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Research Article

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Evaluation of cholinesterase inhibitory and antioxidant activities of free phenolic extract of *Heliotropium. indicum* leaves

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Abstract Alzheimer's disease (AD), one of the leading causes of dementia, is a devastating neurodegenerative condition that primarily affects the brain. It causes memory loss as well as impairments in language and judgment. In this study, the antioxidant and anticholinesterase properties of *Heliotropium. indicum* leaf was investigated. The soluble phenols in dried leaf powder were extracted and the Ellman method was used to assess the ability of the extract to inhibit acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities. DPPH (1, 1-diphenyl-2-picrylhydrazyl) and hydroxyl radical scavenging assays were used to determine the antioxidant activity of the extract. Standard spectrophotometric methods were used to perform quantitative phytochemical (phenolic and flavonoid content) analysis of the leaf extract. The extract inhibited AChE and BChE activity in a dose-dependent manner, with IC_{50} values of 64.99 ± 3.66 and 25.83 ± 1.38 µg/ml, respectively. Furthermore, the scavenging activity of the extract against DPPH (IC_{50} = 42.03 ± 1.33 µg/ml) and hydroxyl (IC_{50} = 50.93± 2.44 µg/ml) radical was lower than that of the standard drug. The extract was discovered to have high phenolic content (307.50 ± 5.04 mg gallic acid equivalent/g dry extract) and flavonoid content (84.75 ± 2.01 mg quercetin equivalent/g dry extract). The significant inhibition of AChE and BChE and potent antioxidant activity in the leaves of *H. indicum* suggest they may be useful in the treatment of Alzheimer's disease.

Keywords Alzheimer's disease, Cholinesterase, *Heliotropium. indicum*, Antioxidant activity

Introduction

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative conditions and the most common type of dementia in which structural and chemical brain disintegration leads to a progressive loss of many aspects of reasoning and behavior [1]. Alzheimer's disease affects around 5.7 million Americans of all ages, with approximately 200,000 persons under the age of 65 experiencing the disease. Records have revealed that deaths from Alzheimer's disease increased dramatically (by approximately 123 %) between 2000 and 2015 [2]. Alzheimer's disease patients frequently experience progressive memory loss and cognitive impairment. None of the known treatments for Alzheimer's disease can slow or stop the illness's progression; they can only treat symptoms. Although there are several contributing variables, aberrant amyloid-beta peptides (Aβ) accumulation remains crucial in the development and course of AD [3-4]. Extracellular amyloid plaques and intracellular neurofibrillary tangles, the two basic histopathological indications of Alzheimer's disease, are seen in vulnerable brain regions such as the hippocampus and cortex. Amyloid plaques are composed of Aβ, the majority of which are 38-43 amino acids long [2]. The role of inflammation in Alzheimer's disease has been documented. Inflammation has been proven in animal

models to impair cognitive performance and produce neuronal injury and synapse loss in vivo and in vitro [5-6]. Pharmaceutical treatment based on natural ingredients is one of the oldest approaches that has been practised throughout human history. Natural products are becoming more popular as people become more concerned about the effects of chemicals on human health and the environment. Surprisingly, there is a natural product-derived medicinal treatment for Alzheimer's disease. Plant-based natural products have been researched extensively as AD therapeutic agents, with seven plant-derived substances identified as anti-Alzheimer medication candidates and AD targets [7].

Heliotropium indicum L. (*Boraginaceae*), often known as cock's comb, is a popular traditional cure for a variety of ailments including stomach pain, convulsion, cataract, conjunctivitis, cold, and high blood pressure [8]. *H. indicum* is a small annual or perennial herb that grows to a height of about 15-50 cm, with opposing leaves and a hairy coating covering the stem and base [9]. This plant has traditionally been used to treat a variety of pathological conditions, including wound healing, bone fracture, eye infection, menstruation disorder, nerve disorder, kidney problem, and for antiseptic purposes [10-12]. Depending on the disease, the plant is prepared and used in various forms such as decoction, powder, cold infusion, poultice, concoction, or squeezing its juice over the affected area. It is used to make soup for postpartum women in some regions of Ghana to treat inflammatory responses and hypertension [8]. Many significant phytochemicals are found in *H. indicum*, including tannins, saponins, steroids, oils, and glycosides [11,13]. The prevalence of *H. indicum* in traditional remedies worldwide has prompted various scientific investigations into its pharmacological action, some of which have resulted in the isolation of pharmacologically important alkaloids. Indicine, indicine-N-oxide, acetyl-indicine, indicinine, heleurine, heliotrine, supinine, supinidine, and lindelofidine are among these alkaloids [14-15]. This study aimed to assess the antioxidant and anticholinesterase activities of H. indicum leaves to determine its relevance for the treatment of AD.

Materials and Methods

Collection and identification of plant sample

The leaves of *Heliotropium. indicum* were collected from a local farmland in Ikere Ekiti South-Western Nigeria. The plant was authenticated by Mr Omotayo (Herbarium curator) at the Department of Plant Science and Biotechnology, Ekiti State University, Ado Ekiti, Nigeria where the voucher specimen was deposited.

Chemicals and reagents

Acetylthiocholine iodide, aluminium chloride, ammonium molybdate, ascorbic acid (AA), bicinchoninic acid, DPPH, Folin–Ciocalteu reagent, Tris-HCl and Triton X-100, Gallic acid (GA) 2-deoxy-D-ribose, thiobarbituric acid and 5,5-dithio-bis-(2-nitro) benzoic acid were obtained from Sigma-Aldrich (Irvine, UK). Other chemicals were of analytical grade.

Extraction of free phenols of *H. indicum* leaves

The extraction of the soluble free phenols was carried out as reported previously by Chu *et al*. [16]. The leaf powder (10 g) was extracted with 80% acetone (1:5 w/v) for 10 h at room temperature. The mixture was filtered through Whatman no 2-filter paper on a Buchner funnel under vacuum. The filtrate was then evaporated on a rotary evaporator under vacuum at 45°C until approximately 90% of the filtrate had been evaporated. The residual solvent was allowed to evaporate in a fume cupboard.

Determination of total phenolic content

The spectrophotometric method was used to determine the total phenolic content [17]. A volume of 1 ml of the sample (1 mg/mL) was mixed with 1 ml of the Folin-phenol Ciocalteu's reagent. After 5 minutes at room temperature, 10 ml of 7% Na_2CO_3 solution was added to the mixture, followed by 13 ml of deionized distilled water, and thoroughly mixed. The mixture was kept in the dark for 90 minutes at 23 ℃ before the absorbance at 750 nm was measured. The total phenolic content was calculated by extrapolating a calibration curve created by preparing a gallic acid (GA) solution. The estimation of the phenolic compounds was carried out in triplicate. The total phenolic content of the dried sample was expressed in milligrams of gallic acid equivalents (GAE).

Determination of total flavonoid content

The total flavonoid content was estimated by the aluminum chloride method described by Yang et al. [18]. The extract (0.5 mL) was mixed with 2.5 mL of distilled water and 150 µL $NaNO_2$ solution (5%). The contents were vortexed for 10 seconds and then left at room temperature for 5 minutes. Thereafter, 300 µL $AlCl_3$ (10%), 1 mL NaOH (1 mM) and 550 µL of distilled water were added. The solution was thoroughly mixed and kept at room

temperature for 15 minutes. The absorbance of each sample was measured at 510 nm. Quercetin (Q) concentrations ranging from 31.25–500.00 µg/mL were prepared and the standard calibration curve was obtained. The total flavonoid content was calculated using a standard Q calibration curve. The results are given in milligrams of quercetin equivalents (QE) per gram of dry extract.

Determination of DPPH radical scavenging activity

The extract's free radical scavenging activity was determined using the previously established in vitro DPPH test [19]. The stock solution was prepared by dissolving 24 mg DPPH in 100 ml of methanol and storing it at 20 °C until needed. Using a spectrophotometer, the working solution was made by diluting DPPH solution with methanol to achieve an absorbance of approximately 0.98 ± 0.02 at 517 nm. At various concentrations (31.25-500 µg/ml), a 3 ml aliquot of this solution was mixed with 100 µl of the sample. The reaction mixture was thoroughly agitated before being incubated in the dark for 15 minutes at room temperature. The absorbance was then measured at 517 nm. The control was created in the same manner as described above but without any sample. The scavenging activity was calculated using the following equation based on the proportion of DPPH radical scavenged:

% DPPH radical scavenging activity = [(Abs control – Abs sample)]/(Abs control) ×100

Determination of hydroxyl radical scavenging activity

The extract's hydroxyl radical scavenging activity was measured using the method described by Islam *et al.* [20]. The technique is based on the detection of the 2-deoxy-D-ribose degradation product by condensation with thiobarbituric acid (TBA). Hydroxyl radical was generated by the Fe^{3+} ascorbate-EDTA-H_2O_2 system (the Fenton reaction). The reaction mixture contains 2-deoxy-D-ribose (2.8 mM); KH2PO4-KOH buffer (20 mM, pH 7.4); FeCl3 (100 µM); EDTA (100 µM); H2O2 (1.0 mM); AA (100 µM); and various concentrations of the test sample or reference standard BHT in a final volume of 1 ml. After 1 hour of incubation at 37 °C, 0.5 ml of the reaction mixture was added to 1 ml of 2.8% trichloroacetic acid, followed by 1 ml of 1% aqueous TBA, and the mixture was incubated at 90 °C for 15 minutes to develop the colour. After cooling, the absorbance of the mixture was measured at 532 nm against a blank solution. The following formula was used to obtain the percentage (%) of hydroxyl radical scavenging ability:

% hydroxyl radical scavenging activity = [(Abs control – Abs sample)]/(Abs control) ×100

Determination of cholinesterase inhibitory activity

The AChE inhibitory assay was carried out using Ellman's *et al* [21] colorimetric approach using acetylthiocholine iodide as a substrate. Rat brains were homogenized in a homogenizer with five volumes of ice-cold homogenization buffer (10 mM Tris-HCl (pH 7.2), 1 M NaCl, 50 mM $MgCl_2$, and 1% Triton X-100) and centrifuged at 10000 g for 30 minutes to obtain the enzyme source. The supernatant that resulted was employed as an enzyme source. All extraction processes were completed at 4 °C. A bicinchoninic acid kit (Sigma Co., St. Louis, MO, USA) with bovine serum albumin as a protein standard was used to assess protein content. The rates of AChE hydrolysis were measured spectrophotometrically. The extractor standard (500 µl) was combined with an enzyme solution (500 µl) and incubated for 15 minutes at 37 °C. Absorbance was measured at 405 nm immediately after adding Ellman's reaction mixture (3.5-ml 0.5 mM acetylthiocholine, 1 mM 5, 5′-dithio-bis (2-nitro benzoic acid)) in a 50 mM sodium phosphate buffer (pH 8.0). Readings were taken every 2 minutes for 10 minutes to ensure that the reaction occurred linearly. The blank reaction was tested by replacing the enzyme with saline. Galanthamine was used as a positive control. The % inhibition of AChE activity was estimated as follows:

% of inhibition of AChE activity= [(Abs control – Abs sample)]/ (Abs control) ×100

The assessment of BChE inhibition was carried out in the same manner as stated previously, with the exception that the enzyme solution was 50 µL and acetylthiocholine iodide was substituted by butyrylthiocholine iodide. The percentage inhibition of BChE activity was estimated using the same formula for AChE activity indicated above.

Statistical analysis

Graph Pad prism was used to analyze the data using One-way analysis of variance (ANOVA). The results were presented as mean SD and values.

Results

***In vitro* cholinesterase enzyme activity**

The extract's AChE and BChE inhibitory activities were measured to assess its potential as an anti-AD drug. Figures 1 and 2 show that the extract inhibited AChE and BChE significantly ($p < 0.05$) when compared to the standard, and

the cholinesterase inhibitory activity was dose-dependent. The AChE inhibitory activity of the extract was found to be 25.71 ± 2.68, 35.86 ± 3.43, 46.72 ± 2.21, 55.11 ± 3.09 and 66.39 ± 2.69 % at a concentration of 31.25, 62.5, 125, 250, and 500 µg/ml, respectively, with an IC_{50} of 64.99 ± 3.66 µg/mL (Table 1 The extract inhibited BChE by 43.69 ± 2.34, 51.57 ± 2.35, 62.01 ± 3.39, 66.89 ± 4.19, 74.33 ± 3.92 % at a concentration of 31.25, 62.5, 125, 250, and 500 µg/ml, respectively, with IC_{50} of 25.83 ± 1.38µg/ml. Galantamine was used as a reference standard in this study. Galantamine's IC50 against AChE and BChE was 41.03 2.36 g/mL and 26.44 1.64 g/mL, respectively (Table 1).

Table 1: IC_{50} values of extract and standards in enzymes inhibitory and radicals scavenging activity assays

	IC_{50} values (µg/ml)			
Sample	**AChE**	**BChE**	**DPPH radical scavenging**	**Hydroxyl radical scavenging**
Extract	64.99 ± 3.66	25.83 ± 1.38	42.03 ± 1.33	50.93± 2.44
BHT	-	-	28.13 ± 1.02	29.72 ± 1.86
Galantamine	41.03 ± 2.36	26.44 ± 1.64	-	-

Each value is expressed as mean ± SD (n = 3).

Table 2: Polyphenolic content of leaf extract of *H. indicum*

Polyphenols	**Extract**
Total Phenolics (mg/Gallic acid Equivalent)	307.50 ± 5.04
Total Flavonoids (mg/Quercetin Equivalent)	84.75 ± 2.01

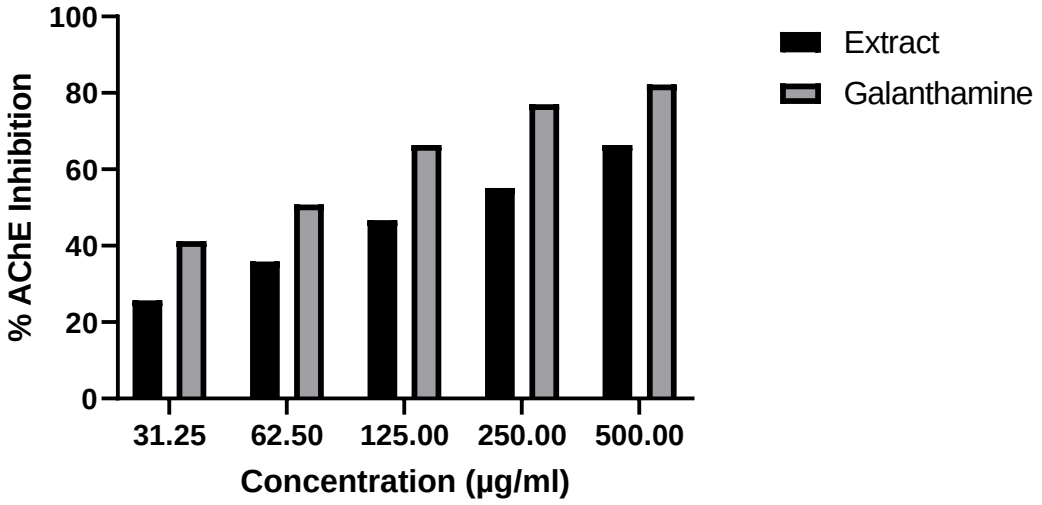

Figure 1: Concentration-dependent response of H. indicum leaf extract and galanthamine against AChE each value is expressed as means± SD (n = 3).

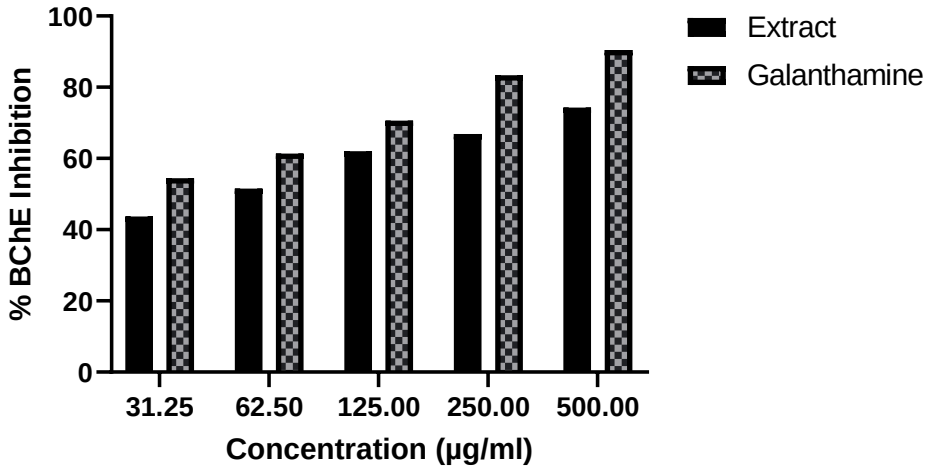

Figure 2: Concentration-dependent response of H. indicum leaf extract and galanthamine against BChE each value is expressed as means± SD (n = 3).

DPPH free radical scavenging activity

The extract's in vitro antioxidant activity was evaluated in comparison to that of the reference antioxidant, BHT. However, there was a dose-dependent relationship between the percentage of DPPH radical scavenging activity. With an IC_{50} 42.03 ± 1.33 µg/ml, the extract had less scavenging activity than the standard, which had an IC_{50} of 28.13 ± 1.02 µg/ml (Figure 3 and Table 1). This suggests that the extract has the ability to attenuate oxidative stress caused by free radicals.

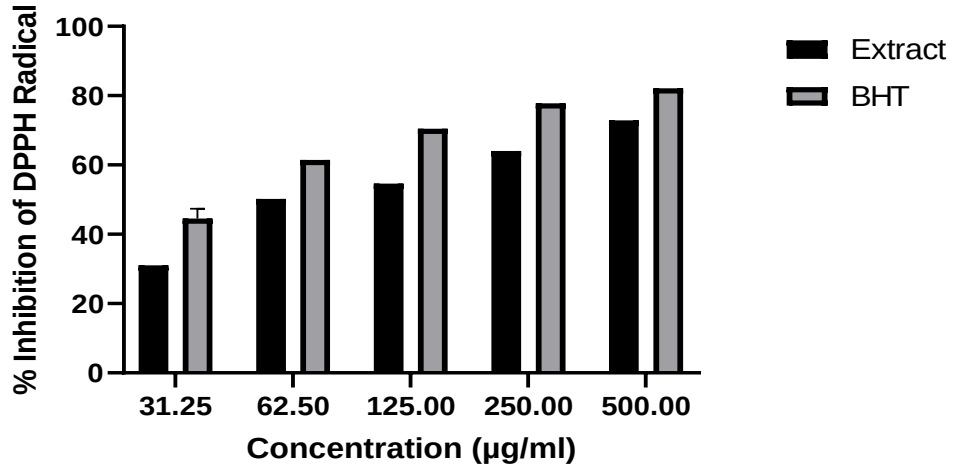

Figure 3: DPPH radical scavenging activity at different concentrations of H. indicum and BHT each value is expressed as means± SD (n = 3)

Hydroxyl radical scavenging activity

The hydroxyl radical is the primary reactive oxygen species that causes lipid oxidation and potentially severe biological damage. This experiment demonstrates how extract and BHT inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe3+ ascorbate - EDTA - H_2O_2 system (the Fenton reaction). At a concentration of 500 µg/ml, the extract and BHT had hydroxyl radical scavenging capacities of 78.90 ± 4.28 % with IC_{50} of 50.93± 2.44 µg/mL and 91.51 ± 4.88% with IC_{50} of 29.72 ± 1.86 µg/ml, respectively (Table 1). Although the results show that extract has a lower capacity for hydroxyl radical scavenging than the standard drug (Figure 4), the extract could still

be a source of antioxidant. Furthermore, the ability of the extract to quench hydroxyl radicals may be directly related to the prevention of lipid peroxidation.

Total phenolic content

The total phenolic content of the extract was determined using the Folin - Ciocalteu's method and calculated as gallic acid equivalent (GAE). The extract's phenolic content was 307.50 ± 5.04 mg GAE /g of dry extract (Table 2).

Total flavonoid content

The total flavonoid content was determined using the aluminum chloride colorimetric method. The total flavonoid content was expressed as Quercetin equivalents (QE). The extract contained 84.75 ± 2.01mg QE /g of dry extract (Table 2).

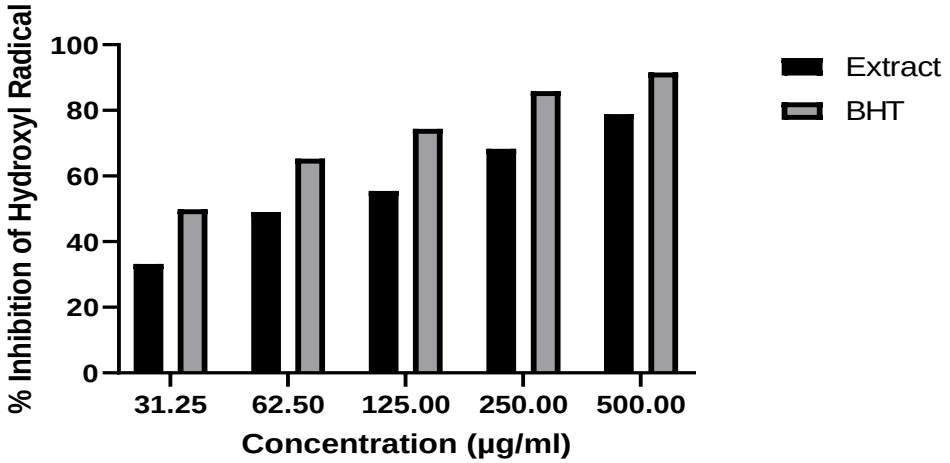

Figure 4: Hydroxyl radical scavenging activity at different concentrations of H. indicum and BHT each value is expressed as means± SD (n = 3).

Discussion

Alzheimer's disease (AD) is a long-term neurodegenerative condition marked by a progressive decline in memory, cognition, and behavior. It typically manifests slowly at first before getting worse over time and eventually resulting in death [22]. Several etiological factors, including genetic abnormalities, a history of head injuries, environmental factors, general lifestyles, depression or hypertension, the deposition of extracellular -amyloid protein (A) and microtubule associated tau protein in the brain, and cholinergic dysfunction, have all been implicated in AD; however, the exact mechanisms by which these factors contribute to the development of AD are not well understood [23]. There are currently no medications that can reverse Alzheimer's disease or any of the other common forms of dementia, but two theoretical methods for treating AD have been developed. The primary progenitors are sequestered in the first strategy to stop AD from developing. This treatment aims to delay the onset of the disease. The second strategy involves symptomatically treating the disease's secondary cognitive symptoms to stave off further cognitive deterioration [24]. Effective treatment plans, however, are implemented based on the severity of the illness and the individuality of each case. Donepezil, galantamine, rivastigmine, and a partial NMDA receptor antagonist are the only cholinesterase inhibitors currently approved by the FDA for treating AD [23].

Cholinesterase inhibitors prevent acetylcholine from being broken down at synapses, thereby increasing the amount of acetylcholine present. Memantine, a partial NMDA receptor antagonist, shields neurons from excitatory damage by glutamate [25]. Few cholinesterase inhibitors are currently used in therapies, and they have a wide range of toxic effects, including anorexia, diarrhoea, fatigue, nausea, gastrointestinal disorders, and cardiovascular disorders [25-26]. Because of this, scientists have shifted their attention to finding new drugs derived from plants, which hold great promise for treating AD. In this investigation, we discovered that extract can significantly inhibit AChE and BChE activities in a dose-dependent manner (Figures. 1 and 2). The extract has been shown to be an effective cholinesterase inhibitor and can be used in the treatment of AD [23]. There is growing evidence that oxidative stress (OS) exposure occurs during the disease progression in AD patients' brain tissues. Glycoxidation, protein oxidation, lipid oxidation, DNA oxidation, and other biological damage brought on by OS are all closely related to the onset of

AD, cancer, diabetes, and other diseases [27]. OS is generally characterized by an imbalanced production of ROS and reactive nitrogen species (RNS). In living cells, free radicals like the superoxide anion radical (O2•-), hydrogen peroxide (H2O2), hydroxyl radical (•OH), singlet oxygen (1O2), alkoxyl radicals (RO•), and peroxyl radicals (ROO•) have a propensity to pair with biological macromolecules like proteins, lipids, and DNA to become stable. As a result of this trend, it has been hypothesized that OS play a role in the pathogenesis of a wide variety of degenerative diseases in humans [23, 27]. Antioxidative defense mechanisms work to eliminate ROS and stop cellular damage by quenching free radicals, guarding against diseases like AD [28]. Such defense mechanisms are essential for shielding living things from the harmful effects of free radical assaults, but when the rate of free radical generation outpaces the effectiveness of this defense mechanism, elevated ROS levels can result, which can cause extensive biological damage [20]. Increased ROS levels contribute to the pathogenesis seen during AD progression. Therefore, we used the DPPH bleaching assay, a standard measure of antioxidant activity [19], to quantify the scavenging potential of the extract.

In this assay, the extent of color change is proportional to the potential and concentration of antioxidant activity, conferred by the hydrogen-donating ability [29]. In our study, the extract showed high scavenging percentage of DPPH, reflecting its potent antioxidant activity (Figure 3). The hydroxyl radical is an extremely damaging ROS formed by successive monovalent reduction of dioxygen (O_2), capable of initiating lipid peroxidation which results in severe cell damage in vivo. The short-lived hydroxyl radical is particularly damaging to the polyunsaturated fatty acid of cell membrane phospholipids with harmful effects on the cell [30]. The extent of color change in this assay is proportional to the potential and concentration of antioxidant activity conferred by hydrogen donating ability [20]. The extract demonstrated a high DPPH scavenging percentage in our study, indicating its potent antioxidant activity (Figure 3). The hydroxyl radical is a highly destructive reactive oxygen species (ROS) formed by the sequential monovalent reduction of dioxygen (O_2), which is capable of initiating lipid peroxidation and leading to severe cell damage in vivo [30]. The extract demonstrated promising hydroxyl radical scavenging activity and could protect deoxyribose in a dose-dependent manner in our study. Evidence shows that the extract's hydroxyl radical activity is directly proportional to its antioxidant activity [20]. (Figure 4). Phenolic and flavonoid compounds are antioxidant that can act as free radical scavengers to prevent cellular damage [23, 30]. Our study found a reasonable quantity of phenolic and flavonoid content, implying that the extract can reduce the risk of various degenerative diseases, including Alzheimer's disease, by eliciting antioxidative activities to prevent OS damage (Table 2). Research has shown that flavonoids can neutralize various oxidizing species, including free radicals [23]. Based on these findings, it appears that the polyphenolic components of the extract may play a role in free radical neutralization and cholinesterase activity inhibition. Additional research is necessary to isolate and characterize the active polyphenol compound that shows promise as a potential drug treatment for AD.

Conclusion

This present study provided experimental evidence that the extract from leaves of *H. indicum* contained substantial amounts of polyphenols and flavonoids and exhibited potential antioxidants and radical scavenging activities by scavenging free radicals as well as effectively inhibiting AChE and BChE activities. Therefore, the plant has promising compounds to be tested as potential drugs for the treatment of diseases resulting from oxidative stress like AD.

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