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NEUROPROTECTIVE POTENTIAL OF PHENOLIC EXTRACTS OF *Capsicum frutescens* AS AN INHIBITOR OF MONOAMINE OXIDASE AND CHOLINESTERASE ACTIVITIES

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ABSTRACT

In this study, we investigated the *in vitro* antioxidant activity of the extracts from ripe and unripe fruits of *Capsicum frutescens*. The effects of the extracts on three enzymes which are associated with Parkinson's disease (PD) were also investigated together with quantitation of total phenol, flavonoid and non-flavonoid contents of the extracts. Activities of mitochondrial monoamine oxidase (MAO), acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) from rat brain were determined in the presence of the extracts. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and OH radicals scavenging abilities, as well as membrane stabilizing effect of the extract via inhibition of Fe^{2+}-induced lipid peroxidation in rat brain tissue homogenate were assessed. The results revealed that the extracts exhibited mitochondrial MAO, AChE and BChE inhibitory effects in concentration-dependent manner, with the unripe fruit extract showing significantly higher ($P<0.05$) inhibitory effects than the ripe fruit extract. However, the ripe fruit extract had significantly higher total phenol and flavonoid contents, as well as exhibited significantly higher ($P<0.05$) DPPH free radical scavenging and membrane stabilizing effects. The observed antioxidant and enzyme inhibitory properties of ripe and unripe pepper (*C. frutescens*) fruits extracts could be an interplay among their constituent polyphenols. This could be a justification for their use as nutraceutical/dietary intervention in the management of neurodegenerative diseases, especially PD. Collectively, these findings suggest that *C. frutescens* could serve as a functional food/nutraceutical in the management of PD and our further studies will be directed towards characterization of the biomolecules involved and their mechanism(s) of action in appropriate models.

Key words: Monoamine oxidase, Cholinesterases, *Capsicum spp*, Neurodegenerative diseases, Functional foods.

INTRODUCTION

Parkinson's disease (PD) ranks the most common neurodegenerative movement disorder in the world (Tamilselvam *et al*., 2013). This pathology is often characterized by a gradual but progressive loss of dopaminergic neurons in the substantia nigra pars compactra (SNpc) as well as aggregation of proteinaceous cytoplasmic inclusions called Lewy bodies (Soldner *et al*., 2009). Several experimental reports have shown that the loss of dopaminergic neurons in PD is as a result of multiple pathological mechanisms including oxidative stress, mitochondria dysfunction, aggregation and accumulation of misfolded or damaged proteins, impaired calcium homeostasis, excitotoxicity, and accumulation of iron (Tamilselvam *et al*., 2013). These impairments result in Parkinson-like symptoms characteristic of PD, which includes bradykinesia, resting tremors, postural instability and muscular rigidity (Moore *et al*., 2005). Risk factors for the development of PD include genetic mutation of several genes, such as parkin (Cookson and Bandman, 2010); inflammation and exposure to various environmental toxicants (Angeline *et al*., 2012). Current therapeutic measures available for the management of PD include the conservation of neuronal dopamine via monoamine oxidase inhibitors, the reduction in PD-induced oxidative stress via antioxidant therapy and symptomatic reduction in PD-associated dementia via the use of cholinesterase inhibitors.

Despite the successes recorded in the development of therapeutic drugs such as monoamine oxidase (MAO) inhibitors for the management of Parkinson's disease (PD), the attendant side effects and huge financial burden they come with has intensified research interest into natural therapeutic measures. In view of this,

plant phenolic extracts have received enormous research attention as one of the most promising phytochemicals with neuroprotective properties. In one study, Sloley *et al.* (2000) reported kaempferol as a MAO inhibitor and potential neuroprotectant in extracts of *Ginkgo biloba* leaves. Also, Akinyemi *et al.* (2016) reported the neuroprotectitve properties of diets supplemented with the phenolic-rich rhizomes of ginger and turmeric in hypertensive rats; while Oboh *et al.* (2016) reported the potential neuroprotective properties of phenolic extract from *Heinsia crinita* leaves as exhibiting antioxidant as well as monoamine oxidase and cholinesterase properties *in vitro*.

Pepper (*Capsicum frutescens*) fruit belongs to the genus Capsicum which includes a varying species of spices known for their nutritional and medicinal properties (Jolayemi and Ojewole, 2013). In folklore medicine, *C. frutescens* fruit, along with other *Capsicum* species have been used in the treatment of different diseases including diarrhoea, asthma, arthritis, muscle cramps and toothache (Jolayemi and Ojewole, 2013). In addition, *C. frutescens* fruits have been reported for their antimicrobial, antioxidant and anti-hyperglycemic properties (Khan *et al.*, 2013). In this study, we investigated the *in vitro* antioxidant properties and inhibitory effect of phenolic extracts from ripe and unripe pepper fruits on some enzymes (mitochondrial monoamine oxidase, acetylcholinesterase and butyrylcholinesterase) that are linked to PD. This is in a bid to evaluating the potentials of *C. frutescens* fruit as a nutraceutical/dietary intervention in the management of neurodegenerative diseases such as PD.

MATERIALS AND METHODS

Materials

Sample Collection and Preparation

The ripe and unripe fruits of pepper (*Capsicum frutescens*), locally referred to as 'ata wewe') were obtained from farm settlements around Iboropa Akoko, Ondo State, Nigeria. Identification was carried out at the Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria. The fruits were washed and thereafter lyophilized. Lyophilized samples were then pulverized and kept for subsequent studies.

Chemicals

Thiobarbituric acid (TBA), 2-deoxyribose, gallic, quercetin, Folin–Ciocalteau's reagent, acetylthiocholine iodide, butyrylcholine iodide, mannitol, benzylamine, semicarbazide, EDTA, sodium dodecyl sulphate (SDS), 1, 1–diphenyl-2-picrylhydrazyl (DPPH), trizma base were procured from Sigma-Aldrich, Inc., (St Louis, MO, USA). Trichloroacetic acid (TCA) and sodium carbonate were sourced from Sigma-Aldrich, Chemie GmbH (Steinheim, Germany). Hydrochloric acid, hydrogen peroxide, benzene, sodium dihydrogen phosphate, disodium hydrogen phosphate, methanol and acetic acid were procured from Scharlab SL, Spain. Aluminum chloride, potassium acetate, iron (II) sulfate, potassium ferricyanide and ferric chloride were sourced from BDH Chemicals Ltd., (Poole, England). Except otherwise stated, water was glass distilled and all other chemicals and reagents were of analytical grade.

Methods

Extraction of Polyphenols

Pepper fruits (ripe and unripe *Capsicum frutescens*) were extracted with 20 volumes of acidified methanol for 48 hours, and thereafter filtered through cheese cloth. The filtrate was reduced under pressure until about 90% of the solvent was evaporated and thereafter lyophilized and stored at -40 °C until further analysis (Oboh and Ogunruku, 2010).

Determination of Inhibitory effect of *C. frutescens* on Mitochondrial Monoamine Oxidase Activity

Preparation of Mitochondria

The rats were immobilized via cervical dislocation and whole brain were quickly excised, placed on ice and weighed. Isolation of brain mitochondria was carried out according to previously reported methods (Satav and Katyare, 1982, Gacche *et al.*, 2011). Whole brain was homogenized in 20 ml of buffer containing 0.3 M mannitol and 0.1 mM EDTA, pH 7.4. The homogenate was centrifuged at 600 x g for 10 min at 4 °C. The supernatant was

collected and centrifuged further at 10,000 x g for 10 min at 4 °C to obtain the brain mitochondria. The mitochondria pellets thus obtained were washed once with 0.25 M sucrose solution containing 0.1 M EDTA, re-suspended in 0.25 M sucrose solution and stored at 4 °C for further studies. Protein concentration was determined based on a modified method of Lowry *et al.* (1951).

Monoamine Oxidase Activity

Monoamine oxidase (MAO) activity of the rat brain mitochondria was determined based on a previously reported method (Green and Haughton, 1961). The reaction mixture contained 25 mM phosphate buffer pH 7, 12.5 mM semicarbazide, 10 mM benzylamine (pH 7), 0.2 mg brain mitochondria isolate and pepper extract (0 – 120 µg/ml) in a total reaction mixture of 1 ml. After 30 min, 1 ml of acetic acid was added. The mixture was boiled for 3 min and centrifuged at 1000 rpm for 5 min. The supernatant (0.75 ml) obtained was mixed with equal volume of 0.05% 2,4–dinitrophenylhydrazine (DNPH) and 2 ml of benzene was added after 10 min incubation at room temperature. The separated benzene layer obtained was thereafter mixed with an equal volume of 0.1M NaOH. The alkaline layer was decanted and heated at 80 °C for 10 min. The orange–yellow colour was measured spectrophotometrically at 450 nm. MAO inhibitory activity of the *C. frutescens* extracts were expressed as percentage inhibition with reference to the control which did not contain the inhibitor.

Determination of Cholinesterases activity *in vitro*

The acetylcholinesterase (AChE) activity was determined according to the method of Ellman *et al.* (1961). The rats were immobilized via cervical dislocation and whole brain were quickly dissected, placed on ice and weighed. The brain was removed, placed on ice and was homogenized in 10 volumes of 0.1 M phosphate buffer, pH 7.0. The homogenate was centrifuged at 4000 rpm for 5 min to yield a low-speed supernatant, which was used for the assay. The reaction mixture contained 200 µl of the low-speed supernatant, 100 µl of a solution of 5, 5-dithiobis-(2-nitrobenzoic) acid (3.3 mM DTNB and 6 mM $NaHCO_3$ in 0.1 M phosphate buffer, pH 7.0), 10 mg/ml pepper extracts (20 - 100µl) and 500µl of 0.1 M phosphate buffer (pH 8.0). After incubation for 20 min at 25 °C, 5 mM substrate (acetylthiocholine iodide/butyrylcholine iodide) was added to initiate the reaction. Enzyme activity was determined by UV-Visible spectrophotometer from the absorbance changes at 412 nm for 3 min at 20 min intervals. The AChE and BuChE inhibitory activities were expressed as percentage inhibition with reference to the control which did not contain the inhibitor.

Lipid Peroxidation assay

Tissue Preparation

The rats were immobilized via cervical dislocation and whole brain were quickly dissected, placed on ice and weighed. The tissue was subsequently homogenized in cold saline (1/10 w/v) with about 10-up-and-down strokes at approximately 1200 rev/min at 4 °C in a Teflon glass homogenizer. The homogenates were centrifuged for 10 min at 1000 x g to yield a low-speed supernatant (S1) fraction that was kept for lipid peroxidation assay (Belle *et al.*, 2004).

Thiobarbituric acid (TBARS) reaction

The lipid peroxidation assay was carried out using the modified method of Ohkawa *et al.* (1979). Briefly 100 µl S1 fraction was mixed with a reaction mixture containing 30 µl of 0.1 M Tris-HCl buffer (pH 7.4), pepper extract (0-100 µl) and 30 µl of 250 µM freshly prepared $FeSO_4$. The volume was made up to 300 µl by water before incubation at 37 °C for 1 hour. The colour reaction was developed by adding 300µl 8.1% SDS (Sodium dodecyl sulphate) to the reaction mixture containing S1, this was subsequently followed by the addition of 500 µl of acetic acid/HCl (pH 3.4) mixture and 500 µl 0.8% TBA (Thiobarbituric acid). This mixture was incubated at 100 °C for 1 hour. TBARS (Thiobarbituric acid reactive species) produced were measured at 532 nm in a spectrophotometer and the absorbance was compared with that of the standard curve prepared using malondialdehyde (MDA).

Determination of DPPH Radical Scavenging Ability

The free radical scavenging ability of the *C. frutescens* extracts against DPPH free radical was evaluated as described by Gyamfi *et al.* (1999).

Briefly, appropriate dilutions of the extracts (1 ml) were mixed with 1 ml 0.4 mM DPPH radicals in methanolic solution. The mixture was left in the dark for 30 min, and the absorbance was taken at 516 nm. The experimental control consisted of 2 ml DPPH solution in the absence of test samples. The DPPH free radical scavenging ability was subsequently expressed as percentage of the control.

Fenton Reaction (Inhibition of Degradation of Deoxyribose)

The ability of the ripe and unripe *C. frutescens* extracts to prevent Fe^{2+}/H_2O_2-induced decomposition of deoxyribose was carried out using the method of Halliwell and Gutteridge (1981). Briefly, appropriate dilution of the extracts was added to a reaction mixture containing 20 mM deoxyribose (120 µl), 0.1 M phosphate buffer (400 µl), 20 mM hydrogen peroxide (40 µl) and 500 µM $FeSO_4$ (40 µl), and the volume was made up to 800 µl with distilled water. The reaction mixture was incubated at 37 °C for 30 min, and the reaction was then stopped by the addition of 0.5 ml of 2.8% trichloroacetic acid (TCA), this was followed by the addition of 0.4 ml of 0.6% thiobarbituric acid (TBA) solution. The tubes were incubated in boiling water for 20 min. The absorbance was read at 532 nm in a spectrophotometer and percentage (%)OH radical scavenging ability was subsequently calculated.

Determination of Total Phenol Content

The total phenol content was determined according to the method of Singleton *et al.* (1999). Briefly, appropriate dilutions of *C. frutescens* extracts were oxidized with 2.5 ml 10% Folin-Ciocalteau's reagent (v/v) and neutralized with 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 in the spectrophotometer. The total phenol content was calculated as gallic acid equivalent.

Determination of Total Favonoid Content

The total flavonoid content of *C. frutescens* extracts was determined using a slightly modified method reported by Meda *et al.* (2005). Briefly, 0.5 ml of appropriately diluted sample extracts were mixed with 0.5 ml methanol, 50 µl 10% $AlCl_3$, 50 µl 1M potassium acetate and 1.4 ml water, and incubated at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm and the total flavonoid content calculated as quercetin equivalent.

Data Analysis

The results of three (3) experiments were pooled and expressed as mean ± standard deviation (SD). Mean values were appropriately analysed and compared using Student's t-test (unpaired) and significance was accepted at $P \leq 0.05$. Also, IC_{50} (effective concentration of extract causing 50% inhibition) values were calculated using nonlinear regression analysis. All statistical analyses were carried out using GraphPad Prism version 5.0 for Windows.

RESULTS

Figure 1 shows the inhibitory effect of the extracts on the activity of monoamine oxidase from the mitochondria of rat brain tissue. As revealed, both extracts were able to inhibit MAO in concentration dependent manner (0 – 120 µg/ml). In table 1 showing the EC_{50} values, unripe extract (52.68±0.04 µg/ml) had a significantly ($P<0.05$) higher inhibitory effect than ripe extract (72.05±0.02 µg/ml). The inhibitory effects of the extracts on acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities are shown in figures 2 and 3. A concentration dependent (20 – 100 µg/ml) inhibition of the cholinesterase activities by the extracts were observed; however, judging by the EC_{50} values, (Table 1), unripe extract had a significantly ($P<0.05$) higher AChE (32.37±0.12 µg/ml) and BChE (63.49±0.12 µg/ml) inhibitory effect than the unripe extract (AChE = 100.50±0.11 µg/ml; BChE = 79.93±0.15 µg/ml).

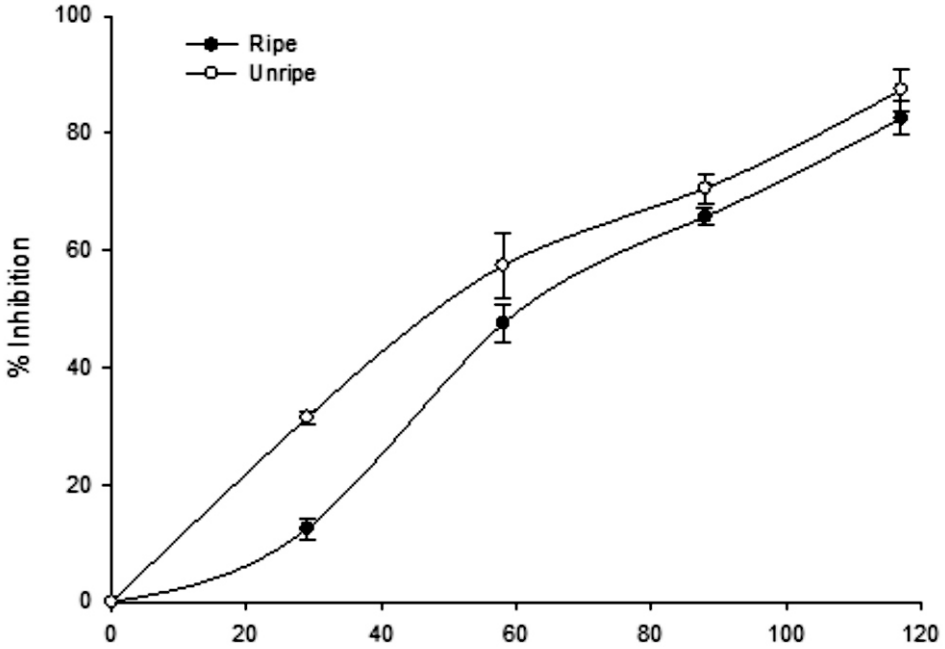

Figure 1: Percentage Inhibition of Brain Mitochondrial Monoamine Oxidase (MAO) Activity as a Function of Concentration of Phenolic Extracts from *Capsicum frutescens*

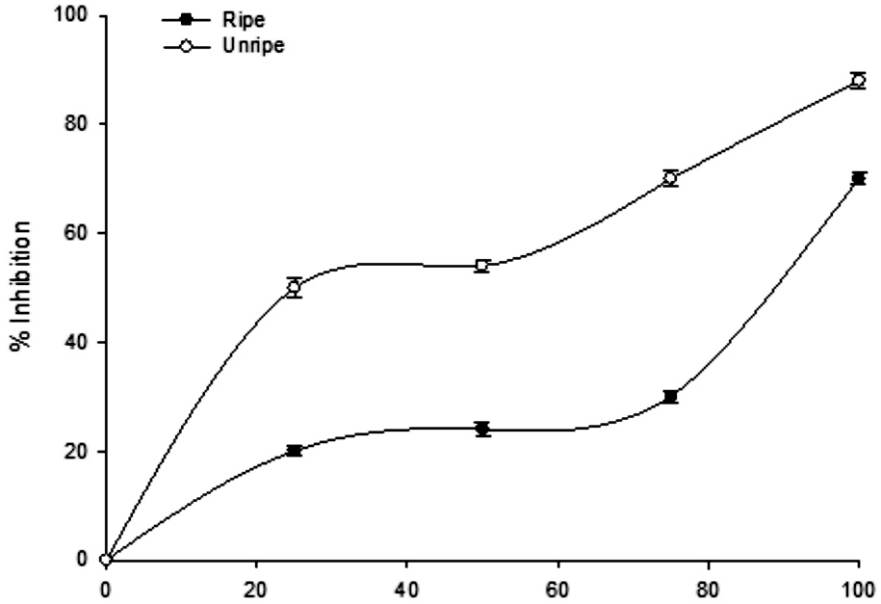

Figure 2a: Percentage Inhibition of Acetylcholinesterase Activity as a Function of Concentration of Phenolic Extracts from *Capsicum frutescens*

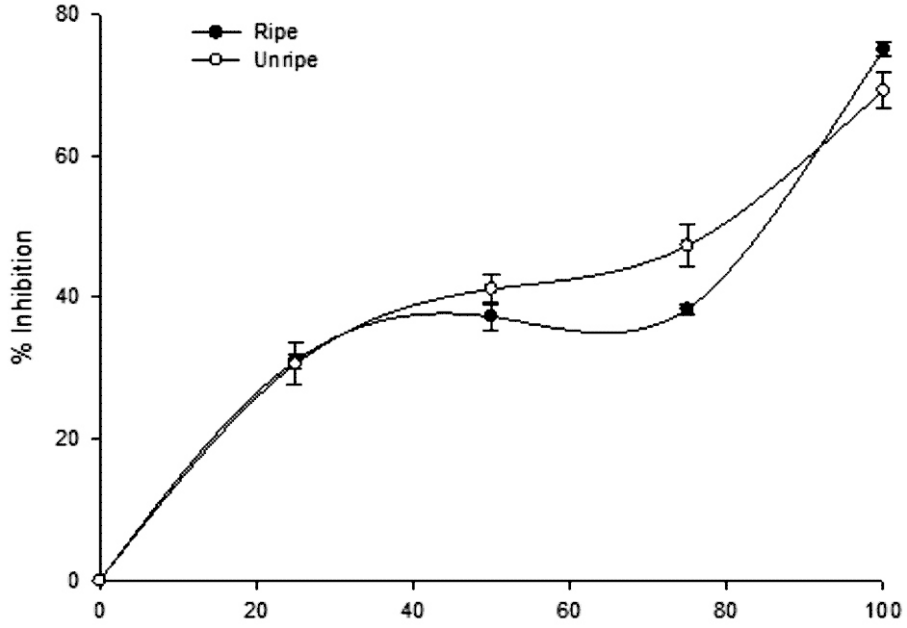

Figure 2b: Percentage Inhibition of Butyrylcholinesterase Activity as a Function of Concentration of Phenolic Extracts from *Capsicum frutescens*

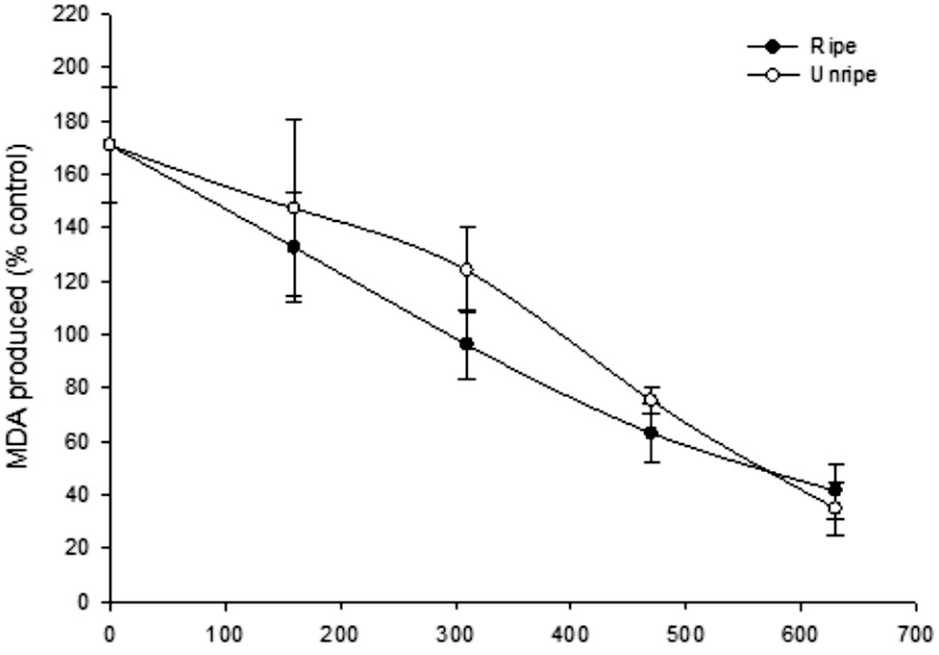

Figure 3: Inhibition of Fe^{2+}-induced Lipid Peroxidation in Rat Brain Tissue by Phenolic Extracts from *Capsicum frutescens*

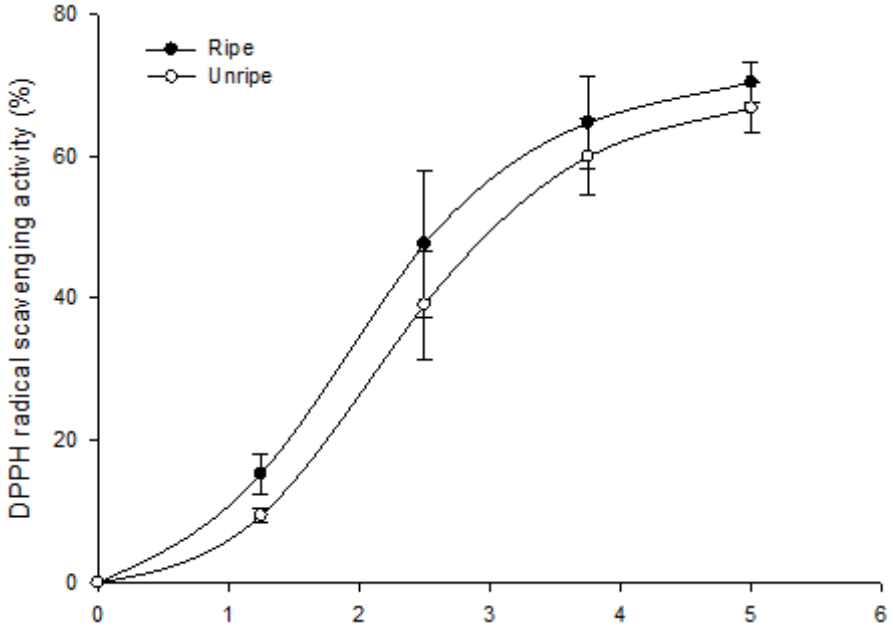

Figure 4: DPPH Radical Scavenging Ability of Phenolic Extracts from *Capsicum frutescens*

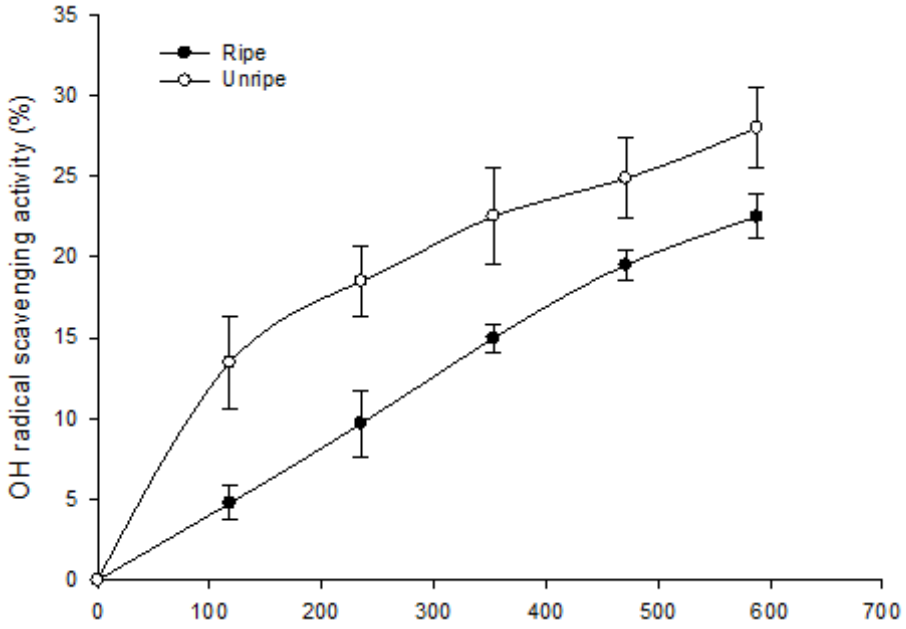

Figure 5: Hydroxyl Radical Scavenging Ability of Phenolic Extracts from *Capsicum frutescens*

Table 1: EC_{50} Values (μg/ml) of Phenolic Extracts from *C. frutescens.*

	Sample	
	Unripe	Ripe
MAO	52.68±0.04[a]	72.05±0.02[b]
AChE	32.37±0.12[a]	100.50±0.11[b]
BChE	63.49±0.12[a]	79.93±0.15[b]
MDA reduction	377.80±1.79[b]	251.20±0.78[a]
DPPH	3.81±0.02[b]	2.95±0.04[a]
OH	131.8±0.04[a]	219.0±0.03[b]

Values represent mean ± standard deviation of triplicate experiments. Values with different superscript letters along the same row are significantly different ($P<0.05$). EC_{50} values were determined by non linear regression analysis of the dose- response curves. MAO-monoamine oxidase, AChE-acetylcholinesterase, BChE-butyrylcholinestase, MDA-malondialdehyde

Table 2: Total Phenol, Total Flavonoid and Non-Flavonoid Phenolic Content of Ripe and Unripe Pepper (*C. frutescens)* Fruits

	Unripe	Ripe
Total Phenol*	86.04±4.16[a]	90.30±3.27[b]
Total Flavonoid**	52.10±0.09[a]	66.67±7.86[b]
Non Flavonoid Phenolics***	33.94±4.16[b]	23.63±11.13[a]

Values represent means ± standard deviation of triplicate experiments Values with different superscript letters along the same row are significantly different ($P<0.05$).
*mg gallic acid equivalent (GAE)/100 g dry weight of sample
**mg quercetin equivalent (QAE)/100 g dry weight of sample
***mg /100 g dry weight of sample

Incubation of Fe^{2+} with rat brain tissue homogenate resulted in a significant ($P<0.05$) increase in rat brain MDA content (171.92±21.62%). However, both extracts significantly ($P<0.05$) reduced brain MDA content in a concentration (100 – 700 μg/ml) dependent manner. The EC_{50} values presented in table 1 showed a significant ($P<0.05$) difference in the inhibitory effect on MDA production of ripe (251.20±0.07 μg/ml), which is higher than the unripe (377.80±0.03 μg/ml) extracts.

C. frutescens extracts scavenged both DPPH and OH radicals in a concentration-dependent manner (1-5 μg/ml and 100-600 μg/ml respectively). According to the EC_{50} values (Table 1), the ripe extract had a significantly higher ($P<0.05$) DPPH (2.95±0.04 μg/ml) scavenging abilities, when compared to the unripe extract (3.81±0.02 μg/ml); however, the unripe extract (131.8±0.04 μg/ml) had a significantly higher ($P<0.05$) OH radical scavenging ability compared to the ripe extract (219.0±0.03 μg/ml).

Concentrations of total phenol, total flavonoid and non-flavonoid contents in *C. frutescens* extracts are presented in table 2. The ripe fruit had significantly higher ($P<0.05$) total phenol (90.30±3.27 mg GAE/100 g), total flavonoid (66.67±7.86 mgQAE/100 g) than the unripe fruit (total phenol = 86.04±4.16 mgGAE/100g; total flavonoid = 52.10±0.09 mgQAE/100g). However, the unripe fruit had higher content of non-flavonoid phenol contents (33.94±4.16 mg/100g) than the ripe fruit (23.63±11.13 mg/100g).

DISCUSSION

Central to the pathology of Parkinson's disease (PD) is the loss of doparminergic neuron at the substantial nigra pars compacta (SNpc) region of the brain (Hauser and Hastings, 2013). This loss of dopaminergic neurons consequently leads to depletion of the neurotransmitter-dopamine (DA) resulting in the characteristic symptoms of PD. One of the reasons for loss of DA is the elevated activity of monoamine oxidase (MAO), which catalyses the oxidation of monoamines such as DA (Hauser and Hastings, 2013). Therefore, one therapeutic strategy for the management of PD is to retard DA oxidation by reducing the activity of MAO via its inhibitors. Consequently, the ability of *C. frutescens* to inhibit the activities of mitochondria MAO activity could be a positive step in serving as a functional food/nutraceutical therapy in the prevention/management of PD; a bioactivity which could probably be attributed to their constituent polyphenols. The therapeutic potentials of plant phenolic extracts for the management of PD by inhibiting MAO activities (*in vitro*) has been reported; in one study, Nwaana *et al.* (2016) reported the MAO inhibitory effect of some commonly consumed green leafy vegetables in southern Nigeria, while Lee *et al.* (2000) has also shown the inhibitory effect of phenolics from Mugwort on mouse brain monoamine oxidase activity.

In the aetiology of neurodegenerative disorders, oxidative stress is regarded both as a causative factor and a resultant condition which leads to loss of neurons (Uttara *et al.*, 2009; Chen *et al.*, 2012; Neidzielska *et al.*, 2016). In PD, elevated MAO activities are often accompanied by excessive free radical generation which ultimately results in oxidative stress (Hauser and Hastings, 2013). As such antioxidant therapies particularly from the diet are considered as critical to effective PD management. Interestingly, the phenolic extracts from *C. frutescens* showed significant antioxidant properties, including DPPH free radical scavenging ability as well as ability to quench the Fenton reaction by scavenging OH radicals. These could therefore, be of therapeutic importance in arresting PD-induced oxidative stress in neuronal cells. This study agrees with previous findings on the antioxidant properties of plant phenolic extracts especially as potential dietary therapeutic measures in the management of neurodegenerative diseases (Yilmaz *et al.*, 2013).

The cholinergic hypothesis of dementia supports the view that elevated activity of acetylcholinesterase is implicative in dementia processes and the use of AChE inhibitors have been able to slow down the progression of dermatan in AD and PD patients. Therefore, in this study, the ability of the phenolic extracts of *C. frutescens* to inhibit the activities of both AChE and BChE could be of therapeutic significance in the management of PD-associated dementia. The ability of plant phenolic extracts to inhibit cholinesterase activities in vitro has been well reported; Oboh *et al.* (2016) reported the cholinesterase inhibitory effect of phenolic extracts from *Heinsia crinita* leaves with potential neuroprotective properties, while Gironés-Vilaplana *et al.* (2012) reported the anticholinesterase properties of a blend of black chokeberry and lemon juice as a dietary intervention for the management of neurodegenerative diseases such as PD and AD attributing the enzyme inhibitory properties of this blend of juice to their constituent phytochemicals which is rich in flavonoids. This study also agrees with that of Oboh *et al.* (2015) on the ability of two varieties of tomato to show potential neuroprotective properties via their anticholinesterase and antioxidant effects.

The membrane stabilizing properties of the extracts is also noteworthy, as both extracts were able to inhibit lipid peroxidation induced by Fe^{2+} in rat brain tissue homogenate. The ability of the extracts to inhibit the lipid peroxidation chain reaction could be as a result of their ability to scavenge OH free radicals and thus attenuate the Fenton reaction processes. Peroxidation of membrane lipids is a consequence of the high generation of ROS in neuronal cells of PD. There is high generation of ROS in mitochondrial of PD subjects which often overwhelms the antioxidant defence status and thus, resulting in oxidative damages of macromolecules. Hence, the ability of the extracts to inhibit lipid peroxidation and offer membrane stabilizing potentials could be another mechanism supporting their therapeutic potentials in the management of PD and other

neurodegenerative diseases. These neuroprotective potentials could be ascribed to the presence of the phytochemicals present in the fruits.

In conclusion, this study has been able to show that phenolic extracts from ripe and unripe pepper (*C. frutescens)* fruits were able to inhibit the activities of rat brain cholinesterases and mitochondria MAO activities *in vitro*. The extracts also exhibited antioxidant properties by scavenging DPPH and OH radicals, as well as exhibit membrane stabilizing properties by inhibiting Fe^{2+} induced lipid peroxidation in rat brain tissue. These observed bioactivities could probably be attributed to their constituent phenolic phytochemicals and could help explain at least in part the mechanisms to justify the use of *C. frutescens* fruits as potential nutraceutical interventions in the prevention/management of neurodegenerative diseases such as PD.

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