

Full Length Research Paper

## Antioxidant activity and protective effects on DNA damage of *Caesalpinia sappan* L. extract

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Several medicinal plants in Northern Thailand have been used for inflammatory symptom and various diseases by traditional practitioners for a long time without the scientific data supported. *Caesalpinia sappan* Linn. is a medicinal plant cultivated in Northern Thailand and has been used for various disease treatment especially inflammatory symptom, arthritis and cancer. The heartwood of *C. sappan* was extracted with 95% ethanol and concentrated under reduced pressure. The extract was studied for free radical scavenging activity on ABTS<sup>•+</sup>, superoxide anion, nitric oxide and total phenolic content. In addition, the extract was studied on protective effect on DNA damage-induced by hydroxyl radical. *C. sappan* extract exerted strong scavenging activity on ABTS<sup>•+</sup> with the VCEAC = 0.5782±0.0042 gram L-ascorbic acid/gram extract and TEAC = 0.9159±0.0055 gram Trolox/gram extract. *C. sappan* extract also exhibited high scavenging activity on superoxide anion with an EC<sub>50</sub> value of 7.73±0.06 µg/ml, which was comparable to the activity of L-ascorbic acid and rutin (EC<sub>50</sub> value of 6.65±0.07 and 7.83±0.13 µg/ml, respectively). Furthermore, it exerted the strong activity on nitric oxide scavenging activity with an EC<sub>50</sub> value of 4.24±0.14 µg/ml. This activity was comparable to curcumin with an EC<sub>50</sub> value of 5.70±0.08 µg/ml. It also contained high amount of phenolic content with the gallic acid equivalent = 0.5540±0.0192 mg gallic acid/mg extract. *C. sappan* extract also showed the protective effect on DNA damage-induced by hydroxyl radical. According to the strong activity on free radical scavenging *in vitro* and protective effect on DNA damage-induced by hydroxyl radical, *C. sappan* has the potential for chemopreventive study.

**Key words:** *Caesalpinia sappan* L., antioxidant activity, DNA damage, superoxide anion, nitric oxide.

### INTRODUCTION

Thailand is a well-known, excellent source of nature-based health products. There are many kinds of medicinal plants and dietary supplements, with 2,000 plant species used as ingredients in traditional medicine prescriptions. Among these, more than 300 plants are frequently used, especially in Northern Thailand. Northern Thailand has many attractive features that would be of interest to horticultural and traditional herbal medicine.

The Northern Thai community has a long history of

extracting medicinal plants for local consumption, both for health promotion and disease treatment. Medicinal plants in Northern Thailand have been used since time immemorial to treat various disorders, offering an alternative to synthetic compounds, as they are considered non-toxic or, at least, less toxic.

In the normal metabolism status, the levels of free radicals and antioxidants in humans are maintained in balance, which is important for sustaining optimal physiological conditions (Temple, 2000). Overproduction of free radicals in certain conditions can cause an imbalance, contributing to disease processes by causing oxidative damage to biomolecules (lipid, DNA and protein) and altering cellular metabolism.

The mechanism for DNA damage, leading to mutation,

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is explained by the attack of hydroxyl radicals on guanine in DNA. Free radicals such as superoxide anion and nitric oxide intervened in the inflammatory process. Nitric oxide can induce cyclo-oxygenase, the rate limiting step enzyme for inflammation process and enhance production of interleukin-1 and tumor necrosis factor (Jang and Murrell, 1998).

*Caesalpinia sappan* L. is a well-known medicinal plant belonging to the Leguminosae family, distributed and cultivated in Southeast Asia as well as in Africa and America. It is a small to medium size, shrubby tree, 4-8 m tall, trunk up to 14 cm in diameter, bark with distinct ridges and many prickles and grayish brown. Many biological activities of *C. sappan* have been reported, such as anti-bacterial and anti-inflammatory activity (Nirajan-Reddy et al., 2004), immunomodulation (Choi et al., 1997), immunosuppression (Ye et al., 2006), hepato-protection (Moon et al., 1992), hypoglycemic activity (Kim et al., 1995), anticomplementary (Oh et al., 1998), antioxidant (Badami et al., 2003) and inhibit lipopolysaccharide-induced nitric oxide production by macrophages (Hikino et al., 1977).

Phenolic compounds are isolated from *C. sappan* such as homoisoflavonoids and the related compounds, protosappanin A (Nagai et al., 1986), protosappanin B (Nagai and Nagumo, 1986), brazilin and brazilein (Miyahara et al., 1986), brazilide A (Yang et al., 2002), 7,3',4'-trihydroxy-3-benzyl-2*H*-chromene (Zhao et al., 2008) and 3'-deoxy-4-*O*-mehtylepisappanol (Fu et al., 2008). Brazilin and brazilein are two major components. Brazilin can induce the expression of hemeoxygenase-1 (HO-1) and against tert-butylhydroperoxide (t-BHP)-induced cell death in House Ear Institute-Organ of Corti 1 (HEI-CO1) cells (Choi et al., 2007). Administration of brazilein after onset of cerebral ischemia reperfusion can reduce the brain infraction area and improve the neurological score (Shen et al., 2007). In Thailand, *C. sappan* is frequently used in traditional medicine formulation in Northern of Thailand especially in Chiang Mai, Nan and Lampang province. It is traditionally used as anti-inflammatory for the treatment of traumatic disease and arthritis. A decoction of the heartwood is traditionally used as coloring agent in food, beverage and cosmetics. However, its efficacy proven with scientific methods, which can be manipulated to give a better understanding of its mechanism of action, was verified in recent time. The objective of this study was to find out and confirm the effects of ethanolic extract of *C. sappan* on antioxidant activity with several methods. At the same time, we measured the total phenolic content of this plant to evaluate the contribution to antioxidant activity. Phenolic compounds are believed to account for major portion of antioxidant activity in many plants (Duthie and Crozier, 2000). In this study, it was the first time to investigate protective effects of *C. sappan* extract on DNA damage-induced by hydroxyl radical to evaluate the potential of *C. sappan* extract for chemopreventive study.

Relationship between total phenolic content and protective effects on DNA damage-induced by hydroxyl radical was also studied.

## MATERIALS AND METHODS

### Chemicals

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, curcumin, nitroblue tetrazolium (NBT), phenazin methosulfate (PMS) and reduced  $\beta$ -nicotinamide adenine dinucleotide (NADH) were purchased from Sigma Chemical Co. (St. Louis, MO). Naphthylethylenediamine dihydrochloride, rutin, sodium nitroprusside (SNP), sulphanilamide and Folin-Ciocalteu reagent were purchased from Fluka Chemical Co. (Switzerland). Plasmid DNA P<sup>UC18</sup> and ethidium bromide were purchased from Vivantis Co. (Malaysia). Other chemicals used, including the solvents, of analytical grade, were purchased from Merck (Darmstadt, Germany).

### Plant material and preparation of extract

*C. sappan* heartwood was collected from San Pa Thong district, Chiang Mai, Thailand in May, 2005. The specimen was compared to herbarium specimen at Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand. The sample was cut into small pieces, dried in a hot air oven (60°C, 48 h) and ground into powder. Sample powder 500 grams was extracted with 95% ethanol using soxhlet's apparatus for 48 h to obtain ethanolic extract. The sample was concentrated and dried by evaporation under reduced pressure.

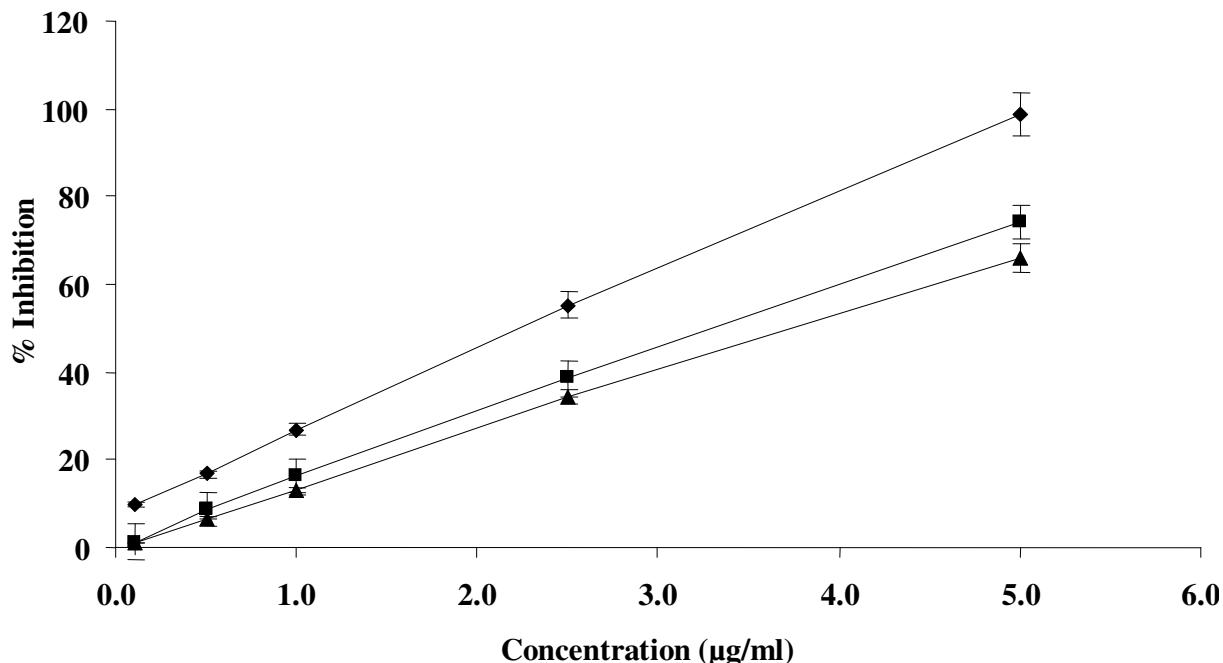
### ABTS free radical cation decolorization assay

ABTS free radical cation decolorization assay was carried out using an improved method with some modification (Re et al., (1999). ABTS<sup>•+</sup> was generated by oxidation of 7.0 mM ABTS with 2.5 mM potassium persulfate. The ABTS<sup>•+</sup> was mixed with the sample at different concentrations (10 to 1,000  $\mu$ g/ml), comparing it to both standard L-ascorbic acid and trolox. After 3 min incubation at room temperature, the color reaction was measured at 734 nm spectrophotometrically. Results of ABTS<sup>•+</sup> decolorization assay were expressed as Vitamin C equivalent antioxidant capacity (VCEAC) and Trolox equivalent antioxidant capacity (TEAC). This index is defined as gram of standard whose antioxidant capacity is equivalent to 1.0 gram of the extract.

### Superoxide anion radical scavenging activity assay

Superoxide anion radicals were generated in a PMS-NADH system by oxidation of NADH and assayed by the reduction of NBT. In this experiment, the superoxide anion radicals were generated in 200  $\mu$ l of PBS buffer (pH 7.4), which contained 2.5  $\mu$ M NADH, 0.5  $\mu$ M NBT, 2.5  $\mu$ M EDTA and sample at different concentrations starting from 10 to 1,000  $\mu$ g/ml.

They were prepared in 96 well plates, compared to standard L-ascorbic acid and rutin. PMS was added to initiate the reaction. After 5 min incubation at room temperature, the color reaction between superoxide anion radical and NBT was measured at 560 nm spectrophotometrically (Yanping et al., 2004).



**Figure 1.** Dose-response curve of ABTS<sup>+</sup> scavenging activity: ▲ = *C. sappan* extract, ■ = Trolox and ◆ = L-ascorbic acid. Data was expressed as mean ± SD. n = 3.

#### Nitric oxide scavenging activity assay

Nitric oxide was generated from sodium nitroprusside (SNP) and measured by the Griess reaction. Nitric oxide interacts with oxygen to produce nitrite ions that can be observed by Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (Maccocci, 1994). Different concentrations of samples and sodium nitroprusside (SNP, 5 mM final concentration) in phosphate buffer saline, pH 7.4, in a final volume of 1 ml were incubated at 25°C for 150 min. A control experiment without samples but with equivalent amount of vehicles was conducted in an identical manner of the sample. After incubation, the reaction mixtures were mixed with Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2% H<sub>3</sub>PO<sub>4</sub>). The absorbance of the chromophore formed during diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine was measured at 540 nm. Curcumin was used as a reference standard (Sreejayan and Rao, 1997).

#### Total phenolic contents

The total phenolic contents of the extract were determined using the Folin–Ciocalteu colorimetric assay. Results are expressed as mg gallic acid equivalent (GAE) per mg of the extract (AOAC, 1990).

#### Protective effect on DNA damage-induced by hydroxyl radical

The reaction mixture contained 50 ng/µl pUC18 plasmid DNA in buffer, 8 µM of Fe(II), 25 µM of H<sub>2</sub>O<sub>2</sub> and different concentrations of tested samples (*C. sappan* extract and epigallocatechin gallate, EGCG). After incubation at 37°C for 60 min, 3 µl of electrophoresis loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycol) was added to the reaction mixture, then an aliquot

(10 µl) was loaded to a 1% agarose gel in 0.5x TBE buffer and electrophoresis was carried out as 100 V for 90 min. Following electrophoresis, gels were stained with ethidium bromide for 30 min. After washing, the bands were visualized under a UV trans-illuminator. Changing of the intensity of the bands are due to DNA strand breakage that leads to a decrease in the proportion of the supercoiled form (form I) and to an increase in the relaxed open-circular form (form II) and the linear double-stranded form (form III). The relative intensities of DNA bands were determined with a gel documentation system (Chenyang et al., 2005).

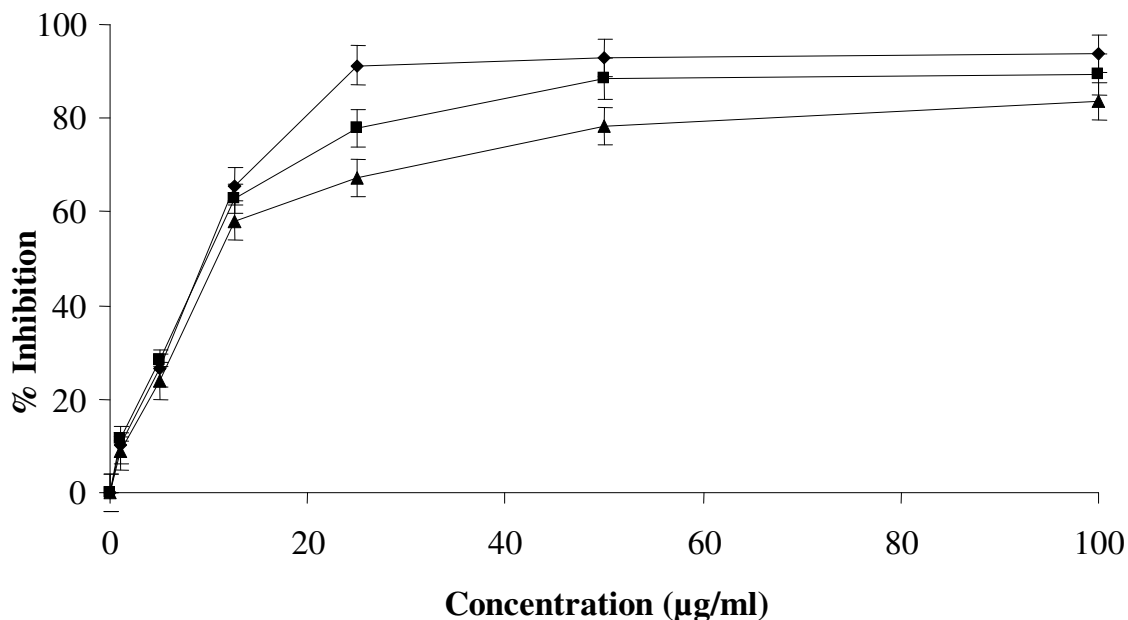
#### Statistical analysis

All results were presented as mean ± SD. The data correlation was obtained by Pearson correlation. Statistical significance was considered at  $p < 0.05$ .

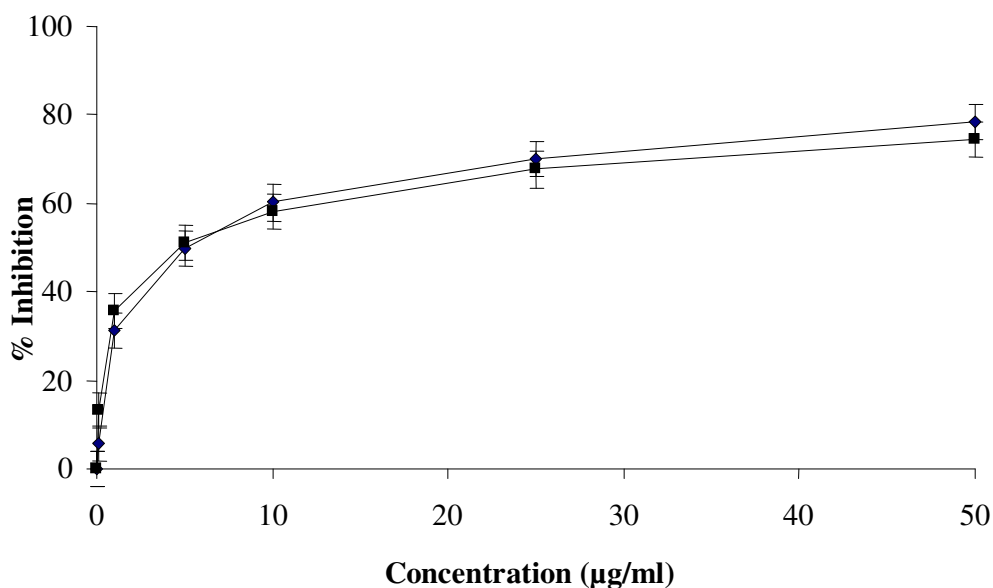
## RESULTS

#### Antioxidant activity

As shown in Figure 1, *C. sappan* exhibited the potent ABTS<sup>+</sup> scavenging activity with the VCEAC =  $0.5782 \pm 0.0042$  gram L-ascorbic acid/ gram extract and TEAC =  $0.9159 \pm 0.0055$  gram Trolox/ gram extract. Furthermore, *C. sappan* exerted strong activity on superoxide anion scavenging activity with an EC<sub>50</sub> value of  $7.73 \pm 0.06$  µg/ml, which was comparable to the activity of L-ascorbic acid and rutin with an EC<sub>50</sub> value of  $6.65 \pm 0.07$  and  $7.83 \pm 0.13$  µg/ml, respectively (Figure 2). Additionally, it exhibited potent activity on nitric oxide scavenging activity



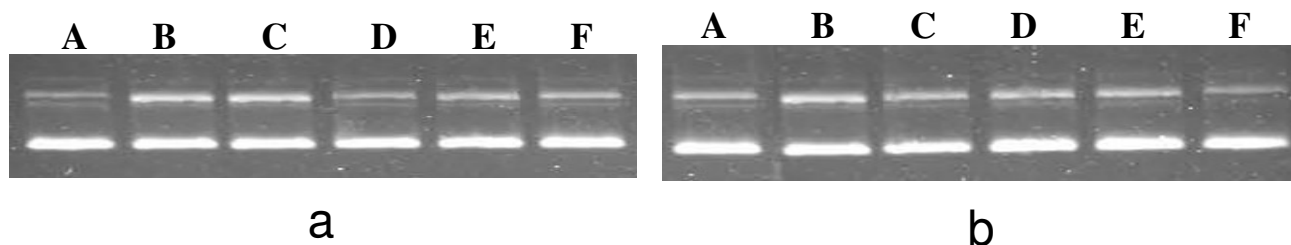
**Figure 2.** Dose-response curve of superoxide anion scavenging activity: ■ = *C. sappan* extract, ◆ = L-ascorbic acid, ▲ = Rutin. Data was expressed as mean  $\pm$  SD. n = 3.



**Figure 3.** Dose-response curve of nitric oxide scavenging activity: ■ = *C. sappan* extract and ◆ = Curcumin. Data was expressed as mean  $\pm$  SD. n = 3.

with an  $EC_{50}$  value of  $4.24 \pm 0.14$   $\mu\text{g/ml}$ , which was comparable to curcumin with an  $EC_{50}$  value of  $5.70 \pm 0.08$   $\mu\text{g/ml}$  (Figure 3). *C. sappan* extract was found to be an efficient scavenger of  $ABTS^{*+}$ , superoxide anion radical and nitric oxide in a dose-dependent manner. The extract had high amounts of phenolic content with the GAE value of  $0.5440 \pm 0.0192$  mg gallic/mg extract. The

total phenolic content was strongly related to superoxide anion and nitric oxide scavenging activity ( $r = 0.999$  and  $0.984$ , respectively,  $p < 0.01$ ). Finally, the total phenolic content was also related to  $ABTS^{*+}$  scavenging activity ( $r = 0.895$ ,  $p < 0.05$ ). The results revealed that the phenolic compounds were the major constituents for antioxidant activity of *C. sappan*.



**Figure 4.** Electrophoresis picture of pUC18 plasmid DNA. The sample was treated by 8  $\mu\text{M}$  Fe(II) and 25  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 60 min with different concentrations of (a) EGCG : (A) control without Fe(II) and  $\text{H}_2\text{O}_2$ ; (B) control with Fe(II) and  $\text{H}_2\text{O}_2$  (C) 1  $\mu\text{g/ml}$ ; (D) 5  $\mu\text{g/ml}$ ; (E) 25  $\mu\text{g/ml}$ ; and (F) 50  $\mu\text{g/ml}$  (b) *C. sappan* extract: (A) control without Fe(II) and  $\text{H}_2\text{O}_2$ ; (B) control with Fe(II) and  $\text{H}_2\text{O}_2$ ; (C) 1  $\mu\text{g/ml}$ ; (D) 5  $\mu\text{g/ml}$ ; (E) 25  $\mu\text{g/ml}$ ; and (F) 50  $\mu\text{g/ml}$ .

**Table 1.** Ratio between supercoiled and relaxed form DNA after treatment with hydroxyl radical and EGCG or *C. sappan* extract

Concentration ( $\mu\text{g/ml}$ )	Ratio between supercoiled and relaxed form DNA	
	EGCG	<i>C. sappan</i> extract
Control without Fe(II) and $\text{H}_2\text{O}_2$	3.80 $\pm$ 0.09	3.65 $\pm$ 0.08
Control with Fe(II) and $\text{H}_2\text{O}_2$	1.46 $\pm$ 0.07*	1.58 $\pm$ 0.09**
1	1.57 $\pm$ 0.07*	1.90 $\pm$ 0.06**
5	3.74 $\pm$ 0.08	3.67 $\pm$ 0.08
25	3.83 $\pm$ 0.09	3.72 $\pm$ 0.06
50	3.87 $\pm$ 0.08	3.69 $\pm$ 0.09

\* and \*\*: Statistically significant at  $P < 0.05$ , using ANOVA with Duncan's test between different concentration of EGCG and *C. sappan* extract, respectively.

### DNA damage protection

The effect of *C. sappan* extract on DNA damage protection was assessed by agarose gel electrophoresis. A representative result was shown in Figure 4b. *C. sappan* extract at a concentration of 5  $\mu\text{g/ml}$  protected pUC18 plasmid DNA from damage induced by Fe(II) plus  $\text{H}_2\text{O}_2$ . The activity was similar to EGCG at the same concentration, as shown in Figure 4a. From this picture, we can observe that the control pUC18 plasmid DNA (without Fe(II) and  $\text{H}_2\text{O}_2$ ) contains mostly supercoiled form small amount of relaxed form (lane A). At the fixed Fe(II) concentration (8  $\mu\text{M}$ ) and  $\text{H}_2\text{O}_2$  concentration (25  $\mu\text{M}$ ), the proportion of supercoiled form decreases, whereas that of the relaxed form increases (lane B).

Table 1 exhibited ratio between supercoiled and relaxed form DNA. *C. sappan* extract exhibited significant protection against DNA damage-induced by hydroxyl radical generated by Fenton reaction at the same concentration comparable to EGCG at 5  $\mu\text{g/ml}$ . The ratio between supercoiled and relaxed form were not significantly different when the concentration of EGCG and *C. sappan* extract more than 5  $\mu\text{g/ml}$ . The total phenolic content was related to protective effects against DNA damage-induced by hydroxyl radical ( $r = 0.885$ ,  $p < 0.05$ ).

### DISCUSSION

Cells and other organs in the human body are constantly exposed to a variety of oxidizing agents and free radicals, some of which are necessary for life. In normal metabolism, the level of oxidants and antioxidants are maintained in a delicate balance by the antioxidant defense system, which is important for sustaining optimal physiological conditions (Temple, 2000). Free radicals have been implicated in many disease conditions. Overproduction of oxidants can cause oxidative stress and oxidative damage to lipid, protein and nucleic acid, which are associated with chronic diseases such as cancer, cardiovascular and neurological disease (Pratico and Delant, 2000). Herbal drugs containing radical scavengers are gaining importance in the treatment of such diseases. Many plants exhibit efficient antioxidant activities owing to their phenolic constituents (Larson, 1988). Epidemiological studies strongly support the idea that increased consumption of phenolic compound-containing foods are associated with health maintenance and the prevention of chronic, age-associated and degenerative diseases, such as cardiovascular and cerebrovascular disease, Parkinson's disease and Alzheimer's disease, as well as many other pathological conditions (Ames et al., 1993; Havsteen, 2002; Johnson, 2004).

In this study, we investigated the antioxidant activity of the ethanolic extract of *C. sappan* by the decolorization of the ABTS<sup>•+</sup>, the scavenging effect on superoxide anion radical and nitric oxide and determination of DNA-damage protection-induced by Fenton reaction. Finally, the total phenolic content was determined by the Folin-Ciocalteu method.

ABTS is one of the radicals generally used for testing the preliminary radical scavenging activity of a compound or plant extract. The ABTS<sup>•+</sup>, generated from oxidation of ABTS by potassium persulfate, is presented as an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants (scavengers of aqueous phase radicals) and of chain-breaking antioxidants (scavengers of lipid peroxyl radicals) (Leong, 2002). In the present study, the *C. sappan* extract exhibited a strong antiradical activity by scavenging ABTS<sup>•+</sup> compared to L-ascorbic acid and Trolox.

Superoxide anion (O<sub>2</sub><sup>•-</sup>), the one-electron reduced form of molecular oxygen, is a precursor to active free radicals that have powerful reactions with biological macromolecules, thereby inducing tissue damage (Halliwell and Gutteridge, 1985). In the PMS-NADH-NBT system, superoxide anion radical, which is generated from dissolved oxygen by the PMS-NADH coupling reaction, reduces NBT. The reduction of absorbance at 560 nm, with antioxidants, indicates the scavenging of superoxide anion in the reaction mixture. Superoxide anion scavenging activity occurred in the following order: L-ascorbic acid > *C. sappan* extract > rutin. These results revealed that the *C. sappan* extract was an efficient scavenger of superoxide anion radical. According to the source of raw material and extraction process, the scavenging effects on superoxide anion was higher than those of Jun et al. (2008), who reported that 95% ethanolic extract of *C. sappan* heartwood showed little scavenging effects on superoxide anion compared to L-ascorbic acid.

In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Moncada et al., 1991). Nitric oxide (NO) is a defense molecule with cytotoxic, microbiocidal and microbiostatic activities. SNP will release nitric oxide when dissolved in PBS solution and reacts with oxygen to form nitrite. SNP solution under aerobic conditions, in the presence of various extracts with Griess reagent, can be used to evaluate the scavenging effect on nitric oxide of the extract. *C. sappan* extract inhibits nitrite formation by competing with oxygen to react with nitric oxide (Figure 3). Due to ABTS free radical cation decolorization assay, superoxide anion and nitric oxide scavenging activity, *C. sappan* extract exhibited the potent radical scavenger. The results corresponded with Wetwitayaklung et al. (2005), who reported that *C. sappan* heartwood exerted high antioxidant activity measured by ABTS free radical cation decolorization assay.

At physiological condition, plasmid DNA is composed of mostly supercoiled form (form I) and a small amount of

the relaxed form (form II). Plasmid DNA is sensitive to damage caused by a variety of agents. When cleavage of one of the phosphodiester chains of the supercoiled DNA (form I) occurs, it produces a relaxed open-circular form (form II). Further cleavage of the circular strand very close to the site of the initial damage produces linear double-stranded DNA molecule (form III) (Spotheim-Maurizot, 1991; Burrows, 1998). The Fenton reagents are composed of Fe(II) and H<sub>2</sub>O<sub>2</sub>. They can produce the hydroxyl radical via initiating and catalyzing the decomposition of H<sub>2</sub>O<sub>2</sub> by Fe(II). Hydroxyl radicals can react with plasmid DNA to produce a relaxed open-circular form. This experiment was the first report for protective effect of *C. sappan* on DNA damage-induced by hydroxyl radical. *C. sappan* extract inhibited hydroxyl radical-mediated damage to pUC18 plasmid DNA at the same concentration of EGCG. Furthermore, the extract exhibited a high amount of phenolic compound content. These findings indicated that the potent antioxidant activity of *C. sappan* extract partly contributed to the amount of phenolic compound contents.

In conclusion, according to these experiments using several activity-evaluation methods, the ethanolic extract of *C. sappan* was found to be a potent radical scavenger. The extract exhibited a strong superoxide anion radical and nitric oxide scavenging activity. The extract was also effective on DNA damage-protection induced by hydroxyl radical. The results of this investigation, which determined the radical scavenging and antioxidant activity of ethanolic extract of *C. sappan*, demonstrates that *C. sappan* extract might be proposed as a dietary supplement or traditional drug for the prevention and/or treatment of conditions that occur due to oxidative damage and can protect DNA damage by hydroxyl radical. Detailed work is currently being undertaken to investigate the purified compounds of this extract with the potent antioxidant activity and chemopreventive potential.

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