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ORIGINAL ARTICLE

Anticancer Potential of *Moringa oleifera* Flower Extract in Human Prostate Cancer PC-3 Cells via Induction of Apoptosis and Downregulation of AKT Pathway

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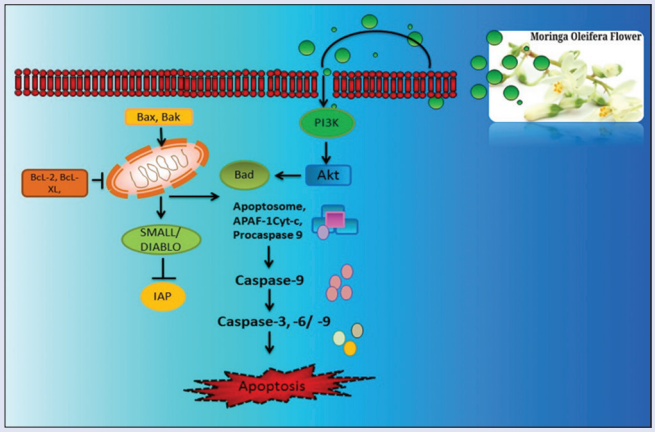
ABSTRACT

Background: *Moringa oleifera* (MO), or the horseradish tree, is a pantropical species that is known by such regional names as benzolive, drumstick tree, kelor, marango, mlonge, mulangay, nébéday, saijhan, and sajna. Over the past two decades, many reports have appeared in mainstream scientific journals describing its anticancer properties of MO. While much of this recent enthusiasm indeed appears to be justified, it is critical to evaluate therapeutic activity of MO on prostate cancer to separate rigorous scientific evidence from anecdote. MO contained active polyphenols such as ellagic acid, gallate, methyl gallate, catechol, kaempferol quercetin, and their derivatives. **Objective:** It is the purpose of this series of brief reviews to critically assess the efficacy of MO flower extract as an antiprostate cancer agent. **Materials and Methods:** The cell viability of the extract was determined using MTT in different time point and cell cycle progression studies was analyzed by flow cytometry. Various markers of apoptosis were evaluated by immunoblotting. **Results:** We observed that prostate cancer cells treated with MO flower extract caused 50% inhibition at a dose of 22.61 µg/mL and 6.25 µg/mL in PC-3 cells at 24 and 48 h, respectively. MO flower extract induced the accumulation of G1 phase cell cycle arrest and apoptosis by annexin V staining. Further, immunoblot detection of PARP cleavage leads to increase the protein expression of caspase-3 activity and Bax indicates induction of apoptosis. **Conclusion:** Together, the results suggest for the first time that administration of MO flower extract inhibits prostate cancer progression in PC-3 cells by interfering AKT pathway.

Key words: Apoptosis, caspases, cell cycle, cell death, natural products

SUMMARY

- Activation of PI3K by *Moringa oleifera* flower extracts leads to the phosphorylation/activation of AKT, leading to the upregulation of prosurvival genes BCL-2 and BCL-XL; which binds and inhibits caspase 3/7/9 required for apoptosis induction in prostate cancer.



Abbreviation used: MO: *Moringa oleifera*; PARP: Poly (ADP-ribose) polymerase; BAD: Bcl-2-associated death promoter; FACS: Fluorescence-activated cell sorting.

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INTRODUCTION

Prostate cancer is the fourth most common cancer in men occurred in economically developing countries.[1] One out of six men will be diagnosed with prostate cancer during their lifetime.[2] Most prostate cancers grow very slowly with no consistent or noticeable symptoms, but when they metastasis, the cancer cells grow more quickly and may spread to other vital organs of the body.[3] The causes of prostate cancer are not well understood; however, the disease is clearly associated with age, resembling an almost inevitable affliction for older men. Besides castration at a young age, which is obviously not a viable option, there is no certain way of preventing prostate cancer.

At hand, there is no widely accepted conventional treatment for the prevention of prostate cancer in high-risk men or for the alleviation of the symptoms associated with enlarged prostate. Conjointly, the treatment can produce weighty side effects, frequently associated with hormones and surgery that might affect the quality of life, including erectile

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dysfunction and urinary incontinence.[4] This significant side effect has generated considerable interest in the potential of alternative treatments including herbal remedies and nutritional supplements for alleviating symptoms and promoting prostate health.[5,6] In the past few years, research has shown that dietary modifications may reduce the chances of developing prostate cancer, prostate cancer recurrence, or reduce the aggression of help slow the progression. A lifelong commitment to a plant-based diet may lower a man's risk of developing prostate cancer and may also affect the progression of the disease. Preliminary evidence indicates dietary and lifestyle changes led to a 4% decrease in prostate cancer and significantly decreased prostate cancer cell growth.[7]

Moringa oleifera (MO) is a pantropical species that is known by such regional names as benzolive, drumstick tree, kelor, and marango. MO is highly honored around the world for its nutritional value and various health benefits. Among those benefits could be MO as cancer treatment, attributed to its rich phytochemicals. MO flower preparations have been cited in the scientific literature as anticancer agent with a range of fairly unique compounds containing 4-(4'-O-acetyl-I±L-rhamno pyranosyloxy) benzyl isothiocyanate, 4-(I±-L-rhamnopyranosyloxy) benzyl isothiocyanate, niazimicin, pterygospermin, and benzyl isothiocyanate.[8,9] Several studies on these compounds had been reported on the anticancer activities, but these studies are merely rested on *in vitro* cell tests.[10,11]

Herein, we sought to undertake a detailed evaluation of the *in vitro* anticancer activity of MO flower extract in PC-3 prostate cancer cells. To the best of our knowledge, there is shortfall of report that presents a thorough mechanistic investigation to develop new, effective, and affordable anticancer drugs for prostate cancer management. With the list of compounds identified, it seemed to be essential to investigate the *in vitro* anticancer effects role of MO flower against prostate cancer by evaluating its effects on cellular viability, cell cycle analysis, and possible cellular mechanisms implicated in its mechanisms of action on PC-3 cells treated with 25 µg/ml of MO flower extract.

MATERIALS AND METHODS

Preparation of *Moringa oleifera* flower extract

The fresh and mature MO flower were obtained from Garden No. 2 at Universiti Putra Malaysia, Selangor, Malaysia, with the voucher specimen (SK 1561/08) and stored in the Institute of Bioscience Herbarium unit. The extracts were prepared by soaking the grated flower in 80% ethanol for 72 h at room temperature after which the process was repeated three times. The supernatant was collected and finally concentrated through rotary evaporator followed by freeze-drying using a lyophilizer to a solid powder form and then it was stored at −20°C until further use.

Cell lines, media, antibodies, and reagents

Prostate cancer (PC-3) cancer cell lines were used in the present study. The medium used to culture these cells was Roswell Park Memorial Institute-1640 medium supplemented with 10% fetal bovine serum and 1% antibiotic (penicillin/streptomycin). Primary antibodies AKT, p-AKT (Cell signaling, USA), Bax, Bcl-2, Caspase-3, Caspase-8, and horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and β-actin was from Sigma Chemical Company, (St Louis, MO, USA).

In vitro cell viability assay

Cells were seeded in ninety-six-well plates and exposed to various concentrations (0.07–100 µg/mL) of MO flower extract. After 24 and 48 h of incubation, cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MTT solution (% mg/mL) was added to each of the treated and control wells and incubated for 4 h at 37°C. The formazan crystals formed was dissolved by adding 100 µl dimethyl sulfoxide to all the wells, then mix well and placed in the dark place at room temperature for 30 min. The magnitude of the purple formazan signal is proportional to the number of viable cells and was observed using 570 nm emission wavelength.

Cell-cycle progression studies by flow cytometry

For the cell-cycle investigation, PC-3 cells were treated with MO flower extract at fixed dose of 25 µg/mL at 24 h and 48 h time points. At the end of incubation period, cells were fixed with 70% ethanol overnight and stained with propidium iodide containing RNase A, followed by data acquisition on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analyses using Flo-Jo software (Ashland, OR, USA).

Immunofluorescence microscopy

PC-3 cells were cultured on coverslips in six well plates, followed by induction of inflammation by Lipopolysaccharide and treatment with 25 µg/mL MO flower of extract. Then, cells were gently washed with phosphate-buffered saline (PBS) and fixed with fixation solution (acetone: ethanol) for 20 min in −20°C. About 1% of BSA solution were used to block the fixed for 20 min at room temperature. Finally, cells were then incubated with a specific dilution (1:250) of Annexin V–FITC at room temperature for 1 h. The cells were washed with PBS followed by staining of nuclei using Hoechst 33342 (TherMO scientific Walthman, MA, USA). The phosphatidylserine exposure was visualized by fluorescent microscope (Olympus, Japan).

Immunoblot analysis

The immunoblot was performed according to the method described by Tan *et al.*[12] Briefly, proteins were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes for 1 h. The membranes were blocked in blocking solution containing 5% of BSA in phosphate-buffered saline containing 1% Tween-20 and incubated with appropriate primary antibodies and then with suitable horseradish peroxidase-conjugated secondary antibodies. Specific target proteins were visualized after added the substrate with enhanced chemiluminescence system (BioTechniques, New York, USA).

Statistical analysis

The comparison between different experimental groups was determined by one-way ANOVA tailed by a *post hoc* Tukey's test. All the experiments were performed in triplicate, and the results were expressed as means ± standard deviation of replicates. Differences were accepted as statistically significant at $P < 0.05$ or less. $P = 0.05$ between each experiments group.

RESULTS

Effect of *Moringa oleifera* flower extract on cell viability in PC-3

The cytotoxicity of MO flower extract was evaluated by treating PC-3 cells (10^5 cells/mL) at various concentrations (0.07–100 µg/mL) for 24 and 48 h using MTT assay. The effective doses were calculated from dose-response curve. Results of the cytotoxicity evaluation against PC-3 cell line of the MO flower extract are shown in Figure 1a and b. MO flower extract exhibited significant activity against the PC-3 cells achieving an IC_{50} value of 8.48 µg/mL and 6.25 µg/mL at 24 h and 48 h, respectively. The standards for cytotoxicity of a crude extract, as established by the

U. S. National Cancer Institute, is an IC_{50} <20 μg/mL in the preliminary assay.[13] Hence, for further cellular and molecular studies, 25 μg/mL of MO flower extract concentrations was considered.

Effect of *Moringa oleifera* flower extract on the cell cycle

The effects of MO flower extract anticancer properties were studied by cell cycle analysis. The distribution of cell cycle phases was expressed in histograms of PC-3 cells after exposed with 25 μg/mL of MO flower extract are mentioned in Figure 2. In untreated, growing PC-3 cells, the G_0/G_1 phases were clearly well defined with many demarcating cells. After 24 h of 12.5 μg/mL MO flower extract treatment, the percentage of cells in the G0/G1 phase significantly decreased from 93.1% to 57.7% compared to control. The cells treated for 48 h, a notable decrease in G0/G1 phase was observed indicating MO flower extract on PC-3 cell cycle arrest appears to be time-independent manner; the higher the treatment time, the lower the percentage of the cells in the G0/G1 phase. It is suggested that the MO flower extract could induced cell cycle arrest in the higher treatment time.

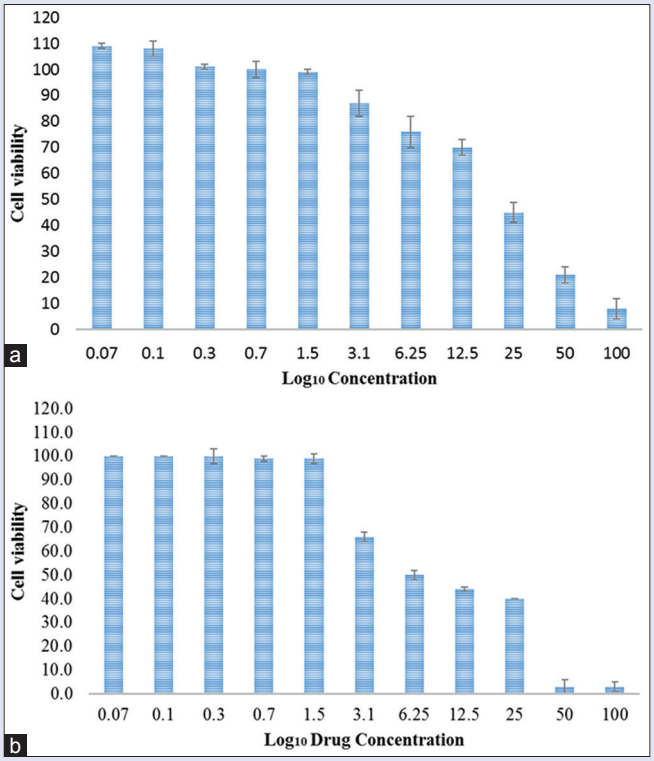

Figure 1: Effects of MO flower extract on PC-3 cell viability for (a) 24 and (b) 48 h. The MTT assay was used to analyze the cell viability and the result represents the average of three independent experiments ± SD. MO: *Moringa oleifera*; SD: Standard deviation; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Apoptosis induction effect of *Moringa oleifera* flower extract on PC-3 cells

PC-3 cells treated with MO flower extract (25 μg/mL) for 24 h and 48 h. Apoptotic cells were observed under fluorescence microscope after staining with Annexin V–FLUOS. Apoptotic cells with Annexin V positive staining were recognized by green plasma membrane. As shown in Figure 3, the higher expression of phosphatidylserine (recognized by green plasma membrane) was observed in cells treated with 25 μg/mL of MO flower extract at 24 h and 48 h. However, the expression was absent in untreated cells indicating MO flower extract have effectively induced apoptosis in PC-3 cells in time-dependent manner.

Moringa oleifera flower extract reduces AKT phosphorylation in PC-3 cells

To scrutinize the apoptotic effect of MO flower extract-induced growth inhibition, the cell death in the MO flower extract-treated PC3 cells was assessed by immunoblot analysis. When the PC3 cells were treated with MO flower extract for up to 72 h, the expression levels of P-AKT protein decreased in a time-dependent manner [Figure 4] concurred with the cell growth kinetics, transformation, invasion, and migration (Shukla *et al.*, 2007). In additional, we also analyzed the expression levels of the Bcl-2 family proteins that are linked for apoptotic signaling.

Moringa oleifera flower extract induces apoptotic cell death via an intrinsic signaling pathway in PC-3 cells

We also analyzed the expression levels of the proteins that play a pivotal role in apoptotic signaling cascade. Treatment of PC-3 with MO flower extract resulted in suppression of Bcl-2, expression and increased Bax,

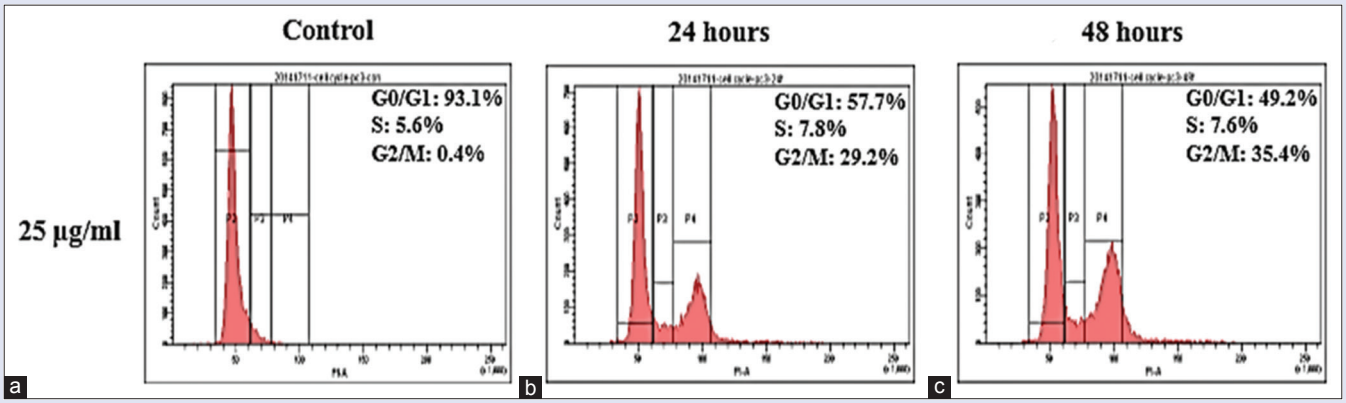

Figure 2: Cell cycle analysis using FACS. PC-3 cells (5×10^5) were preincubated for 24 h and then exposed to MO flower extract by flow cytometer. The collected cells were fixed with 70% cold ethanol until analysis. (a) without treatment, (b) after treatment with 25 μg/mL for 24 h and (c) 25 μg/mL for 48 h. This experiment was carried out thrice independently and the results were expressed in a histogram. FACS: Fluorescence-activated cell sorting; MO: *Moringa oleifera*

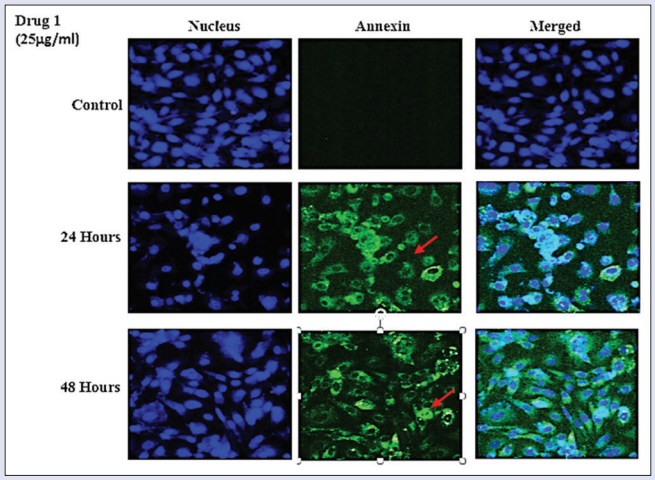

Figure 3: Apoptotic morphological changes in PC-3 were observed by fluorescent microscope (×200). Untreated PC-3 cells (control) showed normal morphology without noticeable apoptosis after 48 h. Early apoptotic features, including chromatin condensation and cell blabbing were witnessed after treatment with 25 µg/ml at 24 and 48 h (bright green fluorescence)

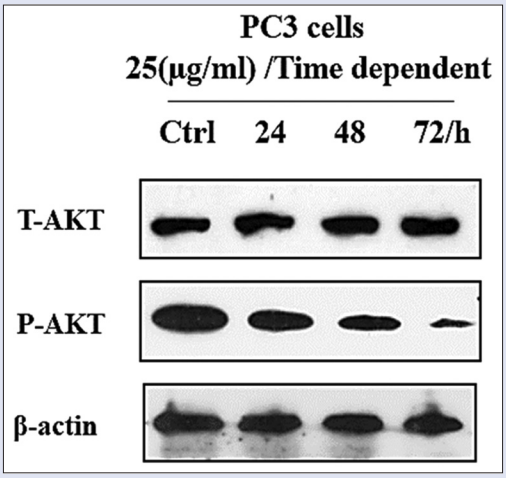

Figure 4: Effects of MO flower extract on the expressions of AKT. PC-3 cells were treated with 25 µg/mL of MO flower extract for 24, 48 and 72 h. The p-AKT and T-AKT cellular levels were analyzed by immunoblotting. β-Actin served as a house keeping gene. MO: *Moringa oleifera*

caspase-3, -8 expressions [Figure 5]. These findings suggest that MO flower extract induces apoptosis and might be involved in the regulation of the mitochondria mediated apoptosis pathway.

DISCUSSION

Programmed cell death or apoptosis is an imperative homeostatic mechanism that balances the cell division, cell death and substantially maintains the appropriate cell number in multicellular organisms.[14] This regulated cellular suicide mechanism is characterized by morphological changes like nuclear condensation, cell shrinkage, membrane blebbing, and DNA fragmentation. Dysregulation of apoptosis has been associated with variety of diseases including development of cancer. Recently, prostate cancer has been associated with a high morbidity and mortality rate with a medium survival of approximately 12–15 months.[15] Many chemotherapeutic agents result in <10% response in advanced prostate carcinoma. Adding, many undesirable side effects sometimes occur during chemotherapy. Natural therapies, plant-derived products in cancer treatment, can induce apoptosis and lessen the adverse side effects. Although scientific evidence from epidemiological and *in vivo* studies suggests that augmented consumption of plant-based diet can lessen the risk of prostate cancer, petite knowledge is available relating to the use of botanicals in averting prostate cancer.[16]

The present study validates the anticancer activities and the underlying mechanisms of MO flower extract on cancer cells of prostate *in vitro*. The inhibitory effects of MO flower extract were observed in MTT assay, and it revealed a strong cytotoxic effect on PC-3 cells. MO flower extract suppressed the PC-3 cells growth in a time-dependent manner. This high cytotoxic activity could be due to phytochemicals present in MO flower extract. Previous study revealed that presence of quinic acid in MO flower is chemo-preventive in nature.[17] Cell cycle and annexin V staining analysis are allied with apoptosis. Treatment with MO flower extract suggests that cell death may be ascribed to the activation of apoptotic pathways as an after effect of the inability of the cells to overcome growth arrest and advance through the cell cycle.[18] Annexin V binding is a major marker of apoptosis. Loss of membrane asymmetry is an early event of apoptosis due to translocation of phosphatidylserine from the inner leaflets to the outer leaflets of the plasma membrane, which is a key feature of apoptotic cells. PC-3 cells treated with MO flower extract displayed bright apple green staining on the cell membrane surface, the typical apoptotic cellular morphology.

Auxiliary investigations attentived on the mechanism of MO flower extract-induced apoptosis of PC-3 cells. The MO flower extract-induced apoptosis of PC-3 cells appeared to be associated to the mitochondria-mediated pathway, grounded on the activation of caspase-3 and-8. Furthermore, immunoblotting analysis showed that MO flower extract induced cleavage of PARP. PARP is one of the main cleavage targets of caspase activation which serves as a marker of cells undergoing apoptosis, suggesting the activation of caspase-dependent apoptotic pathway. Thus, based on the mechanistic basis, we hypothesized that MO flower extract's anticancer properties could be through modulation of cell survival signaling pathways. MO flower extract treatment (1) inhibits the proliferation of human PC-3 cells through induction of apoptosis by altering the expression of apoptotic signaling molecules and (2) results in reduction in the levels of p-AKT, and overexpression of AKT protects cells from MO flower-induced inhibition of cell proliferation implicating a role for AKT signaling in prostate cancer.[19] AKT signaling network is essential to many physiological processes, including cell cycle progression, differentiation, transcription, translation and apoptosis. Activated AKT pathway provides major survival signals to prostate and many other cancer types. AKT is activated by phosphorylation of threonine 308 (Thr 308) and serine 473 (Ser 473). On activation, AKT is translocate into nucleus, leading to increased transcription of antiapoptotic and prosurvival genes such as Bcl-2-associated death promoter (BAD) and Bax that affect the survival state of cell.

Apoptosis is coordinated by the balance between antiapoptotic (Bcl-2) and proapoptotic (Bax) family members. In healthy cells, phosphorylated BAD are sequestered in the cytoplasm, Bcl-2 and Bcl-xL bind to pro-apoptotic Bax proteins to inhibit apoptosis. When cytoplasmic levels of free BAD increase, Bcl-2 and Bcl-xL bind to BAD and release Bax to affect membrane permeability, which in turn results in the sequential activation of caspase and induce a series of signaling cascade for apoptosis.[20] The immunoblot analysis point to that Bcl-2 may not directly contribute in MO flower extract to induce apoptosis in PC-3 cells. This data likewise submits a possibility that MO flower extract perhaps be favorable in inhibiting the development of cancer cells overexpressing Bcl-2. Caspases, the enzymes are a family of cysteine

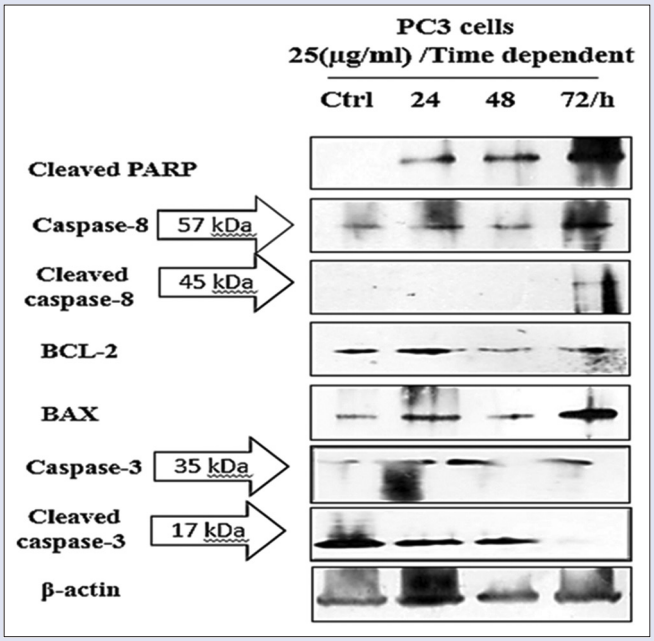

Figure 5: Apoptotic related protein expression in PC-3 cells treated with MO flower extract. The R blots are representative from independent experiments with identical results. The expression levels of Bcl-2 family proteins (Bax and Bcl-2) and mitochondria signaling proteins (caspase-3, -8) were analyzed by immunoblot analysis. β-Actin served as a housekeeping gene. MO: *Moringa oleifera*

proteases, which are involved in apoptosis and cell death through activated by cleavage multiple protein molecules. Previously researchers have been well defined that apoptosis is stimulated by extrinsic and intrinsic pathways and activate caspases including caspase-8 and caspase-3 cascade through extrinsic pathway. In the present study, MO flower extract effectively increased the expression of caspase-8 and caspase-3; therefore, these findings suggested that extract can induce apoptosis through mitochondrial pathway.

CONCLUSION

Based on the results, the findings suggest that MO flower extract has a compelling anticancer effect and causes apoptosis in PC-3 prostate cancer cells *in vitro*. Therefore, MO flower extract has a good profile to be included as a new therapeutic option and expand the synergistic interaction between natural compounds and gene regulation in human cancer. In addition, how MO flower extract function and the other mechanism which may relate to the variations in the cell cycle and justification of the PC-3 cells for caspase-3 downregulation does not associate with apoptosis data and hence should be the subjected for further studies.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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