INFLUENCE OF NITROGEN FERTILIZER MICRO-DOSING ON PHENOLIC CONTENT, ANTIOXIDANT, AND ANTICHOLINESTERASE PROPERTIES OF AQUEOUS EXTRACTS OF THREE TROPICAL LEAFY VEGETABLES

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FULL ARTICLE

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Influence of nitrogen fertilizer micro-dosing on phenolic content, antioxidant, and anticholinesterase properties of aqueous extracts of three tropical leafy vegetables

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Abstract

This work investigated the effect of fertilizer micro-dosing on *in vitro* antioxidant and anti-acetylcholinesterase (AChE) properties of aqueous extracts of leaves of three vegetables (*Solanum macrocarpon* L., *Amaranthus viridis* L., and *Telfairia occidentalis* f. Hooke). Urea was combined (0, 20, 40, and 60 kg/ha) with cow manure (5 t/ha) or without cow manure (80 kg/ha) to grow the leafy vegetables. Significantly higher ($p < .05$) polyphenol extract yield was obtained with 60 kg N/ha when compared with other fertilizer doses. Total polyphenol (510.70–521.50 mg gallic acid equivalent/g) and total flavonoid (609.51–742.50 μg rutin equivalent/g) contents were reduced as fertilizer dose increased. The 2,2-diphenyl-1 picrylhydrazyl radical scavenging and iron reducing properties were enhanced by organic manure while metal ion chelation and anti-AChE activity was highest at the 60 kg N/ha. We conclude that the combined use of organic manure with urea fertilizer led to enhanced antioxidant and anti-AChE activities of the leaf polyphenolic extracts.

Practical applications

The search for natural scavengers of free radicals as food preservatives and nutraceutical agents has intensified in the past decade because of increasing negative reactions to synthetic compounds by consumers. Vegetable leaf polyphenolic compounds used in this work showed free radical scavenging and other antioxidant properties that are comparable to those of butylated hydroxytoluene, a synthetic antioxidant agent. The polyphenol extracts also showed acetylcholinesterase (AChE)-inhibitory property that is similar to that of galanthamine, a drug used for the treatment of Alzheimer's disease. Since oxidative stress is also associated with the development of chronic diseases, the vegetable leaf extracts are potential agents that can be used both as effective food preservatives and bioactive agents against neurodegenerative diseases.

KEYWORDS

acetylcholinesterase, antioxidant, free radical scavenging, nitrogen fertilizer, polyphenols, vegetable leaves

1 | INTRODUCTION

The body produces reactive oxygen species such as hydroxyl, superoxide, peroxide, and hydrogen peroxide for normal functioning and metabolism (Halliwell & Gutteridge, 1999). They are also responsible for normal gene expression and molecular signaling. However, excessive production of these free radicals can lead to oxidative stress and cell damage, which promotes the development of diseases such as cardiovascular disease (CVD), diabetes, cancer, Alzheimer's, and Parkinson's diseases (Aiyegoro & Okoh, 2010; Sen, Chakraborty, Sridhar, Reddy, & De, 2010). Phenols as antioxidants have been found to be effective in the prevention of oxidative stress by scavenging free radicals and delaying the onset of free radical production (Hasnat, Pervin, & Lim, 2013). In the food processing industry, artificial antioxidants

such as butylated hydroxyanisole, butylated hydroxytoluene (BHT), propyl gallate, and tert-butyl hydroquinone have been used as antioxidant food additives but their prolonged use may be toxic (Hasnat et al., 2013; Wanasundara & Shahidi, 1998). Consumer health awareness has led to a high demand for natural antioxidants in contrast to synthetic antioxidants that could negatively affect human health (Sasidharan, Chen, Saravanan, Sundram, & Latha, 2010). Substituting these synthetic antioxidants with naturally occurring plant antioxidants could promote the production of safer food systems.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that usually occurs among the elderly with symptoms that include memory loss and cognitive deficit (Otaegui-Arrazola, Amiano, Elbusto, Urdaneta, & Martinez-Lage, 2014). High oxidative stress in the brain can lead to neuron damage and contribute to AD pathogenesis (Resende et al., 2008; Zhou et al., 2014). However, AD development is also characterized by low acetylcholine (a neurotransmitter) concentration in the brain, which arises due to excessive activities of acetylcholinesterase (AChE). This is because AChE catalyzes rapid hydrolysis of acetylcholine to choline in the brain (Singh et al., 2013). Some drugs such as galanthamine and rivastigmine are AChE inhibitors that have been used in AD treatment but their uses have been limited because they do not actually slow or reverse the disease progression (Williams, Sorribas, & Howes, 2011). Research is now focused on developing naturally occurring compounds from plants as potential AChE inhibitors that could reduce AD progression by boosting acetylcholine concentration and preventing oxidative stress.

Amaranthus viridis L. leaves (*AV*), *Telfairia occidentalis* f. Hooke (*TO*), and *Solanum macrocarpon* L. (*SM*) leaves are popular leafy vegetables that are widely consumed in the West African region for various health benefits (Khandaker, Ali, & Oba, 2008). Several studies have associated the antioxidant properties of vegetables with their ability to neutralize toxic free radicals, thereby reducing the risk of chronic diseases (Dasgupta & De, 2007). Several health benefits of *TO* such as its ability to treat diabetes and anemia, reduce fatigue, and prevent the production of free radicals have been reported (Aderibigbe, Lawal, & Oluwagbemi, 1999; Yang, Lin, & Mau, 2002; Zheng & Wang, 2001). *AV* is rich in betalain identified as rutin, quercetin, amaranthin, amaricin, and hydrohydroxycinnamates. Betalain in amaranth has been found to have anticancer, antiviral, and antioxidant properties (Hussain, Anwar, Hussain Sherazi, & Przybylski, 2008). The hemolytic and anti-inflammatory properties of *SM* have also been reported in literature (Ng, Zainal Abidin, Shuib, & Israf Ali, 2015; Oboh, Ekperigin, & Kazeem, 2005).

Phenolic compounds are the most abundant naturally occurring antioxidants because of their presence in most plant products. They have multifunctional antioxidant properties because of their ability to scavenge free radicals, donate hydrogen, chelate metal ions, break radical chain reactions, and quench singlet oxygen (Hasnat et al., 2013). Plant polyphenols play a key role in the prevention of free radical-mediated lipid oxidation, nucleic acid degradation, DNA alteration and damage of platelet functions. Consumption of vegetables has been associated with reduced incidence of degenerative diseases such as cancer, cardiovascular diseases, Alzheimer disease, diabetes, and hypercholesterolemia as well as improving the immune system, all which are attributed to their antioxidant activities (Sasidharan et al., 2010). Research has shown that synthetic antioxidants have limited use due to the possibility of causing adverse effects on human health, though they are very effective and stable (Nakatani, 1996; Pokorny, 2007). Therefore, recent research interests have been geared toward the use of naturally occurring plant antioxidants instead of the synthetic antioxidants. According to the World Health Organization (WHO), a large percentage of people in developing countries depend mainly on traditional medicine for their health care needs and natural products such as plant extracts provide a wide range of alternatives to drug use (Sasidharan et al., 2010).

Improved vegetable production is associated with fertilizer use as a means of enhancing vegetative growth and healthy development of the leaves. However, the traditional method of fertilizer application involves broad spreading (broadcast) over the soil without consideration for environmental consequences. Therefore, the Micro-Veg Project, funded by the Government of Canada is training vegetable farmers in West Africa on precision application of fertilizer at optimum level, termed "micro-dosing" to supply just the right amount of fertilizer needed by the plant to optimize leaf yields and reduce the potential run-off of excessive nitrogen into the underground water system. To the best of our knowledge, the effects of fertilizer micro-dosing on the potential bioactive properties of the vegetable leaf polyphenols remain unknown. Therefore, the aim of this work was to determine the in vitro antioxidant and anti-AChE activities of polyphenolic extracts from *AV*, *SM*, and *TO* leaves that were cultivated under nitrogen fertilizer micro-dosing.

2 | MATERIALS AND METHODS

2.1 | Materials

The 2,2-diphenyl-1 picrylhydrazyl radical (DPPH), BHT, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid sodium salt (Ferrozine), 2,4,6-tripyridyl-s-triazine (TPTZ), galanthamine, AChE (electric eel), Folin–Ciocalteu phenol reagent, gallic acid, catechin, myricetin, caffeic acid, and rutin were purchased form Sigma Aldrich (Sigma Chemicals, St. Louis, MO). All other reagents were of analytical grade and purchased from Fisher Scientific (Oakville, ON, Canada). *AV*, *TO*, and *SM* were produced at the Micro-Veg Project experimental location, in Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria. The three vegetable species were produced with application of mineral fertilizer according to fertilizer micro-dosing technology using a randomized complete block design with five nitrogen (urea) fertilizer doses replicated four times as follows: 0, 20, 40, 60, and 80 kg of urea/ha. Basal incorporation of manure (cow manure) at 5 t/ha was done on experimental units (2 m × 3 m) that received urea doses at 20, 40, and 60 kg/ha. The 80 kg/ha contained only urea while 0 kg/ha contain only cow manure. Fertilizer was applied to each plot at planting (T1) or two weeks after seedling emergence (T2) to obtain the following samples: 0T1, 0T2, 20T1, 20T2, 40T1, 40T2, 60T1, 60T2, 80T1, and 80T2. Leaves were harvested 25 days after emergence, rinsed in potable water, destalked, dried in a hot air cabinet at 60°C for 8 hr, milled to

fine powder using a Marlex Excella dry mill (Marlex Appliances PVT, Daman, India) and stored at −20°C.

2.2 | Extraction of free polyphenolic compounds

Extraction of the free water soluble polyphenols was carried out according to the method of Alu'datt et al. (2010) with slight modifications. Samples were extracted using distilled water at 1:20 ratio (leaf powder : water) at 60°C for 2 hr in a 500 mL beaker under continuous stirring. The extracts were allowed to cool to room temperature and centrifuged at 10,000 × g for 30 min. The supernatants were filtered through a cheese cloth and the process repeated to obtain a second supernatant. Both supernatants were pooled and concentrated under vacuum in a rotatory evaporator at 60°C. The concentrated extracts were freeze-dried and stored at −20°C.

2.3 | Total phenolic content

The total phenolic content (TPC) of each extract was determined using the Folin–Ciocalteu method (Hoff & Singleton, 1977) with some modifications. A standard calibration curve was prepared using 25–350 μg/mL gallic acid concentration in 50% (v/v) methanol. The polyphenol extracts were also diluted with 50% methanol to a concentration range of 600–1,400 μg/mL. A 0.25 mL aliquot of Folin-Ciocalteu reagent was added to 0.25 mL of gallic acid solution or the sample and then mixed. After standing in the dark at room temperature for 5 min, 0.5 mL of 20% sodium carbonate solution was added followed by 4 mL of double distilled water. The contents were mixed and incubated in the dark for 1 hr. The intensity of the green color was then measured at 725 nm using an Ultospec UV–visible spectrophotometer (GE Healthcare, Montreal, PQ, Canada). TPC was expressed as milligrams gallic acid equivalents (GAE) per gram of dry leaf powder (mg GAE/g).

2.4 | Total flavonoid content

The total flavonoid content (TFC) was determined colorimetrically according to the method described by Nabavi, Nabavi, Ebrahimzadeh, Eslami, and Jafari (2013) with slight modifications. An aliquot of 30 μL extracts in methanol was sequentially mixed with 90 μL of methanol, 6 μL of 10% aluminum chloride, 6 μL of 1 M potassium acetate, and 168 μL of double distilled water, followed by incubation in the dark at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm in a Synergy H4 multi-mode microplate reader (Biotek Instruments, Winooski, Vermont). TFC was calculated as rutin equivalent from a rutin calibration curve (0.05, 0.1, 0.125, 0.25, 0.5, and 1 μg/mL in methanol).

2.5 | High-performance liquid chromatography

The polyphenolic profile of each leaf extract was determined using a 5-micron C18 analytical (250 × 4.6 mm) reverse-phase HPLC column (Phenomenex Inc., Torrance, CA) fitted on a Varian 920/940-LC system (Agilent Technologies, Santa Clara, CA). Polyphenol standards (gallic acid, catechin, rutin, myricetin, and caffeic acid) were dissolved in ethanol at 0.5 mg/mL while 10 mg/mL of the extracts were prepared using 1% (v/v) acetic acid. A 100 μL aliquot of each standard or sample was injected onto the column, which was heated to 37°C. An isocratic gradient was used with 1% acetic acid as elution buffer. The elution time of the peaks obtained from the standards were compared with those of the samples to identify specific polyphenolic compounds.

2.6 | DPPH radical scavenging assay

The scavenging activity of vegetable leaf extracts against DPPH radical was determined using a previously described method (Aluko & Monu, 2003), which was modified for a 96-well clear flat-bottom plate. The leaf extracts were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 containing 1% (w/v) Triton X-100 to give 0.0625, 0.125, 0.25, 0.5, and 0.625 mg phenolics/mL final assay concentrations. DPPH was dissolved in methanol to a final concentration of 100 μM. A 100 μL aliquot of the plant extract solution was mixed with 100 μL of the DPPH solution in the 96-well plate and incubated at room temperature in the dark for 30 min. The absorbance values of the blank and samples were measured at 517 nm using the Synergy H4 multi-mode microplate reader. The blank consisted of buffer only instead of the plant extract while BHT was used as a positive control. The percentage DPPH radical scavenging activity of the extracts was determined using the following equation.

$$\text{DPPH}(\%) = \frac{\text{Abs(blank)} - \text{Abs (samples)}}{\text{Abs (blank)}} \times 100$$

Effective concentration that scavenged 50% of DPPH radicals (EC_{50}, mg/mL) was calculated by non-linear regression from a plot of extract concentration versus percent DPPH scavenged.

2.7 | Metal ion chelation

The metal ion chelating activity (MCA) was measured using a modified method described by Xie, Huang, Xu, and Jin (2008). Briefly, a 1 mL solution of leaf extracts, BHT (final assay concentration of 0.125, 0.25, 0.5, and 1 mg phenolics/mL) or distilled water (blank) was added to 925 mL of water and 0.05 mL of 2 mM $FeCl_2$ in a reaction tube. After mixing, 25 μL of 5 mM Ferrozine solution was added and vortexed thoroughly. The mixture was then allowed to stand at room temperature for 10 min and an aliquot of 200 μL pipetted into a clear bottom 96-well plate. The absorbance of blank and samples was measured at 562 nm using the Synergy H4 multi-mode microplate reader. Percentage metal chelating effect was calculated using the following equation:

$$\text{Metal chelating activity } (\%) = \frac{\text{Abs(blank)} - \text{Abs (samples)}}{\text{Abs (blank)}} \times 100$$

Inhibitor concentration that chelated 50% of ferrous ion (IC_{50}, mg/mL) was calculated by non-linear regression from a plot of extract concentration versus percent MCA.

2.8 | Ferric reducing antioxidant power (FRAP)

FRAP activity was measured using a previously described protocol (Benzie & Strain, 1998), which was slightly modified as follows. The

FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 10 mM TPTZ prepared in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1. The leaf extracts (40 μL) were mixed with 200 μL of working FRAP reagent in a 96-clear well microplate to give 0.0625–1.0 mg phenolics/mL final assay concentration followed by absorbance measurement at 593 nm in the Synergy H4 multimode microplate reader. A standard curve for $FeSO_4 \cdot 7H_2O$ was prepared using a concentration of 25–150 mM. The results were expressed in mM of Fe^{2+} reduced per gram of extract using the calibration curve of the $FeSO_4$ standard.

2.9 | Total antioxidant capacity

Total antioxidant capacity (TAC) of the leaf extracts was evaluated using the phosphomolybdenum method as previously described (Prieto, Pineda, & Aguilar, 1999) with slight modifications. A 0.25 mL aqueous aliquot of the leaf extract was combined with 1 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) to give final concentrations in the 2.5–10 mg phenolics/mL range. The tubes containing the reaction solution were incubated at 95°C for 90 min, allowed to cool to room temperature, and absorbance measured at 695 nm using the Synergy H4 multimode microplate reader with distilled water as blank. A calibration curve was prepared using 2.0–4.5 mg/mL gallic acid and the absorbance of each sample was used to determine the gallic acid equivalent using the standard curve.

2.10 | In-vitro acetylcholinesterase inhibition assay

The assay for AChE activity was conducted using Ellman's colorimetric method with acetylthiocholine iodide (ATCI) as substrate (Khan and Ghani, 2012). Briefly, 25 μL of 15 mM ATCI, 75 μL of 3 mM DTNB, and 50 μL of 50 mM Tris HCl, pH 8.0 containing 2% bovine serum albumin (BSA) were mixed with 25 μL of each extract or galanthamine (10–50 μg/mL) in a microplate. The blank reaction contained all these reagents except the extract or galanthamine. This was followed by addition of 25 μL AChE (0.26 U/mL) dissolved in buffer containing 1% BSA and the reaction samples were incubated at room temperature for 30 min. The absorbance was then measured in the Synergy H4 multimode microplate reader at 405 nm for 20 min at 37°C. Percentage inhibition of AChE activity was calculated as follows.

$$\text{AChE inhibition}\,(\%) = \frac{\text{Ab(B)} - \text{Ab(S)}}{\text{Ab(B)}} \times 100$$

where, Ab (B) is the absorbance of the blank (uninhibited reaction) and Ab (S) is the absorbance of the sample (inhibited reaction).

2.11 | Statistical analysis

Triplicate determinations were used to obtain mean values and standard deviations. For statistical analysis, 3-way analysis of variance (ANOVA) using a model that included vegetable variety (VV), fertilizer dose (FD), and fertilizer application time (FAT) as fixed variables was performed. Duncan's multiple-range test was used to determine the mean treatment differences and significant differences taken at ($p < .05$). IBM SPSS Statistical package (version 24) was used for all statistical analyses.

3 | RESULTS AND DISCUSSION

3.1 | Extract yield, total phenolic content, and total flavonoid content

Aqueous extraction was preferred because initial extraction with solvents or solvent/water mixtures produced insoluble extracts that did not dissolve properly in the aqueous media used for the antioxidant and AChE assays. Fertilizer dose (FD) and vegetable variety (VV) had effects ($p < .05$) on the polyphenol extract yield while there was no significant ($p > .05$) effect of the time of the fertilizer application (Table 1). The polyphenol yields of *TO* and *AV* (32.7 and 32.2) did not differ significantly but were significantly ($p < .05$) higher than that of the *SM* (30.1). Results showed that polyphenol extract yield was significantly higher at 60 kg N/ha compared with other fertilizer doses. The results suggest that the optimum fertilizer dose for optimum polyphenol yield is 60 kg urea N/ha thus implying that precision application of fertilizer may be a useful technique to enhance accumulation of water-soluble compounds but this effect was highly dependent on the type of vegetable. The polyphenolic extracts yields obtained for leaves from plants grown with fertilizer combination (20, 40, and 60 kg urea N/ha containing organic fertilizer) did not differ significantly ($p > .05$) when compared with the extract of leaves from plants raised with only the organic fertilizer (0 kg urea N/ha), except the 60T, which had the highest yields. Leaves harvested from plants grown with only urea fertilizer (80 kg urea N/ha) had the lowest polyphenol extract yield, which suggests that combined use of inorganic and organic fertilizers led to improved content of free leaf polyphenols. It is noteworthy that *TO* had significantly higher ($p < .05$) amounts of TPC and TFC when compared with *SM* and *AV*, especially with the TFC being double and TPC being approximately 27% higher in *TO*. Table 1 also shows that TPC was influenced by fertilizer dosing rates ($p < .05$) with significant decreases when compared with the control (0 kg N/ha). Total phenolics were significantly different between fertilizer treatments with decreases as urea fertilizer level increased to 60 kg N/ha. The results are in contrast to the work of Nguyen and Niemeyer (2008) who observed a higher phenolic content in basil leaves from plants grown under lower nitrogen fertilizer application. This contrast in data trend may be due to the differences in leaf type. However, the current results agree with the work of Onyango et al. (2012) who reported that the TPC tends to decrease with increasing nitrogen applications, and that the extent of this decrease depends on the level and source of nitrogen. The results are also similar to those reported for artichoke where nitrogen fertilizer application led to reduced TPC (Lombardo et al., 2017). Therefore, nitrogen fertilizer application did not promote polyphenol accumulation, which is consistent with the Carbon Nitrogen balance hypothesis which states that synthesis of carbon-based secondary metabolites such as polyphenols is not promoted in the presence of high nitrogen availability (Bryant, Chapin, & Klein, 1983).

TABLE 1 Results from 3-way ANOVA and Duncan's test of the effects of vegetable variety (VV), fertilizer dose (FD), and fertilizer application time (FAT) on antioxidant and anti-acetylcholinesterase properties of aqueous extracts of *Telfairia occidentalis*, (TO), *Solanum macrocarpon* (SM), and *Amaranthus viridis* (AV) leaves[1]

	Source of variation (*F* values)			Mean intensity for VV[2]			Mean intensity for FD (kg urea N/ha)[2]					Mean intensity for FAT[2]	
Parameters	VV	FD	FAT	SM	AV	TO	20	40	60	80	0	Before planting	After planting
DPPH	34.35*	21.5*	6.39*	0.161^{b} (0.002)	0.173^{c} (0.002)	0.156^{a} (0.002)	0.157^{b} (0.002)	0.173^{c} (0.002)	0.162^{b} (0.002)	0.173^{c} (0.002)	0.150^{a} (0.002)	0.161^{a} (0.001)	0.166^{b} (0.001)
FRAP	2,149*	45.5*	62.89*	0.571^{b} (0.004)	0.527^{a} (0.004)	0.851^{c} (0.004)	0.659^{bc} (0.005)	0.595^{b} (0.005)	0.669^{cd} (0.005)	0.647^{b} (0.005)	0.680^{d} (0.005)	0.633^{a} (0.003)	0.667^{b} (0.003)
MCA	1,704*	274.1*	4.15*	0.55^{c} (0.01)	0.19^{a} (0.01)	0.46^{b} (0.01)	0.55^{e} (0.01)	0.34^{b} (0.01)	0.29^{a} (0.01)	0.39^{c} (0.01)	0.41^{d} (0.01)	0.40^{b} (0.00)	0.39^{a} (0.00)
TAC	1,807*	203.2*	2.06	16.29^{a} (0.249)	36.27^{c} (0.249)	32.29^{b} (0.249)	30.25^{d} (0.321)	28.40^{c} (0.321)	34.53^{e} (0.321)	22.37^{a} (0.321)	25.86^{b} (0.321)	28.07^{a} (0.203)	28.49^{a} (0.203)
TFC	3,083*	29.37*	0.93	354.16^{a} (7.48)	506.25^{b} (7.48)	$1,137.71^{c}$ (7.48)	742.50^{c} (9.66)	625.14^{a} (9.66)	680.35^{b} (9.66)	609.51^{a} (9.66)	672.71^{b} (9.66)	670.21^{a} (6.11)	661.88^{a} (6.11)
TPC	5,810.35*	24.19*	13.12	501.88^{b} (0.82)	463.15^{a} (0.82)	585.16^{c} (0.82)	518.37^{b} (1.06)	512.31^{a} (1.06)	510.21^{a} (1.06)	521.50^{b} (1.06)	521.26^{c} (1.06)	515.02^{a} (0.69)	518.44^{a} (0.69)
AChE	190.35*	14.26*	9.43*	13.88^{b} (0.259)	12.90^{a} (0.259)	19.52^{c} (0.259)	15.65^{b} (0.334)	15.13^{b} (0.334)	17.23^{c} (0.334)	13.7^{a} (0.334)	15.46^{b} (0.334)	14.97^{a} (0.211)	15.89^{b} (0.211)
Yield	37.90*	19.21*	8.64	30.10^{a} (0.235)	32.73^{b} (0.235)	32.44^{b} (0.235)	31.43^{b} (0.303)	31.89^{b} (0.303)	33.86^{c} (0.303)	30.22^{a} (0.303)	31.38^{b} (0.303)	31.38^{a} (0.192)	32.15^{a} (0.192)

[1]DPPH, 2,2 diphenyl-1 picrylhydrazyl radical; FRAP, ferric reducing antioxidant power; MCA, metal ion chelating ability; TAC, total antioxidant capacity; TFC, total flavonoid content; TPC, total phenolic content; AChE, acetylcholinesterase activity inhibition; Yield, gross yield of aqueous extracts. Mean intensity values (standard error of the mean) with different letters in the same row (parameter) are significantly different ($p < .05$).
[2]Units: DPPH (Effective concentration that scavenged 50%, EC_{50} mg/mL); FRAP (mmol Fe^{2+}); MCA (Concentration that chelated 50%, IC_{50} mg/mL); TAC (mg/g gallic acid); TFC (μg/g rutin equivalent); TPC (mg gallic acid equivalent/g); AChE (%); Yield (%).
*Significant at $p < .05$.

Figure 1 shows that the fertilizer treatments resulted in only minor variations in TPC content of the *SM* extracts whereas wider variations were observed for the *AV* and *TO* extracts. For example, at the 0 and 20 kg N/ha dose, TPC for *AV* extract increased when the fertilizer was applied after planting whereas the opposite effect was observed at 40 kg N/ha. There was no significant difference between 60 and 80 kg N/ha doses at both fertilizer application time. The TPC of *TO* also showed significantly ($p < .05$) higher value at 80 kg N/ha when urea was applied after planting. From the *TO* results, it was observed that fertilizer application two weeks after planting enhanced TPC in 20, 40, and 80 kg/ha samples while application of fertilizer during planting favored TPC production in 0 and 60 kg/ha. The results are similar to those reported for *Arabidopsis thaliana*, *Lycopersicon esculentum*, and several fruit species (Stewart et al., 2001; Radi, Mahrouz, Jaouad, & Amiot, 2003) where nitrogen fertilizer application produced varying effects on polyphenol accumulation depending on dose and time of application.

Flavonoids are considered the most abundant polyphenolic compounds with catechin, genistein, quercetin, epicatechin, luteolin (or epigenin), butein, and naringenin as typical examples (Quideau, Deffieux, Douat-Casassus, & Pouysegu, 2011). Therefore, flavonoid content is an important parameter to estimate polyphenol accumulation by plants in response to fertilizer treatments. Generally, variations in TFC content (μg rutin/mg) in response to fertilizer dose was less in *SM* extracts (320.63–495.63) when compared with *AV* (164.38–783.13) and *TO* (939.38–1376.88) extracts (Figure 2). The results suggest that in addition to chlorophylls, some of the polyphenolic compounds in the vegetable leaf extracts are probably flavonoids with some of them identified in Figure 3. The extracts had similar polyphenol profile, except the higher levels of myricetin in *AV* and *TO* when compared with *SM*. As shown in Table 1, TFC was influenced by vegetable variety and dose but not fertilizer application time. Reductions in the TFC as the fertilizer dose increased (Table 1) could be attributed to the competition for phenylalanine which is either used to synthesize phenolic compounds or proteins (Jones & Hartley, 1999). As protein accumulates due to higher concentration of nitrogen, the level of phenolic compounds are reduced for a certain amount of phenylalanine (Stewart et al., 2001). The correlation between the nitrogen fertilization and the content of phenolic compounds in these vegetables can be explained by the study carried out by Ibrahim and Jaafar (2011). The study shows that at zero or 90 kg N/ha fertilization dose, phenolics, and especially flavonoids accumulate more in leaves than in other plant parts when compared with a higher dose of 270 kg N/ha. This correlation can also be explained by the protein competition model hypothesis which predicts

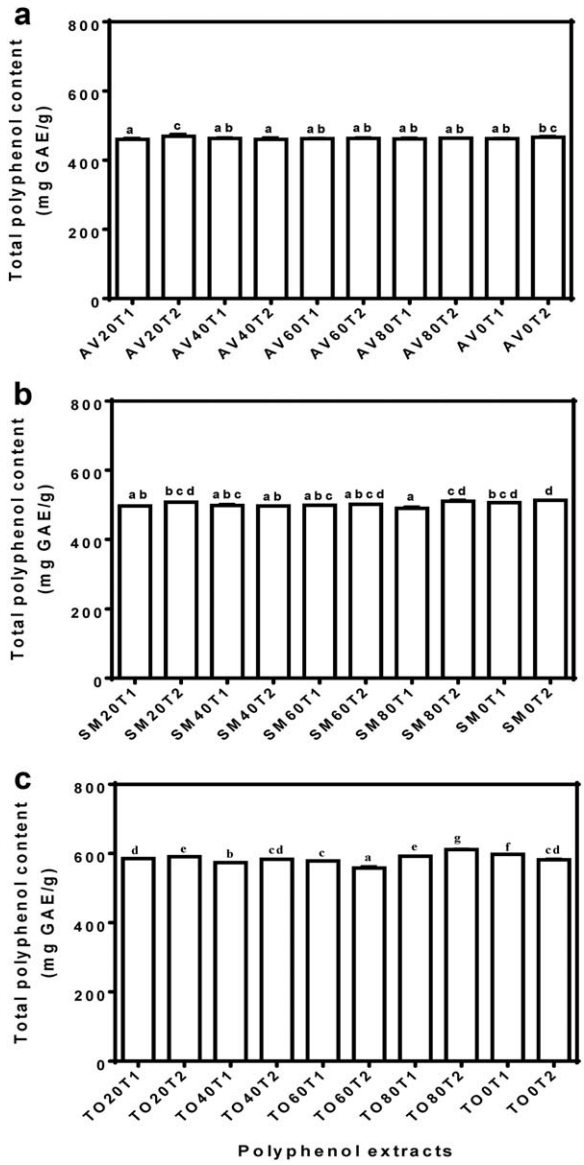

FIGURE 1 Total polyphenol content (expressed as gallic acid equivalent, GAE) of aqueous extracts from the dried leaves of A—*Amaranthus viridis* leaves (AV), B—*Solanum macrocarpon* (SM), and C—*Telfairia occidentalis* (TO). Bars with different letters have significantly ($p < .05$) different mean values. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting

that at high dose of fertilization, there will be an increase in biomass (a consequence of vegetative growth). The increased biomass then leads to reduced synthesis of secondary metabolites such as the polyphenolic compounds (Jones & Hartley, 1999). TFC was highest (742.5) at a lower urea dose of 20 kg N/ha, followed by 60 kg N/ha (680.35), 0 kg N/ha (672.71), and 40 kg N/ha (625.14). The 80 kg N/ha (609.51) has the least value, which suggests that TFC was enhanced by the combined use of urea fertilizer treatment (20–60 kg N/ha) with organic fertilizer or application of organic fertilizer alone (0 kg N/ha) when compared with the urea fertilizer treatment (80 kg N/ha) alone. The study of Ibrahim et al. (2013) also showed that application of organic fertilizer influenced TFC of *Labisia pumila* by 22% when compared with the inorganic fertilizer.

3.2 | Total antioxidant capacity

Total antioxidant capacity (TAC) reflects the synergistic interactions of compounds present in the vegetable leaf extracts and is an index that describes ability of the antioxidants to neutralize preformed free radicals (Van Boekel et al., 2010). The TAC of the extracts increased dose-

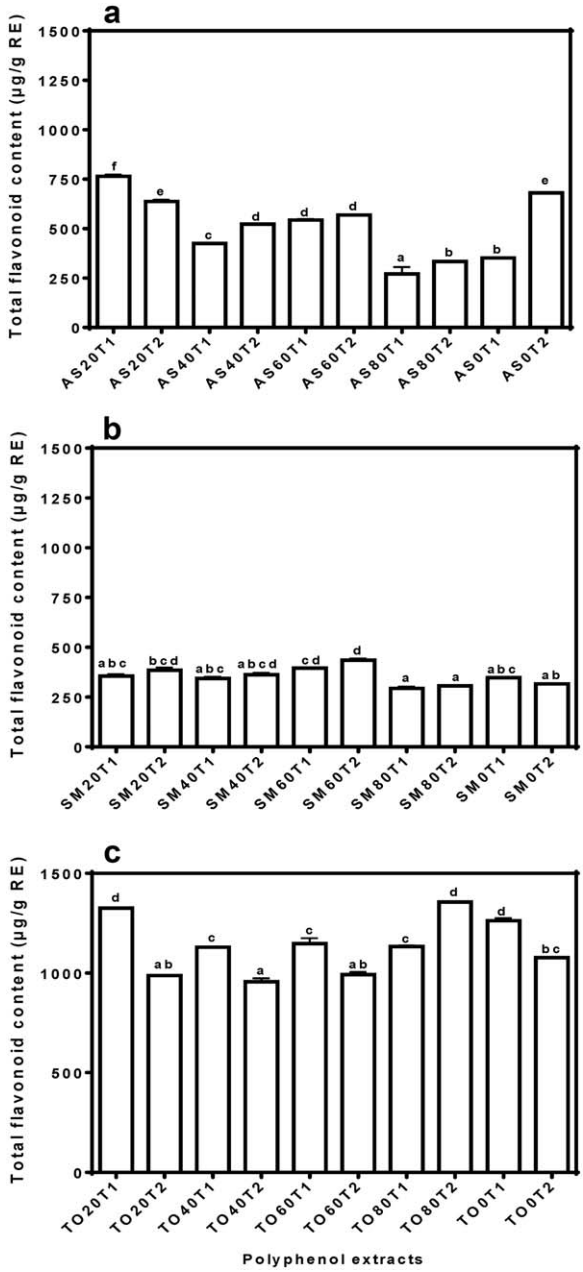

FIGURE 2 Total flavonoid content (expressed as rutin equivalent, RE) of aqueous extracts from the dried leaves of A—*Amaranthus viridis* leaves (AV), B—*Solanum macrocarpon* (SM), and C—*Telfairia occidentalis* (TO). Bars with different letters have significantly ($p < .05$) different mean values. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting

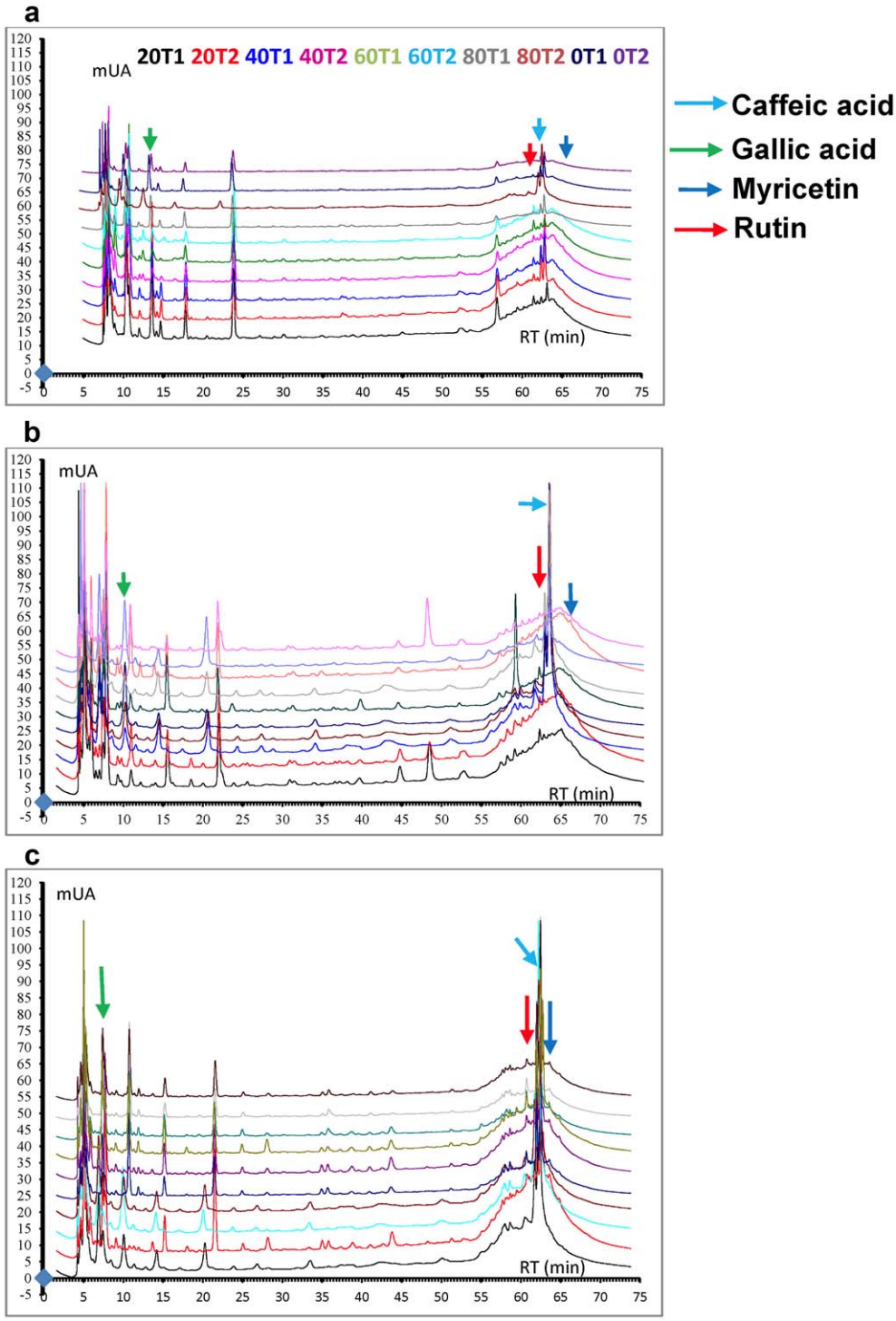

FIGURE 3 HPLC profile of aqueous extracts of dried leaves: A, *Solanum macrocarpon*; B, *Amaranthus viridis*; and C, *Telfairia occidentalis*. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting

dependently for all the vegetable varieties but *SM* extracts were generally the weakest while *AV* extracts were the strongest (Figure 4). TAC of *TO*, *SM and AV* polyphenolic extracts had maximum values at 60 kg N/ha using 10 mg/mL sample concentration. TAC was not dependent on fertilizer application time but vegetable variety and fertilizer dose had significant ($p < .05$) influences (Table 1). *AV* had the highest TAC when compared with *SM* and *TO*. TAC was significantly ($p < .05$) increased (10–34%) by combined application of nitrogen and organic fertilizer (20T–60T) when compared with individual application of inorganic (80T) or organic (0T) fertilizers. Studies have shown that the TAC of plant is basically due to their flavonoids and other polyphenol contents of plants (Potential et al., 2016). However, the TAC showed an

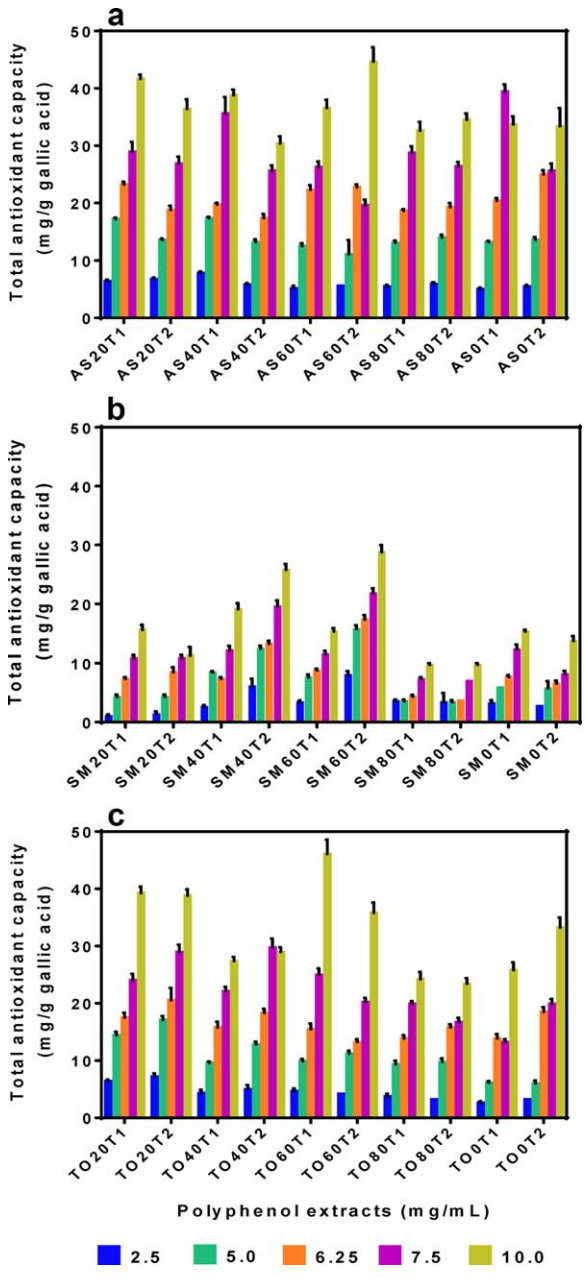

FIGURE 4 Total antioxidant capacity of aqueous extracts from the dried leaves of A—*Amaranthus viridis* leaves (AV), B—*Solanum macrocarpon* (SM), and C—*Telfairia occidentalis* (TO). Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting

opposite trend to those of TPC and TFC, which suggests that the activity of individual compounds may be more important than the synergistic effects of several polyphenols present in the extracts.

3.3 | DPPH radical scavenging assay

DPPH is a stable synthetic compound that has been used in the determination of free radical scavenging activity of various antioxidants (Li, Wu, & Huang, 2009; Viacava, Gonzalez-Aguilar, & Roura, 2014). In the presence of a molecule consisting of a stable free radical (DPPH), an antioxidant with the ability to donate a hydrogen atom will quench the stable free radical, a process that is associated with a change in the absorption and it is measured spectrophotometrically. DPPH radical scavenging activity of the vegetable leaf extracts generally increased up to 0.25 mg/mL polyphenol concentration but declined when concentration increased to 0.5 and 0.625 mg/mL (Figure 5). The initial increases in DPPH radical scavenging activity as polyphenol concentration increased from 0.0625 to 0.25 mg/mL reflects a higher number of hydrogen atoms and electrons that could be donated. However, at 0.5 and 0.625 mg/mL, there may have been increased polyphenol–polyphenol interactions to form oligomers with reduced radical scavenging activity as previously reported for other polyphenolic samples (Plumb, De Pascual-Teresa, Santos-Buelga, Cheynier, & Williamson, 1998; Saint-Cricq de Gaulejac, Vivas, de Freitas, & Bourgeois, 1999). Moreover, hydrogen atom transfer from a polyphenol normally yields a phenolic radical, which is then stabilized by electronic delocalization; an unstabilized phenolic radical essentially becomes a free radical (Quideau et al., 2011). Therefore, the results suggest low stability of the polyphenol radicals, which may have reacted with the reduced form of DPPH to revert back to the radical form, hence reduced antioxidant efficiency at 0.5 and 0.625 mg/mL. Overall, the vegetable extracts had significantly ($p < .05$) lower DPPH scavenging ability than that of BHT, a standard antioxidant compound (Figure 5). The polyphenol extract's effective concentration that scavenged 50% of the DPPH radical (EC_{50}) was used to determine the statistical data shown in Table 1. The DPPH radical scavenging activity of the plant extracts were influenced by vegetable variety, fertilizer dose and fertilizer application time. The DPPH radical scavenging activity was attributed to fertilizer source and rates of nitrogen levels ($p < .05$; Figure 5). At 0.25 mg/mL, the percentage DPPH radical scavenging activity had the highest value for all three vegetable varieties. The EC_{50} results shows that *TO* had the highest DPPH radical scavenging activity value ($EC_{50} = 0.156$ mg/mL) followed by *SM* ($EC_{50} = 0.161$ mg/mL) and *AV* ($EC_{50} = 0.173$ mg/mL). Based on the fertilizer doses, 0 kg N/ha ($EC_{50} = 0.15$ mg/mL) had the highest DPPH radical scavenging activity followed by 20 kg N/ha ($EC_{50} = 0.157$ mg/mL), 60 kg N/ha ($EC_{50} = 0.162$ mg/mL), and the least at 40 kg N/ha and 80 kg/ha treatment ($EC_{50} = 0.173$ mg/mL). The results suggest that the usage of organic fertilizer may enhance the DPPH radical scavenging activity of *TO*, *SM*, and *AV* leaf aqueous extracts and when compared with high urea fertilizer rates. The results also suggest that the combination of urea fertilizer at lower doses with organic fertilizer favored DPPH radical scavenging activity better than high urea dose (80 kg N/ha) only. DPPH radical scavenging assay measures the activity of water-soluble antioxidants (Frankel, Huang, Kanner, & German, 1994). Results obtained from this study do not agree with a previous work that suggested high inorganic N fertilizer application rate (90 kg N/ha) improved the DPPH radical scavenging activity of *Labisia pumila* extracts (Ibrahim et al., 2013). This may be attributed to differences in the vegetable variety used as well as the extraction medium, which was water in this work while *Labisia pumila* was extracted with an aqueous ethanol solution.

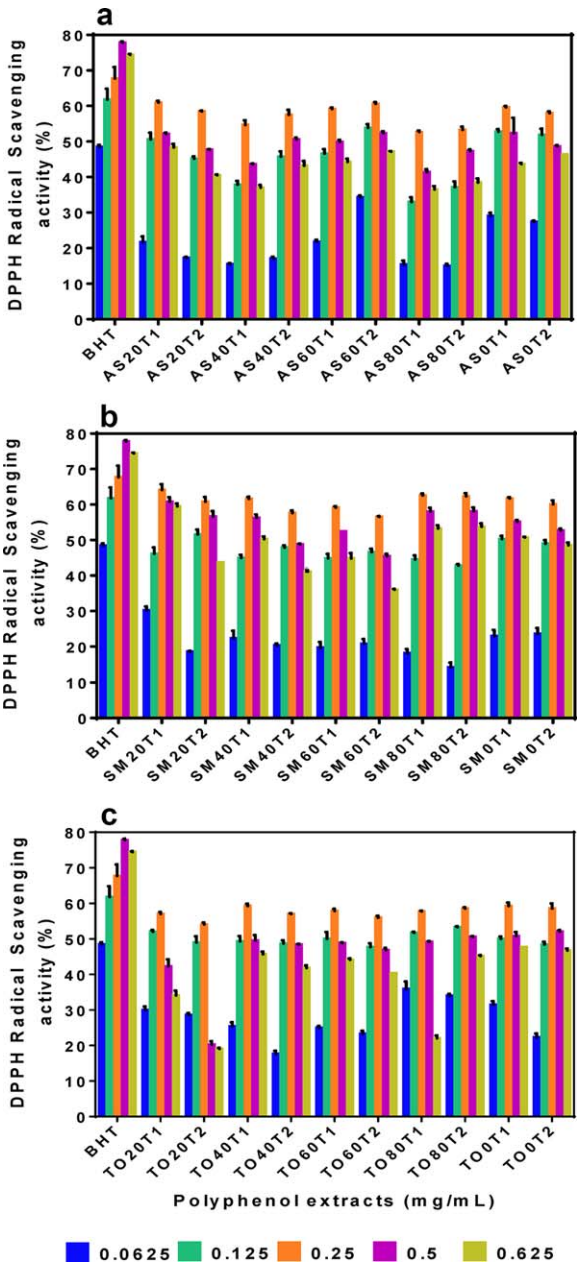

FIGURE 5 DPPH radical scavenging activity of aqueous extracts from the dried leaves of A—*Amaranthus viridis* leaves (AV), B—*Solanum macrocarpon* (SM), and C—*Telfairia occidentalis* (TO). BHT, butylated hydroxytoluene. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting

3.4 | Chelation of metal ions

The presence of transition metals in food or tissues is responsible for the formation of free radicals; therefore, metal chelating agents could reduce the risk of radical-induced damage in the body by stabilizing transition metals (Jayakumar, Thomas, & Geraldine, 2009). Ferrozine forms a stable colored complex with ferrous ion (Fe^{2+}) but these complexes are affected when other chelating agents are present thereby reducing color intensity. The metal chelating activity of the vegetable extracts was enhanced by increased extract concentration up to 0.75 mg/mL but overall effect was not dose-dependent (Figure 6). Metal chelating activity of *SM* and *TO* was increased from 0.125 to 0.75 mg/mL but decreased at 1.0 mg/mL. For *AV* extracts, metal chelating activity increased only up to 0.5 mg/mL while lower values were obtained at 0.75 and 1.0 mg/mL. The lower metal chelating efficiency at high concentrations may be due to polyphenol aggregation through hydrophobic interactions, which would have reduced availability of binding sites (Delimont, Rosenkranz, Haub, & Lindshield, 2017). It is also possible that such polyphenol aggregates become less soluble,

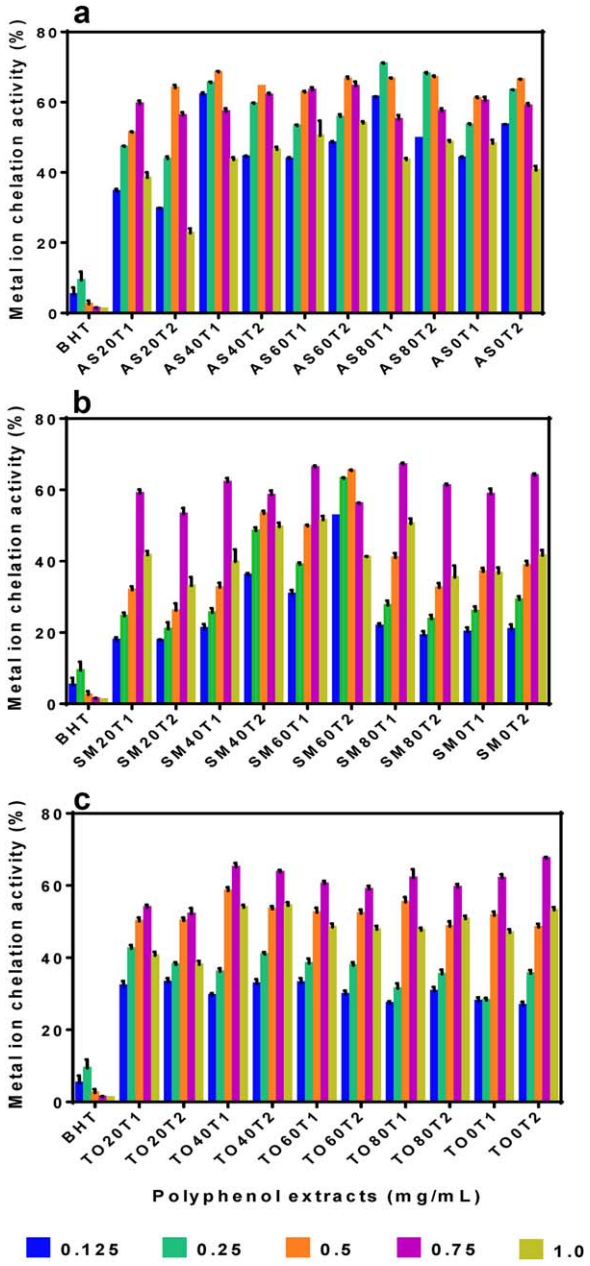

FIGURE 6 Metal ion chelation activity of aqueous extracts from the dried leaves of A—*Amaranthus viridis* leaves (AV), B—*Solanum macrocarpon* (SM), and C—*Telfairia occidentalis* (TO). BHT, butylated hydroxytoluene. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting

which would have minimized interactions with the Fe^{2+}. The metal chelating EC_{50} values were used to determine sources of variation as shown in Table 1. Vegetable variety, fertilizer dose, and fertilizer application time all influenced the metal chelating ability of the polyphenolic extracts. The *AV* extract had significantly ($p < .05$) higher (lowest IC_{50} value) metal chelating ability than the *SM* and *TO* extracts. Therefore, there was no direct relationship between TPC and metal chelating ability, which may be because other factors such as phenolic structure and position of the hydroxyl groups are also important determinants of metal binding (Santoso, Yoshie-stark, & Suzuki, 2004). Nitrogen application at 40–80 kg N/ha led to significantly ($p < .05$) enhanced metal chelating ability (lower EC_{50} values) when compared with the plants that had no nitrogen fertilizer. Application of inorganic fertilizer enhanced the metal chelating ability of the vegetable leaf extracts as shown by the decreases in IC_{50} values for the 20T–60T samples. This is also supported by the lower IC_{50} value for 80T extract (inorganic fertilizer alone) when compared with 0T (organic fertilizer alone). The results suggest that the 40–80 kg N/ha doses enhanced formation of polyphenols with synergistic effects that led to increased metal binding capacity. Overall, the chelating ability of the three vegetable extracts was significantly ($p < .05$) higher than that of BHT. The higher metal chelating ability of the extracts may be due to synergistic interactions between the various polyphenolic compounds when compared with the BHT that is a single compound.

3.5 | Ferric reducing antioxidant power

Ferric reducing antioxidant power (FRAP) assay is based on the reduction of Fe^{3+} to Fe^{2+} and the values obtained are expressed as the concentration of electron-donating antioxidants. Figure 7 shows dose-dependent increases in FRAP for the three vegetable varieties, which indicate concentration-enhanced electron donating ability of the extracts. FRAP was influenced by the vegetable variety, fertilizer dose, and the fertilizer application time (Table 1). The *TO* extract had a significantly higher FRAP value than *SM* and *AV* extracts, which is consistent with the TFC and TPC values. The results are similar to those reported for peanut skins where TFC, TPC, and FRAP had direct correlations (Shem-Tov et al., 2012). The vegetable extracts had significantly ($p < .05$) less FRAP values when compared with BHT; maximum values were achieved at 1.0 mg/mL for vegetables in contrast to 0.5 mg/mL for the BHT. FRAP was influenced by fertilizer source and rates ($p \leq .05$), and followed the same trend with DPPH, where the reducing ability was highest under organic fertilization with the highest activity at 0 kg N/ha, and the lowest activity at 40 kg N/ha. The results are consistent with the findings of Ibrahim et al. (2013) on the impact of nitrogen fertilizer on the antioxidant activity of *Labisia pumila*. The results indicate that as fertilization dose increased, the abilities of the vegetable extracts to reduce ferric ions also increased except for 40 kg N/ha, which has the lowest value and was not significantly different from the 80 kg N/ha. The use of organic fertilizer alone favors the total antioxidant power of these vegetables followed by the combined organic fertilizer treatment with 60 kg urea N/ha. The positive impact of organic fertilizer may be due to increased metabolic activity under

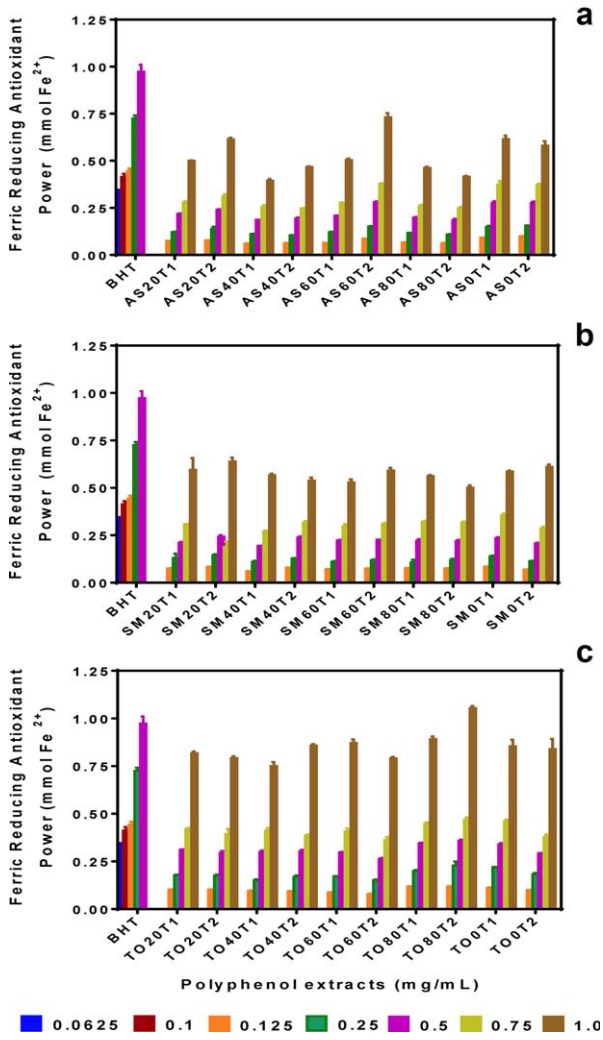

FIGURE 7 Ferric reducing antioxidant power of aqueous extracts from the dried leaves of A—*Amaranthus viridis* leaves (AV), B—*Solanum macrocarpon* (SM), and C—*Telfairia occidentalis* (TO). BHT, butylated hydroxytoluene. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting

these conditions (Woese, Lange, Boess, & Bögl, 1997). It has also been reported that the use of organic fertilizer improves soil properties by increasing soil physical, chemical, and biological properties and prevents soil erosion (Khalil et al., 2007). Higher rate of antioxidant properties obtained with organic fertilizer might be due to the presence of other major and minor elements present in the organic fertilizer since the inorganic fertilizer used in this study supplied only nitrogen. Application of fertilizer two weeks after planting enhanced the rate of ferric ion reduction than those applied during planting, which suggests positive effect on the synthesis of compounds with strong iron-reducing properties.

3.6 | In vitro inhibition of acetylcholinesterase activity

Some neurodegenerative disorders such as dementia and Alzheimer's disease have been suggested to occur as a result of excessive activity

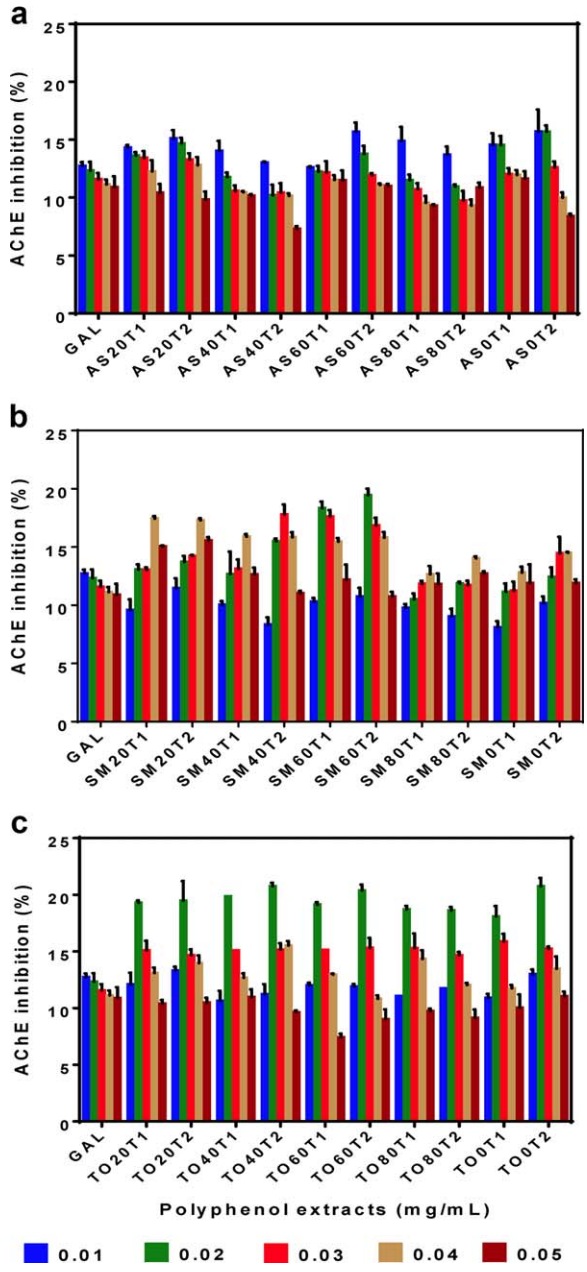

FIGURE 8 Inhibition of acetylcholinesterase (AChE) activity by aqueous extracts from the dried leaves of A—*Amaranthus viridis* leaves (AV), B—*Solanum macrocarpon* (SM), and C—*Telfairia occidentalis* (TO). GAL, galanthamine. Vegetables were grown under different nitrogen fertilizer doses (0, 20, 40, 60, and 80 kg/ha), that were applied before (T1) or after (T2) planting

of acetylcholinesterase, an enzyme that breaks down the neurotransmitter acetylcholine into choline and an acetate group (Pervin et al., 2014; Saravanan & Ponmurugan, 2013). This is due to the reduced availability of acetylcholine, which leads to decreased nerve signal transmission. Therefore, compounds that inhibit acetylcholinesterase activity could boost acetylcholine concentration to facilitate normal nerve functions (Saravanan & Ponmurugan, 2013). All the three vegetable extracts exhibited acetylcholine inhibitory activity in a pattern that was not dose-dependent (Figure 8). AChE inhibition increased from 0.01 to 0.02 mg/mL mostly for the *TO* extracts whereas some of the *SM* extracts had inhibitory values that increased up to 0.03 and 0.04 mg/mL. For the *AV* extracts, AChE inhibition was either highest at 0.01 and 0.02 mg/mL followed by decreases at higher concentrations or highest inhibition occurred at only 0.01 mg/mL. The lack of a dose-dependent inhibition at the concentrations used in this work suggests polyphenol aggregation, which could have reduced ability to interact with AChE protein. Table 1 shows that AChE inhibition by the polyphenol extracts was influenced by vegetable variety, fertilizer dose and fertilizer application time. Generally, the *TO* extracts had significantly ($p < .05$) higher AChE-inhibitory properties than *SM* and *AV* extracts. AChE inhibition by all the polyphenol extracts was very similar from zero N fertilizer application up to 40 kg N/ha dose but then decreased at the 80 kg N/ha dose. The maximum activity occurred at 60 kg urea N/ha in combination with organic fertilizer while the least inhibition occurred at 80 kg urea N/ha (no organic fertilizer). There was no significant difference between the organic fertilizer alone (0 kg urea N/ha) treatment and those of the combined treatments (20 and 40 kg urea N/ha). The result suggests that inhibition of AChE activity may be better achieved with the use of organic fertilizer alone or in combination with urea. The AChE-inhibitory activity of the extracts was more effective when the fertilizer was applied two weeks after planting, which suggests enhanced synthesis of active compounds. The vegetable extracts had significantly ($p < .05$) higher AChE inhibition than galanthamine, the standard drug used for comparison. Therefore, depending on bioavailability, the vegetable extracts could be important sources of compounds that exert neuroprotective effects similar to galanthamine. The AChE-inhibitory values obtained in this work are higher than those reported for the aqueous extract of *Thymus vulgaris*, which was assayed at 0.05 mg/mL concentration but lower than the 37, 37, and 62% for *Corydalis cava* at 0.025, 0.05 and 0.1 mg/mL, respectively (Hasnat et al., 2013).

4 | CONCLUSIONS

Aqueous extracts of the three vegetables had in vitro antioxidant properties that could provide a basis for reducing the damaging effects of free radicals in foods or in human tissues. Nitrogen fertilizer application had a negative influence on the accumulation of polyphenolic compounds, which suggests that the plants channeled excess nutrients toward vegetative growth. However, TAC, metal ion chelation and AChE inhibition were increased up to 60 kg N/ha application when combined with organic fertilizer; this dose may serve as the optimum level for enhanced bioactive properties of the vegetable leaves. The results suggest that the *TO* extracts have the highest antioxidant and anti-AChE potential when compared with *AV* and *SM*. Therefore, the *TO* extracts could serve as suitable ingredients to formulate functional foods and nutraceuticals against oxidative stress and AChE-dependent neurodegenerative disorders. The ability of these vegetables to act as an antioxidant could be attributed to the presence of phenolic compounds such as gallic acid, rutin, myricetin, and caffeic acid. Future studies that use model food systems or appropriate diseased animal

models will be required to confirm the potential benefits of these polyphenolic extracts.

CONFLICT OF INTEREST

The authors declared that they have no conflict of interest.

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