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EVALUATION OF HEPATOPROTECTIVE AND ANTI-INFLAMMATORY BIOMARKERS OF *THEOBROMA CACAO* IN ISOPROTERENOL-INDUCED MYOCARDIAL INFARCTION IN SPRAGUE-DAWLEY RATS

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Abstract

There is a correlation between myocardial injury and liver insufficiency. Dietary products like cocoa with high flavonoids may play a role to boost the integrity of the liver against insults resulting from myocardial injury. Male Wistar rats (200-250g, n = 24) divided into four groups of 6 rats were used for the study. Group 1 was the normal control (placed on a placebo of 0.9% normal saline via oral gavage), Group 2 was the acute myocardial injury group and received a subcutaneous injection of isoproterenol (100mg/kg body weight) twice at an interval of 24 hours to the end of the experiment. Group 3 was administered TC (100mg/kg body weight orally) only for 2 weeks. Group 4 was pretreated with TC (100mg/kg orally) for 2 weeks and then followed by a subcutaneous injection of isoproterenol (100mg/kg body weight) twice at an interval of 24 hours to the end of the experiment. All animals had free access to rat chow and water. At the end of the experimental period, the rats were sacrificed over ketamine anaesthesia, and serum was collected for laboratory investigations of lactate dehydrogenase, troponin, alanine aminotransferase, aspartate transaminase, and alkaline phosphatase. From the study, the administration of ISO resulted in a significant ($p<0.001$) elevation in the serum concentrations of the cardiac biomarkers, troponins, and LDH when compared with the normal control rats, depicting myocardial injury. Pretreatment with cocoa before MI induction preserved the myocardial cells to withstand the insult, with a consequent reduction in the concentrations of LDH and troponins, with values similar to the control group. The serum liver enzymes AST, ALT, and ALP concentration were also significantly elevated above the normal control, indicating hepatotoxicity in the ISO group. The increased serum liver enzymes were reversed in the cocoa treatment groups, indicating the hepatoprotective potentials of *T. cacao. The result* of this study has further strengthened the link between myocardial injury and the liver. It has also provided support for the prophylactic use of dietary *Theobroma cacao* against hepatotoxicity.

1. Introduction

Acute myocardial infarction (AMI) is one of the leading cardiovascular diseases with a high mortality rate (Marschang and Metzier *et al*., 2016). It is diagnosed in every 1 out of 10 patients admitted to the Emergency Unit with a heart attack (Haasenritter *et al*., 2012). In developing countries, a lifestyle change is considered a predisposing factor for the increased mortality rate due to AMI (Rathore *et al*., 2018). AMI distorts the structural, mechanical, electrical, and biochemical properties of the heart (Richardson *et al*., 2015). It can be due to ischaemic heart disease and/or in conjunction with coronary artery disease with resultant deterioration of ventricular function and myocardial necrosis (Liakos and Pariksh, 2018; Aydin and Aydin, 2016). Serum enzymes such as lactate dehydrogenase (LDH), cardiac kinase (CK), Aspartate aminotransferase (AST), malondialdehyde (MDA), and troponins are biomarkers used for the diagnosis of AMI (Danese and Montagnana, 2016; Mythili *et al*., 2015). This is because when there is a decreased coronary blood flow and a consequent deterioration of ventricular function due to myocardial necrosis, the serum concentrations of LDH, CK, AST, MDA, and troponins will significantly increase, indicating tissue damage (Feng *et al*., 2012).

There is a correlation between myocardial injury and hepatic insufficiency (Ekaidem *et al*., 2007). The liver plays a vital role in metabolism, detoxification, and excretion. It metabolizes substances via hydration, condensation, oxidation, reduction, hydrolysis, or conjugation. An alteration in any of these processes may result in liver cell injury (Sriuasfava and Sriuasfava, 2018).

Blockage of blood flow and congestion can manifest in liver damage (Biegus *et al*., 2012), and the damaging effect of myocardial infarction on the liver is multifactorial including a decrease in blood flow to the liver, reduced arterial saturation, and increased hepatic vein pressure (Ambrosy *et al*., 2012). Liver disease possibly may be inflammatory, non-inflammatory, and degenerative. Nutraceuticals in the form of antioxidants, dietary fibres, omega-3 polyunsaturated fatty acids, vitamins, and minerals also play a preventive and curative role in cardiovascular diseases (Choudhary and Tomer, 2013), so do plants with antioxidant properties (Kumar *et al.,* 2017; Viswanatha *et al*., 2010). One such plant with high antioxidant properties is *Theobroma cacao* (Kris-Etherton and Keen, 2002). *Theobroma cacao* is reported to suppress the development of atherosclerotic lesions (Kurosawa *et al*., 2002), decrease platelet hyperactivity (Murphy *et al*., 2003), increase dermal blood flow (Neukam *et al*., 2007), decrease oxidation of LDL cholesterol (Tomaru *et al*., 2007), and promote normal lipid profile (Agwupuye *et al*., 2019). It also inhibits the proliferation of human breast cancer cells and reduces circulating blood sugar levels (RamIjak *et al*., 2012). There is however a paucity of reports on the hepatoprotective potentials of *Theobroma cacao* secondary to myocardial injury.

2. Materials and methods

2.1 Plant Material and Extraction

A Dry Trinitario variety of *Theobroma cacao* seeds was obtained from Cross River State, Nigeria. The variety was identified at the Herbarium unit of the Department of Botany, Cross River University of Technology, Calabar, Nigeria, and assigned a voucher number. About 3kg of de-coated dry cocoa seeds were ground into coarse powder yielding 1.65 Kg of the powder. This was suspended in two litres of ethanol (BDH Ltd Poole, England) and left to percolate for 24 hours at room temperature. The suspension was thereafter filtered with Whatman No. I filter paper. The filtrate was evaporated by hot air oven treatment at 40-45^{O}C to a thick dark gummy crude extract giving a yield of 66g (4.8%). The extract was refrigerated at -4^{O}C until required for use.

2.2 Experimental Animals/Design

Male Sprague Dawley rats (200-250g, n = 24) divided into four groups of 6 rats were used for the study. The animals were kept in plastic cages and a controlled environment (12h light/dark cycles at 27 ± 2°C) for one week for acclimatization before the commencement of the study. Group 1 was the normal control (placed on a placebo of 0.9% normal saline via oral gavage), Group 2 was the AMI group and received a subcutaneous injection of isoproterenol (100mg/kg body weight) twice at an interval of 24 hours to the end of the experiment. Group 3 was administered TC (100mg/kg body weight orally) only for 2 weeks. Group 4 was pretreated with TC (100mg/kg orally) for 2 weeks and then followed by a subcutaneous injection of isoproterenol (100mg/kg body weight) twice at an interval of 24 hours to the end of the experiment. All animals had free access to rat chow and water.

2.3 Induction of Acute Myocardial Injury

Following the method of Boarescu *et al.,* (2018), myocardial infarction was induced by subcutaneous injection of 100mg/kg isoproterenol once for two days with a 24-hour interval between (Gunjal and Puvanakrishnan, 1996). Isoproterenol acts by decreasing the blood flow to the myocardium with consequent hypoxia. The hypoxic state causes a fall in mitochondrial ATP, hence depleting cellular ATP. There is a generation of reactive oxygen species, calcium overload, and phospholipid depletion with attendant lipid peroxidation, tissue inflammation, and structural membrane damage. These result in irreversible damage to the myocardium (Beshel *et al*.,2020). Myocardial infarction induced by isoproterenol is reported to show many metabolic and morphological aberrations in the heart tissue of the experimental animals similar to those observed in human myocardial infarction (Jialal and Sokoll, 2015).

Liver function tests (LFTs) are commonly used in clinical practice to screen for liver disease, monitor the progression of known disease, and monitor the effects of potentially hepatotoxic drugs. The most common LFTs include serum aminotransferases, alkaline phosphatase, bilirubin, albumin, and prothrombin time20. Aminotransferases, such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), measure the concentration of intracellular hepatic enzymes that have leaked into the circulation and serve as a marker of hepatocyte injury. Alkaline phosphatase (AP), γ-glutamyl transpeptidase (GGT), and bilirubin act as markers of biliary function and cholestasis. Albumin and prothrombin reflect liver synthetic function. Increased activities of liver enzymes such as (AST), and alanine aminotransferase (ALT) alkaline transferase (ALP) are indicators of hepatocellular injury. Increased activity of these markers is associated with type 2 diabetes mellitus with a higher incidence of liver function test abnormalities than individuals who do not have diabetes21.

Mild chronic elevations of transaminases often reflect underlying insulin resistance. Anti-diabetic agents have generally been shown to decrease alanine aminotransferase levels as tighter blood glucose levels are achieved. The aminotransferases AST and ALT are normally < 30-40 units/l. Elevations of aminotransferases greater than eight times the upper limit of normal reflect either acute viral hepatitis, ischemic hepatitis, or drug- or toxin-induced liver injury.

2.4 Determination of serum alanine aminotransferase (ALT)

Serum Alanine aminotransferase (ALT), is measured by monitoring the concentration of pyruvate hydrozone formed with 2,4-dinitrophenyhydrozine24. The method is based on the principle that pyruvate (pyruvic acid) formed from the alanine aminotransferase catalysed reaction between –ketoglutarate (oxoglutarate) and L-alanine is coupled with chromogen solution (2,4-dinitrophenyl hydrazine) in an alkaline medium to form

coloured hydrazone, the concentration of which is proportional to the alanine aminotransferase activity as measured with a colorimeter. To 0.05 ml of each serum sample in a test tube was added 0.25 ml of buffer/substrate solution. This was incubated at 37°C for 30 min in a water bath followed by the addition of 0.25 ml of chromogen solution. The content was mixed and allowed to stand for 20min at room temperature. Then 2.5 ml of sodium hydroxide (0.4 N) was added and mixed. The absorbance was read after 5 min against the blank at 540 nm. The blanks were treated as the samples but without the addition of chromogen solution used to stop all the enzymatic reactions. ALT activity (IU/L) was read off from the standard curve 25.

2.5 Determination of serum aspartate aminotransferase (AST)

The determination of the blood serum of Aspartate aminotransferase (AST), is measured by monitoring the concentration of oxaloacetate hydrozone formed with 2,4-dinitrophenylhydrazine24. The method is based on the principle that oxaloacetate (oxaloacetic acid) that is formed from the aspartate aminotransferase catalyzed reaction between alpha-ketoglutarate and aspartate is coupled with chromogen (2,4-dinitrophenyl hydrazine) in alkaline medium to form colored hydrazone. The concentration of the coloured hydrazone is proportional to the aspartate aminotransferase activity and is measured with a colorimeter. To 0.05 ml of each serum sample in a test tube was added 0.25 ml of buffer/substrate solution. The content was incubated at 37°C for 60 min in a water bath followed by the addition of 0.25 ml of chromogen solution. The content was mixed and allowed to stand for 20 min at room temperature after which 2.5 ml of sodium hydroxide (0.4 N) was added and mixed. The absorbance was read after 5 min against blank at 540 nm. The blanks were treated as the samples but without the addition of chromogenic solution used to stop all enzymatic reactions. AST activity (IU/L) was read off from the standard curve 25.

2.5 Determination of serum alkaline phosphatase (ALP)

This measurement of alkaline phosphatase (ALP) followed standard procedure 26. Principle: Phenol released by enzymatic hydrolysis from phenyl phosphate under defined conditions of time, temperature, and pH – is estimated colorimetrically. Technique Test:- 1ml of buffer was mixed with 1ml of phenyl phosphate substrate in a test tube placed in a water bath at 37°C for 3 minutes. 0.1ml of serum was added mixed gently and incubated for exactly 15 minutes, the reaction was stopped by the addition of 0.8ml of 0.5N sodium hydroxide (NaOH). Control: - In a test tube 1ml substrate was mixed with 0.8ml of 0.5N sodium hydroxide, followed by 0.1ml of serum. Standard: - 1.1ml of buffer was mixed with 0.1ml of phenol standard (1mg/100ml) and 0.8ml of 0.5N sodium hydroxide. Blank: - 1.1ml of buffer, 1.0ml of water, and 0.8ml of 0.5N sodium hydroxide were mixed. To all tubes, 1.2ml of 0.5N sodium bicarbonate (NaHCO3) was added with 1ml of Potassium Ferricyanide solution -K3(Fe(CN)6), mixing each tube well after each addition. The successive additions adjusted the pH to develop the color. The 0.0 of reddish–brown colors of 510 nanometers (nM), was read avoiding exposure to strong sunlight.

Calculation:

Serum alkaline phosphatase (King-Armstrong Units/100ml) =

$$\frac{Reading\ of\ an\ Unknown - Reading\ of\ Control}{Reading\ of\ Standard - Reading\ of\ Blank}\ X\ 100$$

3. Data Analysis

Results are expressed as mean ± SEM. Data were analyzed using the GraphPad Prism software (version 6.0). Analysis of variance (ANOVA) followed by Turkey comparison test where the F value was significant. The probability level of $p<0.05$ was accepted as significant.

4. Results

4.1 Troponin and lactate dehydrogenase concentrations in isoproterenol-induced myocardial injury treated with cocoa

Serum concentrations of troponin and lactate dehydrogenase were measured to evaluate myocardial damage. The serum concentration of troponin in the control, MI, cocoa only, and cocoa + MI groups was 0.038 ± 0.00027ng/ml, 0.055 ± 0.0012ng/ml, 0.036 ± 0.00036ng/ml, and 0.036 ± 0.00035ng/ml respectively. The result showed a significant ($p<0.01$) increase in the serum concentration of troponin in the MI group compared to the control group. Administration of cocoa before MI induction significantly ($p<0.01$) decreased troponin concentration when compared with the MI group. Similarly, the mean serum concentration of LDH in control, MI, cocoa only, and cocoa + MI groups was 1465 ± 3.97IU/l, 1653 ± 3.53IU/l, 1430 ± 8.33IU/l, and 1422 ± 18.0IU/l, respectively. This shows a significant ($p<0.01$) increase in the serum concentration of LDH in the MI group when compared to the control. This increase was attenuated ($p<0.01$) by pretreatment with cocoa before MI induction. This is presented in Table 1

Table 1: Serum troponin and lactate dehydrogenase concentration in isoproterenol-induced myocardial injury treated with cocoa.

Cardiac Markers	Control Group	MI Group	Cocoa Only	Cocoa + MI
Troponin (ng/ml)	0.038 ± 0.00027	0.055 ± 0.0012**	0.036 ± 0.00036	0.036 ± 0.00042**c**
Lactate dehydrogenase (IU/I)	1465 ± 3.97	1653 ± 3.53**	1430 ± 8.33	1422 ± 18.0**c**

** = $p<0.01$ compared with control; **c** = $p<0.01$ compared with MI group

4.2 Serum aspartate aminotransferase (AST) concentrations in isoproterenol-induced myocardial injury treated with cocoa

The liver enzyme activity was measured to evaluate the integrity of the hepatocytes following isoproterenol-induced myocardial injury. Serum aspartate aminotransferase (AST) concentrations in the control, MI, cocoa only, and cocoa + MI groups were 50.96 ± 0.13IU/L, 82.65 ± 0.22IU/L, 46.72 ± 0.20IU/L, and 57.98 ± 0.34 respectively. The result showed a significant ($p<0.001$) increase in AST concentration in the MI group when compared with the control, indicating an injury to the hepatocytes following isoproterenol administration. However, pretreatment with cocoa before MI induction resulted in a decreased serum concentration of AST, with values similar to the control group. This is presented in Fig. 1.

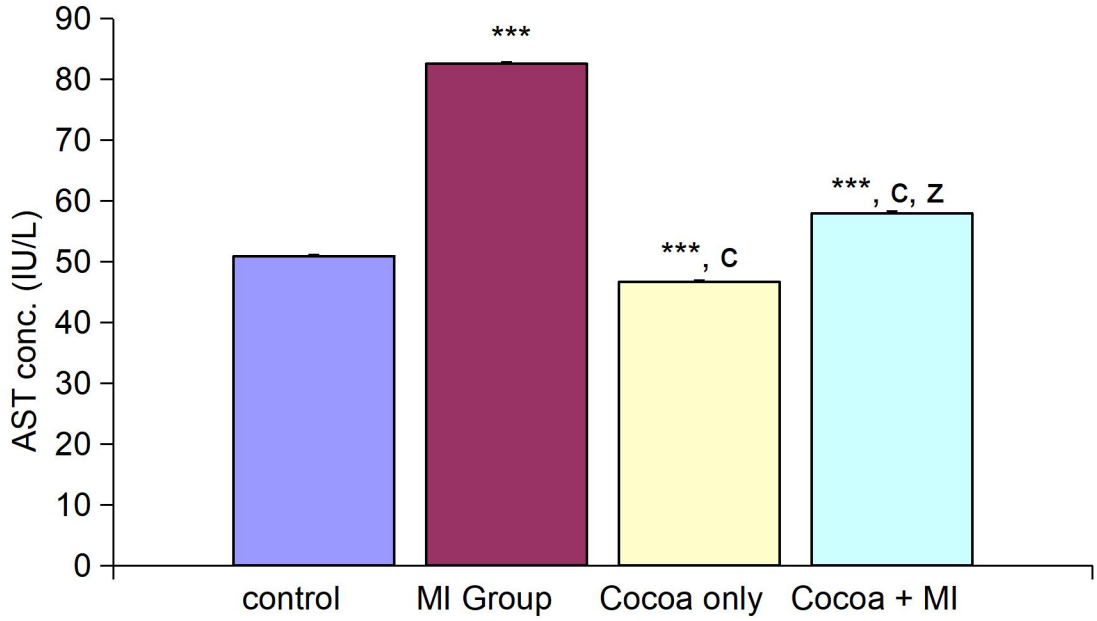

Figure 1: Comparison of aspartate aminotransferase concentrations in the different experimental groups.

Values are expressed as mean + SEM, n = 6.
*** = p<0.001 vs control;
c = p<0.001 vs MI;
z = p<0.001 vs cocoa only

4.3. Serum alanine aminotransferase concentrations in isoproterenol-induced myocardial injury treated with cocoa

Serum alanine aminotransferase (ALT) was also measured to evaluate the integrity of the hepatocytes. The serum ALT concentrations in the control, MI, cocoa only, and cocoa + MI groups were 21.55 ± 0.23IU/L, 38.13 ± 0.32IU/L, 20.37 ± 0.27IU/L, and 29.83 ± 0.25IU/L respectively. The MI group had an increased ($p<0.001$) ALT concentrations when compared with the control, depicting injured hepatocytes following isoproterenol administration. Pretreatment with cocoa prevented an increased surge in serum ALT, depicting less damage to the hepatocytes. This is presented in Fig. 2.

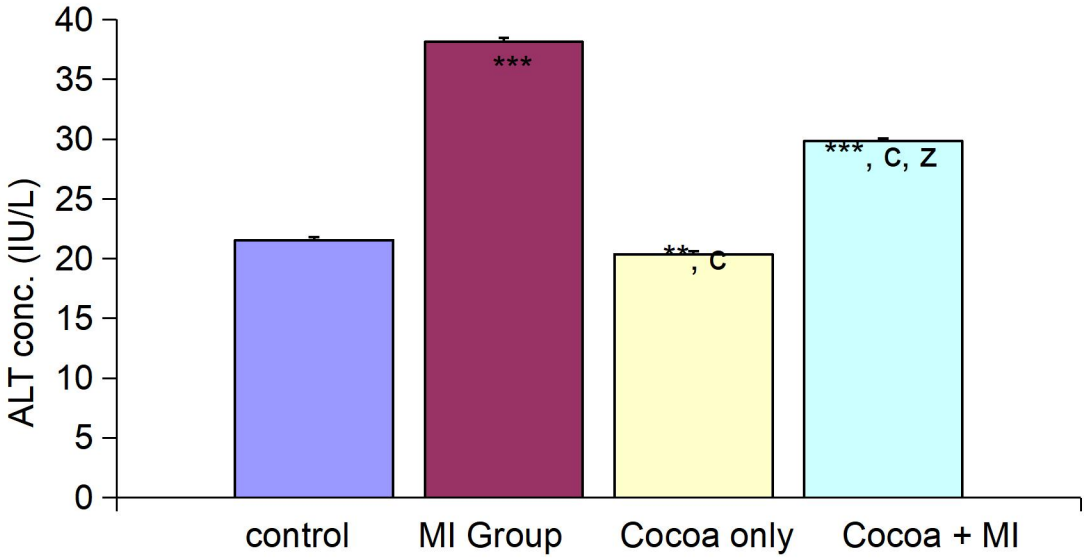

Figure 2: Comparison of alanine aminotransferase concentrations in the different experimental groups.

Values are expressed as mean + SEM, n = 6.
** = p<0.01, *** = p<0.001 vs control;
c = p<0.001 vs MI;
z = p<0.001 vs cocoa only

4.4 Serum alkaline phosphatase concentrations in isoproterenol-induced myocardial injury treated with cocoa

Serum alkaline phosphatase (ALP) was also measured to evaluate the integrity of the hepatocytes. The serum ALP concentrations in the control, MI, cocoa only, and cocoa + MI groups were 57.29 ± 0.22IU/L, 125.19 ± 0.55IU/L, 53.51 ±0.30IU/L, and 71.37 ± 0.44IU/L respectively. The result followed a similar trend with other liver enzymes with a significantly higher ($p<0.001$) ALP concentration in the MI group when compared with the control. The Cocoa pretreatment group showed a lower concentration of ALP concentration when compared with the MI group. ALP values in the pretreated groups are similar to the control group. This is presented in Fig. 3.

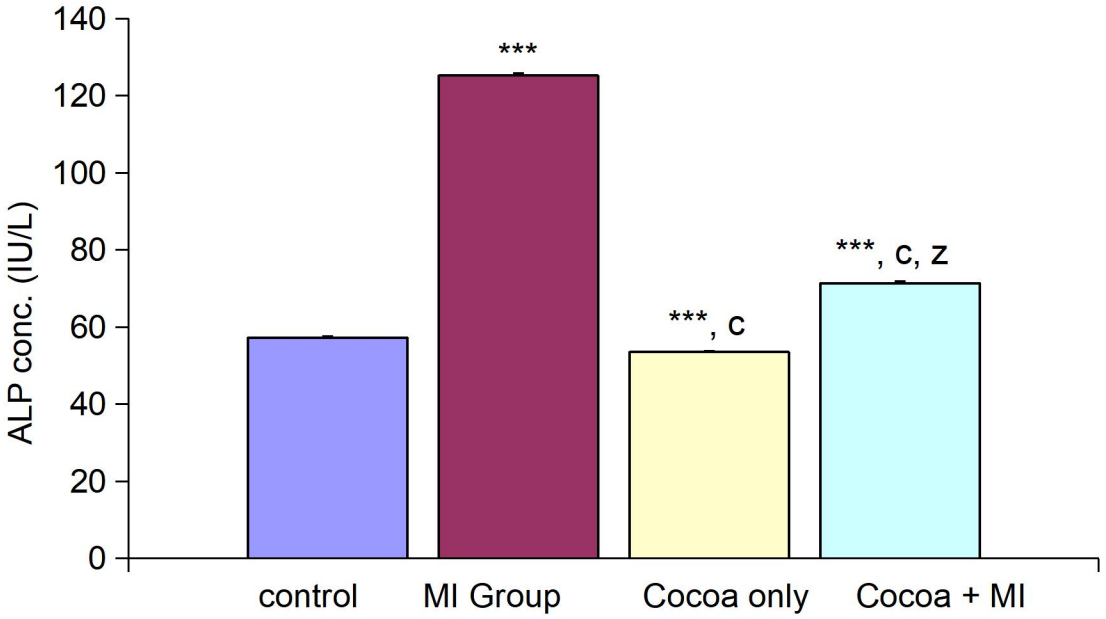

Figure 3: Comparison of alkaline phosphatase concentrations in the different experimental groups.

Values are expressed as mean + SEM, n = 6.
*** = p<0.001 vs control;
c = p<0.001 vs MI;
z = p<0.001 vs cocoa only

5. Discussion

Reports have shown a significant link between myocardial infarction, liver dysfunction, and risk of ischemic hepatitis; a condition that occurs as a result of decreased total hepatic blood flow secondary to low cardiac output, shock, or cardiac arrest (Mason *et al*., 2010; Lightsey and Rockey, 2017). Blockage of blood flow and congestion can manifest in liver damage (Biegus *et al*., 2012). The damaging effect of myocardial infarction on the liver includes a decrease in blood flow to the liver, reduced arterial saturation, and increased hepatic vein pressure (Ambrosy *et al*., 2012). The present study, therefore, evaluated the hepato-protective potentials of *Theobroma* cacao secondary to isoproterenol-induced myocardial injury in rats.

Dietary cocoa ameliorates non-alcoholic fatty liver diseases (Mingyao *et al*., 2021). It also attenuates artemether-lumefantrine-induced hepatotoxicity in non-malarious Guinea pigs (Asiedu-Gyekye *et al*., 2016). Due to its abundant presence of flavonoids epicatechin and procyanidin, cocoa is reported to cause

increased flow-mediated vasodilation (Ottaviani *et al*., 2018; Arteel and Sies, 1999). It has antioxidant and platelet anti-aggregation properties (Rein *et al*., 2000; Zhu *et al*., 2018; Heiss *et al*., 2003). The flavonoids block the "suicide" enzyme cyclooxygenase which breaks down prostaglandins to prevent platelet aggregation (Rice-Evans, 2001).

From the study, the administration of ISO resulted in a drastic and significant elevation in the serum concentrations of the cardiac biomarkers, troponins, and LDH when compared with the normal control rats, depicting myocardial injury (Sudhakumari *et al*., 2012; Asdaq *et al*., 2009). Pretreatment with cocoa before MI induction preserved the myocardial cells to withstand the insult, with a consequent reduction in the concentrations of LDH and troponins, with values similar to the control group.

Following the myocardial injury caused by ISO administration, the serum liver enzymes AST, ALT, and ALP concentration were significantly elevated above the normal control, indicating hepatotoxicity in the ISO group. The increased serum liver enzymes were reversed in the cocoa treatment groups, indicating the hepatoprotective potentials of *T. cacao.*

The result of this study has further strengthened the link between myocardial injury and the liver. It has also provided support for the prophylactic use of dietary *Theobroma cacao* against hepatotoxicity.

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