VOLUME 10 ISSUE 2 2024

ISSN 2454 – 3055

# INTERNATIONAL JOURNAL OF ZOOLOGICAL INVESTIGATIONS

*Forum for Biological and Environmental Sciences*

Published by Saran Publications, India

*International Journal of Zoological Investigations Vol. 10, No. 2, 372-378 (2024)*

**International Journal of Zoological Investigations**

Contents available at Journals Home Page: www.ijzi.net

**Editor-in-Chief: Prof. Ajai Kumar Srivastav**

Published by: Saran Publications, Gorakhpur, India

**ISSN: 2454-3055**

# Anticancer Activity of Siddha Formulation Muthu Chenduram Against Cervical Cancer – *In Vitro* Study

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**Received:** 31st January, 2024; **Accepted:** 18th May, 2024; **Published online:** 12th August, 2024

**https://doi.org/10.33745/ijzi.2024.v10i02.035**

**Abstract:** Globally, cervical cancer is the second most prevalent cause of cancer in women. Despite significant advancements in cancer research, many patients remain incurable due to treatment resistance. Muthu Chenduram has been clinically used for decades to treat various ailments including cervical cancer. The present study was conducted to investigate the anticancer activity of Muthu Chenduram in HeLa cells. The antiproliferative activity of the test drug was investigated using the MTT assay, and the apoptotic effect was measured by the Annexin V FITC assay. The outcome indicates that by increasing the trial drug's concentration, viability and proliferation were reduced in HeLa cells, the lowest viability occurred at 100 µg/ml, at 33.30 ± 0.368%. We found that the $LC_{50}$ value was 66.7653 µg/ml. The flow cytometric method reveals that the percentage of apoptosis increases in treated cells when compared to untreated cells, with 37.58% of the treated cells being late apoptotic, indicating a significant increase in late apoptotic cells. Therefore, the effectiveness of Muthu Chenduram against the HeLa cell line is scientifically validated by the current investigation. To establish its anticancer properties, more *in vitro*, *in vivo*, and clinical investigations are required. It is stated that this study would serve as the groundwork for creating practical approaches and plans for employing Muthu Chenduram to treat cervical cancer.

**Keywords:** Muthu Chenduram, Cervical cancer, HeLa cells, MTT assay, Annexin V FITC

**Citation:** Raghavi M., Karpagambal R., Subathra T., Abarna B., Shanmugapriya P. and Madhavan R.: Anticancer activity of Siddha formulation Muthu Chenduram against cervical cancer – *in vitro* study. Intern. J. Zool. Invest. 10(2): 372-378, 2024.

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## Introduction

The fourth most common cause of mortality is cervical cancer globally among women. According to estimates, 3,420,000 people died from cervical cancer globally in 2020, accounting for 6,004,000 new cases (Sung *et al*., 2021). In the last thirty years, the percentage of young females with cervical cancer has risen from 10% to 40% (Singh *et al*., 2022). The human papillomavirus causes 90% of cases (Papa *et al*., 2017). An HIV-positive woman's risk of cervical cancer is six times greater

than that of HIV-negative women (Stelzle *et al*., 2017). Currently, various therapies are available for treating cancer, such as radiotherapy, chemotherapy, surgery, etc (Bethesda, 2023). Despite advancements in diagnosis and treatment, the incidence rate is still rising in developing countries (Beddoe Ann Marie, 2019). Furthermore, patients frequently seek alternative ways of treatment that have fewer or no adverse effects and can be used in conjunction with conventional treatments (Puthiyedath *et al*., 2022). In recent years, Siddha medicine has become increasingly popular in the management of cancer. The Siddha pathophysiology of cancer suggests that *pitham*, a regulator of digestion and metabolic activity, exists in every cell. The relationship between fire and matter, *Agni* and *Kabam*, in cancer contributes to excessive tissue growth. A metaphoric crisis occurs when *Agni* is reduced, leading to increased *Vatham* and *Kabam* forces promoting proliferation (Jeeva *et al*., 2013). In Siddha literature, many formulations have been mentioned for the treatment of cervical cancer. Among them, *Muthu Chenduram* (MC) is one of the well-known medicines being used clinically for cervical cancer, which is known as *Yoniputtru*, *Karuppai Kazhunthu putru noi* in terms of Siddha. MC is also used to treat *Kiranthi* (Glandular swelling), *Lingaputtru* (Penile cancer), *Kandamalai* (Goitre), *Moola Noi* (Piles), *Pilavai* (Carbuncle), *Araiyappu* (Veneral disease), and *Peenisam* (Diseases of Nose) (Anbarasu,1998). The test drug has been used clinically for a few decades, suggesting its significance in cancer management. Hence, it is necessary to document its efficacy through scientific validation, which has yet to be done. We undertook to explore the anticancer potential of MC against cervical cancer using the HeLa cell line. The antiproliferative and apoptotic activity of MC were determined by the MTT assay and the Annexin V-FITC assay, respectively.

## Materials and Methods

### *Trial drug*:

MC, with batch number SAC 2101 and manufactured in September 2021, was procured from Earth India Naturals Private Limited, a GMP-certified pharmaceutical.

### *HeLa cell culture*:

The HeLa cell line was grown in Dulbecco's modified Eagle's medium (DMEM), which was purchased from the National Centre for Cell Sciences at Pune, India. The cell line received antibiotic treatments and was maintained at 37ºC in a humified 5% $CO_2$ incubator.

### *MTT assay method*:

A confluent monolayer of trypsinised cells, which were two days old, was suspended in a 10% growth medium. The suspension of 100 µl (5 x $10^3$ cells/well) was inoculated in 96-well tissue culture plates and kept at 37 °C in a humidified 5% $CO_2$ incubator. The sample was dissolved in 0.1% DMSO and filtered for sterility through a 0.22 m Millipore syringe. After 24 h, growth media was removed, and compounds were five times serially diluted in DMEM and added to wells. Each concentration of 100 µl was added in triplicates to the respective well, and incubated at 37 °C in a humidified 5% $CO_2$. After 24 h incubation, the sample content was removed, and 30 µl of regenerated MTT solution was added to the test and untreated wells, gently shaken, and incubated in a humidified 5% $CO_2$ incubator for 4 h at 37°C. The supernatant was then removed. After the incubation, 100 µl of MTT solubilization reagent was added, and an inverted phase contrast microscope was used to measure and evaluate the absorbance at 540 nm. The algorithm below was used to compute the percentage of growth inhibition (Raj *et al*., 2003).

% of viability = Mean OD of treated cells x 100/Mean OD of untreated cells

### *Annexin V- FITC through Flow Cytometry*:

The compound MC (66.7653 µg/ml) was treated with the HeLa cell line and subjected to culture for 24 h. There was also untreated control wells kept up. The cell pellet turned into a visible pellet or a white film after the sample was placed in a polystyrene tube. After adding 100 µl of MuseTM

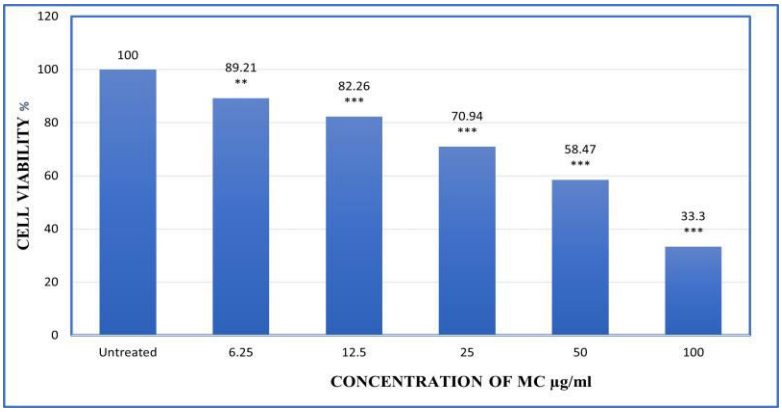


Fig. 1: Dose-dependent cytotoxicity in HeLa cells.

Annexin V and Dead Cell Reagent to each tube and properly mixing them, the tubes were allowed to rest at room temperature in the dark for 20 min. The cells were assessed for apoptosis using Muse FCS 3.0 software, gated against untreated control cells (Ramachandran *et al*., 2017).

## Results

### *Antiproliferative assay*:

The findings demonstrate that MC specifically inhibits proliferation in HeLa cells by raising its concentration. The data is represented as the mean ± standard error of at least three consecutive trials. After performing a one-way ANOVA and Dunnett's test to examine ***p< 0.001 in comparison to the control group, the $LC_{50}$ value (derived from the Effective Dose 50 PLUSV1.0 software) was determined to be 66.7653 µg/ml as shown in Figure 1.

### *Phase-contrast inverted microscopy depicting morphological alterations*:

The HeLa cells underwent morphological alterations after being incubated with MC for 24 h. Condensed nuclei, shrinking cells, blebbing membranes, and apoptotic bodies were all seen in the treated cells as shown in Figure 2.

### *Flow Cytometry assay*:

Comparing the treated cells to the untreated control cells revealed a significant increase in the percentage of apoptotic cells. The percentage of viable cells in the MC-treated cells was 42.68% found to be live, and the remaining cells showed different levels of apoptotic changes in the following percentages: 0.06% were early apoptotic, 37.58% were late apoptotic, and 19.68% were necrotic. Total apoptosis was 37.64%. The present study found that late apoptotic cells showed a substantial rise compared to control cells as shown in Figures 3 and 4.

## Discussion

MC has been clinically used to treat various serious ailments, including cervical cancer and COVID-19. This has emerged as a promising medicine for treating patients with mild symptoms and has been a valuable drug to overcome the pandemic crisis (Neethi *et al*., 2022). MC is a paramount medicine comprising three forms (Metal, mineral, and marine products) of raw drugs from the Siddha System. This includes *Muthu* (Pearl), *Nagam* (Zinc), *Rasam* (Mercury), *Veeram* (*Hydragyrum perchloride*), and *Gandhagam* (Sulphur). Metal- and marine-based compounds have recently seen an increase in demand for cancer treatment (Ndagi *et al*., 2017; Abdullah *et al*., 2009). Previous scientific evidence has shown that some of the compositions of MC have anticancer activity, and they are known to be effective against cancer. Song *et al.* (2022) found that a hydrogel made of poly(γ-glutamic acid) and

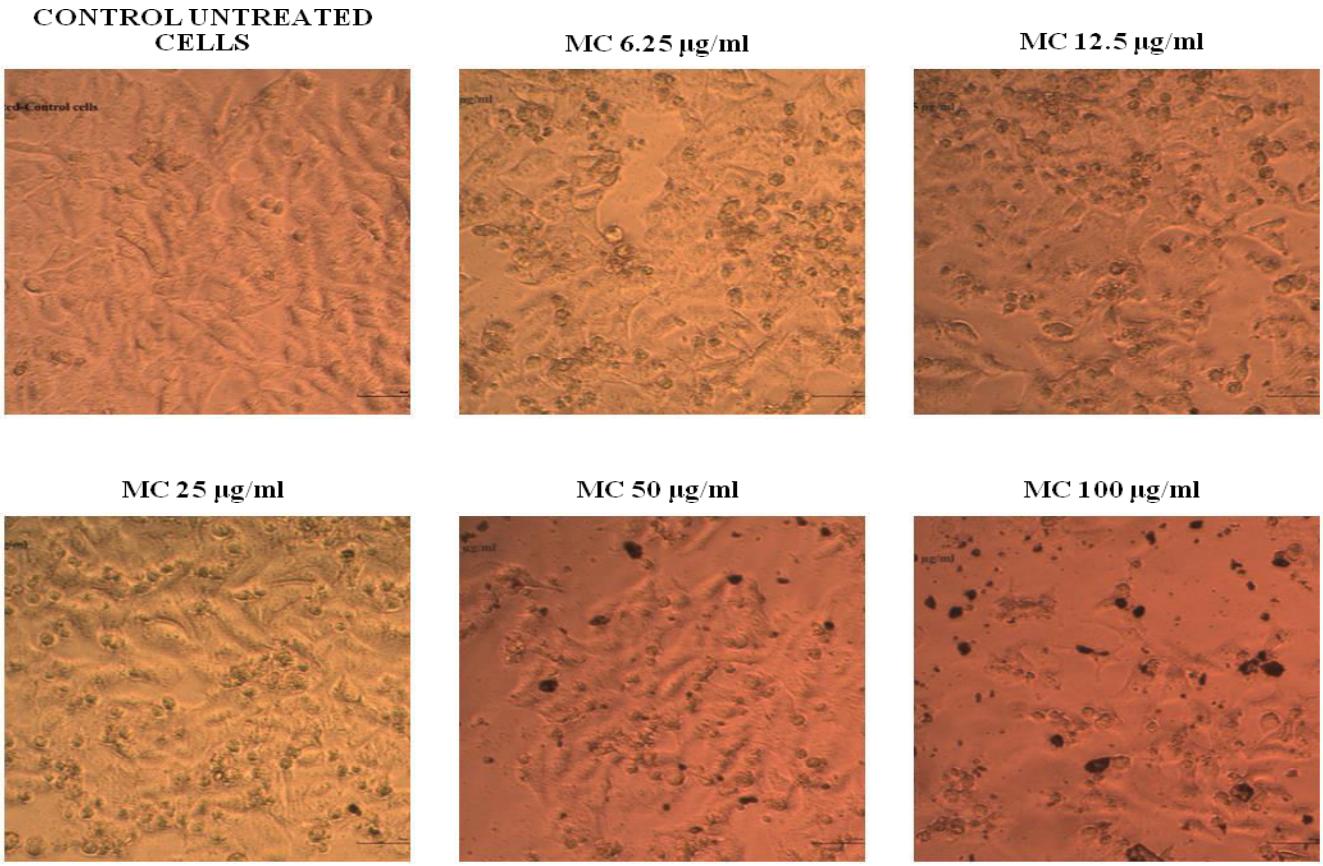


Fig. 2: Phase contrast microscopic images of HeLa cells treated with MC.

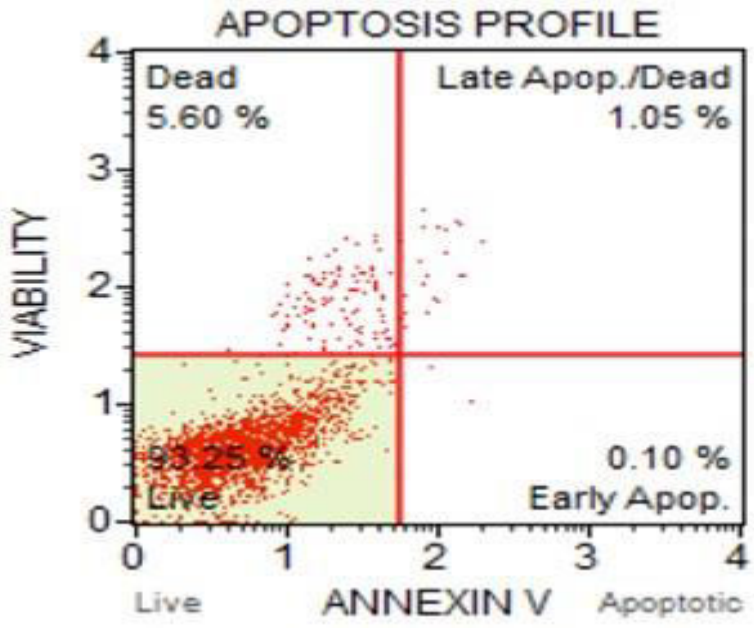


Fig. 3: Untreated cells.

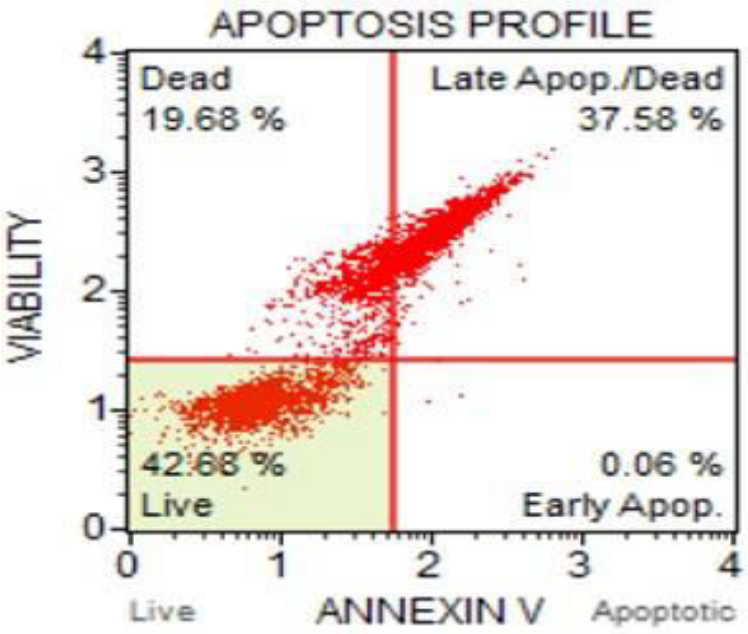


Fig. 4: Cells treated with MC.

pearl extracts effectively treats inflammation-causing human HaCaT cells, demonstrating the anti-inflammatory, anti-scalding, and anti-ulcer properties of pearl. Mousa *et al.* (2023) revealed that zinc oxide nanoparticles may have significant anticancer activity against ovarian cancer, indicating a promising tool for treating ovarian cancer. Riyasdeen *et al.* (2011) suggest that *Rasagenthi mezhugu's* combination of *Rasam and Gandhagam* in chloroform extract can be used to treat prostate, lung, and cervical cancers. Ma *et al.* (2020) discovered that exposure to $HgCl_2$ significantly altered cell morphology, reduced viability, and increased apoptosis in CEK cells. Lee *et al*. (2008) reported that *Gandhagam* inhibited the proliferation of immortalised keratinocytes and oral keratinocytes, suggesting that it has anticancer properties. These research findings prove that the ingredients of MC have anti-cancer potential as an individual drug. It would be better to understand the synergistic effect of these ingredients in our formulation. Thus, we conducted a comprehensive study on MC to explore its potential use as an anticancer agent against cervical cancer. Our study revealed that MC demonstrated potent anticancer activity in the HeLa cell line. Specifically, we employed the MTT and apoptotic assays to assess the anticancer activity of MC. The outcome of this present antiproliferation assay showed that the least viability of cells occurred at a concentration of 100 µg/ml, which shows 33.3 ± 0.368% compared to control cells. It implies a dose-dependent cytotoxic effect on cervical cancer cells.

Annexin V binding assays are highly useful tools for quantifying apoptosis and distinguishing necrotic and apoptotic cells. Apoptosis occurs when the phosphatidylserine inside the membrane is migrated to the outer side of the membrane via annexin V, which binds phosphatidylserine in the presence of $Ca^{2+}$. Propidium iodide, on the other hand, is capable of binding DNA and can enter necrotic and late apoptotic cells. This technique also uses propidium iodide dye and annexin V to detect apoptosis in a cervical cancer cell line (Logue *et al*., 2009). The study found that the percentage of apoptosis significantly increases in treated cells when compared to untreated cells, with 37.58% of the treated cells being late apoptotic, indicating a significant increase in late apoptotic cells. Cervical cancer requires timely and effective treatment, with programmed cell death, playing a crucial role. Apoptosis is a process where cells change, such as

through DNA fragmentation, membrane blebbing, and apoptotic body formation. Apoptotic signaling regulation is crucial for maintaining cell death, survival balance, and genetic integrity. Apoptosis fleeing is a common feature of cancer, it can use different strategies to prevent apoptosis. A disproportion in Bcl-2 proteins' pro- and anti-apoptotic functions, as well as changes to their genetic and epigenetic characteristics, may increase the survival of cancer cells. Understanding the processes behind apoptosis is essential for creating effective medications to target and overcome cancer cell resistance (Xu *et al*., 2018; Nebita *et al*., 2020).

## Conclusion

Based on the *in vitro* study conducted with the HeLa cell line, it has been discovered that MC has significant potential as an anticancer agent. However, further studies are required to confirm its anticancer properties. It is hoped that this research will provide effective approaches for treating cervical cancer using MC.


## Acknowledgements

We express gratitude to the authorities at Department of Nanju Maruthuvam, National Institute of Siddha, Chennai for granting permission to carry out the study.


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