RESEARCH ARTICLE

Editorial Process: Submission:05/14/2025 Acceptance:11/10/2025 Published:11/22/2025

Eco-friendly Extraction of Proanthocyanidin-Rich Compounds from Tamarind Seed Husk and Their Anti-Liver Cancer Activity

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Abstract

Objective: This study aimed to optimize the green extraction of phenolic compounds from TSH and evaluate their antioxidant and anti-liver cancer activities. **Methods:** TSH was extracted using 60% ethanol and distilled water in different temperatures using a 2-level factorial design as the variables, followed by tray drying. Extracts were analyzed for total phenolic content (TPC) using Folin–Ciocalteu reagent as the response and DPPH radical scavenging assay. Oligomeric proanthocyanidins were characterized by high performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC-ESI-QTOF-MS/MS). Cytotoxicity and antimigratory effects on HepG2 cells were assessed using sulforhodamine B (SRB) and wound healing assays. **Result:** The optimal model equation was $TPC = 0.9 - 0.0064A + 0.0059B + (2.61 \times 10^{-5})AB$. Although the ethanolic extract had higher total phenolic content, but the water extract showed stronger antioxidant activity and exhibited significantly stronger cytotoxic activity (IC50 at 72 hours: 44.1±0.9 µg/mL) than ethanolic extract (408.3±9.8 µg/mL) in a time- and dose-dependent manner. Migration of HepG2 cells was also significantly inhibited by the water extract in a dose-dependent manner. **Conclusion:** The water extract of TSH may serve as a promising candidate for functional ingredient development and natural anti-liver cancer applications.

Keywords: Tamarind seed husk- proanthocyanidins- HepG2- green extraction- phytochemicals

Asian Pac J Cancer Prev, **26 (11)**, 4119-4126

Introduction

Liver cancer is among the leading causes of cancer-related mortality worldwide, with hepatocellular carcinoma (HCC) being the predominant form. The increasing incidence and poor prognosis of HCC highlight the urgent need for novel therapeutic agents that are both effective and exhibit minimal side effects. Naturally derived compounds, particularly those rich in polyphenols, have shown promising anticancer potential due to their antioxidative, anti-inflammatory, and antiproliferative properties.

Tamarind seed husk (TSH), a byproduct of tamarind processing, has recently gained attention as a sustainable source of bioactive polyphenols, especially oligomeric proanthocyanidins [1]. These compounds, known for their strong radical-scavenging activity [2], have also demonstrated cytotoxicity against various cancer cell lines [3-5]. Despite its potential, efficient extraction of these bioactives from TSH using eco-friendly and scalable methods remains underexplored.

Previous studies have shown that the composition and yield of phenolic compounds can be significantly influenced by factors such as solvent polarity, extraction temperature, and drying techniques [6, 7]. While ethanol has traditionally been favored for polyphenol extraction, water offers a safer and more sustainable alternative. Moreover, conventional drying processes such as tray drying or vacuum drying may help retain phenolic stability better than high-temperature methods.

To date, limited research has systematically optimized the extraction of proanthocyanidins from TSH using green solvents under controlled conditions while correlating the chemical composition with anticancer bioactivity. Furthermore, the mechanistic role of TSH-derived extracts, particularly in modulating the viability and migration of human liver cancer cells, has not been clearly elucidated.

The aim of this study was to optimize the extraction of phenolic-rich compounds from tamarind seed husk using a two-level factorial design varying solvent and temperature conditions; and to evaluate the biological efficacy of the extracts, focusing on antioxidant capacity, cytotoxicity, and antimigratory effects against HepG2 liver cancer cells. The findings are anticipated to contribute to the development of sustainable extraction technologies and support the therapeutic potential of TSH-derived polyphenols in liver cancer management.

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Materials and Methods

Materials

Tamarind (*Tamarindus indica L.*) seeds were locally sourced from Phayao Province, Thailand. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), gallic acid, methanol, acetonitrile, and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents were of analytical grade and used without further purification. Deionized water was prepared in-house using a two-stage reverse osmosis system.

Methods

The optimal conditions of tamarind seed husk extraction

The extraction protocol was adapted from the method described by Sinchaiyakit et al. [1], with modifications in solvent and temperature conditions to enhance the total phenolic content (TPC). Tamarind seeds were cleaned and oven-dried at 80°C for 1 hour. The seed coats were mechanically separated, ground into a fine powder, and stored at 4°C.

For extraction, powdered husk (TSH) was subjected to either water or 60% ethanol as solvent, and the process was conducted at either room temperature or 60 °C for 1 hour, followed by 24 hours of maceration. Filtration was performed to remove insoluble residues. The aqueous (wTSH) and ethanolic (eTSH) crude extracts were concentrated using rotary evaporation, followed by tray drying. The final dried extracts were stored in amber glass vials at 4°C.

A 2-level factorial design was used to optimize the extraction conditions, where factor A represented the solvent type (water or 60% ethanol) and factor B represented extraction temperature (room temperature or 60°C). Each combination was performed in triplicate. The response variable was TPC (mg gallic acid equivalents [GAE]/g dry weight), modeled by the linear regression equation:

$$\text{TPC (y)} = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 AB$$

where β_0 represents the intercept, β_1 and β_2 correspond to the main effects of solvent and temperature, respectively, and β_3 represents the interaction effect (AB).

Determination of total phenolic compounds by Folin-Ciocalteu method

The TPC was determined using the Folin–Ciocalteu method, as described by Slinkard and Singleton [8]. A 2 mL aliquot of extract was mixed with 1 mL Folin–Ciocalteu reagent and 0.8 mL of 7.5% sodium carbonate. The mixture was incubated for 30 minutes at room temperature, and absorbance was measured at 765 nm. Gallic acid was used to generate a calibration curve (100–500 µg/mL), and TPC was expressed as mg GAE/g dry extract.

Antioxidant capacity analysis

Antioxidant activity was evaluated by DPPH radical scavenging assay, adapted from Bhatnagar et al. [9]. Extracts were prepared at various concentrations (2.5-100 µg/mL) and mixed with 3 mL of 60 µM DPPH ethanolic solution. After incubation at room temperature for 30 minutes, absorbance was recorded at 517 nm. The percentage inhibition was calculated as:

%AOC = [(Ablank− Asample)/ Ablank]× 100

IC_{50} values were calculated and compared to gallic acid as the reference standard (0.625-10 µg/mL).

Bioactive compound identification by High performance liquid chromatography (HPLC) analysis

Following the procedure adapted from Weber et al. [10], wTSE, eTSH, and GSE were each prepared at a final concentration of 1 mg/mL by dissolving in a 1:1 (v/v) mixture of methanol and water. The samples were analyzed using reverse-phase HPLC on a Shimadzu SCL-10AVP system equipped with an LC-20AD pump and SPD-20A UV detector set at 278 nm. Separation was performed on an INERTSIL® column (250 mm × 4.6 mm i.d., 5 µm particle size; GL Sciences Inc., Japan). The mobile phase comprised 0.3% trifluoroacetic acid in water (solvent A) and acetonitrile (solvent B), with a programmed linear gradient as follows: 10-15% B over 45 minutes, 15-60% B over the next 15 minutes, held at 60% B for 20 minutes, reduced to 10% B over 1 minute, and equilibrated at 10% B for 20 minutes. The injection volume was 20 µL, the flow rate was maintained at 0.7 mL/min, and the column temperature was set at ambient conditions. Each chromatographic run was completed within 80 minutes. All analyses were performed in triplicate.

Bioactive compound identification by LC-ESI-QTOF-MS/MS analysis

A 17 mg portion of the crude extract was dissolved in 500 µL of 50% methanol and filtered through a 0.45 µm nylon syringe filter. Chromatographic separation was performed on a Luna C18 column (4.6×150mm, 5µm; Phenomenex, USA) using a binary gradient mobile phase. The system operated under ESI-QTOF negative ionization mode with an injection volume of 5.0 µL and a constant flow rate of 0.5mL/min. The mobile phases consisted of solvent A (5% water + 0.1% formic acid) and solvent B (95% acetonitrile or methanol + 0.1% formic acid). No gradient shift occurred between 30–40 minutes runtime. Method parameters were optimized to detect phenolic compounds with high resolution.

Cytotoxicity Assessment Using SRB Assay

HepG2 human liver cancer cells (ATCC, USA) were cultured according to standard protocols. Cells (1×10^4 cells/well) were seeded into 96-well plates and incubated overnight. Media were then replaced with fresh media containing wTSH or eTSH extracts at various concentrations and incubated for 24, 48, or 72 hours. After treatment, cells were fixed with 10% TCA at 4°C, stained with 0.4% SRB for 30 minutes, and solubilized in 10 mM Tris base. Absorbance was read at 540 nm. Cytotoxicity was calculated relative to untreated controls.

Cell Migration Analysis Using Wound Healing Assay

To assess cell migration, HepG2 cells (2.5×10^5

cells/well) were seeded in 24-well plates and cultured overnight. A scratch wound was created using a sterile pipette tip, followed by washing and treatment with 250 and 500 µg/mL of wTSH or eTSH extracts. Wound closure was imaged at 0 and 72 hours using an inverted microscope (10x). Migration was quantified by comparing residual wound area to the baseline and control.

Results

The optimal conditions of tamarind seed husk extraction

An eco-friendly extraction method for tamarind seed husk (TSH) was successfully optimized by modifying Sinchaiyakit's protocol [1]. A 2-level factorial design revealed significant interactions between solvent type and extraction temperature, with a high model fit ($R^2 = 0.9997$) from the linear regression equation:

$$TPC = 0.9 - 0.0064A + 0.0059B + (2.61 \times 10^{-5})AB$$

The highest TPC was achieved using 60% ethanol at 60 °C, though hot water also produced considerable yields as shown in Table 1. This suggests that both solvent polarity and thermal conditions critically influence polyphenol release. The tray-drying method preserved thermolabile compounds, making the extraction suitable for industrial applications.

Determination of total phenolic compounds by Folin-Ciocalteu method

Using the Folin–Ciocalteu assay, the 60% ethanol extract at 60°C showed the highest TPC (1.061 ± 0.005 mg GAE/g), while water at room temperature yielded the lowest (0.515 ± 0.002 mg GAE/g). Elevated temperature significantly improved yields for both solvents as shown in Table 1., likely due to enhanced cell wall permeability and phenolic solubility. These results emphasize the value of aqueous ethanol and thermal input in maximizing polyphenol recovery.

Antioxidant capacity analysis

DPPH assay results revealed the strongest antioxidant activity in hot water extracts (IC_{50} = 18.8 ± 0.2 µg/mL), closely followed by ethanolic extracts at 60°C (IC_{50} = 19.6 ± 0.2 µg/mL) as shown in Table 2. Lower activity was observed at room temperature. These findings suggest temperature-enhanced diffusion and solubility of low-molecular-weight phenolics in water, such as catechins and protocatechuic acid, which contribute to radical scavenging activity.

Bioactive compound identification by HPLC Analysis

The HPLC method employed effectively detected oligomeric proanthocyanidins in GSE, characterized by a broad peak appearing around a 60-minute retention time. Comparable chromatographic patterns were observed in both wTSH and eTSH extracts, which similarly displayed peak broadening near the 60-minute mark along with a few minor peaks, indicating the presence of additional low-abundance constituents as shown in Figure 1. Notably, the major peak areas in the wTSH and eTSH chromatograms were more prominent compared to GSE,

Table 1. The Total Phenolic Content (TPC) of Tamarind Seed Husk (TSH) Extracted under Different Conditions Using 2-Factorial Design

Green solvents	Temperature (°C)	Yield (%)	Gallic Acid Equivalent (GAE)
100% Deionized water	30 ± 2	21 ± 3	0.515 ± 0.002
100% Deionized water	60 ± 2	26 ± 2	0.771 ± 0.003
40% Deionized water in Ethanol	30 ± 2	35 ± 3	0.852 ± 0.004
40% Deionized water in Ethanol	60 ± 2	43 ± 2	1.061 ± 0.005

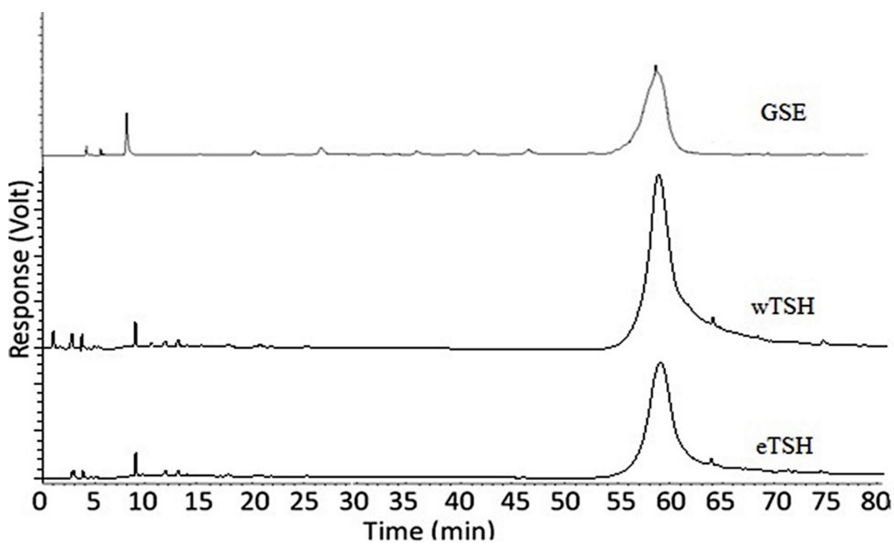

Figure 1. HPLC Chromatograms of GSE, wTSH, and eTSH

Table 2. The Impact of Solvent Type and Extraction Temperature of Tamarind Seed Husk (TSH) Extracts on Free Radical Scavenging Activity Using DPPH Assay

Sample (n = 3)	Temperature (°C)	IC_{50} value (µg/ml)
Water extracts (wTSH)	room temperature	21.7 ±0.2
Water extracts (wTSH)	60 ± 2	8.8 ± 0.2
60% Ethanolic extracts (eTSH)	room temperature	22.4 ± 0.1
60% Ethanolic extracts (eTSH)	60 ± 2	19.6 ± 0.2
Gallic acid	room temperature	5.7 ± 0.1

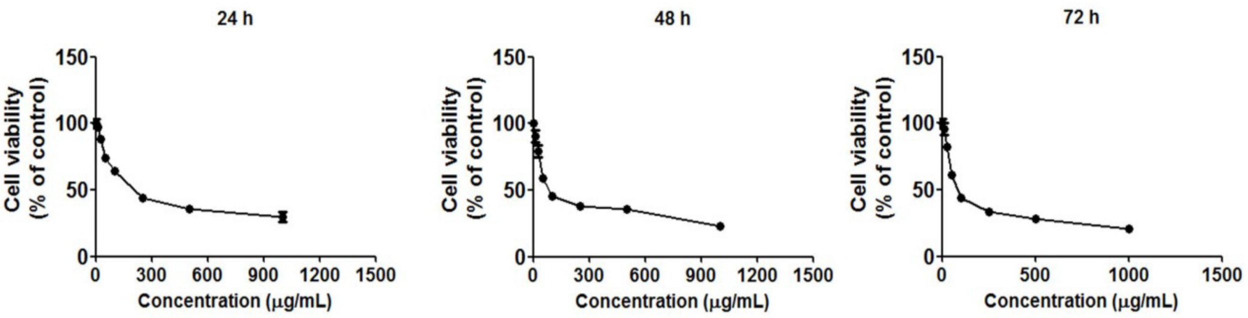

Figure 2. The Evaluating Effect of Water Extract of Tamarind Seed Husk (wTSH) on Cell Viability Over Different Exposure Times (24, 48, and 72 hours) by SRB Assay

suggesting a relatively higher concentration of oligomeric proanthocyanidins in these samples.

Bioactive compound identification by LC-ESI-QTOF-MS/MS Analysis

LC-ESI-QTOF-MS/MS revealed distinct chemical compositions between aqueous (wTSH) and ethanolic (eTSH) extracts. wTSH contained procyanidins (dimers/trimers), catechins, and cinnamtannin A2, correlating with its strong antioxidant and cytotoxic activity as shown in Table 3. eTSH was richer in phenolic acids like 4-hydroxy-3-methoxybenzoic acid as shown in Table 4. These compositional differences explain why TPC alone did not predict antioxidant strength, underscoring the role of specific redox-active polyphenols.

Cytotoxicity Assessment Using SRB Assay

wTSH demonstrated significantly stronger time-dependent cytotoxicity than eTSH. IC_{50} values for wTSH decreased from 216.9 ± 14.3 µg/mL (24 h) to 44.1 ± 0.9 µg/mL (72 h) as shown in Figure 2, while eTSH remained more than 400 µg/mL across time points as shown in Figure 3. These differences may reflect the higher content of flavan-3-ols and procyanidins in wTSH, which are known to induce oxidative stress, mitochondrial dysfunction, and caspase-mediated apoptosis in cancer cells. The results support water extraction as a superior, eco-friendly method for isolating potent bioactives with anticancer potential.

Cell Migration Analysis Using Wound Healing Assay

TSH extracts inhibited HepG2 cell migration in a dose-dependent manner. Treatment with 250 and 500 µg/mL of wTSH and eTSH significantly reduced wound closure after 72 h ($p < 0.01$) as shown in Figure 4 and 5. The highest concentration of wTSH showed the most pronounced inhibition, suggesting interference with cellular motility pathways. These findings reinforce the potential of TSH extracts in suppressing cancer cell metastasis (Table 5).

Discussion

This study demonstrated the successful optimization

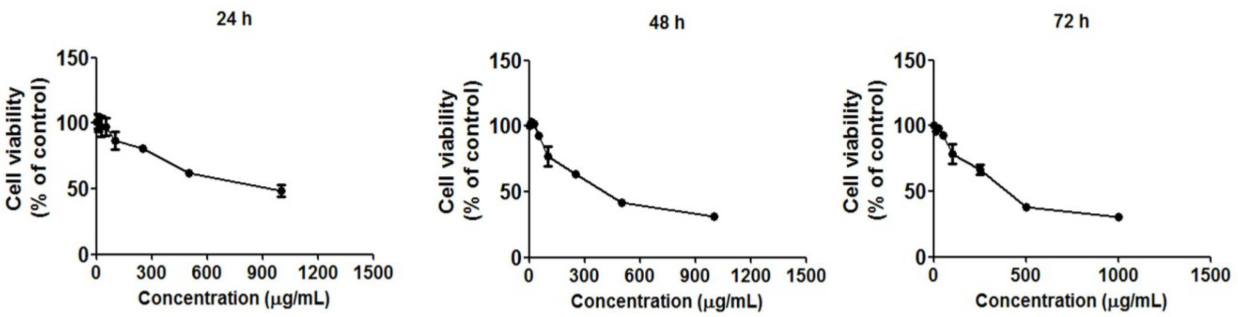

Figure 3. The Evaluating Effect of Ethanolic Extract of Tamarind Seed Husk (eTSH) on Cell Viability Over Different Exposure Times (24, 48, and 72 hours) by SRB Assay

Table 3. The Phytochemical Composition of wTSH Extract from LC-ESI-QTOF-MS/MS Analysis

Retention Time (RT, min)	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentation Pattern	Formula	Tentative identification	Mass error (ppm)
3.143	563.1781	[M+HCOO]-	-	$C_{19}H_{34}O_{16}$	Ciceritol	8.5
3.256	401.1272	[M+HCOO]-	355.1233, 179.0536, 89.0229, 59.0129	$C_{13}H_{24}O_{11}$	4-O-Methylgalactinol	7.14
3.385	387.1119	[M+HCOO]-	341.1074, 179.0541, 89.0230, 59.0129	$C_{12}H_{22}O_{11}$	Sucrose	6.5
3.74	149.0453	[M-H]-	59.0129	$C_5H_{10}O_5$	D-ribose	1.66
5.646	191.019	[M-H]-	85.0277	$C_6H_8O_7$	Citric acid	3.78
8.005	431.1162	[M-H]-	137.0222, 93.0327	$C_{18}H_{24}O_{12}$	Apiosylglucosyl-4-hydroxybenzoate	7.65
8.734	167.0348	[M-H]-	123.0433, 108.0197, 91.0162, 65.0017	$C_8H_8O_4$	4-Hydroxy-3-methoxy benzoic acid	1.09
9.587	153.0191	[M-H]-	109.0273	$C_7H_6O_4$	Protocate chuic acid	1.52
10.584	289.0703	[M-H]-	245.0798, 125.0226	$C_{15}H_{14}O_6$	Epicatechin	5.06
10.712	577.1304	[M-H]-	425.0858, 407.0752, 289.0698, 125.0226	$C_{30}H_{26}O_{12}$	Procyanidin dimer	8.23
11.165	137.0244	[M-H]-	108.02	$C_7H_6O_3$	3,4-Dihydroxybenzal dehyde	0.13
11.331	289.0703	[M-H]-	245.0797, 125.0225, 109.027	$C_{15}H_{14}O_6$	Catechin	5.06
11.44	865.1893	[M-H]-	713.1493, 577.1315, 407.0745,289.0694,125.0223	$C_{45}H_{38}O_{18}$	Procyanidin trimer	10.68
11.979	1153.2474	[M-H]-	-	$C_{60}H_{50}O_{24}$	Cinna tannin A2	12.6

of an eco-friendly extraction method for recovering phenolic compounds from tamarind seed husk (TSH), emphasizing the critical influence of solvent type and extraction temperature [11, 12]. Statistical analysis revealed that these factors significantly impacted the total phenolic content (TPC), with hot water extraction at 60°C yielding the highest recovery among water-based methods. Although ethanol extraction provided slightly higher TPC values [13], hot water extraction offered a sustainable alternative with comparable antioxidant and cytotoxic activities. These findings align with previous reports indicating that elevated temperatures enhance the solubility and diffusion of polyphenols from plant matrices.

The antioxidant capacity (AOC) results, determined by the DPPH assay, further highlighted the importance of extraction conditions. Hot water extracts exhibited superior free radical scavenging activity compared to ethanolic extracts, despite lower overall TPC, suggesting that specific phenolic profiles, rather than total content alone, contribute to antioxidant efficacy. High-performance liquid chromatography (HPLC) analysis further confirmed the presence of oligomeric proanthocyanidins. In alignment with earlier reports, the applied HPLC method

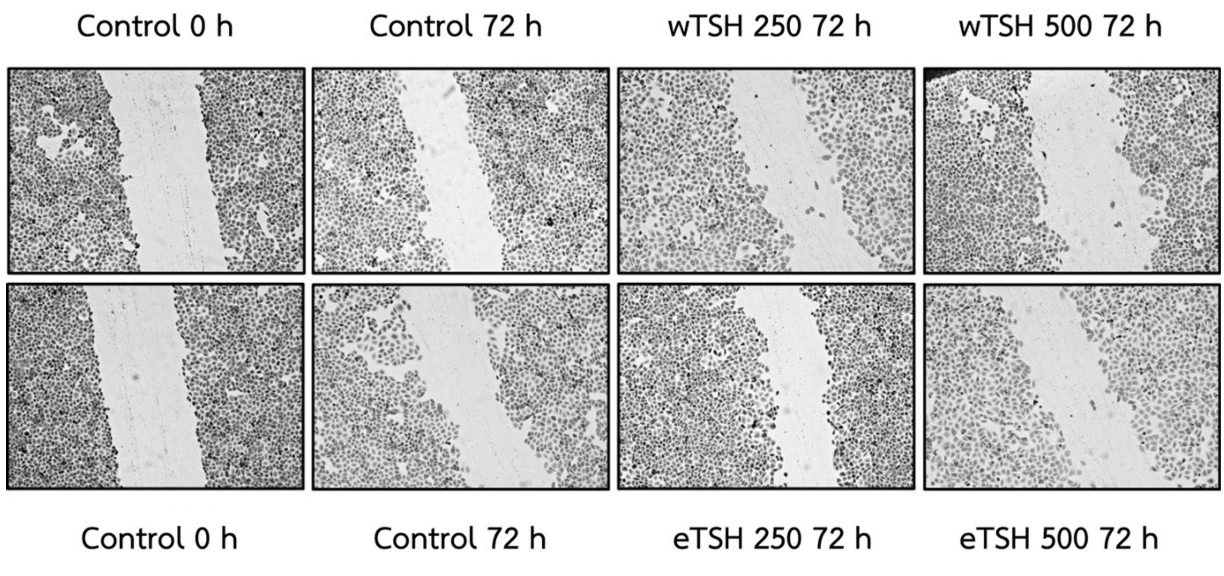

Figure 4. Representative Images from *in vitro* Scratch Wound Healing Assays of wTSH and eTSH Extracts Demonstrating that Cell Migration into the Cell-Free Region at 72 h

Table 4. The Phytochemical Composition of eTSH Extract from LC-ESI-QTOF Analysis

Retention Time (RT, min)	Precursor Ion (m/z)	Product Ion (m/z)	Fragmentation Pattern	Formula	Tentative identification	Mass error (ppm)
3.137	563.1828	[M+HCOO]-	517.1769, 337.1129, 179.0552, 89.0237	$C_{19}H_{34}O_{16}$	Ciceritol	0.16
3.248	401.1302	[M+HCOO]-	355.1242, 179.0549, 89.0235	$C_{13}H_{24}O_{11}$	4-O-Methylgalactinol	-0.34
8.721	167.0351	[M-H]-	123.0443, 93.0339	$C_8H_8O_4$	4-Hydroxy-3-methoxybenzoic acid	1.09
9.58	153.0194	[M-H]-	109.0287, 91.0181	$C_7H_6O_4$	Protocate chuic acid	1.52
11.154	137.0246	[M-H]-	108.0206	$C_7H_6O_3$	3,4-Dihy droxybenzaldehyde	0.13

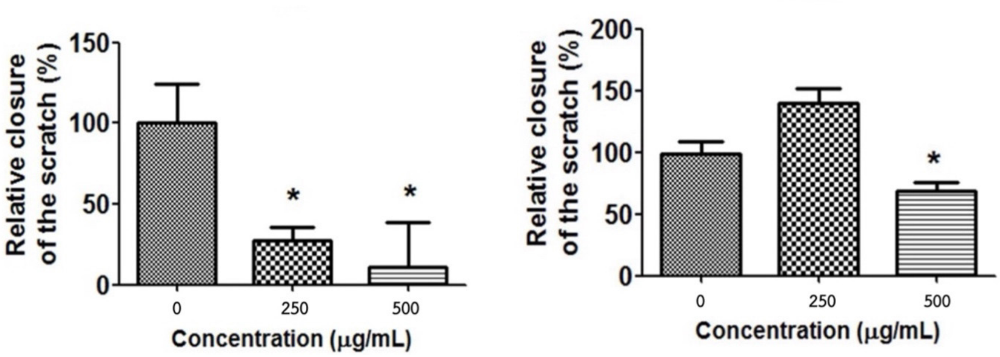

Figure 5. Summary Bar Graph of wTSH (left) and eTSH (right) Extracts Illustrating Percentage Wound Closure at 72 h during the Scratch Wound Assay (*P < 0.01 versus control)

Table 5. The Cytotoxic Effects of wTSH and eTSH Extracts of Tamarind Seed Husk on HepG2 Cells Over Different Exposure Times (24, 48, and 72 hours) from SRB) Assay

TSH extract	IC_{50} at 24 h (µg/ml)	IC_{50} at 48 h (µg/ml)	IC_{50} at 72 h (µg/ml)
wTSH	216.9 ± 14.3	117.0 ± 7.1	44.1 ± 0.9
eTSH	915.2 ± 80.2	419.4 ± 20.7	408.3 ± 9.8

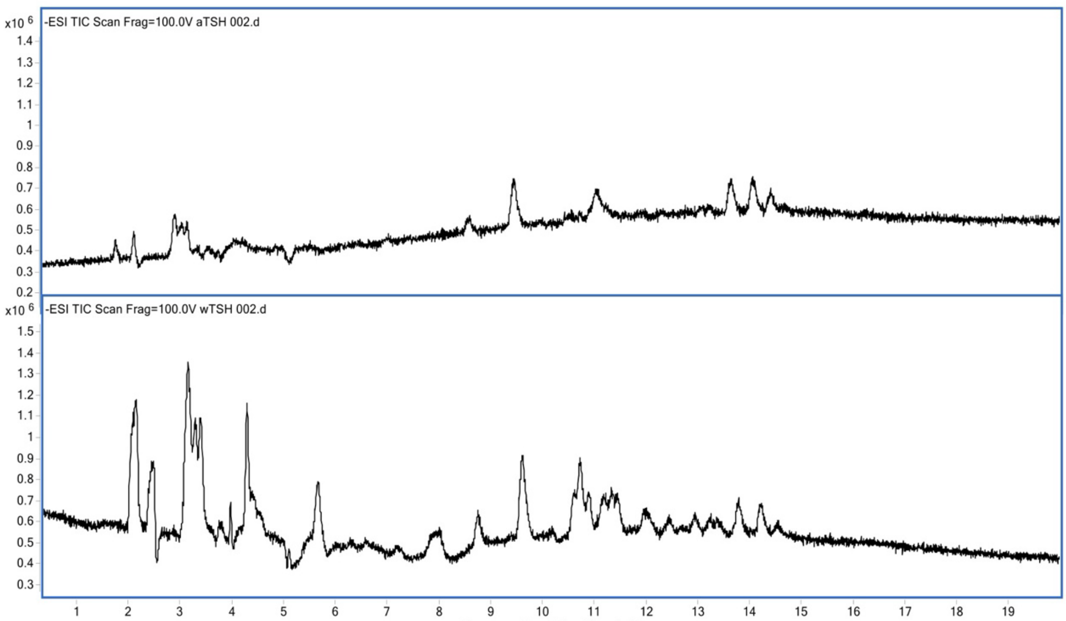

Figure 6. Relative Intensity or Signal Abundance Comparison between eTSH (top) and wTSH (bottom) Extracts from LC-ESI-QTOF-MS/MS Analysis

effectively identified these compounds in grape seed extract (GSE), evidenced by a characteristic broad peak near a 60-minute retention time [13, 14]. Notably, similar chromatographic patterns were observed in both wTSH and eTSH extracts, with comparable peak broadening and additional minor peaks, suggesting complex polyphenolic profiles. Remarkably, the major peak areas in wTSH and eTSH were greater than those in GSE, indicating a higher concentration of oligomeric proanthocyanidins in the TSH-derived samples.

Complementary to HPLC profiling, LC-ESI-QTOF-MS/MS analysis corroborated this by revealing that the water extract contained higher levels of flavan-3-ols, such as catechins and procyanidins, known for their strong antioxidant potential, whereas ethanolic extracts were richer in phenolic acids and sugar alcohol derivatives. This related to the relative intensity or signal abundance of wTSH extract was stronger than eTSH extract as shown in Figure 6. These findings further support the enhanced antioxidant and biological activities observed in the hot water extract [15, 16].

In addition to antioxidant properties, the cytotoxic effects of TSH extracts were evaluated against HepG2 liver cancer cells. The water extract displayed markedly stronger cytotoxic activity than the ethanolic extract, with IC50 values decreasing significantly over time. These results suggest a cumulative apoptotic effect mediated by ROS generation [17-19], inhibition of pro-survival signaling pathways (e.g., PI3K/Akt, NF-κB), and G1-phase cell cycle arrest. The higher cytotoxicity of the water extract can be attributed to its richer content of procyanidins and flavan-3-ols, compounds previously reported to trigger oxidative stress-mediated apoptosis in cancer cells.

Moreover, both extracts demonstrated significant antimigratory effects on HepG2 cells in a concentration-dependent manner, as evidenced by wound healing assays [17, 18]. These findings reinforce the potential role of TSH-derived polyphenols in not only inhibiting cancer cell viability but also suppressing metastatic behaviors.

This study successfully established an optimized, eco-friendly extraction method for tamarind seed husk (TSH) and demonstrated its potential as a valuable source of bioactive polyphenols with both antioxidant and anticancer properties. The factorial design approach clearly identified solvent type and extraction temperature as critical determinants of total phenolic yield. Notably, aqueous extraction at 60°C produced an extract (wTSH) with superior cytotoxic and antioxidant activity compared to ethanolic extracts, despite the latter exhibiting higher total phenolic content. The enhanced biological activity of wTSH is likely attributed to its unique polyphenolic composition, rich in flavan-3-ols and oligomeric proanthocyanidins, which are known to induce apoptosis, cell cycle arrest, and inhibit cancer cell migration. These findings highlight the importance of polyphenol quality and profile over absolute concentration in determining bioactivity. Overall, TSH extracts, particularly those derived via hot water extraction, offer a promising natural resource for developing plant-based anticancer agents and functional food ingredients. Further studies involving bioactivity-guided fractionation, mechanistic exploration, and *in vivo* validation are warranted to fully elucidate their therapeutic potential.

Author Contribution Statement

I am the sole author of this manuscript and was responsible for the conception and design of the study, methodology development, data collection, experimental work, data analysis and interpretation, preparation of figures and tables, writing of the manuscript, and final approval of the version to be submitted for publication.

Acknowledgements

I would like to express my sincere gratitude to the School of Pharmaceutical Sciences, University of Phayao, Phayao, Thailand for their valuable support and provision of research facilities that made this experimental study possible.

Conflict of Interest

The author declare no conflicts of interest related to this research. No financial or personal relationships influenced the study, and there are no competing interests that could affect the integrity of the findings.

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