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Evaluation of *in-vitro* cytotoxicity studies of *Sida rhombifolia* leaves extract

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Abstract:

Recent research into medicinal plants has led to the identification of numerous bioactive compounds with anticancer potential. *Sida rhombifolia*, a traditionally used medicinal herb, was evaluated in this study for its in vitro cytotoxic activity against Dalton's Ascitic Lymphoma (DAL) cell lines using the MTT assay. Various extracts of the leaves—including ethyl acetate, aqueous, and others—were tested to determine their ability to inhibit cell viability. Among them, the ethyl acetate and aqueous extracts exhibited the most significant cytotoxic effects, indicating their potential as sources of natural anticancer compounds. These findings support further investigation into the phytochemical constituents of *Sida rhombifolia* and their mechanism of action in cancer therapy.

Keywords: *Sida rhombifolia,* MTT assay, *Invitro* cytotoxicity, DAL cell lines, Medicinal plants.

Introduction

Cancer remains one of the leading causes of mortality worldwide, and the search for effective, safe, and affordable therapeutic agents continues to be a major focus in biomedical research. Medicinal plants are a promising source of bioactive compounds with anticancer potential. *Sida rhombifolia*, belonging to the family Malvaceae, has been traditionally used for its anti-inflammatory, antimicrobial, and wound healing properties.[1]

Recent studies have highlighted the importance of screening plant-based compounds for cytotoxic activity against cancer cell lines. The MTT assay, a widely used method for assessing cell viability and proliferation, offers a reliable in vitro approach to evaluate the cytotoxic effects of plant extracts.

This study focuses on evaluating the cytotoxic potential of various extracts of *Sida rhombifolia* leaves, particularly ethyl acetate and aqueous extracts, against Dalton's Ascitic Lymphoma (DAL) cell lines. The promising activity observed suggests that *Sida rhombifolia* could serve as a potential source of natural anticancer agents, warranting further research into its active phytoconstituents.

Materials and method

Leaves of Sida rhombifolia were gathered from Sanjivini Botanical Garden in Bhopal, India. Dr. Sayeeda Khatoon, a chemotaxonomist, verified the authenticity of the plants. Dalton ascitic lymphoma cell line, which was generously provided by the Amala Cancer Research Center in Thrissur.

Extraction of dried leaves by using various solvents of increasing polarity

The leaves of Sida rhombifolia were gathered, dried, and pulverized for use in the extraction process. The soxhlet equipment was loaded with 500 grams of the powdered substance. Then, other solvents, ranging from non-polar to polar, such petroleum ether, chloroform, ethyl acetate, and ethanol, were used to extract it. All of the solvents were cleaned up beforehand. Three days were spent using a variety of solvents in a process of continuous hot percolation to extract the desired substance. The cold maceration procedure was used to remove the water. Concentrated extracts were transferred to a 100 ml beaker, and the residual solvent was evaporated in a water bath, reducing the original volume of the extracts by a factor of 10. After that, they were desiccated in a cooler to get rid of any remaining moisture. Airtight containers were utilized to store the dried extracts until they were put to use in further research. [2]

***In-vitro* cytotoxicity study**

Cell Line

Standard cell culture procedures were used to cultivate the Dalton ascitic lymphoma cell line, which was generously provided by the Amala Cancer Research Center in Thrissur and is now housed at the Pharmacology Department at SunRise University in Alwar, where the temperature is maintained at 37 degrees Celsius and the humidity at 5%.

Maintenance of cell line

The following steps were necessary for cell line maintenance [3]

Preparation of cell medium

a) Ingredients

DMEM	10gm
Sodium bi carbonate	2.2gm
HEPES	10ml
Antibiotics	10ml
FBS	100ml

Autoclaved water to make the volume up to 1 lit.

b) Method of Preparation

- Dissolve DMEM and sodium bicarbonate in the aforementioned solution, which was made by adding HEPES to 850 ml of autoclaved water.
- Finally, add 10 ml of the antibiotics and 100 ml of the FBS to the medium, and top it up with autoclaved water to produce 1 liter.
- It should be filtered twice, and then stored at 40 degrees Celsius.

Passaging of cell line

The process of cell passaging or splitting was developed so that cells could be maintained in culture for long periods of time. At 90%-100% confluence, cells should be passed on. [3]

Reagents:

- Ethanol.
- Media with 10% serum and antibiotics.
- Trypsin.
- PBS.

b) Procedure:

• A 37°C water bath was used to gently heat the media and Trypsin.

• Verify if cells in a T flask are 90%-100% confluent using a microscope.

Cleaning the hood with ethanol. The hood's contents, including any bottles or other containers, must be sterilized before use. Ethanol was used to clean the hands. The T flasks were taken out of the incubator and put under the hood to cool down. To get rid of the dead cells, the culture medium was drained from the T flasks, and the T-flasks were washed twice with PBS. The T-75 flask was given 4 cc of trypsin. Under a microscope, we were able to verify that the cells had successfully shed their attachment to the surface. After the cells had been trypsinized, the culture medium was added, and the mixture was incubated in CO2. Every 24 hours of medium was replaced until the T flask was confluent.

Seeding of cells

a) Reagents:

- Ethanol
- Media with 10% serum and antibiotics.
- Trypsin and PBS.

b) Procedure:

We used a 37°C water bath to pre-heat the media and Trypsin.

• Verify 90%-100% confluence by counting cells in a T flask under a microscope.

Ethanol was used to clean the hood.

Specifically, cells were trypsinized.

• A hemocytometer was used for cell counting.

• The cell suspension was diluted with culture media such that 2500–5000 cells could be suspended in 100 l.

Each well of the 96-well plate in which the cells were cultivated had 100 l of cell suspension.

• The CO 2 incubator was used to incubate the plates for 24, 48, and 72 hours, respectively. Borenfreund et al. (1984) report using these plates for drug testing.

Preparation of extract solution

Reagents:

- Culture media.
- DMSO.

Procedure:

• The stock solution with concentration 20mg/ml was prepared by dissolving 2000 mg of powdered extracts in 100 ml of DMSO.

• The extract solution was diluted to a final concentration of 2mg/ml by dissolving 10 ml into 90 ml of culture medium[3].

Treatment with extract solution

Reagents:

- Culture media.
- Extracts stock solution.

Procedure:

Seeded cell plates were delicately extracted from the cozy incubator, where they have been basking in the warm embrace of the friendly environment. The culture media that had been the life support for the cells was unceremoniously discarded, making way for something much more potent and exciting. A well-measured 100µl of the extracts solution was introduced to the plates, sprinkling new life into the cells. In order to serve as a reference, 8 wells were reserved for blank control, as a point of comparison for the experimental values. The plates were then returned to the CO2 incubator for a period of 24 hours, which felt like an eternity to the eager scientists. After the incubation period had elapsed, the plates were gingerly retrieved from the incubator, and the activity of the drug was meticulously scrutinized and evaluated using different cytotoxic assays, meticulously performed according to the well-established protocols established by Borenfreund et al., 1984.

***In-vitro* Cytotoxic Assays**

Various assays were employed to assess the cytotoxicity of extracts against cancer cells, with the aim of determining the most effective treatment options.

MTT Assay

The MTT assay, a widely used standard colorimetric assay, is utilized to measure the activity of enzymes that reduce MTT to formazan, resulting in a distinct purple color. This change in color occurs mainly in the mitochondria of living cells, thereby providing an excellent measure of mitochondrial activity. Furthermore, it is an effective tool for evaluating the cytotoxicity of potential medicinal agents and other toxic substances.

To dissolve the insoluble purple formazan product into a colored solution, a solubilization solution is typically added. This solution can be dimethyl sulfoxide, an acidified ethanol solution, or a solution of the detergent sodium dodecyl sulfate in diluted hydrochloric acid. By measuring the absorbance of this colored solution at a specific wavelength of 500 to 600 nm using a spectrophotometer, the quantity of formazan produced can be quantified. The absorption

maximum is determined by the solvent utilized.

The reduction of MTT to formazan occurs solely when mitochondrial reductase enzymes are active; hence, this conversion is frequently utilized as an indicator of viable living cells. By comparing the quantity of purple formazan produced by cells treated with an agent to the amount produced by untreated control cells, the effectiveness of the agent in causing cell death or altering cell metabolism can be deduced through the production of a dose-response curve. [4]

Thus, the MTT assay is a valuable technique for measuring mitochondrial activity and evaluating the potential toxicity of medicinal agents and other substances. By dissolving the formazan product in a solubilization solution, scientists can accurately quantify the amount of formazan produced by cells and assess their viability. By comparing the quantity of formazan produced by treated and untreated cells, researchers can evaluate the effectiveness of a particular agent in altering cell metabolism or inducing cell death. Overall, the MTT assay is a useful tool in the field of biomedical research and is widely employed to aid in the development of new drugs and medicines.

a) Reagents

- MTT.
- PBS.

b) Procedure

25mg of MTT powder was dissolved in 5ml of PBS and subsequently filtered using a 10ml syringe and syringe filter. The resulting solution was then utilized in an experiment involving incubated cell plates. The first step was to remove the culture media from the plates and replace it with the extract containing culture media. Following this, the plates were again incubated in a CO2 incubator for 24 hours to allow the extract to take action. Five hours before the end of the incubation period, 20µl of MTT solution was added to each well containing cells and the plates were once again incubated at 37ºC for 5 hours. After the incubation period ended, the media was removed and 200µl of DMSO was added to each well. The samples were then subjected to pipetting up and down to dissolve the crystals, followed by transfer to a plate ELISA reader. The next step was to measure the absorbance at 550nm to obtain the optical density. Finally, the % inhibition was calculated using a pre-defined formula. All of these steps were executed with precision and care, and the results of the experiment were highly informative and valuable.

% inhibition = [(OD of untreated)-(OD of drug Treated) /(OD of untreated)] ×100

Results and discussion

In-vitro cytotoxic activity of extract of *Sida rhombifolia* by MTT assay

The findings indicated that PESR, CESR, EAESR, and AQESR had inhibitions of 7.06 ± 0.81, 13.72 ± 3.16, 80.0 ± 2.28, 20.86 ± 3.59, and 82.0 ± 3.12, respectively, as presented in Table 6.4 and Figure 6.1, demonstrating the potency of the inhibitory effects..

Table : *In-vitro* cytotoxic activity of extract of *Sida rhombifolia* by MTT assay

Extract	Concentration	Optical density	% inhibition
-	No treatment	0.3660	0.00 ± 1.31
PESR	200 µg/ml	0.3401	7.06 ± 0.81^{ns}
CESR	200 µg/ml	0.3157	13.72 ± 3.16*
EAESR	200 µg/ml	0.2896	80.86 ± 3.59**
AQESR	200 µg/ml	0.0732	80.0 ± 2.28**

8 wells /group OD at 550 nm,
*P<0. 01 Vs control,
**P<0.001 Vs control.
Values are expressed as mean ± SEM

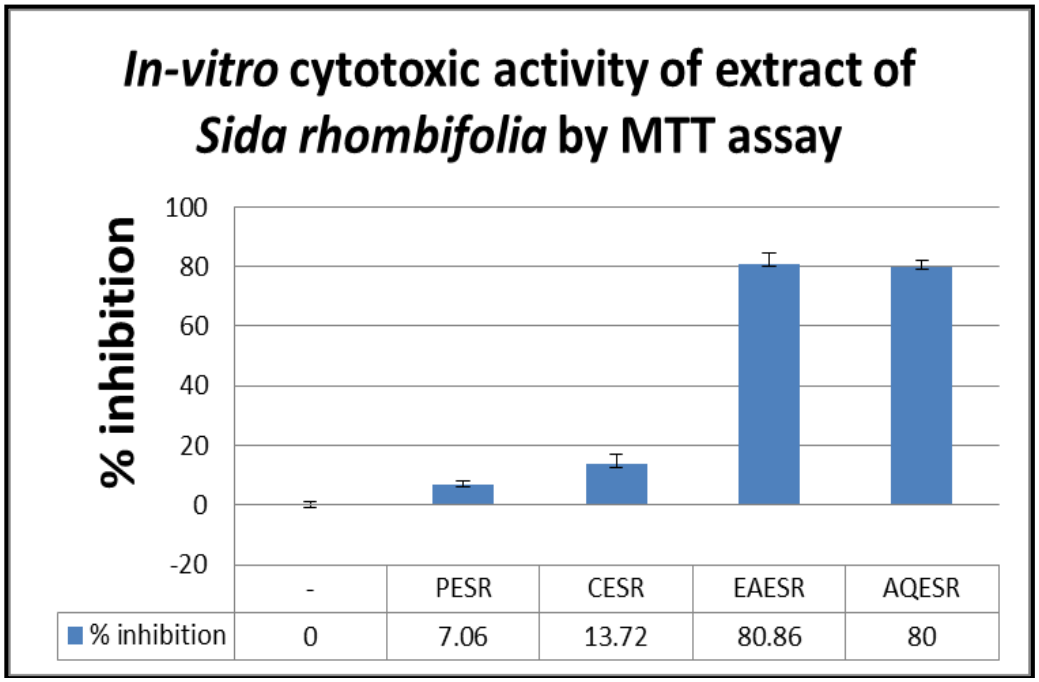

Figure 1: *In-vitro* cytotoxic activity of extract of *Sida rhombifolia* by MTT assay

Conclusion

Recent research on medicinal plants has produced an impressive range of studies on the compounds that inhibit tumors. The use of Sida rhombifolia against DAL indicates its efficacy as an anticancer agent. Various extracts of the plant were tested for in-vitro cytotoxicity against DAL cell lines. The ethyl acetate and aqueous extracts showed significant activity against the cell lines.

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