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Phytochemical Content and Antioxidant Activities of Young and Mature Leaves of *Plukenetia volubilis*. L (Sacha Inchi) Extracted with Different Solvents

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Plukenetia volubilis L. (Sacha Inchi), is a high-value medicinal plant originating from the Amazon region. The leaves of *P. volubilis* are abundant in secondary metabolites, suggesting promising pharmaceutical potential. Despite the presence of secondary metabolites in *P. volubilis* leaves, detailed studies on their specific composition based on their level of maturity and biological relevance remain limited. This study analysed the secondary metabolites in young and mature leaves with hexane, ethanol, and dichloromethane extraction, followed by Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and Total Tannin Content (TTC). Meanwhile, the antioxidant properties were assessed using 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging and Ferric Reducing Antioxidant Power (FRAP) assays. Mature leaves yielded a higher crude extract (23.04%) than young leaves (22.09%). The highest TPC (24.08 ± 2.38 mg GAE/g) was found in ethanol mature leaves extract and the lowest was found in hexane young leaves extract (13.74 ± 0.62 mg GAE/g). The highest TFC value (61.48 ± 0.08 mg QE/g) was found in dichloromethane young leaves extract while the lowest TFC value (24.25 ± 0.52 mg QE/g) was found in hexane mature leaves extract. Besides, TTC was shown the highest in dichloromethane young leaves extract (220.13 ± 0.21 mg/TAE g) and the lowest TTC showed in dichloromethane mature leaves extract (0.08 ± 2.23 mg/TAE g). In the FRAP assay, the ethanol mature leaves extract showed the highest antioxidant activity (13.16 ± 1.80 umol (Fe (II)/g sample), while the ethanol young leaves extract had the lowest antioxidant activity (6.31 ± 0.92 umol (Fe (II)/g sample). In the DPPH assay, the highest antioxidant activity was shown by hexane mature leaves extract with the IC_{50}value of 0.47 mg/mL. Meanwhile, dichloromethane young leaves extract showed the lowest antioxidant activity with an IC_{50} value of 7.85 mg/mL. Dichloromethane mature leaves extract had the highest secondary metabolite content, according to the comparative analysis of all results. These findings demonstrate a statistically significant influence of leaf maturity and solvent polarity on the extraction efficiency and antioxidant activity of *P. volubilis* leaves ($p < 0.05$), indicating their potential as a valuable natural source of antioxidants.

Keywords: *Plukenetia volubilis* L.*,* secondary metabolites, antioxidant properties, young and mature leaf

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Plukenetia volubilis L.*,* commonly known as 'Sacha Inchi', is an oil-rich plant native to the Amazon region. While the seeds have been extensively studied and commercialized for their high omega fatty acid content, the phytochemical potential of the leaves remains largely underexplored. Recent studies indicate that *P. volubilis* leaves contain significant amounts of phenolic and flavonoid compounds, as well as terpenoids, which underlie their antioxidant and therapeutic potentials [1]. Demand for natural antioxidants from plants has increased in response to increasing concerns about the adverse impacts of synthetic antioxidants on human health [2]. Secondary metabolites, notably phenolic compounds and flavonoids, are known for their ability to neutralize free radicals, prevent lipid peroxidation, and provide a variety of health advantages. Leaf maturity and solvent polarity are important factors in determining the extraction efficiency and concentration of bioactive chemicals. Mature leaves usually accumulate more secondary metabolites, whereas young leaves may have distinct profiles of precursors or less glycosylated substances [3]. Solvent selection has a substantial impact on the type and quantity of metabolites extracted, as solvents of varying polarities preferentially dissolve distinct classes of phytochemicals. For example, Wakeel et al. (2019) reported methanol extraction of *Isatis tinctoria*

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yielded the highest total phenolic content (72.45 ± 2.1 mg GAE/g), followed by acetone (54.26 ± 1.9 mg GAE/g) and ethyl acetate (27.38 ± 1.2 mg GAE/g), whereas non-polar hexane extract showed the lowest phenolic content (9.63 ± 0.7 mg GAE/g) [4]. Wakeel et al. (2019) and Do et al. (2014) reported these results indicate that polar solvents such as methanol and ethanol efficiently extract hydrophilic metabolites like phenolics, flavonoids, and tannins, while non-polar solvents primarily recover lipophilic compounds such as terpenoids, fatty acids, and chlorophylls [4,5]. Given these considerations, the purpose of this study is to investigate the secondary metabolite profiles of young and mature *P. volubilis* leaves extracted with hexane, ethanol, and dichloromethane. Furthermore, their antioxidant capabilities were assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. This study offers recent insight on the phytochemical richness and functional potential of *P. volubilis* leaves, contributing to improve the overall value of this underutilized plant resource.

EXPERIMENTAL

Chemicals and Materials

Plant Material Collection

P. volubilis leave samples were collected from the Faculty of Bioresources and Food Industry in Universiti Sultan Zainal Abidin (UniSZA), Besut Campus, 22200 Besut, Terengganu. The leaves were classified into two maturity stages: young and mature. This classification was based on phyllotactic position and morphological criteria. For each plant sampled, the five most apical (terminal) fully expanded leaves on a single branch were designated as young leaves, while the subsequent five leaves located immediately below this group were classified as mature leaves**.**

Sample Preparation and Extraction

According to Tran et al. (2023), the collected *P. volubilis* leaves were rinsed with tap water and air-dried at room temperature overnight [6]. The following day, the leaves were dried in an oven at 40 – 45°C, until completely dried. The dried samples were ground into coarse powder using a grinder, and stored in moisture-proof bags at 25°C. For extraction, 30g of powdered leaf sample was soaked in 300 mL of each solvent (absolute ethanol, hexane, and dichloromethane) and left to macerate for 72 hours with occasional shaking. The extract was filtered using Whatman No. 1 filter paper and concentrated under reduced pressure using a rotary evaporator (Heidoplh, Germany) with the temperature of water bath within 40-45°C. The pressure for hexane, dichloromethane and ethanol are 200-300, 200-400, and 80-120 mbar respectively. The final extract was weighed, labeled, and stored in amber glass vials at 4°C until further analysis.

Characterization Methods

Phytochemical Analysis

The leaves extract of the *P. volubilis.* for each solvent was qualitatively tested for the presence of its chemical constituent.

Total Phenolic Content (TPC) Assay

The total phenolic content (TPC) of *P. volubilis* extracts was analyzed using Folin-Ciocalteu colorimetric method adapted from Ainsworth & Gillespie (2007) with slight modifications to the extract concentrations [7]. A 60 uL aliquot of a 5 mg/mL extract was mixed with 200 uL Folin-Ciocalteu reagent. After 1-2 minutes, 800 uL of 7.5% sodium carbonate was added, and the mixture was incubated for 2 hours. Absorbance was measured at 765 nm using a microplate reader (Fisherbrand™). TPC of samples extracts was calculated using linear regression equation obtained from Gallic Acid Equivalent (GAE) calibration curve, C = cV/m and were expressed as mg GAE (gallic acid equivalents) per gram of extract.

Total Flavonoid Content (TFC) Assay

The total flavonoid content (TFC) assay of *P. volubilis* extracts was adapted from Zakaria et al. (2023) with some modifications to extract concentrations [8]. A mixture of 140 uL sample extract, 150 uL 1 M potassium acetate, and 150 uL 10% aluminium chloride was prepared, and the volume was adjusted to 700 uL with DMSO, then incubated for 30 minutes. About 200 uL of the mixture were transferred to a 96-well plate, and absorbance was measured at 415 nm using a microplate reader (Fisherbrand™). Results were expressed as mg quercetin equivalents per gram of sample.

Total Tannin Content (TTC) Assay

The total tannic content (TTC) analysis was adapted from Ainsworth & Gillespie (2007) with modifications to extract concentrations [7]. The Folin-Ciocalteu colorimetric method was used to analyze the TTC of *P. volubilis* samples. A 600 uL aliquot of a 5 mg/mL extract was mixed with 200 uL Folin-Ciocalteu reagent. After 1-2 minutes, 800 uL of 20% sodium carbonate was added, and the mixture was incubated for 2 hours. Absorbance was measured at 765 nm using a microplate reader (Fisherbrand™), and TTC was calculated from the tannic acid standard curve, expressed as mg tannic acid equivalent per gram of extract.

Antioxidant Analysis

3.5.1 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

The DPPH assay was adapted from Blois (1958) with modifications [9]. A 200 uL 0.1 mM DPPH working solution was added to the 50 uL crude sample in a 96-well plate and incubated in the dark for 30 minutes. Absorbance was measured at 515 nm using a microplate reader (Fisherbrand™). Gallic acid was used as the standard, and the percentage of DPPH scavenging activity was calculated using the formula below:

$$\%\ \text{of DPPH inhibition} = \frac{Control\ absorbance - Sample\ absorbance}{Control\ absorbance}\ x\ 100\%$$

The extract concentration that inhibited 50% of free radicals (IC_{50}) was determined by plotting a graph of the inhibition percentage against the concentration using GraphPad Prism. Antioxidants with stronger scavenging ability have lower IC_{50} values.

Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was adapted from Sachett et al. (2021) with slight modifications [10]. FRAP reagent was prepared by mixing 15 mL acetate buffer (pH 3.6), 1.5 mL TPTZ in 40 mM HCl, and 1.5 mL of 20 mM ferric chloride in a 10:1:1 ratio. A 10 uL sample extract was mixed with 300 uL FRAP reagent, then incubated at 37°C for 30 minutes. Absorbance was read at 593 nm, and results were expressed as µmol Fe^{2+} equivalents per gram of sample (µmol Fe(II)/g sample).

Statistical Analysis

The data obtained were analysed using analysis of variance (ANOVA), and the Tukey test was used to evaluate the significant difference between mean values at the confidence level of 95% *($p < 0.05$)*. Then, Pearson's correlation was carried out to identify the correlation between antioxidant activities, phenolic content, flavonoid content, and tannin content. Each analysis was conducted in triplicate, and the data were analysed in software IBM SPSS Statistics Version 27 and represented as the mean of three independent experiments.

RESULTS AND DISCUSSION

The phytochemical content and antioxidant activities of young and mature *P. volubilis* leaves were evaluated with Total Phenolic Content (TPC), Total Flavonoid Content (TFC), Total Tannin Content (TTC), Ferric Reducing Antioxidant Power (FRAP), and antioxidant activity using DPPH radical scavenging assay (IC_{50}). DPPH and FRAP were employed to ensure a comprehensive assessment of antioxidant potential, as each method provides unique insights into different antioxidant mechanisms.

Table 1. Phytochemical content and antioxidant activities of *P. volubilis* young and mature leaf extracts.

Samples	TPC (mg GAE/g of sample)	TFC (mg QE/g of sample)	TTC (mg TAE/g of sample)	FRAP (µmol Fe(II)/g of sample)	IC_{50} (mg/mL)
MLE	24.08 ± 2.38^{d}	34.40 ± 0.64^{b}	201.08 ± 0.17^{d}	13.16 ± 1.80^{c}	0.65 ± 4.12^{b}
MLD	16.55 ± 1.40^{b}	45.29 ± 0.17^{c}	0.08 ± 2.23^{a}	10.32 ± 0.95^{b}	0.46 ± 3.27^{b}
MLH	15.17 ± 1.73^{ab}	24.25 ± 0.52^{a}	3.30 ± 0.08^{a}	12.43 ± 1.23^{b}	0.47 ± 1.50^{b}
YLE	19.22 ± 0.52^{c}	26.81 ± 0.44^{a}	33.68 ± 2.02^{b}	6.31 ± 0.92^{a}	2.74 ± 1.96^{a}
YLD	17.25 ± 0.77^{bc}	61.48 ± 0.08^{d}	220.13 ± 0.21^{e}	7.14 ± 1.22^{a}	7.85 ± 1.05^{a}
YLH	13.74 ± 0.62^{a}	38.52 ± 0.67^{b}	152.78 ± 1.60^{c}	6.39 ± 0.23^{a}	0.47 ± 1.07^{b}

* Values are shown as means ± standard deviation
* Values in the same column followed by different letters are significantly different ($p < 0.05$).
MLE: Mature leaf in ethanol extract, MLD: Mature leaf in dichloromethane extract, MLH: Mature leaf in hexane extract, YLE: Young leaf in ethanol extract, YLD: Young leaf in dichloromethane extract, YLH: Young leaf in hexane extract.

Total Phenolic Content (TPC) Assay

According to Johari & Khong (2019), total phenolic content (TPC) quantifies the concentration of phenolic compounds, which are known for their antioxidant properties. Plants contain phenolic chemicals with redox characteristics, which allow them to act as antioxidants. From Table 1, mature leaf extracted in ethanol has the highest value of TPC (24.08 mg GAE/g of sample). This finding was aligned with study reported by Johari & Khong (2019) which indicated the bioactivity of the ethanol extract can be related to its higher phenolic content [11]. While, Afshari & Sayyed-Alangi (2017) stated that, ethanol extraction for 18 hours yielded the highest total phenolic content, indicating that ethanol was the most effective solvent for extracting phenolics from *Cressa cretica* leaves. They discovered that the polarity of ethanol matches the polarity of most phenolic compounds, making it a good solvent for phenolic compounds [2]. Then, according to Regolo et al. (2024), ethanol can efficiently solubilize cell wall-bound phenolic derivatives during extraction, which are found at higher concentrations in mature leaf tissues from fruit plants [12].

Total Flavonoid Content (TFC) Assay

From Table 1, young leaf extracted in dichloromethane shows the highest total flavonoid content (61.48 mg QE/g of sample). This is due to young leaves frequently containing hydrophobic flavonoids or precursors of flavonoids that lack glycosylation (attachment to sugar molecules). According to Obia et al. (2023), dichloromethane extracts have been found to be abundant in lipophilic substances such terpenes, alkaloids, and certain flavonoids. Besides, Young leaves often contain non-glycosylated flavonoid precursors (aglycones), which exhibit higher solubility in less polar solvents like dichloromethane due to their lipophilic nature. The significantly higher flavonoid content in young *P. volubilis* leaves extracted with dichloromethane is supported by studies demonstrating that aglycone-rich flavonoids, prevalent in early leaf stages, are more effectively extracted using semi-polar solvents [3]. Zhou (2016) observed that young leaves, being metabolically active, accumulate flavonoid precursors that dissolve better in dichloromethane compared to ethanol or water [13].

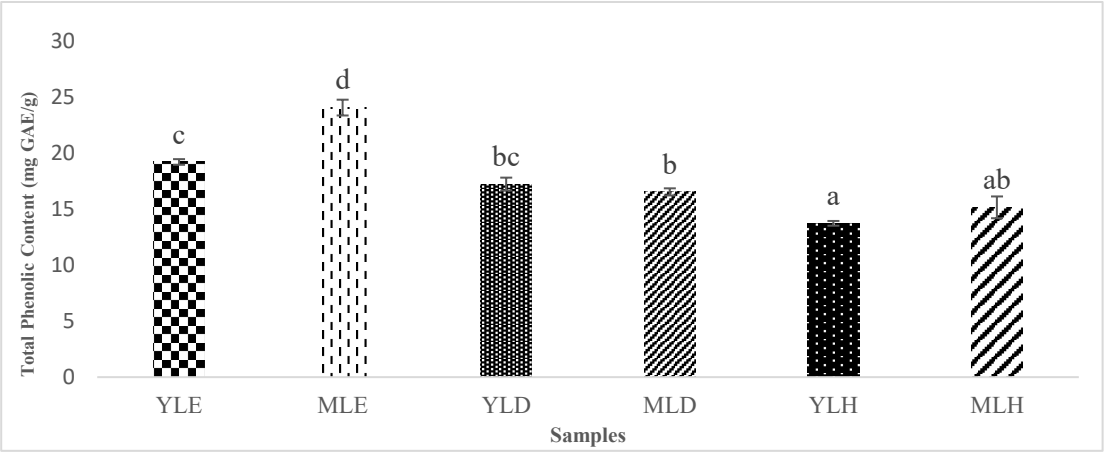

Figure 1. Total Phenolic Content of *P. volubilis* extracts in different solvents.

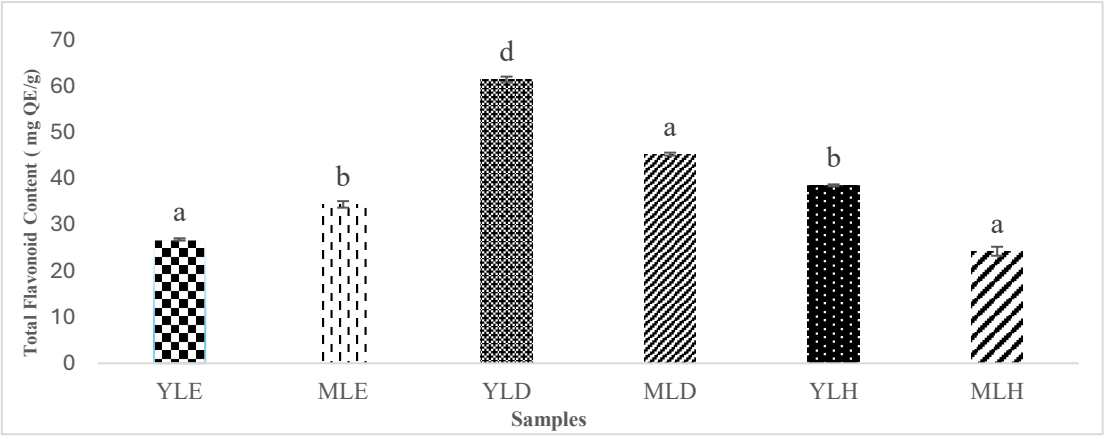

Figure 2. Total Flavonoid Content of *P. volubilis* extracts in different solvents.

Total Tannin Content (TTC) Assay

From Table 1, the highest total tannin content (TTC) was reported in young leaves extracted using dichloromethane (220.13 mg TAE/g of sample). This may be due to the presence of low molecular weight or unbound tannin precursors in young leaves, which are more readily extracted by semi-polar solvents. However, Barbehenn & Constabel (2011) found the tannins in mature leaves tend to be less extracted using dichloromethane because they are more likely to be oxidized, polymerized, and attached to structural cell wall components [14]. This finding is consistent with Yıldırım et al. (2003), who found considerably higher TTC in immature Polygonum leaf extracts using dichloromethane than in mature tissues. These findings indicate that *P. volubilis* younger leaf tissues are more effective source of extractable tannins, especially when utilizing solvents capable of dissolving semi-polar and low-molecular-weight polyphenols [15].

Ferric Reducing Antioxidant Power (FRAP) Assay

From Table 1, mature leaf extracted in ethanolic shows the highest FRAP value (13.16 µmol (Fe (II)/g of sample)). This is supported with previous studies reporting that ethanol-based solvents effectively extract phenolic and flavonoid compounds responsible for ferric-reducing activity. For example, Youn et al. (2018) found a 30% ethanolic leaf extract of *Dendropanax morbifera* showed the highest FRAP activity compared with other solvents [16]. Similarly, Jirakitticharoen et al. (2022) observed that 50% ethanolic extracts demonstrated stronger reducing power than water or hexane extracts [17]. Hardinsyah et al. (2019) also reported that the ethanolic extract of mature leaves exhibited the highest antioxidant potential, suggesting that solvent polarity and leaf maturity significantly influence the antioxidant capacity of plant materials [18]. Similarly, Nobossé et al. (2018) found that mature *Moringa oleifera* leaves extracted with methanol or ethanol demonstrated higher FRAP, which correlated with their elevated phenolic contents [19]. Overall, these findings suggest that ethanol is highly effective solvent for extracting phenolic compounds with strong ferric reducing activity from mature leaves, thereby enhancing the therapeutic potential of *P. volubilis* ethanol extracts.

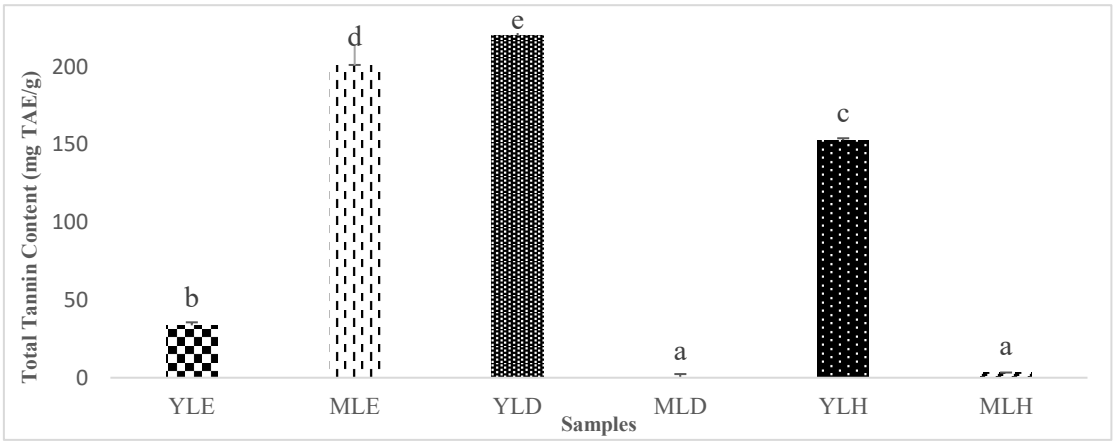

Figure 3. Total Tannin Content of *P. volubilis* extracts in different solvents.

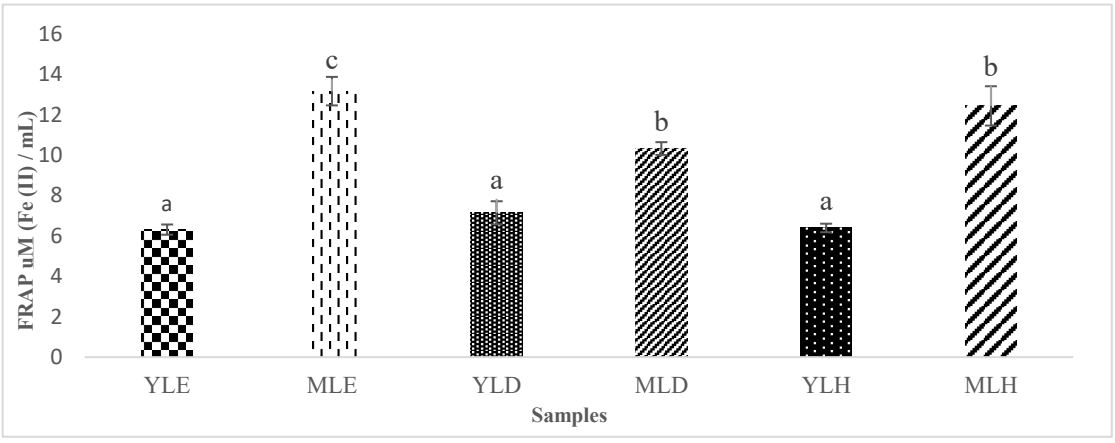

Figure 4. Ferric Reducing Antioxidant Power of *P. volubilis* extracts in different solvents.

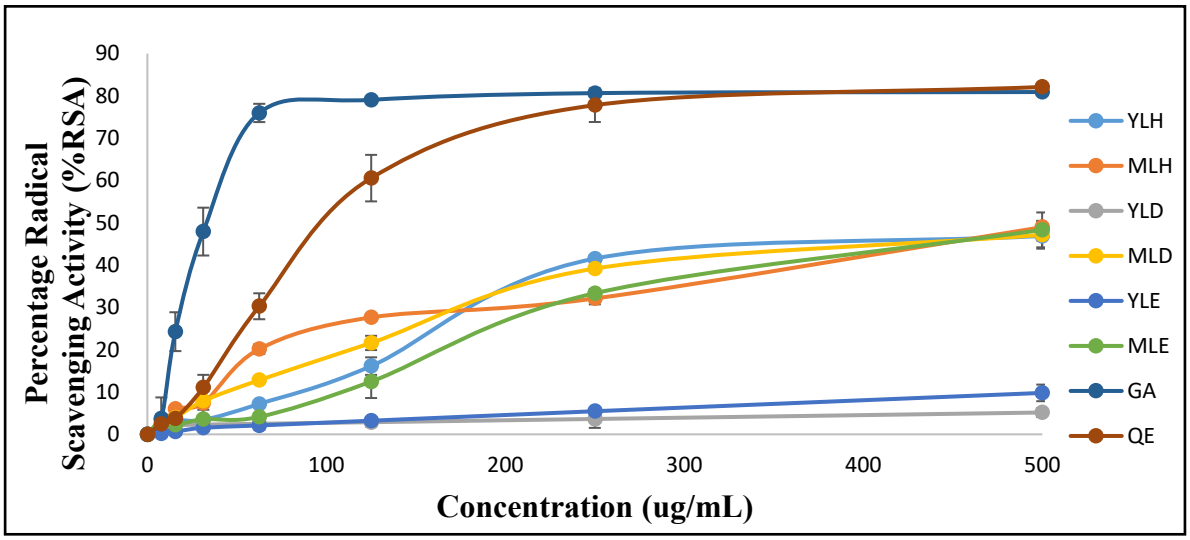

Figure 5. Free radical Scavenging Activity (2.2-diphenyl 1-picrylhydrazyl, DPPH) of *P. volubilis* extracts in different solvents.

Free radical Scavenging Activity (2.2-diphenyl 1-picrylhydrazyl, DPPH) Assay

According to Johari & Khong (2019), scavenging activity of DPPH is predicated on one-electron reduction, which is an indicator of antioxidants' capacity to reduce free radicals [11]. This method involves the discoloration of the stable free radical 2,2-diphenyl-1-picrylhydrazyl in the presence of the antioxidant compound. Table 1 show the mature leaf extracted in hexane exhibited the highest DPPH activity with the lowest IC_{50} value (0.47 mg/mL). This outcome corresponds with Chirinos et al. (2013) stated that the findings that Sacha Inchi contains substantial amounts of lipophilic antioxidants such as tocopherols and phytosterols, which are efficiently recovered by hexane extraction and can contribute to high DPPH scavenging activity [20]. In contrast, the young leaf extracted in dichloromethane showed the lowest activity IC_{50} value (7.85 mg/mL), despite its high flavonoid and tannin content. This suggests that not all flavonoids or tannins contribute equally to antioxidant activity, most likely because of their chemical form or limited reactivity toward DPPH radicals.

Correlation between Secondary Metabolites and Antioxidant Activity

Pearson correlation analysis revealed a significant negative correlation between DPPH radical scavenging activity and IC_{50} value (r = –0.872, p = 0.011), confirming that extracts with lower IC_{50} possessed stronger antioxidant potential. This inverse relationship agrees with observations by Youn (2018) and Johari (2019) that high phenolic concentration is typically associated with low IC_{50} values in leaf extracts [11,16]. However, correlations between secondary metabolites and antioxidant assays were generally weak or statistically non-significant. TPC exhibited a moderate positive correlation with FRAP (r = 0.416, p = 0.412) but a weak negative correlation with DPPH IC_{50} (r = –0.289, p = 0.583). Similarly, TFC showed moderate positive correlation with FRAP (r = 0.601, p = 0.209) and moderate negative correlation with DPPH IC_{50} (r = 0.708, p = 0.115). TTC displayed weak positive correlation with FRAP (r = 0.278, p = 0.599) and weak negative correlation with DPPH IC_{50} (r = –0.352, p = 0.490). Although none of these metabolite assay correlations reached statistical significance (p > 0.05), the positive trends with FRAP and negative trends with DPPH IC_{50} indicate that phenolics, flavonoids, and tannins contribute partially to the reducing and radical scavenging abilities of *P. volubilis* extracts. These results suggest that antioxidant activity is not determined solely by total compound concentration but also by qualitative factors such as compound structure and polarity. As reported by Chen et al. (2020) and Magalhães et al. (2017), structural characteristics such as hydroxyl group placement, degree of polymerization, molecular size, and solubility influence the redox potential and radical scavenging capacity of phenolics [21,22]. Hence, the weak correlations may reflect differences in the structural composition of compounds within extracts. Moreover, Obia et al. (2023) stated the presence of non-phenolic antioxidants, such as terpenoids and alkaloids which particularly in dichloromethane extracts may contribute to total antioxidant capacity [3]. Overall, these findings indicate that antioxidant efficiency in *P. volubilis* depends on both the quantity and the structural diversity of extracted metabolites, reinforcing the importance of compound-specific profiling for future studies.

CONCLUSION

This research presents significant insights into the phytochemical content and antioxidant capacity of *P. volubilis* leaves during two stages of development, young and mature. The results indicated that mature leaves extracted with ethanol exhibited the highest total phenolic content and the most significant antioxidant activity, as assessed by both FRAP and DPPH assays. Young leaves extracted with dichloromethane exhibited highest flavonoid and tannin levels but exhibited lower antioxidant activity. The results represent the significance of phenolic compounds in redox-related antioxidant capacity, particularly within ethanol extracts. The data suggest that phenolics act as the primary antioxidants in this species, although some correlations are non-significant. Mature ethanol leaf extracts may serve as effective sources for developing plant-based antioxidants applicable in pharmaceuticals or nutraceuticals. Future research should focus on identifying bioactive compounds, elucidating their chemical structures, and validating their efficacy through *in vivo* or clinical studies to advance functional food or medicinal applications aligned with global health and sustainability.

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