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Comparative Effects of Ripeness on Monoamine Oxidase (MAO) Inhibition and Anti-oxidative Activity of Amelonado and F3 Amazon Cocoa (*Theobroma Cacao*) Beans *In vitro*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Background: The oxidative deamination of amines and neurotransmitters as typified by the action of monoamine oxidase (MAO) is known to be involved in psychological disorders, oxidative stress, and many other adverse pharmacological reactions. A new series of studies have been geared towards identifying natural inhibitors of monoamine oxidase that also possess strong antioxidant activity. Some psychological disorders have been identified as direct implications of the breakdown of neurotraansmitters by monoamine oxidases. The breakdown, which is biochemically an oxidative deamination, results in the production of reactive species of aldehyde and oxygen. An overexpression or increased activity of monomine oxidase gives rise to an increased production of free radicals. A relatively higher production of free radicals depletes the natural antioxidants in the brain and results in brain disorders. Inhibition of monoamine oxidase has been identified as the major intervention to this pathological process. However, identification of therapeutic interventions that can inibit monoamine oxidase as well as supress the activity of free radicals has been proposed to be a major therapeutic improvement. Cocoa beans elicited significant inhibition against free radical in addition to the substantial antioxidant activity recorded. These findings can be further investigated to identify the major components of cocoa responsible for these molecular interactions.

Keywords: Monoamine oxidase (MAO); Anti-oxidative activity; free radicals; DTTP; ABTS; FRAP.

1. INTRODUCTION

Monoamine oxidases (MAOs) are oxidative mitochondrial flavoenzymes that catalyze the oxidative deamination of catecholamines and serotonin thereby converting them into aldehydes and ketones in a process responsible for cognition [1-3]. During the process of oxidative deamination, they generate H_2O_2, a source of free radical, and reactive species of aldehydes as end [4,5]. Monoamine oxidases are enzymes of the mitochondria and are of two variants with the same function; A and B [5]. The isoforms have different locations, structural conformations, inhibitor awareness, and enzyme-substrate chemistry [6]. The catabolic activity of monoamine oxidase on monoamine neurotransmitters whose oxidative deamination yields H_2O_2 reportedly represents a distinctive source of free radicals in the brain [7].

Cocoa is rich in phenolic antioxidants, surpassing many other foods. The main antioxidants are flavonoids like catechin, epicatechin, and procyanidins [8]. These flavonoids, with their tricyclic structure, have antioxidant properties that combat reactive oxygen species by neutralizing free radicals to prevent them from causing harm, bind to Fe2+ and Cu+, hinder enzymes, and enhance antioxidant defenses [9]. Reactive oxygen species are molecules that are highly reactive and contains oxygen, they are a normal byproduct of oxygen metabolism in the cells but at high levels can be harmful. ROS have several detrimental effects such as DNA damage, protein damage and lipid peroxidation and can also trigger inflammation and these damages have been associated with the development of a number of diseases such as cancer, Alzheimer's disease, Parkinson's disease, and age-related macular degeneration [10,11].

Following global consensus, Oxidative Stress is viewed as a disproportion between the levels of oxidants and antioxidants in a system significantly favouring the oxidants, resulting in interference of redox signaling and cellular homeostasis [12-16]. Overproduction of reactive oxygen species and depletion of molecular antioxidant defense mechanism activates proinflammatory signaling pathway which is capable of damaging crucial biomolecules such as lipids, carbohydrates, nucleic acids, and proteins triggering cell death [17,18]. A lot of studies have identified the involvement of oxidative stress in the development and progression of several chronic illnesses that require immediate and prolonged therapeutic intervention [19].

Inhibition of monoamine oxidase elevates the concentration of the monoamine neurotransmitters present in synapses and monoamine oxidase inhibitors can be employed as therapeutic interventions in pathological situations where the levels of monoamine oxidase are abnormally high. Compounds that inhibit monoamine oxidase terminate the production of neurotoxic free radicals and reactive aldehydes by halting the process of

monoamine deamination and thus preventing oxidative damage to the nerves [20].

Monoamine oxidase inhibitors are widely available and employed clinically as a therapeutic intervention for various neurodegenerative diseases. However, these drugs exhibit various side effects or adverse drug reactions. Therefore, it is necessary to continue the search for new drugs with little or no side effects, to this end, compounds obtained from plants have become crucial [21,22].

Different functional studies have identified cocoa as a prolific source of several biologically active metabolites, majorly, the polyphenols, whose mechanisms of beneficial action have been suggested [23,8,24]. Various phytochemical constituents of the seed have been profiled, with polyphenols being the most numerous groups of compounds [25]. Interestingly, the seed has been associated with numerous biological and therapeutic properties such as anti-oxidative activity, antiproliferative, antiapoptotic, anti-inflammatory, and anti-tumorigenic activity. Furthermore, the therapeutic effect of cocoa in different pathological conditions has been investigated [25].

Herein, two species of cocoa (amelonado and F3 amazon) were analyzed to determine the monoamine oxidase inhibitory property and antioxidant potential of cocoa beans using different *in -vitro* and statistical analyses.

2. APPROACH

2.1 Sample Preparation

Ripe and unripe cocoa (*Theobroma cacao*) pods of two species (Amelonado and F3 Amazon) of Cocoa were obtained from the Cocoa Research Institute of Nigeria (CRIN) Owenna, Ondo road, Ondo State. Analyses were carried out at Functional Foods and Nutraceutical unit (FFNU) laboratory, FUTA (Federal University of Technology) Akure. The cocoa pods were broken and the seeds were extracted and sorted out, the seeds' outer coated layer were peeled and then oven-dried at room temperature (25 ^{o}C). Extracts were collected for both ripe and unripe cocoa species and were tested for monoamine oxidase inhibition and antioxidant activity.

2.2 Aqueous Extract Preparation

Aqueous extract preparation was carried according to Shodehinde and Oboh (2012) with slight modification. Ten gram of each cocoa beans sample was soaked in 100 mL of distilled water for about 24 hours. The mixture filtered and the filtrate was centrifuged for 10 minutes to obtain a clear supernatant liquid. The extract was stored at 4 ^{0}C and used for further analysis. The antioxidant tests and analyses were performed in triplicate and results were averaged.

2.3 Brain Homogenate Preparation

Brain homogenate of cluster rat was prepared according to Thermofisher by weighing 2ml of the brain tissue in a microcentrifuge tube followed by the addition of 500 µL of Cell Lysis Buffer (EPX-99999-000) per 100 mg of tissue and the addition of one 5-mm Stainless Steel Bead, then the tubes were assembled into TissueLyser Homogenize tissue at 25 Hz for 0.5-3 mins which was then centrifuged at 16,000 × g for 10 mins at 4°C.The supernatant was then transferred into a new microcentrifuge tube for futher analysis.

2.4 Total Phenol Content

The total phenol content was determined using Singleton method. A significant mass of the blended dry sample was reacted with 2.5 mL 10 % Folin-Ciocalteau's reagent (v/v) and further by 2.0 mL of 7.5 % sodium carbonate. The mixture was conditioned for 40 minutes at 45 ^{o}C and the absorbance was read at 765 nm in the spectrophotometer [26].

2.5 Total Flavonoid Content

The total flavonoid contents of the ripe and unripe cocoa beans samples were determined using a moderately adjusted method described by Meda *et al.* [27] 0.1 mL of a significant volume of the standard quercetin was diluted with 0.5mL alcohol (methanol), 50 μl of 10% Aluminium Chloride ($AlC1_3$), 50 μL of 1mol/L potassium acetate, and 1.4 mL H_2O. The mixture was conditioned to incubate at 25C for 30 min. Subsequently, the absorbance of the was measured at 415 nm in the spectrophotometer.

2.6 The 2,2-Diphenyl-2-Picrylhydrazyl (DPPH Free Radical Scavenging Ability)

The ability of the samples to scavenge DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical was evaluated, with slight adjustments. 1 mL of 0.4 mM methanolic solution of DPPH ((1,1-diphenyl-

2-picrylhydrazyl) radicals was transferred to a reaction medium (test tube), thereafter, 0.05 mL of test extracts/standard quercetin was added. The mixture was placed in a dark environment for half an hour and the absorbance was measured at 516 nm in the spectrophotometer. The ability of the samples to scavenge DPPH free radicals is then calculated [28].

Percentage inhibition = (Absorbance of control – Absorbance of test) / Absorbance of control × 100

2.7 The Ferric Reducing Antioxidant Property (FRAP)

The $FeCl_3$ solution reducing activity of the cocoa beans extracts was estimated as previously described by Oyaizu [29]. 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 subsequently centrifuged at 805 g for 10 minutes. A volume of 5 mL of the supernatant was mixed with a proportionate volume of water and 1mL of 0.1% ferric chloride. The mixture was conditioned and the reaction was allowed for 30 minutes and the absorbance was subsequently read at 700 nm.

2.8 ABTS (2, 2-azino-bis (3-Ethylbenzothiazoline-6-SulfonicAcid)

In this study, the 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) radical (ABTS) scavenging ability of the samples was estimated using the method described by Re *et al.*, [30]. The ABTS radical was produced by reacting 7 mM ABTS aqueous solution with 2.45mM of Potassium persulfate and stored in a dark environment for 16 hours and subsequently calibrating the absorbance at 734 nm to 0.700 with ethanol. After which, 200 mL of the diluted sample was added to 2.0 mL ABTS solution and the absorbance was measured at 734 nm after 15 minutes.

2.9 Monoamine Oxidases (MAO)

Monoamine oxidase activity was determined using a slightly adjusted method recently described [31]. In a test tube, 0.025 mol/L phosphate buffer (pH 7.0), 0.0125 mol/L semicarbazide, and 10 mmol/L benzylamine were mixed with 100 µl of the brain homogenate. The reaction mixture was incubated for 30 minutes after which acetic acid was added. The mixture was further incubated for 3 minutes at 100 ^{0}C and subsequently centrifuged. 1mL of the supernatant was mixed with a proportionate volume of 2, 4-Dinitrophenylhydrazine, and 1.25 mL of benzene was added after a 10 minutes incubation at 25C. The benzene layer was separated and the reaction mixture was mixed with a proportionate volume of 0.1 N NaOH. The layer was decanted and incubated at 80 °C for 10 minutes. The absorbance of the resulting solution was read at 450 nm in a UV/visible spectrophotometer.

2.10 Lipid Peroxidation (LPO)

The 300 mL of a prepared brain homogenate (rat) was added to 300 mL of 8.1 % sodium dodecyl sulfate (SDS), 500 mL of acetic acid/HCl buffer (pH 3.4), and 500 ml of 0.8 % thiobarbituric acid (TBA). This mixture was incubated at 100°C for 1 hr, subsequently, the absorbance was read at 586 nm (Esterbauer *et al.*, 1991).

2.11 Data Visualization and Analysis

The raw data were computed into graphpad prism 9 for visualization and analysis. Obtained in duplicates, the data are presented as mean ± SD. One-way analysis of variance was employed to analyze the variation in the means and Tukey's multiple comparison test was employed as the post hoc test to compare the ripe and unripe group of both species for statistical difference ($p < 0.05$).

3. RESULTS

3.1 Total Phenol Content

The total phenol content found in both ripe and unripe samples of the two cocoa beans species analyzed (Amelonado and F3 amazon) is presented in Fig. 1. The total phenolic contents of ripe amelonado and ripe F3 amazon cocoa beans were 1.42 ± 0.14 mg/g and 0.267 ± 0.06 mg/g respectively, while unripe Amelonado and unripe F3 amazon cocoa beans were 2.70 ± 0.04mg/g and 2.13 ± 0.10mg/g. The result showed that unripe amelonado had the highest total phenol values ($p < 0.05$) while ripe amazon had the least. Comparatively, the beans of Amelonado species were found to have higher phenolic content than the F3 mazon species.

3.2 Total Flavonoid Content

Fig. 2 shows the total flavonoid content evaluated in both ripe and unripe samples of the two cocoa beans species. The total flavonoid contents of ripe Amelonado and ripe F3 amazon were 0.46 ± 0.04 mg/g and 0.72 ± 0.03 mg/g respectively while the unripe samples of amelonado and unripe F3 amazon were 1.04±0.03mg/g and 1.19 ± 0.01mg/g. The unripe samples of both species had higher flavonoid content than the ripe samples.

3.2.1 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging ability

The radical scavenging ability of the seeds of ripe and unripe samples of amelonado and F3 amazon cocoa beans are presented in Fig. 3. The entrapment of the DPPH radical potentials in the ripe samples (49.33 ± 2.40 % and 42.49 ± 1.02 %) of amelonado and F3 amazon respectively were significantly higher ($p < 0.05$) than the unripe samples (28.57 ± 1.20 % and 21.73 ± 2.19 %) of amelonado and F3 amazon respectively.

3.3 Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing antioxidant power (FRAP) of cocoa beans is given in Fig. 4. The result showed a significant difference ($p < 0.05$) in the values that obtained for the cocoa beans samples. The unripe samples of amelonado and F3 amazon had higher ferric reducing antioxidant power than the ripe samples.

3.3.1 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)

The ABTS scavenging ability of ripe and unripe amelonado and F3 amazon is presented in Fig. 5. There was no significant difference ($p< 0.05$) in the ABTS radical scavenging potential obtained in all the cocoa beans samples.

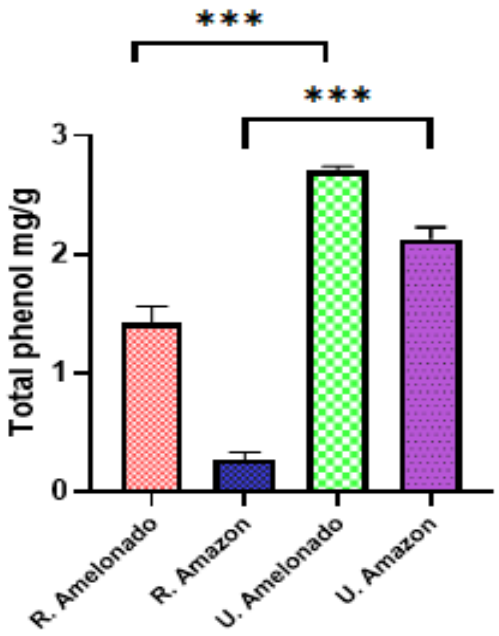

Fig. 1. Total phenol content of ripe and unripe samples of amelonado and F3 amazon species of cocoa beans

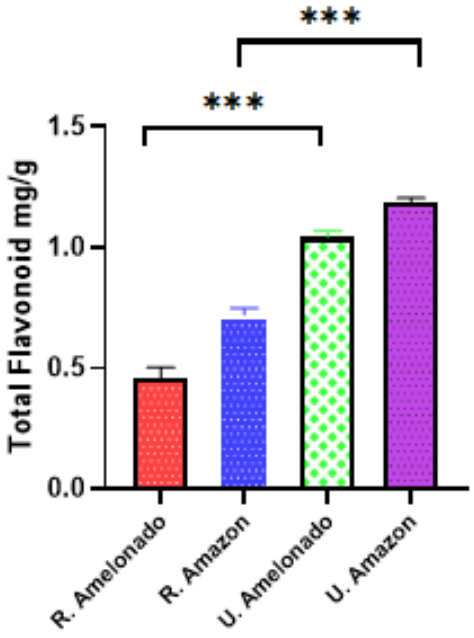

Fig. 2. Total flavonoid content of ripe and unripe samples of amelonado and F3 amazon species of cocoa beans

R – Ripe U – Unripe
Values for the groups are presented in (mean±SD)
Compared groups with (***) are statistically different at p<0.05
Compared groups with (ns) are not statistically different

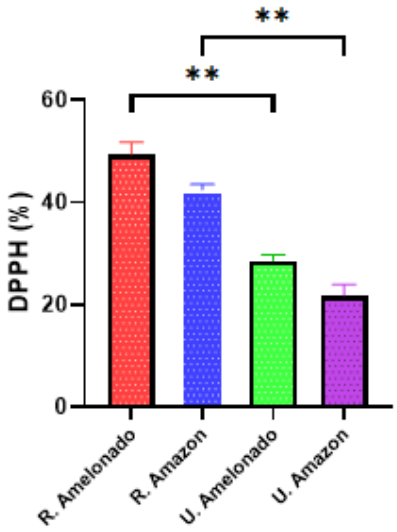

Fig. 3. DPPH radical scavenging ability of ripe and unripe samples of Amelonado and Amazon species of Cocoa

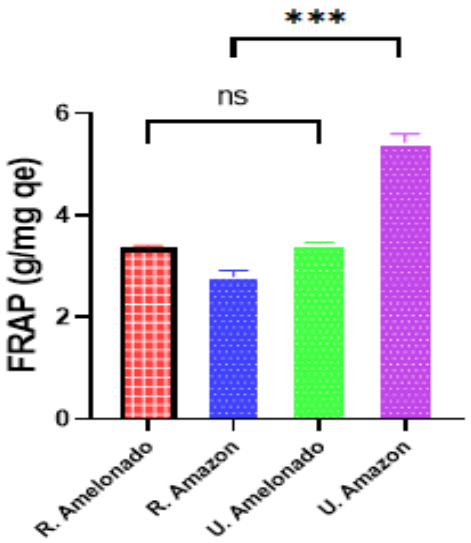

Fig. 4. Ferric reducing antioxidant power of ripe and unripe samples of amelonado and amazon species of cocoa

R – Ripe U – Unripe
Values for the groups are presented in (mean±SD)
Compared groups with (***) are statistically different at p<0.05
Compared groups with (ns) are not statistically different

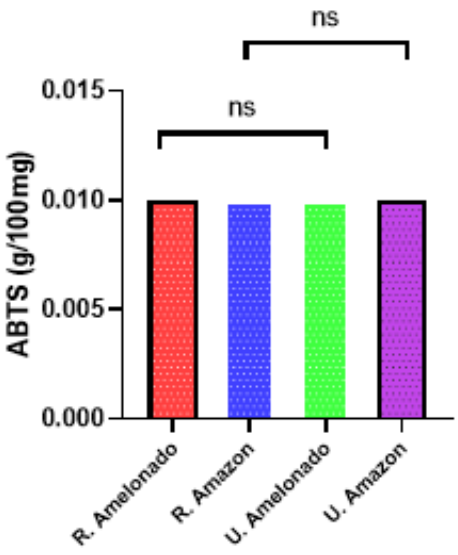

Fig. 5. ABTS radical scavenging ability of ripe and unripe samples of amelonado and amazon species of cocoa

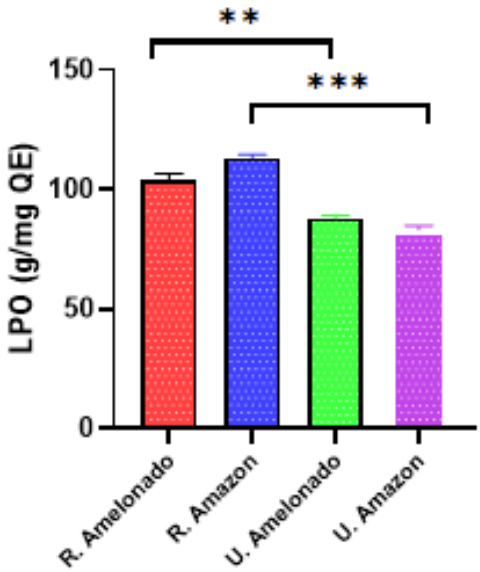

Fig. 6. LPO

R – Ripe U – Unripe
Values for the groups are presented in (mean±SD)
Compared groups with (***) are statistically different at p<0.05
Compared groups with (ns) are not statistically different

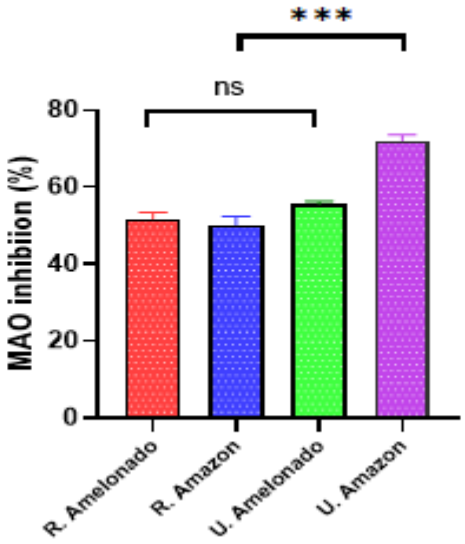

Fig. 7. MAO inhibition of ripe and unripe samples of amelonado and amazon species of cocoa

R – Ripe U – Unripe
Values for the groups are presented in (mean±SD)
Compared groups with (***) are statistically different at p<0.05
Compared groups with (ns) are not statistically different

3.4 Lipid Perodixation

The occurrence of lipid peroxidation with the samples *in situ* is presented in Fig. 6. The observed lipid peroxidation values in the unripe cocoa beans samples of both species (amelonado and F3 amazon) at 87.76 ± 1.50 g/mg and 83.27 ± 1.71 g/mg respectively were lower than the values evaluated in ripe cocoa beans sample (104.08 ± 2.55 g/mg and 113.06 ± 1.68 g/mg respectively).

3.5 Monoamine Oxidase Inhibition

The inhibitory potentials of the samples against monoamine oxidase are shown in Fig. 7. There was significant difference ($p < 0.05$) in the evaluated inhibitory potentials of ripe amelonado and ripe F3 amazon at 51.76 ± 1.78 % and 50.16 ± 2.23 % respectively, while unripe amelonado and unripe F3 amazon values were 55.56 ± 0.82 % and 72.09 ± 1.499 % respectively. The unripe samples (Amelonado and F3 amazon) were found to have higher MAO inhibitory potentials than the ripe samples.

4. DISCUSSION

Neurodegenerative diseases like Alzheimer's disease, depression, and Parkinson disease conventionally incorporate an accumulation of some polypeptides that are toxic to the nerves and are related to the production of reactive oxygen species and subsequently oxidative stress [32]. In some cases, the pathophysiology of these diseases does not only involve oxidative stress but also a disruption in the activity of monoamine oxidase (MAO) [33]. The breakdown of neurotransmitters by monoamine oxidase produces reactive and toxic species of compounds which are the predominant source and cause of oxidative stress in the brain [7].

The inhibition of monoamine oxidase is a conventional target for the development of antidepressants and has been proposed to be linked to a corresponding neuroprotective property [34,35]. A study on the inhibition of monoamine oxidase showed that inhibition of MAO substantially increased the concentration of neurotransmitters which have been suggested to be insufficient in executing synaptic signal processing in depression [36,37]. It was also noted that inhibiting monoamine oxidase may result in some neuroprotective effects [37]. In the light of this, drugs that are efficient in inhibiting monoamine oxidase activity and have strong antioxidant power have been reviewed for these pathologies [38,39]. At the same time, the inhibition of monoamine oxidase may elicit a

corresponding antioxidant activity since a substantial amount of the reactive neurotoxic molecules are produced by monoamine oxidase [40-41]. Cocoa seeds are highly rich in biologically active compounds and they are in high demand in food and drug research due to the high concentration of these compounds [42].

The inhibitory potentials of the samples against monoamine oxidase are shown in Fig. 7. Ripening had no significant effect on the monoamine oxidase inhibitory property of Amelonado species of Cocoa, however, the monoamine oxidase inhibitory property of the ripe seed samples of Amazon species (50.16 ± 2.23%) was significantly lower than the unripe samples (72.09 ± 1.499%). Therefore ripening significantly reduced the monoamine oxidase inhibitory properties of F3 Amazon species of Cocoa.

The result of the *in vitro* analyses carried out on both ripe and unripe cocoa beans samples showed that ripening significantly ($p<0.05$) reduced the flavonoid contents of the cocoa species. It is worthy to note that the cocoa beans of F3 amazon species in this present study had higher flavonoid content than the amelonado species. Different studies have shown that, in addition to their contribution to antioxidant prowess, flavonoids have also shown inhibitory properties against monoamine oxidase. A recent study showed that flavonoids from *H. afrum* and *C. villosus* exhibited Inhibitory potentials against human Monoamine oxidase A and B. The study noted that the monoamine oxidase inhibitory activity of the plants was to a large extent due to the significant concentration of flavonoids [43]. In another study, *H. perforatum* and *P. harmala* were analzyed to determine the monoamine oxidase inhibitory property. The study highlighted that the inhibitors of monoamine oxidase-A in *H. perforatum* are majorly flavonoids [38,39]. Our present finding therefore shows that the potential of unripe cocoa beans of the samples were significantly higher ($p < 0.05$) in inhibiting monoamine oxidase than the ripe cocoa beans.

The analysis of total phenol content showed that ripening reduced the total phenol in cocoa beans as the concentration of total phenol in unripe amelonado was significantly higher ($p < 0.05$) than the value obtained in the corresponding ripe cocoa beans. A similar trend was observed in F3 amazon. Our present finding supports the earlier researched work in which correlation was identified between phenolic components and anti-oxidant activity [44,45].

The analysis of lipid peroxidation inhibition with the cocoa beans samples *in situ* showed that the unripe samples had higher inhibition of lipid peroxidation than the ripe samples. This finding is an indication that unripe samples will be able to prevent cellular injury caused by reactive species better than the ripe samples. This further attests to our prediction that ripening reduces the antioxidant activity of Cocoa.

The ferric reducing ability analysis showed that there was no significant difference between the ferric reducing power of both ripe (3.36 ± 0.04 g/mg) and unripe (3.44 ± 0.03 g/mg) samples of Amelonado species of Cocoa. Ripening does not affect the ferric reducing antioxidant potential of this species. However, ripening the radical scavenging ability analysis showed no significant difference between and among the samples. Ripening has no significant effect on the radical scavenging radical. On the other divide, a different trend was observed for the radical scavenging activity. Ripening was found to increase the radical scavenging activity of both species of cocoa beans samples analyzed.

The results of this present study falls in line with the earlier and current research findings. However a recent study by Kyriacou et al. [46]. demonstrating the effect of ripening on the phenolics content of Ceratonia siliqua L showed that ripening substantially reduced the total phenolics of the plant. Similarly, another study carried out on the degree of antioxidant activity of different samples of olive oil also showed that the polyphenolics and anti-oxidant activity substantially reduced during ripening [47-48].

5. CONCLUSION

The results of this study evidently indicated that ripening changed the biological activity of cocoa beans with respect to antioxidant activity and monoamine oxidase inhibition. Taking an in-depth look at the unripe cocoa beans samples, a significantly higher ($p < 0.05$) change in activity was observed for F3 amazon species during ripening. Of all samples analyzed, unripe amazon seeds had the highest antioxidant activity and possesses the best inhibitory property against monoamine oxidase. Unripe Amelonado possesses significant ($p < 0.05$) inhibitory activity against monoamine oxidase and may be processed and used for therapeutic

intervention against some neurodegenerative diseases, hence, drug discovery efforts should be focused here. However, characterization and further analyses are recommended to identify the major classes of compounds responsible for the inhibitory molecular interaction.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Authors hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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