

**CHEMISTRY AND BIOLOGICAL ACTIVITIES OF THE  
PROANTHOCYANIDINS OF THE INFLORESCENCE OF  
*COCOS NUCIFERA* L, AN AYURVEDIC DRUG IN THE  
TREATMENT OF GYNAECOLOGICAL DISORDERS**

**By**

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award of the Degree of Master of Philosophy in Chemistry in**

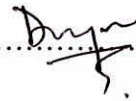
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## DECLARATION

“The work described in this thesis was carried out by me under the supervision of Dr. (Mrs.) C. Padumadasa (Senior Lecturer, Department of Chemistry, Faculty of Applied Sciences, University of Sri Jayewardenepura, Nugegoda), Prof. A. M. Abeysekera ( Senior professor, Department of Chemistry, Faculty of Applied Sciences, University of Sri Jayewardenepura, Nugegoda), Dr. (Ms.) M. G. Thammitiyagoda (Head, Animal Centre, Medical Research Institute, Colombo 8) and a report on this has not been submitted in whole or in part to any university or any other institution for another Degree/Diploma.”

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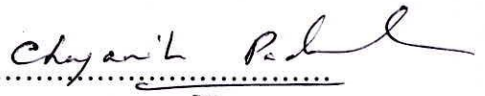
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## LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
ACE	Angiotensin converting enzyme
AQSPA	Aqueous soluble proanthocyanidin fraction
AQSPAF1	30 % aqueous methanol soluble fraction of AQSPA
AQSPAF2	50 % aqueous methanol soluble fraction of AQSPA
BAL	Bronchoalveolar lavage
COX-2	Cyclooxygenase-2
DEPT	Distortionless Enhancement by Polarization Transfer
DHB	2, 5-Dihydroxybenzoic acid
DMAC	4-(dimethylamino)-cinnamaldehyde
DPPH	1,1-diphenyl-2-picrylhydrazyl
DP	Degree of polymerization
DMSO	Dimethyl Sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DUB	Dysfunctional uterine bleeding
EASPA	Ethyl acetate soluble proanthocyanidin fraction
EASPAF1	20 % aqueous methanol soluble fraction of EASPA
EASPAF2	30 % aqueous methanol soluble fraction of EASPA
EASPAF3	50 % aqueous methanol soluble fraction of EASPA
EDR	Endothelium-dependent relaxation
ESI-MS	Electrospray Ionization Mass Spectrometry
FAB-MS	Fast-atom bombardment mass spectrometry

FBS	Fetal bovine serum
FIA-ESI-IT-MS <sup>n</sup>	Flow injection analysis electrospray ionization ion trap tandem mass spectrometry
FSH	Follicle-stimulating hormone
GP	General Practitioner
GnRH	Gonadotropin-releasing hormone
HBSS	Hanks' balanced salt solution containing calcium chloride and magnesium chloride
HeLa cell line	Cervical cancer cell line
HMB	Heavy menstrual bleeding
HPLC	High performance liquid chromatography
HPLC/ESI-MS	HPLC coupled to ESI-MS
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
HSCCC	High-speed counter-current chromatography
IAA	trans-3-indoleacrylic acid
IC50	Half maximal inhibitory concentration
IKK	I $\kappa$ B kinase
I $\kappa$ B	Inhibitor of NF- $\kappa$ B
IL-6	Interleukin 6
iNOS	inducible nitric oxide synthase
LC-MS	Liquid chromatography–mass spectrometry
LC-ESI-MS	Liquid-chromatography-electrospray ionization-mass spectrometry
LDL	Low-density lipoprotein

LNG-IUS	Levonorgestrel-releasing intrauterine system
LSIMS	Liquid secondary ion mass spectrometry
Luminol	3-aminophthalhydrazide
MALDI-TOF-MS	Matrix-assisted laser desorption / ionization - time-of-flight mass spectroscopy
MAPK	Mitogen-activated protein kinase
mDP	Mean degree of polymerization
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MMP	Matrix metalloproteinase
MTT	(3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl tetrazolium bromide)
NADH	$\beta$ -nicotinamide adenine dinucleotide
NBT	Nitro blue tetrazolium
NF- $\kappa$ B	Nuclear factor kappa-light-chain-enhancer of activated B cell
nNOS	neuronal nitric oxide synthases
NP-HPLC	Normal-phase HPLC
NMR	Nuclear magnetic resonance
NSAIDs	Non-steroidal anti-inflammatory drugs
ODC	Ornithine decarboxylase
ONOO <sup>-</sup>	Peroxynitrite
PC3 cell line	Prostate cancer cell line
PhIP	2-amino-1-methyl-6-phenylimidazo (4, 5-b) pyridine
PI-3	Phosphoinositide 3
PMS	Phenazine methosulphate

RDA	Retro Diels-Alder
R <sub>f</sub>	Retention factor
RI	Refractive index
RLU	Relative Light Units
RNI	Reactive nitrogen intermediates
ROS	Reactive Oxygen Species
RP-HPLC	Reverse-phase HPLC
RP-TLC	Reverse-phase TLC
SEC	Size exclusion chromatography
SEM	Standard Error of the Mean
SOR	Superoxide radical
SOZ	Serum Opsonized Zymosan
TEAC	Trolox equivalent antioxidant activity
TGF β1	Transforming growth factor β1
TLC	Thin-layer chromatography
TNF-α	Tumor necrosis factor α
UV	Ultraviolet
UVB	Ultraviolet B
UTIs	Urinary tract infections
WHO	World health organization

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**H.D.K. Dharmadana**

**ABSTRACT**

The immature inflorescence of *Cocos nucifera* L. variety aurantiaca is used by Ayurvedic and traditional medical practitioners for the treatment of menorrhagia in Sri Lanka. A preliminary phytochemical screening carried out by our research group had shown that the inflorescence of *Cocos nucifera* L. contains high levels of proanthocyanidins. The reported presence of proanthocyanidins in *Saraca asoka* which is also used in the treatment of menstrual disorders suggests that proanthocyanidins may play a role in the treatment of menorrhagia. Given the predominance of proanthocyanidins in the inflorescence of *Cocos nucifera* L. we hypothesized that they play a role in controlling menorrhagia by influencing the reproductive hormone levels. The proanthocyanidins of the inflorescence of *Cocos nucifera* L. were extracted with aqueous acetone (70 %) and separated into two fractions: an ethyl acetate soluble proanthocyanidin fraction (EASPA) and aqueous soluble



proanthocyanidin fraction (AQSPA). The total proanthocyanidin yield was 5.66 % on dry weight basis. Purification using chromatography on sephadex LH-20 yielded purified EASPA and AQSPA as off white powders in 0.03 % and 0.26 % respectively. Purified EASPA and AQSPA were characterized by  $^{13}\text{C}$  NMR spectroscopy, ESI-MS, acid catalyzed cleavage studies and thiolysis followed by LC-MS and NMR spectroscopy ( $^1\text{H}$  and  $^{13}\text{C}$  NMR). Acid catalyzed cleavage and thiolysis studies revealed that EASPA and AQSPA are made up of epicatechin and epiafzelechin units. The  $^{13}\text{C}$  NMR analysis of EASPA and AQSPA showed typical signals for epicatechin units and no detectable signals for epiafzelechin units. This indicates that both fractions are comprised mainly of epicatechin units and some epiafzelechin units. This was further confirmed by ESI-MS data for EASPA. The peaks at  $m/z$  579, 867, 1155 and 1443 correspond to molecular masses  $(\text{M}+\text{H})^+$  of epicatechin units with degree of polymerization ranging from 2-5 respectively, while mixed oligomers of epicatechin and epiafzelechin were detected at  $m/z$  563 and 851 as epicatechin-epiafzelechin dimer and epicatechin-epicatechin-epiafzelechin trimer respectively. EASPA (0.33 mg/day) and AQSPA (2.8 mg/day) dissolved in water were administered orally to female rats for 28 consecutive days. Vaginal cytology was performed daily during the study period to identify phases of the oestrous cycle. At the end of the study period oestrogen and progesterone levels were measured and compared with respective control groups (water). In addition, histological changes of endometrium at respective phase were also studied. There is no significant difference in the length of the oestrous cycle, serum estrogen level and endometrium histology between control and test group animals. However, there was a highly significant increase in progesterone levels of the EASPA administered group compared to the control ( $P \leq 0.001$ ), while no significant

difference of progesterone level was observed for AQSPA administered group. The antioxidant, anti-inflammatory and anticancer activity of EASPA and AQSPA were also determined. EASPA showed stronger radical scavenging activity against both DPPH ( $IC_{50} = 11.02 \pm 0.6 \mu\text{g/mL}$ ) and superoxide radical ( $IC_{50} = 26.11 \pm 0.72 \mu\text{g/mL}$ ) than AQSPA with  $IC_{50}$  values of  $21.69 \pm 0.6 \mu\text{g/mL}$  and  $35.48 \pm 0.14 \mu\text{g/mL}$  respectively. However, both fractions showed lower radical scavenging activity against DPPH ( $IC_{50} = 4.3 \pm 0.43$ ) and superoxide radical ( $IC_{50} = 22.56 \pm 0.56$ ) than that of respective positive controls. EASPA also showed strong anti-inflammatory activity ( $IC_{50} = 10.31 \pm 1.11 \mu\text{g/mL}$ ) similar to ibuprofen ( $IC_{50} = 11.2 \pm 1.90 \mu\text{g/mL}$ ) by the oxidative burst assay. The anti-inflammatory activity of AQSPA ( $IC_{50} = 22.34 \pm 1.67 \mu\text{g/mL}$ ) was lower than that of both EASPA and ibuprofen. AQSPA ( $IC_{50} = 14.63 \pm 0.99 \mu\text{g/mL}$ ) exhibited higher cytotoxic activity towards Hela cells than EASPA ( $IC_{50} = 18.78 \pm 0.90 \mu\text{g/mL}$ ), while both AQSPA and EASPA showed moderate cytotoxicity compared with doxorubicin ( $IC_{50} = 0.35 \pm 0.01 \mu\text{g/mL}$ ). The cytotoxicity of both AQSPA ( $IC_{50} = 67.35 \pm 0.16 \mu\text{g/mL}$ ) and EASPA ( $IC_{50} = 44.21 \pm 0.73 \mu\text{g/mL}$ ) against PC3 cells was lower compared to that of doxorubicin ( $IC_{50} = 1.38 \pm 0.16 \mu\text{g/mL}$ ). The distribution of proanthocyanidins in the inflorescence and its change during the maturation of the inflorescence was also studied. Total proanthocyanidin yield in the inflorescence increases with the maturity. The percentage yield of crude EASPA decreases, whereas percentage yield of crude AQSPA increases, with increasing maturity. The variation of proanthocyanidin content of the inflorescence along the length of the inflorescence at different maturity stages and proanthocyanidin content of different floral parts of the inflorescence were determined by the acid butanol assay. There is a significant variation of proanthocyanidin content along the length of the inflorescence ( $p \leq$

0.05). In all stages of development, the middle part contained the highest level of proanthocyanidins. The female flower ( $6.26 \pm 1.26$ ) has a higher proanthocyanidin content than the male flower ( $3.95 \pm 0.48$ ) and the rachilla ( $3.08 \pm 1.79$ ) has a higher proanthocyanidin content than the rachis ( $1.84 \pm 0.12$ ). Result of *in vivo* study suggests that the proanthocyanidins of the inflorescence of *Cocos nucifera* L. exerts its pharmacological effect through the mediation of progesterone, while its antioxidant and anti-inflammatory activities may be important in clinical situations involving menorrhagia. The results of the study on the distribution of proanthocyanidins in the inflorescence support the Ayurvedic practice of using the entire immature inflorescence.