**Abstract.** Background/Aim: Casticin, one of the active components of Vitex rotundifolia L., presents biological and pharmacological activities including inhibition of migration, invasion and induction of apoptosis in numerous human cancer cells in vitro. This study aimed to assess the effects of casticin on tumor growth in a human oral cancer SCC-4 cell xenograft mouse model in vivo. Materials and Methods: Twenty-four nude mice were injected subcutaneously with SCC-4 cells and when palpable tumors reached a volume of 100-120 mm³ the mice were randomly divided into three groups. The control (0.1% dimethyl sulfoxide), casticin (0.2 mg/kg), and casticin (0.4 mg/kg) groups were intraperitoneally injected every two days for 18 days. Tumor volume and body weights were measured every two days. Results: Casticin significantly decreased tumor volume and weight in SCC-4 cell xenograft mice but there was no statistically significant difference between the body weights of control mice and mice treated with 0.2 mg/kg or 0.4 mg/kg casticin. Therefore, the growth of SCC-4 cells in athymic nude mice can be inhibited by casticin in vivo. Conclusion: These findings support further investigations in the potential use of casticin as an oral anticancer drug in the future.

Oral cancer has high mortality and morbidity (1) that appears on the lips, cheeks, tongue, gingiva, the floor of the mouth, hard and soft palate, sinuses, and pharynx (2). Oral squamous cell carcinoma (OSCC) is one of the leading cancers worldwide, representing over 90% of malignant neoplasms of the mouth (3, 4). However, OSCC occurs more frequently in individuals with oral bacterial infections such as higher levels of periodontal pathogenic bacteria in OSCC surfaces (5). In the USA, about 11.3 new cases of oral cancer per 100,000 people are diagnosed every year (6). In Canada,
4,600 new cases of oral cancer are diagnosed per year (7). In Taiwan, 12.1 individuals per 100,000 die annually from oral cancer and it is the fifth most common cancer based on the 2018 report from the Ministry of Health and Welfare, Taiwan, ROC (8), but betel chewing has been recognized to be one of the major factors for oral cancer in Taiwan (9). Currently, the treatments of oral cancer include primary surgery, chemotherapy and radiation therapy or the combination of chemo- and radio-therapy but the outcomes are still unsatisfied because of the side effects; thus, numerous studies have focused on finding new compounds from natural products.

Casticin (3', 5-dihydroxy-3, 4', 6, 7-tetramethoxyflavone), vitexicarpin, is a flavonoid (10, 11) from the Chinese herb Vitex Fructus (10) and is also present in other fruits, herbs, and spices (12). Casticin has long been used as an anti-inflammatory drug in traditional Chinese medicine (13, 14). It has been shown to exert biological and pharmacological effects, especially anti-cancer activities in vitro and in animal models in vivo (15-17). Casticin induced apoptosis in many human cancer cells such as bladder (18), cervical (19) and ovarian cells (20) and...
inhibited cell proliferation in leukemia cells (21) in vitro. Casticin suppressed esophageal cancer cell proliferation and induced apoptosis in vitro, and its in vivo anti-tumor action was shown to be partly mediated via mitochondrial-dependent apoptosis and activation of JNK signaling pathway (22). Furthermore, it has been reported that casticin induced DNA damage and suppressed DNA repair associated proteins in mouse melanoma B16F10 cells (23).

Besides, casticin suppressed EMT in hepatocellular carcinoma and inhibited lung cancer cell migration and invasion in vitro (24) and it also suppressed migration of mouse melanoma cells (25, 26). Moreover, casticin has been shown to present anti-inflammatory effects in preclinical models (13, 27). It has also been shown to inhibit lipopolysaccharide (LPS)-induced lung injury through affecting inflammatory cytokines (TNF-α, IL-6, and IL-1β) (28), and to attenuate liver fibrosis and hepatic stellate cell activation via the inhibition of the TGF-β/Smad signaling pathway (29). Thus, it may have therapeutic potential for inflammatory lung diseases (27).

Recently, we found that casticin promotes immune responses, enhances phagocytosis of macrophages and NK cell activities in animal models in vivo (30). However, the anti-tumor activity of casticin in animal models of human oral cancer cell xenografts is still unclear. Thus, in the present study, the antitumor activity of casticin in xenografted mouse models of SCC-4 human oral cancer cells were investigated. We found that casticin significantly reduced tumor volume in SCC-4 cell xenograft mouse in vivo.

Materials and Methods

Chemicals and Reagents. Casticin with a purity of 99%, cell culture grade dimethyl sulfoxide (DMSO), Tris-HCl, and trypan blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DMEM:F12 medium, fetal bovine serum (FBS), L-glutamine, and antibiotics (penicillin-streptomycin) were purchased from GIBCO®/Invitrogen Life Technologies (Grand Island, NY, USA). The stock solution of casticin (100 mg/ml) was dissolved in DMSO and diluted in cell culture medium before use. DMSO as used as the vehicle at 0.1%.

Cell line and culture. Human oral cancer SCC-4 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, R.O.C.) and cultured based on the supplier’s instructions. Cells were cultured in DMEM:F12 medium containing 10% FBS, 2 mM L-glutamine, and 1% antibiotics (100 Units/ml of penicillin and 100 μg/ml of streptomycin). SCC-4 cells were maintained at 37°C in a humidified atmosphere 5% CO₂ and 95% air in a 75 cm² tissue culture flasks as described previously (31).

Animals and treatments. Twenty-four athymic male mice (CAnN.Cg-Foxn1nu/CrlNarl nude mice) aged six weeks, with 20-25 g body weight, were purchased from the National Laboratory Animal Center, Taipei, Taiwan, and followed the National Institutes of Health Guidelines for Animal Research. All mice were housed in the Animal Center of China Medical University (Taichung, Taiwan, R.O.C.) and were adapted to the environment one week before the experiment. The animal study was approved and issued by the Institutional Animal Care and Use Committee of China Medical University (number: 105-17).

The experimental design is shown in Figure 1. Human oral cancer SCC-4 cells (1×10⁵) in 100 μl mixture containing serum-free DMEM:F12 medium and Matrigel (1:1) were subcutaneously
inoculated into the right hind legs of the 24 mice (32). All mice were randomized into three different treatment groups [control, 0.2 mg/kg (I), and 0.4 mg/kg (II) casticin groups] (n=8 for each group) when the tumor volume reached 100-120 mm$^3$ in each mouse. The tumor volume of the individual mouse was measured with a digital caliper and calculated with the equation: tumor volume=0.523×length×width$^2$ (32). The control animal group (n=8) was intraperitoneally injected every two days for 18 days with 90 μl phosphate-buffered solution (PBS) plus 10 μl DMSO. Experimental groups I and II were intraperitoneally injected every two days for 18 days with 0.2 mg/kg and 0.4 mg/kg casticin, respectively. Tumor growth, tumor volume, and body weight were monitored. After the final drug administration, all mice were sacrificed immediately and dissected for isolating tumor and weight tumor individually, as described previously (32).

**Statistical analysis.** The data are presented as the means±standard deviation (Mean±S.D.). The comparison between casticin-treated and control groups was examined by using one-way ANOVA with Newman-Keuls multi-comparison test. $p<0.05$ was considered to indicate a statistically significant difference between control and experimental groups.

**Results**

**Casticin affected the body weights in xenograft SCC-4 cell-bearing mice.** To determine the antitumor effects of casticin in vivo, SCC-4 cells were subcutaneously injected into the right hind legs of nude mice to establish an SCC-4 cell xenograft tumor model. During treatment with casticin, the body weight of each individual mouse of each group was measured and recorded every two days for a total of 18 days and the results are presented in Figure 2. Figure 2 indicates that the total body weight of each group was not altered following treatment without (control) or with casticin (0.2 mg/kg and 0.4 mg/kg of casticin). Furthermore, each mouse displayed normal behavior, indicative of good tolerability of casticin and no signs of acute or delayed toxicity of casticin in SCC-4 cell xenograft mice.

**Casticin inhibited SCC-4 cell xenograft tumor growth.** When the tumor size reached an approximate volume of 100-120 mm$^3$, the mice were treated with 0.1% DMSO/PBS or casticin (0.2 and 0.4 mg/kg) every two days for 18 days. The tumor size (volume) in each mouse of each group was measured every two days and the results are presented in Figure 3. After 16-days of treatment, casticin at 0.4 mg/kg slightly decreased the rate of increase of tumor volume (Figure 3A) and at the end of treatment, it significantly decreased tumor size (Figure 3B) compared to the control.
Discussion

The current study was based on our previous studies indicating that casticin was cytotoxic (reduced the total number of viable cells) through G2/M phase arrest and induction of apoptosis by caspase- and mitochondria-dependent pathways in SCC-4 cells in vitro (33). However, there was no information regarding the effects of casticin on SCC-4 cells in vivo; thus, we investigated the in vivo antitumor activity of casticin by using a mouse xenograft model. SCC-4 cells were injected into mice and tumor growth was monitored and recorded. The results indicated that after 18-day treatment, casticin suppressed tumor growth based on the reduction of tumor volume by 25% and 40% (Figure 3B) and tumor weight by 50% and 52% (Figure 5B), following treatment with 0.2 and 0.4 mg/kg/day of casticin, respectively. Furthermore, casticin had no significant effect on mouse body weight in subcutaneous xenograft tumors of human oral cancer SCC-4 cells-bearing mice in vivo. Therefore, in the future, we will examine the cytotoxic effects of casticin in vivo in normal mice.

Currently, the preventive and therapeutic protocols for patients with oral cancer depend upon the stage of cancer and the typical clinical treatments of oral cancer include surgery, radiation, and chemotherapy; however, drug resistance and side-effects (toxicity in normal cells) accompany treatment. Therefore, many studies have focused on new approaches or compounds from natural products to overcome the side effects of current chemotherapy drugs including cytotoxicity and drug resistance. Furthermore, we have reported that casticin was cytotoxic for cancer cells; decreased the total number of viable cells, induced cell cycle arrest, and apoptosis of SCC-4 cells in vitro. These findings are in agreement with other reports indicating that casticin suppresses the proliferation of different tumor cells (11, 22, 35, 36). Importantly, casticin had no effects on cell proliferation and prolactin release in non-stimulated primary pituitary cells in vitro (17).

Our results indicated that casticin significantly suppressed the tumor volume (Figure 3) and weight (Figure 5B) in SCC-4 cell xenograft nude mice in vivo and these findings are in agreement with our earlier reports in a human melanoma A375.S2 cell xenografted model in vivo (25).

It is well known that putative drugs should be investigated in cancer cells and then cancer cell xenograft animal models in vivo before used in clinical trials (34, 37-40). Herein, we demonstrate for the first time that casticin has anti-oral tumor potential in an animal model. Further investigations regarding the molecular mechanism of the inhibitory effects of casticin on tumor volume, size, and weight in SCC-4 cell xenograft nude mice in vivo are warranted.

In conclusion, casticin inhibited the growth of ectopic xenograft tumors of SCC-4 cells in vivo.

Conflicts of Interest

The Authors confirm that there are no conflicts of interest regarding this study.

Authors’ Contributions

Data curation: Hung-Sheng Shang, Kuo-Wei Chen, Jiann-Shang Chou, Shu-Fen Peng and Yung-Liang Chen.; Funding acquisition: Hung-Sheng Shang and Yung-Luen Shih.; Methodology: Hung-Sheng Shang, Po-Yuan Chen and Hsieh-Chou Huang.; Validation: Hung-Sheng Shang, Kuo-Wei Chen, Hsu-Feng Lu and Hsin-Yu Chang.; Writing – original draft: Hung-Sheng Shang, Shu-Fen Peng, Yung-Luen Shih and Wen-Wen Huang.; Writing – review and editing: Hung-Sheng Shang, Yung-Luen Shih and Wen-Wen Huang.

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