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Some studies on the neurotoxic effect of palmyrah odiyal flour.

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Abstract

Three decades ago palmyrah *(Borassus flabellifer)* flour (odiyal) was shown to have neurotoxic and hepatotoxic effects. Previous studies have shown that both toxicities can be destroyed by dry heat at 80°C for 45 minutes. This study showed that the toxin, which has been elusive for so long, is probably due to a mixture of molecules requiring synergism. Evidence supporting mitochondrial damage is provided showing that palmyrah flour water extract causes hyperammonaemia and that the urea cycle is affected. There is no evidence for muscle and kidney damage as serum creatinine levels did not change with the neurotoxic symptoms.

Keywords: Palmyrah, Odiyal, Neurotoxic effect, Synergism, Urea cycle.

1. Introduction

The toxic effect of palmyrah *(Borassus flabellifer)* flour was reported as far back as three decades ago [1]. The next significant step was the partial purification of the toxin and establishing that it had a glycoside moiety and a secondary amine with a molecular weight of approximately 1400[2]. The absence of saponins was also shown. Subsequently it was shown that the toxic character was lost on heating at 80°C for 45 minutes[3]. The study [3] also showed that the toxicity decreased on purification and that it resided in the neutral and negatively charged fraction from an ion exchange column. The nature of the toxin remained elusive. Lowered toxic effect was also shown by a precipitate in ethyl acetate fractions of a medium pressure liquid chromatography (MPLC) separation [4]. This precipitate was found to be a complex of a flabelliferin triglycoside, a caroteniod (later identified by phytoene) and a primary amine, which would give a molecular weight around 1500. On this evidence Jansz and others [5] first suggested synergism. It has shown that rats consuming palmyrah flour showed lesions in the mitochondria [6]. The objectives of this study were to attempt to shed more light on (A) the nature of the toxicity-Is it synergism? and (B) the possible mechanism of action.

2. Materials And Methods

2.1 Biological Materials

Weanling male Wistar rats aged four weeks were obtained from the Medical Research institute (MRI) Colombo, animals were caged separately and the groups were selected so that the average weights in each group were similar. The rats were fed on WHO recommended breeding feed [7] and water *ad libitum.* Palmyrah flour was obtained from Kalpitiya through the Palmyrah Development Board.

2.2 Preparation of extract

Palmyrah flour (l00g) was extracted with 1:1 mixture of methanol and water (400ml) and rotary-evaporated to remove methanol. The water extract passed through an ion exchange column (Dowex 50W, H^+ form), eluted to remove negatively charged and neutral molecules (Fr I), washed with distilled water (250 ml) and with methanol: ammonia 1:1 (250 ml) to remove positively charged molecules (Fr II). Fr II was rotary evaporated at 35°C - 45°C to evaporate ammonia and then both fractions were freeze-dried to obtained dry powders. The dry weight of Fr I and Fr II were 2.07g and 1.7g respectively. Both fractions were dissolved in 5ml of distilled water.

Bulk extraction was carried out from 200g of flour, methanol was evaporated as before and the sample was freeze-dried to remove water to obtained 17ml of the extract (Fr 0). The Fr I and Fr II were separated from 200g of flour using above procedure. These were recombined and methanol, ammonia and water removed to obtained 13ml of the extract (Fr III). The Volumes of the Fr 0 and Fr III made up to 40ml with distilled water.

2.3 Thin layer chromatography

This was conducted on silica gel G_{60} using Butanol: Ethanol: Ammonia (7:3:4) and sprayed with anisaldehyde and ninhydrin spray reagents [8].

2.4 Animal model

Basic animal model was as given in [9] where animals were fed on WHO standard rat and mouse breeding feeds [7] with water *ad libitum.*

2.5 Animal Experiments

2.5.1 Preliminary Experiment

Three weanling mice were used for the preliminary study of Fr I and Fr II since neurotoxic effect obeys all or none-effect (two test mice one each for Fr I and Fr II and one control mice). In addition to the normal feed these mice were treated with 0.5ml of the extract (one dose) daily for seven consecutive days. Control mice were treated with 0.5 ml distilled water.

2.5.2 Final Experiment

Eighteen four weeks old male weanling Wistar rats with mean body weight of 70.5g were randomly allocated into three groups control, Fr 0 (crude extract) and Fr III(recombined extractives) (six animal per group) and each day two doses one in the morning and one in the evening 0.5ml of the extract so that each extractive arose from the equivalence of l0g palmyrah flour (the animal each by a rat day) by oesophagal feeding using a sondi needle. When symptoms of neurotoxicity were observed in one group, the blood (2ml) were withdrawn from all the animals from the heart after anesthetizing them.

2.6 Testing of Blood

Serum was separated by centrifuging at 3000rpm for 15 minutes and tested for serum ammonia [10], urea [10] and creatinine [10].

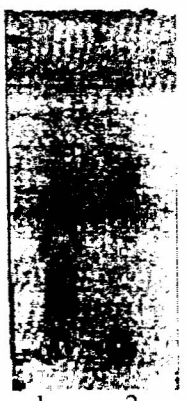
3. Results

3.1 The Neurotoxic effect

The neurotoxic signs such as ruffled coat, muscle incardination, characteristic fits, falling over backwards, immobility of hind limbs and hyper-excitation to touch [3] were shown on Fr 0 (crude extract) and Fr III (recombined extract), but not Fr I and Fr II.

3.2 TLC on fractions

This is shown on Figure 1 (anisaldehyde) and Figure 2 (ninhydrin). There was one ninhydrin spot in Fr II, which coincides with fluorescence under a UV light (566nm). Fr I contained 7-8 flabelliferins (saponin glycoside) spots. Fr II showed amino acids spots. The TLC plates in both sprays on Fr 0 and Fr III were as expected very similar.



Lane 1 Fr I
Lane 2 Fr II
Volume applied 5 ul.

Fig 1: TLC plate after visualized with anisaldehyde for sugars and flabelliferins

Lane 1 FrI
Lane 2 Fr II
Volume applied = 5 ul

Fig 2 : TLC plate after visualized with nirihydrin for amines

3.3 Results of assay of serum

The serum ammonia (Tablel) in Fr III and Fr 0 test groups' animals were significantly different from the control animals ($p= 8 \times 10^{-5}$ and $p= 1.5\ 10^{-4}$ respectively). Table 2 shows that creatinine levels are not significantly different. There was not enough blood from some rats for urea determination but values obtained were as follows.

Fraction (Mean ± Standard deviation)
Fr 0 : 38.4 ±2.0
Fri II : 36.0±1.6
Control : 45.7 ±2.5

Table 1: Serum ammonia levels of weanling rats treated with Fr 0, Fr III palmyrah flour extracts and control rats.

Treatment group	Serum ammonia level Mean ± SD µg/dl	p value compared to control
Test 1 (Fr 0)	68 ± 7.07	8 x 1O-^{5}
Test 2 (Fr III)	54 ± 3.58	1.5 x 10 -4
Control	36 ± 4.23	

n=6
Control = water
SD - Standard deviation
The p value of Fr 0 and Fr III was 1.4×10^{-2}

Table 2: Serum creatinine levels of weanling rats treated with Fr 0, Fr III palmyrah flour extracts and control rats.

Treatment group	Serum ammonia level Mean ± SD µmoles/l	p value compared to control
Test 1 (Fr 0)	26.6 ± 3.52	0.52
Test 2 (Fr III)	24.1 ± 1.96	0.32
Control	25.5 ± 2.07	

n=6
Control = water
SD - Standard deviation
The p value of Fr 0 and Fr III was 1.4×10^{-2}

4. Discussion

It has found that the neutral and negatively charged fraction contained some amines and showed some signs of nurotoxicity [3]. Studying the conditions of separation it was suspected that the ion exchange column was overloaded. This study was repeated with a bed volume three fold the size.

Results showed that both fractions had no neurotoxicity. Recombining these fractions resulted in full neurotoxic symptoms. This is indicated that two or more molecules are responsible for neurotoxicity and was strongly suggestive of synergistic action.

Pathmanathan and coworkers [6] showed by electron microscopy that palmyrah flour caused lesions in the liver mitochondria of Wistar rats. If mitochondria are disrupted this will explain increased AST levels in blood without affecting ALT levels [3]. It could also mean that processes like ATP formation (loss of transmembrane potential) and the urea cycle can be affected.

These studies show that there is a problem of ammonia detoxification and high ammonia could be the cause of the neurotoxic symptoms. The blood urea levels strengthen the primary importance of the urea cycle. There is no significant different in blood creatinine levels showing importantly that although there is muscle incoordination there is no muscle damage. Therefore increase of serum AST could not be from muscles. Further more kidney functioning does not appear to be impaired.

Taking all data into account, there appears to high possibility of the toxin being a complex of a flabelliferin (now identified by Bandara and Jansz as F_B and F_C, unpublished results),

Phytoene (a flabelliferin binding carotenoid) and a primary amine. However isolation of this complex in sufficient quantities to produce statistically significant results will be a Herculean task and would explain why after three decades the toxin has not been structurally elucidated. It probably appears that the neurotoxic effect is one end result of the hepatotoxic effect.

5. Acknowledgements

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6. References

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