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Toxicity Evaluation of Methanol Extract of *Clitoria ternatea* L. Leaf

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ABSTRACT

The *Clitoria ternatea* (Fabaceae) root, seed, and leaf are commonly used in Ayurvedic medicine in Malaysia and Indonesia. The methanol leaf extracts of *C. ternatea* was tested for toxicity by means of brine shrimp lethality test and acute oral toxicity assay. In the brine shrimp lethality test, the leaf extract were non-toxic or showed weak lethality (LC_{50} > 1 mg/ml) at the 6 h, 12 h and 24 h incubation period. Nevertheless, at the 48 h incubation time, the leaf extract exhibited moderate toxicity activity with LC_{50} values of 0.49 mg/ml. In the acute toxicity study using mice, the median lethal dose (LD_{50}) of the extract was found greater than 2000 mg/kg, and we found no pathological changes by means of macroscopic examination of tissues of mice treated with the extract. We conclude that the *C. ternatea* leaf extract is not toxic in mice and brine shrimp.

Keywords: ***Clitoria ternatea*, toxicity study, brine shrimp assay, acute oral toxicity**

INTRODUCTION

Clitoria ternatea L. (Fabaceae), commonly called Shankapushpi in Ayurvedic medicine, is used traditionally to treat various ailments [1]. The roots, seeds and leaves of *C. ternatea* are commonly used in Ayurvedic medicine. The herb had been used for centuries as a memory enhancer, nootropic, antistress, anxiolytic, antidepressant, anticonvulsant, tranquilizing and sedative agent. *C. ternatea* young shoots, leaves, flowers and tender pods are eaten as a vegetable in Kerala (India) and in Philippines [2]. In Malaysia, the flowers used used to impart a bright blue color to rice cakes and the leaves to impart a green color to food. In addition, *C. ternatea* has a relatively well documented neuropharmacological action which justifies its use in Central Nervous System (CNS) diseases in Ayurvedic medicine. *C. ternatea* has been used in 'Medhya Rasayana' a rejuvenating recipe used for the treatment of neurological disorders and thought to strengthen a person's intellectual capacities [2]. *C. ternatea* is a potential candidate for enhancing learning and memory, *Clitoria ternatea* Linn. root extract treatment used during a growth spurt period enhances learning and memory in rats. In traditional medicine transmitted orally or in writing (esp. Ayurveda) various therapeutic effects have been attributed to roots, leaves and seeds of *C. ternatea* [2]. Its extracts possess a wide range of pharmacological activities including antimicrobial, antipyretic, anti-inflammatory, analgesic, diuretic, local anaesthetic, antidiabetic, insecticidal, blood platelet aggregation inhibiting and vascular smooth muscle relaxant properties [2].

Plants are known to generate several chemical constituents, which are naturally toxic to bacteria and fungi. Despite significant progress made in molecular biology and medicinal chemistry that leads to the development of new drugs, plants have been a potential source in the discovery of bioactive compounds for development into clinical agents [4]. In a recent study, leaf extract of *C. ternatea* showed to be effective against *Aspergillus niger*, a pathogenic microorganism which cause significant infection in immunocompromised hosts and severely ill-patients [5].

There is a high degree of concern regarding the safe use of plant extracts and for this reason, preclinical and clinical toxicological evaluation of these extracts are needed [6]. With the aim to assure the safety of the extract, and also due to the scarcity of literature information about *C. ternatea* extract toxicity, an initial toxicological evaluation using brine shrimp assay and an acute oral toxicity of the methanol extract were carried out in this study.

MATERIALS AND METHODS

Plant material

Raw and mature *C. ternatea* leaves were collected in Seberang Jaya, Penang, Malaysia in January 2008. The leaves were then washed with water to remove dirt prior to the drying process (40°C for 3 days). The authencity work was carried out by Dr S. Sreeramanan, a botanist from School of Biological Sciences, Universiti Sains Malaysia where the herbarium was deposited with a voucher number of 11006.

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Preparation of plant extracts

The leaf extract was prepared by maceration of dried powdered plant material in methanol solvent for 3 days [7]. Two hundred g of powdered leaf was macerated in 400 mL methanol under stirring conditions for 72 h. The macerated extract was then filtered through No. 1 Whatman filter paper. The methanol crude extract was vaporized to dryness using the rotary evaporator (BUCHI Rotary Evaporator R-110) and freeze dried.

Experimental animals

Swiss albino mice (20-25g) of both male and female (nulliparous and non pregnant) were used for the assessment of the acute oral toxicity. The animals were kept in a plastic cage with ventilated stainless steel cover and allowed to adjust to the laboratory environment over a period of one week before the commencement of the experiment. The animals were supplied with food and water *ad libitum*. The experimentals protocols were approved by Universiti Sains Malaysia Animals Ethics Committee (USM/PPSF/50(063)JLD 3).

Brine shrimp, Artemia salina assay

The Brine shrimp lethality test was carried out as described by Meyer [8]. Brine shrimp eggs, *Artemia salina* was hatched in artificial seawater (prepared by dissolving 38g of sea salt in 1L distilled water). After 24 h incubation at room temperature (22-29^{o}C), the larvae (nauplii) were attracted to one side of the vessel with a light source and collected with pipette. Extract was dissolved in 2% aqueous Tween 80 (v/v). Seawater was placed in all the test tubes. A two-fold dilution was carried out to obtain a concentration of 10 mg/mL - 0.0195 mg/mL of the extract. A suspension of nauplii containing 15 larvae was counted and added into each test tube and incubated for 24 h. At the 6 h and 12 h, the test tubes were examined and the number of dead nauplii in each tube was counted and recorded. The mortality rate (%) was calculated by dividing the number of dead shrimps with total number shrimps and multiplied with 100 %. Lethality concentration fifty (LC_{50} values) for the extract was calculated using the equation given for the graph. The general toxicity activity was considered weak when the LC_{50} was between 0.5 and 1 mg/mL, moderate when the LC_{50} was between 0.1 and 0.5 mg/mL, and designated as strong when the LC_{50} ranged from 0 to 0.1 mg/ml [9]. Potassium dichromate and 2 % aqueous Tween 80 was use as positive and negative control respectively.

ACUTE ORAL TOXICITY ASSAY

Sighting study

The sighting study was conducted as per OECD-420 guidelines [10]. The animals fasted for 4 hour although still they were allowed free access to water before the commencement of the experiments [11]. Doses of 50, 300 and 2000 mg/kg of leaf extract were given orally to the mice. The control animals received 2% aqueous Tween 80.

Acute oral main study

The acute oral study was performed as per OECD [11] guidelines [12]. The animals will fast for 4 hour although they will be allowed free access to water before the commencement of the experiment. The animals used for the main study will be divided into 4 groups with each group containing six mice. The male control group (A) and female control group (B) will receive 2 % aqueous Tween 80 orally in constant volume of 10 mL/kg. While the male test group (C) and female test group (D) will be treated with 2000 mg/kg of leaf extract.

Observation of animals

All animal were observed individually after dosing at least once during the first 30 minutes periodically during the first 24 hours, with special attention given to first 4 hours and daily thereafter for a total of 14 days. The visual observations included changes in the skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system as well as somatomotor activity and behavioral pattern. Individual weight of animals was determined shortly before the test substance was administered and at least weekly thereafter. Animal found to show sign of toxicity, severe pain or enduring signs of severe distress were humanely killed. All test animals (including those that died during the test or were removed from the study for animal welfare reasons) were subjected for gross necropsy. The appropriate dose was then selected and used for the main study.

Histopathological studies

Slices of liver, kidney, lung, spleen and heart were fixed in 4% formaldehyde, embedded in paraffin wax, sectioned at 8 μm and stained with haematoxylin and eosin. Detailed microscopic examination was carried out in those organs of both control and treatment groups of both sexes.

Data analysis

The mean results of brine shrimp mortality against the logarithms of concentrations were plotted using the Microsoft Excel computer program, which also gives the regression equations. The regression equations were used to calculate LC_{50} value. The data for body weight and organ weight were expressed as the mean ± standard error of the mean (SEM). Body weight evolution and weight of the organs from the control and the test groups were compared using the *t*–test and two way ANOVA run on the software SPSS for Windows.Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Most of the herbal products used in traditional medicine have solid scientific support with regard to their effectiveness. There is little information available on the possible risks of *C, ternatea* to human health Based on their long-term exploit by humans one might expect *C, ternatea* used in traditional medicine to have low toxicity. However, recent investigations have revealed that many plants used as food or in traditional medicine showed toxic effects in *in vitro* assays. This raises concern about the potential toxicity resulting from the application of such plants by the consumers. Therefore, this research work aimed to assess the potential hazards of *C. ternatea* methanol extract by using brine shrimp lethality assay and Acute oral toxicity assay.

Brine shrimp lethality assay

Brine shrimp assay has been an excellent method for preliminary investigations of toxicity and to screen medicinal plants popularly used in traditional medicine. This method was used in this study because it is rapid, inexpensive and only utilizes small amount of extract [4]. Moreover, a positive correlation between the lethality to brine shrimp and the corresponding oral lethal dose in mice of medicinal plants has been demonstrated by Parra *et al.* [13].

The brine shrimp assay has proven to be a convenient system for evaluating the toxicity of plant extract since the larvae sensitive to various compounds in the crude extract. Hence, brine shrimp toxicity assay was carried out using the leaf extract at 6th h, 12th h, 24th h and 48th h incubation time. The lethal concentration (LC_{50}) of the leaf extract for 6 h, 12 h, 24 h and 48 h exposure were > 10 mg/mL, > 10mg/ml, 1.46 mg/ml and 0.49 mg/mL respectively.

Table 1. Toxic effects of the *Clitoria ternatea* leaf methanol extract after 6 h and 12 h using brine shrimp lethality assay.

Extract and Controls	Lethality Concentration fifty (LC50) (mg/ml)			
	6 hr	12 hr	24 hr	48 hr
Leaf	> 10 + 0.21	> 10 + 0.19	1.46 + 0.02	0.49+ 0.01
Potassium dichromate	1.11 + 0.04	0.38 + 0.01	0.024 + 0.001	0.013 + 0.005

The LC_{50} was obtained by linear regression equations. LC^{50} value between 0 and 0.1 mg/ml was considered toxic. Data are mean ± SEM

According to Padmaja [9], the general toxicity activity was considered weak when the LC_{50} was between 500 and 1000 µg/mL, moderate when the LC_{50} was between 100 and 500 µg/mL, and designated as strong when the LC_{50} ranged from 0 to 100 µg/mL. Hence, the toxicity activity of leaf extracts at 6 h, 12 h and 24 h against the Brine shrimps were considered weak. Meanwhile, at 24 h exposure, the leaf extracts exhibited a moderate level of toxicity activity against the Brine shrimps. This preliminary toxicity results show that leaf extract is not toxic to brine shrimps after a short period of exposure. The results of the current study further confirmed the traditional claims of this plant in folk medicine.

Acute oral toxicity assay

Further evidence of the brine shrimp assay results was obtained from acute oral toxicity study in mice. Toxicological evaluation is carried out in various experimental animals to predict toxicity and to provide guidelines for selecting a 'safe' dose in humans. Some of the factors are capable to interfere with the toxicity of medicinal plants extract. The factors include the intrinsic property of the botanical drug or it happens during the process of extract preparation [14].

In this study, the acute oral toxicity of leaf extract of *C. ternatea* was evaluated by employing the OECD [11] guideline that allows the evaluation of lethality potential and possible toxicant effects developed in a short period of time. Extract with 50, 300, and 2000 mg/kg body weight doses were administered as recommended by OECD guidelines [11]. Administration of further higher doses was considered physiologically unsound and is not generally

recommended.

The doses tested in this study did not cause any mortality or any signs of acute toxicity in the tested animals in short term (i.e. 48 h) and long term (i.e. 14 days) observation period. The dose did not cause any appreciable alterations in water and food intake (data not shown) during 2 weeks in both sexes. There were no significant differences (P>0.05) between the weekly mean body weight between control and the animals treated with extract except for day 1 the control group shows heavier mean weight body compared to group treated with 2000 mg/kg body weight of extract for both sex. Furthermore, body weight gain throughout the observation period among the treated animals was comparable to their respective controls. No sex-related differences were evident in either species. In addition, there were no significant differences in the mean organ weight between control and animals treated with 50, 300, and 2000 mg/kg body weight.

Table 2. Mean body weight of mice after 14 days treatment with leaf extract of C. ternatea

Treatment group	Dose (mg/kg)	Weekly mean body weight (g)			Mean weight gain (g)
		Day 1	Day 7	Day 14	
Male	Control	*23.54 ± 0.24	25.73 ±0.72	27.20 ±0.86	3.66 + 0.21
	50	21.33 ± 0.32	24.86 ±0.42	26.83 ±0.66	5.50 + 0.41
	300	24.90 ± 0.22	25.62 ±0.42	26.54 ±0.52	1.64 + 0.10
	2000	*21.55 ± 0.45	24.15 ±0.57	25.18 ± 0.67	3.63 + 0.20
Female	Control	*20.27 ± 0.14	21.79 ± 0.30	23.67 ± 0.42	3.40 + 0.22
	50	22.41 ± 0.55	23.36 ± 0.42	25.45 ± 0.37	3.04 + 0.19
	300	24.50 ± 0.35	25.76 ± 0.51	26.98 ± 0.32	2.48 + 0.17
	2000	*21.47 ± 0.45	22.61 ± 0.55	23.85 ± 0.38	2.38 + 0.20

Data are mean ± SEM; n=6 for control and 2000 mg/kg; * $p < 0.05$.

Table 3. Mean organ weight of mice after 14 days treatment with leaf extract of C. ternatea

Treatment group	Dose (mg/kg)	Mean organ weight (g)				
		Heart	Lung	Liver	Kidney	Spleen
Male	Control	0.13 ± 0.01	0.21 ± 0.01	1.79 ± 0.12	0.39 ± 0.02	*0.20 ± 0.02
	50	0.12 ± 0.02	0.20 ± 0.01	1.64 ± 0.01	0.44 ± 0.01	0.09 ± 0.02
	300	0.13 ± 0.01	0.32 ± 0.02	2.07 ± 0.02	0.43 ± 0.01	0.15 ± 0.02
	2000	0.13 ± 0.00	0.25 ± 0.01	1.98 ± 0.11	0.44 ± 0.03	*0.14 ± 0.2
Female	Control	0.10 ± 0.00	0.18 ± 0.01	1.32 ± 0.06	0.28 ± 0.01	0.08 ± 0.00
	50	0.11 ± 0.01	0.16 ± 0.02	1.14 ± 0.05	0.24 ± 0.01	0.08 ± 0.00
	300	0.11 ± 0.02	0.23 ± 0.02	1.65 ± 0.06	0.33 ± 0.02	0.07 ± 0.00
	2000	0.10 ± 0.01	0.19 ± 0.02	1.29 ± 0.11	0.25 ± 0.15	0.09 ± 0.01

Data are mean ± SEM; n=6 for control and 2000 mg/kg; * $p < 0.05$.

The acute toxicity study indicated that 2 % Tween 80 suspension of *C. ternatea* extract is not toxic when administered orally to experimental animals. Therefore, the acute minimum fatal dose of *C. ternatea* leaf extract for mice is higher than 2000 mg/kg body weight which indicates some high level of safety margin in oral formulation. Gross examination at autopsy and histopathological evaluations of the various organs stained with hematoxylin and

eosin revealed no significant differences (Figure 1; Figure 2) for both sexes. In comparison, the study conducted by Jothy *et al.* [15] revealed similar results using *Cassia fistula* seed extract at dose 5000 mg/kg. No toxic symptoms or mortality were observed in any animals, which lived up to 14 days after the administration of methanol seeds extract at single dose level of 5000 mg/kg body weight in their study. They also reported that the behavioural patterns of animals were observed first 6 hours and then 14 hours after the administration and the animals in both vehicle treated and extract-treated groups were normal and did not display significant changes in behavior, skin effects, breathing, impairment in food intake and water consumption, postural abnormalities and hair loss [15].

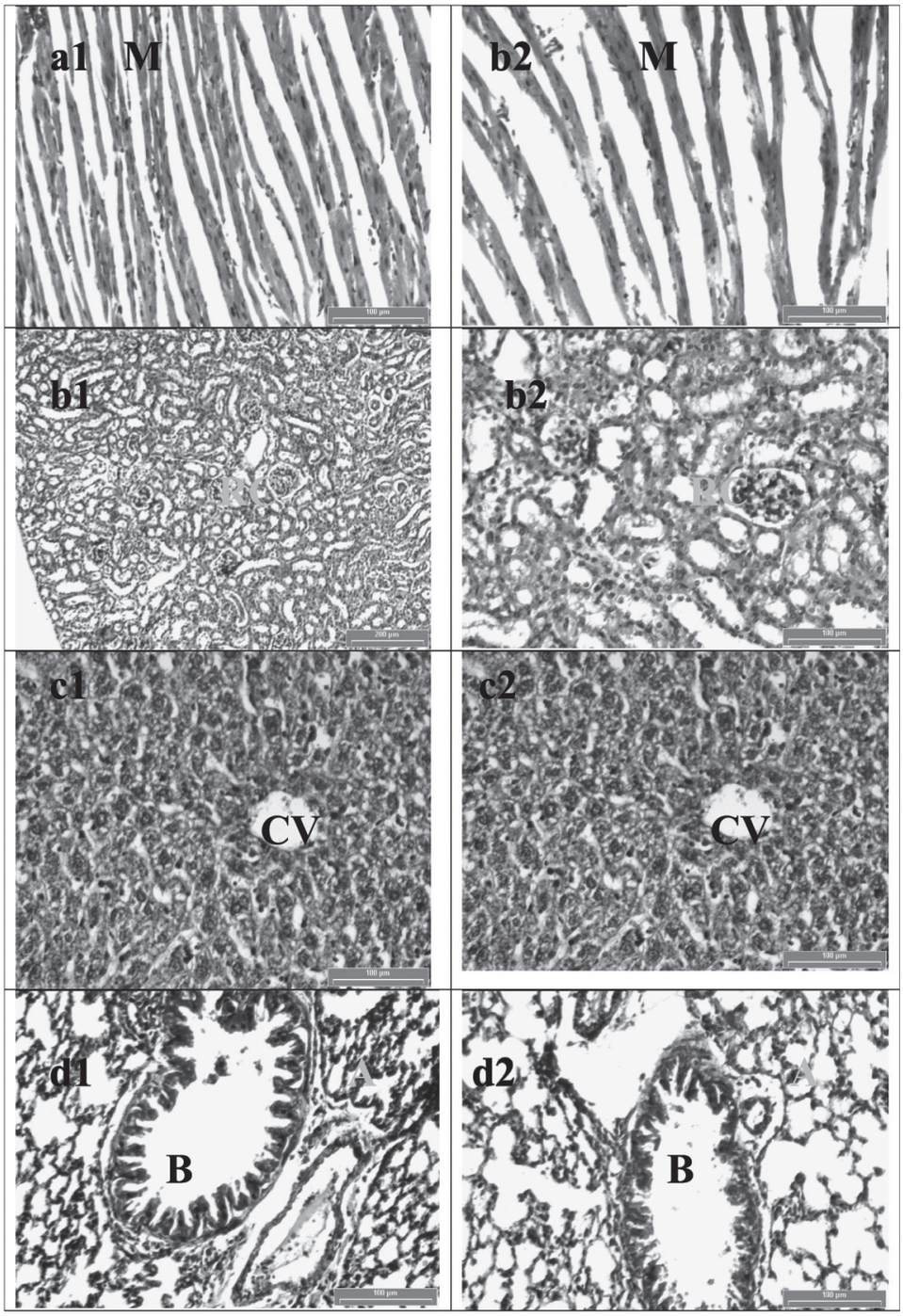

Figure 1. Histological examination of heart (a), kidney (b) liver (c) and lung (d) of female mice. A = alveoli, M = myocardium, B = bronchiole, CV = central vein, RC = renal capsule, 1: treated, and 2: control

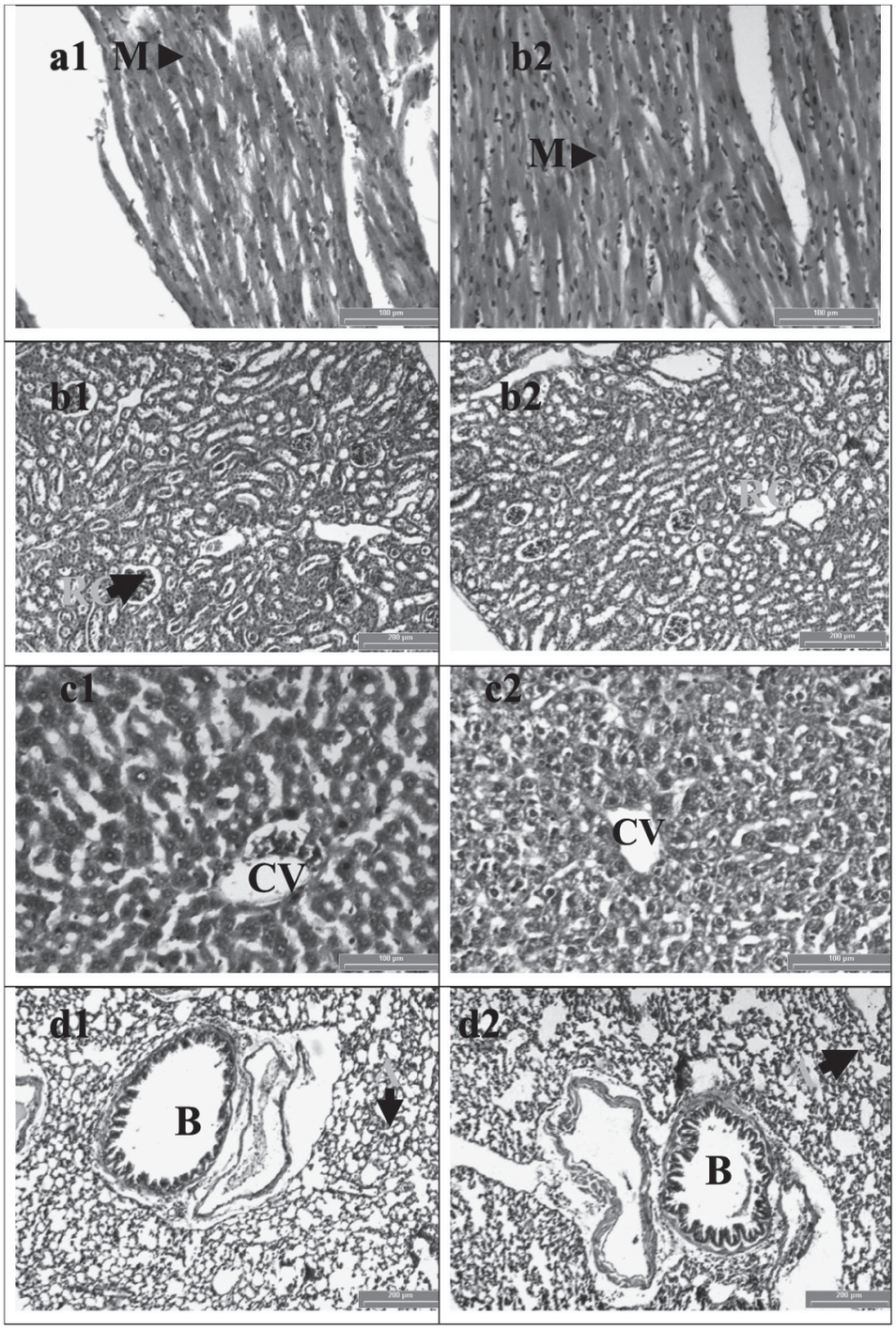

Figure 2. Histological examination of heart (a), kidney (b) liver (c) and lung (d) of male mice. A = alveoli, M = myocardium, B = bronchiole, CV = central vein, RC = renal capsule, 1: treated, 2: control

Based on the results of the brine shrimp lethality test (LC_{50} > 1 mg/ml) and acute oral toxicity studies, it was concluded that a dose of 2000 mg/kg body weight of the extracts of *C. ternatea* leaf extract given orally appeared to be non-toxic. However, there must be a point at which it can be concluded that a test substance is practically non-toxic or non-lethal after an acute exposure. The dose of 2000 mg/kg body weight for acute oral toxicity is generally considered to be very high. Thus *C. ternatea* leaf extracts have a high margin of safety. However, further toxicity studies are needed to determine the effects of this extract on chronic oral toxicity, on animal fetus, on pregnant animals, and their reproductive capacity to complete the safety profile of this extract.

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