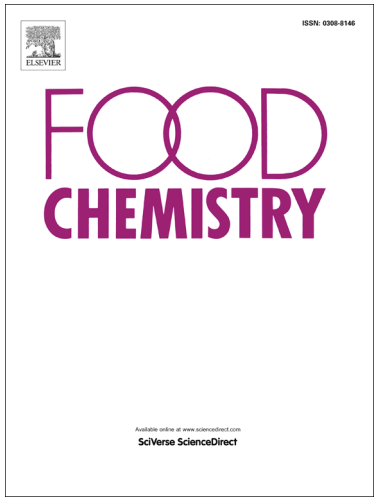

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Functionalities of conjugated compounds of γ–aminobutyric acid with salicylaldehye or cinnamaldehyde

Running title: Functionalities of conjugated compounds of γ–aminobutyric acid….

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ABSTRACT

Aldehydes or ketones can react with amino compounds to form Schiff base adducts, which have been widely studied and shown to exhibit antimicrobial, antioxidant or antiviral activity. Salicylaldehye (SA) and cinnamaldehyde (CA) are components of plant essential oils. γ-Aminobutyric acid (GA) is an important substance in the mammalian central nervous system and responsible for many bioactivities. This study aimed to synthesize functional Schiff base adducts using GA and SA or CA; to study the antimicrobial activity, antioxidant activity and tyrosinase-inhibition activity of these adducts (SA-GA and CA-GA) and their metal complexes—SA-GA-Cu (or Zn) and CA-GA-Cu (or Zn); and to find their applications in food systems. SA-GA and CA-GA both exhibited good antibacterial effects, and so did their Cu complexes. As for antioxidant activity, SA-GA and CA-GA were superior to their metal complexes in most tests. Regarding inhibition of enzymatic browning of mushrooms, both SA-GA-Cu and CA-GA-Cu could inhibit tyrosinase activity effectively.

Keywords：Salicylaldehye, Cinnamaldehyde, γ-Aminobutyric acid, Schiff base, Antimicrobial activity, Antioxidant activity, Tyrosinase, Mushroom browning.

1. Introduction

Most foods are susceptible to microbial spoilage, or biochemical or chemical deterioration. Therefore, a variety of synthetic food additives are used to enhance safety and quality of food products in order to ensure consumer acceptance. As many negative effects have been reported about synthetic food additives (Soni, Carabin, & Burdock, 2005), consumers have grown more concerned about the safety of synthetic food additives. Regarding food preservatives, plant essential oils have been investigated and found to have the potential to be used as natural preservatives (Yang, Liou, Hu, Peng, & Liu, 2013). However, essential oils (EOs) generally have strong characteristic odours and are immiscible with water due to hydrophobicity, which may limit their applications in many food systems.

Aldehydes or ketones can react with amino compounds or amino groups of food proteins to form Schiff base adducts, such as those in the Maillard reaction, which is commonly encountered in food cooking, processing or storage. Schiff base adducts have been widely studied in organic, inorganic and medicinal chemistry and show many biological activities, such as antimicrobial, antioxidant and antiviral activity (Kumar, Dhar, & Saxena, 2009). Although many Schiff base adducts have been synthesized in the literature, few have found applications in food systems because most of the compounds used for the synthesis are not edible or allowed in foods. A recent study used cinnamaldehyde and various amino acids, excluding γ-aminobutyric acid (GA), to generate Schiff

base adducts and investigated their antimicrobial activity *in vitro* and in food systems (Wei, Xiong, Jiang, Zhang, & Ye, 2011). The Schiff base adducts can reduce the odour intensity and increase the solubility of cinnamaldehyde in aqueous systems.

Cinnamaldehyde and salicylaldehye are components of plant EOs, and both are food flavourings assessed by the Flavor and Extraction Manufacturers Association as generally regarded as safe (GRAS) ingredients (Adams et al., 2004; Adams et al., 2005). In addition, Schiff base adducts can be hydrolyzed into their original reactive components in acidic conditions (Cordes & Jencks, 1963). Therefore, the safety of these adducts can be mainly determined by the reactive components after digestion. For example, Schiff base adducts of cinnamaldehyde and amino acids have been investigated and do not show any oral toxicity in animal tests (Wei etal., 2011).

GA is a chief inhibitory neurotransmitter in the mammalian central nervous system. It can regulate neuronal excitability throughout the nervous system. In humans, GA is also directly responsible for the regulation of muscle tone (Watanabe, Maemura, Kanbara, Tamayama, & Hayasaki, 2002). In addition, GA shows relaxation and immunity enhancement effects in humans (Abdou, Higashiguchi, Horie, Kim, Hatta, & Yokogoshi, 2006); it can also reduce the blood pressure in animals and humans (Aoki, Furuya, Endo, & Fujimoto, 2003). As a functional ingredient, GA has been formulated into a number of commercial health-oriented products for use as a dietary supplement. Regarding foods, GA tea is commercially produced in Taiwan with an emphasis on the aforementioned bioactivities (Wang, Tsai, Lin, & Ou, 2006).

In this study, we aimed to synthesize functional Schiff base adducts using GA and salicylaldehye or cinnamaldehyde; to study the antimicrobial activity, antioxidant activity and tyrosinase-inhibition activity of these adducts and their metal complexes; and to find their applications in food systems.

2. Materials and methods

2.1. Chemicals

γ-Aminobutyric acid (GA), salicylaldehye (SA), cinnamaldehyde (CA), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), nitrotetrazolium blue chloride (NBT), tyrosinase (50 KU) and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Potassium peroxidisulfate and ferrous chloride were acquired from Mallinckrodt-Baker, Inc. (Phillipsburg, NJ, USA). Sodium nitroprusside (SNP), sulfanilamide and N-(1-naphthyl)-ethylenediamine dihydrochloride, ferrozine were obtained from Alfa Aesar Co. (Ward Hill, MA, USA). Tryptic soy broth (TSB) (Bacto™), nutrition broth (NB) and agar were obtained from Becton Dickinson & Company (San Jose, CA, USA).

2.2. Microbial strains

94

95 *Staphylococcus aureus* (BCRC10780) and *Escherichia coli* O157:H7 (BCRC14824) were
96 acquired from Bioresource Collection and Research Center (BCRC) at the Food Industry Research
97 and Development Institute, Hsinchu, Taiwan.

98

99 *2.3. Preparation of Schiff base adducts*

100

101 The preparation of Schiff base adducts followed the methods of Wei, Xiong, Jiang, Zhang and
102 Ye (2011) with some modifications. The KOH (0.38 g) was dissolved in methanol (25 ml) and GA
103 (0.59 g) was added to the solution with stirring for complete dissolution. The above solution was
104 reacted at 50°C for 2 h in a round-bottomed flask equipped with a condenser. The mixture was
105 cooled and filtered to obtain the GA-containing filtrate. The methanol solution of SA or CA was
106 prepared by dissolving 0.7 ml SA or 0.84 ml CA in 10 ml methanol placed in a round-bottomed flask.
107 Then the GA-containing filtrate was added dropwise to the methanol solution of SA or CA within 1 h
108 under N_2 gas. The precipitate was formed during the reaction and it was collected after the solvent
109 was evaporated by the gas. Anhydrous ether was added to the precipitate to remove residual reactants
110 and the precipitate was dried under vacuum.

111

112 *2.4. Preparation of metal complexes of the adducts*

Metal (II) nitrates (1 mmol) were dissolved in methanol (10 ml) and the solution was filtered and added dropwise into a (15 ml) methanolic solution of SA-GA or CA-GA (1 mmol). The above mixture was stirred for 2 h. The metal complex obtained was filtered off, washed with methanol and ether; and then dried under vacuum (Nair & Joseyphus, 2008).

2.5. Infrared spectral analysis

The sample was mixed with KBr powder at a 1:100 ratio and dried overnight at 50°C. The sample-KBr pellet was prepared by mechanically pressing the powder mixture. The IR spectra were recorded by a FT-IR spectrophotometer (Prestige 21, Shimazu, Tokyo, Japan) with a scan range of 400-4000 cm^{-1} using KBr as a reference.

2.6. Antimicrobial activity assay for bacteria in vitro

A stock solution (7500 µg/ml) was prepared with SA-GA or CA-GA and its metal ion complexes (Cu^{2+} and Zn^{2+}) dissolved in DMSO. The bacteria from stock cultures were inoculated in 5 ml broth and incubated at 37°C for 24 h to increase their population. The enriched cultures were controlled with dilution to optical density of 1 at 630 nm, which were further diluted with appropriate media for

each microbe to 10^3- 10^4 colony forming units (CFU/ml) as an inoculum. The media for *S. aureus*, *E. coli* O157 were TSB and NB, respectively.

A microbial suspension was mixed with an amount of the test solution (20:1, v/v) at a series of concentrations, and then added to a 96-well microplate. The plate was capped and incubated for 48 h at 37˚C. The minimal bactericidal concentration (MBC) was determined by visually judging the microbial growth in the tubes with a series of broth dilutions. Then, the existence of viable cells was verified by a plate count method. An inoculated growth medium without the test compound was employed as a control. All of the experiments were performed in triplicate and the average values were reported as MBC. The MBC is defined as the lowest concentration of the test solution that results in no viable cells in a culture plate.

2.7. Antimicrobial activity assay in food

White button mushrooms were used for evaluating the antimicrobial activity of the SA-GA-Cu or CA-GA-Cu in a real food system. Each mushroom (50±1 g) was weighed and dipped into a SA-GA-Cu or CA-GA-Cu solution at a concentration of 187 or 375 μg/ml for 10 s and held until no water dripped from the surface. Then, the samples were placed into a sterilized plastic bag and zipped, followed by storage in a chamber at room temperature for 6 h. At sampling time, the sample (16 g) and 144 ml PBS (0.85%) were added into a sterilized container with blades and then homogenized. The homogenate was used for antimicrobial assay. The homogenate was taken for a

series dilution, 1 ml of which was poured onto plate count agar and incubated for 48 h at 37˚C. The population of the microorganisms was determined by counting the number of colonies from the plates with 25-250 colonies and multiplying by the dilution factor. The experiments were performed in triplicate.

2.8. Analysis of antioxidant activity

2.8.1. Scavange of ABTS radical

The ABTS radical assay followed the method of Debnath, Park, Nath, Samad, Park and Lim (2011) with some modifications. ABTS was dissolved in water to make a solution of 7 mM. Then 10 ml of the ABTS solution was mixed with 2 ml potassium peroxidisulfate (14 mM) and 12 ml sodium phosphate buffer (0.01M, pH7.4) to generate ABTS by allowing the mixture to stand in the dark at room temperature for 12-16 h before use. For sample testing, the ABTS solution was diluted with the sodium phosphate buffer to an absorbance of 1 at 734 nm. The test samples (0.1 ml), each at a different concentration, were mixed with 1 ml ABTS solution and reacted for 10 min in dark. The absorbance was recorded at 734 nm. Each sample was measured in triplicate and averaged. This antioxidant effect is given as percentage ABTS scavenging activity that is calculated by the following formula:

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

170 where A_C is the absorbance value of the control and A_S is the absorbance value of the test samples.

171 *2.8.2. Scavenging of nitrite oxide*

172

173 Test samples (0.3 ml) at various concentrations were mixed with 0.88 ml PBS, and sodium
174 nitroprusside (25 mM) in PBS, and then reacted for 2 h at room temperature. A volume of 0.5 ml of
175 the mixture was taken and added to 0.3 ml 5% H_3PO_4 solution containing 1% sulfanilamide, and 0.3
176 ml 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride solution (Marcocci, Maguire, Droylefaix,
177 & Packer, 1994). The absorbance of the reacted sample solution was measured at 570 nm. The
178 scavenging activity is determined according to the following equation:

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

180 where A_C is the absorbance value of the control and A_S is the absorbance value of the test samples.

181

182 *2.8.3. Scavenging of superoxide anion*

183

184 The stock solutions of PMS (30 μM), NADH (936 μM) and NBT (300 μM) were prepared with
185 0.1 M phosphate buffer (pH 7.4). The 50 μl test solution was added into a 96-well microplate

followed by addition of 62.5 µl NBT and 62.5 µl NADH. The mixture was left standing for 2 min for concentration balance. The reaction was started by adding 62.5 µl PMS to the mixture. The reaction mixture was incubated at room temperature for 5 min and absorbance at 560 nm was recorded (Bi et al., 2013). The scavenging activity of superoxide anion is expressed by the following formula:

$$Scavenging\ activity\ (\%) = [(Ac–As)/Ac] \times 100$$

where A_C is the absorbance value of the control and A_S is the absorbance value of the test samples.

2.8.4. Ferric ion chelating activity

In the present assay, 0.5 ml of test solution in a series of concentrations was mixed with 1.6 ml of double de-ionized water, 0.1 ml $FeCl_2$ (2 mM) and 0.1 ml of 5 mM aqueous ferrozine solution. The mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance was measured at 562 nm (Demirtas, Erenler, Elmastas, & Goktasoglu, 2013). EDTA was used as a reference compound for comparison. The calculation was performed using the following formula:

$$Chelating\ activity\ (\%) = [(Ac–As)/Ac] \times 100$$

where A_C is the absorbance value of the control and A_S is the absorbance value of the test sample solution.

204

205 *2.9. Inhibition of tyrosinase in vitro*

206

207 The test sample was dissolved in DMSO and diluted to a series of concentrations. An aliquot of
208 14 μl mushroom tyrosinase (30 U, 40 nM) was pre-incubated with the test sample in 76 μl sodium
209 phosphate buffer (50 mM, pH 6.8) for 10 min at 25°C. Then the 100 μl L-DOPA (4 μM) was added
210 to the sample mixture, which was allowed to stand for 10 min for the formation of dopaquinone. The
211 absorbance was measured at 475 nm by a microplate reader (SpectraMax® M2, Molecular Devices).
212 The inhibition of the enzyme activity was calculated as follows:

213

214 Percent inhibition $(\%) = [(B - S)/B] \times 100$, where B and S were the absorbance for the blank and
215 samples, respectively (Kumar, Yang, Chu, Chang, & Wang, 2010). Ascorbic acid (5 μg/ml) was used
216 as a standard tyrosinase inhibitor for comparison.

217

218 *2.10. Inhibition of browning of mushroom*

219

220 White button mushrooms (*Agaricus bisporus*) with similar size (16±0.5 g; 4±0.2 cm, id) were
221 selected and separated into two groups (10 pieces in each group), one for the treatment and the other
222 as a control. The mushrooms were dipped into a SA-GA-Cu or CA-GA-Cu solution at a

concentration of 187 or 375 µg/ml for 10 s and held until no water dripped from the surface. Each mushroom was weighed before and after the soaking procedure in order to calculate the amount of the active component absorbed by the mushroom. The samples in the control group were subjected to the same procedures except for the double de-ionized water used as the test solution. Each sample in both groups was put in a 100 ml beaker and stored at room temperature. The colour change of samples was monitored at the time period of 0, 4 and 8 h with a colorimeter (CR-200, Minolta, Japan) using a CIE-L*a*b* system for data expression.

2.11. Statistical analysis

Statistical analysis of variance (ANOVA) was conducted using Statistica for Windows (StatSoft, Tulsa, OK, USA). Duncan's multiple range test was used to compare the mean values of data for significant difference at a 95% level.

3. Results and discussion

3.1. Infrared spectra of Schiff base adduct

The IR spectral data of SA-GA or CA-GA and its metal complex are given in Table 1. The C=N

bond can be observed at ν(C-N) 1627-1635 cm^{-1} due to the azomethine stretching vibration, which indicates the successful synthesis of the Schiff base adduct (Guo et al., 2005). Carboxylate (COO^-) stretching occurs at two different spectral regions: an asymmetrical stretching band near 1650-1550 cm^{-1}, and weaker symmetric stretching bands near 1400 cm^{-1} (Gnanasambandam & Proctor, 2000). On complexation in this study, the asymmetric carboxylate stretching $\nu_{asym}(COO^-)$ is shifted from 1610-1618 cm^{-1} to the 1543–1573 cm^{-1} range and the symmetric carboxylate stretching νsym (COO^-) is shifted from 1404-1408 cm^{-1} to 1384 cm^{-1}, indicating the linkage between the metal ion and carboxylate oxygen atom (Nair & Joseyphus, 2008). The spectra of the complexes show broad bands in the 3388–3471 cm^{-1} range, which can be attributed to the stretching vibration of the OH group. This indicates the presence of water molecules in the structure (Hassan, Nassar, Hussien, & Elkmash, 2012). In the complexes, the bands in the 565–586 and 439–470 cm^{-1} range can be attributed to ν(M-N) and ν(M-O), respectively (Nair & Joseyphus, 2008).

3.2. Antimicrobial activity assay for bacteria in vitro

The antimicrobial activity of SA-GA or CA-GA and its metal complex against *S. aureus* and *E. coli* O157：H7 is shown in Table 2. Regarding SA-GA and its metal complexes, these compounds exhibited a better antimicrobial effect against *S. aureus* than against *E. coli* O157：H7. The SA-GA could inhibit *S. aureus* but failed to inhibit *E. coli* O157：H7 at the test concentration of 375 μg/ml. In

contrast, both SA-GA-Cu and SA-GA-Zn could eradicate *E. coli* O157：H7 at 375 μg/ml. This indicates that the metal complex can enhance the antimicrobial activity of SA-GA. Chelation considerably reduces the polarity of the metal ion because of partial sharing of its positive charge with donor groups and possible electron delocalization over the whole chelate ring. Such a chelation could enhance the lipophilic character of the central metal atom, which subsequently favours its permeation through the lipid layer of the cell membrane (Ceyhan, Çelik, Uruş, Demirtaş, Elmastaş, & Tümer, 2011). Moreover, SA-GA-Cu exhibited a greater antimicrobial effect than SA-GA-Zn against *S. aureus*. The reason for the higher antimicrobial activity of Cu^{2+} complex relative to the Zn^{2+} complex may be that Cu^{2+} can form a stronger Cu^{2+}–ligand bond than Zn^{2+}–ligand bond, which increases the lipophilic character of a Cu^{2+} complex compared to a Zn^{2+} complex. In addition, Cu^{2+} has a higher redox activity than Zn^{2+}, which may also account for the higher antimicrobial activity of Cu^{2+} complexes (El-Sherif & Eldebss, 2011).

As with SA-GA, CA-GA also had a good antimicrobial activity against *S. aureus,* which was stronger than that of SA-GA by giving lower MBC values at each corresponding concentration. However, as for the antimicrobial effect against *E. coli* O157：H7, CA-GA could inactivate *E. coli* O157：H7 at 375 μg/ml, but SA-GA could not. The metal complexes of CA-GA also enhanced the antimicrobial activity of CA-GA against *S. aureus.* Noticeably, CA-GA-Cu showed greater antimicrobial activity than CA-GA against *E. coli* O157：H7. In addition, CA-GA-Cu was superior to SA-GA-Cu in suppressing *E. coli* O157：H7. From the results, it seems that Gram (+) bacteria such

as *S. aureus* is more susceptible to the test compounds than Gram (-) bacteria, namely *E. coli* O157：H7. The resistance of Gram (-) bacteria toward antibacterial substances is ascribed to the hydrophilic surface of their outer membrane, which is rich in lipopolysaccharide molecules, forming a barrier to the penetration of numerous antibiotic molecules, and is also relevant to the enzymes in the periplasmic space, which can break down the molecules introduced from outside (Nikaido, 1994; Gao, Van Belkum, & Stiles, 1999).

Since SA-GA-Cu and CA-GA-Cu showed a better antimicrobial activity than the other test compounds in the SA or CA adduct group, both were selected as a representative for each group to further assay their antimicrobial effect in a real food system - mushroom. As the mushrooms were only very briefly (10 s) dipped into the test solutions, the amount of SA-GA-Cu or CA-GA-Cu absorbed into the mushrooms was much lower than that in the test solution, which is listed in Table 3. The initial microbial population in the untreated mushrooms was approximately 10^2 CFU/g. Two concentration levels, 5.8 and 11.6 μg/g, were used for the test. At the concentration of 5.8 μg/g, SA-GA-Cu gave an 82% inhibition ratio of total microorganisms at 2 h; however, the ratio decreased to 67% at 6 h. This decrease may be due to the resuscitation of surviving microorganisms after the administration of the test compound. In contrast, CA-GA-Cu exhibited a 99% inhibition ratio at either 2 or 6 h at the same concentration. Namely, CA-GA-Cu gave a better antimicrobial effect than SA-GA-Cu at this concentration.

At the concentration of 11.6 μg/g, SA-GA-Cu showed a 99% inhibition ratio of the total

299 microorganisms at 2 h and the ratio slightly declined to 93% at 6 h. This result suggests that
300 SA-GA-Cu at this concentration can effectively inhibit >90% microorganisms during 6 h of storage.
301 Comparatively, at the same concentration CA-GA-Cu exhibited a marked antimicrobial effect, which
302 could be observed from the 44% inhibition ratio at the initial time of administrating the test
303 compound (Table 3). CA-GA-Cu could kill or destroy the microorganisms instantly and the damaged
304 organisms seemed to lose their ability to resume growth in the later incubation period. Therefore, the
305 inhibition ratio remains 100% from 2 h to 6 h of storage.

306

307 *3.3. Antioxidant activity*

308

309 *3.3.1. Inhibition of ABTS radical*

310 Assays of 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and
311 1,1-diphenyl-2-picrylhydrazyl (DPPH) are the most popular among various spectrophotometric
312 methods for measuring the antioxidant capacity of foods. ABTS assay is superior to DPPH assay
313 when applied to a variety of plant foods containing hydrophilic, lipophilic and high-pigmented
314 antioxidant compounds (Floegel, Kim, Chung, Koo, & Chun, 2011). Therefore, the ABTS assay was
315 used in this study. The inhibition of ABTS radical activity by the metal complexes of SA-GA or
316 CA-GA is shown in Table 4. Generally, SA-GA and its metal complexes had a stronger scavenging
317 effect than CA-GA and its metal complexes. Noticeably, SA-GA seemed comparable with ascorbic

318 acid in this activity. On the other hand, SA-GA-Cu or SA-GA-Zn did not show any better scavenging
319 activity than SA-GA, which reveals that the metal complexes do not enhance the scavenging effect
320 of SA-GA but slightly reduce it.

321 With respect to CA-GA and its metal complexes, CA-GA had a lower IC_{50} value than that of
322 CA-GA-Cu or CA-GA-Zn, which means that the metal complexes cannot increase the antioxidant
323 activity of CA-GA but slightly decrease it. The effect of metal ions on CA-GA in scavenging ABTS
324 radicals is similar to its effect on SA-GA. In addition, the scavenging activity of CA-GA-Cu is
325 similar to that of CA-GA-Zn. This phenomenon is also applicable to the scavenging activity between
326 SA-GA-Cu and SA-GA-Zn.

327

328 *3.3.2. Inhibition of superoxide anion activity*

329 Inhibition of superoxide anion activity by SA-GA or CA-GA and its metal complexes are listed
330 in Table 4. Regarding SA-GA and its metal complexes, SA-GA exhibited an inhibitory effect with an
331 IC_{50} value of 764 μg/ml. In contrast, SA-GA-Cu and SA-GA-Zn gave IC_{50} values of 5.7 and >6000
332 μg/ml, respectively. This result reveals that the metal complexes do not show a consistent trend in
333 enhancing or reducing the scavenging effect of SA-GA, but manifests that this effect is dependent on
334 the species of the metal ion. In this case, Cu^{2+} enhances the scavenging effect of SA-GA, while Zn^{2+}
335 decreases it. Similarly, with respect to CA-GA and its metal complexes, CA-GA-Cu showed the
336 strongest scavenging effect (IC_{50}: 5.8 μg/ml) among the test compounds, which is comparable with

that (IC_{50}: 5.7 μg/g) of SA-GA-Cu. The IC_{50} value for CA-GA for its inhibitory effect was 1482 μg/ml, which is about twice the value for SA-GA. Meanwhile, as with SA-GA-Zn, CA-GA-Zn did not express any scavenging effect with an IC_{50} value > 6000 μg/ml.

Superoxide dismutases (SODs) are a diverse group of metalloenzymes, and they can catalyze dismutation of two negatively charged superoxide radicals to molecular oxygen and hydrogen peroxide. The redox potential of SOD containing Cu^{2+} and Zn^{2+} (CuZnSOD) during catalysis has been investigated, and the result indicates that copper is the redox partner of the superoxide radical, whereas the oxidative state of Zn^{2+} does not change during the dismutation reaction (Konecny et al., 1999). Several other studies also showed that Cu^{2+}complex of Schiff base adducts can mimic the function of CuZnSOD in dismutating a superoxide anion (Bian et al., 2015; Patel, Patel, Shukla, & Singh, 2013). From these studies, Cu^{2+} seems to play a more important role than Zn^{2+} in scavenging superoxide anion, which is also evidenced from the results of this research.

3.3.3. Inhibition of nitric oxide

Nitric oxide (NO), one of the smallest known bioactive products of mammalian cells, has multiple biological effects. However, a number of disease states are characterized by abnormally high NO production and removing the excess NO could have beneficial effects. In the inhibition of NO radical assay, SA-GA was better than SA-GA-Cu or SA-GA-Zn (Table 4). In contrast, surprisingly CA-GA and its metal complexes did not show any scavenging effect. This may be

attributed to the effect of structural difference. The SA has an OH group on the aromatic ring but CA does not. From literature, a study that compares the reactivity of NO with the metal complexes of Schiff base ligand derived from SA or pyridine-2-carboxaldehyde shows that the pyridine-2-carboxaldehyde-derived ligands do not promote reactivity with NO, whereas, the SA-derived ligands do. Therefore, it is concluded that the substituents on the aromatic moiety have a dramatic effect on the reactivity (Coleman & Taylor, 1980).

3.3.4. Ferrous ion chelating activity

The assay is based on the principle that ferrozine can bind Fe^{2+} rather than Fe^{3+} into a complex that absorbs strongly at 562 nm (Im, Lee, & Löffler, 2013). If the Fe^{2+} is chelated by the test compound, the absorption signal will be reduced. SA-GA or CA-GA shows higher Fe^{2+} chelating activity than its metal complexes (Table 4), which is expected because it is a common characteristic of many synthesized Schiff bases in chelating metal ions (Monier, Ayad, & Abdel-Latif, 2012). However, it is noted that the metal complexes of SA-GA or CA-GA also give certain Fe^{2+} chelating activity even though the ability is lower than that of the metal-free adduct. Several possibilities are hypothesized to explain this. First, Fe^{2+} may compete with the metal ions in the complexed adducts because of relatively high concentration gradient, and partly replace Cu^{2+} or Zn^{2+} in the chelated structure, which may decrease the amount of Fe^{2+} available in the solution for binding ferrozine.

374 Second, because the Cu^{2+} can catalyze the autoxidation of Fe^{2+} to Fe^{3+} (Sayin, 1982), SA-GA-Cu or
375 CA-GA-Cu may also have this ability to convert Fe^{2+} into Fe^{3+}, and thus reduce the amount of Fe^{2+}
376 for the reaction with ferrozine. In contrast, Zn^{2+} does not alter the absorbance of the Fe^{2+}–ferrozine
377 complex (Sayin, 1982); therefore, this may account for the fact that SA-GA-Cu or CA-GA-Cu has
378 greater Fe^{2+} chelating activity than SA-GA-Zn or CA-GA-Zn. Comparatively, SA-GA-Zn has a
379 lower IC_{50} value (207μg/ml) than that (4341μg/ml) of CA-GA-Zn, which may be attributed to the
380 OH group present in the SA structure. The unpaired electrons on the OH group may provide a
381 chelating site for Fe^{2+}, which is not structurally available in the CA.
382

383 *3.3.5. Inhibition of tyrosinase activity*

384 Tyrosinase is a copper dependent enzyme which catalyzes two different reactions using
385 molecular oxygen: the hydroxylation of monophenols to o-diphenols (monophenolase activity) and
386 the oxidation of the o-diphenols to o-quinones (diphenolase activity) (Chen & Kubo, 2002). With
387 respect to SA-GA and its metal complexes, SA-GA-Cu and SA-GA-Zn exhibited a better inhibitory
388 effect on tyrosinase than SA-GA (Table 5). Moreover, SA-GA-Cu is better than SA-GA-Zn at this
389 effect. Similarly, CA-GA-Cu and CA-GA-Zn also gave a stronger inhibitory effect on tyrosinase than
390 CA-GA. In addition, CA-GA-Cu is superior to CA-GA-Zn in exhibiting this effect. From these
391 results, the metal complexes can reinforce the inhibitory ability of SA-GA or CA-GA on tyrosinase,
392 especially the CA-GA. The reason may be that the Cu^{2+} complex of Schiff base adducts could be

attracted to tyrosinase and the Cu^{2+} in the structure could interfere with the electron transferring process at the active site of tyrosinase, which would lead to inactivation of tyrosinase activity. In one study, which used synthetic membranes to immobilize tyrosinase, the researchers found that when Cu^{2+} is chelated on the membrane surfaces, the tyrosinase absorption efficiency can increase due to the effect of binding affinity between the enzyme and the chelated Cu^{2+} (Arıca & Bayramoğlu, 2004).

As for the effect of Zn^{2+} on tyrosinase, it has been suggested in a study that Zn^{2+} can directly form a ligand-binding with tyrosinase at a position near or inside the active site, which induces conformational change that inactivates the enzyme (Han et al., 2007). Although the Zn^{2+} is chelated in the Schiff base adducts, the active site might not be shielded or might be only partially affected by the structure of adducts, depending on the position and orientation of where the Zn^{2+} is present in the structure. Thus, the Zn^{2+} adducts may still retain certain inhibitory effects on the tyrosinase activity. On the other hand, it is probable that the metal ions complexed with the adducts form a more stable or special conformational structure that is favourable for binding tyrosinase and interferes with its activity. The interactive effects of chelators on metal ions have been reported in literature. For example, EDTA and several other metal-ion chelators have been shown to enhance or inhibit iron-dependent lipid peroxidation depending on the types of chelator and the ratio of iron salt to the chelator in the reaction mixture. Regarding the enhancive effect, EDTA can increase the ability of complexed iron to undergo redox cycling and thus accelerate the lipid peroxidation (Gutteridge,

412 1984).

413

414 *3.3.6. Inhibition of mushroom browning*

415 The white button mushroom is widely recognized for its nutritional, organoleptic and medicinal
416 properties, and is highly popular among consumers (Beelman, Royse, & Chikthimmah, 2003).
417 However, the mushrooms are perishable during storage with a shelf life of about 3–4 days. Their
418 commercial value is lost within a few days due to browning, water loss, senescence and microbial
419 attack (Gao, Feng, & Jiang, 2014). As SA-GA-Cu and CA-GA-Cu display good effects on the
420 inhibition of tyrosinase activity *in vitro*, both compounds were used for inhibition of mushroom
421 browning. The colour change of the untreated and treated mushrooms during storage was compared.
422 A colorimeter with a colour scale- CIE-L*a*b* system was used for recording the colour change. L*
423 (0-100) represents lightness from low to high; a* is red, and -a* is green; b* and -b* stand for yellow
424 and blue, respectively. Regarding the colour scale of the control sample, the L* value decreased
425 while the a* and b* values both increased as the storage time increased. Compared with the L*a*b*
426 values of the control sample at the initial time, the colour scale differences (ΔE*) were 4.42 and 8.32
427 for the same sample measured at 4 and 8 h, respectively (Table 6). This indicates that the colour of
428 mushrooms turns darker and consists of more red and yellow components, which is consistent with
429 the browning effect observed visually. When the mushrooms were treated with SA-GA-Cu at 5.8
430 μg/g, the ΔE* values were reduced to 3.57 and 6.85 at 4 h and 8 h, respectively. In addition, the

431 ΔE* values were 2.23 and 4.67 at 4 h and 8 h, respectively, for the samples treated with CA-GA-Cu
432 at the same concentration (Table 6). The results show that SA-GA-Cu and CA-GA-Cu can
433 effectively inhibit the mushroom browning and that CA-GA-Cu gives a stronger effect than
434 SA-GA-Cu, which is consistent with the results obtained from the *in vitro* test of tyrosinase
435 inhibition.

436 Zn and Cu are essential trace elements for human health but over intake of them may cause health
437 risks. Regarding the safety of Zn and Cu intakes, the tolerable upper levels for Zn and Cu are 40 mg/d
438 and 10 mg/d, respectively (Goldhaber, 2003). The effective antimicrobial or antibrowning
439 concentration of the metal complexes used in the mushrooms is ca 12 μg/g. This means that if 200 g
440 (approximately 12 pieces) of the mushrooms are consumed at a meal, the intake of these elements is
441 approximately 2.4 mg, which is lower than the tolerable upper levels of both elements. It is worth
442 noting that the calculation is based on the raw materials; in practice, the mushrooms must be washed
443 or soaked and boiled in water before consumption, which may lead to a marked loss of the added
444 compounds. Therefore, the actual intake of these elements is much lower than the above estimated
445 value. In addition, Zn can compete with Cu and reduce the Cu absorption in digestion (de Romaña,
446 Olivares, Uauy, & Araya, 2011); as a result, one may also use both of the synthesized compounds
447 together as a mixture and adjust to an appropriate ratio between them by taking advantage of the
448 characteristic of Zn that has a higher tolerable upper level. Accordingly, the safety concern can be
449 eliminated when using these adducts and their metal complexes.

4. Conclusions

The Schiff base adducts of GA and SA or CA were synthesized and their functionalities were assessed. These adducts and their metal complexes can impart antibacterial, antioxidant, and/or tyrosinase-inhibition activities. Regarding antibacterial activity, SA-GA and CA-GA both exhibit good antibacterial effects, which can be further enhanced by forming a complex with Cu^{2+} or Zn^{2+}. As for antioxidant activity, SA-GA and CA-GA adducts are more effective than their metal complexes in ABTS, nitric oxide, and ferric ion chelating analyses, whereas their Cu metal complexes are superior to these adducts in superoxide anion analysis. With respect to inhibition of enzymatic browning of mushrooms, both SA-GA-Cu and CA-GA-Cu can inhibit tyrosinase activity effectively. The multiple functionalities of these adducts render them very suitable for preservation of foods such as mushrooms in extension of shelf life.

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588

589 Table 1

590 Infrared spectral data of SA-GA or CA-GA and its metal complexes (cm^{-1})

Compound	υ(C=N)	υ (O-H)	$\upsilon_{asym}(COO^-)$	$\upsilon_{sym}(COO^-)$	υ (M-N)	υ (M-O)
SA-GA	1633	3446	1610	1408	—	—
SA-GA-Cu	1627	3444	1554	1384	574	439
SA-GA-Zn	1631	3427	1543	1384	570	453
CA-GA	1635	3471	1618	1404	—	—
CA-GA-Cu	1633	3388	1570	1384	586	457
CA-GA-Zn	1631	3415	1573	1384	565	470

591 Table 2

592 Antimicrobial activity of SA-GA or CA-GA and its metal complexes against selected bacteria

Compound	MBC (μg/ml)	
	Staphylococcus aureus	*Escherichia coli* O157：H7
SA-GA	93	＞375
SA-GA -Cu	23	375
SA-GA -Zn	46	375
CA-GA	46	375
CA-GA-Cu	11	187
CA-GA -Zn	23	375

593 Table 3

Inhibition ratios of total microorganisms in the mushrooms by SA-GA or CA-GA and its metal complex

Compound	Compound/ mushroom (μg/g)	Inhibition ratio (%)		
		0 h	2 h	6 h
SA-GA-Cu	5.8	0 [a]	82±5 [a]	67±2 [a]
SA-GA-Cu	11.6	0 [a]	99±1 [b]	93±1 [b]
CA-GA-Cu	5.8	0 [a]	99±1 [b]	99±1 [c]
CA-GA-Cu	11.6	44±8 [A]	100±0 [b]	100±0 [c]

[1] Superscripts in the same column with the same letter are not significant different at $p<0.05$

Table 4

Antioxidant activity of SA-GA or CA-GA and its metal complex expressed as IC_{50} (μg/ml) determined by free radical scavenging or metal chelating methods

Compound	ABTS	Superoxide anion	Nitric oxide	Ferric ion
SA-GA	5.7±0.1	764±3	908±23	26±0.1
SA-GA-Cu	12.4±0.2	5.7±0.1	1078±22	105±0.4
SA-GA-Zn	16±0.3	＞6000	1006±9	207±0.8
CA-GA	124.4±1.3	1482±6	＞6000	83±0.3
CA-GA-Cu	162.5±2.8	5.8±0.1	＞6000	111±0.1
CA-GA-Zn	165.7±2.7	＞6000	＞6000	4341±51

Ascorbic acid	2.7±0.01	NA[a]	NA	NA
EDTA	NA	NA	NA	13.2±0.1

[a]NA: not analyzed.

Table 5

Inhibition of tyrosinase activity by the metal complexes of SA-GA or CA-GA

Compound	Concentration range(μg/g)	IC_{50}(μg/g)	Regression equation	R^2
SA-GA	187-1250	899	y=0.001x＋39.16	0.99
SA-GA-Cu	93-750	345	y=0.004x＋22.41	0.95
SA-GA-Zn	93-750	556	y=0.003x＋16.64	0.96
CA-GA	187-1250	459	y=0.002x＋31.63	0.95
CA-GA-Cu	39-312	73	y=0.0071x＋39.65	0.98
CA-GA-Zn	39-312	118	y=0.003x＋42.92	0.97
Ascorbic acid	12-50	22	y=0.073x＋18.16	0.95

Table 6

619 Color change of the mushrooms with or without the treatment of SA-GA-Cu or CA-GA-Cu during

620 storage at room temperature

Treatment	Concentration (μg/g)		Time (h)		
			0	4	8
Control	—	L*	90.81±0.3^{a2}	87.79±1.12^{b}	84.26±0.99^{c}
		a*	0.24±0.03^{a}	0.21±0.04^{a}	1.41±0.16^{b}
		b*	12.33±0.33^{a}	15.55±0.71^{b}	17.33±0.64^{b}
		ΔE*[1]	—	4.42±0.29^{A3}	8.32±0.38^{A}
SA-GA-Cu	5.8	L*	90.86±0.44^{a}	88.43±0.91^{a}	87.06±1.51^{b}
		a*	0.00±0^{a}	0.47±0.04^{a}	1.40±0.28^{b}
		b*	11.63±0.91^{a}	14.2±0.34^{b}	17.15±0.37^{c}
		ΔE*	—	3.57±0.72^{A}	6.85±0.97^{AB}
SA-GA-Cu	11.6	L*	90.44±0.2^{a}	88.63±0.31^{b}	87.09±0.17^{c}
		a*	-0.24±0.06^{a}	0.03±0.01^{b}	0.65±0.04^{c}
		b*	10.23±0.33^{a}	13.68±0.49^{b}	15.89±0.59^{c}
		ΔE*	—	3.91±0.31^{A}	6.64±0.25^{B}
CA-GA-Cu	5.8	L*	90.88±0.33^{a}	88.84±0.98^{ab}	87.38±0.55^{b}
		a*	0.01±0.03^{a}	0.65±0.04^{b}	0.71±0.08^{b}
		b*	12.71±0.58^{a}	13.33±0.48^{a}	15.72±0.65^{b}
		ΔE*	—	2.23±0.6^{B}	4.67±0.08^{C}
CA-GA-Cu	11.6	L*	90.82±0.03^{a}	90.21±0.47^{a}	89.03±0.18^{b}
		a*	-0.03±0.0^{a}	0.16±0.06^{b}	0.46±0.06^{c}
		b*	11.68±0.08^{a}	13.29±0.44^{b}	15.37±1.16^{b}
		ΔE*	—	1.73±0.18^{B}	4.13±0.89^{C}

621 [1] $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{½}$

622 [2] Superscripts in the same row with the same letter are not significant different at $p<0.05$

623 [3] Superscripts in the same column for the ΔE* values with the same capital letter are not significant

624 different at $p<0.05$

625

626 >We synthesize adducts using **γ-**aminobutyric acid and salicylaldehye or cinnamaldehyde.

627 > These adducts have antibacterial, antioxidant, or tyrosinase-inhibition activity.

628 > These adducts are very suitable for preservation of mushrooms.

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630