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Original Research

Anti-cancer activity of asiatic acid against human cholangiocarcinoma cells through inhibition of proliferation and induction of apoptosis

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Abstract: Plant-derived anti-cancer agents have been of considerable interest due to their promising effectiveness with low side effects. Asiatic acid, the main constituent of the medicinal plant *Centella asiatica* (L.) Urban, has a wide range of biological properties such as antioxidant, anti-inflammatory and anti-cancer activities. Cholangiocarcinoma (CCA), which is a malignant tumor of bile duct epithelium, is one of the leading cancers in Southeast Asia, notably the northeast of Thailand where the liver fluke, *Opisthorchis viverrini* predominates. Many *in vitro* and *in vivo* studies have provided evidence supporting that oxidative stress induced by chronic inflammation is involved in CCA genesis with aggressive clinical outcomes. This study was performed to evaluate the cytotoxic effects of asiatic acid on two human CCA cell lines (KKU-156 and KKU-213). Cell viability was determined by a sulforhodamine B (SRB) assay. Morphological changes of the cells were observed by microscopy. Cell apoptosis was detected by flow cytometry using annexin V and propidium iodide (PI) staining. Messenger RNA (mRNA) expression levels of *BAX*, *BCL2* and *Survivin/BIRC5* were analyzed by real-time polymerase chain reaction (PCR). It was found that asiatic acid efficiently suppressed CCA cellular viability via induction of apoptosis. In addition, the occurrence of asiatic acid-induced apoptosis was confirmed by microscopic observation of apoptotic vesicles, down-regulation of anti-apoptotic genes (*BCL2* and *Survivin/BIRC5*) and increased early and late apoptotic cells. Our results showed the chemotherapeutic activities of asiatic acid, suggesting the anti-cancer properties of this compound should be clinically assessed and its supplementation may lead to an improvement of survival of CCA patients.

Key words: Cholangiocarcinoma; Asiatic acid; Plant-derived compound; Cytotoxicity; Apoptosis.

Introduction

The northeast of Thailand, which includes Khon Kaen Province, has the highest prevalence of the liver fluke, *Opisthorchis viverrini*, infection and the highest incidence of cholangiocarcinoma (CCA − cancer of the bile ducts) worldwide (1). Infection of *O. viverrini* is associated with the custom(s) of consuming raw, fermented or partially cooked fresh-water fish, notably fish belonging to the Cyprinid family, which contains the infective life cycle stage called metacercariae. After ingestion, the metacercariae excyst in the duodenum whereupon they migrate to the hepatobiliary tracts where they become egg-producing adult worms (2). The International Agency for Research on Cancer (IARC) has classified *O. viverrini* as a Group 1 carcinogen (*i.e.*, carcinogenic to humans), which represents a direct risk factor for CCA development (3). Chronic inflammation and oxidative stress caused by *O. viverrini* has been shown to be implicated in the pathogenesis of CCA (2, 4-6). CCA is one of the most difficult diseases to diagnose and treat because signs and symptoms of early stage are subtle and non-specific. Most patients present with advanced stages of the disease, leading to a poor prognosis and a very high mortality rate. Cure is possible with surgical resection of patients with initial stages of CCA, who do not have underlying liver or biliary tract disease. However, the 5-year survival rate is still poor, with 60 to greater than 90% post-operative recurrence rates (7, 8). At present, CCA treatments including chemotherapy and radiation therapy are not sufficiently effective. The use of fluorouracil (5-FU), gemcitabine or capecitabine, either alone or in combination with a platinum analogue (*i.e.*, oxaliplatin or cisplatin) has been suggested for CCA treatment. Nevertheless, those regimens have yielded low response rates (9-11). Additionally, elderly patients with CCA are likely to refuse chemotherapy, surgery and radiation therapy, but instead seek out other treatment options, *e.g.*, herbal remedies. Therefore, there is an urgent need to find alternative ways to prevent and treat this silent killer disease and, hence, improve the quality of life of the people, notably poverty-stricken people in the northeast of Thailand and elsewhere in Southeast Asia where *O. viverrini* is distributed.

The use of plant-derived compounds, known as phytochemicals, in cancer prevention and treatment can be beneficial by eliminating or reducing side-effects associated with the chemo-radio therapy. Triterpenoids, a group of polyhydroxy aromatic compounds isolated from diverse medicinal plants, have shown to possess numerous biological activities (12-14). Asiatic acid (Figure 1), a pentacyclic triterpenoid derived from the medicinal herb *Centella asiatica* (L.) Urban, has been

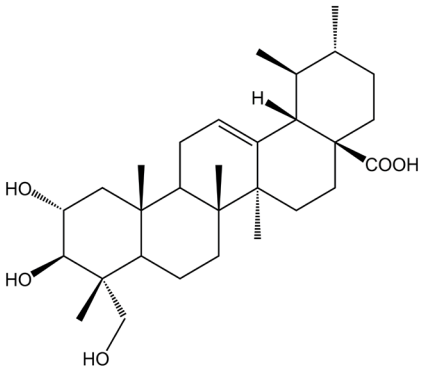

Figure 1. Chemical structure of asiatic acid.

demonstrated to exhibit numerous beneficial effects, such as wound healing, anti-inflammatory, neuroprotective, anti-diabetic, anti-oxidative and anti-cancer activities (15-21).

Accumulated evidence has shown the anti-cancer activities of asiatic acid against various types of cancer cell lines, including breast cancer, colon cancer, prostate cancer, lung cancer, melanoma, hepatoma, glioblastoma and ovarian cancer cells. Different mechanisms have been reported to account for the anti-cancer action of asiatic acid. For instance, asiatic acid has been reported to induce S-G2/M phase cell cycle arrest and apoptosis of human breast cancer cell lines via activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase (MAPK) pathways (22). Asiatic acid-induced apoptosis in human melanoma cells is mediated by reactive oxygen species (ROS) generation and BAX/BCL2 regulation (23), and can promote the apoptosis of human hepatoma and malignant glioma cells through increasing intracellular Ca^{2+} levels (24, 25). In colon cancer cells, asiatic acid has been reported to inhibit cell growth and induce apoptosis via mitochondria-dependent pathway by increasing mitochondrial membrane permeability and cytochrome *c* release (26). Asiatic acid could also perturb the endoplasmic reticulum and alterations in calcium homeostasis to induce cell death of prostate cancer cells (27) and it has been demonstrated to promote the pro-apoptotic effects in human ovarian cancer cells via the suppression of PI3K/Akt/mTOR signaling cascades (28). In lung cancer cells, asiatic acid inhibited cell proliferation and caused cell death mainly through a mitochondrion-mediated pathway. This triterpene also significantly decreased weight and tumor volume in a mouse xenograft model of lung cancer (29). Asiatic acid-induced apoptosis has been proposed to be associated with the modulation of cell cycle progression and induction of apoptosis by targeting multiple molecules, *e.g.,* NF-κB, p38 MAP and ERK kinases, caspases, PARP and BCL2/BAX in the aforementioned cancer types (22-28).

One of the significant hallmarks of cancer cells is to evade apoptosis − a process of programmed cell death. Deregulation of apoptotic pathways is thought to be a significant factor in development and progression of cancer cells. Alterations in the expression of proteins which can either promote or prevent apoptotic cell death such as BCL2, BAX and Survivin/BIRC5 can be used for the detection of apoptosis. In the present study, cytotoxicity effects of asiatic acid on two human CCA cell lines (KKU-156 and KKU-213) were evaluated by sulforhodamine B (SRB) assay, morphological observations and flow cytometry. Messenger RNA (mRNA) expression levels of three genes, including *BAX, BCL2,* and *Survivin/BIRC5* were determined by means of real-time polymerase chain reaction (PCR).

Materials and Methods

Cell cultures

Two human CCA cell lines, KKU-213 and KKU-156, were obtained from Cholangiocarcinoma Research Institute (CARI, Khon Kaen University, Thailand). Both cell types were cultured in Ham's F-12 medium (Invitrogen, CA, U.S.A.) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 g/ml streptomycin (complete media) at 37 °C in a humidified atmosphere of 5% CO_2. A subculture was performed when the cells reached the confluent stage and the media were changed every two days.

Asiatic acid solution preparation

Asiatic acid (Sigma-Aldrich, MO, U.S.A) was dissolved in 100% dimethyl sulfoxide (DMSO) to obtain 2×10^4 µM stock solution, aliquoted and stored at -20 °C until used. An aliquot of the stock solution (2×10^4 µM) was diluted 100-fold with complete media to make 200 µM asiatic acid containing 1% DMSO, which was subsequently diluted 2-fold to with complete media to make 100 µM asiatic acid containing 0.5% DMSO. Each concentration of asiatic acid was prepared by diluting with complete media containing 0.5% DMSO. Final concentration of DMSO was adjusted to 0.5% in all conditions.

Sulforhodamine B (SRB) cell cytotoxicity assay

CCA cell lines (3×10^3 cells in 100 µl/well) were seeded into 96-well plates for 24 h. Both KKU-213 and KKU-156 cells were treated with various concentrations (20-55 µM) of asiatic acid in complete media containing 0.5% DMSO in triplicate for 24 and 48 h. Cell images were taken by an inverted microscope (Zeiss Axiovert 40 CFL) every 24 h. Cytotoxicity was determined using SRB assay (30, 31). Cells were washed with ice-cold phosphate-buffered saline (PBS), fixed by adding 100 µL of chilled 10% trichloroacetic acid (TCA) in PBS to each well and incubated at 4 °C for 24 h. After removal of the TCA, cells were washed with deionized water three times and dried at 60 °C for 30 min. The cells were then incubated with SRB (50 µl per well, 0.4% in 1% acetic acid) in the dark for 45 min at room temperature. The SRB was removed, and the wells washed three times with 1% acetic acid (200 µl) to remove excess stains. The plate was then allowed to dry at 60 °C for 30 min. A volume of 200 µl of Tris-base (10 mM, pH 10.5) was added to each well, followed by incubation for 1 h at room temperature with gentle shaking. The absorbance was determined spectrophotometrically at 540 nm using an ELISA microplate reader (Sunrise, Tecan Austria GmbH). Asiatic acid concentrations required to inhibit 50% cell growth (IC_{50}) were calculated from concentration-effect curves after linear regression analysis.

Annexin-V/propidium iodide (PI) staining assay

CCA cell lines (1×10^5 cells in 2 ml/well) were

plated into 6-well plates for 24 h. Cells were treated with 35, 40 and 45 µM asiatic acid for 24 h. Cells were washed three times with ice-cold PBS and harvested by trypsinization. Cell pellets were stored at -70 °C until used. The Annexin-V-FLUOS Staining Kit (Roche Germany) was used for the detection of apoptotic cells. According to the manufacturer's instructions, cells (KKU-156 or KKU-213) were seeded in six-well plates at 8.0 × 10^4 cells/well and maintained at 37 °C in humidified 5% CO_2 environment for 48 h. Cells were treated with various concentrations of asiatic acid (35, 40 and 45 µM) and incubated for 24 h. Cellular suspensions were harvested, centrifuged at 2000 rpm at 4 °C for 5 min and washed three times with ice-cold PBS. Following centrifugation and removal of supernatant, cell pellets were resuspended in 100 µl of HEPES buffer with 2 µl of annexin-V-fluorescein solution and 2 µl of PI solution. After incubation for 10 min, in the dark, at room temperature, cells were analyzed by flow cytometry on a Becton Dickinson FACSCanto II Flow Cytometer.

Quantification of *BCL2*, *BAX* and *Survivin/BIRC5* mRNA levels by real-time polymerase chain reaction (PCR)

CCA cell lines (1 × 10^5 cells in 2 ml/well) were plated into 6-well plates for 24 h. Cells were treated with 20 µM asiatic acid for 24 h (KKU-213) and 48 h (KKU-156). Cells were washed three times with ice-cold PBS and harvested by trypsinization. Cell pellets were stored at -70 °C until used. Total RNA was isolated from cell pellets with TRIzol® reagent (Invitrogen, CA, U.S.A.) according to the manufacturer's protocol. RNA quality was assessed using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, DE, U.S.A.). Total RNA (2 µg) was converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, U.S.A). Real-time PCR was conducted by using ABI-7500 Real-Time PCR system (Applied Biosystems, CA, U.S.A.). Primer and probe sequences for real-time detection of *BAX* (catalog no. Hs00180269-m1), *BCL2* (catalog no. Hs00608023-m1), *Survivin/BIRC5* (catalog no. Hs04194392-s1) and endogenous control gene *ACTB* (catalog no. Hs99999903-m1) mRNA were purchased from Applied Biosystems. Real-time PCR was performed by using human Taqman pre-developed assay reagents (Applied Biosystems, CA, U.S.A.). Relative mRNA expression levels were analyzed with a cycle threshold (Ct) in the linear range of amplification to β-actin as an internal control.

Statistical analysis

Statistical analysis was performed using the SPSS software version 17.0 (IBM Corporation, U.S.A.). Levels of mRNA expressions were compared by Student's t-test. A $p<0.05$ was considered as statistical significance.

Results

Cytotoxicity of asiatic acid on cholangiocarcinoma cell lines

The cytotoxic effect of asiatic acid on KKU-156 and KKU-213 CCA cells was determined using the SRB method. The principle of SRB assay is based on the ability of SRB, a bright-pink aminoxanthene dye possessing two sulfonic groups, to bind to basic amino acids of the cellular proteins, enabling measurement of whole protein content which is proportional to cell number. The survival of KKU-156 and KKU-213 cells following treatment with increasing concentrations of asiatic acid for 24 and 48 h was assessed (30). KKU-123 and KKU-156 cells were incubated with either culture media containing 0.5% DMSO (control; 0 µM asiatic acid) and 20-55 µM asiatic acid in culture media containing 0.5% DMSO. DMSO at 0.5% concentration did not have any cytotoxic effect to the control cells. The cytotoxic results showed that asiatic acid significantly inhibited the proliferation of both human CCA cells lines, *i.e.*, KKU-156 (Figure 2a) and KKU-213 (Figure 2b), in a dose- and time-dependent manner. The essential asiatic acid concentrations to achieve the half-maximal inhibitory concentrations (IC_{50}) in KKU-156 cell line were 39.7 (± 0.6) and 21.2 (± 0.4) µM at exposure time of 24 h and 48 h, respectively. In the case of KKU-213 cells at 24 h and 48 h, IC_{50} values were 44.6 (± 0.8) and 28.7 (± 0.3) µM, respectively. Both cell lines showed the same trend of IC_{50} values; however, KKU-156 seemed to be more sensitive to asiatic acid treatment than KKU-213, which was consistent with the asiatic acid-induced cellular morphological change observed (Figure 2c and 2d). After treatment of asiatic acid, both human CCA cell lines (*i.e.*, KKU-156 and KKU-213) displayed a characteristic change of apoptosis, *i.e.*, augmented apoptotic vesicles (32). KKU-156 cells were found to undergo apoptosis by exposure to lower concentrations of asiatic acid at 40 µM (24 h) and 20 µM (48 h; Figure 2c), in comparison to KKU-213 cell line, which could trigger induction of apoptosis at 45 µM (24 h) and 35 µM (48 h; Figure 2d).

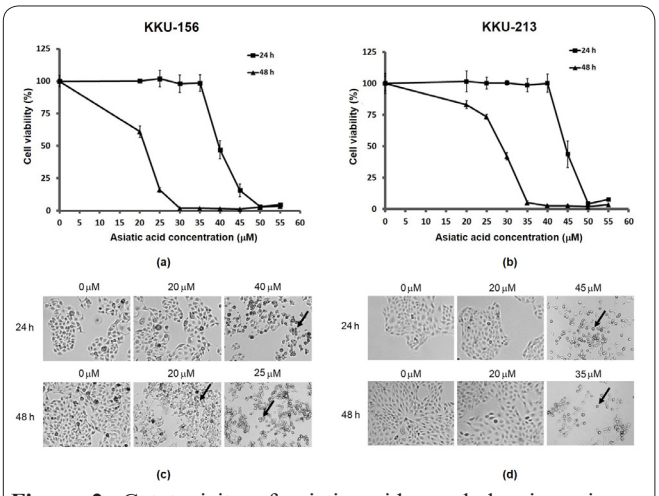

Figure 2. Cytotoxicity of asiatic acid on cholangiocarcinoma (CCA) cell lines. (a) Asiatic acid at different concentrations inhibited the proliferation of KKU-156 human CCA cells in a dose- and time-dependent manner; (b) Asiatic acid at different concentrations inhibited the proliferation of KKU-213 human CCA cells in a dose- and time-dependent manner. Cell growth inhibition of asiatic acid was determined using SRB assay. Both CCA cells were exposed to 0-55 µM of asiatic acid at 24 h (■) and 48 h (▲). Apoptotic vesicles were observed under an inverted microscope after treating cells with various concentrations of asiatic acid for 24 or 48 h in (c) KKU-156 and (d) KKU-123 cell lines. Arrows in (c) and (d) indicated exemplified apoptotic vesicles. Magnification, × 100.

Analysis of apoptosis by flow cytometry

To further evaluate the ability of asiatic acid to induce apoptosis in KKU-156 and KKU-213 CCA cells, flow cytometric analysis was performed using annexin V/PI staining. Annexin V is a protein that binds to phosphatidylserine residues, which are present predominantly in at the inner leaflet of the plasma membrane. In apoptotic cells, the translocation of phosphatidylserine from the inner to the outer surfaces can be detected by staining with fluorochrome-conjugated annexin V. Using annexin V in combination with propidium idodide (PI), a cellular DNA dye, can distinguish live cells, early apoptotic cells, late apoptotic cells and necrotic cells (33). As shown in Figure 3, following asiatic acid treatment, flow cytometric results revealed that apoptosis was induced in a dose-dependent manner in both CCA cell lines, particularly at asiatic acid concentration of 45 µM. In the case of KKU-156 cell line (Figure 3a), the percentage of early apoptotic cells (the lower right quadrant; Q4; annexin V-positive and PI-negative cells) and late apoptotic cells (the upper right quadrant; Q2; annexin V-positive and PI-positive cells) were augmented from 4.3% in untreated cells to 19.5%, 27.0% and 70.2% at asiatic acid concentration of 35, 40 and 45 µM, respectively. Similarly, in asiatic acid-treated KKU-213 cells (Figure 3b), the total population of early and late apoptotic cells was found to be increased from 8.9% in untreated cells to 9.9%, 35.8% and 86.4% for cells subjected to 35, 40 and 45 µM of asiatic acid treatment, respectively. These results showed the ability of asiatic acid to induce apoptosis in human CCA cells.

Effects of asiatic acid on apoptosis-related gene expression of CCA cell lines

Changes in mRNA expression levels of three apoptosis-related genes (*BAX*, *BCL2*, and *Survivin/BIRC5*) in human CCA cell lines during asiatic acid treatment were investigated by real-time PCR. The mRNA expression levels of *BAX* tended to be increased after 48 h treatment with 20 µM asiatic acid in KKU-156 cells as well as after 24 h treatment with 20 µM asiatic acid in KKU-213 cells, in compared with that in untreated cells (Figure 4). On the other hand, in both CCA cells, the mRNA expression levels of *BCL2* and *Survivin/BIRC5* significantly decreased after 24 h or 48 h treatment with 20 µM asiatic acid. These findings showed that the treatment of asiatic acid against CCA cell lines affected the balance of pro- and anti-apoptotic signaling pathways

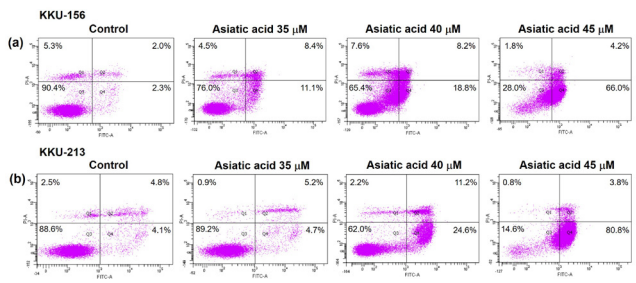

Figure 3. The apoptotic cells were determined by flow cytometry using annexin V (X-axis)/PI (Y-axis) staining in (a) KKU-156 and (b) KKU-213 cell lines. Results shown are representative of two reproducible independent experiments. The lower right quadrant (Q4) indicates early apoptotic cells and the upper right quadrant (Q2) represents late apoptotic cells.

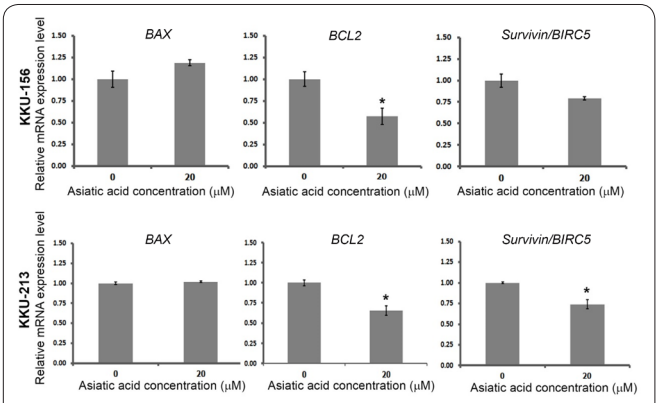

Figure 4. Effects of asiatic acid on the mRNA expression levels of apoptosis-related genes including *BAX*, *BCL2* and *Survivin/BIRC5* in KKU-156 and KKU-213 cell lines, following 48 h or 24 h asiatic acid treatment, respectively. Asterisk (*) represents $p<0.05$ by Student's t-test.

towards apoptosis via down-regulation of anti-apoptotic genes. Moreover, after treatment with 20 µM asiatic acid, increases in *BAX/BCL2* ratios were observed in both cell line types. The *BAX/BCL2* ratios were determined to be 3.3 and 1.6 in the case of KKU-156 and KKU-213 cell lines, respectively.

Discussion

The main finding of our study was that asiatic acid inhibited the growth of human CCA cells. In both CCA cell lines, IC_{50} values obtained in our study are in the same range with those previously reported in other cancer cell types, *e.g.* skin, breast and lung, all with IC_{50} values ranging from 8-80 µM (26-29). KKU-156 cells showed higher sensitivity and lower IC_{50} values than KKU-213 cells when they were treated with asiatic acid. The different IC_{50} in the two different cell lines may be due to differences in cell characteristics and distinct target sites of action as reported in the case human glioma cells (34). It is well established that asiatic acid treatment can affect cancer cell growth by inducing apoptosis in many cell lines (23, 26). In this study, we found that asiatic acid induced apoptosis as clearly shown by increased apoptotic cells detected by flow cytometry using annexin V/PI staining. The different responses detected using flow cytometry based on annexin-V/PI staining were related to microscopic observation of apoptotic vesicles. Our results confirmed that asiatic acid possesses potent growth inhibitory and pro-apoptotic effects against both types of CCA cell lines. In both normal and cancer cells, cell survival and death are delicately controlled by an intricate balance between anti-apoptotic and pro-apoptotic regulators. Molecular analyses were performed to confirm the differential expression of apoptosis-regulatory proteins, including BAX, BCL2 and survivin/BIRC5. Following asiatic acid treatment, real-time PCR showed that there was a slight increase in the expression of the pro-apoptotic molecule *BAX* while there was a significant decrease in the expression of the anti-apoptotic molecules *BCL2* and *Survivin/BIRC5*. Moreover, the ratio of *BAX/BCL2* of each cell line was significantly elevated after exposure to asiatic acid. Changes in expression levels of genes involved in regulation of apoptosis indicate that

asiatic acid induced apoptotic cell death. The asiatic acid-induced apoptosis in CCA can be mediated through alteration of the *BAX/BCL2* ratio and down-regulation of *BCL2* and *Survivin/BIRC5*, and growth suppression by asiatic acid can be due to apoptosis. Our results for CCA cells are in concordance with the previous findings that showed variations of response of apoptosis regulators BCL2, BAX and Survivin/BIRC5 to asiatic acid, for instance in human leukemia, human melanoma and human breast cancer cells (22, 23, 35).

Chronic inflammation and oxidative stress was found to play a key role in CCA carcinogenesis via the induction of DNA damage and cell proliferation, leading to CCA initiation and promotion in animal models by infection with liver fluke (36-38). Moreover, oxidative stress is also involved in CCA progression with poor prognosis and metastasis status of the patients (39, 40). On the other hand, asiatic acid has been shown to have an anti-oxidative effect due to the presence of several hydroxyl groups, an olefin group and a carboxylic acid group (41). Due to its abilities to decrease inflammatory, reduce oxidative stress and induce apoptosis in cancers, asiatic acid which have shown in this study to possess anti-CCA activity may be a good candidate for treatment of this fatal cancer caused by chronic inflammation with liver flukes and oxidative stress. A number of studies have indicated that plant-derived products manifest potent anti-cancer potential via their anti-proliferative and pro-apoptotic effects on CCA, such as curcumin, luteolin, quercetin and EGCG, and β-eudesmol by regulating different signaling modulators, *e.g.* NF-κB and STAT3, involved in tumor development and progression (42-45). Interestingly, in this study, for the first time, we evaluated the anti-cancer activity of asiatic acid on this type of cancer.

In conclusion, this study showed that asiatic acid could inhibit proliferation and induce apoptotic cell death in human CCA cell lines, KKU-213 and KKU-156, as evidenced by: (i) observation of the increased apoptotic vesicles (ii) *in vitro* cytotoxic study (iii) flow cytometry and (iv) decreasing the expression levels of anti-apoptotic signaling molecules *BCL2* and *Survivin/BIRC5*. Our findings provide evidence that the natural product asiatic acid is a promising anti-tumor agent against CCA, suggesting the anti-cancer properties of this compound should be clinically assessed and its supplementation may lead to an improvement of survival in CCA patients.

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Interest conflict

The authors declare no conflict of interest.

Author's contribution

C.S. conceived, designed and performed the experiments; R.T., W.K. and N.A. performed the experiments; P.P. contributed reagents/materials; C.S and R.T. analyzed and interpreted the data; C.S. wrote the paper; all authors contributed to the final approval of manuscript.

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