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The synergistic neurotoxins of palmyrah (*Borassus flabellifer* L.) flour

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ABSTRACT

Palmyrah (*Borassus flabellifer* L.) is consumed in various forms in different parts of Sri Lanka. Although a neurotoxic syndrome had been described in rats it is being consumed by humans. However, the chemical nature of the neurotoxin is unknown. Thus the objective of the present study was to determine the neurotoxic principle using palmyrah seed shoot and bioactivity directed separations. Two primary amines were isolated. These were not toxic individually but toxic when given together showing synergism. Studies of the amines by ^{1}H NMR and ^{13}C-NMR showed a steroidal aglycone (spirostane) linked to a carbohydrate moiety containing three α-rhamnosyl residues and a β-pyranosyl residue. Comparison with known spectral data showed that the β-pyranosyl is likely to be a β-glucosaminosyl which is probably in position 6 of glucose in one compound and most likely to be in the position 3 of glucose in the other. Synergism appears to be at two levels (i.e. at the absorption level) where the high content of neutral saponins present appears to be needed to facilitate absorption of the cationic toxin and at the point of action. The synergistic mechanism of the toxic amines remains to be explained.

Keywords: Palmyrah, neurotoxin, spirostane, saponins, primary amine, synergism.

INTRODUCTION

The seed shoot of palmyrah (*Borassus flabellifer* L.) is eaten, boiled or dried (kottakilangu), but mainly made into flour by milling. This is used in the preparation of many traditional dishes consumed regularly in the North and East of Sri Lanka. The toxic effects of palmyrah flour were first reported in 1971 as a hepatotoxic effect in rats (Arseculeratne et al., 1971). The symptoms were: ruffled coat, apathetic animal, nodding movements of head and uncoordinated spasms of fore-limbs, erect animals on hind limbs, falling over backwards, ataxia and finally death (Arseculeratne et al., 1971). The causative effect was damage to the rat liver mitochondrial membrane (Pathmanathan et al.,

2001). This conclusion was supported by hyperammonaemia and low serum urea (Perera, 2004) and high serum aspartate aminotransferase (AST) (Sumudunie et al., 2004) in rats. Literature also reported neurotoxicity of palmyrah flour (Greig et al., 1980). According to their work neurotoxin was a tertiary amine-saponin of a molecular weight of 1400 (Greig et al., 1980). Toxicity studies carried out in the area of consumption (Arseculeratne et al., 1971) might have led to underutilization of palmyrah flour.

It was reported by Perera (2004) that on separation by ion-exchange chromatography (Dowex), the cationic (amine) fraction was not toxic to Wistar rats on oral administration, unless recombined with the

neutral fraction which is saponin rich and non toxic (Wickramasekara and Jansz, 2003). This is indicative of a synergistic effect, which shows that the saponin rich neutral fraction is needed to disrupt the mucosal membrane (Francis et al., 2002), after which the effective toxin is absorbed. The flour could be detoxified by dry heat at 80 °C for 45 min but not with wet heat (Sumudunie et al., 2004). The toxin was not volatile but dry heating of palmyrah flour causes a marked loss of primary amines (Keerthi et al., 2002). It has to be noted that neurotoxicity in humans has not been reported, and if the structure of the toxin is known it could help expand use of an underutilized resource.

Thus the aim of the study was to solve nearly a four decade old problem *viz.*, the chemical nature of the neurotoxin aided by bioassay directed separation using Wistar rat animal models.

MATERIALS AND METHODS
Seed shoot and animals
Palmyrah seed shoot samples (~10 kg) were obtained from Kalpitiya area in the North-west of Sri Lanka and stored in a freezer (< 0 °C) until use. Moisture of seed shoots was determined using oven dry method (AOAC, 1984; YCO-010 Series, Gemmy Industrial Crop, Taiwan), before commencing extraction procedures.

Inbred male Wistar rats were obtained from Medical Research Institute, Borella, Sri Lanka. Animals were housed in University animal house under standard conditions. Ethical clearance was obtained for the animal experiments from Faculty of Medical Sciences, Ethical Committee before commencement of animal experiments (Ethical committee approval no: 23, 2006).

Chemicals
Grade of chemicals was selected according to the level of separation. General purpose reagents (GPR) were used in preliminary separations up to Medium Pressure Liquid Chromatography (MPLC). From this point onwards analytical grade or spectroscopic grade solvents were employed. Hexane, methylene chloride and methanol were obtained from BDH Poole, England. Ethyl acetate and ammonium hydroxide (specific gravity 0.88) were obtained from

Superchem products (Needham Market/Suffolk) England. Distilled water was obtained from "Manesty" distillation unit (Manesty Machine Ltd, England) and autoclaved before use in intra venous (IV) injections.

Bioactivity directed isolation of active fractions and compounds
Medium pressure liquid chromatography (MPLC)
Palmyrah seed shoots were subjected to the separation procedure given in Figure 1, taking care that each step was well below the detoxification temperature (80 °C). Palmyrah seed shoot extract freeze dried powder (~ 12 g) was mixed with silica (Silica gel G_{60} 0.040-0.063 mm, MERCK, Germany) and packed into a MPLC column ("Separo" column and FMI QD Lab Pump, Sweden). Crude extract of palmyrah seed shoots was tested using animal experiment (see under Animals studies). Compounds were eluted stepwise by modifying the method by Ariyasena et al. (2002a), using 250 ml hexane, 250 ml methylene chloride, 750 ml ethyl acetate and 500 ml methanol respectively. Any extractive remaining in the column was extracted using water. The solvent free extractives were obtained after evaporation of solvents at room temperature. These extractives were further subjected to freeze drying and weights of each fraction obtained. Since hexane and methylene chloride fractions were insoluble in water, single dose animal experiments (see under Animals studies) were carried out using solvent free ethyl acetate, methanol and water fractions only.

Ion exchange column chromatography (IECC)
The methanolic fraction was subjected to further separations as it contained the most potent neurotoxic fraction according to experimental results. A column was set up in order to separate positively charged amines from other components according to the results obtained from animal studies. An amberlite cation resin was used to separate the cationic, anionic and neutral fractions as reported previously (Perera, 2004). The fractions colleted were concentrated under reduced pressure using a rotary evaporator and their respective weights were obtained.

Palmyrah seed shoot

↓

Sorted manually to removal the non edible outer cover

↓

Cut into small pieces and crushed using mortar and pestle

↓

Add 50% methanol

↓

Blend the mixture (Remove residue)

↓

Filtration through nylon cloth

↓

Filtration trough filter paper

↓

Concentrate filtrate using rotary evaporation at $< 40^{o}C$

↓

Freeze dry extractives (A. Ex. I)

↓

Medium pressure liquid chromatography (MPLC)

↓

Freeze dry extractives from different solvents (A. Ex. II)

↓

Ion exchange column chromatography for MeOH fraction

↓

Freeze dry extractives from ion exchange column (A. Ex. III)

↓

Preparative thin layer chromatography I for cationic fraction

↓

Freeze dry extractives from seven fractions (A. Ex. IV)

↓

Preparative thin layer chromatography II for fraction 4

↓

Freeze dry compound A, B and C (A. Ex. V, VI)

↓

Recycle HPLC

Figure 1: Flow diagram for separation of toxic synergists. A. Ex. number - Animal experiment number

Thin layer chromatography (TLC)

Cationic fraction obtained from ion exchange column chromatography (above)

was subjected to preparative TLC-I (prepTLC-I). Preparative TLC plates (2-4 mm thickness) were prepared manually using silica gel $G_{60}PF_{254}$ (MERK, Germany). Separated fractions were introduced to several activated TLC plates in a horizontal line. Plates were developed using butanol: ethanol: ammonium hydroxide (BEN); 7: 5: 5 solvent system. Narrow bands were marked on TLC plate and each band was scraped and collected into separate tubes. Compounds/fractions were collected after re-extraction followed by evaporation of respective TLC bands. Separated solvent free fractions/compounds were then subjected to the animal experiments.

According to the results of the animal experiment IV (under Animals studies) fraction No 4 (with 2-3 compounds), obtained from prepTLC-I was further separated by using preparative TLC-II (prepTLC-II) as above. Isolated compounds from prepTLC-II were named as compound A, B and C. These compounds were tested using animal experiments (under Animals studies). The fractions/compounds obtained during the above chromatographic separations were assessed using ninhydrin, anisaldehyde and other common spray reagents.

Animal studies-Study of toxicity through intra-venous injections of specific freeze dried extractives

Male Wistar rats (body weight range 215–430 g/8–12 weeks old) were used in experiments (I-VI see below) after an acclimatization period of one week. Rats were housed individually in polypropylene cages ($30 \times 22 \times 26$ cm) with a stainless steel lid with a fed hopper and bottle holder. Animals were fed with WHO standard rat and mouse breeding feed *ad libitum* and maintained under standard animal house conditions (Sabourdy, 1988). The separated compounds, after each chromatographic step (MPLC, IECC and TLC) were subjected to standard animal experiments. At least 5 animals were used in each experiment throughout this study, unless otherwise stated. Freeze dried extractives obtained during above separations were dissolved in distilled water and filtered using 0.45 micron syringe filter to prevent complications due to particles. Rats were given appropriate intravenous doses dissolved in less than 1mL of water. The controls were

given only distilled water. The doses for to the body weight of the animal and the ratio of the fractions with respect to its mother fraction. Immediately after dosing rats were observed for reported toxic symptoms at least for 6 hours/day.

Animal experiment I -   for dose curve of methanol water extractives.

Animal experiment II - for hexane, methylene chloride, ethyl acetate, methanol and water extractives.

Animal experiment III - for cationic and neutral (and possibly anionic) fractions.

Animal experiment IV - for seven compound bands from prepTLC I.

Animal experiment V – for individual compound A, B and C obtained from prepTLC II.

Animal experiment VI – for mixture of compounds A and B (2:1 ratio).

Flow diagram for separation of toxic synergist and the conducted animal experiments are indicated in figure 1.

Recycle HPLC

The recycle HPLC was used to further purify the compounds. The separated compounds A and B (with two colourless fluorescent carotenoids as 5-10% impurity) were purified using recycle HPLC column GS 320 (GAIGEL, 21.5 mm i.d. × 500 mm, Japan Analytical Industry Co. Ltd, Tokyo, Japan) with UV detector (JAI UV 310) and refractive index detector (RI-5). Isocratic flow rate was maintained at 5 mL/min using 1:1 methanol: water mixture. An 8-10 mg sample of each compound dissolved in 3 ml HPLC grade methanol was injected to HPLC. More than 6 cycles were conducted for better separations. The separated pure compounds were used for spectroscopic studies.

Spectroscopy

The compounds were dissolved in deuterated pyridine and subjected to NMR experiments (Tetramethylsilane/TMS as an internal reference). ^{1}H-NMR, ^{13}C-NMR, DEPT, HMQC, HMBC and COSY [Distortionless enhancement by polarization transfer (DEPT), [1]H-detected heteronuclear one-bond spectroscopy (HMQC), [1]H-detected heteronuclear multiple-bond spectroscopy (HMBC) and two dimensional [1]H-correlation spectroscopy (COSY)] experiments were

animal experiments were calculated according conducted using Avance AV 600 and Avance AV 500 NMR spectrometers. Infra red spectra in KBr pellets were obtained using a Shimadzu IR 55 spectrophotometer.

RESULTS

Bioactivity guided separation of neurotoxic compound

The moisture content of the raw Palmyrah seed shoot was 51%. A dose of 610 mg/kg/rat/day from freeze dried palmyrah seed shoot methanol: water (1: 1) extractives was found to be lethal to rats within 24 hours and 305 mg/kg/rat/day indicated previously reported toxic symptoms (Animal experiment I). The toxic effects were dose dependent.

Successive MPLC extractions on solvent free methanol water extractives using hexane, methylene chloride, ethyl acetate, methanol and water produced 24, 68, 128, 8462 and 462 mg of extractives per 1 kg raw seed shoot respectively. Experiments indicated that the solvent free methanolic extractive, which was water soluble to be neurotoxic and lethal within 24 hours to rats at a dose of 432 mg/kg/rat/day (Table 1 / Animal experiment II). The prominent symptoms were ruffled coat, uncoordinated spasms, nodding movements of head and of fore-limbs followed by falling over backward and characteristic fits.

Separation of methanol fraction through ion exchange column chromatography yielded 2662 mg of cationic fraction and 3752 mg of neutral (and possibly anionic) fractions for one kilogram of seed shoot. The cationic fraction contained a ninhydrin positive spot which coincided with an anisaldehyde positive spot on TLC with BEN 7: 3: 4 solvent system, while the anionic fraction gave the typical TLC spots of saponins and low retardation factor (R_f) ninhydrin positive spots. Animals administered the cationic fraction intravenously showed the neurotoxic symptoms and death resulted within one day at a dose of 150 mg/kg/rat/day (Animal experiment III). The toxic symptoms and behavior observed from two test groups and control animals were remarkably different.

On prepTLC-I, seven fractions separated and only one fraction (fraction 4)

Table 1: Neurotoxic effects of rats treated with different MPLC extracts of palmyrah seed shoot MeOH water extractive.

Extract (No of animals)	Dose mg/kg/rat/day *	6 hours	Day 1	Day2
Water (n = 7)	170	Neurotoxic symptoms were not observed	Rats were not normal, high excitation, food consumption <5 g/day	Rats were normal, with normal food consumption
Methanol (n = 7)	432		4 rats dead, 3 rats with all neurotoxic symptoms, Not food consumption.	5 rats dead 2 rats remained alive with very low food consumption
Ethyl acetate (n = 7)	3.7		Neurotoxic symptoms were not observed	
Control (n = 7)	Nil			

* Weights of the animals 320- 390 g.

Table 2: Neurotoxic effects of rats treated with different fractions obtained through preparative TLC I (prepTLC-I).

Fraction No	R_f Value	Dose mg/kg/rat/day*	12 hours	Day 1	Day 2	Day 3	Day 4	Day 5
1	0.74-1.00	78	Neurotoxic symptoms were not observed	Neurotoxic symptoms were not observed				
2	0.62-0.74	58						
3	0.53-0.62	34		Mild toxic symptoms were observed				
4	0.48-0.53	24		Low consumption of food, Toxic symptoms were observed		1 death	Toxic symptoms	2 deaths
5	0.30-0.48	22		Neurotoxic symptoms were not observed				
6	0.15-0.30	26						
7	0-0.15	32						

* Weights of the animals 215 - 275 g and n = 5 for each fraction. Control animals - Toxic effects were not observed

had neurotoxic effect (Table 2 / Animal experiment IV). Further separation of this fraction on prepTLC-II yielded three compounds namely A, B and C at R_f 0.65, 0.72 and 0.69 respectively (BEN 7: 5: 5). Compound A and B were ninhydrin positive. None of the compounds were positive to Dragendorff's reagent for tertiary amines. The compound C was inactive to anisaldehyde, phosphomolybdic acid and iodine vapour. The ratio of weights of above three compounds A: B: C was 211:100:28 w/w. Compounds A and B were stable throughout the entire isolation procedure.

Neurotoxic synergism

Pure compounds A, B and C were water soluble. None of them when intravenously administered singly caused neurotoxicity (Animal experiment V). However, recombination of compounds A and B (2:1 ratio) showed neurotoxicity at a dose of 62 mg/kg/rat/day. A dose of 115 mg/kg/rat/day, from above mixture was lethal

to rats (Animal experiment VI). Palmyrah flour usually contains compounds A and B in a ratio of 2: 1. Ruffled coat, uncoordinated spasms and erect immobilized animals were among prominent symptoms observed in treated rats. The toxic effects diminished after termination of dosing.

Spectroscopic studies

Pure compounds were obtained using reverse phase recycle HPLC. Pure compounds A and B were ninhydrin positive. IR spectra had a very broad peak around 3500 cm^{-1} for OH groups in the sugar moieties for both compounds. The comparison of ^{1}H-NMR

(Figure 2) and ^{13}C-NMR spectra of aglycone of compound A (R_f-0.65) with that of a compound isolated from palmyrah flower (inflorescence) by Yoshikawa et al. (2007) is given in Table 3. Comparison clearly suggested spirostane ring structure for compound A. The main differences of A and B were at the signals for anomeric protons (Table 4). Spectral data obtained for ^{13}C NMR of the compound A were identical to the spectral data reported by Yoshikawa et al. (2007), except for the first sugar moiety (Glc). Signal corresponding to position six of the Glc appears around δ 61 in ^{13}C-NMR (Yoshikawa

Table 3: ^{13}C and ^{1}H-NMR comparison for aglycones of two molecules.

Carbon Number	Yoshikawaet al., 2007		This study	
	δ_H (*J* Hz)	δ_C	δ_H (*J* Hz)	δ_C
1	0.98 (m), 1.72 (m)	37.6	0.97 (m), 1.72 (m)	37.5
2	1.83 (m), 2.07 (m)	30.2	1.83 (m), 2.04 (m)	29.9
3	3.86 (m)	78.1	3.87 (m)	78.0
4	2.70 (dd, 11.5, 13.2) 2.79 (dd, 3.8, 13.2)	39.1	2.71 (dd) 2.79 (dd)	38.9
5		141.0		140.8
6	5.33 (br d, ca 5)	121.8	5.33 (br d)	121.8
7	1.51 (m), 1.89 (m)	32.5	1.48 (m), 1.85 (m)	32.2
8	1.52 (m)	31.6	1.62 (m)	31.6
9	0.89 (m)	50.5	0.86 (m)	50.3
10		37.2		37.1
11	1.44 (m), 1.08 (m)	21.2	1.35 (m)	21.1
12	1.69 (m)	40.4	1.68 (m)	39.8
13		41.1		N/D
14	0.97 (m)	55.9	N/D	55.6
15	1.47 (m), 2.02 (m)	33.3	1.48 (m), 1.85 (m)	32.1
16	4.31 (m)	80.9	4.44 (m)	80.4
17	1.59 (dd, 7.3, 8.3)	62.7	1.78 (m)	62.7
18	0.97 (s)	16.7	0.80 (s)	16.3
19	1.04 (s)	19.4	1.03 (s)	19.4
20	2.32 (m)	42.2	N/D	42.4
21	1.02 (d, 6.9)	16.8	0.81 (d, 5.5)	16.3
22		110.7		109.7
23	1.68 (2H, m)	28.4	N/D	N/D
24	1.57 (2H, m)	28.2	N/D	27.5
25	1.63 (m)	30.8	N/D	30.1
26	3.68 (2H, d-like)	69.7	3.63 (m)	70.4
27	0.70 (d, 6.3)	17.3	0.67 (d, 5.2)	17.3

N/D – not detectable.

Table 4: Comparison of the anomeric protons of compound A and B.

^{1}H-NMR Chemical shift of Compound A	^{1}H-NMR Chemical shift of Compound B	Anomeric structure
4.9 (d) $J = 9.2$*	4.6 (d) $J = 8.7$*	β-pyranoside
5.8 (br s) $J < 1.8$	5.8 (br s)	α-rhamnoside
6.4 (br s) $J < 1.8$	6.4 (br s)	α-rhamnoside
6.2 (br s) $J < 1.8$	6.2 (br s)	α-rhamnoside

* The anomeric protons of the β-pyranoside differ.

et al., 2007). This was not observed in the spectrum of compound A possibly because of Glc moiety. It can be concluded that the neurotoxic synergists A and B are steroidal saponins with a spirostane ring system containing a primary amine group probably in the sugar moiety of 4 units comprising, three-α-rhamnosyl and a β-pyranosyl which is not glucose.

DISCUSSION

Traditionally palmyrah flour is made by grinding sun dried or boiled and sun dried palmyrah seed shoots (Balasubramanium et al., 1999). In this study raw seed shoots were used as the starting material to avoid loss of compounds due to processing. The separation protocols were designed to ensure that the processing temperature was below 40 °C. This is important as the toxin is known to be destroyed at 80 °C (Sumudunie et al., 2004). The anionic fraction resulting from ion exchange chromatography contained a high concentration of neutral saponins compared to its cationic fraction. Saponins are capable of damaging biological membranes (Francis et al., 2002). According to Perera cationic fraction is non-toxic when administered orally to Wistar rats (Perera, 2004), but in this study it was found to be toxic when given intravenously. This suggested that the high concentration of the neutral steroids (Wickramasekara and Jansz, 2003) act to facilitate the entry of the cationic toxin in to the blood stream, by damaging the gut mucous membrane.

The mild toxic symptoms observed in fraction n° 3 probably due to a residual effect. Repeated studies on this fraction confirmed absence of specific toxic constituents. Toxic symptoms were observed with the mixture of compound A and B (2:1 ratio) at a concentration of 62 mg/kg/rat/day. However toxic symptoms were not observed when compounds A and B were administered separately at a dose of 100 and 172 mg/kg/rat/day respectively, clearly showing synergistic action.

Naturally occurring ratio of compound A, B and C was found to be 211:100:28. A dose of 24 mg/kg/rat/day from fraction 4 (Animal experiment IV), which contained all three compounds was lethal to rats. If the appeared toxicity is due to compound C, the effective dose for which can be calculated as approximately 2 mg/kg/rat/day. However, a separate experiment indicated that the compound C alone is not toxic even at a dose as high as 40 mg/kg/rat/day. However, the possible contribution of compound C in enhancing synergism cannot be ruled out.

The compounds A and B were ninhydrin positive. The most probable site for an amino group is replacement of a glucosyl OH. Glucosamines are readily available in plants; such molecules can be easily incorporated into secondary metabolites during their biosynthesis. The ^{1}H NMR spectra of compound A and B are similar apart from a chemical shift observed in the ^{1}H NMR signals of anomeric protons. Flabelliferins or Borassoside found in palmyrah usually contain β-glucose as the first sugar moiety attached to the aglycone (Nikawala et al., 2000; Ariyasena et al., 2002b; Yoshikawa et al., 2007). Generally in ^{1}H NMR the anomeric proton of β-glucopyranosyl appears around δ 4.2 to 4.4

(Bax et al., 1984). However, in compounds A and B, a shift has occurred from the normal value to δ 4.9 and 4.6 respectively. Therefore it is likely that the NH_2 is found in both A and B residues on the β-pyranosyl moiety but differing in the position of attachment. The primary amine is most likely to be in position 6' of the β-pyranosyl of A in view of the absence of ^{13}C NMR peak within δ values of 61-63, which corresponds to C–6 of glucopyranosyl. With above spectral evidences and HMBC correlations for the compound A (Figure 4), the structure of A is tentatively suggested as in figure 5.

Similarity of ^1H NMR of compound B (Figure 3) to that the ^1H NMR of compound A suggested similar structures except for NH_2 group. The position 3' on β-pyranosyl in compound B can be hypothesized as a favorable site for NH_2 group even without spectral evidences. This hypothesis cannot be ruled out by available spectral data. It is necessary to highlight that the proposed structures through our findings agree with most of previous reports on the nature of compound causing neurotoxicity. However, more detailed studies on chemistry and the mechanism of action of compounds should be further studied.

There is evidence of neurotoxic effects on the mitochondrial membrane in liver mitochondria (Pathmanathan, 2001). However, the neurotoxic symptoms suggest that mitochondria of the other tissues may be affected, notably that of the nervous system. This damage to the neurons would cause a deficit of ATP which would explain the neurotoxic symptoms, which were clearly
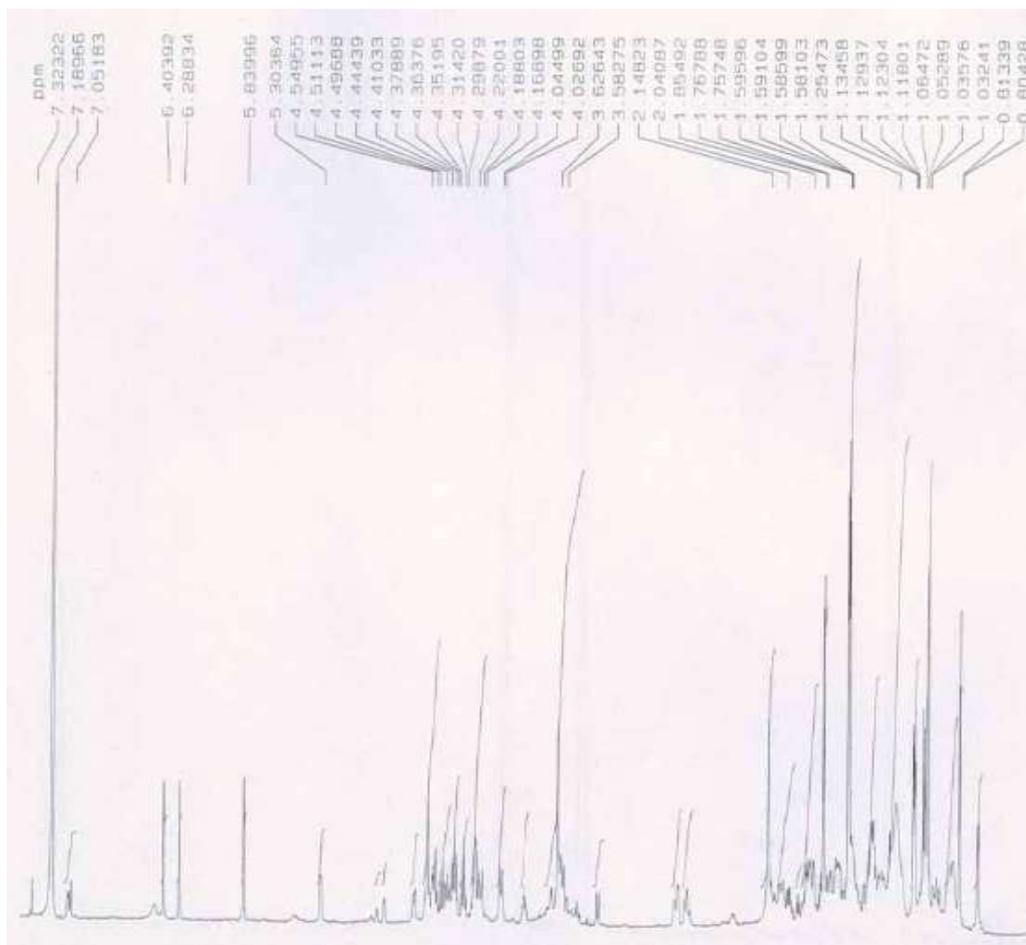
Figure 2: ^1H-NMR spectrum of the compound A (Rf – 0.65).
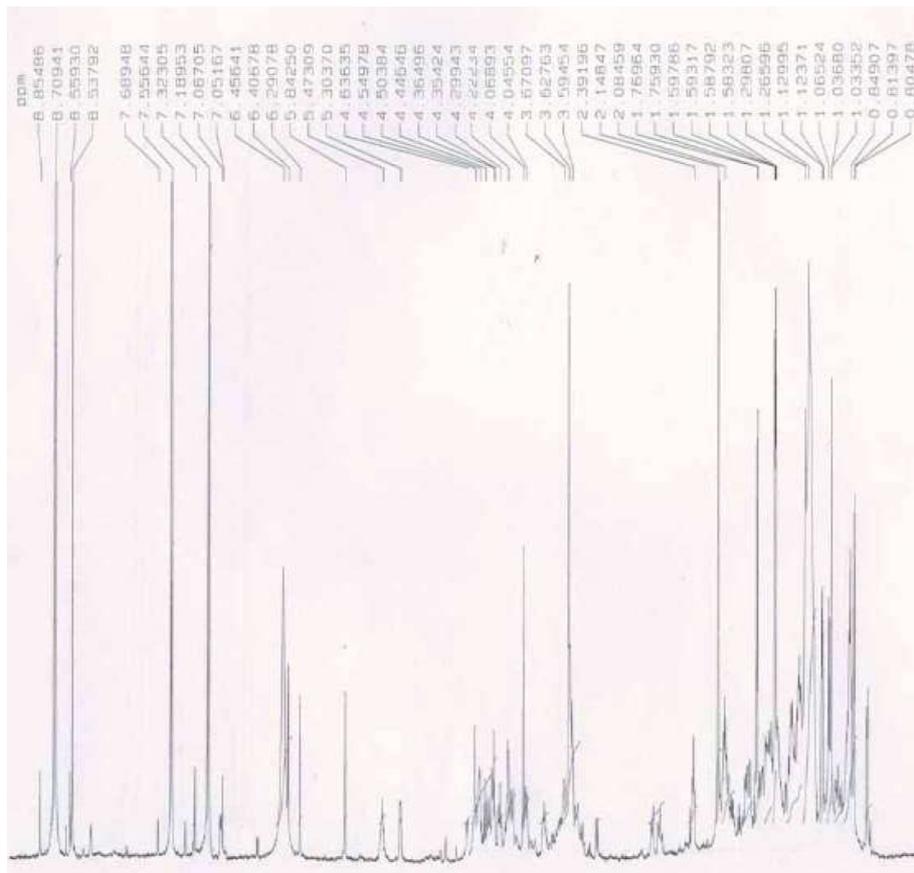
Figure 3: ^{1}H-NMR spectrum of the compound B (Rf – 0.72).
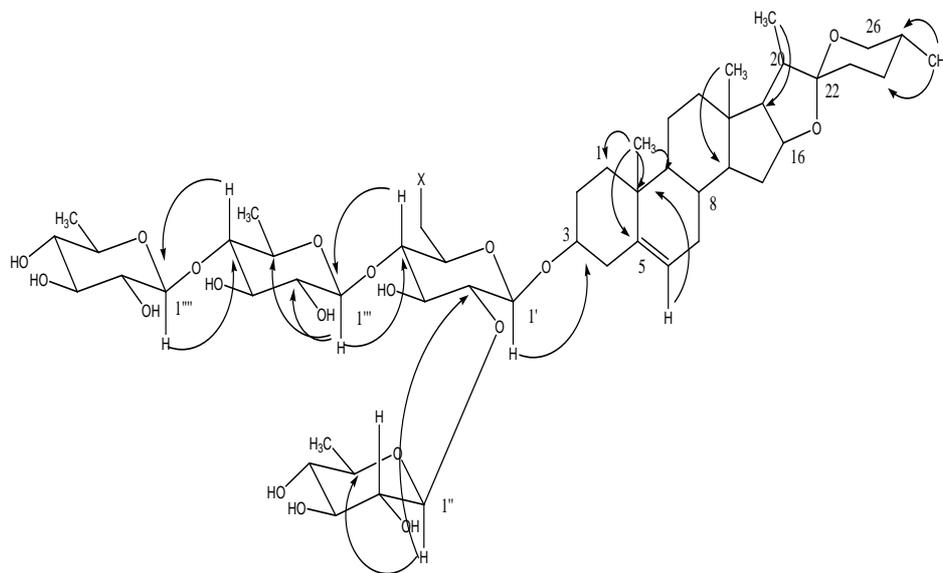
Figure 4: HMBC correlations of neurotoxic compound A.
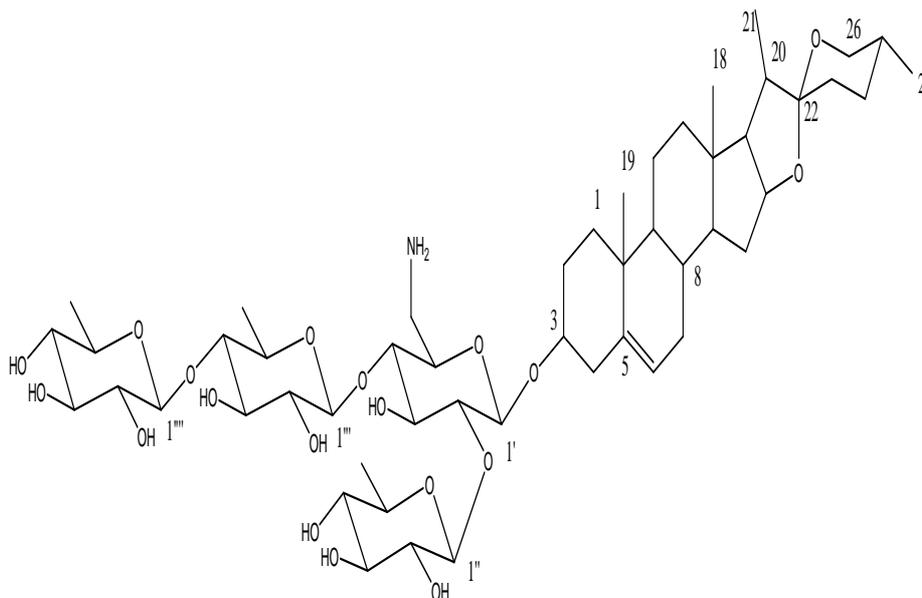
Figure 5: Tentative structure of the neurotoxic compound A.

observed in the above experiments. There are numerous papers to support neurotoxicity as well as hepatotoxicity in rats. But during this study based on our earlier experience and observations, we focused on the very early toxic symptoms which are parallel with other neurotoxicity screening batteries (Kram and Keller, 2001). During the study toxic symptoms appeared within 6-12 hours and death resulted within 24 hours, which is unlikely to be in the case of a hepatotoxicity (Schoental, 1968) but could be due to neurotoxicity (Yeh, 1997).

This study clearly indicates that the toxic compounds are water soluble. Therefore during food processing, detoxification of palmyrah flour can be achieved by washing in addition to heating flour above 80 °C. The washing step can be seen in traditional palmyrah flour food preparations (Balasubramanium et al., 1999). This study provides another possible reason in addition to temperature why neurotoxicity is not reported in the human population. All above findings should encourage the use of underutilized palmyrah flour in food preparations; the processing of which is now in the hands of a section of a population which is economically deprived.

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