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Antioxidant Activity *In Vitro* of Three Constituents from *Caesalpinia sappan* L*

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Abstract: Antioxidant activities of the 95% ethanol extract from *Caesalpinia sappan* heartwood (ECS), protosappanin A, protosappanin B, and brazilein were studied *in vitro*. The inhibition of the formation of malondialdehyde (MDA) and the scavenging of superoxide anions, hydrogen peroxide, and hydroxyl radicals were assayed. The experimental results show that all four substances had antioxidant activity *in vitro* but their capabilities differed for the different indicators. ECS, protosappanin A, and protosappanin B show more inhibition of MDA and scavenging of hydrogen peroxide, while brazilein shows more scavenging of hydroxyl radicals. All the samples show little scavenging of superoxide anions.

Key words: antioxidant; *Caesalpinia sappan* L; protosappanin A; protosappanin B; brazilein

Introduction

Caesalpinia sappan L is a traditional medicine plant produced in India, Myanmar, Vietnam, Sri Lanka, and the Malay Peninsula, and distributesd domestically in China in Yunnan, Guizhou, Sichuan, Guangdong, Guangxi, Fujian, and Taiwan. Many pharmacological activities of *Caesalpinia sappan* L have been reported: vasorelaxation[1], anti-atherosclerosis, analgesic[2], hypoglycemic[3], anti-complementary[4], anti-inflammatory[5], cytotoxic[6,7], anticonvulsant[8], muscle contractile activity[9], spasmolytic activity[10], antibacterial[11,12], antiviral[13], antioxidant[14], and other activities. In addition, extracts of *Caesalpinia sappan* heartwood are used as a pH-sensitive acid-base indicator and a medicinally useful dye[15,16]. Phenolic compounds, which are famous for their antioxidant capability through radical scavenging and inhibition of enzymes responsible for radical production, were isolated from *Caesalpinia sappan* L, such as homoisoflavonoids and the related compounds, protosappanin A, protosappanin B, brazilin, and brazilein[17].

Radicals are ionized molecules or atoms. Superoxide anions ($O_2\cdot^-$) and the hydroxyl radical (OH·) are radicals that are reactive oxygen species (ROS). Radical reactions are activated by enzymatic and non-enzymatic reactions *in vivo*[18]. The pathological state having an imbalance of radical production and elimination leads to weaken antioxidant activity results from radical elimination. In humans, oxidative damage usually does not directly initiate chronic disease but can be a promoter of disease. As a result, interest in the antioxidant activity of the natural compounds is growing[19].

Previous studies on extracts of *Caesalpinia sappan* L with antioxidant effects have measured many indicators including lipid peroxidation (LPO), glutathione (GSH), superoxide dismultase (SOD), catalase (CAT)

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in vivo and xanthine oxidase, $O_2\cdot^-$ and OH· *in vitro*. The present study screened the antioxidant activity *in vitro* of several constituents from *Caesalpinia sappan* L extract, including the 95% ethanol extract of *Caesalpinia sappan* heartwood (ECS), protosappanin A, protosappanin B, and brazilein (Fig. 1). The effects of these four compounds on malondialdehyde (MDA), superoxide anions ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH·) were analyzed experimentally using spectrophotometry.

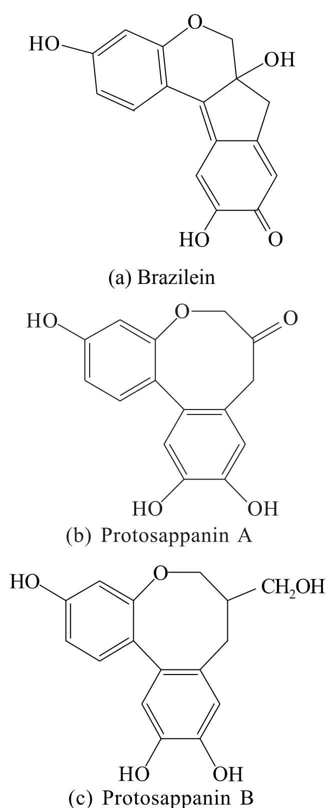


Fig. 1 Chemical structures of three compounds from *Caesalpinia sappan* L

1 Materials and Methods

1.1 Animal materials

Male ICR mice (18-22 g body weight) were purchased from the Beijing Vital-River Experimental Animal Technological Centre. They were housed for 1 week in a 12 h/12 h light/dark cycle in a temperature- and humidity-controlled room and freely fed standard laboratory chows with water *ad libitum*.

1.2 Extracts and reagents

ECS was extracted from the heartwood of *Caesalpinia sappan* L with 95% ethanol (3 times, 1 h/time, 80℃) and concentrated at 50℃ under reduced pressure. Brazilein was extracted from the ECS. The ECS was partitioned between water and ethanol and extracted by petroleum ether. The extract of petroleum ether was concentrated at room pressure to obtain the purified fraction[20]. Protosappanin A and protosappanin B were prepared as described earlier[21]. All three compounds had purities of 98%. They were dissolved in methanol unless otherwise noted. Bovine serum albumin standards and horseradish peroxidase were purchased from Sigma. Other chemicals were analytical grade reagents purchased from Beijing Reagents.

1.3 Methods

1.3.1 Inhibition of the formation of malondialdehyde (MDA)

The thiobarbituric acid (TBA) method[22] was used. The main principle is that MDA decomposed from LPO reacts with TBA to produce a red pigment in acidic and heated conditions. A flaw in this method is that TBA not only reacts with MDA but also with oxidized proteins or nucleic acids to produce a similar red pigment, that is termed the TBA reaction.

Selected quantities of the extracts were added into 0.2 mL mouse brain tissue homogenate and incubated at (37±0.5)℃ for 30 min[23], with vitamin C as positive control and normal saline as the blank. The TBA method was then used with a wavelength of 532 nm with the inhibition ratio calculated as

$$I = [1-(OD_{adm}/OD_{con})]\times 100\% \quad (1)$$

where OD_{adm} is the administration optical density and OD_{con} is the control optical density.

1.3.2 Scavenging of superoxide anions ($O_2\cdot^-$)

The pyorgallol[22] was used with the principle that pyorgallol is autoxidable in an alkaline environment (pH 8.2) and releases $O_2\cdot^-$ with an intermediate product that has strong optical absorption at 420 nm.

4.5 mL of 50 mmol/L, pH 8.3 K_2HPO_4-KH_2PO_4 buffer was added at 25℃ into 10 μL 50 mmol/L pyorgallol and mixed rapidly. The OD value was detected every 30 s at 325 nm. The pyorgallol autoxidation velocity was measured to ensure OD remained approximately at 0.07/min. After administration, the influence of the autoxidation velocity was measured with vitamin C as positive control and the solvent as the blank. Then, the $O_2\cdot^-$ scavenging ratio was calculated as

$$I = [1-(\Delta OD_{adm} / \Delta OD_{bla})]\times 100\% \quad (2)$$

where ΔOD_{adm} is the administration optical density margin and ΔOD_{bla} is the blank optical density margin.

1.3.3 Scavenging of hydrogen peroxide (H_2O_2)

The phenolsulfonphthalein method[22] was used，which is based on H_2O_2 dependent and horseradish peroxidase (HRPO) mediated oxidation of phenol red (PR) producing compounds with altered absorption spectra.

Different amounts of H_2O_2 were added into the phenol red solution, at 37℃ for 5 min. Then 1 mol/L 10 μL NaOH was added to keep pH 12.5. The OD value was detected at the optimal wavelength to establish the standard calibration curve. The H_2O_2 (6 mmol/L) was added into the samples in the linear range of its standard calibration curve at (37±0.5)℃ and incubated for 60 min with the solvent used for control. The change in volume of the H_2O_2 was measured after the sample addition. The H_2O_2 scavenging ratio was calculated as

$$I = [1-(OD_{H_2O_2,\, admn} / OD_{H_2O_2,bla})]\times 100\% \quad (3)$$

where $OD_{H_2O_2,\, admn}$ is the administration H_2O_2 optical density and $OD_{H_2O_2,bla}$ is the blank H_2O_2 optical density.

1.3.4 Scavenging of the hydroxyl radical (OH·)

The 1,10-phenanthroline (Phen) spectrophotometry[24] was used since Phen-Fe^{2+} is a commonly used indicator of redox reactions. The H_2O_2/Fe^{2+} system produces OH· through the Fenton reaction with the Phen-Fe^{2+} oxidated to Fe^{3+}. The OH· can be determined according to the change of the optical absorption at 536 nm.

The samples were added into the Phen-Fe^{2+} solvent system at (37±0.5)℃ and incubated for 60 min. Double distilled water was used as the control with the solvent scavenging and then measured. The apparent scavenging ratio was calculated as

$$I = [(OD_{adm} - OD_{H_2O_2+NaCl}) / OD_{H_2O_2+NaCl}]\times 100\% \quad (4)$$

where OD_{adm} is the administration optical density and $OD_{H_2O_2+NaCl}$ is the optical density of H_2O_2 + NaCl.

1.3.5 Statistical analysis

All the data was analyzed and expressed as mean±S.D. The differences between groups were identified using the two-tailed student's t-test. Differences were considered to be statistically significant at p<0.05, where p means probability.

2 Results

2.1 Inhibition of the formation of MDA

The dose effect of ECS inhibition of MDA *in vitro* is shown in Table 1. The dose effects of protosappanin A, protosappanin B, and brazilein was presented in Table 2. The data in the last column of Table 2 shows that the inhibition of MDA *in vitro* decreased in the order: ECS> protosappanin B>protosappanin A>brazilein. All the samples had a dosage related effect, with the reproducibility of brazilein being not positive, possibly due to its weak anti-oxygen capability.

Table 1 ECS inhibition of the formation of MDA *in vitro*

Group	Final concentration (mg/mL)	$OD_{532\ nm}$	Inhibition (%)
Control	–	0.4850±0.0163	–
Vit. C	0.3680	0.4170±0.0064*	14.02
ECS	0.0161	0.1955±0.0304**	59.69
ECS	0.0121	0.2385±0.0205**	50.82
ECS	0.0061	0.2980±0.0182**	38.56
ECS	0.0048	0.3097±0.0182**	36.14
ECS	0.0024	0.3097±0.0162**	36.14
ECS	0.0012	0.3633±0.0040**	25.09
ECS	0.0006	0.4170±0.0221*	14.02

Note: Data was expressed as mean±SD. n=3. The inhibition ratio was calculated as Eq. (1). Compared with control, *p<0.05, **p<0.01. Vit. C means vitamin C. ECS means 95% ethanol extract from *Caesalpinia sappan* heartwood.

Table 2 Dose effects of ECS, protosappanin A, protosappanin B, and brazilein for inhibition of MDA

Group	Final concentration (mg/mL)	$OD_{532\ nm}$	Inhibition (%)
Control	–	0.375±0.0092	–
ECS	0.006	0.345±0.0219*	36.1
Protosappanin A	0.147	0.295±0.0001**	20.3
Protosappanin A	0.074	0.315±0.0028*	14.9
Protosappanin A	0.037	0.309±0.0078*	16.5
Protosappanin B	0.102	0.298±0.0028**	19.5
Protosappanin B	0.051	0.293±0.0078**	20.8
Protosappanin B	0.025	0.308±0.0028*	16.8
Brazilein	0.074	0.368±0.0148	0.54
Brazilein	0.037	0.376±0.0078	–
Brazilein	0.018	0.373±0.0050	–

Note: Compared with control, *p<0.05, **p<0.01. Data was expressed as mean±SD. n=3. The inhibition ratio was calculated as Eq. (1).

2.2 Scavenging of superoxide anions ($O_2\cdot^-$)

Table 3 presents scavenging capabilities of the 4 samples on $O_2\cdot^-$. There was no direct scavenging effect of ECS, protosappanin A, protosappanin B, and brazilein on $O_2\cdot^-$, (p>0.05) with Vit. C, a positive control showing an effect.

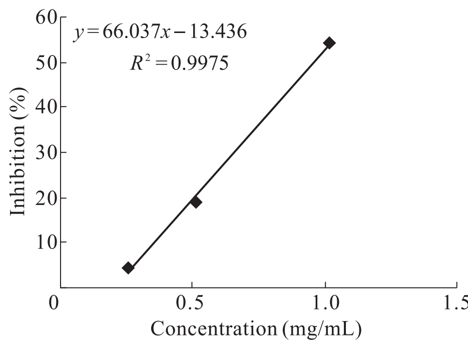
Table 3 ECS, protosappanin A, protosappanin B, and brazilein of scavenging $O_2\cdot^-$

Group	Final concentration (mg/mL)	$\Delta OD_{420\ nm}$
Control	–	0.1100±0.0032
Vit. C	0.368	0.0368±0.0008**
ECS	0.270	0.1138±0.0039
Protosappanin A	0.420	0.1110±0.0012
Protosappanin B	0.290	0.1118±0.0013
Brazilein	0.210	0.1110±0.0012

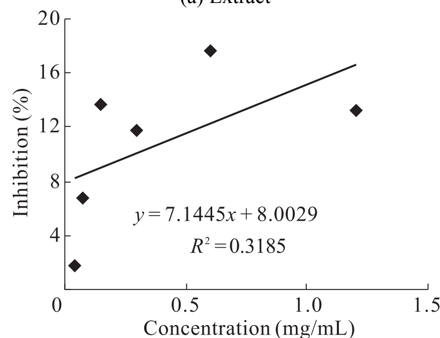
Note: Compared with control, **p <0.01. Data was expressed as mean±SD. n=3.

2.3 Scavenging of hydrogen peroxide

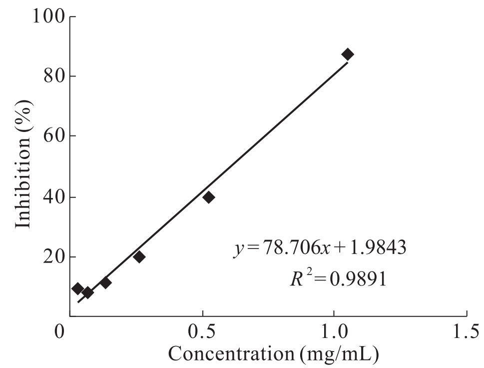
The dose-effects of ECS, protosappanin A, protosappanin B, and brazilein scavenging of H_2O_2 are presented in Fig. 2. All the samples showed the capability to scavenge H_2O_2 *in vitro* with some dose dependent effect, especially protosappanin A and protosappanin B. The order of the scavenging capability was approximately: protosappanin A>protosappanin B>ECS>brazilein.



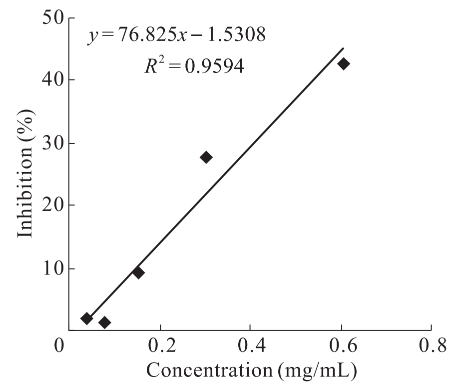
(a) Extract



(b) Brazilein



(c) Protosappanin A



(d) Protosappanin B

Fig. 2 Dose effect of ECS, protosappanin A, protosappanin B, and brazilein for scavenging of H_2O_2. The slopes of the ECS, protosappanin A, and protosappanin B grapes were similar with their higher correlation coefficients showing positive effect-concentration relationships. Both the slope index and the correlation coefficient for brazilein were not as good.

2.4 Scavenging of the hydroxyl radical (OH·)

The direct inhibition of OH· *in vitro* by ECS, protosappanin A, protosappanin B, and brazilein is shown by the results in Table 4. Brazilein had the strongest effect on the scavenging of OH·.

Table 4 ECS, protosappanin A, protosappanin B, and brazilein scavenging of OH·

Group	Final concentration (mg/mL)	$OD_{536\ nm}$	Inhibition (%)
NaCl	–	1.853±0.0099	–
H_2O_2+NaCl	–	0.382±0.0028**	–
H_2O_2+ECS	0.099	0.413±0.0035**,#	8.12
H_2O_2+protosappanin A	0.102	0.476±0.0001**,#	24.61
H_2O_2+protosappanin B	0.117	0.345±0.0063**	–
H_2O_2+Brazilein	0.116	0.634±0.0318**,##	65.97

Note: Compared with NaCl, **p<0.01. Compared with H_2O_2+NaCl, #p<0.05, ##p<0.01. Data was expressed as mean±SD. n=3. The inhibition ratio was calculated as Eq. (4).

3 Discussion and Conclusions

The experimental results show that all four substances had antioxidant activity *in vitro* but their capabilities differed for the different indicators. Although 95% ethanol extract from *Caesalpinia sappan* heartwood showed strong inhibition of the formation of MDA and a good linear dose-effect relation, its capability for scavenging other radicals was weaker. The dose-effect of ECS, protosappanin A, protosappanin B, and brazilein scavenging of H_2O_2 showed that protosappanin A and protosappanin B both had antioxidant activity on H_2O_2 that was consistent with that of ECS. Interestingly, Brazilein showed no antioxidant activity on the indicators except for the scavenging of OH·. None of the substances showed the ability to scavenge $O_2{\cdot}^-$.

These veiled results may occur because the compounds have different functional groups that react in different ways with the radicals. The compounds may have different oxide reactions so that their mechanisms differ. Antioxidant drugs can be divided into preventive antioxidants and chain-breaking antioxidants. Preventive antioxidants transform the harmful substances into harmless or less harmful substances to reduce the formation of radicals and further injury. Chain-breaking antioxidants primarily prevent further oxidant injury by changing the harmful substances, including the formation of radical complexes or the transformation of radicals to non-sensitive compounds. Further experimental support is needed to determine how to classify the antioxidant mechanisms of the compounds tested in this study.

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Todai Week at Tsinghua

“Todai Week at Tsinghua”, a major communication event between Tsinghua University and the University of Tokyo (Todai), started May 19. The University of Tokyo is the first partner institution in Asia with which Tsinghua has cooperated to hold a University Week event.

Tsinghua University President Gu Binglin, University of Tokyo President Hiroshi Komiyama, Ms. Zhang Xiuqin, the Ministry of Education’s Director-General of International Cooperation and Exchange, Mr. Hisashi Michigami, Minister of Japanese Embassy to China, and Tsinghua Vice President Xie Weihe attended the opening ceremony in Tsinghua’s Main Building that morning.

Tsinghua President Gu extended a warm welcome to the University of Tokyo delegation. He noted that, “the Todai Week at Tsinghua will be a platform for further strengthening friendship and collaboration between our two institutions.” He also made the following statement: “I am greatly moved when I know many Todai students in the delegation expressed their wishes for donating blood for the injured people. And on the occasion of last night’s joint student performance, many Todai students donated money for relief efforts and aid to the earthquake area.”

“It is my sincere hope that Todai Week will help promote and strengthen a high level of academic exchange between our two universities. I would especially like to emphasize the importance of the exchange of students and young scholars. This new generation will create new relationships not only between our two universities but also between China and Japan,” said President Komiyama. He later delivered a speech on “Global Sustainability and Vision 2050”.

During Todai Week at Tsinghua, there will be five academic lectures, fourteen symposiums and workshops in the fields of science, engineering, the humanities and social sciences on the Tsinghua campus. The various gatherings will cover a wide range of issues, including Sino-Japanese comparative studies, regional and sustainable development, and frontiers in biotechnology and nanotechnology. More than 100 Todai distinguished professors are invited to Tsinghua to exchange ideas with their Tsinghua counterparts. In addition to academic activities, Todai Week at Tsinghua will also feature exchange activities between students. Over 100 Todai graduate students will participate in the Student Art Performance and many other activities together with Tsinghua graduate students.

The representatives from both universities also planted a tree symbolizing the friendship of the University of Tokyo and Tsinghua in the Friendship Garden of Tsinghua May 19. A Student Exchange Agreement between Tsinghua and the University of Tokyo was also signed that day.

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