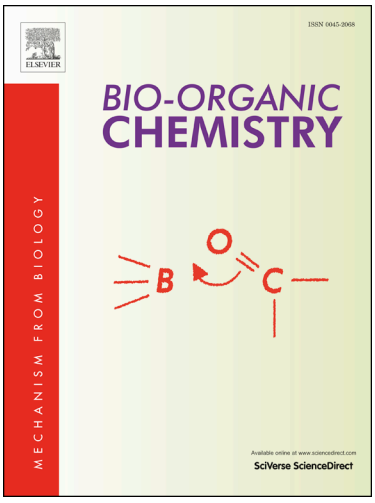


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# Tyrosinase inhibition and anti-melanin generation effect of cinnamamide analogues

Sultan Ullah[a], Chaeun Park[a], Muhammad Ikram[b,c], Dongwan Kang[a], Sanggwon Lee[a], Jungho Yang[a], Yujin Park[a], Sik Yoon[b], Pusoon Chun[d], Hyung Ryong Moon[a,*]

[a]*Laboratory of Medicinal Chemistry, College of Pharmacy, Pusan National University, Busan 46241, South Korea*

[b]*Department of Anatomy, Pusan National University School of Medicine, 49 Busandaehak-ro, Mulgeum-eup, Yangsan-si, Gyeongsangnam-do, 50612, South Korea.*

[c]*Department of Pharmacy, Comsats University Islamabad, Abbottabad Campus, Abbottabad 22060, Pakistan*

[d]*College of Pharmacy and Inje Institute of Pharmaceutical Sciences and Research, Inje University, Gimhae, Gyeongnam 50834, South Korea*

*****Author to whom correspondence should be addressed.

Hyung Ryong Moon, Laboratory of Medicinal Chemistry, College of Pharmacy, Pusan National University, Busan 46241, Republic of Korea. *E-mail address*: mhr108@pusan.ac.kr; Tel.: +82 51 510 2815; Fax: +82 51 513 6754.

**Abstract**

Abnormal melanogenesis results in excessive production of melanin, leading to pigmentation disorders. As a key and rate-limiting enzyme for melanogenesis, tyrosinase has been considered an important target for developing therapeutic agents of pigment disorders. Despite having an (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffold, which plays an important role in the potent inhibition of tyrosinase activity, cinnamic acids have not attracted attention as potential tyrosinase inhibitors, due to their low tyrosinase inhibitory activity and relatively high hydrophilicity. Given that cinnamic acids' structure intrinsically features this (*E*)-scaffold and following our experience that minute changes in the chemical structure can powerfully affect tyrosinase activity, twenty less hydrophilic cinnamamide derivatives were designed as potential tyrosinase inhibitors and synthesised using a Horner-Wadsworth-Emmons reaction. Four of these cinnmamides (**4**, **9**, **14**, and **19**) exhibited much stronger mushroom tyrosinase inhibition (over 90% inhibition) at 25 μM compared to kojic acid (20.57% inhibition); crucially, all four have a 2,4-dihydroxy group on the β-phenyl ring of the scaffold. A docking simulation using tyrosinase indicated that the four cinnamamides exceeded the binding affinity of kojic acid, and bound more strongly to the active site of tyrosinase. Based on the strength of their tyrosinase inhibition, these four cinnamamides were further evaluated in B16F10 melanoma cells. All four cinnamamides, without cytotoxicity, exhibited higher tyrosinase inhibitory activity (67.33 – 79.67% inhibition) at 25 μM than kojic acid (38.11% inhibition), with the following increasing inhibitory order: morpholino (**9**) = cyclopentylamino (**14**) < cyclohexylamino (**19**) < *N*-methylpiperazino (**4**) cinnamamides. Analysis of tyrosinase activity and melanin content in B16F10 cells showed that the four cinnamamides dose-dependently inhibited both cellular tyrosinase activity and melanin content and that their inhibitory activity at 25 μM was much better than that of kojic acid. The results of melanin content analysis well matched those of the cellular tyrosinase activity

analysis, indicating that tyrosinase inhibition by the four cinnamamides is a major factor in the reduction of melanin production. These results imply that these four cinnamamides with a 2,4-dihydroxyphenyl group can act as excellent anti-melanogenic agents in the treatment of pigmentation disorders.

**Key words:** cinnamamide, tyrosinase inhibitor, melanin, anti-melanogenic, docking, B10F16 melanoma cells.

## 1. Introduction

A complicated process of melanogenesis causes skin pigmentation. Melanin is produced in the melanosomes of melanocytes and transferred to keratinocytes in the epidermis [1]. UV initiates melanogenesis, which is a human defence mechanism to produce melanin for protecting skin cells from harmful UV rays [2]. However, abnormal accumulation of melanin causes skin disorders including Riehl melanosis, melasma, and senile lentigo [3, 4]. To decrease the abnormal accumulation of melanin, numerous approaches have been attempted involving the inhibition of tyrosinase, the suppression of melanin transfer from melanocytes to keratinocytes, and the intervention of cell signalling for melanogenesis [5-9]. Although many different chemicals including natural and synthetic substances showed meaningful anti-melanogenic effects in cell-based assays [10-18], most of them caused several side effects, in *in vivo* animal models, or humans, such as an insufficient potency, carcinogenic property, permanent depigmentation, and dermatitis [19-23]. Even though hydroquinone and kojic acid are used as whitening agents in limited concentration in a few countries, these skin-lightening agents are prohibited in most countries due to the risks of undesirable side effects such as possible carcinogenicity in thyroid [24], nephrotoxicity [25], genotoxicity [26] and cytotoxicity to melanocytes [27]. Arbutin is a β-D-glucopyranoside of hydroquinone, which is extracted from the bearberry plant in the genus *Arctostaphylos*. Due to its fewer side effects, arbutin is more widely used as a whitening agent than hydroquinone, and its anti-melanogenic effect is known to be due to the inhibition of tyrosinase activity. Arbutin is hydrolyzed to D-glucose and hydroquinone by the main skin microflora (*Staphylococcus epidermidis* and *Staphylococcus aureus*) [28], enzyme, such as β-glycosidase, and temperature (10% decomposition at 20 $^{o}$C for 5 days) [29]. Therefore, the need to discover novel skin-lightening agents persists.

Tyrosinase is a metalloenzyme carrying two copper atoms at its active site and acts as a multifunctional enzyme involving hydroxylase and oxidase[30]. During melanogenesis, tyrosinase controls two rate-limiting steps: the transformation of L-tyrosine to L-dopa (as a hydroxylase), and the conversion of dopa to dopaquinone (as an oxidase) [31]. Therefore, tyrosinase has been considered a critical target for the treatment of skin disorders associated with abnormal pigmentation. Among several approaches to suppress excessive and abnormal melanogenesis, tyrosinase inhibition studies have been the most studied [10, 11, 14, 32].

Over the past several years, we have synthesized a variety of compounds with an exomethylene type of β-phenyl-α,β-unsaturated carbonyl scaffold to discover new whitening agents (Figure 1). Compounds with the scaffold have been verified to exhibit good tyrosinase inhibition by *in vitro* and/or *in vivo* assays [16, 18, 33-37]. In this study, we focussed on the amide compounds connected to the β-phenyl-α,β-unsaturated carbonyl scaffold to find novel tyrosinase inhibitors, as shown in Figure 1. Cinnamic acid, a naturally occurring compound, can be a handy template for tyrosinase inhibitors due to the presence of this scaffold in its structure. Although caffeic acid, 3,4-dihydroxycinnamic acid, was found to have no tyrosinase inhibitory activity, chlorogenic acid [38, 39], the quinic acid ester of caffeic acid [39], showed mild inhibitory activity, compared with kojic acid. According to our previous cumulative structure-activity relationship data, [35, 36, 40-42] derivatives with a hydroxyl group on the β-phenyl ring of the scaffold generally showed high tyrosinase inhibitory activity, and the number and position of the hydroxyl groups are closely related to the degree of tyrosinase inhibition, depending on the overall structure of the molecules. Therefore, changing the number and position of the hydroxyl groups in cinnamic acids may greatly increase the inhibitory activity of tyrosinase. However, it is difficult for cinnamic acids to be absorbed by the melonocytes present in the epidermal basal layer due to the high polarity of the carboxylic acid functionality. An amide functionality has even less polarity than a

carboxylic acid functionality, and in general, shows more druggable property than an ester functionality in terms of biostability. On the basis of these findings, we designed the amide derivatives of cinnamic acid with at least one hydroxyl group in our search for potent tyrosinase inhibitors (Figure 1). As part of our continuous efforts to find new tyrosinase inhibitors with a cinnamic acid scaffold, we synthesized a series of the amide derivatives of cinnamic acid and evaluated their tyrosinase inhibition using mushroom tyrosinase and the suppression of both tyrosinase activity and melanin production in murine cell systems.

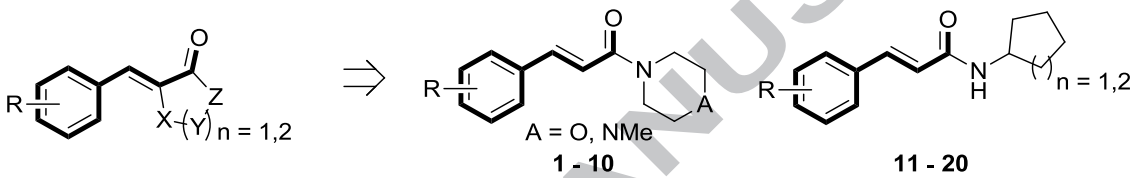


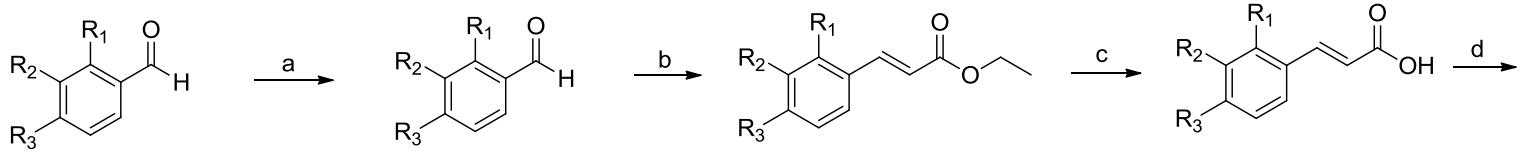

**Figure 1.** An exomethylene type of β-phenyl-α,β-unsaturated carbonyl scaffold and the design of cinnamamide derivatives **1** – **20**.

## 2. Results and Discussion

### *2.1. Chemistry*

For the synthesis of various amide analogues of cinnamic acid, amines such as primary cycloalkylamines and secondary cyclic amines were used in the condensation reaction with cinnamic acid. As shown in Scheme 1, first of all, five benzaldehydes (3-hydroxy-4-methoxybenzaldehyde **21**, 4-hydroxybenzaldehyde **22**, 3,4-dihydroxybenzaldehyde **23**, 2,4-dihydroxybenzaldehyde **24**, and 4-hydroxy-3-methoxybenzaldehyde **25**) with at least one hydroxyl group were reacted with benzyl bromide in the presence of $K_2CO_3$, giving the corresponding benzyl ether compounds **26** – **30** in yields of 95 – 97%, which were converted into the corresponding ethyl cinnamates **31** – **35** in yields of 95 – 98% by treatment with triethyl phosphonoacetate in the presence of a

catalytic amount of DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) and 1.0 equiv. $K_2CO_3$. The configuration of the double bond newly generated through a Horner-Wadsworth-Emmons olefination reaction was determined after hydrolysis of the cinnamates. Treatment with 1N-NaOH produced the corresponding cinnamic acid derivatives **36** – **40** quantitatively. The configuration of the double bond of the cinnamic acids **36** – **40** was assigned as an (*E*)-form, based on the coupling constant of the vinylic protons' peaks. All five cinnamic acids had the coupling constant with 16.0 Hz, implying that the cinnamic acids have the (*E*)-configuration. Cinnamic acids **36** – **40** obtained after hydrolysis were coupled with amines involving primary amines (cyclopentylamine and cyclohexylamine) and secondary amines (4-methylpiperazine and morpholine) with the help of isobutyl chloroformate in the presence of *N*-methylmorpholine to give the corresponding cinnamamides **41** – **60** in yields of 78 – 94%. Debenzylation of **41** – **60** under acidic conditions using acetic acid and *conc*-HCl under reflux produced the desired cinnamamide derivatives **1** – **3**, **5** – **8**, **10** – **13**, **15** – **18**, and **20** in yields of 61 – 76%, but compounds **4**, **9**, **14**, and **19** with a 2,4-dihydroxyl substituent were not obtained. Debenzylation of compounds, **44**, **49**, **54**, and **59**, with a 2,4-dihydroxyl substituent under these acidic conditions provided a mixture of compounds with high polarity. Therefore, milder acidic conditions were needed to prevent the formation of undesired by-products. $BBr_3$ was tried as an alternative acidic reagent for a debenzylation reaction and, surprisingly, produced the corresponding debenzylated cinnamamides **4**, **9**, **14**, and **19** in good yields (62 – 70%). The structures of the twenty final cinnamamides **1** – **20** were confirmed by NMR and mass spectroscopy.

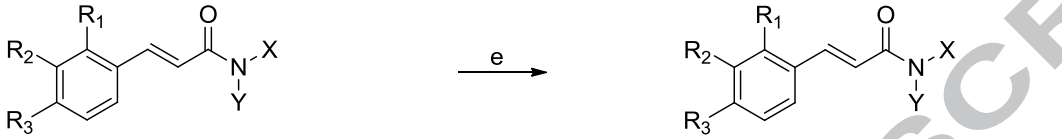



**21**: $R_1$ = H, $R_2$ = OH, $R_3$ = OMe
**22**: $R_1$ = H, $R_2$ = H, $R_3$ = OH
**23**: $R_1$ = H, $R_2$ = OH, $R_3$ = OH
**24**: $R_1$ = OH, $R_2$ = H, $R_3$ = OH
**25**: $R_1$ = H, $R_2$ = OMe, $R_3$ = OH

**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**: $R_1$ = H, $R_2$ = OBn, $R_3$ = OMe
**32**: $R_1$ = H, $R_2$ = H, $R_3$ = OBn
**33**: $R_1$ = H, $R_2$ = OBn, $R_3$ = OBn
**34**: $R_1$ = OBn, $R_2$ = H, $R_3$ = OBn
**35**: $R_1$ = H, $R_2$ = OMe, $R_3$ = OBn

**36**: $R_1$ = H, $R_2$ = OBn, $R_3$ = OMe
**37**: $R_1$ = H, $R_2$ = H, $R_3$ = OBn
**38**: $R_1$ = H, $R_2$ = OBn, $R_3$ = OBn
**39**: $R_1$ = OBn, $R_2$ = H, $R_3$ = OBn
**40**: $R_1$ = H, $R_2$ = OMe, $R_3$ = OBn




**41**: X, Y = $(CH_2)_2NMe(CH_2)_2$, $R_1$ = H, $R_2$ = OBn, $R_3$ = OMe
**42**: X, Y = $(CH_2)_2NMe(CH_2)_2$, $R_1$ = H, $R_2$ = H, $R_3$ = OBn
**43**: X, Y = $(CH_2)_2NMe(CH_2)_2$, $R_1$ = H, $R_2$ = OBn, $R_3$ = OBn
**44**: X, Y = $(CH_2)_2NMe(CH_2)_2$, $R_1$ = OBn, $R_2$ = H, $R_3$ = OBn
**45**: X, Y = $(CH_2)_2NMe(CH_2)_2$, $R_1$ = H, $R_2$ = OMe, $R_3$ = OBn
**46**: X, Y = $(CH_2)_2O(CH_2)_2$, $R_1$ = H, $R_2$ = OBn, $R_3$ = OMe
**47**: X, Y = $(CH_2)_2O(CH_2)_2$, $R_1$ = H, $R_2$ = H, $R_3$ = OBn
**48**: X, Y = $(CH_2)_2O(CH_2)_2$, $R_1$ = H, $R_2$ = OBn, $R_3$ = OBn
**49**: X, Y = $(CH_2)_2O(CH_2)_2$, $R_1$ = OBn, $R_2$ = H, $R_3$ = OBn
**50**: X, Y = $(CH_2)_2O(CH_2)_2$, $R_1$ = H, $R_2$ = OMe, $R_3$ = OBn
**51**: X = H, Y = cyclopentyl, $R_1$ = H, $R_2$ = OBn, $R_3$ = OMe
**52**: X = H, Y = cyclopentyl, $R_1$ = H, $R_2$ = H, $R_3$ = OBn
**53**: X = H, Y = cyclopentyl, $R_1$ = H, $R_2$ = OBn, $R_3$ = OBn
**54**: X = H, Y = cyclopentyl, $R_1$ = OBn, $R_2$ = H, $R_3$ = OBn
**55**: X = H, Y = cyclopentyl, $R_1$ = H, $R_2$ = OMe, $R_3$ = OBn
**56**: X = H, Y = cyclohexyl, $R_1$ = H, $R_2$ = OBn, $R_3$ = OMe
**57**: X = H, Y = cyclohexyl, $R_1$ = H, $R_2$ = H, $R_3$ = OBn
**58**: X = H, Y = cyclohexyl, $R_1$ = H, $R_2$ = OBn, $R_3$ = OBn
**59**: X = H, Y = cyclohexyl, $R_1$ = OBn, $R_2$ = H, $R_3$ = OBn
**60**: X = H, Y = cyclohexyl, $R_1$ = H, $R_2$ = OMe, $R_3$ = OBn

**1**: X, Y = $(CH_2)_2NMe(CH_2)_2$, $R_1$ = H, $R_2$ = OH, $R_3$ = OMe
**2**: X, Y = $(CH_2)_2NMe(CH_2)_2$, $R_1$ = H, $R_2$ = H, $R_3$ = OH
**3**: X, Y = $(CH_2)_2NMe(CH_2)_2$, $R_1$ = H, $R_2$ = OH, $R_3$ = OH
**4**: X, Y = $(CH_2)_2NMe(CH_2)_2$, $R_1$ = OH, $R_2$ = H, $R_3$ = OH
**5**: X, Y = $(CH_2)_2NMe(CH_2)_2$, $R_1$ = H, $R_2$ = OMe, $R_3$ = OH
**6**: X, Y = $(CH_2)_2O(CH_2)_2$, $R_1$ = H, $R_2$ = OH, $R_3$ = OMe
**7**: X, Y = $(CH_2)_2O(CH_2)_2$, $R_1$ = H, $R_2$ = H, $R_3$ = OH
**8**: X, Y = $(CH_2)_2O(CH_2)_2$, $R_1$ = H, $R_2$ = OH, $R_3$ = OH
**9**: X, Y = $(CH_2)_2O(CH_2)_2$, $R_1$ = OH, $R_2$ = H, $R_3$ = OH
**10**: X, Y = $(CH_2)_2O(CH_2)_2$, $R_1$ = H, $R_2$ = OMe, $R_3$ = OH
**11**: X = H, Y = cyclopentyl, $R_1$ = H, $R_2$ = OH, $R_3$ = OMe
**12**: X = H, Y = cyclopentyl, $R_1$ = H, $R_2$ = H, $R_3$ = OH
**13**: X = H, Y = cyclopentyl, $R_1$ = H, $R_2$ = OH, $R_3$ = OH
**14**: X = H, Y = cyclopentyl, $R_1$ = OH, $R_2$ = H, $R_3$ = OH
**15**: X = H, Y = cyclopentyl, $R_1$ = H, $R_2$ = OMe, $R_3$ = OH
**16**: X = H, Y = cyclohexyl, $R_1$ = H, $R_2$ = OH, $R_3$ = OMe
**17**: X = H, Y = cyclohexyl, $R_1$ = H, $R_2$ = H, $R_3$ = OH
**18**: X = H, Y = cyclohexyl, $R_1$ = H, $R_2$ = OH, $R_3$ = OH
**19**: X = H, Y = cyclohexyl, $R_1$ = OH, $R_2$ = H, $R_3$ = OH
**20**: X = H, Y = cyclohexyl, $R_1$ = H, $R_2$ = OMe, $R_3$ = OH

**Scheme 1. Synthesis of cinnamamide derivatives 1 – 20.** 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 **21**, **23** and **25**, 70 $^oC$ for **22**, and **24**, 24 – 36 h; c) 1M-NaOH aqueous solution, 1,4-dioxane, rt, 48 h; d) *i*-$BuO_2CCl$, *N*-methylmorpholine, THF, rt, 30 min, and then, 4-methylpiperazine, morpholine, cyclopentylamine, or cyclohexylamine, rt, 24 h; and e) *c*-HCl, AcOH, reflux, 40 min for **1** – **3**, **5** – **8**, **10** – **13**, **15** – **18**, and **20**, or 4.0 – 8.0 eq. $BBr_3$, DCM, -40 $^0C$, 30 min for **4**, **9**, **14**, and **19**.

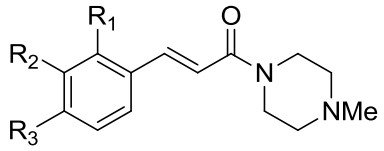
*2.2. Mushroom tyrosinase inhibitory effect of cinnamamide derivatives **1 – 20**.*

The inhibitory effect of the twenty synthesized cinnamamides **1** – **20** on mushroom tyrosinase was evaluated at 25 μM using kojic acid, a well-known tyrosinase inhibitor, as a positive control. As shown in Table 1, all compounds (**2**, **3**, **7**, **8**, **12**, **13**, **17** and **18**) having either a 4-hydroxyl or 3,4-dihydroxyl group on the β-phenyl ring did not show any inhibitory effect regardless of the amide type. Compounds (**1**, **6**, **11**, and **16**) with a 3-hydroxy-4-methoxyl group on the β-phenyl ring exhibited stronger inhibitory activities (from 31.12±7.93 to 60.15±3.51% inhibition) against mushroom tyrosinase than kojic acid (20.57±2.02% inhibition). On the other hand, compounds (**5**, **10**, **15**, and **20**) with a 4-hydroxy-3-methoxyl group on the β-phenyl ring showed similar or more potent inhibitory activity (from 19.33±1.36 to 68.04±8.25% inhibition) than kojic acid, except for compound **15** (no inhibition). Regardless of the amide type, four of the cinnamamides (**4**, **9**, **14**, and **19**) with a 2,4-dihydroxyphenyl group revealed much better inhibitory activity than kojic acid. All four of these cinnamamides showed an inhibitory activity exceeding 90% with an $IC_{50}$ values of 0.14±0.00 μM for **4**, 0.06±0.01 μM for **9**, 0.12±0.00 μM for **14**, and 0.16±0.01 μM for **19**, respectively, while kojic acid showed an $IC_{50}$ value of 30.64±1.27 μM. Notably, although a 4-hydroxyl group alone on the β-phenyl ring of cinnamamides did not show

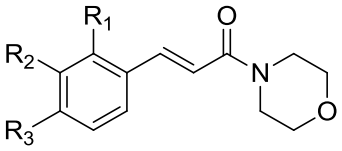
tyrosinase inhibitory activity, the insertion of an additional hydroxyl group into the 2-position of the β-phenyl ring dramatically enhanced the inhibition (**2** *vs.* **4**, **7** *vs*. **9**, **12** *vs*. **14**, and **17** *vs*. **19**). However, the insertion of an additional hydroxyl group into the 3-position did not enhance the tyrosinase inhibition. On the other hand, the insertion of a methoxyl group into the 3-position increased (compounds **5**, **10**, and **20**) or did not influence (compound **15**) the tyrosinase inhibition, depending on their molecular structures. Compounds **1**, **6**, **11**, and **16** have a hydroxyl group at the 3-position of the β-phenyl ring and a methoxyl group at the 4-position of the β-phenyl ring, whereas compounds **5**, **10**, **15**, and **20** have the same substituents in the reverse position. Overall, compounds with a 3-hydroxy-4-methoxy group on the β-phenyl ring exerted stronger inhibitory activities than compounds with a 4-hydroxy-3-methoxy group (**1** *vs.* **5**, **6** *vs*. **10**, and **11** *vs*. **15**). These results suggest that the number and type of the substituents on the β-phenyl ring of cinnamamide derivatives greatly affect the inhibitory activity of tyrosinase.

Because of showing the highest inhibitory activity against mushroom tyrosinase, the four cinnamamide derivatives with a 2,4-dihydroxyphenyl group (**4**, **9**, **14**, and **19**) were used in the following studies involving docking simulation and murine cell-based experiments.

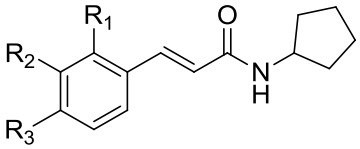
**Table 1.** Substitution patterns, and tyrosinase-inhibitory activities of the synthesized cinnamamide derivatives **1** – **20** and kojic acid



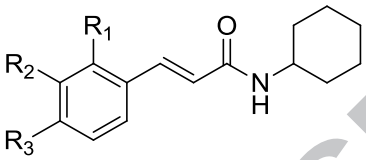

Compounds **1 - 5**




Compounds **6 - 10**




Compounds **11 - 15**



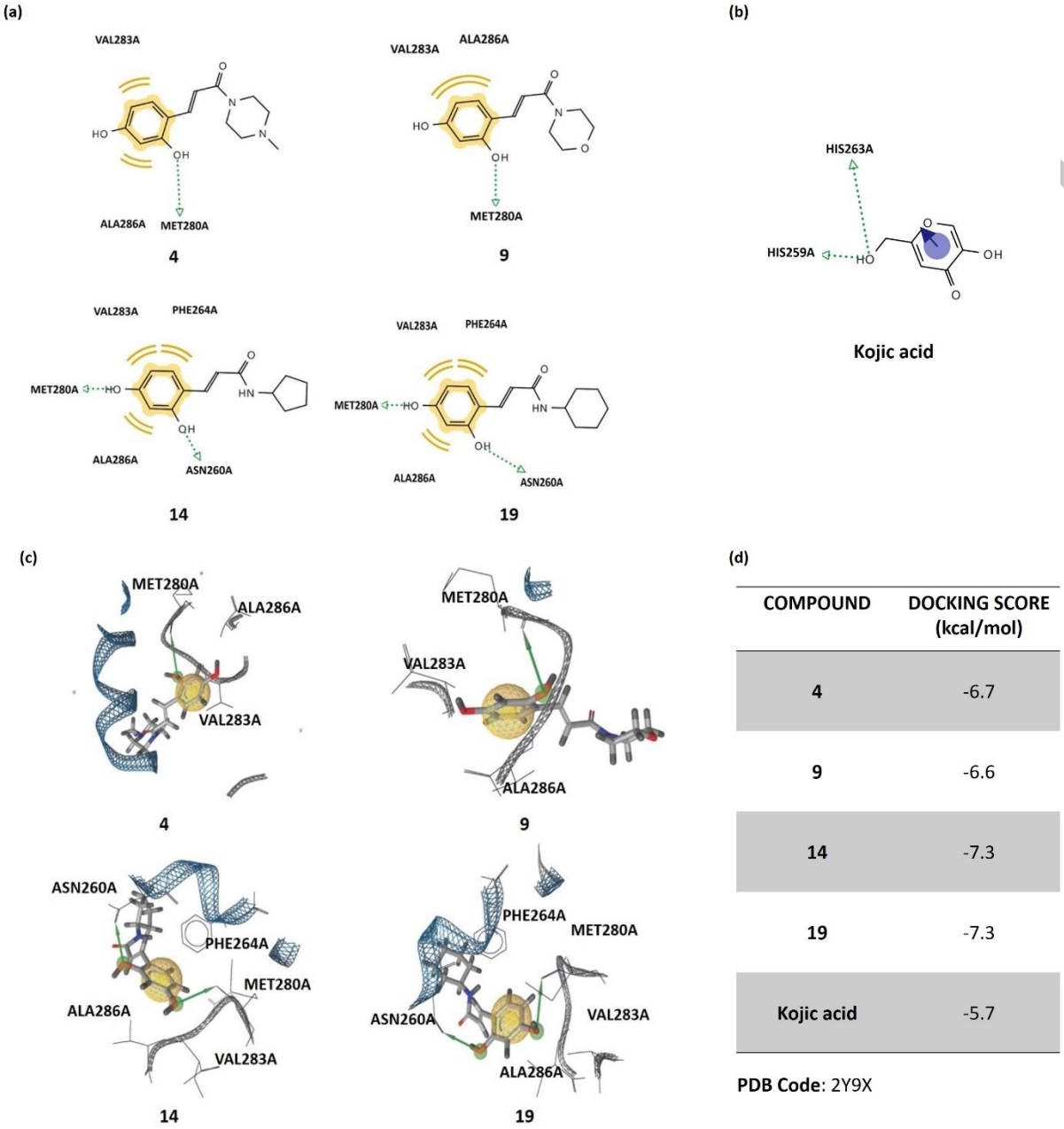

Compounds **16 - 20**

| Compounds | $R_1$ | $R_2$ | $R_3$ | Tyrosinase inhibition (%)[a] |
|---|---|---|---|---|
| **1** | H | OH | OMe | 40.22±4.67 |
| **2** | H | H | OH | NI |
| **3** | H | OH | OH | NI |
| **4** | OH | H | OH | 94.39±0.25 |
| **5** | H | OMe | OH | 19.33±1.36 |
| **6** | H | OH | OMe | 31.12±7.93 |
| **7** | H | H | OH | NI |
| **8** | H | OH | OH | NI |
| **9** | OH | H | OH | 95.74±0.12 |
| **10** | H | OMe | OH | 21.28±3.45 |
| **11** | H | OH | OMe | 60.15±3.51 |
| **12** | H | H | OH | NI |
| **13** | H | OH | OH | NI |
| **14** | OH | H | OH | 94.11±1.38 |
| **15** | H | OMe | OH | NI |
| **16** | H | OH | OMe | 45.61±16.34 |
| **17** | H | H | OH | NI |
| **18** | H | OH | OH | NI |
| **19** | OH | H | OH | 93.78±1.42 |
| **20** | H | OMe | OH | 68.04±8.25 |
| Kojic acid | | | | 20.57±2.02 |

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

*2.3. Docking studies of cinnamamide derivatives **4**, **9**, **14**, and **19** and kojic acid with tyrosinase*

To examine whether the four cinnamamide derivatives bind to the active site of tyrosinase and inhibit the enzyme as the result of the binding, *in silico* docking simulation was carried out using AutoDock Vina 1.1.2 software (The Scripps Research Institute). For tyrosinase ligand preparation, 2D structures of the four cinnamamides were drawn using ChemDraw Ultra 12.0 software (CambridgeSoft Corporation) and converted into the corresponding 3D structures using the energy minimization tool of Chem3D Pro 12.0 software (CambridgeSoft Corporation). The 3D X-ray crystal structure of *Agaricus bisporus* tyrosinase was downloaded from the Protein Data Bank (protein ID: 2Y9X) for docking simulation with the four cinnamamide derivatives.




(d)

| COMPOUND | DOCKING SCORE (kcal/mol) |
|---|---|
| **4** | -6.7 |
| **9** | -6.6 |
| **14** | -7.3 |
| **19** | -7.3 |
| **Kojic acid** | -5.7 |

**PDB Code**: 2Y9X

**Figure 2.** Docking simulation of the four cinnamamides **4**, **9**, **14**, and **19** and kojic acid with tyrosinase and pharmacophore analysis: (a, and b) pharmacophore results for **4**, **9**, **14**, and **19** and kojic acid obtained using LigandScout 4.1.0 showing hydrogen-bonding (green arrow), π-π stacking (violet arrow) and hydrophobic (yellow) interactions, (c) docking simulation results between the four cinnamamide derivatives (**4**, **9**, **14**, and **19**), and mushroom tyrosinase, and (d) docking scores between tyrosinase and **4**, **9**, **14**, **19** or kojic acid.

Compared to the binding affinity (-5.7 kcal/mol) of kojic acid, all four cinnamamide derivatives showed high binding affinities (-6.7 ~ -7.3 kcal/mol), as indicated in Figure 2. According to the pharmacophore analysis results of kojic acid using LigandScout 4.1.0 software, the 4-pyranone ring of kojic acid interacts with the imidazole ring of His263 amino acid residue through π-π stacking and the primary hydroxyl group produces two hydrogen bonds with two amino acid residues, His259 and His263. All four cinnamamide derivatives commonly have a 2,4-dihydroxyl substituent on the phenyl ring. Notably, the 2-hydroxyl groups on the phenyl ring of cinnamamides **4** and **9** belonging to cyclic tertiary amides make a hydrogen bond with Met280 amino acid residue, whereas the 2-hydroxyl groups on the phenyl ring of cinnamamides **14** and **19** belonging to cycloalkyl secondary amides make a hydrogen bond with Asn260 amino acid residue. Further, the 4-hydroxy groups on the phenyl ring of **4** and **9** do not participate in hydrogen bonding, while those of **14** and **19** create hydrogen bonds with the same amino acid, Met280. Also, cinnamamide **4** shows same hydrophobic interactions with two amino acid residues (Val283, and Ala286) as cinnamamide **9**, and cinnamamide **14** shows the same hydrophobic interactions (Phe264, Val283, and Ala286) as cinnamamide **19**. All four cinnamamides commonly form a hydrophobic interaction with two amino acid residues (Val283 and Ala286). These docking simulation results suggest that the cinnamamide analogues bind to the active site of tyrosinase and consequently inhibit tyrosinase.

*2.4. Cell viability of cinnamamide derivatives **4**, **9**, **14** and **19** in B16F10 melanoma cells*

We performed a WST-8 assay to confirm whether cinnamamides **4**, **9**, **14** and **19** are cytotoxic or not. For this purpose, B16F10 melanoma cells were cultured and treated with four different concentrations (0, 5, 10, and 25 μM) of the test cinnamamides. After an incubation of 24 h under a humidified atmosphere, the optical densities were measured on a

microplate reader.

According to Figure 3, no significant cytotoxic effect was found on the melanoma cells at concentrations up to 25 µM, showing that cinnamamides **4**, **9**, **14** and **19** were safe in murine melanoma B16F10 cells. Therefore, all cell-based assays involving cellular tyrosinase and melanin content were carried out at concentrations below 25 µM.

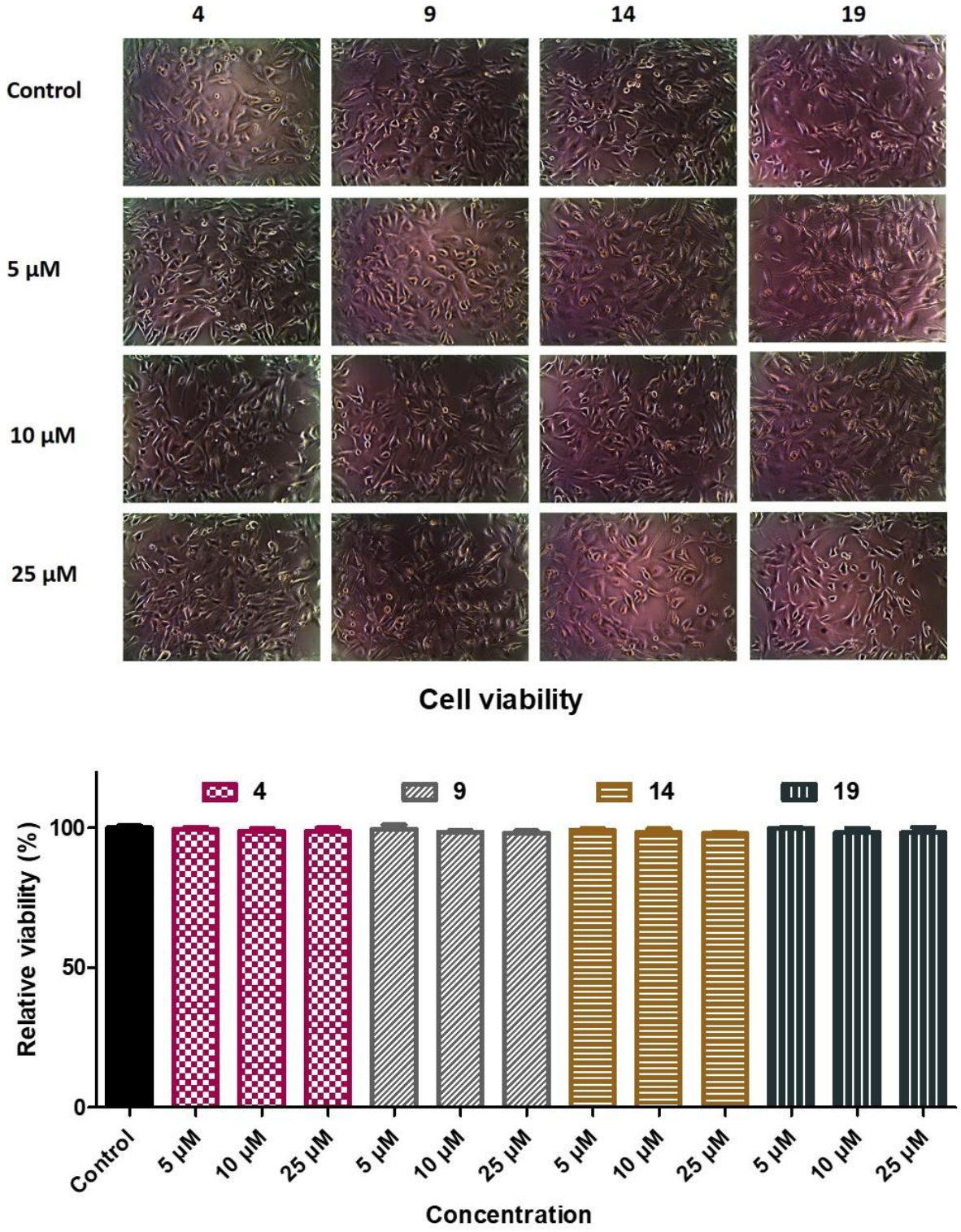



**Figure 3.** Cell viability assay of four cinnamamide derivatives **4**, **9**, **14** and **19** in B16F10 melanoma cells. Cinnamamides were treated at concentrations of 5, 10, and 25 μM. Viabilities are expressed in % to control, and bars represent standard errors.

*2.5. Tyrosinase inhibitory activity of cinnamamide derivatives **4**, **9**, **14** and **19** in α-MSH-stimulated B16F10 melanoma cells*

To determine the tyrosinase inhibitory effect of the four cinnamamide derivatives **4**, **9**, **14** and **19**, α-MSH-stimulated B16F10 melanoma cells were cultured and treated with four different concentrations of test compounds (0, 5, 10, and 25 μM), kojic acid (25 μM ) and arbutin (400 μM). After an incubation of 24 h, the tyrosinase inhibitory effect of compounds on B16F10 cells was determined by measuring the optical densities of the compounds.

According to the results displayed in Figure 4, cinnamamide **4** showed the greatest tyrosinase inhibitory effect among all four cinnamamides, kojic acid and arbutin. The inhibition of tyrosinase activity at 25 μM by the four cinnamamides increased in the order of morpholino (**9**) = cyclopentylamino (**14**) < cyclohexylamino (**19**) < *N*-methylpiperazino (**4**), with 67.33%, 67.67%, 75.89% and 79.67% inhibitions, respectively. Considering that the cinnamamides did not show cytotoxicity at concentrations below 25 μM, as shown in Figure 3, the inhibition of tyrosinase activity was attributed to direct tyrosinase inhibition by the cinnamamides, rather than cytotoxicity. Docking simulation results depicted in Figure 2 also support that the cinnamamides directly inhibit tyrosinase. All four cinnamamide derivatives having a common 2,4-dihydroxyl substituent on the phenyl ring of the cinnamamide exhibited more potent tyrosinase inhibitory activity at 25 μM than kojic acid (38.11%, at 25 μM) and arbutin (49.56%, at 400 μM), two natural tyrosinase inhibitors. Also, the tyrosinase activity was inhibited by the cinnamamide derivatives in a dose-dependent manner. These results indicate that all four cinnamamide derivatives have adequate lipophilicity to penetrate well into the cell membrane.

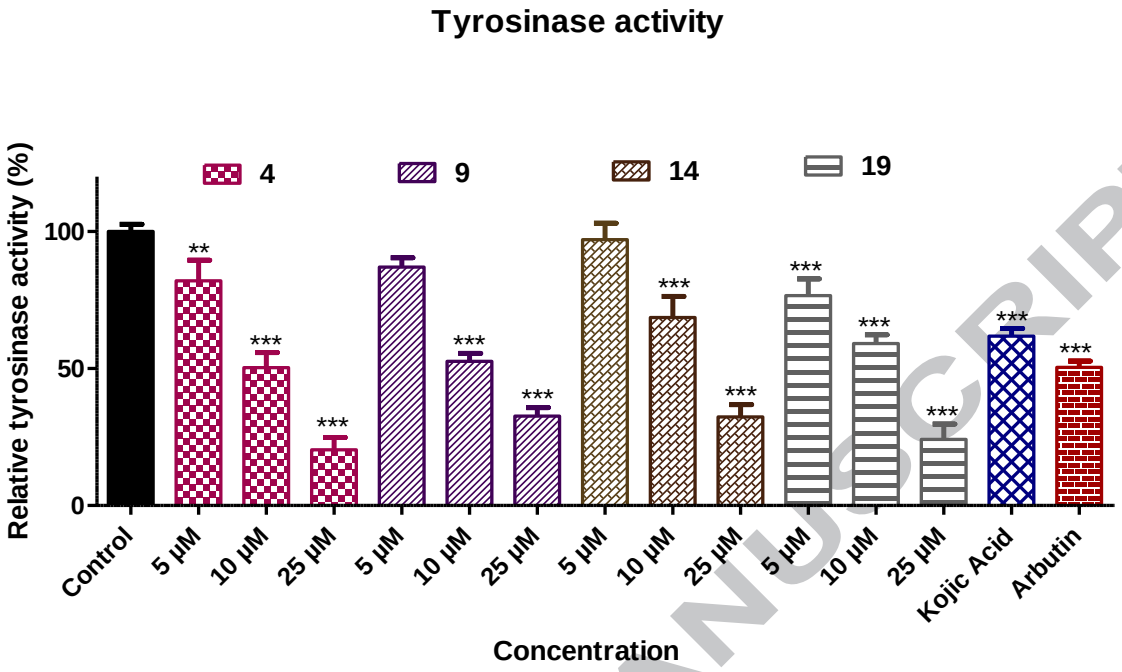



**Figure 4.** Tyrosinase inhibitory activity of cinnamamide derivatives **4**, **9**, **14** and **19** in α-MSH-stimulated B16F10 melanoma cells that were co-treated with the four cinnamamides **4**, **9**, **14**, and **19** (5, 10, and 25 μM), kojic acid (25 μM), or arbutin (400 μM). The asterisks represent the significant difference between the columns. **, $p<0.01$; and ***, $p<0.001$. The bars represent standard errors.

*2.6. Melanin production inhibitory effect of cinnamamide derivatives **4**, **9**, **14** and **19** in α-MSH-stimulated B16F10 melanoma cells*

To observe the melanin production inhibitory effect of the cinnamamide derivatives, melanin content assay was carried out. For this purpose, B16F10 melanoma cells were stimulated by α-MSH and co-treated with cinnamamides **4**, **9**, **14** and **19** in four different concentrations (0, 5, 10, and 25 μM), or kojic acid (25 μM). After an incubation of 24 h, the optical densities were measured to evaluate the melanin production inhibitory effect of the four cinnamamides.

According to the results shown in Figure 5, the four cinnamamide derivatives greatly decreased the melanin content in α-MSH-stimulated B16F10 melanoma cells at 25 μM, as

compared to kojic acid (35.43% inhibition). The inhibition of melanin production by the four cinnamamides increased in the order of cyclopentylamino (**14**) < morpholino (**9**) < cyclohexylamino (**19**) < *N*-methylpiperazino (**4**), with 58.20%, 62.03%, 74.00% and 75.57% inhibitions, respectively. Also, all four cinnamamides decreased melanin content significantly and dose-dependently. The similarity between the inhibition of tyrosinase activity (Figure 4) and the suppression of melanogenesis (Figure 5) implies that inhibition of melanin production is due to inhibition of tyrosinase activity by cinnamamides.

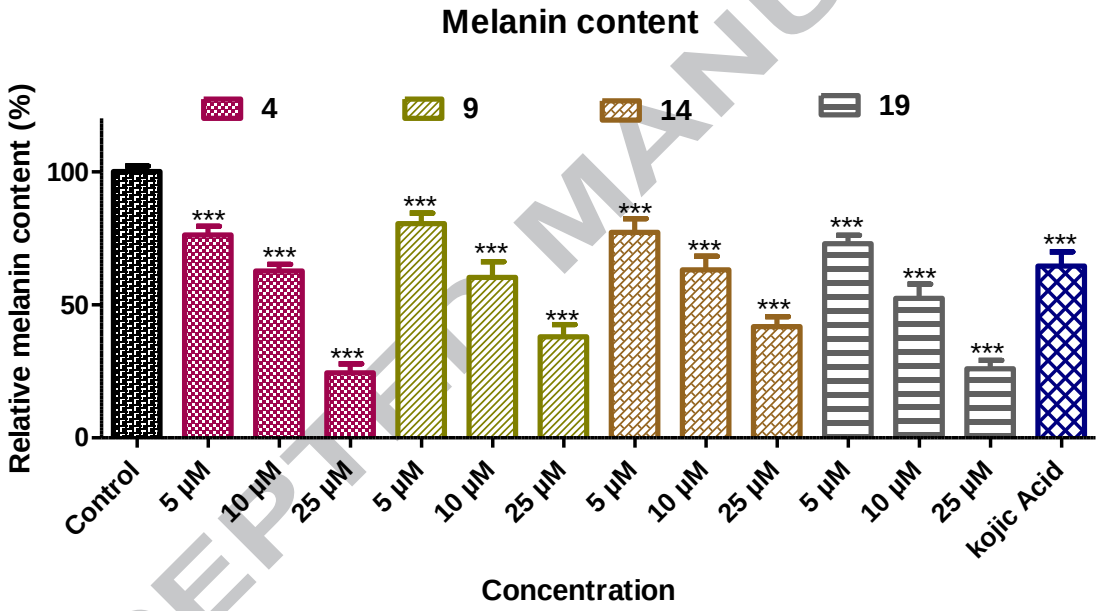



**Figure 5**. Melanin production inhibitory effect of cinnamamide derivatives **4**, **9**, **14** and **19** in α-MSH-stimulated B16F10 melanoma cells. B16F10 cells were stimulated by α-MSH and co-treated with the four cinnamamides **4**, **9**, **14**, and **19** (5, 10, and 25 μM), or kojic acid (25 μM). The asterisks represent the significant difference between the columns. ***, $p<0.001$. The bars represent standard errors.

*2.7. DPPH radical scavenging activity of cinnamamide derivatives **1** – **20***

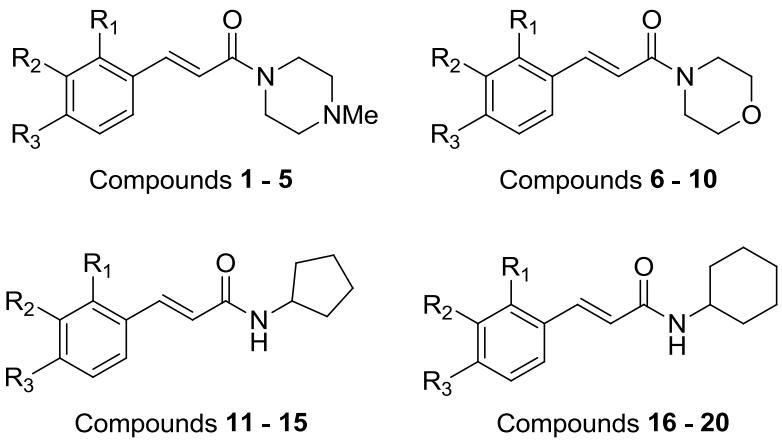
DPPH radical scavenging assay was performed to evaluate the antioxidant activities of cinnamamide derivatives **1 - 20**. In the final concentration of 1 mM, L-ascorbic acid, and

cinnamamides **1** – **20** were added to the DPPH methanol solution and the DPPH radical scavenging activity was determined after 30 min (Table 2).

According to the results shown in Table 2, most of the cinnamamides showed moderate to potent DPPH radical scavenging activity. The DPPH radical scavenging activity is closely related to the structure of the phenyl ring of the cinnamamides, rather than the structure of the amine moiety of the cinnamamides. Cinnamamides (**2**, **7**, **12**, and **17**) with a 4-hydroxyl group on the phenyl ring seldom exhibited DPPH radical scavenging activity and cinnamamides (**1**, **4**, **6**, **9**, **11**, **14**, **16**, and **19**) with either a 3-hydroxy-4-methoxyl or 2,4-dihydroxyl group exhibited weak to mild scavenging activity. High DPPH radical scavenging activity was observed in cinnamamides (**3**, **5**, **8**, **10**, **13**, **15**, **18**, and **20**) with a 3,4-dihydroxyl, or 4-hydroxy-3-methoxyl group. Their DPPH radical scavenging activity (76.40 ~ 85.57%) was comparable to that of L-ascorbic acid (84.64%).

Melanogenesis may result from reactive oxygen species (ROS) which are one of the factors that activate tyrosinase. Considering that cinnamamide derivatives **4**, **9**, **14** and **19** with a 2,4-dihydroxyphenyl group showed low to moderate DPPH radical scavenging activity, the strong anti-melanogenic effect of these cinnamamides may be attributable mainly to direct tyrosinase inhibition, rather than tyrosinase deactivation by antioxidant activity.

**Table 2.** DPPH radical scavenging activities of the synthesized cinnamamide derivatives **1** – **20** and kojic acid



| Compound | DPPH radical scavenging activity (%) | Compound | DPPH radical scavenging activity (%) |
|---|---|---|---|
| **1** | 3.64±0.83 | **11** | 36.69±0.87 |
| **2** | 1.45±1.27 | **12** | 19.83±2.05 |
| **3** | 83.38±0.37 | **13** | 85.57±0.18 |
| **4** | 7.18±0.79 | **14** | 46.52±1.97 |
| **5** | 76.40±0.54 | **15** | 83.71±0.29 |
| **6** | 21.15±0.51 | **16** | 39.25±4.96 |
| **7** | NA | **17** | NA |
| **8** | 83.40±0.56 | **18** | 81.53±0.36 |
| **9** | 35.38±0.89 | **19** | 36.94±0.93 |
| **10** | 82.44±0.26 | **20** | 78.59±0.23 |
| L-ascorbic acid | 84.64±0.32 | | |

Radical scavenging activities were determined 30 min after the addition of cinnamamide derivatives, or L-ascorbic acid to a final concentration of 1.0 mM DPPH solution in methanol. Three independent experiments were performed. Results are presented as the means±SDs of three experiments.

## 4. Conclusion

Cinnamic acid has an (*E*)-β-phenyl-α,β-unsaturated carbonyl scaffold, which is an essential structural moiety for excellent tyrosinase inhibition, but is relatively hydrophilic and difficult to be absorbed on the skin. Conversion of the carboxylic acid functional group in cinnamic acid into an amido group can increase the lipophilicity. Thus, twenty of the cinnamamides were synthesized as tyrosinase inhibitors using a Horner-Wadsworth-Emmons olefination. Of the twenty cinnamamides, four (**4**, **9**, **14**, and **19**) showed a high inhibitory effect on mushroom tyrosinase in excess of 90%. Docking simulation of the cinnamamides and tyrosinase indicated that the four cinnamamides bind more tightly to the active site of tyrosinase than kojic acid. Inhibition of tyrosinase activity and melanin content in B16F10 melanoma cells revealed that the four cinnamamides inhibited both cellular tyrosinase activity and melanin content dose-dependently and more strongly than kojic acid, which was used as a positive control. Similar inhibition patterns were observed in both cellular tyrosinase activity and melanin content, suggesting that the reduction of melanin production

by cinnamamides results from tyrosinase inhibition. Notably, all compounds **4**, **9**, **14**, and **19** which exhibited great anti-melanogenesis had a common 2,4-dihydroxyphenyl group. Comparing the anti-melanogenic effect of the four cinnamamides to that of kojic acid demonstrates the promising potential of the four cinnamamides as anti-whitening therapeutic agents.

## 4. Experimental section

### *4.1. General methods*

All the chemicals and reagents were obtained commercially and used without further purification. Thin layer chromatography (TLC) and column chromatography were conducted on Merck precoated $60F_{245}$ plates and MP Silica 40-63, 60 Å, respectively. The melting points of synthesized compounds were measured in capillary tubes on a Stuart™ melting point apparatus SMP3 (Bibby Scientific, Staffordshire, UK) and are uncorrected. All anhydrous solvents were distilled over CaH and Na/benzophenone. High resolution mass spectroscopy data were obtained on an Agilent Accurate Mass Q-TOF (quadruple-time of flight) liquid chromatography mass spectrometer (Agilent, Santa Clara, CA, USA) in ESI negative mode while low-resolution mass data were also obtained in ESI negative mode on an Expression CMS spectrometer (Advion Ithaca, NY, USA). Infrared spectroscopy data were obtained with a PerkinElmer Frontier FT-IR/FIR Spectrometer (PerkinElmer, Waltham, MA, USA) in ATR (Attenuated total reflection) mode using pure powder. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity INOVA 400 spectrometer or a Varian Unity AS500 spectrometer (Agilent Technologies, Santa Clara, CA, USA) for $^{1}H$ NMR (400 MHz and 500 MHz) and for $^{13}C$ NMR (100 MHz). DMSO-$d_6$, $CDCl_3$, and $CDCl_3$+$CD_3OD$ were used as an NMR solvent for NMR samples. The chemical shift and

coupling constant (*J*) values were measured in parts per million (ppm) and hertz (Hz), respectively. The abbreviations used in the analysis of $^{1}$H NMR data are s (singlet), brs (broad singlet), d (doublet), brd (broad doublet), dd (doublet of doublets), t (triplet), tt (triplet of triplets), brt (broad triplet), q (quartet), quint (quintet), m (multiplet), and brm (broad multiplet).

*4.1.1. General procedure for the synthesis of compounds **26** – **30** [43].*

Benzaldehyde **21** – **25** (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. Volatiles were evaporated on completion of the reaction and the resultant residues were partitioned between dichloromethane and water. The organic layer was dried with anhydrous $MgSO_4$ and evaporated *in vacuo* to provide the products **26** - **30** as a white solid in yields of 95 – 97%. Spectroscopic data can be found in SI.

*4.1.2. General procedure for the synthesis of cinnamic acids **36** – **40** through cinnamates **31** – **35** [44].*

To a stirred solution of compounds **26 – 30** (2.00 g), triethyl phosphonoacetate (1.1 equiv.), and potassium carbonate (2.0 equiv.) in dichloromethane/*N*,*N*-dimethylformamide (15 mL, 2:1) was added a catalytic amount of DBU (0.03equiv., 1,8-diazabicyclo[5.4.0]undec-7-ene). The reaction mixture was stirred at room temperature (compounds **26**, **28**, and **30**) or 70 $^{0}$C (compounds **27**, and **29**) for 24 – 36 h. After removing volatiles, ice water was added to the reaction mixture which was then stirred at room temperature for 30 min. After the generated precipitates were filtered, the filter cake was washed with excess water, and dried to provide ethyl cinnamates **31** – **35** as a white or grey solid in yields of 95 – 98%. The cinnamates **31 – 35** were used in the next step without characterization.

To a stirred solution of cinnamates **31** – **35** (2.57 to 4.11 g) in 1,4-dioxane (20 mL) was added 1N NaOH aqueous solution (8.0 – 16.0 equiv.) and the reaction mixture was stirred at room temperature for 48 h**.** On completion of the reaction, the reaction mixture was then acidified until pH 2 by 2N HCl. The reaction mixture was stirred at room temperature for 30 min to give precipitates. The filter cake obtained from filtration was washed with excess water, and dried to give cinnamic acids **36 – 40** as a white or grey solid in yields of 100%. Spectroscopic data can be found in SI.

*4.1.3. General procedures for the preparation of compounds **41** – **60** [45]***.***

To a solution of cinnamic acids **36 – 40** (100 mg), and isobutyl chloroformate (2.0 equiv.) in anhydrous THF (5 mL) was added *N*-methylmorpholine (2.5 equiv.). After the reaction mixture was stirred at room temperature for 30 min, appropriate amines (4-methylpiperazine, morpholine, cyclopentylamine, and cyclohexylamine: 2.0 equiv.) were added and the reaction mixture was then stirred at room temperature for 24 h. On 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 removed under reduced pressure. The resultant residue was purified by silica gel column chromatography using dichloromethane and methanol (30 – 70: 1) to afford compounds **41 – 60** as a white or yellow solid in yields of 78 – 94%. Spectroscopic data of known compounds can be found in SI.

*4.1.3.1. (E)-3-(3-(Benzyloxy)-4-methoxyphenyl)-1-(4-methylpiperazin-1-yl)prop-2-en-1-oneone (41).* Yellowish white solid; 90% yield; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.55 (d, 1 H, $J$ = 15.5 Hz, 3-vinylic H), 7.44 (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, 2.0 Hz, 6-H), 7.13 (d, 1 H, $J$ = 2.0 Hz, 2-H), 6.86 (d, 1 H, $J$ = 8.5 Hz, 5-H), 6.61 (d, 1 H, $J$ = 15.5 Hz, 2-vinylic H), 5.17 (s, 2 H, benzylic H), 3.90 (s, 3 H, $OCH_3$), 3.76 (brm, 2 H), 3.67 (brm, 2 H), 2.48 (brt, 4 H, $J$ = 5.0

Hz, 3″-H, 5″-H), 2.36 (s, 3 H, $NCH_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 165.8, 151.4, 148.4, 142.9, 137.1, 128.8, 128.2, 128.2, 127.5, 122.4, 114.9, 113.2, 111.7, 71.5, 56.2, 54.8, 46.1, 43.4.

*4.1.3.2. (E)-3-(3,4-Bis(benzyloxy)phenyl)-1-(4-methylpiperazin-1-yl)prop-2-en-1-one (43).* Yellowish white solid; 81% yield; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 7.55 (d, 1 H, $J$ = 15.0 Hz, 3-vinylic H), 7.45 – 7.29 (m, 10 H, 2 xPh), 7.09 (s, 1 H, 2-H), 7.06 (d, 1 H, $J$ = 8.5 Hz, 6-H), 6.90 (d, 1 H, $J$ = 8.5 Hz, 5-H), 6.61 (d, 1 H, $J$ = 15.0 Hz, 2-vinylic H), 5.18 (s, 2 H, benzylic H), 5.18 (s, 2 H, benzylic H), 3.78 (brm, 4 H, 2″-H, 6″-H), 2.61 (brm, 4 H, 3″-H, 5″-H), 2.44 (s, 3 H, $NCH_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 166.8, 150.8, 149.1, 143.3, 137.2, 137.0, 128.8, 128.8, 128.1, 127.4, 127.3, 122.5, 114.9, 114.5, 114.3, 71.7, 71.2, 54.6, 45.9, 43.8.

*4.1.3.3. (E)-3-(2,4-Bis(benzyloxy)phenyl)-1-(4-methylpiperazin-1-yl)prop-2-en-1-oneone (44).* Yellowish white solid; 78% yield; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 7.71 (d, 1 H, $J$ = 15.0 Hz, 3-vinylic H), 7.47 – 7.33 (m, 11 H, $J$ = 7.5 Hz, 6-H, 2 xPh), 6.98 (d, 1 H, $J$ = 15.0 Hz, 2-vinylic H), 6.45 (d, 1 H, $J$ = 2.0 Hz, 3-H), 6.59 (dd, 1 H, $J$ = 9.0, 2.0 Hz, 5-H), 5.08 (s, 2 H, benzylic H), 5.04 (s, 2 H, benzylic H), 3.80 (brm, 2 H), 3.35 (brm, 2 H), 2.56 (brm, 4 H, 3″-H, 5″-H), 2.41 (s, 3 H, $NCH_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 166.7, 161.2, 159.4, 139.9, 136.6, 136.4, 133.2, 128.9, 128.9, 128.6, 128.5, 128.4, 127.7, 117.8, 116.0, 106.4, 100.6, 70.8, 70.4, 54.5, 45.5, 42.9.

*4.1.3.4. (E)-3-(4-(Benzyloxy)-3-methoxyphenyl)-1-(4-methylpiperazin-1-yl)prop-2-en-1-one (45).* Yellow solid; 82% yield; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 7.60 (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), 7.04 (d, 1 H, $J$ = 1.5 Hz, 2-H), 7.03 (dd, 1 H, $J$ = 9.0, 1.5 Hz, 6-H), 6.85 (d, 1 H, $J$ = 9.0 Hz, 5-H), 6.70 (d, 1 H, $J$ = 15.5 Hz, 2-vinylic H), 5.17 (s, 2 H, benzylic H), 3.92 (s, 3 H, $OCH_3$), 3.77 (brm, 2 H), 3.72 (brm, 2 H), 2.50 (brt, 4 H, $J$ = 5.0 Hz, 3″-H,

5″-H), 2.36 (s, 3 H, $NCH_3$); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 165.8, 155.7, 149.8, 143.1, 136.9, 128.8, 128.8, 128.2, 127.4, 121.8, 115.0, 113.7, 110.6, 71.0, 56.3, 54.8, 46.1, 43.6.

*4.1.3.5. (E)-3-(3-(Benzyloxy)-4-methoxyphenyl)-1-morpholinoprop-2-en-1-one (46).* White solid; 91% yield; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 7.58 (d, 1 H, $J$ = 15.5 Hz, 3-vinylic H), 7.44 (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), 7.10 (d, 1 H, $J$ = 8.5 Hz, 6-H) 7.05 (s, 1 H, 2-H), 6.87 (d, 1 H, $J$ = 8.5 Hz, 5-H), 6.58 (d, 1 H, $J$ = 15.5 Hz, 2-vinylic H), 5.16 (s, 2 H, benzylic H), 3.90 (s, 3 H, $OCH_3$), 3.72 – 3.63 (m, 8 H, morpholine-H); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 166.0, 151.6, 148.4, 143.3, 137.1, 128.8, 128.2, 128.2, 127.5, 122.5, 114.4, 113.3, 111.8, 71.6, 67.1, 56.2, 44.1.

*4.1.3.6. (E)-3-(3,4-Bis(benzyloxy)phenyl)-1-morpholinoprop-2-en-1-one (48).* White solid; 94% yield; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 7.58 (d, 1 H, $J$ = 15.0 Hz, 3-vinylic H), 7.46 – 7.29 (m, 10 H, 2 xPh), 7.09 (s, 1 H, 2-H), 7.07 (d, 1 H, $J$ = 8.5 Hz, 6-H), 6.90 (d, 1 H, $J$ = 8.5, 5-H), 6.60 (d, 1 H, $J$ = 15.0 Hz, 2-vinylic H), 5.19 (s, 2 H, benzylic H), 5.18 (s, 2 H, benzylic H), 3.72 – 3.66 (m, 8 H, morpholine-H); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 165.9, 150.8, 149.1, 143.2, 137.2, 137.0, 128.8, 128.8, 128.7, 128.1, 127.5, 127.4, 122.5, 114.7, 114.6, 114.4, 71.8, 71.2, 67.1, 44.3.

*4.1.3.7. (E)-3-(2,4-Bis(benzyloxy)phenyl)-1-morpholinoprop-2-en-1-one (49).* White solid; 90% yield; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 7.74 (d, 1 H, $J$ = 15.5 Hz, 3-vinylic H), 7.47 – 7.33 (m, 11 H, 6-H, 2 xPh), 6.99 (d, 1 H, $J$ = 15.5 Hz, 2-vinylic H), 6.65 (d, 1 H, $J$ = 1.5 Hz, 3′-H), 6.60 (dd, 1 H, $J$ = 8.0, 1.5 Hz, 5′-H), 5.09 (s, 2 H, benzylic H), 5.04 (s, 2 H, benzylic H), 3.57 – 3.33 (brm, 8 H, morpholine-H); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 167.0, 161.3, 159.5, 140.3, 136.6, 136.4, 133.6, 128.9, 128.9, 128.7, 128.6, 128.4, 127.7, 117.8, 115.8, 106.4, 100.6, 70.8, 70.5, 67.0, 44.7.

*4.1.3.8. (E)-3-(3-(Benzyloxy)-4-methoxyphenyl)-N-cyclopentylacrylamide (51).* Yellowish

white solid; 83% yield; $^{1}$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.51 (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), 7.08 (d, 1 H, $J$ = 8.0 Hz, 6-H), 7.04 (s, 1 H, 2-H), 6.85 (d, 1 H, $J$ = 8.0 Hz, 5-H), 6.22 (d, 1 H, $J$ = 15.5 Hz, 2-vinylic H), 5.83 (brs, 1 H, NH), 5.13 (s, 2 H, benzylic H), 4.33 (brm, 1 H, 1″-H), 3.89 (s, 3 H, $OCH_3$), 2.03 (m, 2 H), 1.70 (m, 2 H), 1.62 (m, 2 H), 1.45 (m, 2 H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 166.1, 151.4, 148.4, 141.1, 136.9, 128.8, 128.2, 127.9, 127.6, 122.4, 118.6, 112.9, 111.8, 71.3, 56.2, 51.7, 33.4, 24.0.

*4.1.3.9. (E)-3-(3,4-Bis(benzyloxy)phenyl)-N-cyclopentylacrylamide (53).* Yellowish solid; 81% yield; $^{1}$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.51 (d, 1 H, $J$ = 16.0 Hz, 3-vinylic H), 7.44 – 7.29 (m, 10 H, 2 xPh), 7.09 (d, 1 H, $J$ = 2.0 Hz, 2-H), 7.03 (dd, 1 H, $J$ = 8.5, 2.0 Hz, 6-H), 6.88 (d, 1 H, $J$ = 8.5 Hz, 5-H), 6.23 (d, 1 H, $J$ = 16.0 Hz, 2-vinylic H), 5.84 (brs, 1 H, NH), 5.16 (s, 2 H, benzylic H), 5.15 (s, 2 H, benzylic H), 4.32 (brm, 1 H, 1″-H), 2.03 (m, 2 H), 1.70 (m, 2 H), 1.62 (m, 2 H), 1.45 (m, 2 H); $^{13}$C NMR (500 MHz, $CDCl_3$) $\delta$ 166.1, 150.7, 149.1, 141.0, 137.1, 137.0, 128.7, 128.7, 128.5, 128.1, 127.5, 127.4, 122.5, 118.9, 114.6, 114.1, 71.5, 71.2, 51.7, 33.4, 24.0.

*4.1.3.10. (E)-3-(2,4-Bis(benzyloxy)phenyl)-N-cyclopentylacrylamide (54).* Yellow solid; 78% yield; $^{1}$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.82 (d, 1 H, $J$ = 16.0 Hz, 3-vinylic H), 7.43 – 7.31 (m, 11 H, 6-H, 2 xPh), 6.58 – 6.54 (m, 2 H, 3-H, 5-H), 6.43 (d, 1 H, $J$ = 16.0 Hz, 2-vinylic H), 5.53 (brd, 1 H, $J$ = 4.5 Hz, NH), 5.10 (s, 2 H, benzylic H), 5.02 (s, 2 H, benzylic H), 4.32 (m, 1 H, 1″-H), 2.02 (m, 2 H), 1.71 – 1.59 (m, 4 H), 1.42 (m, 2 H); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 166.7, 161.2, 158.8, 136.7, 136.7, 136.2, 130.7, 128.9, 128.8, 128.4, 128.2, 127.7, 127.5, 119.9, 117.8, 106.6, 101.0, 70.6, 70.4, 51.5, 33.4, 23.9.

*4.1.3.11. (E)-3-(3-(Benzyloxy)-4-methoxyphenyl)-N-cyclohexylacrylamide (56).* Yellow solid; 86% yield; $^{1}$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.51 (d, 1 H, $J$ = 16.0 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* = 7.0 Hz, 4′-H), 7.08 (d, 1 H, *J* = 8.0 Hz, 6-H), 7.05 (s, 1 H, 2-H), 6.84 (d, 1 H, *J* = 8.0 Hz, 5-H), 6.22 (d, 1 H, *J* = 16.0 Hz, 2-vinylic H), 5.75 (brs, 1 H, NH), 5.12 (s, 2 H, benzylic H), 3.88 (m, 4 H, 1″-H, $OCH_3$), 1.96 (m, 2 H, 2″-Ha, 6″-Ha), 1.72 (m, 2 H, 2″-Hb, 6″-Hb), 1.63 (m, 1 H, 4″-Ha), 1.40 (m, 2 H, 3″-Ha, 5″-Ha), 1.19 (m, 3 H, 3″-Hb, 4″-Hb, 5″-Hb); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 165.6, 151.3, 148.4, 141.0, 136.9, 128.8, 128.2, 127.9, 127.6, 122.4, 118.8, 112.7, 111.7, 71.2, 56.2, 48.8, 33.4, 25.7, 25.1.

*4.1.3.12. (E)-3-(3,4-Bis(benzyloxy)phenyl)-N-cyclohexylacrylamide (58).* Yellowish solid; 80% yield; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.51 (d, 1 H, *J* = 15.5 Hz, 3-vinylic H), 7.45 – 7.29 (m, 10 H, 2 xPh), 7.09 (s, 1 H, 2-H), 7.03 (d, 1 H, *J* = 8.0 Hz, 6-H), 6.88 (d, 1 H, *J* = 8.0 Hz, 5-H), 6.22 (d, 1 H, *J* = 15.5 Hz, 2-vinylic H), 5.69 (brs, 1 H, NH), 5.17 (s, 2 H, benzylic H), 5.15 (s, 2 H, benzylic H), 3.89 (brm, 1 H, 1″-H), 1.97 (m, 2 H, 2″-Ha, 6″-Ha), 1.72 (m, 2 H, 2″-Hb, 6″-Hb), 1.63 (m, 1 H, 4″-Ha), 1.40 (m, 2 H, 3″-Ha, 5″-Ha), 1.19 (m, 3 H, 3″-Hb, 4″-Hb, 5″-Hb); $^{13}$C NMR (500 MHz, $CDCl_3$) $\delta$ 165.6, 150.7, 149.0, 141.1, 137.1, 137.0, 128.7, 128.7, 128.5, 128.1, 127.5, 127.4, 127.3, 122.5, 118.9, 114.5, 113.9, 71.5, 71.1, 48.8, 33.4, 25.7, 25.1.

*4.1.3.13. (E)-3-(2,4-Bis(benzyloxy)phenyl)-N-cyclohexylacrylamide (59***).** Yellowish white solid; 79% yield; $^1$H NMR (500 MHz, $CDCl_3$) $\delta$ 7.83 (d, 1 H, *J* = 16.0 Hz, 3-vinylic H), 7.43 – 7.31 (m, 11 H, 6-H, 2 xPh), 6.58 – 6.54 (m, 2 H, 3-H, 5-H), 6.43 (d, 1 H, *J* = 16.0 Hz, 2-vinylic H), 5.43 (brs, 1 H, NH), 5.10 (s, 2 H, benzylic H), 5.03 (s, 2 H, benzylic H), 3.89 (brm, 1 H, 1″-H), 1.96 (m, 2 H, 2″-Ha, 6″-Ha), 1.71 (m, 2 H, 2″-Hb, 6″-Hb), 1.62 (m, 1 H, 4″-Ha), 1.40 (m, 2 H, 3″-Ha, 5″-Ha), 1.17 (m, 3 H, 3″-Hb, 4″-Hb, 5″-Hb); $^{13}$C NMR (100 MHz, $CDCl_3$) $\delta$ 166.1, 161.2, 158.8, 136.7, 136.6, 136.3, 130.7, 128.9, 128.4, 128.2, 127.8, 127.5, 119.9, 117.8, 106.6, 100.9, 70.6, 70.4, 48.5, 33.4, 25.8, 25.1.

*4.1.4. General procedure for the preparation of cinnamamide derivatives **1** – **3**, **5** – **8**, **10** – **13**, **15** – **18** and **20**.*

Compounds **41 – 43, 45 – 48, 50 – 53, 55 – 58** and **60** (80-123 mg) were added in a 25 mL round-bottom flask along with concentrated HCl (1.0 mL) and acetic acid (1.0 mL) in 1:1 ratio and the reaction mixture was refluxed for 40 min. On completion of the reaction, the mixture was neutralised by 2N NaOH and partitioned between dichloromethane and water. The organic layer was dried with anhydrous $MgSO_4$, filtered, and evaporated *in vacuo*. The resulting residue was purified by silica gel column chromatography using dichloromethane and methanol (10 – 30: 1) as the eluent to give cinnamamide derivatives **1** – **3**, **5** – **8**, **10** – **13**, **15** – **18**, and **20** as a white or grey solid in yields of 61 – 76%. Spectroscopic data of known compounds can be found in SI.

*4.1.4.1. (E)-3-(3-Hydroxy-4-methoxyphenyl)-1-(4-methylpiperazin-1-yl)prop-2-en-1-one (1).* White solid (hygroscopic); 68% yield; IR (ATR, $cm^{-1}$): *v* 3343, 2930, 1641, 1584; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.02 (s, 1 H, OH), 7.32 (d, 1 H, *J* = 15.5 Hz, 3-H), 7.13 (d, 1 H, *J* = 2.0 Hz, 2′-H), 7.07 (dd, 1 H, *J* = 8.0, 2.0 Hz, 6′-H), 6.97 (d, 1 H, *J* = 15.5 Hz, 2-H), 6.90 (d, 1 H, *J* = 8.0 Hz, 5′-H), 3.78 (s, 3 H, $OCH_3$), 3.57 (brm, 2 H), 3.52 (brm, 2 H), 2.27 (brm, 4 H, 3″-H, 5″-H), 2.17 (s, 3 H, $NCH_3$); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 165.5, 150.0, 147.1, 143.1, 128.6, 121.4, 115.5, 115.1, 112.5, 56.3, 54.2, 53.7, 44.2; LRMAS (ESI-) *m/z* 275 $(M-H)^-$; HRMS (ESI+) *m/z* $C_{15}H_{21}N_2O_3$ $(M+H)^+$ calcd 277.1547, obsd 277.1541, *m/z* $C_{15}H_{20}N_2NaO_3$ $(M+Na)^+$ calcd 299.1366, obsd 299.1356.

*4.1.4.2. (E)-N-Cyclopentyl-3-(3-hydroxy-4-methoxyphenyl)acrylamide (11).* Yellowish solid; mp: 148.1 – 149.4 $^o$C; 65% yield; IR (ATR, $cm^{-1}$): *v* 3347, 3094, 2942, 1651, 1585, 1534; $^1$H NMR (500 MHz, $CDCl_3$ + a few drops of $CD_3OD$) $\delta$ 7.45 (d, 1 H, *J* = 15.5 Hz, 3-H), 7.06 (d, 1 H, *J* = 1.5 Hz, 2′-H), 6.93 (dd, 1 H, *J* = 8.5, 1.5 Hz, 6′-H), 6.76 (d, 1 H, *J* = 8.5 Hz, 5′-H),

6.26 (d, 1 H, *J* = 15.5 Hz, 2-H), 4.26 (quint, 1 H, *J* = 6.5 Hz, 1″-H), 3.85 (s, 3 H, $OCH_3$), 1.98 (m, 2 H, 2″-H, 5″-H), 1.67 (m, 2 H), 1.58 (m, 2 H), 1.43 (m, 2 H); $^{13}C$ NMR (100 MHz, $CDCl_3$ + a few drops of $CD_3OD$) $\delta$ 166.8, 148.9, 146.1, 141.3, 128.3, 121.8, 118.2, 113.0, 111.0, 56.1, 51.7, 33.1, 24.0; LRMAS (ESI-) *m/z* 260 $(M-H)^-$; HRMS (ESI+) *m/z* $C_{15}H_{20}NO_3$ $(M+H)^+$ calcd 262.1438, obsd 262.1434, *m/z* $C_{15}H_{19}NNaO_3$ $(M+Na)^+$ calcd 284.1257, obsd 284.1253.

*4.1.4.3. (E)-N-Cyclopentyl-3-(4-hydroxy-3-methoxyphenyl)acrylamide (15)***.** Yellowish solid; mp: 188.3 – 189.5 $^oC$; 67% yield; IR (ATR, $cm^{-1}$): *v* 3325, 3096, 2937, 1654, 1613, 1586; $^1H$ NMR (500 MHz, $CDCl_3$ + a few drops of $CD_3OD$) $\delta$ 7.45 (d, 1 H, *J* = 16.0 Hz, 3-H), 6.96 (d, 1 H, *J* = 8.5 Hz, 6′-H), 6.94 (s, 1 H, 2′-H) 6.80 (d, 1 H, *J* = 8.5 Hz, 5′-H), 6.26 (d, 1 H, *J* = 16.0 Hz, 2-H), 4.23 (quint, 1 H, *J* = 7.0 Hz, 1″-H) 3.82 (s, 3 H, $OCH_3$), 1.96 (m, 2 H, 2″-H, 5″-H), 1.65 (m, 2 H), 1.57 (m, 2 H), 1.42 (m, 2 H); $^{13}C$ NMR (100 MHz, $CDCl_3$ + a few drops of $CD_3OD$) $\delta$ 166.7, 147.9, 147.3, 141.4, 127.2, 122.2, 117.8, 115.1, 110.2, 56.0, 51.6, 33.1, 23.9; LRMAS (ESI-) *m/z* 260 $(M-H)^-$; HRMS (ESI+) *m/z* $C_{15}H_{20}NO_3$ $(M+H)^+$ calcd 262.1438, obsd 262.1430, *m/z* $C_{15}H_{19}NNaO_3$ $(M+Na)^+$ calcd 284.1257, obsd 284.1247.

*4.1.4.4. (E)-N-Cyclohexyl-3-(3-hydroxy-4-methoxyphenyl)acrylamide (16).* Yellowish white solid; mp: 193.9 – 195.2 $^oC$; 75% yield; IR (ATR, $cm^{-1}$): *v* 3334, 3140, 2927, 1657, 1619, 1589; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 7.53 (d, 1 H, *J* = 15.5 Hz, 3-H), 7.10 (d, 1 H, *J* = 1.5 Hz, 2′-H), 6.97 (dd, 1 H, *J* = 8.0, 1.5 Hz, 5′-H), 6.79 (d, 1 H, *J* = 8.0 Hz, 5′-H), 6.26 (d, 1 H, *J* = 15.5 Hz, 2-H), 5.86 (brs, 1 H) 3.89 (m, 1 H, 1″-H), 3.89 (s, 3 H, $OCH_3$), 1.96 (m, 2 H, 2″-Ha, 6″-Ha), 1.72 (m, 2 H, 2″-Hb, 6″-Hb), 1.61 (m, 1 H, 4″-Ha), 1.37 (m, 2 H, 3″-Ha, 5″-Ha), 1.19 (m, 3 H, 3″-Hb, 4″-Hb, 5″-Hb); $^{13}C$ NMR (100 MHz, $CDCl_3$) $\delta$ 165.7, 148.3, 146.0, 141.2, 128.5, 121.8, 118.9, 112.9, 110.7, 56.2, 48.8, 33.3, 25.7, 25.1; LRMAS (ESI-) *m/z* 274 $(M-H)^-$; HRMS (ESI+) *m/z* $C_{16}H_{22}NO_3$ $(M+H)^+$ calcd 276.1594, obsd 276.1584, *m/z*

$C_{16}H_{21}NNaO_3$ $(M+Na)^+$ calcd 298.1414, obsd 298.1401.

*4.1.5. General procedure for the preparation of cinnamamide derivatives **4**, **9**, **14**, and **19**.*

To a stirred solution of compounds **44**, **49**, **54**, and **59** (80 mg) in anhydrous dichloromethane (3.0 mL) was added $BBr_3$ (4.0 equiv.) at -40 $^0$C and the reaction mixture was stirred for 40 min. On 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. Volatiles were evaporated under reduced pressure. The resulting residue was purified by silica gel column chromatography using dichloromethane and methanol (10 – 30:1) as the eluent to afford cinnamamide derivatives **4**, **9**, **14**, and **19** as a white solid in yields of 62 – 70%. Spectroscopic data of known compounds can be found in SI.

*4.1.5.1. (E)-3-(2,4-Dihydroxyphenyl)-1-(4-methylpiperazin-1-yl)prop-2-en-1-one (4).* Yellowish white solid; mp: 136.2 – 138.4 $^o$C (decomp.); 67% yield; IR (ATR, $cm^{-1}$): *v* 3344, 3168, 2939, 1640, 1582; $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.92 (s, 1 H, OH), 9.73 (s, 1 H, OH), 7.74 (d, 1 H, *J* = 15.5 Hz, 3-H), 7.48 (d, 1 H, *J* = 8.5 Hz, 6′-H), 6.95 (d, 1 H, *J* = 15.5 Hz, 2-H), 6.32 (d, 1 H, *J* = 2.0 Hz, 3′-H), 6.24 (dd, 1 H, *J* = 8.5, 2.0 Hz, 5′-H), 4.39 (brm, 2 H), 3.47 (brm, 2 H), 3.30 (s, 3 H, $NCH_3$), 3.14 (brm, 4 H, 3′′-H, 5′′-H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 166.4, 161.0, 158.6, 138.9, 130.0, 114.0, 112.5, 108.1, 103.0, 55.4, 54.7, 42.8; LRMAS (ESI-) *m/z* 261 $(M-H)^-$; HRMS (ESI+) *m/z* $C_{14}H_{19}N_2O_3$ $(M+H)^+$ calcd 263.1390, obsd 263.1382, *m/z* $C_{14}H_{18}N_2NaO_3$ $(M+Na)^+$ calcd 285.1210, obsd 285.1205.

*4.1.5.2. (E)-N-Cyclopentyl-3-(2,4-dihydroxyphenyl)acrylamide (14).* White solid; mp: 105.7 – 107.4 $^o$C; 62% yieldm; IR (ATR, $cm^{-1}$): *v* 3296, 3104, 2934, 1640, 1544; $^1$H NMR (500 MHz,

DMSO-$d_6$) $\delta$ 9.80 (brs, 1 H, OH), 9.62 (brs, 1 H, OH), 7.82 (d, 1 H, $J$ = 7.0 Hz, NH), 7.48 (d, 1 H, $J$ = 16.0 Hz, 3-H), 7.18 (d, 1 H, $J$ = 8.5 Hz, 6′-H), 6.41 (d, 1 H, $J$ = 16.0 Hz, 2-H), 6.31 (d, 1 H, $J$ = 2.5 Hz, 3′-H), 6.23 (dd, 1 H, $J$ = 8.5, 2.5 Hz, 5′-H), 4.07 (quint, 1 H, $J$ = 7.0 Hz, 1″-H), 1.79 (m, 2 H, 2″-H, 5″-H), 1.62 (m, 2 H), 1.48 (m, 2 H), 1.37 (m, 2 H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 166.1, 160.2, 158.3, 135.1, 130.0, 118.8, 114.1, 108.0, 103.2, 50.9, 33.1, 24.1; LRMAS (ESI-) *m/z* 246 $(M-H)^-$; HRMS (ESI+) *m/z* $C_{14}H_{18}NO_3$ $(M+H)^+$ calcd 248.1281, obsd 248.1281, *m/z* $C_{14}H_{17}NNaO_3$ $(M+Na)^+$ calcd 270.1101, obsd 270.1095.

*4.2. Biological evaluation*

*4.2.1. Mushroom tyrosinase inhibitory activity of cinnamamide derivatives **4**, **9**, **14** and **19**.*

A standard protocol with minor changes [46] was followed to evaluate the mushroom tyrosinase inhibitory activity of the test compounds. 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 test compounds (final concentration: 25 µM, **4**, **9**, **14** and **19**) was added to a 96-well plate. The plates were incubated at 37 $^0$C for 30 min and the optical densities were measured on a microplate reader (VersaMax$^{TM}$, Molecular Devices, Sunnyvale, CA, USA) at 450 nm. Kojic acid at 25 µM was used as a control. The experiments were repeated three times. The following formula was used to calculate the tyrosinase inhibition:

%Inhibition = [1-(A/B)] X 100)]

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

*4.2.2. In silico docking simulation of cinnamamide derivatives **4**, **9**, **14**, and **19** and tyrosinase*

Docking simulation for the four cinnamamide derivatives, or kojic acid with tyrosinase was performed according to the procedure used in previous work [47, 48]. 3D structures of the cinnamamides and kojic acid were created on ChemDraw 3D Pro 12.0.

Chimera, and AutoDock Vina 1.1.2 softwares were utilised for the docking score calculation between the four cinnamamide derivatives, or kojic acid and tyrosinase. The 3D structure of tyrosinase (*Agaricus bisporus*) was obtained from the Protein Data Bank (PDB ID: 2Y9X). Pharmacophore models were generated on a LigandScout 4.1.0, showing possible interactions such as a hydrogen bond, hydrophobic interaction, and π-π stacking between amino acid residues of tyrosinase and ligands.

*4.2.3. Cell culture*

B16F10 cells (Murine melanoma) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), purchased from Gibco/Thermo Fisher Scientific (Carlsbad, CA, USA), with 100 IU/mL penicillin, and 100 µg/mL streptomycin was used for the cell cultures. Cells were maintained in a standard humidified atmosphere with 5% $CO_2$ at 37 ºC. Assays involving cell viability, tyrosinase activity, and melanin content were carried out on these cells cultured in a 24-well culture plate. All experiments were done in triplicate.

*4.2.4. Cell viability assay in B16F10 melanoma cells*

The WST-8 calorimetric assay was used to measure the cell viabilities at different concentrations of the test compounds [49]. $1x10^4$ B16F10 cells per well were seeded in a 96-well plate and incubated in a standard humidified conditions for cell culture having 5% $CO_2$ at 37°C for 24 h before treating the cells with cinnamamide derivatives ***4***, ***9***, ***14*** *and* ***19***. Cells were further incubated for 24 h after treatment with four concentrations (0, 5, 10, and 25 µM) of cinnamamide derivatives in standard conditions. The following day, a WST-8 reagent was added to the cultured cells at 1:10 ratio and incubated for 30 min to 2 h at 37 °C. The cell viability was calculated using the EZ-Cytox assay (EZ-3000, Daeil Lab Service, Seoul, Korea)

by reading the optical density at 450 nm, in 96-well plates. The experiments were performed in triplicate.

*4.2.5. Tyrosinase inhibitory activity assay in B16F10 melanoma cells*

Tyrosinase activity was evaluated by quantifying the L-DOPA oxidation rate as reported earlier by S.J. Bae et al., with slight modification [50]. Briefly, B16F10 cells at a density of $5x10^4$ cells per well were cultured and allowed to attach to the bottom of a 96-well plate followed by incubation in a humidified environment containing 5% $CO_2$ at 37°C overnight. Before treatment with the test compounds, the control and treatment groups were activated by 1 μM α-MSH. To the treated groups, either cinnamamide derivatives **4**, **9**, **14** and **19** (0, 5, 10, and 25 μM), or kojic acid (25 μM) was added and kept for further 24 h under the standard conditions. The following day, after washing the cells with PBS 2-3 times, 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)) was added to the cells and the mixtures were frozen at -80 °C for 30 min to complete the disintegration of the cells. Cell lysates were centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant (80 μL) of the lysates was mixed with 20 μL of L-dopa (10 mM) in a 96-well plate and incubated for 30 min at 37 °C. Using a microplate reader (Tecan, Männedorf, Switzerland), the optical densities of the solutions were measured at 500 nm. The experiments were repeated in triplicate.

*4.2.6. Determination of melanin content in B16F10 melanoma cells*

The inhibitory effect of the four cinnamamide derivatives on melanin production was determined by the standard protocol with slight modification as previously reported by L.-G. Chen et al., [51]. Briefly, B16F10 cells were cultured in a 24-well plate at a density of $5x10^4$ cells/well in humidified conditions under 5% $CO_2$ incubator at 37 °C. After 24 h of incubation, the cells were activated for melanin production by α-MSH (1 μM). Simultaneously, the treated groups were treated with gradient concentrations (0, 5, 10, and 25 μM) of the four

cinnamamide derivatives, or 25 μM kojic acid and cultured for an additional 24 h under the same conditions. To remove the media content, cells were rinsed with PBS buffer 2-3 times, and 200 μL of 1N NaOH solution was added to extract melanin content. The absorbances of the extracted melanin contents were measured at 405 nm using a microplate reader after transferring the solution to 96-well plates. All experiments were performed in triplicate.

*4.2.7. DPPH radical scavenging activity assay.*

A slightly modified version of a previously reported standard method was used to calculate the DPPH radical scavenging abilities of cinnamamide derivatives **1** – **20** [52]. Briefly, each cinnamamide derivative **1** – **20** (20 μL, 10 mM in DMSO) was added to 180 μL of DPPH methanol solution (0.2 mM) in 96-well plates. L-Ascorbic was utilised as a positive standard material. The mixed solution was incubated for 30 min in 96-wells plate under dark conditions. Absorbances were observed using a VersaMax$^{TM}$ microplate reader at 517 nm. All experiments were repeated in triplicate. The following formula was used to calculate the radical scavenging activities of cinnamamide derivatives **1** – **20**.

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

where Ac represents the absorbance of the non-treated control and As represents the absorbance of the test samples.

*4.2.8. Statistical analysis*

Statistical analysis was determined using GraphPad Prism 5 software (La Jolla, CA, USA). The observed results are displayed as means ± SEMs. Intergroup significance differences were calculated by using one-way ANOVA and Tukey's test. Statistical significance was accepted for *p*-values of $< 0.05$.

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## Highlights

- Twenty less hydrophilic cinnamamide derivatives were synthesized as potential tyrosinase inhibitors via a Horner-Wadsworth-Emmons reaction.
- Four of these cinnmamides (**4**, **9**, **14**, and **19**) exhibited much stronger mushroom tyrosinase inhibition (over 90% inhibition) than kojic acid (20.57% inhibition).
- All four cinnamamides, without cytotoxicity, exhibited higher tyrosinase inhibitory activity and decreased melanin content in B16F10 melanoma cells as compared to kojic acid.
- A docking study revealed that these four cinnamamides bound more strongly to the active site of tyrosinase than kojic acid.

## Ghraphical Abstract