Investigation of proapoptotic effect of *Digera muricata* leaf extract against the skin cancer cell line (B16-F10)

Running Title: Assessment of pro-apoptotic effect of *Digera muricata* leaf extract against the skin cancer cell line

Type of Article: Original Research Article

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ABSTRACT

Introduction: Skin cancers, such as melanoma, basal cell carcinoma, and squamous cell carcinoma, frequently begin as changes in the skin. Cancer research continues to focus on finding tumor-selective and new anticancer drugs with fewer adverse effects. *Digera muricata* is a medicinal herb in the Amaranthaceae family that has antibacterial, antifungal, free radical scavenging function, anti tumor, and other valuable medicinal properties.

Aim: To investigate the pro-apoptotic effect of *Digera muricata* leaf extract against the skin cancer cell line.

Materials and method: MTT assay was used to determine the viability of B16-F10 cells treated with different concentrations (20-200µg) of *Digera muricata* leaf extract. Phase contrast microscopy was used to examine the morphological changes. In addition, the mode of cell death was assessed using AO/EtBr dual staining and observed under a fluorescence microscope. Statistical analysis was performed, keeping the level of significance at $p<0.05$.

Result: The MTT assay revealed that the ethanolic extract of *Digera muricata* leaf had significant cytotoxic and apoptotic potency against the B16-F10 skin cancer cell line, which was validated by significant morphological alterations under phase contrast microscope after 24 hours of treatment. AO/EtBr dual staining results clearly showed the *Digera muricata* leaf extract treatment induced the early apoptotic cells with bright orange areas of condensed or fragmented chromatin in the nucleus. Late apoptotic cells showing uniform bright red nucleus.

Conclusion:

Within the limits of the analysis, it can be inferred that the leaf extract of *Digera muricata* was cytotoxic and triggered cancer cell apoptosis at a concentration of 50 µg/ml within 24 hours. More research is needed to understand the cytotoxicity mechanisms of this plant extract.

Keywords: *Digera muricata*, Skin cancer, Cell viability, Apoptosis

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1. INTRODUCTION

Skin cancers, such as melanoma, Nonmelanocytic Skin Cancer (NMSC), basal cell carcinoma (BCC), and squamous cell carcinoma (SCC), frequently begin as changes in the skin. They may be new swellings or precancerous lesions, which are variations that are not tumors but can develop into cancer in the future (1). The different forms of skin cancer are named for the cells that give rise to them as well as their clinical behavior. Nonmelanocytic Skin Cancer (NMSC) is the most prevalent form of skin cancer in humans. Per year, 2-3 million new cases are recorded globally, with 1.3 million of those occurring solely in the United States. The prevalence rate is expected to more than double in the next 30 years (2). UV sun, ionizing radiation, and some organic carcinogens are the most significant etiological influences. BCC accounts for 80 to 85 % of all NMSC, making it the most prevalent form of skin cancer. Skin cancer patients exhibit common symptoms of persistent sun exposure, such as collagenosis, abnormal pigmentation, wrinkling, telangiectasia, and solar keratosis on sun-exposed areas. Superficial BCC appears red, wrinkled, and scaled, with minor ulcerations on the more exposed skin. They may be circular or oval in shape, with an ill defined border. The center of the tumor can be uniformly fibrotic. Subacute or chronic dermatitis may be caused by a superficial BCC. The other form of NMSC is SCC, which accounts for 15 to 20 % of all NMSCs, has localized destructiveness and tissue invasion, and is more likely to cause death than BCC. The trigger factors that contribute to SCC production are classified as extrinsic factor eg. UVA, ionizing radiation and human papillomavirus (HPV); and intrinsic factors like genodermatoses, preexisting skin lesions and immunosuppression (3). Malignant melanoma (MM) is a cancer that affects the cells that contain the pigment melanin in the skin. Its prevalence is also increasing in areas of light skinned

communities that are excessively exposed to sunlight (4). Cancer is one in a group of diseases that are difficult to cure and in some cases, incurable. Skin cancer is curable if detected and treated in the initial stage (5). The current therapeutic approaches for skin cancer include surgery; basic pharmacological treatment with cisplatin, carboplatin, nitrourea, taxanes, vindesine, and vinblastine; targeted therapy; immunotherapy; interferon; and adoptive cell immunotherapy (ACT). But this comes at the expense of more unpleasant side effects, while the prognosis remains the same. However, using the apoptosis process to destroy cancer cells is well established as an effective and promising approach. The medicinal application of apoptosis is now being seen as a model for the development of antitumor drugs. A number of natural plant extracts and phytochemicals have been shown to cause apoptosis in cancer cell lines. Thus, proapoptotic enzymes engage in a cascade that is initiated in response to proapoptotic signals and ultimately results in cleavage of a series of proteins, culminating in cell disassembly (6). Nowadays, there is a great deal of scientific and industrial curiosity in developing new anticancer agents derived from natural sources. Plants have been used to make well-known anticancer drugs such as paclitaxel camptothecin and podophyllotoxin (7). The efficacy of natural products as anticancer agents was recognised for the first time in 1950 by the United States National Cancer Institute, and since then, several studies have been conducted to discover new natural anticancer agents (8). *Digera muricata* is an Amaranthaceae medicinal herb with antibacterial, antifungal, diuretic, laxative, free radical scavenging activity, anti-tumor, anthelmintic, and other beneficial medicinal properties. It is a 20 to 70 cm tall herb that is common in eastern tropical Africa and subtropical Asia. It is widely spread in Rajasthan, Maharashtra, and Andhra Pradesh in India (9). *Digera muricata* (L.) Mart. is an edible wild herb commonly used among villagers. It is very well known as a herbal treatment for a variety of conditions. This herb is labeled as soothing, astringent of the bowels, and is often used as a laxative in Ayurveda. The leaves are used to cure diabetics. However, the scientific basis for its medicinal use, especially for boiled root infusion given to mothers during childbirth to improve lactation, needs to be assessed. Urinary discharges are treated with the flower and seeds (10). The plant's ethyl alcohol extract is used as diuretics. The whole plant is used to treat digestive system problems. Locals eat the leaves and young shoots of this plant as a vegetable and to alleviate constipation. For kidney stone therapy, the leaf extract is provided once a day. This plant's extract is used to treat biliousness and urinary

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discharges. To avoid pus production, leaf paste is applied locally. The crushed plant is used as a moderate astringent and antibilious in bowel problems. *D. muricata* has been shown to have antioxidant properties that protect kidneys and testes from CCl4-induced toxicity (11). This plant can be used to treat secondary infertility. Hepatic abnormalities have been shown to be linked to secondary infertility (12). The hypogonadism models developed by using CCl4 to cause liver injuries could be the best for studying hypogonadism in rats (13). The entire plant extract enhances blood circulation while still acting as an expectorant. This is a stomachin, antiperiodic, and coolant. Digera muricata is often regarded as a famine food due to its high nutritional content (14-17). The existence of flavonoids in the plant *Digera muricata* has been discovered by phytochemical analysis, and these flavonoids have a variety of effects on the improvement and defense of deficits (18). The existence of flavonoids with mono to poly phenolic groups in the composition can explain the apoptotic behavior of methanolic extract of the plant. Due to the presence of phenolic groups in flavonoids, they have been documented to have pro-apoptotic activity (19). Our team has extensive knowledge and research experience that has translated into high quality publications (20–40). As a result, the current study was undertaken to explore the efficacy of flavonoid rich fractions of *Digera muricata* as an anticancer medication. Hence the main purpose of our study was to assess the proapoptotic effect of *Digera muricata* leaf extract against the skin cancer cell line.

2. MATERIALS AND METHODS

2.1. Reagents

Phosphate Buffered Saline (PBS), Trypsin-EDTA, Fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were bought from Gibco, Canada. Sigma Chemical Pvt Ltd, USA, supplied acridine orange (AO), ethidium bromide (EtBr), Dimethyl Sulfoxide (DMSO), [3-(4,5-dimethylthiazol-2-yl)] 2,5-diphenyl tetrazolium bromide (MTT) and AO/EtBr. The other chemicals used were extra pure molecular grade and obtained from SRL in India.

2.2. Cell line maintenance

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Mus musculus skin melanoma (B16-F10) skin cancer cell lines were obtained from the NCCS, Pune. T25 culture flasks containing DMEM combined with 10% FBS and 1% antibiotics were used to grow the cells. The cells were placed at 37°C in a humidified environment of 5% CO2. The cells were trypsinized and passaged until they reached confluency.

2.3. Preparation of the Herbal Extract

The current research made use of *Digera muricata* leaf powder obtained from IMPCOPS (Chennai, India). 50g of *Digera muricata* powder was immersed in 500 ml of 95% ethanol and maintained in static condition at room temperature for three days. The solution was filtered using primitive filter paper, then using Whatmann paper. After rota evaporation of the fine filtrate, 3g of the substance was collected. The cumulative ethanol extract was condensed in a vacuum evaporate and preserved at 4^oC immediately.

2.4. Cell viability (MTT) assay

The cell viability of *Digera muricata* extract treated B16-F10 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. B16-F10 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 37^oC. Then the cells were treated with *Digera muricata* in different concentrations (0, 20, 40, 60, 80, 100 and 200 µg) 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 37^oC 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. The intensity of the colour 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

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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}: 60μg/ml) for further studies. Analysis of cell morphology changes by a phase contrast microscope. $3x10^4$ cells were seeded in 6 well plates and treated with *Digera muricata* for 24hrs. 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 mode of cell death by AO/EtBr dual staining

The effects of *Digera muricata* in B16-F10 cell death were also determined by AO/EtBr dual staining as described previously (Cury-Boaventura et al., 2004). The cells were treated with *Digera muricata* for 24 h and then the cells were harvested, washed with ice cold PBS. The pellets were resuspended in 5 μl of acridine orange (1 mg/ml) and 5 μl of EtBr (1 mg/ml). The apoptotic changes of the stained cells were observed by using a fluorescence microscope. Statistical analysis was done & the level of significance was set at $p<0.05$.

2.7. Statistical analysis

Statistical analyses were performed using one-way ANOVA followed by Student–Newman–Keul's (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

3.1. Effect of *Digera muricata* leaf extract on cell viability of skin cancer cell line

The MTT test was used to determine the cytotoxic capability of *Digera muricata* leaf extract in skin cancer cells. For 24 hours, the cells were treated with different doses of *Digera muricata* leaf extract (0, 20, 40, 60, 80, 100, and 200 μg/ml). At the 24 hour time period, *Digera muricata* leaf extract dramatically reduced the viability of B16-F10 cancer cells compared to control

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(Figure 1). With increasing concentration, the fraction of viable cells decreased steadily. At the concentration of 60 μg/ml, we detected a 50% growth inhibition. As a result, the IC^{50} dosage of 60 μg/ml was considered for further investigations.

3.2. The effect of *Digera muricata* on cell morphology

An inverted phase contrast microscope was used to examine the cell morphology of *Digera muricata* leaf extract-treated skin cancer cells. The B16-F10 cells were treated with *Digera muricata* leaf extract (60 g/ml) for 24 hours, and compared to the untreated cells, the treated cells displayed substantial morphological alterations, such as cell shrinkage and reduced cell density which are characteristics of an apoptotic cell (Figure 2). Apoptosis-inducing cells also showed morphological alterations such as rounder cells that shrank and lost contact with nearby cells. Some sensitive cells were even detached from the surface of the plates.

3.3. Pro-apoptotic effect of *Digera muricata* leaf extract in lung cancer cells (AO/EtBr staining).

The induction of apoptosis in *Digera muricata* leaf extract-treated skin cancer cells was confirmed by Acridine orange/Ethidium bromide (AO/EtBr) dual staining. The nuclear morphology of apoptotic cells is assessed using AO/EtBr dual staining. For 24 hours, the cells were treated with *Digera muricata* leaf extract (60 g/ml). Following treatment, the cells were stained for 20 minutes with both AO/EtBr stain and examined under fluorescence microscopy. The results revealed that AO stained both living and dead cells, but EtBr exclusively stained those that had their membrane integrity disrupted. Green stained cells indicate live cells, yellow stained cells indicate early apoptotic cells, and orange stained cells indicate late apoptotic cells. Control cells in this study were uniformly green, but *Digera muricata* leaf extract-treated cells displayed yellow, orange, and red signals (Figure 3). These findings support the hypothesis that *Digera muricata* leaf extract causes apoptosis in skin cancer cells.

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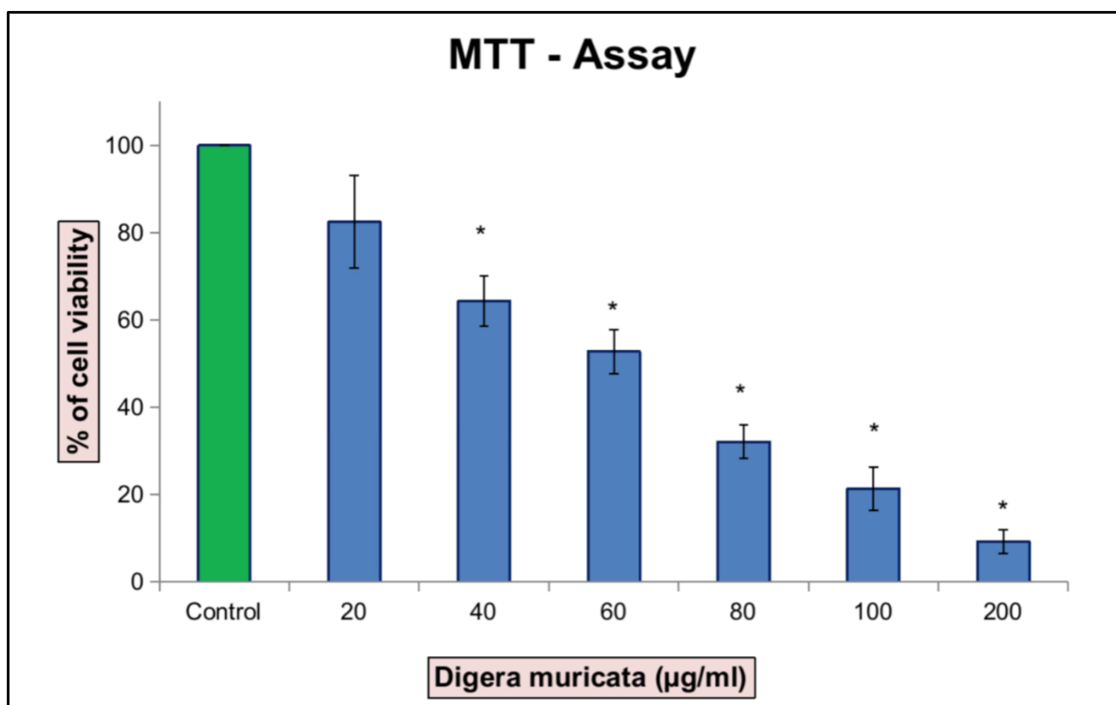

Figure 1: *This bar graph represents the investigation of the cytotoxic effect of Digera muricata leaf extract against the skin cancer cell line. The proapoptotic effects of Digera murica on B16-F10 cells was determined by MTT assay. The cells were treated with different concentrations of the plant extract (0, 20, 40, 60, 80, 100 and 200 µg) for 24hrs. The inhibitory concentration (IC^{50}) dose was observed to be 60µg/ml (p value: 0.0051) and this value was fixed for further experiments. * represents statistical significance between control versus treatment groups at $p < 0.05$ level using Student's–Newman–Keul's test. From the figure we can interpret that except for 20 µg/ml, the statistical significance was observed in 40, 60, 80, 100 and 200 µg/ml concentration of the plant extract.*

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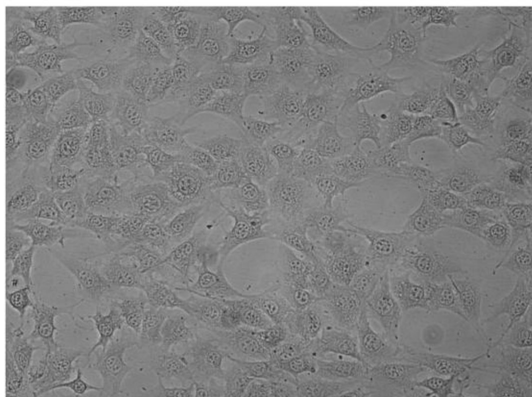
B16-F10

20X Control | Ethanolic extract of Digera muricata leaf (50 µg/ml)



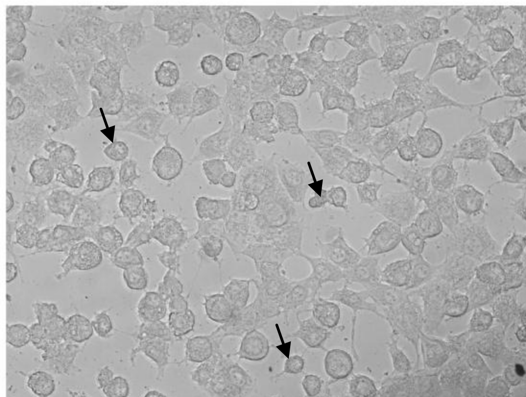


Figure 2: *This figure represents the effect of ethanolic extract of Digera muricata leaf on the cell morphological changes caused in skin cancer cell line (B16-F10) after 24 hours, which was observed in the phase contrast microscope at 20x magnification. From the figure we can interpret that the number of cells decreased after the treatment and the cells exhibited cell shrinkage and cytoplasmic membrane blebbing.*

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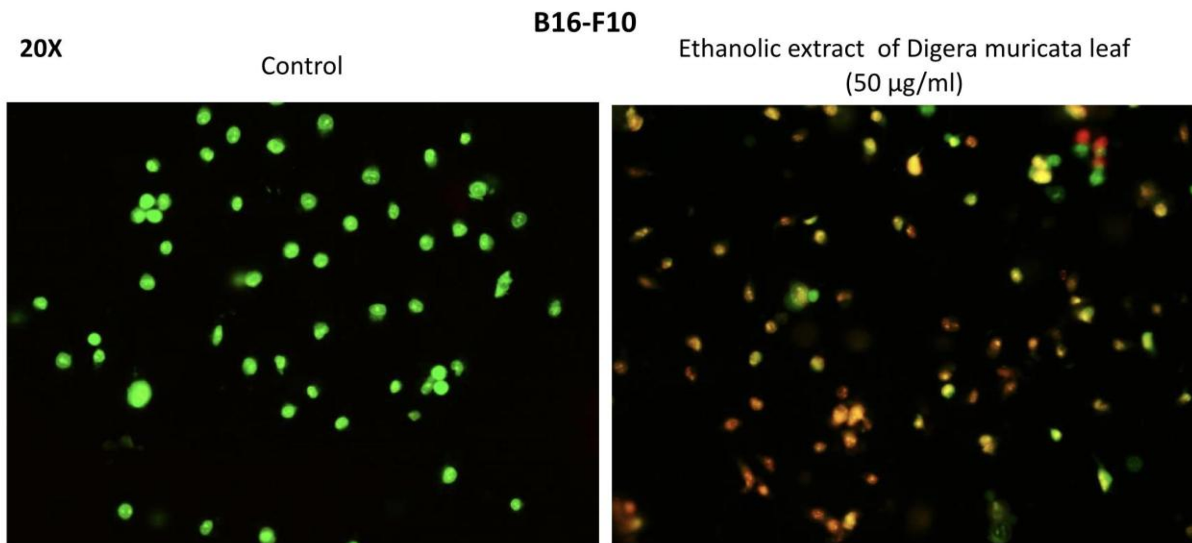

Figure 3: *This figure represents the induction of apoptosis in Digera muricata leaf extract treated skin cancer cell line (B16-F10) viewed under a fluorescence microscope after acridine orange and ethidium bromide dual staining (AO/EtBr) of ethanolic extract of Digera muricata treated cells (50µg). The viable cell will possess a uniform bright green nucleus. Early apoptotic cells will have bright orange areas of condensed or fragmented chromatin in the nucleus. Late apoptotic cells will have uniform bright red nucleus.*

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4. DISCUSSION

The current research looked at the ability of *Digera muricata* leaf extracts to induce apoptosis in B16-F10 cells and their pro-apoptotic function for the first time. Anticancer agents are screened using a variety of techniques. The 3-(4,5-dimethylthiazol-2yl)-2,4 diphenyltetrazolium bromide (MTT) assay, which is an easy and effective tool for evaluating anticancer agents, is one of the techniques used. The MTT cell proliferation assay tests the rate of cell proliferation and, alternatively, it evaluates the loss of viable cells as a result of metabolic activities such as apoptosis or necrosis (41). Tetrazolium salt reduction is now generally accepted as a valid method of examining cell proliferation. Dehydrogenase enzymes reduce the yellow tetrazolium MTT to produce reducing equivalents such as NADH and NADPH in metabolically active cells. Spectrophotometric methods can be used to solubilize and quantify the intracellular purple formazan that results. Lower absorbance values suggest a slower rate of cell proliferation, while higher absorbance values indicate a faster rate of cell proliferation (42).

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The results of MTT assay in our study showed that except for 20 µg/ml, the statistical significance was observed in 40, 60, 80, 100 and 200 µg/ml concentration of the plant extract displaying a strong pro apoptotic activity on the skin cancer cell line. The inhibitory concentration (IC^{50}) dose was observed to be 60µg/ml as shown by the results of MTT reduction assays. Another analysis using *D. muricata* crude extract revealed dose dependent inhibition at all concentrations taken, with a negligible decrease in percentage proliferation (90.83%) at 25 g/ml and a significant decrease in percentage proliferation (29.68%) at 250 g/ml (43). Using the MTT assay, several herbals and phytochemicals have been shown to have cytoprotective properties (44). Flavonoids, alkaloids, terpenoids, saponins, coumarins, tannins, cardiac glycosides, and anthraquinones were found in different fractions of the D. muricata (45). In *Digera muricata* methanol extract, the percentages of flavonoids (5.26±0.09), saponins (3.13±0.11), alkaloids (0.64±0.01) and tannins (0.35±0.14) had been determined in a study (46). Previous studies have shown a link between flavonoids and reduced cancer risk, with intake of flavonoid-rich vegetables and fruits (47). As a result, the substantial activity of the plant's methanolic extract may be attributed to the induction of cell death by apoptosis, as shown by our findings.

In addition, morphological modifications were detected using a phase contrast microscope, which revealed a reduction in the number of cells following treatment, as well as cell shrinkage and cytoplasmic membrane blebbing. During apoptosis, the interplay of caspase substrate cleavage determines key morphological changes such as chromatin condensation, nuclear remodeling, and membrane blebbing (48). After a 24-hour treatment with the extract and a positive control, morphological changes such as cell rounding and some sensitive cells detaching from the surface were observed. *Digera muricata* extract induced early apoptotic changes in B16-F10 skin cancer cells, according to these morphological changes.

One effective technique for anticancer drug production is to induce apoptosis in cancer cells. Many substances derived from plants have been studied for their ability to induce apoptosis (49). The apoptosis inducing effect of extract from *Digera muricata* was confirmed by AO/EtBr dual staining assays to see if the extract's anticancer activity was dependent on an apoptotic pathway. In double sequential AO/EtBr staining, the ethanolic extract of *Digera muricata* treated cells (50µg) clearly showed apoptotic morphous such as bright orange areas of condensed or fragmented chromatin in the nucleus indicating the presence of early apoptotic cells while the late apoptotic cells having a uniform bright red nucleus was observed using fluorescent microscope. Previous studies support the findings of this present study. Since apoptosis is considered a priority in the development of anticancer drugs, these findings affirm *Digera muricata* leaves extract as a possible source of chemotherapeutic agents (36, 50-63). This is not an anomaly, as many readily available chemotherapeutic agents and folk medicinal plants induce cell apoptosis to combat cancer (64). However, more rigorous studies are needed to thoroughly validate this claim.

5. CONCLUSION

Within the limitations of the study it can be concluded that the *Digera muricata* plant extract was cytotoxic and induced apoptosis to the cancer cells at 50µg/ml concentration in a 24 hours time point. However more research is needed to understand the mechanisms of anti-cancer potential of this *Digera muricata*.

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