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Exploring the Anti-Cancer Potential of Siddha Formulation *Vaalai Rasa Mezhugu* (VRM) on TM3 Testicular Cancer Cells: An In Vitro Study

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ABSTRACT: Testicular cancer (TC) is common in men aged 14 to 44 and has been increasing for the past two decades. The Siddha practice of medicine contains many herbo-mineral formulations for cancer treatment. One such formulation is *Vaalai Rasa Mezhugu*, it is widely used to treat various cancers including testicular cancer. But scientific proof of the therapeutic efficacy of *Vaalai Rasa Mezhugu* is not available. Hence current study was carried out to evaluate the anti-cancer property of *Vaalai Rasa Mezhugu* using TM3 cell lines, by estimating its antiproliferative activity through MTT assay and Apoptosis by annexin V flow cytometry. The findings revealed a dose-dependent cytotoxic effect (LC₅₀ value-4.2163 µg/ml), significant late apoptotic effect (52.90%). These findings reveal the utility of *Vaalai Rasa Mezhugu* in testicular cancer. Future clinical study will be conducted to evaluate safety and efficacy of *Vaalai Rasa Mezhugu* in human subjects which will in turn highlight its role as a novel anti-cancer drug from Traditional medicine.

1. INTRODUCTION

Cancer is a major health issue, with Testicular cancer (TC) being the most common type among men aged 14 to 44, following prostate and lung cancers. Its incidence has risen in Western countries over the past twenty years. Both genetic and environmental factors contribute to the development of testicular cancer, with cryptorchidism identified as the most significant risk factor (Cheng et al., 2018). Testicular cancer is usually diagnosed in men aged 20 to 34 and is believed to originate from an early lesion called carcinoma in situ of the testis, also known as “Intratubular Germ Cell Neoplasia (IGCN) or Testicular Intraepithelial Neoplasia (TIN)” (Ziglioli et al., 2011).

In 2020, the “International Agency for Research on Cancer (IARC)” reported 74,458 new cases of testicular cancer all over the world with varying incidence rates from 3 to 12 new cases per 100,000 males per year in Western countries. In Asia and the Middle East, a total of 20,651 cases of testicular cancer were documented. The five countries with the highest numbers of

cases were India, China, Japan, Turkey, and Indonesia, reporting 4,638 (22.7%), 4,502 (21.8%), 2,458 (11.9%), 1,605 (7.8%), and 1,497 (7.2%) cases, respectively (Giona, 2022). Radical inguinal orchiectomy is commonly used as a primary treatment for testicular tumors which involves excision of the involved testis along with spermatic cord. After surgery, treatment may involve observation only; retroperitoneal lymph node dissection; radiation therapy, and chemotherapy (Shaw, 2008). Chemotherapy, radiation, and surgery are effective standard treatments but they lead to debilitating side effects impacting quality of life for many patients. Siddha medicine is a traditional system of medicine widely practiced in India especially its southern region, which focused on health promotion and management of various chronic diseases.

Traditional medicine systems have an important role in fulfilling global healthcare requirements. India has richly diversified traditional healthcare practices namely, Siddha, Ayurveda and others, collectively referred to as Ayush. Siddha system is one of the oldest Indian indigenous traditions having

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number of formulations with evidence-based anticancer activity for prevention and treatment of various cancers. *Vaalai Rasa Mezhu* (VRM) is a Siddha herbo-mineral medicine indicated for conditions such as throbbing pain (*Soolai*), arthritis (*Mudakku vaatham*), cervical lymphadenitis (*Kandamaalai*), fistula (*Pauttiram*), and lymph node-related neoplasia (*Araiyappu*). While Siddha physicians have clinically utilized VRM in managing testicular cancer, there is no scientific evidence for its effectiveness (Balarammaiy, 1980; Sambasivampillai, 1991). *Rasam* (Hydrargyrum), *Veeram* (Hydrargyrum perchloride), and *Pooram* (Hydrargyrum subchloride) are the ingredients of major importance in VRM. Numerous studies demonstrated anticancer potentials by these metals. Hence, the present study attempts at evaluating the growth inhibitory effect of *Vaalai Rasa Mezhu* (VRM) on the human testicular cancer cell line TM3.

2. MATERIALS AND METHODS

2.1. Selection of the test drug

The test drug “*Vaalai Rasa Mezhu*” is one of the Herbo mineral formulations for arthritis and tumor which is indicated in the Siddha literature “Siddha marunthu sei perumuraigal” written by V. Balarammaiy (V. Balarammaiy et al 1980).

2.2. Collection of raw drugs

The raw drug was purchased in a Country shop, in Chennai and authenticated by the “Department of Gunapadam, National Institute of Siddha, Chennai” (certified No. NISMB6652024, GUN/AUT/07/24).

2.3. Ingredients of *Vaalai Rasa Mezhu*

The ingredients of the VRM are listed in Table 1.

Table 1
Ingredients of *Vaalai Rasa Mezhu*.

S. No	The vernacular name of the ingredients	Botanical name/Chemical name	Quantity
1.	Purified Veeram	Hydrargyrum perchloride	10gm
2.	Purified Vaalai rasam	Hydrargyrum	10gm
3.	Purified Pooram	Hydrargyrum subchloride	10gm
4.	Elam	<i>Elettaria cardamomum</i>	10gm
5.	Kirambu	<i>Syzygium Aromaticum</i>	10gm
6.	Varagarisi	<i>Paspalum scrobiculatum</i>	35gm

2.4. Preparation of *Vaalai Rasa Mezhu*

Initially, the Rasam was mixed with betel leaf juice and blended until a smooth consistency was achieved. Next, the Veeram and Pooram were added to the mixture. Then, the clove (*Kirambu*) and cardamom (*Elam*) were shallow fried and ground into a fine powder. These powders were combined with *Varagarisi* flour and blended thoroughly. Finally, a small amount of castor oil was mixed in, and the mixture was ground

until it achieved a semisolid, wax-like texture. The final product (VRM) was stored in an airtight container (Balarammaiy, 1980).

2.5. In-vitro evaluation of Anticancer Activity

2.5.1 Cell Line

The TM3 cell line (TC) was originally obtained from the National Centre for Cell Sciences (NCCS), Pune, India, and subsequently cultured in Dulbecco’s modified Eagle’s medium (DMEM).

2.5.2 MTT Assay

Cells were cultured in 25 cm² flasks containing DMEM supplemented with “10% FBS, L-glutamine, sodium bicarbonate, and an antibiotic solution (Merck, Germany)”. The cells were maintained in a humidified 5% CO₂ incubator at 37°C (NBS Eppendorf, Germany). Cell viability was assessed using the MTT assay after direct observation under an inverted phase contrast microscope. Confluent cells were trypsinized, suspended in 10% growth media, and seeded into 96-well plates (5x10³ cells/well). VRM stock solution (1 mg/mL in 0.1% DMSO) was prepared, filtered (0.22 μm Millipore syringe filter), and serially diluted (100, 50, 25, 12.5, 6.25 μg/mL in DMEM). Triplicates of each concentration (100 μL) were added to wells, and incubated at 37°C in a humidified 5% CO₂ incubator. Untreated control cells were maintained in parallel. After 24 hours, cells were examined using an inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera), and microscopic observations were photographed. Cytotoxicity markers included cell shape changes, granulation, and vacuolization. Divya and Pushpa (2021); Jerard et al. (2020); Mosmann (1983); Sooraj et al. (2024).

2.5.3 Apoptosis by Annexin V Flow Cytometry

The TM3 cell line was cultured using established methods. Upon reaching confluency, cells were exposed to the LC₅₀ concentration (4.2163 μg/mL) of the sample and incubated for 24 hours. Untreated control wells were maintained in parallel. For apoptosis analysis, cells were transferred to 12 × 75 mm polystyrene tubes (minimum 1 × 10⁶ cells per tube). Then centrifugation (5 minutes, 3000 RPM) was done and the resultant supernatant was carefully removed without disturbing the cell pellet. Next, 100 μL of Muse TM Annexin V & Dead Cell Reagent was added to each tube, mixed gently (3-5 seconds), and incubated in the dark at room temperature for 20 minutes. Flow cytometry analysis was performed using Muse software. Cells were gated against untreated controls and evaluated for apoptosis using Muse FCS 3.0 software (Ciniglia et al., 2010).

3. RESULTS

3.1. In-vitro Cytotoxic Effect Determination by MTT Assay

Cytotoxic effects of VRM on cancer cells at varying concentrations demonstrating dose-dependent cell viability reduction in Figure. 1

3.2. Cytotoxicity Assay by MTT Method

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

Table 2

Percentage viability of *Vaalai Rasa Mezbugu* at various concentrations.

Sample Concentration ($\mu\text{g/mL}$)	Percentage viability
Control	100
6.25	25.88
12.5	21.82
25	18.10
50	15.25
100	10.97

3.3. Apoptosis by Annexin – V Flow Cytometry

VRM treated induces apoptosis in cancer cells, with notable differences between untreated and treated groups in Figure 3.

The early apoptotic percentage was determined to be 0.10% and the late apoptotic change was found as 4.15%. The total Apoptotic percentage was found to be in the minimal amount of 4.25%.

In the sample treated cells, the early apoptotic percentage was found to be 3.05% and the late apoptotic was found to be 49.85%, 2.05 % constituted the debris resulting in a total apoptosis rate of 52.90%.

4. DISCUSSION

Standard care for testicular cancer which includes surgical intervention, chemotherapy, and radiotherapy has challenges that cannot be overlooked. Invasive methods may cause infertility or ejaculatory problems (Cheng et al., 2018). Those who get chemotherapy often suffer from nausea, vomiting, and even develop long-term effects (Huddart et al., 2018). Damage to blood vessels an increase in the occurrence of secondary tumors and the heart disease risk are all side effects brought about by radiotherapy (Travis et al., 2010). In addition, each of these interventions might have a deep psychological and emotional impact on the patients aggravating their wellbeing (Smith et al., 2017). Hence it is essential to find new treatments with lesser side effects.

The Siddha system of medicine comprises three fundamental concepts: vali (movement), azhal (conversion), and iyam (form). In essence, azhal is important in cellular digestion and metabolism. It is the overbalance of azhal over iyam (fire over water), which primarily is the feature of tumors, that

leads to uncontrolled tissue proliferation. Azhal being low thus creates a state which leads to an excess of the forces of vali and iyam. This may lead to problems like migration owing to the discontinuation of *Viyana vaayu*, *Samana vaayu*, and *Suzhumunai naadi*. Every individual possesses Thegi which is the very unique body constitution of an individual and is a trait dominated by the body time dominated by the particular doshas (*Vatham*, *Pitham*, *Kapham*, and any combinations of these). This constitution also determines how individuals react to the environment, the diseases, and the medications given. To maintain health, an individual must avoid extreme levels of doshas, as is the case with cancer, the most affected doshas being *Kapham* and *Vatham* tend to aggravate tumor proliferation and dispersal (Subathra et al., 2022).

The MTT assay results indicated that the % of cell viability is inversely proportional to the concentration. At 6.25 $\mu\text{g/mL}$, it is 25.88, while at 100 $\mu\text{g/mL}$, it is 10.97. This indicates that cytotoxicity has increased in a dose-dependent approach. It results was 4.2163 $\mu\text{g/mL}$. This value represents the lethal concentration where 90 % of cells get inhibited upon treatment with the test drug. Annexin V binding assays are used in the detection of apoptosis and prompt discrimination from necrosis, allowing differentiation between late-stage apoptotic and necrotic cells. When annexin V is combined with propidium iodide, it produces an efficacious method to detect and measure apoptosis of testicular cancer cells. The latter was quantified and the fold increase in cell death compared to control represents the apoptotic response of these cells towards treatment with half-maximal lethal concentrations, LC_{50} , (i.e. 90% of cells die at this concentration). Apoptosis was carefully detailed utilising annexin V flow cytometry to provide key information about the cellular response to treatment and, hence, the efficacy of a given therapy. The results indicated that late apoptotic changes in the control group were 4.15%, while the drug-treated group showed a significant increase to 49.85%. Overall, the total apoptotic percentage rose from 4.25% in the control group to 52.90% in the drug-treated group, demonstrating a marked increase in cell death due to the drug's apoptotic activity.

Some of the ingredients in this formulation such as *Rasam* (Hydrargyrum), *Veeram* (Hydrargyrum perchloride), and *Pooram* (Hydrargyrum subchloride) were shown to be cytotoxic and anti-proliferative effects. The anticancer potential of a Siddha preparation of *Rathinagarasa Mezbugu* (RNM), containing *Rasam* was analysed using the MCF-7 cancer cell line and the study showed dose-dependent growth inhibition with nuclear damage in cancer cells (Parvathy et al., 2023). Rajalakshmi et al. (2014) in their research on *Veera Mezbugu*, showed cytotoxicity at 1000 $\mu\text{g/ml}$ against EAC cell lines was 74.05%. These extracts were studied by researchers as *Rasa Karpoora kuligai* (RSK) with *Pooram* as the main ingredient to exhibit cytotoxic and apoptotic properties in HeLa cells, thereby reducing cell growth, dose-dependently modulating apoptotic pathways (Manjari et al., 2016). Semalatha S et al. Anti-cancer and anti-tumor effect of *Bhramasthiram* with *Rasam* against

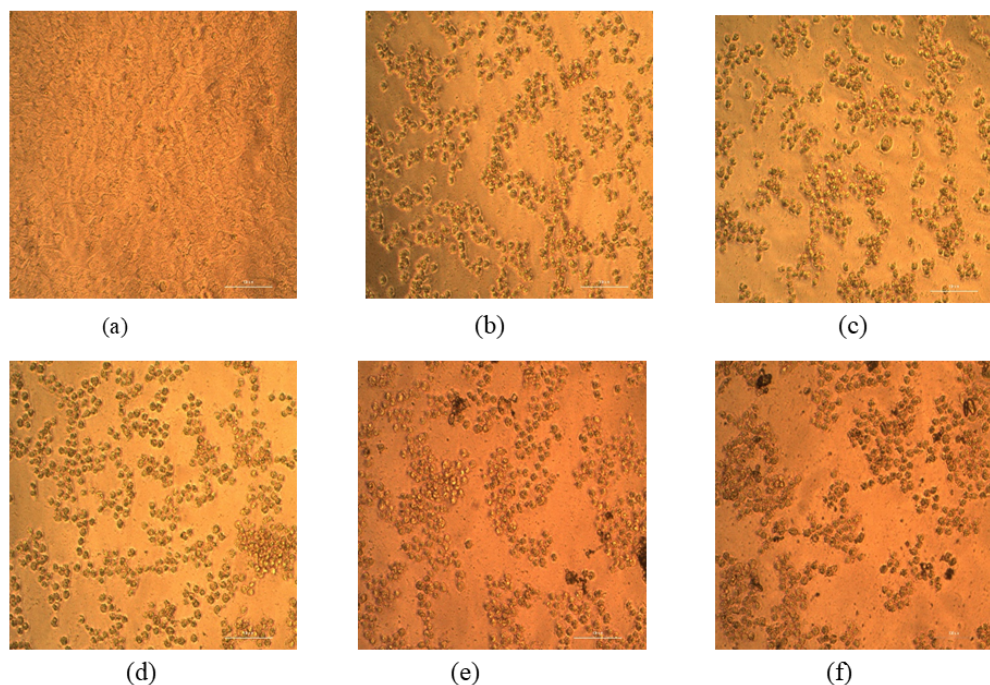


Figure 1. Cancer cells treated with different concentrations of VRM a) Control b) VRM - 6.25 µg/mL, c) VRM - 12.5 µg/mL, d) VRM - 25 µg/mL, e) VRM - 50 µg/mL, f) VRM - 100 µg/mL.

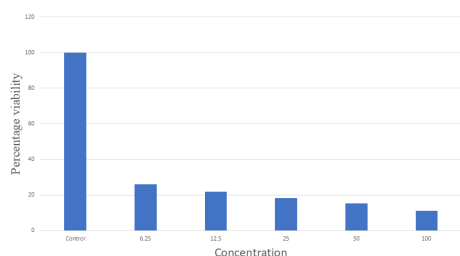


Figure 2. The cytotoxicity of VRM against TM-3 cells was evaluated using the MTT assay, with a graphical representation of results. Cell viability percentages are plotted on the Y-axis, while the X-axis represents varying VRM concentrations. Triplicate experiments yielded mean \pm SE values. Statistical analysis employing one-way ANOVA and Dunnett's test revealed significant differences ($*p < 0.001$) compared to control groups. The LC₅₀ value, signifying 50% cell mortality, was computed using ED50 PLUS V1.0 software, yielding a value of 4.2163 µg/mL.

Human KB oral cell line and HeLa cells were also reported by using MTT assay (Semalatha, 2022). In this study, VRM along with MTT assay results, and Annexin V flow cytometry confirmed that VRM is responsible for its anticancer activity.

5. CONCLUSION

Vaalai Rasa Mezhu showed a concentration-dependent decrease in cell viability, indicating substantial cytotoxic activity. The LC₅₀ value confirmed the ability to initiate apoptosis, and evidence from flow cytometry showed a high percentage of late apoptosis in the treated group. However, further *in vivo* and clinical investigations are needed to prove its therapeutic effect on testicular cancer.

CONFLICTS OF INTEREST

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

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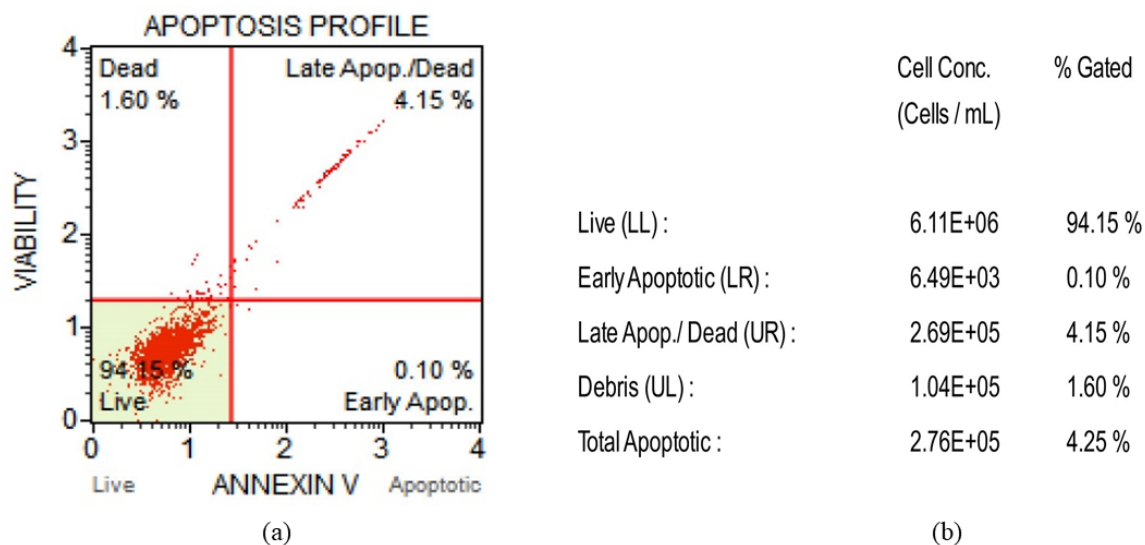


Figure 3. 3(a) Apoptosis profile: untreated control cells, (b) Cell distribution of cells in control cells.

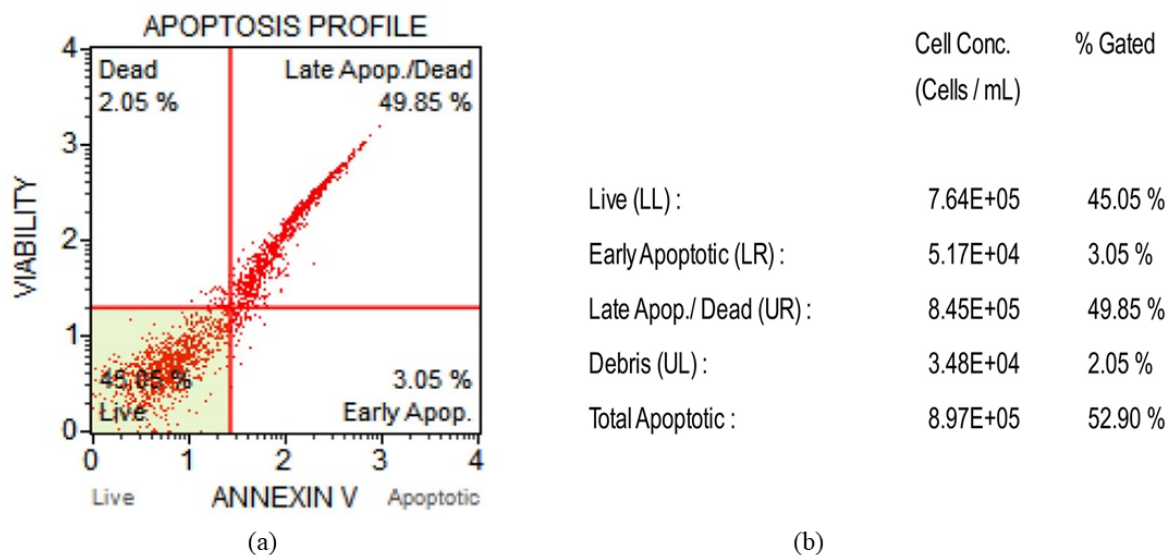


Figure 4. 4(a) Apoptosis profile: untreated control cells, (b) Cell distribution of cells in control cells.

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AUTHOR CONTRIBUTIONS

Conceptualization: SR; Medicine Preparation: SR; Data collection and compilation: SR; Manuscript Writing: SR, SA, KAP and SSG; Proofreading and editing: SR, SA, KAP SSG and AB.

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