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HPLC-DAD fingerprinting analysis, antioxidant activities of *Tithonia diversifolia* (Hemsl.) A. Gray leaves and its inhibition of key enzymes linked to Alzheimer's disease

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ABSTRACT

Tithonia diversifolia (Hemsl.) A. Gray leaves have long been used to manage neurodegenerative diseases without scientific basis. This study characterized the phenolic constituents, evaluated the antioxidant properties of phenolic extracts from *T. diversifolia* leaves used as traditional medicine in Africa and its inhibition of key enzymes linked to Alzheimer's disease. The extract was rich in phenolic acids (gallic acid, chlorogenic acid, caffeic acid and *p*-coumaric acid) and flavonoids (apigenin) and had 1,1-diphenyl-2-picryl-hydrazil radical scavenging abilities (IC_{50} = 41.05 μg. mL^{-1}), 2,2-Azino-bis3-ethylbenthiazoline-6sulphonic acid radical scavenging ability (IC_{50} = 33.51 μg. mL^{-1}), iron chelation (IC_{50} = 38.50 μg. mL^{-1}), reducing power (Fe^{3+}-Fe^{2+}) (7.34 AAEmg/100 g), inhibited acetylcholinesterase (IC_{50} = 39.27 μg mL^{-1}) and butyrylcholinesterase (IC_{50} = 35.01 μg mL^{-1}) activities. These results reveal the leaf as a rich source of phenolic compounds with antioxidant and cholinesterase inhibitory activity.

1. Introduction

Humans are unavoidably and continually affronted by different environmental stresses caused by reactive oxygen species, which induce pathological processes of many neurodegenerative diseases, including Parkinson's and Alzheimer's [1]. The brain and nervous systems are understood to be principally susceptible to oxidative stress due to limited antioxidant capacity, utilization of metabolic oxygen, failure of the neurons to synthesize glutathione and high lipid content [2,3]. Hence, epidemiological studies have indicated one useful way to avoid or manage neurodegenerative diseases via consumption of foods rich in antioxidants [4]. Alzheimer's disease (AD) is the common form of age-related dementia and is described as a gradual and sinister neurodegeneration of the nervous system that ultimately leads to a slow decline of cognitive function and dementia [5]. While the etiology of Alzheimer's disease is not fully understood, inhibition of cholinergic enzymes like acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities has been recognized as an acceptable treatment against Alzheimer's disease [6]. The commonly used synthetic drugs in the treatment of Alzheimer's disease includes the acetylcholinesterase inhibitors which includes donepezil, tacrine and rivastigmine; however, they are limited in use because of their adverse effects. Thus, recent efforts have focused on plant phytochemicals as natural sources of acetylcholinesterase and butyrylcholinesterase inhibitors with little or no side effects that could be used in managing neurodegenerative diseases [6,7].

Tithonia diversifolia (Hemsl.) A. Gray is considered to be a medicative plant that is loosely utilized to treat numerous diseases [8]. It is usually referred to as Mexican sunflower, bush helianthus or "*sepeleba*" (Yoruba). Studies have shown that extracts from the plant exhibited anti-malaria, anti-diarrhoea, anti-inflammatory, antibacterial, anti-proliferation properties and treatment of haematomas and wounds [8]. The leaf is reported to contain sesquiterpene lactones taginin C, diversifolin, diversifolin methyl ether and tirotundin as bioactive

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compounds against inflammation [9]. The antioxidant potentials and inhibitory effects of *Tithonia diversifolia* aqueous extract on some pro-oxidant (Fe^{2+} and sodium nitroprusside) induced lipid peroxidation in rat brain homogenates had been reported by Ojo and Ojo [10]. Therefore, this study sought to assess its phenolic composition and evaluate the inhibitory property of *Tithonia diversifolia* leaf on the key enzymes (acetylcholinesterase and butyrylcholinesterase) linked to Alzheimer's disease.

2. Materials and methods

2.1. Plant material

Leaves of *Tithonia diversifolia* were freshly purchased in a local market, Ado-Ekiti, Nigeria. The plant was authenticated and documented by a senior taxonomist at the herbarium (UHAE/2014/086) unit of the Department of Plant Science, Ekiti State University, Nigeria. Leaf samples were instantly washed and air-dried for 4 weeks. Dried leaves were ground to powder and then kept in a sealed vessel for further investigation. All plant names in this manuscript were formatted consistently with the newest revision in "The Plant List" (www.theplantlist.org), and correspond to the good practices in publishing studies on herbal materials, as described by [11].

2.2. Chemicals and reagents

All chemicals used were of analytical grade. Methanol, acetone, acetic acid, gallic acid, *p*-coumaric acid, chlorogenic acid and caffeic acid were purchased from Merck (Darmstadt, Germany). Acetylcholine iodide, butyrylcholine iodide 5,5′-dithio-bis (2- nitrobenzoic acid) (DTNB), were sourced from Sigma-Aldrich, Chemie GmbH (Steinheim, Germany). Apigenin was acquired from Sigma Chemical Co. (St. Louis, MO, USA). High performance liquid chromatography with diode array detection (HPLC-DAD) was performed with a Shimadzu Prominence Auto Sampler (SIL-20 A) HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC-20 AT reciprocating pumps connected to a DGU 20 A5 degasser with a CBM 20 A integrator, SPD-M20 A diode array detector and LC solution 1.22 SP1 software.

2.3. Phenolic extract preparation

Fifty grams (50 g) of powdered *Tithonia diversifolia* leaves was soaked in 120 mL of 80% acetone for 72 h. It was then filtered using a cheese cloth, concentrated to a small volume to remove acetone using rotary evaporator. It was then transferred to a 500 mL beaker and placed in a water bath (40 °C) to evaporate to dryness. The yield was 12.5% and the extract was kept in a closed container in a refrigerator at 4 °C for further studies.

2.4. Quantification of phenolic compounds

2.4.1. Determination of total phenol content

The total phenol content of the leaf extract was assessed (as gallic acid equivalent) as described by [12]. In short, 200 µL extract dissolved in 10% DMSO (240 µg. mL^{-1}) was incubated with 1.0 mL of Folin Ciocalteau chemical agent (diluted 10 times) and 800 µL of 0.7 mol. L^{-1} Na_2CO_3 for 30 min at ambient temperature. Absorbance was read at 765 nm using a spectrophotometer. All readings were repeated in triplicate and results expressed as mg GAE/ 100 g dry aqueous extracts.

2.4.2. Determination of flavonoid content

The flavonoid content of the extract was determined using the method reported by [13]. Briefly, 0.5 mL of appropriately diluted sample was mixed with 0.5 mL methanol, 50 µL 10% $AlCl_3$, 50 µL 1 M potassium acetate and 1.4 mL water, and incubated at room temperature for 30 min. Absorbance of the mixture was read at 415 nm. All experiments were in triplicates. A standard curve was plotted with quercetin and the total flavonoid content of the extract was expressed as quercetin equivalent.

Table 1
Total phenol content, total flavonoid content and reducing properties (FRAP) values of aqueous phenolic extract of *Tithonia diversifolia*.

Parameters	Value
Total phenol content (GAEmg/100 g)	251.63 ± 0.47
Total flavonoid content (QEmg/100 g)	98.21 ± 0.08
Ferric reducing antioxidant property (AAEmg/100 g)	7.34 ± 1.22

Values represent means ± standard mean of error of triplicate readings.
AAE = Ascorbic Acid Equivalent.
QE = Quercetin Equivalent.
GAE = Gallic Acid Equivalent.

2.5. Determination of antioxidant activities

2.5.1. Reducing property

The reducing property of the leaf extract was evaluated by reduction of $FeCl_3$ solution as described by [14]. A 2.5 mL aliquot of extract was mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The solution was incubated for 20 min at 50 °C in a water bath and 2.5 mL of 10% trichloroacetic acid was added. The sample was then centrifuged at 650 g for 10 min and 5 mL of the supernatant was mixed with an equal volume of water and 1.0 mL, 0.1% $FeCl_3$. The above process was also applied to a standard ascorbic acid solution, and the absorbance read at 700 nm. The reducing ability was calculated as percentage inhibition.

2.5.2. Iron (Fe^{2+}) chelation assay

The Fe^{2+} chelating ability of the leaf extract was determined by employing a modified procedure by [3]. Freshly made 500 µmol L^{-1} $FeSO_4$ (150 µL) was added to a solution containing 168 µL of 0.1 mol L^{-1} Tris-HCl (pH 7.4), together with 218 µL saline and an aqueous extract (20–100 µg/mL). The solution was incubated for 5 min, followed by addition of 13 µL of 0.25% (w/v) of 1, 10-phenanthroline. Absorbance was read at 510 nm. (EDTA was used as a standard for evaluating metal chelating activity (EDTAE)).

2.5.3. DPPH free radical scavenging ability

The free radical scavenging ability of the extract against DPPH free radical was assessed using the method described by [15]. Briefly, appropriate dilution of the extract (1 mL) was mixed with 1 mL 0.4 mM DPPH solution in methanol. The mixture was left in the dark for 30 min and the absorbance was read at 516 nm. The DPPH radical scavenging assay was calculated with respect to the control (Vitamin C was used as a standard for evaluating DPPH activity). DPPH scavenging ability (%) = [(Abscontrol – Abssapmles)/Abscontrol] × 100

2.5.4. 2,2-Azino-bis3-ethylbenthiazoline-6sulphonic acid (ABTS·) radical scavenging ability

The ABTS· scavenging ability of the phenolic extracts was assessed based on the method described by [16]. The ABTS· was produced by reacting 7 mM ABTS aqueous solution with $K_2S_2O_8$ (2.45 mM, final concentration) in the dark for 16 h and altering the absorbance 734 nm to 0.700 with ethanol. 200 µL of suitable dilution of phenolic extracts were added to 2.0 mL ABTS· solution and the absorbance was read at 734 nm after 15 min. (Vitamin C was used as a standard for evaluating ABTS activity)

2.6. Determination of cholinesterase activity

Inhibitory activity of AChE was evaluated by an adapted colorimetric method as described by [17]. The AChE activity was determined

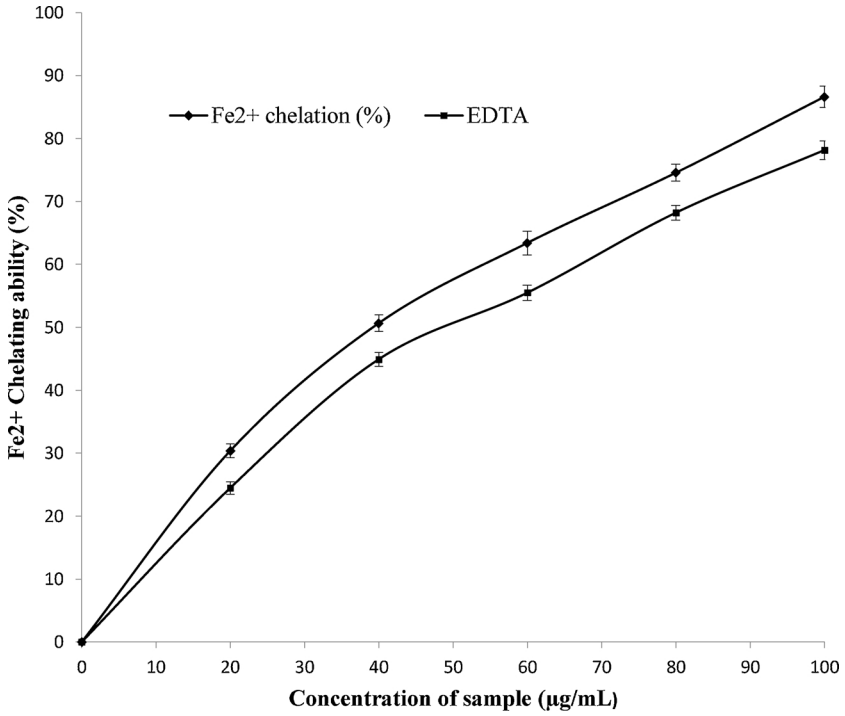

Fig. 1. Fe^{2+} chelating ability of phenolic extract from *Tithonia diversifolia* leaves. Data expressed as mean ± SEM (n = 3).

Table 2
IC_{50} values for Fe^{2+} chelating ability, DPPH·, ABTS· as well as acetylcholinesterase and butyrylcholinesterase inhibitory activities.

Sample	IC_{50} (µg/ml)
Fe Chelation	38.50 ± 0.05
DPPH·	41.05 ± 0.02
ABTS·	33.51 ± 0.01
Acetylcholinesterase	39.27 ± 0.12
Butyrylcholinesterase	35.01 ± 0.01

Values represent means ± standard mean of error.

Table 3
Analysis of the compounds present in *Tithonia diversifolia* extract.

Compounds	*Tithonia diversifolia*	
	mg/g	%
Gallic acid	1.28 ± 0.01[a]	0.12
Chlorogenic acid	7.95 ± 0.02[b]	0.79
Caffeic acid	5.18 ± 0.02[c]	0.51
p-Coumaric acid	0.53 ± 0.01[d]	0.05
Apigenin	5.09 ± 0.02[e]	0.50

Results are expressed as mean ± standard deviations (SD) of three determinations.
Averages followed by different letters differ by Tukey test at $p < 0.05$.

in a mixture containing 200 µL of a solution of AChE (0.415 $U.mL^{-1}$ in 0.1 M phosphate buffer, pH 8.0), 100 µL of a solution of 5,5′ -dithiobis (2-nitrobenzoic) acid (DTNB) (3.3 mM in 0.1 M phosphate-buffered solution, pH 7.0) containing $NaHCO_3$ (6 mM), phenolic extracts of *Tithonia diversifolia* leaves, and 500 µL of 4 phosphate buffer, pH 8.0. After incubation for 20 min at 25 °C, 100 µL of 0.05 mM acetylcholine iodide solution was added as the substrate, and AChE activity was assessed as change in absorbance at 412 nm for 3 min at 25°C using a spectrophotometer. Inhibition of BChE was evaluated by an adjusted colorimetric method as described by [18]. The BChE activity was assessed in a mixture comprising 200 µL of a solution of BChE (0.415 $U.mL^{-1}$ in 0.1 M phosphate buffer, pH 8.0), 100 µL solution of 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB) (3.3 mM in 0.1 M phosphate-buffered solution, pH 7.0) containing $NaHCO_3$ (6 mM), phenolic extracts, and 500 µL of 4 phosphate buffer, pH 8.0. After incubation for 20 min at 25 °C, 100 µL of 0.05 mM butyrylcholine iodide solution was added as substrate, and BChE activity was determined as change in absorbance at 412 nm for 3 min at 25 °C using a spectrophotometer. (Prostigmine was used as a standard for evaluating cholinesterase activity). The AChE and BChE inhibitory activities were expressed as percent inhibition (%).

2.7. HPLC analysis

Reverse phase chromatographic analyses were carried out under gradient conditions using C18 column (4.6 mm × 250 mm) packed with 5 µm diameter particles. The mobile phase with a flow rate of 0.6 mL/min consisted of a mixture of solvents: A (water/acetic acid, 98:2% v/v) and B (methanol/water/acetic acid, 70:28:2% v/v) using the following linear eluting gradient: 0–3 min: 0% B in A; 3–25 min: 30% B in A; 25–43 min: 50% B in A; 43–55 min: 60% B in A; 55–60 min: 80% B in A; 60–65 min: 50% B in A; and 65– 69 m in. 0% B in A; following the method described by [19] with some modifications. The wavelengths used were 254 nm for gallic acid, 327 nm for chlorogenic acid, caffeic acid and p-coumaric acid, and 366 nm for apigenin. *T. diversifolia* extract (10 mg mL^{-1}) and mobile phase were filtered through 0.45 mm membrane filter (Millipore) and then degassed by ultrasonic bath prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase in a concentration range of 0.030–0.250 mg. mL^{-1}. Chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–600 nm). All chromatography operations were carried out at ambient temperature in triplicate.

2.8. Data analysis

Results of the three replicates were pooled and expressed as the

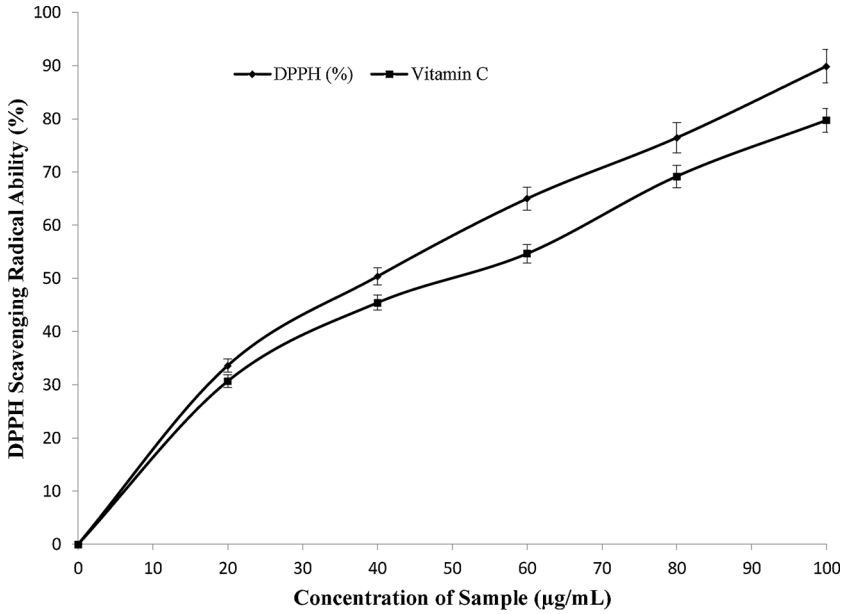

Fig. 2. DPPH· scavenging ability of phenolic extract from *Tithonia diversifolia* leaves. Data expressed as mean ± SEM (n = 3).

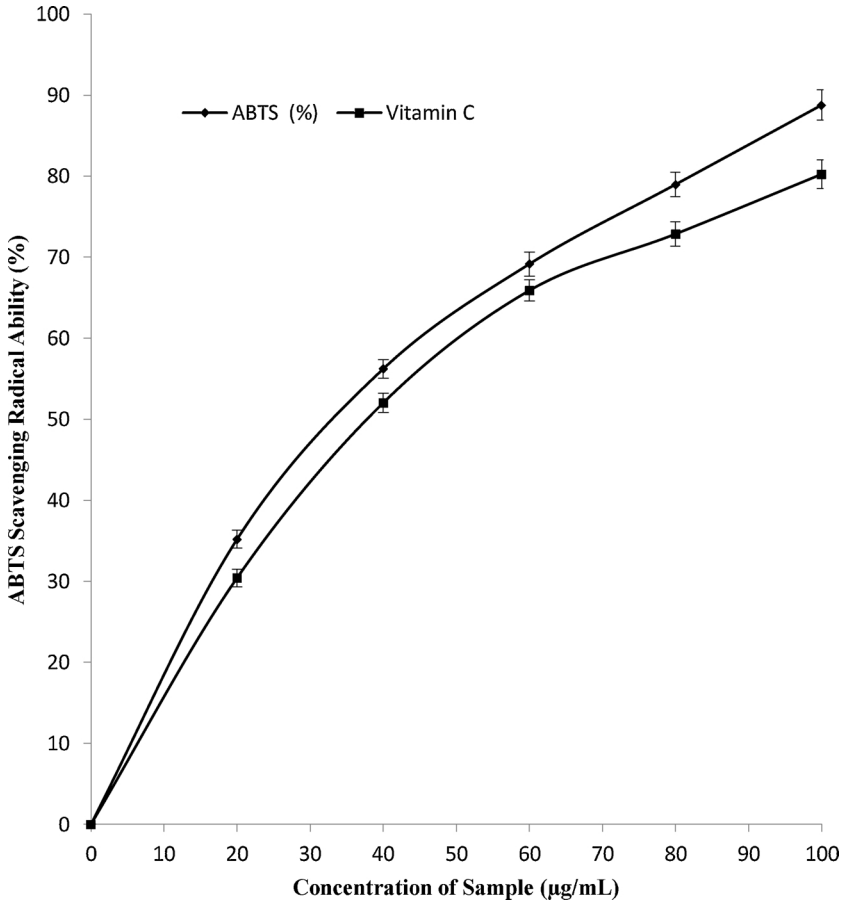

Fig. 3. ABTS· scavenging ability of phenolic extract from *Tithonia diversifolia* leaves. Data expressed as mean ± SEM (n = 3).

mean ± SEM. Differences between groups of HPLC were assessed by an analysis of variance model and Tukey's test. The level of significance for the analyses was set at $p < 0.05$. These analyses were performed by using the free software R version 3.1.1. [20].

3. Results

3.1. Phenolic and flavonoid content

The total phenol and flavonoid content of phenolic extract are presented in Table 1. The results revealed that *Tithonia diversifolia* had a

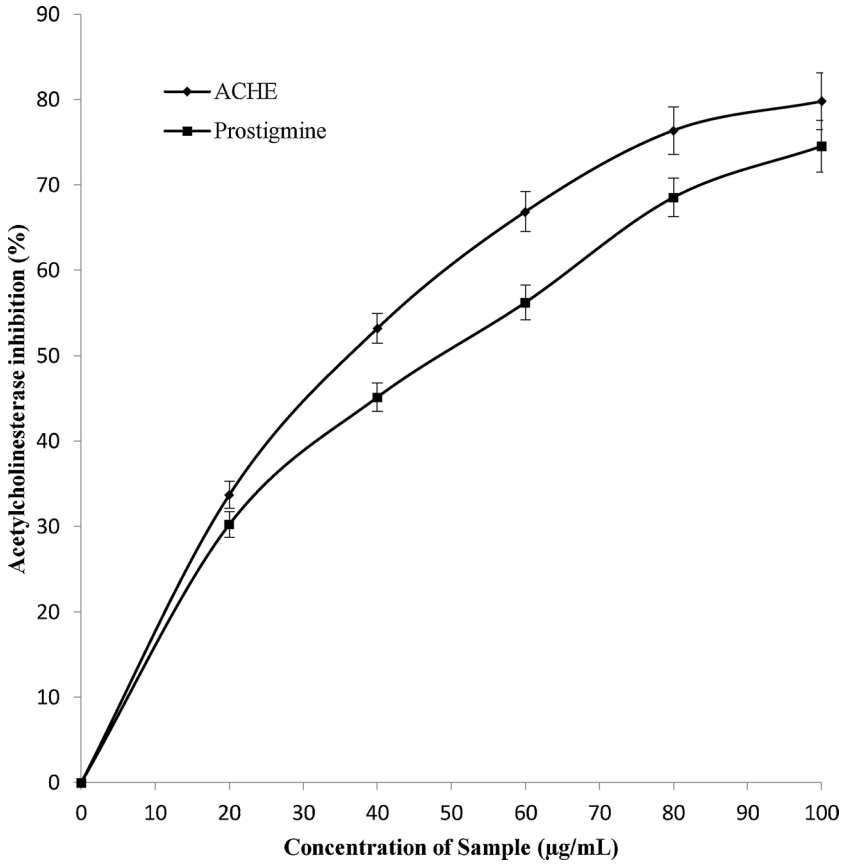

Fig. 4. Acetylcholinesterase inhibition by phenolic extract from *Tithonia diversifolia* leaves. Data expressed as mean ± SEM (n = 3).

total phenol content of 251.63 mg g^{-1} GAE and total flavonoid content of 98.21 mg g^{-1} QUE.

3.2. Reducing property

The reducing ability of phenolic extracts from *Tithonia diversifolia* is presented in Table 1. The results revealed that the phenolic extract had a reducing property (7.34 mg g^{-1} AAE).

3.3. Iron (Fe^{2+}) chelating ability

Fe^{2+} chelating ability of phenolic extracts from *Tithonia diversifolia* is presented in Fig. 1 with its IC_{50} values (38.50 µg. mL^{-1}) in Table 2. This result revealed that the phenolic extract exhibited metal chelating activity in a concentration-dependent manner better than the standard EDTA as shown in Table 3.

3.4. DPPH scavenging activity

DPPH radical scavenging ability is displayed in Fig. 2, with its IC_{50} values (41.05 ± 0.02 µg mL^{-1}) in Table 2. This showed that the phenolic extracts scavenged free radicals in a concentration-dependent manner (20–100 µg mL^{-1}) better when compared to vitamin C standard.

3.5. 2,2-Azino-bis3-ethylbenthiazoline-6sulphonic acid (ABTS·) radical scavenging ability

The free radical scavenging ability of the extract was evaluated using the moderately stable ABTS radical (ABTS·) and displayed in Fig. 3 with IC_{50} (33.51 µg. mL^{-1}) values. The results showed that the phenolic extract quenched ABTS radical in a concentration-dependent manner (20–100 µg. mL^{-1}) when compared with standard vitamin C.

3.6. Acetylcholinesterase (AChE) and butyrylcholinesterase inhibitory properties

Acetylcholinesterase inhibitory properties of *Tithonia diversifolia* phenolic extract is displayed in Fig. 4. The result revealed that AChE activity was inhibited in a concentration-dependent manner (20–100 µg. mL^{-1}), having an IC_{50} value of 39.27 µg. mL^{-1} as showed in Table 2 when compared to prostigmine with corresponding IC_{50} value of 50.02 µg. mL^{-1}. The ability of the phenolic extract to inhibit butyrylcholinesterase activity *in vitro* is displayed in Fig. 5. The result revealed that the extract had a high BChE inhibitory activity with IC_{50} value of 35.01 µg. mL^{-1} than prostigmine with relatively IC_{50} value of 48.56 µg. mL^{-1} as revealed in Table 2.

3.7. HPLC-DAD analysis of phenolic composition

The phenolics (flavonoids and phenolic acids) composition of *T. diversifolia* leaves as quantified using HPLC-DAD are presented in Table 3. The major phenolic acids were gallic acid, chlorogenic acid, caffeic acid and *p*-coumaric acid, while, apigenins were the major flavonoids. The result showed that the leaves had high levels of phenolic acids of pharmacological importance (Fig. 6).

4. Discussion

Phenolic compounds can protect the human body from free radicals, whose formation is associated with the natural metabolism of aerobic cells [21]. The phenolic compounds are strong antioxidants capable of removing free radicals, chelating metal catalysts, activating antioxidant enzymes, reducing α- tocopherol radicals and inhibiting oxidases [22].

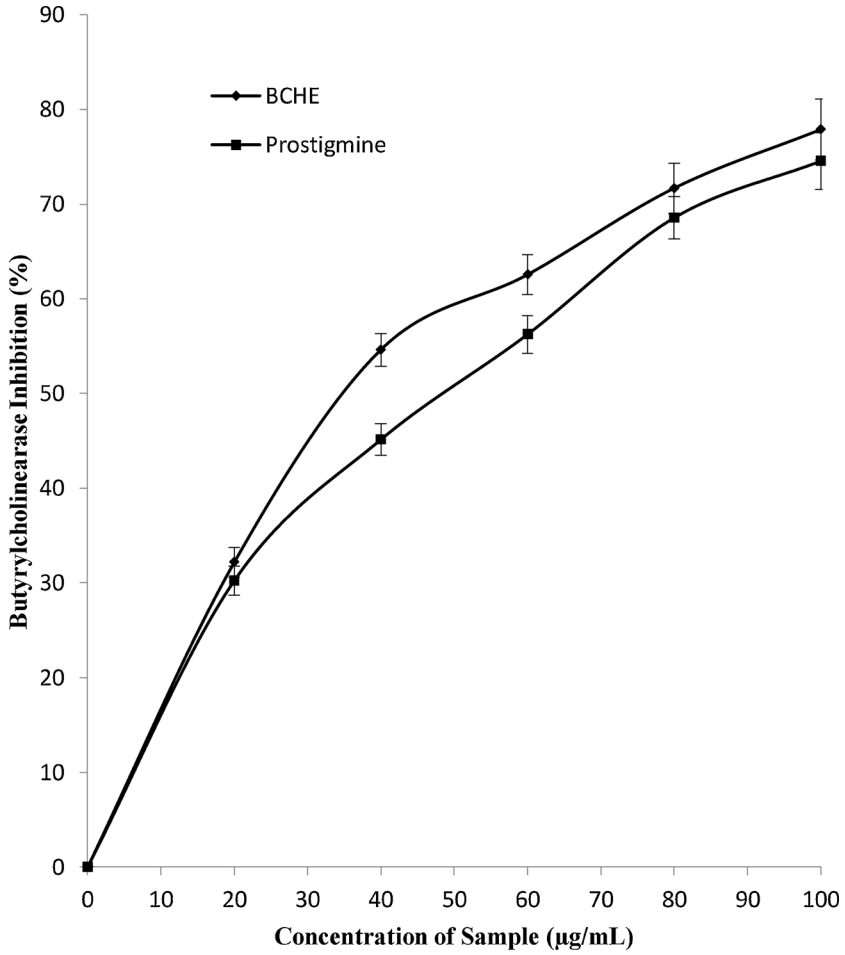

Fig. 5. Butyrylcholinesterase inhibition by phenolic extract from *Tithonia diversifolia* leaves. Data expressed as mean ± SEM (n = 3).

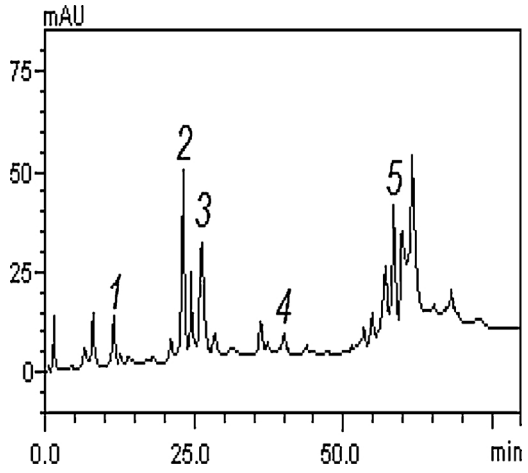

Fig. 6. Representative high performance liquid chromatography profile of *Tithonia diversifolia* extract, detection UV was at 325 nm. Gallic acid (peak 1), chlorogenic acid (peak 2), caffeic acid (peak 3), *p*-coumaric acid (peak 4) and apigenin (peak 5).

The antioxidant properties of the extract further illustrate the potential role of flavonoids antioxidants that could reduce cellular oxidative stress [23].

The reducing power values obtained were moderately high. This may be an indication that the plant extracts possess antioxidant potentials. The higher the absorbance value of the extract, the higher the antioxidant capacities [24,25].

Metal ions which induce oxidative stress have been linked to Alzheimer's disease [4]. By chelating Fe^{2+}, the generation of hydroxyl radicals in the Fenton reaction may be attenuated and thus prevent possible damage to biomolecules [26]. Accumulation of iron has been reported to lead to an increase in free radicals and development of oxidative stress [10].

Phenolics play a vital role in scavenging DPPH radicals by donating electrons or hydrogen to stabilize the radical [15,23,27]. This finding agrees with several earlier studies of positive correlations between total phenol content and DPPH free radical scavenging ability of several plants [24,28].

The results of the ABTS radical scavenging ability of the extracts of *T. diversifolia* and vitamin C showed that the extracts are able to scavenge ABTS radicals, however, *T. diversifolia* had a higher ABTS radical scavenging ability than vitamin C. These ability of the leaf might be due to the hydrogen donating ability of the phenolics present in the extract [23,29]. Hence, the antioxidant mechanism exhibited by *T. diversifolia* leaf could play some part in the prevention of oxidative stress-induced neurodegeneration.

Inhibition of the enzymes linked to Alzheimer's disease with medication has been linked to some side effects such as headache, diarrhoea, drowsiness and vomiting. When AChE is inhibited, acetylcholine degradation in the brain becomes impossible [30,31]. The subsequent increase in brain neurotransmitter acetylcholine concentrations enhance communication between nerve cells and this may reverse the symptoms of Alzheimer's disease temporarily [31,32]. Furthermore, extracts of *T. diversifolia* leaf has higher AChE and BChE inhibitory activities than the standard drug prostigmine. Thus, inhibition of AChE is considered a promising mechanism for the treatment of Alzheimer's

disease. The AChE and BChE inhibition was in agreement with earlier reports showing plant phytochemicals significantly improved cognitive performance and memory [5,33].

High amounts of chlorogenic acid (7.95 ± 0.02 mg g^{-1}) and caffeic acid (5.18 ± 0.02 mg g^{-1}) in *T. diversifolia* may be linked to the biological effects in Alzheimer's disease. The antioxidant properties of plant foods have been associated with presence of an array of important phenolic and nonphenolic phytochemicals including phenolic acids and flavonoids [4,34]. Chlorogenic acid occurs principally in plant foods and has several biological effects such as antioxidant, anti-mutagenic, anti-carcinogenic, anti-inflammatory and endothelial protection [35,36]. Identified phenolic compounds are well acknowledged as potential antioxidants, free radical scavengers, metal chelation agents and inhibitors of lipid peroxidation [27,37,38]. These herbs showed potential for management of Alzheimer's disease as they inhibited cholinergic enzymes (acetylcholinesterase and butyrylcholinesterase) in a concentration-dependent manner and exhibited radical scavenging ability due to the phytochemicals present in the extract.

5. Conclusion

The results suggest that, *T. diversifolia* leaf has potential application as functional food, rich in phenolic compounds and exhibited antioxidant and anti-cholinesterase activity showing a stronger activity than standard drug prostigmine. Hence, the potential mechanism through which the extracts employed their neuroprotective properties may be by inhibiting cholinesterase activities and thwarting oxidative-stress-induced neurodegeneration.

Conflict of interest

The authors declare that there is no conflict of interests whatsoever throughout the compilation of the manuscript.

Transparency document

The Transparency document associated with this article can be found in the online version.

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