Synthesis of 4-(5-(2,3-Dimenthoxyphenyl)-3-(4-Methoxyphenyl)-4,5-Dihydro-1*H*-Pyrazol-1-y1) Benzenesulfonamide as a Promosing Tyrosinase Inhibitor Candidate

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Abstract

In this study, titled compound **5** has been successfully synthesized with 93% yield. The pyrazoline compound was obtained from the cyclocondensation reaction of 4-hydrazinylbenzenesulfonamide **3** with chalcone (*E*)-3-(2,3-dimethoxyphenyl)-1-(4-methoxyphenyl)prop-2-en-1-one **4** under basic conditions. The molecular structure was confirmed through analysis of FTIR, NMR and HRMS spectroscopic data. Furthermore, its tyrosinase enzyme inhibitory activity was determined through in vitro assay against tyrosinase of *Agaricus bisporus*. However, the pyrazoline compound **5** showed lower inhibitory activity than the positive control, kojic acid, whereas the IC_{50} value of the compound **5** is higher than that of kojic acid. The compound **5** IC_{50} value was 262.15 μM, while kojic acid IC_{50} value was 88.52 μM.

Keywords: *hyperpigmentation; melanin; pyrazoline; tyrosinase*

Introduction

Tyrosinase is a copper-containing enzyme that can be found in animals, plants, and microorganisms. Tyrosinase enzyme has an important role in the process of melanogenesis, where it is the main speed-limiting enzyme in enzymatic browning and melanin synthesis[1]. The tyrosinase enzyme also shows monophenolase and diphenolase activity, which catalyzes the hydroxylation reaction of L-tyrosine to L-DOPA and the oxidation of L-DOPA to dopaquinone, which then undergoes nonenzymatic polymerization to produce dark pigments[2],[3]. In humans, excessive tyrosinase activity causes accelerated melanin formation in the skin, thereby triggering hyperpigmentation effects such as freckles, melasma, aging, and melanoma[4]. Meanwhile, from the perspective of agriculture, excessive tyrosinase activity will reduce the commercial value of fruits and vegetables due to changes in color, taste and nutritional characteristics. Thus, a tyrosinase inhibitor is needed to overcome the problems associated with the browning reaction by the tyrosinase enzyme.

Compounds capable of inhibiting the browning activity of the enzyme tyrosinase have attracted worldwide interest from researchers focused in different fields including medicine, biology, agricultural sciences, chemistry, and pharmacology. Tyrosinase inhibitors capable of inhibiting melanin biosynthesis such as kojic

acid and hydroquinone have been used as cosmetic agents to control hyperpigmentation and freckle formation[5],[6]. However, the use of kojic acid compounds in this context has been associated with some serious side effects, including erythema and contact dermatitis[7],[8]. In addition, the use of hydroquinone compounds as whitening agents in cosmetics has been banned by several countries because they are cytotoxic and increase the risk of developing cancer[9]. Therefore, further research is needed to identify natural and biocompatible tyrosinase inhibitors that can be used as whitening agents and anti-browning agents in the cosmetic and food industries.

Many efforts have been devoted to the search for effective tyrosinase inhibitors via the synthetic pathway. Some of them are polyphenol derived compounds, such as flavanones, chalcones, resveratrol and its analogues, *N*-benzilbenzamide, and benzoate ester derivatives have been studied intensively[10]. In addition, the search for tyrosinase inhibitors from a compound containing a heterocyclic ring has also been reported, one of which is a pyrazoline derivative compound[11]. Pyrazoline is a five-ring heterocyclic compound consisting of two nitrogen atoms and three carbon atoms which is known to have various biological activities such as protein glycation inhibitor, antibacterial, antifungal, anticancer, antidepressant, anti-inflammatory, antituberculosis and antioxidant[12]. However, the number of publications reporting pyrazoline compounds as inhibitors of the tyrosinase enzyme is still limited. Thus, further research is needed on the synthesis of this pyrazoline derivative to obtain a more effective and safe tyrosinase inhibitor.

Based on the description above, we reported the synthesis of 1,3,5-triaryl pyrazoline derivatives from the reaction between an unsaturated α,β-keto compound with hydrazine containing a sulfonamide group. Furthermore, the activity of the compound was tested *in vitro* against the fungal tyrosinase enzyme.

Experimental

Materials

The materials used are chalcone (E)-3-(2,3-dimethoxyphenyl)-1-(4-methoxyphenyl)prop-2-en-1-one, sulfanilamide (SigmaAldrich), tin(II)chloride (AR Chemical), sodium nitrite (Merck), sodium hydroxide (Merck), acid chloride (SmartLab), absolute ethanol (Merck), universal pH indicator, TLC plate GF$_{254}$ (Merck), silica gel 60 (0.063-200 mm) (Merck), deionized water and organic solvents.

Instruments

The reaction was conducted in sealed-vessel reactor Monowave50 (AntonPaar, Austria). TLC was monitored under UV lamp (Cole Parmer UV Lamp 254 and 366 nm), melting point was measured using Fisher John's melting point apparatus (SMP 11-Stuart ®), purity of final compound was determined using HPLC (UFLC Prominance-Shimadzu LC Solution, UV detector SPD 20AD), bond vibration frequencies was detected using FTIR spectrophotometer (FTIR Shimadzu, IR Prestige-21), molecular weight was detected using mass spectrometer (Water LCT premiere XE positive mode), proton and carbon chemical shift was determined using NMR spectrometer (Agilent 500 MHz with DD2 console system) and a microplate reader.

Synthesis procedures

Synthesis of 4-hydrazinylbenzenesulfonamide

Mixture of sulfanilamide **1** (20 mmol) and concentrated hydrochloric acid (10 mL) was added with 20 g of crushed ice of deionized water. The mixture is then placed in an ice bath. To the mixture was added a solution of sodium nitrite (20 mmol) in 2 mL of deionized water dropwise while stirring with a magnetic stirrer. The mixture was stirred vigorously until the solution becomes clear, which indicates that the diazotation reaction has been completed. Then the azo intermediate compound is reduced by pouring tin(II)chloride (10 g) dissolved in 10 mL of hydrochloric acid into the mixture while maintaining a temperature of 0°C.

The mixture was stirred continuously until a white precipitate was formed. After completion, the precipitate was filtered using a vacuum to obtain hydrazine **3**.

Synthesis of pyrazoline compound

Chalcone (*E*)-3-(2,3-dimethoxyphenyl)-1-(4-methoxyphenyl)prop-2-en-1-one **4** (1 mmol) and 4-hydrazinylbenzenesulfonamide **3** (1 mmol) were dissolved in absolute ethanol (10 mL). Sodium hydroxide solution (3N, 50 mol%) was added to the mixture, then reacted using a sealed-vessel reactor at 80°C for 60 minutes. The reaction was monitored using TLC every 60 minutes. After the chalcone and hydrazine had completely reacted, the reaction mixture was added 10 mL of distilled water, and the pH of the mixture was neutralized by adding hydrochloric acid. The mixture is allowed to stand in the refrigerator until a maximum precipitate is formed. The precipitate was filtered using vacuum, washed with cold water and methanol, then dried in the oven to obtain target compound.

Tyrosinase inhibitory activity assay

Blank control (B0)

In a 96-well plate, 70 μL of phosphate buffer pH 6.5 was added with 30 μL of the enzyme tyrosinase 333 U/mL, then incubated at 37°C for 5 minutes. Then 110 μL of phosphate buffer pH 6.5 was added and incubated at 37°C for 30 minutes. After 30 minutes, the absorbance was measured at a wavelength of 492 nm with a microplate reader.

Blank (B1)

In a 96-well plate, 70 μL of phosphate buffer pH 6.5 was added with 30 μL of the enzyme tyrosinase 333 U/mL, then incubated at 37°C for 5 minutes. Then 110 μL L-tyrosine 2 mM was added and incubated at 37°C for 30 minutes. After 30 minutes, the absorbance was measured at a wavelength of 492 nm with a microplate reader.

Sample control (S0)

In a 96-well plate, 70 μL of sample (in phosphate buffer) was added with 30 μL of the enzyme tyrosinase 333 U/mL, then incubated at 37°C for 5 minutes. Then 110 μL of phosphate buffer pH 6.5 was added and incubated at 37°C for 30 minutes. After 30 minutes, the absorbance was measured at a wavelength of 492 nm with a microplate reader.

Sample (S1)

In a 96-well plate, 70 μL of sample (in phosphate buffer) was added with 30 μL of the enzyme tyrosinase 333 U/mL, then incubated at 37°C for 5 minutes. Then 110 μL L-tyrosine 2 mM was added and incubated at 37°C for 30 minutes. After 30 minutes, the absorbance was measured at a wavelength of 492 nm with a microplate reader. The inhibition for each enzyme assay was calculated as follows:

$$\% \text{ Inhibition} = \frac{\text{ABS}_{control} - \text{ABS}_{sample}}{\text{ABS}_{control}} \times 100$$

Each experiment was carried out in triplicate (n=3). The IC_{50} value, which is the concentration of pyrazoline and kojic acid compounds required to inhibit enzyme activity by 50%, was calculated from linear regression of ln concentration vs % inhibition graph.

Results and Disscusion

Synthesis and characterisation

Titled compound **5** was succesfully synthesized as shown in Figure 1. Firstly, hydrazine compound was synthesized using a reported method with minor modifications[13]. Preparation of hydrazine compounds using sulfanilamide **1** starting compounds, which in this sulfanilamide is an aniline derivative so that the amine group can be converted into hydrazine. The amine group is converted to hydrazine through a diazotation reaction, which forms a nitrogen atom with another nitrogen azo intermediate compound (N≡N) **2** with the help of sodium nitrite under acidic conditions. This reaction must be carried out at 0 °C to prevent decomposition[14].

Furthermore, the azo compound **2** is reduced with an appropriate reducing agent such as tin(II)chloride to form hydrazine **3** (-NH-NH2).

Afterwards, the titled pyrazoline compound was prepared using methodology has been reported with minor modifications[15],[16].
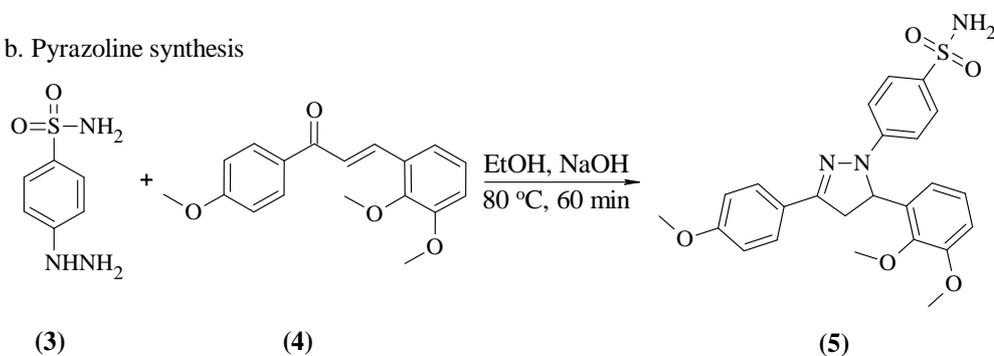
a. Hydrazine synthesis
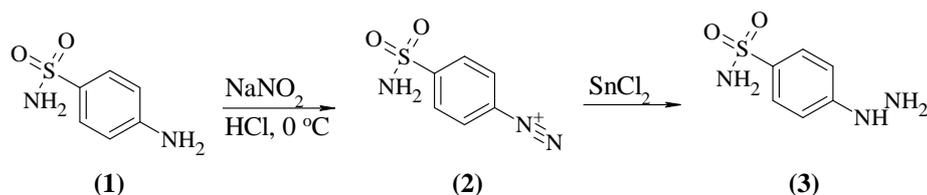
b. Pyrazoline synthesis

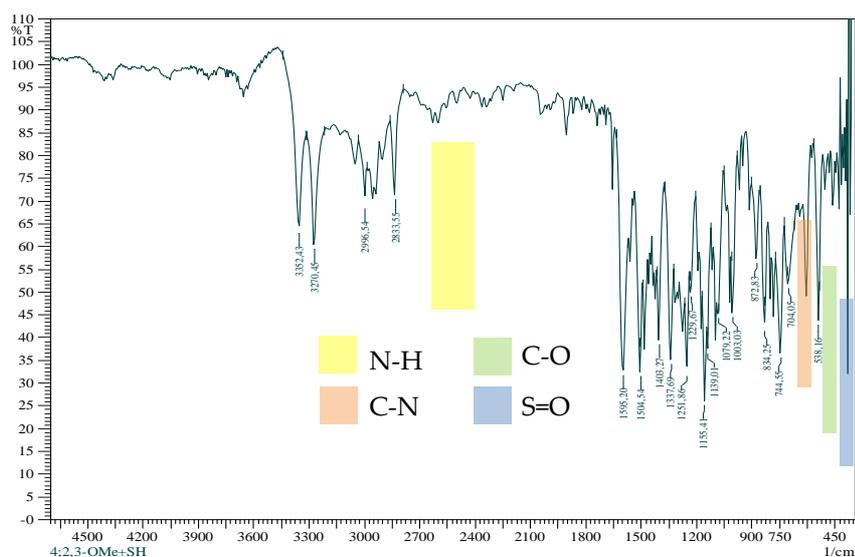**Figure 1.** Synthesis route for compound **5**.

Figure 2. Pyrazoline compound on FTIR spectrum.

Hydrazine **3** compound that has been obtained is reacted with the chalcone **4**. Synthesis of pyrazoline compounds from chalcone can be carried out in both acidic and basic conditions. In this regard, basic condition was chosen in this study because apart from helping catalyze the reaction, bases also increase the solubility of hydrazine which has a fairly low solubility. The pyrazoline compound will be formed through a cyclocondensation reaction to obtain compound **5** with 93% yield.

FTIR spectrum shows typical vibration of compound **5** (Figure 2). The peaks at wavenumbers of 3352 cm^{-1} and 3270 cm^{-1} show the stretching vibration of the NH_2 bond of the sulfonamide group. Furthermore, the absorption at a wavenumber of 1403 cm^{-1} shows the stretching vibration of the C-N bond of the pyrazoline ring. The absorption at a wavenumber of 1251 cm^{-1} shows the stretching vibration of the C-O bond of the methoxy substituent bound to the phenyl ring and at a wavenumber at 1155 cm^{-1} shows the stretching vibration of the S=O of sulfonamide group. The presence of N-H and S=O bond vibrations from the sulfonamide group and C-N bond vibrations from the pyrazoline ring and the absences of a peak around 1650 cm^{-1} which indicates the C=O bond vibration from chalcone, indicates targeted pyrazoline compound has been formed.

In the HNMR spectrum of compound **5**, the characteristic of 1,3,5-trisubstituted pyrazoline is obrserved by appearance of the ABX pattern (Figure 3). This pattern arises because of presence of stereocenter at C5 causes different chemical shifts. Signal of H_A and H_B protons were found as doublets of doublets at δ_H 3.15 ppm and δ_H 3.89 ppm, respectively. The orientation of this peak occurs because of the difference in the value of the coupling constant from geminal and vicinal protons, resulting in a breakdown of the peaks with the appropriate coupling constant values. The proton H_A appears with the value of the geminal coupling constant J_{AB} = 17.1 Hz and the vicinal coupling constant J_{AX} = 5.8 Hz. While the proton H_B has a geminal coupling constant J_{AB} = 17.1 Hz and a vicinal coupling constant J_{BX} = 12.3 Hz. Similar to H_A and H_B protons, H_X proton also appears as a doublet of doublet peaks at δ_H 5.63 ppm. The proton coupling constants H_X are both vicinal couplings, because the protons H_A and H_B are bonded to different carbons, the vicinal coupling constants are J_{BX} = 12.3 Hz and J_{AX} = 5.8 Hz.
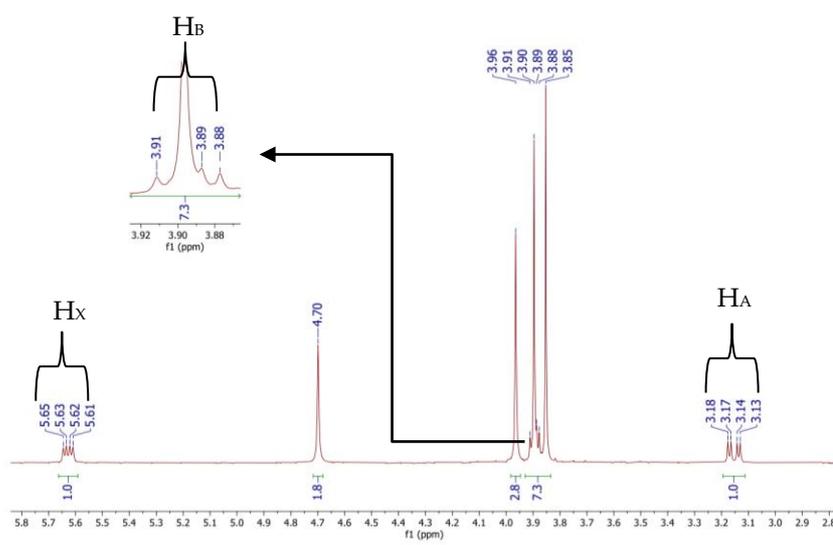

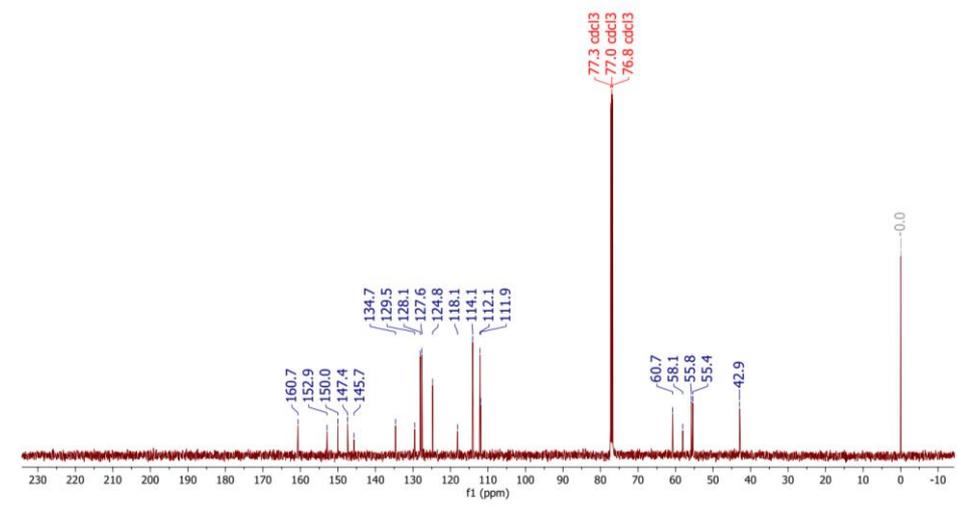**Figure 3.** Pyrazoline compound on ^{1}H NMR spectrum.

Figure 4. Pyrazoline compound on ^{13}C NMR spectrum.

The CNMR spectrum of compound **5** also shows the number of signals corresponding to the number of 24 total carbon atoms (Figure 4). The presence of a pyrazoline ring was confirmed by the presence of a signal at δ_C 42.9 ppm indicating a C4 carbon atom and singal at δ_C 55.4 ppm indicating C5 carbon. The sp^2 carbon of the pyrazoline ring appears in the large chemical shift region at δ_C 160.7 ppm chemical shift. The summary of the interpretation of the HNMR and CNMR spectra is presented in Table 1.

Furthermore, the formation of target molecule was confirmed through mass spectroscopy spectrum (Figure 5). The peaks [M+H]+ of target compound was found at m/z 468.1598 with high intensities (cal. for $C_{24}H_{26}N_3O_5S$: 468.1593). The peak observed at m/z 935.2815 is possibly a dimer that formed during the process. In addition, the difference between [M+H]+ calculated and obtained is 0.0005. It means less than 5 ppm and it was very pure. There are no fragmentations in HRMS.

Tyrosinase inhibitory activity assay

Pyrazoline **5** was evaluated its tyrosinase inhibitory activity through monophenolase activity assay. In this assay we used L-tyrosine as a substrate and kojic acid as a positive control. The principle of this assay is that the L-tyrosine substrate through an enzymatic reaction will go through an oxidation reaction to produce dopachrome which absorbs UV waves at a wavelength of 492 nm, so it can be quantified through spectrophotometric analysis[17]. A good inhibitor will inhibit the formation of dopachrome so that the absorption of the sample mixture at that wave will be of smaller value than blank mixure. By means, the higher absorption of the sample solution the higher quantity of dopachrome is formed, in other words the enzyme inhibition does not occur.

In the initial stage, the samples were screened at a concentration of 500 ppm and 250 ppm. The results of screening for compound **5** showed a fairly good percentage of inhibition at a concentration of 250 ppm (> 80%). Then compound **5** was further tested by conducting tests on several concentrations of 250, 125, 62.5, 31.25, and 15.625 ppm. Through linear regression of % inhibition vs. ln concentration curve, the IC$_{50}$ values of compound **5** were determined.

The assay results showed that the synthesized compound **5** inhibited the oxidation of L-tyrosine poorly. Its inhibitory activity was lower than that of kojic acid, where the IC_{50} values of compound **5** and kojic acid were 262.15 µM and 88.62 µM, respectively (Table 2). The difference between the IC_{50} values of compound **5** and kojic acid is almost 3 times with value over 200 µM indicate the compound is inactive[18].

Table 1. Interpretation of proton and carbon NMR spectra of compound **5**
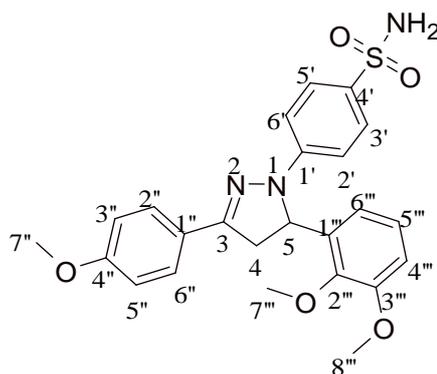
Position	δ_H (ppm) (Multiplisity, J)	δ_C (ppm)
NH₂		-
3	-	160.7
4	3.15 (dd, J = 17.1, 5.8 Hz, 1H) 3.89 (dd, J = 17.1, 12.3 Hz, 1H)	58.1
5	5.63 (dd, J = 12.3, 5.8 Hz, 1H)	60.8
1'	-	124.8
2'	7.06 (d, J = 8.7 Hz, 2H)	114.1
3'	7.69 (d, J = 9.0 Hz, 2H)	128.1
4'	-	147.4
5'	7.69 (d, J = 9.0 Hz, 2H)	128.1
6'	7.06 (d, J = 8.7 Hz, 2H)	114.1
1''	-	129.5
2''	7.69 (d, J = 9.0 Hz, 2H)	127.6
3''	6.93 (d, J = 9.0 Hz, 2H)	112.2
4''	-	145.7
5''	6.93 (d, J = 9.0 Hz, 2H)	112.2
6''	7.69 (d, J = 9.0 Hz, 2H)	127.6
7''	3.90 (s, 3H)	42.9
1'''	-	134.7
2'''	-	152.9
3'''	-	150.0
4'''	6.67 (dd, J = 7.9, 1.4 Hz, 1H)	124.8
5'''	6.95 (t, J = 8.0 Hz, 1H)	118.1
6'''	6.85 (dd, J = 8.2, 1.4 Hz, 1H)	111.9
7'''	3.96 (s, 3H)	55.8
8'''	3.85 (s, 3H)	55.4
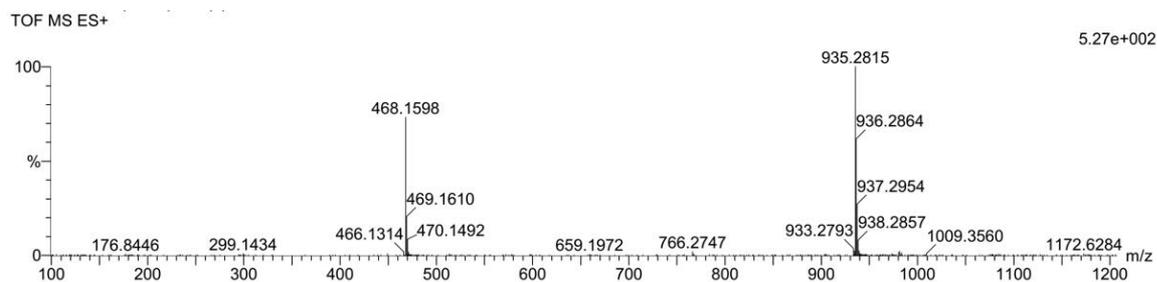
Figure 5. Pyrazoline compound on HRMS spectrum.

Table 2. IC$_{50}$ values of synthesized compound and kojic acid

Compound	IC$_{50}$ (μM)
Pyrazoline 6	262.15
Kojic acid	88.62

Conclusions

Pyrazoline compound **5** has been successfully synthesized with a satisfactory yield of 93%. The elucidation of FTIR, NMR and HRMS spectroscopic data confirmed the chemical structure. Tyrosinase inhibition activity assay showed that compound **5** considered inactive with an IC$_{50}$ value of 262 μM.

Acknowledgments

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