Research article

The effect of ginger extract on expression of Gli1 and Patched-1 genes involved in breast cancer signaling pathway under *in vitro* condition

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ABSTRACT

Introduction and Aim: Breast cancer ranks among the leading causes of death in women worldwide. Ginger has shown potential efficacy against certain cancer types, surpassing conventional therapies such as chemotherapy and radiation. However, its molecular mechanisms remain less understood.

Materials and Methods: In this study, MCF-7 cancer cells were cultured and treated with various concentrations of aqueous ginger extract (20, 30, 45, 65 µg/mL) for 12, 48, and 72 hours. The effects were assessed through gene expression analysis and cell vitality assays (MTT).

Results: The cell vitality test revealed a direct correlation between cytotoxicity and extract concentration. Concentrations exceeding 30 µg/mL exhibited significant cell death (IC50 of 104.03 µg/mL). Gene expression analysis demonstrated an increase in Patched-1 and a decrease in Gli1 expression with rising extract concentrations. The maximum Patched-1 expression occurred at 65 µg/mL after 72 hours. Patched-1 (oncogene) and Gli1 (tumor suppressor) are pivotal genes in the hedgehog (Hh) signalling pathway associated with breast cancer.

Conclusion: It appears that ginger compounds play a substantial role in regulating cancer progression by influencing key components of the hedgehog signalling pathway.

Keywords: Ginger extract; breast cancer; gene expression analysis; Patched-1; Gli1.

INTRODUCTION

Breast cancer stands as the most prevalent cancer among women globally, with approximately 1 in 8 women facing a breast cancer diagnosis in their lifetime (1,2). In Iran, it ranks as the second most common cancer after skin cancer, yet claims the top spot among women, with an annual increase of 7000 cases (3). Amidst the heterogeneous nature of this disease, recent decades have witnessed the development of targeted therapies, including hormone therapy for estrogen receptor (ER) expression and the use of trastuzumab to inhibit HER2 signalling (4).

Innovative approaches to cancer treatment have led to a growing interest in herbal consumption, given their minimal side effects (5). With advancements in molecular science, several anticancer herbs have emerged, demonstrating their potential to inhibit cancer-activating enzymes, stimulate DNA repair mechanisms, enhance protective enzymes, induce antioxidant actions, and boost the body's immunity (6). Among these, ginger, derived from the *Zingiber officinale* plant, has gained prominence in chemotherapy (7,8). Ginger extract has exhibited significant antifungal (9), antioxidant (10), and anti-tumor effects (11). Key compounds responsible for these biological activities include shogaols, geraniol, pardols, geranyl acetate, sesquiterpenes, pyrogallol, zingiberol, curcumene, β-sesqui- phellandrene, and β-bisabolene (12,13).

Numerous studies in recent years have explored the preventive effects of ginger on cancer cell proliferation under in vitro and in vivo conditions (14-18). Regarding ginger's impact on breast cancer, studies have demonstrated that [6]-gingerol inhibits metastasis of MDA-MB-231 human breast cancer cells, resulting in a 16% reduction in invasion and motility at a ginger extract concentration of 10µM (19). Moreover, using chitosan-coated nanostructured lipid carriers (CS-NLCs) for (6)-gingerol delivery to MCF-7 breast cancer cells has demonstrated significant cytotoxic activity against MCF-7 cancer cells, while showing minimal impact on human umbilical vein endothelial (HUVEC) normal cell line (20).

Given the significance and complexity of cancers, coupled with the limitations and diverse side effects of conventional treatments, experimental studies aimed at comprehending pathways leading to cancer suppression and drug development, particularly utilizing herbal remedies, hold significant value. The primary objective of this study is to analyse the therapeutic effects of ginger extract on breast cancer cell death and explore its impact on the expression of genes involved in signalling pathways. By investigating the influence of ginger extract on the expression of major genes associated with breast cancer signalling pathways, we aim to contribute to the reduction of side effects associated with chemical drugs in breast cancer treatment.

MATERIALS AND METHODS

Aqueous ginger extract preparation

To obtain the aqueous ginger extract, 10g of finely powdered dried ginger was dissolved in a 70% ethanol solution with a ratio of 1:25. The mixture was left undisturbed in a dark environment for 48 hours. Subsequently, the solution underwent filtration using a fine filter, and the resulting extract was concentrated using a Rotary evaporator. The concentrated extract was carefully transferred to small culture plates using a sampler. In the final step, the plates were subjected to drying in an oven maintained at a temperature of 40°C for a duration of two days to ensure complete dryness. The residual layer on each plate was meticulously separated using a scalpel and reserved for cell treatment procedures.

Cell culture and ginger extract treatments

In this investigation, the MCF-7 breast cancer cell line obtained from the Iranian Biological Resource Center (IBRC) was employed. For cell culture, a media blend consisting of a 1:1 ratio of Dulbecco Modified Eagle Media (DMEM) High Glucose and DMEM F12, supplemented with 10% fetal bovine serum (FBS) and 1% Pen Strep antibiotic (100U/mL penicillin; 50µg/mL streptomycin), was utilized. The MCF-7 cell line was cultured in 75ml cell culture flasks and maintained in an incubator at a temperature of 37°C, humidity set at 95%, and 5% CO_2.

The impact of ginger extract was systematically examined across varying dosages and time frames, encompassing two biological replicates. Sequential cell passages were executed, with approximately 5 million cells cultured in each 75ml filtered flask. Following a 24-hour incubation period and confirmation of cell adhesion to the flasks, each flask received treatments with concentrations of 20, 30, 45, and 65µg/mL of ginger extract. Subsequent assessments were conducted after 12, 48, and 72 hours, respectively, maintaining consistency across two biological replicates. The control group comprised flasks with MCF-7 cells devoid of ginger extract treatment.

For cell viability assessments, images were captured using an inverted microscope at X200 magnification over consecutive days. To facilitate cell counting and passaging, the culture media in the flasks were aspirated, and the cells were washed with PBS (Phosphate Buffered Saline) buffer. Following a 3-minute trypsinization and incubation period, cells were centrifuged for 5 minutes at 1500 rpm to generate a cell suspension. Cell staining and counting were performed using a 1:1 ratio of cell suspension and trypan blue on a Neobar lam, observed through an optical microscope.

Cell viability analysis

The cytotoxic effect of ginger extract on cell viability was assessed through the MTT assay (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue), and the IC50 (concentration of extract causing 50% growth inhibition) was determined. In this assay, approximately 5000 cells from the cell suspension, both with and without ginger extract (control samples), were introduced into each well of a 96-well flat-bottom cell culture plate.

Initially, cells in each well were incubated with culture media (totalling 200 microliters per well) for 24 hours at 37°C, 5% CO2, and 95% humidity. Subsequently, the culture media was removed, and varying concentrations of ginger extract, dissolved in autoclaved deionized water, were added in doses of 5, 10, 20, 30, 45, 65, 70, 80, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, and 1000µg/mL to each well with fresh cell culture media. The cells were then incubated for an additional 72 hours.

For each concentration, three wells of the 96-well plate were utilized, and three wells served as control samples (containing only culture media without extract). Subsequently, the culture media was aspirated, and the cells were incubated in an MTT solution (20µg/mL) for 4 hours. After dissolving formazan crystals using 100µl of DMSO and 10µl of glycine buffer, light absorbance was measured at 570 nm using a fluorescence plate reader (Bio-Tek Instruments; Winooski, VT). The percentage of cell viability and cytotoxicity index were calculated using the following formula (21):

$$\text{Cell Viability } (\%) = \frac{\text{A 570 nm of Treated Cell}}{\text{A 570 nm of Control Cell}} \times 100$$

$$\text{Growth Inhibition } (\%) = \frac{\text{OD of Control Cell} - \text{OD of Treated Cell}}{\text{OD of Control Cell}} \times 100$$

RNA extraction

The separation of cells in each flask was initiated by Trypsin-EDTA, followed by centrifugation with a phosphate buffer to remove the culture media. Subsequently, the cells were carefully transferred to RNase-free microtubes. RNA extraction from the cultured cells was conducted using the RNX-plus kit (DENAzist, Iran), following the company's instructions with minor adjustments. DNase I (0.5µg/mL of DNase I for 30 mins. at 37°C) was employed to eliminate DNA contaminations. All extracted RNA samples were subjected to quantitative and qualitative assessment using UV spectro-photometry (Nanodrop 2000c, Thermoscientific, USA) and Agarose gel electrophoresis.

cDNA synthesis

cDNA synthesis was done by use of cDNA synthesis kit (Thermoscientific, USA) according to company instructions. As brief, cDNA was synthesized using RevertAid First Strand cDNA Kit, Fermentas 10000 U of M-MuLV Reverse transcriptase enzyme, oligo (dt)

and Random Hexamer primers in the volume of 20 microliter (in specific times and temperature according to kit) in thermocycler device.

Primer designing and qRT-PCR

For primer design (Table 1), the mRNA sequences of Gli1 and Patched-1 genes were retrieved from Genbank NCBI with accession numbers NM_001160045 and NM_001083605. Primers were designed using Primer3 Software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) based on precise parameters. The specificity and accuracy of the designed primers were confirmed through BLAST analysis and conventional PCR assay, ensuring a single peak in melting curve analysis and a single band in high-resolution agarose gel electrophoresis.

Real-time PCR was conducted to investigate the expression and copy number of genes using 5X HOT FIREPol EvaGreen master mix (Solis BioDyne, Estonia) as per the company's instructions and the QIAGEN thermocycler instrument (ROTOR GENE Q, Germany). Temperature cycles were executed as follows: initial denaturation at 95°C for 10 minutes, followed by 45 cycles, including denaturation at 95°C for 30 seconds, primer annealing at 60°C for 15 seconds, and extension at 72°C for 20 seconds. The final stage comprised a concluding extension at 72°C for 10 minutes.

GAPDH and β-actin genes were utilized as reference genes to calculate the relative expression level of Gli1 and Patched-1 genes (22). The relative expression level of genes was calculated using the $2^{-\Delta\Delta CT}$ formula in comparison to reference control genes (23).

Table 1. Primers designed for gene expression analysis

Gene	Primer Sequence F/R
GAPDH	(F)5'-CTCCTCACAGTTGCCATGTAGAC-3'
	(R)5'-GGTTGAGCACAGGGTACTTTATTG-3'
Beta actin	(F)5'-AAATGCTTCTAGGCGGACTATG-3'
	(R)5'-AACAAATAAAGCCATGCCAATC-3'
GLI-1	(F)5'-GGGCAACATGAGTGTCTTACTG-3'
	(R)5'-TTAGGAAATGCGGTCTGTGATG-3'
Patched-1	(F)5'-CTCCCTCTCTTAAATGGAGCAG-3'
	(R)5'-TCCTACATAATGCCTGCCTCTAC-3'

Statistical analysis

For the statistical significance analysis of relative gene expression levels compared with reference genes, a completely randomized design was employed as a factorial experiment. Mean values of relative expression levels were further compared using LSD and Duncan tests (P=0.05, P=0.01) utilizing SPSS version 16.0.

RESULTS

Cytotoxicity effect of ginger extract on MCF-7 cancer cell line

MTT analysis revealed a significant increase in cytotoxicity of MCF-7 cancer cells after 72 hours of incubation, particularly at concentrations exceeding 30µg/ml of the ginger extract. At this concentration, cell death reached 25% (Table 2). A clear correlation was established between cell cytotoxicity and extract concentration, with an observed increase in cell death as the extract concentration escalated. The calculated IC50 was determined to be 104.03µg/mL (Fig. 1).

Effect of time on Gli1 and Patched-1 gene expression

The results of Patched-1 gene expression analysis, compared with control genes at 12, 48, and 72 hours, and across various concentrations of ginger extract, demonstrated an increase in the expression of the Patched-1 gene up to 45µg/mL. However, beyond this concentration, a significant decrease in its expression was observed (Figs. 2A and 2B). Specifically, concentrations other than 20µg/mL of ginger extract resulted in significant changes ($p<0.01$) in Patched-1 gene expression. Notably, the concentration of 20µg/mL exhibited similar results to the control treatment. Conversely, in the case of the Gli1 gene, an increase in extract concentration led to a reduction in its expression.

As depicted in Fig. 2A, there were no changes in the expression of the Gli1 gene compared to the control treatment up to a concentration of 30µg/mL for the initial 12 hours. However, an increase in concentration to 45 and 65µg/mL significantly decreased gene expression ($p<0.05$ and $p<0.01$, respectively). In the 48-hour treatment with the extract, although the results for concentrations of 30 and 45µg/mL were statistically similar (Fig.2B), it can be inferred that at higher extract concentrations, a significant decrease in Gli1 gene expression occurred compared to the control treatment.

Fig. 2C illustrates Gli1 and Patched-1 relative gene expressions after 72 hours at concentrations of 20, 30, 45, and 65µg/mL. A clear positive relationship between gene expression and extract concentration was observed after 72 hours of treatment. This relationship was significant for both Gli1 and Patched-1, indicating an ascending and descending trend with concentration for Patched-1 and Gli1, respectively. According to the Duncan test, the decrease and increase in gene expression for Gli1 and Patched-1, respectively, at a concentration of 65µg/mL of ginger extract compared with the control treatment was significant ($p<0.01$).

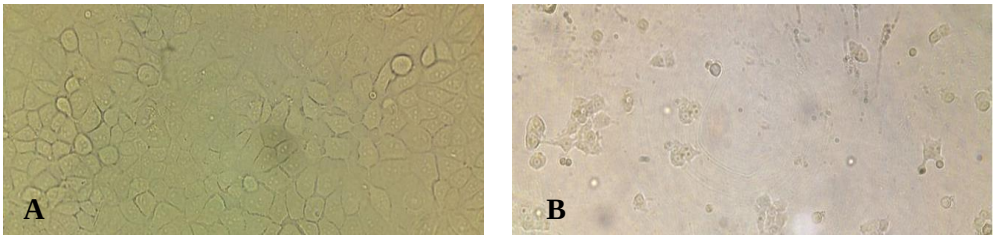

Fig.1: MCF-7 breast cancer cell line. A) Control treatment (no ginger extract), and B) Concentration of 104.03µg/mL ginger extract (IC_{50}).

Table 2. Effect of different ginger extract concentrations on light absorbance of MCF-7 cell line according to MTT assay in comparison with control samples.

Concentration (µg/mL)	Aqueous extract	
	Absorbance (in 570 nm)	Growth inhibition (%)
5	0.980±0.057	2.0±5.7
10	0.960±0.053	4.0±5.3
15	0.925±0.117	7.5±1.7
20	0.847±0.053	15.3±5.3
30	0.748±0.019*	25.2±1.9
45	0.661±0.041*	33.9±4.1
65	0.591±0.055*	40.9±5.5
70	0.577±0.034*	42.3±3.4
80	0.554±0.047**	44.6±4.7
100	0.518±0.031**	48.2±3.1
150	0.457±0.055**	54.3±5.5
200	0.419±0.030**	58.1±3.0
300	0.370±0.034**	63.0±3.4
400	0.339±0.039**	66.1±3.9
500	0.316±0.014**	68.4±1.4
600	0.299±0.014**	70.1±1.4
700	0.285±0.029**	71.5±2.9
800	0.274±0.028**	72.6±2.8
900	0.264±0.039**	73.6±3.9
1000	0.256±0.033**	74.4±3.3
Control	1.000±0.142	

Each value shows the mean plus or minus standard error of three independent samples.**: P<0.01,*: P<0.05

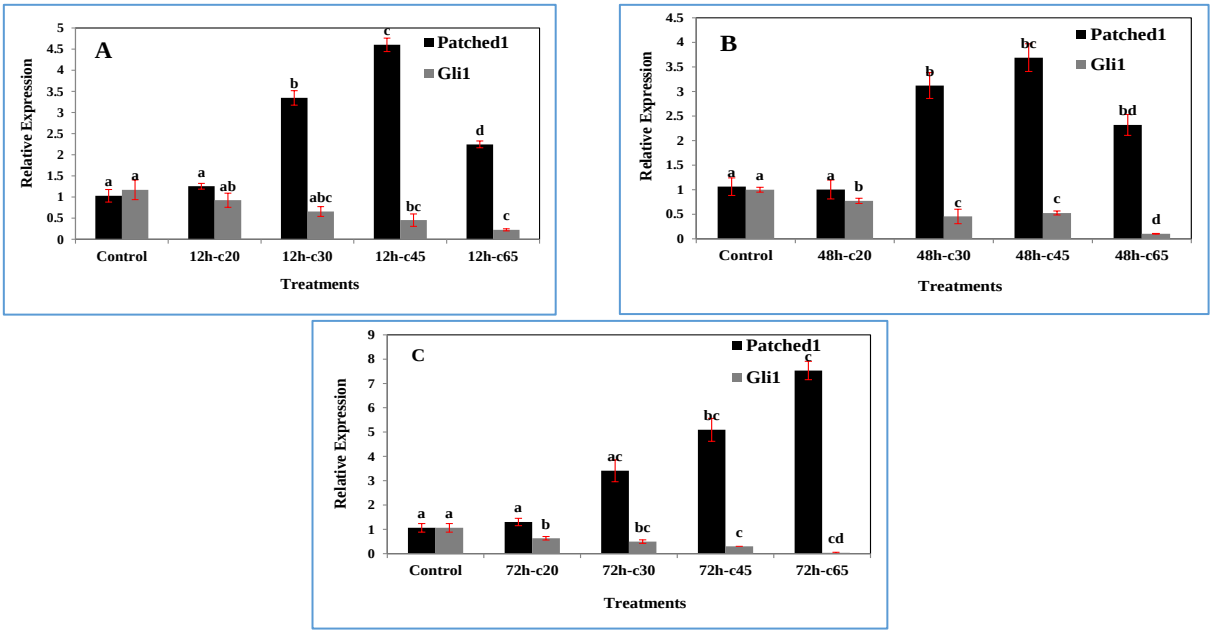

Fig.2: Gli1 and Patched-1 gene expressions after A) 12, B) 48, and C) 72 hours in concentrations of 20, 30, 45, and 65µg/mL of ginger extract.

Effect of various concentrations of extract on Gli1 and Patched-1 gene expression

The results of relative expression analysis for Gli1 and Patched-1 genes at concentrations of 20, 30, 45, and 65µg/mL of ginger extract are depicted in Fig.3. The findings indicate that changes in the expressions of Gli1 and Patched-1 genes over various time points and concentrations (20, 30, 45, and 65µg/mL) were generally not significant, except for 65µg/mL of extract at 72 hours for the Patched-1 gene and at 48 and 72 hours for the Gli1 gene. Overall, the results suggest that time does not substantially alter gene expression for both genes at the specified extract concentrations.

Data correlation from gene expression analysis and cytotoxicity test (MTT)

The correlation between data obtained from the relative expression analysis of Gli1 and Patched-1 genes and the cytotoxicity test (MTT) is illustrated in Fig. 4. The data from the cell viability test and relative expression analysis of Gli1 and Patched-1 genes exhibit a very high correlation (r=0.98 for Patched-1 and r=0.97 for Gli1). In low extract concentrations, Patched-1 expression is low and increases with the rise in extract concentration. Simultaneously, cell viability decreases with an increase in extract concentration, as clearly depicted in the diagram.

Conversely, in low extract concentrations, Gli1 gene expression is high. As the extract concentration increases, there is a decrease in cell viability, accompanied by a reduction in gene expression. The results demonstrate that data from cell culture experiments are consistent with the results of molecular experiments and exhibit a high negative correlation. Furthermore, since Gli1 and Patched-1 genes are two of the main genes involved in the breast cancer signalling pathway known as the Hedgehog Signalling Pathway (24, 25), it is evident that ginger extract significantly affects this pathway.

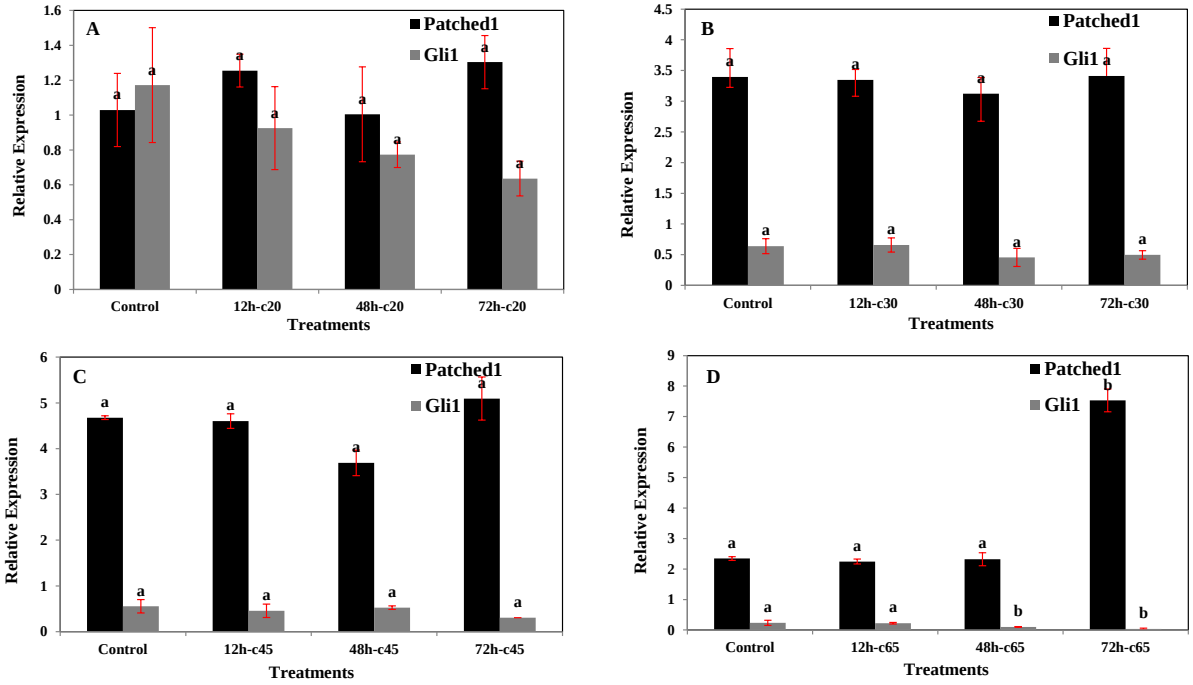

Fig.3: Gli1 and Patched-1 relative genes expression in concentrations of A) 20, B) 30, C) 45, and D) 65µg/mL of ginger extract after 12, 48, and 72 hours.

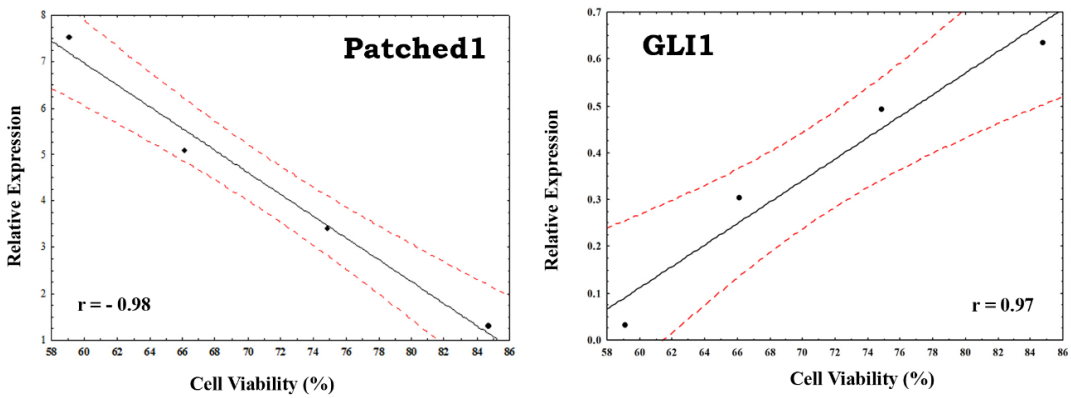

Fig.4: Data correlation of gene expression analysis and cytotoxicity test (MTT) (black and red lines show trend line and the 95% significance interval, respectively)

DISCUSSION

In the pursuit of effective drugs that selectively target cancer cells while sparing healthy ones, a comprehensive understanding of the cellular and molecular mechanisms underlying cancer creation and progression is imperative. Several studies in recent years have explored the therapeutic properties of ginger and its positive impact on cancer treatment. However, before considering the application of ginger extract in cancer treatment or recovery from side effects, it is crucial to elucidate its mechanism of action. One approach is the quantitative evaluation of the effects of ginger extract on the expression of genes involved in cancer signalling pathways, especially breast cancer.

This study focused on the analysis of the effects of ginger extract on breast cancer cell death, specifically examining genes in the signalling pathway (Patched-1 and Gli1). The results of this study revealed a pronounced cytotoxic effect of ginger extract on MCF-7 breast cancer cells, along with alterations in the expression of genes associated with the breast cancer signalling pathway.

The relationship between MCF-7 cell cytotoxicity and extract concentration was established, indicating a significant increase in cell death when the extract concentration exceeded 30µg/mL, with an IC50 calculated to be 104.03µg/mL. Gene expression analysis demonstrated that an increase in extract concentration led to elevated Patched-1 gene expression, peaking at 65µg/mL after 72 hours. These findings align with previous studies on Patched-1 expression in breast cancer.

Examination of various ginger extract concentrations highlighted that at low concentrations (20µg/mL), the results were comparable to the control treatment, while other concentrations induced significant alterations in Patched-1 gene expression at a significance level ($p<0.01$). Conversely, an increase in extract concentration resulted in decreased Gli1 gene expression, consistent with existing literature on Gli1 expression in breast cancer.

CONCLUSION

The effects of ginger extract on Gli1 and Patched-1 gene expressions were found to be more dependent on concentration rather than time. This study establishes that ginger extract can influence tumor-suppressor genes (Patched-1) and oncogenes (Gli1) in the breast cancer signaling pathway, known as the Hedgehog Signaling Pathway. The molecular and cellular insights provided by this study contribute to the understanding of the pharmaceutical effects of ginger and its potential application as an anticancer drug.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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