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THE RANGE OF STEROIDAL SAPONINS OF PALMYRAH FLOUR: COULD THEY CONTRIBUTE TO TOXIC EFFECTS ON CONSUMERS

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Abstract

The shoot flour (Odiyal) of the Palmyrah palm (Borassus flabellifer) is a starchy staple in the North of Sri Lanka, despite showing evidence of bioactivity and toxicity in animal and in-vitro studies. It appeared possible that the flabelliferins (steroidal saponins) could play some role in some of the reported toxicities. It was decided to separate the flabelliferins by medium pressure liquid chromatography (MPLC) and preparative thin layer chromatography (TLC). In all, 9 flabelliferins were isolated, 6 of which were found to be similar to those previously separated from palmyrah fruit pulp, as judged by R_f on TLC, MPLC eluent gradients and hydrolytic product pattern of naringinase. The bitter flabelliferin on palmyrah flour differed from that of fruit pulp and was closely associated with a primary amine. This combination showed neurotoxic effects on Wistar rats.

keywords: : palmyrah, odiyal, steroidal, saponins, neurotoxicity, toxic amine.

1 Introduction

The seed of the palmyrah palm (*Borassus flabellifer L*) on germination produces a starch containing shoot from which a flour can be prepared [1]. This is called odiyal [2] and is a starchy staple from which many traditional recipes are prepared in the North of Sri Lanka where 10-11 million trees grow [2]. Many studies on toxic effect of

this flour using animal models and *in-vitro* studies have been reported. Some key references are those reporting hepatotoxicity[3], neurotoxcity [4], mutagenic effects[5], clastogenic effects [6], and immunosuppresion [7]. In none of these studies, with the exception of an immunosuppressive dammarane (triterpenoid)[7], have the active constituents been characterized. It has been reported that the neurotoxic agent was a compound of molecular weight 1400 containing quaternary amine and glycosidic moiety[4], however evidence was incomplete. There has been no dedicated study on saponins of palmyrah flour (odiyal) although passing mention of these compounds had been made previously [8][9]. One report stated that odiyal had no saponins[4]. The objective of this study was to separate and tentatively identify the saponins of odiyal flour by comparing MPLC, TLC, and enzyme hydrolysis data of this study of those obtained from flablliferins of palmyrah fruit pulp [10][11][12] the structures of which had been elucidated previously spectroscopically [10][11]. Problems of separating the bitter flabelliferins of odiyal led to an associate of a primary amine and flabelliferin.

2 Materials and methods

Unboiled palmyrah flour (odiyal), the raw material, was obtained from the Palmyrah Development Board, Colombo, Sri Lanka. The shoot of palmyrah seed was collected from Kalpitiya in the North-West of Sri Lanka from which consumers in Colombo, Sri Lanka obtained their palmyrah flour for the purpose of preparation of some food recipes.

2.1 Extraction of flabelliferins

Batches of flour (50g) of particle size 100 μm was soaked in water (200ml) and blended several times over a period of 6h. Methanol (analytical grade) (200 ml) was added and the mixture was homogenized using a Waring blender and left to stand for 16h. The extract was filtered, firstly through poplin cloth and then through Whatman No 4 filter paper. The methanol of the filtrate was evaporated at 35°C and water at 40°C using a rotor evaporator under reduced pressure until the volume was reduced to 5ml. The concentrate (5ml) was extracted with ethyl acetate (4 x 5ml), giving an ethyl acetate extract containing the flabelliferins with shorter chain carbohydrate moieties. The residue contained flabellferin tri and tetraglycosides. Flabelliferins were separated by MPLC[11] using the apparatus of Baeckstrom [11] (Separo, Stockholm, Sweden) using a column of 25 mm id and length 50 mm containing silica gel G_{60} [0.040-0.003mm] from Merck, Germany (via Kebo lab, Uppsala, Sweden) A gradient of n-hexane, dichloromethane, ethyl acetate, methanol, and water was used for both the ethyl acetate and water extract separately using a dilution factor of 0.5 and number of dilutions for each solvent composition of [6]. Fractions

(9ml) were collected and monitored using n-butanol: ethanol: aq. NH_3 (sp gr 0.88) [BEN] ratio of 7:3:4. TLC was conducted as reported previously and spots were visualized with anisaldehyde reagent[9]. Fractions containing one spot were pooled and purified flabelliferins obtained. When a mixture of flabelliferins was observed, then either repeated MPLC (with increased dilution factor) or preparative TLC (silica gel G_{60}- 300 μm) with the same solvent system described above was used. All flabelliferins contained a fluorescent impurity which was removed by preparative TLC on silica gel G_{60} using n-butanol:acetic acid:water (4:1:5-upper layer) [BAW]

2.2 Separation of amine

A ninhydrin positive compound (spray containing 1% ninhyrin in acetone which was heated at 100°C for 10 min) was found to co-chromatograph with what turned out to be the bitter flabelliferin. This "associate", from MPLC was dissolved in water (20 ml) and 10g of cation exchange resin Dowex - 50W (H^+ form), Sigma, St Louis Mo, USA was added. This was separated at 2000 rpm for 5 min in a centrifuge (Kubota 5100, Japan) The supernatant contained the flabelliferin. The amine was flushed out from the resin by methanolic NH_4OH (1:1) and concentrated by evaporation under reduced pressure at 30°C (NH_3) and by freeze-drying (H_2O)

2.3 Enzyme hydrolysis

Pure flabelliferins (approximately 1mg) were incubated with naringinase (ex. *Penicillium decumbens*) from Sigma, St Louis Mo, USA (0.3 units) in a volume of 0.5 ml acetate buffer (pH 4.45, 0.05M) at 37°C. Aliquots of 5μl and 10 μl were spotted on TLC plate at zero time, 0.5, 1.5, 3, 4, 6, and 20h respectively and TLC conducted and sprayed with anisaldehyde [9]
In the case of the bitter flabelliferin enzymatic hydrolysis was conducted using a heat stable α-amylase (100μl, 1140 BAA units) ex. *Bacillus licheniformis* (from Novo, Aarhus, Denmark). In this case pH was maintained at 6.0 using 0.1M phosphate buffer (1 ml) and temperature was 90°C. Aliquots were spotted on TLC and spots detected as for naringinase hydrolysis.

2.4 Studies with rats

Adult female Wistar rats (200g - 250g) which were inbred at the Medical Research Institute (MRI), Colombo, Sri Lanka were used for this study. Only 3 rats were used as the neurotoxic effect was consistently observed when present. The rats were fed with standard WHO feed [11] and water add libitum. Extractives (2.5 ml) were administered by a syringe with a sondi needle into the oesophagus. In the case of the flabelliferin amine "associate" a single dose extractive (from an equivalent of 17 g flour was administrated) while in the case of the separated amine 2 doses

per day for 3 days each containing extractive from an equivalent of 8 g flour was administered to make conditions closer to previous studies where 6-8 g of palmyrah flour is consumed by rats per day [4]. The rats were observed for the neurotoxic symptoms described previously.

3 Results

The ethyl acetate extract of palmyrah flour yielded 5 flabelliferins, while the water extract yielded 4 flabelliferins. Total yield of crude flabelliferin was more than 2%. The flabelliferins were dominated by spot at R_f 0.64 and a spot at 0.5 (bitter flabelliferin).

The tentative identification of flabelliferins were made using

(1) Rf value [10][11] on TLC, where the flabelliferins gave their characteristic disc shaped green spot on spraying with anisaldehyde and heating.

(2) Specific elution gradient for recovery on MPLC [10][11]

(3) Hydrolysis pattern with naringinase when comparing with hydrolytic pattern of flabelliferins [10][12] whose structure had been elucidated spectroscopically [10][11].

The bitter flabelliferin posed a major problem as its association with an amine changed its R_f value in both TLC solvents systems as well as made it less soluble in methanol. Until the amine (ninhydrin positive) was separated by absorption on an ion-exchange resin, a true R_f could not be obtained. Although bitter, it's naringinase hydrolysis pattern was different from the bitter compound in palmyrah fruit pulp. This difference was confirmed by the finding that the bitter flabelliferin could not be hydrolyzed by heat stable α amylase, unlike the bitter flabelliferin of fruit pulp which was debittererd by cleavage of an α-1,4 bond [9][10]. While flabelliferin tetraglycosides are normally soluble in methanol until this flabelliferin was separated from an associated amine it was only water soluble, which made its separation from other flabelliferins easier.

This amine on TLC gave a similar high R_f to some common amino acid derived amines. The bitter flabelliferin amine mixture produced neurotoxic symptoms on Wistar rats. The symptoms were as follows.

All three rats showed malaise, mucous salivation and upright posture reminiscent of symptoms described by Greig for neurotoxicity[4].

In the case of the flabelliferin - amine "association" a single dose isolated from 17g palmyrah flour was sufficient to bring about the effect in 3 days after which the rats returned to normal. Once the flabelliferin was removed and the amine from 8g palmyrah flour orally administered for 3 days (twice a day) the effect, which

Table 1: Tentative identification and enzyme hydrolysis pattern of flabelliferins isolated from palmyrah flour.

TLC Rf	MPLC Eluent Gradient	Naringinase Hydrolysis Fragments	Tentative identification
0.42	Methanol: Ethyl acetate	Glucose** Diglycoside* Triglycoside*	F- I (Ref 9)
0.50	Methanol: Ethyl acetate (30:70)	Rhamnose** Triglycoside* Diglycoside*	Tetraglycoside Rha terminus (bitter flabelliferin of palmyrah flour)
0.53	Methanol: Ethyl acetate (30:70)	Rhamnose** Diglycoside*	FB (Ref 12)
0.57	Methanol: Ethyl acetate (30:70)	No hydrolysis	FC (Ref 10,12)
0.63	Methanol:Ethyl acetate (20:80)	Glucose ** Diglycoside* Monoglycoside*	New Flabelliferin Triglycoside
0.64	Methanol:Ethyl acetate (20:80)	Monoglycoside*	FD (Ref 12) Diglycoside
0.68	Methanol:Ethyl acetate (10:90)	Glucose ** Aglycone**	FE (Ref 12) Diglycoside
0.78	Ethyl acetate:Methylene Chloride (40:60)	Glucose** Spot at Rf-0.94 Aglycone**	Interpretation difficult probably a new flabelliferin See text
0.85	Ethyl acetate:Methylene Chloride(80:20)	Glucose** Aglycone**	FF (Ref 8,13) Monoglycoside

** By standards

* From Rf value of previous studies [8][9][10][11][12], and sugars released from hydrolysis

was not severe, lasted only as long as the day after administration was terminated. After which the rats returned to normal. It should be noted that this specimen of palmyrah flour when consumed by rats at a consumption rate of 8g per day produced neurotoxic symptoms in 5-6 days.

4 Discussion

Palmyrah flour (odiyal) yielded as many as 9 flabelliferins. It appeared that , 6 of them were the same as those found in palmyrah fruit pulp.

Table I, column I gives the R_f values (BEN), which for flabelliferins in this solventsystem is known to be inversely proportional to chain length of the polar carbohydrate moiey [9][10]. That is with respect to R_f value, monoglycoside> diglycoside > triglycoside > tetraglycoside. The only sugars identified in the flabelliferins of palmyrah fruit pulp (PFP) in the past were rhamnose and glucose [9][10][11][12]. Presence of rhamnose increases the R_f value. According to data report previously

in PFP [10][11][12], the flabelliferin from palmyrah flour have R_f values consistent with, F_B triglycoside[12], MW 868 [12](11) F_C triglycoside, MW 868 (111) F_D diglycoside [12], MW, 722 [12](IV) F_E diglycoside, MW, 734[11] (V) F_F monoglucoside, MW 578 [11].

According to R_f value, the spot at R_f 0.42 was probably a tetraglycoside. Two other new flabelliferins appeared at R_f 0.63 and R_f 0.78. These values indicated tri and monoglycosides respectively. In column 2 of Table 1 the elution gradients of flabelliferins on MPLC is shown. They were consistent with conclusions reached on R_f value and result on MPLC for flabelliferins of PFP [11].

Column 3 gives the naringinase (a β rhamnosidase and β glucosidase at pH 4.45) hydrolysis products. Naringinase gave rise to the release of smaller glycosides, rhamnose, glucose and the aglycose. Whether the released glycoside is tri, di, or mono is judged by Rf value and comparison with corresponding hydrolysis data of flabelliferins from PFP [10]. Such hydrolysis data support the identification of F_B, F_C, F_D, which have identical patterns when obtained from PFP. Results from naringinase hydrolysis of F_E and F_F are consistent with structures elucidated spectroscopically [8][11]. The bitter flabelliferin of palmyrah flour has a distinctly different α-amylase and naringinase hydrolysis patterns from the bitter flabelliferin (F-II) of PFP[10]. What is clear is that palmyrah flour contains at least [9] flabelliferins and the bitter flabelliferin of palmyrah flour differs from that of PFP. Clearly more spectroscopic data is needed to confirm further conclusions. Of most interest is the bitter flabelliferin. This is on account of its association with an amine, which is fluorescent and small enough to show the volatility observed. Its R_f value is similar to mines like tyramine, phenyl ethylamine and 1,5 diaminopentane in BAW (4:1:1). This would give the amine a MW in the range of 150 and explain the apparent volatility of the amine. It is uncertain how the bitter flabelliferin form a stable association with amine.

The findings so far are consistent with those of Grieg and co-workers[4] who postulated a tentative structure for the neurotoxin as a glycoside with molecular weight (using Sephadex G-25) of 1400 and a quaternary N^+. It is felt that with new data at hand that Greigs neurotoxin [4] was the adduct or strong association o the bitter flabelliferin (MW 1030) and the amine (MW 100-150) which is very close to the estimated MW by Sephadex gel chromatography (an approximate method). The flabelliferin would have given rise to the glycoside identified previously [4]. Another difference is that Grieg and co-workers [4] postulated a quaternary amine on the basis that the neurotoxin could not be isolated in organic solvents after making the solution alkaline. This is explained by the fact that amine concerned is a small water-soluble molecule but a primary amine (ninhydrin positive). It is possible that the role of the flabelliferin is to bind the amine and make it non-volatile. The decrease in toxic symptoms on removal of the flabelliferin could be due to reduced toxicity due to volatility. The association would explain the stability of the toxin

to heat in solution[5] (non-volatile association) in boiled palmyrah shoot and in steamed palmyrah (trapping in starch matrix) but loss of toxicity on dry heating of flour to 80°C for 45 min (K.A.V. Sumudunie, 2001 personal communication)
Grieg and co-workers[4] pointed out that neurotoxicity appeared after a lag period of 4-5 days. They stated that one possibility was that the neurotoxin per se was not present in plamyrah flour but had to be metabolized to a form permeable to neural cells. That is the bitter flabellifaerin containing adduct in the palmyrah flour could be considered a "pro-toxin" (cf pro-drug). If the metabolic transformation was not possible in humans, it would explain why the neurotoxic effect is not shown by major consumers of palmyrah flour and its recipes.
Further studies are needed on the role of the flabelliferins of palmyrah flour on consumers, taking account that the flour is used frequently in food preparations especially in the North of Sri Lanka.

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