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Biological evaluation of coumarin derivatives as mushroom tyrosinase inhibitors

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ABSTRACT

A series of coumarin derivatives were synthesised and their inhibitory effects on the diphenolase activity of mushroom tyrosinase were evaluated. The results showed that some of the synthesised compounds exhibited significant inhibitory activities. Especially, 2-(1-(coumarin-3-yl)ethylidene)hydrazinecarbothioamide bearing thiose-micarbazide group exhibited the most potent tyrosinase inhibitory activity with IC_{50} value of 3.44 μM. The inhibition mechanism analysis of 2-(1-(coumarin-3-yl)-ethylidene)hydrazinecarbothioamide and 2-(1-(6-chlorocoumarin-3-yl)ethylidene)-hydrazinecarbothioamide demonstrated that the inhibitory effects of the compounds on the tyrosinase were irreversible. Preliminary structure activity relationships' (SARs) analysis suggested that further development of such compounds might be of interest.

1. Introduction

Tyrosinase (monophenol or *o*-diphenol, oxygen oxidoreductase, EC 1.14.18.1, syn. polyphenol oxidase), also known as polyphenol oxidase (PPO), is a copper-containing monooxygenase that is widely distributed in microorganisms, animals, and plants (Song et al., 2006). Tyrosinase catalyses by involving molecular oxygen in two distinct reactions: in the hydroxylation of monophenols too-diphenols (monophenolase) and in the oxidation of *o*-diphenols to *o*-quinones (diphenolase) (Chen, Liu, & Huang, 2003). Due to the high reactivity, quinines could polymerise spontaneously to form high molecular weight brown-pigments (melanins) or react with amino acids and proteins to enhance brown colour of the pigment produced (Matsuura, Ukeda, & Sawamura, 2006). Hyperpigmentations, such as *senile lentigo*, melasma, freckles, and pigmented acne scars are of particular concern to women. The treatment usually involves the use of medicines or medicinal cosmetics containing depigmenting agents or skin whitening agents (Tripathi et al., 1992). In clinical usage, tyrosinase inhibitors are used for treatments of dermatological disorders related to melanin hyperaccumulation and are essential in cosmetics for depigmentation (Schallreuter et al., 2009; Wood et al., 2009). For example, age spots and freckle were caused by the accumulation of an excessive level of epidermal pigmentation (Thanigaimalai et al., 2010).

Previous reports confirmed that tyrosinase was one of the main causes of most fruits and vegetables quality loss during post harvest handling and processing, leading to faster degradation and shorter shelf life (Yi et al., 2010). Tyrosinase has also been linked to Parkinson's and other neurodegenerative diseases (Zhu et al., 2011). In insects, tyrosinase is uniquely associated with three different biochemical processes, including sclerotisation of cuticle, defensive encapsulation and melanisation of foreign organism, and wound healing (Ashida & Brey, 1995). These processes provide potential targets for developing safer and effective tyrosinase inhibitors as insecticides and ultimately for insect control. Thus, the development of safe and effective tyrosinase inhibitors is of great concern in the medical, agricultural, and cosmetic industries. However, only a few such as kojic acid, arbutin, tropolone, and 1-phenyl-2-thiourea (PTU) (Fig. 1) are used as therapeutic agents and cosmetic products (Battaini, Monzani, Casella, Santagostini, & Pagliarin, 2000).

Coumarin derivatives are an important class of compounds, widely present in plants, including edible vegetables and fruits (Curini, Cravotto, Epifano, & Giannone, 2006; Rai et al., 2010). Coumarin derivatives are of great interest due to their diverse structural features and versatile biological properties, such as anti-inflammatory, antioxidant, vasorelaxant, cytotoxic, anti-HIV, antitubercular and antimicrobial (Belluti et al., 2010; Chimenti, Bizzarri, Bolasco, & Secci, 2010; Kostova, 2006; Neyts et al., 2009; Ostrov et al., 2007; Roussaki, Kontogiorgis, Hadjipavlou-Litina, Hamilakis, et al., 2010; Upadhyay & Mishra, 2010). In particular, their antibacterial, antifungal and anticancer activities make the compounds attractive for further derivatisation and screening as novel therapeutic agents (Khode, Maddi, Aragade, Palkar, & Ronad, 2009). The literature survey revealed that compounds with thiourea moieties have been reported to demonstrate a wide range of pharmacological activities, which include antibacterial, antifungal, anticonvulsant and tyrosinase inhibitory activity. Such as phenylthioureas, alkylthioureas and 1,3-bis-(5-methanesulfonylbu-

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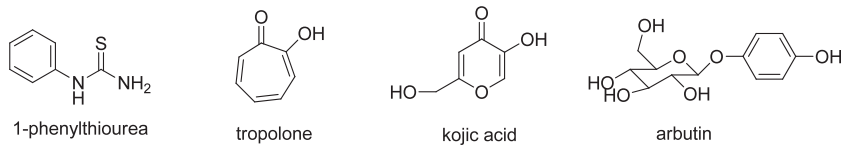


Fig. 1. Chemical structure of known tyrosinase inhibitors.

tyl)thiourea, displayed weak or moderate tyrosinase inhibitory activity (Ley & Bertram, 2001). More recently, our investigations also demonstrated that thiosemicarbazide derivatives exhibited potent inhibitory activities against mushroom tyrosinase (Liu, Yi, Wan, Ma, & Song, 2008). During recent years, extensive studies on the pharmacology of coumarin derivatives have been reported, but the tyrosinase inhibitor activities of this kind of compounds have hardly ever appeared in the literature. Stimulated by these results, in the present investigation, we synthesised a series of coumarin derivatives bearing thiosemicarbazide moieties or ester groups, their inhibitory activities against mushroom tyrosinase were evaluated using kojic acid as a comparing substance. Meanwhile, the structure–activity relationships of these compounds were also primarily discussed. The aim of the present study was the discovery of safe and efficient compounds as food additives or food preservatives, which can offer a clue to the design and synthesis of novel tyrosinase inhibitors.

2. Materials and methods

2.1. Chemical reagents and instruments

Melting points (m.p.) were determined with WRS-1B melting point apparatus and the thermometer was uncorrected. NMR spectra were recorded on Bruker 400 spectrometersat 25 °C in $CDCl_3$ or DMSO-d_6. All chemical shifts (δ) are quoted in parts per million downfield from TMS and coupling constants (*J*) are given in hertz. Abbreviations used in the splitting pattern were an follows: s = singlet, d = doublet, t = triplet, q = quintet, m = multiplet, LC–MS spectra were recorded using the LCMS-2010A. All reactions were monitored by TLC (Merck Kieselgel 60 F254) and the spots were visualised under UV light. Infrared (IR) spectra were recorded as potassium bromide pellets on VECTOR 22 spectrometer.

Tyrosinase, L-3, 4-dihydroxyphenylalanine (L-DOPA) and kojic acid were purchased from Sigma–Aldrich Chemical Co. Other chemicals were purchased from commercial suppliers and were dried and purified when necessary.

2.2. General procedures for the synthesis of substituted 3-acetylcoumarin

2.2.1. General

Piperidine (5 mol%) was added to the mixture of substituted salicylaldehyde (1 mmol) and ethylacetoacetate (1.1 mmol) in dry CH_3CN (10 ml), then the reaction mixture was stirred for about 4 h at the room temperature. The progress of the reaction was monitored by TLC. After completion of the reaction, formed precipitate was collected by filtration and washed with cold CH_3CN, then dried under vacuum to provide the substituted 3-acetylcoumarin.

2.2.2. 3-Acetylcoumarin (**1**)

Yield 76%. Yellow solid, mp 120.7–122.1 °C. IR (KBr): 3078, 3026, 1723, 1675, 1598, 1556, 1441, 1406, 1351, 1297, 1220, 1198, 1162, 1098, 967, 762 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 8.49 (s, 1H, C═CH), 7.65–7.63 (m, 2H, Ph-H), 7.37–7.32 (m, 2H, Ph-H), 2.71 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.41 (1C), 159.17 (1C), 155.27 (1C), 147.39 (1C), 134.33 (1C), 130.17 (1C), 124.92 (1C), 124.48 (1C), 118.20 (1C), 116.63 (1C), 30.49 (1C).

2.2.3. 6-Chloro-3-acetylcoumarin (**2**)

Yield 89.87%. Yellow solid, mp 213.1–213.9 °C. IR (KBr): 2946, 1726, 1611, 1469, 1412, 1355, 1278, 1134, 1082, 970, 829, 765 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 8.40(s, 1H, C═CH), 7.63 (dd, H, *J* = 8.5 Hz, 2.5 Hz, Ph-H), 7.60 (d, 1H, *J* = 2.5 Hz, Ph-H), 7.33 (d, 1H, *J* = 8.5 Hz, Ph-H), 2.72 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.41 (1C), 158.57 (1C), 152.23 (1C), 145.39 (1C), 141.08 (1C), 132.12 (1C), 128.65 (1C), 127.56 (1C), 126.94 (1C), 118.02 (1C), 16.14 (1C).

2.2.4. 6-Bromo-3-acetylcoumarin (**3**)

Yield 94.10%. Yellow solid, mp 229.1–229.9 °C. IR (KBr): 3039, 1732, 1675, 1608, 1550, 1415, 1351, 1233, 1201, 1063, 980, 832, 768 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$): δ 8.40 (s, 1H, C═CH), 7.78 (dd, H, *J* = 8.5 Hz, 2.5 Hz, Ph-H), 7.74 (d, 1H, *J* = 2.5 Hz, Ph-H), 7.27 (d, 1H, *J* = 8.5 Hz, Ph-H), 2.72 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 195.41 (1C), 158.62 (1C), 152.71 (1C), 145.32 (1C), 140.77 (1C), 134.69 (1C), 131.12 (1C), 126.65 (1C), 120.88 (1C), 118.67 (1C), 116.18 (1C), 16.16 (1C).

2.3. The general procedure for the synthesis of substituted 2-(1-(coumarin-3-yl) ethylidene)hydrazinecarbothioamide compound

2.3.1. General

The appropriate substituted 3-acetylcoumarin (10 mmol) was dissolved in anhydrous ethanol (20 ml), thiosemicarbazide (10 mmol) and acetic acid (0.5 ml) were added to the above system. The reaction mixture was refluxed for 6 h. The reaction was monitored by TLC. After completion of the reaction, the reaction mixture was cooled to room temperature. The appearing precipitate was filtered and recrystallised from 95% alcohol to obtain the corresponding substituted 2-(1-(coumarin-3-yl) ethylidene)hydrazinecarbothioamide compound.

2.3.2. 2-(1-(Coumarin-3-yl)ethylidene)hydrazinecarbothioamide (**4**)

Yield 85.38%. Yellow solid, mp 214.2–216.7 °C. IR (KBr): 3385, 3235, 3155, 1716, 1598, 1499, 1428, 1367, 1290, 1236, 1111, 1069, 970, 861, 765 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 10.61 (s, 1H, NH), 8.46 (s, 1H, C═CH), 8.39 (s, 1H, NH_2), 7.94 (s, 1H, NH_2), 7.76 (d, *J* = 8.5 Hz, H, Ph-H), 7.65 (t, 1H, *J* = 8.5 Hz, Ph-H), 7.44 (d, 1H, *J* = 8.5 Hz, Ph-H), 7.38 (t, 1H, *J* = 8.5 Hz, Ph-H), 2.38 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.72 (1C), 159.56 (1C), 154.07 (1C), 146.39 (1C), 142.55 (1C), 132.83 (1C), 129.56 (1C), 126.25 (1C), 125.20 (1C), 119.39 (1C), 116.41 (1C), 16.47 (1C).

2.3.3. 2-(1-(6-Chlorocoumarin-3-yl)ethylidene)hydrazinecarbothioamide (**5**)

Yield 87.68%. Yellow solid, mp 232.0–232.9 °C. IR (KBr): 3417, 3235, 3138, 1732, 1707, 1588, 1502, 1479, 1451, 1428, 1287, 1233, 1204, 1130, 1092, 954, 925, 871, 832, 772 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 10.49 (s, 1H, NH), 8.44 (s, 1H, NH_2), 8.41 (s, 1H, C═CH), 8.44 (s, 1H, NH_2), 7.82 (s, H, Ph-H), 7.68 (dd, 1H, *J* = 8.5 Hz, 2.5 Hz, Ph-H), 7.47 (d, 1H, *J* = 8.5 Hz, Ph-H), 2.45 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.31 (1C), 158.64 (1C),

151.96 (1C), 145.37 (1C), 140.63 (1C), 131.79 (1C), 128.35 (1C), 127.63 (1C), 126.82 (1C), 120.40 (1C), 117.97 (1C), 15.87 (1C).

2.3.4. 2-(1-(6-Bromocoumarin-3-yl)ethylidene) hydrazinecarbothioamide (**6**)

Yield 89.15%. Yellow solid, mp 231.9–232.9 °C. IR (KBr): 3404, 3222, 3135, 1720, 1588, 1495, 1467, 1422, 1236, 1233, 1204, 1101, 1060, 957, 861, 826, 775 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 10.60 (s, 1H, NH), 8.44 (s, 1H, NH_2), 8.40 (s, 1H, C═CH), 7.90 (s, 1H, NH_2), 7.80 (s, H, Ph-H), 7.68 (dd, 1H, *J* = 8.5 Hz, 2.5 Hz, Ph-H), 7.41 (d, 1H, *J* = 8.5 Hz, Ph-H), 2.24 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.30 (1C), 158.58 (1C), 152.63 (1C), 145.32 (1C), 140.71 (1C), 134.53 (1C), 130.93 (1C), 126.73 (1C), 120.80 (1C), 118.35 (1C), 116.14 (1C), 15.86 (1C).

2.4. The general procedures for the synthesis of substituted coumarin-3-carboxylic acid ester compounds

2.4.1. General

The substituted coumarin-3-carboxylic acid ethyl esters (Scheme 2) were prepared by Knoevenagel reaction. To a mixture of diethyl malonate (1 mmol) and the appropriate salicylaldehyde (1 mmol) in ethanol (10 ml) was added piperidine (5 mol %) and the reaction mixture was stirred at the room temperature. The progress of the reaction was monitored by TLC. After completion of the reaction, the solvent was removed under vacuum and the residue was purified by chromatography. The obtained substituted coumarin-3-carboxylic acid ethyl ester (2 mmol) was dissolved in 10% NaOH (50 ml), then 3 N HCl (50 ml) was added the mixture. The suspension was filtered and dried under vacuum to provide substituted coumarin-3-carboxylic acid. The coumarin-3-carboxylic acid (2 mmol) was added to thionyl chloride (30 ml), the reaction mixture was refluxed for about 2 h, the thionyl chloride was removed under vacuum. The desired substituted coumarin-3-carbonyl chloride was obtained. To a mixture of substituted coumarin-3-carbonyl chloride (2 mmol) and toluene (20 ml) or ethyl ether (20 ml) was added dropwise appropriate alcohol (2 mmol) and then the reaction mixture was refluxed for about 12 h. The solvent was removed under vacuum and the residue was purified by chromatography.

2.4.2. Ethyl coumarin-3-carboxylate (**7**)

Yield 85.06%. Yellow solid, mp 93.1–96.2 °C. IR (KBr): 3055, 2972, 1764, 1697, 1608, 1559, 1479, 1447, 1371, 1297, 1242, 1204, 1130, 1028, 983, 916, 797 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.52 (s, 1H, C═CH), 7.66–7.60 (m, 2H, Ph-H), 7.37–7.30 (m, 2H, Ph-H), 4.44 (q, *J* = 7.2 Hz, 2H, CH_2), 1.43 (t, *J* = 7.2 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.97 (1C), 157.94 (1C), 155.08 (1C), 148.50 (1C), 134.30 (1C), 129.46 (1C), 124.80 (1C), 118.28 (1C), 117.82 (1C), 116.71 (1C), 61.91 (1C), 14.15 (1C).

2.4.3. Ethyl 6-chlorocoumarin-3-carboxylate (**8**)

Yield 88.00%. White solid, mp 173.7–176.5 °C. IR (KBr): 3068, 2972, 1752, 1697, 1614, 1556, 1470, 1364, 1287, 1239, 1207, 1021, 996, 839, 791 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.43 (s, 1H, C═CH), 7.59 (s, 1H, Ph-H), 7.57 (d, *J* = 8.4 Hz, 1H, Ph-H), 7.32 (d, *J* = 8.4 Hz, 1H, Ph-H), 4.44 (q, *J* = 7.2 Hz, 2H, CH_2), 1.42 (t, *J* = 7.2 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.62 (1C), 156.00 (1C), 153.44 (1C), 147.08 (1C), 134.11 (1C), 130.10 (1C), 128.41 (1C),119.50 (1C), 118.79 (1C), 118.23 (1C), 62.18 (1C), 14.16 (1C).

2.4.4. Ethyl 6-bromocoumarin-3-carboxylate (**9**)

Yield 67.45%. White solid, mp 171.0–172.3 °C. IR (KBr): 3068, 2972, 1752, 1710, 1614, 1595, 1550, 1467, 1409, 1364, 1284, 1236, 1207, 1018, 989, 858, 791 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.43 (s, 1H, C═CH), 7.76–7.71 (m, 2H, Ph-H), 7.27 (d, *J* = 8.8 Hz, 1H, Ph-H), 4.45 (q, *J* = 7.2 Hz, 2H, CH_2), 1.43 (t, *J* = 7.2 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.59 (1C), 155.89 (1C), 153.92 (1C), 146.94 (1C), 136.88 (1C), 131.49 (1C), 119.49 (1C), 119.32 (1C), 118.50 (1C), 117.30 (1C), 62.18 (1C), 14.17 (1C).

2.4.5. Ethyl 7-hydroxycoumarin-3-carboxylate (**10**)

Yield 51.04%. Yellow solid, mp 177.5–179.9 °C. IR (KBr): 3462, 2991, 1745, 1678, 1614, 1444, 1380, 1297, 1242, 1217, 1140, 1034, 970, 842, 797 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 11.06 (s, 1H, OH), 8.51 (s, 1H, C═CH), 8.08 (d, *J* = 8.8 Hz, 1H, Ph-H), 6.87–6.84 (m, 2H, Ph-H), 4.43 (t, *J* = 7.2 Hz, 2H, CH_2), 1.42 (t, *J* = 7.2 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.81 (1C), 156.00 (1C), 153.62 (1C), 147.83 (1C), 134.30 (1C), 129.55 (1C), 128.94 (1C), 119.63 (1C), 119.27 (1C), 118.65 (1C), 61.87 (1C), 14.51 (1C).

2.4.6. Coumarin-3-carboxylic acid (**11**)

Yield 69.76%. White solid, mp 203.6–204.3 °C. IR (KBr): 3052, 1745, 1691, 1614, 1489, 1431, 1371, 1223, 1207, 1037, 983, 826, 800, 768 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 12.32 (s, 1H, COOH), 9.00 (s, 1H, C═CH), 7.82–7.76 (m, 2H, Ph-H), 7.51–7.47 (m, 2H, Ph-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.09 (1C), 162.39 (1C), 154.56 (1C), 151.48 (1C), 135.76 (1C), 130.49 (1C), 126.25 (1C), 118.45 (1C), 117.20 (1C), 114.89 (1C).

2.4.7. 6-Chlorocoumarin-3-carboxylic acid (**12**)

Yield 93.18%. White solid, mp > 300 °C. IR (KBr): 3110, 3046, 1755, 1726, 1617, 1582, 1479, 1409, 1374, 1342, 1249, 1210, 1150, 1079, 967, 880, 791 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 13.41 (s, 1H, COOH), 8.70 (s, 1H, C═CH), 8.05 (s, 1H, Ph-H), 7.78–7.75 (m, 1H, Ph-H),7.50 (d, *J* = 9.2 Hz, 1H, Ph-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.71 (1C), 164.52 (1C), 158.09 (1C), 146.88 (1C), 134.18 (1C), 132.57 (1C), 116.47 (1C), 113.25 (1C), 111.48 (1C), 102.19 (1C).

2.4.8. 6-Bromocoumarin-3-carboxylic acid (**13**)

Yield 67.45%. White solid, mp 208.3–210.3 °C. IR (KBr): 3436, 3049, 1764, 1681, 1611, 1553, 1476, 1415, 1364, 1242, 1204, 1140, 1066, 964, 874, 804 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 13.41 (s, 1H, COOH), 8.70 (s, 1H, C═CH), 8.05 (s, 1H, Ph-H), 7.78–7.75 (m, 1H, Ph-H),7.50 (d, *J* = 9.2 Hz, 1H, Ph-H);^{13}C NMR (100 MHz, DMSO-d_6) δ 164.71 (1C), 164.52 (1C), 158.09 (1C), 146.88 (1C), 134.18 (1C), 132.57 (1C), 116.47 (1C), 113.25 (1C), 111.48 (1C), 102.19 (1C).

2.4.9. 7-Hydroxycoumarin-3-carboxylic acid (**14**)

Yield 93.58%. Yellow solid, mp 263.4–265.3 °C. IR (KBr): 3158, 2834, 1726, 1710, 1611, 1572, 1499, 1454, 1396, 1367, 1322, 1278, 1220, 1137, 1028, 993, 852, 794 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 12.79 (s, 1H, COOH),11.22 (s, 1H, OH), 8.69 (s, 1H, C═CH), 7.76 (d, *J* = 8.8 Hz, 1H, Ph-H), 6.88 (dd, *J* = 8.8 Hz, 1H, Ph-H), 6.77 (d, *J* = 8.8 Hz, 1H, Ph-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 164.69 (1C), 164.48 (1C), 158.08 (1C), 157.46 (1C), 149.88 (1C), 132.49 (1C), 114.53 (1C), 112.95 (1C), 111.08 (1C), 102.09 (1C).

2.4.10. Methyl coumarin-3-carboxylate (**15**)

Yield 89.65%. White solid, mp 156.8–158.7 °C. IR (KBr): 3046, 2946, 1758, 1694, 1614, 1559, 1470, 1431, 1355, 1300, 1242, 1204, 1085, 1005, 832, 794 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.47 (s, 1H, C═CH), 7.61–7.58 (m, 2H, Ph-H), 7.33–7.26 (m, 2H, Ph-H), 3.96 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.25 (1C), 155.8 (1C), 147.69 (1C), 147.63 (1C), 134.29 (1C), 134.26 (1C), 130.19 (1C), 128.49 (1C),118.75 (1C), 118.26 (1C), 53.04 (1C).

*2.4.11. Methyl 6-bromocoumarin-3-carboxylate (**16**)*

Yield 86.50%. Yellow solid, mp 176.8–178.9 °C. IR (KBr): 3039, 2972, 1748, 1697, 1617, 1588, 1556, 1473, 1435, 1377, 1287, 1236, 1204, 999, 880, 816, 794 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.47 (s, 1H, C═CH), 7.76–7.20 (m, 2H, Ph-H), 7.27 (d, *J* = 8.8 Hz, 1H, Ph-H), 3.97 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.26 (1C), 155.91 (1C), 154.00 (1C), 147.50 (1C), 137.06 (1C), 131.56 (1C), 119.29 (1C), 119.13 (1C), 118.54 (1C), 117.40 (1C), 53.06 (1C).

*2.4.12. Methyl 7-hydroxycoumarin-3-carboxylate (**17**)*

Yield 93.58%. White solid, mp 116.2–117.2 °C. IR (KBr): 3055, 2956, 1745, 1694, 1614, 1563, 1451, 1361, 1313, 1268, 1246, 1217, 1153, 996, 919, 797 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 9.96 (s, 1H, OH), 8.55 (s, 1H, C═CH), 7.66–7.60 (m, 2H, Ph-H), 7.36–7.32 (m, H, Ph-H), 3.95 (s, 3H, CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 163.69 (1C), 156.64 (1C), 155.23 (1C), 149.04 (1C), 134.42 (1C), 129.52 (1C), 124.85 (1C), 118.00 (1C), 117.85 (1C), 116.78 (1C), 52.86 (1C).

*2.4.13. Pentyl coumarin-3-carboxylate (**18**)*

Yield 53.28%. Yellow solid, mp 133.4–134.5 °C. IR (KBr): 3055, 2956, 2927, 1764, 1691, 1620, 1563, 1470, 1415, 1383, 1351, 1294, 1246, 1207, 1085, 989, 829, 788 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 8.71 (s, 1H, C═CH), 8.08 (d, *J* = 8.8 Hz, 1H, Ph-H), 7.79–7.58 (m, 2H, Ph-H), 7.50 (d, *J* = 8.8 Hz, 1H, Ph-H), 4.72 (t, *J* = 6.8 Hz, 2H, CH_2), 2.52–2,50 (m, 2H, CH_2), 1.73–1.66 (m, 2H, CH_2), 1.37–1.31 (m, 2H, CH_2), 0.92 (t, *J* = 6.8 Hz, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.95 (1C), 156.05 (1C), 153.65 (1C), 147.81 (1C), 134.31 (1C), 129.61 (1C), 128.93 (1C), 119.67 (1C), 119.37 (1C), 118.69 (1C), 65.81 (1C), 28.25 (1C), 27.95 (1C), 22.24 (1C), 14.31 (1C).

*2.4.14. Isopropyl 6-chlorocoumarin-3-carboxylate (**19**)*

Yield 67.45%. Yellow solid, mp 82.1–82.6 °C. IR (KBr): 3046, 2982, 2959, 1748, 1604, 1563, 1473, 1457, 1374, 1297, 1249, 1210, 1137, 1101, 1005, 922, 864, 797 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.38 (s, 1H, C═CH), 7.60–7.57 (m, 2H, Ph-H), 7.32 (d, *J* = 8.8 Hz, 1H, Ph-H), 5.30–5.24 (m, 1H, CH), 1.40 (d, *J* = 6.4 Hz, 6H, $2CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.00 (1C), 155.97 (1C), 153.41 (1C), 146.56 (1C), 134.09 (1C), 130.08 (1C), 128.47 (1C), 120.00 (1C), 118.91 (1C), 118.23 (1C), 69.99 (1C), 21.76 (2C).

*2.4.15. Isopropyl 6-bromocoumarin-3-carboxylate (**20**)*

Yield 69.75%. White solid, mp 217.3–218.8 °C. IR (KBr): 3065, 2972, 1745, 1710, 1611, 1563, 1473, 1409, 1367, 1290, 1246, 1207, 1101, 1005, 970, 877, 829, 794 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.37 (s, 1H, C═CH), 7.75–7.70 (m, 2H, Ph-H), 7.27 (d, *J* = 8.8 Hz, 1H, Ph-H), 5.30–5.24 (m, 1H, CH), 1.40 (d, *J* = 6.4 Hz, 6H, $2CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 161.99 (1C), 155.93 (1C), 153.90 (1C), 146.41 (1C), 136.76 (1C), 131.45 (1C), 119.95 (1C), 119.38 (1C), 118.25 (1C), 117.30 (1C), 70.02 (1C), 21.78 (2C).

*2.4.16. Isopropyl 7-hydroxycoumarin-3-carboxylate (**21**)*

Yield 61.36%. White solid, mp 204.7–205.9 °C. IR (KBr): 3062, 2975, 1742, 1704, 1620, 1563, 1473, 1419, 1364, 1290, 1246, 1207, 1105, 1009, 967, 832, 791 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 9.98 (s, 1H, OH), 8.44 (s, 1H, C═CH), 7.64–7.59 (m, 2H, Ph-H), 7.34–7.30 (m, 1H, Ph-H), 5.28–5.23 (m, 1H, CH), 1.39 (d, *J* = 6.5 Hz, 6H, $2CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.34 (1C), 156.70 (1C), 155.05 (1C), 148.03 (1C), 134.21 (1C), 129.48 (1C), 124.82 (1C), 118.66 (1C), 117.87 (1C), 116.69 (1C), 69.63 (1C), 21.80 (2C).

*2.4.17. Pentyl 7-hydroxycoumarin-3-carboxylate (**22**)*

Yield 56.18%. Yellow solid, mp 53.2–55.0 °C. IR (KBr): 3046, 2949, 1764, 1723, 1604, 1447, 1367, 1294, 1242, 1207, 1124, 1009, 919, 861, 797 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 9.87 (s, 1H, OH), 8.49 (s, 1H, C═CH), 7.64–7.59 (m, 2H, Ph-H), 7.35–7.30 (m, 1H, Ph-H), 4.43 (t, *J* = 6.5 Hz, 2H, CH_2), 1.80–1.74 (m, 2H, CH_2), 1.41–1.36 (m, 4H, $2CH_2$), 0.93 (t, *J* = 7.0 Hz, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.15 (1C), 156.59 (1C), 155.18 (1C), 148.33 (1C), 134.23 (1C), 129.46 (1C), 124.77 (1C), 118.51 (1C), 117.90 (1C), 116.76 (1C), 66.08 (1C), 28.26 (1C), 28.01 (1C), 22.43 (1C), 13.96 (1C).

2.5. Tyrosinase assay

The spectrophotometric assay for tyrosinase was performed according to the method reported by our groups with some slight modifications (Liu et al., 2008; Yi et al., 2010). Briefly, all the synthesised compounds were screened for the diphenolase inhibitory activity of tyrosinase using L-DOPA as substrate. All the compounds were dissolved in DMSO. The final concentration of DMSO in the test solution was 2.0%. Phosphate buffer, pH 6.8, was used to dilute the DMSO stock solution of test compounds. Thirty units of mushroom tyrosinase (0.5 mg/ml) were first pre-incubated with the compounds, in 50 mM phosphate buffer (pH 6.8), for 10 min at 25 °C. Then the L-DOPA (0.5 mM) was added to the reaction mixture and the enzyme reaction was monitored by measuring the change in absorbance at 475 nm of formation of the DOPA chrome for 1 min. The measurement was performed in triplicate for each concentration and averaged before further calculation. IC50 value, a concentration giving 50% inhibition of tyrosinase activity, was determined by interpolation of the dose–response curves. The percent of inhibition of tyrosinase reaction was calculated as following:

$$\text{Inhibition rate } (\%) = [(B-S)/B] \times 100$$

Here, the B and S are the absorbances for the blank and samples. Kojic acid was used as reference standard inhibitors for comparison.

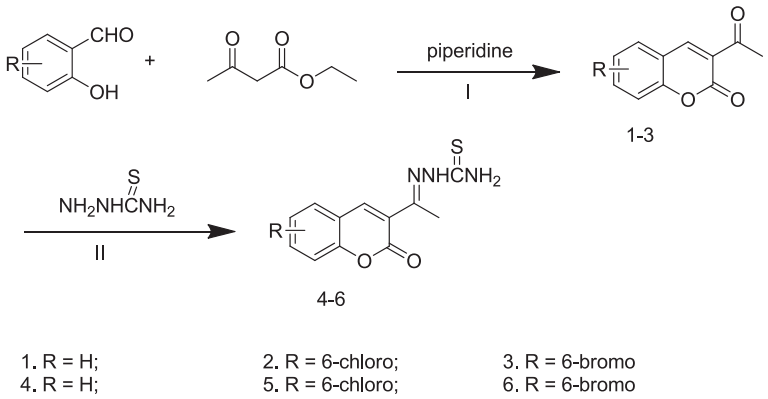
3. Results and discussion

3.1. Synthesis

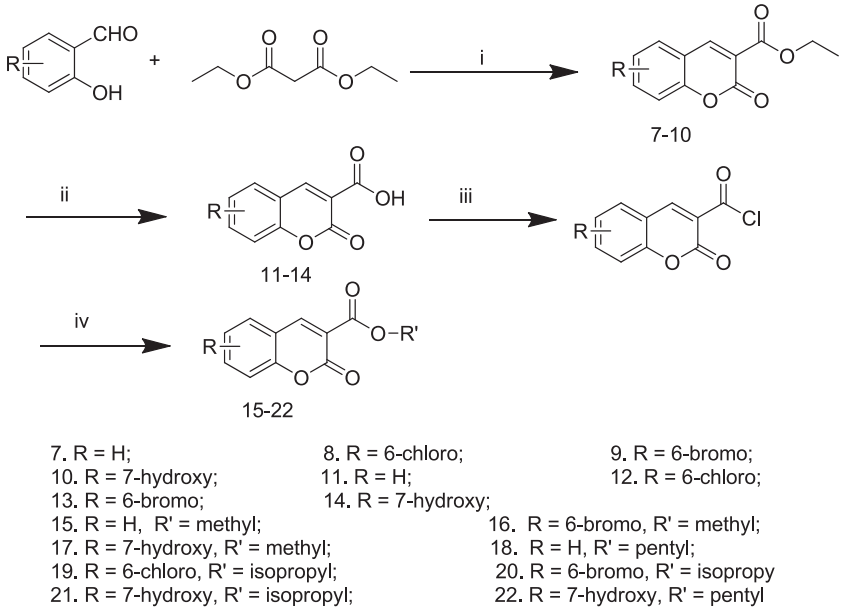
According to the general procedure shown in Scheme 1, substituted coumarin was synthesised by Knoevenagel condensation. In presence of piperidine as catalyst, the reaction of substituted salicylaldehyde and ethyl acetoacetate was carried out at room temperature. In present of acetic acid as catalyst, the obtained substituted coumarin was further converted into it is thiosemicarbazide derivative. The yields of these compounds were from moderate to good (Chimenti et al., 2010; Chimenti, Bizzarri, Bolasco, Secci, & Chimenti, 2007; Liu et al., 2008; Wanare et al., 2010). According to the general procedure shown in Scheme 2, the substituted coumarin-3-carboxylate ethyl ester was obtained from substituted salicylaldehyde and diethyl malonate by the Knoevenagel condensation. The substituted coumarin-3-carboxylate ethyl ester was further converted into the target compound by the hydrolysis reaction, chlorination reaction, esterification reaction in turn (Chimenti et al., 2009; Khode et al., 2009).

3.2. Tyrosinase inhibitory activity

For evaluating the tyrosinase inhibitory activity, all the synthesised compounds were subjected to tyrosinase inhibition assay with L-DOPA as substrate, according to the method reported by our groups with some slight modifications. The tyrosinase inhibitory activities of kojic acid was ever reported, therefore, it was selected as comparing substance. The IC_{50} values of coumarin derivatives



Scheme 1. Synthesis of coumarin compounds bearing thiosemicarbazide moieties. Reagents and conditions: (I) piperidine, acetonitrile, RT, 1–4 h. (II) Acetic acid, ethanol, reflux, 6 h.



Scheme 2. Synthesis of the substituted 2-oxo-2H-coumarin-3-carboxylate esters. Reagents and conditions: (i) piperidine, ethanol, RT. (ii) 10% NaOH/3 N HCl. (iii) Thionyl chloride, reflux. (iv) Appropriate alcohol/toluene or ethyl ether, reflux.

against tyrosinase were summarised in Table 1, and IC_{50} values of all these compounds were determined from logarithmic concentration–inhibition curves and given as means of three experiments.

Our results showed that compounds **3**–**6** and **20** exhibited potent inhibition on mushroom tyrosinase with IC_{50} values ranged from 3.44 to 195.03 μM. Especially, compounds **4**–**6** bearing thiosemicarbazide showed more potent inhibitory activities than the other compounds. In addition, compound **4** demonstrated more potent inhibitory activity than the reference standard inhibitor kojic acid. Most of the coumarin-3-carboxylic acid analogues showed low or no activities against tyrosinase. The results may be related to the structure of tyrosinase, which contained a type-3 copper centre with a coupled dinuclear copper active site in the catalytic core. Tyrosinase inhibition of compounds **4**–**6** depended on the competency of the sulfur atom to chelate with the dicopper nucleus in the active site, and tyrosinase would lose its catalysing ability after forming complex (Gerdemann, Eicken, & Krebs, 2002).

When comparing to the tyrosinase inhibitory activities of substituted 3-acetylcoumarin, compounds with halogen atom exhibited more potent inhibitory activities than the 3-acetylcoumarin, with the increase of lipophilicity of the compound, the inhibitory activities increased gradually. These results showed that the inhibitory activities of 3-acetylcoumarin and it is halogen substituted analogues ralated to the lipophilicity. However, 2-(1-(coumarin-3-yl)ethylidene) hydrazinecarbothioamide showed the most potent inhibitory activity in all their homologues, and with the increase of the radius of halogen atom, the inhibitory activities decreased gradually. In addition, the inhibitory activities of the 2-(1-(coumarin-3-yl)ethylidene)hydrazinecarbothioamide and it is homologues were more potent than the inhibitory activities of 3-acetylchroman-2-one and it is homologues. This result might be related to different inhibitory mechanism. Since the 2-(1-(coumarin-3-yl)ethylidene) hydrazinecarbothioamide and it is homologues mainly depended on chelation of the sulfur atom with the active centre of tyrosinase, the increase of the the radius of halogen atom might cause stereohindrance for the inhibitors approaching the active site of the enzyme. However, substituted 3-acetylcoumarins are similar to benzaldehyde-type inhibitors, the tyrosinase inhibitory mechanism of this type of inhibitors, come from

Table 1
Tyrosinase inhibitory activities and yields of the synthesised compounds.

Compounds	Yield (%)	CLog P[d]	IC_{50} (μM)[a]	Percent of inhibition[c]
1	76	0.9114	–	8.3%
2	89.87	1.7644	–	42.45%
3	94.10	1.9144	195.03	
4	85.38	1.5506	3.44	
5	87.68	2.4036	34.32	
6	89.15	2.5536	114.68	
7	85.06	1.91	–	NA[b]
8	88.00	2.623	–	23.2%
9	67.45	2.773	–	12.88%
10	51.04	2.17114	–	NA[b]
11	69.76	1.54175	–	43.38%
12	93.18	2.26375	–	38.57%
13	67.45	2.41375	–	29.68%
14	93.58	1.43245	–	48.4%
15	89.65	1.381	–	33.33%
16	86.50	2.244	–	NA[b]
17	93.58	1.64214	–	NA[b]
18	53.28	3.497	–	33.32%
19	67.45	2.932	–	15.62%
20	69.75	3.082	131.05	
21	61.36	2.48014	–	13.9%
22	56.18	3.75814	–	NA[b]
Kojic acid			23	

[a] Values were determined from logarithmic concentration–inhibition curves (at least eight points) and are given as means of three experiments.
[b] Not active at 200 μM concentration.
[c] Percent of inhibition of tyrosinase reaction at the 200 μM.
[d] Value of CLog P was obtained by ChemBioDraw Ultra 12.0.

the ability to form a Schiff base with a primary amino group in the enzyme (Kubo et al., 1999). The increase of lipophilicity might be benefited to the formation of Schiff base.

As shown in Table 1, coumarin-3-carboxylic acid and all of the substituted coumarin-3-carboxylic acids exhibited certain inhibitory activities against tyrosinase at the concentration of 200 μM. However some of the coumarin-3-carboxylic acid esters and the substituted coumarin-3-carboxylic acid esters lost their inhibitory activities against tyrosinase. The results indicated that the carboxyl group might be effective group to the interaction of compound with the active site of tyrosinase. From Table 1, comparing the ethyl esters of coumarin-3-carboxylic acid and their substituted analogues, only the ethyl esters of coumarin-3-carboxylic acid with halogen atom showed weak inhibitory activities at the concentration of 200 μM. Among the methyl esters of coumarin-3-carboxylic acid, only the methyl coumarin-3-carboxylate exhibited inhibitory activity at the concentration of 200 μM. The isopropyl ester of coumarin-3-carboxylic acid and it is substituted analogues had certain inhibitory activity against tyrosinse, especially, isopropyl 6-bromocoumarin-3-carboxylate with the IC_{50} value of 131.05 μM showed the most potent activity than the other ester compounds. For the pentyl ester compounds, the inhibitory percent of pentyl coumarin-3-carboxylate is 33.32% at the concentration of 200 μM, and pentyl 7-hydroxycoumarin-3-carboxylate exhibited no inhibitory activity at the same concentration. These results suggested that the ester group structure might be an important factor for the improvement of inhibitory activity.

3.3. Inhibitory mechanism

The inhibitory mechanisms of the selected compounds **4**, **5** on mushroom tyrosinase for the oxidation of L-DOPA were determined. Figs. 2 and 3 showed the relationship between enzyme activity and concentration in the presence of different concentrations of the above-mentioned compounds. The results showed that the plots of V versus [E] gave a family of parallel straight lines with the same slopes. These results demonstrated that the inhibitory effects of compound **4**, **5** on the tyrosinase were irreversible, suggesting that substituted 2-(1-(coumarin-3-yl)ethylidene)-hydrazinecarbothioamide compounds effectively inhibited the enzyme by binding to its binuclear active site irreversibly. The result may be related to the structure of tyrosinase, within the structure, there are two copper ions in the active centre of tyrosinase and a lipophilic long-narrow gorge near to the active centre. Compound **4** or **5** could exhibit strong affinity for copper ions in the active centre and form a reversible non-covalent complex with the tyrosinase, and this then reacts to produce the covalently modified "dead-end complex" (Tsou, 1988).

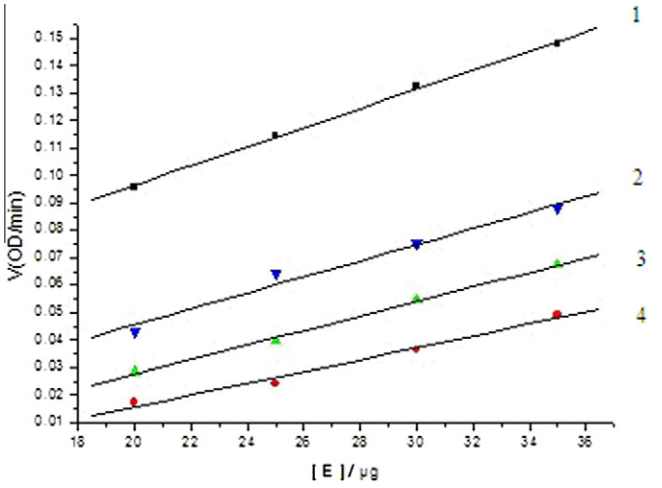


Fig. 2. The effect of concentrations of tyrosinase on its activity for the catalysis of L-DOPA at different concentration of compound **4**. The concentrations of compound **4** for curves 1–4 are 0, 25, 50 and 100 μmol/l, respectively.

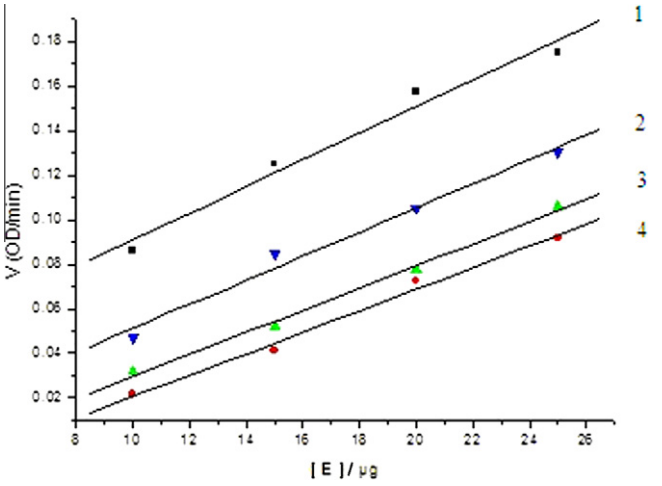


Fig. 3. The effect of concentrations of tyrosinase on its activity for the catalysis of L-DOPA at different concentration of compound **5**. The concentrations of compound **5** for curves 1–4 are 0, 25, 50 and 100 μmol/l, respectively.

The present investigation reported that coumarin derivatives had potent inhibitory effects on the diphenolase activity of mushroom tyrosinase. Interestingly, compound **4** was found to be the most potent inhibitor with IC_{50} value of 3.44 μM. Preliminary structure activity relationships (SARs) analysis indicated that (1) thiosemicarbzide moiety might play an important role in determining their inhibitory activities, because of the sulfur atom could chelate with the dicopper nucleus in the active site, and tyrosinase would lose its catalysing ability after forming complex; (2) carboxyl group might be effective group to the interaction of

compound with the active site of tyrosinase; (3) the ester group structure might be an important factor for the improvement of inhibitory activity. The inhibition mechanism analysis of compounds **4**, **5** demonstrated that the inhibitory effects of the two compounds on the tyrosinase were irreversible.

Acknowledgments

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