Research Article

Evaluation of Anti-MRSA and Xanthine Oxidase Inhibition Activities of Phenolic Constituents from *Plumula nelumbinis*

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Isolation of metabolites from *Plumula nelumbinis* led to the discovery of eleven compounds, including six flavonoids and five phenolderivatives. Their structures have been determined on the basis of chemical and spectroscopic data. Most of them, such as compounds **1**, **4**, **6**, **8**, and **10**, have shown inhibitory activity against hospital-acquired methicillin-resistant *Staphylococcus aureus* (HA-MRSA). MICs of compound **8** against SA-200195 and SA-300150 were 2 µg/mL and 8 µg/mL, respectively. And the antioxidant activity of isolated compounds was determined by checking the scavenging activity against three different radicals: 2,2-diphenyl-1-picryldrazyl (DPPH) radical, hydroxyl radical ($\cdot\text{OH}$), and superoxide anion ($\text{O}_2^{\cdot-}$), as well as xanthine oxidase inhibition. All flavonoids showed strong antioxidant activity. And compound **6** displayed the highest inhibitory effect against xanthine oxidase with IC_{50} value of 8.2 µg/mL.

1. Introduction

Plumula nelumbinis is the dried young cotyledon and radicle of ripe seed of *Nelumbo nucifera* Gaertn (Nymphaeaceae). It is a well-known traditional herbal heat-clearing and detoxifying medicine which is frequently used to clear the heart fire, stop bleeding, and arrest seminal emission in many countries [1–3]. *Plumula nelumbinis* contains various alkaloids and flavonoids [4]. Previously, rutin, hyperin, and galuteolin were reported as the antioxidative flavonoids. The extraction of *Plumula nelumbinis* showed antioxidant effect on superoxide radical and hydroxyl radical [5].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most common cause of nosocomial infections. MRSA is defined as difficult-to-treat strain of *Staphylococcus aureus* which resists to almost all antibiotics [6]. However infections caused by MRSA have been a major threat to public health in hospitals and the community during the past decade. And there were few studies about anti-MRSA effect of *Plumula nelumbinis*. So the discovery of new anti-MRSA agents will greatly help improve healthcare safety.

2. Experimental

2.1. General Experimental Procedures and Chemicals. All melting points were determined on a Fisher-Johns apparatus and are uncorrected. Optical rotations were measured with a WZZ-2S digital polarimeter spectrophotometer. ^1H , ^{13}C NMR, DEPT, ^1H - ^1H COSY, NOESY, HSQC, HMBC, and HSQC-TOCSY spectra were taken with Bruker AM-400 and DRX-500 spectrometer in CD_3OD or $\text{DMSO}-d_6$. Negative ion FABMS and HRFABMS were recorded using a matrix of triethanolamine on a JEOL SX-102A spectrometer.

Xanthine, xanthine oxidase, 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), deoxyribose, nitroblue tetrazolium (NBT), and phenazine methosulphate (PMS), were obtained from Sigma Chem. Co. (St. Louis, USA). And other chemicals used were obtained from China National Medicines Co. Ltd. (Beijing, China).

2.2. Plant Material. *Plumula nelumbinis*, the buds of *Nelumbo nucifera* Gaertn, were purchased from Fujian Medicine Co.

Ltd, Fujian Province, People's Republic of China, in September 2007 and identified by Professor Ke-Cuo He, College of Plant Protection, Fujian Agriculture and Forestry University. A voucher specimen (No. 070923) was deposited in the College of Plant Protection, Fujian Agriculture and Forestry University.

2.3. Extraction and Isolation. The air-dried *Plumula nelumbinis* (5.0 kg) were powdered and extracted exhaustively by maceration with MeOH at room temperature. The extract solution was concentrated under diminished pressure to afford 110 g of dried extract. The extract was subjected to column chromatography over macroporous adsorbent resin D101 (Shanghai Anlande Co. Ltd.) and eluted with H₂O and MeOH, and MeOH eluent was concentrated to afford MeOH fraction (52 g). MeOH fraction submitted to column chromatography over silica gel and eluted with a gradient CHCl₃-MeOH (100:1-2:1) to afford ten fractions. Fr. 3 (2.1 g) submitted to Sephadex LH-20 column chromatography eluted with MeOH to afford three fractions. Afterwards, SFr. 3 (265 mg) was analyzed by normal-phase semipreparation HPLC with CHCl₃-acetone (9:1) to yield **8** (26.7 mg) and **9** (97.4 mg). Fr. 6 (6.2 g) as mentioned above was subjected to step-gradient silica gel column chromatography with a solvent consisting of CHCl₃-MeOH to afford six fractions. And then, SFr. 2 (356 mg) was subjected to column chromatography over RP-18 and eluted with a gradient 40%-90% MeOH. Further purified by RP-18, semipreparation HPLC eluted with 75% MeOH to yield **1** (52.7 mg) and **4** (43.7 mg). Fr. 7 (308 mg) was purified over RP-18 with 80% MeOH to yield **6** (12.8 mg) and **11** (39.0 mg). Fr. 8 (620 mg) was purified over RP-18 with 65% MeOH to yield **2** (82.3 mg) and **5** (72.1 mg). Fr. 9 (3.9 g) was subjected to step-gradient silica gel column chromatography with a solvent consisting of CHCl₃-MeOH to afford **10** (49.3 mg), **3** (38.0 mg), and three fractions. SFr. 3 (216 mg) was purified by Sephadex LH-20 and then purified by RP-18 semipreparation HPLC eluted with 75% MeOH to yield **7** (22.6 mg).

2.4. Bacterial Strains and Media. Five HA-MRSA strains were provided by Fujian Medical University Union Hospital. All strains were cultured on nutrient agar before determination of MIC values. Mueller-Hinton broth (MHB) was used for susceptibility tests.

2.5. Susceptibility Testing against HA-MRSA. Minimum inhibitory concentration (MIC) values were determined by standard microdilution procedures in duplicate, as recommended by the National Committee for Clinical Laboratory Standards Guidelines [7]. Concentration of test compounds ranged from 4 to 128 $\mu\text{g/mL}$. An inoculum density of 5×10^5 – 1×10^6 cfu of each test strain was prepared by blood counting chamber. MHB (100 μL) was dispensed into 1–6 wells of a 96-well microtiter plate (Nunc, 300 μL volume per well). All compounds were dissolved in DMSO before diluting into MHB. And the highest concentration of DMSO remaining after diluting (3% v/v) caused no evident inhibition of HA-MRSA growth. Then, compound **1** was dispensed into

well 1 (128 $\mu\text{g/mL}$) and serially diluted across the plate (64–4 $\mu\text{g/mL}$). Then the inoculum (100 μL) was added into wells 1–6. And susceptibility testing of other compounds was determined as described above, leaving 2 wells empty for growth control of each strain and 2 wells being free of inoculum served as the sterile control in a 96-well microtiter plate. Then the plates were incubated at 37°C for 18 h. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; sigma) was dissolved in phosphate buffered saline (PBS, 5 mg/mL), and it was used to detect bacterial growth by color change from yellow to dark blue. The MICs were defined as the lowest concentration which yielded no visible bacterial growth. All samples were tested in triplicate.

2.6. Xanthine Oxidase Inhibition Assay. The xanthine oxidase inhibition activity was evaluated by the slightly modified method of the previously reported method [8]. The conversion of xanthine to uric acid was calculated according to the increasing absorbance at 290 nm. Test compounds were dissolved in a small amount of DMSO (final concentration < 1%) and diluted with phosphate buffer (20 mM, pH 7.5). Test solutions were prepared by adding xanthine (final concentration 50 μM), hydroxylamine (final concentration 200 μM), EDTA (final concentration 100 μM), and test compounds in various concentrations. The reaction was started by adding 2.5 mU/mL xanthine oxidase in a phosphate buffer solution (200 mM, pH 7.5). The mixture was incubated for 30 min at 37°C. The reaction was terminated by adding 100 μL of HCl (0.58 M) and the variation of absorbance was measured against a blank which was prepared in the same way except that the xanthine oxidase was replaced by buffer solution. The absorbance of the mixture was measured at 290 nm. And a test mixture containing no test compounds was prepared to measure the total uric acid production. Allopurinol was used as a reference inhibitor. All tests were carried out in triplicate. The half-maximal inhibitory concentration (IC_{50}) was calculated by linear regression analysis.

2.7. DPPH Free Radical Scavenging Assay. 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH), a stable free radical, shows strong absorption at 515 nm (in ethanol), as a deep violet color. The concentration of DPPH was 300 μM . The test compounds were dissolved in DMSO and DPPH solution was prepared in ethanol. After incubation, decrease in absorption was measured at 515 nm by using a multiplate reader [9]. Percent radical scavenging activity of compounds was determined in comparison with a DMSO treated control group. The compounds were done in triplicate. The following formula was used as calculating % radical scavenging activity:

$$\% \text{ RSA} = \left(\frac{\text{OD}_{\text{control}} - \text{OD}_{\text{test compound}}}{\text{OD}_{\text{control}}} \right) \times 100. \quad (1)$$

2.8. Hydroxyl Radicals Scavenging Assay. Hydroxyl radicals scavenging activity was evaluated using the hydroxyl radical system generated by the Fenton reaction [10]. The reaction mixture containing 2 mM deoxyribose, 0.1 mM ferric chloride, 0.1 mM ascorbic acid, 0.1 mM EDTA, and 2 mM

FIGURE 1: Structure of compounds 1–11.

H₂O₂ in 20 mM phosphate buffer (pH 7.4) was added to every concentration of the test compounds. The reaction was started by the addition of H₂O₂. After incubation at 37°C for 3 h, the reaction was stopped by adding 0.75 mL of 3.0% trichloroacetic acid and 0.75 mL of 1.0% of 2-thiobarbituric acid in 50 mM NaOH. Then the solution was boiled for 10 min and cooled in water. The absorbance of the solution was measured at 520 nm. Scavenging capacity was expressed as inhibitory concentration of test compound required to produce 50% inhibition of hydroxyl radicals (IC₅₀ values).

2.9. Superoxide Anion Radical Scavenging Assay. Superoxide anion radical scavenging activities of test compound were determined by monitoring the competition of those with NBT for the superoxide anion generated by the PMS-NADH system [11]. Superoxide anion radical was generated in 20 mM Tris-HCl buffer (pH 7.8) containing 50 μM nitroblue tetrazolium (NBT), 10 μM phenazine methosulphate (PMS), and test compounds in various concentrations. After at 25°C for 5 min, the reaction was started by adding 78 μM NADH. Blue chromogen, formed due to NBT reduction, was read at 560 nm. Results were expressed as percentage of inhibition.

3. Results and Discussion

The MeOH extract and isolated compounds 1–11 were screened for anti-MRSA and xanthine oxidase inhibition activities. The MeOH extract was proved to be effective with MIC at 64, 64, 32, 64, and 32 μg/mL, respectively, against these five HA-MRSA strains and with IC₅₀ = 76.5 μg/mL against xanthine oxidase. In order to substantiate the result and find out which compound inhibited HA-MRSA, the extract was subjected to column chromatography over silica gel, RP-18, Sephadex LH-20, and semipreparation HPLC to yield the active compounds 1–11.

3.1. Structural Elucidation of Compounds 1–11. Structural elucidation of compounds 1–11 obtained from *Plumula*

nelumbinis (Figure 1) makes them identified as follows: six flavonoids, namely, quercetin-3-*O*-β-D-glucopyranoside (1), isorhamnetin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (2) [12], acacetin-3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (3) [13], isovitexin (4) [14], lonicerin (5) [15], quercetin (6) [16]. Five phenolderivatives, namely, phenol-1-*O*-β-D-glucopyranoside (7) [17], 4-hydroxybenzyl alcohol (8), 4-hydroxyphenethyl alcohol (9) [18], 4-hydroxycinnamic acid (10), 4-*O*-β-D-glucopyranosyl-trans-cinnamic acid (11) [19] were obtained from *Plumula nelumbinis*. The structures determination of compounds 1–11 was established using NMR spectral method and their spectral data were compared to previous literature values.

3.2. MICs of Compounds 1–11 against HA-MRSA. The five HA-MRSA strains were isolated from patients in the hospital. Among the isolated compounds, compound 8 exerted anti-MRSA activities against all the strains tested with MIC values ranging from 2 to 64 μg/mL which has not been reported yet. What is more, the inhibitory effect of compound 8 was obviously observed by treating it against strain SA-200195 (2 μg/mL) and SA-300150 (8 μg/mL), respectively. Compounds 1, 4, 6, and 10 showed weak anti-MRSA effect against five HA-MRSA strains. Other compounds did not show any statistical significance in anti-MRSA activity (Table 1).

3.3. Xanthine Oxidase Inhibition Activity. All isolates were tested by the xanthine oxidase inhibitory activity assay (Table 1). Compounds 1–6 that were isolated from the methanol extract of *Plumula nelumbinis* showed xanthine oxidase inhibitory activity. In addition to the known xanthine oxidase inhibitory flavone [20], compounds 6 showed the most effective inhibitory activity with IC₅₀ value of 8.2 μg/mL. IC₅₀ of the others flavonoids ranged from 23.9 to 68.9 μg/mL as shown in Table 1. Interestingly, compound 4 showed lower xanthine oxidase inhibitory activity than the others flavonoids, because of the presence of 6-*C*-glycosyl

TABLE 1: Xanthine oxidase inhibitory activity, scavenging effect, and susceptibility of HA-MRSA to compounds 1–11.

Cpd ^a	Xanthine oxidase IC ₅₀ (μg/mL)	MIC (μg/mL)					Scavenging effect IC ₅₀ (μg/mL)		
		SA-100180 ^b	SA-100220	SA-200195	SA-211510	SA-300150	DPPH	•OH	O ₂ ^{•−}
1	24.6	>128	64	32	64	64	2.3	273.9	150.7
2	23.9	>128	>128	64	>128	128	5.8	264.1	158.9
3	28.2	>128	>128	64	>128	>128	3.4	266.7	167.4
4	68.9	128	64	32	64	64	23.3	348.5	186.3
5	37.4	>128	128	64	>128	64	9.2	236.8	143.9
6	8.2	64	64	32	32	16	1.1	206.7	123.4
7	>100	>128	>128	>128	>128	>128			
8	>100	64	64	2	64	8			
9	>100	>128	>128	128	>128	>128			
10	>100	32	32	32	64	32			
11	>100	>128	>128	128	>128	>128			
Allopurinol	3.2								
Rifampicin		2	1	1	2	1			
Ascorbic acid							1.3	166.5	111.9

^acpd: compound.^bSA-100180, SA-100220, SA-200195, SA-211510, and SA-300150 are all methicillin-resistant *Staphylococcus aureus* (HA-MRSA).

groups. The IC₅₀ value of allopurinol, which coassayed in the study as positive control, was shown to be 3.2 μg/mL.

3.4. DPPH Scavenging Activity. The DPPH scavenging activity of tested compounds is shown in Table 1. The antioxidative activity of the flavonoids (1–6) was analyzed using the scavenging radicals activity test. All compounds showed potent DPPH scavenging activity. Compound 6 was more potent than ascorbic acid, with IC₅₀ values of 1.1 μg/mL. Other compounds with IC₅₀ values ranged from 2.3 to 23.3 μg/mL. And ascorbic acid was used as positive control. The results indicated that a carbonyl group at C-4 and a double bond between C-2 and C-3 were essential for DPPH scavenging activity.

3.5. Hydroxyl Radicals Scavenging Activity. Increased production of reactive oxygen species (ROS) beyond the body's antioxidant capacity gives rise to oxidative stress. Much of the oxidative damage to biomolecules is induced by hydroxyl radical (•OH), the most reactive one among ROS species [21]. As described in Table 1, compounds 1–3, 5, and 6 showed higher hydroxyl radical scavenging activity than 4 in a concentration-dependent manner. The result provided evidence that flavonoids from *Plumula nelumbinis* had significant effect on scavenging hydroxyl radical.

3.6. Superoxide Anion Radical Scavenging Activity. Superoxide anion scavenging abilities of tested compounds were estimated using PMS-NADH system (NBT method). Results were used to indicate the superoxide productivity. Compounds 1–3, 5, and 6 showed higher superoxide anion

scavenging activity, and this activity was decreased in the order 6 > 5 > 1 > 2 > 3. Specifically, compound 9 showed the highest scavenging activity (IC₅₀ = 123.4 μg/mL). Moreover, other compounds showed the scavenging activities by IC₅₀ value which ranged from 143.9 to 248.3 μg/mL. The IC₅₀ value of ascorbic acid was shown to be 111.9 μg/mL.

4. Conclusion

Flavonoids have been shown to act as scavengers of various oxidising species, 2,2-diphenyl-1-picryldrazyl radical (DPPH), superoxide anion (O₂^{•−}), and hydroxyl radical (•OH). Previous studies [22] have revealed that a catechol moiety in B-ring is the molecular structural basis of the antioxidant activity. Meanwhile, a carbonyl group at C-4 and a double bond between C-2 and C-3 are also important characters for high antioxidant activity in flavonoids. Similarly, isoflavones are often more active than flavones because of the stabilization effects of the 4-carbonyl and 5-hydroxyl in the former [23].

Some flavonoids also show inhibitory effect on the enzyme activity, such as NADH-oxidase and xanthine oxidase. As the reoxidation of xanthine oxidase, both superoxide radicals and hydrogen peroxide are produced. In a previous structure-activity study, Cos et al. [20] found that flavonols showed lower inhibitory activity than flavones. And hydroxyl groups at both C-3 and C-3' were essential for high superoxide scavenging activity. The flavonoids could be classified into three groups according to their ability: inhibit xanthine oxidase, scavenge for superoxide radicals, or show no activity. And C-glycosyl groups at C-6 and C-8 strongly decreased the inhibitory effect on xanthine oxidase. This indicated

that steric interactions could reduce the inhibitory effect of flavonoids on xanthine oxidase.

In a number of structure-activity studies, flavonoids have been tested for their anti-MRSA effect [24]. Flavonoids not only exert a direct antibacterial effect but also show a potentiation effect against MRSA. The active isoflavones from *Lupinus argenteus* [25], genistein, orobol, and biochanin A reduced the MIC of berberine (16-fold) and norfloxacin (4-fold). As results described above, compound **8** showed anti-MRSA effect against each strain, and compound **9** did not show similar effect. A difference of one methylene group between the two compounds was detected. However the mechanisms of the different anti-MRSA activity of the two compounds require further elucidation.

Conflict of Interests

There is no conflict of interests among all authors.

Acknowledgments

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