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Sub-acute oral toxicity study of methanol leaves extract of *Catharanthus roseus* in rats

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ABSTRACT

Objective: To examine the sub-acute (14 d) oral toxic effects of methanol leaves extract of *Catharanthus roseus (C. roseus)* (Family: Apocynaceae) on liver and kidney functions in Sprague Dawley (SD) rats. **Methods:** Twenty four female SD rats were used throughout the experiment. The first group was orally treated with distilled water and served as control, whereas the remaining three groups were orally treated with single dose daily of 0.1 g/kg, 0.5 g/kg, 1 g/kg of *C. roseus* extract, respectively for 14 d. Cage-side observations were done daily. Any animal died during the experiment was dissected for gross organ examination. Body weight changed, food consumption and water intake were recorded weekly. Blood was collected via cardiac puncture on day-15 and used for determination of serum levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatinine and urea. The relative organ weights were also measured. All results were expressed as mean ± S.E.M and analysed using Dunnett's test. The level of significance was set at $P<0.05$ when compared to the control group. **Results:** Repeated oral administration of 0.5 g/kg and 1 g/kg of methanol leaves extract of *C. roseus* caused mortality and diarrhoea in rats after few days of treatment. There were no significant changes observed in serum biochemical markers, body weight changed, water and food intake and relative organ weight in rats treated with a single dose daily of 0.1 g/kg of *C. roseus* extract treatment for 14 d when compared to control group. **Conclusionds:** Fourteen days repeated oral administration of 0.1 g/kg of methanol leaves extract of *C. roseus* was safe in female SD rats without causing any significant damages to liver and kidney.

1. Introduction

Catharanthus roseus (C. roseus) (Family: Apocynaceae) or previously known as *Vinca rosea* is an herbaceous plant which probably originated from Madagascar[1]. In Malaysia, it is commonly known as periwinkle or Kemunting Cina[2]. In other countries, Kemunting Cina is also known as *thenbanmahnyoban* (Myanmar) and *prevenche de Madagascar* (French)[3]. Madagascan periwinkle is a flowering plant and it is placed in the group of dicotyledons. Each flower has 5 petals which are joined at the base to form a tube. In Malaysia, the decoction of this plant is used to treat diabetes, reduce blood pressure, insomnia and cancer[3]. Vincristine and vinblastine are clinically used as chemotherapeutic agents for acute lymphoblastic leukaemia, lymphomas, multiple myeloma, Hodgkin's disease and testicular carcinoma[4].

Several oral toxicity studies have been reported on the different parts of *C. roseus* in experimental animals. Fourteen days oral administration with 4 g/kg of ethanol flower extract of *C. roseus* did not produce any toxicity signs and mortality in both male and female rats. In another oral toxicity, significant liver damage was observed in rabbits after receiving 0.1 g/kg of aqueous leaf extract of *C. roseus* for 9 d[5]. Recently, one study reported that the oral LD_{50} of methanol leaf extract of *C. roseus* was 2.1 g/kg in mice[6]. However, the information regarding the safety of methanol leave extract of *C. roseus* are still inadequate. The objective of this study is to examine the toxicity after repeated (14 d) oral administration of methanol leaves extract of *C. roseus* on the functions of liver and kidney in Sprague Dawley (SD) rats. The present study was conducted according to toxicity testing guideline described by Organisation for Economic

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Co-operation and Development (OECD).

2. Materials and methods

2.1. Chemicals

All the chemicals used were at analytical grade. Diethyl ether and methanol were obtained from Chemolab Supplies Sdn. Bhd., Malaysia.

2.2. Plant materials

The methanol leaves extract of *C. roseus* used in this experiment was supplied by Universiti Sains Malaysia (USM), Penang. Plant leaves were ground to a homogenous powder in a Wiley mill (no. 20 mesh) after drying in an oven (35 ℃). The dried powdered leaves were then extracted with methanol by using soxhlet apparatus. After the solvent was removed under reduced pressure, portion of the concentrated extract was spray-dried.

2.3. Experimental animals

A total of 24 healthy Sprague Dawley female rats (12 weeks old; weighing (220 ± 20) g were used in this experiment and they were bred and kept in the animal house in UCSI University. They were housed at a room temperature maintained at (25 ± 1) ℃ with 12 hours light: 12 h dark cycle. Food and water were provided *ad libitum*. Animals were handled according to the guideline in Ethics Clearance Form (Animals), Faculty of Pharmaceutical Sciences, UCSI University.

2.4. Protocol of sub-acute oral toxicity study

Sub-acute oral toxicity study was conducted in accordance to OECD 407 guideline[7]. All the animals were then randomly divided into four groups with each group consisted of 6 rats. The first group was orally treated with distilled water (vehicle) and served as control group, whereas the remaining three groups were orally treated with single dose daily of 1 g/kg, 0.5 g/kg and 0.1 g/kg methanol leaves extract of *C. roseus*, respectively up to 14 d. All of the animals were closely observed for the first four hours after each dosing to examine any toxic signs and lethality[8]. If there is any death during the experimental period, the animal will be dissected for gross examination. Body weight change, food consumption and water intake on day-0, -3, -7 and -14 during the experimentation period were recorded. After day-14, the animals were subjected to overnight fasting (at least 16 h) period and the blood samples were collected via cardiac puncture on day-15. All the animals were dissected to determine the relative organ weight and grossly examined for any abnormalities. All blood serum samples were sent to the UCSI University Pathology Laboratory within the same day and analysed by using Hitachi 912 automatic analysing machine. Several serum biochemical parameters such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine and urea were determined by using the protocol described by Roche Diagnostics reagent kits[9].

2.5. Statistical analysis

All results were expressed as mean ± standard error of mean (SEM) and analysed using Dunnett's test[10]. The level of significance was set at $P<0.05$ when compared to the respective control group.

3. Results

Repeated oral administration of 1 g/kg and 0.5 g/kg of *C. roseus* extract caused diarrahoe in female rats after 5th dosing and 9th dosing, respectively. Mortality was observed in rats treated with 1 g/kg of methanol leaves extract of *V. rosea* after 9th dosing and half of the rats (n=3) received 0.5 g/kg *C. roseus* extract died after 12 d treatment. Gross nescropy on the died rats treated with 1 g/kg extract showed black spots on the surface of livers and significant ($P<0.05$) elevation of relative liver (2.3 folds) and kidney (1.6 folds) weights compared to control group (Table 1). A significant ($P<0.05$) elevation to 3.5 folds in serum ALP and 1.9 folds of relative liver weight was observed in the rats treated with 0.5 g/kg extract for 14 d when compared to the control group (Table 2). Food and water intake were generally decreased in rats treated with 0.5 g/kg and 1 g/kg of extracts (Table 3). Rats that orally treated with a single dose daily of 0.1 g/kg of *C. roseus* up to 14 d developed no remarkable adverse effects during the experimentation. All the serum biochemical markers for liver and kidney functions, relative organs weight, body weight changed, food and water consumption showed no significant differences between 0.1 g/kg *C. roseus* treatment group and control group (Table 1-3).

Table 1

Effect of methanol leaves extract of *C. roseus* on the relative organs weight and lethality in rats.

Doses (g/kg)	Relative organ weight (g/100 g)					Lethality	Remarks
	Liver	Kidney	Spleen	Lung	Heart		
Control	2.45±0.18	0.68±0.06	0.26±0.04	0.77±0.10	0.33±0.03	0	No significant change was observed.
1.0	5.62±0.51*	1.12±0.06*	0.05±0.05	1.20±0.11	0.53±0.05	6/6	First rat to have diarrhoea after 5th day. All rats died on day-9. Five animals had black spots on liver surface.
0.5	4.75±0.73*	0.83±0.07	0.29±0.04	0.82±0.12	0.47±0.07	3/6	First rat to have diarrhoea after 9th day. Three rats died on day-12.
0.1	2.40±0.06	0.67±0.06	0.25±0.05	0.67±0.09	0.34±0.01	0	No significant damage was observed.

n = 6 (initial number of animals); Values are expressed in mean ±S.E.M.; analysed using Dunnett's test; *$P<0.01$ significant difference as compared to control group.

Table 2

Effect of methanol leaves extract of *C. roseus* on liver and kidney functions in rats.

Dose (g/kg)	Liver function test			Kidney function test	
	ALP (IU/L)	ALT (IU/L)	AST (IU/L)	Urea (mmol/L)	Creatinine (μ mol/L)
Control	45.00±6.11	55.20±5.03	162.20±13.31	5.84 ±0.27	61.60±1.81
0.5	160.50±60.50* (*n*=3)	66.00±10.00 (*n*=3)	170.50±44.50 (*n*=3)	5.95±0.05 (*n*=3)	62.00±2.00 (*n*=3)
0.1	58.20±4.41	52.80±5.00	186.40±19.29	6.02±0.50	63.40±2.58

n = 6 (initial number of animals); Values are expressed in mean ±S.E.M.; analysed using Dunnett's test; **P*<0.05 significant difference as compared to control group.

Table 3

The Effect of methanol leaves extract of *V. rosea* on the body weight, food consumption and water intake in rats.

Groups	g/kg	Day 0	Day 3	Day 7	Day 14
Body weight (g)	Control	237.5±20.9	244.5±19.5	252.1±18.3	261.4±18.1
	1.0	227.6±22.7	220.2±22.6	193.0±23.2*	(*n*=0)
	0.5	218.3±23.4	215.7±22.3	209.6±56.6	280.2±17.3 (*n*=3)
	0.1	240.5±19.0	240.0±15.2	247.0±15.2	250.4±14.5
Food Consumption (g/rat/d)	Control	27.1±1.2	29.9±0.6	35.5±2.0	32.3±0.8
	1.0	26.5±1.3	25.6±1.6*	32.1±3.8	(*n*=0)
	0.5	28.7±1.3	23.8±0.6*	22.8±1.5**	29.2±0.5 (*n*=3)
	0.1	29.3±1.8	26.6±2.7	30.4±1.1	30.4±1.1
Water consumption (mL/rat/d)	Control	12.3±0.1	13.5±0.5	14.8±0.3	261.4±18.1
	1.0	12.8±0.7	7.5±1.2**	1.7±1.0**	(*n*=0)
	0.5	11.5±0.1	8.9±0.5**	9.3±0.3**	12.5±0.5** (*n*=3)
	0.1	12.6±0.3	12.5±0.2	13.1±0.1	11.5±1.6

n=6 (initial number of animals); Values are expressed in mean ±S.E.M; Analysed using Dunnett's test; **P*<0.05 and ***P*<0.01 significant difference as compared to control group.

4. Discussion

Toxicological studies are the platform for hazard identification stage of safety assessment[11]. Acute exposure to methanol leaves extract of *C. roseus* at doses from 0.1 g/kg to 1 g/kg to female rats did not cause any mortality and adverse effects 24 h post first dose treatment. This could suggest that the oral LD_{50} value of methanol leaves extract in rats was greater than 1 g/kg. However, prolonged treatment of *C. roseus* extract could enhance the toxicity in rats. The *C. roseus* extract was highly toxic at doses of 0.5 g/kg and 1 g/kg and caused the rats to have diarrahoe and to die after a few days of treatment. Gross examination on the livers with obvious black spots on the liver surface and significant increased in relative liver weight from the died rats after treating with high dose of *C. roseus* revealed the targetted toxic effects on this plant on rat livers. Dirrahoea was one of the adverse effects due to imbalanced in absorptive and secretory mechanims in the gastroinstetinal system[8]. The evalation of approximately 3.5 folds in serum ALP with normal AST and Alt levels observed in rats treated with 0.5 g/kg of *C. roseus* extract is clinically related to obstruction of bile ducts[12].

Results obtained from sub-acute repeated oral toxicity study showed that 0.1 g/kg of *C. roseus* extract was safe in female SD rats. No mortality, adverse effects and damages on the liver and kidney funtions were observed. Thus, the non-observable adverse effects level (NOAEL) of methanol leaves extract of *C. rosues* in rats was 0.1 g/kg. The NOAEL is the highest dose level that have no adverse findings observed related to the treatment[7]. From the World Health Organisation guideline, 14 d treatment in rats is equivalent to the single dose or repeated dose lesser than 7 d in humans[13]. Curry *et al* has previously calculated interspecies dose conversion from rats weighing 100 g body weight to humans with 60 kg body weight. The equivalent surface area dosage conversion factor from rat to humans was 1/7[14]. Therefore, methanol leaves extract of *C. roseus* at 0.1 g/kg (or 100 mg/kg) that orally administered to SD rats was equivalent to oral dose of *C. roseus* extract for human usage at 14.3 mg/kg (0.1 g/kg/7). Hence, single dose or repeated oral dosing less than one week of 14.3 mg/kg of *C. roseus* methanol leaves extract in humans is expected to have the same effects as observed in the present repeated dosing of 100 mg/kg of *S. crispus* ethanol leaves extract up to 14 d in female SD rats. This information could serve as a reference to other researchers for starting dose selection in human clinical trials. However, the correlation from experimental animals to humans need to be further elucidated by considering the species differences in toxico/pharmacokinetic aspects.

Several compounds such as alkaloids, flavonoids, tannins, triterpenes and soponins have been idetified from the leaves from *C. rosues*[15]. As been previously reported by Upmanya *et al*, a single dose of intravenous administration of 1 mg/kg of vincristine to male Wistar rats produced significant elevation of serum levels AST, ALT and ALP[16]. Any injury to the liver cells (hepatocytes) by the test substances would cause these hepatic enzymes to leak into the circulation and rise in the serum levels[17]. In additional to that, vinblastine and vincristine have been reported to be mainly metabolised by CYP 3A4 isoenzyme in the human livers to produce an active metabolite which

is called desacetylvinblastine[18]. The possible adverse effects observed on the rat liver surface in this toxicity study could be related to the metabolic activation by phase I drug metabolising enzymes. Biotransformation of a xenobiotic and endogenous compounds by drug metabolising enzymes such as cytochrome P450 can signifcantly alter its toxicity by producing reactive metabolites[19]. Another possible reason of causing mortality in rats received repeated dosing of 1 g/kg and 0.5 g/kg of *C. roseus* extract was due to the accumulation of vinca alkaloids and its bioactive metabolites in rat body system. Vinca alkoloids have been reported to have slow metabolism rate in liver and excretion through kidney which makes the alkaloids to accumulate in the body for longer period[20]. Thus, the toxic effects of test subtance could be varied based on the concentration of substance, duration of treatment and route of administration. Other than the toxic effects on liver functions, both vincristine and vinblastine have been reported to cause neurotoxicity that influences the motor and neuron functions along with bone marrow depression in humans[21]. This toxicity study could be further studied for understanding the mechanistic adverse effects of methanol leaves extract of *C. rosues* and ultimate accurate prediction human drug toxicity by incorporating long term toxicity, drug metabolism, toxicokinetics studies. Individual compound that responsible for adverse effects needs to be elucidated as well.

In conclusion, prolonged treatment by repeating dosing of 0.5 g/kg and 1 g/kg extract to rats for a few days caused diarrahoea and martality. Only 0.1 g/kg of *C. roseus* extract was safe without affecting both kidney and liver functions in female SD rats after 14 d treatment.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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