**Anti-Tyrosinase Kinetics and Antibacterial Process of Caffeic Acid
N-nonyl ester in Chinese Olive (*Canarium album*) Postharvest**

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Abstract

Enzymatic browning and bacterial putrefaction are mainly responsible for quality losses of Chinese Olive (*Canarium album*) postharvest and lead to very short shelf life on average. Screening anti-browning and anti-bacterial agents is important for preservation of Chinese Olive. Caffeic acid N-nonyl ester (C-9) and Caffeic Acid N-Heptyl ester (C-7) was synthesized as inhibitors of tyrosinase, which is a key enzyme in browning process. The compound of C-9 could inhibit the activity of tyrosinase strongly and its IC_{50} value was determined to be 37.5 μ M, while the compound of C-7 had not inhibitory ability. Kinetic analyses showed that compound of C-9 has been a reversible inhibitory mechanism below 50 μ M and been irreversible mechanisms above 50 μ M. For the reversible inhibitory mechanism, the values of inhibitory constants (K_I and K_{IS}) were determined to be 24.6 and 37.4 μ M, respectively. The results of Chinese Olive fruit postharvest showed that the compound of C-9 could effectively anti-browning and anti-bacterial putrefaction. In addition, this compound had strong antibacterial activities against *S. aureus*, *E. coli*, *B. subtilis* and *Salmonella*. Therefore, C-9 could be a potential anti-browning and anti-bacterial reagent.

KEYWORDS: Chinese Olive, Tyrosinase, Preservation, Postharvest treatments, Aseptic, Anti-browning, Anti-bacterial.

1. Introduction

Chinese Olive (*Canarium album*), a native species in the southeast of China, has been found with relatively broad in traditional medicine material that has some pharmacological functions[1]. Some previous researches reported that the dried fruits of Chinese Olive has activity of anti-bacterium, anti-virus, anti-inflammation and detoxification[2]. Like the Mediterranean olive (*Olea europaea* L.), Chinese olive fruit was a fusiform drupe, the fruit flesh has the characteristics of strong bitter and astringent tastes[3]. Chinese olive (*Canarium album* L.), one native and well-known tropical fruit tree in the southeast of China, contain a large amount of phenolic compounds and possess great pharmacological activities[4]. Browning and bacterial putrefaction was mainly responsible for the quality loss of Chinese Olive postharvest[5].

Caffeic acid, an analogue of cinnamic acid, has been found in plants such as the seeds of *Cuscuta chinensis* [6]. Recently, various functions has been identified for caffeic acid and its derivatives, including anti-tumour [7], anti-inflammatory [8], and anti-oxidation [9] effects and as a prophylactic for malathion-induced neuropeptides [10]. In addition, many caffeic acid analogues also had great potential as anti-fungal and anti-bacterial agents [11]. Huleihel M et.al found that the caffeic acid phenethyl ester could effect some biomarker in bacteria by FTIR microspectroscopy[12]. Caffeic acid also could present antibacterial properties by pH dependent[13]. In particular, caffeic ester derivatives, such as caffeic acid phenethyl ester, with diverse functions had attracted considerable attention recently[14]. We had synthesized many types of caffeic ester analogues and studied the different biological activities of analogues. In those studies, we observed an effect of C-9 and C-7 on tyrosinase.

Tyrosinase (EC 1.14.18.1) was a multifunctional copper-containing oxidase that

was widely founded in microorganisms [15]. Tyrosinase was found in many species of bacteria and usually associated with melanin synthesis [16]. Tyrosinase was ubiquitously distributed in most species and essential for pigmentation. The active site of tyrosinase was characterized by a pair of anti-ferromagnetically coupled copper ions [17]. Tyrosinase catalysed the hydroxylation of phenols to catechols and the oxidation of catechols to quinones [18]. This catalytic process was very important. Enzymatic browning was a major factor in the Maillard reaction. Browning contributes to quality loss in foods and usually reduces the sensory properties of foods. There were associated changes in colour, smell and nutrients, which resulted in a short shelf-life and market value [19]. Tyrosinase inhibitors had been established as important constituents of anti-browning agents and had potential uses as food additives [20]. In our previous papers, alkyl 3,4-dihydroxybenzoates [21], chlorobenzaldehyde thiosemicarbozones [22] and cardol triene [23] had strong inhibitory effects on mushroom tyrosinase.

Due to the importance of tyrosinase inhibitors, the aim of our work was to identify novel compounds that inhibit tyrosinase. The inhibition of the diphenolase activities of tyrosinase was an important criterion used in the evaluation of tyrosinase inhibitors. Therefore, we synthesized new types of caffeic acid derivatives and investigated the kinetics of the inhibition on tyrosinase by C-9, which was an unvalued derivative of caffeic acid. We measured the kinetic parameters and inhibition constants characterizing the mechanism of inhibition. Furthermore, we found that C-9 has two different inhibitory mechanisms within two concentration ranges. That was quite different from other tyrosinase inhibitors we previously studied [23-25]. We also found that C-9 had stronger bacteriostatic activity than previously identified compounds [26]. These data may provide the basis for novel tyrosinase

inhibitors and potent new food additives or antibacterial agents.

2. Materials and methods

2.1. Materials

2.1.1. Raw Materials

Olive fruits for the main analysis and bioassays were generously provided by Prof. He-Tong Lin from the collection of varieties maintained at Fuzhou (Fujian Agriculture and Forestry University, Fujian Province, China).

2.1.2. Reagents

Mushroom tyrosinase (EC 1.14.18.1) was purchased from Sigma-Aldrich (St. Louis, MO). The activity of the enzyme is 5037 U/mg. 3,4-dihydroxy-L-phenylalanine (L-DOPA) was purchased from Aldrich (St. Louis, MO, USA). Caffeic acid and n-nonyl alcohol were the products of TCI Chemicals Co. Ltd. (Shanghai, China). The purities of these chemical compounds are 99.99% and 99.99%, respectively. The positive control Streptomycin Solution was purchased from Becton Dickinson Medical Devices Co. Ltd. (Shanghai). *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Agrobacterium tumefaciens* and *Salmonella* were collected from a colony preserved at -80 °C at the Fujian Academy of Agricultural Sciences. All other reagents were analytical grade products obtained from Aldrich (St. Louis, MO, USA). The water was redistilled and ion-free.

2.2. Methods

2.2.1 Synthesis method

This compound was prepared by a reaction of caffeic acid with nonyl ester in a solution of acetone and trichloromethane. A mixture of caffeic acid (30 mM) with nonyl ester (30.5 mM) in 60 mL of acetone and trichloromethane with

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) (30 mM) and 4-dimethylaminopyridine (DMAP) (0.05 mM) solution was refluxed at 85 °C for 5-7 h and then cooled to 25 °C. The yellow viscous oily liquid was collected and purified on a silica column. The products were purified by recrystallization from trichloromethane and methanol and were identified by ESI-MS and ¹H NMR data. ESI-MS data were obtained on a Bruker Esquire-LC. The ¹H NMR data were acquired on a 600 MHz NMR spectrometer (AV600) from Bruker.

2.2.2. Anti-tyrosinase Assay.

The activities of mushroom tyrosinase were determined by a previously reported method [27] using a Beckman UV-800 spectrophotometer. C-9 was first dissolved in dimethyl sulphoxide (DMSO) and diluted into DMSO solutions of the inhibitors in different concentrations. The inhibitory effects of the inhibitors on the diphenolase activity of tyrosinase were determined by a previously reported method [28]. The effect of different concentrations of mushroom tyrosinase on the catalysis of DOPA at different concentrations of C-9 was determined as follows. The assay conditions were a 3-mL reaction system containing 0.05 M phosphate sodium buffer, pH 6.8, 0.5 mM L-DOPA and different concentrations of C-9 (0, 10, 20, 30, 40, 50, 60 and 70 μM). The final concentration of DMSO was kept at 3.3% in the reaction media. The inhibition types of the inhibitors on the diphenolase activity of mushroom tyrosinase were determined as follows. The concentrations of inhibitors were unchanged, and the concentrations of substrates (L-DOPA for the diphenolase activity assay) were changed. The concentrations of L-DOPA ranged from 0.20 to 1.00 mM and 0.25 to 0.67 mM. The final concentration of mushroom tyrosinase was 6.67 μg/ml in the diphenolase activity assay. Controls without inhibitor but containing 3.3% DMSO were routinely included. The inhibition types of the inhibitors on the enzyme were

assayed by Lineweaver-Burk plots, and the inhibition constants were measured by the plot of the intercept versus the concentration of the inhibitor. The extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC_{50}). The inhibition type was assayed by a Lineweaver-Burk plot, and the inhibition constant was determined by the second plots of the apparent K_m/V_m or $1/V_m$ versus the concentration of the inhibitor. The reactions were executed at a constant temperature of 30 °C.

2.2.3. Fluorescence Quenching

Fluorescence quenching means the decrease of fluorescence intensity between the fluorescent and solute molecules. It was usually used to study the interaction between conformation of protein molecules and small molecules[29]. Cary Eclipse fluorescence spectrophotometer was used to record the fluorescence intensities with an excitation wavelength of 280 nm and emission slit widths of 5 nm [30]. C-7 and C-9 did not have any fluorescence phenomenon at this excitation wavelength. In this study, the compound was added in 0.2 mg/ml tyrosinase solution to detect the fluorescence intensity changes and the final concentrations of inhibitor range from 25 to 75 μ M.

2.2.4. Preservation of Chinese Olive

The Olive fruits were harvest from Min-Hou district of Fuzhou country of Fujian province, China, at mature stage. Then, it was transported the fruits under ambient conditions within 4 hours to the laboratory. Any mechanical damage Olive fruits were excluded and the size of fruits was selected. The fruits were washed with bacteria free water in 3 times and air-dried. The selected fruits were dipped in each solution for 15 mins and air-dried, then packed in 0.015 mm thick polyethylene bags(30 fruit per bag) and stored at $(37\pm1)^{\circ}\text{C}$ and $80\%\pm5\%$ relative humidity. The fruits dipped in bacteria

free water were used as control. The concentration of 25 μ M and 75 μ M of C-9 were used. The concentration of 75 μ M of C-7 was used as contrast. There were three replicates each per treatment. The polyphenol oxidase (PPO) was the measured with previous method with little modified [31]. We chose the catechol as the substrate. The surface colour (b*) of Chinese Olive was measured with an ADCI-60-C colorimeter (Beijing Chen-Tai-Ke Instrument Co.Ltd., Beijing, China). To analyse the b* value, each fruit was measured at three equidistant points of the cap (n = 12 measurements per replicate). B* value of 0 and 100 percent green and yellow, respectively[32]. A white calibration plate was used for calibration (X=82.15; Y=86.86; Z= 90.50). The percentage of browning and bacterial putrefaction was observed and count 3 replicated group with statistic analysis.

2.2.5. Antimicrobial Assay

The antimicrobial assay was executed in tryptone beef extract agar, at pH 7.2, with an inoculum of $1-2 \cdot 10^5$ cells/mL. The antimicrobial activities of C-9 were determined using the agar well diffusion method following a published procedure with slight modifications [33]. The culture medium was mixed with the given microorganism by spreading the bacterial inoculum in the culture medium. Tubes (150-mm diameter) were punched in the agar and filled with C-9 at different concentrations. Control tubes, containing neat DMSO (negative control) and the standard antibiotic streptomycin sulphate (1000 U/mL), were also run parallel. The bacteria were incubated at 37 °C for 24 h. Antimicrobial activities were assessed by measuring the diameter of the zone of inhibition for the respective drug. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were tested by broth micro-dilution methods. Serial 2-fold dilutions of the C-9 were prepared in DMSO, and 30 μ L of each dilution was added to 3 mL of the medium with an inoculum of

1-2·10⁵ cells/mL and under the same culture conditions. After the cultures were incubated at 37 °C for 24 h, MIC was determined as the lowest concentration of the C-9 that demonstrated no visible growth. After the determination of the MIC, 100-fold dilutions with drug-free medium from each tube showing no turbidity were incubated at 37 °C for 48 h. The MBC was the lowest concentration of the C-9 that did not show visible growth in the drug-free cultivation conditions.

2.2.6. Full Absorption Scanning Study

We carried out this experiment according to the method [34] with modifications and in combination with broth micro-dilution methods. We chose *A. tumefaciens* as the corresponding control, which could not be inhibited by C-9. *B. subtilis*, *E. coli*, and *S. aureus* were chosen as the test samples. The samples began at the same initial bacterium concentration and were treated with different concentrations of C-9 (0, 0.01, 0.1 and 1 mM). The full absorption spectra of the bacterium were taken at 2, 4, 6, 8, 12, 24 and 48 hours. The full absorption spectra were recorded using a Beckman DU-800 spectrophotometer.

2.2.7. Tyrosinase Docking With the Ligand Assay

Molecular operation environment 2008 software (MOE) was used for protein-ligand docking in this research. According to recent studies [30], the structure of the tyrosinase was used as the initial model for docking simulations after removal of the caddie protein, the exogenous ions, and water molecules (PDB ID: 2y9w). The 3D structures of the ligands and C-9 were prepared with Chembiodraw Ultra 14.0. Before dock simulation, the structure models of the protein and ligands were energy minimized by using the energy minimization module of the MOE. In the meantime, hydrogens were added to the models of the protein and ligands with the protonate 3D module. For molecular docking, the refinement module was set to force field and

retain of the first and second scoring were set to 30. We used the MM/GBVI binding free energy scoring to rank the docking results, where a more negative value reflects a stronger interaction. Other parameters used were the default settings of the software. The docked conformation with the highest score was selected to analyse the mode of binding between C-9, its relative quinones and the protein.

2.2.8. Statistical Analysis

Statistical analysis was carried out using SPSS software (version 19.0; IBM, Inc., New York, USA). The results are reported as the mean $\pm$ SEM. After testing the data for normal distribution and equal variance, the differences between two groups were analysed by unpaired t-tests. The differences between multiple groups were analysed by one-way ANOVA. We repeated each assay in triplicate. A $P < 0.05$ was considered statistically significant.

3. Results and Discussion

3.1 ¹H NMR and mass spectrometry

The products were yellow viscous oily liquid and dissolved in DMSO. The followings were the data of ¹H NMR and LC-MS spectra of the compounds.

Nonyl (E)-3-(3,4-dihydroxyphenyl)acrylate (C-9): ¹H NMR (DMSO-d₆, TMS, 600 MHz): δ (ppm) 7.35 (OH, 2H, d), 7.02 (C₆H₃, 1H, s), 6.95 (C₆H₃, 1H, d), 6.75 (C₆H₃, 2H, d), 6.15 (CH=C, 1H, d), 4.10 (CH-O, 2H, t), 1.42-1.35 (CH=C, 2H, m), 1.25 (CH₂C, 12H, s), 0.86 (CH₃, 3H, t); ESI-MS: m/z (100%) = 307.51 (M⁺, DMSO) (see **Figure 1A** for the structure).

Heptyl (E)-3-(3,4-dihydroxyphenyl)acrylate (C-7): ¹H NMR (DMSO-d₆, TMS, 600 MHz): δ (ppm) 7.35, 7.33 (OH, 2H, d), 7.03 (C₆H₃, 1H, s), 6.95 (C₆H₃, 1H, d), 6.76 (C₆H₃, 2H, d), 6.19 (CH=C, 1H, d), 4.10 (CH-O, 2H, t), 1.42-1.35 (CH=C, 2H, m), 1.25 (CH₂C, 8H, s), 0.96, 0.97, 0.98 (CH₃, 3H, t); ESI-MS: m/z (100%) = 278.15

(M +, DMSO) (see **Figure 1B** for the structure).

3.2 Different effects of C-9 and C-7 on activity of mushroom tyrosinase.

When using L-DOPA as the assay substrate for diphenolase activity, the progression curve of the enzyme reaction should be a line passing through the origin with different slopes that indicates diphenolase activity. There was no lag period for the enzyme catalysing the oxidation of L-DOPA. In our investigation, C-9 and C-7 were used as an inhibitor of the activity of mushroom tyrosinase for the oxidation of L-DOPA. The inhibitory concentration effect on the diphenolase activity decreased with increasing inhibitor concentration. When the concentration of C-9 reached 60 μ M, enzyme activity was inhibited by 78% (**Figure 3A**), indicating that C-9 exhibited a potent inhibitory effect on diphenolase activity with dose-dependence. The IC_{50} value of C-9 on the diphenolase activity of the tyrosinase was 37.5 μ M. But the C-7 didn't work at the same concentration.

3.3. Inhibition mechanism of C-9 on the diphenolase activity of mushroom tyrosinase.

The inhibition mechanism of C-9 on mushroom tyrosinase for the oxidation of L-DOPA was studied. As in our previous paper, we determined whether the inhibitor is reversible or irreversible depending on whether increasing the inhibitor concentration resulted in a decreased slope of the line. If the data reveal a family of straight lines all passing through the origin, the inhibition of diphenolase by the inhibitor is reversible [26]. If the data reveal a family of parallel lines, the inhibition of diphenolase by the inhibitor is irreversible [23]. One tyrosinase inhibitor can have only one type of inhibition mechanism. In this paper, the plots of the remaining enzyme activity versus the concentrations of enzyme in the presence of different concentrations of C-9 below 50 μ M gave a family of straight lines, which all passed

through the origin (**Figure 3B**). When the concentration of C-9 was higher than 50 μM , the plots of the remaining enzyme activity tended to form a family of parallel lines (**Figure 3B**). In other words, the data indicate that the inhibition of diphenolase by C-9 was reversible when the concentration of C-9 was below 50 μM and was irreversible by concentrations above 50 μM . The presence of C-9 can bring down the amount of the efficient enzyme when the concentration was increasing, consistent with our later docking experiment results. The inhibition type of C-9 on the diphenolase activity of the enzyme was determined (**Figure 3C**). With increasing concentrations of C-9, the K_m increased and the V_{max} decreased. Double-reciprocal plots yielded a family of straight lines intersecting at the second quadrant. Therefore, the inhibition belonged to mixed-I type. In this type, the competitive effect was stronger than the uncompetitive effect, which indicated that this compound inhibited the enzyme-substrate complex less than did the free enzyme.

Tyrosinase mixed type inhibition kinetics usually follow the **equations**:

Based on mixed type inhibition kinetics, the kinetic parameters for C-9 can be analysed by using the following equations.

In the absence and presence of mixed type inhibitor, the kinetic function is:

$$v_0 = \frac{V_m \cdot [S]}{K_m + [S]} \quad v_i = \frac{V_m \cdot [S]}{K_m \left(1 + \frac{[I]}{K_I}\right) + [S] \cdot \left(1 + \frac{[I]}{K_{IS}}\right)} \quad (1)$$

The linear of v is:

$$\frac{1}{v} = \frac{K_m}{V_m} \cdot \left(1 + \frac{[I]}{K_I}\right) \cdot \frac{1}{[S]} + \frac{1}{V_m} \left(1 + \frac{[I]}{K_{IS}}\right) \quad (2)$$

The inhibition rate is:

$$i\% = \frac{K_m \frac{[I]}{K_I} \cdot [S] \frac{[I]}{K_{IS}}}{K_m \left(1 + \frac{[I]}{K_I}\right) + [S] \cdot \left(1 + \frac{[I]}{K_{IS}}\right)} \times 100\% \quad (3)$$

When $i\%$ reaches 50%, $[I] = IC_{50}$. Therefore:

$$\frac{1}{2} = \frac{K_m \frac{IC_{50}}{K_I} \cdot [S] \frac{IC_{50}}{K_{IS}}}{K_m \left(1 + \frac{IC_{50}}{K_I}\right) + [S] \cdot \left(1 + \frac{IC_{50}}{K_{IS}}\right)} \times 100\% \quad (4)$$

Equation 4 is converted to:

$$[I]_{50} = \frac{K_m + [S]}{\frac{K_m}{K_I} + \frac{[S]}{K_{IS}}} \quad (5)$$

The slope of part a in part b of **Figure 4** is:

$$\text{Slope} = \frac{K_m}{V_m} \left(1 + \frac{[I]}{K_I}\right) \quad (6)$$

The K_I calculated by the straight-line formula in Figure 4 part b is:

$$Y = 0.0001531 * X + 0.003766$$

$$K_I = \text{Intercept} / \text{slope} = 24.6 \mu\text{M}$$

The intercept of part a shown in part c of **Figure 2(C)** is:

$$\text{Intercept} = \frac{1}{V_m^{app}} = \frac{1}{V_m} \left(1 + \frac{[I]}{K_{IS}}\right) \quad (7)$$

The K_I can be calculated by the straight-line formula in Figure 4 part b:

$$Y = 0.0001655 * X + 0.006196$$

$$K_{IS} = \text{Intercept} / \text{slope} = 37.44 \mu\text{M}$$

The values of K_I and K_{IS} by eq 6 and eq 7 were determined to be 24.6 μM and 37.44 μM , respectively (**Table 1**). According to the experimental conditions and eq 5, we could obtain a theoretical value of $IC_{50} = (0.624 + 0.50) / (0.624 / 0.0246 + 0.50 / 0.374) = 0.371 \text{ mM}$, which was extremely close to the test value (37.5 μM).

3.4. Anti-browning and anti-bacterial putrefaction in preservation of Chinese Olive postharvest

We chose the storage temperature of 37°C to accelerated the enzymatic browning and bacterial putrefaction during preservation process of Chinese Olive postharvest. The results were shown in **Figure 2**. Obviously, the group of 75 μM C-9 treated has greater green and lower PPO activity than the other group(**Figure 2A, 2B, 2C**).The group of 75 μM C-9 treated was also effectively anti-bacterial putrefaction(**Figure 2D**). Contrasting the other group, the group of 75 μM C-9 treated can be more effectively anti-browning and anti-bacterial putrefaction (**Figure 2E, 2F, 2H, 2G**). The results was shown that the irreversible inhibitory mechanism of C-9 was also effect on PPO on Chinese Olive and inhibition of bacterial putrefaction.

3.5. Antimicrobial experiment of C-9.

We studied the antimicrobial activities of C-9 on *B. subtilis*, *E. coli*, *S. aureus*, *Salmonella*, *K. pneumonia*, *P. aeruginosa* and *A. tumefaciens*. The results were shown in **Figure 3** and **Table 2**. Specifically, the bacteriostatic activities were assayed by taking 1000 U/mL streptomycin sulphate as the positive control. C-9 had a significant inhibitory effect against *E. coli*, *S. aureus*, *Salmonella* and *K. pneumonia*. At the same time, the compound could inhibit the proliferation of *B. subtilis* and *S. aureus* to different degrees, but less than the positive control. No obvious effect of C-9 against *P. aeruginosa* and *A. tumefaciens* was observed. DMSO had no obvious inhibition on the

proliferation of these seven different types of bacteria. We also used a broth dilution method [26] to test the antimicrobial activities of C-9 against the seven bacteria. The results were listed in **Table 3**. C-9 was effective against *B. subtilis*, *E. coli*, *S. aureus*, *Salmonella* and *K. pneumonia*. The antimicrobial activity against *B. subtilis*, *E. coli*, *S. aureus* and *Salmonella* was more effective with the same MIC of 10 mM and with the same MBC of 10 mM, while the MIC and the MBC against *K. pneumonia* were, respectively, 100 mM and 100 mM (**Table 3**).

3.6. Full absorption detection of microbial growth metabolism with or without C-9

In order to further understand the antibacterial mechanism of C-9, we also studied the full absorption spectra of the bacterium at different sampling times. We used a concentration of C-9 lower than the MIC to avoid full inhibition. The results were shown in **Figure 4**. Increasing concentrations of C-9 not only changed the intensity of absorption but also induced an absorption peak at 400 nm for *B. subtilis*, *E. coli* and *S. aureus*. According to FG Carmona et al. [35], caffeic acid could be transformed into the corresponding quinone with tyrosinase and had an absorption peak at 400 nm. We speculated that the peak represents the accumulation of nonyl (E)-3-(3,4-dioxocyclohexa-1,5-dien-1-yl)acrylate from C-9 (see the structure in **Figure 1C**) and other quinones. In addition, there was not a peak at 400 nm in the corresponding control for *A. tumefaciens*, which could not be inhibited by C-9.

3.7. Analysis of molecular docking of C-9 and its catalysate.

Docking simulations were further performed to understand the mechanism underlying the potent anti-tyrosinase activities of C-9. C-9 (see the structure in **Figure 1A**) is similar to L-DOPA (see the structure in **Figure 1B**). The docking modes of C-9, C-9 before and after catalysis of nonyl (E)-3-(3,4-dioxocyclohexa-1,5-dien-1-yl)acrylate

were examined in the enzyme catalytic site. The docked conformations revealed that C-9, C-9 in catalysis formation and nonyl (E)-3-(3,4-dioxocyclohexa-1,5-dien-1-yl)acrylate (see **Figure 1C** for the structure) could form metal interactions with the di-copper irons of the enzyme (**Figure 6B**). After accumulation of the C-9 product, the catalyst of C-9 could be changed (**Figure 6A** and **Figure 6C**). The dihydroxyl group of the C-9 and C-9 in catalysis could also chelate the copper ions in the enzyme active site. Interestingly, the corresponding quinone that was the transformation of C-9 into the enzyme could also strongly bind the pocket mouth of the enzyme active site, including His263, His244, Glu256, His85, Val 283 and Gly 281 (**Figure 6B**).

4. Conclusion

In the work we present here, we synthesized C-9 and determined its inhibitory mechanism of mushroom tyrosinase, as well as their anti-browning and anti-bacterial putrefaction in Chinese Olive postharvest. We also reported a new method to analyse antibacterial mechanisms. The results showed that C-9 could strongly inhibit tyrosinase activities by a novel inhibitory mechanism and be effectively anti-browning in Chinese Olive postharvest. Tyrosinase has two enzymatic activities, ortho-monophenoloxidase and polyphenoloxidase activities, and uses many phenols and catechols as substrates [36]. We synthesized the C-9 to screen for a new type tyrosinase inhibitor. Although an increasing number of tyrosinase inhibitors have been reported every year, most cannot be used because of their low individual activities or side effects. For example, 2-(4-hydroxyphenoxy)-tetrahydro-6-(hydroxymethyl)-2H-pyran-3,4,5-triol (Aarbutin) ($IC_{50} = 30 \text{ mM}$) and kojic acid ($IC_{50} = 0.023 \text{ mM}$) were eliminated in clinical trials. Tropolone represents one of the most efficient tyrosinase inhibitors reported ($IC_{50} = 0.4 \text{ }\mu\text{M}$), but the serious side

effects limited its medicinal use [37]. In this work, C-9 could effectively inhibit the diphenolase activity of mushroom tyrosinase. The inhibitory potency of C-9 ($IC_{50} = 37.5 \mu\text{M}$) on diphenolase activity was more potent than the benzaldehyde thiosemicarbozones that we previously investigated ($IC_{50} = 3800 \mu\text{M}$) [24]. Similarly, Giuseppe Battaini et al. [38] determined the formation constants of the catalyst-inhibitor complexes with tyrosinase for kojic acid. Therefore, it could be supposed that complexes would be formed between C-9 compounds and tyrosinase when both substrates were mixed together in solution. In fact, the catechol part of the C-9 compounds and the copper ion of tyrosinase could be the centre of complexation, based on the active centre structure of tyrosinase. In this complex, the active centre of tyrosinase could coordinate with two phenolic hydroxyl group molecules at the same time in two opposite directions (see **Figure 1C and 5**). It was suggested that C-9 could be catalysed and was formed between the hydrogen atom on 3-OH and 4-OH in the catechol portion of the molecule, which was beneficial for decreasing the molecular energy state. C-9 was investigated as a novel reversible-irreversible mixed type inhibitor. It is rare that one tyrosinase inhibitor has both reversible and irreversible inhibitory functions at different concentration ranges, indicating that the binding of inhibitor and tyrosinase depends on the concentration of inhibitors. The value of K_{IS} and K_I is very close, indicating that the affinity of the inhibitor for the free enzyme is similar to that of the inhibitor for the enzyme-substrate complex. In addition, C-9 also had antimicrobial activity against *B. subtilis*, *E. coli*, *S. aureus*, *Salmonella* and *K. pneumonia*. It was found that C-9 could inhibit the proliferation of these five different types of microbes to different extents (**Figure 5**). The results showed that C-9 had a broad spectrum of antimicrobial activity. It could suppress the growth of G^+ and G^- bacteria. However, the inhibitory effect was poor for *P.*

aeruginosa and *A. tumefaciens*, whose tyrosinase activities did not function well [17]. The reason C-9 could inhibit the growth of microorganisms may be the same as that for caffeic acid. Recently, more and more tyrosinase inhibitors have been reported as antibacterial agents, such as 5-benzylidene barbiturate derivatives [39], leaf extracts [40] and even lactoferrin hydrolysates [41], yet without clear mechanisms. We offered a new method that combines the docking assay with a UV-scanning assay. The results revealed that C-9 could inhibit tyrosinase reversibly and bind with catalyst centre at concentrations below 50 μ M. These data may provide the basis for developing novel preservation method of Chinese Olive and developing new aseptic for other fruits.

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ABBREVIATIONS USED

C-9, caffeic acid n-nonyl ester; C-7, caffeic acid n-heptyl ester; DMAP, 4-dimethylaminopyridine; EDC·HCl, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; DMSO, dimethyl sulphoxide; L-DOPA, 3,4-dihydroxy-L-phenylalanine; IC_{50} , the inhibitor concentrations leading to 50% activity lost; K_I , equilibrium constant of the inhibitor combining with the free enzyme; K_{IS} , equilibrium constant of the inhibitor combining with the enzyme-substrate complex; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

References

- [1] L.L. Zhang, Y.M. Lin, Journal of Zhejiang University. Science. B, 9 (2008) 407-415.
- [2] D.J. Guo, H.L. Cheng, S.W. Chan, P.H. Yu, Inflammopharmacology, 16 (2008) 201-207.
- [3] Z. He, W. Xia, Food chemistry, 105 (2007) 1307-1311.
- [4] Z. He, W. Xia, J. Chen, European Food Research and Technology, 226 (2008) 1191-1196.
- [5] J.H. Zhou, G.F. Zhong, Z.Z. Lin, H.L. Xu, Journal of Food Agriculture & Environment, 10 (2012) 505-508.
- [6] S. Donnapee, J. Li, X. Yang, A.-h. Ge, P.O. Donkor, X.-m. Gao, Y.-x. Chang, Journal of ethnopharmacology, 157 (2014) 292-308.
- [7] K. Yasuko, N. Tomohiro, M. Sei-Itsu, L. Ai-Na, F. Yasuo, T. Takashi, Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism, 792 (1984) 92-97.
- [8] Y.-J. Chen, M.-S. Shiao, S.-Y. Wang, Anti-cancer drugs, 12 (2001) 143-149.
- [9] P. Michaluart, J.L. Masferrer, A.M. Carothers, K. Subbaramaiah, B.S. Zweifel, C. Koboldt, J.R. Mestre, D. Grunberger, P.G. Sacks, T. Tanabe, A.J. Dannenberg, Cancer research, 59 (1999) 2347-2352.
- [10] R. Rezg, B. Mornagui, J.S. Santos, F. Dulin, S. El-Fazaa, N.B. El-Haj, R. Bureau, N. Gharbi, Environmental science and pollution research international, 22 (2014) 6198-6207.
- [11] N. Aziz, S. Farag, L. Mousa, M. Abo-Zaid, Microbios, 93 (1997) 43-54.
- [12] M. Huleihel, V. Pavlov, V. Erukhimovitch, Journal of photochemistry and photobiology. B, Biology, 96 (2009) 17-23.
- [13] M.P. Almajano, R. Carbo, M.E. Delgado, M.H. Gordon, Journal of food science, 72 (2007) C258-263.
- [14] M.F. Tolba, H.A. Omar, S.S. Azab, A.E. Khalifa, A.B. Abdel-Naim, S.Z. Abdel-Rahman, Critical reviews in food science and nutrition, 40 (2014) 1549-7852.
- [15] E.I. Solomon, U.M. Sundaram, T.E. Machonkin, Chemical reviews, 96 (1996) 2563-2606.
- [16] M. Fairhead, L. Thöny-Meyer, New biotechnology, 29 (2012) 183-191.
- [17] H. Claus, H. Decker, Systematic and applied microbiology, 29 (2006) 3-14.
- [18] L.M. Mirica, M. Vance, D.J. Rudd, B. Hedman, K.O. Hodgson, E.I. Solomon, T.D. Stack, Science, 308 (2005) 1890-1892.
- [19] M. Martinez, J.R. Whitaker, Trends in Food Science & Technology, 6 (1995) 195-200.
- [20] Z.Z. Pan, Y.J. Zhu, X.J. Yu, Q.F. Lin, R.F. Xiao, J.Y. Tang, Q.X. Chen, B. Liu, J Agric Food Chem, 60 (2012) 10784-10788.
- [21] Z.Z. Pan, H.L. Li, X.J. Yu, Q.X. Zuo, G.X. Zheng, Y. Shi, X. Liu, Y.M. Lin, G. Liang, Q. Wang, Q.X. Chen, J Agric Food Chem, 59 (2011) 6645-6649.
- [22] Z.C. Li, L.H. Chen, X.J. Yu, Y.H. Hu, K.K. Song, X.W. Zhou, Q.X. Chen, J Agric Food Chem, 58 (2010) 12537-12540.
- [23] J.X. Zhuang, Y.H. Hu, M.H. Yang, F.J. Liu, L. Qiu, X.W. Zhou, Q.X. Chen, J Agric Food Chem, 58 (2010) 12993-12998.
- [24] L.H. Chen, Y.H. Hu, W. Song, K.K. Song, X. Liu, Y.L. Jia, J.X. Zhuang, Q.X. Chen, J Agric Food Chem, 60 (2012) 1542-1547.
- [25] W.M. Chai, Y. Shi, H.L. Feng, L. Xu, Z.H. Xiang, Y.S. Gao, Q.X. Chen, J Agric Food Chem, 62 (2014) 6382-6389.
- [26] Y.J. Zhu, K.K. Song, Z.C. Li, Z.Z. Pan, Y.J. Guo, J.J. Zhou, Q. Wang, B. Liu, Q.X. Chen, J Agric Food Chem, 57 (2009) 5518-5523.
- [27] Y.F. Lin, Y.H. Hu, H.T. Lin, X. Liu, Y.H. Chen, S. Zhang, Q.X. Chen, J Agric Food Chem, 61 (2013) 2889-2895.
- [28] Q.X. Chen, I. Kubo, J Agric Food Chem, 50 (2002) 4108-4112.
- [29] D. Kim, J. Park, J. Kim, C. Han, J. Yoon, N. Kim, J. Seo, C. Lee, J Agric Food Chem, 54 (2006) 935-941.
- [30] X.X. Chen, Y. Shi, W.M. Chai, H.L. Feng, J.X. Zhuang, Q.X. Chen, PloS one, 9 (2014) e91809-e91809.
- [31] R.H. Hbaieb, F. Kotti, R. Garcia-Rodriguez, M. Gargouri, C. Sanz, A.G. Perez, Food chemistry, 174 (2015) 240-247.
- [32] J. Joas, Y. Caro, M.N. Ducamp, M. Reynes, Postharvest Biology and Technology, 38 (2005) 128-136.
- [33] M. Owais, K. Sharad, A. Shehbaz, M. Saleemuddin, Phytomedicine : international journal of phytotherapy and phytopharmacology, 12 (2005) 229-235.
- [34] M. Jimenez-Atienzar, J. Cabanes, F. Gandia-Herrero, F. Garcia-Carmona, Biochemical and biophysical research communications, 319 (2004) 902-910.
- [35] F. Carmona, E. Pedreño, J. Galindo, F. Cánovas, Analytical biochemistry, 95 (1979) 433-435.

- [36] P. Han, C.-Q. Chen, C.-L. Zhang, K.-K. Song, H.-T. Zhou, Q.-X. Chen, *Food chemistry*, 107 (2008) 797-803.
- [37] V. Kahn, A. Andrawis, *Phytochemistry*, 24 (1985) 905-908.
- [38] G. Battaini, E. Monzani, L. Casella, L. Santagostini, R. Pagliarin, *JBIC Journal of Biological Inorganic Chemistry*, 5 (2000) 262-268.
- [39] Q. Yan, R. Cao, W. Yi, Z. Chen, H. Wen, L. Ma, H. Song, *European journal of medicinal chemistry*, 44 (2009) 4235-4243.
- [40] H.Y. Lai, Y.Y. Lim, S.P. Tan, *Bioscience, biotechnology, and biochemistry*, 73 (2009) 1362-1366.
- [41] H. Abe, K. Kawase, S. Kobayashi, H. Miyakawa, H. Saito, S. Shimamura, M. Takase, Y. Tamura, M. Tomita, K. Yamauchi, in, *Google Patents*, 1993.

CAPTION OF FIGURES

Figure 1. The structure of CA-9 (A), CA-7 (B), corresponding Quinone of CA-9 (C) , L-DOPA (D).

Figure 2. The preservation of Chinese Olive treated with C-9 and C-7 in postharvest. (A) is the inhibition of PPO which is extracted from Chinese Olive treated with different concentrations of CA-9, CA-7 and bacteria free water in different storage days. (B) is the aberration change of Chinese Olive in b^* at different concentrations of CA-9, CA-7 and water in different storage days. (C) is the browning percentage of Chinese Olive in different storage time. (D) is the percent of bacterial putrefaction of Chinese Olive in different storage time. The character of Chinese Olive treated with bacteria free water (E), 25 μ M CA-9 (F), 75 μ M CA-7 (G) and 75 μ M CA-9 (H) in 3rd day.

Figure 3. The inhibitory mechanism of C-9 and C-7.

The inhibition of CA-9(●) and CA-7(○) on the activity of mushroom tyrosinase for the catalysis of DOPA (A). The Effect of concentrations of mushroom tyrosinase on its activity for the catalysis of DOPA at different concentrations of CA-9(B). Concentrations of CA-9 for curves 0-7 were 0, 10, 20, 30, 40, 50, 60 and 70 μ M, respectively. Lineweaver-Burk plots for inhibition of CA-9 on mushroom tyrosinase for the catalysis of DOPA at different concentrations as substrate and different concentrations of CA-9. The Concentrations of L-DOPA ranged from 0.20 to 1.00 mM and 0.25 to 0.67 mM, respectively. Concentration of CA-9 in part (a) for curves 1-5 was 0, 10, 20, 30 and 40 μ M, respectively; the enzyme concentration 20 μ g/3ml. The data were taken after reaction 30s. Secondary plots of the slopes (b) and the intercepts (c) of the straight lines from the part a. The line was drawn using linear least squares fit (C). The fluorescence quenching experiment of compounds C-7 (E)

and C-9 (F) Fluorescence absorption phenomena of the compounds.

Figure 4. Antimicrobial activity of C-9 at different concentrations.

The concentrations of C-9 for dishes 1-4 were 0.01, 0.1, 1 and 10 mM, respectively. a, positive control with 1000 U/mL of streptomycin sulfate and penicillin; b, negative control with DMSO.(A) is for the inhibitory character and (B) is for statistic analysis.

Figure 5. Full length Scan of bacterium in different time treated by CA-9 at different concentrations.

The concentrations of CA-9 were 0, 0.01, 0.1 and 1 mM, respectively. The sampling time was 2, 4, 6, 8, 10, 12, 24, 48 hours.

Figure 6. Binding mode of the lowest energy docked conformation found for the ligand with tyrosinase residues.

Figure 1

Figure 2

Figure 3

Figure 4

A

B

Figure 5

Figure 6

Table 1. Kinetic constants and inhibition constants of C-9 and C-7 on mushroom tyrosinase

Constants		C-9	C-7
IC_{50}		37.5 μ M	-
Inhibition	Reversible ^(a)	Irreversible ^(b)	-
Inhibition Type	Mixed	-	-
K_I	24.6 μ M	-	-
K_{IS}	37.4 μ M	-	-

Table 1. Kinetic constants and inhibition constants of C-9 and C-7 on mushroom tyrosinase. (a), concentration of inhibitor lower than 40 μ M; (b), concentration of inhibitor higher than 50 μ M; -, constants could not be determined.

Table 2. Antimicrobial Activity of Nonyl-3,4-Dihydroxycinnamate (C-9)

Concentration (mM)	<i>Staphylococcus aureus</i>	<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Salmonella</i>	<i>Pseudomonas aeruginosa</i>	<i>Klebsiella rhinoscleromatis</i>	<i>Agrobacterium tumefaciens</i>
a	+++	-	+++	-	+++	+++	+++
b	-	-	-	-	-	-	-
0.01	+	±	++	±	-	-	-
0.1	+	+	++	+	-	±	-
1	++	+	+++	++	-	±	-
10	++	++	+++	++	-	++	±

Table 2. Antimicrobial Activity of C-9. a, positive control with 1000 U/mL of streptomycin and penicilin; b, negative control with DMSO; +++, antimicrobial zone is above 12 mm in diameter; ++, antimicrobial zone is between 10 mm and 12 mm; +, antimicrobial zone is less than 10 mm; ±, antimicrobial zone is faint; -, no inhibition.

Table 3. MIC and MBC (mM) of C-9.

Bacteria	MIC (mM)	MBC (mM)
<i>Staphylococcus aureus</i>	10	10
<i>Escherichia coli</i>	10	10
<i>Bacillus subtilis</i>	10	10
<i>Salmonella</i>	10	10
<i>Pseudomonas aeruginosa</i>	>100	Not tested
<i>Klebsiella rhinoscleromatis</i>	100	100
<i>Agrobacterium tumefaciens</i>	>100	Not tested