Original Research Article

EFFECT OF METHANOL EXTRACT OF *CLINACANTHUS NUTANS* ON SERUM BIOCHEMICAL PARAMETERS IN RATS

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ABSTRACT

The present study aims to evaluate *in vitro* free radical scavenging activity of methanol extract of *Clinacanthus nutans* and the effect of the extract on liver and kidney function parameters in normal rats. The free radical scavenging activity was estimated by using DPPH assay. The methanol extract was administered orally at a single dose of 2 g□kg−1 of body weight to female Sprague Dawley rats, and were monitored for 14 days. Blood samples were collected from each rat on day-15 after overnight fasting under light ether anesthesia. Blood serum was separated by centrifugation and used for biochemical analyses. The extract showed dose-dependent free radical scavenging activity with IC_{50} value of 1.33 ± 0.001 mg/ml. Single dose oral administration of *C. nutans* leaves extract at 2 g/kg dose did not produce any toxic signs or deaths in female SD rats. There were no significant ($P > 0.05$) differences in alanine aminotranferease (ALT**),** aspartate aminotransferase **(**AST**)** , alkaline phosphatase (ALP), total bilirubins, urea and creatinine levels between treatment rats and control rats. Infra red (IR) analysis of the extract showed the presence of C-O stretch, C=O stretch and OH band. The UV-visible absorption spectrum confirmed that the extract contains UV-active compounds.

Keywords: *Clinacanthus nutans*, Biochemical parameters, Free radical scavenging, FT-IR, Serum,

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INTTRODUCTION

Clinacanthus nutans (Burm. f.) Lindau (Acanthaceae) is a medicinal herb widely distributed throughout the tropical regions including Southeast Asia and China [1]. *Clinacanthus nutans* is widely used as medicine to treat nettle rash, dysentery, heals burns, scalds, insect stings, cures oral inflammatory symptoms and fever and it is categorised as a vital medicinal plant for human's health [2]. In Sukajadi village of West Java, Indonesia, the leaves of *C. nutans* is traditionally used as a medicine to treat dysentery [3]. *C. nutans* leaf extracts was reported for the treatment of genital herpes and varicella zoster were reported [4].

Herbal medicines are considered safe hence their safety profiles are not adequately documented. Furthermore, herbal medicinal products are not covered under drug-regulatory criteria in many countries. Toxicological evaluations using Organisation for Economic Cooperation and Development (OECD) guidelines need to be undertaken to establish safety profiles of herbal medicines [1]. The present study describes *in vitro* free radical scavenging activity and acute effect

of methanol extract of *Clinacanthus nutans* on serum biochemical parameters in female Sprague Dawley (SD) rats. The study also includes Fourier transform infrared (FT-IR) and Ultraviolet-visible (UV-vis) spectroscopy fingerprints of the extract.

MATERIALS AND METHODS

Chemicals and reagents

Gallic acid, sodium carbonate anhydrous, sodium nitrate, potassium bromide, aluminium trichloride, sodium hydroxide, Folin-Ciocalteu's phenol reagent, and methanol, were from Merck (Germany). Quercetin and 2,2-diphenyl-1-picryhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were of analytical or HPLC grade. Water used was of Millipore quality.

Plant materials

Four kilograms of *C. nutans*, authenticated by Jabatan Perhutanan Negeri Sembilan, were brought from Yik Poh Ling Herbal Farm, Seremban. The leaves were separated from the stalks and air dried under shade for a week. The dried leaves were grinded into powder using blender (Waring, Malaysia). A voucher specimen is kept at the Department of Pharmaceutical Chemistry, UCSI University.

Plant extraction

The powdered dried leaves (255 g) were macerated with methanol (1 l) in covered conical flasks for 3 days at room temperature (25 ± 2 °C). The solution was stirred using magnetic stirrer. The mixture was filtered using filter paper (Double Rings, China) and concentrated using rotary evaporator (Büchi, Switzerland) at 40 °C. The crude extract was freeze-dried. The crude freeze-dried extract was kept in a desiccator filled with silica desiccant until further analysis.

UV-visible spectrophotometry

Perkin-Elmer double beam UV-visible spectrophotometer Lambda 25 with 1 cm matched quartz cells was used for spectral analysis and measurement of the UV-Vis spectra. *C. nutans* methanolic extract was reconstituted with methanol into final concentration of 1 mg/ml. Sample (1 mg/ml) in 1.0 cm optical path quartz cell (PerkinElmer, USA) was scanned from 200 to 700 nm. Methanol was used as blank.

Fourier transform infrared spectroscopy *(*FT-IR*)*

The FT-IR spectroscopy analysis of the freeze-dried extract was recorded in potassium bromide (KBr) disc. A small portion of KBr salt was transferred and ground in an agate mortar. Powder of KBr was transferred to compressor and compressed to become a thin disc. FT-IR spectroscopy in the mid-IR range of 4000 – 400 cm spectrum was recorded on Thermo Nicolet Avatar 350 FT-IR spectrometer.

Estimation of total phenolic content

Total phenolic content of the extract was determined using Folin–Ciocalteu reagent . Concentration of the extract prepared was 10 mg/ml. Sample was prepared by mixing 0.4 ml of 10 mg/ml of extract with 0.4 ml of Folin–Ciocalteu reagent (50% v/v) and 2 ml of 15% sodium carbonate. Sample was diluted with deionised water (1.2 ml) prior incubation for 2 h at room

temperature. Gallic acid (GA) was used to plot standard calibration curve. Six concentrations of GA standard solution ranging from 0.1 to 0.6 mg/m1 were prepared to obtain the standard calibration curve. Absorbance of each sample was measured at 760 nm using UV-Vis Spectrophotometer. All tests were performed three times and averaged. Total phenolic content of the extract was expressed as milligrams of gallic acid equivalents (GAE) per 1 g of dry plant extract.

Estimation of total flavonoid content

Total flavonoid content of the extract was determined using aluminium trichloride . Samples were prepared by mixing 0.1 ml of extract (10 mg/ml) with 0.3 ml of deionised water followed by 0.03 ml of 5% sodium nitrate and incubated for 5 min before 0.03 ml of 10% aluminium trichloride was added into the mixture. After 5 min, 0.2 ml of 1 mM sodium hydroxide solution was added and diluted with deionised water to a total volume of 2 ml. Concentrations of quercetin solution ranging from 0.2 to 0.8 mg/ml were used to obtain the standard calibration curve. Absorbance of each sample was measured at 510 nm using spectrophotometer. All tests were performed three times and averaged. Total flavonoid content of the extract was expressed as milligrams of quercetin equivalents per 1 g of plant extract.

Free radical scavenging activity

Free radical scavenging activity of the extract was measured using 1,1-diphenyl-2-picryl-hydrazil, DPPH (Sigma-Aldrich, USA). DPPH solution (0.1 mM) was prepared in methanol and 2 ml of the solution was mixed with 0.2 ml of various concentrations of extract, ranging from 0.25 to 10 mg/ml. The final volume of the solution was adjusted with methanol to the final volume of 3 ml and incubated in dark area at room temperature for 30 min. The absorbance of the sample solution was measured using spectrophotometer at 517 nm against methanol as blank. Quercetin (1 mg/ml) was used as positive control. The absorbance of samples was compared with control, consisting 2 ml DPPH solution and 1 ml of methanol [5]. All tests were performed three times. The activities of the extracts were calculated using the following formula:

Free radical scavenging activity = (Ac – As) / Ac x 100%

where Ac is absorbance value of control and As is absorbance value of samples.

Acute (1 day) oral toxicity

A total of 12 young male SD rats aged between 10-12 weeks old were used for the evaluation of acute oral toxicity of methanol extract of *C. nutans* leaves according to OECD 423 guideline [6]. All the rats were randomly assigned into two groups with six animals per group. First group was served as control and treated with distilled water (as vehicle) while the second group was served as treatment group that received single dose (1 day treatment) of 2 g/kg of *C. nutans* extract. Limit dose at 2 g/kg was chosen based on the recommendation given by OECD 423 guideline [6]. Cage-side observation was conducted for 14 days to see the appearance of any toxic signs due to *C. nutans* treatment. Body weight changes, food and water intake were recorded on day-0, day-3, day-7 and day-14. Blood samples were collected from each rat on day-15 after overnight fasting under light ether anesthesia. Blood serum was separated by centrifugation and used for biochemical analyses at UCSI University Path Lab (Kuala Lumpur, Malaysia). All the rats were sacrificed to obtain relative organs weight for liver, kidney, lung, spleen and heart.

Serum Biochemical parameters

The following serum biochemical parameters were estimated, urea, and creatinine for kidney function; alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilurubin for liver function. The serum samples were analysed by using Roche Cobas C311 chemistry analyzer (Roche, Switzerland).

Statistical Analysis

Results were presented as mean ± standard deviation. Results for acute toxicity study were analysed using Student's t-test. $p<0.05$ was considered as significant difference when compared to the respective control group.

RESULTS AND DISCUSSION

Extraction

Maceration was used to obtain crude extract from the dry leaf powder of *C. nutans*. Maceration is a simple and cost-effective extraction technique which gives good and selective extraction. It uses limited volume of solvents. However, it requires the analytes to be sufficiently soluble into the solvent in order to be extracted from the leaves. To improve efficacy of extraction, the mixture of solvent and leaves powder were stir constantly throughout the extraction process. This ensures the concentrated solution is dispersed and accumulated around the surface of the particles, thereby bringing fresh menstruum to the particle surface for further extraction [7].

Percentage yield of 15.29 % w/w was obtained by extraction of leaf powder (255.00 g) through maceration using methanol as solvent. The containers used for the maceration were covered with aluminium foil to reduce possibility of photo-degradation of the extracts.

UV-Visible spectrophotometry

The UV-vis absorption spectrum of the extract is shown in Figure 1. The extract was found to contain compounds which absorb UV. UV-vis spectrum of the extract showed two peaks (λ_{max}) at 272.58 and 334.95 nm. This indicates the presence of specific chromophores in the structure of the constituents of the extracts that absorb at these particular wavelengths. The UV-Vis spectra of flavonoids (flavones) have been reported to exhibit two major peaks between 240 – 400 nm due to excitation of π-electrons [8].

Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectroscopy was useful to detect characteristic peaks and functional groups of compounds present in *C. nutans* methanolic extract. FT-IR spectrum of the extract is shown in Figure 2. The spectrum showed -OH band at frequency range 3200-3600 cm^{-1}, C-H stretch at frequency range of 2800-3000 cm^{-1}, C=O stretch at frequency of 1628 cm^{-1} and C-O stretch at frequency of 1050-1400 cm^{-1}. The presence of -OH band and C=O stretch suggests that the constituents in the extract have -OH group and C=O group as functional groups. Phenolic and flavonoid compounds are compounds containing hydroxyl and carbonyl group as functional groups.

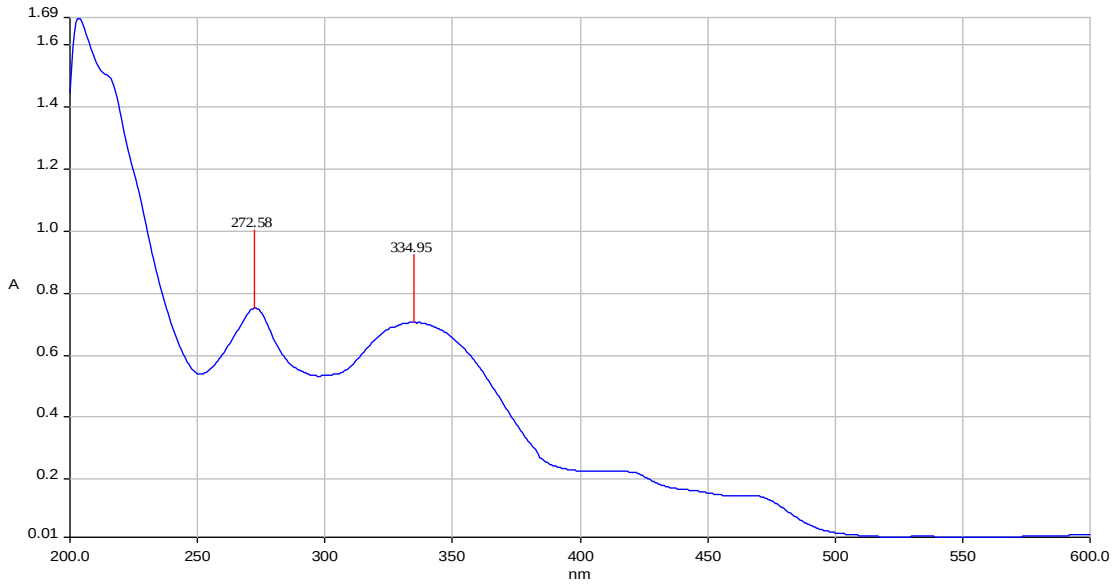

Figure 1: UV-Visible spectrum of *Clinacanthus nutans* leaf extract.

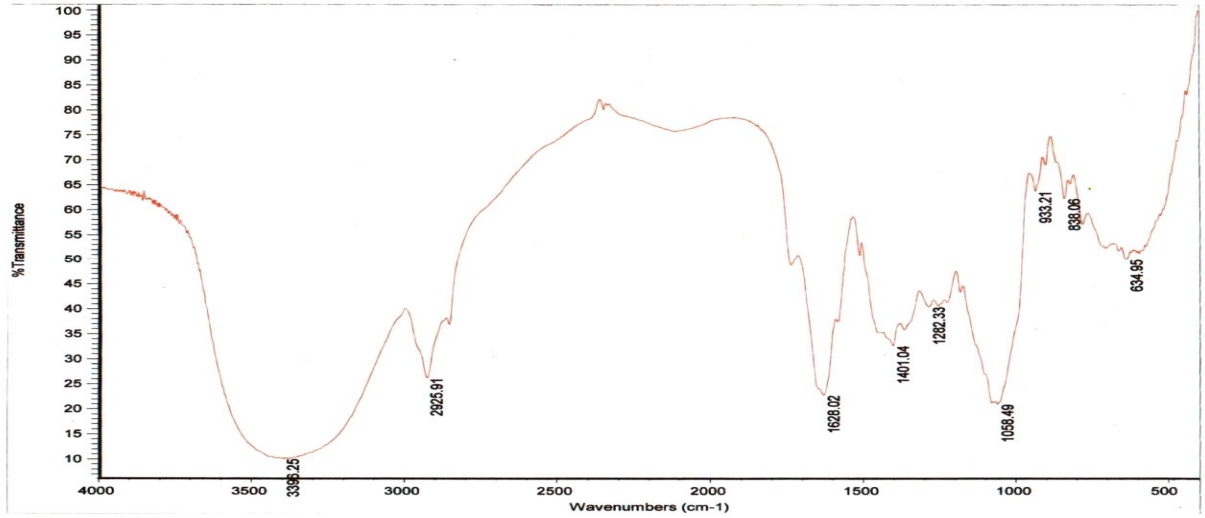

Figure 2: FT-IR spectra of *Clinacanthus nutans* leaf extract.

Estimation of total phenolic contents

Total phenolic content of the extract was 0.78 ± 0.005 mg GA/g dry extract. Phenolic compound is one of the important secondary metabolites and bioactive compounds of plant. The estimated total phenolic content in *C. nutans* can be associated with its functions. Polyphenols are bioactive constituents present in food plants which are very important in the control and prevention of tissue damage by activated oxygen species due to their antioxidant effects [9].

Phenolic compounds are reported to increase bile secretion, reduces blood cholesterol and lipid levels and inhibit microbial growth. Phenolic compounds are also crucial for plant growth and plant reproduction. [10]

Estimation of total flavonoid content

Total flavonoid content of the extract was 0.21 ± 0.005 mg QE/g. Total phenolic content was higher compare to total flavonoid content in extract. Flavonoid is a sub-class of phenolic and known with its polyphenolic structures. Flavonoids play important role in protecting biological systems against harmful effects of oxidative processes on macromolecules. Flavonoids are reported posses antiulcer activity, hepatoprotective activity, anti-inflammatory activity, anti-diabetic effects, vasorelaxant process, anti-atherosclerotic effects, anti-thrombogenic effects, cardioprotective effects and anti-neoplastic activity [11].

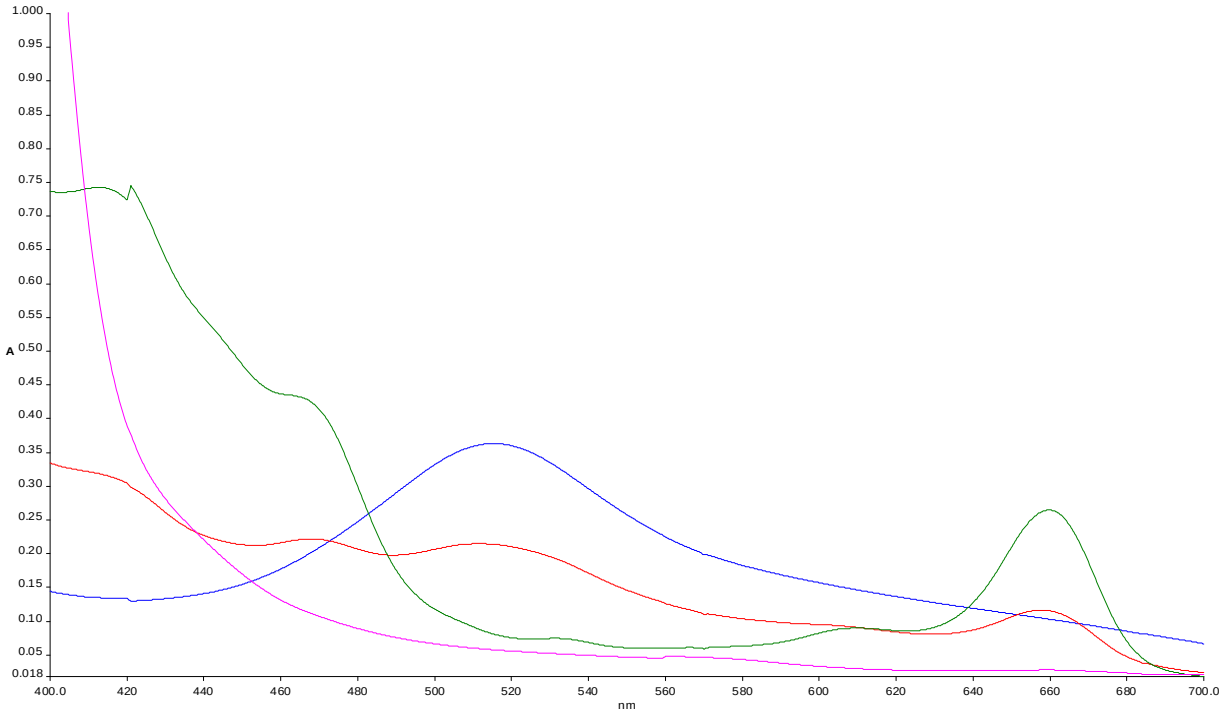

Figure 3: UV- Visible spectra of DPPH solution (0.1 mM) and 60 min after mixing with methanol extract of *C. nutans* (1 mg/ml, 3 mg/ml), quercetin (1 mg/ml) .

Free Radical Scavenging Activity

Scavenging of the stable free radical diphenylpicrylhydrazyl (DPPH) radical is one of the known methods to evaluate antioxidant activity of extracts and active compounds *in vitro*. It is characterised as stable free radical by virtue of the delocalisation of spare electron over the molecule as whole. The reduction of DPPH causes the decolouration the purple colour of DPPH to deep violet colour with loss of absorbance at 517 nm [12]. In this study, The *C. nutans* methanolic leaves extract showed dose-dependent free radical scavenging activity with IC_{50} value of 1.33 ± 0.001 mg/ml. The free radical scavenging activity of the extract was lower compare to the positive reference compound, quercetin (1 mg/ml). The spectral change of DPPH solution after mixing it with the solution of the extracts and positive reference compound

(quercetin) is shown in Figure 3**.** Quercetin at 1mg/ml which has lowest absorbance value, is the most potent antioxidant compare to the extract at 1 mg/ml and 3 mg/ml.

The presence of phenolic and flavonoid compounds in the extract contributed to the antioxidant activity of the extract. Phenolics and flavonoids are known as anti-oxidant agents and act as free radical oxidation terminator [13]. Phenolic compounds have many phenolic hydroxyl groups and as a result have many active points in radical scavenging. Though the presence of phenol structure render compounds effective free radical inhibitors, hydrogen atoms not existing as phenolic groups have been recorded to work for radical scavenging [14] .The FT-IR spectrum of the extract showed a broad peak near 3400 cm^{-1} due to -OH stretching vibration. UV-vis spectrum of the extract showed two major between 240 -400 nm which indicate the presence of flavonoids in the extract [8]. These observations confirm the existence of -OH moieties which have the ability to terminate propagation of chain carrying radicals by acting as H-atom donor.

Acute (1 day) oral toxicity

No mortality and treatment related changes in general state, behavior and external appearance was observed in the *C. nutans* treated female rats up to the 2 gkg^{-1} single dose. Body weight, food and water consumption showed no treatment-related alterations at the end of 14 days observation period (Table 1). The extract did not produce any toxic signs or deaths hence the 50% lethal dose must be higher than 2 g□kg−1.

Medicinal plants serve as the source of traditional medicine to treat various diseases. Therefore, medicinal plants play an important role as complementary and alternative medicine as there is growing acceptance of traditional medicine for certain diseases Traditional healers utilised the active ingredients of the medicinal plants to achieve therapeutic activity. It is estimated that at least 25% of modern medicines are derived from medicinal plants, directly or indirectly [15].

Table 1: Acute (1 day treatment) effect of methanol extract of *Clinacanthus nutans nutans* leaves on body weight, food consumption and water intake in female rats.

Grouping (g/kg)	Day-0	Day-3	Day-7	Day-14
	Body Weight Changed (g)			
Control	155.8±7.24	173.3±10.0	188.3±17.03	241.1±17.56
2g/kg *C. nutans*	153.3±12.63	176.6±11.4	196.3±10.88	244.5±12.76
	Water Intake (ml/rat/day)			
Control	37.5±1.88	38.33±1.91	45.0±2.25	31.67±1.58
2g/kg *C. nutans*	35.0±1.75	40.83±2.04	42.5±2.12	30.0±1.50
	Food Consumption (g/rat/day)			
Control	23.11±1.16	26.03±1.30	28.82±1.44	19.21±0.96
2g/kg *C. nutans*	21.58±1.08	23.81±1.19	28.85±1.44	21.24±1.06

Data are expressed as mean ± standard deviation (n= 6), Data was analysed by using Student's t-test . $p<0.05$ was considered as significant difference when compared to the respective control group.

Effect of the extract on biochemical parameters

In the present study, there were no significant differences in AST, ALT, ALP, total bilirubins, urea and creatinine levels between treatment and control group (Table 2). Hence, *C. nutans* does not cause either hepatic or renal toxicity. This was further supported by gross pathology on the liver and kidney and measurement of relative organ weight whereby no treatment related difference in lesions or relative organ weights was observed (Figure 4).

Table 2: Acute effect of methanol extract of *Clinacanthus nutans* on serum biochemical parameters in female rats.

Parameters (Unit)	**Groupings**	
Kidney Function Tests	**Control**	***C. nutans* (2.0 g/kg)**
Urea (mM)	4.8±0.74	4.1±0.64
Creatinine (µM)	44±4.76	43±2.16
Liver Function Tests	**Control**	***C. nutans* (2.0 g/kg)**
Alkaline Phosphatase (IU/L)	302.0±66.2	303.8±59.1
Alanine Aminotransferase (IU/L)	49.3±8.22	53.3±14.41
Aspartate Aminotransferase (IU/L)	177.2±14.43	222.3±55.78
Total Bilurubin (µM)	1.0±0.31	0.7±0.22

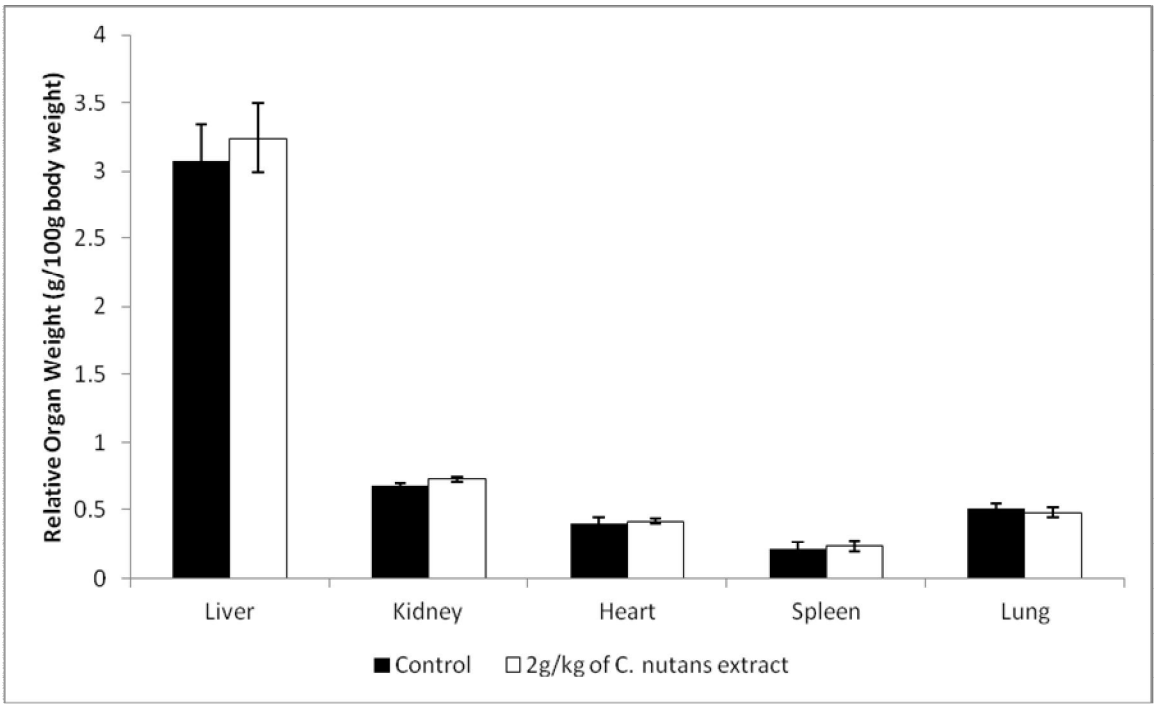

Figure 3: Acute (1 day treatment) effect of methanol extract of *Clinacanthus nutans* leaves on relative organs weight in female SD rats.

Data are expressed as mean ± standard deviation (n= 6). Data was analysed by using Student's t-test . $p<0.05$ was considered as significant difference when compared to the respective control group.

Based on the results obtained from this study, it was concluded that a dose of 2 g/kg of the methanol extract of *C. nutans* leaves given orally to female rats appeared to be non toxic and the oral LD_{50} value is higher than 2 g/kg in female rats. This study provides important preliminary data on the acute toxicity profile of *C. nutans* and further investigation on the long term effect of this plant would be necessary.

CONCLUSION

In conclusion, treatment of female rats with a single dose (1 day treatment) of 2 g/kg of *C. nutans* extract showed no lethality and adverse effects in female rats. The extract demonstrated in vitro free radical scavenging activity. The qualitative UV-visible spectroscopy analysis confirmed that the extract contain compounds which are UV-active. The FT-IR analysis of the extract showed that the chemical constituents in the extract contain hydroxyl and carbonyl functional groups. The leaves of the plant can be explored as non-toxic natural antioxidant by the pharmaceutical industries. Characterisation of the bioactive components of the extract by liquid chromatography mass spectrometry is in progress.

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