doi.org/10.36721/PJPS.2022.35.2.REG.553-559.1

The seed extract of *Oroxylum indicum* suppresses cell proliferation, migration and promotes apoptosis in cervical cancer cells

Benjaporn Buranrat[1]* and Supavadee Boontha[2]
[1]Faculty of Medicine, Mahasarakham University, Muang, Maha Sarakham, Thailand
[2]School of Pharmaceutical sciences, University of Phayao, Maeka, Phayao, Thailand

Abstract: The herb *Oroxylum indicum* has been used for treating several diseases and also has anticancer activity. This research examined the anticancer effects of ethanolic extract of *O. indicum* seeds on HeLa cervical cancer cells and investigates underlying mechanisms. The data indicated that the extract inhibited HeLa cells growth with low IC_{50} values and arrested the cell cycle at G0/G1 phase at a dose of 50 µg/mL. Moreover, this extract produced a marked increase in the apoptosis of cancer cells which was demonstrated by acridine orange/ethidium bromide (AO/EB) staining and was significant at a dose of 250 µg/mL for 24 h. Correlating with apoptotic data by flow cytometric analysis, the results indicated that viable cells were significantly reduced and late apoptotic cells were induced starting at the dose of 50 µg/mL of the extract. These extracts significantly induced cancer cell apoptosis which was mediating through reduction of mitochondrial function by stimulating intracellular reactive oxygen species (ROS) formation. Furthermore, the extract caused inactivation of cervical cancer cell migration and was detected with the wound healing method. The data in this study strongly indicated that *O. indicum* seed extract has powerful activity against cervical cancer.

Keywords: *Oroxylum indicum,* cell cycle arrest, apoptosis, migration, mitochondrial membrane potential.

INTRODUCTION

Over several decades, scientists have found new sources of anticancer agents from natural products which can be rich sources of active compound which powerful anticancer action and low systemic toxicity. *Oroxylum indicum*, Bignoniaceae family, is a famous vegetable food in several Asian countries including Thailand (Rojsanga *et al*., 2020). Outstandingly, *O. indicum* has been reported for treating many disorders such as acute and chronic bronchitis, cough, pharyngitis and stomach pain (Rojsanga *et al*., 2020). Several parts of *O. indicum*, stem bark, leaf, fruit, and seed have been testified for anticancer properties in many cancer types such as liver, leukemia, breast and cervical cancer cells (Nik Salleh *et al*., 2020).

Our previous study indicated that the edible parts such as leaves and fruit, have potent anticancer antimigratory activities on human MCF-7 breast cancer cells through suppressing the mevalonate pathway (Buranrat *et al*., 2020). Moreover, stem bark has high potency on MDA-MB-231 estrogen receptor-negative breast cancer cells to stimulate apoptosis by targeting specific on cancer cells without being harmful for normal cells (Kumar *et al*., 2012). The total flavonoids from *O. indicum* seed extract may possibly demonstrate significant action on suppression of tumor growth through modulating PI3K/Akt/PTEN signaling pathway (Li *et al*., 2018). The other mechanism that has been reported is *via* down-regulating HPV18 oncoproteins E6 and E7 and a significant induction caspase 8 and caspase 3 levels (Wahab *et al*., 2019). However, there is limited information of the activities of seed extract of *O. indicum* on HeLa cervical cancer cells which would be interesting to explore.

The present study investigated the antiproliferative, apoptotic and antimigratory effects of *O. indicum* seed extract on HeLa human cervical cells to gain insights regarding the underlying mechanism (s) that interfere with mitochondrial function and generate reactive oxygen species (ROS) in HeLa cell lines.

MATERIALS AND METHODS

Plant and extraction

O. indicum seed (specimen number: MSUT-7226) was obtained in May 2017 from Maha Sarakham Province, Thailand and extracted with ethanol as in previous studies (Buranrat *et al*., 2018). The yield of ethanolic extract was 14.75% from dry weight. The total flavonoid content was 65.47±5.26 and phenolic compound was 124.69±7.89 µg/g in the extract.

Cell lines, cell culture and sulforhodamine B (SRB) method

The human HeLa cervical cancer cell line was obtained from the American Type Culture Collection (HeLa229, CCL-2.1™, ATCC; Manassas, VA, USA) and maintained according to the supplier's instructions. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium with 10% fetal bovine serum (FBS) plus Penicillin G/Streptomycin. The cytotoxic effects on cancer cells of the seed extract of *O. indicum* and cisplatin

**Corresponding author:* e-mail: buranrat@gmail.com

(an anticancer drug) were investigated using the SRB method. Briefly, cancer cells were seeded ($1x10^4$ cells/well) on 96-well plates and the next day, seed extract (10-250 µg/mL) or cisplatin (0-50 µM) were added, exposing for three incubation times of 24-72 h, fixing with 10% trichloroacetic Acid (TCA), staining with 0.4% SRB, solubilizing with 10 mM Tris base buffer, and optical density was read at 540 nm.

Colony formation method

The formation of colonies was examined by a colony formation assay. Briefly, the method involved seeding 500 cells on 6-well plate followed by exposure to the extract (0-250 µg/mL) or cisplatin (0-50 µM) for 24 h and culture for further ten days. The colonies of cancer cells were stained, incubated with 0.5% crystal violet for 1 h, captured and the colonies formation were counted.

Cell cycle arrest

For cell cycle analysis, 6-well plates were seeded at $2.5x10^5$ cells/well and incubated with seed extract (50-250 µg/mL) for 24 h. Afterwards, cells were washed in phosphate buffered saline (PBS) for three times, collected in a 0.5 mL tube and then the cells were fixed in 70% ethanol for overnight. Next, the pellet cells were incubated in propidium iodide (PI) solution (Cat No. 550825, BD Biosciences, CA, USA) at 4°C for 30 min in dark. The cells were measured by flow cytometer with excitation at 488 nm and emission at 600 nm (BD Biosciences, CA, USA) using BD Accuri C6 Plus software and the resulting fluorescent signals were displayed as histograms.

Acridine orange/Ethidium bromide (AO/EB) staining

For apoptosis analysis, 96-well plates were seeded with $2.5x10^5$ cells/well and exposed to seed extract (50-250 µg/mL) for 24 h. Next, cells were washed in PBS buffer and stained with AO/EB mixture (1 µg/mL) at room temperature for 15 min. The cell image was captured using an inverted fluorescence microscope and long-pass emission filters of 480 and 535 nm (CKX53 Olympus, *20x* magnification).

Apoptosis

For apoptosis analysis, 6-well plates were seeded with $2.5x10^5$ cells/well and then incubated in seed extracts (50-250 µg/mL) for 24 h. Then, the cancer cells were washed in PBS, collected in 0.25% trypsin-EDTA, 100 µl binding buffer added and mixed with 5 µl of Annexin V-FITC and 1.5 µl of PI solution (Cat No. 558547, BD Biosciences, CA, USA). Next, stained cancer cells were incubated for 15 min at room temperature in the dark. Finally, viable, early apoptotic, late apoptotic, necrotic cells, were measured by a flow cytometer with excitation of Annexin V-FITC at 488 nm and PI at 552 nm (BD Biosciences, CA, USA) within 60 min using BD Accuri C6 Plus software.

Mitochondrial function

The modification of the mitochondrial function or mitochondrial membrane potentials ($\Delta\Psi m$) were determined by JC-1 dye (Cat. No. 1-800-346-9897, Cayman Chemical, Michigan, USA). 6-well plates were seeded with $2x10^5$ cells/well of HeLa cells and incubated with seed extract (50-250 µg/mL) for 24 h, trypsinized, loaded with 100 µL DMEM medium containing 5 µL JC-1 assay reagent for 30 min at 37°C in the dark, and finally added 400 µL of DMEM medium. Mitochondrial membrane potential was determined by flow cytometric analysis (BD Biosciences, CA, USA) using BD Accuri C6 Plus software. In each graph, the y-axis represents healthy cells as JC-1 aggregates, the x-axis represents unhealthy cells as JC-1 monomer and the downward shift of fluorescence from JC-1 aggregates to JC-1 monomer shows the decrease of mitochondrial function. The excitation of JC-1 is at 488nm, excitation of JC-1 monomer at 530 nm and JC-1 aggregates at 590 nm.

Reactive oxygen species (ROS) formation

For ROS formation assay, 6-well plates were seeded with $2x10^5$ cells/well of HeLa cancer cells and incubated with the seed extract (50-250 µg/mL) for 24 h. Then cells were trypsinized, and new complete DMEM medium added with 25 µM 2′,7′-dichlorofluorescin diacetate, DCF-DA, (Cat.no. D6883, Sigma Merck KGaA, Darmstadt, Germany) for 30 min at 37°C in the dark. ROS generation was determined by flow cytometry with excitation at 498 nm and emission at 522 nm (BD Biosciences, CA, USA) using BD Accuri C6 Plus software and fluorescent signals were displayed as histograms.

Wound healing method

For the wound healing method, 6-well plates were seeded with $2x10^5$ cells/well of HeLa cells were exposed to seed extracts (25-250 µg/mL) and the wound image was captured at 0 and 48 h. The distance of wound was measured and calculated and compared between the control and treatment groups.

STATISTICAL ANALYSIS

Data was obtained from three independent experiments (n=3) and represented as the mean±SE. Student's *t-test* was used to compare between treatment and control groups by GraphPad Prism 5 (GraphPad Software, Inc.) Statistically significant difference were considered at <0.05.

RESULTS

The effects of O. indicum on HeLa cervical cancer cells proliferation, colony formation, and cell cycle arrest

The seed extract and cisplatin demonstrated significant antiproliferative action on HeLa cells in a time- and dose-dependent manner (fig. 1A and C).

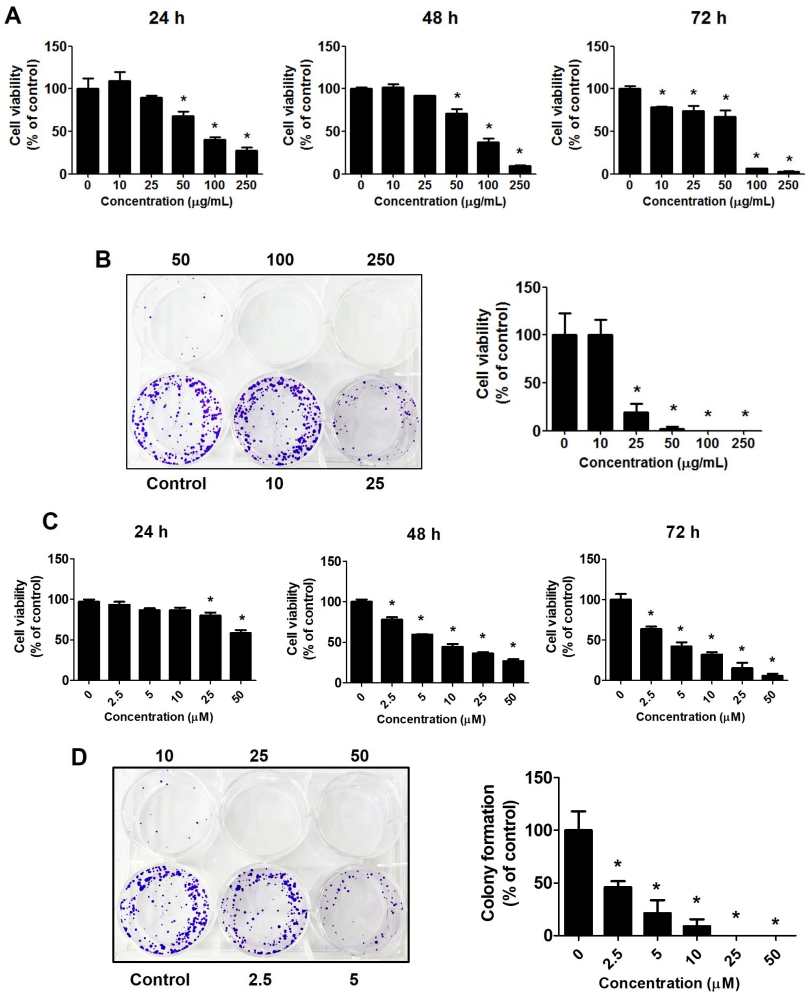
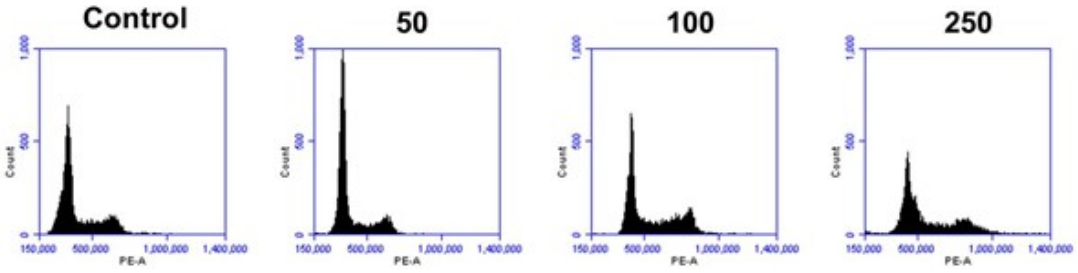


Fig. 1: The *O. indicum* effects on HeLa cell viability and colony formation. (A and C) 1x10^4 cells/well of HeLa cells were incubated to the *O. indicum* extract (10-250 µg/mL) or cisplatin (0-50 µM) for 24-72 h, and cell viability measured using the SRB method. (B and D). 500 cells/well of HeLa cells were incubated with the extract or cisplatin and also stained with crystal violet. Data are represented as mean±SE (n=3) and the p-value set at < 0.05.



Groups	G0/G1 phase	S phase	G2/M phase
0	67.9±2.35	14.6±1.23	17.4±1.98
50	71.1±3.63*	11.6±2.25	14.4±3.74
100	54.9±3.14*	11.2±1.14	27.3±1.69*
250	51.1±2.36*	12.3±0.97	18.5±2.14

Fig. 2: The *O. indicum* effects on HeLa cell cycle arrest. 2.5x10^5 cells/well of HeLa cells were incubated with the *O. indicum* extract (50-250 µg/mL) for 24 h, and cell cycle arrest measured using flow cytometric analysis. Data are represented as mean±SE (n=3) and the p-value set at < 0.05.

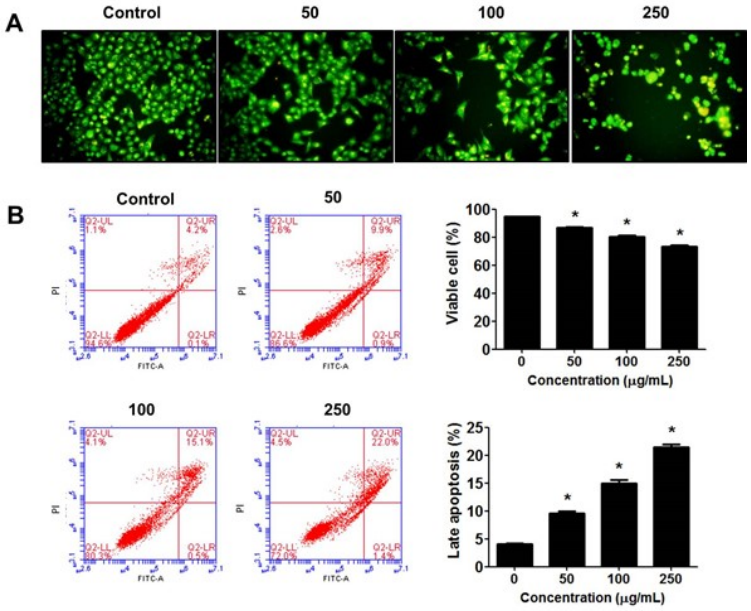


Fig. 3: The *O. indicum* effects on HeLa cell apoptosis. (A) $1x10^4$ cells/well of HeLa cells were incubated with the *O. indicum* extract (50-250 µg/mL) for 24 h, stained with AO/EB dye, and captured cells by inverted microscopy (*20x* magnification). (B) $2.5x10^5$ cells/well of HeLa cells were incubated with the *O. indicum* extract, stained with PI and Annexin V-FITC, and apoptosis measured by flow cytometric analysis. Data are represented as mean ± SE (*n*=3) and the *p*-value set at < 0.05.

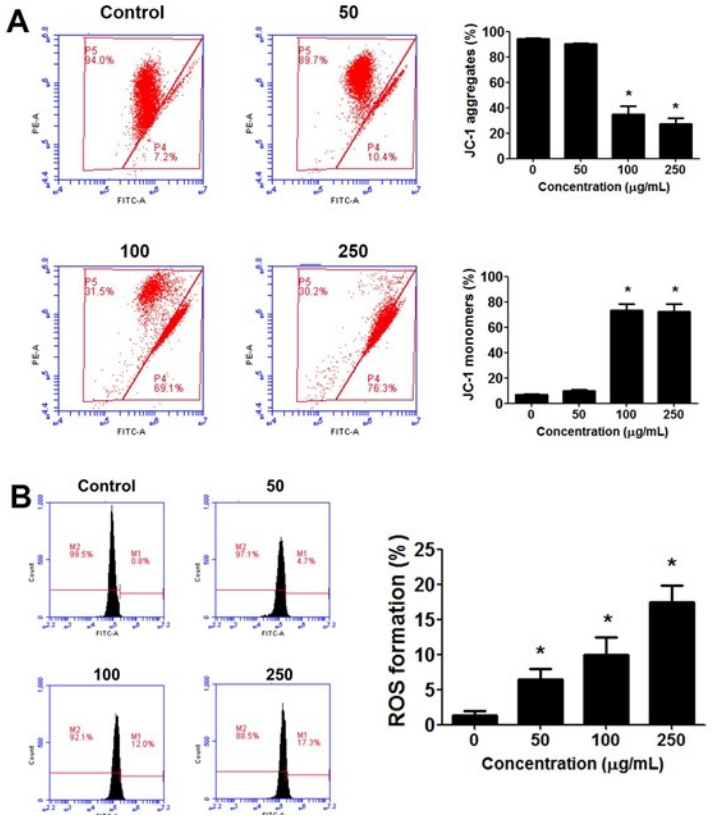


Fig. 4: The *O. indicum* effects on mitochondrial membrane potential and ROS formation. (A) $2.5x10^5$ cells/well of HeLa cells were incubated with the extract (0-250 µg/mL) for 24 h, stained with JC-1, and mitochondrial membrane potential measured by flow cytometry. (B) $2.5x10^5$ cells/well of HeLa cells were incubated with the extract and exposed to crystal violet. Data are represented as mean ± SE (*n*=3) and the *p*-value set at < 0.05.

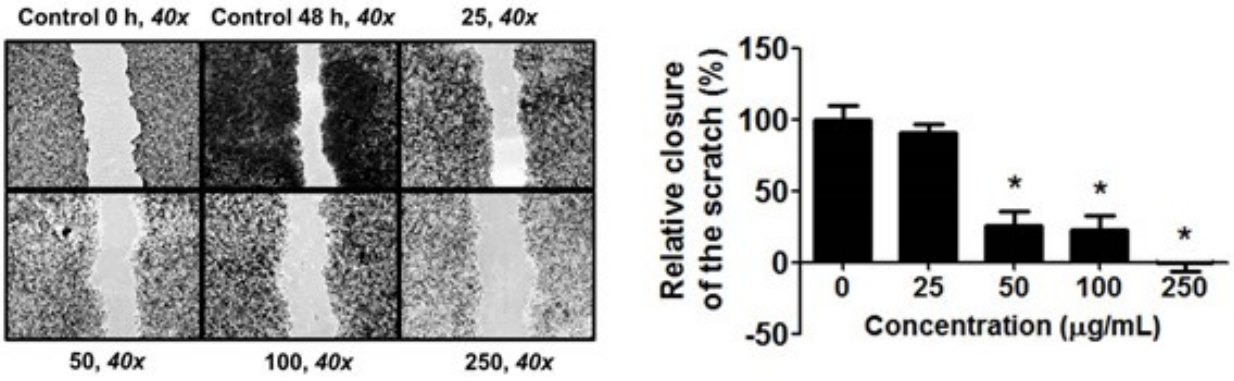


Fig. 5: The effect of *O. indicum* on HeLa cell migration. $2.5x10^5$ cells/well of HeLa cells were made a wound by 0.2 mL tip and treated with extract for 48 h. Data are represented as mean ± SE (n=3) and the p-value set at < 0.05.

The IC_{50} values of the extract were 103.84±8.39, 86.05±5.81, 44.71±1.08 µg/mL and cisplatin were 74.73±7.62, 9.66±0.43, 4.23±0.62 µM for 24, 48, 72 h, respectively.

The result indicated that *O. indicum* extract significantly suppressed the formation of colonies which was related to reduced cell proliferation. The IC_{50} value of colony formation of the extract was 14.95±1.59 µg/mL and for cisplatin was 7.28±1.47 µM at 24 h treatment (fig. 1B and D) and the result indicated that IC_{50} was less than with the SRB assay.

The extract caused induction of HeLa cells death and it was thus interesting to further examine these effects on cell cycle progression. After treating the cells with 50 µg/mL extract for 24 h there was a significant stoppage of the cell cycle at G0/G1 phase (fig. 2) with the percentage values of 67.9±2.35, 71.1±3.63, 54.9±3.14, and 51.1±2.36% for 0, 50, 100 and 250 µg/mL extract, respectively. Accumulation at G2/M phase showed the maximal activity at the dose of 100 µg/mL of the extract and the percentage was 17.4±1.98, 14.43±3.74, 27.3±1.69 and 18.5±2.14% for 0, 50, 100 and 250 µg/mL. This data specified that the *O. indicum* extract-mediated death of HeLa cells via stopping the cell cycle progression at G0/G1 or G2/M-phase, depending on different concentrations.

The effects of O. indicum on cervical cancer cells apoptosis, mitochondrial membrane potential, and ROS formation

To explore the effects of *O. indicum* on the activation of apoptotic action, AO/EB staining was used. The data indicated that exposing with 50, 100 and 250 µg/mL of the extract for 24 h caused a significant reduction of viable cell number in a dose-dependent manner. Interestingly, at the dose of 250 µg/mL morphological changes occurred to HeLa cells with nuclear condensation or nuclear fragmentation and this indicated the apoptosis had occurred (fig. 3A).

To confirm the *O. indicum* effect on apoptosis with AO/EB staining and to distinguish between viable, early apoptosis, late apoptosis and necrosis; treated-cells were stained with PI and Annexin V-FITC and then examined by flow cytometry. The data showed a dose-dependent decrease in the percentage of viable cells (left lower quadrant) and further increase in the percentage of late apoptotic cells (right upper quadrant) was observed. The percentage of late apoptotic HeLa cells of 0, 50, 100 and 250 µg/mL extract was 4.07±0.15, 9.57±0.42, 14.9±0.72 and 21.43±0.55%, respectively (fig. 3B).

Additionally, to investigate the effects of *O. indicum* extract on mitochondrial function, JC-1 staining followed by flow cytometric analysis was used. Treating HeLa cells with *O. indicum* extract caused a reduction of JC-1 aggregate (healthy cells) fluorescence intensity from 94.4±0.46% in untreated cells to 90.27±0.81, 36.63±4.67, and 27.1±6.51% of 50, 100 and 250 µg/mL extract, respectively (fig. 4A) and the results showed increased JC-1 monomers (unhealthy cells) intensity. This data indicated that the reduction of the mitochondrial function after exposing with *O. indicum* extract caused induction of apoptosis in cervical cancer cells.

The ability of the *O. indicum* extract mechanism to inhibit cell viability or induce apoptosis was evaluated through ROS production and DCF-DA staining with flow cytometric analysis. At each concentration of the extract (50-250 µg/mL) there was increased ROS formation in a dose-dependent manner, showing that the level of apoptosis was augmented (fig. 4B). These results were consistent with those of the AO/EB, Annexin V-FITC, and JC-1 staining to assess cell apoptosis, indicating that the *O. indicum* extract induced HeLa cells apoptosis. This may be based on its capability to eradicate cervical cancer cells.

The effects of O. indicum on cervical cancer cells migration

To explore the *O. indicum* effect on the migration of HeLa cells, wound-healing (scratch motility) was used.

The data are shown in fig. 5. *O. indicum* extract inhibited rapid movement of HeLa cells compared with the untreated control group in a dose-dependent manner; with a 48 h incubation period the IC_{50} value was 39.09±4.54 µg/mL. Based on the above, *O. indicum* extract at low concentration could suppress migration HeLa cervical cancer cells.

DISCUSSION

For several decades, studies of natural/herbal compounds have revealed valuable sources of anticancer agents to suppress cancer cells growth and migration with higher potency and lower toxicities. Significant effort has been made to identify vegetable foods or medicinal herbs that have a broad-spectrum activity to kill several tumors with one compound. One of the most necessary properties of anticancer agents is the power to eliminate uncontrollable cell growth and resistance to cancer cell death. However, anticancer agents must have the lowest toxic effects to normal cells. Many studies have reported the cytotoxic action of extracts of *O. indicum* can defeat cancer cells and that cytotoxic action on normal cells is low. Thus, this study focused on the *O. indicum* effects in the HeLa cervical cancer cells, based on growth inhibition, apoptosis induction, and migratory suppression.

In this regard, *O. indicum* extract is one of the capable agents with promising potential to be established as a new plant-based anticancer agent. A number of studies have reported that *O. indicum* was able to demonstrate antiproliferative effects against nasopharyngeal cancer, human breast cancer cells, leukemia cells, colorectal carcinoma cells and cervical cancer cells (Costa-Lotufo *et al*., 2005; Lalou *et al*., 2013; Moirangthem *et al*., 2012; Siriwatanametanon *et al*., 2010; Zazali *et al*., 2013). These results found that ethanolic seed extracts of *O. indicum* caused induction of HeLa cells death and cell cycle arrest with low IC_{50} values. For cytotoxic effects, our previous studies showed that four parts of *O. indicum*; the leaves, fruit, bark and seed, caused induction of MCF-7 cancer cells death and inhibition of cell migration (Buranrat *et al*., 2018). Further, two edible parts, the leaves and fruit, indicated high potent anti-breast cancer cell growth effects by inhibiting Rac1 expression levels (Buranrat *et al*., 2020). It is likely that *O. indicum* preferentially destroyed breast cancer cells compared to normal cells as revealed by suppression of migration assay (Kumar *et al*., 2012).

The other important aspect of this study is that we have observed apoptotic effects of *O. indicum* extracts on HeLa cells and the data indicated that seed extracts induced apoptosis, as observed by staining with AO/EB dye and Annexin V-FITC and PI. Furthermore, mitochondria function plays a crucial role in healthy cells and in survival of cancer cells survival and are the major organelle for the ROS production (Bhatti *et al*., 2017). Recent study has showed that the mechanism of action of many anticancer agents or herbal medicines are realized due to the formation of high levels of ROS, which lead to the mitochondrial membrane dysfunction and subsequent induction of apoptosis (Bhatti *et al*., 2017). Consequently, extract of *O. indicum* seed has high efficacy to induce HeLa cells apoptosis through producing intracellular ROS which ultimately leads to enhanced mitochondrial dysfunction and damage. Additionally, total flavonoids from *O. indicum* could significantly induce apoptosis in cancer cells by interfering in survival signaling pathway, PI3K/Akt/PTEN (Li *et al*., 2018). *O. indicum* had several mechanisms of action for the induction of cervical cancer cells death.

Lastly, the data indicated significant highly potent effects of *O. indicum* on of HeLa cells migration suppression with low IC_{50} values. As in our previous study, *O. indicum* leaf and fruit extracts caused suppression of MCF-7 cell migration with decreasing matrix metallopeptidase 9 (MMP-9) protein levels and also significantly decreased MMP-9 and intercellular adhesion molecule 1 (ICAMP1) gene expression. Consistently, *O. indicum* exhibited beneficial effects after testing metastatic potential by wound healing assay (Kumar *et al*., 2012). Hence, *O. indicum* extracts could be an appropriate source for emerging agents for the treatment of aggressive cervical cancer. Further study needs to deeply explore the mechanisms of action of *O. indicum* extracts in cervical cancer and, importantly, to perform testing to *in vivo*.

CONCLUSION

The present study provided evidence that ethanolic seed extracts of *O. indicum* inhibited cell proliferation, induced apoptosis and suppressed migration in HeLa cells *via* inducing ROS formation and inhibiting mitochondrial function. These results require further pharmacological exploration with in depth of phytochemical or active compounds analysis and investigations of the underlying mechanism of action on HeLa cells. *O. indicum* is one of potential active plant medicines useful in prevention/treating cervical cancer.

ACKNOELEDGEMENT

This work was financially supported by Mahasarakham University (Fast Track 2020).

REFERENCES

Ahn HJ, Kim KI, Kim G, Moon E, Yang SS and Lee JS (2011). Atmospheric-pressure plasma jet induces apoptosis involving mitochondria via generation of free radicals. *PloS One,* **6**(11): e28154.

Bhatti JS, Bhatti GK and Reddy PH (2017). Mitochondrial dysfunction and oxidative stress in metabolic disorders - A step towards mitochondria based therapeutic strategies. *Biochim. Biophys Acta Mol. Basis Dis.,* **1863**(5): 1066-1077.

Buranrat B, Noiwetch S, Suksar T and Ta-Ut A (2020). Inhibition of cell proliferation and migration by *Oroxylum indicum* extracts on breast cancer cells via Rac1 modulation. *J. Pharm. Anal.,* **10**(2): 187-193.

Buranrat B, Noiwetch S, Suksar T, Ta-ut A and Boontha S (2018). Cytotoxic and antimigration effects of different parts of *Oroxylum Indicum* extract on human breast cancer MCF-7 cells. *Science & Technology Asia,* **23**(4): 42-52.

Chan DC (2012). Fusion and fission: Interlinked processes critical for mitochondrial health. *Annu Rev Genet.,* **46**: 265-287.

Costa-Lotufo LV, Khan MT, Ather A, Wilke DV, Jimenez PC, Pessoa C, De Moraes ME and De Moraes MO (2005). Studies of the anticancer potential of plants used in Bangladeshi folk medicine. *J. Ethnopharmacol.,* **99**(1): 21-30.

Dinda B, SilSarma I, Dinda M and Rudrapaul P (2014). *Oroxylum indicum* (L.) Kurz, an important Asian traditional medicine: From traditional uses to scientific data for its commercial exploitation. *J. Ethnopharmacol.,* **161**: 255-278.

Gokhale M and Bansal YK (2006). An avowal of importance of endangered tree *Oroxylum indicum* (Linn.) Vent. *Nat. Prod. Rad.,* **5**(2): 112-114.

Kumar N, CijoGeorge V, Suresh PK and Ashok Kumar R (2012). Cytotoxicity, apoptosis induction and anti-metastatic potential of *Oroxylum indicum* in human breast cancer cells. *Asian Pac. J. Cancer Prev.,* **13**(6): 2729-2734.

Lalou C, Basak A, Mishra P, Mohanta BC, Banik R, Dinda B and Khatib AM (2013). Inhibition of tumor cells proliferation and migration by the flavonoid furin inhibitor isolated from *Oroxylum indicum*. *Curr. Med. Chem.,* **20**(4): 583-591.

Li NN, Meng XS, Men WX, Bao YR and Wang S (2018). Total Flavonoids from *Oroxylum indicum* Induce Apoptosis via PI3K/Akt/PTEN Signaling Pathway in Liver Cancer. *Evid Based Complement Alternat Med.,* pp.1-9.

Moirangthem DS, Talukdar NC, Bora U, Kasoju N and Das RK (2012). Differential effects of *Oroxylum indicum* bark extracts: antioxidant, antimicrobial, cytotoxic and apoptotic study. *Cytotechnology,* **65**(1): 83-95.

Nik Salleh NNH, Othman FA, Kamarudin NA and Tan SC (2020). The biological activities and therapeutic potentials of baicalein extracted from *Oroxylum indicum*: A systematic review. *Molecules,* **25**(23): 1-23.

Rojsanga P, Bunsupa S and Sithisarn P (2020). Flavones contents in extracts from *Oroxylum indicum* seeds and plant tissue cultures. *Molecules,* **25**(7): 1-8.

Siriwatanametanon N, Fiebich BL, Efferth T, Prieto JM and Heinrich M (2010). Traditionally used Thai medicinal plants: *In vitro* anti-inflammatory, anticancer and antioxidant activities. *J Ethnopharmacol.,* **130**(2): 196-207.

Wahab NH, Din NAM, Lim YY, Jamil NIN and Mat NFC (2019). Proapoptotic activities of *Oroxylum indicum* leave extract in HeLa cells. *Asian Pac. J. Trop. Biomed.,* **9**(8): 339-345.

Zazali KE, Abdullah H and Jamil NIN (2013). Methanol extract of *Oroxylum indicum* leaves induces G1/S cell cycle arrest in HeLa cells via p53-mediated pathway. *Int. J. Med. Plants Res.,* **2**(7): 225-237.