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Synthesis and Antibacterial Activity of Dihydromyricetin-Co(II)

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Dihydromyricetin is a natural food additives with good prospects in food industry, But its little research is on dihydromyricetin complex with better effects. Antibacterial agent is very important in food industry, but little attention has been devoted to the relationship between dihydromyricetin-metal complex and antibacterial activity. In this paper, dihydromyricetin-Co(II) has been prepared and the interaction of dihydromyricetin-Co(II) complx with antibacterial activity was investigated by tube double dilution method. The results indicate that the dihydromyricetin-Co(II) conditional stability constant is 6.47×10^{13} L^2 mol^{-2} and its antibacterial activity could effectively inhibit the growth of *Candida albicans*. The minimum antimicrobial dosage of dihydromyricetin-Co(II) against *Candida albicans* was 12.5-25 μg/mL. The minimum bactericidal dosage was 25-50 μg/mL. The result showed dihydromyricetin-Co(II) against *Candida albicans* was stronger than that of dihydromyricetin.

Keywords: Dihydromyricetin-Co(II) complex, Flavonoid, Antibacterial activity.

INTRODUCTION

Ampelopsis grossedentata, a wild plant in South China, is a flavonol-rich plant source. Dihydromyricetin as a major flavonoid attract great interest for its important function in food industry and biochemistry (Fig. 1). Flavonoids are important components of function food and are reported to have beneficial effects such as the cardiovascular, as well as antibacterial, antioxidant, antihypertensive, analgesic properties, *etc*.[1,2]. Due to flavonoid stabile conjugated π electrons with coordination oxygen atoms having the characteristic of quite strong chelation and the chemistry research indicating that the biological activity of flavonoid metal complex is better than itself mostly[3,4]. It is very important to study the flavonoid metal complex and its function on the food industry[5]. However, there is a less literature[6,7] on dihydromyricetin metal complex and the antibacterial activity of the dihydromyricetin metal complex. The transition metal ions with 3*d* electronic structures have effect for such as tromethamine, as well as sterilization, anticoagulant, analgesia, *etc*.[8-9]. It is expected that dihydromyricetin combined with cobalt ion might result in increase of antibacterial activity and bioactivity of dihydromyricetin. In this study, dihydromyricetin-cobalt complex was prepared. Its physicochemical properties and antibacterial activity were investigated.

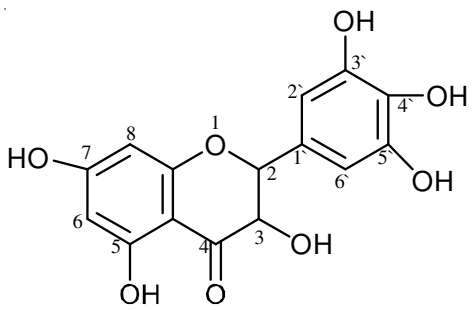

Fig. 1. Structural formula of dihydromyricetin

EXPERIMENTAL

Leaves of *A. grossedentata* for this study were bought in Guangxi, China. Culture medium were purchase from Tianhe Microorganism Regent Co., Ltd (Hangzhou, China). Other chemicals were of analytical grade.

Preparation of dihydromyricetin: Dihydromyricetin was from *Ampelopsis grossedentata* extacted with ethanol reflux extraction[7-10]. Leaves of *Ampelopsis grossedentata* were extracted with 65 % ethanol for 1.5 h and then fitered through the gause. The filtrate was left to stand for 24 h under 0-4 °C temperature after being condensed by Rotary Evaporators. The filtered precipitate was colleted and purified to provide the white crystal by recrystallizing from water and petroleum ether and acetone and water in order. After being dried, the dihydromyricetin was determined by Shimadzu LC-10A HPLC system

consisted of C18 column, gradient method was constant current, detection wavelength at 296 nm, column temperature was 30 °C, detection was a UV detector. The dihydromyricetin product with purity of 95 % by high performance liquid in Fig. 2 and Table-1.

Preparation of dihydromyricetin-cobalt complex: Dihydromyricetin (0.6404 g) (2 mmol) was stirred in 50 mL alcohol at a room temperature until dissolved fully. Sodium acetate was added to adjust the pH amount to 7.5 and temperature was kept under 60 °C for 0.5 h. Cobaltous chloride (1 mmol) was added to mixture to keep being stirred and temperature was kept under 60 °C for 6 h. After the reaction finished, the temperature was cooled down to the room temperature. And then, the precipitate formed was isolated by filtration and washed repeatedly with absolute ethyl alcohol and dried under the vacuum in 40 °C. The resultant black power was collected as dihydromyricetin-cobalt complex.

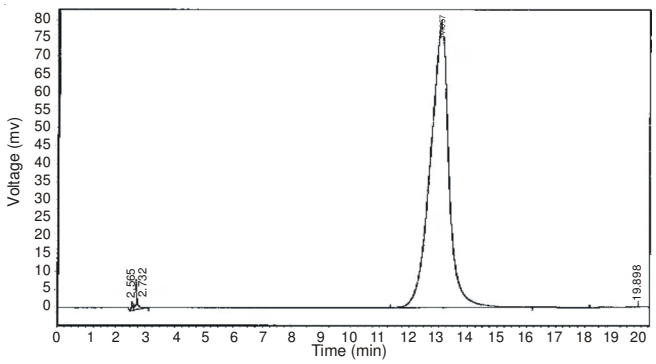

Fig. 2. HPLC of dihydromyricetin

TABLE-1
ANALYSIS DATES OF DYHYDROMYRICETIN HPLC

Peak number	Retention time	Peak height	Peak area	Content
1	2.565	1076.689	4905.117	0.1379
2	2.732	1530.230	18645.084	0.5242
3	13.057	78800.258	3530133.500	99.2424
4	19.898	139.710	3397.000	0.0955

Determination of conditional stability constant: The complex stability constant was measured according to the relationship between the molar ratio of each component in the complex and absorbance empolying a published method of equimolar continuous change method and molar ratio method by UV-visible spectrophotometer[11].

IR analysis: IR analysis was performed on a JASCO-FI-IR-4100 infrared spectrophotometer (Tianjin, China) by the KBr method.

Antibacterial activity tests: The complex and ligand (dihydromyricetin) were tested for their *in vitro* antimicrobial activity against *Staphylococcus aureus* and *E. coli* and *Candida albicans* using the serial two-fold tube dilution method. The complex and ligand were dissolved in SWFI (sterile water for injection) containing 1 % Tween-80 as solubilizer to obtain testing solution with concentration of 400 μg mL^{-1}. *Staphylococcus aureus* and *E. coli* bacteria were cultured on Muller-Hinton Agar plate (37 °C, 24 h) and *Candida albicans* was cultured on Sabouraud agar plate (28 °C, 48 h). After that, the suspensions were diluted with 0.5 McFarland standard turbidity and diluted again to 10^5 CFU mL by broth. A positive control (containing inoculums but no tested compounds) and negative control (containing tested compounds but no inoculums) were included on each micro tubes. The contents of the tubes were mixed and the micro tubes were incubated at 37 °C for 24 h and 28 °C for 48 h, respectively under microaerophilic conditions. The each organism was tested 3 times on different days to measure the reproducibility of the test. Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the compounds under test that inhibit the visible growth of microorganisms when the latter is at optimal concentration[12]. Minimum bactericidal concentration (MBC) was also determined for complex. The MBCs were determined according to the recommendation of CLSI (formerly National Committee for Clinical Laboratory Standards)[13] with some modifications. After MIC determination bacterial cultures were subcultured in plates of solid media and 0.1 mL sample of broth from each well with no growth were transferred on to plain Muller-Hinton agar plates for 20 h incubation at 37 °C and *Candida albicans* was cultured on Sabouraud agar plate (28 °C, 48 h). The antibiotic concentration inducing a 99.9 % loss of viability was defined as minimum bactericidal concentration[14]. Each assay was repeated at least three times to confirm the reproducibility.

RESULTS AND DISCUSSION

The production method of dihydromyricetin in this study was very simple and the purity of dihydromyricetin obtained was more than 95 % in Fig. 2. We prepared the complex according to different quantity ratio of dihydromyricetion and cobalt-ion, such as 0.25, 0.5, 1, 1.5, 2, 2.5, 3. The results showed that when the ratio was 2, the complex absorbance was maximum and the complex was the most stable by equimolar continuous change method and molar ratio method. the relationship between the molar ratio of each component in the complex and absorbance was shown in Fig. 3. We prepared dihydromyricetin-cobalt complex in term of the quantity ratio 2. The obtain complex was used for the following structural analysis and antimicrobial activity.

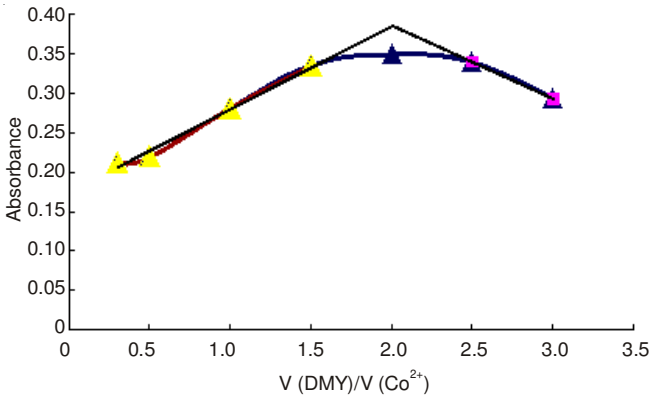

Fig. 3. Absorption curve by equimolar continuous variation method

Determination of conditional stability constant: The stable maximum absorbance on the basis of the experiment (A_0) and the maximum absorbance in term of theory (A_{max}) at

the composition of Co(II):DMY = 1:2 was 0.350 and 0.387 (Fig. 3). The DMY-Co(II) degree of ionization of 0.0956 is given by

$$\alpha + \frac{A_0 - A_{max}}{A_0}$$

The conditional stability constant ($K_s = 6.47 \times 10^{13}$ L^2 mol^{-2}) was calculated according to the following formula:

$$K_S = \frac{1-\alpha}{4c^2\alpha^3}$$

where α is the degree of ionization of complex. c is the complex concentration at the maximum absorbance.

IR analysis: The infrared spectra of dihydromyricetin and dihydromyricetin-cobalt(II) were shown in Figs. 4 and 5. The IR spectral studies provide important clues regarding the dihydromyricetin and its cobalt complex.The ligand stretching frequency of the carbonyl and phenolic hydroxyl groups involved in coordination undergo shift which can be taken as an evidence of coordination. The IR spectrum of dihydromyricetin shows characteristic absorption bands of the carbonyl groups at 1641 cm^{-1} ($\nu_{C=O}$) and at 1271 cm^{-1} (ν_{C-O-C}) while for dihydromyricetin-cobalt these signals are placed at 1618 cm^{-1} and at 1265 cm^{-1}, respectively. The metallic ion in coordination with oxygen causes carbonyl bond to be weaker. In IR spectrum of complex analysis, the metal absorption band at 660 cm^{-1} (ν_{O-Co}) as a new peaks was observed. These observation suggested that some weak physical interactions between dihydromyricetin and cobalt ion took place during the formation of the complex.

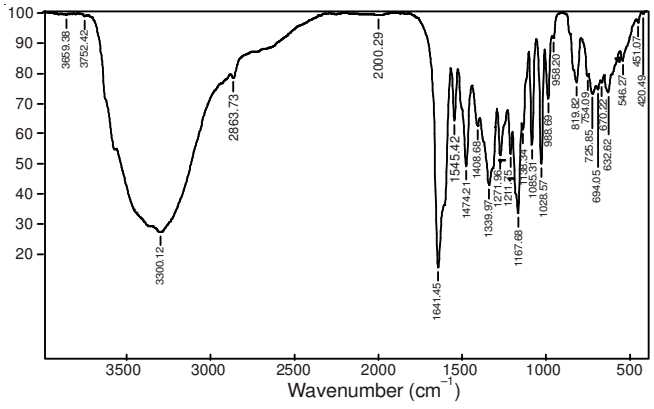

Fig. 4. IR spectra of dihydromyricetin

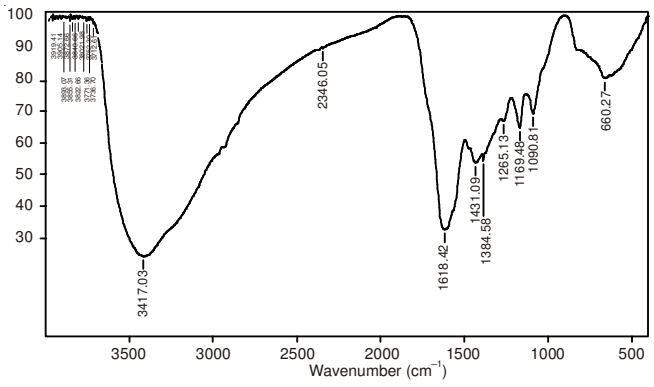

Fig. 5. IR spectra of dihydromyricetin-cobalt(II)

Antibacterial screenig: The antibacterial activity of the complex was qualitatively determined by experiment in which three bacteria, namely *Staphylococcus aureus* (ATCC25923), *E. coli* (ATCC25922) and *Candida albicans* (ATCC10231) using the serial two-fold tube dilution method. The susceptibility of certain strains of bacterium towards dihydromyricetin and dihydromyricetin-cobalt(II) was judged by measuring the MIC and MBC. The results of these investigations are listed in Table-2. It can be seen that dihydromyricetin-cobalt(II) is slightly inferior to ligand (dihydromyricetin) against *Staphylococcus aureus* and *E. coli*, However, the complex exhibits stronger antibacterial activity against *Candida albicans* than the ligand. This is explained by Tweedy`s chelation theory[15]. Significant MIC values of compound indicated 25 μg/mL according to results in Table-2 and MBC values indicated 50 μg/mL against *Candida albicans* in Table-2 and in Fig. 6. On chelation, the polarity of the Co(II) ion reduced to a greater extent due to the overlap of the ligand orbitals and partial sharing of the positive charge of the Co(II) ion with the donor groups. Further, it increases the dolocalization of π electrons over the whole chelate ring and hence enhances the liposolubility of DMY and further enhances the penetration of the DMY-Co(II) into the lipid membrance. It was likely to the structure of complex was more steady and stronger interaction with *Candida albicans* than the others. It is perceived that the factors such as solubility, conductivity, dipole moment and cell permeability may also contribute for the increased activity of the complexes[16-18].

TABLE-2
MINIMUM INHIBITORY CONCENTRATION DATA AND MINIMUM BACTERICIDAL CONCENTRATION DATA OF DMY AND DMY-Co (MICROGRAM PER LITER)

Pathogenic bacteria	MIC(μg mL^{-1})		MBC (μg mL^{-1})		Drug control	Positive control	Negative control
	DMY	DMY-Co	DMY	DMY-Co			
Staphylococcus aureus	100	400	200	>400	-	+	-
E.coli	200	400	400	>400	-	+	-
Candida albicans	>400	25	>400	50	-	+	-
ATCC25923	100	400	200	>400	-	+	-
ATCC25922	400	400	400	>400	-	+	-
ATCC10231	>400	25	>400	100	-	+	-

Note: "-" represents "no bacteria growth"; "+" represents "bacteria growth"

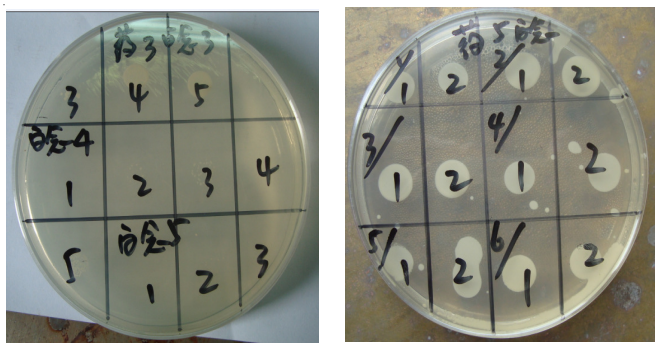

Fig. 6. Bactericidal experiments of DMY-Co(II) (drug 3) and DMY(drug 5) against *Candida albicans*

Conclusion

In conclusion, a new dihydromyricetin-cobalt(II) complex was readily synthesized and determined by IR. The dihydromyricetin-Co(II) conditional stability constant is determined by UV-visible spectrophotometer. Antibacterial active results showed that the complex exhibited stronger antimicrobial activity against *Candida albicans* compared to dihydromyricetin. This will be significant for further development of dihydromyricetin and improvement of antibacterial drug.

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