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ORIGINAL ARTICLE

Induction of Cell Death and Apoptosis of *Oroxylum Indicum* Extract With Gemcitabine On Human Cholangiocarcinoma Cells Through Reducing Epidermal Growth Factor Receptor

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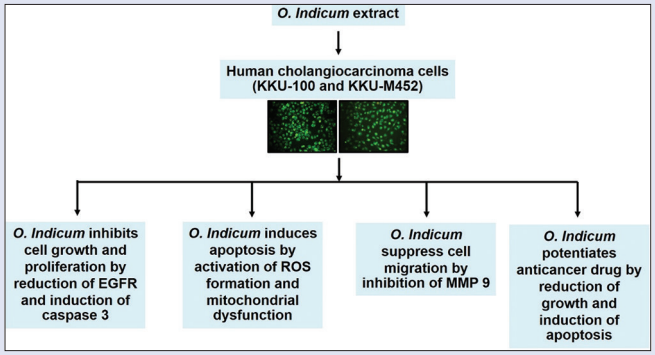
ABSTRACT

Background: Cholangiocarcinoma (CCA), epithelial bile duct cancer, is deadly cancer with a very poor prognosis and standard anticancer drugs strategy remains poor and unfavorable outcomes. **Objectives:** In this study, we examined how *Oroxylum indicum* leaf extract in combination with gemcitabine (anticancer drug) affects the viability, apoptosis, and migration of CCA cells. **Materials and Methods:** Two CCA cells, KKU-100 and KKU-M452, were incubated with *O. indicum* or gemcitabine or in combination for 24-72 hr and these effects were explored by using the sulforhodamine B, colony formation, cell cycle arrest, ROS formation, mitochondrial function, migration, and Western blot analysis. **Results:** *O. indicum* expressively suppressed the growth of both CCA cells at 72 hr with IC_{50} values were 9.88 ± 1.36 and 8.56 ± 1.02 μg/mL for KKU-100 and KKU-M452 cells, with the number of cells were decreased by dose-dependent manner. Further, the extract caused the reduction of colony formation and then suppressed the cells at G0/G1 phase for KKU-100 and S to G2/M phase for KKU-M452 cells. At 250 μg/mL, two CCA cells generated ROS formation along with decreasing mitochondrial function and then led to induce cancer cells apoptosis. Furthermore, *O. indicum* extract inhibited CCA cell migration via decreasing MMP-9 expression levels. The mechanism of its action was significant suppressed EGFR and caspase 3 levels. Combination group of *O. indicum* plus gemcitabine found that *O. indicum* potentiated gemcitabine to inhibit cell viability, induce apoptosis, increase ROS formation, decrease mitochondrial function, and suppress EGFR expression. **Conclusion:** *O. indicum* leaf extracts with potentiation of gemcitabine can reduce CCA cell growth, induce apoptosis, and cell migration through decreasing EGFR and caspase 3 expression. *O. indicum* could be useful for enhancing the activity of anticancer drugs displayed to prevent or treat CCA.

Key words: Cholangiocarcinoma. Reactive oxygen species (ROS), migration, mitochondrial dysfunction, *Oroxylum indicum*, proliferation

SUMMARY

- *O. indicum* inhibited CCA cells growth by reduction of number of cells and colony formation along with suppression cell cycle arrest.
- *O. indicum* suppressed CCA cell migration via decreasing MMP-9 expression levels.
- The mechanism of *O. indicum* was suppressed EGFR and caspase 3 levels.
- *O. indicum* in combination with gemcitabine, anticancer drugs, also showed the additive effects by inhibiting cell viability, inducing apoptosis, increasing ROS formation, decreasing mitochondrial function, and suppressing EGFR expression.
- *O. indicum* could be useful for defeating CCA.



Abbreviations used: AO/EB: Acridine orange/ethidium bromide; CCA: Cholangiocarcinoma; DMEM: Dulbecco's modified Eagle's medium; EGFR: Epidermal growth factor receptor; FBS: Fetal bovine serum; MMP: Matrix metallopeptidase; ROS: Reactive oxygen species; SRB: Sulforhodamine B.

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INTRODUCTION

Cholangiocarcinoma (CCA) is a bile duct cancer that arises from bile duct epithelial cells and is reported as a rare case in worldwide; however, in Thailand, CCA was demonstrated as a serious public health problem, still associated with high incidence and mortality rates.[1] Presently, CCA patients approximately 10% present with the initial-stage disease are considered a surgical procedure,[2] and chemotherapeutic treatment is the option for patients who cannot have surgery.[3] Unfortunately, CCA is commonly resistant to standard chemotherapeutic agents by altering itself to tolerance mechanisms,[4] and the effects are stills disappointing, with a low efficacy and survival rate. New, high potency alternative agents are urgently required to manage CCA. Interestingly, there have been several recent reports on the use of traditional medicine with or without chemotherapeutic drugs for relief of suffering from several cancer, including CCA.[5]

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The important advantages of the natural medicinal are their low adverse effects and cost, therefore, *Oroxylum indicum* was more interested to explore cancer treatment. *O. indicum*, Bignoniaceae family, is a common vegetable in many regions of Thailand as cooked for foods and vegetable beside the dish. It has been reported for traditional medicine in many diseases, especially cancer.[6] From the results obtained from pharmacological activities, *O. indicum* has been demonstrated in antiinflammation, antihelmintic, antimicrobial activities, hepatotoxicity protection, immunostimulation, and anticancer activity.[7] Several parts of *O. indicum* were determined the anticancer effects such as the leaf, stem bark, root, and seed; however, edible parts such as leaf extract still have less information. Further, *O. indicum* has been reported the several active compounds including chrysine, baicalein, oroxylin A and oroxylin B,[6] However, less data is found on the edible parts activities such as the young leaves of *O. indicum* on CCA.

From our previous research demonstrated that *O. indicum* leaf and fruit extract had a high power to against human breast cancer cells with induction of apoptosis by increasing caspase 3 activity along with reduction of migration via inhibiting migration through MMP-9 reduction.[8] For the other cancer cells, *O. indicum* extract activated cervical cancer cells apoptosis by showing blebbing, DNA fragmentation and apoptotic bodies,[9] and stimulation of caspase 8 and caspase 3 activation with upregulating p53, pRb, Fas, and FasL,[10] finally the cancer cells death occurred. Confirmation with cell cycle arrest analysis, it has been demonstrated that *O. indicum* activated the growth arrest of cancer cells at G1/S phase via p53-mediated pathway stimulation.[9] Moreover, *O. indicum* activated apoptosis and suppressed migration in human breast cancer aggressive cells (MDA-MB-231). *O. indicum* extract could be useful for treating cancer progression/aggression in late stages of malignancy.[11] *In vivo* study indicated that *O. indicum* extract from stem bark displayed cytotoxic effects compared with anticancer drugs, vincristine.[12]

Several mechanisms of anticancer agents have been related to growth suppression and apoptosis induction, epidermal growth factor receptor (EGFR) was more interested. EGFR is a receptor of tyrosine kinases in HER family and the final step of signal pathway provides the cell proliferation induction and apoptosis suppression.[13] Upregulation of EGFR expression has been implied in the pathogenesis of many cancer types and it has been related to resistance to chemotherapeutic drugs.[14] Consequently, EGFR is an attractive target for anticancer treatments. As resistance and high toxicity to chemotherapeutic drugs of CCA is a major role in the unsuccessful treatment of the disease.

Therefore, this present work explored the *O. indicum* effects on cancer cell death, migration, and apoptosis, and further determined the in combination effects of *O. indicum* with gemcitabine, an anticancer drug. We investigated the mechanism (s) responsible for these activities with the detection of EFGR and caspase 3 expressions.

MATERIALS AND METHODS

Plant extraction

Leaves of *O. indicum* were obtained in July 2020 from Maha Sarakham Province, Thailand and a voucher specimen was deposited (MSUT_7226 at Faculty of Sciences, Mahasarakham University. The extracts were made as previously described.[7] In brief, leaves of *O. indicum* (250 mg) were dried and macerated twice with 95% ethanol (1 L) for seven days. Then, the extracts were filtered, evaporated, lyophilized, and then stored at -20°C. The percentage of yield was 9.05%, and the extract was standardized to quantify total phenolic compound (gallic acid) and total flavonoid contents (rutin), which were 45.14 ± 4.35 and 29.56 ± 7.85 mg/g, respectively.

Cell culture and sulforhodamine B (SRB) assay

The two human CCA cells, KKU-100 (Slowly growing cells) and KKU-M452 (Rapidly growing cells), were kindly provided by the Faculty of Medicine, Khon Kaen University, which was established from CCA patients in Northeastern Thailand. All cells were grown in DMEM with 1% antibiotics and 10% fetal bovine serum (FBS).

For cytotoxic assay, 1×10^4 cells/well of cancer cells were plated in 96-well culture plates for 24 hr. Next, the cells were exposed to *O. indicum* (0-250 μg/mL) for 24-72 hr. Afterward, 10% trichloroacetic acid was added, 0.4% SRB solution stained the cells, and 10 mM Tris base solubilized the cells. Measurement of O.D. by microplate reader at 540 nm.

To determine the effect of *O. indicum* in combination with gemcitabine, and anticancer drugs, the method was still used SRB assay as described previously. Briefly, the cells were seeded and then *O. indicum* extract or gemcitabine or in combination with vary gemcitabine concentrations; however, the concentration of *O. indicum* extract was fixed concentration (50 μg/mL). After 24-hr incubation period, cell viability was measured by SRB method.

Acridine orange/ethidium bromide (AO/EB) assay

To explore the cell morphology and cell number after exposure with *O. indicum* extract, AO/EB staining was performed. Cancer cells approximately 1×10^4 cells/well were cultured in 96-well culture plates for 24 hr and exposed to *O. indicum* (0-250 μg/mL) for 24 hr. After that, cancer cells were added with 1 μg/mL of each dye (AO/EB) for 15 min, and photographed by fluorescence phase-contrast inverted microscope (*20x* magnification, CKX53, Olympus, USA).

Colony formation assay

To determine the colony-forming ability after exposure with *O. indicum* extract and colony formation was performed. Cancer cells approximately 500 cells/well were seeded in 6-well plates for 24 hr and further incubated with *O. indicum* (0-250 μg/mL) for 24 hr. After 15 days, methanol was added to fix the cells, and 0.5% crystal violet was added to stain the cells. The photos were photographed and colonies were counted.

Cell cycle distribution assay

To explore the distribution of cell cycle after exposing with *O. indicum* extract, flow cytometry was performed. Cancer cells approximately 2.5×10^5 cells/well were seeded in 6-well plates for 24 hr and incubated with *O. indicum* (0-250 μg/mL) for 24 hr, and further cells were mixed with PI dye for 30 min. The distribution of cell cycle was measured by the DNA content using the flow cytometry BD Accuri C6 Plus software.

Reactive oxygen species (ROS) formation assay

To determine the generation of ROS after exposing to *O. indicum* extract, flow cytometry was performed. Cancer cells approximately 2.5×10^5 cells/well were seeded in 6-well plates for 24 hr, incubated with *O. indicum* (0-250 μg/mL) for 24 hr and further resuspended in DCF-DA fluorescent probe for 30 min. The intensity of fluorescence of ROS was determined using the flow cytometry using the flow cytometry BD Accuri C6 Plus software.

In combination treatment, after cancer cells were plated for 24 hr and then *O. indicum* extract (50 μg/mL) or gemcitabine (10 μM) or in combination with two compounds were added for 24 hr. At the end of treatment, ROS formation was evaluated by flow cytometry.

Mitochondrial function assay

To determine the function of mitochondria after exposing to *O. indicum* extract, flow cytometry was performed. Cancer cells approximately

2.5×10^5 cells/well were seeded in 6-well plates for 24 hr, incubated with *O. indicum* (0-250 µg/mL) for 24 hr and then resuspended in JC-1 probe for 30 min. Fluorescence intensity in cells was determined using the flow cytometry using the flow cytometry BD Accuri C6 Plus software.

In combination treatment, after cancer cells were plated for 24 hr and then *O. indicum* extract (50 µg/mL) or gemcitabine (10 µM) or in combination with two compounds were added for 24 hr. At the end of treatment, mitochondrial function was evaluated by flow cytometry.

Wound healing assay

To determine the cancer cells' migration after exposure with *O. indicum* extract and Wound healing assay was performed. Cancer cells approximately 2.5×10^5 cells/well were seeded in 24-well plates for 24 hr, made a wound with scratching by 0.2 mL pipette tips, treated with *O. indicum* (0-250 µg/mL) for 24 hr and the images were photographed at 0, 24 (KKU-M452), and 72 (KKU-100) hr by an inverted phase-contrast light microscope (Olympus, CKX53, 20x magnification). The wound healing was calculated as the difference in the area between 0 and 24, and 72 hr divided by the width of the wound.

Gelatin zymography assay

To determine the cancer cells' migration after exposing to *O. indicum* extract, gelatin zymography assay was performed. Cancer cells approximately 2.5×10^5 cells/well were seeded in 24-well plates for 24 hr, incubated with *O. indicum* (0-250 µg/mL) in 0% FBS medium for 24 hr and then media was collected. Measurement of protein concentration by Bradford's reagent and 20 µg protein was subjected to 0.01% gelatin (w/v) in 10% SDS-PAGE-containing. Gels were exposed to developing buffer overnight, stained with 0.25% Coomassie Brilliant Blue R-250, and added destaining buffer to wash the gel. The clear band was photographed and evaluated the band density by using a ChemiDoc™ MP imaging system.

Matrigel migration assay

To determine the cancer cells' migration after exposing to *O. indicum* extract, matreigel migration assay was performed. Cancer cells approximately 2×10^4 cells/well were seeded into Transwell chamber with 0% FBS of DMEM medium containing *O. indicum* (0-250 µg/mL) for 24 hr, and the lower chamber was contained 10% FBS DMEM medium. The cells number in three selected areas were counted.

Apoptotic assay

To determine the apoptosis after exposing to *O. indicum* extract, a flow cytometry assay was performed. Cancer cells were seeded in 6-well plates (2.5×10^5 cells/well) for 24 hr, incubated with *O. indicum* extract (50 µg/mL) or gemcitabine (10 µM) or in combination with two compounds were added for 24 hr. After that, the cancer cells were added with Annexin V-FITC/PI dye in dark at room temperature for 15 min and the fluorescence intensity in cells was determined using the flow cytometry using the flow cytometry BD Accuri C6 Plus software.

Western blot assay

To explore the EGFR and caspase 3 protein expression levels after exposing to *O. indicum* extract, Western blot assay was performed. Cancer cells were plated in 6-well culture plates (2.5×10^5 cells/well) for 24 hr, and incubated with *O. indicum* extract (0-250 µg/mL) for 24 hr. The cells were extracted and protein solution was collected. The total proteins approximately 20 µg were loaded onto 10% SDS-PAGE and run with electrophoresis. Then, the protein in gel was transferred to PVDF membranes, blocked with 3% BSA and incubated with primary antibodies including EGFR (1:1000) and caspase 3 (1:1000) overnight at 4°C. After washing, further, the membrane was exposed to a secondary antibody (1:2,500) for 2 hr. The bands were photographed using a ChemiDoc™ MP imaging system after exposing them with ECL substrate. Protein expression levels were compared with beta-actin expression.

In combination treatment, after cancer cells were plated for 24 hr and then *O. indicum* extract (50 µg/mL) or gemcitabine (10 µM) or in combination with two compounds were added for 24 hr. EGFR protein expression was measured by Image Lab software.

Statistical analysis

The data are presented as mean ± SE and tested the differences between control and treatment groups were evaluated by a student unpaired *t-test*. Statistically significant if *P* values were less than 0.05 compared to the untreated control.

RESULTS

Cell viability and cell morphology

These results indicated that the growth of CCA cells was decreased in a dose- and time-dependent manner, with 72 hr IC_{50} values of 9.88 ± 1.36 µg/mL in KKU-100 cells and 8.56 ± 1.02 µg/mL in KKU-M452 cells, respectively [Figure 1a and b]. Further, *O. indicum* extracts also reduced the cells number of CCA cells, especially in KKU-M452 cells [Figure 1c and d]. This extract exhibited cytotoxic and antigrowth effects against the CCA cells.

Colony formation and cell cycle arrest

This study we used only KKU-100 cells to represent colony formation assay because KKU-M452 cells did not form the colony. The data indicated that *O. indicum* extracts decreased colony formation in a dose-dependent manner and at 100-250 µg/mL of the extract did not detect the colony-forming ability [Figure 2a]. Further, to confirm the cytotoxic effects of this extract and cell cycle arrest was studied. This extract caused induction of cell cycle arrest at G0/G1 phase significant at the dose of 100 and 250 µg/mL in KKU-100 cells along with the arrest of the cell distribution at s to G2/M phase in KKU-M452 cells. These extracts inhibited colony formation along with stopping the cell cycle distribution. [Figure 2b and c].

ROS formation and mitochondrial membrane potential

From the data indicated that *O. indicum* extract was found to activate ROS generation and at the dose of 250 µg/mL showed the highest activity with 7.4% in KKU-100 cells and 5.6% in KKU-M452 cells, respectively [Figure 3a and b]. Furthermore, the leaf extract caused inhibit the mitochondrial function in dose-dependent manner, 0, 50, 100, 250 µg/mL, increasing the JC-1 monomer (unhealthy cells) from 3.27 ± 0.06, 9.53 ± 0.15, 11.60 ± 0.70, and 12.03 ± 0.85% in KKU-100 cells along with 9.93 ± 2.70, 23.83 ± 3.38, 38.57 ± 8.85, 61.93 ± 3.98% in KKU-M452 cells, respectively [Figure 3c and d]. *O. indicum* extract stimulated ROS production in two CCA cells and reduced mitochondrial function, especially in KKU-M452 cells.

Cell migration

For CCA migration, these extracts suppressed migration in a dose-dependent manner. This *O. indicum* extract significantly decreased the wounds closing compared with the control group at a dose of 50-250 µg/mL [Figure 4a and b]. Confirmation with Matrigel migration assay, this data also confirmed that these extracts decreased the number of CCA migrated cells to the lower chamber with significant at the dose of 50-250 µg/mL in both of KKU-100 and KKU-M452 cells [Figure 4c and d]. Afterward, these extracts strongly

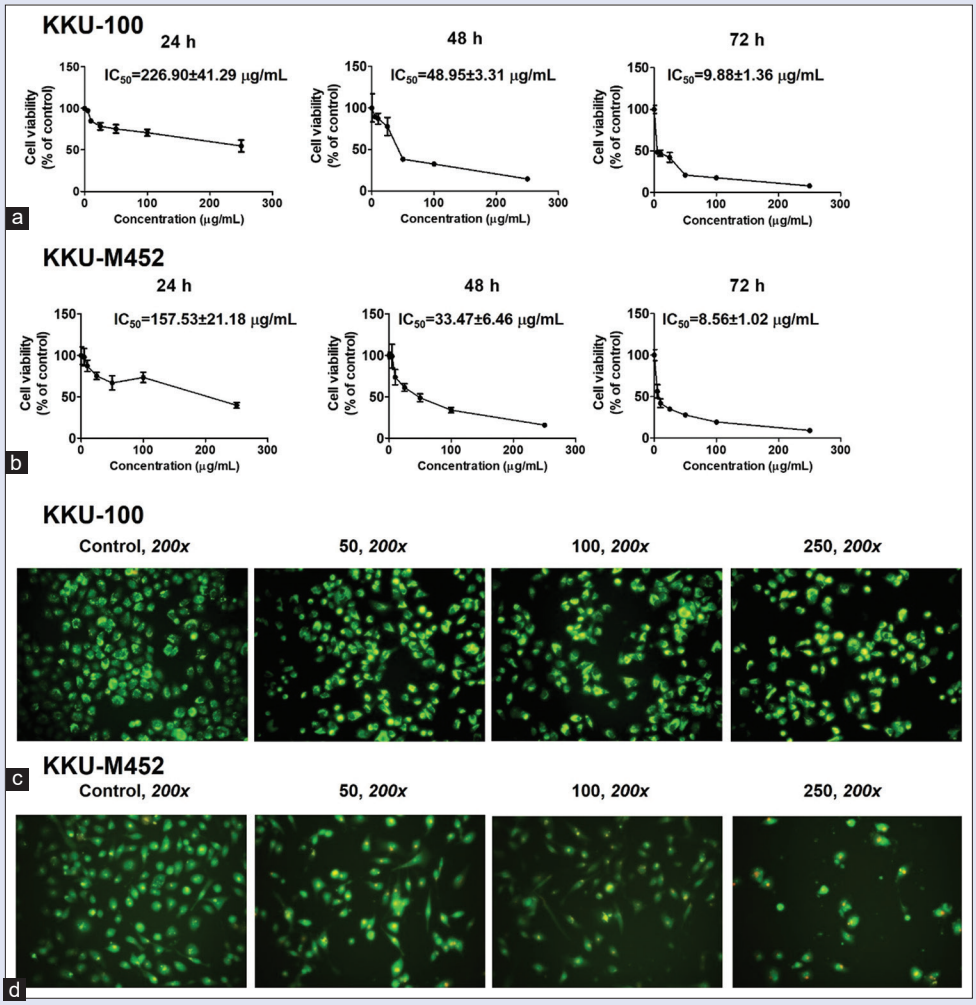


Figure 1: Cytotoxic effects of *O. Indicum* on CCA cells. (a and b) KKU-100 and KKU-M452 cells were exposed to the extract for 24-72 h and proliferation was investigated using a SRB assay. (c and d) CCA cells were stained with AO/EB and observed the morphology by fluorescence inverted microscopy (*20x* magnification). Mean ± SE (*n* = *3*). **P* < 0.05

inhibit matrix metalloproteinase (MMP) 9 protein expression that secreted to the medium [Figure 4e-f]. Best from the results obtained, *O. indicum* extract decreased both of CCA cell migration via decreasing MMP 9 expression.

EFGR and caspase 3 expression levels

EFGR and caspase 3 expression levels were used only KKU-100 cells to represent the CCA cells because the responsibility of two CCA cells to *O. indicum* was showed the same effects. The expression levels of EGFR were significantly reduced in a dose-dependent manner from 50 to 250 μg/mL, while the caspase 3 protein expression levels were upregulated compared with the untreated control group [Figure 5a-c]. *O. indicum* against CCA cells by reduction protein-related cell proliferation, EGFR and induction protein-related cell apoptosis, caspase 3 levels.

O. indicum in combination with gemcitabine on cell proliferation, apoptosis, and ROS formation

From the great effects of *O. indicum* on CCA cell death and further the potentiation effects of *O. indicum* with anticancer drugs, gemcitabine, was more interested to explore. We still used only KKU-100 cells in combination effects to explore the cell's growth, apoptosis, and ROS formation. By measuring CCA cell death under various doses of *O. indicum* and gemcitabine after 24 hr, the IC_{50} values of *O. indicum* and gemcitabine were expressed to be 158.02 ± 86.24 μg/mL and 53.53 ± 14.08 μM and in combination was 0.91 ± 0.51 μM [Figure 6a]. Further, to evaluate the apoptotic induction by flow cytometry indicated that combination treatment stimulated KKU-100 cells apoptosis both in early and late apoptosis compared with extract and gemcitabine treatment alone [Figure 6b]. Additionally, the mechanism of apoptosis was explored by ROS formation, when *O. indicum* and gemcitabine were exposed together, these data found the activation of ROS formation related to apoptotic stimulation was activated compared with control and drug treatment alone [Figure 6c].

O. indicum in combination with gemcitabine on mitochondrial function and EGFR expression

In combination effects showed great activity to induce CCA cell death and apoptosis, therefore, the mechanism of action was explored by mitochondrial dysfunction and EGFR expression levels. For mitochondrial function, *O. indicum* and gemcitabine treatment alone caused induction of mitochondrial dysfunction as represent the JC-1 monomers induction and in combination was expressed as the potentiation effects compared with control and treatment alone [Figure 7a]. For EGFR expression, EGFR level was significantly decreased when compared with treatment alone [Figure 7b].

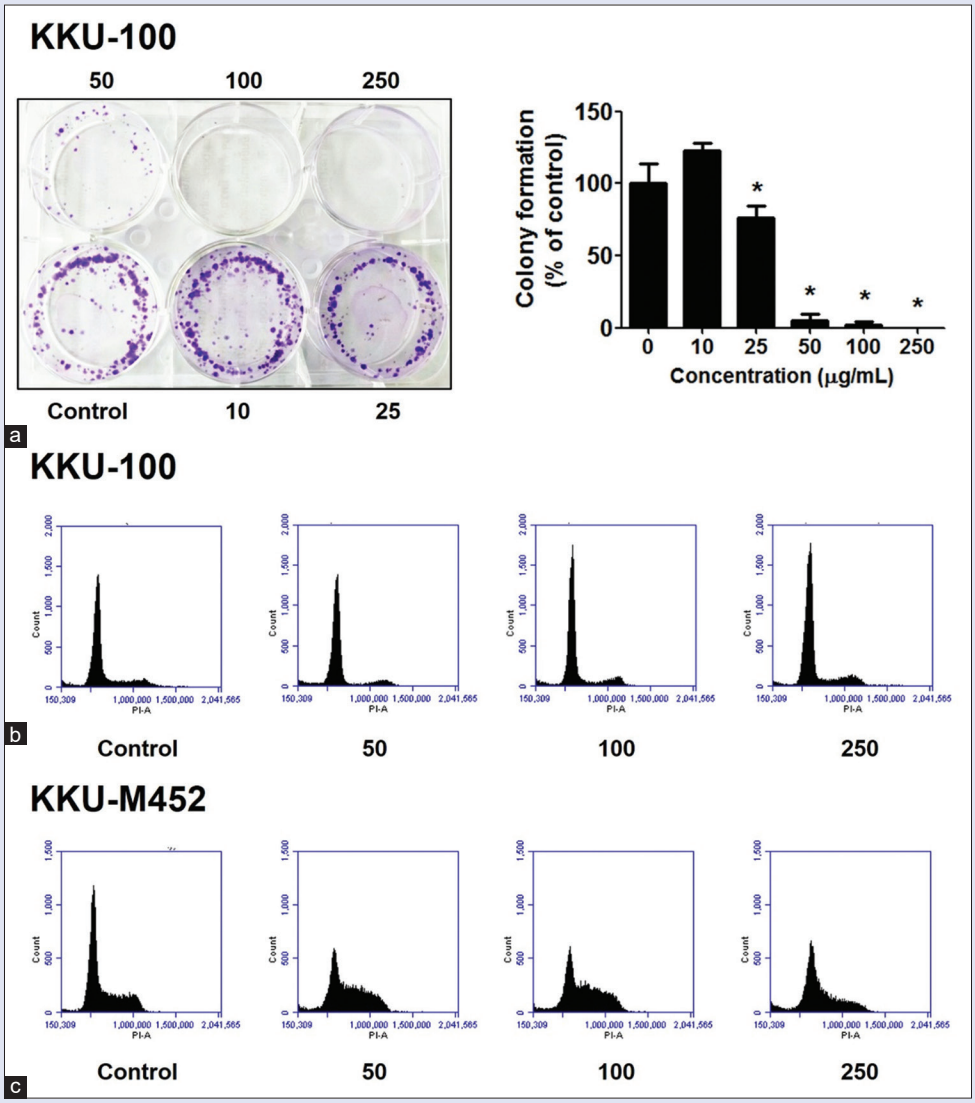
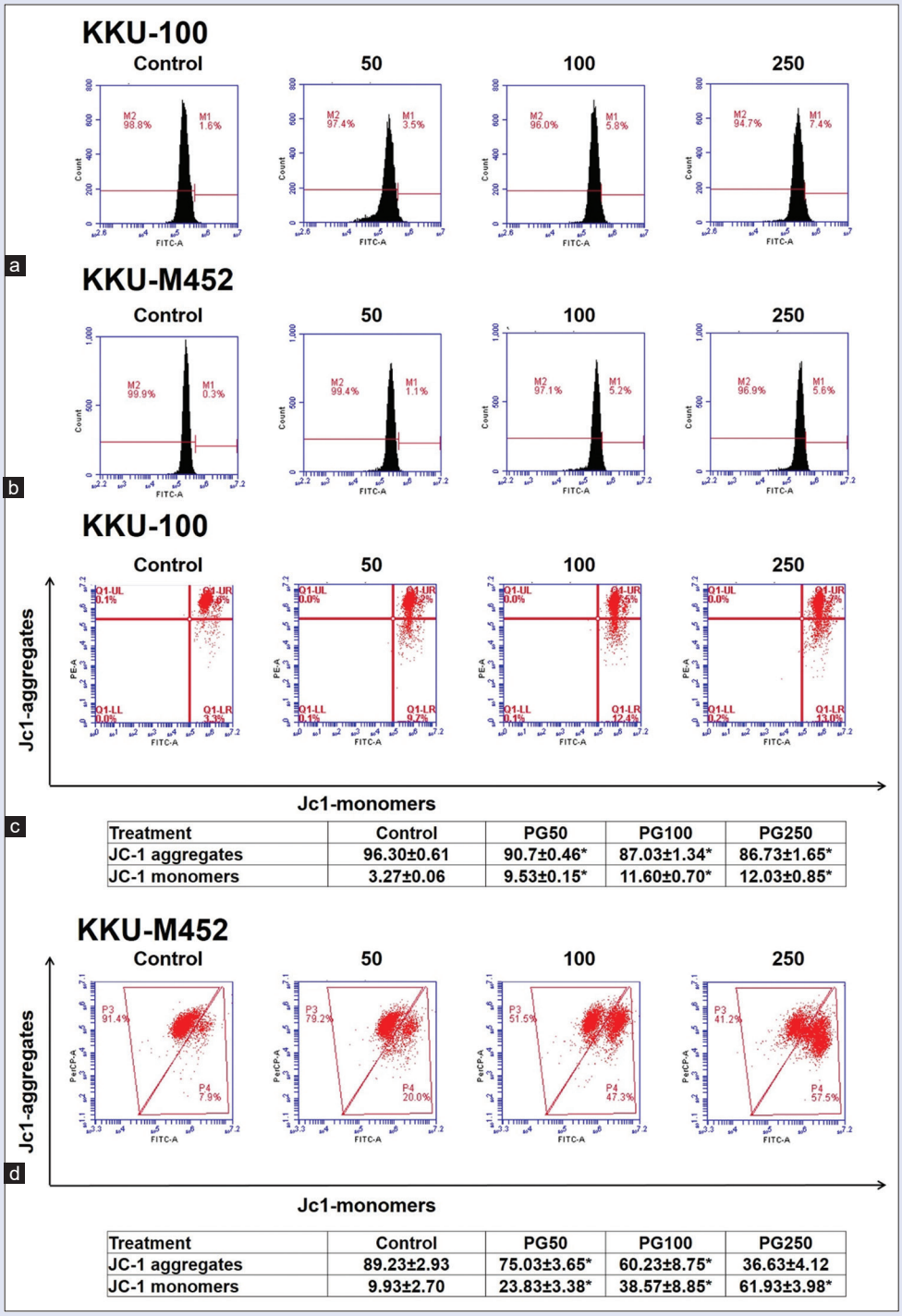


Figure 2: *O. Indicum* effects on colony formation and cell cycle distribution of in CCA cells. (a) KKU-100 cells were incubated with *O. Indicum* (0-250 µg/mL) for 24 hr, and colony formation was counted. (b and c) CCA cells were incubated with *O. Indicum* (0-250 µg/mL) for 24 hr, stained with PI solution, and measured the cell cycle distribution by flow cytometry. Mean ± SE ($n = 3$). *$P < 0.05$

DISCUSSION

CCA is a bile duct cancer with an aggressive phenotype and a very poor prognosis. Presently, the seeking of new anticancer agents from natural compounds with high efficacy and low side effects for CCA treatment is urgently required. In our previous studies, we found that *O. indicum* leaf extract could suppress cell growth and proliferation in breast cancer cells. In this study, we further confirmed the *O. indicum* extract on other cancer cells, CCA was more interested. The data demonstrated that *O. indicum* extract has anti-CCA properties in both two cell types with slowly (KKU-100) and rapidly (KKU-M452) growth and migration, as evidenced by the suppression of cell growth through arresting cell cycle distribution. Apoptosis activation was indicated through ROS stimulation and mitochondrial dysfunction. Migratory ability reduction was reported via decreasing MMP 9 level. *O. indicum* on two CCA cell types showed the same anticancer effects. Therefore, in the next experiment, we will be used just only KKU-100 cells to represent CCA cells. The mechanism of *O. indicum* showed that its suppressed EGFR level along with induction of caspase 3 level, leading to CCA cell death and apoptosis. Furthermore, the potentiation effects of *O. indicum* with anticancer drugs, gemcitabine, were explored. Combination group, *O. indicum* increased gemcitabine effects on growth inhibition, apoptosis induction, ROS formation, mitochondrial dysfunction, and EGFR downregulation in CCA cells. Therefore, *O. indicum* extract had more potency in the treatment and prevention of CCA.

The growth inhibitory effects of *O. indicum* extract was explored in several cancer cell types with different mechanism; however, it still less information on CCA cells. Several studies have indicated that *O. indicum* suppressed growth effects defeat many cancer types including cervical, nasopharyngeal, leukemia, breast, and colorectal carcinoma. Apoptosis is one of the main mechanisms of cell death in cancer treatment via arresting cell cycle distribution, generating ROS, and reducing mitochondrial function. Cervical cancer cells found that the methanolic extract of *O. indicum* indicated the growth arrest of cancer cells at G1/S phase with correlating p53 induction pathway[9] as shown in our study, these extracts caused induction of cell cycle arrest at G0/G1 phase in KKU-100 cells and at G2/M phase in KKU-M452 cells. Further, *O. indicum* induced breast cancer cells apoptosis by activation



Treatment	Control	PG50	PG100	PG250
JC-1 aggregates	96.30±0.61	90.7±0.46*	87.03±1.34*	86.73±1.65*
JC-1 monomers	3.27±0.06	9.53±0.15*	11.60±0.70*	12.03±0.85*

Treatment	Control	PG50	PG100	PG250
JC-1 aggregates	89.23±2.93	75.03±3.65*	60.23±8.75*	36.63±4.12
JC-1 monomers	9.93±2.70	23.83±3.38*	38.57±8.85*	61.93±3.98*

Figure 3: *O. Indicum* effects on ROS formation and mitochondrial function CCA cells. (a and b) CCA cells were incubated with *O. Indicum* (0-250 µg/mL) for 24, stained with DCF-DA solution, and ROS formation was measured by flow cytometry. (c and d) Cancer cells were incubated with *O. Indicum* for 24 hr, stained with JC-1 solution, and mitochondrial function was measured. Mean ± SE ($n = 3$). *$P < 0.05$

of ROS formation and induction of caspase 3 activity[8] as mentioned from these data. Mitochondrial dysfunction was detected in this study in a dose-dependent manner like as in oral cancer HSC-3 cells with reducing mitochondrial membrane potential. *O. indicum*-induced growth inhibition and apoptosis induction via ROS formation and mitochondrial dysfunction.

The previous studies demonstrated that the *O. indicum* extract suppressed cancer cell migration with very low concentration. Human breast cancer cells were significantly attenuated migration through reduction of MMP 9 protein and gene levels after exposure to *O. indicum* extracts.[8] With the less information on *O. indicum* on migration; however, the Wound healing assay indicated that *O. indicum* stem bark extract had more potent antimigratory activities in both estrogen-positive receptor and estrogen-negative receptor after scratching.[11] Further, *O. indicum* suppressed the cancer cells migration *in vivo* study was more interested to be explored in the further.

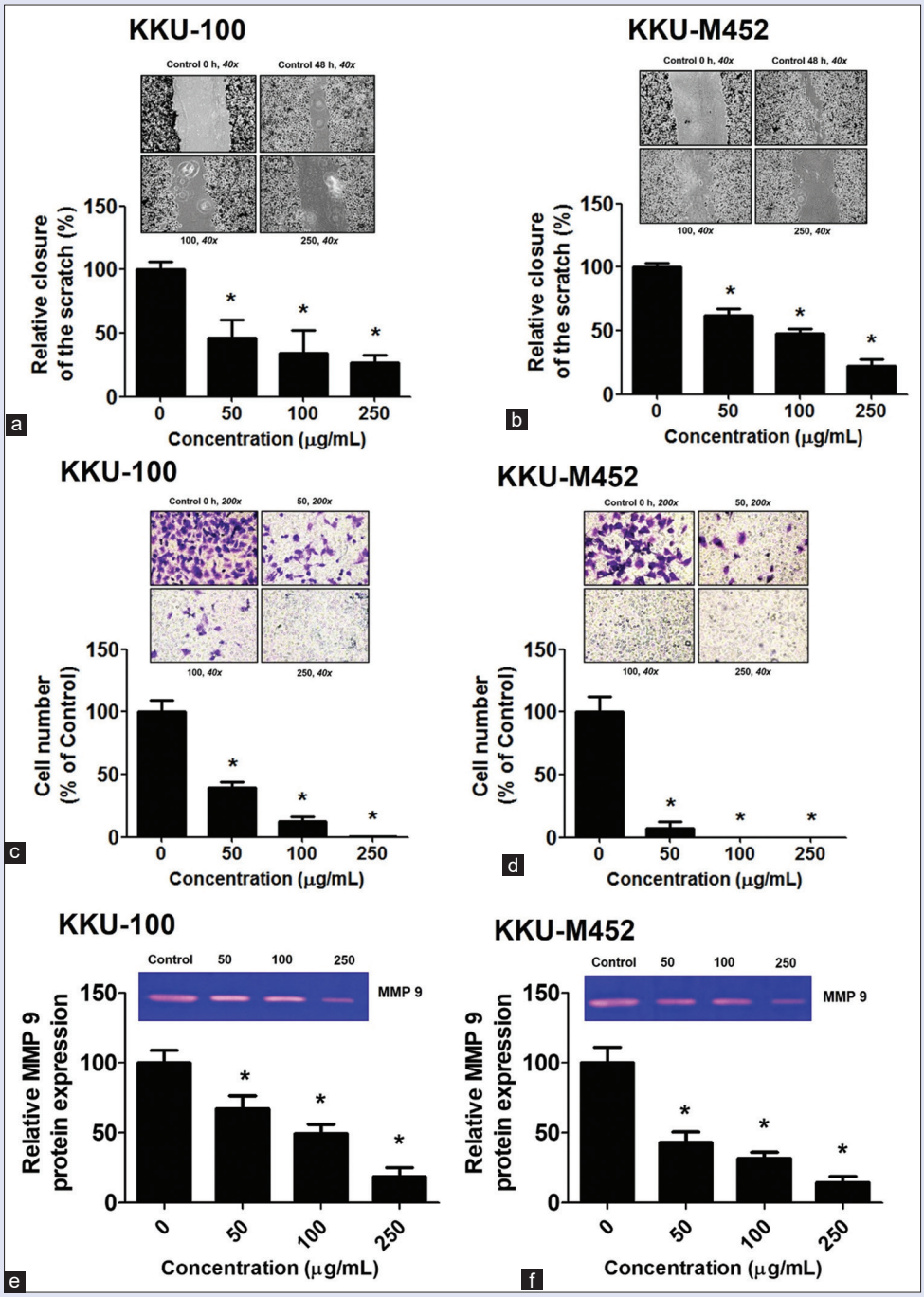


Figure 4: *O. Indicum* effects on cell migration in CCA cells. (a and b) CCA cells were incubated with *O. Indicum* (0-250 μg/mL) for 24 and 72 hr in KKU-M452 and KKU-100 cells., and Wound healing was captured by inverted microscopy. (c and d) cancer cells were treated with *O. Indicum* (0-250 μg/mL) for 24 hr, and migrated cells were captured by inverted microscopy. (e and f) cancer cells were treated with *O. Indicum* (0-250 μg/mL) for 24 hr and the medium was run electrophoresis for detecting MMP-9 expression. Mean ± SE ($n = 3$). *$P < 0.05$

Especially, the over-expression of EGFR has been demonstrated to activate abnormal growth, inhibit apoptosis, and induce migration. Therefore, an active compound that reduce the activation of EGFR has become a key point in this work. This finding provided that *O. indicum* significantly decreased EGFR by dose-dependent manner along with induction of caspase 3 levels, leading to induce CCA cell death. The results as shown in genistein effects, genistein inhibited the CCA cells growth by reducing the activation of EGFR and then apoptosis is occurred.[15] The important role of caspase 3, is it is the important activator of apoptosis[16] and this data revealed that *O. indicum* displayed the greatest action on the stimulation of caspase 3 expression levels. In conclusion, *O. indicum*-induced cell death and apoptosis via decreasing EGFR and inducing caspase 3 protein levels.

However, the exact mechanisms by which *O. indicum* plus anticancer drugs activates cell death and apoptosis are fewer data. Standard chemotherapy play an important role in the CCA treatment. Nevertheless, the most commonly used chemotherapeutic agents, gemcitabine, beneficial effect has provided a very limited increasing survival rate

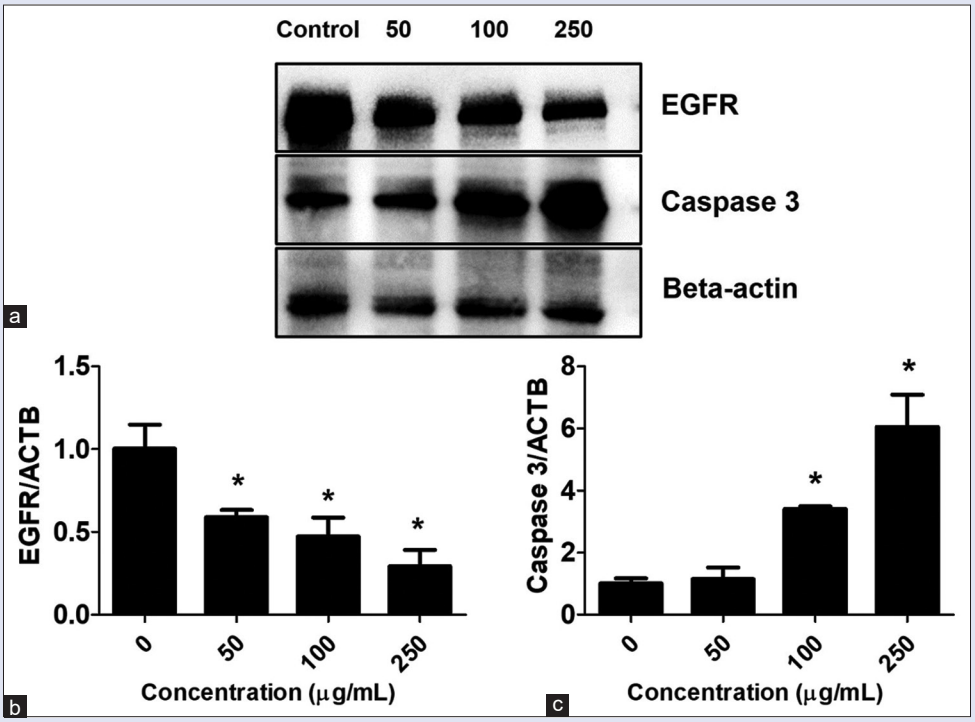


Figure 5: *O. Indicum* effects on EFGR and caspase 3 protein expression in CCA cells. For Western blot analysis assay, (a-c) KKU-100 cells were treated with *O. Indicum* (0-250 μg/mL) for 24 hr, and subjected to Western blot analysis. The levels of targeting protein were normalized using beta-actin as a loading control. Mean ± SE ($n = 3$). *$P < 0.05$

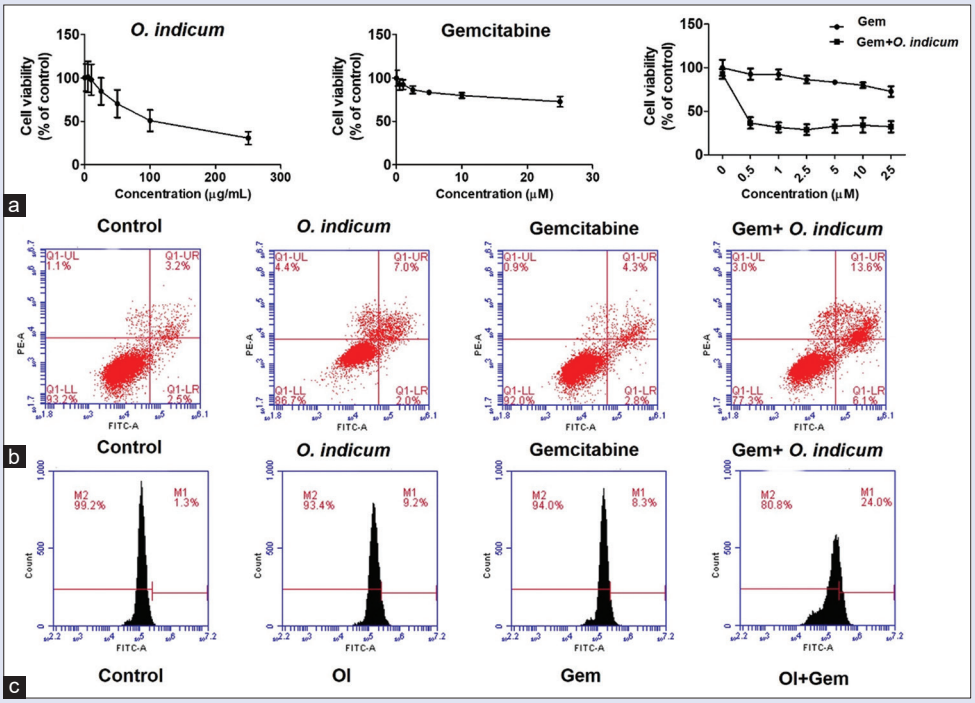


Figure 6: *O. Indicum* in combination with gemcitabine, anticancer drug, effects on cytotoxicity, apoptosis, and ROS formation in CCA cells. (a) KKU-100 cells were exposed to *O. Indicum* (0-250 μM), gemcitabine (0-25 μM), and in combination of *O. Indicum* (50 μg/mL), gemcitabine (0-25 μM), for 24 h and proliferation was investigated using a SRB assay. (b) KKU-100 cells were incubated with *O. Indicum* (50 μg/mL) in combination with gemcitabine (10 μM), stained with Annexin-V FITC/PI solution, apoptosis was measured by flow cytometry. (c) KKU-100 cells were treated, stained with DCF-DA solution, ROS formation was measured by flow cytometry. Mean ± SE ($n = 3$). *$P < 0.05$

of CCA patients.[17] Hence, the strategies of clinical management that increase the gemcitabine efficacy in battling CCA are immediately desired. *O. indicum* combined with gemcitabine presented the greatest effect on the stimulation of CCA cell death, induction of apoptosis by

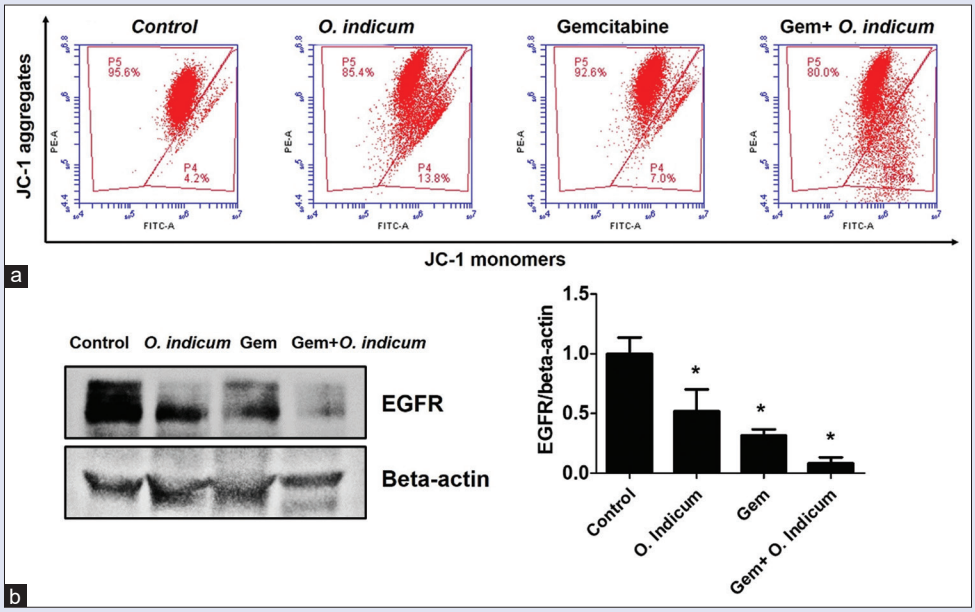


Figure 7: *O. Indicum* effects on mitochondrial function and EGFR protein expression in KKU-100 cells. (a) KKU-100 cells were incubated with *O. Indicum* (50 μg/mL) in combination with gemcitabine (10 μM), stained with JC-1 solution, mitochondrial function was measured by flow cytometry. (b) KKU-100 cells were incubated with *O. Indicum* (50 μg/mL) in combination with gemcitabine (10 μM), and EGFR expression was detected by Western blotting. Mean ± SE ($n = 3$). *$P < 0.05$

activation of ROS formation and reduction of mitochondrial function correlated with EGFR suppression. In the further, the *O. indicum* effects will be a candidate for anticancer therapies with or without standard chemotherapeutic agents remains to be seen. The study of the dose of toxic effect *in vivo* study is must be needed, the studies in clinical must be developed very thoughtfully. Hence, the discovery the dosage of *O. indicum* will be critical for CCA treatment.

CONCLUSION

The *O. indicum* leaf extracts display a powerful cytotoxic effect, apoptotic action and antimetastatic activity. Our data obtains a basis for the management and application of *O. indicum* leaf extract to be further examined and used for treating/preventing CCA.

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Conflicts of interest

There are no conflicts of interest.

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