Bio-safety evaluation of cycas seed extract on Tilapia, *Oreochromis mossambicus* by oxidative metabolism

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ABSTRACT

The effect of cycas seed extracts (CSE) was observed in tilapia (Oreochromis mossambicus) by exposing them to various concentrations (1, 2, and 5%). The oxidative enzymes, depletion and accumulation in the liver and dorsal muscles were studied on acute (48h/2 days) and chronic (15 and 30 days) conditions. Compared to control, increase ($p<0.01$) in activity of lactate dehydrogenase (LDH) (EC 1.1.1.27) and decrease ($p<0.01$) in activity of succinate dehydrogenase (SDH) (EC 1.3.99.1) following CSE exposed fishes were found. Significant ($p<0.01$) dose dependent depression in SDH and the elevation in LDH, after the acute and chronic conditions in both liver and muscle tissues are favouring an aerobic metabolism in CSE stressed fishes to meet the energy demands. These changes were appeared to favour a less efficient anaerobic metabolism probably due to the inability of tissues in treatment fishes to derive sufficient oxygen for normal metabolic functions. Nonetheless, it's triggered the anaerobic metabolism and arrests the kreb's cycle activities. The present bio-intensive study may give an attention towards the Cycas plant extracts in order to develop a very cheap biopesticide.

Keywords: Cycas seed extracts, *Oreochromis mossambicus*, oxidative enzymes, Succinate dehydrogenase, Lactate dehydrogenase, biopesticide.

INTRODUCTION

In recent decades many of the investigations for the pest management have been conducted to replace the synthetic chemicals with the natural and economical compounds (Kelm *et al.*, 1997; Casida and Quistad, 1998; Peta Devanand and Usha Rani, 2008; Gahukar, 2010). The use of plant derivatives for pest control was common in the tropics before the advent of synthetic pesticides (Saxena, 1987; Casida and Quistad, 1998). Some of the biologically active plant extracts have also been studied for their potential efficiency in minimizing the extent of insecticidal pollution and reducing the cost of operation. Many plant species of various families have been reported to exhibit insecticidal, growth disrupting and synergistic action, nearly 2000 to 2400 species are possess the insecticidal properties (Klocke, 1989; Baskaran and Narayanasamy, 1995a). And over 100 insects belonging to 10 different orders and another 100 non-insect pests can be controlled successfully by using plant products (Gahukar, 2010). In many cases the plants have a history of usage as folk remedies and are still used to kill or repel insects.

The widely used traditional pest control practises in agriculture are largely plant extract-based bio-pesticides. Besides the Cycas male flower affected the landing of Brown Plant Hopper (BPH) of rice (Baskaran and Narayanasamy, 1995b) and *Cycas circinalis* flower controls the pest named Ear head bug of rice (Narayanasamy, 2006) however the seeds of cycas (*Cycas circinalis* L.; Family: Cycadaceae) contain a toxin, cycasin which has been shown to be a potent hepatotoxin and carcinogen in experimental animals (Desai, 2000). Cycasin is a glycoside and was isolated form cycads in 1950. Large amounts provoke liver failure; small amounts are carcinogenic. Methazoxymethanol is derived from cycasin and is neurotoxic. Another toxin is BMAA, an aminoacid which resembles BOAA, beta-N-oxalylamino-L-alanine (see lathyrism). Ince and Codd (2005) stated that BMAA is one of two cycad chemicals with known neurotoxic properties. Importantly, in the toxic and infectious hypothesis: a role of cyanobacteria in the production of endogenous β-N-methylamino-l-alanine is discussed by Stipa *et al.*, (2006); the other is cycasin, a proven developmental neurotoxin.

In modern fish culture practices, plant products like rotenone, jugulone, pyrethrin and pyrethroids are used to remove predatory and weed fishes from rearing ponds. The toxic action of these plant products have been observed to be of short duration depending on the concentration of toxin (Ramanujam and Radha, 1980). In India total 112 plants were reported for their piscicidal

action (Chopra *et al*., 1949) and majority of it contains saponin, alkaloids, glycosides and essential oils (Chopra *et al.*, 1956). The ways by which piscicidal toxins, possibly bring about the fishes to a 'Stupefied intoxicated or paralysed' state have been suggested by earlier works are entering the blood-stream thereon spreading to vital organs, the central nervous system where they impair respiratory reactions in mitochondria or cause paralysis; preventing O_2 uptake by lowering surface tension between water and gills, acting on blood they might cause haemolysis by affecting the muscle activity. Fish being the inhabitant of closed environment becomes a useful model in assessing the effect of plant extracts on physiological, biochemical and immunological parameters (Vashist *et al*., 2000). However, lactate dehydrogenase (Enzyme code/EC 1.1.1.28), here after LDH is a parameter widely used in toxicology an in clinical chemistry to diagnose cell, tissue and organ damage (Kaplan and Pesce, 2009) as well Succinate dehydrogenase (Enzyme code/ EC 1.3.99.1), here after SDH too.

In general, any stress inducing substance will affect the respiratory metabolism of fish. Any alteration in the intermediary metabolism due to stress is bound to affect the activity of oxidative enzymes like SDH, LDH etc. Nevertheless, the LDH is an important glycolytic enzyme which is present virtually in all invertebrate tissues (Kaplan and Pesce, 2009). It is involved in carbohydrate metabolism and has been used as an indicative criterion of exposure to chemical stress (Diamantino *et al*., 2001) and the alterations of normal LDH activity pattern were found the oxygen stress, after exposure (Wu and Lam, 1997). LDH is a parameter widely used in toxicology and in clinical chemistry to diagnose the cell, tissue, and organ damage. However, the potential of this enzyme as an indicative criterion in invertebrate toxicity tests has been scarcely explored (Ribeiro *et al*. 1999). The effect of CSE on the target organs of liver and muscle of fish were examined to assess the changes in SDH and LDH (RajaMamannan, 2002). Since the toxicity and negative environmental effects of organochlorine and organo-phosphorus insecticides are notably increased, a bio-safety approach to evaluate the effects of plant derived substances is urgent. However the present study is to examine the cycas seed extracts (CSE) on some oxidative enzymes of euryhaline fresh water teleost, *Oreochromis mossambicus*, which is extensively available locally in order to develop a very cheap biopesticide.

MATERIALS AND METHODS

Preparation of Cycas Seed Extract (CSE)

The fruits (seeds) of Cycas species (Plate 1) were taken and air dried for one week and powdered material of 2-3 kg (large scale) mixed with organic solvent acetone and minced with an electric blender for one minute. The extract was filtered through muslin and whatman filter paper No.40 with suction. The extracts were taken in soxhelate at 65±2°C refluxing for 6h. and distilling the solvents under reduced pressure and evaporated to dryness (taken as 100% concentration) (see, for examples, R. J. P. Cannell, *Natural Products Isolation*, Humana Press, 1998 and Plant Extracts and Dermatological uses thereof, Behr *et al*.,).

Plate 1: Cycas tree with bunch of seeds in the campus of Govt. Arts College, Coimbatore.

Fish collection and treatment

O. mossambicus with body-length and weight 9-11cm and 12-15g respectively, were purchased from a local aquatic breeding base in Coimbatore. Prior to the experiments, the fishes were acclimated for 15 days to water, which had been under the animal house conditions (28±1°C), with the total mortality of fish near zero. The shrimp were housed in glass aquaria containing 20 liters of well-aerated weathered tap water in 7.6 pH level and DO of 7.4 mg O_2/ l and fish feed (dry pellets for freshwater fish and groundnut cake) was fed once daily in the morning. The glass aquaria was cleaned and replenished with water once daily during both acclimation and experimentation in order to eliminate metabolites and excretory products for the survival of the fishes well, without any disease problem. The live weight of the fishes was taken before the experiments. The fishes were blotted with paper towels and the weights were taken with a sensitive physical balance to the nearest milligram. Sex was not taken into consideration.

After acclimatization, fish were divided into four groups of twelve fishes each (n=12) and kept in glass aquaria at the rate of about 3.4g/l fish/water. Each group corresponded to an exposure concentration, while the unexposed group (Group 1) served as control samples: 0.1 mg/l (Group 2), 0.2 mg/l (Group 2) and 0.5 mg/l (Group

3). Fish were randomly selected for the experiment, water pH was 7.0 ± 0.6 and temperature 28±1°C was maintained. After the acute (48h/2 days) and chronic (15 & 30 days) exposure, four groups of four fishes each were taken out for parallel samples. Fish were weighed, dissected and their livers and dorsal muscle tissues were separated after rinsing in physiological salt water. About 0.30g of liver and dorsal muscle tissue were blotted to remove the adhering blood, rinsed in 0.25M sucrose solution and homogenized after addition of the same medium about 10% using a power driver Teflon tipped glass homogenizer under cold conditions. Following completion of homogenization, the homogenate was centrifuged for 30min at 12,000rpm to remove cellular debris. This homogenization technique has been shown to be proficient in cellular and mitochondrial disruption in fishes. The post lysosomal supernatant portion was centrifuged at 25,000rpm for 60min for and the microsomal pellet so obtained was washed, re-centrifuged and then re-suspended in 0.25M sucrose solution at 0-4^{o}C for enzyme assays. The supernatant was assayed for lactate dehydrogenase (LDH, EC 1.1.1.27) and succinate dehydrogenase (SDH, EC 1.3.99.1) activities. LDH activity was determined using a modification of the procedure described by Bergmeyer *et al*., (1965). SDH activity was determined using a technique developed by Singer (1984).

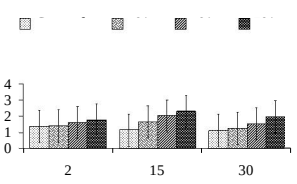
Statistical analysis

All data were analyzed using SPSS (Version 16). Main effects of treatment, interval and their interaction were analyzed by one-way analysis of variance (ANOVA). Significant differences between means were found by Tukey's test. Significance was established at $p < 0.05$.

RESULTS AND DISCUSSION

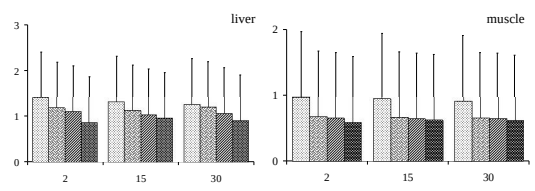
Significant ($p<0.01$) effect of CSE exposures, in terms of dose and duration on the activities of dehydrogenases (lactate and succinate) in liver and dorsal muscles was observed. Compared to control, there was decrease in activity of SDH and increase in activity of LDH till 5% CSE concentration. The activity of SDH and LDH level was scored high in the muscles than liver. In the liver, SDH activities varied significantly across the three CSE exposure level ($F=53.0$, df= 3,32,35, $p=0.00$). Compared to control, the mean value of depression level in SDH are significant at $p<0.01$. In muscle tissues, SDH activities varied significantly across the CSE exposure level ($F=797.0$, df= 3,32,35, $p=0.00$). However, compared to control SDH activity on 5% CSE exposures are significantly more depleted than 1% ($p<0.01$) and in 2% ($p<0.01$), but the activity in 1% and 2% CSE exposure levels have similar depletion rate at $p>0.26$ (Fig 1).

In the liver, LDH activities varied significantly across the three CSE exposure level ($F=70.0$, df= 3,32,35, $p=0.00$). LDH activity in 5% CSE exposure levels are significantly increased more than 1% ($p<0.01$) and on 2% ($p<0.01$), but the activity in 1% and 2% CSE exposure levels have not similar enhance (1%=$p>0.11$, 2%= $p>0.29$). In muscle tissues, LDH activities varied significantly across the CSE exposures ($F=22.0$, df= 3,32,35, $p=0.00$). Compared to control, LDH activity in 5% CSE exposures are significantly more enhanced than 1% ($p<0.01$) and in 2% ($p<0.01$), but the activity in 1% and 2% CSE exposure levels have similar depletion at $p>0.26$ (Fig. 1).



I) Succinate dehydrogenase

Mean Value (μ moles of formazan/mg protein/hr)



II) Lactate dehydrogenase

Time after the end of exposure (days)

Figure 1. Significant changes of oxidative enzymes in acute and chronic conditions of CSE exposure over control.

Cycads studies are scanty with regard to toxicity effects on the targeted animals. However recent studies done by HUANG *et al*., (2010) stated fern and gymnosperm have a great significance for application as new pesticides due to their particular status in plant toxicology and co-evolution with insects, where the insecticidal activities of methanol extracts from the *Cycas acuminatissima* (roots) resulted in higher than 50% of mortalities in 4th instar larvae of *Aedes albopictus* at 24h after treatment and possessed insecticidal activities against the adult of *Musca domestica* at 48h (2 days) after treatment with higher than 90% mortalities. There are many commercial botanical insecticides used in China originated from angiosperms, whereas insecticidal activities of fern and gymnosperm were ignored for a long time because they are usually not fed by crop pests (He *et al*. 2004).

In this study, the toxic effects of CSE on the oxidative enzymes in *O. mossambicus* acute (48h/2days) to chronic (30days) conditions induced rapid changes and seems to overcome the toxic effects of the exposure. But no clear cut variation was recorded for any concentration. The depletion of the key citric acid cycle enzyme SDH indicates impairment of oxidative phase of glucose metabolism and accumulation of intermediates of kreb's cycle metabolities. The suppression of SDH activity in acute and chronic conditions indicates derailment of metabolic cycle and reliance on anaerobic glycolysis for energy synthesizing mechanisms. Thus there was a shift in energy metabolism of the fish from aerobiosis to anaerobiosis. This may also be due to the out come of mitochondrial disruption leading to a decrease in activities of oxidative enzymes and an increase in glycolytic enzymes. The induced decrease of SDH activity can be attributed to the ability of CSE to inhibit mitochondrial enzymatic activities, Anastasi and Bannister (1990) who demonstrated inhibition of this enzyme in fish muscles after chronic intoxication with permethrin, which are a synthetic pyrethroid and thus an insecticide with an entirely different action from organophosphorus insecticides.

Relative to pre-exposure (control) levels the decreased synthesis of SDH and increased accumulation of LDH probably affects the energy synthesizing machinery of the cells. These changes were appeared to favour a less efficient anaerobic metabolism probably due to the inability of tissues in treatment fishes to derive sufficient oxygen for normal metabolic functions. Therefore it is perceptible that CSE may have induced toxicity on targeted organs. Nonetheless, it's triggered the anaerobic metabolism and arrests the kreb's cycle activities. In fish, apart from their well known cholinesterase (ChE) inhibitory effect, pesticides and plant derived extracts modify the activity of several other metabolic enzymes (Mukhopadhyay and Dehadrai, 1980a, b; Sastry and Sharma, 1980; Joshi and Desai, 1981; Natarajan, 1984, 1985; Aaltonen *et al.*, 2000). The insecticidal activity of the plant extracts, of *Cycas acuminatissima* (leaves) mortalities of the tested insects were found higher than 40% at 48 h after exposure (HUANG *et al*., 2010). Hence, present bio-intensive study was also concluded that it may give an attention towards the *Cycas* plant extracts, in order to develop a very cheap bio-pesticide.

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REFERENCE

Aaltonen, T. M., Jokinen, E. I., Lappivaara, J., Markkula, S. E., Salo, H. M., Leppanen, H. and Lammi, R. 2000. Effect of primary and secondary-treated blached kraft mill effluents on the immune system and physiological parameters of roach. *Aquatic Toxicology,* **51**: 55-67.

Anastasi, A., Bannister, T. V. 1990. Effect of pesticide on Mediterranean fish muscle enzyme. *Acta Adriatica,* **21**: 119-136.

Baskaran, V. and Narayanasamy, P. 1995a. *Traditional Pest Control*. Caterpillar Publications, Mariyappa Nagar, Tamil Nadu, India.

Baskaran, V. and Narayanasamy, P. 1995b. Tackle the agricultural pests problems the traditional way key note papers and extended Abstracts of 2nd congress on *Traditional Sciences and technologies of India,* Anna University, Chennai. P.6.

Bergmeyer, H., Bernt, E., Hess, B. 1965. *Methods in Enzymatic Analysis*. New York: Academic Press, pp 736-741.

Casida, J. E. and Gary, B. and Quistad, G. B. 1998. Golden age of insecticide research: past, present, or future? *Annual Review of Entomology,* **43**: 1-16.

Chopra, R. N., Badhwar, R. L., and Ghosh, S. 1949. *Poisonous Plants of India*. Government of India Press, Calcutta. **1:** 762.

Chopra, R. N., Nayar, S. L., and Chopra, I. C. 1956. *Glossary of Indian Medicinal Plants*. C.S.I.R., New Delhi. **34**: 82- 94.

Desai, B. B. 2000. *Handbook of Nutrition and Diet*. New. York: Marcel Dekker Inc. pp. 797. ISBN 0-8247-0375-8.

Diamantino, T. C., Almeida, E., Soares, A. M. V. M., and Guilhermino, L. 2001. Lactate dehydrogenase activity as an effect criterion in toxicity test with *Daphhnia magna* straus. *Chemosphere,* **45**: 553-560.

Gahukar, R. T. 2010. Role and perspective of phytochemicals in pest management in India. *Current Science,* 98 (**7**): 897-899.

He, T. P., Wen, Y. G., Wen, X. F., Li, J. G. 2004. The plant resources in Shiwandadashan Mountain natural reserve of Guangxi. *Chinese Wild Plant Resources*, **1**: 23-26. (in Chinese)

HUANG Su-qing, ZHANG Zhi-xiang, LI You-zhi1, LI Yu-xia1, and XU Han-hong. 2010. Anti-Insect Activity of the Methanol Extracts of Fern and Gymnosperm. *Agricultural Sciences in China*, 9 (**2**): 249-256.

Ince, P. G.and Codd, G. A. 2005. Return of the cycad hypothesis - does the amyotrophic lateral sclerosis/ parkinsonism dementia complex (ALS/PDC) of Guam have new implications for global health? *Neuropathology and Applied Neurobiology*, **31:** 345–353.

Joshi, U. M. and Desai, A. K. 1981. Effects of sublethal concentration of monocrotophos on acid and alkaline phosphatase activities in the tissue of freshwater fish, *Tilapia mossambica*. *Journal of Animal Morphology and Physiology*, **28**: 221-228.

Kaplan, L. A. and Pesce, A. J. (Eds.). 2009. *Clinical Chemistry*: *Theory, Analysis and Correlation*. Mosby-Year Book, Inc., Missouri. Pp 1200.

Kelm, M. A., Nair, M. G. and Schutzki, R. A. 1997. Mosquitocidal compounds from *Magnolia salicifolia*. *International Journal of Pharmacognacy*, **35**: 84–90.

Klocke, J. A. 1989. Plant compounds as source and models of insect-control agents. In: Hostettmann, K. (Ed.), *Economic and Medicinal Plant Research*. Academic Press, London, pp. 103–144.

Mukhopadhyay, P.K., and Dehadrai, P.V. 1980b. Biochemical Changes in the air-breathing cat-fish, *Clarius batrachus* (Linn) exposed to malathion. *Environmental Pollution*. A**22**: 149-158.

Mukhophadhyay, P.K., and Dehadrai, P.V. 1980a. A studies on air-breathing catfish, *Clarius batracus* (Linn) under sub lethal malathiron exposure. *Indian Journal of Experimental Biology,* **18**: 400-404.

Narayanasamy, P. 2006. Traditional knowledge of tribals in crop protection. Indian *Journal of Traditional Knowledge*, 5(**1**): 64-70.

Natarajan, G. M. 1984. Effect of sub lethal concentration of metasystox on selected oxidative enzymes, tissue respiration and haemotology of the fresh water air-breathing fish *Channa Striata*. *Pesticide Biochemistry and Physiology,* **21**: 194-198.

Natarajan, G. M. 1985. Introduction of branchial enzymes in snake head (*Channa Striatus*) by oxydemeton-methyl. *Pesticide Biochemistry and Physiology*, **23**: 41-46.

Peta Devanand and Usha Rani, P. 2008. Biological potency of certain plant extracts in management of two lepidopteran pests of *Ricinus communis L. Journal of Biopesticides*, **1**(2): 170 – 176.

RajaMamannan, M. A. 2002. Enzymological responses of a fresh water fish *Orecochromis mossambicus* [Peters] to Cycas Seed Extract exposure. M. Phil., Dissertation. Bharathiar University, Coimbatore-46.

Ramanujam, S. N. and Radha, B. K. 1980. Studies on piscicidal plants of North-Eastern India. Hope for an indigenous plant poison for nursery management. *Current Science,* 49 (**6**): 251-252.

Ribeiro, S., Guilhermino, L., Sousa, J. P., Soares, A. M. V. M. 1999. Novel bioassay based on acetylcholinesterase and lactate dehydrogenase activities to evaluate the toxicity of chemical to soil isopods. *Ecotoxicology and Environmental Safety,* **44**: 287-293.

Sastry, K. V., and Sharma, K. 1980. Diazion effect on the activities of brain enzymes from *Ophiocephalus* (*Channa*) *punctatus*. *Bulletin of Environmental Contamination and Toxicology*, **24**: 326-332.

Saxena, K. 1987. Indigenous crop Protection practices in sub-Saharan East Africa. In: *Database of Natural Crop Protectant Chemicals* (DNCPC). URL: http:www.ipmafrica.htpm

Singer, T. 1984. Determination of the activity of succinate, NADH, chlorine, and alpha-glycerophosphate dehydrogenase. *Methods Biochemical Analysis*, 22: 123-175.

Stipa, G. 2006. Sporadic amyotrophic lateral sclerosis as an infectious disease: A possible role of cyanobacteria? *Medical Hypotheses*, **67**: 1363–1371.

Vashist, S., Kumari, I. S., and Jamil, K. 2000. The study of alteration in respiratory metabolism of teleost fish *Tilapia mossambica* due to the effect of polluted waters. *Proceedings of the National Academy of Sciences*, India **70** (B): 229-235.

Wu, R. S. S., and Lam, P. K. S. 1997. Glusoce-6-phosphate dehydrogenase and lactate dehydrogenase in the greenlipped mussel (*Perna viridis*). Possible biomarker for hypoxia in the marine environment. *Water Research* **31**: 2797-2801.

Yasmeen and Saxena, S. K. 1990. Rhizosphere and rhizoplane mycoflora of *Adiantum incisum*. *Indian Fern Journal* **7**: 21–23.

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