*The Journal of Animal & Plant Sciences* 19(4): 2009, Pages: 174-178
**ISSN: 1018-7081**

# EFFECTS OF COCOA POWDER ON OXIDANT/ ANTIOXIDANT STATUS IN LIVER, HEART AND KIDNEY TISSUES OF RATS

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## ABSTRACT

The aim of study was to asses the oxidative status in terms of lipid peroxidation and antioxidant enzymes in different tissues of orally administered cocoa-treated rats. For this purpose 12 Male Albino Wistar rats were randomly divided into 2 groups; each group consists of 6 rats. Group I remain untreated, comprises healthy rats. Group II received cocoa powder (1g/kg b.w.) orally for 21 consecutive days. Oxidative status was evaluated by Catalase, GSH, SOD, MDA and 4-HNE, in Liver, Heart and Kidney tissues. The cocoa-treated rats showed significantly increased level of GSH in liver and heart tissue, Catalase in liver and heart, SOD in liver, MDA in liver, similarly decreased level of GSH in kidney tissue, 4-Hydroxynonenal (4-HNE) in liver and heart tissues. Body weight was decreased. No mortality was observed at this dose. The results explain the beneficial antioxidant effects and reveal that cocoa powder enhanced the antioxidant enzymes level in liver and heart tissues while no remarkable changes was observed in kidney tissue.

**Key Words**: Antioxidant enzymes, Cocoa, Oxidative status, Kidney, Liver, Heart.

## INTRODUCTION

Cacao beans (*Theobroma cacao*) have been used world wide as a major ingredient of cocoa and chocolate (Meng *et al.*, 2009). Cocoa bean is loaded with the polyphenols such as quercetin (including its glucoside), clovamide, deoxyclovamide and procyanidin, Epicatechin, (+)-catechin (Thomspson *et al.*, 1972, Sanbongi *et al.,* 1998, Hammerstone *et al.*1999).Research indicates that the flavonoids, a class of polyphenols, has antioxidant characteristics with potential health benefits that may reduce the risk of cardiovascular disease and cancer (Arts and Hollman 2005, Allen *et al.,* 2008). The specific antioxidants in chocolate (i.e., cocoa flavanols) include catechin and epicatechin, which are single flavanol molecules structurally similar to the antioxidants found in grapes and tea (Raloff, 2000).Cocoa can substantially increase a person's energy level, since it contains two stimulating methylxanthines - a significant amount of theobromine and a small amount of caffeine (Keen 2001, Sorond *et al.,* 2008). PEA (phenylethylamine) is a chemical found in cocoa/cacao beans which increases the activity of neurotransmitters (brain chemicals) in certain areas of the brain which control the ability to focus attention and stay alert (Lee *et al.,* 2003, Crew *et al.,*2008). Cocoa also appears to have anti-aging and anti-inflammatory properties. Cocoa is a good source of the minerals magnesium, sulphur, calcium, iron, zinc, copper, potassium, and manganese; plus some of the B Vitamins. Cocoa enhanced clot prevention afforded by cocoa flavanols (Rein *et al.,* 2000). Cocoa has antioxidant compounds is described previously, therefore the purpose of the study was to examine the effect of Cocoa powder in antioxidant enzymes in different tissues and also evaluate the changes in oxidative status in liver, heart and kidney tissues in rats .

## MATERIALS AND METHODS

**Animals & diet:** Wistar albino rats of male sex (200–250g b.w.), purchased from the animal house of ICCBS (International Center for Chemical and Biological Sciences), Karachi, Pakistan, for the study. Animals were acclimatized to the laboratory conditions one week before the start of experiment and caged in a quite temperature controlled room (23± 4$^{o}$C). Rats had free access to water and standard rat diet. The experiments were conducted in accordance with ethical guidelines for investigations in laboratory animals.

**Study Design: Twelve (**12) male Albino Wistar rats were randomly divided into 2 groups (n=6).
Group I: Control group remains Untreated
Group II: Cocoa-treated rats, administered (1g/kg, b.w.) orally for 21 consecutive days.

**Sample Collection:** After 8 weeks treatment, animals were anesthetized and decapitated. Liver, heart and kidney were excised, trimmed of connective tissues, rinsed with saline to eliminate blood contamination, dried by blotting with filter paper and weighed. The tissues were then kept in freezer at -70°C until analysis.

**Liver Homogenate preparation:** Liver were perfused with saline and homogenized in chilled potassium chloride (1.17%) using a homogenizer. The homogenates were centrifuged at 800 g for 5 minutes at 4°C to separate the nuclear debris. The supernatant so obtained was centrifuged at 10,500 g for 20 minutes at 4°C to get the post mitochondrial supernatant which was used to assay SOD, CAT, GSH and MDA, HNE activity.

**Heart Homogenate preparation:** Homogenates were prepared on ice in the ratio 4 g tissue for 16 ml of phosphate p H 7.5, containing 1 mM/L $Na_2$ EDTA, 10 ml of 500 mM/L BHT (butylated hydroxytoluene)in acetonitrile was added to prevent formation of new peroxides during the assay. The homogenates were centrifuged at 2000 minutes at $4^0$ C and frozen at -$70^0$C until analysis.

**Kidney Homogenate preparation:** Kidney homogenates were obtained by using a tissue homogenator, Ultra Taurax T-25 Polytron, at $4^o$ C. The homogenates (1:10 w/v) were prepared by using a 100 mmol KCl buffer (7.0 p H) containing EDTA 0.3 mM, All homogenates were centrifuged at 600 g for 60 minutes at $4^o$C and the supernatant was used for biochemical assays.

**ANALYTICAL METHODS**

**Estimation of Catalase activity:** Catalase activity was assayed by the method of (Sinha *et al.,* 1972)**.** Briefly, the assay mixture consisted of 1.96 ml phosphate buffer (0.01 M, pH 7.0), 1.0 ml hydrogen peroxide (0.2 M) and 0.04 ml homogenate (10%) in a final volume of 3.0 ml. 2 ml dichromate acetic acid reagent was added in 1 ml of reaction mixture, boiled for 10 min, cooled. Changes in absorbance were recorded at 570 nm**.**

**Estimation of Super Oxide Dismutase levels:** Levels of SOD in the cell free supernatant was measured by the method of **(**Kono *et al.,* 1978). Briefly, Solution A: Briefly, 1.3 mL of solution a (0.1 mM EDTA containing 50 mM $Na_2CO_3$, pH 10.5). Solution B: 0.5 mL of solution B (90 mm NBT - nitro blue tetrazolium dye).Solution C: 0.1 mL of solution C (0.6% TritonX-100 in solution A). Solution D: 0.1 ml of solution D (20 mM Hydroxylamine hydrochloride, pH 6.0) was mixed and the rate of NBT reduction was recorded for one minute at 560 nm. 0.1 ml of the supernatant was added to the test cuvette as well as reference cuvette, which do not contain solution D. Finally, the percentage inhibition in the rate of reduction of NBT was recorded as described above. One enzyme unit was expressed as inverse of the amount of protein (mg) required inhibiting the reduction rate by 50% in one minute.

**Estimation of GSH:** GSH activity was determined by the procedure of Carlberg and Mannervik 1985. The assay solution contained 10% BSA (bovine serum albumin), 50 mM potassium phosphate buffer (pH 7.6), 2 mM NADPH, and 20 mM oxidized glutathione. Absorbance at 340 nm was recorded at a temperature of $25^0$ C. The activity was calculated using the molar coefficient for NADPH of 6.22 $\mu mol^{-1}$ x $cm^{-1}$ and expressed in U/g of tissue.

**Estimation of MDA:** The malonyldialdehyde (MDA) was determined spectrophotometrically according to the method by Ohkawa *et al.,* 1979. Briefly, the reaction mixture consisted of 0.2 ml of 8.1% sodium dodecyle sulphate, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid was added to 0.2 ml of 10%(w/v) of homogenate. The mixture was brought up to 4.0 ml with distilled water and heated at 95°C for 60 minutes. After cooling with tap water, 1.0 ml distilled water and 5.0 ml of the mixture of n-butanol & pyridine (15:1 v/v) was added and centrifuged. The organic layer was taken out and its absorbance was measured at 532 nm and compared with those obtained from MDA standards.

**Estimation of 4-HNE:** The 4-HNE content was measured by the method of Kinter *et al.,* 1996. Briefly, the assay mixture consisted of 2 ml of filtrate with 1 ml of 2, 4 Dinitrophenyl hydrazine and kept for 1 hour at room temperature. Sample was extracted with hexane, and the extract was evaporated at $40^o$C. Cooled sample was re-suspended with 2 ml of methanol and was measured at 350 nm against methanol blank.

**Statistical analysis:** Results are presented as mean ± SD. Statistical significance and differences from control and test values were evaluated by Student's t-test at $p<0.01$.

## RESULTS AND DISCUSSION

**Effect of Cocoa powder on Body weight:** Mean body weight of cocoa-treated rats was slightly decreased as compare to Day $1^{st}$ (Figure1). The rats

were weighed at every 3rd day before cocoa administration.

**Effect of Cocoa Powder on Oxidative Status in Liver tissue:** In present study, the Cocoa-treated rats showed increased level of liver GSH (61.73±5.45, P<0.01), Catalase (5.75±0.12, P<0.01), SOD (54.12±10.48, P<0.01), MDA (28.73±7.16, P<0.01) and decreased level of 4-HNE (465.38±97.55, P<0.01) (Table 2) as compared to control.

**Effect of Cocoa Powder on Oxidative Status in Heart tissue:** The Cocoa-treated rats showed increased level of GSH (9.05±0.55, P<0.01), Catalase (2.56±0.54, P<0.01) and decreased level of 4-HNE (170.82±74.87, P<0.01) as compared to control. (Table 3). While SOD and MDA showed no significant results.

**Effect of Cocoa Powder on Oxidative Status in Kidney tissue:** Table 4 showed decreased GSH level (2.63±0.38, P<0.01) as compared to control. Tissue Catalase, SOD, MDA and 4-HNE showed no significant results.

Oxygen radical production, which increases with clinical progression of diseases, involves increased lipid peroxidation, as a result of which there are cellular membrane degeneration and DNA damage. Extent of lipid peroxidation cloud be determined by estimation of the final lipid peroxidation products- malondialdehyde and 4-hydroxynonenal, compounds known to produce protein cross-linking through Schiff's base with DNA and DNA damage (Sharma *et al.*, 2001).

In present study, we determined the oxidative status of Liver, Heart and Kidney tissues after prolonged administration of Cocoa powder in rats. Prolonged administration of cocoa showed accumulation of MDA in heart and liver tissues and 4-HNE in kidney tissue, implicated oxidative stress (Table 2, 3). It has been reported that malondialdehyde is a well-characterized mutagen (Esterbauer *et al.,* 1990) that reacts with deoxyguanosine to form a major endogenous adduct with DNA in human livers (Chaudbery *et al.,* 1994). 4-hydroxynonenal was found to be genotoxic in primary cultures of rat hepatocytes at low concentrations, which might occur under in vivo conditions of oxidative stress (Esterbauer *et al.*, 1990). The 4-Hydroxynonenal (4-HNE) is a cytotoxic α, β-unsaturated acyl aldehyde that is naturally produced from lipid peroxidation and cleavage in response to oxidative stress and aging. Such reactive lipids covalently modify cellular target proteins, thereby affecting biological structure and function (Bennaars-Eiden *et al.,* 2002). It is found throughout animal tissues, and in higher quantities during oxidative stress due to the increase in the lipid peroxidation chain reaction, due to the increase in stress events.4-HNE has been hypothesized by several researchers to play a key role in cell signal transduction, in a variety of pathways from cell cycle events to cellular adhesion (Awasthi *et al.,* 2004) .

Increased SOD level in liver and increased Catalase level in liver and heart tissue was observed (Table 2 & 3). Increased superoxide dismutase activity could augment superoxide radical dismutation, thus leading to intensification of hydrogen peroxide generation (Szatrowski and Nathan 1991). The biochemical function/ 'purpose' of the CAT activity is 'scavenging' of toxic $H_2O_2$, representing the reactive oxygen species (ROS), having adverse effect towards almost all cell compounds including membrane lipids, DNA and proteins. Participation of the Catalase activity in antioxidant defence has been shown for spectrum of organisms, from bacteria to mammals (Cadens 1989, Sies 1997, Isik *et al.,* 2007, Kirkman and Gaetanic. 2007). Recently it has been demonstrated that superoxide anion as well as alkoxyl peroxyland radicals could inactivate one of the antioxidant enzymes -catalase and reduce the effectiveness of cells to defend against free radical damage (Mayo *et al.,* 2003). SOD is the major antioxidant enzyme that provides the body's first enzymatic step in the defense system against oxidative stress. In patients with chronic heart failure, endothelial-bound SOD activity was substantially reduced. Diminished SOD levels were closely linked with increased vascular oxidative stress, which contributes to endothelial dysfunction in chronic heart failure (Landmesser *et al.,* 2002).

Catalase is used by cells to defend against the toxic effects of hydrogen peroxide, which is generated by various reactions and/or environmental agents or by the action of superoxide dismutase, enzymes while detoxifying superoxide anion (Michiels *et al.,* 1994).

The increased level of GSH in liver and heart tissue was observed (Table 2 & 3). This type of increased GSH was observed previously in hypercholesterolemic animals in comparison with normocholesterolemic ones, which might reflect an adaptation of the liver to oxidative stress because high intracellular GSH levels promote better survival under such conditions (Dikinson *et al.*,2003, Kurosawa *et al.*,2005, Ruzaidi *et al.*, 2005). Conversely, increased activity of these enzymes as a result of polyphenol intake has been reported in the literature (Young *et al.,* 2000, Analikumar *et al.,* 2001) suggesting an enhanced

protection of the liver against oxidative stress situations by these antioxidants. This study showed the prevention of age-related decline on enzyme activities or their recovery by antioxidant supplementation in animals subjected to oxidative stress (Analikumar *et al.*, 2001, Park *et al.*, 2002, Molina *et al.*, 2003).

The speculated findings of this study showed that chronic administration of cocoa markedly changed the oxidative and anti oxidative status in liver tissue, which might be responsible for weight fluctuation, while slightly changing was observed in heart tissue and no alteration in kidney tissue. Cocoa can be used in therapeutic terms as it enhances the antioxidant enzymes activity in liver and heart tissues. Moreover, increased antioxidant enzymes level by Cocoa may be subjected a beneficial response in oxidative stress.

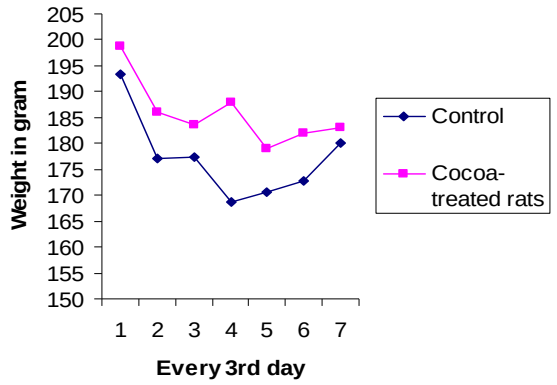


**Figure1: Effect of Cocoa powder on Body weight in Control and Cocoa-treated rats:**

**Table 2: Effect of Cocoa Powder on Oxidative Status in Liver tissue**

| Parameters | Control | Cocoa-treated |
|---|---|---|
| Catalase (mmol/g tissue) | 1.38±0.24 | 5.75*±0.12 |
| SOD (U/g tissue) | 13.23±2.63 | 54.12*±10.48 |
| GSH (U/g tissue) | 10.17±1.34 | 61.73*±5.45 |
| MDA (nmol/ g tissue) | 4.60±1.95 | 28.73*±7.16 |
| 4-HNE (nmol/ g tissue) | 770.82±271.87 | 465.38*±97.55 |

Values are mean ± SD significant difference between Control and Cocoa-treated rats by Student's t-test;*P<0.01

**Table 3: Effect of Cocoa Powder on Oxidative Status in Heart tissue**

| Parameters | Control | Cocoa-treated |
|---|---|---|
| Catalase (mmol/g tissue) | 1.36±0.32 | 2.56*±0.54 |
| SOD (U/g tissue) | 24.63±3.42 | 19.53±7.59 |
| GSH (U/g tissue) | 3.67±2.52 | 9.05*±0.55 |
| MDA (nmol/ g tissue) | 4.55±0.49 | 4.90±0.80 |
| 4-HNE (nmol/ g tissue) | 470.19±68.47 | 170.82*±74.87 |

Values are mean ± SD significant difference between Control and Cocoa-treated rats by Student's t-test;*P<0.01

**Table 2: Effect of Cocoa Powder on Oxidative Status in Kidney tissue**

| Parameters | Control | Cocoa-treated |
|---|---|---|
| Catalase (mmol/g tissue) | 0.53±0.05 | 0.46±0.17 |
| SOD (U/g tissue) | 28.76±3.05 | 29.36±5.76 |
| GSH (U/g tissue) | 4.25±0.90 | 2.63*±0.38 |
| MDA (nmol/ g tissue) | 1.81±0.55 | 1.25±0.17 |
| 4-HNE (nmol/ g tissue) | 298.85±19.6 | 306.81±48.03 |

Values are mean ± SD significant difference between Control and Cocoa-treated rats by Student's t-test;*P<0.01

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