Chapter

Unsweetened Natural Cocoa Powder: A Potent Nutraceutical in Perspective

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Abstract

Unsweetened natural cocoa powder is a pulverized high-grade powder of compressed solid blocks which remains after extraction and removal of the cocoa butter. The authors determined the elementary composition of UNCP, investigated its effect on nitric oxide levels, toxicity, and its protective effect on the heart, kidney, and liver during simultaneous administration with high dose (HD) artemether/lumefantrine (A/L). Macro- and microelements in UNCP were analyzed with energy dispersive x-ray fluorescence spectroscopy (EDXRF). Adult male guinea pigs were administered various doses of UNCP alone and also simultaneously with A/L. Phytochemical analysis of UNCP showed the presence of saponins, flavonoids, tannins, cardiac glycosides, and 38 macro- and microelements. Histopathological analysis showed no toxic effect on the heart, liver, kidney, lungs, testis, and spleen. Administration of various doses of UNCP increased white blood cell counts and lymphocyte count ($p > 0.05$) compared with the controls. Additionally, UNCP and A/L combination caused an increase in nitric oxide levels when compared with the control group and restores some hematological disorders induced by the 3-day HD A/L administration. Even though UNCP appears to be relatively safe, care should be taken due to the high content of copper element to avoid the possibility of intestinal lining erosion.

Keywords: unsweetened cocoa powder, artemether-lumefantrine, malaria, *Theobroma cacao*, nutraceutical, dark chocolate

1. Introduction

Nutraceuticals, a word coined by DeFelice [1], can be described as any nontoxic food extract scientifically proven to have health benefits for the treatment and prevention of certain diseases.

Natural products have been a good source of drug leads from time immemorial for as long as history has been recorded. The most common among these natural products are plants, which have been exploited for their medicinal purposes [2–4]. Interestingly, many consider plant products to be devoid of adverse effects; thus, the demand for herbal remedies has been on the increase in industrialized

countries as it has been with developing countries [5]. Currently, extensive research is being carried out on natural products including plants from the rain forests, among other places for their potential medicinal value as well as potential toxic effect [6].

One such plant that has generated a lot of interest is *Theobroma cacao,* belonging to the class *Equisetopsida* and family *Malvaceae*. For some decades now, Ghana and Cote D'voire, two neighboring countries in West Africa, have been the world's leading producers of cocoa. Seeds from the fruit of *Theobroma cacao* are used to make cocoa powder and chocolate. Cocoa seeds (or the powder) are major nutraceuticals in Ghana and most parts of Africa. As food, it is consumed by most indigenous people in the raw state (the bean or the pod), as dark chocolate, and as a beverage prepared with powder obtained from cocoa beans. The cocoa powder is prepared by the removal of cocoa butter from the beans by fermentation, which is then followed by the following processes: drying and bagging, winnowing, roasting, grinding, and pressing. After the final process, solid blocks of compressed cocoa are obtained (press cake), which are pulverized into a powder to produce a high-grade cocoa powder. Most of the marketed natural cocoa powder in Ghana is sweetened, even though it has been proven that regular intake of unsweetened natural cocoa powder as a beverage has immense health benefits. Pharmacologically, cocoa is known to exert antioxidant [7], anti-inflammatory [8], antimalarial [2, 3], and anti-asthmatic properties [8, 9].

1.1 Composition of unsweetened cocoa powder

The chemical composition of cocoa powder is well documented. Cocoa contains water-soluble polyphenols (also called flavanols), which include catechins, epicatechin, procyanidins, anthocyanins, and leukoanthocyanins [10]. The antioxidant properties of cocoa are partly ascribed to its structural characteristics, and this is the basis of its role as a free-radical scavenger [10]. Quantitative analysis of unsweetened natural cocoa powder (UNCP) has shown that flavanol contents do not change during different manufacturing processes [11, 12]. Several polyphenols such as 14 N-phenylpropenoyl-L-amino acids, N-[4′-hydroxy-(E)-cinnamoyl]-L-tryptophan, and N-[4′-hydroxy-3′-methoxy- (E)-cinnamoyl]-L-tyrosine have also been found to be present in cocoa powder [13–15].

1.1.1 Phytochemical analysis

A study conducted to ascertain the phytochemical components of unsweetened cocoa powder in Ghana used various pharmacopoeias tests to confirm the presence of the various components. To test for saponins, about 0.5 g of the UNCP was added to water in a test tube and shaken to observe foam formation. To test for the presence of tannins, about 0.5 g of UNCP was dissolved in 80% of aqueous methanol (10 mL). Freshly prepared iron III chloride solution was added and observed for a color change. The presence of alkaloids was confirmed by performing the Dragendorff's, Mayer's, and Wagner's reagent tests as previously described [16].

To identify flavonoids in the UNCP, about 0.1 g of the sample was added to 80% ethanol and filtered. Subsequently, magnesium turnings were added to the filtrate, followed by concentrated HCl. A color change was observed within 10 min. A test for cardiac glycosides was conducted by dissolving about 0.5 g of UNCP in chloroform (2 mL) in a test tube, after which concentrated sulfuric acid was carefully added down the side of the test tube to form a lower layer. This result obtained

confirmed work by Oracz et al. [11], Andres-Lacueva et al. [12], and Stark et al. [17] that the flavanol contents of cocoa does not change during different manufacturing processes.

1.1.2 Micro- and macro-elemental composition

Besides the phytochemical components, micro- and macro elements have been shown to be present in most plant products. The most toxic of these are the heavy metals. These have the potential to interfere with the availability of secondary metabolites [18]. For the safety of consumers, the World Health Organization states maximum permissible levels of heavy metals in raw plant materials for only cadmium (0.3 mg kg^{-1}), arsenic (1 mg kg^{-1}), and lead (10 mg kg^{-1}) [19].

To establish the elemental components of marketed UNCP, energy dispersive X-ray fluorescence method was used. Briefly, samples of the powder were well dried and pelleted using Licowax C micropowder PM-Hoechstwax as binder. Simultaneous measurements of the elemental components were measured with a SPECTRO X-Lab 2000 spectrometer (Geological Survey Department, Accra, Ghana) as previously described [20]. The results revealed a total of 38 elements, of which 12 were macro, and are listed in **Table 1**.

1.2 Toxicity of unsweetened cocoa powder

Rarely are toxicological studies conducted on nutraceuticals. This could be because such products are generally termed "natural" and perceived to be devoid of any adverse effects. Interestingly, cardiotoxic and teratogenic potentials have been reported for cocoa [21, 22]. Against this background, a study was conducted to elucidate the safety of cocoa powder in Sprague Dawley (SD) rats. The experimental procedure for this study was approved by the departmental ethical and protocol review committee and the Noguchi Memorial Institute for Medical Research, University of Ghana. Institutional Animal Care and Use Committee also approved the protocol for the study. The study was conducted in accordance with international ethical guidelines.

The design of the experiment was such that the rats were randomly assigned to either the experimental group or the control group. There were 20 rats in all, 10 in each group. All rats had access to water and food except for a 12-hour fasting period before the administration of the unsweetened natural cocoa powder. The experimental group of rats received UNCP at a dose of 2000 mg/kg, while the control group received an equal volume of distilled water only. After the study period, effects of UNCP on selected organs, as well as on biochemical indices of blood, are described below.

1.2.1 The effect of UNCP on the body and relative weight of organs

The effect of UNCP on the body and relative weight of organs was determined as described previously [20]. Selected organs, the lungs, heart, liver, spleen, kidney, intestines, and testis, were excised. The organs were then placed in ice-cold saline to wash off blood. They were trimmed of fat, connective tissues, blot dried, and then weighed on a balance. The organ-to-body weight index, OBI, was determined as the ratio of organ weight and the body weight of the animal before sacrifice × 100. Body weight of rats was also taken dosing, a week after dosing on day 7 and before sacrificing them on day 14 (**Tables 2** and **3**).

	Element	Mean/SD mg/4000 mg
Macroelements	Na	2.4666 ± 0.00
	Mg	33.0133 ± 0.02
	Al	14.0093 ± 0.01
	Si	15.3880 ± 0.02
	P	64.3866 ± 0.00
	S	30.9120 ± 0.00
	Cl	2.3616 ± 0.00
	K	149.0667 ± 0.03
	Ca	11.0146 ± 0.00
	Ti	0.0232 ± 0.00
	Mn	0.4093 ± 0.00
	Fe	1.0309 ± 0.00
Microelements	V	0.2320 ± 1.73
	Cr	0.4200 ± 17.44
	Co	0.0108 ± 0.10
	Ni	0.0638 ± 1.16
	Cu	0.2984 ± 1.71
	Zn	0.4086 ± 0.74
	Ga	0.0024 ± 0.00
	As	0.0020 ± 0.00
	Rb	0.1698 ± 0.49
	Sr	0.1064 ± 0.20
	Y	0.0016 ± 0.00
	Zr	0.0125 ± 0.42
	Nb	0.0070 ± 0.29
	Mo	0.0044 ± 0.00
	Sb	0.0043 ± 0.06
	I	0.0133 ± 0.15
	Cs	0.0232 ± 0.10
	Ba	0.0620 ± 5.81
	La	0.0480 ± 0.00
	Ce	0.0849 ± 4.97
	Hf	0.0148 ± 0.17
	Ta	0.0213 ± 0.06
	Pb	0.0036 ± 0.00
	Bi	0.0024 ± 0.00
	Th	0.0020 ± 0.00
	U	0.0112 ± 0.10

Table 1.
Mean and standard deviation (SD) of measured elements (mg/4000 mg).

1.2.2 Histopathology examination

The histopathology examination was performed as previously reported by Asiedu-Gyekye et al. [20] The lungs, heart, liver, spleen, small intestinal organs, and testis were placed in a 10% buffered formaldehyde solution for 24 h. Tissue samples from the organs were paraffin embedded and sectioned at 5 μm thickness. The sectioned tissues were stained with hematoxylin and eosin (H&E) and evaluated under a light microscope (Olympus BX 51TF) for histological changes.

1.2.3 The effect of UNCP on hematological parameters

The effect of UNCP on hematological parameters was studied as described previously by Asiedu-Gyekye et al. [20]. Blood (2 mL) from euthanized SD rats was drawn out by cardiac puncture. This was then transferred into ethylene diamine tetra acetic acid (EDTA) test tubes. An automated hematology analyzer was used for the evaluation. Peripheral blood smear was used to examine the nature of blood cells.

DAY	CTRL	UNCP	*p* value
Day 1	112.5 ± 12.50	142 ± 8.000	0.0526
Day 7	115.0 ± 10.00	142.0 ± 7.176	0.3618
Day 14	112.5 ± 7.500	135.0 ± 7.000	0.0522

Table 2.
Changes in body weight of SD rats after administration of unsweetened natural cocoa powder solution.

ORGANS	CTRL	UNCP	*p* value
Liver	8.605 ± 4.225	7.454 ± 1.263	0.3260
Kidney	0.6000 ± 0.05000	0.5500 ± 0.02739	0.3618
Heart	8.605 ± 4.225	7.454 ± 1.263	0.3534
Lungs	0.8500 ± 0.05000	0.7600 ± 0.02915	0.3618
Spleen		7.454 ± 1.263	0.0829
Testis		1.224 ± 0.02502	0.3618

Table 3.
Changes in organ weight of SD rats after administration of unsweetened natural cocoa powder solution.

1.2.4 The effect of UNCP on serum biochemistry

The effect of UNCP on serum biochemistry was studied as described previously by Asiedu-Gyekye et al. [20]. Blood (1 mL) of sacrificed rats was collected via cardiac puncture. The blood sample was allowed to stand for a while, this was then centrifuged at 4000 rpm for 15 minutes using a Wiperfuge centrifuge. The serum was then collected for the measurement of the biochemical parameters (**Table 4**).

Herbal medicines are thought to have no side effects or the potential to cause harm due to their natural origins and are often considered as healthy food supplements and not drugs. Additionally, most herbs used for medicinal purposes lack specific instructions concerning dose, frequency, and route of administration. Evaluating UNCP as nutraceutical, the assumption was that the average African weighs 60.70 kg.

Lead and arsenic limits permissible by the WHO are 0.00016 and 0.0010 mg/kg, respectively [23]. Heavy metals determined in UNCP were Pb—0.0036 mg and As—0.002 mg corresponding to 0.0002 and 0.0001 mg/kg, respectively, which are far below WHO guidelines. There was a high content of copper (0.2984 mg per 4 g UNCP) observed which should be of concern especially when high doses of UNCP are consumed. This is due to the fact that copper has been shown to play a role in the pathogenesis of Wilson's syndrome and liver damage [16, 24], while the high content of chromium could have beneficial effect in the management of diabetes mellitus and cardiovascular disorders [18, 25, 26]. Thus, the relationship between these elements, nutrition, and medicine observed is indicative of the fact that micro- and macro-elements of herbal products does influence certain body processes and hence should not be envisaged always as contaminants.

Body weight changes between the control group and the experimental group were observed on day 1, day 7, and day 14. However, with respect to dosing, they were found not to be statistically significant ($p > 0.05$). Body weight of the SD rats decreased by 8.3 and 22.2% ($p < 0.05$) in the 2nd week after dosing for the control and test groups, respectively. Although the decrease in body weight with the control animals and the test animals is consistent with the corresponding decrease in their food and water intake, that for the test animals might partly be due to the ability of UNCP to react with nutrients in the body including stored fat, carbohydrate, and protein.

Parameter	UNITS	CTRL	UNCP	*p* value
Creatinine	μmol/L	43.25 ± 2.925	40.61 ± 1.158	0.3618
Urea UV	mmol/L	8.605 ± 4.225	8.854 ± 1.263	0.2880
Bilirubin total	μmol/L	0.795 ± 0.0144	0.625 ± 0.165	0.3960
ALT	U/L	125 ± 0.722	120 ± 5.01	0.1999
Albumin	g/L	40.5 ± 0.442	39.0 ± 0.692	0.2046
AST	U/L	2.49 ± 0.358	2.52 ± 0.541	0.3263
Total protein	g/L	73.1 ± 1.02	70.6 ± 3.09	0.6061
Triglycerides	mmol/L	1.28 ± 0.160	0.943 ± 0.116	0.1185
Bilirubin direct	μmol/L	0.555 ± 0.0240	1.38 ± 0.489	0.1791
ALP	U/L	707 ± 7.86	535 ± 40.7	0.0103
GGT	U/L	1.20 ± 0.200	2.40 ± 1.44	0.4316
HDL cholesterol	mmol/L	0.560 ± 0.0372	0.755 ± 0.0349	0.0060
Cholesterol	mmol/L	2.08 ± 0.0854	2.15 ± 0.129	0.6616
LDL cholesterol	mmol/L	1.27 ± 0.0740	0.934 ± 0.124	0.0810
Na^+	mmol/L	137 ± 0.479	133 ± 0.477	0.0002
K^+	mmol/L	5.75 ± 0.132	6.44 ± 0.293	0.1098
Ca^{2+}	mmol/L	0.845 ± 0.0132	0.858 ± 0.0180	0.5200

Table 4.
Changes in serum biochemistry of SD rats after administration of unsweetened natural cocoa powder solution.

A reduction of 10.90% ($p > 0.05$) in the organ weight was observed in the test group as compared to the control group. These variations in the relative organ weight of the control and experimental groups of SD rats were not significantly different.

High-density lipoproteins (HLD) increased slightly with a p value <0.05, a decrease in the level of triglycerides and low density lipoprotein (LDL) cholesterol of the UNCP group ($p > 0.05$) was observed. In comparison with that of the control, the cholesterol levels remained relatively unchanged. This is consistent with the speculation that UNCP possess lipid lowering abilities.

Hematological results (**Table 5**) revealed a decrease (28.44%, $p > 0.05$) in the level of platelet in the UNCP group in comparison with the controls. Polyphenols in cocoa have been found to reduce platelet count. Neutrophil and lymphocyte polymorph of white blood cells showed a slight decrease of 8.02 and 18.73%, respectively ($p > 0.05$).

Histopathology evaluation of the organs studied; liver, kidney, heart, lungs, spleen, and the testis of the animals that received UNCP showed no toxic effect as compared to that of the control group. UNCP solution therefore is not likely to have toxic effects on the kidney when administered in a single oral high dose of 2000 mg/kg. Notable changes were observed on the small intestines in the form of erosions of the mucosal lining of the villi (**Figure 1**). These effects were, however,

Parameter	Ctrl	UNCP	*p* value
WBC	8.605 ± 4.225	7.454 ± 1.263	0.3618
Neut. number	2.020 ± 0.4400	1.858 ± 0.3836	0.4109
Lymph number	5.985 ± 3.435	4.864 ± 1.111	0.3422
Mono. number	0.3700 ± 0.220	0.3740 ± 0.1225	0.4936
Eosin. number	0.2250 ± 0.125	0.3520 ± 0.07439	0.2045
Baso. number	0.0050 ± 0.005	0.0060 ± 0.002449	0.4228
Neut.%	27.65 ± 8.450	25.38 ± 4.450	0.4021
Lymph.%	65.80 ± 7.600	63.90 ± 6.119	0.4348
Mono.%	4.000 ± 0.6000	5.700 ± 1.642	0.2828
Eosin.%	2.500 ± 0.2000	4.940 ± 0.9553	0.0941
RBC	7.360 ± 1.060	7.156 ± 0.5533	0.4290
HGB	12.20 ± 1.500	12.38 ± 1.048	0.4646
HCT	36.05 ± 4.050	37.82 ± 3.269	0.3877
MCV	49.20 ± 1.600	52.76 ± 0.9405	0.0515
MCH	16.65 ± 0.3500	17.28 ± 0.2354	0.1037
MCHC	33.80 ± 0.4000	32.76 ± 0.3696	0.0862
RDW-CV	17.05 ± 3.150	15.74 ± 1.105	0.3106
RDW-SD	27.00 ± 2.000	27.70 ± 1.064	0.3746
PLT	696.5 ± 324.5	498.4 ± 166.3	0.2855

Table 5.
Changes in hematological indices in rats after receiving 2000 mg/kg bwt of UNCP.

not observed in the control animals those that received equivalent volumes of the vehicle (distilled water). This could be due to the high concentration of proanthocyanidins contained in the 2000 mg/kg dose of UNCP (approximately 2.5 g in man). Proanthocyanidins have been found to instigate the destruction of the mucosal lining of the gastrointestinal tract.

1.2.5 Conclusion

In conclusion, the aqueous solution of unsweetened natural cocoa powder administered at the single oral high dose of 2000 mg/kg appears to be relatively safe in male SD rats. Caution should however be taken when using UNCP especially in high quantities or amounts since it is capable of causing considerable damage to the mucosal lining of the small intestines.

1.2.6 Reproductive toxicity

Since UNCP is widely consumed by individuals in their reproductive period of life, it is worth investigating the generative toxicity, genotoxic, and aspects of the reproductive toxicity potential of UNCP in both male and female white wistar rats. As a preliminary study, the genotoxic potential of UNCP was assessed using the DNA comet method. DNA breaks in the rectal epithelium, liver, bone marrow, and kidney of the mice were quantified and compared with the negative control (2% starch). Pre-implantation loss, viable fetuses, corpora lutea and post-implantation

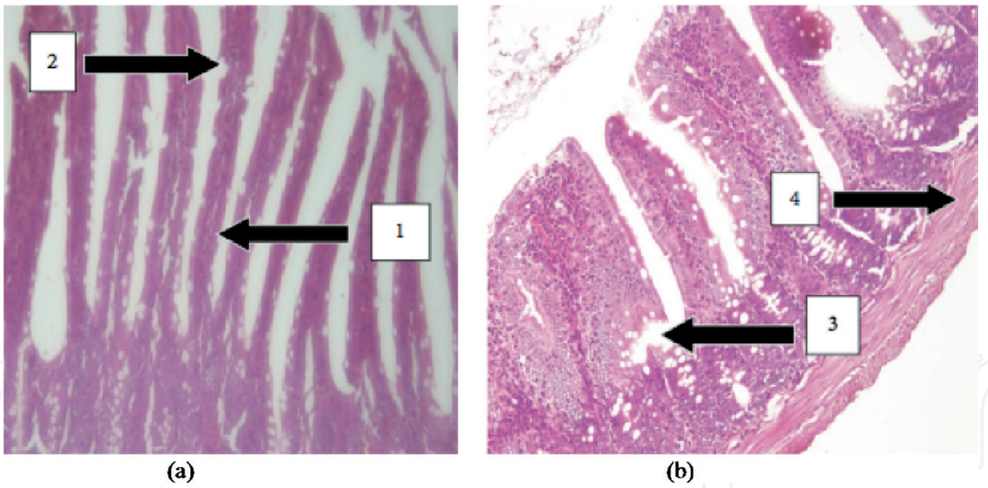

Figure 1.
H&E stained sections of the small intestine of 20× magnification. (a) Small intestine sections of control male SD rates showing villi (1) goblet cells (2) basement membrane with no proliferation (4). (b) Small intestine sections of the group treated with UNCP showing moderate changes and erosion of the mucosal lining of the villi (3).

loss, and implantation sites/number of implants were assessed. Results show that UNCP does not exhibit any reproductive toxicity potential.

1.3 Organoprotective effect of unsweetened cocoa powder against high-dose artemether-lumefantrin-induced effects

Artemisinin and its derivatives, derived from *Artemisia annua* (**sweet** wormwood), have impressive parasiticidal properties *in vivo* and *in vitro* [27, 28]. Despite the efficacy of artemisinin in reducing the malaria parasite load in the body, monotherapy was discouraged in an attempt to delay or prevent the development of drug resistance. Artemisinin-based combination therapy (ACT) was recommended by the WHO for use in the treatment of malaria after resistance was developed to the quinine derivatives used at the time [29, 30]. Increased therapeutic efficacy was reported to be associated with the use of the combination. Treatment failure to these therapies is suspected, and in recent years, some countries have considered increasing the dose of the A/L in treatment in order to arrest the issue of resistance [31], but increase in dose implies that there will be increased side effects, adverse reactions, and hepatotoxic effects [32]. In fact, there are already concerns about frequent usage of A/L on some organ systems [28]. Considering the fact that, so far, A/L is one of the most effective combination therapies, the issue of drug-induced hepatotoxicity needs to be addressed. Another effect of A/L is its effect on nitric oxide levels, where it has been found to reduce nitric oxide levels. However, other studies show that A/L increase nitric oxide levels as a compensatory mechanism in cases of reduced nitric oxide levels [28]. Additionally, some studies of artemether in rats have shown changes in the hematological profile of the rats [33]. It is therefore suspected that artemether may aggravate anemia in malaria patients. In another study, A/L was found to reduce red blood cell count (RBC), HGB, and packed cell volume (PCV) in patients taking treatment [34].

Ghana is the second producer of cocoa in the world. It is therefore not surprising that many consume cocoa beverage at one time or the other during the day. It is also one of the endemic regions for malaria. Thus, a high possibility of consuming a cocoa product while being treated for malaria using any of the ACTs is

recommended by WHO. Artemether/lumefantrine (A/L) is one of the combination therapies used more frequently for treating malaria in Ghana.

It is interesting to note that anecdotal reports of the ability of cocoa to prevent malaria exist. Following these reports, regular intake of cocoa powder as a beverage has been shown to be associated with reduction in the incidence of episodic malaria and its use as diet-mediated malaria prophylaxis [3]. The antiplasmodial activity of different fractions especially the nonpolar solvent fractions have also been confirmed. Thus, simultaneous consumption of cocoa beverage during antimalarial treatment with A/L is expected to have dual benefits such as rapid clearance of the malaria parasites.

Regular intake of unsweetened natural cocoa powder as a beverage has immense health benefits including both cardiovascular and neurodegenerative disorders, reduces platelet aggregation, and improves lipid profile [25, 35].

Determination of the major elemental composition and toxicity of unsweetened cocoa powder was conducted. Another set of projects were aimed at assessing whether UNCP will worsen or is able to prevent some common cardiovascular and renal side effects associated with the use of A/L, its effect on nitric oxide levels and to assess its hepatoprotective potential against A/L-induced liver toxicity during their simultaneous ingestion in male guinea pigs, and finally to investigate the effect of UNCP on the hematological parameters and NO levels during high-dose (HD) A/L administration.

The methods used in the assessment of the parameters listed above are as described previously by Asiedu-Gyekye et al. [26] as follows: 30 adult male guinea pigs weighing between 300 and 350 g were purchased from the Animal Experimentation Department of the Noguchi Memorial Institute for Medical Research, University of Ghana, Legon. The guinea pigs were acclimatized to laboratory environment (20–23°C) with a 12 h light-darkness cycle for 7 days prior to experimentation. The guinea pigs had access to standard laboratory diet and water *ad libitum*. The adult male guinea pigs were randomly assigned to five groups with each group containing six guinea pigs, 500 mg/kg per body weight, respectively, for 14 days, with two other groups serving as controls. One control group (negative control group [NCG]) received 75 mg/kg body weight A/L, and the other group was given distilled water (vehicle control group [VCG]).

The dosage regimen was as follows:

Group 1: Control (distilled water only)/vehicle control (CTRL).
Group 2: 75 mg/kg A/L(last 3 days)/negative control(COARTEM).
Group 3: Cocoa 300 mg/kg (14 days) + 75 mg/kg A/L (last 3 days) (300).
Group 4: Cocoa 900 mg/kg (14 days) + 75 mg/kg A/L (last 3 days) (900).
Group 5: Cocoa 1500 mg/kg (14 days) + 75 mg/kg A/L (last 3 days) (1500).
The guinea pigs were, thus, observed daily for a total period of 14 days.

Conversion of animal doses to HED was based on basal surface area. HEDs were calculated according to a study by Reagan-Shaw et al. [24] and Asiedu-Gyekye et al. [26] using the formula:

Human equivalent dose (HED) (mg/kg) = Animal dose (mg/kg) × (Human km/Human km).

The value of km factors (i.e., body weight, kg/surface area, m^2) for adult and guinea pigs to be 37 and 8, respectively, and an average weight of Ghanaian to be 70.0 kg.

After day 14, the guinea pigs were euthanized with 50 mg/kg chloroform by exsanguination, and 2 ml of blood was sampled by cardiac puncture and transferred into EDTA-2 k test tubes for immediate analysis.

1.3.1 Potential of unsweetened natural cocoa powder to attenuate high-dose artemether-lumefantrine-induced hepatotoxicity in nonmalarious guinea pigs

1.3.1.1 Biochemical assays

Blood samples were collected from the descending aorta and aliquoted into EDTA-2K tubes and plain tubes, respectively, at the end of the dosing period. This was done after euthanization of the animals under ether anesthesia. The EDTA blood was immediately analyzed for hematological parameters using the SYSMEX Hematology Autoanalyzer (Kobe, Japan), while sera prepared from blood in plain tubes were used for biochemical examinations including clinical chemistry measurements such as alkaline phosphatase (ALP), alanine aminotransferase (ALT) or glutamic pyruvic transaminase (SGPT) levels, serum glutamic oxaloacetic transaminase (SGOT) or aspartate transaminase, and gamma glutamyl transpeptidase (GGT). These were measured as liver function tests (LFT), which gives an indication of the state of the liver.

Nitric oxide levels were also measured using the Griess Reagent System. The total nitric oxide kit by R&D Systems was used in this study, and the assay reported previously by Bryan and Grisham [36] was employed for this purpose. In this assay, nitrate is converted to nitrite by nitrate reductase after which colorimetric detection of nitrite as an azo dye is carried out. The Griess reaction involves a two-step diazotization reaction, acidified NO_2^- to produce a nitrosating agent, which then reacts with sulfanilic acid to produce the diazonium ion. The diazonium then couples to *N*-(1-naphthyl) ethylenediamine to form azo-derivatives, which are chromophoric. These azo-derivatives are absorbed within the range of 540–570 nm.

Elevation of serum and plasma enzymes such as alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate transferase (AST) has been shown to be reliable markers of acute hepatocellular damage. This occurs due to hepatocyte membrane distortion leading to membrane leakage of the hepatocyte cytosolic contents. AST is abundant in the cardiac muscles, skeletal muscles, kidneys than in the liver; thus, ALT is the most reliable marker.

This study revealed that A/L increased ALT, AST, GGT, and bilirubin levels but witnessed a reduction in albumin and total protein levels indicating the presence of hepatotoxicity (**Figures 2**–**4**). The increases in AST and ALT were found to be dose dependent.

Usually in patients with hepatotoxicity, ALT levels increase by more than three times the upper limit of normal, ALP levels also increase by more than twice the upper limit or total bilirubin more than twice when associated with increased ALP or ALT. It is important to mention that liver damage could manifest with either predominately initial alanine transferase elevation (hepatocellular) or initial alkaline phosphatase rise (cholestatic). The two are, however, not mutually exclusive.

The synthetic function of the liver can be assessed by the levels of total protein and albumins. Administration of A/L leads to a decrease in total protein and albumin levels supporting the hepatotoxic effects of A/L (**Figures 5**–**7**).

UNCP significantly increased the levels of total proteins, which further supports its hepatoprotective effect. Elevation of albumin levels after administration of UNCP was significant. For the first time, this study reports the hepatoprotective effect of UNCP against A/L-induced hepatic damage in guinea pigs.

1.3.1.2 Histopathological studies

Guinea pigs were euthanized, and their livers were swiftly excised and washed with 0.9% saline. The livers were stored in 10% neutral buffered formaldehyde.

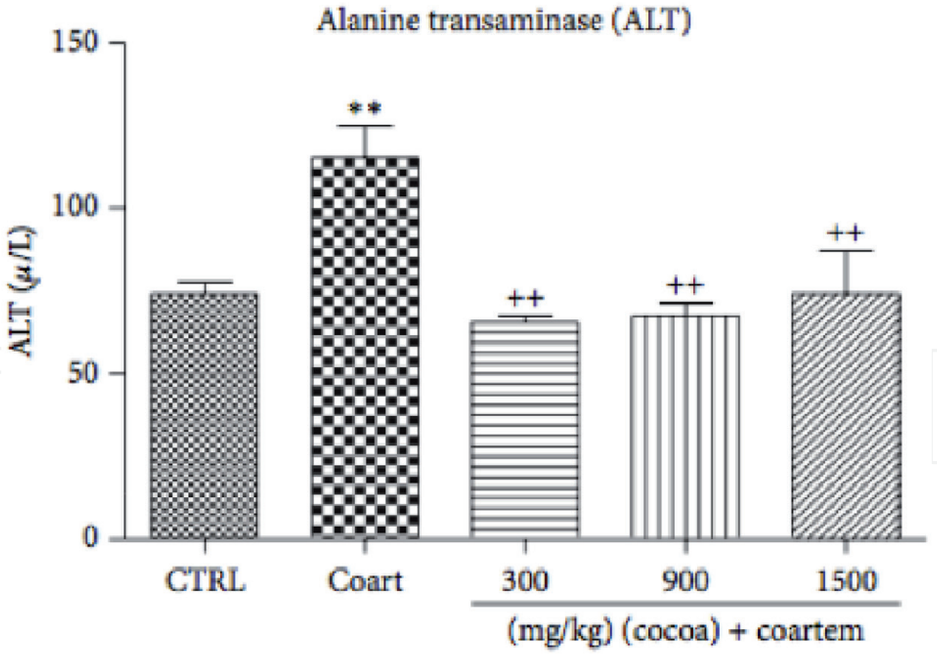

Figure 2.
*Changes in ALT levels of guinea pigs during a 14-day administration of UNCP followed by a 3-day A/L administration. The differences among the means were analyzed using one-way analysis of variance (ANOVA) followed by Newman-Keuls post hoc analysis, where ** means $p < 0.001$ when compared with the control (distilled water) and $^{++}p < 0.001$ when compared with the A/L group.*

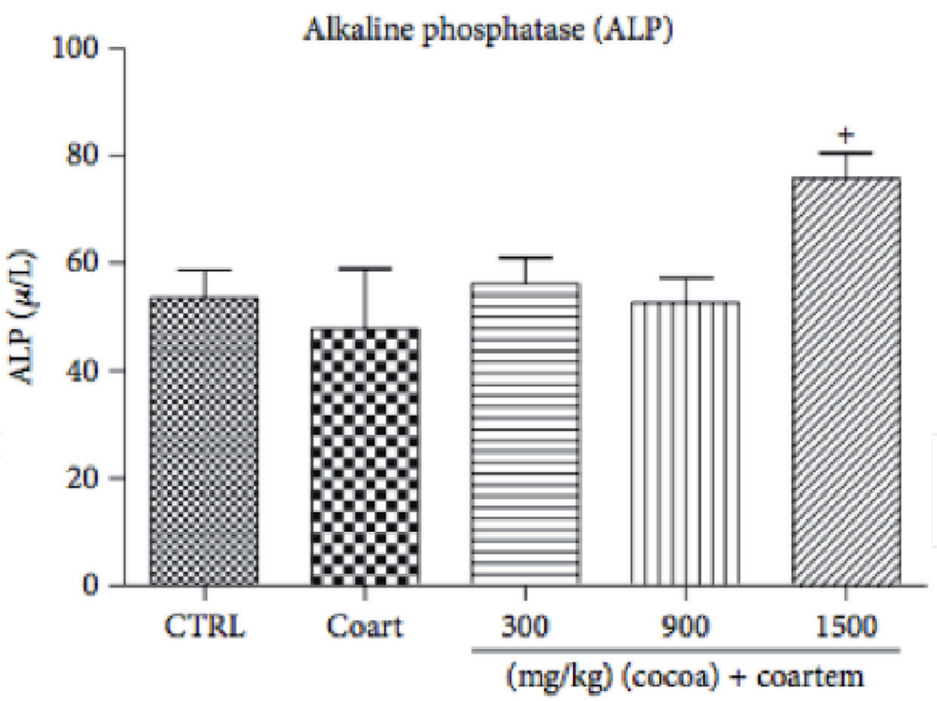

Figure 3.
Changes in ALP levels of guinea pigs during a 14-day administration of UNCP followed by a 3-day A/L administration of UNCP followed by a 3-day A/L administration. The differences among the means were analyzed using one-way ANOVA followed by Newman-Keuls post hoc analysis, where $^{+}$ means $p < 0.05$ when compared with the A/L group.

The liver tissues were then cut and sectioned using a microtone into 2 μm thick liver slices and stained with hematoxylin-eosin for examination. The stained tissues were observed with an Olympus microscope (BX-51) and photographed by INFINITY 4 USB Scientific Camera (Lumenera Corporation, Otawa, Canada).

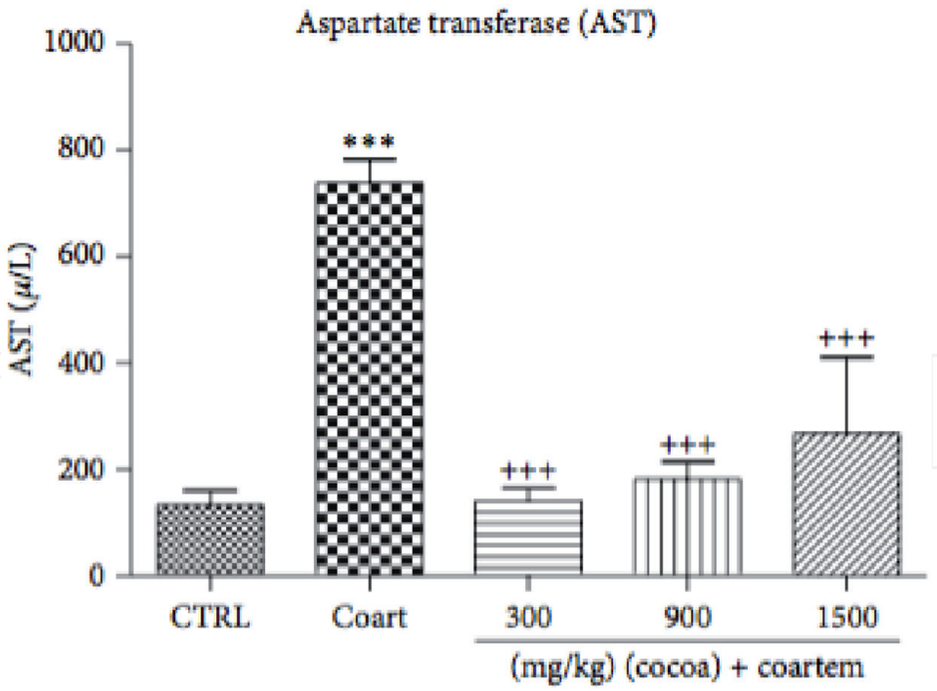

Figure 4.
*Changes in AST levels of guinea pigs during a 14-day administration of UNCP followed by a 3-day A/L administration. The differences among the means were analyzed using one-way ANOVA followed by Newman-Keuls post hoc analysis, where *means $p < 0.05$, ** means $p < 0.001$ when compared with the control (distilled water) and ++ $p < 0.001$ when compared with the A/L group.*

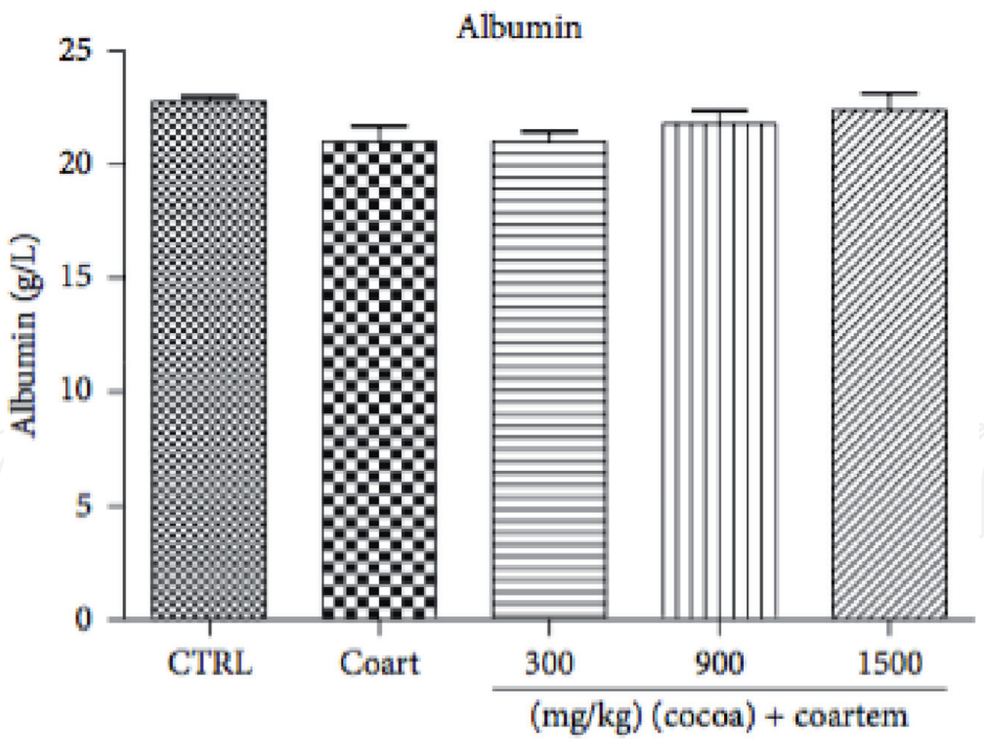

Figure 5.
Changes in albumin levels of guinea pigs during a 14-day administration of UNCP followed by a 3-day A/L administration. The differences among the means were analyzed using one-way ANOVA followed by Newman-Keuls post hoc analysis.

The hepatoprotective effect of UNCP was further confirmed in the histological data presented above, where very insignificant abnormalities were observed in the group that received UNCP before A/L. Damaged liver tissues in animals that received A/L alone was observed, which is evidenced by disturbed (necrotic)

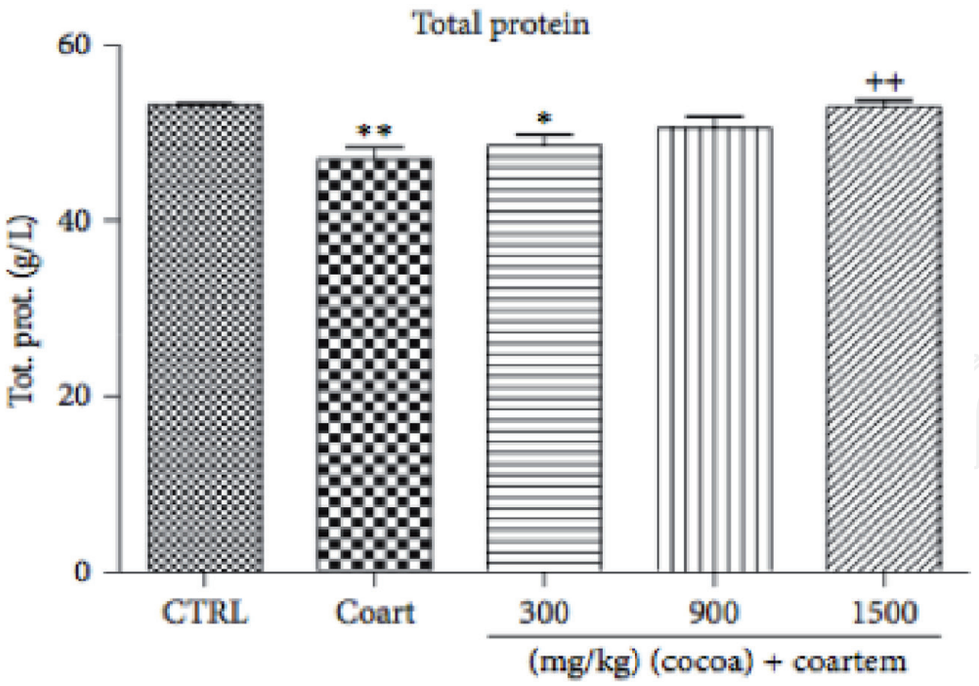

Figure 6.
*Changes in total protein levels of guinea pigs during a 14-day administration of UNCP followed by a 3-day A/L administration. The difference among the means were analyzed using one-way ANOVA followed by Newman-Keuls post hoc anlaysis, where * means p < 0.05, ** means p < 0.001 when compared with the control (distilled water) and ++p < 0.001 when compared with A/L group.*

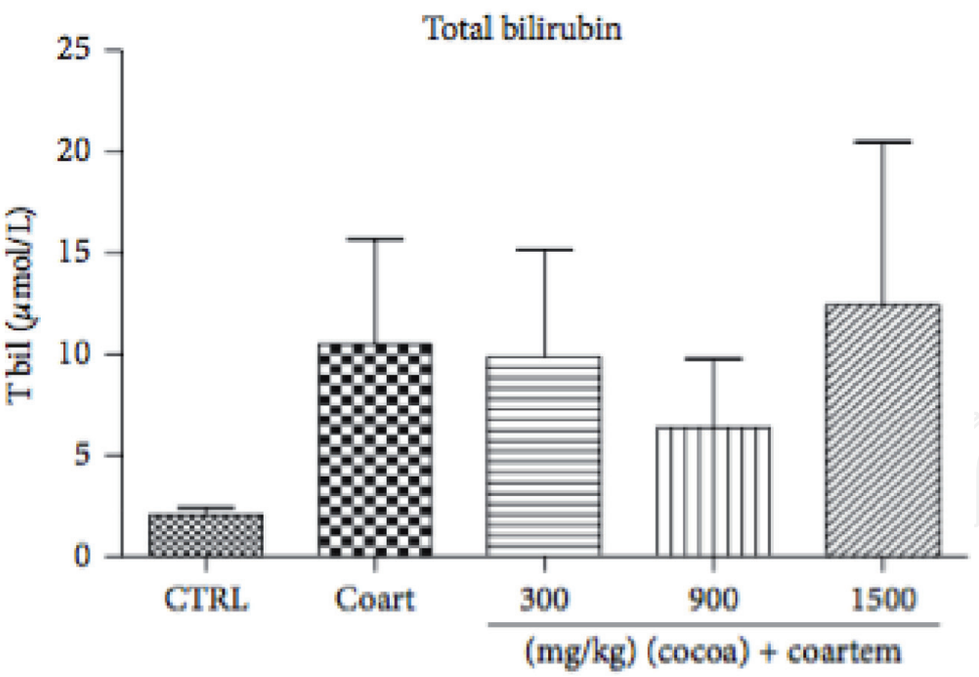

Figure 7.
Changes in total bilirubin levels of guinea pigs during a 14-day administration of UNCP followed by a 3-day A/L administration. The differences among the means were analyzed using one-way ANOVA followed by Newman-Keuls post hoc analysis.

liver parenchyma (NeLP), a highly congested and dilated central vein (CCV), and lymphocytic infiltration (LYM) in all animals (**Figure 8(b)**). Administration of UNCP before A/L reduced the extent of liver damage evidenced by the undisturbed liver parenchyma with an uncongested but dilated central vein (mild liver damage).

1.3.1.3 Conclusion

Unsweetened natural cocoa powder has hepatoprotective potential during high-dose A/L administration. The simultaneous consumption of UNCP and A/L is not likely to result in liver injury or dysfunction. However, care must however be taken during high daily consumption due to the high copper content.

1.3.2 Cardio and renal toxicity

Artemether-lumefantrine is one of the fixed-dose combination therapies recommended by the WHO for the treatment of malaria falciparum in Africa. Administration of this medication, however, generates free radicals that have the potential of causing cellular damage and other organ toxicity, characteristic being cardio-hepato- and renal toxicity.

It is speculated that simultaneous consumption of natural cocoa during antimalarial treatment with A/L is expected to rapidly clear the malaria parasites as well as ameliorate the A/L-induced toxic injury to heart and kidneys. Thus, the consumption of natural antioxidants such as found in cocoa could be beneficial in rectifying such damage in humans. The study also aimed at assessing whether UNCP will worsen or is able to prevent some common cardiovascular and renal side effects associated with the use of A/L. To establish the protective effect on the heart and

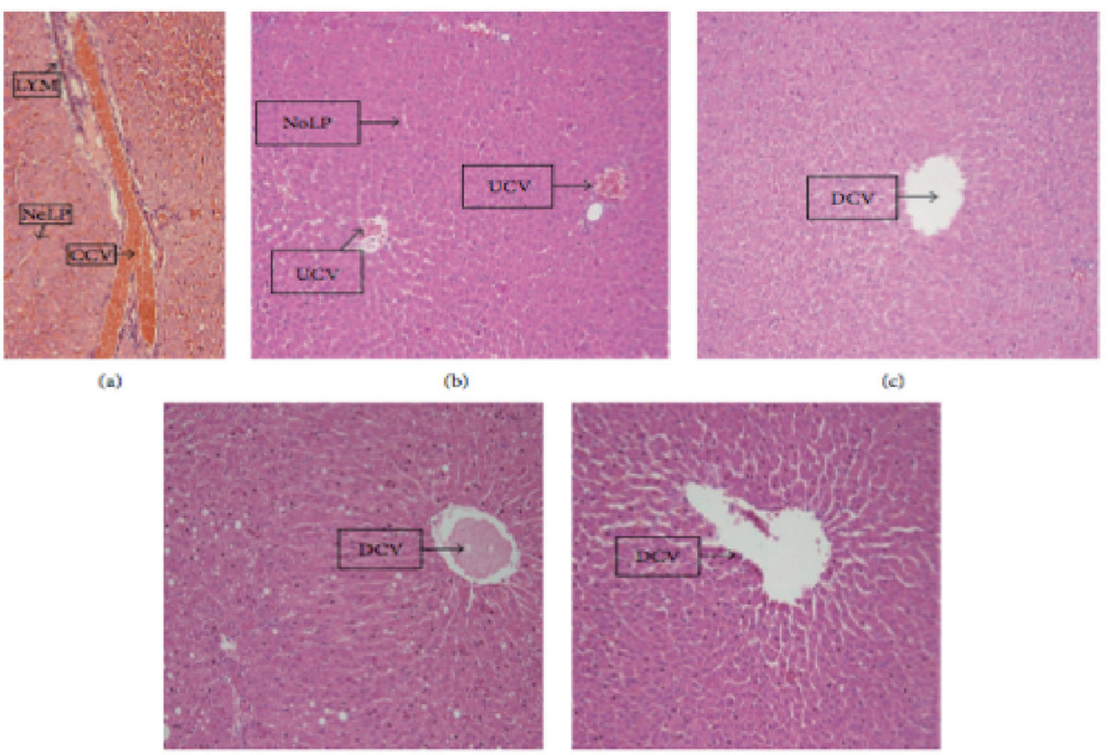

Figure 8.
Microtome sections of liver from guinea pigs that received (a) only a 3-day HD A/L administration (75 mg/kg/bwt) showing disturbed (necrotic) liver parenchyma (NeLP), a highly congested and dilated central vein (CCV), and lymphocytic infilteration (LYM). (b) Distilled water, control showing undisturbed liver parenchyma (NoLP) with uncongested central veins (UCV). Note the regularity of liver cell plates, microcirculatory zones, and sinusoids. (c) A 14-day UNCP administration (300 mg/kg/bwt) followed by a 3-day A/L administration (75 mg/kg/bwt) showing undisturbed liver parenchyma with uncongested but dilated central vein (DCV). (d) A 14-day UNCP administration (900 mg/kg/bwt) followed by a 3-day A/L administration (75 mg/kg/bwt) showing undisturbed liver parenchyma with uncongested but dilated central vein (DCV). (e) A 14-day UNCP administration (1500 mg/kg/bwt) followed by a 3-day A/L administration (75 mg/kg/bwt) showing undisturbed liver parenchyma with uncongested but dilated central vein (DCV) (H&E stain ×40).

kidney against (A/L) administration, various disease markers were assayed after concomitant administration of cocoa and artemether/lumefantrine (A/L).

The biochemical assays were performed as reported previously by Asiedu-Gyekye [16]. Blood samples collected in plain gel tubes were allowed to clot. It was then centrifuged at 3000 rpm for 15 min, sera removed and stored at −20°C. The sera were analyzed for biochemical parameters such as the total cholesterol, triglycerides, high density lipoproteins (HDL), low density lipoproteins, very low density lipoproteins (VLDL), creatinine, urea, blood electrolytes, creatine kinase (CK), and aspartate transferase [35, 37]. Measurements were carried out on the Selectra Junior Autoanalyzer (Vital Scientific BV, Version 04, Netherlands).

Euthanized guinea pigs were dissected, their hearts and kidneys were removed. The tissues were kept in 10% buffered formalin. The tissues were embedded in paraffin wax and sectioned at 4 μm thickness, stained with hematoxylin-eosin. The light microscope was employed for the histological studies of the study animals as well as two control groups. A total of 30 photomicrographs at a magnification of ×40 were used for each group.

1.3.2.1 Biochemical assays

In the medium and high UNCP dose groups, there was a decrease in the mean serum levels of low density lipoprotein by 11.6 and 10.6% ($p < 0.05$), respectively, in comparison with the negative control group (NCG) (0.662 ± 0.269 mmol/L) [16].

A dose-dependent increment was observed for both VLDL and triglycerides upon administration of UNCP [16]. The triglyceride changes were as follows: controls (1.075 ± 0.360 mmol/L), A/L-administered group (0.966 ± 0.619 mmol/L), 300 mg/kg UNCP (0.980 ± 0.391 mmol/L), 900 mg/kg UNCP (1.208 ± 0.317 mmol/L), 1500 mg/ kg UNCP (1.478 ± 0.487 mmol/L) ($p < 0.05$) [16].

Groups, which received 900 mg/kg UNCP, showed 12.9% ($p < 0.05$) increase in the mean serum levels of high density lipoprotein (HDL) in comparison with the NCG 0.148 ± 0.046, ($p < 0.05$) [16].

The levels of coronary risk was high in animals that received 1500 mg/kg UNCP exhibited high levels of coronary risk (11.778 ± 1.167), but those on 900 mg/kg UNCP showed low levels (8.470 ± 2.624) in comparison with the NCG (9.08 ± 2.894, $p < 0.05$) [16].

Significant changes in serum levels of total cholesterol, low density lipoproteins cholesterol, and triglycerides were not observed after administration of both A/L and UNCP. It must however be noted that lipid profile including cholesterol in general takes considerable time to show significant changes even with cholesterol lowering agents. The authors conclude that the 14-day administration of cocoa may not have been long enough to produce significant changes expected.

1.3.2.2 Creatine kinase (CK)

Asiedu-Gyekye's group observed a significant difference in the mean levels of CK in VCG (598.0 ± 382.425 μmol/L) and NCG (1039.0 ± 749.494 μmol/L) [16]. The groups that received 300 mg/kg UNCP, 900 mg/kg UNCP, and 1500 mg/kg UNCP had their CK as follows: 552.2 ± 399.968 μmol/L, 318.5 ± 122.516 μmol/L, and 366.8 ± 174.921 μmol/L, respectively (**Figure 8**). The LD, MD, and HD cocoa groups hence reduced the creatine levels by 46.9, 69.3, and 64.7%, respectively ($p < 0.05$).

Creatine kinase (CK) or creatine phosphokinase is a marker of damaged tissue. Myocardial injury is often associated with an increase in CK levels. In this study, it was observed that animals that received 900 mg/kg bwt + A/L had 69.3% reduction

in serum CK showing the greatest mitigating activity against coartem toxicity. CK plays a crucial role in the conversion of creatine to phosphocreatine and adenosine diphosphate; thus, it might also protect or enhance myocardial bioenergetics.

1.3.2.3 Renal function test

Asiedu-Gyekye's group reported a reduction in urea by 53% in 1500 mg/kg when compared with the VCG ($p < 0.05$) [16]. Those of groups 3 and 4 were reduced by 14 and 10.64% in comparison with the VCG.

For the creatinine levels, significantly increments was observed; a 24.08% increase in NCG compared with VCG and decrease of 21.27, 17.54, and 11.05% in groups 3, 4, and 5, respectively, when compared with group 1 ($p < 0.05$) [16] .

The sodium, potassium, and chloride levels remained relatively unchanged in all study groups when compared with the control that received distilled water only [16].

The 1500 mg/kg group showed a significant reduction in urea levels as compared to the Coartem® only group. Creatinine levels decreased in all the groups compared with the control group. The authors attribute the observed effects to the antioxidant and nephroprotective effects of cocoa. Guinea pigs that received only the 75 mg/kg Coartem® group showed high levels of renal damage.

1.3.2.4 Histopathological examination

Aspartate transferase, one of the enzymes mentioned earlier in this chapter, is distributed mostly in the heart followed by the liver and skeletal muscles. Elevated serum aspartate transferase values are hence indicative of cellular injury and may present in myocardial disease, shock, hypoxia, among others. The group administered with of distilled water +A/L showed a significantly increased serum levels of aspartate transferase. The reverse (significant reduction) was observed in all animals administered unsweetened natural cocoa powder extract.

These observations are corroborated by histopathological examination of the myocardial tissues of the guinea pigs (figure not shown), where tissue sections from animals that received only A/L 75 mg/kg showed evidence of inflammation and degeneration of the myocardial tissue. Normal cardiac tissue structures of myocardial tissue were observed in animals administered UNCP extract except those of animals that received 900 mg/kg UNCP where there was a single case observed with suspected ongoing tissue necrosis at the initial stages. Similar observations of the cardioprotective effect have also been made by other researchers.

Photomicrographs of myocardial tissues of animals from the different experimental groups revealed patchy areas of congestion, edema, extensive nuclear, and tissue degeneration leading to loss of microstructure of myocardial tissues for animals receiving 75 mg/kg A/L [16]. Animals that received 300 mg/kg UNCP and the control group retained the normal branching of myocardial cells characteristics [16].

Coronary risk ratio is an important indicator of cardiovascular health. Assessment of it showed that groups on 1500 mg/kg cocoa +A/L were at a higher risk compared with the control groups [16]. This observation corroborates the findings that although cocoa possesses many health benefits, high level intake could be deleterious, an effect believed to be caused by (−) epigallocatechin-3-gallate.

1.3.2.5 Conclusion

Unsweetened Natural Cocoa Powder showed renoprotective and cardioprotective potential during high-dose A/L administration [16]. It therefore suggests that

simultaneous ingestion of A/L and UNCP may be beneficial to the heart and kidney. However, regular intake of large quantities of UNCP could be deleterious to health because of the high content of copper.

1.3.3 Hematological changes and nitric oxide levels

Hematological parameters are one of the vital indices monitored during malaria treatment. Thus, in this study, the effect of UNCP on the hematological parameters and NO levels during high-dose (HD) A/L administration was investigated.

The nitrite concentration in the plasma was measured as an index of NO levels by Griess reagent system (South Africa) according to the manufacturer's instruction.

The results obtained from this study showed that A/L administration decreased the levels of white blood cell(WBC) count, lymphocyte count, hemoglobin (HGB), Red blood cell (RBC) count, and platelet counts in the group that received Coartem® (negative control group). Normally, these reduced indices imply bone marrow depression, autoimmune hemolytic anemia, systemic lupus, or severe hemorrhage.

Asiedu-Gyekye's group reported a reduction of 31.87% in the WBC count of the NCG (Coartem®) in comparison with the vehicle control group($p > 0.05$) [26]. Administration of 300, 900, and 1500 mg/kg body weight of UNCP restored the WBC levels during concomitant administration with A/L ($p = 0.1158$) [26].

Prophylactic administration of UNCP with A/L at doses of 300, 900, and 1500 mg/kg body weight restored the decreased levels of the RBC count by 4.17, 5.55, and 12.55%, respectively ($p > 0.05$).

Administration of UNCP at doses of 300, 900, and 1500 mg/kg body weight restored not just the WBC levels during concomitant administration with A/L but also other hematological parameters like the hemoglobin levels, red blood cell counts, platelet counts, and all other parameters measured.

Nitric oxide (NO) is known to have hepatoprotective and cardioprotective effects. Upon concomitant administration of A/L and UNCP at doses of 300, 900 and 1500 mg/kg, respectively. The NO observed by Asiedu-Gyekye's group was dose dependent, with 300 mg/kg of UNCP exhibiting the highest increase of 149.71% in NO ($p < 0.05$), 900 mg/kg gave a 34.25% ($p < 0.05$), and the 1500 mg/kg dose of UNCP showed 4.88% increment in NO ($p < 0.05$) [26]. The observed moderate nitric oxide increases that are beneficial could be attributed to the flavonoid content of the unsweetened natural cocoa.

Reference to this work is: [26].

1.3.3.1 Conclusion

UNCP restored some hematological disorders induced by high-dose A/L in guinea pigs by causing a significant increase in lymphocyte and platelet (PLT) levels at a dose of 1500 mg/kg. There was also an increase in NO with different doses of UNCP administration as a sequel to A/L dosing, which suggests that the combination (A/L and UNCP) is safe and advantageous. This study indicates the health benefits of daily ingestion of UNCP to prevent deleterious effects of A/L for the management of malaria.

1.3.3.2 Anti-asthma potential

Theobroma cacao has a lot of potential. UNCP was also investigated on its ability to help manage bronchial asthma. Anecdotal reports indicated that regular

consumption of UNCP was accompanied by prevention and reduction of asthmatic episodes. Bronchial asthma is prevalent in Ghana and West Africa. Due to its phytochemical composition and the presence of theobromine and theophylline, there could be a high possibility of a bronchodilatatory effect, the experiment was carried on in guinea pigs with prednisone and the drug for comparison. This study was a bronchial asthma model induced via the introduction of ovalbumin sensitization. The results showed a reduction in the brochoconstriction, inflammatory responses, and eosinophilia infiltration [9].

1.3.3.3 In vitro antimalarial activity of natural cocoa powder

Anecdotal reports from Ghana suggest that a daily intake of a beverage of natural unsweetened cocoa powder could protect an individual against *Plasmodium falciparum* malaria. However, as at then, there was no known scientific report linking the consumption of cocoa or its products to the reduction in malaria incidence. Thus, a research conducted to determine this antiplasmodial activity and elucidate possible mechanisms of this activity. An *in vitro* inhibitory studies of extracts and fractions of cocoa powder against *P. falciparum* revealed that the nonpolar extract (chloroform, ethyl acetate, and petroleum ether) had better antiplasmodial activity than the polar extract [2]. The chloroform extract was the most active, with 50 and 90% inhibition concentration at 48.3 ± 0.9 and 41.7 ± 7.8 μg/mL, respectively. The ring-stage of *P. falciparum* treated with chloroform of natural cocoa powder showed a decline in growth. These results suggested that natural cocoa powder has measurable direct inhibitory activity against *P. falciparum*.

These studies attest to the additional future health benefits of unsweetened natural cocoa powder when consumed on daily basis especially its organoprotective role and also in attenuating high-dose artemether-lumefantrine organ effects. The antimalarial potential of cocoa is very promising especially in Africa where malaria is endemic, thus could be very beneficial in the management of uncomplicated malaria with very limited adverse effects on the major organs and reproductive system.

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