In vitro Antioxidant Assay of Cocoa (*Theobroma cacao*) Oil and Cake

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Abstract

Numerous studies have highlighted the nutritional and pharmacological importance of cocoa. In this current study, three in vitro *assay methods were used to investigate the antioxidant activity of cocoa oil and cake: scavenging effect on 2,2-diphenyl-1-picryhydrazyl radical (DPPH), hydroxyl radical generated from hydrogen peroxide and peroxide oxidation by ferric thiocynate method. It was revealed that cocoa oil and cake possessed significant antioxidant activity ($P<0.05$) when compared with antioxidant standards: butylated hydroxyl anisole (BHA), ascorbic acid and α-tocopherol used in the assay. At 1.0-0.0625 mg/ml, the oil had percentage inhibition of 65.0-73.6% while cake had percentage inhibition of 75.4-88.7% in DPPH assay however, the % inhibition was between 98.6-98.9% for cocoa oil and 92.6-98.2% for cocoa cake at the same concentration in the hydroxyl radical scavenging assay while in the ferric thiocyanate assay, percentage inhibition of cocoa oil was approximately 94% at 0.005 - 0.4 mg/ml which is better than the activity of cocoa cake, 91% at the same concentration. The results indicate that inhibition was better with the hydroxyl radical scavenging assay and that different antioxidative mechanism is involved in the free radical scavenging activities of cocoa. The results of this study further support the medicinal and nutritional values of cocoa.*

Keywords: *Antioxidant, cocoa, 2, 2-diphenyl-1-picrylhydrazyl radical, hydroxyl radical, peroxide oxidation.*

Introduction

Theobroma cacao otherwise known as cacao or cocoa tree is a small evergreen 4–8 m tall in the family Sterculiaceae or Malvaceae and is native to tropical America but can also be found in almost all regions of the world. The pod contains 20 to 60seeds, usually called "beans", embedded in a white pulp. Cocoa bean is the dried and fully fermented fatty seed of *Theobroma cacao* from which cocoa cake and cocoa oil (butter) are extracted. Their most noted active constituent is theobromine.

Raw cocoa also contain a high level of flavonoids, specifically epicatechin, which has been observed to have beneficial cardiovascuar effects on health, the benefits extend to the brain and have important implications for learning and memory (Bergmann 1969; Coe1994; Coe and Michael 1996; McNeil 2006). Researchers also found that the Kuna Indians living on the islands had significantly lower rates of heart disease and cancer compared to those on the mainland who do not drink cocoa as on the islands and that foods rich in cocoa also appear to reduce blood pressure due to the presence of alkaloids, theobromine, caffeine and polyphenolics that are abundant in cocoa powder. Cocoa powder is also rich in nutritional high caloric fat, protein and carbohydrate. Numerous studies on cocoa medicinal and nutritional properties as anti-bacteria, anti-malaria, anti-parasites, anti-hepatocarcinogenesis amongst others have been reported in literature. The leaf is also effective in the treatment of scurvy,

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rheumatism, pile, indigestion and blood sugar control (Smith *et al*. 1996; Cragg *et al*. 1997; Motamayor *et al*. 2002; Ali 2004; Buijsse 2006; Bayard *et al*. 2007; Taubert *et al*. 2007; Keogh *et al*. 2007; Sofowora 2008).

The objective of this research work is to carry antioxidant screening on the oil (butter) and cake obtained from cocoa by using three out *in vitro* assay methods: scavenging effect on 2, 2-Diphenyl-1-picrylhydrazyl radical (DPPH), hydroxyl radical generated by hydrogen peroxide and peroxide oxidation by ferric thiocynate method. Butylatedhydroxyanisole (BHA), ascorbic acid and α-tocopherol are used as reference standards (Soares *et al*. 1997; Koleva *et al*. 2002; Gow-chin and Pin-Der 1994).

Antioxidant screening is carried out because the oxidative damage caused by free radicals could be related to aging and diseases, such as diabetes, cancer amongst others (Halliwell and Gutteridge 1984; Potterat 1997). Free radicals in the form of reactive oxygen and nitrogen (ROS/N) species are an integral part of normal physiology. Over-production of these reactive species can occur, due to oxidative stress brought about by the imbalance of the bodily antioxidant defense system and free radical formation. These reactive species can react with biomolecules, causing cellular injury and even death as a result of the development of chronic diseases such as hypertension, heart failure, cancer, diabetes, infections, inflammation, asthma, aging, depression, memory loss, rheumatism and those that involve the cardio-and cerebrovascular systems (Gurdip *et al*. 2007). Antioxidants might help in the prevention of these disorders/diseases. Antioxidant may be defined as a molecule capable of slowing or preventing the oxidation of other molecules and reduce the action of ROS/N in tissue damage. Antioxidants are often added to foods to prevent the radical chain reactions of oxidation, and they act by inhibiting the initiation and propagation step leading to the termination of the reaction and delay the oxidation process (Shahidi *et al*. 1992). The consumption of fruits and vegetables (Peschel *et al*. 2006) containing antioxidants has also been found to offer protection against these diseases. Different testing approaches including physical, chemical and biochemical methods have been employed for investigation and characterization of natural antioxidants (Bors *et al*. 1991; Potterat 1997; Oloyede *et al*. 2010a).

Materials and Methods

Chemicals and Reagents

Ethyl acetate, hexane, methanol, butanol, chloroform, hydrochloric acid, ammonia solution, naphthol, bismuth nitrate, potassium iodide, sodium hydroxide, copper acetate, NaOH, sodium chloride, copper sulphate pentahydrate, ferric chloride, conc. tetraoxosulphate (VI) acid, conc. HCl, ammonia solution, sodium potassium tartarate, linoleic acid, ammonium thiocynate, ethanol, ferrous chloride, hydrochloric acid, potassium chloride, glacial acetic acid, disodium hydrogen phosphate, and dihydrogen potassium phosphate were all BDH general purpose chemicals and distilled prior to use. Dimethylsulphoxide (M&B, England), hydrogen peroxide (Merck, Germany) and 2, 2 -diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, butylatedhydroxylanisole (BHA) and α-tocopherol were obtained from Sigma Chemical Co (St Louis, MO).

Equipment and Apparatus

UV-Visible spectrophotometer (Unico 1200 & Perkin Elmer lambda 25 models, Germany), Soxhlet apparatus, Mettler analytical balance H80 (UK), Water Bath (Gallenkamp), Rotavapor RII0 (Buchi, England), silica gel GF_{254} (precoated aluminium sheets, Merck, Germany), pH meter (Jenway model).

Reference Standards

Ascorbic acid, Butylated hydroxyanisole (BHA) and α-Tocopherol for antioxidant activity.

Sample preparation

Cocoa seed was obtained from Cocoa Research Institute of Nigeria (CRIN) Ibadan, Oyo State, Nigeria in July 2010. It was weighed and then pulverized using laboratory milling machine.

Extraction/Partitioning Procedure

The powdered cocoa (500 g) was extracted with 5 lt (x3) of hexane to remove lipids. The procedure yielded 210.23 g of defatted cocoa powder. The defatted cocoa powder was extracted with a mixture of acetone and water (70:30 v/v) using soxhlet apparatus. The aqueous extract was re-extracted with hexane to remove residual lipids. The hexane layer contains the fatty acid. The acetone layer was collected and concentrated with the aid of a Bucchi rotavapor and stored in a desiccator prior to further analysis. Thin Layer Chromatography (TLC) was employed using silica gel 60 F_{254} precoated plates and solvent system: Ethyl acetate/methanol (8:2) to detect antioxidant activity by using 2, 2-diphenyl-1-picrylhydrazylradical (DPPH) as a spray reagent. Yellow coloration on the spots on the TLC plates indicates that cocoa has antioxidant activity. Quantitative antioxidant screening was carried out on cocoa oil and cake using the following spectrophotometric experiments; scavenging effect on DPPH, hydroxyl radical generated by hydrogen peroxide and peroxide oxidation by ferric thiocynate method.

In vitro Antioxidant Screening

Scavenging Effect on DPPH

The ability to scavenge free radicals is one of the stable and reliable *in vitro* methods that can be used to determine antioxidant activity. The antioxidant activity or the capacity to scavenge the "stable" free radical DPPH was determined using the DPPH free – radical scavenging method. A 3.94 mg of 2, 2-diphenyl-1-picryhydrazyl radical (DPPH), a stable radical was dissolved in methanol (100ml) to give a 100 µm solution. To 3.0 ml of the methanolic solutions of DPPH was added 0.5 ml of each of the fractions with doses ranging from 1.0 mg/ml to 0.0625 mg/ml (Gulcin *et al*. 2002; Mutee *et al*. 2010; Oloyede *et al*. 2010a). The decrease in absorption at 517 nm of DPPH was measured 10 minutes later. The actual decrease in absorption was measured against that of the control and the percentage inhibition was also calculated. The same experiment was carried out on ascorbic acid, butylated hydroxylanisole (BHA) and α-tocopherol which are known antioxidants. All tests and analyses were run in triplicates and the results obtained were averaged. The radical scavenging activity (RSA) was calculated as the percentage inhibition of DPPH discoloration using the equation below:

$$\% \text{ inhibition} = \{(A_{DPPH} - A_S)/A_{DPPH}\} \times 100, \quad (2)$$

where A_S is the absorbance of the solution and A_{DPPH} is the absorbance of the DPPH solution (Hatano *et al*. 1988).

Scavenging Effect on Hydrogen Peroxide

Extracts of cocoa were spectro-photometrically determined for hydroxyl radical scavenging effect at 285 nm. A solution of 2 mM hydrogen peroxide was prepared in phosphate buffered-saline (PBS) pH 7.4. The fractions at the following concentrations; 1.0 - 0.0625 mg/ml was added to the H_2O_2 solution. Decrease in absorbance of H_2O_2 at 285 nm was determined in a UV/Visible spectrophotometer 10 minutes later against a blank solution containing the test extract in PBS without H_2O_2. All tests were run in triplicates and averaged (Soares *et al*. 1997; Oloyede and Farombi 2010). The same experiment was carried out on Butylatedhydroxyanisole (BHA), ascorbic acid and α-tocopherol which are known antioxidant standards.

Antioxidant Activity by Ferric Thiocynate Method

The antioxidant activity of cocoa oil and cake was again determined by ferric thiocynate method (Collee *et al*. 1989). 10 mg of each extract was dissolved separately in 99.5% of ethanol and various concentrations (0.00625 - 0.8 mg/ml) were prepared. A mixture of a 2 ml of sample in 99.5% ethanol, 2.0 ml of 2.51% linoleic acid in 99.5% ethanol, 4 ml of 0.05 M phosphate buffer (pH 7.0) and 2 ml of water was placed in a vial with a screw cap and placed in an oven at 60^0C in the dark. To 0.1 ml of this sample solution, 10 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocynate were added. After the addition of 0.1 ml of 2 x 10^{-2} M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance of the red colour which developed

was measured in 3 min at 500 nm. The control and standards were subjected to the same procedures as the samples, except that for the control, only solvent was added, and for the standard, sample was replaced with the same amount of butylatedhydroxyanisole (BHA), ascorbic acid and α-tocopherol (reference compounds) (Oloyede *et al*. 2010b). All test and analysis were run in triplicates and the results obtained were averaged. The inhibition of lipid peroxidation in percentage was calculated using this equation:

$$\% \text{ Inhibition} = 1 - (A_1/A_2) \times 100, \quad (1)$$

where A1 was the absorbance of the test sample and A2 was the absorbance of control reaction.

Statistical Analysis

Group data were expressed as mean value ± SEM and was calculated for each parameter, each parameter was analyzed separately using ANOVA followed by Dunnets '*t*' test. A *P*-value of <0.05 was considered statistically significant. Graphs and charts were plotted using Microsoft Excel 2007 software.

Results and Discussion

Antioxidant Activity

The *in vitro* antioxidant activities of cocoa oil and cake were determined by the following methods: scavenging effect on 2, 2-diphenyl-1-picryhydrazyl radical (DPPH), on hydroxyl radical generated by hydrogen peroxide and ferric thiocynate (FTC) method. The results are presented in Tables 1-3 and Figs. 1-3.

Scavenging Effects on DPPH

The reduction in absorbance of DPPH at 517 nm caused by the samples was measured in triplicate after 10min. DPPH is known to be a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares *et al*. 1997). The tested samples showed very good activity when compared to the standard used (Table 1). There was decrease in absorption at 517 nm as the concentration is decreased indicating that cocoa oil and cake have hydrogen donating ability or ability to scavenge free radical. The observation was further corroborated by the calculated percentage inhibition. At 1.0-0.0625 mg/ml the oil had percentage inhibition of 65.0-73.6% while cake had percentage inhibition of 75.4-88.7%. These activities though lower than the activities ascorbic acid and butylatedhydroxylanisole (BHA) were better than the activity of α-tocopherol.

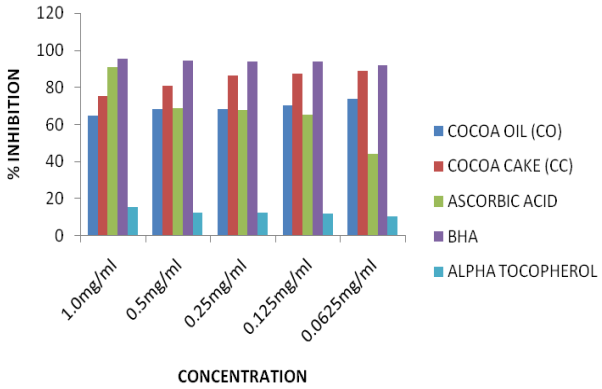

Fig. 1. DPPH Free radical scavenging activity of cocoa oil, cocoa cake and standards.

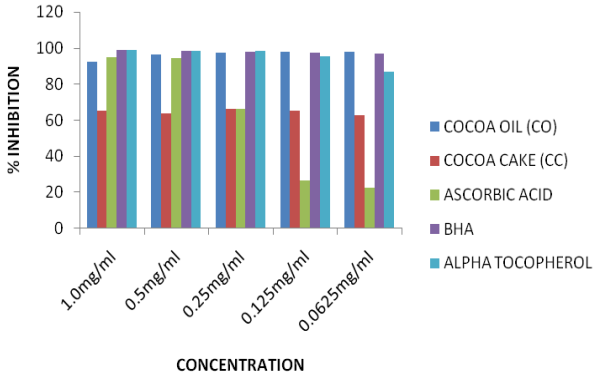

Fig. 2. H_2O_2 Free radical scavenging activity of cocoa oil and cake and standards at 285 nm measured in triplicate.

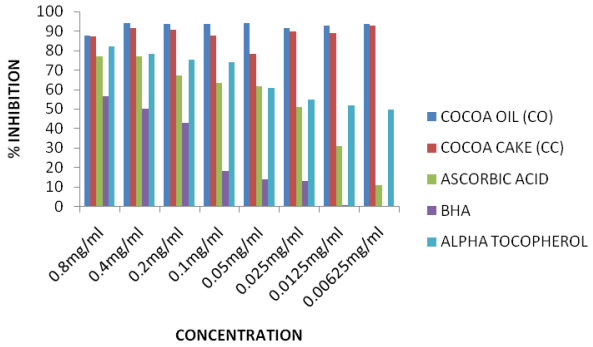

Fig. 3. Peroxide oxidation of cocoa oil, cocoa cake and standards at 500 nm measured in triplicate.

Cocoa oil and cake therefore possess good activities as free radical scavengers (Fig. 1) (Gow-chin *et al*. 1994; Alan and Miller 1996).

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Table 1. Absorbance values from scavenging effect of cocoa oil and cake on DPPH at 517 nm*.

CONC (mg/ml)	CO	CC	ASCORBIC ACID	BHA	ALPHA TOCOPHEROL
1.0	0.1307±0.015	0.929±0.001	0.0843±0.010	0.0370±0.006	0.6800±0.029
0.5	0.1288±0.023	0.720±0.002	0.2893±0.128	0.0460±0.006	0.7040±0.003
0.25	0.1288±0.045	0.507±0.005	0.5147±0.015	0.0650±0.003	0.7207±0.012
0.125	0.1254±0.032	0.480±0.002	0.2977±0.124	0.0483±0.002	0.7047±0.007
0.0625	0.1173±0.001	0.428±0.001	0.3200±0.082	0.0490±0.004	0.7070±0.007

*Absorbance measurement for CO (cocoa oil) and CC (Cocoa cake), ascorbic acid, BHA and α-Tocopherol at 517 nm.

Table 2. Scavenging effect of hydroxyl radical generated by H_2O_2 of cocoa oil, cocoa cake and standards at 285 nm*.

Conc. (mg/ml)	CO	CC	Ascorbic Acid	BHA	Alpha Tocopherol
1.0	0.2785±0.021	0.0401±0.021	0.1952±0.001	0.0413±0.016	0.0321±0.045
0.5	0.1305±0.001	0.0411±0.002	0.2078±0.012	0.0617±0.019	0.0633±0.032
0.25	0.1071±0.003	0.0418±0.005	1.2645±0.119	0.0740±0.015	0.1552±0.061
0.125	0.0869±0.041	0.0435±0.012	2.7586±0.049	0.0947±0.003	0.1807±0.015
0.0625	0.0685±0.003	0.0463±0.034	2.9236±0.211	0.1126±0.014	0.4940±0.017

*Absorbance measurement of cocoa oil (CC), cocoa cake (CC), ascorbic acid, BHA and α-Tocopherol at 285 nm.

Table 3. Peroxide oxidation of cocoa oil and cake at 500 nm using the Ferric thiocyanate method*.

CONC (mg/ml)	CO	CC	ASCORBIC ACID	BHA	ALPHA TOCOPHEROL
0.8	0.094±0.006	0.095±0.003	0.173±0.008	0.326±0.006	0.133±0.004
0.4	0.046±0.003	0.064±0.001	0.173±0.008	0.375±0.008	0.164±0.006
0.2	0.048±0.003	0.069±0.002	0.245±0.008	0.431±0.008	0.184±0.009
0.1	0.047±0.004	0.092±0.006	0.275±0.006	0.616±0.005	0.195±0.023
0.05	0.046±0.000	0.163±0.217	0.287±0.050	0.647±0.004	0.294±0.004
0.025	0.065±0.001	0.077±0.002	0.367±0.004	0.653±0.008	0.340±0.069
0.0125	0.053±0.001	0.084±0.002	0.516±0.008	0.747±0.003	0.360±0.005
0.00625	0.049±0.000	0.053±0.001	0.668±0.002	0.750±0.001	0.377±0.008

*Absorbance measurement of CO (Cocoa oil) and CC (Cocoa cake), ascorbic acid, BHA and α-Tocopherol at 500 nm.

Scavenging Effects on Hydrogen Peroxide (H_2O_2)

Cocoa oil and cake had the ability to scavenge hydroxyl radical, the activity was significant (P<0.05) when compared with antioxidants like ascorbic acid, Butylated hydroxyanisole (BHA) and α-tocopherol. The absorbance values decreases (1.0-0.0625 mg/ml) as the concentration is decreased (Table 2) unlike in the standards where absorbance values increases as the concentration is decreased.

Figure 2 shows the percentage inhibition of cocoa oil and cake on hydroxyl radical scavenging activity at 1.0 - 0.0625 mg/ml. The samples scavenging activities were significant (P<0.05) with all the standards at these concentrations. The % inhibition was between 98.61-98.95% for cocoa oil and 92.61-98.18% for cocoa cake at the entire concentrations used (Fig. 2). These activities were better than that of ascorbic acid but comparable with Butylated hydroxyanisole (BHA) and α-tocopherol (Fig. 2). The results obtained showed that cocoa oil and cake scavenge hydroxyl radicals in a concentration dependent manner. And that the activities were better when compared with the DPPH assay, which may be as a result of the highly reactive nature of hydroxyl radicals indicating that the mode of action involved using different radicals varies. Therefore cocoa oil and cake are sources of antioxidant agents especially polyphenolic compounds previously reported in the plant which have the ability to scavenge the highly reactive hydroxyl radicals and stopping the chain reactions in biological macromoecues (Namiki 1990; Lugasi *et al*. 1999).

Antioxidant Activity by Ferric Thiocyanate Method (FTC)

The effect of cocoa oil and cake on the oxidation of linoleic acid and the amount of peroxide which oxidized ferrous chloride ($FeCl_2$) to a reddish ferric chloride ($FeCl_3$) pigment was determined in by the FTC method. As the antioxidant activity increases, concentration of peroxide decreases. The autoxidation of linoleic acid without the sample or use of antioxidant standards was accompanied by a very rapid increase in peroxide value. Cocoa oil and cake at various concentration (0.00625 – 0.8 mg/ml), showed antioxidant activities in a concentration dependent manner. Significant differences (P<0.05) were obtained in peroxide values of the control and linoleic acid containing the samples or reference standards (Table 3).

Cocoa oil showed better antioxidative activity than the cake but both were better in activity than the standards: butylated hydroxyanisole (BHA), ascorbic acid and α-tocopherol. As a function of percentage inhibition cocoa oil had approximately 94% inhibition at 0.005 – 0.4 mg/ml which is better than the activity of cocoa cake 91% at the same concentration however both activities were better than all the standards BHA, ascorbic acid and α-tocopherol (Fig. 3).

This current study revealed that different antioxidative mechanisms were involved in the free radical scavenging activities of cocoa oil and cake. They were most effective in scavenging hydroxyl radical which is known to cause oxidative damage to biological macromolecules. The oxidative process is possible because oxygen can be transformed through metabolic activity into more reactive forms such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radicals, collectively called reactive oxygen species (Halliwell and Gutteridge 1984; Bors and Saran 1991; Sies 1997; Koleva *et al*. 2002; Wolf *et al*. 2005). Cocoa oil and cake screened in this current study has the ability to scavenge reactive radicals especially the hydroxyl radical and can therefore be employed in the therapy of diseases involving free radical reactions. This result therefore supports the nutritional and medicinal application of cocoa as having beneficial cardiovascular effects on health, preventing heart disease and cancer.

Conclusion

Three *in vitro* antioxidant screening: scavenging effect on 2, 2-diphenyl-1-picryhydrazyl radical (DPPH), on hydroxyl radical generated by hydrogen peroxide and peroxide oxidation by ferric thiocynate used in this assay to determine the activity of cocoa oil and cake at low concentration shows that they

have significant antioxidant activities (P<0.05) when compared with antioxidant standards ascorbic acid, BHA and α-tocopherol and could be beneficial in the treatment of ailments resulting from oxidative stress such as Parkinson's disease, Alzheimer's disease, malaria, cancer, cardiovascular disorders, bacterial and viral infections, inflammation, coronary heart disease and stroke.

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