Bulletin of Pure and Applied Sciences
Zoology (Animal Science), Vol.43B, No.2S,
July-December 2024: P. 867-878

Print version ISSN 0970-0765
Online version ISSN 2320-3188

Original Research Article
Content Available online at: www.bpasjournals.com

VALIDATION OF *IN-VITRO* ANTI-CANCER ACTIVITY OF SIDDHA METALLO-MINERAL FORMULATION *NANDHI MEZHUGU* AGAINST TM3 CELL LINES.

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Abstract:

Background: Testicular cancer is the most common malignancy among men between 14 and 44 years of age, and its incidence has risen over the past two decades. *Nandhi Mezhugu*is a Siddha herbo-mineral formulation widely used to treat various cancers and testicular cancer is one among them. Due to lack of scientific evidence, the present study was envisioned to evaluate the anti-cancer activity of *Nandhi Mezhugu* in the TM3 cell lines.

Methods: TM3 cell lines were used to assess the antiproliferative activity of *Nandhi Mezhugu* using the MTT assay, apoptosis by Acridine Orange (Ao) and Ethidium Bromide (Etbr) Double Staining and Annexin V flow cytometry.

Results: *Nandhi Mezhugu* produced dose-dependent cytotoxic effects against TM3 cell lines with an LC50 value of 102.2946 µg/mL. Further investigation, using Apoptosis by

Ao and EtBr showed the apoptotic changes by colour change and chromatin condensation. Annexin V flow cytometry, equivalently revealed the late apoptotic effect, with a percentage value of 21.40.

Conclusion: Thus, the current study evaluates the effectiveness of *Nandhi Mezhugu* against Testicular cancer. Future RCT or Clinical trials will explore the effectiveness and safety of *Nandhi Mezhugu* in human subjects

Keyword: Anti-cancer, Annexin V Flowcytometry, MTT assay, *Nandhi Mezhugu,* Siddha, TM3 cell lines.

1.Introduction:

Cancer is one of the most severe diseases affecting people, with testicular cancer being the most prevalent type among men aged 14 to 44 next to prostrate and lung cancers. Over the past two decades, its incidence has been increased in Western countries [1]. Both genetic and environmental factors play a role in the development of testicular cancer, with cryptorchidism being the most significant risk factor. Testicular cancer is most commonly diagnosed in men between 20 and 34 years old. It is believed to originate from an early lesion known as carcinoma in situ of the testis, also referred to as Intratubular Germ Cell Neoplasia (IGCN) or Testicular Intraepithelial Neoplasia (TIN) [2]. In 2020, the International Agency for Research on Cancer (IARC) reported 74,458 new cases globally. The incidence varies widely around the world, ranging from 3 to 12 new cases per 100,000 males per year in Western societies [3]. The main treatment for testicular tumors is radical inguinal orchiectomy, which involves the removal of the testicle and spermatic cord. After this procedure, additional treatment options may include observation, retroperitoneal lymph node dissection, radiation therapy, and chemotherapy [4]. While chemotherapy, radiation, and surgery are standard and effective treatments for testicular cancer, they can significantly affect a patient's quality of life due to their debilitating side effects. Siddha medicine, a traditional medical system, focuses on promoting health and addressing a variety of chronic illnesses.

In ancient Siddha literature, cancer is described using various terms such as *'Puttru,'* '*Vippuruthi*,' and '*Kazhalai*,' with testicular cancer specifically referred to as '*Andaputtru*.' Siddha medicine may hold significant potential in oncology as a preventive measure, anti-cancer treatment, and adjunct to chemotherapy, as it aims to improve the patient's quality of life and reduce the impact of the disease. Siddha texts outline several formulations for cancer treatment, and various in-vitro studies have explored the anti-cancer properties of certain remedies. For instance, the Siddha metallo-mineral formulation *Nandhi Mezhugu*, mentioned in Siddha Vaidiya Thirattu, is indicated for various illnesses, including testicular cancer [5]. *Nandhi Mezhugu* has been investigated for its anti-cancer activity against cervical cancer using HeLa cell lines[6] and has undergone toxicity studies to ensure its safety[7].

Despite these investigations, there is still insufficient evidence to confirm *Nandhi Mezhugu's* efficacy in treating testicular cancer. To address this gap, we evaluated the anti-cancer effects of *Nandhi Mezhugu* on TM3 cell lines using the MTT assay, apoptosis analysis via Acridine Orange and Ethidium Bromide dual staining, and

Annexin V flow cytometry.

2. Materials and methods:

The test drug *Nandhi Mezhugu* was procured from a GMP certified pharmaceuticals, Tamil Nadu. The study was done in Biogenix Research Centre laboratory in Trivandrum.

2.1 Cell Line:

At the National Centre for Cell Sciences (NCCS), Pune, India, the TM3 cells (Testicular cancer cell line) were initially procured and it was then maintained in Dulbecco's modified Eagles medium, DMEM.

2.2 MTT Assay:

A 25 cm^2 tissue culture flask containing DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany), and an antibiotic solution containing 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 2.5 µg/ml of amphoteracin B was used to cultivate it. Cell lines that were cultured were maintained at 37ºC in an NBS Eppendorf, Germany, humidified 5% CO2 incubator.

The MTT assay method was used to assess the vitality of the cells after they were directly observed using an inverted phase contrast microscope. Using trypsinization, a confluent monolayer of cells that was two days old was suspended in 10% growth media. A 96-well tissue culture plate was seeded with 100µl of the cell suspension **(**5x103 cells/well), which was then incubated at 37ºC in a humidified 5% CO2 incubator. Using a cyclomixer, 1 mg of *Nandhi Mezhugu* was weighed and dissolved in 1ml of 0.1% DMSO. To guarantee sterility, the sample solution was filtered using a 0.22 µm Millipore syringe filter. The growth medium was removed after 24 hours, and each freshly prepared test compound *Nandhi Mezhugu* in DMEM was serially diluted five times by a two-fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of DMEM). Three duplicates of each concentration of 100µl were added to each well, and the mixture was then incubated at 37ºC in an incubator with 5% CO2 that was humidified. Control cells that had not been treated were also kept.

Following a 24-hour treatment period, the entire plate was examined using an inverted phase contrast tissue culture microscope (Olympus CKX41 equipped with an Optika Pro5 CCD camera), and the microscopic observations were captured as photographs. Any discernible alterations in the cell's shape, such as rounding or contracting, granulation, and vacuolization in the cytoplasm of the cells, were regarded as markers of cytotoxicity. [8,9]

2.3 Determination of Apoptosis by Acridine Orange (Ao) and Ethidium Bromide (Etbr) Double Staining:

TM3 cells after attaining sufficient confluency**, LC50 concentration of sample-(102.2946µg/mL)** was added and incubated for 24 hours. Untreated control cells were also maintained. After incubation**,** the cells were washed by 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 blue filter of fluorescent microscope (Olympus CKX41 with Optika Pro5 camera). [10]

The cells were then distinguished into four categories which differentiates the living cells from the early apoptotic, late apoptotic and the necrotic cells.

2.4 Apoptosis by Annexin V Flow Cytometry:

The TM3 cell line was cultivated using the conventional techniques previously mentioned. Once sufficient confluency was reached, LC50 concentration of the sample, **102.2946µg/mL**was added, and the mixture was incubated for a full day. Additionally, untreated control wells were kept up. The sample of cells was moved into a polystyrene tube measuring 12 by 75 mm. For fixation in a tube, 1×106 cells are the bare minimum advised. After that, the samples were centrifuged for five minutes at 3000 RPM. The particle was not disturbed during the removal of the supernatant. The cell pellet either produces a visible pellet or a white film on the tube bottom following centrifugation.

Add 100 µL of the Muse TM Annexin V & Dead Cell Reagent to each tube in the tubes. After fully mixing the tubes for three to five seconds with a medium-speed pipette or vortex, they were allowed to sit at room temperature in the dark for twenty minutes. Muse flow cytometry software was used to evaluate the cells in a flow cytometer. Using Muse FCS 3.0 software, cells were gated against untreated control cells and examined for apoptosis [11].

3. Results:

3.1 *In-vitro* Cytotoxic Effect Determination by MTT Assay

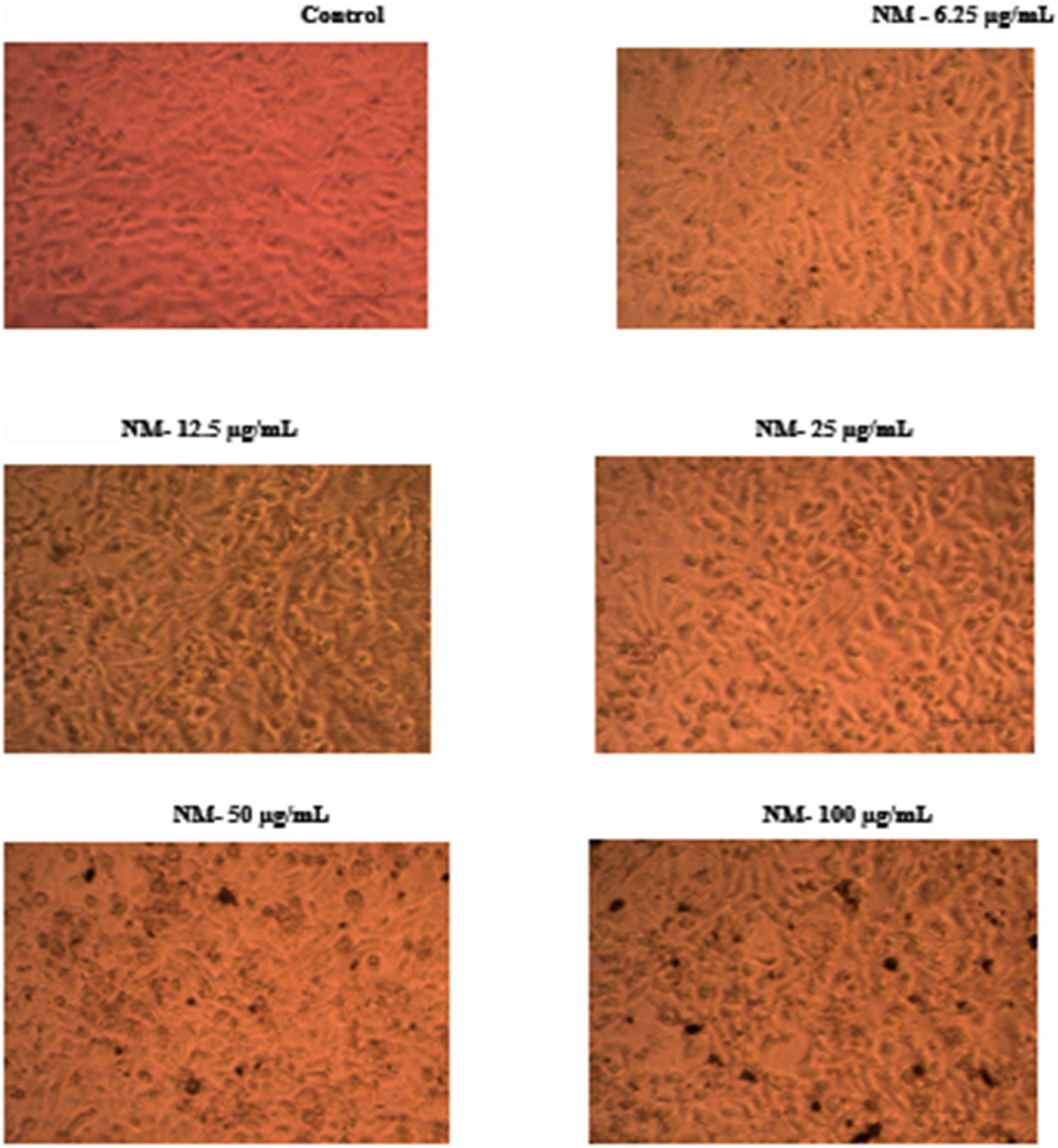

Figure1. Represents the ohase contrast images obtained during the MTT Assay.

Table - 1: Percentage viability of *Nandhi Mezhugu* at various concentrations

Sample Concentration (µg/ml)	Percentage Viability
CONTROL	100.00
Nandhi Mezhugu -6.25 µg/mL	92.7473
Nandhi Mezhugu -12.5 µg/mL	85.0953
Nandhi Mezhugu -25 µg/mL	73.1108
Nandhi Mezhugu -50 µg/mL	63.653
Nandhi Mezhugu -100 µg/mL	54.7532

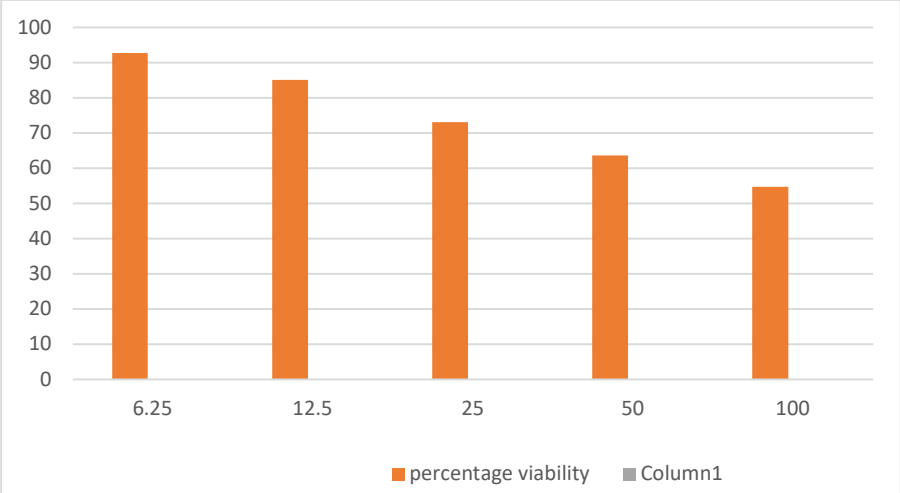

Figure: 2 - Graphical representation depicting the cytotoxic effect of *Nandhi Mezhugu* by MTT assay- Along Y axis Percentage viability, Along X axis varied concentration of *Nandhi mezhugu*. All experiments were done in triplicates and results represented as Mean+/- SE. One-way ANOVA and Dunnets test were performed to analyse data. ***p< 0.001 compared to control group.

The LC50 Value is the 50% of cell death at a specific concentration, it was calculated using ED50 PLUSV1.0 Software and found to be **102.2946 µg/mL**

3.2 Apoptosis by Acridine Orange (Ao) And Ethidium Bromide (Etbr) Double Staining:

Fig: 3.a Control **Fig. 3.b. Sample Treated**

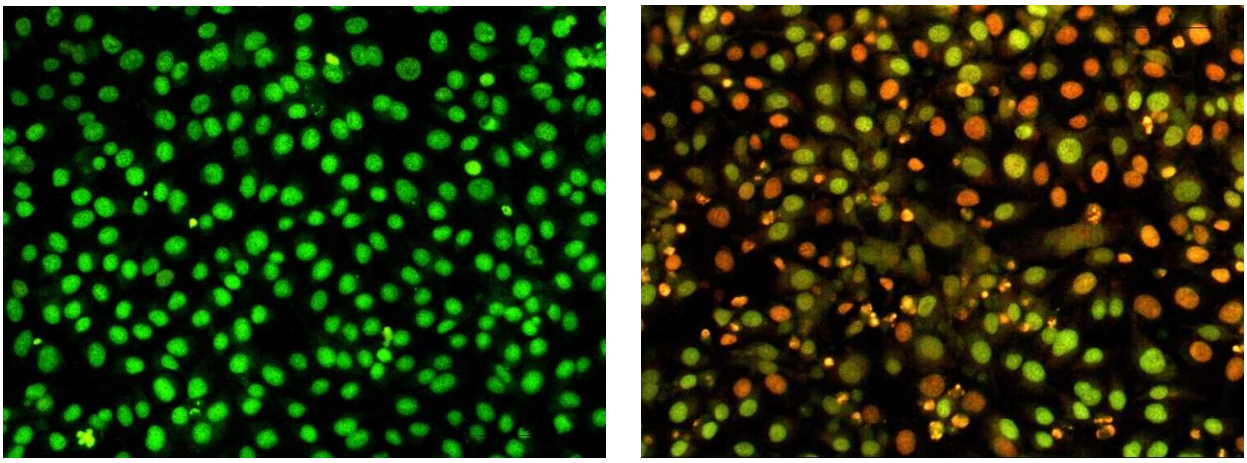

Fig. 3. a,b Represents the phase contrast images obtained during the double staining method

- 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)
- Necrotic cells (uniformly orange-stained cell nuclei).

3.3 Apoptosis by Annexin – V Flowcytometry:

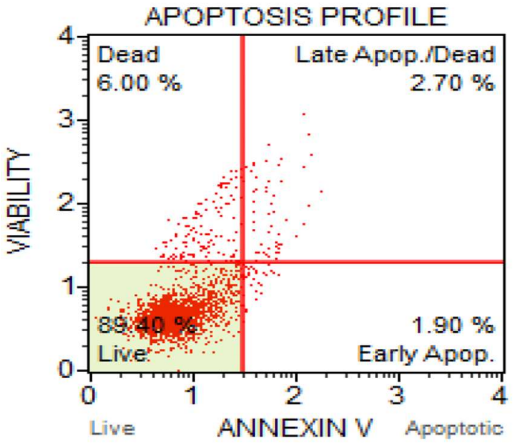

Fig 4.a: Apoptosis profile of untreated control cells

	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	2.24E+06	89.40 %
Early Apoptotic (LR) :	4.75E+04	1.90 %
Late Apop./ Dead (UR) :	6.75E+04	2.70 %
Debris (UL) :	1.50E+05	6.00 %
Total Apoptotic :	1.15E+05	4.60 %

Fig 4.b: Cell distribution in control cells

- The early apoptotic percentage was determined to be 1.90% and the late apoptotic changes was founds as 2.70%. Total Apoptotic percentage was found to be in minimal amount 4.60%

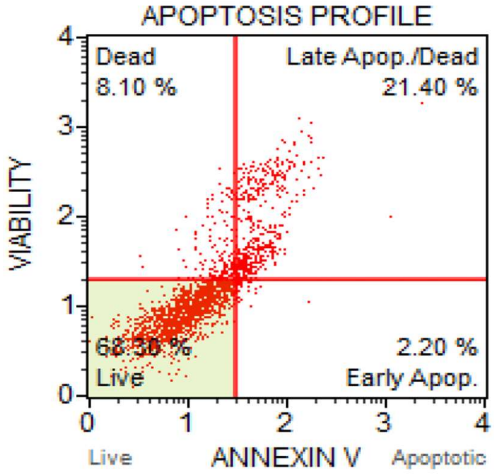

Fig 4.c: Apoptosis profile of cells treated with sample

	Cell Conc. (Cells / mL)	% Gated
Live (LL) :	1.64E+06	68.30 %
Early Apoptotic (LR) :	5.29E+04	2.20 %
Late Apop./ Dead (UR) :	5.14E+05	21.40 %
Debris (UL) :	1.95E+05	8.10 %
Total Apoptotic :	5.67E+05	23.60 %

Fig 4.d: Cell distribution in cells treated with sample

- In the sample treated cells the early apoptotic percentage was found to be 2.20% and the late apoptotic was found to be 21.40%, 8.10 % constituted the debris resulting in the total apoptosis rate of 23.6.

4. Discussion:

Current treatment for testicular cancer primarily involves Cisplatin-based combination chemotherapy, alongside advancements in post-chemotherapy surgical procedures and diagnostic techniques, which have significantly enhanced long-term survival for many patients. However, challenges remain, including controversial issues related to clinical stage I disease management, salvage chemotherapy, post-chemotherapy surgery, and the adoption of innovative imaging studies. Relapses after salvage chemotherapy generally carry a poor prognosis, and the optimal treatment strategy remains unclear [12]. Despite the success of these treatments, high rates of metastasis and recurrence can ultimately lead to death, and the adverse effects, such as sexual and urological dysfunction, can severely impact quality of life [13,14]. Thus, it is crucial to seek alternative therapies that are both effective and associated with fewer side effects. Currently, cancer research is focusing on both conventional treatments and traditional medicines. An integrated approach that combines conventional medicine with traditional remedies may be essential in addressing this challenging disease.

The Siddha system of medicine is based on three fundamental principles: *vali* (movement), *azhal* (transformation), and *iyam* (structure). In the context of cancer, *azhal* plays a crucial role as a regulator of cellular digestion and metabolic activity. An imbalance between *azhal* and *iyam* (the relationship between fire and water) contributes to uncontrolled tissue growth, a hallmark of cancer. When *azhal* is diminished, a metaphorical crisis ensues, leading to an over activation of *vali* (movement) and *iyam* (structure) forces, the dysregulation of *Viyana vaayu (Paravukal), Samana vaayu (Nadukkal), and Suzhumunai naadi* derangement (Vital force) can lead to metastasis of these cellular proliferations. Each individual has a unique *Thegi* (body constitution), with doshas predominant as a single dosha (*Vatham, Pitham, or Kapham*) or combinations (*Vathapitham, Pithakapham, Kabhavatham, Vathapithakapham*). *Thegi* influences responses to the environment, including susceptibility to diseases and drug reaction. Balanced doshas result in unique positive traits, while imbalances lead to specific vulnerabilities. So, maintain balance among these doshas is crucial for holistic health and well-being. In cancer, changes in *Kapham* results in weakened immunity and compromised tumour integrity, while *Vatham* contributes to tumour growth and its ability to spread [15]. This perspective, grounded in Siddha principles, provides a distinctive insight into the pathophysiology of cancer and suggests potential approaches for therapeutic intervention. Several compound formulations have been indicated for testicular cancer and *Nandhi Mezhugu,* comprising of several ingredients acts on the deranged vital forces and rearranges them by acting through its hot potency. [5].

From the results of MTT Assay, the percentage of cell viability decreases with an increase in the concentration of the test drug. In 6.25 µg/mL it is found to be 92.7473 whereas at 100, it was found to be 54.7532 µg/mL. This shows the cytotoxicity has increased in the dose-dependent manner. The LC_{50} value was found to be **102.2946 µg/mL.** The LC_{50} value represents the lethal concentration, at which 50 percent of the cells were inhibited on treatment with the test drug.

From the results of EtBr and Acridine orange double staining method, all nuclei

in control (appearing as green in epifluorescence) showed a regular spherical structure and chromatin organization. Whereas in the sample treated with the LC_{50} value, several nuclear apoptotic bodies containing the fragmented DNA were visible, appearing as green fluorescent patches (early apoptosis, in an advanced apoptotic stage the disintegration of apoptotic bodies lead to total nuclear DNA fragmentations typical of late apoptosis (orange to red stained nuclei) and necrotic cells (uniformly orange-stained cell nuclei) [16].

Annexin V binding assays are a crucial tool for accurately measuring apoptosis and differentiating it from necrosis, allowing for the identification of necrotic and late-stage apoptotic cells. By combining annexin V and propidium iodide, researchers can reliably detect and quantify apoptosis in testicular cancer cells. The technique was employed to assess the apoptotic response of testicular cancer cells to a treatment, with the LC50 value indicating the concentration at which 50% of cells undergo apoptosis. The extent of apoptosis was then comprehensively evaluated using annexin V flow cytometry, providing valuable insights into the cellular response to treatment and the efficacy of the therapeutic approach. The results of apoptosis Annexin V flow cytometry reveal that when compared to the control and drug-treated groups, the late apoptotic changes in the control group were found to be 2.70% whereas in the drug-treated group, it is found to be 21.80%. The total apoptotic percentage significantly increased from 4.60 to 23.60%. This shows a significant increase in cell death in the drug-treated group when compared to the control group, proving the apoptotic activity. From this, it is evident that the test drug *Nandhi Mezhugu* possesses significant anti-cancer activity and by future studies for instance in-vitro, in-vivo studies and clinical trials should be conducted to accentuate its potential in the treatment of testicular cancer.

5.Conclusion:

The test drug *Nandhi mezhugu* showed a significant effect in the cytotoxicity decreasing the cell viability in dose-dependent manner and the images of the chromatin condensation and orange stained revealed the apoptosis occurred. Late apoptosis percentage from the results of flow cytometry in the test drug treated group with LC_{50} value revealed their potency to induce apoptosis to a certain extent. Further in-vivo and clinical trials should be carried out to prove its therapeutic effect in the management of testicular cancer.

6.Reference:

1. Cheng L, Albers P, Berney DM, et al. Testicular cancer. Nat Rev Dis Primers. 2018;4(1):29. Published 2018 Oct 5. doi:10.1038/s41572-018-0029-0

2.Ziglioli F, Maestroni U, Dinale F, Ciuffreda M, Cortellini P. Carcinoma in situ (CIS) of the testis. Acta Biomed. 2011;82(2):162-169.

3. Giona S. The epidemiology of testicular cancer. Exon Publications. 2022 Sep 12:107-16.

4. Shaw J. Diagnosis and treatment of testicular cancer. American Family Physician. 2008 Feb 15;77(4):469-74.

5. Dr.K.N.Kuppuswamy Muthaliyar.,Dr.K.S.Uthamarayan.,Siddha Vaithiya Thirattu.,Indian

medicine and homeopathy.,2009.,Pg.no.168.
6. Girija NS, Neethi B, Sakthimanipriya L, Sinekha MA, Shanmugapriya P, Meenakumari R. In-Vitro Cytotoxic, Antiproliferative and Apoptotic Activity of Siddha Formulation Nandhi Mezhugu(NM) Against Hela Cell Line. Applied Biochemistry and Biotechnology. 2024 Mar;196(3):1612-22
7. Kantham, Lakshmi & G, Ganapathy & Suba, V. & Srinivasan, Morkonda & A, Geetha. (2017). Acute, Sub-Acute (28-Days), And Sub-Chronic (90-Days) oral Toxicity Studies Of Nandhi Mezhugu. International Journal of Research in Ayurveda & Pharmacy. 8. 182-189. 10.7897/2277-4343.082110.

8. Jerard C, Michael BP, Chenicheri S, Vijayakumar N, Ramachandran R. Rosmarinic Acid-Rich Fraction from Mentha arvensis Synchronizes Bcl/Bax Expression and Induces Go/G1 Arrest in Hepatocarcinoma Cells. Proc Natl Acad Sci, India, Sect B Biol Sci. 2020 Sep;90(3):515–22
9. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. Journal of Immunological Methods. 1983 Dec;65(1–2):55–63.
10. Zhang, J.H., YU, J., Li, W.X. and Cheng, C.P. (1998) Evaluation of Mn2+ stimulated and Zn2+ inhibited apoptosis in rat corpus luteal cells by flow cytometry and fluorochromes staining. *Chin. J. Physiol. 41(2):121-126.*

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

12. Chovanec M, Cheng L. Advances in diagnosis and treatment of testicular cancer. bmj. 2022 Nov 28;379.

13. Abbas Z, Rehman S. An overview of cancer treatment modalities. Neoplasm. 2018 Sep 19;1:139-57

14. Frumovitz, M., Sun, C. C., Schover, L. R., Munsell, M. F., Jhingran, A., Wharton, J. T., Eifel, P., Bev- ers, T. B., Levenback, C. F., Gershenson, D. M., & Bodurka, D. C. (2005s). Quality of life and sexual functioning in cervical cancer survivors. *Journal of Clinical Oncology, 23*(30), 7428–7436. https://doi. org/10.1200/JCO.2004.00.3996

15. Subathra T, Gomathi R, Shanmugapriya P, Ramamurthy M, & Meenakumari R. A review on chithiramoola kuligai – therapeutic approach for cervical cancer in accordance with siddha principles. *Journal of population therapeutics and clinical pharmacology*, *29*(04), 3919–3926. https://doi.org/10.53555/jptcp.v29i04.6366

16. Ciniglia C, Pinto G, Sansone C, Pollio A. Acridine orange/Ethidium bromide double staining test: A simple In-vitro assay to detect apoptosis induced by phenolic

compounds in plant cells. Allelopathy Journal. 2010;26(2):301-8.