Trop J Nat Prod Res, December 2025; 9(12): 6319 – 6325

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Induction of JUN and FOS Expression by Gallic Acid Derivatives in MCF-7 Breast Cancer Cells: An in Silico and RT-PCR Study

Ade Arsianti[1,2], Anisah Firda Rachmani[3], Aryo Tedjo[1,2]*, Norma Nur Azizah[2], Shun Hirota[4], Hiroki Tanimoto[5]

[1]*Department of Medical Chemistry, Faculty of Medicine, Universitas Indonesia, Salemba Raya 6, Jakarta 10430, Indonesia*
[2]*Drug Development Research Cluster, Indonesian Medical Education and Research Institute, Faculty of Medicine, Universitas Indonesia, Salemba Raya 6, Jakarta 10430, Indonesia*
[3]*Biomedical Science, Faculty of Medicine, Universitas Indonesia, Salemba Raya 6, Jakarta 10430, Indonesia*
[4]*Division of Materials Science, Graduate School of Science and Technology, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan*
[5]*Faculty of Pharmaceutical Sciences, University of Toyama, 2630 Sugitani, Toyama, 930-0194 Japan.*

ARTICLE INFO

Article history:
Received 15 September 2025
Revised 14 November 2025
Accepted 20 December 2025
Published online 01 January 2026

ABSTRACT

Breast cancer remains the leading malignancy worldwide and continues to cause high morbidity and mortality. Although chemotherapy, hormonal therapy, immunotherapy, and targeted treatments are available, these therapeutic options frequently induce adverse effects and contribute to resistance, limiting long-term outcomes. Gallic acid and its derivatives have been widely reported to exert cytotoxic and pro-apoptotic effects on various cancer cell lines, including MCF7 breast cancer cells. However, the underlying molecular mechanisms, particularly those involving apoptosis-related gene modulation, have not been fully elucidated. This study investigated the expression changes of key apoptosis-related genes in MCF7 cells following treatment with two gallic acid derivatives, N-octyl gallamide and N-tert-butyl gallamide, using integrated in silico and in vitro approaches. Differentially expressed genes (DEGs) were identified from the GSE158788 dataset using GEO2R. Protein–protein interaction networks were constructed using Cytoscape, and KEGG pathway enrichment was performed to determine relevant signaling pathways. JUN and FOS, identified as hub genes, were validated using quantitative RT-PCR in MCF7 cells treated with IC_{50} and $2\times IC_{50}$ concentrations of the test compounds. DEG and PPI analyses identified JUN and FOS as key hub genes associated with gallic acid–mediated apoptosis. RT-PCR results demonstrated that N-octyl gallamide significantly upregulated JUN (ΔΔCTs 1.25 ± 0.251; $p < 0.05$) and FOS (1.82 ± 0.691; $p < 0.05$), exceeding the effects of tamoxifen. In contrast, N-tert-butyl gallamide did not significantly alter JUN or FOS expression (ΔΔCTs 0.76 ± 0.053; $p > 0.05$). N-octyl gallamide exhibits strong potential as an anticancer agent through JUN and FOS activation in MCF7 cells. These findings highlight its promise for further development as an alternative or adjuvant breast cancer therapy.

***Keywords*:** differentially expressed genes, gallic acid, N-octyl gallamide, breast cancer, apoptosis, qRT-PCR

Introduction

Breast cancer is the most commonly diagnosed cancer worldwide and ranks among the leading causes of cancer-related mortality. In Indonesia, it remains the cancer with the highest incidence and the second-leading cause of cancer death.[1] Its persistent clinical burden highlights the need for more effective and safer therapeutic options.[2] Although current treatment strategies, including chemotherapy, radiotherapy, hormonal therapy, immunotherapy, and combined modalities, have improved patient outcomes, their effectiveness is often limited by systemic toxicity, poor selectivity, or the development of drug resistance.[3] These limitations justify the exploration of new anticancer agents with better safety and mechanistic specificity. Gallic acid (3,4,5-trihydroxybenzoic acid), a polyphenolic compound found in plants, has demonstrated antioxidant, anti-inflammatory, antimicrobial, and cytotoxic properties in various cancer models.[5-7] Its anticancer effects occur through mechanisms such as caspase activation and increased ROS generation.[5-7] However, its hydrophilic nature restricts membrane permeability, reducing its bioactivity.[8] Structural modification through alkyl substitution has been reported to enhance lipophilicity and strengthen cytotoxic potential by improving interactions with key cancer-related proteins, including CASP3, CASP7, JUN, and AKT1, in MCF-7 breast cancer cells.[6,7] Preliminary studies indicate that gallic acid derivatives such as N-octyl gallamide, N-tert-butyl gallamide, and N-isoamyl gallamide induce apoptotic responses although their molecular mechanisms and downstream gene targets remain unclear.[9]

High-throughput platforms, including microarray and RNA sequencing, facilitate large-scale identification of biomarkers and mechanisms involved in cancer progression and drug response.[10,11] Gene Expression Omnibus (GEO) provides access to publicly available transcriptomic datasets for such analyses.[12] Differentially expressed gene (DEG) analysis enables the identification of key genes and signaling pathways influenced by bioactive compounds.[11] Studies have used DEG analysis to investigate the effects of gallic acid in metabolic disorders and cervical cancer, showing its ability to modulate apoptosis, ferroptosis, and necroptosis.[13,14] However, to date, no DEG-based investigation has evaluated the transcriptomic effects of gallic acid derivatives in MCF-

*Corresponding author. E mail: aryo.tedjo@ui.ac.id
Tel: +62-8998452818

Citation: Arsianti A, Rachmani AF, Tedjo A, Azizah NN, Hirota S, Tanimoto H. Induction of JUN and FOS Expression by Gallic Acid Derivatives in MCF7 Breast Cancer Cells: An in Silico and RT-PCR Study. Trop J Nat Prod Res. 2025; 9(12): 6319 – 6325 https://doi.org/10.26538/tjnpr/v9i12.53

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

7 cells. Therefore, this study was designed to identify DEGs in the GSE158788 dataset to characterize gene expression changes induced by gallic acid derivatives in MCF-7 cells. In silico analysis using the KEGG database was performed to determine key signaling pathways and hub genes, which were validated experimentally using real-time PCR to confirm their relevance at the molecular level. To the best of our knowledge, this study is the first to integrate DEG analysis with KEGG-based signaling pathway exploration to elucidate the transcriptomic effects of gallic acid derivatives (i.e., N-octyl gallamide, N-tert-butyl gallamide, and N-isoamyl gallamide) in MCF-7 breast cancer cells, with in vitro RT-PCR validation of key regulatory genes. Its integrative workflow provides new insight into the molecular mechanisms influenced by these compounds and identifies potential gene targets for further therapeutic development.

Materials and Methods

Gene Expression Data Analysis of Cancer Related to Gallic Acid

Gene expression analysis was conducted using the GSE158788 dataset retrieved from the GEO database. GEO2R (NCBI, USA) was used to identify DEGs with $p < 0.05$ and $\log_2FC > 0.5$ (up) or < -0.5 (down). Enrichment and pathway analyses were performed using ShinyGO v0.76, while apoptosis- and cancer-related pathways were confirmed using KEGG (2024 release). Overlapping genes among time points were visualized using Venny v2.1. Analyses were conducted in triplicate (n = 3).

Protein–Protein Interactions

DEGs were subjected to functional enrichment using Enrichr (2024 update). Protein–protein interaction (PPI) networks were constructed with Cytoscape v3.10.3 integrated with STRING v12.0 (confidence score ≥0.90). Hub genes were identified using CytoHubba (MCC and Closeness Centrality algorithms), and modules were evaluated using MCODE.

Primer Design

Target mRNA sequences were retrieved from NCBI Gene (Entrez). Primer-BLAST and Primer3Plus v3.2 were employed for primer design with the following criteria: product size 70–200 bp, primer length 20 ± 2 bp, Tm 57–63 °C, GC content 40%–60%, ΔTm ≤2–3 °C, and Homo sapiens (ID: 9606). Primer structure and stability were assessed using NetPrimer to exclude hairpins and high-energy dimers.[17]

Validation of Target Gene Expression in MCF7 Cells

MCF7 cells were cultured in T-75 flasks containing complete medium composed of DMEM, 10% FBS, 1% penicillin–streptomycin, and antifungal amphotericin B. Cells were incubated in a 5% CO_2 incubator at 37 °C for 2–3 days. Once the culture reached 80% confluence, the cells were harvested and subcultured. The medium in the T-flask was removed and replaced with 4 mL of 0.25% trypsin-EDTA, followed by incubation for 5 minutes in a CO_2 incubator. Subsequently, 8 mL of complete medium was added to inactivate trypsin. The cell suspension was transferred into a 15 mL conical tube and centrifuged at 1,500 rpm for 10 minutes. The supernatant was discarded, and the cell pellet was resuspended in 1 mL of complete medium.[18] For cell counting, 10 µL of the suspension was mixed with 10 µL of trypan blue, and 10 µL of this mixture was loaded into a hemocytometer and observed under a microscope. A total of 500,000 cells were seeded into each well of a six-well plate containing complete medium and incubated for 24 hours. The medium was then replaced with medium containing the test compounds (N-octyl gallamide, N-tert-butyl gallamide, and tamoxifen) at IC_{50} and $2\times IC_{50}$ concentrations, with 0.1% DMSO serving as the control. Cells were incubated for an additional 24 hours in a CO_2 incubator. RNA isolation was performed using the Isolation reagent (No.RC112-01, Vazyme, China). After 24 hours of treatment with the test compounds, the medium was replaced with lysis buffer, and the cell lysates were transferred into microtubes containing gDNA filter columns and centrifuged at 12,000 rpm for 30 seconds. The filtrates were mixed with 125 µL of absolute ethanol and transferred into RNA columns, followed by centrifugation. The washing step was performed twice with buffer, and the RNA column was transferred to an RNase-free microtube and eluted with RNase-free ddH_2O before final centrifugation. The obtained RNA was either used immediately for RT-PCR or stored at –85 to –65 °C. RNA purity was assessed using nuclease-free water as a blank, with absorbance measured at 260/280 nm and 260/230 nm. RNA was considered pure if the 260/280 ratio ranged from 1.8 to 2.0 ng/mL. Reverse transcription was performed to synthesize cDNA strands from the isolated RNA using a HiScript III RT Supermix qPCR kit (Vazyme, R323-01). The RNA input concentration was 50 ng/µL, with reaction conditions of 37 °C for 15 minutes and 85 °C for 5 seconds. The resulting cDNA was either used immediately for RT-PCR or stored at –20 °C. Quantitative RT-PCR (qRT-PCR) was carried out using a master mix containing SYBR Green reporter, forward and reverse primers, cDNA template, and nuclease-free water, followed by preparation of the reaction mix for each sample tube. The resulting data were analyzed, and relative gene expression levels were determined using the Livak (ΔΔCt) method.[19]

Results and Discussion

Gene Expression Data Analysis of Cancer Related to Gallic Acid

The GSE158788 dataset represents RNA-Seq data from HeLa cells treated with gallic acid (50 µg/mL) to examine its effect on cell apoptosis. This dataset was utilized to identify the "common DEGs" as targets for predicting gene expression responses of MCF7 breast cancer cells to gallic acid. DEG analysis was performed using GEO2R with thresholds of $Log2FC \geq 0.5$ and the adjusted p-value ≤ 0.05. Comparisons were conducted between the control group and gallic acid–treated groups at 2, 4, 6, and 9 hours. From the KEGG signaling pathway mapping, nine pathways related to breast cancer and apoptosis were identified, with three overlapping pathways (Table 1).

Table 1: Groups of Pathways Related to Breast Cancer and Apoptosis Based on DEG Analysis from the GSE158788 Dataset

Gene Group	Related Pathway
Apoptosis	hsa04668 TNF signaling pathway hsa04064 NF-kappa B signaling pathway hsa04141 Protein processing in endoplasmic reticulum hsa04210 Apoptosis
Breast cancer	hsa04310 Wnt signaling pathway hsa04330 Notch signaling pathway hsa04110 Cell cycle hsa04915 Estrogen signaling pathway hsa03440 Homologous recombination hsa04010 MAPK signaling pathway
Both	hsa04115 p53 signaling pathway hsa04151 PI3K-Akt signaling pathway

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

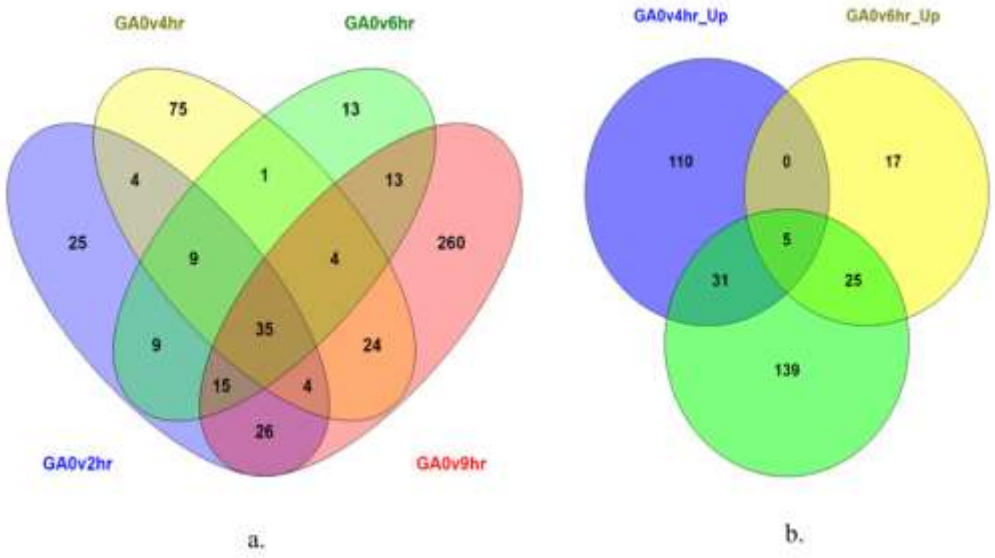

Figure 1: Venn diagram of DEGs identified in pathways related to breast cancer and apoptosis: (a) upregulated genes and (b) downregulated genes. GA0v2hr represents the control group compared to the 2-hour treatment group, GA0v4hr to the 4-hour treatment group, GA0v6hr to the 6-hour treatment group, and GA0v9hr to the 9-hour treatment group.

Genes from these pathways were filtered using ShinyGo, with 127 downregulated genes yielded in the first experimental group and no upregulated genes. In the second group, 156 genes were downregulated and 146 upregulated; in the third group, 99 genes were downregulated and 47 upregulated; and in the fourth, 381 genes were downregulated and 200 upregulated (Figure 1). A Venn diagram revealed 35 genes common to all downregulated groups and 5 common genes in the upregulated groups (Figure 1; Table 2).

Table 2: DEGs Involved in Apoptosis and Breast Cancer Signaling Pathways

Gene Symbol	Log2(FC)*	Gene Symbol	Log2(FC)*
JUN	3.421	NFKB2	1.485
GADD45A	3.291	DUSP5	1.475
EFNA3	2.938	CHRM1	1.426
EFNA1	2.925	DDIT3	1.365
HSPA6	2.800	DUSP6	1.361
PTGS2	2.700	FOS	1.296
DUSP10	2.624	SOCS3	1.157
BCL2L11	2.409	PMAIP1	1.137
IRF1	1.911	MAP2K6	-1.073
DUSP1	1.888	NF1	-0.927
CDKN1A	1.810	CALML4	-0.657
TNFAIP3	1.764	INSR	-0.579
IL6	1.747	PRKCA	-0.530

*FC =FoldChange

The most highly upregulated gene was JUN (Log2FC = 3.421), which is involved in the Wnt, TNF, estrogen, and apoptosis pathways. The most significantly downregulated gene was MAP2K6 (Log2FC = –1.073), consistently identified in the TNF and mitogen-activated protein kinase (MAPK) pathways. The 10 most upregulated genes were consistently observed across all groups, while only MAP2K6 was consistently found among the downregulated genes.

Protein–Protein Interactions

The physical and functional relationships among DEGs were analyzed using STRING with a confidence level of 0.999 and visualized through Cytoscape, which generated a PPI network consisting of 40 nodes and 215 edges (Figure 2). MCODE analysis divided this network into clusters representing groups of proteins that closely interact with one another. Three clusters were identified, reflecting protein complexes or components of biological signaling pathways. The first cluster consisted of 15 genes, with NFKBIA as the central node, including CDKN1A, PTGS2, SOCS3, IL6, FOS, JUN, and others. The second cluster comprised ATF4, GADD45A, BCL2L11, and PPP1R15A, while the third cluster included EFNA3, EFNA1, and INSR.

The cluster with the highest connectivity received the highest score from MCODE (Figure 3). To identify key hub genes in the PPI network, CytoHubba analysis was performed using the MCC and Closeness algorithms. Both methods highlighted the 10 genes with the highest connectivity (Figure 3), among which JUN and FOS consistently ranked in the top two positions. These genes encode the Jun and Fos proteins, subunits of the AP-1 transcription factor, which form a dimeric structure directly involved in the cell-cycle (G1/S) progression signaling pathway.[20] Thus, the integration of DEG and PPI analyses indicates that JUN and FOS may serve as important biomarkers in the apoptotic response of breast cancer cells to gallic acid derivatives.

Primer Design

The primers designed for JUN and FOS genes (Table 3) exhibited characteristics suitable for qPCR analysis. As shown in Table 4, the primer lengths ranged from 20 to 22 nucleotides, with GC content between 40% and 60%, providing sufficient binding stability without increasing the risk of undesirable secondary structures. The product sizes for JUN (121 bp) and FOS (181 bp) were within the optimal range (70–200 bp) for RT-PCR, ensuring efficient amplification. The analysis also showed that the melting temperature differences (ΔTm) between forward and reverse primers were within the range of 2–3 °C, enabling efficient annealing at a single reaction temperature.

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Table 3: Primer Design Results of JUN and FOS

Gene	Primer	Sequence	PCR Product (bp)
JUN	Forward	5'-GCCAACATGCTCAGGGAACA-3'	121
JUN	Reverse	5'-CCCCGACGGTCTCTCTTCAA-3'	
FOS	Forward	5'-GGGGCAAGGTGGAACAGTTA-3'	181
FOS	Reverse	5'-AGGTTGGCAATCTCGGTCTG-3'	

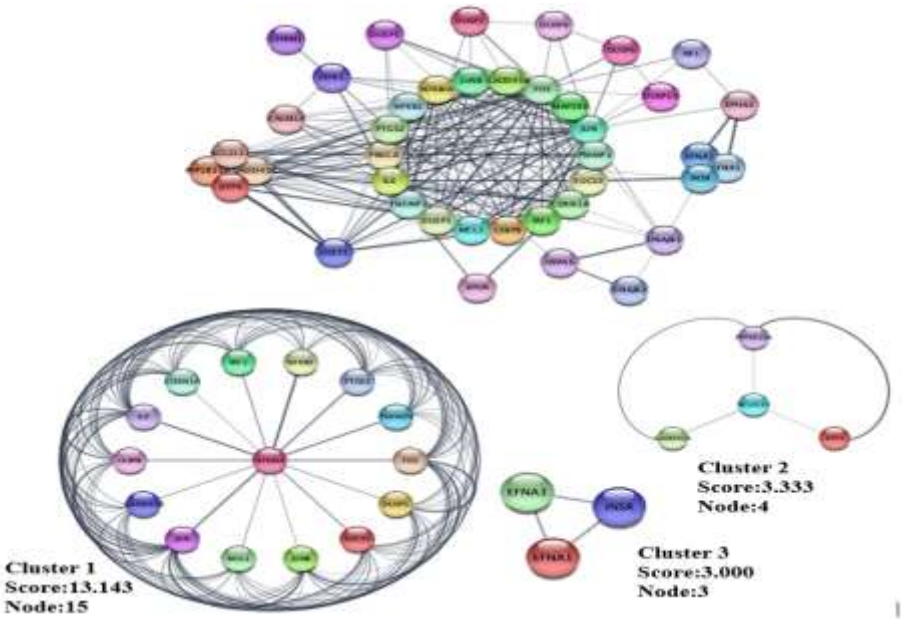
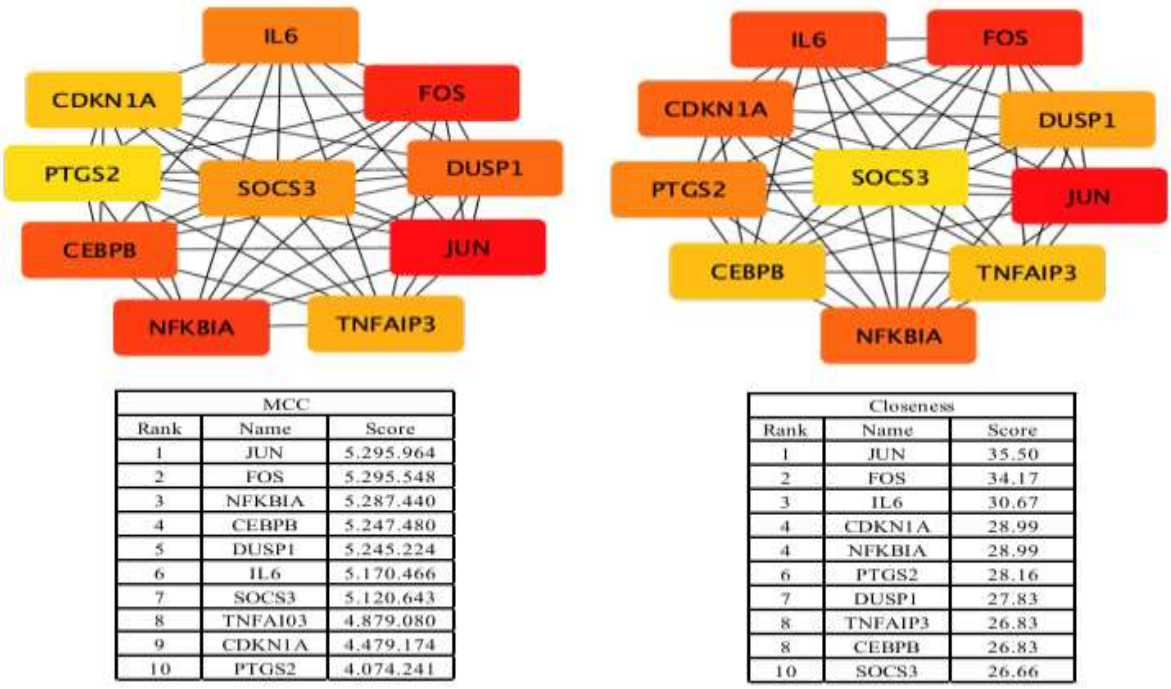


Figure 2: Network visualization of common gene interactions and three closely related gene clusters.



MCC		
Rank	Name	Score
1	JUN	5.295.964
2	FOS	5.295.548
3	NFKBIA	5.287.440
4	CEBPB	5.247.480
5	DUSP1	5.245.224
6	IL6	5.170.466
7	SOCS3	5.120.643
8	TNFAI03	4.879.080
9	CDKN1A	4.479.174
10	PTGS2	4.074.241

Closeness		
Rank	Name	Score
1	JUN	35.50
2	FOS	34.17
3	IL6	30.67
4	CDKN1A	28.99
4	NFKBIA	28.99
6	PTGS2	28.16
7	DUSP1	27.83
8	TNFAIP3	26.83
8	CEBPB	26.83
10	SOCS3	26.66

Figure 3: Results of PPI centrality analysis using MCC and closeness scoring parameters.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Validation of Target Gene Expression in MCF7 Cells

In vitro assays using the half-maximal inhibitory concentration (IC_{50}) of N-octyl gallamide, N-tert-butyl gallamide, and tamoxifen in MCF7 cells were determined based on previous studies and are summarized in Table 4.[9] IC_{50} and $2\times IC_{50}$ doses of each compound were subsequently applied to MCF7 cells, with 0.1% DMSO serving as the negative control.

Table 4: IC50 and 2×IC50 Concentrations of the Gallic Acid Derivates and Tamoxifen for MCF7 Cells

No	Compound	Concentration (µg/mL)	
		IC_{50}	$2\times IC_{50}$
1	Tamoxifen	4.64 ± 0.08	9.28 ± 0.08
2	N-Octyl galamide	57.73 ± 0.44	115.46 ± 0.44
3	N-tert-butyl galamide	83.88 ± 4.09	167.76 ± 4.09

The RNA concentration and purity indices of the isolates obtained from treated cells were first determined, with the results presented in Table 5. Overall, the purity ratios of RNA isolates ranged from 1.89 to 2.01, and RNA concentrations ranged from 109 to 195 ng/µL. Based on these results, all RNA isolates met the purity criteria, with 260/280 ratios within the acceptable range of 1.7–2.0. The gene expression analysis (Figure 4) showed that JUN and FOS expression levels in MCF7 cells treated with N-octyl gallamide and tamoxifen were higher compared to the control group (0.1% DMSO), with the most pronounced upregulation observed in the N-octyl gallamide group. According to ANOVA statistical analysis, N-octyl gallamide significantly increased relative JUN and FOS expression ($p < 0.05$) at the IC_{50} concentration compared to the control, with expression relative values (ΔΔCTs) of 1.25 ± 0.251 for JUN and 1.82 ± 0.691 for FOS. Meanwhile, tamoxifen induced a significant increase in FOS expression (ΔΔCTs 2.33 ± 0.444) ($p < 0.05$) at both tested concentrations. In contrast, N-tert-butyl gallamide did not significantly alter JUN or FOS expression (ΔΔCTs 0.76 ± 0.053, $p > 0.05$) at either concentration compared to the control. JUN and FOS are the major components of the activator protein-1 (AP-1) transcription factor complex, which regulates cell proliferation, differentiation, stress response, and apoptosis.[20]

Table 5: RNA Concentration and Purity (A260/280) of MCF7 Cells Treated with Test Compounds

No	Treatment Isolate	Concentration (ng/2µL)		Average	Purity (A260/280)		Average
		1	2		1	2	
1	Tamoxifen IC50	312.20	309.10	310.65	1.99	1.97	1.98
2	Tamoxifen 2×IC50	325.70	317.40	321.55	2.01	2.00	2.00
3	Ter-butyl Galamide IC50	342.20	333.30	337.75	2.01	1.99	2.00
4	Ter-butyl Galamide 2×IC50	329.40	317.60	323.50	1.97	1.98	1.98
5	Octyl-Galamide IC50	222.80	217.40	220.10	1.92	1.90	1.91
6	Octyl-Galamide 2×IC50	217.40	220.50	218.95	1.91	1.89	1.90
7	DMSO (control)	376.90	363.90	370.40	2.00	2.01	2.01

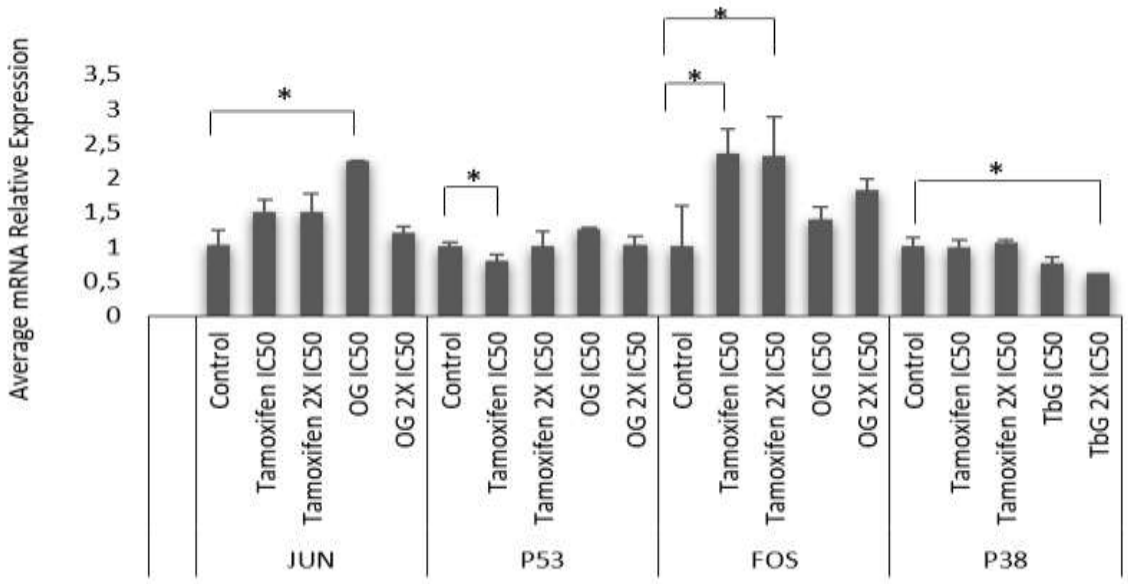


Figure 4: Distribution of relative mRNA expression levels of JUN and FOS compared to the control group following treatment with Tamoxifen, Octyl Galamide (OG), and Ter-butyl Galamide (TbG) at two test concentrations.

The activity of AP-1 is tightly controlled by c-Jun N-terminal kinase (JNK), a member of the MAPK family, through phosphorylation-dependent stabilization and enhancement of c-Jun transcriptional activity.[21] Activation of this axis commonly occurs in response to oxidative stress, DNA damage, or exposure to cytotoxic phytochemicals or chemotherapeutic agents. In cancer biology, AP-1 signaling may exert dual functions; however, under apoptotic pressure, JUN/FOS activation frequently promotes pro-apoptotic transcriptional programs. In this study, treatment of MCF7 breast cancer cells with selected gallic acid derivatives, particularly N-octyl gallamide, resulted in significant upregulation of JUN and FOS, indicating activation of the JNK/AP-1 pathway.

This observation aligns with earlier findings demonstrating that gallic acid and its derivatives can modulate apoptosis-related signaling cascades. Previous reports have shown that gallic acid downregulates anti-apoptotic BCL2 expression in MCF7 cells[26] and induces caspase-8 activation in HL-60 leukemia cells,[27] suggesting that phenolic derivatives can initiate cell death via both intrinsic and extrinsic apoptotic pathways. The ability of these compounds to modulate upstream regulators of apoptosis supports the relevance of the JUN and FOS induction observed in the present work. The comparison with tamoxifen provides important mechanistic insight.

Tamoxifen is known to induce apoptosis in cells with high BCL2 expression and has been reported to activate p38 kinase and c-Jun phosphorylation, subsequently triggering caspase-8 activation.[22,23] Additionally, tamoxifen can increase phosphorylated JNK levels, which further reinforces its mechanistic link to the MAPK/AP-1 axis.[25] This convergence of pathways underscores the importance of JUN and FOS as downstream effectors of apoptosis-related MAPK signaling in endocrine therapy and suggests that gallic acid derivatives may share similar cytotoxic mechanisms.

A key finding of this study is that N-octyl gallamide produced a stronger upregulation of JUN and FOS compared with tamoxifen, indicating more potent activation of the AP-1–mediated apoptotic response. This enhanced activity is particularly relevant considering that resistance to breast cancer therapy is frequently associated with upregulation of anti-apoptotic proteins such as BCL2. BCL2 overexpression accelerates breast cancer progression and contributes to treatment resistance and that therapeutic regimens targeting BCL2 can significantly improve treatment outcomes.[28] The robust induction of JUN and FOS by N-octyl gallamide suggests that this compound may effectively counteract BCL2-mediated survival mechanisms, thereby increasing apoptotic susceptibility in breast cancer cells.

Conclusion

This study demonstrated that N-octyl gallamide significantly upregulates JUN and FOS expression in MCF7 breast cancer cells, indicating activation of AP-1–associated signaling pathways that may lead to apoptosis. Compared with tamoxifen, N-octyl gallamide exhibited a stronger effect on the expression of these key transcriptional regulators, supporting its potential as an alternative or adjuvant therapeutic candidate for breast cancer. In contrast, N-tert-butyl gallamide did not produce significant changes in target gene expression, which emphasizes the influence of alkyl chain structure on biological activity. Overall, these findings reinforce the relevance of gallic acid derivatives in the development of mechanism-based anticancer agents. Further investigations are needed to validate the effects of N-octyl gallamide at the protein level, determine its impact on downstream apoptotic pathways, and evaluate possible synergistic effects with standard therapies such as tamoxifen or BCL2-targeting compounds. In vivo studies, pharmacokinetic profiling, and structural optimization will be essential to advance N-octyl gallamide toward preclinical development as a promising, mechanism-driven anticancer candidate.

Conflict of Interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgements

We thank Hibah Penelitian Tesis Magister Tahun Anggaran 2023 (Grant Number: NKB-1096/UN2.RST/HKP.05.00/2023) for supporting the study.

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