The Antioxidant Activity of *Caesalpinia sappan* L. Heartwood in Various Ages

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Abstract

The heartwood of *Caesalpinia sappan* L. (Caesalpiniaceae) is traditionally used in China and Ayurveda. In Thailand it is used as coloring agent in beverage, food, garment and cosmetics. The objective of this study is to find out which ages of wood giving effective natural antioxidant. The heartwood extracts in various ratio of extraction solution CH_3OH:CH_2Cl_2 and various ages were measured for the amount of brazilin and antioxidant activity. The antioxidant activity was obtained by trolox equivalent antioxidant capacity (TEAC) assay. The CH_3OH:CH_2Cl_2 (1:3) was the best ratio for extraction solution and gave extracts with high amount of brazilin and antioxidant property. The older heartwood had higher quantities of antioxidant activity. The ages of the heartwood was not an important factor for amount of brazilin and its derivatives.
Keywords: Antioxidant acivity, *Caesalpinia sappan* L., brazilin, TEAC

Introduction

In recent years, the govermental authorities and consumers are considerably interested in food safety. They have arranged the campaign for avoiding synthetic food colouring agent and have persuaded for replacing them with natural source colours. These attempts have led to focus on *Caesalpinia sappan* L. (Family Caesalpiniaceae) which its heartwood give natural red colour. The heartwood is pale red, hard, heavy with even and fine texture. A decoction of the heartwood is traditionally used as coloring agent in bevarage, food, garment and cosmetics. It is mixed with the extracts of *Carthamus tinctorius* L., *Crocus sativus* L., *Cinnamomum loureirii* Nees., *Jasminum sambac* (L)Ait., *Mesua ferrea* L., *Nelumbo nucifera* Gaertn., and so on for indigenous coloring solution called Namya-utai, which has antithrist and cardiotonic properties. In Ayurveda, the heartwood is used for wound healing, treatment of ulcers, diarrhoea, epilepsy, diabetes, etc. In traditional Chinese medicine, it is used as analgesic and anti-inflammation for the treatment of traumatic disease and menstrual disorders. Flavonoids (Namikoshi and Saitoh, 1987; Namikoshi et al., 1987; Namikoshi, Nakata and Saitoh, 1987) and phenolic (Fuke et al., 1985; Saitoh et al., 1986) such as 4-*O*-methylsappanol, protosappanin A (Nagai et al., 1986), protosappanin B (Nagai and Nagumo, 1986), protosappanin E, brazilin (Kim et al., 1997), brazilein, caesalpin J (Miyahara et al., 1986), triterpenoid and steroid (such as campesterol, stigmasterol, β-sitosterol) were isolated from the wood. The hepatoprotection (Moon et al., 1992), immunomodulation (Choi et al., 1997), hypoglycemia (Kim et al., 1995; Moon et al., 1988), anticomplementary (Oh et al., 1998), anticonvulsant (Baek et al., 2000), anti-inflammatory, antibacterial (Nirajan Reddy et al., 2003), antioxidation (Badami et al., 2003; Yingming et al., 2004), and other biological activities of sappan have been reported.

Its *in vitro* antioxidation (in 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide and oil oxidation) and *in vivo* antioxidation have been evaluated. These results showed that the sappan crude extracts have high antioxidant property. Hence, the antioxidant activity and amount of brazilin equivalent of the extracts from heartwood of sappan in various ages were investigated in this study. The aim of the study was to find out which ages of wood giving effective natural antioxidant and calculating the amount of phenolic compounds in sappan in brazilin equivalent. Since brazilin is the major substance in heartwood of sappan, which gives red colour. The calculation of antioxidant and

amount of phenolic compounds are methods for choosing the suitable ages of the wood for commercial production.

Materials and methods

Plant

The trunk heartwood (in 2, 4, 6, 10 years old) and branch heartwood (at 30 years old) of *Caesalpinia sappan* L. were collected from Huay Kha Khaeng, Uthaithani province by a researcher of Faculty of Pharmacy, Silpakorn University in Febuary, 2004. The voucher specimens were deposited in the Department of Pharmacognosy, Silpakorn University in Nakorn-Pathom, Thailand.

Chemicals

$ABTS^{2-}$, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate), was obtained as sulfonic acid from Sigma. Trolox, (+/-) -6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, 97%, was purchased from Aldrich. L-Ascorbic acid was purchased from Fisher Scientific UK Limited (Loughborough, UK). Brazilin Lot No.2956 was purchased from Anatech Ltd. US. Potassium persulfate from Asia Pacific Specialty Chemicals Limited. Namya-Utai (Utaitip, Lot No.46063) was received from Osotspa Co., Ltd., Bangkok. Absolute ethanol, methanol, dichloromethane were purchased from Merck. Bidistilled water was produced by our laboratory.

Preparation of sappan extract

Commercial sappan powder (10 mesh) 10 g was macerated in 400 mL of CH_3OH:CH_2Cl_2 mixture in various ratio (1:0, 3:1 and 1:3). These macerations were done in triplicate in each solvent extraction. They were shaken at room temperature for 7 days. The extracts were evaporated under vacuum to dryness. The percent yield of the extracts from each solvent extraction was calculated. The crude extract was crushed into powder and then kept in a dessicator.

Ten grams sappan powder of 2, 4, 6, 10 and 30 years old were separately macerated in 400 mL of CH_3OH:CH_2Cl_2 (1:3). These macerations were done in triplicate for each type of sappan powder. They were shaken at room temperature for 7 days. The extracts were evaporated under vacuum to dryness. The percent yield of the extracts from each source of sappan powder was calculated. The crude extract was crushed into powder and then kept in a dessicator.

Preparation of brazilin calibration curve

Brazilin was used as a standard substance for preparing calibration curve in concentration range between 0.25-1.5 mg/10 ml in phosphate buffer pH 7.4. All experiments were done in triplicate for each concentration. Phosphate buffer pH 7.4 was used as blank and absorbance of standard brazilin solution was measured at 541 nm.

Calibration curve was plotted between absorbance and concentration of brazilin, and the regression coefficient (r^2) was calculated.

Sample preparation

All kind of samples were done in triplicate for each concentration. All of these substances were diluted with phosphate buffer. The final concentration of samples was obtained as follows.

Namya-Utai was prepared in 100 µl/10 ml.The CH_3OH, CH_3OH:CH_2Cl_2 (3:1) and CH_3OH:CH_2Cl_2 (1:3) extracts from commercial sappan were prepared in 0.5 mg/10 ml. The 30, 10, 6, 4 and 2 years sappan extracts were prepared in 0.5 mg/10 ml.

Preparation of $ABTS^{\cdot+}$ solution (Re et al., 1999)

An $ABTS^{\cdot+}$ solution was prepared by mixing an equal volume of 7 mM $ABTS^{2-}$ in water with 4.9 mM potassium persulfate in water. The solution was protected from light and stored at room temperature for 12-16 hrs. $ABTS^{\cdot+}$ formation was checked its absorbance (A) at 734 nm. The absorbance of $ABTS^{\cdot+}$ was equilibrated to 0.7 ($\pm$ 0.02) by diluting with water at room temperature.

Preparation of calibration curve

Trolox was used as a standard substance. The calibration curve in concentration range between 0-17.27 $x10^{-3}$ mg/50 µL was prepared. The experiments were done in triplicate for each concentration.

For calibration, standard solution (50 µl) was mixed with of $ABTS^{\cdot+}$ solution (3 ml). Absorbances were measured at 734 nm at 6 min, giving Atrolox. Absorbance of solvent was prepared by mixing absolute ethanol (50 µl) with $ABTS^{\cdot-}$ solution (3 ml) and monitored its absorbance at 6 min.

Calculation of antioxidant capacity

Calibration curve was plotted between % inhibition of absorbance at t=6 min and concentration of trolox. The regression coefficient (r^2) was calculated from the curve.

$$\% \text{ inhibition } T \quad \frac{\text{A solvent - A compound}}{\text{A solvent}} \times 100$$

When

% inhibition T = % inhibition of $ABTS^{\cdot+}$ absorbance that was inhibited by trolox.

Preparation of samples for measuring antioxidant (brazilin, Namya-Utai and sappan extracts)

All experiments were done in triplicate in each concentrations. All of these substances were diluted with absoluted ethanol. Their final concentration of samples per 50 µl are presented as follows.

Vitamin C was prepared in concentration range of 0-13.21 $x10^{-3}$ mg/50 µl.

Brazilin was prepared in concentration range of 0-0.04 mg/50 µl.

Namya-Utai was prepared in concentration range of 0-4 µl/50 µl.

The CH_3OH, CH_3OH:CH_2Cl_2 (3:1) and CH_3OH:CH_2Cl_2 (1:3) extracts of commercial sappan were prepared in concentration range 0-20 µg/50 µl.

The 30, 10, 6, 4 and 2 years sappan extracts were prepared in concentration range 0-20 mg/50 µl.

Measurement the absorbances of samples

All experiments were done in triplicate. Sample solution 50 µl was mixed with $ABTS^{\cdot+}$ solution 3 ml. Absorbances were measured at 734 nm at 6 min. Absorbance of solvent was prepared by mixing absolute ethanol 50 µl with $ABTS^{\cdot+}$ solution 3 ml and monitored its absorbance at 6 min.

The curve of each sample was plotted between % inhibition of absorbance at t=6 min and concentration of each sample.

Calculation for brazilin equivalent

The absorbances of the extracts at the equal concentration were taken into the calibration curve of brazilin for giving the equivalent amount of brazilin.

Calculation for TEAC

$$TEAC = \frac{\%\ \text{inhibition of sample}}{\%\ \text{inhibition of trolox}}$$

TEAC is the ratio of % inhibition of sample to %inhibition of trolox at the same concentration of sample and trolox.

Apparatus

Agilent 8453E UV-Visible Spectroscopy System

Results

The calibration curve of brazilin was shown in Figure 1a. The graph equation was $A = 1.4497[C_B] + 0.0332$, when A= absorbance. From this equation, the amount of brazilin and its derivatives in sappan extracts were calculated as brazilin equivalent as shown in Table 1.

Table 1 The % yield of extracts

Type of extracts	% yield of extracts [a]
Commercial sappan (1:0) [b]	11.1±0.88
Commercial sappan (3:1) [b]	11.9±0.18
Commercial sappan (1:3) [b]	8.66±0.24
Sappan 30years old [c, d]	3.44±0.81
Sappan 10years old [c, e]	5.15±0.41
Sappan 6 years old [c, e]	4.27±0.72
Sappan 4 years old [c, e]	2.84±0.22
Sappan 2 years old [c, e]	5.07±0.63
Namya-utai	-

Note:
[a] mean ± SD, n=3
[b] The ratio in parentheses are the ratio of CH_3OH:CH_2Cl_2
[c] an CH_3OH:CH_2Cl_2 (1:3) extract
[d] branch heartwood
[e] trunk heartwood

The results of antioxidant activity of trolox (Figure 1b), vitamin C (Figure 1c), brazilin (Figure 1d), extracts (Figure 2a-b) and Namya-utai (Figure 3) are presented in % inhibition curves. All equations of the curve and their r^2 are shown in Table 2.

Table 2 The slope, intercept and r^2 of % inhibition equations from TEAC method.

Type of substances and extracts	Equation		
	slope [a]	intercept	r^2
Trolox	5.0248	- 0.9708	0.9985
Vitamin C	7.3681	- 3.0745	0.9935
Brazilin	1.7726	+ 1.044	0.9969
Commercial sappan (1:0) [b]	3.8644	+ 1.808	0.9967
Commercial sappan (3:1) [b]	4.0318	+ 1.406	0.9984
Commercial sappan (1:3) [b]	3.8972	+ 1.600	0.9980
Sappan 30 years old [c, d]	4.1608	+ 1.502	0.9969
Sappan 10 years old [c, e]	4.6128	+ 1.962	0.9959
Sappan 6 years old [c, e]	4.2690	+ 3.182	0.9846
Sappan 4 years old [c, e]	4.0498	+ 1.904	0.9945
Sappan 2 years old [c, e]	4.5444	+ 3.458	0.9886
Namya-utai	8.0248	+ 3.866	0.9865

Note : [a] [b] The slope of all equations have p-value less than 0.01.
[c] The ratio in parentheses are the ratio of CH_3OH:CH_2Cl_2
[d] an CH_3OH:CH_2Cl_2 (1:3) extract
[e] branch heartwood
trunk heartwood

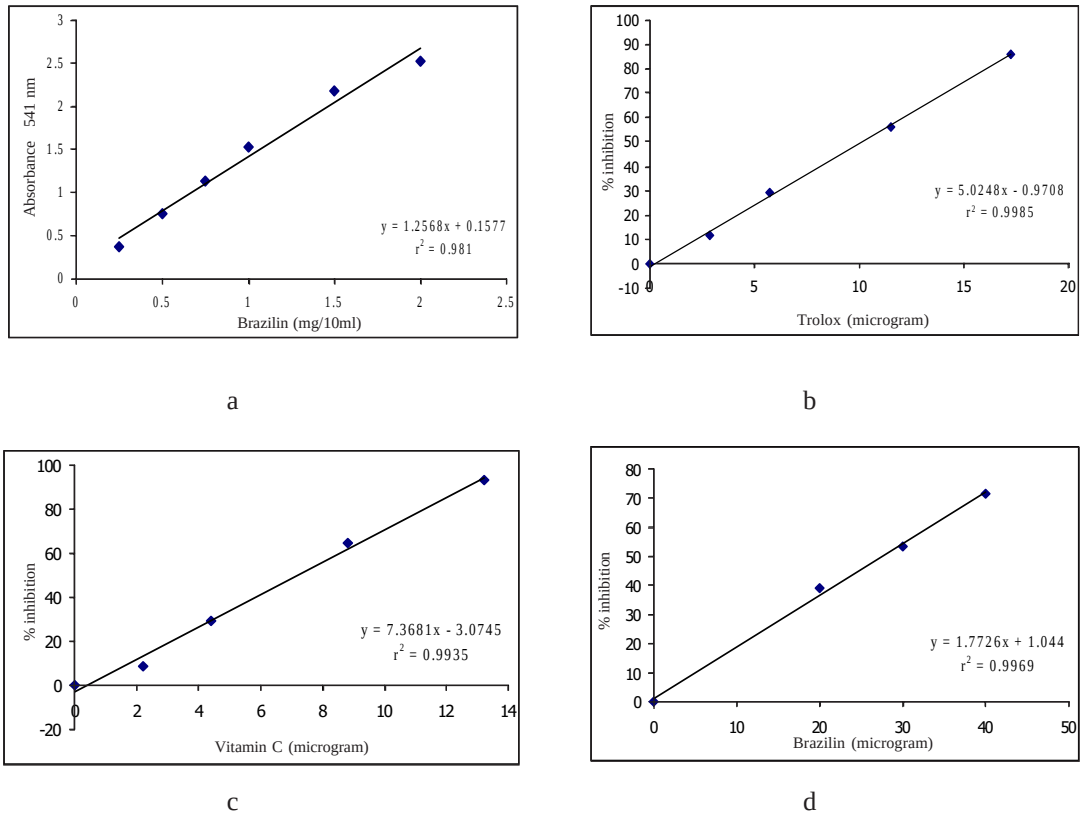
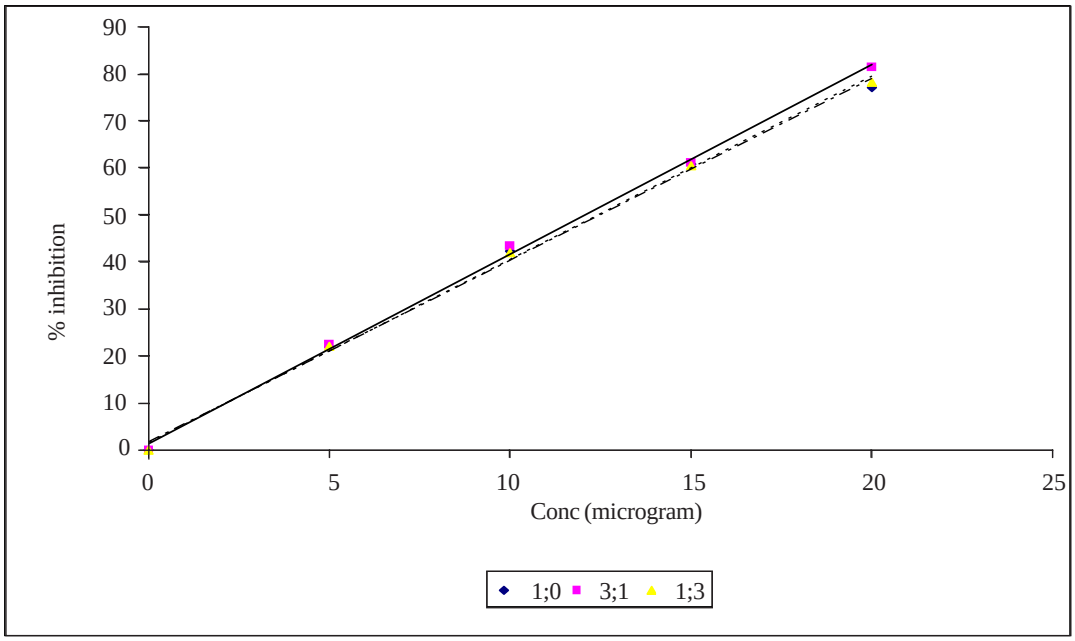
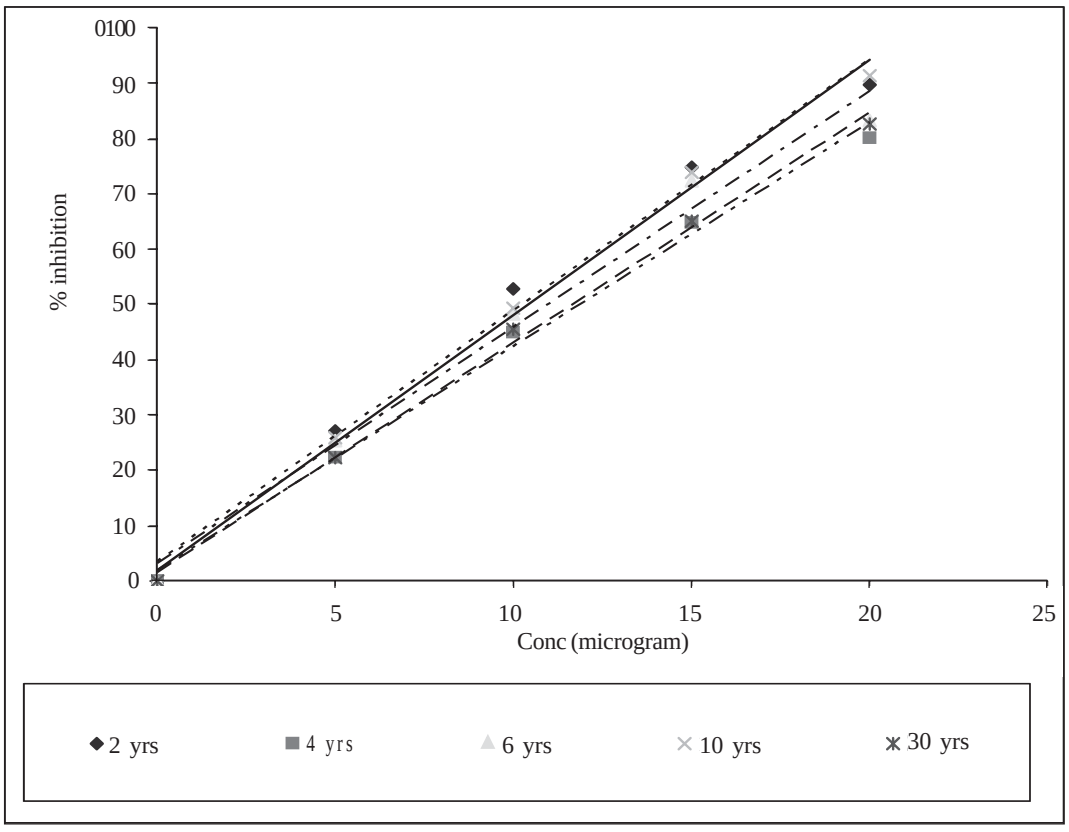


Figure 1 a) The calibration curve of brazilin in phosphate buffer pH7.4, b) The standard % inhibition curve of trolox, c) The % inhibition curve of vitamin C, d) The % inhibition curve of brazilin.





b

Figure 2 The % inhibition curve of the crude extracts obtained from commercial sappan using CH_3OH, CH_3OH:CH_2Cl_2 (3:1), CH_3OH:CH_2Cl_2 (1:3) (Figure 2a) as extraction medium, and from the sappan [in CH_3OH:CH_2Cl_2 (1:3)] in various ages (Figure 2b).

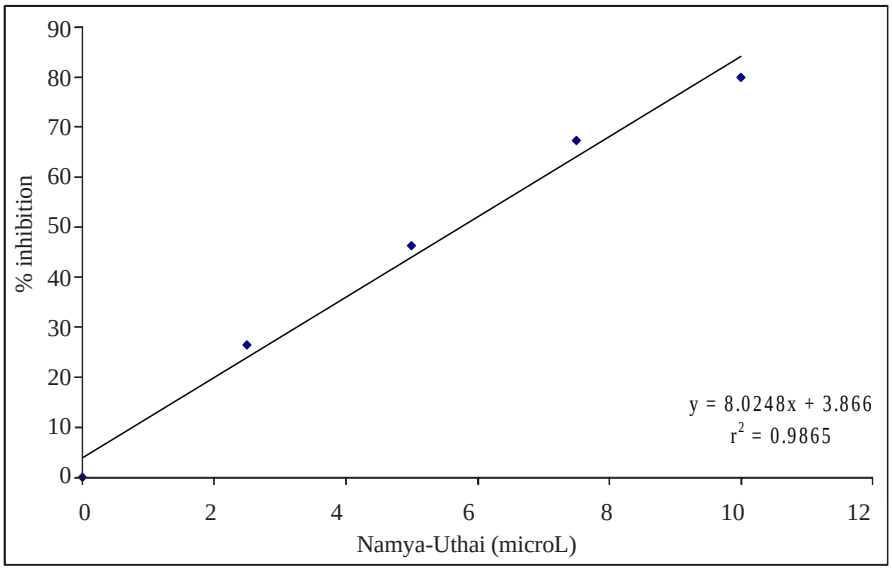


Figure 3 The % inhibition curve of Namya-utai.

The extracts have strong antioxidant activities as shown in low IC_{50} values (Table 3). IC_{50} value is the concentration of extract that shows 50% inhibition. The IC_{50} value of each extract was calculated from equation in Table 2. The antioxidant activity of each extract at equal concentration was calculated in comparable to trolox (see Table 3). The extracts showed 0.74-0.99 antioxidant activity compared to trolox. Brazilin has low antioxidant activity (0.34 compared to trolox). The commercial sappan extract using CH_3OH:CH_2Cl_2 (1:3) as extracing solvent gave higher amount of brazilin and antioxidant activity than commercial sappan extracts using CH_3OH and CH_3OH:CH_2Cl_2 (3:1) as extracing solvent. The older heartwood of the trunk, the higher antioxidant activity was found, except for 2-years old trunk heartwood extract which showed the strongest antioxidant activity. The 30-years old branch heartwood extract showed lower antioxidant activity than the 10-years old trunk heartwood. The commercial sappan extract gave lower antioxidant activity than the heartwood extract. Namya-utai presented the highest antioxidant activity.

From graphs, their slopes showed % inbition that changed per unit concentration of extracts, but high slopes did not represent the high antioxidant activities. Since the intercepts also had an influence.

Table 3 The IC_{50} value and antioxidant activity of trolox, vitamin C, brazilin and extracts and amount of brazilin equivalent (mg) in 1 mg extract

Type of substances and extracts	IC_{50}	antioxidant activity (equivalent to Trolox) TEAC	amount of brazilin equivalent (mg) in 1 mg extract
Trolox	10.1427 μg	1	
Vitamin C	7.2031 μg	1.4327	
Brazilin	28.7960 μg	0.3385	
Commercial sappan (1:0) [a]	13.4065 μg	0.7474	1.772
Commercial sappan (3:1) [a]	12.7501 μg	0.7896	1.782
Commercial sappan (1:3) [a]	12.4192 μg	0.8181	1.930
Sappan 30 years old [b, d]	11.6559 μg	0.8747	2.244
Sappan 10 years old [b, e]	10.4141 μg	0.9758	2.066
Sappan 6 years old [b, e]	10.9670 μg	0.9308	2.294
Sappan 4 years old [b, e]	11.8761 μg	0.8604	2.940
Sappan 2 years old [b, e]	10.2416 μg	0.9923	2.192
Namya-utai	5.7489 μl	1.7068	0.866 [c]

Note: [a] The ratio in parentheses are the ratio of CH_3OH:CH_2Cl_2
[b] an CH_3OH:CH_2Cl_2 (1:3) extract
[c] in 1 μl of Namya-utai solution
[d] branch heartwood
[e] trunk heartwood

Discussion

The observed antioxidant activity in this study supported the ethanomedical use of sappan and in hepatoprotective and anti-inflammation. The antioxidant activity of Namya-utai was high because it was made from a combination of herbal drugs. Its antioxidant property may not only come from sappan. Its total antioxidant activity represented the combined effect of all ingredients. However, the amount of all ingredients in the Namya-utai solution was not labeled. The commercial sappan extracts indicated lower antioxidant activity than sappan at any ages. This could explain that commercial sappan powder did not only come from the heartwood. It may be produced from heartwood and bark of sappan since the bark was always an adulterant in commercial sappan powder. From commercial sappan extracts, CH_3OH: CH_2Cl_2 (1:3) is the best extracting solution among (1:0) and (3:1) due to higher amount of brazilin and antioxidant activity than those macerated from CH_3OH and CH_3OH: CH_2Cl_2 (3:1).

Brazilin, a phenolic heterocyclic compound, the main constituent in sappan heartwood exhibited lower antioxidant activity than the extracts at the same concentration. It indicated that brazilin was not the only compound that had antioxidant property in sappan heartwood. The total antioxidant activity of the extract came from combined effect of lots of compounds in sappan, such as flavonoids and other phenolics. Brazilin might not have the strongest antioxidant activity in the extracts. Then the high brazilin equivalent in 30 years old and 4 years old extract did not correspond the high antioxidant activity (see Table 1). In this experiment, the amount of brazilin was calculated in a comparative method. The result showed the combined amount of brazilin and its derivatives, because it was not only brazilin that could absorb visible light at 541 nm. From the result of brazilin equivalent, the amount of brazilin and its derivatives vary upon ages.

The age of the heartwood was a factor for strong antioxidant activity, except for the 2 years old extract. It may be that the 2 years old extract would have higher amount of other compounds that had antioxidant activity. Finally, trunk was better than branch in giving an antioxidant activity.

Conclusion

Sappan heartwood had high antioxdant activity. It was used as traditionally coloring agent in bevarage, food and cosmetics. Its high effect of antioxidant activity may protect other ingredients in recipe from oxidation reaction. At any ages of sappan, the amount of brazilin equivalent and the antioxidant activity are very high.

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