Antioxidant activity and protective effect of *Clitoria ternatea* flower extract on testicular damage induced by ketoconazole in rats[*]

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Abstract: Background: Ketoconazole (KET), an antifungal drug, has adverse effects on the male reproductive system. Pre-treatments with antioxidant plant against testicular damage induced by KET are required. The flowers of *Clitoria ternatea* (CT) are proven to have hepatoprotective potential. However, the protective effect on KET-induced testicular damage has not been reported. Objective: To investigate the protective effect of CT flower extracts with antioxidant activity on male reproductive parameters including sperm concentration, serum testosterone level, histopathology of the testis, and testicular tyrosine phosphorylation levels in rats induced with KET. Methods: The antioxidant activity of CT flower extracts was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) assays. Male rats were treated with CT flower extracts (10, 50, or 100 mg/kg BW) or distilled water via a gastric tube for 28 d (preventive period: Days 1–21) and induced by KET (100 mg/kg BW) via intraperitoneal injection for 7 d (induction period: Days 22–28). After the experiment, all animals were examined for the weights of the testis, epididymis plus vas deferens and seminal vesicle, serum testosterone levels, sperm concentration, histological structures and diameter of testis, and testicular tyrosine phosphorylation levels by immunoblotting. Results: The CT flower extracts had capabilities for DPPH scavenging and high reducing power. At 100 mg/kg BW, the extract had no toxic effects on the male reproductive system. Significantly, in CT+KET groups, CT flower extracts (50 and 100 mg/kg BW) alleviated the reduction of reproductive organ weight parameters, testosterone levels, and sperm concentration. In addition, CT flower extracts gave protection from testicular damage in KET-induced rats. Moreover, in the CT100+KET group, CT flower extracts significantly enhanced the expression of a testicular 50-kDa tyrosine phosphorylated protein compared with that of other groups. Conclusions: *C. ternatea* flower extracts possessing antioxidant activity are not harmful to the male reproductive system and can protect against testicular damage in KET-induced rats.

Key words: *Clitoria ternatea* (CT) flower extracts, Antioxidant activity, Ketoconazole (KET), Testicular damage,
            Testicular tyrosine phosphorylation
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1 Introduction

   Ketoconazole (KET) is an antifungal drug commonly used for treatment of advanced prostate cancer (Rodriguez and Acosta, 1995; Kinobe *et al.*,

2006). However, adverse effects of KET on the male reproductive system in both humans and animals have been documented. Significantly, KET was reported to decrease the weight of male reproductive organs, especially the testes, and to reduce epididymal sperm concentration and serum testosterone levels (Pon, 1987; Adams *et al.*, 1998; Marwaha and Maheshwari, 1999; Amin, 2008). Moreover, Amin (2008) has also reported damage to rat testes induced by KET intra-peritoneal (i.p.) injection (i.e., atrophy of seminifer-ous tubules, degeneration of Sertoli and germ cells, and sloughing of germ cells). Interestingly, such pa-thological changes in KET-treated rat testes could be prevented by pretreatment and co-administration with an antioxidant plant extract (Amin, 2008). Recently, there have been studies searching for non-toxic me-dicinal plants with antioxidant activity for use in the prevention of testicular damage. Our recent study suggested that an increase in phosphorylation levels of testicular proteins caused by administration of antioxidant plant extracts could be ascribed to tes-tosterone production and spermatogenesis (Iamsaard *et al.*, 2013).

Clitoria ternatea (CT) Linn. (called "Unchan" in Thailand) is a climbing vine bearing purple or white flowers. Every part of the CT vine has been syste-matically analyzed and revealed to have various me-dicinal properties (Patil and Patil, 2011). In Thailand, the purple flowers are used for many medical pur-poses and it is believed that they have antoxidant capacity. Mukherjee *et al.* (2008) reviewed the use of CT in Ayurvedic medicine and found that CT has been commonly used in anti-stress, anti-depressant, anti-microbial, and anti-inflammatory treatments. Moreover, CT has been shown to have significant hepatoprotective effects on drug-induced liver dam-age (Nithianantham *et al.*, 2011). To our knowledge, the potential protective effects of CT flower extracts against KET-induced testicular damage in rats have not been reported. Therefore, the aim of this study was to evaluate the antioxidant activities and potential protective effects of aqueous CT flower extracts on KET-induced testicular damage in rats.

2 Materials and methods

2.1 Chemicals and reagents

KET (200 mg/tablet; KETOHIM) was pur-chased from a local drug store. Folin-Ciocalteau's reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), acetate buffer, 2,4,6-tripyridyl-*s*-triazine (TPTZ), and ferric chloride ($FeCl_3$) were purchased from Sigma-Aldrich Co., Ltd. (Thailand). Ascorbic acid and other chemicals were of analytical grade.

2.2 Plant collection and extraction

The fresh purple CT flowers were collected from Khon Kaen Province, Thailand, in June to July 2012. Species identification was confirmed by Prof. Pranom CHANTARANOTHAI, a plant taxonomist, from Khon Kaen University (KKU), Thailand. Voucher specimens of CT were kept in the KKU Herbarium (No. *Jaturon Burawat* 01 [KKU]). In the extraction process, the CT flowers were washed with distilled water and air dried for two days. The dried flowers were crushed with a hammer mill crusher (AEG IP54 Lbi 07, Germany) for 30 min. Then, the crushed sample (3 kg) was extracted with 10 L of distilled water and boiled at 95–100 °C for 30 min. The soluble extract was filtered through nylon. The filtrate was dried using lyophylization. The extraction yield of aqueous extract from CT flowers was 3.97 g.

2.3 DPPH radical scavenging assay and deter-mination of half maximum inhibition concentra-tion (IC_{50})

The radical scavenging activity of the CT aqueous extract was determined using DPPH assay (Brand-Williams *et al.*, 1995). Briefly, five concentrations of the CT flower extracts were prepared from a concen-trated extract stock solution to provide a concentra-tion plot. Each concentration of CT flower extracts (2 ml) was mixed with 2 ml DPPH in 0.004% (v/v) methanol and incubated at room temperature in dark for 30 min. The absorbance was recorded at 517 nm using an ultraviolet-visible (UV/VIS) spectrophoto-meter (Jasco V530, Japan). Ascorbic acid (1, 3, 5, 8, and 14 μg/ml) was used as the positive standard. All samples were measured in triplicate. The scavenging activity of the CT flower extracts or standard was calculated as the percent inhibition of DPPH radical scavenging activity using a standard formula as (absorbance of control−absorbance of sample)×100%/ absorbance of control. Data were expressed as the IC_{50} calculated from the point of 50% inhibition against the concentration (μg/ml) plot.

2.4 Ferric reducing antioxidant power (FRAP) assay

Reducing power capacity was determined using the FRAP assay (Benzie and Strain, 1996). Briefly, 0.1 ml of five different concentrations of CT flower extracts were mixed with 3 ml of FRAP reagent (containing 300 mmol/L acetate buffer, 10 mmol/L TPTZ, and 20 mmol/L $FeCl_3$, in a volume ratio of 10:1:1). The CT mixture was incubated in dark for 20 min and the absorbance was recorded at 593 nm using a UV/VIS spectrophotometer. For standard preparation, six concentrations of ascorbic acid (5.76, 9.60, 17.28, 24.00, 32.00, and 48.00 µg/ml) were plotted to determine its reducing power.

2.5 Animals and treatment regime

Male Sprague-Dawley rats (180–200 g) were purchased from the National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand. The study was approved by the Animal Ethics Committee of KKU, based on the Ethics of Animal Experimentation of the National Research Council of Thailand (ref. No. 0514.1.12.2/93). Thirty-six rats were divided into six groups and each group (*n*=6) was treated as shown in Table 1.

2.6 Histopathological examination of the testes

On the day after termination of the CT-KET co-administration, all rats were euthanized by cervical dislocation and sacrificed to collect the male reproductive organs (i.e., testis, epididymis plus vas deferens, and seminal vesicle). These organs were subsequently removed of fats and weighed. To examine the testicular damage, testes were fixed in 10% (v/v) formalin in phosphate buffered saline (PBS)

(pH 7.4), embedded in paraffin, sectioned at 4–6 µm thickness, and stained with hematoxylin-eosin (Iamsaard *et al.*, 2013). All photographs were captured by a Nikon light ECLIPSE E200 microscope equipped with a DXM1200 digital camera. ImageJ was used to measure and calculate approximate average diameters of seminiferous tubules in four different axes (50 tubules per animal).

2.7 Plasma testosterone assay

At the end of the experiment, all animals were sacrificed to expose the left ventricle of the heart. Blood was collected by puncture of the left ventricular chamber using 1 ml of heparin to prevent blood clotting. The blood was centrifuged at 5000 r/min at 4 °C for 10 min to separate the plasma serum from blood cells. The plasma testosterone concentration was assayed by enzymatic immunoassay at the Radiology Unit, Srinagarind Hospital, Faculty of Medicine, KKU, Thailand.

2.8 Epididymal sperm concentration

Mature sperm were collected from the left epididymis and vas deferens. Epididymal sperm fluid was dipped and re-suspended in 1 ml PBS (37 °C, pH 7.4) and centrifuged (500*g*, 37 °C, 5 min) to wash and separate the mature sperm pellet from its fluid. To analyze the epididymal sperm concentration, the sperm pellets were re-suspended with 1 ml 0.3% (v/v) bovine serum albumin (BSA)-KSOM (potassium-enriched simplex optimized medium; EmbryoMax KSOM Powdered Mouse Embryo Culture Medium; Millipore catalogue No. R-MR-020P-5D). In triplicate preparations, the sperm solutions (1:20 dilution) were used to count mature sperm using a Neubauer counting chamber and to calculate their concentration (Iamsaard *et al.*, 2013).

Table 1 Treatment on each group of rats

Group	Treatment	
	Days 1–28 (via a gastric tube)	Days 22–28 (intraperitoneal injection)
Negative control	Distilled water, 1 ml	Saline, 0.5 ml
KET (positive control)	Distilled water, 1 ml	KET (100 mg/kg BW), 0.5 ml
CT100	CT flower extracts (100 mg/kg BW), 1 ml	Saline, 0.5 ml
CT10+KET	CT flower extracts (10 mg/kg BW), 1 ml	KET (100 mg/kg BW), 0.5 ml
CT50+KET	CT flower extracts (50 mg/kg BW), 1 ml	KET (100 mg/kg BW), 0.5 ml
CT100+KET	CT flower extracts (100 mg/kg BW), 1 ml	KET (100 mg/kg BW), 0.5 ml

BW: body weight; KET: ketoconazole (saline-dissolved). The KET group (positive control) is designed based on Amin (2008). Days 1–21 are the preventive period and Days 22–28 are the induction period

2.9 Western blot analysis for testicular tyrosine protein phosphorylation

As described by Iamsaard *et al.* (2013), briefly, the left testis was homogenized with RIPA buffer (Cell Signaling Technology, Inc., USA) containing a cocktail of protease inhibitors. The testicular homogenate was then centrifuged at 12 000 r/mim for 10 min to collect testicular lysate. The total protein concentration of the lysate was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., USA). Total proteins (60 µg) collected from triplicate samples were separated by 10% (0.1 g/ml) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto nitrocellulose membranes to detect the intensity of tyrosine phosphorylation using the 4G10 primary antibody (1:3000; Millipore Co., USA). For standard samples, BSA (AMRESCO®, USA) was used as the negative control and epidermal growth factors (EGFs) (Millipore Co., USA) as the positive control. In the detection of phosphotyrosine proteins, the enhanced chemiluminescence (ECL) substrate was applied before visualization under gel doct 4 (ImageQuant 400, GH Healthcare, USA). To quantify the levels of phosphorylation, the ImageJ program was used to analyze the relative intensity of phosphorylated protein bands among groups.

2.10 Statistical analysis

One-way analysis of variance (ANOVA) and *t*-test were used to examine the significance of differences among sets of data, and between pairs of data points using Sigma Stat program (Version 3.1.1). All quantitative results were expressed as mean±standard deviation (SD).

3 Results

3.1 Antioxidant activity of CT flower extracts

In the DPPH scavenging assay, the results showed that CT flower extracts possessed a concentration-response relationship in DPPH scavenging activity, using ascorbic acid as a positive control. Compared with the IC_{50} of ascorbic acid [(5.34±0.09) µg/ml], the IC_{50} of CT was (84.15± 1.50) µg/ml ($y=0.0686x+45.017$, $R^2=0.98$). For the FRAP assay calibrated with standard ascorbic acid ($y=0.007x+0.3769$, $R^2=0.9802$), the reducing power of the CT flower extracts was (0.33±0.01) mmol/mg ascorbic equivalent. These results demonstrate that the CT flower extracts used for preventive experiments in this study possessed antioxidant capacity.

3.2 Weights of male reproductive organs

After treatment for 28 consecutive days, the body weights of control and experimental groups were not different ($P>0.05$). In contrast, all reproductive organs in KET-treated rats weighed significantly less than those of the controls ($P<0.05$; Table 2). We found that the CT flower extracts did not affect the weights of reproductive organs. In addition, all doses of CT flower extracts could prevent the weight loss of the testis in rats induced with KET (Table 2). Moreover, 50 and 100 mg/kg BW CT flower extracts significantly improved the weights of the epididymis plus vas deferens in KET-induced rats ($P<0.05$). Only 100 mg/kg BW CT flower extracts prevented the weight loss of the seminal vesicle in the KET group ($P<0.05$).

Table 2 Values of analyzed parameters on the reproductive system of control and experimental rats

Group	Weight (g)			Testicular diameter (µm)	Serum testosterone level (ng/ml)	Sperm concentration ($\times 10^6$ cells/ml)
	Testis	Epididymis plus vas deferens	Seminal vesicle			
Control	3.77±0.19	0.66±0.04	1.24±0.14	304.00±7.30	1.58±0.10	33.46±4.30
KET	3.19±0.30*	0.53±0.06*	0.85±0.14*	265.25±1.77*	0.99±0.16*	20.67±2.01*
CT100	3.69±0.11	0.63±0.04	1.17±0.19	291.50±2.44	1.54±0.23	28.67±3.84
CT10+KET	3.75±0.08	0.56±0.12*	0.86±0.23*	269.50±7.97*	1.47±0.25	21.17±2.70*
CT50+KET	3.76±0.02	0.63±0.06	0.91±0.19*	300.75±6.26	1.73±0.16	31.00±3.45
CT100+KET	3.70±0.23	0.65±0.06	1.10±0.15	303.25±5.78	1.41±0.23	29.46±4.07

* Significant differences ($P<0.05$) as compared with the control group. Data are expressed as mean±SD ($n=6$).

3.3 Protective effects of CT flower extracts on KET-induced testicular damage

The effects of CT flower extracts on testicular damage were examined by observing histopathological structures (Fig. 1). The result showed shrunken seminiferous tubules in the KET group compared with the controls (Figs. 1a and 1b). This finding was corroborated by their diameters (Table 2). In the KET group, the seminiferous tubules had very minor pathological effects (in fewer than 10% of tubules) including sloughing of germ cells, early cell
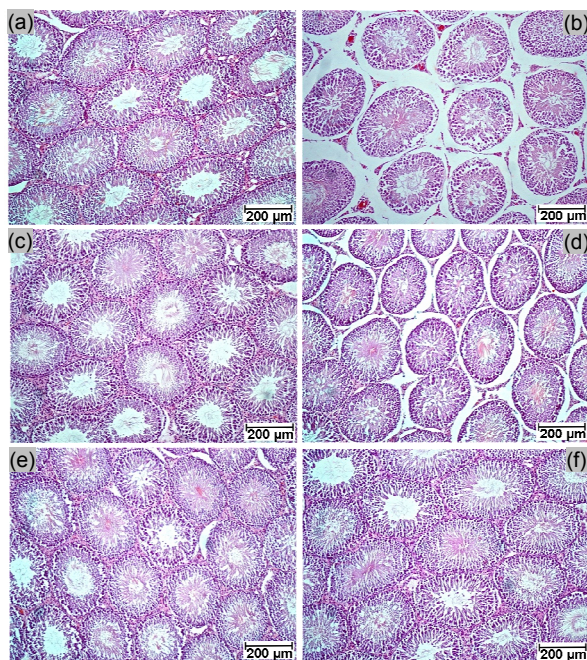

Fig. 1 Photographs showing rat testicular histology (H&E) from a representative section
(a) Control; (b) KET; (c) CT100; (d) CT10+KET; (e) CT50+KET; (f) CT100+KET

degeneration with giant cells, and late cell degeneration (Fig. 2). Some shrunken tubules with significantly reduced diameters (Table 2) and other abnormal seminiferous tubules could be found in the CT10+KET group (Fig. 1d). However, no histopathology or reduction in seminiferous tubule diameter was observed only in rats of the CT100, CT50+KET, and CT100+KET groups (Table 2; Figs. 1c, 1e, and 1f).

3.4 Effects of CT flower extracts on serum testosterone levels and epididymal sperm concentrations

Table 2 shows that only the KET and CT10+KET groups had a significant decrease in serum testosterone levels compared with the controls. In contrast, 100 mg/kg BW CT flower extracts did not affect such levels, whereas 50 and 100 mg/kg BW CT flower extracts increased the testosterone hormone in KET-induced rats (Table 2). Similarly, not only the CT group but also the CT50+KET and CT100+KET groups showed no differences in epididymal sperm concentration compared with the controls (Table 2).

3.5 Effects of CT flower extracts on testicular tyrosine protein phosphorylation

Following immuno-Western blotting, four major phosphorylated proteins (50, 55, 60, and 65 kDa) of rat testicular lysate were clearly detected in both control and experimental groups. For densitometry analyses, we found that the relative intensity of the 55, 60, and 65 kDa phosphorylated proteins was not significantly different (data not shown). Interestingly, a testicular 50-kDa phosphorylated protein was especially intense in the CT100+KET group compared with other groups (Fig. 3).
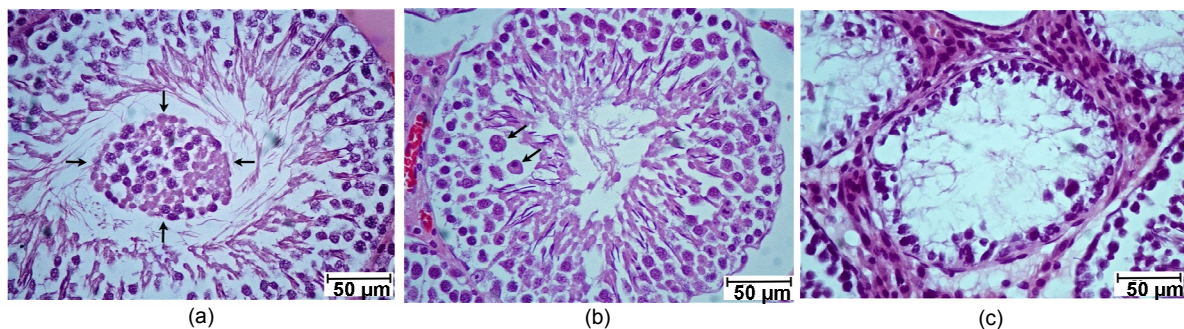

Fig. 2 Photographs showing histopathogy (H&E) of seminiferous tubules observed in KET-induced rats from representative sections
(a) Sloughing of germ cells (arrows); (b) Early cell degeneration with giant cell (arrows); (c) Late cell degeneration
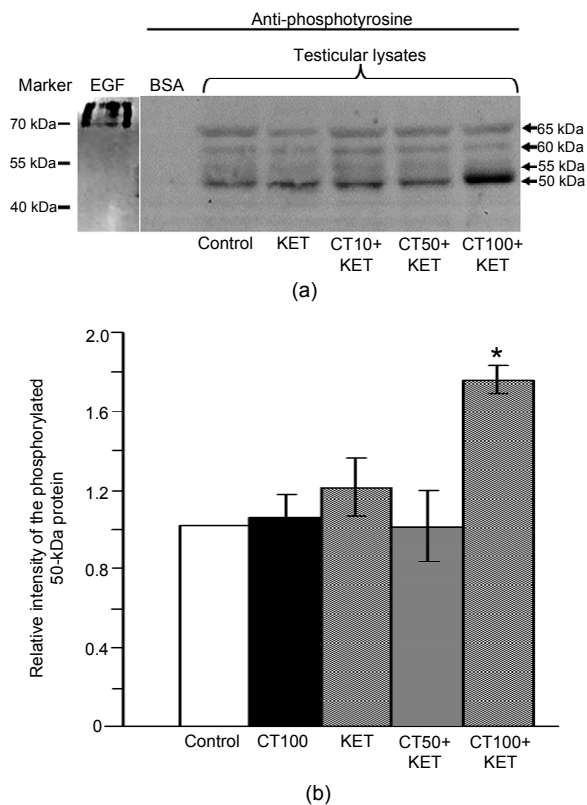
Fig. 3 **Immuno-Western blot analysis for tyrosine protein phosphorylation levels in testicular lysates (a) and relative intensity of the phosphorylated 50-kDa protein of testicular lysate (b)**

In (a), 60 μg protein is for each lane of control, KET, and CT+KET groups. Bovine serum albumin (BSA) and epidermal growth factor (EGF)-like growth factor were used as negative and positive controls for phosphotyrosine antibody, respectively. In (b), data are expressed as mean±SD (n=3). * P<0.05 vs. control

4 Discussion

The results in Table 2 suggest that high concentrations of CT flower extracts (100 mg/kg BW) were not harmful to male reproductive parameters. By FRAP assay, the antioxidant activity of the CT aqueous extract in this study was comparable to the ferric reducing power of ascorbic acid. In addition, a group of polyacylated anthocyanins in this blue flower is already identified and crystallized (Honda and Saito, 2002; Hiromoto *et al.*, 2013). Consistent with previous reports (Adams *et al.*, 1998; Amin, 2008), in the present study, KET affected reproductive organ weight, sperm concentration, and serum testosterone levels (Table 2). Moreover, CT flower extracts have been shown to exhibit high cytotoxic activity against the breast cancer cells (Akter *et al.*, 2014). The non-toxicity of the CT flower extracts and testicular toxicity of KET induction were further confirmed by histological observations in the testis tissues (Figs. 1b and 2). However, the testicular pathology observed in Fig. 2, which was similar to that described by Amin (2008), was not quantified among groups because it was found in less than 10% of rats in the KET group and was rarely found in the low-dose group of the CT flower extracts. It seemed that testicular damage in our study was less severe than that in the previous study (Amin, 2008). This may caused by that KET in our study was prepared from a tablet, while KET (Nizoral) in Amin (2008) was from a stock solution. In CT50+KET and CT100+KET groups, the pre-treatment of CT followed by co-administration with CT and KET significantly maintained male reproductive organ weights, except for that of the seminal vesicle in the CT50+KET group (Table 2). Similarly, CT+KET rats retained normal serum testosterone levels, sperm concentration, seminiferous tubule diameters, and testicular architecture compared with the control group (Table 2; Fig. 1). These results clearly demonstrate a potential protective effect of CT flower extracts on testicular damage of rats induced by KET.

It has been shown that KET can cause hepatotoxicity and testicular toxicity which was revealed by histopathology and biochemistry results (Amin and Hamza, 2005; Amin, 2008). In rats, KET-induced testicular damage was demonstrated to be associated with alterations to testicular levels of malondialdehyde, glutathaione, catalase, and superoxide dismutase (Amin, 2008). In addition, this oxidative stress damage and antioxidant depletion in rat testis were shown to be remarkably protected by an antioxidant plant extract (Amin, 2008). Unfortunately, the extract in the present study has not shown such associations, but it was assumed to have the same action since it had high antioxidant activity. Increasingly, post-translational phosphorylated tyrosine proteins are being shown to be involved in many biological processes including sperm production, capacitation, and acrosome reaction (Morales *et al.*, 2007; Bailey, 2010; Fardilha *et al.*, 2011; Yamashita *et al.*, 2011). For the first time, we attempted to explain changes in

testicular tyrosine protein phosphorylation levels between control and experimental groups induced with KET (Fig. 3). However, the four major tyrosine-phosphorylated proteins present in the testicular lysate were not different from those of the control (Fig. 3), suggesting that testicular damage by KET induction was not associated with tyrosine phosphorylated proteins. In this study, the blot was cropped to demonstrate only testicular proteins against tyrosine phosphorylated antibody. Interestingly, a 50-kDa tyrosine phosphorylated protein showed high expression only in the CT100+KET group (Figs. 3a and 3b), but not in the CT100 or lower-dose CT+KET groups. In contrast, the relative intensities of the other three tyrosine phosphorylated proteins (65, 60, and 55 kDa; Table 2) were not significantly different from each other or the control (data not shown). The possible role of over-expression of a 50-kDa tyrosine phosphorylated protein in KET-induced testicular damage in rats reduced by CT flower extracts is still unclear. Similar to a previous report, this could be partially explained if this 50-kDa protein is responsible for testosterone production (Iamsaard *et al.*, 2013).

In conclusion, CT extract has antioxidant activity, which is harmless and can reduce KET-induced testicular damage in rats.

Compliance with ethics guidelines

Sitthichai IAMSAARD, Jaturon BURAWAT, Pipatpong KANLA, Supatcharee ARUN, Wannisa SUKHORUM, Bungorn SRIPANIDKULCHAI, Nongnut UABUNDIT, Jintanaporn WATTATHORN, Wiphawi HIPKAEO, Duriya FONGMOON, and Hisatake KONDO declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要：

本文题目：蝶豆花提取物的抗氧化活性及其对酮康唑诱导大鼠睾丸损伤的保护作用

Antioxidant activity and protective effect of *Clitoria ternatea* flower extract on testicular damage induced by ketoconazole in rats

研究目的：研究蝶豆花提取物的抗氧化活性及其对酮康唑（KET）诱导雄性大鼠睾丸损伤的保护作用。

创新要点：对蝶豆花提取物保护酮康唑引起的雄性动物睾丸损伤进行研究，为减轻抗真菌药物酮康唑的生殖毒性提供理论依据和解决途径。

研究方法：采用 2,2-二苯基-1-苦肼基自由基（DPPH）分析法和亚铁还原能力实验法（FRAP）来测定蝶豆花提取物的抗氧化活性。在为期 28 天的动物试验中，前 21 天为预防期，第 21 至 28 天为 KET 诱导期。整个试验期中，对雄性大鼠进行蝶豆花提取物（0、50、100 mg/kg BW）灌胃饲养；在诱导期，同时腹腔注射 KET (100 mg/kg BW)。试验结束后，测定睾丸、附睾和输精管以及精囊的重量、血清睾酮水平、精子浓度、组织结构、睾丸的直径和睾丸酪氨酸磷酸化水平。

重要结论：蝶豆花提取物具有清除 DPPH 自由基能力和较高的还原能力，其在 100 mg/kg BW 下对雄性大鼠生殖系统无毒。50、100 mg/kg BW 蝶豆花提取物能改善 KET 引起的生殖器官重量下降、血清睾酮水平和精子浓度，降低 KET 引起的睾丸损伤。与其他组相比，100 mg/kg BW 蝶豆花提取物能显著提高睾丸中 50-kDa 的酪氨酸磷酸化蛋白的表达。由此可见，具有抗氧化活性的蝶豆花提取物不会损伤雄性生殖系统，而且具有保护 KET 诱导大鼠睾丸损伤的作用。

关键词组：蝶豆花提取物；抗氧化活性；酮康唑（KET）；睾丸损伤；酪氨酸磷酸化