Effective Compounds From *Caesalpinia sappan* L. on the Tyrosinase In Vitro and In Vivo

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Abstract

*Caesalpinia sappan* L. has been used as an herbal medicine to treat skin damage as a facial cleanser. In this study, 8 known compounds (1-8), (3R,4S)-3-(3′,4′-hydroxybenzyl)-3,4-dihydro-2′,3′-dimethyl-3H-[1,3]dioxolo [4,5-c]chromen-7-ol (1), brazilin (2), protosappanin A (3), protosappanin C (4), protosappanin B (5), caesalpin J (6), sappanone B (7), and sappanchalcone (8), were isolated from the 70% ethanol extract of *C. sappan*. The effects of 8 compounds and extracts of *C. sappan* on tyrosinase were assayed in vitro and in vivo. The results indicated that compounds 1, 2, 4, and 7 had activating effects on the tyrosinase. The experiments of enzyme kinetics showed that compounds 3 and 6 were competitive inhibitors on tyrosinase, while compound 6 was anticompetitive inhibitor. The 70% ethanol extract of *C. sappan* could reduce the contents of tyrosinase in rat serum, ie, the 70% ethanol extracts of *C. sappan* could inhibit the formation of melanin in vivo. Compounds 2, 3, 5, and 6 promoted the formation of tyrosinase in rat serum, while compound 7 inhibited the synthesis of tyrosinase in rat serum.

Keywords

*Caesalpinia sappan* L., chemical constituents, tyrosinase, flavonoids, inhibitory type

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*Caesalpinia sappan* L. known as red wood, a member of the family Leguminosae, is widely distributed in China, India, Burma, and other countries. The dried heartwood of *C. sappan* has been used as traditional Chinese medicine for the treatment of various diseases including traumatic injury, fractures and muscle injury, blood stasis stagnation pain, dysmenorrhea, amenorrhea, postpartum blood stasis, and carbuncle swelling. Pharmacological researches revealed that *C. sappan* had many bioactivities, such as inhibition of melanin production, anti-inflammation, antioxidant, antibacterial effects, and immune regulation.

Chemical constituents and pharmacological investigations of *C. sappan* heartwood had showed that the main bioactive components of *C. sappan* were phenolic compounds such as brazilin, protosappanin, and chalcone.²⁻⁷ Brazilin and 4-O-methylsappanol from the ethanol extracts of *C. sappan* exhibited effects on melanin synthesis in human malignant melanoma cell line (HMV-II).⁴ It was found that brazilin possessed stronger anti-inflammatory effect than hematoxylin and berberine hydrochloride.⁵⁻⁶ Brazilin exhibited hypoglycemic effect in diabetic animals through amelioration of glucose metabolisms in insulin-responsive tissues.⁸ Another study also found that brazilin could reduce the bromotrichloromethane (BrCCl₃)-induced toxicities on hepatocytes and depress BrCCl₃-induced microsomal calcium sequestration.⁹ In addition, the ethyl acetate, methanol, and water extracts of *C. sappan* heartwood exhibited strong antioxidant activity by the method of 1,1-diphenyl-2-picryl hydrazyl (DPPH) and nitric oxide.¹⁰ It was also reported that the extract of *C. sappan* inhibited cancer cells in vitro and in vivo.¹¹ The aqueous extract of *C. sappan* could prolong the allograft survival time in murine skin allograft induced by immune tolerance.¹² It was also found that methanolic and 50% ethanolic extracts of *C. sappan*

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heartwood showed inhibitory effects on *Propionibacterium acnes* and lipase activity.\(^{13}\)

However, the effects of chemical constituents of *C. sappan* on tyrosinase activity and the inhibitory type have been rarely reported. At present, only Mitani et al.,\(^4\) Hridya et al.,\(^4\) Laksmiani et al.,\(^15\) and Chang et al.\(^16\) have reported it. Therefore, we investigated the chemical constituents of *C. sappan* and examined their effects on tyrosinase activity, types of inhibition, and mechanism in vitro and in vivo.

Eight compounds (1-8) were isolated from *C. sappan* (Figure 1), identified as (3R,4S)-3-(3′,4′-hydroxybenzyl)-3,4-dihydro-2″,3″-dimethyl-3H-[1,3]dioxolo[4,5 c]chromen-7-ol (1),\(^17\) brazilin (2),\(^18,19\) protosappanin A (3),\(^20\) protosappanin C (4),\(^21,22\) protosappanin B (5),\(^22\) caesalpin J (6),\(^23\) sappanone B (7),\(^24\) and sappanchalcone (8),\(^21,22\) by comparing their spectral data with those reported in the literatures. And, their tyrosinase activation rates in vitro were shown in Tables 1 and 2.

![Chemical structures of 8 compounds isolated from *Caesalpinia sappan*.](image)

**Figure 1.** Chemical structures of 8 compounds isolated from *Caesalpinia sappan*.

| Table 1. Activation Rate of Tyrosinase of Different Fractions of *Caesalpinia sappan*. |
|-----------------------------------------|---------------------------------|------------------------------|-----------------|-----------------|-----------------|
| Total extract                          | 441.9                           | 420.5                        | 50.9            | 17.0            | 1.3             |
| Water elution                          | 41.1                            | 36.7                         | 6.8             | 8.4             | −0.1            |
| 20% Methanol                           | 627.1                           | 407.5                        | 81.0            | 50.2            | 20.8            |
| 40% Methanol                           | 17.1                            | −42.0                        | −6.1            | −0.3            | −2.4            |
| 60% Methanol                           | 53.5                            | 8.9                          | −18.5           | −14.1           | −9.7            |
| Methanol                               | −78.6                           | −72.1                        | 23.6            | 2.7             | −23.0           |
| Kojic acid                             | −95.6                           | −95.3                        | −93.1           | −86.3           | −84.4           |

*Note*: Kojic acid was used as the positive control.
In Table 1, the total extract, water elution fraction, and 20% methanol eluted fraction of *C. sappan* could remarkably activate tyrosinase. The effects of total extract and 20% methanol eluted fraction on tyrosinase activation rate were increased gradually with the increase of their concentrations. Overall, the order of their activation activities was 20% methanol eluted fraction > total extract > water elution fraction. The effects on tyrosinase activity of 40% and 60% methanol eluted fractions changed from inhibition to activation with the increase of their concentrations. The methanol-eluted fraction showed inhibitory activity at 0.0625, 1.000, and 2.000 mg/mL and activation activity at 0.125 and 0.25 mg/mL.

In Table 2, compounds 2 and 7 could remarkably activate tyrosinase, and compounds 3 and 6 showed inhibition activity on tyrosinase, and the inhibition activity of compound 3 on tyrosinase was enhanced along with the increase of their concentrations. Compounds 1, 4, 5, and 8 showed opposite effects when the concentrations were different. Among them, compound 1 had inhibition activity on tyrosinase at low concentration but had activation activity at high concentration, while the effect of compound 8 was opposite. Compound 4 exhibited activation activity except at the concentration of 0.0625 mg/mL.

According to the results of tyrosinase activity in vitro, compounds 3 and 6 showed effective inhibitory activity on tyrosinase. Therefore, inhibitory mode and mechanism of these compounds on tyrosinase were determined.

In Figure 2 and Table 3, $K_i$ was increased and $V_{max}$ was unchanged after compound 3 was added into the reaction system, indicating that the inhibition mode of compound 3 on tyrosinase was competitive inhibition. Compound 3 and l-dopa competed for tyrosinase activity center, so that the reaction rate was decreased. Both $K_i$ and $V_{max}$ were reduced after compound 6 was added into the reaction system. It illustrated that the inhibitory mode of compound 6 on tyrosinase was anticompetitive inhibition. Compound 6 was bound to a tyrosinase and l-dopa complex to form a complex which could not be decomposed further, so that both $K_i$ and $V_{max}$ were reduced. $K_i$ was used to represent the dissociation constant between the enzyme and the inhibitor. The inhibitory effect of inhibitors enhanced along with the increase of the value. And the $K_i$ value of compound 3 was smaller than that of compound 6, indicating that compound 3 has stronger inhibitory effect on tyrosinase than that of compound 6, which was consistent with tyrosinase activity assay.

The contents of tyrosinase in rat serum after administration of different fractions (total extraction, water elution, 20% methanol part, 40% methanol part, and 60% methanol part) and compounds 2, 3, and 5 to 7 were examined and the results were shown in Figures 3 and 4.

In Figure 3, the contents of tyrosinase in all of sample administration groups were lower than that in the control group except for the positive control group. Among them, there was no significant difference between the 40% and 60% methanol eluted fraction. The $K_i$ value of compound 3 was smaller than that of compound 6, indicating that compound 3 has stronger inhibitory effect on tyrosinase than that of compound 6, which was consistent with tyrosinase activity assay.

The contents of tyrosinase in rat serum after administration of different fractions (total extraction, water elution, 20% methanol part, 40% methanol part, and 60% methanol part) and compounds 2, 3, and 5 to 7 were examined and the results were shown in Figures 3 and 4.

In Figure 3, the contents of tyrosinase in all of sample administration groups were lower than that in the control group except for the positive control group. Among them, there was no significant difference between the 40% and 60% methanol eluted fraction.
methanol-eluted fractions compared with the control group. The total extract of *C. sappan* and 100% methanol-eluted fractions were significantly lower than that of the control group (*p* ≤ .05). Water elution and the 20% methanol-eluted fraction were significantly lower than control group (*p* ≤ .001). The results demonstrated that 70% ethanol extract of *C. sappan* could reduce the content of tyrosinase in rat serum.

In Figure 4, the contents of tyrosinase in all of administration group of serum were higher than that of the control group in addition except 7. Among them, the content of tyrosinase in compound 5 group was significantly higher than that of the control group (*p* ≤ .05), while the content of tyrosinase in compound 7 group was significantly lower than that of the control group (*p* ≤ .05). The content of compound 6 group was significantly higher than that of the control group (*p* ≤ .01). The contents of tyrosinase in compounds 2 and 3 groups were significantly higher than that of the control group (*p* ≤ .001). Except for compound 7, the contents of tyrosinase in other 5 drug administration groups were higher than that of the positive control group. Among them, compounds 5 and 6 showed no significant difference compared with the positive control group; the content of tyrosinase in compound 2 group was significantly higher than that of the positive control group (*p* ≤ .05); the content of tyrosinase in compound 3 group was significantly higher than that of the positive control group (*p* ≤ .01).

Tyrosinase is one of the key enzymes involved in melanin synthesis. If its activity is inhibited, the production of melanin will be reduced. Compounds with these activities could be used in skin whitening and treated the face pigmentation sickness, such as freckle, chloasma, and senile plaque. While, the increase of tyrosinase activity can promote melanin synthesis to treat vitiligo and albinism. Most of the previous studies have reported that flavonoid compounds acted as potent tyrosinase inhibitors. Studies have found that brazilin could inhibit melanin formation in human melanoma cell line (HMV-II) with the half-maximal effective concentration (EC$_{50}$) of 3.0 ± 0.5 µM. The results of this study were different from the reference, the discrepancy may be related to different administration modes of brazilin, experimental models, administration doses, etc. Iida K found that using l-tyrosine instead of l-dopa as substrate, the compounds exhibited a stronger inhibitory effect. Forsythiaside isolated from dichloromethane fraction of *Forsythia suspensa* showed activation effect on tyrosinase activity in vitro, while the effect on tyrosinase activity in B16 cells was inhibited. The above researches showed that different administration modes of some compounds, different experimental models, and different dosages of the drug may affect the results of enzymatic reaction.

Enzyme kinetics experiments showed that the type of inhibition of compound 3 on the tyrosinase was the competitive inhibition, indicating that they only bind with the free enzyme and reduce the binding between dopa and enzyme, so as to achieve the inhibitory effect on tyrosinase. The type of inhibition of compound 6 on tyrosinase was anticompetitive inhibition, indicating that enzyme inhibitors only bind to the enzyme-substrate complex.

Compounds 1, 2, 4, and 7 had activation effect on the tyrosinase activity in vivo. Enzyme kinetics experiments showed that the type of inhibition of compounds 3 on tyrosinase was competitive inhibition, while that of compound 6 was anticompetitive inhibition. Compounds 2, 3, 5, and 6 could promote the formation of tyrosinase in rat serum, and compound 7 could inhibit the synthesis of tyrosinase in rat serum. It was suggested that compounds 2, 3, 5, and 6 could improve the activity

**Table 3.** The $K_m$, $V_{max}$, and $K_i$ of Compounds.  

| Sample     | $K_m$ (mM) | $V_{max}$ (mM/s) | $K_i$ (M) |
|------------|------------|-----------------|-----------|
| Control    | 1.8        | $2.7 \times 10^{-3}$ |           |
| Compound 3 | 8.4        | $2.7 \times 10^{-3}$ | $3.9 \times 10^{-2}$ |
| Compound 6 | 1.1        | $1.7 \times 10^{-3}$ | $2.0 \times 10^{-1}$ |

**Figure 3.** Contents of tyrosinase in rat serum samples after administration of different fractions of *Caesalpinia sappan* (*n* = 3). Compared with the control group: *P* ≤ .05, **P* ≤ .01, and ***P* ≤ .001. Compared with the positive control group: *P* ≤ .05, **P* ≤ .01, and ***P* ≤ .001.
of tyrosinase in rat serum to promote the formation of melanin, and thus alleviate the white lesions of the body. Compound 7 and total extract reduced the formation of melanin by inhibiting the activity of tyrosinase and finally improved pigmentation.

**Experimental**

**General**

NMR spectra were measured on Bruker Ascend TM 400 superconducting nuclear magnetic resonance (NMR) spectrometer (Bruker Co., Germany). A CXTH LC-3000 (Beijing Innovation Technology Co., Ltd., Beijing, China) and Waters-2489 (Waters Co., Milford, MA, United States) preparative high-performance liquid chromatography (HPLC) system equipped with a YMC-C18 column (250 × 10 mm, 5 µm) were used for isolation. D101 macroporous resin was purchased from Tianjin City Fu Yu Fine Chemical Co., Ltd., Tianjin, China; GRP-9270 type water jacket incubator from Shanghai Senxin Experimental Equipment Co. Ltd., Shanghai, China; Multiskan GO microplate reader from Thermo Electron Inc, United States; AL104 type electronic balance and DELTA 320 type pH meter from Mettler-Toledo Inc, Columbus, OH, United States; and TGL-16gR high-speed desktop refrigerated centrifuge from Shanghai Anting Scientific Instrument Factory, Shanghai, China.

GF254 thin layer chromatography silica gel, silica gel H, 40 to 80 mesh and 200 to 300 mesh silica gel, was purchased from Qingdao Ocean Chemical Co., Ltd., Qingdao, Shandong, China; Sephadex LH-20 from Pharmacia Co., Sweden; 1-3-(3,4-dihydroxyphenyl) alanine from Alfa Aesar, 10102261, Haverhill, MA, United States; tyrosinase from Worthington Biochemical Corporation, 35K14431, Lakewood, NJ, United States; kojic acid from A Johnson Matthey Company, 10134934; Royston, United Kingdom; dimethyl sulfoxide (DMSO) from Tianjin City Fu Yu Fine Chemical Co., Ltd., Tianjin, China; 50 SPF female SD (sprague dawley) rats, weighing 220 ± 20 g, were purchased from the Experimental Animal Management Center of Henan Province with the license number of SCXK (Henan 2015-0004).

**Plant Material**

The heartwood of *C. sappan* L. was purchased from a medicine market in November 2014 and identified by Professor Changqin Li from Henan University. The specimen was deposited in Henan University.

**Extraction and Isolation**

*Caulacarpus sappan* heartwood (2.2 kg, dry weight) was extracted with 70% ethanol at 50°C for 3 times (2, 1, and 1 hour; 10, 6, and 6 L for each time, respectively) to obtain the total extract. The total extract was filtered and evaporated to dryness under reduced pressure with a rotary evaporator. The total extract was chromatographed on D101 macroporous resin by eluting with water, 20% methanol, 40% methanol, 60% methanol, and methanol, respectively. Then, 5 fractions were obtained, namely, water elution fraction, 20%, 40%, 60%, and 100% methanol eluted fractions with yields of 12.0, 26.3, 71.2, 45.7, and 48.3 g, respectively.

The 40% methanol eluted fraction (35.0 g) was subjected to flash column chromatography (CC) (40-80 mesh silica gel) eluting with petroleunum ether-acetone (v/v = 50:1-1:1) to obtain 4 fractions (Fr.3.1-Fr.3.4). Fr.3.1 (0.796 g) was further subjected to CC over silica gel, eluted by gradient system of dichloromethane-methanol (50:1-30:1, v/v), to obtain Fr.3.1a. Subfraction Fr.3.1a was chromatographed over a column of
silica gel, eluted with a gradient system of dichloromethane-methanol (70:1-50:1, v/v) to obtain Fr.3.1a. Fr.3.1a was purified by CC over Sephadex LH-20 using dichloromethane-methanol (1:1) as solvent to yield compound 2 (90.4 mg). Fr.3.2 (1.57 g) was further subjected to CC over silica gel, eluted with chloroform-acetone to afford Fr.3.2a (20:1) and then Fr.3.2a was subjected to CC over Sephadex LH-20, using dichloromethane-methanol (1:1) as eluent, to obtain compound 1 (5.7 mg).

Fr.3.3 (1.85 g) was subjected to CC over silica gel, eluted with a gradient system of dichloromethane-acetone (8:1-4:1, v/v), to obtain compound 3 (5 mg) and 2 fractions (Fr.3.3-a-b). Fr.3.3a was applied to pressure-reducing CC, using dichloromethane-methanol (30:1) as eluent and then purified by CC over Sephadex LH-20 (acetone eluent) to obtain compound 4 (30.9 mg). Fr.3.3b was subsequently subjected to medium-pressure preparative liquid chromatography (silica gel, 30:1 dichloromethane-methanol eluent) and purified by CC over Sephadex LH-20 (1:1 dichloromethane-methanol eluent) to obtain compound 5 (91.6 mg).

Fr.3.4 (9.85 g) was further separated on silica gel H medium pressure liquid chromatography, using petroleum ether-ethyl acetate (10:1-1:1) as eluent, to obtain 2 fractions (Fr.3.4-a-b). Fr.3.4a was subsequently subjected to pressure-reducing CC over silica gel, eluted with a gradient system of dichloromethane-ethyl acetate (40:1-4:1), to obtain compound 3 (11.3 mg) and compound 7 (8.4 mg). Fr.3.4b was subjected to CC over silica gel, eluted with a gradient system of dichloromethane-methanol (50:1-10:1, v/v) and then subjected to CC over Sephadex LH-20, eluting with dichloromethane-methanol (1:1), to obtain compound 2 (14 mg).

The 60% methanol-eluted fraction (30.0 g) was subjected to a silica gel CC (40-80 mesh) eluted with dichloromethane-methanol (100:0-3:2, v/v) to afford 3 fractions (Fr.4.1-Fr.4.3). Fr.4.1 was separated by recrystallization to yield Fr.4.1a (250 mg). Fr.4.1a was further purified by HPLC, eluted with methanol-water gradient [methanol-H₂O, 60:40-68:32 (0-8 minutes) → 68:32-80:20 (8-20 minutes) → 80:20-100:0 (20-40 minutes), v/v] to obtain compound 8 (34 mg). Fr.4.2 (905.3 mg) was subjected to silica gel H medium pressure liquid chromatography (chloroform-acetone, 40:1-1:1, v/v), silica gel H atmospheric CC (chloroform-methanol, 30:1, v/v), and purified by Sephadex LH-20 gel CC (chloroform-methanol, 1:1, v/v) to obtain compound 3 (50 mg).

Fr.4.3 was subjected to flash CC on 40 to 80 mesh silica gel and eluted with dichloromethane-acetone (v/v = 20:1-1:1) to afford Fr.4.3a, and subsequently separated by preparative HPLC, eluted with methanol-water gradient (methanol: 0-20 minutes, 50%-70%; 20-45 minutes, 70%-90%) as the mobile phase, 29.5 to 34.5 minutes as retention time to obtain compound 9 (90 mg), 37 to 40 minutes as retention time to obtain compound 10 (5.5 mg).

**Tyrosinase Activity Assay in Vitro**

Ninety-six-well microplate was used to assay tyrosinase activity according to the method reported.³⁰ The percentage of activation of the enzymatic activity was calculated as follows: tyrosinase activation activity was expressed as activation rate at a certain concentration. The formula for the activation rate of tyrosinase is as follows:

\[
\text{Tyrosinase activation rate (\%)} = \left( \frac{A - B}{C - D} - 1 \right) \times 100
\]

where \(A\) is the absorbance of sample with added test sample, substrate, and tyrosinase; \(B\) is the absorbance of sample with added test sample and substrate; \(C\) is the absorbance of sample with added DMSO, substrate, and tyrosinase; and \(D\) is the absorbance of sample with added DMSO and substrate.

According to the reaction system, phosphate buffer saline (PBS) (45 µL, pH 6.8), samples (5 µL) with the concentration of 2, 1, 0.25, 0.125, and 0.0625 mg/mL, and aqueous solution of tyrosinase (0.2 U/mL, 25 µL) were successively added in 96-well microplate, and incubated at 30°C for 10 minutes. Then, levodopa solution (0.5 mg/mL, 25 µL) was added to the mixture and oscillated at 30°C for 5 minutes, and the absorbance was measured at 492 nm. At the same time, the sample solution without adding the enzyme as sample blank, without adding the sample as blank control, and adding inhibitor kojic acid as positive control were carried out under the same conditions.

**Tyrosinase Inhibitory Mode and Mechanism**

According to Michaelis-Menten equation, a series concentrations (2.5-1.25/8 mmol/L) of dopa solution were prepared, the amount of tyrosinase was immobilized, pH 6.8, 25 µL dopa solution as substrate, cultivated for 10 minutes at the constant temperature of 30°C, and then tyrosinase (25 µL) was added with different concentrations of substrate, the initial reaction velocities were determined by \(1/[S]\) as the abscissa and \(1/V\) as the ordinate to produce a straight line, from which the \(K_m\) and \(V_{max}\) values were then calculated.³¹,³²

**Tyrosinase Activity in Vivo**

The SD female rats were randomly divided into 16 groups, with 3 rats in each group. These 16 groups were divided into 2 categories (I and II), one for 6 extracts of \(C.\) sappan groups (I) and the other one for 6 isolated compounds groups (II). Each category included blank group, positive control group, and control group. The rats in control group (I) were given equal volume of distilled water and 0.0115 g/kg of vitamin C, respectively. The rats in control group (II) were intraperitoneally administered 1 time for 7 days, and the single injection dose was calculated as 5 mg/kg. The blank
group (II) and the positive control group (II) were given an equal volume of 5% glucose injection and 1 mg/mL of vitamin C injection which was diluted by 5% glucose injection, respectively. The rats were fasted for 12 hours before the last drug administration and killed 1 hour after the last drug administration for collecting the blood sample from the abdominal aortas. The blood samples were rested for 2 to 3 hours at room temperature, and sequentially centrifuged 20 minutes at 3000 rpm. Then, the upper serum was taken to deposit at ~40°C.

There were 4 parallel samples including the original serum of 3 SD rats in each group and the mixed serum with equal amount of serum. The contents of tyrosinase in rat serum of 3 SD rats in each group and the mixed serum with equal amount of serum. The contents of tyrosinase in rat serum were determined according to the manufacturer recommendations instruction manual.36

Declaration of Conflicting Interests
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