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Determination of enzymatic and non-enzymatic antioxidants in fresh beans of *Theobroma Cacao* (L.) and *Coffee Arabica* (L.)

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Abstract
In the present study, laboratory evaluations were made to quantitatively assess enzymatic and non-enzymatic antioxidants in fresh beans of *Theobroma cacao* and *Coffee arabica.* Enzymatic antioxidants like Superoxide dismutase, Catalase, Glutathione peroxidase, Glutathione-S-Transferase, Glutathione Reductase, Glucose-6-Dehydrogenase, Polyphenol Oxidase and non-enzymatic antioxidants like Total reduced glutathione, Vitamin C were estimated quantitatively. Antioxidant enzymes are capable of stabilizing, or deactivating free radicals before they attack cellular components. The results obtained from the present study provides evidence of *Theobroma cacao* and *Coffee arabica* contains various antioxidants and this justifies the use of plant species as traditional medicine for treatment of various diseases. The finding of this study suggests that these seed extracts could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing various diseases. The results are very much encouraging but scientific validation is necessary before being put into practice.

Keywords: Enzymatic, Non-enzymatic antioxidants, *Theobroma cacao, Coffee arabica*

1. Introduction
The importance of oxidation in the body and in foodstuffs has been widely recognized. Oxidative metabolism is essential for the survival of cells. A side effect of this dependence is the production of free radicals and other reactive oxygen species that cause oxidative changes. There is increasing evidence for the involvement of such species in a variety of normal *in vivo* regulatory systems [1]. When an excess of free radicals is formed, they can overwhelm protective enzymes such as superoxide dismutase, catalase and peroxidase and cause destructive and lethal cellular effects (*e.g.*, apoptosis) by oxidizing membrane lipids, cellular proteins, DNA and enzymes, thus shutting down cellular respiration.
Furthermore, reactive oxygen species seem to influence cell signalling pathways in ways that are only now being unravelled [2, 3]. Oxidation can also affect foods, where it is one of the major causes of chemical spoilage[4] resulting in rancidity and/or deterioration of the nutritional quality, colour, flavour, texture and safety of foods[5]. It is estimated that half of the world's fruit and vegetable crops are lost [6] due to postharvest deteriorative reactions.
Defence mechanisms against the effects of excessive oxidations are provided by the action of various antioxidants and the need to measure antioxidant activity is well documented. Oxidative stress in humans arises from an imbalance in the antioxidant status (reactive oxygen species *versus* defence and repair mechanisms). The endogenous defences are enzymes such as superoxide dismutase, catalase and glutathione peroxidase, plus vitamin E, uric acid and serum albumins. Besides these defences, consumption of dietary antioxidants is also important.
An antioxidant may be defined [6] as 'any substance that when present at low concentrations, compared with those of the oxidizable substrate significantly delays or inhibits oxidation of that substrate'. Antioxidant activity cannot be measured directly but rather by the effects of the antioxidant in controlling the extent of oxidation. Some of the antioxidants are exogenous in nature and are obtained from food. Examples include antioxidants like Vit. C, Vit. E, glutathione etc., Vitamin C is an important dietary antioxidant, it significantly decreases the adverse effect of reactive species such as reactive oxygen and nitrogen species that can cause oxidative damage to macromolecules such as lipids, DNA and proteins which are implicated in chronic diseases including cardiovascular disease, stroke, cancer, neurodegenerative diseases and cataractogenesis [7]. Vitamin E is a fat soluble antioxidant that stops the production of reactive oxygen species formed when fat undergoes oxidation [8]. Glutathione is body's master antioxidant and one of the most important cleansing and healing agents. Glutathione blocks free radical damage and help to recycle Vitamins E and C, therefore plays a key role in their function.

1.1 *Theobroma cacao* L
Theobroma cacao L. belongs to family: Sterculiaceae. Cocoa solid, cocoa butter, and chocolate are all rich sources of antioxidants [9]. Epidemiological studies show an inverse association between the consumption of cocoa and the risk of cardiovascular disease [10, 11] The likely mechanisms are antioxidant activity, improvement in endothelial function, vascular function, and insulin sensitivity; as well as attenuation of platelet reactivity and reduction in blood pressure [12, 13].
The medicinal use of chocolate has a long history in North America dating to the 16th century [14]. In the 1600s, it was argued that "chocolate" should be considered a medicine because it changed a patient's health. The pharmacologically active ingredients of cocoa seeds include amines, alkaloids,

fatty acids, polyphenols (including flavonoids), tyramine, magnesium, phenylethylamine, and N-acylethanolamine [15, 16].

Cocoa is rich in polyphenols that have beneficial effects on cardiovascular disease [17] in cocoa, the polyphenols of particular interest are flavanols, a subclass of flavonoids. Cocoa has been reported to be a source of natural antioxidants [18] the free radical scavengers that preserve cell membranes, protect DNA, prevent the oxidation of low-density lipoprotein (LDL) cholesterol that leads to atherosclerosis, and prevent plaque formation in arterial walls [19] The antioxidant activity of cocoa has been attributed to the procyanidins and their monomeric precursors, epicatechin and catechin, which inhibit oxidation of LDL [20, 21, 22]. Dark chocolate and cocoa inhibit LDL oxidation and increase high-density lipoprotein (HDL)-cholesterol concentrations [23, 24].

1.2 *Coffee arabica* L.

Coffee arabica, the scientific name for coffee tree. It belongs to family, Rubiaceae. The first cultivated species of coffee is native to Northeastern Africa. Coffee can have a stimulating effect on humans due to its caffeine content. Coffee bean is the herbal supplement. Coffee bean is a powerful cardiac and respiratory stimulant that increases heartbeat and blood flow and acts as a bronchodilator to improve breathing.

The pharmaceutical industry incorporates the caffeine in coffee bean into many commercial painkillers, where its stimulating effects help to rush such substances as aspirin and paracetamol into the system and enhance efficacy. Recent reports indicate that coffee bean helps to relieve migraine headache. In homeopathic and alternative medicine, coffee has long been used to relieve tension headaches and reduce hyperactivity.

Coffee bean is considered as central nervous system stimulant. As a brain stimulant, it helps to increase cerebral activity. According to the research, caffeine is a cognitive stimulant that helps to reduce levels of the protein called beta-amyloid in the brain, whose accumulation is responsible for Alzheimer's disease. Coffee bean has been used to control vomiting and ease nausea. The caffeine in coffee bean may be of great help in treating diabetes. Coffee may ward off liver damage. The chlorogenic acid and caffeic acid in coffee bean work as powerful antioxidants that absorb free oxygen radicals and prevent destructive, free radical or oxidative damage to tissues or cells.

Keeping all these pharmacological benefits, the main objective of present study is to determine various enzymatic and non-enzymatic antioxidants present in fresh beans of *Theobroma cacao* and *Coffee arabica*.

2. Materials and Methods

Sample Collection and Processing

Fresh beans of Cacao and Coffee were collected from Wayanad district of Kerala during the month of April. The freshly plucked beans were washed with ice cold saline, finely chopped and homogenised with ice cold 0.IM Tris buffer (1g/2ml). The supernatant was used for the analysis of the enzymic antioxidants like Superoxide dismutase, Catalase, Glutahione peroxidase, Glutathione reductase, Glucose 6 phosphate dehydrogenase and Polyphenol oxidase, and non-enzymatic antioxidants like Total reduced glutathione and Vitamin C.

i) Assay of Superoxide Dismutase (SOD)

The assay of superoxide dismutase was done according to the method of [25]. In this method, 1.4ml aliquots of the reaction mixture (comprising 1.11 ml of 50 mM phosphate buffer of pH 7.4, 0.075 ml of 20 mM L-Methionine, 0.04ml of 1% (v/v) Triton X-100, 0.075 ml of 10 mM Hydroxylamine hydrochloride and 0.1ml of 50 mM EDTA) was added to 100µl of the sample extract and incubated at 30ºC for 5 minutes. 80 µl of 50 µM riboflavin was added and the tubes were exposed for 10 min to 200 W-philips fluorescent lamps. After the exposure time, 1ml of Greiss reagent (mixture of equal volume of 1% sulphanilamide in 5% phosphoric acid) was added and the absorbance of the color formed was measured at 543 nm. One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50% of nitrite formation under assay conditions. The values are expressed as units/mim/mg protein.

ii) Assay of Catalase (CAT)

Catalase activity was assayed by the method of [26]. The enzyme extract (0.5 ml) was added to the reaction mixture containing 1ml of 0.01 M phosphate buffer (pH 7.0), 0.5 ml of 0.2 M H_2O_2, 0.4 ml H_2O and incubated for different time period. The reaction was terminated by the addition of 2 ml of acid reagent (dichromate/acetic acid mixture) which was prepared by mixing 5% potassium dichromate with glacial acetic acid (1:3 by volume). To the control, the enzyme was added after the addition of acid reagent. All the tubes were heated for 10 minutes and the absorbance was read at 610 nm. Catalase activity was expressed in terms of µmoles of H_2O_2 consumed/min/mg protein.

iii) Assay of glutathione peroxidase (GPx)

Glutathione peroxidase was assayed according to the method of [27] with slight modifications. The reaction mixture consisting of 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.1 ml of 10mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H2O2, 0.2 ml of water and 0.5 ml of plant extract was incubated at 0, 30, 60, 90 seconds respectively. The reaction was terminated with 0.5 ml of 10% TCA and after centrifugation; 2 ml of the supernatant was added to 3 ml of phosphate buffer and 1ml of DTNB reagent (0.04% DTNB in 1% sodium citrate). The color developed was read at 412 nm and the enzyme activity is expressed in terms of µg of glutathione oxidised/min/mg protein.

iv) Assay of glutathione s transferase (GST)

Glutathione transferase activity using 2, 4 dichloronitrobenzene as substrates was assayed spectrophotometric ally essentially as described by [28]. The cuvettes (final volume of 3.0 ml) contained 0.1 M phosphate buffer (pH 6.5), 1 mM GSH and 1 mM of chlorodinitrobenzene and 20 μl of appropriately diluted plant extract from the different sources. Change in absorbance at 340 nm was followed against a blank containing all reactants excepting enzyme protein, Specific activity was expressed as µmoles of CDNB-GSH conjugate formed/min/mg protein.

v) Assay of glutathione reductase activity (GR)

Glutathione reductase is assayed using the procedure given by [29] Glutathione reductase catalyses the reduction of oxidised glutathione (GSSG) to reduced glutathione (GSH)

and is assayed by measuring the decrease in absorbance at 340 nm. 0.2 mL of sample, 1.5 mL of buffer (0.3 M phosphate buffer, pH 6.8), 0.5 mL EDTA, 0.2 mL GSSG and 0.1 mL NADPH were added. The decrease in optical density of the enzyme was measured against that of the blank at 340 nm. The enzyme activity is calculated in terms of µmoles of glutathione oxidised/min/mg protein.

vi) Assay of Glucose 6 Phosphate Dehydrogenase (G6PDH)

Glucose 6 phosphate dehydrogenase is assayed using the procedure given by [30], by measuring the increase in absorbance at 340 nm when NADP reduces to NADPH. This reaction takes place when electrons are transferred from glucose 6 phosphate to NADP in the reaction catalysed by Glucose 6 Phosphate dehydrogenase.0.4 mL of Tris - HCl buffer, 0.2 mL of NADP, 0.2 mL of magnesium chloride, 1.0 mL water and 0.2 mL of enzyme was taken in a cuvette. The reaction was started by the addition of 0.2 mL of glucose 6 phosphate and the increase in OD was measured at 340 nm. The activity was expressed in terms of units/ mg protein, in which one unit is equal to the amount of enzyme that brought about a change in OD of 0.01/min. G6PDH concentration was expressed as µmoles/g tissue.

vii) Assay of polyphenol oxidase (PPO)

Assay of Polyphenol oxidase activity was carried out according to the procedure of [31]. To 2.0 ml of plant extract and 3.0ml of distilled water added and mixed together. 1.0ml of catechol solution (0.4mg/ml) added to the above solution and the reactants were quickly mixed. The enzyme activity was measured as change in absorbance/min at 490nm.PPO concentration was expressed as µmoles/g tissue.

viii) Estimation of Reduced Glutathione (TRG)

The amount of reduced glutathione in the samples was estimated by the method of [32]. 1ml of the sample extract was treated with 4.0 ml of metaphosphoric acid precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g EDTA and 30 g NaCl dissolved in 100ml water). After centrifugation, 2.0 ml of the protein-free supernatant was mixed with 0.2 ml of 0.4 M Na2HPO4 and 1.0 ml of DTNB reagent (40 mg DTNB in 100 ml of aqueous 1% tri sodium citrate). Absorbance was read at 412 nm within 2 minutes. GSH concentration was expressed as µg/mg protein.

ix) Estimation of Vitamin C

Estimation of Vit. C was carried out according to the procedure of [31]. The assay mixture for vitamin C consisted of 0.1 ml of brominated sample extract, 2.9 ml of distilled water, 1 ml of 2% DNPH reagent and 1-2 drops of thiourea. After incubation at 37ºC for 3 h, the orange-red osazone crystals formed were dissolved by the addition of 7 ml of 80% sulphuric acid and absorbance was read at 540 nm after 30 minutes. Vitamin C concentration was expressed in terms of µg/mg plant tissue.

Statistical Analysis

All the analyses were performed in triplicate and the results were statistically analyzed and expressed as mean (n=3) ± standard deviation (SD).

3. Results

Estimation of enzymatic antioxidants

The levels of enzymatic antioxidants like SOD, CAT, GPx, GST, GR, G6PDH, PPO values is shown in table 1.

Table 1: Levels of enzymatic antioxidants in fresh beans of *Theobroma cacao* and *Coffee Arabica*

S. No	Antioxidants	*Theobroma cacao*	*Coffee arabica*
1.	Superoxide Dismutase	1.89 ± 0.04	1.97 ± 0.02
2.	Catalase	3.18 ± 0.02	2.49 ± 0.07
3.	Glutathione Peroxidase	1.20 ± 0.03	1.73 ± 0.02
4.	Glutathione-S-Transferase	6.25 ± 0.21	7.12 ± 0.14
5.	Glutathione Reductase	1.54 ± 0.06	0.93 ± 0.03
6.	Glucose 6 Phosphate Dehydrogenase	1.12 ± 0.09	1.20 ± 0.01
7.	Polyphenol Oxidase	6.56 ± 0.23	4.89 ± 0.15

Values are expressed as mean ± SD (n =3)

Units:- SOD-Units/min/mg protein, CAT- µmoles of H_2O_2 consumed/min/mg protein, GPx- µg og glutathione oxidized/min/mg protein, GST- µmoles of CDNB-GSH conjugate formed/min/mg protein, GR- µmoles of glutathione utilized/min/mg protein, G6PDH- µmoles/g tissue, PPO- µmoles/g tissue.

Estimation of non-enzymatic antioxidants

The levels of enzymatic antioxidants like TRG and Vit.C values is shown in table 2.

Table 2: Levels of non-enzymatic antioxidants in fresh beans of *Theobroma cacao* and *Coffee Arabica*

S. No	Antioxidants	*Theobroma cacao*	*Coffee arabica*
1.	Total reduced glutathione	1.70 ± 0.08	0.62 ± 0.04
2.	Vitamin C	1.31 ± 0.06	1.22 ± 0.03

Values are expressed as mean ± SD (n =3)

Units:- TRG- µ/mg plant tissue, Vit. C- µ/mg plant tissue.

4. Discussion

Oxidative stress is essentially an imbalance between these of free radicals and the ability of the body to counteract or detoxify their harmful effects through neutralization by antioxidants. These free radicals can damage cell membranes and lipoproteins by a process called as lipid peroxidation. Proteins may also be damaged by ROS/NOS, [33] leading to structural changes and loss of enzyme activity. Free radicals

may cause DNA strand breaks which can cause cell mutation. The body has several mechanisms to counteract these attacks by using DNA repair enzymes and or [34] antioxidants. If not regulated properly; oxidative stress can induce a variety of chronic and degenerative diseases.

Antioxidant enzymes are capable of stabilizing, or deactivating free radicals before they attack cellular components. They act by reducing the energy of the free radicals or by giving up some of their electrons for its use, thereby causing it to become stable. In addition, they may also interrupt with the oxidizing chain reaction to minimize the damage caused by free radicals. For the past decade, countless studies have been devoted to the beneficial effects of antioxidant enzymes. By reducing exposure to free radicals and increasing the intake of antioxidant enzyme rich foods or antioxidant enzyme supplements, our body's potential to reducing the risk of free radical related health problems is made more palpable [35]. Antioxidant enzymes are, therefore, absolutely critical for maintaining optimal cellular and systemic health and well-being.

Superoxide dismutase (SOD, orgotein) is a ubiquitous enzyme that has received attention because of its therapeutic activity and because of claims that its ingestion may improve health and lengthen the human lifespan. A highly reactive superoxide free radical is generated as a toxic metabolite in a wide range of normal biological reactions that reduce oxygen. Since the superoxide radical is toxic to normal living cells, the enzyme superoxide dismutase, which is present in all cells, catalyzes the conversion of superoxide to the harmless components oxygen and hydrogen peroxide.

The catalase enzyme is so critical to our health that it is found in nearly every living organism on the planet that is exposed to oxygen. This antioxidant enzyme can catalyze the conversion of hydrogen peroxide into water and oxygen. Hydrogen peroxide is a by-product of cell metabolism, which serves some useful functions including healthy immune response. Catalase has one of the highest rates of turnover when compared to all other enzymes. In other words, one catalase enzyme can change 40 million molecules of hydrogen peroxide into water and oxygen in just one second. In fact, catalase enzymes act to protect our cells, counteracting and balancing the continual production of hydrogen peroxide.

Glutathione, a major non-protein thiol in living organisms, plays a central role in coordinating the body's antioxidant defense processes. Excessive peroxidation causes increased glutathione consumption. Reduced thiols have long been reported to be essential for recycling of antioxidants like vitamin E and vitamin C [36].GPx is one of the most important enzymes in the body with antioxidant properties. Levels of GPx in the body are closely linked with that of glutathione, the master antioxidant. Glutathione (GHS for short) is a tripeptide that not only protects the cells against ill effects of pollution; it is also acts as your body's immune system boosters. It is present in high concentrations in the cells and plays a pivotal role in maintaining them in reduced state lest they suffer damage by oxidation (from free radicals). The role as antioxidant is particularly important for brain as it is very sensitive to presence of free radicals.

Glutathione S-transferases (GSTs), a family of cytosolic multifunctional enzymes. It catalyzes the conjugation of glutathione with a variety of reactive electrophilic compounds, thereby neutralizing their active electrophilic sites and subsequently making the parent compound more water soluble. Reduced *glutathione* (GSH), a tripeptide with a free sulfhydryl group, is required to combat oxidative stress and maintain the normal reduced state in the cell. Oxidized glutathione (GSSG) is reduced by NADPH generated by glucose 6-phosphate dehydrogenase in the pentose phosphate pathway. Indeed, cells with reduced levels of glucose 6-phosphate dehydrogenase are especially sensitive to oxidative stress. This stress is most acute in red blood cells because, lacking mitochondria, they have no alternative means of generating reducing power.

Vitamin C is regard as the first line natural antioxidant defense in plasma and a powerful inhibitor of LPO [37]. Vitamin C is a water soluble antioxidant. It acts as a free radical scavenger. It scavenges peroxyradicals[38]. Vitamin C protects non-smokers against the harmful effects of ROS from passive smoking [39]. It has been found in the chloroplast, cytosol, vacuole and extracellular compartments of the plant cells and shown to function as a reluctant for many free radicals [40].

5. Conclusion

Oxidative stress plays a major role in the pathogenic of many disorders including aging, cancer, diabetes, alzheimer's, strokes, viral infections (that cause airway epithelial inflammation), neurodegenerative processes (including cell death, motor neuron diseases and axonal injury) and infraction, and brain edema. Antioxidant enzyme plays an important role in protecting oxidative injury to the body. One of the therapeutic approach by which these disorders can be prevented is to increase the levels of these enzymes (SOD, CAT, GPx etc.) in the body by interventions which may include increases intake of dietary supplements rich in antioxidants/antioxidant enzymes and regular exercise. Based on all these finding it is suggested that this plant is a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the oxidative stress related degenerative diseases such as cancer and various other human ailments.

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