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Liver Toxicity of Raw and Processed *Cycas circinalis* (*Madu*) Seed Flour: An Animal Study

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ABSTRACT

Various parts of Cycas circinalis (Sinhala: Madu) are used as food or in food preparation in many countries. Flour made from mature dried seeds is used in Sri Lanka to prepare starchy staples such as pittu and roti. However, the seeds are reported to contain hepato-toxins known as cycasins. This study was conducted to observe the liver toxicity, if any, of foods made incorporating cycas seed flour as data on effects of processing on liver toxicity are not available. The study was conducted with pittu and roti made by incorporating cycas seed flour obtained from soaked and dried seeds. Toxicity of pittu, roti and raw flour were observed by assessing the behavior of animals, liver enzymes and histopathological changes in liver sections of the mice fed diets made by incorporating pittu, roti and raw flour for 28 days. Significant elevations in liver enzymes ($p<0.05$) (reference AST 195± 38, ALT 21.2± 1.1 IU/L, raw flour AST 345± 71, ALT 50.8± 3.1 IU/L; pittu AST 406± 68, ALT 39.2± 1.1 IU/L; roti AST 333± 31, ALT 28.8± 1.5 IU/L) were observed in rats fed cycas flour incorporated diets. Fatty inclusions, few scattered lymphocytes and mild perpendicular inflammations were observed in the liver sections of all test groups. However, the elevation of liver enzymes and histopathological changes were significantly low ($p<0.05$) in mice fed with roti incorporated diet when compared to other test groups. Hence, the present study suggests that dry heat processing is detoxifying hepato-toxins in seeds to a certain extent. However, the present study confirms that continuous feeding of raw or processed cycas flour containing food could lead to liver parenchymal cell damage.

KEYWORDS: *Cycas circinalis, liver toxicity, cycasins, roti, pittu*

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1 INTRODUCTION

Cycads, the predominant flora of Permian era, are rapidly diminishing in number all around the world (Goel and Khuraijam, 2015). Cycads often grow in rocky soils underexposed conditions where other plants are unable to compete (Spencer, 1990). Cycad seeds are brightly coloured (red, brown, green) and have the appearance of edible fruits. The mature seed has a fleshy outer husk covering a thin, stony shell that protects the starchy kernel (Goel and Khuraijam, 2015).

There is evidence for the usage of cycads as food in the past. The common names of some cycads, which are related to ordinary food varieties [i.e. wild pineapple *(Macrozomia),* wild date *(Encephalartos),* wild sago (*Zamia floridana),* false sago palm (*C. circinalis*), bread palm (*C. rumphii*) and sago palm (C. *revoluta)* etc.], reveal the profuse usage of cycads as foods by humans. Among cycad species, *C. nathorstii* (Mudannayake *et al*, 2015) and *Cycas zeylanica* (Lindstorm and Hill, 2002) are native to Sri Lanka but restricted to certain parts of the island and the seeds are consumed as food by the residents in those areas. Since the distant past, *C. circinalis* seed flour has been used by the Sri Lankans in the preparation of two starchy staples, "*pittu*" and "*roti*" (both food items were made by mixing seed and rice flour, scraped coconut, water and salt; *pittu* is cooked by steaming and *roti* is made by dry heat). According to folklore, consumption of foods made from *Cycas circinalis* to treat Protein Energy Malnutrition (PEM) had been a long standing practice in Sri Lanka, although the nutritional quality of this plant had not been scientifically investigated. Cycad seeds are the staple food of the people in Guam, Irian Jaya and Kii Peninsula and also they use cycad seeds for medicinal and recreational purposes (Spencer, 1990).

Though *C. circinalis* seeds are being consumed as food, several studies have revealed the presence of neurotoxins, carcinogens and toxic chemicals which cause detrimental effects on the liver. Methylazoxymethanol (MAM) and beta-N-methylamino-L-alanine (BMAA) are the active forms of the well-known seed chemical substances called cycasins which are responsible for the toxic effects. MAM is a potent hepato-toxin carcinogen and a neuro-teratogen which had also elicited an overt interruption to the cerebellar development of mice in a previous study (Sieber *et al*., 1980; Kisby and Spencer, 2011; Kobayashi and Matsumoto, 1965). BMAA is an excitant neurotoxin that produces a motor system disorder in primates after repeated sub convulsive dosing (Spencer, 1990). The smallest dose of crude cycad materials that induced palpable abdominal tumor masses, ascites, tumors in the liver, kidneys and intestines was 2.5mg/ day when the study was performed with rat models (Laqueur *et al*., 1963). Previous studies reveal the occurrence of hepatomas with prolonged consumption of cycasin while renal and intestinal neoplasms required only short term consumption (Hirono *et al*., 1968; Laqueur, 1964). Large intestinal bacterial flora was an essential factor for the

formation of MAM and BMAA as they are needed to convert the plant's primary toxic chemicals to MAM and BMAA (Laqueur *et al*., 1967).

Many studies have also revealed that *C. circinalis* seeds are responsible for unique neurological disorders [Amyotrophic Lateral Sclerosis- Pakinsonism Dementia (ALS PD)] common to some areas as Guam and Irian Jaya where these seeds are consumed as a staple. Although detoxification methods such as slicing and soaking, soaking and sun drying, leaching etc. have been identified by the Australian aboriginals, still the risk of the occurrence of neurological disorders due to consumption of *C. circinalis* seeds as a staple has not been diminished (Spencer, 1990).

Hence, the proved positive correlation between the neuro-degenerative disorders (ALS-PD), hepato-toxicity and the usage of un-processed or improperly processed cycads has established a demur on the safe use of this seed. The objective of the present study was to assess the liver toxicity in mice, if any that could arise with continuous consumption of *pittu* and *roti* made out of *C. Circinalis* flour in comparison to the consumption of unprocessed seed flour. Since *pittu* and *roti* are made using wet and dry heat respectively, the toxicity will reflect the effect of different processing methods on seed flour as well.

2 MATERIALS AND METHODS

2.1 Materials

Cycas circinalis seeds (~10 kg) were purchased from a wholesale dealer in Colombo, Sri Lanka. Vegetative parts of the plant (herbarium sheet) were identified and confirmed by the National Herbarium, Royal Botanical Garden Peradeniya, Sri Lanka with reference to specimen number 4723. Wheat flour and rice flour used for food preparation were purchased from a retail outlet in Colombo, Sri Lanka. Mature scraped coconuts (8-10 months maturity) were used in the preparation of *pittu* and *rotti*. Vitamin and mineral mix for animal food formulae was purchased from Hexagon Nutrition (Pvt) Ltd., Nasik, India.

2.2 Preparation of Seed Flour and Food

a) Preparation of unprocessed (raw) seed flour: Dried *C. circinalis* seeds were soaked (0.2 kg /L of water for 12 hours) and the excess water drained. The seeds were sun-dried until hard and made into flour (particle size 0.3 – 0.5 mm).

b) *Pittu* preparation: Raw cycas seed flour, rice flour and scraped coconut were mixed in 1:1:0.7 ratio with salt to taste. The mixture was loosely packed into a *pittu bamboo* and steamed for 10 min (wet heat).

c) *Roti* preparation: Raw cycas seed flour, wheat flour and scraped coconut (1:1:0.7) were mixed and made into a dough with adequate water and salt. The dough (50g) was flattened (diameter 15 cm and the thickness 0.5 cm) and cooked

on a heated flat pan by turning sides every 2 min for 10 min (dry heat).

The oven dried foods from b) and c) were made into flour by milling and sieving through a 0.3-0.5 mm mesh to obtain the test samples required for the animal study and the unprocessed seed flour (a) was used as a control in the animal study.

2.3 Animal Models

Twenty ICR laboratory male mice, aged 28 days from the same colony, were obtained from Medical Research Institute, Colombo, Sri Lanka. Animals were kept in the Animal House of Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka under standard conditions that would not interfere with normal growth. On the first day, mice were put into separate cages and the standard mice diet (Subourdy, 1988) and water *ad libitum* were provided for 3 days for acclimatization of animals. On the fourth day, the mice were divided into 4 groups (5 mice/ group) so that the weights of mice in each group were not significantly different ($P<0.05$) and the difference between the mean weights of the groups did not exceed 5g.

2.4 Preparation of Diets

The three test diets were prepared to provide ~10% protein from *roti*, *pittu* and raw flour. Control diet with unprocessed seed flour was prepared by adding raw seed flour ~850 g/ 1 kg food. The diet with *pittu* flour was prepared by adding 910 g of *pittu* flour in 1 kg food. The diet with *roti* flour was prepared by adding 816 g *roti* flour in 1 kg food. The reference group was provided with a standard casein incorporated diet (10g/day/mouse). Other ingredients were added according to the standard mice diet for protein quality estimation, described in AOAC methods.

The vitamin mix used for the diet preparation contained vitamin A, D, E, menadione, choline, p-aminobenzoic acid, inositol, niacin, Ca, pantothenate, riboflavin, thiamin HCl, pyridoxine HCl, folic acid, biotin and vitamin B_{12}. The mineral mix used for the diet preparation was composed of NaCl, KI, KH_2O_4, $MgSO_4$, $CaCO_3$, $FeSO_4.7H_2O$, $MgSO_4.H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ and $CoCl_2.6H_2O$. Glucose, corn starch, cellulose, corn oil, vitamin and mineral mix were the other ingredients of the diets (AOAC, 2000).

2.5 Toxicity Study

The two test groups were provided with the appropriate *pittu* and *roti* test diets while the reference group and the control groups were fed with the reference diet and the control diets respectively, according to AOAC Official method 960.48 (10g food/ mouse/ day). The study was carried out for 28 days. During the study period, the mice were observed daily to identify any abnormal behaviours, convulsions or any overt toxic effects. Weight changes of the mice were estimated at the end of every week. Food intake was recorded every other day. At the end of 28 days, rats were euthanized by overexposure to ether. Blood was taken by a heart puncture and serum AST (Aspartate transaminase) and ALT (Alanine transaminase) levels were

determined by enzymatic assay kits (BIOLABO SA, Maizy, France) at the Biochemistry Research Laboratory of Faculty of Medical Sciences, University of Sri Jayewardenepura. Weights of the livers and kidneys were measured and liver histopathological studies were carried out at the Pathology Laboratory of Faculty of Medical Sciences, University of Sri Jayewardenepura, after sectioning, staining (H and E) and observing liver sections under the light microscope. The histopathological images of the test and control groups were compared with each other.

2.6 Ethical Clearance

Ethical approval (Approval No.431/09) for the study was obtained from the Ethics Review Committee of the Faculty of Medical Sciences, University of Sri Jayewardenepura, Nugegoda, Sri Lanka.

2.7 Statistical Analysis

The results are expressed as mean±standard deviations. The significant differences between the mean AST and ALT levels of the groups were analyzed by ANOVA using SPSS version 13.

3 RESULTS & DISCUSSION

The total feed intake of the groups is stated in table 1. *Cycas* unprocessed seed flour fed group and the *roti* fed group had significantly high and low ($p<0.05$) feed intakes respectively compared to other groups. No behavioural changes or convulsions were observed in any of the rats throughout the study period. A significantly low ($p<0.05$) liver and kidney weights were observed in all test and control groups compared to the reference group. The body weight: organ weight ratios were also significantly ($p<0.05$) lower in these groups compared to the reference group (Table 1).

Table.1: Mean body weights at the end of the study, liver and kidney weights (g), body weight: organ weight ratios

Group	Total feed intake*	Mean weight change (g)*	Weights of liver Mean±SD	Body weight: liver weight	Weights of kidney Mean±SD	Body weight: kidney weight
Reference	463	+15.0	1.8 ± 0.38^{a}	0.02^{c}	0.5 ± 0.08^{e}	0.005^{g}
Unprocessed (control)	827	9.6	1.3 ± 0.17^{b}	0.01^{d}	0.4 ± 0.08^{f}	0.002^{h}
Pittu	520	4.8	1.1 ± 0.19^{b}	0.01^{d}	0.3 ± 0.93^{f}	0.002^{h}
Roti	353	5.0	1.0 ± 0.29^{b}	0.01^{d}	0.3 ± 0.11^{f}	0.004^{h}

Values presented as mean± standard deviation. Values with different superscriptions (a, b, c, d, e, f, g & h) in the same column are significantly different ($p<0.05$) compared to reference; * Senadheera and Ekanayake, 2016

Literature indicates that cycas seed flour contains about 2.3% of cycasin (Campbell and Mickelson, 1966) and thus according to the mean feed intake of the groups, unprocessed seed flour, *pittu* and *roti* groups had consumed approximately 1.4g/ rat/ day, 0.9g/ rat/ day and 0.6g/ rat/ day of cycasin. When an oral single dose of cycasin was administered to rats at a dose ranging from 50-400 mg/kg BW, liver damage had been observed due to DNA fragmentation in liver cells (Cavanna *et al*, 1979). As the cycasin content/ rat/ day in the present study was not as higher as in the study carried out by Cavanna and researchers (1979), an acute change in liver enzymes was not observed.

Alanine transaminase (ALT) is an enzyme prominent in hepatocytes and the incline of serum ALT indicates liver cell damage. Aspartate transaminase (AST) is an enzyme prominent in skeletal muscles, heart muscles, liver and red blood cells which could be significantly elevated mainly with haemolysis and muscle catabolism. Due to the proven data on hepatotoxicity caused by cycasins, ALT and AST levels were estimated in the present study to observe any toxicity with consumption of foods made of cycas seed flour. Both AST and ALT levels of control and test groups showed significantly ($p<0.05$) higher values when compared to the reference group (Table 2).

Table.2: Serum AST and ALT concentrations of mice

Group	**AST (IU/L)** **Mean ± SD**	**ALT (IU/L)** **Mean ± SD**
Reference	195 ± 38^{bc}	21.2 ± 1.1^{bc}
Unprocessed (control)	345 ± 71^{ac}	50.8 ± 3.1^{ac}
Pittu	406 ± 68^{ab}	39.2 ± 1.1^{ab}
Roti	333 ± 31^{abc}	28.8 ± 1.5^{abc}

[a]Statistically significant ($p<0.05$) when compared with the Reference group; [b]Statistically significant ($p<0.05$) when compared with the unprocessed flour group; [c]Statistically significant ($p<0.05$) when compared with the *pittu* group; SD-Standard Deviation; ALT-Alanine transaminase; AST- Aspartate transaminase

The significantly high ALT and AST levels in mice fed with test diets (continuously) compared to the reference group could be due to these hepato-toxic cycasin present in *C. circinalis* flour. This could be further proved by the elevated

ALT levels in test groups which were proportionate to the cycasin contents in the diets. Among the test diet, mice fed with *roti* diet had a significantly low increase ($p<0.05$) in AST and ALT thus indicating comparatively less damage to the liver cells. This could be due to low intake of toxins as the feed intake in the *roti* diet fed group was less or could be due to the destruction of hepato-toxins by the dry heat used in the preparation of *roti*.

Although there is a direct correlation between liver weights and feed intake of the animals (Anyika *et al*., 2009), significantly low liver and kidney weights of the test group animals could not be due to this reason as body weight: organ weight ratios were also lower in these groups. A study has revealed ingestion of cycasin by the rats, produce hepato-toxic effects, glycogen, RNA, and phospholipid depletion, cellular necrosis and haemorrhage (Williams and Laqueur, 1965) which could contribute to low liver weights.

Livers of the animals in the reference group showed a typical mammalian liver architecture (Figure 1(a)). However, in all the test groups fatty inclusions (steotosis – macrovesicular), a few scattered lymphocytes and mild perpendicular inflammations were observed in the liver sections. These observations were significant in the *pittu* fed group compared to the *roti* fed group.

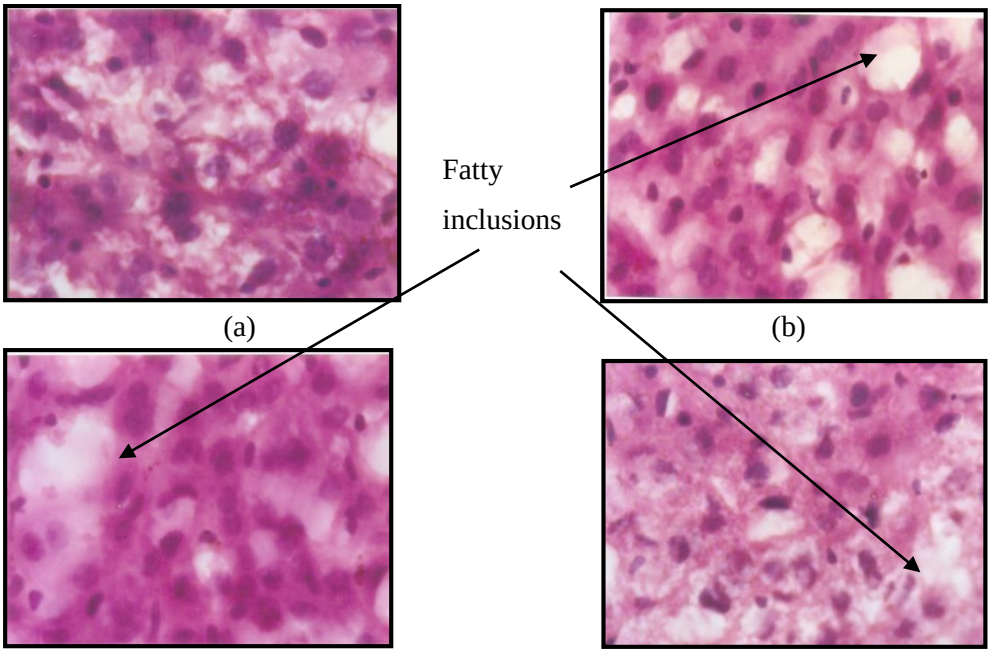

Figure.1: Photomicrographs of livers stained with Haematoxylin–Eosin (10x100) (a) mouse fed with reference diet (b) mouse fed with *pittu* diet (c) mouse fed with *roti* diet (d) mouse fed with diet with soaked flour

Low body weight gain observed in all test groups [4.8 g-9.6 g] compared to reference group [15 g] could also be due to the derangement of the liver cells which affect the metabolic activities. Nutrient depletion in serum owing to liver damage and low quality proteins in cycas seeds (Senadheera and Ekanayake, 2016) could have led to muscle catabolism increasing the AST levels in serum.

The change in common liver architecture further indicates liver toxicity. Thus the high ALT and AST levels, reduction in liver weights and histology of liver confirms the presence of cycasin in the local strains of *Cycas circinalis*. However, as *roti* fed group had elicited comparatively lower toxic parameters it can be concluded that dry heat processing can destroy the toxins in cycas seed flour to a certain extent. However, in Sri Lanka, toxicity due to foods prepared with *Cycas circinalis* flour is not reported. This could be attributed to the fact that these foods are not frequently consumed by Sri Lankans and also the method of food preparation leading to reduce the toxic effects.

Another reason for the reported acute toxicity in animals and the lack of reported acute toxicity in humans could be the lower production, thus lower absorption of toxins by the human large intestine (as toxins are produced mainly by gut flora) compared to the animals like rats who could produce toxins in a higher rate as microbial metabolism of foreign substances in the gut is high (Karali, 1995).

4. CONCLUSIONS

Serum enzyme profiles and the histopathology of the atrophied mice liver show fatty changes and lymphocyte accumulation, indicating liver toxicity in mice due to frequent consumption of *C. circinalis* raw and processed seed flour. Dry heat treatment appears to detoxify liver toxins in seeds to a significant extent as seen in mice fed with *roti* diet. However, due to low feed intake in *roti* fed group, this fact is not apparent. Thus *C. circinalis* seed flour cannot be recommended for frequent consumption due to observed toxicity.

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