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Original Article

Soft coral Lobophytum crassum extract inhibits migration and induces apoptosis capabilities in human oral squamous cell carcinoma cells

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KEYWORDS
Oral squamous cell carcinoma;
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Abstract *Background/purpose:* The extract of the soft coral *Lobophytum crassum* has shown an anti-cancer activity in various cancer cells. However, its effect on the oral squamous cell carcinoma cell (OSCC) lines remains unclear. The purpose of this study is to investigate the anti-cancer effects of the extract of *L. crassum* (C127) on the OSCC cells.
Materials and methods: This study evaluated the effects of the soft coral extract of *L. crassum* (C127) on SAS and Ca9-22 cells, cell viability, migration ability, and apoptosis. Electric cell-substrate impedance sensing (ECIS) was parallelly used on SAS cells to confirm the results.

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Lobophytum crassum;
Marine-derived compounds

Results: C127 affected cell viability, morphology, and migration ability in both cell lines. In SAS cells, C127 inhibited cell viability in a dose dependent manner ($P < 0.001$) and induced apoptosis at 10 μg/mL ($P < 0.05$). In addition, C127 significantly inhibited migration ability on both cell lines in a dose-dependent manner ($P < 0.001$).
Conclusion: This study illustrated the potential of marine-derived compounds of *L. crassum* on its activity against OSCC cells.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is one of the most common cancers worldwide. Although the survival rates are higher for early-stage HNSCC, generally, the five-year survival rate ranges from approximately 40%–60%.[1] The acquired resistance in HNSCC cells resulted from the utilization of chemotherapies causes the poor treatment efficacy.[2] Therefore, the discovery of potential compounds especially natural ones for developing adjuvant or a combination chemotherapies has been focused for improving the therapy outcome in HNSCC.[3,4]

Scientists have been interested in marine natural products due to their distinct, unique structural diversifications and a wide range of medicinal potentialities based on novel mechanisms of action.[5] Since fifties, over a dozen medicines have been produced from or inspired by marine organisms, such as sponge and mollusk.[5] Given the newly discovered metabolites in soft corals display various bioactivities, soft corals have emerged as a hotspot for anti-cancer drug research.[6,7] The cembranoid metabolites sourced from the soft coral genera have shown anti-cancer, anti-bacterial, and anti-inflammatory activities.[8] However, the studies of the marine organism derived products in oral cancer research are limited.

In the *Lobophytum* genus, more than 15 of them have been investigated chemically.[9] *Lobophytum crassum* is one of the species has shown its therapeutic potential with more than ten cembranoid diterpene compounds exhibiting anti-cancer effects.[6,10,11] The extract of *L. crassum* exhibited a cytotoxic effect in leukemia cell lines and hepatocellular carcinoma.[7] One of its active compound 13-acetoxysarcocrassolide has shown an anti-proliferation effect in the oral squamous cell carcinoma (OSCC) from tongue via apoptosis and a noncanonical pathway.[12] Furthermore, the compound crassolide derived from wild type *L. crassum* has shown its toxicity in breast cancer cell lines.[13] Yet, the cytotoxicity of individual compound varies among different cell lines.[7,13] Compared to individual compounds, the extract of *L. crassum* has shown a relatively even cytotoxic effect among cell lines, which indicates the individual metabolites might have the coordinating effects on cancer cells.[7]

Since the aquaculture techniques of soft coral has improved in recent years, aquaculture *L. crassum* can be reproduced in a laboratory for a large amount, which could be a reliable resource for the development of marine drug. However, the effects of the extract of *L. crassum* in OSCC cell line, SAS cells, have not been studied yet. Therefore, for exploring the therapeutic potential of marine organism derived products on oral cancers, this study aimed to investigate the effects of the extract of *L. crassum* (C127) on the oral squamous cell carcinoma cells, SAS cells.

Materials and methods

Extract of the soft coral *L. crassum*

The soft coral extract C127 was kindly provided by Professor Mei-Chin Lu at National Dong Hwa University. The extraction of C127 was described in the previous publication.[7] Briefly, the aquaculture soft coral of *L. crassum* was freeze-dried then was thoroughly extracted using ethyl acetate. The residue from the reduced pressure evaporation of the ethyl acetate extract was dissolved in DMSO to produce the C127 solution with a concentration of 12.8 mg/mL.

Cell culture

The tongue squamous cell carcinoma cell line, SAS cells, and human gingival squamous carcinoma cell line, Ca9-22 cells, were acquired from the Bioresource Collection and Research Centre in Taiwan. SAS cells were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), and DMEM was for the culturing of CA9-22 cells. Media were enriched with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin mixture. Cells were maintained at a temperature of 37 °C in an environment with 5% CO_2 and adequate humidity. The culture medium was refreshed every other day, and cells were utilized for further passages or specific experiments upon reaching approximately 80% confluency. For the imaging studies, photographs of the cells were captured 24 h post the application of varying concentrations of C127 extract.

Cell viability

PrestoBlue (Thermo Fisher Scientific, Waltham, MA, USA) assay was employed to measure cell viability. Cells were exposed to varying concentrations of C127 (0, 1, 3, 10, and 30 μg/mL), and the viability assay was conducted at 24 h, 48 h, and 72 h. At each time point, viability dye was added

to the culture medium at a dilution of 1:10. The absorbance readings were taken at 570 nm and 600 nm. The cell viability percentage was determined using the reduction percentage of PrestoBlue, calculated by comparing the experimental readings against control.

Impedance measurements via electric cell-substrate impedance sensing

The ECIS ZΘ system, including its components, was sourced from Applied BioPhysics, located in Troy, NY, USA. The system utilized 8W1E arrays for the experiments, which comprise 8 wells each featuring a 250 μm diameter gold electrode for sensing. The ECIS method records the impedance between the cell substrate and the electrode, translating into measurements of cell attachment and spreading. A cell density of 1.25×10^5 cells/cm^2 was seeded into each well. When reaching confluency, cells were cultured for 24 h, followed by treating with a series concentration of C127 for additional 24 h. Impedance measurements were then taken across a range of frequencies to observe changes in cell morphology. These impedance measurements aid in the identification of morphological characteristics such as resistance in the cleft between cells and substrate (α) and resistance in the resistance cell–cell junction (Rb).[14]

Wound healing assay

Cells were seeded in 24-well plates and cultured to reach confluence. An 1 mL sterile pipette tip was then used to create a scratch across the monolayer of cells. Cells were then cultured with indicated concentration of C127 in serum free medium. The images were taken at 0, 6, and 12 h to monitor cell movement, and the wound area was quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Apoptosis assay

The Annexin V/PI staining method was utilized to determine and quantify apoptotic cells within the SAS cell populations following the manufacturer's guidelines (abcam, Cambridge, UK). The percentage of apoptotic cells was measured using FACSCalibur (BD, Becton Drive Franklin Lakes, NJ, USA).

Western blot

Proteins were extracted using RIPA buffer, and concentrations were determined via BCA Protein Assay. Proteins were separated by SDS-PAGE, transferred to PVDF membranes (Millipore, Billerca, MA, USA), and probed with anti-PARP (Cell Signaling Technology, Danvers, MA, USA). The signals were then quantified by the SignalBoostTM Immunodetection Enhancer kit (Calbiochem, San Diego, CA, USA) and ImageJ software (National Institutes of Health, Bethesda, MA, USA) respectively.

Statistics

Data were statistically analyzed using one-way ANOVA to identify significant differences between control and treated groups followed by post-hoc Tukey's multiple comparisons tests. *P*-value of less than 0.05 indicated statistical significance.

Results

C127 inhibits cell viability of OSCCs

Fig. 1 shows the optimal frequency for detecting SAS cells is at 4 kHz where the increased resistance caused by the cells were most significant. The result of ECIS at the end of the 24 h treatment is consistent to the viability assay (Fig. 2A). As shown in Fig. 2A and B, C127 exhibited significant toxicity to SAS cells and Ca9-22 cells at the concentrations of 10 and 30 μg/mL. The 72 h IC_{50} values of C127 against SAS and Ca9-22 cells were 5.4 ± 2.2 and 15.7 ± 1.4 μg/mL respectively. Parallelly, ECIS was used to analyze cell viability and morphology by detecting the resistance change. First, the multiple frequency scanning was conducted by ECIS for optimizing the current frequency for detecting SAS cells. In addition, the real-time ECIS data also showed the dynamics of viability during the experiment (Fig. 2C).

C127 changes the morphology

In the phase-contrast images of cells, both types of cells expended mildly at low concentrations (1 and 3 μg/mL) and exhibit a shrunken morphology without detaching at the

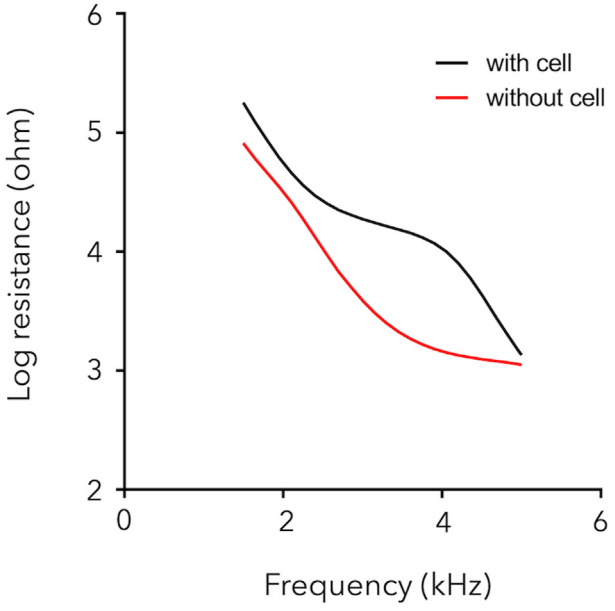

Figure 1 The multiple frequency scanning was conducted by ECIS for optimizing the current frequency for detecting cell behaviors. 4,000 Hz is the optimized frequency for detecting SAS cells.

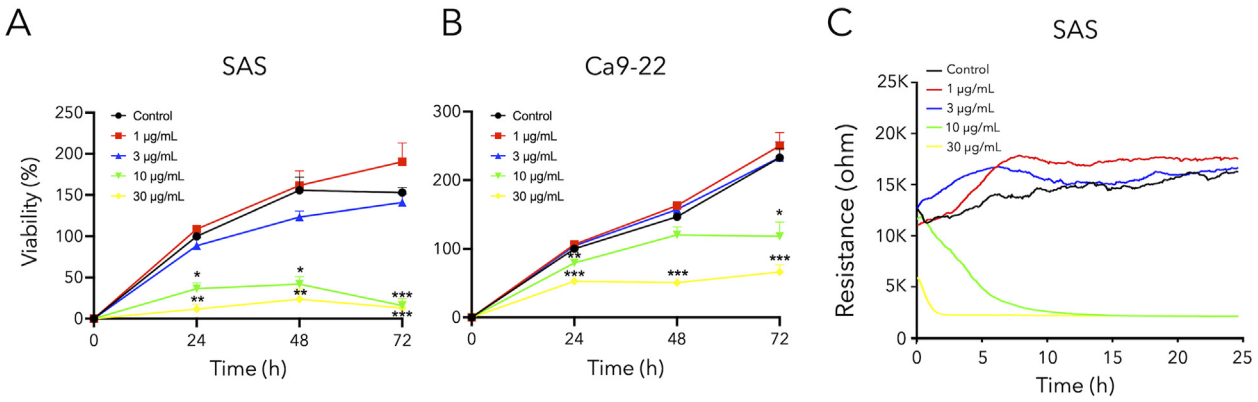

Figure 2 Cell viability assay of (A) SAS cells and (B) Ca9-22 cells on C127 treatment for 72 h at the indicated concentrations by PrestoBlue assay. (C) 24 h viability assay of SAS cells by ECIS system.

concentration above 10 μg/mL after 24 h (Fig. 3A). At low concentrations of C127, the increase in cell size is also shown in the ECIS results with the increased Rb value (Fig. 3B) and alpha value (Fig. 3C) which resulted from the cell–cell junction and cell-substrate adhesion respectively. Consistently, decreased Rb value and alpha value were observed at higher concentrations of C127 (10 and 30 μg/mL) (Fig. 3B and C), which reflects the shrunken morphology shown in Fig. 3A. However, there is no statistical difference between groups.

C127 inhibits the ability of migration

A wound healing assay was employed to assess the effect of C127 on OSCC migration. As depicted in Fig. 4A—SAS cells treated with C127 exhibited significantly reduced migration compared to the control group at concentrations exceeding 3 μg/mL, at both the 6 h ($P < 0.05$) and 12 h ($P < 0.001$) time points. Similarly, Ca9-22 cells displayed inhibited migration at higher concentrations (≥10 μg/mL) at 6 h ($P < 0.01$) and at the concentrations above 3 μg/mL after 12 h ($P < 0.001$).

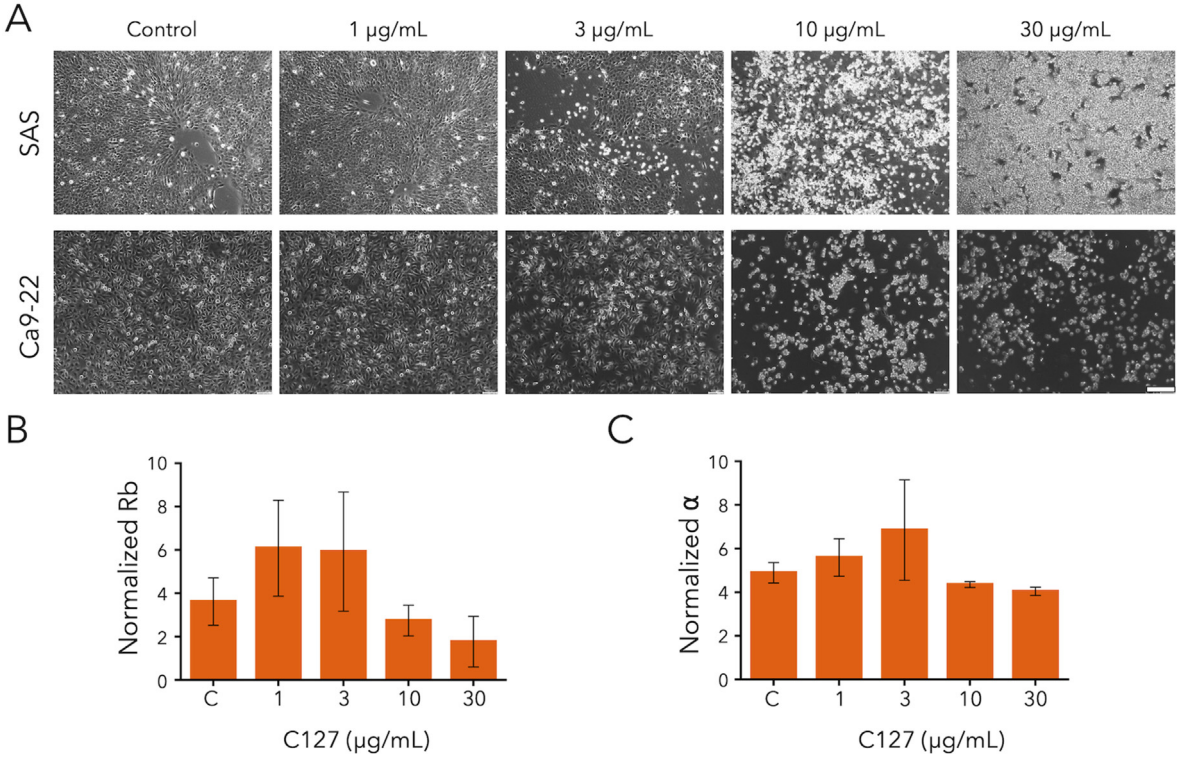

Figure 3 (A) Morphology of SAS and Ca9-22 cells after a 24-h-culture at a series concentration of C127. Scale bar, 200 μm. (B) Rb value revealed the resistance resulted from the cell–cell junction which decreased at 10 and 30 μg/mL. (C) Alpha value shows the cell-substrate adhesion level. The lower value of α showed the decreased cell-substrate adhesion at 10 and 30 μg/mL.

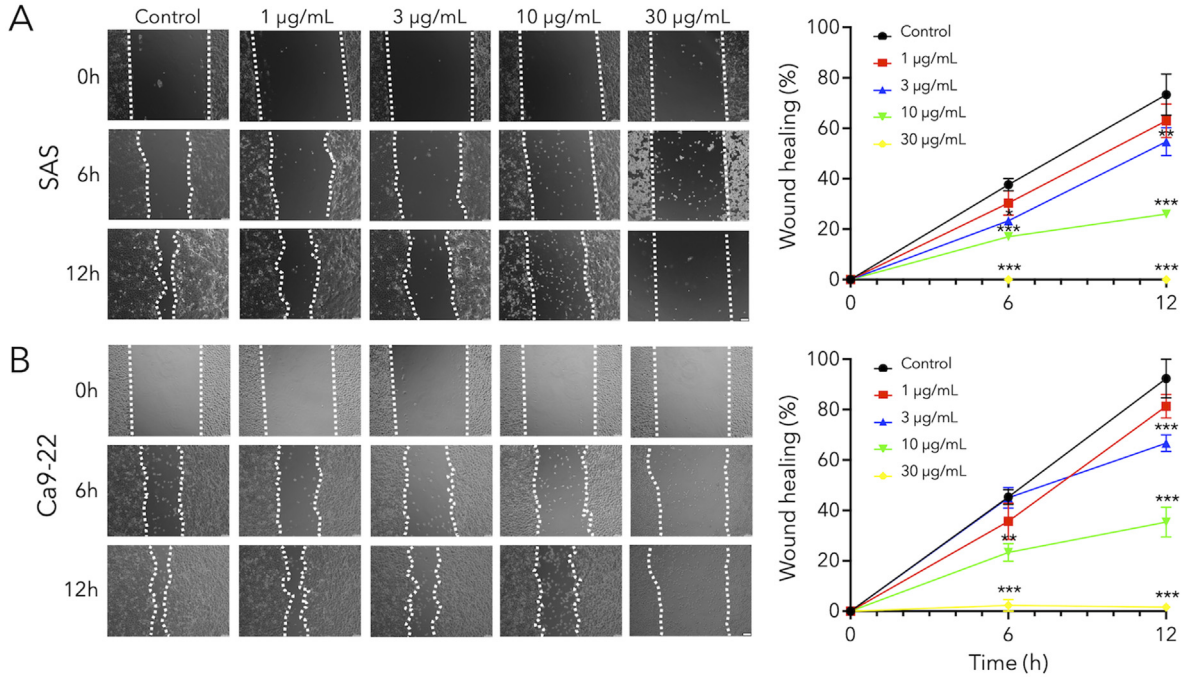

Figure 4 The analysis of the effect of C127 on migration ability in (A) SAS cells and (B) Ca9-22 cells. Scale bar, 100 μm *$P < 0.05$, **$P < 0.01$, ***$P < 0.01$ compared to control group.

These observations suggest that C127 impedes the migratory ability of OSCCs in a dose-dependent manner.

C127 affects the micromotion of SAS cells

In the cell micromotion assay, normalized resistance curves were measured with multiple frequency-scan after treated with different concentrations of C127 for 24 h 4 kHz was found to be a proper frequency for analyzing how SAS cells reacts to C127 at which the resistance of SAS cells changed distinctively at different concentrations of C127 (Fig. 5A). It has been shown that cell micromotion detected by ECIS is correlated to cell metabolic activity.[15] The micromotion of SAS cells in response to different concentrations of C127 were detected by ECIS (Fig. 5B). By calculating the fluctuation of cells in each group, the cell activities of SAS cells significantly decreased in a dose dependent manner (Fig. 5C).

Apoptosis is involved in C127-induced cell death

Flow cytometry analysis with Annexin V staining revealed a significant increase in the percentage of apoptotic SAS cells following a 24-h incubation with C127, in a dose-dependent manner (Fig. 6A and B). The percentage of cells in both early and late stages of apoptosis was significantly increased at concentrations of 10 and 30 μg/mL compared to the control group ($P < 0.05$; $P < 0.001$). Western blot analysis further corroborated these findings (Fig. 6C). Cleaved poly (ADP-ribose) polymerase (PARP), a marker of caspase-dependent apoptosis, was significantly elevated in SAS cells treated with C127 compared to the control group, indicating activation of the apoptotic pathway ($P < 0.05$).

Discussion

Marine drugs have been studied for over half a century.[5] Although the cytotoxic effect of *L. crassum* has been widely studied in various types of cancer cell lines, the effects of *L. crassum* derived products in OSCCs remain largely unclear. This study investigated the effects of the extract of the soft coral *L. crassum* on cell proliferation and migration ability in SAS and Ca9-22 cells. It has been reported the major bioactive compound in *L. crassum*, 13-acetoxysarcocrassolide, inhibits cell proliferation and migration in Ca9-22 cells.[12] In our study, the aligned results were observed in higher concentration of C127 treated groups (Figs. 2 and 4). Consistently, reduced cell viability was reported in two lung cancer cell line treated with crassolide extracted from wild type *L. crassum*.[16]

In our study, apoptosis in SAS cells was trigged by C127 with the increased level of cleaved PARP (Fig. 6). This result is consistent with that in human lung cancer cells treated with crassolide extracted from *L. crassum*. Crassolide has shown to induce apoptosis by triggering the reactive oxygen species-mediated endoplasmic reticulum stress.[16] However, previous study has reported 13-acetoxysarcocrassolide also triggered apoptosis via a non-canonical pathway with the involvement of Keap1/Nrf2 pathway in OSCCs, which might result from the unique structural features of marine derived products.[12] In addition, Nrf2 is required for the cell migration in various cancer cells.[17,18] The involvement Keap1/Nrf2 pathway in the induction of apoptosis and the inhibition of cell migration of C127 need to be further investigated in oral cancers.

Interestingly, at a concentration of 3 μg/mL, C127 significantly inhibited the migration ability of both SAS and Ca9-22 cells for 12 h (Fig. 4), despite not exhibiting a

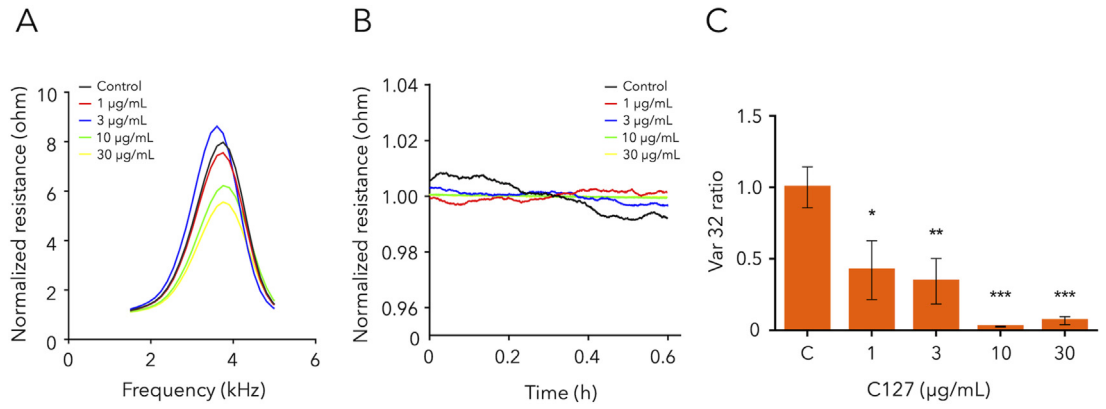

Figure 5 (A) The multiple frequency scanning for identify the optimal frequency. The resistance change is distinct between groups, and the frequency at 4 kHz is suitable for the analysis of cell micromotion in the presence of C127. (B) RTC detection and (C) Var32 analysis showed the activity of SAS cells after treated with C127 for 24 h at the indicated concentrations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Each value is expressed as a percentage of the control.

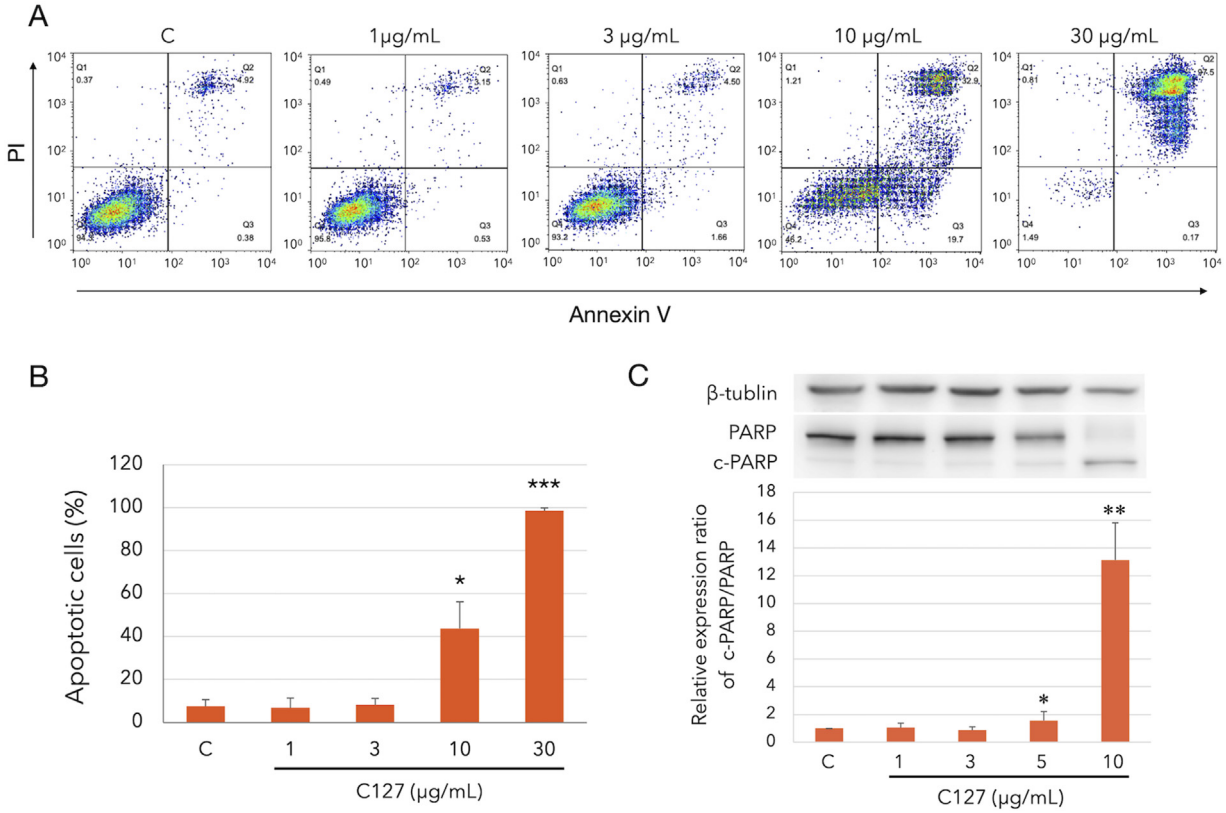

Figure 6 (A) Flow cytometry analysis with Annexin V staining to assess apoptosis in SAS cells treated with different C127 concentrations (1, 3, 10, 30 μg/mL) for 24 h. (B) Percentage of apoptotic cells (combining early and late stages) after 24-h incubation with C127. (C) Western blot result of the relative expression ratio of cPARP/PARP. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to control group.

cytotoxic effect on SAS cells at this concentration (Fig. 2). This observation is noteworthy considering SAS cells are generally considered a more aggressive OSCC lineage compared to Ca9-22 cells.[19] Furthermore, the IC_{50} value of C127 against SAS cells was significantly lower than that against Ca9-22 cells ($P < 0.01$), indicating a selective targeting effect. This selectivity is reminiscent of other compounds like paclitaxel, evodiamine, and kaempferol.[20] However, further studies are warranted to elucidate the underlying mechanisms responsible for the selective anti-migration effect of C127 in OSCC cells.

A reduction of cell micromotion can be observed accompanied with cytoplasmic shrinkage when apoptosis is induced.[21] C127 at the concentrations above 10 μg/mL induced apoptosis in SAS cells (Fig. 6), and also caused a decrease of overall resistance (Fig. 2C). In general, Var32

ratio reflects the decrease of cell fluctuation in apoptosis. We found that the Var32 ratio reduced significantly in a dose dependent manner (Fig. 6), which revealed that C127 affected cell micromotion without inhibiting cell viability and the migration ability.

In conclusion, the variety of marine life and their distinct biochemical profiles has resulted in the discovery of a plethora of biologically active natural compounds produced from marine species, making them an important source for developing new medications. This study demonstrated the potential of marine-derived substances, specifically *L. crassum*, on its activities against the cancer cell line, SAS cells.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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References

1. Tiwana M, Wu J, Hay J, Wong F, Cheung W, Olson R. 25 year survival outcomes for squamous cell carcinomas of the head and neck: population-based outcomes from a Canadian province. *Oral Oncol* 2014;50:651—6.
2. Patil VM, Noronha V, Joshi A, et al. Phase I/II study of palliative triple metronomic chemotherapy in platinum-refractory/early-failure oral cancer. *J Clin Oncol* 2019;37:3032—41.
3. Roh JL, Kim EH, Jang H, Shin D. Nrf2 inhibition reverses the resistance of cisplatin-resistant head and neck cancer cells to artesunate-induced ferroptosis. *Redox Biol* 2017;11:254—62.
4. Lin SR, Chang CH, Hsu CF, et al. Natural compounds as potential adjuvants to cancer therapy: preclinical evidence. *Br J Pharmacol* 2020;177:1409—23.
5. Haque N, Parveen S, Tang T, Wei J, Huang Z. Marine natural products in clinical use. *Mar Drugs* 2022;20:528.
6. Kao CY, Su JH, Lu MC, et al. Lobocrassins A—E: new cembrane-type diterpenoids from the soft coral Lobophytum crassum. *Mar Drugs* 2011;9:1319—31.
7. Peng BR, Lu MC, El-Shazly M, Wu SL, Lai KH, Su JH. Aquaculture soft coral Lobophytum crassum as a producer of anti-proliferative cembranoids. *Mar Drugs* 2018;16:15.
8. Nurrachma MY, Sakaraga D, Nugraha AY, et al. Cembranoids of soft corals: recent updates and their biological activities. *Nat Product Bioprospecting* 2021;11:243—306.
9. Rajendra S, Raghunathan C. A new species and new record of the soft coral genus Lobophytum (Octocorallia: Malacalcyonacea: Sarcophytidae) from the Andaman Islands, India. *Thalassas* 2024 (in press).
10. Lee NL, Su JH. Tetrahydrofuran cembranoids from the cultured soft coral Lobophytum crassum. *Mar Drugs* 2011;9:2526—36.
11. Lai KH, You WJ, Lin CC, El-Shazly M, Liao ZJ, Su JH. Anti-inflammatory cembranoids from the soft coral Lobophytum crassum. *Mar Drugs* 2017;15:327.
12. Liu YC, Peng BR, Hsu KC, et al. 13-Acetoxysarcocrassolide exhibits cytotoxic activity against oral cancer cells through the interruption of the Keap1/Nrf2/p62/SQSTM1 pathway: the need to move beyond classical concepts. *Mar Drugs* 2020;18:382.
13. Tsai KC, Chen CS, Su JH, Lee YC, Tseng YH, Wei WC. The blockade of mitogen-activated protein kinase 14 activation by marine natural product crassolide triggers ICD in tumor cells and stimulates anti-tumor immunity. *Mar Drugs* 2023;21:225.
14. Liao HY, Huang CC, Chao SC, et al. Real-time monitoring the cytotoxic effect of andrographolide on human oral epidermoid carcinoma cells. *Biosens Bioelectron* 2022;12:304.
15. Opp D, Wafula B, Lim J, Huang E, Lo JC, Lo CM. Use of electric cell—substrate impedance sensing to assess in vitro cytotoxicity. *Biosens Bioelectron* 2009;24:2625—9.
16. Lai KM, Wang JH, Lin SC, et al. Crassolide induces G2/M cell cycle arrest, apoptosis, and autophagy in human lung cancer cells via ROS-mediated ER stress pathways. *Int J Mol Sci* 2022;23:5624.
17. Chang CW, Chen YS, Tsay YG, et al. ROS-independent ER stress-mediated NRF2 activation promotes warburg effect to maintain stemness-associated properties of cancer-initiating cells. *Cell Death Dis* 2018;9:194.
18. Tanaka Y, Hamada S, Matsumoto R, Taguchi K, Yamamoto M, Masamune A. Nrf2 expression in pancreatic stellate cells promotes progression of cancer. *Am J Physiol Gastrointest Liver Physiol* 2021;321:G378—88.
19. Yang CM, Chang HS, Chen HC, et al. Low C6orf141 expression is significantly associated with a poor prognosis in patients with oral cancer. *Sci Rep* 2019;9:4520.
20. Ogasawara M, Matsubara T, Suzuki H. Screening of natural compounds for inhibitory activity on colon cancer cell migration. *Biol Pharm Bull* 2001;24:720—3.
21. Huang CC, Tung TH, Huang CC, et al. Electrochemical assessment of anticancer compounds on the human tongue squamous carcinoma cells. *Sensors* 2020;20:2632.