

Research Article



Sub-Acute Toxicity Study of *Cycas circinalis.L* and *Ionidium suffruticosum* Ging in Wistar Albino Rats

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ABSTRACT

Pharmacological and toxicological evaluations of medicinal plants are essential for drug development. *Cycas circinalis* and *Ionidium suffruticosum* are the two herbs which had been used since ancient times for treating various ailments traditionally in Indian system of Medicine (Siddha Medicine), but the medicinal effects of both the herbs were yet to be proved scientifically. The present study was done to evaluate the sub-acute toxicity of both the medicinal herbs as a preliminary part in proving its medicinal effect on maintaining the fertility of male. OECD guidelines 407 were followed for the sub-acute toxicity study. A total of 42 male Wistar rats were randomly divided into seven groups of 6 animals in each group for 3 dosages up to 28 days and each group was administered 250 mg/kg, 500 mg/kg, 1000 mg/kg body weight of *C.circinalis* and *I.suffruticosu* methanolic extracts. The hematological and biochemical parameters were analyzed and compared between the control and experimental group, there was only slight variations were noted and was found to be within the standard laboratory values and statistical analysis of the parameters showed insignificance. The histopathological examination of various vital organs of both control and experimental groups were compared and no abnormal architecture, morphological disturbances, desquamation, disorganization, infiltration and no destruction of the tissues were noted. The sub-acute toxicity study proves that the ethanolic extract of *C.circinalis* and *I.suffruticosum*, when given orally at concentration up to 1000mg/kg body weight did not exhibit any toxicological effect and proved to be safe until 1000mg/kg.

Keywords: *Cycas circinalis*, *Ionidium suffruticosum*, Siddha Medicine, Sub-acute toxicity, OECD guidelines 407, Histopathology, Toxicology.

INTRODUCTION

Herbal medicines have been popular from ancient times among people and in recent years, a multilateral approach has emerged on using medicines which is of herbal origin.¹ Medicinal herbs may cause some irretrievable tissue damage through its unwanted side effects. Evaluating toxic effects of medicinal herbs by performing experimental tests on animal models will have an effective advantage on identification and recognition of medicine's harmful effects on humans.²

On the other hand, recognizing the damages on different tissues and organs of body, following the use of medicinal herbs will provide an appropriate strategy in order to specify the consumption of these drugs. Determining the dosage of medicines is very important too, for overdose of herbal medicines does not cure the illness and also cause irreparable damages.³

Herbal medicines as preparations derived from naturally occurring plants with medicinal or preventive properties are a major component in all indigenous medicine.

The World Health Organization (WHO) estimates that 4 billion people, about 80% of the world's population, use herbal medicines for some aspect of primary health care.⁴ It is estimated that 70% of indigenous populations in developing countries rely on phytotherapy⁵ and 25% of drugs in typical western pharmacies are plant derived.⁶ The realities on ground pose challenges to scientists and

governments of developing countries with respect to the development and production of standardized herbal medicines. WHO has advocated for the proper identification, sensible exploitation, scientific development and appropriate utilization of herbal medicines which provide safe and effective remedies in medicare.⁵

Pharmacological and toxicological evaluations of medicinal plants are essential for drug development.⁷⁻⁹ The present study was done to evaluate the subacute toxicity of two medicinal herbs such as *Cycas circinalis* and *Ionidium suffruticosum*. OECD guidelines 407 were followed for the sub-acute toxicity study.¹⁰

Cycas circinalis .L (Family –Cycadaceae), a sago palm commonly known as Madana Kaman in Tamilnadu. The genus is native of eastern and southeastern Asia and is cultivated in many tropical and subtropical areas for ornamental purpose.¹¹ The male sago cone has aphrodisiac activity.¹² *C.circinalis* is considered to be an invigorating and nutritive tonic for people emaciated by famine or disease, In siddha system of medicine, the male cone was used to improve maleness. Both male and female cones were having narcotic, stimulant, and aphrodisiac activity. It has been reported that β-N-methylamine, L.alanine from *C.circinalis* are being implicated in the pathogenesis of human neurological disorders like lathyrism and amyotrophic lateral sclerosis. Male cones are used as stimulant; seeds are used as aphrodisiac as well as to improve sperm production.



Ionidium Suffruticosum (Ging). (Family – Violaceae) known as padmavathi, padmcharini or purusharathna in Sanskrit, is an important medicinal plant in the Indian system of medicine. *I.suffruticosum* is a small perennial herb found in India, Ceylon, tropical Asia, Africa and Australia.¹³ Traditionally the herb is used as an aphrodisiac, demulcent, tonic, diuretic, urinary tract infections, diarrhea, leucorrhea, dysuria and sterility.¹⁴ Various phytoconstituents such as dipeptide, alkaloids, aurantiamide acetate, isoarborinol and β sitosterol were isolated from this plant.¹⁵

MATERIALS AND METHODS

Extraction of *Cycas circinalis* and *Ionidium suffruticosum*

The *Cycas circinalis* (Cc) dry male cones specimen was identified as per Siddha literature, authenticated and collected from the trees directly and air dried in shadow for 10 days and simultaneously the *Ionidium suffruticosum* (Is) plant was also identified as per Siddha literature, authenticated and collected, washed with distilled water and air dried for 10 days.

The dried male cones of *Cycas* and *Ionidium* (whole plant) was powdered using pulverizer and passed through sieve to get fine powder.

The 50 grams of powder of plant material of Cc and Is were successively extracted with 500ml of absolute ethanol solvents by hot continuous percolation method in Soxhlet apparatus for 24 hrs. The extracts were concentrated by using hot water bath and subjected to drying in an hot air oven.¹⁶ The percentage yield of Cc and Is extracts was found to be 7.14 and 8.42 respectively.

Animal selection and drug administration

Forty two male rats were randomly divided into seven groups of 6 animals in each group for 3 dosages of both the herbs extract and one group was kept as control. The protocol of the toxicity study was approved by the Institutional Animal Ethics Committee of Saveetha University (IAEC. NO. ANAT.005/2012). Each group was administered 250 mg/kg, 500 mg/kg, 1000 mg/kg body weight of *C.circinalis* and *I.suffruticosum* extracts respectively and control group administered sterile water simultaneously. The extracts were administered orally using oral gavage tube for 28 days.

Collection of blood and tissue samples

On the 29th day all the rats were anesthetized and shaved on the ventral aspect of the neck and the skin was incised to about 2 cm in length and opened. The jugular vein was traced out and using 21 gauge needle and a disposable 3ml syringe, 2ml of blood was withdrawn.

Serum was separated by centrifugation for about 3000 rpm after the coagulation of blood for 40 minutes. The serum was collected using micropipette from the samples and the hematological parameters and biochemical markers were analyzed as per the standard procedure.¹⁷ The rat was cut open by midline thoraco abdominal

incision and followed by transcardial perfusion using 4% paraformaldehyde in 0.1 M phosphate buffered saline on the left ventricle and the right atrium was cut open.

Flickering of limbs, blanching of tissues with consequent hardening of the whole body of the animal indicates that perfusion made was complete. The vital organs such as brain, liver, kidney, lungs, heart, stomach, intestine, testes, and skeletal muscles were collected carefully by fine dissection as per standard procedure.¹⁸ The collected organs were fixed in formalin for 48 hrs. Samples tissues were collected from each organ and processed.

Histological Techniques

The fixed tissues were dehydrated using 70% alcohol, 90% alcohol, absolute alcohol I, followed by absolute alcohol II, with each change for 2-4 hours. The dehydrated tissues were then cleared using Xylene with 2 changes for 3 hours followed by impregnation of cleared tissues in molten paraffin wax at 60° C with 2 changes of wax for 3 hours.

The wax impregnated tissues were now embedded in paraffin wax. Three sections were taken using rotary microtome and then sections were mounted on slides and left for drying in hot plate at 40° C. The slides with tissue section were dewaxed using xylene followed by removal of xylene by taking it to absolute alcohol I & II, 95% alcohol, 70% alcohol and then at last taken to water for staining.^{19,20}

Haematoxylin and Eosin Staining

Tissue sections after hydration transferred to haematoxylin and left for 10 minutes, then slides transferred to slide washing tray and washed until the sections were blue and placed in running tap water for about 10 minutes at pH of 8.

After bluing of sections they were dipped into acid alcohol where they are agitated for few seconds and then returned to slide washing tray until the section become blue again. The slides were counterstained in 1% eosin for 2 minutes and then rinsed in water which differentiates the eosin stain.

The stained slides now dehydrated using different grades of alcohol for 10 to 15 seconds and then cleared using xylene for 10 to 15 seconds followed by mounting using DPX and cover slip.¹⁹ The histopathological examination of various vital organs of both control and experimental groups were compared for the toxicological effects.²¹

RESULTS AND DISCUSSION

The hematological parameters and biochemical markers were analyzed and tabulated as Mean \pm SEM.

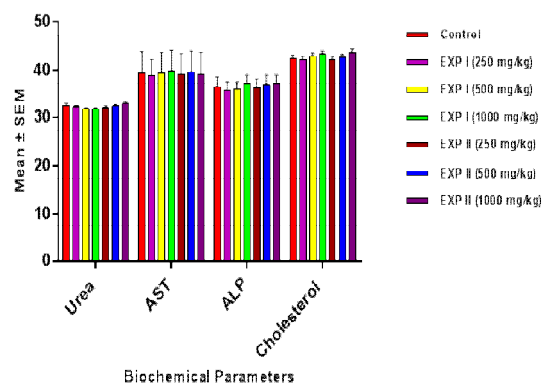
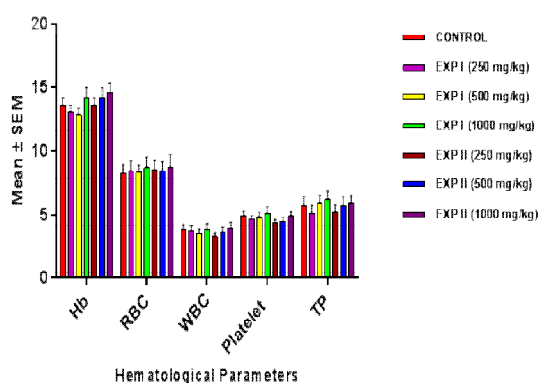
The parameters were compared between the control and Experimental I group (administered *Cycas circinalis*), followed by comparison of same control with Experimental II group of rats (administered with *Ionidium suffruticosum*) (Table 1).



Table 1: Hematological and Biochemical parameters

Parameters	Control	Experimental I			Experimental II		
		250 mg/kg	500 mg/kg	1000 mg/kg	250 mg/kg	500 mg/kg	1000 mg/kg
Hb (mg/dl)	13.6±0.6	13.1±0.5	12.9±0.5	14.2±0.8 ^{a**}	13.6±0.6	14.2±0.76	14.6±0.8 ^{b*}
RBC (×10 ⁶ /μl)	8.31±0.72	8.51±0.8	8.39±0.6	8.77±0.8 [#]	8.62±0.7	8.52±0.71	8.84±0.9 [#]
WBC (×10 ³ /μl)	3.77±0.48	3.71±0.4	3.54±0.3	3.84±0.5 [#]	3.27±0.3	3.63±0.4	3.92±0.5 [#]
Platelet (×10 ⁵ /μl)	4.98±0.37	4.7±0.31	4.84±0.4	5.22±0.48 [#]	4.37±0.30	4.54±0.32	4.94±0.36 ^{b*}
Total Protein (g/dl)	5.77±0.71	5.21±0.6	5.98±0.6	6.21±0.69 [#]	5.30±0.54	5.74±0.71	5.94±0.6 [#]
Urea (mg/100ml)	32.6±0.33	32.2±0.3	31.8±0.28	31.8±0.3 [#]	32.14±0.28	32.48±0.3	32.9±0.29 [#]
Creatinine (mg/dl)	0.32±0.04	0.31±0.03	0.38±0.04	0.38±0.04 [#]	0.33±0.03	0.34±0.04	0.39±0.04 [#]
AST (U/l)	39.4±4.31	38.9±3.29	39.4±4.1	39.8±4.2 [#]	39.2±4.1	39.7±4.12	39.24±4.32 [#]
ALP (U/l)	36.4±2.21	35.7±1.9	35.9±1.6	37.2±1.8 [#]	36.2±2.01	36.8±2.2	37.2±1.8 [#]
Cholesterol (mg/dl)	42.4±0.60	42.2±0.6	42.8±0.58	43.2±0.6 ^{a*}	42.2±0.6	42.6±0.54	43.6±0.58 [#]

Values are expressed as Mean ± SEM, n = 6, # - non significant, * -significant, ^{a*}P<0.05, ^{a**}P<0.01 & ^{a***}P< 0.001 control groups compared to Experimental I group, ^{b*}P<0.05, ^{b**}P<0.01& ^{b***}P< 0.001 control groups compared to Experimental II group. Experimental I – administered C.circinalis extract, Experimental II – administered I.suffruticosum extract. Statistical analysis – One Way ANOVA.



HB – Haemoglobin, RBC – Red Blood Cell, WBC – White Blood Cells, TP –Total Protein, Control groups compared to Experimental groups. Experimental I – administered C.circinalis extract, Experimental II – administered I.suffruticosum extracts.

AST – Aspartate Transaminases, ALP– Alkaline Phosphatase, Control groups compared to Experimental groups. Experimental I – administered C.circinalis extract, Experimental II – administered I.suffruticosum extracts.

Graph 1: Hematological Parameters of Sub-acute Toxicity Study

Graph 2: Biochemical Parameters of Sub-acute Toxicity Study

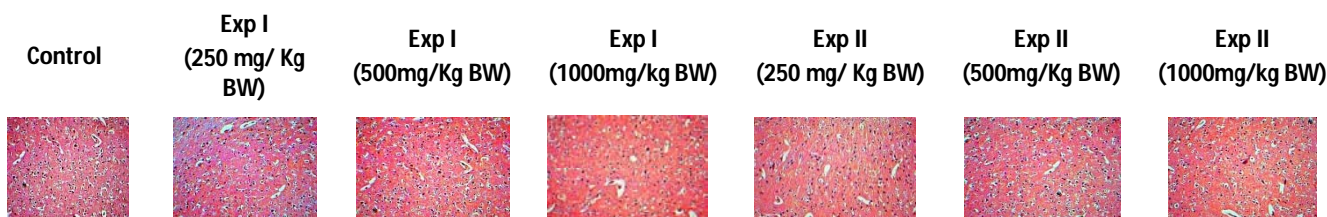


Figure 1: Histopathological Examination of Brain H & E stain (10X)

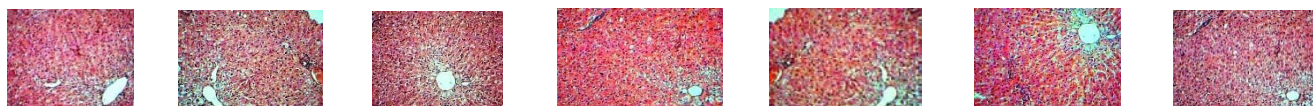


Figure 2: Histopathological Examination of Liver H & E stain (10X)

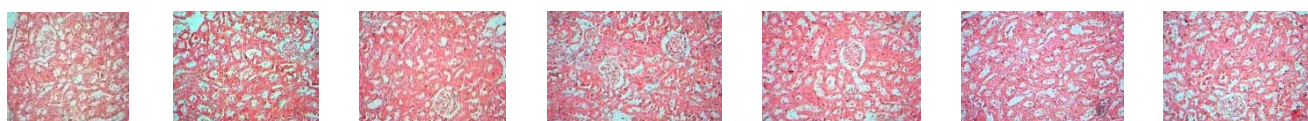


Figure 3: Histopathological Examination of Kidney H & E stain (10X)

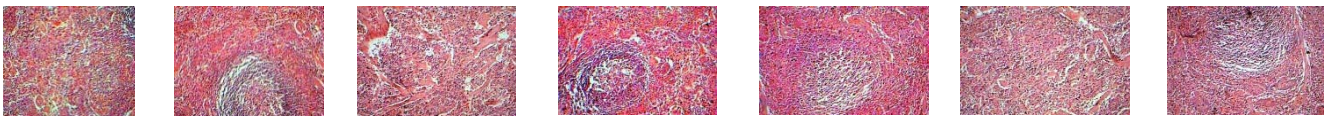


Figure 4: Histopathological Examination of Spleen H & E stain (10X)

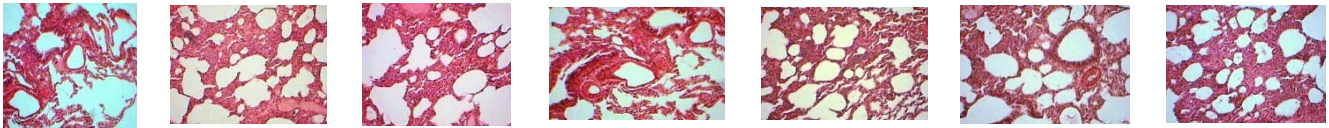


Figure 5: Histopathological Examination of Lung H & E stain (10X)

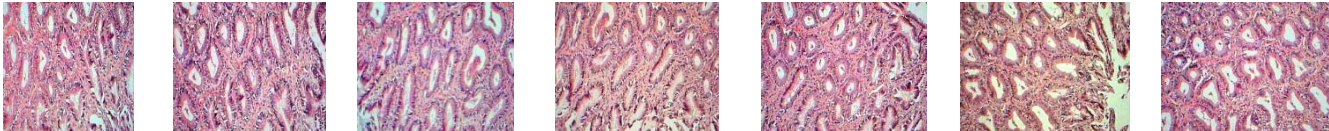


Figure 6: Histopathological Examination of Stomach H & E stain (10X)

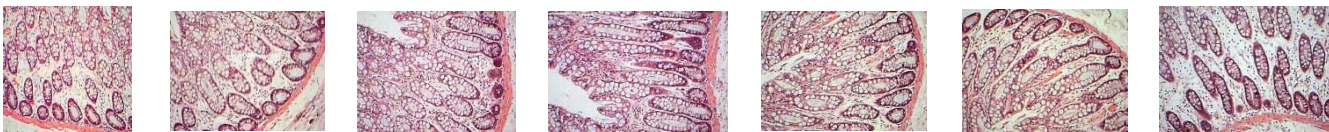


Figure 7: Histopathological Examination of Intestine H & E stain (10X)

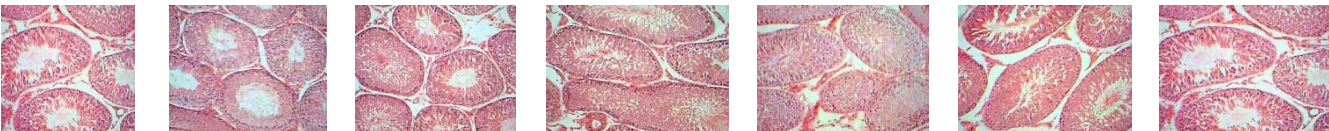


Figure 8: Histopathological Examination of Testes H & E stain (10X)

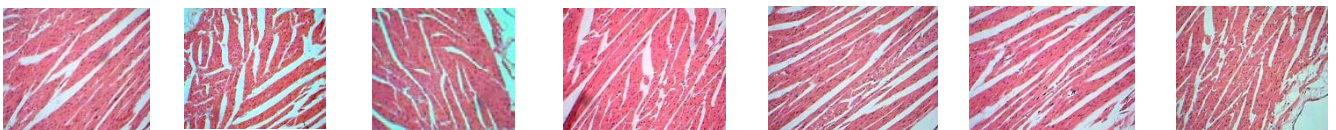


Figure 9: Histopathological Examination of Heart H & E stain (10X)

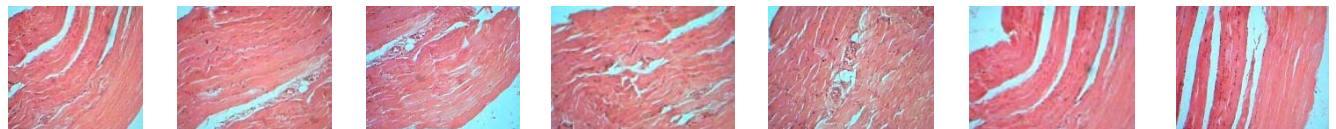


Figure 10: Histopathological Examination of Skeletal Muscle H & E stain (10X)

Figure I: Histopathological Examination of various tissues of sub-acute toxicity study

Experimental I – administered *C. circinalis*, Experimental II – administered *I. Suffruticosum*, Histopathological examination of various tissues when compared to normal control group rats tissues did not show any abnormal architecture, with morphological disturbances, desquamation, disorganization, infiltration and destruction of the tissues.

The hematological and biochemical parameters when compared between the control and experimental group only slight variations were noted and which was found to be within the standard laboratory values (Table 1) (Graph 1 & 2) and statistical analysis (One Way ANOVA) of the parameters showed insignificance as the values were within the same range except hemoglobin and platelets which was found to be significant. Aspartate Transaminases (AST) and Alkaline Phosphatase (ALPs) are good indices of liver damage. There were no deleterious

changes found in the level of transaminases and ALPs in serum of drug treated groups when compared with control animals. The level of transaminases and ALP usually rises in acute liver damage. AST is also present in red blood cells, cardiac muscles, skeletal muscles, kidney and brain tissue, and may be elevated due to damage to these sources as well. AST is defined as a biochemical marker for the diagnosis of acute myocardial infarction. Equally, there was also no marked change in creatinine, when compared to the control (Graph 2), creatinine is known as an effective indicator of renal function and any rise in creatinine levels will be observed if there was a marked damage to functional nephrons.

Thus, the results recorded in this study suggest that ethanolic extract of Cc and Is did not affect the renal function. The liver is the site of cholesterol disposal or degradation and the major site of synthesis. Since, no

significant changes were observed in cholesterol levels in this study (Table 1 & Graph 2), it suggests that Cc and Is extract had no effects on the cholesterol metabolism of the rat.²²

The histopathological examination of different organs of both control and experimental groups were compared and no abnormal architecture, morphological disturbances, desquamation, disorganization, infiltration and destruction of these tissues were noted (Figure 1). Brain showed normal architecture without any infiltration and cerebral oedema. Neuron cell bodies were displayed on a loose fibrillary background. Prominent perineuronal and perivascular halos or clearing (Virchow Robbins spaces) were seen in both the control and drug treated rats brain (Fig 1). The liver of drug treated animals showed normal histological feature at 250, 500 and 1000 mg/kg with reserved hepatic architecture, hepatocytes arranged as radial plates. No degeneration of hepatocytes, no cytoplasmic inclusions in hepatocytes, focal steatosis, sinusoidal spaces did not show any vascular congestion. The central vein did not show any congestion and no inflammation of portal tract when compared with control animals (Fig 2).²³

The kidney of drug treated rats showed normal glomerular tufts displayed on a background containing tubules and there is no necrosis of tubular epithelium in the kidney, no evidences of focal tubular casts and no glomerular hyperaemia in kidney when compared to the control rats kidney (Fig 3).²³ The spleen appears to consist of discrete lymphatic nodules called the white pulp, embedded in a red matrix called the red pulp. The splenic stroma showed engorgement of sinusoids by red blood cells and showed normal white pulp with a central arteriole surrounded by periarteriolar lymphoid sheath and red pulp which is permeated by an interconnected network of sinuses, lined by endothelial cells resting upon a basement membrane with numerous narrow slits (Fig 4).²⁴ The lung tissues were normal in all the drug treated groups. The alveolar wall consists of three tissue components, surface epithelium (Type I & II pneumocytes), supporting tissue (reticular, collagenous, elastic fibres) and blood vessels. The alveolar air spaces were surrounded by interstitium containing few blood vessels and inflammatory cells and showed no focal lesions, alveolar epithelium were normal (Fig 5). The mucosa of stomach is thrown into prominent folds and consists of gastric glands, the submucosa made up of connective tissues and large blood vessels. There were no mucosal erosions observed in stomach of drug treated rats when compared to control (Fig 6).²⁴

The mucosa of large intestine lined by simple columnar epithelium with goblet cells, the mucosal linings were normal in both control and drug treated group (Fig 7).²⁴ Testes showed cut sections of seminiferous tubules with normal germinal epithelium in various stages of development and spermatozoa in the lumen of the tubules. Leydig cells were seen in the interstitium (Fig

8).²⁵ The heart was normal in the drug treated group with cardiac myocytes were arranged in interlacing and parallel array and their nuclei were spindle shaped and elongated (Fig 9). The skeletal muscle fibres were elongated, unbranched cylindrical cells, with multiple nuclei arranged at the cell periphery. The skeletal muscle showed normal histopathological presentation in all drug treated groups when compared to control groups (Fig 10).

CONCLUSION

The *C.circinalis* and *I.suffruticosum* does not produce any toxic effect in male albino rats with no adverse histopathological presentations. Findings of the sub-acute toxicity tests suggest that Cc and Isis practically non-toxic when administered orally for an extended period at therapeutic doses. The drugs at these doses showed potential for boosting components of the immune system and protecting the liver, kidney and cardiovascular system. These findings provide a justification for specially designed studies to further investigate these possible beneficial activities. The sub-acute toxicity study proves that ethanolic extract of *C.circinalis* and *I.suffruticosum* when given orally at concentration of 1000mg / kg body weight, did not exhibit any toxicological effect and proved to be safe until 1000mg/kg.

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