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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH



# Inhibition of melanin production by sesquiterpene lactones from *Saussurea lappa* and their analogues

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## Abstract

This research is aimed towards the discovery of a novel skin whitening agent from sesquiterpene lactones. We screened nine sesquiterpene lactones from *Saussurea lappa* root extracts, costunolide (**1**), $\alpha$-, $\beta$-, γ-cyclocostunolides (**2**, **3**, **4**), santamarine (**5**), reynosin (**6**), arbuscirin A (**7**), dehydrocostus lactone (**8**), and 11$\beta$, 13-dehydrocostus lactone (**9**), for their melanin inhibitory activity in B16 melanoma cells. This study was focused on the inhibition of the extracellular melanin content. The screening results revealed that only $\alpha$-cyclocostunolide (**2**) showed notable inhibition of the melanin production, particularly the extracellular melanin content, with an $IC_{50}$ value of 5.75 µM. In addition, structure-activity relationship (SAR) of 12 sesquiterpene lactone analogues with reference to their melanin inhibitory activity was also investigated. However, none of the analogues were found to show any significant melanin inhibitory activity. To the best of our knowledge the above study is the first SAR investigation involving the melanin inhibitory activity of sesquiterpene lactones. It established that eudesmanolide-type lactone having a double bond between C3 and C4 showed the most efficient melanin inhibition. Additionally, the presence of a hydroxyl, ketone or ester moiety as the C1-substituent markedly decreased the melanin inhibitory activity. Thus, these findings suggest that $\alpha$-cyclocostunolide (**2**) might be useful in the therapy of skin disorders.

**Keywords** Melanin inhibition · Sesquiterpene lactones · *Saussurea lappa* · Structure-activity relationship · $\alpha$-Cyclocostunolide

## Introduction

Melanogenesis is a well-known physiological process for melanin production in the epidermal melanosome of melanocytes and its transportation to the keratinocytes (Riley 1997). Eumelanin and pheomelanin are the two types of melanin pigments whose main function is the protection of the skin from ultraviolet (UV) radiations (Briganti et al. 2003; Thanigaimalai et al. 2012). However, abnormal overproduction and accumulation of melanin can lead to skin diseases like freckles, melasma, senile lentigines, and inflammation, which may ultimately result in skin cancer (Ohguchi et al. 2003; Chadwick et al. 2012; Huang et al. 2012).

Tyrosinase is the key melanocyte-specific enzyme. It catalyzes the two rate-limiting steps of melanin synthesis, which are hydroxylation of L-tyrosine to *o*-diphenol (L-DOPA) and subsequent oxidation of L-DOPA to dopaquinone (Chang 2012). The development of melanogenesis inhibitors from both natural and synthetic compounds is an interesting research field because only a few potential agents, without side effects, are presently in use in medical and cosmetic products (Karioti et al. 2007; Bae et al. 2012).

In our previous study, we had isolated and synthesized sesquiterpene analogues from *S. lappa* as potent TNF-$\alpha$ inhibitors for the treatment of inflammation (Choodej et al. 2018). Sesquiterpene lactones, a large group of natural products, display a wide variety of biological activities like antifungal, antibacterial, antiulcer, anti-inflammatory, anticancer, and anti-melanogenesis effects (Yamahara et al. 1985; Li et al. 2005; Rao et al. 2007; Choi et al. 2008; Rasul et al. 2012). Up till now, a structure-activity relationship (SAR) study for sesquiterpene lactones with reference to their anti-melanogenesis activity has not been reported. In our

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continuing search for new melanogenesis inhibitors, natural sesquiterpene lactones, costunolide (**1**), $\alpha$-, $\beta$-, $\gamma$-cyclocostunolide (**2**, **3**, **4**), santamarine (**5**), reynosin (**6**), arbuscirin A (**7**), dehydrocostus lactone (**8**), and 11$\beta$, 13-dehydrocostus lactone (**9**) (Fig. 1) were evaluated as melanogenesis inhibitors in B16 melanoma cells. Twelve newly synthesized sesquiterpene lactones were also assayed (Fig. 2).

## Materials and methods

### Plants

The roots of *S. lappa* were purchased (August 2015) from a herbal medicine store (Marma-Samunprai) in Thailand (Choodej et al. 2018).

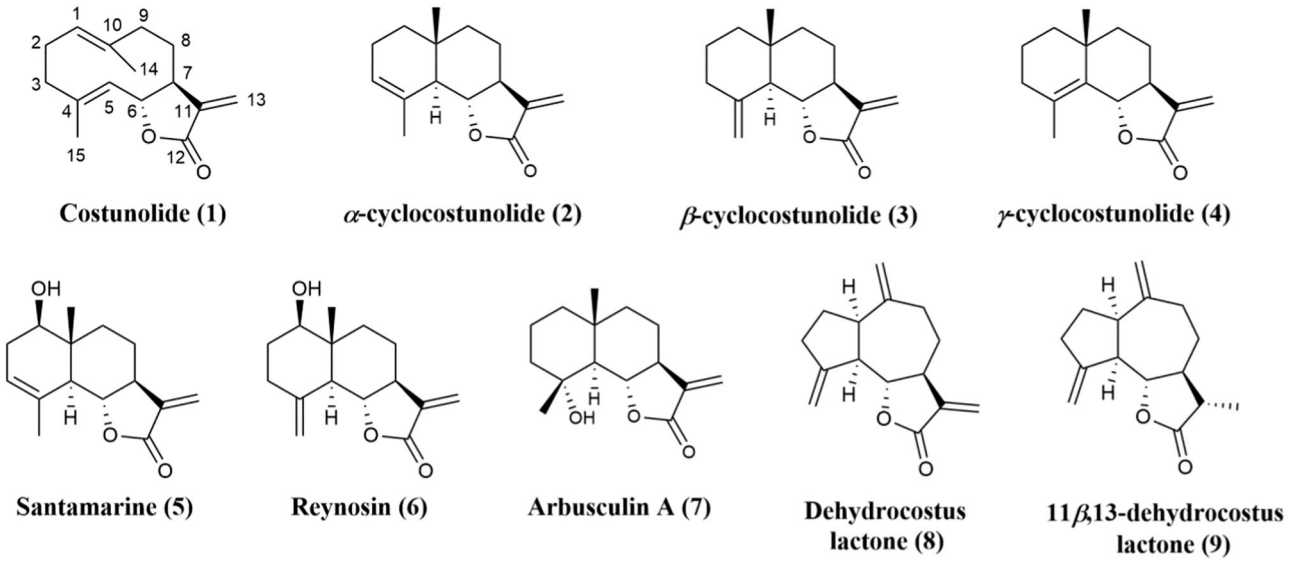


**Fig. 1** Structure of natural sesquiterpene lactones (**1**–**9**)

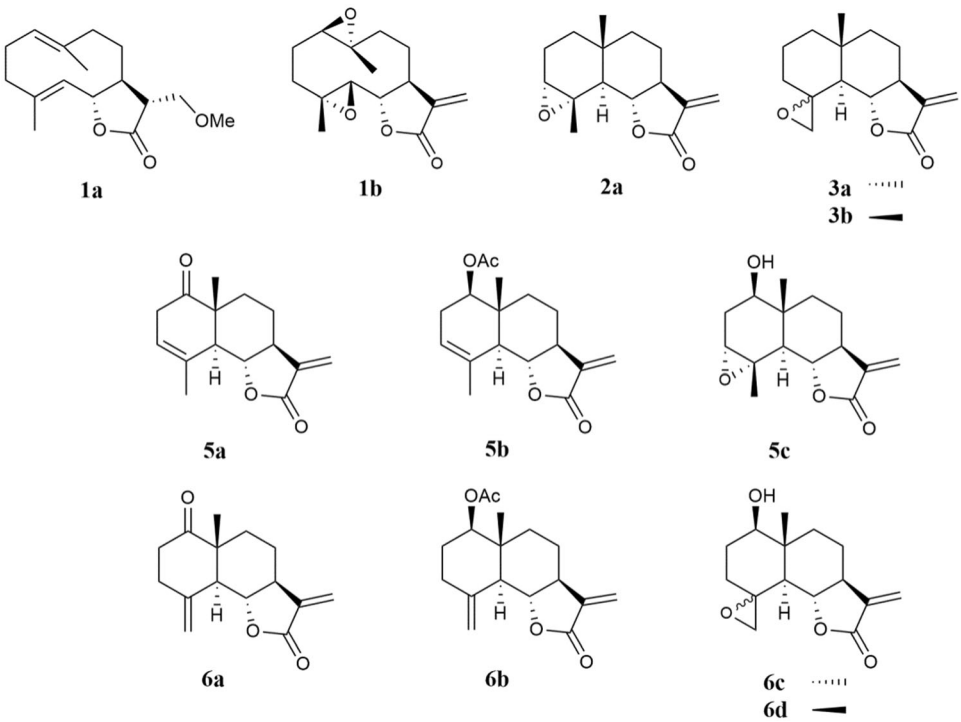


**Fig. 2** Structure of synthesized sesquiterpene lactones (**1a**–**6d**)

### Sesquiterpene lactones and semi-synthetic analogues

Sesquiterpene lactones and semi-synthetic analogues were isolated and synthesized using methods reported in our previous report (Choodej et al. 2018).

### Cell culture

The murine melanoma B16F0 cell line, purchased from DS Pharma Biomedical (Osaka, Japan), was cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were cultured at 37 °C in a humidified atmosphere of 5% $CO_2$.

### Measurement of cellular melanin content

Melanin content was measured based on a previously described method, with slight modifications (Yamauchi et al. 2015). Briefly, cells were added to a 24-well plate at a density of $5.0 \times 10^3$ cells/well and incubated at 37 °C for 24 h. After various concentrations of samples and arbutin (a positive control) were added, the cells were incubated for an additional 72 h (control treated with DMSO). The medium was collected and 200-µL aliquots were loaded into a 96-well plate. Then extracellular melanin content was measured at 510 nm using a microplate reader. After that the cells were washed twice with phosphate-buffered saline (PBS), lysed in 600 µL of 1 M NaOH and heated at 100 °C for 30 min to solubilize the melanin. The cell lysate (250 µL) was added to a 96-well microplate, and the intracellular melanin content was determined at 405 nm using a microplate reader. All the experiments were performed in triplicate. Melanin production has been expressed as a percentage, compared to the control cell containing only DMSO.

### Cell viability

Measurement of cell viability was determined using the microculture tetrazolium technique as previously described (Yamauchi et al. 2014). B16 cells were seeded into a 24-well plate at a density of $5.0 \times 10^3$ cells/well. After the cells were incubated with the test compounds at various concentrations for 72 h, MTT solution [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide in PBS (5 mg/mL), 50 µL] was added to each well and incubated for an additional 4 h. The culture medium was then removed and replaced with 1.0 mL of isopropyl alcohol, containing 0.04 M HCl, to dissolve the formazan crystals. Subsequently, a 150 µL aliquot was transferred to a 96-well plate and the absorbance was measured at 590 nm using a microplate reader. All the experiments were performed in triplicate. Cell viability has been expressed as a percentage, compared to the control cell containing only DMSO.

### Statistical analysis

All the data was calculated using the GraphPad Prism software, version 7.00.

## Results and discussion

In this study, nine natural sesquiterpene lactones (Fig. 1) were examined for melanin inhibitory effect in B16 melanoma cells. The cells were treated with different concentrations of each compound (2.5–25 µM) for 72 h. Both intracellular and extracellular melanogenesis was measured through the cell viability study using the MTT solution (Table 1). Among the tested compounds, $\alpha$-cyclocostunolide (**2**) was found to be the most potent melanogenesis inhibitor. It also showed no toxicity at the highest concentration tested (10 µM). The cell viability slightly decreased to 70% at 15 µM. The $\alpha$-cyclocostunolide has been reported to reduce nitric oxide (NO) and TNF-α overproduction in LPS-stimulated macrophages (Zhao et al. 2008). As shown in Fig. 3, treatment with various concentrations (2.5, 5.0, 7.5, and 10 µM) of the compound **2** significantly decreased the extracellular melanin content in a dose-dependent manner with an $IC_{50}$ value of 5.75 µM for the extracellular melanin content. Particularly, melanin inhibitory potency at 10 µM was 1.6-fold greater than arbutin, a skin lightening agent, at 730 µM. The intracellular melanin content was also lower than in the case of arbutin and DMSO as a control. However, previous studies suggested that the intracellular melanin usually secreted into the culture medium and thus its level was 23 times lower than the extracellular melanin level (Laskin et al. 1982; Yamauchi et al. 2015). Therefore, the total melanin content was lowered by compound **2**. Moreover, compound **2** did not inhibit the mushroom tyrosinase (Batubara et al. 2010). This indicated that melanin inhibition was not caused by tyrosinase inhibition, which otherwise would have affected the protein expression at a post-translational modification.

$\beta$-Cyclocostunolide (**3**) and $\gamma$-cyclocostunolide (**4**) did not appreciably decrease the melanin content in B16 cells, despite their structural similarity with compound **2**. Costunolide (**1**), a major constituent of *S. lappa* exhibiting a broad range of biological activities (Pandey et al. 2007; Yang et al. 2010), showed cytotoxicity in B16 melanoma cell at 10 µM by up to 50% cell viability (Table 1). However,

**Table 1** Effect of nine natural sesquiterpene lactones from *S. lappa* root on melanin inhibition

| Compounds | Concentration (μM) | Cell viability (%) | Intracellular melanin content (%) | Extracellular melanin content (%) |
|---|---|---|---|---|
| **1** | 10.0 | 49.48 ± 0.17*** | 76.59 ± 1.74* | 27.58 ± 1.73** |
| | 5.0 | 74.85 ± 0.44*** | 83.70 ± 0.27*** | 44.75 ± 1.12*** |
| | 2.5 | 98.15 ± 1.24*** | 95.73 ± 4.02 | 71.27 ± 1.37*** |
| **2** | 10.0 | 91.88 ± 4.80** | 68.65 ± 1.48*** | 21.71 ± 0.65*** |
| | 5.0 | 96.08 ± 2.23*** | 75.26 ± 3.65*** | 25.10 ± 5.61* |
| | 2.5 | 107.65 ± 1.03*** | 76.29 ± 4.73** | 67.55 ± 3.72** |
| **3** | 12.5 | 86.49 ± 0.33*** | 85.20 ± 1.09*** | 59.82 ± 1.38*** |
| | 6.25 | 90.50 ± 4.27 | 94.94 ± 3.19** | 95.10 ± 5.32 |
| | 3.125 | 92.08 ± 12.54 | 99.72 ± 2.33** | 98.93 ± 3.74 |
| **4** | 25.0 | 69.11 ± 1.72*** | 81.92 ± 0.15*** | 85.38 ± 0.56 |
| | 12.5 | 89.23 ± 3.58 | 93.77 ± 1.00*** | 92.70 ± 0.24*** |
| | 6.25 | 101.50 ± 1.59*** | 96.31 ± 4.21 | 99.24 ± 1.38** |
| **5** | 25.0 | 69.77 ± 0.94*** | 68.71 ± 0.81*** | 56.48 ± 1.40** |
| | 12.5 | 87.97 ± 3.31*** | 101.81 ± 0.61** | 91.40 ± 1.68*** |
| | 6.25 | 95.67 ± 0.66** | 102.94 ± 2.44** | 92.03 ± 3.57 |
| **6** | 25.0 | 55.08 ± 2.71*** | 102.21 ± 0.56** | 35.49 ± 5.25 |
| | 12.5 | 85.27 ± 5.10 | 103.84 ± 1.87*** | 95.84 ± 2.20** |
| | 6.25 | 92.87 ± 0.66** | 108.10 ± 2.44** | 99.73 ± 0.59*** |
| **7** | 25.0 | 87.96 ± 6.67 | 101.10 ± 1.26*** | 96.43 ± 1.29** |
| | 12.5 | 94.89 ± 9.06 | 112.42 ± 0.59*** | 101.06 ± 2.80** |
| | 6.25 | 99.10 ± 0.51*** | 116.16 ± 3.77 | 105.40 ± 1.05*** |
| **8** | 25.0 | 93.29 ± 0.52*** | 104.46 ± 5.23 | 94.09 ± 1.47*** |
| | 12.5 | 96.17 ± 1.04*** | 100.95 ± 4.29 | 89.49 ± 8.48 |
| | 6.25 | 101.79 ± 8.18 | 106.49 ± 1.01*** | 107.03 ± 1.02** |
| **9** | 25.0 | 100.56 ± 4.98 | 105.46 ± 2.49 | 75.57 ± 2.99** |
| | 12.5 | 101.07 ± 0.82*** | 107.04 ± 1.63*** | 94.15 ± 1.49*** |
| | 6.25 | 104.50 ± 8.21 | 104.30 ± 5.41 | 98.25 ± 1.12*** |
| **Arbutin**[a] | 730 | 103.31 ± 0.75*** | 81.62 ± 2.00** | 36.36 ± 1.53*** |

Data are expressed as mean ± SD ($n = 3$)

*$p < 0.033$, **$p < 0.002$, ***$p < 0.001$ vs. control

[a]Positive control

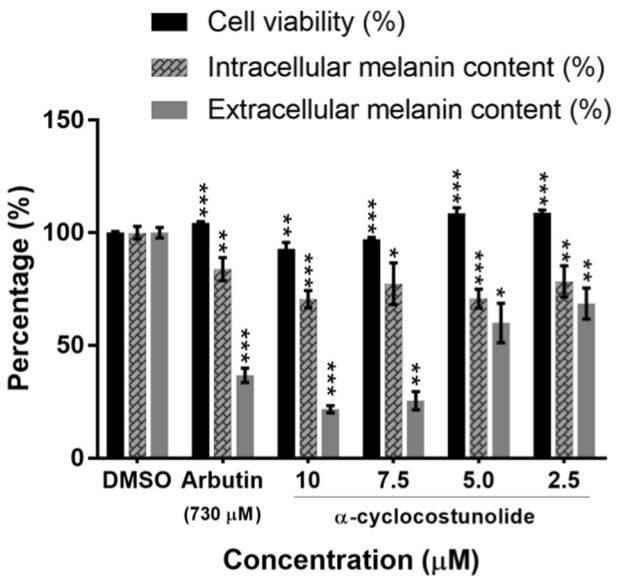


**Fig. 3** Melanin inhibitory effect of α-cyclocostunolide (**2**) on B16 melanoma cells Data are expressed as mean ± SD ($n = 3$). *$p < 0.033$, **$p < 0.002$, ***$p < 0.001$ vs. control

guaianolide-type compound **8** and **9** exhibited no inhibitory effect.

In order to examine the structural-activity relationship (SAR) of α-cyclocostunolide (**2**), 12 sesquiterpene lactone analogues were tested for melanin inhibitory activity (Table 2). Both compounds **1a** and **1b** belong to the germacranolide-type which is the same as costunolide (**1**). They were nontoxic to B16 cells, and thus were insignificant in the inhibition of melanogenesis. On the basis of the above observations, we hypothesized that the double bond between C3 and C4 of the eudesmanolide-skeleton would be the key structural feature for the presence of melanin inhibitory activity in these molecules.

To investigate this hypothesis, compound **2** was subjected to epoxidation. The resulting epoxide **2a**, showed no melanin inhibitory activity which confirmed that the double bond between C3 and C4 was essential for inhibition of melanogenesis. Likewise, the replacement of the C1-substituent with hydroxyl (**5**), ketone (**5a**) or ester (**5b**) groups also led to a decrease in the melanin inhibitory effect, as summarized in Fig. 4.

**Table 2** Effect of twelve synthetic sesquiterpene lactones on melanin inhibition

| Compounds | Concentration (μM) | Cell viability (%) | Intracellular melanin content (%) | Extracellular melanin content (%) |
|---|---|---|---|---|
| **1a** | 12.5 | 100.30 ± 0.01*** | 108.84 ± 0.44*** | 99.51 ± 2.06* |
| | 25 | 105.27 ± 8.50 | 106.32 ± 1.00*** | 94.75 ± 1.60** |
| **1b** | 12.5 | 89.90 ± 0.94** | 104.61 ± 3.09 | 98.15 ± 1.35** |
| | 25 | 96.41 ± 0.48*** | 94.72 ± 1.80*** | 99.74 ± 0.68*** |
| **2a** | 12.5 | 81.36 ± 0.70*** | 89.87 ± 0.07*** | 93.07 ± 0.03*** |
| | 25 | 66.67 ± 4.05 | 75.31 ± 0.58** | 67.27 ± 0.87*** |
| **3a** | 12.5 | 105.47 ± 4.37 | 103.63 ± 0.61*** | 99.51 ± 0.34*** |
| | 25 | 101.62 ± 6.33 | 100.27 ± 6.89 | 96.09 ± 1.22** |
| **3b** | 12.5 | 106.27 ± 7.44 | 98.27 ± 0.38*** | 96.04 ± 1.90** |
| | 25 | 108.30 ± 5.64 | 97.56 ± 2.60** | 99.14 ± 2.40* |
| **5a** | 12.5 | 103.62 ± 4.42 | 114.39 ± 0.86** | 103.74 ± 2.79* |
| | 25 | 100.66 ± 4.36 | 113.69 ± 2.38 | 99.54 ± 1.51** |
| **5b** | 12.5 | 91.40 ± 0.63*** | 97.94 ± 2.44* | 97.68 ± 2.49* |
| | 25 | 49.11 ± 2.48* | 78.56 ± 1.06** | 47.26 ± 0.51*** |
| **5c** | 12.5 | 87.25 ± 2.90*** | 96.24 ± 1.12** | 94.50 ± 0.33*** |
| | 25 | 70.42 ± 1.90*** | 91.52 ± 1.44** | 77.54 ± 0.07** |
| **6a** | 12.5 | 93.58 ± 2.69 | 100.23 ± 3.30 | 101.59 ± 1.00*** |
| | 25 | 75.30 ± 0.63*** | 85.71 ± 2.31* | 84.08 ± 0.97*** |
| **6b** | 12.5 | 107.91 ± 4.46 | 91.41 ± 2.58* | 101.12 ± 1.92** |
| | 25 | 100.46 ± 7.61 | 70.95 ± 1.12 | 96.62 ± 1.76** |
| **6c** | 12.5 | 96.17 ± 0.51*** | 91.31 ± 0.65** | 104.60 ± 0.46*** |
| | 25 | 73.97 ± 12.34 | 88.48 ± 1.02** | 73.85 ± 2.93 |
| **6d** | 12.5 | 100.01 ± 7.83 | 100.51 ± 3.20 | 102.12 ± 0.91*** |
| | 25 | 86.34 ± 1.20*** | 88.73 ± 5.60*** | 96.41 ± 0.53*** |

Data are expressed as mean ± SD ($n = 3$)

*$p < 0.033$, **$p < 0.002$, ***$p < 0.001$ vs. control

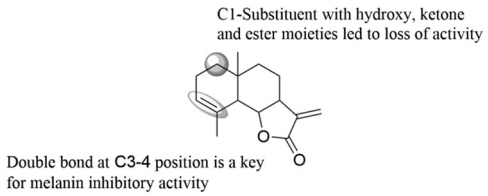


**Fig. 4** Summary of SAR of the eudesmanolide skeleton for melanin inhibitory activity

Furthermore, compounds **3a**–**b**, **5c**, and **6a**–**d**, which are epoxide analogues of *β*-cyclocostunolide (**3**), santamarine (**5**) and reynosin (**6**) respectively, did not show any significant inhibition of the melanin production in the B16 melanoma cells.

## Conclusions

In this work nine sesquiterpene lactones **1**–**9** were isolated from the root extract of *S. lappa* and screened for melanin inhibitory activity in B16 melanoma cells. Out of these nine compounds only *α*-cyclocostunolide (**2**), was found to inhibit melanogenesis in the B16 melanoma cells with an $IC_{50}$ value of 5.75 μM. The data on cell viability indicated that inhibition of intra- and extracellular melanogenesis was caused by compound **2** without inducing cell death. The melanin inhibitory effect of the synthetic analogues of the sesquiterpene lactones was also investigated. However, none of the analogues showed any melanin inhibitory activity. Based on the structure-activity relationship, we concluded that the double bond between the C3 and C4 position was important for melanin inhibitory activity. In contrast, the introduction of C1-substituent with hydroxyl, ketone or ester groups led to the loss of melanin inhibitory activity, seemingly due to steric hindrance. From the above findings, *α*-cyclocostunolide (**2**) have been identified as a potential candidate for melanin inhibition. Further studies on the mechanism of melanin inhibition, such as expression of tyrosinase in melanogenesis, are currently underway.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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