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Flabelliferins of naringinase debittered palmyrah fruit pulp

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Abstract

Palmyrah (*Borassus flabellifer L*) fruit pulp was debittered with the enzyme naringinase. At least three of the 4 natural flabelliferins including the bitter principle (F-II) and an antimicrobial principle (F_B) were hydrolysed and two spots (F_x and F_y) appeared on Tlc. F_x was a pure saponin triglycoside M. W. 868. Spectroscopic examination of F_y showed it to be an impure mixture of saponins and steroid(s). On separation with MPLC, 3 isolates were obtained including a free steroid which corresponded to stigmast-5en-3βol. F_x and F_y had no bitterness or antibacterial activity and also had little or no foam stabilizing and haemolytic activity, unlike F-II and F_B. While the properties of F_x were very similar to F_C, a natural flabelliferin (MW 868), F_y contained the products of naringinase hydrolysis of the bitter flabelliferin (F-II) and the antimicrobial flabelliferin (F_B).

Key Words : **Palmyrah, *Borassus flabellifer*, Flabelliferins, Naringinase, Anti-bacterial effect, Debittering.**

1. Introduction

Palmyrah fruit pulp (PFP) is vastly underutilized due to the presence of a bitter principle (flabelliferin-F-II, a steroidal saponin of M. W. 1030[1]). Fruit pulp from Hambantota in the south of Sri Lanka contains 4 natural flabelliferins including F-II[2] (bitter compound). Among these flabelliferins also is an antimicrobial (both bacteria and yeast) triglycoside flabelliferin (MW 868) called F_B[3]. Naringinase has been shown to hydrolyse F-II[1] and debitter the fruit pulp. Debittering fruit pulp reverses the inhibitory effect of bitter PFP on growth of young ICR-mice[4].

The objectives of this study were to understand the changes occuring in debittering by naringinase, to fulfill the general objective of expanding utilisation of palmyrah fruit pulp, on which ongoing work is mainly conducted in Sri Lanka.

2. Materials And Methods

Fruit Pulp

The pulp of ripe fresh palmyrah fruits collected from Hambantota, Sri Lanka were extracted with water (1:1), manually.

Debittering

Debittering was carried out under sterile conditions using naringinase (ex. *Penicillium decumbens* from Sigma, USA) The enzyme preparation was incubated with PFP (1mg. g^{-1} PFP) at pH 5 and 37^0C for 24 hours.

Purification of product flabelliferins

The crude flabelliferins after debittering were extracted and purified as described previously[2] using flash chromatography as the final step.

Thin Layer Chromatography

The product flabelliferins were subjected to Tlc analyses on silica gel pre-prepared plates (100 μm) using butanol: ethanol: NH_3 (sp gr 0.88) (7:2:5) as solvent system[1]

Haemolysis and froth tests

These tests were conducted as described previously[2]

Anti-bacterial test

This test was carried out by the Bauer-Kirby method as described previously[3].

Spectroscopy

FAB/MS was conducted as reported previously[1] in negative mode.

GC/MS analysis was carried out using a Varian GC-4000. A Finnigan Mat MS SSQ-7000 Mass spectrometer with direct inlet was used. ^{1}HNMR, ^{13}CNMR and 2D NMR was performed using a Bruker-DMX at 300^0 K in a solution of $CDCl_3$ using TMS as internal reference.

MPLC

Medium pressure liquid chromatography was performed using Separo columns with FMI pump model QD-O-ssy with a reservoir to create a continuous gradient. The gradient of the solvent system used in succession

was CH_2Cl_2: MeOH, 99.8 : 2, 99.6 : 04 ; 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; 0.4 : 99.6; 0.2 : 99.8 20 ml of each combintation was used. Three isolates were obtained at 75-85 ml (free steroid), 165-170 ml and 180-190 ml.

3. Results

Naringinase action

From the naringinase debittered PFP, after separation of crude flabelliferins, 2 spots were observed on tlc with Rf 0.40 - 0.43 (termed F_x) and 0.83 (termed F_y). These were isolated by flash chromatography.[1]

Properties of F_x and F_y

F_x and F_y were not bitter to taste and had no haemolytic activity on fresh human red blood cells. F_y did not stabilize foam. Foam stability of F_x at 1mg ml^{-1} was very low (foam height, 2 mm; stability, 5 h). They had no antibacterial activity by the Bauer-Kirby method at 1.25 mg. $disc^{-1}$ on *Staphylococcus aureus* (NCTC 8532) *Staphylococcus epidermidis* (NCTC 4276), *Escherichia coli*_(NCTC 10148) *Pseudomonas aeruginosa* (NCTC 10662), *Proteus rettigeri*_(NCTC 7475), *Acinetobacter_calcoaceticus* (NCTC 5866). The latter shows that not only the bitter flabelliferin (F-II) but also the antimicrobial flabelliferin (F_B) is hydrolysed by naringinase. Rf values indicate that the carbohydrate moiety of some flabelliferins are degraded by naringinase.

It had been known for sometime that naringinase could debitter PFP[1]. This study provides some insight into the mode of hydrolysis and the products of debittering.

The product flabelliferin F_x had a MW of 868 by FAB/MS (Fig. 2) it could not have been the product of naringinase hydrolysis of the bitter flabelliferin (F-II), as F-II had a MW of 1030 with a rhamnose terminus.[1] It was not F_B (MW 868) as it did not share its antibacterial properties.[3] It had similar Rf[2], MW[2] and properties[3] to F_C (MW 868). It is therefore probably unhydrolysed F_C. Like F_C[2], Fx had a rhamnose terminus.

While it appeared that there was no Tlc spot corresponding to F-II and F_B, the high Rf spot Fy on FAB/MS analysis showed a molecular ion peak at 722 corresponding to a diglycoside (1 rha + 1 glu). ^{1}HNMR, however showed that Fy was impure. It had on ^{1}HNMR, 3 signals for sugars at δ= 4.3, 4.4 and 4.5. However, the contribution steroidal and carbohydrate signals on separate integration were in the stoichiometric ratio of approximately only 1:1, indicating that it is likely that F_y is a mixture of diglycosides, monoglycosides and a steroid(s).

The study shows that naringinase hydrolyses not only the bitter F-II but also the antimicrobial FB into smaller steroidal glycosides. This would enable treated PFP (which has 16% sugar)[6] to be used for both, beverages and as a base for alcoholic fermentation. The removal by hydrolysis of F-II is important as bitter PFP is an inhibitor to weight gain in young ICR mice[4] and is an inhibitor to the Na^+/K^+ ATP[ase7]

Spectroscopic Analysis

^{1}HNMR analysis of F_x showed it to contain 3 saccharide signals at $\delta = 4.15$ (βglucose), $\delta = 4.65$ (β rhamnose) and $\delta = 4.45$ (not identified but from FAB-MS derived MW, it should be rhamnose). A clear rhamnose duplet is seen at $\delta = 1.3$-1.35 on ^{1}HNMR (Fig. 1) (Bax et al., 1984)[5]

FAB/MS analysis (Fig. 2) showed that F_x and M. W. of 868 (saponin striglycoside) with a rhamnose terminus. NMR and FAB/MS analysis showed that F_y was impure. MPLC resulted in 3 isolates. One of these was a steroid of M. W. 414 (Fig. 3). This data together with 2D-nmr and ^{13}C-nmr indicated that the steroid is consistent with stigmast-5en - 3β01. This was probably not the aglycone but a free steroid identified by Jeyaratnam[6]

4. Discussion

Jeyaratnam (1986)[6] showed that the steroidal aglycone of palmyrah fruit pulp was spirost-5en -3β01(25R) (MW 414). In that study, monoglycosides of the saponins were reported in fruits collected from Jaffna in the North of Sri Lanka. Fruits collected from the North-West of Sri Lanka[1] contained the bitter flabelliferin F-II (MW 1030, Rha terminus) which corresponded to a tetraglycoside (2 Rha, 2 Glu) of a steroid of MW 414.

The fruit pulp used in this study was collected from Hambantota in the south of Sri Lanka. This had F-II (the bitter flabelliferin)[2] and also 3 other natural flabelliferins F_B (anti-microbial)[3] F_c[2] and F_d[2].

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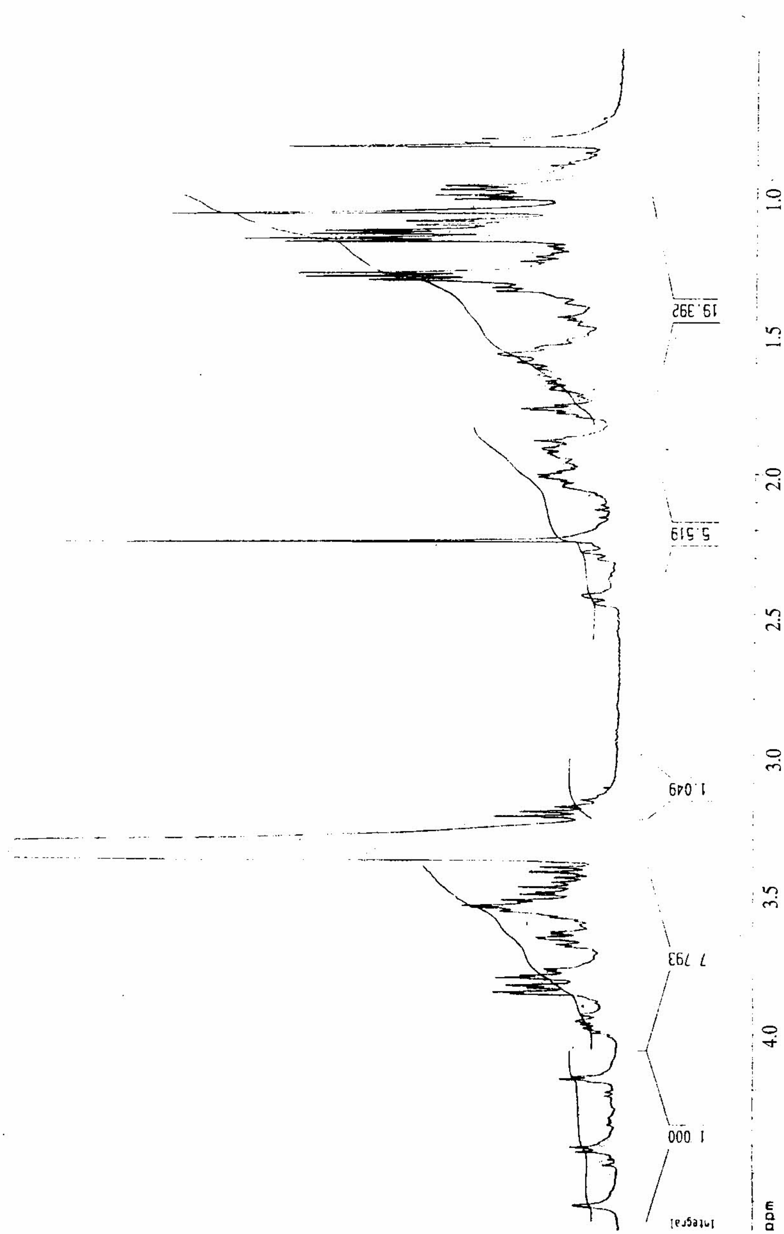

Fig. 1. ^{1}H nmr of F_x

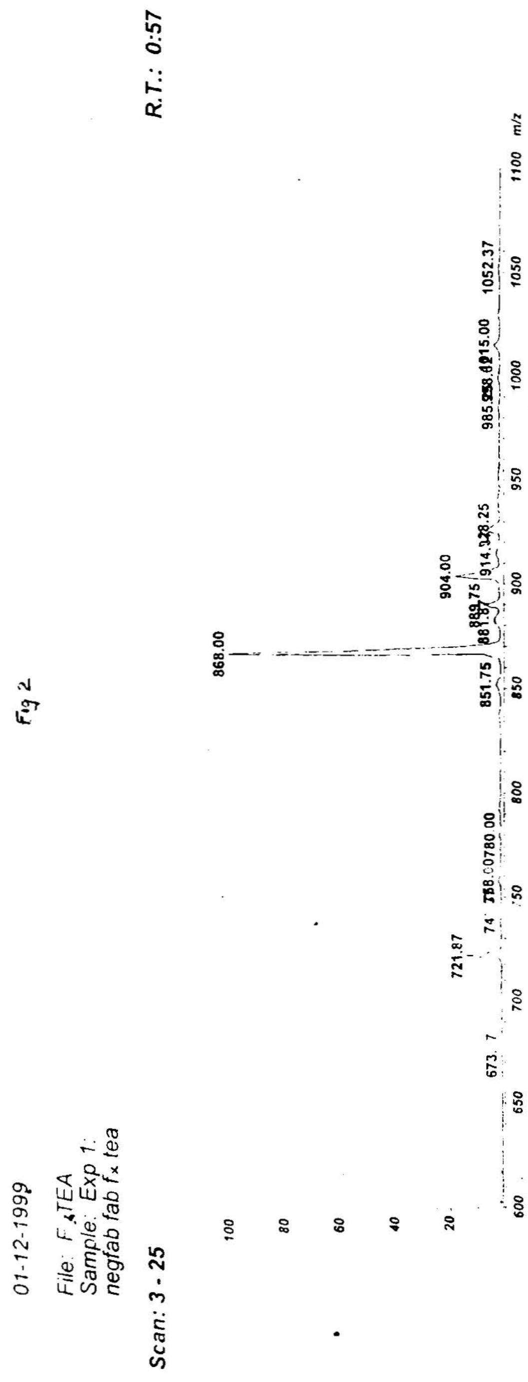

Fig. 2. FAB/MS of F_x

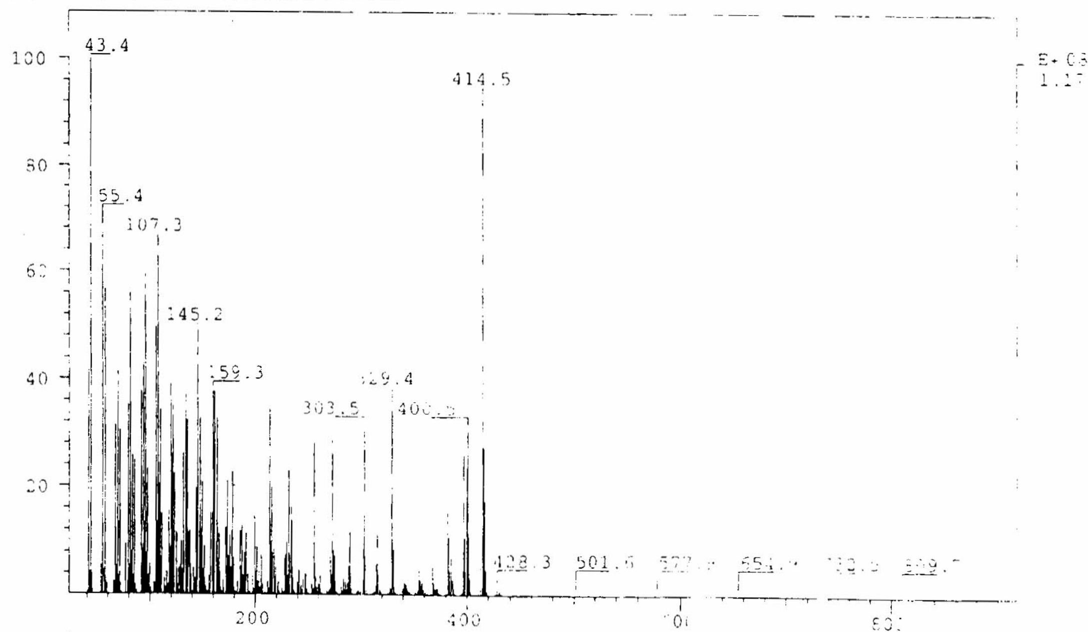

Fig. 3. MS of steroid (aglycone)