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Cytotoxic Activity of Phenolic Compound Isolated From *Pandanus Odorattissimus* against (MDA-MB-231) Breast Cancer Cell Lines

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***Abstract*-** The present study reports potential activities like cytotoxic, genotoxic and apoptotic activity of phenolic compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate isolated from methanolic extract of *Pandanus odorattissimus*. The compound showed a significant cytotoxic effect on MDA-MB-231 cell line. Exposure of the compound reduced the viability of MDA-MB-231 cells after 24, 48 and 72 hours, the compound exerted a significant cytotoxic effect on MDA-MB-231 cells. The compound also induced significant DNA damage. The results of comet assay with pattern of the MDA-MB-231 cells has shown an intact head and complete absence of DNA fragments in the form of tail suggesting that the doses are not genototoxic. The phenolic compound at concentration of 20 µg/mL, showed an increase in percentage tail of DNA upto 5.87 units when compared to control 5.05.

***Keywords*:** Cytotoxic activity, Comet assay, *Pandanus odorattissimus* L, MDA-MB-231 cells.

I. INTRODUCTION

Cancer is a multi-step disease incorporating physical, environmental, metabolic, chemical and genetic factors [1]. Breast cancer is the most commonly occurring cancer in women, comprising almost one-third of all malignancies [2]. It accounts for approximately 25% of all female malignancies with a higher prevalence in developed countries. Breast cancer is the second leading cause of cancer-related death among females in the world [3].

Development of breast cancer cell targeting drug without affecting the normal cells is a challenging task in the field of cancer drug discovery. The limited success of clinical therapies including radiation, chemotherapy, immunomodulation and surgery in treating cancer, as evident by the high morbidity and mortality rates, which also can produce severe side effects such as bone marrow depression, lecukopenia, anaemia, alopecia, and hyperuricaemia, teratogenicity, carcinogenicity, and also its reduced spermatogensis in men, amenoria in women because of this indication there is an imperative need of new cancer management. Herbal Plants have long been shown to be excellent and reliable sources for the development of novel anti-cancer drugs [4]. Use of natural products and its derivatives in the development of anticancer drugs are increasing all over the world because of lesser side effects as compared to synthetic drugs[5,6,7]. As more than 60% of the chemotherapeutic drugs are developed from plants and their derivatives. Medicinal plants are potential sources of natural products exhibiting anti-proliferation and anti-metastatic properties[8].

Pandanus odoratissimus L is said to be a restorative, deodorant, indolent and phylactic promoting a feeling of well being and acting a counter to tropical lassitude. It may be chewed as a breath sweetener or used as a preservative in foods. It is also said to posses healthful properties, including antiviral, anti-allergy, antiplatelet, anti-inflammatory, antioxidant and antitumor activity [9].

Aim of present study was to evaluate the cytotoxic, genotoxic and apoptotic activity of phenolic compound isolated from *pandanus odoratissimus.*

II. MATERIAL AND METHODS

2. 1. Chemicals

Dimethyl sulfoxide (DMSO), Dulbecco's Modified Eagle's Medium (DMEM), Fetal bovine serum (FBS), Trypan blue, Triton X-100, Ethyl methane suphonate (EMS), Normal Melting Agarose (NMA), Low Melting Point Agarose (LMPA) and Ethidium Bromide (EtBr) were purchased from Sigma Chemicals India Ltd, ion free Phosphate Buffered Saline (PBS) was purchased from Hi-Media, India. 3,5-dinitrosalicylic acid was obtained from Merck. EMS known for mutagenic behaviour was used as positive control.

2. 2. Collection of plant material

Aerial leaf parts of *P. odorattissimus* L. were collected from Gurmitkal, near Gulbarga, north Karnataka, India. The botanical identification was made by Dr. Shiddamallya N, Scientist, National Ayurveda Dietetics Research Institute (NADRI), Bangalore. A voucher specimen was deposited in department (RRCBI- 12749).

2. 3. Preparation of plant extracts

The extract was obtained by infusion and maceration from 200g of plant material. The material was weighed chopped and extracted with solvent. The infusion was prepared with 50 gm of dried leaves in 2 × 200 ml of methanol respective to its temperature and solid matters were removed by filtration. After this preliminary step, the same plant material was extracted in boiling distilled water at the same condition and the maceration was done following the aforementioned process at room temperature 28ºC overnight. The solvent was removed by rotary evaporation. The yield (w/w) of the infusion and maceration of methanol was 3.78 and 1.78 respectively in terms of newly collected plant material.

2. 4. Cytotoxicity assay

The preliminary cytotoxicity analysis was done by using methyl tetrazolium (MTT). A mitochondrial enzyme in the living cells, namely succinate hydrogenase, cleaves the tetrazolium ring, converting the methyl tetrazolium (MTT) to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. The assay detects living, but not dead cells and signal generated is dependent on the degree of activation of the cells. This method was therefore used to measure cytotoxicity, proliferation or activation.

The colorimeter assay that measures the reduction of 3-(4-dimethylthiasol-2-yl)-2,4-diphenyltetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored, formazan product. The cells are then solubilised with an organic solvent (DMSO or isoproponal) and released solubilized formazan reagent is measured spectrophotometrically [10,11].

MDA-MB-231 cells were maintained in RPMI 1640 containing 1 mmol/L L-glutamine, supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) penicillin/ Streptomycin and incubated at 37 ºC in humidified 5% CO2 incubator. To 180 µL aliquots in six replicates of cell suspension (5× 105/mL) seeded to 96 well polystyrene tissue culture plates, 15µL aliquots of compound 4-(4-(3,4-dimethoxyphenyl) hexahydrofuro [3,4-c]furan-1-yl)-2-methoxyphenyl acetate solutions (1.25, 2.5, 5.0, 10, and 20 µg/mL) were added to each well using distilled water as solvent.

Cells incubated in culture medium alone served as a control for cell viability (untreated wells). All chemical exposures were carried in 96 well tissue culture plates for the purpose of chemical dilutions. Cells were placed in the humidified 5% CO2 incubator at 37 ºC for 24, 48 and 72 hours respectively. After incubation 20 µL aliquots of MTT solution (5 mg/mL in PBS) were added to each well re-incubated for 4 hour at 37 ºC, followed by low centrifugation at 800 rpm for 5 minutes. Then, the 200 µL of supernatant culture medium was carefully aspirated and 200 µL aliquots of dimethylsulfoxide (DMSO) were added to each well to dissolve the formazan crystals followed by incubation for 10 minutes to dissolve air bubbles.

The culture plate was placed on a Biotex Model micro-plate reader and the absorbance was measured at 550 nm. The amount of colour produced is directly proportional to the number of viable cells. All assays were performed in six replicates for each concentration and means ±
SD values were used to estimate the cell viability.

The cell viability rate was calculated as the percentage of MTT absorption as follows:

2. 5. Cell viability assay

For the determination of cytotoxic activity of compound, viable cell numbers were determined using the trypan blue exclusion method [12]. Trypan blue dye exclusion was added to all cultures in a ratio of 1:1. The extract treatments and preparations

were examined under the standard light microscope at 100× magnification. In principle, viable cells exclude an acid dye such as trypan blue; its uptake is indicative of irreversible membrane damage proceeding cell death. The ratio of live cells to dead cells (cell viability) was also determined. Standard curves were prepared and 50% cytotoxic concentrations of extracts (CC50) which caused a 50% decrease in cell viability were derived.

2. 6. Determination of DNA damaging effects of the extracts (Alkaline comet assay)

The comet assay is a versatile and sensitive method for measuring single-strand and double-strand breaks in DNA [13]. Among the variety of methods developed for detecting DNA damage, comet assay or single cell gel electrophoresis (SSGE) assay is often used since it is fast, convenient and easy to apply. It is particularly attractive as a method to undertake *in vivo* and *in vitro* studies [14,15]. The genotoxic effect of the extracts was determined by alkaline comet assay.

The cell pellets were obtained by centrifugation (5000 rpm, 5 mins) which were again resuspended in 100 µL of PBS. To this 100 µL of 1% LMPA (low melting point agar) was added, mixed well at 37 ºC and finally layered on top of the end frosted slides that were precoated with 1% normal melting point agarose. Now cover slips (24 mm X60 mm) were placed on top of the slides and were kept at 4 ºC. After 5 mins the cover slips were removed and 100 µL of 0.5% LMPA was again layered on top of the slides before placing the cover slips back and keeping the slides at 4 ºC again. After overnight lysis at 4 ºC in freshly prepared lysing solution (2.5 M of NaCl, 100 mM of EDTA, 10 mM of Tris and 1% Triton X-100, pH 10) slides were kept in electrophoretic unit, filled with chilled and freshly prepared electrophoresis buffer (1 mM Na2EDTA and 300 mM NaOH, pH 13).

The slides were left in (the electrophoresis solution) for 30 mins to allow unwinding of DNA. Following the unwinding, electrophoresis was performed for 30 mins at 0.7V/cm using a power supply. To prevent DNA damage from stray light, all the steps starting from single cell preparation were performed under dim light. After electrophoresis, the slides were immediately neutralized with 0.4M of Tris buffer (pH 7.5) for 5 mins each. The slides were then stained with EtBr (20 mg/mL:75 µL pre slide) for 10 mins in dark. After staining, the slides were dipped once in the chilled distilled water to remove the excess stain and subsequently, fresh cover slips were kept over them.

The slides were examined with 3-4 hours, using an image analysis system attached to fluorescent microscope. The image was transferred to a computer through a charge couple device camera and analyzed using komet 5.0 software. All the experiments were conducted in triplicates and the slides were prepared in duplicates. 25 cells per slide equaling 150 cells per group were randomly captured at a constant depth of the gel, avoiding the cells present at the edges and superimposed comets.

III. RESULTS

Structure of the compound

4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate

(**Image -1**).

3. 1. MTT assay

The MTT assay to examine the cytotoxic effect of phenolic compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on MDA-MB-231 cells for 24, 48 and 72 hours, respectively. Data generated from these studies clearly indicates that exposure of the compound 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate exposure significantly reduced the viability of HL-60 cells. After 24, 48 and 72 hours of exposure, the compound exerted a significant cytotoxic effect on MDA-MB-231 cells, showing LD50 values of 12.24 ± 0.5 µg/mL for 24 h **(Graph - A)**, 10.25 ± 0.3 µg/mL for 48 h **(Graph - B),** and 5.2 ± 0.6 µg/mL for 72 h **(Graph - C)**. The effect of phenolic compound seems to be time dependent with regard to cell viability in different doses of compound it was compared with control **(Graph - D)**.

3. 2. Comet Assay

Cytotoxicity assessment is way to find out the immediate response of cells, which come in contact with a foreign particle where as the genotoxicity of those reveals a long term response. Thus it is very important to investigate the genotoxicity of those concentrations that revels the cytotoxicity. The figures show the percentage DNA pattern of MDA-MB-231 cells treated with different concentrations of isolated compound. The percentage of DNA value of control cells was recorded to be 5.05 units. There was a very slight increase in the samples treated with particles shows value percentage of DNA at 5.20, 5.35% units, 5.42 units, 5.68 units and 5.87 units corresponding to 1.25 µg/mL, 2.50 µg/mL, 5.0 µg/mL, 10.0 µg/mL and 20.0 µg/mL respectively of phenolic compound. The resultant comet pattern of the MDA-MB-231 cells also showed an intact head and

complete absence of DNA fragments in the form of tail **(Fig a, b, c, d, e & f)**, suggesting that the doses were not genototoxic. The metabolic compound at 20 µg/ml, concentration showed increase in percentage tail DNA 6.01 units when compared to control 5.05. This increase in percentage of tail DNA pattern was also found non significant when analyzed with ANOVA **(Image - II)**.

IV. DISCUSSION

In the present study, we have examined the cytotoxic effect of phenolic compound on MDA-MB-231 cells. Data from this study clearly indicates that the phytochemical compound isolated from methanolic extract is highly cytotoxic to MDA-MB-231 cells, showing LD50 values of 12.24 ± 0.5 µg/mL, 10.25 ± 0.3 µg/mL and 5.2 ± 0.6 µg/mL for 24, 48 and 72 hours of exposure, respectively. Recently, it has been observed that the compound is cytotoxic to human liver carcinoma (HepG2) cells, showing an LD50 of 8.55 ± 0.58 µg/ml after 72 hours of exposure [16]. We found that low dose of the compound has induced minimal toxicity in MDA-MB-231 cells upon 72 hours of exposure. Interestingly, such doses are similar to the therapeutically effective concentrations of compound which have been shown to induce remission in APL patients with minimal toxicity. Clinically, the standard dose for the treatment of patients with APL is 0.15 mg/kg per day which yields a maximum dose of 2-3 µM of compound in the plasma. High levels of the compound (5 µg/mL and higher for 72 hrs) induce more than 50% of cell mortality [17].

In the present study, the results indicate that high levels of phenolic compounds exposure of MDA-MB-231 cells has inhibited cell proliferation and induced mortality in a dose and time - dependent manner. Such effects have been observed with other test models, as well as clinically studied [18,19]. Current study proves the potentiality of isolated compound as anticancerous agent.

V. CONCLUSIONS

4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate when checked for the cell viability in duration of 24, 48 and 72 hours, duration the 48 hrs duration has shown a significant inhibition of cell viability. Thus the inhibition of cancer after the treatment of pure extract of phenolic compounds on the MDA-MB-231 cell line indicates that the compound is a potent drug for the cancer treatment. The phenolic compounds exposure of MDA-MB-231 cells has inhibited cell proliferation and induced mortality in a dose and time - dependent manner.

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CONFLICT OF INTEREST

The authors declare no conflict of interest in this article.

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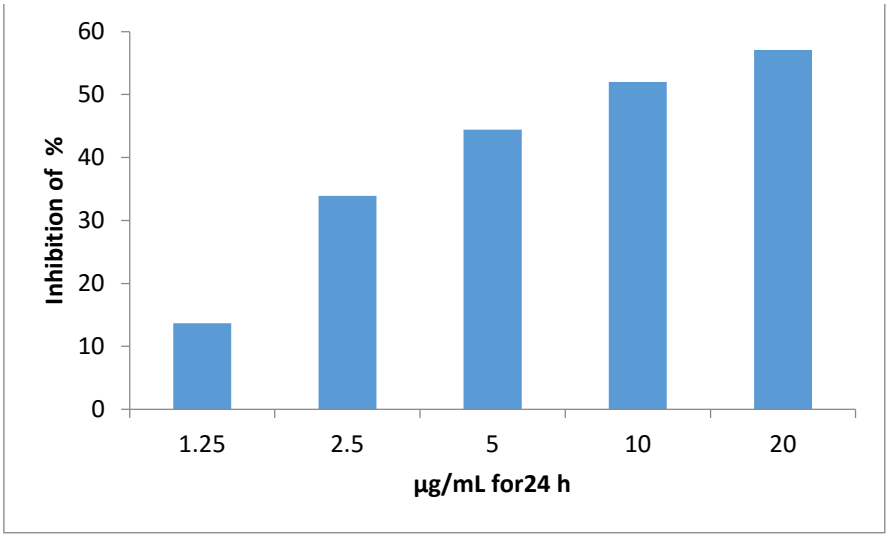
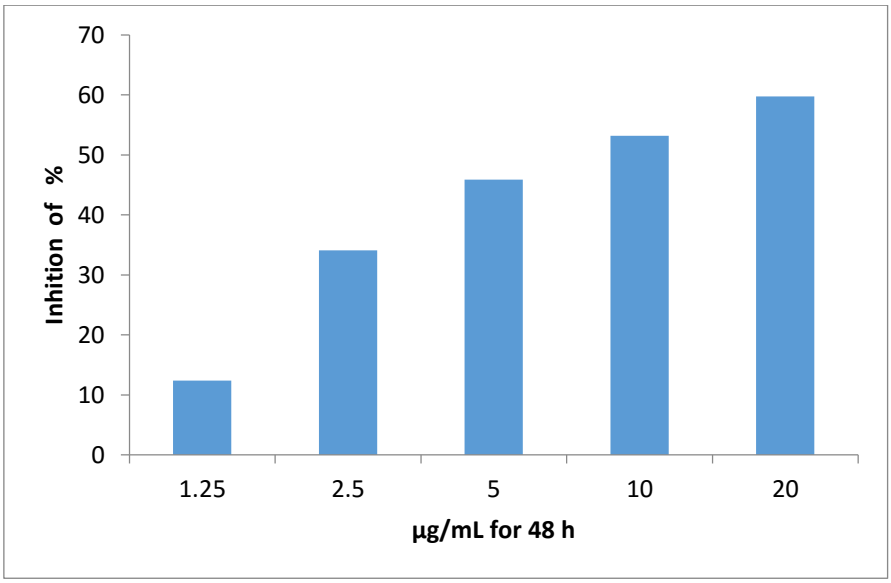
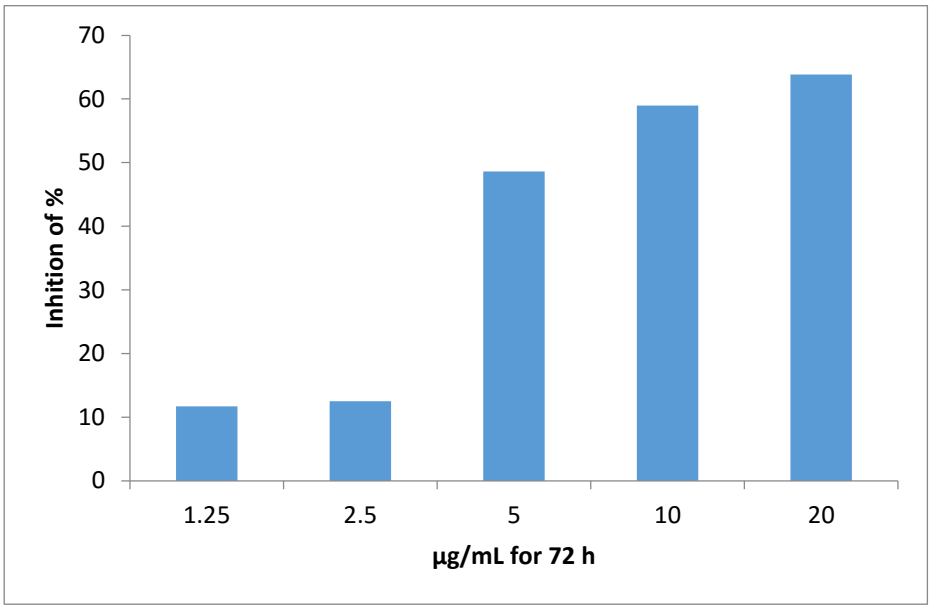


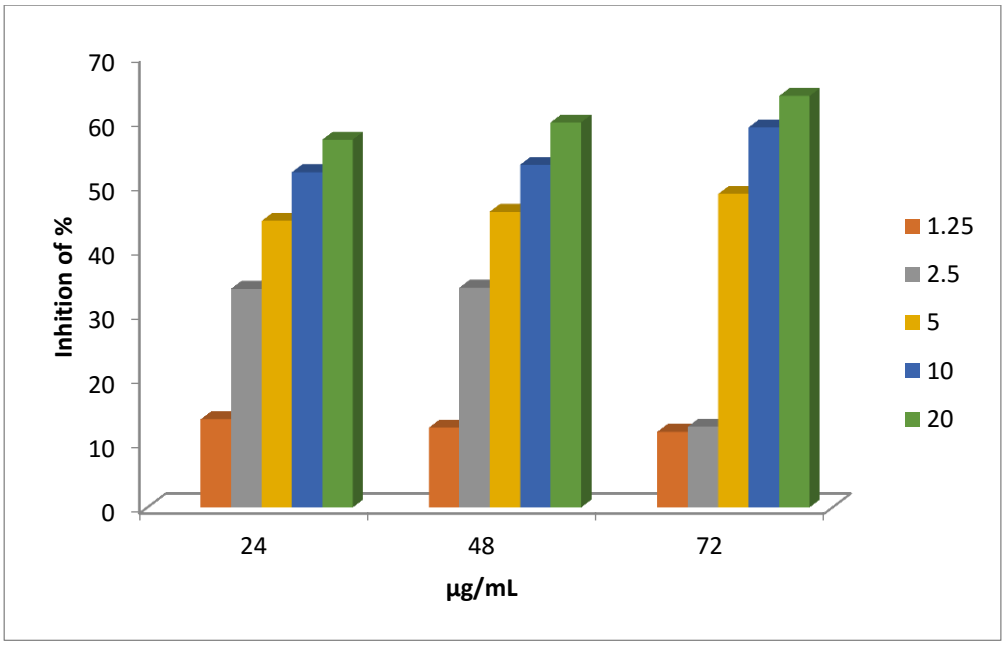
Graph 1: The cytotoxic effect of phenolic compound 4-(4-(3,4-dimethoxyphenyl) hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on MDA-MB-231 cells for 24 hours.



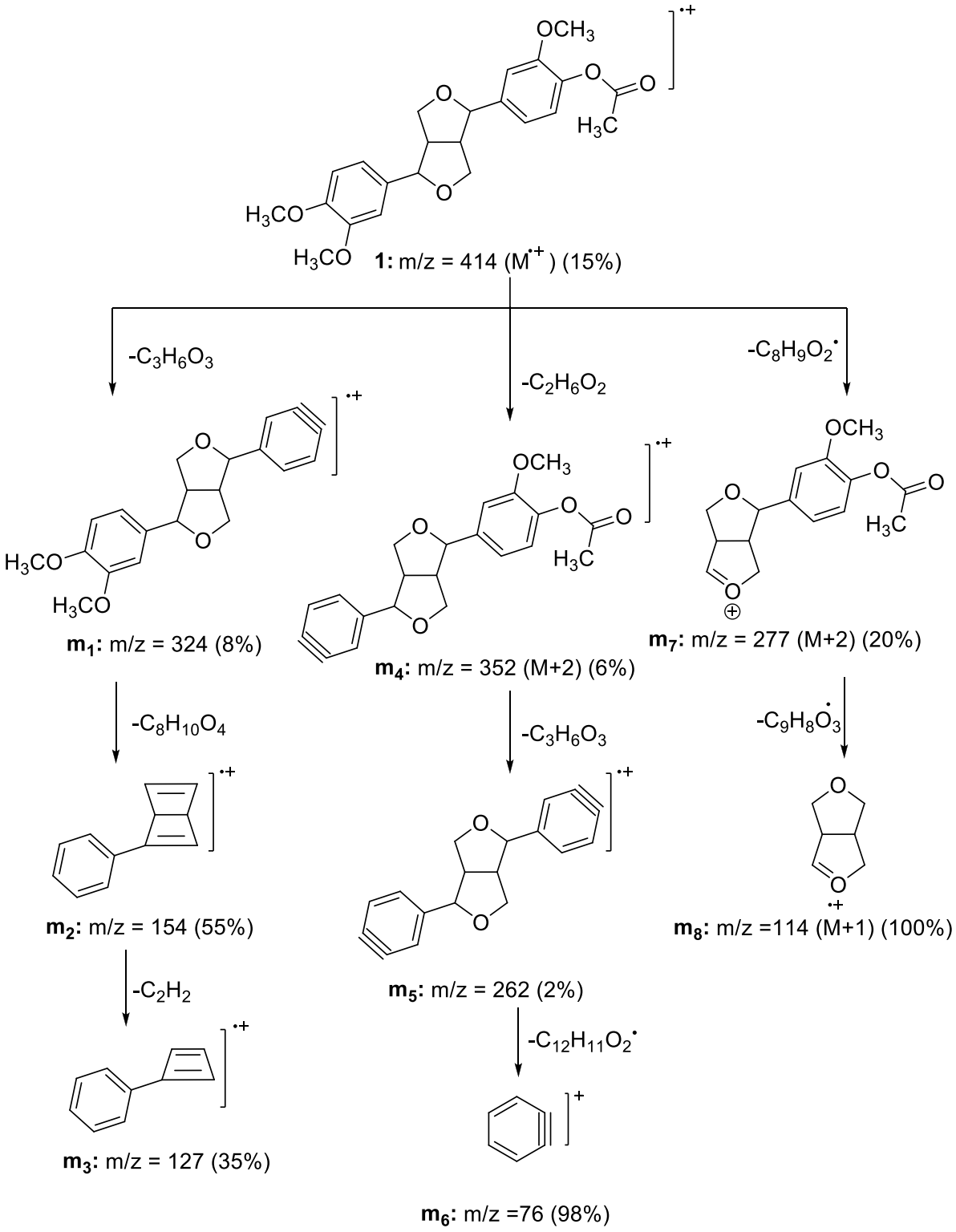
Graph 2: The cytotoxic effect of phenolic compound 4-(4-(3,4-dimethoxyphenyl) hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on MDA-MB-231 cells for 48 hours.



Graph 3: The cytotoxic effect of phenolic compound 4-(4-(3,4-dimethoxyphenyl) hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on MDA-MB-231 cells for 72 hours



Graph 4: The cytotoxic effect of phenolic compound 4-(4-(3,4-dimethoxyphenyl) hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate on MDA-MB-231 cells for 24, 48 and 72 hours



(Scheme-1)

Image I: 4-(4-(3,4-dimethoxyphenyl)hexahydrofuro[3,4-c]furan-1-yl)-2-methoxyphenyl acetate

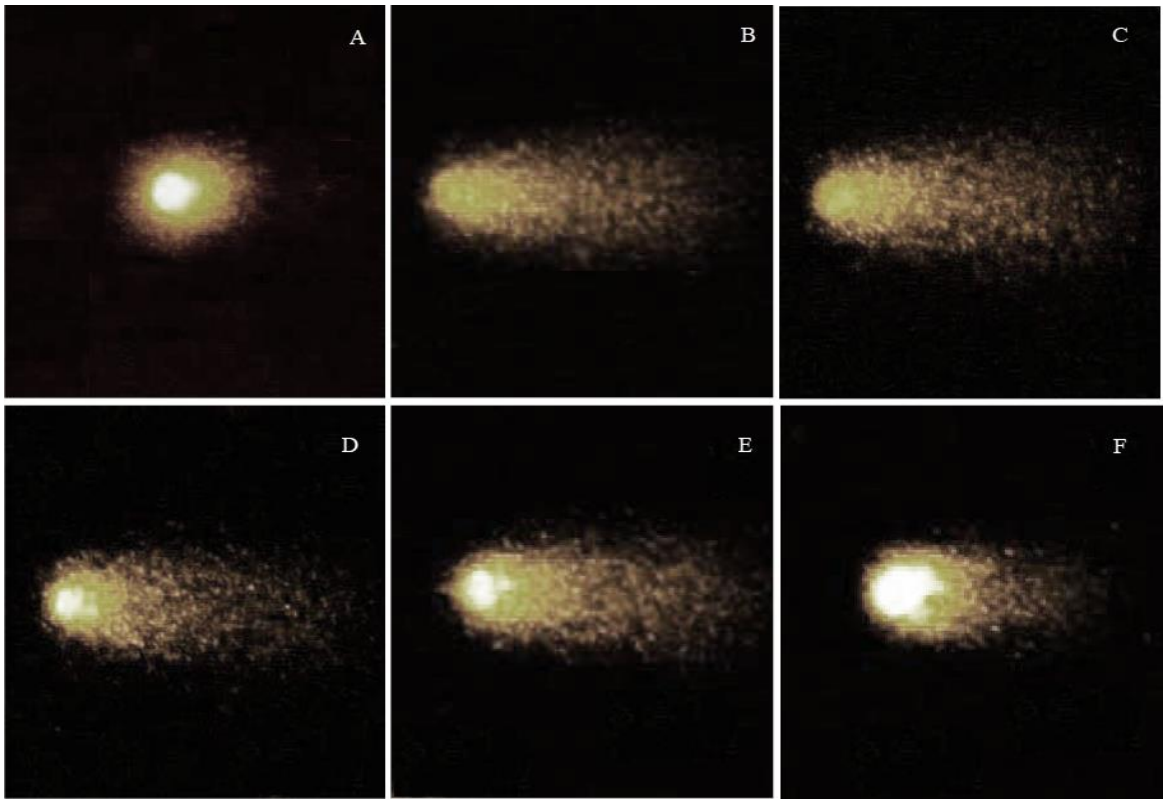


Image II: The figures show the percentage DNA pattern of MDA-MB-231 cells treated with different concentrations of isolated compound. A = control, B =1.25 µg/mL, C = 2.50 µg/mL, D = 5.0 µg/mL, E = 10.0 µg/mL and F = 20.0 µg/mL respectively of phenolic compound.

