J. Natn. Sci. Coun. Sri Lanka 1998 26(1): 9-18

FLABELLIFERINS - STEROIDAL SAPONINS FROM PALMYRAH (*BORASSUS FLABELLIFER* L.) FRUIT PULP

1. Isolation by flash chromatography, quantification and saponin related activity.

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(Received: 02 July 1997; accepted: 02 January 1998)

Abstract: Four saponins (flabelliferins) from palmyrah (*Borassus flabellifer* L.) fruit pulp from specimens collected from Hambantota in the South of Sri Lanka, were separated and isolated by flash chromatography. A method for quantifying flabelliferins by tlc-densitometry was developed. FAB/MS data showed that of the four natural flabellilferins separated , one flabelliferin (F-II) corresponds to the bitter flabelliferin tetraglycoside previously isolated from fruits collected in Kalpitiya in the North - West of Sri Lanka. The other three flabelliferins isolated were labelled F_B, F_C & F_D. Of these F_B and F_C were (steroidal triglycosides of molecular weight 868) and F_D an impure steroidal diglycoside . It appears that the steroidal aglycone was similar to that isolated previously (MW = 414). Of the four flabelliferins isolated F_B was most effective in foam stabilisation while F_B and F_C had the highest haemolytic action as judged by spectrophotometry.

Keywords: FAB/MS, flabelliferins, froth test, haemolysis, palmyrah, saponins, tlc-densitometry.

INTRODUCTION

Palmyrah (*Borassus flabellifer* L.) is a characteristic feature of the landscape in the dry zones of Northern and Southern Sri Lanka. There are over 10 million trees growing over an area of 60 kha with a potential yield of 15 kt palmyrah fruit pulp per annum.[1] Although the fruit pulp is consumed in a number of traditional recipes,[1] the presence of a bitter principle detracts from more extensive use.[2] The bitter principle isolated from the North-West of Sri Lanka has been identified to be a steroidal saponin (flabelliferin II).[3] It contains 2 glucoses and 2 rhamnoses in its carbohydrate moiety and has a molecular weight of 1030.[3] In addition to this saponin, another saponin (F-I) which was not bitter, with molecular weight 1062 (tetraglucoside) was also identified. The aglycone of these steroidal saponins was first isolated and characterized (as spirost - 5 en - 3β-ol (25 R)) by Jayaratnam.[1] Detailed studies by ir, nmr and ms by Jayaratnam[1] show that it had a molecular weight of 414. Jayaratnam also isolated two other saponins of this steroidal aglycone from palmyrah fruit pulp from Jaffna. These were a monorhamnoside, and a monoglucoside.[1] However no reference was made in that study to bitterness or a bitter saponin.

Debittering by the enzyme naringinase on F-II was previously reported.[3] In pursuing these studies on debittering, toxicity and detoxification, due to the inability to collect samples from the sources mentioned above, specimens were collected from Hambantota in the South of Sri Lanka. Attempts to purify these saponins by the methods previously described[3,4] were unsuccessful. Therefore a new method of separation was worked out. In this study we also report a tlc densitometric method of quantifying flabelliferins. Using the purified flabelliferins, studies were also conducted on haemolysis and the froth test.

METHODS AND MATERIALS

Palmyrah fruits : The fruits were collected from Hambantota district. The pulp was extracted manually in the laboratory.

Preparation of crude bitter extract: Fruit pulp portions (200g) were extracted with methanol (200 ml), fats and carotenoids extracted with petroleum ether 60 - 80°C (320 ml x2) and concentrated following the procedure reported previously.[3]

Removal of sugars: The crude extract contained in addition to the saponins, glucose, fructose and sucrose.[1,3,4] The extract was desugared using dry cellulose chromatography.[3] Desugaring was also possible by fermenting the palmyrah fruit with yeast prior to extraction of fruit pulp with methanol.[4]

Flash chromatography: This was conducted using a column of 21 cm length and 2.5 cm diameter using Silica gel G_{60} F_{254}. Pressure was 1.5 bar and inert gas N_2. The solvent used was n-butanol, ethanol, aq NH_3 (sp gr 0.88) in a ratio of 7:3:4. Fractions of 1 ml were collected. Fractions were monitored on silica gel - G_6 plates (300 μm) in the same solvent system but in a ratio 7:2:5. Spots were visualized using anisaldehyde - sulphuric acid reagent.[3]

Tlc-densitometry: Quantification was carried out after tlc (as above) using 100 μm silica gel G_{60} F_{254} pre-prepared plates (Merck FGR) and visualising with spraying (with anisaldehyde reagent) and heating in an oven at 120°C for 10 min. A Shimadzu CS - 900 dual wave length flying spot densitometer was used for quantification. This was equipped with an integrater and chart recorder. Operating details are as follows : Lamp, Tungsten iodide; Scan mode, zig-zag; swing width, 10mm; wavelength, 440nm. Standard curves were plotted using solutions of 5mg.ml^{-1} flabelliferin using 5 μl - 25 μl spots. Scanning of spots for standard curves was horizontal and plots were made in terms of μg saponin vs % peak area.

Fast atom bombardment / Mass spectrometry: This was carried out as reported previously[3] in a negative mode using either triethanolamine or thioglycerol as matrix.

Froth test: The purified flabelliferins and the desugared mixture (2 mg each) contained in 2.0 ml 0.1M phosphate buffer (pH 6.5) in test tubes (16 mm in diameter) was shaken vigorously for 30 sec. The height of froth and time taken to settle were measured.

Haemolysis: To human blood (0.9 ml) drawn just before use, was added 0.1 ml sodium citrate (36.5 gl^{-1}) and diluted with 0.2M phosphate buffer (pH 7.4) to 50 ml.[5] A solution of purified flabelliferins in phosphate buffer (5mg ml^{-1}) in aliquots of 1.0, 0.6 and 0.2 ml respectively and phosphate buffer 1.0, 1.4 and 1.8 ml respectively was added to 1 ml blood preparation to give a total volume of 3 ml. This was incubated in glass test tubes at 30°C and then centrifuged for 30 min at 1500 rpm. The absorbance of the supernatant was measured in an ELICO - SL 150 UV/Vis spectrophotometer at 578 nm.

Debittering: This was carried out on the desugared flabelliferins and palmyrah fruit pulp using either naringinase[3] *or* Hitempase$_{2XL}$ (a heat stable bacterial α-amylase ex. *Bacillus licheniformis* obtained from Quest International). Hitempase, 0.1 ml (114,800 alpha amylase units. ml^{-1}) was added to 100 ml of undiluted (liquid) fruit pulp at pH 6.5 and the mixture heated at 95°C for 10 min. The same procedure on a smaller scale was followed using crude flabelliferin mixture (5mg) in 1 ml 0.1M phosphate buffer (pH 6.5). Aliquots of 5 - 10 µl were run on tlc (as described previously) both before and after incubation, with the enzyme.

RESULTS

Purification of flabelliferins: The purification technique reported previously[3] on samples from the Northern Province[3] failed to separate the bitter principle F - II. This was due to the presence of a mixture of saponins. Desugaring by fermenting the fruit pulp with DCL yeast[4] followed by desugaring the extracted fruit pulp also did not yield purified saponins.

Flash chromatography resulted in the separation of 4 flabelliferins (which we have termed F - II, F_B, F_C and F_D) in yields of approximately 230 mg, 25 mg, 11 mg and 6 mg from 200g fruit pulp. They eluted at 70-90 ml, 45-55 ml, 35-45 ml and 10-20 ml respectively and had Rf values on tlc at 0.35, 0.39, 0.41 and 0.49 respectively for F-II, F_B, F_C and F_D. F - II was most bitter to taste. It appears that elution had not been sufficient to isolate F - I reported previously[3] but a spot corresponding to F - I was seen on tlc. Results of relative bitterness of the isolated flabelliferin and Rf value are shown in Table 1.

Quantification: Using tlc-densitometry linear standard curves were obtained for the purified flabelliferins, plotting % peak area vs µg flabelliferin in the range of 25-100 µg in the case of F- II, F_B and F_D. Results from F_C were not satisfactory. Using this principle it is possible to quantify saponins in a desugared mixture.

Table 1: Rf and relative bitterness of flabelliferins.

Flabelliferin	Rf	Relative Bitterness of isolate
F-II	0.35	most bitter
F_B	0.39	slightly bitter
F_C	0.41	not bitter
F_D	0.49	slightly bitter
Crude	-	bitter - sweet

A typical separation (scanned vertically) of a crude mixture is shown in Figure 1. Here separation is incomplete but shows F-I, F-II, $F_B + F_C$ and F_D in the peak area ratio of 12.4 : 27.4 : 12.7 : 7.9. In quantifying the flabelliferins the standards and sample must be run on the same tlc plate.

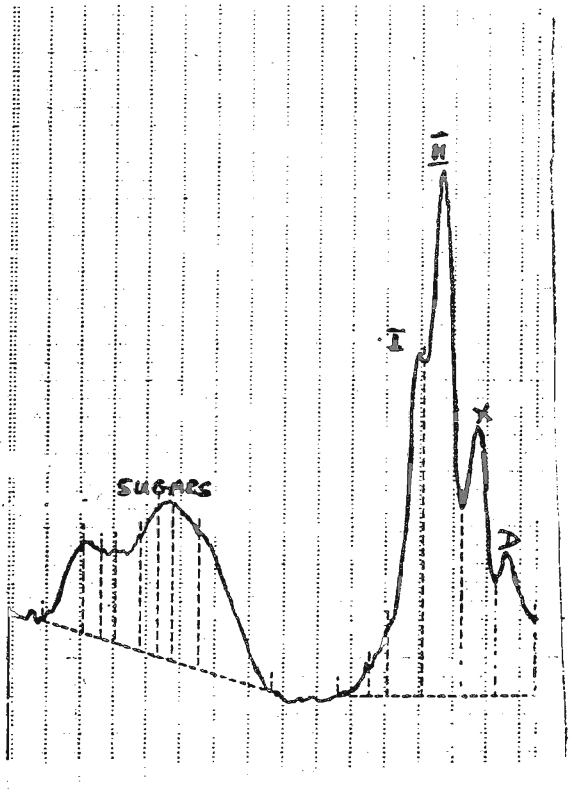

Figure 1: Separation of flabelliferins by tlc-the densitometric patern. I - Flabelliferin I, II - Flabelliferin II, X - Flabelliferin F_B+F_C, D- - Flabelliferin F_D.

FAB / MS data: Spectra of F_B and F_C are shown in Fig. 2 and Fig. 3. Both show a molecular weight of 868. The spectrum of F - II (MW = 1030) is very similar to that reported previously[3] while F_D is probably impure, containing diglycosides. Table 2 summarises the main results from FAB/MS.

Table 2: Data from FAB/MS and interpretation.

Flabelliferin	MW	Other peaks	Interpretation
F - II	1030	884,722 i.e(1030-146) and(884-162)	Tetraglycoside Terminal deoxy hexose (Rhamnose) followed by hexose (glucose)
F_B	868	721 (868-147)	Triglycoside Terminal deoxy hexose (rhamnose)
F_C	868	722 (868-146)	Triglycoside Terminal deoxy hexose (rhamnose)
F_D	722*	-	Diglycoside

* Some doubt as FAB/MS spectum indicated 721

Table 3: Froth test.

Flabelliferin	Height of foam (mm)	Settling time (h)
F - II	7	3.5
F_B	10	25
F_C	5	5
F_D	no foam	-
Crude	10	25

For details *see* Methods and Materials.

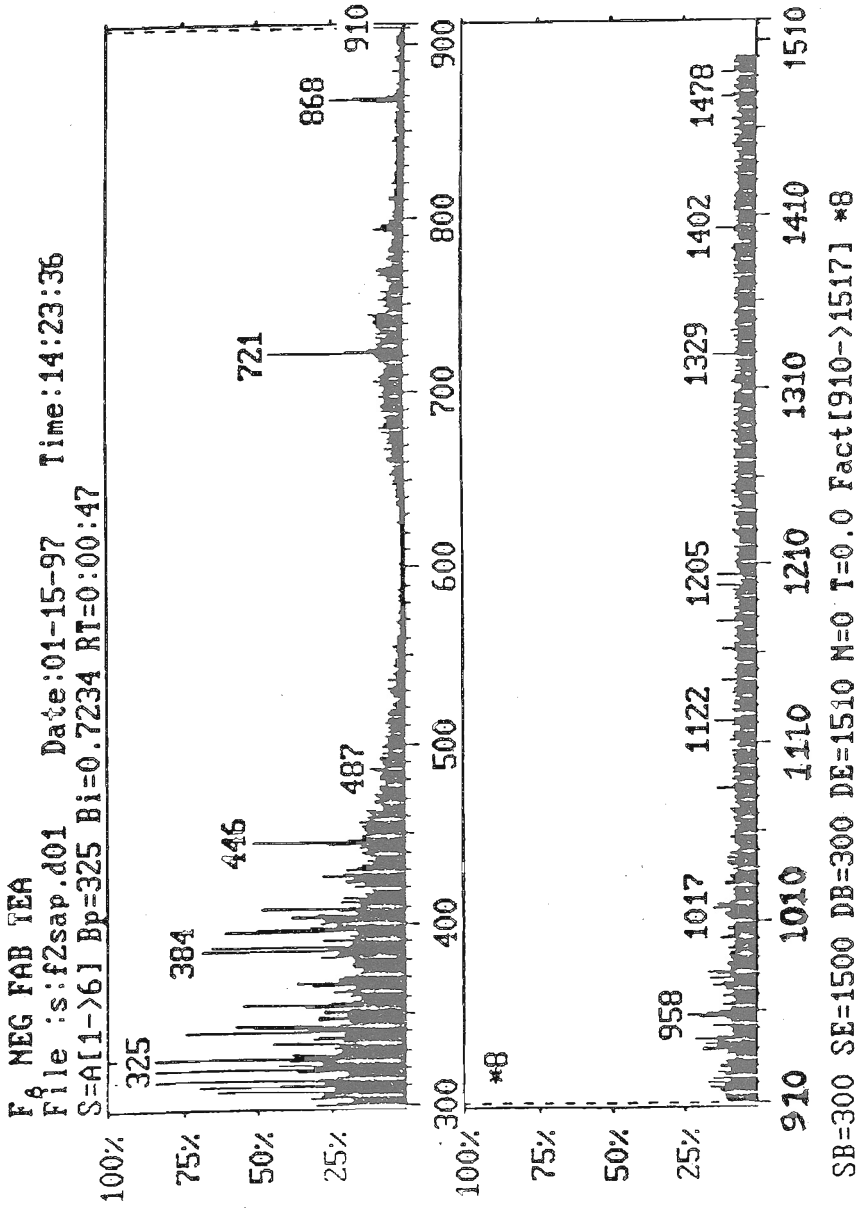

Figure 2: FAB/MS of Flabelliferin F_β.

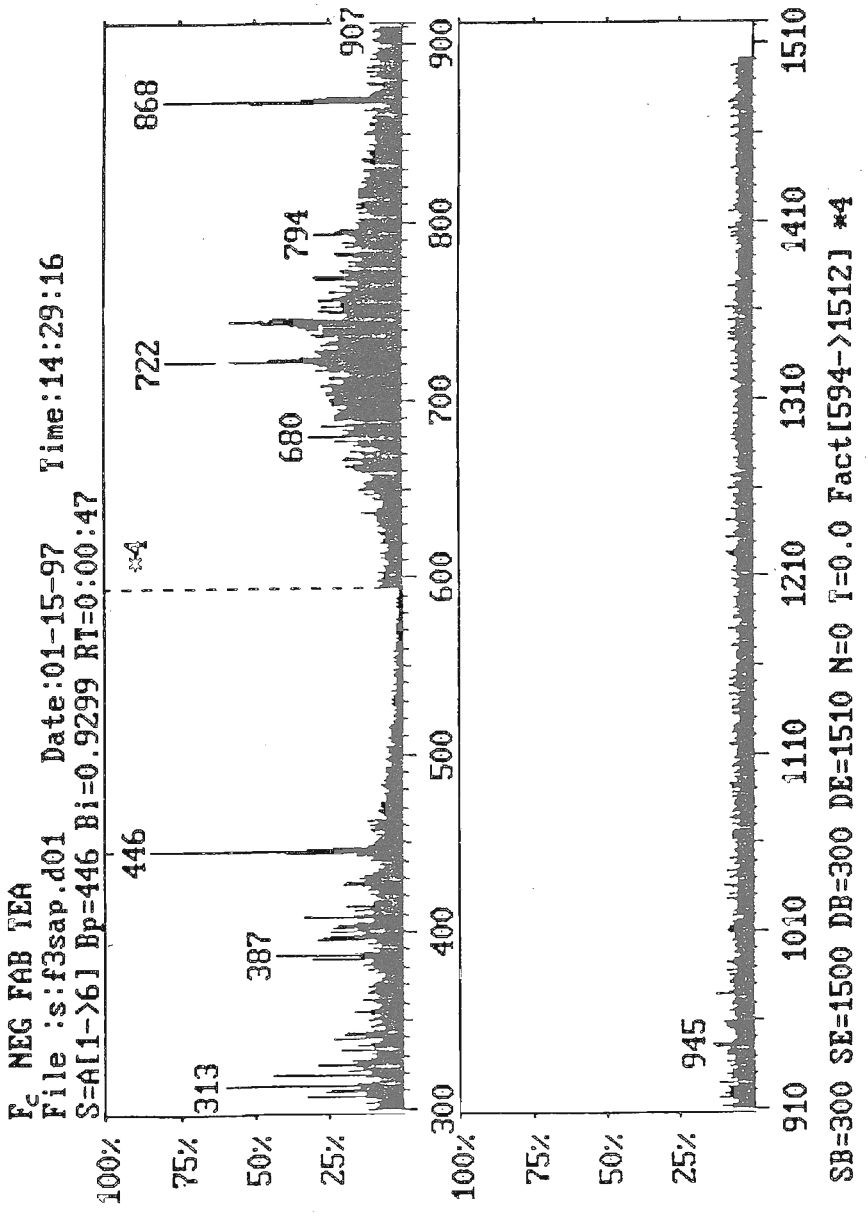

Figure 3: FAB/MS of Flabelliferin F_C

Hydrolysis: As reported previously,[3] the enzyme preparation naringinase debitters palmyrah fruit pulp. On crude extracts this hydrolysis shifts the major saponin tlc spots to higher Rf values. Hitempase also eliminates bitterness in both fruit pulp and crude extracts. Tlc of the hydrolysed crude extracts showed loss of spots corresponding to F-I and F- II.

Froth test: Results are given in Table 3. Of the isolated saponins F_B was most potent.

Haemolysis: Results of the spectrophotometric assay of released haemoglobin are given in Table 4. In the course of this study it was found that the history of the blood sample was important and that haemolysis can increase 4-5 fold if the blood suspension was stored even one day in a refrigerator (5 - 8°C).

Table 4: Haemolysis by UV absorbance in supernatant at 578 nm.

Flabelliferin	Absorbance at 578 nm			
	Control	1.67mg ml^{-1}	1mg ml^{-1}	0.33mg ml^{-1}
F - II	0.02	0.05	0.03	0.01
	0.01	0.04	0.02	0.01
F_B	0.02	1.27*	0.90	0.04
	0.02	1.00*	0.79	0.02
F_C	0.01	1.09*	0.84	0.03
	0.01	1.22*	0.88	0.01
F_D	0.01	1.00	0.02	0.02
	0.01	1.28	0.02	-

* Calculation after dilution

Average for complete haemolysis = 1.10*

DISCUSSION

Previous studies[1,3] on hydrolysates of flabelliferins yielded two sugars *viz.* rhamnose and glucose. Previous studies[1,3] based on nmr and ms showed the molecular weight of the steroidal aglycone was probably 414. FAB/MS data of this study confirms the presence of flabelliferin II reported previously to contain 2 rhamnoses and 2 glucoses in its carbohydrate moiety.[3] This is the very bitter compound reported previously.[3] These samples also yielded 3 other saponins F_B, F_C, and F_D in order of increasing Rf value which had not been reported naturally previously. Their separation was made possible by flash chromatographic techniques. According to FAB/MS data F_B and F_C (different Rf values) had the

same molecular weight 868, which if assuming the same molecular weight of aglycone, are triglycosides and have a carbohydrate moiety containing two rhamnoses and one glucose with rhamnose terminii. Since the two compounds elute separately on flash chromatography and have different Rf values on tlc, they possibly differ in the sequence of sugars in the carbohydrate moiety. Flabelliferin D (Rf 0.49) is probably impure and appears to have a molecular weight of about 722 corresponding to a diglycoside with one rhamnose and one glucose.

Since samples from different sources *viz.*, Jaffna,[1] Kalpitiya[3] and Hambantota show saponins with different carbohydrate moieties it appears that there is considerable diversity among cultivars/stands. Even if it is assumed that : (1) glucose and rhamnose are the only sugars in the carbohydrate chain, (11) the carbohydrate chain is four saccharides or less, this leaves room for a plethora of possible flabelliferins. The tlc-densitometric technique will be useful as a spot test for such diversity. This would be important in the context that these saponins have different haemolytic and foam stabilising activity and also have varying bioactivity against growth of yeast.[6] The flabelliferins appear to retard alcoholic fermentation[7] in boiled palmyrah fruit pulp. This inhibitory effect is reversed by hydrolysis by the enzyme naringinase[7] with the loss of F - I and F - II.[7] The diversity in bioactivity of the isolated flabelliferins gives the tlc-densitometric assay larger importance. Studies on bioactivity using both purified flabelliferins and palmyrah fruit pulp are proceeding.

Acknowledgement

We thank the Natural Resources, Energy and Science Authority for a grant RG/95/C/13, the International Programme in Chemical Sciences (IPICS) Sweden, for a grant SRI : 07, Prof. Per-Erik Jansson of the Clinical Research Centre, Huddige Hospital, Sweden for FAB/MS data and T.M.S.G. Tennekoon, University of Sri Jayewardenepura for assistance on densitometry.

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