

## EVALUATION OF CYTOTOXIC POTENTIAL OF DIGERA MURICATA LEAF EXTRACT ON LUNG CANCER CELL LINE

**Running Title:** Assessment on cytotoxic activity of ethanolic leaf extract of *D.muricata* on lung cancer cell line.

### ABSTRACT

Lung cancer is the second most frequent cancer, accounting for one out of every five male cancers and one out of every nine female cancers. Treatment for lung cancer is determined by the disease's cell type, the extent to which it has spread, and the patient's overall health. It is common knowledge that tumours impart resistance to chemotherapeutic medicines or radiation in part owing to apoptotic pathway dysfunction in cancer cells. *Digera muricata* (*D.muricata*) has been used as medicinal remedies for various ailments due to its antioxidant, anti-inflammatory, anti-bacterial, anti-tumor activity. The objective of the study was to examine the cytotoxic activity of ethanolic leaf extract of *D.muricata* on lung cancer cell lines. The cytotoxic potency of *D.muricata* leaf extract was carried out by MTT assay against the lung cancer cell line (A-549). Different concentrations of *D.muricata* ethanolic leaf extract (25-150 $\mu$ g) were treated for 24h. Furthermore, the morphological changes were analysed using phase contrast microscopy. Pro-apoptotic and nuclear morphological changes in *Digera muricata* ethanolic leaf extract treated cells were examined using DAPI staining. The ethanolic leaf extract of *D.muricata* showed the dose dependent cytotoxic potency against the A549 cell line which confirmed with greater morphological changes upon 24 hrs treatment. The MTT assay clearly showed that the *D.muricata* treatment has significantly reduced the cell viability when the concentration was increased for 24hrs. We observed IC-50 dose at 50  $\mu$ g/mL concentration. DAPI staining clearly showed condensed chromatin and fragmented nuclei in treated lung cancer cells. All these results clearly showed that ethanolic extract of *Digera muricata* treatment significantly inhibited the cell proliferation and induced apoptosis in lung cancer cells.

**Keywords:** *Digera muricata*, Lung cancer, Cytotoxicity, Apoptosis.

## INTRODUCTION

With the increasing demand for natural alternatives to medical care, more plants are being studied world wide for various medicinal benefits (1). It is thus important that plants with therapeutic benefits are documented. Health care professionals and organizations are developing new methods and medicines abiding by the legacy of traditional medicine to replace the chemical treatment otherwise provided (2,3). The adverse effects of long term drugs is the fulcrum sliding people to searching for alternatives such as natural or traditional medicine (4,5). *Digera muricata* is one such plant with various medicinal properties. *Digera muricata* is also known as latmahuria. It is a wild and edible plant belonging to the Amaranthaceae family. Different parts of the plant are known to serve different functions (6). Urinary discharges are treated with the seeds and flowers (7). It is also used as an astringent and laxative for the bowels. The roots of the herb are boiled and infused into water. This infusion is given to new mothers to improve lactation. The herb is most commonly used for renal disorders in folk medicine. There is also evidence to show that the free radicals cause injuries to the kidneys (8). *D.muricata* is used to treat carbon tetrachloride induced toxicity (9).

Research on new medication to treat lung cancer is done using lung cancer cell lines. There is a current collection of more than 200 lung cancer cell lines. This collection was possible because of a systemic approach to initiate and characterise cell lines from small and non-small cell lung cancer. The lung cancer cell lines are widespread and are at ready disposal for use by any researcher. This allows multiple researches to be performed and helps catalyse a vast number of biomedical discoveries. There are major similarities between lung tumors and lung cancer cell lines (10). The main purpose for research using lung cancer cell lines is due to the sharp increase in lung cancer cases over the century. Lung cancer is the most common cause of death due to

cancer among women and men worldwide. The main causative or risk factors for lung cancer are smoking, asbestos, carbon inhalation and other occupational hazards like radon and environmental tobacco smoke (10). In India, it is the second most common type of cancer which can be fatal to both males and females. Unlike other cancer diagnoses, lung cancer is usually detected in the later stages and is usually associated with distant metastasis among both males and females as of the statistics from 2020 (11).

Molecular and cellular pathogenesis have been studied in detail over the past few decades. It has been deduced that the changes of tumor suppressor genes and abnormal proto-oncogenes play a role in angiogenesis and development of lung cancer. Theories also suggest molecular abnormalities leading to respiratory lesions. It is predicted that new pathways and targets may be discovered in lieu of the complex genetic involvement for development of lung cancer (12). This calls for newer clinical assessment methods and newer treatment options. New generation diagnosis for lung cancer involves the identification of early metabolic markers for early detection of lung cancer. In the near future, software tools like genomics, imageology, metabolomics need to be developed for analysing the mechanism of lung cancer (13). Cancer is a lethal condition and needs holistic and systemic care to enter remission. Thus, preventive measures must be strictly followed and early cancer markers must be easily identified. Chemotherapy leads to various deleterious side effects and may also be a risk factor for future cancer. Due to such effects, popularity for natural cure and medication has recently increased (14). Many plant concentrates have been discovered to have cytotoxic effects against various cancer cells (15). Finding the most efficient plant extract for the specific cancer cell lines is of utmost importance (16,17). Our team has extensive knowledge and research experience that has translated into high quality publications (18–38). Therefore, the present study is aimed to check for the cytotoxic effect of ethanolic leaf extract of *D.muricata* against lung cancer cell lines(39) (40) (41) (42).

## **MATERIALS AND METHODS**

### **Reagents:**

DMEM (Dulbecco's Modified Eagle Medium), Phosphate Buffered Saline (PBS), Trypsin-EDTA, Fetal bovine serum (FBS), were purchased from Gibco, Canada. DAPI, Dimethyl sulfoxide (DMSO), [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), were purchased from Sigma Chemical Pvt Ltd, USA. All other chemicals used were extra pure of molecular grade and were purchased from SRL, India.

### **Cell Line maintenance**

Lung cancer cell lines (A549) were obtained from the NCCS, Pune. The cells were grown in T25 culture flasks containing DMEM supplemented with 10% FBS and 1% antibiotics. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Upon reaching confluency, the cells were trypsinized and passaged.

### **Preparation of the herbal extract**

Leaf of *D.muricata* obtained from IMPCOPS (Chennai, India) was used for the present study. About 50g of *D.muricata* powder was soaked in 500 mL of 95% ethanol and kept at room temperature for 3 days in a static condition. Then the solution was filtered with crude filter paper followed by whatmann paper. Fine filtrate was subjected to rota evaporation after that 3g of the material was obtained. The total ethanol extract was concentrated in a vacuum evaporate and immediately stored at 4°C.

### **Cell viability (MTT) assay**

The cell viability of plant extract treated A549 cells was assessed by MTT assay. The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. A549 cells were plated in 48 well plates at a concentration of  $2 \times 10^4$  cells/well 24 hours after plating, cells were washed twice with 500µl of serum-free medium and starved by incubating the cells in serum-free medium for 3 hours at 37°C. After starvation, cells were treated with different concentrations of *D.muricata* ethanolic leaf extract (25 - 150µg) for 24 hours. At the end of treatment, the medium from control and treated cells were discarded and 200µl of MTT containing DMEM (0.5 mg/ml) was added to each well. The cells were then

incubated for 4h at 37°C in the CO<sub>2</sub> incubator. The MTT containing medium was then discarded and the cells were washed with 1x PBS. The crystals were then dissolved by adding 200µl of solubilization solution and this was mixed properly by pipetting up and down. Then the formazan crystals formed were dissolved in dimethyl sulfoxide (200µl) and incubated in dark for an hour. Then the intensity of the color developed was assayed using a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed as the percentage of control cells cultured in serum-free medium. Cell viability in the control cells without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells]×100.

### **Morphology study**

Based on MTT assay we selected the optimal doses (IC-50: 50µg/ml) for further studies. Analysis of cell morphology changes by a phase contrast microscope. 3×10<sup>4</sup> cells were seeded in 6 well plates and treated with *D.muricata* (50µg/ml) for 24h. At the end of the incubation period, the medium was removed and cells were washed once with a phosphate buffer saline (PBS pH 7.4). The plates were observed under a phase contrast microscope.

### **Determination of nuclear morphological changes of cells (DAPI staining)**

For the nuclear morphological analysis, after the treatment period, the monolayer of cells was washed with PBS and fixed with 3% paraformaldehyde for 10 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature and incubated with 0.5µg/ml of DAPI for 5 min. The apoptotic nuclei (intensely stained, fragmented nuclei, and condensed chromatin) were viewed under a fluorescent microscope.

### **Statistical analysis**

All data obtained were analyzed by Student's-t-test using MS-Excel, represented as mean ± SD for triplicates. The level of statistical significance was set at p<0.05.

## **RESULTS**

### **Effect of *D.muricata* leaf extract on the cell viability of lung cancer cell line**

MTT assay was used to assess the cytotoxic potential of the leaf extract of *D.muricata* on the lung cancer cell lines. Various concentrations of the extract (25-150 µg/ml) were tested against the lung cancer cell line for 24 hours. The results of the assay suggested that there was a significant decrease in the viability of lung cancer cell lines (A-549) when treated with *D.muricata* leaf extract when compared to the control for 24 hours. The viability of the cells gradually decreased with increasing concentration of the leaf extract. It was observed that 50% growth inhibition occurred at a concentration of 50 µg/ml. Thus, the IC-50 dose considered for further experiments was 50 µg/ml. (Figure1)

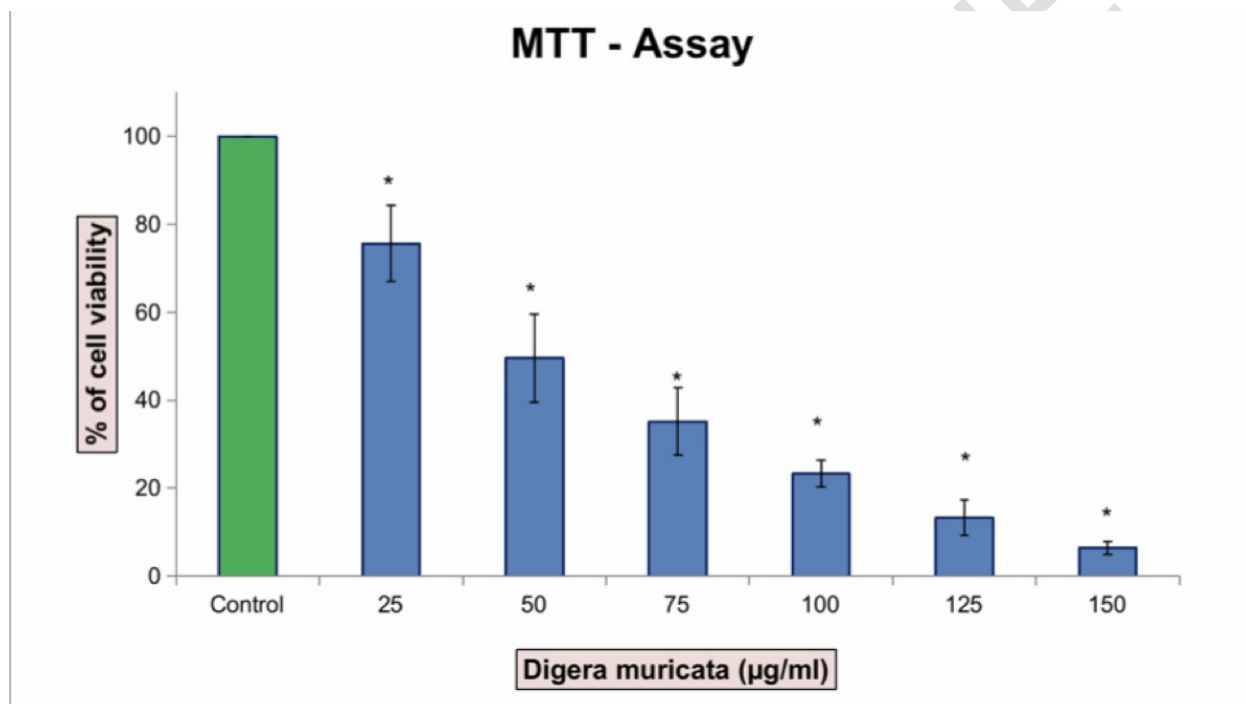
### **The effect of *D.muricata* leaf extract on cell morphology**

Phase contrast microscopy was used to analyse the changes in cell morphology of the lung cancer cell lines when treated with *D. muricata* leaf extract. The changes in cell morphology among the lung cancer cell lines treated with 50 µg/ml concentration of *D.muricata* leaf extract was compared with the control group for 24 hours. The results suggest that the treated cells showed characteristic changes similar to that of apoptotic cells- shrinkage of cells with reduced cell density. Some cells also showed other changes much like that of apoptotic cells like cell rounding and loss of contact with adjacent cells. Some cells detached themselves from the base of the plates (Figure 2).

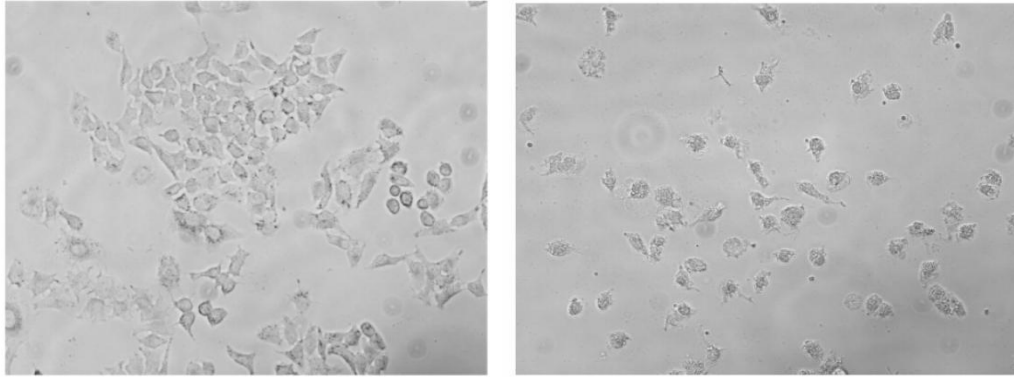
### **Pro-apoptotic effect of *D.muricata* leaf extract in lung cancer cell lines (DAPI staining)**

DAPI staining is used to analyse the induction of apoptosis among the lung cancer cell lines treated with *D.muricata* leaf extract (50 µg/ml) after 24 hours. Fluorescence microscopy was

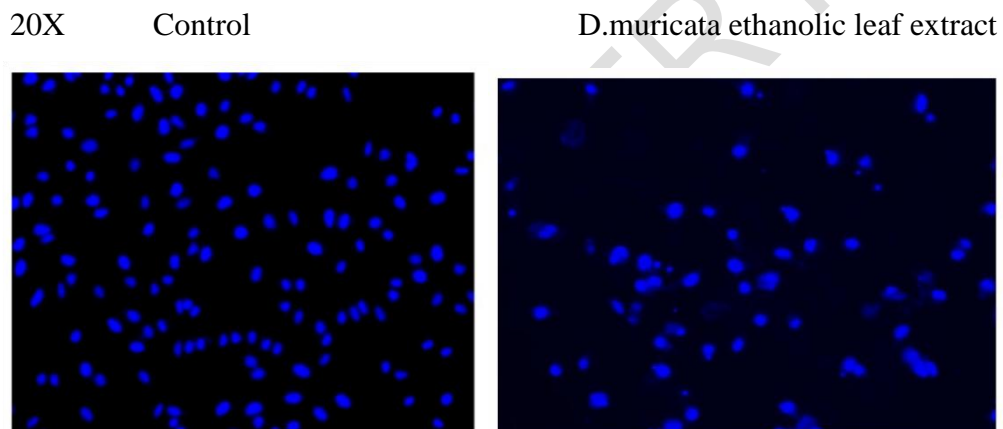
used to check the pro-apoptotic effect post staining. The results suggested that the cancer cell lines treated with the leaf extract showed condensation of chromatin along with nuclear fragmentation. These changes are suggestive of characteristic apoptotic activity when compared to the control group which had round nuclei (Figure 3).



**Figure 1:** The cytotoxic effects of *D.muricata* on A549 cells was determined by MTT assay. The Cells were treated with different concentrations (0, 25, 50, 75, 100, 125 and 150 µg) for 24hrs. The 50% of inhibition observed in concentration of 50 µg/ml, ( $p$  value: 0.0215) which has been taken as  $IC_{50}$  value and fixed for further experiments. Data are shown as means  $\pm$  SD ( $n = 3$ ). \* compared with the control-blank group,  $p < 0.001$



**Figure 2:** Represents the morphological changes in lung cancer cell line upon without and with treatment of *D.muricata* at 50  $\mu\text{g}/\text{mL}$  for 24hrs by phase contrast microscope at 20x magnification



**Figure 3:** Induction of apoptosis in *Digera muricata* leaf extract treated lung cancer cell line. The nuclei were stained with DAPI staining and observed under a fluorescence microscope. The treated cells clearly showed condensed chromatin and nuclear fragmentation

## DISCUSSION

Based on the MTT assay, it can be inferred that there was a significant cytotoxic activity of the ethanolic extract of *D.muricata* against lung cancer cell lines. The cytotoxic potential of the ethanolic extract was found to be statistically significant in comparison with the control group. It was identified that the inhibitory concentration for this ethanolic extract of *D.muricata* is 50µg/ml. The cytotoxic potential of this plant extract may be owing to the presence of substances known as flavonoids, present in the ethanolic extract. The flavonoids are composed of mono or poly phenolic groups. These phenolic groups dispense the cytotoxic potential to flavonoids (43). The results obtained from this study are supported by the results from previous studies. A study done by Mehwish et al compared the various bioactivities of different plant extracts. It was found that *D. muricata* had the most concentration of flavonoid content and thus best cytotoxic activity (44). A similar study conducted in Bangladesh checked the various bioactivities of *D.muricata* and concluded that apart from its antidepressant, anti diarrhea, analgesic activities, it also has good cytotoxic potential (45).

The cell morphology test compared the differences in cell morphology of the control and the cell morphology of the cell lines treated with the ethanolic extract of *D.muricata*. It was observed that the number of cells decreased after treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing. Previous studies also analysed the changes in cellular morphology of the cancer cell line. Their studies also confirmed similar cellular changes with increased free floating cells (46,47). The DAPI staining apoptosis test result suggests that there was condensation of chromatin and nuclear fragmentation which occurred owing to the process of apoptosis (48). These changes were observed under fluorescent microscopy. These results obtained in our study are supported by previous similar studies done. Most anticancer effects are based on apoptotic activity of the extracts or the chemotherapeutic agents (49,50). However, future *in vivo* studies need to be performed for better understanding and usage of the extract as a cytotoxic agent.

## **CONCLUSION**

Overall, the present study results demonstrated that the plant extracts of *D.muricata* were cytotoxic and induced apoptosis to the cancer cells at 50µg/ml concentration for 24h incubation period(34) (51) (52) (53) (54) (55) (56) (57) (58) (59) (60) (61) (62) (63) (64). However more research is needed to understand the mechanisms of cytotoxicity of this plant extract.

#### **COMPETING INTERESTS DISCLAIMER:**

**Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.**

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