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IN-VITRO EVALUATION OF THE ANTICANCER POTENTIAL OF IRUNELLI KARPAM, A CLASSICAL SIDDHA FORMULATION, AGAINST TRIPLE-NEGATIVE BREAST CANCER CELLS

S. Kanishka [*1], S. M. Kirubhakaran [2] and S. Mathukumar [3]

Sri Sairam Siddha Medical College and Research Centre [1], Chennai - 600132, Tamil Nadu, India.
Sattam Sarntha Maruthuvamum Nanju Maruthuvamum, Sri Sairam Siddha Medical College and Research Centre [2], Chennai - 600132, Tamil Nadu, India.
Board of Unani, Siddha, Sowa Rigpa [3], NCISM.

Keywords:

Irunelli Karpam, Siddha medicine, Triple-negative breast cancer, MDA-MB-231, Cytotoxicity, Integrative oncology

Correspondence to Author:
S. Kanishka

CRRI, Bachelor of Siddha Medicine and Surgery, Sri Sairam Siddha Sri Sairam Siddha Medical College & Hospital Sai Leo Nagar, West Tambaram, Poonthandalam, Sirukalathur, Chennai - 600044, Tamil Nadu, India.

E-mail: kanishkasiddha@gmail.com

ABSTRACT: Breast carcinoma continues to pose significant healthcare challenges globally, contributing substantially to oncological morbidity and mortality rates, thereby highlighting the urgent need for enhanced therapeutic strategies with improved safety profiles. Traditional Siddha medicine offers valuable resources for the development of potential anticancer compounds. *Irunelli Karpam*, a classical Siddha formulation composed of purified sulfur and *Phyllanthus emblica* Linn., was evaluated for its cytotoxic activity against the human triple-negative breast cancer cell line (MDA-MB-231) *in-vitro*. The formulation was tested at concentrations of 12.5–200 µg/mL using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The findings revealed a progressive decline in viability across the tested concentrations, from 94.61% (12.5 µg/mL) to 12.48% (200 µg/mL), yielding a calculated half-maximal inhibitory concentration of 47.17 µg/mL. Microscopic assessment identified the hallmark features of programmed cell death, including cellular contraction, spherical transformation, and loss of substrate adhesion. The current results demonstrate the substantial growth-inhibitory capacity of *Irunelli Karpam* against MDA-MB-231 cells which make it a complementary therapeutic agent, warranting further mechanistic studies, toxicity profiling, and preclinical validation.

INTRODUCTION: Globally, cancer ranks as the second most common cause of mortality, accounting for approximately 16% of all deaths annually [1]. Data from the United States documented over 600,000 cancer-attributed fatalities [2, 3]. In women, breast cancer is the most prevalent malignancy and one of the leading causes of cancer related mortality.

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Triple-negative breast cancer presents a particularly challenging clinical scenario, because of the lack of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) receptor expression, a phenotype that limits therapeutic targeting options and correlates with an unfavorable prognosis. Despite advances in chemotherapy, radiotherapy, and targeted treatments, long-term survival remains constrained by toxicity, cost, and therapeutic resistance [4, 5].

These limitations highlight the need for safer and more effective alternatives to these drugs. Recent comprehensive reviews have systematically evaluated natural compounds against triple-negative breast cancer, identifying multiple plant-

derived agents with promising cytotoxic activity [6]. Natural extracts have demonstrated the ability to modify cancer cell migration through mitochondrial metabolism alterations [7], suggesting multi-targeted mechanisms of action that may overcome resistance patterns observed with conventional single-agent therapies.

Traditional systems of medicine, such as Siddha, offer a vast repository of plant- and mineral-based formulations with potential anti-cancer properties. The integration of Ayurvedic and traditional medicine principles with modern cancer therapeutics represents an emerging paradigm in integrative oncology [8]. Several Siddha preparations, including *Poorna Chandhrodhayam Chendhooram* and *Rasagandhi Mezhugu* have been scientifically validated for their cytotoxic effects against cancer cell lines, suggesting that classical formulations may provide novel therapeutic leads [9, 10].

The principal ingredient of *Irunelli Karpam*, *Phyllanthus emblica* (Indian gooseberry), contains abundant ascorbic acid, polyphenolic compounds, and tannins, with extensively characterized antioxidant and immune-modulating activities [11]. Recent investigations on related *Phyllanthus* species have confirmed selective cytotoxic effects against breast cancer cell lines, with minimal toxicity toward normal mammary epithelial cells [11, 12]. When processed using traditional purification methods, sulfur yields bioavailable forms that exhibit anti-inflammatory and antimicrobial properties. The synergistic interaction between the herbal and mineral components in Siddha is hypothesized to enhance their therapeutic efficacy, while minimizing toxicity.

This classical herbo-mineral preparation is documented in The Siddha Formulary of India for managing dermatological conditions, including various rashes and scabies [13]. Preliminary observations and recent investigations suggest that its pharmacological spectrum may extend beyond dermatological disorders, with potential activity against abnormal cellular growth [14, 15]. Despite its traditional use and preliminary bioactivity reports, a systematic evaluation of the anticancer potential of *Irunelli Karpam* remains limited in the literature. Therefore, this study aimed to evaluate the *in-vitro* cytotoxic activity of *Irunelli Karpam* in the MDA-MB-231 human breast cancer cell line using the MTT assay. By bridging traditional knowledge, with modern scientific validation.

MATERIALS AND METHODS:

Preparation of *Irunelli Karpam*: The Siddha formulation *Irunelli Karpam* was prepared using purified elemental sulfur (*Nellikai andhakam*) and fresh *Phyllanthus emblica* fruit (*Nellikai*) juice, The authenticity of both raw materials, amla **Fig. 1A** and sulfur **Fig. 1B**, was verified by the *Gunapadam* Pharmacology Department, Sri Sairam Siddha Medical College and Research Centre, Chennai, India.

Sulfur was purified following the classical procedure described in *Gunapadam Thathu Jeeva Vaguppu* [16] **Fig. 1C**. After purification, sulfur was finely powdered and freshly extracted amla juice was gradually incorporated at a 1:2 (w/v) ratio, with continuous trituration until a homogeneous paste was obtained **Fig. 1D**. The preparation was standardized according to The Siddha Formulary of India (1992) [17]. The formulation was further authenticated and characterized according to previously published analytical and biochemical reports on *Irunelli Karpam*, which confirmed its compositional consistency and safety profile [14, 18].

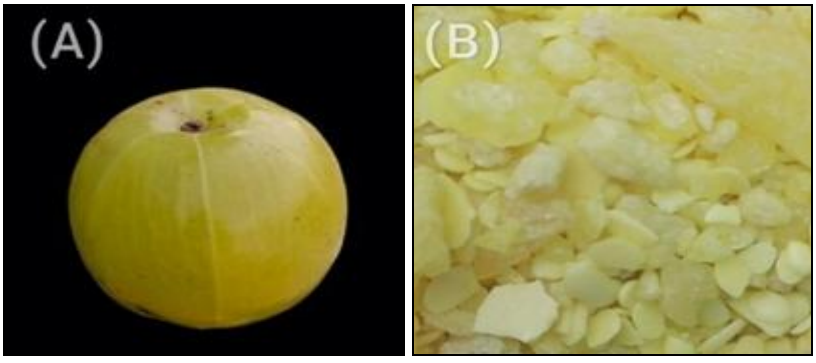

FIG. 1: RAW DRUGS, PURIFIED DRUGS AND MEDICINE PREPARED

Physicochemical and Preliminary Characterization: Heavy metal analysis performed using Atomic Absorption Spectroscopy (AAS) revealed the presence of sulfur (major component), along with trace amounts of iron, manganese, copper, and lead within permissible limits as per Ayurvedic Pharmacopoeia standards [18]. The formulation exhibited marked antioxidant capacity *in-vitro* (DPPH radical scavenging and FRAP assays), demonstrating dose-dependent free radical scavenging activity [19], suggesting potential cytoprotective effects. Particle size analysis and scanning electron microscopy should be considered in future studies to characterize the nanoparticulate nature of the formulation.

Cell Line and Culture Maintenance: MDA-MB-231 human breast adenocarcinoma cells were obtained from NCCS, Pune, India and were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (FBS) and antibiotics, within a 37°C incubator maintaining 5% CO_2 atmosphere. Regular passaging ensured that the cells remained in exponential growth phase.

Evaluation of Anticancer Activity: Cytotoxic activity was evaluated using an MTT-based colorimetric assay [20]. MDA-MB-231 cells were plated in 96-well culture plates (1×10^4 cells per well) and allowed to grow for 48 h in a humidified incubator (37°C, 5% CO_2) until reaching 70-80% confluency. The medium was replaced with fresh DMEM containing different concentrations of the test formulation, and the cells were incubated for 24 h. All assays were conducted with three technical replicates, and the data are represented as mean ± SD. The IC_{50} value was calculated using non-linear regression analysis with appropriate statistical software. Morphological alterations in the treated and untreated cells were observed under a digital inverted microscope at 20× magnification and photographed. After the treatment exposure, the culture medium was aspirated, and the cultures were rinsed three times with the phosphate-buffered saline (pH 7.4). MTT solution was then added into each well, followed by dark incubation at 37°C for 2 h. The resulting formazan precipitate was solubilized using 100 µL DMSO, and optical density readings were obtained at 570 nm using a Bio-Rad spectrophotometer.

Calculation of Cell Viability: Percentage viability was calculated by comparing sample absorbance to control absorbance values, expressed as:

$$\% \text{ Viability} = (\text{Sample } OD_{570} / \text{Control } OD_{570}) \times 100$$

Statistical Analysis: Data processing was performed using statistical analysis software, with viability percentages derived from triplicate measurements across two separate experimental runs. Dose-response data were analyzed to estimate IC_{50} values using non-linear regression, and group differences were evaluated using one-way analysis of variance (ANOVA) with appropriate post-hoc comparisons, with $p < 0.05$ considered statistically significant.

RESULT:

Morphological Observations: The cytotoxic effects of *Irunelli Karpam* on MDA-MB-231 cells were assessed using microscopic examination. The control group cells exhibited a characteristic epithelial architecture with spindle morphology, maintained membrane integrity, and formed confluent monolayers. Treatment with *Irunelli Karpam* induced dose-dependent morphological alterationsin the cells. At lower concentrations (12.5–25 µg/mL), cells exhibited mild shrinkage

and reduced density. Higher concentrations (50–200 µg/mL) caused pronounced cytotoxic effects, including cell rounding, detachment, and loss of cell confluence. At 200 µg/mL, extensive cytoplasmic vacuolation and nuclear condensation were evident, along with the appearance of apoptotic bodies and cellular debris in the medium, suggesting late-stage apoptotic or necrotic changes in the cells.

The highest dose tested (200 µg/mL) resulted in extensive disruption of cell architecture, indicating strong cytotoxic activity. These observations are consistent with previously reported effects of Siddha nanoformulations, such as *Poorna Chandhrodhayam Chendooram*, which also demonstrated Concentration-dependent morphological alterations in MDA-MB-231 cells.

Cell Viability (MTT Assay):

TABLE 1: CONCENTRATION-DEPENDENT CYTOTOXIC EFFECT OF *IRUNELLI KARPAM* ON MDA-MB-231 CELLS AFTER 24-HOUR TREATMENT (VALUES REPRESENT MEAN OF TWO INDEPENDENT EXPERIMENTS CONDUCTED WITH THREE REPLICATES)

S. no.	Concentrations (µg/mL)	Absorbance	Cell Viability (%)	% Inhibition
1	Control (0)	0.761	100	-
2	12.5	0.72	94.6	5.4
3	25	0.6	78.8	21.2
4	50	0.35	46.3	53.7
5	100	0.2	26.7	73.3
6	200	0.101	12.48	87.5

The cytotoxic effects of *Irunelli Karpam* were quantified using the MTT assay. A progressive decline in cellular viability was observed with increasing dosage, consistent with the microscopic findings. As shown in **Table 1**, cellular viability declined from 100% in the untreated controls to 12.48% at the highest concentration (200 µg/mL). Specifically, the viabilities were 94.61%, 78.84%, 46.32%, and 26.67% at 12.5, 25, 50 and 100 µg/mL. An IC_{50} value of 47.17 µg/mL was observed. The representative data from the absorbance measurements provided in **Table 1** confirm the reproducibility across the experimental replicates. The dose-response curve plotted in **Fig. 2** illustrates the progressive decline in cell viability with increasing formulation concentration, highlighting the potent cytotoxicity of the formulation. These results are comparable to those reported for other Siddha nanoformulations on MDA-MB-231 cells.

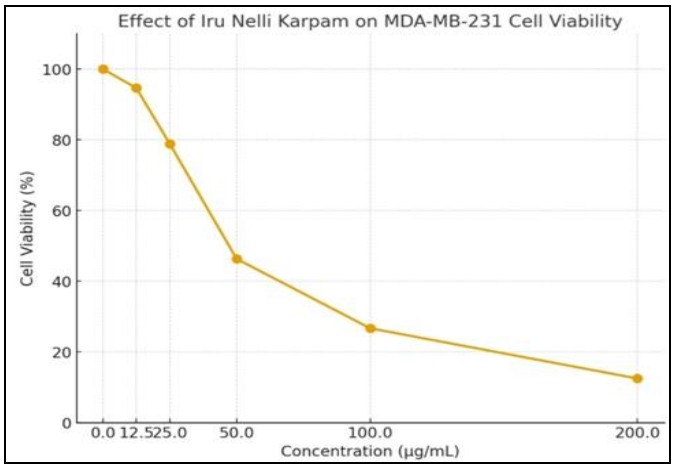

FIG. 2: VIABILITY OF CANCER CELLSUPON INCREASING THE TEST FORMULATION

For comparative context, the cytotoxic potency of *Irunelli Karpam* (as shown in **Fig. 3**, IC_{50} = 47.17 µg/mL) is within the range of other traditional herbo-mineral formulations tested against MDA-MB-231 cells. Although lower than that of standard anticancer drugs such as doxorubicin (IC_{50} ≈ 1–2 µg/mL), it indicates promising moderate activity with a likely reduced systemic toxicity potential.

Representative Microscopy: Representative microscopic images **Fig. 3** further illustrates the

cytotoxic effects, including cell shrinkage, rounding, and detachment at higher concentrations of the extract. The morphological alterations observed in this study closely mirrored those reported for other Siddha nanoformulations, reinforcing the potential of *Irunelli Karpam* as an effective anticancer agent.

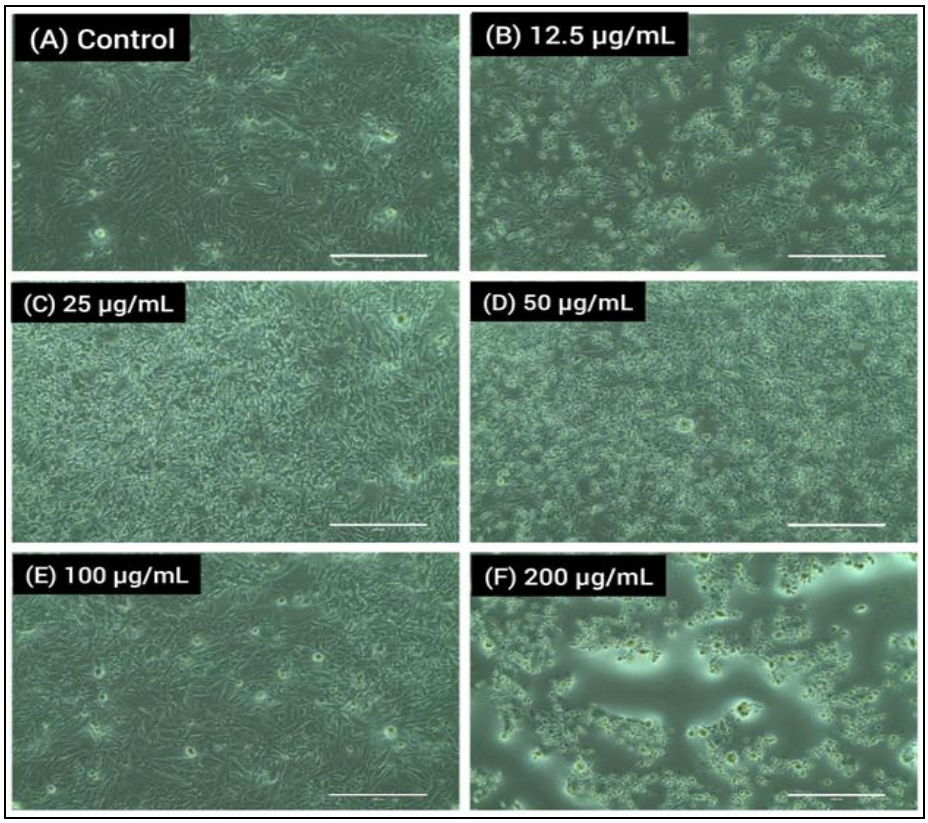

FIG. 3: REPRESENTATIVE PHOTOMICROGRAPHS SHOWING MORPHOLOGICAL CHANGES IN MDA-MB-231 CELLS FOLLOWING 24-HOUR TREATMENT WITH *IRUNELLI KARPAM* AT VARIOUS CONCENTRATIONS (12.5–200 µG/ML) COMPARED TO THE UNTREATED CONTROL IMAGES. (DEMONSTRATING THE PROGRESSIVE CYTOTOXIC EFFECTS INCLUDING CELL SHRINKAGE, ROUNDING AND DETACHMENT AT HIGHER CONCENTRATIONS)

IC_{50} Determination and Dose-Response Analysis: Fig. 4 illustrates the half-maximal inhibitory concentration (IC_{50}) of *Irunelli Karpam* against MDA-MB-231 cells, which was 47.17 µg/mL. The dose-response curve exhibited a sigmoidal pattern characteristic of concentration-dependent cytotoxicity, with a steep decline in viability observed between 25 and 100 µg/mL, indicating a narrow therapeutic window that warrants careful dose optimization in subsequent studies.

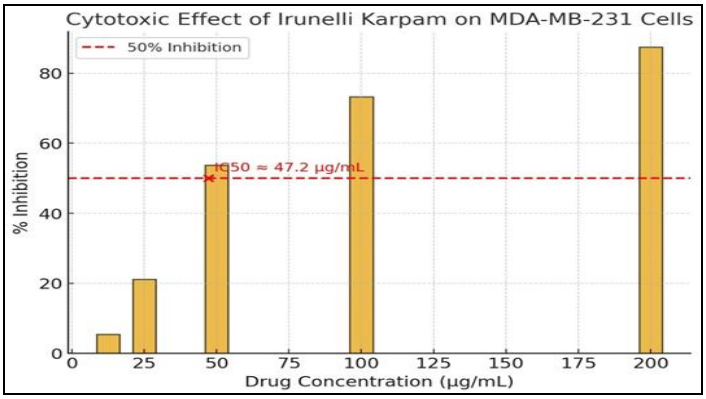

FIG. 4: INCREASE IN CELL INHIBITION PERCENTAGE UPON INCREASING THE TEST FORMULATION CONCENTRATION

Statistical Analysis: All data are expressed as mean ± standard deviation (SD) for three independent replicates per group.

The standard deviation was calculated using the formula in the **Fig. 5.**

$$S = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \overline{x})^2}{n-1}}$$

FIG. 5: FORMULA FOR CALCULATING STANDARD DEVIATION

Where, n = 3.

TABLE 2: EFFECT OF *IRUNELLI KARPAM* ON MDA-MB-231 CELL VIABILITY (MTT ASSAY, 24 H). VALUES ARE EXPRESSED AS MEAN ± SD (N=3). STATISTICAL COMPARISONS WERE MADE BETWEEN THE UNTREATED CONTROL AND TREATED GROUPS USING THE TWO-TAILED UNPAIRED T-TEST. NS = NOT SIGNIFICANT (P > 0.05); ** P < 0.01; *** P < 0.001

S. no.	Concentration (µg/mL)	Mean Cell Viability (%) ± SD	p-value (vs Control)	Significance
1	0 (Control)	100.00 ± 0.99	-	-
2	12.5	94.61±0.18	0.18	Ns
3	25	78.84 ± 0.55	0.07	Ns
4	50	46.32 ± 0.84	0.008	**
5	100	26.68±0.55	0.001	***
6	200	12.48 ± 1.12	0.0004	***

As in the **Table 2,** calculated SD values ranged from 0.18% to 1.12 %, indicating excellent reproducibility (coefficient of variation < 10%).

Inter-group statistical comparisons were performed using the two-tailed unpaired Student's t-test. Significant reductions in cell viability were observed from 50 µg/mL ($p < 0.01$), with highly significant effects at 100 µg/mL ($p = 0.001$) and 200 µg/mL ($p = 0.0004$).

The IC_{50} value of *Irunelli Karpam* was 47.17 µg/mL, confirming a clear, dose-dependent cytotoxic effect.

Summary Statement: Overall, these findings demonstrate that *Irunelli Karpam* induces concentration-dependent cytotoxicity in MDA-MB-231 cells. The combined decline in viability and concurrent morphological alterations substantiate the potential utility of these compounds as therapeutic agents against breast cancer.

DISCUSSION: The present investigation demonstrated the quantifiable cytotoxic efficacy of *Irunelli Karpam* when tested against the MDA-MB-231 breast carcinoma cell line under *in-vitro* conditions. Untreated control cells exhibited typical epithelial morphology, maintaining elongated spindle-shaped structures and intact cell confluence. In contrast, exposure to increasing concentrations of the formulation resulted in progressive cytotoxic alterations, including cell shrinkage, rounding, membrane blebbing, and detachment, which are hallmarks of loss of viability and apoptotic induction. Quantitative analyses confirmed these observations. Cell viability decreased correlating with applied concentrations, with minimal reduction at lower concentrations and a marked decline at higher concentrations. At 200 µg/mL, the viability was reduced to 12.48%, highlighting the potency of the formulation. This consistent agreement between morphological changes and viability data suggests that *Irunelli Karpam* compromises cellular integrity and metabolic activity proportionally to dosage levels.

Mechanistic Insights: The cytotoxic mechanism of *Irunelli Karpam* may involve multiple pathways.

In addition, the formulation likely induced apoptosis through the activation of intrinsic mitochondrial pathways. Evidence from comparable Siddha and herbal formulations shows the upregulation of pro-apoptotic markers (Bax and caspase-3) and downregulation of anti-apoptotic proteins (Bcl-2). These changes lead to mitochondrial membrane depolarization and DNA fragmentation which are hallmarks of apoptosis. Reactive oxygen species (ROS) generation may also contribute to oxidative-stress-mediated cytotoxicity [21, 22]. Recent mechanistic studies have elucidated the complex role of ROS in vascular-related diseases and cancer progression[21], demonstrating that ROS-mediated pathways represent a critical intersection between oxidative stress and programmed cell death in cancer therapeutics [23]. While the formulation exhibits antioxidant properties in cell-free systems [15], its

pro-oxidant effects in cancer cells can trigger selective cytotoxicity [21, 22]. At higher concentrations, certain phytochemicals, including those present in *Phyllanthus emblica*, may shift from antioxidant to pro-oxidant activity, leading to oxidative stress–mediated cytotoxicity. At higher concentrations, Compounds such as gallic acid and ellagic acid can generate reactive oxygen species (ROS) that overwhelm the antioxidant defenses of cancer cells, leading to oxidative damage and apoptosis [23].

The incorporation of purified sulfur in the formulation may further enhance the anticancer potential of this formulation. Compounds incorporating sulfur influence redox homeostasis, suppress tumor proliferation by interfering with cell cycle progression, and induce apoptosis through mitochondrial pathways. Additionally, the ability of sulfur to form bioactive complexes with organic molecules may contribute to the improved bioavailability and targeted delivery of active phytoconstituents. Previous standardization studies of *Irunelli Karpam* have confirmed acceptable heavy metal limits and biochemical consistency [14, 18], supporting its safety for pharmacological evaluation.

Comparison with Related Work: Recent evaluations of other Siddha preparations have further substantiated the anticancer potential of traditional formulations, including *Thangauram* against HeLa cells [24] and *Vaalai Rasa Mezhugu* against testicular cancer cells [25]. Additionally, Siddha prebiotic medicines have shown promise in managing chemo-radiotherapy-induced side effects in cancer patients [26], suggesting broader applications beyond direct cytotoxicity. Comparable Ayurvedic and TCM formulations such as *Triphala* and Baicalein, also exhibit ROS-mediated apoptosis, highlighting a shared mechanistic rationale across traditional systems. The emerging field of integrative oncology emphasizes the scientific validation of traditional medicine systems through rigorous experimental methodologies [27, 28]. Contemporary reviews have systematically documented the phytotherapeutic efficacy of herbal medicines in cancer treatment [28], demonstrating that plant-derived compounds exhibit diverse mechanisms of action including apoptosis induction, cell cycle arrest, and immune modulation. Standardization protocols for traditional formulations [27]ensure reproducibility and quality control, bridging traditional knowledge with evidence-based practice.

Limitations and Future Work: Several constraints warrant acknowledgment. The experimental scope encompassed only MDA-MB-231 cells, without a comparative assessment using normal epithelial cell lines to establish selective toxicity indices. Although *Phyllanthus emblica* (Amla) extracts exert marked cytotoxicity against breast cancer cell lines while exhibiting minimal or no toxicity toward normal human breast epithelial cells (MCF-^{10}A), indicating selective anticancer activity [11, 12], no published cytotoxicity data are available for purified or elemental sulfur on normal breast epithelial cells (MCF-10A). Existing studies have examined highly purified sulfur on oral keratinocytes or sulfur nanoparticles, underscoring the need for direct evaluation of normal mammary cells [29, 30].

Hence, future studies on *Irunelli Karpam* should include normal cell lines (e.g., MCF-10A breast epithelial cells) to assess selective toxicity. An SI > 2 indicates selective toxicity toward cancer cells, thereby strengthening therapeutic relevance [6]. Second, the precise molecular mechanisms underlying the observed cytotoxicity, including specific apoptotic markers (caspase activation, PARP cleavage, and Annexin V staining), cell cycle distribution, and ROS generation, were not investigated and warrant further detailed mechanistic studies. Third, the complex nature of the formulation makes it challenging to attribute its effects to specific ingredients. Finally, *in-vivo* validation using animal models is essential prior to clinical translation.

Compared to other Siddha formulations evaluated against MDA-MB-231 cells, *Irunelli Karpam* demonstrated competitive cytotoxic activity. *PoornaChandhrodhayamChendhooram* also exhibited notable efficacy against MDA-MB-231 cells 9 while *Rasagandhimezhugu* showed antiproliferative effects on multiple cancer cell lines 10. The IC_{50} of 47.17 µg/mL observed in this study positions *Irunelli Karpam* as a moderately potent agent, although direct comparisons are limited by methodological variations between

studies. Standardization of assay protocols and comparative evaluations using standard chemotherapeutic agents (e.g., doxorubicin and paclitaxel) would provide valuable context for interpreting these results. Overall, this study highlights the relevance of integrating traditional Siddha formulations into contemporary cancer research. By demonstrating its selective cytotoxicity against breast cancer cells, *Irunelli Karpam* emerges as a promising candidate for future mechanistic studies and preclinical evaluations aimed at developing safer and more affordable therapeutic options.

CONCLUSION: The present investigation establishes the initial systematic documentation of the cytotoxic efficacy of *Irunelli Karpam* against TNBC cellular models under controlled *in-vitro* conditions. The formulation exhibited concentration-dependent cytotoxicity, with an IC_{50} of 47.17 µg/mL, accompanied by characteristic morphological changes indicative of cell death. These findings validate the traditional use of Siddha preparations and support their potential role in integrative oncology research.

However, translation from bench to bedside requires substantial research efforts. Priority areas include:

1. Elucidation of molecular mechanisms using flow cytometry, western blotting, and gene expression profiling techniques.
2. Assessment of selectivity using normal cell lines and calculation of selectivity indices.
3. Identification and quantification of active phytochemical constituents.
4. Pharmacokinetic and toxicological evaluations in animal models.
5. Investigation of potential synergistic effects with conventional chemotherapy.

By bridging traditional wisdom with modern scientific rigor, this study contributes to the growing evidence base for Siddha medicine in cancer therapeutics and opens avenues for developing safer, more accessible, and culturally relevant treatment modalities for breast cancer. Mechanistic evaluation through apoptotic assays (Annexin-V, caspase activity), ROS quantification, and cell cycle profiling should be prioritized to substantiate the observed effects. Phytochemical profiling using HPTLC or GC–MS could also help link active constituents to biological effects.

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Ethical Approval: This study did not involve human participants or animal subjects. Cell line experiments were conducted in accordance with institutional biosafety guidelines.

Data Availability: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Generative AI and AI-Assisted Technologies in the Manuscript Preparation Process: The author used Claude (Anthropic, 2024 version) to assist with grammar improvement while ensuring that the intellectual content, analysis, and interpretations were entirely original and authored by the researcher.

CONFLICT OF INTEREST: The authors declare no conflicts of interest related to this study.

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