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Antioxidant Capacity and Toxicity Screening of *Cinnomomum iners* Standardized Leaves Methanolic Extract

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Abstract: This study investigated the antioxidant capacity and safety parameters (toxicity) of *Cinnomomum iners* standardized leaves methanolic extract (CSLE) to provide information regarding long term usage of this plant. CSLE exhibited high total phenolic (211.94 ± 12.04 mg GAE g^{-1} plant material) and flavonoid (13.38 ± 0.08 mg CE g^{-1} plant material) contents. The antioxidant activity of CSLE was evaluated using DPPH, H_2O_2 and reducing power assay. CSLE showed potent antioxidant activity with IC₅₀ value of 0.5 ± 0.26 mg mL^{-1} and 1.00 ± 0.31 mg mL^{-1} in DPPH assay and H_2O_2 study, respectively. The electron donating capability (reducing power) of CSLE was found to be higher than standard antioxidant (vitamin E). The toxicity screening of CSLE was conducted using brine shrimp assay, acute toxicity screening and histopathologic study. An acute toxicity study was carried out by using OECD guideline 423. The LC₅₀ and LD₅₀ values of CSLE were found to be 2.59 ± 0.3 mg mL^{-1} and > 5000 mg kg^{-1} , respectively. The organ body weight of the CSLE treated mice shows no considerable difference with control group mice. This shows that CSLE does not affect the weight of the mice. No mortality or significant signs of acute toxicity was observed during the 14 days observation period. On the day 14, all the mice were dissected and organ as heart, liver, kidney, spleen and lung were withdrawn for the calculation of organ body index. The organ body index obtained showed that no significant difference with control. Withdrawn organ was subjected to histopathological study. Histopathological analysis of organs did not show any pathological changes. The results obtained in present study indicate that CSLE is very good antioxidant source with high margin of safety.

Key words: Total phenolic, flavonoid, reducing power, histopathologic study

INTRODUCTION

For centuries, medicinal plants are the basis for the treatment of various diseases (Riditid *et al.*, 2008). Nearly 80% of people living in developing countries still depend on plant based traditional medicine for their primary health care and almost three fourths of the herbal drugs used worldwide were derived from medicinal plants (Verma and Singh, 2008). Natural antioxidants present in the plants are closely related with their ability to treat various diseases. Hence, antioxidant assays are widely used for assessing medicinal properties of plant material (Ozsoy *et al.*, 2008). However, numerous herbal products and herb derived-products could lead to organ damage after consumption. They might be toxic, mutagenic and carcinogenic (Schimmer *et al.*, 1994, 1988). Therefore, it is very important to confirm the safety of the herbal product.

Cinnomomum iners is a genus of evergreen trees and shrubs belonging to the family, Lauraceae. According to Agroforestry Tree Database *Cinnomomum iners* is

commonly found in India, Burma (Myanmar), Indo-China, Thailand, Peninsular Malaysia, Sumatra, Java, Borneo, Sulawesi and the Southern Philippines. It is a medium-sized evergreen tree found throughout lowland and hill forest of Malaysia. It is locally known as kayu manis hutan, medang kemangi and teja with respect to the aroma of crushed leaves. The bitter taste of this plant is due to the presence of terpenes. With respect to the bitter characteristic of this plant, it is widely practiced as traditional medicine to relieve fever, for digestive system and appetite problem (Pengelly, 2004). In addition, this plant has been used to relieve headache, breathing problem and cough. The major bioactive compounds of this plant are saponin, terpene, cinnamic aldehyde and eugenol (Choi *et al.*, 2003). The ethnobotanical reports offer information on the medicinal properties of *Cinnomomum iners* root extract that include details on their antiparasmodial activity and cytotoxicity (Omar *et al.*, 2007). Furthermore, an amylase inhibitor property of this plant has been revealed (Iida *et al.*, 1997). Researchers from India reported that oil obtained from stem bark of

Cinnomomum iners contain 1, 8-cineole as a major component that possess antinociceptive and anti-inflammatory activity (Baruah *et al.*, 2001). The antioxidant activity of essential oil of *Cinnomomum iners* has also been reported (Phutdhawong *et al.*, 2007). Recently, the analgesic properties of this plant leaves have been published by our research team (Mustaffa *et al.*, 2010). In this context, this work was undertaken to determine the antioxidant capacity and safety parameters of *Cinnomomum iners* standardized leaves methanolic extract. The results of this investigation may be useful to provide information regarding long term usage of this plant.

MATERIALS AND METHODS

Chemicals: All reagents were used of analytical grade. Propylene glycol (propane-1, 2-diol), Folin-Ciocalteu, sodium carbonate (Na_2CO_3), polysorbate-80 (Tween 80), aluminium chloride (AlCl_3), 2,2-Diphenyl-2-picryl-hydrazyl (DPPH), potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$], ferric chloride (FeCl_3) and dimethyl sulphoxide (DMSO) were purchased from Fisher Scientific (Loughborough, UK). Diethyl ether, vitamin C (ascorbic acid), vitamin E (α -tocopherol), hydrogen peroxide (H_2O_2) and trichloroacetic acid (TCA) were obtained from Merck (Darmstadt, Germany). Gallic acid and catechin were purchased from Sigma-Aldrich (St Louis, MO, United States). Sodium nitrate (NaNO_3) and sodium hydroxide (NaOH) were obtained from Fluka, United States.

Plant material: *Cinnomomum iners* leaves were collected at USM (Universiti Sains Malaysia). The plant leaves were washed with water to remove dirt prior to the drying process. The leaves then crushed into the fine powder. An initial quality evaluation of the powdered material was carried out to validate its authenticity and also to ensure quality by using techniques as adopted from WHO guidelines on quality control of herbal medicines (WHO, 2005). The authenticity work was carried out by botanist from School of Biological Sciences, Universiti Sains Malaysia where the plant material was deposited. The voucher specimen number is 11014. This project was carried out in research laboratory at Centre for Drug Research, Universiti Sains Malaysia, Malaysia from May 2009 to August 2009.

Methodology

Extraction: Powdered dried leaves (100 g) of the plant were macerated in methanol (500 mL) for 3 days. The leaves extract was filtered and concentrated under reduced pressure at 40°C in a rotary evaporator. The

concentrated extract obtained was placed in the oven for 3 days at 40°C to remove the remaining methanol. The dried extract was standardized according to validated GC-MS method (Mustaffa *et al.*, 2010). *Cinnomomum iners* standardized leaves methanolic extract (CSLE) was found to contain 0.048 mg (4.8%) of beta-caryophyllene in 1 mg of plant leaves extract. CSLE was kept at 4°C and dissolved in methanol for the antioxidant activity or cosolvent (propylene glycol-Tween 80-water = 4:1:4) for the toxicity studies. For the brine shrimp toxicity studies CSLE was dissolved in 5% DMSO.

Determination of total phenolic content: Total phenols estimation was determined according to the method of Wolfe *et al.* (2003). CSLE (125 μL) was added to Folin-Ciocalteu reagent (500 μL) prior to vortex-mixing of the mixture. Then the mixture was incubated for 6 min at 20°C in water bath. Subsequently Na_2CO_3 solution [1.25 mL, 7% (w/v)] and 3 mL of distilled water were added to the mixture. The mixture was further incubated for 90 min at room temperature. Finally, the absorbance was measured at 760 nm by using UV-160A Shimadzu spectrophotometer. The mean ($\pm\text{SD}$) results of triplicate analyses were expressed as mg gallic acid equivalents per g plant material (GAE g^{-1}).

Determination of total flavonoid content: Total flavonoid content was determined by using method of Sakanaka *et al.* (2005). CSLE (250 μL) was added with distilled water (1.25 mL) and NaNO_2 [75 μL , 5% (w/v)] prior to the incubation at room temperature for 6 min. Subsequently, AlCl_3 solution [150 μL , 10% (w/v)] was added and the mixture was further incubated for 5 min before the addition of NaOH (0.5 mL, 1 M). Thereafter, distilled water (275 μL) was added and vortex-mixed. Finally, the absorbance was measured at 510 nm in spectrophotometer and the measurement was compared to a standard curve of catechin and expressed as mg catechin equivalents per g plant material (CE g^{-1}). The data represent the mean of triplicate analyses.

Antioxidant screening

2,2-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging method: DPPH scavenging activity of CSLE extract was determined by the modified method of Brand-Williams *et al.* (1995). CSLE (77 μL , $0.2\text{--}1.4\text{ mg mL}^{-1}$) was added to DPPH ($6 \times 10^{-5}\text{ mol L}^{-1}$) and vortex-mixed. Thereafter, the mixture was allowed to stand in the dark for 15 min at room temperature. Subsequently, the absorbance was measured at 515 nm using spectrophotometer and DPPH scavenging activity was calculated by using the following formula:

$$\text{Inhibition (\%)} = \frac{A_c - A_t}{A_c} \times 100$$

where, A_c is the absorption of control ($t = 0$) and A_t is the absorption of tested extract or standard solution ($t = 15$ min). DPPH solution without plant extract or standard served as the control. Vitamin C was used as the standard antioxidant.

Hydrogen peroxide (H_2O_2) decomposition: H_2O_2 scavenging activity of CSLE extract was determined according to the method of Ruch *et al.* (1989). In brief, CSLE (2.0 mL, 0.2-1.4 mg mL⁻¹) or standard was added to H_2O_2 (1.2 mL, 40 mM), prepared in phosphate buffer (pH 7.4). The mixture was allowed to stand for 10 min at room temperature. Then, the absorbance was recorded immediately at 230 nm using spectrophotometer. The scavenging activity was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{A_c - A_t}{A_c} \times 100$$

where, A_c is the absorbance of the control and A_t is the absorbance of the extract. H_2O_2 solution without the plant extract or the standard served as the control and vitamin C was used as the positive control.

Reducing power assay: Reducing power of CSLE was determined according to the method described by Oyaizu (1986). In brief, CSLE (0.5 mL, 0.5-2.5 mg mL⁻¹) or standard with different concentration was added with sodium phosphate buffer (0.5 mL, 0.2 M, pH 6.6) and $K_3Fe(CN)_6$ solution [0.5 mL, 1% w/v], then the tubes were vortex-mixed for 15 s. Next, the mixture was allowed to stand in water bath at 50°C for 20 min. Thereafter, TCA [0.5 mL, 10% (w/v)] was added and the mixture was centrifuged at 3000 rpm for 10 min. Supernatant layer solution (0.5 mL) was added with distilled water (0.5 mL) and $FeCl_3$ [0.1 mL of 0.1% (w/v)]. Finally, the formation of blue coloured complex was measured at 700 nm in spectrophotometer. Vitamin E was used as standard antioxidant. All samples were prepared in triplicate. Increased absorbance of the reaction mixture indicates increased reducing power.

Toxicity studies

Brine shrimp toxicity assessment: Brine shrimp (*Artemia salina*) lethality bioassay technique referring to the modified method of Krishnaraju *et al.* (2005) was applied for the determination of general toxicity of plant extract. In brief, brine shrimps eggs were hatched in a glass beaker filled with saline on the day prior to the experiment. Stock solution of the CSLE at 1 g mL⁻¹ was prepared by

dissolving the extract in 5% DMSO solution. From the stock solution, different concentrations of CSLE (0.5-6 mg mL⁻¹) were added into test tubes containing artificial sea water. The final volume of each test tube was 5 mL. Subsequently, 10 newly hatched shrimp was added to each test tube and incubated at room temperature for 24 h. The percentage of mortality was calculated after 24 h. Lethality concentration of extract that kills 50% of the shrimps (LC_{50}) was calculated. The same procedure using potassium dichromate served as positive control. All the experiments were performed in triplicate.

Acute toxicity evaluation: An acute toxicity study was carried out by using OECD (2001b) guideline 423. CSLE was administered orally to Swiss albino mice at doses of 300, 2000 and 5000 mg kg⁻¹. Cosolvent was used as control. The behavioural changes (abdominal constriction, hyperactivity, sedation, grooming), mortality and body weight were observed for 14 days. The animal body weight was compared with control group at day 1, 7 and 14. On the day 14, necropsy was carried out on the animal under diethyl ether anaesthesia and organs such as liver, kidney, lung, heart and spleen were withdrawn for the histological procedure. The organ body index of tested groups was calculated and compared with control group:

$$\text{Organ body index (\%)} = \frac{\text{Organ weight (g)}}{\text{Body weight (g)}} \times 100$$

Histopathological studies: After the animals were sacrificed, postmortem examination was performed according to Tsung *et al.* (2005). All the organs were slice into small pieces and preserved with 4% formalin before further treatment. Then the organs were dehydrated using methanol followed by waxing and clearing process. Thereafter, the tissues were embedded in paraffin and processed into 4-5 μ m thick sections and fixed in slides. Finally, samples were stained using hematoxylin-eosin and assessed for any tissue damage under photomicroscope.

Statistical analysis: Data were expressed as mean \pm SEM for acute toxicity studies and Mean \pm SD for antioxidant assay and brine shrimp lethality evaluation. Data were analyzed by SPSS version 12.0.1 software programme. Statistically significant difference between groups was calculated by the application of Analysis of Variance (ANOVA) followed by Dunnett's test. Tukey test was used for independent pair-wise comparison between two groups. A difference was considered significant at $p < 0.05$. IC_{50} and LC_{50} values were calculated from the linear regression line.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content: The Folin-Ciocalteu reagent was used to estimate total phenols present in the extract and the value was expressed as Gallic Acid Equivalents (GAE). The total phenolic content was 211.94 ± 0.04 mg GAE per g plant material. The flavonoid content of CSLE was 13.38 ± 0.08 mg CE per g plant material.

Antioxidant screening

DPPH free radical scavenging method: The free radical scavenging activity of CSLE has been evaluated by using the DPPH free radical. DPPH assay has been widely used for the screening of scavenging activity of antioxidant because it is a rapid and sensitive method to detect hydrogen donating ability of plant extracts at low concentrations (Baumann *et al.*, 1979). The antioxidant quality of an extract is determined by the IC_{50} value. A low IC_{50} value indicates strong antioxidant activity. From the result obtained (Table 1), CSLE exhibited IC_{50} value of 0.5 ± 0.26 mg mL^{-1} . The IC_{50} value of natural antioxidants, vitamin C was 6.12 ± 0.14 $\mu g mL^{-1}$. Although the IC_{50} value of CSLE was greater than reference antioxidant (vitamin C) it was comparable to other plant with good antioxidant activity (*Centella asiatica*, 0.2 mg mL^{-1} ; *Pisonia alba*, 0.18 mg mL^{-1} ; *Orthosiphon stamineus*, 0.21 mg mL^{-1} ; *Mentha arvensis*, 0.22; *Ocimum basilicum*, 0.19) (Subashree *et al.*, 2009; Zuraini *et al.*, 2008). This indicates that CSLE have bioactive constituents which act as hydrogen donor to stabilize free radical.

Hydrogen peroxide (H_2O_2) decomposition: Hydrogen peroxide can deactivate a few cellular enzymes in the body by direct oxidation of essential thiol (-SH) groups. The toxic effect of hydrogen peroxide is mainly caused by its reaction with Fe^{2+} or Cu^{2+} ions which are responsible for forming harmful hydroxyl radicals which may cause oxidative DNA damage (Miller *et al.*, 1993). CSLE was capable of scavenging hydrogen peroxide which is shown in Table 1 (IC_{50} of 1.00 ± 0.31 mg mL^{-1}). This suggests that CSLE is capable in accelerating the conversion of H_2O_2 to H_2O to prevent accumulation of hydrogen peroxide in body. The H_2O_2 scavenging activity of vitamin C was found to be 22.00 ± 0.2 $\mu g mL^{-1}$ (Table 1).

Reducing power assay: The reducing power of a compound generally depends on its ability in donating electrons. The reducing power of CSLE was monitored by its reduction capability of Fe^{3+} /ferricyanide complex to a ferrous form (Zou *et al.*, 2004). As illustrated in Fig. 1,

Table 1: IC_{50} values of *Cinnomomum iners* standardized leaves methanolic extract (CSLE) and standard antioxidant (vitamin C)

Results	$IC_{50} \pm SD$	
	DPPH	H_2O_2
CSLE (mg mL^{-1})	0.50 ± 0.04	1.00 ± 0.31
Vitamin C ($\mu g mL^{-1}$)	6.12 ± 0.07	22.00 ± 1.20

Values are expressed as Mean \pm SD, n = 3

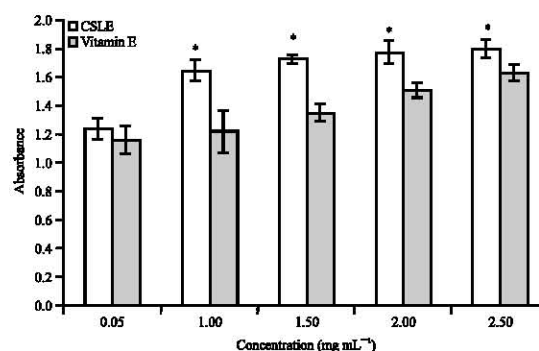


Fig. 1: Reducing power of *Cinnomomum iners* standardized leaves methanolic extract (CSLE) compared to vitamin E. Data presented as means \pm standard deviation of three replicate. * $p < 0.05$, significant increase from vitamin E

CSLE showed a significantly higher reducing power than standard antioxidant (vitamin E) at 1.00-2.50 mg mL^{-1} (Reducing power of CSLE at 1 mg mL^{-1} , 1.65, 1.5 mg mL^{-1} , 1.72, 2.0 mg mL^{-1} , 1.77, 2.5 mg mL^{-1} , 1.80) (Reducing power of vitamin E at 1 mg mL^{-1} , 1.22, 1.5 mg mL^{-1} , 1.35, 2.0 mg mL^{-1} , 1.51, 2.5 mg mL^{-1} , 1.63). This goes to say that CSLE has a good reducing ability and its reducing power increased in a concentration dependent manner. In addition, the reducing power of CSLE was found to be as good as *Smilax excelsa* leaves extract (5.0 mg mL^{-1} : Absorbance; 1.6) which is used as natural antioxidant source in Turkey (Ozsoy *et al.*, 2008). Flavonoids and phenolic compounds might contribute to the remarkable reducing activity of CSLE as phenolic hydroxyl groups present in them act as a reducing agent by donating electron (Shahidi *et al.*, 1992).

Toxicity studies

Brine shrimp toxicity assessment: Brine shrimp lethality assay is a preliminary toxicity screening that enables determination of medium lethal concentration (LC_{50}) values for the extract (Massele and Nshimo, 1995). In this bioassay, high LC_{50} was recorded (2.59 ± 0.3 mg mL^{-1}) compared to potassium dichromate standard (0.014 ± 0.21 mg mL^{-1}) (Table 2). LC_{50} value more than 1 mg mL^{-1} is considered safe for consumption

(Meyer *et al.*, 1982). This simply means that CSLE may be safe for consumption since the LC_{50} values is about two fold higher than standard values for toxicity.

Acute toxicity evaluation: Acute toxicity assessment is the first step in the toxicological analysis of herbal drugs. CSLE at doses of 300, 2000 and 5000 $mg\ kg^{-1}$ had no adverse effect on the behavioural responses of the tested mice up to 14 days of observation. There was no mortality

Table 2: LC_{50} values of *Cinnomomum iners* standardized leaves methanolic extract (CSLE) and positive control (potassium dichromate) using brine shrimp (*Artemia salina*) lethality assay

Results	$LC_{50} \pm SD$
CSLE ($mg\ mL^{-1}$)	2.590 ± 0.3
Potassium dichromate ($\mu g\ mL^{-1}$)	0.014 ± 0.21

Values are expressed as Mean \pm SD, n = 3

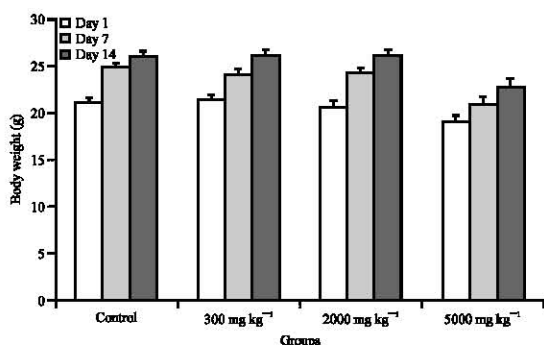


Fig. 2: Body weight of Swiss albino mice at day 1, 7 and 14 for acute toxicity study of control group and *Cinnomomum iners* standardized leaves methanolic extract (CSLE) treated mice at 300, 2000 and 5000 $mg\ kg^{-1}$. Data presented as Means \pm SEM, n = 5. * $p < 0.05$, significant increase from control group

observed at all the tested doses. None of the used doses affect the weight of the mice. The LD_{50} of CSLE was therefore estimated to be more than 5000 $mg\ kg^{-1}$; thus CSLE was regarded as non toxic plant. The mice body weight in tested groups (dosing of 300, 2000 and 5000 $mg\ kg^{-1}$ of CSLE) at day 1, 7 and 14 had no significant difference with the control group (Fig. 2). Body weight of control group at day 1; 21.09 g, day 7; 24.80 g, day 14; 25.95 g. Body weight of mice treated with 300, 2000 and 5000 $mg\ kg^{-1}$ of CSLE at day 1; 19-20 g, day 7; 20-24 g, day 14; 22-26 g.

There is no significant difference of calculated organ body index of mice treated with CSLE at 300, 2000 and 5000 $mg\ kg^{-1}$ with the control group (Fig. 3) [organ body index for control group (heart; 0.0204, spleen; 0.031, liver; 0.253, kidney; 0.047, lung; 0.039)], [organ body index for 300, 2000 and 5000 $mg\ kg^{-1}$ CSLE treated mice (heart; 0.018-0.023, spleen; 0.026-0.028, liver; 0.242-0.285, kidney; 0.045-0.054, lung; 0.039-0.042)]. This indicates CSLE did not affect the body weight and organ weight of mice up to 5000 $mg\ kg^{-1}$. This revealed that CSLE did not produce organ swelling, atrophy or hypertrophy.

Histopathological studies: The microscopic examination of internal organs did not find any abnormalities in the 5 $g\ kg^{-1}$ CSLE (Fig. 4b; heart, Fig. 5b; kidney, Fig. 6b; lung, Fig. 7b; spleen, Fig. 8b; liver) as compared to the control group (Fig. 4a; heart, Fig. 5a; kidney, Fig. 6a; lung Fig. 7a; spleen, Fig. 8a; liver). In addition there were not any alterations noticed in the microscopic examinations (Fig. 4b-8b). Microscopic assessment in the lower dose was not performance in agreement with the OECD guideline that considers the microscopic evaluation of organs in low dose group as unnecessary when no

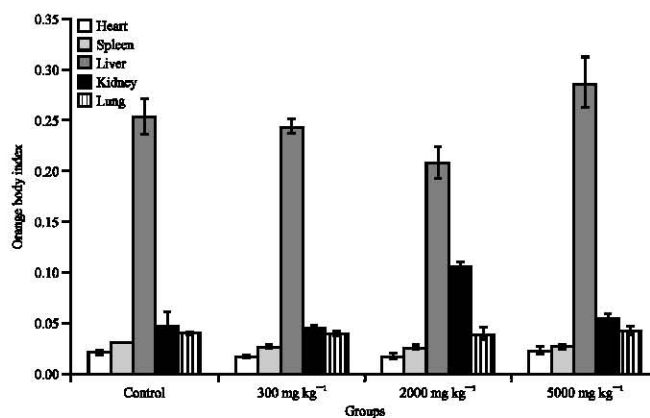


Fig. 3: Organ body index of Swiss albino mice of control group and *Cinnomomum iners* standardized leaves methanolic extract (CSLE) treated mice at 300, 2000 and 5000 $mg\ kg^{-1}$. Data presented as Means \pm SEM, n = 5. * $p < 0.05$, significant increase from control group

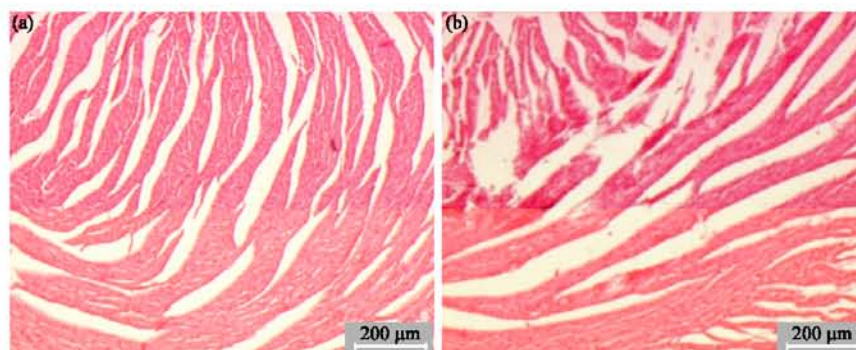


Fig. 4: (a, b) A representative for control group and 5000 mg kg⁻¹ CSLE treated mice heart

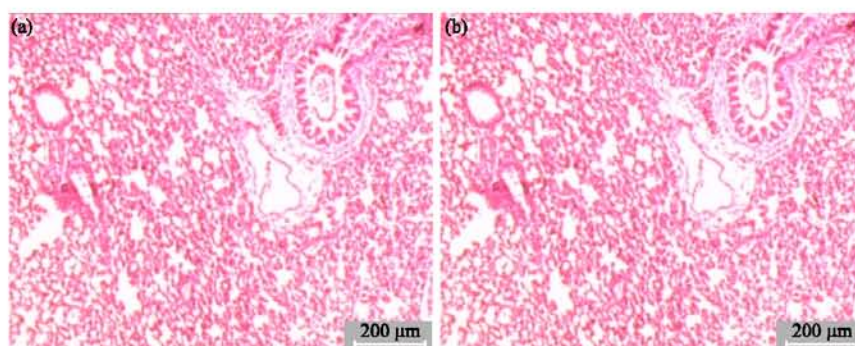


Fig. 5: (a, b) A section for control group and 5000 mg kg⁻¹ CSLE treated mice kidney

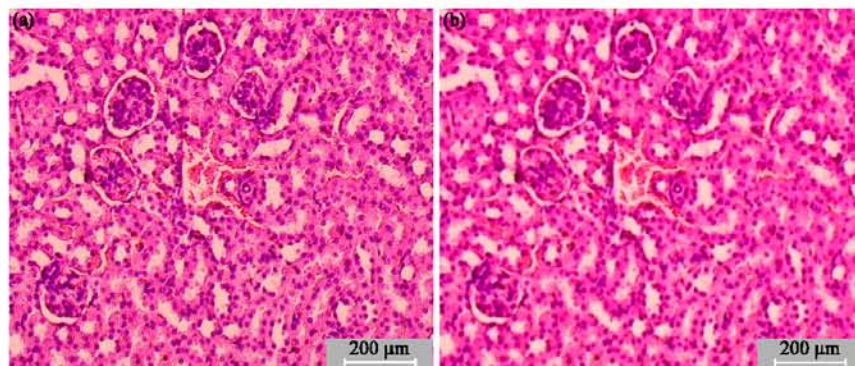


Fig. 6: (a, b) A section for control group and 5000 mg kg⁻¹ CSLE treated mice lung

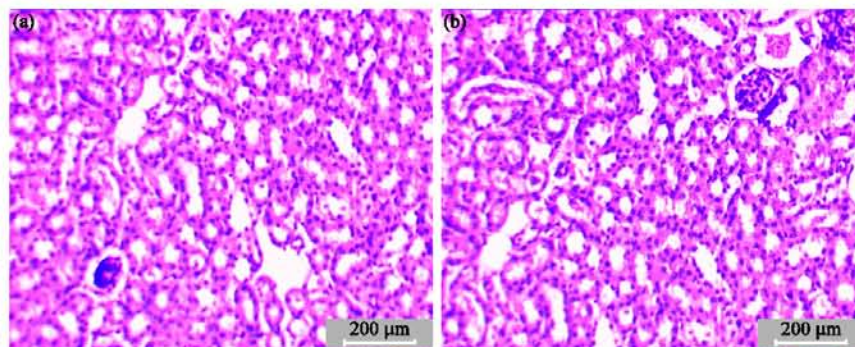


Fig. 7: (a, b) A representative for control group and 5000 mg kg⁻¹ CSLE treated mice spleen

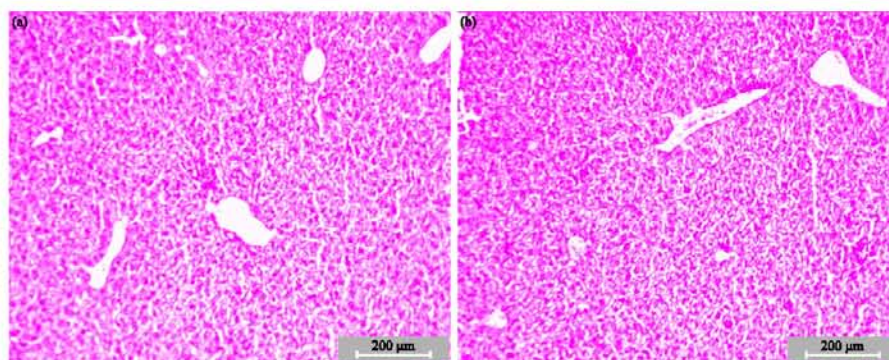


Fig. 8: (a, b) A section for control group and 5000 mg kg⁻¹ CSLE treated mice liver

histopathological abnormalities are found in the high dose group (OECD, 2001a). The present investigation shows that CSLE is non-toxic at least up to maximum dose of 5 g kg⁻¹ b.wt. acutely. Although this short term acute toxicity study revealed no gross organ toxicity, longer term use might result in organ damage. So, further studies are necessary to study long term toxicity.

CONCLUSION

In conclusion, our investigation clearly showed CSLE offer protection against oxidative damage of free radicals. In addition CSLE did not induce any short term toxicological effects with LD₅₀ and LC₅₀ values falls in safety margin. However, further long term toxicity studies of CSLE are required for the consumption of CSLE as natural antioxidants in near future.

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