RESEARCH PAPER

Assessment of mutagenic and genotoxic activity of *Caesalpinia sappan* L. extract by Ames test and micronucleus assay

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Keywords
Caesalpinia sappan
genotoxic activity
mutagenic activity
Ames test
micronucleus assay

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Abstract

The mutagenic activity of the water extract of sappan heartwood (*Caesalpinia sappan* L.) was evaluated by Ames test. A plate incorporation method with and without the rat liver mixture (S9 mix) using *Salmonella typhimurium* strains TA98 and TA100 as indicator strains was applied in the present study. This plant extract exhibited an antibacterial potential at the concentration above 2.0 mg/plate either with or without S9 mix. However, it showed no mutagenic activity at the concentration between 0.25 and 2.5 mg/plate. The genotoxicity of the extract from sappan heartwood was further assayed by a cytokinesis – block micronucleus test using Chinese Hamster lung fibroblast (V79 cell line). It was found that the extract showed the cytotoxic effect to V 79 cell line at 400 and 200 µg/ml in the presence and absence of S9 mix, respectively, but was revealed to have no toxic effect to DNA observed at the concentration between 5 and 200 µg/ml.

Introduction

Sappan wood (*Caesalpinia sappan* L.) has been used in Thai traditional medicine for a long time, especially in the herbal formula used to treat a number of ailments. Sappan wood extract exhibited antimicrobial (Mohan *et al*, 2001, Niranjan *et al*, 2003, Xu and Lee, 2004), antioxidant activities (Badami *et al*, 2003, Yingming *et al*, 2004, Wetwitayaklung *et al*, 2005), hepatoprotection (Moon *et al*, 1992, Srilakshmi *et al*, 2010) and also antidiabetic (Moon *et al*, 1998, Kim *et al*, 1995). For the antimicrobial activity, the water extract of sappan wood was demonstrated to have the inhibitory effect against a number of microorganisms e.g., *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium* but not to *Candida albicans* (Suttisawad and Thanaisawanyangkura, 2006). Its antimicrobial activity was confirmed when 1 % of its water extract added to chilli paste was able to prolong the shelf life of this paste to be at least 6 months by limiting the number of contaminants to be less than stated by the guideline of Department of Medical Science, Ministry of Public Health, Thailand for food products. The water extract of sappan wood tends to be useful as a preservative in chilli pastes. The extract showed neither acute nor sub acute toxicity when was conducted in both male and female rat (Sireeratawong *et al*, 2010). However, further safety information relating to gene and others of this extract is still needed before its promotion for public use. The objective of the present study was to assess the effect of the sappan heartwood water extract on mutagenicity using Ames test and micronucleus formation using a micronucleus frequency assay.

Materials and Methods

Media and chemicals: Oxoid nurient broth No. 2 (Oxoid, Ltd. England), Vogel-Bonner minimal glucose agar and Top agar were in-house prepared. Mitomycin C and cytochalasin B were purchased from Sigma Aldrich, USA. Giemsa stain, DMEM medium and fetal bovine serum were purchased from Gibco Invitro gen Coperation, USA. Cyclophospha-mide (Endoxan) were purchased from Baxter Oncology Gmb H, Germany.

Sappan heartwood and extraction: Sappan wood was purchased from Vejapong Osoth retail shop in Bangkok and identified by an expert Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University. The extraction was conducted by cutting 1 kg of sappan heartwood into small pieces and boiled with 10 liters of water for 30 min. After filtration, the marc was subsequently boiled following the same procedure 3 times until exhausted. The filtrates were combined and freeze-dried. The dry power was kept in a tight container. For working use, a part of this powder was dissolved in distilled water to 100 mg/ml and stored at -20°C.

Bacterial Strains: *Salmonella typhimurium* histidine auxotrophs tester strains TA98 (his D3052,rfa, uvrB ,pKM 101) and TA100 (his G46,rfa, uvrB , pKM 101) kindly provided by National Cancer Institute, Bangkok, Thailand was received as the frozen stocks. They were genotypic characterized regarding to Maron and Ames (1983).

Fraction S9 and S9 Mix: Rat liver homogenate containing metabolic enzyme (S9 fraction) was prepared as previously described (Matsushima, 1976) at the Department of Biochemistry, Faculty of Medicine, Chiang Mai University, Thailand. The components of S9 mix included 8 mM magnesium chloride, 33 mM potassium chloride, 5 mM G6P, 4mM NADP, 100 mM sodium phosphate (pH7.4), and 10% S9 fraction (v/v).

Cell culture: V79 cell line (Chinese hamster lung fibroblast cells) was cultured in the DMEM growth medium supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic.

Ames Test: The mutagenic effect of the extract was assayed using Ames test. The test was based on a plate incorporation method (Maronand Ames, 1983) using *S. typhimurium* strains TA98 and TA100 in the presence and absence of metabolic activation. Briefly, the sappan heartwood extract at 0.25, 0.5, 1.0, 2.0 and 2.5 mg/plate was added onto the tester strains in the absence and presence of exogenous metabolic activation (rat liver S9 mix) according to the recommendation of OECD guideline 471 (OECD,1997). Distilled water was used as the negative control. Standard mutagens, 2-(2-furyl-3-(5-nitro-2-furyl) acrylamide (AF2) and benzo(a)pyrene (BP), were used as the positive controls for TA98 and TA 100 without S9 mix and with S9 mix, respectively. The experiment was conducted in triplicate samples and repeated twice.

According to the generally accepted procedure for this test, the substance is interpreted to be mutagenic if it causes doubling of the number of his+ revertants comparing to the control (Brusick, 1987). Statistical analysis was performed using the independent-sample *t* test. Significance of the data was considered at $p < 0.05$.

Cytokinesis-block proliferation index (CBPI) and Micronucleus (MN) frequency assays: The cytokinesis-block micronucleus assay was performed following the final validated protocol (Fenech, 2000) with slight modification. Briefly, V79 cells (5×10^4 cells) were incubated with 5, 10, 50 ,100 and 200 µg/ml of sappan heartwood extract in the presence and absence of S9 mix at 37°C for 3 h. Distilled water was used as the negative control, while mitomycin C at 1 µg/ml and cyclophosphamide at 20 µg/ml were the positive controls. The media were refreshed, and 3µg/ml of cytochalasin B was added at 18 h as the first mitotic division blocker. before the treated-cells were harvested after 24-h incubation. The treated cells were trypsinized and prepared as the monolayer on glass slides using cytospin equipment. After staining with 10% Giemsa solution, the cells were examined under the microscope (400X magnification). Cytokinesis-block proliferation index (CBPI) was calculated from the sum amounts of mononucleated + 2x binucleated, and + 3x multinucleated cells in each group divided by total number of the cells. Micronucleus (MN) frequency was determined under the microscope (1000X magnification) from the number of micronucleus, resulted from acentric fragmented chromosomes during anaphase stage, per 1000 binucleated cells. Percent cytostasis effect was evaluated from cell division inhibition using the following equation:

$$\% \text{ cytostasis} = 100 - 100\frac{(\text{CBPI}_\text{T} - 1)}{(\text{CBPI}_\text{c} - 1)}$$

where CBPI_T= CBPI from the test compound and CBPI_C = CBPI from the vehicle control.

All experiments were performed in triplicate. Data were analyzed by one way ANOVA using SPSS program and Tukey multiple comparison test.

Significance of the data was considered at $p<0.05$**.**

Results and Discussion

The assessment of the mutagenic activity of sappan heartwood water extract was performed based on the results obtained in the Ames test and the properties of cytotoxicity and genotoxicity that were those obtained with CBPI-MN assays in V79 cells.

Mutagenicity test was conducted using *S. typhimurium* strains TA98 and TA100. Sappan extract at all tested concentrations (0.25, 0.5, 1.0, 2.0 and 2.5mg/plate) both in the presence and absence of S9 mix did not show any significant increase in the number of revertant bacterial colonies either in TA98 or TA100 when compared to the negative control (Table 1 and Figure 1).

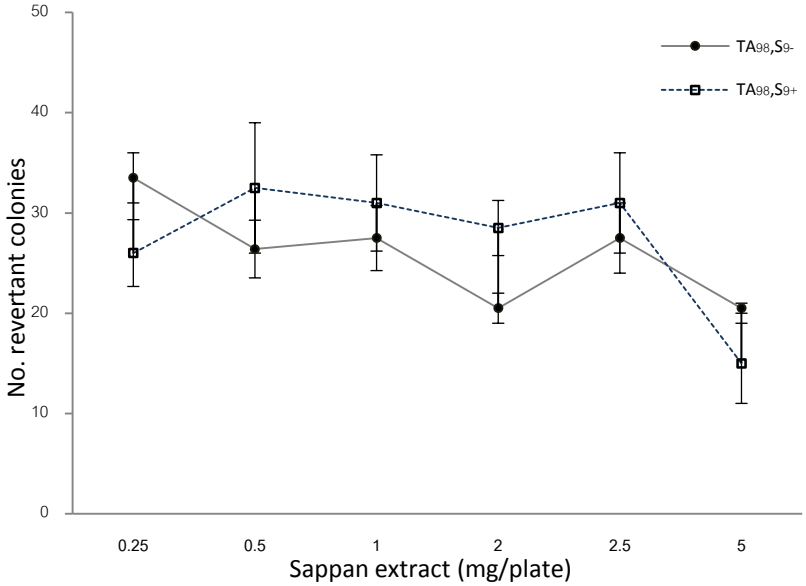
Table 1 Ames test results for mutagenic activity of sappan extract on *S. typhimurium* TA98 and TA100 in the presence (+S9) and absence (-S9) of S9 mix for metabolic activation

Treatment (Concentration)	Average of revertant bacterial colonies (colonies per plate, mean±SD)			
	TA98		TA100	
	-S9	+S9	-S9	+S9
Distilled H_2O	32.2±4.8	29.5±4.3	157.5±11.8	154.2±8.5
AF-2				
0.01 µg/plate	ND*	ND	715.8±46.0**	ND
0.10 µg/plate	731.5±22.5**	ND	ND	ND
BP				
1.25 µg/plate	ND	ND	ND	1270±278.3**
5.0 µg/plate	ND	615.8±165.1**	ND	ND
Sappan Extract				
0.25 mg/plate	33.5±2.5	26.0±3.3	138.3±25.3	141.8±21.8
0.5 mg/plate	26.4±2.9	32.5±6.5	152.0±25.6	135.0±20.0
1.0 mg/plate	27.5±3.3	31.0±4.8	150.0±24.6	166.3±32.4
2.0 mg/plate	20.5±1.5**	28.5±2.8	118.0±28.0	159.0±31.0
2.5 mg/plate	27.5±3.5	31.0±5.0	100±4.0	135.5±17.5

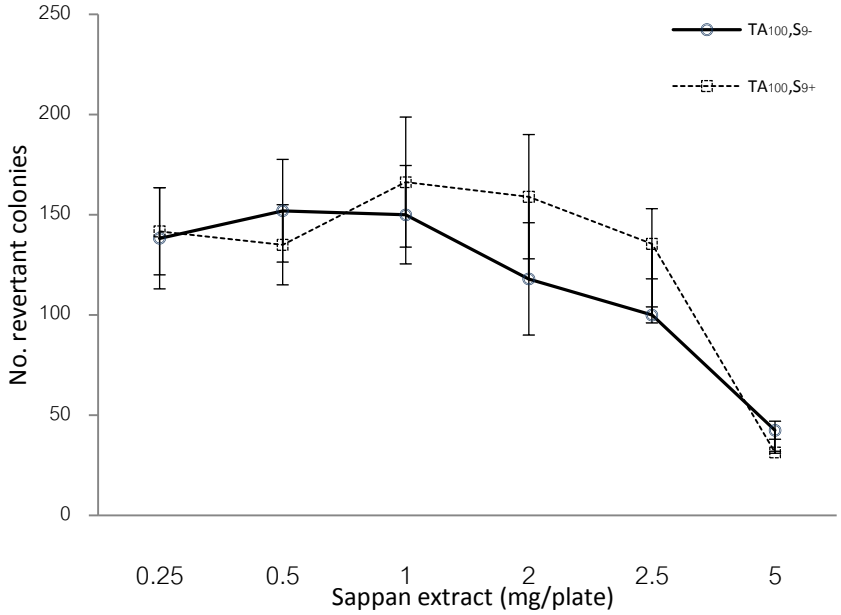
*Not determined

***p*<0.05 = significant difference from the control by the independent-sample T test

The well-known mutagens, AF-2 and BP, which were used as the positive controls exhibited significant increase in revertant bacterial colonies in comparison with the negative control (Table 1). The obtained results indicated sappan extract produced neither frame shift mutation in strain TA98 nor base pair mutation in strain TA 100 in the presence and absence of S9mix (Maron and Ames, 1983; OECD 1997). However, the plant extract at the concentration higher than 2.0 mg/plate had the antibacterial activity both in the presence and absence of S9 mix. Regarding to this antimicrobial activity, Mohan *et al* (2001) had demonstrated a broad-spectrum antibacterial activities of *C. sappan* extract and showed tannins and alkaloids were likely to be the bioactive substances.



(a)



(b)

Figure 1 Mutagenic activity of the plant extract to *S. typhimurium* TA98 (a) and TA100 (b) in the presence and absence of S9 mix

Table 2 Genotoxic effect of sappan extract on V79 cells after treatment in the presence (+S9) and absence (-S9) of S9 mix

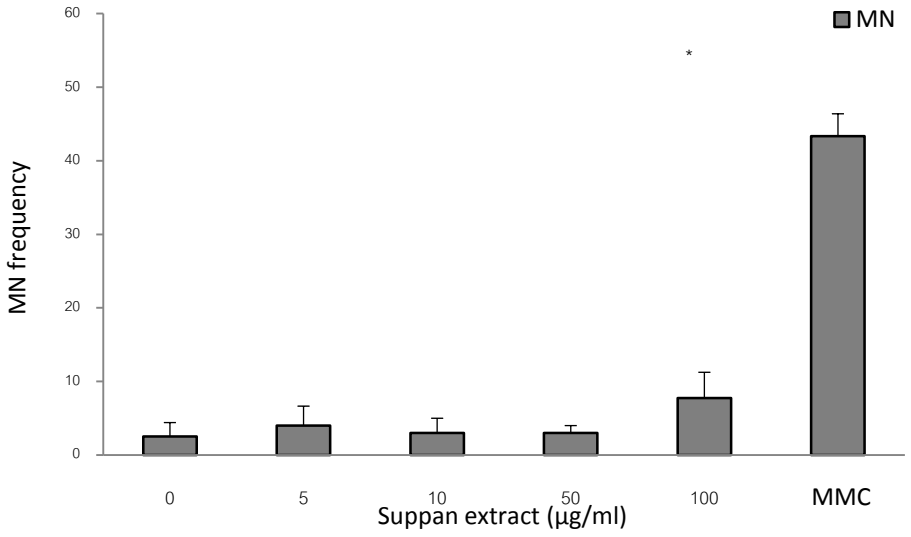
Treatment	CBPI (mean±SD)		MN frequency (mean±SD)	
	-S9	+S9	-S9	+S9
Distilled H_2O	1.68±0.02	1.78±0.08	2.50±1.91	3.0±2.71
Mitomycin C				
1 µg/ml	1.37±0.25	ND*	43.33±3.06**	ND
Cyclophosphamide				
20 µg/ml	ND	1.27±0.18**	ND	50.33±8.50**
Sappan extract				
5 µg/ml	1.69±0.03	1.80±0.02	4.00±2.65	3.75±2.36
10 µg/ml	1.66±0.01	1.75±0.03	3.00±2.00	2.00±1.80
50µg/ml	1.63±0.02	1.71±0.05	3.00±1.00	3.50±3.00
100 µg/ml	1.40±0.12**	1.69±0.06	7.75±3.50	3.25±2.22
200 µg/ml	<1.09	1.48±0.07	ND	7.67±3.51
400 µg/ml	ND	1.0±0.00**	ND	ND

*Not determined

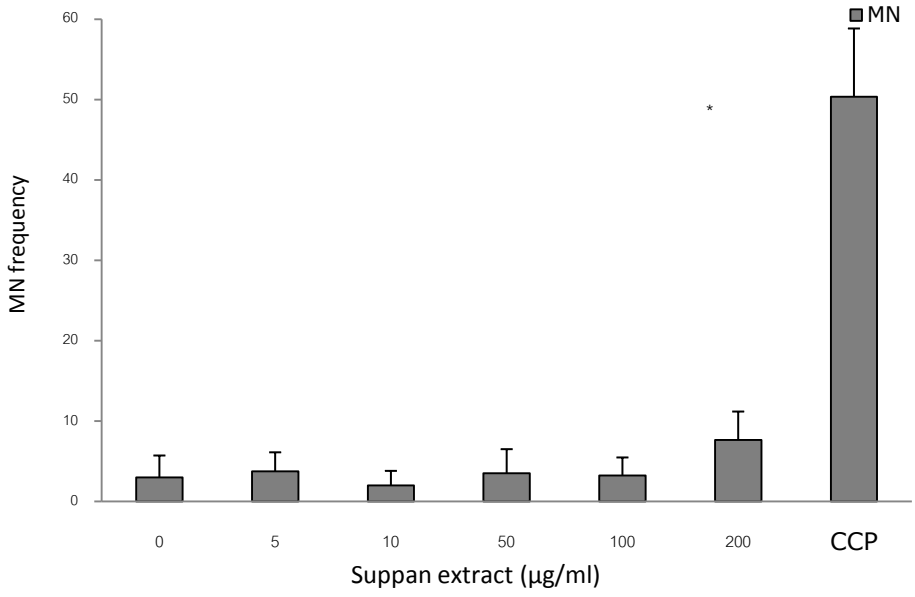
**p<0.05 = significant difference from the control by one way ANOVA, Turkey multiple compassion test

In the present study, the well established *in vitro* CBPI-MN assay was used to determine the capability of the sappan extract in inducing chromosomal damage by measuring the formation of micronucleus in binucleated V79 cells. Results indicated the concentrations ranged between 5 and 400 µg/ml of the plant extract induced no MN as the frequency of MN in the tested cells exposed to this extract did not increase in comparison to the control group (Table 2 and Figure 2). According to OECD guideline (OECD 2007), genotoxicity of the compounds are determined from CBPI of the treated cells after adding of cytochalasin B, the inhibitor to actin polymerization that stops the interphase of cell division. In the presence of CBPI and MN frequency assays, it is likely to conclude that the water extract from sappan heartwood has no genotoxic effect. On the contrary, the cytostasis effect or cytotoxicity of this extract was found as more than 50% of V79 cell line was affected by the sappan extract at 200 and 400 µg/ml in both the absence and presence of S9 mix (Table 3 and Figure 3). There was a study showed the cytotoxic activity of ethanolic extract of *C. sappan* L. on A-549, lung cancer cell line (Hemalatha *et al* 2011). The inhibition percentage with regard to cytotoxicity was found to be 87 % at 1000 µg/ml with IC_{50} value of 49±0.03 µg/ml.

The present study clearly showed the lack of *in vitro* mutagenic and genotoxic activity but with the possessing of antibacterial activity of the water extract from sappan. In conjunction with the *in vivo* evaluation on acute and subacute toxicity of the similar extract in rats (Sireeratawong *et al*, 2010), it is likely to predict the safety use of this extract as the preservative in food products or beneficial herb in the herbal formula for disease treatment/phytotherapy.



(a)



(b)

Figure 2 Effect of plant extract at various concentrations in induction of micronucleus in hamster lung fibroblast V79 cell in the absence (a) and presence (b) of S9 mix

Table 3 Cytostasis effect of sappan extract on V79 cell

Treatment	% Cytostasis	
	-S9 condition	+S9 condition
Mitomycin C		
1 µg/ml	46.27	ND*
Cyclophosphamide		
20 µg/ml	ND	78.94
Sappan extract		
5 µg/ml	-	-
10 µg/ml	2.99	3.85
50µg/ml	5.98	10.26
100 µg/ml	41.80	11.54
200 µg/ml	>86.57	39.75
400 µg/ml	ND	100

*Not determined

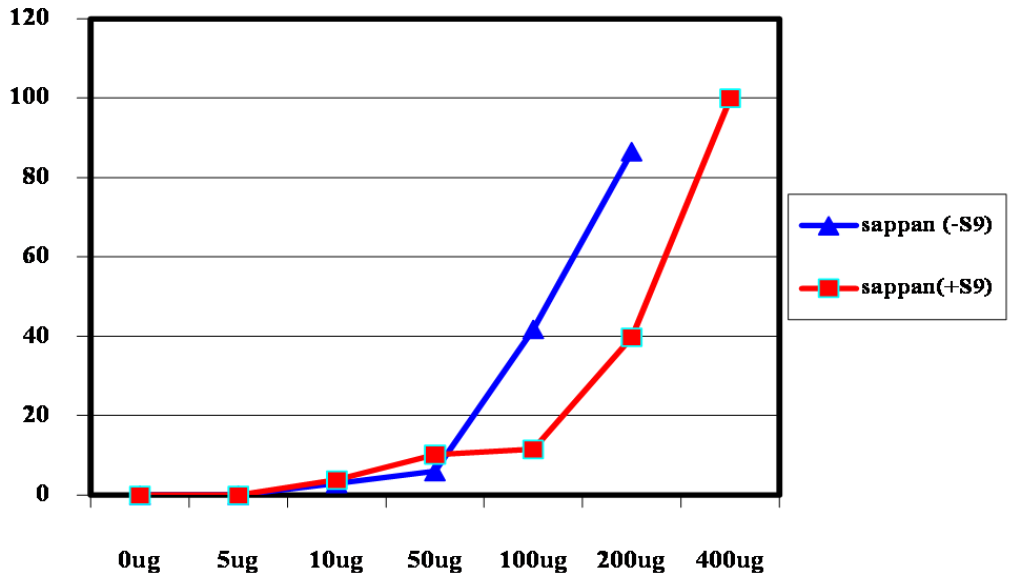


Figure 3 Comparison of cytotoxicity exhibited by the plant extract on cytostasis inhibition of hamster lung fibroblast V79 cells in the presence and absence of S9

Conclusion

The water extract from sappan heartwood was not mutagenic under the tested doses assayed by Ames test using *S. typhimurium* TA98 and TA100 as the tester strains in the presence and absence of S9 mix but it showed the antimicrobial activity. This plant extract was neither toxic to DNA as the MN frequency did not increase in V79 cells under the tested doses in both the presence and absence of S9 mix. However, it showed the extract was cytotoxic to this kind of cells as over 50% of cells were affected at high concentration both with and without S9 mix.

Acknowledgement

This research project was supported by Food and Drug Administration, Ministry of Public Health.

References

Badami S, Moorkoth S, Rai SR, Kannan E and Bhojraj S. Antioxidant activity of *Caesalpinia sappan* heartwood. Biol Pharm Bull. 2003;26:1534-1537.

Hemalatha K, Sunitha D and Satyanarayana D. Cytotoxic activity of ethanolic extracts of *Caesalpinia sappan* Linn. and *Anaona squamosa* Linn. in 549 cell line. Int J Pharm&Ind Res. 2001;1:318-320.

Kim YM, Kim SG, Khil LY and Moon CK. Brazilin stimulates the glucose transport in 3T3-L1 cells. Planta Medica. 1995;61:297-301.

Maron DM and Ames BN. Revised methods for the Salmonella mutagenicity test. Mutat Res. 1983;113:173-215.

Matsushima T, Sawamura M, Hara K and Sugimura T. A safe substitute for polychlorinated biphenyls as an inducer of metabolic activation system. In DeSerres FJ, Fouts JR, Bend JR and Philpot RM (Eds) *In vitro* metabolic activation in mutagenesis testing. Amsterdam: Elsevier 1976;pp 85-88.

Mohan G, Anand SP and Doss A. Efficacy of aqueous and methanol extracts of *Caesalpinia sappan* L. and *Mimosa pudica* L. for their potential antimicrobial activity. South As J Biol Sci. 2001;1:48-57.

Moon CK, Park KS, Kim SG, Won HS and Chung JH. Brazillin protects cultured rat hepatocytes from BrCC13-induced toxicity. Drug Chem Toxicol. 1992;15:81-91.

Moon CK, Chung JH, Lee YM, Lee SH. Hwang GS, Park KS *et al.* Effects of brazilin on erythrocyte deformability and its related biochemical factors in streptozotocin induced diabetic rats. Arch Pharm Res. 1988;11:149-154.

Niranjan RV., Ravikanth V., Jansi LV., Suryanarayan MU. and Venkateswarlu Y. Inhibitory activity of homoisoflavonoids from *Caesalpinia sappan* against *Beauveria bassiana*. Fitoterapia 2003;74:600-602.

OECD. OECD Guidelines for the testing of chemicals no. 471: Bacterial reverse mutation test. Paris:OECD Publishing. 1997.

OECD. OECD Guidelines for the testing of chemicals no. 487: *In vitro* Mammalian cell micronucleus test. Paris:OECD Publishing. 2010.

Sireeratawong S, Piyabhan P, Singhalak T, Wongkrajang Y, Temsiririrkkul R, Punsrirat J, *et al*. Toxicity evaluation of sappan wood extract in rats. J Med Assoc Thai. 2010;93(Suppl 7):550-557.

Srilakshmi VS, Vijayan P, Raj PV, Dhanaraj SA and Chandrashekhar HR. Hepatoprotective properties of *Caesalpinia sappan* Linn. Heartwood on carbon tetrachloride

induced toxicity. Indian J Exp Biol. 2010;48:905-910.

Suttisawad N and Thanaisawanyangkura S. Antimicrobial acivity of *Caesalpinia sappan* L. as preservative in chilli-paste. A special project for BSc. In Pharmacy, Bangkok: Faculty of Pharmacy, Mahidol University, 2006.

Wetwitayaklung P, Phaechamud T and Keokitichai S. The antioxidant activity of *Caesalpinia sappan* L. heartwood in various ages. Naresuen University J. 2005; 13:43-52.

Xu HX and Lee SF. The antibacterial principle of *Caesalpinia sappan*. Phytother Res. 2004;18:647-651.

Yingming P, Ying L, Hengshan W and Min L. Antioxidant activity of several Chinese medicine herbs. Food Chem. 2004;88:347-350.