



Influence of Different Solvents on Antioxidant Properties, Total Phenolics, and Flavonoids from *Piper betle* Leaves

Nattawoot Rattanathamwat^{1*}, Supharat Duanyai¹, Korakoj Pethassanayothin¹, Wachirasak Kienwong², Julalak Jarujutarat², Janyawan Janyatham², Pasit Tinnam², Piya Rontlaong², and Atchara Kaewnoi³

¹ Faculty of Science and Technology, Bansomdejchaopraya Rajabhat University, Bangkok, 10600, Thailand

² Faculty of Engineering and Industrial Technology, Bansomdejchaopraya Rajabhat University, Bangkok, 10600, Thailand

³ Graduate School, Bansomdejchaopraya Rajabhat University, Bangkok, 10600, Thailand

* Correspondence: nattawoot.ra@bsru.ac.th

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Abstract: This study investigated the effects of different solvents (95% ethanol, DCM, acetone, and hexane) and extraction durations (3, 5, and 7 days) on the antioxidant activity, total phenolic content (TPC), and total flavonoid content (TFC) of *Piper betle* L. (yellow betel) leaves collected from Samut Sakhon Province, Thailand. The extracts were prepared from both fresh and dried leaves by maceration. Antioxidant activity was evaluated using DPPH, ABTS, and FRAP assays, while TPC and TFC were determined using the Folin-Ciocalteu and AlCl₃ colorimetric methods, respectively. The results showed that dried leaves consistently yielded higher extraction percentages than fresh leaves. Acetone extracts demonstrated superior antioxidant activities, with the lowest IC₅₀ values in both DPPH and ABTS assays (3.65 µg/mL and 1.37 µg/mL, respectively), and the highest FRAP values (16.77 mM FeSO₄ equivalent/mg extract). Moreover, acetone extracts of fresh leaves (3-day extraction) exhibited the highest TPC (1,222.419 mg GAE/g extract) and TFC (51.70 mg QE/g extract). Statistical analysis based on analysis of variance (ANOVA) revealed that solvent type was the most significant factor affecting extraction yield, TPC, TFC, and antioxidant activities ($p < 0.05$). In contrast, extraction duration had little effect beyond 3 days, and leaf condition (fresh versus dried) significantly affected several response variables. These findings demonstrate that solvent polarity and extraction duration are key determinants of phenolic and flavonoid recovery and antioxidant-related properties of *Piper betle* L. leaf extracts under the investigated experimental conditions.

Keywords: *Piper betle* L.; solvent extraction; antioxidant activity; total phenolic content; total flavonoid content

1. Introduction

Piper betle L., commonly known as betel leaf, is a traditional medicinal plant widely cultivated in central and southern Thailand. The leaves are consumed fresh or boiled as a herbal remedy and have been traditionally used for cooling purposes and relief of common ailments, including digestive discomfort, respiratory symptoms, and skin conditions. Pharmacological studies have demonstrated that betel leaves possess antibacterial, anti-inflammatory, and antifungal activities, as well as antioxidant potential, derived from compounds such as eugenol, hydroxychavicol, β-carotene, and ascorbic acid. These bioactive compounds may inhibit lipid peroxidation and related oxidative

processes that generate free radicals [1]. Previous research confirmed that *P. betle* leaves contain phenolic compounds with medicinal properties [2]. Further studies reported that phenolic constituents exhibited strong antibacterial activity, supporting their investigation as natural preservative candidates. Parkpoom et al. [3] investigated the antimicrobial and antioxidant properties of betel leaf extracts and found that ethanolic and propylene glycol extracts inhibited several pathogenic microorganisms, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans*. The ethanolic extract exhibited the strongest inhibition against *S. aureus*, with an inhibition zone of 2.93 ± 0.28 cm at 12.50 mg/mL, and showed a minimum inhibitory concentration. *P. betle* leaves are rich in phenolic and phenylpropanoid compounds, particularly hydroxychavicol, eugenol, and chavibetol, which are considered key contributors to their biological activities. MIC (MIC) and MBC) values of 3.125 and 6.25 mg/mL, respectively. In addition to antimicrobial activity, ethanolic extracts also demonstrated antioxidant potential, as measured by DPPH, ABTS, and FRAP assays, with reported values of $3,653.01 \pm 57.72$ μ g/mL, $17,261 \pm 697.00$ μ g/mL, and $3,616.99 \pm 41.49$ mg Trolox equivalents/g dw, respectively. The total phenolic content (TPC), determined by the Folin–Ciocalteu method, was reported to be 538.33 ± 13.48 mg GAE/g dw. These findings collectively indicate that betel leaf extracts are rich in bioactive compounds associated with antimicrobial and antioxidant activities. *P. betle* leaves contain a diverse range of phenolic and phenylpropanoid compounds, particularly hydroxychavicol, eugenol, and chavibetol, whose differences in polarity and molecular structure play a critical role in their solubility and extractability in solvents of varying polarity. Hydroxychavicol is a highly polar phenolic compound due to the presence of multiple hydroxyl groups, whereas eugenol and chavibetol exhibit semipolar characteristics. Differences in polarity and solubility of these compounds influence their extractability in solvents with varying polarity, thereby affecting the phenolic content and antioxidant activity of the resulting extracts.

The choice of solvent is a critical factor influencing the extraction yield of phytochemicals from medicinal plants. Borges et al. [4] studied the extraction of *Olea europaea* (olive leaves) and *Acacia dealbata* (flowers) using various solvents, including water, methanol, ethanol, acetone, DCM, and hexane, in combination with different techniques such as maceration, Soxhlet extraction, ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE). Their results indicated that polar solvents, such as ethanol, methanol, and acetone, yielded higher phenolic compound yields and stronger antimicrobial activity than nonpolar solvents, highlighting the importance of solvent polarity in phenolic extraction. Additionally, the use of assisted extraction techniques further enhanced extraction efficiency. In a solvent-comparison study, El Mannoubi et al. [5] compared the efficiency of 80% ethanol and 80% acetone in extracting bioactive compounds from *Opuntia stricta*. The results showed that 80% ethanol yielded the highest overall extract, whereas 80% acetone extracts contained higher levels of phenolics and flavonoids and exhibited stronger antioxidant activity, suggesting that extraction yield and bioactive compound recovery do not necessarily correlate. In another study, Chatepa et al. [6] evaluated the effect of different solvents on the extraction of *Azadirachta indica* (neem) and *Vernonia amygdalina* (bitter leaf). Their findings revealed that the optimal solvent varied by plant species: methanol and water were most effective for neem, whereas different extraction patterns were observed for bitter leaf, indicating that plant matrix characteristics influence solvent efficiency. Piper betle L. Banik et al. [7] compared acetone and ethanol extracts and reported that both solvents produced extracts with antibacterial activity. However, acetone extracts were more effective against certain bacterial strains, whereas ethanolic extracts contained higher phenolic contents, demonstrating solvent-dependent differences in bioactivity profiles. Using different solvent systems, Venugopalan et al. [8] reported that ethanol and methanol extracts of *P. betle* leaves exhibited stronger antioxidant activities than hexane or aqueous extracts. Consistent with these observations, Nouri et al. [9] demonstrated that ethanolic extracts of betel leaves yielded higher phenolic contents and stronger antioxidant activities than methanolic extracts. Collectively, these studies demonstrate that solvent polarity is a dominant factor governing phenolic and flavonoid extraction efficiency and antioxidant activity, although the optimal solvent may vary depending on plant species, plant matrix characteristics, and extraction conditions.

However, previous studies on *P. betle* extraction have mainly focused on limited solvent systems or single extraction conditions. Comparative investigations that simultaneously evaluate the effects of solvent polarity, extraction duration, and plant material condition (fresh versus dried leaves) under consistent

experimental conditions remain limited. As a result, a systematic understanding of how these factors collectively influence phenolic and flavonoid recovery and antioxidant activity in *P. betle* leaves is still lacking.

Given this background, the present study was designed to investigate the effects of different solvents and extraction conditions on the antioxidant activity, total phenolic content (TPC), and total flavonoid content (TFC) of *Piper betle* leaves. This study was conducted to evaluate how solvent polarity and extraction duration influence the extraction efficiency and antioxidant-related properties of phenolic and flavonoid compounds under the studied conditions, to provide fundamental scientific information to support a systematic comparison of extraction parameters.

2. Materials and Methods

2.1 Materials

Fresh *Piper betle* L. leaves were collected from Laksong Subdistrict, Ban Phaeo District, Samut Sakhon Province, Thailand. The plant material was taxonomically identified as *Piper betle* L. (Piperaceae) by a botanist at the Department of Botany, Faculty of Science, Chulalongkorn University. A voucher specimen (No. A 18147) was deposited at the Herbarium of the Department of Botany, Faculty of Science, Chulalongkorn University (BCU), Bangkok, Thailand. Analytical grade (AR) solvents and chemicals were used in this study, including ethanol (RCI Labscan, Thailand), hexane (RCI Labscan, Thailand), dichloromethane (DCM) (RCI Labscan, Thailand), acetone (RCI Labscan, Thailand), 2,2-diphenyl-1-picrylhydrazyl (DPPH; RCI Labscan, Thailand), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma-Aldrich, USA), **ascorbic acid** (RCI Labscan, Thailand), sodium carbonate (Sigma-Aldrich, USA), Folin-Ciocalteu reagent (Sigma-Aldrich, USA), gallic acid (Sigma-Aldrich, USA), potassium persulfate (RCI Labscan, Thailand), 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ; Sigma-Aldrich, USA), ferric chloride (Sigma-Aldrich, USA), and quercetin (Sigma-Aldrich, USA).

2.2 Extraction of Betel Leaves Using Different Solvents by Maceration Method

The maceration method was employed for extraction, as previously reported [10, 11]. Fresh leaves were washed with distilled water and used immediately, while dried leaves were dried in a hot-air oven at 60 °C for 24 h until constant weight was achieved. Fresh and dried leaves were cut into small pieces, weighed, and soaked in different solvents (95% ethanol, DCM, acetone, and hexane) at a 1:10 (w/v) ratio (1 g sample per 10 mL solvent). Samples were extracted at room temperature (25 ± 2 °C) for 3, 5, and 7 days. The mixtures were filtered through Whatman No. 1 filter paper, and the solvents were evaporated under reduced pressure using a rotary evaporator at 40 °C. The concentrates were freeze-dried and dried to constant weight to minimize residual solvents before storage. The dried extracts were weighed, and the extraction yield (%) was calculated as shown in Equation (1): Fresh leaves were used to represent the native physicochemical state of the plant material and to minimize degradation of moisture-sensitive bioactive compounds. In contrast, dried leaves were included for comparison, as drying is commonly applied during post-harvest processing and may influence extraction efficiency. Although moisture content was not quantitatively determined, oven-dried leaves (60 °C, 24 h) were used to ensure consistent moisture reduction before extraction. The maceration durations of 3, 5, and 7 days were selected to evaluate the time-dependent extraction behavior of phenolic and flavonoid compounds under mild conditions at room temperature. Previous studies have demonstrated that maceration time plays a critical role in determining phenolic composition and antioxidant capacity, with measurable changes commonly observed within the early to intermediate stages of extraction [12,13]. Comparative studies have reported that maceration periods ranging from a few days to one week are commonly used to investigate extraction kinetics, including time points at 1, 3, 7, and 14 days [14,15]. However, prolonged maceration may lead to partial degradation or oxidation of phenolic compounds, particularly when extraction is extended beyond the optimal duration [12,16]. Therefore, the maximum maceration period in this study was limited to 7 days to balance extraction efficiency and chemical stability, while enabling systematic comparison of extraction yields, phenolic, flavonoid, and antioxidant activities at different time points [12,15,17]. All maceration experiments were conducted at room temperature (25 ± 2 °C) under dark conditions to minimize potential photo-degradation of phenolic compounds. Extraction was performed in closed glass containers to prevent solvent evaporation and reduce oxidative exposure. The mixtures were gently agitated

manually once daily to enhance solvent penetration and mass transfer during maceration. The solvent-to-sample ratio of 1:10 (w/v) was selected based on previous maceration studies, which reported that this ratio provides sufficient solvent volume to facilitate solute diffusion and achieve extraction equilibrium under mild conditions, while avoiding excessive solvent consumption and enabling meaningful comparisons of extraction yield and phenolic recovery across different extraction times [14,15].

$$\text{Yield (\%)} = \frac{\text{Weight of dried extract (g)}}{\text{Weight of initial leaves (g)}} \times 100 \quad (1)$$

2.3 Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) of the extracts was determined using the Folin–Ciocalteu method with gallic acid as a standard, as described by Singleton and Rossi [18]. Extract solutions of various concentrations were prepared. For the assay, 20 μL of extract solution was mixed with 100 μL of Folin–Ciocalteu reagent diluted 1:10 with deionized water, followed by the addition of 80 μL of 7.5% (w/v) sodium carbonate (Na_2CO_3), giving a final volume of 200 μL . TPC values were calculated by interpolating absorbance values from the gallic acid calibration curve, correcting for dilution factors, and expressing them as mg GAE/g extract. The mixtures were thoroughly mixed and incubated in the dark at room temperature for 30 min. Absorbance was measured at 760 nm using a microplate reader. All measurements were carried out in triplicate ($n = 3$). The phenolic content of the extracts was determined from the gallic acid calibration curve and expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract).

2.4 Determination of Total Flavonoid Content (TFC)

The total flavonoid content was determined using the aluminum chloride colorimetric method with quercetin as the standard, as described by Chang et al. [19]. A quercetin standard stock solution was prepared at 100 $\mu\text{g/mL}$ and diluted to obtain standard solutions at 10, 20, 30, 40, and 45 $\mu\text{g/mL}$. Extract samples were prepared at a concentration of 400 $\mu\text{g/mL}$. For the assay, 100 μL of either the extract sample or quercetin standard solution was mixed with 100 μL of 2% aluminum chloride (AlCl_3) solution. The mixtures were incubated at room temperature for 10 min, and absorbance was measured at 415 nm using a microplate spectrophotometer (Perkin Elmer®, EnSight 3400). Each experiment was conducted in triplicate ($n = 3$). The flavonoid content was calculated from the quercetin standard calibration curve and expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g extract).

2.5 DPPH Radical Scavenging Capacity Assay

The antioxidant activity of the extracts was evaluated using the DPPH radical scavenging capacity assay according to the method described by Brand-Williams et al. [20]. Ascorbic acid (vitamin C) was used as a positive control and standard antioxidant reference for comparison. A stock solution of the sample was prepared at 200 $\mu\text{g/mL}$, and serial two-fold dilutions were made to obtain five concentrations: 100, 50, 25, 12.5, and 6.25 $\mu\text{g/mL}$. Different initial extract concentrations were used for each antioxidant assay because the assays differ in sensitivity, reaction kinetics, and linear response range. Therefore, the concentration ranges were selected based on preliminary tests and established literature protocols to ensure measurable inhibition within the dynamic range of each method and to allow accurate determination of IC_{50} values. For the assay, 100 μL of each sample solution was mixed with 100 μL of methanolic DPPH solution (152 μM). The reaction mixtures were incubated in the dark at room temperature for 30 min, after which absorbance was measured at 517 nm using a microplate spectrophotometer (Perkin Elmer®, EnSight 3400). The percentage of radical scavenging activity was calculated according to Equation (2):

$$\text{radical scavenging(\%)} = \frac{(A_{\text{control}} - A_{\text{blank control}}) - (A_{\text{sample}} - A_{\text{blank sample}})}{(A_{\text{control}} - A_{\text{blank control}})} \times 100 \quad (2)$$

where:

A_{sample} = absorbance of the extract sample mixed with DPPH

$A_{\text{blank sample}}$ = absorbance of the extract sample mixed with methanol

A_{control} = absorbance of the DPPH solution without extract

$A_{\text{blank control}}$ = absorbance of methanol alone

All measurements were performed in triplicate ($n = 3$). IC_{50} values were determined by fitting concentration–response curves using nonlinear regression analysis (four-parameter logistic model) with GraphPad Prism version 10.

2.6 ABTS Radical Cation Decolorization Assay

The antioxidant activity of the extracts was evaluated using the ABTS radical cation decolorization assay. According to the method described by Re et al. [21]. Ascorbic acid was used as a standard antioxidant reference for comparison. A stock solution of 7 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was prepared and mixed with 2.45 mM potassium persulfate at a 1:0.5 (v/v) ratio. The mixture was incubated in the dark for 12–16 h to generate $ABTS^{\bullet+}$ radicals. Before use, the $ABTS^{\bullet+}$ solution was diluted with ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm.

Sample solutions were prepared at different concentrations (100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 $\mu\text{g/mL}$ in ethanol). For the assay, 20 μL of each sample solution was added to a 96-well plate, followed by 180 μL of the $ABTS^{\bullet+}$ solution. The mixture was shaken gently and incubated in the dark for 6 min. Absorbance was then measured at 734 nm ($n = 3$) using a microplate spectrophotometer (Perkin Elmer®, EnSight 3400). The percentage of inhibition was calculated using Equation (3):

$$\text{inhibition(\%)} = \frac{A_c - A_s}{A_c} \times 100 \quad (3)$$

where:

A_c = absorbance of the control ($ABTS^{\bullet+}$ solution without extract)

A_s = absorbance of the test sample containing the extract

2.7 Ferric Ion Reducing Antioxidant Power (FRAP) Assay

The antioxidant activity of the extracts was further evaluated using the ferric ion reducing antioxidant power (FRAP) assay, as described by Benzie and Strain [16]. Ferrous sulfate (FeSO_4) was used as a standard reference compound to construct a calibration curve for FRAP quantification. The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 20 mM ferric chloride (FeCl_3), and 10 mM 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ) in 40 mM hydrochloric acid (HCl) at a ratio of 10:1:1 (v/v/v).

Sample solutions were prepared at a concentration of 400 $\mu\text{g/mL}$ in methanol. For the assay, 20 μL of each extract was added to a 96-well plate, followed by 180 μL of FRAP reagent. The mixture was gently shaken and incubated for 4 min at room temperature, after which absorbance was measured at 593 nm ($n = 3$) using a microplate spectrophotometer (Perkin Elmer®, EnSight 3400). The absorbance value was calculated according to Equation (4):

$$\text{Absorbance} = A - B - C \quad (4)$$

where:

A = absorbance of the sample mixed with FRAP reagent

B = absorbance of the sample mixed with the acetate buffer

C = absorbance of methanol mixed with FRAP reagent

The blank-corrected absorbance obtained from Equation (4) was used to calculate the FRAP value by interpolation from the ferrous sulfate (FeSO_4) standard calibration curve. The concentration of FeSO_4 equivalents was determined using the linear regression equation derived from the calibration curve. The FRAP value was determined by comparing the absorbance of the test samples with the calibration curve of ferrous sulfate (FeSO_4) in the concentration range of 0.06–0.20 mM. Results were expressed as millimoles of ferrous sulfate equivalents per milligram of extract (mM FeSO_4 equivalent/mg extract). Different sample concentrations were applied across assays because each method differs in sensitivity, reaction mechanism, and linear response range. Radical scavenging assays (DPPH and ABTS) require serial dilutions to determine IC_{50} values accurately, whereas reducing power-based assays (FRAP) and colorimetric complex formation assays (TPC and TFC) require sufficiently high concentrations to generate absorbance values within the linear range of their respective calibration curves.

2.8 Statistical Analysis

All experiments were conducted in triplicate, and the results were expressed as mean $\pm$ standard deviation (SD) ($n = 3$). All instruments were calibrated according to the manufacturer's instructions before analysis, and appropriate blanks were applied in all assays. A full factorial experimental design was applied to evaluate the effects of solvent type, extraction time, and leaf condition (fresh and dried) on extraction yield, as well as the effects of assay type, solvent, extraction time, and leaf condition on antioxidant activities. Analysis of variance (ANOVA) was performed to determine significant differences among groups, followed by Duncan's multiple range test. An independent-samples t-test was used to compare fresh and dried leaf samples. Statistical analyses were carried out using SPSS Statistics (version 21.0), with significance set at $p < 0.05$.

3. Results and Discussion

3.1. Percentage Yield of Piper betle Leaf Extracts

The extraction yields of *Piper betle* L. leaves under two conditions (fresh and dried) using four different solvents (95% ethanol, DCM, acetone, and hexane) with maceration times of 3, 5, and 7 days are presented in Table 1. Overall, the extraction yield was influenced by both the physical condition of the plant material and the polarity of the extraction solvent. As shown in Table 1, dried leaves consistently produced significantly higher extraction yields than fresh leaves across all solvents and maceration times ($p < 0.05$). The highest extraction yield was obtained from dried leaves extracted with 95% ethanol for 3 days ($8.30 \pm 0.20\%$ w/w), followed by acetone extraction of dried leaves at the same maceration time ($7.17 \pm 0.62\%$ w/w). From a chemical perspective, drying alters the plant matrix by disrupting cell walls and reducing intracellular water content, thereby facilitating solvent penetration and improving the mass transfer of extractable compounds. In addition, removing moisture prevents dilution of the extraction solvent, thereby increasing the effective concentration gradient driving solute diffusion. Consequently, dried plant materials generally provide higher extraction yields than fresh samples, as reported in several phytochemical extraction studies [10, 17].

Table 1. Percentage yield (% w/w) of extracts from fresh and dried *Piper betle* leaves obtained with different solvents at various maceration times (3, 5, and 7 days).

Solvent/days	% Yield	
	Fresh leaves	Dried leaves
3 days		
95% Ethanol	4.51 ± 0.08^a	$8.30 \pm 0.20^{b*}$
DCM	1.47 ± 0.11^b	$6.32 \pm 0.50^{c*}$
Acetone	2.84 ± 0.20^b	$7.17 \pm 0.62^{b*}$
Hexane	0.57 ± 0.24^c	$4.08 \pm 0.25^{c*}$
5 days		
95% Ethanol	5.87 ± 0.15^a	$7.92 \pm 0.74^{b*}$
DCM	2.31 ± 0.53^b	$5.17 \pm 0.35^{c*}$
Acetone	2.78 ± 0.02^b	$6.45 \pm 0.89^{b*}$
Hexane	0.29 ± 0.09^c	$4.82 \pm 0.54^{c*}$
7 days		
95% Ethanol	4.93 ± 0.31^a	$8.08 \pm 0.16^{b*}$
DCM	1.77 ± 0.23^b	$5.68 \pm 0.48^{c*}$
Acetone	3.13 ± 0.18^b	$6.67 \pm 0.15^{b*}$
Hexane	0.44 ± 0.02^c	$4.75 \pm 0.13^{c*}$

Values are expressed as mean $\pm$ SD ($n = 3$).

Different lowercase letters (a–c) within the same column indicate significant differences among solvents within the same leaf condition (fresh or dried) and extraction time, as determined by one-way ANOVA followed by Duncan's multiple range test ($p < 0.05$).

* indicates significant differences between fresh and dried leaves within the same solvent and extraction time, determined by an independent-samples t-test ($p < 0.05$).

Among the solvents tested, 95% ethanol and acetone produced higher extraction yields than DCM and hexane, reflecting the greater solubility of moderately polar phenolic and phenylpropanoid compounds in polar and semi-polar solvents. *Piper betle* leaves are rich in hydroxylated phenolic constituents, which can be efficiently solubilized through hydrogen bonding and dipole–dipole interactions with ethanol and acetone, whereas non-polar solvents such as hexane exhibit limited affinity for these compounds, resulting in substantially lower extraction yields. Similar solvent-dependent trends have been widely reported for phenolic-rich plant materials [4, 10–11]. When comparing maceration times within the same solvent system, extending the extraction period from 3 to 7 days did not result in a substantial increase in extraction yield. This suggests that the extraction equilibrium was reached during the early stages of maceration. Regarding extraction duration, the limited effect of prolonged maceration on extraction yield indicates that most soluble phytochemicals were released during the initial stages of extraction. Once diffusion equilibrium between the plant matrix and solvent phase is achieved, further extension of maceration time does not significantly enhance yield. Prolonged extraction may even increase the risk of oxidative degradation or polymerization of phenolic compounds without providing additional extraction benefits [16–17]. Based on the analysis of variance (ANOVA) of the experimental design for the extraction of compounds from *Piper betle* L. leaves, three main factors were evaluated: solvent type, maceration time (days), and leaf condition. The ANOVA results are summarized in Table 2.

Table 2. Analysis of variance (ANOVA) of factors affecting the extraction yield (% yield) of *Piper betle* leaves

Source	DF	AdjSS	AdjMS	F-Value	P-Value
Model	23	408.409	17.757	118.42	0.000
Linear	6	396.166	66.028	440.34	0.000
Solvent	3	155.814	51.938	346.37	0.000
Time	2	0.036	0.018	0.12	0.888
Leaf condition	1	240.316	240.316	1602.66	0.000

Table 2 shows that the overall ANOVA model for extraction yield was statistically significant ($p < 0.001$). Solvent type and leaf condition significantly affected extraction yield ($p < 0.05$), confirming that both solvent polarity and sample pretreatment play critical roles in determining extraction efficiency. In contrast, maceration time within the investigated range (3–7 days) did not significantly affect extraction yield ($p = 0.888$), indicating that extending the extraction period beyond the initial stage provides limited additional benefit. These results suggest that solvent polarity and leaf condition are the primary determinants of extraction efficiency, whereas extraction time plays a relatively minor role within the investigated range. Overall, the results demonstrate that the extraction yield of *P. betle* leaves is primarily governed by the physical condition of the plant material and solvent polarity, whereas maceration duration within the investigated range plays a comparatively minor role. These findings provide essential baseline information for optimizing extraction conditions before evaluating antioxidant activity and phytochemical content in subsequent analyses.

3.2 Total Flavonoid Content (TFC) and Total Phenolic Content (TPC)

The total flavonoid content (TFC) of extracts obtained from fresh and dried *Piper betle* leaves was determined using a quercetin calibration curve. The calibration curve exhibited good linearity, with a coefficient of determination (R^2) of 0.9988, and the regression equation was $y = 0.0104x - 0.0107$ (Figure 1). Total phenolic content (TPC) was determined using gallic acid as the standard reference, and the corresponding calibration curve showed excellent linearity ($R^2 = 0.9994$) with the regression equation $y = 0.0452x + 0.0118$ (Figure 2). The quantified TFC and TPC values under different extraction conditions are summarized in Table 3.

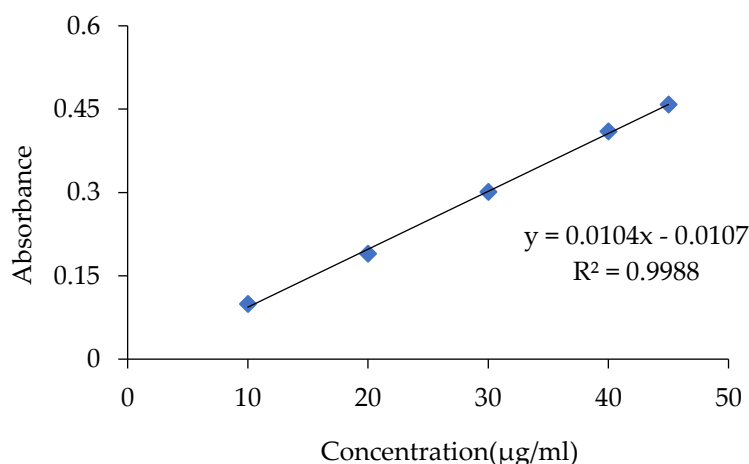


Figure 1. Standard calibration curve of quercetin

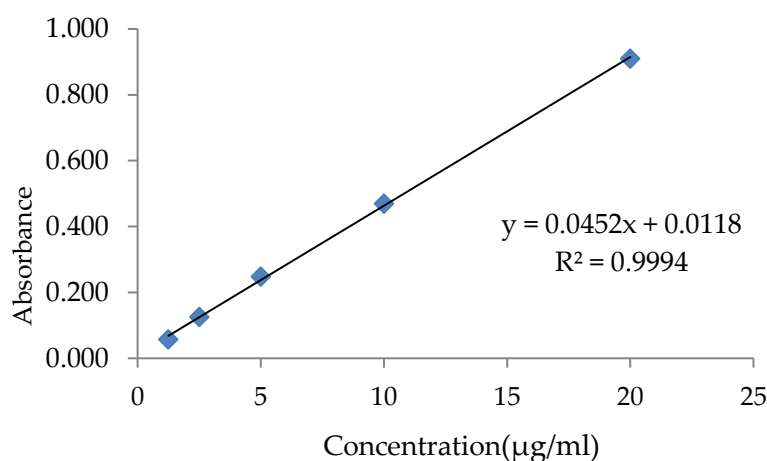


Figure 2. Standard calibration curve of gallic acid

The total flavonoid content (TFC) and total phenolic content (TPC) of *Piper betle* leaf extracts obtained under different extraction conditions are summarized in Table 3. The results clearly demonstrate that sample condition (fresh vs. dried leaves), solvent polarity, and maceration duration affected both TFC and TPC values. Under fresh leaf conditions, acetone extracts consistently exhibited the highest TFC and TPC values, particularly at shorter maceration periods (3 days). For example, the fresh acetone extract obtained after 3 days showed the highest TFC (51.71 ± 15.51 mg QE/g extract) and the highest TPC ($1,222.419 \pm 24.27$ mg GAE/g extract) among all tested solvents (Table 3). This can be attributed to the intermediate polarity of acetone, which enables efficient solubilization of a broad range of flavonoids and phenolic compounds, including phenolic acids and polyphenols containing multiple hydroxyl groups [22]. Previous studies have reported that *Piper betle* L. leaves are rich in phenolic compounds, particularly eugenol, hydroxychavicol, chavicol, and allyl pyrocatechol, which possess strong **electron-donating capacity** and contribute to antioxidant-related properties [23]. In the present study, the acetone extract of fresh leaves obtained after 3 days of maceration exhibited the highest total phenolic content ($1,222.42 \pm 24.27$ mg GAE/g extract). Although this value exceeds the leaf type, it should be noted that TPC values are expressed as gallic acid equivalents (GAE), which reflect the total reducing capacity of the extract rather than the absolute phenolic concentration. Moreover, the Folin–Ciocalteu assay is not specific to phenolic compounds and may respond to other reducing substances present in fresh plant tissues, potentially leading to elevated GAE values [24]. The high TPC observed in the acetone

extract may be attributed to hydroxychavicol-rich phenolic fractions, whose molecular structures facilitate stronger electron-transfer reactions than those of gallic acid under alkaline assay conditions. In addition, acetone has been reported to be highly effective in extracting these phenolics and in inhibiting polyphenol oxidase (PPO) activity in fresh betel leaves, thereby reducing enzymatic degradation during short extraction periods [25]. However, when maceration time was extended to 5–7 days, TPC values decreased, which may be associated with oxidation or degradation of phenolic compounds in fresh leaves with high moisture content during prolonged extraction. Overall, these results highlight the importance of solvent selection and extraction duration in preserving redox-active phenolic compounds in *Piper betle* leaf extracts.

DCM extracts also showed relatively high TFC and TPC values compared with ethanol and hexane. Although DCM is less polar than acetone, it is effective in extracting flavonoid aglycones and semi-polar phenolic compounds, which contribute to its moderate to high extraction efficiency [10]. In contrast, hexane consistently yielded the lowest TFC and TPC values, reflecting its nonpolar nature and limited ability to dissolve phenolic compounds with polar functional groups. This observation agrees with previous reports indicating that non-polar solvents are unsuitable for phenolic extraction [11]. A comparison between fresh and dried leaf extracts showed that TPC values were generally higher in dried leaves, especially when ethanol or DCM was used as the extraction solvent (Table 3). The increased TPC in dried samples can be explained by moisture removal, which increases the relative concentration of phenolic compounds on a dry-weight basis and enhances solvent penetration into plant tissues. In addition, drying may disrupt cell walls and vacuolar structures, facilitating the release of bound phenolics during extraction [17]. In contrast, TFC values tended to be higher in fresh leaf extracts, particularly for acetone and DCM at shorter maceration times. Many flavonoids are known to be sensitive to heat, oxygen, and prolonged exposure to air, and partial degradation or oxidation may occur during drying, leading to reduced flavonoid content in dried samples [17]. This explains why fresh leaves often retained higher TFC values, whereas dried leaves showed a relative decrease in flavonoid content despite higher TPC values.

Maceration duration also influenced TFC and TPC, although the effect was not linear. In several solvent systems, higher TFC and TPC values were observed at shorter or intermediate extraction times (3–5 days), while prolonged maceration (7 days) did not consistently enhance compound recovery. This trend suggests that the extraction equilibrium was reached early, and extended maceration may lead to oxidation or degradation of phenolic and flavonoid compounds rather than increased extraction efficiency. Similar observations have been reported for plant polyphenol extraction using maceration techniques [12,15]. Although TFC and TPC represent different classes of phytochemicals, the results in Table 3 indicate a general positive association between TFC and TPC, particularly in acetone and DCM extracts. This relationship is chemically reasonable, as flavonoids constitute a major subclass of phenolic compounds, and extraction conditions that favor phenolic solubilization often enhance flavonoid recovery. However, the lack of a strict linear relationship between TFC and TPC across all solvents and conditions suggests that solvent selectivity and compound stability play important roles in determining the relative abundance of flavonoids versus total phenolics [20, 22].

Overall, the results demonstrate that acetone was the most effective solvent for the simultaneous extraction of flavonoids and phenolic compounds, whereas ethanol and DCM were comparatively more effective for maximizing total phenolic content (TPC) in dried leaf samples. The observed differences between fresh and dried leaves further highlight the importance of sample pretreatment and extraction parameters in preserving and recovering bioactive compounds from *Piper betle* L. Although the hydrodistilled essential oil exhibited measurable TPC values (Table 3), these results should be interpreted with caution. Essential oils are primarily composed of volatile, non-polar to semi-polar constituents, such as terpenoids and phenylpropanoids, rather than classical phenolic compounds. Moreover, the Folin–Ciocalteu assay is known to respond to various reducing substances, including certain non-phenolic constituents, which may contribute to apparent TPC values in essential oils. Therefore, direct quantitative comparison of TPC between essential oil and solvent-based extracts is not appropriate, and the essential oil data are presented mainly for qualitative reference. Based on the analysis of variance (ANOVA) of the extraction experiment designed with three

factors, namely solvent type, maceration time, and leaf type, the results are summarized in Table 4. The analysis of variance (ANOVA) results for total flavonoid content (TFC) (Table 4) showed that solvent type, maceration time, and leaf type significantly affected flavonoid content ($p < 0.05$). Solvent type exerted a strong influence on TFC ($F = 131.85$), indicating the importance of solvent polarity in flavonoid extraction. Maceration time also showed a significant but weaker effect ($F = 3.72$), while leaf type had the greatest impact ($F = 239.06$), confirming clear differences between fresh and dried leaves.

Table 3. Total Flavonoid Content (TFC) and Total Phenolic Content (TPC)

Solvent/ days	TFC		TPC	
	mg QE/g extract		mg GAE/g extract	
	Fresh leaves	Dried leaves	Fresh leaves	Dried leaves
3 days				
95% Ethanol	18.662 ± 1.22 ^{cA*}	13.813 ± 1.54 ^{cB**}	463.300 ± 5.35 ^{bA}	526.475 ± 75.63 ^{cAB}
DCM	42.873 ± 3.51 ^{bA**}	7.177 ± 0.14 ^{aA*}	232.289 ± 26.59 ^{dA**}	717.257 ± 47.47 ^{bA*}
Acetone	51.705 ± 15.51 ^{aA*}	18.697 ± 2.08 ^{bA**}	1,222.419 ± 24.27 ^{aA*}	816.323 ± 46.86 ^{aB**}
Hexane	7.371 ± 0.93 ^{dB**}	2.006 ± 0.41 ^{bB*}	283.161 ± 9.62 ^{cA*}	204.019 ± 11.37 ^{dB**}
5 days				
95% Ethanol	15.385 ± 1.64 ^{bA}	14.796 ± 0.23 ^{abB}	380.273 ± 11.67 ^{bB**}	573.550 ± 43.17 ^{cA*}
DCM	29.355 ± 3.04 ^{aB*}	12.983 ± 0.15 ^{bB**}	210.656 ± 33.95 ^{cAB**}	706.686 ± 82.17 ^{bA*}
Acetone	33.656 ± 0.74 ^{aC*}	17.082 ± 2.94 ^{aAB**}	606.637 ± 31.81 ^{aB**}	1,198.083 ± 14.73 ^{aA*}
Hexane	14.080 ± 2.63 ^{bA*}	4.208 ± 0.33 ^{cC**}	203.024 ± 14.57 ^{cB}	229.953 ± 10.71 ^{dA}
7 days				
95% Ethanol	13.608 ± 2.04 ^{cB**}	27.123 ± 4.41 ^{aA*}	230.998 ± 18.89 ^{bC*}	436.873 ± 51.39 ^{cB*}
DCM	39.647 ± 3.13 ^{bB*}	8.359 ± 0.45 ^{cC**}	184.108 ± 4.64 ^{cB**}	811.406 ± 27.10 ^{aA*}
Acetone	43.948 ± 0.83 ^{aB*}	16.009 ± 1.16 ^{cB**}	638.594 ± 37.63 ^{aB}	592.380 ± 21.17 ^{bC}
Hexane	2.823 ± 0.24 ^{dC**}	9.466 ± 0.96 ^{bA*}	218.879 ± 6.43 ^{bC**}	244.088 ± 10.98 ^{dA*}
Hydrodistillation				
Essential oil	N/D	N/D	352.360 ± 6.93	504.523 ± 2.27

Values are expressed as mean ± SD (n = 3).

Different lowercase letters (a–d) within the same column indicate significant differences among solvents at the same extraction time and condition, determined by one-way ANOVA followed by Duncan's multiple range test ($p < 0.05$).

Different uppercase letters (A–C) within the same row indicate significant differences among extraction times for the same solvent and condition, determined by Duncan's test ($p < 0.05$).

* and ** indicate significant differences between fresh and dried leaves within the same solvent and extraction time, determined by an independent-samples t-test ($p < 0.05$).

Table 4. Analysis of variance (ANOVA) of factors affecting total flavonoid content (TFC) of *Piper betle* leaves

Source	DF	AdjSS	Adj MS	F-Value	P-Value
Model	23	13444.0	584.52	42.92	0.000
Linear	6	8744.4	1457.41	107.01	0.000
Solvent	3	5387.3	1795.76	131.85	0.000
Time	2	101.3	50.66	3.72	0.031
Leaf condition	1	3255.9	3255.85	239.06	0.000

Similarly, the ANOVA results for total phenolic content (TPC) are presented in Table 5, which revealed that all three factors significantly influenced phenolic recovery ($p < 0.001$). Solvent type showed the most pronounced effect ($F = 1398.11$), followed by leaf type ($F = 251.87$) and maceration time ($F = 238.68$). These results emphasize that phenolic extraction is strongly governed by solvent polarity and sample condition.

Table 5. Analysis of variance (ANOVA) of factors affecting total phenolic content (TPC) of *Piper betle* leaves

Source	DF	AdjSS	AdjMS	F-Value	P-Value
Model	23	10276769	446816	373.90	0.000
Linear	6	5883739	980623	820.53	0.000
Solvent	3	5012295	1670765	1398.11	0.000
Time	2	570460	285230	238.68	0.000
Leaf condition	1	300985	300985	251.87	0.000

Overall, the ANOVA analyses confirm that solvent selection and leaf pretreatment are the dominant factors controlling both TFC and TPC, whereas maceration time plays a secondary but still significant role in determining phytochemical recovery from *Piper betle* leaves.

3.3 Antioxidant Activities of Extracts by DPPH, ABTS, and FRAP Assays

The antioxidant activities of *Piper betle* leaf extracts obtained from fresh and dried samples were evaluated using three complementary assays: DPPH radical scavenging, ABTS radical cation decolorization, and ferric reducing antioxidant power (FRAP). These assays were selected because they represent different antioxidant mechanisms, including hydrogen atom transfer (HAT), single-electron transfer (SET), and overall reducing capacity [rotary evaporator]. Extracts were prepared using four solvents of varying polarity (95% ethanol, DCM, acetone, and hexane) and maceration times of 3, 5, and 7 days. The antioxidant activities are summarized in Table 6.

As shown in Table 6, acetone extracts consistently exhibited the strongest radical scavenging activity in both DPPH and ABTS assays, particularly for fresh leaves. The lowest DPPH IC₅₀ values were observed for fresh leaf extracts obtained with acetone after 5 and 7 days of maceration, at 3.665 ± 0.26 and 3.653 ± 0.41 µg/mL, respectively. These values were comparable to those of dried leaves extracted with acetone for 3 days (IC₅₀ = 3.948 ± 0.19 µg/mL), indicating that solvent polarity played a more dominant role than extraction time in determining radical scavenging capacity. In the ABTS assay, dried leaves extracted with acetone for 3 days showed the strongest antioxidant activity, with an IC₅₀ value of 1.368 ± 0.09 µg/mL. This observation suggests that moderate drying may enhance the accessibility of certain antioxidant compounds by disrupting plant cell structures and improving solvent penetration. However, prolonged maceration beyond 3–5 days did not enhance antioxidant activity and, in several cases, resulted in higher IC₅₀ values, indicating reduced radical scavenging efficiency. These findings suggest that extended extraction times may promote partial degradation, oxidation, or transformation of phenolic and flavonoid compounds, particularly under prolonged exposure to solvent and oxygen. Similar trends have been reported by Alam et al. [13] and Sarma et al. [14], who demonstrated that intermediate extraction times and medium-polarity solvents yielded optimal antioxidant activities in *Piper betle* and other medicinal plants. It should be noted that the IC₅₀ values obtained from DPPH and ABTS assays reflect chemical radical scavenging capacity and do not directly represent biological antioxidant potency. Consistent with the FRAP results, these findings further support that acetone effectively extracts electron-donating phenolic compounds with strong reducing capacity.

Table 6. Antioxidant activities of betel leaf extracts determined by DPPH, ABTS, and FRAP assays

Solvent/ days	Antioxidant Activities by Different Assays					
	DPPH IC ₅₀ (µg/mL)		ABTS IC ₅₀ (µg/mL)		FRAP assay (mM FeSO ₄ equivalent/mg extract)	
	Fresh leaves	Dried leaves	Fresh leaves	Dried leaves	Fresh leaves	Dried leaves
3 days						
95% Ethanol	6.834 ± 1.43 ^{aA}	8.353 ± 1.45 ^{bAB}	2.702 ± 0.25 ^{aA}	2.361 ± 0.23 ^{bA}	5.337 ± 0.25	0.221 ± 0.07
DCM	13.150 ± 0.49 ^{bA**}	4.294 ± 0.19 ^{aA*}	3.926 ± 0.14 ^{bA**}	3.472 ± 0.10 ^{cB*}	4.010 ± 0.04	6.558 ± 2.61
Acetone	6.480 ± 1.33 ^{aA**}	3.948 ± 0.19 ^{aA*}	2.520 ± 0.22 ^{aA**}	1.368 ± 0.09 ^{aA*}	7.451 ± 1.67	11.059 ± 0.58
Hexane	11.500 ± 0.51 ^{bA*}	17.057 ± 0.67 ^{cA**}	2.780 ± 0.48 ^{aA*}	8.832 ± 0.06 ^{cC**}	3.551 ± 0.08 ^{dA*}	0.791 ± 0.01 ^{dA**}
5 days						
95% Ethanol	6.110 ± 0.72 ^{bA}	6.564 ± 0.27 ^{bA}	4.118 ± 0.13 ^{bcB**}	2.307 ± 0.09 ^{aA*}	7.037 ± 0.16	8.910 ± 0.10
DCM	15.066 ± 2.07 ^{cA**}	8.940 ± 0.94 ^{cB*}	3.980 ± 0.48 ^{bA}	3.602 ± 0.66 ^{bB}	6.188 ± 0.24	1.539 ± 0.03
Acetone	3.665 ± 0.26 ^{aA*}	5.146 ± 0.73 ^{aB**}	2.771 ± 0.28 ^{abC**}	1.966 ± 0.14 ^{aB*}	13.048 ± 2.40	13.578 ± 0.38
Hexane	11.940 ± 1.02 ^{dA*}	19.717 ± 0.09 ^{dB**}	4.554 ± 0.07 ^{cB*}	7.553 ± 0.37 ^{dB**}	0.749 ± 0.01 ^{dB}	0.733 ± 0.02 ^{dB}
7 days						
95% Ethanol	6.645 ± 0.09 ^{bA*}	9.457 ± 1.11 ^{bB**}	5.707 ± 0.58 ^{bcC**}	3.731 ± 0.15 ^{cB*}	6.645 ± 0.23	3.888 ± 0.03
DCM	15.743 ± 1.50 ^{dA*}	7.083 ± 1.38 ^{aB*}	11.880 ± 0.43 ^{dB**}	2.039 ± 0.19 ^{aA*}	5.055 ± 0.10	8.344 ± 0.10
Acetone	3.653 ± 0.405 ^{aA*}	5.645 ± 0.69 ^{aB**}	3.224 ± 0.21 ^{aC**}	2.836 ± 0.07 ^{bC*}	11.872 ± 0.62	16.772 ± 0.27
Hexane	12.187 ± 0.42 ^{cA*}	20.317 ± 0.65 ^{cB**}	6.126 ± 0.31 ^{bcC**}	2.956 ± 0.25 ^{bA*}	5.060 ± 0.13 ^{dC**}	0.741 ± 0.06 ^{dB*}
Hydrodistillation						
Essential oil						
VitC	6.232 ± 0.55	5.993 ± 0.12	2.293 ± 0.24	2.623 ± 0.14	11.695 ± 4.91	5.610 ± 3.72

Values are expressed as mean ± SD (n = 3).

Different lowercase letters (a–d) within the same column indicate significant differences among solvents at the same extraction time and condition, determined by one-way ANOVA followed by Duncan's multiple range test (p < 0.05).

Different uppercase letters (A–C) within the same row indicate significant differences among extraction times for the same solvent and condition, determined by Duncan's test (p < 0.05).

* and ** indicate significant differences between fresh and dried leaves within the same solvent and extraction time, determined by an independent-samples t-test (p < 0.05).

In the FRAP assay, antioxidant activity was evaluated based on the ability of extracts to reduce Fe^{3+} -TPTZ to Fe^{2+} -TPTZ, reflecting their electron-donating capacity [16]. Quantification was performed using a ferrous sulfate (FeSO_4) standard calibration curve. The calibration curve exhibited excellent linearity over the tested concentration range ($R^2 = 0.9997$), confirming the analytical reliability of FRAP measurements and supporting the validity of the FRAP values reported in Table 3 (Figure 3). Consistent with the DPPH and ABTS results, acetone extracts demonstrated the highest reducing power among all solvent systems. The strongest FRAP activity was observed in dried leaves extracted with acetone for 7 days (16.772 ± 0.27 mM FeSO_4 equivalent/mg extract) and in fresh leaves extracted with acetone for 5 days (13.048 ± 2.40 mM FeSO_4 equivalent/mg extract). These results indicate that acetone efficiently extracts phenolic compounds with strong reducing capacity, particularly high-molecular-weight polyphenols and flavonoids capable of electron donation. The superior performance of acetone can be attributed to its intermediate polarity, which allows efficient solubilization of both polar and semi-polar antioxidant compounds. This observation is consistent with previous reports by Benzie and Strain [16] and Dai and Mumper [10], who demonstrated that acetone or aqueous acetone systems are particularly effective for extracting polyphenols and proanthocyanidins with high reducing power from various plant materials. When comparing fresh and dried leaves, antioxidant activity exhibited solvent- and assay-dependent variations. In general, fresh leaf extracts showed stronger radical scavenging activity (lower IC_{50} values) in DPPH and ABTS assays, whereas dried leaf extracts occasionally exhibited higher FRAP values. This discrepancy reflects the different chemical principles underlying each assay. While DPPH and ABTS primarily measure free radical scavenging ability, FRAP reflects total reducing capacity, which may be influenced by the concentration of stable phenolic polymers formed or concentrated during drying [16, 21]. Extraction time had a limited positive effect on antioxidant activity. In most cases, extending maceration from 3 to 7 days did not significantly enhance antioxidant performance and, in some cases, reduced activity. This suggests that equilibrium between solute diffusion and solvent saturation was reached at earlier extraction times, and prolonged maceration may promote oxidative degradation of bioactive compounds [13, 15].

Overall, the antioxidant activities determined by DPPH, ABTS, and FRAP assays showed a clear association with the total phenolic content (TPC) and total flavonoid content (TFC) of the extracts. Extracts exhibiting higher TPC and TFC values generally demonstrated stronger antioxidant activities, as evidenced by lower IC_{50} values in the DPPH and ABTS assays and higher reducing power in the FRAP assay. This trend was particularly evident in acetone and DCM extracts, which consistently showed both elevated phenolic/flavonoid contents and enhanced antioxidant performance. These observations support the well-established role of phenolic acids and flavonoids as major contributors to antioxidant activity, owing to their capacity to donate electrons or hydrogen atoms and stabilize free radicals. Similar relationships between phenolic content and antioxidant capacity have been widely reported in plant extracts, indicating that variations in antioxidant activity are closely linked to qualitative and quantitative differences in phenolic composition rather than extraction yield alone [10,16]. Although formal correlation analysis and chromatographic fingerprinting techniques (e.g., TLC or HPLC) were not performed, the consistent solvent- and time-dependent trends observed across TPC, TFC, and antioxidant assays suggest qualitative differences in phenolic composition among extracts and support a positive association between phenolic/flavonoid content and antioxidant activity in *Piper betle* leaf extracts [10,16]. Collectively, these results demonstrate that antioxidant activity in *Piper betle* leaf extracts is governed by a complex interplay between solvent polarity, extraction time, and the chemical nature of the extracted phytochemicals.

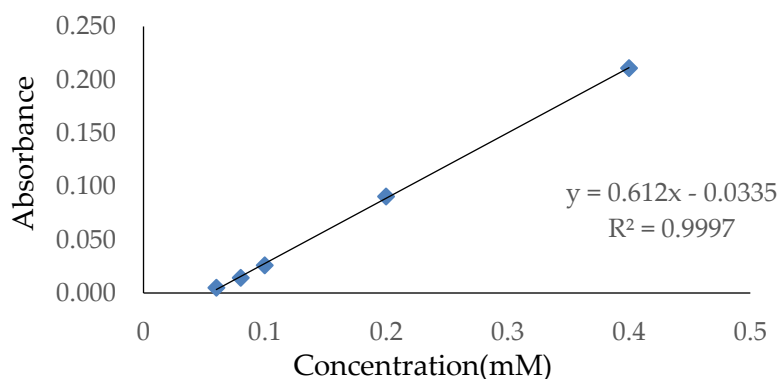


Figure 3. Standard calibration curve of ferrous sulfate

Analysis of variance (ANOVA) was performed to evaluate the effects of four experimental factors—assay type, solvent type, extraction time, and leaf condition—on the antioxidant activity of *Piper betle* leaf extracts. The ANOVA results are summarized in Table 7.

Table 7. Analysis of variance (ANOVA) of factors affecting antioxidant activities of *Piper betle* leaf extracts

Source	DF	AdjSS	AdjMS	F-Value	P-Value
Model	71	4733.41	66.668	152.08	0.000
Linear	8	1432.93	179.117	408.61	0.000
Assays	2	1103.99	551.994	1259.23	0.000
Solvent	3	227.16	75.721	172.74	0.000
Time	2	97.64	48.818	111.37	0.000
Leaf condition	1	4.15	4.145	9.46	0.003

As shown in Table 7, the overall model was statistically significant ($p < 0.001$), indicating that the selected factors collectively influenced antioxidant activity. Among the main effects, assay type (DPPH, ABTS, and FRAP) had the strongest influence on antioxidant values ($F = 1259.23$, $p < 0.001$), reflecting the distinct chemical mechanisms underlying each assay. Solvent type (ethanol, DCM, acetone, and hexane) also had a significant effect ($F = 172.74$, $p < 0.001$), confirming that solvent polarity plays a critical role in extracting antioxidant-active compounds. In addition, extraction time (3, 5, and 7 days) significantly affected antioxidant activity ($F = 111.37$, $p < 0.001$), suggesting time-dependent changes in the recovery or stability of bioactive compounds. Although to a lesser extent, leaf condition (fresh vs. dried leaves) also showed a statistically significant influence ($F = 9.46$, $p = 0.003$), indicating that post-harvest processing affects antioxidant-related properties. Overall, the ANOVA results demonstrate that antioxidant activity in *Piper betle* leaf extracts is significantly governed by assay methodology, solvent selection, extraction duration, and leaf condition, supporting the solvent- and time-dependent trends observed in the experimental data. Following the observed significant differences in antioxidant activities and phytochemical contents among solvents and extraction times as revealed by ANOVA, Pearson's correlation analysis was performed using dried leaf extracts to minimize variability associated with moisture content and to elucidate better the relationships between total phenolic/flavonoid contents and antioxidant activities.

Table 8. Pearson's correlation coefficients between total phenolic/flavonoid contents and antioxidant activities of dried betel leaf extracts.

Parameter	DPPH IC ₅₀	ABTS IC ₅₀	FRAP
TFC	−0.53	−0.61*	0.40
TPC	−0.50	−0.44	0.59*

* $P < 0.05$

Pearson's correlation analysis of dried leaf extracts ($n = 12$) revealed a significant positive correlation between total phenolic content (TPC) and FRAP values ($r = 0.59$, $p < 0.05$), indicating that phenolic compounds contribute to the reducing power of the extracts. In addition, total flavonoid content (TFC) showed a significant negative correlation with ABTS IC_{50} values ($r = -0.61$, $p < 0.05$), highlighting the role of flavonoids in radical scavenging activity.

4. Conclusions

This study systematically evaluated the effects of solvent polarity, extraction duration, and leaf condition on the recovery of phenolic and flavonoid compounds and their associated antioxidant activities in *Piper betle* leaves. Among the solvents tested, acetone consistently exhibited the highest extraction performance, particularly for fresh leaves, yielding the maximum total phenolic content ($1,222.42 \pm 24.27$ mg GAE/g extract) and total flavonoid content (51.70 ± 15.51 mg QE/g extract) at 3 days of maceration, which also corresponded to strong antioxidant activities across DPPH, ABTS, and FRAP assays. In contrast, hexane extracts showed the lowest extraction yields, phenolic and flavonoid contents, and antioxidant activities under all conditions, confirming that non-polar solvents are unsuitable for the recovery of phenolic-rich antioxidant compounds from *P. betle* leaves. Dried leaves generally produced higher extraction yields, especially with ethanol, whereas fresh leaves favored higher flavonoid recovery and radical scavenging capacity, indicating that leaf pretreatment significantly influences extraction outcomes depending on the targeted phytochemical class. Statistical analysis (ANOVA) confirmed that solvent type was the most influential factor affecting extraction yield, TPC, TFC, and antioxidant activity ($p < 0.05$), followed by leaf condition, while extraction time beyond 3 days provided no substantial improvement and, in some cases, resulted in reduced bioactivity, likely due to oxidative degradation of phenolic compounds during prolonged maceration. Correlation analysis further supported these findings by demonstrating that phenolic and flavonoid contents were significantly associated with the antioxidant activities of dried betel leaf extracts, particularly reducing power and radical scavenging capacity. Therefore, a medium-polarity solvent (acetone) combined with short maceration time (3 days) represents the optimal extraction condition for maximizing phenolic- and flavonoid-associated antioxidant properties of *P. betle* leaves under the investigated conditions. The main limitations of this study include the use of maceration as the sole extraction technique, a limited solvent range, and the absence of compound-specific profiling. In addition, moisture content was not quantified during the comparison of fresh and dried leaves. Nevertheless, this study provides statistically supported and condition-specific evidence that medium-polarity solvents are most effective for extracting antioxidant-active phytochemicals from *P. betle* leaves and offers a solid foundation for future optimization using application-safe solvent systems and advanced extraction or analytical techniques.

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The research team sincerely hopes that the findings of this study will be beneficial to betel farmers and can be further developed for industrial applications in the future.

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preparation, Nattawoot Rattanathamwat and Supharat Duanyai.; writing—review and editing, Nattawoot Rattanathamwat and Supharat Duanyai.; project administration, Wachirasak Kienwong and Nattawoot Rattanathamwat.; funding acquisition, Wachirasak Kienwong and Nattawoot Rattanathamwat. In addition, all authors have read and agreed to the published version of the manuscript.

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References

- [1] Pelczar, M. J.; Chan, E. C. S.; Krieg, N. R. *Microbiology: Concepts and Applications*; McGraw-Hill: New York, 1993.
- [2] Akter, K. N.; Karmakar, P.; Das, A.; Anonma, S. N.; Shoma, S. A.; Sattar, N. M. Evaluation of antibacterial and anthelmintic activities with total phenolic contents of *Piper betle* leaves. *Avicenna J. Phytomed.* **2013**, *4*(5), 320–329. <https://doi.org/10.22038/ajp.2014.2483>
- [3] Parkpoom, T.; Chuntree, K.; Komutiban, O. Antimicrobial and antioxidant capacity of leaf extract of *Piper betle* L. for development to cosmetics; Faculty of Science and Technology, Suan Dusit University: Bangkok, 2020.
- [4] Borges, A.; Abreu, A. C.; Ferreira, C.; Saavedra, M. J.; Simões, M. Comparison of techniques and solvents on the antimicrobial and antioxidant potential of *Olea europaea* and *Acacia dealbata* extracts. *Microorganisms* **2020**, *8*(4), 595. <https://doi.org/10.3390/microorganisms8040595>
- [5] El Mannoubi, I.; Ben Ali, Y.; Ben Hmida, S.; M'Hamdi, M.; Ben Salem, R. Impact of different solvents on extraction yield, phenolic and flavonoid contents, and antioxidant activities of *Opuntia stricta* fruit. *Bull. Natl. Res. Cent.* **2023**, *47*, 31. <https://doi.org/10.1186/s42269-023-00991-3>
- [6] Chatepa, L. E. C.; Banda, E. L.; Tembo, Y.; Malunga, E.; Gondwe, T.; Gondwe, E.; Mbewe, A. R. Effects of solvent extraction on the phytoconstituents and antioxidant activities of *Azadirachta indica* and *Vernonia amygdalina*. *Heliyon* **2024**, *10*(10), e30242. <https://doi.org/10.1016/j.heliyon.2024.e30242>
- [7] Banik, A.; Zerín, T.; Sultana, R. Comparative phytochemical and antibacterial properties of *Piper betle* leaf extracts from Barguna and Moheshkhali, Bangladesh. *Iran. J. Med. Microbiol.* **2020**, *14*(2), 125–132. <https://doi.org/10.30699/ijmm.14.2.125>
- [8] Venugopalan, S.; Sharma, A.; Venugopalan, V.; Gautam, H. Comparative study on the antioxidant activities of different solvent extracts from *Piper betle* leaves. *Biomed. Pharmacol. J.* **2015**, *8*(1), 49–56.
- [9] Nouri, L.; Nafici, M.; Mehrbod, P. Phytochemical, antioxidant, antibacterial, and α -amylase inhibitory activities of betel leaf extracts: Effect of ethanol vs methanol. *Ind. Crops Prod.* **2014**, *62*, 901–907. <https://doi.org/10.1016/j.indcrop.2014.09.020>
- [10] Dai, J.; Mumper, R. J. Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Molecules* **2010**, *15*(10), 7313–7352. <https://doi.org/10.3390/molecules15107313>
- [11] Do, Q. D.; Angkawijaya, A. E.; Tran-Nguyen, P. L.; Huynh, L. H.; Soetaredjo, F. E.; Ismadji, S.; Ju, Y. H. Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *J. Food Drug Anal.* **2014**, *22*(3), 296–302. <https://doi.org/10.1016/j.jfda.2013.11.001>
- [12] Damijanić, K.; Đermić, E.; Poljuha, D.; Sladonja, B. Effects of maceration duration on the phenolic composition and antioxidant capacity of 'Teran' (*Vitis vinifera* L.) wine. *Agric. Conspectus Sci.* **2012**, *77*(2), 103–107.
- [13] Alam, M. B.; Park, N. H.; Song, B. R.; Lee, S. H. Antioxidant potential-rich betel leaves (*Piper betle* L.) exert depigmenting action by triggering autophagy and downregulating MITF/tyrosinase in vitro and in vivo. *Antioxidants* **2023**, *12*(2), 374. <https://doi.org/10.3390/antiox12020374>
- [14] Sarma, C.; Rasane, P.; Kaur, S.; Singh, J.; Singh, J.; Gat, Y.; Garba, U.; Kaur, D.; Dhawan, K. Antioxidant and antimicrobial potential of selected varieties of *Piper betle* L. (betel leaf). *An. Acad. Bras. Cienc.* **2018**, *90*(4), 3871–3878. <https://doi.org/10.1590/0001-3765201820170746>

- [15] Laloo, N.; Terblanche, U.; Ssemakalu, C. C.; Pillay, M. Optimizing the extraction of phenolic compounds and antioxidant activity of *Carpobrotus edulis* (L.) L. Bolus using response surface methodology. *Sci. Rep.* **2024**, *14*, 2187. <https://doi.org/10.1038/s41598-024-52187-7>
- [16] Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: The FRAP assay. *Anal. Biochem.* **1996**, *239*(1), 70–76. <https://doi.org/10.1006/abio.1996.0292>
- [17] Naczek, M.; Shahidi, F. Extraction and analysis of phenolics in food. *J. Chromatogr. A* **2004**, *1054*(1–2), 95–111. <https://doi.org/10.1016/j.chroma.2004.08.059>
- [18] Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic–phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*(3), 144–158.
- [19] Chang, C. C.; Yang, M. H.; Wen, H. M.; Chern, J. C. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J. Food Drug Anal.* **2002**, *10*(3), 178–182. <https://doi.org/10.38212/2224-6614.2748>
- [20] Brand-Williams, W.; Cuvelier, M. E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *LWT—Food Sci. Technol.* **1995**, *28*(1), 25–30. [https://doi.org/10.1016/S0023-6438\(95\)80008-5](https://doi.org/10.1016/S0023-6438(95)80008-5).
- [21] Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*(9–10), 1231–1237. [https://doi.org/10.1016/S0891-5849\(98\)00315-3](https://doi.org/10.1016/S0891-5849(98)00315-3)
- [22] Barros, L.; Carvalho, A. M.; Ferreira, I. C. F. Antioxidant activity and total phenolic content of different extracts from *Rosa canina* L. fruits. *Food Chem. Toxicol.* **2011**, *49*(2), 423–426. <https://doi.org/10.1016/j.fct.2010.11.008>
- [23] Singh, A.; Kumar, R.; Kumar, S.; Giri, S. S. Investigation of antioxidant and antimicrobial activities of various extracts of *Piper betle* L. leaves. *Food Chem. Adv.* **2023**, *3*, 100536. <https://doi.org/10.1016/j.focha.2023.100536>
- [24] Everette, J. D.; Bryant, Q. M.; Green, A. M.; Abbey, Y. A.; Wangila, G. W.; Walker, R. B. A thorough study of reactive species, reaction capacities, and physical parameters of 10 common antioxidant assays. *J. Agric. Food Chem.* **2010**, *58* (14), 8139–8144. <https://doi.org/10.1021/jf100825p>
- [25] Sundang, M.; Hussain, M. S.; Kuntadi, S. A.; Pin, C. H. Antioxidant activity, total phenolic and total flavonoid content of *Piper betle* L. leaves from different locations in Malaysia. *Malays. J. Fundam. Appl. Sci.* **2012**, *8*(1), 1–6. <https://doi.org/10.11113/mjfas.v8n1.115>