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Anti-Proliferative and cytotoxic effects of *Azima tetraacantha* Lam. On cervical cancer cell line (HeLa) and human peripheral lymphocyte (HPL)

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Abstract

Azima tetraacantha Lam. (Family: Salvadoraceae, Common name: Needle bush) is distributed along the coastal areas of India. It is a spiny, evergreen shrub with a tendency to scramble. In the present study, the anti-cancer as well as cytotoxic properties of methanol leaf extract of *Azima tetraacantha* was determined, *in vitro*, against the cancer cell line HeLa and Human peripheral Lymphocyte (HPL) respectively. MTT assay was used to determine cell viability and LDH leakage assay was used to determine the cell permeability in both HeLa and HPL cells. A dose dependant cell proliferation inhibitory activity was observed for the methanol extract ($IC_{50} = <100\mu\text{g/ml}$) against HeLa. However, the extract remained less toxic to normal cells, HPL. Thus, the present study revealed that the methanolic extract of *Azima tetraacantha* possess chemoprotective effect and can be used as a potent agent in treating cancer.

Keywords: *Azima tetraacantha*, anti-proliferative, cytotoxic, methanol extract.

1. Introduction

Cervical cancer is the principal cause of cancer-related mortality in women of the developing countries that contribute more than 85% of global disease burden [1]. Anti-proliferative *in vitro* screening remains an efficient tool for anti-cancer drug discovery. Studies are being conducted worldwide using traditional medicinal plants that will possibly lead to the discovery of new natural therapeutic agents with fewer side effects for cancer treatments.

The present study focuses on the anti-cancer as well as cytotoxic properties of methanol leaf extract of *Azima tetraacantha* Lam. (Fig. 1). The plant is a rambling spinous shrub belonging to the family Salvadoraceae. It is characterized by the presence of four thorns at right angles to each other at each node. The leaves, root and root bark have medicinal properties and are used among different people in Kerala. The leaves are administered to women after confinement as part of mother care treatment. Since leaves are being used traditionally for the treatment of women specific problems, the leaf samples were chosen to evaluate the anti-cancer effect on cervical cancer cells (HeLa) and cytotoxic effect on human peripheral lymphocytes (HPL), *in vitro*. MTT assay was used to determine cell viability and LDH leakage assay was used to determine the cell permeability in both HeLa and HPL cells.



Fig 1: Habit of *Azima tetraacantha* Lam.

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2. Materials and Methods

2.1 Plant material

Leaves of *Azima tetracantha* were collected from Vembayam, Trivandrum. A voucher specimen has been submitted in the Herbarium, Dept. of Botany (KUBH 5813). The leaves were cleaned and freed from foreign materials. They were then minced, air dried and powdered [2].

2.2 Preparation of Extracts

The powdered samples were extracted with methanol (ME) using the Soxhlet apparatus. The extract obtained was subsequently concentrated under reduced pressure and the residue collected. The extractive value was calculated and the residue was used for qualitative screening of secondary metabolites.

2.3 HeLa Cell Culture

HeLa cell lines were procured from the National Center for Cell Sciences (NCCS), Pune, India. The cells were cultured in Eagle's media, supplemented with 20% heat inactivated Foetal Bovine Serum (FBS), antibiotics (penicillin and streptomycin) and dissolved sodium bicarbonate. The pH was maintained at 7.4. Trypsin was added to the cultured cell lines for cell segregation and incubated for 30 minutes at 37 °C. The cells were harvested by scraping, for further studies.

2.4 Lymphocyte Culture Medium

HPL's were cultured in RPMI 1640 (Himedia) media, supplemented with 10% heat inactivated FBS (Himedia), antibiotics (Penicillin and Streptomycin). Phytohaemagglutinin (PHA) (Himedia) was used as the stimulant for cell proliferation. The culture was filtered using 0.2 µm pore sized cellulose acetate filter (Sartorius, Japan) in completely aseptic conditions. Lymphocytes were separated from the blood using Hisep (Lymphocyte separation medium, Himedia) and diluted to 10⁵ cells/ml and used for studies

2.5 MTT Assay [3, 4, 5]

MTT cell proliferation assay is a safe, sensitive, *in vitro* assay for the measurement of cell proliferation. MTT assay was performed as described earlier. Cell culture was plated in a 96 well plate and was incubated at 37 °C in a humidified 5% CO₂/95% air. The cells were then treated with methanolic leaf extract of *A. tetracantha*, at varying concentrations (100-1500µg/ml). The cell culture suspension was mixed with 50µl MTT solution, after four hours incubation. The culture plates were incubated for three hours at 37 °C. After incubation, 100 µl DMSO was added and then re-incubated for 30 minutes. The optical density of each well sample was measured with a microplate reader, at 540 nm, using DMSO as blank. The percentage viability was calculated using the formula

$$\% \text{ viability} = (\text{OD of test} - \text{OD of blank}) / (\text{OD of control} - \text{OD of Blank})$$

2.6 Lactate Dehydrogenase (LDH) Leakage Assay

Plasma membrane integrity of both HeLa and HPL cells were assessed using LDH leakage assay (Grivell and Berry, 1996) [6]. Known concentrations of methanol extract (100-1500 µg/ml) from the growth medium of experimental cultures were added to a 1ml cuvette containing potassium phosphate buffer,

NADH solution and sodium pyruvate solution. This was mixed thoroughly. The blank solution was prepared by adding potassium phosphate buffer and the sample. The absorbance of the solution was read at 340nm in a spectrophotometer. The intensity of the color was considered to be proportional to LDH leakage. The results are expressed in enzyme units (U/ml).

2.7 Statistical analysis

All results were mean of triplicate measurements ± standard deviation. IC₅₀ was calculated by Probit Analysis using SPSS software version 17 at level of significance is $p < 0.05$.

3. Results

The leaf powder after extraction with methanol yielded 7.8± 0.15% residue. On qualitative, secondary metabolite screening, the extract showed the presence of high amounts of terpenoids.

3.1 MTT Assay

The present study shows that the cell viability of HeLa was inversely proportional to the concentration of methanol extract treated (Graph 1). The results clearly confirmed that the exposure of HeLa cells at different concentrations resulted in decrease of cell proliferation in a dose dependent manner. The viability decreased more than 70% at 100µg ml⁻¹. IC₅₀ was found to be 44.26µg ml⁻¹ against HeLa cells. However, the viability of normal cell line (HPL) remained undisturbed by the methanol extract of *A. tetracantha*.

3.2 LDH Leakage Assay

The effect of methanol extract of *A. tetracantha*, on cell permeability of HeLa cell lines and HPL cells are presented in table 1. The amount of LDH in the media was quantified as enzyme units. LDH was higher when the extract was treated against HeLa than HPL.

3.3 Morphological observations

The effect of different concentrations (100-1500µg/ml) of methanol extract on HeLa cells was observed using phase contrast of an inverted microscope. The phase contrast observations in HeLa cells revealed that the morphology of cells varied considerably, with an increase in concentration. Untreated HeLa cells (Control) revealed cells displaying their normal shape and lesser number of degrading cells (Fig. 2a). On the other hand, cells treated with different concentrations of methanol extract exhibited varying morphological changes like nuclear fragmentation and chromatin condensation, finally resulting in formation of apoptotic bodies (Fig. 2b-e). The representative image of HPL is shown in Figure 3.

Graph 1. Percentage cell viability of HeLa and HPL cells using MTT assay

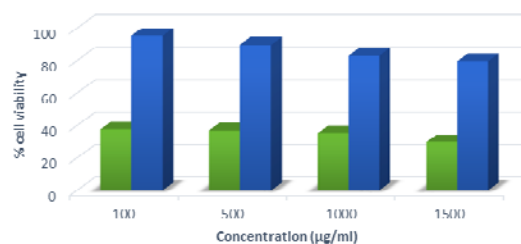


Table 1: Effect of methanol extract of *A. tetraantha*, on cell permeability of HeLa and HPL cells

Concentration of methanol extract (µg/ml)	Enzyme unit (U/ml)	
	HeLa	HPL
Control	0.009±0.01	0.000±0.00
100	0.0186±0.33	0.002±0.34
500	0.550±0.15	0.008±0.73
1000	0.661±0.64	0.011±0.67
1500	0.763±0.46	0.019±0.43

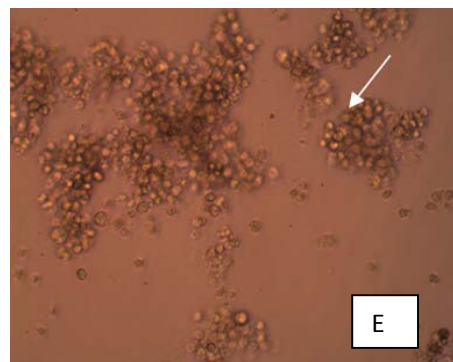
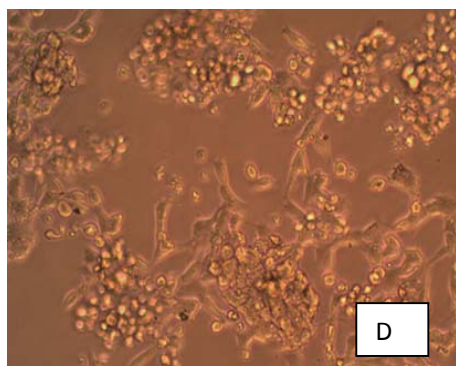
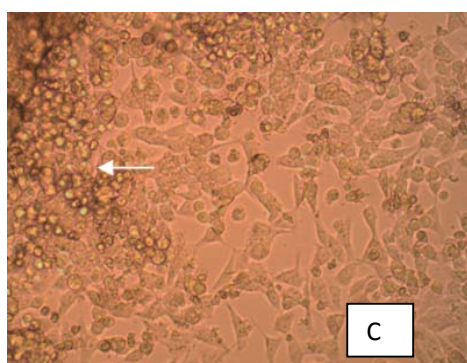
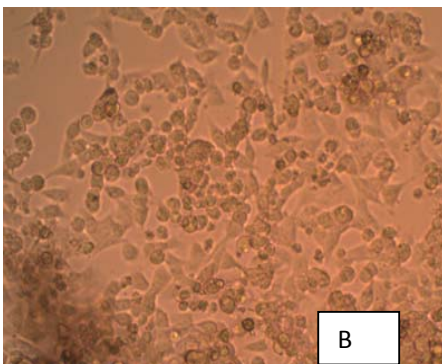
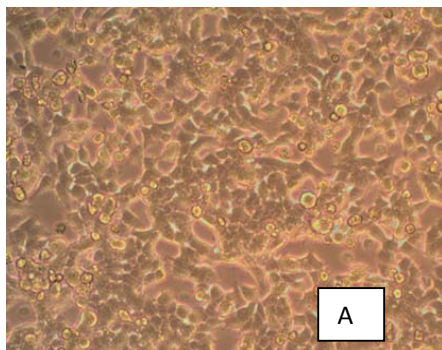
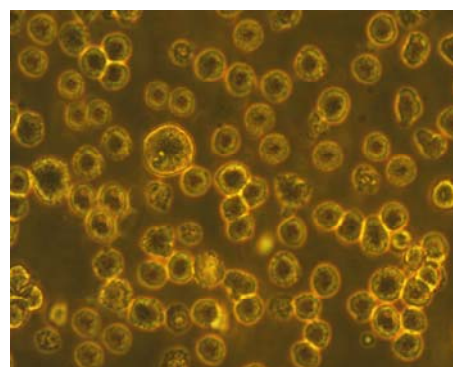
**Fig 2:** Effect of varying concentrations of methanol extract on HeLa cells

Fig 2.2a. Control Fig 2.2b. 100 µg ml⁻¹ Fig 2.2c. 500 µg ml⁻¹
 Fig 2.2d. 1000 µg ml⁻¹ Fig 2.2e. 1500 µg ml⁻¹.
 Arrow mark indicates apoptotic cells

**Fig 3:** Effect of methanol extract on HPL cells

4. Discussion

4.1 MTT Assay

MTT assay is a well-established *in vitro* method for cytotoxicity against cancer cell lines and hence it was utilized to determine the selective activity of the methanol extract. MTT assay measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase. The production of the resultant formazan appears to be proportional to the level of energy metabolism in the cells [4, 7]. Therefore, it is possible to measure the metabolically activated cells, even in the absence of cell proliferation. The amount of formazan produced is proportional to the amount of MTT in the incubation medium. This method was used to determine the anti-proliferative effect of methanolic extract of *A. tetraantha* on HeLa cells and HPLs.

Dose- dependent decrease in cell proliferation was observed in HeLa cells when treated with methanol extract of *A. tetraantha*. A similar dose dependent cell proliferation inhibitory activity was also reported for methanolic extracts of *Jatropha curcus* and *J. gossypifolia* against HeLa cells. The significant inhibition of HeLa cell growth was shown at an IC₅₀ of 98.18 µg/ml and 110.6 µg/ml respectively for both the plants [8]. A linear decrease in cell viability and linear increase in cell inhibition was observed in HeLa cells along with a concomitant increase in concentration of methanol extract of *A. tetraantha*. In this study, the concentration required to inhibit 50% of HPL cells was found to be much greater than

1500 µg/ml. This suggests that the methanol extract is not toxic to normal cells.

4.2 LDH Leakage Assay

LDH leakage is considered as a marker of cytotoxicity. LDH leakage assay monitors the integrity of the plasma membrane and is sensitive and easy to perform^[9]. This assay was used to determine the effect of the extract on the integrity of the plasma membrane of cultured HeLa and HPL cells. This is based on the conversion of lactate to pyruvate in the presence of LDH with parallel reduction of NAD.

The methanol extract of *A. tetraacantha* was capable of killing HeLa cells more effectively, by increasing the cell permeability leading to increased cell death. The release of LDH to the media was in a concentration dependent manner. Only very low amounts of LDH were released when the methanol extract of *A. tetraacantha* was treated against the normal cells, HPL. This observation also suggests the lesser damage done by methanol extract to the lymphocytes, HPLs. It has been well documented that lactate dehydrogenase levels, as a marker of necrosis in the cell medium, elevate when cells are exposed to high concentration of anticancer agents^[10, 11].

The decreased release of LDH by HPL is a result of its anti-cytotoxic activity, while an increased amount of LDH in the media is suggestive of the anti-cancerous potency of methanol extract of *A. tetraacantha*, against HeLa cells. This is in support of the results of MTT assay against the cervical cancer cells, HeLa.

5. Conclusion

In conclusion, the results obtained from both MTT and LDH leakage assay, it is indented that the methanol extract is effective in inhibiting the proliferation and in inducing cell death in the cervical cancer cell line, HeLa cells. The study also shows that the methanol extract does not affect the growth and metabolism of normal cells. Since both the aspects are the most suitable attributes of a chemotherapeutic drug, this potential plant should be considered during further drug development.

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