International Journal of Pharmacy
Journal Homepage: http://www.pharmascholars.com

Research Article **CODEN: IJPNL6**

SUB-ACUTE (28 DAYS) TOXICITY STUDY OF METHANOL LEAVES EXTRACT OF *CLINACANTHUS NUTANS* IN RATS

Jin Han Chin*, Gabriel Akyirem Akowuah, Mandumpal Chacko Sabu, Shaik Ibrahim Khalivulla

Faculty of Pharmaceutical Sciences, UCSI University, No. 1, Jalan Menara Gading, 56000, Cheras, Kuala Lumpur, Malaysia

***Corresponding author e-mail:** jhchin@ucsiuniversity.edu.my

ABSTRACT

Clinacanthus nutans (Family: Acanthaceae) or locally known as "Sabah snake grass" in Malaysia, is used for the treatment of herpes simplex infection, herpes zoster infection, diabetes mellitus, skin rash, dysuria and cancer. The objective of this study was to evaluate the safety of methanol leaves extract of *C. nutans* by sub-acute 28-day (repeated doses) oral administration to male Sprague Dawley (SD) rats. First group was orally treated with distilled water and served as control (n=6), whereas the remaining treatment groups (n=6) were orally treated with a single dose daily of 300 mg/kg, 600 mg/kg and 900 mg/kg of *C. nutans* extract, respectively for 28 days. Cage-side observations were done daily. The animal body weight, food consumption and water intake were recorded weekly. Blood was collected *via* cardiac puncture on day-29 and used to perform biochemical analyses, i.e. liver function tests, kidney function tests, total protein, glucose, lipid profile and haematology evaluation. The relative organ weights were also measured. All results were expressed as mean ± standard deviation and analysed using Dunnett's test. From the results obtained, repeated oral administration of methanol leaves extract of *C. nutans* (300 mg/kg, 600 mg/kg and 900 mg/kg) for 28 days did not cause mortality or adverse effects in rats. No significant change was observed in the body weight changed, water and food intake between the treatment group and control group. However, significant increase in serum total proteins (p<0.01), albumin/globulins ratio (p<0.01) and relative liver weight (p<0.01) were observed in male rats after 28 days treatment with 900 mg/kg of *C. nutans*. In conclusion, 28 days oral administration of methanol leaves extract of *C. nutans* up to 900 mg/kg was safe in male SD rats without causing any adverse effects. The Non-Observable-Adverse-Effect-Level (NOAEL) for sub-acute (28 days) effects of methanol leave extract of *C. nutans* in male SD rats was 900 mg/kg *bw*.

KEY WORDS: *Clinacanthus nutans*; NOAEL; Serum biochemical markers; Toxicity

INTRODUCTION

Clinacanthus nutans (Burm. f.) Lindau is classified under family of Acanthecaea. This small shrub is commonly known as "Sabah snake grass" or "belalai gajah" in Malaysia, has stimulated public interest in the recent years due to its high medicinal values [1]. *C. nutans* has been reported to have antiviral, anti-oxidant, anticancer and anti-inflammatory [2]. In Thailand, the uses of *C. nutans* topical preparations like cream and lotion to relieve insect bites, skin inflammation, lesions caused by the varicella-zoster virus and genital herpes in patients have been proven in a clinical trial [3]. Several bioactive constituents such as β-sitosterol, stigmasterol, C-glycosyl flavones, Orientin, vitexin, sulfur containing glycosides and glycoglycerolipids have been isolated and identified from the leaves of *C. nutans* [4-5]. The safety for this plant is not well established although it has been traditionally used for treating certain diseases and for health promotion. A study conducted by Chavalittumrong *et al*., (1995) on acute (1 day) study of 1.3 g/kg and sub-chronic (90 days) effect of 1.0 g/kg of ethanol leaves extract of *C. nutans* in mice and rats indicated that *C. nutans* did not produce any adverse effect in both species [6]. Up to date, the toxicity, drug interaction and adverse effects

after consumption of methanol leave extract of *C. nutans* are not completely known. Toxicological testing using animals is one the essential preclinical studies to examine the possible *in vivo* effects of test substances on the biological system and the severity of the of the effect that is present. The objective of the present study was to examine the possible sub-acute toxicity effects after 28 days oral administration of methanol leaves extract of *C. nutans* at 300 mg/kg, 600 mg/kg and 900 mg/kg in male Sprague Dawley rats in accordance to OECD 407 guideline. The objective could be achieved by analysing serum biochemical biomarkers and measurement of relative organs weights, body weight changed, food and water consumption throughout the experiment.

METHODOLOGY

Chemicals: Methanol and diethyl ether were supplied by Merck, Darmstadt, Germany.

Plant Material and Extraction: The fresh leaves of *C. nutans* were purchased from herbal park located at Pantai, Negeri Sembilan, Malaysia. The plant was authenticated by the Institute of Biological Sciences, Universiti Putra Malaysia. The fresh leaves were separated from the stems and air-dried under the shade for a week. The dried leaves were then blended into fine pieces with the blender. The fine dried leaves were macerated in methanol at room temperature for 3 days. The extraction was carried out repeatedly until the last extract became colourless. The extract collected was filtered through filter paper. The methanol extracts were concentrated as methanol solvent was evaporated using rotary evaporator under reduced pressure at 40^{o}C. Then, the crude extract was freeze-dried at -50^{o}C. Percentage yield from the extraction was calculated. The extract was then stored in the desiccator until further usage [7].

Sub-acute (28 days) Oral Toxicity Study: The present toxicity study was carried out in accordance to OECD 407 guideline (2008) [8]. All the procedures were approved by faculty ethical research committee (Proj-In-FPS-EC-13-001). Male albino rats aged 10 weeks ± 1 week old from Sprague Dawley strain with body weight between 150-170g were used in this oral toxicity study. All rats were acclimatised in an animal holding room maintained at 25-26^{o}C for 5 days prior to the experiment. Animals were randomly assigned into 4 groups with six rats in each group (n=6). First group was served as a control group which received distilled water as the vehicle. Second, third and fourth treatment groups were received single daily dose of 300 mg/kg, 600 mg/kg and 900 mg/kg via the oral route for consecutive 28 days. All the doses (300 mg/kg, 600 mg/kg and 900 mg/kg) were prepared freshly by reconstituting the methanol leaves extract of *C. nutans* in distilled water as vehicle. Starting dose at 300 mg/kg was selected based on the recommendation given by OECD 407 guideline. All rats, including the control group were monitored closely to see the appearance of toxic signs and symptoms. Body weight, food and water consumption were recorded weekly. Overnight fasting for 16 hours was performed to all rats after the last dose treatment at day-28. At day-29, approximately 2 to 3 ml of blood samples were collected from each animal through cardiac puncture under mild diethyl ether anesthesia. Blood samples were sent to UCSI University pathology laboratory within the same day of blood collection for analyses. The blood samples were used to determine the haematology parameters (haemoglobin, total white blood count, haematocrit, and platelet counts). The serum samples were used for biochemical analysis (aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total bilirubins, urea, creatinine, sodium and potassium, glucose, total protein, including ratio albumin/globulin, Total cholesterol and triacylglycerol. All rats were euthanised and the vital organs such (liver, kidney, lung, heart and spleen) were removed. Gross examination on the exterior surface of organs was carried out and the relative organ weight for each organ was determined.

Statistical Analysis: All data were presented as mean ± standard deviation. All results were analysed by using One-way ANOVA followed by post-hoc, Dunnett's test. Dunnett's test was used to compare the difference between treatment groups with the respective control group. Statistical difference was considered to be significant at $p<0.01$.

RESULTS

The percentage yield of methanolic extract obtained from 510.00 g of finely blended dried *C. nutans* leaves was 15.29 % w/w. Based on the cage-side observation, the rats that received the *C. nutans* treatment did not demonstrate noticeable toxic signs and symptoms at any of the doses tested in this study. There were no significant changes in body weight, food consumption and water intake in rats after treatment with *C. nutans* for 28 days at any doses (Figure 2). After 28 days of treatment, male rats treated with *C. nutans* extract at the dose of 900 mg/kg showed a significant increase in relative liver weight ($p<0.01$), total proteins ($p<0.01$) and albumin/globulin ratio ($p<0.01$) when compared to

the control group (Figure 1 & 3). In additional to that, treatment of methanol leaves extract of *C. nutans* at 300 mg/kg and 600 mg/kg for the duration of 28 days did not alter serum biochemical markers for liver and kidney functions as well as glucose, protein and lipid profiles and haematology parameters in rats when compared to the control group (Figure 3-5).

DISCUSSION

Liver and kidney are the prime targets for drugs and chemicals ingested through the oral route [9]. In general, elevation of the serum levels of AST, ALT, GGT or total bilirubins indicates hepatic damage while the elevation of serum levels of urea and creatinine indicates kidney failure [10]. Based on the biochemical analysis, the *C. nutans* extract treated rats and control rats exhibited insignificant changes in the serum levels of AST, ALT, GGT, total bilirubins, urea, creatinine, sodium and potassium. Serum biochemical analysis revealed that 28 days oral administration of *C. nutans* methanol leaves extract did not cause damage to the livers and kidney. This was further supported by insignificant difference was observed in relative organ weight for liver and kidney obtained from *C. nutans* treated animals and control rats.

Normally, the relative organ weight for liver and kidney will increase due to swelling if the test substance is inducing damage to the respective organs [10]. Haematological parameters were included in the present study to examine the effect of *C. nutans* treatment on blood production and formation in rats. Haematological analysis is diagnostically importance index in assessing the physiological and pathological status of health of an organism after exposure to toxic compounds.[11] Based on the haematological analysis, the concentration of red blood cells (RBC), haemoglobins, platelets, haematocrit and total white blood cells (TWBC) showed insignificant difference between treatment and control group. This indicated that *C. nutans* extracts (300 mg/kg, 600 mg/kg and 900 mg/kg) did not interfere with the hematopoiesis and leucopoiesis in rats. Based on the results obtained, treatment with 900 mg/kg (p<0.01) of *C. nutans* leaves extract for 28 days elevated total serum protein level, ratio albumin & globulin and relative liver weight in male SD rats. Serum proteins play important functions in maintaining water distribution between blood and tissues [12].

Most of the serum proteins are synthesised in the liver and albumin is the major protein, accounting for 55-60% of the serum protein. Elevation of total protein in the blood is an importance marker in diagnosing acute inflammatory processes and tissue injuries [13-14]. Acute inflammatory is defined as the initial response of the body to tissue injury by releasing autacoids and it is usually proceeding with the development of the immune response [15-16]. However, the elevation of 7.7 % total proteins and 10% of albumin/globulin ratio in rats treated with 900 mg/kg was not associated with the significant changes in total white blood cells and other parameters measured. Higher ratio of albumin over globulin indicating the concentration of albumin in blood is higher than globulins. Based on this observation, the possibility of *C. nutans* treatment causing acute inflammatory could be ruled out. In general, induced inflammation in rats will decrease the rate of albumin synthesis by reducing the concentration of albumin mRNA [17].

Hence, the possible explanation of the elevation of total proteins in the blood might be due to the effect of *C. nutans* leaves extract in inducing the biosynthesis of albumin and other proteins such as enzymes in rat liver. This effect of *C. nutans* on the rat's liver was further supported by the increment of relative liver weight. However, the actual mechanism that responsible for the biosynthesis of albumin in rat liver remains unknown. Although the level of total protein and relative liver weight were significantly higher in rats treated with *C. nutans* at 900 mg/kg, but these animals did not present either sign of toxicity or organ damages. This suggested that the repeated oral administration of the *C. nutans* methanol leaves extract (300 mg/kg, 600 mg/kg and 900 mg/kg) for duration of 28 days was safe in rats. However, precaution step should be made of the possibility drug-albumin interaction after treating with 900 mg/kg of *C. nutans* extract.

These drugs are including salicylate, warfarin, indomethacin, digitoxin, phenytoin and some penicilins [17]. Laboratory animal toxicity study is carried out with the aim to extrapolate the doses and effects on humans. There are few formulations available for converting dose and treatment duration from animals to humans. No-observed-adverse-effect-level (NOAEL) is defined as the highest dose where no adverse treatment-related findings are observed in animals due to treatment [9]. Based on the results obtained, the NOAEL for methanol leaves extract of *C. nutans* was determined as 900 mg/kg in male SD rats. According to the general research and evaluation of traditional medicine guideline described by World Health Organisation (2000), four weeks or equivalent to 28 days treatment duration for the toxicity study of test substance in animals is

equivalent to expected clinical usage of one week in humans [18]. Reagan-Shaw *et al* (2008) has reported on the dose translation from animal to human studies [19]. Based on this normalisation method, human equivalent dose (HED in mg/kg) is calculated by multiplying 900 mg/kg of *C. nutans* tested in rats by the K_m factor (6) for a rat with 150 g body weight and then divide by the K_m factor (37) for a human with 60 kg body weight. Thus, this calculation gives the HED of *C. nutans* methanol leaves extract as 145.9 mg/kg for a human with 60 kg body weight.

CONCLUSION

In conclusion, results obtained from the repeated dose 28-day oral administration of *C. nutans* methanol leave extract in rats suggested that the extract was devoid from the toxic effect or organ damage although an elevation of serum protein profiles and relative liver weight was observed in male rats treated with 900 mg/kg of *C. nutans*. The NOAEL for 28 days of *C. nutans* methanol leave extract in male rats was 900 mg/kg. Further study on the chronic (90 days) effect of *C. nutans* needs to be carried out to confirm the safety of long term usage of *C. nutans*.

ACKNOWLEDGEMENT

Authors would like to thank the financial support provided by the Centre of Excellence for Research, Value Innovation and Entrepreneurship (CERVIE), UCSI University (Proj-In-FPS-003) to complete this study.

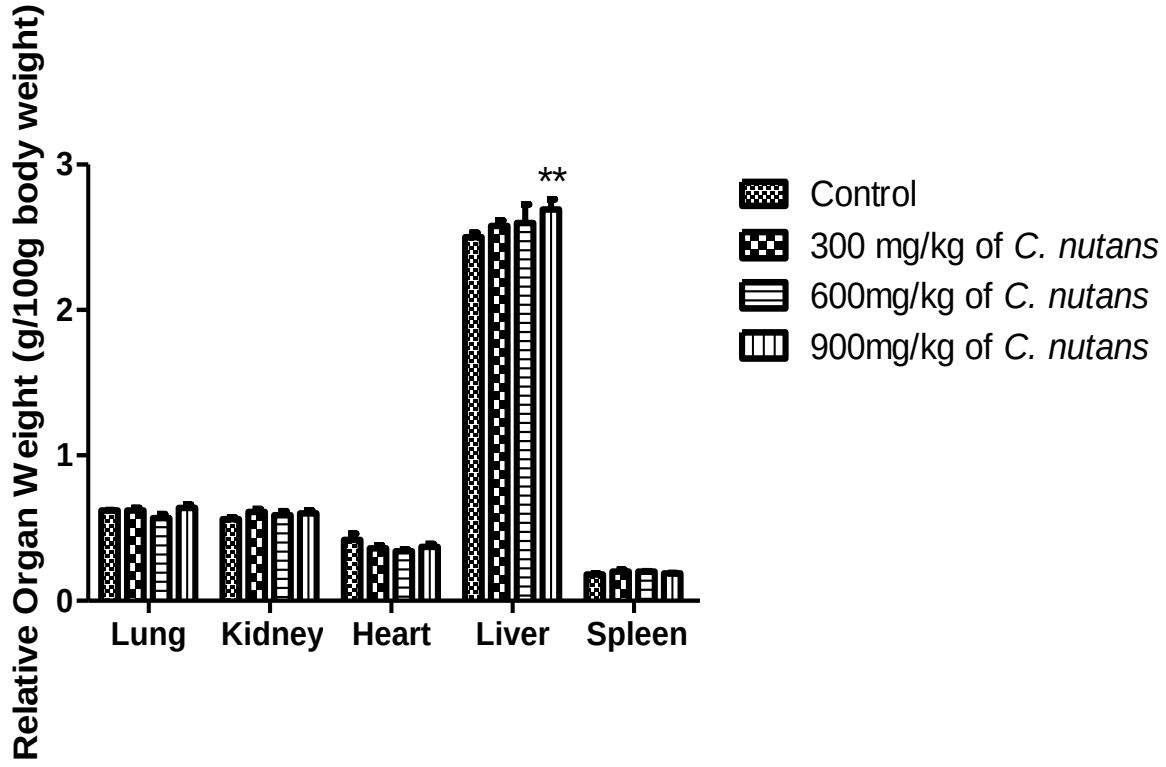

Figure 1: Effect of 28 days oral administration of methanol leaves extract of *Clinacanthus nutans* on relative organ weight in male SD rats *(N=6, Data = mean ± standard deviation; Results were analysed using Dunnett's test; **p<0.01 compared to control)*

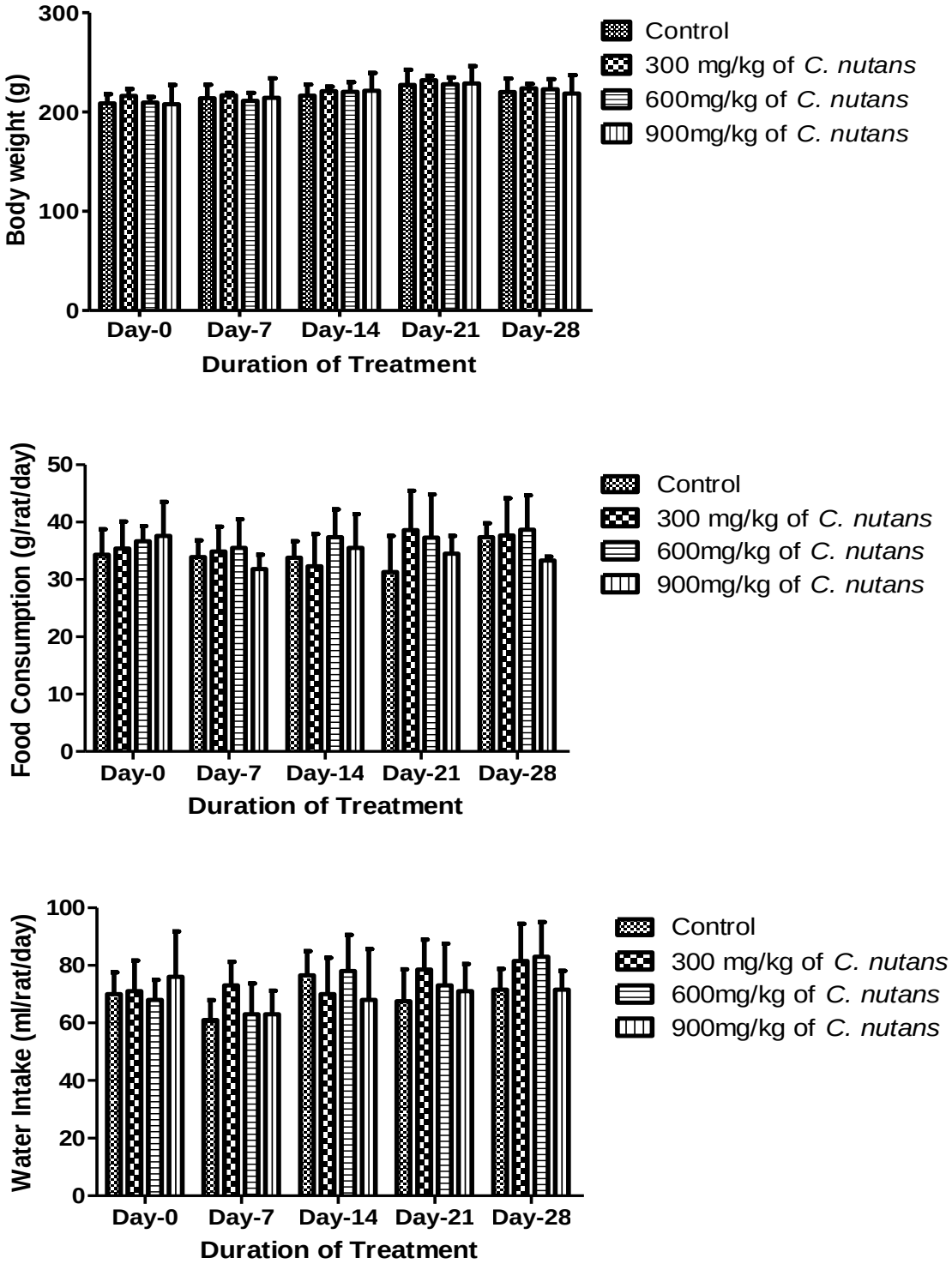

Figure 2: Effect of 28 days oral administration of methanol leaves extract of *Clinacanthus nutans* on body weight, food and water intake in male SD rats. (*N=6, Results = mean ± standard deviation; Results were analysed using Dunnett's test)*

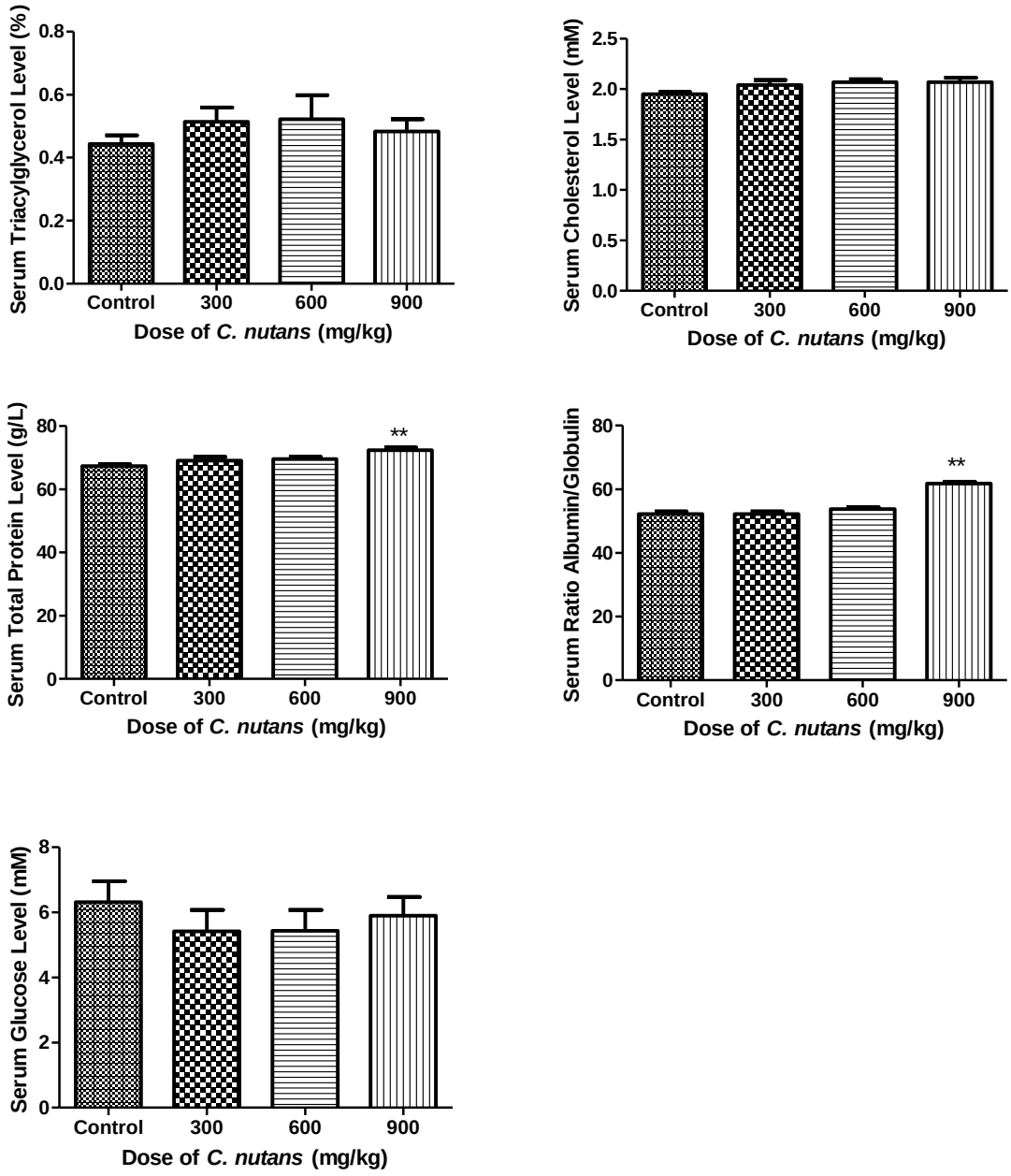

Figure 3: Effect of 28 days oral administration of methanol leaves extract of *Clinacanthus nutans* on serum levels of glucose, protein and lipids in male Sprague Dawley rats *(N=6, Data = mean ± standard deviation; Results were analysed by Dunnett's test; ** p<0.01 significant difference compared to control group)*

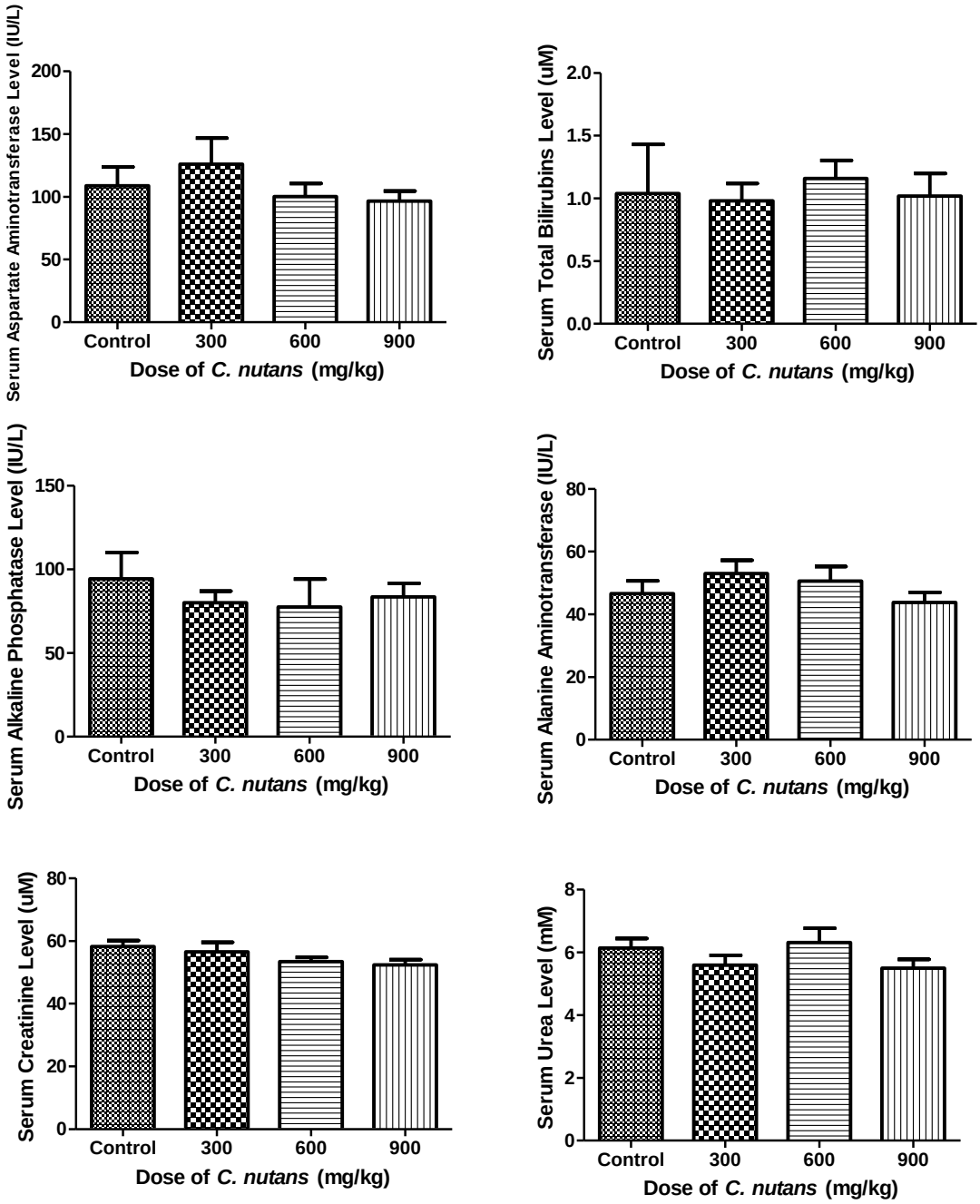

Figure 4: Effect of 28 days oral administration of methanol leaves extract of *Clinacanthus nutans* on serum levels of AST, ALT, ALP, Total bilirubins, urea and creatinine in male Sprague Dawley rats *(N=6, Data = mean ± standard deviation; Results were analysed by Dunnett's test)*

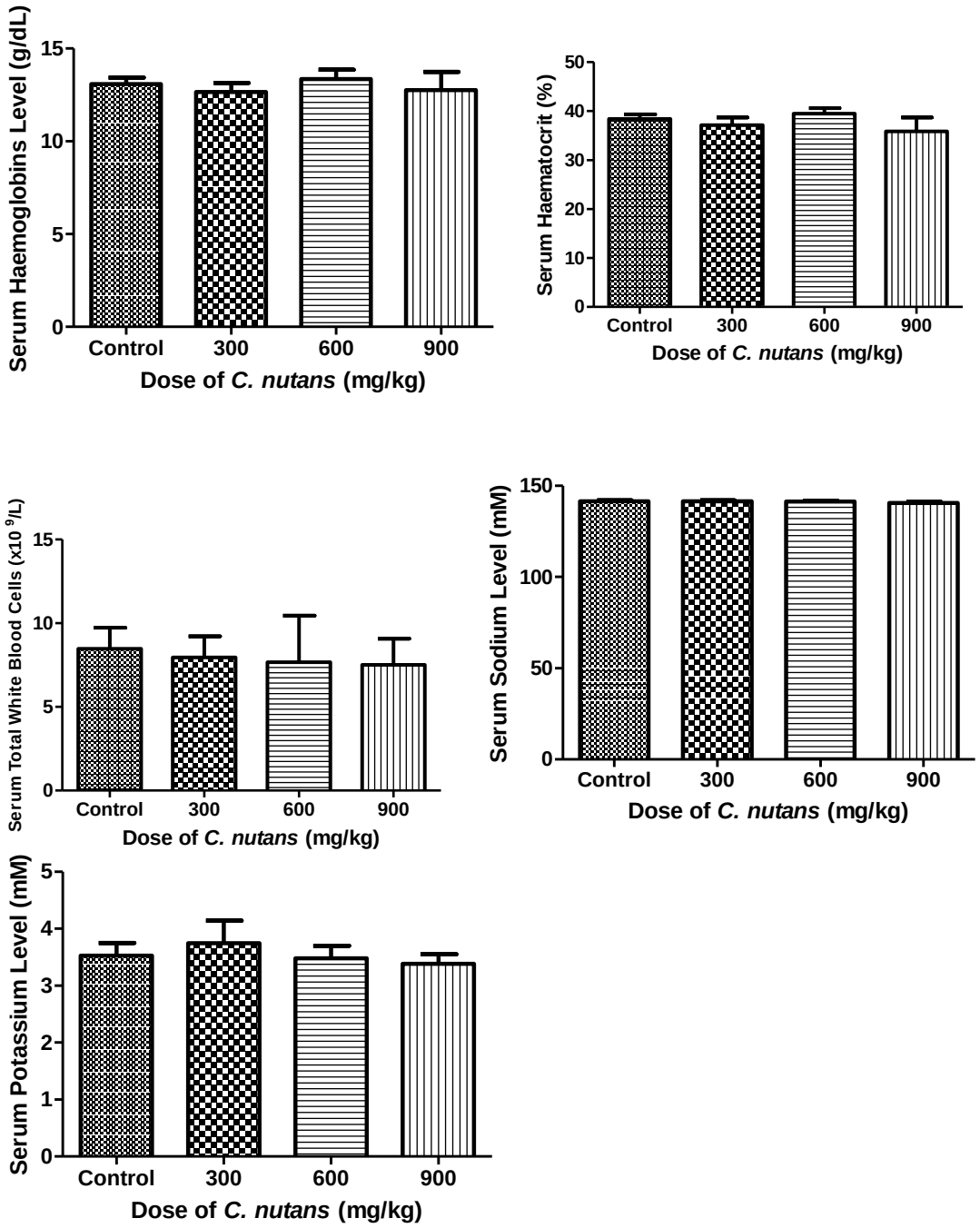

Figure 5: Effect of 28 days oral administration of methanol leaves extract of *Clinacanthus nutans* on haematology parameters and serum levels of electrolytes in male SD rats *(N=6, Data = mean ± standard deviation; Results were analysed using Dunnett's test)*

REFERENCES

1. Yong YK, Tan JJ, The SS, Mah SH, Ee GCL, Choing HS, Ahmad Z. Evid Based Complement Alternat Med, 2013; 1-8.
2. Sakdarat S, Shuyprom A, Pientong C, Ekalaksananan T, Thongchai S. Bioorg. Med. Plants, 2009; 17: 1857-60.
3. Kongkaew C, Chaiyakunapruk N. Complement therapies in medicine, 2011; 19(1): 47-53.
4. Sakdarat S, Shuyprom A, Dechatiwongse Na Ayudhya T, Waterman PG, Karagianis G. Thai J Phytopharm, 2006; 13(2): 13-24.
5. Teshima K, Kaneko T, Ohtani K, Kasai R, Lhieochaiphant S, Picheansoonthon C. Phytochem, 1998; 48(5): 831-5.
6. Chavalittumrong P, Attawish A, Rungsamon P, Chuntapet P. Bull Dep Med Serv (Thai), 1995; 37: 323-38.
7. Akah PA, Okolo CE, Ezike AC. Afr J. Biotechnol, 2009; 8(10): 2389-93.
8. OECD 407 Guidelines for the testing of chemicals. Repeated dose 28-day oral toxicity study in rodents. 2008.
9. Timbrell JA. Principles of Biochemical Toxicology. 4th ed., New York; Informa Healthcare: 2009.
10. Adeneye AA, Olagunju JA, Elias SO, Olatunbosun DO, Mustafa AO, Adeshile OI. Intl. J. of Appl. Res. in Nat. Prod, 2008; 1(3): 29-42.
11. Adeneye AA, Ajagbonna OP, Adeleke TI, Bello SO. J. Ethnopharmacol, 2006; 105: 374-9.
12. Lieberman M, Marks AD. Mark's Basic Medical Biochemistry: A clinical approach. 3rd ed., Philadelphia; Lippincott Williams & Wilkins: 2009.
13. Murray RK. Plasma proteins and immunoglobulins. In: Murray RK, Bender DA, Botham KM, Kennelly PJ, Rodwell VW and Weil PA (eds.). Harper's Illustrated Biochemistry, New York; McGraw Hill Lange: 2009, pp. 568-9.
14. Vasile M, Teren O, Ciupina V, Turcu G. Rom J Respiration Physiol, 2009; 61: 121-8.
15. Furst DE, Munster T. Nonsteroidal anti-inflammatory drugs, disease-modifying antirheumatic drugs, nonopioid analgesics & drugs used in gout. In: Katzung BG (ed). Basic & Clinical Pharmacology, New York; McGraw Hill: 2001, pp. 596-7.
16. Santos JA, Arruda A, Silva MA, Cardoso CAL, Vieira MDC, Kassuya CAL. J. Ethnopharmacol, 2012; 144: 802-5.
17. Nicholson JP, Wolmarans MR, Park GR. British J. Anaesthesia, 2000; 85(4): 599-610.
18. General Guidelines for methodologies on research and evaluation of traditional medicine. Geneva: World Health Organization. 2000.
19. Reagen-Shaw S, Nihal M, Ahmad N. The FASEB J, 2008; 22(3): 659-61.