# ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF *THEOBROMA CACAO*

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THIS DISSERTATION IS PRESENTED AS PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF BACHELOR OF SCIENCE WITH HONOURS



INDUSTRIAL CHEMISTRY PROGRAMME
SCHOOL OF SCIENCE AND TECHNOLOGY
UNIVERSITY MALAYSIA SABAH

APRIL 2007

PUMS 99:1

UNIVERSITI MALAYSIA SABAH

**BORANG PENGESAHAN STATUS TESIS@**

JUDUL: Antioxidant & Antimicrobial activities of theobroma cacao

Ijazah: Sarjana muda kimia (Industri)

SESI PENGAJIAN: 2004/2005

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# DECLARATION

I declare that this dissertation entitled Antioxidant and Antimicrobial Activities of Theobroma Cacao and the work presented in it is my own. Sources of finding reviewed here in have been duly acknowledged.

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## ABSTRACT

*Theobroma Cacao* has been well known for its high content of polyphenols. In this study fresh and dry cocoa beans were collected from Malaysian Cocoa Board, Sabah and extracted with 70% acetone-aqueous to give crude extracts. The total polyphenol content of these crude extracts was determined by using the Folin-Ciocalteu reagent, antioxidation properties was evaluated using Ferric thiocyanate (FTC) method while the antimicrobial property was evaluated using the disc diffusion method. It showed that fresh (57 mg GAE / g) cocoa beans contained more phenolic content than dry (48 mg GAE/ g) cocoa and both samples displayed a moderate antioxidation activity compared to butylated hydroxytoulene (BHT) a synthetic antioxidant. The extracts were active against *S. aureus* and *B. cereus* as shown by the clear inhibition zone of 14.5 mm and 15.5 mm for fresh cocoa beans and 10.0 mm and 10.5 mm for dry cocoa beans respectively. Fresh cocoa beans were found to have high phenolic content with moderate antioxidation and potent antimicrobial activities. Thus, these cocoa beans can be developed into new neutraceuticals or pharmaceuticals products.

## *AKTIVITI ANTIOKSIDA DAN ANTIMIKROORGANISMA THEOBROMA CACAO*

## ABSTRAK

*Theobroma Cacao dikenali mempunyai kandungan fenolik yang tinggi. Dalam kajian ini biji koko segar dan biji koko kering yang diperolehi dari Lembaga Koko Malaysia, Sabah diekstrak dengan menggunakan 70% aseton-akueus untuk menjadikanya ekstrak mentah. Jumlah kandungan fenolik dalam biji koko ditentukan dengan menggunakan Folin-Ciocalteu reagen, sifat antioksida pula ditentukan dengan menggunakan kaedah Ferrik thiocyanate (FTC) sementara antimikroorganisma ditentukan dengan menggunakan kaedah disk difusi. Keputusan menunjukkan kandungan fenolik dalam biji koko segar (57 mg GAE / g) adalah lebih tinggi berbanding dengan biji koko kering (48 mg GAE/ g) dan kedua-dua sampel ini menunjukkan aktiviti anti-oksida yang sederhana berbanding dengan butylated hydroxytoulene (BHT). Kedua-dua ekstrak ini adalah aktif dalam melawan bakteria S. aureus dan B. cereus dengan zon perencatan 14.5 mm dan 15.5 mm bagi biji koko segar dan 10.0 mm dan 10.5 mm bagi biji koko kering. Keputusan menunjukkan biji koko segar mengandungi kandungan fenolik yang tinggi, antioksida yang sederhana dan berpotensi sebagai antimikroorganisma. Jadi, biji koko ini boleh berkembang sebagai produk neutraketical atau farmaketical.*

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| | |
|---|---|
| m | milligram |
| g | gram |
| kg | kilogram |
| μm | micrometer |
| mm | millimeter |
| mL | millilitter |
| cm | centimeter |
| μL | microlitter |
| L | litter |
| °C | degree celsius |
| % | percentage |
| v/v | volume per volume |
| h | hour |
| BHT | butylated hydroxytoulene |
| FTC | Ferric thiocyanate |
| FAO | Food and Agriculture organization |
| DMSO | Dimethyl sulfoxide |
| *S.aureus* | *Staphylococcus aureus* |
| *B.cereus* | *Bacillus cereus* |
| *E. colli* | *Escherichia coli* |
| GAE | Gallic acid equivalent |

# CHAPTER 1

# INTRODUCTION

## 1.1 Chronic Disease and Cocoa

In 2001, 59 % of the 56.5 million deaths annually and 45.9 % of the global disease are caused by chronic diseases such as cardiovascular, diabetes, obesity, cancers and respiratory diseases. It has been stated by World Health Organization (WHO) that, by 2020, chronic diseases will be the cause for almost three-quarters of all deaths in worldwide. By 2010, cardiovascular disease will be the leading cause of death in developing countries. The number of new cases annually is estimated to rise from 10 million to 15 million by 2020 (WHO, 2002).

Dietary factors are responsible for about 30% of all cancers and cardiovascular disease in Western countries and up to 20% in developing countries such as Asian countries. A diet low in energy-dense foods that are high in saturated fats and sugars and abundant in fruit and vegetables, together with an active lifestyle are among the key measures to prevent chronic disease (WHO and Food and Agriculture Organization, 2003). Studies suggests that people with high dietary intakes of fruits and vegetables are less affected by cardiovascular or cancer than people who have low dietary intakes of these foods (Ferrari and Torres, 2003). A low intake of fruits and vegetables is estimated to cause about 19 % of cancer, and about 31% of heart disease and 11% of strokes worldwide and 2.7 million deaths are caused by low fruit and vegetable intake (WHO and Food and Agriculture Organization, 2003).

There are several mechanisms, to protect this disease such as reduced plasma cholesterol, modulation of lipid and lipoprotein metabolism, estrogenic effects, modulation of enzymes, such as Phase I and Phase II enzymes of the detoxification pathway, or modulation of apoptosis (Schramm and German, 1998). However, one major hypothesis in the protection against radical oxygen species modulated damage of biological molecules, including DNA, lipids, proteins and carbohydrate, is by antioxidant micronutrients such as vitamin C, vitamin E, beta-carotene, selenium or non-nutrient phytochemicals, such as polyphenols (Bravo, 1998).

Among phytochemicals, polyphenols constitute one of the most widely distributed groups of substances in the plant kingdom, with more than 8000 phenolic structures currently known (Bravo, 1998). They occur in a variety of fruits, vegetables, nuts, seeds, flowers, bark, beverages, and even some manufactured food as a component of the natural ingredients used. Polyphenols have become an intense focus of research interest because of their health-beneficial effects in the treatment and prevention of cancer, cardiovascular disease, and other pathologies (Wollgast and Anklam, 2000). A different polyphenol-rich foods and beverages, such as grape products, cocoa or tea produce beneficial effects with comparable patterns including improved endothelial function, anti-thrombotic, anti-atherogenic and anti-hypertensive effects. The possible mechanisms of these effects are multiple and may be different depending on the active polyphenols provided by each food or beverage (Stoclet *et al.*, 2004)

Cocoa is particularly rich in polyphenols, the total polyphenol content of the bean being estimated to be 6% to 8% by weight of the dry bean. The pigment cells in cocoa bean contain approximately 65%-70% of polyphenols and 3% of anthocyanins by weight (Forsyth, 1955). Flavan-3-ols had been identified as the major antioxidant components in the cocoa bean (Steinberg *et al.*, 2003).

Many studies suggest that, dietary supplementation with flavonoid-rich cocoa may be cardio protective because of their interference in many pathophysiological mechanisms associated with atherosclerosis. Possible beneficial effects by cocoa flavonoids includes antioxidant properties, improvement in endothelial function, blood pressure lowering, decreased platelet activation and function and modulation of immune function and inflammation (Mary and Marguerite, 2004).

## 1.2 Objectives of Study

The objectives of this study were:

- To determine the total content of polyphenols in fresh and dried cocoa beans
- To evaluate the antioxidative property of cocoa
- To evaluate the antimicrobial activities of the extracts against pathogenic bacteria (*Staphylococcus aureus*, *Bacillus cereus* and *Escherichia coli*)
- To compare the total phenolic content, antioxidant and antimicrobial activities of fresh and dry cocoa beans

**1.3 Scope of Study**

Fresh and dry cocoa beans were used for this study. The samples were collected from Malaysian Cocoa Board, Sabah. Total phenolic content was determined by using Folin-Ciocalteu method and the antioxidative property in cocoa was evaluated by ferric thiocyanate method. Antimicrobial screening was done by using disc diffusion method.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Discovery and Development of Cocoa

*Theobroma cacao* is the scientific name given to cocoa in 18th century by a Swedish Botanist, Carolus Linnaeus. It was cultivated over 3000 years ago by the original inhabitants of Central America and Northern South America. The original discover of the Americas, Columbus was made aware of cocoa plant and bean. After his fourth voyage and returned to Spain in 1502, he introduced cocoa to Spain. The cocoa tradition reached Italy and France after some hundred years and in the middle of the $17^{th}$ century shops selling cocoa were opened in the United Kingdom (Weisburger, 2001).

In 1828, chocolate powder was produced by pressing cocoa butter from roasted and ground cocoa beans and then in the middle of the $19^{th}$ century a London firm produced commercial chocolates. The cultivation of cocoa plants spread to West Africa, which currently produce about 70% of the world's total production, the remainder coming from Central and South America, West Indies and small amount in tropical areas of Asia (Weisburger, 2001).

## 2.2 Varieties of Cocoa

There are many varieties of cocoa, which are divided into three main groups (Wollgast and Anklam, 2000). They are:

- Criollo - This variety is a native to Central America. It is very small, fragile and sensitive to disease, making it harder to grow than other varieties.

- Forastero – It originates from the Amazon basin. It is easier to grow, but it has a more bitter taste than Criollo. Today about 70% of the cacao trees are Forastero.

- Trinitario – It originates from the island of Trinidad. This is a cross between Criollo and Forastero. About 15-20% of the cacao trees today belong to this variety.

## 2.3 Raw Materials of Cocoa

Cocoa beans are made up from pulp, testa, cotyledon and radicle. The pulp of cocoa beans contents water ($H_2O$), glucose ($C_6H_{12}O_6$), pentosans, citric acid and potassium salts. The cotyledon is build up from cellulose (2-3%), starch (5%), sucrose (2-3%), fat (30-32%), proteins (8-10%), theobromine (2-3%), caffeine (1%), acids(1%) and polyphenols (5-6%) (MARDI, 1990).

Fat provides a source of energy. Cocoa and chocolate contain fat in a form of cocoa butter. The digestibility and assimilation of cocoa butter is quite high. Fat is an important part of the diet of most heterotrophs (Segall *et al.*, 2005). Figure 2.1 shows the chemical structure of a triglyceride, an example of fat.

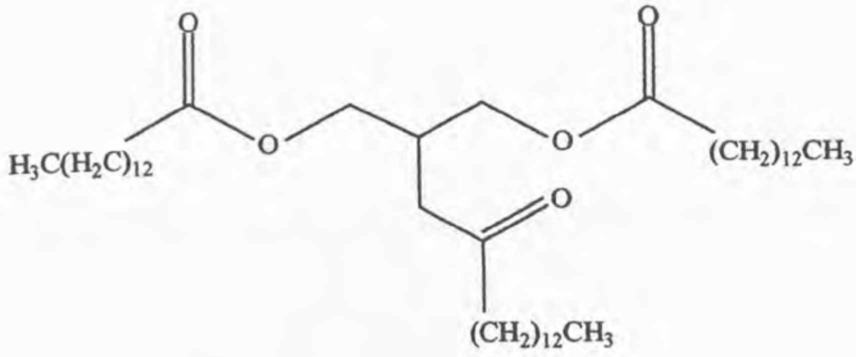



**Figure 2.1** The chemical structure of a triglyceride.

Theobromine and caffeine belongs to alkaloid molecules known as methylxanthines present in cocoa. Methylxanthines naturally occur in many plants species and include theophylline. Caffeine is a xanthine alkaloid compound that acts as a stimulant in humans. Cocoa contains a small amount of caffeine. Methylxanthines have physiological effects on various body systems, including the central nervous, cardiovascular, gastrointestinal, respiratory, and renal systems. Alkaloid is an amine thus it reacts with acid to yield a soluble salt. Alkaloid beneficial effects are included analgesic, anti-thrombotic, anti-atherogenic, anti-hypertensive and anti-tumor (Fasihuddin and Rasmah, 1993). The chemical structure of theobromine and caffeine are shown in Figure 2.2 and Figure 2.3.

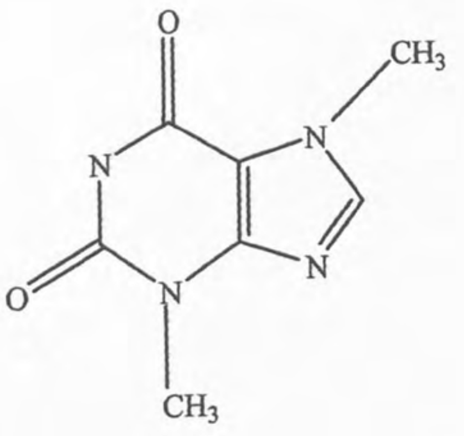


**Figure 2.2** The chemical structure of theobromine.

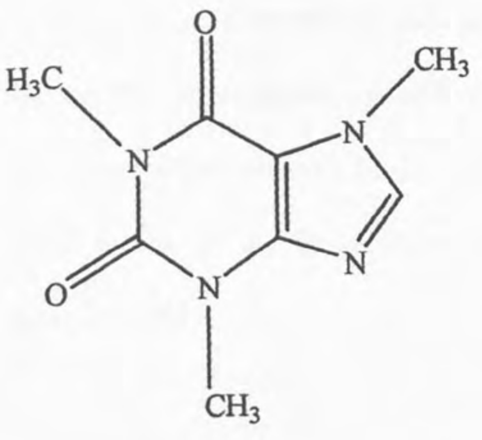


**Figure 2.3** The chemical structure of caffeine.

## 2.4 Fermentation and Drying Process of Cocoa

A correct fermentation and drying of cocoa beans, which is carried out in the countries of origin is essential to the development of suitable flavors. A simple chemical reaction takes place during fermentation process are shown in the Figure 2.4.

$$\underset{\text{(Sugar)}}{C_6H_{12}O_6} \longrightarrow \underset{\text{(Alcohol)}}{2C_2H_5OH} + \underset{\text{(Carbon Dioxide)}}{2CO_2} + \underset{\text{(Energy)}}{\text{18 Calorie}}$$

**Figure 2.4** The chemical reaction occurs during the fermentation process (Suhaimi, 1994).

The fermentation and drying process reduce the overall level of polyphenol especially anthocyanins in cocoa beans (Pettipher, 1986). Polyphenols in the cocoa beans undergoes oxidation and polymerization process to form complex tannins during the fermentation process. During drying stage, enzymatic or spontaneous oxidation of polyphenols leads to the formation of melanin and melanoproteins and these results in the characteristic of browning fermented cocoa beans (Kim and Keeney, 1984).

## 2.5 Polyphenols

### 2.5.1 Biosynthesis and Chemistry of Polyphenols

Phenolic compounds or polyphenols are the most numerous and widely distributed groups in plant kingdom with more than 8000 phenolic structures currently known (Bravo, 1998). Polyphenols are the secondary metabolism product of plants. They form biogenetically from two main primary synthetic pathways. There are shikimate and acetate pathway. The steps are shown in Figure 2.5.

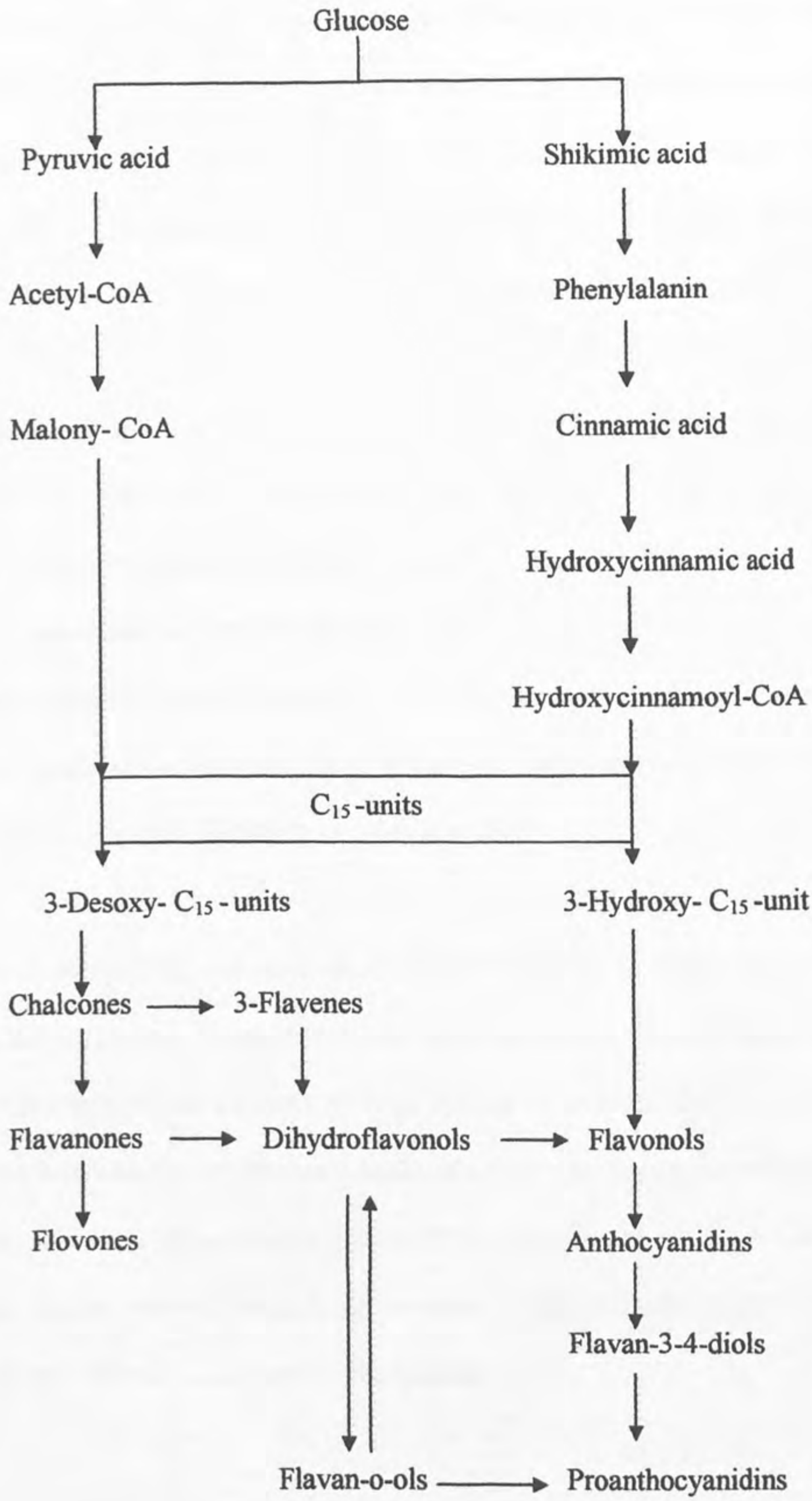



**Figure 2.5** Biosynthesis and interconnection of flavonoids (Wollgast and Anklam, 2000).

Acetic acid and shikimic acid are formed from glucose metabolism. Acetic acid in active form acetyl-CoA later form malonyl-CoA, is the starting point of fatty acid synthesis in a primary pathway and the secondary pathway of the synthesis of the a ring flavonoids. Aromatic acid amino is the product of the primary shikimate pathway but the product degradation leads into the phenylpropanoid pathway. The phenylpropanoid pathway is considered as a secondary pathway. The phenylpropanoid is essential to the survival of terrestrial plants providing plant constituents such as a lignin with its important mechanical and structural role. Furthermore, phenylpropanoid-derived compounds have distinct roles in the physiology of plants (e.g., as signaling compounds within the plant and as factors controlling male sterility and regulating hormonal activity) and their inter-relations with other organisms (e.g., as defensive compounds against micro-organisms and signalling compounds between plants and other organisms) (Wollgast and Anklam, 2000).

Natural polyphenols can range from simple molecules to highly polymerized compounds and they occur in conjugated form with one or more sugar residues linked to hydroxyl groups or direct linkages of sugar unit to an aromatic carbon atom. The sugar unit can be presented as monosaccharides, disaccharides or oligosaccharides and glucose is the common sugar residue (Bravo, 1998). Polyphenol can be divided into 10 different classes with different basic structure. Table 2.1 illustrates the basic chemical structure of main polyphenolic compounds.

**Table 2.1** Basic chemical structure of main classes of polyphenolic compounds (Bravo, 1998).

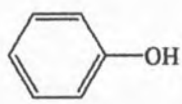
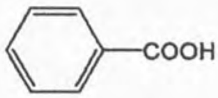
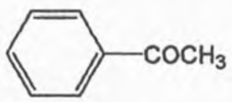
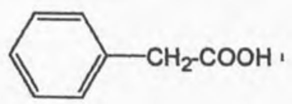
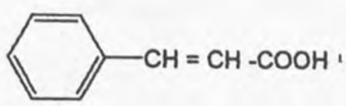
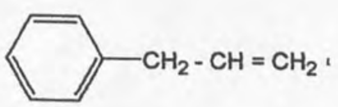
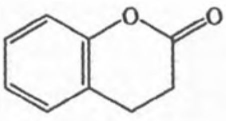
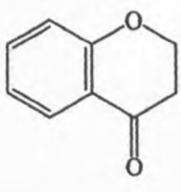
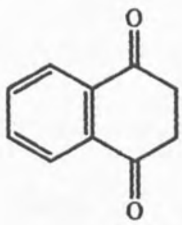
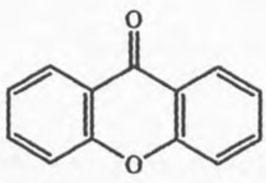
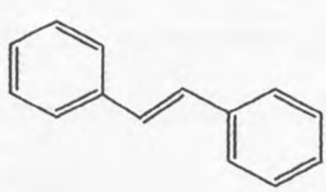
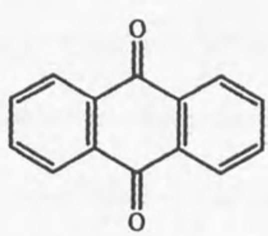
| Class | Basic skeleton | Basic structure |
|---|---|---|
| Simple phenols | $C_6$ |  |
| Benzoquinones | $C_6$ |  |
| Phenolic acid | $C_1 - C_6$ |  |
| Acetophenones | $C_6 - C_2$ |  |
| Phenylacetic acid | $C_6 - C_2$ |  |
| Hydroxycinnamic acid | $C_6 - C_3$ |  |
| Phenylpropenes | $C_6 - C_3$ |  |

**Table 2.1** Continue.

| | | |
|---|---|---|
| Coumarines,isocoumarines | $C_6 - C_3$ |  |
| Chromones | $C_6 - C_3$ |  |
| Naphtoquinones | $C_6 - C_4$ |  |
| Xanthones | $C_6 - C_1 - C_6$ |  |
| Stilbenes | $C_6 - C_2 - C_6$ |  |
| Anthraquinones | $C_6 - C_2 - C_6$ |  |

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