CHEMICAL CONSTITUENTS AND ANTIOXIDANT ACTIVITIES OF *CLEISTOCALYX NERVOSUM* FRUITS IN *IN VITRO* AND *IN VIVO* MODELS

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ABSTRACT

Cleistocalyx nervosum var. *paniala*, or Ma-kiang in Thai, is a purplish red fruit cultivated in northern Thailand. Its flesh is edible and is used to make juice, wine and jam. The previous study reported that the aqueous extract of *C. nervosum* pulp showed antioxidant activity and anticarcinogenesis. The present study investigated the fruit's chemical constituents and antioxidant activities, both *in vitro* and *in vivo*. Both aqueous and ethanol extracts (95% v/v) were used. The amount of polyphenols, flavonoids, cyanidin-3-glucoside and DPPH radical-scavenging capacity of the ethanolic extract were greater than those of the aqueous extract. The 95% ethanol extract of *C. nervosum* was further investigated for its safety and antioxidant activity in animal models. The administration of the ethanolic extract of *C. nervosum* at 5 g/kg bw was safe in wistar rats. Various concentrations of *C. nervosum* ethanolic extract at 100, 300 and 1000 mg/kg bw were intragavage fed to male wistar rats for 90 days. The ethanolic extract significantly decreased malondialdehyde (the end-product of lipid peroxidation) levels in liver, but not in serum. It also induced hepatic glutathione peroxidase activity. The results of this study showed that 95% ethanolic extract of *C. nervosum* contains a high content of anthocyanin that has antioxidant capacities both in *in vitro* and *in vivo* models.

Keywords: *Cleistocalyx nervosum,* Anthocyanin, Antioxidant activity

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องค์ประกอบทางเคมีและฤทธิ์ต้านอนุมูลอิสระในหลอดทดลองและสัตว์ทดลองของผลมะเกี๋ยง

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บทคัดย่อ

มะเกี๋ยง (*Cleistocalyx nervosum* var. *paniala*) เป็นผลไม้ที่มีสีม่วงแดงปลูกมากในเขตภาคเหนือของประเทศไทย เนื้อของผลมะเกี๋ยงสามารถรับประทานทั้งในรูปสดและแปรรูปเป็นน้ำผลไม้ ไวน์ และแยม มีรายงานพบว่าเนื้อของผลมะเกี๋ยงที่สกัดด้วยน้ำมีฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ป้องกันการเกิดมะเร็งตับ งานวิจัยครั้งนี้เป็นการศึกษาองค์ประกอบทางเคมีที่สำคัญและฤทธิ์ต้านอนุมูลอิสระของสารสกัดจากผลมะเกี๋ยงทั้งในหลอดทดลองและสัตว์ทดลอง โดยใช้สารสกัดมะเกี๋ยงทั้งที่ได้จากการสกัดด้วยน้ำและ95% เอธานอล จากการศึกษาพบว่าปริมาณสารประกอบฟีนอลิก ฟลาโวนอยด์ ไซยานิดิน-3-กลูโคไซด์(แอนโธซัยยานิน) และฤทธิ์ต้านอนุมูลอิสระในหลอดทดลองโดยใช้วิธี DPPH ของสารสกัดมะเกี๋ยงที่สกัดด้วยเอธานอลสูงกว่าสารสกัดที่สกัดด้วยน้ำ ดังนั้นจึงศึกษาความปลอดภัยและฤทธิ์ต้านอนุมูลอิสระของสารสกัดมะเกี๋ยงที่สกัดด้วยเอธานอลในสัตว์ทดลองต่อไป จากการศึกษาความเป็นพิษเฉียบพลันของสารสกัดมะเกี๋ยงที่สกัดด้วยเอธานอลในหนูวิสต้าร์เพศเมีย 14 วัน พบว่าสารสกัดมะเกี๋ยงความเข้มข้น 5 กรัมต่อกิโลกรัมน้ำหนักตัวมีความปลอดภัยในสัตว์ทดลอง และเมื่อทำการป้อนสารสกัดมะเกี๋ยง 100, 300 และ 1000 มิลลิกรัมต่อกิโลกรัมน้ำหนักตัวให้แก่หนูวิสต้าร์เพศผู้เป็นเวลา 90 วัน พบว่าสารสกัดมะเกี๋ยงที่สกัดด้วยเอธานอลสามารถลดปริมาณมาลอนไดอัลดีไฮด์ (ผลผลิตสุดท้ายของ lipid peroxidation)ในตับแต่ไม่มีผลในซีรั่ม นอกจากนี้ยังเพิ่มการทำงานของเอนไซม์ glutathione peroxidase ในตับหนูอีกด้วย ผลของการวิจัยนี้แสดงให้เห็นว่าสารสกัดมะเกี๋ยงที่สกัดด้วย 95% เอธานอล ประกอบด้วยสารกลุ่มแอนโธซัยยานินปริมาณสูงและมีฤทธิ์ต้านอนุมูลอิสระทั้งในหลอดทดลองและในสัตว์ทดลอง

คำสำคัญ: *Cleistocalyx nervosum*, แอนโธซัยยานิน, ฤทธิ์ต้านอนุมูลอิสระ

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INTRODUCTION

Free radicals play an important role in the mechanisms underlying a number of chronic diseases. They can attack and damage cellular macromolecules, including nucleic acids, lipids, proteins and carbohydrates, leading to altered enzymatic activity, cellular signaling, and vital protein function. Oxidative DNA damage caused by free radicals initiates the multistage carcinogenesis process, starting with DNA mutation and accumulation of genetic events resulting in dysplastic cellular appearance, deregulated cell growth and finally turn to carcinoma.[1] In atherogenesis, reactive oxygen species initiate a radical chain reaction leading to reduced nitric oxide availability, enhancement of proinflammatory cytokines and chemical modification of lipoproteins.[2] In diabetes, free radicals can stimulate glycation of proteins, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes.[3] However, living organisms can defend themselves via antioxidant systems to counteract reactive radical species. Antioxidant systems include antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, as well as small molecules including glutathione, uric acid and bilirubin.[1] Epidemiological studies have suggested that vegetables and fruits containing such compounds that have preventative effects for cancer, diabetes, cardiovascular diseases and Alzheimer's disease.[4,5] Plant polyphenols, a large group of natural antioxidants ubiquitous in vegetables and fruits, can ameliorate oxidative stress. They trap and scavenge free radicals, regulate nitric oxide, decrease leukocyte immobilization, induce apoptosis, inhibit cell proliferation and angiogenesis as well as exhibit phytoestrogenic activity.[6]

Anthocyanins, common flavonoids found in plants, have a basic flavylium cationic structure, which can be transformed to quinonoidal bases through proton transfer reactions. The latter form plays an important role in the antioxidant action of anthocyanins.[7] The promoting effect of anthocyanins on ARE-regulated phase II enzyme expression seems to be a critical point in modulating the defense system against oxidative stress. Anthocyanins induce the activation of phase II enzymes through the antioxidant response element pathway against oxidative stress-induced apoptosis.[8] Many studies have suggested that anthocyanins have potent anti-inflammatory, anti-carcinogenic and antioxidant properties.[4,5,7,8]

Cleistocalyx nervosum var. *paniala*, common name Ma-kiang, is a purplish red fruit cultivated in northern Thailand. Its flesh is edible and is used to make juice, wine and jam. The rich purplish red color of

the aqueous extract is characterized by an anthocyanin profile. The most common anthocyanin is cyanidin-3-glucoside. Previous study has demonstrated that the methanol extract of *C. nervosum* seed has antigenotoxic effects against chemical induced mutagenesis.[9] *C. nervosum* extracts can stimulate human lymphocyte activity, which could be useful for modulating immune functions of the body.[10] The aqueous extract of *C. nervosum* has chemopreventive effects on the early stages of rat hepatocarcinogenesis via enhancement of antioxidant enzymes and decreased oxidative stress.[11,12] The present study aimed to investigate the chemical constituents and antioxidant activities of *C. nervosum* using both *in vitro* and *in vivo* models.

MATERIALS AND METHODS

Materials

Diethyl ether, 95% (v/v) ethanol, sodium bicarbonate and trichloroacetic acid were purchased from BDH (England); β-NADPH was purchased from Oriental Yeast Co., Ltd. (Japan); Catechin, gallic acid, glutathione reductase, hemin and thiobarbituric acid were purchased from Sigma-Aldrich Chemical Co. (U.S.A); Folin & Ciocalteu's phenol reagent was purchased from Fluka A.G. (Switzerland); Glutathione (Reduced form) was purchased from Wako (Japan); Potassium chloride was purchased from Carlo-Erba (Italy).

Plant material

Fruits of *C. nervosum* were collected from the Horticulture Research Center Region 1, Office of Agricultural Research and Development, Department of Agriculture, Thailand, in July-August, 2010. The plant was identified and confirmed by comparison with voucher specimens of known identity (Punvittayagul and Taya 1), which are deposited at the Queen Sirikit Botanic Garden, Chiang Mai, Thailand. The pulp was manually removed from the seeds, weighed and stored in a freezer at -20 ^{o}C until extraction.

Preparation of *C. nervosum* extracts

The flesh of *C. nervosum* was ground and extracted with distilled water or 95% (v/v) ethanol. The extracts were centrifuged at 3,000 rpm for 15 min, and filtered through a Whatman no.1 filter paper. The extracts were then evaporated and lyophilized. The crude extracts were stored at -20 ^{o}C until use.

Determination of chemical constituents in *C. nervosum* extracts

The total phenolic compound content in each extract was measured by the method of Singelton *et al*. **(**1999**)**. Extracts were added to Folin-Ciocalteu reagent and 7% (w/v) Na_2CO_3. Then, the solution mixtures were incubated for 15 min at 45 ^{o}C. The absorbance of each sample was measured at

760 nm using a spectrophotometer. Gallic acid was used to calibrate the standard curve and the total phenolic content was expressed as mg of gallic acid equivalents (GAE) per 100 g of fresh weight of the sample. [13]

The total flavonoid content was evaluated using the method described by Moksimovic *et al*. **(**2005**)**. The sample extract was incubated with 5% (w/v) $NaNO_2$ for 10 min before adding 10% (w/v) $AlCl_3.6H_2O$. After the mixture was allowed to stand at room temperature for 10 min, 1 M NaOH was added. The absorbance of the reaction mixture was measured at 532 nm using a spectrophotometer. Catechin was used to calibrate the standard curve and total flavonoids content expressed as mg of catechin equivalents (CE) per 100 g of fresh weight of the sample. [14]

The content of cyanidin-3-glucoside was analyzed by HPLC with an Allure C18 column (250x4.6 mm). The mobile phase was an aqueous 10% formic acid as solvent A and the mixture of acetonitrile: water: formic acid (5:4:1) as solvent B. The gradient profile was run with a flow rate of 1.0 ml/min for 30 min. The detection wavelength was 280 nm.[15]

Determination of *in vitro* antioxidant activity by DPPH free radical-scavenging assay

Different concentrations of each extract (1 mg/ml) were added to 0.3 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) in MeOH solution in a 96-well microplate and allowed to stand at room temperature for 30 min. The resulting colored solution was measured by spectrophotometer at a wavelength 517 nm. Methanol was used as a negative control while 1-100 mg/ml of ascorbic acid was used as a positive control. The percent of inhibition was calculated using the equation, % Inhibition = $(C - S)/C$ x 100 where C is the net absorbance of the negative control and S is the net absorbance of the sample. Percent inhibition was plotted against concentration and the equation for the straight line was used to obtain the IC_{50} value. A lower IC_{50} value indicates greater antioxidant activity.[16]

Animals

All rats were obtained from the National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand. Female wistar rats, age 8 weeks old, weight approximately 200 g, were used in acute oral toxicity tests. Male wistar rats, age 4 weeks old, were examined for *in vivo* antioxidant activity. The rats were housed under standard environmental conditions of 24 ^{o}C and 12:12 hr dark-light cycle and allowed free access to water and diet. The experimental protocol was approved by The Animal Ethics Committee of Faculty of Medicine, Chiang Mai University.

Acute toxicity test

The acute oral toxicity test was carried out according to the Organization for Economic Co-operation and Development (OECD) guidelines for Testing of Chemicals number 425.[17] The study was comprised of two groups of 5 female rats. The treated group was received 5000 mg/kg bw of ethanolic extract of *C. nervosum* given once orally. The control group received 10 ml/kg bw of water. The experimental animals were observed for 30 min after treatment, followed by observation for 8 hr and once daily throughout the experiment. All rats were sacrificed using an overdose of diethyl ether after 14 days. The internal organs were observed and weighed.

Antioxidant study in rats

Thirty-two rats were divided into 4 groups. Group 1 received 4 ml/kg bw of distilled water as a vehicle control. Groups 2 to 4 received the ethanolic extract of *C. nervosum* at doses of 100, 300 and 1,000 mg/kg bw for 90 days, respectively. All rats were weighed once a week. The amount of diet and water consumption was measured twice a week. At the end of the study, the rats were anesthetized under diethyl ether and sacrificed. The whole blood was taken from the abdominal vein. The liver, spleen and kidney tissues were removed and weighed. Livers were frozen and stored at -80 ^{o}C.

Determination of lipid peroxidation in liver tissues and serum

The liver homogenate was precipitated in 50% Trichloroacetic acid and centrifuged at 6,000 rpm, 4 ^{o}C for 20 min. Two ml of 0.67% thiobarbituric acid was added to the supernatant and the mixture was heated to 100 ^{o}C for 10 min. The reaction was stopped by placing on ice and adding butanol. After centrifugation at 3,000 rpm for 15 min, the resulting solution was measured spectrophotometrically at 532 nm. The amount of malondialdehyde (MDA), the end-product of lipid peroxidation in thiobarbituric acid reactive substance (TBARS) assay, was quantified.[18]

Serum was mixed with 3 N sulfuric acid and 10% phosphotungstric acid. The mixture was centrifuged at 3,000 rpm for 15 min. The sediment was resuspended in thiobarbituric acid reagent, mixed and heated to 100 ^{o}C for 1 hr in a water bath. The reaction was stopped by placing on ice and adding butanol. After centrifugation at 3,000 rpm for 15 min, the resulting solution was measured spectrophotometrically at 532 nm. The amount of TBARS was quantified and used as an index of lipid peroxidation.[19]

Determination of total glutathione

The liver homogenate was centrifuged at 14,000 rpm for 30 min at 4 ^{o}C. Two hundred µl of supernatant were deproteinized with 5% metaphosphoric acid

and the mixture was centrifuged at 14,000 rpm for 30 min. The 200 µl of reaction mixture was composed of deproteinized supernatant, 10 mM sodium phosphate buffer containing 5 mM EDTA (pH 7.5), 4 mM β-NADPH, 6 U glutathione reductase and 10 mM DTNB. After incubation for 30 min, the resulting solution was read by spectrophotometry at a wavelength of 405 nm using a microplate reader. Glutathione concentration in samples was calculated using a calibration curve and expressed as nmol/mg protein.[20]

Preparation of microsome and cytosol of rat liver

One gram of liver sample was homogenized and centrifuged at 10,000 g for 30 min at 4°C. The resulting supernatant was then centrifuged at 100,000 g for 60 min at 4°C to obtain the cytosolic and microsomal fractions. Total protein concentration in each sample was measured by the Lowry method using a bovine serum albumin calibration curve and expressed as mg protein/ml.[21]

Catalase activity assay

The reaction mixture contained 30 mM H_2O_2, 50 mM phosphate buffer, pH 7.0 and sample for a total volume of 750 µl. Catalase activity was estimate by the decrease in absorbance of H_2O_2 at 240nm.[22]

Glutathione peroxidase activity assay

The reaction mixture contained 0.1 M Tris-EDTA buffer (pH 8.0), 0.1 M GSH, 2 mM β-NADPH, 7 mM *tert*-butylhydroperoxide, 10 U Glutathione reductase and 10 µl of sample. The oxidation of β-NADPH was followed by measuring in the decrease in the absorbance at 340nm.[23]

Glutathione reductase activity assay

The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 1.2 mM GSSG and 1.2 mM β-NADPH. Decrease in the absorbance of β-NADPH at 340 nm was monitored spectrophotometrically.[24]

Heme oxygenase-1 activity assay

The reaction mixture containing 50 mM of the substrate hemin, microsomal fraction and rat liver cytosol as a source of biliverdin reductase, 2 mM glucose-6-phosphate, 0.2 U/ml glucose-6-phosphate dehydrogenase, 0.8 mM NADPH and 100 mM potassium phosphate buffer containing $MgCl_2$, pH 7.4 was incubated at 37 °C for 1 hr. The reaction was stopped with chloroform and the chloroform layer was measured spectrophotometrically. Bilirubin formation was calculated from the difference in absorption between 460 and 530nm.[25]

Statistical analysis

All experiments were conducted independently at least three times. Results were expressed as mean ± SD. Statistical significance of difference between groups was determined by one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. A *p*- value < 0.05 was regarded as significant.

RESULTS

The amounts of chemical constituents and antioxidant capacity of *C. nervosum* extracts are shown in Table 1. The ethanolic extract presented higher amounts of total phenolic compounds, total flavonoids and cyanidin-3-glucoside when compared to those of the aqueous extract. The DPPH- free radical scavenging activity of aqueous and ethanolic extracts of *C. nervosum* is demonstrated in Figure 1. The ethanolic extract (IC_{50} was 1.68±0.51mg/ml) exhibited higher antioxidant capacity than the aqueous extract (IC_{50} was 5.68±2.17mg/ml). Therefore, the ethanolic extract of *C. nervosum* was further investigated in animal models.

Table 1 Chemical constituents of aqueous and ethanolic extracts of *C. nervosum*

Chemical compound [a]	Aqueous extract	Ethanolic extract
Total phenolic compounds (mg eq GAE/g extract)	9.22±1.67	35.67±6.29
Total Flavonoids (mg eq CE/g extract)	5.23±3.60	13.39±5.73
Cyanidin-3-glucoside (ug/mg extract)	3.37±0.42	25.28±2.28

[a] measured for 3 times.

Data are expressed as mean±SD

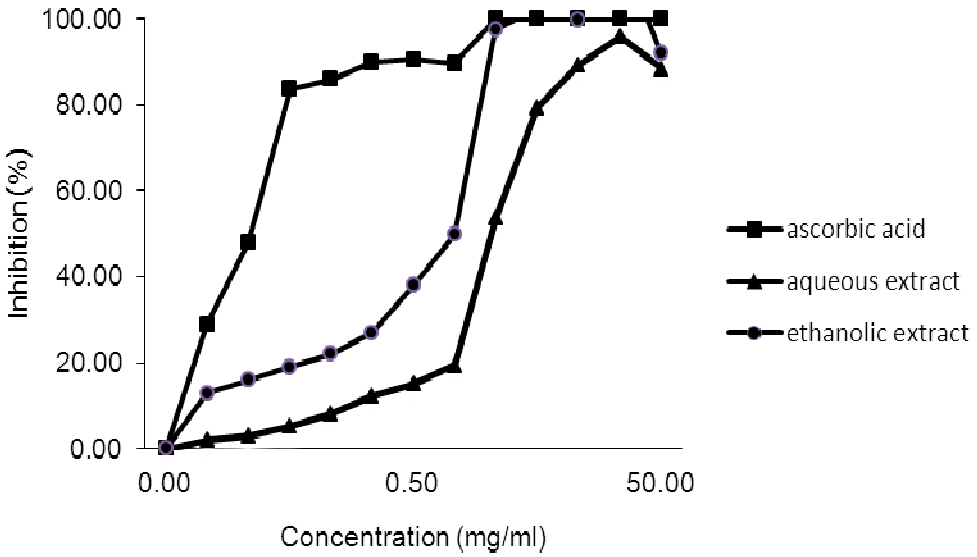


Figure 1 Free radical scavenging of aqueous and ethanolic extracts of *C. nervosum* by the DPPH assay: (□) ascorbic acid; (▲) ethanolic extract of *C. nervosum*; (x) aqueous extract of *C. nervosum*. Results are mean ± SD.

The ethanolic extract of *C. nervosum* did not result in any mortality to rats and no toxic effect was observed throughout the 14 days study period. The body weights, relative organ weights and consumption of diet and water of the treated rats were not significantly different as compared to the control rats (Table 2). In addition, gross examination of internal organs of all rats did not reveal any abnormalities.

The effects of 90 days-administrations of 100, 300 and 1000 mg/kg bw of *C. nervosum* ethanolic extract on oxidative stress and the antioxidant activity in rats are shown in Table 3 and Figure 2. There were no significant differences in body weight change, food and water consumption and vital organ sizes among the treated groups and a vehicle control group (data not shown). The levels of TBARS in liver were significantly decreased (p<0.05) in rats treated with *C. nervosum* ethanolic extract compared to control rats. In contrast *C. nervosum* ethanolic did not affect on TBARS contents in serum. Hepatic glutathione of treated rats was not significantly different when compared to control group. Treatment of 100 and 1000 mg/kg bw the ethanolic *C. nervosum* extract resulted in significant increase in glutathione peroxidase activity (p<0.05), but not of 300 mg/kg bw. While, the ethanolic *C. nervosum* extract did not affect the other antioxidant enzymes including heme oxygenase-1, glutathione reductase and catalase.

Table 2 General observation and relative organ weights of control and *C. nervosum* extract treated rats in the acute toxicity study

Observation	Control (N=5)	5000mg/kg bw of *C. nervosum* ethanolic extract (N=5)
Initial body weight (g)	196±8.94	196±6.52
Final weight (g)	229±8.21	236±4.18
Body weight change (percent)	16.94±4.35	20.52±4.59
Food intake (g/rat/day)	15.83±2.58	16.67±2.04
Water intake (ml/rat/day)	16.67±3.42	21.25±9.45
Heart (g/100g bw)	0.32±0.03	0.32±0.03
Lung (g/100g bw)	0.47±0.04	0.46±0.09
Thymus (g/100g bw)	0.17±0.04	0.17±0.03
Liver (g/100g bw)	3.51±0.24	3.54±0.27
Pancreas (g/100g bw)	0.40±0.09	0.38±0.10
Adrenal gland (mg/100g bw)	30.62±6.52	51.36±10.09
Spleen (g/100g bw)	0.23±0.03	0.26±0.03
Kidneys (g/100g bw)	0.70±0.04	0.67±0.14
Stomach (g/100g bw)	0.46±0.06	0.50±0.05
Ovary (mg/100g bw)	70.04±22.49	75.38±8.37
Fallopian tube (g/100g bw)	0.19±0.06	0.17±0.02

Data are expressed as mean±SD (Number of rats in each group = 5).

Table 3 Effect of ethanolic extract of *C. nervosum* on the levels of serum malondialdehyde and hepatic malondialdehyde, total glutathione and heme oxygenase-1 activity in rats

Treatment	MDA in serum (nmol)	MDA in liver (nmol/mg protein)	Hepatic GSH (nmol/mg protein)	HO-1 (umol/min/mg protein)
Distilled water	4.412±1.74	0.1301±0.004	22.13±3.76	20.70±5.65
C. nervosum 100 mg/kg bw	4.245±1.74	0.0803±0.001*	21.02±5.08	20.79±6.03
C. nervosum 300 mg/kg bw	3.611±1.21	0.0883±0.017*	18.60±4.22	19.03±5.28
C. nervosum 1000 mg/kg bw	4.628±2.86	0.0603±0.001*	17.86±6.01	17.61±3.55

Data are expressed as mean±SD (Number of rats in each group = 8).
MDA = malondialdehyde, GSH = glutathione and HO-1 = heme oxygenase-1.
*Significant difference from control at $p<0.05$.

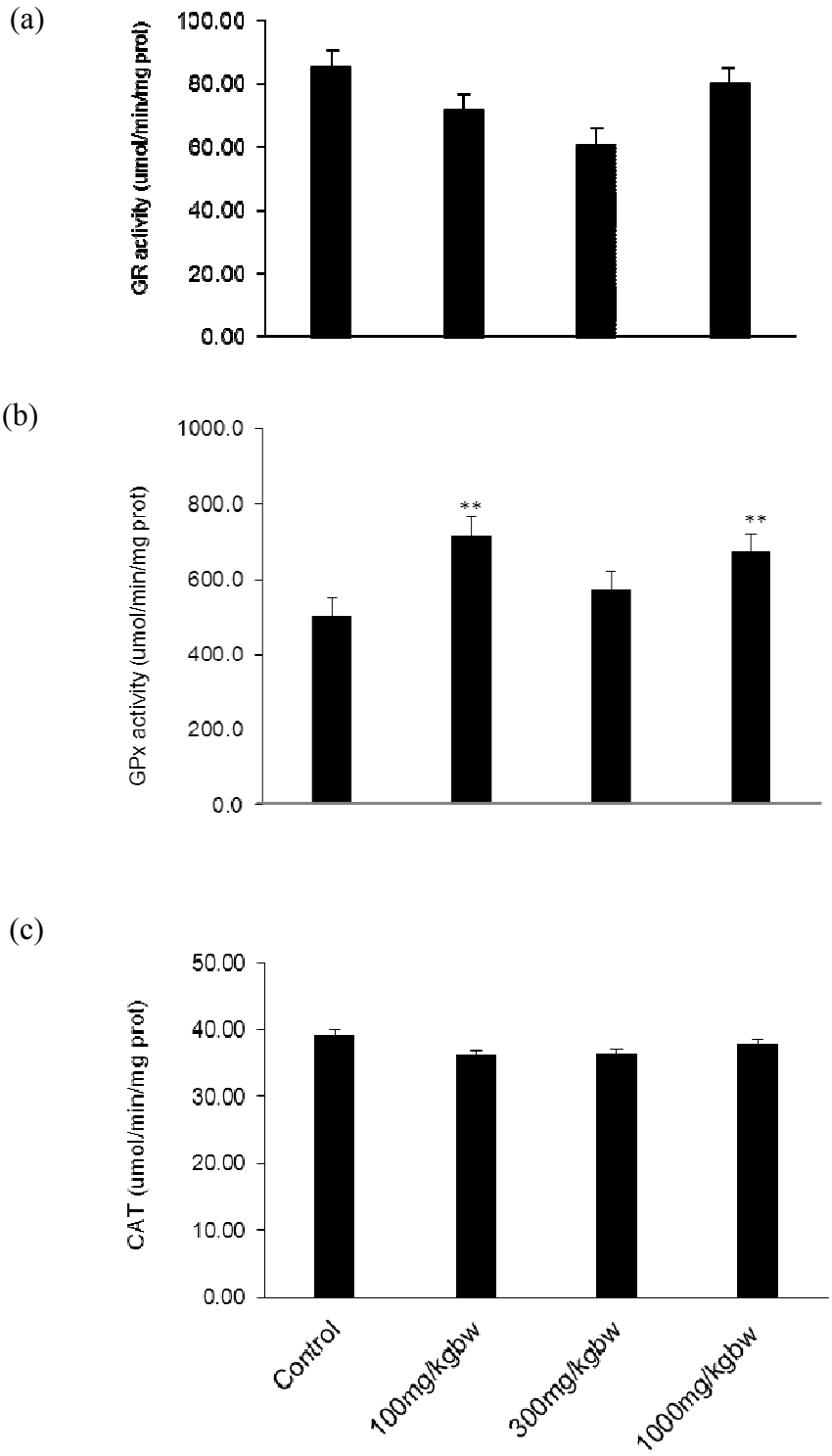


Figure 2 Effect of ethanolic extract of *C. nervosum* on antioxidant enzyme activities in rat livers. (a) glutathione reductase (b) glutathione peroxidase (c) catalase. Results are mean±SD (Number of rats in each group = 8). ***p*<0.05 vs. control

DISCUSSION AND CONCLUSION

The recent study clearly demonstrated that the amounts of polyphenols, flavonoids and cyanidin-3-glucoside as well as the DPPH radical-scavenging capacity were detected at higher level in the ethanolic extract than the aqueous extract of *C. nervosum*. These results support previous findings, that ethanol concentration has a critical role in the extraction of soluble components from different natural products.[26] This may be due to the fact that the *C. nervosum* has more nonpolar chemical constituents than polar ones, thus favoring the less polar solvent. The *in vitro* antioxidant activity was correlated with the content of total phenolic compounds, total flavonoid compounds and anthocyanins. This agrees with previous studies reporting that these compounds may take part in antioxidant capacity involving scavenging of free radicals and/or the chelating iron generating Fenton reaction.[27-28] Glycosylation and hydroxylation of the anthocyanidin backbone affects antioxidant activity. It has been reported that cyanidin is the most common anthocyanidin and the 3-glucoside is the most active antioxidant anthocyanin.[29]

In the experiments involving subchronic treatment with the ethanol extract, the concentrations of TBARS (indicators of lipid peroxide production) were significantly decreased in livers of treated rats while hepatic glutathione peroxidase (GPx) activity was increased. Most tissue damage is considered to be mediated by free radicals attacking membranes through peroxidation of unsaturated fatty acids.[30] The concentration of lipid peroxidation products may reflect the degree of oxidative stress. It has been reported that dietary polyphenols induce the antioxidant/detoxifying enzymes by modulating cellular signaling processes such as Nuclear factor-kappa B (NF-κB) activation, activator protein 1 (AP-1) DNA binding, glutathione biosynthesis, phosphatidylinositol-3-kinase (PI3K)/Akt pathway, Mitogen-Activated Protein Kinase (MAPK) protein activation and the translocation into the nucleus of NF-E2-related factor 2 (Nrf2).[31] GPx is an antioxidant enzyme involved in the detoxification of hydrogen and lipid peroxides and also acts as a peroxynitrite reductase. Shull and his colleagues reported an increase in GPx mRNA at higher concentrations of hydrogen peroxide.[32] Another study suggested a protective role for GPx in diabetes mellitus-associated atherogenesis.[33] In this study, the activities of GPx in rats treated with 100 and 1000 mg/kg bw of the ethanolic extract were enhanced, but not with 300 mg/kg bw. Therefore, the increased activity of GPx might be a part of decreasing of MDA levels in ethanolic extract treated rats. On the other

hand, the antioxidative effect of *C. nervosum* ethanolic extract could be due to its polyphenols with hydroxyl groups, which can act as reducing agents. Ethanolic extract of *C. nervosum* treatment did not affect total glutathione, glutathione reductase, catalase or heme oxygenase-1 in rats. This may suggest that under normal physiological conditions, there is a critical balance in the generation of oxygen free radicals and antioxidant defense systems. Thus, the formation of antioxidant enzymes is related to changes in the levels of free radicals.

In conclusion, the 95% ethanolic extract of *C. nervosum* contains a high content of anthocyanin that showed relatively good antioxidant capacities both in *in vitro* and *in vivo* models. In addition, *C. nervosum* with antioxidant capacities may be acting as naturally occurring antioxidant agents.

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