Original Research Article

Anticancer effect of *digera muricata* leaf extract against prostate cancer cell lines

Running title: Anticancer effect of *Digera muricata* leaf extract on PC-3 cell line

The type of article: Original Research Article

ABSTRACT

Background: Prostate cancer has become a major health problem globally during the last few decades. It is the second most frequently diagnosed cancer in men worldwide and the fifth most common cancer overall. Chemotherapy or other cancer treatments including androgen depletion therapy, show high toxicity and cause serious side effects in cancer patients. Thus, there have been many studies conducted to find various natural products as potential anticancer drug candidates with low toxicity and fewer side effects for the treatment and prevention of prostate cancer. The *Digera muricata* of the genus *Digera Forssk* and family Amaranthaceae Juss. is a wild edible plant. The presence of phenol, flavonoids, alkaloids, terpenes, sterols, tannins, glycosides and lignins is seen in *Digera muricata* leaf extract, which are reported to have cytotoxic activities.

Aim of the Study: The aim of this study is to assess the cytotoxic effects of *Digera muricata* leaf extract on prostate cancer cell line.

Materials and methods: The cytotoxic potency of *Digera muricata* leaf extract was carried out by MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide) assay against the prostate cancer (PC-3) cell line. PC-3 cells were treated with different concentrations of *Digera muricata* leaf extract (25-150µg/ml) for 24h. Furthermore, the morphological changes were analysed using phase contrast microscopy and nuclear morphological changes examined using DAPI (4′,6-diamidino-2-phenylindole) staining under the fluorescence microscopy

Results: The MTT assay showed decreased cell vitality with increased concentration of *Digera muricata* leaf extract. The morphological study showed that the number of cells decreased after

treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing. The treated cells also showed condensed chromatin and nuclear fragmentation

Conclusion: Within the limits of this study it can be demonstrated that the leaf extract of *Digera muricata* were cytotoxic and induced apoptosis to the cancer cells.

KEYWORDS: *Digera muricata*, prostate cancer, cell viability, Cytotoxicity, Apoptosis

1. INTRODUCTION

Prostate cancer is a major health problem and is the second most frequently diagnosed cancer in men worldwide and the fifth most common cancer overall[1]. Prostate cancer primarily affects elderly males with more than three quarter of the cases affecting men above the age of 65. Prostate cancer has become a major health problem globally during the last few decades. There has been lower incidence of prostate cancer in India and non resident Indians when compared to the Western population. Within India, prostate cancer has a wide disparity in disease characteristics, incidence, and mortality, with the urban population being more increasingly affected [2]. Incidence of prostate cancer is increasing in various parts of India. Changes in the diagnostic criteria and modalities, awareness levels of the public, and changing lifestyles may be responsible for much of the observed change [3].

The prostate is a gland located between the bladder and the external genital organ of the males. The prostate is present in front of the rectum. The urethra runs through the center of the prostate which aids in the excretion of urine. The function of the prostate is to secrete fluid that nourishes and protects sperm. Androgens are required for growth of the cancerous cells in the early stages but eventually moves on to an androgen-independent stage, and progresses despite androgen extirpation [4]. The pathological mechanism at a molecular level is poorly understood for androgen-independent cancer progression. Among prostate cancer cell lines, the PC-3 cell line is known to be corresponding to androgen-independent cancer cells [5, 6]. Chemotherapy or other cancer treatments including androgen depletion therapy, show high toxicity and cause serious side effects in cancer patients. Drug resistance and low anticancer efficacy also pose difficulties in the clinical treatment [7, 8]. For these reasons, there have been many studies conducted to find

various natural products as potential anticancer drug candidates with low toxicity and fewer side effects for the treatment and prevention of prostate cancer.

The *Digera muricata* of the genus Digera Forssk and family Amaranthaceae Juss is a wild edible plant commonly known as 'Cancali soppu'. It is native to Egypt and eastern Kenya but can be commonly seen distributed throughout India. In Ayurveda the herb is considered to be a cooling astringent to the bowels and is also used as a laxative. The flowers and seeds are used to treat urinary discharges [9]. while the boiled root infusion is given to the mother after parturition for lactation purposes [10]. The presence of phenol, flavonoids, alkaloids, terpenes, sterols, tannins, glycosides and lignins in *Digera muricata* leaf extract was observed on conducting phytochemical tests [11].

Tetrachloride-induced nephrotoxicity can cause the generation of reactive oxygen species which are in accumulation of amyloids, lipid peroxidation and thus can lead to kidney injuries [12-14]. *Digera muricata* is seen to have antioxidant properties which acts as a defence mechanism against toxicity induced by carbon tetrachloride and thus may have a role in the therapeutic treatment of free radical mediated diseases [15]. Cytotoxicity is the ability of a material to be toxic to a cell and induce apoptosis to that cell [16, 17]. The cytotoxicity of extract of the plants may be due to the presence of flavonoids having mono to poly phenolic groups in the structure. The flavonoids have phenolic groups which are reported to have cytotoxic activities. Previous studies have been done to assess the association between flavonoids and cancer risk and it has been proven that there is a decrease in cancer risk with consumption of vegetables and fruits rich in flavonoids [18-36]. . Thus in this study, we are assessing the cytotoxic effects of *Digera muricata* leaf extract on prostate cancer cells.

2. MATERIAL AND METHODS

2.1. Reagents:

DMEM (Dulbecco's Modified Eagle Medium), Phosphate Buffered Saline (PBS), Trypsin-EDTA, Fetal bovine serum (FBS), were purchased from Gibco, Canada. Dimethyl sulfoxide (DMSO), [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), DAPI, were

purchased from Sigma Chemical Pvt Ltd, USA. All other chemicals used were extra pure of molecular grade and were purchased from SRL, India.

2.2. Cell line maintenance:

PC-3 Prostate cancer cell lines were obtained from the National Center for Cell Science (NCCT), 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% CO2. Upon reaching confluency, the cells were trypsinized and passaged.

2.3. Preparation of the Herbal Extract:

Digera muricata Leaf powder obtained from The Indian Medical Practitioners' Cooperative Pharmacy & Stores (IMPCOPS) (Chennai, India) was used for the present study. About 50g of *Digera 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 whatman 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 and immediately stored at 4˚C [37].

2.4. Cell viability (MTT) assay:

The cell viability of *Digera muricata* extract treated PC-3 cells was assessed by MTT assay [38]. The assay is based on the reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells. PC-3 cells were plated in 48 well plates at a concentration of $2x10^4$ 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 37ºC. After starvation, cells were treated with *Digera muricata* leaf extract in different concentrations (25 -150µg) for 24 hours. At the end of treatment, the medium from control and *Digera muricata* 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 37ºC in the CO_2 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 medium 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.

2.5. 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. 3×104 cells were seeded in 6 well plates and treated with *Digera muricata* (concentration for MCF-7 cells) 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.

2.6. Determination of nuclear morphological changes of cells (DAPI staining):

For the nuclear morphological analysis, 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 [38].

2.7. Statistical analysis:

Statistical analyses were performed using one-way ANOVA followed by Student–Newman–Keuls (SNK) tests for comparison between treatment values and control values. Data were expressed as mean ± SEM. The level of statistical significance was set at $p<0.05$.

3. RESULTS

In the current study, we analysed the effect of *Digera muricata* leaf extract on prostate cancer cell lines. [Figure 1] shows the MTT assay which determines the cytotoxic effect of the leaf extract on the cancer cells. Here, the cancer cell activity was inhabited in a dose dependent

manner by the leaf extract of *Digera muricata*. The Cells were treated with increasing concentrations of the leaf extract (0, 25, 50, 75, 100, 125 and 150µg) for 24hrs. The 50% of inhibition observed in concentration of 50µg/ml. Maximum cancer cell viability is seen with the control (100%) while the minimum viability is seen with 150µg/ml (10%). [Figure 2] depicts the morphological changes in the prostate cancer cell lines after treatment with *Digera muricata* at 50 µg/mL. Here, the cell number decreased after treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing. [Figure 3] depicts the nuclear changes in the treated cancer cells. The cells treated with the leaf extract showed condensed chromatin and nuclear fragmentation, characteristic features of apoptosis.

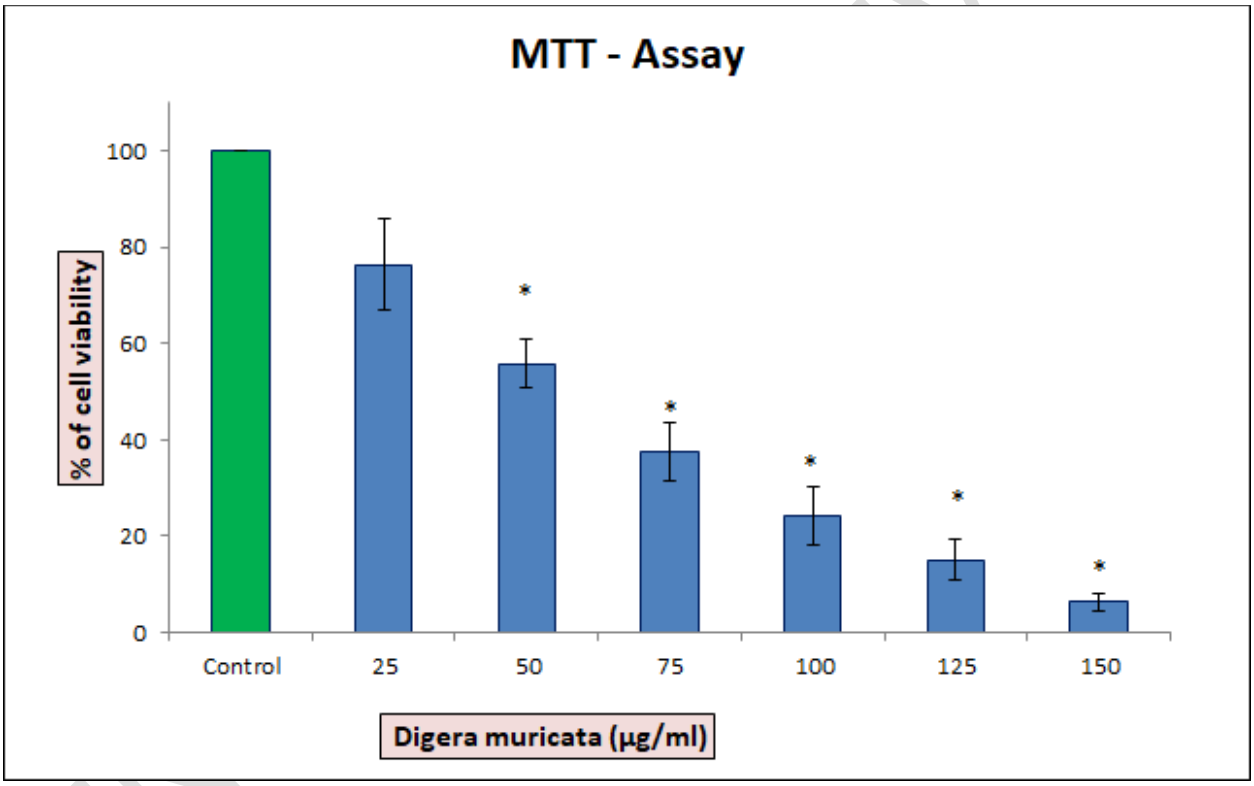

*Figure 1: The cytotoxic effects of Digera muricata leaf extract on PC-3 prostate cancer cell line was determined by MTT assay. The Cells were treated with different concentrations (0, 25, 50, 75, 100, 125 and 150µg) for 24hrs. The 50% of inhibition observed in concentration of 50µg/ml, (p value: 0.0033) which has been taken as IC50 value and fixed for further experiments. **

represents statistical significance between control versus treatment groups at p< 0.05 using Student's-Newman-Keuls test

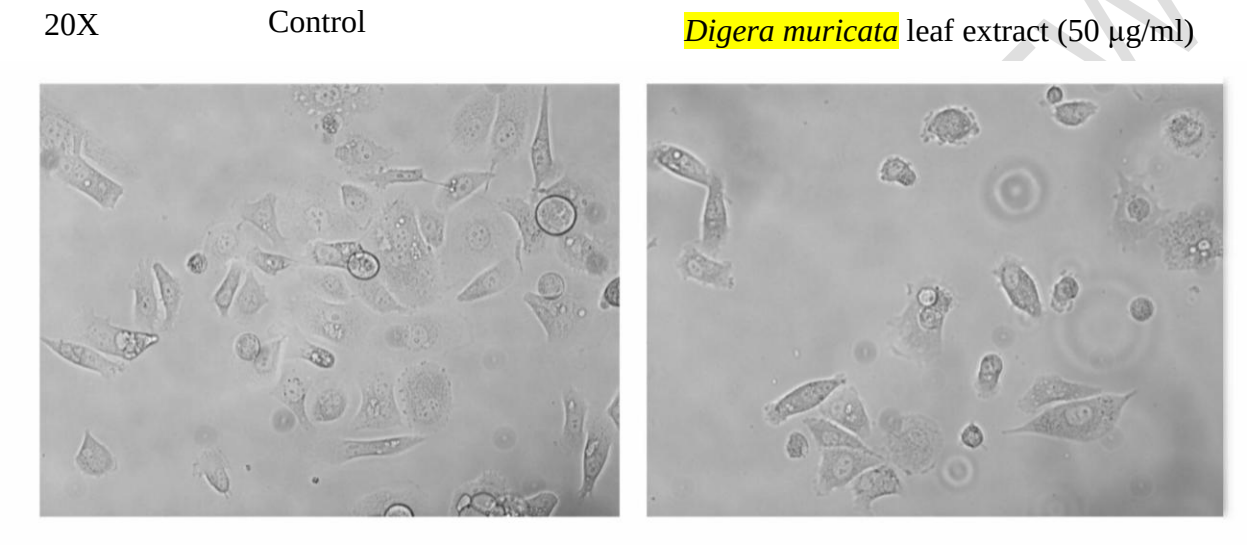

Figure 2: Represents the morphological changes in prostate cancer cell line with and without treatment of Digera muricata at 50 µg/mL for 24hrs by phase contrast microscope at 20x magnification. Here, the number of cells decreased after treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing.

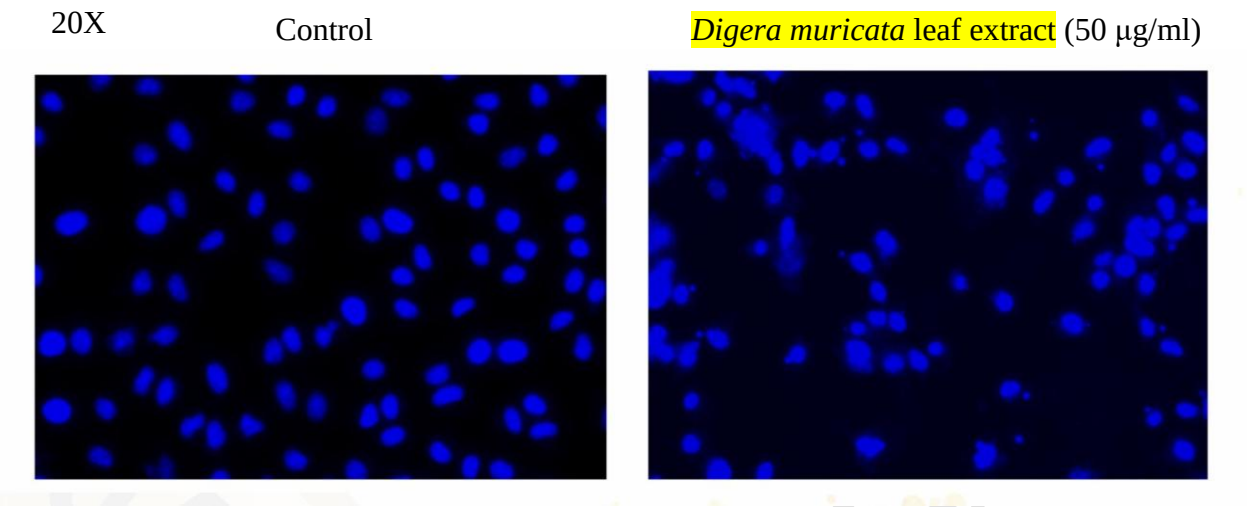

Figure 3: This figure represents the DAPI staining of PC-3 prostate cancer cell line upon, without and with treatment of Digera muricata leaf extract at 50 µg/ml for 24 hrs. The nuclei were stained with DAPI staining and observed under a fluorescence microscope. The treated cells clearly showed condensed chromatin and nuclear fragmentation.

4. DISCUSSION

Treatment for cancer including chemotherapy has been proven to have many side effects. Many new chemotherapeutic agents have been developed and are undergoing clinical trials. However, they have a narrow therapeutic spectrum considering their toxicity. Thus, many researches have been done to develop anticancer compounds derived from natural sources [39]. Certain plants contain natural compounds called phytochemicals which have anticancer effects and can be used to prevent cancer. These phytochemicals include phenol, flavonoids, alkaloids, terpenes, sterols, tannins, glycosides and lignins [40].

In our study, based on the MTT assay, the cell viability is seen to decrease with the increased viability of the leaf extract. Maximum cancer cell viability is seen with the control (100%) while

the minimum viability is seen with 150µg/ml (10%). IC-50 dose was observed at 50 µg/ml [Figure 1]. The morphology tests showed that the number of cells decreased after treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing [Figure 2]. This is a feature of cell apoptosis. The reason for cytoplasmic blebbing is not quite clear. A few hypotheses suggest that the cytoplasmic blebs can represent the structural and functional communication of the cell with the neighbouring cells to initiate phagocytosis or it also may be a way to deplete ATP in order to mix compartments to aid in cellular packaging or it may be a prerequisite for the formation of apoptosis bodies [41]. Also, the nuclei were stained with DAPI staining and observed under a fluorescence microscope. The treated cells clearly showed condensed chromatin and nuclear fragmentation which are features of apoptosis [Figure 3].

In our study the cell viability decreased with increased concentration of the leaf extract. This is in concordance with previous researches where the extract of *Digera muricata* showed inhibition of cancer cells at all concentrations in a dose dependent manner with a significant number of decrease in percentage proliferation against lung cancer cell lines [40, 42]. A study by Shazia et al., on cervical and lung cancer cell lines provide similar results. It is suggested that the methanolic and aqueous fraction of the *Digera muricata* leaf extract played an important role in its anticancer effect. Here the anticancer activity was at its peak in the extract containing methanol which indicated that most of the active components were extracted using methanol [40, 42,43]. The cytotoxic changes that were observed were cell aggregation, cell rounding and cell death[44,45].

In the above study, the presence of flavonoids in the extract seems to have an important role in the anticancer effect of *Digera muricata*. In a similar study, Ashokkumar et al., stated that the activity of inhibition of the cancerous cells can be because of the interaction of the metabolic nature of the cancer cells with the nature of the compounds found in each crude extract or may be due to the effectiveness of some enzymes which act as antioxidants mainly in cancer cells [46]. While not many studies have been done regarding the anticancer effects of *Digera muricata*, there have been studies highlighting the antioxidant, antiinflammatory and antibacterial of the plant [47-50]. The limitation is that the study does not involve any in vivo

study, so its effect is not assessed. This paves way for various future studies such as to view the drug action in in vivo studies and also to know about the side effects of the extract.

5. CONCLUSION

Within the limits of this study it can be demonstrated that the leaf extract of *Digera muricata* were cytotoxic and induced apoptosis to the prostate cancer cells at 50 µg/ml concentration. . However more research is needed to find out the active principle compound from this plant and understand the molecular mechanisms of cytotoxicity effect of *Digera muricata* plant derived compounds against various cancer cells.

NOTE:

The study highlights the efficacy of "ayurveda which is an ancient tradition, used in some parts of India. This ancient concept should be carefully evaluated in the light of modern medical science and can be utilized partially if found suitable.

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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