ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: https://ijrps.com

# Baicalein-rich Fraction of *Oroxylum indicum* Leaves induces Apoptosis by Repressing E6 and E7 Expression in HPV-associated Cervical Cancer Cell Lines

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*Article History:*

Received on: 02.10.2018
Revised on: 17.12.2018
Accepted on: 19.12.2018

*Keywords:*

Apoptosis,
Baicalein,
E6,
E7,
*Oroxylum indicum*,
p53,
pRb

ABSTRACT

*Oroxylum indicum* (*O. indicum*) has been implicated as a promising anti-cancer agent for cancer treatment including cervical cancer. It has been shown that this plant was able to inhibit the proliferation of cancer cells by acting as anti-human papillomavirus (HPV) and an apoptosis inducer. The therapeutic anti-cancer properties of *O. indicum* is strongly postulated due to its major chemical constituents such as chrysin, oroxylin A and baicalein. In this present study, the baicalein-rich fraction was extracted to elucidate its anti-cancer activity against cervical cancer cells, SiHa (HPV 16 positive) and HeLa (HPV 18 positive) cells. Utilizing preparative thin layer chromatography (PTLC) (n-hexane: ethyl acetate; 50:50), this fraction was prepared from the methanolic crude extract of *O. indicum* and subjected to high-performance liquid chromatography (HPLC) for baicalein quantitation. Biological activities of this fraction were tested using methylene blue assay and western blot to observe the expression of HPV oncoproteins; E6 and E7, and the tumour suppressor proteins; p53 and pRb. From the $IC_{50}$ values obtained for both SiHa and HeLa cell lines, the baicalein-rich fraction was the most potent compound compared to cisplatin and the methanol crude extract of *O. indicum*. After 24 hours treatment period, western blot analysis showed that MCE- and BRF-treated SiHa and HeLa cells exhibited anti-HPV effects by down-regulating the expression of E6 and E7 which explain the induction of apoptosis through the up-regulation of p53 and pRb. The overall data suggested that baicalein-rich fraction from *O. indicum* has anti-proliferative activity and strongly induced apoptosis in treated cervical cancer cell lines *via* E6 and E7 repression. Therefore, baicalein isolated from *O. indicum* can be further exploited as a potential anti-cancer candidate for cervical cancer treatment.

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ISSN: 0975-7538
DOI: https://doi.org/10.26452/ijrps.v9iSPL2.1751

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## INTRODUCTION

Persistent infection of human papillomavirus (HPV) shows a strong association with cervical cancer development, a life-threatening disease which accounts as the third leading cause of death among women worldwide (American Cancer Society, 2016). The high-risk types of HPV especially HPV 16 and HPV 18 are responsible for approximately more than 50 % of multistage carcinogenesis of cervical carcinoma reported worldwide including Malaysia (Bosch *et al.,* 1995; Petry, 2014; Raub *et al.,* 2014). Previous biological studies have identified E6 and E7 as the major viral oncoproteins that play a key role in HPV-

associated cervical carcinoma tumorigenesis (Bathula and Rangaswamy, 2015). Both HPV proteins perform the carcinogenic activities by modulating the products of tumour suppressor genes, hence causing the deregulation of cellular proliferation (Narisawa-Saito and Kiyono, 2007).

HPV E6 protein targets p53 tumour suppressor protein, a negative regulator of cell cycle, and facilitates its degradation through the ubiquitin-mediated pathway. Once activated, p53 rapidly initiated DNA repair and apoptosis signalling pathway in response to the genotoxic stress and DNA synthesis impairment (Mellert and Espinosa, 2013). Moreover, p53 directly participate in the intrinsic pathway of apoptosis which leads to the permeabilization of mitochondrial outer membrane through the interaction with multiple members of Bcl-2 family and caspase activation (Fridman and Lowe, 2003). Deprivation of p53 protein hinders the growth detention and apoptosis process as p53 functions in inducing cell cycle arrest and initiate cell death in the presence of cytotoxic damage (Accardi *et al.,* 2011). The oncogenic properties of HPV E6 protein translated from both HPV 16 and HPV 18 genomes were demonstrated *in vitro* by the formation of an E6/wild-type p53 complex with the help of E6-associated protein ligase (E6AP) (Scheffner *et al.,* 1990). Additionally, p53 activation is also inhibited by E6 viral oncoprotein through the blockage of the alternate reading frame p14 (p14/ARF) pathway (Khoronenkova and Dianov, 2011) and alternatively with the aid of histone acetyltransferase, hADA3 (Kumar *et al.,* 2002).

Another major HPV oncoprotein, HPV E7, is a necessary co-factor for cellular transformation and enhances viral pathogenesis (Boulet *et al.,* 2007). Low frequency of E7 expression was able to immortalize primary human epithelial cells (Longworth and Laimins, 2004; Morandell *et al.,* 2012). The anti-apoptotic activities of E7 were shown through the deregulation of cell cycle control by targeting another product of the tumour suppressor gene, retinoblastoma protein (pRb). Without growth-promoting signals, pRb binds to the E2F transcription factor to stop E2F from stimulating its target gene, which is required for the cell cycle entrance to S phase leading to cellular proliferation. E2F will be released from the binding in the presence of growth-promoting signals through the phosphorylation of pRb, a process mediated by cyclin-dependent kinase (CDK) activity (Godefroy *et al.,* 2006). In HPV-infected cells, E7 performs an analogous function and cause the phosphorylation of pRb and disrupt the pRb/E2F complexes. The disruption releases active E2F proteins and allows the transcription of essential proteins for cell cycle progression (Ganguly and Parihar, 2009). E7 is also capable to hinder apoptosis in HPV-associated cancer cells by upregulating the inhibitor of apoptosis (IAP) protein and by suppressing the activation of caspase-8, a key role caspase in the extrinsic pathway of apoptosis (Petry, 2014). This carcinogenic action of HPV oncoproteins induced genome instability and lead to cancerous cell development with uncontrolled cell propagation. Therefore, the down-regulation of E6 and E7 proteins may be a promising therapeutic approach with the restoration of p53 and pRb expression to stabilize the cell proliferation/cell death balance.

As to date, chemotherapy and radiotherapy are still the main strategies to combat cervical cancer. However, the major side effects introduced by these treatments were the biggest drawback and aroused medical concerns among healthcare practitioners (Torre *et al.,* 2016). This hindrance drove the interest of current researchers to investigate the anti-cancer potentials of among plants as they are considered as less toxic with fewer side effects (Bishayee and Sethi, 2016).

*Oroxylum indicum*, a traditional medicinal plant that possesses a long history of use in Ayurveda and Chinese medicine, have drawn the attention with its breakthrough potential as an anti-cancer agent (Deka *et al.,* 2013). Several *in vitro* studies have shown that *O. indicum* exerted positive cytotoxic effects on various cancer cells. Treatment with the plant extract increased cellular DNA fragmentation and inhibited the migration of metastatic breast cancer cells (MDA-MB-231) (Kumar *et al.,* 2012), induced cell cycle arrest by reducing the cell proportion in G1 phase in promyelocytic leukaemic cells (HL-60) (Kumar *et al.,* 2007), and promoted the development of apoptosis characteristic features such as nuclear fragmentation and cytoplasmic membrane blebbing in HeLa cells (Moirangthem *et al.,* 2013; Zazali *et al.,* 2013). This therapeutic potential is logically hypothesized due to the presence of flavonoid abundance in this plant namely baicalein, oroxylin A and chrysin (Lalrinzuali *et al.,* 2015; Zaveri *et al.,* 2008). Baicalein, a common flavonoid, is also available in other plants such as *Scutellaria baicalensis* and *Scutellaria lateriflora* (Gao *et al.,* 2008; Li and Chen, 2005). Baicalein isolated from these plants was extensively studied to assess its potential as anti-cancer agent against multiple cancer cell lines such as breast (An *et al.,* 2016), colorectal (Kim *et al.,* 2012), hepatoma (Chen *et al.,* 2009), and ovarian (Chen *et al.,* 2013) cancer cells. However, the information regarding the anti-proliferative effect of baicalein isolated from *O. indicum* is still limited. Baicalein from *O. indicum* was shown to exert cytotoxic effect against promyelocytic leukaemic cells (Kumar *et al.,* 2007)

and colorectal carcinoma cells (Lalou *et al.,* 2013). In this study, the therapeutic potential of baicalein from *O. indicum* leaves as a promising anti-cancer agent in HPV-associated cancer was the main focus.

In this present study, the anti-HPV and apoptosis-inducing effects of the baicalein-rich fraction extracted from *O. indicum* were investigated on cervical cancer cells; SiHa and HeLa cell lines with positive HPV 16 and HPV 18. The ability of the fraction in down-regulating the HPV oncoproteins and upregulating the disturbed tumour suppressor proteins were determined with Western blotting analysis.

## MATERIALS AND METHODS

### Plant Sampling

The leaves of *O. indicum* were collected from Tumpat, Kelantan, Malaysia and was identified by a botanist, Dr. Shamsul Khamis from the Faculty of Science and Technology, National University of Malaysia and deposited in the herbarium of the International Islamic University Malaysia (PIIUM 0276).

### Preparation of Baicalein-rich Fraction from *O. indicum*

Grinded dried leaves of *O. indicum* (70 g) were subjected to serial exhaustive extraction in Soxhlet apparatus with methanol after defatted with n-hexane, affording 9.66 g of methanol crude extract (MCE). The resulting extract was subjected to preparative thin layer chromatography (PTLC) to isolate the baicalein-rich fraction (BRF). All analytical grade reagents used in this process were acquired from HmBG, Germany.

Thin layer chromatography (TLC) silica gel 60 $F_{254}$ plates (20 cm x 20 cm) (Merck, Germany) were used for PTLC. Standard solution of baicalein (Sigma-Aldrich, USA) and sample solution of crude extract were spotted in the form of bands at approximately 15 mm from the lower edge of the plates. Linear ascending development was carried out in a TLC glass chamber with n-hexane: ethyl acetate (50:50, *v/v*) as the mobile phase. Developed plates were air-dried at room temperature and were visualized under ultra-violet (UV) lamp at 254 nm wavelength. The $R_f$ value of the band which appeared from the crude extract sample was compared with the band of baicalein and was marked accordingly. The band was scrapped and then eluted using absolute methanol, filtered and dried at room temperature. The powder obtained from this process was kept at 4°C and subjected to HPLC for baicalein quantitation.

### Quantitation of Baicalein by HPLC

Baicalein concentration in MCE and BRF from fraction obtained *via* PTLC were quantified by HPLC. Complete separation of constituents was achieved on a Waters 2690 separations module equipped with 4.6 x 150 mm i.d. Zorbax Eclipse Plus C18 5 µm column. The mobile phase consisted of water, methanol, acetonitrile, and orthophosphoric acid at a ratio of 60:30:38:1, v/v/v/v respectively. The injection volume was 10 µl, and the isocratic method was run for 15 minutes at 1 ml/min flow rate under room temperature and detected at 262 nm.

### Cell Culture

SiHa and HeLa human cervical cell lines carrying the genome of HPV 16 and HPV 18 respectively, and a normal cell line NIH/3T3 were obtained from the American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified Eagle Media (DMEM) (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, USA) and were incubated at 37°C with 5% $CO_2$.

### Anti-proliferative Assay

Approximately $5x10^4$ cells/ml were seeded in each of the 96-well microtiter plates, incubated overnight and treated with various concentrations of the fraction ranging from 0.39 µg/ml to 100 µg/ml for 72 hours. The antiproliferative assay was conducted using methylene blue (Merck, Germany) assay described by Zazali et al. (2013). The absorbance was measured at 660 nm using a microplate reader. The untreated cells worked as a negative control while the cells treated with cisplatin (Tokyo Chemical Industry, Japan) served as positive control. The cells were also treated with MCE, to compare the anti-proliferative activity with BRF. The $IC_{50}$ values obtained for each treatment were used for subsequent experiments. A normal cell, NIH/3T3, an embryonic mouse fibroblast cell was used to test the toxicity for each treatment.

### Cell Stimulation

SiHa and HeLa cells were seeded in 12-well plate with $3x10^6$ cells/ml for each well. The cells were incubated overnight at 37°C with 5% $CO_2$. The cells were then treated with the respective MCE, BRF and cisplatin treatments for 24 hours using the $IC_{50}$ values obtained from the antiproliferative assay. Untreated cells were referred to as negative control.

### Western Blot Analysis

Cells were lysed in radioimmune precipitation assay (RIPA) buffer (Thermo Fisher Scientific,

USA) containing 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0. Protein concentrations were determined using the Pierce Bicinchoninic Acid (BCA) protein assay kit (Thermo Fisher Scientific, USA) and separated through 10% SDS-polyacrylamide gel electrophoresis before transferred to polyvinylidene difluoride (PVDF) membranes. Then, 1% non-fat dry milk (Sunlac, Malaysia) dissolved in TBST buffer containing 25mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3 and 0.05% Tween 20 were used to block the membranes. Primary antibodies specific to E6 and E7 for HPV 16, E6 and E7 for HPV 18, p53 (Santa Cruz Biotechnology, USA) and pRb (Genetex, USA) were applied to the membranes. Lastly, the visualization of adherent proteins was done by incubating the membrane with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology, USA) before projected to the chemiluminescence system (reagents from Nacalai Tesque, Japan).

### Statistical Analysis

Statistical analyses were performed using the one-way analysis of variance (ANOVA) ($p$<0.05) with Bonferroni's post-hoc test for untreated cells versus cells treated with MCE, BRF and cisplatin for each tested antibody. The correlation coefficient was also tested to determine the relationship between the reductions of HPV E6/E7 with the elevation of p53/pRb. Statistical analysis was computed GraphPad Prism 6.0. Each experiment had three independent replicates and the data were expressed as mean ± standard deviation (SD; $n$=3). Statistical significance was considered as follows, $p$<0.05 one-way ANOVA; *$p$<0.05, **$p$<0.01, ***$p$<0.001 for Bonferroni test comparing untreated cells versus cells treated with MCE, BRF and cisplatin for each tested antibody.

## RESULTS AND DISCUSSION

### Isolation of BRF from MCE *via* PTLC

As demonstrated in Figure 1.A, the baicalein peak was the fourth highest among all other peaks (11.15 % peak area) which represented its abundance as one of the major compounds in *O. indicum* leaves. Following isolation with PTLC, baicalein became the highest peak in BRF with another four unidentified compounds with up to 75 % of peak area (Figure 1.B). The content of baicalein in BRF was calculated from the regression equation of the calibration curve (Y=mx+c, where m is the slope and c as the intercept point value). The regression equation of the curves and their coefficient of determination ($R^2$) was mentioned as Y=40104x-491952, 0.9965 (Figure 1.D). From the equation, baicalein concentration was determined as 13 ppm and 26 ppm in MCE and BRF respectively.

Based on a phytochemical analysis done on different parts of *O. indicum*, the presence of flavonoids was strongly positive in the fresh leaves extract followed by the fruits and seeds (Samatha *et al.,* 2012). Since the solubility of flavonoid compounds increased with the increasing polarity of solvents used for extraction, polar solvents namely methanol, ethanol and ethyl acetate were frequently used to extract the compounds (Sultana *et al.,* 2009). Therefore, methanolic extraction was applied in this study to isolate the targeted flavonoid, baicalein from *O. indicum* leaves crude extract. PTLC was used because this technique is simple, affordable and required less expertise compared to other purification methods (Luitel *et al.,* 2010). The technique has been used in several other studies to isolate chemical constituents such as baicalein from *O. indicum* crude extract (Ali *et al.,* 1998; Islam *et al.,* 2010; Luitel *et al.,* 2010).

### Anti-proliferative Activity of MCE and BRF on SiHa and HeLa Cell Lines

The $IC_{50}$ values obtained from each treatment (MCE, BRF and cisplatin) as listed in Table 1, were within the limits of concentration recommended by the United States National Cancer Institute plant screening programme. Plant extracts with $IC_{50}$ of lower than 20 µg/ml are considered as highly cytotoxic (Laishram *et al.,* 2015).

As presented in Figure 2 and Table 1, MCE treatment showed a better cytotoxic effect on HeLa cells with $IC_{50}$ of 6.06 ± 1.75 µg/ml, compared to cisplatin ($IC_{50}$ = 7.00 ± 2.80 µg/ml). This discovery was in agreements with a study by Zazali et al. (2013). In this *in vitro* study, BRF was observed to inhibit the growth and survival of SiHa and HeLa cell lines at $IC_{50}$ of 7.62 ± 1.17 µg/ml and 2.41 ± 1.59 µg/ml respectively. These values were lower than that obtained for MCE and cisplatin treatment which suggests that BRF exhibited more cytotoxic effects compared to other treatments. BRF was more potent than MCE as demonstrated by Badisa et al. (2006) in which the pure compound was found to exert better anti-cancer effects against cancer cell lines compared to the crude extract. This finding proposed that a higher percentage of baicalein in BRF increased the anti-proliferative ability of *O. indicum* in inhibiting cancer cells proliferation providing evidence for baicalein as a potent anti-cancer agent. Moreover, undetectable $IC_{50}$ (>100 µg/ml) for MCE and BRF treatment on the normal cell, NIH/3T3 proposed the cytoselective activity of this natural treatment,

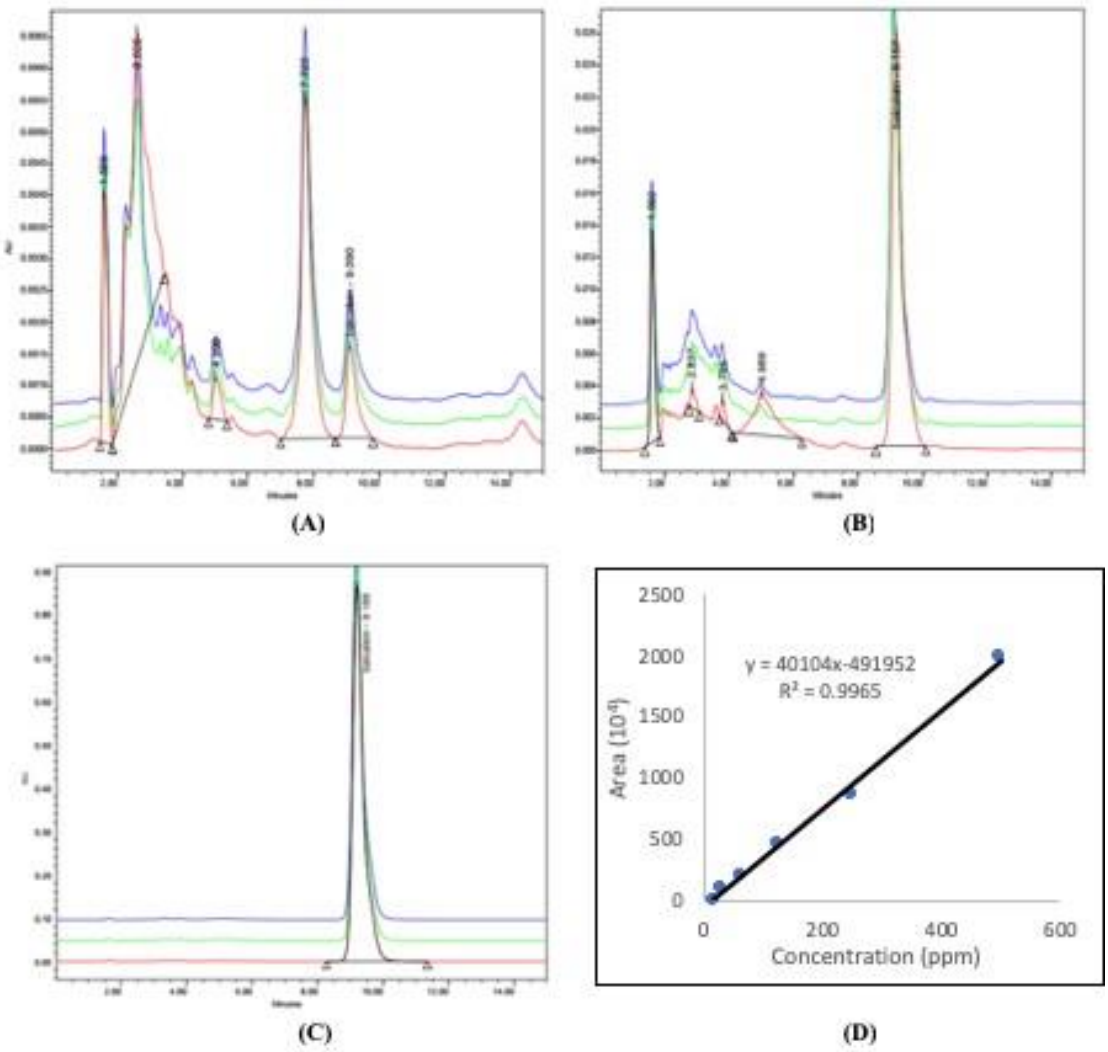


**Figure 1: Chromatogram of baicalein concentration in (A) MCE, (B) BRF, (C) standard baicalein. (D) is the calibration curve of baicalein standard compound**

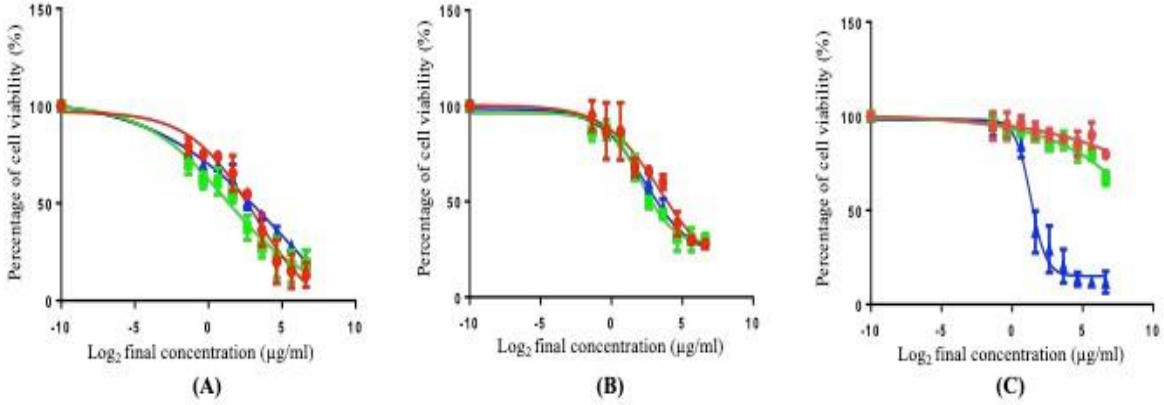


**Figure 2: Anti-proliferative activity of MCE, BRF and cisplatin in (A) SiHa, (B) HeLa and (C) NIH/3T3 cells**

**Table 1: IC50 values for MCE, BRF and cisplatin treatment in SiHa, HeLa and NIH/3T3 cells**

| Treatment | $IC_{50}$ (µg/ml) | | |
|---|---|---|---|
| | SiHa | HeLa | NIH/3T3 |
| MCE | 13.93 ± 1.85 | 6.06 ± 1.75 | NA |
| BRF | 7.62 ± 1.17 | 2.41 ± 1.59 | NA |
| Cisplatin | 8.75 ±1.20 | 7.00 ± 2.80 | 2.30 ± 1.18 |

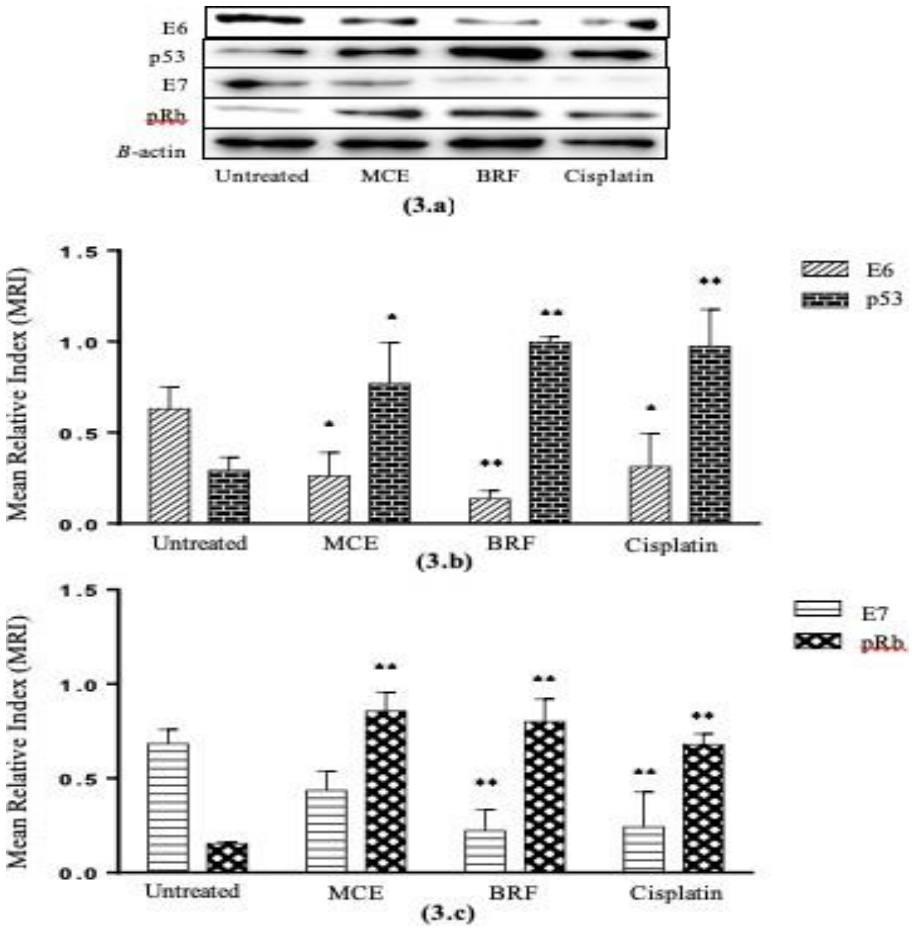


**Figure 3: The suppression of E6 and E7 oncogenes and modulation of tumour suppressor proteins, p53 and pRb in SiHa cells (HPV 16) treated with MCE, BRF and cisplatin**

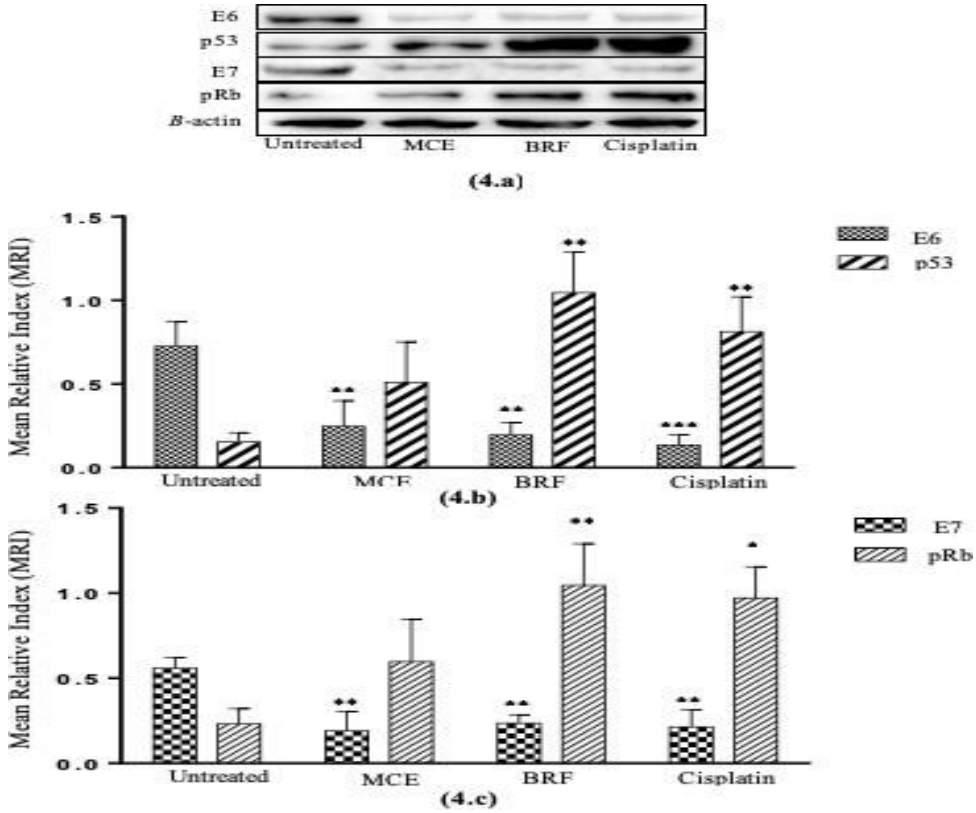


**Figure 4: The suppression of E6 and E7 oncogenes and modulation of tumour suppressor proteins, p53 and pRb in HeLa cells (HPV 18) treated with MCE, BRF and cisplatin**

unlike cisplatin which still exerts a low $IC_{50}$ value (2.30 ± 1.18 µg/ml). The cytoselective capability is an important criterion for the development of an anti-cancer agent to ensure minimal side effects during and after the treatment period (Kumar *et al.,* 2013). This phenomenon is a beneficial advantage of *O. indicum* which strengthen its ability as a safe and selective anti-cancer agent.

**Anti-HPV and Apoptosis-inducing Effects of MCE and BRF on Treated Human Cervical Cancer Cell Lines**

Western blot analysis revealed a substantial reduction of E6 and E7 HPV oncoproteins for both HPV 16 (Figure 3) and HPV 18 (Figure 4) model. The expression of E6 and E7 for both cancer cells were also markedly repressed after 24 hours treatment period with MCE and BRF. Down-regulation of these oncogenic proteins remarkably restored the expression of p53 and pRb in SiHa and HeLa cells. These results suggested that specific suppression of HPV E6/E7 oncoproteins could possibly stimulate the p53/pRb pathway in HPV-associated cervical cancer cells.

The established causative action of E6 and E7 inhibiting the function of tumour suppressor proteins, p53 and pRb respectively allows cancerous progression in infected cervical area (Bathula *et al.,* 2015). The p53 protein functions to induce growth arrest by blocking the cell cycle at the G1 phase and initiates the mitochondria-induced apoptosis pathway once triggered by cellular stress exposure (Mellert and Espinosa, 2013). Meanwhile, pRb acts by blocking the action of E2F proteins, which functions as the transcription factor for the genes required in promoting cell division (Godefroy *et al.,* 2006). Interference action of E7 hindered the pRb action and released the E2F from E2F/pRb complexes, which then provide a sustainable proliferation signalling, as one of the hallmarks of cancer (Yuan *et al.,* 2012).

Despite the mainstream reactions of HPV E6/E7, both oncoproteins also work on additional cellular target to provide a favourable condition for cellular transformation in the host cells. HPV 16 E6 and E7 were able to suppress the expression of type-1 interferons (IFNs) in host cells hence allowing viral evasion from antigen recognition by deteriorating the co-stimulatory signals by inflammatory cytokines (Conesa-Zamora, 2013). Consequently, immune tolerance induction occurred and provide a gateway for the virus to evade the host immune response (Sasagawa *et al.,* 2012). Other than that, both viral proteins were also found to be involved in the inhibition of death receptor-mediated apoptosis by binding itself to the death effector domain/procaspase-8 complex and accelerating their degradation. This tumorigenic action hindered the formation of the death-inducing signalling complex (DISC), a crucial component in the extrinsic pathway of apoptosis (Yuan *et al.,* 2012). E6 and E7 also help in delaying the epithelial cells differentiation to abuse the host's cell DNA machinery as a step to synthesize required proteins for viral assembly. This infected epithelial cells then brought to the rise of dysplasia, warts and tumour (Paavonen, 2007). Thus, overexpression of the major HPV oncoproteins, E6 and E7, resulted in transformed cells resistance against apoptosis, which is another major hallmark of carcinogenesis (Ganguly and Parihar, 2009).

In our study, treatment with BRF and MCE markedly reduce the overexpression level of E6 and E7 and enhanced the level of p53 and pRb proteins in both SiHa and HeLa cells. A better anti-cancer action was shown by BRF as the cells treated with the fraction expressed the lowest HPV 16 E6/E7 and HPV 18 E6 compared to MCE-treated cells. The similar trend was also observed for p53 in SiHa and p53/pRb for HeLa in which the expression level of the tumour suppressor proteins was highest in the cells treated with BRF. In contrast, the repression of HPV 18 E7 in HeLa treated with MCE was slightly better than the BRF-treated HeLa cells. This pattern was also observed for the expression of pRb in SiHa cells in which a slightly higher level of pRb expression was detected in MCE-treated cells compared to BRF. A previous study has shown that methanol extract of *O. indicum* leaves dramatically elevate p53 level in HeLa cells in a dose-dependent manner (Zazali et al. 2013). Baicalein isolated from *Scutellaria radix* also demonstrated anti-angiogenesis effect by elevating p53/pRb signalling pathway as a way to induce cell cycle arrest in the human umbilical vein endothelial cells (HUVECs) (Ling *et al.,* 2011). The p53 level was also remarkably elevated in human hepatoma J5 cells exposed to baicalein treatment (Kuo *et al.,* 2010). Moreover, the expression of component proteins in the p53-mediated pathway apoptosis (reduced expression of Bcl-2 anti-apoptotic protein and upregulation of Bax pro-apoptotic protein) was also observed in mice bearing U14 cervical cancer treated with baicalein confirming the anti-tumour activity of the flavonoid (Peng *et al.,* 2011).

In the correlation analysis to determine the association between the reduction of HPV E6/E7 and the elevation of p53/pRb, all relationships of HPV E6/p53 and HPV E7/pRb a non-significant strong negative relationship in both SiHa and HeLa cells. For SiHa cells, the correlation coefficients ($r$) for HPV 16 E6/p53 and HPV 16 E7/pRb were -0.9255 and -0.7986 respectively. For HeLa cells,

the correlation coefficients (*r*) for HPV 18 E6/p53 and HPV 18 E7/pRb were -0.8642 and -0.7965 respectively. The high value of *r* (r>-0.76) presented from both cells was considered as an excellent non-parallel relationship in the tested pathway (E6/p53 and E7/pRb) indicating that the suppression of E6 and E7 are associated with the restoration of p53 and pRb respectively. Thus, the anti-HPV and apoptosis-inducing activities of BRF isolated from *O. indicum* leaves were proven throughout this study. The action of BRF was considered better than MCE in performing the anti-proliferative activity and in modulating certain apoptosis molecules.

## CONCLUSION

BRF extracted from *O. indicum* leaves attenuated the growth and survival of cervical cancer cells greater compared to MCE without interfering with the proliferation of normal cells. It acted as anti-HPV mediator by down-regulating the expression of HPV 16 and HPV 18 oncoproteins, E6 and E7. BRF also induced cell death in SiHa and HeLa cells through the restoration of p53 and pRb expression level. As a conclusion, we postulated that baicalein from *O. indicum* could be one of the best candidates as a new alternative for anti-cancer agent against cervical cancer, a major life-threatening disease.

## Acknowledgement

This work was supported by the Fundamental Research Grant Scheme (203/PPSK/6171191) provided by the Ministry of Higher Education, Malaysia. The authors thank Universiti Sains Malaysia for the facilities provided to conduct the research and Dr. Soraya Shafawati Mohamad Tahier from Universiti Malaysia Terengganu and Dr. Noor Izani Noor Jamil from Universiti Sains Malaysia for their guide and assistance.

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