Integrative Cancer Therapies
Volume 18: 1–11

DOI: 10.1177/1534735419828829
journals.sagepub.com/home/ict
SAGE

Combined Effect of *Moringa oleifera* and Ionizing Radiation on Survival and Metastatic Activity of Pancreatic Cancer Cells

Lior Hagoel[1], Prof. Akiva Vexler[1], Dr. Lital Kalich-Philosoph[1], Dr. Gideon Earon[1]*, Dr. Ilan Ron[1], Dr. Alex Shtabsky[1], Dr. Silvia Marmor[1], and Dr. Shahar Lev-Ari[1]*

Abstract

Background: Radiotherapy is one of the main treatments for malignancies. Radioresistance is a major obstacle in this treatment, calling for new treatments to improve radiotherapy outcome. Herbal medicine has low toxicity and could be a source for new radio-enhancing agents. *Moringa oleifera* (moringa) is a well-known medicinal plant with antiproliferative and antimetastatic properties. Possible mechanisms of moringa anticancer activity may be related to the expression of PARP-1, Bcl-2, COX-2, p65, p-IκB-a, and others. **Purpose:** The aims of the present study were to investigate effect of moringa alone and combined with radiation on survival and metastatic activity of pancreatic cancer cells and on tumor growth. **Methods and Results:** The combination of moringa and radiation significantly inhibited PANC-1 cell survival in a dose-dependent manner, as tested by clonogenic and XTT assays. Moreover, standard transwell cell migration/invasion assays demonstrated reduced metastatic activity of these cells. Pyruvate mitigated the inhibitory effect of combined treatment on cell survival. Flow cytometry of moringa-treated cells revealed induction of apoptosis. Western blot analysis found that the combined treatment decreased expression of the pro-apoptotic protein Bcl-2, and downregulated the key component of DNA repair pathways PARP-1 and the NF-κB-related proteins IκB-α, p65-subunit, and COX-2. Moringa significantly inhibited growth of subcutaneous tumors generated by PANC-1 cells in nude mice. Immunohistochemical analysis demonstrated moringa's antiproliferative and antiangiogenic effects. **Conclusions:** Moringa decreased pancreatic cancer cell survival and metastatic activity and significantly inhibited tumor growth. The combination of moringa plus radiation resulted in an additional inhibitory effect that provided the rationale for further investigation of this combination as a novel strategy to overcome pancreatic cancer cell radioresistance.

Keywords

pancreatic cancer, moringa, radiation, cell survival, metastatic activity, tumor growth

Submitted May 9, 2018; revised November 22, 2018; accepted December 13, 2018

Introduction

Pancreatic cancer is an aggressive type of cancer with metastatic behavior and the fourth leading cause of cancer death in the United States.[1] Extensive local tumor invasion and early systemic dissemination are its principal hallmarks.[2] Because of difficulties in early diagnosis and limited efficient therapeutics, most patients with pancreatic cancer have poor probability of survival.[3] Approximately 19% of pancreatic cancer patients survive 1 year after diagnosis and 4% for 5 years, making this disease the most lethal of all cancers. Treatment options for pancreatic cancer vary with the disease stage and the general health of a patient, and they include surgery, chemotherapy, and irradiation. Metastatic pancreatic tumors are highly chemoresistant. The combination of gemcitabine and FOLFIRINOX or paclitaxel is standard treatment for metastatic pancreatic

[1]Tel Aviv Sourasky Medical Center, Tel Aviv, Israel

*equal contribution.

Corresponding Author:
Shahar Lev-Ari, Laboratory of Herbal Medicine and Cancer Research, Institute of Oncology, Tel-Aviv Sourasky Medical Center, 6 Weizmann Street, Tel-Aviv 64239, Israel.
Email: shaharl@tlvmc.gov.il

cancer; however, it offers only a moderate increase in median survival.[3,4]

Despite the advances in radiotherapy, many cancer patients still sustain locally recurrent disease after radiotherapy, calling for new treatment strategies that would enhance the efficacy of radiotherapy. Attention has been drawn to herbal medicine in the quest for new radio-enhancing agents with a low toxicity profile. Herbs and their bioactive metabolites have been reported to be antineoplastic in both experimental and clinical studies.[5] *Moringa oleifera* (moringa) is one of the best known and most widely distributed and naturalized species of family Moringacceae. In medicine, different extracts from nearly every part of this plant, including leaves, root, bark, gum, fruit (pods), flowers, seeds, and seed oil, have been used for treatment of various diseases, including cancer.[6] Moringa is rich in phenols, caffeoylquinic acid, β-sitosterol, quercetin, keampferol, vitamins, and minerals, especially essential amino acids and β-carotene.[7] It has been reported that aqueous extract of moringa had potent antiproliferative activity on human cancerous pancreatic cells.[8] Moreover, the leaf and bark alcohol extracts of moringa possess anticancer activity that can be used to develop new drugs for treatment of breast and colorectal cancers.[9]

The exact antitumor mechanism of moringa activity has not fully established, but it has been suggested that the moringa effect on pancreatic cancer cells is correlated to reduction of the overall expression of key NF-κB family proteins, inducing apoptosis and thereby generating cell death.

Drug combinations are being increasingly used in treating the most severe diseases, such as cancer. The aims of those combinations are to decrease toxicity, minimize the induction of drug resistance, and achieve additional therapeutic effect.

To date, there have been no reports demonstrating the efficacy of combining ionizing radiation with moringa as a potential novel approach to enhance the effectiveness of conventional pancreatic cancer therapy. Therefore, the present study aimed to investigate the cytotoxicity of *M oleifera* aqueous leaf extract on pancreatic cancer cells PANC-1, as well as to evaluate the combined effect of radiation with moringa and explore possible mechanisms of the combined treatment.

Materials and Methods

Preparation and Chemical Analysis of Moringa Aqueous Leaf Extract

Moringa trees grow in a rich mineral soil in the Dead Sea area. Leaves of *M oleifera* were received from Moringa Arava Ltd, Israel. The aqueous leaf extract (moringa) was prepared by mixing 1 g dried and powdered leaves with 10 mL boiling water for 5 minutes and then filtered twice through sterile filter paper. This stock solution of moringa (100 mg/mL) was stored at 4°C during the experiments and diluted in a culture medium immediately before the experiments.[8] Gas chromatography-mass spectrometry analyses of moringa was performed by BACTOCHEM (Israel) for quality and batch-to-batch consistency (Table 1). Among the substances found were heptadecane (238 mg/kg) and stigmasterol (91 mg/kg), both of which demonstrate anticancer activity.

Table 1. Gas Chromatography-Mass Spectrometry Analysis of Moringa.

Name of the Substance—Identification by NIST Library	Semi Quantitative[a] (mg/kg)
n-Hexadecangic acid	149.12
Octadecanoic acid	72.61
Vitamin E	368.37
Testosterol	461.17
Stigmasterol	91.14
Phytol	73.54
Octadecatrienoic acid	725.12
Campesterol	139.33
Hexadecane	46.59
a-Amyrin	128.88
17-Pentatriacontene	378.45
Heptadecane, 9-octyl-	238.84
Tetracosane, 3-ethyl-	109.36
5-Androsten-17.a-ethynyl-3.},17.8.-diol	289.02
13-Methyl-Z-14-nanacosene	83.71
3.7,11,15-Tetramethyl-2-hexadecen-1-ol	55.06

Abbreviation: NIST, National Institute of Standards and Technology.
[a]Analysis was performed by BACTOCHEM (Israel).

Cell Line

The human pancreatic epithelioid carcinoma cell line PANC-1, which originated from a 56-year-old Caucasian male, was obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% heat-inactivated (56°C for 30 minutes) fetal bovine serum (Life Technologies, Israel) and 1% penicillin-streptomycin antibiotics (Biological Industries, Beit-Haemek, Israel). The cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

In Vitro Studies

Irradiation of Cells In Vitro

The cells were plated in 60 mm culture plates or 96 micro well plates and irradiated with a single 2, 4, or 6 Gy dose. A

linear accelerator was operated at a 6 MeV photon beam at a dose rate of 418 cGy/min. The sample anterior distance was 100 cm. A bolus gel layer (1-cm thick) was placed above the plates.

Clonogenic Survival Assay

Cell survival was evaluated using a very sensitive clonogenic assay. After careful pipetting, a known number of cells (200-500 cells/plate) were seeded in 60 mm tissue culture plates. Nonirradiated and irradiated cells were grown for 10 to 14 days, allowing the surviving cells to produce macroscopic colonies each consisting of 50 or more cells. The colonies were washed with phosphate-buffered solution (PBS), fixed with 70% methanol for 10 minutes at room temperature (RT), and stained with Giemsa stain (Beckman Coulter Inc, Brea, CA) for 5 to 10 minutes at RT. The stained colonies were counted by light microscopy. The surviving fraction was calculated as a ratio of colonies produced by treated cells to colonies produced by nontreated cells taking into account plating efficiency (PE; the ratio of colonies produced by nontreated cells to the number of seeded cells). Each variant of the experiment was performed in triplicate and repeated 3 times.

XTT (Colorimetric Tetrazolium Salt) Assay for Cell Survival

Cell survival was also evaluated using XTT-based cell proliferation assay.[8] PANC-1 cells seeded in 96 micro well plates (1.5-2 × 10^3 cells/well) and incubated for 24 hours were treated with moringa and/or irradiated. After 72 hours of incubation, the cultures were incubated for 1 to 3 hours with XTT, and the strongly colored formazan product was measured at a wavelength of 450 nm (Sunrise plate reader, Switzerland). Each plate included blank wells that contained media and XTT, while the control wells contained nontreated cells and fresh medium. Each variant of the experiment was performed in triplicates and repeated at least 3 times.

Flow Cytometry Analysis of Apoptotic Cells

The cells were seeded in 60 mm plates and treated with moringa and/or irradiated and allowed to grow for 24 hours. The cells were collected, washed twice with PBS, and underwent centrifugation. The cell pellets were then resuspended in binding buffer and double stained with fluorescein isothiocyanate-conjugated annexin V (Annexin V-FITC) and propidium iodide (PI) using an apoptosis detection kit (BioVision Inc, Milpitas, CA). The samples were incubated in the dark for 5 minutes and analyzed by BD FACSDiva software using BD FACSCanto 2 (BD Bioscience) instrument. The experiment was repeated 3 times.

Western Blot Analysis of Selected Proteins Expression

The cells (3-5 × 10^5 cells in 60 mm plates) were maintained at 37°C in 5% CO_2 humidified atmosphere. The culture medium was replaced with a medium containing moringa and/or irradiated at doses of 2 and 4 Gy. Expression of selected proteins was determined using Western blot analysis as described previously.[8] The cells were harvested and homogenized in lysis buffer on ice using the proteo JET mammalian cell lysis reagent (Fermentas Life sciences, Israel). The lysates were centrifuged at 13 000 *g* at 4°C for 20 minutes. Protein concentration was determined using Bio-Rad kit (Bio-Rad, Hercules, CA). The probes (50 μg of protein) were separated on polyacrylamide gel and transferred onto a nitrocellulose membrane. The membranes with selected proteins were incubated at RT for 1 hour with primary antibody against PARP-1, Bcl-2, COX-2, p65, p-IκB-α, and β-actin, and then with mouse anti-rabbit immunoglobulin G-horseradish peroxidase and goat anti-mouse immunoglobulin G-horseradish peroxidase (Santa Cruz Biotechnology Inc, Santa Cruz, CA). All blots were analyzed using SuperSignal West Pico Chemiluminescent substrate.

Transwell Cell Migration and Invasion Assays

Cell migration was assayed using a modified Boyden chamber (according to the manufacturer's instructions; Greiner Bio-One GmbH, Germany) with an 8 μm pore size membrane in a 24-well plate (Nunclon, Sigma-Aldrich, St Louis, MO). DMEM (600 μL) and 10% fetal bovine serum were added to the lower part of the chambers. PANC-1 cells (5 × 10^5 cells/mL) in 100 μL of serum-free DMEM with different concentrations of moringa were placed in the upper part of the chambers. The cells were incubated at 37°C for 24 hours. The culture media were discarded and the top side of each transwell chamber membrane was scraped with a wet cotton swab to remove the nonmigrated cells. The migrated cells were fixed by 70% ethanol and stained with Giemsa stain (Beckman Coulter Inc). The average number of migrated cells was counted from 6 randomly selected microscopic fields at ×40 magnification using ImageJ software. The migration index of the treated cells was calculated relative to the controls. The assay was carried out in duplicate, and the experiment was repeated 3 times. Cell invasion was assayed exactly as transwell cell migration assay (see above), but the Boyden chamber membrane was covered with Matrigel at 3 mg/mL concentration.

Evaluation of Reactive Oxygen Species (ROS) Contribution to Moringa/Radiation-Induced Cell Death

To assess the effect of ROS on moringa and radiation-induced cell death, PANC-1 cells were plated in 96-wells plates and allowed to attach overnight. The cells treated with moringa and/or irradiated were then incubated in the medium with or without sodium pyruvate (1 mM). Cell survival was determined using XTT assay after 72 hours of incubation.[8,9]

Statistical Analysis of In Vitro Experiments

The results of the in vitro experiments were represented as an average of 2 to 4 experiments, and each arm was typically performed in triplicate. The mean values and standard errors were calculated for each point from pooled normalized data. The significance of difference between the arms was determined using a 2-tailed Student *t* test with unequal variance, and significance was set at $P < .05$ (*), $P < .01$ (**), or $P < .001$ (***).

In Vivo Studies

Animals

Immune deficient athymic CD-1 nude mice were used for a xenograft ectopic tumor model. The mice, 6- to 7-week-old females, were obtained from the Harlan Animal Production Area (Jerusalem, Israel) and were housed in a laminar flow cabinet under pathogen-free conditions in standard vinyl cages (5 mice/cage) with air filter tops. Cages, bedding, and water were autoclaved. The local Ethics Committee for Accreditation of Laboratory Animal Care approved all facilities in accordance with the current regulations and standards of the Israeli Ministry of Health. Also, in accordance with the Ethics Committee regulations, the experiment was stopped when the mice became moribund, when the tumors reached 2 cm in their widest diameter, or when the weight of the mice decreased by 15% from the initial weight. The mice were sacrificed by a lethal dose of CO_2 at the end of the experiment. The developed tumors were evaluated at the Pathology Department of the Tel-Aviv Sourasky Medical Center.

Ectopic (Subcutaneous) Tumor Model in Nude Mice

PANC-1 cancer cells harvested and resuspended at a concentration of 1×10^6 cells/0.2 mL PBS were injected subcutaneously into the flank area of the mice. After 2 weeks when tumor volume was about 20 to 30 mm^3, the tumor-bearing mice were randomized into 4 treatment groups of 5 mice each. Moringa (0.5, 1.0, and 1.5 mg/g, 200 µL/mouse) was injected intraperitoneally twice weekly for 6 weeks. The development and progression of tumors in mice was followed-up by measurements with a digital caliper twice weekly. The mice were autopsied, and the tumor specimens underwent pathologic evaluations. The effect of each given treatment was evaluated based on the calculation of tumor volume (v) using the following formula[10,11]:

$$v = a\,(\text{large diameter}) \times b^2\,(\text{small diameter}) \times \pi/6.$$

Pathology and Immunohistochemistry of Developed Tumors

Tumor samples were formalin-fixed and embedded in paraffin for routine hematoxylin and eosin and immunohistochemical (CD 31, Ki 67, IκB-a, p65, clone DO7 of p53 [mutant and wild-type], and Bcl-2) staining. All sections were dehydrated, mounted on coverslips, and viewed under a light microscope. Antigen retrieval was performed for 40 minutes at 95°C in citrate buffer, pH = 6.0, 6.4 M sodium citrate dehydrate, and 1.6 M citric acid monohydrate. The slides were cooled at RT for 20 minutes and washed 3 times for 3 minutes with Tris-buffer, pH 7.6, 0.15 M sodium chloride, and 0.05 M Trizma HU. The slides were peroxidase blocked for 5 minutes, washed as described above, and then incubated for 30 minutes with the primary antibody, followed by the secondary antibody (visualization reagent), followed by the substrate-chromogen solution (3,3-diaminobenzi-dine), and finally counterstained with hematoxylin. Staining was quantified between "0" and "3+" according to the Dako instructions (Petach Tikva, Israel). The analysis of the slides was performed in collaboration with Drs Alex Shtabsky and Silvia Marmor (TASMC pathologists).

Statistical Analysis of In Vivo Experiments

The results for *in vivo* experiments were analyzed by the 2-way analysis of variance and Tukey's multiple comparison test, with comparisons of all pairs of the tested treatments. The statistical analysis was performed using the Dunnett test and the GraphPadPrizm 6.0 software. The differences in tumor size between the different treatment arms were considered statistically significant when $P < .05$.

Results

Inhibitory Effect of Radiation and Moringa on Pancreatic Cancer Cell Survival

The effect of radiation and moringa alone and in combination on PANC-1 cell survival was evaluated by radio-sensitive clonogenic assay (Figure 1). The irradiation of PANC-1

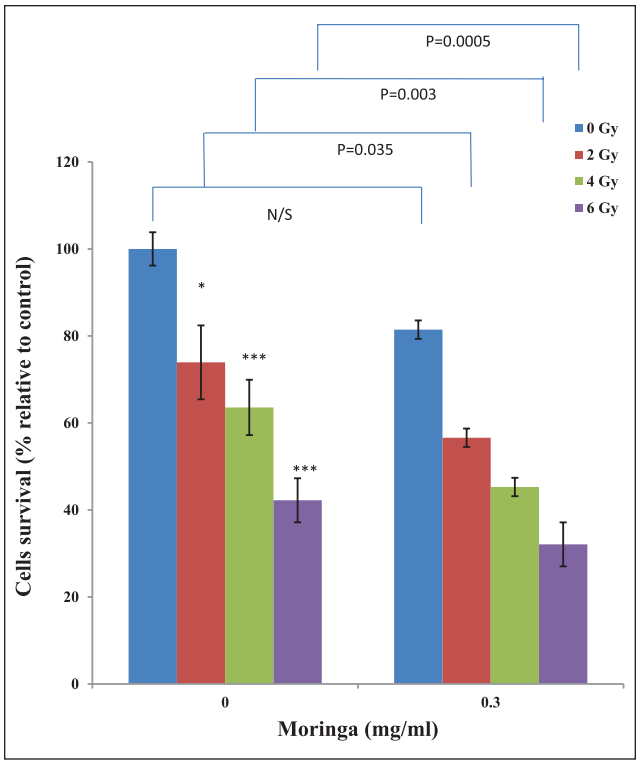

Figure 1. Effect of moringa and radiation alone and in combination on survival of PANC-1 cells. Cell survival was evaluated by clonogenic assay. Cells treated with 0.3 mg/mL moringa for 30 minutes were irradiated with 2, 4, and 6 Gy. Data are mean ± SE values from 5 experiments performed in triplicate. The effect of radiation alone was analyzed by *t* test (*$P < .05$; **$P < .01$; and ***$P < .001$). The efficacy of combined treatment (radiation and moringa, 0.3mg/mL) was compared with the effect of the corresponding radiation dose alone.

cells resulted in significant dose-dependent inhibition of cell survival: it was 74% at a dose of 2 Gy and only 42% at a dose of 6 Gy. The treatment of PANC-1 cells with moringa also induced a dose-dependent decrease in cell survival (Figure 1). The inhibitory effect of all combinations of moringa with the tested radiation doses was more significant than the effect of each treatment alone. The combination of radiation and moringa (0.3 mg/mL) resulted in an additive effect on cell survival: 0.3 mg/mL moringa inhibited 19% of PANC-1 cells, while it inhibited 43% of the cells when combined with 2 Gy, 55% with 4 Gy, and 68% with 6 Gy. The combined effect of radiation and moringa was also evaluated by XTT cell survival assay. The inhibitory effect of each treatment alone and their combinations was less significant but the regularities of cell survival were similar.

The cytoprotective role of pyruvate against ROS-induced cell death is well documented.[12] To examine whether the inhibitory effect of moringa and radiation on PANC-1 cell survival was also related to ROS products, the cells were exposed for 72 hours with or without 1 mM sodium pyruvate. The addition of 1 mM pyruvate in the medium did not change the effect of moringa on cell survival (data not shown). As expected, pyruvate reduced the effect of radiation alone as well as the effect of radiation combined with moringa on PANC-1 cell survival (Figure 2).

Moringa Combined With Radiation Reduced Metastatic Activity of PANC-1 Cells

Cell migration and invasion were evaluated by transwell cell migration and invasion assays using membranes that were either untreated or covered with Matrigel. Irradiation of PANC-1 cells with 4 Gy reduced migration and invasion to 76.4% relative to control. Moreover, moringa (1.8 mg/mL) reduced cell migration (61.6%) and invasion (63.7%). The combined treatment inhibited migration and invasion of PANC-1 cells more significantly: the combination of 4 Gy with 1.8 mg/mL moringa inhibited migration by 56.4% and invasion by 39.8% (Figure 3A and B).

Combination of Moringa and Radiation Induced Apoptosis in PANC-1 Cells

To explore whether the combination of moringa and radiation can cause cell death through the induction of apoptosis, PANC-1 cells that were double stained by Annexin V-FITC and PI were analyzed by flow cytometry (Figure 4, Table 2). The cells stained only with Annexin V-FITC underwent early apoptosis, the cells stained with Annexin V-FITC and PI underwent late apoptosis, and the cells stained only with PI underwent necrosis. Flow cytometry analysis of 4 Gy-irradiated PANC-1 cells revealed that 5.3% underwent early apoptosis, 7% underwent late apoptosis, and 10.1% underwent necrosis. The cells treated with 1.8 mg/mL moringa showed an increase in the percentage of late apoptosis (18%) and necrosis (19.6%).

Moringa Combined With Radiation Downregulated Expression of PARP-1, Bcl-2, COX-2, and p65 Proteins

Expression of selected proteins important for cell behavior was evaluated using Western blot analysis (Figure 5). Moringa combined with radiation decreased the expression of all proteins tested (PARP-1 [the key component of DNA repair pathways], Bcl-2 and COX-2 [apoptosis-related proteins], and NF-κB-related proteins [p65]) more significantly than each treatment alone, mainly at a dose of 4 Gy combined with 1.8 mg/mL moringa.

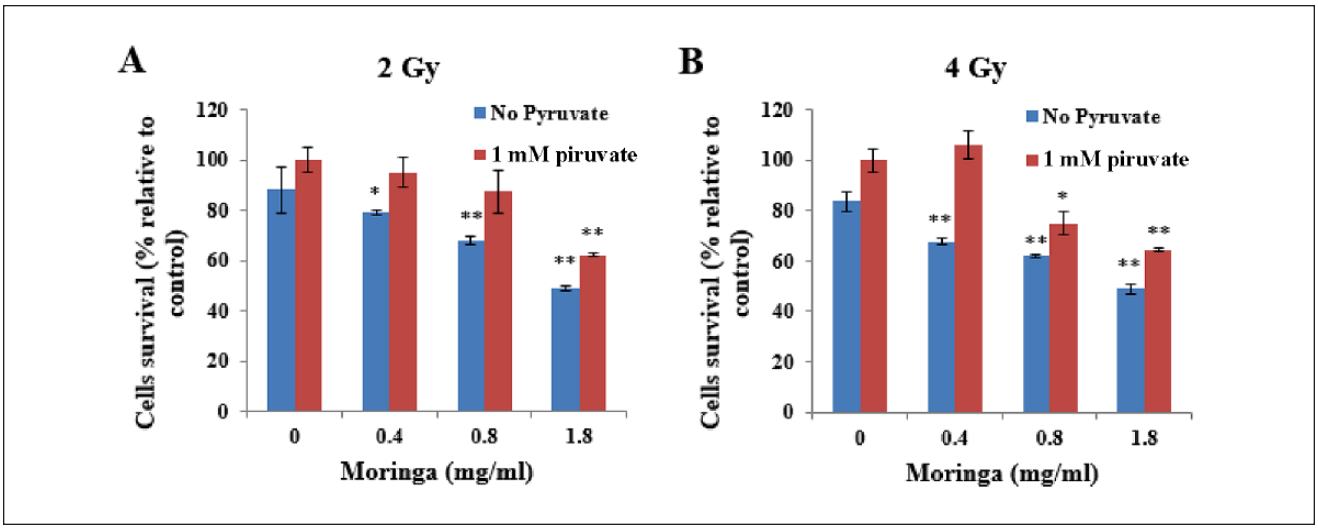

Figure 2. Effect of pyruvate on survival of irradiated and moringa treated PANC-1 cells. Cells irradiated with 2 Gy (A) or 4 Gy (B) were exposed to moringa with or without 1 mM pyruvate. Cell survival was assessed by XTT assay. Data are mean ± SE values from 3 experiments performed in triplicate (*P < .05; **P < .01; and ***P < .001).

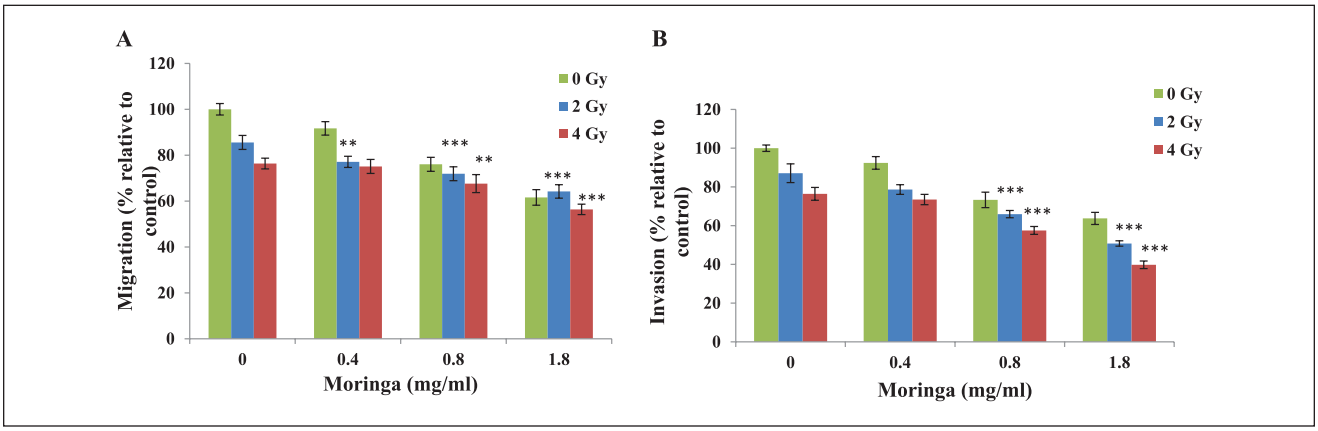

Figure 3. Effect of moringa and radiation alone and in combination on metastatic activity of PANC-1 cells. Cells irradiated with 2 or 4 Gy were treated with moringa. Migration (A) and invasion (B) of cells was determined by transwell cell migration/invasion assay. The graphs represented the averaged data of treated cells relative to the untreated control cells. Data are mean ± SE values from 3 experiments performed in triplicate (*P < .05; **P < .01; and ***P < .001).

Moringa Inhibited Pancreatic Tumor Growth in Nude Mice

The subcutaneous implantation of 1×10^6 PANC-1 cells in immune-deficient athymic CD-1 nude mice (females, age 6-7 weeks) resulted in tumor development in >80% of the mice. After 2 weeks, tumor-bearing mice were divided into 4 treatment groups (5 mice/group). Moringa was administered intraperitoneally twice weekly. All tumors increased in size, but the rate of growth was different (Figure 6). The tumor volume (158 ± 18 mm^3) in the nontreated mice increased more than ~6 times relative to the initial value while the tumor volume (104 ± 25 mm^3) in mice treated with 1.5 mg/mL moringa increased only ~3 times. The difference between tumor size in the control group and the group treated with 1.5 mg/mL moringa was statistically significant (P = .034, 2-way analysis of variance). The tumor volume at the end of the experiment was smaller also in the groups treated with 0.5 and 1.0 mg/mL moringa than that of the control group, but the difference between the treated and untreated groups was not significant (P = .347 and P = .276, respectively). The Dunnett multiple comparison (performed at the last 2 time points) and GraphPad Prizm 6.0 software confirmed these findings.

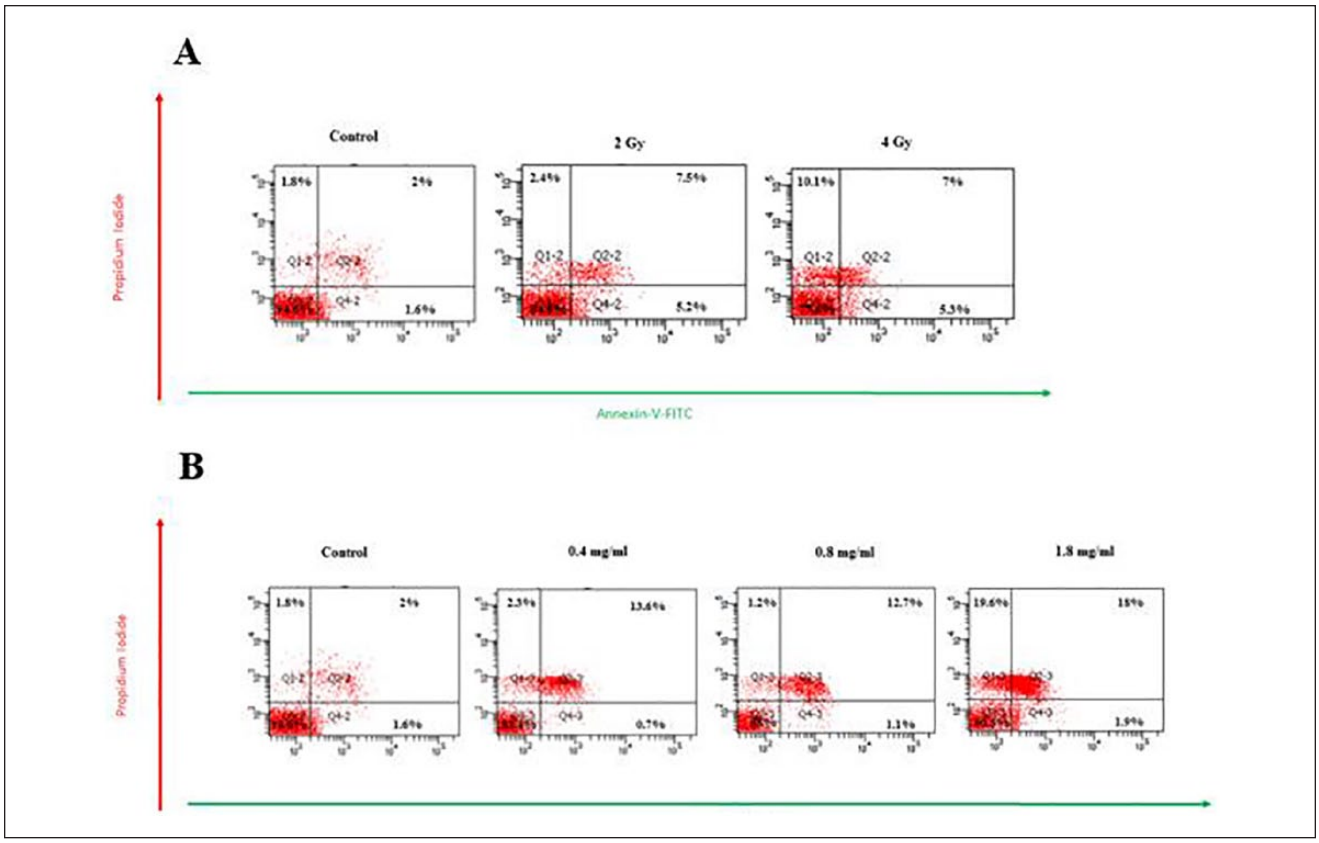

Figure 4. Moringa and radiation induced apoptosis in PANC-1 cells. Cells were irradiated with 2 Gy and 4 Gy (A) or treated with moringa (B). Apoptosis was assessed 24 hours posttreatment using Annexin-V-FITC/PI double staining and analysis by flow cytometry.

Table 2. Radiation and Moringa Induced Apoptosis in PANC-1 Cells[a].

	Radiation (Gy)			Control	Moringa (mg/mL)		
	Control	2	4		0.4	0.8	1.8
Necrosis	1.8	2.4	10.1	1.8	2.3	1.2	19.6
Early apoptosis	1.6	5.2	5.3	1.6	0.7	1.1	1.9
Late apoptosis	2	7.5	7	2	13.6	12.7	18

[a]Flow cytometry analysis of irradiated and moringa-treated PANC-1 cells.

Moringa Demonstrated Antiproliferative and Antiangiogenic Activity in Tumors

Hematoxylin and eosin staining of tumors revealed nuclear atypical mitosis. The expression of the nuclear protein Ki-67 strongly correlated with tumor growth. Immunohistochemical analysis of Ki-67 showed that moringa inhibited tumor progression (Figure 7A vs B). Pathological angiogenesis plays an important role in proliferation and in the spread of cancer cells, and the expression of the CD31 endothelial cell-specific marker is proportional to angiogenesis. Immunohistochemical analysis of CD31 found a difference between the nontreated and the moringa-treated tumors, but that difference did not reach a level of significance (Figure 7C vs D).

Effect of Moringa on Expression of Bcl-2 and IκB-α in Tumors

We also investigated the effect of moringa on the expression of Bcl-2, one of anti-apoptotic Bcl-2 family proteins, and IκB-α, an inhibitor of NF-κB. The results revealed that moringa decreased the expression of both proteins (Figure 7E vs F, G vs H).

Discussion

To the best of our knowledge, this is the first time that the combination of radiation and moringa demonstrated a significant inhibitory effect on pancreatic cancer cell survival and metastatic activity. Radiotherapy is one of the major

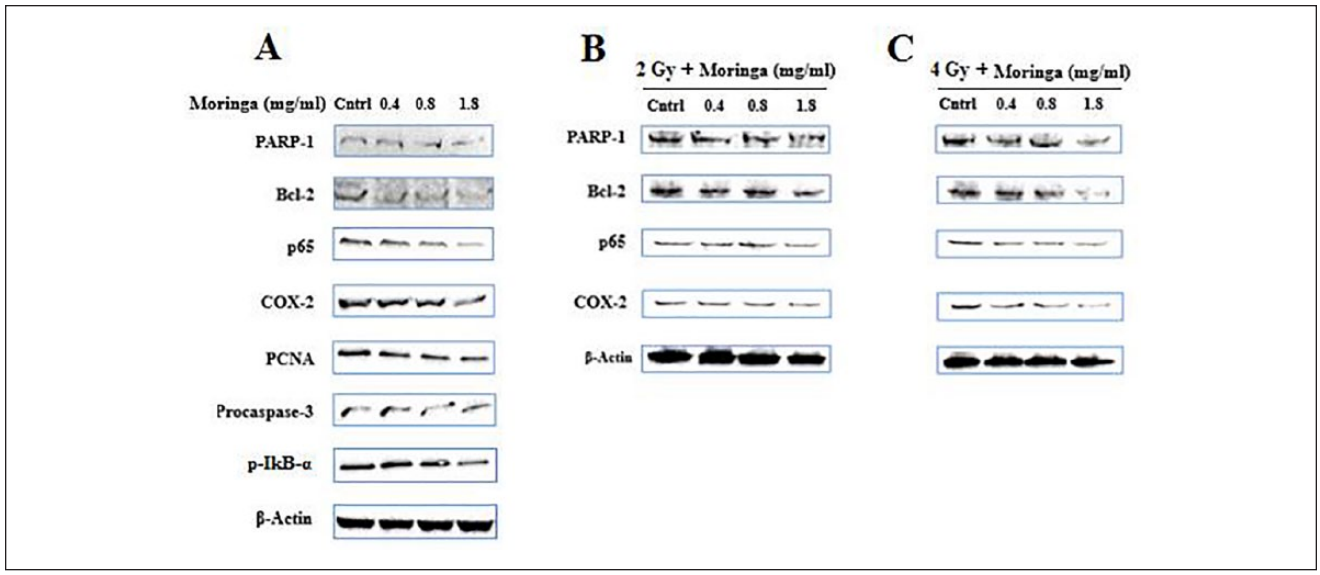

Figure 5. Combined effect of moringa and radiation on expression of selected proteins in PANC-1 cells. Cells nonirradiated (A) or irradiated with 2 Gy (B) or 4 Gy (C) were treated with moringa. Cell lysates were subjected to Western blot analysis with PARP-1, Bcl-2, COX-2, and p65 antibodies. β-Actin was used as a control for protein loading.

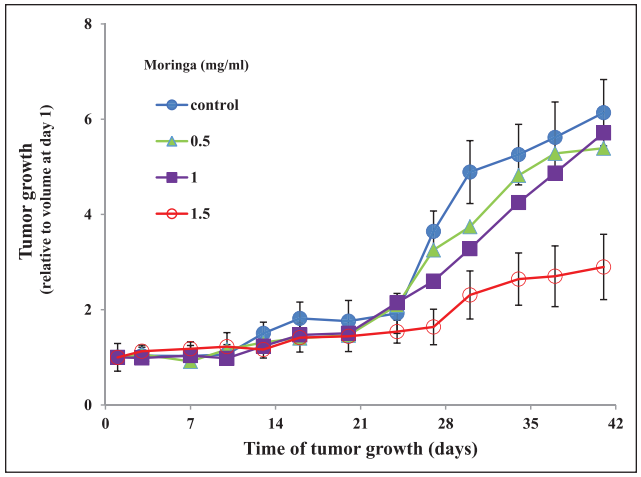

Figure 6. Effect of moringa on growth of tumors generated by PANC-1 cells. Cells (1.0×10^6 cells/mouse) were implanted subcutaneous into athymic nude mice. Tumor bearing mice were divided in 4 groups according treatment with moringa administered intraperitoneally twice a week. Tumor size was measured twice a week and tumor volume was calculated for each mouse.

treatments for pancreatic cancer, but it has limited efficiency.[2] Irradiation of radioresistant PANC-1 cells with a dose of 2 Gy resulted in an only 20% decrease in cell survival (Figure 1). Based on our previous study,[8] we hypothesized that moringa could enhance the efficacy of radiotherapy. As expected, the combination of moringa and radiation decreased cell survival more significantly than each treatment alone (Figure 1).

It is well known that irradiation of cancer cells results in a transient generation of ROS/RNS (reactive nitrogen species) oxidative stress and consequent DNA damage that leads to cell death. In order to evaluate the effect of ROS on moringa and radiation-induced cell death, PANC-1-treated cells were co-exposed to sodium pyruvate since it is known to protect cells from ROS-induced cell death.[12] Our findings showed that pyruvate mitigated the inhibitory effect of radiation alone and in combination with moringa on cell survival (Figure 2), but it did not change the effect of moringa on PANC-1 cell survival. These findings suggested that one of the mechanisms underlying radiotherapy efficiency in PANC-1 cells is ROS production, and that moringa does not induce cell death through ROS production.

Metastases are the most common cause of death from pancreatic cancer.[13] Progression of pancreatic cancer to the metastatic stage is characterized by altered cellular plasticity, increased motility and invasiveness, downregulation of cell-cell contact, and elevated expression and activation of matrix-degrading proteins.[14] Qian et al[15] showed that irradiation of PANC-1 cells with 5 Gy and 10 Gy increased motility and migration of the cells. Contrarily, our findings indicated that irradiation of PANC-1 cells had a slight inhibitory effect on cell invasion and that it also suppressed migration (Figure 3). These results were the first demonstration that moringa decreased PANC-1 cell migration and invasion in a dose-dependent manner (Figure 3). Moringa combined with radiation decreased PANC-1 cell migration and invasion more significantly than each treatment alone (Figure 3). The irradiation of cells generated free radicals and induced direct DNA damage that leads to apoptosis and necrosis in various cancer cells.[16-18]

Pancreatic cancer cells have been shown to be resistant to radiation: for example, 6 Gy irradiation of PANC-1 cells

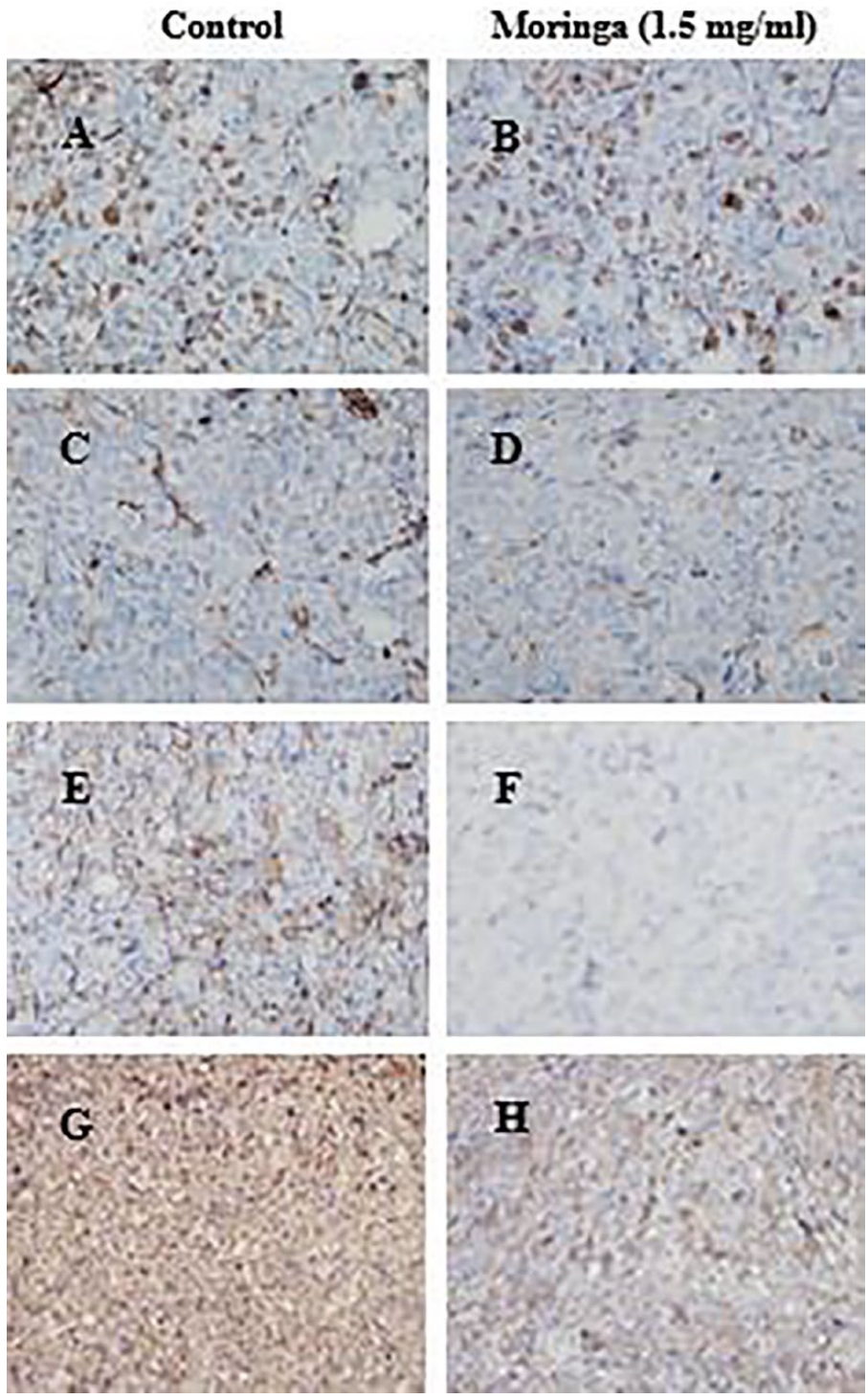

Figure 7. Effect of moringa (1.5 mg/mL) on expression of selected proteins in tumors. Immunohistochemistry of nontreated and treated tumor sections stained for different markers: (A and B) Ki-67 marker of proliferation; (C and D) CD31 marker of angiogenesis; (E and F) Bcl-2 antiapoptotic protein; and (G and H) IκB-α NF-κB inhibitor protein. Magnification ×400.

resulted in only 15% apoptosis.[19] We also found that 4 Gy irradiation of PANC-1 cells induced 12.3% apoptosis and 10.1% necrosis (Figure 4A), and that moringa (1.8 mg/mL) induced 18% apoptosis and 20% necrosis (Figure 4B). These data show that irradiation and moringa caused cell death through the induction of apoptosis and necrosis.

Bcl-2-family proteins play a key role in cell death through positive and negative regulators of apoptosis. Alterations in the expression and function of these proteins contribute to the pathogenesis and progression of human cancers, including pancreatic cancer. Downregulation of antiapoptotic proteins enhanced the effect of radiation on cancer cells, including PANC-1 cells. We also found a significant inhibitory effect of moringa on Bcl-2 expression in PANC-1 cells irradiated by 4 Gy (Figure 5).

COX-2 protein is known to be upregulated by radiation.[20,21] It was suggested that inhibition of COX-2 by celecoxib may potentiate radiotherapy by increasing cellular radiosensitivity.[22,23] We also found that radiation upregulated COX-2 in PANC-1 cells: specifically, the expression of COX-2 following 4 Gy treatment was lower than after 2 Gy (Figure 5). The combination of moringa and radiation resulted in more significant inhibition of COX-2 expression than the inhibitory effect of each treatment alone. Downregulation of Bcl-2 and COX-2 could be one of the mechanisms of moringa's radiosensitizing activity.

Cellular resistance to chemotherapeutic drugs and radiation is linked to NF-κB expression. Active NF-κB was shown to strengthen the cancer cells' ability to survive, while suppression of NF-κB led to the induction of apoptosis and cell death. On activation following various stimuli, such as radiation, the NF-κB inhibitor IκB-α is phosphorylated and degraded through ubiquitin-mediated proteolysis. As a result, the heterodimer p50/p65 translocates to the nucleus where it acts as a transcription factor for a large number of proteins, including well-described antiapoptotic proteins. Prasad et al[24] found that the expression of the p65 subunit is upregulated after exposure to 2 Gy. The exposure of PANC-1 cells to moringa was also shown in our laboratory to reduce the overall expression of key NF-κB family proteins (p65 and IκB-α).[8] Moreover, radiation upregulated p65 expression, as demonstrated by the expression of p65 following 4 Gy being lower than after 2 Gy (Figure 5). It has been reported that inhibition of NF-κB-related proteins improves the apoptotic response to radiation in pancreatic carcinoma.[25,26] Therefore, considering the profound link between NF-κB activation, radioresistance, and pancreatic carcinoma pathogenesis, the ability of moringa to downregulate and modulate NF-κB signaling by decreasing the p65 subunit may be used for increasing the susceptibility of pancreatic cells to radiation. Inhibition of the NF-κB signaling cascade and apoptosis-related proteins by the combination of moringa with radiation explains, at least in part, its inhibitory effect on the survival of the pancreatic cancer cell PANC-1.

PARP-1 is a crucial component of the base excision repair complex required for DNA single-strand and double-strand break repair, and its activation is one of the earliest responses to DNA damage caused by radiation.[27] PARP-1 activity is also essential in the upstream regulation of radiation-induced NF-κB activation.[28] Therefore, downregulation of PARP-1 may delay the repair of DNA damage and increase sensitivity to radiation. Moringa was found to induce PARP-1 cleavage in alveolar epithelial cells.[29] Our current data also indicated that the combination of radiation and moringa inhibited PARP-1 expression (Figure 5).

The *in vivo* part of our study demonstrated that moringa significantly inhibited pancreatic tumor growth in a dose-dependent manner (Figure 6). Immunohistochemistry revealed reduced antiproliferative (a decrease of the Ki-67 proliferation marker) and antiangiogenic (a decrease of the CD31 endothelial cell-specific marker) activity of moringa as well as decreased Bcl-2 and IκB expressions (Figure 7).

Conclusions

Moringa decreased pancreatic cancer cell survival and metastatic activity and significantly inhibited tumor growth. The moringa/radiation combination resulted in an additional inhibitory effect that provides a rationale for further investigation of this combination as a novel strategy to overcome the radioresistance of pancreatic cancer cells. According to our findings, the possible mechanisms of moringa's anticancer activity may be related to changes in the expression of PARP-1, Bcl-2, COX-2, p65, and p-IκB-a.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article. The study was supported by the Chaya and Kadish Shermeister Endowment, and by Donation from Mr. Diter Luffler.

References

1. Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. *Lancet*. 2011;378:607-620.
2. Li J, Wientjes MG, Au JL. Pancreatic cancer: pathobiology, treatment options, and drug delivery. *AAPS J*. 2010;12:223-232.
3. Conroy T, Desseigne F, Ychou M, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med*. 2011;364:1817-1825.
4. Van Hoff D, Ervin T, Arena FP, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med*. 2013;369:1691-1703.

5. Engdal S, Klepp O, Nilsen OG. Identification and exploration of herb-drug combinations used by cancer patients. *Integr Cancer Ther*. 2009;8:29-36.
6. Farooq F, Rai M, Tiwan A, et al. Medicinal properties of *Moringa oleifera*: an overview of promising healer. *J Med Plants Res*. 2012;6:4368-4374.
7. Suphachai C. Antioxidant and anticancer activities of *Moringa oleifera* leaves. *J Med Plants Res*. 2014;8:318-325.
8. Berkovich L, Earon G, Ron I, Rimmon A, Vexler A, Lev-Ari S. *Moringa oleifera* aqueous leaf extract down-regulates nuclear factor-kappaB and increases cytotoxic effect of chemotherapy in pancreatic cancer cells. *BMC Complement Altern Med*. 2013;13:212.
9. Jung IL. Soluble extract from *Moringa oleifera* leaves with a new anticancer activity. *PLoS One*. 2014;9:e95492. doi:10.1371/journal.pone.0095492
10. Al-Asmari AK, Albalawi SM, Athar MT, Khan AQ, Al-Shahrani H, Islam M. *Moringa oleifera* as an anticancer agent against breast and colorectal cancer cell lines. *PLoS One*. 2015;10:e0135814. doi:10.1371/journal.pone.0135814
11. Feldman J, Goldwasser R, Mark S, Schwartz J, Orion I. A mathematical model for tumor volume evaluation using two-dimensions. *J Appl Quant Methods*. 2009;4:455-462.
12. Wang X, Perez E, Liu R, Yan LJ, Mallet RT, Yang SH. Pyruvate protects mitochondria from oxidative stress in human neuroblastoma SK-N-SH cells. *Brain Res*. 2007;1132:1-9.
13. Stathis A, Moore MJ. Advanced pancreatic carcinoma: current treatment and future challenges. *Nat Rev Clin Oncol*. 2010;7:163-172.
14. Fujita M, Otsuka Y, Imadome K, Endo S, Yamada S, Imai T. Carbon-ion radiation enhances migration ability and invasiveness of the pancreatic cancer cell PANC-1, in-vitro. *Cancer Sci*. 2012;103:677-683.
15. Qian LW, Mizumoto K, Urashima T, et al. Radiation-induced increase in invasive potential of human pancreatic cancer cells and its blockade by a matrix metalloproteinase inhibitor CGS27023. *Clin Cancer Res*. 2012;8:1223-1227.
16. Ahire V, Kumar A, Mishra KP, Kulkarni G. Ellagic acid enhances apoptotic sensitivity of breast cancer cells to γ-radiation. *Nutr Cancer*. 2017;69:904-910.
17. Dai Y, Desano J, Qu Y, et al. Natural IAP inhibitor embelin enhances therapeutic efficacy of ionizing radiation in prostate cancer. *Am J Cancer Res*. 2011;1:128-143.
18. Shirai Y, Shiba H, Iwase R, et al. Dual inhibition of nuclear factor kappa-B and Mdm2 enhance the antitumor effect of radiation therapy for pancreatic cancer. *Cancer Lett*. 2016;370:177-184.
19. Wang ZM, Lu J, Zhang LY, et al. Biological effects of low-dose-rate irradiation of pancreatic carcinoma cells in vitro using 125I seeds. *World J Gastroenterol*. 2015;21:2336-2342.
20. Steinauer KK, Gibbs I, Ning S, French JN, Armstrong J, Knox SJ. Radiation induces upregulation of cyclooxygenase-2 (COX-2) protein in PC-3 cells. *Int J Radiat Oncol Biol Phys*. 2000;48:325-328.
21. Sobolewski C, Cerella C, Dicato M, Ghibelli L, Diederich M. The role of cyclooxygenase-2 in cell proliferation and cell death in human malignancies. *Int J Cell Biol*. 2010;2010:215158.
22. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science*. 1998;281:1322-1326.
23. Koki A, Masferrer JL. Celecoxib: a specific COX-2 inhibitor with anticancer properties. *Cancer Control*. 2002;9(2 suppl):28-35.
24. Prasad A, Mohan N, Chandrasekar B, Meltz ML. Activation of nuclear factor kappa B in human lymphoblastoid cells by low-dose ionizing radiation. *Radiat Res*. 1994;138:367-372.
25. Li F, Sethi G. Targeting transcription factor NF-kappa B to overcome chemoresistance and radioresistance in cancer therapy. *Biochim Biophys Acta*. 2010;1805:167-180.
26. Papademetrio DL, Lompardía SL, Simunovich T, et al. Inhibition of survival pathways MAPK and NF-κB triggers apoptosis in pancreatic ductal adenocarcinoma cells via suppression of autophagy. *Target Oncol*. 2016;11:183-195.
27. Sancar A, Lindsey-Boltz LA, Unsal-Kaçmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu Rev Biochem*. 2014;73:39-85.
28. Veuger S, Hunter JE, Durkacz BW. Ionizing radiation-induced NF-kappaB activation requires PARP-1 function to confer radioresistance. *Oncogene*. 2009;28:832-842.
29. Tiloke C, Phulukdaree A, Chuturgoon AA. The antiproliferative effect of *Moringa oleifera* crude aqueous leaf extract on cancerous human alveolar epithelial cells. *BMC Complement Altern Med*. 2013;13:226.