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In vitro Antiproliferative and Cytotoxic potential of the Siddha formulation - Neeradi Muthu Vallathi Mezhugu - Against HeLa Cervical cancer cell line

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Abstract

Background: Cervical cancer is the fourth most prevalent illness in women all over the world, killing around a quarter million people each year. Conventional therapy risks the emergence of drug resistance, recurrence, and metastases. Hence, high-efficacy, low-side-effect medicines are needed. Siddha, an Indian traditional medicine, can help find new cervical cancer treatments. **Objective:** The primary purpose of the present research is to screen the anti-proliferative and anti-apoptotic potential of the Siddha formulation *Neeradi Muthu Vallathi Mezhugu* (NMVM) using MTT and double staining techniques against the human cervix adenocarcinoma cell line (HeLa). **Methods:** The percentage viability of HeLa cells was determined by the MTT, acridine orange, and ethidium bromide staining assay model after incubation with test drug NMVM at concentrations of 6.25, 12.5, 25, 50 and 100 µg/ml. **Results:** Results of the MTT assay signifies that the lowest viability was observed at 100µg/ml (31.23 ± 0.63), followed by 50 µg/ml and 25µg/ml, with percentage viability of 52.64 ± 0.51 and 69.99 ± 1.74 found in MTT assay. The corresponding IC_{50} value of the test drug NMVM was 151.34 µg/ml. Results of the apoptotic assay showcase the representation of normal morphology in the control group with a major emittance of green fluorescence which indicates the existence of viable cells. **Conclusion:** Based on the current study's findings, it was determined that the siddha formulation NMVM has potential anticancer capabilities against the HeLa cells; nevertheless, more research is required before the medicine can be recommended for therapeutic use.

Keywords: Anticancer, Cervical cancer, Cytotoxic, HeLa cell line, MTT assay, Neeradi Muthu Vallathi Mezhugu, Siddha Formulation.

1. INTRODUCTION

The worldwide burden of dealing with cancer has resulted in a greater emphasis on research into cancer therapies such as chemotherapy and surgery. The choice of treatment method, whether surgery, radiation, or chemotherapy, is based on the severity of the disease at diagnosis. Several of these approaches aim to interfere with the signalling pathways that promote cell growth and survival [1]. Common cytotoxic

chemotherapeutic drugs like doxorubicin and cisplatin, for example, can cause programmed cell death (apoptosis) in cancer cells [2]. Among women all around the world, cervical cancer ranks as the fourth largest cause of mortality from the disease [3,4]. It is projected that there were 570,000 new instances of cervical carcinoma in women throughout the world in 2018 and that the disease claimed the lives of 311,000 women (especially middle-aged women) [5,6].

Common approaches for slowing the progression of cancer include aggressive therapies including radiation, chemotherapy, and surgical resection that are employed based on the severity of the disease [7]. Chemotherapy is the most belligerent treatment among them which is untolerated by most patients owed of its side effects [8]. Hence, it's important to work together to find better ways to treat cervical cancer.

Uncontrolled cell proliferation, invasion, migration, and metastasis are all hallmarks of cancer's multistage, highly complicated progression [9]. The use of apoptosis-inducing medicines to stop tumour growth, invasion, and metastasis in human cancer is common practice. Herbal remedies have been used centuries to cure various illnesses, including cancer [10]. Traditional medicines increasingly incorporate the use of medicinal plants [11]. Their many potential uses have also piqued the current medical community's attention. They provide an unclear wellspring of biologically active molecules that may be used to postpone the onset of, or perhaps treat, diseases like cancer. Researchers are encouraged to explore the anticancer qualities of medicinal plants by the increased prevalence of tumours and the trend towards traditional system [12,13].

There has been a considerable reduction in healthcare issues due to the widespread adoption of the siddha medical system, which has been used for centuries [14]. The primary principle of Siddha treatment is identifying and treating the vatha, pittha, and kaba imbalances at the root of any health problem. Any imbalance in the tridosha, as taught by traditional medicine, can cause metabolic changes that ultimately appear as a disease [15]. The primary purpose of the present research is to screen the anti-proliferative and anti-apoptotic potential of the siddha formulation *Neeradi Muthu Vallathi Mezhugu* (NMVM) using MTT and double staining techniques.

2. MATERIALS AND METHODS

2.1. Cell line and culture

The human cervical adenocarcinoma (HeLa) cell line was procured from the National Institute of Cell Research in Pune, India. The cells were kept alive in a nutrient-rich medium called Dulbecco's modified eagle media supplemented with 10% foetal bovine serum and 1% penicillin-streptomycin. The optimal conditions for development occurred at 37 degrees Celsius in a humidified incubator with 5% carbon dioxide.

2.2. *In vitro* cell viability by MTT assay

After trypsinizing the monolayer of cells was brought down to 1.0 x 10^5 cells/ml in a medium supplemented with 10% foetal bovine serum. The diluted suspension of cell (50,000 cells per well) was put on a 96-well microtiter plate. When a partial monolayer had developed after 24 hours, then the supernatant was taken off by flicking, the monolayer was washed with media, and 100 µl of test medicines of varying concentrations were added to the

microtiter plates. After that, we incubated the plates at 37 degrees Celsius for 48 hours in a 5% carbon dioxide environment. Following incubation, the test solutions were removed from each well and added 100 µl of MTT (5 mg/10 ml of MTT in PBS). Plates were reserved in a 37°C, 5% CO2 for 4 hours in incubator. The 100 µl of DMSO was added after removal of supernatant, then the plates were slightly shaken to scatter the generated formazan. In order to determine the absorbance, a 570 nm wavelength microplate reader was used. The concentration of test drug required to prevent cell growth are derived by using dose-response curves for each cell line by 50% (IC_{50}) values [16,17].

Cell viability (%) = (Abs of treated cells/Abs of control cells) ×100

2.3. Assessment of Apoptosis by Acridine Orange (AO) and Ethidium Bromide (EtBr) Double Staining

Following 24 hours of treatment with the IC_{50} dosage of the test drug NMVM (151.34 µg/mL), the cells were rinsed with cold PBS and stained with a combination of Acridine Orange (100 µg/ml) and Ethidium Bromide (100 µg/ml) at room temperature for 10 minutes. After two rounds of washing with 1X PBS, the stained cells were analysed under a fluorescence microscope using a blue filter (Olympus CKX41 with Optika Pro5 camera) [18]. The cells were classified as either alive (normally green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with condensation or fragmentation), or necrotic (brown or black nucleus) (uniformly orange-stained cell nuclei).

3. RESULTS

3.1. Result Analysis on Cytotoxic potential of NMVM by MTT assay

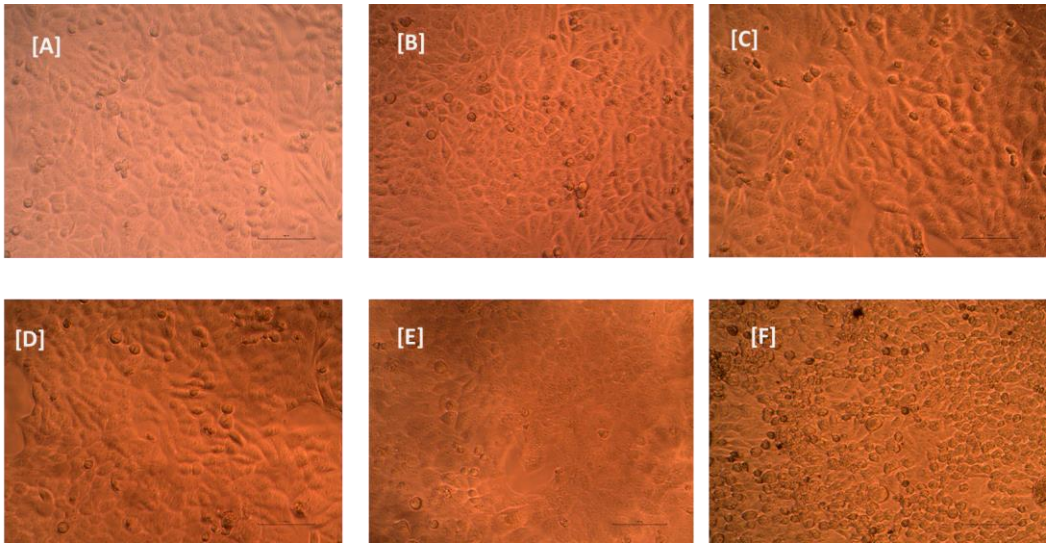
Cytotoxic potential of the trial drug NMVM was assessed by using an MTT assay, from the study, it was apparent that the lowest viability was observed at 100µg/ml (31.23 ± 0.63), followed by 50 µg/ml and 25µg/ml, with percentage viability of 52.64 ± 0.51 and 69.99 ± 1.74 found in MTT assay. The corresponding IC_{50} value of test drug NMVM was 151.34 µg/ml. (Table 1 and Figure 1.)

Table 1: Percentage of cell viability and IC_{50} value of NMVM against HeLa cells.

Culture condition	% cell viability	IC_{50} (µg/ml)
Control – Untreated	100.00 ± 0.0	151.34
NMVM 6.25 µg/ml	92.42 ± 2.35	
NMVM 12.5 µg/ml	84.59 ± 0.59	
NMVM 25 µg/ml	69.99 ± 1.74	
NMVM 50 µg/ml	52.64 ± 0.51	
NMVM 100 µg/ml	31.23 ± 0.63	

Results represented as Mean ± SD. One-way ANOVA and Dunnets test were performed to analyse data.

Figure 1: Morphology of the control and drug-treated HeLa cells in MTT assay

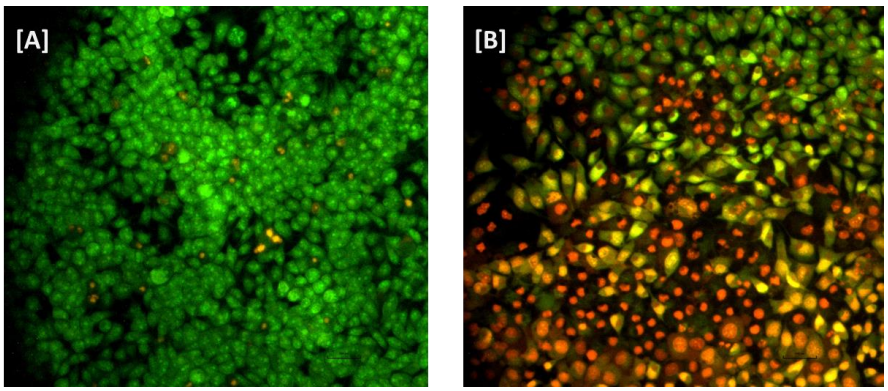


Morphology of control and drug-treated wells subjected to MTT assay visualized under the inverted microscope: (A) Untreated control HeLa cells, (B) NMVM 6.25 µg/ml, (C) NMVM 12.5 µg/ml, (D) NMVM 25 µg/ml, (E) NMVM 50 µg/ml and (F) NMVM 100 µg/ml.

3.2. Result from analysis on Apoptosis assessment using Double staining technique

Apoptotic potential of the trial drug NMVM was ascertained at the predicted IC_{50} value of 151.34 µg/ml using DNA-specific staining techniques. Results showcase the representation of normal morphology in the control group with a major emittance of green fluorescence which indicates the existence of viable cells. Test group evident the distribution of both apoptotic and necrotic cells with orange-stained nuclei. As shown in Figure 2.

Figure 2: Double staining cell morphological analysis of the control and drug-treated HeLa cells



Morphology of control and drug-treated HeLa cells subjected to Acridine orange (AO)-ethidium bromide (EB) DNA

specific staining: (A) Untreated control HeLa cells and (B) NMVM incubated HeLa cells.

4. DISCUSSION

Cancer is the second chief cause of mortality after coronary heart disease and stroke worldwide according to data compiled by the WHO. A combined total of 15.2 million fatalities worldwide are caused by ischemic heart disease and stroke. However, > 9.6 million people lost their lives to the disease in 2018 [19]. Despite recent medical advancements, cancer therapy remains a significant burden, making the study of novel methods for combating the disease an appealing area of biomedical research [20]. Because of the negative consequences of standard cancer therapies like chemotherapy and radiation therapy, scientists are always on the lookout for alternative drug sources with more specificity and fewer adverse effects.

An incidence of 528,000 and 266,000 deaths occur due to cervical cancer each year, due to the disease [21]. In developed countries, screening has greatly reduced the incidence and mortality rates of cervical cancer; however, it remains high (i.e., 80%) in developing countries, where the diagnosis age of patients is slightly decreasing and the patients are at the advanced stage of disease (70%) [22,23].

In recent years, bioactive chemicals found in nature that can suppress, slow, or reverse the multistage carcinogenesis process with low toxicity have received increased research interest. Sixty percent or more of the currently used anticancer medicines have their roots in nature [24]. As apoptosis controls cell death, the lysosomes and cell membranes are not damaged, hence there is no outflow of internal contents. It's desirable for anti-tumour medications to have no effect on inflammation or subsequent damage. Anti-cancer medication research has shifted its focus to include the quest for molecules that can induce apoptosis in tumour cells [25].

The uterine endothelial cells known as HeLa are among the most well-known cell lines. Because of their ubiquitous cell surface receptors, HeLa cells may be utilised to examine a wide range of cytokines. In addition to being useful for research into new drugs, these cells can also shed light on how those drugs will perform in the body. Cell viability techniques, such as the MTT test, are routinely practiced to determine cytotoxicity, cell viability, and proliferation by altering tetrazolium salt to formazan crystals [26]. In the present investigation the cytotoxic potential of the trial drug NMVM was evaluated by using an MTT assay, it was evident from the study that the lowest viability was observed at 100µg/ml (31.23 ± 0.63), followed by 50 µg/ml and 25µg/ml, with percentage viability of 52.64 ± 0.51 and 69.99 ± 1.74 found in MTT assay. The corresponding IC_{50} value of NMVM was 151.34 µg/ml.

Apoptosis is considered by morphological alterations in cells and nucleus (cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation) [27,28]. Recently scientists focused on alternative medicine for effective apoptotic induction drugs [29]. In the present investigation, the apoptotic potential of the trial drug NMVM was ascertained at the predicted IC_{50} value of 151.34 µg/ml using DNA-specific staining techniques. Results showcase the representation of normal morphology in the control group with a major emittance of

green fluorescence which indicates the existence of viable cells. Test drug-treated cells evident the distribution of both apoptotic and necrotic cells with orange-stained nuclei.

5. CONCLUSION

In the last three decades, the incidence of cervical carcinoma among young women is increased exponentially. Drugs that prevent cell growth and encourage apoptosis are often used in cancer treatment. The practice of traditional medicines to manage cancer is part of that tradition's long and illustrious track record. The findings of the MTT and DNA staining assays in the current study show that NMVM significantly inhibits the growth of HeLa cells, with the IC50 value being 151.34 g/ml. This led us to the conclusion that the Siddha formulation has promising anticancer effects and shall be suggested for the therapy of cervical cancer with the preceding clinical evaluation.

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CONFLICT OF INTERESTS

Nil

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