

Cytotoxicity effect of ethanolic extract of *Mikania glomerata* against A549 human lung cancer cell line

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Abstract

The genus *Mikania* is the largest of its kind in the family *Eupatorieae* (*Asteraceae*), with more than 430 species concentrated mainly in the tropical regions, among which, *Mikania glomerata* is a most common herb generally employed in the treatment of respiratory disorders. The *Mikania glomerata* plant leaf extracts were used for anticancer study against A549 human lung cancer cell line. The solid – liquid extraction method has been employed to prepare the ethanol extract of *Mikania glomerata* through soxhelt apparatus method. To check the anti-proliferative effect of this extract, the extract chosen was tested for cell viability on the lung cancer cells A549 in different concentrations. Cell viability was evaluated by MTT assay for 24 hour and 48 hours. The LD₅₀ value was calculated for the level of cell death and its effectiveness. The dose-dependent manner plays an important role in the treatment of lung cancer cell lines. The present study suggests that *Mikania glomerata* may be used as an alternative anticancer agent and further research is needed to improve the effectiveness.

Keywords: *Mikania glomerata*, Cytotoxicity study, Ethanol extract, A549 human lung cancer cell line.

1. Introduction

For many years herbal medicines have been used and are still used in developing countries as the primary source of medical treatment. Plants have been used in medicine for their natural antiseptic properties. Thus, research has developed into investigating the potential properties and uses of terrestrial plants extracts for the preparation of potential nanomaterial based drugs for diseases including cancer [1]. Medicinal plants have been used for thousands of years in folk medicines in Asian and African populations and many plants are consumed for their health benefits in developed nations. According to the World Health Organisation (WHO) some nations still rely on plant-based treatment as their main source of medicine and developing nations are utilising the benefits of naturally sourced compounds for therapeutic purposes [2]. Compounds which have been identified and extracted from terrestrial plants for their anticancer properties include polyphenols, brassinosteroids and taxols.

Plant-derived drugs are desired for anticancer treatment as they are natural and readily available. They can be readily administered orally as part of patient's dietary intake. Also, being naturally derived

compounds from plants they are generally more tolerated and non-toxic to normal human cells [3]. The *Mikania* plant species are very important in the traditional medicines for several purposes. The pharmacological effects of *Mikania* genus are attributed mainly to the presence of coumarin (1,2-benzopyrone) and derivatives. However, other metabolites showed to produce significant pharmacological effects. Recent studies report the presence of coumarin, triterpenes/steroids, flavonoid glycosides, dihydrocoumarin, o-coumaric acid, kaurenoic acid, cinnamoylgrandifloric acid, stigmasterol, cupressenic acid, isopropiloxi-grandifloric acid, isobutiloxi-grandifloric acid, kaurenol, spathulenol, caryophyllene oxide, syringaldehyde, saponins, tannins[4-7]. Ferreira and Oliveira [8] demonstrated new constituents, which were isolated from the leaves of *Mikania* sp.: taraxerol, lupeol, transmelilotoside, cismelilotoside, adenosine, patuletin 3-O- β -d-glucopyranoside, kaempferol 3-O- β -d-glucopyranoside, quercetin 3-O- β -d-glucopyranoside, methyl-3,5-di-O-caffeoyl quinate and 3,3',5-trihydroxy-4',6,7-trimethoxyflavone. This study aims to scrutinize the cytotoxic effect of ethanolic extract of *Mikania*

glomerata plant leaves against the A549 human lung cancer cell line.

2. Materials and methods

2.1 Plant Material

The *Mikania glomerata* plant leaves were collected from Coimbatore, Tamil Nadu, India. The botanical identity of the species was authenticated at the Botanical Survey of India, Southern Regional Centre, Coimbatore.

2.2 Solvent Extraction

The *Mikania glomerata* leaves were shade-dried and coarsely powdered using a pulverizer. 1 KG of *M. glomerata* powder was successively extracted with ethanol (80°C) using Soxhlet apparatus. The solvent was removed by vacuum distillation in a rotatory evaporator at 60°C. The extract was filtered through Whatman No. 1 filter paper and concentrated on a water bath to a syrupy mass. The dried substance was dissolved in ethanol and stored in cold room for future use.

2.3 A549 Human lung cancer cell line

The A549 lung cancer cell line was obtained from the American Type Culture Collection (Rockville, MD). This is a hypotriploid human cell line with the modal chromosome number of 66, occurring in 24% of cells. Cells with 64 (22%), 65, and 67 chromosome counts also occurred at relatively high frequencies; the rate with higher ploidies was low at 0.4%. There were 6 markers present in single copies in all cells. They include der(6)t(1;6) (q11;q27); ?del(6) (p23); del(11) (q21), del(2) (q11), M4 and M5. Most cells had two X and two Y chromosomes. However, one or both Y chromosomes were lost in 40% of 50 cells analyzed. Chromosomes N2 and N6 had single copies per cell; and N12 and N17 usually had 4 copies. A549 cells could synthesize lecithin with a high percentage of desaturated fatty acids utilizing the cytidine diphosphocholine pathway. The cells are positive for keratin by immunoperoxidase staining. The base medium for this cell line was ATCC-formulated RPMI-1640 Medium, Catalog No. 30-2001. To make the complete growth medium, the following components was added to the base medium: fetal bovine serum to a final concentration of 10%. Temperature: 37°C; Atmosphere: air, 95%; carbon dioxide (CO₂), 5%. The culture medium was removed and discarded. The cell layer was briefly rinsed with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contain trypsin inhibitor. 2.0 to 3.0 mL of Trypsin-EDTA solution was added to flask and cells were observed under an inverted microscope until cell layer was dispersed (usually within 5 to 15 minutes). 6.0 to 8.0 mL of complete growth medium was added and the cells were aspirated by gently pipetting. Appropriate

aliquots of the cell suspension were added to new culture vessels. The cultures were incubated at 37°C.

2.4 Cell proliferation assay

All cells were grown as described earlier. For cell proliferation effects, four different concentrated samples (10, 20, 30, 40 µg/mL) were seeded at 20,000 cells per flask. All cells were grown for 24 hours and cell counts were taken. The cell lines study was used to screen the activity of ethanolic extract of *Mikania glomerata* leaves against for A549 human lung cancer cell line.

2.5 Cells counting using haemocytometer

Assessment of cell viability and distinction of cell types was carried using Neubauer counting chamber. Cover-slip was moistened and affixed to the haemocytometer. Care was taken to ensure that the cover-slip and haemocytometer were clean and grease-free. Alcohol was used for cleaning purpose. A small amount of trypan blue-cell suspension was transferred to one of the chambers of the haemocytometer by carefully touching the cover slip at its edge with the pipette tip and allowing each chamber to fill by capillary action. Overfilling or under filling of the chamber was avoided.

2.6 Determination of the number of cells (total and viable)

The cells were viewed under a microscope at 100x magnification. Focusing was done to see a grid of 9 squares. Then the microscope was focused on one of the 4 outer squares in the grid. Adjustments were made so that the square contained 16 smaller squares. Cell counting was carried in the four 1 mm corner squares. Procedure was repeated based on either concentration or dilution. Trypan Blue is the "vital stain"; excluded from live cells. Live cells appear colourless and bright (refractile) under phase contrast. Dead cells stain blue and are non-refractile. The cells were counted as per the procedure illustrated. 4 corner squares were counted and the average was calculated. Each large square of the haemocytometer, with cover-slip in place, represents a total volume of 0.1 mm³ (1.0 mm X 1.0 mm X 0.1 mm) or 10⁻⁴ cm³. Since 1 cm³ is equivalent to approximately 1 ml, the total number of cells per ml was determined using the following calculations:

$$\% \text{ Cell Viability} = [\text{Total Viable cells (Unstained)} / \text{Total cells (Viable + Dead)}] \times 100.$$

$$\text{Viable Cells/ml} = \text{Average viable cell count per square} \times \text{Dilution Factor} \times 10^4$$

$$\text{Average viable cell count per square} = \text{Total number of viable cells in 4 squares} / 4.$$

2.7 Assessment of cell density and evaluation of viable cells

The samples were diluted to appropriate concentrations and the absorbance of the sample was

measured by means of spectrophotometer at 550 nm. Consistent wavelength must be used while measuring the cell density of a particular cell line. An independent calibration curve was generated for each cell line [9]. After spectrophotometer analysis, actual counting of cells was executed using counting chamber.

2.8 Fluorescence assay for cell cycle distribution

At the end of 24 hours after treatment, the cells were smeared on to glass microscope slides, fixed with methanol and air dried. The cells were then stained with propidium iodide (0.1 µg/mL) and scored under fluorescence microscope fitted with PI filter. A total of 100 cells were scored for intensity of DNA staining in the nucleus. Initially the slides were scored for determining the 1x fluorescence intensity using intensity tool from Adobe Photoshop. This was determined by counting the cells and the majority of cells will fall into this category. This is the G1 cells fluorescence intensity. Based on this intensity other phases of cell cycle were determined

2.9 Clonogenic survival assay

The clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone. This cell is then said to be clonogenic. For cell proliferation effects, cells were seeded at 20,000 cells per flask. Three flasks were used per concentration. All cells were grown for 24 hours. The various treatment conditions on A549 human lung cancer cell line with different concentration of ethanolic extract of *Mikania glomerata*. At the end of 24 hours' treatment, the cells were removed and plated on to separate flasks. Trypsinize the stock flask of cells containing the cells that have to be tested. Make sure that the cells are in single-cell suspension and obtain an accurate cell count. The cells were counted using a hemocytometer. Using a Pipette, add 20,000 cells (the cell number can vary depending on the cell type) to the 5 mL of medium in each T-25 flask. Shake gently to distribute the cells evenly. The flasks were then incubated for 12-14 days at 37°C. The flasks were then stained with Gentian violet and scored for number of colonies.

Clonogenic assay serves as a useful tool to test whether a given cancer therapy can reduce the clonogenic survival of tumor cells. A colony is defined as a cluster of at least 50 cells which can often only be determined microscopically. Clonogenic assay is the method to determine cell reproductive death after treatment with ionizing radiation, but can also be used to determine the effectiveness of other cytotoxic agents. The following protocol has been modified from a published version[10].

2.9.1 Apoptosis

Apoptosis induction was determined by measuring Annexin V activity using Annexin V apoptosis kit. The kit was sourced from Promega corporation, Southapton, UK and consisted of Annexin V buffer and lyophilized Annexin V substrate. Cells were seeded at the concentration of 10^4 cells per well in 96 well microtitre plate and incubated for 48 hours. Aliquot of 100 µL of fresh media containing appropriate concentration of extracts were transferred to the assigned respective wells. Staurosporine 0.1 µg/mL was used as positive control and untreated wells were treated as negative control. Total of six wells were assigned for each treatment. The plate was allowed to equilibrate to room temperature after 24 hours of incubation prior to performing the assay. 100 µL of Annexin V reagent was added to each well and mixed for 60 seconds and incubated for further 1 hour at room temperature. An aliquot of 100 µL of contents from each well was transferred to white-walled 96 well plate. The light emitted was measured by Packard lumiscount microplate luminometer and measurement was recorded using THERMOmax™ plate reader linked to a computer using SoftMax Pro software. The results are depicted in the tabular form.

2.9.2 MTT assay

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a yellow tetrazole), is sliced by mitochondrial dehydrogenase of viable cells, resulting in a purple formazan product that could be measured spectrophotometrically. The formazan product formed is in proportion to the number of viable cells and inversely proportional to the amount of cytotoxicity. The absorbance of formazan product can be measured at a wavelength between 510 and 600 nm using a spectrophotometer [11-13]. The positive control treatment consisted of 1 % phenol. Positive control reagents are chemicals known to bring a standard positive response in a test system. The positive control chemical is used to show that the test system is responding satisfactorily and that the test is valid. The negative control used consisted of the sterile distilled water. Qualitative and quantitative evaluations of viability of cells were determined using MTT assay. Cells were seeded at the density of approximately 1×10^5 cells per well in 96 well plates in 100 µL medium. After an incubation of 48 hours' culture medium was removed and replaced with aliquots of 100 µL of medium containing appropriate concentration of extracts particles. 1 mg/mL of phenol was treated as positive control and untreated cells served as negative control. Total of six wells were assigned for each treatment. The cells were treated with the test material and positive control for 24 hours.

Following the incubation period the cells were examined microscopically to assess the morphological alterations indicative of qualitative evaluation of toxicity. A 100 μ L of MTT reagent was added to each well and mixed at least for 60 seconds and the plates were incubated for approximately 4 hours. 100 μ L of Dimethyl Sulfoxide (DMSO) was transferred to each well to lyse the cells. Absorbance of the lysate was measured at 560 nm to determine the quantitative evaluation of cytotoxicity using the THERMOmax™ plate reader linked to a computer using SoftMax Pro software.

The culture medium was removed from the plate and the wells were washed with phosphate buffered saline. Aliquots (100 μ L) of the culture medium containing appropriate concentrations of the test article, positive controls, and negative control were applied to the plate wells. Plate was incubated at approximately 37°C in a humidified atmosphere of 5% (v/v) CO₂ in air for approximately 24 hours. Following the incubation period, the cells on the plate was examined microscopically to assess any morphological alterations indicative of toxicity. All measurements were recorded using the THERMOmax™ plate reader linked to a personal computer using SoftMax Pro software. Programming of the plate reader included setting up a template to which blanks, controls, empty and test article wells were assigned. The raw data were exported and analysis performed using Microsoft Excel. Statistical analysis of Mean and standard deviations (SD) were calculated and viability of test item treated cultures was compared to the negative control cultures. The assay was normally considered acceptable for the evaluation of the test results valid if the following criteria were met: No significant cytotoxicity was observed in the negative controls. The positive control compound induces a statistically significant cytotoxicity compared to the negative controls. The test extract is declared as a cytotoxic

substance if, there was a noticeable increase (>50%) in cytotoxicity even after normalization to the negative control.

3. Results and Discussion

The genus *Mikania* sp. is among the list of best-selling natural products in the world because it's biological activity. Different classes of compounds were previously isolated from *Mikania* sp., which can be associated to the pharmacological potential observed and related to the plant. Different compounds were search in extracts using a variation of solvent polarity [hexane, chloroform, ethyl acetate ethanol and ethanol/water (1:1)]. Therefore, all compounds identified in extracts of *Mikania* sp. by both techniques are important because many have proven to induce antitumor activity and may also act synergistically. Coumarin was identified in all extracts. Studies in various tumor cell lines have pointed to coumarin and its derivatives as potential substances for cancer treatment [14-18]. Other compounds with antitumor activity were identified in the extracts analyzed: curcumene, psoralen, kaurenoic acid, scopoletin, o-coumaric acid, pinene [19-23].

3.1 Cell proliferation assays

Initial cell counts were done in the triplicate culture vessels (Mean value of 20,429 cells) and 24hrs counts were made to ensure cell proliferation in the taken test cell line (Mean value of 52,051 cells) (Table 1). Control cell lines with a mean value of 46,585 cells were arranged without the test material to evaluate the inhibitory effect. A significant reduction in the number of cells in the culture flasks after exposure to 20 μ g/ml, 30 μ g/ml and 40 μ g/ml of alcoholic extract of test species with respective mean values of 29,907, 31,165 and 30,644 were observed. The results indicated the anti- cell proliferative effect of alcoholic extract of *Mikania glomerata* on A459 cells.

Table 1: Cell proliferation assays (A459 cells) in the presence of ethanolic extracts of *M. glomerata*

	Cell counts per flask				
	Culture 1	Culture 2	Culture 3	Mean	SD
Initial cell count	20291	20214	20782	20429	308
24 hr counts	50116	52974	53064	52051	1677
Control	45688	45856	48211	46585	1411
10 μ g/ml	54831	48397	33472	45567	10957
20 μ g/ml	20067	32528	37126	29907	8826
30 μ g/ml	24273	35464	33757	31165	6029
40 μ g/ml	34033	20493	37406	30644	8951

3.2 Cell cycle distribution analysis

Cell cycle distribution pattern of A459 human lung cancer cell line in the presence of alcoholic extracts of *M. glomerata* are the follows. (a) The cells in the quiescent state (PreG1 or Go) has increased in

30 μ g/ml (24 hr) and 40 μ g/ml (24 hr) of the extract, when compared to the control. (b) No significant change in Gap 1 (G1) phase in all the test doses of the extract. (c) A marginal decrease in synthesis phase (S) was observed in the distribution of cells in 30 μ g/ml

(24 hr) and 40 µg/ml (24 hr), when evaluated against the control. A slight decrease in Gap 2/ Mitosis (G2/M) phase was observed in 10 µg/ml (24 hr) and 20 µg/ml (24 hr). A 78 % and 65% drop in G2/M phase was noticed in 30 µg/ml (24 hr) and 40 µg/ml (24 hr) respectively (Table 2).

Table 2: Cell cycle distribution analysis (A459 cells) in the presence of ethanolic extracts of *M. glomerata*

	Distribution of cells in various cell cycle phases			
	Pre G1	G1	S	G2/M
Distribution at seeding	1.38	74.10	8.96	15.56
Control (24 hr)	1.86	72.30	4.20	21.65
10 µg/ml (24 hr)	1.93	75.09	5.54	17.45
20 µg/ml (24 hr)	1.02	76.23	4.77	17.99
30 µg/ml (24 hr)	2.95	83.61	7.30	6.14
40 µg/ml (24 hr)	3.12	80.00	9.27	7.61

3.3 Clonogenic survival assay

The clonogenic cell survival assay determines the capacity of a cell to proliferate indefinitely, thereby maintaining its reproductive ability to form a large colony or a clone (Figure 1). The test was conducted in triplicate culture plates with A459 human lung cancer cell line using alcoholic extract of *Mikania glomerata* and the results were observed after 24hrs of insult in graded concentrations (10 µg/ml, 20 µg/ml, 30 µg/ml and 40 µg/ml) (Table 3). A replica of culture

plates without the alcoholic extract of *Mikania glomerata* was prepared to serve as control with a mean value 72 clones. The assay exhibited a considerable decrease in clone formation in the 30 µg/ml dose of the extract exhibiting a mean value of 40 clones. A mean of 71% reduction in clone formation was noticed in 40 µg/ml concentration of the extract. The alcoholic extract of *M. glomerata* in higher dosage significantly affected colony formation in A459 cells.

Figure 1: Clonogenic survival assays in the presence of extracts of *M. Glomerata*

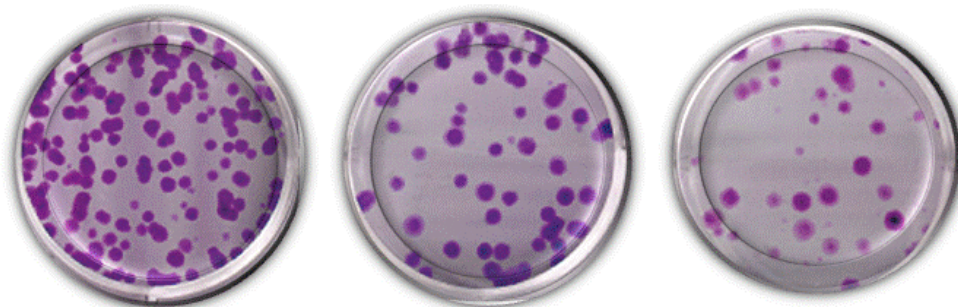


Table 3: Clonogenic survival assays (A459 cells) in the presence of ethanolic extracts of *M. glomerata*

	Colonies per plate				
	Culture 1	Culture 2	Culture 3	Mean	SD
Control (24 hr)	91	55	69	72	18.1
10 µg/ml (24 hr)	48	93	58	66	23.6
20 µg/ml (24 hr)	72	70	67	70	2.5
30 µg/ml (24 hr)	57	41	23	40	17.0
40 µg/ml (24 hr)	11	40	13	21	16.2

3.4 Apoptosis study

Apoptosis, or programmed cell death, is a normal physiologic process for removal of unwanted cells. This event was detected on A459 human lung cancer cell line using fluorochrome-labeled Annexin V after 24 hr exposure of alcoholic extracts in graded concentrations (Table 4). The Phosphatidylserine (PS) levels (RFUs) in cells from the untreated controls served as a baseline indicator for normal Phosphatidylserine levels. The cultures treated with the

positive control, staurosporine, 0.1µg/mL produced a significant increase in Relative Fluorescence Unit (RFU), indicating release of Phosphatidylserine to the cell surface and thereby demonstrating the initiation of apoptosis. The results exhibited a significant apoptosis activity following treatment with 10, 20, 30 and 40 µg/ml (24 hr) of alcoholic extract of *Mikania glomerata*. From the experiment, it is understood that the investigated material in alcoholic solvent induced apoptosis in A459 cells.

Table 4: Apoptosis using Annexin V levels (A459 cells) in the presence of ethanolic extracts of *M. glomerata*

	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Mean	SD
Untreated	2423	2123	2563	2500	2332	2383	2387	153
10 µg/ml (24 hr)	4273	4543	4268	3902	4373	4094	4242	222
20 µg/ml (24 hr)	4025	4218	4091	4181	4527	4279	4220	175
30 µg/ml (24 hr)	4508	4140	4129	4432	4470	4242	4320	171
40 µg/ml (24 hr)	4514	4253	4399	4113	4421	4483	4364	153
Staurosporine 0.1 µg/mL	4687	5204	4594	5467	5380	4718	5008	386

3.5 Cytotoxicity using MTT assay

The optical density (OD) of untreated cells had a mean value of 0.836 ± 0.09 (Table 5). The positive control (phenol 1%) had a mean OD value of 0.207 ± 0.04 indicating the validity of the experiment. Exposure of cultures to the extract for 24hrs in the concentrations 10 µg/ml, 20 µg/ml, 30 µg/ml and 40

µg/ml had a mean OD value of 0.815 ± 0.09 , 0.797 ± 0.06 , 0.86 ± 0.11 and 0.64 ± 0.04 respectively demonstrating significant cytotoxicity in the higher tested doses. The results thus proved the cytotoxic nature of *M. glomerata* extract in alcoholic form in higher concentration to A459 cells.

Table 5: Cytotoxicity using MTT assay (A459 cells) in the presence of ethanolic extracts of *M. glomerata*

	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Mean	SD
Untreated	0.9222	0.7827	0.7879	0.8798	0.7027	0.9383	0.836	0.092
10 µg/ml (24 hr)	0.7965	0.7391	0.7495	0.8355	0.784	0.9858	0.815	0.09
20 µg/ml (24 hr)	0.7438	0.8013	0.8976	0.8132	0.7108	0.8171	0.797	0.065
30 µg/ml (24 hr)	0.8282	0.7068	0.7533	0.9398	0.9765	0.9568	0.86	0.114
40 µg/ml (24 hr)	0.5247	0.6808	0.7901	0.7099	0.6755	0.5039	0.647	0.111
Phenol 1%	0.2329	0.2075	0.1403	0.1735	0.1052	0.2077	0.178	0.048

4. Conclusion

The results of anticancer effect of ethnolic extract of *Mikania glomerata* have explained that the extracts actively inhibit the proliferation of A549 human lung cancer cell line with moderate extent. This study can be indicated that the primary and secondary metabolites of ethnolic extract of *Mikania glomerata* play an important role in the cytotoxic study. Further studies in future including other tumor cell lines to be scrutinized its inhibitory level with different solvent extracts of *Mikania glomerata*.

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