

Extraction Time Modulates Ginger Phytochemicals and Selective Cytotoxicity in Hormone Receptor-Positive Breast Cancer Cells

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History

- Submission Date: 14-04-2026;
- Review completed: 20-05-2026;
- Accepted Date: 14-06-2026.

DOI : 10.5530/pj.2026.18.150

Article Available online

<http://www.phcogj.com/v18/i3>

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ABSTRACT

Introduction: Ginger (*Zingiber officinale* Roscoe) contains bioactive phytochemicals with potential anticancer properties; however, the influence of hot-water reflux extraction time on phytochemical composition and selective cytotoxicity against hormone receptor-positive breast cancer cells remains unclear. **Methods:** Ginger powder was extracted by hot-water reflux at 98-100 °C for 30, 60, 90, and 120 minutes (GS-30, GS-60, GS-90, and GS-120). Concentrations of [6]-gingerol and [6]-shogaol were determined using high-performance liquid chromatography with ultraviolet detection (HPLC-UV). Cytotoxicity against MCF-7 breast cancer cells and normal human dermal fibroblasts (NHDF) was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The half-maximal inhibitory concentration (IC₅₀), half-maximal cytotoxic concentration (CC₅₀), and the selectivity index (SI) were calculated. **Results:** Increasing the extraction time resulted in progressively higher concentrations of both [6]-gingerol and [6]-shogaol, with GS-120 containing the highest levels of these marker compounds. However, GS-30 and GS-60 exhibited greater cytotoxicity toward MCF-7 cells, with IC₅₀ values of 112.47 ± 6.03 µg/mL and 112.93 ± 5.52 µg/mL, respectively, compared with 323.13 ± 42.99 µg/mL for GS-90 and 404.07 ± 14.86 µg/mL for GS-120. The SI values were 7.5, 8.6, 2.27, and 2.03 for GS-30, GS-60, GS-90, and GS-120, respectively, with GS-60 exhibiting the greatest selectivity toward breast cancer cells over normal fibroblasts. **Conclusions:** Although prolonged extraction increased the concentrations of [6]-gingerol and [6]-shogaol, it did not enhance selective anticancer activity. These findings suggest that the selective cytotoxic effects of hot-water ginger extracts are influenced by the overall phytochemical profile rather than the abundance of individual marker compounds. Integrating phytochemical characterization with biological evaluation may facilitate the optimization of extraction conditions for developing ginger extracts with improved selective anticancer activity.

Keywords: Ginger; Hot-water extraction; Gingerol; Shogaol; Breast cancer; Cytotoxicity

INTRODUCTION

Breast cancer remains the most frequently diagnosed malignancy among women worldwide and a leading cause of cancer-related mortality. According to GLOBOCAN 2022, approximately 2.3 million new cases and more than 670,000 deaths were reported globally¹. Hormone receptor-positive breast cancer, including Luminal A and Luminal B subtypes, accounts for approximately 70-75% of all breast cancer cases and generally responds well to endocrine therapy^{2,3}. Recent advances, such as the addition of cyclin-dependent kinase (CDK) 4/6 inhibitors in high-risk early disease and the use of oral selective estrogen receptor degraders in advanced disease, have improved outcomes^{4,5}. However, treatment resistance, recurrence, and long-term adverse effects remain important clinical challenges. Therefore, safe and naturally derived compounds with anticancer potential are recommended as promising alternatives.

Ginger (*Zingiber officinale* Roscoe) has long been used as both food and traditional medicine and contains numerous bioactive phytochemicals, including gingerols, shogaols, paradols, zingerone, flavonoids, and other phenolic compounds^{6,7}. Among these constituents, [6]-gingerol and [6]-shogaol are recognized as major marker compounds and have been reported to possess antioxidant, anti-inflammatory, antiproliferative,

and pro-apoptotic properties⁷. Previous studies have demonstrated that ginger-derived compounds suppress breast cancer cell proliferation through multiple mechanisms, including induction of apoptosis, modulation of oxidative stress, inhibition of NF-κB signaling, mitochondrial dysfunction, and cell-cycle arrest^{8,9}. Ginger extracts have also demonstrated chemopreventive potential¹⁰. Collectively, these findings suggest that ginger phytochemicals may contribute to breast cancer prevention and serve as complementary therapeutic agents.

Extraction conditions play an important role in determining the phytochemical composition and biological properties of herbal extracts^{11,12}. Factors such as temperature, solvent type, extraction duration, and extraction method can influence both the recovery and transformation of bioactive constituents. Recent optimization studies have shown that microwave-assisted extraction (MAE) with aqueous ethanol enables the rapid recovery of gingerols and shogaols¹³. Ultrasound-assisted extraction (UAE) provides efficient recovery under moderate thermal conditions¹⁴. Comparative studies of ultrasound, magnetic agitation, maceration, and reflux extraction further demonstrated that extraction technique and duration significantly affect the concentrations of [6]-gingerol, [6]-shogaol, as well as total phenolic content and antioxidant capacity¹⁵. Hot-water extraction is particularly

Cite this article: Kasama K, Keerakarn S, Jarasphol R, Somboon R, Ariya S, Siripat A. Extraction Time Modulates Ginger Phytochemicals and Selective Cytotoxicity in Hormone Receptor-Positive Breast Cancer Cells. *Pharmacogn J.* 2026;18 (3): 324-329.

attractive because water is safe, inexpensive, environmentally friendly, and suitable for pharmaceutical and nutraceutical applications¹¹. Reflux extraction provides controlled heating conditions while minimizing solvent loss during prolonged processing. Thermal treatment may also promote the dehydration of gingerols into shogaols, thereby altering phytochemical profiles and biological activities^{16,17}. Collectively, these findings highlight the importance of optimizing extraction conditions to enhance the efficacy of ginger extracts.

Despite growing interest in the anticancer potential of ginger, the effects of hot-water reflux extraction time on phytochemical composition and selective cytotoxicity have not been systematically investigated. Previous studies have primarily focused on maximizing the recovery of bioactive compounds; however, the relationship between extraction time, phytochemical composition, and selective cytotoxicity toward cancer cells remains poorly understood. In addition, it remains unclear whether higher concentrations of marker compounds, such as [6]-gingerol and [6]-shogaol, are associated with greater anticancer activity. Therefore, the present study examined the effects of different hot-water reflux extraction times on the phytochemical composition and selective cytotoxicity of ginger powder extracts against hormone receptor-positive breast cancer cells. The concentrations of [6]-gingerol and [6]-shogaol were determined using high-performance liquid chromatography with ultraviolet detection (HPLC-UV). Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays in MCF-7 breast cancer cells and normal human dermal fibroblasts (NHDF), and the selectivity index (SI) was calculated to assess preferential cytotoxicity toward cancer cells. By combining phytochemical analysis with biological evaluation, this study provides a better understanding of how extraction time influences both the chemical composition and selective anticancer activity of hot-water ginger extracts. These findings may help optimize extraction conditions for the future development of ginger extracts as potential therapeutic agents for breast cancer.

MATERIALS AND METHODS

Preparation of Ginger Extracts

Commercial 100% instant ginger powder (Doi Kham Brand, Thailand) obtained from a single manufacturing lot was used throughout the study to minimize raw material variability. Ginger powder (20 g) was mixed with 200 mL of purified water and extracted by hot-water reflux at 98–100°C using a solid-to-liquid ratio of 1:10 (w/v). Four extraction durations were investigated: 30, 60, 90, and 120 min, designated as GS-30, GS-60, GS-90, and GS-120, respectively. Following extraction, insoluble residues were removed by filtration through No.4 filter paper. The filtrates were adjusted to a final volume of 200 mL with purified water and spray-dried at an inlet temperature of 170°C and an outlet temperature of 85–95 °C. The dried extracts were collected and stored in airtight containers until further analysis.

HPLC-UV Analysis of [6]-Gingerol and [6]-Shogaol

The concentrations of [6]-gingerol and [6]-shogaol were determined by HPLC-UV using a modified method based on previous reports^{17,18}. Authentic [6]-gingerol and [6]-shogaol reference standards (CATO, China; purity ≥98% for both standards) were used for calibration and analyte identification. Approximately 500 mg of each spray-dried extract was dissolved in methanol and adjusted to a final volume of 5 mL. The solutions were sonicated for 10 min and filtered through a 0.22 µm nylon syringe filter before analysis.

Chromatographic separation was performed using a reversed-phase C18 column (250 × 4.6 mm, 5 µm particle size) maintained at 30°C. The mobile phase consisted of water containing 0.1% acetic acid (solvent A) and acetonitrile containing 0.1% acetic acid (solvent B).

Gradient elution was performed from 40% to 80% solvent B over 20 min, followed by re-equilibration to the initial condition at 25 min. The flow rate was 1.0 mL/min, the injection volume was 10 µL, and UV detection was carried out at 280 nm. Calibration curves were prepared using authentic [6]-gingerol and [6]-shogaol standards across a concentration range of 1–100 mg/L, with seven levels: (1, 5, 10, 25, 50, 75, and 100 mg/L). Linearity was observed for both analytes, with coefficients of determination (R^2) of 0.99998. Chromatographic identity was confirmed by comparison with authentic standards, with [6]-gingerol and [6]-shogaol eluting at approximately 8.8 and 15.0 min, respectively. The limits of detection (LOD) and quantification (LOQ) were 10 and 50 mg/kg, respectively. Analytical repeatability was evaluated from triplicate HPLC-UV measurements of each extract solution, with relative standard deviation (RSD) values ranging from 0.02% to 0.66% for [6]-gingerol and from 0.22% to 0.75% for [6]-shogaol.

Cell Culture

Human hormone-receptor positive breast cancer cells (MCF-7; ATCC, Manassas, VA, USA) and normal human dermal fibroblasts (NHDF; PromoCell, Heidelberg, Germany) were used as representative cancer and normal cell models, respectively. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cell cultures were maintained in a humidified incubator at 37°C with 5% CO₂. Cell morphology was observed using a Zeiss Primovert inverted microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).

Cytotoxicity and Selectivity Assays

Cytotoxic activity was evaluated using the MTT assay as described previously¹⁹. Spray-dried extracts were dissolved in deionized water to prepare 500 mg/mL stock solutions and sterilized through a 0.22 µm syringe filter. MCF-7 and NHDF cells were seeded in 96-well plates (5 × 10³ cells/well) and incubated overnight. Cells were treated with GS-30, GS-60, GS-90, and GS-120 at final concentrations of 15.625–1000 µg/mL for 24 h. After treatment, the medium was replaced with 0.5 mg/mL MTT solution and incubated for 2 h at 37°C. Formazan crystals were dissolved in DMSO, and absorbance was measured at 570 nm using a Tecan Spark microplate reader (Tecan Austria GmbH, Grödig, Austria). Cell viability was expressed relative to untreated controls. Half-maximal inhibitory concentration (IC₅₀) values for MCF-7 cells and half-maximal cytotoxic concentration (CC₅₀) values for NHDF cells were calculated by nonlinear regression using GraphPad Prism 8.0.1 (GraphPad Software, San Diego, CA, USA). Cancer selectivity was evaluated using the selectivity index (SI), defined as CC₅₀ (NHDF)/IC₅₀ (MCF-7), with higher SI values indicating preferential cytotoxicity toward cancer cells²⁰.

Statistical Analysis

Data from HPLC-UV and cytotoxicity assays are expressed as mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism version 8.0.1 (GraphPad Software). Differences among extraction groups were assessed using one-way ANOVA followed by Tukey's multiple-comparison test, under the assumptions of normal distribution and equal variances. Statistical significance was defined as $p < 0.05$.

RESULTS

Effect of Extraction Time on Solid Content, Extraction Yield, and Spray-Dried Powder Recovery

Hot-water reflux extraction of ginger powder at 98–100°C for 30, 60, 90, and 120 min produced extraction yields ranging from 91.5% to 93.2%

Table 1. Solid content, extraction yield, and spray-dried powder recovery of ginger extracts prepared at different extraction durations.

Sample	Extraction time (min)	Solid content (%)	Extraction yield (%)	Spray-dried powder (g)
GS-30	30	9.32	93.2	12.48
GS-60	60	9.15	91.5	12.59
GS-90	90	9.22	92.2	12.54
GS-120	120	9.17	91.7	11.92

Values were obtained from a single extraction experiment.

Table 2. Contents of [6]-gingerol and [6]-shogaol in ginger extracts prepared at different extraction durations.

Sample	[6]-Gingerol (mg/kg extract)	[6]-Shogaol (mg/kg extract)	Shogaol/Gingerol ratio
GS-30	591.76 ± 3.93	180.59 ± 0.80	0.305
GS-60	678.31 ± 0.16	250.70 ± 0.56	0.370
GS-90	672.83 ± 2.99	262.12 ± 1.37	0.390
GS-120	876.62 ± 4.13	347.79 ± 2.61	0.397

Values are expressed as mean ± SD from three replicate measurements of a single extract preparation.

Table 3. Half-maximal cytotoxic concentration (CC₅₀), half-maximal inhibitory concentration (IC₅₀), and selectivity index (SI) of ginger extracts.

Sample	NHDF (CC ₅₀ , µg/mL)	MCF-7 (IC ₅₀ , µg/mL)	SI
GS-30	850.83 ± 99.14	112.47 ± 6.03	7.57
GS-60	971.59 ± 247.55	112.93 ± 5.52	8.60
GS-90	733.70 ± 42.97	323.13 ± 42.99	2.27
GS-120	819.37 ± 26.31	404.07 ± 14.86	2.03

Values are expressed as mean ± SD from three biological replicates.

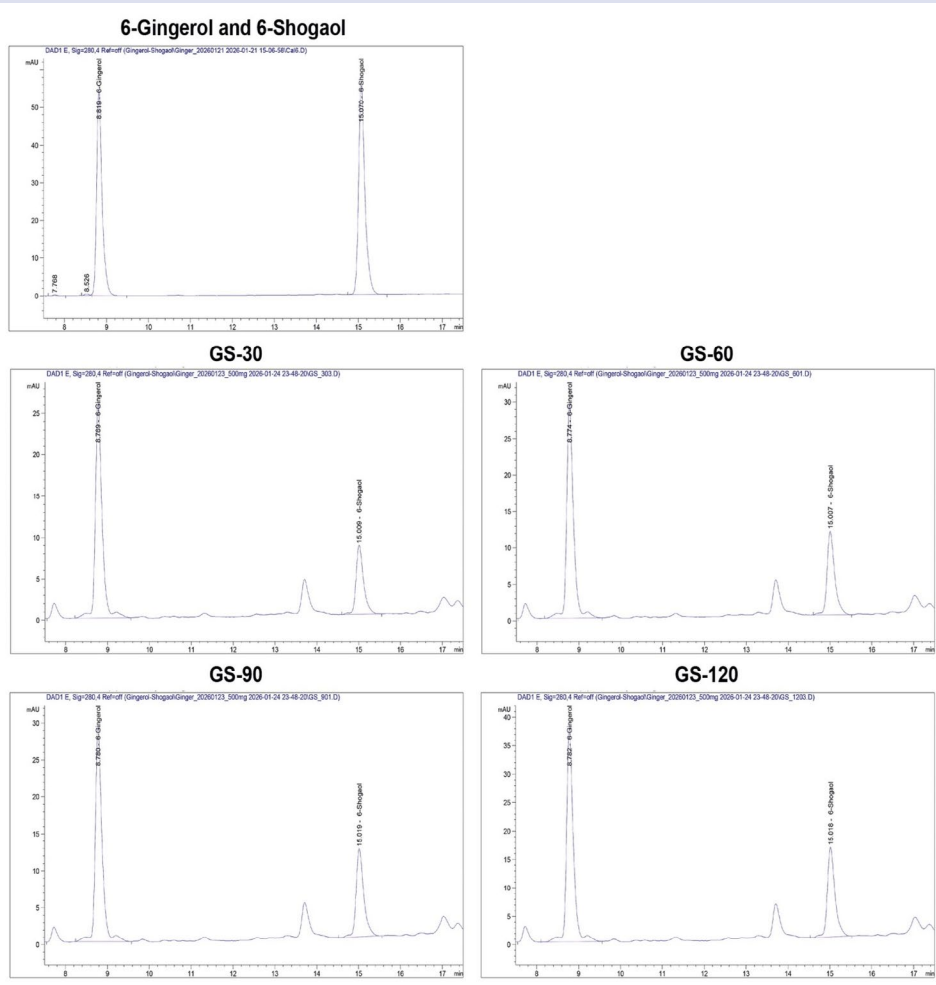


Figure 1. Representative HPLC chromatograms of mixed standards of [6]-gingerol and [6]-shogaol and ginger extracts obtained after extraction for 30 min (GS-30), 60 min (GS-60), 90 min (GS-90), and 120 min (GS-120).

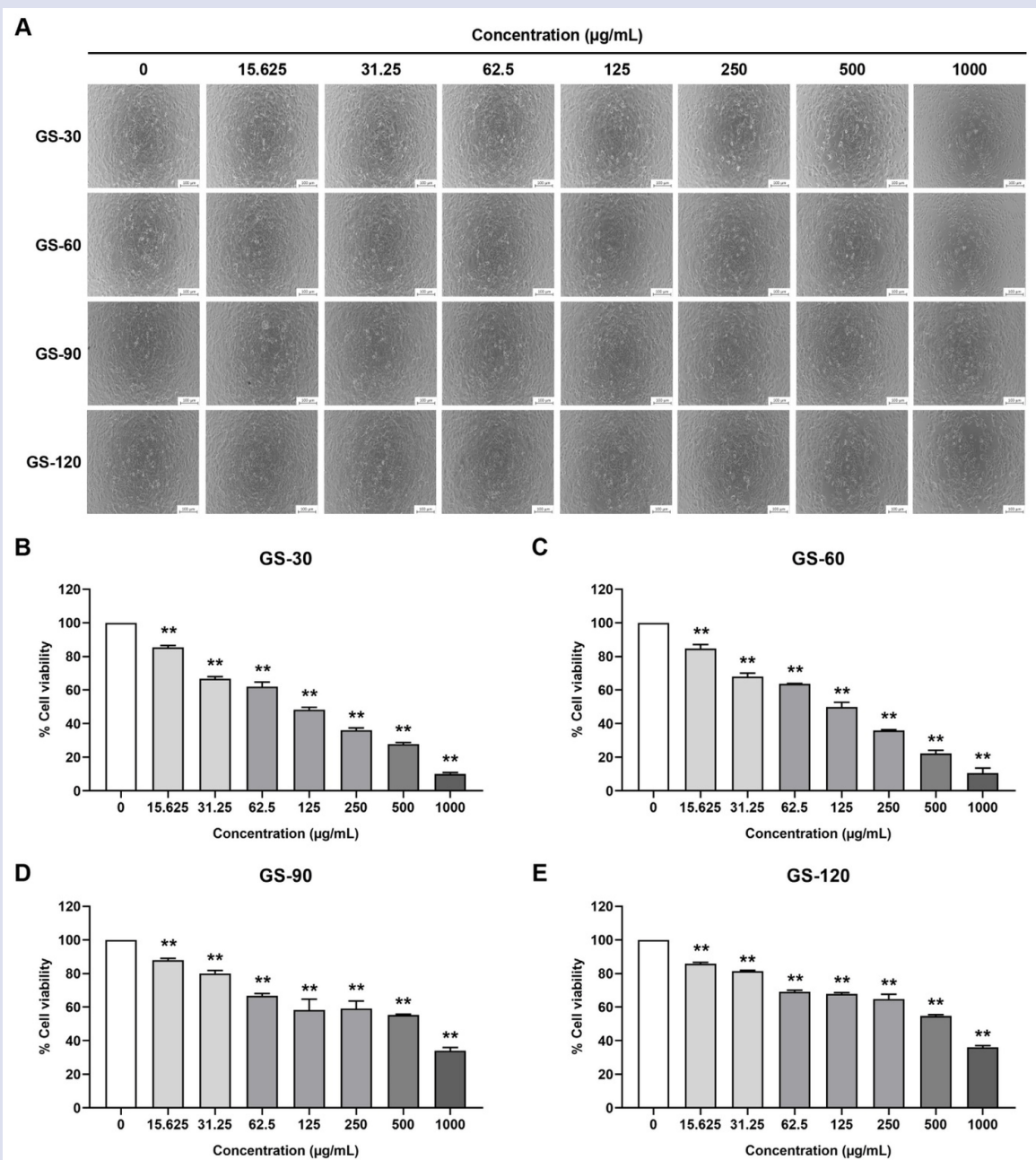


Figure 2. Cytotoxic effects of ginger extracts against MCF-7 cells. (A) Representative microscopic images of MCF-7 cells after treatment with different ginger extracts. (B-E) Percentage cell viability of MCF-7 cells after 24 h treatment with GS-30 (B), GS-60 (C), GS-90 (D), and GS-120 (E), determined by MTT assay. Bar graph represents mean $\pm$ SD from three biological replicates. ** $p < 0.001$ vs. untreated control (0 $\mu\text{g/mL}$).

(Table 1). The highest spray-dried powder recovery was obtained from GS-60, whereas GS-120 yielded the lowest powder weight. Overall, extraction efficiency remained comparable among the four extraction conditions.

Effect of Extraction Time on [6]-Gingerol and [6]-Shogaol Contents

Representative HPLC chromatograms of ginger extracts are shown in Figure 1, with quantitative data summarized in Table 2. The peaks assigned to [6]-gingerol and [6]-shogaol eluted at approximately 8.8 and 15.1 min, respectively, corresponding to the retention times of the authentic standards. The analysis indicated that GS-120 contained the

highest concentrations of [6]-gingerol and [6]-shogaol, whereas GS-30 contained the lowest. GS-120 exhibited higher concentrations of both compounds compared with GS-30 and GS-60. Extraction at 60 and 90 min yielded comparable [6]-gingerol levels. Overall, extended extraction was associated with increased concentrations of both marker compounds.

Cytotoxic Activity and Selectivity Index

The cytotoxic activity of ginger extracts against MCF-7 breast cancer cells and NHDF was evaluated using the MTT assay. All extracts reduced MCF-7 cell viability with increasing concentration (Figure 2). GS-30 and GS-60 showed IC_{50} values of $112.47 \pm 6.03 \mu\text{g/mL}$ and $112.93 \pm 5.52 \mu\text{g/mL}$, respectively, while GS-90 and GS-120 exhibited

higher IC₅₀ values (Table 3). GS-30 and GS-60 demonstrated greater cytotoxic potency, whereas GS-120, despite containing the highest levels of [6]-gingerol and [6]-shogaol, did not exhibit the lowest IC₅₀.

In NHDF cells, all extracts displayed CC₅₀ values higher than their corresponding IC₅₀ values. GS-60 showed the highest SI, followed by GS-30, while GS-90 and GS-120 had lower values (Table 3). These findings suggest that shorter extraction times yield ginger extracts with stronger anticancer activity and greater selectivity toward cancer cells.

DISCUSSION

This study demonstrated that hot-water reflux extraction time influenced both the phytochemical composition and biological properties of ginger powder extracts. Although prolonged extraction increased [6]-gingerol and [6]-shogaol concentrations, higher levels of these marker compounds did not correspond to greater anticancer activity or selectivity. These findings emphasize the importance of integrating phytochemical characterization with biological evaluation when optimizing extraction conditions for herbal extracts.

The extraction times of 30, 60, 90, and 120 min were selected to represent short to prolonged reflux conditions commonly reported for hot-water extraction of medicinal plants. These time intervals allowed evaluation of the progressive effects of extraction duration while maintaining constant extraction temperature and solvent conditions. Although no preliminary optimization experiment was performed to determine the optimal extraction time, the selected durations provided a practical range for investigating changes in phytochemical composition and biological activity associated with prolonged heating.

Extraction yields remained relatively constant among the four extraction groups; however, quantitative HPLC-UV analysis revealed that concentrations of both [6]-gingerol and [6]-shogaol increased with extraction time, particularly in GS-120. Previous studies have shown that thermal processing facilitates the release of intracellular phytochemicals into the extraction medium^{11,12}. In addition, prolonged heating may promote dehydration reactions that convert gingerols into shogaols^{16,17}. Similar effects have also been reported using MAE, UAE, and organic solvent extraction, where extraction conditions strongly influence phytochemical recovery and biological activity¹³⁻¹⁵. Collectively, these findings indicate that extraction conditions affect not only extraction efficiency but also the resulting phytochemical composition.

Despite GS-120 containing the highest concentrations of [6]-gingerol and [6]-shogaol, GS-30 and GS-60 exhibited greater cytotoxicity toward MCF-7 cells, with GS-60 demonstrating the highest SI. These results suggest that increased concentrations of the two quantified marker compounds alone were not associated with enhanced biological activity under the present experimental conditions. Similar observations have been reported for several herbal extracts, in which biological activity cannot be explained solely by the abundance of one or two major constituents²¹. However, because only [6]-gingerol and [6]-shogaol were quantified and no mechanistic experiments were performed, the present findings should be interpreted cautiously. Additional evidence is required before concluding that overall phytochemical composition directly determines selective cytotoxicity.

One possible explanation is that prolonged extraction alters the relative abundance of multiple phytochemicals that collectively influence biological activity. In addition to [6]-gingerol and [6]-shogaol, ginger contains numerous bioactive compounds, including paradols, zingerone, gingerdiones, flavonoids, terpenoids, and phenolic acids^{6,7}. These compounds have been reported to possess antioxidant, anti-inflammatory, and anticancer activities and may interact synergistically

or antagonistically within crude extracts. Consequently, changes in the concentrations of unmeasured constituents during prolonged heating may influence the overall biological activity of the extracts. Since these compounds were not quantified in the present study, this explanation remains speculative and warrants further investigation using comprehensive metabolomic profiling together with bioactivity-guided fractionation.

The present study has several strengths. Extraction conditions were carefully controlled, phytochemical characterization was performed using HPLC-UV, and selective cytotoxicity was evaluated in both breast cancer and normal human cells, allowing calculation of the SI. Nevertheless, several limitations should be acknowledged. First, only a single extraction experiment was performed, which limits assessment of extraction reproducibility. Second, only two marker compounds were quantified, and other potentially bioactive constituents were not characterized. Third, biological activity was evaluated using only the MTT assay, which measures metabolic activity but does not distinguish among mechanisms such as apoptosis, cell-cycle arrest, or other forms of cell death. Additional assays, including apoptosis analysis, cell-cycle analysis, colony formation, migration, and mechanistic studies, would strengthen the biological conclusions. Fourth, only one hormone receptor-positive breast cancer cell line (MCF-7) was examined. Future studies should include additional molecular subtypes, including HER2-positive and triple-negative breast cancer cell lines, to determine whether the observed effects are broadly applicable. Finally, comprehensive phytochemical characterization using metabolomics, together with mechanistic investigations, will be necessary to clarify how extraction conditions influence phytochemical composition and selective anticancer activity and to support the rational development of ginger extracts with therapeutic potential.

CONCLUSION

This study demonstrated that hot-water reflux extraction time influenced the phytochemical composition and biological properties of ginger powder extracts. Although prolonged extraction increased [6]-gingerol and [6]-shogaol concentrations, higher marker-compound levels did not correspond to greater anticancer activity or improved selectivity. Instead, GS-60 exhibited the most favorable biological activity, showing potent cytotoxicity against MCF-7 cells and the highest SI. These findings suggest that the selective anticancer activity of ginger extracts may be influenced by the overall phytochemical composition rather than the abundance of individual marker compounds. Further studies involving comprehensive phytochemical profiling and mechanistic investigations are warranted to confirm these observations. Overall, this study highlights the importance of integrating phytochemical characterization with biological evaluation when optimizing extraction conditions for the development of ginger extracts with therapeutic potential.

ACKNOWLEDGMENTS

This study was supported by research funding from Mae Fah Luang University. Partial financial support was provided by the Cancer and Immunology Research Center (CIRC) grant, Mae Fah Luang University, and publication support from Mae Fah Luang University is gratefully acknowledged. The authors also thank the School of Medicine and the School of Anti-Aging and Regenerative Medicine, Mae Fah Luang University, Chiang Rai, Thailand, for their academic support. In addition, we acknowledge the Bioscience Innovation Division, PTT Innovation Institute, PTT Public Company Limited, Thailand, and the Cancer and Immunology Research Center (CIRC), Mae Fah Luang University, for providing technical assistance and laboratory support.

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Cite this article: Kasama K, Keerakarn S, Jarasphol R, Somboon R, Ariya S, Siripat A. Extraction Time Modulates Ginger Phytochemicals and Selective Cytotoxicity in Hormone Receptor-Positive Breast Cancer Cells. *Pharmacogn J.* 2026;18 (3): 324-329.