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Methanolic extract of Karo-derived *Trigonella foenum-graecum* induces apoptosis and cell-cycle arrest in breast cancer cells through modulation of PI3K/AKT/mTOR signaling

Cicik Suriani[1*], Fauziyah Harahap[1], Syahmi Edi[1], Ahmad Shafwan S Pulungan[1], Armansyah Maulana Harahap[2*]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Medan, Indonesia
[2]Department of Physical Education, Health and Recreation, Faculty of Sports Science, Universitas Negeri Medan, 20221, Indonesia

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ABSTRACT

Introduction: Breast cancer therapy often faces challenges with drug resistance and severe side effects, highlighting the urgent need for novel therapeutic agents. *Trigonella foenum-graecum* (TFG) contains steroid sapogenins with potential as anti-cancer agents. This study assessed the methanolic extract of TFG (METFG) against eight breast cancer cell lines. The novelty lies in uncovering p53-independent mechanisms and demonstrating its capacity to overcome initial therapeutic resistance by simultaneously blocking PI3K/AKT/mTOR survival pathways.

Methods: TFG seeds were extracted using methanol, and the chemical components were analyzed via GC-MS. Cytotoxicity across eight breast cancer cell lines was evaluated using MTT assays across 24 and 48 hours. Furthermore, apoptosis, cell cycle progression, and the phosphorylation of DNA damage and PI3K/AKT/mTOR marker proteins were analyzed using flow cytometry and Western blot.

Results: METFG demonstrated significant time-dependent cytotoxicity, reducing the IC_{50} in MCF7 -/-p53 cells from 172.5±2.4 µg/mL at 24 hours to 134.3±7.2 µg/mL at 48 hours. The extract successfully induced apoptosis and triggered a cell-cycle phase shift, increasing the percentage of G2/M-phase cells to 29% in MDA-MB cell lines. Additionally, METFG significantly reduced phosphorylation of CHK1, CHK2, AKT, and mTOR, suggesting a potential inhibition of the DNA damage response and cell survival signaling.

Conclusion: The endemic Karo METFG exhibits significant cytotoxic activity by inducing apoptosis, promoting cell cycle arrest, and suppressing DDR and PI3K/AKT/mTOR pathways. Future investigations must assess its selective toxicity against normal mammary epithelial cells to establish its safety.

Implication for health policy/practice/research/medical education:
This study revealed the potential of *Trigonella foenum-graecum* methanol extract as a possible alternative therapy for breast cancer by inducing apoptosis, inhibiting cell cycle progression, and suppressing key signaling pathways in cancer cells. This encourages collaboration among research, clinical practice, and education to improve sustainable cancer management, support further observation, and develop herbal treatments for breast cancer. However, preclinical and clinical investigations are needed.

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Introduction

Breast cancer is the most commonly diagnosed cancer globally and one of the leading causes of cancer deaths in women worldwide (1). With high incidence and mortality rates, breast cancer has a major impact on public health and creates an urgent need for more effective and innovative approaches to prevention and therapy. Current breast cancer treatment approaches, such as surgery, chemotherapy, radiotherapy, and hormone therapy, have been successful in improving patient survival rates (2).

***Corresponding authors**: Cicik Suriani, Email: ciciksuriani@unimed.ac.id and Armansyah Maulana Harahap, Email: armansyah.maulanahr@gmail.com

However, these therapies also often cause serious side effects, including damage to healthy tissue, drug resistance, and decreased quality of life. Therefore, research continues to identify new therapeutic agents that are highly effective while posing a lower risk of side effects (3).

Natural products are the primary focus in the search for new anticancer agents because they contain diverse bioactive compounds with pharmacological properties, including anticancer activity. Phytochemical compounds such as flavonoids, alkaloids, terpenoids, and phenolic acids are known to affect various molecular pathways involved in breast cancer cell growth and proliferation. These effects include induction of apoptosis, suppression of cell proliferation, and inhibition of metastasis through regulation of complex mechanisms, such as the PI3K/AKT/mTOR and JAK/STAT pathways, as well as modulation of estrogen signaling, which plays an important role in hormone receptor-positive breast cancer (4,5). The efficacy of natural compounds in the treatment of breast cancer is evident from the success of several plant-derived drugs, for example, paclitaxel extracted from the *Taxus brevifolia* tree, which is widely used in chemotherapy and acts by inhibiting cancer cell division (6,7). In addition, compounds such as catechins, genistein, and resveratrol, found in various plant products, are also being investigated for their ability to induce apoptosis and inhibit tumor angiogenesis, with relatively low toxicity compared to conventional chemotherapy (8,9).

The development of natural product-based anticancer drugs, including compounds from traditional medicinal plants, is an important alternative in breast cancer therapy. This approach is expected to provide safer, more effective therapy while reducing the burden of side effects for patients (10,11). *Trigonella foenum-graecum* has traditionally been used in the treatment of various disorders, and modern research is beginning to reveal the bioactive potential of this plant, which is particularly relevant for breast cancer (12,13). Extracts from this plant exhibit important anti-inflammatory, antimicrobial, antioxidant, and antiviral properties, as well as anticancer effects, with great potential in breast cancer. Phytochemical analysis of *T. foenum-graecum* identified various secondary metabolites such as diosgenin, yamogenin, tigogenin, gitogenin, and sarsasapogenin (14). These compounds are known to have therapeutic capabilities that can affect key molecular pathways in breast cancer due to their phytoestrogenic nature (15). For example, diosgenin and phenolic compounds play a role in modulating signaling pathways that regulate cancer cell proliferation and induction of apoptosis (16). In particular, these compounds can target the PI3K/AKT/mTOR pathway, a pathway that is critical in the regulation of growth, survival, and drug resistance in breast cancer cells (17).

In addition, the antioxidant and anti-inflammatory potential of *T. foenum-graecum* also helps inhibit the tumor microenvironment that supports breast cancer progression. By suppressing chronic inflammation and oxidative stress, this plant extract may help slow tumor progression and improve the effectiveness of conventional therapies. Its antiviral ability also adds value as a multifunctional agent in breast cancer therapy, given that some viruses can play a role in cancer initiation and progression (17).

These overall bioactive effects, derived from diverse phytochemicals such as diosgenin, yamogenin, tigogenin, gitogenin, and sapogenin, make *T. foenum-graecum* an attractive candidate for further research as a supportive therapy or an autonomous anticancer agent, specifically in the context of breast cancer (18). This approach enables the development of therapies that target key molecular mechanisms of breast cancer, induce programmed cell death, inhibit cell cycle progression, and suppress signaling pathways involved in cancer cell growth and survival (19). Recent findings highlight the potential of *T. foenum-graecum* as a medicinal source, prompting further research into its anticancer activity. The focus of this study was to evaluate the anticancer effects of *T. foenum-graecum* methanol extract on breast cancer by testing eight different breast cancer cell lines (20,21). Cytotoxicity and its impact on cell survival were analyzed using the MTT assay. Furthermore, the mechanism of action of this plant was traced through the induction of apoptosis, cell-cycle changes, and the expression of key checkpoint proteins such as CHK1 and CHK2. This study also highlights the role of the PI3K/mTOR signaling pathway in breast cancer cell survival and proliferation. This comprehensive approach can deepen the understanding of the therapeutic potential of *T. foenum-graecum* as a novel, effective anticancer agent in breast cancer.

Materials and Methods

Plant materials

Seeds of *T. foenum-graecum* were harvested from Karo District, North Sumatra, Indonesia, in July 2025. Botanical identification of the plant was carried out by a botanist in the field of Pharmacognosy and Phytochemistry at the Faculty of Mathematics and Natural Sciences, University of North Sumatra. Voucher specimens (UM/HI-FMIPA/202501/2) were deposited in the herbarium of FMIPA, University of North Sumatra, for future reference. After collection, sections were air-dried at ambient temperature and protected from direct sunlight to maintain the integrity of heat- and light-sensitive phytochemicals. After drying, the plant material is meticulously pulverized into a powder with particle size not exceeding 0.5 mm to enhance extraction and increase the yield of bioactive chemicals.

Extraction of plant materials

The seeds of *T. foenum-graecum* were extracted with methanol, following the procedure described in previous studies (21). The extraction process resulted in a final yield

of 25% w/w. In brief, 100 g of dried plant material, crushed into a powder, was soaked in 1000 mL of 99.7% purity methanol at room temperature for 72 hours. The soaking container was wrapped in aluminum foil to protect it from light, and the mixture was stirred continuously for optimal extraction. After the soaking process was complete, the solution was filtered using Whatman number 1 filter paper. The methanol extract was then concentrated using a rotary evaporator under a low pressure of about 12 mbar, while maintaining the water bath temperature at 50 °C to protect the active components. After the methanol had evaporated completely, the semi-solid extract was stored in a 50 mL Falcon tube, tightly covered with aluminum foil to protect it from light, and kept at 4 °C to maintain its stability. The phytochemical profile of this methanol extract was analyzed using a derivatization-based GC-MS technique (22,23).

Cell lines and culture conditions

In this study, to comprehensively address the heterogeneity of breast cancer, a panel of eight human breast cancer cell lines obtained from the Research Innovation and Agency of Indonesia (BRIN) was utilized. This panel was carefully selected to represent distinct molecular subtypes: hormone-dependent, ER-positive models (MCF7 and T47D); a HER2-positive model (SK-BR-3); and an aggressive triple-negative breast cancer (TNBC) model (MDA-MB). Furthermore, to specifically investigate the role of the p53 tumor suppressor, we employed isogenic variants of the MCF7 and T47D lines: MCF7 +/+ p53 (wild-type) and MCF7 -/- p53 (knockout), as well as T47D +/+ p53 and T47D -/- p53. The inclusion of these specific p53 variants is highly relevant, as it allows for a detailed comparative analysis to determine whether the extract's mechanisms—particularly its capacity to induce specific phases of cell cycle arrest—are dependent on functional p53 signaling or operate via uniquely uncharacterized p53-independent pathways. All cells were cultured using RPMI 1640 or DMEM media, each containing 10% fetal bovine serum (FBS), 100 units/mL streptomycin, and 100 units/mL penicillin. Cell culture was performed at 37 °C under a 5% carbon dioxide atmosphere. When the culture reached a confluency of about 80%, the cells were subcultured every 72 hours as needed (24,25).

Cytotoxicity test

To test the cytotoxic properties of the methanolic extract of *T. foenum-graecum*, a stock solution was prepared by dissolving 20 mg of extract into 1 mL of DMSO. From this stock solution, various concentrations ranging from 12.5 to 200 µg/mL were prepared and then applied to breast cancer cells grown in 96-well plates. Each well contained 2.5×10^3 cells and was performed in triplicate to ensure data reliability. Next, the cell culture was incubated for 24 or 48 hours, as specified in the experimental design. After the incubation period, 5 mg/mL MTT solution in phosphate-buffered saline (PBS) was added to each well. Incubation was continued at 37 °C for 2 to 4 hours to allow the MTT reduction to formazan crystals. Afterward, 100 µL of DMSO was added to dissolve the formazan crystals, and the absorbance was measured at 570 nm using a spectrophotometer. Cell proliferation was assessed by comparing the absorbance of the treatment group with that of the negative control, which used DMSO alone. As a positive control, cisplatin was used to confirm the cytotoxicity assay's effectiveness. This method facilitated quantitative evaluation of the effect of extract concentration on cancer cell viability. In summary, this crucial step precisely quantified the IC_{50} value, which served as the fundamental baseline for all subsequent mechanistic assays to evaluate the anticancer activity of *T. foenum-graecum* extract (26).

Cell cycle analysis

Cancer cells that had been cultured in a six-well plate to reach 70-75% confluence level were then treated for 48 hours with *T. foenum-graecum* extract at IC_{50} concentration. After the treatment, the cells were collected and preserved in 70% ethanol solution stored overnight at -20 °C to maintain cell condition for further analysis. The next day, the cells were rinsed to remove residual ethanol, then stained with propidium iodide (PI), which stains cell DNA, and with RNase-free DNase to digest cellular RNA, so that only DNA would be analyzed. Staining lasts for 30 minutes to allow the dye to penetrate the cell membrane optimally (7,27).

The distribution of cells at different stages of the cell cycle was determined using flow cytometry. This method allows quantitative measurement of PI-bound DNA in each cell, which is then interpreted to determine the proportion of cells in the G0/G1, S, and G2/M phases of the cell cycle. This information is important for assessing how *T. foenum-graecum* extract affects cell proliferation and progress during the cell division cycle (28,29).

Apoptosis and necrosis assay

Cells were cultured in six-well plates to 70% confluence and exposed to *T. foenum-graecum* extract at an IC_{50} concentration for 48 hours. After treatment, cells were washed with PBS and stained using Annexin V and PI according to protocol. Analysis of apoptosis and necrosis was performed by flow cytometry using fluorescence detection at 530/30 nm for Annexin V and 615 nm for PI. Cells were divided into four groups: live, early apoptotic, late apoptotic, and necrotic cells based on staining patterns and analyzed using FlowJo software (30,31).

Western blot analysis

Cells were grown in six-well plates to approximately 70% confluence and exposed to *T. foenum-graecum* extract for 48 hours. After treatment was completed, cells were washed twice with PBS, then lysed with RIPA buffer (1X)

containing a protease inhibitor cocktail to protect proteins from degradation. The resulting protein concentration was measured using the Bradford method. A total of 30 micrograms of protein was then separated by SDS-PAGE using a 10% gel. The electrophoresed proteins were transferred to a nitrocellulose membrane. The membrane was blocked for 1 hour at room temperature with 5% BSA to prevent nonspecific antibody binding. After blocking, the membrane was washed with Tris-buffered saline containing 0.1% Tween 20, and then incubated overnight at 4 °C with specific primary antibodies against Cyclin B1, CDK1, CHK1, phospho-CHK1, CHK2, phospho-CHK2, AKT, phospho-AKT, mTOR, phospho-mTOR, and actin. All primary antibodies were applied at a specified dilution ratio of 1:1000. The following day, the membranes were incubated with anti-rabbit or anti-mouse secondary antibodies at a 1:2000 dilution for 1 hour at room temperature. Target proteins were visualized using a chemiluminescence kit, and protein band densities were analyzed with Bio-Rad Image Lab software. Actin was used as a loading control for data normalization, thereby improving the accuracy of protein analysis results (32,33).

Statistical analysis

All experiments were conducted in three independent biological replicates. Data are presented as mean ± SD. Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test in GraphPad Prism, with significance defined at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

Results

Chemical constituents of the methanol extract of *Trigonella foenum-graecum*

GC-MS derivatization analysis of *T. foenum-graecum* methanol extract identified various bioactive compounds with potential cytotoxic and anticancer effects. The extract contained compounds such as the major compounds sapogenin, diosgenin, yamogenin, phytol, DHA (docosahexaenoate), and the phytosterols campesterol and stigmasterol. These compounds are known as plant metabolites that can inhibit cancer cell proliferation and trigger apoptosis. For example, diosgenine and yamogenine are metabolites that play a role in suppressing tumor growth, especially in breast cancer-dependent hormonal tumors. Terpenoid compounds such as α-pinene and phytol also exhibit cytotoxicity in various cancer cell models. In addition, DHA, as an omega-3 fatty acid, has antiproliferative and pro-apoptotic effects. Phytosterols such as campesterol and stigmasterol modulate cancer cell growth and death. In addition to these compounds, the metabolite quinic acid, a cyclic polyol that plays a role in antiproliferative and antioxidant activities, was also identified, indicating its possible contribution to the extract's biological effects. Detailed data, including the comprehensive gas chromatography–mass spectrometry (GC–MS) chromatogram and the relative abundance percentages of each identified bioactive compound, are provided in Table S1 and Figure S1. In conclusion, the analytical profile explicitly confirms that the methanol extract of *T. foenum-graecum* is rich in diverse phytochemicals associated with cytotoxic properties.

Cytotoxicity of methanolic extract of *Trigonella foenum-graecum* on BC cell lines

The cytotoxic efficacy of the *T. foenum-graecum* extract across the tested cell lines at 24 and 48 hours, expressed as IC_{50} values, is summarized in Table 1. Analysis showed that cytotoxicity increased over time, as evidenced by lower IC_{50} values at 48 hours than at 24 hours (Figure 1). At 24 hours of treatment, the methanolic extract effectively induced cytotoxicity in MCF7 -/- p53, MCF7 +/+ p53, and MCF7 cells, while MDA-MB, SKBR 3, and T47D cells did not show significant changes (Table 1). The initial insensitivity in some of these cell lines indicated an initial resistance to the extract. However, after 48 hours, IC_{50} values decreased significantly in most sensitized cells, indicating reduced cell proliferation. For example, the

Table 1. Cytotoxic effects of *Trigonella foenum-graecu*m methanolic extract (METFG) on breast cancer cell lines following 24-hour and 48-hour exposure

Cell line	METFG IC_{50} (µg/mL) ± SD (24 h)	METFG IC_{50} (µg/mL) ± SD (48 h)	Tamoxifen IC_{50} (µg/mL) ± SD (24 h)	IC Tamoxifen IC_{50} (µg/mL) ± SD (48 h)
MCF7 -/- p53	172.5±2.4	134.3±7.2**	46.8±1.9	40.6±4.4*
MCF7+/+ p53	244.1±42.5	172.3±24.2*	28.4±1.6	25.3±4.5
MDA-MB	Unresponsive	166.6±8.2	122.2±3.4	25.1±2.1***
MCF-7	281.0±13.4	161.0±16.3**	31.2±2.5	15.60±4.4*
TE7D +/+ p53	242.2±31.2	153.2±0.4*	70.3±3.5	41.3±5.3*
T47D -/- p53	251.3±28.1	164.1±4.4*	24.2±2.2	16.3±9.6
SK-BR-3	Unresponsive	Unresponsive	126.8±6.6	19.0±4.6***
T47D	Unresponsive	Unresponsive	92.5±3.1	42.2±3.3***

Data are presented as mean ± SD from three independent biological replicates. IC50: Half-maximal inhibitory concentration. 'Unresponsive' indicates that cell viability did not decrease by 50% even at the highest concentration tested. Statistical significance between 24 hours and 48 hours of exposure is indicated by *P< 0.05, **$P < 0.01$, ***$P < 0.00.1$

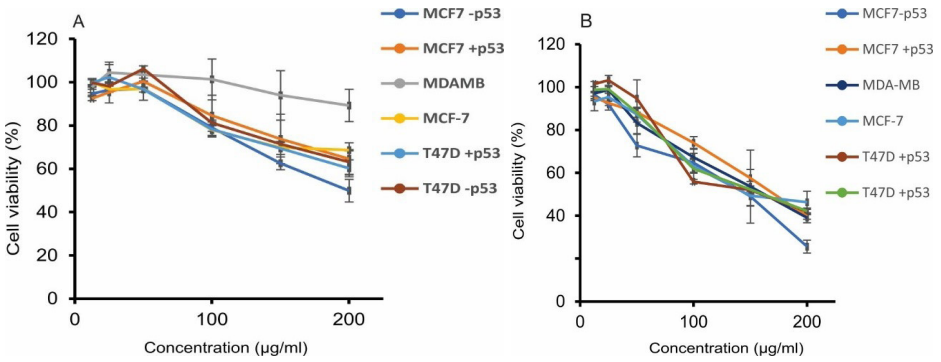

Figure 1. Inhibitory effects of increasing concentrations of *Trigonella foenum-graecum* extract on the viability of various breast cancer cell lines after (A) 24 hours and (B) 48 hours of incubation.

IC_{50} value in MCF7 -/- p53 cells dropped from 192.5 ± 2.7 µg/mL at 24 hours to 137.3 ± 7.9 µg/mL at 48 hours. Similarly, MCF7 +/+ p53, MCF7, and T47D all showed decreased IC_{50} values after 48 hours of exposure. MDA-MB cells, which did not respond after 24 hours, began to show sensitivity with an IC_{50} value of 161.6 µg/mL at 48 hours, indicating that long-term exposure to the extract could overcome initial resistance in some cancer cell lines. In contrast, SK-BR-3 and T47D cells remained unresponsive to the extract at both observation times, reflecting continued resistance to the treatment (Figure 1). A positive control assay with tamoxifen was performed to assess assay precision and reliability.

Trigonella foenum-graecum promotes cell death in breast cancer cells

The effects of *T. foenum-graecum* methanolic extract on apoptosis and necrosis were evaluated using Annexin V-FITC and PI double labeling, analyzed by flow cytometry. This method distinguishes between live cells, early apoptosis, late apoptosis, and necrosis, thus elucidating the mechanism of cell death induced by the extract. The percentage of cells in each phase of death is summarised in Table 2. The extract treatment significantly decreased the proportion of live cells in all cell lines compared with the control and DMSO treatments, confirming its cytotoxic effect. Both apoptosis

Table 2. Percentage of apoptotic and necrotic cells in breast cancer cell lines after treatment with *Trigonella foenum-graecum* methanolic extract

Breast cancer cell lines	State	Viable (%) (Q4)	Early apoptosis (%) (Q3)	Late apoptosis (%) (Q2)	Necrosis (%) (Q1)
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
MCF7	Control	95.9 ± 3.11	0.32 ± 0.06	0.81 ± 0.53	2.93 ± 2.52
	Treated	78.3 ± 6.22**	4.38 ± 2.1*	12.7 ± 7.36**	4.62 ± 1.61
	DMSO	98.95 ± 0.64	0.12 ± 0.11	0.2 ± 0.2	0.7 ± 0.33
MDA-MB	Control	95.45 ± 0.11	0.42 ± 0.06	1.36 ± 0.32	2.75 ± 0.27
	Treated	87.6 ± 2.33*	0.95 ± 0.06*	3.8 ± 2.79*	7.68 ± 1.57**
	DMSO	94.5 ± 0.71	0.58 ± 0.13	1.67 ± 0.55	3.27 ± 0.29
MCF7 +/+ p53	Control	98.05 ± 0.53	0.12 ± 0.06	0.98 ± 0.58	0.84 ± 0.11
	Treated	84.7 ± 2.55**	1.56 ± 0.13*	10.03 ± 2.88**	3.67 ± 0.47*
	DMSO	94.9 ± 1.84	0.76 ± 0.27	3.48 ± 1.25	0.83 ± 0.31
MCF7 -/- p53	Control	98.35 ± 2.26	2.04 ± 1.36	1.82 ± 1.1	0.92 ± 0.21
	Treated	85.95 ± 2.23**	3.79 ± 1.02	7.05 ± 2.79**	3.21 ± 1.57*
	DMSO	95.15 ± 2.3	2.02 ± 1.4	2.12 ± 0.81	0.73 ± 0.1
T47D +/+ p53	Control	98.8 ± 0.14	0.36 ± 0.05	0.49 ± 0.0	0.35 ± 0.05
	Treated	82.55 ± 0.95**	2.27 ± 1.42*	8.31 ± 0.64*	6.84 ± 3.01*
	DMSO	98.35 ± 0.25	0.66 ± 0.13	0.69 ± 0.07	0.34 ± 0.04
T47D -/- p53	Control	98.85 ± 0.25	0.27 ± 0.11	0.35 ± 0.15	0.53 ± 0.01
	Treated	77.5 ± 5.02***	5.59 ± 2.3**	9.89 ± 2.98**	7.0 ± 4.32*
	DMSO	98.65 ± 0.11	0.38 ± 0.04	0.46 ± 2.98	0.54 ± 0.02

Data are categorized into Q1 (necrosis), Q2 (late apoptosis), Q3 (early apoptosis), and Q4 (viable cells), each reflecting a distinct stage of cellular status as determined by flow cytometry analysis. Cells were treated with the methanolic extract at their respective IC_{50} concentrations for 48 hours. DMSO (Dimethyl sulfoxide) was used as the negative vehicle control. Data are presented as mean percentages ± SD from three independent experiments. Significant differences compared to the untreated control group are denoted as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

and necrosis occurred in treated cells, with apoptosis as the major pathway (Figure 2). Early apoptosis was significantly increased in several cell lines; for example, in MCF7 -/- p53, the percentage of early apoptosis rose from 0.27% in control to 5.59% after treatment, while in MCF7 it increased from 0.32% to 4.38%. A significant increase in late apoptosis also occurred, especially in MCF7 and MCF7 -/- p53, reaching 12.7% and 9.89%, respectively. In addition, MDA-MB and T47D cell lines showed increased apoptotic activity post-treatment, with apoptotic rates of approximately 5% in MDA-MB, 11% in T47D +/+ p53, and 10% in T47D -/- p53, indicating the important role of apoptosis in the cytotoxic effect of the extract. Necrosis was also significantly increased in certain cell lines, such as MCF7 +/+ p53 and MCF7 -/- p53, which showed an increase in the necrotic cell population to 6.845% and 7.00%, respectively, after treatment. Other cell lines, such as MDA-MB and T47D +/+ p53, also showed necrosis rates of approximately 8% and 4%.

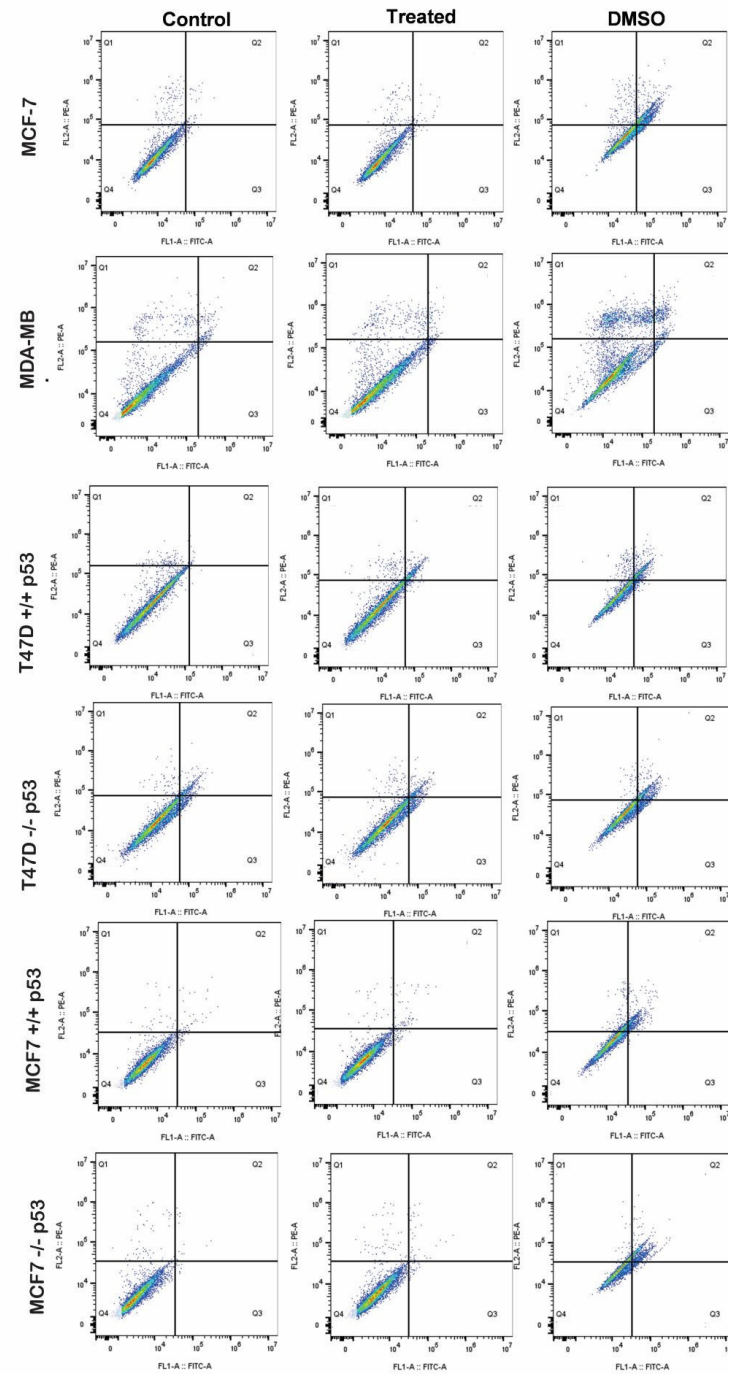

Figure 2. The effect of *Trigonella foenum-graecum* methanol extract on the percentage of viable cells (Q4), early apoptosis (Q3), late apoptosis (Q2), and necrosis (Q1) in breast cancer cell lines, analyzed by flow cytometry. Control cells were cultured in standard media, while treated cells were exposed to the extract at their respective IC_{50} concentrations for 48 hours. DMSO was used as a negative control.

Trigonella foenum-graecum extract induces cell-cycle arrest in BC cell lines

Cell cycle analysis of breast cancer cell lines treated with *T. foenum-graecum* extract at IC_{50} concentrations revealed significantly altered cell cycle distributions, with variations among cell lines. In untreated MDA-MB cells, most cells were in the G1 phase (about 69%), while the S and G2/M phases were relatively low (Figure 3).

After treatment, the proportion of cells in the G1 phase decreased dramatically to about 40%. In comparison, the proportions in the S and G2/M phases increased to about 28% and 29%, respectively, indicating cell cycle arrest at the S and G2/M checkpoints. This pattern of cycle-phase shift was also observed in the T47D +/- p53 cell line, where treatment decreased the number of cells in G1 and increased those in S and G2/M. In T47D -/- p53, increased G2/M arrest was also observed, suggesting that this extract could inhibit the cell cycle regardless of p53 status. Other changes also appeared in MCF7 cells, with wild-type p53 cell lines showing G1 arrest post-treatment, while those without p53 (knockout) showed greater G2/M arrest. Cyclin B and CDK1 proteins, which regulate the G2/M phase transition, were decreased after treatment, indicating G2/M phase arrest. Similarly, Cyclin D and

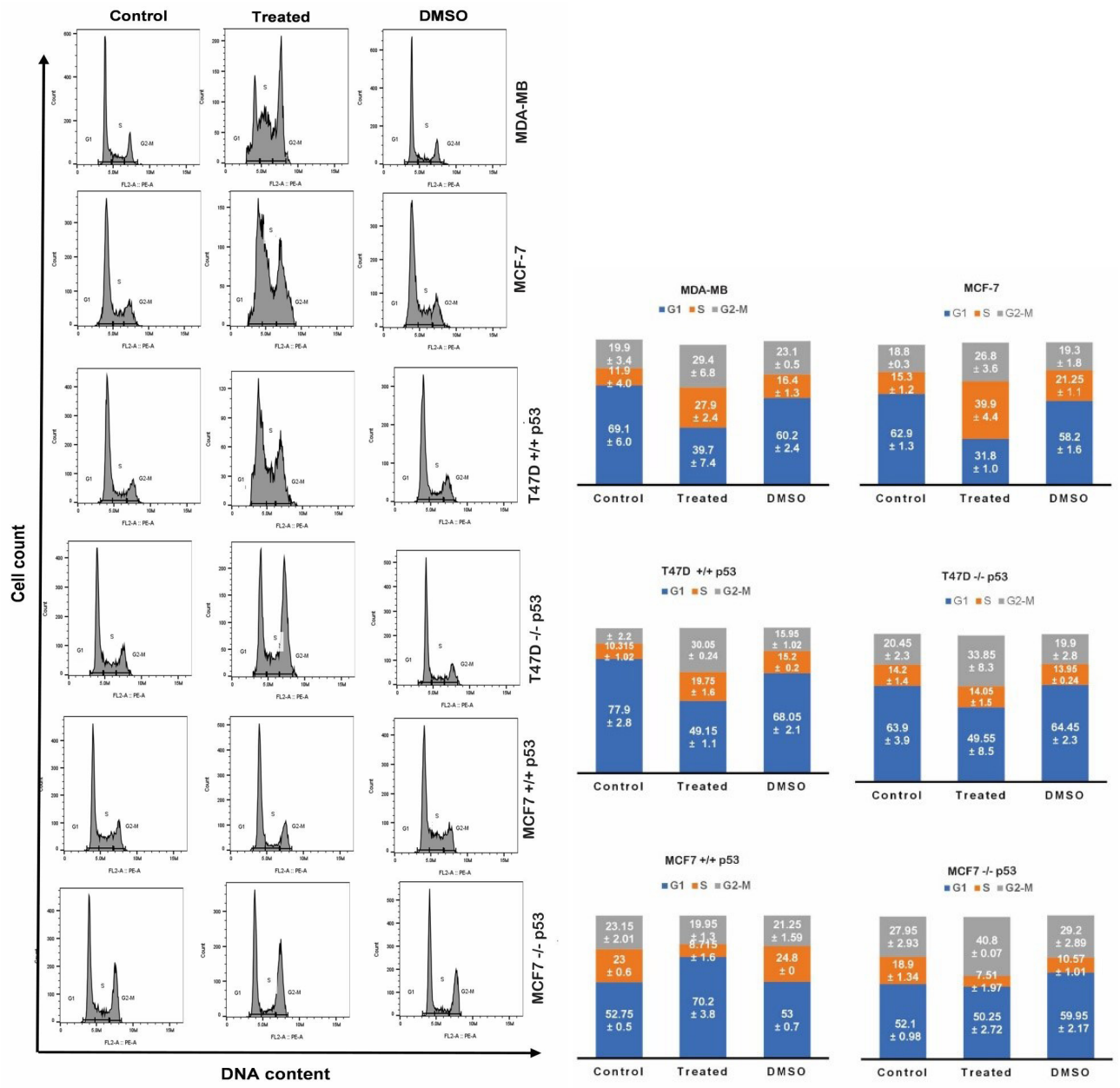

Figure 3. Cell cycle analysis of breast cancer (BC) cell lines after treatment with *Trigonella foenum-graecum* extract at respective IC_{50} concentrations for 48 hours. (A) Representative flow cytometry chromatograms of the cell cycle distribution. (B) Mean percentages of cells in G1, S, and G2/M phases from three independent experiments.

CDK4, which play a role in the G1-to-S phase transition, were decreased, especially in MCF7 +/+ p53 cells, reinforcing the G1-phase arrest (Figure 4). The difference in response between p53 wild-type and knockout cells confirms the role of the p53 complex in controlling the cell cycle.

Effects of *Trigonella foenum-graecum* extract on CHK1/CHK2 and PI3K/AKT/mTOR pathways

Treatment with *T. foenum-graecum* methanolic extract decreased the levels of phosphorylated CHK1 and CHK2, indicating a significant impact on the DNA damage response (DDR) pathway. CHK1 and CHK2 play important roles in cell cycle arrest and DNA repair. Phosphorylation of these proteins normally occurs in response to genotoxic stress to maintain genomic stability. Although direct quantification of DNA damage (such as γ-H2AX assessment) was not performed in this study, the observed reduction in CHK1 and CHK2 phosphorylation indicates targeted suppression of downstream DDR checkpoint signaling. Instead of merely acting as a primary genotoxic agent that activates the DDR, METFG effectively paralyzes cancer cells' DNA repair machinery. By abrogating these downstream checkpoints, the extract forces cells to bypass repair mechanisms, thereby significantly increasing their susceptibility to apoptosis. Phytochemicals in the extract, such as terpenoids, sterols, and phenolics, are known to interfere with signaling pathways that regulate DDR (Figure 5).

The PI3K/AKT/mTOR pathway, which is often active

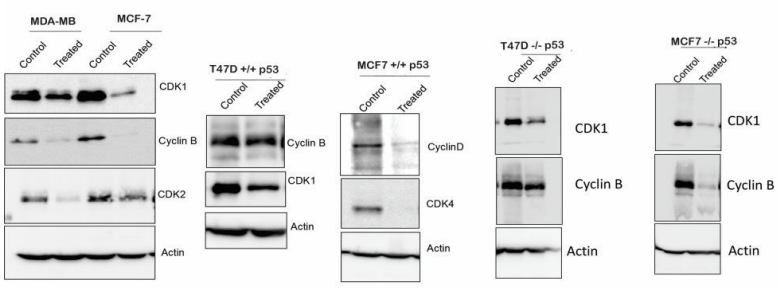

Figure 4. Protein expression levels of cyclins and cyclin-dependent kinases in breast cancer cell lines. Western blot analysis showing the protein levels of Cyclin B, Cyclin D, CDK1 (Cyclin-dependent kinase 1), CDK2, and CDK4. Cells in the 'Treated' group were exposed to the methanolic extract of *Trigonella foenum-graecum* at their respective IC_{50} concentrations for 48 hours, whereas the 'Control' group received no extract. Actin was used as an internal loading control.

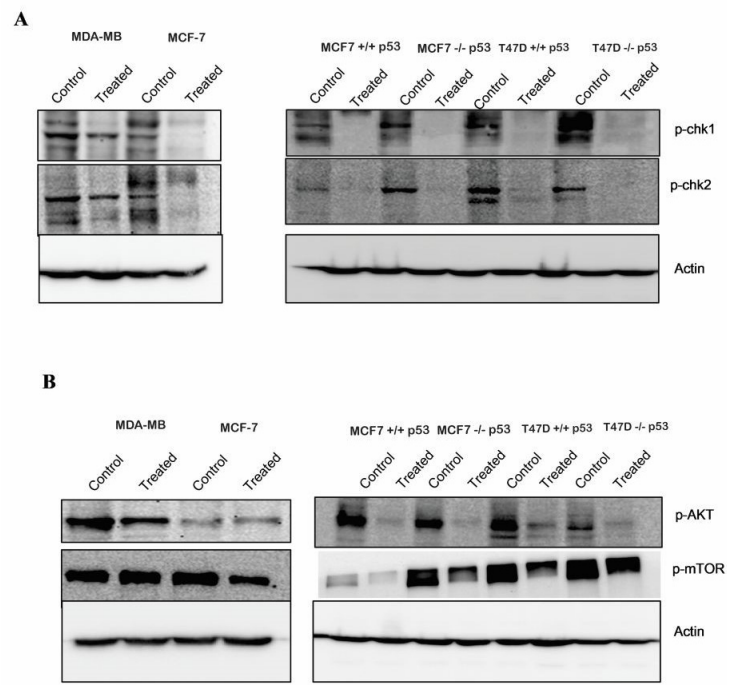

Figure 5. Effect of *Trigonella foenum-graecum* extract on the modulation of protein levels involved in breast cancer signaling pathways. Western blot analysis showing (A) protein levels of phosphorylated CHK1 (p-CHK1) and phosphorylated CHK2 (p-CHK2), and (B) protein levels of phosphorylated AKT (p-AKT) and phosphorylated mTOR (p-mTOR). Cells in the 'Treated' group were exposed to the methanolic extract of T. foenum-graecum at their respective IC50 concentrations for 48 hours, whereas the 'Control' group received no extract. Actin was used as an internal loading control.

in various cancers, controls cell growth, survival, and proliferation. After treatment with the extract, the phosphorylation levels of AKT and mTOR proteins decreased in most breast cancer cell lines tested, suggesting inhibition of the pathway. The different responses across cell lines indicate that the extract selectively inhibits certain components of the PI3K/AKT/mTOR pathway, depending on the cellular environment.

Discussion

This study provides an in-depth understanding of the anticancer properties of the methanolic extract of *T. foenum-graecum* against various breast cancer cell lines, using a comprehensive approach. Using a series of methods, such as detailed phytochemical analysis to identify the various bioactive compounds contained in the extracts, cytotoxicity assays that measure the ability of the extracts to reduce cell viability specifically, and apoptosis evaluation to understand the mechanism by which cell death is triggered. This study also involved cell cycle analysis to detect cell arrest or changes in cell distribution in their life cycle phases (34). Equally important, exploration of critical signaling pathways was conducted to uncover how the extract interacts with proteins and molecular regulators that determine cancer survival and proliferation. Among the bioactive compounds, diosgenin, yamogenin, and sapogenin emerged as major components that act as potent agents in inducing tumor cell death. These compounds are known to disrupt cellular metabolism through mitochondrial dysfunction, leading to decreased ATP production and activation of intrinsic death pathways, which further promote the apoptotic process (35).

Diosgenin, an important anticancer lignan in this extract, has been reported to inhibit cancer cell proliferation by triggering cell cycle arrest, for example, through activation of the p53 pathway, which then triggers apoptosis (36). This indicates that p53 status affects the specific phase of the cell cycle that is arrested by *T. foenum-graecum* extract (36). This cell cycle disruption is likely related to decreased expression of cyclin and CDK proteins, which are important for cell cycle regulation. Terpenoid compounds such as α-pinene and phytol also contribute to the anticancer activity of the extract; α-pinene is known to suppress tumor cell growth by inducing oxidative stress and interfering with survival signaling pathways such as PI3K/Akt. In addition, docosahexaenoic acid (DHA), an omega-3 fatty acid, plays an important role through different but complementary mechanisms (37,38). Although less active than other major metabolites, phytosterols such as campesterol and stigmasterol contribute to the richness of the extract's anticancer profile by altering cell membrane dynamics and cholesterol metabolism, potentially attenuating signaling pathways important for cell growth. These phytosterols are known to induce apoptosis and inhibit cancer cell proliferation, possibly by activating cell death receptors or modulating the AKT/mTOR pathway, leading to cell cycle arrest and induction of autophagy (37,39).

Yamogenin and its derivatives exhibit potent anticancer activity by inducing apoptosis and regulating oxidative stress and mitochondrial function across various cancers, including breast, colon, and prostate cancer. The free radical-scavenging antioxidant properties of these compounds amplified the antiproliferative effects of the extracts. Thus, overall, this study confirms that the methanolic extract of *T. foenum-graecum* is a rich and complex source of phytochemicals with varied and complementary mechanisms of action, which are collectively capable of inhibiting the growth and survival of breast cancer cells through a multifaceted set of molecular processes, showing great potential as an effective and natural anticancer therapeutic candidate (40). Collectively, the anticancer efficacy of the methanol extract appears to arise from its bioactive constituents, which promote apoptosis, generate oxidative stress, and inhibit critical pathways that support tumor survival and progression. These interactions likely enable simultaneous modulation of multiple cellular targets, thereby increasing the overall therapeutic potential (41,42).

Trigonella foenum-graecum extract showed time-increasing cytotoxic effects on various breast cancer cell lines, with higher efficacy levels at 48 hours versus 24 hours. Cell lines such as MCF7 (with both normal and mutant p53) and T47D showed significant sensitization to this extract, characterized by a marked decrease in IC_{50} values with longer exposure times. Although MDA-MB cells showed a delayed response, this indicated that prolonged exposure could overcome the initial resistance that emerged (43,44). In contrast, cell lines SW480 and SW620 showed no response, indicating inherent baseline resistance (45). The differences in response among these cell lines, particularly the resistance exhibited by SK-BR 3 and T47D, illustrate the complexity of cancer biology and the important roles of genetic and phenotypic factors in the development of treatment resistance. Such resistance is associated with increased CXCR4 protein expression, which is part of the CXCR4/CXCL12 axis known to drive therapeutic resistance. In addition, variations in response between cell lines may be influenced by genetic differences, such as p53 status, KRAS mutations, or other molecular characteristics that affect drug sensitivity. Flow cytometry analysis also showed that this extract primarily induced apoptosis in these cancer cell lines, with an increase in the population of cells undergoing early and advanced apoptosis, indicating activation of the intrinsic apoptotic pathway (46). This mechanism of cell death through apoptosis is more beneficial than necrosis because it reduces inflammation and minimizes damage to surrounding tissues, which is very important in cancer therapy to avoid adverse side effects (47).

Furthermore, treatment with the extract correlated

with the suppression of the PI3K/AKT/mTOR pathway, a critical network regulating cell survival and growth that is frequently hyperactivated in cancer. The observed decrease in the phosphorylation levels of AKT and mTOR indicates an attenuation of this signaling cascade, which coincides with the reduced cell proliferation and increased apoptosis seen in our assays. However, it is important to note that these current findings are primarily correlative. To conclusively establish whether METFG directly targets these specific kinases or indirectly modulates them through upstream mediators, further mechanistic validations, such as pathway rescue experiments, specific PI3K inhibition controls, and targeted gene knockdown studies, are required (48).

Typically, genotoxic agents activate CHK1 and CHK2 to halt the cell cycle and facilitate DNA repair. However, our findings reveal a paradoxical decrease in CHK1/CHK2 phosphorylation, indicating that METFG functions primarily by uncoupling the DDR signaling pathway. Since direct quantification of DNA double-strand breaks was not performed, we postulate that the extract's primary mechanism relies heavily on inhibiting downstream repair kinases rather than inducing extensive initial DNA damage. This profound inhibition blocks activation of cell-cycle checkpoints, leaving cancer cells defenseless against basal or induced cellular stress. Consequently, this leads to the accumulation of unresolved genomic errors, which forcefully facilitates apoptotic cell death (49). In addition, the extract inhibited the PI3K/AKT/mTOR pathway, which plays a key role in regulating cell survival and growth and is often activated in cancer (50). Decreased phosphorylation of AKT and mTOR indicates suppression of these pathways, leading to reduced cell proliferation and increased apoptosis. The selective inhibition observed across various cellular pathways suggests that the *T. foenum-graecum* extract may affect specific components of those pathways, depending on the cellular environment (51).

These findings collectively suggest that *T. foenum-graecum* exerts anticancer effects through multiple mechanisms, including induction of apoptosis, cell cycle arrest, and inhibition of the PI3K/AKT/mTOR pathway. To further develop the therapeutic potential of *T. foenum-graecum*, additional research is needed to isolate and characterize the specific compounds responsible for its anticancer effects. Exploring the synergistic effects of these compounds may increase effectiveness while minimizing the possibility of toxicity. In addition, *in vivo* studies using appropriate animal models are essential for evaluating the extract's pharmacokinetics, bioavailability, therapeutic efficacy, and safety profile in complex biological systems. These investigations will provide important insights into the translational potential of *T. foenum-graecum* as a novel anticancer agent and lay the foundation for future clinical trials.

While this study provides comprehensive in vitro evidence regarding the anticancer properties of the *T. foenum-graecum* methanolic extract, several limitations must be acknowledged. First, the current research relies primarily on standard cell viability and flow cytometry assays without direct, real-time visualization of the extract's cellular distribution and morphological impact. Therefore, future studies should employ advanced imaging techniques, such as EVOS fluorescence or phase-contrast microscopy, to precisely observe how the plant extract binds to, enters, and localizes within cancer cells. Second, we have not yet specifically evaluated the synergistic activity between individual isolated bioactive compounds. Subsequent research must focus on isolating and characterizing these specific compounds to evaluate their direct synergistic effects and minimize potential toxicity. Most importantly, in vivo studies using appropriate animal models are essential for evaluating the extract's pharmacokinetics, bioavailability, therapeutic efficacy, and safety profile in complex biological systems, ultimately laying a crucial foundation for future clinical trials.

Conclusions

The methanol extract of *T. foenum-graecum* exhibits moderate but significant cytotoxic activity against breast cancer cells by inducing programmed cell death (apoptosis) and prominently disrupting the cell cycle. Crucially, these biological effects are closely correlated with the suppression of the PI3K/AKT/mTOR signaling pathways, which are vital to cancer cell survival. Although further direct target validations, such as knockdown or pathway rescue studies, are necessary to definitively map the exact causal molecular interactions, the highly diverse phytochemical content in this extract, primarily comprising active sesquiterpenoids and phenolic compounds, clearly contributes to its substantial therapeutic efficacy. These findings open the door to developing *T. foenum-graecum* as a potential source of bioactive ingredients for breast cancer therapy and encourage further research to explore its clinical applications in greater depth.

Conflict of interests

The authors declare that they have no financial and personal relationships with other persons or organizations that could improperly influence our work, no professional or other personal interest of any kind in any product, service, and/or company that could be construed as influencing the position presented in, or review of, the manuscript.

Author's contribution

Conceptualization: Cicik Suriani and Armansyah Maulana Harahap.
Data curation: Cicik Suriani and Armansyah Maulana Harahap.
Formal analysis: Ahmad Shafwan S Pulungan.

Funding acquisition: Cicik Suriani.
Investigation: Fauziyah Harahap.
Methodology: Armansyah Maulana Harahap.
Project administration: Syahmi Edi.
Resources: Cicik Suriani and Armansyah Maulana Harahap.
Software: Fauziyah Harahap.
Supervision: Fauziyah Harahap.
Validation: Syahmi Edi.
Visualization: Ahmad Shafwan S Pulungan.
Writing–original draft: Armansyah Maulana Harahap.
Writing–review & editing: Armansyah Maulana and Fauziyah Harahap.

Declaration of AI-assisted tools in the writing procedure

The authors affirm that AI-based tools (GPT-4, and QuillBot) were used solely for language refinement and to enhance text readability, under the authors' strict supervision. All scientific content, interpretations, and conclusions were developed entirely by the authors, who take full responsibility for the manuscript's accuracy. No images were created or manipulated using AI tools.

Ethical considerations

All ethical issues, including plagiarism, research misconduct, data fabrication, falsification, and duplicate or redundant publication, were carefully considered and fully avoided by the authors.

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Supplementary files

Supplementary file 1 contains Table S1 and Figure S1.

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