

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

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 MAO that also possess strong antioxidant activity. Herein, the effect of ripening was documented on the MAO inhibitory potential and antioxidant power of two cocoa species (amelonado and F3 amazon). The pods of two cocoa were collected, broken to reveal coated beans. The cocoa beans were evaluated for the total phenol, total flavonoid, DPPH (2,2-diphenyl-2-picrylhydrazyl), FRAP (Ferric reducing antioxidant property), ABTS (2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), lipid peroxidation, and inhibition of monoamine oxidase (MAO) activities. The results showed that ripening changed the biochemical activities of both species. Ripening reduced the antioxidant power of both species and the monoamine inhibitory potential of the amazon species.

Practical Application

Some psychological disorders have been identified as direct implications of the breakdown of neurotransmitters by monoamine oxidases (MAO). 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 MAO 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 MAO has been identified as the major intervention to this pathological process. However, identification of therapeutic interventions that can inhibit MAO as well as suppress 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.

Introduction

Monoamine oxidases (MAOs) are oxidative mitochondrial flavoenzymes that catalyze the oxidative deamination of catecholamines and serotonin thereby converting them into alkanals and alkanones in a process responsible for cognition (Aljanabi *et al.*, 2021; Tripathi & Mazumder, 2021; Maggiorani *et al.*, 2017). During the process of oxidative deamination, they generate H₂O₂, a free radical, and reactive species of alkanals as end products (Umbarkar *et al.*, 2015; Datta & Bhattacharjee, 2020). Monoamine oxidases are enzymes of the mitochondria and are of two variants with the same function; A and B (Datta & Bhattacharjee, 2020). The isoforms have different locations, structural conformations, inhibitor awareness, and enzyme-substrate chemistry (Duim *et al.*, 2015). The catabolic activity of MAO on monoamine neurotransmitters whose oxidative deamination yields H₂O₂ reportedly represents a distinctive source of free radicals in the brain (Soto-Otero *et al.*, 2001).

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 (Sies, 2020). 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 (Bhatt *et al.*, 2020; Cakmak & Gulcin 2019). 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 (García-Sánchez *et al.*, 2020).

Inhibition of MAO elevates the concentration of the monoamine neurotransmitters present in synapses and MAO inhibitors can be employed as therapeutic interventions in pathological situations where the levels of Monoamine oxidase are abnormally high. Compounds that inhibit MAO 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 (Kristal, 2001).

MAO 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. (Tripathi *et al.*, 2018; Mazumder *et al.*, 2020)

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 (Quiñones *et al.*, 2011, Lee *et al.*, 2003, Gültekin-Özgülven *et al.*, 2016). Various phytochemical constituents of the seed have been profiled, with polyphenols being the most numerous groups of compounds (Cinar *et al.*, 2021). 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 (Cinar *et al.*, 2021).

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

Methods

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 °C).

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 °C and used for further analysis. The antioxidant tests and analyses were performed in triplicate and results were averaged.

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 $16,000 \times g$ for 10 mins at 4°C . The supernatant was then transferred into a new microcentrifuge tube for further analysis.

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-Ciocalteu's reagent (v/v) and further by 2.0 mL of 7.5 % sodium carbonate. The mixture was conditioned for 40 minutes at 45°C and the absorbance was read at 765 nm in the spectrophotometer (Singleton *et al.*, 1999).

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.* (2005). 0.1 mL of a significant volume of the standard quercetin was diluted with 0.5 mL alcohol (methanol), $50\mu\text{L}$ of 10% Aluminium Chloride (AlCl_3), $50\mu\text{L}$ of 1 mol/L potassium acetate, and 1.4 mL H_2O . The mixture was conditioned to incubate at 25°C for 30 min. Subsequently, the absorbance of the was measured at 415 nm in the spectrophotometer.

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 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 (Gyamfi *et al.*, 1999).

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

Absorbance of control

The Ferric Reducing Antioxidant Property (FRAP)

The FeCl₃ solution reducing activity of the cocoa beans extracts was estimated as previously described by Oyaizu (1986). 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.

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

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.*, 2000). 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.

Monoamine Oxidases (MAO)

MAO activity was determined using a slightly adjusted method recently described (Green and Haughton, 1961). 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 °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.

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).

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 \pm 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 ($p < 0.05$).

UNDER PEER REVIEW

Results

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

Figure 2 shows the Total flavonoid content evaluated in both ripe and unripe samples of the two cocoa beans species. The total flavonoid contents of R. Amelonado and R. 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 ± 0.03 mg/g and 1.19 ± 0.01 mg/g. The unripe samples of both species had higher flavonoid content than the ripe samples.

The DPPH radical scavenging ability of the seeds of ripe and unripe samples of amelonado and F3 amazon cocoa beans are presented in figure 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.

The ferric reducing antioxidant power (FRAP) of cocoa beans is given in Figure 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.

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

The occurrence of lipid peroxidation with the samples *in situ* is presented in figure 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).

The inhibitory potentials of the samples against monoamine oxidase are shown in Figure 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.

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 (Li *et al.*, 2013). 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) (Carraro Junior *et al.*, 2021). The breakdown of neurotransmitters by MAO produces reactive and toxic species of compounds which are the predominant source and cause of oxidative stress in the brain (Soto-Otero *et al.*, 2001).

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 (Baker *et al.*, 2019; Youdim *et al.*, 2006). A study on the inhibition of MAO 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 (Blier 2016; Matveychuk *et al.*, 2021). It was also noted that inhibiting monoamine oxidase may result in some neuroprotective effects (Matveychuk *et al.*, 2021). In the light of this, drugs that are efficient in inhibiting MAO activity and have strong antioxidant power have been reviewed for these pathologies (Gulcan & Orhan, 2020). At the same time, the inhibition of MAO may elicit a corresponding antioxidant activity since a substantial amount of the reactive neurotoxic molecules are produced by MAO (Herraiz *et al.*, 2018). 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 (Kongor *et al.*, 2016).

The inhibitory potentials of the samples against monoamine oxidase are shown in figure 7. Ripening had no significant effect on the MAO inhibitory property of Amelonado species of Cocoa, however, the MAO 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 MAO 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 MAO. A recent study showed that flavonoids from *H. afrum* and *C. villosus* exhibited Inhibitory potentials against human MAO A and B. The study noted that the MAO inhibitory activity of the plants was to a large extent due to the significant concentration of flavonoids (Larit *et al.*, 2018). In another study, *H. perforatum* and *P. harmala* were analyzed to determine the MAO inhibitory property. The study highlighted that the inhibitors of MAO-A in *H. perforatum* are majorly flavonoids (Herraiz & Guillén, 2018). Our present finding therefore shows that the potential of unripe cocoa beans of the samples were significantly higher ($p < 0.05$) in inhibiting MAO 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 (Cook & Samman, 1996; (Ge *et al.*, 2021).

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 FRAP 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 ABTS radical scavenging ability analysis showed no significant difference between and among the samples. Ripening has no significant effect on the ABTS radical scavenging radical. On the other divide, a different trend was observed for the DPPH radical scavenging activity. Ripening was found to increase the DPPH 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.* 2021 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 (Wang *et al.*, 2021).

Conclusion

The results of this study evidently indicated that ripening changed the biological activity of cocoa beans with respect to antioxidant activity and MAO 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 MAO. Unripe Amelonado possesses significant ($p < 0.05$) inhibitory activity against MAO and may be processed and used for therapeutic intervention against some neurodegenerative diseases. However, characterization and further analyses are recommended to identify the major classes of compounds responsible for the inhibitory molecular interaction.

Declaration

Ethics approval and consent to participate

Not Applicable

Consent for Publication

Not Applicable

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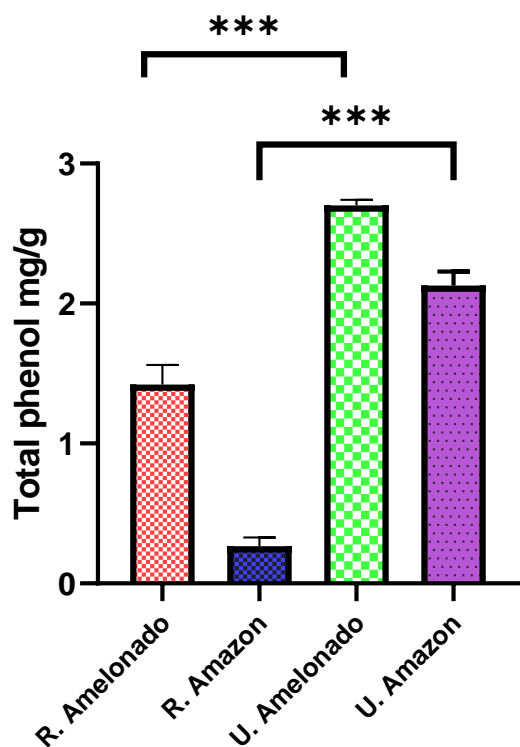


Figure 1: Total phenol content of ripe and unripe samples of amelonado and F3 amazon species of cocoa beans

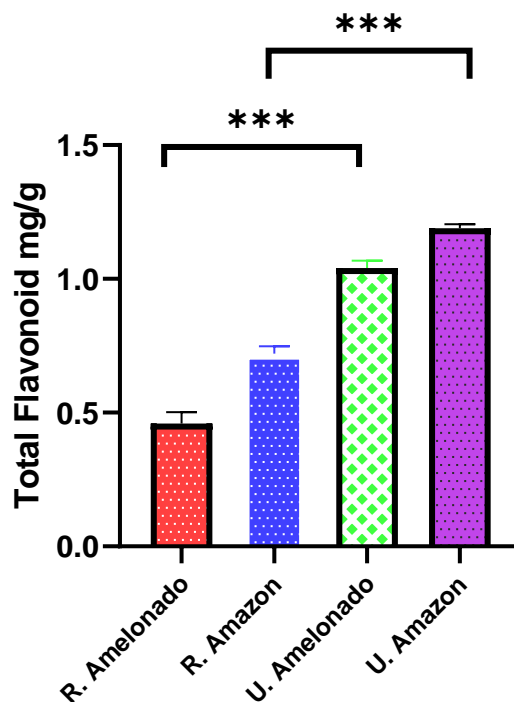


Figure 2: Total flavonoid content of ripe and unripe samples of amelonado and F3 amazon species of cocoa beans

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

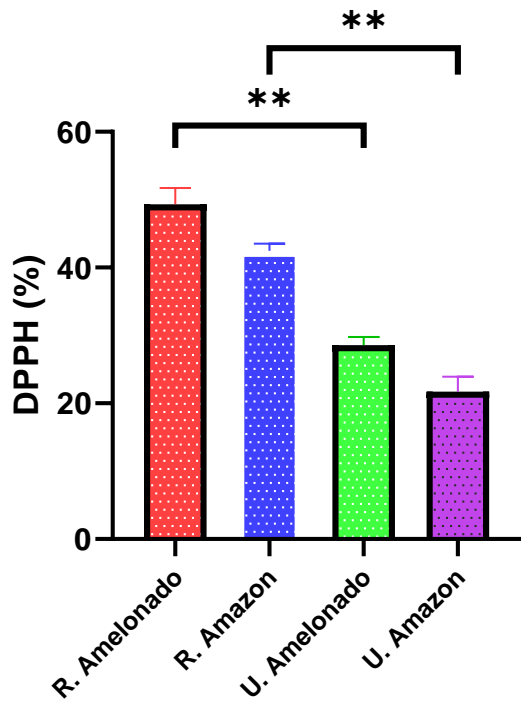


Figure 3: DPPH radical scavenging ability of ripe and unripe samples of Amelonado and Amazon species of Cocoa

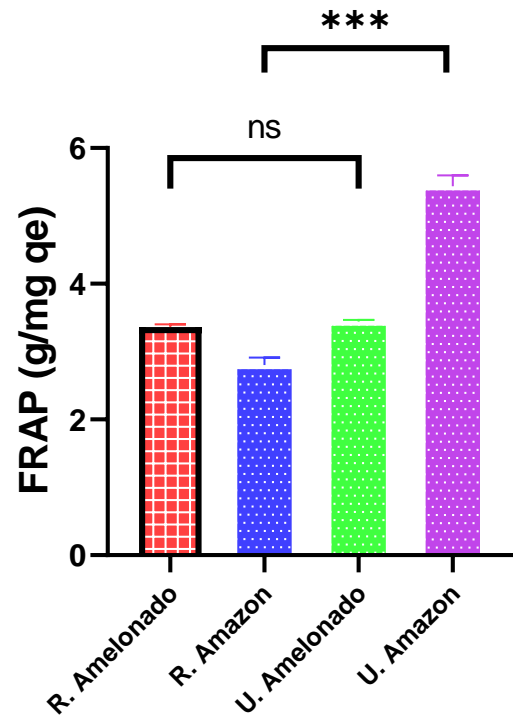


Figure 4: Ferric Reducing Antioxidant power of ripe and unripe samples of Amelonado and Amazon species of Cocoa

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

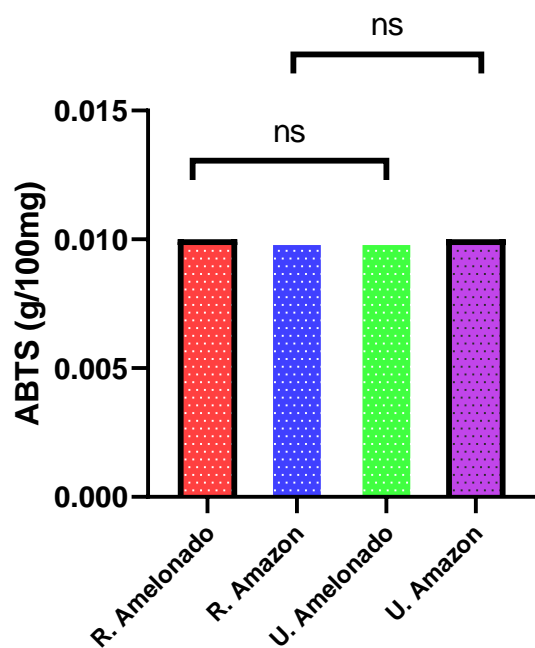


Figure 5: ABTS radical scavenging ability of ripe and unripe samples of Amelonado and Amazon species of Cocoa

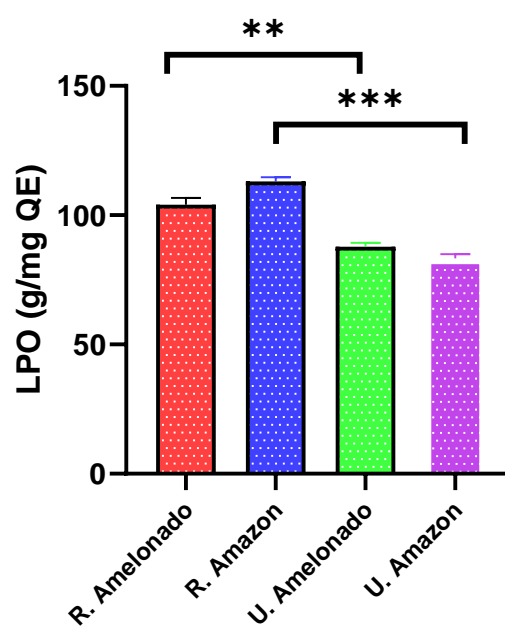


Figure 6: LPO

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

UNDER REVIEW

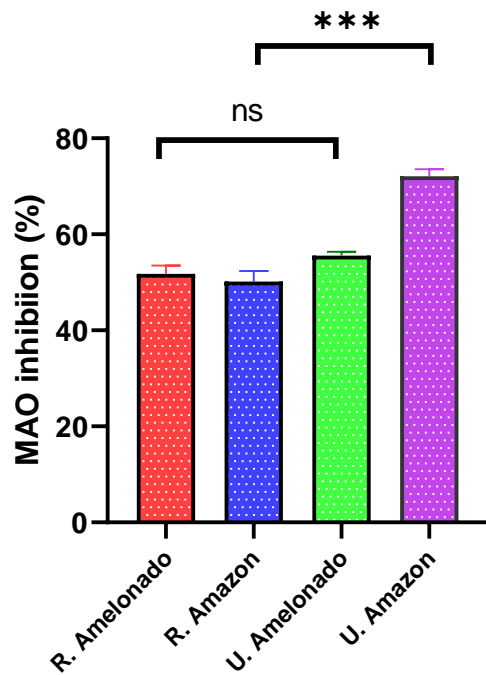


Figure 7: MAO INHIBITION of ripe and unripe samples of Amelonado and Amazon species of Cocoa

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