ORIGINAL ARTICLE

Association between *Piper betle's* Leaf Venation Patterns and Its Antioxidant Status – A Preliminary Study

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ABSTRACT

Background: In Malay tradition, "sirih bertemu urat" (converging veins) and "sirih tidak bertemu urat" (non-converging veins) are terms used for betel leaves on the same plants but with different pattern. Morphologically, the converging-vein leaves exhibit the left and right veins (nerves lateral) meet at the central vein, while non-converging vein leaves lack this characteristic. Despite limited scientific evidence supporting their efficacy, Malay Traditional Medicine favors the use of *P. betle* 's converging-vein leaves for medicinal purposes. Therefore, this study aims to investigate the antioxidant status of extracts from both converging- and non converging-vein betel leaves by analyzing phenolic compounds and antioxidant activity. **Methods:** The leaves were subjected to a maceration process in an incubator shaker using 80% methanol to obtain the extract. The total phenolic content was measured using the Folin-Ciocalteu method, and antioxidant activity was evaluated through DPPH and FRAP assays. **Results:** Statistical analysis revealed significant differences ($p < 0.05$) in the phenolic content between *P. betel*'s converging- and non converging-vein leaves, "indicating that *P. betel*'s converging-vein leaves have a higher phenolic content compared to *P. betel*'s non converging-vein leaves". The results obtained from the DPPH and FRAP assays also suggested that *P. betel*'s converging-vein leaves may have higher antioxidant activity compared to the non converging-vein. **Conclusion:** The variations in leaf morphology within the same plant species might influence the leaf's total phenolic compounds which in turn reflect its antioxidant properties. Thus, for medicinal purposes, it is important for researchers and practitioners to note the variation in leaf's venetion pattern when selecting betel leaves in order to obtain the optimal outcome.

Keywords:
Piper betle, venation pattern, antioxidant, 'bertemu urat'

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INTRODUCTION

Piper betle L. belongs to the family of Piperaceae with a hundred varieties that can be found all over the world. It is commonly known as betel leaf or betelvine. Betel leaf can be found in eastern and central Peninsular Malaysia and is known as"sirih". *P. betle* commonly used in traditional medicine as recorded in Malay medical manuscripts, traditional Chinese medicine, and the Indian Ayurvedic medicinal system. Based on Kitab Tib MS 174, betel leaves can heal coughs, remove body odor and bad breath, treat abscesses, and decrease vaginal discharge (Aziz & Yunus, 2019). Medicinal used of *P. betle* were also written in MSS 2999 Kitab Tib (Hussain, 2015) for the treatment of blurred vision, heartburn, lethargy, leucorrhea, and orthostatic hypotension. Moreover, in the Ayurvedic medicinal system, *P. betle* has historically been used to treat a variety of illnesses, including the common cold, bronchial asthma, cough, stomachalgia, and rheumatism, as well as boils, bad breath, constipation, conjunctivitis, and gum swelling (Biswas, 2022).

These traditional indications were aligned with scientific data. Jaiswal et al. (2014) showed that the leaves has a very high antioxidant content due to the presence of phenolic compounds known as hydroxyl-chavicol (4-allyl pyrocatechol). Hydroxyl-chavicol was found to be a highly effective scavenger of receiving oxygen species (ROS). Some other major phenolic compounds are eugenol and isoeugenol. Hydroxyl-chavicol, eugenol and isoeugenol were discovered to have protective properties against oxidative DNA damage and facilitate in the inhibition of lipid peroxidation. Eugenol was also mentioned by Savsani et. al (2020) to exhibit the most powerful antioxidant activity. *P. betle* also contains a variety of phytochemical compounds, including alkaloids, flavonoids, saponins, tannins, and steroids (Aara, 2020).

Moreover, it is important to note that in Malay tradition, only "sirih bertemu urat" leaves are used for the purpose of medicine. "Sirih bertemu urat" leaves referred to the leaves where the left and right veins (lateral veins) meet at the central vein (converge-vein). As for "sirih tidak bertemu urat" leaves, the left and right veins do not meet

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each other at the central veins creating the off-set pattern on the leaves (non converge-vein). Even though both types of these leaves possess different morphologies, they originate from the same plant. According to Yusoff (2017), the converge-vein leaves typically can be found in the middle section of the plant, where it is rooted for attachment. Salleh (2014) stated that in the customary engagement ceremony of Malay culture, the use of *P. betle*'s converge-vein leaves is discouraged due to their significant medicinal value, as evidenced by various Malay medical manuscripts. Various Malay medical manuscripts indicate that when using betel leaf, it is highly recommended to use converge-vein leaves as also mentioned in the treatment of eye diseases based on Ramuan Obat EAP153/9/4 (Mohd Shafri, 2021). Typically, betel leaves that deemed suitable for traditional Malay medicinal purposes are darker in color, medium in size, and positioned to face sunlight (Yusoff, 2017).

While traditional medicine attributes superior healing properties to the converge-vein leaves of *P. betle*, there is a notable lack of scientific evidence to validate these claims. Ekosari and Sugiarto (2013) reported that converge-vein leaves of *P. betle* possessed a flavonoid content 10 ppm higher than non converge-vein leaves. However, their findings are constrained by a lack of methodological transparency regarding the measurement techniques used. Consequently, a significant gap remains in the scientific literature concerning whether venation patterns truly dictate the antioxidant profile of the plant. This study, therefore, aims to investigate the impact of *P. betle* venation patterns on phenolic content, which serves as the primary driver of its medicinal and antioxidant properties."

MATERIALS AND METHODS

Sample Collection

The *P. betle* leaves were a kind gift from Encik Ganesan, owner of the *P.betle* farm where the leaves were picked [Kampung Pintu Padang, Raub (3°49'47.0"N 101°53'53.0"E)]. A total of 250 g of fresh "bertemu urat" leaves and 270 g of "tidak bertemu urat" leaves were collected. The leaf-picking were done from 11:45 am to 12:30 pm. The temperature and light intensity at the collection site were recorded respectively, using smartphone apps for temperature measurement and a lux meter (Mastech, Hong Kong) for light intensity measurement. The collected leaves were packed in plastic bags and transported to the laboratory where they were directly washed and dried.

Sample Preparation

The betel leaves were initially washed with distilled water and left to air-dry at room temperature for 10 days. During this period, the leaves were weighed daily until there was constant weight for subsequent 3 days. By using a blender (Panasonic, Japan), the leaves were grounded before the extraction process.

Extraction Process

A 2L solution of 80 % methanol was prepared using a combination of methanol and distilled water in an 8:2 ratio as the solvent extract. Each gram of powdered leaves was combined with 25 ml of the 80% methanol solution in a conical flask. The resulting mixture underwent a maceration process in an incubator shaker (Infors HT, Switzerland), set at 30°C and 200 rpm, for a duration of 18 hours. The macerated mixture was filtered twice using filter paper (Smith, USA). The samples were stored in a chiller at 4 °C until transported to the Natural Product lab, Kulliyyah of Science, for solvent drying. For solvent drying and extract concentration, a rotary evaporator (Buchi, Switzerland) was utilized. The solvent was concentrated using the rotary evaporator. The concentrated extract was subsequently stored at 4 °C until its further use.

Total Phenolic Content (TPC)

TPC was measured using the Folin-Ciocalteu (Jaiswal et. al 2014). 200 µL of the leave extracts were mixed with 1 ml of newly diluted (10-fold) Folin-Ciolateu. The mixture was incubated at room temperature for 8 minutes. 3 ml of 7.5 % sodium carbonate was added inside the mixture and was shaken vigorously, followed by incubation in the dark for 1 hour. The absorbance of the sample was measured 765 nm using UV-Vis spectrophotometer (Secomam, France). Gallic acid between 50-500 mg/l was used to create the standard curve. The test was done in triplicate. Since the extracts were in liquid form, instead of presenting the result as mg gallic acid equivalent (GAE)/g crude extract, the result was presented as mg gallic acid equivalent (GAE)/ ml extract. Total phenolic content in the samples were expressed by the following formula (Gentscheva et al., 2021):

$$\text{Gallic acid equivalent} = \left(\frac{C\ X\ Vs}{Ve}\right) \quad (1)$$

Where, C = Concentration of gallic acid from the calibration curve (mg/L)
Vs = Volume of sample used in assay (L)
Ve = Volume of extract (ml)

DPPH Radical Scavenging Activity

The assay was based on Jaiswal et al. (2014) with slight modifications. 100 ml of 2,2-Diphenyl-1-picryl hydrazyl (DPPH) solution was prepared (in 80 % methanol). A series of sample dilution was prepared. Then, 100 µL of the diluted samples were mixed with 3.9 ml of 0.004 % DPPH solution, followed by 30 minutes incubation in the dark. Absorbance were read at 515 nm using UV-Vis spectrophotometer (Secomam, France). 80% methanol was used as blank. A sample blank sample containing the same amount of methanol and DPPH was also used. Ascorbic acid was used to plot the standard curve. Since the level of antioxidant was inversely proportional to the absorbance measured, free radical scavenging activities were expressed as percent inhibition (%) based on ascorbic acid equivalent and determined using the formula below:

Percent inhibition of DPPH scavenging activity,

$$= \left(\frac{A\,c - A\,s}{Ac}\right) \text{ x } 100\% \qquad (2)$$

Where, Ac = the absorbance of control
As = the absorbance of sample.

Ferric Reducing Antioxidant Potential (FRAP) Assay

The procedure was performed based on the method described by Jaiswal et al. (2014). To prepare the FRAP assay, 200 mL of 300 mM acetate buffer (pH 3.6) was combined with 20 mL of 20 mM ferric chloride hexahydrate (dissolved in distilled water) and 20 mL of 10 mM 2,4,6-tri-(2-pyridyl)-s-triazine (TPTZ) (dissolved in 40 mM HCl). A standard graph for ferrous sulphate (FeSO4) was created using various concentrations ranging from 0.1 mM to 1 mM. To perform the assay, 0.8 mL of the FRAP solution was mixed with 24 µL of each standard or sample concentration. After a 4-minute incubation period, the absorbance at 593 nm was measured using a UV-Vis spectrophotometer (Secomam, France). The ferrous sulphate equivalent concentration was calculated using the standard graph.

Statistical Analysis

The antioxidant status between the extracts of *P.betle* “bertemu urat” and *P. betle* “tidak bertemu urat” was determined by independent t-test using SPSS statistical software version 20 for windows. The value of *p* less than 0.05 (p<0.05) was considered as statistically significant.

RESULTS

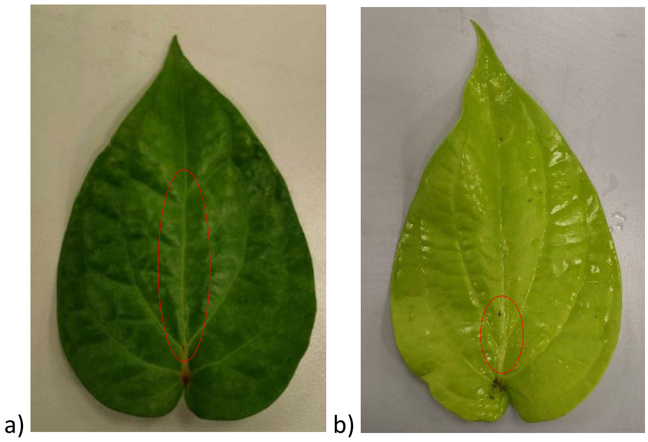

Figure 1: (a) Leaf morphology of *P. betle* “bertemu urat”, that showed the left and right veins (nerves lateral) meet at the central vein; (b) Leaf morphology of *P. betle* “tidak bertemu urat”, that showed the left and right vein did not meet at the central vein.(Label the central vein)

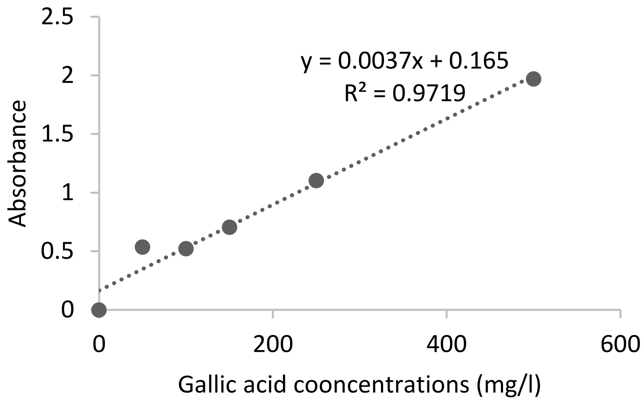

Figure 2: Gallic acid standard curve

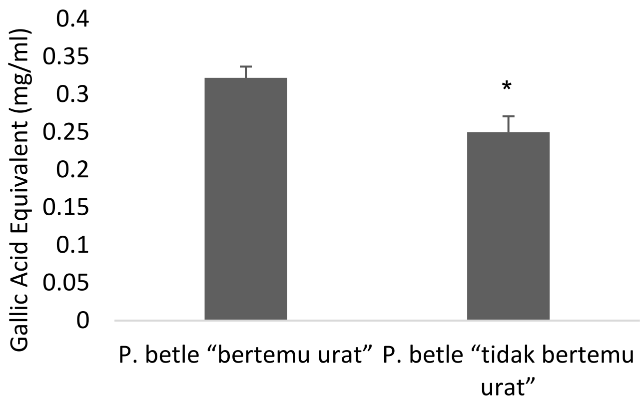

Figure 3: Total Phenolic Content (TPC) of *P. betle* leaf *extracts* expressed in gallic acid equivalent. Data are expressed as mean ± SEM. *Indicates *p*<0.05 (t-test) as compared to the bar on the left. *P. betle* ‘bertemu urat’ leaves contain significantly higher phenolic content as compared to the *P. betle* ‘tidak bertemu urat’ leaves.

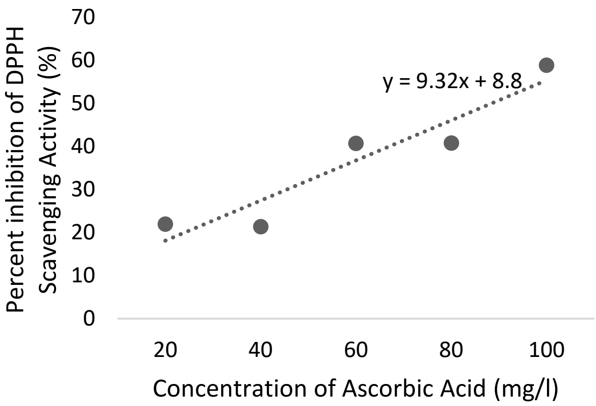

Figure 4 : Ascorbic acid's standard curve

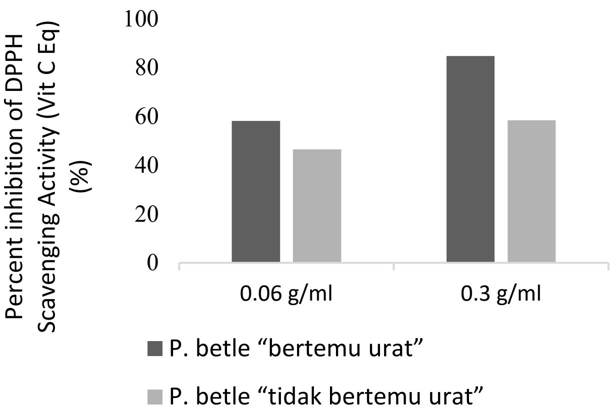

Figure 5 : Percent inhibition of DPPH radicals scavenging activities of *P. betle extracts* expressed in ascorbic acid equivalent

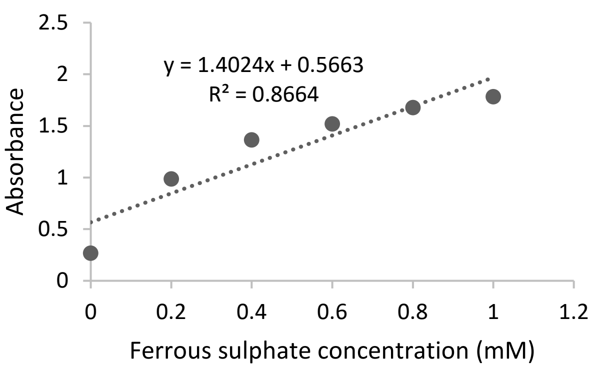

Figure 6 : Ferrous sulphate calibration curve

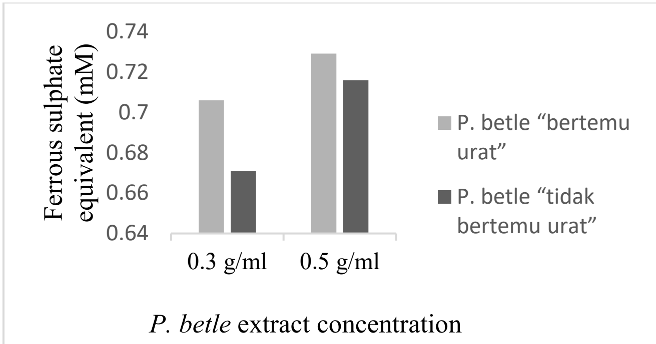

Figure 7 : Antioxidant activities of *P. betle extracts* expressed in ferrous sulphate equivalent

DISCUSSION

In this study, 80 % methanol was used as solvent to extract the phytochemicals. Previous study (Jaiswal et al., 2014) had shown that 80% methanol was the most effective solvent for extracting total phenolic content because the addition of 20% water improved the extraction efficiency and yielded higher antioxidant compounds. In order to remove the solvents after maceration, we used the rotary evaporator that removed all the methanol. Due to some limitations, the extracts were not freeze-dried in order to remove the water content. Thus, the precise extraction yield could not be determined. The initial concentration of the extracts were determined based on the dry-weight of *P. betle* after grounding (the volume of ethanol added for maceration is based on the weight) in total volume of the extracts gained after the rotary evaporator method. Thus, this could lead to inaccuracy in our data.

As for initial comparison, we observed the morphological differences between the leaves of *P. betle* converge- and non converge-vein. The two type of leaves exhibit notable differences in morphology, as documented by Ekosari & Sugiarto (2013). The authors describe the converge-vein leaves of *P. betle* as having a symmetrical shape, where the veins on both sides meet at the main vein, while the veins of non converge-vein leaves do not converge at the main vein. This indicates that the observation of this study aligns with their findings. Furthermore, the non converge vein of *P. betle* leaves have an oblong shape, often longer and with a slender tip, typically displaying a length-to-width ratio of 1:7. On the contrary, *P. betle* "bertemu urat" leaves have a sharper and slightly curved (cuspidate) tip, with a length-to-width ratio of 1:5.

Then, the biochemical assays were conducted. The total phenolic content (TPC) assay used the Folin-Ciocalteau reagent that reacts with the hydroxy group in phenolic ions, reducing heteropoly acid (phosphomolybdate-phosphotungstate) and forming a dark blue molybdenumtungsten complex that has the absorbance of 765 nm (Martono et al., 2019). Thus, the presence of phenolic contents in the test samples were corresponded to the increase of the absorbance. To quantify the total phenolic compounds in the sample extracts, gallic acid was used as a standard (Figure 1) due to its purity and stability as a phenol (Hilma et al., 2018). As shown in figure 2, extract of *P.betle* converge-vein leaves showed higher phenolic content as compared to non converge-vein leaves.

Previous studies suggested that total phenolic content in *P. betle* varies based on the species and also the geographical origin of the plant sample (Jaiswal et al., 2014; Makarova et al., 2021). Additionally, other factors that may influence the phenolics compound and antioxidant properties of the plants include the individual chemotypes, environmental changes, the harvesting stage (floral budding stage, blooming stage, or fruit set stage), geographical origin, and the age of the plant (Al-Rimawi et al. 2014, Makarova et al., 2021). Moreover, these plant properties may also be influence by light intensity and temperature that highly associated with geographical region. In addition to that, this study aim to determine whether leaf's venation pattern also could affect the biochemical properties of plant sample. This is of particular importance because variations in total phenolic content could be the factor that lead to variations in antioxidant activity of plant extracts. Further biochemical assays, DPPH and FRAP assays showed this pattern.

In DPPH assay, the absorbance of the extracts was compared to that of ascorbic acid's standard and the DPPH radical scavenging activities were expressed in ascorbic acid equivalent. Ascorbic acid was used as the standard because it is a natural occurring effective antioxidant with the ability to donate hydrogen atom to form a relatively stable ascorbyl free radical (Olszowy-Tomczyk, 2021). DPPH compound are free radicals. The compound is colored and has maximum absorbance at 517 nm. Compound with antioxidant properties able to transfer electron to DPPH radicals and cause discoloration of the solution. Thus, lower absorbance value is relative to higher antioxidant properties of a test compound. Our result showed that extract of *P. betle*'s converge-vein leaves showed a trend of higher free radicals scavenging activities as compared to non converge-vein leaves at both concentrations tested (0.06 g/ml and 0.3 g/ml) (Figure 4).

As for FRAP assay, it was based on the ability of antioxidant to reduce the complex of ferric ions (Fe^{3+})-ligand to the intensely blue ferrous complex (Fe^{2+}). The blue complex has absorbance at 593 nm. Increase in absorbance of the samples indicate increase in antioxidant activity (Munteanu and Apetrei, 2021). Antioxidant activities of the *P. betle* extracts were expressed as ferrous sulphate equivalents of Fe^{2+} based on the ferous sulphate calibration curve. Based on our result, the extract of *P. betle's* converge-vein leaf extractshowed a trend of higher free radicals scavenging activities as compared to non converge-vein leaf extract at both concentrations tested (0.3 g/ml and 0.5 g/ml) (Figure 7).

Based on previous studies, the arrangement of the lateral veins (leaf venation patterns) on a plant leaf is associated with several physiological function of the plants such as water and nutrient transport (Review: Roth-Nebelsick, 2001), the utilization of water by the leaf (Kawai and Okada, 2015) and photosynthesis (Brodribb et al., 2007). Thus, leaf with different vein pattern, such as symmetrical or asymmetrical pattern as shown *P. betle*, could have different function or biochemistry activities that could lead to differences in the productions of the phytochemicals.

IC_{50} for the *P. betle* extracts were unable to be determined due to the limitations mentioned earlier. Nevertheless, Uddin et al. (2015) reported an IC_{50} value of 151.36 (mg/L), while Nur Sazwi et al. (2013) obtained an IC_{50} value of 179.5 ± 93.1 (mg/L) for *P.betle* leaf extract. Conversely, Phongpaichit (2007) recorded IC_{50} value of 9.04 ± 0.09 mg/L for ascorbic acid. This indicate that *P. betle* extract has much lower antioxidant capacity as compared to vitamin C. Medicinal properties of *P. betle* were recorded not only in Malay Medical Manuscript but also in many of the scientific literatures encompassing a wide area of pathological conditions (Biswas et. al. 2022).

It is possible that there are other properties of *P. betle* extract that contributes to its medicinal properties apart from its radical scavenging activities. One possible aspect is signal transduction. Several scientific articles on natural products reported that certain phytochemicals have anticancer properties by inhibiting the MAPK-signaling pathway. The pathway regulates many of mammalian cell's physiological functions including proliferation and differentiation and is one of the targets for anticancer drug development (Shi et al. 2024).

CONCLUSION

In conclusion, results from this preliminary study might suggest that *P. betle* extract from leaves that are converge-vein had higher antioxidant properties as compared to non converge-vein. Further studies need to be done to validate this data but hopefully this result will pave the way to further explore the medicinal potential of *P. betle* and give more insight to researchers on relationship between leaf venation patterns and medicinal properties.

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