

**Chemical and biological studies of the
inflorescence of *Cocos nucifera* L., used
in Ayurveda for the treatment of
menorrhagia**

by

Ekanayake Arachchige Charitha Priyadarshani

M. Phil.

2019

**Chemical and biological studies of the
inflorescence of *Cocos nucifera* L., used in
Ayurveda for the treatment of
menorrhagia**

by

Ekanayake Arachchige Charitha Priyadarshani

Thesis submitted to the University of Sri Jayewardenepura for the
award of the Degree of Master of Philosophy

Declaration of the Candidate

The work described in this thesis was carried out by me under the supervision of Dr. C. Padumadasa (principal supervisor), Prof. A. M. Abeysekera, Dr. B. Seneviratne, Dr. S. Padumadasa and Dr. M. G. Thammitiyagodage and report on this has not been submitted in whole or in part to any university or any other institution for another Degree / Diploma.

C. P. Ekanayake

Signature:.....

Date:.....

Declaration by the Supervisor

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.

Certified by:

1. Principal Supervisor : C. Padumadasa

Signature:

Date:

2. Supervisor : A. M. Abeysekera

Signature:

Date:

3. Supervisor : B. Seneviratne,

Signature:

Date:

4. Supervisor : M. G. Thammitiyagodage

Signature:

Date:

5. Supervisor : S. Padumadasa

Signature:

Date:

Certification by the supervisor

We certify that the candidate has incorporated corrections, additions and amendments recommended by the examiners in this version of the M. Phil. thesis.

Certified by:

1. Principal Supervisor : C. Padumadasa

Signature: Date:

2. Supervisor : A. M. Abeysekera

Signature: Date:

3. Supervisor : B. Seneviratne,

Signature: Date:

4. Supervisor : M. G. Thammitiyagodage

Signature: Date:

5. Supervisor : S. Padumadasa

Signature: Date:

Table of Contents

1. Introduction	1
2. Literature review	3
2. 1. <i>Cocos nucifera</i> L.	3
2. 1. 1. Classification of <i>Cocos nucifera</i> L.	4
2. 1. 2. Inflorescence of <i>Cocos nucifera</i> L.	6
2. 1. 3. Uses of <i>Cocos nucifera</i> L. in ethnobotanical medicine	8
2. 1. 4. Biological activities of <i>Cocos nucifera</i> L.	9
2. 1. 5. Phytochemistry of <i>Cocos nucifera</i> L.	11
2. 2. Proanthocyanidins	11
2. 2. 1. Structure of proanthocyanidins	12
2. 2. 2. Nomenclature of proanthocyanidins	22
2. 2. 3. Distribution of proanthocyanidins in the plant kingdom	23
2. 2. 4. Biological activities of proanthocyanidins	23
2. 2. 4. (i). Anti-oxidative activity of proanthocyanidins	23
2. 2. 4. (ii). Anti-bacterial and anti-viral properties of proanthocyanidins	25
2. 2. 4. (iii). Anti -cancer activity of proanthocyanidins	26
2. 2. 4. (iv). Cardio protective properties of proanthocyanidins	27
2. 2. 4. (v). Progestogenic activity of proanthocyanidins	28
2. 2. 5. Analytical methods to quantify proanthocyanidins	28
2. 2. 5. (i). Vanillin assay	28
2. 2. 5. (ii). DMAC [4-(dimethylamino)-cinnamaldehyde] method	29
2. 2. 5. (iii). Prussian blue assay and Folin Denis method	30
2. 2. 5. (iv). Protein precipitation method	31
2. 2. 5. (v). Acid butanol method	31
2. 2. 6. Structure elucidation of proanthocyanidins	35
2. 3. Toxicity studies of test materials	36
2. 3. 1. Importance of toxicity studies	36
2. 3. 2. Types of toxicity studies	37
2. 3. 2. (i). <i>In vitro</i> methods	37
2. 3. 2. (ii). <i>In vivo</i> methods	38

2. 3. 3. Animal models.....	40
2. 3. 4. Routes of test material administration	41
2. 3. 5. Biomarkers.....	42
2. 3. 6. Systemic toxicology studies.....	43
2. 3. 6. (i). Acute toxicity study	43
2. 3. 6. (i). (i). The median lethal dose (LD ₅₀)	43
2. 3. 6. (i). (ii). Main test and limit test.....	44
2. 3. 6. (ii). Subacute and chronic toxicity studies of test materials	45
2. 4. Plant toxins	51
2. 4. 1. Glycosides.....	53
2. 4. 1. (i). Cardiac glycosides	53
2. 4. 1. (i). (ii). Natural sources of cardiac glycosides	54
2. 4. 1. (i). (iii) Structure of cardiac glycosides	55
2. 4. 1. (i). (iv). Analytical methods for determination of cardiac glycosides	58
2. 4. 1. (i). (iv). Chemical tests for investigation of cardiac glycosides.	59
2. 4. 1. (i). (vi). Pharmacological actions of cardiac glycosides.....	63
2. 4. 1. (i). (vii). The structure activity relationships	66
2. 5. Menorrhagia.....	67
3. Materials and methods	69
3. 1. Instrumentation.....	69
3. 2. Chromatography	69
3. 3. Chemicals	70
3. 4. Plant material.....	70
3. 5. Statistical analyses.....	71
3. 6. Extraction, purification and characterization of ethyl acetate soluble proanthocyanidins (EASPA) of the immature inflorescence of <i>Cocos nucifera</i> L.	72
3. 6. 1. Prussian blue test	73
3. 6. 2. Acid catalyzed cleavage test.....	74
3. 7. Acute and subacute toxicity studies of the ethyl acetate soluble proanthocyanidins (EASPA) of immature inflorescence of <i>Cocos nucifera</i> L. in rats	74
3. 7. 1. Acute oral toxicity study.....	75
3. 7. 1. (i). Biochemical parameters and haematology	76
3. 7. 1. (ii). Histopathological studies	77

3. 7. 2. Subacute toxicity study	78
3. 7. 2. (i). Biochemical parameters and haematology	79
3. 7. 2. (ii). Histopathological studies	79
3. 8. Acute and subacute toxicity studies of the aqueous extract used in Ayurveda (AE) of immature inflorescence of <i>Cocos nucifera</i> L. in rats	80
3. 8. 1. Preparation of aqueous extract used in Ayurveda (AE) of the immature inflorescence of <i>Cocos nucifera</i> L.	80
3. 8. 2. Acute oral toxicity study	80
3. 8. 2. (i). Histopathological studies	81
3. 8. 3 Subacute toxicity study	81
3. 8. 3. (i). Histopathological studies	82
3. 8. 3. (ii). Biochemical parameters and haematology	82
3. 9. Investigation of the immature inflorescence of <i>Cocos nucifera</i> L. for cardiac glycosides	83
3. 9. 1. Investigation of the methanol extract of the immature inflorescence of <i>Cocos nucifera</i> L. for cardiac glycosides.....	83
3. 9. 2. Investigation of the aqueous extract used in Ayurveda (AE) of the immature inflorescence of <i>Cocos nucifera</i> L. for cardiac glycosides	84
3. 9. 2. (i). Keller- Killiani test.....	86
3. 9. 2. (ii). Libermann – Burchard test.....	87
3. 9. 3. Investigation of the immature inflorescence of <i>Cocos nucifera</i> L. for cardiac glycosides by thin layer chromatography (TLC)	88
3. 9. 4. Investigation of interferences of phenolics for the TLC analysis of cardiac glycosides.....	89
3. 10. Determination of proanthocyanidin content of the aqueous extract used in Ayurveda (AE) of the immature inflorescence of <i>Cocos nucifera</i> L.	90
3. 10. 1. Acid butanol assay	90
3. 10. 2. Preparation of the standard curve for the acid butanol assay	91
3. 11. Isolation of phenolic compounds of the immature inflorescence of <i>Cocos nucifera</i> L.	91
3. 11. 1. Separation of non-proanthocyanidin phenolics	91
3. 11. 2. Thin layer chromatography of NP	92
3. 11. 3. Preparative thin layer chromatography of NP	93

3. 11. 4. High performance liquid chromatography (HPLC) analysis of the isolated phenolic compound (X).	93
4. Results	95
4. 1. Extraction, purification and characterization of ethyl acetate soluble proanthocyanidins (EASPA) of the immature inflorescence of <i>Cocos nucifera</i> L.	95
4. 2. Acute and subacute toxicity studies of the ethyl acetate soluble proanthocyanidins (EASPA) of immature inflorescence of <i>Cocos nucifera</i> L. in rats	97
4. 2. 1. Acute toxicity study	97
4. 2. 2. Subacute toxicity study	102
4. 2. 2. (i). Mortality, general signs of toxicity and food and water consumption of rats	102
4. 2. 2. (ii). Biochemical parameters and haematology	103
4. 2. 2. (iii). Macropathology and relative organ weight (ROW) of rats	103
4. 2. 2. (iv). Histopathological studies	104
4. 3. Acute and subacute toxicity studies of the extract used in Ayurveda (AE) of the immature inflorescence of <i>Cocos nucifera</i> L. in rats	109
4. 3. 1. Acute toxicity study	109
4. 3. 2. Subacute toxicity study	113
4. 3. 2. (i). Mortality, general signs of toxicity and food and water consumption of rats	113
4. 3. 2. (ii). Biochemical parameters and haematology	114
4. 3. 2. (iii). Macropathology and relative organ weight (ROW) of rats	114
4. 3. 2. (iv). Histopathological studies	115
4. 4. Investigation of the immature inflorescence of <i>Cocos nucifera</i> L. for cardiac glycosides	120
4. 4. 1. Investigation of methanol and aqueous extract of immature inflorescence of <i>Cocos nucifera</i> L. for cardiac glycosides	120
4. 4. 2. Investigation of the immature inflorescence of <i>Cocos nucifera</i> L. for cardiac glycosides by thin layer chromatography (TLC)	124
4. 4. 3. Investigation of interferences of phenolics for the TLC analysis of cardiac glycosides	125
4. 5. Determination of the proanthocyanidin content of the Ayurvedic decoction of the immature inflorescence of <i>Cocos nucifera</i> L.	125
4. 6. Isolation of phenolic compounds of the immature inflorescence of <i>Cocos nucifera</i> L.	127

5. Discussion	131
5. 1. Extraction and purification of ethyl acetate soluble proanthocyanidins (EASPA) of the immature inflorescence of <i>Cocos nucifera</i> L.	131
5. 2 Acute and subacute toxicity studies of the ethyl acetate soluble proanthocyanidins (EASPA) of immature inflorescence of <i>Cocos nucifera</i> L. in rats.....	133
5. 3. Acute and subacute toxicity studies of aqueous extract used in Ayurveda (AE) of the immature inflorescence of <i>Cocos nucifera</i> L. in rats.....	140
5. 4. Investigation of the immature inflorescence of <i>Cocos nucifera</i> L. for cardiac glycosides	147
5. 4. 1. Investigation of the methanol extract of the immature inflorescence of <i>Cocos nucifera</i> L. for cardiac glycosides.....	147
5. 4. 2. Investigation of the Ayurvedic decoction (aqueous extract) of the immature inflorescence of <i>Cocos nucifera</i> L. for cardiac glycosides.....	152
5. 4. 3. Investigation of the immature inflorescence of <i>Cocos nucifera</i> L. for cardiac glycosides by thin layer chromatography (TLC).....	154
5. 4. 4. Investigation of interferences of phenolics for the TLC analysis of cardiac glycosides.....	155
5. 5. Determination of the proanthocyanidin content of aqueous extract used in Ayurveda (AE) of the immature inflorescence of <i>Cocos nucifera</i> L.....	156
5. 6. Isolation of phenolic compounds of the immature inflorescence of <i>Cocos nucifera</i> L.	159
6. Conclusions	161

List of Tables

Table 01. Effect of EASPA on average body weight in acute toxicity study.	98
Table 02. Effect of EASPA on percentage bodyweight gain in acute toxicity study (%).	99
Table 03. Effect of EASPA on serum haematological parameters in acute toxicity study.	99
Table 04. Effect of EASPA on serum biochemical parameters in acute toxicity study.	100
Table 05. Effect of EASPA on relative organ weight in acute toxicity study.	100
Table 06. Effect of EASPA on average body weight in subacute toxicity study.....	104
Table 07. Effect of EASPA on percentage body weight gain in subacute toxicity study.	104
Table 08. Effect of EASPA on average food intake in subacute toxicity study.	105
Table 09. Effect of EASPA on average water intake in subacute toxicity study.....	105
Table 10. Effect of EASPA on serum haematological parameters in subacute toxicity study.	106
Table 11. Effect of EASPA on serum biochemical parameters in subacute toxicity study.	107
Table 12. Effect of EASPA on relative organ weights in subacute toxicity study.	107
Table 13. Effect of AE on average body weight in acute toxicity study.	110
Table 14. Effect of AE on percentage body weight gain in acute toxicity study.....	110
Table 15. Effect of AE on average food intake in acute toxicity study.	110
Table 16. Effect of AE on average water intake in acute toxicity study.....	111
Table 17. Effect of AE on relative organ weight in acute toxicity study.....	111
Table 18. Effect of AE on average body weight in subacute toxicity study.....	115
Table 19. Effect of AE on percentage body weight gain in subacute toxicity study	115
Table 20. Effect of AE on average food intake in subacute toxicity study.....	116
Table 21. Effect of AE on average water intake in subacute toxicity study.	116
Table 22. Effect of AE on heamatologicala parameters in subacute toxicity study.....	117
Table 23. Effect of AE on biochemical parameters in subacute toxicity study.	118
Table 24. Effect of AE on relative organ weight in subacute toxicity study.	118
Table 25. Color change of different fractions of the immature inflorescence of <i>Cocos nucifera</i> L. with Keller Killiani, Libermann-Burchard, Kedde, Prussian blue and acid cleavage tests.....	121

List of Figures

Figure 01. The inflorescence of <i>Cocos nucifera</i> L.....	7	
Figure 02. Basic skeleton of diphenylpropanoids	13	
Figure 03. Principal naturally occurring flavan-3,4-diols.....	14	
Figure 04. Chemical structures of typical flavan-3-ol units.....	16	
Figure 05. Proanthocyanidin dimers showing B type and A type linkages.	19	
Figure 06. The reaction mechanism of the acid butanol assay	33	
Figure 07. Tetracyclic ring system of the steroid nucleus	56	
Figure 08. Cardenolide	Figure 09. Bufadienolide.....	57
Figure 10 . The chemical reaction of the Kedde test	60	
Figure 11. Reaction mechanism of Libermann-Burchard reaction.....	61	
Figure 12. Schematic diagram of different maturity stages of inflorescences within the coconut palm.	71	
Figure 13 Chemical structure of epicatechin units in EASPA.....	95	
Figure 14. Effect of EASPA on histomorphologies of vital organs in test group rats in acute toxicity study (H and E Stain, 100 x).	101	
Figure 15. Effect of EASPA on histomorphologies of vital organs in test group rats in subacute toxicity study (H and E Stain, 100 x).....	108	
Figure 16. Effect of AE on histomorphologies of vital organs in test group rats in acute toxicity study (H and E Stain, 100 x).....	112	
Figure 17. Effect of AE on histomorphologies of vital organs in test group rats in subacute toxicity study (H and E Stain, 100 x).....	119	
Figure 18 (a). Photographs on color change of different fractions of IC with Keller Killiani test.....	122	
Figure 19. TLC chromatograms of the methanolic extract of the IC free from phenolic compounds.	124	
Figure 20. TLC chromatogram of the low molecular weight phenolic fraction of the IC.	125	
Figure 21. The calibration plot of proanthocyanidins from the inflorescence of <i>Cocos nucifera</i> L.	126	
Figure 22 a. TLC chromatograms of non-proanthocyanidin phenolic fraction (NP) with the mobile phase I	128	
Figure 23. HPLC chromatogram of A	130	

Abbreviations

Abbreviation	Explanation
AE	Aqueous extract
ABTS	2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
ALP	Alkaline phosphatase
AOAC	Association of official analytical chemist
ALT	Alanine transaminase
AQSPA	Aqueous soluble proanthocyanidins
AST	Aspartate aminotransferase
CHL/IU cells	Mammalian cell cultures
COX	Cyclooxygenase
CPCSEA	Committee for the purpose of control and supervision of experiments on animals
CTA	Chloramine-trichloroacetic acid
EASPA	Ethyl acetate soluble proanthocyanidins
EDTA	Ethylene diamine tetra acetate
DP	Degree of polymerization
DMAC	4-dimethylamino-cinnamaldehyde
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	Ferric reducing ability of plasma
FLU-A	Influenza A
GC-MS	Gas chromatography couple to a mass spectrophotometer
GSPE	Seed extract of <i>Vitis vinifera</i>
HB	Haemoglobin

HPLC	High performance liquid chromatography
HCN	Prussic acid
HMB	Heavy menstrual bleeding
IDA	Iron deficiency anemia
LD ₅₀	Median lethal dose
LOX	Lipoxygenase
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
NMR	Nuclear magnetic resonance
NP	Non-proanthocyanidin phenolic fraction
NP-PEG	Natural product-polyethelene glycol
IUPAC	International union of pure and applied chemistry
LNG-IUS	Levonorgestrel releasing intrauterine system
PIV	Parainfluenza virus
RBC	Red blood count
RDW-CV	Red blood cell distribution width
RSV	Respiratory syncytial virus
SV-40	simian virus 40
SAM	Shoot apical meristem
SPSS	Statistical package for social sciences
THP-1	Human monocytic cell line
TLC	Thin layer chromatography
MCP-1	Monocyte chemo attractant protein 1
MPV	Mean platelet volume
VCAM-1	Vascular cell adhesion protein 1

OECD	Organization for economic co-operation and development
PCT	Procalcitonin
ROW	Relative organ weight
RS	Ring system
WBC	White blood count

Acknowledgement

This research project was financially supported by the Research Grant; ASP/01/RE/SCI/25, University of Sri Jayewardenepura, Sri Lanka and without it, this work would not have been a success.

I would like to express my heartiest gratitude to my principal supervisor, Dr. C. Padumadasa who consistently allowed this project to be my own work, but guided me in the correct path whenever she thought I needed it. As well I would like to thank my supervisor, Prof (Emeritus). A. M. Abeysekera, for his precious and patient guidance to me for this research project. I would like to thank, my supervisors, Dr. M. G. Thammitiyagodage, Dr. S. Padumadasa and Dr. B. Seneviratne for their continuous advices to carry out this project.

My sincere thank also goes to technical officers in Department of Chemistry, Faculty of Applied Sciences, University of Sri Jayewardenepura who always assisted to carry out my laboratory work.

I express my profound gratitude to my colleagues, Dr. Ureshani Karunaratne, Dr. Kalpani Rathnayake, Thiloka Kariyawasam, Udari Kodithuwakku and Dr. Dayangi Hemalika who were always there for me with helping hands. As well, I am grateful to my parents, sister and brother for their unfailing support and continuous encouragement throughout my years of study. Finally, I would like to thank my loving husband, Eng. Subhash Dananjaya, without whose infinite patience, continuous encouragement and constant support, this could never be completed.

Thank you.

Chemical and biological studies of the inflorescence of *Cocos nucifera* L., used in Ayurveda for the treatment of menorrhagia.

Ekanayake Arachchige Charitha Priyadarshani

ABSTRACT

Ayurvedic and traditional medical practitioners of Sri Lanka use the decoction of the immature inflorescence of *Cocos nucifera* L. variety aurantiaca for the treatment of menorrhagia. The extraction, purification, characterization and progestogenic effect of ethyl acetate soluble proanthocyanidins (EASPA) of this inflorescence have previously been reported. This finding is very significant as progestogens are widely used in the treatment of menorrhagia in western medicine.

EASPA, being a potential drug candidate in relation to treatment of menorrhagia was evaluated for its safety using acute and subacute toxicity studies in Wistar rats. Acute and subacute toxicity studies of EASPA were carried out according to OECD guidelines 423 and 407 respectively is reported herein. In the acute toxicity study, a single dose of EASPA (2000 mg/kg body weight) was orally administered to rats and monitored for 14 days. In the subacute toxicity study, rats were orally administered with EASPA daily for 28 days at doses of 1.75, 3.5, 7 and 14 mg/kg body weight. Rats in the acute or subacute toxicity study did not exhibit any mortality, clinical signs of toxicity, changes in haematological, biochemical and their histopathological investigations compared to those of control group rats. According to the results of acute toxicity, the LD₅₀ of EASPA was estimated to be greater than 2000 mg/kg body weight. Considering the results of subacute toxicity study, it is possible to suggest that the oral administration of EASPA daily for 28 days was well tolerated up to the dose, 14 mg/kg in rats.

Acute and subacute toxicity of aqueous extract used in Ayurveda (AE) of the immature inflorescence of *Cocos nucifera* L. for the treatment of menorrhagia was evaluated for its safety in Wistar rats in order to compare the toxicity results with those of the EASPA. Acute and subacute toxicity studies of AE was carried out similar to those of EASPA. Dose levels of 150, 300, 600 and 1200 mg/kg body weight of AE were used for the subacute toxicity study of AE. All treated rats in both acute and subacute toxicity studies did not show any mortality or signs of toxicity. However, in the acute toxicity study, histopathological examination of liver of treated rats showed some signs of toxicity indicating hepatotoxic nature of this decoction at the tested dose. The LD₅₀ of this AE was estimated to be greater than 2000 mg/kg body weight. Results of subacute toxicity study, suggest that the oral administration of AE daily for 28 days was well tolerated up to the dose, 1200 mg/kg in rats.

Cardiac glycosides are known as a major cardio toxins in plants. Therefore, investigation of the immature inflorescence of *Cocos nucifera* L. for the presence or absence of cardiac glycosides was carried out using chemical and chromatographic methods. According to results, cardiac glycosides were absent in this inflorescence. Proanthocyanidin content of the AE was determined using acid butanol method as an attempt to standardize it. The determined proanthocyanidin content of this AE was 619.6 mg in EASPA equivalence. Further, preliminary attempt to isolate phenolic compounds of the immature inflorescence of *Cocos nucifera* L. is reported herewith.