

**Analysis and standardization of an Ayurvedic medicinal oil
(‘Pinda’ oil) and the measurement of partition coefficients of
some of its constituents**

By

Chandani Ranasinghe

Thesis submitted to the University of Sri Jayewardenepura for
the award of the Degree of Doctor of Philosophy in Chemistry

on 31st March 2011

DECLARATION

The work described in this thesis was carried out by me under the supervision of Prof. A.M. Abeysekera (Department of Chemistry, University of Sri Jayewardenepura) and Prof. G.M.K.B. Gunaherath (Department of Chemistry, The Open University of Sri Lanka) and a report on this has not been submitted in whole or part to any university or any other institution for another Degree/Diploma.


.....

Chandani Ranasinghe

DECLARATION

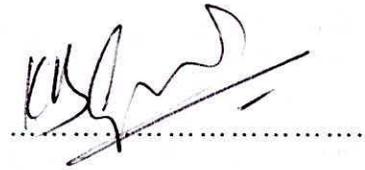
We certify that the above statement made by the candidate is true and that this thesis is suitable for submission to the University for the purpose of evaluation.

Supervisors:



Prof. A.M. Abeysekera

Date:.....19/11/2012



Prof. G.M.K.B. Gunaherath

Date:.....19/11/2012

I certify that the candidate has incorporated all corrections, amendments and additions recommended by the examiners.

A. M. Abeysekera
.....

Prof. A. M. Abeysekera

22/12/2012
.....

Date

TABLE OF CONTENTS

Tables, figures and schemes	v
Acknowledgements	xi
Abstract	xiii
Abbreviations	xvi
1. INTRODUCTION	1
1.1 Globalization of Ayurveda	1
1.2 Ayurvedic drug industry	4
1.3 Standardization of Ayurvedic drugs	6
1.3.1 Approaches to standardize Ayurvedic drugs	7
1.3.2 Currently available methods for standardization of herbal drugs	8
1.3.3 Chemometric evaluation of herbal drugs	11
1.3.4 Therapeutic standardization of herbal drugs	12
1.4 Ayurvedic medicinal oils ('Thaila')	12
1.4.1 'Pinda' oil	14
1.4.2 Chemistry of raw materials used in 'Pinda' oil and the biological activities of their constituents	17
1.5 Partition coefficients (P) of compounds	30
1.5.1 Measurement of log P	32
1.5.2 Applicability of predicted log P values	35
1.6 Work described in this thesis	36

2. MATERIALS AND METHODS	37
2.1 General experimental procedures	37
2.2 Isolation and characterization of secondary plant metabolites of plant raw materials of 'Pinda' oil	39
2.2.1 Isolation and characterization of constituents of <i>R. cordifolia</i>	40
2.2.2 Isolation and characterization of constituents of <i>G. glabra</i>	46
2.2.3 Isolation and characterization of constituents of <i>C. buchanani</i>	51
2.2.4 Isolation and characterization of constituents of sesame oil	54
2.3 Qualitative analysis of 'Pinda' oil for its major constituents	56
2.3.1 Laboratory preparation of 'pseudo-medicinal' oils	57
2.3.2 Partitioning of oils	57
2.3.3 Identification of secondary plant metabolites in fractions of oils	58
2.4 Standardization of 'Pinda' oil	59
2.4.1 Quantitative extraction of anthraquinones from 'Pinda' oil	59
2.4.2 HPLC analysis of 'Pinda' oil	60
2.4.3 Determination of the concentrations of anthraquinones in 'Pinda' oil	61
2.4.4 Validation of the HPLC method	63
2.4.5 Quantitative analysis of anthraquinones in market samples 'Pinda' oil	64
2.4.6 Statistical analysis of the results	65
2.5 Study of the industrial preparation process of 'Pinda' oil	65
2.5.1 Industrial preparation process of 'Pinda' oil	65
2.5.2 Sampling	66

2.5.3 Analysis	67
2.6 Evaluation of the effect of the aqueous extraction step on the composition of 'Pinda' oil	67
2.7 Determination of log P [log (partition coefficient)] of some constituents of plant materials used to make 'Pinda' oil	68
2.7.1 Determination of log P by HPLC method	68
2.7.2 Determination of log P of purpurin by the shake flask method	70
2.7.3 Determination of log P by calculation methods	73
3. RESULTS AND DISCUSSION	74
3.1 Isolation and characterization of major constituents of <i>R. cordifolia</i>	75
3.2 Isolation and characterization of major constituents of <i>G. glabra</i>	87
3.3 Isolation and characterization of major constituents of <i>C. buchanani</i>	95
3.4 Isolation and characterization of major constituents of sesame oil	102
3.5 Qualitative analysis of 'Pinda' oil for its major constituents	106
3.6 TLC profiles of 'Pinda' oil	109
3.7 Standardization of 'Pinda' oil	113
3.8 Quantitative extraction of anthraquinones from 'Pinda' oil	119
3.9 Analysis of 'Pinda' oil by RP-HPLC	121
3.9.1 Method development	121
3.9.2 Validation of the method	122
3.9.3 Analysis of market samples of 'Pinda' oil	129
3.10 Studies on the industrial preparation process of 'Pinda' oil	133
3.11 Evaluation of the effect of the aqueous extraction step on the composition of 'Pinda' oil	137

3.12 Determination of the partition coefficients (log P) of constituents of plant material used to make 'Pinda' oil	139
3.12.1 Determination of log P by HPLC method	139
3.12.2 Determination of log P by shake flask method	144
3.12.3 Comparison of calculated log P values with experimentally determined values	147
4. CONCLUSION	148
5. REFERENCES	149
6. APPENDICES	168

TABLES, SCHEMES AND FIGURES

LIST OF TABLES

Table 1.1: Some natural products used in modern drugs	3
Table 1.2: Raw materials and uses of different types of 'Pinda' oil	14
Table 1.3: Uses of <i>Rubia cordifolia</i> in different medicinal systems	18
Table 1.4: Biological activities shown by constituents and extracts of <i>Rubia cordifolia</i>	20
Table 1.5: Biological activities of constituents/extracts of <i>Glycyrrhiza glabra</i>	23
Table 1.6: Biological activities of constituents/extracts of <i>Cryptolepis buchanani</i>	26
Table 1.7: Some examples for different software developed for log P estimation	35
Table 2.1: λ_{\max} of the anthraquinones and their retention times in acetonitrile:1% aqueous formic acid (65:35)	61
Table 2.2: Concentrations of the standards (alizarin, xanthopurpurin, purpurin and rubiadin)	63
Table 2.3: Chronological order of steps of manufacturing process of 'Pinda' oil and withdrawal of samples	66
Table 2.4: Reference compounds used to construct the calibration curve and their log P values (at 25 °C)	69
Table 2.5: Constituents of plants whose retention times were measured for log P calculation	70
Table 2.6: Concentrations of purpurin solutions	71
Table 3.1.1: Major compounds isolated from the methanol extract of stems and	

roots of <i>R. cordifolia</i> and some of their physical properties	76
Table 3.1.2: NMR (600 MHz, DMSO- <i>d</i> ₆) data for 1-hydroxy-2-methyl-9,10-anthraquinone	77
Table 3.1.3: NMR (DMSO- <i>d</i> ₆ , 600 MHz) assignments for munjistin methyl ester	81
Table 3.2.1: Major compounds isolated from stems of <i>G. glabra</i> their physical appearance and the melting points	88
Table 3.2.2: NMR (DMSO- <i>d</i> ₆ , 600 MHz) data for betulinic acid	90
Table 3.2.3: NMR data (DMSO- <i>d</i> ₆ , 600 MHz) for formononetin	92
Table 3.3.1: Major compounds isolated from methanol extract of stems of <i>C. buchanani</i> and some of their physical properties	96
Table 3.3.2: NMR (DMSO- <i>d</i> ₆ , 300 MHz) data of isovanillin	98
Table 3.3.3: ¹³ C NMR shifts (125 MHz, CDCl ₃) of α-amyrin acetate and lupeol acetate	101
Table 3.4.1: NMR (DMSO- <i>d</i> ₆ , 600 MHz) data for sesamolin	104
Table 3.4.2: NMR (DMSO- <i>d</i> ₆ , 600 MHz) data for sesamin	106
Table 3.6.1: Major components isolated/detected from raw materials and their availability in ‘Pinda’ oil	110
Table 3.9.1: Replicate measurements of peak areas of anthraquinones and their RSD	126
Table 3.9.2: Replicate measurements of concentrations of anthraquinones	127
Table 3.9.3: Results of addition - recovery experiments	127
Table 3.9.4: Concentrations of four anthraquinones in six market samples of ‘Pinda’ oil	129
Table 3.10.1: Concentrations of four anthraquinones in samples drawn at	

different times during heating process after addition of sesame oil in the manufacture of 'Pinda' oil	135
Table 3.11.1: Comparison of oils prepared according to the two methods (a) and (b) with respect to the peak areas of anthraquinones	138
Table 3.12.1: log P values of the compounds isolated from the plant raw materials of 'Pinda' oil	142
Table 3.12.2: log P values of purpurin according to the 'slow-stirring' method	146

LIST OF FIGURES

Fig. 1.1: Examples for phytochemical leads for modern day drugs	4
Fig. 1.2: Worldwide trade in herbal remedies	5
Fig. 1.3: Plant raw materials used in the preparation of 'Pinda' oil	16
Fig. 1.4: Industrial preparation of 'Pinda' oil in cauldrons of 1000 L capacity	16
Fig. 1.5: Radical scavenging activity of anthraquinones	21
Fig. 3.1.1: ^1H NMR spectrum of 1-hydroxy-2-methyl-9,10-anthraquinone	78
Fig. 3.1.2: ^1H NMR spectrum of purpurin 2 methyl ether	79
Fig. 3.1.3: ^1H NMR spectrum of munjistin methyl ester	80
Fig. 3.1.4: gHMBC correlations of munjistin methyl ester	81
Fig. 3.1.5: ^1H NMR spectrum of rubiadin	82
Fig. 3.1.6: ^1H NMR spectrum of xanthopurpurin	84
Fig. 3.1.7: ^1H NMR spectrum of purpurin	85
Fig. 3.2.1: ^1H NMR spectrum of formononetin	92
Fig. 3.6.1: TLC of 'Pinda' oil to detect triterpenes present in 'Pinda' oil	111
Fig. 3.6.2: TLC of 'Pinda' oil to detect anthraquinones present in 'Pinda' oil	111
Fig. 3.6.3: TLCs of 'Pinda' oil to detect a coumarin and flavonoids present in 'Pinda' oil	111
Fig. 3.7.1: Chemical structures of different sorbents used	116
Fig. 3.7.2: Possible intramolecular H-bonding in pseudopurpurin and H bonding involving free silanol groups of reversed phase silica gel	117
Fig. 3.8.1: Example for peak splitting - Sample: alizarin	121

Fig. 3.9.1: Chromatogram of an extract of 'Pinda' oil	122
Fig. 3.9.2: Standard curves for Alizarin, purpurin, rubiadin and xanthopurpurin	125
Fig. 3.9.3: Calculation of signal to baseline noise ratio with the help of peak heights	128
Fig. 3.9.4: Dendrogram for cluster analysis of market samples corresponding to single linkage with Euclidean distances	131
Fig. 3.9.5: Dendrogram for cluster analysis of market samples corresponding to complete linkage with Euclidean distances	131
Fig. 3.9.6: Dendrogram for cluster analysis of market samples corresponding to average linkage with Euclidean distances	131
Fig 3.10.1: Concentration of anthraquinones in oil phase during the heating process in the manufacture of 'Pinda' oil.	136
Fig. 3.11.1: Chromatograms (at 254 nm wave length) of laboratory prepared 'oil' samples	138
Fig. 3.12.1: Calibration curves of log P vs. log K of reference compounds	141
Fig. 3.12.2: Comparison of log P in two mobile phases	143
Fig. 3.12.3: Calibration graph for standard solutions of purpurin in <i>n</i> -octanol	146

LIST OF SCHEMES

Scheme 2.1: Scheme showing partitioning of methanol extract of plant material	40
Scheme 2.2: Scheme showing partitioning of 'Pinda' oil and 'pseudo-medicinal' oils	58

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Professor A.M. Abeysekera and Professor G.M.K.B. Gunaherath for the opportunity given to me to carry out this work under their careful supervision. I owe them very much for their guidance, constant support and valuable advice through extensive discussions. Their constructive criticism and comments during the writing up of the thesis had been of immense help.

My sincere thanks are due to Professor U.G. Chandrika, for providing me with the HPLC facility and teaching me how to operate the instrument. She extended her helping hand voluntarily at all times and provided me good company and lots of good ideas.

I appreciate the assistance given by Dr. Ranjala Ratnayake to obtain spectroscopic data and for the suggestions made to solve problems regarding HPLC. Her quick supply of polyamide enabled me carry out my research work uninterrupted.

The assistance given by Mr. Chitraka Wickramarachchi in statistical analysis of the data is gratefully acknowledged.

I wish to acknowledge the technical support given to me by Messers. Siripala Senadeera, Sagara Dias and Sisira Weerasinghe of the Department of Chemistry, University of Sri Jayewardenepura.

I gratefully acknowledge National Science Foundation for the financial support and Link Natural Products Limited for the supply of authenticated raw materials, 'Pinda' oil samples and for permitting me to study the industrial manufacturing process in their factory.

It is a pleasure to thank all my colleagues at The Open University of Sri Lanka, who supported me in numerous ways, with a special mention of Professor Sukumal Wimalasena who helped me in the final stages of preparing the thesis.

Finally I would like to thank my family for their understanding, patience and for being very supportive throughout the course of this study.

Analysis and standardization of an Ayurvedic medicinal oil ('Pinda' oil) and the measurement of partition coefficients of some of its constituents

Chandani Ranasinghe

ABSTRACT

'Pinda' oil is a potent and widely used Ayurvedic medicinal oil for the treatment of inflammations and a variety of dermatological conditions such as eczema, itchy skin, cracked skin and red skin. In the manufacture of 'Pinda' oil, the constituents of an aqueous extract of *Rubia cordifolia*, *Glycyrrhiza glabra*, and *Cryptolepis buchanani* are incorporated into sesame oil.

With the commercialization of Ayurvedic drug manufacture, the need for quality control and standardization of drugs has arisen to ensure the quality, potency and the efficacy of these drugs, for the benefit of the consumer.

Analysis and standardization of 'Pinda' oil was a challenging task due to the difficulty in obtaining a suitable extract of secondary plant metabolites incorporated into the oil devoid of fatty matter.

'Pinda' oil was subjected to liquid-liquid partitioning and TLC profiles ('fingerprints') of the fractions thus obtained were developed. The secondary plant metabolites incorporated in to the oil were identified with the help of the major compounds isolated from the three plants used to make 'Pinda' oil. The introduction to this thesis reviews the chemistry and biological activities of these three plants and a compilation of natural products isolated from them is included in Appendix 2.

The five anthraquinones pseudopurpurin, alizarin, purpurin, xanthopurpurin and rubiadin the triterpenoids β -sitosterol, lupeol acetate and α -amyrin acetate, the flavonoids liquiritigenin and isoliquiritigenin and the simple phenolic scopoletin were identified as the major constituents incorporated into 'Pinda' oil.

A phenolic extract comprising of the five anthraquinones was obtained by solid phase extraction of 'Pinda' oil using polyamide as the sorbent. Fats were removed by eluting with *iso*-octane. A RP-HPLC method was developed to quantify the anthraquinones present in this phenolic extract. The chromatograms were obtained by isocratic elution of the phenolic extract using acetonitrile : 1% formic acid in water (65:35) as the mobile phase. Peaks corresponding to the four anthraquinones alizarin, purpurin, xanthopurpurin and rubiadin displayed the required purity levels for quantification.

The precision and the accuracy of the above method were found to lie within the acceptable ranges. Therefore this method was considered a good method for standardizing different market samples of 'Pinda' oil in terms of the anthraquinones present in *Rubia cordifolia*. Analysis showed a wide variability among the samples indicating the need for standardization and quality control of the drug.

The industrial manufacturing process was studied in relation to the rates of incorporation of the four anthraquinones of concern into the oil. Xanthopurpurin showed a very slow incorporation into oil in the initial stage of heating while all four anthraquinones reached their concentration maxima by 46 hours of heating. The experiments done by direct extraction of raw material with oil showed lower concentration levels of anthraquinones of concern in the oil thus validated the need for the aqueous extraction step in the traditional preparation process of manufacture.

Measurement of partition coefficients (P) of the most abundant constituents of plant materials were done to study whether there was a correlation between the log P values and concentrations of compounds in the oil. No correlation could be observed.