IJBPAS, August, 2023, 12(8): 3758-3766

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

IN-VITRO ANTI-CANCEROUS ACTIVITY OF *VITHU RASA MEZHUGU* – A SIDDHA HERBO-MINERAL FORMULATION

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Received 16th Sept. 2022; Revised 25th Oct. 2022; Accepted 15th Nov. 2022; Available online 1st Aug. 2023

https://doi.org/10.31032/IJBPAS/2023/12.8.7360

ABSTRACT

In India, Non-Communicable Diseases were estimated to account for 63% of all deaths, and cancer (CA) was one of the leading causes (9%). Cervical cancer is the fourth most frequent cancer in women with an estimated 604,000 new cases in 2020. Of the estimated 342,000 deaths from cervical cancer in 2020, about 90% of these deaths occur in low- and middle-income countries. In this In-vitro study, the anti-cancer effect on the HeLa cell line was evaluated. Cancer cells were exposed to various concentrations of the Siddha formulation *Vithu Rasa Mezhugu* (VRM) (6.25 – 100 µg/ml) for 24 hours. The results have shown that *Vithu Rasa Mezhugu* (VRM) induced cytotoxicity in cancer cells in a dose-dependent manner, as measured by MTT assay. It was found that the number of cells decreases as the dose increases and at approximately 109.985517µg/mL dose, 50% of the cells (HeLa cells) were less as compared to the normal control. LC50 was found to be 109.9 µg/ml which restricted the proliferation of the HeLa cell lines after 24 hours. Apoptosis effect of VRM by AO and EtBr Double Staining results revealed that VRM has promising cell apoptotic and necrotic activity, which may help in the destruction of cancer cells in the treatment of cervical cancer.

Keywords: *Vithu Rasa Mezhugu*, A Siddha Herbo-mineral formulation, In-vitro anti-cancer activity, HeLa cell line

INTRODUCTION:

Globally, Non-Communicable Diseases (NCDs) accounted for 71% of total deaths. In India, NCDs were estimated to account for 63% of all deaths, and cancer (CA) was one of the leading causes (9%) **[1]**. Nowadays incidence of these NCDs is gradually increasing due to the sedentary lifestyle, environmental factors, etc.,

One of the studies reported that the projected number of patients with cancer in India is 1,392,179 for the year 2020, and the common 5 leading sites are breast, lung, mouth, cervix uteri, and tongue. The majority of the cases with cancer were diagnosed at the locally advanced stage for breast (57.0%), cervix uteri (60.0%), head and neck (66.6%), and stomach (50.8%) cancer, whereas in lung cancer, distant metastasis was predominant among males (44.0%) and females (47.6%) **[2]**. Worldwide, cervical cancer is the fourth most frequent cancer in women with an estimated 604,000 new cases in 2020. Of the estimated 342,000 deaths from cervical cancer in 2020, about 90% of these deaths occur in low- and middle-income countries **[3]**.

Treatment for cervical cancer depends on disease extent at diagnosis and locally available resources and might involve radical hysterectomy or chemoradiation, or a combination of both **[4]**. Prolonged therapy of Cancer drugs can cause many complications like hair loss, loss of appetite or taste changes, fatigue, anemia, and sleep disturbances.

To overcome these circumstances and also reduces the complications of synthetic drugs and surgical treatments, there is a need to establish an effective drug from the Siddha system of Medicine. In Siddha, higher-order medicines like *Parpam*, *Chenduram*, and *Mezhugu* are used in the treatment and management of CA which is mainly prepared from the ingredients of *Semecarpus anacardium* and *Mercury*. *Vithu Rasa Mezhugu* (VRM) **[5]** is one of the higher-order medicines which is prepared from *Rasam* (Mercury) and *Serankottai* (*Semecarpus anacardium*). VRM is indicated for *Linga putru* (penile cancer), *Yoni putru* (cervical cancer), *Vaayu* (arthritis), *Soolai* Noi (painful condition), *Vatha noi* (musculo skeletal diseases), *Ranangal* (ulcers), *Sorigal* (scabies), *Veekam* (edema), *Kattigal* (tumor), *Thoal vatham* (periarthritic shoulder), *Kuthikal vatham* (calcaneal spur), *Asthi kudaichal* (pain in bony joints), *Karalai kattina soolai* (pain due to restriction of joints), and *Kiranthi vagaigal* (syphilitic ulcers). In this study, an attempt has been made to validate the natural-based Siddha herbo-mineral formulation VRM for its effect on the cervical cancer cell line.

MATERIALS AND METHODS:

Preparation of VRM:

Rasam (Mercury) and *Serankottai* (*Semecarpus anacardium*) were bought from an authorized country raw drug shop at Parrys corner in Chennai, Tamil Nadu. The raw drugs were identified and authorized by botanical and pharmacological experts from the National Institute of Siddha. Purified *Serankottai* (*Semecarpus anacardium*) and purified *Rasam* (mercury) were ground in a stone mortar (*kalvam*) for 6 hours until it attains waxy consistency. Then it was carefully collected and stored in an airtight container for study purposes.

Invitro Anti-cancer Effect Determination by MTT Assay:

The Experimental Procedure was done for *Vithu Rasa Mezhugu* at Biogenix Research center, Thiruvananthapuram, Kerala.

HeLa (Human cervical cancer) cell line was initially procured from National Centre for Cell Sciences (NCCS), Pune, India, and maintained Dulbecco's modified Eagles medium, DMEM (Sigma Aldrich, USA). The cell line was cultured in a 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany), and the antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

The viability of cells was evaluated by direct observation of cells by an Inverted phase contrast microscope and followed by the MTT assay method.

Cells seeding in 96 well plate:

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^3$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of compound stock:

1mg of sample was weighed and dissolved in 1mL 0.1% DMSO using a cyclomixer. The sample solution was filtered through 0.22 µm Millipore syringe filter to ensure sterility.

Anticancer Evaluation:

After 24 hours the growth medium was removed, freshly prepared each compound in 5% DMEM was five times serially diluted by two-fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of 5% DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Non-treated control cells were also maintained.

Anticancer Assay by Direct Microscopic observation:

The entire plate was observed after 24 hours of treatment in an inverted phase-contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observations were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation, and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Anticancer Assay by MTT Method:

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization.

After 24 hours of the incubation period, the sample content in wells ware removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (Dimethyl sulphoxide, DMSO, Sigma Aldrich, USA) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using a microplate reader at a wavelength of 540 nm **[4]**.

The percentage of growth inhibition was calculated using the formula:

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

b.2. Determination of Apoptosis by Acridine Orange (AO) and Ethidium Bromide (EtBr) Double Staining [6]

HeLa cell line was initially procured from National Centre for Cell Sciences (NCCS), Pune, India, and maintained Dulbecco's modified Eagles medium, DMEM (Sigma aldrich, USA).

The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate, and the antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

Principle

DNA-binding dyes Acridine Orange (AO) and Ethidium Bromide (EtBr) (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells. AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into a double-stranded nucleic acid (DNA). EtBr is taken

up only by non-viable cells and emits red fluorescence by intercalation into DNA.

Procedure

After treatment with LD 50 concentration of sample for 24 hours, the cells were washed with cold PBS and then stained with a mixture of AO (100 µg/ml) and EtBr (100 µg/ml) at room temperature for 10min. The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope in a blue filter of a fluorescent microscope (Olympus CKX41 with Optika Pro5 camera).

The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation), and necrotic cells (uniformly orange-stained cell nuclei).

Statistics:

All experiments were done in triplicates and results were represented as Mean+/- SE. One-way ANOVA and Dunnett's test were performed to analyze data. ***p< 0.001 compared to control group, **p< 0.01 compared to control group, ns – non significant compared to control group.

RESULTS AND DISCUSSION:

Invitro Anticancer Effect of *Vithu Rasa Mezhugu* on HeLa cell line:

Table 1: Anticancer effect of VRM on HeLa cell line

Sample Concentration (µg/mL)	OD value I	OD value II	OD value III	Average OD	Percentage Viability
Control	0.6385	0.6476	0.6529	0.6463	100.00
SAMPLE – VRM					
6.25	0.6235	0.6294	0.6327	0.6285	97.25
12.5	0.5814	0.5725	0.5763	0.5767	89.24
25	0.5635	0.5514	0.5625	0.5591	86.51
50	0.471	0.4425	0.4586	0.4574	70.77
100	0.373	0.3678	0.3506	0.3638	56.29

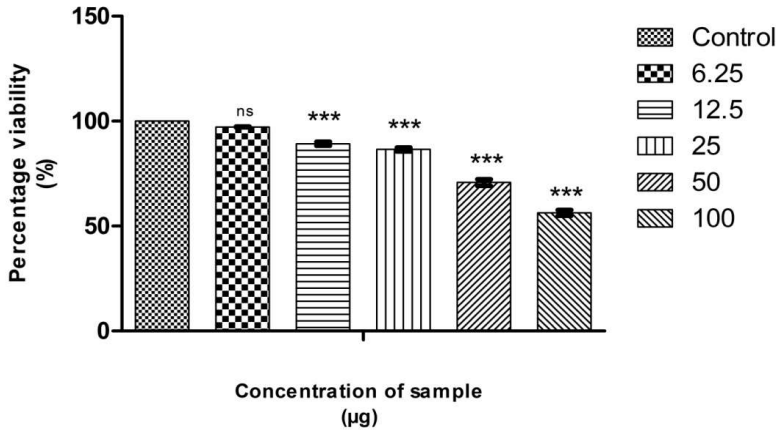

Figure 1: Graphical representation depicting the Anti-cancer effect of VRM by MTT assay- Along Y-axis Percentage viability, Along X-axis different concentrations of VRM.

Phase contrast images of HeLa cells in the treatment of VRM shown in following pictures:

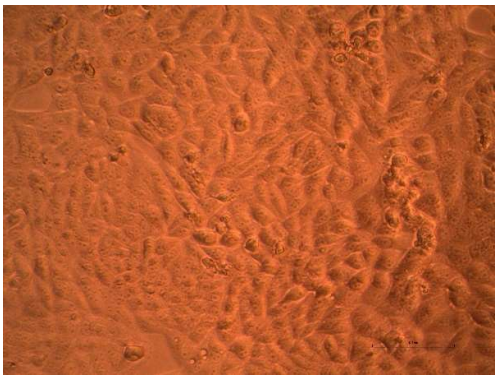

Figure 1.1. Control

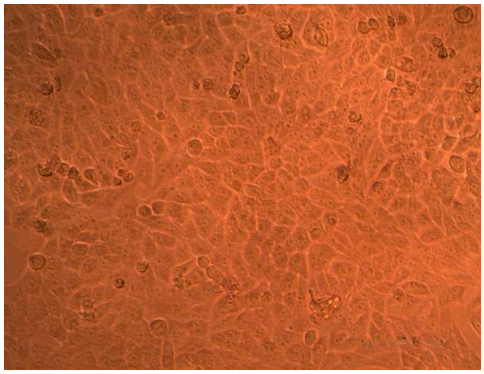

Figure 1.2. VRM 6.25 µg/mL

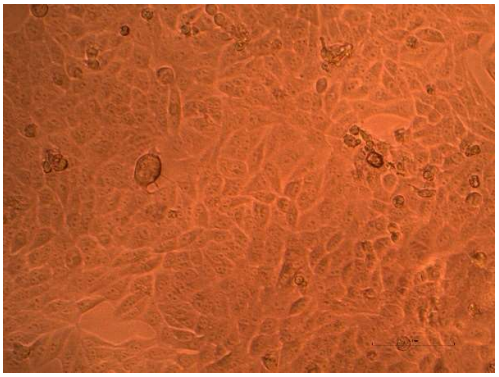

Figure 1. 3. VRM 12.5 µg/mL

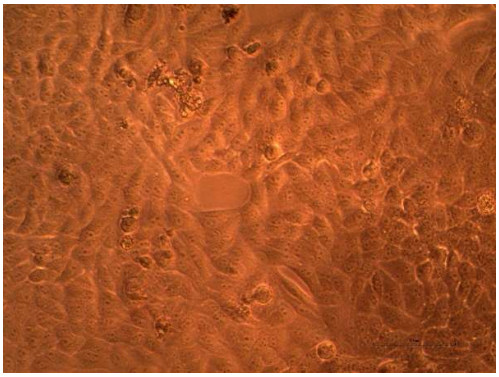

Figure 1.4. VRM 25 µg/mL

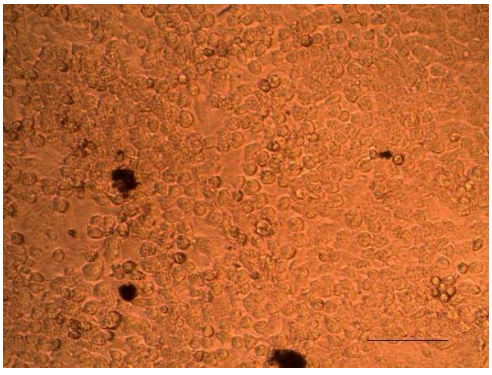

Figure 1.5. VRM 50 µg/mL

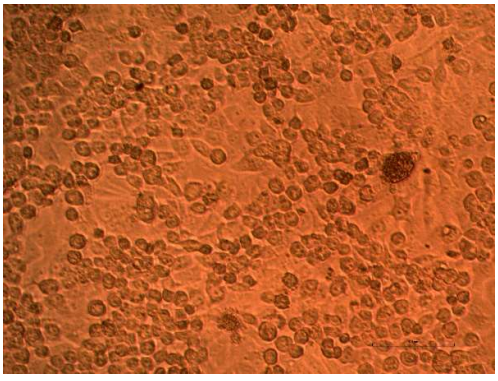

Figure 1.6. VRM 100 µg/mL

b.2. Determination of Apoptosis by Acridine Orange (AO) and Ethidium Bromide (EtBr) Double Staining:

Control and VRM-treated (24 h) HeLA cells stained with acridine orange (AO) and ethedium bromide (EtBr) shown in **Figure 2.1 and 2.2**.

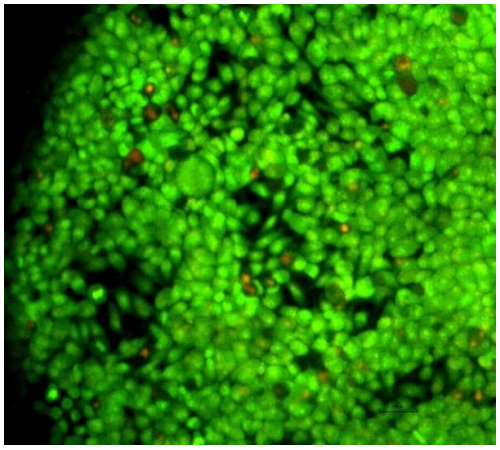

Figure 2.1. Control

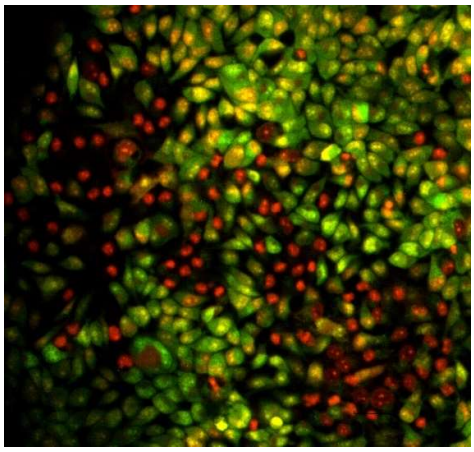

Figure 2.2. VRM

Anticancer effect of VRM on HeLa cell line:

The experiment was screened at different concentrations to determine the LC50 using MTT assay. A chart was plotted using the % cell viability on Y-axis and the concentration of the test sample on the X-axis (Fig.1) VRM at different doses (6.25 – 100 µg/ml) was administered for 24 hrs. It was found that the number of cells decreases as the dose increases and at approximately 109.985517µg/mL dose, 50% of the cells (HeLa cells) were less as compared to normal control as shown in **Figure 1.1 to 1.6**.

The percentage of cell viability was determined by calculating the OD values of the treated against the control. The absorbance values were measured by using a microplate reader at a wavelength of 540 nm. Comparison values are made on a basis of 50% inhibition of growth in treated cells with specific agents. The percentage of growth inhibition was found to be increasing with increasing concentrations of the test drug. This confirms that the trial medicine VRM has a potent anti-cancerous effect and can be used in the treatment of cervical cancer. Likewise, one of the study results exhibited that good cytotoxic activity in a hydroalcoholic extract of *Semecarpus anacardium* in both HeLa and SiHa cell lines due to the presence of toxic flavones **[7]**. The high flavonoid content in *Semecarpus anacardium* may be responsible for its antimutagenic and antiproliferative activity, regulation of cell signalling and cell cycle, and inhibition of angiogenesis, thus it is having efficacy in the prevention and treatment of cancers **[8]**.

Apoptosis effect of VRM by AO and EtBr Double Staining:

The results obtained with AO & EB double staining of control and VRM treated cells are shown in fig.no:2.1, 2.2. The control cells fluoresced uniformly green and had normal features. Most of the cells treated with VRM showed uniformly

orange-stained cell nuclei and cell lysis which indicates necrosis. Some of the cells showed late apoptotic (orange-stained nuclei with 92 chromatin condensation or fragmentation) and indicated apoptotic features such as cell shrinkage, chromatin condensation, nuclear fragmentation, and apoptotic body formation. A few cells indicated early apoptotic features observed as a bright green nucleus with condensed or fragmented chromatin. The results of AO/EB staining also exhibited a high number of apoptotic cells on treatment with VRM compared with control cells. Thus, it is concluded that VRM has promising cell apoptotic and necrotic activity, which may help in the destruction of cancer cells in the treatment of cervical cancer.

CONCLUSION:

Thus, it can be concluded that the Siddha formulation ***Vithu Rasa Mezhugu*** **(**VRM) promisingly has cytotoxic activity against human cervical cancer cells and can be used in the treatment and management of cervical cancer.

Acknowledgments:

The authors acknowledge the support provided by the department of Gunapadam, National Institute of Siddha to carry out this preliminary research work.

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