
**The Diversity, Bioactivity and Structural
studies of flabelliferins from palmyrah
(*Borassus flabellifer* L.) fruit pulp**

**By
Darshika Dilleny Ariyasena**

M. Phil. 2002

Declaration

“The work described in this thesis
was carried out by me
under the supervision of professor E. R. Jansz
and professor A. M. Abeysekera
and

I certify that this thesis does not incorporate without
acknowledgement, any material previously submitted for a degree
or diploma in any university or higher educational institution in Sri
Lanka or abroad and to the best of my knowledge and belief, it does
not contain any material previously published or written by another
person except where due reference is made in the text”

DD Ariyasena

D. D. Ariyasena

27/06/02

Date

Certification

We certify that the above statement
made by the candidate
is true and that this thesis is suitable
for submission to the university
for the purpose of evaluation



Professor E. R. Jansz



Professor A. M. Abeysekera



**The Diversity, Bioactivity and Structural
studies of flabelliferins from palmyrah
(*Borassus flabellifer L.*) fruit pulp**

By

Darshika Dilleny Ariyasena

Department of Chemistry
Faculty of Applied Sciences
University of Sri Jayawardenepura

Thesis submitted to the
University of Sri Jayawardenepura
for the award of the degree, Master of Philosophy
In Applied Chemistry

January 2002

Table of Contents

<i>List of tables</i>		X
<i>List of figures</i>		XI
<i>List of plates</i>		XIV
Abbreviations		XV
Acknowledgements		XVI
Abstract		XVIII
1	Introduction	
1.1	General introduction	1
1.2	Scope of study	3
2	Literature Review	
2.1	The palmyrah palm	6
2.1.1	Utilizable products of palmyrah palm	6
2.1.1.1	Non – edible products	6
a	Trunk / stem	6
b	Petiole	6
c	Sheath	6

d	Stalk	7
e	Leaf	7
f	Seed	7
2.1.1.2	Edible products	9
a	Sap based products	9
i	Fresh palmyrah sap	9
ii	Palmyrah sweet toddy	9
iii	Bottled palmyrah sweet toddy	10
iv	Palmyrah toddy	10
v	Bottled palmyrah toddy	10
vi	Palmyrah jaggary	10
vii	Palmyrah treacle	11
viii	Palmyrah candy	11
ix	Palmyrah palm sugar	12
x	Palmyrah arrack	12
xi	Palmyrah palm sugar products	12
b	Palmyrah tuber based products	13
i	Raw dried tuber	13
ii	Tuber flour	13
iii	Tuber starch	14

iv	Boiled and dried tuber	14
v	Palmposha	14
vi	Biscuits	15
vii	Porridge (instant)	15
viii	Debittered tuber flour	15
c	Fruit based products	16
2.1.2	Studies on varieties	16
2.2	Past work on palmyrah flour	19
2.3	Past work on palmyrah fruit pulp (PFP)	22
2.4	Occurrence of steroidal saponins	26
2.5	Methods of separation	29
2.6	Methods of structural analysis	31
2.6.1	Fast Atom Bombardment – Mass Spectrometry (FAB-MS)	31
2.6.2	Electrospray Ionization – Mass Spectrometry (ESI-MS)	32
2.6.3	Gas chromatography - Electron Impact - Mass Spectrometry (GC-EI-MS)	33
2.6.4	Nuclear Magnetic Resonance (NMR) Spectroscopy	35
2.7	Theory of densitometry	36
2.8	Determination of the substitution of the monosaccharide units	37

2.9	Animal models for bioactivity	38
2.9.1	Formulation of animal diets	38
2.9.2	Selection of animals	40
2.9.3	Suitable experimental conditions	40
3	Materials and methods	
3.1	Reagents, solvents, special chemicals and enzymes	42
3.1.1	Water	42
3.1.2	Solvents	42
3.1.3	Special chemicals	42
3.1.4	Enzymes	42
3.2	Palmyrah fruit pulp	42
3.2.1	Collection of palmyrah fruits	42
3.2.2	Extraction and storage of palmyrah fruit pulp (PFP)	43
3.2.3	Debittering of fruit pulp	43
3.3	Isolation of crude flabelliferins	43
3.4	Techniques to separate flabelliferins	45
3.4.1	Solvent gradient column chromatography	45
3.4.2	Chromatotron	46
3.4.3	Medium Pressure Liquid Chromatography	48

3.4.3.1	Isolation of flabelliferins from crude flabelliferin mixtures using MPLC	48
a	Pre – adsorption of the sample	48
b	Column packing	48
c	Creating the gradient	50
	Step – 1, Crude separation	50
	Step – 2, Further separation	51
	Step – 3, Purification of flabelliferins	51
3.4.3.2	Isolation of flabelliferins from raw PFP using MPLC	52
a	Pre – adsorption of the sample	52
b	Column packing	53
c	Creating the gradient	53
3.4.4	Simple solvent extraction	54
3.4.5	Flash chromatography	54
3.5	Monitoring the fractions	54
3.5.1	Thin Layer Chromatography (TLC)	54
3.5.2	Spray reagents	55
3.6	Structural elucidation	55
3.6.1	Nuclear Magnetic Resonance Spectroscopy	55
3.6.2	Electrospray Ionization – Mass Spectrometry	55

3.6.3	Gas chromatography - Electron Impact - Mass Spectrometry	56
3.6.4	Gas Chromatography	56
3.7	Diversity of flabelliferins	57
3.7.1	Collection of palmyrah fruits	57
3.7.2	Typing of the fruits	57
3.7.3	TLC / Densitometry	57
3.8	Animal Studies	58
3.8.1	Animal model - 1	58
3.8.2	Animal model - 2	60
3.9	Determination of reducing sugars	61
3.9.1	Nelson method	61
3.10	Extraction and quantification of carotenoids	63
3.11	Determination of fruit colour and taste	64
3.11.1	Colour of the fruit pulp	64
3.11.2	Taste of the fruit pulp	64
3.12	Sugar linkage analysis	64
3.12.1	Experimental procedure	65
3.13	Derivatization by trimethylsilylation	66
3.13.1	Experimental procedure	66

4	Results	
4.1	Separation and purification of flabelliferins	67
4.1.1	Crude separation	67
4.1.2	Purification of flabelliferins	67
4.1.2.1	Simple solvent extraction	67
4.1.2.2	Flash chromatography	68
4.1.2.3	Solvent gradient column chromatography	68
4.1.2.4	Chromatotron	68
4.1.2.5	Medium Pressure Liquid Chromatography/ Isolation of flabelliferins from crude flabelliferin mixtures	69
a	Crude separation	69
b	Further separation	69
c	Final separation	70
4.2	Structural elucidation	70
4.2.1	Structure of aglycone	70
4.2.2	Structure of F _B	75
a	ESI-MS data	75
b	Sugar linkage position-by methylation analysis	75
c	NMR data	78
4.2.3	Structure of F _D	82

a	ESI-MS data	82
b	Sugar linkage position-by methylation analysis	82
c	NMR data	89
4.2.4	Structure of F _F	93
a	Sugar linkage position-by methylation analysis	93
b	NMR data	93
4.2.5	Partial structure of F _E and F _N	99
4.3	Diversity in flabelliferin profiles	106
4.3.1	Morphology	106
4.3.2	Flabelliferin profile	110
4.4	Miscellaneous studies	110
4.4.1	Carotenoid content	110
4.4.2	Raw PFP separation with MPLC	115
4.5	Animal Studies	116
4.5.1	Animal model - 1	116
4.5.1.1	Debittering	116
4.5.1.2	Weight gain	117
4.5.1.3	Other results	118
4.5.2	Animal model - 2	118
4.5.2.1	Sugar content	118

4.5.2.2	Flabelliferin profile	119
4.5.2.3	Weight gain	120
5	Discussion	122
	References	140
	Publications and communications of this study	150
	Appendix- 1	152
	Appendix- 2	171

List of Tables

Table-2.1	The fruit and the pulp characteristics of the varieties of Alankudah (AL) and Mandalakudah (MK)	17
Table-2.2	The fruit and seed characteristics of Alankudah (AL) and Mandalakudah (MK) palmyrah selections	18
Table-2.3	Composition of PFP	23
Table-2.4	Feed formula of WHO recommended rat/mouse breeding feed	39
Table-3.1	Amounts of Silica gel and water to coat rotors of different thicknesses	47
Table-3.2	Flow rate and the relative pump scale for different thicknesses of rotors	47
Table-3.3	Feed formula of the test feed, based on the WHO recommended rat/mouse breeding feed	59
Table-4.1	Colour, taste, % dry weight and the total flabelliferins by dry weight for each sample of different types.	109
Table-4.2	Flabelliferin profiles of different palmyrah fruit types.	111
Table-4.3	Carotenoid content and palmyrah fruit type	113
Table-4.4	Comparison of weight gains – Animal model - 1	117
Table-4.5	Sugar content of PFP	119
Table-4.6	Flabelliferin profile	119
Table-4.7	Comparison of weight gains – Animal model – 2	121

List of Figures

Figure-2.1	Structure of the free steroid(stigmast-5en-3 β ol) by Jayaratnam (1986)	27
Figure-2.2	Structure of the aglycone (spirost-5en-3 β ol) by Jayaratnam (1986)	27
Figure-4.1	Gas chromatogram of the TMS-derivertized aglycone	72
Figure-4.2	Mass spectrum of the TMS-derivertized aglycone	73
Figure-4.3	Mass spectrum of the TMS-derivertized β -sitosterol	74
Figure-4.4	ESI-MS spectrum of F _B	76
Figure-4.5	ESI-MS spectrum of F _B	77
Figure-4.6	Gas chromatogram of methylated F _B , by Hakomori method	79
Figure-4.7	Mass spectrum of 1,5-di- <i>O</i> -acetyl-6-deoxy-2,3,4-tri- <i>O</i> -methylhexitol	80
Figure-4.8	Mass spectrum of 1,2,4,5-tetra- <i>O</i> -acetyl-3,6-di- <i>O</i> -methylhexitol	81
Figure-4.9	¹³ C – NMR spectrum of F _B	83
Figure-4.10a	Expansion of ¹ H-NMR spectrum of F _B (4.20 ppm-5.50 ppm)	84
Figure-4.10b	Expansion of ¹ H-NMR spectrum of F _B (0.70 ppm-2.70 ppm)	85
Figure-4.11	HMQC spectrum of F _B	86

Figure-4.12	ESI-MS spectrum of F _D	87
Figure-4.13	ESI-MS spectrum of F _D	88
Figure-4.14	Gas chromatogram of methylated F _D , by Hakomori method	90
Figure-4.15	Mass spectrum of 1,4,5- tri- <i>O</i> -acetyl- 2,3,6- tri- <i>O</i> -methylhexitol	91
Figure-4.16	Mass spectrum of 1,5- di- <i>O</i> - acetyl- 6- deoxy- 2,3,4- tri- <i>O</i> -methylhexitol	92
Figure-4.17a	Expansion of ¹ H-NMR spectra of F _D (4.20 ppm – 5.50 ppm)	94
Figure-4.17b	Expansion of ¹ H – NMR spectrum of F _D (0.65 ppm – 2.65 ppm)	95
Figure-4.18	HSQC spectrum of F _D	96
Figure-4.19	Gas chromatogram of methylated F _F , by Hakomori method	97
Figure-4.20	Mass spectrum of 1,5-di- <i>O</i> -acetyl-2,3,4,6-tetra- <i>O</i> -methylhexitol	98
Figure 4.21a	Expansion of ¹ H-NMR spectrum of F _F (3.10 ppm- 5.57 ppm)	100
Figure-4.21b	Expansion of ¹ H-NMR spectrum of F _F (0.70 ppm- 3.20 ppm)	101
Figure-4.22	¹³ C – NMR spectrum of F _F	102
Figure-4.23	HSQC spectrum of F _F	103
Figure-4.24	ESI-MS spectrum of F _N and F _E	104
Figure-4.25	ESI-MS spectrum of F _E	105
Figure-4.26	Densitometric scan of Naththandiya – 1 PFP	112

Figure-4.27	The visible spectrum of the carotenoids from Polonnaruwa-2 PFP	114
Figure-5.1	The structure of the aglycone	130
Figure-5.2	The structure of F _B	131
Figure-5.3	The structure of F _D	133
Figure-5.4	The structure of F _F	134

List of Plates

Plate-1.1	A juvenile palmyrah tree	2
Plate-1.2	An adult palmyrah grove	2
Plate-1.3	A mature female inflorescence	4
Plate-2.1	Some products of palmyrah leaf	8
Plate-3.1	Palmyrah fruit pulp enmeshed in fibre	44
Plate-3.2	Extracted palmyrah fruit pulp	44
Plate-3.3	MPLC apparatus	49
Plate-4.1	Morphological type- I	107
Plate-4.2	Morphological type- II	107
Plate-4.3	Morphological type- III	108
Plate-4.4	Morphological type- IV	108

Abbreviations

AOAC	Association of Official Analytical Chemists
¹³C-NMR	Carbon Nuclear Magnetic Resonance spectrometry
EI/MS	Electron Impact - Mass Spectrometry
ESI-MS	Electron Spray Ionization - Mass Spectrometry
FAB/MS	Fast Atom Bombardment - Mass Spectrometry
F-I	Flabelliferin tetraglucoside in Kalpitiya
F-II	Bitter flabelliferin tetraglycoside
F_B	Anti bacterial flabelliferin triglycoside
F_C	Inactive flabelliferin triglycoside
F_D	Inactive flabelliferin diglycoside
F_E	New flabelliferin
F_F	New flabelliferin
F_M	New flabelliferin
F_N	New flabelliferin
F_O	New flabelliferin
GC	Gas Chromatography
Glc	Glucose
¹H-NMR	Proton Nuclear Magnetic Resonance spectrometry
HMQC	Heteronuclear Multiple Quantum Coherence
HSQC	Heteronuclear Single Quantum Coherence
MPLC	Medium Pressure Liquid Chromatography
MW	Molecular Weight
PFP	Palmyrah fruit pulp
R_f	Retardation Factor
Rha	Rhamnose
TMS	Tetra Methyl Silane
TLC	Thin Layer Chromatography

Acknowledgements

I take this opportunity to express my heartiest gratitude to my supervisor, Prof. E. R. Jansz (Department of Biochemistry, Faculty of Medical Sciences, University of Sri Jayawardenepura), who guided me, advised me and always encouraged me in this work. His own enthusiasm and commitment had been a great inspiration enabling me to successfully complete this work through many hardships. I am eternally grateful to him for his invaluable contribution towards my success.

I extend my warmest gratitude to Prof. A. M. Abeysekera (Department of Chemistry, Faculty of Applied Sciences, University of Sri Jayawardenepura), also my supervisor for his constant support, guidance and invaluable advice in this work.

I am indebted to Prof. Peter Baeckstrom (Department of Organic Chemistry, Royal Institute of Technology, Stockholm, Sweden), for his expertise and all the support in MPLC separations and also Prof. Per-Erik Jansson (Analytical Unit, Karolinska Institute, Huddinge, Sweden), for his professionalism, invaluable advice and guidance in obtaining spectral data.

I express my gratitude to Dr. S. Jayasekara and Dr. M. Thammitiyagoda (Animal Section, Medical Research Institute, Colombo) for their constant support and guidance in animal studies and also the staff of the Animal Section, Medical Research Institute, Colombo, during this time for their kind assistance.

I am pleased to acknowledge the contribution of staff of the Biochemistry laboratory, (Faculty of Medical Sciences, University of Sri Jayawardenepura) and the Research laboratory (Faculty of Applied Sciences, University of Sri Jayawardenepura) and all those who worked with me in the laboratory during this time for their willing assistance.

My special thanks go to Dr. (Mrs) Malin Akerblom, Director International Program In Chemical Sciences (IPICS), Uppsala University, Sweden for providing the opportunity to conduct research work at the Royal Institute of Technology, Stockholm, Sweden and Karolinska Institute, Huddinge, Sweden.

I am grateful to Dr. (Mrs) S. Yatawara (Department of Chemistry, Faculty of Applied Sciences, University of Sri Jayawardenepura), Mr. J. K. Nikawala (Department of Chemistry, Faculty of Applied Sciences, University of Sri Jayawardenepura and Palmyrah Development Board, Colombo) for their contribution in completion of this work.

Financial Assistance provided by National Science Foundation (NSF) (RG/99/C/3) and International Program In Chemical Sciences (IPICS) (SRI:07) are gratefully acknowledged.

Finally, I thank my parents and my husband for their constant support extended to me in achieving my goals and this is dedicated to them.

Abstract

Palmyrah fruit pulp (PFP) has a total potential production of 15- 20 k tonnes. annum⁻¹. However, the bulk of PFP goes to a waste on account of: (1) inadequate basic knowledge in processing and (2) the presence of a bitter principle and bioactive factors now collectively known as flabelliferins. These are steroidal saponins. The overall objective of this study was to collect chemical data of flabelliferins from PFP to promote its wider utilization. There are two possible major avenues for utilization: (1) debittering the fruit pulp to give jams, cordials etc., (2) fermenting the fruit pulp to obtain potable alcohol.

The bitter flabelliferin, F-II, a tetraglycoside is an inhibitor to the Na⁺/K⁺ pump. The triglycoside F_B is a microbial inhibitor (yeast and selected bacteria). The triglycoside F_C and the diglycoside F_D are inactive flabelliferins. F-I was reported to be a tetraglucoside. In order to support the utilization of PFP, it was important to study the flabelliferin profiles of varying fruit pulps and attempt to correlate them to an easily distinguishable feature of the fruit or the fruit pulp so as to aid in the selection of the best mode of processing for utilization.

The study showed that flabelliferin profiles of specimens collected varied considerably in morphology. There was a common type, type- I (size, medium to big; colour, black; pericarp, rough with brown longitudinal striations; distal side, black) and three other types, type- II, type- III and type- IV. There was no correlation between flabelliferin profile and morphological type, colour of fruit pulp, carotenoid content (total absorbance)

and the carotenoid present (λ_{\max}). As expected bitter fruit pulps contained large amount of F-II. There was great diversity in flabelliferin profile ranging from 2 flabelliferins (for example, Chilaw) to more than 10 flabelliferins (for example, Polonnaruwa). There appeared to be a correlation with location but this line of study was not pursued.

The diversity of flabelliferin profiles made previously documented procedures of separation not universally applicable to all PFP's. When the flabelliferin mixture is complex (for example, the Polonnaruwa sample containing at least 10 flabelliferins) a better separation technique was needed. Therefore other procedures; solvent gradient chromatography, chromatotron, selective solvent extraction and medium pressure liquid chromatography (MPLC) were employed. MPLC was the most successful technique and flabelliferins could be separated from the most complex mixtures and also from a specific fluorescent flabelliferin-binding agent. However, depending on flabelliferin profile, other techniques had specific value.

The flabelliferin F_B (antimicrobial triglycoside) and the flabelliferin F_D (inactive diglycoside) and three new flabelliferins (F_N , triglycoside of MW 884; F_E , diglycoside of MW 738 and F_F , monoglucoside of MW 579) were separated from pooled specimens of PFP collected from Ampara, Anamaduwa, Polonnaruwa and Mannar. Starting from 200 g of PFP, 88.8 mg of F_B , 52.3 mg of F_D , 7.8 mg of F_E , 30.6 mg of F_F and 5.6 mg of F_N were isolated by an MPLC technique.

Starting from 200g of PFP from Jaffna, 300mg of F-II was isolated using a chromatotron. Selective solvent extraction using ethyl acetate from a methanol extract is a simple method of isolating F_D from flabelliferin profiles containing triglycosides and tetraglycosides. A technique based on direct MPLC (without methanol extraction, petroleum ether cleaning, acetone extraction and dry cellulose chromatography) was worked out to separate not only the flabelliferins in their pure state but also the carotenoids and the free sugars in PFP. This has the advantage of not subjecting materials to heat and usage of a lesser amount of chemicals. In addition it is less time consuming and gives a yield 2.4 fold that of the indirect method of isolation.

The flabelliferins were hydrolyzed by trifluoro acetic acid (0.1M) and the aglycone was confirmed as β -sitosterol by GC/EI/MS studies of the trimethyl silyl derivative and also by ¹H-NMR and ¹³C-NMR.

Elucidation of the structure of F_B was important due to its potential for use as an antimicrobial agent. The F_B was analyzed by Hakomori method followed by GC/EI/MS and also by NMR. It showed that the sugar chain was linked to β - sitosterol by the anomeric C of glucose (β configuration), which is linked to two rhamnoses (both were of α anomeric configuration) by 1 \rightarrow 2 and 1 \rightarrow 4 linkages. F_B therefore has a branched glycosidic moiety. Similarly, the structure of F_D (diglycoside) showed that the sugar chain was linked to β - sitosterol by the anomeric C of glucose. Glucose is linked to a rhamnose by α -1 \rightarrow 4 linkages. The anomeric C of glucose (β configuration) was linked to the β -

sitosterol of F_F (monoglucoside). Though the linkage positions of F_E, and F_N could not be determined due to insufficient data. It was found that in F_N (MW 884), the carbohydrate moiety consists of two glucoses and a rhamnose with a glucose terminus. The sugar moiety is attached to the sapogenin (β - sitosterol) by the other glucose. Similarly, F_E (MW 738) showed a carbohydrate moiety of two glucoses.

The last part of the study concerned the effect of debittering with naringinase on the nutritive status of ICR mice. Results showed that incorporation of bitter PFP at 10 % level in WHO recommended standard rat/ mice breeding feed, reduced weight gain significantly compared to the control (p=0.029). Debittering of PFP reversed the effect (p=0.88) with respect to control. As debittering not only hydrolyzed F-II but also F_B, this was not proof that F-II was the causative agent for reducing weight gain. Using data from the morphological study, two special natural PFP's were detected. One contained F-II but not F_B (bitter PFP) and the other contained only F_B and not F-II (non- bitter PFP). Results showed that the bitter PFP reduced weight gain compared to control (p=0.021) but the non-bitter increased weight gain compared to control (p=0.014). It is concluded that, provided F-II is absent, PFP at 10 % level does not have a weight reducing effect. On the contrary it appears to be growth promoting. This may be due to its carotenoid content.

The Diversity, Bioactivity, Chromatographic and Spectroscopic studies of palmyrah (*Borassus flabellifer* L.) fruit pulp

Darshika Dilleny Ariyasena

ABSTRACT