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In vitro cytotoxicity and radiomodifying effects of aqueous extracts of *Moringa oleifera* (Lam)

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ABSTRACT

Moringa oleifera (*M. oleifera*) is renowned for its range of essential nutrients and bioactive compounds. However, the toxicity and potential radiation modifying effects of extracts from its entities are not well characterized. The cytotoxic and radiomodulatory effects of aqueous extracts obtained from seeds, leaves and flowers of *M. oleifera* were evaluated. Cytotoxicity and radiation modifying effects of extracts were assessed in apparently normal Chinese hamster lung fibroblasts (V79 cells), using the colony forming assay. The free radical scavenging activity of each extract was also assessed, using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. At relatively low concentrations of 6.25–100 µg/ml, the seed extract showed about 20% cytotoxicity and yielded radiation sensitizing factors ranging from 1.23±0.07 to 2.38±0.76. The leaf extract was non-toxic at concentrations of up to 50 µg/ml, but produced ~12% cell kill at 100 µg/ml. With little or no cytotoxicity on its own, the leaf extract was radiosensitizing, with radiation modifying factors ranging from 1.30±0.18 to 1.50±0.26. At all tested concentrations, the flower extract was non-toxic, but significantly enhanced cell growth in unirradiated cultures, showing no effect on cellular radiation response. With the exception of the leaf extract which had a maximum of only 9% free radical scavenging capacity, the other extracts showed no potential as radical scavengers. Aqueous extracts of seeds, leaves and flowers of *M. oleifera* may potentially be clinically useful as cytotoxic agents, radiosensitizers, and wound healing promoters.

Introduction

Radiation therapy is one of the most preferred treatment modalities for cancer. Promising approaches to optimize radiotherapy response involves concurrent therapies to enhance the local control with both radiosensitizers and radioprotectors [1]. Radiosensitizers may enhance the tumor-killing efficacy of radiation whilst limiting toxicity to normal cells. The commercial potential of radioprotectors resides in the contexts of their dual (clinical and non-clinical) utilities. Clinically, radioprotectors are used during radiotherapy to ameliorate collateral radiation damage to surrounding non-malignant tissue. Their non-clinical usage is a consequence of unplanned irradiation incidences, such as, occupational exposure and as a countermeasure for civil protection against terrorist activities.

New radiosensitizers and radioprotectors with high respective efficacies in targeting tumor or normal cells to selectively increase tumor cell killing during radiotherapy would bring tremendous gains into clinical radiotherapy. The possibility of one non-toxic compound or potion acting both as a radiosensitizer and a radioprotector in radiation therapy would be an even more attractive and beneficial strategy, given that systemic toxicity of current radioprotective and radiosensitizing drugs limits their applications.

Many protectants are very efficient scavengers of water-derived free radicals and, therefore, compounds with the ability to effectively quench or scavenge free radicals are good candidates for moderating the harmful effects of ionizing radiation [2,3]. When these pharmacological agents are present in the biological system prior to radiological procedures, they are capable of neutralizing highly reactive oxygen-derived free radicals produced by ionizing radiation, thereby obviating radiation-induced cellular damage [4,5]. Natural compounds, particularly those from edible plants, are seen as viable candidates to screen for pharmacological radiomodulators because they are generally safe, non-toxic and less expensive [6]. Plants constitute an enormous repository of natural antioxidants [7], and these reserves could offer leads for the development of novel, effective, non-toxic, cost-effective and attractive strategies for radiotherapy and biological radiation protection.

Moringa oleifera (Lam), a member of the family Moringaceae is well-known for its range of essential nutrients and bioactive compounds. Particularly, for its rich antioxidant pool, the fact that it is almost ubiquitous throughout the world except for temperate regions, and its profound medicinal and antioxidant properties are well documented [8-11].

The present study is an effort to identify and evaluate the *in vitro* radioprotective and radiosensitizing effects of *M. oleifera* in apparently normal Chinese hamster lung fibroblasts (V79 cells). Here, aqueous extracts of flowers, leaves and seeds from *M. oleifera* were obtained by sequential exhaustive

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extraction (SEE) of the dried pulverized plant materials with solvents of increasing polarity: n-hexane, dichloromethane, ethyl acetate, and ethanol. The marc from each plant part was then resuspended and extracted in water to afford the aqueous extract. The extracts were also assessed for their DPPH free radical scavenging capacities as an index of their antioxidant potential.

Materials and methods

Chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH) (cat #: D9132) and L-ascorbic acid (cat #: A0278) were purchased from Sigma-Aldrich (USA). All other chemicals and reagents were of analytical grade.

Plant material and preparation of aqueous extracts

Fresh leaves, seeds and flowers of *Moringa oleifera* were collected from Bawku (Upper-East Region, Ghana), authenticated by a certified curator, and a voucher specimen (JAM001/14) was prepared and deposited at the Ghana Herbarium (University of Ghana). Samples were shade dried at room temperature (28–31°C). To obtain the aqueous extracts used for this study, the shade dried parts of *M. oleifera* (leaves, seeds and flowers) were pulverized and 20.19, 26.21 and 18.18 g of powdered leaves, seeds and flowers, respectively, were separately subjected to 30 cycles of sequential exhaustive extraction (SEE) for 6 h. For each successive cycle, 200 ml of each solvent of increasing polarity, namely, n-hexane, dichloromethane, ethyl acetate and ethanol were used according to the classical Soxhlet technique. This method consists of continuous extraction of the plant material, while fresh amounts of solvent are delivered, leading to total extraction. Solvents were removed from each extract under reduced pressure in a BÜCHI Rotavapor R-200 rotary evaporator (Flawil, Switzerland) to obtain concentrated crude extracts. These extracts were then allowed to dry in an open air oven at 31°C to constant weights and were stored at 4°C for future use. In the current study, an aqueous extract from each plant part was prepared by re-suspending the spent marc, obtained after the ethanol step of the SEE, in 200 ml distilled water on a magnetic stirrer for 24 h at room temperature. The resulting extracts were centrifuged at 3 000 *rpm* for 10 min at ambient temperature, and the supernatants were filtered through a Whatman No. 1 filter paper and dried under reduced pressure. The dried extracts were stored at 4°C until required.

Cell line and culture maintenance

Chinese hamster lung fibroblast (V79) cells were routinely cultured in Eagle's Minimum Essential Medium, EMEM (Sigma-Aldrich, USA), containing 10% heat-inactivated fetal bovine serum (FBS) (HyClone, UK), penicillin (100 U/ml), streptomycin (100 µg/ml) (Lonza, Belgium), and maintained at 37ºC in a humidified atmosphere (95% air and 5% CO_2). Cultures were maintained by passaging weekly.

Extract cytotoxicity and radiomodulatory assays

Monolayers of exponentially growing V79 cells in 25-cm^2 tissue culture flasks were trypsinized to give single-cell suspensions and seeded in 25-cm^2 culture flasks. To evaluate potential toxicity of each extract, 300 and 500 cells were seeded per flask (in triplicate) for unirradiated cultures in the absence and presence of extract, respectively. To determine the radiomodulatory effects of the various extracts, a second set of cell cultures was prepared, in which 400 and 600 cells were seeded per flask (in triplicate) for cultures to be irradiated to 2 Gy without and with extract, respectively. The cultures were then incubated for 4–5 h to allow the cells to attach. For extract treatment, stock extract solutions were prepared in water and sterile filtered through 0.22-µm nylon filter membranes. Each extract was then diluted in sterile growth medium and added to the corresponding cell cultures at concentrations of 0, 6.25, 12.5, 25, 50, and 100 µg/ml. Crude aqueous extracts of *M. oleifera* from the flower (MF), leaf (ML) and seed (MS) were evaluated. The second set of cell cultures was immediately irradiated to 2 Gy at a dose rate of 1 Gy/min, using a Faxitron MultiRad 160 (Faxitron Bioptics, Tucson, AZ, USA).

Cytotoxicity and modulation of radiosensitivity in V79 cells was assessed on the basis of clonogenic cell survival. Immediately after treatment with extracts and irradiation, the cell cultures were re-incubated for 6 days. Cultures were then terminated and colonies fixed in glacial acetic acid:methanol:water (1:1:8, v/v/v), stained in 0.01% amido black in fixative, washed in tap water, air-dried, and counted. Three independent experiments were performed for each dose point and the mean (±SE) surviving fractions were determined. The interaction between extracts and X-irradiation (2 Gy) was expressed as a radiation modifying factor (*RMF*), defined as the ratio of the mean cell surviving fractions (*SF*) in the absence and presence of inhibitors, as follows:

$$RMF = SF(2\text{ Gy})/SF([\text{extract}] + 2\text{ Gy}) \quad (1)$$

The criteria for inhibition, no effect, and enhancement of radiosensitivity by inhibitors are $RMF<1.0$, $RMF=1.0$ and $RMF>1.0$, respectively.

DPPH free radical scavenging assay

To evaluate the crude extracts for their potential as free radical scavengers, a modified version of the method reported by Abderrahim and colleagues was employed [12]. Stock solutions of the aqueous extracts were prepared by dissolving the dried crude extract powders in water and the resultant solutions were sterilized by filtration. Ten microliters of a working stock of each extract in EMEM was dispensed into corresponding wells in 96-well Corning microplates, on ice, and 200 µl of a 0.06 mM solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in ethanol was added to give final extract concentrations of 0, 6.25, 12.5, 25, 50 and 100 µg/ml. The decrease in absorbance after 30 min incubation at room temperature was determined at 517 nm, using a Synergy Multidetection Multiplate Reader (Bio-Tek Instruments Inc., Vermont, USA). L-ascorbic acid, at the same concentrations as extracts, was used as a positive control for antioxidants. For each agent and concentration, triplicate wells were prepared and the mean absorbance was used to calculate the percent free radical scavenging, as follows:

$$\%DPPH\ scavenging\ activity = [(A_0-A_1)/A_0]\times 100\% \quad (2)$$

where A_0 and A_1 represent the DPPH absorbance in the absence and presence of extract, respectively.

Data analysis

Statistical analyses were performed using the GraphPad Prism (GraphPad Software, San Diego, CA, USA) computer program. Unless otherwise stated, data were presented as the mean (±SE) from at least 3 independent experiments. For each experiment and data point, 3 replicates were assessed.

Results

Cytotoxicity and radiomodulatory effects of crude aqueous extracts of *M. oleifera*

Cytotoxicity data for the V79 cells, based on clonogenic cell survival, in the presence and absence of the seed extract of *M. oleifera* at varying concentrations with or without radiation exposure are presented in Fig. 1. In unirradiated cultures, the extract was found to be toxic at all concentrations, with an average of about 20% cell kill. Exposure of cell cultures to seed extract prior to irradiation to 2 Gy resulted in an extract concentration-dependent reduction in cell survival. The SF2 decreased from 0.60 to 0.33 when extract concentration increased from 0 to 100 µg/ml (Fig. 1). This elevation in radiosensitivity translated to significant radiation modifying factors ranging from 1.23±0.07 at 6.25 µg/ml of extract to 2.38±0.76 at 100 µg/ml (Table 1).

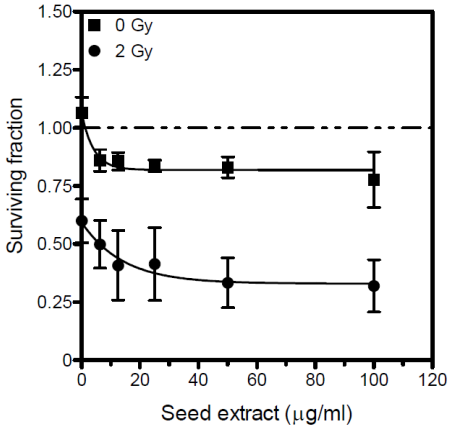

Figure 1. Cytotoxicity and radiomodulation of varying concentrations of an aqueous extract of *M. oleifera* seeds in V79 cells. Cell cultures were pre-treated with extract and immediately irradiated with X-rays to 0 and 2 Gy, respectively, and cell survival was assessed on the basis of colony forming efficiency. Symbols represent the mean surviving fraction ± SE from three independent experiments

When the cell cultures were treated with leaf extract alone, no cytotoxic effect was seen for extract concentrations of up to 50 µg/ml (Fig. 2). A 12% cell kill was, however, apparent at 100 µg/ml. In the presence of non-toxic extract concentrations (6.25–50 µg/ml), cells were radiosensitized, yielding radiation modifying factors ranging from 1.30±0.18 to 1.50±0.26 (Table 1). A higher leaf extract concentration of 100 µg/ml did not result in an increase in radiosensitization.

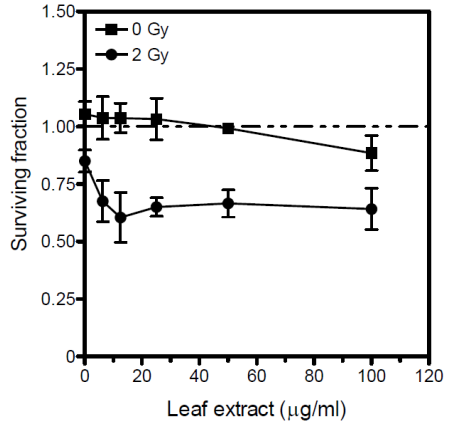

Figure 2. Cytotoxicity and radiomodulation of varying concentrations of an aqueous extract of *M. oleifera* leaves in V79 cells. Cell cultures were pre-treated with extract and immediately irradiated with X-rays to 0 and 2 Gy, respectively, and cell survival was assessed on the basis of colony forming efficiency. Symbols represent the mean surviving fraction ± SE from three independent experiments

Table 1. Summary of radiation dose-modifying data for V79 cells treated with three aqueous extracts from *Moringa oleifera*. RMF_2 denotes the radiation modifying factor at 2 Gy, presented as the mean ± SE from three independent experiments

Extract	Concentration (µg/ml)	RMF_2
Seed	6.25	1.23 ± 0.07
	12.5	1.96 ± 0.71
	25	2.00 ± 0.78
	50	2.26 ± 0.75
	100	2.38 ± 0.76
Leaf	6.25	1.30 ± 0.18
	12.5	1.50 ± 0.26
	25	1.32 ± 0.15
	50	1.30 ± 0.14
	100	1.37 ± 0.18
Flower	6.25	1.08 ± 0.01
	12.5	1.06 ± 0.01
	25	1.06 ± 0.06
	50	1.09 ± 0.03
	100	1.00 ± 0.06

Over the entire concentration range employed in this study, the flower extract of *M. oleifera* was observed to be non-toxic and was found to enhance cell growth in unirradiated cultures (Fig. 3). Treatment of cell cultures with flower extract, at concentrations of 6.25–100 µg/ml, yielded about 25% higher plating efficiency in comparison with untreated cultures. Exposure of cells to flower extract prior to irradiation did not affect radiosensitivity, with the SF2 being constant at about 0.75 regardless of extract concentration. The corresponding radiation modifying factors were essentially constant and were found to range between 1.00±0.06 to 1.09±0.03 (Table 1).

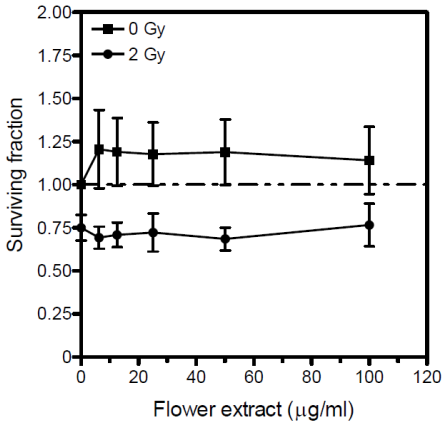

Figure 3. Cytotoxicity and radiomodulation of varying concentrations of an aqueous extract of *M. oleifera* flowers in V79 cells. Cell cultures were pre-treated with extract and immediately irradiated with X-rays to 0 and 2 Gy, respectively, and cell survival was assessed on the basis of colony forming efficiency. Symbols represent the mean surviving fraction ± SE from three independent experiments

Assessment of different extracts as DPPH free radical scavengers

To evaluate potential free radical scavenging activities of the *M. oleifera* extracts, samples were tested for their capacity to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. L-ascorbic acid was used as a standard reference antioxidant. The data presented in Fig. 4 demonstrates the highly efficient free radical scavenging activity of the standard antioxidant (ascorbic acid). The percentage of DPPH free radicals scavenged rose sharply with increasing ascorbic acid concentration to reach a plateau of ~97% at 50 µg/ml. The seed and flower extracts displayed no scavenging activity at all, while the leaf extract demonstrated a weak free radical scavenging ability, reaching a maximum of 9% at the highest extract concentration tested (Fig. 4).

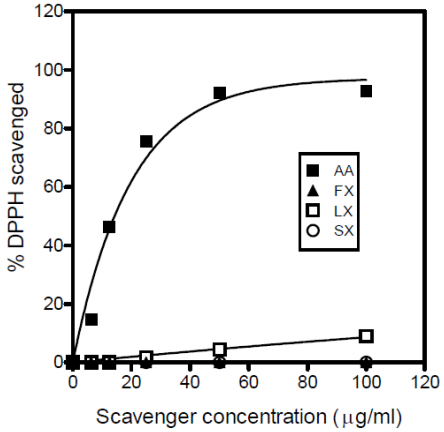

Figure 4. Cytotoxicity and radiomodulation of varying concentrations of an aqueous extract of *M. oleifera* flowers in V79 cells. Cell cultures were pre-treated with extract and immediately irradiated with X-rays to 0 and 2 Gy, respectively, and cell survival was assessed on the basis of colony forming efficiency. Symbols represent the mean surviving fraction ± SE from three independent experiments

Discussion

M. oleifera extracts have been studied extensively for their antioxidant properties [10]. Several benefits have been demonstrated for *M. oleifera* extracts. Leaf extracts of *M. oleifera* have been reported to protect against radiation-induced damage [8], and promote wound healing [13]. One of the basic goals of radiation oncology is to selectively increase the real or effective dose of radiation to the tumor to enhance therapeutic gain. Pharmacologically, this objective is attainable either through differential radiosensitization of tumor cells relative to normal tissue, or radioprotection of surrounding normal tissues relative to malignant tissues. On the radiation protection front, radioprotectors are being proposed as pharmacological countermeasures to minimize radiation-induced damage to individuals chronically exposed to low doses of ionizing radiation as a complementary line of protection [3-5], and to protect the civilian population against inadvertent and maliciously planned radiological events.

In the present study, crude aqueous extracts obtained from flowers, leaves, and seeds of *M. oleifera*, were evaluated for their potential cytotoxicity and radiomodulatory properties. For this, we assessed V79 cell survival using the colony forming assay. From the data presented in Fig. 1, the crude seed extract of *M. oleifera* is toxic to V79 cells and significantly enhances cellular radiation response, giving dose-modifying factors of ~1.23–2.38 (Table 1). Significantly high cytotoxicity has been demonstrated for aqueous seed extracts of *M. oleifera* in a fish model [14] and a variety of microbial systems [15], albeit at much higher extract concentrations. The radiosensitization seen here is consistent with the finding that aqueous seed extract of *M. oleifera* has no free radical scavenging capacity (Fig. 4), and therefore does not protect cells from the cytotoxic effects of ionizing radiation (Fig. 1).

Of note is the finding that the crude aqueous leaf extract of *M. oleifera* is non-toxic in V79 cells at concentrations of up to 50 µg/ml, but renders the cells more radiosensitive (Fig. 2). A marginal free radical scavenging activity was also noted (Fig. 4). The current findings for leaf extract of *M. oleifera* contrast with several studies demonstrating significant antiproliferative and cytotoxic effects in human tumor cells [16], strong antioxidant properties in both *in vitro* and *in vivo* systems [17], and radioprotection in a mouse model [18,19]. The attractive property of the radiosensitizing effect of the leaf extract, as depicted in Fig. 2, is that it occurs at low concentrations. These results seem to highlight the potential utility of leaf extract of *M. oleifera* as a radiosensitizer at sub-cytotoxic concentrations, and are consistent with a report by Berkovich et al. that leaf extracts of *M. oleifera* increase the efficacy of the chemotherapeutic drug cisplatin in human pancreatic cancer cells [20]. However, in the investigations of Berkovich and co-workers, leaf extract concentrations of over a 100-fold of those used in the current study were employed [20]. The radiosensitization seen at non-toxic concentrations is of significant clinical interest, and further investigations to explain the possible underlying mechanisms are warranted. One plausible reason for the observed radiosensitization is that at low concentrations the principles responsible for the radiosensitization may be present in active monomeric entities but may dimerize at higher extract concentrations, thereby, dousing out their bioactivity. However, this notion cannot be corroborated by the radiosensitization illustrated for very high leaf extract concentrations [20]. Also, the present data are not supported by the radioprotection exhibited by the leaf extract of *M. oleifera* in a murine model [8]. The possibility of the leaf extract of *M. oleifera* possessing both radiosensitizing and radioprotecting modes of action, akin to those reported for Curcumin [21], requires further elucidation.

The data presented in Fig. 3 clearly show that the flower extract is not only non-toxic on its own, but also enhances cell survival and proliferation. Furthermore, pre-treatment of cell cultures with flower extract of *M. oleifera* does not affect radiosensitivity (Table 1). The absence of a radiomodulatory effect for the flower extract is supported by the lack of a free radical scavenging activity, as demonstrated in Fig. 4. The pro-proliferative and pro-survival properties of the flower extract might have significant implications for wound management, especially in preoperative radiotherapy which is often besieged with quantifiable negative wound healing complications [22-25]. All concentrations of aqueous flower extracts tested here were sub-cytotoxic and appeared to provide cells with a biologically favorable environment for survival and proliferation. Although enhanced fibroblast proliferation in an unirradiated wound might promote healing, accelerated proliferation following radiation exposure appears to correlate with the development of wound healing morbidity or subcutaneous fibrosis [26]. The absence of a radiomodulatory effect when V79 cells were pre-treated with the flower extract (Fig. 3) seems to suggest that its presence

may not adversely affect the capacity of irradiated fibroblasts to contribute to wound healing. Significant wound healing activity has been demonstrated for the aqueous extract of *M. oleifera* leaves [13]. For now, the mechanism of action for the observed pro-proliferative and pro-survival activity of the flower extract is unclear and warrants further investigation.

Conclusion

The present investigation has demonstrated the possibility that at relatively low concentrations, the aqueous extract of *M. oleifera* seeds may potentially act both as a cytotoxic agent and radiosensitizer. It is also shown that at low concentrations, the leaf extract is non-toxic, but appears to exhibit a radiosensitizing effect and could potentially be used as a safe tumor radiosensitizer. The study further highlights that the aqueous extract of *M. oleifera* flowers promotes cell growth and does not interfere with cellular radiosensitivity, making it a potential candidate agent for the management of wound healing.

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Conflict of interest

The authors report no conflicts of interest.

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