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Studies on a hyper-haemolytic compound of palmyrah flour (Odiyal)

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Abstract

Palmyrah (*Borassus flabellifer* L.) flour (Odiyal) is well known to contain toxins. A new saponin was isolated from the medium pressure liquid chromatography (MPLC) fraction between 90% - 100% MeOH:EtOAc extract and crystallized. On subjecting to Micro-Time of Flight (Micro-TOF) mass spectrometry it was found to have a molecular weight of 1534, containing the common steroidal aglycone of odiyal of molecular weight 414, 5 ramhnosyl and 1 glucosyl residues. The first fragment corresponding to MW 228 may be an uncommon fatty acid derivative. Testing for haemolysis on human red blood cells (RBC) showed the compound to be hyper-haemolytic with a haemolytic index 37 fold that of the flabelliferin B (F_B) which had been the previously reported steroidal saponin RBC haemolyser with highest activity from palmyrah.

Key words: Palmyrah, odiyal, hyper-haemolytic agent, steroidal saponin derivative

Introduction

Palmyrah (*Borassus flabellifer* L.) flour (odiyal) is known to have a number of toxic effects. These include mutagenic (anderson and poulson, 1985), clastogenic (Kangwnpong *et al.*, 1981), neurotoxic (Arseculeratne *et al.*,1971), cytotoxic (Keerthi, 2008), mosquito larvaecidal (Keerthi *et al.*, 2007) and anti-microbial (Nikawala *et al.*, 1998a) effects. Most of these effects are due to saponins (Nikawala *et al.*, 1998b). During the course of isolation of the dengue mosquito larvaecidal saponins, by serendipity, another pure saponin was isolated. The objective of this study was to: (i) test the compound for bioactivity since

it did not correspond to any of the above activities and (ii) determine the structure by mass spectrum.

Materials and Methods

Materials

Palmyrah flour originating from Kalpitiya was obtained from the Palmyrah Development Board sales outlet.

Isolation

The compound was isolated during bio-activity directed separation of the dengue mosquito larvaecide (Keerhi *et al.*, 2007). The separation was conducted using Medium pressure liquid chromatography (MPLC) (Ariyasena *et al.*, 2002). The solvent gradient fraction between 90% - 100% methanol: ethyl acetate mixture contained a white solid that (was not larvaecidal and) crystallized on evaporating. Compound was re-crystallized by dropwise addition of ethyl acetate into ethanol solution until cloudiness appears and then keeping the mixture at low temperature (0-8°C).

Mass spectrometry

The re-crystallized pure compound (1mg dissolved in MeOH) was subjected to mass spectroscopic analysis. The mass spectrum was obtained using Bruker Micro Time of Flight Mass Spectrophotometer (microTOF) operating under positive mode (Keerthi, 2008).

Haemolysis

Since the mass spectrum showed potential for amphipathic character, the compound was tested for haemolysis of human RBC. Stock solutions from Na_2HPO_4 and NaH_2PO_4 each 0.4M were prepared. Portions of Na_2HPO_4 (81 mL) and NaH_2PO_4 (19 mL) were mixed and diluted to 200 mL. A portion (0.9 mL) of blood was mixed with 0.2 mL sodium citrate and diluted to 50 mL using pH 7.4, 0.1 M phosphate buffer. The above were mixed and incubated at 31°C and absorbance measured at 578 nm after centrifuging (1500 RPM for 20 min). After standing for 1 hour for the haemolysis, the percentage haemolysis (%H) was calculated using the following formula (Nikawala et al., 1998b).

$$\%H = 100\% \ (Ab - Ab_{Control}) \ / \ (Ab_{100} - Ab_{Control})$$

Ab - Absorbance of the sample

$Ab_{Control}$ - Absorbance of the control

Ab_{100} - Absorbance of the 100% haemolysed sample

Haemolysis index was calculated following the method of Nikawala et al., (1998b) for flabelliferins.

Results

Haemolysis

Results are summarized in Tables 1 and 2. Haemolysis was observed from fractions containing compounds with RF values 0.72, 0.63, 0.54, 0.40 and 0.27. The compound with RF value 0.40 in BEN (7:3:4) solvent system was identified as the compound with highest haemolytic activity. The calculated haemolytic index was 37,000, which was 37 fold higher than for reported hyperhaemolytic flabelliferin F_B, which previously was highest recorded haemolyser for a saponin from palmyrah.

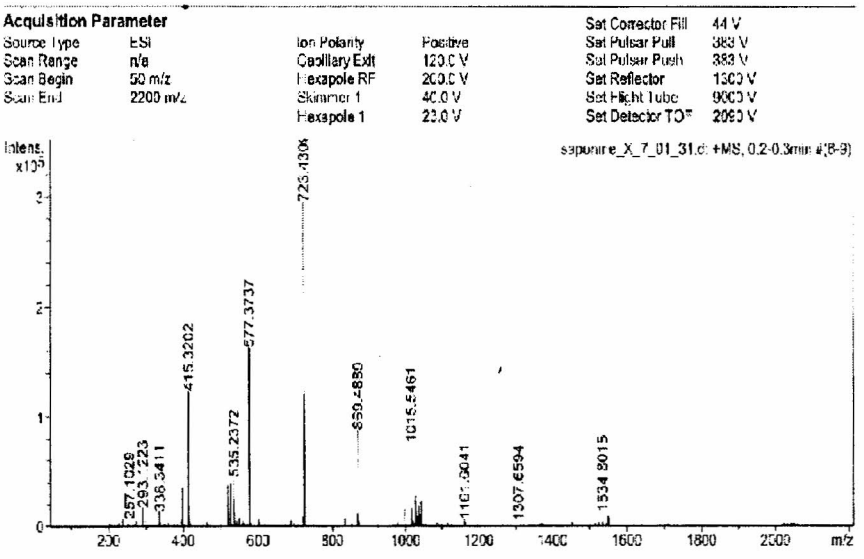

Figure 1: Micro TOF mass spectrum of hyper-haemolytic compound

Mass spectrometry

Re-crystallisation resulted in ~ 5 mg of off-white crystals. TOF mass spectrometry (Figure 1) gave a steroidal glycoside with a MW of 1534. A fragmentation peak at (M -228) followed by loss of fragments of 146 × 5 representing 5 rhamnoses followed by a loss of fragment of 162. Thus leaving room for aglycone of a molecular weight of 414. The mass fragmentation pattern had the following peaks at 1307 (M + H –$C_{16}H_{20}O$), 1161 (M + H – $C_{16}H_{20}O$ – Rha), 1015 (M + H –$C_{16}H_{20}O$ – 2 Rha), 869 (M + H – $C_{16}H_{20}O$ – 3 Rha), 732 (M + H –$C_{16}H_{20}O$ – 4 Rha), 577 (M + H –$C_{16}H_{20}O$ – 5 Rha) and 415 (M + H –$C_{16}H_{20}O$ – 5 Rha - Glc) were observed.

Table 1: Haemolysis observed with human RBC using two different concentrations of haemolytic compound

Fraction No (Test tube)	Absorbance at 578 nm	
	6 µg/ml	33 µg/ml
1 (1- 12)	No haemolysis observed	No haemolysis observed
2 (13 - 24)		
3 (25 - 36)		
4 (37 - 44)		
5 (45 - 46)		
6 (47 - 49)		
7 (50 - 56)		0.198
8 (57 - 60)	0.321	0.865
9 (61)	0.004	0.105
10 (62 – 64)	0.003	0.069
11 (65 – 68)	No haemolysis observed	
12 (69 – 75)		

Table 2: Haemolysis observed with the compound before re-crystallisation at different concentrations

Concentration of compound µg/ml	Absorbance at 578 nm	Percentage Haemolysis (% H)
3	0.140	0.16
6	0.321	0.37
9	0.402	0.46
12	0.465	0.54
15	0.523	0.60
18	0.573	0.66
21	0.602	0.70
24	0.789	0.91
27	0.838	0.97
30	0.821	0.95
33	0.865	1.00

Discussion

Previous studies (Arseculeratne et al., 1971; Nikawala et al., 1998a; Nikawala et al., 1998b) indicated that the aglycone of the steroidal saponins of odiyal flour to be either β-sitosterol or spirostane (both have a molecular weight of 414). Therefore the aglycone of the hyper-haemolytic compound is one of the above two. The 5 fragments of 146 correspond to a deoxy-sugar and 162 corresponds to a pyranosyl. The only sugars in the saponins of palmyrah are rhamnosyl and glucosyl (Keerthi, 2008). The derivative in this type of saponin is usually a fatty acid (Chung et al., 2007; Rubnob et al., 2001; Sultana and Afolayan, 2007; Nguyen et al., 2004). However the peak corresponding to MW 228 was indicating that this may be an uncommon fatty acid derivative. This type of molecule with high amphipathic character will account for its hyper-haemoliticity.

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