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Fatty Acid Profile of Young Leaflets of *Cycas circinalis* L. and the Effect on Selected Serum Parameters in Wistar Rats

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Abstract

Young leaflets of *Cycas circinalis* L. (Cycadaceae) are used as delicacy and medicine in Sri Lanka, Philippines, Indonesia, and Malaysia. However, only few pharmacological or chemical studies have been done to evaluate the reported medicinal importance of the species. In the present study, albino Wistar rats were tested against the young leaflets of *C. circinalis* to determine the changes in the activities of specific liver enzymes such as alanine amino transferase (ALT), aspartate amino transferase (AST), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT) and creatinine. The fatty acid constituents present were analyzed using gas chromatography–mass spectrometry (GC-MS). When the albino Wistar rats were fed daily with 1.0 g of young leaflets of *C. circinalis* over a period of four weeks, the body weight increment was significantly higher in test group than the control. Slight elevation of ALP and GGT levels were observed, however, the difference was non-significant. The AST concentration was found to be significantly lower ($p<0.05$) in test group than the control (55.1±3.7 U/L). The ALT activity in rats fed with *C. circinalis* leaflets was slightly lower than those of the control. However, the difference was not statistically significant. The n-Hexadecanoic acid (palmitic acid) was found to be one of the most abundent fatty acids present in young *C. circinalis* leaves. Palmitic acid has been shown to alter aspects of the central nervous system responsible for the secretion of insulin and to suppress the body's natural appetite-suppressing signals from leptin and insulin - the key hormones involved in weight regulation. It could be suggested that the weight gain by albino Wistar rats fed with young *C. circinalis* leaves may be due to the presence of high level of palmitic acid or their derivatives in the leaves.

Keywords: creatinine, enzyme, liver, n-Hexadecanoic acid, plant

Introduction

Cycadaceae is a family with nine genera and nearly 100 species. Collectively, the plants are referred to as cycads. Asian as well as Southwest Pacific nations are reported to have been using different *Cycas* sp. for medicinal as well as culinary purposes. Young leaflets which are still rolled are used as a vegetable in Sri Lanka, Philippines, Indonesia, and Malaysia (Thieret, 1958). Babu et al. (2013) reported the presence of alkaloid, flavonoids, amino acids, and triterpenoids with 40% yield in ethanolic solvent in dry male cones of *C. circinalis.* Antibacterial activity of *C. circinalis* ovules against some of the human and plant pathogenic bacteria were also reported (Kalpashree & Raveesha, 2013; Moawad et al., 2013). However, studies on the reported medicinal importance of young cycas leaflets are scarce.

The juice of cycad seeds when used to treat skin ulcers of mice had shown faster healing but the animals died early when compared to the untreated ones due to liver tumors (O'Gara et al., 1964). Amyotropic lateral sclerosis has been observed in humans who consumed kernel flour of cycad seeds as well as animals such as cattle that fed on grasslands with cycads (Bell et al., 1967). The presence of neurotoxins in cycad flour has been studied by many researchers (Duncan et al., 1990; Duncan et al., 1992; Banack & Cox, 2003; Brenner et al., 2003).

Serum concentrations of liver enzymes are used as markers of liver function. Alanine aminotransferase (ALT) is a liver-specific enzyme found in the cytosol of hepatocytes. Aspartate aminotransferase (AST) is also similar to ALT and serum levels of AST and ALT become elevated whenever disease processes affect liver cell integrity. Moreover, ALT elevation persists longer than those of AST activity. Alkaline phosphatase (ALP) is found in many tissues, including bone, liver, intestine, kidney and placenta. Serum ALP measurements are of particular interest in the investigation of two groups of conditions: hepatobiliary disease and bone diseases associated with increased osteoblastic activity. The gamma glutamyl transferase (GGT) activity is elevated mainly in obstructive hepatobiliary diseases. Interconversion of phosphocreatine and creatinine is a particular feature of the metabolism processes of muscle contraction. Creatine and phosphocreatine partially convert to the

waste product, creatinine. Thus, the amount of creatinine produced is related to the muscle mass (and body weight), age, sex, diet or exercise and does not greatly vary from day to day. Since creatinine is endogenously produced and released into body fluids at a constant rate and its plasma levels are maintained within narrow limits, its clearance can be measured as an indicator of glomerular filtration rate which in turn reflects on renal function.

In the present study, albino Wistar rats were tested following administration of the young leaflets of *C. circinalis* to determine the changes in specific liver enzyme activities such as ALT, AST, ALP, GGT. Serum creatinine and the fatty acids present were also analyzed.

Materials and Methods

Healthy male albino Wistar rats weighing 380.0 – 415.0 g were used as the model animals. Rats were housed individually under standard environmental conditions at the Animal House. The standard World Health Organization (WHO) recommended diet was given and water was supplied *ad libitum*. All animal experiments were carried out following ethical clearance by the Institutional Ethics Review Committee, and international laws and guidelines (WHO, 2003) were followed. The experimental rats were divided into two groups with eight animals in each. Group I serving as control, was given only the standard laboratory rodent feed; and Group II, the test group was fed with standard laboratory rodent feed and 1.0 g of fresh young *C. circinalis* leaflets per kg body weight daily at 1500 hours over a period of four weeks. The rats were allowed to eat the finely chopped leaflets which were placed in hot water for five minutes. Distilled water was given to the control animals at the same time. The changes in body weights were measured weekly using a weighing scale (Shimadu UX 620H, Japan). At the end of four weeks, blood samples (1.0 mL) were drawn from the lateral vein of rats under mild anesthesia with diethyl ether to determine the changes in enzyme concentrations. Following bleeding, blood was centrifuged at 3500 rpm (Hermle Z-206A, Germany) and the serum was separated and kept at -20^0C for further analysis. Throughout the study period, animals were observed for behavioral changes or any visible signs of toxicity.

Determination of alanine amino transferase (ALT) concentration

The reagent with 2-oxoglutarate (15.0 mmol/L), L-alanine (500.0 mmol/L), LDH (≥1600 UI/L), NADH (≤0.18 mmol/L) and Tris Buffer (100.0 mmol/L) with pH 7.5±0.1 at 30^0C was diluted with demineralized water and mixed gently until complete dissolution. One mL of reagent was transferred to a temperature-controlled cuvette (1.0 cm path length) and it was brought to 37^0C. Serum sample (100.0 µL) was added and subsequently mixed. A timer was started and initial absorbance was recorded after one minute at 340 nm in a spectrophotometer (Shimadzu 240, Japan). Absorbance was again recorded at every minute for a period of three minutes.

Concentration of ALT in IU/L = (ΔAbs/min) x 1746

Determination of aspartate amino transferase (AST) concentration

A reagent with EDTA (5.0 mmol/L), 2-oxoglutarate (12.0 mmol/L), L-alanine (200.0 mmol/L), MDH (495.0 UI/L), LDH (820.0 UI/L), NADH (≤0.18 mmol/L) and Tris Buffer (80.0 mmol/L) under a pH 7.80±0.1 at 30^0C was diluted with demineralized water which was mixed gently until complete dissolution. One mL of the reagent was pipetted into the cuvette and brought into 37^0C. Serum sample (100.0 µL) was added and subsequently mixed. A timer was started and initial absorbance was recorded after one minute at 340 nm in a spectrophotometer. Absorbance was again recorded at every minute for three minutes.

Concentration of AST in IU/L = (ΔAbs/min) x 1746

Determination of alkaline phosphatase (ALP) concentration

A buffer with 1.0 mol/L diethanolamine buffer pH 10 (25^0C) and 0.5 mmol/L $MgCl_2$ was mixed gently with 10.0 mmol/L p-nitrophenyl phosphate substrate to obtain complete dissolution. One mL of the reagent was pipetted into the cuvette and brought into 37^0C. Serum sample (100.0 µL) was added and subsequently mixed. A timer was started and initial absorbance was recorded after one minute at 405 nm through a spectrophotometer. Absorbance was again

recorded at every minute for three minutes. This procedure was repeated for each specimen.

Concentration of ALP in IU/L = (ΔAbs/min) x 5450

Determination of gamma glutamyl transferase (GGT) concentration.

A buffer with 62.0 mmol/L of glycylglycine and 95.0 mmol/L TRIS at pH 8.1was mixed with 2.0 mmol/L L-G-glutamyl-p-nitroanilide (GPNA) substrate until completely dissolved. One mL of the reagent was pipetted into the cuvette and brought into 37^0C. Serum sample (100.0 μL) was added and subsequently mixed. A timer was started and initial absorbance was recorded after one minute at 340 nm in a spectrophotometer. Absorbance was again recorded every minute for three minutes.

Concentration of γ GT in IU/L = (ΔAbs/min) x 2121

Determination of serum creatinine concentration

A base with 6.4 mmol/L of disodium phosphate and 150 mmol/L NaOH was mixed with 0.75 mmol/L sodium dodecyl sulphate and 4.0 mmol/L of picric acid at pH 4.0 to achieve complete dissolution. One mL of the reagent was pipetted into the cuvette and brought into 37^0C. Serum sample (100.0 μL) was added and subsequently mixed. A timer was started to obtain record of the initial absorbance after one minute at 340 nm through a spectrophotometer. Absorbance was again recorded every minute for 3 minutes.

Concentration of creatinine in mg/dl = (A2 – A1) Assay / (A2 – A1) Standard × 2 mg/dl

Fatty acid analysis of *C. circinalis*

Samples were air dried for two days and dried samples were ground to obtain a coarse powder. Fifty grams (50.0 g) of dried sample was used in extraction. A mixture of petroleum ether, chloroform, and methanol (1:1:1) was used as the solvent. The sample was extracted in 200.0 mL of solvent in a Soxhlet extractor at a temperature range of 45-85ºC. The procedure was repeated five times until a clear color was obtained. The samples obtained were evaporated to dryness using the

rotary evaporator (BUCHI-R124) at 45^0C with pressure reduction starting from 300 mbar. The concentrated sample was dissolved in 50.0 mL of the solvent. The extract (2.0 µL) was injected to the GC-MS machine (Agilent7890A) with reference library.

Conditions of the GC-MS machine are given below:

Column - Agilent 7890A GC (30 m × 0.25 mm, thickness 0.25 µm); Amount injected – 2.0 µl; Injector temperature – 280ºC; Maximum temperature – 350ºC; Column flow – 1.0 mL/min; Column pressure – 11.567 psi; Column thickness – 0.25 µm; Average velocity – 25.028 cm/s; Detector – 5975C inert XLEI/CI MS detector; Hold up time – 1.0539 min. GC-MS system conditions are:

	Rate (ºC/min)	Value (ºC)	Hold time (min)	Run time (min)
Initial	0.0	70	4	4
Ramp	10.0	280	4	30

Chromatograms were observed and the chemical constituents present in the sample were identified in comparison with the library compounds.

Statistical analysis

Statistical analyses were carried out using one-way ANOVA followed by Duncan's multiple comparison tests. Student's *t* test was done to determine the statistical significance between groups. P values <0.05 were considered statistically significant.

Results and Discussion

Table 1 summarizes the results of the changes in enzyme concentrations in test and control albino Wistar rats following 4 weeks of daily feeding with 1.0 g of fresh young *C. circinalis* leaflets. The results indicate that weight gain in test animals is significantly higher ($p<0.05$) in the test group than the control rats (Figure 1).

Table 1. Liver enzyme profile (IU/L) and creatinine (mg/dl) concentrations of albino Wistar rats following 4 weeks of feeding with young *C. circinalis* leaflets.

	ALT	AST	ALP	GGT	Creatinine
Control	23.9±4.3	55.1±3.7	80.1±0.7	26.6±2.8	1.6±0.8
Test	22.2±4.6	49.2±4.4*	80.9±0.5	29.6±2.9	1.4±0.9
LSD 5%	0.02	0.11	0.01	0.01	0.01

* $p<0.05$ when compared to Control

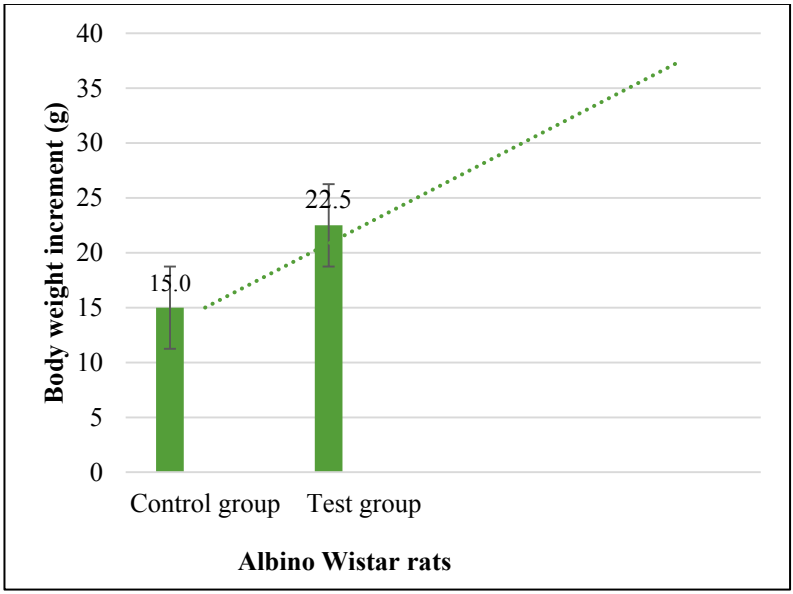

Figure 1. Differences in the body weight increment of albino Wistar rats following the administration of young *C. circinalis* leaflets for a period of four weeks.

Serum levels of ALT are measured clinically as part of a diagnostic liver function test to determine the state of the liver. Increased concentrations of ALT are rarely observed in conditions other than parenchymal liver diseases and its elevation persists longer than those of AST activity (Hultcrantz & Gabrielsson, 1993; Lee & Brady, 2009).

In the present study, it was observed that the ALT concentration in rats fed with *C. circinalis* young leaflets was slightly lower (22.2±4.6 IU/L) than those of the control group (23.9±4.3 IU/L) but the difference was non-significant. Both groups had enzyme concentrations within the normal range.

The AST concentration was found to be significantly lower in test group (49.2±4.4 IU/L) than the control group (55.1±3.7 IU/L). It is found in the liver, cardiac muscles, skeletal muscle, kidney, brain and red blood cells and is used as a marker of liver function as well as a part of the cardiac enzyme profile (Robert & Tomas, 2011). However in the present study, it was observed that when the rats were fed with young leaves of *C. circinalis,* the concentration of AST was significantly reduced.

Serum ALP measurements are of particular interest in the investigation of two groups of conditions: hepatobiliary disease (hepatitis, cirrhosis or malignancy) and bone diseases such as rickets due to vitamin D deficiency, Paget's disease, hyperparathyroidism, and metastatic carcinoma.

Cycas toxicosis has been described in people and many animals, including dogs, sheep, and cattle. It is diagnosed based on a history of known exposure such as observed ingestion, identification of chewed plants, identification of plant material in vomitus, etc. and compatible clinical signs. Elevated levels of cycasin, beta-methylamino-L-alanine had been found in the livers of animals that ingest cycad palms. It had also been found to cause 32.1% mortality rate in dogs with clinical signs (Cheeke, 1998; Hooper, 1978). Increased concentrations of ALP had been observed in these studies on cycad toxicity whereas in the current study, no significant differences were observed in concentrations of ALP in the test and control rats.

The gamma-glutamyl transferase (GGT) is more sensitive than ALP and transaminases in detecting obstructive jaundice, cholangitis and cholecystitis. Only moderate elevation occurs in infectious hepatitis, chronic alcoholism or drug use. Decreased GGT activity is found in cases of hypothyroidism. In the present study, there was no statistically significant difference in GGT concentration of test and control groups.

Creatinine clearance is measured as an indicator of the glomerular function. In the present study, it was observed that there was no statistically significant difference between the creatinine levels of test and control groups indicating that *C. circinalis* young leaflets have no possible adverse effect on the kidney function of rats. The GC-MS analysis (Figure 2) indicated the presence of the following compounds in *C. circinalis* young leaves with more than 80% similarity as shown in Tables 2 and 3.

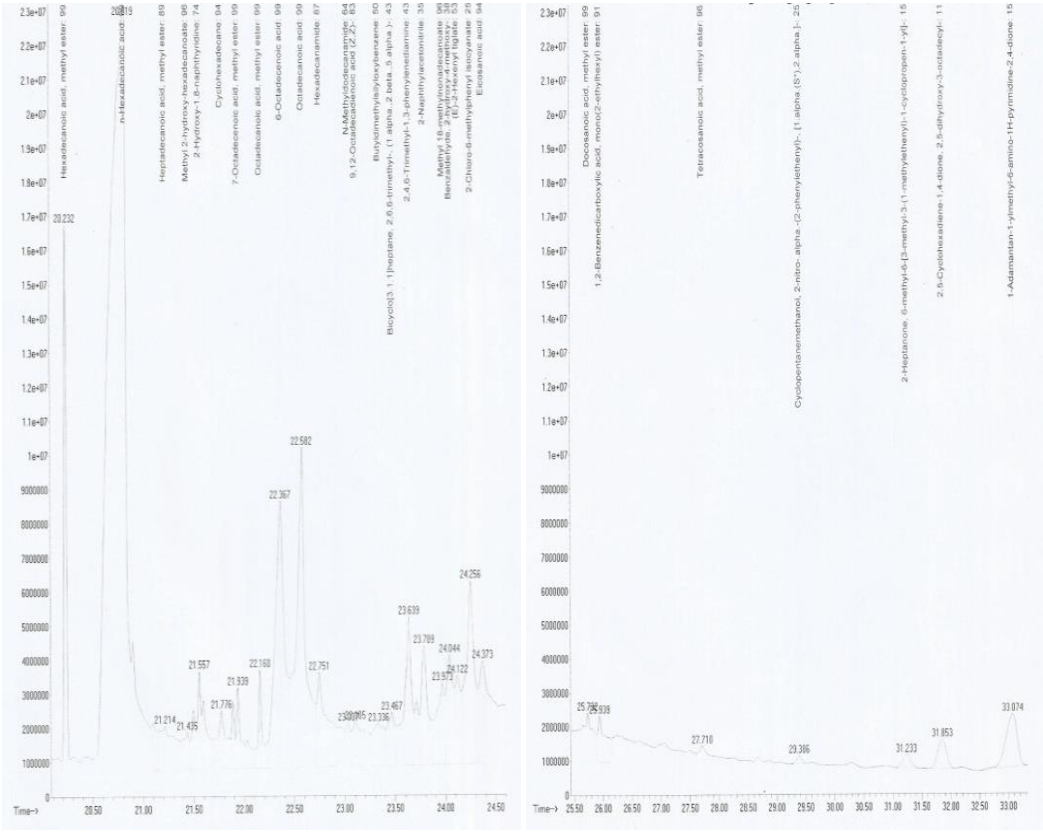

Figure 2. GC-MS chromatograms of young leaves of *C. circinalis*

Table 2. Fatty acids present in young leaves of *C. circinalis* with over 90% similarity.

Compound Name	Molecular weight	% Similarity
6-Octadecenoic acid	22.367	99
7-Octadecenoic acid, methyl ester	21.939	99
Docosanoic acid, methyl ester	25.732	99
Dodecanoic acid	16.290	99
Hexadecanoic acid, methyl ester	20.232	99
n-Hexadecanoic acid	20.819	99
9-Octadecenoic acid	22.582	99
Octadecanoic acid, methyl ester	22.160	99
Tetradecanoic acid	18.541	99
Tetracosanoic acid, methyl ester	27.710	96
Methyl-2-hydroxy-hexadecanate	21.435	96
Methyl-18-methylnonadecanoate	23.973	96
Pentadecanoic acid	19.579	96
Eicosanoic acid	24.373	94
Pentadecanoic acid, methyl ester	29.192	94
1,2-Benzenediol	11.630	94
1,2-benzenedicarboxylic acid, mono (2-ethylhexyl) ester	25.939	91

Table 3. Fatty acids present in young leaves of *C. circinalis* with over 80% similarity.

Compound Name	Molecular weight	% Similarity
Heptadecanoic acid, methyl ester	21.214	89
9,12-octadecadienoic acid	23.105	83
Methyl tetradecanoate	18.116	80

The n-hexadecanoic acid was found in high concentrations in young *C. circinalis* leaflets. Structural and kinetic studies have revealed that n-hexadecanoic acid is an inhibitor of phosholipase A_2 and hence could be considered as an antiinflammatory substance which could be used in the treatment of rheumatic symptoms (Aparna et al., 2012). Palmitic acid has been shown to alter aspects of the central nervous system responsible for the secretion of insulin and to suppress the body's natural appetite-suppressing signals from leptin and insulin which are the key hormones involved in weight regulation (Dixon et al., 2004; Anneken et al., 2006; Benoit et al., 2009). Therefore it could be suggested that the weight gained by albino Wistar rats fed with young *C. circinalis* leaflets may be due to the presence of high level of palmitic acid or their derivatives in the young leaflets.

A study conducted with weanling rats fed with high carbohydrate diets containing cis-12-octadecenoic acid and trans-9, trans-12-octadecadienoic acid (Oleic acid) indicated that six hepatic enzymes involved in lipid metabolism and the concentrations of those enzymes were elevated during the study (Ernken et al., 1987).

Conclusion and Recommendations

Overall, as judged by the concentrations of liver enzymes and creatinine, it is evident that administration of young leaflets of *C. circinalis* does not exert any adverse effect on the liver and kidney function of Wistar rats. Further studies would be done to determine long term toxicity and bioactivity guided fractionation of the extracts of leaflets would also be carried out.

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