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Research Article

EVALUATION OF GROWTH INHIBITORY POTENTIAL OF *MORINGA OLEIFERA* FLOWERS ON PC3 CELL LINES

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ABSTRACT

Objective: PC3 cell lines are the classical *in vitro* androgen-independent models of prostate cancer with high metastatic potential. *Moringa oleifera* is a predominant Indian nutritional plant with high medicinal value. The objective of the study was to investigate the flowers of this traditional plant for their potential to inhibit the growth of cancer cells in PC3 cell lines. Methods: The growth response of cancer cells to the methanol extract of *M. oleifera* flowers was analyzed by MTT (3-4,5-dimethylthiazol-2-yl 2,5-diphenyl tetrazolium bromide) assay and compared to that of known common standard therapeutics. Similarly, the plant material was also tested for its effect on normal cells. Results: While Turmeric and Curcumin showed cancer cell growth inhibition at higher concentrations, *M. oleifera* flower extract exhibited a gradual dose-dependent decrease in the percentage of cancer cell growth from 0.01µg/ml onwards with its concentration being 46.91 µg/ml for fifty percent growth inhibition (GI_{50}). Also, while the plant extract did not affect the cell viability of normal cells, the other two showed insignificant changes. Conclusion: The results suggested the presence of potent anticancer compounds in the *M. oleifera* flower extract, non-toxic to normal cells, but responsible for its effective growth inhibition of PC3 cells. This further signified the application of *M. oleifera* flowers as the efficacious source of natural therapeutics against androgen-independent prostate cancer.

Keywords: PC3 cell lines, androgen-independent prostate cancer, Moringa oleifera, MTT assay, GI50

INTRODUCTION

There is an accepted relationship between oxidative stress and physiopathology of several chronic diseases. Therefore, the plant materials with potent antioxidant activities and protective phytochemicals may be relevant for the treatment of oxidative stress related diseases such as cancer. Though boosting up the immune system can be protective against cancer, the presence of antioxidants exhibit an additional anti-carcinogenic effect by offering protection against Deoxyribonucleic acid (DNA) damage [1].

Moringa oleifera Lam. is a multifunctional versatile plant with an impressive range of economic, health and nutritional potentials. Folk medicine supports the use of *M. oleifera* flowers for treating cancerous tumors [2].The methanolic extract of *M. oleifera* flowers has been shown to have significant antioxidant capacity [3]. Having known this fact, it is decided to investigate further its effect on the human prostate cancer (PC3) cell lines which are the classical *in vitro* androgen-independent models of prostate cancer with high metastatic potential [4,5]. These cell lines seem to be useful in investigating the biochemical changes in advanced staging of the disease and in assessing their response to chemotherapeutic agents. The type of cancer cells involved and their innate differences from healthy cells are at the crux of any cancer therapy or diagnostic approach [6]. Uncontrolled cellular proliferation being a fundamental aberration in cellular behavior during carcinogenesis, growth rate measurement seems to be one of the important indicators for cancer treatment.

Curcumin and its source turmeric are used for comparison in the study. Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-hepata diene-3,5-dione; diferulolylmethane] is a major constituent of the yellow spice turmeric derived from the rhizomes of *Curcuma* species. For centuries, it has been used in Asian food [7]. The ethanobotanical, pharmacognostic, phytochemical and pharmacological properties of turmeric and Curcumin have been extensively reviewed [8]. Although a few mechanisms including inhibition of tyrosine kinases, sensitization to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mediated apoptosis, have been implicated in the biological effects of Curcumin [9,10], its primary molecular target and mechanism of action remain to be clarified.

The present study is undertaken for the first time to investigate the effect of the methanol extract of the *M. oleifera* flowers on the growth of PC3 cells which may further facilitate an insight into the anticancer potential of this plant material against androgen-independent prostate cancer.

MATERIALS AND METHODS

Plant material and extraction

Methanol extract of *M. oleifera* flowers collected from Palayamkottai, Tamilnadu, India, with better antioxidant potentials and higher content of phytochemicals was the subject of the study [3]. Fresh flowers of *M. oleifera* were collected during the season February to March from the farms of Palayamkottai. They were identified and authenticated by Dr. Sasikala Ethirajulu, Assistant Director (Pharmacognosy), Siddha Central Research Institute, Chennai. The flowers were cleaned and shade dried for 5 days. The dried material was powdered and subjected to direct extraction.
Using Direct method of extraction [11], 10 grams of air dried powder of the flower sample was extracted with 100 ml of methanol in shaking condition. The process was repeated 3 times with the same material but using fresh solvent. The solvent was removed by condensation. The extracted residue was used for analysis.

Cell lines and culture conditions

The human prostate cancer PC3 cell lines and Vero cell lines derived from the kidney of African green monkey, the commonly used mammalian normal cell lines were purchased from National Centre for Cell Sciences (NCCS), Pune. The cells were grown in 25cm × 25cm × 25cm tissue culture flasks containing Dulbecco's Modified Eagle Medium (DMEM) /Hams F12 nutrient mix as culture medium supplemented with 10% Fetal Calf Serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin and grown at 37 °C under a humidified atmosphere of 95% air and 5% Carbon-di-oxide (CO_2).

Cells were regularly passaged and maintained before including for the experiment.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [12]

MTT assay was performed to study the growth response of the cancer cells in PC3 cell lines as well as the cell viability of normal Vero cells, to the methanol extract of *M. oleifera* flowers and compare them with that of the standard compound, Curcumin and its natural source, Turmeric. The method described by Carmichael was followed and the percentage of cell viability was based on the determination of accumulated formazan derivative in treated cells at 570 nm with respect to the untreated ones.

When the cell density in the culture flask reached 70-80% confluence, the cells were trypsinized, seeded in 96-well plates at varying cell number according to the size and shape of the cell, between 5000 and 10000 cells per well in 100 µL and incubated for 24 hours in CO_2 incubator. Test samples of dilutions 100, 10, 1, 0.1, 0.01 µg/ml were added to the cells in 100 µL volume. Dimethyl sulphoxide (DMSO) was the solvent used to maintain the concentrations. The plates were further incubated for 48 hours in the CO_2 incubator. MTT solution was composed of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide at 5 mg/ml in phosphate buffered saline (1.5 mM Potassium dihydrogen phosphate, 6.5 mM Disodium hydrogen phosphate, 137 mM Sodium chloride, 2.7 mM Potassium chloride; pH 7.4), from this solution 50µl was pipetted out into each well. The plate was further incubated for 2.30 hours and the medium was carefully decanted. The formazan crystals were air dried in dark place and dissolved in 100 µL DMSO. The plates were mildly shaken at room temperature and the absorbance was measured at 570 nm using Synergy H4 microplate reader (BioTek USA) with Gen5 software. Measurements were taken in quadruplicates.

From the optical density (OD) values, the percentage growth of the PC3 cells with the addition of the samples was calculated based on the formula: Percentage growth = $100x[(T-T_0)/(C-T_0)]$ if T was greater than or equal to T_0. If T was less than T_0, Percentage growth = $100x[(T-T_0)/T_0)]$, where T was the OD of the test, T_0 was the OD at time zero and C was the OD of the negative control. From the percentage growth, a dose response curve was generated and concentration at 50% growth inhibition (GI_{50}) values of the test samples along with concentration at total 100% growth inhibition (TGI) and lethal concentration at -50% growth inhibition (LC_{50}) values were interpolated from the growth curves.

Percentage of cell viability of Vero cells was calculated using the formula:

% cell viability = $Absorbance_{570}$ of treated cells / $Absorbance_{570}$ of control cells x 100.

RESULTS AND DISCUSSION

Effect on cell viability

MTT assay was employed to assess cell viability after exposure to test material of interest. The assay was based on the ability of mitochondrial succinate-tetrazolium reductase system to convert yellow tetrazolium salt to purple formazan dye. Reports of previous studies had suggested the application of this assay for the quantitation of growth modulating effects on cultured prostate cancer cell lines and a dose-dependent reduction of MTT converting activity with the treatment of antineoplastic agents [13]. These studies highlighted significant correlations of the MTT results with that of thymidine incorporation assay and direct DNA measurements. They also revealed the suitability of MTT assay to large-scale chemosensitivity testing and discrimination between cytostatic and cytotoxic drug effects providing additional information on the mode of action of the drugs tested, with a high degree of precision and ease. Such applications of MTT assay were reflected in the results of the present study.

Absorbance values of the test samples with Turmeric, Curcumin and *M. oleifera* flower extract in the dilution range of 100, 10, 1, 0.1, 0.01 µg/ml and that of the respective negative controls on PC3 cells were obtained as presented in the Table 1. The absorbance values seemed to increase gradually with the dilution of *M. oleifera* flower extract from 100 µg/ml to 0.01 µg/ml, except at 1 µg/ml and 0.1 µg/ml where the values did not show significant discrepancy. But irregularities with a sudden decrease were observed in the lower concentration range in case of Turmeric (0.1 µg/ml) and Curcumin (0.01 µg/ml). Though the absorbance values varied significantly among each sample for different concentrations and between samples for higher concentrations (100 µg/ml and 10 µg/ml), they did not show significant difference at lesser concentrations (1 µg/ml, 0.1 µg/ml and 0.01 µg/ml) between the samples.

Table 1: Absorbance values of Negative Control and Test (T) on PC3 cells

Content/Sample	Turmeric	Curcumin	*M. oleifera* flowers
T(100 µg/ml)	0.250 ± 0.014	0.066 ± 0.006	0.287 ± 0.010
T(10 µg/ml)	0.402 ± 0.009	0.355 ± 0.007	0.373 ± 0.006
T(1 µg/ml)	0.416 ± 0.012	0.414 ± 0.010	0.408 ± 0.008
T(0.1 µg/ml)	0.404 ± 0.023	0.417 ± 0.020	0.408 ± 0.006
T(0.01 µg/ml)	0.405 ± 0.044	0.416 ± 0.010	0.417 ± 0.007
Negative Control	0.430 ± 0.031	0.430 ± 0.020	0.413 ± 0.008

Results are mean ± SD of quadruplicates. T values are statistically significant at $**p<0.01$ when compared between different concentrations, but between samples, p=0 at 100 µg/ml and 10 µg/ml but p>0.1 for other concentrations. Absorbance at Time zero (T_0) = 0.220 ± 0.008.

Based on the absorbance values, the percentage growth of PC3 cells was predicted for the given concentration range of the samples added and plotted as shown in Fig. 1. These values showed significant difference between the samples only at 100 µg/ml but not at lesser concentrations. In general, the percentage growth curve seemed to be dose dependent but irregularities were noted in case of Curcumin and Turmeric at lesser concentrations as like their absorbance values.

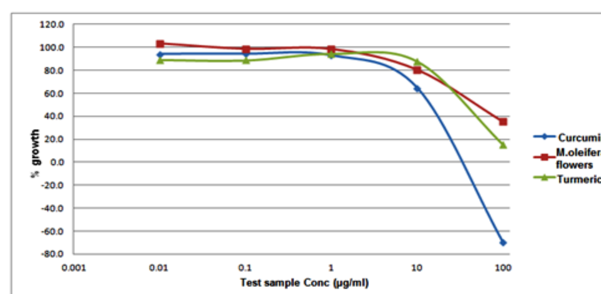

Fig. 1: Percentage growth of PC3 cells against test samples at different concentrations

From the percentage growth values, the remaining percentage was taken as the percentage of PC3 cell growth inhibition by test samples. Concentration of the test samples at 50% growth inhibition (GI_{50}), 100% growth inhibition (Total growth inhibition TGI) and -50% growth inhibition (Lethal concentration LC_{50}) were determined from the percentage growth graph. For Curcumin, GI_{50}, TGI and LC_{50} values were found to be 12.83 µg/ml, 30.20 µg/ml and 71.10 µg/ml respectively. GI_{50} values of Turmeric and *M. oleifera* flower extract were 33.19 µg/ml and 46.91 µg/ml respectively while their TGI and LC_{50} values were above 100 µg/ml. Growth inhibitory concentration values of *M. oleifera* flower extract was closer to that of Turmeric than Curcumin. This indicated that the crude extract of the plant material inhibited the growth of cancer cells at greater concentrations compared to the active principle compounds present in them. This further suggested a better and greater inhibition of PC3 cell growth expected by the active principle compounds present in the methanol extract of *M. oleifera* flowers.

While observing the percentage growth values at various concentrations of the test samples, a few interesting points were noted. *M. oleifera* flower extract showed a gradual dose dependent decrease in percentage growth of PC3 cells from 0.01µg/ml to100 µg/ml. But, in case of Turmeric, the percentage growth increased from 0.01µg/ml to 1 µg/ml and then decreased drastically. Similarly, in case of Curcumin, the percentage growth increased from 0.01 µg/ml to 0.1 µg/ml and then decreased. The value decreased abruptly at 100 µg/ml showing a negative value of -70.23 ± 2.48%. This suggested the predominance of cytotoxicity over cytostasis with the application of Curcumin. This was further proved by the morphological changes observed with the growth inhibition of the PC3 cells by Turmeric, Curcumin and *M. oleifera* flower extract as depicted in Fig. 2, Fig. 3 and Fig. 4 respectively. Variations were observed both in the shape and number of cells. A steady decrease in the cell count with very little changes in the cell shape was observed with the increase in concentration of *M. oleifera* flower extract. But the shape of the cells shrank tremendously in case of Turmeric and Curcumin with the increasing concentrations. The cell shrinkage was maximum at 100 µg/ml of Curcumin. Thus, Curcumin might be capable of destroying the prevailing cancer cells rather than preventing or inhibiting their further growth. But for controlling the growth of highly metastazing cells like PC3 cells, cytostatic bioactives which could inhibit cancer cell growth might be more beneficial than the cytotoxic bioactives which could destroy the cancer cells. Also, the probability of risk associated with the cytotoxic therapeutics on normal cells seemed to be high as evidenced in the observation of these chemicals on normal cells. Based on these observations, it could be considered that *M. oleifera* flower extract was cytostatic and not cytotoxic even at 100 µg/ml and therefore could be more suitable to manage PC3 cells than Turmeric or Curcumin.

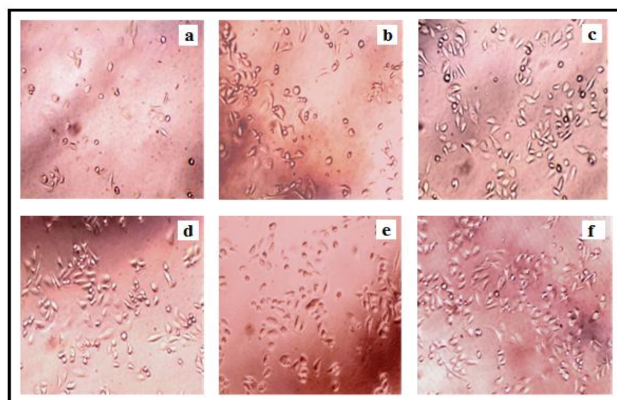

Fig. 2: Growth inhibition of PC3 cells by Turmeric at (a)100µg/ml (b)10µg/ml (c)1µg/ml (d)0.1µg/ml (e)0.01 µg/ml as seen through inverted microscope at 10X magnification. (f)Negative control.

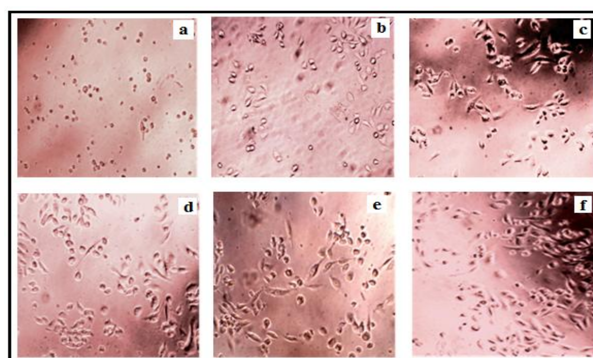

Fig. 3: Growth inhibition of PC3 cells by Curcumin at (a)100µg/ml (b)10µg/ml (c)1µg/ml (d)0.1µg/ml (e)0.01 µg/ml as seen through inverted microscope at 10X magnification. (f)Negative control.

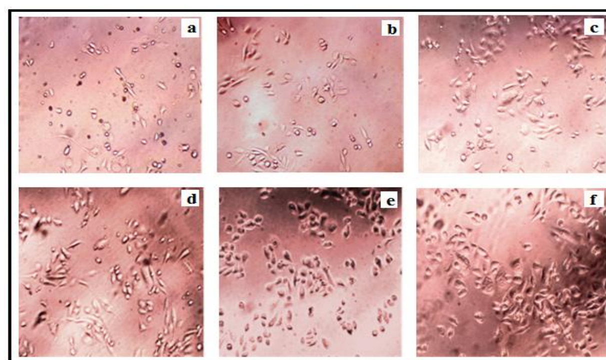

Fig. 4: Growth inhibition of PC3 cells by *M. oleifera* flower extract at (a)100µg/ml (b)10µg/ml (c)1µg/ml (d)0.1µg/ml (e)0.01 µg/ml as seen through inverted microscope at 10X magnification. (f)Negative control.

On observing the effect of the test samples on normal cells, a few more interpretations were made supporting the above finding. It was found that all the three extracts tested on normal cells did not show significant changes in the cell viability percentage values till 1µg/ml. But, while these values remained almost the same even for higher concentrations (10µg/ml and 100µg/ml) of *M. oleifera* flower extract, a minor reduction was observed at these concentrations of Turmeric as well as Curcumin as given in Table 2. The decrease in the cell viability was more with Curcumin than Turmeric. Also, while the morphology of the cells remained unchanged with the addition of *M. oleifera* flower extract at all concentrations, there were notable changes with the addition of Turmeric and Curcumin at 10µg/ml and 100µg/ml as shown in Fig. 5. When the values were analyzed statistically, significant difference was observed only in case of Curcumin among all concentrations (p=0.016) as well as among higher concentrations (p=0.056). But on comparing the three samples, the values were not statistically significant at all the concentrations studied. These results suggested that though the changes were not major, they reflected the possible toxicity associated with Curcumin at higher concentrations. Also, they highlighted the non-toxic nature of the *M. oleifera* flower extract on normal cells even upto 100 µg/ml.

Results are mean ± SD of quadruplicates. When compared between different concentrations (0.01-100µg/ml), p=0.383 for Turmeric, p=0.016 for Curcumin and p=1 for *M. oleifera* flowers. When compared between higher concentrations (1-100µg/ml), p=0.287 for Turmeric, p=0.056 for Curcumin and p=0.998 for *M. oleifera* flowers. When compared between samples, p=0.999 for 0.01µg/ml, p=1 for 0.1µg/ml, p=0.999 for 1µg/ml, p=0.409 for 10µg/ml and p=0.090 for 100µg/ml.

Table 2: Effect of test samples on the cell viability of Vero cells

Test Sample	Percentage cell viability				
	100μg/ml	10μg/ml	1μg/ml	0.1μg/ml	0.01μg/ml
Turmeric	95.44 ± 4.20	97.23 ± 3.72	100.20 ± 4.10	100.10 ± 4.70	100.19 ± 3.90
Curcumin	92.22 ± 3.72	95.36 ± 4.90	100.30 ± 3.41	100.19 ± 2.56	100.22 ± 2.78
M.oleifera flowers	100.26 ± 2.90	100.18 ± 3.63	100.30 ± 3.22	100.13 ± 3.90	100.32 ± 3.10

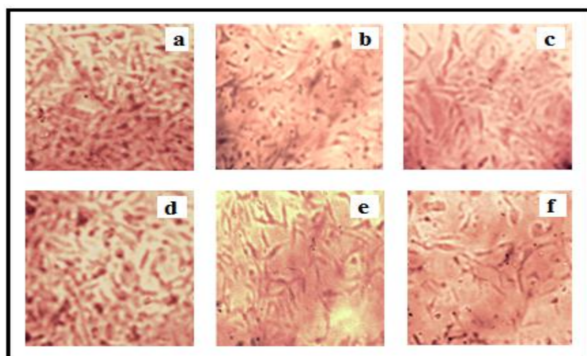

Fig. 5: Changes in the morphology and cell viability of Vero cells by Turmeric at (b)10μg/ml (c)100μg/ml, and by Curcumin at (e)10μg/ml (f)100μg/ml (a)Negative control for Turmeric, and (d) Negative control for Curcumin.

PC3 cell growth inhibition

The results observed suggest that the growth inhibitory activity of the methanolic extract of *M. oleifera* flowers on PC3 cells may be due to its antioxidant potential [3]. This is supported by reports which explain that antioxidants can prevent tumor initiation and act as protective agents [14]. Previous findings also have supported the existence of correlation between antiproliferative effects and antioxidant activities [15,16].

The interesting growth inhibitory activity proves that the methanolic extract of *M. oleifera* flowers is a promising source of bioactive compounds. The growth inhibition of PC3 cells without any significant change in the normal cells may be due to the sensitivity of the PC3 cell line to the active compounds in the methanolic extract of *M. oleifera* flowers or to the tissue specific response of the extract [17]. As far it is known, there are no reports on the growth inhibitory activity of this plant material on PC3 cells.

A balance between cell growth, cell differentiation and cell death through apoptosis, is essential for normal cellular activity. But, this balance is greatly disturbed for cancer cells which results in uncontrolled cell growth. The growth reduction of PC3 cells by the methanol extract of *M. oleifera* flowers reflects a certain shift of the signaling balance in the treated cells.

It is known that many plant-derived components modulate ERK (Extracellular signal-regulated kinases) activities in order to elicit their antineoplastic actions [18,19,20]. Therefore, it may be speculated that since the methanolic extract of *M. oleifera* flowers can inhibit the growth of PC3 cells, it may bring about changes in ERK activity which may be responsible for the growth inhibitory effects.

Earlier studies have been attempted to study the effect of ethanolic extract of *M. oleifera* on HL-60, CEM, HCT-8 and B-16 tumor cell lines. The effect seems not to be related to membrane disruption and is found to be inactive in the mouse erythrocyte hemolytic assay. The extract also seems to be inactive in the brine shrimp lethality assay [21].

Previous findings have shown that 4-(4'-*O*-acetyl-a-L-rhamnopyranosyloxy)benzyl isothiocyanate and the related compound niazimicin, obtained from *M. oleifera* are potent inhibitors of phorbol ester (TPA)-induced Epstein-Barr virus early antigen activation in Burkitt's lymphoma cells [22,23]. Isothiocyanates 1 and 2 from *M. oleifera* seeds are also evaluated for their growth inhibitory effects on A549 non-small cell lung adenocarcinoma and HCT 116 colon carcinoma cell lines [24]. It is also found that ingestion of *Moringa* seed pod extracts can prevent skin tumor [25]. Organic solvent extracts of the dried leaves of *M. oleifera* are shown to have antitumor effects on myeloma cells [26]. Another study has proved the anticancer potentials of the aqueous extracts of *M. oleifera* on HeLa cells with the IC_{50} value of 70 μg/ml [27]. A more recent study has indicated the anticancer activity of ethanolic extract of *M. oleifera* alone and in combination with doxorubicin on HeLa cancer cells with the strongest activity shown by the combination of 250 nM doxorubicin and 250 μg/mL extract [28]. Even though documentation for the anticancer activity of *M. oleifera* is available, the detailed mechanism of action and its role in prostate cancer therapy are yet to be scientifically elucidated.

Potential for Differentiation therapy

The conventional therapies for cancer are often non-specific and highly toxic. A potentially less toxic approach which is now beginning to show translational promise in the clinical setting is the 'differentiation therapy'. This approach is based on the effect of the desired drug that causes the malignant cells to undergo terminal differentiation instead of killing the tumor cells [29]. In this therapy, cells of most tumors, including prostate cancers, are blocked at an early stage of cellular differentiation and that certain agents can bypass or correct this block *in vitro* [30]. These studies have been found to be promising in the treatment of human myeloid leukemia [31]. Such systems with PC3 cell lines have been described [32]. The therapy is highly proved in the treatment of acute promyelocytic leukemia with all-trans retinoic acid [33]. The therapy may provide an alternative for treatment of cancers that do not respond to hormonal manipulations or cytotoxic chemotherapy. From the observations in the present study, it may be suggested that the methanol extract of *M. oleifera* flowers may induce differentiation which further enable the cancerous cells to be more sensitive to hormonal therapy.

Earlier records have indicated that treatment of cancerous cells with non-toxic differentiation inducers have resulted in dose-dependent inhibition of cell proliferation with no significant inhibitory effects on normal cells or skin fibroblasts [34]. These suggest that nontoxic differentiation inducers present in the methanol extract of *M. oleifera* flowers if used alone or in combination with other antitumor agents, may possibly provide a feasible approach for the treatment of advanced prostate cancer.

CONCLUSION

This study clearly demonstrates the potent growth inhibitory effect of the *M. oleifera* flowers on PC3 cells, besides their non-toxicity to normal cells. Although *in vivo* studies may be warranted to validate the efficacy, this experimental design has predicted a promising potential to treat androgen-independent prostate cancer with the bioactives in the methanol extract of *M. oleifera* flowers.

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