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Protective effect of *Sasa veitchii* extract against *all-trans*-retinoic acid-induced inhibition of proliferation of cultured human palate cells

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ABSTRACT

Cleft palate is the most common facial birth defect worldwide. It is caused by environmental factors or genetic mutations. Environmental factors such as pharmaceutical exposure in women are known to induce cleft palate. The aim of the present study was to investigate the protective effect of *Sasa veitchii* extract against medicine-induced inhibition of proliferation of human embryonic palatal mesenchymal cells. We demonstrated that *all-trans*-retinoic acid inhibited human embryonic palatal mesenchymal cell proliferation in a dose-dependent manner, whereas dexamethasone treatment had no effect on cell proliferation. Cotreatment with *Sasa veitchii* extract repressed *all-trans*-retinoic acid-induced toxicity in human embryonic palatal mesenchymal cells. We found that cotreatment with *Sasa veitchii* extract protected *all-trans*-retinoic acid-induced cyclin D1 downregulation in human embryonic palatal mesenchymal cells. Furthermore, *Sasa veitchii* extract suppressed *all-trans*-retinoic acid-induced *miR-4680-3p* expression. Additionally, the expression levels of the genes that function downstream of the target genes *(ERBB2* and *JADE1)* of *miR-4680-3p* in signaling pathways were enhanced by cotreatment with *Sasa veitchii* extract and *all-trans*-retinoic acid compared to *all-trans*-retinoic acid treatment. These results suggest that *Sasa veitchii* extract suppresses *all-trans*-retinoic acid-induced inhibition of cell proliferation via modulation of *miR-4680-3p* expression.

Keywords: *Sasa veitchii*, cleft palate, microRNA, *all trans*-retinoic acid

Abbreviations:
*at*RA: *all-trans*-retinoic acid
Cu-Chl: copper chlorophyllin
CCND1: cyclin D1
DEX: dexamethasone
ERBB2: Erb-B2 receptor tyrosine kinase 2
HEPM: human embryonic palatal mesenchymal
JADE1: jade family PHD finger 1
miRNA: microRNA
SE: *Sasa veitchii* extract

INTRODUCTION

Cleft palate is one of the most common birth defects worldwide (affects approximately 1:700 babies). Since cleft palate affects speech, hearing, and feeding capabilities, patients with cleft palate receive multidisciplinary care, which includes not only physical repair and treatment, but also speech and psychiatric therapy for a long time.[1,2] However, long-term treatment is expensive for the patient. Therefore, it is important to prevent the onset of cleft palate.

The etiology of cleft palate is multifactorial and involves genetic and environmental factors. For example, disruption of signaling pathways, including bone morphogenetic protein, sonic hedgehog protein, and WNT signaling, induces cleft palate.[3,4] A systematic review has revealed that more than 130 genes are associated with cleft palate.[5] Environmental factors such as cigarette smoking, alcohol consumption, and medication use are well-known risk factors for cleft palate, as they lead to suppression of the expression of specific genes or signaling pathways.[6,7] As for medicine, the association with *all-trans* retinoic acid (*at*RA) and cleft palate is well-established.[8] *at*RA is a derivative of vitamin A that plays a crucial role in a variety of biological processes, including cell proliferation, differentiation, and extracellular matrix production.[9] While *at*RA is widely prescribed in the treatment of cancer and skin disease,[10,11] long-term use of *at*RA is associated with birth defects including cleft palate.[12,13] Although *at*RA-induced cleft palate is believed to involve many signaling pathways, such as transforming growth factor/Smad, bone morphogenetic protein, and mitogen-activated protein kinase,[14-16] it is still unclear how *at*RA causes cleft palate. Moreover, although novel gene mutations associated with cleft palate have been reported, it remains unclear how genetic and environmental factors affect cleft palate.

In humans, the palate develops from two primordia: the primary and the secondary palates. Formation of the secondary palate is initiated during the 6th week of gestation and maturation occurs during the 10th week. The two developing palatal shelves present on each side of the tongue are directed downward starting from the 6th–7th week. During the initial part of the 7th–8th week, the tongue is withdrawn from between the shelves, and subsequently, the shelves elevate and fuse with each other above the tongue and with the primary palate. Finally, the medial epithelial seam disintegrates either by apoptosis, migration toward epithelial triangles on both the oral and the nasal sides, or epithelial–mesenchymal transition, and thereby, palatal fusion is completed by the 10th week.[17,18] Any failure in the occurrence of these processes leads to cleft palate. Folic acid is known to decrease the risk of cleft palate by reducing the incidence of fusion defects and attenuating oxidative stress.[19-21] Although dietary intake of folic acid is the first choice to prevent cleft palate, searching for an alternative method is also important for patients with anomalies in folic acid metabolism. Kampo formula is commonly prescribed for pregnant women and is popular for the treatment of various diseases. Although Kampo formula has not been reported to protect against cleft palate, we hypothesized that Kampo formula and other naturally derived products might have protective effects against cleft palate.

Sasa veitchii belongs to the Gramineae family and has been used as a folk medicine and health-promoting food in Asia. In Japan, its leaves are used as food-wrapping material to prevent food from rotting. *Sasa veitchii* extract (SE) shows multiple protective effects, including antitumor, antihyperglycemic, antioxidant, and antiviral activities.[22-27] Moreover, SE is known to decrease the risk of craniofacial diseases such as periodontal diseases and gingivitis. Therefore, SE might show protective effects against other craniofacial diseases as well. In the present study, we examined the protective effects shown by SE against *at*RA- or dexamethasone (DEX)-induced inhibition

of proliferation of human embryonic palatal mesenchymal (HEPM) cells.

MATERIALS AND METHODS

Cell culture

HEPM cells were obtained from the JCRB Cell Bank (JCRB9095, Osaka, Japan) and maintained in Minimum Essential Medium Eagle-alpha modification (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Biowest, Paris, France) and penicillin/streptomycin (Fujifilm Wako Pure Chemical Corporation) at 37 °C in a humidified atmosphere with 5% CO_2.

Preparation of SE

SE was obtained from Sunchlon Co, Ltd. (Nagano, Japan). One milliliter of SE was made from 2.82 g of *Sasa veitchii* leaves according to the company data. The procedure to prepare an SE sample was shown in Supplementary Figure S1 and previously reported.[28]

Cell proliferation assay

HEPM cells were plated in 96-well plates at a density of 5,000 cells per well and treated with various concentrations of *at*RA (Fujifilm Wako Pure Chemical Corporation), DEX (Fujifilm Wako Pure Chemical Corporation), SE (Sunchlon), or copper chlorophyllin (Nacalai Tesque, Kyoto, Japan). After 48 h of treatment, cell viability was evaluated by the Alamar Blue assay (Bio-Rad Laboratories, Hercules, CA).

Quantitative RT-PCR

HEPM cells were plated on 35-mm cell culture dishes at a density of 400,000 cells per plate and treated with 100 μM *at*RA and 0.03% SE. After 48 h of treatment, total RNA was extracted using the QIAshredder and miRNeasy Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol (n = 3–5). Reverse transcription-PCR conditions have been previously described.[29] The levels of the target mRNAs were normalized according to the β-actin levels. The oligonucleotide sequences of the primers used were as follows: human β-actin (NM_001101): sense, 5′-ACCTTCTACAATGAGCTGCGTG-3′, and antisense, 5′-TGGGGTGTTGAAGGTCT-CAAAC-3′; human Erb-B2 receptor tyrosine kinase 2 (ERBB2; NM_004448): sense, 5′-ACCTG-GATGACAAGGGCTG-3′, and antisense, 5′-CGCTTGATGAGGATCCCAAAG-3′; and human jade family PHD finger 1 (JADE1; NM_199320): sense, 5′-CGGGAGCAGGATGTCTTATTTAGG-3′, and antisense, 5′-TGTTCCTGGACTTTGCACACAG-3′. For the analysis of microRNAs (miR-NAs), 25 ng of total RNA was reverse-transcribed using miRNA reverse transcription reaction kit (Poly A Polymerase, RTase Mix, and 5× Reaction Buffer; GeneCopoeia, Rockville, MD) and incubated for 60 min at 95 °C and 5 min at 85 °C. miRNA expression was examined using an All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia) according to the manufacturer's instructions. Probes for *miR-4680-3p* (HmiRQP2296) and U6 (HmiRQP9001) were obtained from GeneCopoeia. The PCR conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 sec, 60 °C for 20 sec, and 72 °C for 10 sec. The amounts of the target miRNAs were normalized according to the quantity of the miRNA encoding U6.

Apoptosis assay

HEPM cells were plated in 24-well plates at a density of 50,000 cells per well and treated with 100 μM *at*RA and/or 0.03% SE. After 48 h of treatment, apoptosis-positive cells were

detected using Apotracker Green (BioLegend, San Diego, CA) according to the manufacturer's instructions. The nuclei were counterstained with Hoechst 33342 (Nacalai Tesque). A total of six fields were used for the quantification of apoptosis-positive cells.

Western blot analysis

HEPM cells (35mm dish) were homogenized with 100 μL ice-cold RIPA buffer (Nacalai Tesque) containing a protease inhibitor and centrifuged (18,000 × *g* for 20 min at 4 °C). The resulting supernatants were collected, and protein levels were determined using a BCA protein assay kit (Nacalai Tesque). Protein samples (10 μg) were subjected to 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Rabbit anti-cleaved caspase-3 polyclonal antibody (1:2,500 dilution; Cell Signaling Technology, Beverly, MA), mouse anti-cyclin D1 (CCND1) monoclonal antibody (1:1,000 dilution; Santa Cruz Biotechnology, Dallas, TX), mouse anti-CCNA (1:1,000 dilution; Santa Cruz Biotechnology), anti-mouse CCNB (1:1,000 dilution; Santa Cruz Biotechnology), anti-mouse CCNE (1:1,000 dilution; Santa Cruz Biotechnology), and anti-mouse β-actin monoclonal antibody (1:2,500 dilution; MBL, Aichi, Japan) were used as primary antibodies for immunoblotting. A peroxidase-conjugated anti-rabbit immunoglobulin G (IgG; Cell Signaling Technology) and peroxidase-conjugated anti-mouse IgG was used as a secondary antibody (1:5,000 dilution). The immunoreactive bands were visualized by Western Blot Hyper HRP Substrate (Takara Bio, Shiga, Japan). Band intensity was measured using Image J software (NIH, Bethesda, MD).

Statistical analyses

All statistical analyses were performed using the SPSS software (version 24.0; SPSS, Chicago, IL). Multiple comparisons were evaluated using one-way analysis of variance (ANOVA) followed by the post-hoc Tukey-Kramer test. Statistical significance was set at $p < 0.05$.

RESULTS

Pharmaceutical exposure during pregnancy can potentially induce cleft palate in the fetus via suppression of cell proliferation.[6,30-32] First, we conducted cell proliferation assays to analyze the process of medicine-induced inhibition of cell proliferation in HEPM cells. Various concentrations of *at*RA and DEX (Fig. 1A and 1B) were used in these assays. Our findings revealed that the treatment of HEPM cells with 100 μM *at*RA inhibited cell proliferation (Fig. 1A), whereas DEX had no effect on cell proliferation (Fig. 1B). DEX is known to interfere with the cell cycle (G1/S phase) in various cell lines.[6] However, we did not observe any defects in cell proliferation following treatment with DEX. In the future, it will be important to refine the experimental protocol to effectively induce inhibition of cell proliferation using DEX.
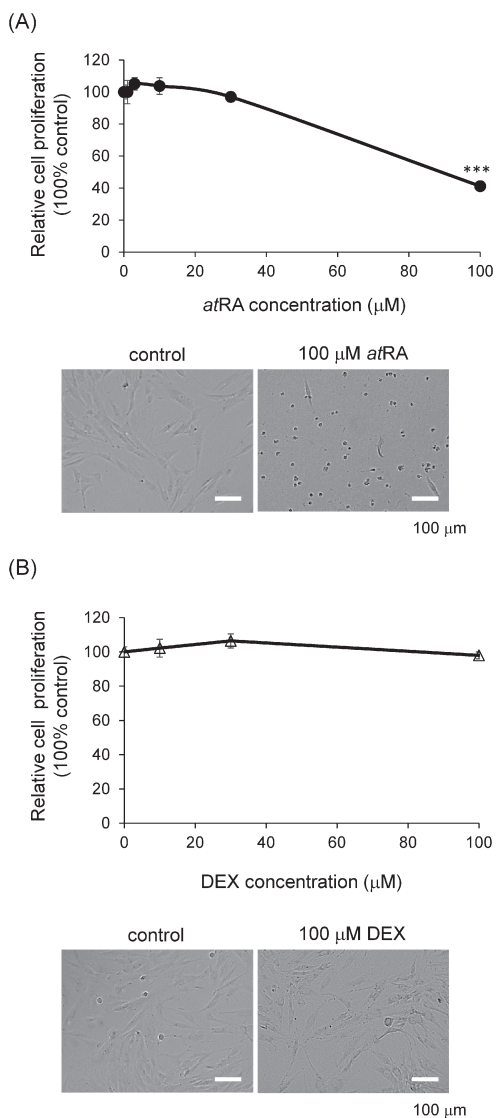
(A)

(B)

Fig. 1 Effects of *at*RA and DEX on proliferation of HEPM cells
Fig. 1A: Proliferation of HEPM cells treated with *at*RA (0–100 μM) for 48 h. Scale bar, 100 μm.
Fig. 1B: Proliferation of HEPM cells treated with DEX (0–100 μM) for 48 h. ***$p < 0.001$ versus control.
    Scale bar, 100 μm.
*at*RA: *all-trans*-retinoic acid
DEX: dexamethasone
HEPM: human embryonic palatal mesenchymal

We examined the protective effects of SE against *at*RA-induced toxicity in HEPM cells. The concentrations of SE (0.003, 0.01, and 0.03%) were selected according to the results of our previous study since these concentrations were not affected HEPM cell numbers.[28] We observed

that treatment with 100 μM *at*RA markedly reduced the proliferation of HEPM cells, whereas cotreatment with SE reversed *at*RA-induced toxicity in a dose-dependent manner (Fig. 2A).
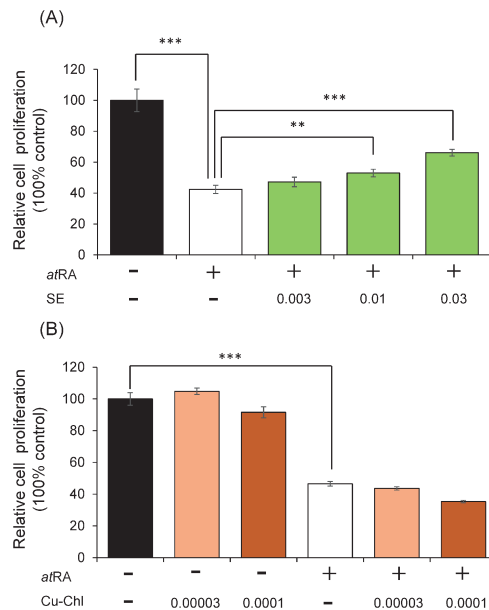

Fig. 2 Protective effect of SE against *at*RA-induced inhibition of proliferation of HEPM cells
Fig. 2A: Proliferation of HEPM cells treated with 100 μM of *at*RA and various concentrations (0.003–0.03%) of SE. **$p < 0.01$ and ***$p < 0.001$.
Fig. 2B: Effect of Cu-Chl against 100 μM *at*RA-induced inhibition of HEPM cell proliferation. ***$p < 0.001$.
*at*RA: *all-trans*-retinoic acid
Cu-Chl: copper chlorophyllin
HEPM: human embryonic palatal mesenchymal
SE: *Sasa veitchii* extract

It is widely accepted that copper chlorophyllin (Cu-Chl: 0.25%) is the main component of SE.[25-27] Consequently, our hypothesis centered on the notion that the protective effect of SE is due to the presence of Cu-Chl. We observed that treatment with Cu-Chl at concentrations of 0.00003% and 0.0001% (which were 2.5 and 8.25 times higher than the concentration of Cu-Chl in 0.03% SE, respectively) had no effect on cell proliferation. Furthermore, cotreatment with Cu-Chl and *at*RA failed to alleviate the toxic effect of *at*RA in HEPM cells (Fig. 2B). Therefore, we concluded that the protective effect of SE is derived from ingredients other than Cu-Chl.

*at*RA is known to inhibit cell proliferation through induction of apoptosis and cell cycle arrest in mice and cell line.[33-36] We investigated the involvement of apoptosis and cell cycle upon treatment with *at*RA and SE in HEPM cells. As shown in Fig. 3A, we observed that *at*RA treatment activated apoptosis-positive cells, while cotreatment with SE did not alleviate apoptosis. Moreover, cleaved caspase 3 activity induced by *at*RA remained unchanged upon treatment with SE (Fig. 3B), indicating that SE has no effect on *at*RA-induced apoptosis in HEPM cells. In contrast, we found that *at*RA treatment downregulated the levels of CCNA, CCNB, and CCND1, while it had no affect on CCNE1 levels (Fig. 3B). Although CCNA and CCNB level remained unchanged following cotreatment with SE, it successfully restored the levels of CCND1 (Fig.

3B). These results indicate that SE protects against *at*RA-induced cell cycle arrest associated with CCND1 in HEPM cells.
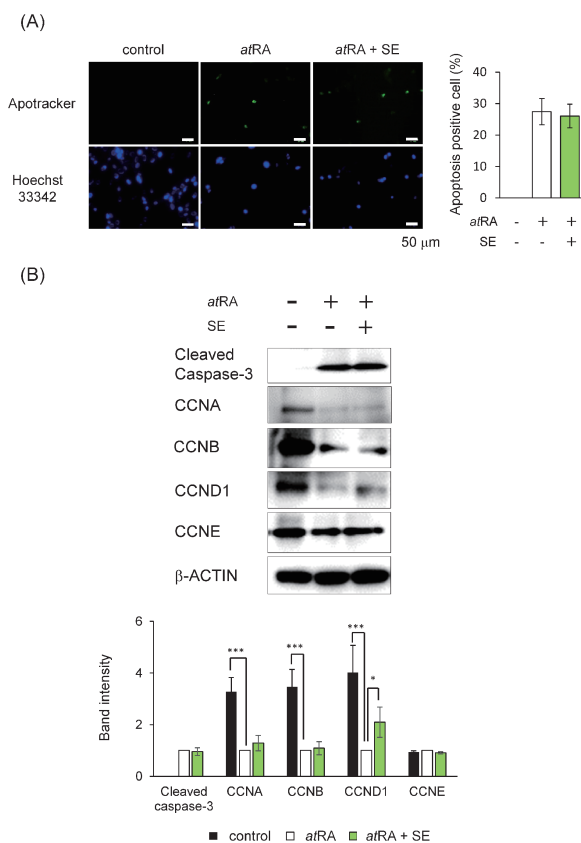

Fig. 3 Involvement with apoptosis and cell cycle by treatment with of SE against *at*RA in HEPM cells

Fig. 3A: Apotracker staining (green) of HEPM cells after treatment with 100 µM *at*RA and 0.03% SE for 48 h. The nuclei were counterstained with Hoechst 33342 (blue). Scale bar, 50 µm.
Fig. 3B: Immunoblotting of HEPM cells after treatment with 100 µM *at*RA and 0.03% SE for 48 h. β-actin was served as an internal control. *$p < 0.05$ and ***$p < 0.001$ versus *at*RA.
*at*RA: *all-trans*-retinoic acid
HEPM: human embryonic palatal mesenchymal
SE: *Sasa veitchii* extract
CCND1: cyclin D1

Finally, we measured *miR-4680-3p* expression level and its downstream genes (*ERBB2* and *JADE1*). Yoshioka et al demonstrated that *at*RA-induced inhibition of HEPM cell proliferation occurs through the regulation of *miR-4680-3p-ERBB2/JADE1* expression,[32] while our study found that the upregulation of *miR-4680-3p* expression occurs following treatment with *at*RA (Fig. 4A). Additionally, we demonstrated that SE downregulated the expression of *miR-4680-3p* in HEPM cells. To further investigate the effects of *miR-4680-3p* on the genes that function downstream of *ERBB2* and *JADE1*, we conducted a quantitative RT-PCR analysis, which identified that *at*RA

treatment suppressed *ERBB2* and *JADE1* expression (Fig. 4B). Moreover, the decrease in levels of *ERBB2* and *JADE1* was reversed through the cotreatment with SE. These results indicate that SE exerts a protective effect via modulation of *miR-4680-3p-ERBB2/JADE1* expression.
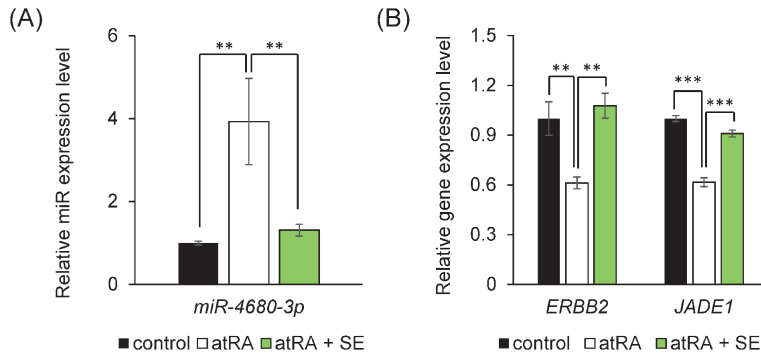

Fig. 4 SE suppresses *at*RA-induced *miR-4680-3p* expression in HEPM cells

Fig. 4A: Quantitative RT-PCR for analysis of *miR-4680-3p* expression after treatment of HEPM cells with 100 µM *at*RA and 0.03% SE for 48 h. **p < 0.01.

Fig. 4B: Quantitative RT-PCR for analysis of *ERBB2* and *JADE1* expression after treatment of HEPM cells with 100 µM *at*RA and 0.03% SE for 48 h. **p < 0.01 and ***p < 0.001.

*at*RA: *all-trans*-retinoic acid

ERBB2: Erb-B2 receptor tyrosine kinase 2

HEPM: human embryonic palatal mesenchymal

JADE1: jade family PHD finger 1

SE: *Sasa veitchii* extract

DISCUSSION

In this study, we examined the protective effects of SE against *at*RA and DEX-induced toxicity in HEPM cells. We demonstrated that administration of 100 µM *at*RA markedly reduced the cell proliferation, whereas cotreatment with SE reversed *at*RA-induced toxicity in a dose-dependent manner. Moreover, *at*RA-induced CCND1 reduction was reversed through treatment with SE. Since the SE that is investigated in our investigation is available to purchase in Japan as over-the-counter medicine, it may be an easy measure to reduce the onset of cleft palate.

It is reported that Cu-Chl (0.25 %) is the main component of SE.[25-27] Cu-Chl has a variety of potential effects, including antimutagenic, anticarcinogenic, and antioxidant activities.[37] Based on the report that the values of total antioxidant status in patients with cleft lip and palate were lower than those in the control group (health people),[38] we hypothesized that the protective effect of SE is due to the presence of Cu-Chl. However, our results failed to alleviate the toxic effect of *at*RA by cotreatment with Cu-Chl in HEPM cells. Therefore, we concluded that the protective effect of SE was due to the presence of ingredients other than Cu-Chl. This conclusion is corroborated by the results of our previous study, in which we analyzed SE by 2D-high performance liquid chromatography and observed broad peaks in the chromatogram.[39] *Sasa* species include various phenolic compounds, which have antioxidant capacity.[40,41] In addition, Ko et al reported that phenolic compounds differ by the methodology of extraction and harvest time.[42] In the future, we need to measure the content of phenolic compounds from SE and identify the active components of SE. First, we plan to evaluate the fractionated components.

Recent reports suggest that miRNAs are involved in the etiology of cleft palate.[43,44] miRNAs are endogenous small non-coding RNAs (18–24 nucleotides long) that negatively regulate the expression of their target genes.[45] Suzuki and Li et al analyzed genes involved in the formation of cleft palate and identified miRNAs associated with the development of cleft palate in humans.[5,46] They found that the overexpression of *miR-133b*, *miR-140-5p*, *miR-374a-5p*, and *miR-4680-3p* inhibited HEPM cell proliferation via downregulation of the genes that function further along the signaling pathways. Additionally, *at*RA suppressed HEPM cell proliferation through the regulation of *miR-4680-3p-ERBB2/JADE1* expression.[32] Moreover, as it is known that natural compounds such as propolis and curcumin alter the expression levels of specific miRNAs related to oxidative stress and cell proliferation in human cell lines,[47,48] we hypothesized that the protective effect of SE would also be due to *at*RA-induced modulation of *miR-4680-3p* expression and its downstream genes (*ERBB2* and *JADE1*). ERBB2 is a member of the ERBB receptor tyrosine kinase family, which includes the epidermal growth factor receptor.[49] The binding of ligands to receptors induces the homo- or heterodimerization of receptors and activates the kinase domain that induces downstream signaling cascades, such as mitogen-activated protein kinase/extracellular signal-regulated kinase and phosphatidylinositol-3 kinase/protein kinase B/mechanism of rapamycin pathways, which are crucial for cell proliferation, migration, and differentiation.[50,51] In addition, *at*RA inhibits cell proliferation of HEPM cells by upregulating *miR-4680-3p* expression, which leads to the suppression of ERBB2 expression, and consequently, downregulation of the downstream genes that function in the extracellular signal-regulated kinase 1/2 signaling pathway.[32] JADE1 is a transcription factor that has two variants: JADE1-L (the long form that comprises 842 amino acids) and JADE1-S (the short form that lacks a 333-amino-acid-long C-terminal fragment).[52] The inhibition of JADE1 (both JADE1-L and JADE1-S) expression by siRNA-knockdown, suppressed DNA synthesis in cultured epithelial cell lines and primary fibroblasts.[53] Treatment of HEPM cells with *at*RA downregulates JADE1 expression, leading to decreased cell proliferation via downregulation of CCND1 expression.[32] In the present study, we demonstrated that upregulation of *miR-4680-3p* and downregulation of *ERBB2* and *JADE1* expression by *at*RA was modulated by cotreatment with SE. Yoshioka et al previously reported that ERBB2 and JADE1 inhibition using siRNA suppressed cell proliferation of HEPM cells through downregulating CCND1.[32] Moreover, we found that treatment with SE increased CCND1 levels and *ERBB2/JADE1* expression (Supplementary Figure S2). These results suggest that SE alleviates *at*RA-induced toxicity by regulating *miR-4680-3p-ERBB2/JADE1-CCND1* expression (Fig. 5).
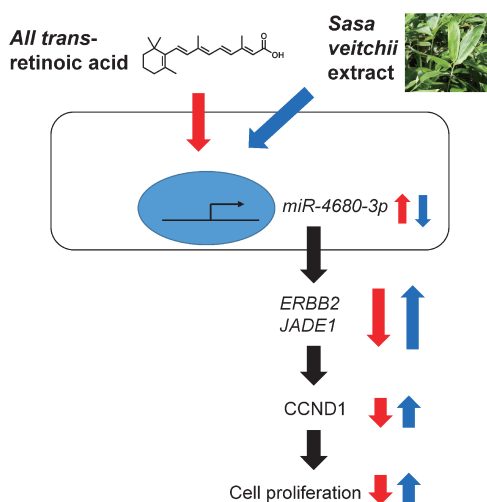
Fig. 5 Proposed mechanism of SE against *at*RA-induced cell proliferation inhibition
ERBB2: Erb-B2 receptor tyrosine kinase 2
JADE1: jade family PHD finger 1
CCND1: cyclin D1

The present study has some limitations. (1) Active components of SE: The SE used in this study was composed of many ingredients. Despite the main component of SE being 0.25% Cu-Chl, we did not observe alleviation of *at*RA-induced inhibition of cell proliferation on cotreatment of the cells with Cu-Chl. In future studies, the active components of SE should be explored from the fractionated components. (2) miRNA expression levels: We used other researchers' datasets for analysis in the present study. Although their datasets assisted with the identification of miRNA-gene networks, other miRNAs may have also been affected by the treatment with *at*RA and SE. We need to conduct miRNA-seq to elucidate the mechanism of *at*RA-induced inhibition of cell proliferation and examine the protective effect of SE. (3) In vitro experiments: In the present study, we conducted in vitro experiments. The extent to which SE reaches the embryo remains unclear, as there is currently no reported information on its absorption, distribution, metabolism, and excretion in adults. Furthermore, to comprehensively understand the molecular mechanism of cleft palate, it is essential to consider not only mesenchymal cells but also epithelial cells. However, it is worth noting that the epithelial cells near the palatal shelves are not yet fully developed at this stage. In future studies, in vivo experiments should be performed to evaluate the protective effects of SE.

In conclusion, this is the first report demonstrating the protective effect of SE against *at*RA-induced inhibition of HEPM cell proliferation. The SE (Sunchlon) we used in this study is an over the counter medicine in Japan and is available for consumption by pregnant women. Our results may be helpful in developing a prevention strategy for cleft palate and subsequently, reduce the rates of cleft palate in Asia.

ACKNOWLEDGMENT

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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SUPPLEMENTARY INFORMATION
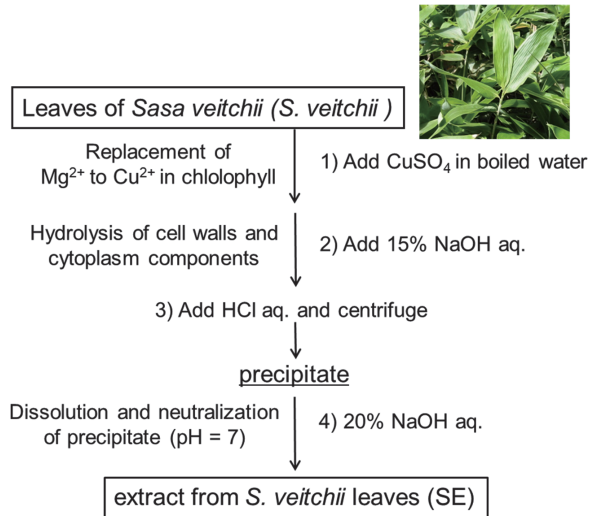
Supplementary Fig. S1   Extraction procedure
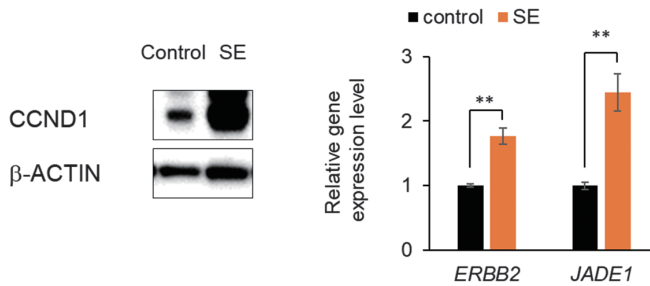
Supplementary Fig. S2   Involvement with cyclin D1 and miR-4680-3p downstream genes by treatment with of *Sasa veitchii* extract (SE) in HEPM cells

Immunoblotting or qPCR of HEPM cells after treatment with 0.03% SE for 48 hr. β-actin was served as an internal control.
CCND1: cyclin D1
ERBB2: Erb-B2 receptor tyrosine kinase 2
JADE1: jade family PHD finger 1