EXTRACTION AND IDENTIFICATION OF PHYTOSTEROLS FROM *Dioscorea alata* L. AND *Colocasia esculenta*

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DECLARATION

The materials in this thesis are original except for quotations, excerpts, summaries and references, which have been duly acknowledged.

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SUJATHA KRISHNAN
PS 2002-001-480
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ABSTRAK

PENGESTRAKKAN DAN PENENTUAN PHYTOSTEROLS DARIPADA *Dioscorea alata* L. DAN *Colocasia esculenta*

Matlamat utama penyelidikan ini adalah untuk mengekstrak dan mengenalpasti kehadiran phytosterol dalam *Dioscorea alata* L. dan *Colocasia esculenta*. Phytosterol mempunyai struktur yang sama dengan kolesterol tetapi penambahan kumpulan *etil* atau *metil* serta ikatan ganda dua pada bahagian rantainya adalah berubah bagi setiap jenisnya. Sebatian ini terdapat secara semulajadi di dalam semua benda hidup. Pengestrakkan dan pengenalpastian phytosterol dalam *Dioscorea alata* L. dan *Colocasia esculenta* yang dinyatakan boleh meningkatkan aplikasi penyelidikan dalam Sains dan Teknologi sebagai terbitan ubat-ubatan yang terbaru. Metodologi dalam analisis ini terdiri daripada saponifikasi, untuk menukar acylglicerol kepada asid sabun, dan seterusnya bahan yang tidak disaponifikasikan akan diekstrak dengan menggunakan pelarut organik. Ekstrak tersebut telah dianalisiskan dengan menggunakan Kromatografi Lapisan Nipis, Kromatografi Cecair Berupaya Tinggi dan Kromatografi Gas dengan Spektrometer Jirim. Kajian ini menunjukkan pengekstrakan phytosterol daripada *Dioscorea alata* L. adalah 15% lebih banyak berbanding dengan *Colocasia esculenta*. Selain daripada itu, *Dioscorea alata* L. juga menunjukkan sumber yang baik untuk mengekstrak β-sitosterol daripada phytosterol kasar. Kesan phytosterol terhadap kolesterol dikaji ke atas 40 ekor tikus dan 20 ekor ayam. Kesan phytosterol menunjukkan bahawa kolesterol serum terutamanya pada LDL dan kolesterol keseluruhan menurun sebanyak 44.64 % dan 44.89% masing-masing dengan $p<0.05$ ($r=0.993$), selepas membekalkan phytosterol dengan makanan hariannya selama empat minggu. Selain daripada itu, kesan penambahan phytosterol dalam pemakanan ayam yang mula bertelur menyebabkan kolesterol pada kuning telur menurun sebanyak 5% ($p<0.05$) berbanding dengan tanpa bekalan phytosterol dalam pemakanan harian. Sebagai kesimpulan, dalam kajian ini, phytosterol telah menunjukkan sebagai agen penurun kolesterol semulajadi dalam haiwan yang dapat menambahkan nilai penggunaannya dalam kehidupan harian.

ABSTRACT

EXTRACTION AND IDENTIFICATION OF PHYTOSTEROLS FROM *Dioscorea alata L.* and *Colocasia esculenta*

The aim of this study is to extract and identify the phytosterols from Dioscorea alata L. and Colocasia esculenta and explore the possible commercial application. Phytosterol structures are similar to that of cholesterol but with an extra methyl or ethyl group and double bond in the side chain. These molecules are found naturally in all living organisms. Phytosterols extracted from the yam species can be used as additive to cooking oil, margarine, butter, salad dressing, cosmetic, conditioning creams or merely consumed in capsules forms. Our method for extraction of phytosterols in yam species requires a saponification process, which results in the conversion of the acylglycerols into fatty acid soaps. After saponification, the non-saponifiable fraction was separated from the above soaps by organic solvent (hexane) and the extract was analyzed using Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas Chromatography – Mass Selective Detector (GC-MSD). The effect of phytosterols on cholesterol levels was studied on mice and chicken. The results showed that Dioscorea alata L. contained 15% higher phytosterols as compared with Colocasia esculenta. The Dioscorea alata L. was found to contain higher β-sitosterol as compared with Colocasia esculenta. When mice were fed with phytosterols, the total cholesterol levels in their serum was found to be reduced by 44.89% and the low density lipoprotein (LDL) by 44.64% with $p<0.05$ ($r=0.993$). In phytosterol feeding experiments, chicken (layers) produced eggs in which the cholesterol levels were lowered by 5% ($p<0.05$). In conclusion, the sterols from plants were found to act as natural cholesterol-lowering agents in animals.

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LIST OF ABBREVIATIONS

BR	Brassicasterol
CA	Campesterol
CC	Column Chromatography
CEC	Capillary Electro-Chromatography
CH	Cholesterol
DAD	Diode Array Detector
PDA	Photodiode Array Detector
ELSD	Evaporative Light Scattering Detector
FID	Flame Ionization Detector
FIV	Feline Immunodeficiency Virus
GC	Gas Chromatography
GC-MSD	Gas Chromatography-Mass Selective Detector
HDL	High Density Lipoprotein
HMG-CoA	3-Hydroxy-3-methyl-glutaryl-coenzyme A
HPLC	High Performance Liquid Chromatography
HX	Hexane
HIV	Human Immunodeficiency Virus
IEC	Ion-Exchange Chromatography
IPA	Isopropanol
KOH	Potassium hydroxide
LDL	Low Density Lipoprotein
LLC	Liquid-Liquid Chromatography

LSC	Liquid-Solid Chromatography
MeCN	Acetonitrile
MS	Mass Spectrometry
MW	Molar Mass
NMR	Nuclear Magnetic Resonance
ODS	Octadecylsilane
RA	Rheumatoid Arthritis
R_f	Retardation factor
RI	Refractive Index
RTs	Relative Retention Times
SEC	Size-Exclusion Chromatography
SFE	Supercritical Fluid Extraction
SI	Sitosterol
SIM	Single Ion Monitoring
SPE	Solid-Phase Extraction
ST	Stigmasterol
TLC	Thin Layer Chromatography
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Sterols are hydroxylated steroids, a class of polyisoprenoid molecules found naturally in almost all living organisms (Hertherington and Steck, 1999). Sterols are constituents of the cell membranes; found in all cellular organisms to carry out their physiological functions. Sterols found in plants called "phytosterols". They are biogenetic precursors of numerous metabolites including plant steroid hormones (Breinholder *et al.*, 2002).

The first sterol structure was identified in 1932 and their stereochemical aspects were elucidated in the 1950s. Phytosterols have structures similar to cholesterol but posses an additional side chain at C-24 (Abidi, 2001). Phytosterols are minor components of all vegetable oils comparing major portions of the unsaponifiable fraction of the oils (Dunford and King, 2000).

Yams are a major food crop in many tropical countries including Malaysia. Amongst the yams are a group of plants belonging to the Genus *Dioscorea* in the Family of *Dioscoreaceae*. They are the most important native edible tubers grown in Terengganu and Kelantan (Northern Malaysia). Yams have also been cultivated as a primary food source for starch in Western Africa, India, the Pacific Islands and Southeast Asia. The main varieties in Malaysia are *Dioscorea alata* (Greater Yam), *Dioscorea esculenta* (Asiatic Yam) and *Colocasia esculenta* (Chinese yam) (Sayed, 2001). A large number of the yam species is used as medicine by the Chinese for asthma, urinary tract problems and rheumatic pain. The African yam has been

acclaimed as a miracle drug for the treatment of many skin conditions and for boosting the immune system (Kaimal, 1999).

The effects of phytosterols on health have been the subject of various studies. If consumed 3 g/day, the risk of heart disease reduces 15-40%. Phytosterols also have been investigated for toxicology and estrogenic potential. Even the consumption of 25 g or more per day, no adverse effects have been noted. However, persons with genetic condition called phytosterolaemia or sitosterolaemia, the consumption of phytosterols effect their health (Hicks *et al.*, 2000) and these patients are strongly urged to avoid intake of dietary phytosterols.

Several studies (Hallikainen *et al.*, 2000; Hendriks *et al.*, 1999 and Law, 2000) have shown that supplementation of stanol and sterol esters slightly reduces the absorption of β-carotene (pro-vitamin A), lycopene and α-tocopherol (vitamin E). However, an important role of phytosterols is to avoid the oxidation of LDL-cholesterol. Normen *et al.*, (2000) have found that esterified soy sterol and β-sitosterol ester inhibited cholesterol absorption.

Some phytosterols supplemented food products have been manufactured. These include margarine, corn oil and cooking oil for the reduction of LDL-cholesterol and increase energy expenditure thus preventing weight gaining (Hicks *et al.*, 2001).

1.2 Objectives of the Research

The main objectives of the research project are:-

(i) To extract the phytosterols from *Dioscorea alata* L. and *Colocasia esculenta* using solvent extraction and saponification.

(ii) To identify the extracted sterols using Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC) and Gas Chromatography-Mass Selective Detector (GC-MSD). The identification of

phytosterols from yam species will boost the commercial significance of yam production. They are marketable raw materials with their sufficiently nutritious content of phytosterols.

(iii) To analyse and compare the presence of phytosterols from two different types of yam for possible commercial extraction of β-sitosterol and stigmasterol.

(iv) To investigate the cholesterol lowering effect of phytosterols on mice.

(v) To show the effect of feeding the identified phytosterols on the cholesterol content in egg yolk of *Rhode Island Red* chicken.

CHAPTER 2

LITERATURE REVIEW

2.1 Sterols

Sterols are important components of the lipid systems of all living organisms. They are hydroxylated steroids and a class of polyisoprenoid molecules, derived from mevalonic acid. Sterols are constituents of the cell membranes of all living cells and therefore they occur in nature. However, the role of the phytosterol has not been investigated fully (Parish, 1997). Sterols have a total of 27-30 carbon atoms and a side chain with ≥7 carbon atoms is attached at the carbon-17 (Abidi, 2001).

Almost, all steroids are hydroxylated at C-3 including phytosterols. In the animal kingdom, the steroids are profound importance being used as hormones, coenzymes and pro-vitamins. The sterols such as stigmasterol, ergosterol, cholesterol and fucosterol are natural triterpene with 30 carbon atoms. The terpenes exist in various groups of natural products such as hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpene (C_{15}), diterpene (C_{20}), sesterterpene (C_{25}), triterpene (C_{30}) and tetraterpenes (C_{40}). However, Sesterterpene (C_{25}) is very rare in nature(Kaufman *et al.*, 1999 and Ahmad and Raji, 1999).

Their structures are related but vary on the different types of side chains and different numbers and positions of C=C (double bonds). They are crystalline, unsaponifiable alcohols with high melting points and show very similar physical and chemical properties. These substances are insoluble in water and soluble in non-polar organic solvents. They are also heat-resistant, odourless, and flavourless.

Plant fats and oils contain phytosterols which are present in pure or esterified form, or conjugated as glycosides (Careri *et al.*, 2001). About 25-80% of sterols are

esterified with fatty acids. Generally, the phytosterols can be categorized into three subclasses: (i) 4,4-desmethylsterols (cycloartenol), (ii) 4α-methylsterols (obtusifoliol) and (iii) 4,4-dimethylsterols (campesterol, sitosterol and Δ5-avenasterol) (Abidi, 2001).

2.2 Phytosterols

Plant sterols or phytosterols are natural component of edible vegetable oils. These phytochemicals are similar in structure to cholesterol but differing only in the number of carbon atoms or double bonds between carbon atoms in the side chain (Figure 2.1). The major phytosterols are β-sitosterol, campesterol and stigmasterol. (Eskin, 1999).

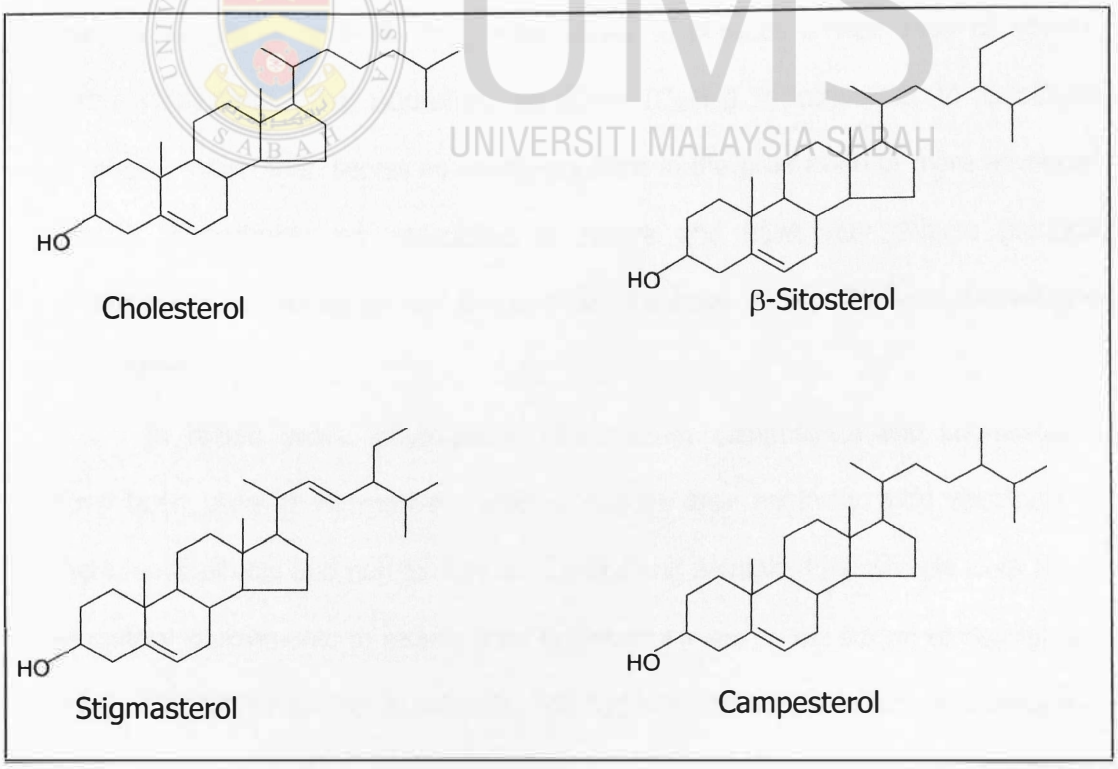

Figure 2.1 Structures of Common Plant Sterols and Cholesterol (Abidi, 2001).

Apparently, the sterols occurring in vegetable oil are mainly desmethylsterols. In other words, the edible oils contain much smaller number of 4-methylsterols and 4,4-desmethylsterols (Aparicio and Apricio-Ruiz, 2000). The most abundant sterol components present in the sterol fraction of vegetable oils (i.e. coconut, canola, cocoa, corn, cottonseed, linseed, olive, palm, peanut, rice bran, sesame, soybean, and sunflower) are mixtures of campesterol, sitosterol, stigmasterol and 5-avenasterol. However, a high percentage of 5-avenasterol is found in coconut oil. The other minor component of sterol is brassicasterol (Abidi, 2001).

2.3 Uses of Phytosterols

Epidemiological studies have shown that diets rich in vegetables and fruits reduce the risk of developing various types of cancer, cardiovascular disease, diabetes and other common illnesses (Pegel, 1997). While humans have the ability to synthesis cholesterol, plants have the biogenetic ability to produce a vast array of sterols, rarely cholesterol. The isoprenoid squalene ($C_{30}H_{50}$) is converted to lanosterol ($C_{29}H_{48}O$), which then serves as an intermediate in the production of more advanced sterols. Phytosterols are ubiquitous in nature and have very diverse practical applications (Hertherington and Steck, 1999; Kaufman *et al.*, 1999 and Ahmad and Raji, 1999).

In recent years, phytosterols (β-sitosterol, campesterol and stigmasterol) have been used in alternative medicine due to their relatively wide spectrum of therapeutic effects and non-toxicity in humans and animals. Phytosterols were taken as dietary supplements to reduce total cholesterol levels in the serum of normal and mildly hypercholesterolaemic subjects. The hypocholesterolemic effect of phytosterols may be explained by three mechanisms that include (i) inhibitions of cholesterol