Effects of *Cymbopogon citratus* Stapf Water Extract on Rat Antioxidant Defense System

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Background: *Cymbopogon citratus, Stapf (CCS) is commonly known as lemon grass. Previous studies showed that it has a strong antioxidant property and have been traditionally used as analgesic, antipyretic, antiseptic in Southeast Asia. However, the effect of CCS on antioxidant defense system has not been demonstrated.*
Objective: *The present study was conducted to investigate the effects of CCS water extract on rat antioxidant defense system, especially on the expression of γ-glutamylcysteine ligase (γ-GCL) and heme oxygenase-1 (HO-1).*
Material and Method: *The CCS water extract was screened for its phytochemical contents and antioxidant activity in vitro. Moreover, the extract was studied in rats to evaluate its effects in vivo. Male Sprague-Dawley rats aged eight weeks (250±20 g) were orally administered with CCS at 250, 500 and 1,000 mg/kg/day for one month.*
Results: *The extract contained flavonoids (496.17 mg gallic acid/g CCS extract) and phenolic compounds (4,020.18 mg catechin/g CCS extract). The scavenging activity (DPPH assay) of the extract was demonstrated by EC_{50} of 917.76±86.89 μg/ml whereas the EC_{50} of the potent antioxidant, vitamin C was 31.22±1.84 μg/ml. In the animals, the protein expression of antioxidant enzymes, γ-GCL and HO-1 was significantly increased in the high dose-treated animals (1,000 mg/kg/day). This was consistent with elevation of serum total antioxidant capacity.*
Conclusion: *Taken together, the present study provides evidence that CCS water extract exhibits antioxidant activity and antioxidant enzymes induction in vivo.*

Keywords: *Cymbopogon citratus Stapf, Antioxidant, γ-glutamylcysteine ligase, Heme oxygenase-1*

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Nowadays, people are more concerned about their health. Treatment or supplements with medicinal plants appear to be an alternative approach. The antioxidant property of plant extracts might have them considered as natural antioxidants in nutraceutical preparations. Cumulative evidence in experimental animals and clinical studies have shown that supplementation of antioxidants could be useful in prevention and management of reactive oxygen species-induced cellular damage, which is implicated in the development of the aging process and several chronic diseases such as cardiovascular diseases and cancers[1,2].

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Besides intrinsic antioxidant activity, one of the potential strategies for protecting cells from oxidative damage of several antioxidant compounds is the induction of antioxidant enzymes, especially heme oxygenase 1 (HO-1) and γ-glutamylcysteine ligase (γ-GCL)[3,4]. HO-1 is an enzyme responsible for heme degradation and exerts cytoprotective effect in several pathological states such as heart failure[5] and hypertension[6]. Similar to HO-1, γ-GCL has a potential to protect cells against damage caused by oxidative stress. The enzyme catalyzes the rate-limiting step of the synthesis of glutathione (GSH) which is the most abundant intracellular antioxidant molecule[7].

Cymbopogon citratus Stapf (CCS), commonly known as lemon grass, is a widely used herb in tropical countries. It has been shown effective in the treatment of fever, infection, headache and rheumatic pain. Furthermore, the pharmacology properties of particular parts of CCS have been reported. The leaves decoction has been shown to have antioxidant property whereas

the stalk has been shown to have an artery relaxation effect. Moreover, anti-mutagenic and anti-carcinogenic effects of CCS ethanol extract were reported[8]. Thus, it is the authors' interest to investigate the effects of CCS extract consumption on rat antioxidant defense system.

Material and Method

Plant material

Fresh CCS specimens were purchased from local market in Pathumthani, Thailand during December 2013. The collected plant materials (whole plant) were washed thoroughly in water, cut into small pieces, boiled in water and then filtered. The filtrates were concentrated by rotary vacuum evaporation and then lyophilized with a freeze dryer. The extract was kept at -80°C for analysis.

Total phenolic content assay

The CCS extract was screened for the presence of some phytoconstituents. The total phenolic content of the CCS extract was determined using the Folin-Ciocalteu reagent[9]. The reaction mixture contained 100 µl of diluted extract, 500 µl of freshly prepared diluted Folin-Ciocalteu reagent and 400 µl of 7.5% sodium carbonate. Mixtures were kept in the dark at ambient conditions for 30 min to complete the reaction. The absorbance at 756 nm was measured. Gallic acid was used as standard, and the results were expressed as mg gallic acid/g CCS extract.

Total flavonoid content assay

Total flavonoid content was determined using aluminum chloride according to a previously described method[10] using catechin as a standard. The plant extract (0.5 ml) was added to 1.5 ml of 95% ethanol followed by 100 µl of 10% aluminum chloride, 100 µl of 1M potassium acetate and 2,800 µl of distilled water. After 30 min incubation, the absorbance was measured at 510 nm. The results were expressed as mg catechin/g CCS extract.

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay

The DPPH radical-scavenging activity test was applied following the method described previously[11]. Briefly, 0.08 mM DPPH was prepared in 100% ethanol. DPPH solution, Tris buffer solution and 80% ethanol were mixed in order to obtain a 1:1:1 ratio for 1.8 ml. Then, the plant extract (0.6 ml) was added and incubated for 30 min in the dark. The absorbance was read at 525 nm. The free radical-scavenging activity of vitamin C was also measured to compare the relative potency of the extract.

Experimental animals and design

Twenty-four adult male Sprague-Dawley rats weighing about 200-250 g were obtained from the National Animal Center, Mahidol University, Thailand. The animals were housed in specific standard laboratory condition for one week. The conditions were a temperature-controlled environment (25±1°C), 55±5% relative humidity and with regular 12 hours light/12 hours dark cycles. All animals were fed with standard rat chow diet and water ad libitum. The animals were divided into 4 groups, each of which consisted of 6 rats. The animals in group 1 reserved as normal control were orally administered vehicle solution (distilled water) for 30 days. The animals in groups 2-4 were orally administered CCS extract at the doses of 250, 500 and 1,000 mg/kg/day, respectively, for 30 days. CCS extract was dissolved in distilled water. The protocol for the present study was approved by the Animal Research Committee of Thammasat University, Thailand with the license number AE 009/2013.

Serum total antioxidant capacity assay (TAC)

The measure of serum TAC considers the cumulative action of all the antioxidants present in serum, thus providing an integrated parameter to identify antioxidant condition in the body. Total antioxidant capacity (TAC) in serum was assessed with Abcam's total antioxidant capacity assay kit (ab65329, Abcam, Cambridge, UK) as per described datasheet. Trolox (a water-soluble derivative of vitamin E (6-hydroxy-2.5.7.8-tetramethylchroman-2-carboxylic acid) was used to create standard curve following the kit protocol procedure.

Measurement of liver and kidney function markers

The blood was taken from the rat by cardiac puncture and centrifuged at 2,000 rpm 4°C for 10 min to separate the serum. The levels of liver function markers, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and kidney function markers, including blood urea nitrogen (BUN) and serum creatinine (Cr), were measured by the laboratory unit at Thammasat University Hospital.

Histopathological examination of rat liver

The rat liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The serial

sections were cut 5 mm thick and stained with hematoxylin-eosin (H&E) and examined under a photomicroscope.

Western blot analysis

Western blot analysis was used to determine the expression levels of HO-1, γ-GCL and β-actin. The fresh liver was washed with PBS, lysed with RIPA buffer [150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), 50 mM glycerophosphate, 20 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na3VO4] with addition of protease inhibitor cocktail (M221: Amresco, OH, USA) at 4°C for 15 min and transferred into a microtube. After vigorous vortex mixing, the suspension was centrifuged at 12,000 g for 20 min, and supernatant was collected and stored at -20°C until use. The protein samples (20 μg) were mixed with SDS loading buffer and subjected to separation by electrophoresis in 8-10% SDS polyacrylamide gel. The bands were blotted onto a PVDF membrane. The membranes were blocked with 5% (w/v) skimmed milk powder in Tris buffered saline (TBS) containing 0.1% Tween-20 at room temperature for one hour. The PVDF membranes were incubated with goat polyclonal anti-human HO-1 (sc-7695), rabbit polyclonal anti-human γ-GCL (sc-28965) and goat polyclonal anti-human β-actin (sc-8432 HRP) antibodies in PBS at 4°C overnight. After washing with PBS, the blots were incubated with the HRP-conjugated secondary antibodies (anti-rabbit IgG-HRP sc-2004 and anti-goat IgG-HRP sc-2354) at room temperature for one hour. After removal of the secondary antibodies and TBS buffer washes, the blots were incubated in ECL substrate solution (Super Signal West Pico Chemiluminescent Substrate: ThermoScientific, IL, USA). The densities of the specific protein bands were visualized and captured by Image Quant™ 400 (GE HealthCare).

Statistical analysis

Data are presented as mean ± SEM. An analysis of variance (ANOVA) with Duncan post-hoc test was used to determine significant differences between each experimental group. An ANOVA on rank test was also performed for non-parametric test. The level of significance was set at $p<0.05$.

Results

Phytochemical screening and antioxidant activity

The water extract of CCS was screened for the presence of some phytoconstituents. The extract contained tannins, flavonoids and phenolic compounds but lacked alkaloids. The CCS extract contained total phenolic compounds of 4,020.18 mg catechin/g CCS extract and total flavonoids of 496.17 mg gallic acid/g CCS extract. Moreover, the extract of CCS was also screened for its possible antioxidant activity through DPPH method. The scavenging activity of the extract was shown by EC_{50} of 917.76±86.89 mg/ml whereas the EC_{50} of the potent antioxidant, vitamin C, was 31.22±1.84 mg/ml.

General toxicity of the CCS extract

During the administration of the extract, the general appearance and behavior of the animals were monitored daily, and body weight was measured weekly. The changes in body weight were comparable in the three groups of animals (data not shown). To assess liver toxicity, the histopathological examination of the rat liver was performed. No abnormality was observed in any of the animals (Fig. 1). In addition, the weight of major internal organs, including liver, kidney and heart did not differ among the groups (Table 1). In addition, treatment with the CCS extract did not affect liver and kidney function as shown by normal serum AST, ALT, BUN and Cr levels (Table 2). These results indicated that the CCS water extract administration was generally safe at a dose up to 1,000 mg/kg/day.

Effects of CCS extract consumption on antioxidant system

Serum TAC was significantly elevated in the CCS-treated animals when compared to the control group, $p<0.05$ (Fig. 2). To investigate whether the effect

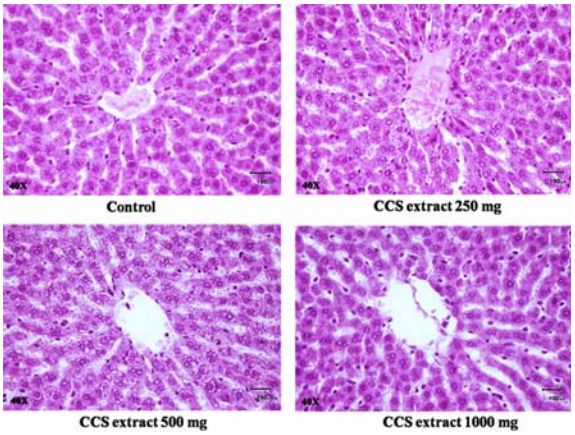

Fig. 1 Effect of CCS extract on the histopathological of the rat liver. Representative slides from corresponding groups.

Table 1. Relative organ weight of rat after 30 days of CCS treatment. Value are mean ± SEM (n = 6).

	Heart (g/100 g BW)	Kidney (g/100 g BW)	Pancreas (g/100 g BW)	Liver (g/100 g BW)
Control	0.38±0.01	0.70±0.03	0.30±0.02	3.40±0.33
CCS 250 mg/kg/day	0.39±0.01	0.73±0.03	0.34±0.03	3.29±0.15
CCS 500 mg/kg/day	0.42±0.06	0.78±0.03	0.37±0.0.04	3.33±0.23
CCS 1,000 mg/kg/day	0.34±0.06	0.73±0.02	0.38±0.0.03	3.28±0.28

Table 2. Biochemical parameters in rat serum after 30 days CCS treatment. Value are mean ± SEM (n = 6).

	AST (U/L)	ALT (U/L)	BUN (mg/dL)	Cr (mg/dL)
Control	20.33±0.61	0.38±0.07	194.50±15.08	65.50±3.42
CCS 250 mg/kg/day	19.33±0.80	0.43±0.62	193.83±8.98	77.67±8.45
CCS 500 mg/kg/day	21.50±1.98	0.38±0.09	196.83±24.01	64.17±2.75
CCS 1,000 mg/kg/day	18.00±1.59	0.39±0.04	191.50±13.00	58.83±2.87

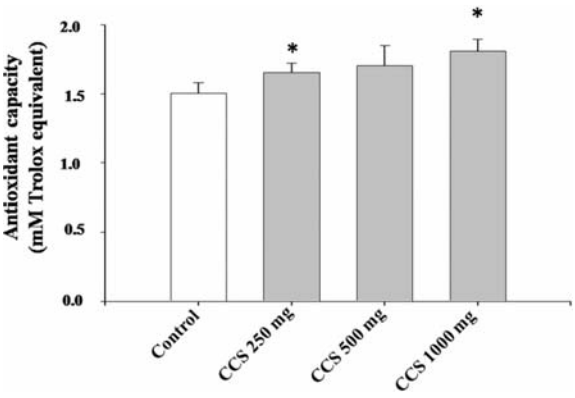

Fig. 2 Effect of CCS water extract on total antioxidant capacity in rat serum. The x axis is the weight of CCS water extract. Each bar represents the mean ± SEM, n = 6 animals. *p<0.05 compared with the control.

of CCS on increased serum TAC was due to the induction of antioxidant and cytoprotective enzymes, protein expression analysis was performed. Consistent with the serum TAC level, the expression of γ-GCL and HO-1 proteins tended to increase with all doses of the CCS treatment whereas a significant difference was found only in the high-dose treatment group (1,000 mg/kg/day), as shown in Fig. 3.

Discussion

A body of evidence suggests that diverse phytochemicals with antioxidant properties can protect against oxidative damage by directly neutralizing reactive oxidants and/or possibly increasing the capacity of endogenous antioxidant defenses.

In the present phytochemical screening, the extract contains flavonoids, phenolics and tannins but lacks alkaloids. In addition, the radical-scavenging activity of the CCS extract is demonstrated. The antioxidant effects of natural products have been reported to be mostly due to phenolic compounds[12], which are also found in the CCS extract. Moreover, flavonoids have been reported to possess antioxidant and anti-inflammatory activities against the cytotoxicity of linoleic acid hydroperoxide in rat phenochromocytoma PC12 cells[13]. The present study of Gali H et al (1996) discovered that tannins also had potent antioxidants and anti-inflammatory activities in mouse skin in vivo[14]. Thus, the scavenging activity of the CCS extract could be due to the presence of one or more active principles in the plant.

In the present study, besides directly reacting with reactive oxygen species as mentioned above, the CCS extract has ability to upregulate γ-GCL and HO-1 protein expression consistently with the elevation of serum TAC. A number of studies have demonstrated that the expression of protective enzymes, including HO-1 and γ-GCL, can be induced by dietary antioxidants via activation of redox-sensitive transcriptional factors[15,16]. The γ-GCL has a potential to protect cells against damage caused by oxidative stress. The enzyme catalyzes the rate-limiting step of the synthesis of GSH, which is the most abundant intracellular antioxidant molecule. Previous studies have demonstrated that dietary compounds such as berry extract and beta-

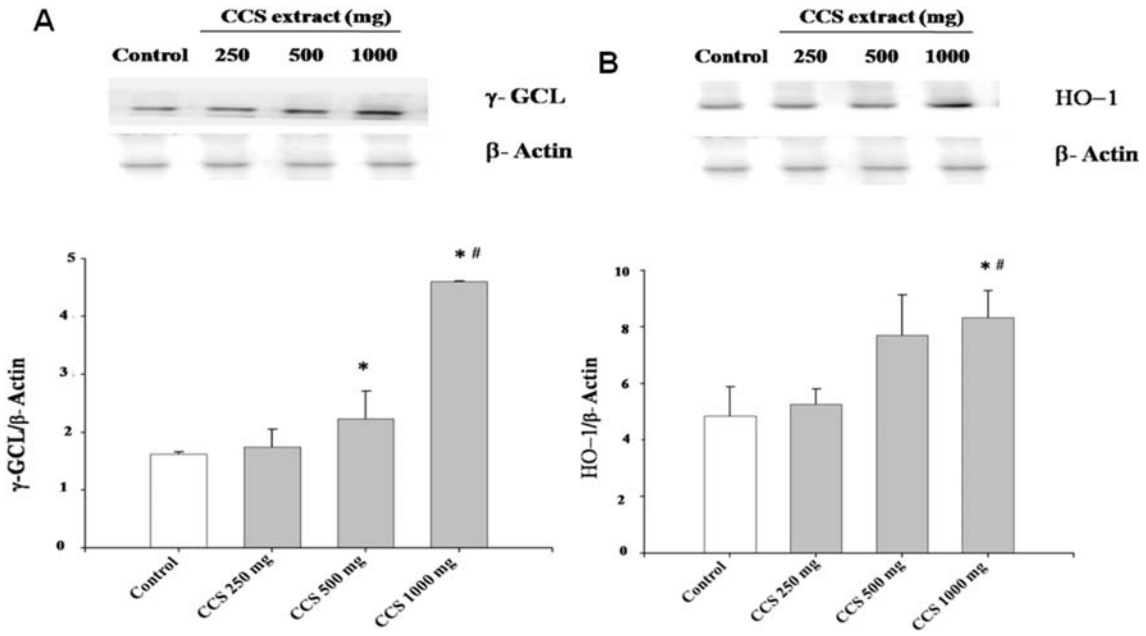

Fig. 3 Western blot analysis of HO-1 and γ-GCL in rat liver. The effects of CCS extract treatments on γ-GCL expression (A) and HO-1 expression (B) were analyzed by Western immunoblotting. The y axis is the ratio of antioxidant protein expression and internal control protein expression. The x axis is the weight of CCS water extract. Each bar represents the mean ± SEM, n = 6 experiments. * $p<0.05$ compared with the control; # $p<0.05$ compared with 250 mg treated group. The figure shown was representative from 6 animals with similar results.

carotene can induce the expression of γ-GCL[17,18].

HO-1 is an enzyme responsible for heme metabolism and plays critical roles as a cytoprotective and anti-inflammatory enzyme. HO-1 protein expression has been shown to be upregulated by several antioxidant compounds such as chrysin, apigenin, luteolin in order to protect cells from oxidative damage[19]. The up-regulation of HO-1 gene expression by plant extract such as *Syzygium formosanum*, a Taiwanese plant that contains several phenolic compounds, has been demonstrated[20].

For 30-day oral CCS consumption, the results of general toxicity has shown that the CCS extract is relatively safe as histopathological examination of the liver revealed no apparent abnormalities, and there were no changes in serum biochemical parameters. In summary, the antioxidant effects of the CCS extract have been evidenced, and no toxicities have been found. Therefore, dietary supplements with CCS extract may provide a health benefit on antioxidant defense system. However, further pharmacological evaluations are required to identify and isolate the active compounds in the extract as well as elucidating their mechanisms of action. CCS could be developed to be a dietary supplement, studies of long-term consumption and toxicology should be required.

Conclusion

The present study has provided clear evidence for the antioxidant activity of CCS as demonstrated in the assay of free radical-scavenging activity. In addition to the direct antioxidant effect, the extract of CCS was able to induce the body's endogenous antioxidant and cytoprotective system.

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Potential conflicts of interest

None.

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ผลของสารสกัดตะไคร้ด้วยน้ำต่อระบบต้านอนุมูลอิสระในหนู

นันทิยา สมภาร, ศุภเกต แสนทวีสุข, จริญญาพร เนาวบุตร, อัจราพร แถวหมอ

ภูมิหลัง: ตะไคร้ (Cymbopogon citratus, Stapf), มีรายงานการศึกษาว่าเป็นพืชที่มีฤทธิ์ต้านอนุมูลอิสระสูง รวมทั้งถูกนำมาใช้ในการรักษาอาการปวดเมื่อย อาการไข้และระงับเชื้อ ในประเทศแถบเอเชียใต้ อย่างไรก็ตามยังไม่มีรายงานการศึกษาผลของสารสกัดตะไคร้ต่อระบบต้านอนุมูลอิสระ

วัตถุประสงค์: เพื่อศึกษาผลของสารสกัดตะไคร้ด้วยน้ำต่อระบบต้านอนุมูลอิสระในหนู โดยเฉพาะอย่างยิ่งการแสดงออกของแกมม่ากลูตามิลซีสทีอีนไลเกสและฮีมอ็อกซี่จีเนสเอนไซม์

วัสดุและวิธีการ: สารสกัดตะไคร้ถูกนำมาวิเคราะห์ปริมาณสาร phytochemical และความสามารถในการต้านอนุมูลอิสระในหลอดทดลอง รวมทั้งประเมินผลของสารสกัดในหนูเพศผู้พันธุ์ Sprague-Dawley อายุ 8 สัปดาห์ น้ำหนัก 250±20 กรัม โดยป้อนสารสกัดขนาด 250, 500 และ 1,000 มิลลิกรัม/กิโลกรัม/วัน เป็นเวลา 1 เดือน

ผลการศึกษา: สารสกัดตะไคร้มีส่วนประกอบของสารกลุ่ม flavonoids (496.17 มิลลิกรัม gallic acid/กรัม) และสารกลุ่ม phenolic (4,020.18 มิลลิกรัม/กรัม) การศึกษาฤทธิ์กำจัดอนุมูลอิสระด้วยวิธี DPPH assay พบว่าสารสกัดมีค่า EC_{50} 917.76±86.89 ไมโครกรัม/มิลลิลิตร ในขณะที่ค่า EC_{50} ของวิตามินซี ซึ่งเป็นสารต้านอนุมูลอิสระที่มีฤทธิ์แรงมีค่าเท่ากับ 31.22±1.84 ไมโครกรัม/มิลลิลิตร การศึกษาในสัตว์ทดลองพบว่าการแสดงของโปรตีนซึ่งเป็นเอนไซม์ในระบบต้านอนุมูลอิสระคือ แกมม่ากลูตามิลซีสทีอีนไลเกสและฮีมอ็อกซี่จีเนส มีระดับสูงขึ้นในสัตว์ทดลองกลุ่มที่ได้รับสารสกัดขนาดสูงคือ 1,000 มิลลิกรัมต่อกิโลกรัมต่อวัน ซึ่งสัมพันธ์กับระดับ total antioxidant capacity ในซีรัม

สรุป: ผลการศึกษาครั้งนี้แสดงให้เห็นถึงฤทธิ์ของสารสกัดตะไคร้ในการกำจัดอนุมูลอิสระโดยตรงและยังสามารถกระตุ้นการแสดงออกของเอนไซม์ ในระบบต้านอนุมูลอิสระในหนูได้