RESEARCH ARTICLE

In vitro Evaluation of Cytotoxic Activities of Essential Oil from *Moringa oleifera* Seeds on HeLa, HepG2, MCF-7, CACO-2 and L929 Cell Lines

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Abstract

***Moringa oleifera* Lam. (Moringa*ceae*) is widely consumed in tropical and subtropical regions for their valuable nutritional and medicinal characteristics. Recently, extensive research has been conducted on leaf extracts of *M. oleifera* to evaluate their potential cytotoxic effects. However, with the exception of antimicrobial and antioxidant activities, little information is present on the cytotoxic activity of the essential oil obtained from *M. oleifera* seeds. Therefore, the present investigation was designed to investigate the potential cytotoxic activity of seed essential oil obtained from *M. oleifera* on HeLa, HepG2, MCF-7, CACO-2 and L929 cell lines. The different cell lines were subjected to increasing oil concentrations ranging from 0.15 to 1 mg/mL for 24h, and the cytotoxicity was assessed using MTT assay. All treated cell lines showed a significant reduction in cell viability in response to the increasing oil concentration. Moreover, the reduction depended on the cell line as well as the oil concentration applied. Additionally, HeLa cells were the most affected cells followed by HepG2, MCF-7, L929 and CACO-2, where the percentages of cell toxicity recorded were 76.1, 65.1, 59.5, 57.0 and 49.7%, respectively. Furthermore, the IC_{50} values obtained for MCF-7, HeLa and HepG2 cells were 226.1, 422.8 and 751.9 µg/mL, respectively. Conclusively, the present investigation provides preliminary results which suggest that seed essential oil from *M. oleifera* has potent cytotoxic activities against cancer cell lines.**

Keywords: Anticancer agents - cytotoxicity - medicinal plants - *Moringa* - seed oil

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Introduction

Cancer is one of the most harmful diseases leading to human death in developing as well as developed countries. In 2012, 14.1 million cancer cases have been reported worldwide, from which 52.5% cases occurred in men and 45.4% cases were in women (Sultana et al., 2014). The number of cancer cases is estimated to increase by about 70% in 2035 (Ferlay, 2014). Breast and cervical cancers are two of the most common cancer causes in women worldwide (WCR, 2008; Farshori et al., 2013; Srisuwan et al., 2014). In Saudi Arabia, there was a significant increase in the age-standardized incidence rate (ASIR) female breast cancer between 2001 and 2008, with the eastern province recording the highest overall ASIR, at 26.6 per 100,000 women (Alghamdi et al., 2013). On the other hand, the occurrence of cervical cancer in Saudi Arabia is very low, ranking 11th between all female cancers, and accounts only for 2.4% of all new cases reported (Alsbeih et al., 2013). Colon cancer, the third most common cancer worldwide, accounts for the fourth occurring cancer among all age groups in Saudi Arabia (Al-Kuraya et al., 2006). Liver cancer is considered as the third cause of cancer mortality worldwide (Farooq et al., 2013). In Saudi Arabia, liver cancer accounts for about 10 and 4% of total cancers in males and females, respectively (Al-Ahmadi and Al-Zahrani, 2013).

Standard cancer treatment protocols depended largely on chemotherapy and/or radiotherapy, as well as surgical intervention (Huang et al., 2014). However, due to sever physical and psychological side effects of such standard treatment methods as well as recent advancements and developments in the field of isolation and identification of phytochemical compounds, there have been an increasing trend during the last two decades toward the application of traditional herbal medicine as a potential source for anticancer agents (Awodele et al., 2012; Shahat et al., 2013). Nowadays, several anticancer agents, currently used in clinical trials, have been isolated and identified form plants and their constituents. Vinblastine, camptothecin, homoharringtonine, ellipticine and paclitaxel have been isolated from *Catharanthus roseus* G. Don. (Apocynaceae), *Camptotheca acuminate* Decne (Nyssaceae), *Cephalotaxus harringtonia* var.

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drupacea (Sieb and Zucc.) (Cephalotaxaceae), *Bleekeria vitensis* A.C. Sm. and *Taxus brevifolia* Nutt. (Taxaceae), respectively (Bakar et al., 2010; Kuete et al., 2011; Sultana et al., 2014).

The flowering plant family Moringaceae contains only the genus *Moringa* with 13 species distributed from tropical to subtropical regions and ranging in size from tiny herbs to massive trees (Janick and Paull, 2008). The most widely known species is the *M. oleifera* Lam., also known as horseradish tree, drumstick tree, melonge or malunggay (Awodele et al., 2012). Beside their valued nutritional characteristics, different parts of *M. oleifera* (leaves, flowers, park, pods, stem, roots and seeds) have been reported to possess different biological properties, i.e. antimicrobial, antitrypanosomal, hypotensive, antiulcer, hypocholesterolemic, antispasmodic, antioxidant, anti-inflammatory as well as anti-cancer (Sreelatha et al., 2011; Awodele et al., 2012; Satish et al., 2013; Tiloke et al., 2013; Vongsak et al., 2013a; 2013b; Hannan et al., 2014). *M. oleifera* seeds contain 33-41% w/w vegetable oil (Rashid et al., 2008). The seed oil of *M. oleifera* resembles olive oil and is widely used in cooking, lighting, hairdressing as well as soap and perfume industries (Ghazali and Mohammed, 2011). Moreover, it has been found to exhibit antimicrobial and antioxidant activities (Siddhuraju and Becker, 2003; Chuang et al., 2007).

Taking into consideration the lack of anticancer studies on seed oils from *Moringa*, to the best of our knowledge, the present investigation was designed to evaluate the possible anti-proliferative activity of seed oil obtained from *M. oleifera* on human cervical, hepatocellular, breast, colon cancers as well as mouse fibroblast cell lines.

Materials and Methods

Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM) culture medium containing 4.5 g/L glucose was purchased from AppliChem, Darmstatt, Germany. Fetal bovine serum (FBS), 0.25% Trypsin-EDTA solution and antibiotic/antimycotic solution were purchased from GIBCO® Invitrogen, Life Technologies, USA. Other chemicals and reagents were of cell culture grade and were purchased from Sigma-Aldrich Chemical Company, St. Louis, MO, USA. Disposable culture ware and consumable materials were procured from Corning, NY, USA.

Preparation of seed oils

Commercial purified oil of *M. oleifera* seeds was kindly provided by AsiaNet Trading Est., Riyadh, KSA. The oils were extracted by cold pressing in order to retain all the aromatic, chemical and nutritional characteristics of the oil. The purified oil was used to prepare a stock solution of 1 mg/mL in DMSO. The working seed oil solutions were prepared by serially diluting the stock oil solution with the cultivation medium. Finally, the working solution sets were filtered using 0.22 μm sterile syringe filters (Millipore, USA).

Cell lines and cultivation conditions

HeLa, human cervical cancer; HepG2, human hepatocellular carcinoma; MCF-7, human breast cancer; CACO-2, Caucasian colon adenocarcinoma and L929, mouse fibroblast cell lines, were purchased from Sigma-Aldrich Chemical Company, USA. Cells were cultivated in DMEM, supplemented with 10% FBS, 100x, 1% antibiotic/antimycotic solution and 3.6 g/L $NaHCO_3$. Cells were routinely sub-cultured and propagated in a humidified CO_2 incubator (ShelLab, USA) at 5% CO_2, 37°C and 95% humidity. Upon testing the effect of seed oil on cell viability, cells were trypsinized, centrifuged and washed twice with sterile PBS buffer solution. The viable cell count and viability were estimated using Trypan blue exclusion method (Al-Sheddi et al., 2014). Cell batches with viabilities above 95% were used to run the cytotoxicity assays.

Treatments and experimental design

The tested different cell lines were used to evaluate the cytotoxic effects of *M. oleifera* seed oil. Cells were treated with different concentrations of the prepared seed oil ranging from 15.6 μg/mL to 1 mg/mL. After cell treatment, the cytotoxic activities of the tested seed oils were screened using 3-(4,5-dimethylthiazol-2-yl),5-biphenyl tetrazolium bromide (MTT). Control sets were treated with DMSO only (final concentration not exceeding 0.5%). In addition, the morphological changes were assessed using phase contrast inverted microscope.

Cytotoxicity assay

The percentage of cell viability was evaluated with MTT according to the method described by Siddiqui et al. (2008). In brief, after trypsinization and washing, cells were resuspended and were used to inoculate 96 well culture plates at a final concentration of 10^4 cells/100 μl/well. The plates were incubated at 5% CO_2 and 37°C to allow cell adherence. After 24h, the medium was replaced with fresh medium containing different concentrations from the prepared seed oil serial dilutions, and were allowed to grow for another 24h. After incubation, 10 μl MTT (5 mg/mL in PBS) were added to each well, and the plates were further incubated for 4h. The supernatants were then discarded and 200 μl of DMSO were added to each well and mixed to dissolve the precipitated dark blue formazan crystals. The absorbance of formazan, directly proportional to the number of living cells, was read at 550 nm using a microplate reader (Thermo Scientific, USA). The percentages of viability (%V) and inhibition (%I) were calculated as:

$$\%V=100(A_t/A_c)$$
$$\%I=100\ [1-(A_t/A_c)]$$

where A_t and A_c are the absorbances for treated and control cells, respectively. IC_{50} values were defined as sample concentration inhibiting 50% of cell growth, and they were obtained from the linear regression of the calibration curve.

Morphological examination

Cells treated with different concentrations of seed oil were microscopically examined for morphological changes using an inverted contrast microscope (Nikon Eclipse T500, Japan) at 10x magnification.

Statistical analysis

The obtained data were analyzed with the help of SPSS 9.0, and the results were given as mean±SD of three replicates. The mean comparison between different evaluated groups was performed using ANOVA one-way analysis of variance. Statistical significance was defined when $p<0.05$.

Results

Cytotoxicity assay

The cytotoxic effect of the *M. oleifera* essential oil on different cell lines (HeLa, HepG2, MCF-7, CACO-2 and L929) has been evaluated using standard MTT assay. The different cell lines have been treated with increasing concentrations (15-1000 μg/mL) for 24h. The obtained results illustrated in Figure 1A-E show clearly, that increasing the concentration of the essential oil adversely decreased the viability of the treated cell lines, with a highly significant effect ($p<0.001$). Moreover, the highest concentration applied (1 mg/mL) gave the highest decrease in cell viability in all cell lines, where the viability recorded 23.9±0.85, 34.93±0.32, 40.48 ±6.61, 50.28±5.86 and 42.99±0.17 for HeLa, HepG2, MCF-7, CACO-2 and L929 cells, respectively. Furthermore, the obtained results, for all cell lines, also revealed that the effect on cell viability was not statistically significantly changed upon treating the cells with either 15.6 and 31.3 μg/mL or 62.5 and 125 μg/mL. Additionally, it can be clearly observed that treatment of HeLa and MCF-7 cells with either 31.3 or 62.5 μg/mL showed no statistically significant difference. On the other hand, CACO-2 cells showed no statistically significant difference in cell viability when treated with a concentration ranging from 31.3 to 250 μg/mL.

The results in Figure 1F compares between the highest percentages of toxicity obtained for different cell lines at 1 mg/mL of essential oil. It can be clearly noted that HeLa cells were the most affected cells followed by HepG2, MCF-7, L929 and CACO-2, where the percentages of cell toxicity recorded 76.05, 65.07, 59.52, 57.02 and 49.72%, respectively.

The concentration of essential oil required to inhibit 50% of the cells is recorded in Table 1. The results showed that the IC_{50} value obtained for MCF-7 cells was 226.1 μg/mL, while the values recorded for HeLa and HepG2 cells were higher by 1.9-and 3.3-folds. On the other hand, the IC_{50} values for CACO-2 and L929 cells were higher than 1 mg/mL.

Morphological examination

Figure 2 shows the morphological changes occurred in HepG2, MCF-7, L929 as well as CACO-2 cells upon treatment with different concentrations of *M. oleifera* seed oil. Increasing the seed oil concentration (>125 μg/mL) resulted in a drastic change in the morphological characteristics of the tested cell lines, which was proportional to the applied concentration. Cells started to shrink and lose their capacity to adhere to the surface of the cultivation plate. Moreover, at the highest applied concentration, cells appeared rounded and were completely floated in comparison to the control morphology.

Table 1. IC_{50} Values Obtained for Different Cell Lines as Affected by Essential Oil From *M. oleifera* Seeds.

L929	CACO-2	MCF-7	HepG2	HeLa	Cell Line
>1000.0	>1000.0	226.1	751.9	442.8	IC_{50} (μg/mL)

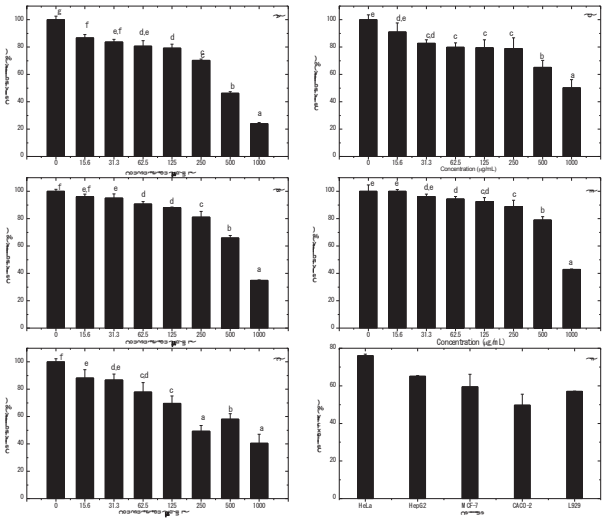

Figure 1. Effect of Different Concentrations of *M. oleifera* Seed Oil on the Viability of Different Cell Lines (A: HeLa, B: HepG2, C: MCF-7, D: CACO-2, E: L929, F: Toxicity on Different Cell Lines at 1 mg/mL). Data are expressed as means±SD. Dissimilar alphabets within the same cell line indicate significantly different at $p<0.05$ using one-way ANOVA

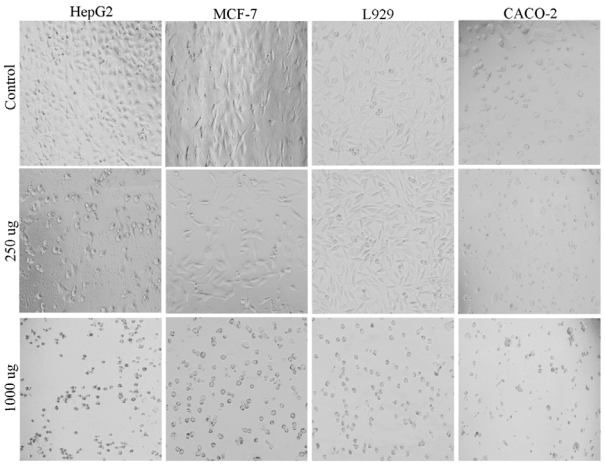

Figure 2. Morphological Changes in Different Cell Lines as Affected by Different Concentrations of *M. oleifera* Seed Oil after 24h. Images are captured using inverted contrast microscope (Nikon Eclipse T500) at 10x magnification

Discussion

Natural medicinal plants and their constituents provide the pharmaceutical industry with more than 60% of the compounds exhibiting potent cytotoxic activities against cancer cells (Cragg and Newman, 2005). *M. oleifera*, growing in tropical and subtropical climates, has been highly appreciated for its nutritional benefits. The leaves of *M. oleifera* are rich in proteins, minerals and vitamins A, B and C, and they contain all essential amino acids (Janick

and Paull, 2008). During the past two decades, *M. oleifera* leaves have been shown to possess different biological activities; antimicrobial, anti-inflammatory, antioxidant, anti-parasitic as well as anticancer (Gasparotto et al., 2001; Anwar et al., 2007; Ferreira et al., 2011; Awodele et al., 2012). Although *M. oleifera* has been widely investigated for its anticancer activity, scarce information can be found in the literature regarding the cytotoxic activity of its seed essential oil. Therefore, the present investigation has been carried out to evaluate the anti-proliferative activity of *M. oleifera* seed oil on different cell lines; i.e. HeLa, HepG2, MCF-7, CACO-2 and L929. Our results showed that increasing the concentration of seed oil greatly decreased the viability of different cell lines depending on the concentration applied after 24h of incubation. These results are in accordance with those of Al-Oqail et al. (2013), who obtained a dose-dependent response of different concentrations of fenugreek seed oil on HEp2, MCF-7, WISH and Vero cells. Moreover, our obtained results showed variation among different cell lines when treated with different concentrations of seed oil. In such case, HeLa cells showed the highest decrease in cell viability followed by HepG2, MCF-7, L929 and CACO-2. The obtained results are in agreement with those of Heo et al. (2014), who also evaluated anticancer effects of different parts of indigo plant on different cell lines (Hek-293, HCT-116, HeLa, MCF-7, Hep3B, SNU-1066 and SNU-601). They concluded that the plant extract effectively reduced the cell viability of different cell lines depending on the extract concentration as well as cell type. Generally, the cytotoxic effects of the essential oil obtained from *M. oleifera* seeds can be attributed to the presence, nature and composition of the oil. Saturated and unsaturated fatty acids constitute 49.1 and 50.9% of *M. oleifera* seed essential oil (Abdulkarim et al., 2005). Among these fatty acids, octadecenoic, hexadecanoic, octadecanoic and docosanoic acids correspond for 70.0, 6.8, 6.5 and 5.8%, respectively. Recently, unsaturated fatty acids have been reported to exhibit anticancer activity against many malignant cell lines *in vitro*, as well as reducing the tumor growth and cancer incidence in animal models (Scheim, 2009). Furthermore, Mustafa et al. (2004) investigated the anticancer activities of different fatty acid analogs of podophyllotoxin on SK-MEL, KB, BT-549, SK-OV-3 and HL-60 cell lines. Their fatty acid analogs incorporated 10-hydroxydecanoic, 12-hydroxydodecanoic, 15-hydroxypentadecanoic, 16-hydroxyhexadecanoic, 12-hydroxyoctadec-Z-9-enoic, eicosa-Z-5,8,11,14-tetraenoic, eicosa-Z-8,11, 14-trienoic, eicosa-Z-11,14-dienoic, eicosa-Z-11-enoic and eicosanoic acids. They found that their analogs, except for eicosa-Z-11-enoic and eicosanoic acids, exhibited cytotoxic effects against the different cancer cell lines. Additionally, Ravichandran and Johnson (1999) suggested that the antitumor effect of essential fatty acids is related to the increased lipid peroxidation leading to increased production of free radicals, which finally lead to the modification of membrane fatty acid composition of tumor cells.

The effect of different concentrations of seed oil on the morphology of different cell lines showed a concentration dependent-effect where the cells started to lose their normal morphological characteristics as well as adhering capacity, and finally leading to cell detachment and death. These results are in accordance with those of Vijayarathna and Sreenivasan (2012), who evaluated the cytotoxic effects of *Elaeis guineensis* on MCF-7 and Vero cells. They found that both cell lines showed morphological alterations with cell blebbing and vacuolation and suggested autophagy as a mechanism for cell death.

Finally, from the aforementioned results, it can be concluded that seed essential oil of *M. oleifera* has statistically significant cytotoxic effects on HeLa, HepG2, MCF-7, CACO-2 and L929 cells. Moreover, the effect was proportional to the applied concentration, with HeLa, HepG2 and MCF-7 cell lines as the most affected cell lines. Additionally, the present work provides a preliminary platform for further investigation of the possible mechanism and role of *M. oleifera* seed oil on cancer cell lines.

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