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In vitro Cytotoxic studies of Adhatoda vasica leaves on human lung adenocarcinoma cell line (A549)

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ABSTRACT:
Justicia adhatoda L Whole part methanolic extract (JAWME) is one of the traditional plants with well-known medicinal values prescribed widely to treat many diseases. As globally, we look towards the use of non-toxic plant based products with conventional medicinal usage and the developing countries depends on herbal medicines for their primary health care needs. The aim of this study is to explore the in vitro cytotoxicity of the methanol extract of *Justicia adhatoda L* Whole part (JAWME) on human lung adenocarcinoma cell line (A549) using MTT assays. The results show a significant inhibition percentage on human lung adenocarcinoma cell line (A549) was observed in both assays. In MTT assay the IC50 values was 176.2µg/ml on methanol extracts respectively. Thus, both MTT assays can be used for cytotoxic screening of leaves of JAWME towards the development of modern drug.

KEYWORDS: MTT assay, human lung adenocarcinoma cell line (A549), IC_{50} value, cytotoxic activity.

INTRODUCTION:

MTT mechanism
Since last many years, plants have beneficial activity in different type of diseases producing in human beings. As per WHO calculate that about 80% of the world's inhabitants problem should treated by medicinal herbal drug for their primary health care. Plants have long history used in the treatment of cancer. Many bioactive compounds of *Catharanthus roseus, Angelica Gigas, Podophyllum peltatum, Taxus brevifolia, Podophyllum emodii, Ocrosia elliptica*, and *Campototheca acuminata* have been used in the treatment of advanced stages of various malignancies. There are various medicinal plants reported to have anti-cancer as well as anti-inflammatory activity in the Ayurvedic system of medicine. [1,2]
Malabar nut (Justicia adhatoda L.) is belongs to the Acanthaceae family of tiny sub-herbaceous, evergreen plants. Because of its unique phytochemistry, this plant is recognised as a well-known treatment in the Ayurvedic and Unani schools of medicine. Despite being common around the world, it is most usually found in tropical areas of Burma, Malaysia, Sri Lanka, India, and south-east Asia. This plant thrives in waste places with stony, dry soil and generally low moisture levels. The main chemical components of Justicia adhatoda are vasicine and vasicinone, in addition to an essential oil. [3,4,5}The chemical composition of Justicia adhatoda shows that it contains alkaloids, polyphenolics, glycosides and phytosterols while its major constituents are quinazoline alkaloids having vasicine as its principal alkaloid. The phytochemical analysis of essential oil obtained from leaves of Justicia adhatoda showed the presence of numerous chemical constituents such as phytosterols, anthraquinones, alkaloids, polyphenols, flavonoids, saponins and triterpenoids containing N-oxides of vasicine, vasicine, maiontone and deoxyvasicine.[6,7] Due to these chemical compounds, this plant shows several biological activities such as antidiabetic, anti-bacterial, anti-

inflammatory, anti-malarial, anti-oxidant, anti-mutagenic, respiratory stimulant and bronchodilatory activities along with cardio-protective, anti-ulcer, insecticidal, allopathic, hepatoprotective and anti-cholinesterase potentials thus used in several commercial products. Therefore potential *Justicia adhatoda L* Whole part methanolic extract (JAWME) were selected for the further research work on assessment of antioxidant properties including the composition of their antioxidant components like ascorbic acid, total phenol content, total flavonoid content and wound healing potential of *Justicia adhatoda L* Whole part methanolic extract (JAWME) [8,9,10].

2. Material and Methods

2.1 Plant collection and preparation

Plant material was collected locally from medicinal garden of Veer Bahadur Singh Purvanchal University, Jaunpur-222003, India. Identified and authenticated was done by scientist-E Arti Grag, Botanical Survey of India, Central Regional Centre, Praygraj, U.P. The samples are preserved in the institutional herbarium with accession numbers *Justicia adhatoda* L. accession no. 104530 for future reference [11].

2.2 Extraction of plant material

Plant material was extracted by using cold maceration method; plant samples were collected, washed, rinsed and dried properly. Powder form of plant sample was extracted with different organic solvents (petroleum ether, ethyl acetate, and methanol) and allows standing for 4-5 days each. The extract was filtered using filter paper to remove all unextractable matter, including cellular materials and other constituents that are insoluble in the extraction solvent. Extract was transferred to beaker and evaporated; excessive moisture was removed and extract was collected in air tight container. Qualitative analysis of extracts of different solvents was carried out to find out the presence of various phytoconstituents [11,13]. Extraction yield of all extracts were calculated using the following equation below:

Percentage Yield = $\frac{\text{Actual yield}}{\text{Theoretical yield}} \times 100$

Qualitative Phytochemical Estimation of Extracts

Detailed phytochemical testing was performed to identify presence or absence of different phytoconstituents in extracts by using standard procedures**.** The extracts were subjected to following tests

Reagents

Trypan blue (Hyclone, Lot no: JRH27098), Sodium bicarbonate (MP Biomedicals, Lot No: 2048J), EDTA (MP Biomedicals, Lot No: 6941H), DPBS (Dulbecoo's phosphate buffer saline) (MP Biomedicals, Lot No: C1290), Trypsin (Invitrogen, Lot No: 1376596), MTT Salt

Cell proliferation kit

MTT (Roche applied sciences, Cat. No. 11 465 007 001) Media DMEM (Dulbecoo's Modified Eagels medium, high glucose), DMEM (Dulbecco's Modified Eagels medium, low glucose), FBS (Fetal Bovine Serum) (Bioclot, Lot No: 07310)

Glasswares and plastic wares

96-well micro titer plate, Tissue culture flasks, Falcon tubes, Reagent bottles

Equipments

Fluorescence inverted microscope (Leica DM IL), Biosafety cabinet classII (Esco), cytotoxic safety cabinet (Esco), CO2 incubator (RS Biotech, mini galaxy A), Sciences; Veer Narmad South Gujarat University, Surat by Dr. Minoobhai Parabia, Dr. Ritesh Vaidh.

Cytotoxicity Screening Cell line used:

Adenocarcinoma cell line (A549), ELISA plate reader (Thermo), Micropipettes (Eppendorff), RO water system (Millipore)

Processing of plant material:
The collected *Justicia adhatoda L.* Whole part was manually cleaned to remove course impurities and washed thoroughly with distilled water and shade dried. The dried plant materials were uniformly grinded using mechanical grinder to make a crude powder stored in airtight container for further use.

2. Microculture tetrazolium (MTT) assay

Principle
This Colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, colored formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.[14,15,16]

Preparation of culture media for cell line culture
Dulbecco's Modified Eagle Medium with High Glucose (DMEM-HG) supplemented with 10% Foetal Bovine Serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin antibiotic solution and was used for culturing MCF-7- Human Breast cancer cell line (Catalogue - ATCC® HTB-22™). Minimum essential Medium (MEM)+F12 medium supplemented with 10% Foetal Bovine Serum (FBS), 1% L-glutamine, 1% penicillin-streptomycin antibiotic solution was used to culture SH-SY5Y- Homo sapiens bone marrow neuroblast. (Catalogue - ATCC® CRL2266™). 1X Dulbecco's Phosphate Buffered Saline (DPBS), 0.25% Trypsin-EDTA solution, MTT reagent, Dimethyl Sulfoxide (DMSO), antibiotics, media etc. were all purchased from HiMedia, India.

Procedure
The monolayer cell culture was trypsinized and the cell count was adjusted to 3-lakhcells/ml using medium containing 10% newborn calf serum. To each well of 96 well microtitre plates, 0.1ml of diluted cell suspension was added. After 24 hours, when the monolayer formed the supernatant was flicked off and 100 µl of different test compounds were added to the cells in microtitre plates and kept for incubation at 37ºC in 5 % CO2 incubator for 72 hour and cells were periodically checked for granularity, shrinkage, swelling. After 72 hour, the sample solution in wells was flicked off and 50µl of MTT dye was added to each well. The plates were gently shaken and incubated for 4 hours at 37°C in 5% CO_2 incubator. The supernatant was removed, 50 µl of Propanol was added, and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 490 nm25. The percentage growth inhibition was calculated using the formula below: The percentage growth inhibition was calculated using following formula,
%cell inhibition= 100-{(At-Ab)/(AcAb)}x100
Where,
At= Absorbance value of test compound
Ab= Absorbance value of blank
Ac=Absorbance value of control

Data interpretation
Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes.
%cell survival= {(At-Ab)/ (Ac-Ab)} x100
Where,
At= Absorbance value of test compound
Ab= Absorbance value of blank
Ac=Absorbance value of control % cell inhibition= 100-cell survival

Statistical analysis

The results of the data were expressed as the mean ± standard error of 6 independent determinations in two separate experiments. Statistical data was performed by using one-way
ANOVA while Significance of result is calculated from p value.

RESULT:

The extraction of *Justicia adhatoda L* whole part was performed and yield of extract was found to be 8.1%. The colors of extracts like Pet. Ether-yellow, Ethyl Acetate- green, Methanol-green of *Justicia adhatoda L* whole part was found.

Phytochemical screening test

Phytochemical screening tests were performed using methanolic extract of *Justicia adhatoda L* whole part (JAWME) and was found to be JAWME extract contains Glycosides, Alkaloids, Amino acids, Carbohydrates, Glycosides, Phenolic compounds, Tannins, Saponins, Flavonoids, Proteins. The bioactive compounds provide semi-qualitative information on the active constituents of the extract. Phytochemical investigation of PAMW revealed the presence of diverse phytoconstituents in methanolic extract Table 1. Therefore JAWME was taken further in-vivo study.

Table 1: Results of phytochemical screening test

S. No.	Experiment	Result		
		Pet. Ether Extract	**Ethyl Acetate**	**Methanol**
Test for Carbohydrates				
+1.	Molisch's Test	-	-	+
2.	Fehling's Test	-	-	+
3.	Benedict's Test	-	-	+
4.	Bareford's Test	-	-	+
Test for Alkaloids				
1.	Mayer's Test	-	+	+
2.	Hager's Test	-	+	+
3.	Wagner's Test	-	+	+
4.	Dragendroff's Test	-	+	+
Test for Terpenoids				
1.	Salkowski Test	-	-	+
2.	Libermann-Burchard's Test	-	-	+
Test for Flavonoids				
1.	Lead Acetate Test	-	+	+
2.	Alkaline Reagent Test	-	+	+
3.	Shinoda Test	-	+	+
Test for Tannins and Phenolic Compounds				
1.	$FeCl_3$ Test	-	+	+
2.	Lead Acetate Test	-	+	+
3.	Gelatine Test	-	+	+
4.	Dilute Iodine Solution Test	-	+	+
Test for Saponins				

1.	Froth Test	+	-	-
Test for Protein and Amino acids				
1.	Ninhydrin Test	-	-	+
2.	Biuret's Test	-	-	+
3.	Million's Test	-	-	+
Test for Glycosides				
1.	Legal's Test	-	-	+
2.	Keller Killani Test	-	-	+
3.	Borntrager's Test	-	-	+

+ = Components present

The cytotoxicity activity is carried out by using MTT assay. Natural product substances have historically served as the most significant source of new leads for pharmaceutical development. Adenocarcinoma cell line (A549) were seeded and exposed to *Justicia adhatoda L.* Whole part of methanol extract extracts at 10, 20, 40, 60, 80, 160, 320 µg/ml of concentration. In MTT assay shows dose-dependent inhibition of cell proliferation in adenocarcinoma cell line (A549) by the *Justicia adhatoda L.* Whole part of methanol extract extracts (Figure 1). Table 2shows the *Justicia adhatoda L.* Whole part of methanol extract exposure demonstrated a maximum decrease in cell growth by MTT assay on Adenocarcinoma cell line (A549)of 67.20% inhibition at 300µg/ml concentration by methanolic (S1) and compared to DMSO control treatment. The IC50 value for MTT has been determined for methanolic 176.2µg/ml using Graph Pad Prism 6 software.

The reduction of tetrazolium salts is broadly believed as a consistent and reliable way to study cell proliferation. The effect of test extract on the cellular proliferation and viability were determined using 3-(4, 5Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay method. [20] Reduction of tetrazolium by dehydrogenase enzymes is character of metabolically active cell that produces NADH and NADPH. The formazan product (purple crystals) has low aqueous solubility (Fig. 1). Formazan dissolved in dimethyl sulfoxide (DMSO) was quantified and the intensity of the product color was measured at 570 nm. This was directly proportional to the number of living cells in the culture. Untreated cells (basal) were used as control of viability (100 %) and the results were expressed as % viability (log) relative to control

Table 2: Percentage of cell inhibition of *Justicia adhatoda L.* Whole part of methanol extract against human lung adenocarcinoma cell line (A549)

Compound name	Conc. µg/ml	OD at 590nm	% Inhibition	IC50 µg/ml
Control	0	0.739	0	
S1	25	0.656	11.27	**176.2**
	50	0.593	19.76	
	100	0.498	32.57	
	200	0.405	45.13	
	400	0.279	62.19	

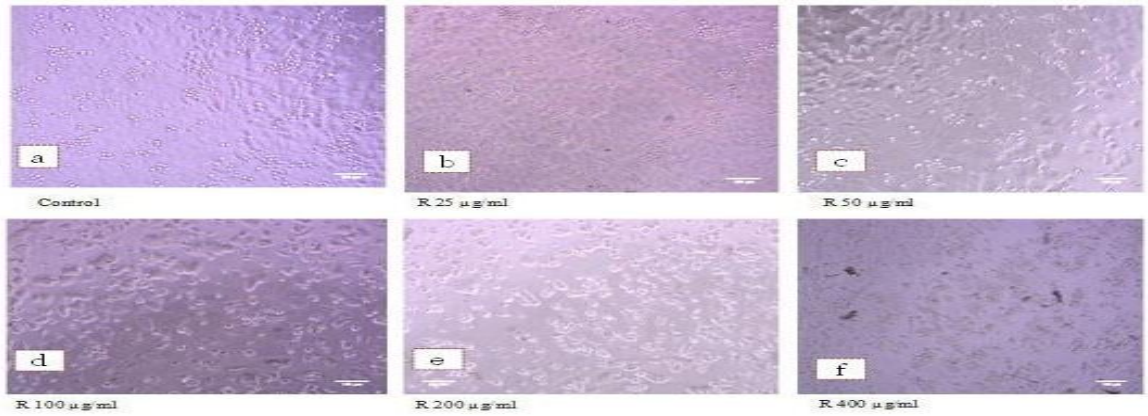

Figure 1: Percentage of cell inhibition of *Justicia adhatoda L.* Whole part of methanol extract against human lung adenocarcinoma cell line (A549)

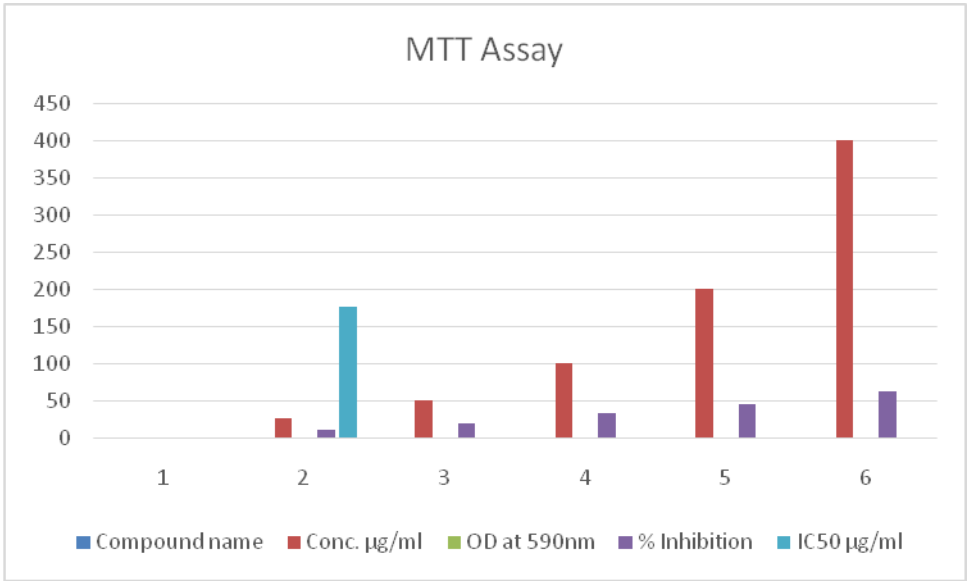

Figure 2: Graph inhibition percentage of various *Justicia adhatoda L.* Whole part of methanol extract concentrations

DISCUSSION:

Humankind have been benefitted by plants, herbs, and ethnobotanicals since early days and still used worldwide for health promotion and to treat diseases. Herbal medicines, due to less toxicity, have great demand across the globe. Natural sources and plants are the basis of modern medicine; thereby largely contribute to the preparation commercial drugs. At present the most reliable and available in-vitro screening techniques used to evaluate the anticancer activity of herbal formulations on cancer cell lines are MTT assay. The MTT is used for quantitative and for qualitative analysis, respectively. The MTT assay is colorimetry method of analysing the color reduction of reagent to estimate cell viability. It determines the cytotoxicity by mitochondrial dehydrogenase activities in living cells.

Cancer is one of the most dreadful diseases worldwide that increases at a progressive rate. Public awareness towards phytopharmaceuticals gains the importance of phytoconstituents as therapeutic agents to provide defensive mechanism. The presence of secondary metabolites effectively inhibits the growth of cancerous cells. The results will be helpful for pharmaceuticals to develop the drug from plant resource which is ecofriendly to treat the cancer patients. *Adhatoda vasica* leaves extracts having number of potential bioactive compounds. These all of potential bioactive compounds have been found to inhibit growth of many cancer cells. They act as an anti-proliferative agent against tumor cells, especially human lung adenocarcinoma cell line (A549). It plays its role by inducing apoptosis. Thymus vulgaris inhibits growth

of human breast cancer and colorectal cancer this is due to presence of phytochemicals like polyphenolic compounds.

Every system of medicine emphasized the importance of most secondary metabolites from herbal plants were used to cure enumerous diseases like diabetics, cancer, arthritis etc. Hence, phytoconstituents can continue to be used as ideal sources for anticancer drug formulations. In this study, the MTT methods were used to evaluate the cytotoxicity potential of the *Justicia adhatoda L.* Whole part of methanol extract. Results from those assays revealed that the *Justicia adhatoda L.* Whole part of methanol extract explored a very good percent of cell inhibition with increasing concentration of the bioactive component of the test material.

CONCLUSION:

We conclude that the *in vitro* studies reduce the usage of animals for clinical trials. It helps to assess the larger number of compounds with minimum quantity quickly. The findings suggest that the *Justicia adhatoda L.* Whole part of methanol extract has a potential promising novel anticancer activity against lung cancer cell line. Thus, the overall results indicate the potential use of this indigenous tree as a novel chemotherapeutic agent for the treatment of lung cancer. This study may help in opening new avenue for cancer research as well as plant-based cancer treatments which may bypass the problems incurred due to chemo or radiation therapy and higher chemical doses

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