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Research Article

Moringa oleifera Extracts Induce Cholangiocarcinoma Cell Apoptosis by Induction of Reactive Oxygen Species Production

*Leelawat S[1], Leelawat K[2,3]

[1]*Faculty of Pharmacy, Rangsit University, Patumthani, 12000, Thailand*
[2]*Department of Surgery, Rajavithi Hospital, Bangkok, 10400, Thailand*
[3]*College of Medicine, Rangsit University, Bangkok, 10400, Thailand*

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ABSTRACT

To date, there are no effective chemotherapeutic treatments for cholangiocarcinoma. The anticancer effects of *Moringa oleifera* extracts have been demonstrated in various kinds of cancers. Therefore, we evaluated the anticancer effects of *M. oleifera* extracts on cholangiocarcinoma cells. In this study, RMCCA1 and KKU100 human hilar cholangiocarcinoma cell lines were treated with *Moringa* extracts and examined for cell viability and apoptosis. Factors that contribute to cancer-cell survival in response to stress were assayed, including mitochondrial membrane potential changes and reactive oxygen species (ROS) production. The results showed that *Moringa* extracts inhibited cell proliferation and induced cell apoptosis. In addition, treatment with *Moringa* leaf and seed extracts (MLE and MSE) induced a marked decrease in mitochondrial membrane potential and an increase in reactive oxygen species (ROS) production in cholangiocarcinoma cells. Consequently, pretreatment with N-acetylcysteine (NAC), an antioxidative agent, completely inhibited the cytotoxicity of MLE and MSE in both cholangiocarcinoma cell lines. In conclusions, these results indicate that the *Moringa* extracts induce cytotoxicity in cholangiocarcinoma cells through the induction of ROS accumulation and mitochondria dysfunction. These findings suggest that after future studies, *M. oleifera* may serve as a potential therapeutic agent for cholangiocarcinoma.

Key words: *Moringa oleifera*, cholangiocarcinoma, apoptosis, reactive oxygen species

INTRODUCTION

Cholangiocarcinoma (CCA) is a cancer arising from the bile duct epithelium. This cancer is one of the most aggressive malignant tumors and is associated with local invasiveness, as well as a high rate of metastasis[1,2]. Three-year survival rates of 35% to 50% can be achieved only in a subset of patients with negative histological margins at the time of surgery[3]. Unfortunately, patients with cholangiocarcinoma are often diagnosed at an advanced stage. These patients with advanced cholangiocarcinoma have a median overall survival of only 9 months. Palliative therapeutic approaches consisting of percutaneous and endoscopic biliary drainage are typically used for these patients, as there is no effective chemotherapeutic treatment for this type of cancer[2-4]. The identification of the novel biological compounds against cholangiocarcinoma cells is therefore notably important.

Moringa oleifera has been shown to provide a wide spectrum of medicinal uses, as well as a strong nutritional value. Different parts of this plant, such as the leaves, roots and seeds, act as anti-inflammatory, diuretic, anti-hypertensive, cholesterol-lowering, hepatoprotective and antibacterial agents[5-7]. Recent study has demonstrated that leaf extracts from M. oleifera have strong anti-proliferative and potent apoptosis-inducing effects against human nasopharyngeal cancer cells (KB cells)[8]. However, there is no study examining the effects of M. oleifera on cholangiocarcinoma cells. Therefore, this study investigated the anticancer activity of M. oleifera on human cholangiocarcinoma cell lines.

MATERIALS AND METHODS

Moringa oleifera extraction: M. oleifera pods and leaves were collected from Pathumthani province, Thailand. Pods were separated into 3 parts (pod husk, pulp and seed). All samples were dried at 55°C and ground to powder. Five-gram powder samples were macerated 5 times with 200 ml of 70% ethanol for 48 hours. Extract solutions were dried by rotary evaporation and freeze-drying. All Moringa extracts were stored at -20°C for further assays.

Cell cultures: The human cholangiocarcinoma cell lines, RMCCA1[9] and KKU100[10], were grown in HAM's F12 (Gibco, Grand Island, NY, USA) medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C in a 5% CO_2 humidified atmosphere.

Cell proliferation assay: Cells were seeded in 96-well culture plates at a density of 10,000 cells per well followed by the addition of the Moringa pod husk, pulp, leaf and seed extracts in various concentrations or the vehicle (DMSO). The cells were subsequently incubated for 24 hours before applying WST-1 cell proliferation assay

*Author for correspondence: E-mail: surang.l@rsu.ac.th

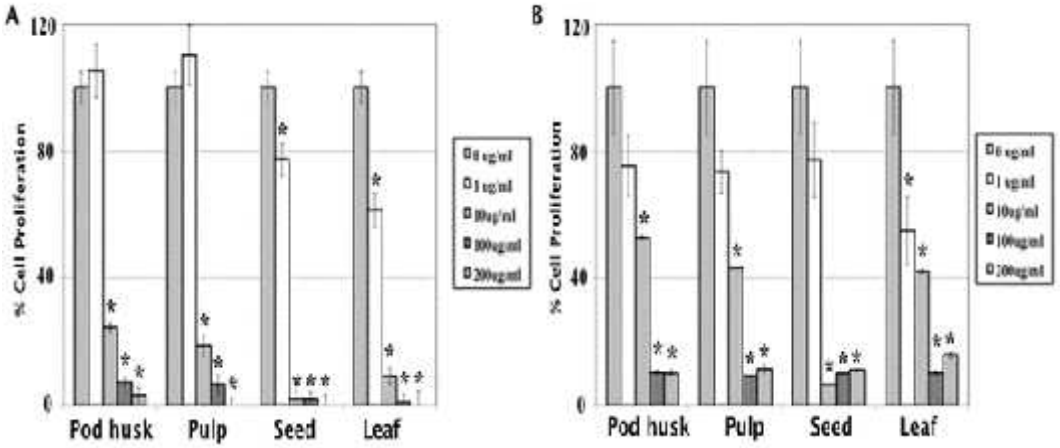

Fig. 1: Moringa extracts inhibit proliferation in cholangiocarcinoma cells
RMCCA1 (A) and KKU100 (B) cells were treated with Moringa pod husk, pulp, leaf and seed extracts at various concentrations (0-200 µg/ml) for 24 hours. Effects on cell proliferation were measured by WST-1 and analyzed by spectrophotometric analysis (absorbance = 450 nm). Results are shown as the mean ± SE of three independent experiments, where the optical density value from vehicle-treated cells was set as 100% of proliferation. (*, $p < 0.001$ compared with the vehicle-treated cells). *; $p < 0.05$ compared with the control cells

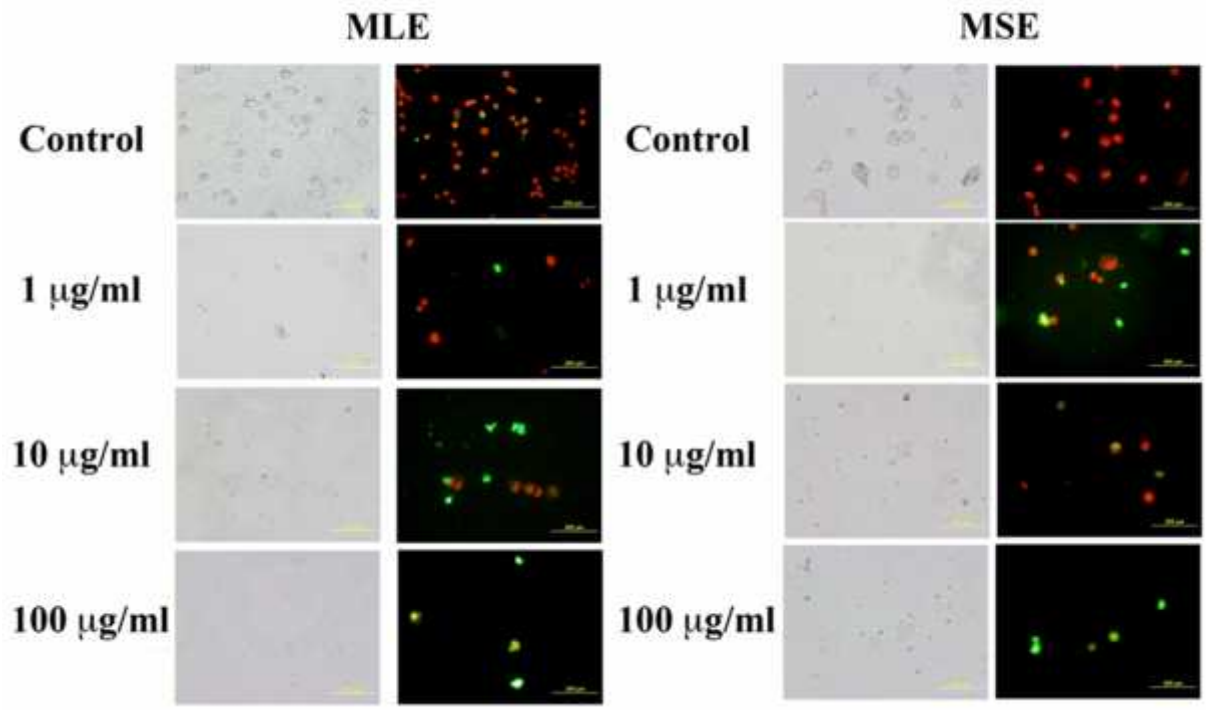

Fig. 2: Moringa extracts induce apoptosis in cholangiocarcinoma cells
RMCCA1 cells were treated with 1, 10 and 100 µg/ml MLE, MSE or vehicle. Cell apoptosis was determined with an Apo-BrdU TUNEL assay kit and counterstained with propidium iodide. Left panels show phase contrast images of RMCCA1 cells, while right panels show fluorescence images. Apoptotic cells were detected by an increase of green florescence signal.

reagent (Roche Diagnostics, Laval, Quebec) according to the manufacturer's recommendations. The percentage of cell proliferation was calculated relative to untreated cells.

TUNEL assay**:** Apoptosis was determined by TUNEL assays using an Apo-BrdU TUNEL assay kit (Invitrogen Co., Carlsbad, CA) following the manufacturer's instructions. Briefly, cells were fixed with 1% paraformaldehyde and ice-cold 70% ethanol for 30 minutes. Next, fixed cells were labeled with BrdUTP using terminal deoxynucleotide transferase (TdT) at 37°C for 60 minutes and stained with Alexa Fluor 488-labeled anti-BrdU antibody for 30 minutes at room temperature. To score for apoptosis, cells were counterstained with propidium iodide and counted under a fluorescent microscope at 400X magnification to determine the percentage of apoptotic cells per experimental condition.

Table 1: The percentage of apoptotic cells in RMCCA1 treated with MLE or MSE

	Control	1 µg/ml	10 µg/ml	100 µg/ml
MLE (mean ±SD)	4.3 ± 3.51	15.3 ± 7.61	76.5 ± 8.32*	88.7 ± 6.24*
MSE (mean ±SD)	5.2 ± 3.12	9.8 ± 6.13	81.0 ± 11.02*	92.7 ± 7.32*

**; p <0.05 compared with the control cells*

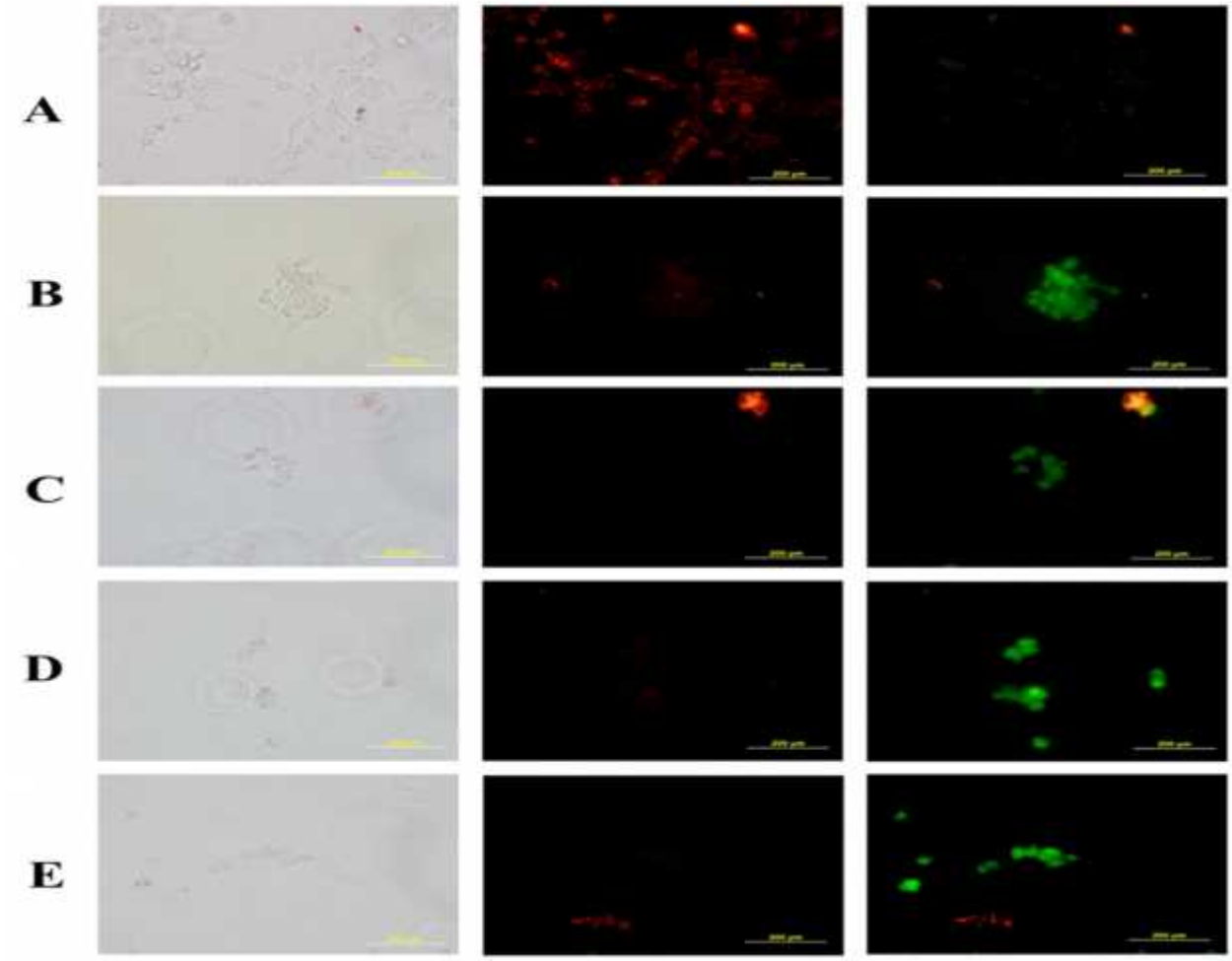

Fig. 3:Moringa extracts induce a marked decreased in mitochondrial membrane potential in cholangiocarcinoma cells Left panels show phase contrast images of KKU100 cells. Middle panels show red fluorescence images, while right panels show green fluorescence images. Loss of mitochondrial membrane potential was detected by a decrease of red fluorescence with a concomitant increase of green florescence. The experimental design is described in the material and method section. (A) untreated cells; (B) cells treated with 10 µg/ml MLE; (C) cells treated with 100 µg/ml MLE; (D) cells treated with 10 µg/ml MSE and (E) cells treated with 100 µg/ml MSE.

Mitochondrial-Membrane Potential (Δψm) assay

The detection of the mitochondrial permeability transition events provides an early indication of the initiation of cellular apoptosis. This process is typically defined as a collapse of the electrochemical gradient across the mitochondrial membrane, as measured by the change in the mitochondrial membrane potential (Δψ). The DePsipher™ Kit (Trevigen Inc, Gaithersburg, MD, Germany) was used to detect Δψ disruption. This kit uses a unique cationic DePsipher™ dye (5,5'6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanineiodide) to indicate the loss of the mitochondrial potential. The dye readily enters cells and fluoresces brightly red in its multimeric form within healthy mitochondria. In apoptotic cells, the mitochondrial membrane potential collapses, and the dye cannot accumulate within the mitochondria. In these apoptotic cells, the dye remains in the cytoplasm as a green fluorescent monomeric form. Apoptotic cells, showing primarily green fluorescence, are easily differentiated from healthy cells that show red fluorescence, which can be analyzed by fluorescence microscopy.

Following the manufacturer's protocols, cells were seeded in 12-well plates at a density of 2×10^5 cells/mL and

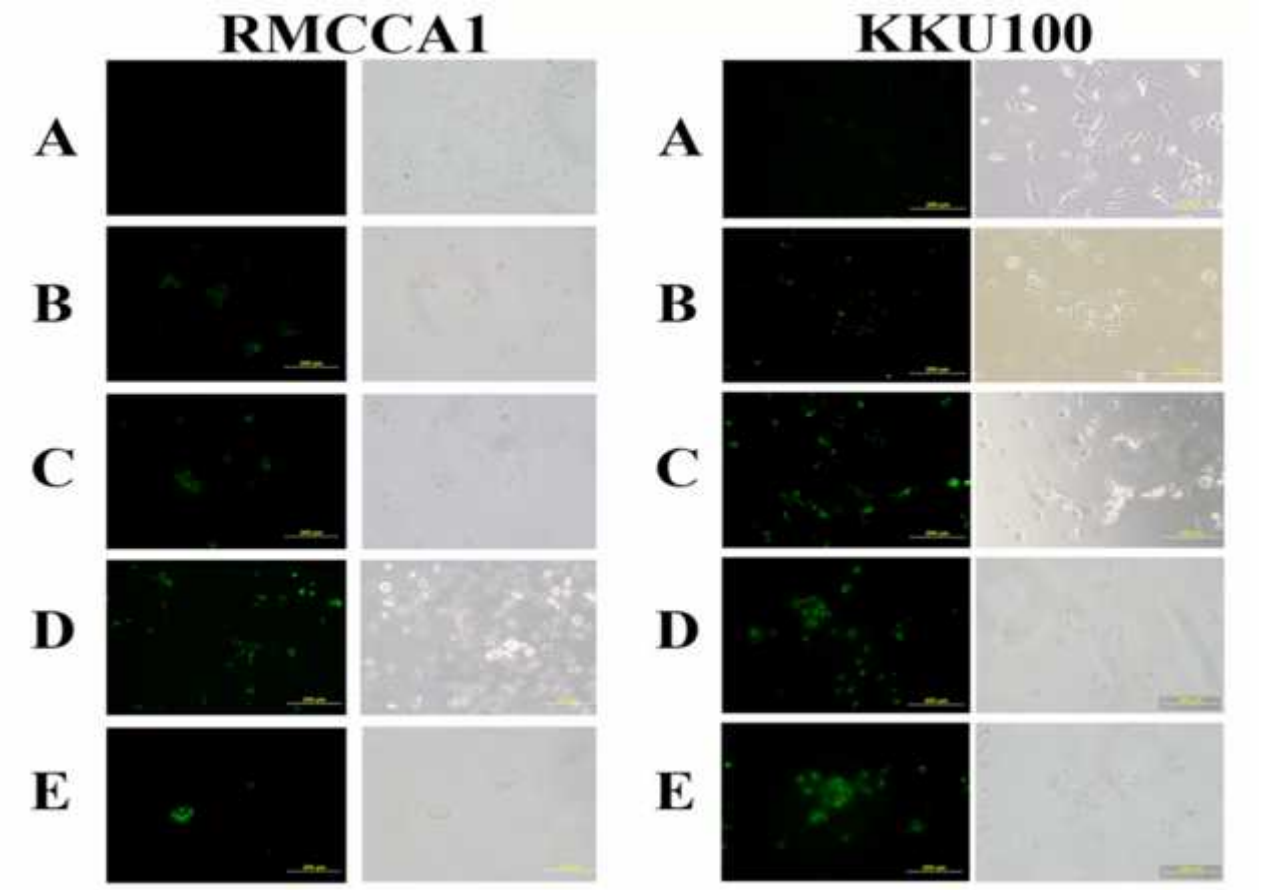

Fig. 4: Moringa extracts induce accumulation of ROS in RMCCA1 and KKU100 cholangiocarcinoma cells
Right panels show phase contrast images of cholangiocarcinoma cells, while left panels show green fluorescence images. Accumulation of ROS was detected by an increase of green florescence. The experimental design is described in the materials and methods section. (A) untreated cells; (B) cells treated with 10 µg/ml MLE; (C) cells treated with 100 µg/ml MLE; (D) cells treated with 10 µg/ml MSE and (E) cells treated with 100 µg/ml MSE.

treated with Moringa leaf and seed extracts (MLE and MSE) at doses of 0, 10 and 100 µg/mL for 24 h. Next, 1 ml of incubation buffer containing 1 µl DePsipher™ dye was added to each well, and plates were incubated for 20 min at 37°C in a 5% CO_2 incubator. Next, cells were analyzed under a fluorescent microscope at 400X magnification.

Detection of reactive oxygen species (ROS) production: ROS were detected using an Image-iT™ LIVE Green Reactive Oxygen Species Detection Kit (Molecular Probes, Eugene, OR, USA). Cholangiocarcinoma cells were seeded in 12-well plates at a density of 2×10^5 cells/mL and treated with MLE and MSE at doses of 0, 10 and 100 µg/mL for 24 h. Next, cells were incubated with 3 µM Carboxy-H_2DCFDA for 40 minutes at 37°C in the dark, and ROS were visualized under a fluorescence microscope at 400X magnification.

Statistical analysis: Experiments were all performed in triplicate, and quantitative data were described as means with standard deviation (SD). Data between three or more groups were compared using a one-way analysis of variance (ANOVA) followed by the Dunnett's post hoc test. A p value of less than 0.05 was considered to be statistically significant.

Results and discussion

The effect of *Moringa* extracts on cholangiocarcinoma cell proliferation: WST-1 assays were performed to investigate the effects of Moringa pod husk, pulp, leaf and seed extract on the proliferation of two cholangiocarcinoma cell lines. RMCCA1 and KKU100 cells were treated with Moringa pod husk, pulp, leaf and seed extracts at concentrations of 1, 10, 100 and 200 µg/ml or vehicle (PBS). After 24 h of incubation, the proliferation of cholangiocarcinoma cells was significantly inhibited by Moringa pod husk, pulp, leaf and seed extracts in a dose-dependent manner (Figure 1). The IC_{50} values of Moringa pod husk, pulp, leaf and seed extracts for RMCCA1 cells were 5.2, 4.0, 1.8 and 2.5 µg/ml respectively. The IC_{50} values of Moringa pod husk, pulp, leaf and seed extracts for KKU100 cells were 12.1, 5.8, 1.4 and 2.9 µg/ml respectively.

The effect of *Moringa* extracts on cholangiocarcinoma cell apoptosis**:** Moringa leaf and seed extracts (MLE and MSE) that had high antiproliferative activities were selected to evaluate whether the antiproliferative activity was associated with the induction of cholangiocarcinoma cell apoptosis. A TUNEL assay was performed to study the mechanism by which Moringa extracts induced cholangiocarcinoma cell apoptosis. MLE, MSE or control vehicle (PBS) were added to RMCCA1 cells and incubated for 24 h. Apoptotic cells were measured through an

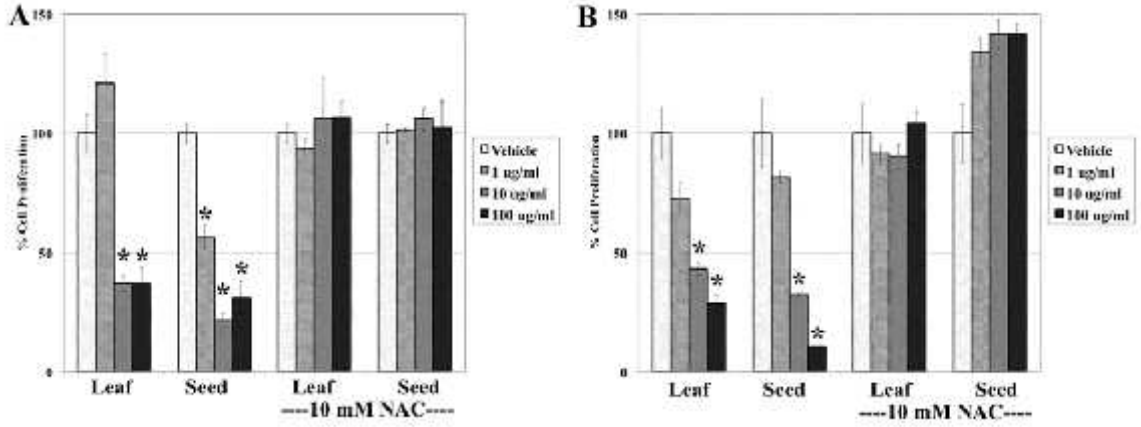

Fig. 5: N-acetylcysteine inhibits the cytotoxicity of Moringa extracts in cholangiocarcinoma cells
RMCCA1 (A) and KKU100 (B) cells were pre-treated with 10 mM NAC and incubated with MLE and MSE at various concentrations (0-100 µg/ml) for 24 hours. Effects on cell proliferation were measured by WST-1 and analyzed by spectrophotometric analysis (absorbance = 450 nm). Results are represented as the mean ± SE of three independent experiments, where the optical density value from vehicle-treated cells was set as 100% of proliferation. (, p <0.001 compared with the vehicle-treated cells).*

increase of green fluorescence (Figure 2). As shown in Table 1, the number of apoptotic cells after exposure to 10 and 100 µg/ml MLE and MSE significantly increased.

The effect of *Moringa* extracts on mitochondrial-membrane potential ($\Delta\psi_m$) of cholangiocarcinoma cells: We analyzed the effects of Moringa extracts on changes of mitochondrial membrane potential, which is a known indicator apoptotic cell death initiation. DePsipher™ dye staining showed that control cells had heterogeneous red fluorescent staining of the mitochondria. Upon exposure to MLE and MSE, KKU100 cholangiocarcinoma cells showed a marked decrease in $\Delta\psi_m$, as evidenced as a disappearance of red fluorescence or an increase of green fluorescence in most cells. Some cells were devoid of red fluorescence, which is an indication of the loss of $\Delta\psi_m$ and severe of cellular damage (Figure 3).

The effect of MLE and MSE on the generation of ROS in cholangiocarcinoma cells: Previous study indicated that the enhanced killing of cancer cells by Moringa oleifera extracts involves the induction of ROS production[8]. Accordingly, we next determined whether Moringa extracts led to the generation of ROS in cholangiocarcinoma cells. Reactive oxygen species were detected via Carboxy-H_2DCFDA, a cell-permeable, non-fluorescent fluorescein derivative. In the presence of cellular oxidants, this molecule is oxidized and produces a green-fluorescence indicative of ROS production that is detectable by fluorescence microscopy.

As shown in Figure 4, little ROS generation was detected in RMCCA1 and KKU100 cells treated with vehicle (PBS). However, ROS generation increased following treatment with 10 and 100 µg/ml MLE and MSE for 24 h, correlating with the reduction in cell viability seen with these compounds at the same concentration.

N-acetylcysteine inhibits the cytotoxicity of *Moringa* extracts in cholangiocarcinoma cells: N-acetylcysteine (NAC), an antioxidative agent[11], was used to evaluate whether the cytotoxicity of *Moringa* extract related to the generation of ROS. RMCCA1 and KKU100 cholangiocarcinoma cells were pre-treated with 10 mM NAC followed by incubation with 10-100 µg/ml MLE and MSE. Cell proliferation was determined by WST1 assay. As shown in Figure 5, pretreatment with NAC completely inhibited the cytotoxicity of MLE and MSE in both cholangiocarcinoma cell lines. Indeed, pre-incubation with 10 mM NAC increased the proliferation of KKU100 treated with MSE. These results suggest that *Moringa oleifera*-induced cytotoxicity in cholangiocarcinoma cells relates to the generation of ROS.

DISCUSSION

In this study, we used two cholangiocarcinoma cell lines, RMCCA1 and KKU100, which are derived from cholangiocarcinoma patients, to study the anticancer effects of Moringa extracts in vitro. These cell lines exhibited resistance to chemotherapeutic agents, such as oxaliplatin, even at high concentrations (100-200 µM)[12,13]. The antiproliferative and apoptotic effects of Moringa pod husk, pulp, leaf and seed extracts in these human cholangiocarcinoma cell lines were identified. These findings are consistent with a previous study demonstrating that Moringa leaf extracts display significant anticancer activities against human cancer (KB) cells[8]. In this study, we demonstrate for the first time that not only leaf extracts but also extracts from other parts of Moringa oleifera display significant anticancer activities. However, Moringa leaf extracts displays the highest antiproliferative activity against cholangiocarcinoma cells.

Many natural compounds including dioscin and isoalantolactone were demonstrated to kill cancer cells selectively by targeting ROS metabolism[14,15]. Moringa oleifera is rich in fairly unique glycoside compounds, known as glucosinolates and isothiocyanates (benzyl isothiocyanate (BITC))[5,6,16]. The antitumor effects of the Moringa plant became evident after the recent publication

of study demonstrating that benzyl isothiocyanate (BITC) induce apoptosis in cancer cells in vitro[17]. BITC induces apoptosis in human cancer cells through the production of reactive oxygen species (ROS), as the result of an inhibition of complex III of the mitochondrial respiratory chain [18,19]. In addition, previous studies have demonstrated that ROS accumulation inhibits HSP90 function, activates JNK, and induces cell cycle arrest, as well as apoptotic and necrotic cell death[20]. In this study, we demonstrate that M. oleifera induces ROS accumulation in cholangiocarcinoma cells. Moreover, we found that the scavenging of ROS by NAC completely inhibited a M. oleifera-induced decrease in cholangiocarcinoma cell viability. Therefore, we conclude that the accumulation of ROS represents the underlying mechanism by which M. oleifera induces cytotoxicity of cholangiocarcinoma cells.

CONCLUSIONS

To the best of our knowledge, this present study is the first report demonstrating the anticancer effects of M. oleifera in cholangiocarcinoma cells. We suggest that these results may indicate a potential approach to cholangiocarcinoma therapy.

Competing interests

The authors declare that they have no competing interests.

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