EFFECT OF NATURAL COCOA POWDER SUPPLEMENTATION ON OXIDATIVE STRESS AND HEMATOLOGICAL INDICES IN HEALTHY GHANAIAN ADULTS

A THESIS SUBMITTED BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF A MASTER OF PHILOSOPHY (M. PHIL) DEGREE IN PHYSIOLOGY

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DECLARATION

BY CANDIDATE

This thesis is submitted to the University of Ghana (UG), School of Graduate Studies through the College of Health Sciences, Medical School, Department of Physiology. This study is the result of my own research, which I carried out under supervision in the Department of Physiology. I hereby declare that, except for references to other people's work, which has been duly acknowledged. This thesis presents finding original to me and has not been submitted either completely or in part for the award of any other degree in this or another University.

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DEDICATION

In humble but grateful acknowledgement to God Almighty, I dedicate this work to my loving parents and wonderful brothers, loved ones and mentors whose moral, financial, spiritual, familial support and encouragement brought me this far.

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I thank the Almighty God for His Grace, protection, wisdom and sustenance throughout my life.

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ABBREVIATIONS AND SYMBOLS

ABTS: Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

AGEs: Advanced Glycosylation End-products

Akt: Protein kinase B

Apo: Apolipoprotein

B: Basophils

cGMP: Cyclic guanosine monophosphate

COX: Cyclooxygenase

CYP: Cytochrome P450

DPPH: 2,2-Diphenyl-1-pycrylhydrazyl

E: Eosinophils

eNOS: Endothelial nitric oxide synthase

GSH-Px: Glutathione peroxidase

Hct: Hematocrit

Hb: hemoglobin

HDL: High-density lipoprotein

HO-1: Heme-oxygenase 1

Ig: Immunoglobulin

IL: Interleukin

L: Lymphocytes

Leu: Leukocytes

LDL: Low-density lipoprotein

LT: Leukotrienes

M: Monocytes

MAPK: Mitogen-activated protein kinase

MDA: Malondialdehyde

MCV: Mean cell volume

MCH: Mean cell hemoglobin

MCHC: Mean cell hemoglobin concentration

MEK: Mitogen-activated protein kinase kinase

MPO: Myeloperoxidase

NADPH: Nicotinamide adenine dinucleotide phosphate

NF-κB: Nuclear factor-κB

NO: Nitric oxide

NOX: NADPH oxidase

PBMCs: Human peripheral blood mononuclear cells

PI3 K: Phosphoinositide 3-kinase

PLA: Phospholipase A_2

RBC: Red blood cells

ROS: Reactive oxygen species

SAHS: School of Allied Health Sciences

VCAM: vascular cell adhesion molecule; CD106

TNF-α: Tumor necrosis factor-α

VEGF: Vascular endothelial growth factor

VCAM-1 vascular cell adhesion molecule 1

VLA-4: Very late antigen-4

VSMC: Vascular smooth muscle cell.

ABSTRACT

Background: Natural cocoa powder (NCP) has been recognized to possess significant amounts of procyanidin flavonoids, methylxanthines, catechin and epicatechin with healthful effects. Stimulus for this work was to validate claims that NCP was a healthful product best for the aged with cardiovascular health problems and also for daily consumption for all age groups as a preventive measure. Literature review does not show any work done in healthy adult Ghanaians involving the long term supplementation of NCP as a baseline study before further research into disease areas. Several 'foreign' studies used chocolate due to its more acceptable taste.

Aim and objective: To evaluate the long term effect of NCP supplementation on selected oxidative stress makers and hematological indices in healthy adult Ghanaians.

Methodology: Seventeen (12 males and 5 females) subjects selected by random sampling from the staff and students of SAHS, Korle-Bu completed the study. NCP was consumed as a beverage twice daily before meals for 12 weeks. pre- and post- supplementation blood draw, anthropometry, hematological indices and analysis with biochemical assays such as advanced glycated end-products (AGEs), vascular cell adhesion molecule1 (VCAM-1), tumour necrosis factor alpha (TNF-α), glutathione peroxidase (GPx), malondialdehyde (MDA), heme oxygenase 1 (HO-1) was done

Results: The mean age of the subjects was 30.8 ± 10.2 years, mean pre- skeletal muscle was 35.18 ± 10.30% and post- skeletal muscle 37.64 ± 8.90%, there was a significant increase (p = 0.031), mean pre- resting metabolism was 1475 ± 270 Kcal and the mean post- resting metabolism was 1530 ± 206 Kcal, this showed significant increase (p = 0.028). The mean pre- GSH-Px was 1.73 ± 0.18 ng/L and post- GSH-Px 1.96 ± 0.10 ng/L, there was statistically significant increase (p = 0.001). The mean pre- TWBC was 5.36 ± 1.7 x 10^9/L and post- TWBC

6.04 ± 2.6 x 10^9/L. There was a significant increase (p = 0.031). Mean pre- Hb was 13.48 ± 1.40 g/dl and post- Hb 13.95 ± 1.50 g/dl. There was a significant increase after the supplementation (p = 0.016). Mean pre- Hct was 31.58 ± 9.5% and post- Hct 36.01 ± 7.6%. There was significant increase after the intervention (p = 0.001). Mean WBC differentials (Monocytes (M), Eosinophils (E) & Basophils (B)) the mean pre- M was 5.76 ± 1.40% and post- M 6.51 ± 1.90%. There was a significant increase (p = 0.039). Mean pre- E was 0.97 ± 0.80% and post- E 1.45 ± 1.20%. There was a significant increase after the supplementation (p = 0.005). Mean pre- B was 0.33 ± 0.30% and post- B% 0.46 ± 0.30% which showed significant increase (p = 0.006) respectively. Mean pre- MDA was 0.69 ± 0.10 ng/L and post- MDA 0.64 ± 0.10 ng/L, there was a statistically significant decrease (p = 0.033). Mean overall weight pre- was 66.86 ± 10.61 kg and post-supplementation was 65.19 ± 10.45 kg, showing significant decrease (p = 0.009). Mean pre- BMI was 23.01 ± 3.8 kg/m^2 was higher than the mean post- BMI 22.96 ± 3.5 kg/m^2 showing a statistically significant decrease (p = 0.006). Mean pre- SBP was 124.53 ± 11.30 mmHg and post- SBP 121.12 ± 9.10 mmHg. There was a significant decrease (p = 0.001). The mean pre- DBP was 80.12 ± 9.60 mmHg and post- DBP 78.24 ± 9.10 mmHg, there was significant decrease after the 12 weeks supplementation (p = 0.003). Mean pre- MCH was 33.48 ± 12.8 pg and post- MCH 31.42 ± 3.8 pg, there was a significant decrease (p = 0.001). Mean pre- MCHC was 47.66 ± 16.70 g/dl and post- MCHC 40.38 ± 9.60 g/dl. There was a significant decrease after the supplementation (p = 0.001).

Conclusion: Long term supplementation with NCP caused significant changes in the anthropometric, hematological indices and biochemical markers. Furthermore, this was accompanied by significant increase in antioxidant levels. Natural cocoa powder therefore

reduced oxidative stress and improved the biochemical markers and hematological indices. The long term daily consumption is therefore recommended.

CHAPTER ONE

1.0 GENERAL INTRODUCTION

Though Ghana produces 26% of the world's cocoa; there is still a paucity of scientific documentation of the healthful effects of cocoa in humans. Ghana's cocoa bean is the gold standard brand but little is known about its uses in health and disease states. Cocoa beans are more popular for making chocolate due to its more palatable taste compared to the more astringent cocoa powder. Most of the foreign studies are on chocolates with more additives and less potency than natural cocoa powder (NCP). NCP is the least patronized of the cocoa products due to its astringent taste despite its healthful properties. As a struggling lower middle income economy with overburdened healthcare system battles daily with preventable chronic diseases such as malaria, tuberculosis, obesity, cardiovascular diseases, diabetes mellitus, neurodegenerative diseases etc. Hence this study to validate its effects, formulate reformative policies and modify lifestyles towards phytotherapy and healthy living.

Cocoa contains flavonoids in the highest proportion compared to green and black tea, apples, gingko, red wine etc. Flavonoids are natural antioxidants concentrated in foods such as cocoa counteracting the potentially destructive accumulation of prooxidants causing oxidative stress. Oxidative stress accompanies a wide spectrum of clinically important disorders, including atherosclerosis, ischemia/reperfusion, aging, diabetes mellitus, hypertension, heart diseases and obesity etc. Although reactive oxygen species (ROS) can activate signalling pathways that contribute to ischemic preconditioning and cardioprotection, high levels induce adverse structural modifications. This study seeks to

validate and establish the effects of NCP supplementation on oxidative stress, antioxidant status, cell membrane stability (endothelial function and RBC hemolysis), vasodilatation, anti-inflammation, hypotensive and anti-degradative in healthy Ghanaian adults.

1.1 BACKGROUND OF THE STUDY

Polyphenols are beneficial compounds found in many foods, fruits, vegetables, teas and wines (Keen *et al*., 2005). Cocoa polyphenols such as catechins, epicatechins and their polymers like procyanidins collectively called flavonoids have potent antioxidant properties (Hopper *et al*., 2008). They exert antioxidant and anti-inflammatory effects, anti-thrombotic, anti-coagulant, cardiotonic, anti-microbial, CNS-stimulant, myorelaxant properties etc. (Min-Hsiung *et al*., 2010). Studies have ascribed the health benefits of cocoa consumption to its high flavonoid content. NCP contains the highest levels of total antioxidant capacity when compared to all other kinds of edible cocoa products. Cocoa consumption plays a preventative role in the cardiovascular system (Keen *et al*., 2005) and guards against tumoural and carcinogenic processes (De *et al*., 2000).

Few interventional studies offer little data related to their effect in the healthy state, whereas several ‘foreign’ studies have reported some favourable changes in biomarkers assessing antioxidant status but very few findings related to inflammatory, vascular adhesion, heme degradation, hypotensive, lipid peroxidation biomarkers ##. In moderation, NCP offers strong antioxidant effects in combination with a sensational experience involving psycho-activity, mood and taste. The benign profile of its fatty acids in combination with the absence of sugar and additives like milk and preservatives should

lessen concerns about its adverse effects. The modulation of oxidative stress by cocoa polyphenols has been demonstrated in many experimental models such as rats and guinea pigs with little or none in humans. Cocoa inhibits LDL oxidation *in vitro* (Vinson *et al*., 1999, Serafini *et al*., 2003), increases plasma antioxidant capacity (Osakabe *et al*., 2001) and decreases formation of reactive intermediates (Rein *et al*., 2000).

However, studies dealing with the effects of long-term NCP supplementation on cell membrane stability, inflammation, antioxidant status and oxidative stress involving heme-degradation *in vivo* are non-existent in the indigenous Ghanaian setting. This study focuses on the antioxidant, cell membrane stabilizing, anti-inflammatory, anti-aging, vasodilatory, rejuvenating and anti-degradative effects in healthy adult Ghanaians.

1.2 PROBLEM STATEMENT

The global use of phytotherapy in health care delivery has taken centre stage due to the realization of its medico-socioeconomic importance but there is little or no documented scientific baseline data in apparently healthy Ghanaians. There is paucity of documented baseline scientific data and empirical evidence on the effects of natural foods such as NCP in healthy adults to build upon, especially in the Ghanaian indigenous setting (McCarthy, 2010). Besides the constituents of NCP, trace elements such as copper, iron, chromium, magnesium, selenium and vanadium as well as vitamins A, C, E, folate and the B group among others already established make it a healthy supplementation for healthy living. Established scientific data to support claims of benefits such as its antioxidant capacity, cell membrane stabilizing, vasodilatory, anti-aging, nutritive and

anti-degradative status in the indigenous human population is as yet unavailable, hence this study.

The main objective of this study is to determine and compare the 12 week pre- and post-supplementation effect of NCP beverage on selected oxidative stress markers and hematological indices among healthy Ghanaian adults and thereby set a baseline data with empirical evidence to substantiate claims of its effects in the physiologic free-living state.

1.3 JUSTIFICATION

Given Ghana's status as a leading producer of the world's cocoa beans, ignorance as to the healthful benefits and utility of this major cash crop despite its availability, potential cost-effectiveness and medico-socioeconomic impact is unacceptable. Coupled with this, Ghana is bedevilled by preventable debilitating diseases such as malaria, anemia, sickle cell anemia, tuberculosis, malnutrition, cardiovascular diseases etc. NCP supplementation can be a regime for healthy living, hence the need for in-depth and diverse studies into its phytotherapeutic attributes. Before now no long term quasi-experimental intervention study on the effect of NCP among healthy adults has been done, let alone involving bio-oxidative stress and inflammation in a single pilot study. The routine has been with the effect of chocolate on cholesterol and blood pressure due to its more acceptable taste. This is a pilot study undertaken in apparently healthy Ghanaian adults and the first of its kind portraying such an array of assays as malondialdehyde (MDA), tissue necrosis factor alpha (TNF-α), advanced glycated end products (AGEs), Glutathione peroxidase (GSH-Px), vascular cell adhesion molecule 1 (VCAM-1) and heme oxygenase 1 (HO-1).

Additionally, there is a global trend towards phytotherapy and nutriceuticals where food nutrients and supplements are taken to cure or manage disease.

1.4 HYPOTHESIS

There will be no significant change in the selected markers of oxidative stress and hematological indices before and after 12 week supplementation of NCP in healthy Ghanaian adults.

1.5 AIM

To evaluate the effect of 12 weeks NCP supplementation on markers of oxidative stress and hematological indices in healthy Ghanaian adults

1.6 SPECIFIC OBJECTIVE

To measure and compare the pre- and post-supplementation levels of NCP on the following markers of oxidative stress: vascular cell adhesion molecule 1 (VCAM-1) (cell membrane stability), Malondialdehyde (MDA) (Oxidative stress – pro-oxidant), Advanced Glycosylation End-products (AGEs) (Lipid peroxidation and aging), heme-oxygenase 1 (HO-1) (RBC hemolysis), Glutathione peroxidase (GSH-Px) (Antioxidant status) and Tumour Necrosis Factor alpha (TNF-α) (Tissue damage) and hematological indices (leukocytes (L%), red cell (RBC) count, hemoglobin (Hb), hematocrit (Hct) as well as the calculated indices mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC).

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 BOTANICAL AND COMMON NAMES

'*Theobroma cacao'* was coined by the Swedish botanist Carl Linnaeus in 1753, as published in his famous book *Species Plantarum*. Theobroma means "food of the gods" in Latin and Aztec languages, also in Greek "god" (*Theo*) and "food" (*Broma*). Cacao is derived from the 'Nahuatl', an Aztec word for bitter water.

Kingdom: Plantae (Angiosperms) **Order**:Malvaceae

Class: Equisetopsida **Family**: Sterculiaceae (sterculia)

Subclass: Magnoliidae **Subfamily**: Byttnerioideae

Superorder: Rosanae **Genus**: Theobroma (Cuatrecasas, 1964)

Some common names include: Cacao tree, Chocolate tree, Cocoa Semen, Cocoa Testae, Theobroma, Theobroma sativum, Theobromine, Kakau (Arabic), Arbore De Cacao, Arvore-Da-Vida, Cacaoeiro, Cacau-Da-Mata, Cacueiro (Brazil) (Lim, 2012).

2.2 COCOA VARIETIES

2.2.1 CRIOLLOS

The beans are considered to have a finer flavor than the other varieties and referred to as "flavor beans" due to their unique flavor characteristics. They are not disease-resistant, and hence hard to grow and keep healthy (Galleano *et al*., 2009).

2.2.2 FORASTERO

This is a wild species of which the Amelonado are the most extensively planted. Amelonado sub-varieties include Comum in Brazil, West African Amelonado in Africa, Cacao Nacional in Ecuador and Matina or Ceylan in Costa Rica and Mexico. Its disease resistant property makes it the economic favorite of top producing countries. This species has a richer "chocolate" flavor and referred to as the "bulk beans" (Galleano *et al*., 2009)

2.2.3 TRINITARIO

This species is considered to be among the Forasteros although they are a hybrid cross between Criollo and Forastero. It is highly sought after for its highly unique flavor and bulk (Galleano *et al*., 2009).

2.3 GEOGRAPHICAL DISTRIBUTION

There is tremendous variation among experts regarding the origins of cocoa. The Orinoco Valley of Venezuela, the Brazilian Amazon, or that it is native to Central America are possible locations (Galleano *et al*., 2009). Up until recently experts have based their claims on the use of cocoa throughout history. DNA sampling is now helping to sort out the geographical origin issue (Kilham and Helene, 2007). This remarkable tool will help to better understand the path cocoa has traveled throughout time till date. Though *Theobroma cacao* surely originated from South America, the tree is now cultivated in virtually every tropical area around the equator.

2.4 DESCRIPTIONAL OVERVIEW OF COCOA

According to the Encyclopedia of Life, Cocoa (*Theobroma cacao*) is described as a small evergreen tree in the Sterculiaceae family. It grows to about 4 – 8 m (15 - 26 feet) tall and is native to the tropical region of the Americas. Recent studies of *Theobroma cacao* genetics show that the plant originated in the Amazon and was distributed by man throughout Central America and Meso-America. Its seeds were used to make chocolate and other cocoa products such as cocoa powder, butter and liquor (Cuatrecasas, 1964).

Cocoa trees flourish in wet humid tropical regimes within 10° north and south of the equator in the lowlands. They are tolerant of temperature variations between 22 - 32°C. Cocoa prefers areas with mean annual rainfall of 1250 – 3000 mm. In its native habitat; cocoa grows best with the partial overhead shade of rubber and palm trees. It can tolerate brief periods of drought (with <100 mm rain) though it is more sensitive to water-logging but can withstand flooding. Cocoa thrives in rich, organic well-drained, moist, deep soils. An ideal cocoa soil should be at least 1.5 m deep, clay content 30 – 40 % with top soil having 2 % carbon, a cation exchange capacity of <120 mmol/kg and a base saturation of 35 %. Cocoa is grown on soils with a wide range of pH from 6 - 7.5 where major nutrients and trace elements will be available (Cuatrecasas, 1964).

The leaves of the cocoa tree are alternate, entire, unlobed, 10 - 40 cm (4 - 16 in.) long and 5 -20 cm (2 - 8 inches) broad. It is about the only cash crop that is hand crafted from its planting to harvesting (de-podding) stages then to the processing (drying, roasting, fermentation even bagging and storage) as opposed to other mechanizable processes.

Cocoa beans are technically not beans or legumes, but rather the seeds of the fruit of the *Theobroma cacao* tree. The pod-shaped fruit is botanically classified as baccate-like (berry-like) and each pod produces 35-50 seeds surrounded by thick sweet pulp (Cuatrecasas, 1964). The pod and pulp surrounding the beans constitute the fruit of cocoa. Its main characteristic is that the flowers and fruits are produced on stem of plant. The fruit of cocoa is similar to the shape of the rugby or American football. After harvest and de-podding, cocoa seeds and their surrounding fruit pulp are typically placed in heaps or boxes and fermented under the influence of naturally occurring microbes that multiply using the sugar from the pulp as an energy source. The seeds are then dried in the sun or temperately in wood fired ovens. The seeds then have their thin coats removed from the embryonic tissue, or roasted and milled depending on the mode of transportation. This is then packaged and shipped to the cocoa processors for finished products such as cocoa powder, chocolates and others.

Natural cocoa powder (NCP) is produced by the mechanical pressing of a sizeable amount of fat (cocoa butter) from the cocoa liquor. This pressed material is then crushed or ground and packaged into containers for the end user. For the purpose of this study the natural cocoa powder supplement is packaged as Golden Tree Royale Natural Cocoa Powder, manufactured by Cocoa Processing Company (CPC), Tema, Ghana.

2.5 PROCESSING AND PREPARATION OF NCP

Step 1 - Pods are harvested and de-podded, beans are cleaned, fermented and dried.

Step 2 – Bean roasting of the beans enhances the flavor and color.

Step 3 – Winnowing to remove the bean shells leaving the cocoa nibs.

Step 4 - Nibs undergo alkalization usually with potassium carbonate.

Step 5 – Alkalized nibs are then milled to create cocoa liquor

Step 6 - Cocoa liquor is pressed to extract the cocoa butter, leaving the cocoa presscake.

Step 7 - The presscake is grinded and pulverized to form the natural cocoa powder, which is then packaged for retail.

2.6 PICTORIAL DESCRIPTION OF COCOA



Image 1: Mature cocoa pods ripening on the stems of the cocoa trees


Image 2a: Fresh cocoa pods and beans. Image 2b: Dry cocoa pods and beans

2.7 CULTURAL AND MEDICINAL HISTORY OF COCOA

2.7.1 CULTURAL HISTORY OF COCOA

Cocoa has been regarded as precious food for health and longevity, usually consumed by royalty. The story of cocoa began as a fermented alcoholic drink, discovered in the Ulua

Valley of present day Honduras, between 1400 - 1100 BC (Maugh, 2007). It became an integral part of Aztec culture in Mexico – epitomized by the consumption habits of King Montezuma who was alleged to drink 50 cups of *xocoatl* a day. The Aztecs believed that the cocoa tree was brought by "the god of air", '*Quetzalcoatl'*, after man was driven from the equivalent of the Garden of Eden, the '*quachahuatl*' tree (Dand, 1997). They also attributed religious significance being consumed with spices, honey, and sometimes maize in addition. The beans were used as currency for trade by barter where hundred beans were traded for a slave among the Central Americans (Pomeranz and Topik, 2004). Mayans by 600 AD commenced large scale plantations.

Hernándes Cortés visited King Montezuma in 1519 and was struck by the popularity and religious significance attached to '*xocoatl'*. He "wrote to Charles I about a 'divine drink that banished tiredness and the Spanish court accepted the drink. The discovery of sugar greatly enhanced the taste. Cortés planted cocoa during his travels and soon after, the Spaniards began large-scale cultivation. By the 17th century, cocoa had spread to the British, French and Dutch territories in Central America. It arrived in Brazil during the 18th century, then São Tomé in 1822, island of Fernando Po (Equatorial Guinea) West Africa around 1854.

The spread of its cultivation in Ghana started when Tetteh Quarshie, a native of Osu, Accra, and his apprentice Adjah travelled to Fernando Po working as blacksmiths. He returned in 1879 with cocoa pods and established a farm at Akwapim Mampong where farmers bought pods to propagate (Phytochemical properties of Cocoa, 2013). Currently,

Ghana is Africa's second largest cocoa producer after Côte d'Ivoire with about 450 000 tons and an annual average growth rate of 1.6 % (Galleano *et al*., 2009).

2.7.2 MEDICINAL HISTORY OF COCOA

The use of cocoa as a primary remedy and a vehicle to deliver other medicines originated in the New World and diffused to Europe in the mid-1500s. With '*kakaw*' derived from the original Olmec and subsequent Mayan language now christened 'cocoa'. Colonial era documents like Badianus Codex of 1552 (Emmart, 1940) which noted the use of cocoa flowers to treat fatigue, whereas the Florentine Codex of 1590 (León-Portilla, 2002) offered a prescription of cocoa beans, maize and the herb '*tlacoxochitl*' (*Calliandra anomala*) to alleviate fever, panting of breath and treat the faint of heart. Subsequently manuscripts produced in Europe and New Spain between the 16th - 20th centuries revealed over 100 medicinal uses. Three consistent roles can be identified as:

1) To treat emaciated patients to gain weight

2) To stimulate nervous systems of apathetic, exhausted or feeble patients

3) To improve digestion and elimination where cocoa countered the effects of stagnant or weak stomachs, stimulated kidneys and improved bowel function (Dillinger *et al*., 2000). Other medical conditions/complaints treated with cocoa and its related products include anemia, loss of appetite, mental fatigue, poor breast milk production, tuberculosis, fever, gout, kidney stones, aging, sexual dysfunction and low virility. Cocoa paste was also a medium of drug administration as well as used to mask the bitter taste of other pharmacological additives. In addition, preparations of cocoa bark, oil (cocoa butter),

leaves and flowers were used to treat burns, bowel dysfunction, wounds, cuts and skin irritations (Dillinger *et al*., 2000, Katz *et al*., 2011).

2.8 PHYSICO-CHEMICAL CHARACTERISTICS OF NCP

2.8.1 PHYSICAL PROPERTIES OF NCP

Certain physical properties of NCP are important to both the consumers and manufacturers. Taste and color vary significantly depending on the source of cocoa beans and its processing techniques. NCPs have a characteristic light brown color. The ability to produce cocoa powders that are darker in color is of importance in the food industry as it yields a desirable rich dark brown color to confectionary. Specifically, alkalized cocoa has less bitterness and solubility compared to the untreated, unsweetened NCP.

2.8.2 COLOR OF NATURAL COCOA POWDER

The degree of color of NCP can be measured using the Hunter "L" scale. Miller *et al*. (2008) published that the Hunter "L" values for natural cocoa powders ranged from 38 - 41 exhibiting light brown colors. In contrast, alkalized cocoa powders had "L" values ranging from 16 - 35 depending on the degree of alkalization.

2.8.3 TASTE AND NATURE OF COCOA POWDER

Cocoa has a nutty, raw beany/green/bitter malta/caramel flavored taste, a distinguishably chocolaty aroma, a fine free flowing powder based on the fermentation and acidity indices (physical, titratable and volatile acidity), butterfat content, melting point,

polyphenols, purines and sugars used to characterize the beans as well as pyrazines and associated aromatic compounds (Sukha and Butler, 2006, Amores *et al*., 2007).

2.8.4 THERMOSTABILITY OF COCOA

Theobromine and polyphenols content of Cocoa have been found to be highly stable upon heating between 100°C and 120°C. Cocoa is stable at regular temperatures for food processing.

2.9 CHEMICAL PROPERTIES OF COCOA

Cocoa beans are virtually inedible because of their high concentration of polyphenols, which gives the distinct astringent taste. The polyphenol content decreases appreciably throughout the different processing techniques or stages (Rusconi and Conti, 2010). When cocoa liquor is exposed to intense pressure and temperature, cocoa butter is extracted resulting in a fine grinded powder (Monagas *et al*., 2009, Mellor *et al*., 2010, Andújar *et al*., 2012). An indication of the composition of natural cocoa powder (NCP) is as follows, though variation depending on the degree of roasting, fermentation, alkalization and pressing processes: Moisture 3.0%, Cocoa butter 11.0%, Ash 5.5%, Water soluble ash 2.2%, Alkalinity of water soluble ash as K_2O in original cocoa 0.8%, Phosphate (as P_2O_5) 1.9%, Chloride (as NaCl) 0.04%, Ash insoluble in 50% HCl 0.08%, Shell (calculated to unalkalized nib) 1.4%, Total nitrogen 4.3%, Nitrogen (corrected for alkaloids) 3.4%, Protein, Nitrogen corrected for alkaloids 21.2%, Theobromine 2.8% (Ribeiro *et al*., 2012). There also are typical phytochemicals found in *Theobroma cacao*:

the bean which is concentration of this largely contains flavanols, flavonoids, methylxanthines, reducing sugars, saponins, alkaloids and tannins.

Table 2.1: The Nutrient Content of the Study Cocoa powder

Nutrient/100 g powder		Nutrient/100 g powder	
Energy (kcal)	560	Total catechins (mg)	556.8
Protein (g)	7.1	Catechins (mg)	99.2
Carbohydrates (g)	53.4	Epicatechins (mg)	227.0
Fat (g)	35.7	Gallocatechins (mg)	16.4
Myristic acid (g)	0.5	Epigallocatechins (mg)	11.9
Palmitic acid (g)	6.9	Catechin gallates (mg)	9.4
Stearic acid (g)	7.5	Epicatechin gallates (mg)	0.6
Oleic acid (g)	8.0	Epigallocatehingallates (mg)	43.7
Linoleic acid (g)	1.0	Procyanidins (mg)	148.6
Linolenic acid (g)	0.1		

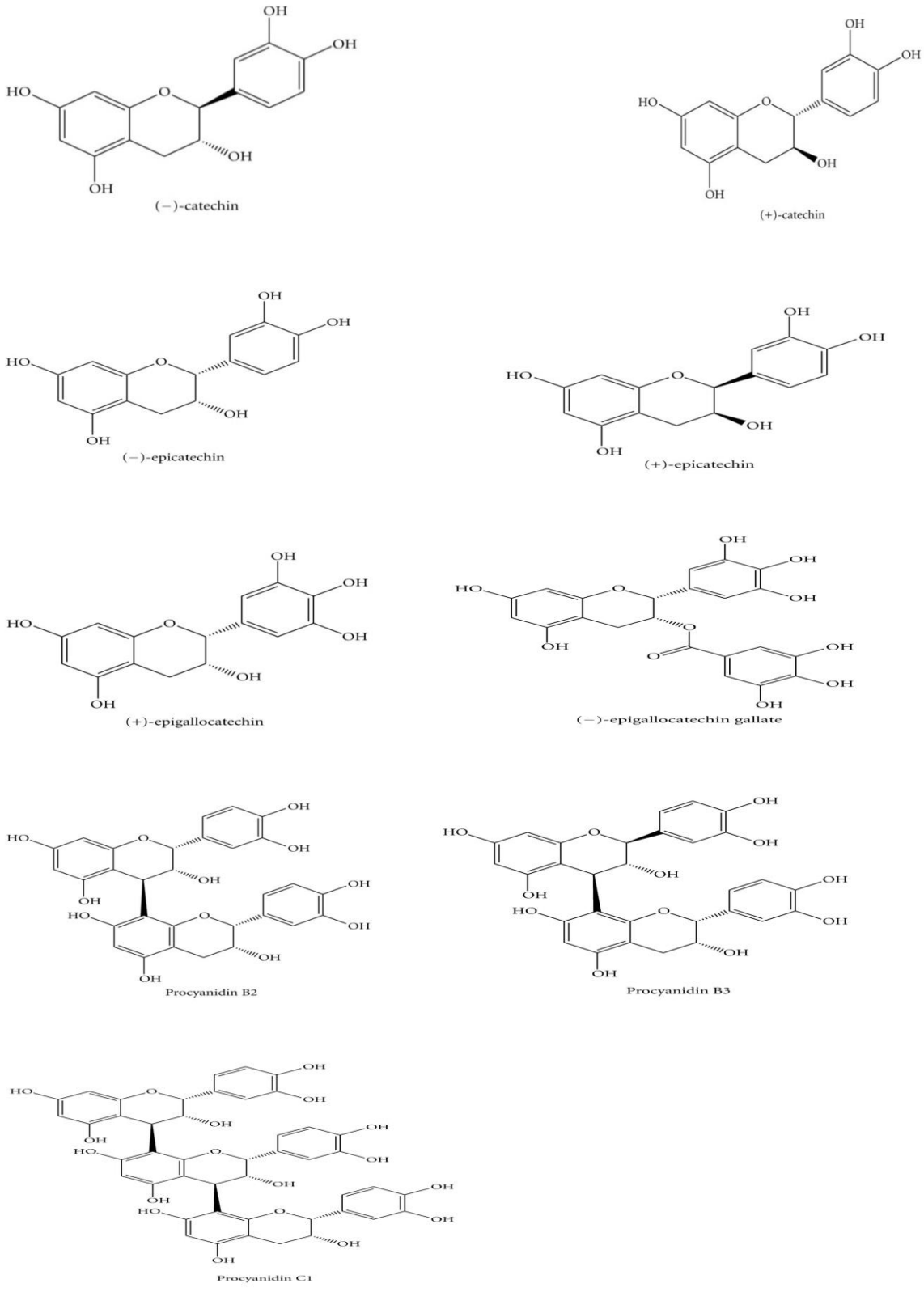


Figure 2.1: Chemical structure of major cocoa phenolics.

2.9.1 OTHER CHEMICAL CONSTITUENTS OF NCP

Natural cocoa powder contains protein, carbohydrates, fats like myristic acid, palmitic acid, stearic acid, oleic acid, linolenic acid, catechins, epicatechins, procyanidins and a variety of others like methyl xanthine theobromine (about 1 % of total weight) and caffeine (<0.1 %). In addition to this are serotonin, histamine, salsolinol, methyltetrahydroisoquinoline, phenethylamine, telemethylhistamine, spermidine, p-tyramine, 3-methyloxytyramine, tryptamine and spermine (Andújar *et al*., 2012).

2.10 PHARMACOLOGICAL ACTIVITY OF COCOA

The basic structural feature of flavonoids is the 2-phenyl-benzopyrane or flavane nucleus, which consists of two benzene rings (A and B), linked through a heterocyclic pyrane ring (C). Flavonoids inhibit a variety of eukaryotic enzymes and have a wide range of activities. Enzyme inhibition has been postulated to be due to the interaction of enzymes with different parts of the flavonoid molecule, e.g. carbohydrate, phenyl ring, phenol and benzopyrone ring (Havsteen, 1983). Flavones, also a class of flavonoids are potential blood circulatory enhancers in the brain suggesting that cocoa is potentially useful as a natural anti-depressant, anti-sclerotic and preventive against *Helicobacter Pylori* infections. The effects of cocoa in man are under-researched, owing possibly to the assumption that it is behaviourally inert. Toxicological research in animals using cocoa cannot be extrapolated to humans for several reasons despite the alarming results such as theobromine poisoning in dogs, cats and livestock (Tarka, 1982). Caffeine is also a comparative agent but its underlying mechanisms are still unclear. Of all constituents proposed to play a role in our

craving for chocolate, caffeine is the most convincing, followed by theobromine. Most other substances are unlikely to exude a psychopharmacological effect owing to the extremely low concentrations or its inability to reach the blood-brain barrier, whilst chronic chocolate craving and addiction can be explained by means of a culturally determined ambivalence towards chocolate (Smit, 2011).

Cocoa flavonoids possess several other pharmacological activities like anti-ageing, anti-bacterial, anti-fungal, anti-inflammatory, anti-diabetic, anti-hepatotoxic, anti-allergic, anti-thrombotic, anti-hypertensive, anti-tumor, CNS stimulant, myorelaxant, cardiotonic, nutritive and vasodilator properties (Osakabe *et al*., 2001). They also show lipooxygenase, cyclooxygenase, protein kinase C, tyrosine kinase activities (Shohaib *et al*., 2011). Flavonoids are inhibitory against phosphodiesterase causing an increase in c-AMP level yielding symptoms like vasodilatation (but vasoconstriction in the brain), palpitation, diuresis and bronchodilation. Furthermore, they promote fat metabolism which is strongly believed to be synergistic with caffeine and theobromine in accelerating fat breakdown (Smit *et al*., 2004, Smit, 2011).

Major dietary sources of flavonoids include tea (green and black), red wine, apples, tomatoes, cherry, onions, thyme, parsley, soya beans, other legumes, grape fruits, oranges, lemons, ginkgo, dark chocolate and Neem (*Azadirachta indica*) (Sokpor *et al*., 2012). Flavonoids are also strong inhibitors of xanthine oxidase (XO), indicated in the treatment of atherosclerosis, gout, hyperuricemia and reperfusion injury. They have also been shown to inhibit the growth of various cancer cell lines like prostate cancer *in vitro* and reduce the

tumor development (Nair *et al*., 2004). The aldo-reductase inhibitive property of flavonoids is useful in diabetes-induced retinopathy and cataract. Catechins specifically act as anti-ulceric agents by inhibiting the H+/ K ATPase (Shohaib *et al*., 2011).

2.11 BIOAVAILABILITY OF COCOA POLYPHENOLS

Cocoa polyphenols like epicatechins are well absorbed with maximum plasma concentration 2 hours after consumption and about 20% of the consumed epicatechin excreted in the urine (Williamson, 2009). In the case of dimers, Holt *et al*. (2002) described the presence of procyanidin dimers, especially B2 (epicatechin-(4β-8)-epicatechin) in peripheral blood of healthy adults who had consumed cocoa beverage likewise within two hours.

2.12 GASTRIC DEGRADATION OF COCOA POLYPHENOLS

There have been contradictory findings concerning the gastric stability of cocoa polyphenols. Spencer *et al*. (2000) reported that in the acidic gastric milieu, procyanidin oligomers are hydrolyzed to mixtures of epicatechin monomers and dimers. However, Rios *et al*. (2002) observed that during gastric transit, procyanidins were remarkably stable in the stomach environment, reached the small intestine intact and were available for absorption or metabolism. In addition, their limited absorption in the small intestine influenced the digestive process or the gut physiology. Consequently, some of the health effects of procyanidins may be associated with the formation by microflora of low-molecular-weight metabolites once the procyanidins reach the colon. This hypothesis has been corroborated by Gonthier *et al*. (2003) who demonstrated that while procyanidins of

high molecular weight were not present in plasma or urine, the monomers and aromatic acids produced during their metabolism by intestinal microflora can be found in the latter. Adding that catechin was well absorbed in the small intestine with recoveries of 20 – 40 % (Gonthier *et al*., 2003, Andújar *et al*., 2012).

2.13 MECHANISM OF ACTION OF COCOA CONSTITUENTS

Research shows that theobromine inhibits fat accumulation while cocoa polyphenols increases fat metabolism as shown in Fig. 2.2. Recent findings indicate that cocoa contains naturally occurring γ-butyric acid (GABA) which is beneficial in lowering elevated blood pressure and calming nervousness. This health promoting effects are augmented by the synergisms of theobromine and polyphenols. In addition to this, the relaxatory effect of cocoa is attributed to GABA. Detailed mechanism of action still remains unclear.

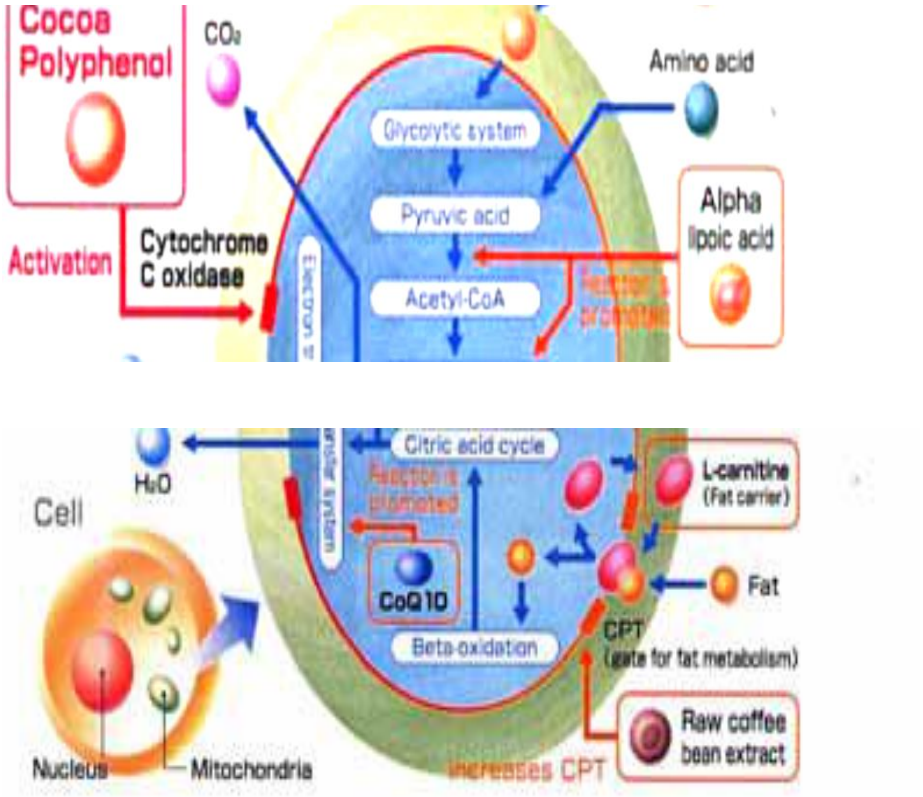


Figure 2.2: Mechanism of Action of Cocoa (Gallaneo et al., 2009)

2.14 DOCUMENTED RESEARCHES AND USES OF COCOA

Table 2.1: Effects of cocoa products on metabolism, cardiovascular diseases, inflammation and cancer prevention

(a)

Metabolic and endocrine disorders

	Assays	Effects
In vitro	Pancreatic α-amylase, lipase & secreted PLA_2 inhibitions (Gu *et al*., 2011)	(i) Cocoa dose-dependently inhibited pancreatic α-amylase, lipase and secreted PLA_2, showing inhibitory activity against PLA_2.
	HepG2 & Caco2 (Yasuda *et al*., 2011)	(i) (+)-catechin, (−)-EC, procyanidin B2, procyanidin C1, cinnamtannin A2 ↑Apo A1 ↓Apo B due to upregulation of SREBPs & ↑LDL receptor activity
In vivo	Randomized crossover trial of 19 hypertensive patients with impaired glucose tolerance (Grassi *et al*., 2008)	(i) Flavanol-rich dark chocolate (DC) (100 g/day for 15 days): (a) ↓insulin resistance, SBP & DBP, TC & LDL-C (b) ↑insulin sensitivity, β-cell function & FMD
	Randomized, crossover feeding trial in 42 high-risk volunteers (Khan *et al*., 2012)	(i) Chronic cocoa consumption (42 g/day for 4 weeks): (a) significant ↑of phase II metabolites (glucuronide, sulfate conjugates) in 24 h urine (b) ↑HDL-cholesterol (c) ↓LDL-cholesterol
	Meta-analysis of 8 randomized controlled trials involving 215 participants (Jia *et al*., 2010)	(i) Short-term cocoa consumption: (a) ↓LDL & TC (depending on the amount of cocoa consumed and the health status of participants)

Randomized, single-blind, crossover study with 14 overweight/obese subjects (Almoosawi *et al*., 2010)	(i) 20 g of DC with 500 mg of polyphenols and then 20 g of DC with 1000 mg of polyphenols or vice versa for 2 weeks separated by a 1-week washout period: (a) both doses were equally effective in reducing FBG levels, SBP & DBP
Randomized, placebo-controlled double-blind crossover trial (Mellor *et al*., 2010)	(i) High polyphenol chocolate (16.6 mg of ECs in 45 g): (a) ↑HDL-C (b) ↓LDL-C, TC/HDL ratio High-polyphenol chocolate is effective in improving the atherosclerotic cholesterol profile in patients with diabetes

(b)

Cardiovascular diseases

Assays	Effects
42 high-risk patients in a randomized crossover feeding trial for 4 weeks (Monagas *et al*., 2009)	(i) 40 g of cocoa powder with 500 mL skim milk/day: (a)↓VLA-4, CD40 & CD36 in monocytes (b) ↓serum concentrations of P-selectin & ICAM-1
20 healthy subjects followed a balanced diet for 4 weeks; day 14 - 27, introduced daily 45 g of DC (860 mg of polyphenols, of which 58 mg were EC) or white chocolate (WC) (5 mg polyphenols, undetectable EC) (Spadafranca *et al*., 2010)	(i) 2 h after DC intake (860 mg of polyphenols, of which 58 mg were EC): (a) detectable EC levels were observed (b) less DNA damage to mononuclear blood cells (c) no effect on plasma total antioxidant activity (ii) Effects were no longer evident after 22 h: DC

Blinded parallel-design study with 32 healthy subjects consuming 234 mg cocoa phenolics a day for 28 days (Murphy *et al*., 2003)	(i) ↑Plasma EC & catechin concentrations by 81% & 28% respectively (ii) ↓Platelet function
Double-blind, randomized study with 22 heart transplant recipients (Flammer *et al*., 2007)	(i) 2 h after ingestion of 40 g of flavonoid-rich DC (0.27 mg/g of catechin & 0.9 mg/g of EC, with a total polyphenol content of 15.6 mg of EC equivalents per gram): (a) ↑coronary artery diameter (b) improved endothelium-dependent coronary vasomotion (c) ↓platelet adhesion
Crossover, single blind study with 20 healthy subjects and 20 smokers who received 40 g of DC (cocoa > 85%) or milk chocolate (MC) (cocoa < 35%) (Carnevale *et al*., 2012)	(i) Smokers: (a) ↑platelet recruitment, platelet formation of ROS and eicosanoids, and NOX2 activation (ii) Smokers + DC group: (a) ↓platelet ROS & NOX2 activation decreased significantly (iii) Healthy +DC: (a) platelet variables did not change (iv) MC (smokers and healthy): (a) no changes detected in either of the groups treated with MC
Single oral administration of a natural flavonoid-enriched cocoa powder (50–600 mg/kg) in spontaneously hypertensive rats (Cienfuegos-Jovellanos *et al*., 2009)	Antihypertensive effect in hypertensive rats without modifying the arterial blood pressure in normotensive rats. No dose-response effect was observed
50 male Kurosawa and Kusanagi-hypercholesterolemic rabbits received 100 g/day of standard diet or cacao liquor polyphenol diet (Akita *et al*., 2008)	(i) Polyphenol-treated group: (a) ↓area of atherosclerotic lesions in the aortas of the polyphenol-treated group was significantly smaller than in the control group (b) preserved parasympathetic nervous tone

	(c) no differences in the plasma lipid concentrations
6-month clinical trial with 36 pre-hypertensive healthy adult volunteers, at content levels of 120–139 & 80–89 mmHg (Ried *et al*., 2009)	(i) 50 g of DC/day: (a) no significant differences were observed in the blood pressures of the treated and control groups

(c)

Anti-inflammatory properties

In vitro assays	Polyphenol	Effect
Unstimulated & LPS-stimulated PBMCs (Sanbongi *et al*., 1997)	Different flavanol fractions (20 µg/mL)	(i) Monomers-pentamers: ↑of LPS-induced synthesis of IL-1β, IL-6, IL-10 & TNF-α (ii) Long- & short-chain flavanol fractions: ↑the production of GM-CSF in the absence of LPS & the expression of CD69 & CD83
Macrophages (Desch *et al*., 2010)	(i) Epicatechin (58 µg/mL) (ii) Cocoa extract (50 µg/mL) (iii) Isoquercitrin (18.6 µg/mL)	(i) ↓MCP-1 &TNF-α at the transcriptional level (ii) ↓TNF-α, IL-1α & IL-6 mRNA levels (iii) ↓NO secretion
In vivo/Ex vivo	Polyphenol	Effect
DSS-induced ulcerative colitis in Balb/C mice (Andújar *et al*., 2012)	Cocoa polyphenol-enriched extract (500 mg/kg)	(i) *In vivo*: ↓weight loss, improves normal stool consistency, ↓visible blood in feces, ↓neutrophil infiltration, ↓NO production, ↓COX-2 expression, ↓STAT3 and STAT1α phosphorylation (ii) *Ex vivo* in peritoneal macrophages: ↓IL-6, IL-1β &TNF-α (iii) *In vitro* in LPS-stimulated RAW 264.7

		cells: inhibits NF-κB
Human PBMCs from 18 healthy volunteers (Vázquez-Agell et al., 2013)	Cocoa powder (40 g) with either milk or water	(i) Cocoa + water: (a) ↓NF-κB activation in leukocytes & adhesion molecules (b) ↓sICAM-1 (c) ↓E-selectin (ii) Cocoa + milk: (a) no change in NF-κB activation (b) ↓sICAM-1 (iii) Milk: (a) ↑NF-κB activation
RLE cells *in vitro* & *ex vivo* (Lee *et al*., 2010)		(i) Cocoa polyphenol extracts dose-dependently (10 –100 μM) attenuated in vitro hydrogen peroxide-induced (a) inhibition of GJIC (b) phosphorylation & internalization of connexin 43 (c) accumulation of ROS & activation of ERK (ii) *Ex vivo* in RLE cell lysates (a) inhibits hydrogen peroxide-induced MAPK/ MEK1 activity

(d)

Cancer prevention

In vitro

Cell line	Effects
Caco-2 cells (Carnésecchi *et al*., 2002)	(i) Treatment of cells with 50 μg/mL of procyanidin-enriched extracts: (a) ↓cell growth by 70%, blocking the cell cycle in the G_2/M phase (b) ↓activities of ornithine decarboxylase and S-adenosylmethionine decarboxylase & ↓the intracellular pool of polyamines

HepG2 cells (Martín *et al*., 2008)	(i) Pre-treatment of cells subjected to oxidative stress with 0.05–50 μg/mL of cocoa polyphenol extract for 2 or 20 h: (a) completely prevented cell damage & enhanced the activity of antioxidant enzymes (b) recovered levels of GSH (c) prevented in a dose-dependent fashion the ↑ROS
JB6 P+ cells (Kang *et al*., 2008, Kim *et al*., 2010)	(i) Cocoa procyanidin fraction (5 μg/mL) & procyanidin B2 (40 μM) inhibit (a) TPA-induced neoplastic cell transformation at 47 and 93% respectively (b) phosphorylation of MEK, ERK & p90 ribosomal s6 kinase (c) COX-2 expression (d) AP-1 & NF-κB activation & the TPA induced (ii) Cocoa polyphenol extract (5–20 μg/mL) (a) ↓TNF-α-induced upregulation of VEGF by ↓TNF-α-induced activation of AP-1 & NF-κB (b) ↓TNF-α-induced phosphorylation of Akt & ERK (c) ↓PI3K activity by binding PI3K directly (d) ↓TNF-α-induced MEK1 activity
RLE cells *in vitro* and *ex vivo* (Lee *et al*., 2010)	(i) Cocoa polyphenol extracts dose-dependently (10–100 μM) attenuated *in vitro* H_2O_2-induced (a) inhibition of GJIC (b) phosphorylation & internalization of connexin 43 (c) accumulation of ROS & activation of ERK (ii) *Ex vivo* in RLE cell lysates (a) inhibits H_2O_2-induced MAPK/MEK1 activity

In vivo

Animals	Effects
Wistar Han rats (5 weeks old) pre-treated for 2 weeks with a cocoa-rich diet and injected with azoxymethane once a week for 2 weeks (Rodríguez-Ramiro *et al*., 2011)	(i) The cocoa-rich diet (1 g of polyphenol/kg of diet): (a) anti-proliferative effects in azoxymethane-induced colon cancer: ↓ERK, Akt & cyclin D1 (b) pro-apoptotic effects: ↓Bcl-X_L levels and ↑levels of Bax & caspase-3 activity

Andújar *et al*., 2012

Other documented cocoa studies done include properties such as cardiotonicity (Ried *et al*., 2009), increased muscle recovery and energy metabolism (Karp *et al*., 2006), immuno-modulation (Abril-Gil *et al*., 2012). In addition to these cocoa is said to have vasodilatory (Recio-Rodriguez, 2012), hypotensive effects (Hollenberg *et al*., 2009), aphrodisiac (Afoakwa *et al*., 2008), antiseptic (Addai, 2010), pro-insulinemic (Jalil *et al*., 2009), nutritive (Golomb *et al*., 2012), anti-malarial (Amponsah *et al*., 2012), anti-thrombotic (Rein *et al*., 2000), anti-inflammatory (Balzer, 2008), CNS stimulant (Akita *et al*., 2008) and platelet reactivity (Flammer *et al*., 2007) properties. Cocoa also showed its hypo-lipidemic ability to decrease rat TC, LDL-C, RBC, triglycerides, increase WBC and platelet counts (Abrokwah *et al*., 2009), weight control and/or anti-obesity effects in humans (Matsui *et al*., 2005, Ferrazzano *et al*., 2009), cardiopulmonary and cerebrovascular functions (Karp *et al*., 2006, Sorond *et al*., 2008), retards age-related brain impairments and improves mood (Strandberg *et al*., 2008), suppresses appetite (Harper *et al*., 2007), decreases LDL lipid peroxidation, stimulates detoxification enzymes

(Maskarinec, 2009), slows tooth decay and gum damage (Tomofuji *et al*., 2009) as well as provides ocular (Osakabe *et al*., 2001) and UV protection (Williams *et al*., 2009).

2.15 COCOA AND OXIDATIVE STRESS

Electrons donated to molecular oxygen leads to an increase of superoxide anion (O_2.-) production i.e. ROS. Large quantities of ROS lead to cellular damage of lipids, membranes, proteins and DNA. ROS initiates lipid peroxidation leading to formation of lipid-derived reactive aldehydes associated with oxidative disease due to its high abundance and strong reactivity (Wang *et al*., 2000). Dietary and environmental factors promote free radical (FR) and ROS formation depleting cellular antioxidants (Kelly *et al*., 1995). ROS have physiological functions such as activation and modulation of signal transduction pathways (Monteiro and Stern, 1996), alteration of activities of redox-sensitive transcription factors (Manna *et al*., 1998) and regulation of mitochondrial enzyme activities (Nulton-Persson and Szweda, 2001). ROS levels are reduced by antioxidant defense and increased by transition metals such as iron (Fe), copper (Cu) or exogenous agents like ionizing radiation or ozone.

Oxidative modification of LDL plays a key role in the initiation of atherogenesis (Steinberg *et al*., 1989). Studies have also shown that flavonoids prevent LDL oxidation *in vitro* by scavenging radical species or sequestering metal ions (Morel *et al*., 1994, Salah *et al*., 1995). Kondo *et al*., (1996) reported that cocoa prolongs the lag time of LDL oxidation in a concentration-dependent manner. Waterhouse *et al*. (1996) found that at 5 µmol gallic acid equivalents/L cocoa phenols inhibited LDL oxidation by 75%, whereas red wines inhibited

LDL oxidation by 37-65%. The ability of flavonoids to prevent LDL oxidation in vivo depends on their bioavailability and binding to lipoproteins. Wang *et al* (2000) reported a reduction in plasma lipid peroxidation 2 and 6 hours after procyanidin-rich chocolate consumption. The consumption of cocoa fiber with a hypercholesterolemic diet improved the lipid profile, suggesting that cocoa fiber contributes to a reduction in cardiovascular risk (Lecumberri *et al*., 2007). Flavonoids react with nitric oxide (NO) (van Acker *et al*., 1995) and superoxide (Girard *et al*., 1995) to protect against oxidation and nitration reactions (Haenen *et al*. 1997, Pannala *et al*. 1997). Mathur *et al*. (2002) demonstrated reduced LDL oxidation after cocoa supplementation for 6 weeks compared to unsupplemented subjects. Baba *et al*. (2007) indicated that after 4 weeks of varying dosages of supplementation with cocoa powder, subjects showed decreased plasma LDL cholesterol, oxidized LDL and apo-B concentration respectively compared to the baseline. 12-weeks supplementation of cocoa powder to normo- and mildly hyper-cholesterolemic subjects showed decreased LDL oxidation and increased plasma HDL cholesterol compared to the controls (Mathur *et al*., 2002). Cocoa supplementation in healthy males significantly prevented LDL oxidation (Osakabe *et al*., 2001). However, no long-term studies in Ghana have evaluated the effects of cocoa in whole or part on oxidative modifications, cell membrane stability, inflammation, heme degradation, antioxidant status and blood profile in healthy humans, hence this study.

2.16 COCOA AND ANTIOXIDANT ACTIVITY

The antioxidant system is composed of compounds such as:- superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione (GSH), glutathione

reductase (GSSH), glucose-6-phosphate dehydrogenase (G6PD) (Melster and Anderson, 1983), glutaredoxin and thioredoxin systems (Lundberg *et al*., 2001), uric acid, vitamins A, C, E as well as protein and DNA repair enzymes (Holmgren, 1985, Hedley and Chow, 1994, de Zwart *et al*., 1999, Droge, 2002). The cocoa flavanol content affecting the antioxidant activity is dependent on the cocoa-processing techniques such as fermentation, alkalization, roasting and even bean species used (Arteel *et al*., 2000), though the study supplement is already processed. The antioxidant properties of cocoa flavanols are partially ascribed to their structural characteristics, including the oligomeric chain length and the stereochemical features of the molecule. These structural characteristics of flavanols represent the molecular basis for their radical-scavenging property (Kofink *et al*., 2007, Radojčić *et al*., 2009). Long term feeding studies of cocoa showed an increase in total plasma antioxidant capacity and a reduction in susceptibility to oxidative injuries (Keen *et al*., 2005). For the anti-oxidative activity of cocoa, SOD mimicking activity and the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of cocoa are highly exploited.

2.16.1 OXYGEN RADICAL ABSORBANCE CAPACITY (ORAC) VALUE OF NATURAL COCOA POWDER (NCP)

ORAC values are expressed in micromoles of Trolox Equivalents per 100 grams of sample (laboratory measure). The antioxidant value of dry unsweetened Cocoa powder in ORAC units is: 25,653 μmol TE/100g. Below are examples of foods with their ORAC values (Haytowitz and Bhagwat, 2010).

Top antioxidant foods	ORAC value per 100g
Unprocessed cocoa powder	26000
Acai berry	18500
Processed dark chocolate	13120
Prunes	5770
Raisins	2830
Blueberries	2400
Blackberries	2036
Strawberries	1540
Spinach raw	1260
Broccoli	890
Red grapes	739

The values for antioxidant activity, measured with the aid of 2,2′-azino-bis(3-ethyl benzthiazoline-6-sulphonic acid) ($ABTS^+$) and DPPH methods, were 1128 and 836 mg/serving respectively, expressed as vitamin C equivalent antioxidant capacity. When compared with red wine, green and black tea, the relative activity of cocoa was 1.8, 2.5, and 4.4 times higher, respectively, in the $ABTS^+$ test, and 1.7, 2.4, and 4.3 times higher, respectively, in the DPPH test. Hence, on a per-serving basis, cocoa has higher flavonoid content and antioxidant capacity than red wine (2 times), green tea (2 - 3 times), and black tea (4 - 5 times) respectively (Andújar *et al*., 2012).

2.17 COCOA AND CELL MEMBRANE STABILITY

The mechanism of action involved in membrane stability include uncoupling of oxidative phosphorylation (ATP biogenesis linked to respiration), inhibiting denaturation of protein, erythrocyte membrane stabilization, lysosomal membrane stabilization, fibrinolytic activities and platelet aggregation. Flavonoids exert profound stabilizing effect on lysosomal membrane both *in vivo* and *in vitro*, while tannins stabilize erythrocyte and biological macromolecular membranes by binding to cations (Oyedapo *et al*., 2010). Using

flow mediated dilation (FMD) of the brachial artery; a single dose of cocoa drink increased nitric oxide (NO) in human plasma thus improving endothelial function (Heiss *et al*., 2007). Balzer *et al*. (2008) also reported that cocoa ingestion improved basal FMD by 30% without changes in endothelial function, blood pressure, heart rate and glycemic control among diabetics. Cocoa procyanidins exhibited endothelium-dependent relaxation (EDR) through activation of nitric oxide synthase (NOS) activity in rabbit aortic rings in vitro (Karim *et al*., 2000, Jalil *et al*., 2009).

2.18 COCOA AND INFLAMMATION

Suppression of the innate immune response inflammatory cytokine production is one of Cocoa's healthful benefits (Sanbongi *et al*., 1997, Mao *et al*., 2003). The effect of cocoa against Aβ-induced apoptosis may be due to the dual effects of mitochondrial and membrane protection. Due to its strong chemo-preventive activities, the additional benefit of anti-neurotoxicity suggests that daily consumption of cocoa may also provide added health benefit by reducing the risk of neurodegenerative diseases such as atherosclerosis, Alzheimer's disease (Heo and Lee, 2005).

2.19 COCOA, HEMATOLOGICAL INDICES AND PLATELET FUNCTION

Platelet activation in response to endothelial injury leads to thrombus formation in damaged arteries (Linden and Jackson, 2010). They are also important mediators of inflammation and play a central role in atherogenesis. The development of drugs interfering with platelet–vessel wall interaction at various stages of thrombus formation is crucial to

managing cardiovascular risk. Based on epidemiological studies, diets rich in flavonoids can reduce the risk of arterial thrombosis (Arts and Hollman, 2005).

The moderate consumption of cocoa-derived products, especially NCP significantly improves platelet function (Flammer *et al*., 2007). Significant inhibition of platelet aggregation and activation has been found upon the acute or chronic intake of dietary cocoa flavanols (Heptinstall *et al*., 2006). Studies show that the intake of 100 mg of cocoa flavanols induced a 3 – 11% reduction in platelet aggregation. The physiological relevance of the beneficial effects on platelets after supplementation with cocoa has been estimated to be comparable to standard doses of aspirin (~80 mg) clinically used as anti-aggregant therapy (Pearson *et al*. 2002).

Very few human studies have examined the effects of polyphenols-rich beverages such as tea, coffee and alcoholic beverages on hematological indices and platelet function without consensual results. Consumption of one litre per day of black tea was shown to inhibit platelet activation by 4 – 10% (Steptoe *et al*. 2007). Natella *et al*. demonstrated that the acute intake of 200 ml of coffee had significant anti-aggregatory effects (Natella *et al*., 2008). The chronic intake of polyphenols may result in only a relatively low inhibition of platelet aggregation under shear stress (Ostertag *et al*., 2010), all these had varying results for the hematological indices. Though other fruit juices such as orange, grapefruit and sea buckthorn taken daily for 7–10 days in healthy volunteers failed to reduce *ex vivo* platelet activity (Eccleston *et al*., 2002). There is little or no indigenous studies on the effect of natural cocoa powder on the hematological indices after 12 weeks supplementation in apparently healthy subjects, hence this study.

2.20 OVERVIEW OF RELEVANT BIOMARKERS

2.20.1 ADVANCED GLYCATED END PRODUCTS (AGEs)

AGEs reduce the bioavailability and activity of endothelium-derived NO. Because NO inhibits many of the mechanisms that contribute to atherosclerosis such as leukocyte adhesion to the vessel wall, vascular smooth muscle growth, platelet adhesion and aggregation (Bucala *et al*., 1991, Asahi *et al*., 2000). Hogan *et al*., (1992) demonstrated that matrix-bound and serum AGEs inhibits the anti-proliferative effects of NO. Vasoconstriction in diabetes and its complications results in AGEs' reduction of NO activity (Bucala *et al*., 1991, Tan *et al*., 2002). Several mechanisms by which AGEs reduce NO activity have been proposed. One suggests that AGEs reduce the half-life of endothelial NO synthase (eNOS) mRNA through an increased rate of mRNA degradation and reduced eNOS activity (Rojas *et al*., 2000). Another mechanism proposes that AGEs impair NO production via the binding of chronic myeloid leukemia (CML) residues to endothelial AGE receptors. This causes decrease in phosphorylation of serine residues in eNOS resulting in deactivation of the enzyme (Chakravarthy *et al*., 1998, Xu *et al*., 2003). AGEs deactivates endothelium-derived NO (eNO) and reduces the endothelial production of prostacyclin (PGI2) (Yamagishi *et al*., 1998). It also enhances the expression of endothelin-1, via NF-κB, in bovine aortic endothelial cells incubated with erythrocytes from patients with type 2 diabetes mellitus (Quehenberger *et al*., 2000). AGE-bound RAGE in the endothelium results in the production of reactive oxygen intermediates, triggered through the activation of NADPH oxidase. AGE-RAGE interaction stimulates the production of ROS activating a range of signaling pathways which include activation of NF-κB (Yan *et al*., 1994, Wautier *et al*., 2001).

2.20.2 HEME OXYGENASE 1 (HO-1)

Heme oxygenase (HO) is the rate-limiting enzyme in heme degradation. It catalyzes the oxidation of heme to generate several biologically active molecules - carbon monoxide (CO), biliverdin, and ferrous ion (Maines, 1983). The endogenously produced CO serves as a second messenger affecting several other cellular functions including inflammation, proliferation and apoptosis (Maines, 1983, Ryter *et al*., 2006). Biliverdin is subsequently reduced to bilirubin, both of which have antioxidant properties. Ferrous iron induces ferritin expression important for iron sequestration. There are three isoforms in the HO family - HO-1, HO-2, and HO-3. They are products of different genes and thus regulated differently. HO-1 is normally expressed at low levels in most tissues/organs except the spleen; however, it is highly inducible in response to a variety of stimuli to protect cells against oxidative stress and inflammatory injury (Ryter *et al*., 2006).

In inflammation the upregulation of HO-1 results in suppression of immune effector functions. HO-1 induction by cobalt protoporphyrin decreases the lympho-proliferative response and differentiation of cytotoxic T cells (Woo *et al*., 1998). Given the anti-inflammatory function of HO-1 and its expression in atherosclerotic lesions, supports its role in atherogenesis (Wang *et al*., 1996). HO-1 overexpression by pharmacological inducers or viral gene transfer inhibits atherogenesis in hypercholesterolemic animal models (Ishikawa *et al*., 2001a, 2001b; Juan *et al*., 2001). On the other hand, genetic ablation of both apo E and HO-1 in mice accelerates the development of atherosclerosis and exacerbates lesion formation, demonstrating unequivocally an essential role of HO-1 in protecting against atherogenesis (Yet *et al*., 2003). HO-1 expression in macrophages

increases antioxidant protection and decreases inflammatory components of atherosclerotic lesions. Decreased HO-1 expression in macrophages correlates with increased pro-inflammatory cytokines expression, such as monocyte chemo-attractant protein 1 (MCP-1) and interleukin 6 (IL-6), scavenger receptor A (SR-A) expression, and foam cell formation (Orozco *et al*., 2007). Anti-atherogenic effects of a number of mediators including statins are mediated through HO-1 induction (Lee *et al*., 2004, Heeba *et al*., 2009). Collectively, these studies establish that HO-1 is critical in anti-inflammation and protection against vascular diseases that result from the manifestations of inflammation, such as atherosclerosis (Chen *et al*., 2001). Cocoa is said to exert part of its beneficial effects through activation of Nrf2 and an increase in the neuroprotective HO-1 enzyme (Shah *et al*., 2010).

2.20.3 MALONDIALDEHYDE (MDA)

Malondialdehyde (MDA) is a natural product formed in all mammalian cells as a product of lipid peroxidation. It is a highly reactive three carbon dialdehyde produced as a byproduct of polyunsaturated fatty acid peroxidation and arachidonic acid metabolism (Acris antibodies, 2012). MDA readily combines with several functional groups of molecules including proteins, lipoproteins and DNA. It reacts with DNA to form adducts to deoxyguanosine and deoxyadenosine (Acris antibodies, 2012). The major adduct to DNA is called pyrimidopurinone M1G which appears to be a major endogenous DNA adduct in human beings that may contribute significantly to cancer linked to lifestyle and dietary factors (Acris antibodies, 2012). MDA modified proteins show altered physicochemical behavior and antigenicity. MDA is toxic and has been implicated in aging mutagenesis,

carcinogenesis, diabetic nephropathy and radiation damage. Increased expression of MDA has been reported in the brains of Alzheimer's patients. Antibodies to MDA will help to visualize the MDA adducts. Cocoa powder-treated rats showed significantly increased level of MDA in their liver tissues after 21 days (Noori *et al*., 2009).

2.20.4 VASCULAR CELL ADHESION MOLECULE (VCAM-1)

The vascular cell adhesion molecule 1 (VCAM-1) mediates leukocyte rolling and adhesion to the endothelium during VCAM-1-dependent eosinophil infiltration into the lung in experimental ovalbumin-induced asthma (Chin *et al*., 1997), as well as T-cell infiltration across the blood-brain barrier (BBB) in experimental allergic encephalomyelitis (Baron *et al*., 1993). VCAM-1-dependent migration is important *in vivo* because in several diseases leukocytes migrate on VCAM-1. Because of this critical role for VCAM-1 in these diseases, targeting of VCAM-1 or its ligand VLA-4 has been used to treat clinical diseases (Abdala-Valencia *et al*., 2011). During inflammation, adhesion molecules regulate recruitment of leukocytes to inflamed tissues. It is reported that VCAM-1 activates extracellular regulated kinases 1 and 2 (ERK1/2), but the mechanism for this activation is unknown. Leukocyte binding to VCAM-1 triggers signaling events in endothelial cells that are critical during VCAM-1-dependent trans-endothelial migration (TEM) (Abdala-Valencia *et al*., 2011). It activates the endothelial cell NADPH oxidase NOX2 which catalyzes the release of low levels of ROS (1 µM H_2O_2) (Matheny *et al*., 2000). H_2O_2 diffuses through membranes to oxidize and transiently activate endothelial cell-associated protein kinase Cα (PKCα). PKCα then phosphorylates and activates endothelial cell protein tyrosine phosphatase 1B (PTP1B). VCAM-1 signals through ROS, PKCα, and PTP1B are

required for VCAM-1-dependent leukocyte TEM *in vitro* (Deem *et al*., 2007). Nicotinamide adenine dinucleotide phosphate-oxidase 2 (NOX2) and ROS are also required for VCAM-1-dependent leukocyte recruitment *in vivo* (Berdnikovs *et al*., 2009, Abdala-Valencia *et al*., 2011, Keshavan *et al*., 2005).

2.20.5 TUMOUR NECROSIS FACTOR – ALPHA (TNF-α)

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic inflammatory cytokine. Most organs appear to be affected by TNF-α, the cytokine serves a variety of functions, many of which are not yet fully understood. The cytokine possesses both growth stimulating properties and growth inhibitory processes, and it appears to be self-regulatory properties. For instance, TNF-α induces neutrophil proliferation during inflammation, but it also induces neutrophil apoptosis upon binding to the TNF-R55 receptor (Murray *et al*., 1997). TNF-α is produced by several cell types especially macrophages. Tracey and Cerami (1993) suggested that two beneficial functions of TNF-α have led to its continued expression. First, the low levels aid homeostasis maintenance by regulating the body's circadian rhythm. Next, it promotes the remodeling or replacement of injured and senescent tissue by stimulating fibroblast growth.

Additional functions of TNF-α include its role in the immune response to microbial invasions as well as necrosis of specific tumors. It also acts as a key intermediary in the local inflammatory immune response. TNF-α is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection. TNF-α secreted by the macrophage causes blood clotting which serves to contain the infection. Without TNF-α, mice infected with gram

negative bacteria experience septic shock (Janeway *et al*., 1999). Although TNF-α causes necrosis of some types of tumors, it promotes the growth of other types of tumor cells. High levels of TNF-α is said to correlate with increased risk of mortality (Rink and Kirchner, 1996). TNF-α participates in both inflammatory disorders of inflammatory and non-inflammatory origins (Strieter *et al*., 1993). Exogenous and endogenous factors from microbes stimulate production of TNF-α and other cytokines. Lipopolysaccharide from bacteria cell walls is a potent stimulus for TNF-α synthesis (Tracey and Cerami, 1993). Victims of septic shock may experience fever, falling blood pressure, myocardial suppression, dehydration, acute renal failure and then respiratory arrest (Tracey and Cerami, 1993). Increase in cytokine production to an extent that it escapes the local infection, or enters the bloodstream develops into sepsis, body organs fail and death results (Janeway *et al*., 1999). Monocyte chemo-attractant protein 1 (MCP-1) and TNF-α were significantly and dose-dependently reduced by cocoa extract, this effect was shown to be higher than that produced by equivalent concentrations of epicatechin but was lower than that produced by isoquercitrin. Cocoa extract added prior to cell activation resulted in a significant inhibition of TNF-α secretion. Both cocoa extract and epicatechin decreased TNF-α, IL-1α and IL-6 mRNA expression, suggesting that their inhibitory effect on cytokine secretion is produced partially at the transcriptional level (Ramiro *et al*., 2005). Cocoa extract also significantly decreased NO secretion in a dose-dependent manner and with a greater effect than that produced by epicatechin. In conclusion, our study shows that cocoa flavonoids not only inhibit NO release from macrophages but also down-regulate inflammatory cytokines and chemokines (Ramiro *et al*., 2005).

2.20.6 GLUTATHIONE PEROXIDASE (GSH-Px)

Several proteins in mammalian cells can metabolize hydrogen peroxide and lipid hydroperoxides in the body. These proteins include four selenium-containing glutathione peroxidases that are found in different cell fractions and tissues of the body. Selenoperoxidases are involved in cell antioxidant systems. However, they also have more subtle functions in ensuring the regulation and formation of arachidonic acid metabolites that are derived from hydroperoxide intermediates. The range of biological processes, which are potentially dependent on optimal selenoperoxidase activity in mammals, emphasizes the importance of achieving adequate selenium intake in the diet. Molecular techniques have allowed overexpression of GSH-Px in mammalian cell lines, from which it has become clear that lipid hydroperoxides also have an important function as activators of lipooxygenase and cyclooxygenase participate in inflammation, and act as signal molecules for apoptotic cell death and receptor-mediated signal transduction at the cellular level.

CHAPTER THREE

3.0 METHODOLOGY

3.1 STUDY SITE

The study subjects were recruited from the School of Allied Health Sciences (SAHS), College of Health Sciences, University of Ghana, Korle-Bu. Laboratory assays were performed at the Chemical Pathology Unit Laboratory, SAHS; Virology Laboratory and Physiology Department, both under University of Ghana Medical School (UGMS) and the Central Laboratory of the Korle-Bu Teaching Hospital, Accra.

3.2 ETHICAL ISSUES

Ethical clearance was sought and obtained from the Ethical and Protocol Review Committee of the University of Ghana Medical School (UGMS), Korle-Bu with reference number MS.Et/M.8-P.4.2/2011-1012. Written informed consent was obtained from all the participants (refer to the Appendix). The study strictly adhered to the Declaration of Helsinki principles and guidelines concerning human subjects (2008).

3.3 STUDY DESIGN

This was a therapeutic intervention with single group assignment as the intervention model. It was a randomized longitudinal and comparative study design with endpoint classification for evaluation and efficacy. The intervention type used in this study was a food supplement Golden Tree Royale® natural “unsweetened” cocoa powder labeled with a 5-digit FDB/DK code. There was open label masking where both the investigator and subjects were aware

of the supplement was employed. The primary purpose of this study was to validate the effect of cocoa supplementation on healthy people and encourage the daily intake of NCP for healthy living. The supplementation period was between August – November, 2012.

3.4 STUDY SUBJECTS

The fifty-four (54) subjects were age and weight matched apparently healthy Ghanaians. There were 31 males and 23 females aged between 21 - 55 years. They were randomly selected from the consenting staff and students of SAHS who were at post as at the time of the study and domiciled in the SAHS hostel without restriction to age, gender or socioeconomic status.

3.5 SAMPLING TECHNIQUE

The sampling technique used for this study was the simple random sampling method**.**

3.6 INCLUSION AND EXCLUSION CRITERIA

3.6.1 INCLUSION CRITERIA

- Subjects who had not been hospitalized or seen medical personnel with disease conditions for at least 2 months prior to the start of the study.
- Staff and students of SAHS to enable strict monitoring and optimum compliance.
- Subjects who underwent the wash-out pre-selection stage and were advised to reduce to the minimum consumption of flavonoid-rich diets [such as tea (green and black), coffee, red wine, onions, beans, soybeans, seafood, oranges and grape juice] and cocoa-related products throughout the study period.

- Non-alcoholics, non-smokers, non-cocoa allergists, non-pregnant, non-menopausal, healthy participants with body mass indices between 18.5 and 24.99 kg/m^2, so as to allow for the comparison of the baseline characteristics between the two BMI categories.

3.6.2 EXCLUSION CRITERIA

- Participants with self-reported Hb AS, Hb SC and Hb SS genotypes as well as β- or α-thalassemic traits.
- Participants already on any other antioxidant therapy and medications known to affect growth or nutritional status (e.g. growth hormone and glucocorticoid therapy), herbal supplements, active smokers, morbidly obese, alcoholics, allergists to cocoa or dairy products, lactose or caffeine intolerants.
- Participants younger than 18 or older than 55 years of age.
- Participants with heart rhythms other than sinus rhythm and very high physical activity levels
- Participants with chronic illnesses such as CVD, hypertension, bleeding disorders.

3.7 SAMPLE SIZE DETERMINATION

The variables used to calculate the power of the study was oxidative damage, cell membrane stability, inflammation, antioxidant status, anthropometrics and blood indices. In order to obtain a probability of 95 %, the study detected treatment differences at a two-sided 0.05 significance level, assuming the treatment difference was 16 and within-subject

standard deviation of the response variable was 10. Using the statistical formula for the determination of sample size in comparative studies of two groups, $n = 4\sigma^2 (z_{crit} - z_{pwr})^2 / d^2$ where σ - standard deviation of each group (assumed to be 10), z_{crit} - confidence level 95 % (1.96), z_{pwr} statistical power at 95 % (1.645), d - minimum expected difference between the means was assumed to be 16, Thus the total sample size for the study was

$n = 4 \times 10^2 (1.96 + 1.645)^2 / 16^2$

n = 20 subjects but for the purpose of this study, a surplus sample size of 54 subjects to make up for the drop-outs.

3.8 EXPERIMENTAL DESIGN

Structured questionnaires were administered screening for physical activity levels, use of other dietary supplements, intake of cocoa-related products and alcohol, medical history, as well as their anthropometry. They were assigned to the Pre-intervention (Pre-) group on day 1 to begin consumption of the natural cocoa powder (NCP) beverage.

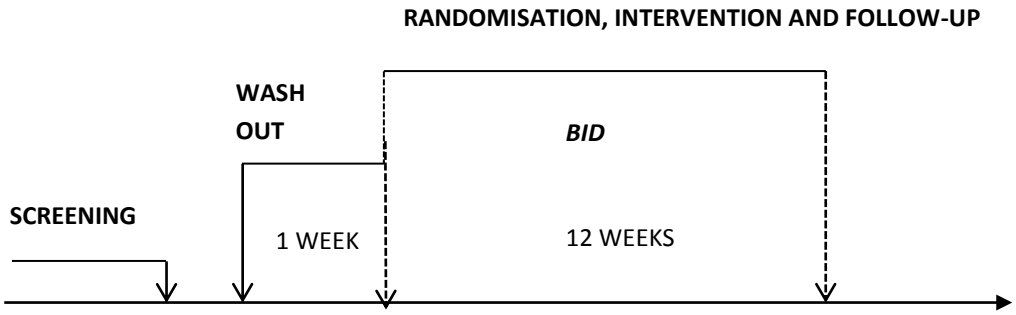
Routine anthropometric measurements and biochemical analyses was performed on Week 0 (Day 1) and referred to as the Pre-supplementation group, and on the day after completion of the supplementation Week 12 (Day 85) referred to as the Post-supplementation group (each subject in the test group was a respective control at Week 0 Day 1).

3.9 PREPARATION OF NATURAL COCOA POWDER BEVERAGE

Two heaped tablespoonfuls (each weighing 15 g, total fat of 17 %DV, 450 mg flavonoids, 612 kcal /2568 KJ per 100g) of Golden Tree™ Royale® NCP was reconstituted in 200 mL

mug with warm water. Subjects drank NCP beverage twice daily for 12 weeks. The first serving was taken at 7.00 am in the morning (breakfast) and the second at 8.00 pm in the evening (dinner). The extent of efficacy *in vivo* was dependent on the individual absorption, metabolism, distribution and excretion rates.

A. OUTLINE OF EXPERIMENTAL DESIGN



CONSENT	+	-
QUESTIONNAIRES	+	-
ANTHROPOMETRY	+	+
BLOOD DRAW	+	+
COMPLIANCE	+	+
NCP SUPPLEMENTATION	+	+

3.10 ANTHROPOMETRY

Height and weight to the nearest millimeter and 0.1 kilogram respectively of each subject in light clothing were measured using a stadiometer and digitalized scale. Subjects also had their percentage body and visceral fat, percentage skeletal muscle, body mass index (BMI; in kg/m^2) and basal metabolic rate (BMR) measured to the nearest whole number with the TBF-501 Tanita Corp bio-electrical impedance fat

analyzer (IL, USA). Participants were asked to stand barefooted with their knees straightened, their heels placed together against the backdrop and the head positioned such that the plane that passes between the upper margin of the ear canal and the lower border of the eye (Frankfurt Plane) was parallel to the floor.

3. 10b BLOOD PRESSURE DETERMINATION

The systolic, diastolic blood pressures and pulse rates were measured with the digital Lumiscope Co, Inc. auto-inflating blood pressure monitor (NJ, USA). Each subject in a comfortably seated position after 15 minutes in warm room with the cuff (large size 14.2 - 17.7" (26 - 45cm)) placed at the left upper arm of each subject, 2 - 3cm above the antecubital vein.

3.10c BLOOD SAMPLE ANALYSIS

The baseline full blood count (hematological indices) and serum biochemical ELISA markers namely: malondialdehyde (MDA), glutathione peroxidase (GPx), heme oxygenase (HO-1), advanced glycated end-products (AGEs), vascular cellular adhesion molecule (VCAM-1), tumour necrosis factor alpha (TNF-α) obtained from MyBioSource® Inc. San Diego, California, USA were assessed. The same parameters were measured again at the end of the 12 weeks NCP supplementation. The fixed time for the 5ml fasting blood sample collection on the stipulated visits was 7:00 am. Two-thirds of the blood samples about were shared into the serum separator tubes and centrifuged at 3000 revolutions per minute for 5 minutes, 37°C and the serum aliquoted into labelled Eppendorf tubes stored at a temperature of – 20°C until subsequent

analyses. However, the remaining one-third of the blood samples was collected into EDTA tubes were analysed immediately for the hematological indices.

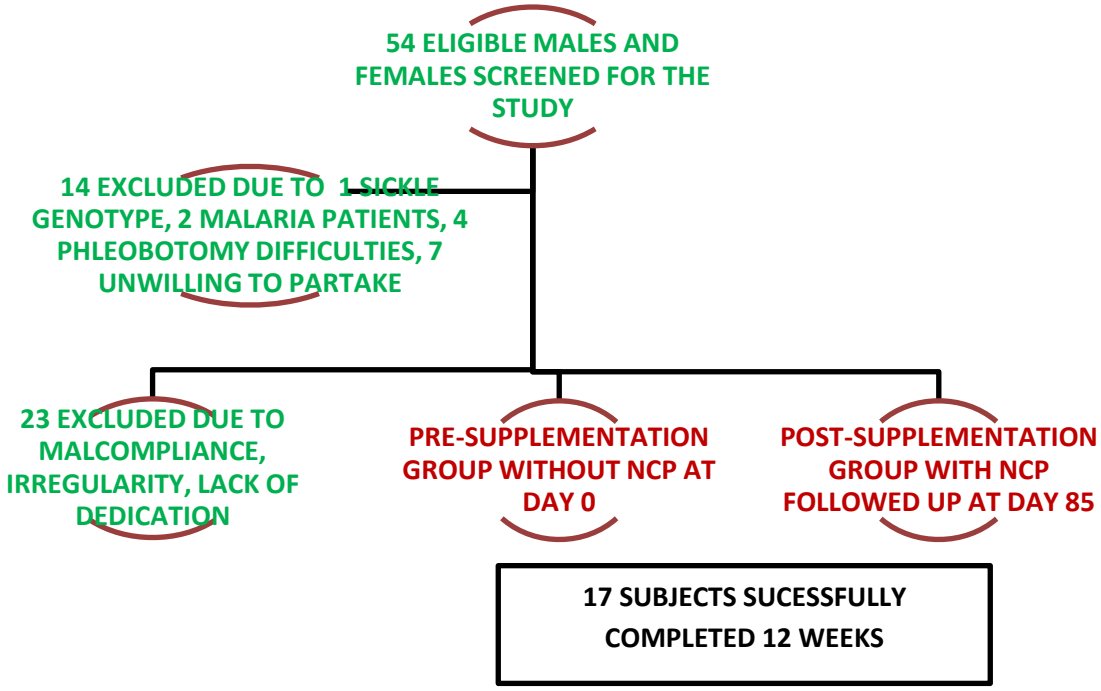
3.11 PROCEDURE FOR BLOOD SAMPLE COLLECTION

Each subject after 15 minutes of sitting comfortably in warm room had the tourniquet placed on the left upper arm 2 - 3cm above the antecubital vein, blood was drawn from the vein using sterile syringe in the presence of trained phlebotomists according to the procedure described by Grassi *et al*. (2008). The blood samples were collected in the morning between the hours of 7 - 9 am.

3.11.1 SAMPLE STORAGE

Gel separator tubes were used to collect the serum after the samples were allowed to clot for 30 minutes before centrifugation at approximately 1000 x g for 15 minutes at 2 - 8°C using the Hettich EBA 20, (Zentrifugen, Germany) tabletop centrifuge. All the samples were stored at - 20°C in freezers with an alternative power supply to avoid repeated freeze-thaw cycles due to power failure.

B. CONSORT ILLUSTRATING PARTCIPANT FLOW AND STUDY DESIGN



3.12 LABORATORY ASSAYS

3.12.1 HUMAN MALONDIALDEHYDE (MDA) ELISA:

3.12.1.1 PRINCIPLE OF THE ASSAY

This assay employs the competitive inhibition enzyme immunoassay technique. An antibody specific to MDA had been pre-coated onto a microplate. Standards or samples were added to the appropriate microtiter plate wells with HRP-conjugated MDA and incubated for an hour. A competitive inhibition reaction was launched between MDA (Standards or samples) and HRP-conjugated MDA with the pre-coated antibody specific for MDA. The more amount of MDA in the samples, the less antibody bound by HRP-conjugated MDA. The substrate solutions were then added to the wells. The

color obtained was a direct opposite to the amount of MDA in the sample. The intensity of the color was measured after there was no further color change.

3.12.1.2 DETECTION RANGE, SPECIFICITY AND SENSITIVITY OF MALONDIALDEHYDE (MDA)

The standard curve concentrations used for the ELISA was 40, 10, 2, 0.4 and 0.1 µg/ml respectively. This assay recognized only human MDA. No significant cross-reactivity or interference was observed. The minimum detectable dose of human MDA is typically less than 0.04µg/ml. The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero.

3.12.1.3 ASSAY PROCEDURE

- All reagents and samples were thawed to room temperature before use and assayed in duplicates. The reagents were added using pipettes without making direct contact with the inner wall of the well.
- A blank well was set without any solution, while 50µl of Standard or Sample was added into each well. 50µl of HRP-conjugate was also added to each well with the exception of the Blank well, then mixed thoroughly and incubated for 1 hour in a Baird & Tatlock incubator (Essex, UK) at 37°C. The microtiter plate was then placed on the white surface on the workbench to ensure visibility of any color change.
- During the manual washing, each well was filled with about 200µl Wash Buffer using a fabricated manifold dispenser, allowed to stand for 10 seconds, then

emptied by immediate inversion into the sink to prevent over-spilling onto the well surface and dapped with paper napkin to absorb all liquid excesses for about 2 minutes. This process was repeated three (3) times. 50µl of Substrate A and Substrate B were then added to each well, mixed thoroughly by gentle shaking then wrapped in foil and kept in the dark to prevent drafts and temperature fluctuations after which the plate was incubated for 15 minutes at 37°C.

- 50µl of Stop Solution was added to each well. The first four wells containing the highest concentration of standards developed the obvious blue color after thorough mixing.
- The optical density (OD) of each well was determined within 10 minutes, using a Labsystems Multiskan microtiter plate reader (Amesham, UK) set to 450 nm and attached to a printer.

3.12.1.4 CALCULATION OF RESULTS

The average of the duplicate readings for each standard, Blank, and sample was calculated and subtracted from the optical density of the Blank. A standard curve was created by plotting the mean absorbance for each standard on the x-axis against the concentration on the y -axis and drawing a best fit curve through the points on the graph. The data was linearized by plotting the log of the MDA concentrations versus the log of the OD. The best fit line was determined by regression analysis.

3.12.2.0 VASCULAR CELL ADHESION MOLECULE (VCAM-1) ELISA

3.12.2.1 PRINCIPLE OF ASSAY

The microtiter plate provided in this kit had been pre-coated with an antibody specific to VCAM-1. Standards or samples were then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for VCAM-1 and avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. TMB (3, 3', 5, 5' tetra methyl-benzidine) substrate solution was added to each well. Only the wells containing VCAM-1, biotin-conjugated antibody and enzyme-conjugated avidin exhibited color change. The enzyme-substrate reaction was terminated by the addition of the sulphuric acid solution; the color change was measured spectrophotometrically by the microplate reader at a wavelength of 450 ± 2 nm. The concentration of VCAM-1 in the samples was then determined by comparing the OD of the samples to the standard curve.

3.12.2.2 DETECTION RANGE, SPECIFICITY AND SENSITIVITY OF VCAM-1

The detection range was between 15.63 - 1000 ng/ml. The standard curve concentrations used was 1000, 500, 250, 125, 62.5, 31.25 and 15.63 ng/ml respectively. This plate coated antibodies recognized only human VCAM-1. No significant cross-reactivity or interference was observed. The minimum detectable dose of human VCAM-1 is < 3.9 ng/ml. The sensitivity or Lower Limit of Detection (LLD) of the assay was defined as the lowest protein concentration that could be differentiated from zero.

3.12.2.3 ASSAY PROCEDURE

- The same protocol as described in the section 3.12.1.3 was used after which 100µl of Sample was added to each well and then covered with the adhesive strip to prevent spilling and incubated for 2 hours at 37°C.
- The liquid was removed (poured out by immediate inversion of the plate) from each well, but not washed, then 100µl of Biotin-antibody working solution was added to each well.
- After incubation for an hour at 37°C, the Biotin-antibody working solution appeared cloudy. This was then warmed up to room temperature and mixed gently by gently shaking until solution appeared uniform.
- Each well was then washed using the manual manifold dispenser and the wash buffer. The process was repeated for a total of three washes, in which each well was filled with Wash Buffer (200 µl) and allowed to stand for a minute. The contents were removed by immediately inverting/flicking the plate over a sink, followed immediately by patting the plate on an absorbent paper towel to absorb any excess fluid, this was very essential for accurate results.
- 100µl of HRP-avidin working solution was added to each well; the microtiter plate was covered with a new adhesive strip and incubated for 1 hour at 37°C.
- The aspiration process was repeated and washed three times as aforementioned.
- 90µl of TMB Substrate was added to each well and incubated for another 15 - 30 minutes at 37°C. The plates were kept away from drafts and other temperature fluctuations in a dark place.

- 50µl of the Stop Solution was then added to each well, the first four wells containing the highest concentration of standards developed an obvious blue color. This was done by gently tapping the plate to ensure thorough mixing and uniform color change.
- The optical density of each well was determined within 30 minutes using a microtiter plate reader set to 450 nm.

3.12.2.4 CALCULATION OF RESULTS

This procedure was similar to the aforementioned process described under section 3.12.1.4.

3.12.3.0 ADVANCED GLYCATED END PRODUCTS (AGEs) ELISA

3.12.3.1 PRINCIPLE OF ASSAY

The AGEs enzyme linked immunosorbent assay applies a technique called a quantitative sandwich immunoassay. The microtiter plate provided had been pre-coated with a polyclonal antibody specific for AGE. Standards or samples were added to the wells, if AGE was present, it would bind to the antibody pre-coated wells. In order to quantitatively determine the amount of AGE present in the samples, a standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody, specific for AGE was added to each well to “sandwich” the AGE immobilized on the plate. The microtiter plate underwent incubation, and then the wells were thoroughly washed to remove any unbound components. A and B substrate solutions were then added to each well. The enzyme (HRP) and substrate were allowed to react over short metal azides.

Upon disposal, sink drain was flushed with large volumes of water to prevent azide accumulation.

3.12.3.2 SENSITIVITY AND SPECIFICITY

The sensitivity in this assay was 1.0 pg/mL. This assay had high sensitivity and excellent specificity for detection of AGE. No significant cross-reactivity or interference between AGE and analogues was observed. Limited by current skills and knowledge, it is impossible to complete the cross-reactivity detection between AGE and all the analogues, therefore cross reaction may still exist in some cases.

3.12.3.3 CALCULATION OF RESULTS

This procedure was similar to that under section 3.12.1.4 except that the standard curve was used to determine the amount of the unknown sample by plotting the average OD (450 nm) for each standard on the vertical (y) axis against the concentration on the horizontal (x) axis, and a best fit curve drawn through the points on the graph. The mean OD value for each standard and sample was calculated and subtracted from the mean value of blank control before the interpretation of results.

3.12.4.0 GLUTATHIONE PEROXIDASE (GSH-PX) ELISA

3.12.4.1 PRINCIPLE OF ASSAY

The coated well immune-enzymatic assay for the quantitative measurement of GSH-PX utilized a polyclonal anti-GSH-PX antibody and GSH-PX-HRP conjugate. The assay sample and buffer were incubated together with GSH-PX-HRP conjugate in pre-coated

plate for one hour. After the incubation period, the contents of the wells were decanted and washed five times. The wells are then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction formed a blue colored complex. A stop solution was added to stop the reaction, which turned the solution yellow. The intensity of color was measured spectrophotometrically at 450nm in a microplate reader. The intensity of the color was inversely proportional to the GSH-PX concentration since GSH-PX from samples and GSH-PX-HRP conjugate compete for the anti-GSH-PX antibody binding site. Since the number of sites is limited, as more sites are occupied by GSH-PX from the sample, fewer sites are left to metal azides. Upon disposal, the sink drains were flushed off with a large volume of water to prevent azide accumulation. All universal and local regulations for disposal were observed.

3.12.4.2 QUALITY CONTROL FOR ELISA ASSAY

A. The kit components were refrigerated at - 20°C when not in use. All reagents and components were thawed to room temperature in an incubator before use or opening the foil bags. Once the desired number of strips was removed, the bag was immediately resealed and stored at - 8°C to maintain its integrity.
B. As much as possible, the use of badly hemolyzed or lipemic sera was avoided and all reagents were capped when not in use. Standards, controls and samples were all run in duplicates. Reagent lots from other kits were clearly labeled and not haphazardly mixed.
C. When pipetting reagents, a consistent order (north-south direction) of addition from well-to-well was maintained to ensure equal incubation times for all the wells.

D. Absorbances were read within 2 hours of assay completion. The provided controls were run with every assay.

E. All residual wash liquid was drained from the wells by efficient manual decanting followed by dabbing the plate on absorbent paper napkins. The absorbent paper was never put directly into the wells.

F. Stabilized chromogen is light sensitive thus prolonged exposure to light was avoided. And so was its contact with metal to prevent color change.

3.12.4.3 SAMPLE COLLECTION AND STORAGE

The sample collection and storage for GSH-Px assay was similar to that described under section 3.12.3.2.

3.12.4.4 SENSITIVITY AND SPECIFICITY

The sensitivity and specificity for this assay though limited by current skills and knowledge was similar to that in section 3.12.3.3.

3.12.4.5 SAFETY PRECAUTIONS

The kit contained materials with minute quantities of sodium azide which reacts with lead and copper plumbing to form explosive compounds.

3.12.4.6 CALCULATION OF RESULTS

The standard curve was obtained from a similar procedure described in section 3.12.1.4

3.12.5.0 HEME OXYGENASE 1 (HO-1) ELISA

3.12.5.1 PRINCIPLE OF ASSAY

The pre-coated well, immuno-enzymatic assay for the quantitative measurement of HO-1 utilized a polyclonal anti-HO-1 antibody and HO-1-HRP conjugate. The assay sample and buffer were incubated together with HO-1-HRP conjugate in pre-coated plate for one hour. After the incubation period, the contents of the wells were decanted and washed five times. The wells were then incubated with a substrate for HRP enzyme. The product of the enzyme-substrate reaction formed a blue colored complex. Finally, a stop solution was added to stop the reaction, which then turned the solution yellow. The intensity of color was measured spectrophotometrically at 450 nm in a microplate reader. The intensity of the color was inversely proportional to the HO-1 concentration, since HO-1 from samples and HO-1-HRP conjugate competed for the anti-HO-1 antibody binding site. The number of sites was limited since more sites were occupied by HO-1 from the sample, thus, fewer sites were left to bind HO-1-HRP conjugate.

3.12.5. QUALITY CONTROL

This is similar to that described under section 3.12.4.2 except that the stabilized chromogen was light sensitive thus prolonged exposure to light was avoided. Also direct contact between stabilized chromogen and metal was avoided to prevent color change.

3.12.5.3 SENSITIVITY AND SPECIFICITY OF HO-1

This is similar to that described under section 3.12.3.3.

3.12.5.4 CALCULATION OF RESULTS

The same procedure as described in section 3.12.1.4.

3.12.6.0 TUMOUR NECROSIS FACTOR ALPHA (TNF-α) ASSAY

3.12.6.1 PRINCIPLE OF ASSAY

An anti-human TNF-α coating antibody adsorbed onto microwells was used. Human TNF-α present in the sample or standard was bound to the antibodies adsorbed to the microwells. A biotin-conjugated anti-human TNF-α antibody was added and bound to human TNF-α captured by the first antibody. Following incubation the unbound biotin-conjugated anti-human TNF-α antibody was removed during the wash step. Streptavidin-HRP was added and bound to the biotin-conjugated anti-human TNF-α antibody. Following incubation, the unbound Streptavidin-HRP was removed during the wash step, and the substrate solution reactive with HRP was added to the wells. A colored product was formed in proportion to the amount of human TNF-α present in the sample or standard.

3.12.6.3 PREPARATION OF REAGENTS

Buffer concentrates were brought to room temperature and diluted before starting the test procedure.

3.12.6.3.1 WASH BUFFER

The entire content (50 ml) of the Wash Buffer Concentrate was poured into a clean 1000 ml graduated cylinder, brought to a final volume of 1000 ml with glass-distilled water and mixed gently to avoid foaming. The pH of the final solution was adjusted to

7.4 and transferred to a clean wash bottle and stored at 20 - 25°C, since the Wash Buffer was only stable for 30 days.

3.12.6.3.2 ASSAY BUFFER

Five (5) ml of the Assay Buffer Concentrate (20x) was poured into a clean 100 ml cylinder and brought to a final volume of 100 ml with distilled water, mixed gently to avoid foaming and stored at 2 - 8°C. The Biotin-Conjugate was used within 30 minutes after dilution. A 1:100 dilution of the concentrated Biotin-Conjugate solution was made with Assay Buffer (1x) in a clean plastic tube.

3.12.6.3.3 STREPTAVIDIN-HRP

This was used within 30 minutes after 1: 200 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1x) in a clean plastic tube.

3.12.6.3.4 HUMAN TNF-ALPHA STANDARD

The reconstitution of human TNF-α standard was done by addition of distilled water to the reconstitution volume stated on the label of the standard vial. This was swirled gently to ensure homogeneous solubilization (concentration of reconstituted standard = 1000 pg/ml). The standard was not stored but used immediately after reconstitution. Standard dilutions were prepared directly in the tubes.

3.12.6.3.5 EXTERNAL STANDARD DILUTION

Seven (7) tubes were labeled one for each standard point S1, S2, S3, S4, S5, S6 and S7 then prepared 1: 2 serial dilutions for the standard curve. 225 µl of Sample Diluent was pipetted into each tube. Another 225 µl of reconstituted standard (concentration = 1000

pg/ml) was pipetted into the first tube, mixed and labeled S1, (concentration of standard 1 = 500 pg/ml). 225 µl of this dilution was pipetted into the second tube, mixed thoroughly and labeled S2 before the next transfer. These serial dilutions were repeated 5 more times creating the points of the standard curve while the Sample Diluent served as the blank.

3.12.6.3.6 CONTROLS

This was reconstituted by adding 800 µl distilled water to lyophilized controls, swirled gently to ensure complete and homogeneous solubilization. The stored reconstituted controls were aliquoted at - 20°C.

3.12.6.3.7 ADDITION OF COLOUR-IMPARTING REAGENTS (DYES)

Distinctive colors to each step of the ELISA procedure were made clearly visible by the addition of colorful dyes. This procedure did not interfere with the test results but was designed to enable efficient test performance. The dye solutions provided (Blue-, Green-, and Red-Dye) were added to the reagents to impart their color.

3.12.6.3.8 TEST PROTOCOL

The procedure was similar to that described in section 3.12.6.3.5 except that 100 µl of Sample Diluent was added in duplicate to all standard wells. 100 µl of prepared standard (concentration = 1000 pg/ml) in duplicate was added into well A1 and A2. The wells A1 and A2 were washed manually as aforementioned (concentration of standard 1, S1 = 500 pg/ml), and 100 µl added to wells B1 and B2 respectively. This procedure was continued 5 times, creating two rows of human TNF-α standard dilutions ranging

from 500.0 - 7.8 pg/ml. 100 µl of the contents from the last microwells (G1, G2) was used. 100 µl of Sample Diluent was added in duplicate to the blank wells. 50 µl of Sample Diluent and sample was added to the wells each in duplicate. 50 µl Biotin-Conjugate was added to all the wells and then covered with a film and incubated for 2 hours, The strips were washed 4 times and 100 µl of the diluted Streptavidin-HRP was added, including the blank and covered again with the film and incubated for 1 hour after which the strips were washed another 4 times and 100 µl of TMB Substrate Solution added to all wells and incubated for 10 minutes in the dark. The color change was monitored and the substrate reaction stopped before positive wells were no longer visible. As recommended, 100 µl of the stop solution was added when the highest standard developed a dark blue color to inactivate it. The results were read immediately on the microplate reader using 450 nm as the primary wavelength.

3.12.6.4 CALCULATION OF RESULTS

The average absorbance values were calculated for each set of duplicate standards and samples in a similar fashion to that described in section 3.12.1.4 except that duplicates were within 20 per cent of the mean value. The concentrations read from the standard curve were multiplied by the dilution factor (x 2).

3.13 GENERAL PRECAUTIONS TAKEN DURING THE STUDY

- All chemicals were considered as potentially hazardous thus handled only by trained laboratory personnel in accordance with the principles of good laboratory

practice. Suitable protective clothing such as laboratory overalls, safety goggles and gloves were worn. Care was taken to avoid contact with skin or eyes.

- The reagents were intended for research use only and not for use in diagnostic or therapeutic procedures. Care was taken not to mix or substitute reagents with those from other lots or sources or use reagents beyond label expiration date.
- Kit reagents were not exposed to strong light during storage or incubation and pipetting was not done by mouth.
- Eating, drinking or smoking was avoided in areas in the laboratory where kit reagents or samples are handled.
- Care was taken to avoid contact of substrate solution with oxidizing agents, metal and splashing or generation of aerosols.
- Disposable pipette tips and clean, dedicated reagent trays for dispensing the conjugate and substrate reagent were used to avoid microbial contamination or cross-contamination of reagents or specimens which would invalidate the test. Glass-distilled water or deionized water was used for reagent preparation.
- Care was taken to decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents.
- Liquid wastes not containing acid and neutralized waste were mixed with sodium hypochlorite in volumes such that the final mixture contained 1.0% sodium hypochlorite and allowed to stand for 30 minutes for effective decontamination. Liquid waste containing acid was neutralized prior to the addition of sodium hypochlorite.

3.14 DATA HANDLING

The data obtained was entered using Microsoft Excel and Statistical Package for Social Sciences (SPSS) version 20. The results were summarised and presented using descriptive statistics. Means and standard deviations were calculated for the age, height, BMI, resting metabolism, percentage body and visceral fat, total white blood cells, neutrophils, BP and heart rate of the subjects. Serum levels of MDA, GPx, HO-1, VCAM-1, AGEs and TNF-α were summarised and presented as means and standard deviations. Also, tables and graphs were used to present the blood indices.

3.15 STATISTICAL ANALYSIS

Paired *t*- and Wilcoxon tests were used to compare differences between two means. The parametric data was tested using the paired *t* test. The Wilcoxon signed-rank test was used for data not satisfying assumptions of normality. The Pearson's chi-square test for independence was used to discover if there was relationship between two variables. Analyses were performed using SPSS (version 20, IBM SPSS software for Windows 2010). Statistical significance was accepted at $p < 0.05$. Data was shown in tabular form as mean ± standard deviation.

CHAPTER FOUR

4.0 RESULTS

4.1 GENERAL CHARACTERISTICS OF SUBJECTS

Of the Fifty four (54) subjects recruited into the study, thirteen (13) withdrew before completion for reasons unrelated to the study while twenty (20) were removed from the study due to malcompliance. At the end of the intervention, the attrition rate calculated as sample size (week 0 – week 12) / week 0 × 100, 54 – 17 / 54 × 100 = 68.5 % therefore only 31.5 % of the week 0 sample size successfully completed the study.

The supplement, Golden Tree Royale® Natural Cocoa Powder (NCP) was largely well-tolerated though four (4) subjects reported mild nausea, increased hunger sensation, frequent eating, frequent urination, following consumption and withdrew on this basis. Seventeen (17) subjects successfully completed the study. Twelve (12) of them were males and five (5) females. For the females, the mean height was 164.4 ± 9.9 cm, mean weight 62.54 ± 16.20 kg, mean systolic blood pressure 119.4 ± 8.4 mmHg, mean diastolic blood pressure 79.0 ± 7.9 mmHg, mean pulse rate of 74.4 ± 2.9 beats per minute (bpm), mean BMI 22.82 ± 3.80, the mean resting metabolism was 1345 ± 179 kcal, mean percent skeletal muscle 29.56 ± 6.3%, the mean percent visceral fat 4.6 ± 2.6%. While for the males, the mean height was 173.42 ± 7.40 cm, mean weight 68.66 ± 7.50 kg, mean systolic blood pressure 121.83 ± 9.70 mmHg, mean diastolic blood pressure 77.92 ± 9.80 mmHg, mean pulse rate 70.0 ± 5.8 bpm, mean BMI 23.02 ± 3.60, mean resting metabolism was 1599 ± 165 kcal, mean percent skeletal muscle 41.01 ± 7.6%, the mean percent visceral fat 4.33 ± 2.90%.

The age of the subjects ranged from 21 to 55 years with a mean age of 30.8 ± 10.2 years, mean height of 170.9 ± 8.70 cm, mean weight of 65.19 ± 10.45 kg, mean BMI 23.0 ± 3.50, mean pulse rate of 71.29 ± 5.40 bpm, mean systolic blood pressure 121.11 ± 9.11 mmHg and mean diastolic blood pressure of 78.24 ± 9.06 mmHg. The characteristics of the pre- and post- supplementation groups are summarized in Table 4.1 below.

Table 4.1: Pre- and Post-supplementation means of general characteristics

Parameter	Pre-	Post-	*p*-value
Weight (kg)	**66.86 ± 10.61**	**65.19 ± 10.45**	**0.009***
BMI (kg/m^2)	**23.01 ± 3.80**	**22.96 ± 3.53**	**0.006***
SBP (mmHg)	**124.53 ± 11.25**	**121.12 ± 9.11**	**0.162**
DBP (mmHg)	**80.12 ± 9.62**	**78.24 ± 9.06**	**0.271**
Pulse (bpm)	**70.18 ± 8.8**	**71.29 ± 5.4**	**0.550**

*** Significant at 5%**

Table 4.1 shows the results of the general characteristics. Values are given in mean ± standard deviation

In the general characteristics category, the mean pre- mean weight 66.86 ± 10.61 kg was higher than the mean post- weight 65.19 ± 10.50 kg. This showed statistically significant decrease after the supplementation (p = 0.009). The mean pre- BMI 23.01 ± 3.8 kg/m^2 was higher than the mean post- BMI 22.96 ± 3.53 kg/m^2 showing a statistically significant decrease (p = 0.006). The mean pre- systolic blood pressure 124.53 ± 11.25 mmHg was higher than the mean post- systolic blood pressure 121.12 ± 9.11 mmHg but did not show any statistically significant change. The mean pre- diastolic blood pressure 80.12 ± 9.62 mmHg was higher than the mean post- diastolic blood pressure 78.24 ± 9.06 mmHg,

though this did not show any significant difference after the 12 weeks supplementation. The mean pre- pulse 70.18 ± 8.80 bpm was lower than the mean post- pulse 71.29 ± 5.40 bpm. However, this did not show any significant difference.

4.2 FAT PARAMETERS OF THE SUBJECTS

In the fat parameters category, the mean pre- resting metabolism 1475 ± 270 kcal was lower than the post- 1530 ± 206 kcal, showing significant difference between week 0 and 12 (p = 0.028). The mean pre- percent skeletal muscle 35.18 ± 10.30% was lower than the mean post- percent skeletal muscle 37.64 ± 8.90%. This also showed a significant difference after the intervention (p = 0.031). The mean pre- percent visceral fat 4.41 ± 2.90% was lower than the mean post- percent visceral fat 4.47 ± 3.70%. This did not show any statistically significant difference. The mean pre- percent body fat 23.3 ± 12.60% was slightly lower than the post- percent body fat 23.0 ± 12.80%. This did not show any statistically significant change (Table 4.2).

Table 4.2: Pre- and Post-supplementation means of fat parameters

Parameters	Mean	SD	*p*-value
Resting metabolism (Kcal) Pre-	**1475**	**270**	
Post-	**1530**	**206**	**0.028***
Skeletal muscle (%) Pre-	**35.18**	**10.30**	
Post-	**37.64**	**8.90**	**0.031***
Visceral fat (%) Pre-	**4.41**	**2.90**	
Post-	**4.47**	**3.70**	**0.745**
Body fat (%) Pre-	**23.3**	**12.6**	
Post-	**23.0**	**12.8**	**0.407**

Wilcoxon's signed rank test ***Significant at 5%**

Table 4.2 shows the overall results of the supplementation for fat parameters. Values are expressed as mean ± standard deviation.

4.3 HEMATOLOGICAL INDICES OF THE SUBJECTS

In the hematological indices category, the mean pre- Hb 13.48 ± 1.40 g/dl was lower than the mean post- Hb 13.95 ± 1.50 g/dl. There was a significant increase after the supplementation (p = 0.016). The mean pre- Hct 31.58 ± 9.5% was lower compared to the mean post- Hct 36.01 ± 7.6%. There was statistically significant difference after the intervention (p = 0.001). The mean pre- RBC count 4.16 ± 0.7 x 10^{12}/L was lower than the mean post- RBC count 4.62 ± 0.4 x 10^{12}/L. The increase after the intervention was not statistically significant. The mean pre- MCH 33.48 ± 12.8pg was higher than the mean post- MCH 31.42 ± 3.8pg. There was statistically significant decrease after the intervention (p = 0.001). The mean pre- MCV 76.14 ± 18.30 fl was lower than the post- MCV 78.02 ± 14.90 fl but it was not statistically significant. The mean pre- MCHC 47.66 ± 16.70 g/dl

was higher than the mean post- MCHC 40.38 ± 9.60 g/dl. There was a statistically significant decrease after the supplementation (p = 0.001). The mean pre- TWBC 5.36 ± 1.7 x 10^9/L was higher than the mean post- TWBC 6.04 ± 2.6 x 10^9/L. There was a statistically significant increase (p = 0.031). The mean pre- M% 5.76 ± 1.40% was lower than the mean differential post- M% 6.51 ± 1.90%. There was statistically significant increase in the differential monocytes (p = 0.039). The mean pre- E% 0.97 ± 0.80% was lower than the mean post- E% 1.45 ± 1.20%. There was statistically significant increase in the E% after the supplementation (p = 0.005). The mean pre- B% 0.33 ± 0.30% was lower than the mean post- B% 0.46 ± 0.30% which showed significant change after the supplementation (p = 0.006). Also the mean pre- N% 56.77 ± 12.70% was higher than the mean post- N% 55.79 ± 14.50%. However, the difference after the supplementation was not statistically significant. The mean pre- L% 40.77 ± 9.6% was lower than the post- L% 41.87 ± 10.2%. However, this change did not show any statistically significant difference (Table 4.3).

Table 4.3: Pre- and post-supplementation of the mean hematological indices

Parameter	Mean	SD	*p*-value
Hb g/dl			
Pre-	**13.48**	**1.40**	**0.016***
Post-	**13.95**	**1.50**	
Hct %			
Pre-	**31.58**	**9.50**	**0.001***
Post-	**36.01**	**7.60**	
RBC x 10^{12}/L			
Pre-	**4.16**	**0.70**	**0.287**
Post-	**4.62**	**0.40**	
MCV fl			
Pre-	**76.14**	**18.30**	**0.381**
Post-	**78.02**	**14.90**	
MCH pg			
Pre-	**33.48**	**12.80**	**0.001***
Post-	**31.42**	**3.80**	
MCHC g/dl			
Pre-	**47.66**	**16.70**	**0.001***
Post-	**40.38**	**9.60**	
TWBC x 10^{9}/L			
Pre-	**5.36**	**1.70**	**0.031***
Post-	**6.04**	**2.60**	
N%			
Pre-	**56.77**	**12.70**	**0.943**
Post-	**55.79**	**14.50**	
L%			
Pre-	**40.77**	**9.60**	**0.155**
Post-	**41.87**	**10.20**	
M%			
Pre-	**5.76**	**1.40**	**0.039***
Post-	**6.51**	**1.90**	
E%			
Pre-	**0.97**	**0.80**	**0.005***
Post-	**1.45**	**1.20**	
B%			
Pre-	**0.33**	**0.30**	**0.006***
Post-	**0.46**	**0.30**	

Wilcoxon's signed rank test ***Significant at 5%**

Table 4.3 shows the results for hematological indices after the supplementation. The values are expressed as mean ± standard deviation.

4.4 SELECTED OXIDATIVE STRESS MARKERS OF THE SUBJECTS

This category compares the effect of NCP beverage on selected markers of oxidative stress at pre- (week 0, day 1) and post- (week 12, day 85) supplementation period in the healthy Ghanaian adults. The mean pre- MDA 0.69 ± 0.10 ng/L was higher than the mean post- MDA 0.64 ± 0.10 ng/L. The MDA after the supplementation showed a decrease that was statistically significant (p = 0.033). The mean pre- GSH-Px 1.73 ± 0.18 ng/L was lower than the mean post- GSH-Px 1.96 ± 0.10 ng/L. This showed a statistically significant increase after the supplementation (p = 0.001). The mean pre- VCAM-1 0.53 ± 0.13 mg/L was slightly higher than the mean post- VCAM-1 0.44 ± 0.18 mg/L, though the decrease was not statistically significant. The mean pre- AGEs 0.261 ± 0.07 ug/L was slightly lower than the mean post- AGEs 0.263 ± 0.12 ug/L, though the difference was not statistically significant. The mean pre- HO-1 1.74 ± 0.35 mg/L was lower than the mean post- HO-1 1.85 ± 0.22 mg/L. This increase was not considered statistically significant. The mean pre- TNF-α 0.31 ± 0.32 ng/L was lower than the mean post- TNF-α 0.46 ± 0.56 ng/L but this increase did not show any statistical significance (Table 4.4).

Table 4.4: Pre- and post- supplementation means of selected oxidative stress markers

Analyte	Mean	SD	P-value
MDA (ng/L) Pre-	**0.69**	**0.10**	**0.033***
Post-	**0.64**	**0.10**	
GSH-Px (ng/L) Pre-	**1.73**	**0.18**	**0.001***
Post-	**1.96**	**0.10**	
VCAM-1 (mg/L) Pre-	**0.53**	**0.13**	**0.068**
Post-	**0.44**	**0.18**	
AGEs (ug/L) Pre-	**0.261**	**0.07**	**0.538**
Post-	**0.263**	**0.12**	
HO-1 (mg/L) Pre-	**1.74**	**0.35**	**0.554**
Post-	**1.85**	**0.22**	
TNF-α (ng/L) Pre-	**0.31**	**0.32**	**0.981**
Post-	**0.46**	**0.56**	

Wilcoxon's signed rank test ***Significant at 5%**

Table 4.4 shows the results of the selected oxidative stress markers after the supplementation. The values are expressed as mean ± standard deviation. TNF-α - tumor necrosis factor alpha; MDA - malondialdehyde; AGEs - advanced glycated end products; GSH-Px - Glutathione peroxidase; VCAM-1 - vascular cell adhesion molecule 1; HO-1 - heme oxygenase 1.

4.5 COMPARISON BY GENDER OF GENERAL CHARACTERISTICS, FAT PARAMETERS, SELECTED OXIDATIVE STRESS MARKERS AND HEMATOLOGICAL INDICES

In the vital characteristics category where 12 out of 17 were males and the rest females, the differences between the mean weight, systolic blood pressure, diastolic blood pressure,

pulse rate and BMI after the supplementation were not statistically significant. As shown in Table 4.6, the gender stratified mean resting metabolism, percentage skeletal muscle and body fat in the fat parameters category showed statistically significant difference. The mean resting metabolism 1607.00 ± 169.1 kcal of the males was higher than the mean resting metabolism 1345.20 ± 178.5 kcal of the females. There was statistically significant difference between gender and resting metabolism (p = 0.027). The mean percent skeletal muscle 41.01 ± 7.6% was far higher than the mean percent skeletal muscle 29.56 ± 6.3%. There was statistically significant difference between them (p = 0.029). The mean body fat 19.01 ± 11.4% was very low compared to the mean body fat 32.58 ± 11.5% of the females. There was statistically significant difference between gender and body fat (p = 0.035). In Table 4.6, the gender stratified hematological indices category did not not show any statistically significant differences except in the mean hemoglobin and the calculated indices, mean cell hemoglobin concentration. the mean hemoglobin (Hb) 14.52 ± 1.30 g/dL of the males was higher than the mean Hb 12.58 ± 0.60 g/dL of the females. There was statistically significant diference between them (p = 0.005). The mean cell hemoglobin concentration (MCHC) 42.50 ± 10.50 g/dL of the males was higher than the mean MCHC 35.28 ± 3.90 g/dL of the females. There was statistically significant difference between gender and MCHC (p = 0.045). In the selected oxidative stress markers category, after the supplementation and subsequent stratification by gender there was no statistically significant difference between them. The overall mean differences between the general characteristics, fat parameters, selected oxidative stress markers and the hematological indices did not show any statistically significant differences (Table 4.5).

Table 4.5: Comparison by gender and fat parameters

Body parameters	Gender	Mean	SD	p-value
Resting metab. (kcal)	M	1607	169	0.027*
	F	1345	179	
Skeletal muscle (%)	M	41.01	7.6	0.029*
	F	29.56	6.3	
Body fat (%)	M	19.01	11.4	0.035*
	F	32.58	11.5	

Mann Whitney's test ***Significant at 5%**

Table 4.5 shows the results of gender stratified fat parameters. The values are expressed as mean ± standard deviation. Only the statistically significant parameters are shown.

Table 4.6: Comparison by gender and hematological indices

Parameter	Gender	Mean	SD	p-value
Hb (g/dl)	M	14.52	1.30	0.005*
	F	12.58	0.60	
MCHC (g/dl)	M	42.50	10.50	0.045*
	F	35.28	3.90	

Mann Whitney's test ***Significant at 5%**

Table 4.6 shows the gender stratified hematological indices result after thesupplementation. The values are expressed as mean ± standard deviation. Only the statistically significant parameters have been shown.

CHAPTER FIVE

5.0 DISCUSSION

Oxidative stress which is primarily the imbalance between prooxidants and antioxidants may develop resulting in oxidative damage and consequently decreased immune function, increased fatigue and decrease physical performance. The possible pathways of physiologic oxidative stress includes the transient and acute muscular deoxygenation, which resembles the ischemia-reperfusion, xanthine oxidase activation, catecholamine autoxidation and NADPH oxidase activation, prostanoid metabolism, phagocyte respiratory burst activity, disruption of iron containing proteins and altered calcium homeostasis. Mechanical stress due to routine daily activities such as walking etc. is also another hypothesis used to explain the increment of free radicals and the cumulative reactive oxygen and nitrogen species (RONS) (Mahdavi *et al*., 2012). The muscle tissue damage caused by varying levels of force from stressful work, initiates the inflammation process that eventually produces oxygen free radicals and lipid peroxidation. In addition, contractions may cause micro-tears both in muscle and in the vascular endothelium, which also affect the migration of white cells.

Research in the context of oxidative stress after long term free-living supplementation with NCP is virtually non-existent in indigenious scientific documentation to compare with. To the best of my knowledge, there is no short term study that has examined effect of natural cocoa powder supplementation on the selected oxidative stress markers used in this study and hematological indices in apparently healthy Ghanaian adults, hence this study.

The subjects' age ranged from 21 to 55 years with a mean of 30.8 ± 10.2 years indicative of the productive age bracket of the Ghanaian population. A similar age group was studied by Ried *et al*. (2009) though the researchers used dark chocolate. From the present study, the mean height of the overall subjects was 170.9 ± 8.7 cm, mean weight 65.19 ± 10.45 kg, mean BMI 22.96 ± 3.53, mean pulse rate of 71.29 ± 5.40 bpm, mean systolic blood pressure 121.12 ± 9.11 mmHg and mean diastolic blood pressure of 78.24 ± 9.06 mmHg. This representation indicates subjects in healthy physiologic state. This study showed a decrease in weight for the overall subjects after the supplementation and this decrease also reflected in the BMI which showed statistically significant decrease. The high cocoa-content chocolate study by Di Renzo *et al*. (2012) in pregnant women showed that modest daily consumption contributed to reduced blood pressure, glycemic and liver patterns without affecting weight gain. The subjects enrolled were between the overweight to the moderately obese group. The fat content of 35.7g/100g powder might also account for this alteration. In terms of the fat parameters, when the data was stratified according to gender significant differences were observed in percentage body fat in the females due to the body distribution of adipose tissue and the role of hormones. However, the percentage resting metabolism and skeletal muscle showed significant differences in the males, naturally because of their muscle bulk and hormonal influence.

Few human studies have examined the effects of polyphenols-rich beverages on hematological indices without consensual results. These indices are very helpful as the first line of defence in the immune system and indicative of the state of the cardivascuar system. These findings could be brought about by the nutritive abilities and macronutrient

composition of cocoa in the form of NCP. When data stratified by gender only the Hb and MCHC in males showed significant differences which is a physiologic tendency based on gender.

Studies in cocoa-related supplementation have shown that intervention duration of less than 1 week to as much as 12 weeks produce positive outcomes of its antioxidant ability. Several studies have reported predominantly high antioxidant activity of cocoa (Keen *et al*., 2005, Kofink *et al*., 2007, Radojčić *et al*., 2009, Andújar *et al*., 2012), which was also observed to be significant in the present study. In addition to this, the significant antioxidant activity which verifies the antioxidant capabilities of cocoa were in agreement with work done in rat models (Abrokwah *et al*., 2009). Statistically significant differences between the pre- and post-supplementation in the present study were observed with the two main oxidative stress markers – glutathione peroxidase (GSH-Px, a natural antioxidant) and malondialdehyde (MDA, a natural prooxidant). The explanation for elevated GSH-Px effect should be from the antioxidant properties of the polyphenols in cocoa. These antioxidants in the form of dietary polyphenols have the ability to modulate cellular sensor(s) for oxidative stress and thereby increase NO bioavailability (McMahon *et al*., 2004, Mann *et al*., 2007), which causes vasodilation, resulting in the clearance and prevention of the deposition of excess cholesterol in the blood vessels. Thus consuming NCP can serve as a heathy preventive measure against lifestyle conditions like atherosclerosis, cardiovascular risk factors, obesity etc. This is in agreement with work done by Sarria *et al*., (2013) in which increased antioxidant activity, hypoglycemic and

anti-inflammatory effects in healthy and hypercholesterolemic individuals was observed without weight gain.

The present findings in which MDA levels were significantly reduced was in accordance with that of Wang *et al* (2000) who also reported a reduction in plasma lipid peroxidation 2 and 6 hours after procyanidin-rich chocolate consumption. Lecumberri *et al*. (2007) also observed that the consumption of cocoa fiber with a hypercholesterolemic diet improved the lipidemic profile, suggesting that cocoa fiber contributes to a reduction in cardiovascular risk, as flavonoids react with the superoxide free radical (Girard *et al*., 1995). This justifies the free-living study design employed in the present study which was to mimick the recommended routine long term consumption. In this case the subjects were restricted from consuming antioxidant rich foods like teas (green and black), oranges, tomatoes etc. In addition to this, the ingestion of other food substances did not alter the active ingredients in cocoa . Mathur *et al*. (2002) also showed reduced lipoprotein oxidation after cocoa supplementation for 6 weeks compared to unsupplemented subjects. This is in agreement with a study by Baba *et al*. (2007) who indicated that after 4 weeks of varying dosages of supplementation with cocoa powder, subjects showed decreased plasma lipid peroxidation compared to the baseline. In another study 12-weeks of cocoa powder supplementation to normo- and mildly hyper-cholesterolemic subjects showed decreased LDL oxidation and increased plasma HDL-C compared to the controls (Mathur *et al*., 2002). This formed the basis for the 12 weeks duration used in this study, though intermediate assessment midway through the supplementation period would have been helpful. Osakabe *et al*., (2001) also showed that cocoa supplementation in healthy males

significantly reduced LDL oxidation. It is possible that the higher dose and frequency of the NCP may have had significant effect on MDA production. Noori *et al*., in 2009 using cocoa powder-treated rat models also showed significantly decreased levels of MDA in their liver tissues after 21 days. This observation was in agreement with the current findings. Regarding the long-term supplementation, the findings from this study were consistent with other studies. Mathur *et al*., (2002) demonstrated that cocoa products reduced lipoprotein oxidative susceptibility but did not affect the biomarkers of inflammation. Also, Osakabe *et al*., (2001) showed that the daily cocoa powder with sugar intake reduced the susceptibility.

A previous study showed that supplementation with cocoa increases WBC concentration (Abrowkwah *et al*., 2009). In the present study, Hb ($p = 0.016$), Hct ($p = 0.001$), TWBC ($p = 0.031$), M% ($p = 0.039$), E% ($p = 0.005$), B% ($p = 0.006$) and the calculated indices, MCH ($p = 0.001$), MCHC ($p = 0.001$) showed statistically significant changes. This profile shows that the supplementation with natural cocoa powder boosted the immune system, since these cells are the first line of defense against infection. This observation is supported by the observed immunoprotective ability of cocoa made by Addai, (2010). Furthermore, these results were consistent with the report of other studies in animal models by Abrowkwah *et al*., (2009), though this showed an improvement with significant increment in Hb and Hct in the healthy individuals indicating the healthy state of the circulatory system.

The beneficial effects of nitric oxide (NO) modulation include the regulation of blood pressure, lowering of NO-affected hypercholesterolemia and monocyte endothelial adhesion (van Acker *et al*., 1995). There were no statistically significant differences in the other selected oxidative stress markers such as VCAM-1 (vasculoendothelial adhesion) which is in agreement with Balzer *et al*. (2008) who also reported that cocoa ingestion improved basal flow-mediated dilatation (FMD) by 30% without changes in endothelial function, blood pressure, heart rate and glycemic control among diabetics. But this study showed significant changes in the blood pressure and heart rate. As well as the rate of mitochondrial oxidant production from females being significantly lower than that from males (Vina *et al*., 2011) could be possible contributory factors to gender, hormonal and muscular activities compared to the obtained results considering the higher male sample size in this study. In addition, the protective effect of estrogen against oxidant production on the vascular function in addition to up-regulating the expression of eNOS (Vina *et al*., 2011) could have influenced the outcome. The present study did not show any statistically significant difference after the gender stratification.

The other selected oxidative stress markers such as AGEs (aging and apoptosis) reduced but did not show any statistically significant change which indicates that the endogenous NO inhibited its activity. Civelek *et al*. (2010) in their study with streptozotocin-induced diabetic rats, showed increased levels of AGEs which is in agreement with the present study that the antioxidant supplementation to reduce the aging and apoptotic processes. HO-1 (heme degradation) also did not show any statistical significance though its anti-inflammatory function protects against oxidative stress. It may have contributed to the

significant antioxidant outcome which correlates with decreased pro-inflammatory cytokines expression, such as monocyte chemo-attractant protein 1 (MCP-1) and interleukin 6 (IL-6), scavenger receptor A (SR-A) expression, and foam cell formation (Orozco *et al*., 2007). This is also in agreement with work done by Ryter *et al*. (2006) involving carbon monoxide applications. TNF-α did not show significant changes in this study though it slightly increased because its low level aids the maintenance of homeostasis by regulating the body's circadian rhythm and promotes the remodeling or replacement of injured and senescent tissue by stimulating fibroblast growth as well as its role in immune response to microbial invasions as well as necrosis of specific tumors (Tracey and Cerami, 1993).

5.1 CONCLUSION

Findings from this study indicated that the 150 mg/100g of natural cocoa powder (NCP) beverage supplementation consumed twice daily for 12 weeks was well-tolerated and showed statistically significant effect on some oxidative stress parameters, precisely the prooxidant-antioxidant balance in the apparently healthy adult Ghanaians. The significant increase in the antioxidant (GSH-Px) and decrease in prooxidant (MDA) markers may be due to the presence of the flavoniods such as epicatechin and catechin, proanthocyanidins, methylxanthines which have known antioxidant properties in addition to the innate physiologic reactive oxygen and nitrogen species (RONS) defense system.

In summary, the body's prooxidant-antioxidant system was augmented by the antioxidant supplementation with NCP which is in agreement with the claims of cocoa's healthful benefits and should be encouraged for long term supplementation in people of all ages for daily healthy living.

5.2 RECOMMENDATIONS

1. The present findings validated the claim that cocoa in the form of NCP has healthful properties such as the antioxidant, nutritive, immunomodulatory and hypotensive abilities, thus can be consumed daily by all age groups safely without any adverse effects. This study will go a long to influence health policies to encourage the use of the readily available scientifically tested food consumed as medicine. Furthermore it will help reduce the socioeconomic impact of the over-burden healthcare system by modifying lifestyle. Also this study will help encourage the consumption of the unsweetened cocoa powder as opposed to the expensive, heavily refined and less potent cocoa products such as chocolate. NCP could be made a healthy option to the various teas on the markets by for example, including NCP in the school feeding program.
2. Further human intervention studies should be done in Ghana in strict clinical trial conditions or during controlled repeated consumption of the supplement for short, intermediate and longer duration (of about 24 weeks) with a larger sample size in different population subgroups (e.g. smokers, older adults, obese subjects, subjects with pathologic conditions such as type 2 diabetes, obesity and hypertension etc.).

3. This study could be repeated in apparently healthy individuals with a more comprehensive battery of assays such as lipid profile and peroxidation, glycemic status to obtain an all inclusive indigenous reference study.

5.4 LIMITATIONS OF THE STUDY

1. Methodological limitations which may not have been adequately controlled for other food constituents in the natural cocoa powder or as free-living subjects exercising their right to food choices despite their consent and voluntariness to the study design may also have had an effect on the outcome.

2. Failure to investigate other parameters and other biochemical tests was due to logistic challenges, limited financial resources, high cost of the kits and equipment as well as the strict working timelines for the completion of the MPhil. Program research project.

3. The high attrition rate which necessitated over 200% mop-ups surplus of the sample size encountered due to the astringent taste of the beverage, the chronic administration and strict adherence to compliance.

4. The need for an improved study design (randomized controlled trial) and a more comprehensive phenotyping of the volunteers before selection for study participation to permit the use of probability theories to express the likelihood that any difference in outcome between treatment groups merely indicates chance, as well as eliminate selection bias and confounding factors in treatment assignment.

5. Pro-industry findings since the natural cocoa powder used in this study was sponsored by a prominent food company as opposed to non-profit or other sources.

6. Therapeutic misconceptions where the subjects believed that they were certain to receive treatment that is best for them personally; that is, they do not understand the difference between research and treatment.

7. This study treated the subjects without the confounding factors associated with gender thus should be repeated in a gender transformative study designed to verify its role.

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APPENDIX

Ethical clearance letter

UNIVERSITY OF GHANA MEDICAL SCHOOL
COLLEGE OF HEALTH SCIENCES
ACADEMIC AFFAIRS OFFICE

Phone: +233-0302-666987-8
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P O Box 4236
Accra
Ghana

My Ref. No: **MS-AA/C.2/Vol.12[A]**

Your Ref. No.

20th July, 2012

Miss Elsie Amedonu
Dept. of Physiology
UGMS, Korle-Bu.

ETHICAL CLEARANCE

Protocol Identification Number: MS-Et/M.8-P.4.2/2011-2012

The Ethical and Protocol Review Committee of the University of Ghana Medical School on 20th July, 2012 unanimously approved your research proposal.

TITLE OF PROTOCOL: **"Effect of Natural Cocoa Powder Beverage Consumption in Apparently Healthy Ghanaians"**

PRINCIPAL INVESTIGATOR: Miss Elsie Amedonu

This approval requires that you submit six-monthly review reports of the protocol to the Committee and a final full review to the Ethical and Protocol Review Committee at the completion of the study. The Committee may observe, or cause to be observed, procedures and records of the study during and after implementation.

Please note that any significant modification of this project must be submitted to the Committee for review and approval before its implementation.

You are required to report all serious adverse events related to this study to the Ethical and Protocol Review Committee within seven (7) days verbally and fourteen (14) days in writing.

As part of the review process, it is the Committee's duty to review the ethical aspects of any manuscript that may be produced from this study. You will therefore be required to furnish the Committee with any manuscript for publication.

Please always quote the protocol identification number in all future correspondence in relation to this protocol.

Signed:.....
PROFESSOR ANDREW A. ADJEI
(CHAIRMAN, ETHICAL AND PROTOCOL REVIEW COMMITTEE)

cc: Dean
Head of Department
Research Office

INFORMED CONSENT FORM

Name of principal investigator: Elsie Stephanie Afidelali Amedonu

Name of Institution: University of Ghana Medical School (UGMS), Korle-Bu, Accra

Name of sponsor: School of Allied Health Sciences (SAHS), College of Health Sciences, University of Ghana

Project title: Baseline study of the effect of natural cocoa beverage consumption on apparently healthy Ghanaians.

I …… have been invited to take part in this research. I have been told of the purpose and procedure of this study which is to answer the questions raised about the effect of natural cocoa powder beverage on apparently healthy Ghanaians. I will be asked a few routine questions about personal information such as my educational and familial background, past medical history etc. I will not be reimbursed monetarily for participating in this research study.

The risk or dangers and discomforts might involve the pain of blood collection. The study team will try to reduce the chances of those risks happening by employing trained phlebotomist but on the occurrence of anything untoward the participant will be provided free medical care at the hospital. The arm will be cleaned before blood collection and new needles will be used for each participant. I promise to prepare and take the natural cocoa powder beverage in compliance with the research study.

STUDY QUESTIONNAIRE

Name (surname in blocks):

Date: Questionnaire number:

Blood group: Date of birth:

Age: Sex:

Date of recruitment: Contact Number:

Email: …… Address: ……

Height (m): ……. Weight (kg): …… BMI: …….

BP: …… Pulse: …… Marital status: …….

Number of children: …… Level of education: …….

Occupation: …….. Course and level: ……

Economic status: Family history:

Personal history: Medical history:

Do you have health insurance (NHIS): If No why?

Do you consume alcohol …… how much? ……

Are you taking any dietary supplements? ……. Which ones? ……..

How often do you consume cocoa and cocoa-related products? …….

What are some of these brands? ……

What type of beverages do you consume?

Number of times you drink in a day:

What health beverages do you drink most often?

If not a health beverage consumer, why?

What do you think might make you become more interested in health beverages?

What times of day do you drink health beverages?

What benefits do you get from health beverages?

Do you prepare your own health beverages or do you buy them?

What overall experience around the health beverage would you like to have that is missing?

How would you describe natural cocoa powder beverage?

What type of food do you mostly consume?

How many times you eat in a day:

Can you do an accurate food recall for past 24 hours?

Can you do a 3 day food recall?

Do you have any general allergies?

Are you allergic to cocoa and cocoa-related products?

What do you know about the use of plants for the cure and/or management of diseases?

Do you think this Natural Cocoa Powder product has brought an obvious change?

In what way do you think this happened?

What do you have to say about your physical activity level?

Rate yourself on a scale of 1- 10 (10 being the highest):

How would you consider your general appearance e.g. weight?

Are you interested in the outcome of the research?

How compliant and dedicated are going to be on this project?

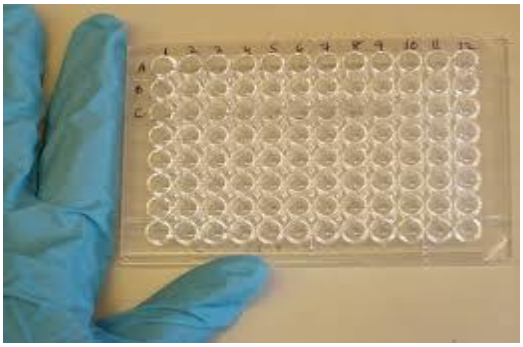
Would you need a daily or otherwise reminder? Pls indicate by text, email, call, visit etc.

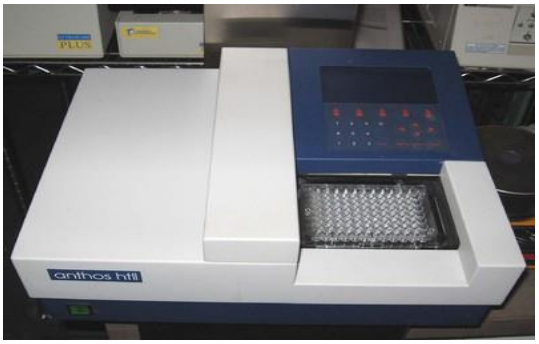
Do you foresee any challenges to the successful execution of the project eg heaters, electricity?

Any other information

Hb: ……... g/dl, MCV: ……..…… fl, MCH ……..……. pg, MCHC ……... g/dl

Total WBC……..….% Differentials ……% WBC……..……%





AGEs 2

MULTISKAN MS
PRIMARY EIA
V. 1.5-0
ABSORBANCE MODE
CONTINUOUS MOVEMENT
FILTER 450

ABSORBANCES
3X. XXX 19XX XX:XX:XX

	1	2	3
A	0.112	0.212	0.212
B	0.139	0.191	0.210
C	0.191	0.239	0.409
D	0.263	0.217	0.214
E	0.422	0.233	0.277
F	0.681	0.211	0.765
G	1.444	0.321	0.295
H	0.267	0.152	0.328

	4	5	6
A	0.175	0.312	0.247
B	0.277	0.174	0.300
C	0.302	0.202	0.278
D	0.249	0.257	0.188
E	0.310	0.222	0.148
F	0.391	0.219	0.695
G	0.279	0.258	0.436
H	0.213	0.227	0.271

	7	8	9
A	0.282	0.049	0.063
B	0.203	0.042	0.043
C	0.047	0.041	0.040
D	0.051	0.052	0.041
E	0.038	0.037	0.043
F	0.036	0.040	0.051
G	0.035	0.047	0.042
H	0.033	0.060	0.044

	10	11	12
A	0.064	-0.001	-0.001
B	0.042	-0.001	-0.001
C	0.041	-0.001	-0.001
D	0.043	-0.001	-0.001
E	0.071	-0.000	-0.001
F	0.040	-0.000	-0.001
G	0.040	-0.001	-0.001
H	0.042	-0.001	-0.001

AGEs 1

MULTISKAN MS
ABSORBANCE MODE
CONT
FILTER 450 MOVEMENT

ABSORBANCES
3X. XXX 19XX XX:XX:XX

	1	2	3
A	0.110	0.212	0.211
B	0.137	0.191	0.210
C	0.185	0.237	0.409
D	0.263	0.217	0.214
E	0.420	0.232	0.275
F	0.667	0.210	0.361
G	1.430	0.319	0.293
H	0.266	0.152	0.325

	4	5	6
A	0.174	0.313	0.247
B	0.276	0.174	0.297
C	0.321	0.199	0.277
D	0.248	0.256	0.188
E	0.310	0.221	0.148
F	0.387	0.216	0.688
G	0.276	0.257	0.435
H	0.209	0.226	0.270

	7	8	9
A	0.282	0.049	0.060
B	0.201	0.042	0.043
C	0.042	0.041	0.040
D	0.047	0.053	0.040
E	0.037	0.038	0.043
F	0.036	0.040	0.051
G	0.035	0.048	0.042
H	0.033	0.060	0.044

	10	11	12
A	0.067	-0.001	-0.001
B	0.042	-0.001	-0.001
C	0.041	-0.001	-0.001
D	0.043	-0.001	-0.001
E	0.070	-0.001	-0.001
F	0.040	-0.001	-0.001
G	0.040	-0.001	-0.001
H	0.042	-0.000	-0.001

VCAM-1

MULTISKAN MS
PRIMARY EIA
V. 1.5-0
ABSORBANCE MODE
CONTINUOUS MOVEMENT
FILTER 450

ABSORBANCES
3X. XXX 19XX XX:XX:XX

	1	2	3
A	0.078	0.627	0.551
B	1.948	0.654	0.558
C	1.600	0.556	0.516
D	1.541	0.414	0.523
E	1.141	0.745	0.534
F	0.864	0.691	0.488
G	0.3	0.1	0.1
H	0.365	0.141	0.548

	4	5	6
A	0.617	0.754	0.636
B	0.545	0.581	0.558
C	0.494	0.378	0.150
D	0.772	0.475	0.150
E	0.546	0.468	0.151
F	0.636	0.714	0.478
G	0.565	0.419	0.172
H	0.559	0.760	0.538

	7	8	9
A	0.655	0.042	0.060
B	0.468	0.047	0.043
C	0.042	0.040	0.052
D	0.053	0.043	0.048
E	0.040	0.054	0.048
F	0.039	0.042	0.043
G	0.042	0.040	0.051
H	0.043	0.042	0.046

	10	11	12
A	0.078	-0.000	-0.000
B	0.047	-0.000	-0.000
C	0.043	-0.001	-0.001
D	0.050	-0.001	-0.001
E	0.042	-0.000	-0.000
F	0.043	-0.001	-0.001
G	0.043	-0.000	-0.001
H	0.041	-0.001	-0.001

~~MAS~~ MDA

MULTISKAN MS 16/01/13
PRIMARY EIA
V. 1.5-0
ABSORBANCE MODE
CONTINUOUS MOVEMENT
FILTER 450

ABSORBANCES
3X. XXX 19XX XX:XX:XX

	1	2	3
A	0.135	0.232	0.347
B	1.791	0.256	0.289
C	1.157	0.250	0.271
D	0.677	0.203	0.281
E	0.242	0.234	0.264
F	0.145	0.374	0.233
G	2.530	0.345	0.251
H	0.223	0.290	0.243

	4	5	6
A	0.337	0.249	0.352
B	0.292	0.327	0.279
C	0.255	0.220	0.264
D	0.215	0.276	0.260
E	0.242	0.294	0.266
F	0.289	0.192	0.264
G	0.274	0.241	0.316
H	0.242	0.297	0.237

	7	8	9
A	0.264	0.290	0.356
B	0.359	0.293	0.283
C	0.335	0.282	0.268
D	0.245	0.256	0.332
E	0.306	0.214	0.378
F	0.291	0.323	0.279
G	0.327	0.295	0.298
H	0.216	0.355	0.250

	10	11	12
A	0.204	0.305	0.375
B	0.290	0.348	0.305
C	0.249	0.284	0.417
D	0.293	0.410	0.343
E	0.361	0.363	0.243
F	0.276	0.302	0.454
G	0.251	0.419	0.353
H	0.286	0.361	0.329

GPX 16/01/13

MULTISKAN MS
PRIMARY EIA
V. 1.5-0
ABSORBANCE MODE
CONTINUOUS MOVEMENT
FILTER 450

ABSORBANCES
3X. XXX 19XX XX:XX:XX

	1	2	3
A	0.118	1.669	1.612
B	2.202	1.585	1.738
C	1.496	1.572	1.379
D	1.178	1.511	1.267
E	0.611	1.446	1.283
F	0.453	1.570	1.647
G	0.342	1.709	1.687
H	1.619	1.733	1.612

	4	5	6
A	1.802	2.092	1.365
B	1.856	1.926	1.997
C	1.880	2.098	1.996
D	1.755	1.958	1.777
E	1.819	1.818	1.803
F	1.768	1.820	1.674
G	1.926	1.747	1.642
H	1.837	1.806	2.022

	7	8	9
A	2.143	1.974	2.178
B	2.070	2.105	1.969
C	1.949	2.049	1.853
D	1.895	2.055	2.021
E	1.984	1.984	1.943
F	1.871	1.731	1.973
G	1.733	1.808	2.030
H	1.872	1.992	1.810

	10	11	12
A	1.963	1.913	2.032
B	1.926	2.327	1.778
C	1.947	2.049	1.899
D	2.131	1.891	2.026
E	1.876	1.917	2.033
F	1.994	1.990	0.068
G	2.059	1.926	1.058
H	1.700	1.898	0.050

TNF-α 25/07/13

MULTISKAN MS
PRIMARY EIA
V. 1.5-0
ABSORBANCE MODE
CONTINUOUS MOVEMENT
FILTER 450

	1	2	3
A	0.046	0.110	0.140
B	2.303	0.117	0.082
C	1.427	0.105	0.158
D	0.862	0.109	0.178
E	0.506	0.142	0.073
F	0.306	0.134	0.074
G	0.213	0.175	0.127
H	0.158	0.169	0.109

	4	5	6
A	0.092	0.074	0.078
B	0.164	0.082	0.076
C	0.177	0.099	0.074
D	0.073	0.074	0.069
E	0.076	0.094	0.072
F	0.121	0.085	0.063
G	0.081	0.080	0.086
H	0.191	0.904	0.084

	7	8	9
A	0.205	0.000	0.000
B	0.147	-0.000	-0.000
C	0.035	0.000	0.000
D	0.035	0.000	0.000
E	0.037	-0.000	-0.000
F	0.035	0.000	0.000
G	0.035	0.000	0.000
H	0.035	0.000	-0.000

	10	11	12

HO -1

MULTISKAN MS
PRIMARY EIA 17/01/13
V. 1.5-0
ABSORBANCE MODE
CONTINUOUS MOVEMENT
FILTER 450

ABSORBANCES
3X. XXX 19XX XX:XX:XX

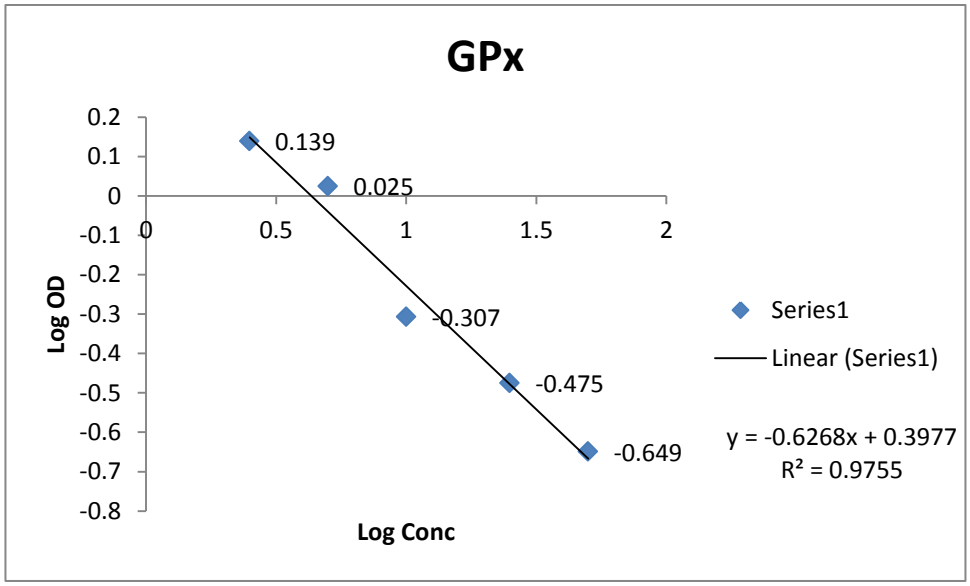
	1	2	3
A	0.107	1.833	1.279
B	2.439	1.612	1.779
C	1.807	2.026	2.333
D	1.368	1.188	1.968
E	0.771	2.175	1.943
F	0.555	1.429	1.255
G	0.276	2.068	1.768
H	2.012	0.968	2.217

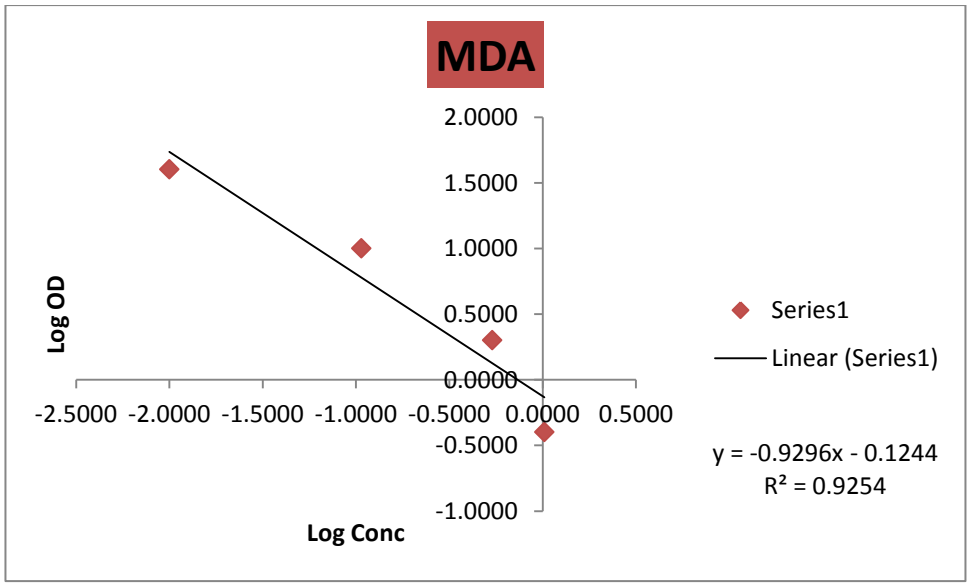
	4	5	6
A	1.759	1.493	1.690
B	1.958	2.033	1.628
C	2.120	2.106	2.078
D	1.992	2.134	1.573
E	1.805	1.651	1.737
F	2.051	2.047	2.007
G	2.084	1.868	1.677
H	2.106	1.909	1.800

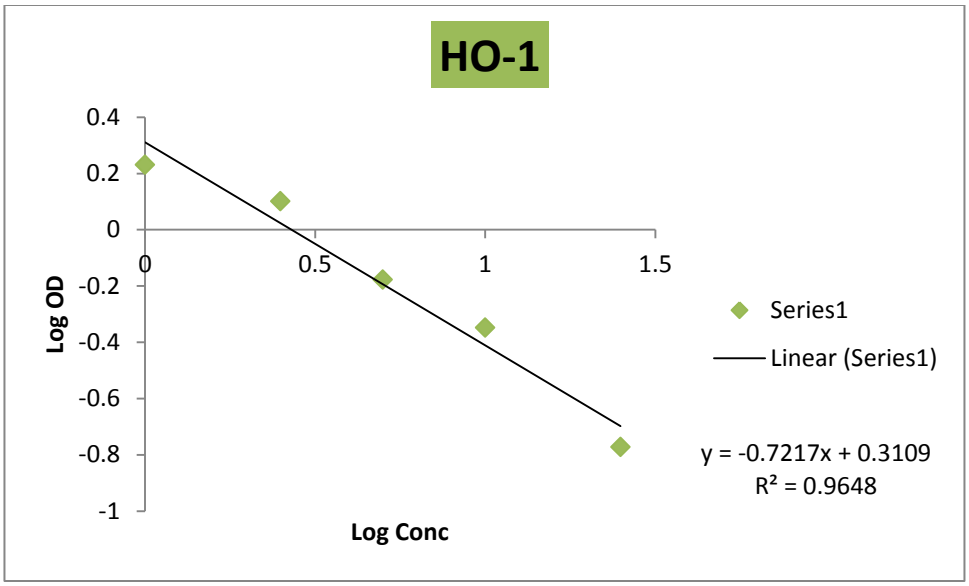
	7	8	9
A	1.648	0.040	0.040
B	1.473	0.042	0.052
C	0.042	0.038	0.039
D	0.040	0.044	0.042
E	0.046	0.035	0.038
F	0.046	0.039	0.045
G	0.039	0.040	0.038
H	0.044	0.036	0.036

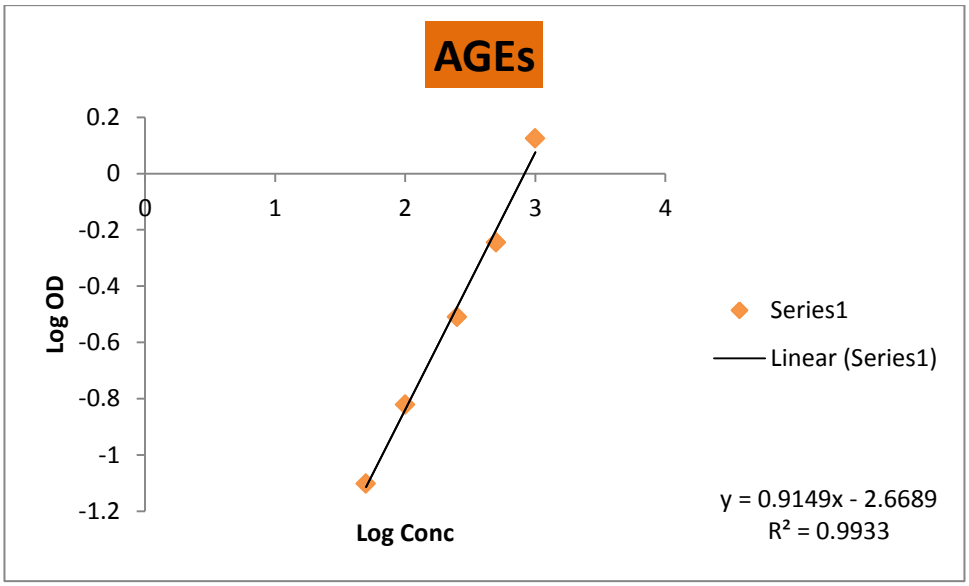
	10	11	12
A	0.042	-0.001	-0.001
B	0.034	-0.001	-0.001
C	0.041	-0.001	-0.001
D	0.047	-0.001	-0.001
E	0.037	-0.000	-0.000
F	0.034	-0.000	-0.000
G	0.033	-0.000	-0.000
H	0.040	-0.000	-0.000

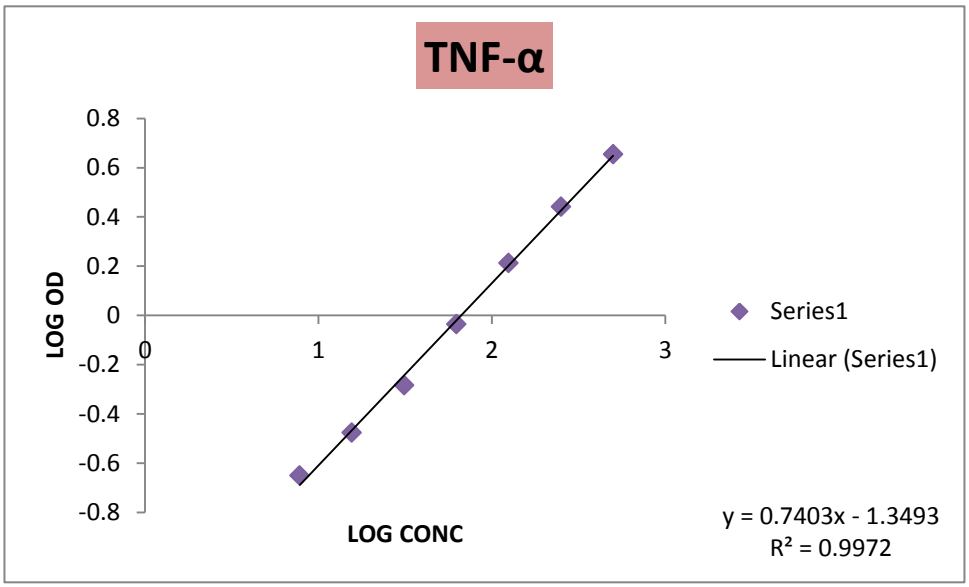
Graphical Representations of the ELIZA assays for further calculations

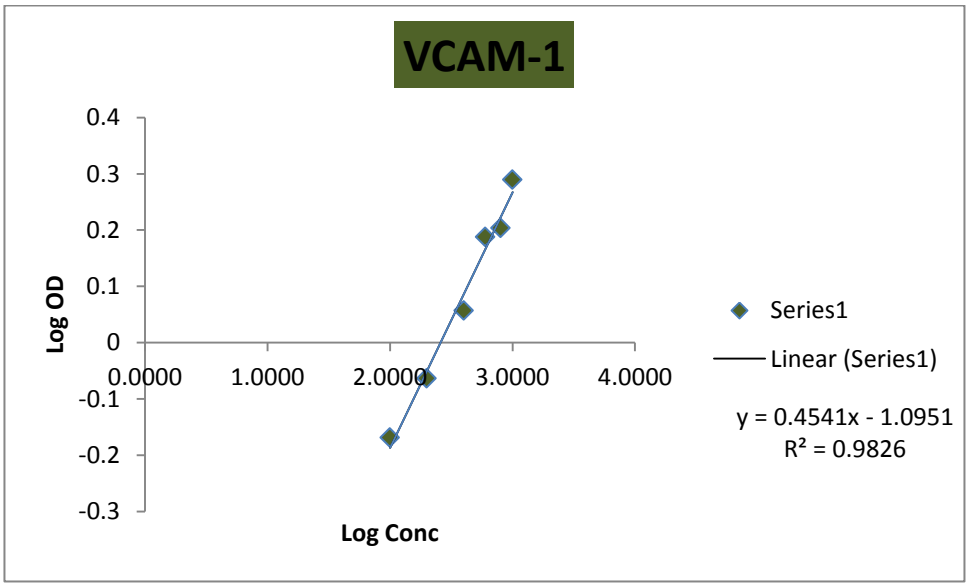












***p*-values based on paired t- and Wilcoxon tests**

THE OVERALL PAIRED t-TEST (n=17)

Parameters	Pre-	Post-	p-value
Weight	65.19±10.45	66.86±10.61	0.005*
Pulse	70.18±8.77	71.29±5.41	0.398
BMI	23.01±3.81	22.96±3.53	0.937
Rest. Metab.	1475.23±270.22	1530±206.73	0.135
Visceral fat	4.41±2.85	4.47±3.66	0.894
Body fat	23.26±12.59	23.00±12.79	0.802
Hb	13.48±1.38	13.95±1.45	0.020*
Hct	31.95±1.45	36.01±7.60	0.005*
MCV	76.14±18.34	78.02±14.97	0.249

Overall Wilcoxon tests

Skeletal Mx	8.13±122.00	15.50±31.00	0.001*
RBC	9.73±146.00	3.50±7.00	0.025*
MCH	9.50±95.00	8.29±58.00	0.599
MCHC	2.00±4.00	9.43±132.00	0.047*
TWBC	9.38±122.00	7.75±31.00	0.000*
L%	8.88±106.50	9.30±46.50	0.000*
B%	8.31±108.00	6.00±12.00	0.000*
E%	8.64±121.00	7.50±15.00	0.000*
N%	11.14±78.00	7.50±75.00	0.000*
M%	5.76±1.35	6.51±1.90	0.022*
SBP	124.53±11.25	121.12±9.11	0.001*
DBP	80.12±9.62	78.24±9.06	0.003*