https://doi.org/10.47552/ijam.v16i1.5194 ISSN No: 0976-5921

*International Journal of Ayurvedic Medicine, Vol 16 (1), 2025; 191-196*

# *In-vitro* Evaluation of the Siddha Formulation Mahaveera Mezhugu (MVM) for Anti-Cancer Activity Against MCF-7 Human Breast Cancer Cells

**Research Article**

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## Abstract

Over one in ten new cases of cancer detected in women are related to breast cancer, making it the most common disease diagnosed in this demographic. It is the second leading cause of cancer-related deaths worldwide for women. The many cancer treatments and their potential adverse effects made people aware of the need to look for safe, affordable substitutes for cancer treatment. The Siddha system can offer a lot of formulations in the management of breast cancer. There are also many literary works that facilitate this. Efficacy of *Mahaveera Mezhugu* (MVM) is a Herbo-mineral siddha preparation it is indicated for *Vatharogam, Megarogam*. This MVM has been used for siddha clinically to treat breast cancer. but there is no scientific data to validate this medicine. Hence this study aims to evaluate the anticancer potential of *Mahaveera Mezhugu* through an *in-vitro* study in the MCF-7 Human Breast cell line, determined by dimethyl sulphoxide (DMSO, MTT and Annexin V- Fitc apoptosis by flow cytometry assay. $LC_{50}$ concentration was found at 5.1879371 µg/mL. The Siddha system, which combines many well-researched drugs, has shown clinically significant improvements in the treatment of breast cancer. Further research and large randomized control trial are needed to determine the effects of the drug.

**Keywords:** *Mahaveera mezhugu, Anti-cancer activity, MCF-7 cell line, MTT assay, Annexin V, Breast cancer.*

## Introduction

An important cause of death across the globe is cancer. The discovery of new drugs is essential to help reduce the death rate from cancer. Humans have continuously strived to reduce mortality and morbidity due to cancer, with the help of various advancements in science, technology and enhanced research to gain knowledge about various human neoplastic diseases. A variety of cancers are reported in human beings. Among the most prevalent is breast cancer neoplasms, in 2022, there were 2.3 million women diagnosed with breast cancer and 6,70,000 deaths globally (1). It is the most common cancer among female Indians (2).

Chemotherapy for breast cancer is an established and recommended treatment regimen. However, the biggest concerns of chemotherapy drugs and radiotherapy are their inherent side effects that make patients shift towards other effective and alternative therapies. In a retrospective study conducted from January 2006 to December 2015, it was found that the most prevalent complications of contemporary treatment included neutropenia, sepsis, and anaemia, with lung cancer, breast cancer, and non-Hodgkin lymphoma being the most frequently represented cancers (3,4). This is where the Traditional system of medicine plays a crucial role in meeting the demands of global health care. India is well-known for its traditional medicine systems, which include Siddha, Ayurveda and various other traditional medicine systems. The Siddha system of medicine is one of the oldest Indian medicinal systems with several medicinal formulations that possess evidence for preventing and treating various types of cancers (5).

The most common type of breast cancer begins in the milk-production ducts, called ductal carcinoma but cancer may also occur in the lobules or other parts of breast tissues (6). *Mahaveera Mezhugu (*MVM*)* is one of the Siddha herbo-mineral drug indicated for *Vatharogam, Megarogam* and clinically used in the management of breast cancer by Siddha physicians but currently, there is no scientific evidence for it (7). Another add-on factor is that the formulation (MVM)

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consists of *Veeram* (Hydrargyrum perchloride), *Pooram* (Hydrargyrum subchloride), *Lingam* (Red sulphide of mercury) and *Chithira moolam* (*Plumbago zeylanica*), each of which has already been proved to have tremendous potential for a cancer cure in various scientific studies (8). Hence, the purpose of this study was to assess the growth inhibitory effect of *Mahaveera Mezhugu* (MVM) in the human breast cancer cell line MCF-7.

## Materials and Methods

### Trial Drug

*Mahaveera Mezhugu* (MVM) is a classical Siddha Herbo mineral formulation mentioned in "*Siddha Vaidhiya Thirattu"*. The drug was procured from a GMP-certified pharma company.

### Ingredients of *Mahaveera Mezhugu*

Table 1: Provides a detailed overview of the components of MVM.

**Table: 1 Ingredients of *Mahaveera Mezhugu***

| S. No | Vernacular name | Botanical name/ Chemical name |
|---|---|---|
| 1 | *Veeram* | Hydrargyrum perchloride |
| 2 | *Pooram* | Hydrargyrum subchloride |
| 3 | *Lingam* | Cinnabar |
| 4 | *Chukku* | *Zingiber officinale.* Roscoe |
| 5 | *Milagu* | *Piper nigrum* L. |
| 6 | *Thippili* | *Piper longum* L. |
| 7 | *Chithramoolam* | *Plumbago zeylanica* L. |
| 8 | *Kungumapoo* | *Crocus sativus* L. |
| 9 | *Korosanai* | Ox bile |
| 10 | *Pachaikarpooram* | Borneo camphor |
| 11 | *Murungaipattai Saaru* | *Moringa oleifera* Lam. |

### Preparation of Mahaveera Mezhugu

First, the *Veeram* was purified with the juice of Moringa bark until it completely blended. After that, the drugs *Veeram*, *Lingam*, *Pooram*, *Thirikadugu* and *Chithiramoolam* were ground with honey and milk. Finally, the drugs *Kunguma poo*, *Korosanai*, and *Pachai karpooram* were added to the above mixture and ground well until it reached a Mezhugu (semisolid/wax) form. The medicine was then stored in an airtight container (7).

Dosage: 1-2 Payaru alavu (65mg-130mg) /Bd

Adjuvant: Honey

### In-vitro Evaluation of Anticancer Activity

#### *Cell Line*

The MCF-7 cell line was initially procured from the "National Centre for Cell Sciences (NCCS) in Pune" India and later maintained in "Dulbecco's Modified Eagle's Medium" (DMEM).

#### *Cell Culture and Cytotoxicity Assessment*

Cells were grown in 25 $cm^2$ flasks containing "Dulbecco's Modified Eagle Medium (DMEM)" enriched with 10% FBS, L-glutamine, sodium bicarbonate, and an antibiotic solution (Merck, Germany)". The cultures were maintained at 37°C in a humidified incubator with 5% $CO_2$ (New Brunswick Scientific division of Eppendorf, Germany). Cell viability was evaluated using the MTT assay following direct observation under an inverted phase contrast microscope. Once the cells reached confluence, they were trypsinized, resuspended in 10% growth medium, and plated into 96-well plates at a density of $5x10^3$ cells per well.

A stock solution of MVM (1 mg/mL in 0.1% Dimethyl Sulfoxide) was prepared, filtered through a 0.22 µm Millipore syringe filter, and serially diluted in DMEM to concentrations of 100, 50, 25, 12.5, and 6.25 µg/mL. Each concentration (100 µL) was added in triplicate to the wells, followed by incubation at 37°C in a humidified 5% $CO_2$ environment. Untreated control cells were cultured under the same conditions. After 24 hours, the cells were examined under an inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera), and images of the observations were captured. Cytotoxicity was assessed based on morphological changes such as cell shape alterations, granulation, and vacuolization (9,10,11).

Using the following formula, the percentage of growth inhibition was determined:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control group}} * 100$$

### Apoptosis Analysis Using Annexin V Flow Cytometry

The MCF-7 cell line was cultured following standard protocols. Once the cells reached confluency, they were treated with the $LC_{50}$ concentration of the sample (5.1879371 µg/mL) and incubated for 24 hours. Untreated control wells were maintained simultaneously for comparison. For apoptosis evaluation, the cells were transferred into 12 × 75 mm polystyrene tubes, ensuring a minimum of $1 \times 10^6$ cells per tube. The tubes were centrifuged at 3000 RPM for 5 minutes, and the supernatant was carefully discarded without disrupting the cell pellet.

Subsequently, 100 µL of Muse™ Annexin V & Dead Cell Reagent was added to each tube, gently mixed for 3-5 seconds, and incubated at room temperature in the dark for 20 minutes. Flow cytometric analysis was conducted using Muse software, with cells gated against untreated controls. Apoptotic events were quantified and analyzed using Muse FCS 3.0 software (12,13)

## Results

Cytotoxic effects of MVM on cancer cells treated with various concentrations of cell viability percentage in Table 1 and Figure 1& 2.

https://doi.org/10.47552/ijam.v16i1.5194 ISSN No: 0976-5921

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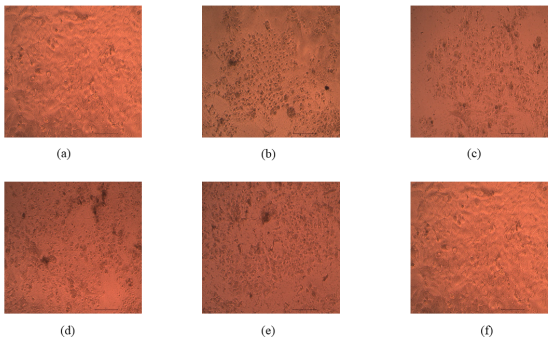
**Table: 2 Percentage viability of Mahaveera Mezhugu at various concentrations**

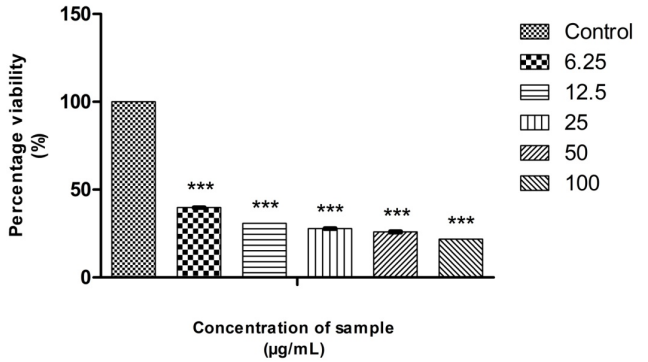
| MVM Concentration µg/mL | Percentage viability |
|---|---|
| Control | 100 |
| 6.25 | 39.76*** |
| 12.5 | 30.81*** |
| 25 | 27.86*** |
| 50 | 25.94*** |
| 100 | 21.82*** |

**Figure: 1 Morphological Comparison of MCF-7 Human Breast cell line treated with test drug MVM at varying concentrations a) Control b) MVM - 6.25µg/mL, c) MVM - 12.5µg/mL, d) MVM - 25µg/mL, e) MVM - 50µg/mL, f) MVM - 100µg/mL.**




The data were expressed as mean ± standard deviation (SD). Statistical evaluation was performed using one-way analysis of variance (ANOVA), followed by Dunnett's test for post-hoc comparisons. A p-value of ***p < 0.001 relative to the control group was considered statistically significant.

**Figure: 2 Anti-cancer activity of MVM on cell viability of MCF-7 Human Breast cell line**




The data were expressed as mean ± standard deviation (SD). Statistical evaluation was performed using one-way analysis of variance (ANOVA), followed by Dunnett's test for post-hoc comparisons. A p-value of ***p < 0.001 relative to the control group was considered statistically significant.

**Apoptosis by Annexin – V Flow cytometry**

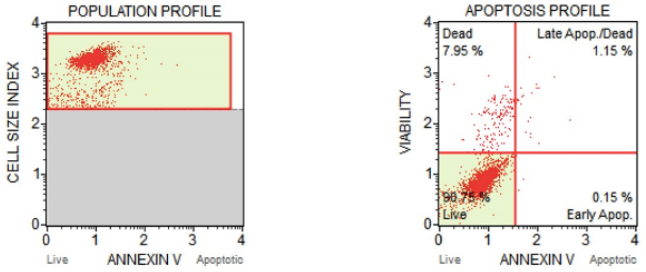
MVM treatment induces apoptosis in cancer cells, as evident from the significant differences observed between the untreated groups shown in Figure 3 and Table 3.

The early apoptotic percentage was determined to be 0.15% and the late apoptotic change was found as 1.15%. The total Apoptotic percentage was found to be in the minimal amount of 1.30%.

**Table 3: Cell distribution in cells untreated with MVM**

| Parameter | Cell Concentration (Cells/mL) | % Gated |
|---|---|---|
| Live (LL) | 1.16E+06 | 90.75% |
| Early Apoptotic (LR) | 1.92E+03 | 0.15% |
| Late Apoptotic/Dead (UR) | 1.47E+04 | 1.15% |
| Debris (UL) | 1.02E+05 | 7.95% |
| Total Apoptotic | 1.66E+04 | 1.30% |

**Figure:3 Apoptosis profile of Untreated Cells (a) Population (b) control**




(a) (b)

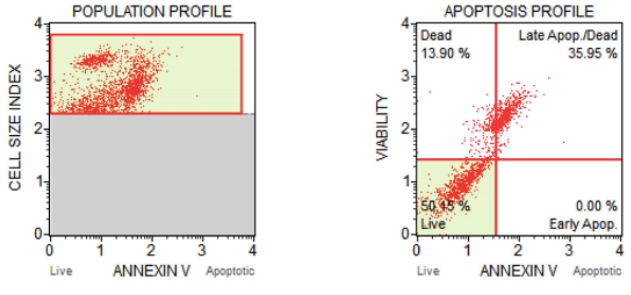
MVM treatment induces apoptosis in cancer cells, as evident from the significant differences observed in treated groups shown in Figure 4 and Table 4.

In the MVM-treated cells the early apoptotic percentage was found to be 0.00% and the late apoptotic was found to be 35.95%, 13.95 % constituted the debris resulting in the total apoptosis rate of 35.95%.

**Table 4: Cell distribution in cells treated with MVM**

| Parameter | Cell Concentration (Cells/mL) | % Gated |
|---|---|---|
| Live (LL) | 5.48E+05 | 50.15% |
| Early Apoptotic (LR) | 0.00E+00 | 0.00% |
| Late Apoptotic/Dead (UR) | 3.93E+05 | 35.95% |
| Debris (UL) | 1.52E+05 | 13.90% |
| Total Apoptotic | 3.93E+05 | 35.95% |

**Figure:4 Apoptosis profile of treated Cells (a) Population (b) MVM profile**




(a) (b)

## Discussion

Breast cancer treatments, while effective, can present some side effects. Chemotherapy may cause fatigue, hair loss, and reduced blood cell counts, with potential long-term risks such as heart damage (14). Endocrine therapies, such as tamoxifen and aromatase inhibitors, can lead to cognitive impairment, menstrual dysfunction, and an increased risk of osteoporosis (15,16). Radiation therapy may contribute to accelerated aging. Surgical interventions, especially for low-risk cases like "Ductal carcinoma in situ (DCIS)" might result in overtreatment. Despite the development of new drugs, some studies suggest limited population-level benefits, highlighting the need for reassessment of treatment strategies (17). It is important to find alternative treatments that work well while causing fewer side effects. Cancer research today focuses on both advanced medical treatments and traditional healing methods. Combining standard therapies with traditional remedies in a balanced way might be key to addressing this challenging disease.

The Siddha system of medicine is based on three core principles: *Vali* (movement), *Azhal* (conversion), and *Iyam* (form). *Azhal* plays a key role in cellular digestion and metabolism. An imbalance where *azhal* (fire) dominates *iyam* (water) can lead to uncontrolled tissue growth, a hallmark of tumours. Conversely, low *azhal* levels can disrupt the balance of *vali* and *iyam*, causing issues like cell migration due to interruptions in vital energy pathways (*Viyana vaayu, Samana vaayu,* and *Suzhumunai naadi*).

Each person has a unique body constitution, known as Thegi, influenced by the *doshas—Vatham, Pitham, and Kapham*—and their combinations. This constitution affects how individuals respond to their environment, illnesses, and treatments. To stay healthy, it is essential to maintain balanced *doshas*. In cancer, the *doshas Kapham* and *Vatham* are often imbalanced, contributing to tumour growth and spread (18).

In this study, the potential of a novel treatment was investigated using the MTT Assay to determine its effectiveness against the MCF-7 human breast cancer cell line. By screening the experiment at different concentrations, the $LC_{50}$ value of MVM was calculated, offering insights into its cytotoxic potential and therapeutic viability. MTT assay was done to check the cytotoxic effects of the drug and from the results, it can be observed that the compound induced a dose-dependent decrease in cell viability. MVM at a minimal concentration of 6.25ug/ml reduced the cell viability to 40%. The percentage of growth inhibition was found to increase with increasing concentration of test drug *MVM* The viability of the cell was observed to decrease serially at the concentration ranging from 21.82% at 100µg/ml, 25.94% at 50µg/ml, 27.86%, at 25µg/ml, 30.81% at 12.5µg/ml, 39.76% at 6.5µg/ml respectively. The $LC_{50}$ test sample in the MCF-7 Cell line is found to be 5.1879371µg/mL.

The above results indicates that the trial drug MVM possesses significant anti-cancerous properties. The apoptotic effects of the trial drug MVM were determined by Annexin V FITC flow cytometry which depends on the externalization of phosphatidyl serine and binding of fluorescent tagged Annexin to it. MCF-7 cell lines were cultured as per the standard procedures and treated with MVM at 5.1879371µg/mL. It was observed that at concentrations of 5.1879371µg/mL *MVM,* there was a significant increase in the percentage of cells undergoing apoptosis compared to the untreated control cells. In the sample of cells treated with *MVM*, 50.5% were found to be live and different levels of apoptotic changes were noted in the following percentages 0.00 %-early apoptotic and 35.95%- late apoptotic, 13.90% - debris, Total apoptotic is 35.95%.

The phase contrast analysis of the cells depicts considerable morphological changes corresponding to membrane blebbing and apoptotic bodies which demonstrate the potent anti-proliferative effects of the compound. The study revealed that the late apoptotic cells were significantly increased in the drug-treated cells, compared to the control cells. From the results, it can be observed that when compared with untreated control the percentage of live cells was reduced to 50% and the percentage of late apoptotic cells has been increased to 35.95%. These findings confirm the induction of apoptosis in cancer cells within 24 hours of incubation when compared with untreated control cells.

In addition to this, the cytotoxic and anti-proliferative effects of the majority of the ingredients in this formulation have been documented such as *Veeram* (Hydrargyrum perchloride), *Pooram* (Hydrargyrum subchloride), *Lingam* (Red sulphide of mercury) and *Chithira moolam* (*Plumbago zeylanica*). A study on the anticancer activity of a Siddha formulation *Pancha Paasana Chendhuram* (PPC) with *Lingam* (Red sulphide of mercury) as one of its constituents on MCF-7 Cells revealed dose-dependent inhibition of MCF-7 cell proliferation along with inducing nuclear damage in cancer cells (19). S Rajalakshmi *et al* evaluated the anti-cancer activity of siddha drugs *Veera Rasa Padhangam (VRP)* and *Panchamuga Chendhuram (PMC) containing Veeram and Lingam respectively* with the standard drug taxol using Cell Viability Assay. The *in vitro* study demonstrated that siddha drugs VRP and PMC the standard drug Taxol (20).

P Rajalakshmi *et al* studied the anti-cancer activity of *Veera Mezhugu* against the EAC cell line using the cell viability assay that demonstrated 74.05% cytotoxicity at a concentration of 1000 µg/ml (21)**.** Manjari *et al* studied the anticancer activity of *Rasa Karpoora kuligai (RSK) with Pooram as one of the main ingredients, against* HeLa cell line. The findings collectively suggested dose-dependent reduction in cell growth, significant cytotoxic and apoptotic effects on HeLa cells, potentially through modulation of apoptotic pathways and cell cycle arrest (22)**.** Plumbagin (PLB), a naturally present naphthoquinone constituent isolated from the roots of the medicinal plant *Plumbago zeylanica*, exhibits anticancer activity against a variety of cancer cell lines including breast cancer (23). Another study reported the anticancer activity of Plumbagin against Human breast cancer MCF-7 Cell line with potent anti-proliferative effects by inducing G1 phase cell cycle arrest (24). M Ravichandran *et al*

*investigated* the *in vitro* antiproliferative activity of *Chitramoola kuligai* containing *Plumbago zeylanica* against Cervical cancer in the HeLa cell line (25). It enumerated the anti-proliferative effect in a dose-dependent manner and the IC50 value was obtained at 83µg/ml**.** Hence it can be concluded that the chemical compounds and phytoconstituents present in the ingredients of the trial drug MVM and from the results of MTT Assay and Annexin V flowcytometry, it is evident that the test drug MVM are likely responsible for its anticancer activity.

## Conclusion

*Mahaveera Mezhugu* has demonstrated a dose-dependent ability to inhibit cell growth and shows significant potential in promoting late-stage apoptosis and inducing cell death in MCF-7 breast cancer cells. Findings from the in-vitro experiments indicate that this Siddha formulation possesses strong anticancer properties and could play a role in managing breast cancer, which predominantly affects women. However, since this study was limited to in-vitro analysis, further in-vivo research and clinical trials across various phases are necessary to fully evaluate its effectiveness and potential in breast cancer treatment.

**Acknowledgement**

The authors acknowledge the support and facilities provided by the National Institute of Siddha, Tambaram sanatorium, and our Center for Research on Molecular Biology and Applied Science, Trivandrum for their support in this study.

**Funding**

The author(s) received no financial support for the research, authorship, and/or publication of this article

**Conflict of interest**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Abbreviations**

**MVM**- Mahaveera Mezhugu; **GMP**- Good Manufacturing Practices; **NCCS**- National Centre for Cell Sciences; **DMEM**- Dulbecco's Modified Eagle's Medium; **DMSO**- dimethyl sulphoxide; **DCIS**- Ductal carcinoma in situ; **OD**- Optical Density; **$CO_2$**- Carbon dioxide; **FBS**-Fetal Bovine Serum

**Author contribution**

Conceptualization: SR; Medicine Preparation: SR; Data collection and compilation: SR; Manuscript Writing: SR, SSG, AB, MN & TR; Proofreading and editing: SR, SSG, SD, AB, MN, SSK & TR

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