Anti-proliferative Activity of Crude Extract and Fractions Obtained from *Digera muricata* on HeLa Cell Lines of Human Cervix and A<sub>549</sub> Cell Lines of Human Lung

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ABSTRACT

**Aim:** *Digera muricata* (Linn.) of family Amaranthaceae is an ethanobotanically important plant species traditionally used against various disorders. **Materials and Methods:** Cytotoxic potential of methanolic extract and its fractions were investigated against HeLa and A<sub>549</sub> cell lines. Crude extract of *D. muricata* was prepared in methanol by continuous hot soxhlation technique. Crude extract was fractionated into two organic and one aqueous fraction by the help of column chromatography. 3-(4,5-dimethylthiazol-2yl)-2,4 diphenyltetrazolium bromide assay was used to evaluate the reduction of viability of the cancer cell lines. Cell viability was inhibited by crude extract of *D. muricata* in a dose dependent manner ranging from 25 µg/ml to 250 µg/ml. **Results:** Apoptosis assays using nucleic acid stains namely propidium iodide (PI) exclusion assay and hoestch/PI assay were performed by the help of fluorescence microscopy. Morphological analysis was done by calculation of apoptotic ratio and percentage apoptosis. **Conclusion:** Our results suggest that methanolic and aqueous fraction of the extract of *D. muricata* can be a good source of cytotoxic compounds.

**Keywords:** 3-(4,5-dimethylthiazol-2yl)-2,4 diphenyltetrazolium bromide assay, A<sub>549</sub> cell line, cytotoxic, *Digera muricata*, HeLa cell line

INTRODUCTION

*Digera muricata* (L.), Family Amaranthaceae, wild edible plant commonly known as “Latmahuria” is commonly distributed throughout India. In Ayurveda, the herb is considered as a cooling, astringent to the bowels and also used as a laxative. The flowers and seeds be used to treat urinary discharges. Boiled root infusion is given to mothers after child birth for lactation purpose. *D. muricata* is also used to treat renal disorders in folk medicine practices. The generation of reactive radical species has been implicated in carbon tetrachloride-induced nephrotoxicity which are involved in lipid peroxidation and accumulation of dysfunctional proteins, leading to injuries in kidneys. The treatment with the plant augments the antioxidant defense mechanism against carbon tetrachloride induced toxicity and provides evidence that it may have a therapeutic role in free radical mediated diseases.

The phytochemical investigation on the plant *D. muricata* has revealed the presence of flavonoids in it that have a diverse effect on improvement and protection of deficits. Hence, the present work was undertaken to investigate the effect of crude extract and flavonoid rich fractions of *D. muricata* as an anticancer drug.

Cancer belongs to a group of disorders that are difficult to be treated and are sometimes incurable. Nowadays, considerable scientific and commercial interests have increased for discovering new anticancer agents with natural sources. Plants have been sources of the well known anticancer drugs such as camptothecin, podophyllotoxin and paclitaxel. The potential of natural products as anticancer agents was recognized for the first
time in the 1950 by the U.S. National Cancer Institute, and after that many investigations have been performed to find out new natural anticancer agents. Different methods are used for screening of anticancer agents. One of the techniques is 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide (MTT) assay which is a simple and reliable method for evaluation of anticancer agents.

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT is reduced by metabolically active cells, by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means.

The MTT cell proliferation assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis; it measures the reduction in cell viability.

The number of assay steps has been minimized as much as possible to expedite sample processing. The MTT reagent yields low background absorbance values in the absence of cells. For each cell type, the linear relationship between cell number and signal produced is established, thus allowing an accurate quantification of changes in the rate of cell proliferation. Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation and a higher absorbance rate indicates an increase in cell proliferation.

Cervical cancer is an important area of action for any cancer control program because of the burden of disease and the potential for effective prevention via screening. Cervical cancer comprises approximately 12% of all cancers in women. It is the second most common cancer in women worldwide but the commonest in developing countries such as Indonesia. Annual global estimates around the year 2000 are for 470,600 new cases and 233,400 deaths from cervical cancer annually. 80% of these cases occur in developing countries. Incidence of cancer in Indonesia was estimated to be 100 per 100,000 people per year or about 200,000 population per year, and cervical cancer is the first most common cancer in women of Indonesia. Cervical cancer is malignant neoplasm of the cervix uteri or cervical area. It may present with vaginal bleeding, but symptoms may be absent until the cancer is in its advanced stages. The most important risk factor in the development of cervical cancer is infection with a high-risk strain of human papilloma virus. Symptoms of advanced cervical cancer may include: Loss of appetite, weight loss, fatigue, pelvic pain, back pain, leg pain, single swollen leg, heavy bleeding from the vagina, leaking of urine or feces from the vagina, and bone fractures. Natural compounds from plants have provided many effective anticancer agents in current use. Currently, over 50% of drugs in clinical trials for anticancer activity have been isolated from natural sources.

Worldwide, lung cancer is another most common cancer in terms of both occurrence and mortality, with the highest rates in Europe and North America. Despite rapid advances in diagnostic and operative techniques, lung cancer remains one of the most difficult human malignancies to treat. The American Cancer Society estimates that 214,440 persons in the United States developed lung cancer in 2009, with 159,390 deaths. Lung cancer-dependent deaths constituted 30% (men) and 26% (women) of the estimated total cancer-related deaths in 2009. Data indicate that while the overall incidence of lung cancer is declining, it continues to rise in women. The relative 5-year survival ratio of the patients that had lung or bronchus cancer from 1995 to 2001 was still quite low (15%) and was not improved very much compared to the 1970’s (12%). From the 1950s, the incidence of lung adenocarcinoma started to rise relative to other types of lung cancers.

Recently, multiple new chemotherapeutic agents have been developed, and some are in clinical trials. Although some of them have produced promising results, their therapeutic spectrum is narrow along with toxicity. This toxicity problem at therapeutic concentration induced scientists to search for anticancer compounds derived from natural sources such as phytochemicals. Keeping this in mind, the aim of the present work was to explore the anticancer property of D. muricata on A549 human lung cancer cell lines and help cell lines of cervical cancer.

MATERIALS AND METHODS

Plant material

The whole plant of D. muricata was collected in the month of August, 2011 from fields behind the Faculty of Pharmacy, Integral University, Lucknow, U.P, India. For identification and taxonomic authentication, sample of plant material was given to National Botanical Research Institute, Lucknow, India, which confirmed the authenticity of the plant specimen (voucher specimen no. NBRI/263/2011). The
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A fresh plant was used for the examination of macroscopic and microscopic characters whereas the dried powder was used for the determination of physicochemical parameters. Preliminary phytochemical investigation was done as per standard methods.

**Preparation of extract**

The whole plant was dried in an oven at 50 ± 0.5°C up to approximately 5-7% moisture content. This powder was extracted with methanol in a soxhlet apparatus for 24 h. The extract was concentrated under reduced pressure by a rotary vacuum evaporator. This extract was then fractionated by column chromatography into three flavonoid rich fractions namely chloroform, ethylacetate and residual water. The methanolic extract and all the fractions were dissolved in Di-methyl-sulphoxide to prepare a series of concentrate in a range of 25-250 μg/ml. This extract and its fractions were screened for their anticancer activity.

**Cytotoxicity assays**

**Cell lines**

Two cancerous cell lines HeLa (human cervix) and A549 (human lung carcinoma) were obtained from cell repository at NCCS, Pune.

**Reagents and chemicals**

HeLa cell line was cultured in Eagle’s minimal essential medium (MEM, Himedia) with 2.0 mM L-glutamine, 1.5 g/L NaHCO₃, 0.1 mM non-essential amino acids and 1.0 mM sodium pyruvate and supplemented to contain 10% fetal calf serum (Himedia). Similarly, A₅₄⁹ cell was cultured in Ham’s F12K medium (Himedia) with 2.0 mM L-glutamine adjusted to contain 1.5 g/L NaHCO₃ and 10% fetal calf serum (Himedia).

MTT reagent, phosphate buffer saline, dimethyl-sulphoxide, propidium iodide (PI) and Hoechst stain kit, paraformaldehyde, triton.

**Microculture tetrazolium toxicity assay**

The cytotoxic potential was evaluated using the MTT assay.Cells were grown at 37°C in 5% CO₂ incubator. The medium was replaced with fresh supplemented medium containing different concentrations (25-250 μg/ml) of methanolic extract and fractions of the plant obtained during fractionation by column chromatography. Cells were then incubated at 37°C for 48 h. After incubation, 10 μl of a 5 mg/ml stock solution of MTT in phosphate buffer solution (PBS) was added to each well containing the cells and incubated again for 4 h. Then, the supernatant without the cells was aspirated from each well, and 100 μl of dimethyl sulfoxide was added to dissolve the dark blue formazan crystals resulting from MTT reduction by homogenization in plate shaker. The extent of MTT reduction to formazan within cells was measured by taking absorbance at 550 nm using a scanning microplate reader (Biorad, Model 680). The percentage cell proliferation was calculated by using the following formula:

\[
\text{Percentage cell proliferation} = \left( \frac{\text{Absorbance of control} - \text{Absorbance of treated}}{\text{Absorbance of control}} \right) \times 100
\]

**Statistical analysis**

Data of cell proliferation were expressed as the mean ± standard deviation from three independent experiments. One-way ANOVA and Dunnett’s multiple comparison tests were performed using Graph Pad prism (version 5.01 Software Inc. 2013) for the significance test, using a \( P \leq 0.05 \).

**Morphological examination of cancer cells**

**Calculation of the apoptotic ratio**

The apoptotic cells were scored by counting the cells with condensed chromat and fragmented nuclei under inverted fluorescent microscope using G-2A filter at ×400 magnification.

**Calculation of the percentage apoptosis**

By scoring the number of apoptotic cells with that of the total number of cells involved in the experiment, percentage apoptosis was measured which indicated the level of apoptosis induced by the test drug. Freshly stained cell suspension was dropped into a glass slide and covered by a cover slip. Slides were observed under phase contrast microscope. The percentages of viable, apoptotic, and necrotic cells were determined in more than 200 cells by help of hemocytometer.

**PI exclusion assay for necrosis**

First, the cell suspension was aspirated and washed by 100 μl PBS, 2-3 times followed by 50 μl of 4% paraformaldehyde (50 μl). It was left as such for 15 min. It was then treated with 50 μl triton for 20 min. After being washed with 100 μl of PBS, 1 μl of PI stain was added. Then, it was kept in the dark for 10 min and visualized under fluorescence microscope.
Hoechst/PI nuclear staining and fluorescence microscopy

First, the cell suspension was aspirated and washed by 100 µl of PBS, 2-3 times, followed by 75 µl of 4% paraformaldehyde and kept at room temperature for 15 min. It was then treated with Methanol and kept for 20 min at room temperature. After being washed with 100 µl of PBS, 1 µl of PI stain and 1 µl of Hoechst stain was added and kept for 15 min. It was then visualized under fluorescent microscope after rinsing with PBS. The morphology of viable, apoptotic and necrotic cells was visualized under fluorescence microscope.

RESULTS

Effect of crude methanolic extract of *D. musica* against A₅₄₉ cell lines

Crude extract of *D. musicata* showed inhibition at all concentrations in a dose dependent manner with a nominal amount of decrease in percentage proliferation (90.83%) at a concentration of 25 µg/ml and a marked decrease in percentage proliferation (29.68%) at a concentration of 250 µg/ml. A marked decrease in percentage proliferation was found at higher concentrations (between 100 and 250 µg/ml), however, this decrease in percentage proliferation gradually slowed down and finally became more or less constant beyond 250 µg/ml.

Effect of crude methanolic extract of *D. musica* against HeLa cell lines

Crude extract of *D. musicata* showed inhibition at all concentrations in a dose dependent manner with a minimum decrease of percentage proliferation (72.88%) at a concentration of 25 µg/ml and a maximum decrease in percentage proliferation (27.61%) at a concentration of 250 µg/ml. A marked decrease in percentage proliferation was found at higher concentrations (250 µg/ml) amounting to a constant beyond this value.

Effect of the chloroform fraction of *D. musicata* against A₅₄₉ and HeLa cell lines

The chloroform fraction of *D. musicata* showed effect against A₅₄₉ cell lines in a dose dependent manner with a nominal decrease of percentage proliferation (95.83%) at a concentration of 25 µg/ml and a decrease of up-to 27.62% proliferation at a concentration of 250 µg/ml. A significant decrease of percentage proliferation was found at higher concentrations (250 µg/ml). The Chloroform fraction of *D. musicata* showed effect against HeLa cell lines in a dose dependent manner with a nominal decrease of 88.6% proliferation at a concentration of 25 µg/ml and a decrease of up-to 35.48% proliferation at a concentration of 250 µg/ml.

Effect of ethylacetate fraction of *D. musicata* against A₅₄₉ and HeLa cell lines

The ethylacetate fraction of *D. musicata* showed effect against A₅₄₉ cell lines in a dose dependent manner with a marked decrease of 59.5% proliferation at a concentration of 25 µg/ml and an increase of up-to 24.95% proliferation at a concentration of 250 µg/ml. A significant decrease was found at higher concentrations (250 µg/ml).

Effect of the aqueous fraction of *D. musicata* against A₅₄₉ and HeLa cell lines

The aqueous fraction of *D. musicata* showed effect against A₅₄₉ cell lines in a dose dependent manner with a marked decrease of 78.08% proliferation at a concentration of 25 µg/ml and an increase of up-to 35.66% proliferation at a concentration of 250 µg/ml. A significant decrease was found at higher concentrations (250 µg/ml).

PI - Exclusion assay for necrosis

Due to their extensive membrane damage, necrotic cells were quickly stained by short incubations with PI. A number of necrotic cells could be seen in the photomicrograph that resulted in increased permeability of the stain. Necrotic debris was seen which increased with concentration (Figures 4-6).

Hoechst/PI double staining and fluorescence microscopy

Apoptotic cells showed a high Hoechst staining and a low PI staining, since they initially tend to exclude PI (Figure 7).
Necrotic cells were brightly stained with PI while healthy cells were dimly stained by Hoechst and not stained by PI. Live cells showed only a low level of fluorescence; apoptotic cells showed a higher level of blue fluorescence, and dead cells showed low-blue and high-red fluorescence. Late apoptotic cells exhibited an orange nucleus showing condensation of chromatin while necrotic cells displayed an orange nucleus with intact structure. This assay provided a useful quantitative evaluation and was done 3 times. The staining pattern resulted from the simultaneous use of these dyes made it possible to distinguish normal, apoptotic, and dead cell populations by fluorescence microscopy.

Figure 1: Percentage proliferation of cancerous cells A549 after 24 h after treatment with methanolic extract and its fractions obtained from *Digera muricata* evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,4 diphenyltetrazolium bromide assay at indicated concentrations. Values are mean ± standard deviation from three independent experiments. Triplicates of each treatment group were used in each independent experiment. Results were found to be statistically significant difference as compared to control ($P < 0.001$ for each).

Figure 2: Percentage proliferation of cancerous cells HeLa after 24 h after treatment with methanolic extract and its fractions obtained from *Digera muricata* evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,4 diphenyltetrazolium bromide assay at indicated concentrations. Values are mean ± standard deviation from three independent experiments. Triplicates of each treatment group were used in each independent experiment. Statistically significant difference as compared to control ($P < 0.001$ for each).

Figure 3: The morphological changes as seen through phase contrast microscope in various doses of methanolic extract of *Digera muricata*. 
DISCUSSION

The results of cytotoxicity study of methanolic extract of * Digera muricata* showed significant cytotoxicity against both the cell lines, but more for HeLa cell lines. The cytotoxicity of methanolic extract of the plants may be due to the presence of flavonoids having mono to poly phenolic groups in the structure. The flavonoids have been reported for their cytotoxic activity due to the presence of phenolic groups. The anticancer activity was at its peak in methanolic extract indicating that most of the active components were extracted with methanol. Cytotoxic changes observed was cell aggregation, cell rounding and cell death. The overall results indicated the promising baseline information for the potential uses of the methanol extracts of the plants as an anticancer agent.

The association between flavonoids and reduced cancer risk has been reported in previous studies that show a decrease in cancer risk with consumption of vegetables and fruits rich with flavonoids. Results of this study are in accordance with this finding since the phytochemical screening showed the presence of flavonoids in the extract and its fractions. The anti-proliferative activity of total flavonoids and alkaloids isolated from different plants has been reported. The significant activity of methanolic extract of the plant can be due to the induction of cell death by apoptosis that was shown in our results.

Our apoptotic test results would support the previous studies especially nuclear condensation and cell shrinkage which were clearly observed. This is not an exception since many commercially available chemotherapeutic agents and folk medicinal plants exert their anticancer effect by inducing cell apoptosis. However, this needs further comprehensive studies to be fully substantiated.

The results suggest a possible selectivity and better activity of the extract against cervical cancer cell lines in comparison to lung cancer cell lines, as observed for the cisplatin compounds, which are preferentially used for testicular and ovarian cancer. The selectivity of action could be related to the differences in morphology and physiology between tested cell lines, although this is not yet proven.
The results are very encouraging and can promote a specific treatment for both the cancers. Although, it has not been reported in the scientific literature as a cytotoxic agent, the activity presented here may be related to the major presence of flavonoids in the extract, or even its performance in synergy with other constituents such as phenolic and flavonic constituents identified in this extract.

**CONCLUSION**

In the present study, we have observed that methanolic extract of *D. muricata* showed significant cytotoxicity against both the cell lines, but more for HeLa cell lines. Crude methanolic extract of *D. muricata* inhibited the cell viability in a dose dependent manner ranging from 25 μg/ml to 250 μg/ml. Our results from this *in vitro* study suggests that the methanolic and aqueous fraction of the extract of *D. muricata* have significant anti-proliferative activity against HeLa and A549 cell lines. On the basis of the present findings, it can be concluded that the crude extract of *D. muricata* can be a good source of cytotoxic compounds.

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**REFERENCES**


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