



Research article

Safflospermidines from the bee pollen of *Helianthus annuus* L. exhibit a higher *in vitro* antityrosinase activity than kojic acidPhanthiwa Khongkarat^a, Rico Ramadhan^b, Preecha Phuwapraisirisan^c, Chanpen Chanchao^{d,*}^a Program in Biotechnology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand^b Department of Chemistry, Faculty of Science and Technology, Airlangga University, Surabaya 60115, Indonesia^c Department of Chemistry, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand^d Department of Biology, Faculty of Science, Chulalongkorn University, 254 Phayathai Road, Bangkok 10330, Thailand

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ABSTRACT

Background: Ozone deterioration in the atmosphere has become a severe problem causing overexposure of ultraviolet light, which results in humans in melanin overproduction and can lead to many diseases, such as skin cancer and melasma, as well as undesirable esthetic appearances, such as freckles and hyperpigmentation. Although many compounds inhibit melanin overproduction, some of them are cytotoxic, unstable, and can cause skin irritation. Thus, searching for new natural compounds with antityrosinase activity and less/no side effects is still required. Here, bee pollen derived from sunflower (*Helianthus annuus* L.) was evaluated.

Materials and methods: Sunflower bee pollen (SBP) was collected from *Apis mellifera* bees in Lopburi province, Thailand in 2017, extracted by methanol and sequentially partitioned with hexane and dichloromethane (DCM). The *in vitro* antityrosinase activity was evaluated using mushroom tyrosinase and the half maximal inhibitory concentration (IC₅₀) is reported. The antioxidation activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and reported as the half maximal effective concentration. Two pure compounds with antityrosinase activity were isolated by silica gel 60 column chromatography (SG₆₀CC) and high performance liquid chromatography (HPLC), and their chemical structure deduced by Nuclear Magnetic Resonance (NMR) analysis.

Results: The DCM partitioned extract of SBP (DCMSBP) had an antityrosinase activity (IC₅₀, 159.4 µg/mL) and was fractionated by SG₆₀CC, providing five fractions (DCMSBP1–5). The DCMSBP5 fraction was the most active (IC₅₀ = 18.8 µg/mL) and further fractionation by HPLC gave two active fractions, revealed by NMR analysis to be safflospermidine A and B. Interestingly, both safflospermidine A and B had a higher antityrosinase activity (IC₅₀ of 13.8 and 31.8 µM, respectively) than kojic acid (IC₅₀ of 44.0 µM). However, fraction DCMSBP5 had no significant antioxidation activity, while fractions DCMSBP1–4 showed a lower antioxidation activity than ascorbic acid.

Conclusion: Safflospermidine A and B are potential natural tyrosinase inhibitors.

1. Introduction

Melanin is dark pigment that is produced in the epidermis and is important for skin protection against DNA damage from ultraviolet (UV) light. However, repeated overexposure to UV irradiation can lead to the overproduction of melanin or hyperpigmentation, which eventually causes undesirable aesthetic problems, such as age spots, freckles, and melasma (Hernández-Barrera et al., 2008). In addition, it can cause detrimental damage, leading to ageing and skin cancer (Briganti et al., 2003; Blume-Peytavi et al., 2016).

Tyrosinase (TYR), which catalyzes the hydroxylation of L-tyrosine and the oxidation of 3,4-dihydroxy-L-phenylalanine (L-DOPA), is a key

enzyme in melanin production (Körner and Pawelek, 1982). Moreover, tyrosinase-related protein-1 (TRP-1) and -2 (TRP-2) are involved in the oxidation of L-tyrosine (Riley, 1997), while TYR, TRP-1, and TRP-2 are the rate-limiting enzymes in melanogenesis (Wu et al., 2000). Thus, tyrosinase inhibitors can potentially be used to reduce the melanin content in skin (Kim and Uyama, 2005).

However, a more effective and safer tyrosinase inhibitor is still required because some of the current tyrosinase inhibitors have been reported to cause cell cytotoxicity or some other side effects, such as irritation, skin peeling, redness, or skin sting. For example, although hydroquinone was reported to be an effective *in vitro* and *in vivo* tyrosinase inhibitor, it is unfortunately cytotoxic to melanocytes and has the

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side effect of hypopigmentation resulting in vitiligo (O'Donoghue, 2006; Manini et al., 2009).

Natural products are one of the main sources in the search for tyrosinase inhibitors, where, for example, they have included caffeine from camellia pollen (Yuanfan et al., 2019), ellagic acid from nuts, soft fruits, and other plant tissues (Pitchakarn et al., 2013), and phloretin from apples (Chen et al., 2019a; Wang et al., 2018). Bee pollen has been reported to be an alternative source for potential nutritional and medical applications, since it exhibits many bioactivities, such as neurotoxicity protection and treatment (Ben Bacha et al., 2019), anti-inflammatory and antinociceptive activity (Lopes et al., 2019), and antibacterial and pro-regenerative effects (Schuh et al., 2019). Like other bee products, the bioactivities of bee pollen depended mainly on the plant origin and its geographical region (Arruda et al., 2013). In addition, the reported activity following fractionation is influenced by the extraction methods, extraction solvents, extraction numbers and extraction times (Li et al., 2019), as well as the assay conditions.

In this work, bee pollen from monofloral sunflower (*Helianthus annuus* L.) plantations was evaluated. Although native to North America (Hernández et al., 2019), extensive monocultures of sunflowers are widely cultivated in several countries, including Thailand, for oil seed production, and indeed oilseed sunflower accounts for 90% of the crop value globally (Hladni, 2016). These monocultures require honeybees for crop pollination, which results in the ability to easily obtain large amounts of sunflower pollen from the bees.

In the present study, we evaluated the antityrosinase and anti-oxidation activities of monofloral sunflower bee pollen (SBP), starting from a crude extract to two highly enriched compounds with antityrosinase activity.

The plant origin of the bee pollen was first observed under scanning electron microscopy (SEM) to confirm by morphology it was sunflower pollen. After that, the dried SBP was extracted by organic solvents, and partitioned by chromatography screening for antityrosinase activity against mushroom tyrosinase and L-DOPA in comparison to kojic acid (positive reference control). In addition, the anti-oxidation activity was assayed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) with ascorbic acid as the reference control. The chemical structure of the two obtained active pure compounds was analyzed by Nuclear Magnetic Resonance (NMR) spectroscopy.

2. Materials and methods

2.1. Sample collection

The SBP from *Apis mellifera* was collected from bees foraging in extensive monocultures of sunflower in Lopburi province, Thailand in 2017. After drying in an oven (Memmert, Germany) at 40 °C, it was stored at 25 °C until used.

2.2. Identification of the bee pollen by morphology

A sample of the SBP was sent to a SEM laboratory service at Scientific and Technological Research Equipment Center of Chulalongkorn University, where SEM and Energy Dispersive X-ray Spectrometry (6610LV; Tokyo, Japan) was used. The morphology of the bee pollen was observed under SEM at 1,000 × magnification in comparison to reference pollen descriptions.

2.3. Fractionation of SBP: crude methanol (MeOH) extract

The fractionation was performed with some modification from Chantarudee et al. (2012). The SBP (140 g) was mixed with 800 mL of MeOH, shaken at 100 rpm, 15 °C for 18 h in a shaking incubator (SI-23MC, China), and then centrifuged at 5,500 × g, 4 °C for 15 min in a

superspeed centrifuge (Sorvall RC6 Plus, Germany). The supernatant was collected, while the solid residue was re-extracted three more times in the same manner. All four supernatants were combined together (~3.2 L) and evaporated under reduced pressure and a maximum temperature of 40–45 °C using a rotary evaporator (Heidolph, Germany) to give the crude MeOH extract of SBP (CSBP). This was kept at -20 °C in the dark until used.

2.4. Partitioning of the CSBP

The CSBP was sequentially partitioned by hexane (low polarity) and dichloromethane (DCM; medium polarity). The CSBP (113.85 g, section 2.3) was dissolved in 250 mL of MeOH, the volume selected such that the extract was no longer sticky. The mixture was then poured into a separating funnel and partitioned with an equal volume of hexane. After the two phases were clearly separated, the upper phase (hexane part) was removed and collected. The lower MeOH phase was re-partitioned a further twice with an equal volume of hexane in the same manner. The hexane extracts were pooled together and evaporated as in section 2.3 to give the hexane-partitioned extract of CSBP (HXSBP).

Meanwhile the residual hexane-extracted MeOH phase (350 mL) was extracted with an equal volume of DCM three times in the same manner, with the lower DCM phase being harvested, pooled and evaporated as in section 2.3 to give the DCM-partitioned extract of CSBP (DCMSBP). Finally, the residual hexane- and DCM-extracted MeOH phase was evaporated as in section 2.3 to give the MTSBP extract. All three partitioned extracts were tested for antityrosinase and anti-oxidation activities as detailed in sections 2.5 and 2.6, respectively.

2.5. *In vitro* antityrosinase activity

The *in vitro* antityrosinase activity was determined as previously reported (Zhang et al., 2015) with minor modification. Five different concentrations of the respective test sample dissolved in dimethyl sulfoxide (DMSO) were prepared. The reaction mixture contained 120 µL of 2.5 mM L-DOPA in 80 mM phosphate buffer pH 6.8 (PB), 30 µL of PB and 10 µL of the sample solution in DMSO. The mixture was mixed and pre-incubated at 25 °C for 10 min, before 40 µL of 165 units/mL mushroom tyrosinase in 80 mM PB was added and incubated at 25 °C for 5 min. The absorbance was then measured at 475 nm (A_{475}) by a microplate reader (Sunrise, Tecan, Austria). In parallel, kojic acid was used as a positive reference standard of a diphenolase inhibitor. Each sample was performed and measured in triplicate and the data is reported as the mean ± one standard deviation (SD). The inhibition of tyrosinase (%), or antityrosinase activity, was calculated using Eq. (1);

$$\% \text{ Tyrosinase inhibition} = \frac{[(A-B) - (C-D)]}{(A-B)} \times 100, \quad (1)$$

where A is the A_{475} after incubation without the test substance, B is the A_{475} after incubation without the test substance and tyrosinase, C is the A_{475} after incubation with the test substance and tyrosinase, and D is the A_{475} after incubation with the test substance but without tyrosinase.

The inhibitory concentration at 50% (IC_{50}) was obtained using Microsoft Excel 2016 after plotting a graph of the tyrosinase inhibition (%) against the concentration of the respective sample and using linear and nonlinear regression and a correlation of both parameters.

2.6. *In vitro* anti-oxidation activity

The anti-oxidation activity of each sample was determined *in vitro* using the DPPH assay modified from Chantarudee et al. (2012). Five different concentrations of the respective test sample were prepared in DMSO. For each concentration, 20 µL of sample was mixed with 80 µL of 0.15 mM DPPH in MeOH and incubated at 25 °C for 30 min. The absorbance at 517 nm (A_{517}) was then measured. In parallel, ascorbic

acid (vitamin C) was used as the reference standard. The antioxidation activity (%) was calculated from Eq. (2);

$$\text{Antioxidation activity (\%)} = \frac{(\text{ABS control} - \text{ABS sample})}{\text{ABS control}} \times 100 \quad (2)$$

where ABS control and ABS sample are defined as the A_{517} of the control and sample, respectively. The data are reported as the mean \pm SD.

The effective concentration at 50% (EC_{50}) was obtained using Microsoft Excel 2016 after plotting the antioxidation activity (%) against the concentration of each sample and using linear and nonlinear regression formula and a correlation of both parameters.

2.7. Enrichment of active components

2.7.1. Silica gel 60 column chromatography ($SG_{60}CC$)

The partitioned extract providing the highest antityrosinase activity was further fractionated by $SG_{60}CC$ using a 500-mL column. The partitioned extract (4.93 g) was dissolved in 40 mL of MeOH and combined with 10 g of rough SG_{60} . After drying, it was gradually poured over the surface of the packed $SG_{60}C$ and then eluted in 500 mL of DCM followed by 2 L of 10: 1 (v/v) DCM: MeOH and 1 L of MeOH, collecting 10 mL fractions. Each fraction was analyzed by one-dimensional thin layer chromatography (1D-TLC). Fractions with similar chemical profile were pooled and evaporated as per section 2.3.

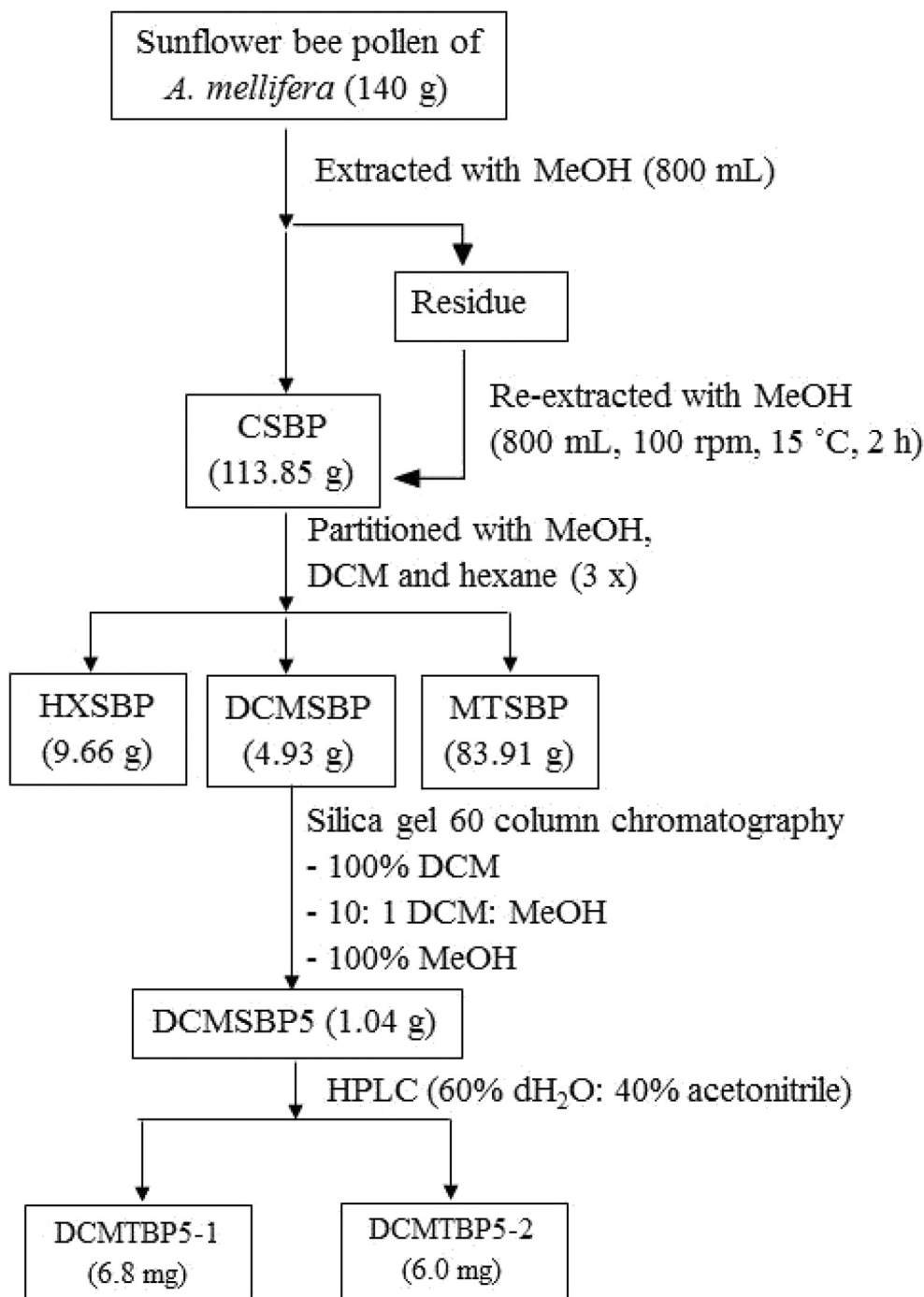


Figure 1. Summarized extraction and enrichment procedures for the SBBP.

2.7.2. Identification of likely chemically similar fractions by 1D-TLC analysis

A TLC plate of $5 \times 5 \text{ cm}^2$ was used as the immobile phase. The sample (section 2.7.1) was spotted onto the solvent front line using a capillary tube, dried at $25 \text{ }^\circ\text{C}$ and resolved against the appropriate mobile phase, such as 10: 1 (v/v) DCM: MeOH and 10: 0.5: 1 (v/v) DCM: ethyl acetate: MeOH. The TLC plate was dried, dipped in anisaldehyde and heated over a hot plate and then visualized by ultraviolet light at a wavelength of 254 nm. Fractions displaying the same pattern of chemical compounds were pooled and tested for tyrosinase inhibitory activity.

2.7.3. High performance liquid chromatography (HPLC) fractionation

To further fractionate the selected fractions (section 2.7.2), the HPLC method reported by Lv et al. (2015) was further developed and modified. The optimal operating condition (data not shown) was found using a SB-PHENYL column ($5 \mu\text{m}$, $9.4 \times 250 \text{ mm}$), loading $10 \times 10 \mu\text{L}$ aliquots of the respective sample (100 mg/mL in MeOH) with a column temperature of $25 \text{ }^\circ\text{C}$ and eluting in an isocratic mobile phase (1 mL/min) of milli Q H_2O and acetonitrile (ACN) ranging from 0:100 to 70:30 (v/v) H_2O : ACN. The eluted fractions were detected by UV-visible spectroscopy at 254 nm (A_{254}). The retention time of the extract was determined.

2.8. Chemical structure analysis

The active compounds that had been enriched to potential purity (section 2.7.3) were characterized by NMR spectroscopy. Briefly, the evaporated sample was dissolved in an appropriate deuterated solvent (MeOH- D_4 , Merck) at a ratio of 5 mg compound: $600 \mu\text{L}$ deuterated solvent, transferred to an NMR tube and shaken until homogeneous. The NMR spectra were recorded by a Bruker Avance III HD 500 spectrometer, operated at 500 MHz for ^1H and 126 MHz for ^{13}C nuclei using tetramethylsilane as the internal standard. The chemical shift value in δ (ppm) was assigned with reference to the signal or the residual protons in the deuterated solvents. Chemical shifts and J coupling values are reported in ppm and Hz, respectively. The molecular weight of active fractions was analyzed by a microTOF focus II mass spectrometer using electrospray ionization.

The overall procedure of the SBP enrichment for antityrosinase and antioxidation activities is summarized schematically in Figure 1.

2.9. Statistical analysis

Using the SPSS program version 19.0, data which were derived from three independent repeats in each experiment are presented as the mean \pm one standard deviation (1 S.D.). They were analyzed by one way analysis of variance (ANOVA). The significance of difference was accepted at the $p < 0.05$ level.

3. Results

The plant origin of the collected bee pollen was determined from its morphology under SEM, using known SBP as a reference material. The morphology of the bee pollen was homogeneous (Figure 2), spherical in shape with three farrows (tricolporate pollen) and spines (Figure 2), consistent with that of sunflower pollen (Lin et al., 2016).

The SBP was then extracted in MeOH to give 113.85 g of CSBP, a yield of 81.3% (Table 1), as a turbid brown oil in color.

The CSBP was then further partitioned sequentially by hexane and DCM to yield the three crude partitioned extracts (HXSBP, DCMSBP, and MTSBP for the hexane, DCM, and residual MeOH extracts of CSBP, respectively). Their yields and appearances are summarized in Table 1. These partitioned extracts were tested for their antityrosinase and antioxidation activities.

For the antityrosinase activity, using the *in vitro* mushroom tyrosinase inhibitory assay, the obtained absorbance was converted to the tyrosinase inhibition activity (%), and are presented as the mean \pm SD in Table 1. Only DCMSBP had a significant antityrosinase activity ($\text{IC}_{50} = 159.4 \mu\text{g/mL}$), but this was over 18-fold less effective than kojic acid ($\text{IC}_{50} = 8.6 \mu\text{g/mL}$). Nonetheless, its value was still much better than the other partitioned extracts ($\text{IC}_{50} > 500 \mu\text{g/mL}$).

For the antioxidation activity, determined using the DPPH assay, no significant antioxidant activity was detected for all three crude extracts (EC_{50} values $> 1,000 \mu\text{g/mL}$) compared to ascorbic acid (EC_{50} of $89.8 \mu\text{g/mL}$).

Since the DCMSBP provided the best antityrosinase activity ($\text{IC}_{50} = 159.4 \mu\text{g/mL}$), the sample (4.93 g) was further enriched using SG_{60}CC . A total of 155 fractions were collected, but after pooling fractions with a similar 1D-TLC plate profile five different fractions (DCMSBP1–5) were

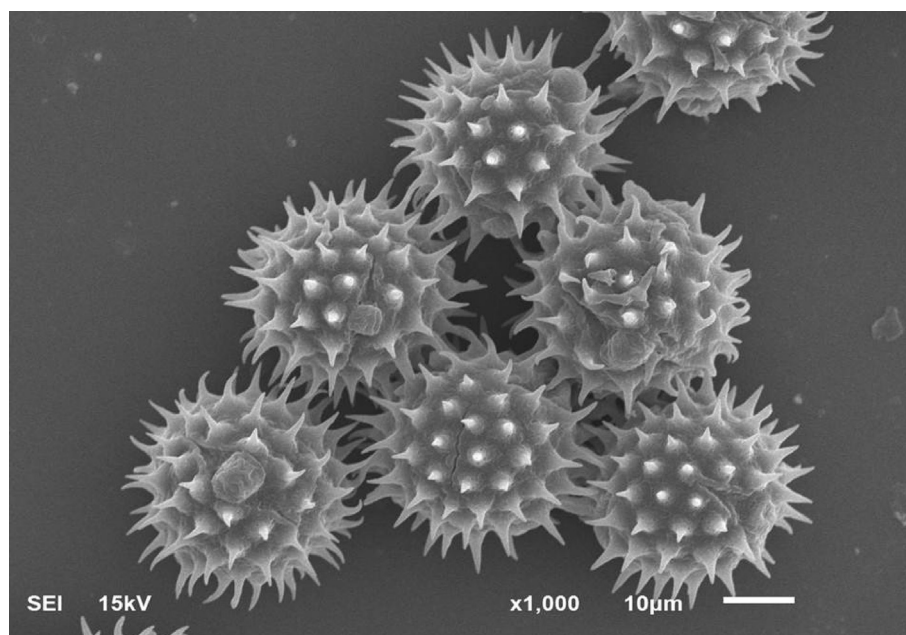


Figure 2. The morphology of SBP, as observed under SEM at $1,000 \times$ magnification.

Table 1. The IC₅₀ and EC₅₀ values of the crude and partitioned extracts.

Sample	Weight (g)	Yield (%)	Character	IC ₅₀ (µg/mL)	EC ₅₀ (µg/mL)
Crude extract					
CSBP	113.85	81.32	Turbid brown oil	-	-
Partitioned extract					
MTSBP	83.91	73.70	Dark brown oil	>500 ^c	>1,000 ^b
DCMSBP	4.93	4.33	Sticky dark brown solid	159.39 ^b	>1,000 ^b
HXSBP	9.66	8.48	Dark brown oil	>500 ^c	>1,000 ^b
Reference controls					
Ascorbic acid	-	-	-	-	89.82 ^a
Kojic acid	-	-	-	8.58 ^a	-

Remark: Data are shown as the mean from three replicates. Means within a column with a different superscript letter are significantly different ($p \leq 0.05$; One way ANOVA).

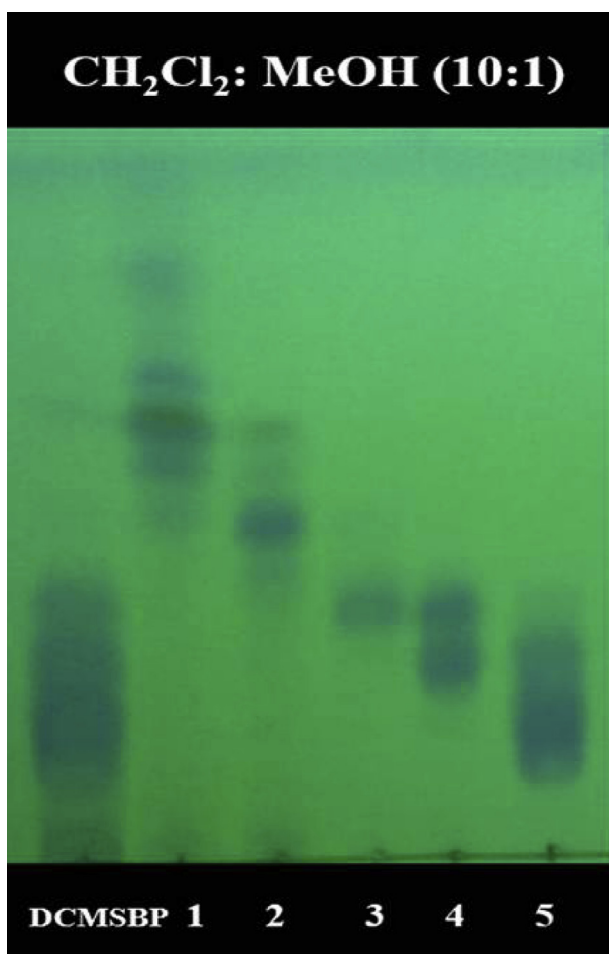


Figure 3. Representative 1D-TLC plate showing the chemical profile of DCMSBP and the five fractions (DCMSBP1–5) obtained after SG₆₀CC and pooling fractions with a similar 1D-TLC profile.

obtained (Figure 3). Their weight and characteristics are recorded in Table 2.

Pooled fractions DCMSBP1–5 were separately tested for the antityrosinase activity (%) at a concentration of 50 µg/mL. At this concentration, fraction DCMSBP5 provided the highest antityrosinase activity at $52.4 \pm 4.6\%$, followed by DCMSBP4 at $34.6 \pm 5.4\%$, which were both lower than that for kojic acid (Figure 4). The plot of the concentration against antityrosinase activity for DCMSBP5 and kojic acid is shown in Figure 5.

The IC₅₀ value for the antityrosinase activity of DCMSBP5 was 18.8 µg/mL, which was significantly (1.8-fold) higher (less effective) than that of kojic acid (IC₅₀ of 10.4 µg/mL), but it was 8.5-fold lower (more effective) than the parental DCMSBP extract (IC₅₀ of 159.4 µg/mL), suggesting that active compound(s) had been enriched by the SG₆₀CC. Thus, fraction DCMSBP5 was further fractionated by HPLC.

The HPLC was eluted with an isocratic gradient of 0:100 to 70:30 (v/v) H₂O: ACN, where DCMSBP5 was separated into seven peaks, but the two main peaks eluting in 60:40 (v/v) H₂O: ACN at a retention time of 22.217 and 23.991 min (Figure 6) were the only ones found to have antityrosinase activity. These two fractions (DCMSBP5-1 and DCMSBP5-2) were defined as compounds 1 and 2, respectively. Their weight and characters are summarized in Table 3.

After enrichment to potential purity, the chemical structures of compounds 1 and compound-2 were characterized by ¹H- and ¹³C-NMR spectroscopy and mass spectrometry. Compound 1 was obtained as a white powder with a molecular ion peak [M + Na]⁺ at a m/z of 606.2576. The ¹H-NMR spectrum showed two sets of cis-olefinic hydrogen signals at δ 6.58 and 5.92/5.8 ppm and a set of trans-olefinic hydrogen signals at δ 7.45 and 6.37 ppm, three sets of *p*-substituted phenyl signals at δ 7.39, 7.21, 6.77, and 6.71 ppm and methylene proton signals on the spermidine backbone. The ¹³C-NMR spectrum also showed three sets of carbon signals of the carbonyl group at 172.04, 170.59, and 169.33 ppm, two sets of signals of carbon in three aromatic rings between 116.4 and 131.2 ppm, two sets of olefinic carbons at 121.0, 121.8, 137.8, and 134.9 ppm, and a signal of carbon on the spermidine backbone between 47.78 and 25.69 ppm, which was consistent with the structure of *N*¹-(*E*)-*N*⁵,*N*¹⁰-(*Z*)-tri-*p*-coumaroyl spermidine or safflopermidine B.

In the case of compound 2, it was obtained as a white powder with a molecular ion peak [M + Na]⁺ at a m/z of 606.2522. The ¹H-NMR showed two sets of trans-olefinic hydrogen signals at δ 7.42 and 6.38 ppm and a set of trans-olefinic hydrogen signals at δ 6.58 and 5.93/5.81 ppm, plus three sets of *p*-substituted phenyl signals and seven methylene proton signals similar to compound 1. The ¹³C-NMR spectrum also showed carbon signals that were similar to the corresponding carbon signals in compound 1, suggesting that compound 2 was consistent with the structure of *N*¹,*N*¹⁰-(*E*)-*N*⁵-(*Z*)-tri-*p*-coumaroyl spermidine or safflopermidine A. Therefore, after analysis, compounds 1 and 2 were ascribed as safflopermidine B and A, respectively (Figure 7).

Compound 1

*N*¹-(*E*)-*N*⁵,*N*¹⁰-(*Z*)-Tri-*p*-coumaroyl spermidine or safflopermidine B (Figure 5): White powder; HR-ESI-MS m/z: 606.2576 [M + Na]⁺; ¹H-NMR (500 MHz, Methanol-*d*₄) δ: 7.45 (d, 1H, 15.7 Hz), 7.40/7.35 (m, 4H), 7.24–7.17 (m, 2H), 6.77 (m, 2H), 6.71 (m, 3H), 6.57 (m, 4H), 6.37 (q, 1H, 15.7 Hz), 5.92 (m, 1H), 5.8 (q, 1H, 12.5 Hz), 3.45 (m, 2H), 3.35 (m, 2H), 3.18 (m, 2H), and 1.88–1.31 (m, 8H). ¹³C-NMR (126 MHz,

Table 2. The weight, yield (compared to DCMSBP) and character of the pooled fractions (DCMSBP1–5) obtained after pooling fractions with a similar 1D-TLC profile.

Fraction	Weight (g)	Yield (%)	Character
DCMSBP1	0.51	10.34	Sticky dark brown solid
DCMSBP2	0.76	15.42	Sticky dark brown solid
DCMSBP3	0.25	5.07	Sticky dark brown solid
DCMSBP4	0.67	13.59	Sticky dark brown solid
DCMSBP5	1.04	21.10	Pale yellow solid

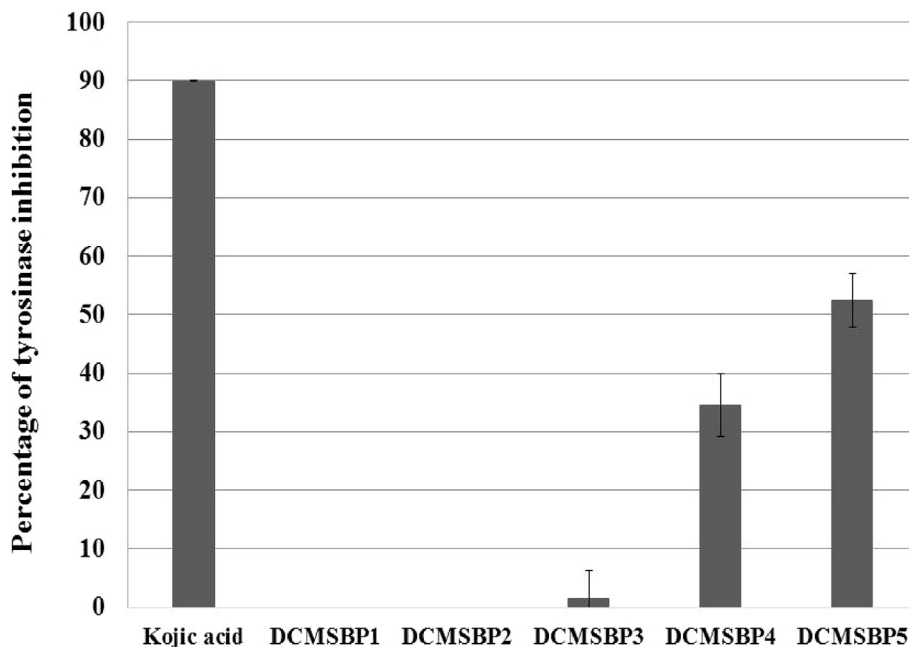


Figure 4. Antityrosinase activity (%) of fractions DCMSBP1–5 and kojic acid at 50 µg/mL. Data are shown as the mean ± 1SD, derived from three replicates.

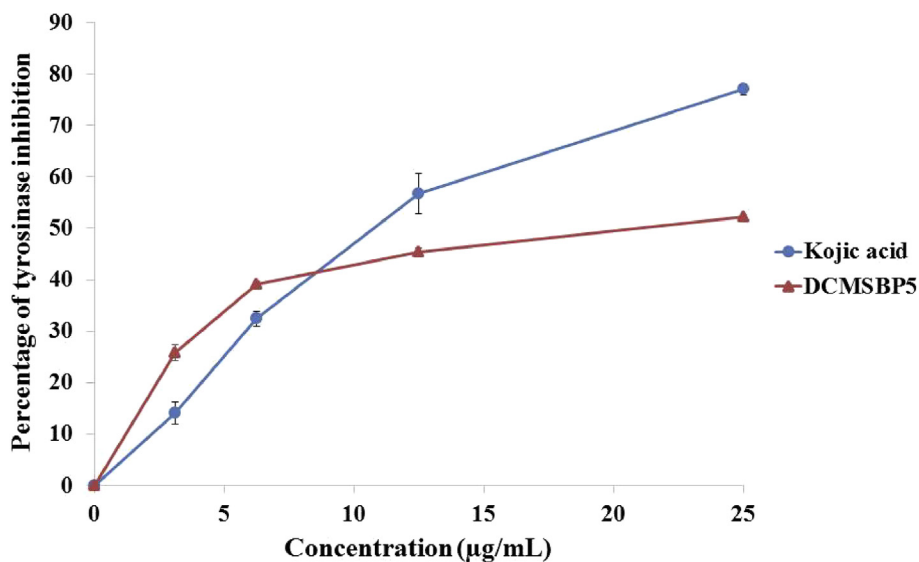


Figure 5. Antityrosinase activity (%) of fraction DCMSBP5 compared to kojic acid. Data are shown as the mean ± 1SD.

MeOD) δ: 172.04, 170.59, 169.33, 160.70, 159.40, 141.86, 137.78, 134.90, 132.18, 131.20, 130.59, 128.20, 127.63, 121.77, 120.96, 118.40, 116.77, 116.40, 116.03, 47.78, 46.02, 44.08, 40.05, 39.87, 38.26, 37.89, 37.74, 29.77, 28.35, 27.91, 27.31, 27.01, 25.78, and 25.69.

Compound 2

*N*¹,*N*¹⁰-(*E*)-*N*⁵-(*Z*)-Tri-*p*-coumaroyl spermidine or safflospermidine A (Figure 5): White powder; HR-ESI-MS *m/z*: 606.2522 [M + Na]⁺; ¹H-

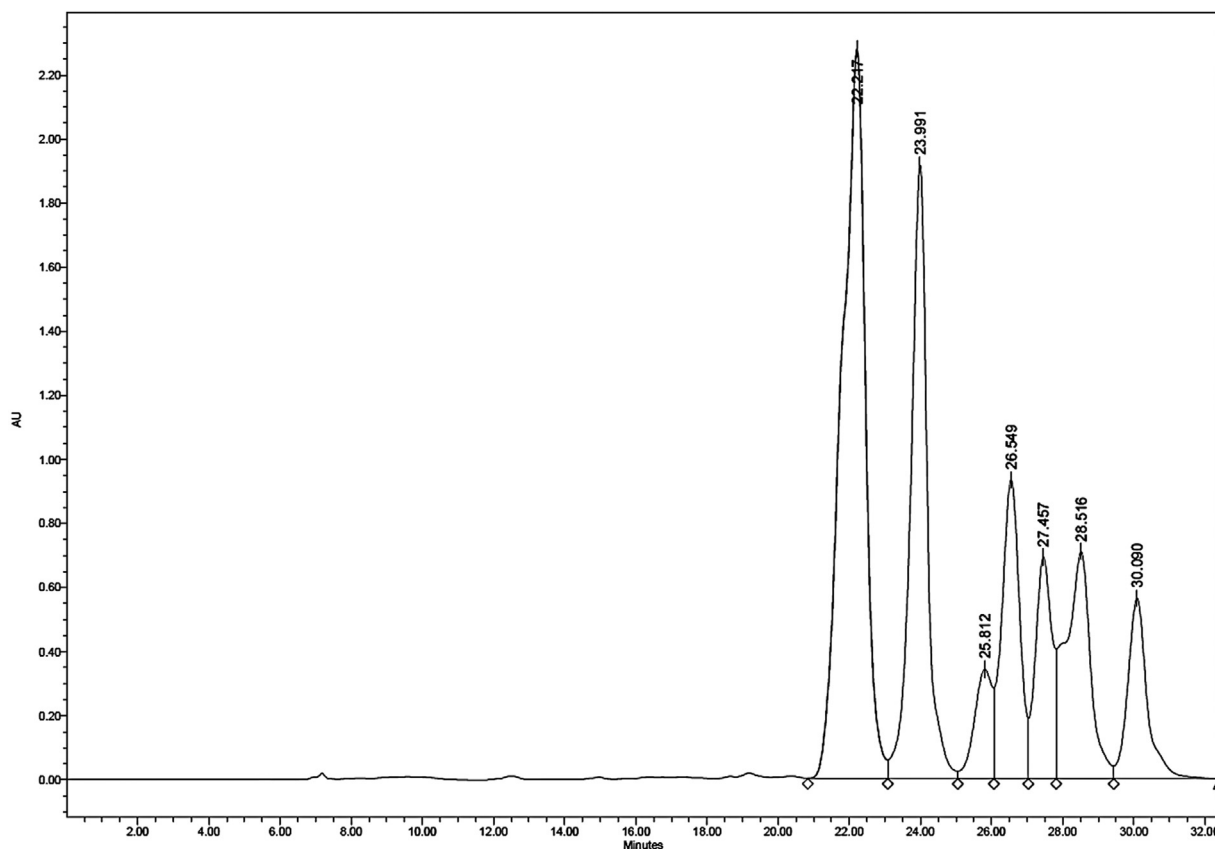


Figure 6. The HPLC chromatogram of DCMSBP5 showing the elution of DCMSBP5-1 and DCMSBP5-2 at a retention time of 22.217 and 23.991 min, respectively.

Table 3. The IC₅₀ value of fraction number DCMSBP5-1 and DCMSBP5-2 after HPLC.

Fraction	Weight (mg)	Yield (%) *	Character	IC ₅₀ (μM)
DCMSBP5-1 (Compound 1 or safflospermidine B)	5.3	26.5	White solid	13.6 ^a
DCMSBP5-2 (Compound 2 or safflospermidine A)	4.8	24.0	White solid	31.8 ^b
Kojic acid**	-	-	-	44.0 ^c

Remark: *HPLC fractionation of DCMSBP5 was performed with 20 injections of 10 μL of 100 mg/mL DCMSBP5.

**The IC₅₀ values were calculated from a nonlinear regression and are shown as the mean. Means with a different superscript letter are significantly different ($p \leq 0.05$; One-way ANOVA).

NMR (500 MHz, Methanol-d₄) δ: 7.42 (m, 7H), 7.20 (m, 1H), 6.78 (m, 4H), 6.71 (m, 2H), 6.58 (m, 1H), 6.38 (m, 2H), 5.93/5.81 (m, 1H), 3.10–3.60 (m, 8H) and 1.30–1.95 (m, 6H). ¹³C-NMR (126 MHz, MeOD) δ: 172.04, 169.30, 160.68, 159.41, 141.87, 134.90, 132.17, 131.20, 130.58, 128.06, 127.61, 121.79, 120.96, 118.42, 116.77, 116.40, 47.58, 46.03, 44.08, 40.12, 39.88, 38.17, 37.91, 29.77, 28.06, 27.86, 26.95, and 25.69.

Safflospermidine A and B were separately tested for their *in vitro* antityrosinase activity, with the IC₅₀ values given in Table 3.

Fractions DCMSBP1–5 were also tested for their antioxidation activity at 1,000 μg/mL, with the results shown in Figure 8. Fraction DCMSBP2 provided the highest antioxidation activity at $87.8 \pm 1.2\%$, followed by (in order) DCMSBP3, DCMSBP4, DCMSBP1, and DCMSBP5.

The EC₅₀ values were estimated and are reported in Table 4 in comparison to ascorbic acid as the standard reference. Fraction DCMSBP2 had the lowest EC₅₀ value (538.3 μg/mL) of the samples, which was much (ca. 7.5-fold) higher (less active) than that for ascorbic acid (71.5 μg/mL). However, its EC₅₀ value was still much lower than that for DCMSBP3, DCMSBP4, and DCMSBP1.

The antioxidation activity of DCMSBP5 ($15.5 \pm 3.8\%$) was lower than that of the parental DCMSBP extract ($23.06 \pm 3.11\%$), whereas fractions DCMSBP1–4 had a greater antioxidation activity (lower EC₅₀ value) than the parental DCMSBP (Figure 8 and Table 4). However, since their antioxidation activity was much lower than that of ascorbic acid, they were not further fractionated.

4. Discussion

In this research, SBP was chosen because of the large area of sunflower monocrops grown for oil in Thailand. In those areas, pollinators, especially honeybees, are needed for pollination. The plant origin of bee products is important for quality control, including any potential health risk for consumers (Kast et al., 2018). Morphological analysis under SEM has been widely used to identify plant pollen types (Kast et al., 2019), and here confirmed that the bee pollen in this work originated from sunflower (Figure 2). Thus, it would be safe to consume with the nutritive value of pollen protein. The protein content and amino acid compositions of SBP have been reported previously (Taha et al., 2019). Beside nutrition, bee pollens are widely known as a natural product consisting of various biologically active substances which depend mainly on botanical origin (Dukhanina et al., 2006; Mosaic et al., 2019).

In this work, the antityrosinase and antioxidant activity of SBP was focused on instead. The antityrosinase activity of SBP was fractionated to apparent purity of two main compounds, safflospermidine A and B (although these may not be the only such compounds). Safflospermidine

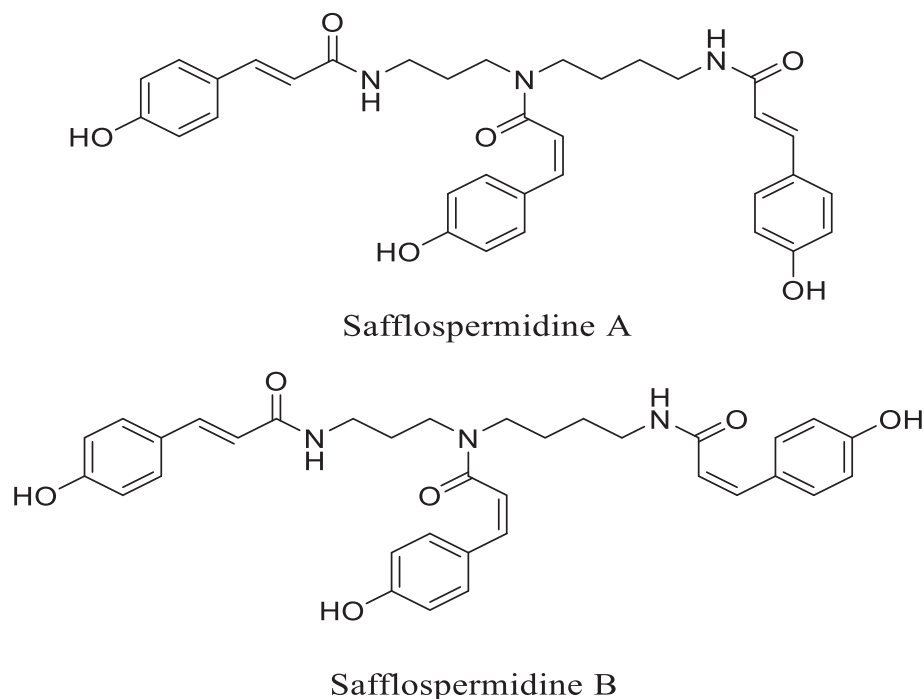


Figure 7. Chemical structure of safflospersmidine A and B, which matches that deduced for compounds 2 and 1, respectively.

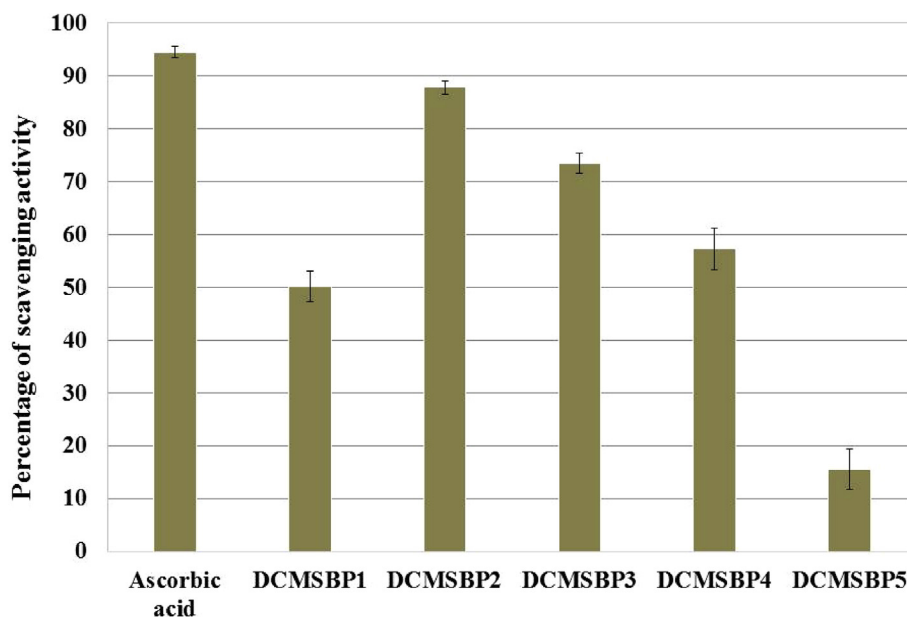


Figure 8. Antioxidation activity of fractions DCMSBP1–5 at 1,000 µg/mL compared to that for ascorbic acid (1,000 µg/mL). Data are shown as the mean ± 1SD, derived from three replicates.

B had a higher antityrosinase activity than safflospersmidine A, and both compounds were more active than the reference standard kojic acid (Table 3) using the L-DOPA assay. Since the commercial tyrosinase was purified from mushroom, and is highly homologous with mammalian tyrosinases (Chang, 2009), it is likely that the method used in this work can be used as a screening method for searching for human tyrosinase inhibitors.

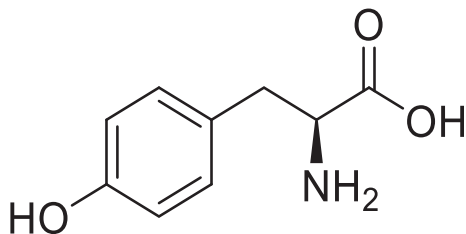
With respect to the relationship between the chemical structure and antityrosinase activity, the active compounds obtained in this study were polyamine derivatives, as spermidine conjugated with *p*-coumaroyl moieties (Figure 6). Considering the *p*-coumaroyl moieties of the polyamine derivatives, the structures and orientations are somewhat similar

to tyrosine (Figure 9). Therefore, spermidine derivatives, such as those extracted from SBP, may bind to the active site of tyrosinase like a lock and key. Since they block tyrosine and L-DOPA as well, tyrosine and L-DOPA would not be converted to dopachrome and melanin. Thus, the *p*-coumaroyl moieties of spermidine derivatives may play a key role in the inhibition of tyrosinase. These data are consistent with a study on the tyrosinase inhibitory activity of polyamine derivatives from the bee pollen of *Quercus mongolica*, where the polyamine derivatives with *p*-coumaroyl moieties showed an antityrosinase activity, and the authors concluded that polyamines with phenolic groups were good tyrosinase inhibitors. However, the inhibitory activity might be different depending on the number and type of phenolic moieties (Kim et al., 2018).

Table 4. The EC₅₀ values of the pooled fractions (DCMSBP1-4) after SG₆₀CC.

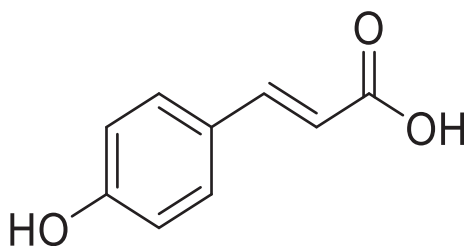
Sample	EC ₅₀ value (μg/mL)*
DCMSBP1	986.0 ^e
DCMSBP2	538.5 ^b
DCMSBP3	633.0 ^c
DCMSBP4	822.5 ^d
Ascorbic acid	71.5 ^a

Remark: *The EC₅₀ values were calculated from a nonlinear regression and are shown as the mean, derived from three replicates. Means with a different superscript letter are significantly different ($p \leq 0.05$; One way ANOVA).

**Figure 9.** Chemical structure of tyrosine.

Moreover, the antityrosinase activity of *p*-coumaric acid (Figure 10), which was similarly presented in the two spermidine compounds isolated in this work from SBP, has been reported before (An et al., 2010), where the *p*-coumaric acid acted as a strong inhibitor of human and murine tyrosinase compared with kojic acid and arbutin. In addition, *p*-coumaric acid inhibited human tyrosinase at much lower concentrations than those required for the inhibition of murine tyrosinase. Kinetic analysis revealed that *p*-coumaric acid was a mixed type (for tyrosine) or a competitive (for L-DOPA) inhibitor of human tyrosinase. The antimelanogenic effect of *p*-coumaric acid was observed in human epidermal melanocytes exposed to UVB, where *p*-coumaric acid was a potent and selective inhibitor of human tyrosinase and so potentially useful as a hypopigmenting agent (An et al., 2010).

The relationship between both antityrosinase and antioxidation activities of bee pollen from acorn trees (*Quercus acutissima*, Fagaceae) has been reported (Kim et al., 2015), where they were both mainly correlated to the phenolic content. The same relationship between both bioactivities and the correlation to the phenolic content has also been reported from the hydrosol (cinnamaldehyde and benzaldehyde as the main compounds) of *Cinnamomum osmophloeum* Kanehira leaves (Ho et al., 2019). In addition, kojic acid, *p*-coumaric acid, and quercetin all showed this phenomena (Chen et al., 2019b; Lee et al., 2018). However, some compounds were reported to contain either an antityrosinase or an antioxidation activity. For example, 1,3,5,6-Tetrahydroxy-9H-xanthen-9-one exhibit only an antioxidation activity, while 1,3,7-trihydroxy-9H-xanthen-9-one, 1,3-dihydroxy-5,7-dinitro-9H-xanthen-9-one, 1,3-dihydroxy-6-(trifluoromethyl)-9H-xanthen-9-one, and 7-(2,4-difluorophenyl)-1,3-dihydroxy-9H-xanthen-9-one had only an antityrosinase activity (Zhou et al., 2018).

**Figure 10.** The chemical structure of *p*-coumaric acid.

Although the SBP had only a significant antityrosinase activity, this was at least in part due to the safflospermidine A and B content. However, an antioxidant activity was previously found in the bee pollen from chestnuts, roses, and *Schisandra chinensis* (Sahin and Karkar, 2019; Yang et al., 2019; Shen et al., 2019). Thus, identification of the plant origin of bee pollen is necessary in order to get the targeted bioactivity.

Besides sunflowers, safflospermidine A and B have also been isolated from the florets of *Carthamus tinctorius* (Jiang et al., 2008), but their antityrosinase activity is firstly reported in this work.

Overall, it can be concluded that safflospermine A and B in SBP are potential candidates as tyrosinase inhibitors compared to kojic acid. Both compounds may be useful in cosmetic therapeutics to reduce hyperpigmentation. However, in the future, it is required to test the safety of using these compounds. The cytotoxicity of both compounds should be investigated at the cellular level, such as initially in the B16F10 melanoma cell. In addition, skin irritation can be performed in mice (Ko et al., 2013).

Declarations

Author contribution statement

Preecha Phuwapraisirisan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Chanpen Chanchao: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Phanthiwa Khongkarat: Performed the experiments; Wrote the paper.

Rico Ramadhan: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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