

Antimutagenic Potential of *Piper sarmentosum* Leaf Extracts: Insights from Culinary Preparation

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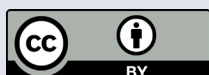
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ABSTRACT

OBJECTIVE *Piper sarmentosum* Roxb., a traditional vegetable in Lanna and Thai cuisine, is valued for its flavor but its health-promoting properties after cooking remain unclear. This study investigated the phytochemical profiles, antioxidant capacity, and mutagenicity of its leaf extracts prepared to mimic typical culinary practices.

METHODS Three *P. sarmentosum* extracts were prepared: an ethanolic extract of uncooked leaves (PEE), a hot water extract simulating curry broth (PHWE), and an ethanolic extract of boiled leaf residues representing the cooked leaves in curry (PREE). Their chemical composition, antioxidant activity, and antimutagenic effects were evaluated.

RESULTS The extracts showed distinct phytochemical patterns. PEE and PREE were rich in phenolics and flavonoids, yielding strong antioxidant activity in ABTS and FRAP assays. PHWE contained higher levels of condensed tannins, reflecting the extraction of water-soluble compounds. None of the extracts showed mutagenicity in the Salmonella assay. Notably, PEE and PREE displayed significant antimutagenic activity against aflatoxin B₁ (AFB₁) in *Salmonella typhimurium* strains TA98 and TA100 with metabolic activation.

CONCLUSIONS *P. sarmentosum* leaves retain considerable antioxidant and antimutagenic potential after cooking, highlighting their value as a safe and functional food ingredient with potential health benefits.

KEYWORDS *P. sarmentosum*, wild betel, Salmonella mutation assay, antioxidant, antimutagenicity

INTRODUCTION

Piper sarmentosum Roxb., commonly known as “wild betel” or “cha-plu” in Thai, is a perennial creeping herb native to Southeast Asia. It is valued both as a traditional medicinal plant and as a culinary ingredient, particularly in Thai and Lanna cuisine (1). The fresh leaves are often consumed raw in traditional snacks such as “Miang

Kham” or incorporated into cooked dishes such as “Kaeng Khae”, a northern Thai curry (Figure 1).

Phytochemical studies have shown that *P. sarmentosum* contains diverse bioactive constituents, including phenolic acids, flavonoids, tannins, xanthophylls, and essential oils (2). These compounds have been associated with a wide range of biological activities, including antimicrobial,

anti-inflammatory, antioxidant, and anticancer properties (3–5). The leaves, in particular, are a rich source of natural antioxidants owing to their high levels of flavonoids and related phenolic compounds (6, 7). Notable flavonoids such as quercetin, naringenin, myricetin, and apigenin (5) are well documented for their anticancer potential through modulation of multiple molecular pathways, including apoptosis, autophagy, NF- κ B, JAK/STAT, and tumor suppressor signaling (8).

Beyond their antioxidant potential, extracts of *P. sarmentosum* leaves have demonstrated therapeutic properties such as anti-inflammatory, antimicrobial, and cardioprotective effects (4, 9–11). For example, methanolic extracts have been shown to protect against stress-induced gastric ulcers via antioxidant and anti-inflammatory mechanisms (12), while aqueous extracts exhibit *in-vivo* anti-nociceptive and anti-inflammatory effects (13) and *in-vitro* cytoprotective activity against oxidative stress (14). Importantly, aqueous extracts have also been reported to be non-mutagenic, supporting the safety of *P. sarmentosum*

for dietary and medicinal use (15).

Despite increasing interest in natural antioxidants and functional foods, limited information is available on the phytochemical composition and biological activities of *P. sarmentosum* following traditional cooking processes. In everyday cuisine, the leaves are often boiled at 100 °C for 15–30 minutes when prepared in soups or curries. To address this knowledge gap, the present study simulated culinary preparation to obtain three types of extracts: PEE (ethanolic extract of uncooked leaves), PHWE (hot water extract simulating curry broth), and PREE (ethanolic extract of boiled leaf residues representing the cooked leaves in curry). These extracts were analyzed for their phytochemical composition, antioxidant activity, mutagenicity, and antimutagenicity, providing new insights into the functional properties of *P. sarmentosum* and its potential applications in food and health product development. A summarized workflow diagram illustrating the experimental process is presented in Figure 2.



Figure 1. Photographs of *P. sarmentosum* leaves (A), the northern Thai snack Miang Kham (B), and the northern Thai curry Kaeng Khae (C).

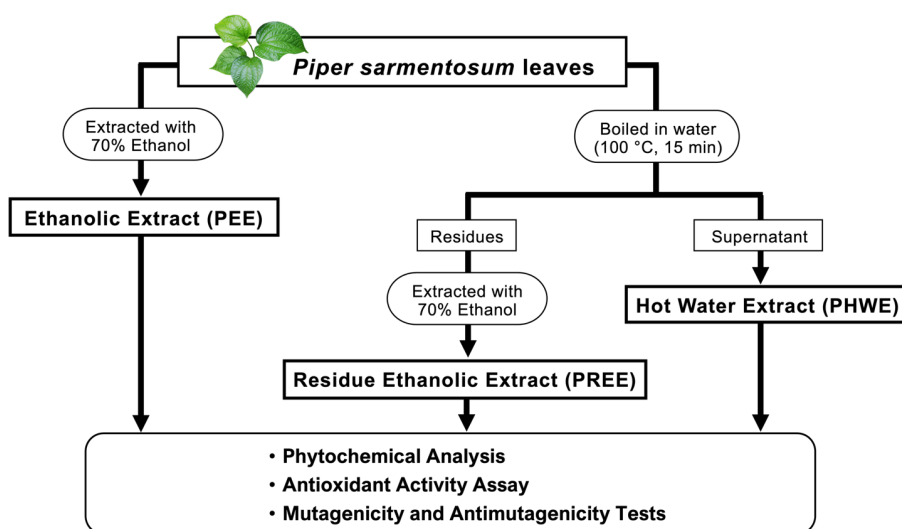


Figure 2. Schematic overview of the experimental workflow in this study

METHODS

Preparation of *P. sarmentosum* Roxb. extracts

Fresh *P. sarmentosum* leaves were collected from Terdwalai Farm, Chiang Mai, Thailand, and deposited at the Faculty of Pharmacy, Chiang Mai University (voucher specimen no. 0023333). The leaves were washed, chopped, and dried at 60°C in a hot-air oven, then ground into powder. This powder was extracted with 70% ethanol (1 g : 10 mL), filtered, concentrated, and freeze-dried to obtain the ethanolic extract (PEE).

To mimic traditional cooking, fresh leaves were boiled in an open-lid stainless-steel vessel with boiling water for 15 min and the temperature was maintained using a thermostatically controlled hot plate. The decoction was filtered and freeze-dried to obtain the hot-water extract (PHWE). The boiled leaf residues were then oven-dried at 60°C, ground into powder, and extracted with 70% ethanol (1 g : 10 mL). The extract was concentrated and freeze-dried to obtain the residue ethanolic extract (PREE). All extracts (PEE, PHWE, and PREE), collectively referred to as *P. sarmentosum* leaf extracts (PSLEs), were stored at -20°C until use.

Total phenolic content

Total phenolic content of PSLEs was determined using a modified Folin-Ciocalteu colorimetric assay (16). Extracts were mixed with Folin-Ciocalteu reagent and incubated at room temperature for 10 min. Sodium carbonate was then added, and the mixture was incubated at 45°C for 15 min. Absorbance was measured at 765 nm, and phenolic content was calculated from a gallic acid calibration curve, expressed as mg gallic acid equivalents per gram of extract (mg GAE/g extract).

Total flavonoid content

Total flavonoid content of PSLEs was determined using a modified aluminum chloride colorimetric assay (16). Extracts were mixed with 5% sodium nitrite and 10% aluminum chloride, incubated at room temperature for 20 min, followed by sodium hydroxide addition and 5 min further incubation. Absorbance was measured at 532 nm, and flavonoid content was calculated from a catechin standard curve, expressed as mg catechin equivalents per gram of extract (mg CE/g extract).

Total chlorophyll and carotenoid determination

Total chlorophyll and carotenoids in PSLEs were determined using a modified acetone extraction method (17). Extracts were homogenized in 80% acetone and incubated at 4°C for 3 h, followed by centrifugation at 9,000 rpm and 4°C for 5 min. Absorbance of the supernatants was measured at 480, 510, 645, and 663 nm. Concentrations were calculated using following equations and expressed as mg per gram of extract (mg/g extract).

$$\text{Total chlorophyll (mg/L)} = 20.2(A_{645}) + 8.02(A_{663})$$

$$\text{Total carotenoids (mg/L)} = 7.6(A_{480}) - 1.49(A_{510})$$

Tannins determination

Hydrolysable tannins were quantified by acid hydrolysis. PSLEs were mixed with sulfuric acid and incubated at 85°C for 20 h. After centrifugation (3,000 × g, 5 min), the supernatant was adjusted to pH 5.5 with ethanolamine and ammonium acetate, incubated at 4°C for 48 h, then reacted with potassium iodate at 30°C for 50 min. Absorbance was measured at 525 nm, and results were expressed as mg methyl gallate equivalents per gram of extract (mg MGE/g extract).

Condensed tannins were determined using a modified acidified vanillin assay (18). PSLEs were mixed with vanillin reagent (vanillin in methanol with concentrated HCl), incubated at 30°C for 30 min, and absorbance was recorded at 500 nm. Values were expressed as mg catechin equivalents per gram of extract (mg CE/g extract).

High-performance liquid chromatography (HPLC) analysis

HPLC profiling of PSLEs was performed on an Agilent system (Agilent Technologies, Santa Clara, CA, USA) equipped with a Zorbax Eclipse Plus C18 column (4.6 × 250 mm, 5 µm) and a photodiode array detector (19). For phenolics, the mobile phase comprised 3% acetic acid in water (A) and methanol (B). A gradient from 90% to 10% A was applied over 50 min, followed by 10 min re-equilibration at 90% A/10% B. The injection volume was 10 µL, flow rate 1 mL/min, and detection at 280 nm. Compounds were identified by retention time and spectral matching, confirmed by co-injection (spiking) with authentic standards, and quantified against calibration curves.

For flavonoids, the mobile phase was 1% acetic acid in water (A) and 1% acetic acid in methanol (B). Elution was run from 90% to 0% A over 40 min, followed by 5 min re-equilibration (90% A/10% B). Detection was at 280 and 329 nm, with peak identification and quantification performed as described above.

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

Antioxidant capacity of PSLEs was determined by the ABTS radical scavenging assay. Extracts were mixed with ABTS radical working solution and incubated at room temperature in the dark for 6 min. Absorbance was measured at 734 nm, with Trolox as the standard. Results are expressed as inhibitory concentration at 50% (IC₅₀) values.

Ferric reducing antioxidant power (FRAP) assay

FRAP was measured using a reagent containing 2,4,6-tripyridyl-s-triazine and 20 mM ferric chloride in sodium acetate buffer (pH 3.6). Extracts were incubated with the reagent at room temperature for 4 min, and absorbance was read at 593 nm. Ferrous sulfate was used to generate a standard curve, and antioxidant activity was expressed as mg of ferrous sulfate equivalents per milliliter (mg FeSO₄/mL), with Trolox as a positive control.

Salmonella mutation assay

The mutagenic potential of PSLEs was evaluated using the *Salmonella typhimurium* strains TA98 and TA100, with and without metabolic activation ($\pm$ S9). Test extracts were mixed with either the S9 mix (+S9, liver fraction and cofactors) or sodium phosphate buffer (–S9), followed by addition of the bacterial culture. After 20 min incubation at 37°C with shaking, the mixture was combined with top agar containing L-histidine and D-biotin and overlaid on minimal glucose agar plates. Plates were incubated at 37°C for 48 h, and revertant colonies were counted. DMSO served as the negative control for PEE and PREE, while distilled water was used for PHWE. Positive controls were 2-aminoanthracene (2-AA) for +S9 and 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) for –S9. The mutagenic index (MI) was calculated. Extracts with MI higher than 2 were considered potentially mutagenic.

Antimutagenicity was assessed against aflatoxin B₁ (AFB₁; 25 ng/plate) in the presence of S9. For –S9 conditions, AF-2 (50 ng/plate) was used with TA98 and sodium azide (NaN₃; 1000 ng/plate) with TA100. PSLEs were mixed with the mutagens, followed by the addition of bacteria and either S9 mix (+S9) or buffer (–S9). After 20 min incubation at 37°C, the mixtures were plated as above. Revertant colonies were counted after 48 h, and antimutagenic activity was expressed as the percentage inhibition relative to the mutagen control. The degree of antimutagenicity was classified according to the criteria shown in Table 1.

Statistical analysis

The data are reported as mean $\pm$ SD, except for the results obtained from mutagenicity in the *Salmonella* mutation assay, which was expressed as mean $\pm$ SEM values and performed in three independent experiments. The statistical significance of differences between groups was analyzed by one-way analysis of variance (ANOVA) and Tukey's HSD in GraphPad Prism software version 9.5.1, and $p < 0.05$ was considered statistically significant.

RESULTS

Appearance, yield, and phytochemical composition of *P. sarmentosum* leaf extracts

The characteristics of the PEE, PHWE, and PREE are summarized in Table 2. PEE and PREE were obtained as dark brown powders, whereas PHWE was black, reflecting differences in solvent polarity and compound solubility. Among the extracts, PEE and PHWE showed higher yields compared to PREE, consistent with the sequential extraction process in which water removed a portion of soluble phytochemicals before subsequent ethanol extraction of the residue.

Phytochemical profiling revealed that PEE contained the highest total phenolic content

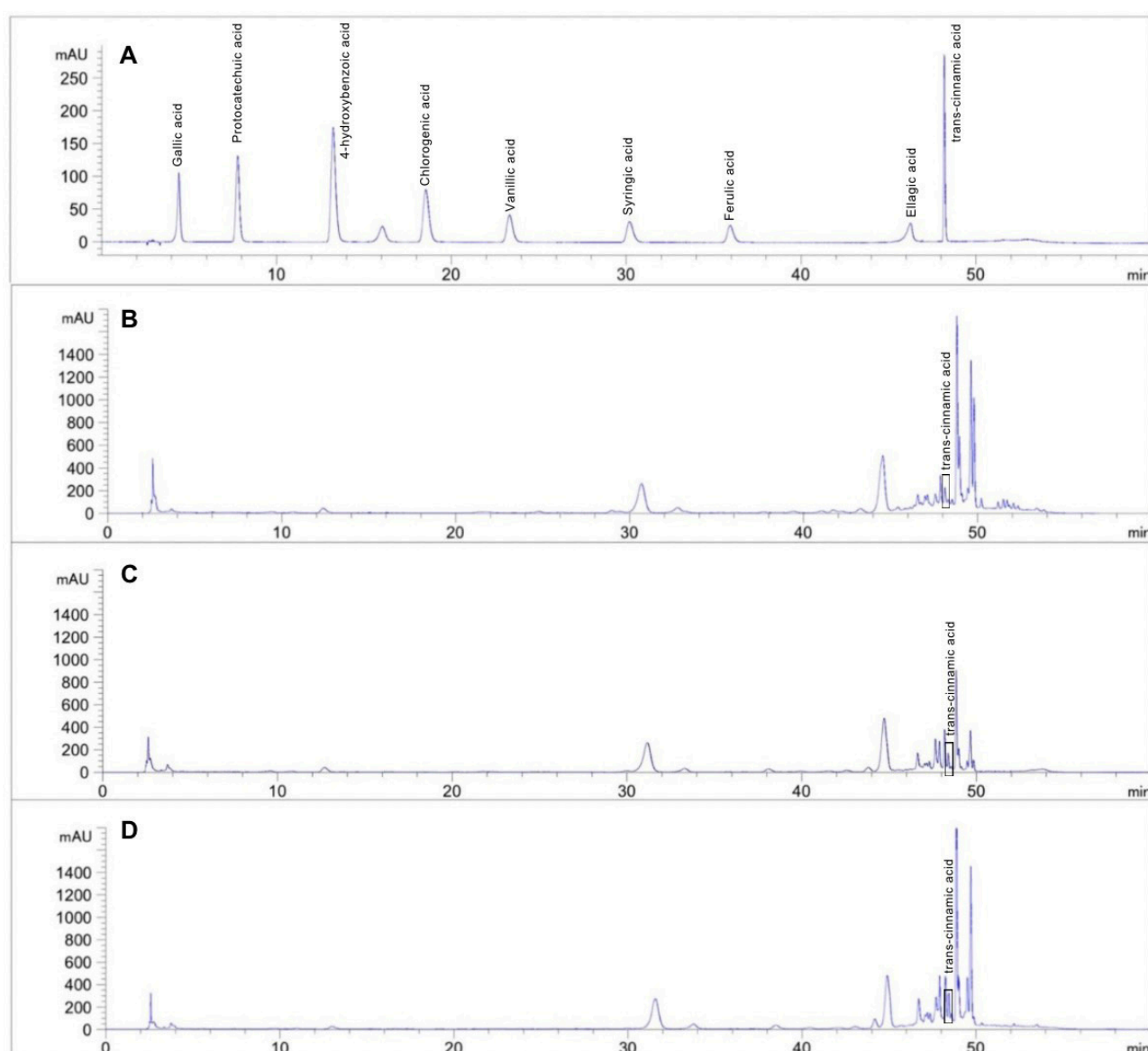
Table 1. Classification of antimutagenicity efficacy

% inhibition	Antimutagenicity
More than 90%	Very strong antimutagenicity
71-90%	Strong antimutagenicity
51-70%	Moderate antimutagenicity
31-50%	Mild antimutagenicity
30-0%	No antimutagenicity
Less than -30%	Co-mutagenicity

Table 2. Appearance, yield and phytochemical composition of PSLEs

Parameter	PEE	PHWE	PREE
Color of extract powder	Dark brown	Black	Dark brown
Yield (%w/w of fresh leaves)	3.77±1.00	3.26±0.70	1.76±0.20
Total phenolic content (mg GAE/g extract)	51.51±15.67	41.14±7.55	48.12±6.24
Total flavonoid content (mg CE/g extract)	14.25±2.20	4.10±0.44	12.35±2.11
Chlorophylls (mg/g extract)	7.86±1.69	0.25±0.03	11.27±0.73
Carotenoids (mg/g extract)	2.44±0.63	0.17±0.02	3.58±0.26
Hydrolysable tannins (mg MGE/g extract)	1.50±0.52	0.48±0.10	1.64±0.17
Condensed tannins (mg CE/g extract)	23.16±1.93	133.12±8.24	41.52±2.09
Trans-cinnamic acid (mg/g extract)	0.32±0.00	0.62±0.00	0.72±0.00
Apigenin (mg/g extract)	3.59±0.14	0.45±0.02	0.22±0.01

GAE, gallic acid equivalent; CE, catechin equivalent; MGE, methyl gallate equivalent

**Figure 3.** HPLC chromatograms of reference standard phenolic acids (A), PEE (B), PHWE (C), and PREE (D). The trans-cinnamic acid peak is observed at a retention time of 48.3 min.

(TPC) and total flavonoid content (TFC), followed by PREE, while PHWE exhibited the lowest values. PREE was particularly rich in chlorophylls and carotenoids, whereas PHWE showed the greatest amount of condensed tannins, consistent with the water solubility of these compounds.

HPLC analysis of phenolic acids (Figure 3) revealed compositional differences among the extracts. Common peaks were observed at retention times of approximately 31 and 45 min across all samples, suggesting the presence of shared hydrophilic constituents. However, peaks at approximately 49–50 min were more prominent in PEE and PREE, indicating that these extracts contained higher levels of hydrophobic compounds. Similarly, HPLC analysis of flavonoids (Figure 4) showed compositional differences among the

extracts, with common peaks observed at retention times of approximately 24, 30, and 38 min across all samples. Peaks at approximately 38–39 min were more prominent in PEE and PREE. Further quantitative analysis revealed that PREE contained the highest trans-cinnamic acid content (0.72 ± 0.00 mg/g extract), whereas PEE was the richest source of apigenin (3.59 ± 0.14 mg/g extract), far exceeding levels in PHWE and PREE.

Antioxidant activities of *P. sarmentosum* leaf extracts

The antioxidant capacities of PSLEs were assessed using ABTS radical scavenging and FRAP assays, with Trolox as a reference standard (Table 3). Among the extracts, PEE exhibited the strongest ABTS scavenging activity ($IC_{50} = 31.9 \pm 4.4$ mg/mL),

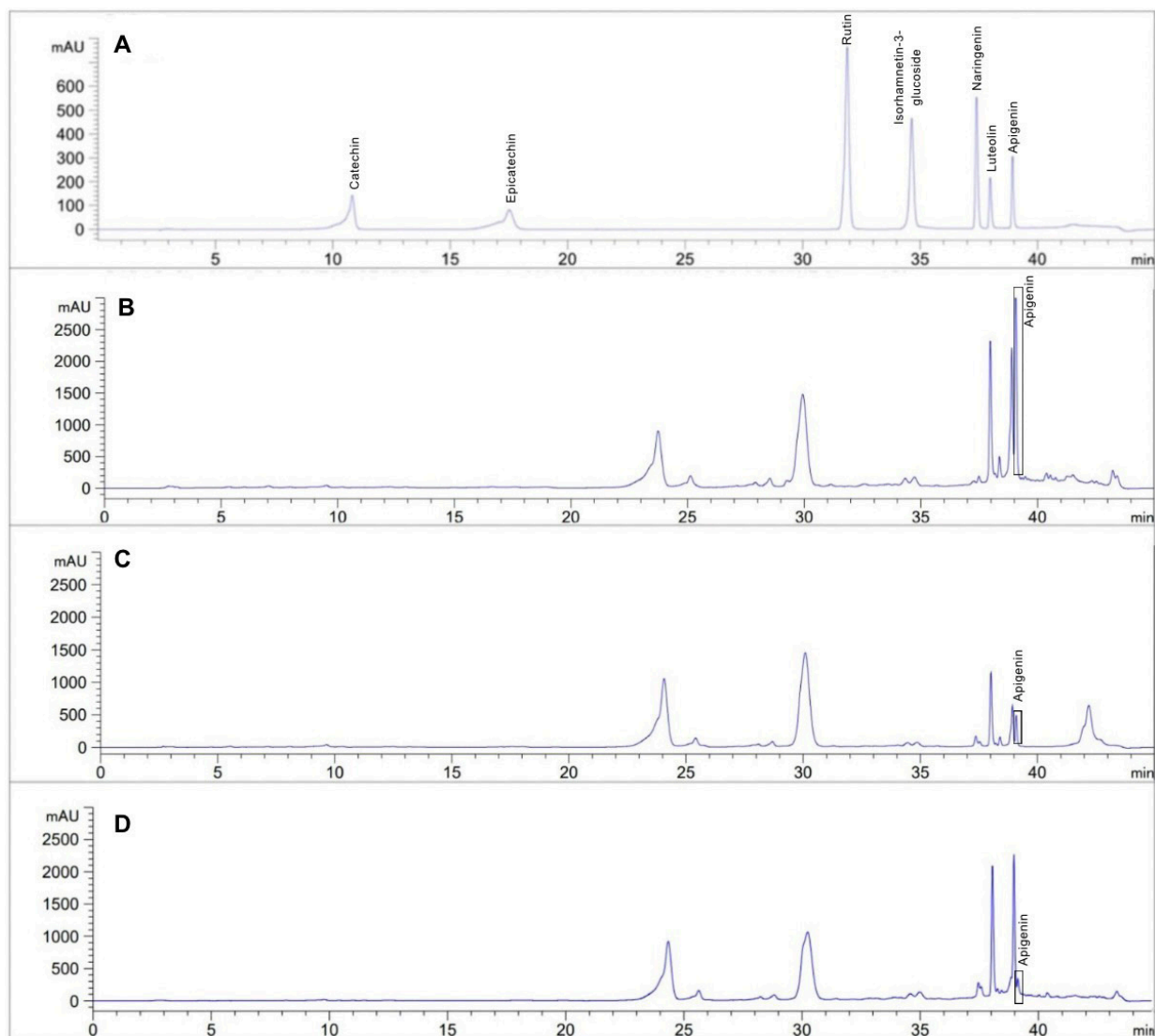


Figure 4. HPLC chromatograms of reference standard flavonoids (A), PEE (B), PHWE (C), and PREE (D). The apigenin peak is observed at a retention time of 38.9 min.

whereas PHWE showed the weakest effect. This trend was consistent with the phytochemical profiles, as extracts with higher phenolic and flavonoid contents (PEE and PREE) displayed stronger antioxidant activities.

In agreement, FRAP analysis revealed that PEE possessed the highest reducing power (0.12 ± 0.03 mg FeSO₄/mL), followed by PREE and PHWE. These findings suggest that phenolic and flavonoid constituents contribute significantly to the antioxidant potential of PSLEs.

Mutagenicity and antimutagenicity of *P. sarmentosum* leaf extracts

The mutagenic potential of PSLEs was assessed using the Salmonella mutation assay (Figure 5). None of the extracts (PEE, PHWE, PREE) produced a significant increase in revertant colonies in strains TA98 or TA100, either in the presence (+S9) or absence (-S9) of metabolic activation. All mutagenic indices remained below 2.0, indicating no detectable mutagenic activity. The larger standard deviation observed in the mutagenicity results for TA98 (Figures 5A and 5B) but not for

Table 3. Antioxidant capacities of PSLEs

Extract	ABTS	FRAP*
	IC ₅₀ (mg/mL)	(mg FeSO ₄ /mL)
Trolox	0.35±0.01	0.14±0.03
PEE	31.90±4.36	0.12±0.03
PHWE	44.67±4.67	0.08±0.02
PREE	34.70±10.30	0.10±0.03

*FRAP values represent activity equivalent to 0.05 mg/mL Trolox and 1 mg/mL PSLEs.

TA100 (Figures 5C and 5D) can be attributed to the lower baseline revertant counts typical of TA98 compared to TA100, resulting in greater relative variability.

Antimutagenicity testing against AFB₁-induced mutagenesis in the presence of metabolic activation (+S9) showed that both PEE and PREE significantly suppressed revertant colony formation in a clear dose-dependent manner, with inhibition consistently above 30% at all tested concentrations (Figure 6). At concentrations of 1,000–5,000 µg/plate, both extracts exhibited marked antimutagenic activity exceeding 70%, indicating strong protective potential. At the

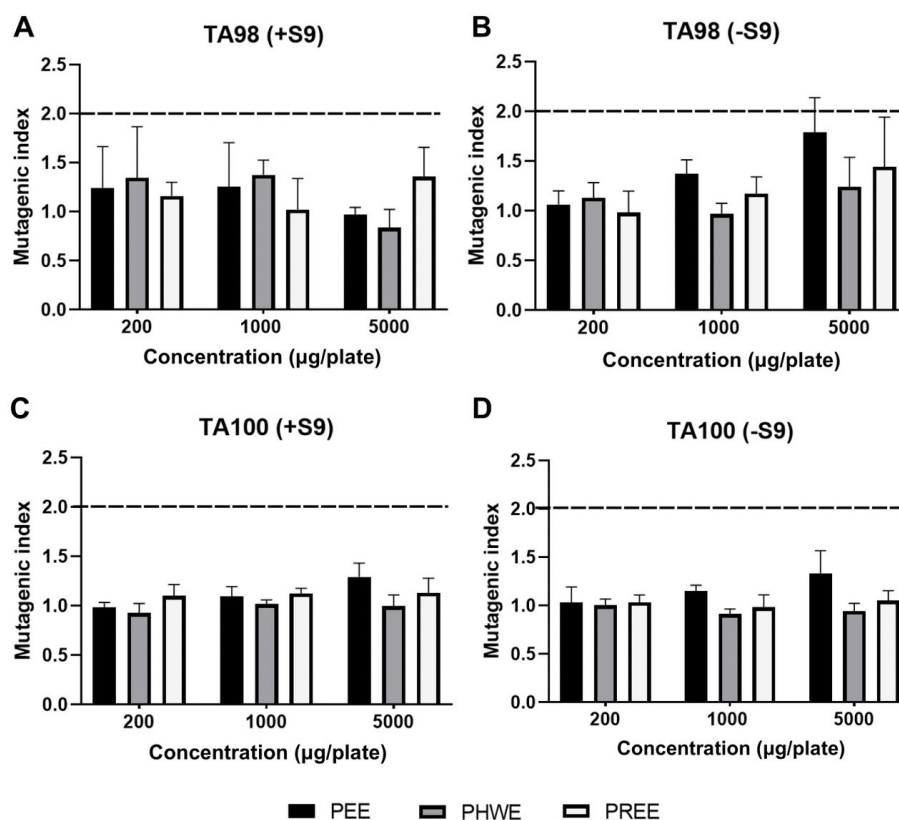


Figure 5. Mutagenicity of PSLEs in *S. typhimurium* strains TA98 with (A) and without S9 (B) and TA100 with (C) and without S9 (D). Dashed lines at 2 indicate thresholds for mutagenicity.

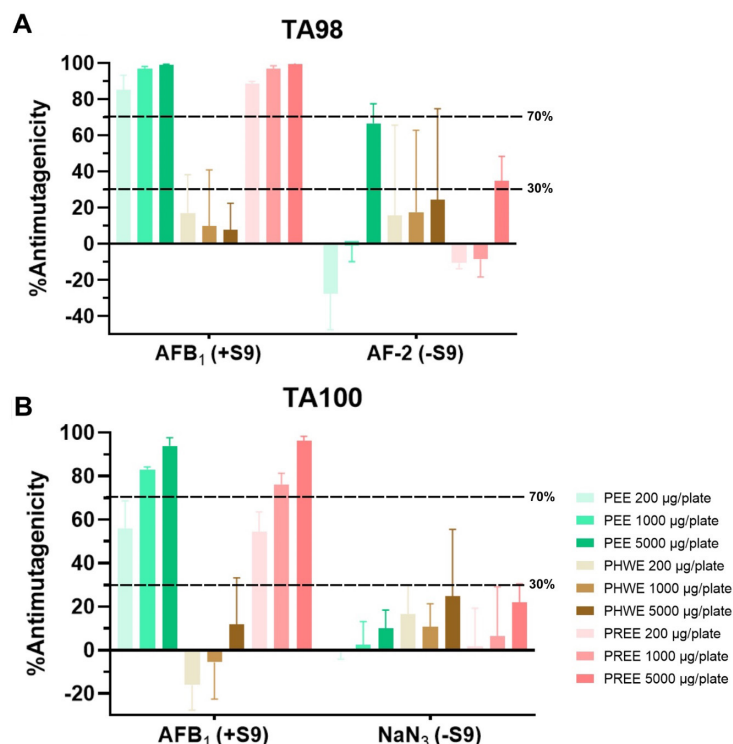


Figure 6. Antimutagenicity of PSLEs in *S. typhimurium* against mutagens: AFB₁ with S9 and AF-2 without S9 in TA98 strain (A) and AFB₁ with S9 and NaN₃ without S9 in TA100 strain (B) dashed lines at 30% and 70% indicate thresholds for anti-mutagenicity and strong anti-mutagenicity, respectively.

highest concentration (5,000 µg/plate), PEE and PREE achieved inhibition greater than 90%, demonstrating very strong protective effects in both TA98 and TA100 strains. In contrast, PHWE exhibited no detectable antimutagenic activity under the same experimental conditions.

In the absence of metabolic activation (–S9), none of the extracts achieved more than 30% inhibition of NaN₃-induced (TA100) or AF-2-induced (TA98) mutagenesis at 200–1,000 µg/plate. However, at 5,000 µg/plate, both PEE and PREE suppressed AF-2-induced mutagenesis in TA98 by more than 30%. Under these conditions, PEE achieved an antimutagenicity of 66%, while PREE reached 34%, indicating moderate and mild protective effects, respectively. The larger standard deviation observed for PHWE antimutagenicity in Figures 6A and 6B may reflect the extract's matrix properties, including potential for partial precipitation, which can affect plating efficiency and interactions with the S9 metabolic activation system, thereby increasing plate-to-plate variability in addition to the inherent biological variability of the Salmonella assay.

DISCUSSION

Piper sarmentosum Roxb. (Cha-plu) is an aromatic herb commonly consumed in Lanna and Thai cuisine. This study investigated the phytochemical composition, antioxidant, and anti-mutagenic activities of *P. sarmentosum* leaf extracts prepared to reflect traditional culinary forms: PEE (ethanolic extract of uncooked leaves), PHWE (hot water extract simulating curry broth), and PREE (ethanolic extract of boiled leaf residues representing the cooked leaves in curry).

Ethanol, with both polar and non-polar properties, enabled extraction of a broad spectrum of bioactive compounds, while hot water primarily extracted hydrophilic molecules. Consistently, PEE and PREE showed higher TPC and TFC, whereas PHWE was enriched in condensed tannins. HPLC profiling identified trans-cinnamic acid and apigenin as major phenolic and flavonoid constituents, respectively, both recognized for antioxidant, anti-inflammatory, antimutagenic, and anticancer activities (20–22). These findings are consistent with previous reports showing that ethanolic extracts of *P. sarmentosum* are rich in phenolic acids and flavonoids, particularly cinnamic acid derivatives and apigenin (23).

PREE was further distinguished by high chlorophyll and carotenoid contents, pigments with well-documented antioxidant and disease-preventive properties (24, 25). These hydrophobic compounds are efficiently extracted in ethanol, explaining their prevalence in PEE and PREE.

The strong antioxidant activities observed can be attributed to the abundance of phenolics and flavonoids, which act via hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms. The ABTS assay reflects both HAT and SET, while FRAP captures SET alone (26). The higher phenolic and flavonoid contents along with hydrophobic pigments (chlorophylls/carotenoids) in PEE and PREE corresponded with stronger ABTS and FRAP activities, whereas PHWE, which contained higher condensed tannins and lower flavonoid levels explained its weaker radical scavenging and reducing power. PEE and PREE consistently exhibited superior activity, likely due to their higher levels of trans-cinnamic acid, apigenin, chlorophylls, and carotenoids. These results align with previous studies reporting potent antioxidant effects of *P. sarmentosum*, driven by its diverse phytochemical profile (6).

The mutagenicity assessment using the Salmonella mutation assay (TA98 and TA100) demonstrated that none of the extracts induced an increase in revertant colonies, with or without S9 activation. As these strains represent frameshift (TA98) and base-pair substitution (TA100) mutations in the histidine operon (27), the results confirm the absence of mutagenic activity, supporting the safety of *P. sarmentosum* for consumption.

Notably, under metabolic activation, both PEE and PREE strongly inhibited AFB₁-induced mutagenesis, achieving more than 90% inhibition at 5,000 µg/plate, indicating very strong antimutagenic potential. In contrast, their activity against direct-acting mutagens (AF-2 and NaN₃) without S9 was limited, with only mild to moderate inhibition at the highest dose. This selectivity suggests that the protective effects are preferentially exerted through modulation of metabolic activation pathways rather than direct interaction with mutagens. The protective effects are likely mediated by trans-cinnamic acid, apigenin, and carotenoids, all of which have been reported to suppress mutagen-induced mutagenesis (28–31). These compounds may interfere with mu-

tagen bioactivation pathways or enhance DNA protection mechanisms. Phenolic acids and flavonoids are also known to counteract environmental mutagens such as AFB₁ (32). The superior antimutagenicity of PEE and PREE compared to PHWE can be explained by not only higher total phenolic content but also the presence of specific flavonoids (e.g., apigenin) and hydrophobic antioxidants; water extraction for PHWE preferentially enriches highly polymerized tannins that may exhibit limited antimutagenic efficacy in the Salmonella system. Although regression analysis of individual constituent compounds was not conducted in the present study, comparative evidence from the literature suggests that these phytochemicals are likely responsible for the strong antimutagenic activity observed in both PEE and PREE. However, definitive confirmation of their causal roles will require bioassay-guided fractionation and detailed mechanistic investigations.

The observed differences in antimutagenic potency among the three extracts, despite their similar phytochemical profiles, can be attributed to variations in constituent concentrations and their synergistic interactions. PEE and PREE consistently demonstrated stronger antioxidant and antimutagenic effects, suggesting that not only the presence but also the ratios and synergistic interactions of bioactive compounds determine functional efficacy. This agrees with earlier findings that combinations of phenolics and flavonoids can exert enhanced bioactivity (33). From a culinary perspective, the boiled leaf residue (PREE) retained substantial phytochemicals and bioactivities, suggesting that brief cooking, as in soups or curries, preserves functional compounds at levels comparable to the uncooked leaves.

CONCLUSIONS

In summary, *P. sarmentosum* leaf extracts prepared to represent common culinary forms exhibited distinct phytochemical characteristics and bioactivities. The PEE and PREE extracts contained higher levels of total phenolics, flavonoids, and hydrophobic pigments (chlorophylls and carotenoids), corresponding with stronger antioxidant capacities in both ABTS and FRAP assays. In contrast, PHWE was enriched in condensed tannins but showed comparatively lower antioxi-

dant activity. None of the extracts demonstrated mutagenicity in the Salmonella assay. Notably, PEE and PREE markedly inhibited AFB₁-induced mutagenesis under metabolic activation, likely due to the combined influence of elevated phenolic and flavonoid contents and the presence of specific compounds such as trans-cinnamic acid, apigenin, and other hydrophobic antioxidants. These findings indicate that *P. sarmentosum* retains substantial functional potential following brief culinary processing, and that specific phenolic and flavonoid constituents may play key roles in its antimutagenic effects.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

AUTHOR CONTRIBUTIONS

N.S.: investigation, data curation, visualization, writing – original draft preparation; B.L.: investigation, data curation; S.T.: data curation, validation, supervision; T.C.: IBC approval, supervision; R.W.: conceptualization, methodology, supervision; O.K.: conceptualization, methodology, data curation, validation, supervision, writing – review & editing.

DATA AVAILABILITY STATEMENT

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

INSTITUTIONAL REVIEW BOARD STATEMENT

Not applicable.

INFORMED CONSENT STATEMENT

Not applicable.

DECLARATION OF GENERATIVE AI IN SCIENTIFIC WRITING

The authors used ChatGPT to assist with improving the readability and language of this manuscript. All content was subsequently reviewed, revised, and approved by the authors, who take full responsibility for the final version.

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