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Studies on the Toxicology of the Palmyrah Palm (*Borassus flabellifer* L): Part I. A Bioassay for the Neurotoxin

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Abstract : The lethal effect of partially purified preparations of the neurotoxin of the palmyrah palm (*Borassus flabellifer* L) on tadpoles of the tree frog (*Rhacophorus leucomystax maculatus* Schneider) was used as the basis of the bioassay for this toxin. The partially purified neurotoxin had an LD50 of 54.1+ 17.9 μg/ml on 12-15 mm tadpoles during a 4 day test period. Heat treatment at 100°C for 10 min, of aqueous solutions of the toxin did not alter its LD50. The time course of tadpole mortality with 1 to 4 LD 50 of this preparation showed a delayed response, deaths having occurred on the second, third and fourth days contrasting with more rapid deaths which were maximal on the first or second day, produced by crude preparations of the neurotoxin. No histological abnormalities were detected in the liver, brain or spinal cord of intoxicated tadpoles.

1. Introduction

Flour from the young shoot of the palmyrah palm (*Borassus flabellifer* L) which is consumed by humans in some Asian and African countries both as a food and as a traditional medicine, has previously been reported to be hepatotoxic and neurotoxic to experimental rats.[2,4] A neurotoxin from this flour was partially purified by Greig and co-workers.[3] The monitoring of the purification of the neurotoxin needed a convenient bioassay and the latter authors used weanling or adult rats in which the neurotoxic fractions reproduced the characteristic neurotoxic effects which result from the feeding of this flour. They further showed that the lethal response of brine shrimp larvae (*Artemia salina*) could be used to quantitate the neurotoxin.

A tadpole bioassay system has been described[1] for the quantitation of aflatoxin. This test was also found to be capable of providing histological confirmation of aflatoxicity through the development of characteristic abnormalities in the nuclei of the hepatocytes of the intoxicated tadpoles. This test system was investigated as a possible alternative bioassay for the neurotoxin of the palmyrah palm and we report that the neurotoxin was found to be lethal to tadpoles with LD50 values which were appreciably lower than those reported for the brine shrimp larvae.

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2. Experimental

2.1 Tadpoles. The tadpoles of the tree frog (*Rhacophorus leucomystax maculatus* Schneider) were found on preliminary tests to be more sensitive to the lethal action of the palmyrah neurotoxin than the commonly available tadpoles of the toad *Bufo melanostictus* Gray.

Fresh egg nests of the tree frog were obtained from garden ponds and were incubated in laboratory tanks with water plants, at 24°-26°C. Each nest provided 100-200 larvae of similar size and age, for replicate assays.

2.2 Method. This was essentially similar to that used for the bioassay of aflatoxin.[1] Five tadpoles of equal total body length (10-20 mm) were placed in 10 ml of distilled water without added food, in each of a series of sloped, unstoppered McCartney bottles; one bottle with 5 tadpoles was used for each dose level. At least one bottle without added toxin was used as a control for each set of tests. The bottles were incubated at room temperature (24°-26°C) under normal conditions of lighting (daylight and artificial light) prevalent in the laboratory. Untreated (control) tadpoles were actively motile and showed no histological abnormalities in the liver and central nervous system after the test period of 4 days.

2.3 Quantitation. Assays in which the control tadpoles showed deaths within the 4 day test period, were abandoned. This often happened if the room temperature rose to over 30°C. Dead tadpoles in the treated bottles were removed immediately on detection. The final mortality was counted after the 4 day test period. LD50 values were calculated by the method of Reed and Muench.[5]

2.4 Toxin preparations. Partially purified neurotoxin was a gift from Dr. J. B. Greig (MRC Toxicology Unit, Surrey, England). This product was a pale yellow solid, readily soluble in distilled water to yield a clear solution with a pH of 7.5-8. This material represented approximately 023.% by weight of the original flour.

The lethality of this preparation on tadpoles was compared with that of crude, methanolic extracts of the neurotoxin,[3] from raw shoots of palmyrah. The brown, plastic, dark yellow solid from the raw shoots represented approximately 8.7% of the weight of the original flour. The latter extracts were soluble in distilled water yielding opalescent solutions with a pH of 5.6-6. Solutions (10-15 mg/ml) in distilled water were adjusted to pH 7.6 before addition to the tadpoles, in a doubling dilution series. After initial, gentle manual agitation, further agitation was provided by the constant motion of the tadpoles.

Confirmation of the toxicity of these solutions was made by oral intubation to rats, in doses equivalent to 10 grams of the flour per rat per day. Production of the characteristic neurotoxic symptoms was used to confirm, qualitatively, the neurotoxicity of these preparations.

2.5 Histology. Separate batches of tadpoles, treated with approximately 1 to 4 LD50, were fixed fresh, on the second and fourth day, for histological examination. Control tadpoles taken on the same days, were used for comparison. Step-serial, coronal and sagittal sections of paraffin embedded larvae were stained with haematoxylin and eosin (H & E) for examination of the central nervous system and the liver. Survivors from toxin assays were also examined histologically.

3. Results

3.1 Lethality. Partially purified neurotoxin had a mean LD50 (from 10 titrations) of 53.8 ± 20.1 μg/ml on 12 mm tadpoles. With 15 mm tadpoles, a mean LD50 of 54.7 ± 11.1 μg/ml was obtained from 4 titrations. The mean overall LD50 (14 titrations) for 12-15 mm tadpoles was 54.1 ± 17.9 μg/ml.

Replicate titrations of the same solution of neurotoxin on the same batch of tadpoles gave a mean LD50 of 68.5 ± 11.2 μg/ml (4 titrations), with a coefficient of variation of 16%.

Crude neurotoxin from raw shoots had a mean LD50 (5 titrations) on 12 mm tadpoles, of 32.2 ± 7.4 μg/ml. Dose response curves with the purified and crude preparations of neurotoxin, are shown in Figure 1.

3.2 Time Course of the lethal response. With purified neurotoxin mortality commenced on the second and third days and was a maximum on the 4th or 5th days whereas with crude preparations deaths were maximal on the first or second days of treatment (Figures 2 and 3).

3.3 Thermostability of the neurotoxin. Heat treatment at approximately 100°C for 10 min, of the aqueous solutions of the purified and crude preparations of neurotoxin, did not alter the lethal end points, confirming the observations of Greig *et al.*[3], that the neurotoxin is thermostable.

3.4 Histology. Neither with 1 LD50 at 2 days nor with four LD50 at 2 and 4 days of treatment, were any significant histological abnormalities detected in the liver or central nervous system of toxin treated tadpoles.

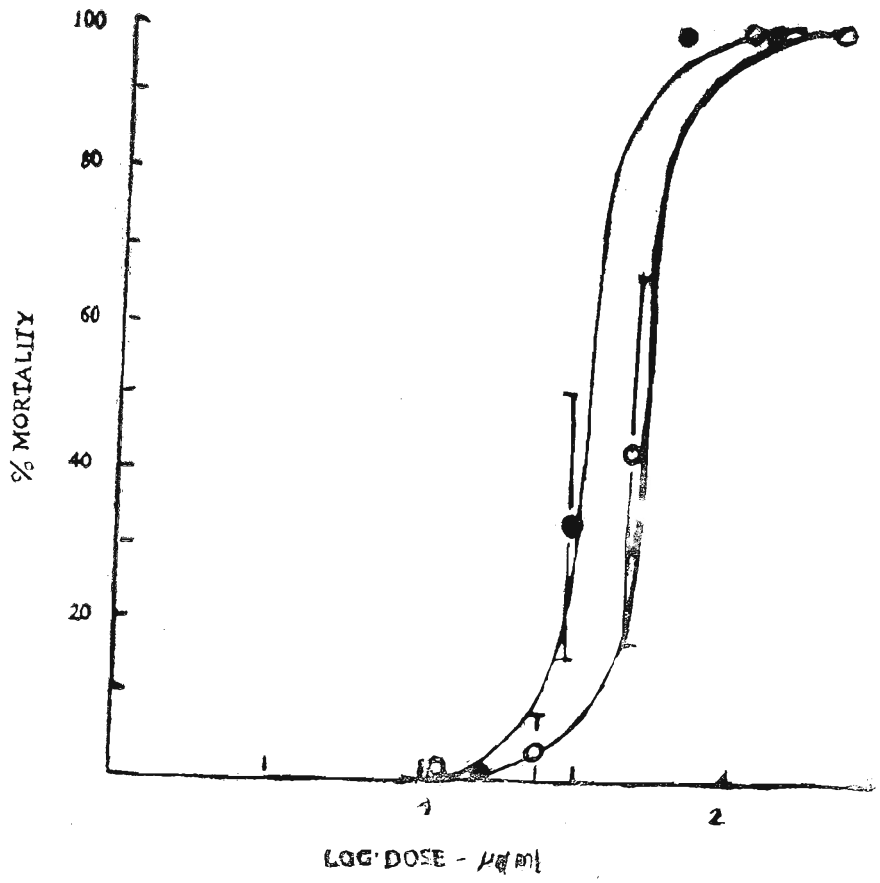

Figure 1. Log-dose mortality curves with crude neurotoxin from raw palmyrah shoots –●–; partially purified neurotoxin –O–. Pooled data from replicate titrations on 20 tadpoles (15mm) per dose with purified neurotoxin and on 15 tadpoles (15mm) per dose with crude neurotoxin. Vertical bars indicate ± 1 SD.

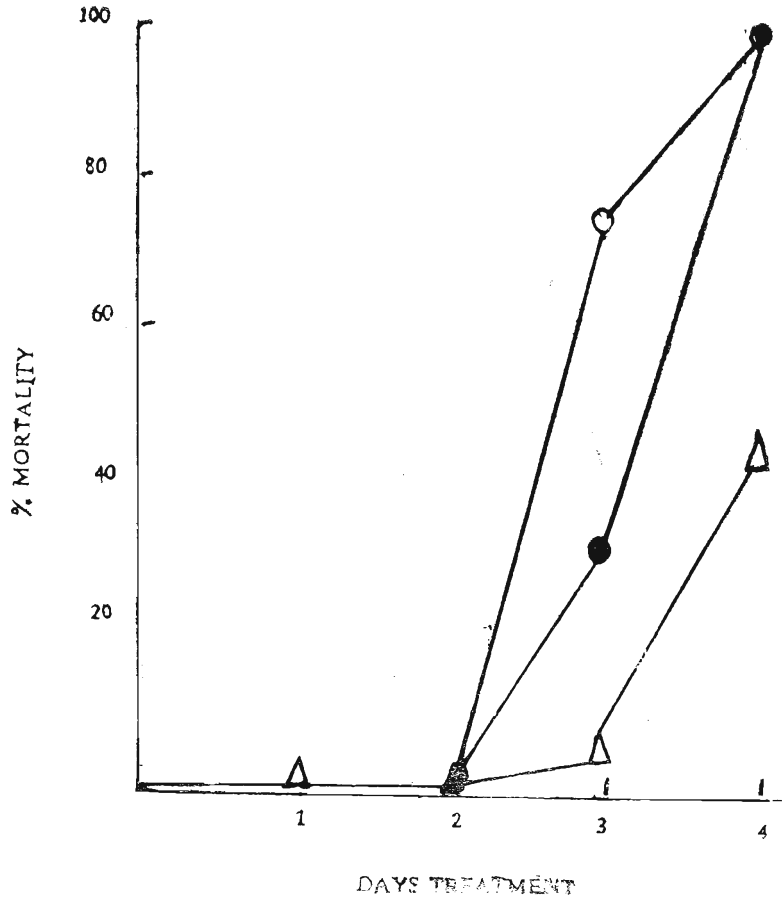

Figure 2. Time course of mortality of 12 mm. tadpoles treated with purified neurotoxin over a 4 day period ; 160 μg/ml –O– ; 80 μg/ml –●– ; 40 μg/ml –Δ–. LD 50 of toxin 54.7μg/ml.

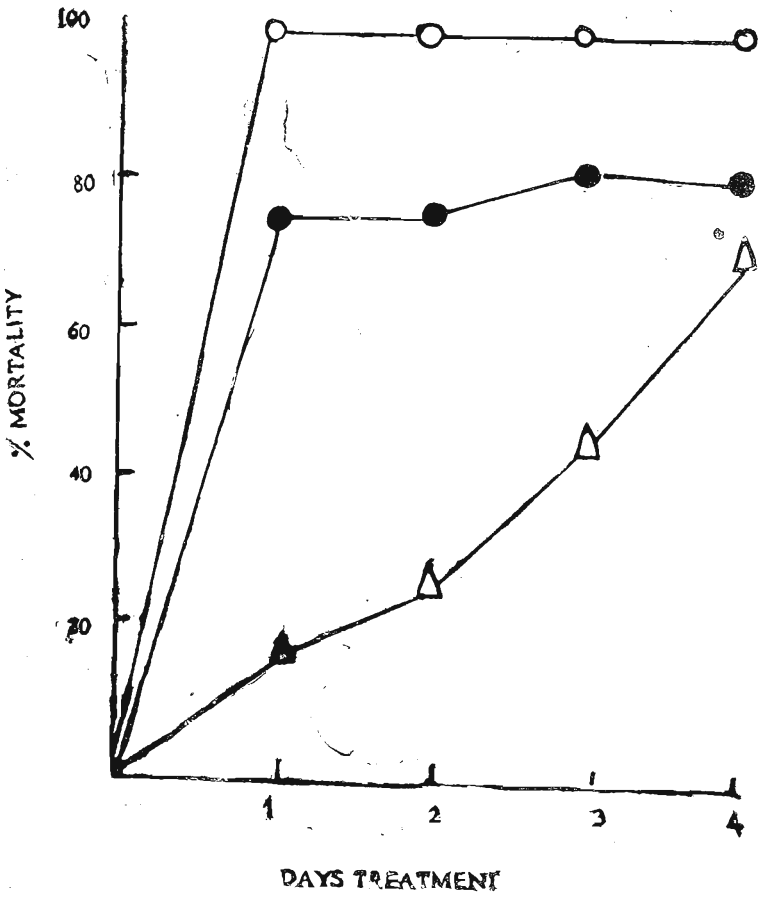

Figure 3. Time course of mortality of 12 - 18 mm tadpoles treated with crude preparations of neurotoxin from raw palmyrah shoots over a 4 day period : 200 μg/ml –O– ; 100 μg/ml –●–; 50 μg/ml –Δ–. LD 50 of toxin 21.3/μg/ml.

4. Discussion

The tadpole bioassay of aflatoxin had the advantage, apart from simplicity and sensitivity, of providing histological confirmation of aflatoxicity through the development of characteristic nuclear abnormalities in the intoxicated hepatocytes. This advantage is however absent in the tadpole bioassay of the palmyrah neurotoxin, since no histological abnormalities were seen in the neurotoxin treated tadpoles. In this respect the test is comparable to the brine shrimp larval bioassay of the neurotoxin, in which no histological confirmation of neurotoxicity is possible. However, one advantage of the tadpole bioassay is that the LD50 of the partially purified neurotoxin is approximately 180-fold lower on tadpoles than on brine shrimp larvae, which was reported as 1.81 mg/200 μl.[3] A disadvantage of the tadpole method is that the natural availability of the tree frog larvae depends on rainfall; egg nests were scarce during dry weather. The possibility of breeding the larvae in laboratory tanks is being investigated.

The difference in the time course of mortality between tadpoles treated with the partially purified neurotoxin and those treated with crude neurotoxin, might suggest the existence in crude preparations of a synergistic factor (s) which accelerated the lethality of the neurotoxin.

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