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ORIGINAL ARTICLE

Toxicity Evaluation of *Camellia sinensis* var. *assamica* and Its Fermented Miang Product

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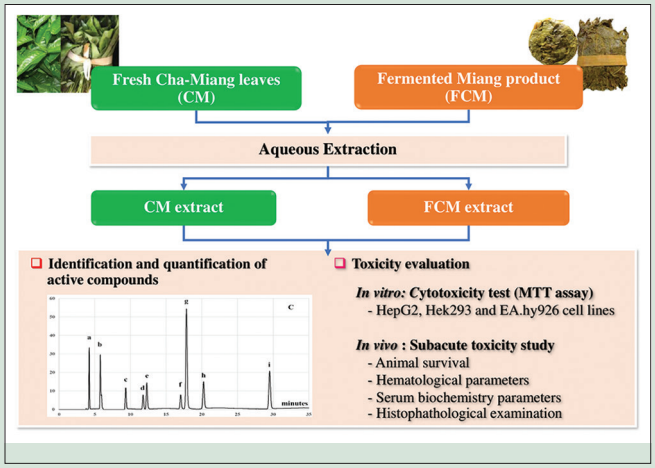
ABSTRACT

Introduction: *Camellia sinensis* var. *assamica* (Assam tea or Cha-Miang [CM]) is widely accepted to be beneficial to health. The tea leaf is used to produce various fermented tea products such as black tea (China, India) and Miang (Thailand). Despite its medicinal properties, toxicological information regarding certain tea variety and its fermented Miang product, especially its long-term toxicity, is currently limited. This study aimed to evaluate the potential toxicity of the extract of fresh resh Cha-Miang leaves (CM) and its fermented Miang product (FCM) in both *in vitro* and *in vivo* models. **Materials and Methods:** Cytotoxic effect on cell viability of HepG2, HEK293, and EA.hy926 cell lines incubated with CM or FCM extract for 24 h and 48 h was investigated by the MTT assay. After that, 14-day repeat oral toxicity test was performed on Wistar rats. **Results:** No *in vitro* cytotoxic effect of CM and FCM extract was found in the tested cell types, at a dose up to 1000 µg/ml. For *in vivo* study, both CM and FCM extracts at a dose of 300 mg/kg/day did not produce any sign or symptom of toxicity; no mortality was observed. Furthermore, investigation of hematological parameters, blood chemistry, body weight, and organ weight in the treated rats revealed no significant difference compared with that of normal controls. **Conclusion:** The results suggested that crude extract of CM and FCM at the doses used in this study are safe, however, further evaluation of possible chronic toxicity is recommended.

Key words: *Camellia sinensis* var. *assamica,* cytotoxicity, fermented tea product, Miang, subacute toxicity

SUMMARY

- Gallic acid, catechin, epicatechin, gallocatechin, epicatechingallate, epigallocatechin, epigallocatechingallate, and caffeine are found to be the major polyphenol compounds in Cha-Miang (CM) and fermented Miang (FCM) extract
- The CM and FCM extract did not affect clear toxic cell death to all the tested cell types (HepG2, Hek293, and EA.hy926 cell line), with an inhibitory concentration more than 1000 µg/ml
- Subacute oral toxicity studies of CM and FCM extract at a dose of 300 mg/kg BW/day for 14 days were safe to the rats as evaluated by clinical condition of the animals including animal survival, body weights, organ weights, hematological parameters, serum biochemistry parameters, as well as histopathological examination
- The oral administration of CM and FCM extract at the doses used in this study is safe to consume and can be used for further study.



Abbreviations Used: CM: Fresh Cha-Miang leaves; FCM: Fermented Miang product; HepG2 cell: Human hepatocellular carcinoma cell lines; Hek293 cell: Human embryonic kidney 293 cell lines; EA.hy926 cell: Human umbilical vein cell lines; IC_{50}: Inhibitory concentration for 50% of cell viability; LD_{50}: Lethal dose for 50% of the animal test population; MTT: 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; FBS: Fetal bovine serum; DMSO: Dimethyl sulfoxide; CO_2: Carbondioxide; C: Catechin (C); EC: Epicatechin ; ECG: Epicatechingallate; EGC: Epigallocatechin; EGCG: Epigallocatechingallate; GC: Gallocatechin; GCG:

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INTRODUCTION

Tea (*Camellia sinensis*) is presently cultivated around the world, especially China, India, Laos, Thailand, Vietnam, and Myanmar.[1,2] The tea beverage is the second favorite drink only after water[3] and has been classified into the following three types according to manufacturing process: green tea, oolong tea, and black tea.[4] *Camellia sinensis* var. *assamica* is one of the major tea varieties used to produce tea beverage, especially black tea. In northern Thailand, this type of tea is also used as a material to produce traditional fermented chewing snack product namely "Miang,"[5] and thus also called the tea leaf as "Cha-Miang (CM)." Unlike other tea products, Miang is made through a unique fermentation

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process.[6] The conventional methods in the production of Miang vary among regions. In general, tea leaves are tied into a small bundle, streamed, and then naturally fermented in a bamboo basket for several days or up to a year.[5,7] Analysis of chemical constituents found that, fresh tea leaves contain very high amounts of catechins (60%–70% of total polyphenol content).[8] The research reported that products obtained in the production process of Miang, including fresh leaves, fermented product, and wastewater, contain flavonoids and polyphenols as the main component. These polyphenols are composed of catechin and catechin derivatives similar to those found in other tea varieties,[9] but obviously differ in the quantity of active constituents.[5,6] Typical consumption of green and black tea infusion is considerably safe and has a positive health impact.[10-12] A major concern of tea consumption is associated to its possible hepatotoxicity.[13,14] Several studies also showed that catechins exert pharmacological properties such as antioxidant, anticancer, antihypertensive, anti-inflammatory, anti-hyperlipidemic, hypocholesterolemic, anti-diabetic, anti-obesity, and cardioprotective effects.[15-19] Moreover, the iron-binding property of catechin compounds in green tea may be responsible to exhibit antioxidant activity in iron-loaded rats.[20,21] Although Miang may contain chemical constituents like those of fresh tea leaf, studies focusing on its biological properties and possible toxicity have been done mostly only in the test tube level.[22] Thus, the present study aims to identify the possible toxicity of the extract of fresh CM and its fermented Miang product (FCM) both in *in vitro* and *in vivo* tests.

MATERIALS AND METHODS

Preparation of crude extracts of Cha-Miang and fermented product

Fresh CM leaf and locally made fermented Miang (traditional nonfilamentous fermented tea leaf) sample[5] were collected from Pang Ma-O village, Chiang Dao District, Chiang Mai, Thailand, during September 2016. The village is one of the most important areas in Thailand that grows Assam tea and has a long history of Miang production. Specimens were dried at 50°C in a hot air oven for 24 h. The obtained samples were subjected to grounding and tested for moisture content. The moisture content of <5% is acceptable, otherwise humid sample was continuously dried for the next 24 h to complete dryness. Dried powders were kept at −20°C until use in further study.

Extraction of the products was performed in hot distilled water, at 100°C using a shaking incubator at 120 rpm for 1 h. The extract was then passed to a filter membrane (Whatman No. 1), and the clear supernatant was collected and subjected to drying using a spray drying machine. Unless otherwise described, the dry extract was resuspended to desired concentration in deionized water (for chemical analysis), phosphate-buffered saline (PBS) (for *in vitro* study), or distilled water (for *in vivo* study).

Chromatographic analysis of catechin and related compounds

Identification and contents of catechin (C) and related compounds, namely epicatechin (EC), epicatechingallate (ECG), epigallocatechin (EGC), epigallocatechingallate (EGCG), gallocatechin (GC), gallocatechingallate (GCG), gallic acid (GA), and caffeine, were analyzed by reverse-phase high-performance liquid chromatography (HPLC) using an Agilent 1200 series HPLC instrument (Agilent Technologies, USA) equipped with a multi-wavelength detector according to the method of Saenjum *et al.*[23] The condition was carried out using a Symmetry Shield RP18 column (4.6 mm × 250 mm, 5-μm particle diameters) (Waters Corporation, USA). The mobile phase consists of 10% acetonitrile in 0.1% acetic acid and deionized water at the flow rate of 1.0 ml/min with the detection wavelength at 280 nm.

Cell lines and culture conditions

The human liver cancer cell line (HepG2), human embryonic kidney 293 cell line (Hek293), and human umbilical vein cell line (EA.hy926) were obtained from the American Tissue Culture Collection (ATCC, VA, USA). The cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin solution at 37°C in an atmosphere of 5% CO_2. The cells were subcultured when reach 70%–80% confluence twice each week.

MTT assay of cytotoxicity

The CM and FCM extracts were evaluated for cytotoxic activity in HepG2, Hek293, and EA.hy926 cell lines. The cells were plated in 96-well culture plates at a concentration of 3×10^3 cells/well. After 24 h, the cells were treated with either CM or FCM extracts (final concentration of 0–1000 μg/ml in PBS) and then incubated for further 24 h or 48 h at 37°C. Three individual experiments were done in triplicate sample. At the end of the treatment, the medium was discarded, and the cells were washed with PBS (200 μL). Thereafter, cell viability was assessed using practical MTT assay, as previously described.[24] Briefly, after the medium was aspirated, serum-free medium containing MTT (0.5 mg/mL) was added and incubated for 4 h at 37°C in a CO_2 incubator. Finally, the obtained MTT-formazan product was dissolved by adding 100 μL of DMSO. The spectrophotometrical absorbance of purple formazan dye was measured at 540/630 nm (STECTROstar Nano, BMG Labtech, Germany) zeroed against the blank well for each well. Calculation of percentage of cell viability at each concentration was done by comparison against positive control (untreated cell at a concentration of 0 μg/ml set as 100% viability). 1% DMSO was used as a negative control for each assay.

Animals

A total of 15 male Wistar rats (200–250 g), at an initial age of 6–8 weeks, were obtained from the National Animal Center, Salaya Campus, Mahidol University, Thailand, and acclimated for 7 days before starting the experiment. All rats were housed in standard controlled cages (4 rats/cage) and fed with standard laboratory diet (CP082) and free water *ad libitum*. The experimental animals were housed under controlled temperature rooms at (25°C ± 2°C) and lighting in a 12 h light/12 h dark cycle (lights on 06.00–18.00) hours. All animals achieved humane supervision in accordance with the recommended Care and Use of Laboratory Animals house, the protocol published by the Ethics Committees at the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (Permit number: 88/2559).

Experimental design and protocol

The limit test was performed to determine the LD_{50} value and the dose for subacute toxicity study. As per OECD test Guideline 420 (fixed dose procedure),[25] initially, a single dose of CM or FCM (300 mg/kg) was orally administered to the rat and then based on no observed mortality and morbidity, a dose of 2000 mg/kg BW was administered to another five rats. All the rats were observed critically for any mortality and toxic signs for 14 days.

The 14-day repeat dose experiment was performed at a dose of 300 mg/kg BW/day. Before the test, the animals were kept under the standard condition and without food for 12 h before dosing but had permissive free water. The animals were divided randomly into three groups comprising five rats in each group and fed with the same

normal chow diet (CP082) throughout the experimental period: NC group: control rats fed with 10 ml/kg vehicle (distilled water), CM group: rats were subjected to oral gavage with 300 mg/kg BW/day of CM extract, and FCM group: rats received 300 mg/kg BW/day, *p.o.* of FCM extract. The rats were closely observed for the first 30 min, then 4 h after feeding. The diet was provided after 1–2 h of dosing. After the survival of the treated rats, another day of administration was continued with the same dose under the same standard conditions for the additional rats. All the groups were observed for any toxic effect and then at regular intervals for 14 days. The surviving rats were observed to determine the toxic reaction onset. Weights of rats were recorded along with all necessary data.

Blood chemistry and hematological examination

At the end of the experiment (14 days), the rats were anesthetized with pentobarbital (100–150 mg/kg body weight). Blood samples were collected by hepatic vein puncture before incision of the abdomen in a plain. Whole blood was used for hematological examination of complete blood count (CBC), operated by Clinical Laboratory, Small Animal Hospital, Faculty of Veterinary Medicine, Chiang Mai University, Thailand. Serum was separated and subjected to analysis of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (Cr), total bilirubin, direct bilirubin, total protein, and albumin. These serum parameters were assayed using Randox Diagnostic reagent under automated clinical chemistry analyzer (RX Monaco, Randox Laboratories, UK). according to the manufacturer's instruction.

Histopathological examination

The vital organs (liver, kidney, heart, spleen, and pancreas) were removed after sacrificing rats by cervical dislocation. The organ was weighed before subsequently preserving it in 10% formalin for further histopathological examination using hematoxylin and eosin stain. Tissue sectioning, embedding, and staining were performed at the Department of Pathology, Faculty of Medicine, Chiang Mai University, Thailand.

Statistical analysis

Values were given as the mean ± standard deviation from triplicate samples of three independent experiments. Comparison of differences between the treatment groups was performed using a one-way ANOVA of GraphPad Prism software version 5.0 (GraphPad Software Inc., La Jolla, CA, USA) followed by Tukey's test. P <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Miang is a traditional fermented tea made from fermentation of Assam tea (CM) leaves with mixed microbial culture involving lactic acid bacteria and yeast. Different chemical compositions depending on various fermentation processes affect their bioactivities, which need careful safety assessment.

Identification and quantification of active compounds

The total phenolic content of CM and FCM extracts was determined in triplicate using Folin–Ciocalteu's phenol reagent according to the method described elsewhere[26] using a spectrophotometer (STECTROstar Nano, BMG LABTECH, Germany) set at 765 nm. The total phenolic content of CM and FCM extract was 2700.56 ± 12.73 and 1539.44 ± 30.71 mg gallic equivalent/g extract, respectively.

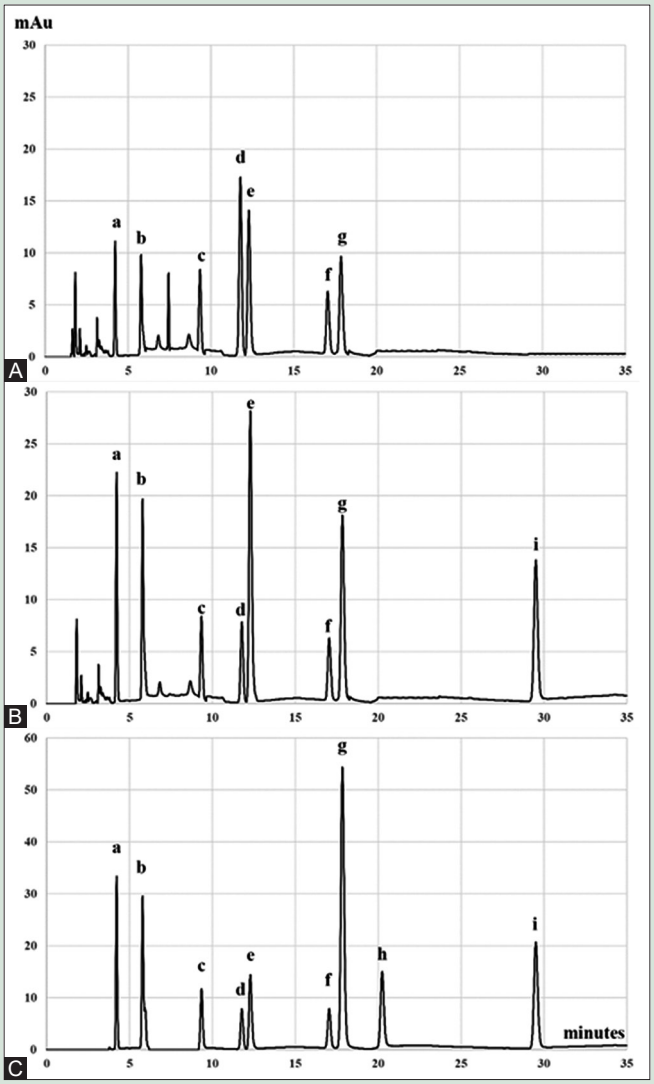

Figure 1: High-performance liquid chromatography chromatograms of representative (A) CM extract, (B) FCM extract, and (C) mixed authentic catechin standards. The peaks are (a): gallic acid; (b): gallocatechin; (c): epigallocatechin; (d): caffeine; (e): catechin; (f): epicatechin; (g): epigallocatechin gallate; (h): gallocatechin gallate; and (i): epicatechin gallate. CM: Cha-Miang;CM: fresh Cha-Miang leaves; FCM: fermented Miang product

Table 1: The amount of catechin and related compounds in fresh tea leaf (Cha-Miang) and fermented product (Miang) extracts

Compounds	Amount (mg/g extract)	
	CM	FCM
Gallic acid	0.60±0.10	1.58±0.14
Gallocatechin	0.55±0.12	2.64±0.17
Epigallocatechin	2.28±0.20	4.32±0.26
Caffeine	23.56±0.38	13.53±0.42
Catechin	12.64±0.47	18.19±0.39
Epicatechin	2.15±0.26	3.56±0.22
Epigallocatechin gallate	0.82±0.13	4.92±0.33
Gallocatechin gallate	ND	ND
Epicatechin gallate	ND	1.05±0.18

The amount of catechin and related compounds found in CM and FCM extracts was calculated from the peak area of sample compared to authentic standard. All values are expressed as mean±SD (n=3). ND: Not detectable; CM: fresh Cha-Miang leaves; FCM: fermented Miang product SD: Standard deviation

Traditional fermentation process used during the production of Miang product causes changes in the amount of catechins in the FCM extract compared with the original CM. The major polyphenol compounds found in CM and FCM extract composed of GA, C, EC, GC, ECG, EGC, EGCG, and caffeine. Representative HPLC chromatograms of CM extract; FCM extract; and a standard mixture of the seven catechins, GA, and caffeine are shown in Figure 1. The concentration was calculated by comparing the peak area of the analyte in the sample with the peak area of the authentic standard of a known concentration. The results are presented in Table 1. FCM extract had increased catechin content and lower caffeine content compared to CM. Epicatechin gallate, a catechin derivative, which is non-detectable in CM extract, was also detected.

Cytotoxicity of Cha-Miang and FCM extracts in cultured cell lines

In vitro cell-based cytotoxicity assays with cultured cell lines have been developed and are widely used to rapidly screen the cytotoxic activity of several compounds. Primary human cell culture is accepted as a gold material to determine cell toxicity as it is closely related to normal cells. Due to limited availability, and high cost consumption of fresh primary cell culture, cancer cell lines have been extensively employed as a human cell model for toxicity studies. HepG2 cell is considered a suitable system for assessing the hepatotoxicity of chemical compounds[27,28] due to its homogeneity and consistency of cellular features and its ability to maintain its properties *in vitro*. HEK293 cell is a specific cell line originating from human embryonic kidney cells which had been widely used in cell biology as well as nephrotoxicology studies.[29] EA.hy926 cell is one of the endothelial cell (EC) models frequently used in pharmacotoxicological study of different xenobiotic compounds.[30] In this sense, HepG2, HEK293, and EA.hy926 cell lines were employed to this cytotoxicity assessment as representative hepatocyte, renal, and endothelial vein cells, respectively.

The cytotoxicity test results revealed the relative viability of the cells treated with different concentrations of CM extract for 24 h- and 48 h-incubation times [Figure 2]. Cell survival tends to decrease in a dose-dependent manner. The CM extract, at the concentration used, did not affect clear toxic cell death to all the tested cell types, with an inhibitory concentration (IC_{50}) >1000 μg/ml. This finding was consistent with that of cytotoxic study of green and black tea using HepG2 cell, as previously reported by Sun *et al.*[31]

FCM extracts appeared to have different efficacy of growth inhibition of HepG2 and HEK293 cells, at 24 and 48 h incubation, respectively. In addition, the treatment of all cell types with FCM extract exhibited dose–response inhibition of cell proliferation, both at 24 and 48 h of incubation. Due to the lack of published data on the toxicity studies of Miang products, our study reported for the first time the effect of the product on cell viability. At the dose 1000 μg/ml on 24 h incubation, FCM extract causes 22% reduction of viability on HepG2 cell. For 48 h incubation at the same dose, 30% reduction of HEK293 cell viability was observed. According to the criteria of U.S. National Cancer Institute,[32]

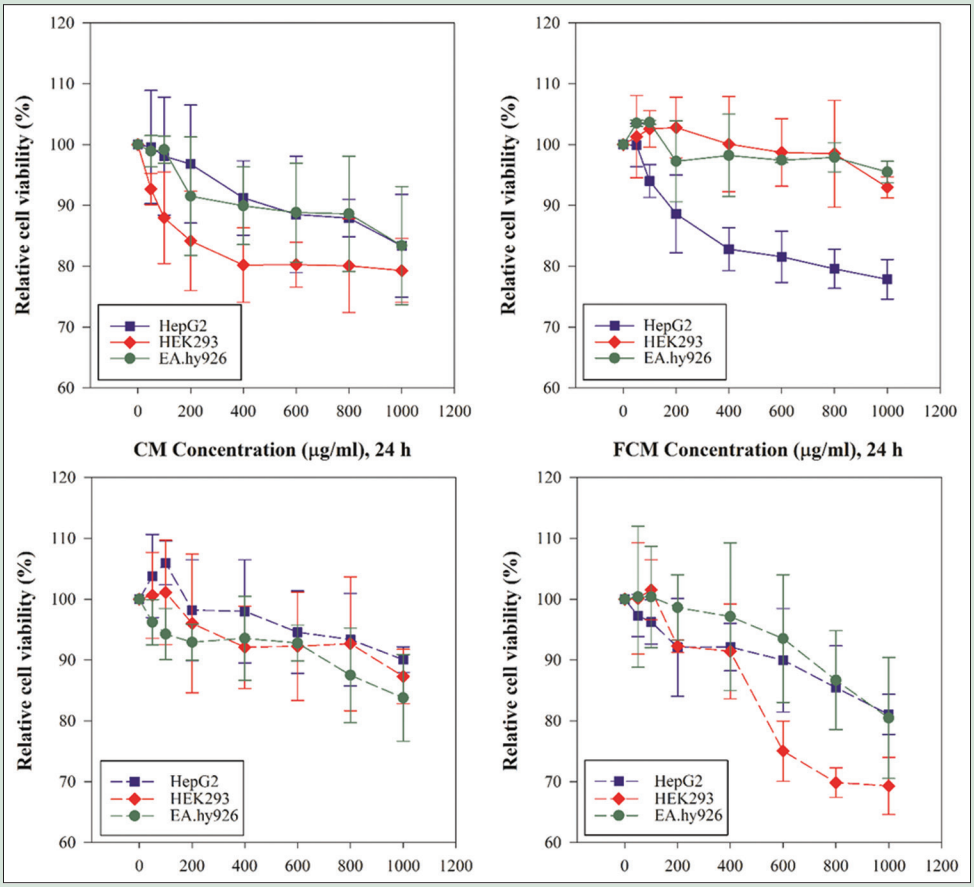

Figure 2: Effect of CM and FCM treatment on the extent of cell growth and viability of the indicated cell lines, determined by MTT assay. Cells (3×10^3 cells/well) were exposed to various concentrations (0–1000 μg/ml) of CM and FCM extracts for 24 h or 48 h. Thereafter, cell viability was assessed using practical MTT assay. Three individual experiments were done in triplicate sample. Cell growth and viability tended to decrease in a dose-dependent manner. Either CM or FCM extract, however, was considered non-toxic to the tested cells, with IC_{50} value >1000 μg/ml. CM: fresh Cha-Miang leaves; FCM: fermented Miang product

crude extract which has IC_{50} >20 μg/ml is considerably to have no toxicity. This study, thus, suggested the noncytotoxic effect of CM and FCM extracts to representative liver, kidney, and umbilical vein cells.

Animal toxicity test

On observation of acute toxicity test using rats following a single oral administration of CM or FCM extract at 300 or 2000 mg/kg body weight, there were no significant changes in body weight, clinical signs, mortalities, or macroscopic necropsy examination of any organs at post-mortem in rats following the administration of the extract at any dose tested. However, rats that received 2000 mg/kg BW were slightly more active compared to untreated control (data not shown).

Repeated administrations of either CM or FCM extract at 2000 mg/kg by oral gavage for 14 days caused some side effects. Rats showed signs of agitation after day 3 and became extremely tired afterward. Two of the five rats in the group treated with CM and FCM extract died at day 7 and day 8, respectively. These side effects seem to have likely caused by excessive amounts of caffeine intake. Thus, the treatment was stopped, and the remaining rats were removed from the study.

Subacute toxicity study was performed again by lowering dosage at 300 mg/kg/day, for 14 consecutive days. At the end of the study period, the animals showed no signs of abnormal behavior, though the rats treated with either CM or FCM extract had increased activity when compared to NC group. No significant change in food intake and body weight gain was found when compared with the NC group. No mortality occurred throughout the study period. In parallel with previous reports, administration of fresh and fermented tea extract at dose levels of up to 1250 mg/kg/day did not affect changes in body weight, food consumption, as well as animal behavior.[33-35] These findings suggested nonlethal consumption of CM and FCM extracts at a dose lower than 2000 mg/kg BW, which may provide useful information for further clinical use.

Toxicity test to hematology system of Cha-Miang and FCM extract in rats

Neither CM nor FCM extract at a dose of 300 mg/kg body weight showed significant toxic effects on the hematology system, which was manifested in the level of CBC parameters, including white blood cell (WBC) count, red blood cell (RBC) count, and platelet (PLT) count. Moreover, there was no significant change in the value of anemic markers including hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin (MCH), and MCH concentration when compared with NC group. Levels of determined parameters are indicated in Table 2. These results are concordant with those of Hsu *et al.*[35] Furthermore, there were studies which reported that repeated dose 90-day oral toxicity studies of nonfermented and fermented tea extract at dose levels 250 to 1250 mg/kg BW also showed no significant differences in hematological parameters compared with the control group.[33,34,36-38]

Clinical chemistry parameters

The values for serum biochemical constituents and enzymes in the rat administered with CM and FCM extract for 14 days are presented in Figure 3. Compared with the NC group, treatment with either CM or FCM extract did not cause significant change in the biochemical parameters of liver (ALT) and kidney function (BUN, globulin, bilirubin, and direct bilirubin). On the other hand, levels of AST, total protein, albumin, and creatinine of treated groups statistically significantly decreased ($P < 0.05$). The decrease in levels, however, is still in the normal range. These results are also in agreement with a previous study, which demonstrate that the administration of nonfermented and fermented tea extract at dose 1250 mg/kg/day for 4 and 13 weeks did not affect difference in the biochemical parameters of liver and kidney function compared with the control group.[34,35] Our study suggested that there was no exert hepatic or renal damage, following 14-day repeated administration of CM and FCM extracts.

Table 2: Hematological parameters of the rats after 14-day repeat oral administration test

Assay	Parameter	(Unit)	Group of experiments		
			NC	CM	FCM
WBC count	WBC	$\times10^3$/μl	2.59±0.47	2.86±0.89	3.46±1.40
	Neu%	%	19.85±1.21	24.55±3.41	21.50±0.46
	Lym%	%	74.35±0.52	70.95±4.21	72.00±0.92
	Mon%	%	4.15±0.64	2.90±1.15	4.80±0.81
	Eos%	%	1.65±1.33	1.60±0.35	1.70±0.35
	Bas%	%	0.00±0.00	0.00±0.00	0.00±0.00
	Neu#	$\times10^3$/μl	0.51±0.07	0.69±0.12	0.74±0.29
	Lym#	$\times10^3$/μl	1.93±0.36	2.06±0.75	2.51±1.06
	Mon#	$\times10^3$/μl	0.10±0.00	0.08±0.01	0.16±0.04
	Eos#	$\times10^3$/μl	0.05±0.04	0.04±0.02	0.06±0.03
	Bas#	$\times10^3$/μl	0.00±0.00	0.00±0.00	0.00±0.00
RBC count	RBC	×106/μl	6.62±1.07	7.42±0.28	7.94±0.33
	Hb	g/dl	12.40±1.50	13.40±0.46	13.90±0.35
	Hct	%	39.00±7.04	43.45±2.37	45.80±2.54
	MCV	fl	58.90±1.15	58.55±0.98	57.65±0.87
	MCH	pg	18.85±0.75	18.05±0.06	17.55±0.29
	MCHC	g/dl	32.05±1.91	30.85±0.64	30.40±0.92
	RDW-CV	%	13.55±0.06	13.05±0.17	13.25±0.64
	RDW-SD	fl	32.75±0.40	31.25±0.98	31.30±1.85
PLT count	PLC	$\times10^3$/μl	693.00±0.08	741.00±1.18	720.00±0.23
	MPV	fl	5.55±0.17	5.35±0.017	5.60±1.12
	PDW		15.00±0.00	15.00±0.23	15.15±0.17
	PCT	%	0.38±0.04	0.39±0.00	0.40±0.05

Data are presented as mean±SD (n=5). Statistical analysis for a possible effect of CM, or FCM treatment was evaluated as compared with the NC group. No statistically significant change was found. NC: Normal control; CM: fresh Cha-Miang leaves; FCM: fermented Miang product; SD: Standard deviation; MCH: Mean corpuscular hemoglobin; MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration; RDW: Red cell distribution width; PDW: Platelet distribution width; MPV: Mean platelet volume; PLC: Platelet count; WBC: White blood cell; RBC: Red blood cell; Hb: Hemoglobin; Hct: Hematocrit

Effects of Cha-Miang and FCM extract on vital organ histology

There is no change in the weight of various vital organs, neither in the CM- or FCM-treated rats compared with a normal control group. Histological examination study of the vital organs including the liver, kidney, spleen, heart, and pancreas on autopsy did not reveal any abnormalities. Represent microscopic (×40) pattern of tissues is shown in Figure 4.

Histopathological studies of the liver sections in the NC group showed the normal appearance of the central vein, ECs, and hepatocytes (H). Similar normal appearance was found in rats administered with either 300 mg/kg/day of CM or FCM extract for 14 consecutive days. Similarly, histopathological studies of other organs including kidney, spleen, heart, and pancreas sections of rats treated with a dose of 300 mg/kg CM or FCM showed no different appearance compared with the controls.

The liver and kidney play fundamental roles in the metabolism and secretion of drugs or plant products.[39,40] Exogenous chemicals and their metabolites might preliminarily result in toxicity or damage on this organ, and, further, effect on other vital organs.[41] In the present study, histopathological examination of the tissues section from the treated rats showed no change in the microscopic structure. The result was also accompanied by the

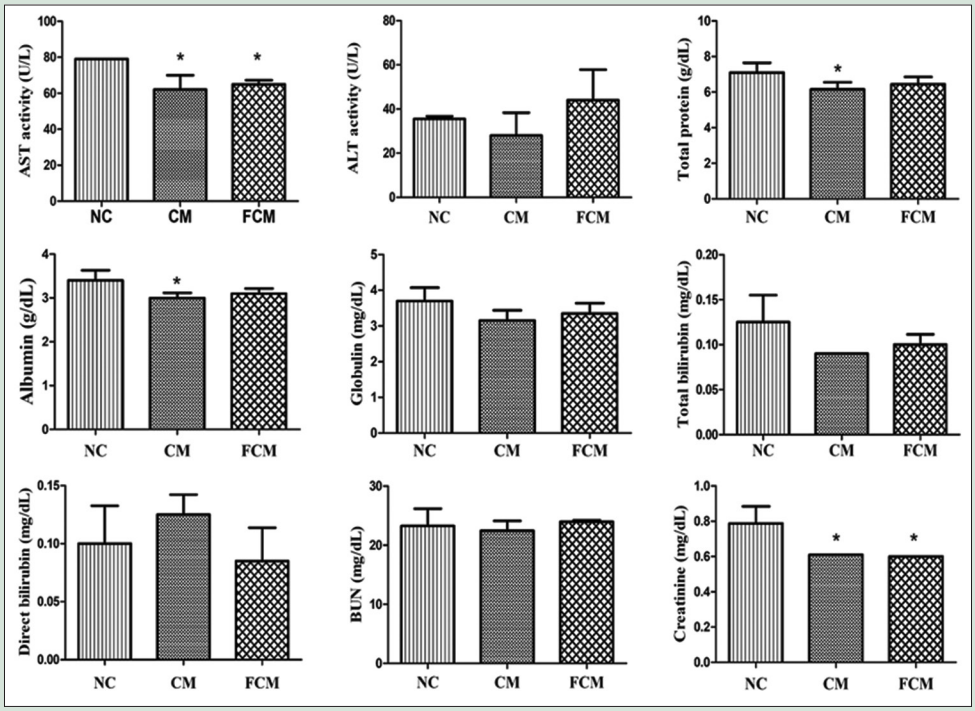

Figure 3: Plasma biochemical parameters of liver and renal function in rats administered with CM or FCM extract at a dose of 300 mg/kg BW/day for 14 consecutive days were compared with those of normal control (NC group). No pathological change was observed in the rats treated with either CM or FCM extract. Data are expressed as mean ± standard deviation ($n = 5$). *$P < 0.05$ compared with NC group. NC: Normal control; CM: fresh Cha-Miang leaves; FCM: fermented Miang product

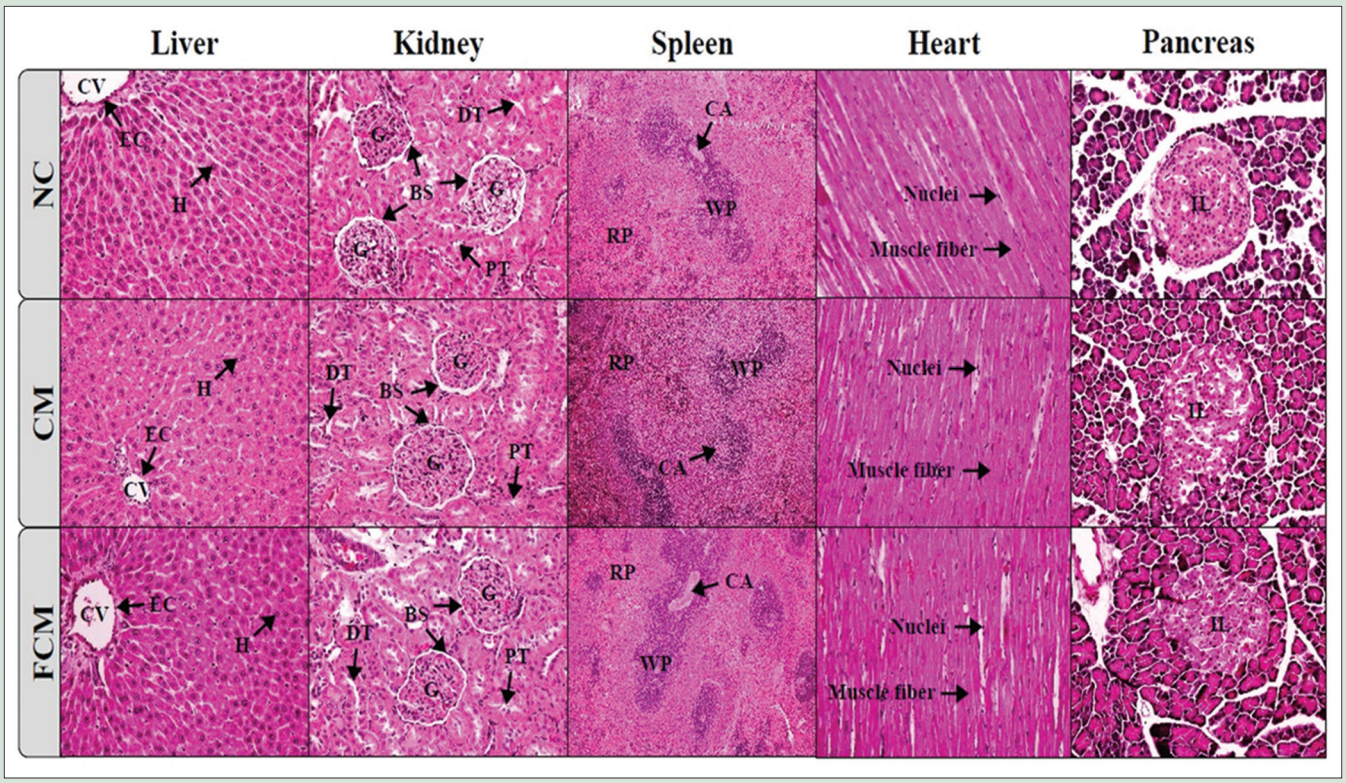

Figure 4: Representative microphotographic tissue of vital organs of the rats after treated with CM or FCM extract for 14 consecutive days. Histological examination study (H and E stain) of the vital organs including the liver, kidney, spleen, heart, and pancreas on autopsy did not reveal any abnormalities as normal appearances seen when compared to NC group. NC: Normal control; CV: Central vein; EC: Endothelial cells; H: Hepatocytes; PT: Proximal tubule; DT: Distal tubule; G: Glomeruli; BS: Bowman's space; CA: Central arteriole; WP: White pulp; RP: Red pulp; IL: Islet of Langerhans; CM: fresh Cha-Miang leaves; FCM: fermented Miang product

non-adverse effects of the extract in any of the biochemical markers (such as ALT, AST, BUN, creatinine, albumin, globulin, and bilirubin). This absence of pathological changes, however, results from a short period of administration of CM or FCM extract. Although it brings the supportive evidence of nonacute toxicity of the extracts, a possible chronic toxic side effect of the extracts might not yet be seen.

CONCLUSION

The results observed herein provide supporting evidence that CM and FCM extracts are generally safe, proven by either *in vitro* or *in vivo* toxicity test. The fermented product at a dose of 300 mg/kg BW, when administered to the rats, did not cause serious toxic side effects to cytology, hematology system, and hepatic and kidney function. In addition, no sign of toxicity in other tissue organs (spleen, heart, and pancreas) was found. The study thus provides scientific evidence to support the use of this plant extract as safe to consume and can be used for further study. Nevertheless, more subchronic or chronic toxicity evaluation is recommended to warrant the use of these products.

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Conflicts of interest

There are no conflicts of interest.

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