed with 176.5 µL PBS and 176.5 µL $K_3Fe(CN)_6$. The mixtures were incubated at 50 °C for 20 min and subsequently added with 176.5 µL TCA. The resulting mixtures were centrifuged for 10 min at 650 xg. Then, 273 µL of the supernatant were mixed with 273 µL deionized H2O and 55 µL $FeCl_3$. The absorbance was read at 700 nm and the FRAP value was calculated following the equation: FRAP value = $[(A_1 - A_2)/(A_0 - A_2)]$ x 2, where A_0 is the absorbance of the positive control, A_1 is the absorbance of the plant extracts and standards, and A_2 is the absorbance of the blank.

Statistical analysis

Data were expressed as mean ± standard deviation. One-way analysis of variance (ANOVA) and Tukey's Honest Significant Difference (HSD) were employed to determine significant differences ($p \leq 0.05$) using GraphPad Prism 9.5.0.

Results and Discussion

Phytochemical estimation of the plant extracts

Estimation of the total phenolics content (TPC) and total flavonoids content (TFC) (Table 2) in the plant extracts was done using colorimetric assays. TPC was expressed as milligrams of gallic acid equivalents per gram of the dry weight sample (mg GAE/ g DW). TFC was expressed as milligrams of quercetin equivalents per gram of the dry weight sample (mg QE/ g DW).

Table 2. Total phenolic and total flavonoid content of crude methanolic extracts

Species	Total phenolic content (mg GAE/g DW)	Total flavonoid content (mg QE/g DW)
Guettardella microphylla	12.20 ± 0.25^{a}	3.81 ± 0.20^{a}
Kanapia monstrosa	6.89 ± 0.06^{b}	2.70 ± 0.06^{b}
Psychotria luzoniensis	16.36 ± 0.87^{c}	3.54 ± 0.23^{ac}
Pyrostria subsessilifolia	11.25 ± 0.41^{a}	3.43 ± 0.20^{c}
Pyrostria triflora	17.17 ± 0.83^{c}	1.86 ± 0.04^{d}
Timonius ternifolius	5.47 ± 0.38^{b}	2.16 ± 0.21^{d}

Values with different superscripts (a – e) within the same column show significant difference ($p \leq 0.05$). Data were expressed as mean ± standard deviation, n=5. GAE – gallic acid equivalents; QE – quercetin equivalents.

As shown in Table 2, *P. triflora* gave the highest TPC (17.17 mg GAE/g DW) and *T. ternifolius* showed the lowest value (5.47 mg GAE/g DW). Comparison of the TPC values also indicated that *P. triflora* and *P. luzoniensis*; *G. microphylla* and *P. subsessilifolia*; and *K. monstrosa* and *T. ternifolius* showed statistically comparable ($p > 0.05$) estimation of TPC. Estimation of the TFC indicated non-significant difference ($p > 0.05$) in *G. microphylla*, *P. luzoniensis,* and *P. subsessilifolia*. Interestingly, *P. triflora* had the lowest TFC despite having the highest TPC value.

The identification of phenolics and flavonoids in plants has been highly valued for pharmaceutical and medical applications due to their ability to alleviate oxidative stress-induced tissue damage associated with chronic diseases (Del Rio *et al.,* 2013; Tungmunnithum *et al.,* 2018). In numerous studies, both TPC and TFC are often correlated with antioxidant capacity (Aryal *et al.,* 2019; Liaqat *et al.,* 2021). Phenolic compounds are major plant antioxidants acting by scavenging free radicals, chelating metal ions that catalyze ROS formation, or stimulating endogenous antioxidant synthesis in cells. Moreover, phenolics are strong natural antioxidants generally regarded as safer than their synthetic counterparts (Boora *et al.,* 2014; Zeb, 2020). Flavonoids are plant-derived exogenous polyphenolic compounds. The antioxidant mechanisms of flavonoids can be achieved by the suppression of ROS formation via interaction with enzyme function; direct scavenging of ROS; and chelation of metal ions such as iron and copper to prevent the development of free radicals (Procházková *et al*., 2011; Kumar and Pandey, 2013; Amir Aslani and Ghobadi, 2016).

DPPH Free Radical Scavenging Activity

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay is used to provide an initial assessment of the radical scavenging potential of the test compounds. Antioxidant activity is evaluated using colorimetric analysis, wherein a color change from purple to yellow indicates the conversion of the DPPH• radical to DPPH, a more stable product, by an antioxidant compound through hydrogen or electron donation (Cornago *et al.*, 2011; Khatun *et al.*, 2020; Yu *et al.*, 2020). As shown in Figure 2, *P. luzoniensis* (96.40 % ± 0.44) demonstrated the highest radical scavenging activity and is statistically comparable to the standards ($p > 0.05$). *G. microphylla* (53.68 % ± 1.01) and *T. ternifolius* (54.46 % ± 0.30) both showed comparable activities, and were found to be moderately active.

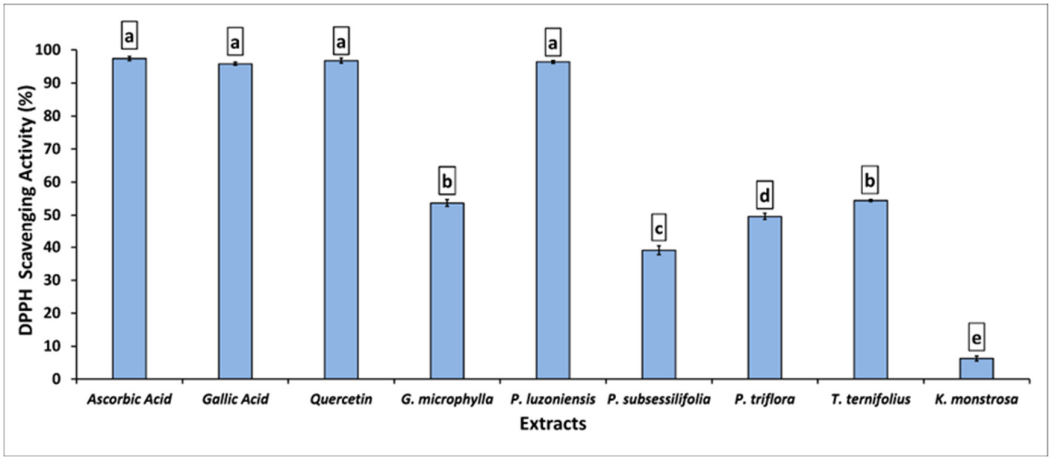

Figure 2. DPPH radical scavenging activity of the crude plant extracts and standards at 1 mg/mL
Values with different superscripts (a – e) show significant difference at $p < 0.05$. Data were expressed as mean ± SD, n=5.

The high DPPH radical scavenging activity of *P. luzoniensis* may be attributed to its identified phenolic and flavonoid compounds. Quercetin 3-O-rutinoside, quercetin-3-O-glucopyranoside, kaempferol 3-O-β-D-apiosyl-(1→2)-β-D-glucopyranoside, asperuloside, and (6S,9R)-roseoside were isolated from leaf ethanolic extracts of *P. luzoniensis* (Ramil *et al.,* 2020). These compounds were previously described to possess antioxidant and other important pharmacological properties (Zhang *et al.,* 2004; Choung *et al.,* 2017; Fitzpatrick and Woldemariam, 2017; Habtemariam, 2019; Manzione *et al.,* 2020).

Hydrogen peroxide scavenging activity

The hydrogen peroxide radical scavenging assay measures the capacity of compounds or plant extracts to diminish the H_2O_2 and dissociate it into oxygen and water (Duh *et al.,* 1999; Wettasinghe and Shahidi, 1999). In the human body, hydrogen peroxide is a non-radical oxidant that occurs as a by-product of biochemical metabolism in cells (Fernando and Soysa, 2015). This compound can be formed through catalysis by the enzyme superoxide dismutase (SOD) via a dismutation reaction from superoxide anions. Likewise, H_2O_2 can be directly synthesized in the presence of oxidases such as urate oxidase, glucose oxidase, and D-amino acid oxidase (Phaniendra *et al.,* 2014; Martemucci et al., 2022). Despite the relatively low reactivity of H_2O_2, its ability to diffuse freely across biological membranes and relatively long half-life are causes for concern. Upon interaction with Fe^{2+} and Cu^{2+} ions within the cell, it can generate highly reactive hydroxyl radicals through the Fenton reaction, which in turn, can induce oxidative stress and cause cellular damage (Ofoedu *et al.,* 2021). The overexpression of H_2O_2 is also shown to be associated with mitochondrial dysfunction, inflammatory responses, and certain life-threatening diseases (Singh *et al.,* 2019).

As shown in Figure 3, *G. microphylla* expressed the highest percentage of H_2O_2 scavenging activity (88.56 % ± 4.81) and was significantly higher ($p < 0.05$) compared to the standard ascorbic acid. The % H_2O_2 scavenging activity of *K. monstrosa* (39.81 % ± 7.90) and *P. luzoniensis* (53.48 % ± 3.13) were statistically comparable to the standard ($p > 0.05$). *P. subsessilifolia* (67.11 % ± 5.05), *P. triflora* (62.11 % ± 4.04) and *T. ternifolius* (75.34 % ± 2.19) all exhibited similar scavenging activity and were significantly higher ($p < 0.05$) compared to the standard.

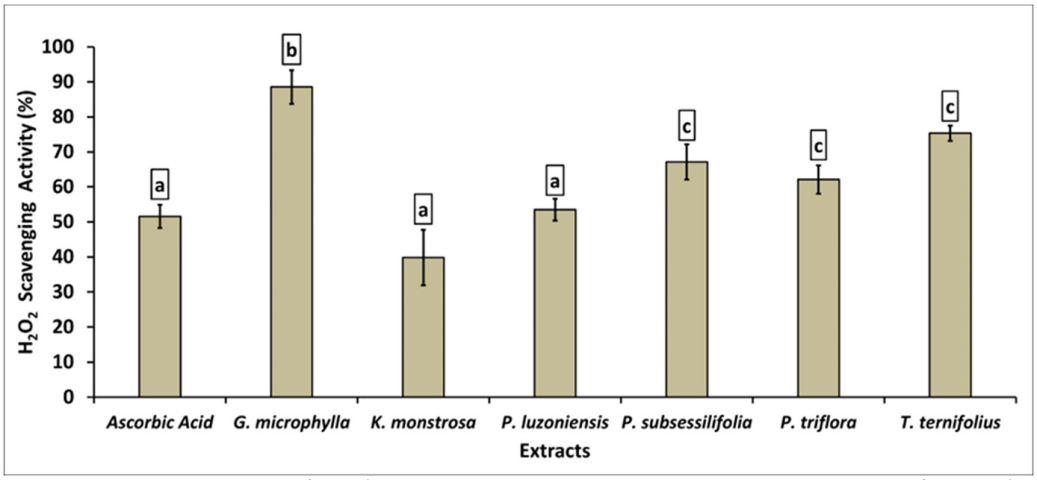

Figure 3. Hydrogen peroxide (H_2O_2) scavenging activity of the plant extracts and ascorbic acid (standard) at 1 mg/mL

Values with different superscripts (a – c) show significant difference at $p < 0.05$. Data were expressed as mean ± SD, n=5.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay involves the formation of Fe^{2+}-1,10-phenanthroline complex. In the presence of H_2O_2, Fe^{2+} is oxidized to $Fe^{3+,}$ and a hydroxyl radical is produced (Treml and Šmejkal, 2016) resulting in the reduced formation of the Fe^{2+}-1,10-phenanthroline complex. Upon addition of an antioxidant, there is increased formation of Fe^{2+}-1,10-phenanthroline complex due to a decreased production of hydroxyl radical and conversion of Fe^{2+} to Fe^{3+} (Kim *et al.,* 2020). The hydroxyl radical is considered the strongest oxidant and the most reactive free radicals among the ROS. Owing to its extremely short half-life, it immediately attacks proteins, DNA, lipids, or any kind of biomolecule it encounters. Results suggest that *T. ternifolius* may potentially be a good source of potent antioxidants that can quench hydroxyl radicals, which may be useful in the prevention of lipid peroxidation and other harmful effects of hydroxyl radicals in cells.

As shown in Figure 4, *T. ternifolius* had the highest hydroxyl radical scavenging activity (81.28 % ± 4.69) and is statistically comparable to the ascorbic acid standard ($p > 0.05$). *P. luzoniensis* (62.45 % ± 1.49) showed the second-highest hydroxyl radical scavenging activity. *G. microphylla* (27.81 % ± 11.53), *P. subsessilifolia* (39.26 % ± 1.09), and *P. triflora* (28.15 % ± 1.88) all exhibited comparable hydroxyl radical scavenging activity. *K. monstrosa* showed the lowest hydroxyl radical scavenging activity at 12.59 % ± 3.42.

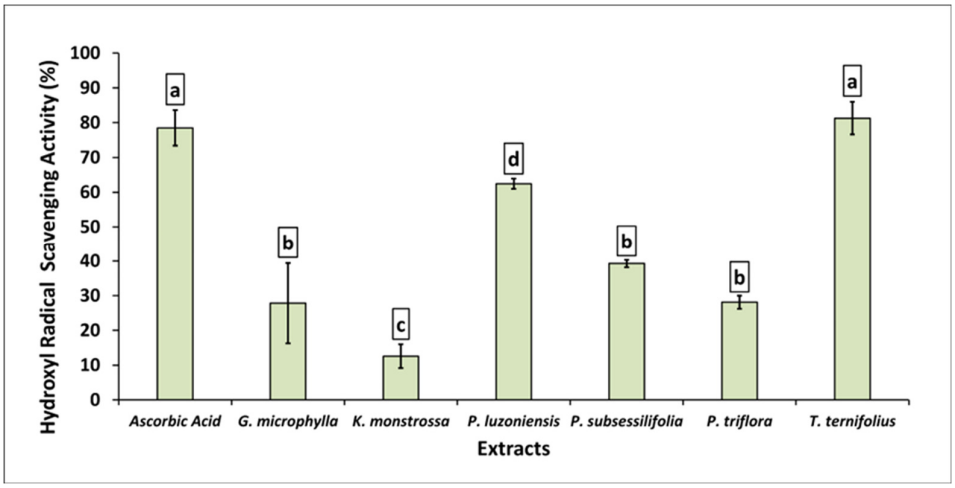

Figure 4. Hydroxyl radical scavenging activity of the plant extracts and standard at 1 mg/mL
Values with different superscripts (a – d) show significant difference at $p < 0.05$. Data were expressed as mean ± SD, n=5.

Nitric oxide (NO) scavenging assay

Under aerobic conditions, NO is unstable and reacts with oxygen to produce the stable nitrite ion (NO_2^-) (Weitzberg *et al.,* 2010). The latter product is estimated using Greiss reagent, which allows colorimetric characterization of nitrite concentration (Csonka *et al.,* 2015). In the presence of NO scavengers, NO production is inhibited via direct competition with oxygen, which is indicated by a diminished absorbance of the formed purple chromophore at 546 nm (Sarwar *et al.,* 2015). Although NO is involved in facilitating normal physiological processes in the human body such as neurotransmission, vasodilation, and inflammatory reactions, evidence has shown that excessive NO production contributes to oxidative damage (Etim *et al.,* 2013). Additionally, the involvement of NO in neural processes such as neurotransmitter release, neuronal excitability, learning, and memory, implicates its role in the pathogenesis of neurodegenerative disorders (Ebrahimzadeh *et al.,* 2010).

The nitric oxide scavenging activity of the plant extracts was assessed in 250, 500, and 1000 µg/mL concentrations (Figure 5). *P. luzoniensis* demonstrated significantly high nitric oxide scavenging across all three concentrations (250-1000 µg/mL), with activity ranging from 93.54 to 98.95% NO scavenging activity. *P. subsessilifolia, P. triflora*, and *T. ternifolius* had similar activities in the 250-500 µg/mL concentrations. A dose-dependent NO scavenging activity was observed among the six plant extracts.

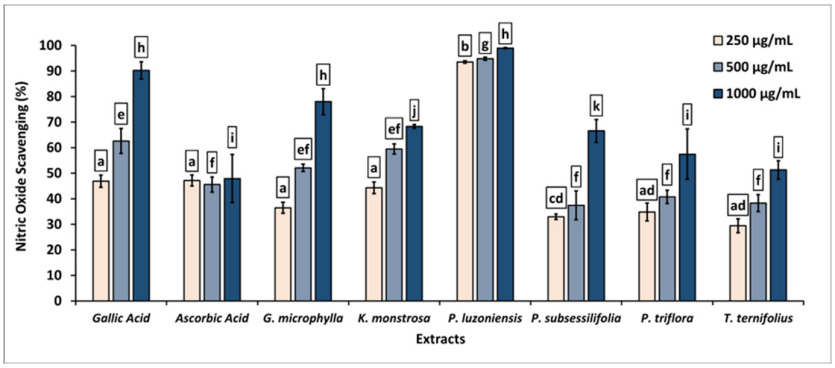

Figure 5. Nitric oxide scavenging activity of the plant extracts and standards
Values with different superscripts (a – k) show significant difference at $p < 0.05$. Data were expressed as mean ± SD, n=5.

ABTS radical cation assay

The ABTS antioxidant assay measures the ability of compounds or plant extracts to scavenge the radical cation 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$). The antioxidant activity of a potential scavenger is determined by its ability to reduce $ABTS^{\bullet+}$ to ABTS as indicated by the diminished absorbance of the pre-formed radical at 734 nm (Karasakal, 2015). Suggested processes potentially involved in the ABTS scavenging activity of antioxidants are the hydrogen atom transfer/single electron transfer (HAT/SET) reaction mechanism, stepwise electron transfer-proton transfer (ET-PT), and concerted electron-proton transfer (CEP) mechanism with water as the proton acceptor (Ilyasov *et al.,* 2020).

The ABTS radical scavenging activity of the plant extracts was assessed in 125, 250, 500, and 1000 µg/mL concentrations (Figure 6). Trolox in 62.5, 125, 250, and 500 µg/mL concentrations was used as the standard. *P. luzoniensis* at 1000 µg/mL showed 97.33% ABTS radical scavenging activity and is comparable to Trolox at the highest (500 µg/mL) concentration. *G. microphylla* at 1000 µg/mL showed the second highest ABTS radical scavenging activity (52.15 % ± 2.84). Both species had high phenolic contents (Gorinstein *et al.,* 2003; Rajurkar and Hande, 2011) which are likely to contribute to the elevated ABTS scavenging activities. Apart from *T. ternifolius*, the ABTS radical scavenging activities of all plant extracts are in a dose-dependent manner.

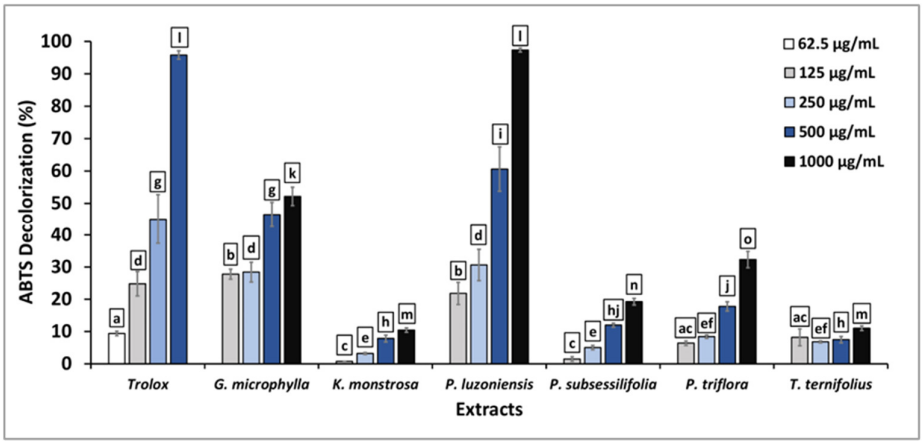

Figure 6. Free Radical Scavenging by ABTS Radical Assay of the plant extracts and Trolox (standard). Values with different superscripts (a – o) show significant difference at $p < 0.05$. Data were expressed as mean ± standard deviation, n=5.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay measures the antioxidant activity of a compound or plant extracts by donating an electron to reduce ferric ions (Fe^{3+}) into ferrous ions (Fe^{2+}) (Yu *et al.,* 2020; Liaqat *et al.,* 2021). Addition of $FeCl_3$ to the ferrous form results in the formation of a Prussian blue-colored complex. Thus, reducing power is measured by the formation of Prussian blue at 700 nm. Depending on the reducing power of the antioxidant, the sample color changes from yellow to green or blue. A higher reducing power is indicated by a higher absorbance value (Koksal *et al.,* 2011).

The ferric reducing antioxidant potentials of the plant extracts were expressed in FRAP values as shown in Figure 7. *P. luzoniensis* showed the highest FRAP values in both 500 µg/mL (1.85 ± 0.08) and 1000 µg/mL (2.12 ± 0.13) concentrations. *P. triflora* also exhibited a comparable FRAP value (1.88 ± 0.06) to the standard at 1000 µg/mL. *G. microphylla* and *P. subsessilifolia* showed comparable FRAP values at 500 µg/mL.

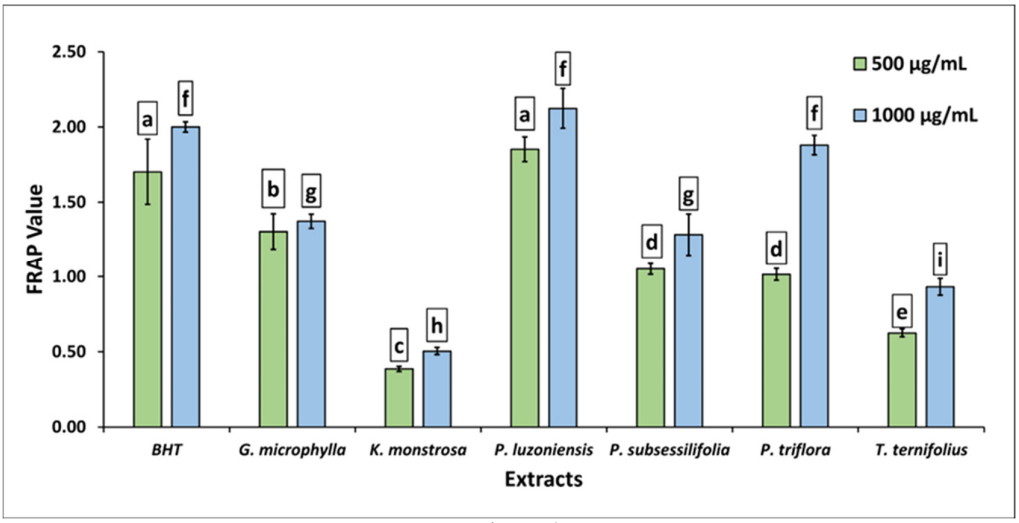

Figure 7. Ferric Reducing Antioxidant Power (FRAP) Assay of the plant extracts using butylated hydroxytoluene (BHT) as standard

Values with different superscripts (a – i) show significant difference at $p < 0.05$. Data were expressed as mean ± standard deviation, n=5.

The plant extracts showed varying levels of antioxidant activities in the assays employed. Results revealed that *P. triflora* had the highest TPC but the lowest TFC. *G. microphylla* exhibited the highest TFC and strongest inhibitory activity against hydrogen peroxide (H_2O_2). *P. luzoniensis* manifested the highest scavenging activity against DPPH, ABTS radicals, and ferric ions. In contrast, *P. subsessilifolia*, *K. monstrosa*, and *T. ternifolius* demonstrated lower levels of scavenging activity in some assays. This suggests that plant species may show good antioxidant potential in specific antioxidant assays, but may perform poorly or moderately in others, reflecting the various mechanisms behind the antioxidant activities of bioactive compounds present. This may also indicate that compounds present in plant species may respond differently to various radicals or oxidants. Hence, plant species showing low antioxidant activity may be subjected to other antioxidant assays following a different mechanism. In addition, TPC and TFC may not always be indicative of an extract's antioxidant activity. Factors such as oxidation conditions, nature of the compounds, and interactions with other compounds, which may have antagonistic or synergistic effects (Hidalgo *et al.* 2010), should be taken into consideration (Amarowicz *et al.,* 2004; Chaves *et al.,* 2020).

Furthermore, the use of different concentrations may yield significant descriptive results. Concentration-dependent antioxidant capacities and the approximate concentration at which scavenging activities are most effectively induced can be further investigated.

Conclusions

This study provides a pioneering report on the antioxidant activities of Philippine Rubiaceae species *G. microphylla, P. triflora, P. subsessilifolia, P. luzoniensis, T. ternifolius*, & *K. monstrosa*. Results revealed that the leaf crude methanolic extracts of *G. microphylla, P. triflora, P. subsessilifolia, P. luzoniensis, T. ternifolius*, & *K. monstrosa* possessed notable antioxidant activities that could impede the overproduction of free radicals. Thus, these species could be potential sources of natural antioxidants that may be used in nutraceutical and pharmaceutical applications.

Authors' Contributions

Conceptualization: GJDA and MAT; Methodology: JDJE, JMMB, KMSG, METP, CJAT and GGAH; Formal analysis: JDJE, JMMB, KMSG, METP, CJAT, GGAH and MAT; Investigation: JDJE, JMMB, KMSG, METP, CJAT, GGAH and MAT; Data curation: JDJE, JMMB, KMSG, METP, CJAT and GGAH; Writing – original draft preparation: JDJE, JMMB, KMSG, METP and CJAT; Writing – review and editing: GGAH, GJDA and MAT; Resources: MAT; Project administration: GJDA and MAT. All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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