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Proapoptotic activities of *Oroxylum indicum* leave extract in HeLa cells

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ABSTRACT

Objective: To examine the proapoptotic properties of *Oroxylum indicum* methanol extract on cervical cancer cells.

Methods: Methylene blue assay was used to determine the IC_{50} value of the extract. Western blotting assays were done to analyze the expression of HPV oncoproteins (HPV18 E6 and E7) and apoptotic molecules (caspase-3 and caspase-8). Reverse transcriptase PCR assays were performed to determine genetic alteration of tumor suppressors *p53* and *pRb* and apoptosis markers *Fas* and *FasL*. Enzyme-linked immunosorbent assay (ELISA) was done to determine the expression of cytokine levels (IL-6 and IL-12).

Results: The determination of IC_{50} value indicated a higher anti-proliferative activity of the extract compared to cisplatin. After 24 hours of treatment, Western blot analysis showed that treated HeLa cells exhibited a significant down-regulation of HPV18 oncoproteins E6 and E7, and a significant induction of caspase-8 and caspase-3 activation level. Meanwhile, the mRNA expressions of *p53*, *pRb*, *Fas* and *FasL* were significantly upregulated in treated cells. Moreover, ELISA showed an increased IL-12 and decreased IL-6 production after *Oroxylum indicum* treatment.

Conclusions: The methanol extract of *Oroxylum indicum* has an anti-proliferative activity and proapoptotic potential. It induces localized-immunity improvements by altering cytokine production in HPV-positive cervical cancer cells.

1. Introduction

Cervical cancer is the second most common cancer among women worldwide[1]. Approximately, 90% of it is etiologically caused by a persistent infection of human papillomavirus (HPV). HPV 16 and HPV 18 are the most common types identified in the multistage carcinogenesis of reported cervical cancer[2,3].

The main mechanism of HPV oncogenicity is by modulating apoptosis. In this manner, E6 and E7 oncoproteins of HPV act cooperatively by interfering the functions of cellular tumor suppressor proteins, p53 and retinoblastoma protein (pRb) respectively. HPV oncoproteins also avoid apoptosis by suppressing the caspase activation, a key element in intrinsic and extrinsic apoptosis pathway. Despite the deregulation of apoptosis, chronic inflammation initiated by the persistent HPV infection has been implicated with the development of cervical cancer[4]. HPV-induced chronic inflammation is closely associated with the imbalance secretion of pro-inflammatory cytokines such as

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interleukin-6 (IL-6), and anti-inflammatory mediators such as IL-12[5]. Due to the adverse effects caused by chemically synthetic drugs, the development of new anticancer drugs originated from natural products has drawn scientist's attention[6]. *Oroxylum indicum* (*O. indicum*), a medium-sized tree that belongs to the family of Bignoniaceae, possesses variety of medicinal importance[7]. Previously, *O. indicum* extract showed a high cytotoxic effect on HeLa cells by showing the prominent characteristic features of apoptotic cells such as cytoplasmic membrane blebbing, nuclear fragmentation and apoptotic bodies[8-10]. These findings suggested proapoptotic ability of *O. indicum* extract against cancer cells. However, the fundamental molecular mechanism remained poorly understood. Therefore, we investigated the effects of *O. indicum* methanol extract on the expression of pro-apoptosis molecule, viral oncoproteins, and cytokine production to determine the underlying mechanism related to the apoptosis induction effect of the extract.

2. Materials and methods

2.1. Preparation of methanol extract

The leaves of *O. indicum* were collected at Tumpat, Kelantan, Malaysia. The voucher specimen (PIIUM 0276) was identified by Dr. Shamsul Khamis from Universiti Kebangsaan Malaysia and deposited in the herbarium. Dried plant leaves were grinded and extracted with petroleum ether and methanol using soxhlet apparatus as described by Zazali *et al*.[10].

2.2. Cell lines and culture conditions

Cervical cancer cell line (HeLa) was obtained from American Type Culture Collection. Cells were cultured in cell culture flasks with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were incubated in humidified incubator supplemented with 5% (v/v) CO_2 at 37 ℃.

2.3. Anti-proliferative assay

HeLa cells were seeded in 96-well plates (5×10^4 cells/well) and were maintained at 37 ℃ in humidified incubator supplied with 5% (v/v) CO_2. After 80% confluence, the cells were treated with methanol extract of *O. indicum* at final concentration of 0.39-99 μg/mL. The cell viability was determined through methylene blue assay (MBA) after 72 hours of stimulation[10]. Cisplatin was used as a positive control. The half maximal inhibitory concentration (IC_{50}) was chosen for subsequent experiments.

2.4. Cells treatment

HeLa cells were seeded in 12-well plate (3×10^6 cells/mL) and were maintained at 37 ℃ in humidified incubator supplied with 5% (v/v) CO_2. By using the IC_{50} value obtained from anti-proliferative assay, cells were treated with the corresponding value of concentration for 24 h. Untreated cells were used as a control. Pellet of the cells was harvested after centrifugation at 1 500 rpm for 5 min.

2.5. Western blot analysis

Cells were lysed by adding 100 μL of radioimmunoprecipitation assay buffer to the cell pellet and incubated overnight at 4 ℃. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (120 μg protein per well) and transferred to polyvinylidene fluoride membrane (0.2 μm). The membrane was incubated with primary caspase-8 (1:500 dilution), caspase-3 (1:500 dilution), HPV18 E6 (1:500 dilution) and HPV18 E7 (1:500 dilution) overnight at 4 ℃, followed by incubation with a secondary antibody (1:2 000 dilution) (Santa Cruz, USA) at room temperature for 2 h. The protein of interest was visualized using an enhanced chemiluminescence system (Nacalai Tesque, Japan). β-actin expression was used as an internal control.

2.6. Reverse transcriptase-polymerase chain reactions (RT-PCR)

The total RNA of cells was extracted using RNAeasy® Plus Mini Kit (Qiagen, Texas, USA) and the procedure was carried out according to manufacturer's instruction. The complementary DNA (cDNA) was then synthesized by using Omniscript® Reverse Transcription kit (Qiagen, USA). PCR was performed using Taq DNA polymerase and the following primers in Table 1. The PCR for *p53* and *pRb* was performed under different conditions. For *p53*, it was performed at 94 ℃ in 5 min for the first denaturation, 35 cycles at 94 ℃ in 30 s for denaturation, 60 ℃ in 60 s for annealing, 72 ℃ in 60 s for extension, and another 10 min at 72 ℃ for the final extension. While for *pRb*, it was performed at 94 ℃ in 5 min for the first denaturation, 40 cycles in 60 s at 94 ℃ for denaturation, 30 s at 53 ℃ for annealing, 45 s at 72 ℃ for extension, and 7 min at 72 ℃ for the final extension. For *Fas* and *FasL*, the amplification was performed at 94 ℃ for 3 min for denaturation, cycle denaturation for 10 s at 92 ℃, annealing for 30 s at 55 ℃ and extension at 72 ℃ for 60 s followed by 40 cycles of amplification. *β-actin* was used as an internal reference gene to normalize the expression of the target genes.

Table 1. Primer sequences for *p53*, *pRb*, *Fas* and *FasL*.

Gene	Forward primer	Reverse primer	Size (bp)
p53	5'-TTGGGAGTAGATGGAGCCT- 3'	5'-AGAGGCAAGGAAAGGTGATA- 3'	313
pRb	5'-GTTATCAATACCACCAGGGAG-3'	5'-CAAATCTGAAACACTATAAAGCC- 3'	443
Fas	5'-CAGAACTTGGAAGGCCTGCATC- 3'	5'-TCTGTTCTGCTGTGTCTTGGAC- 3'	660
FasL	5'-ACACCTATGGAATTGTCCTGC-3'	5'-GACCAGAGAGAGCTCAGATACG-3'	311
β-actin	5'-AAATCTGGCACCACACCTTC-3'	5'-CCATCTCTTGCTCGAAGTCC-3'	432

2.7. Cytokine ELISA

A total of 3×10^6 cells/mL of HeLa cells were seeded in a 12-well plate and incubated at 37 ℃ in humidified incubator supplemented with 5% (v/v) CO_2. Cells were treated with and without the presence of stimulant, TNF- α and *O. indicum* extract. For set of stimulants, 10 ng/μL of TNF- α was added for 1 h prior to treatment. The same IC_{50} value obtained from anti-proliferative assay was used to treat the cells for the next 24 h after adding the stimulant. Untreated cells were used as a control. After 24 hours of treatment, supernatant was collected after centrifugation at 1 500 rpm for 5 min. The production of IL-6 and IL-12 proteins in supernatant was determined by ELISA using human IL-6 ELISA MaxTM Set Deluxe Kits (BioLegend, USA) and human IL-12 ELISA MaxTM Set Deluxe Kits (BioLegend, USA), according to manufacturer's instruction.

2.8. Statistical analysis

The data obtained from independent experiments (n=3) were presented as the means ± standard deviation (mean±SD). The independent t-test was used to analyse the differences between treated and control groups for Western blot analysis and RT-PCR. Meanwhile, one-way ANOVA was used to analyse data collected from ELISA. Probability values P<0.05 were considered statistically significant.

3. Results

3.1. Anti-proliferative activity of O. indicum

The anti-proliferative activity of *O. indicum* extracts on HeLa cells was measured based on IC_{50} value obtained from MBA and as presented in Figure 1. Treatment with lower IC_{50} values indicated a higher anti-proliferative action. Results showed that the extract possessed inhibitory effects on HeLa cells proliferation with lower IC_{50} [(6.25±1.06) μg/mL] compared to cisplatin [(9.75±1.15) μg/mL].

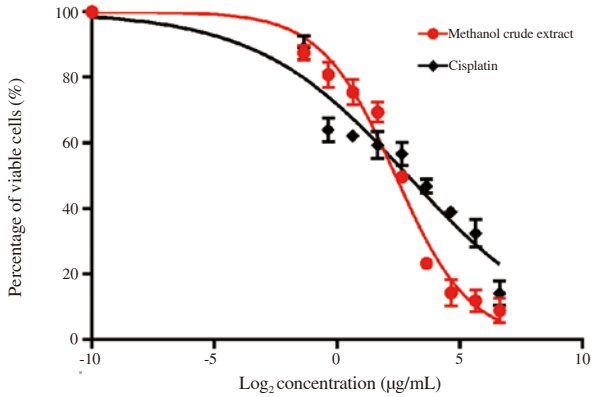

Figure 1. Anti-proliferative effect of *Oroxylum indicum* methanol extract and cisplatin on HeLa cells analysed by methylene blue assays after 72 h.

3.2. Protein expression by Western blot analysis

Western blotting was used to study the potential changes induced by *O. indicum* treatment in treated HeLa cells. Results obtained from the analysis showed that treatment with methanol extract of *O. indicum* was able to significantly reduce the expression of HPV18 E6 (Figure 2a) and E7 (Figure 2b) oncoproteins through the protein detection with expected size 16 kDa and 21 kDa, respectively. Meanwhile, Western blotting analysis also demonstrated a significant upregulation of caspase-3 (Figure 2c) and caspase-8 (Figure 2d) activation after 24 hours of treatment.

3.3. mRNA expression analysis by RT-PCR

The mechanism of apoptosis in treated-HeLa cells was further examined through the inspection of essential proteins in controlling the apoptosis pathway *via* detection of their respective mRNA level by RT-PCR. Based on the result obtained, *O. indicum* methanol extract treatment was able to significantly elevate the transcriptional level of both tumor suppressors *p53* (Figure 3a) and *pRb* (Figure 3b), and apoptosis-related molecules *Fas* (Figure 3c) and *FasL* (Figure 3d) by comparing the mRNA level in untreated and treated HeLa cells.

3.4. Cytokine production analysis by ELISA

ELISA analysis was used to detect the production of IL-12 and IL-6 in the supernatant of treated and untreated cells. An increased IL-12 production was observed in the treated/unstimulated cells (Figure 4a). Upon TNF- α stimulation, the IL-12 production did increase in untreated/stimulated cells and treated/stimulated cells compared to untreated/unstimulated cells (Figure 4a). On the other hand, a significant decreased in IL-6 production was observed upon treatment with *O. indicum* extract (Figure 4b). After TNF- α stimulation, the production of IL-6 was significantly increased compared to untreated/unstimulated cells. However, the production of the cytokine significantly decreased in treated/unstimulated and treated/stimulated cells (Figure 4b). The overall results suggest that the methanol extract of *O. indicum* is capable in inducing and decreasing the production of IL-12 and IL-6, respectively.

4. Discussion

As cancer has become a leading cause of death worldwide, an advancement in the treatment and control of cancer progression is a must. A major disadvantage brought by synthetic drugs increases the demanding needs to explore the potential of traditional medicine for the development of new anti-cancer drugs.

In this regard, *O. indicum* extract is one of the possible candidates with promising potentials to be developed as a new plant-based anti-cancer agent. A number of studies have reported that *O. indicum* was able to demonstrate anti-proliferative effects against nasopharyngeal cancer[11], human breast cancer cells[12], leukemia cells[13], colorectal carcinoma cells[14] and cervical cancer cells[8-10]. In this study, we examined the efficiency of methanol extract of the plant since

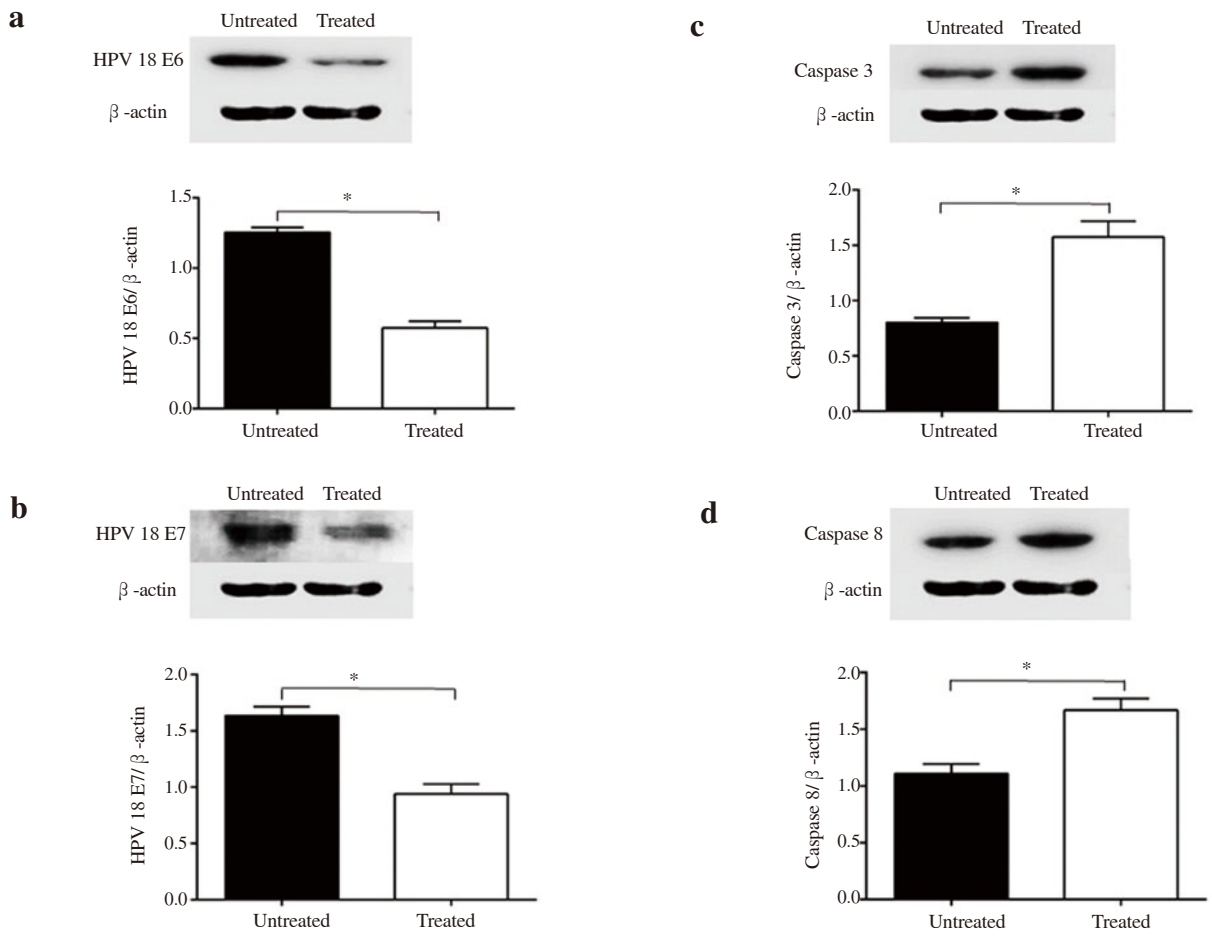

Figure 2. Expression of HPV oncoproteins and apoptotic molecules in HeLa cells treated with *Oroxylum indicum* extract. (a) HPV18 E6 (b) HPV18 E7 (c) caspase-3 (d) caspase-8. Detection of proteins was determined by Western blot analysis after 24 h. β -actin was used as an internal control. Graph represents the mean relative index for expression level of targeted proteins in treated and untreated HeLa cells. The signal density was normalized with β -actin. Values are expressed as mean ± SD from three independent experiments. $^{*}P < 0.05$ is considered significant.

previous studies proposed that the methanol extract showed greater cytotoxic potential compared to an aqueous extract in selected tested cell lines[10,15]. Herein, our present study also confirmed that this extract significantly possessed better inhibitory effects on HeLa cells proliferation [IC_{50}: (6.25±1.06) μg/mL] with a lower dose compared to cisplatin [IC_{50}: (9.75±1.15) μg/mL]. This similar trend of anti-proliferative activity was also observed previously and indeed, methanol extract of *O. indicum* leaves has selectively promoted death in cervical and breast cancer cells without interfering the proliferation of normal cells[10,16]. These findings were closely related to the flavonoid compound extracted in methanol as this polar solvent was able to extract the highest flavonoid content from *O. indicum* compared to other solvents such as petroleum ether, benzene and chloroform by referring to the previous phytochemical studies[9,17]. It has been reported that *O. indicum* contains high flavonoid bioactive compound with apoptosis inducer properties such as chrysin, baicalein and oroxylin A[18–20]. Therefore, the aim of this present study was to investigate the effect of *O. indicum* methanol extract as an apoptosis inducer and the association of the effects with apoptosis pathway in HeLa cells treated with the extract.

The cytotoxic and apoptotic effects of *O. indicum* treatment were supported by decrease of E6 and E7 viral oncoproteins expression in HeLa cells. These oncoproteins were directly associated with the interferences of p53 and pRb functions, respectively. E6 functions were best-characterized by its ability to promote ubiquitin-dependent degradation of p53, a crucial protein to activate the expression of various regulators needed for the cell cycle arrest and apoptosis. The degradation of p53 makes it not available to act as "stop" signal for cell division and thus leads to the genomic instability[21]. Meanwhile, E7 interferes the action of pRb by associating with its hypophosphorylated form and binds to E2F. The binding results in the release of E2F from the pRb-E2F complex. E2F protein subsequently activates essential genes required for the DNA replication and cell cycle progression[22]. Therefore, a significant reduction level of E6 and E7 expressions in treated HeLa cells explained the restoration of both tumor suppressor gene expressions. The expression of *p53* and *pRb* at transcriptional level was found to be increased in treated cells compared to untreated cells. This finding also proposed the involvement of *O. indicum* treatment with the cellular processes which are responsible to regulate the gene expression of *p53* and *pRb*. Hence, the down-regulation of these key oncogenes could be the contributing factor to enhance the sensitivity of HeLa cells in inducing apoptosis when treated with *O. indicum* methanol extract. Similarly, these significant changes in HeLa cells

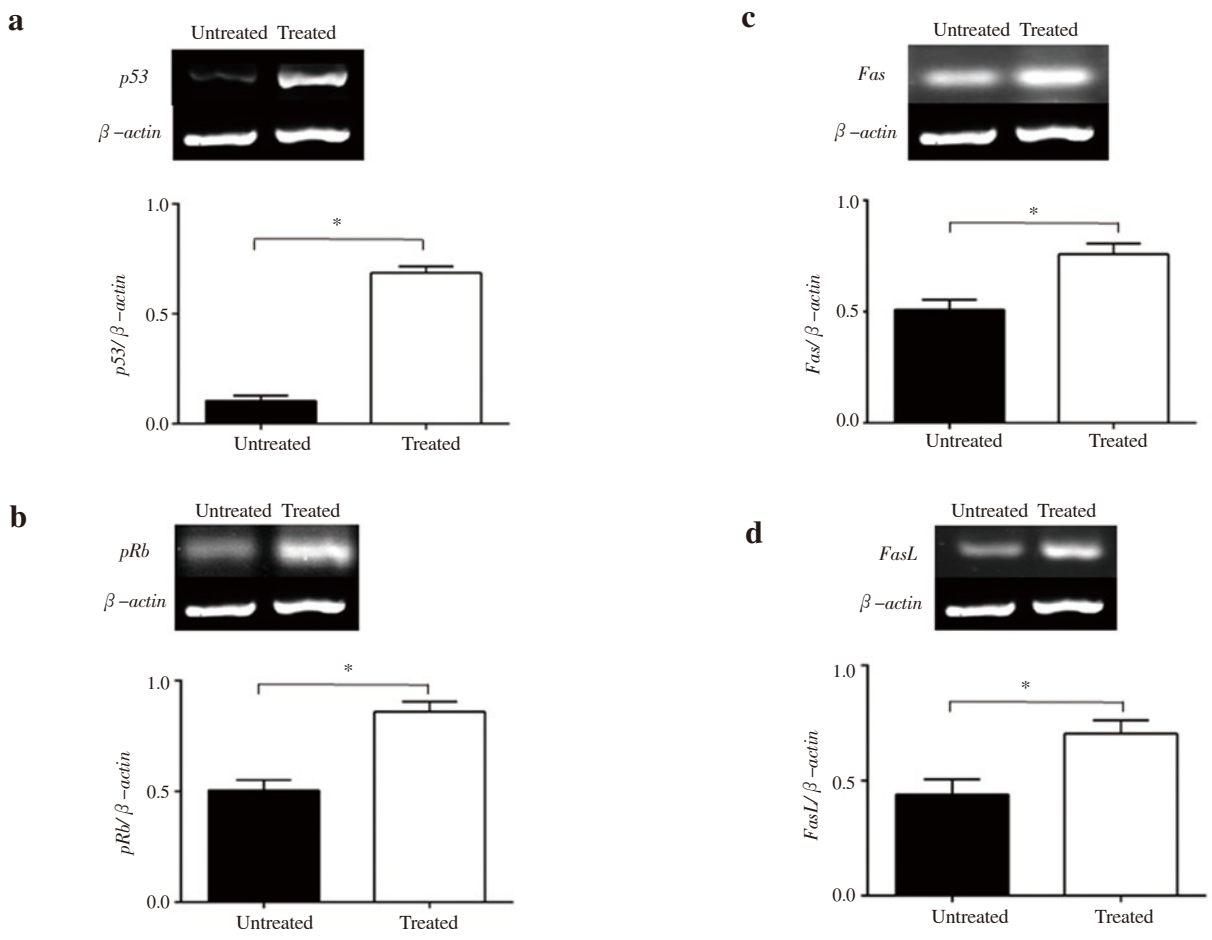

Figure 3. mRNA expression of tumor suppressor proteins and apoptotic molecules in HeLa cells treated with *Oroxylum indicum* extract. (a) *p53* (b) *pRb* (c) *Fas* (d) *FasL*. Detection of mRNA level was determined by RT-PCR analysis after 24 h. *β –actin* was used as an internal control. Graph represents the mean relative index for expression level of targeted genes in treated and untreated HeLa cells. The signal density was normalized with *β –actin*. Values are expressed as mean ± SD from three independent experiments. $^{*}P < 0.05$ is considered significant.

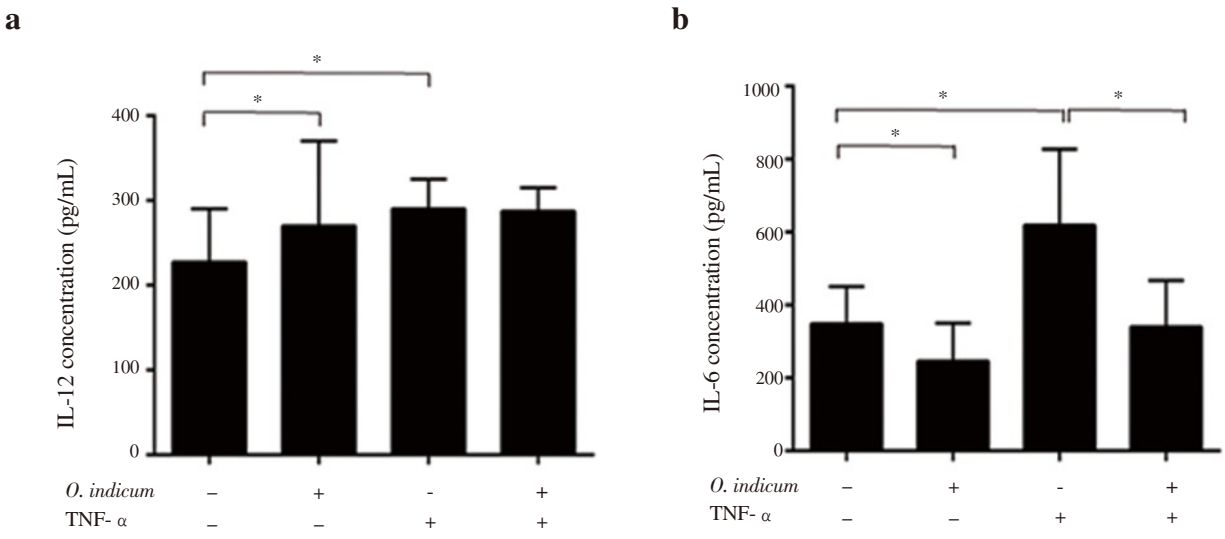

Figure 4. Expression of cytokine level in HeLa cells. (a) IL-12 (b) IL-6. Values are expressed as the mean ± SD from three independent experiments. $^{*}P < 0.05$ is considered significant.

were also observed in the cells treated with extract of *Curcumin longa* and *Phyllantus emblica*[23,24].

On the other hand, membrane death receptor-mediated apoptosis plays a significant role in extrinsic pathway of apoptosis. Trimerization of Fas, after binding to its death ligand, Fas ligand (FasL) will eventually trigger the extrinsic apoptosis pathway. This event promotes the recruitment of Fas-associated death domain and procaspase-8 and leads to the formation of death-inducing signalling complex[25]. Oligomerization occurs at the complex and causes active caspase-8 to be released. Subsequently, caspase-8 induces activation of caspase-3 as its main target. Caspase-3 is an executional caspase that is capable in cleaving a variety of cellular substrate and proteins and leads to the DNA damage and the irreparable damage which eventually causes apoptosis induction[26].

In HPV-related cervical cancer, low levels of Fas, FasL and caspase-8 were detected as a result of inhibition by HPV oncogene proteins in order to facilitate the evasion of cancer cells from host immune system[27]. It was found that HPV E5 protein down-regulated the mRNA expression of Fas and FasL[28]. In addition, E6 and E7 hinder the activation of caspase-8 by degrading the procaspase-8[29]. In this present study, the expressions of *Fas*, *FasL*, caspase-8 and caspase-3 were shown to be remarkably upregulated after *O. indicum* treatment. Increment in the expression of *Fas* and *FasL* significantly restored the activated caspase-8 level in treated HeLa cells. The high expression level of caspase-8 as the downstream target in death receptor-mediated pathway explained the aggregation of caspase-3 activation in cervical cancer cell lines after treatment period. Moreover, caspase-8 also can act as a molecular linker bridging between extrinsic pathway and intrinsic pathway of apoptosis by cleaving the Bcl-2 family member Bid. Bid then will bind to a proapoptotic protein, Bax, which results in the release of cytochrome c as an initiator for mitochondrial pathway[30]. Both pathways resulted in the cleavage of procaspase-3 to release the active form of caspase-3, elucidating the upregulation of caspase-3 level in the cells with *O. indicum* treatment. These findings strongly suggested the proapoptotic function of *O. indicum* treatment by inducing death receptor-mediated apoptosis pathway by elevating the expression of *Fas* and *FasL* and concurrently inducing mitochondria-mediated pathway through the activation of caspase-8 and caspase-3, similar to the effect of ginsenoside Rh2, a ginseng saponin, on treated HeLa cells[31]. The upregulation of these molecules has been targeted by available anti-cancer drugs and become one of the targets in finding an alternative cancer therapy[32].

As for cytokines, their release has played an important role in malignant transformation, proliferation, survival and other neoplastic mechanisms[33]. Two cytokines were chosen for this study, IL-12 and IL-6 as both cytokines have been shown to serve as potential biomarkers to assess the risk of invasive cancer and metastasis[34]. Low level of IL-12 was detected in cancer cells due to the inhibition action of immunosuppressive factors such as interleukin 10 (IL-10) and prostaglandin E2[35]. In this recent study, elevation of IL-12 level was shown after *O. indicum* treatment. Upregulation of IL-12 will provide an effective anti-cancer immune response by enhancing the synthesis of IFN- γ, a cytokine responsible in stimulating monocytes, fibroblast and endothelial cells to induce the secretion of anti-angiogenic chemokines[34]. IL-12 secretion also may provide an anti-tumor immunity by induction of Th1 adaptive immunity and cytotoxic T lymphocyte responses against cancer cells[36]. Overexpression of this cytokine significantly reduced the tumor growth rate in melanoma, thricostatin-A mammary adenocarcinoma, glioma, and C26 colon carcinoma cells[35]. Therefore, the ability of *O. indicum* to elevate IL-12 is a potential therapeutic strategy for cancer management as it provides strong protection against tumor angiogenesis and progression of metastasis.

In contrast, IL-6 production was reported to be reduced in treated cells. Treatment with *O. indicum* extract can strongly suppress the IL-6 production. Even under stimulated conditions where the IL-6 was produced almost two times higher than unstimulated cells, the extract was still capable to decrease the IL-6 production to a similar level with treated/unstimulated cells (*O. indicum* alone). IL-6 is the most clinically relevant and studied cytokines in both chronic inflammation and genital malignancies, including cervical cancer[37]. IL-6 is the most decontrolled cytokine expressed in cancer environment and its overexpression was observed in many forms of solid tumor[38]. The pivotal function of IL-6 in promoting metastatic progression and tumor growth is the worst detrimental effect from its pro-angiogenic activities. The vascular endothelial cell migration leads to the propagation of existing blood vessels that allows the tumors to possess their own blood supply and to be characterized by endothelial tip sprouting at the adjacent areas[39]. This cytokine is also capable of modulating the activities of tumor-related immune cells by inhibiting monocytes and dendritic cells differentiation[40]. As we correlate with the evidence reported by previous studies regarding the function of IL-6, we can deduce that the extract is capable in reducing the carcinogenic IL-6 in cervical cancer cells. The result revealed a good approach as the crude extract does have some anti-cancer properties in HeLa cells.

Conflict of interest statement

We declare that there is no conflict of interest.

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