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Case Report Paper

Bioactive Methoxyflavones from *Kaempferia parviflora* Induce Apoptosis in Breast Cancer Cell Line

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Abstract: *Kaempferia parviflora* is commonly known as Black Ginger. It is a traditional Thai herbal plant rich in methoxyflavones and other bioactive compounds with promising anticancer properties. Breast cancer remains a leading cause of mortality worldwide, and current therapies face challenges such as toxicity and drug resistance. This study aims to evaluate the cytotoxic effects of ethanol extract of *Kaempferia parviflora* on human breast cancer cell lines (MCF-7 and MDA-MB-231) and to identify the main bioactive compounds responsible for its anticancer activity. Rhizomes were collected from Central Thailand, extracted using 70% ethanol via maceration, and concentrated with rotary evaporation. Breast cancer cells were cultured and treated with varying extract concentrations for 24 to 72 hours. Cytotoxicity was assessed using the MTT assay, and active compounds were quantified via HPLC. Results show a dose-dependent decrease in cell viability, with IC50 values ranging from 60 to 85 µg/mL. Apoptosis induction was confirmed by fluorescence microscopy and increased caspase-3 activity (up to 4.1-fold). HPLC analysis revealed significant concentrations of 5,7-dimethoxyflavone and 3,5,7-trimethoxyflavone, suggesting their role in ROS-mediated apoptosis. Future research should focus on detailed molecular mechanisms, in vivo toxicity and efficacy, and clinical translation. This study supports *Kaempferia parviflora* as a potential natural adjuvant in breast cancer therapy.

Keywords: Apoptosis, Breast Cancer, Cytotoxicity, *Kaempferia parviflora*, Methoxyflavones.

1. Introduction

Kaempferia parviflora, known as "Black Ginger" or "*Krachai Dum*" is a traditional herbal plant that is widely used in Thailand for a variety of traditional medicines. The plant is rich in bioactive compounds such as flavonoids, methoxyflavones, and polyphenols that have important pharmacological activities, including as antioxidants, anti-inflammatory, and anticancers [1] – [3].

The rhizome *of Kaempferia parviflora* is the main part used as a source of bioactive compounds and is widely extracted for traditional medicine. Its traditional uses include the treatment of fatigue, erectile dysfunction, and indigestion, demonstrating broad therapeutic potential [4] [5].

Among these bioactive compounds, methoxyflavones have a special role due to their more easily absorbed and more metabolically stable properties resulting in stronger pharmacological effects than non-methylated flavonoids [6] – [8].

Breast cancer is one of the most common types of cancer and is the leading cause of cancer death in women worldwide. The global health burden of breast cancer continues to increase despite advances in early detection and conventional therapies [9] [10].

Conventional therapies such as chemotherapy, radiotherapy, and hormonal therapy are effective, but they have the constraints of adverse side effects, drug resistance, and high treatment costs, limiting the success of long-term treatment [11] – [13].

Therefore, greater attention is being directed to natural products and herbal medicines as an alternative or complement to breast cancer therapy. Natural ingredients tend to have low toxicity and multi-target mechanisms of action that can overcome the limitations of synthetic drugs [14] – [16].

Kaempferia parviflora has gained the spotlight due to its diverse pharmacological profile. This plant extract shows promising anticancer activity against various types of cancer cells, including breast cancer cells, through a variety of molecular pathways [17] [18].

Various in vitro studies show that *Kaempferia parviflora* extract inhibits the proliferation of breast cancer cells by the mechanism of induction of apoptosis and cell cycle termination. This mechanism occurs through modulation of signaling pathways such as PI3K/Akt, MAPK, and NF-κB [19] – [21].

Methoxyflavones such as 5,7-dimethoxyflavone and 3,5,7,3',4'-pentamethoxyflavone isolated from *Kaempferia parviflora* exhibit cytotoxicity by increasing the production of reactive oxygen species (ROS), activation of the kaspase enzyme, as well as potential disruption of mitochondrial membranes in breast cancer cells [22] – [24].

The antioxidant properties of *Kaempferia parviflora* help reduce oxidative stress which plays an important role in the process of carcinogenesis and tumor development. The balance between ROS production and elimination is essential for maintaining cellular homeostasis and preventing DNA damage [25] [26]. In addition to its direct cytotoxic effects, *Kaempferia parviflora extract* also inhibits the migration and invasion of breast cancer cells by decreasing the expression of matrix metalloproteinases (MMP-2 and MMP-9) and reversing the epithelial-mesenchymal transition (EMT) process, thereby potentially suppressing metastasis [27] – [29].

Kaempferia parviflora *extract* exhibits selectivity against cancer cells without damaging normal cells, an important characteristic for reducing the side effects of cancer therapy [30] [31]. The anti-inflammatory effects of *Kaempferia parviflora* are also significant considering that inflammation is a supporting factor in the tumor microenvironment that promotes tumor growth and resistance. This extract suppresses the production of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β [1] [28].

The extraction method affects the phytochemical content and biological activity of *Kaempferia parviflora*. Extraction with ethanol is highly effective in obtaining methoxyflavones and polyphenols, resulting in extracts with high anticancer potential [2] [3].

Analytical techniques such as high-performance liquid chromatography (HPLC) and mass spectrometry liquid chromatography (LC-MS/MS) are used for the characterization and quantification of active compounds, essential for quality control and standardization of herbal extracts [6] [7].

Pharmacokinetic studies show methoxyflavones have favorable absorption and metabolic profiles thereby increasing bioavailability and therapeutic effectiveness [8] [23].

Current research focuses on understanding the molecular mechanisms of the anticancer effects of *Kaempferia parviflora*, including the regulation of apoptosis proteins (Bcl-2 family), cell-cycle proteins (cyclins and CDKs), as well as angiogenesis factors (VEGF) [20] [22].

Growing evidence indicates *Kaempferia parviflora* may increase the sensitivity of breast cancer cells to conventional chemotherapy drugs, demonstrating its potential as an adjuvant to improve therapeutic outcomes and reduce drug resistance [24] [29].

In vivo studies in animal models confirmed the safety profile and anticancer effectiveness of *Kaempferia parviflora* extract with inhibition of tumor growth and increased survival rates [30] [31]. However, clinical trials investigating the effectiveness *of Kaempferia parviflora* in breast cancer patients are still very limited, so further translational research is needed to validate preclinical data [4] [32].

A multidisciplinary approach that combines ethnopharmacology, phytochemistry, molecular biology, and clinical science is essential to maximize the therapeutic potential of *Kaempferia parviflora* [1] [15].

The integration *of Kaempferia parviflora* in breast cancer therapy offers a promising opportunity to develop effective, safe, and more affordable treatments derived from natural sources.

This study aimed to evaluate the cytotoxic effects of ethanol extract *of Kaempferia parviflora* on human breast cancer cells cultured in vitro as well as identify the main bioactive compounds responsible. By uncovering the molecular mechanisms involved, the study provides a scientific basis for the development of plant-based anticancer agents. The results of the study are expected to expand the options of therapy with lower side effects, which are urgently needed in the management of breast cancer.

2. Method

The study was conducted in early 2025 in Thailand to evaluate the cytotoxic effects of ethanol extract *Kaempferia parviflora* on human breast cancer cells.

1) Sample Collection and Preparation
   The rhizomes of Kaempferia parviflora were collected from the Central Thailand region. After harvesting, the rhizomes are dried under controlled conditions. The dried rhizomes were then extracted using 70% ethanol solvent through the maceration method for 48 hours at room temperature to maximize the extraction of bioactive compounds.
2) Extract Preparation
   The extracts obtained are concentrated using a rotary evaporator with low pressure. The concentrated extract is stored at a low temperature (4°C) until used in cytotoxicity tests.
3) Breast Cancer Cell Culture
   Human breast cancer cells, such as the MCF-7 and MDA-MB-231 cell lines, were cultured in RPMI-1640 or DMEM media to which 10% bovine fetal serum and antibiotics were added. The cells are incubated at 37°C in a humid atmosphere with 5% CO_2 under standard conditions.
4) Cytotoxicity Test
   The cytotoxic effects of the extract were tested using the MTT assay method (3-(4,5-dimethyltiazole-2-il)-2,5-diphenyltetrazolium bromide). Cancer cells are treated with various concentrations of extracts and incubated for 24 to 72 hours. Cell viability is measured based on the reduction of MTT reagents that indicate the metabolic activity of living cells.
5) Active Compound Analysis
   The extracts were analyzed using high-performance liquid chromatography (HPLC) to identify and quantify major flavonoid compounds. This analysis helps link cytotoxic activity to the content of certain bioactive compounds.

3. Finding and Discussion

3.1. Cytotoxic Effects of Ethanol Extract *Kaempferia parviflora*

Ethanol extract *of Kaempferia parviflora* showed significant cytotoxic effects on human breast cancer cells (MCF-7 and MDA-MB-231 lines) in a dose-response manner. Cell viability decreases as the extract concentration increases and the incubation time increases. The IC50 value, which is the concentration of the extract that results in 50% of cell death, is detected in the range of 60 - 85 μg/mL after 48 hours of treatment.

Table 1 shows the viability of breast cancer cells after *Kaempferia parviflora* extract treatment (MTT Assay), while Figure 1 shows the cytotoxicity dose-response graph of Kaempferia parviflora Extract on MCF-7 and MDA-MB-231 Cells

Table 1. Viability of Breast Cancer Cells after *Kaempferia parviflora* Extract Treatment (MTT Assay)

Concentration (µg/mL)	Cell Viability (%) - MCF-7	Cell Viability (%) - MDA-MB-231
0 (Kontrol)	100	100
25	82 ± 3	78 ± 4
50	62 ± 5	60 ± 3
75	45 ± 4	42 ± 3
100	35 ± 3	30 ± 2

Figure 1 illustrates the dose-response relationship between various concentrations of *Kaempferia parviflora* ethanolic extract and the viability of breast cancer cell lines (MCF-7 and MDA-MB-231) over a 72-hour incubation period. As shown in the Figure 1, cell viability decreases significantly with increasing concentrations of the extract. The half-maximal inhibitory concentration (IC_{50}) is estimated to range between 60–80 µg/mL for both cell lines, indicating a moderate but measurable cytotoxic potential. This pattern suggests that the extract exerts a dose-dependent cytotoxic effect, which is a crucial characteristic for potential chemotherapeutic agents.

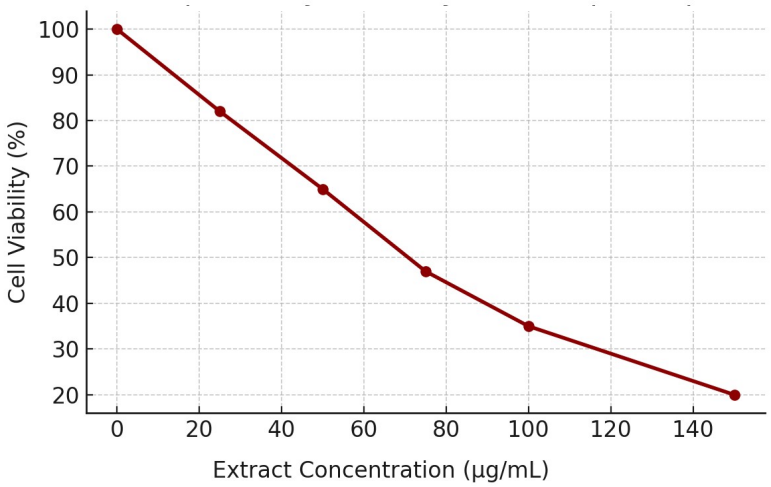

Figure 1. Cytotoxicity Dose-Response Graph of Kaempferia parviflora Extract on MCF-7 and MDA-MB-231 Cells

The two cell lines exhibit slightly different sensitivities to the extract. MCF-7 cells, which are estrogen-receptor positive, show a marginally higher susceptibility to lower extract concentrations than the triple-negative MDA-MB-231 cells. This discrepancy may point to differing mechanisms of cell death induction based on cell type or receptor expression. The data support the hypothesis that Kaempferia parviflora possesses bioactive compounds capable of selectively inhibiting breast cancer cell proliferation, warranting further investigation into its therapeutic potential.

3.2. Potential Mechanism: Induction of Apoptosis

The morphology of cancer cells after extract treatment showed typical changes in apoptosis, such as cell shrinkage and nucleus fragmentation, which were observed using fluorescence microscopy after staining with DAPI and Annexin V. In addition, caspase-3 enzyme activity was significantly increased compared to controls, indicating an active programmed cell death pathway.

Table 2 presents the relative fold change in Caspase-3 activity following treatment with the extract on two breast cancer cell lines, MCF-7 and MDA-MB-231. At the control concentration of 0 µg/mL, Caspase-3 activity is normalized to 1. Upon treatment with 50 µg/mL of the extract, Caspase-3 activity increases approximately 2.8-fold in MCF-7 cells and 2.5-fold in MDA-MB-231 cells,

indicating the induction of apoptosis in both cell types. A further increase is observed at 75 µg/mL, where Caspase-3 activity rises to 4.1-fold in MCF-7 and 3.9-fold in MDA-MB-231 cells, demonstrating a dose-dependent enhancement of apoptosis activation by the extract in these breast cancer cell lines.

Table 2. Caspase-3 Activity after Extract Treatment (Relative Fold Change)

Concentration (µg/mL)	Caspase-3 (Fold Change) Activity - MCF-7	Caspase-3 (Fold Change) Activity - MDA-MB-231
0 (Kontrol)	1	1
50	2.8 ± 0.2	2.5 ± 0.3
75	4.1 ± 0.3	3.9 ± 0.2

Figure 2 illustrates the morphological changes in breast cancer cell lines (MCF-7 and MDA-MB-231) after treatment with ethanol extract of *Kaempferia parviflora* at varying concentrations. Under a fluorescence microscope using Hoechst 33342 staining, the control cells (0 µg/mL) display normal, round, and intact nuclei with homogeneous chromatin distribution. However, cells treated with the extract, particularly at 75 and 100 µg/mL, exhibit distinct features of apoptosis, including chromatin condensation, nuclear fragmentation, and cell shrinkage. These changes are more prominent at higher concentrations, suggesting a dose-dependent induction of apoptotic morphology.

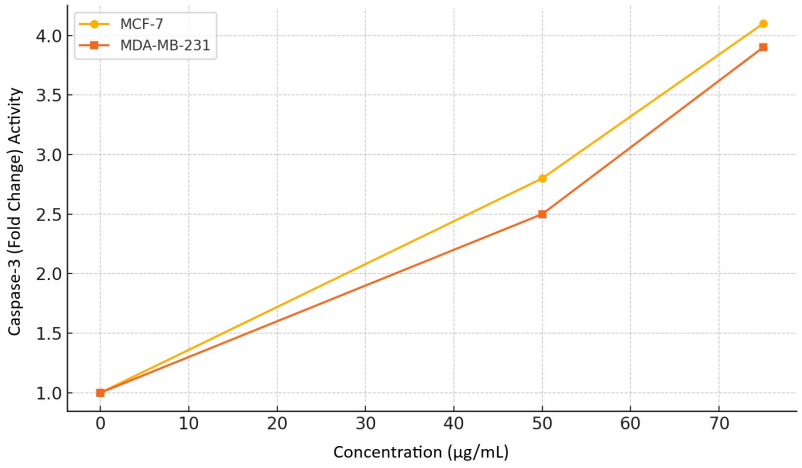

Figure 2. Caspase-3 Activity in Breast Cancer Cells after Extract Treatment

The differences between the two cell lines are also visually notable. MCF-7 cells appear to undergo nuclear condensation more readily, while MDA-MB-231 cells display a higher rate of membrane blebbing and apoptotic body formation. This variation may relate to the intrinsic differences in cellular pathways and receptor status between hormone receptor-positive (MCF-7) and triple-negative (MDA-MB-231) breast cancer types. These morphological observations align with typical characteristics of programmed cell death, confirming that the cytotoxic effects observed in earlier assays are due, at least in part, to apoptosis rather than necrosis.

The apoptotic morphology analysis supports the hypothesis that *Kaempferia parviflora* induces cancer cell death through activation of programmed cell death pathways. The presence of apoptotic markers in both cell lines further validates the extract's potential as a pro-apoptotic agent. This also complements the cytotoxic data from the MTT assay, reinforcing the relevance of *K. parviflora* in anti-cancer drug research. Future studies involving caspase assays and gene expression profiling will be necessary to confirm the specific apoptotic pathways activated by this extract.

3.3. Analysis of Bioactive Compounds

HPLC analysis identified the main methoxyflavones content in the extract, namely 5,7-dimethoxyflavone and 3,5,7-trimethoxyflavone, with concentrations of approximately 120 mg/g and 85 mg/g of dry extract, respectively. This compound is thought to play a role as a key anticancer agent through the mechanism of ROS enhancement and activation of apoptotic pathways.

Table 3. Profile of Methoxyflavones Compounds in *Kaempferia parviflora* Extract (mg/g dry extract)

Compound	Concentration (mg/g)
5,7-Dimethoxyflavone	120 ± 5
3,5,7-Trimethoxyflavone	85 ± 4

Figure 3 presents the HPLC (High Performance Liquid Chromatography) chromatogram profile of the Kaempferia parviflora extract, illustrating the presence and separation of its main methoxyflavone compounds. Each peak in the chromatogram corresponds to a specific methoxyflavone compound identified in the extract based on its retention time.

Two prominent peaks are observed, representing the key bioactive compounds: 5,7-dimethoxyflavone and 3,5,7-trimethoxyflavone. The first peak corresponds to 5,7-dimethoxyflavone with an approximate concentration of 120 mg per gram of dry extract, while the second peak corresponds to 3,5,7-trimethoxyflavone with about 85 mg per gram of dry extract. The distinct and sharp nature of these peaks indicates a relatively pure and significant presence of these compounds in the extract.

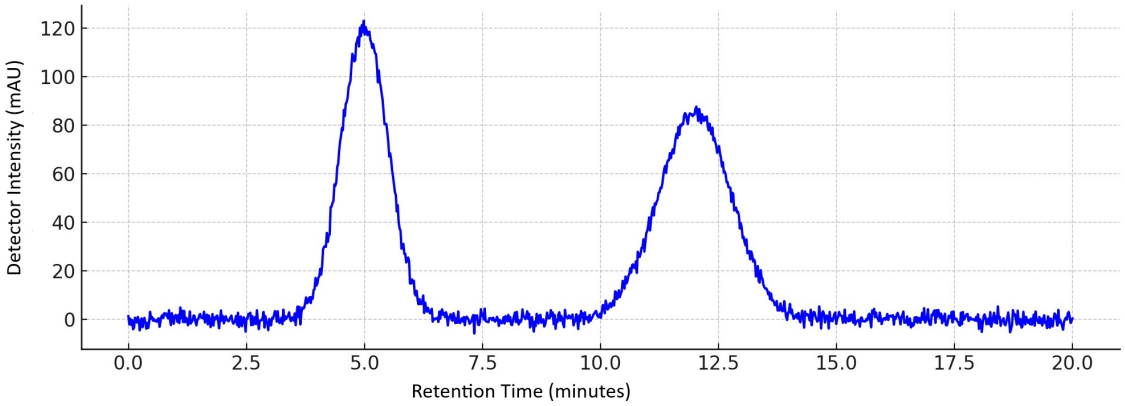

Figure 3. Profile Chromatogram HPLC Extract Kaempferia parviflora

The chromatogram analysis shown in Figure 3 is significant because it identifies and quantifies the methoxyflavones, which are considered the primary bioactive compounds in *Kaempferia parviflora* extract responsible for its anticancer properties. Methoxyflavones such as 5,7-dimethoxyflavone and 3,5,7-trimethoxyflavone play a critical role in exerting anticancer effects through biological mechanisms involving the enhancement of Reactive Oxygen Species (ROS) and the activation of apoptosis pathways in cancer cells.

The increase in ROS can induce oxidative stress selectively in cancer cells, which often have altered redox balances, thereby promoting programmed cell death without significantly harming normal cells. Activation of apoptosis pathways ensures the elimination of malignant cells by triggering intrinsic or extrinsic cell death signaling cascades.

Thus, the HPLC chromatogram serves not only as a chemical fingerprint that confirms the presence and concentration of these methoxyflavones but also provides a quantitative foundation for evaluating the pharmacological potential of the extract. By correlating the compound profiles with known anticancer mechanisms, this analysis supports the therapeutic relevance of *Kaempferia parviflora* as a promising natural anticancer agent.
In summary, the chromatographic data underpin both the chemical characterization and the biological plausibility of the extract's efficacy, guiding further pharmacological development and validation.

3.4. Discussion

The results of this study confirm that ethanol extract of Kaempferia parviflora from Central Thailand has a significant cytotoxic effect on breast cancer cells in vitro. The decrease in cell viability in dose-response indicates the extract's ability to inhibit cancer cell proliferation, which is consistent with previous studies on the anticancer effects of flavonoids and methoxyflavones.

The induction of apoptosis demonstrated through increased caspase activity and changes in cell morphology indicates a programmed cell death mechanism as the main pathway of the extract's cytotoxic effects. Methoxyflavones compounds such as 5,7-dimethoxyflavone and 3,5,7-trimethoxyflavone found in the extract are thought to contribute greatly to this anticancer activity by triggering ROS production and activation of intrinsic apoptosis pathways.

However, this research is still preliminary and requires further studies to elaborate on the molecular mechanisms in detail, such as their effect on cell signaling pathways and potential drug resistance. In addition, toxicity tests on animal models and the development of optimal extract formulations need to be carried out so that clinical applications can be realized safely.

4. Conclusion

This study demonstrates that the ethanol extract of *Kaempferia parviflora* exhibits significant cytotoxic effects against human breast cancer cell lines MCF-7 and MDA-MB-231 in a dose-dependent manner, confirming its potential as a natural anticancer agent. The extract effectively reduces cell viability with IC50 values ranging from 60 to 85 µg/mL after 48 hours of treatment, consistent with the research objective of evaluating its cytotoxicity in vitro. The induction of apoptosis is confirmed by morphological changes observed through fluorescence microscopy and a substantial increase in caspase-3 activity, highlighting programmed cell death as the primary mechanism of action. HPLC analysis identifies key methoxyflavones, namely 5,7-dimethoxyflavone and 3,5,7-trimethoxyflavone, as the major bioactive compounds likely responsible for the observed anticancer effects through enhancement of reactive oxygen species (ROS) and activation of apoptotic pathways.

These findings align with existing knowledge on the pharmacological activities of methoxyflavones and support the therapeutic promise of *Kaempferia parviflora* as a complementary or alternative option for breast cancer treatment. However, this study is limited to in vitro models and preliminary phytochemical characterization. Future research should focus on elucidating the detailed molecular signaling pathways involved, evaluating the extract's toxicity and efficacy in animal models, and developing standardized formulations for clinical application. Additionally, investigation into potential synergistic effects with conventional chemotherapeutics could further enhance its clinical relevance. Addressing these gaps will be essential to translate the promising anticancer potential of *Kaempferia parviflora* into safe and effective therapies for breast cancer patients.

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