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Research Article

Anticancer Effect of Osmanthus Heterophilus Leaf Extract Through Inhibition of mTOR Phosphorylation And Upregulation of Cytokeratin 18 in Head and Neck Squamous Cell Carcinoma

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Abstract

Background: Head and neck squamous cell carcinoma (HNSCC) generally begins in the squamous cells of the head and neck mucosal surfaces, and are the sixth most common cancers worldwide. The leaf extract of *Osmanthus heterophyllus* contains lignans and secoiridoids, which have various biological activities including anticancer activity. This study aimed to investigate the potential anticancer effects of *O. heterophyllus* leaf extract on HNSCC FaDu cells and its underlying mechanism. **Methods:** The extract of *O. heterophyllus* leaves was obtained using a Dionex™ ASETM 350 Accelerated Solvent Extractor. After treating FaDu cells with the extract for 24 h, cell viability was evaluated using an MTT assay, and apoptosis-related proteins, mammalian target of rapamycin (mTOR) phosphorylation, and cytokeratin (CK) 18 expression were detected by western blotting. Cells were stained with an Annexin-V/PI double-staining assay using an Arthur image-based cytometric assay. **Results:** *O. heterophyllus* ethanol extract (OHEE) significantly reduced FaDu cell viability, increased cleaved caspase 3 and PARP, and also increased Annexin-V/PI double-stained cells. The expression of CK18 by OHEE increased while that of phospho-mTOR decreased, and the expression of CK18 decreased when mTOR phosphorylation was restored by glucose. **Conclusions:** OHEE induces apoptosis in HNSCC FaDu cells, which is associated with the regulation of mTOR and CK18.

Keywords: *Osmanthus Heterophyllus*; Head and Neck Squamous Cell Carcinoma (HNSCC); Anticancer; Mtor, Cytokeratin 18

Introduction

Cancer is a major public health concern owing to the high attendant mortality and high economic burden borne by patients and society [1, 2]. Cancer is a complex multifactorial disease influenced by interactions between the host and its environment, which is characterized by continuous angiogenesis, uncontrolled replication, apoptosis evasion, tissue invasion, and metastasis [3, 4]. The therapeutic modalities for cancer include surgery, radiation therapy, chemotherapy, hormone-based therapy, stem cell therapy, and dendritic cell-based immunotherapy [5]. Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, for which smoking and alcohol consumption are the principal risk factors [6]. Surgery, radiation therapy, and chemotherapy constitute the principal treatment methods for HNSCC, depending on the location and stage of the tumor. However, the prognosis remains poor despite advances in diagnostic procedures and treatment [7-9].

The mammalian target of rapamycin (mTOR), which regulates biological processes, such as cancer, immunity, metabolism, and aging, is an essential protein kinase of the PI3K family that forms a subunit of the mTOR 1 and 2 complexes. mTOR regulates protein synthesis, autophagy, and lipid metabolism. Moreover, it is involved in various neoplasms such as HNSCC, breast, lung, colon, cervical, genital, and bone cancers since it is an important signaling system in tumor apoptosis, the cell cycle, and cancer cell proliferation [10]. Dysregulation of mTOR signaling due to genetic alterations leads to metabolic reprogramming, resulting in several cancers including HNSCC [11]. The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mTOR pathway is affected by several validated mutated genes that are responsible for the activation of mTOR signaling in HNSCC [12, 13]. PIK3CA is a frequently identified mutation in HNSCC that activates PI3K [14].

Loss of the phosphatase and tensin homolog (PTEN) protein is more common in poorly differentiated tumors [15, 16] and advanced tumor stages [17, 18]. Additionally, hyper-activation of PI3K-mediated mTOR signaling leads to loss of PTEN expression [19]. HRAS mutations can also reportedly cause abnormal activation of mTOR [20, 21]. Conditional inhibition of TGFBR1 and PTEN in mouse models of HNSCC has also confirmed an association between transforming growth factor (TGF)-β and the PI3K-mTOR pathway [22]. Therefore, several studies have sought to devise methods to inhibit the abnormally activated PI3K/AKT/mTOR pathway in HNSCC [23]. Studies investigating the role of mTOR inhibitors in HNSCC have focused on rapamycin, rapamycin derivatives, temsirolimus, and everolimus, which showed inhibitory effects in in vitro and in vivo organ transplant models [24].

The Oleaceae family is a rich source of iridoid, secoiridoid, phenylpropanoid, and lignan glycosides [25, 26]. Three secoiridoid glycosides and 10 lignan glycosides are found in the leaves of *Osmanthus heterophyllus*, which belongs to this family [27, 28]. Lignans are secondary metabolites found in plants and have various biological activities, such as anticancer effects [29], while secoiridoids have anti-allergic, anti-arthritic, anti-bacterial, anti-coagulant, anti-inflammatory, wound healing, and anticancer activities [30].

No study has reported the anticancer activity of the *O. heterophyllus* leaf extract. Therefore, this study aimed to investigate the anticancer activity and mechanism of action of *O. heterophyllus* leaf extract on HNSCC.

Materials and Methods

Preparation of the Plant Extract

The leaves of O. heterophyllus were collected from the Jeollanam-do Forest Resources Research Institute (126°49’21.0”E longitude and 35°00’22.7”N latitude), in accordance with the Wild Animal and Plant Protection Act of Korea. First, the leaves of O. heterophyllus were washed and dried; subsequently, 5 g of the leaves were extracted with water, prethanol A, and water/prethanol A mixtures using the ASETM 350 Accelerated Solvent Extractor (Dionex™, Sunnyvale, CA, USA). The extraction process was performed at 70 ? for 5 min, followed by a reaction time of 15 min for a single cycle. The extracts were filtered using Whatman No. 42 filter paper (150 mm) and a 0.45-μm syringe filter. Thereafter, the extracts were concentrated using a rotary evaporator (SB-1300, EYELA, Tokyo, Japan) and lyophilized using a freeze dryer (FDCF-12012, OPERON, Gyeonggi-do, Republic of Korea). The lyophilized extracts were prepared at a concentration of 100 mg/mL for each extraction solvent, after which they were frozen and stored at a temperature of –20 °C.

Cell Culture

The human hypopharyngeal carcinoma cell line, FaDu, was purchased from the Korean Cell Line Bank (Seoul, Republic of Korea) and cultured in Minimum Essential Medium (Gibco®, Grand Island, NY, USA). The Human Embryonic Kidney (HEK) 293T, and Human Keratinocyte (HaCaT) cell lines were cultured in Dulbecco’s Modified Eagle Medium (Gibco®, Grand Island, NY, USA). Subsequently, 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin (Gibco®, Grand Island, NY, USA) were added to the medium, followed by culture at 37 °C under 5% CO2.

MTT Assay

FaDu cells were seeded at a concentration of 5×10^4 cells/well in 48-well plates. Cells cultured overnight were treated with various concentrations of *O. heterophyllus* extract. After incubating the extract for 24 h, 50 µL of a 5 mg/mL stock solution of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Aldrich) was added to each well and incubated at 37 °C for 2 h. Thereafter, the supernatant was removed, and 200 µL of dimethyl sulfoxide was added to each well, followed by melting on a horizontal shaker for 20 min. Optical density was determined by measuring the absorbance at 570 nm using a microplate reader (Biochrom Asys UVM 340, Cambridge, UK).

AuthurTM Image-based Cytometric Assay

The cells were seeded in 6-well plates at 1.2×10^6 cells/well. Cells cultured overnight were treated with different concentrations of *O. heterophyllus* extract in 50% prethanol A. After 24 h of extract treatment, the cells were washed with phosphate-buffered saline (PBS) and treated with Trypsin-EDTA solution (Gibco®, Grand Island, NY, USA). The harvested cells were washed with cold PBS and suspended in annexin-binding buffer (1X). Subsequently, 5 µL of Annexin V and 1 µL of 100 µg/mL PI solution (Molecular Probes, OR, USA) were added to 100 µL of the cells, and incubated at 25 °C for 15 min. The stained samples were pipetted and analyzed using an Arthur™ image-based cytometer (NanoEnTek Inc., Seoul, Republic of Korea).

Western Blot Analysis

The FaDu cells were plated in 100-mm dishes at a concentration of 1.2×10^6 cells/well. The cells were cultured overnight and treated with *O. heterophyllus* ethanol extract (OHEE) at different concentrations. After culturing for 24 h, the cells were washed twice with PBS. Radioimmunoprecipitation assay buffer (Biosolution Co., Ltd., Seoul, Republic of Korea) containing protease inhibitor cocktail III (ProGEN, Gyeonggi-do, Republic of Korea) was added to the cells, which were then scraped off the ice. The cell lysate was centrifuged at 16,000 × g at 4 °C for 30 min to separate the supernatant, which was used for protein expression analysis. Using the Pierce™ Rapid Gold BCA Protein Assay Kit (Thermo Fisher, Waltham, MA, USA), 30 µg of quantified protein was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, USA). The membrane was blocked with 5% skim milk in tris-buffered saline with 0.1% Tween® 20 detergent (TBST) for 1 h at room temperature, washed thrice with TBST, and incubated with the following primary antibodies overnight at 4 °C: antibodies against PARP (#9542, 1:1000, Cell Signaling Technology®), cleaved caspase-3 (#9664, 1:1000, Cell Signaling Technology®), β-actin (#3700, 1:5000, Cell Signaling Technology®), mTOR (#2983, 1:1000, Cell Signaling Technology®), phosphorylated (p)-mTOR (#5536, 1:1000, Cell Signaling Technology®), and cytokeratin (CK) 18 (1:5000, Santa Cruz Biotechnology). The membrane was washed four times with TBST buffer, and reacted with secondary anti-mouse HRP (#7076, 1:5000, Cell Signaling Technology®) and anti-rabbit HRP antibodies (#7074, 1:5000, Cell Signaling Technology®). West-Q Femto Clean ECL solution (W3680-010, GenDEPOT, Texas, USA) was used to detect protein luminescence, and the protein band levels were obtained using a UVITEC Alliance Q9 Micro Chemiluminescent Imaging System (Cambridge, England).

Statistical Analysis

Data were expressed as the mean ± standard deviation, and differences between groups were performed using one-way ANOVA followed by Tukey's post hoc analysis (IBM SPSS version 20.0, Armonk, NY, USA). p-values < 0.05 were considered statistically significant.

Results

Effect of *O. Heterophyllus* Leaf Extract on Cell Viability of HNSCC FaDu Cells

We treated the specimens with different doses of the extract and performed the MTT assay to determine the anticancer activity of the *O. heterophyllus* leaf extract in HNSCC FaDu cells. The survival rate of cancer cells reduced significantly depending on the extraction solvent (water or prethanol A) and concentration of the extract. The viability was reduced to 70.28 ± 2.13%, 52.18 ± 9.12%, 45.68 ± 6.2%, 51.68 ± 2.45%, and 65.77 ± 1.87% in 100 µg/mL of water, and 30%, 50%, 70%, and 95% prethanol A extract, respectively (Fig. 1). Therefore, the experiment was performed using 50% prethanol A extract (OHEE50), which greatly reduced the viability of the cells.

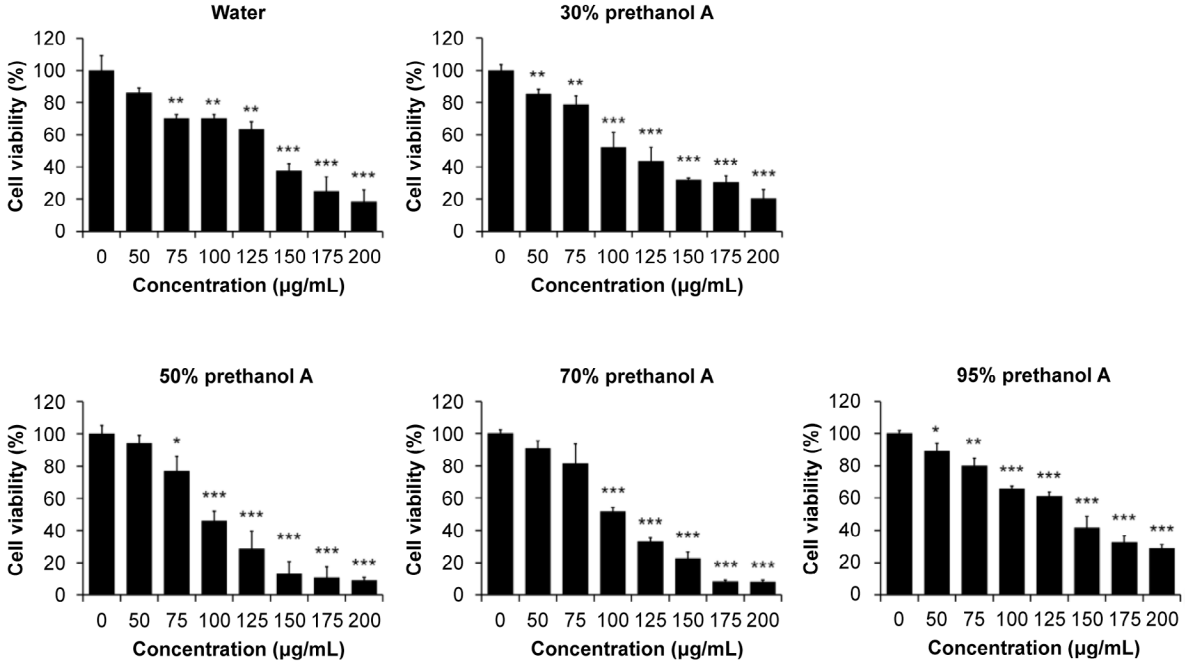

Figure 1: Inhibitory effect of the extracts obtained from *O. heterophyllus* leaves using different concentrations of prethanol A on the viability of HNSCC cells. HNSCC FaDu cells were plated and treated with various concentrations of extracts in water and prethanol A. Cell viability was measured via the MTT assay. All data are presented as the mean ± SD. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to the control.

OHEE Reduces Cell Viability through Apoptosis

After treatment with OHEE50, the cells were stained with Annexin-V/PI, and analyzed using the ArthurTM image-based cytometer to confirm apoptosis. The proportion of viable cells reduced significantly by 66.0 ± 2.52% and 34.5 ± 1.00% after treatment with 150 and 200 μg/mL of OHEE50, respectively, compared to the control group (Fig. 2A). Subsequently, to determine whether OHEE induced apoptosis in FaDu cells, the expression of apoptosis-related proteins was analyzed using western blot analysis. These results confirmed that the decrease in cell viability induced by OHEE50 was caused by apoptosis mediated by marked elevation in the levels of cleaved PARP and caspase 3 (Fig. 2B). Thereafter, the MTT assay was performed using the HEK 293T and HaCaT cells to confirm whether the OHEE50-induced reduction in FaDu cell viability was replicated in normal cells. However, the viability of the HEK 293T and HaCaT cells was not reduced (Fig. 2C).

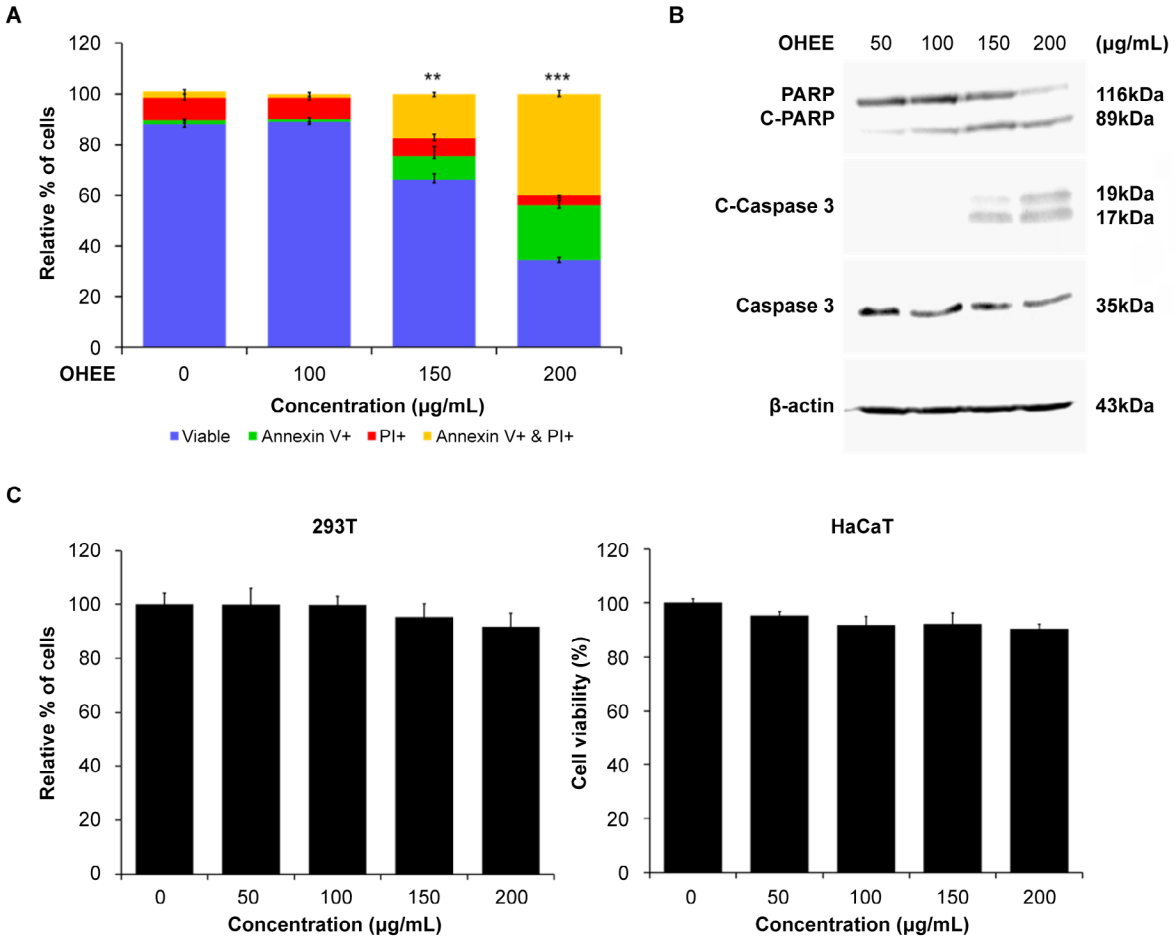

Figure 2: Apoptosis of HNSCC FaDu cells induced by 50% prethanol A extract of *O. heterophyllus* leaves (OHEE50). The cells were treated with the indicated concentrations of OHEE50 for 24 h. (A) Western blotting was used to analyze the expression of apoptosis-related proteins. (B) Apoptotic cell death was analyzed using Arthur image-based cytometry after annexin V and PI staining. Data are expressed as the mean ± SD. The Y-axis represents the percentage of cells that stained positive for annexin V (green), PI (red), annexin V and PI (yellow), or negative for annexin V and PI (blue). **$p < 0.01$, ***$p < 0.001$ compared to viable cells (C) Immortalized Human Embryonic Kidney (HEK 293T) cells and Keratinocytes (HaCaT) were plated and treated with OHEE50. Cell viability was measured using the MTT assay.

OHEE Regulates mTOR Phosphorylation and CK18 Expression

mTOR phosphorylation was reduced in FaDu cells with OHEE-induced apoptosis, and cell viability was restored when mTOR phosphorylation was increased, suggesting that mTOR phosphorylation is associated with FaDu cell survival [31]. Therefore, we investigated whether OHEE50 affects mTOR phosphorylation using western blot analysis. Our results showed that mTOR phosphorylation was markedly reduced by OHEE50 treatment (Fig. 3A), consistent with a previous study that reported that mTOR phosphorylation is reduced during FaDu cell death [31]. In contrast to mTOR phosphorylation, CK18 expression was markedly increased by OHEE50 treatment (Fig. 3B).

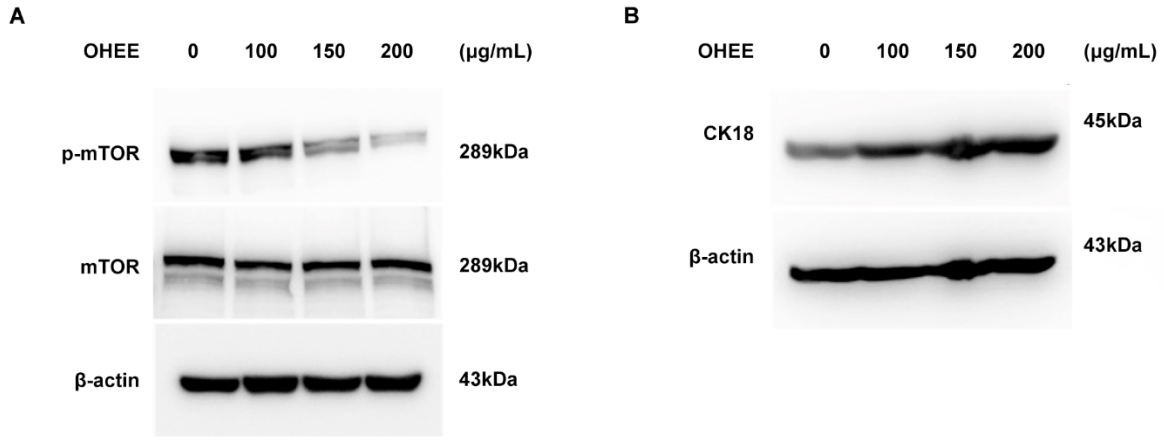

Figure 3: Treatment with OHEE induces apoptosis through the regulation of mTOR and CK18 expression. FaDu cells are treated with the indicated concentration of OHEE50 for 24 h. The cell lysates were analyzed by western blotting using anti-phospho-mTOR, anti-mTOR, anti-CK18, and β-actin antibodies.

OHEE Induces Apoptosis by Increasing CK18 Expression

Glucose can regulate mTOR activity and promote cancer cell survival [32, 33]. Western blot analysis confirmed that mTOR phosphorylation increased when FaDu cells were treated with glucose. The phosphorylation of mTOR by glucose increased significantly depending on the glucose concentration, whereas CK18 expression was significantly decreased (Fig. 4A). Subsequently, phosphorylation of mTOR, which was reduced by OHEE, was restored by glucose, and the change in CK18 expression were consistent with previous results (Fig. 4B). In addition, to confirm that the increase in phosphorylation of mTOR by glucose and the decrease in CK18 expression restored cell viability, the MTT assay was performed after treatment with glucose and OHEE50. The viability of FaDu cells, which was reduced at each of the different concentrations of OHEE, improved significantly depending on the glucose concentration (Fig. 4C).

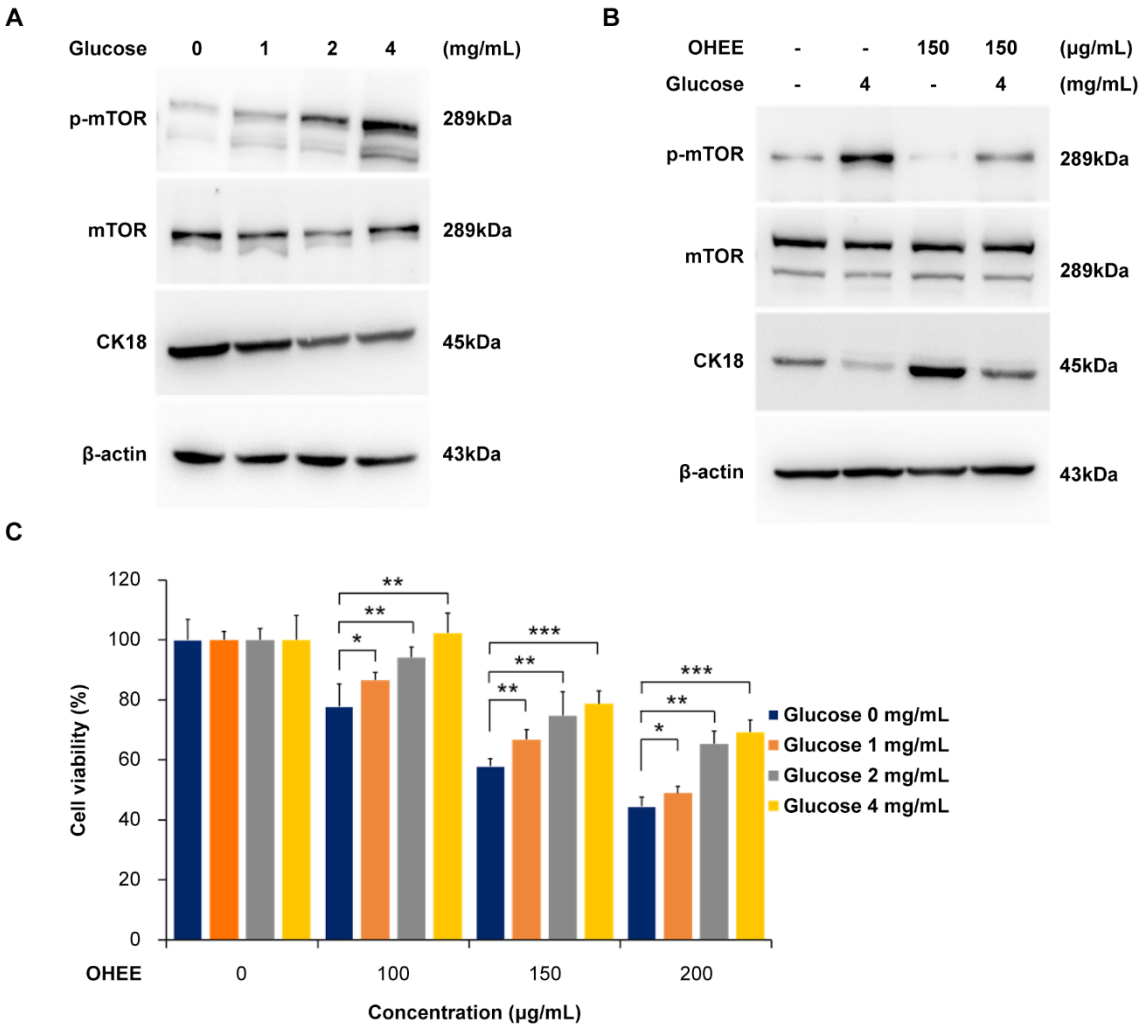

Figure 4: OHEE-induced apoptosis is regulated by CK18. (A) FaDu cells were cultured in a medium containing glucose for 24 h. (B) The cells were pretreated with glucose (4 mg/mL) for 24 h and treated with OHEE50 (150 μg/mL) for 24 h. Cell lysates were prepared and analyzed by western blot analysis using anti-phospho-mTOR, anti-mTOR, anti-CK18, and β-actin antibodies. (C) After the cells were pretreated with the indicated concentrations of glucose for 24 h, they were treated with OHEE50 at the indicated concentrations for 24 h. Cell viability was analyzed via the MTT assay. Data are presented as the mean ± SD. Statistical analyses were performed using one-way ANOVA. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

OHEE Reduces the Viability of Various Cancer Cell Lines

The effect of OHEE50 in reducing the viability of HNSCC cells in various cancer cell lines was confirmed using the MTT assay. Depending on the concentration of OHEE, cell viability significantly decreased in the cervical (HeLa), colon (HCT116), non-small cell lung (NCI-H1299), breast (MDA-MB-231), and gastric cancer cell lines (AGS) (Fig. 5).

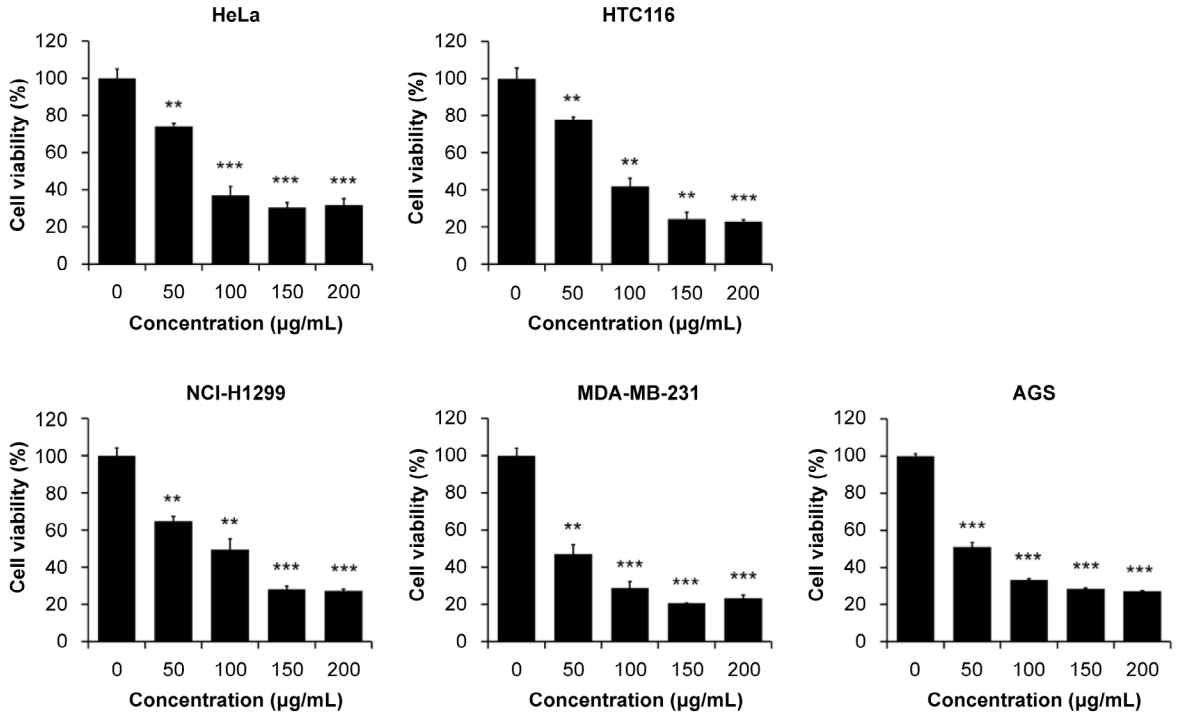

Figure 5: Inhibitory effects of OHEE on the viability of other cancer cells. The cells were plated and treated with various concentrations of OHEE50. Cell viability was measured by MTT assay. Data are presented as the mean±SD. **p<0.01, ***p<0.001 compared with the control.

Discussion

Chemotherapy exerts various pharmacological effects on cancer cells depending on the type of neoplasm; however, various adverse effects arising from the toxicity of anticancer agents have been identified. Therefore, to minimize the side effects and maximize the therapeutic effect of chemotherapy, researchers have attempted to develop anticancer drugs using natural plants products [34]. Therefore, our study focused on the anticancer effects of the leaf extract of *O. heterophyllus* in HNSCC.

Extracts obtained in both water and various concentrations of prethanol A significantly reduced the viability of HNSCC FaDu cells. This study used OHEE50, which was the most effective at inhibiting cell viability, and determined that the extract reduced cell viability through apoptosis. This was evidenced by the increased expression of cleaved caspase-3 and PARP, and increase in the number of annexin V/PI-stained cells. Furthermore, we found that mTOR phosphorylation was reduced by OHEE and that the reduction in cell viability was attenuated by the restoration of mTOR phosphorylation by glucose. These results are consistent with studies that reported that decreased phosphorylation of mTOR induces apoptosis in HNSCC cell lines [31]. CK18 expression was also increased or decreased when Akt1 was overexpressed or knocked down, respectively; CK18 expression was also increased by overexpression of Akt2, but not by knockdown, and was also mitigated by PI3K inhibitors. These results suggest that CK18 targets Akt in the PI3K/Akt pathway in epithelial carcinoma cells [35]. Consequently, the PI3K/Akt/mTOR pathway, which confers a competitive growth advantage, metastatic ability, angiogenesis, and treatment resistance in cancer, is activated [36], and since Akt can regulate CK18, it can be inferred that mTOR can regulate CK18. However, mTOR has not been reported to regulate CK18 expression in cancer cells. Furthermore, CK18 mRNA levels are increased in epithelial-specific knockout models of mTOR [37], mTOR signaling is abnormally activated in aged or degenerated retinal pigment epithelium, and CK18 expression is reduced [38]. These studies confirmed that CK18 can be regulated by mTOR; however, CK18 and mTOR seemed to have opposite expression behaviors in cancer cells, contradicting the assumption that CK18 is positively regulated by mTOR in cancer cells. Our results showed that CK18 expression increased when OHEE decreased mTOR phosphorylation, and CK18 expression decreased when

mTOR phosphorylation was restored by glucose. These results demonstrate that OHEE-induced apoptosis is the result of increased expression of CK18 by decreased phosphorylation of mTOR; thus, this is the first study to show that CK18 and mTOR may have opposite effects in cancer cells, contrary to prior assumptions about CK18 and mTOR. In addition, OHEE exerts anticancer effects by reducing cell viability in cervical, colon, non-small cell lung, breast, and gastric cancer cell lines.

Thus, this study showed that the extract obtained from *O. heterophyllus* leaves is a potential anticancer agent for HNSCC. However, further studies are needed to investigate the components of the extract that are responsible for the anticancer effects.

Conclusions

OHEE decreased cell viability and induced apoptosis in HNSCC FaDu cells by regulating mTOR and CK18. This study lays the foundation for formulating OHEE-based anticancer agents and contributes to the discovery of CK18-targeting components in OHEE.

Disclosure

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable in this section.

Availability of data and materials: The datasets supporting the conclusions of this article are available in the manuscript.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: DHJ performed the main experiments and data analyses and drafted the manuscript. DSO performed the data analysis. HWK and JK performed the data analysis and edited the manuscript. EC designed the study, performed the data analysis, supervised the research progress, and edited the manuscript. All the authors have read and agreed to the published version of the manuscript.

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