



Effect of Methanol and Ethyl Acetate Leaf Extracts of *Osbeckia octandra* L. (Heen Bovitiya) on the Apoptosis and Migration of Human Oral Squamous Cell Carcinoma Cell Lines

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Abstract

Background Cancer has become a leading disease all over the world, and much attention is paid to prevention and management. Oral cancer is common in South Asian countries. Many therapeutic drugs are tested for the treatment of cancer. *Osbeckia octandra* is an endemic plant in Sri Lanka that is commonly used by traditional medical practitioners which has shown to possess anticancer properties.

Objectives The study was conducted to examine the anticancer potential of *Osbeckia octandra* chemical-based leaf extracts using an in vitro cell culture model of human oral squamous cell carcinoma (OSCC) cells and to delineate the possible molecular pathways involved in the process.

Methods Cells were cultured and treated with three concentrations (0.3, 3, and 30 µg/mL) of leaf extracts which were prepared using methanol (OMLE), ethyl acetate (OEALE), and hexane (OHLE) extraction protocols. The cell viability/cytotoxicity and cellular migration potentials were analyzed using standard assays. RNA was extracted from the treated cells and reverse transcribed to synthesize cDNA. Key genes involved in the *BCL2* pathway were analyzed.

Results Cell viability was decreased with increasing concentrations in the OMLE and OHLE showing the dose-dependent impact of two extracts on cancer cell viability. 30 µg/mL indicated the lowest ($p < 0.05$) cell viability while 3 µg/mL concentration in the OEALE reported the highest cytotoxicity. Moreover, 3 and 30 µg/mL of OMLE and OEALE significantly impaired the cell migration in the wound healing assay. Moreover, *BCL2* gene expression was significantly altered by the 30 µg/mL concentration of the same extracts.

Conclusion The results of this study depict the methanol and ethyl acetate extracts of *O. octandra* possess anticancer activities on OSCC cells probably via *BCL2* pathway. Further in-depth studies are warranted to examine the exact bio-active compounds and the underneath mechanisms.

Keywords *Osbeckia octandra* · Oral squamous cell carcinoma · Anticancer · *BCL2*

Introduction

Cancers in the oral cavity, pharynx, and larynx account for the one of the most frequent types of cancers worldwide [1]. Oral cancer is malignancy occurring in the epithelium of the oral cavity, and most of them are oral squamous cell carcinomas (OSCCs). However, it can be regarded as the most frequent type of cancer in certain specific geographical regions, especially in Southeast and South Asia [2]. In the Sri Lanka, it is the most common cancer among males and the National Cancer Registry, reported as 11.6% of all reported cancers as oral cancer is with a high mortality rate of 4 deaths per day [3, 4]. The high prevalence is related to the influence of risk factors, especially tobacco and betel quid chewing which is very prevalent in Indian subcontinent. Despite recent

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advances in diagnosis of oral cancer and management, prognosis remains low. 5-year survival rate of oral cancer is as low as 50% without a change in the last few decades and hence requires innovative management modalities to combat oral cancer beyond surgical/ oncological management [5].

With the advancement of biomedical research, many novel drugs of natural product origin are tested as treatments for cancers. Usage of herbal plants in the investigating of anticancerous effects is a novel trend in the field of cancer research. *Osbeckia octandra* L. which is locally called “Heen Bovitiya” belongs to the family Melastomataceae. It is an endemic plant to Sri Lanka which is widely used in traditional medicine to treat liver-related disorders, especially jaundice [6]. Although the hepato-protective ability of *Osbeckia* has been investigated by many researchers, anticancerous effect of *Osbeckia* has not been deeply evaluated. Recent findings of ours and others have shown promising results regarding the anticancer property of *Osbeckia* water-based leaf extract and crude extracts *via* inducing apoptosis, metastasis, and DNA damage [7]. Objective of the present study was to investigate the anticancer potential of *Osbeckia octandra* chemical-based extracts, namely *Osbeckia octandra* methanol-based leaf extract (OMLE), *Osbeckia octandra* ethyl acetate-based leaf extract (OEALE), and *Osbeckia octandra* hexane-based leaf extract (OHLE) on OSCC cell lines and to identify a potential pathway. An established oral squamous cell carcinoma cell line derived from squamous cell carcinoma of the tongue, YD-10B, was used as the *in vitro* model [8].

Material and Methods

Plant Extract Preparation

Plant samples were collected from an herbarium. The Curator of the Royal Botanical Gardens in Peradeniya authenticated them. (Ref No- *Osbeckia octandra* specimen No UB 89). The fractionation extraction from the leaves was performed as described in the literature earlier [9]. Healthy fresh leaves were cleaned well with distilled water, air-dried for 2–3 hours, and powdered. *O. octandra* leaf powder (100 g) was extracted using hexane, ethyl acetate, and methanol sequentially by sonication at room temperature [10]. Solvents were filtered, evaporated using a rotary evaporator, and dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions of 3 mg/mL concentration and stored at -20°C until further use.

Cell Culture

The human squamous cell carcinoma cell line (YD-10B) which was originally established at the Oral Cancer

Research Institute, Yonsei University College of Dentistry, South Korea, was kindly gifted by them. The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) medium with standard supplements [8]. As supplements, 5% (v/v) of fetal bovine serum (FBS), 2% (v/v) of penicillin and streptomycin (P&S) mixture, 1% (v/v) of amphotericin, and 1% (v/v) of L-glutamine (all were purchased from Sigma-Aldrich, St.Louis, Missouri, USA) were used. Cells were cultured under standard cell culture conditions at 37°C temperature and in a humid atmosphere with 5% CO_2 in the air [11].

Cytotoxicity/Cell Viability Assay

The cytotoxicity assay was performed using Trypan Blue (Sigma-Aldrich, St.Louis, Missouri) cell staining solution as described earlier after treating with the extracts [7]. Cells at 70% confluency were treated with 0 $\mu\text{g/mL}$, 0.3 $\mu\text{g/mL}$, 3.0 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$ of OHLE, OEALE and OMLE for 24 hrs. OEALE and OMLE solutions were prepared by dissolving stock solutions in cell culture media. The concentrations were selected based upon the previous literature of similar studies [12]. Doxorubicin, a chemotherapeutic drug, was used as the positive control with a concentration of 5 μM . After the desired culture period, cells were dislodged enzymatically and stained with Trypan blue and counted using a hemacytometer under an upright light microscope [13]. The viability was described as a percentage of total cells (viable cells plus dead cells).

Cell Migration Assay

A standard wound healing assay was used to analyze the effect of OHLE, OEALE, and OMLE on the cell migration. The wound healing assay is a proxy to identify the cancer metastasis potential [14]. YD-10 B cells were seeded in $4 \times 10^4/\text{mL}$ initial cell concentration on 24-well plates and allowed to grow until confluency. Then, a scratch was created in the cell layer using a pipette tip and treated with different types of *O. octandra* extracts with the concentrations of 0.3 $\mu\text{g/mL}$, 3.0 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$, and doxorubicin. Cultures were imaged at 0 hrs, 6 h, 12 h, and 24 h of post-treatment using inverted microscope (IX73, Olympus, Japan) with the imaging software Cell Sense[®] (Olympus, Japan). Images obtained were analyzed by ImageJ[®] software (National Institutes of Health and Laboratory Optical and Computational Instrumentation, USA), and cell migration potential was assessed as wound healing percentages using a method described elsewhere [15, 16].

Gene Expression Analysis

Total RNA was extracted from the treated cells after treatments using TRIzol method as described in the literature [17]. The extracted RNA was quantified and reverse transcribed to synthesize cDNA. The relative mRNA expression levels of *B-cell lymphoma-2 (BCL2)*, *BCL2-antagonist of cell death (BAD)*, and *B-cell lymphoma-2-associated-x protein (BAX)* genes were evaluated using semi-quantitative RT-PCR with the relevant primers [18]. The housekeeping gene β -actin was used to normalize the expression data [19].

Primers	Primer sequences	Annealing <i>T</i>
β -actin	(Forward) 5'-TCA GCA AGC AGG AGT ATG-3'	60 °C
	(Reverse) 5'-GTC AAG AAA GGG TGT AAC G-3'	
BAD	(Forward) 5'-CCC AGA GTT TGA GCC GAG TG-3'	58 °C
	(Reverse) 5'-CCC ATC CCT TCG TCG TCC T-3'	
BAX	(Forward) 5'-CCC GAG AGG TCT TTT TCC GAG-3'	58 °C
	(Reverse) 5'-CCA GCC CAT GAT GGT TCT GAT-3'	
BCL2	(Forward) 5'-CGG TTC AGG TAC TCA GTC ATC C-3'	60 °C
	(Reverse) 5'-GGT GGG GTC ATG TGT GTG G-3'	

After PCR amplifications, products were run in 12% gels and the gel images were obtained using an automated gel documentation system (Vilber Lourmat, France). Captured images were analyzed using ImageJ2x software to quantify the intensities of the bands and analyze the gene expression semi-quantitatively [20]. Intensity values obtained from the β actin gene were used to normalize the expression.

Statistical Analysis

Complete randomized design was used as the experimental model of the study, and the data were statistically analyzed using Minitab software using a one-way ANOVA. Means were compared to the control using Dunnett's multiple comparison test while a probability value of $p \leq 0.05$ was considered as statistically significant.

Results

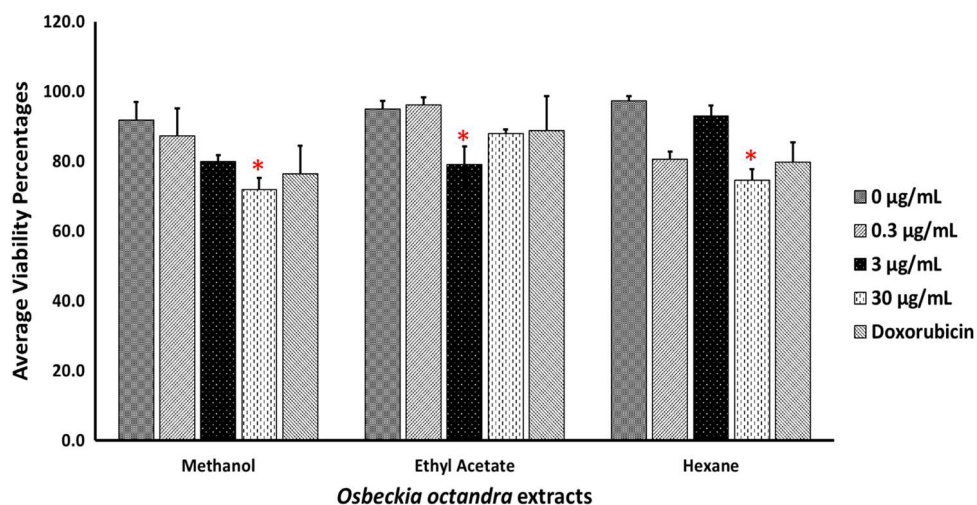
Cytotoxic potential

Cell viability was significantly decreased with the increasing concentrations of OMLE showing dose dependency with 30 μ g/mL as the lowest ($p < 0.05$) cell viability percentage (Fig. 1). A significantly lower ($p < 0.05$) cell viability was observed treatment groups which were exposed to 3 μ g/mL concentration of the OEAL while treatment with OHLE extract also indicated a significant reduction in the viability of YD-38 cells in related to the 30 μ g/mL treatment group (Fig. 1).

Morphological Changes

The morphology of YD-10B cells was changed with the increasing dose of *O. octandra* treatments (Fig. 2). The number of visible vacuoles increased showing that cells had undergone a stress condition. The cell mat structure was disturbed and that may be due to the reduction in cell viability. Shrinkage of the cell mat in 30 μ g/mL treatment was observed in OMLE (Fig. 2). Membrane blebbing, shrinkage of cells, and increase in visible vacuoles were increased with the increasing *Osbeckia octandra* concentrations.

Fig. 1 Cell viability of YD-10 B cells (%) after 24 h incubation with different types of *Osbeckia octandra* extracts. With different concentrations (0 μ g/mL, 0.3 μ g/mL, 3 μ g/mL and 30 μ g/mL) and positive control, doxorubicin (5 μ M). *Denotes statistical significance when $p < 0.05$



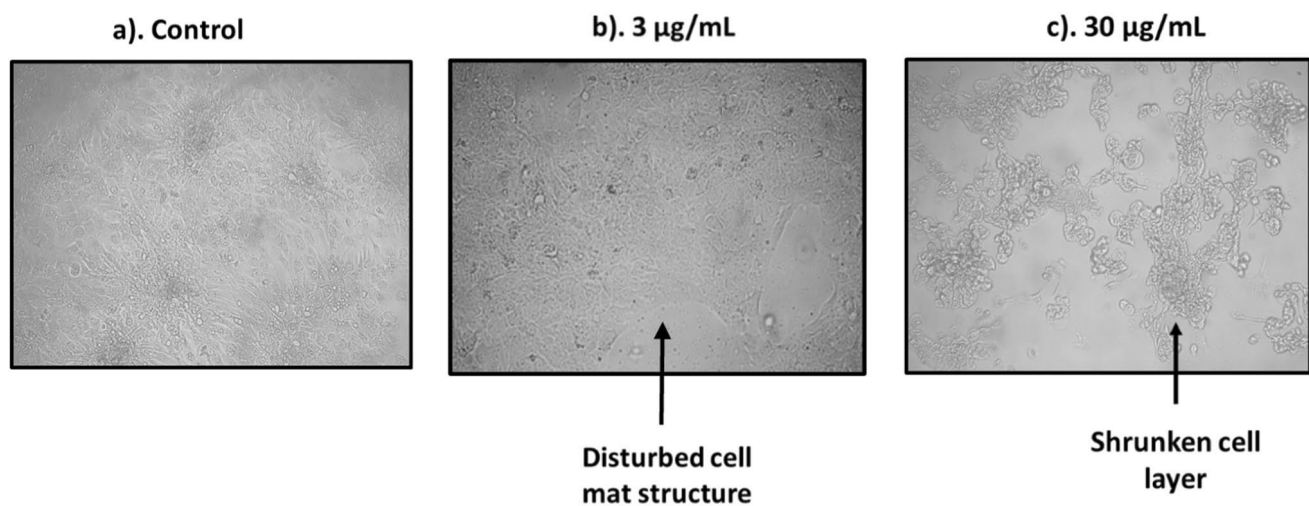


Fig. 2 A Morphology of YD-10 B cells under microscope after the treatment with OMLE. Microscopic view ($\times 200$) of YD-10 B cells treated with different concentrations of OMLE. Control (a), 3 $\mu\text{g/mL}$ (b), 30 $\mu\text{g/mL}$ (c)

Cell Migration Analysis by Wound Healing Assay

A significant reduction in the wound healing rate was reported in relevant to the 3 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$ concentrations of both OMLE and OEALE indicating the treatments hampered the cell migration plus proliferation (Fig. 3). The other two concentrations, 3 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$, of OMLE had significantly reduced ($p < 0.05$) the rate of healing compared to the non-treated control, and doxorubicin control as well. However, OHLE did not exhibit influence on the wound healing.

Gene Expression Analysis

Three cell survival and apoptotic pathway genes *BAX*, *BAD*, and *BCL2* were semi-quantitatively analyzed in the present study. There was a significantly lower ($P < 0.05$) relative *BCL2* mRNA expression at 30 $\mu\text{g/mL}$ treatment group compared to the negative control (0 $\mu\text{g/mL}$) group

in OMLE (Fig. 4). Moreover, the 30 $\mu\text{g/mL}$ treatment group also showed a significantly higher mRNA expression related to *BAX* gene expression as well ($P < 0.05$). None of the treatment groups indicated any significance in the relative expression of the *BAD* gene when compared with the negative control.

The mRNA Expression of OEALE

As shown in Fig. 5, 30 $\mu\text{g/mL}$ concentration of OEALE depicted a significantly lower value in *BCL2* gene expression while none of any treatment groups indicated a significant change in the *BAX* and *BAD* mRNA expression.

The mRNA expression of OHLE

As depicted in Fig. 6, there were not any significant changes in the relative mRNA expression values of all the genes, *BCL2*, *BAD*, and *BAX* in the OHLE.

Fig. 3 Wound healing rates ($\mu\text{m/h}$) of YD-10B cells with different types of *O. octandra* extracts. Treatments are 0 $\mu\text{g/mL}$, 0.3 $\mu\text{g/mL}$, 3.0 $\mu\text{g/mL}$, 30.0 $\mu\text{g/mL}$ of *O. octandra* OMLE, OEALE, OHLE, and 5 μM of doxorubicin (positive control). *Denotes statistical significance when $p < 0.05$ compared to negative control. **Denotes statistical significance when $p < 0.05$ compared to positive control.

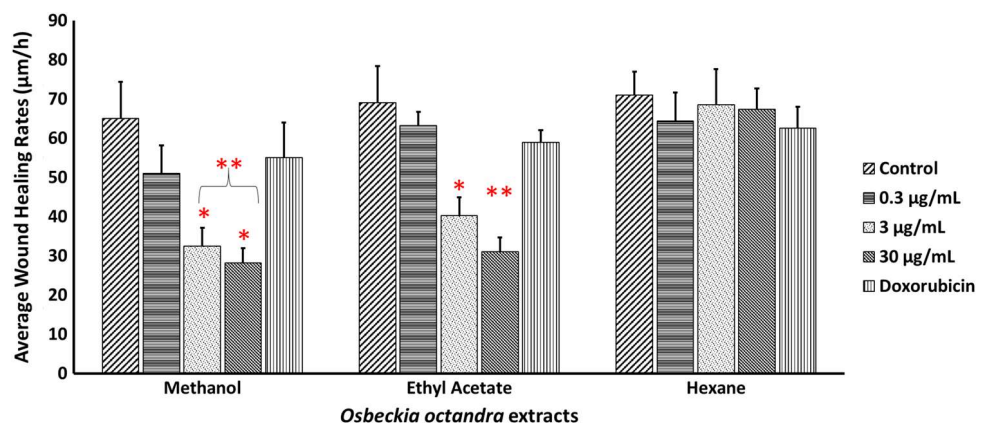


Fig. 4 *BCL2*, *BAX*, and *BAD* gene expression in OMLE-treated YD-10B cells. Semi-quantified relative expression of *BCL2*, *BAX*, and *BAD* gene in YD-10 B cells treated with OMLE. Treatment groups were 0.3 $\mu\text{g/mL}$, 3 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$, and doxorubicin 5 μM . *Indicates the statistical significance when $p < 0.05$ compared to positive control

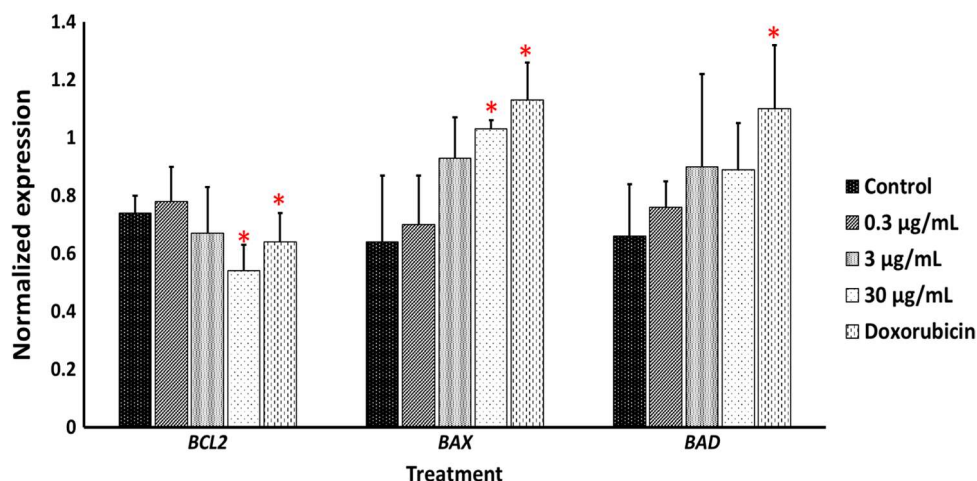


Fig. 5 *BCL2*, *BAX*, and *BAD* gene expression in OEAL-treated YD-10B cells. Semi-quantified relative expression of *BCL2*, *BAX*, and *BAD* gene in YD-10 B cells treated with OEAL. Treatment groups were 0.3 $\mu\text{g/mL}$, 3 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$, and doxorubicin 5 μM . *Indicates the statistical significance when $p < 0.05$ compared to positive control

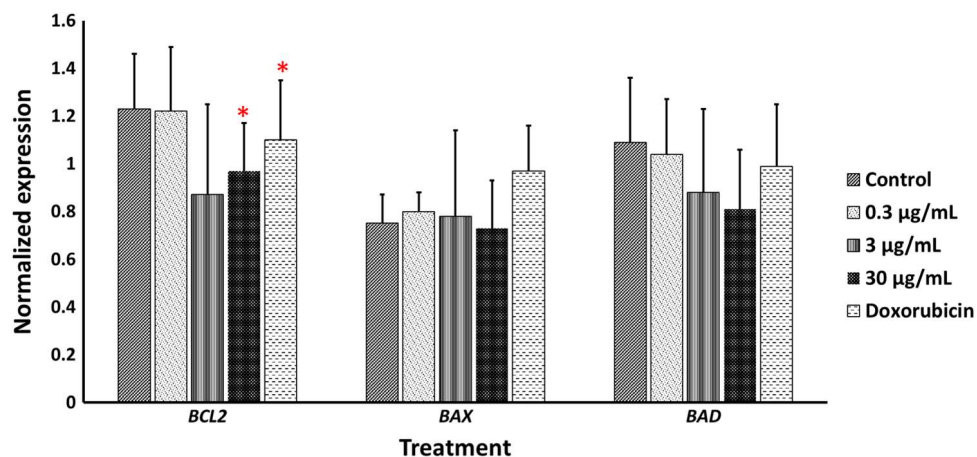
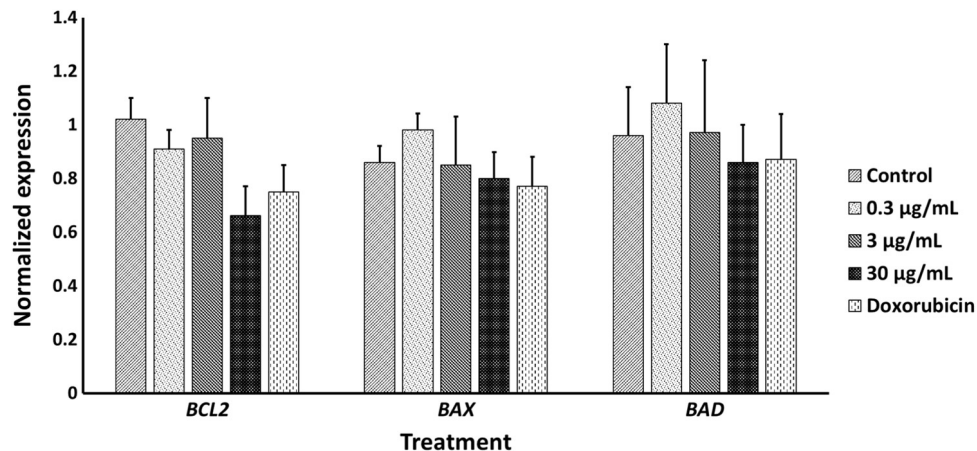


Fig. 6 *BCL2*, *BAX*, and *BAD* gene expression in OHLE-treated YD-10B cells. Semi-quantified relative expression of *BCL2*, *BAX*, and *BAD* gene in YD-10 B cells treated with OHLE. Treatment groups were 0.3 $\mu\text{g/mL}$, 3 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$, and doxorubicin 5 μM . Statistical significance, $p < 0.05$



Discussion

Cancer is formed due to various causes including genetic and epigenetic regulations of the genes that normally play a role in cell proliferation. These changes can lead to uncontrolled cell growth. Even though there is a huge advancement in the modern medical sector, plants or herbal medicine still make

an important contribution to healthcare. Recent studies have highlighted the importance of herbal medicine in protection against the development of cancer as well as cancer suppression ability. With the optimistic results obtained from our previous OWLE study, we conducted the present study to find out the same anticancer property *via* chemical extraction of *O. octandra* and their treatment efficacy by gene

expression analysis. Plant-based extracts or their various extracted compounds possess qualities in impairing cancer cells *via* different mechanisms, such as immunity enhancement, apoptosis inducement, reducing the drug resistance and angiogenesis inhibition [21].

The YD-10B oral squamous carcinoma cells were cultured and treated with 0, 0.3, 3, and 30 $\mu\text{g/mL}$ concentrations. Chosen concentrations were not toxic to the healthy cells as previous *in vivo* studies have indicated. Studies have demonstrated that even the use of crude extract of *O. octandra* 500 mg/kg body weight per day is physiologically non-toxic to Wistar rats [6].

The treatment group of 30 $\mu\text{g/mL}$ of both OMLE and OHLE has effectively reduced the cancer cell viability while a low dose (3 $\mu\text{g/mL}$) of OEALE has reported a significant reduction in comparison with the negative control. In addition, all these significant treatment groups have performed way better than the positive control, doxorubicin. The results obtained are kindred to a similar experiment which was done using curcumin to treat the same cell line, YD-10B, as the present study [21]. In that study, they have suggested that the apoptotic pathway of YD-10B cells induced by curcumin is *via* inducing DNA damage. Our previous experiment also showed that the OWLE possesses the ability to induce DNA damage in carcinoma cells, and hence, we can assume OMLE, OHLE, and OEALE have the same potential.

Carcinoma cells possess the features of rapid and uncontrolled proliferation, undifferentiating leading to loss of function and metastasis [22]. Moreover, metastasis could be identified as the leading cause of carcinoma-related mortality, and thus, an efficient anticancer agent should have the ability to reduce metastasis concurrently while inducing cell apoptosis. To measure the effect on metastasis altering by *Osbeckia* extracts, a wound healing assay was performed under *in vitro* conditions. Figure 3 depicts 3.0 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$ concentrations of OMLE and OEALE have significantly reduced the wound healing rate which may indicate its potential to lower the cancer metastasis while affecting the cancer cell migration. These two treatments have performed even better than the positive control, doxorubicin as well. However, some studies have identified that the oral squamous carcinoma cells develop resistance to doxorubicin to some extent [23].

When comparing the results obtained from the viability and cell migration assays, OMLE and OEALE have performed better compared to the OHLE in the action of anticancer and their effects are more promising in affecting the metastasis rather than reducing cell viability (Fig. 1 vs Fig. 3). The overall effect of the two extracts may be due to both viability reduction by increased apoptosis as well as by reduction in metastasis potential.

With the advancement of medical research, gene expression profiling has emerged as an interesting new technology

to identify gene-based classifiers that correlate with diagnosis, prognosis, or prediction of response treatment of a cancer. These molecular markers mostly are the proteins that are produced at a higher rate by the cancer cells. Three cell survival and apoptotic pathway genes *BAX*, *BAD*, and *BCL2* were semi-quantitatively analyzed in the present study to understand the mechanisms of actions of *O. octandra* leaf extracts.

BCL2 protein is the most prominent anti-apoptotic protein expressed in OSCC. Expression of *BCL2* might be used as an indicator for OSCC. It contributes to the development of cancer and developing resistance to current anticancer treatments. As depicted in Figs. 4 and 5, the OMLE and OEALE have affected the *BCL-2* gene expression by significantly reducing the gene expression at concentrations of 30 $\mu\text{g/mL}$. Thus, both OMLE and OEALE can be identified as potent *BCL2* inhibitors. The exact mechanism that involved *BCL-2* in the process of inhibiting apoptosis is still uncertain. However, it has been revealed that *BCL-2* has been localized to the areas of contact between the outer and the inner mitochondrial membranes; therefore, it is worth suggesting that it has the ability in protecting the cell from apoptosis *via* altering mitochondrial function [24]. The exact molecular signaling pathway/s that OMLE and OEALE affect the *BCL2* gene in oral cancer cells require further investigations.

The *BAX* gene could be identified as a pro-apoptotic gene, and it has been revealed that the higher expression of *BAX* is associated with better overall survival and lower recurrence rates in OSCC [25]. According to Fig. 4, the treatment group 30 $\mu\text{g/mL}$ of OMLE has exhibited significantly higher *BAX* expression levels while OEALE and OHLE didn't show any significance as depicted in Figs. 5 and 6. Thus, OMLE affects oral cancer cell suppression by increasing the pro-apoptotic properties *via* *BAX*. The *BAD* gene is also considered a pro-apoptotic gene as same as the *BAX* gene. Furthermore, *BAD* inhibits cancer cell proliferation and higher *BAD* expression is associated with longer disease-free survival [26], as the results obtained from the study neither of the extracts have exhibited any significance.

Previous qualitative analysis of phytochemicals in *O. octandra* leaves has revealed that it contains phenols, tannins, flavonoids, steroids, and alkaloids and also possesses a high number of phenolic compounds ranging from 450 to 600 mg/GAE/g [7, 27]. They are plant metabolites characterized by the presence of several phenol groups, and some of them are very reactive in neutralizing free radicals by donating a hydrogen atom or an electron, chelating metal ions in aqueous solutions. Even though the phenolic compounds are mainly correlated with the antioxidant property, certain shreds of evidence show that they are cytotoxic as well [28]. Therefore, it can be hypothesized that the anticancer effect of *O. octandra* may be due to the presence of a higher number of phenolic compounds, but further comprehensive studies

must be carried out regarding the exact molecules which are responsible. In addition, phytochemicals such as terpenoids and tannis which are available in *O. octandra* leaves are also identified as promising anticancer agents [29].

Considering all the facts, OMLE and OEAL could be identified as propitious agents for developing a therapeutic or preventive drug for oral carcinoma and further in-depth studies are warranted.

Conclusion

Osbeckia octandra methanol-based leaf extract (OMLE) and *Osbeckia octandra* ethyl acetate-based leaf extract (OEAL) induce anticancer activity in YD-10B oral carcinoma cells via enhancing cell mortality and reducing cell migration. The *BCL-2* and *BAX* gene expression pathways are altered in their mode of action. Moreover, further in-depth studies related to the isolation and identification of exact bioactive compound/s which are responsible for the anticancer property of *O. octandra* may be beneficial in the pathway of developing anticancer drugs in future studies.

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Declarations

Conflict of interest The authors report no declarations of interest.

Ethical Approval The YD-10B cells used in the current study were originally established at the Oral Cancer Research Institute, Department of Oral Pathology, Yonsei University College of Dentistry, Seoul, Korea, in the year 2005 and are commercially available in the Korean Cell Line Bank. Hereby we would like to declare that the current study used already established commercial cancer cell lines for the in vitro studies. No primary human or animal cells or tissues were used in the present study. Therefore, the authors have not obtained any ethical clearance for the use of commercial cell lines.

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