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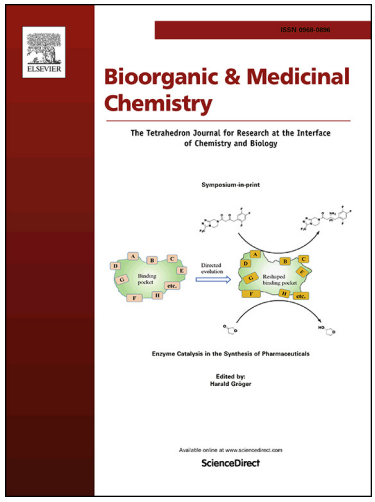

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Design, synthesis and anti-melanogenic effect of cinnamamide derivatives

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Abstract

Pigmentation disorders are attributed to excessive melanin which can be produced by tyrosinase. Therefore, tyrosinase is supposed to be a vital target for the treatment of disorders associated with overpigmentation. Based on our previous findings that an (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffold can play a key role in the inhibition of tyrosinase activity, and the fact that cinnamic acid is a safe natural substance with a scaffolded structure, it was speculated that appropriate cinnamic acid derivatives may exhibit potent tyrosinase inhibitory activity. Thus, ten cinnamamides were designed, and synthesized by using a Horner-Emmons olefination as the key step. Cinnamamides **4** (93.72% inhibition), **9** (78.97% inhibition), and **10** (59.09% inhibition) with either a 2,4-dihydroxyphenyl, or 4-hydroxy-3-methoxyphenyl

substituent showed much higher mushroom tyrosinase inhibition at 25 μM than kojic acid (18.81% inhibition), used as a positive control. Especially, the two cinnamamides **4** and **9** having a 2,4-dihydroxyphenyl group showed the strongest inhibition. Docking simulation with tyrosinase revealed that these three cinnamamides, **4**, **9**, and **10**, bind to the active site of tyrosinase more strongly than kojic acid. Cell-based experiments carried out using B16F10 murine skin melanoma cells demonstrated that all three cinnamamides effectively inhibited cellular tyrosinase activity and melanin production in the cells without cytotoxicity. There was a close correlation between cellular tyrosinase activity and melanin content, indicating that the inhibitory effect of the three cinnamamides on melanin production is mainly attributed to their capability for cellular tyrosinase inhibition. These results imply that cinnamamides having the (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffolds are promising candidates for skin-lighting agents

Key words: cinnamamide, melanin, tyrosinase inhibitor, docking, B10F16 melanoma cells.

1. Introduction

Melanin is a generic term for pigment complexes present in the tissues of plants and animals. In humans, melanin is biosynthesized in the melanosome of melanocytes present in the epidermal basal layer and melanin production is generally initiated by exposure to UV light.[1] In plants, melanin is produced in excess when the mixture of tyrosinase and tyrosinase substrates is contacted with oxygen by destruction of the cell structure, such as exfoliation of the agricultural product peels such as vegetables and fruits, or external physical impacts. Melanin is a major factor in determining the colour of the skin, pupil and hair.[2] Their colour is mainly determined by the amounts and ratios of eumelanin and pheomelanin. Eumelanin and pheomelanin are synthesized through different synthetic pathways from dopaquinone, a common intermediate in the melanin biosynthesis process.[2,3]

Melanin can block UV rays and protect skin cells from harmful UV radiation. However, local

melanin deposits on the skin, such as senile spots, cause cosmetic problems.[4-6] In addition, melanin causes browning of agricultural products,[7] causing damage to flavour and tissue, which ultimately lowers the marketability. Thus, compounds that effectively inhibit melanogenesis can be used as therapeutic agents for skin diseases such as hyperpigmentation, cosmetics, and antibrowning agents for agricultural products in the food industry.

Melanin is synthesized through chemical and enzymatic reactions starting from L-tyrosine, and several enzymes are involved in melanin biosynthesis, including tyrosinase, which is widely distributed in nature, from bacteria to plants and humans.[8] Tyrosinase is involved in three chemical reactions in the melanin biosynthesis process, and in the rate-limiting step which oxidizes L-tyrosine to dopaquinone.[9] Thus, tyrosinase has been widely recognized as an important target enzyme for the development of whitening agents. This enzyme is a metalloenzyme with two copper (II) ions at the active site and it acts as an oxidase that oxidizes the phenolic ring.[10] Therefore, some researchers have attempted to develop tyrosinase inhibitors by synthesizing a copper chelator.[11,12]

For the past several years, our laboratory has synthesized a variety of phenolic compounds mimicking the chemical structure of natural substrates, L-dopa and L-tyrosine, for tyrosinase and has evaluated their tyrosinase inhibitory activity to find competitive tyrosinase inhibitors that bind to the active site of tyrosinase more strongly than L-tyrosine and L-dopa.[13-18] From these studies, we concluded that compounds with an (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffold exhibit profound tyrosinase-inhibitory activity. Cinnamic acid, a natural compound isolated from cinnamon or blossom,[19] has been reported to have extensive physiological activities[20-27] and also to show mild tyrosinase inhibitory activity.[28]

Cinnamic acid has the (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffold in view of the molecular structure and is not cytotoxic.[29] Additionally, it showed potential towards to the inhibition of tyrosinase although the potency was mild. Amide bonds are widely present in many medicines

available in the market and are used as important functional groups because they are structurally stable and can act as hydrogen bond donors and/or receptors. Therefore, we targeted a variety of cinnamamide derivatives to find potent tyrosinase inhibitors.

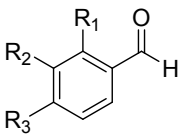
In this study, as part of our ongoing efforts to find novel tyrosinase inhibitors derived from natural products and featuring the (*E*)-scaffold, we synthesized a series of cinnamamide analogues and examined their anti-tyrosinase activity using mushroom tyrosinase and their anti-melanogenic effect through cell-based assays using a murine cell line.

2. Results and Discussion

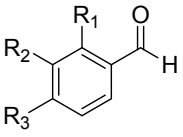
2.1. Chemistry

Our strategy for cinnamic acid derivatization was to synthesize stable cinnamamides with various substituents on the β–phenyl ring, which could be easily prepared from Horner-Emmons olefination of benzaldehydes having various substituents in appropriate phenyl positions with triethyl phosphonoacetate followed by saponification and coupling reaction with secondary cyclic amines such as pyrrolidine and piperidine. According to the structure-activity relationship (SAR) data we have collected, compounds having at least one hydroxyl group on the β–phenyl ring of the (*E*)-motif, that is, 4-hydroxyphenyl, 4-hydroxy-3-methoxyphenyl, 2,4-dihydroxyphenyl, 3-hydroxy-4-methoxyphenyl and 3,4-dihydroxyphenyl, generally showed potent inhibition towards tyrosinase. Therefore, cinnamamide derivatives with these five phenyl rings were synthesized. First, the hydroxyl groups of five benzaldehydes (3-hydroxy-4-methoxybenzaldehyde (**11**), 4-hydroxybenzaldehyde (**12**), 3,4-dihydroxybenzaldehyde (**13**), 2,4-dihydroxybenzaldehyde (**14**), and 4-hydroxy-3-methoxybenzaldehyde (**15**)) were protected as benzyl ethers, giving compounds **16** – **20**, as shown in Scheme 1. Horner-Emmons olefination between aldehydes **16** – **20** and triethyl phosphonoacetate in the presence of bases (K_2CO_3, and a catalytic amount of DBU (1,8-diazabicyclo[5.4.0]undec-7-ene)) afforded ethyl

cinnamates **21** – **25**, which were hydrolysed to produce the corresponding cinnamic acids **26** – **30**. After the activation of the carboxylic acid functionality of **26** – **30** to the corresponding mixed anhydrides using isobutyl chloroformate in the presence of *N*-methylmorpholine, treatment of two secondary cyclic amines, pyrrolidine and piperidine, afforded the corresponding cinnamamide derivatives **31** – **35** and **36** – **40**, respectively. Finally, debenzylation was conducted under reflux and acidic conditions using concentrated HCl and acetic acid to produce the corresponding cinnamamide analogues **1** – **3**, **5** – **8**, and **10**. However, these acidic conditions did not afford cinnamamides **4** and **9** with the 2,4-dihydroxyphenyl group, but rather produced a mixture of compounds with high polarity. Use of BBr_3 as an alternative Lewis acid for removal of the benzyl groups of **34** and **39**, interestingly, produced the desired cinnamamides **4** and **9** with the 2,4-dihydroxyphenyl moiety in a moderate yields. The structures of the synthesized cinnamamides **1** – **10** were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and mass spectroscopy. The geometry of the double bond of the final compounds was assigned as an (*E*)-form on the basis of *J* values (15.0 – 15.6 Hz) of the vinylic peaks in the ^{1}H NMR spectra.



a



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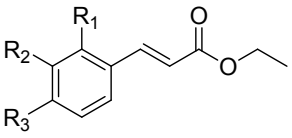

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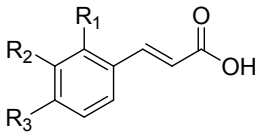
12: R_1 = H, R_2 = H, R_3 = OH
13: R_1 = H, R_2 = OH, R_3 = OH
14: R_1 = OH, R_2 = H, R_3 = OH
15: R_1 = H, R_2 = OMe, R_3 = OH

16: R_1 = H, R_2 = OBn, R_3 = OMe
17: R_1 = H, R_2 = H, R_3 = OBn
18: R_1 = H, R_2 = OBn, R_3 = OBn
19: R_1 = OBn, R_2 = H, R_3 = OBn
20: R_1 = H, R_2 = OMe, R_3 = OBn

21: R_1 = H, R_2 = OBn, R_3 = OMe
22: R_1 = H, R_2 = H, R_3 = OBn
23: R_1 = H, R_2 = OBn, R_3 = OBn
24: R_1 = OBn, R_2 = H, R_3 = OBn
25: R_1 = H, R_2 = OMe, R_3 = OBn

c



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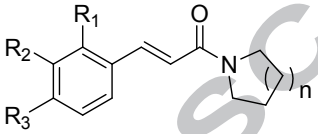

e

26: R_1 = H, R_2 = OBn, R_3 = OMe
27: R_1 = H, R_2 = H, R_3 = OBn
28: R_1 = H, R_2 = OBn, R_3 = OBn
29: R_1 = OBn, R_2 = H, R_3 = OBn
30: R_1 = H, R_2 = OMe, R_3 = OBn

31: n = 1, R_1 = H, R_2 = OBn, R_3 = OMe
32: n = 1, R_1 = H, R_2 = H, R_3 = OBn
33: n = 1, R_1 = H, R_2 = OBn, R_3 = OBn
34: n = 1, R_1 = OBn, R_2 = H, R_3 = OBn
35: n = 1, R_1 = H, R_2 = OMe, R_3 = OBn
36: n = 2, R_1 = H, R_2 = OBn, R_3 = OMe
37: n = 2, R_1 = H, R_2 = H, R_3 = OBn
38: n = 2, R_1 = H, R_2 = OBn, R_3 = OBn
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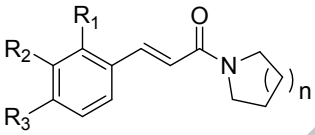
40: n = 2, R_1 = H, R_2 = OMe, R_3 = OBn



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3: n = 1, R_1 = H, R_2 = OH, R_3 = OH
4: n = 1, R_1 = OH, R_2 = H, R_3 = OH
5: n = 1, R_1 = H, R_2 = OMe, R_3 = OH
6: n = 2, R_1 = H, R_2 = OH, R_3 = OMe
7: n = 2, R_1 = H, R_2 = H, R_3 = OH
8: n = 2, R_1 = H, R_2 = OH, R_3 = OH
9: n = 2, R_1 = OH, R_2 = H, R_3 = OH
10: n = 2, R_1 = H, R_2 = OMe, R_3 = OH

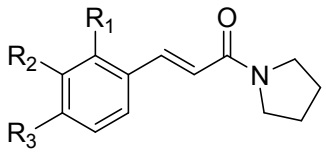
Scheme 1. Synthetic scheme of cinnamamide derivatives 1 – 10. Reagents and conditions: a) BnBr, K_2CO_3, CH_3CN, reflux, 24 h; b) $(EtO)_2P(=O)CH_2CO_2Et$, K_2CO_3, DBU (cat. amount), DCM, DMF, rt for **11**, **13**, and **15**, or 70 °C for **12**, and **14**, 24 – 36 h; c) 1N-NaOH aqueous solution, 1,4-dioxane, rt, 48 h; d) *i*-BuO_2CCl, *N*-methylmorpholine, THF, rt, 30 min, and then, pyrrolidine, or piperidine, rt, 24 h; and e) *c*-HCl, AcOH, reflux, 40 min, or DCM, 4 – 8 equiv. BBr_3, -40 ^{0}C, 30 min.

*2.2. Mushroom tyrosinase inhibitory effect of cinnamamides **1 – 10**.*

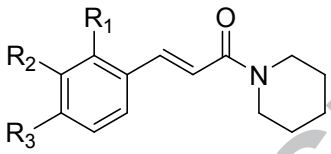
The inhibitory effects of the ten synthesized cinnamamide derivatives on mushroom tyrosinase were examined at 25 μM using kojic acid as a positive control, as depicted in Table 1. Overall, cinnamamide derivatives **6** – **10** with piperidine exhibited slightly higher inhibition than cinnamamide derivatives **1** – **5** with pyrrolidine, except for compounds **4** and **9** with a 2,4-dihydroxy group. Compounds, **1** and **6**, with a 3-hydroxy-4-methoxy group, showed slightly greater inhibitory activity with 26.42±3.49 and 36.70±6.52% inhibition, respectively, than kojic acid (18.81±1.25% inhibition). According to our cumulative SAR data, compounds with a 3-hydroxy-4-methoxyphenyl group generally showed stronger inhibition than compounds with a 4-hydroxy-3-methoxy group.[16,18,30,31]. Interestingly, therefore, compound **10** (59.09±1.40% inhibition) with a 4-hydroxy-3-methoxy group showed more potent inhibitory activity than compound **6** (36.70±6.52% inhibition) with a 3-hydroxy-4-methoxy group. Compounds **2** and **7**, with a 4-hydroxy group, exhibited similar or slightly less inhibition (13.65±3.78 and 19.07±3.74% inhibitions) than kojic acid. Introduction of an additional hydroxyl group at position 3 of the β–phenyl group of compounds **2** and **7** reduced the tyrosinase inhibitory activities, such that compounds **3** and **8** with a catechol (3,4-dihydroxyphenyl) group revealed weak to no inhibition against mushroom tyrosinase. On the other hand, introduction of an additional hydroxyl group at position 2 of the β–phenyl group of compounds **2** and **7** dramatically increased their tyrosinase inhibitory activities, such that compounds **4** and **9** with a 2,4-dihydroxy group exhibited the highest inhibitory activity with 93.72±0.25 and 78.97±0.16% inhibitions, respectively. This result is consistent with our cumulative SAR data showing that compounds having a 2,4-dihydroxyphenyl group generally show potent tyrosinase inhibitory activities.[32-36] These results imply that fine changes in the chemical structure can have a very large effect on the tyrosinase activity. Because

cinnamamides **4**, **9**, and **10** revealed the most potent inhibitions against mushroom tyrosinase, these three compounds were used in the following studies involving cell-based assays.

Table 1. Substitution patterns, and tyrosinase-inhibitory activities of the synthesized cinnamamide derivatives **1** – **10** and kojic acid



Compounds **1** - **5**



Compounds **6** - **10**

Compound	R^1	R^2	R^3	Tyrosinase inhibition (%)[a]
1	H	OH	OMe	26.42±3.49
2	H	H	OH	13.65±3.78
3	H	OH	OH	3.41±2.25
4	OH	H	OH	93.72±0.25
5	H	OMe	OH	24.00±4.90
6	H	OH	OMe	36.70±6.52
7	H	H	OH	19.07±3.74
8	H	OH	OH	12.43±4.29
9	OH	H	OH	78.97±0.16
10	H	OMe	OH	59.09±1.40
Kojic acid				18.81±1.25

[a]Tyrosinase inhibition was assayed at 25 μM using L-tyrosine as a substrate. Results are presented as mean ± SEMs.

*2.3. Docking simulation of cinnamamides **4**, **9**, **10** and kojic acid with tyrosinase.*

Docking simulation was performed using AutoDock Vina 1.1.2 software (The Scripps Research Institute) to investigate whether cinnamamides **4**, **9**, and **10** directly inhibit tyrosinase by binding to its active site. For tyrosinase ligand preparation, the energy minimization tool of Chem3D Pro 12.0 software (CambridgeSoft Corporation) was used to convert the 2D structures of the three cinnamamides into the corresponding 3D structures. The 3D structure of *Agaricus bisporus* tyrosinase from the Protein Data Bank (ID: 2Y9X) was used for docking simulation of the cinnamamides. All three cinnamamide derivatives showed stronger binding affinities with -6.8 ~ -6.9 kcal/mol than kojic acid (-5.7 kcal/mole), used as a positive control, as depicted

in Figure 1d. Interactions involved in docking simulation between tyrosinase and ligands, **4**, **9**, and **10** were identified using LigandScout 4.1.0 software. Kojic acid forms π-π stacking with His263 and two hydrogen bonds between the hydroxyl group of kojic acid and His259 and His263 of tyrosinase (Figure 1b). On the other hand, cinnamamide **4** creates three hydrogen bonds (His85, His 263, and Met280), and cinnamamides **9** and **10** form only one hydrogen bond with Met280 (Figure 1a). Especially, the 4-hydroxyl group on the phenyl ring of **4** interacts with two histidine residues (His85, His263), like the hydroxyl group of kojic acid. All three cinnamamide derivatives interact with Val283 in a hydrophobic manner, cinnamamides **4** and **9** commonly exhibit additional hydrophobic interactions with Ala286, and cinnamamide **10** exhibits additional hydrophobic interactions with Phe264. These results indicate that the three cinnamamide derivatives can bind to the active site of tyrosinase effectively and more highly inhibit the tyrosinase activity than kojic acid.

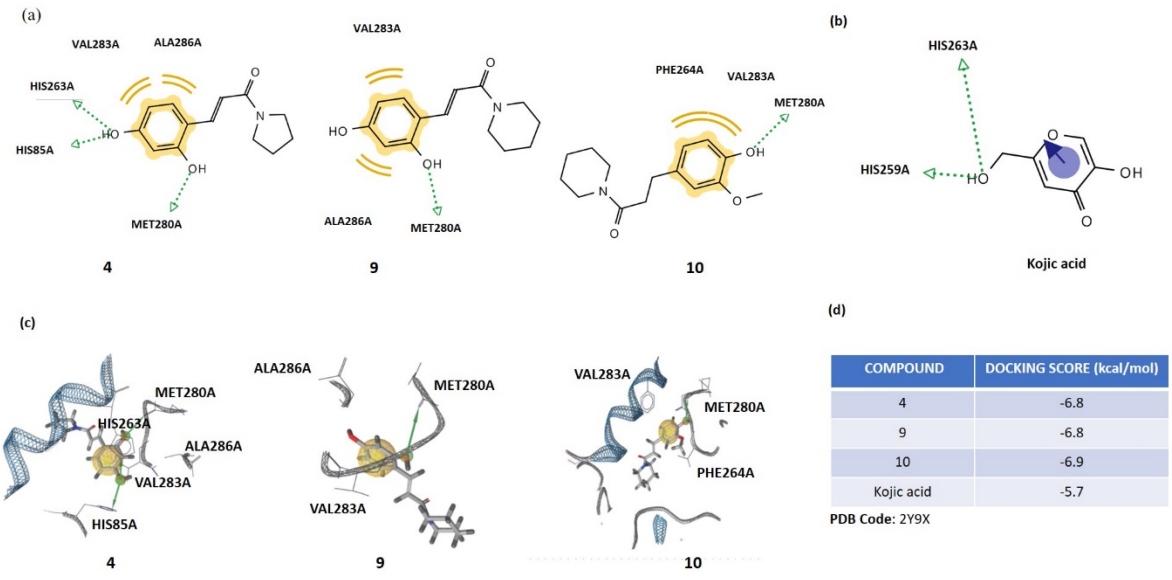


Figure 1. Docking simulation of **4**, **9**, **10** and of kojic acid with tyrosinase and pharmacophore analysis: (a, and b) Pharmacophore results for **4**, **9**, **10** and kojic acid, indicating possible hydrogen-bonding (green arrow), π-π stacking (violet arrow) and hydrophobic (yellow) interactions, (c) docking simulation results between **4**, **9**, or **10** and mushroom tyrosinase, and

(d) docking scores between tyrosinase and **4**, **9**, **10** or kojic acid.

*2.4. Cell viability of cinnamamides **4**, **9** and **10** in B10F16 melanoma cells*

The cell viabilities of cinnamamides **4**, **9** and **10** were determined in B10F16 melanoma cells by performing WST-8 assay. The three test cinnamamides **4**, **9** and **10** were treated at four different concentrations (0, 5, 10, and 25 μM) with melanoma cells and incubated for 24 h. Up to 25 μM, no prominent changes occurred in melanoma cell viability, as shown in Figure 2. Thus, these concentrations were chosen for use in subsequent cell-based assays to evaluate tyrosinase inhibition and anti-melanogenesis.

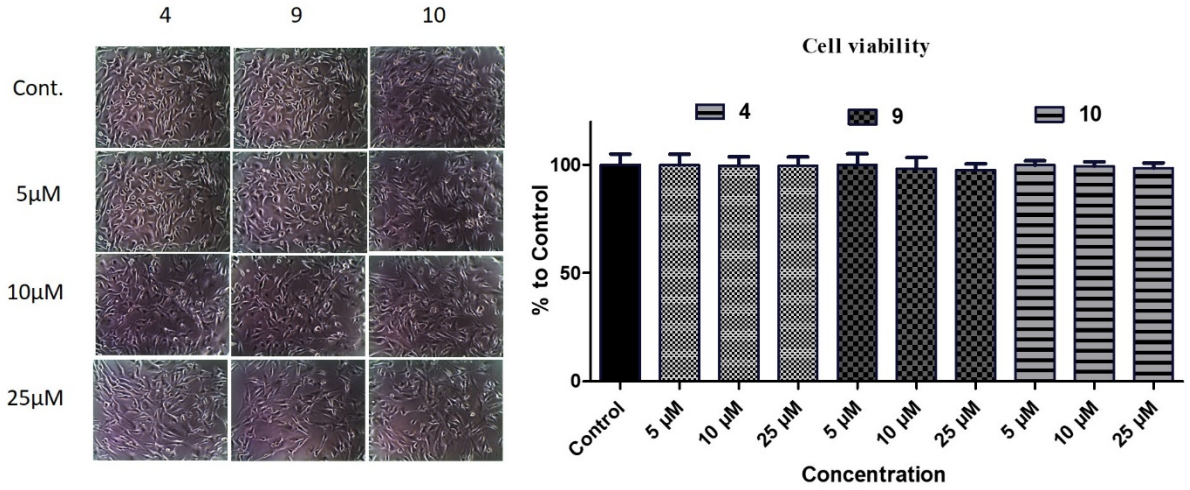


Figure 2. Cell viability assay of cinnamamides **4**, **9** and **10** in B16F10 melanoma cells. Cinnamamides were treated at concentrations of 5, 10, and 25 μM. Viabilities are expressed in % to control, and bars represent standard errors.

*2.5. Tyrosinase inhibitory effect of cinnamamides **4**, **9** and **10** in α-MSH-stimulated B16F10 melanoma cells*

α-MSH-stimulated B10F16 melanoma cells were treated with four different concentrations (0, 5, 10, and 25 μM) of the three cinnamamides **4**, **9** and **10**. The cells were incubated for 24 h. The results of tyrosinase inhibition are indicated in Figure 3.

According to the results, cinnamamides **4** and **10** exhibited the highest tyrosinase inhibitory

effect in α-MSH-stimulated B10F16 melanoma cells at all three concentrations and each cinnamamide inhibited tyrosinase activity in a concentration-dependent manner. Cinnamamide **9** also suppressed the activity of tyrosinase greater than kojic acid and arbutin (400 μM), used as positive controls, at 25 μM. The tyrosinase activity inhibitions at concentrations of 10 and 25 μM were 38.33%, and 76.67% for cinnamamide **4**, 23.33%, and 64.67% for cinnamamide **9**, and 44.00%, and 73.33 % for cinnamamide **10**, respectively. The tyrosinase inhibition of the three cinnamamides **4**, **9** and **10** at a concentration of 25 μM was much higher than that of kojic acid (36.67%) at 25 μM and arbutin (51.67%) at 400 μM. These results demonstrate that our previous findings showing that compounds with 2,4-dihydroxyphenyl groups exhibit generally strong tyrosinase inhibition against mushroom tyrosinase can be applied to tyrosinase inhibition in B16F10 cells.

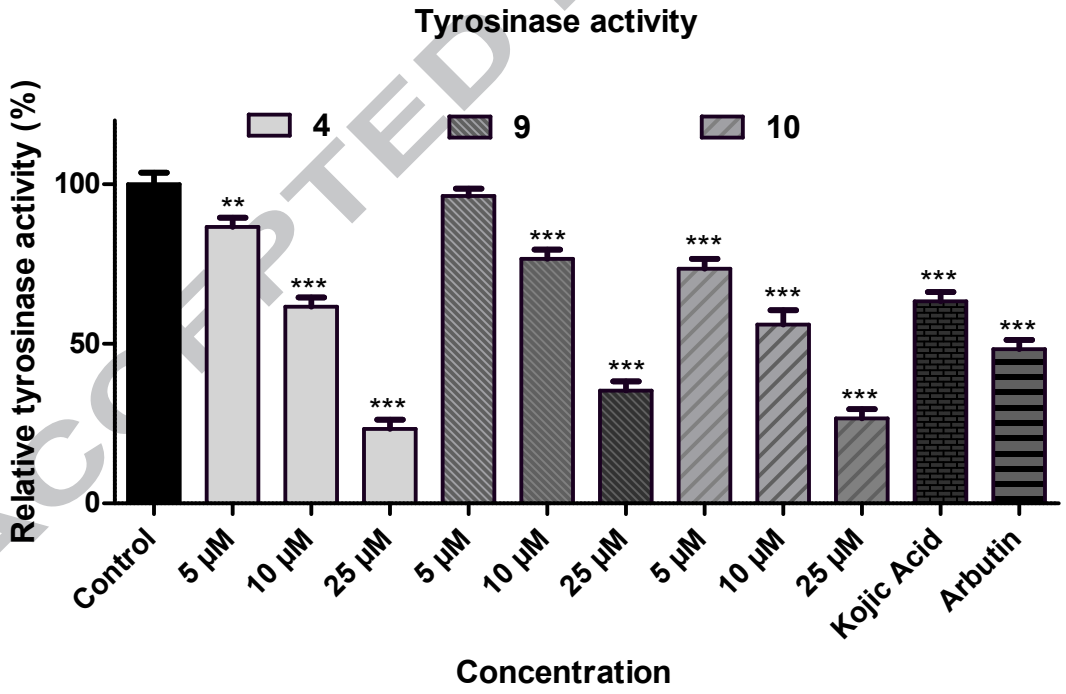


Figure 3. Tyrosinase inhibitory activity of cinnamamides **4**, **9** and **10** in α-MSH-stimulated B16F10 melanoma cells that were co-treated with cinnamamides **4**, **9**, and **10**, kojic acid (25 μM) or arbutin (400 μM). The asterisks represent the significance difference between the

columns: **, $p<0.01$; and ***, $p<0.001$. The bars represent standard errors.

*2.6. Anti-melanogenic effect of cinnamamides **4**, **9** and **10** in α-MSH-stimulated B16F10 melanoma cells*

B10F16 melanoma cells were stimulated by α-MSH to determine the effect of the three cinnamamides **4**, **9** and **10** on the melanin content. The α-MSH-stimulated B16F10 melanoma cells were co-treated with the three cinnamamides or kojic acid. After incubation for 24 h, optical densities were measured to evaluate the effect of the three cinnamamides on the melanin content in α-MSH-stimulated melanoma cells.

According to the results shown in Figure 4, all three cinnamamides exhibited strong decreases in the melanin content compared to kojic acid at 25 μM in α-MSH-stimulated B16F10 melanoma cells. Of the three cinnamamides, cinnamamide **4** showed the highest melanin content reduction (78.30%) at 25 μM, followed by cinnamamides **10** (72.67%) and **9** (61.80%); all three reductions were superior to that of kojic acid (37.63% at 25 μM). All three cinnamamides decreased melanin content in a dose-dependent manner and showed a decrease in melanin content at 10 μM equal to or greater than that of kojic acid (25 μM). Considering the very large similarity between the residual tyrosinase activity depicted in Figure 3 and the melanin content shown in Figure 4, the anti-melanogenic effect of the three cinnamamides appears to be mainly due to the tyrosinase inhibitory activity.

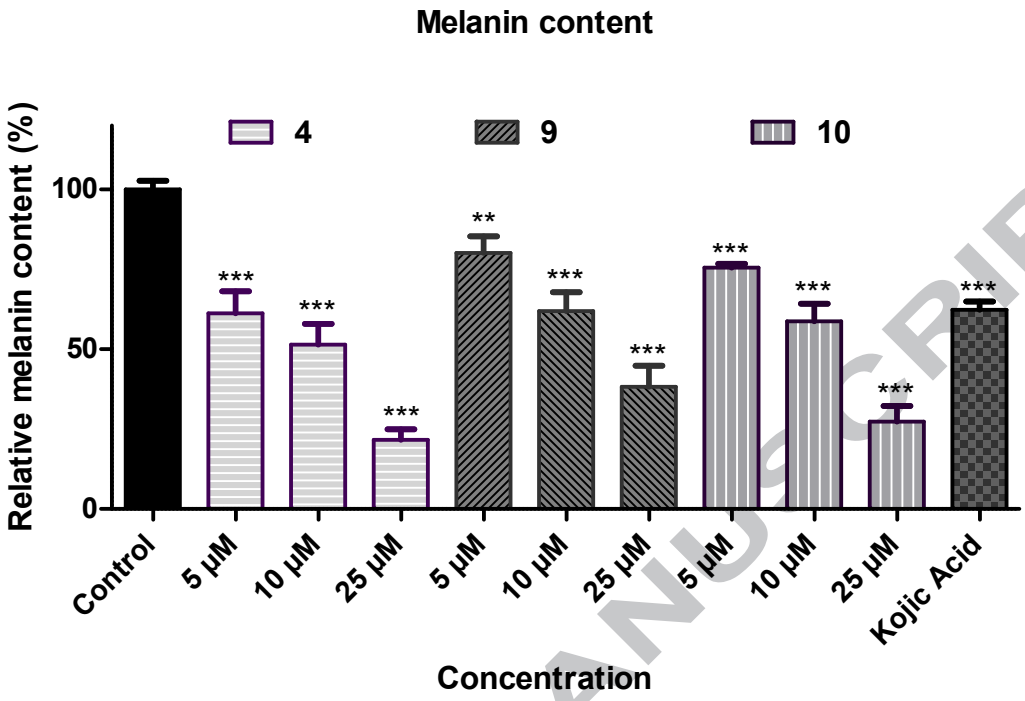


Figure 4. Anti-melanogenic effect of cinnamamides **4**, **9** and **10** in α-MSH-stimulated B16F10 melanoma cells that were co-treated with cinnamamides **4**, **9**, and **10** or kojic acid (25 μM). The asterisks represent the significant difference between the columns: **, $p<0.01$; and ***, $p<0.001$. The bars represent standard errors.

*2.7. DPPH radical scavenging activity of cinnamamides **1 – 10***

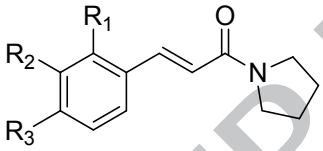
The radical scavenging activity of the cinnamamides was determined by performing DPPH assay. Cinnamamides **1** – **10** and L-ascorbic acid were added at a final concentration of 1 mM to a DPPH methanol solution and radical scavenging activities were measured after 30 min. The results are listed in Table 2.

According to our previous studies, compounds having a 3,4-dihydroxyl or 4-hydroxy-3-methoxyl substituent on the β–phenyl ring of the (*E*)-scaffold generally exhibit a high DPPH radical scavenging activity, whereas compounds that lose the substituent at position 3 exhibit dramatically decreased DPPH radical scavenging activity.[15,16] Like our previous findings, cinnamamides **3**, **5**, **8**, and **10** with 3,4-dihydroxyphenyl and 4-hydroxy-3-methoxyphenyl

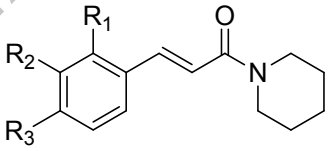
groups exhibited high DPPH radical scavenging activity (83.16%, 82.09%, 82.85%, and 80.69%, respectively) similar to that of L-ascorbic acid (84.64%), whereas cinnamamides **2**, and **7** with no substituent at the 3-position on the phenyl ring showed very low scavenging activity.

Reactive oxygen species (ROS) is known to induce melanogenesis through tyrosinase activation.[37] Therefore, the inhibitory effect of cinnamamide **10** on the melanin production shown in Figure **4** seems to be due to both the indirect inhibition of tyrosinase by antioxidant activity and the direct inhibition of tyrosinase.

Table 2. DPPH radical scavenging activities of the synthesized cinnamamide derivatives **1** – **10**, and kojic acid



Compounds **1** - **5**



Compounds **6** - **10**

Compound	DPPH radical scavenging activity (%)	Compound	DPPH radical scavenging activity (%)
1	34.19±0.24	**6**	37.59±0.09
2	3.51±1.09	**7**	0.91±0.48
3	83.16±0.05	**8**	82.85±0.49
4	22.33±1.11	**9**	30.67±1.71
5	82.09±0.21	**10**	80.69±0.28
		L-ascorbic acid	84.64±0.32

The radical scavenging activities were measured at 30 min after the addition of cinnamamides with a final concentration of 1.0 mM to the DPPH methanol solution. The presented results are the means±SDs of three independent experiments.

3. Conclusion

Cinnamamides with an (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffold were designed

as potential tyrosinase inhibitors. Cinnamamides **1** – **10** were prepared using Horner-Emmons olefination as a key step. Of the 10 cinnamamides, three, namely **4**, **9** and **10**, exhibited greatly superior tyrosinase inhibition against mushroom tyrosinase than that of kojic acid. In docking simulations using tyrosinase, all three cinnamamides showed stronger binding affinities than kojic acid and pharmacophore analysis revealed that these cinnamamides bind to the same binding site as the natural substrates of tyrosinase. The three cinnamamides effectively suppressed melanin content and inhibited tyrosinase activity to extents far superior to that achieved by kojic acid in B16F10 murine melanoma cells without cytotoxicity. These results support the promising potential of cinnamamides for use as anti-melanogenic agents in pigmentation disorders.

4. Materials and Methods

4.1. General methods

^{1}H NMR and ^{13}C NMR data were obtained by a Varian Unity INOVA 400 spectrometer or a Varian Unity AS500 spectrometer (Agilent Technologies, Santa Clara, CA, USA) using DMSO-d_6 and $CDCl_3$ as solvents. All chemical shifts were measured in parts per million (ppm) versus residual solvent or deuterated peaks (δ_H 7.24 and δ_C 77.0 for $CDCl_3$, δ_H 2.50 and δ_C 39.7 for DMSO-d_6). Coupling constants are represented in hertz. The following abbreviations are used for ^{1}H NMR: singlet (s), broad singlet (brs), doublet (d), doublet of doublets (dd), broad doublet (brd), triplet (t), broad triplet (brt), quartet (q) or multiplet (m). Low-resolution and high-resolution mass data were recorded on an Expression CMS (Advion Ithaca, NY, USA) and Agilent Accurate Mass Q-TOF liquid-chromatography mass spectrometer (Agilent, Santa Clara, CA, USA), respectively. All reactions were conducted under a nitrogen atmosphere and monitored by thin layer chromatography (TLC; Merck precoated $60F_{245}$ plates). All anhydrous solvents were distilled over Na/benzophenone or CaH_2 before use.

4.1.1. General procedure for the preparation of compounds 16 – 20.

Benzaldehydes **11 – 15** (5.00 g), benzyl bromide (1.0 equiv.), potassium carbonate (1.0 equiv.) and acetonitrile (50 mL) were added to a 250 mL round-bottom flask and the reaction mixture was refluxed for 24 h. Acetonitrile was evaporated on completion of the reaction and the resulting residues were partitioned between dichloromethane and water. The dichloromethane layer was dried with $MgSO_4$, filtered, and evaporated under reduced pressure to give the products **16 - 20** as a white or grey solid in yields of 95 – 97%.

4.1.1.1 3-(Benzyloxy)-4-methoxybenzaldehyde (16). White solid, 97% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.82 (s, 1 H, CHO), 7.47 (dd, 1 H, J = 8.0, 2.0 Hz, 6-H), 7.47 – 7.44 (m, 3 H, 2-H, 2′-H, 6′-H), 7.38 (t, 2 H, J = 7.5 Hz, 3′-H, 5′-H), 7.32 (t, 1 H, J = 7.5 Hz, 4′-H), 7.00 (d, 1 H, J = 8.0 Hz, 5-H), 5.19 (s, 2 H, benzylic H), 3.96 (s, 3 H, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.0, 155.2, 148.9, 136.5, 130.2, 128.8, 128.3, 127.7, 127.1, 111.6, 111.0, 71.1, 56.4.

4.1.1.2. 4-(Benzyloxy)benzaldehyde (17). White solid, 97% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.89 (s, 1 H, CHO), 7.84 (d, 2 H, J = 8.5 Hz, 2-H, 6-H), 7.44 (d, 2 H, J = 8.0 Hz, 2′-H, 6′-H), 7.41 (t, 2 H, J = 8.0 Hz, 3′-H, 5′-H), 7.35 (t, 1 H, J = 7.5 Hz, 4′-H), 7.08 (d, 2 H, J = 8.5 Hz, 3-H, 5-H), 5.15 (s, 2 H, benzylic H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.0, 163.9, 136.1, 132.2, 130.3, 128.9, 128.5, 127.7, 115.3, 70.5.

4.1.1.3. 3,4-Bis(benzyloxy)benzaldehyde (18). White solid, 96% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.81 (s, 1 H, CHO), 7.49 (d, 1 H, J = 2.0 Hz, 2-H), 7.48 – 7.36 (m, 9 H, 6-H, 2′-H, 3′-H, 5′-H, 6′-H, 2″-H, 3″-H, 5″-H, 6″-H), 7.34 – 7.30 (m, 2 H, 4′-H, 4″-H), 7.02 (d, 1 H, J = 8.0 Hz, 5-H), 5.26 (s, 2 H, benzylic H), 5.22 (s, 2 H, benzylic H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.0, 154.4, 149.4, 136.7, 136.4, 130.5, 128.9, 128.8, 128.3, 128.2, 127.5, 127.2, 126.9, 113.3, 112.5, 71.2, 71.0.

4.1.1.4. 2,4-Bis(benzyloxy)benzaldehyde (19). Gray solid, 95 % yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.39 (s, 1 H, CHO), 7.84 (d, 1 H, J = 9.0 Hz, 6-H), 7.45 – 7.38 (m, 8 H, 2′-H, 3′-H,

5′-H, 6′-H, 2″-H, 3″-H, 5″-H, 6″-H), 7.37 – 7.34 (m, 2 H, 4′-H, 4″-H), 6.64 (dd, 1 H, J = 9.0, 2.0 Hz, 5-H), 6.60 (d, 1 H, J = 2.0 Hz, 3-H), 5.14 (s, 2 H, benzylic H), 5.11 (s, 2 H, benzylic H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 188.5, 165.4, 162.9, 136.1, 136.1, 130.7, 129.0, 128.9, 128.6, 128.5, 127.8, 127.5, 119.7, 107.2, 100.3, 70.6, 70.4.

4.1.1.5. 4-(Benzyloxy)-3-methoxybenzaldehyde (20). White solid, 97% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.84 (s, 1 H, CHO), 7.45 – 7.42 (m, 3 H, 2-H, 2′-H, 6′-H), 7.40 – 7.37 (m, 3 H, 6-H, 3′-H, 5′-H), 7.33 (t, 1 H, J = 7.5 Hz, 4′-H), 6.99 (d, 1 H, J = 8.0 Hz, 5-H), 5.25 (s, 2 H, benzylic H), 3.95 (s, 3 H, OCH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.1, 153.8, 150.3, 136.2, 130.5, 128.9, 128.4, 127.4, 126.8, 112.6, 109.5, 71.1, 56.3.

4.1.2. General procedure for the synthesis of compounds 26 – 30 via compounds 21 -25.

To a stirred solution of compounds **16 – 20** (2.00 g), triethyl phosphonoacetate (1.1 equiv.), and potassium carbonate (2.0 equiv.) in dichloromethane/*N*,*N*-dimethylformamide (2:1, 15 mL) was added a catalytic amount of DBU (0.03 equiv.). The reaction mixture was stirred at room temperature (compounds **11**, **13**, and **15**) or 70 ^{0}C (**12**, and **14**) for 24 – 36 h. After evaporation of dichloromethane, ice water was added to the reaction mixture which was then stirred for 30 min to give precipitates. After filtration, the filter cake was washed with a plenty of water, and dried to produce ethyl cinnamates **21** – **25** as a white or gray solid in yields of 95 – 98%. The ethyl cinnamates were used directly in the next reaction without characterization.

Compounds **21** – **25** (2.57 to 4.11 g) were added to a 100 mL round-bottom flask and 1,4-dioxane (20 mL) and 1N-NaOH aqueous solution (8.0 – 16.0 equiv.) were added subsequently. The reaction mixture was stirred at room temperature for 48 h**.** After completion of the reaction, the reaction mixture was acidified until pH 2 by 2N-HCl aqueous solution. The reaction mixture was stirred at room temperature for 30 min and then the resultant solid was filtered. The filter cake was washed with a plenty of water, and dried to give the cinnamic acid

derivatives **26 – 30** as a white or grey solid in yields of 100%.

4.1.2.1. (*E*)-3-(3-(Benzyloxy)-4-methoxyphenyl)acrylic acid (26). White solid, 100% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.19 (brs, 1 H, COOH), 7.49 (d, 1 H, J = 16.0 Hz, 3-vinylic H), 7.45 (d, 2 H, J = 7.5 Hz, 2′-H, 6′-H), 7.41 (d, 1 H, J = 2.0 Hz, 2´-H), 7.38 (t, 2 H, J = 8.0 Hz, 3′-H, 5′-H), 7.32 (t, 1 H, J = 7.5 Hz, 4′-H), 7.20 (dd, 1 H, J = 8.0, 2.0 Hz, 6´-H), 6.98 (d, 1 H, J = 8.0 Hz, 5´-H), 6.40 (d, 1 H, J = 16.0 Hz, 2-vinylic H), 5.12 (s, 2 H, benzylic H), 3.78 (s, 3 H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.5, 151.7, 148.6, 144.7, 137.6, 129.1, 128.6, 128.5, 127.6, 123.7, 117.4, 112.5, 112.4, 70.5, 56.3.

4.1.2.2. (*E*)-3-(4-(Benzyloxy)phenyl)acrylic acid (27). White solid, 100% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.25 (brs, 1 H, COOH), 7.61 (d, 2 H, J = 9.0 Hz, 2-H, 6-H), 7.52 (d, 1 H, J = 16.0 Hz, 3-vinylic H), 7.43 (d, 2 H, J = 7.5 Hz, 2′-H, 6′-H), 7.38 (t, 2 H, J = 7.5 Hz, 3′-H, 5′-H), 7.31 (t, 1 H, J = 7.5 Hz, 4′-H), 7.02 (d, 2 H, J = 8.0 Hz, 3-H, 5-H), 6.36 (d, 1 H, J = 16.0 Hz, 2-vinylic H), 5.14 (s, 2 H, benzylic H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.5, 160.6, 144.3, 137.4, 130.6, 129.1, 128.6, 128.4, 127.7, 117.4, 115.8, 70.0.

4.1.2.3. (*E*)-3-(3,4-Bis(benzyloxy)phenyl)acrylic acid (28). White solid, 100% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.22 (brs, 1 H, COOH), 7.47 (d, 1 H, J = 16.0 Hz, 3-vinylic H), 7.46 -7.41 (m, 5 H, 2-H, 2′-H, 6′-H, 2″-H, 6″-H), 7.37 (t, 2 H, J = 7.5 Hz, 3′-H, 5′-H), 7.36 (t, 2 H, J = 7.5 Hz, 3″-H, 5″-H), 7.30 (t, 2 H, J = 7.5 Hz, 4′-H, 4″-H), 7.17 (dd, 1 H, J = 8.5, 2.0 Hz, 6-H), 7.05 (d, 1 H, J = 8.5 Hz, 5-H), 6.40 (d, 1 H, J = 16.0 Hz, 2-vinylic H), 5.18 (s, 2 H, benzylic H), 5.16 (s, 2 H, benzylic H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.5, 150.7, 148.9, 144.6, 137.8, 137.6, 129.1, 129.1, 128.5, 128.5, 128.3, 128.1, 123.5, 117.7, 117.5, 114.5, 113.5, 70.6, 70.5.

4.1.2.4. (*E*)-3-(2,4-Bis(benzyloxy)phenyl)acrylic acid (29). Grey solid, 100% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.10 (s, 1 H, COOH), 7.77 (d, 1 H, J = 16.0 Hz, 3-vinylic H), 7.62 (d, 1 H, J = 9.0 Hz, 6-H), 7.45 – 7.31 (m, 10 H, 2 x Ph), 6.81 (d, 1 H, J = 2.0 Hz, 3-H), 6.65

(dd, 1 H, J = 9.0, 2.0 Hz, 5-H), 7.38 (d, 1 H, J = 16.0 Hz, 2-vinylic H), 5.19 (s, 2 H, benzylic H), 5.14 (s, 2 H, benzylic H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.8, 162.1, 158.8, 139.3, 137.3, 137.3, 130.6, 129.2, 129.1, 128.7, 128.6, 128.5, 128.4, 117.3, 116.6, 107.8, 101.2, 70.5, 70.2.

4.1.2.5. (*E*)-3-(4-(Benzyloxy)-3-methoxyphenyl)acrylic acid (30). White solid, 100% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.17 (brs, 1 H, COOH), 7.50 (d, 1 H, J = 16.0 Hz, 3-vinylic H), 7.42 (d, 2 H, J = 7.5 Hz, 2′-H, 6′-H), 7.38 (t, 2 H, J = 7.5 Hz, 3′-H, 5′-H), 7.32 (t, 1 H, J = 7.5 Hz, 4′-H), 7.32 (d, 1 H, J = 2.0 Hz, 2-H), 7.16 (dd, 1 H, J = 8.0, 2.0 Hz, 6-H), 7.04 (d, 1 H, J = 8.0 Hz, 5-H), 6.43 (d, 1 H, J = 16.0 Hz, 2-vinylic H), 5.11 (s, 2 H, benzylic H), 3.80 (s, 3 H, OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.5, 150.3, 149.9, 144.7, 137.4, 129.1, 128.6, 128.5, 128.0, 123.1, 117.5, 113.7, 111.2, 70.4, 56.3.

4.1.3. General procedure for the preparation of compounds 31 – 40.

A solution of compounds **26 – 30** (100 mg), isobutyl chloroformate (2.0 equiv.), and *N*-methylmorpholine (2.5 equiv.) in anhydrous THF (5 mL) was stirred at room temperature for 30 min. Then cyclic amine (pyrrolidine and piperidine) (2.0 equiv.) was added to the reaction mixture which was then stirred at room temperature for 24 h. After completion of the reaction, the reaction mixture was partitioned between ethyl acetate and water. The organic layer was washed with brine, dried with $MgSO_4$ and filtered. The filtrate was evaporated in reduced pressure. The resultant residue was purified by column chromatography using methylene chloride and methanol (30 – 70:1) as the eluent. The products **31 – 40** were obtained as a white or yellow solid in yields of 80 – 91%.

4.1.3.1 (*E*)-3-(3-(Benzyloxy)-4-methoxyphenyl)-1-(pyrrolidin-1-yl)prop-2-en-1-one (31). Yellowish solid, 80% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57 (d, 1 H, J = 15.2 Hz, 3-vinylic H), 7.42 (d, 2 H, J = 8.0 Hz, 2′-H, 6′-H), 7.34 (t, 2 H, J = 8.0 Hz, 3′-H, 5′-H), 7.26 (t, 1 H, J = 8.0 Hz, 4′-H), 7.09 (d, 1 H, J = 8.0, 2.0 Hz, 6-H), 7.03 (d, 1 H, J = 2.0 Hz, 2-H), 6.84 (d, 1 H,

J = 8.0 Hz, 5-H), 6.45 (d, 1 H, *J* = 15.2, 2-vinylic H), 5.18 (s, 2 H, benzylic H), 3.87 (s, 3 H, OCH_3), 3.59 – 3.53 (m, 4 H, 2″-H, 5″-H), 1.95 – 1.88 (m, 4 H, 3″-H, 4″-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.1, 151.3, 148.4, 141.6, 137.1, 128.8, 128.4, 128.2, 127.6, 122.4, 116.9, 113.3, 111.7, 71.5, 56.2, 46.7, 46.2, 26.4, 24.6.

4.1.3.2. (*E*)-3-(4-(Benzyloxy)phenyl)-1-(pyrrolidin-1-yl)prop-2-en-1-one (32). Yellowish white solid, 91% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.66 (d, 1 H, *J* = 15.5 Hz, 3-vinylic H), 7.48 (d, 2 H, *J* = 8.5 Hz, 2-H, 6-H), 7.43 (d, 2 H, *J* = 7.5 Hz, 2′-H, 6′-H), 7.39 (t, 2 H, *J* = 7.5 Hz, 3′-H, 5′-H), 7.33 (t, 1 H, *J* = 7.5 Hz, 4′-H), 6.96 (d, 2 H, *J* = 8.5 Hz, 3-H, 5-H), 6.60 (d, 1 H, *J* = 15.5 Hz, 2-vinylic H), 5.08 (s, 2 H, benzylic H), 3.60 (brt, 4 H, *J* = 6.0 Hz, 2″-H, 5″-H), 1.94 – 1.91 (m, 4 H, 3″-H, 4″-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.3, 160.2, 141.9, 136.7, 129.7, 128.8, 128.4, 128.3, 127.7, 116.4, 115.3 70.2, 46.6, 25.8.

4.1.3.3. (*E*)-3-(3,4-bis(Benzyloxy)phenyl)-1-(pyrrolidin-1-yl)prop-2-en-1-one (33). White solid, 80% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 (d, 1 H, *J* = 15.6 Hz, 3-vinylic H), 7.43 – 7.25 (m, 10 H, 2 x Ph), 7.07 (d, 1 H, d = 2.0 Hz, 2-H), 7.04 (dd, 1 H, *J* = 8.8, 2.0 Hz, 6-H), 6.86 (d, 1 H, *J* = 8.8 Hz, 5-H), 6.46 (d, 1 H, *J* = 15.6 Hz, 2-vinylic H), 5.15 (s, 2 H, benzylic H), 5.15 (s, 2 H, benzylic H), 3.55 (brt, 4 H, *J* = 6.4 Hz, 2″-H, 5″-H), 1.90 – 1.85 (m, 4 H, 3″-H, 4″-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.0, 161.0, 137.7, 136.7, 136.5, 132.6, 128.9, 128.8, 128.4, 128.3, 127.7, 118.7, 118.1, 106.3, 100.7, 70.7, 70.4, 46.4, 46.0, 26.2, 24.6.

4.1.3.4. (*E*)-3-(2,4-bis(Benzyloxy)phenyl)-1-(pyrrolidin-1-yl)prop-2-en-1-one (34). White solid, 84% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (d, 1 H, *J* = 16.0 Hz, 3-vinylic H), 7.45 – 7.28 (m, 11 H, 6-H, 2 x Ph), 6.81 (d, 1 H, *J* = 16.0 Hz, 2-vinylic H), 6.60 (d, 1 H, J = 2.0 Hz, 3-H), 6.55 (dd, 1 H, *J* = 8.0, 2.0 Hz, 5-H), 5.04 (s, 2 H, benzylic H), 5.03 (s, 2 H, benzylic H), 3.49 (brm, 2 H, 2″-H, 5″-H), 3.17 (brm, 2 H, 2″-H, 5″-H), 1.80 (brm, 4 H, 3″-H, 4″-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.1, 161.0, 159.3, 137.9, 136.7, 136.5, 132.7, 128.9, 128.8, 128.4, 128.4, 128.4, 127.4, 118.6, 118.1, 106.3, 100.7, 70.7, 70.4, 46.3, 46.1, 26.1, 24.5.

4.1.3.5. (*E*)-3-(4-(Benzyloxy)-3-methoxyphenyl)-1-(pyrrolidin-1-yl)prop-2-en-1-one (35). Yellowish white solid, 89% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (d, 1 H, J = 15.5 Hz, 3-vinylic H), 7.42 (d, 2 H, J = 7.5 Hz, 2′-H, 6′-H), 7.36 (t, 2 H, J = 7.5 Hz, 3′-H, 5′-H), 7.30 (t, 1 H, J = 7.5 Hz, 4′-H), 7.06 – 7.03 (m, 2 H, 2-H, 6-H), 6.85 (d, 1 H, J = 9.0 Hz, 5-H), 6.57 (d, 1 H, J = 15.5, 2-vinylic H), 5.17 (s, 2 H, benzylic H), 3.91 (s, 3 H, OCH_3), 3.60 (brt, 4 H, J = 6.0 Hz, 2″-H, 5″-H), 1.85 (brm, 4 H, 3″-H, 4″ H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.2, 155.5, 149.9, 142.3, 136.9, 128.8, 128.2, 127.4, 121.8, 116.6, 113.7, 110.9, 71.1, 56.3, 46.1, 25.5.

4.1.3.6. (*E*)-3-(3-(Benzyloxy)-4-methoxyphenyl)-1-(piperidin-1-yl)prop-2-en-1-one (36). Yellow solid, 87% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (d, 1 H, J = 15.5 Hz, 3-vinylic H), 7.45 (d, 2 H, J = 7.5 Hz, 2′-H, 6′-H), 7.37 (t, 2 H, J = 7.5 Hz, 3′-H, 5′-H), 7.30 (t, 1 H, J = 7.5 Hz, 4′-H), 7.10 (dd, 1 H, J = 8.5, 1.5 Hz, 6-H), 7.05 (d, 1 H, J = 1.5 Hz, 2-H), 6.86 (d, 1 H, J = 8.5 Hz, 5-H), 6.65 (d, 1 H, J = 15.5 Hz, 2-vinylic H), 5.17 (s, 2 H, benzylic H), 3.90 (s, 3 H, OCH_3), 3.59 (brt, 4 H, J = 5.0 Hz, 2″-H, 6″-H), 1.69 – 1.65 (m, 2 H, 3″-Ha, 5″-Ha), 1.62 – 1.58 (m, 4 H, 3″-Hb, 4″-H, 5″-Hb); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.7, 151.3, 148.4, 142.4, 137.1, 128.8, 128.5, 128.2, 127.6, 122.3, 115.5, 113.2, 111.7, 71.5, 56.2, 45.1, 26.4, 24.8.

4.1.3.7. (*E*)-3-(4-(Benzyloxy)phenyl)-1-(piperidin-1-yl)prop-2-en-1-one (37). Yellowish white solid, 89% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (d, 1 H, J = 15.5 Hz, 3-vinylic H), 7.45 (d, 2 H, J = 9.0 Hz, 2-H, 6-H), 7.42 (d, 2 H, J = 7.5 Hz, 2′-H, 6′-H), 7.39 (t, 2 H, J = 7.5 Hz, 3′-H, 5′-H), 7.33 (t, 1 H, J = 7.5 Hz, 4′-H), 6.96 (d, 2 H, J = 9.0 Hz, 3-H, 5-H), 6.77 (d, 1 H, J = 15.5 Hz, 2-vinylic H), 5.09 (s, 2 H, benzylic H), 3.62 (brt, 4 H, J = 5.5 Hz, 2″-H, 6″-H), 1.69 – 1.66 (m, 2 H, 3″-Ha, 5″-Ha), 1.62 – 1.58 (m, 4 H, 3″-Hb, 4″-H, 5″-Hb); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.9, 160.1, 142.5, 136.8, 129.5, 128.8, 128.6, 128.3, 127.7, 115.3, 115.1, 70.3, 45.6, 26.4, 24.8.

4.1.3.8. (*E*)-3-(3,4-Bis(benzyloxy)phenyl)-1-(piperidin-1-yl)prop-2-en-1-one (38). White solid, 85% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (d, 1 H, J = 15.5 Hz, 3-vinylic H), 7.46 –

7.29 (m, 10 H, 2 x Ph), 7.09 (d, 1 H, *J* = 1.5 Hz, 2-H), 7.06 (dd, 1 H, *J* = 8.5, 1.5 Hz, 6-H), 6.90 (d, 1 H, *J* = 8.5 Hz, 5-H), 6.66 (d, 1 H, *J* = 15.5 Hz, 2-vinylic H), 5.18 (s, 4 H, 2 x benzylic H), 3.60 (brt, 4 H, *J* = 5.0 Hz, 2″-H, 6″-H), 1.69 – 1.66 (m, 2 H, 3″-Ha, 5″-Ha), 1.62 – 1.58 (m, 4 H, 3″-Hb, 4″-H, 5″-Hb); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.8, 150.6, 149.0, 142.8, 137.3, 137.0, 129.1, 128.8, 128.7, 128.1, 127.6, 127.4, 122.4, 115.4, 114.6, 114.4, 71.8, 71.2, 45.5, 26.4, 24.8.

4.1.3.9. (*E*)-3-(2,4-Bis(benzyloxy)phenyl)-1-(piperidin-1-yl)prop-2-en-1-one (39). White solid, 81% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, 1 H, *J* = 15.5 Hz, 3-vinylic H), 7.47 – 7.33 (m, 11 H, 6-H, 2 x Ph), 7.02 (d, 1 H, *J* = 15.5 Hz, 2-vinylic H), 6.64 (d, 1 H, *J* = 2.0 Hz, 3-H), 6.59 (dd, 1 H, *J* = 8.5, 2.0 Hz, 5-H), 5.07 (s, 2 H, benzylic H), 5.05 (s, 2 H, benzylic H), 3.43 (brm, 4 H, 2″-H, 6″-H), 1.63 – 1.58 (m, 2 H, 3″-Ha, 5″-Ha), 1.49 - 146 (m, 4 H, 3″-Hb, 4″-H, 5″-Hb); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.7, 161.0, 159.2, 139.1, 136.7, 136.5, 132.8, 128.9, 128.9, 128.5, 128.4, 128.3, 127.8, 118.1, 116.7, 106.3, 100.7, 70.7, 70.4, 45.4, 26.3, 24.9.

4.1.3.10. (*E*)-3-(4-(Benzyloxy)-3-methoxyphenyl)-1-(piperidin-1-yl)prop-2-en-1-one (40). Yellow solid, 88% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.58 (d, 1 H, *J* = 15.5 Hz, 3-vinylic H), 7.43 (d, 2 H, *J* = 7.5 Hz, 2′-H, 6′-H), 7.36 (t, 2 H, *J* = 7.5 Hz, 3′-H, 5′-H), 7.30 (t, 1 H, *J* = 7.5 Hz, 4′-H), 7.04 (d, 1 H, J = 1.5 Hz, 6-H), 7.03 (dd, 1 H, *J* = 9.0, 1.5 Hz, 2-H), 6.85 (d, 1 H, *J* = 9.0 Hz, 5-H), 6.74 (d, 1 H, *J* = 15.5 Hz, 2-vinylic H), 5.18 (s, 2 H, benzylic H), 3.92 (s, 3 H, OCH_3), 3.62 (brt, 4 H, *J* = 5.5 Hz, 2″-H, 6″-H), 1.69 – 1.65 (m, 2 H, 3″-Ha, 5″-Ha), 1.62 – 1.58 (m, 4 H, 3″-Hb, 4″-H, 5″-Hb); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.8, 149.8, 149.8, 142.9, 136.9, 129.0, 128.8, 128.2, 127.4, 121.7, 115.4, 113.7, 110.8, 71.1, 56.3, 45.5, 26.4, 24.8.

4.1.4. General procedure for the preparation of cinnamamides 1 – 3, 5 – 8 and 10.

Compounds **31 – 33**, **35 – 38** and **40** (80 mg) were added to a 25 mL round-bottom flask, along with concentrated HCl and acetic acid (1:1, 2 mL), and the reaction mixture was

refluxed for 40 min. After completion of the reaction, the mixture was neutralised by 2 N NaOH aqueous solution and partitioned between methylene chloride and water. The organic layer was dried with anhydrous $MgSO_4$, and filtered and the filtrate was evaporated in reduced pressure. The resulting residue was purified by column chromatography using methylene chloride and methanol (10 – 30:1) as the eluent. Cinnamamides **1** – **3**, **5** – **8** and **10** were produced as a yellow or white solid in yields of 67-74 %.

4.1.4.1. (*E*)-3-(3-Hydroxy-4-methoxyphenyl)-1-(pyrrolidin-1-yl)prop-2-en-1-one (1). Yellow solid, 70% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.60 (d, 1 H, J = 15.6 Hz, 3-H), 7.17 (d, 1 H, J = 1.2 Hz, 2′-H), 6.98 (dd, 1 H, J = 8.0, 1.2 Hz, 6′-H), 6.79 (d, 1 H, J = 8.0 Hz, 5′-H), 6.54 (d, 1 H, J = 15.6 Hz, 2-H), 3.87 (s, 3 H, OCH_3), 3.56 (t, 4 H, J = 6.4 Hz, 2″-H, 5″-H), 1.90 (brm, 4 H, 3″-H, 4″-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.4, 148.5, 146.1, 142.1, 129.0, 121.7, 116.8, 113.1, 110.8, 56.2, 46.4, 26.4, 24.6; LRMAS (ESI-) *m/z* 246 $(M-H)^-$.

4.1.4.2. (*E*)-3-(4-Hydroxyphenyl)-1-(pyrrolidin-1-yl)prop-2-en-1-one (2). Yellowish white solid, 72% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56 (d, 1 H, J = 15.0 Hz, 3-H), 7.34 (d, 2 H, J = 7.5 Hz, 2′-H, 6′-H), 6.81 (d, 2 H, J = 7.5 Hz, 3′-H, 5′-H), 6.49 (d, 1 H, J = 15.0 Hz, 2-H), 3.55 (brm, 4 H, 2″-H, 5″-H), 1.96 (brm, 2 H, 3″-H), 1.87 (brm, 2 H, 4″-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.1, 159.1, 142.8, 129.9, 126.8, 116.0, 115.0, 47.0, 46.4, 26.2, 24.5; LRMAS (ESI-) *m/z* 216 $(M-H)^-$.

4.1.4.3. (*E*)-3-(3,4-Dihydroxyphenyl)-1-(pyrrolidin-1-yl)prop-2-en-1-one (3).[38] White solid, 68% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.37 (brs, 1 H, OH), 9.00 (brs, 1 H, OH), 7.28 (d, 1 H, J = 15.5 Hz, 3-H), 7.03 (s, 1 H, 2′-H), 6.93 (d, 1 H, J = 8.0 Hz, 6′-H), 6.72 (d, 1 H, J = 8.0 Hz, 5′-H), 6.62 (d, 1 H, J = 15.5 Hz, 2-H), 3.57 (t, 2 H, J = 6.5 Hz, 2″-H), 3.35 (t, 2 H, J = 6.5 Hz, 5″-H) 1.88 (quint, 2 H, J = 6.5 Hz, 3″-H), 1.77 (quint, 2 H, J = 6.5 Hz, 4″-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.6, 148.0, 146.1, 141.4, 127.2, 121.3, 116.8, 116.3, 115.2, 46.5, 46.2, 26.3, 24.6; LRMAS (ESI-) *m/z* 232 $(M-H)^-$.

4.1.4.4. (*E*)-3-(4-Hydroxy-3-methoxyphenyl)-1-(pyrrolidin-1-yl)prop-2-en-1-one (5). Yellow solid, 71% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.64 (d, 1 H, J = 15.5 Hz, 3-H), 7.10 (d, 1 H, J = 8.0 Hz, 6′-H), 7.00 (s, 1 H, 2′-H), 6.91 (d, 1 H, J = 8.0 Hz, 5′-H), 6.57 (d, 1 H, J = 15.5 Hz, 2-H), 3.91 (s, 3 H, OCH_3), 3.61 (brm, 4 H, 2″-H, 5″-H), 1.95 (brm, 4 H, 3″-H, 4″-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.3, 147.6, 146.9, 142.2, 128.0, 122.1, 116.3, 115.0, 110.3, 56.2, 46.6, 26.2, 24.6; LRMAS (ESI-) *m/z* 246 $(M-H)^-$.

4.1.4.5. (*E*)-3-(3-Hydroxy-4-methoxyphenyl)-1-(piperidin-1-yl)prop-2-en-1-one (6). Yellowish white solid, 72% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.56 (d, 1 H, J = 15.2 Hz, 3-H), 7.14 (s, 1 H, 2′-H), 6.97 (d, 1 H, J = 8.4 Hz, 6′-H), 6.79 (d, 1 H, J = 8.4 Hz, 5′-H), 6.72 (d, 1 H, J = 15.2 Hz, 2-H), 3.88 (s, 3 H, OCH_3), 3.60 – 3.58 (m, 4 H, 2″-H, 6″-H) 1.63 - 1.58 (m, 6 H, 3″-H, 4″-H, 5″-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.9, 148.2, 146.0, 142.6, 129.2, 121.7, 115.7, 112.8, 110.8, 56.2, 45.8, 26.4, 24.8; LRMAS (ESI-) *m/z* 260 $(M-H)^-$.

4.1.4.6. (*E*)-3-(4-Hydroxyphenyl)-1-(piperidin-1-yl)prop-2-en-1-one (7). Yellowish white solid, 74% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.59 (d, 1 H, J = 15.0 Hz, 3-H), 7.34 (d, 2 H, J = 8.5 Hz, 2′-H, 6′-H), 6.87 (d, 2 H, J = 8.5 Hz, 3′-H, 5′-H), 6.72 (d, 1 H, J = 15.0 Hz, 2-H), 3.65 – 3.60 (m, 4 H, 2″-H, 6″-H), 1.69 - 1.58 (m, 6 H, 3″-H, 4″-H, 5″-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.6, 158.9, 143.6, 129.8, 127.1, 116.2, 113.8, 47.5, 43.9, 26.8, 25.9, 24.7; LRMAS (ESI-) *m/z* 230 $(M-H)^-$.

4.1.4.7. (*E*)-3-(3,4-Dihydroxyphenyl)-1-(piperidin-1-yl)prop-2-en-1-one (8).[39] White solid, 67% yield. ^{1}H NMR (500 MHz, $CDCl_3$ + a few drops of CD_3OD) δ 7.39 (d, 1 H, J = 15.0 Hz, 3-H), 7.02 (s, 1 H, 2′-H), 6.88 (d, 1 H, J = 8.0 Hz, 6′-H), 6.76 (d, 1 H, J = 8.0 Hz, 5′-H), 6.64 (d, 1 H, J = 15.0 Hz, 2-H), 3.56 – 3.54 (m, 4 H, 2″-H, 6″-H), 1.63 – 1.53 (m, 6 H, 3″-H, 4″-H, 5″-H); ^{13}C NMR (100 MHz, $CDCl_3$ + a few drops of CD_3OD) δ 166.9, 147.2, 144.9, 144.1, 127.4, 121.3, 115.4, 114.5, 113.4, 47.5, 43.9, 26.4, 25.9, 24.5; LRMAS (ESI-) *m/z* 246 $(M-H)^-$.

4.1.4.8. (*E*)-3-(4-Hydroxy-3-methoxyphenyl)-1-(piperidin-1-yl)prop-2-en-1-one (10).

Yellowish white solid, 73% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.59 (d, 1 H, J = 15.2 Hz, 3-H), 7.07 (dd, 1 H, J = 8.0, 1.6 Hz, 6′-H), 6.97 (d, 1 H, J = 1.6 Hz, 2′-H), 6.88 (d, 1 H, J = 8.0 Hz, 5-H), 6.71 (d, 1 H, J = 15.2 Hz, 2-H), 3.90 (s, 3 H, OCH_3), 3.61 – 3.59 (m, 4 H, 2″-H, 6″-H) 1.64 – 1.55 (m, 6 H, 3″-H, 4″-H, 5″-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.9, 147.5, 146.9, 143.0, 128.1, 122.0, 115.0, 114.9, 110.1, 56.2, 45.4, 43.9, 26.4, 24.8; LRMAS (ESI-) *m/z* 275 $(M-H)^-$; HRMS (ESI-) *m/z* $C_{15}H_{18}NO_3$ $(M-H)^-$ calcd 260.1292, obsd 260.1299.

4.1.5. General procedure for the preparation of cinnamamides 4 and 9.

To a 25 mL round-bottom flask, a solution of compounds **34** and **39** (80 mg) in anhydrous methylene chloride (3 mL) was added under a nitrogen atmosphere. BBr_3 (4.0 equiv.) was added to the reaction mixture at -40 ^{0}C which was then stirred for 30 min. After completion of the reaction, the mixture was neutralised by the addition of an equal amount of pyridine to the used BBr_3, and then an equal amount of methanol was added to the mixture. The reaction mixture was evaporated under reduced pressure. The resultant residue was purified by column chromatography using methylene chloride and methanol (10 – 30:1) as the eluent. Cinnamamides **4** and **9** were obtained as a white solid in yields of 65 – 67%.

4.1.5.1. (*E*)-3-(2,4-Dihydroxyphenyl)-1-(pyrrolidin-1-yl)prop-2-en-1-one (4). White solid, 65% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.86 (s, 1 H, OH), 9.64 (s, 1 H, OH), 7.62 (d, 1 H, J = 15.5 Hz, 3-H), 7.39 (d, 1 H, J = 8.0 Hz, 6′-H), 6.69 (d, 1 H, J = 15.5 Hz, 2-H), 6.32 (d, 1 H, J = 2.5 Hz, 3′-H), 6.23 (dd, 1 H, J = 8.0, 2.5, 2 Hz, 5′-H), 3.53 (t, 2 H, J = 7.0 Hz, 2″-H), 3.35 (t, 2 H, J = 7.0 Hz, 5″-H), 1.89 (quint, 2 H, J = 6.5 Hz, 3″-H), 1.77 (quint, 2 H, J = 6.5 Hz, 5″-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.2, 160.6, 158.4, 136.8, 130.2, 115.7, 114.2, 108.1, 103.1, 46.5, 46.2, 26.3, 24.6; LRMAS (ESI-) *m/z* 232 $(M-H)^-$; HRMS (ESI-) *m/z* $C_{13}H_{14}NO_3$ $(M-H)^-$ calcd 232.0979, obsd 232.0987.

4.1.5.2. (*E*)-3-(2,4-Dihydroxyphenyl)-1-(piperidin-1-yl)prop-2-en-1-one (9). White solid, 67% yield. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.81 (s, 1 H, OH), 9.64 (s, 1 H, OH), 7.64 (d, 1

H, J = 15.5 Hz, 3-H), 7.44 (d, 1 H, J = 8.5 Hz, 6′-H), 6.92 (d, 1 H, J = 15.5 Hz, 2-H), 6.31 (d, 1 H, J = 2.0 Hz, 3′-H), 6.22 (dd, 1 H, J = 8.5, 2.0 Hz, 5′-H), 3.51 (brm, 4 H, 2″-H, 6″-H), 1.60 – 1.43 (m, 6 H, 3″-H, 4″-H, 5″-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.8, 160.5, 158.3, 137.7, 130.1, 114.3, 113.8, 108.0, 103.1, 46.6, 43.2, 27.2, 26.2, 24.9; LRMAS (ESI-) m/z 246 $(M-H)^-$; HRMS (ESI-) m/z $C_{14}H_{16}NO_3$ $(M-H)^-$ calcd 246.1136, obsd 246.1146.

4.2. Biological experiments

4.2.1. Mushroom tyrosinase inhibition assay

Cinnamamide derivatives **1 – 10** were assessed for mushroom tyrosinase inhibition with slight modification of a known method.[40] A 200 µL mixture of 20 µL of tyrosinase solution (1,000 U/mL), 170 µL of substrate solution (14.7 mM phosphate buffer, 293 µL L-tyrosine solution) and 10 µL of the test compounds (final concentration: 25 µM) was added to a 96-well plate. The plates were incubated at 37 ^{0}C for 30 min and the optical densities were calculated using a microplate reader (VersaMax^{TM}, Molecular Devices, Sunnyvale, CA, USA) at 450 nm. Kojic acid (25 µM) and arbutin (400 µM) were used as positive controls. The experiments were replicated three times. The following formula was used to calculate the tyrosinase inhibition:

$$\%\text{Inhibition} = [1-(A/B) \text{ X } 100)]$$

where A represents the absorbance of the test compounds and B the absorbance of the non-treated control.

*4.2.2. In silico docking simulation of cinnamamides **4**, **9**, and **10** with tyrosinase*

Docking simulation of the three cinnamamides **4**, **9**, and **10**, or kojic acid with tyrosinase enzyme was performed according to the procedure used in previous work[41,42]. 3D structures of the cinnamamides and kojic acid were created on Chem3D Pro 12.0 software. AutoDock Vina 1.1.2, and Chimera softwares were used for the docking score calculation between the test

compounds and tyrosinase enzyme. The 3D structure of tyrosinase (*Agaricus bisporus*) was obtained from Protein Data Bank (PDB ID: 2Y9X). Pharmacophore models were created on a LigandScout 4.1.0, indicating possible interactions between ligands and the amino acid residues of tyrosinase.

4.2.3. Cell culture

Murine B16F10 melanoma cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), obtained from Gibco/Thermo Fisher Scientific (Carlsbad, CA, USA), with 100 IU/mL penicillin, and 100 µg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 ºC. Cell viability, melanin content and tyrosinase activity assays were performed on cells cultured in either a 96-well plate or 24-well dish. All experiments were performed in triplicate.

4.2.4. Cell viability assay in B16F10 melanoma cells

Cell viabilities were determined using WST-8 assay[43]. B16F10 melanoma cells were seeded in a 96-well plate at a density of $1x10^4$ cells per well and incubated in a humidified condition with 5% CO_2 at 37 °C for 24 h. The three test cinnamamides **4**, **9** and **10** at four different concentrations (0, 5, 10, and 25 µM) were added to the cells and further incubated for 24 h under the above mentioned conditions. The following day the cultured cells were treated with WST-8 reagents and incubated for 30 min to 2 h at 37 °C and the cell viability was assessed using the EZ-Cytox assay (EZ-3000, Daeil Lab Service, Seoul, Korea) in 96-well microtiter plates by reading at an OD of 450 nm. The experiments were replicated three times.

4.2.5. Tyrosinase activity assay in B16F10 melanoma cells

The tyrosinase activity assay was performed with minor modification of the standard procedure[44]. Briefly, B16F10 cells at $5x10^4$ cells per well were cultured in a 96-well plate followed by incubation in a humidified environment containing 5% CO_2 at 37°C. After 24 h,

the cells were treated either with α-MSH (1 μM) and the three test cinnamamides **4**, **9** and **10** (0, 5, 10, 25 μM), or with α-MSH (1 μM) and kojic acid (25 μM) and further incubated for 24 h under the above mentioned conditions. The following day the cells were washed with PBS 2-3 times and then broken with lysis buffer (100 μL containing 50 mM PBS (90 μL, pH 6.8), 0.1 mM PMSF (5 μL), and 1% Triton X-100 (5 μL)). The lysates of the cells were then frozen at -80 °C for 30 min followed by centrifugation at 12,000 rpm for 30 min at 4 °C. Supernatant of the lysates was transferred to a 96-well plate and 80 μL of supernatant with 20 μL of 10 mM L-dopa were incubated at 37 °C for 30 min. Optical densities were measured at 500 nm by using a microplate reader (Tecan, Männedorf, Switzerland). The experiments were performed in triplicate.

4.2.6. Anti-melanogenesis assay in B16F10 melanoma cells

To assess the inhibitory effect of the three test cinnamamides **4**, **9** and **10** on melanin production, melanin content assay was performed using slight modification of the standard method previously reported.[45] Briefly, B16F10 cells were seeded at a density of $5x10^4$ cells/well in a 24-well plate and the cells were cultured in 5% CO_2 incubator at 37 °C. After 24 h, the cells were treated with α-MSH (1 μM) plus either four different concentrations of the three test cinnamamides (0, 5, 10, and 25 μM), or kojic acid (25 μM) and further incubated for 24 h under the above mentioned conditions. The cells were washed with PBS buffer 2-3 times to remove the media content and incubated with 1 N NaOH solution (200 μL) to dissolve melanin. After transferring the solution to 96-well plates, the absorbances of the dissolved melanin solution were measured at 405 nm using a microplate reader. All experiments were performed in triplicate.

4.2.7. DPPH radical scavenging activity assay.

A previously reported method with slight modification was adopted to determine the DPPH radical scavenging abilities of the test cinnamamides **1** – **10**.[46] Briefly, 180 μL of a

DPPH methanol solution (0.2 mM) was mixed with 20 μL of each cinnamamide **1 – 10** (10 mM in DMSO) in 96-well plates. L-Ascorbic acid was used as a positive standard reference material. Next, the mixed solution in 96-wells plates was incubated in the dark for 30 min and the absorbances were measured at 517 nm using a VersaMax™ microplate reader. All experiments were performed in triplicate. The following formula was used to calculate the DPPH scavenging activities of **1 – 10**:

scavenging activity (%) = [(Ac–As)/Ac × 100]

where Ac is the absorbance of the non-treated control and As is the absorbances of the cinnamamides.

4.2.8. Statistical analysis

GraphPad Prism 5 software (La Jolla, CA, USA) was used to determine the statistical analysis. All experiments were performed in triplicate. Calculated results are shown as means ± SEMs. Intergroup significance differences were calculated by using one-way ANOVA and Tukey's test. Statistical significance was considered at < 0.05 of two-sided *P*-values.

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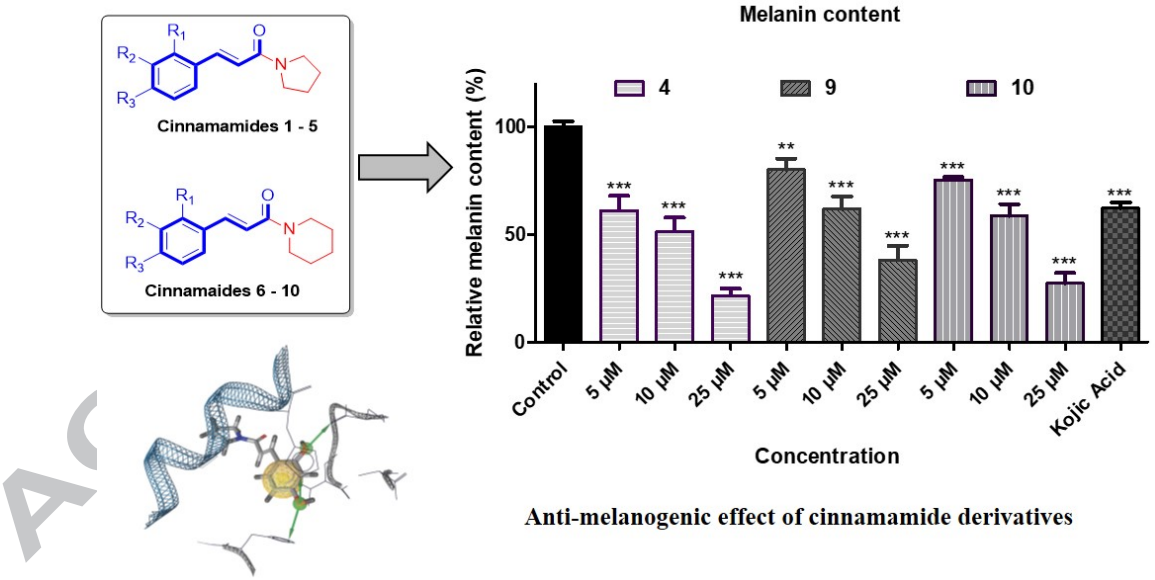
Graphical abstract



Anti-melanogenic effect of cinnamamide derivatives