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SEPARATION TECHNIQUES OF FLABELLIFERINS FROM PALMYRAH (*Borassus flabellifer L.*) FRUIT PULP

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Abstract

The flabelliferins (steroidal saponins) of palmyrah are of importance due to their significance in food and bioactivity. There are a plethora of flabelliferins and their separation and quantification is a challenge. This paper discusses the methods of purifying flabelliferins and determining the flabelliferin profiles of varying Palmyrah fruit pulps (PFP).

Flabelliferins were separated by a number of techniques, namely flash chromatography, chromatotron, selective solvent extraction, solvent gradient chromatography and medium pressure liquid chromatography (MPLC). The best overall system of separation was MPLC although depending on flabelliferin profile, the other techniques had specific value.

keywords :Palmyrah, bitter principle, MPLC, solvent gradient, flabelliferin profile

1 Introduction

Steroidal saponins from palmyrah were first reported by Jayaratnam[1] who identified a monoglucoside and a monorhamnoside of spirost-5-en-3βol. Jansz and

2.3 DE-SUGARING

This was carried out in two ways, (1) by fermenting the sugars in the fruit pulp using *Saccharomyces cervisiae* (2×10^7 cells 100^{-1} *ml* PFP) for 4 days and omitting the acetone step[2] or (2) by dry cellulose chromatography[2] either with or without the acetone step.

2.4 PURIFICATION OF FLABELLIFERINS

2.4.1 Flash chromatography

Flash chromatography was carried out according to the method described by Nikawala and coworkers[3].

2.4.2 MPLC (Medium Pressure Liquid Chromatography)

MPLC apparatus of Baeckstrom[7] with Separo columns, FMI pump (model QD-O-ssy) and a reservoir to create a continuous gradient was used in a solvent gradient system with CH_2Cl_2 and methanol (99.8:0.2, 99.6:0.4, 98.2:0.8, 96.8:3.2, 93.75:6.25, 3:1, 1:1, 1:3, 6.25:93.75, 3.2:96.8, 0.8:98.2, 0.4:99.6, 0.2:99.8). The isolates were obtained at 75-85 *ml* (free steroid), 165-170 *ml* and 180-190 *ml*.

2.4.3 Solvent gradient column chromatography

The column (2.5 *cm* in diameter) was packed with silica gel (230-400 mesh and average pore diameter 60Å) in hexane to a length of 25.0 *cm*. After the introduction of the sample on to the column bed, the polarity of the mobile phase was increased gradually using CH_2Cl_2, ethyl acetate and methanol in different ratios (100:0:0, 90:10:0, 75:25:0, 50:50:0, 25:75:0, 10:90:0, 0:100:0, 0:90:10, 0:80:20, 0:70:30, 0:60:40, 0:50:50, 0:45:55, 0:40:60, 0:35:65, 0:30:70, 0:25:75, 0:20:80, 0:15:85, 0:10:90, 0:0:100). One (1) *ml* fractions were collected. Thin layer chromatography was carried out to confirm the presence of each flabelliferin.

2.4.4 Chromatotron (Harrison Research, model 8924, made in U.S.A)

The rotors should be coated with silica gel G60 PF_{254} of a thickness of either 1*mm*, 2*mm* or 4*mm* depending on weight of the sample. The pump (Lab Pump Jr-FLUID metering Inc, model RHSY, made in U.S.A)was set at a flow rate according to the thickness of the rotor that was being used,

coworkers[2] isolated the bitter principle of palmyrah fruit pulp (PFP) from Kalpitiya and identified it as a tetraglycoside of the same aglycone and coined the term flabelliferin. The bitter principle was called flabelliferin-II (F-II) and another saponin, a tetraglucoside was called flabelliferin-I. This isolation was based on dry cellulose chromatography (desugaring) and crystallisation. Nikawala *et al.*[3] found that the earlier technique of isolation did not work on complex mixtures of the flabelliferins and had to resort to flash chromatography to separate a mixture of 4 flabelliferins which other than F-II contained F_B (an antimicrobial flabelliferin triglycoside[4]), F_C (a non-bioactive flabelliferin triglycoside) and F_D a non-active flabelliferin diglycoside). Further study by this group showed that there was a large variation in flabelliferin profiles depending on (I) source of PFP and (II) enzymes used for the purpose of debittering[5]. Further the existence of a large variety of flabelliferins meant that there was no universally applicable technique for their isolation. However, the diversity of flabelliferins could be assessed by simple TLC techniques[2]. It is becoming very clear that some of the flabelliferins are bioactive and the end use of PFP could be dictated by the flabelliferins it contains. For example "non-bitter" PFP[6] could be easily utilized as a cordial. Bitter PFP not containing F_B or other antimicrobial factors could be used as substrates for yeast fermentation (ethanol production[7]). Those containing anti-yeast flabelliferins and bitter principle need to be enzymatically hydrolysed[2][8][9][10] before utilization as food particularly the very bitter PFP which can be debittered by a relatively cheap heat stable α-amylase[9]. Therefore many different end uses could be visualized.
The objective of the study was to examine different methods of flabelliferin isolation and attempt to select methods matching flabelliferin profiles of different pulps. The paper is meant to convey to the other researchers, the options available for flabelliferin isolation and quantification.

2 MATERIALS AND METHODS

2.1 FRUITS

Ripe palmyrah fruits of four morphologically distinct types were collected from 20 locations (Jaffna (2), Mannar, Ampara (2), Chilaw, Batticaloa, Puttalam, Trincomalee, Vavuniya, Hambantota, Kurunegala, Anamaduwa (2), Naththandiya, Polonnaruwa (2), Kalpitiya, Anuradhapura (2)) of Sri Lanka. PFP was extracted manually with distilled water in the ratio of 1:2 (v/v).

2.2 EXTRACTION OF CRUDE FLABELLIFERINS

Crude flabelliferins were extracted by the method of Jansz and coworkers[2] using extraction with methanol, cleaning with petroleum ether and extracting the residue in acetone.

	Pump Scal
1 *mm* layer: 2-4 *ml/min*	90-180
2 *mm* layers: 6-8 *ml/min*	250-350
4 *mm* layers: 8-10 *ml/min*	350-410

After passing hexane (100 *ml*) to wash out the new plate, the sample dissolved in minimum amount of methanol was introduced. Hexane (100 *ml*) was passed again before increasing the polarity gradually using CH_2Cl_2, ethyl acetate and methanol in different ratios, as described in solvent gradient column chromatography (section 2.4.3) and 1 *ml* fractions were collected. Thin layer chromatography was conducted with all the fractions in order to confirm the separation.
As there was an impurity exactly overlapping on top of all the separated flabelliferin fractions, each fraction was subjected to another chromatotron run on new rotors with a solvent system of of iso-propanol: ethyl acetate (1:1).

2.4.5 Selective solvent extraction

Crude flabelliferin mixture was dissolved in 20 *ml* of water and then extracted to ethyl acetate using a separating funnel. TLC was conducted to confirm the separation.

2.5 THIN LAYER CHROMATOGRAPHY

TLC was carried out on pre-prepared silica gel G60 plates (Merck) using n-butanol, ethanol and ammonia in the ratio of 7:3:4 or 7:2:5 and visualized with anisaldehyde spray reagent[2].

2.6 TLC PROFILES AND QUANTIFICATION

The TLC plates after visualizing was scanned (see figure-1 and figure-2)using three types of densitometers for comparison; (a) Shimadzu[3] at 500 *nm* (b) Advantech[6] (digital, model DM 303, Advante Toyo Kaisha Ltd, Japan) at 300 *nm* and (c) Pharmacia Biotech computerized densitometer (Compaq Deskpro 2000 5/233 MMX computer, Sharp scanner JX 330, Image Master 1D Elite ver 2.01, Hoefer Pharmacia Biotech Inc, U.S.A) at 500 *nm*. The plates were scanned ensuring that the spots lay within the range of a linear standard curve obtained by plotting peak area verses μg flabelliferins in each case. This yielded a method of quantification.

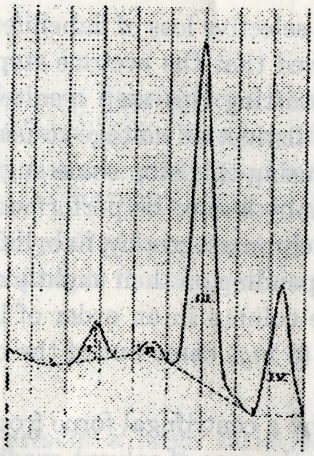

Figure 1: Densitometric scanning of Shimadzu densitometer.

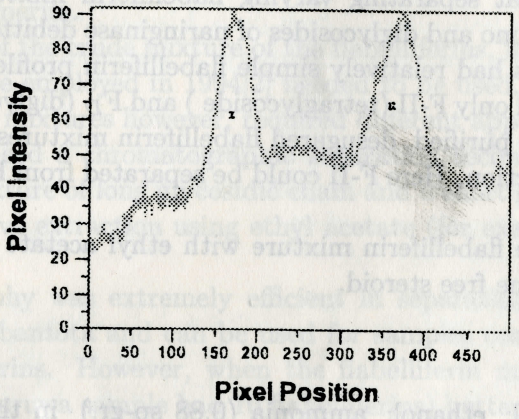

Figure 2: Denistometric scanning of Pharmacia Biotech computerized densitometer
Legends: I - F_D II - F-II

3 RESULTS

3.1 SEPARATIONS

The techniques could be used for various purposes for example, quantification of flabelliferins by TLC/densitometer before and after fermentation. This did not alter flabelliferin profile significantly showing that yeast did not produce enzymes to hydrolyze flabelliferins. It was feared that the acetone extraction step which was done

to reduce sugars might cause a selective loss of flabelliferins and produce artifactural profiles. However, results showed that the acetone step did not selectively lose any given type of flabelliferin. Omitting this step resulted in very slow dry cellulose chromatography. This gives a distinct advantage to use the acetone extraction step originally used[2]. Even after using this step some sugars still remained and therefore dry cellulose chromatography had to be performed.

Using solvent gradient column chromatography, flabelliferins were separated as bands and eluted from the column depending on their slightly different polarities. The compounds were eluted out of the column in an order of increasing polarity and there by the least polar free steroid (6 mg) came out of the column first and any residual sugar last.

The flabelliferins were eluted by a centrifugal force from the chromatotron in an order of increasing polarity as in the case of solvent gradient column chromatography. Further separations using iso-propanol and ethyl acetate as the cleaning-up solvent system, resulted in pure flabelliferins, F-II, F_C and F_D (the particular sample did not contain F_B).

MPLC was effective at separating varying flabelliferin mixtures as well as free steroids, aglycones, mono and diglycosides of naringinase debittered[2] mixed flabelliferins. Some samples had relatively simple flabelliferin profiles for example those from Chilaw contained only F-II (tetraglycoside) and F_D (diglycoside). These could be separated from the purified, desugared flabelliferin mixtures by the simple technique of solvent extraction. Here F-II could be separated from F_D by extracting F_D into ethyl acetate.

Partition of the crude flabelliferin mixture with ethyl acetate yielded the saponin diglycoside, F_D and the free steroid.

3.2 TLC

The system n-butanol: ethanol: ammonia (0.88 sp-gr.) in the ratio of 7:2:5 was found to be the best for separating flabelliferins (table-1). However, when flabelliferin profiles were dominated by short glycosidic chain flabelliferins, the same system in the ratio of 7:3:4 was preferred.

Table-1 : Rf values

Flabelliferin	Rf value
F-II	0.35
F_B	0.39
F_C	0.41
F_D	0.47

Solvent system, n-butanol: ethanol: ammonia (0.88 spgr) in the ratio of 7:2:5.

3.3 QUANTIFICATION

In order to quantify flabelliferins a densitometer had to be used to scan the TLC plates. The best resolution of flabelliferins for quantification was by Shimadzu (zig-zag mode) at 500 *nm* and this was extremely good when the number of flabelliferins in a fruit pulp was large (figure-1). If PFP contained a small number of flabelliferins, the computerized densitometer yielded impressive quantitative scans (figure-2). Using these scans and a standard curve it was possible to quantify flabelliferins.

4 DISCUSSION

Fruit pulps from palmyrah present a large variation in the types of flabelliferin (steroidal saponins) profiles depending on the origin of the fruit pulp[6][7][9]. This variation not only presented difficulties in having one uniform technique for isolation of flabelliferins but also is the determining factor for the end-use of the fruit pulp. In this study we compared the various extraction and purification techniques to assess their merits and demerits.

In the preparation of the crude mixture of the flabelliferins, it became clear that the traditional technique employed in 1994[2] needed to be used.

Separation of crude mixtures however, required different approaches. Samples from Kalpitiya did not need a chromatographic separation technique[2]. Samples containing a simple mixture of long glycosidic chain and a short glycosidic chain can be separated by selective extraction using ethyl acetate (for example the sample from Chilaw).

Flash chromatography was extremely efficient in separating the four flabelliferin mixtures from Hambantota and can be used for samples containing similar or less number of flabelliferins. However, when the flabelliferin mixture is complex (for example the Polonnaruwa sample had 10 flabelliferins) better separation techniques were needed. Solvent gradient chromatography fullfilled this need. The same solvent system could be used faster with the chromatotron and refined further using MPLC.

The importance of these separation techniques lie in the fact that pure flabelliferins are needed for testing for bioactivity. Already it is known that F-II (tetraglycoside) is growth retarding (in mice)[6][8] and an inhibitor to the Na^+/K^+ pump[5] and further that F_B (triglycoside)is an inhibitor of yeast and bacteria[4]. There may be others that are also bioactive. We also know that some are inactive[4] and this poses interesting structural activity relationships which may have important theoretical implications.

In addition the study has shown that densitometers can be used for quantification. It appears that the specific wavelength sophisticated densitometers are the best for complex flabelliferin profiles while more simple densitometers are sufficient for simple flabelliferin mixtures (four or less number of flabelliferins).

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