Pharmacogn. Mag.
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ORIGINAL ARTICLE

Cratoxylum formosum Leaf Extracts Inhibit Growth, Induce Apoptosis, and Decrease Metastasis Of Hela Human Cervical Cancer Cells

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Submitted: 26-Sep-2021 Revised: 28-Feb-2022 Accepted: 11-Mar-2022 Published: 07-Jul-2022

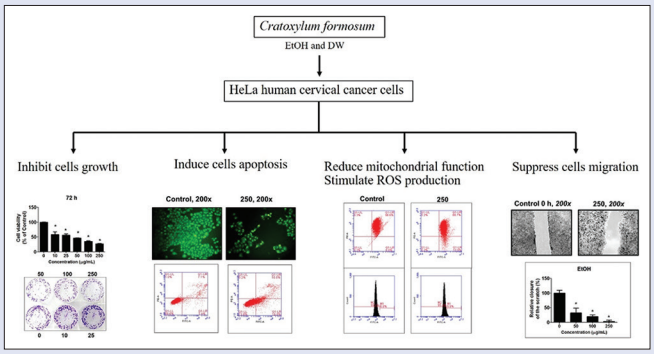
ABSTRACT

Background: *Cratoxylum formosum* (CF) is being used in Asian countries to treat disease and for eating purposes. **Objectives:** In the present study, the young leaf extract of CF was extracted by distilled water (DW) and 95% ethanol and examined on anti-cancer actions in cervical cancer cells. **Materials and Methods:** CF was determined the cytotoxic effects by sulforhodamine B, colony formation, cell cycle arrest, apoptosis, ROS formation, and mitochondrial function assay. **Results:** The data revealed that the Distilled water (DW) and ethanol (EtOH) extract had potent cytotoxicity against HeLa cells, and EtOH extract had more potency than DW extract. Inhibition of colony-forming ability was related with cell growth and at 250 mg/mL of CF was showed the highest activity. In addition, cancer cell distribution was stopped at the G0/G1 phase after incubating with 250 µg/mL of both extracts. The extracts potently induced cancer cell apoptosis properties in a dose-dependent manner. DW extract induced late apoptosis from 4.7%, 6.2%, 7.8%, and 8.0% (0, 50, 100, 250 µg/mL); however, EtOH extract induced early apoptosis from 3.4%, 5.7%, 6.1% and 16.0%, respectively. Consistent with apoptotic effects, EtOH extract reduced mitochondrial function and the higher effects were found at 250 µg/mL concentrations and related to the ROS production. The two CF extracts powerfully inhibited the migratory abilities of HeLa cells. The data indicated that EtOH of CF extract had more potency against HeLa cells than DW extract. **Conclusion:** CF extracts are potent against HeLa cells proliferation, induces apoptosis, and suppresses migration; use of this plant for cervical cancer treatment should be encouraged.

Key words: Apoptosis, cervical cancer cells, *cratoxylum formosum (CF)*, growth, metastasis

SUMMARY

- The DW and EtOH extracts of CF leaves were investigated for anti-cancer activity on cervical HeLa cancer cells.
- The anti-cancer activities were demonstrated by inhibiting growth, colony formation, and arresting cell cycle distribution.
- Apoptosis was induced by morphological changes, late apoptotic cell induction, ROS production, and decrease of mitochondrial function.
- The extract suppressed cancer cell migration.
- The CF leaves extract exhibited significant results in human cervical cancer cells treatment.



Abbreviations used: *CF: Cratoxylum formosum*, EtOH: Ethanol, DW: Distill water, SRB: Sulforhodamine B, AO: Acridine orange, EB: Ethidium bromide

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DOI: 10.4103/pm.pm_442_21

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INTRODUCTION

Natural plants from South East Asian countries including Thailand have been used in several pharmacological activities to defeat many diseases such as inflammation,[1] bacterial,[2] fungal[3] and microbial infections,[4] and cancer.[5,6] Many varied species of vegetables or plants are used in prevention and treatment of many cancer types.[7] *Cratoxylum formosum* (CF, Teaw) belonging to the Guttiferae family, composes of many types of chemical constitutes such as formosumone A, vismione D, toxyloxanthone B, chlorogenic acid, phenolics, flavonoids, which have powerful scavenging activity of free radical.[8] Previously, CF extract was reported to cause anti-cancer effects on apoptosis induction and cell proliferation inhibition in many cancer cell types.[5,6,9] There is significantly little information on the action of CF on cervical cancer, and the available mechanism of action for the anti-cancer effects is still limited.

Various types of cancer cells were defeated by CF via cell growth inhibition, apoptosis induction and migratory suppression from the several parts of CF. CF extract suppressed many cancer cells proliferation, including breast cancer,[10] cervical cancer,[5] colon cancer, liver cancer,[11] and oral cancer,[12] by dose-dependent manner. The better CF effects had more selectivity actions on cancer cells than normal cells, as shown in leukemia cancer cells. CF induced DNA break, changed morphology,

Cite this article as: Buranrat B. *Cratoxylum formosum* leaf extracts inhibit growth, induce apoptosis, and decrease metastasis of HeLa human cervical cancer cells. Phcog Mag 2022;18:296-303.

and activated cell death in cancer cells than normal cells.[13] Moreover, the researcher reported that EtOH and CF extract had an intense effect on liver cancer cells by inducing cancer cell deaths and correlating with apoptotic induction.[9] Further, CF induced apoptosis by induction of caspase cascade on caspase-3, -8 and -9, reduction of the mitochondrial membrane potential, and stimulation of apoptosis, which saw many apoptotic bodies.[9] Our previous study of CF extract suggested that this extract had more potency on the human breast cancer and liver cancer cells with the powerful actions.[10,11] However, cervical cancer has limited information, and the broad spectrum's action of CF should be explored further.

Cervical cancer is the third most common cancer and is the fourth leading cause of cancer death in women worldwide.[14] Although the screening program of cervical cancer is very effective, incidence and mortality rates are still high in young women making it a public health problem.[15] The standard treatment improves the survival rate of cervical cancer, but the chemotherapies are accompanied by high toxicities.[16] Consequently, the improvement of harmless and successful beneficial compounds for cervical cancer treatment has been need of the hour. Subsequently, natural compounds are being screened to discover new anti-cancer drugs. As there is inadequate data on the effect of CF on human cervical cancer cells (HeLa), we needed to investigate the comparative effects of young leaf of CF from distill water (DW) and EtOH extract on HeLa cancer cell death, apoptosis and migration, and the mechanisms of action responsible.

MATERIALS AND METHODS

Plant extraction

CF leaf was collected in May 2014 at Udon Thani province, as in the previous study,[10] and a specimen number was SKP083030601. In the extraction method, CF leaf was dried, extracted with distilled water (DW) and 95% ethanol, filtered, evaporated, lyophilized, and stored in a freezer. The yield was 15.63% for DW extraction and 9.36% for EtOH extraction.

Cell cultures and sulforhodamine B (SRB) method

Human cervical HeLa cancer cell lines were purchased from the ATCC and cultured in 10% fetal bovine serum (FBS) of DMEM medium with penicillin and streptomycin. The SRB method was performed to examine the effect of CF on HeLa cell growth. HeLa cancer cells, approximately 1×10^4 cells/well were plated in a 96-well plate and the next day, DW and EtOH of CF extract were mixed in each well for 24 hr, 48 hr, and 72 hr. Next, cancer cells were exposed with 10% TCA, stained with 0.4% SRB, and solubilized with 10 mM Tris base. Absorbance density was noted as 540 nm by spectrophotometer.

Colony formation method

To examine the effect of CF on cell replication, the clonogenic method was performed. HeLa cancer cells, approximately 500 cells/well, were plated in 6-well plates and the next day, cells were mixed with CF extract for 24 hr. The cancer cells were changed to a new complete DMEM medium, cultured for 10 days, then fixed cells with 100% methanol and finally exposed with 0.5% crystal violet at room temperature for 1 hr. The formation of colonies of HeLa cells were viewed, counted, and compared with control groups.

Cell cycle arrest

In order to examine the effect of CF on cancer cell, distribution and cell cycle arrest by flow cytometric analysis was used. HeLa cancer cells, approximately 2.5×10^5 cells/well, were plated in 6-well culture plates overnight and further treated with CF extract for 24 hr. Following treatment, the cells were detached, washed and fixed in 70% ethanol at -20°C for 24 hr. The pellet cells were incubated with a 500 µL propidium iodide (Cat No. 550825, BD Biosciences, CA, USA) for 30 min at 4°C in the dark. After that, the cell cycle distribution was performed using a flow cytometer (BD Biosciences, CA, USA) using BD Accuri C6 Plus software, and fluorescent signals were displayed as histograms.

Apoptosis

To examine the CF effect on apoptosis, acridine orange (AO)/ethidium bromide (EB) staining and Annexin V-FITC/PI double staining by flow cytometric analysis were performed. For AO/EB staining, HeLa cancer cells, approximately 1 x 10^4 cells/well, were plated in 96-well plates and the next day, cancer cells were incubated with the extract for 24 hr. After that, the cells were washed and incubated with AO (1 µg/mL) and EB (1 µg/ml) for 15 min in the dark at room temperature. The cells were observed in fluorescent inverted microscopy (Olympus, 20 × magnification).

For flow cytometry, HeLa cancer cells, approximately 2.5×10^5 cells/well, were plated in 6-well culture plates overnight and further treated with CF extract for 24 hr. Following treatment, the cells were collected, centrifuged, and mixed with 500 µL of binding buffer and additionally added with 5 µL of Annexin V-FITC and PI (Cat No. 558547, BD Biosciences, CA, USA) was mixed into the cells suspension and then incubated for 15 min at room temperature. After that, the cells suspension was mixed with 1.5 µL of a PI. The fluorescence intensity was measured using a flow cytometer (BD Biosciences, CA, USA), and the percentage of apoptotic rates were calculated using BD Accuri C6 Plus software.

Mitochondrial function by flow cytometric analysis

JC-1 solution and flow cytometric method were used to examine the effect of CF on mitochondrial function. HeLa cancer cells, approximately 2.5×10^5 cells/well, were plated in 6-well culture plates overnight and further treated with CF extract for 24 hours. Following treatment, the suspension cells were incubated with 5 µL of JC-1 solution (Cat. No. 1-800-346-9897, Cayman Chemical, Michigan, USA) in 100 µL assay buffer, at 37°C for 15 min. After that, the cells were added to 400 µL of assay buffer and then subjected to a flow cytometer (BD Biosciences, CA, USA). Fluorescence intensity was measured using BD Accuri C6 Plus software.

Reactive oxygen species formation by flow cytometric analysis

DCF-DA solution and flow cytometric method were used to examine the effect of CF on ROS formation. HeLa cancer cells, approximately 2.5×10^5 cells/well, were plated in 6-well culture plates overnight and further treated with CF extract for 24 hr. Following treatment, the cancer cells were washed once with PBS, collected, added to assay buffer with 5 µL of DCF-DA (Cat.no. D6883, Sigma Merck KGaA, Darmstadt, Germany); it was then added in 500 µL of complete DMEM medium and incubated at 37°C for 15 min. The cancer cells were then loaded onto a flow cytometer (BD Biosciences, CA, USA). Fluorescence intensity was measured using BD Accuri C6 Plus software.

Wound healing assay

A wound healing assay was used to examine the effect of CF on cancer cell migration. HeLa cancer cells, approximately 2.5×10^5 cells/well were plated in 24-well culture plates overnight Next, the cells were scratched by 0.2 mL pipette tips for making a wound, and further mixed with CF extract for 48 hr. Images were then captured at 0 hr and 48 hr via inverted microscopy (4 × magnification). The denuded area determined

the distance of wound healing closing. The wound distance (%) was measured, calculated and compared with control groups.

Statistical analysis

Statistical comparison of the control and CF treatment groups were performed using the Student's *t*-test, and the values were expressed as the Mean ± SE. of three determinations. *$P < 0.05$.

RESULTS

CF effects on HeLa cell growth

To determine the anti-cancer effects of CF leaf from DW and EtOH on cervical HeLa cancer cells growth and SRB method, colony formation, and cell cycle arrest were used. For SRB results indicated that CF extraction inhibited HeLa cells' cell viability by both concentration- and time-dependent manner [Figure 1a and b]. At 72 hr incubation period, inhibitory concentration (IC_{50}) values of DW extract was 236.67 ± 31.54 µg/mL and of EtOH extract was 37.00 ± 6.65 µg/mL [Table 1]. The results indicated that CF extract caused inhibition of cell viability, and EtOH had stronger anti-cancer activity than DW extract.

The colony formation method was performed to confirm the CF effect on HeLa cell growth. We found that DW and EtOH extracts reduced the colony-forming ability in a dose-dependent manner at 24 hr incubation period, with IC_{50} values of 335.17 ± 37.33 µg/mL for DW extract and 103.55 ± 16.25 µg/mL for EtOH extract [Figure 1a and b, Table 2]. In conclusion, both CF extract demonstrated the cytotoxic action and antiproliferative effect on HeLa cells.

Table 1: The IC_{50} values and Emax of CF extract on HeLa cell viability

Groups of treatment	Incubation periods (h)	IC_{50} (µg/mL)	Emax
DW extract	24	386.07±85.56	28.22±4.62
	48	285.00±18.19	30.03±1.67
	72	236.67±31.54	38.13±7.05
EtOH extract	24	126.67 ± ± 18.07	53.96±4.98
	48	99.67±5.03	55.94±5.67
	72	37.00±6.56	73.13±2.01

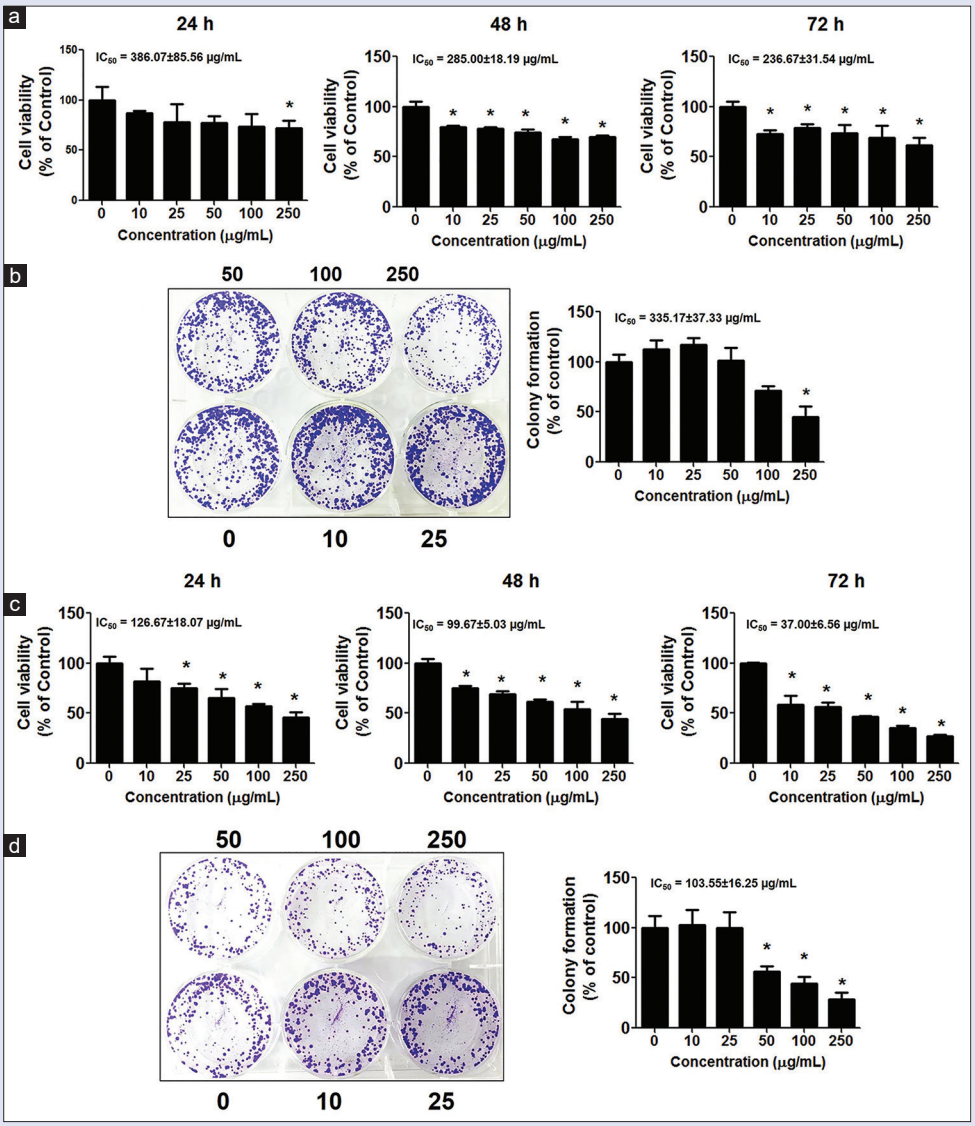

Figure 1: CF effects on cell viability and colony formation in HeLa cells. HeLa cells were exposed to various concentrations of DW (a) and EtOH extract (c) (0-250 µg/mL) for 24–72 hr; cell viability was then measured by SRB method. 500 cells/well of HeLa were exposed to DW (b) and EtOH extract (d) (0–250 µg/mL) for 24 hr, further cultured for 15 days, stained with crystal violet, and then counted. Values are expressed as mean ± SE. *$P < 0.05$

To demonstrate the possible mechanism of CF-induced cell death induction in HeLa cells. The results confirmed that upon treatment with CF extract, the G0/G1 phase distribution increased between 60.83%, 59.19%, 63.95%, and 67.57% for 0, 50, 100, 250 µg/mL of DW extract, 60.35%, 63.85%, 64.23%, and 68.55% of EtOH extract, respectively [Figure 2a and 2b]. CF leaf extract exhibited induction at G0/G1 phase of cell cycle arrest, especially at high-dose of CF extracts.

CF effects on HeLa cell apoptosis

AO/EB staining and Annexin V-FITC and PI double staining were used to examine the CF extract on cell apoptosis. The AO/EB fluorescence microscopy revealed that viable cells were decreased after treatment with both of CF extract by dose-dependent manner. Still, there were fewer abnormal nuclei in the CF extract when compared with the control treatment group [Figure 3a and 3b].

Further, to analyze the percentage of apoptotic rate of CF-treated cervical cancer HeLa cells, Annexin V-FITC/PI double staining was performed by flow cytometric analysis. The data indicated that as the concentration of DW extract was induced, the percentage of late apoptotic cells in the HeLa was increased [Figure 3a and 3b] from 4.7%, 6.2%, 7.8%, and 8.0% of 0, 50, 100, 250 µg/mL, respectively. EtOH extract demonstrated that this extract caused induction of early apoptotic cells from 3.4%, 5.7%, 6.1%, and 16.0%, respectively, and late apoptotic rate as well [Figure 3a and 3b]. These data demonstrated that CF extracts induced apoptosis of HeLa cells efficiently.

Table 2: The IC_{50} values and Emax of CF extract on HeLa cell replication

Groups of treatment	IC_{50} (µg/mL)	Emax
DW extract	335.17±37.33	54.77±10.64
EtOH extract	103.55±16.25	71.01±6.64

CF effects on mitochondrial function and ROS formation

Modifications in mitochondrial membrane potential (MMP) play a significant role in cancer apoptosis, so to examine the CF effect on mitochondrial function using the JC-1 fluorescent dye probe. The data found that MMP was interrupted as demonstrated by a reduction in JC-1 aggregates, healthy cells, and increased JC-1 monomers, unhealthy cells [Figure 4a and 4b]. At 250 µg/mL, DW extract increased JC-1 monomers by approximately 7.0% compared to untreated control cells by approximately 0.1%. EtOH extract had higher potency to activate mitochondrial dysfunction than DW extract. The JC-1 monomers were found 10.9% at the 250 µg/mL dose.

Based on the ROS production of CF, the percentage changes of reactive oxygen species (ROS) levels after treating with CF extract in HeLa cells were investigated using a DCF-DA fluorescence probe and analyzed by flow cytometry. After DCF-DA staining, the data exhibited that the ROS intensity of green fluorescence (DCF) in the right-hand side was markedly enhanced in EtOH extract of CF-induced HeLa cells compared to the untreated control groups [Figure 4a and 4b]. It can be concluded that CF extract induced HeLa cancer cell apoptosis by reducing mitochondrial membrane potential and increasing ROS formation.

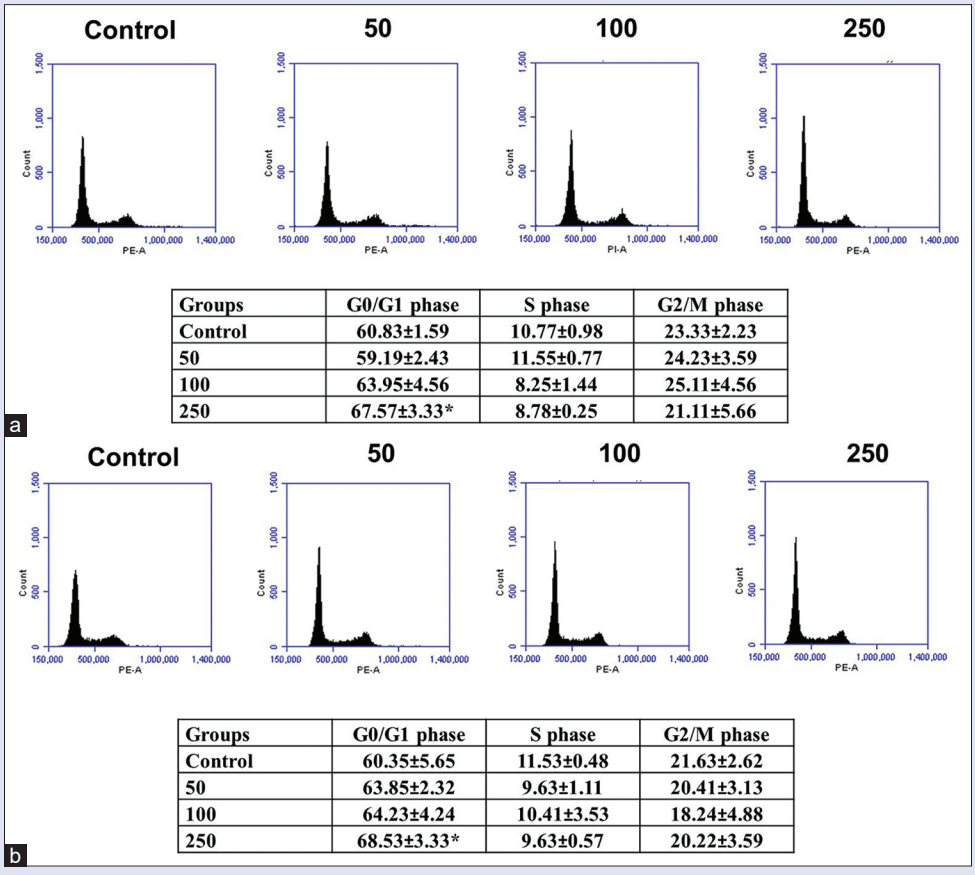

Figure 2: CF effects on cell cycle arrest in HeLa cells. HeLa cells were exposed to CF extract (0–250 µg/mL) with DW (a) and EtOH (b) extract for 24 hr and then cell cycle arrest was analyzed via flow cytometric method. Values are expressed as mean ± SE. *$P < 0.05$

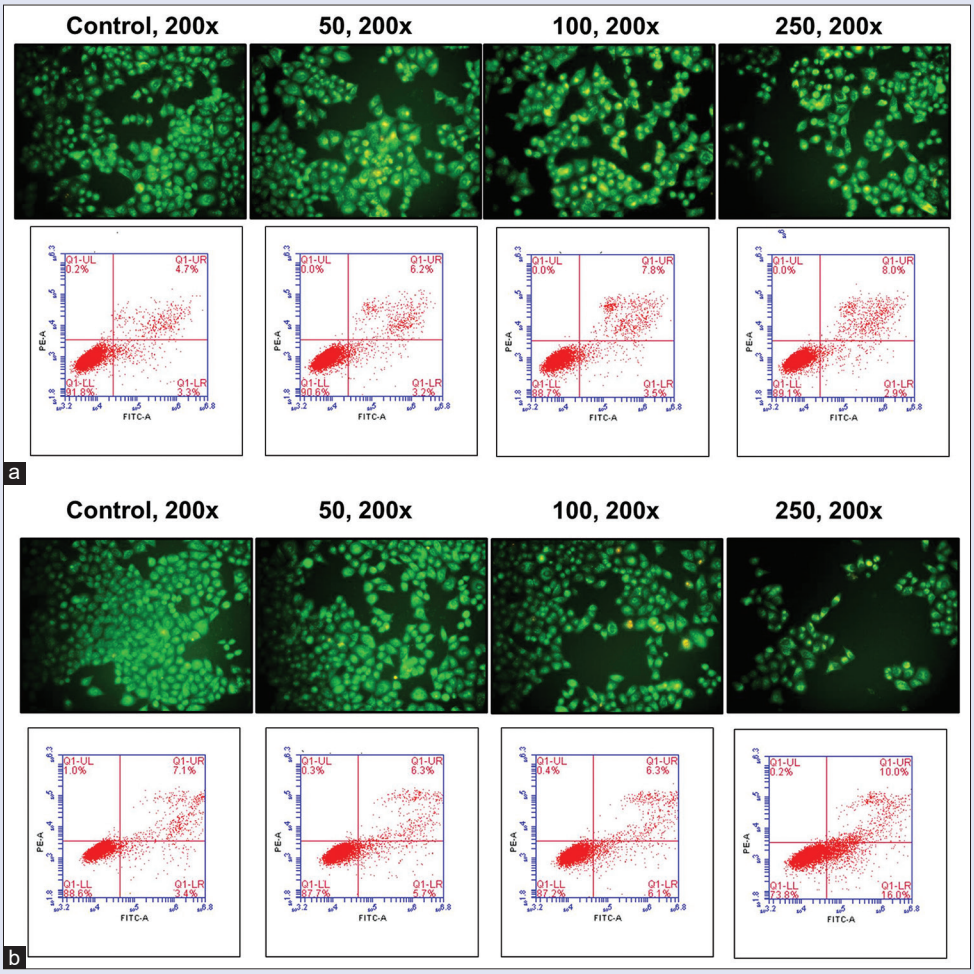

Figure 3: CF effects on apoptosis in HeLa cells. HeLa cells were exposed to CF extract (0–250 µg/mL) with DW (a) and EtOH (b) extract for 24 hr; the images were captured and apoptosis was analyzed by flow cytometric method. *$P < 0.05$

CF effects on HeLa cell migration

The wound healing method was used to examine the CF extracts on HeLa cancer cell migration. The data demonstrated that CF strongly suppressed HeLa cells migration by DW and EtOH extract by a dose-dependent manner, with IC_{50} values of 135.19 ± 38.63 µg/mL for DW extract and 24.32 ± 8.16 µg/mL for EtOH extract [Figure 5a–d and Table 3]. It can be concluded that CF extract can consequently inhibit HeLa cancer cell migration.

Table 3: The IC_{50} values and Emax of CF extract on HeLa cell migration

Groups of treatment	IC_{50} (µg/mL)	Emax
DW extract	135.19±38.63	71.72±10.64
EtOH extract	24.32±8.16	96.54±4.17

DISCUSSION

Previous studies have validated the relationship between cancer cell proliferation, apoptosis, and migration by using Thai plants or natural compounds. It has been demonstrated to serve as anti-cancer agents in several cancer types with high efficacy and expected low toxicities.[5,17–19] Our previous studies indicated that the EtOH extracts of CF leaves have strongly appreciable cytotoxicity in MCF-7 breast and HepG2 liver cancer cells, underlying apoptosis mechanism along with migratory inhibition.[10,11] This study explored the anti-cancer efficacy of CF extracts from DW and EtOH on HeLa cervical cancer cells, demonstrating its mode of action by apoptosis. The data indicated that EtOH extract strongly suppressed HeLa cells growth in the company with cell cycle arrest, induced apoptosis, reduced mitochondrial function, and further inhibited migration as well. CF leaf extract caused strong defects on several cancer cell types, including cervical cancer cells.

CF extract from EtOH strongly suppressed cervical cancer cell viability by dose-dependent manner and cell replication. This finding is in agreement with results of previous studies,[10,11] in which CF of EtOH extract inhibited cells proliferation on breast MCF-7 cells (IC_{50} = 53.74 ± 3.02 µg/mL) with low IC_{50} values at 48 hr less than cervical HeLa cells (IC_{50} = 99.67 ± 5.03 µg/mL) < liver HepG2 cells (IC_{50} = 124.90 ± 6.86 µg/mL). CF extract had cytotoxic effects in many cancer cell types, and breast cancer had more sensitivity to CF extract; however, CF had anti-cancer effects with low IC_{50} values. Further, CF extracts had more potent growth inhibition on cholangiocarcinoma cells in a dose-dependent manner with IC_{50} values of 12.1 ± 5.6 µg/mL,[6] and inhibited the cell cycle distribution at the G2/M phase and then suppressed Cdc25A and cyclin A levels. On the other hand, our data indicated that CF extracts caused induction of cancer cells death by arresting at G0/G1 phase. CF extract had broad spectrum effect on several cancer cell types with low dose concentration.

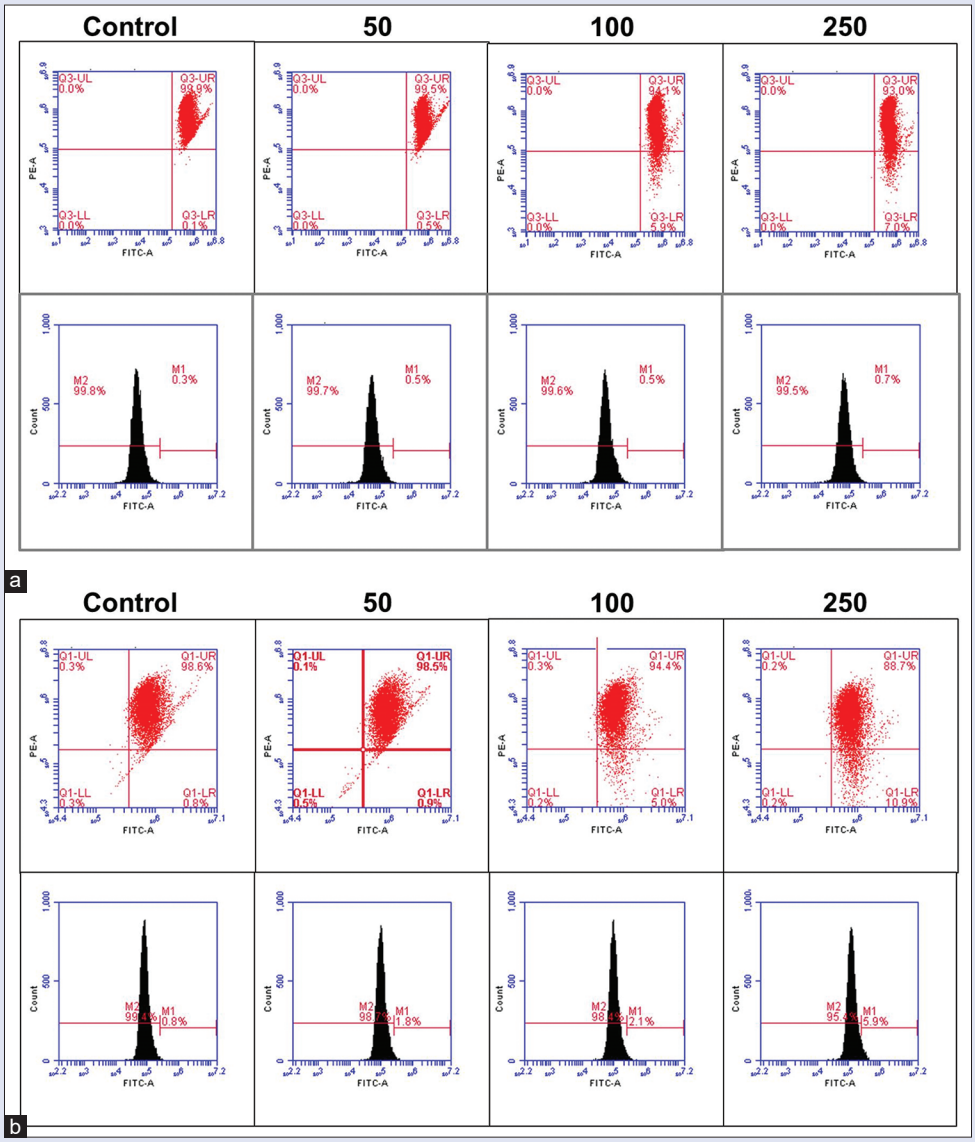

Figure 4: CF effects on mitochondrial function and ROS formation in HeLa cells. HeLa cells were exposed to CF extract (0–250 µg/mL) with DW (a) and EtOH (b) extract for 24 hr; images were captured and apoptosis was analyzed via flow cytometric method. *$P < 0.05$

Apoptosis of cancer cells was detected in anti-cancer drug's mechanism.[20–22] Unsuccessful apoptosis was associated with resistance and tumorigenesis. Apoptosis can be identified by morphological changes with membrane blebbing, DNA condensation, and the formation of apoptotic bodies by AO/EB staining.[23] Our results indicated that EtOH extract had detected more morphological changes at the dose of 250 µg/mL and confirmed by staining with PI and Annexin V-FITC. Likely, CF extract stimulated cholangiocarcinoma cells apoptosis by a dose-dependent manner, reducing STAT3 and NF-κB expression.[6] Moreover, HepG2 cells apoptosis occurred through intrinsic and extrinsic caspase-dependent pathways in mitochondria in time- and concentration-dependent manners by powerfully activating the levels of caspase-3, -7, -8, and -9, reducing the function of mitochondria, and triggering apoptotic bodies production and DNA stand break.[9]

Mitochondrial dysfunction and ROS formation are the most common mechanisms for cancer cell death and apoptosis.[24] Our study indicated that CF significantly induced ROS formation and reduced the mitochondrial membrane potential (MMP), causing apoptosis and cell growth reduction in breast and liver cancer cells.[10,11] Confirmation in HeLa cells, EtOH CF extract, caused induction of ROS production level and then reduced mitochondrial function in dose-dependent manner at 24-hr incubation period. The mitochondria plays a major role in healthy cells and cancer cell survival and is also a binding site for ROS generation.[25] Nevertheless, a high level of ROS can be represented as a model of cytotoxicity and cause cancer cell death.[26] The BA6 was exposed to lung cancer cells and showed significant increase in both cellular ROS and mitochondrial ROS, which lead to reduced MMP.[27] CF extract induced intracellular ROS formation, further inducing loss of mitochondrial function, and finally stimulated cervical cancer cell death and apoptosis.

Cancer metastasis is an essential characteristic of aggressive or progressive cancer cells. Metastasis is involves multiple steps such as adhesion, invasion, and migration of cancer cells.[28] Our study indicated that CF significantly decreased migration of breast and liver cancer cells by inhibiting MMP9 expression.[10,11] Additionally, CF

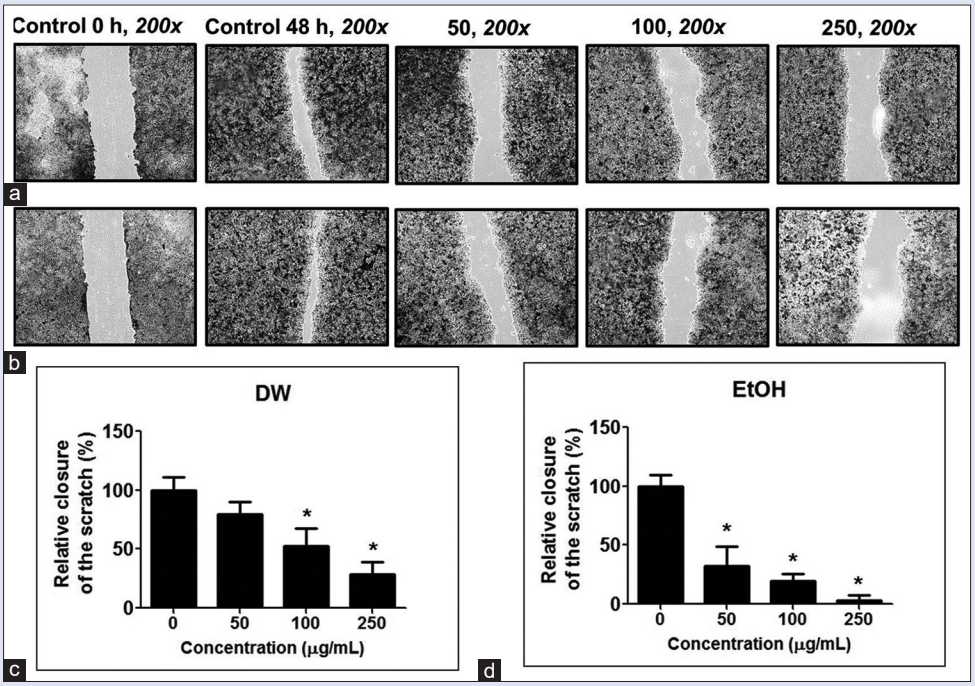

Figure 5: CF effects on HeLa cells migration after HeLa cancer cells were scratched and exposed to various concentrations of CF for 48 hr. Shown are the images of wound healing for DW (a), EtOH (b), and graph of wound healing assay (c and d). Determination of wound healing is captured under inverted microscopy and compared between control and treatment groups. Values are expressed as mean ± SE. *$P < 0.05$

effects on HeLa cells migration indicated that DW and EtOH extract decreased the cancer cells migration in dose-dependent manner, especially EtOH extract. Truly, plant extracts or compound or chemical agents with the more capability to inhibit the metastasis-related steps could be a potential candidate for cancer chemoprevention and chemotherapy. The CF extracts powerfully blocked the migration and invasion activities of cholangiocarcinoma cells by suppressing STAT3 activation.[6] CF extract had several effects on cervical cancer cells including cell death induction, apoptosis stimulation, and migratory suppression. Further, CF will be useful to prevent and treat cervical cancer.

CONCLUSION

This study has demonstrated that DW and EtOH extract from edible CF leaf suppresses HeLa cervical cancer cell growth and cell replication ability. CF extract also suppresses the distribution of cell cycle at G0/G1 phase. Moreover, CF extract caused apoptosis induction, which was accompanied by decreasing mitochondrial membrane potential and increasing ROS generation. Additionally, the CF extracts attenuated cervical cancer cell migration in a dose-dependent manner. They showed that the dose of suppression was less than those of IC_{50} values of cell viability and colony formation assay. These data demonstrated a theory for CF and other Thai medicinal plants to be further examined as sources of new compounds to treat/prevent cervical cancer; nevertheless, the exact mechanisms of action need to be explored.

Acknowledgements

The authors would like to express their gratitude to Faculty of Medicine, Mahasarakham University for providing facilities.

Financial support and sponsorship

This research project was financially supported by Mahasarakham University 2021 (Grant No. 2021).

Conflicts of interest

There are no conflicts of interest.

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