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Effect of Ripening on Cholinergic Enzymes and Antioxidant Potentials of Pepper Fruit (*Dennettia tripetala*)

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ABSTRACT

This study was designed to determine the effect of ripening of pepper fruit on some key enzymes linked with neurodegenerative diseases and their antioxidant potentials. Fresh matured ripe and unripe pepper fruits were collected. The effects of ripening on key enzymes [monoamine oxidase (MAO), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)] linked with neurodegenerative diseases, their phytochemicals (total phenol and total flavonoid) contents and antioxidant [Ferric reducing antioxidant properties (FRAP), Fe^{2+} chelating ability, and 2,2- diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability] of the pepper fruit extracts were assessed. The results revealed that there is no significant difference in monoamine oxidase and butyrylcholinesterase inhibition of both ripe and unripe pepper fruit extracts, whereas there is a significant difference in acetylcholinesterase inhibition of ripe and unripe pepper fruit extracts. The total phenol (20.45 mg/g) contents of ripe pepper fruit was significantly higher ($P < 0.05$) than that of unripe pepper fruit (16.75 mg/g), while there was no significant difference in the total flavonoid content. Also, unripe pepper fruit extracts had higher antioxidant activities, as observed from the results of DPPH*, and Fe^{2+} chelating ability. The inhibition of cholinesterase and monoamine oxidase, as well as antioxidant properties of the ripe and unripe pepper fruit could make them good dietary means for the management of neurodegenerative disorders. However, there was not much difference between the enzymes (monoamine and cholinesterases) inhibition and antioxidant potentials of ripe and unripe pepper fruits.

Keywords: Ripening, Pepper fruit, *Dennettia tripetala*, neurodegenerative, antioxidant

INTRODUCTION

Medicinal plants have been in use since time immemorial as remedies for memory loss and aging. Sometimes, these plants are used by man as food or in form of medicine to modify the functioning of the central nervous system (CNS) (Elufioye *et al.*, 2012). Neurodegenerative disorders like Alzheimer's disease (AD), Parkinson disease (PD), depression, schizophrenia and others are associated with impairments in learning and memory (Jewart *et al.*, 2005; Adewusi *et al.*, 2010). Reduction in cognitive and mental functions ailment commonly known as Alzheimer's disease (AD) are related with the loss of cortical cholinergic neurotransmission (Lai *et al.*, 2013). The most widely accepted biochemical theory of neurodegenerative diseases is the cholinergic hypothesis (Lai *et al.*, 2013). Acetylcholinesterase (AChE), butyrylcholinesterase (BChE), monoamine oxidase (MAO), and oxidative stress have been identified as the therapeutic targets in the management of neurodegenerative conditions (Youdim *et al.*, 2006). The effect of action of

MAO inhibitors in the management of depression and other neurodegenerative conditions such as Alzheimer's disease (AD) and Parkinson's disease (PD) have been established (Riederer *et al.*, 1989). Excessive MAO activity has been linked to increased generation of free radicals in the brain and consequently neuronal damage (Thomas, 2000).

Dennettia tripetala G. Baker (Annonaceae), Pepper fruits is a woody aromatic tree with simple leaves and abundant fruits widely found in the tropical rainforest and sometimes in Savana region of Nigeria (Okwu *et al.*, 2005). It is locally called *ako* in Edo, *nkarika* in Ibibio, *opipi* in Idoma, *mmimi* in Igbo and *ata igbere* in Yoruba language.

Fruit ripening is a very complex developmental process by which a plant organ suffers profound physiological, structural and biochemical transformations (Alos *et al.*, 2019). The mature fruits of *Dennettia tripetala* constitute the main edible portions; its fruits appear red when ripe (Figure 1a) and green when unripe (Figure 1b). It has a peppery spicy taste which usually serves as a mild stimulant to the consumer (Keay, 1989; Ndukwu and Nwadibia, 2006; Oyemitan *et al.*, 2006). In folk medicine, leaves and root of *Dennettia tripetala* in combination with other medicinal plants are used to treat various ailment including fever, typhoid, worm infestation, infantile convulsion, vomiting and stomach upset (Oyemitan *et al*., 2008). Its leaves are also used in pepper soup delicacies and as condiment in some local dishes for pregnant and postnatal women to aid uterine contraction (Okwu and Morah, 2004; Achinewhu *et al*., 1995).

Figure 1a Ripe Pepper fruits

Figure 1b Unripe Pepper fruits

The physiological changes that accomplish ripening which brings about changes in pigments have been reported to impart significantly on the quantity of total phenol contents of *Dennettia tripetala* (Adedayo *et al*. (2010). Hence, this study aims to determine the effects of ripening on the inhibitory abilities of anticholineterases, monoamine oxidase and antioxidant properties of Pepper fruit (*Dennettia tripetala*).

MATERIALS AND METHODS

Chemicals

Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Semicarbazide and Benzylamine were obtained from Sigma-Aldrich (St. Louis, MO USA), 2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 1, 1-diphenyly-2-picrylhydrazyl (DPPH) and 1, 10- phenanthroline were obtained from Fluka chemie, GmbH, Hannover Germany. Acetic acid was procured from BDH Chemical Ltd., (Poole, England). Except otherwise stated, all other chemicals were

obtained from standard chemical suppliers and were of analytical grade.

Sample treatment and preparation

Matured ripe and unripe pepper fruits were sorted and washed with distilled water and chopped into small pieces with the aid of a table knife and air dried at room temperature. The dried samples were blended into coarse powder and defatted using n-Hexane. The residues were dried and used for the extraction of the samples. 40 g of the defatted pepper fruits were soaked in 200 mL in 70% methanol and filtered through Whatman No 1 filter paper. The filtrate was then evaporated to dryness and kept in a refrigerator at about 4ºC for further use.

Enzyme assays

Monoamine oxidase (MAO) Assay.

The effect of the pepper fruit extracts on MAO activity was carried out according to Green and Haughton, (1961) with slight modification. In brief, the reaction mixture contained 25 mM phosphate buffer, 12.5 mM semicarbazide, 10 mM benzylamine (pH adjusted to 7.0), tissue homogenate and appropriate dilutions of extract in a total reaction volume of 2.0 mL. After 30 min, 1 mL of acetic acid was added and boiled for 3 min in boiling water bath. From the resultant solution, 1.0 mL was mixed with equal volume of 0.05% of 2, 4-DNPH and 2.5 mL of benzene was added after 10 min incubation at room temperature. After separating the benzene layer it was mixed with equal volume of 0.1 N NaOH. Alkaline layer was decanted and heated at 80°C for 10 min. The orange-yellow colour developed was measured at 450 nm against blank.

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) assay

The ability of pepper fruit extracts to inhibition of acetylcholinesterase (AChE) was assessed by a modified colorimetric method (Perry *et al*. 2000). The AChE activity was determined in a reaction mixture containing 200 µL of AChE solution (EC 3.1.1.7) in 0.1 M phosphate buffer pH 8.0, 100 µL of a solution of 3.3 mM 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB), Extracts (0 –100 µl) and 500 µL of phosphate buffer, pH 8.0. After incubation for 20 min at 25ºC, acetylthiocholine iodide (100 µL of 0.05 mM solution) was added as the substrate, and AChE activity monitored at 412 nm for 3.0 min at 25ºC. Likewise, 100 µL of butyrylthiocholine iodide was used as a substrate to assay for BChE enzyme, while all the other reagents and conditions were the same. The AChE and BChE inhibitory activity was expressed as percentage inhibition activity.

DPPH free radical scavenging ability

The free radical scavenging ability of the extracts against DPPH (1,1-diphenyl–2 picrylhydrazyl) free radical was evaluated as described by Gyamfi *et al.* (1999). Briefly, an appropriate dilution of the ripe and unripe pepper fruit extracts (1.0 mL) were mixed with 1.0 mL of 0.4 mmol L^{-1} methanolic solution containing DPPH radicals. The mixture was left in the dark for 30 min and the absorbance was measured at 516 nm. The DPPH free radical scavenging ability was subsequently calculated with respect to the reference (which contains all the reagents without the test sample).

Fe^{2+} chelation assay

The Fe^{2+} chelating ability of the ripe and unripe pepper fruit extracts were determined using a modified method of (Minotti and Aust, 1987) with a slight modification by (Puntel *et al.,* 2005). Freshly prepared 500 µmol L^{-1} $FeSO_4$ (150 µL) was added to a reaction mixture containing 168 µL of 0.1 mol L^{-1} Tris-HCl (pH 7.4), 218 µL saline and the extracts (0 – 100 µL). The reaction mixture was made up to 987 µL with distilled water and incubated for 5 min, before the addition of 13 µL of 0.25% 1; 10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in a spectrophotometer. The Fe^{2+} chelating ability was subsequently calculated.

Determination of total phenol content

The total phenol content was determined on the ripe and unripe pepper fruit extracts using the method reported by Singleton *et al*. (1999). Appropriate dilution of the extracts were oxidized with 2.5 mL of 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°C and the absorbance was measured at 765 nm. The total phenol content was subsequently calculated using Gallic acid as standard.

Determination of total flavonoid content

The total flavonoid content of the extracts was determined using a slightly modified method reported by Meda *et al.* (2005). Briefly, 0.5 ml of appropriately diluted sample was mixed with 0.5 mL methanol, 50 µL of 10% $AlCl_3$, 50 µL of 1.0 mol L^{-1} potassium acetate and 1.4 mL water, and allowed to incubate at room temperature for 30 min. Thereafter, the absorbance of the reaction mixture was subsequently measured at 415 nm. The total flavonoid was calculated using quercetin as standard.

Determination of ferric reducing antioxidant power (FRAP) assay

The reducing property of the pepper fruit extracts was determined by assessing the ability of the extract to reduce $FeCl_3$ solution as described by Oyaizu (1986). A 2.5 ml aliquot was mixed with 2.5 mL of 200 mmol L^{-1} sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50ºC for 20 min and then 2.5 ml of 10% trichloroacetic acid was added. This mixture was centrifuged at 650 rpm for 10 min. 5 mL of the supernatant was mixed with an equal volume of water and 1 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm and ferric reducing antioxidant property was subsequently calculated using ascorbic acid as standard.

Data Analysis

The results of replicate readings were pooled and expressed as means ± standard deviation. Significance was accepted at $P<0.05$. The IC_{50} (extract concentration causing 50% inhibition in the activities of enzyme) value was calculated using nonlinear regression analysis.

RESULTS AND DISCUSSION

The results of the IC_{50} of acetylcholinesterase, butyrylcholinesterase and monoamine oxidase inhibitory activities of the ripe and unripe extracts of *Dennettia tripetala* (pepper fruits) are presented in the Table 1. The ability of the *Dennettia tripetala* extracts to inhibit monoamine oxidase (MAO) activity *in vitro* is shown in Figure 2; the result revealed that both ripe and unripe extracts were able to inhibit monaoamine oxidase activity in a dose-dependent manner. However, the ripe *Dennettia tripetala* (IC_{50} = 0.316 mg/ml) had higher monoamine oxidase inhibitory activity, while the unripe *Dennettia tripetala* (IC_{50} = 0.333 mg/ml) had the lower inhibitory ability. MAO activity has been shown to result in higher free radicals generation (Baker *et al*., 2012) and their inhibitors have been linked to reduction in the production of reactive oxygen species in previous studies (Thomas, 2000). Therefore, the ability of both ripe and unripe pepper fruits to inhibit MAO could be of great important in the prevention or management of neurodegenerative disorders.

Table 1. IC_{50} values of DPPH radical scavenging ability, Fe^{2+} chelating ability, AChE, BChE and MAO inhibitory activities of the unripe and ripe pepper fruits extracts

IC_{50} (mg/mL)	DPPH	Fe^{2+}	AChE	BChE	MAO
Unripe	2.875 ± 0.063^{a}	0.388 ± 0.008^{a}	0.056 ± 0.002^{a}	0.054 ± 0.002^{a}	0.333 ± 0.013^{a}
Ripe	3.088 ± 0.067^{a}	0.395 ± 0.009^{a}	0.072 ± 0.003^{b}	0.057 ± 0.004^{a}	0.316 ± 0.014^{a}

Values represent mean ± standard deviation of replicate readings.
Values with the same superscript letter along the same column are not significantly different (p<0.05).

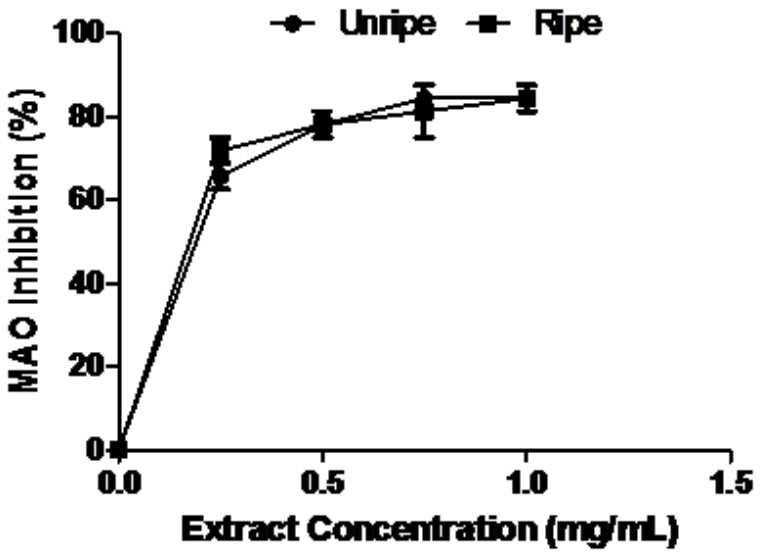

Figure 2. Monoamine oxidase inhibitory activity of ripe and unripe pepper fruit extract.

The abilities of the ripe and unripe pepper fruit extracts to inhibit acetylcholinesterase and butyrylcholinesterase activities were investigated and the result is presented in Figure 3 and 4 respectively. The result revealed that both ripe and unripe pepper fruit extracts inhibited acetylcholinesterase in a dose-dependent manner. However, the unripe pepper fruit extracts (IC_{50} = 0.056 mg/ml) had significantly ($p < 0.05$) higher acetylcholinesterase inhibitory activity, while the ripe pepper fruit (IC_{50} = 0.072 mg/ml) had lower inhibitory effect. Also, BChE inhibition is very important in AD management as the expression level of BChE rises with the progression of the degenerative condition (Lane *et al*., 2006; Fernandez-Bachiller *et al*., 2012). The result of this study revealed that both ripe and unripe pepper fruit extracts inhibit butyrylcholinesterase in a dose dependent manner. However, the unripe extract (IC_{50} = 0.054 mg/ml) had higher butyrylcholinesterase inhibitory activity, while the ripe IC_{50} = 0.057 mg/ml) had lower effect.

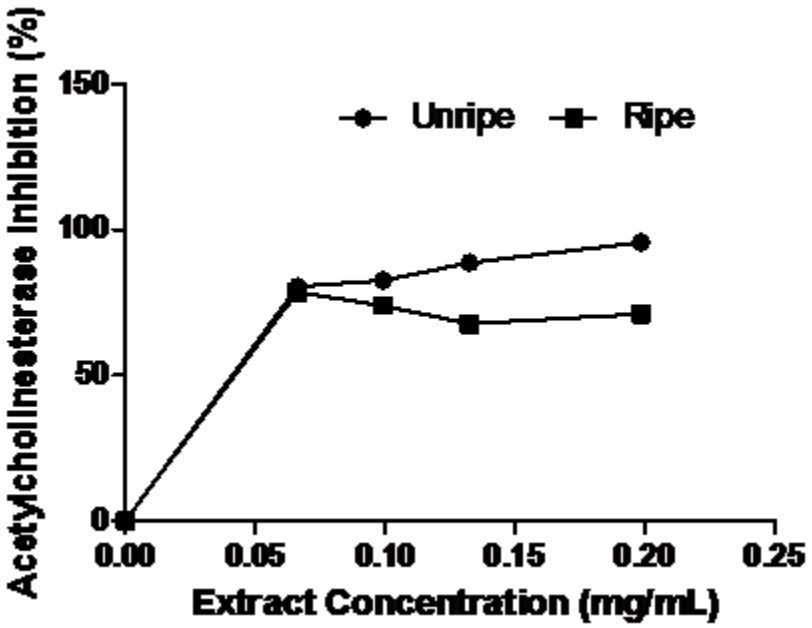

Figure 3. Acetylcholinesterase inhibitory activity of ripe and unripe pepper fruit extract.

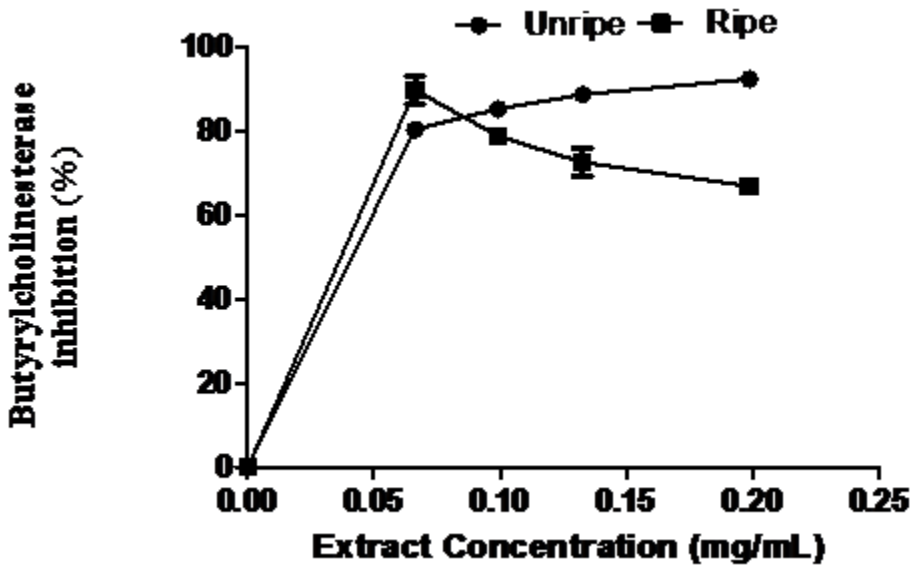

Figure 4. Butyrylcholinesterase inhibitory activity of ripe and unripe pepper fruit extract.

Furthermore, several evidences have shown that oxidative stress is intimately involved in age-related neurodegenerative diseases, many studies have examined the positive benefits of antioxidants to block or reduce neuronal death occurring in the pathophysiology of these disorders (Ramassamy, 2006). The ability of both ripe and unripe *Dennettia tripetala* to scavenge

DPPH free radical is presented in Figure 5 and their IC_{50} in Table 2. The results revealed that the scavenging abilities of both extracts against DPPH radical were concentration-dependent. However, the result revealed that unripe pepper fruit had higher free radical scavenging ability than that ripe pepper fruit. This implies that both ripe and unripe *Dennettia tripetala* could be capable of scavenging free radicals which induces oxidative stress and age-related neurodegenerative diseases.

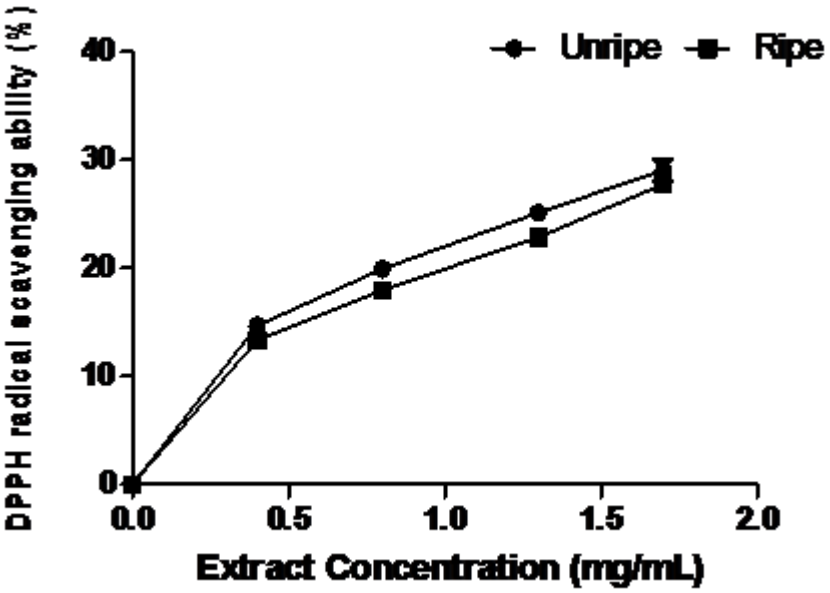

Figure 5. DPPH Radical Scavenging activity of ripe and unripe pepper fruit extract.

Table 2. Total phenol (mg GAE/g) and total flavonoid (mg QE /g) and Ferric reducing antioxidant property **(**FRAP**)** of ripe and unripe pepper fruits

Sample	Total phenol (mg GAE/g)	Total flavonoid (mg QE/g)	FRAP (mgAAE/g)
Unripe	16.75 ± 1.84^{a}	4.08 ± 0.46^{a}	17.66 ± 0.29^{a}
Ripe	20.45 ± 3.50^{b}	3.68 ± 0.11^{a}	16.24 ± 0.29^{a}

Values represent mean ± standard deviation.
Values with the same superscript letter along the same column are not significantly different ($p< 0.05$).

Accumulation of iron has been shown to be very common in the brain of AD patients (Crichton *et al*., 2011), which usually lead to the generation of radicals through the Fenton reaction (Markesbery and Carney, 1999). Synthetic iron chelators have the disadvantage of high toxicity and difficulty crossing the blood brain barrier (Hider *et al*., 1994). However, the extracts of pepper fruits used in this study are rich in phenolic compounds which are iron chelators and can cross the blood brain barrier (Youdim *et al*., 2006; Abd ElMohsen., 2002). The result obtained in this study revealed the ability of ripe and unripe pepper fruit extracts to chelate Fe^{2+} as shown in Figure 6. The result showed that all the extracts were able to chelate Fe^{2+} in a dose dependent manner. Extracts of unripe pepper fruit (IC_{50} = 0.388 mg/ml) had higher Fe^{2+} chelating ability,

while ripe pepper fruit extract (IC_{50} = 0.395 mg/ml) had the lower chelating ability.

Most of the antioxidant potential of medicinal plants is due to the redox properties of phenolic compounds, which enable them to act as reducing agents, hydrogen donors and singlet oxygen scavengers (Hakkim *et al*., 2007). The results of the total phenol and flavonoid contents, and ferric reducing antioxidant properties (FRAP) of the ripe and unripe *Dennettia tripetal*a are presented in Table 2. The result revealed that the ripe *Dennettia tripetala* extract had the highest total phenol content (20.45 mg GAE/g) comparing with the unripe pepper fruit extract that had 16.75 mg GAE/g total phenol content. The high total phenolics content of the ripe pepper fruit extract agreed with earlier report on the phenolics content of ripe and unripe pepper fruits (Adedayo *et al*., 2010). Likewise, unripe had the higher flavonoid content (4.08 mgQE/g) compared to that of the ripe extract that had 3.68 mgQE/g. Flavonoids have also been reported to be responsible for antioxidant activity, since they act on pathways and enzymes involved in anti-inflammatory processes (Araújo *et al*., 2008). Also, the hydrogen-donating substituents (hydroxyl groups) attached to the aromatic ring structures of flavonoids enable them to undergo a redox reaction, which in turn, help them scavenge free radicals (Brand-Williams *et al*., 1995).

The result of ferric reducing ability power of ripe extract (16.24mg/g) is lower than that of unripe extract (17.66mg/g), this result is in agreement with what Adedayo *et al*. (2010) reported for aqueous extracts of pepper fruit.

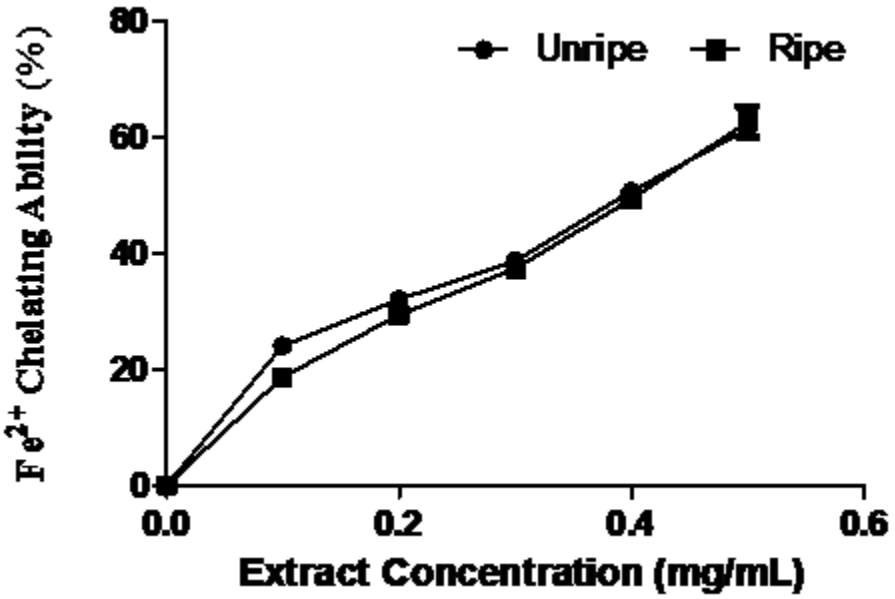

Figure 6. Iron (II) chelating ability of ripe and unripe pepper fruit extract.

CONCLUSION

The results of this study in the long run revealed that ripening of *Dennettia tripetala* had no significance effect on enzymes linked with neurodegenerative diseases, and antioxidant activities of both ripe and unripe pepper fruits. Therefore, consumption of both ripe and unripe pepper fruits could be helpful in the prevention and/or management of neurodegenerative diseases.

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