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Original Research

Scavenging Free Radical Potential and Electron Donor Capacity of *Piper betle* L. (Piperaceae): Comparative Analysis of Antioxidant Activity Across Geographic Locations

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Abstract:

Reactive oxygen species (ROS) induce oxidative stress, leading to disease and cellular injury. Betel, or *Piper betle* L., is rich in phenolic and flavonoid compounds which has antioxidant properties. This study evaluated the free radical scavenging potential and electron-donation capacity of *P. betle* L. leaf extracts from various locations to determine their antioxidant efficacy. The ethyl acetate extract of betel leaves from Cianjur demonstrated the highest electron-donating ability-measured using the ferric reducing antioxidant power (FRAP) method, and the greatest free radical scavenging activity-assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method, with values of 611.603 ± 26.565 and 2.231 ± 0.072 mg AEAC/g, respectively. Betel leaves from different regions demonstrated stronger potential as electron donors to enhance antioxidant capacity than as free radical scavengers. Overall, *P. betle* leaves are recognized as a potent natural source of antioxidants.

Keywords: Antioxidant assays; DPPH; FRAP; *Piper betle* L.; Piperaceae; Reactive oxygen species (ROS); Total flavonoid content (TFC); Total phenolic content (TPC))

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1. Introduction

Medicinal plants continue to play a crucial role in the discovery of bioactive compounds for pharmaceutical, nutraceutical, and cosmetic applications. Due to their abundance of phenolic compounds, flavonoids, alkaloids, and terpenoids, medicinal plants have garnered increasing scientific interest as natural sources of antioxidants and therapeutic agents. The rising demand for plant-based products has further underscored the importance of identifying botanical resources with consistent phytochemical profiles and biological activities [1, 2].

The growing demand for plant-based products has further emphasized the importance of identifying botanical resources with consistent phytochemical profiles and biological activities. Free radicals and reactive oxygen species (ROS) are highly unstable molecules that can cause oxidative stress, resulting in inflammation, cellular damage, and various chronic diseases. Numerous factors, including pollutants, ultraviolet (UV) radiation, and metabolic processes, contribute to the generation of free radicals [3]. The body depends on antioxidants to neutralize these harmful molecules and prevent oxidative damage. Antioxidants protect cells from oxidative stress

by stabilizing free radicals through electron donation [4]. Among medicinal plants widely utilized in Southeast Asia, *Piper betle* L. has gaines considerable attention because of its traditional uses and diverse pharmacological properties. Indonesia is home to a vast diversity of plants, many of which have been traditionally used for medicinal purposes. One such plant is *P. betle* L., commonly known as betel. *P. betle* L. belongs to the family Piperaceae, a large family comprising approximately 3,600 species distributed mainly throughout tropical and subtropical regions worldwide. Members of this family are recognized for producing a broad spectrum of secondary metabolites, including phenolics, flavonoids, lignans, alkaloids, and essential oils, which contribute to their antioxidant, antimicrobial, anti-inflammatory, anticancer, and other pharmacological activities. Consequently, Piperaceae has become an important source of medicinal plants for pharmaceutical and cosmetic development [5, 6]. This herbal plant has been utilized in traditional medicine across various cultures, particularly in Southeast Asia and South Asia. In Indonesia, it is known by different regional names such as ranub (Aceh), sedah (Bali), sere (Madura), ganjang or gapura (Bugis), suruh (Java), and sirih (Palembang, Minangkabau) [7]. The variety of names reflects the widespread use and cultural significance of this plant in Indonesian society. Betel has long been used in various traditions, including the practice of chewing betel leaves, which is believed to strengthen teeth and reduce bad breath [8]. In modern medicine, betel leaves are used as active ingredients in health and skincare products, including antiseptics, mouthwash, and wound-healing creams. *P. betle* is reported to contain a wide range of phytochemicals and beneficial secondary metabolites, such as hydroxychavicol, α-pinene, eugenol, β-caryophyllene, chavibetol, α-cadinene, β-sitosterol, piperol A and B, piperbetol, and methyl piperbetol [9]. These compounds have been widely recognized for their broad spectrum of biological activities, including anti-inflammatory effects, free radical scavenging, inhibition of lipid peroxidation, and antioxidant properties [10]. Other secondary metabolites found in betel leaves, such as phenols, glycosides, steroids/triterpenoids, tannins, and flavonoids, also exhibit antioxidant activity [7]. Flavonoids and phenols can delay or inhibit oxidation reactions caused by free radicals [11].

Betel leaf extract is renowned for its diverse pharmacological properties, including antibacterial, analgesic, anti-inflammatory, antioxidant, antiproliferative, and antidiabetic effects. These benefits are attributed to the bioactive compounds present in the leaves, making them an important natural remedy in both conventional and alternative medicine [6]. Due to the presence of hydroxychavicol, betel leaf extract exhibits antiproliferative effects on MCF-7 cells and enhances catalase activity by scavenging free radicals such as H_2O_2, superoxide radicals, and hydroxyl radicals. Additionally, this extract can increase the body's antioxidant enzyme activity [12].The antidiabetic properties of betel leaf extract have been attributed to its ability to reduce cortisol levels in the body. Cortisol is a hormone released during stress that elevates glucose production beyond normal levels [13].

In this study, we aim to compare the free radical scavenging potential and electron-donating capacity of betel leaf extracts from three different regions in West Java, Indonesia, as well as to profile the flavonoid markers in the selected extracts. Since betel is a native plant of Southeast Asia and is widely used as a raw material for herbal remedies, cosmetics, and antiseptics in Indonesia, this study seeks to provide new insights that can support the pharmaceutical industry in standardizing these raw materials.

2. Experimental

2.1 Reagents and materials

The materials used included betel (*P. betle* L.) leaves as the plant sample and various solvents such as distilled water, toluene, chloroform, absolute ethanol (96.0%), *n*-hexane, ethyl acetate, and analytical-grade methanol. Several reagents were also utilized, including iron (III) chloride, sodium carbonate, sodium acetate, gallic acid, quercetin, and Folin-Ciocalteu reagent. Additionally, ascorbic acid, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), and DPPH reagent were used for antioxidant activity testing.

2.2 Apparatus

Analytical balance, ruler, crucible, oven, furnace, filter paper, funnel, azeotropic distillation apparatus, boiling stones, stoppered flask, flat dish, flat-bottomed weighing bottle, desiccator, heater, test tubes, beakers, mortar and pestle, UV lamp (Camag), pycnometer, Eppendorf tubes, UV-Visible spectrophotometer (Thermo Fisher Scientific, Inc.; Trace 1300), cuvettes, micropipettes, HPLC (Shimadzu Corporation), TLC plates GF_{254}, and reflux apparatus.

2.3 Sample collection

Samples of betel (*P. betle* L.) (Fig. 1) were obtained from three areas in West Java, Indonesia: Ciwidey (7.0849 °S, 107.4461 °E), Cianjur (6.8193 °S, 107.1425 °E), and Lembang (6.8145 °S, 107.6230 °E) (Fig. 2), all of which are situated within a relatively homogenous altitude range.

Specifically, samples were collected from Lebak Muncang Village in Ciwidey District, Bandung Regency (1,100 m a.s.l.); Cikadu District in Cianjur Regency (950 m a.s.l.); and Wangunharja Village in Lembang District, West Bandung Regency (1,200 m a.s.l.). All three regions have a tropical climate, providing similar environmental conditions. Plant identification was conducted at the Herbarium Bandungense Laboratory, Biology Study Program, School of Life Sciences and Technology, Bandung Institute of Technology. Five kilograms of fresh betel leaves were collectedfrom each location. The leaves were thoroughly rinsed, finely chopped, and oven-dried for 48 hours at a temperature of 40-50 °C. The dried leaves were then ground into a coarse medicinal powder

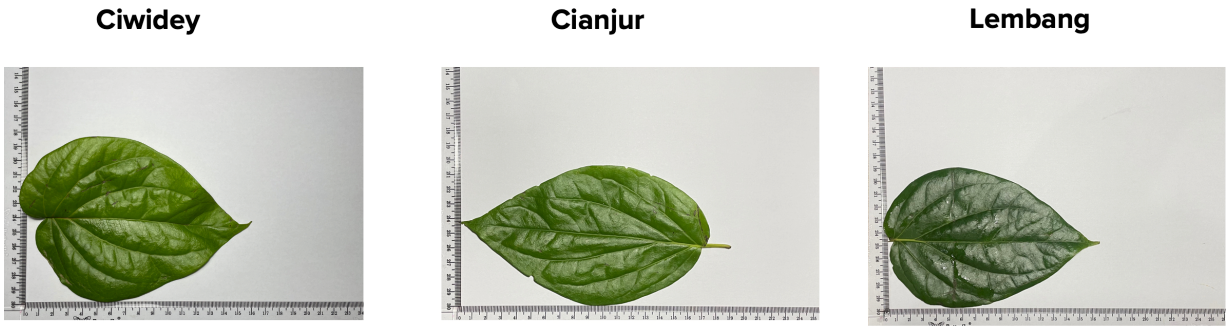

Figure 1. *Piper betle* L. samples collected from three locations in West Java, Indonesia, including Ciwidey, Cianjur, and Lembang.

and stored in sealed containers under dry conditions.

2.4 Preparation of *P. betle* L. leaf extracts

Crude powdered betel (*P. betle* L.) leaves were collected from three different regions—Ciwidey, Cianjur, and Lembang—with 300 g obtained from each location. The extraction was performed using the reflux method with three solvents of increasing polarity: *n*-hexane, ethyl acetate, and ethanol. Each extraction was conducted three times, and the resulting extracts were concentrated using a rotary evaporator to produce a thick extract.

2.5 Phytochemical characterization of *P. betle* L. leaf extracts

2.5.1 Preparation of solution A

A total of 5.0 g of betel leaf crude drug was combined with 100 mL of distilled water and simmered for 15 minutes. The hot mixture was then filtered, and the resulting filtrate was referred to as solution A, which was used for the qualitative tests of flavonoids, tannins, quinones, and saponins.

2.5.2 Flavonoid test

Five milliliters of solution A were transferred into a test tube, followed by the addition of 0.1 g of magnesium powder, 1 mL of concentrated hydrochloric acid, and 5 mL of amyl alcohol. The mixture was shaken and allowed to separate. The development of a yellow to orange color in the upper layer indicated the presence of flavonoids [14].

2.5.3 Tannin test

Solution A was divided into three separate test tubes, each containing 5 mL. Tube I received a 5% ferric chloride solution, tube II was treated with a 10% gelatin solution, and tube III was treated with Stiasny's reagent. The appearance of a bluish-black or green tint upon the addition of ferric chloride indicated the presence of phenolic compounds. In tube II, the formation of a white precipitate upon the addition of gelatin confirmed the presence of tannins.In tube II, the formation of a white precipitate upon addition of gelatin confirmed the presence of tannins. In tube III, heating the mixture after adding Stiasny's reagent resulted in a pink precipitate, indicating the presence of catechin tannins. The solution from tube III was then filtered, and 1 mL of 1 M sodium acetate and 1 mL of a 5% ferric chloride solution were added to the filtrate. The development of a bluish-black color confirmed the presence of gallic tannins [14].

2.5.4 Quinone test

Five milliliters of solution A were placed into a test tube and combined with 1 N sodium hydroxide solution. The appearance of a red color indicated the presence of quinones in the sample [14].

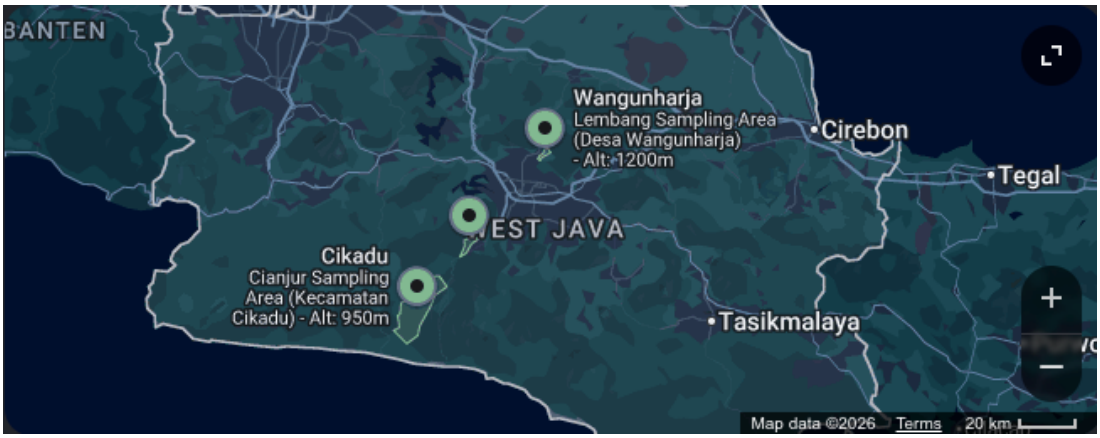

Figure 2. Geographical locations of *Piper betle* L. sampling sites in West Java, Indonesia (Ciwidey, Cianjur, and Lembang), and representative leaf samples collected from each location.

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2.5.5 Saponin test

A test tube containing 10 mL of solution A was shaken vertically for 10 seconds. The formation of stable foam, ranging from 1 to 10 cm in height and remaining intact for 10 minutes, indicated the presence of saponins. The foam remained stable upon the addition of a few drops of 2 N hydrochloric acid (Ministry of Health Indonesia, 1995).

2.5.6 Steroid/triterpenoid test

One gram of betel leaf crude drug was mixed with 20 mL of ether and ground in a mortar, followed by filtration. The filtrate was transferred to an evaporating dish and allowed to evaporate. A few drops of Liebermann-Burchard reagent were then added. The appearance of a green-blue color suggested the presence of steroids, while a reddish-purple color confirmed the presence of triterpenoids [14].

2.6 Quantitative phytochemical analysis of *P. betle* L. leaf extracts

2.6.1 Total phenol content

Total phenolic content was determined using the Folin-Ciocalteu method. Gallic acid served as the reference standard and was prepared as a stock solution at a concentration of 1000 µg/mL, which was then diluted to obtain a concentration range of 60-130 µg/mL. A 50 µL aliquot of gallic acid solution at various concentrations was mixed with 500 µL of Folin-Ciocalteu reagent (10%) and 400 µL of sodium carbonate (1.0 M) in an Eppendorf tube. After 30 minutes of incubation, absorbance was measured at 765 nm using a UV-Vis spectrophotometer. The absorbance values obtained were used to construct a gallic acid calibration curve. Methanol combined with sodium carbonate and Folin-Ciocalteu reagent was used as the blank.

The same method was used to measure the total phenolic content in betel leaf extracts. Each extract was dissolved in analytical-grade methanol to prepare a stock solution with a concentration of 10,000 µg/mL, which was then further diluted to a working concentration range of 250-1250 µg/mL, depending on the sample response. Measurements were performed six times for each extract, and the total phenolic content was determined using the linear regression equation of the gallic acid calibration curve. The results were expressed as gallic acid equivalents (GAE) in mg per gram [15].

2.6.2 Total flavonoid content

The aluminum (III) chloride reagent method was used to determine the total flavonoid content. A stock solution of quercetin at 500 µg/mL served as the reference standard and was further diluted to a working concentration range of 40-110 µg/mL. Then, 300 µL of methanol, 560 µL of distilled water, 20 µL of aluminum (III) chloride, and 20 µL of 1 M sodium acetate were combined with a 100 µL aliquot of quercetin solution at various concentrations. The samples were incubated for 30 minutes and measured using a UV-Vis spectrophotometer at a wavelength of 415 nm. Using the same method as the reference standard, the total flavonoid content of betel leaf extracts was determined. The extracts were dissolved in analytical-grade methanol at a concentration of 1000 µg/mL and then diluted to 2500-5000 µg/mL. The analysis was performed six times, and the total flavonoid content was calculated using the linear regression equation from the quercetin calibration curve. Results were expressed as mg quercetin equivalent (QE) per gram of extract [16].

2.7 Assessment of antioxidant activity

2.7.1 DPPH assay

The DPPH radical scavenging method, as described by Celep et al., was used to measure antioxidant activity, with ascorbic acid serving as the reference standard [17]. A stock solution containing 50 µg/mL of DPPH was prepared. Then, 20 mg of ascorbic acid was dissolved in 100 mL of analytical-grade methanol to prepare the ascorbic acid stock solution. From this stock, standard solutions were prepared by diluting 10, 12.5, 15, 20, and 30 µL with analytical-grade methanol to a final volume of 125 µL.

Subsequently, 750 µL of the DPPH solution was added to each standard solution, and the mixtures were incubated in the dark for 30 minutes. Absorbance was measured at 517 nm using a UV-Vis spectrophotometer. A calibration curve was generated to determine the regression equation for ascorbic acid. Extract samples were prepared by dissolving the extracts in analytical-grade methanol, followed by filtration. The resulting filtrates were treated under the same conditions as the ascorbic acid standards. Measurements were performed in six replicates for each extract, and the percentage of DPPH radical scavenging activity was calculated. The antioxidant activity of the extracts was determined by applying the scavenging percentage values to the ascorbic acid regression equation. Results were expressed as ascorbic acid equivalent antioxidant capacity (AEAC) in mg AEAC/g sample [17].

2.7.2 FRAP assay

The FRAP assay was used to measure antioxidant activity, following a modified method described by Özyürek et al. (2008) [18]. Solutions were prepared as follows: 200 µg/mL ascorbic acid standard, 5380 µg/mL $FeCl_3 \cdot 6H_2O$, 3120 µg/mL TPTZ, and acetate buffer at pH 3.6. The FRAP working solution (710 µg/mL) was prepared by mixing $FeCl_3 \cdot 6H_2O$, TPTZ, and acetate buffer in a 1:1:10 ratio. The sample extract solution (10,000 µg/mL) was prepared and filtered. To determine antioxidant activity, an ascorbic acid standard curve was generated using serial dilutions of the stock solution with volumes of 35, 30, 27.5, 25, 22.5, 20, 17.5, 15, 12.5, and 10 µL, each tested in triplicate. Each diluted standard was brought to a total volume of 500 µL with distilled water, followed by the addition of 500 µL of FRAP reagent. The mixture was incubated in the dark for 30 minutes and measured at 595 nm using a UV-Vis spectrophotometer. A calibration curve was constructed, and the regression equation was

determined with an R^2 value of at least 0.99.
For sample antioxidant capacity measurement, 12.5 µL of the extract solution was diluted with purified water to a final volume of 500 µL, followed by the addition of 500 µL of FRAP reagent. The mixture was incubated in the dark for 30 minutes, and absorbance was measured at 595 nm. The antioxidant activity of the samples was calculated using the ascorbic acid calibration curve and expressed as mg AEAC/g sample [18].

2.8 Quantitative correlation analysis of *P. betle* L. leaf extracts

To assess relationship between the flavonoid content and antioxidant activity, a quantitative correlation study was conducted using statistical analysis. Pearson's correlation coefficient (r) was calculated using SPSS 20 for Mac to determine the strength and direction of the relationship between TFC and antioxidant activity (DPPH and FRAP assays). The correlation was interpreted based on the following criteria: weak (0.00-0.30), moderate (0.31-0.60), strong (0.61-0.90), and very strong (0.91-1.00). Statistical significance was set at $p < 0.05$. Additionally, statistical tests were performed to identify significant differences among the extract samples. The data were analyzed to investigate the relationship between flavonoid and phenolic contents and the antioxidant activity of betel leaf extracts. This analysis included analysis of variance (ANOVA), conducted using Minitab software, to assess the significance of differences among the samples.

2.9 Qualitative correlation analysis of *P. betle* L. leaf extracts

A qualitative correlation analysis was performed to identify the correlation between phenolic and flavonoid content in the extracts and their antioxidant activity. This analysis utilizedthin-layer chromatography (TLC) GF_{254} plates, which were sprayed with various visualization reagents. Citroborate reagent was used to detect flavonoids, which appeared as yellow, green or blue fluorescentspots under UV light at a wavelength of 366 nm. Folin–Ciocalteu reagent was employed to identify phenolic compounds, indicated by the formation of grayish-blue spots.

2.10 HPLC analysis of *P. betle* L. leaf extracts

The selected extract exhibiting significant antioxidant activity was further analyzed using HPLC with an LC-20AD Shimadzu system (Shimadzu, Japan). Separation was performed using a LiChrospher 100 RP-C18 stationary phase. The mobile phase consisted of Water Pro Injection (Eluent A) and Methanol HPLC (Eluent B), applied with the following gradient program: a linear increase from 40% to 60% Eluent B over 5 minutes, followed by an increase to 70% at 10 minutes, and then a return to 40% at 15 minutes. Detection was carried out using a UV/Vis SPD-20A detector at 360 nm.
Various flavonoids, including quercetin, apigenin, apigenin-7-*O*-glycoside, rutin, and kaempferol, were used as standards to identify flavonoid compounds in the selected extract. The concentration of flavonoid compounds was determined using the one-point method, with each concentration analyzed in triplicate to ensure accuracy and reproducibility.

3. Results and discussion

3.1 Phytochemical characterization of *P. betle* L. leaf extracts

The phytochemical screening of betel leaf crude drug samples from different sites is presented in Table 1. Based on the results of this table, crude betel leaf samples from Ciwidey, Cianjur, and Lembang contained flavonoids, phenols, tannins, steroids/triterpenoids, and quinones in all samples. However, saponins and alkaloids were not detected in any of the samples.

Table 1. Phytochemical screening of dried betel leaves from different sites.

Component	Ciwidey	Cianjur	Lembang
Flavonoid	+	+	+
Phenol	+	+	+
Saponin	–	–	–
Tannin	+	+	+
Steroid/triterpenoid	+	+	+
Coumarin	+	+	+
Quinone	+	+	+
Alkaloid	–	–	–

+: detected; –:not detected.

3.2 Quantitative phytochemical analysis of *P. betle* L. leaf extracts

3.2.1 Total phenolic content (TPC)

The gallic acid calibration curve was used to determine the total phenol content (TPC). The regression equation for TPC was y = 0.0052x + 0.00529, with an R^2 value of 0.9971. The total phenol content (TPC) of the extracts varied significantly among different sample locations and extraction solvents (Table 2). The highest TPC was observed in the ethyl acetate extract from Cianjur (537.641 ± 16.436 mg GAE/g), followed by the *n*-hexane extracts from Cianjur (418.923 ± 2.195 mg GAE/g) and Ciwidey (394.308 ± 8.381 mg GAE/g). In contrast, ethanol extracts exhibited the lowest TPC values, ranging from 61.814 ± 4.892 mg GAE/g to 69.964 ± 1.633 mg GAE/g. The high TPC value in Cianjur's ethyl acetate extract indicates that the phenolic compounds in the betel extracts from the three tested regions are predominantly semi-polar compounds, such as phenolic acids. According to the solubility principle, semi-polar compounds like phenolic acids are soluble in semi-polar solvents such as ethyl acetate. This result is consistent with findings by Wongsa et al. (2012), who reported that aqueous extracts of *Piper sarmentosum*, a species from the same genus (Piperaceae), exhibited significantly lower TPC (10.98 ± 0.25 mg GAE/g), highlighting the limited efficiency of polar solvents in extracting phenolic compounds from betel leaves [19]. Additionally,

Table 2. Quantitative analysis of total phenolic content (TPC) of *P. betle* L. leaf extracts (mg GAE/g).

Sample	*n*-Hexane	Ethyl acetate	Ethanol
Ciwidey	394.308 ± 8.381^{ax}	282.218 ± 5.729^{ay}	69.964 ± 1.633^{az}
Cianjur	418.923 ± 2.159^{bx}	537.641 ± 16.436^{by}	64.122 ± 7.858^{abz}
Lembang	62.583 ± 2.435^{cx}	76.686 ± 4.716^{cy}	61.814 ± 4.892^{bx}

[a-c]: Different letters within the same column indicate significant differences ($p < 0.05$); [x-z]: Different letters within the same row indicate significant differences ($p < 0.05$).

Sundang et al. (2014) reported the total phenolic content of *P. betle* L. methanolic extract as 34.29 mg QE/g (expressed as catechin equivalent), providing a useful reference point for comparison with the present study [20].

These studies also highlight the impact of growth location to the TPC of betel leaves. Overall, betel leaves from Cianjur exhibited the highest TPC values compared to those from Lembang and Ciwidey. Although these three locations are situated at similar altitudes, the phenolic contents remained different, suggesting that other factors contribute to this variation. The variation in TPC among these regions may be related to differences in soil type, rainfall intensity, humidity, and temperature. Therefore, when the same plant grows in different regions, its growth may vary, resulting in differences in chemical content across locations. In another study, Irawan et al. reported a TPC of 758.534 ± 0.003 mg GAE/g in the ethanolic extract of *P. betle* L. var. *nigra*, a different variant of the same species investigated in the present study (*P. betle* L. var. *viridis*), indicating that phenolic content may vary significantly between different variants of *P. betle* [21]. In three different cultivars of *P. betle* L., Harini et al. (2018) reported TPC values ranging from 95.04 to 127.33 mg gallic acid equivalent (GAE) per 100 g of sample [22]. Based on the study by Sonphakdi et al. (2024), the TPC of *P. betle* which was extracted with methanol and ethanol were reported as 130.07 ± 4.46 mg GAE/g and 147.69 ± 1.82 mg GAE/g, respectively, indicating a high presence of phenolic compounds in both solvents [23]. According to Nguyen et al. (2020), the total phenolic content of *P. betle* L. extracts varied significantly depending on the solvent used [24]. The ethanol extract had the highest phenolic concentration (249.96 ± 6.42 mg GAE/g), followed by methanol (115.04 ± 3.60 mg GAE/g), ethyl acetate (124.54 ± 3.84 mg GAE/g), and hexane (55.30 ± 1.13 mg GAE/g).

The total phenolic content data were analyzed using one-way ANOVA, followed by Tukey's post hoc test, to identify significant differences in total phenolic content across all extracts, categorized by solvent type and cultivation location. The phenolic concentration at each location varied considerably depending on the extraction solvent used. Samples from Ciwidey and Cianjur showed significant differences across all three extracts (*n*-hexane, ethyl acetate, and ethanol), indicating that solvent polarity significantly influences the extraction of phenolic compounds. In contrast, the ethyl acetate extract from the Lembang sample exhibited a significant difference ($p < 0.05$) compared to the n-hexane and ethanol extracts, which did not differ statistically. The n-hexane and ethyl acetate extracts showed notable variations among the three cultivation regions ($p < 0.05$). For the ethanol extract, there was no significant difference between Ciwidey and Cianjur or between Lembang and Cianjur, while Ciwidey and Lembang differed significantly ($p < 0.05$).

3.2.2 Total flavonoid content (TFC)

A calibration curve for quercetin was used to calculate the total flavonoid content (TFC). The regression equation for TFC was y = 0.0074x − 0.0418, with an R^2 value of 0.9937. Total flavonoid content (TFC) followed a similar trend, with the highest value detected in the ethyl acetate extract from Lembang (41.899 ± 3.525 mg QE/g), followed by Cianjur (39.679 ± 1.589 mg QE/g) (Table 3). Meanwhile, *n*-hexane and ethanol extracts showed relatively lower flavonoid concentrations across all locations. The TFC results in this study were noticeably higher than those reported in other studies. For example, Nursamsiar et al. (2023) reported that the ethanol extract of *P. betle* obtained through reflux extraction contained 6.4 mg QE/g of flavonoids, while the ethanol extract of Piper crocatum had 16.1 mg QE/g [25]. These samples were collected from Moncongloe, South Sulawesi, Indonesia, which has distinct environmental conditions compared to West Java. Flavonoid biosynthesis is influenced by environmental factors such as soil composition, altitude, humidity, and temperature; therefore, TFC may vary by location. Another study by

Table 3. Quantitative analysis of total flavonoid content (TFC) of *P. betle* L. leaf extracts (mg QE/g).

Sample	*n*-Hexane	Ethyl acetate	Ethanol
Ciwidey	22.404 ± 2.634^{axy}	21.287 ± 1.508^{ax}	24.629 ± 0.860^{ay}
Cianjur	26.007 ± 2.998^{abx}	39.679 ± 1.589^{by}	18.481 ± 0.797^{bz}
Lembang	29.755 ± 1.849^{bx}	41.899 ± 3.525^{by}	22.017 ± 0.855^{cz}

[a-c]: Different letters within the same column indicate significant differences ($p < 0.05$); [x-z]: Different letters within the same row indicate significant differences ($p < 0.05$).

Sundang et al. (2014) reported that the total flavonoid content of *P. betle* L. methanolic extract was 31.08 mg QE/g, expressed as quercetin equivalent, serving as a reference value for comparison in the current study [20]. In three different cultivars of *P. betle* L., Harini et al. found that the total flavonoid content ranged from 51.72 to 61.08 mg catechin equivalent (CE) per 100 g of sample, indicating variation in flavonoid accumulation among the cultivars studied [22]. According to [24], the total flavonoid content of *P. betle* extracts varied significantly depending on the solvent used. The ethyl acetate extract showed the highest amount (29.07 ± 0.96 mg RE/g), closely followed by methanol (34.55 ± 1.10 mg RE/g) and ethanol (27.82 ± 1.25 mg RE/g), while the hexane extract contained the least (10.72 ± 0.17 mg RE/g). Poudel et al. (2024) reported that the total flavonoid content in *P. betle* methanolic extract ranged from 5.39 to 106.13 mg quercetin equivalent, with an average of 39.67 mg, indicating wide variation in flavonoid levels depending on extraction or sample conditions [26].

In accordance with the TPC data, the total flavonoid content (TFC) was statistically analyzed using one-way ANOVA to determine significant variations in TFC among extracts categorized by solvent type and cultivation location. The analysis revealed substantial differences both within each site and between the various extraction solvents (x, y). At a single site, the Ciwidey sample showed significant differences between the ethyl acetate and ethanol extracts, whereas the *n*-hexane extract did not differ significantly from either the ethyl acetate or ethanol extracts (x, y). The Cianjur and Lembang samples exhibited more pronounced variations across all solvents (x, y, z).

The *n*-hexane extract exhibited a significant difference between Ciwidey (a) and Lembang (b), while Cianjur (ab) was intermediate and did not differ significantly from either location. The ethyl acetate extract exhibited no significant difference between Cianjur and Lembang (b, b), whereas Ciwidey differed significantly from both ($p < 0.05$). The ethanol extract exhibited the greatest variation among locations (a, b, c), indicating that environmental factors markedly influence the flavonoid concentration in betel leaf extracts when polar solvents are used.

3.2.3 Evaluation of *in vitro* antioxidant activity

The antioxidant activity of plant extracts from three locations was analyzed based on their ability to scavenge free radicals (DPPH assay) and donate electrons to enhance antioxidant capacity (FRAP assay). The results were expressed as antioxidant capacity in terms of ascorbic acid equivalents (mg AEAC/g) (Table 4 and Table 5).

In the DPPH analysis, the ethyl acetate extract from Cianjur exhibited the highest antioxidant activity (2.231 ± 0.072 mg AEAC/g), followed by the ethyl acetate extract from Lembang (1.511 ± 0.138 mg AEAC/g). The extract with the lowest antioxidant activity was the *n*-hexane extract from Lembang (0.822 ± 0.052 mg AEAC/g). These results indicate that the type of solvent significantly influences the extraction of antioxidant compounds, with ethyl acetate being the most effective solvent for extracting phenolic and flavonoid compounds responsible for antioxidant activity. This finding aligns with several previous studies comparing the impact of solvent type on antioxidant activity. Irawan et al. (2024) [21] investigated the antioxidant activity of the ethanol extract of *P. betle* L. var. *nigra* using both DPPH and FRAP assays, where the DPPH radical scavenging method yielded an IC_{50} value of 115.562 ± 0.14 mg/L, indicating the concentration required to neutralize half of the DPPH free radicals. Similarly, Harini et al. (2018) [22] reported that the antioxidant activity of methanolic extracts from three different *P. betle* L. cultivars, assessed using the DPPH method, exhibited IC_{50} values ranging from 65 ± 0.20 mg/L to 96.63 ± 0.54 mg/L, reflecting varying free radical scavenging capacities among cultivars. Sonphakdi et al. (2024) [23] also found that *P. betle* extracts obtained using ethanol and methanol exhibited strong antioxidant activity, with IC_{50} values as low as 0.03 mg/mL, highlighting their excellent radical scav-

Table 4. DPPH antioxidant activity of *P. betle* L. leaf extracts (mg AEAC/g).

Sample	*n*-Hexane	Ethyl acetate	Ethanol
Ciwidey	1.499 ± 0.030^{ax}	1.221 ± 0.020^{ay}	1.578 ± 0.037^{az}
Cianjur	1.526 ± 0.011^{ax}	2.231 ± 0.072^{by}	1.499 ± 0.029^{bx}
Lembang	0.822 ± 0.052^{bx}	1.511 ± 0.138^{cy}	1.328 ± 0.022^{cz}

$^{a\text{-}c}$: Different letters within the same column indicate significant differences ($p < 0.05$); $^{x\text{-}z}$: Different letters within the same row indicate significant differences ($p < 0.05$).

Table 5. FRAP antioxidant activity of *P. betle* L. leaf extracts (mg AEAC/g).

Sample	*n*-Hexane	Ethyl acetate	Ethanol
Ciwidey	415.003 ± 24.198^{ax}	603.401 ± 26.565^{ay}	78.050 ± 2.247^{az}
Cianjur	602.055 ± 41.107^{bx}	611.603 ± 26.565^{ax}	97.625 ± 6.165^{by}
Lembang	444.790 ± 32.621^{ax}	264.344 ± 19.477^{by}	123.022 ± 3.914^{cz}

$^{a\text{-}c}$: Different letters within the same column indicate significant differences ($p < 0.05$); $^{x\text{-}z}$: Different letters within the same row indicate significant differences ($p < 0.05$).

enging potential. Likewise, Nguyen et al. (2020) [24] reported that methanol extracts showed the strongest radical scavenging activity (IC_{50} of 16.96 ± 0.62 µg/mL), slightly outperforming ethanol (20.57 ± 0.48 µg/mL) and ethyl acetate (20.97 ± 1.02 µg/mL) extracts, while the *n*-hexane extract showed the weakest activity (IC_{50} of 93.96 ± 3.76 µg/mL). Furthermore, Poudel et al. (2024) [26] revealed that the methanolic extract of Piper had an IC_{50} value of 96.93 µg/mL, indicating moderate antioxidant activity based on its DPPH radical scavenging ability.

The DPPH antioxidant activity data were statistically analyzed using one-way ANOVA, followed by Tukey's post-hoc test, to identify significant differences in antioxidant activity. Significant variations were observed both among solvent types within the same site and among sites using the same solvent. At each site, all three Ciwidey extracts demonstrated statistically significant differences ($p < 0.05$) (x, y, z). In the Cianjur samples, the *n*-hexane and ethanol extracts showed no significant differences, whereas the ethyl acetate extract differed significantly from both. Similarly, the Lembang samples displayed considerable variation among extracts ($p < 0.05$).

For the *n*-hexane extract, no significant difference was observed between Ciwidey and Cianjur (a, a), while the Lembang sample (b) showed a significant difference from both Ciwidey and Cianjur. The ethyl acetate and ethanol extracts demonstrated significant variations across all three locations (a, b, c). Therefore, we conclude that although these three locations are situated at similar altitudes, the DPPH antioxidant activity differed due to variations in the metabolites present in each extract.The presence of significant differences among solvent types at the same locations (Ciwidey and Lembang) indicates that the choice of extraction solvent has a greater influence on antioxidant activity than the cultivation site. Conversely, the lack of substantial variation in the ethyl acetate extract across locations suggests that this solvent effectively extracts antioxidant compounds regardless of environmental conditions. In contrast, the antioxidant activity of the *n*-hexane and ethanol extracts was significantly affected by location, as evidenced by marked inter-location differences. This finding suggests that the compounds extracted by these solvents—nonpolar compounds in *n*-hexane and polar compounds in ethanol—vary considerably depending on the environmental conditions of the cultivation site. The FRAP assay showed a similar trend, with the highest antioxidant activity observed in the ethyl acetate extract from Cianjur (611.603 ± 26.565 mg AEAC/g), followed by the ethyl acetate extract from Ciwidey (603.401 ± 26.565 mg AEAC/g). The lowest antioxidant activity was recorded in the ethanol extract from Lembang (123.022 ± 3.914 mg AEAC/g). The significant variation in antioxidant activity across extracts and locations can be attributed to differences in total phenolic and flavonoid content, as well as environmental factors affecting metabolite production. Some of these environmental factors include altitude, rainfall intensity, humidity, and temperature. Therefore, when the same plant grows in different regions, its growth may vary, resulting in differences in chemical content among locations and consequently different antioxidant activities. According to Irawan et al. (2024) [21], the ferric reducing antioxidant power (FRAP) assay yielded an EC50 value of 119.273 ± 0.385 mg/L, representing the extract concentration required to reduce ferric ions by 50%. Similarly, Harini et al. (2018) [22] reported that methanolic extracts of three *P. betle* L. cultivars demonstrated ferric-reducing antioxidant power ranging from 3254.90 ± 171.97 µg/g to 6791.86 ± 96.31 µg/g ascorbic acid equivalents, indicating notable variation in antioxidant potential among cultivars. Jaiswal et al. (2014) [27] found that the FRAP antioxidant activity of 80% ethanol extracts of *P. betle* ranged from 0.24 to 0.70 mmol Fe^{2+} Eq/g, indicating moderate reducing power depending on the variety. In the same study, 80% ethyl acetate extracts exhibited stronger antioxidant capacity, with FRAP values ranging from 0.21 to 1.35 mmol Fe^{2+} Eq/g, suggesting that ethyl acetate is more effective in extracting compounds with high reducing potential. Additionally, Sazwi et al. (2013) reported that the aqueous extract of *P. betle* showed notable antioxidant capacity, with a FRAP value of 357.3 ± 129.5 µmol Fe(II)/mg [28].

The FRAP antioxidant activity data were analyzed using one-way ANOVA followed by Tukey's post hoc test to identify significant differences among antioxidant activity levels. Marked disparities were observed both among solvent types within the same locations and between locations using the same solvent. At each site, the three Ciwidey extracts exhibited significant differences ($p < 0.05$) (x, y, z). In the Cianjur samples, the n-hexane and ethanol extracts showed no significant difference, whereas the ethyl acetate extract differed significantly from both. Similarly, in the Lembang samples, all extracts exhibited substantial variation ($p < 0.05$). For the *n*-hexane extract, no significant difference was found between Ciwidey and Cianjur (a, a), whereas the Lembang sample (b) differed significantly from both. The ethyl acetate and ethanol extracts exhibited notable variations across all three locations (a, b, c). The pronounced differences among solvent types within a single location (Ciwidey and Lembang) suggest that solvent type has a greater influence on antioxidant activity than cultivation site. Conversely, the absence of substantial variation in the ethyl acetate extract among locations indicates that this solvent consistently extracts antioxidant compounds regardless of environmental variation. In contrast, the antioxidant activity of the *n*-hexane and ethanol extracts was significantly affected by location, reflecting regional variations in the solubility and availability of non-polar and polar compounds, respectively, influenced by environmental conditions. When comparing the two assays, the FRAP assay generally yielded higher AEAC values than DPPH, suggesting that the extracts contain compounds more effective at reducing ferric ions than scavenging free radicals. This difference may be attributed to the presence of reducing agents, such as phenolic and

flavonoid compounds, which contribute substantially to antioxidant potential. These findings are consistent with the TPC and TFC data, which showed that ethyl acetate extracts, particularly those from Cianjur and Lembang, had the highest levels of phenolic and flavonoid compounds. Overall, the results reinforce the strong correlation between polyphenolic content and antioxidant activity, highlighting the pivotal role of phenolic and flavonoid compounds in antioxidant mechanisms.

3.3 Quantitative correlation analysis of *P. betle* L. leaf extracts

Flavonoid and phenolic compounds can act as free radical scavengers (DPPH assay) and electron donors that enhance antioxidant capacity (FRAP assay). The correlation between total phenolic content (TPC) and total flavonoid content (TFC) with antioxidant activity in betel leaf extracts was analyzed using Pearson correlation, and the results are presented in Table 6.

Table 6 indicates that the correlations between TPC and TFC with antioxidant activity—measured by free radical scavenging ability (DPPH assay) and electron-donating capacity (FRAP assay)—were positive and ranged from strong to very strong across all betel leaf extracts from different locations. Overall, the FRAP assay exhibited a stronger correlation with phenolic and flavonoid contents than the DPPH assay, suggesting that phenolic and flavonoid compounds in betel leaf extracts play a crucial role in antioxidant potential through an electron transfer mechanism. The correlation coefficients ranged from 0.62 to 0.98 for phenolic compounds and from 0.77 to 0.96 for flavonoid compounds, demonstrating their significant contribution to antioxidant activity. Among the extracts, ethanol and ethyl acetate extracts exhibited the highest correlation coefficients, particularly in the FRAP assay, with r values exceeding 0.90. This suggests that these extracts contain phenolic and flavonoid compounds that contribute more effectively to ferric ion reduction. Meanwhile, the correlations in the DPPH assays were slightly lower but remained strong, indicating that radical scavenging activity is also influenced by these compounds, although other non-phenolic antioxidants may contribute as well. According to a study by Poudel et al. (2024) [26], TPC and DPPH radical scavenging activity were found to be strongly correlated, with an R^2 value of 0.9939. Furthermore, a high correlation (R^2 = 0.9890) was found between TFC and DPPH. The correlation between the two antioxidant assays (as free radical scavengers and electron donors increasing antioxidant capacity) was also analyzed and is presented in Table 7.

The results presented in Table 7 indicate a strong to very strong correlation, suggesting that both assays produce consistent patterns in evaluating antioxidant capacity. Ethanol extracts exhibited the highest correlation coefficients, particularly in the FRAP analysis, with values exceeding 0.90. This finding suggests that phenolic and flavonoid compounds in the ethanol extract of betel leaves play a crucial role in antioxidant potential through an electron transfer mechanism. Ethyl acetate extracts also showed strong correlations, confirming their effectiveness in contributing to antioxidant activity. In

Table 6. Pearson correlation coefficient results (antioxidant activity *vs.* TPC and TFC).

Antioxidant assay	Extract	Pearson correlation coefficient (r)	
		Total phenolic	Total flavonoid
DPPH	*n*-Hexane Ciwidey	0.821***	0.922****
	n-Hexane Cianjur	0.79***	0.841***
	n-Hexane Lembang	0.968****	0.896***
	Ethyl acetate Ciwidey	0.62***	0.775***
	Ethyl acetate Cianjur	0.816***	0.869***
	Ethyl acetate Lembang	0.875***	0.839***
	Ethanol Ciwidey	0.764***	0.756***
	Ethanol Cianjur	0.886***	0.962****
	Ethanol Lembang	0.89***	0.711***
FRAP	*n*-Hexane Ciwidey	0.93****	0.874***
	n-Hexane Cianjur	0.986****	0.819***
	n-Hexane Lembang	0.953****	0.83***
	Ethyl acetate Ciwidey	0.938****	0.989****
	Ethyl acetate Cianjur	0.909****	0.951****
	Ethyl acetate Lembang	0.801***	0.961****
	Ethanol Ciwidey	0.781***	0.775***
	Ethanol Cianjur	0.977****	0.869***
	Ethanol Lembang	0.901****	0.883***

: strong, *: very strong.

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Table 7. Pearson correlation coefficient results of *P. betle* L. leaf extracts (DPPH *vs.* FRAP).

DPPH	Pearson correlation coefficient (r)
	FRAP
n-Hexane Ciwidey	0.886***
n-Hexane Cianjur	0.783***
n-Hexane Lembang	0.890****
Ethyl acetate Ciwidey	0.774***
Ethyl acetate Cianjur	0.752***
Ethyl acetate Lembang	0.789***
Ethanol Ciwidey	0.923***
Ethanol Cianjur	0.871***
Ethanol Lembang	0.941***

: strong; *: very strong.

contrast, correlation values for the DPPH method varied among extracts. While ethanol and ethyl acetate extracts showed high correlation values, n-hexane extracts displayed comparatively lower values, indicating that non-polar compounds may contribute less to the antioxidant mechanisms detected by these methods.

3.4 Qualitative phytochemical analysis of *P. betle* L. leaf extracts

The qualitative correlation between phenolic and flavonoid compounds and antioxidant activity was investigated to assess the contribution of these bioactive compounds to the antioxidant mechanisms of betel leaf extracts. The compounds in the extracts were separated based on their polarity using thin-layer chromatography (TLC). The resulting chromatograms were visualized using specific detection reagents: 10% Folin-Ciocalteu reagent for phenolic compounds (indicated by a blue-gray color), citroborate reagent for flavonoids (under UV light at 366 nm), 0.2% DPPH solution for free radical scavenging activity (yellow spots), and 0.8% FRAP reagent for Fe^{3+}-TPTZ reducing activity (blue color). This approach enabled a visual assessment of the correspondence between the spot patterns of phenolic and flavonoid compounds and the antioxidant activities detected by DPPH and FRAP assays, providing insights into their potential roles in the overall antioxidant properties of the extracts (Fig. 3, Fig. 4 and Fig. 5).

The qualitative analysis of flavonoids, phenols, and antioxidant activity using thin layer chromatography (TLC) provides insights into the presence and distribution of bioactive compounds in samples from Ciwidey (CW), Cianjur (CA), and Lembang (LM). In the flavonoid test (using citroborate reagent), visible spots indicated the presence of flavonoid compounds. Variations in spot intensity and distribution across samples suggested differences in flavonoid content, likely influenced by environmental factors such as soil characteristics and

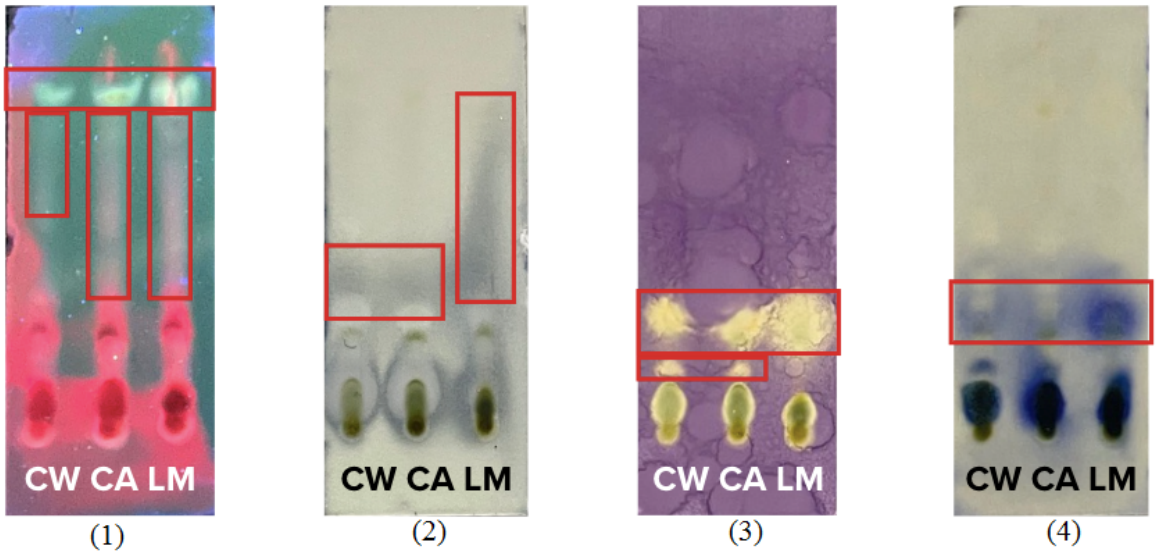

Figure 3. Thin layer chromatogram of *n*-hexane betel leaves extracts; stationary phase silica gel GF_{254}; mobile phase: *n*-hexane-ethyl acetate (9:1); (1) sprayed with citroborate; (2) sprayed with Folin-Ciocalteu; (3) sprayed with DPPH 0.2%; (4) sprayed with FRAP 0.8%. (CW: Ciwidey; CA: Cianjur; LM: Lembang).

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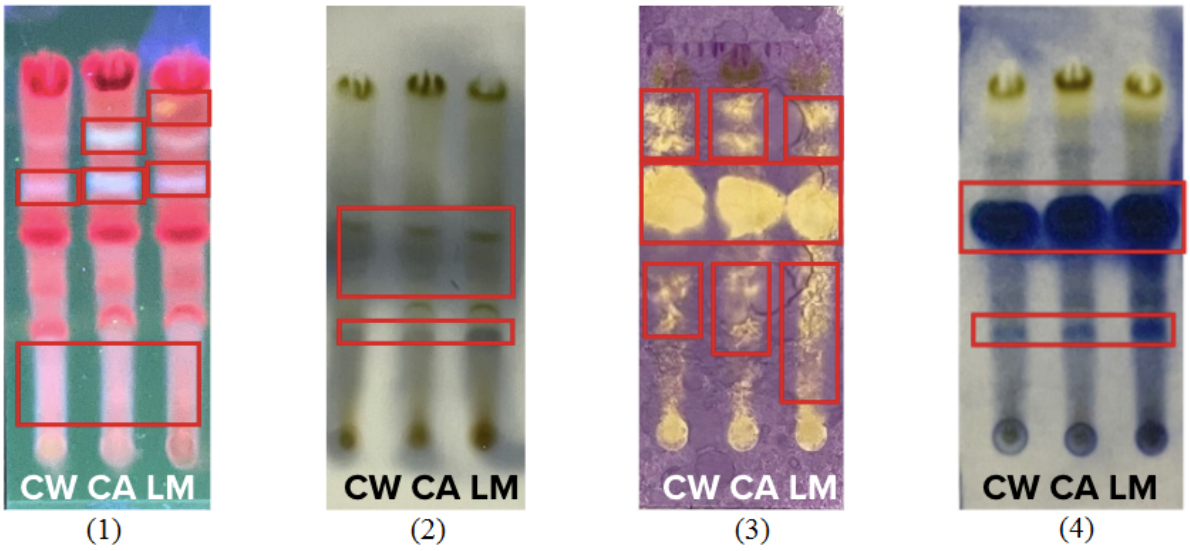

Figure 4. Thin layer chromatogram of ethyl acetate betel leaves extracts; stationary phase silica gel GF_{254}; mobile phase: chloroform-methanol (9:1); (1) sprayed with citroborate; (2) sprayed with Folin-Ciocalteu; (3) sprayed with DPPH 0.2%; (4) sprayed with FRAP 0.8%. (CW: Ciwidey; CA: Cianjur; LM: Lembang).

climate conditions in each region. The phenol test (using Folin-Ciocalteu reagent) revealed distinct spot patterns, confirming the presence of phenolic compounds. Differences in spot intensity among the samples suggested variation in phenolic compound content, which correlated with potential antioxidant capacity. In the antioxidant activity tests (DPPH and FRAP), visible spots indicated the presence of antioxidant compounds that interact with the respective reagents. The intensity of the spots in the DPPH assay suggested variations in radical scavenging activity, whereas the FRAP assay highlighted differences in the reducing power of the samples. The correlation between the presence of flavonoids and phenols with antioxidant activity confirmed the overall antioxidant potential.

3.5 HPLC analysis of *P. betle* L. leaf extracts

High-performance liquid chromatography (HPLC) analysis was performed to quantify flavonoid compounds in the samples by comparing retention times and peak areas (area under curve: AUC) with those of standard solutions. The ethyl acetate extract of Cianjur betel leaves was selected for flavonoid identification and quantification because it exhibited the highest antioxidant activity according to both DPPH and FRAP assays. The flavonoid standards used in this study included apigenin, rutin, quercetin, apigenin-7-*O*-glucoside, and kaempferol. These compounds were chosen based on previous reports of their presence in *P. betle* leaves [5], as well as their well-established antioxidant properties and significance as major representatives of flavonoid subclasses.

Based on Fig. 6, the sample exhibited two peaks with

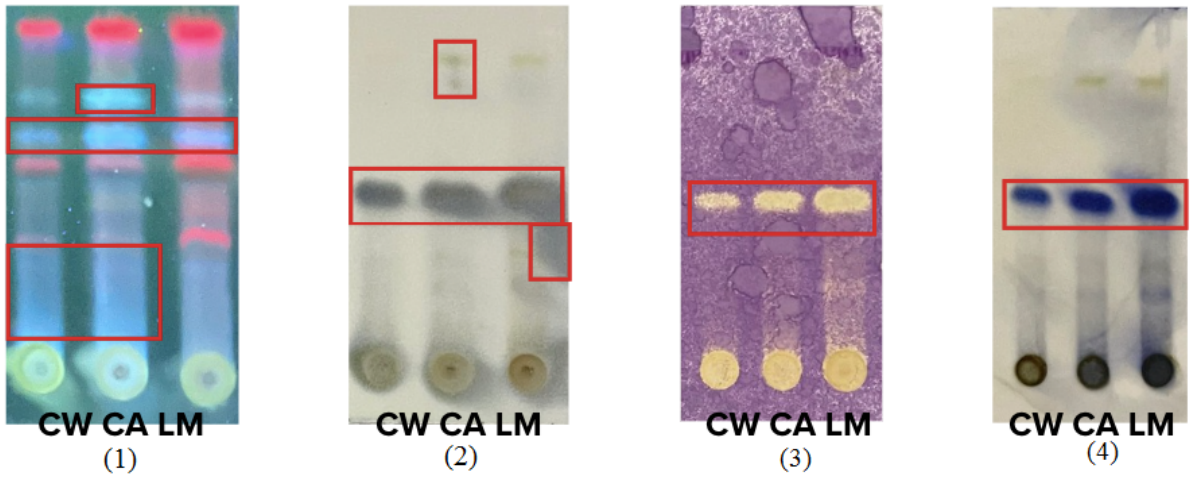

Figure 5. Thin layer chromatogram of ethanol betel leaves extracts; stationary phase silica gel GF_{254}; mobile phase: chloroform-methanol (9:1); (1) sprayed with citroborate; (2) sprayed with Folin-Ciocalteu; (3) sprayed with DPPH 0.2%; (4) sprayed with FRAP 0.8%. (CW: Ciwidey; CA: Cianjur; LM: Lembang).

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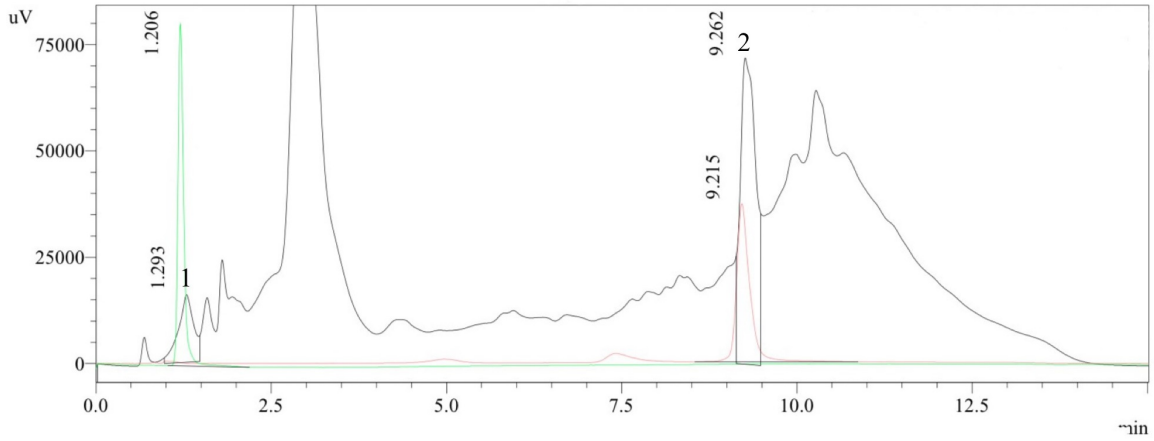

Figure 6. HPLC chromatogram of Cianjur ethyl acetate extract and standards; black line = sample 25000 µg/mL; green line = rutin 25 ppm; red line = apigenin 100 ppm.

retention times similar to those of rutin and apigenin standards. Therefore, the ethyl acetate extract of Cianjur betel leaves was identified to contain both compounds. Subsequently, the one-point calibration method was used to quantify the amounts of these compounds in the selected extract, and the results are presented in Table 8. The flavonoid components in the sample were quantified using HPLC by comparing the retention times and peak areas (AUC) of the compounds with those of reference standards. One peak in the sample chromatogram exhibited a retention time closely matching that of the apigenin standard, with an average content of 9.3146 ± 0.2885 mg/g and low variability, indicating high analytical reliability. The elevated apigenin concentration suggests strong antioxidant potential. Another peak corresponded to the rutin standard, showing an average content of 0.5919 ± 0.0581 mg/g. These results are consistent with those reported by Purba and Paengkoum (2019) [5], who demonstrated that aqueous, methanolic, ethanolic, and chloroform extracts of *P. betle* leaves contained both rutin and apigenin. These compounds are known to neutralize free radicals through electron donation mechanisms, further supporting the observed antioxidant activity of the betel leaf extract.

Table 8. Determination of rutin and apigenin in ethyl acetate betel leaves extract from Cianjur.

Compound	AUC		Average (mg/g)
	Reference	Sample	
Rutin	463962	243275	0.5919 ± 0.0581
		286393	
		292844	
Apigenin	487856	1117053	9.3146 ± 0.2885
		1114446	
		1176655	

4. Concluding remarks

This study confirms that extraction solvents and geographical origin significantly influence the phenolic and flavonoid content, as well as the antioxidant activity measured by free radical scavenging (DPPH assay) and electron donation capacity (FRAP assay) of betel leaf extracts. The ethyl acetate extract from Cianjur exhibited the highest antioxidant activity (DPPH: 2.231 ± 0.072 mg AEAC/g; FRAP: 611.603 ± 26.565 mg AEAC/g) and total phenolic content (537.641 ± 16.436 mg GAE/g), while the ethyl acetate extract from Lembang showed the highest overall flavonoid concentration (41.899 ± 3.525 mg QE/g). A strong positive correlation between phenolic and flavonoid content and antioxidant activity further supports their role as key contributors. The Cianjur ethyl acetate extract contained rutin (0.5919 ± 0.0581 mg/g) and apigenin (9.3146 ± 0.2885 mg/g). These findings highlight that betel leaves from Cianjur have strong potential as a natural antioxidant source for herbal and cosmetic applications. To the best of our knowledge, this study is among the first to comprehensively compare the phytochemical composition and antioxidant activity of *P. betle* L. collected from different geographical locations in West Java using complementary antioxidant assays (DPPH and FRAP), phytochemical profiling, and HPLC analysis. These findings provide new insights into the influence of geographical origin on the phytochemical quality of *P. betle* and emphasize the importance of cultivation site selection for obtaining plant materials with superior antioxidant properties. Therefore, the present study may contribute to the standardization of *P. betle* as a raw material for herbal medicines, cosmetic formulations, and other pharmaceutical products. Future studies should investigate the effects of seasonal variation, soil characteristics, and climatic factors on phytochemical accumulation, as well as evaluate the *in vivo* antioxidant efficacy and safety of the selected extracts to further support their industrial applications. Further *in vivo* studies are recommended to confirm the activity and elucidate the underlying mechanisms.

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Authors contributions
JBFL performed experiments, analyzed and interpreted data and wrote the first manuscript. IF conceived and designed the study. IF, ARC, RH, DR and HP analyzed and interpreted data and revised the manuscript. IF and RH confirmed the authenticity of all the raw data. All authors have read and approved the final manuscript.

Availability of data and materials
The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Conflict of interests
The authors assert that they do not have any identifiable conflicting financial interests or personal relationships that might be perceived to influence the work presented in this paper.

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