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Effect of fermentation on the antioxidant potential and bioactive compounds of cocoa (*Theobroma cacao*) spp.

Sidiqat Adamson Shodehinde[*1], Veronica Oluwatoyin Odubanjo[1], Blessing Moyinoluwa Oladunjoye[1], Victor Ayodeji Oladimeji[1], Samuel Olawale Olubode[1], Precious Ayorinde Akinnusi[1], and Ganiyu Oboh[2]

[1]*Department of Biochemistry, Faculty of Science, Adekunle Ajasin University, P.M.B. 001, Akungba Akoko. Nigeria;*
Email:** ***sidiqat.shodehinde@aaua.edu.ng

[2]*Functional Foods and Nutraceutical Unit, Department of Biochemistry, Federal University of Technology, Akure. Ondo State. 340001, Nigeria;*

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ABSTRACT

Background & Aim: Cocoa has long been prized for its high concentration of antioxidants and bioactive compounds. Fermentation is one of the processes involved in the manufacturing of cocoa. Fermentation has a substantial effect on the antioxidant potential as well as the bioactive compounds of cocoa beans according to studies. This present study sought to determine and compare the effect of fermentation on phenolic contents and antioxidant potentials of cocoa beans.

Experimental: The experiment was accomplished by following the procedures. The cocoa pod was broken and the beans were extracted and separated into 3 groups. The cocoa beans of each group were wrapped with polyethylene and then subjected to different hours of fermentation (24, 72 and 120 hours). After aqueous extraction, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability assay, the ferric reducing property (FRAP) assay, the 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, total phenol content assay, total flavonoid content assay were carried out using standard procedures.

Results: The research found that fermentation enhances DPPH radical scavenging capacity, boosts ferric reducing antioxidant potential, has no effect on ABTS radical scavenging ability, decreases phenolic content, and increases flavonoid content of cocoa beans after a series of biochemical assays.

Recommended applications/industries: cocoa has long been thought to aid in the prevention of disease because of its antioxidant properties. However, various industrial procedures that it goes through during manufacture, including fermentation, may have an impact on its antioxidant capability. Understanding the effects of fermentation on cocoa could lead to the development of more efficient manufacturing methods, potentially increasing cocoa's antioxidant potential.

1. Introduction

Carolus Linnaeus, the inventor of modern-day taxonomic plant categorization, originally classified cocoa, which is botanically known as *Theobroma cacao*, in the mid-1700s. The term "cacao" comes from the Olmec and subsequent Mayan languages (Kakaw) (Dillinger *et al.*, 2000).

Cacao is an evergreen tree with "cauliflorous" flowers (and later fruits) that protrude directly from the woody branches and trunk. The plant reaches a height of 4–8 meters, with the occasional exception of 20 meters (Afoakwa, 2016). The plant produces Branches ranging in height from 1 to 1.5 meters, huge leaves, and

inflorescence on the trunk and branches. The fruits (which are commonly referred to as pods) are 10–32 cm long and spherical to cylindrical in shape (Maximova *et al*., 2003). The pods are indehiscent and contain 20–60 seeds grouped in five rows, which are popularly referred to as beans (Bhattacharjee and Kumar, 2007). Unripe pods are white, green, or red, but when completely matured, they turn green, yellow, red, or purple (Maximova *et al*., 2003).

Fig. 1. Diagram showing Cocoa (*Theobroma cacao*) (Sanusi and Oluyole, 2005).

Cocoa production is significant in Latin America, particularly in Ecuador, Brazil, Peru, Colombia, the Dominican Republic, and Mexico. It is also used as an export item in countries like Peru (Ramos-Escudero *et al*., 2021). Cocoa production is also highly common in other Nigerian states, such as Ondo and Ekiti.

By-products from cocoa are made in the field-processing chain, which includes the removal of cocoa beans from the cocoa pod husk, followed by fermentation and drying for storage and transport (Vásquez et al., 2019). The fruit is composed of bean shell, pod husk, and pulp apart from the cocoa seed, which represents about 70–80% of the fruit in dry weight (Nair, 2010)

Beans from ripe pods are removed and fermented to increase the biochemical activity necessary for flavor development, but this process destroys seed viability. Cacao seeds have a limited viability period of 10–13 weeks and require up to 50% moisture for germination (Bhattacharjee and Kumar, 2007).

They are prized not only in the food sector, but also in the pharmaceutical and cosmetic industries (Whitlock *et al*., 2001), due to their high concentration of bioactive chemicals and antioxidant potential. Interest in cocoa has increased dramatically in recent years as a result of its potential health advantages. By scavenging free radicals or chelating transition metal ions, which lowers their ability to produce reactive oxygen species, antioxidants from cocoa can postpone cellular damage (Oracz and Żyżelewicz, 2020) and at the same time protect against diseases such as coronary heart disease, cancer, and neurological disorders (Whitlock *et al*., 2001; Oracz and Żyżelewicz, 2020; Hazirah *et al*., 2013)

Several studies have shown that processing methods such as fermentation, drying, alkalization, and roasting of cocoa beans results in considerable changes in the chemical makeup of the finished products (Rodriguez-Campos *et al*., 2012; Albertini *et al*., 2015; Suazo *et al*., 2014). During processing, naturally occurring antioxidants (Sanbongi *et al*., 2019; Ren *et al*., 2003; Wollgast and Anklam, 2000; Arlorio *et al*., 2008) undergo significant modifications in their chemical structure, which may affect their bioactivities (Oracz and Żyżelewicz, 2020). Contact with the oxidative enzymes polyphenol oxidase (PPO) and peroxidase during fermentation and drying of cocoa beans might result in oxidative destruction of polyphenols. Enzymatic oxidation of monomeric flavan-3-ols to semi-quinones and quinones. Furthermore, these oxidation products polymerize into insoluble tannins with a high molecular weight (Whitlock *et al*., 2001).

This study is aimed to determine and compare the effect of fermentation on the phenolic content and antioxidant activities on cocoa beans *in vitro*.

2. Materials and Methods

2.1. Chemical reagents

Chemicals and reagents used such as gallic acid, Folin–Ciocalteau's reagent was procured from Sigma-Aldrich, Inc., (St Louis, MO), trichloroacetic acid (TCA) was sourced from Sigma-Aldrich, Chemie GmbH (Steinheim, Germany), dinitrophenyl hydrazine (DNPH) from ACROS Organics (New Jersey, USA), methanol and acetic acid were sourced from BDH Chemicals Ltd., (Poole, England), thiourea, $CuSO_4.5H_2O$, H_2SO_4, sodium carbonate, $AlCl_3$, potassium acetate, Tris-HCl buffer, sodium dodecyl sulfate, $FeSO_4$, quinolinic acid, and sodium nitroprusside were of analytical grade while the water was glass distilled.

2.2. Collection and processing of cocoa beans seed (CBS)

Cocoa (*Theobroma cacao*) pods of the species 'Amelonado' were obtained from Cocoa Research

Institute of Nigeria (CRIN) Owena, Ondo State with longitude 7.1961 ^{o}N and latitude 5.0195 ^{o}E in the forest zone of Southwest of Nigeria. Analyses were carried out at Functional Foods and Nutraceuticals Unit (FFNU) laboratory, Federal University of Technology, Akure. The cocoa pod was broken and the beans were extracted and sorted out to peel the outer coated layer. The beans were separated into 3 groups. The cocoa beans of each group were wrapped with polyethylene and tied to generate enough heat that will ferment the beans. The samples were then subjected to different hours of fermentation (24, 72 and 120 hours). At the expiration of the period of each group, the tied polythene was removed, washed, dried at 25 ^{o}C and later blended into powder. The powder of each sample was kept in a tight container for future analysis.

2.2.1. Aqueous extract preparation

Aqueous extract preparation was carried according to Shodehinde and Oboh (2012) with slight modification. Ten grams of each cocoa beans sample was soaked in 100 mL of distilled water for about 24 h. The mixture was filtered and the filtrate was centrifuged for 10 minutes to obtain a clear supernatant liquid. The extract was stored at 4 ^{o}C and used for further analysis. The antioxidant tests and analyses were performed in triplicate and results were averaged.

2.3. Antioxidant assays

2.3.1. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging ability

The free radical-scavenging ability of the fermented and unfermented Amelonado against DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical was evaluated, with modifications. To 1 mL of 0.4 mM methanolic solution of DPPH radicals, 0.05 mL of test fermented and unfermented Amelonado was added. The mixture was left in the dark for 30 min and the absorbance was measured at 516 nm in the spectrophotometer. The procedure was done for Amelonado fermented for 24, 72, and 120 h, respectively, which was subsequently compared with the result of the unfermented Amelonado.

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of the test})}{\text{Absorbance of control}} \times 100$$

2.3.2. The ferric reducing property (FRAP)

The reducing activity of the processed Amelonado was determined by assessing the ability to reduce $FeCl_3$ solution. A 2.5 mL aliquot was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was then centrifuged at 805g for 10 min, a volume of 5 mL of the supernatant was mixed with an equal volume of water and 1mL of 0.1% ferric chloride. The absorbance was measured at 700 nm in the spectrophotometer after allowing the solution to stand for 30 min.

2.3.3. The 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)

2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) radical (ABTS) scavenging ability of processed Amelonado was determined according to the method described by (Re *et al*., 2000). The ABTS was generated by reacting 7 mM ABTS aqueous solution with $K_2S_2O_8$ (2.45 mM final concentration) in the dark for 16 hours and adjusting the absorbance at 734 nm to 0.700 with ethanol. Thereafter, 200 mL of fermented and unfermented Amelonado were added to 2.0 mL ABTS solution and the absorbance was measured at 734 nm after 15 minutes using a spectrophotometer. The Trolox equivalent antioxidant capacity (TEAC) was subsequently calculated using Trolox as the standard.

The procedure was also done for Amelonado fermented for 24, 72, and 120 h, respectively, which was subsequently compared with the result of the unfermented Amelonado.

2.3.4. Total phenol content

The total phenol content was determined, appropriate aqueous extracts of processed Amelonado were oxidized with 2.5 mL 10% Folin-Ciocalteau's reagent (v/v) and neutralized by 2.0 mL of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45 ^{o}C and the absorbance was measured at 765 nm in the UV spectrophotometer.

The procedure was also done for Amelonado fermented for 24, 72, and 120 h respectively which was subsequently compared with the result of the unfermented Amelonado.

2.3.5. Total flavonoid content

The total flavonoid content of the processed Amelonado was determined, 0.1 mL of the appropriate volume of the sample/standard quercetin was mixed with 0.5 mL methanol, 50 µL of 10% Aluminium Chloride (Al_2C1_3), 50 µL of 1 mol/L potassium acetate, and 1.4 mL water. The reaction mixture was incubated at room temperature for 30 min. Thereafter, the absorbance of the reaction mixture was measured at 415 nm in the spectrophotometer.

The procedure was also done for Amelonado fermented for 24, 72, and 120 h respectively which was subsequently compared with the result of the unfermented Amelonado.

2.4. Statistical Analysis

The statistical analysis was used to calculate the standard deviation and one-way ANOVA using GraphPad prism 9. Furthermore, the means were compared to check for the statistical difference using Tukey's multiple comparison tests. The significant level was established at $P<0.05$.

3. Results and discussion

Cocoa has long been known to have substantial antioxidant properties and the potential to protect against a variety of ailments. Cocoa flavonoids and phenols have also been shown to reduce blood pressure, enhance blood flow to the brain and heart, prevent blood clots, and fight cell damage. Since cocoa is a plant that goes through a variety of processing conditions before being consumed, including fermentation, it's critical to look into the impact of these procedures on antioxidant activity, as well as phenolic and flavonoid content.

The study employed different assay techniques to determine the effect of fermentation on cocoa bean seeds which were fermented for 24, 72, and 120 h, respectively. The result of the fermented cocoa was compared with a result from unfermented cocoa which was also subjected to the same assay technique as shown and discussed below.

3.1. The DPPH radical scavenging ability of fermented and unfermented amelonado cocoa beans extract determined by DPPH assay.

The DPPH radical scavenging ability of aqueous extract of fermented and unfermented amelonado is presented in Figure 2.

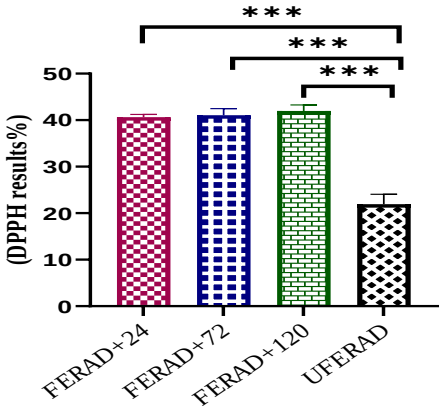

Figure 2. The DPPH radical scavenging ability of fermented and unfermented amelonado cocoa beans extract. Values are presented in mean ± standard deviation at $P<0.05$. FERAD: fermented amelonado; UFERAD; unfermented amelonad. ***: significant difference in mean compared at $P<0.05$.

DPPH is a stable nitrogen-centered free radical which can be used to evaluate the antioxidant activity of natural products by measuring the radical quenching capacity in a relatively short time (Prathapan *et al*., 2011). The use of DPPH radical scavenging activity provided an easy and rapid inference to evaluate antioxidant activity (Silva *et al*., 2005).

The DPPH radical scavenging ability of aqueous extract of fermented and unfermented Amelonado presented in Figure 2 shows that there was an increase in scavenging potential of fermented amelonado cocoa compared to the unfermented amelonado, this means that fermentation of amelonado increases its potential to scavenge for free radicals produced from DPPH. Fig 2 shows the entrapment of the DPPH radical was 40.63 ± 0.59%, 41.07 ± 1.41 %, and 41.96 ± 1.30% after fermentation time of 24, 72, 120 h, respectively, compared to the unfermented amelonado with its entrapment ability at 21.95 ± 2.12%. The result shows that there was a significant difference ($P<0.05$) between the fermented amelonado and unfermented amelonado. Therefore, it can be concluded that an increase in fermentation time can be proportional to an increase in the DPPH free radical scavenging ability of cocoa beans.

3.2. The ferric reducing antioxidant properties (FRAP) of fermented and unfermented amelonado cocoa beans extract.

The ferric reducing antioxidant properties of fermented and unfermented Amelonado beans are shown in Figure 3.

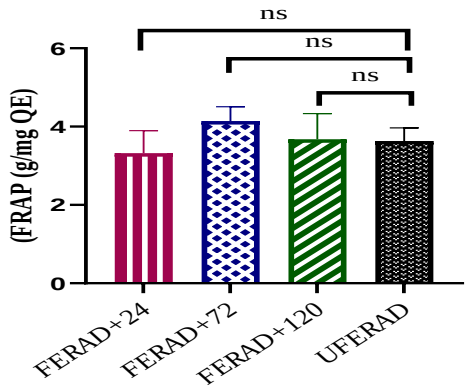

Figure 3. The ferric reducing antioxidant properties (FRAP) of fermented and unfermented amelonado cocoa beans extract. Values are presented in mean ± standard deviation at P<0.05. FERAD: fermented amelonado; UFERAD; unfermented amelonad. ns: no significant difference in mean compared.

The ferric reducing antioxidant properties (FRAP) assay is done to check the prepared cocoa beans' ability to reduce Fe^{3+} to Fe^{2+}(Gülçin *et al*., 2011).

Statistical analysis shows that there is no significant difference ($p < 0.05$) between the fermented amelonado (FERAD+24 = 3.32 ± 0.57, FERAD+72 = 4.14 ± 0.03, FERAD+120 = 3.68 ± 0.65) and the unfermented amelonado (UNFERAD = 3.63 ± 0.33).

This means that fermentation does not affect the ferric reducing antioxidant properties of cocoa.

3.3. The ABTS radical scavenging ability of fermented and unfermented amelonado cocoa beans.

The ABTS radical scavenging ability on fermented and unfermented amelonado cocoa is displayed in Figure 4.

ABTS assay also known as Trolox equivalent antioxidant capacity (TEAC) is used to measure the relative ability of antioxidants of samples to scavenge the ABTS generated in the aqueous phase. ABTS is also frequently used by the food industry and agricultural researchers to measure the antioxidant capacities of samples (Awika *et al*., 2003).

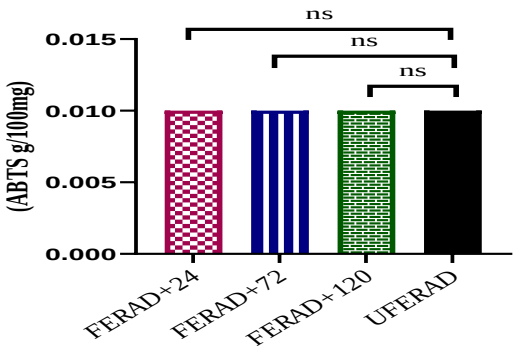

Figure 4. The ABTS radical scavenging ability of Fermented and unfermented amelonado cocoa beans. Values are presented in mean ± standard deviation at P<0.05. FERAD: fermented amelonado; UFERAD; unfermented amelonad. ns: no significant difference in mean compared.

The statistical analysis resulted that all samples have a standard error of zero, which can also predict that there is no significant difference between the fermented and unfermented amelonado ABTS scavenging ability. This signifies that fermentation does not affect the ABTS scavenging ability of cocoa.

3.4. Yields of total phenolic contents of fermented and unfermented amelonado

Figure 5 shows the total phenol contents of the fermented and unfermented amelonado cocoa extract.

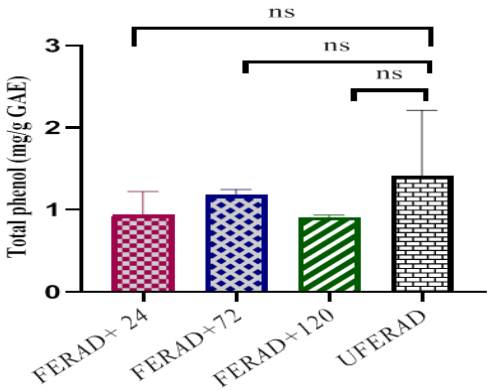

Figure 5. Yields of total phenolic contents of fermented and unfermented amelonado. Values are presented in mean ± standard deviation at P<0.05. FERAD: fermented amelonado; UFERAD; unfermented amelonad. **ns=** no significant difference in mean compared.

Phenolic compounds are ubiquitous secondary metabolites in plants (Fereidon and Ambigaipalan, 2015). They are known to play important roles which

include antioxidant scavenging and prevention of diseases.

Figure 5 shows the effect of fermentation on the phenolic content of fermented and unfermented Amelonado seed.

Statistical analysis showed that there is no significant difference (P<0.05) between fermented amelonado (for 24, 72 and 120 h) and the unfermented amelonado (UNFERAD = 1.42 ± 0.79).

This signifies that fermentation does not affect the total phenol content of cocoa.

3.5. Yields of total flavonoid contents of fermented and unfermented amelonado

Figure 6 shows the total flavonoid content found among cocoa beans subjected to different fermentation times.

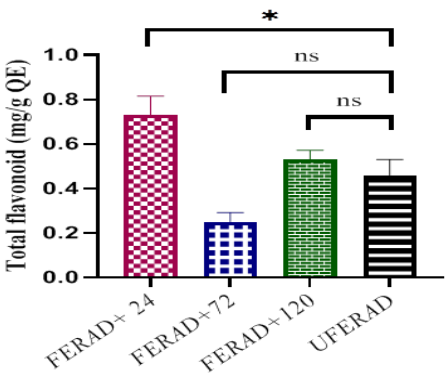

Figure 6. Yields of total flavonoid content of fermented and unfermented amelonado. Values are presented in mean ± standard deviation at P<0.05. FERAD: fermented amelonado; UFERAD; unfermented amelonad. ns: no significant difference in mean compared. ***** : significant difference in mean compared.

Flavonoids are plant secondary metabolites widely distributed in the plant kingdom which has been reported to show antioxidant activities. Flavonoids have a considerable effect on human nutrition and health (de Lira Mota *et al.*, 2009). The effect of fermentation on the flavonoid content of Amelonado seed was evaluated as shown in Figure 6. The statistical analysis showed that there was a significant difference (P<0.05) between the flavonoid content of amelonado fermented for 24 h (0.73 ± 0.08) compared to unfermented amelonado (0.46 ± 0.07). This implies that there was higher flavonoid content in amelonado that was fermented for 24 h compared to unfermented amelonado. However, the analysis also revealed that there was no significant difference (P<0.05) between amelonado fermented for 72 h (0.25 ± 0.04) as well as 120 h (0.53 ± 0.04) in comparison to unfermented amelonado. This signifies that fermentation for 24 hours causes an upregulation in the flavonoid content of amelonado. It also signifies that fermentation did not affect the flavonoid content of amelonado fermented for 72 hours and 120 hours respectively.

4. Conclusion

After the whole analysis, it can be concluded that an increase in fermentation time can be proportional to increase DPPH free radical scavenging ability of cocoa. Fermentation has no effect on ferric reducing antioxidant properties, ABTS radical scavenging ability and total phenol content of cocoa beans. Fermentation also causes an upregulation in the flavonoid content of cocoa beans fermented for 24 h and has no effect on the flavonoid content of amelonado fermented for 72 and 120 h.

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