Assessment of genotoxicity and antigenotoxicity of an aqueous extract of *Cleistocalyx nervosum* var. *paniala* in *in vitro* and *in vivo* models

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ABSTRACT

*C. nervosum* var. *paniala*, an edible fruit found in Northern Thailand, contains high amounts of phenolic compounds with *in vitro* antioxidant activity. The aqueous extract of the ripe fruit was evaluated for its safety and beneficial effects using genotoxicity and toxicity tests. The *C. nervosum* extract was not only non-mutagenic in *Salmonella typhimurium* strains TA98 and TA100 in the presence and absence of metabolic activation, but exhibited also moderate antimutagenic effects against aflatoxin B1 and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline-induced mutagenesis. Electrospray ionization-mass spectrometric analysis revealed the major anthocyanins, which included cyanidin-3,5-diglucoside, cyanidin-3-glucoside and cyanidin-5-glucoside. The administration of *C. nervosum* at concentration of 5,000 mg/kg bw did not induce acute toxicity in rats. A liver micronucleus test was performed to detect clastogenicity and anticlastogenicity. The extract in the dose of 1,000 mg/kg did not cause micronucleus formation in the liver of rats. Furthermore, in rats administered 100–1,000 mg/kg of the extract, no anticlastogenic effect against diethylnitrosamine-induced hepatic micronucleus formation was observed. These studies provide data concerning the safety and antimutagenic potency of an aqueous extract of *C. nervosum* fruit.

KEY WORDS: *Cleistocalyx nervosum* var. *paniala*, acute toxicity, Ames test, liver micronucleus assay

Introduction

Anthocyanins occur ubiquitously in flowering plants and confer the bright red, blue and purple colors to fruits and vegetables. Epidemiological studies have suggested that the consumption of anthocyanins lowers the risk of cardiovascular disease, diabetes, arthritis and cancer, due at least in part to anti-oxidant and anti-inflammatory activities (Prior & Wù, 2006). Potential cancer chemopreventive activities of anthocyanins revealed in *in vitro* studies included radical scavenging activity, stimulation of phase II detoxifying enzymes, reduced cell proliferation, inflammation, angiogenesis, invasiveness and induction of apoptosis and differentiation (Caillet et al., 2012; Galvano et al., 2004). Anthocyanins were shown to exhibit anticarcinogenic activity against multiple cancer cell types *in vitro* and tumor types *in vivo* (Galvano et al., 2004; Stoner et al., 2008).

*C. nervosum* var. *paniala* family Myrtaceae is a native plant found in Northern Thailand, with an orange-red fruit that is commonly consumed either as fresh fruit or is used in fruit products. Previous studies have reported that cyanidin-3-glucosides were found in the ripe fruit of *C. nervosum* (Jansom et al., 2008). There are but few data concerning the biological activity of *C. nervosum* fruit *in vitro* or *in vivo*, though *Cleistocalyx operculatus*, which belongs to the same genus, has been investigated for its biological activities. The buds of *C.
*C. nervosum* var. *paniala* extracts by means of the Ames test. Clastogenic and anticlastogenic effects were also observed in rat liver using the micronucleus assay. Because *C. nervosum* is extensively consumed as a fresh fruit, it is desirable to determine the limits of toxicity for large amounts. Acute toxicity test was therefore also assessed in rats.

### Materials and methods

#### Chemicals

All chemicals and solvents used were of analytical grade. The standard mutagens used included 2-(2-furyl)-3-(5-nitro-2-furyl)-acrylamide (AF-2), 2-aminoanthracene (2-AA), and 2-amino-3,4-dimethyliimidazo[4,5-f] quinoline (MeIQ); these were purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Aflatoxin B1 (AFB1) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cyanidin-3-O-glucoside was purchased from Apin Chemicals Ltd. (Oxfordshire, UK). Collagenase type IV and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen Corporation (USA). All solvents used in the analysis of anthocyanin constituents were of HPLC grade.

#### Plant material

Ripe fruits of *Cleistocalyx nervosum* var. *paniala* were collected during July-August, 2008 from Tambon Choeng Doi, Amphur Doi Saket, Chiang Mai, Thailand. This plant was identified and confirmed by comparing it with voucher specimens of known identities (QBG 7290, QBG 17340, QBG 25139) deposited at the Queen Sirikit Botanic Garden, Chiang Mai, Thailand. The pulp was manually separated from seeds, weighed and stored at −20°C until use.

Sample preparation

One hundred gram of *C. nervosum* pulp was ground with 50 ml of distilled water using a blender. The mixture was centrifuged at 1,000 g for 15 min and filtered through a filter paper. The filtrate was dried using a lyophilizer. The aqueous extract was a purplish-red solid with final yield equivalent to 8.48 g.

### Determination of total phenolic and flavonoid contents and condensed tannins

The total phenolic content was determined via the Folin-Ciocalteu reagent method (Singleton et al., 1999). The absorbance of the sample was measured at 765 nm, and results were expressed as mg gallic acid equivalent (GAE)/g fresh weight (fw). Total flavonoid content was determined using the aluminum chloride colorimetric method. The absorbance at 532 nm was used and expressed as mg catechin equivalent (CE)/100 g fw (Maksimovic et al., 2005). Finally, the condensed tannin was assessed by the vanilin assay (Butler et al., 1982). After spectrophotometric analysis at 550 nm, the amount of condensed tannins was calculated as mg CE/100 g fw.

### Quantification of anthocyanins by High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC-ESI-MS)

The *C. nervosum* aqueous extract was dissolved in 0.1% formic acid in methanol and filtered through filter paper and the remaining methanol was evaporated under reduced pressure.

A 2.1 × 150 mm Halo column (Agilent Technologies, USA) with a particle size of 2.7 μm was used. The mobile phase consisted of water containing 0.5% formic acid (solvent A) and methanol (solvent B), with gradient elution started at 85:15 (A:B). The HPLC effluent was passed through the photodiode array detector (PAD), which was set to monitor at wavelengths of 254 and 520 nm. After separation, the HPLC effluent was delivered into a single quadrupole mass spectrometer (Agilent Technologies, USA) via orthogonal atmospheric pressure ionization (API)-electrospray interface. The optimum electrospray ionization (ESI) conditions were as follows: ionization mode, positive; nebulizer pressure, 32 psi; drying gas flow rate, 10 L/min; drying gas temperature, 350°C; and capillary voltage, 4,000 V. Helium was used as a collision gas and a fragmentor voltage of 130 V was used for the collision-induced dissociation (CID). The quadrupole temperature was 100°C and the electron multiplier voltage was 2,650 V.

The relative contents of anthocyanins in the extract of *C. nervosum* were obtained by the LC-ESI-MS technique. Naphtholphthalein was used as an internal standard. Peak area normalization was utilized to determine the relative contents of the individual anthocyanin components. Three replicates of sample extract were analyzed.

### In vitro mutagenic and antimutagenic assays by Ames test

Both TA98 and TA100 of *Salmonella typhimurium* were kindly provided by Dr. Takehiko Nohmi, National Institute of Health, Japan. Both tests were performed rapidly and are relatively inexpensive. They are appropriate for estimating the carcinogenic effects of the standard short-term genotoxicity tests include bacterial mutation assays (Ames test), micronucleus tests and chromosomal aberration tests. These tests can be performed rapidly and are relatively inexpensive. They are appropriate for estimating the carcinogenic effects of the chemicals and as guidelines for long-term carcinogenicity tests (Kirkland et al., 2006). Such genotoxicity tests have also been modified for detecting antigenotoxicity of natural products (Inboot et al., 2004). The standard short-term genotoxicity testing to identify genotoxic or carcinogenic effects of the test compound (Rao et al., 2004). The standard short-term genotoxicity tests include bacterial mutation assays (Ames test), micronucleus tests and chromosomal aberration tests. These tests can be performed rapidly and are relatively inexpensive. They are appropriate for estimating the carcinogenic effects of the chemicals and as guidelines for long-term carcinogenicity tests (Kirkland et al., 2006). Such genotoxicity tests have also been modified for detecting antigenotoxicity of natural products (Inboot et al., 2012).

In *C. nervosum*, the essential oil and ethanol extract of *C. nervosum*, isolated from buds of *C. nervosum*, were of HPLC grade. Most essential oil components have also been modified for detecting antigenotoxicity of natural products (Inboot et al., 2012).

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Anesthetized with diethyl ether. The internal organs were subjected to partial hepatectomy on day 22. On day 26, the liver was incised and then washed with phosphate buffer saline. Finally, the isolated hepatocytes were washed in 10% buffered formalin and re-suspended in the same solution. Hepatocyte suspensions were mixed with 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) stain solution for fluorescent microscopy. The number of micronucleated hepatocytes was counted and recorded, based on analysis of 2,000 hepatocytes from each animal under a fluorescent microscope. The criteria for micronucleated hepatocyte scoring were described elsewhere (Cliet et al., 1989). Percentages of mitotic index (MI), indicative of mitotic activity, were calculated from mitotic cells in counted hepatocytes.

For the anticlastogenic assay, the protocol used in this study was modified from that described previously (Charoensoin et al., 2010). Male rats were divided into 4 groups. The pre- and co-treatments with 100–1,000 mg/kg of C. nervosum extract concomitant with 30 mg/kg diethyl nitrosamine administration were performed on days 15 and 18. Partial hepatectomy was performed on day 22 and all rats were sacrificed by diethyl ether anesthesia and liver perfusion on day 26.

## Results

### Chemical constituents in an aqueous extract of C. nervosum

The total phenolic compounds were 181.16±0.59 mg GAE/100 g fw. The amounts of total flavonoids and condensed tannins were 54.86±3.45 mg CE/100 g fw and 1,902.72±183.63 mg CE/100 g fresh weight, respectively. The UV-Vis spectrum measured at 520 nm of C. nervosum showed three separate peaks at retention times 17.80, 20.83, and 42.82 min, which corresponded to anthocyanins indicated that cyanidin-3-glucoside, cyanidin-3,5-diglucoside, and cyanidin-5-glucoside, respectively (Figure 1). The percentage of relative contents among the assigned anthocyanins indicated that cyanidin-3-glucoside accounted for the major anthocyanin of C. nervosum with a relative content of 73.48±19.26% (Table 1).

### In vitro mutagenic and antimutagenic activities of aqueous extract of C. nervosum

For the test doses no killing effect or sign of cytotoxicity was observed for either TA98 or TA100 strains of *S. typhimurium*. The numbers of TA98 and TA100 revertants before calculating the percentage inhibition. As mentioned for the mutagenic assay, the preincubation technique was modified in the antimutagenic assay. In brief, the mixtures consisting of solvent alone or 100–200 mg/ml of extract in the presence of AFB1, MeQ, or AF-2 were preincubated with overnight culture of TA98 strain with or without S9 mix at 37 °C for 20 min before top agar addition. The His⁺ revertant colonies were analyzed after 37 °C incubation for 48 hr. Triplicate plates per run were assayed. As shown in Table 1, the antimutagenic effect of the C. nervosum extract was dose-dependent.

| Retention time(min) | Structural assignment of anthocyanins | [M]+ | ESI-Mass spectrum | Relative Content in extract (%) |
|---------------------|--------------------------------------|------|------------------|--------------------------------|
| 17.80               | cyanidin-3,5-diglucoside              | 611  | 287,449, 611     | 0.75±0.26                      |
| 20.83               | cyanidin-3-glucoside                  | 449  | 287,449          | 73.48±19.26                    |
| 42.82               | cyanidin-5-glucoside                  | 449  | 287,449          | 25.77±19.01                    |

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His+ revertant colonies from plates treated with 0.8–200 mg/ml of the extract under present and absent metabolic activation were not significantly different from negative control (Table 2). This suggested that the dose of 0.8–200 mg/ml of *C. nervosum* extract was non-mutagenic to the strains of *S. typhimurium*, either with or without metabolic activation. Considering the antimutagenicity of *C. nervosum* extract in the TA98 strain (Table 3), co-treatment of MeIQ and 100–200 mg/ml of the extract in the presence of S9 mix showed dose-dependent antimutagenic activity, and the highest dose exhibited significant reduction (*p*<0.05) of the percentage of revertant colonies (% inhibition = 62.3±18.0) in comparison with the positive control. However, the percentage of revertant colonies observed from 100–200 mg/ml of the extract co-treated with AF-2 in the absence of S9 mix was not significantly different from the positive control. The number of revertant colonies by co-incubation of AFB1 with the extract was slightly lower compared with the positive control, indicating a possible weak antimutagenic effect.

**Evaluation of acute toxicity of aqueous extract of *C. nervosum***

None of the animals showed any changes in general appearance after administration of 5,000 mg/kg of the extract.

**Table 2. Mutagenic activity for aqueous extract of *C. nervosum***

| Treatment | TA 98 Average of His+ revertant colonies | TA100 Average of His+ revertant colonies |
|-----------|------------------------------------------|----------------------------------------|
| AF-2 2 ug/ml | 286±32.6 | 834±184.3 |
| 2-AA 10 ug/ml | N.A. | 612±186.9 |
| DW 50 ul | 32±6.3 | 143±34.8 |
| *C. nervosum* extract 0.8 mg/ml | 32±6.1 | 138±39.2 |
| *C. nervosum* extract 4 mg/ml | 32±9.0 | 137±41.7 |
| *C. nervosum* extract 20 mg/ml | 32±8.8 | 133±31.4 |
| *C. nervosum* extract 100 mg/ml | 31±8.0 | 141±44.2 |
| *C. nervosum* extract 200 mg/ml | 37±15 | N.A. |

The His+ revertant colonies are expressed as Mean±SEM of three independent replicates.

**Table 3. Antimutagenic activity assay for aqueous extract of *C. nervosum***

| Test compounds | AF-2 1 ug/ml | AFB1 0.16 ug/ml | MeIQ 0.05 ug/ml |
|----------------|-------------|-----------------|----------------|
| Standard mutagen | 2.15±1.26 | 0.15±0.00 | 0.15±0.00 |
| *C. nervosum* extract 100 mg/ml | 2.68±0.84 | 0.29±0.26 | 0.29±0.26 |
| *C. nervosum* extract 150 mg/ml | 2.68±0.84 | 0.29±0.26 | 0.29±0.26 |
| *C. nervosum* extract 200 mg/ml | 10.17±3.23 | 0.45±0.06 | 0.45±0.06 |
| *C. nervosum* extract 1,000 mg/kg | 10.15±2.05 | 0.38±0.17 | 0.38±0.17 |

The number of spontaneous revertant colonies range from 17±2.4–21±3.7. Parentheses indicated the percentage inhibition of the extract against each mutagen.

* Significant difference in comparison with positive control (*p*<0.05)

**Table 4. Clastogenicity and anticlastogenicity of an aqueous extract of *C. nervosum***

| Treatment | MNHEPs/1,000 | Mitotic index (%) |
|-----------|--------------|-------------------|
| 5% Tween 80 | 2.15±1.26 | 0.15±0.00 |
| C. nervosus 1,000 mg/kg | 2.68±0.84 | 0.29±0.26 |
| DEN + 5% Tween 80 | 12.61±2.05* | 0.50±0.27 |
| DEN + *C. nervosum* 100 mg/kg | 10.17±3.23 | 0.45±0.06 |
| DEN + *C. nervosum* 200 mg/kg | 10.15±2.05 | 0.38±0.17 |
| DEN + *C. nervosum* 1,000 mg/kg | 10.88±2.79 | 0.54±0.41 |

MNHEPs/1,000 (Micronucleated hepatocytes per 1,000 hepatocytes) are expressed as Mean±SD.

*Significant difference from negative control (5% Tween 80 as vehicle), *p*<0.05
aqueous extract. There were no deaths during the 14-day observation period, and gross examination of internal organs of all rats did not reveal any abnormality.

**In vivo clastogenic and anticlastogenic effects of aqueous extract of C. nervosum**

The clastogenicity of *C. nervosum* extract was examined by a micronucleus assay in regenerating rat livers. Four days after partial hepatectomy, the growth rate of the treated rats was not significantly different from that of the control group. The mean number of micronucleated hepatocytes and mitotic indices was not significantly different in rats treated with 1,000 mg/kg of the test compound compared with control rats (Table 4). This suggested that the *C. nervosum* extract was non-mutagenic in rat liver. While the biological activities of anthocyanins from fruits and vegetables have been extensively studied, their anti-clastogenicity against diethylnitrosamine (DEN), a hepatocarcinogen in animals and humans, remains unknown. In the present study, the DEN-administered rats had significantly greater numbers of micronuclei and a higher mitotic index compared with control rats. The dosed rats had an average body weight similar to that of the control rats. The analysis of micronucleated hepatocytes of rats administered 100–1,000 mg/kg of *C. nervosum* extract showed that the numbers of micronucleated hepatocytes of DEN-treated rats were not affected by any of the doses of the extract tested compared with control rats (Table 4).

**Discussion**

Recently, our research group reported the effect of an aqueous extract of *C. nervosum* on antioxidant systems in the rat liver, including total glutathione, glutathione peroxidase, catalase, and heme oxygenase-1 activities, indicating a possible biphasic effect on oxidative status of the rat liver (Taya et al., 2009). Subsequently, we studied the composition and *in vivo* biological effects of *C. nervosum* fruits related to their antioxidant properties. We found that the aqueous extract of *C. nervosum* was rich in cyanidin-3-glucoside with a relative content of 73.48±19.26%. The LC-ESI-MS spectra indicated that the structures were consistent with the authentic standards and those presented in previous reports, which included cyanidin-3,5-diglucoside, cyanidin-3-glucoside, and cyanidin-5-glucoside, respectively (Figure 1) (Tian et al., 2005; Jansom et al., 2008).

In the studies of *in vitro* mutagenicity and antimutagenicity, *C. nervosum* extracts were not only non-mutagenic but also antimutagenic. Co-treatment of MelQ and the extract in the presence of S9 mix showed dose-dependent antimutagenic activity, and the highest dose exhibited significant reduction (*p*<0.05) of the percentage of revertant colonies in comparison with the positive control. The number of revertant colonies by co-incubation of AF1 with the extract was slightly different compared with the positive control, indicating a weak antimutagenic effect (Table 3). Anthocyanins and phenolic compounds were reported to exhibit antioxidant, antimutagenic and anticarcinogenic properties (Caillet et al., 2012; Galvano et al., 2004). Our results agree with previous reports in which natural anthocyanins acted as antimutagens. “It has been reported that anthocyanins from colored maize modulate the mutagenic activity of 2-aminoanthracene (2-AA) in *S. typhimurium* TA98 and TA100 through the inhibition of base-change mutation (Mendoza-Díaz et al., 2012). Additionally, the anthocyanin colors from foods inhibited the reverse mutation induced by food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) in the presence of rat liver microsomal activation system in a dose dependent manner (Aoki et al., 2004).”

With regard to these findings, the protective activity could be explained as the result of the major anthocyanins. The mechanism of inhibition of the mutagen-activating enzymes is the protection of the hot-spot region of the gene from mutation. Biotransformation of MelQ and AF1 are mediated by CYP1A2 and CYP3A4 in human and rat liver cells, respectively (Forrester et al., 1990; Turesky et al., 2002). Therefore, cyanidin glucosides in *C. nervosum* possibly modulate the key enzymes by means of activity inhibition.

While the biological activities of anthocyanins from fruits and vegetables have been extensively reported, their anticlastogenicity against diethylnitrosamine (DEN), a known hepatocarcinogen in animals and humans, has not been previously investigated. We found that DEN-administered rats had significantly greater numbers of micronuclei and higher mitotic indices compared with control rats (Table 4). This result was consistent with experiments using DEN as a clastogen inducing hepatic micronuclei in rats and mice (Tates et al., 1980; Clet et al., 1989). DEN was thus used as a model inducer in the experiment for anticlastogenic activity of *C. nervosum* extract in rat liver. The analysis of micronucleated hepatocytes of rats administered *C. nervosum* extract showed that the extract did not affect the number of micronucleated hepatocytes of DEN-treated rats compared with control rats (Table 4). When consumed, anthocyanins are absorbed in the stomach of rats and appear in both portal and systemic plasma (Passamonti et al., 2003). The anthocyanin concentration of systemic blood may reflect its distribution and existence in target organs, especially the liver as a major site for metabolism. Passamonti et al. proposed a portal blood-liver transport mechanism of anthocyanins, which was related with an organic anion carrier called bilitranslocase in HepG2 cells (Passamonti et al., 2005). They also determined the concentration of anthocyanins in portal and systemic plasma and in liver of rats given a mixture of grape anthocyanins, suggesting that most anthocyanins were absorbed in parent structures. Due to the fact that the *in vitro* antimutagenic assay used was carried out by bacterial mutation, the biotransformation was not entirely similar to that occurring in test animals. Additionally, the influences of microbial metabolism after upper intestinal absorption and metabolizing enzymes in the liver, along with loss and decrease of bioavailability.
of the active compounds, were possible reasons for the differences in the results from in vitro and in vivo antimutagenic observations.

In conclusion, our results indicated that anthocyanins isolated from the C. nervosum var. paniola fruit were not only safe in acute toxicity test, but even displayed antimutagenicity in vitro. These observations will be beneficial for the development of this species as a medicinal plant.

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