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Preparation and inhibitory effect of salicin dimethyl ether in Laryngeal cancer cells through the apoptosis activation

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Laryngeal cancer is detected commonly worldwide, and it ranks second highest in incidence among the respiratory tract neoplasms following only head and neck squamous cell cancer. In the present study salicin dimethyl ether was synthesized and evaluated against laryngeal cancers cells for anticancer property. MTT assay was used for the measurement of changes in TU686 and Tu212 cell proliferation while as induced apoptosis was detected by flow cytometry. Protein expression was determined by western blotting and expression of mRNA by RT-PCR assay. In the present study salicin dimethyl ether was synthesized by the reaction of salicin with methyl iodide using sodium hydride as base. Salicin dimethyl ether treatment led to a significant decrease in TU686 and Tu212 cell proliferation in a dose-dependent manner. In TU686 and Tu212 cells salicin dimethyl ether treatment caused a significant increase in cell apoptosis and elevated caspase-3 activity. Treatment with salicin dimethyl ether led to a prominent reduction in Bcl-2 protein expression in TU686 and Tu212 cells at 72 h. Salicin dimethyl ether treatment led to a prominent decrease in p-PI3K and p-Akt protein expression in TU686 and Tu212 cells, compared to the untreated cells. A significant increase in miR-15a expression in TU686 and Tu212 cells was observed on treatment with salicin dimethyl ether at 72 h. In summary, the current study demonstrates that salicin dimethyl ether, a synthetic derivative of salicin, suppresses proliferation of TU686 and Tu212 cells. The underlying mechanism involves induction of apoptosis, inhibition of PI3K/Akt pathway and promotion of miR-15a expression. Therefore, salicin dimethyl ether may be used for inhibition of laryngeal cancer growth, however, *in vivo* studies need to be conducted to confirm the effect.

Keywords: synthesis, condensation, ether, laryngeal cancer, apoptosis

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Abbreviations: HNC, head and neck cancers; HPV, human papillomavirus; LSCC, Laryngeal squamous cell carcinoma

INTRODUCTION

Laryngeal cancers constitute one-third of all the head and neck tumors and contribute significantly to morbidity and mortality. These cancers are common in patients with high smoking history who are also susceptible to the cancers of aero-digestive tract. There are different subsites in the larynx from which these cancers evolve, and all these cancers have different implications in symptomatic presentation, spreading pattern and treatment paradigm. The disease is highly curable and larynx-preserving at an early stage by surgery or radiation monotherapy, whereas the worst outcomes with little chances of larynx-preservation are reported in the late-stage of the cancer (Koroulakis *et al*., 2021).

Laryngeal cancer is detected commonly throughout the world with approximately 600 000 new cases each year (Bray *et al*., 2018). It accounts for approximately 20% of all cases of head and neck cancer in which 40% of patients are diagnosed with advanced disease. The incidence of laryngeal cancer has increased, and 90 000 patients die each year (Siegel *et al*., 2019). Similar to the other types of head and neck cancers (HNC), consumption of alcohol and smoking are the main reasons associated with Laryngeal squamous cell carcinoma (LSCC). It is reported that squamous cancers of the oropharynx (generally tonsils and tongue base) are caused by human papillomavirus (HPV) (Agalliu *et al*., 2016; D'Souza *et al*., 2007) and evidence has emerged that HPV infection is also associated with an increase in risk of LSCC (Chen *et al*., 2017). In laryngeal cancer patients the rate of HPV infection has been found to vary between 3% to 85% (Lindeberg *et al*., 1999). The patients with HPV-induced HNC are generally found to be in a young age group and major risk factors identified include 16, 18, and 31 types of HPV (D'Souza *et al*., 2010). Although studies have demonstrated that LSCC is associated with HPV infection (Aaltonen *et al*., 2002), its role in the disease is yet to be fully understood (Syrjänen, 2005). There has been no significant improvement in the survival rate of laryngeal cancer patients despite drastic improvement in surgical techniques, radiation therapy instrumentation and chemotherapeutic agents over the past three decades (Divi *et al*., 2010). Instead, a decrease in survival rate of the patients has been recorded due to a rapid increase in air pollution. There is an urgent need for early diagnostic techniques and development of novel treatment strategies so that mortality associated with laryngeal cancer can be prevented (Ampil & Nguyen, 2014). Moreover, studies shall be conducted to evaluate the mechanism at a molecular level underlying the laryngeal cancer for the development of an effective treatment. It has been confirmed that laryngeal cancer is induced by many factors, including intake of alcohol, air pollutants, smoking and hazardous chemicals (Janssens *et al*., 2014). At a molecular level, phosphoinositide-3 kinase (PI3K)/protein kinase

B (Akt), Bcl-2, etc., have been identified to be involved in the development of laryngeal cancer (Vachhani *et al.*, 2014). Numerous tumors are related to the altered expression of miRNAs in the tumor cells compared to the adjacent wild-type tissues (Yu & Li, 2015). It is reported that early diagnosis and progression of tumors can be made possible by the miRNA levels analysis of the cancer cells (Zhang *et al.*, 2014). Diagnosis, metastasis, progression and recurrence of several types of tumors is associated with the miRNAs as the molecular markers (Zhang *et al.*, 2014).

Salicin, a natural product extracted from the bark of White Willow, has been found to possess analgesic and anti-inflammatory properties. Pharmacodynamics have revealed that salicylic acid formed from the salicin within the body is actually responsible for anti-inflammatory and pain-relieving properties (Pilotto *et al.*, 2004). It also inhibits COX-2 activity, just like that of aspirin, but has no anticoagulant potential (Hawkey, 2004). Recently it has been demonstrated that induction of COX-2 acts as a critical event in inflammatory disorders (Vane *et al.*, 1994). It is further supported by the fact that effective inhibition of inflammatory responses is observed in animal models using selective COX-2 inhibitors, *in vivo* (Chan *et al.*, 1995). Aspirin (acetylsalicylic acid) acts as a nonselective COX inhibitor and is deacetylated rapidly to form salicylic acid in the blood (Mitchell *et al.*, 1995). It is reported that salicylic acid has virtually no inhibitory effect on purified COX-1 or COX-2, but it targets synthesis of prostaglandin in the intact cells (Wu *et al.*, 1991). Salicin isolated from the extract of white willow bark has been found to be effective in treating knee and/or hip osteoarthritis pain (Bigler *et al.*, 2001) as well as back pain (Macarthur *et al.*, 2005). In the present study salicin dimethyl ether was synthesized and evaluated against laryngeal cancer cells for anticancer property.

MATERIALS AND METHODS

Synthesis of salicin dimethyl ether

To a solution of salicin (1000 mg) in acetonitrile solvent was added methyl iodide (2 equivalents) and sodium hydride (1.2 equivalent) as base. The reaction mixture was stirred for 24 h till completion as monitored by thin layer chromatography. The reaction mixture was charged on silica gel column through which a mixture of solvents (hexane and dichloromethane) in 90:10 ratio was passed to purify the product. The product formed was characterized by ^{1}H NMR, ^{13}C NMR and HRMS techniques.

Cell culture

The TU686 and Tu212 laryngeal carcinoma cell lines were procured from the American Type Culture Collection (ATCC) Center and cultured overnight in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The medium contained 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.) and was mixed with penicillin (100 μM) and streptomycin (100 μM). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2.

MTT assay

TU686 and Tu212 cells were distributed in 96-well plates containing DMEM medium mixed with FBS at 2×0^{5} cells/well density. Following the overnight incubation, the cells were treated with 1 to 20 μM concentration of salicin dimethyl ether for 72 h at 37°C in a humidified atmosphere containing 5% CO_2. Then 50-μl solution of MTT dye (5 mg/ml; Sigma-Aldrich) was put into each well and cells were incubated for 4 h more. Afterwards, 150 μl of dimethyl sulfoxide (Invitrogen) was added to the wells, the plates were agitated for 15 min and then optical density measurements were made using a Versamax microplate reader at 487 nm.

Flow cytometric assay

TU686 and Tu212 cells at 2 x 10^{6} cells/well density were cultured in 6-well plates containing DMEM-medium, 10% FBS and 1 and 20 μM salicin dimethyl ether. The cells were cultured at 37°C in a humidified 5% CO_2 atmosphere for 72 h and then washed in ice-cold PBS before being harvested. After re-suspending in 1X binding buffer, the cells were stained for 40 min in 5 μl Annexin V-fluorescein isothiocyanate under complete dark conditions at 4°C. Then propidium iodide (10-μl) was put into each well of the plate and apoptosis induction was detected by flow cytometry (BD C6 flow cytometer; BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis

TU686 and Tu212 cells (2×10^{6} cells/well) were cultured in 6-well plates containing 1 and 20 μM salicin dimethyl ether mixed with DMEM-medium at 37°C under 5% CO_2 atmosphere. Cells were treated with radioimmuno-precipitation assay lysis buffer (Beyotime), harvested and subsequently centrifuged at 4°C for 15 min at 12000×*g*. Protein content was estimated in supernatant using the bicinchoninic acid assay (Beyotime) as per the manufacturer's instructions. Proteins were separated by loading ~50 μg/lane samples on 10% SDS-PAGE and subsequently transferred to a PVDF nitrocellulose membrane (Sigma-Aldrich). Incubation of the membranes was performed with primary antibodies overnight at 4°C. Membranes were washed with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% skimmed milk at room temperature. Following washing, incubation of the membranes was carried out with an anti-mouse or anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Inc.) at 37°C for 1 h. The protein blots were detected using enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc., USA) and analyzed using Quantity One software 3.0 (Bio-Rad Laboratories). The primary antibodies used were as follows: anti-caspase-3, anti-Bcl-2, anti-PI3K, anti-p-Akt, anti-Akt (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-β-actin (Sangon Biotech Co., Ltd., Shanghai, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

TU686 and Tu212 cells (2×10^{6} cells/well) were cultured in 6-well plates containing 1 and 20 μM salicin dimethyl ether mixed with DMEM-medium at 37°C under 5% CO_2 atmosphere. The TRIzol® reagent (Invitrogen) was used for the extraction of total RNA and TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) for the reverse transcription into cDNA. The TaqMan MicroRNA assay (Applied Biosystems) was used for quantitative-PCR of the 1.0 μl cDNA samples according to the manufacturer's protocol. Quantification of the gene expression was made using the 2-ΔΔCq method (Zubillaga-Guerrero *et al.*, 2015). The primer sequences used are given in Table 1.

Table 1. Sequence of primers used in RT-PCR assay.

Gene	Forward	Reverse
miR-15a	5′-GCTAGC AGCACATAATGGTTTGTG-3′	5′-GTGCAG GGTCCGAGGTATTC-3′
U6	5′-GTGCAGGGTCCGAGGTATTC-3′	5′-AACGCTTCACGAATTTGCGT-3′

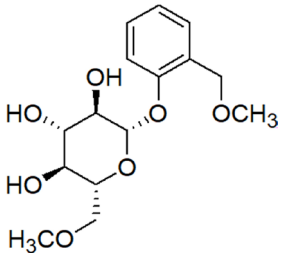

Figure 1. Preparation of Salicin dimethyl ether.

Statistical analysis

The data expressed are the mean ± standard deviation for triplicate measurements. Differences between the groups were determined using one-way analysis of variance ANOVA and Bonferroni's post *hoc test*. Differences were taken as statistically significant at $P<0.05$.

RESULTS

Preparation of salicin dimethyl ether

Salicin dimethyl ether was synthesized by the reaction of salicin (1 equivalent) with methyl iodide (2 equivalents) in acetonitrile solvent using sodium hydride as base. The reaction progressed smoothly at room temperature and product formation was complete in 12 h. Purification of the product (89% yield) on silica gel column was followed by characterization using ^{1}H NMR, ^{13}C NMR and HRMS techniques (Fig. 1). Salicin dimethyl ether was stored in desiccator at room temperature till use in the study for anticancer property against laryngeal cancer cells.

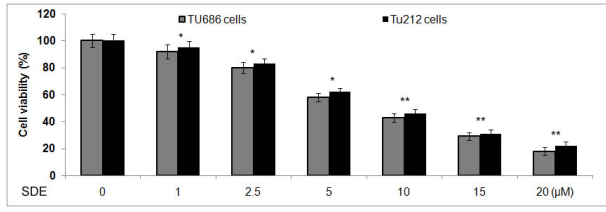

Figure 2. Effect of salicin dimethyl ether on proliferative potential of TU686 and Tu212 cells.
Salicin dimethyl ether was added to the cultures of TU686 and Tu212 cells at 1 to 20 μM concentrations and incubation was continued for 72 h. Proliferation changes in the cell cultures were evaluated by MTT assay. *$P<0.05$ and **$P<0.01$ *vs.* untreated cells.

Salicin dimethyl ether inhibits TU686 and Tu212 cancer cell proliferation

TU686 and Tu212 cells were treated with salicin dimethyl ether at 1 to 20 μM concentrations for 72 h to evaluate the changes in proliferation (Fig. 2). Salicin dimethyl ether treatment led to a significant decrease in TU686 and Tu212 cell proliferation compared to the untreated cells. Reduction in proliferative potential of TU686 and Tu212 cells on treatment with salicin dimethyl ether was found to be dose-dependent.

Salicin dimethyl ether increases TU686 and Tu212 cell apoptosis

TU686 and Tu212 cells were treated with salicin dimethyl ether at 1 and 20 μM concentrations for 72 h to detect apoptosis induction (Fig. 3). Salicin dimethyl ether treatment led to a significant increase in TU686 and Tu212 cell apoptosis compared to the untreated cells.

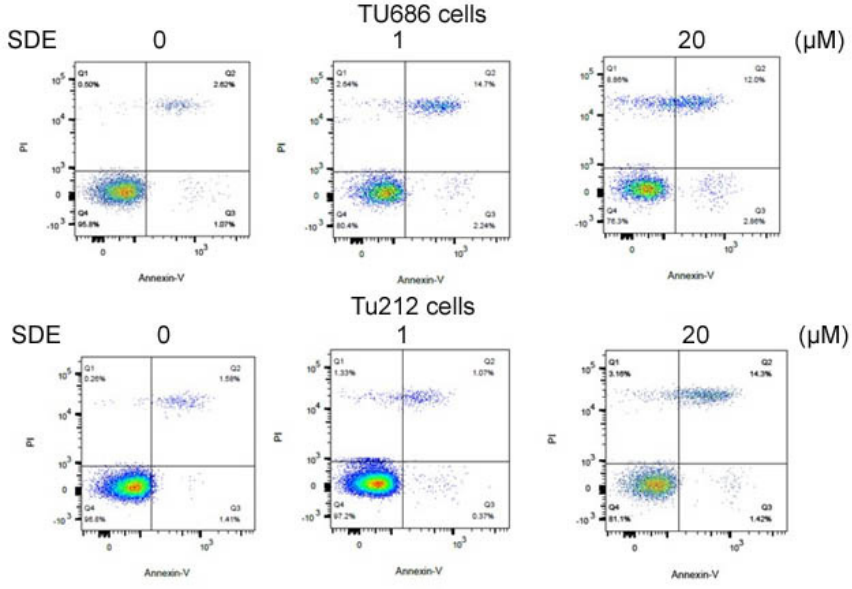

Figure 3. Effect of salicin dimethyl ether on apoptosis of TU686 and Tu212 cells.
Salicin dimethyl ether was added to the cultures of TU686 and Tu212 cells at 1 and 20 μM concentrations and incubation was continued for 72 h. Apoptosis induction in the cell cultures was detected by flow cytometry assay. *$P<0.05$ and **$P<0.01$ *vs.* untreated cells.

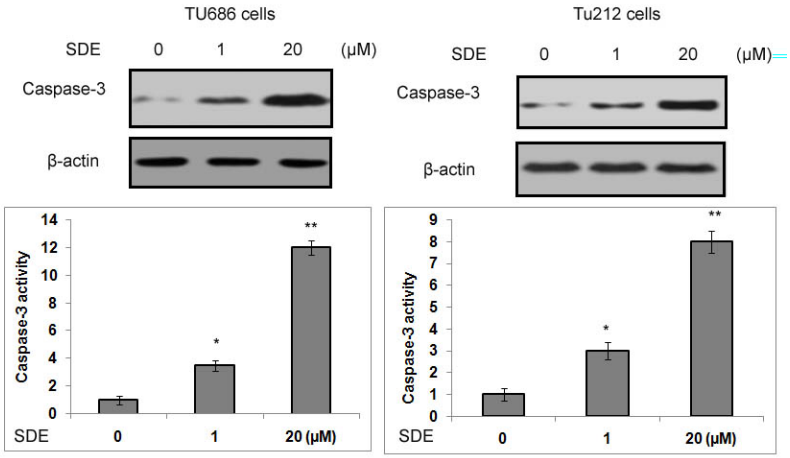

Figure 4. Effect of salicin dimethyl ether on caspase-3 activity in TU686 and Tu212 cells.
Salicin dimethyl ether was added to the cultures of TU686 and Tu212 cells at 1 and 20 μM concentrations and incubation was continued for 72 h. Caspase-3 activity in the cell cultures was determined by western blotting assay. *P<0.05 and **P<0.01 *vs*. untreated cells.

Increase in TU686 and Tu212 cell apoptosis by salicin dimethyl ether treatment was significantly (P<0.05) higher at 20 μM compared to 1 μM concentration.

Salicin dimethyl ether promotes caspase-3 activity in TU686 and Tu212 cells

In TU686 and Tu212 cells, caspase-3 activity was assessed at 72 h of salicin dimethyl ether treatment by western blotting (Fig. 4). Salicin dimethyl ether treatment led to a prominent increase in caspase-3 activity in TU686 and Tu212 cells compared to the untreated cells. Increase in caspase-3 activity in TU686 and Tu212 cells by salicin dimethyl ether treatment was higher at 20 μM compared to 1 μM concentration.

Salicin dimethyl ether suppresses Bcl-2 expression in TU686 and Tu212 cells

In TU686 and Tu212 cells, Bcl-2 protein expression was assessed at 72 h of salicin dimethyl ether treatment by western blotting (Fig. 5). Salicin dimethyl ether treatment led to a prominent reduction in Bcl-2 protein expression in TU686 and Tu212 cells compared to the untreated cells. Suppression of Bcl-2 protein expression in TU686 and Tu212 cells by salicin dimethyl ether treatment was higher at 20 μM concentration compared to 1 μM concentration.

Salicin dimethyl ether suppresses PI3K and Akt protein expression in TU686 and Tu212 cells

In TU686 and Tu212 cells PI3K and Akt protein expression was assessed at 72 h of salicin dimethyl ether treatment by western blotting (Fig. 6). Salicin dimethyl ether treatment led to a prominent decrease in PI3K and Akt protein expression in TU686 and Tu212 cells compared to the untreated cells. Suppression of PI3K and Akt protein expression in TU686 and Tu212 cells by salicin dimethyl ether treatment was higher at 20 μM compared to 1 μM concentration.

Salicin dimethyl ether promotes miR-15a expression in TU686 and Tu212 cells

In TU686 and Tu212 cells miR-15a expression was determined at 72 h of salicin dimethyl ether treatment by the RT-PCR assay (Fig. 7). Salicin dimethyl ether treatment led to a significant increase in miR-15a expression in TU686 and Tu212 cells compared to the un-

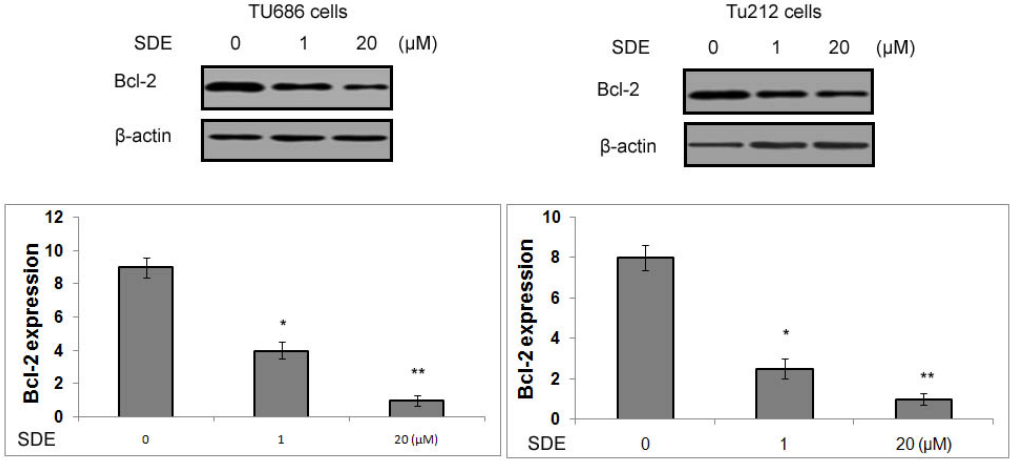

Figure 5. Effect of salicin dimethyl ether on Bcl-2 protein expression in TU686 and Tu212 cells.
Salicin dimethyl ether was added to the cultures of TU686 and Tu212 cells at 1 and 20 μM concentrations and incubation was continued for 72 h. Bcl-2 protein expression in the cell cultures was determined by western blotting assay. *P<0.05 and **P<0.01 *vs*. untreated cells.

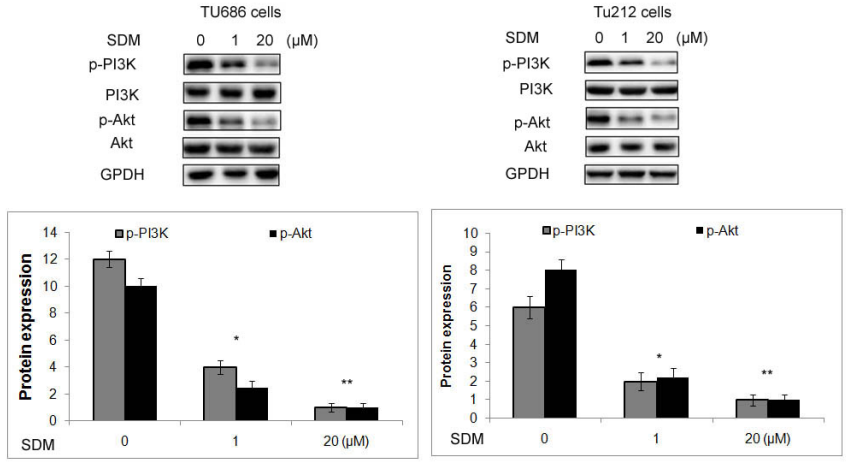

Figure 6. Effect of salicin dimethyl ether on PI3K and Akt protein expression in TU686 and Tu212 cells.
Salicin dimethyl ether was added to the cultures of TU686 and Tu212 cells at 1 and 20 μM concentrations and incubation was continued for 72 h. Expression of PI3K and Akt proteins in the cell cultures was determined by western blotting assay. *P<0.05 and **P<0.01 *vs.* untreated cells.

treated cells. Increase in miR-15a expression in TU686 and Tu212 cells by salicin dimethyl ether treatment was higher at 20 μM compared to 1 μM concentration.

DISCUSSION

Laryngeal caner is detected commonly in head and neck region of the human beings as a malignant tumor (Gilbert *et al.*, 2010). The annual incidence of laryngeal cancer has shown a significant increase over past few decades and one of the reasons is believed to be the elevation in concentration of air pollutants (Mouw *et al.*, 2012). Recurrence of the tumor or metastasis are responsible for death of the patients after treatment using radiotherapy, surgery and chemotherapy (Koskinen et al., 2007). Thus, laryngeal cancer continues to be a major life threat to human life, despite advanced surgical procedures, radiotherapy and chemotherapeutic agents (Hamilton *et al.*, 2013). In the current study, salicin dimethyl ether was synthesized by the reaction of salicin with methyl iodide in the presence of sodium hydride for evaluation against laryngeal cancer cells for the development of novel treatment. The reaction produced salicin dimethyl ether in an excellent yield at room temperature over 12 h. The initial data showed that salicin dimethyl ether treatment significantly reduced TU686 and Tu212 cell proliferation compared to the untreated cells. Moreover, it was observed that reduction in proliferative potential of TU686 and Tu212 cells on treatment with salicin dimethyl ether was dose-dependent.

Apoptosis activation for controlling tumor growth is regulated by many genes, which include Bcl-2 family (Hosseini *et al.*, 2015). It is known that Bcl-2 plays a prominent role in preventing cell death by inhibiting the activation of the apoptotic pathway (Hosseini *et al.*, 2015). However, Bax and antagonists of Bcl-2 homologous genes increase cell death by promoting the apoptotic pathway (Besbes *et al.*, 2015). It is reported that Bcl-2 expression facilitates survival of the cells having damaged DNA, which leads to an accumulation of mutations and consequently tumor development (Moldoveanu *et al.*, 2014). *In vivo* studies have shown that PI3K/Akt pathway is associated with anti-apoptotic mechanism through the up-regulation of protein synthesis and increased glucose metabolism (Vachhani *et al.*, 2014). Abnormality in signal transduction is related with excessive cell growth, increased proliferation and consequently formation of tumors (Bitting *et al.*, 2013). The PI3K/AKT pathway has also been shown to be involved in laryngeal cancer growth where expression of p-AKT indicates PI3K/AKT activation. Activation of AKT presents failure of therapeutic agents against head and neck cancer, and its inhibition promotes radio-sensitivity (Gupta *et al.*, 2002). Several genes and non-coding RNAs have been reported to regulate the progression of laryngeal cancer through PI3K/AKT pathway. For example, proliferation of laryngeal cancer cells is promoted by miR132 through targeting FOXO1 leading to the activation of PI3K/

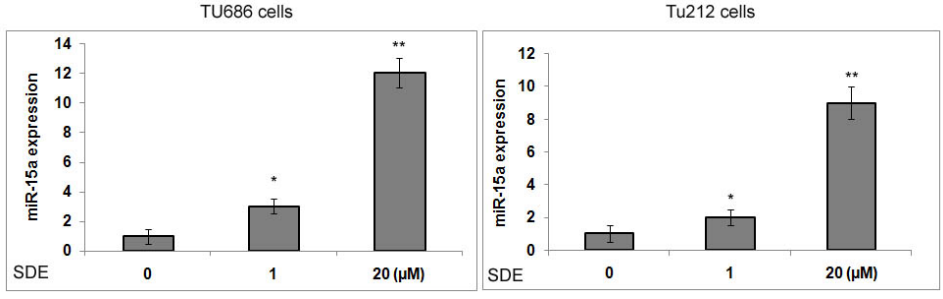

Figure 7. Effect of salicin dimethyl ether on miR-15a expression in TU686 and Tu212 cells.
Salicin dimethyl ether was added to the cultures of TU686 and Tu212 cells at 1 and 20 μM concentrations and incubation was continued for 72 h. Expression of miR-15a in the cell cultures was determined by RT-PCR assay. *P<0.05 and **P<0.01 *vs.* untreated cells.

AKT pathway (Lian *et al.*, 2016). TRA2β increases proliferation, promotes metastasis and inhibits apoptosis of laryngeal cancer cells by activating PI3K/AKT pathway (Ni *et al.*, 2019). Thus, PI3K/Akt pathway has a main role in cellular survival and is therefore also known as the pathway of anti-apoptosis (Simpson *et al.*, 2015). The present study explored the association of salicin dimethyl ether with apoptosis activation and related factors, including caspase-3 activity, Bcl-2 protein expression, PI3K and Akt pathway in TU686 and Tu212 cells. It was found that salicin dimethyl ether treatment led to a significant increase in TU686 and Tu212 cell apoptosis compared to the untreated cells. Salicin dimethyl ether treatment for 72 h also led to a prominent increase in caspase-3 activity in TU686 and Tu212 cells. The expression of Bcl-2 protein in TU686 and Tu212 cells showed a prominent reduction on treatment with salicin dimethyl ether for 72 h. Moreover, salicin dimethyl ether treatment led to a remarkable decrease in PI3K and Akt protein expression in TU686 and Tu212 cells. Thus, these findings indicated that salicin dimethyl ether exhibits inhibitory effect on laryngeal cancer cells through the activation of apoptosis, up-regulation of pro-apoptotic factors and downregulation of anti-apoptotic pathway.

Expression of miR-15a has been found to reduce viability of laryngeal cancer cells by inducing cell apoptosis through targeting PI3K/Akt expression (Skawran *et al.*, 2008). Inhibitory effect of therapeutic agents on laryngeal cancer cells is reversed by the knockdown of miR-15a expression (Seo *et al.*, 2014). In the present study salicin dimethyl ether treatment led to a significant increase in miR-15a expression in TU686 and Tu212 cells. Moreover, increase in miR-15a expression in TU686 and Tu212 cells by salicin dimethyl ether treatment was higher at 20 μM concentration compared to 1 μM concentration.

CONCLUSION

In summary, the current study demonstrates that salicin dimethyl ether, a synthetic derivative of salicin, suppresses proliferation of TU686 and Tu212 cells *in vitro*. The underlying mechanism of salicin dimethyl ether in the inhibition of laryngeal cancer cell growth involves induction of apoptotic pathway, inhibition of PI3K/Akt pathway and promotion of miR-15a expression. Therefore, salicin dimethyl ether may be developed as a therapeutic agent for the regulation of laryngeal cancer growth, however *in vivo* studies need to be conducted to confirm the effect.

Limitation of the study

The present study is only conducted using cell lines *in vitro*, therefore salicin dimethyl ether needs to be screened against laryngeal cancer growth *in vivo* in animal models. The toxicity and bioavailability of the compound in animal models of laryngeal growth is yet to be evaluated.

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