

**ASPECTS OF THE CHEMISTRY AND
ANTIMICROBIAL ACTIVITY OF
FLABELLIFERINS OF PALMYRAH
FRUIT PULP**

By

Janaka Keerthi Nikawala

M.Phil. 2000


**ASPECTS OF THE CHEMISTRY AND ANTIMICROBIAL
ACTIVITY OF FLABELLIFERINS OF PALMYRAH FRUIT PULP**

By

JANAKA KEERTHI NIKAWALA

Thesis submitted to the University of Sri Jayewardenepura for the award of the degree
Master of Philosophy in Applied Chemistry

The work described in this thesis
was carried out by me under the
supervision of Prof. E. R. Jansz,
Prof. A. M. Abeysekera and Dr.
(Mrs) S. C. Wijeyaratne and a
report on this has not been
submitted to any University for
another degree.


.....
J. K. Nikawala
26/06/2000



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.

Prof. E. R. Jansz

Prof. A. M. Abeysekera

Dr. (Mrs.) S. C. Wijeyaratne

TABLE OF CONTENTS

	page
<i>List of Tables</i>	v
<i>List of Figures</i>	vi
<i>List of Plates</i>	viii
ABBREVIATIONS	ix
ACKNOWLEDGEMENTS	x
ABSTRACT	xii
1. INTRODUCTION	1
1.1 Background, Justification and Scope of the thesis	3
2. REVIEW OF LITERATURE	4
2.1 Description of plant	4
2.2 Utilizable parts of the plant	10
2.2.1 Roots	10
2.2.2 Stem (trunk)	12
2.2.3 Leaves	12
2.2.4 Inflorescence	14
2.2.5 Seeds and shoot	14
2.2.6 Neera	15
2.2.7 Fruits	16
2.2.7.1 Young fruits	16
2.2.7.2 Ripe fruits	18
2.2.7.3 Palmyrah fruit pulp	18
2.2.8 Products from fruit pulp	24

2.3 Bitter compounds	25
2.3.1 The sensation of bitterness	25
2.3.2 Some bitter compounds	25
2.3.3 Factors affecting bitterness	26
2.4 Bitter principle in palmyrah fruits	26
2.5 Methods of debittering fruits and vegetables	27
2.6 Past work on Palmyrah fruit pulp saponins and steroids	28
2.7 Use of spectroscopy in a saponin analysis	29
2.7.1 NMR	30
2.7.2 FAB-MS	30
3. MATERIALS AND METHODS	31
3.1 Collection of palmyrah fruits	31
3.2 Extraction and storage of the fruit pulp	31
3.3 Extraction of crude flabelliferins	34
3.3.1. Alcoholic extraction	34
3.3.2. Aqueous acid extraction	35
3.4 Extraction of flabelliferins from fermented palmyrah fruit pulp	36
3.4.1 Without added yeast	36
3.4.2 Use of yeast	37
3.5 Concentration of flabelliferins	38
3.6 Purification of flabelliferins	39
3.6.1 Acetone extraction	39
3.6.2 Dry cellulose chromatography	39
3.6.3 Flash chromatography	39
3.6.4 MPLC	40
3.6.4.1 Apparatus	40
3.6.4.2 Packing materials	40
3.6.4.3 Column fitting	43

3.6.4.4	Dry packing column procedure	44
3.6.4.5	Sample application	45
3.6.4.6	Pre-adsorption method	45
3.6.4.7	Flow rates	47
3.6.4.8	Preparation of the step-wise gradient solvents	47
3.7	TLC densitometry	49
3.8	Haemolysis	49
3.9	Froth test	50
3.10	Effect of flabelliferins on alcoholic fermentation	50
3.11	Effect of flabelliferins on bacterial growth	52
3.12	Effect of flabelliferins on yeast growth	52
3.12.1	Composition of the synthetic medium	53
3.13	Debittering of flabelliferins	55
3.14	Isolation of debittered flabelliferins	56
3.15	Bioactivity of debittered flabelliferins	56
3.16	Spectroscopic studies	57
3.16.1	FAB/MS	57
3.16.2	Mass Spectrometry	57
3.16.3	NMR	57
4.	RESULTS	58
4.1	Extraction and concentration of crude flabelliferins	58
4.2	Purification of flabelliferins	58
4.3	Bitterness	59
4.4	Haemolysis	60
4.5	Froth test	60
4.6	FAB/MS data	60
4.7	Quantification	69

4.8	Studies on growth of yeast	75
4.9	Effect on alcoholic fermentation	75
4.10	Effect on bacterial growth	85
4.11	Debittering of flabelliferins	95
4.12	Isolation of debittered flabelliferins	95
4.12.1	Isolation of free steroid	95
4.13	Bioactivity of debittered flabelliferins	97
4.14	Haemolysis by F _X and F _Y	97
4.15	Spectroscopic studies	98
4.15.1	F _X	98
4.15.2	F _Y	98
4.16	Structure of steroid	104
4.17	Net results of Naringinase debittering	104
5.	DISCUSSION	108
6.	CONCLUSION	124
7.	REFERENCES	125
8.	PUBLICATIONS AND COMMUNICATIONS FROM THIS STUDY	130
9.	APPENDICES	131

LIST OF TABLES

Table 1.1	Palmyrah population in Sri-Lanka	5
Table 2.1	Composition of palmyrah fruit pulp (PFP)	19
Table 2.2	Mineral composition of PFP	20
Table 2.3	Sugar composition of PFP	21
Table 2.4	Sugars of PFP	21
Table 4.1	R _f and bitterness of flabelliferins	59
Table 4.2	Release of haemoglobin from human red blood cells	61
Table 4.3	Results of froth test	62
Table 4.4	Summary of data from FAB/MS and interpretation	63
Table 4.5	Summary of effect of flabelliferins on fermentation efficiency	84
Table 4.6	Effect of crude flabelliferins on alcoholic fermentation	85
Table 4.7	Test for anti-bacterial action	86
Table 4.8	Anti-bacterial activity of F _B	87
Table 4.9	Haemolysis by debittered flabelliferins	97
Table 4.10	Comparison of F _C and F _X	99
Table 4.11	Assigned ¹ H and ¹³ C chemical shifts for stigmast- 5en- 3βol (24αEt)	107
Table 5.1	Molecular weights of flabelliferins	111
Table 5.2	Comparison of ¹³ C-NMR data	120
Table 5.3	Summary of characteristics of F _B , F-II, F _C & F _X	121
Table 5.4	Types and possible end use of PFP.	122

LIST OF FIGURES

Figure 3.1	Alcohol extraction technique (from PFP)	34
Figure 3.2	Aqueous acid extraction technique (from PFP)	35
Figure 3.3	Extraction of flabelliferins from fermented PFP (no added yeast)	36
Figure 3.4	Extraction of flabelliferins from fermented PFP (added yeast)	37
Figure 3.5	Concentration of flabelliferins	38
Figure 3.6	The MPLC system	41
Figure 3.7	The standard curve for the Nelson Method	51
Figure 4.1	FAB/MS of Flabelliferin - F _B	64
Figure 4.2	FAB/MS of Flabelliferin - F _C	65
Figure 4.3	FAB/MS of Flabelliferin - F-II	66
Figure 4.4	FAB/MS of Flabelliferin - F _D	67
Figure 4.5	¹ HNMR spectrum of Flabelliferin - F-II	68
Figure 4.6	Densitometric standard curves for F-II, F _B , and F _D	70
Figure 4.7	A typical densitometric scan (horizontal) for F-II	71
Figure 4.8	A typical densitometric scan (horizontal) for F _B	72
Figure 4.9	A typical densitometric scan (horizontal) for F _D	73
Figure 4.10	A typical vertical scan of a crude mixture	74
Figure 4.11	Effect of F-II on growth of <i>Sacchromyces cerevisiae</i>	76
Figure 4.12	Effect of F _B on growth of <i>Sacchromyces cerevisiae</i>	77
Figure 4.13	Effect of F _C on growth of <i>Sacchromyces cerevisiae</i>	78
Figure 4.14	Effect of F _D on growth of <i>Sacchromyces cerevisiae</i>	79
Figure 4.15	Effect of F _B on alcoholic fermentation	80
Figure 4.16	Effect of F-II on alcoholic fermentation	81
Figure 4.17	Effect of F _C on alcoholic fermentation	82
Figure 4.18	Effect of F _D on alcoholic fermentation	83
Figure 4.19	Flabelliferin profile on TLC before and after debittering	96
Figure 4.20	FAB/MS of flabelliferin-F _X	100
Figure 4.21	¹ HNMR spectrum of flabelliferin-F _X	101
Figure 4.22	FAB/MS of flabelliferin-F _Y	102

Figure 4.23	^1H NMR spectrum of flabelliferin - F _Y	103
Figure 4.24	Mass Spectrum of steriod	105
Figure 4.25	^1H NMR spectrum of steriod	106
Figure 5.1	Stigmast-5en-3 β ol (24R)	108
Figure 5.2	Spirost-5en-3 β ol (24R)	109

LIST OF PLATES

PLATE 2.1	A Palmyrah grove	11
PLATE 2.2	A ten year old Palmyrah tree	13
PLATE 2.3	A ripe fruit from Kalpitiya	17
PLATE 3.1	A ripe fruit from Hambantota	32
PLATE 3.2	Palmyrah fruit pulp (Hambantota)	33
PLATE 3.3	Photograph of MPLC system of Baeckstrom	42
PLATE 3.4	Apparatus for removing last traces of solvents	46
PLATE 4.1	Effect of F_B on <i>Staphylococcus aureus</i>	88
PLATE 4.2	Effect of F_B on <i>Staphylococcus epidermidis</i>	89
PLATE 4.3	Effect of F_B on <i>Escherichia coli</i>	90
PLATE 4.4	Effect of F_B on <i>Pseudomonas aeruginosa</i>	91
PLATE 4.5	Effect of F_B on <i>Proteus rettigeri</i>	92
PLATE 4.6	Effect of F_B on <i>Acinetobacter calcoaceticus</i>	93
PLATE 4.7	Effect of F_B (in different concentrations) on <i>Staphylococcus aureus</i>	94

ABBREVIATIONS

AOAC	- Association of Official Analytical Chemists
PFP	- Palmyrah fruit pulp
F-I	- Flabelliferin tetraglucoside in Kalpitiya
F-II	- Bitter flabelliferin tetraglycoside
F _B	- Anti-microbial flabelliferin triglycoside
F _C	- Inactive flabelliferin triglycoside
F _D	- Inactive Flabelliferin diglycoside
F _X	- Low Rf naringinase flabelliferin product
F _Y	- High Rf naringinase flabelliferin product
FAB/MS	- Fast Atom Bombardment Mass Spectrometry
MW	- Molecular weight
¹ HNMR	- Proton Nuclear Magnetic Resonance
MPLC	- Medium Pressure Liquid Chromatography
Rf	- Retardation factor
Tlc	- Thin layer chromatography
NCTC	- National Collection Typed Cultures
rha	- rhamnose
glu	- glucose
TMS	- Trimethylsilyl

ACKNOWLEDGEMENTS

The successful completion of my post-graduate work was made possible by the efforts of many people. I take this opportunity to express my heartfelt gratitude to Prof. E.R. Jansz, my supervisor, who had been a tower of strength with his support and encouragement and proper guidance to complete my work successfully. It is my bounden duty to show my deep appreciation and gratitude to him. I am privileged to receive the supervision of Prof. Jansz who is a pioneer and an expert in bioorganic experimental research in Sri-Lanka.

I especially wish to express my deepest gratitude to my supervisor Prof. A. M. Abeysekera for his invaluable advice, kind guidance and professional expertise throughout my study, in spite of his other commitments.

I am deeply indebted to Dr.(Mrs.) S.C. Wijeyaratne, also my supervisor, for the constant support and encouragement she offered during the course of this investigation.

I am very much indebted to the Mr.A. M. Paciyathan, Chairman, Palmyrah Development Board for providing me the golden opportunity of undertaking this research project and also for granting me leave.

I extend my gratitude to NARESA (RG/95/C/13) for providing funds and Dr. (Mrs.) Malin Akerblom Director, International Programme In Chemical Sciences, Uppsala University, Sweden for providing facilities for experiments at Royal Institute of Technology, Stockholm, Sweden.

I am grateful to Prof. Peter Baeckstrom, Ass. Prof. (Mrs.) Ulla Jacobsson, Ass. Prof. (Mrs.) A. K. B. Karlsson and Prof. Per Erik Janssen for their guidance and assistance in various ways during my stay in Sweden.

To Dr. A. Amerasekera and Mr. Wimal Padmasiri I owe a deep debt of gratitude for the NMR interoperation at University of Colombo.

I also extend my thanks to Mr. R. Guhanesan, Research Officer, PDB for encouragement. Special thanks are extended to Dr. (Mrs.) S. Jayasekara and the staff of the Animal center, Medical Research Institute, and Mr. T. M. S. G. Tennekoon and Mr. S. Rangoda all the non-academic staff members of the laboratories in the departments of Biochemistry, Chemistry, and Botany of the University of Sri Jayewardenepura.

I extend my gratitude to Mrs. Oranee Jansz for proof reading.

I am forever grateful to my parents and wife who helped in ways which are too many to list.

ABSTRACT

Palmyrah fruit pulp is available in excess of 15 –20 Metric tons.per. annum. It is under-utilized due to a bitter principle, which had been tentatively identified as a saponin, that is a tetraglycoside of spirost-5en-3 β ol (24R). The aim of this study was to investigate the bitter principle and debittering of palmyrah fruit pulp obtained from Hambantota.

The flabelliferins of palmyrah fruit pulp collected from Hambantota were isolated by methanol extraction, cleaned with petroleum ether and extracted into acetone, followed by dry cellulose chromatography and flash chromatography. Four Flabelliferins were obtained and were called F-II, F_B, F_C, and F_D. F-II the bitter compound was confirmed as saponin tetraglycoside M.W. 1030 with a rha. terminus. This saponin showed average haemolysis, slight foam stabilization and was an inhibitor of yeast at 250 μ g/ml. Also isolated were F_B and F_C saponin triglycosides M. W. 868 with a rhamnose terminus. Both were haemolytic and foam stabilizing (more by F_B), F_B was also highly active against growth of yeast and alcoholic fermentation by *Saccharomyces cerevisiae* strain S11-F₃ and six strains of bacteria. F_C a saponin triglycoside did not show such bio-activity. F_D was a saponin diglycoside that also did not show bio-activity.

The crude flabelliferins could be debittered by naringinase which yielded two saponin spots termed F_X and F_Y. F_X was a triglycoside possibly identical or very similar to F_C. F_Y was an impure mixture containing at least 3 to 4 compounds. On separation by MPLC, one of these compounds was a steroid M.W. 414. The MS and NMR spectra of the steroid were consistent with stigmast-5 en - 3 β ol (24 α Et).

The other compound F_Y probably arose from F-II, F_B and F_D, (smaller glycosides). It is considered that debittering not only hydrolyses F-II but also F_B, thus destroying both bitterness and anti-microbial activity. In addition it is possible to debitter some samples of palmyrah fruit pulp with a cheap enzyme heat stable α - amylase and mixture of amylo glucosidase and pullulanase.