

Evaluation of cholinesterase inhibitory and antioxidant potential of *Wedelia chinensis*: possible implications in alleviating Alzheimer's disease

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Abstract

Background

Wedelia chinensis has been reported as a folk medicine for the treatment of different diseases including neurodegenerative disease. Although the plant has been studied well for diverse biological activities, the effect of this plant in neurological disorder is largely unknown. The present study was undertaken to evaluate the cholinesterase inhibitory and antioxidant potential of *W. chinensis*.

Methods

The extract and fractions of the plant were evaluated for acetylcholinesterase and butyrylcholinesterase inhibitory activity by modified Ellman method. The antioxidant activity was assessed in several *in vitro* models/assays such as reducing power, total antioxidant capacity, total phenolic and flavonoid content, scavenging of 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radical and hydroxyl radical, and inhibition of brain lipid peroxidation. Chromatographic and spectroscopic methods were used to isolate and identify the active compound from the extract.

Results

Among the extract and functions, aqueous fraction (AQF) and ethylacetate fraction (EAF) exhibited high inhibition against acetylcholinesterase (IC_{50} : $40.02 \pm 0.164 \mu\text{g/ml}$ and $57.76 \pm 0.370 \mu\text{g/ml}$) and butyrylcholinesterase (IC_{50} : $31.79 \pm 0.182 \mu\text{g/ml}$ and $48.41 \pm 0.053 \mu\text{g/ml}$). Similarly, the EAF and AQF had high content of phenolics and flavonoids and possess strong antioxidant activity in several antioxidant assays including DPPH and hydroxyl radical scavenging, reducing power and total antioxidant activity. They effectively inhibited the peroxidation of brain lipid *in vitro* with IC_{50} values of $45.20 \pm 0.995 \mu\text{g/ml}$ and $25.53 \pm 0.042 \mu\text{g/ml}$, respectively. A significant correlation was observed between total flavonoids and antioxidant and cholinesterase inhibitory activity. Activity guided chromatographic separation led to the isolation of a major active compound from the EAF and its structure was elucidated as apigenin by spectral analysis.

Conclusions

The potential ability of *W. chinensis* to inhibit the cholinesterase activity and peroxidation of lipids suggest that the plant might be useful for the management of AD.

Background

Wedelia chinensis, locally known as Bhoringoraj, belongs to the family Asteraceae. It is grown in Dhaka, Mymensingh, Tangail, Patuakhali, Barisal and sporadically in some other areas of Bangladesh. The plant is traditionally used to treat liver enlargement, jaundice and other ailments of the liver and gall bladder. It is also used in the treatment of Juvenile arthritis, rheumatic fever, cough, cephalagia, diseases of skin, uterine hemorrhage and menorrhagia. The leaves of the plant are often used for dyeing grey hair, promoting hair growth and tonic [1, 2]. It has been reported that the decoction of the plant is used for strengthening the nervous system and to treat multiple sclerosis [3]. The plant is indicated for many ailments in traditional Ayurvedic and Unani system of medicine. Biological investigations of this plant have shown that the plant possesses multiple pharmacological effects such as anti-cancer, anti-hepatotoxic, anti-inflammatory, anti-microbial and anti-oxidant activities [4, 5, 6, 7, 8]. In a neuropharmacological study, the plant exhibited a protective role in D-galactose induced neuronal cell loss and CNS depressant activity in mice [9, 10]. Phytochemical study reported the isolation of four active compounds, such as wedelolactone, indole-3-carboxylaldehyde, apigenin and luteolin, of which the latter two compounds are flavonoids [9]. Although the plant has demonstrated a magnitude of therapeutic activities, the protective effect of this plant in neurodegenerative diseases is largely unknown.

Degeneration of the central nervous system results in a variety of neurological disorders including Alzheimer's disease (AD). AD is the most devastating neurodegenerative disorder of the elderly people and the most common cause of dementia. Cholinergic neuron, which is involved in the regulation of memory and cognition, is severely impaired in AD. The most remarkable features found in AD are cholinergic dysfunction associated with a progressive decline in neurotransmitter acetylcholine (ACh) [11]. Therefore, inhibition of cholinesterases, which catalyze the hydrolysis of acetylcholine, is the promising therapeutic approach in AD. In addition, oxidative stress has been detected as one of the common neurotoxic pathway in neurodegenerative diseases including AD. It has been shown that A β protein, which is excessively generated in AD, can produce reactive oxygen species including free radical, and make an imbalance between ROS and antioxidant system leading to oxidative stress [12, 13]. Free radicals are capable of attacking most of the cellular biomolecules such as DNA, protein, and lipid. Among the oxidized molecules, increased peroxidation of has been found in the brain of AD which can be determined as thiobarbituric reactive species [14, 15]. The increased peroxidation might result from the relative abundance of polyunsaturated fatty acid in the brain. Plant derived antioxidants are reported to protect the oxidative stress-induced neurotoxicity in AD [16].

This study was an attempt to evaluate the cholinesterase inhibitory and antioxidant activities of the extracts of *W. chinensis* using *in vitro* models and to isolate and characterize the active constituent.

Methods

Chemicals

Donezepil, galantamine, acetylthiocholine and S-butyrylthiocholine, DPPH (2,2'-diphenyl-1-picrylhydrazyl), ammonium molybdate, Folin-Ciocalteu reagent, thiobarbituric acid (TBA), trichloroacetic acid (TCA), 2-deoxy-D-ribose, 5,5'-dithio-bis-(2-nitro) benzoic acid (DTNB), triton X-100, aluminum chloride, potassium ferricyanide, and Tris-HCl

were procured from Sigma-Aldrich, Germany. Catechin, ascorbic acid and gallic acid were obtained from Wako Pure Chemical Company Ltd., Japan. Methanol, ethylacetate, chloroform and petroleum ether were purchased from Active Fine Chemicals Limited, Dhaka, Bangladesh. All other chemicals, unless specified, were of analytical grade.

Animals and collection of brain

Mice were used only for collection of brain as a source of crude acetylcholinesterase enzyme. Swiss Albino Mice having 5-6 weeks were purchased from the Animal House, Jahangirnagar University, Savar, Dhaka and were caged in the experimental room. A standard diet and water ad libitum were given to the mice. The study was carried out in compliance with the ARRIVE guidelines. The international ethical guidelines were followed to deal with the laboratory animals. The procedures were approved by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC) of the University of Rajshahi, Bangladesh (*Ethical clearance number: 102*).

To collect brain from mice, mice were anesthetized with sodium pentobarbital (30 mg/kg; intraperitoneal injection; Taj Pharmaceuticals Ltd., India) and then sacrificed by cervical dislocation. Cervical dislocation was performed by a trained person. Brains were taken quickly; washed in ice cold saline and homogenized in 50 mM Tris buffer, PH 7.4 to yield the crude acetylcholinesterase enzyme [17].

Collection and extraction of plant materials

The whole plant was collected from the district of Natore, Bangladesh in March 2016 and authenticated by Professor Dr. A.H.M. Mahbubur Rahman, Department of Botany, Rajshahi University, where a voucher specimen (accession no. 370) have been deposited.

Crude methanol extract (CME, 18.5 g) was prepared from plant powder (500 g) of *W. chinensis* by hot extraction with methanol in a soxhlet apparatus. The extract (10 g) was then concentrated *in vacuo* with a rotary evaporator and suspended in water. It was then sequentially partitioned with solvents of increasing polarity such as petroleum ether, chloroform, ethylacetate and water by the method as described earlier [17] to yield the corresponding petroleum ether (PEF, 3.2 g), chloroform (CLF, 2.5 g), ethylacetate (EAF, 1.4 g) and aqueous (AQF, 2.9 g) fractions. All the fractions were preserved in a refrigerator at 4°C until further use.

Phytochemical analysis

Phytochemical screening of the plant extract

Qualitative tests were performed to identify the classes of phytochemicals such as flavonoids, glycosides, alkaloids, tannins, saponins, and steroids in the different fractions by the methods as described earlier [18].

Quantitation of total phenolic content (TPC)

Folin-Ciocalteu method was used to determine the the total phenolic content of the extractives of *W. chinensis* as described [19]. To a mixture of 2.5 ml of 10% Folin-Ciocalteu reagent and 2.5 ml of 7.5% sodium carbonate solution, 0.5 ml sample was added and left in the dark for 20 minutes at 25⁰C. The absorbance of the reaction mixture was recorded by a spectrophotometer at 760 nm. A standard curve was obtained for gallic acid and the phenolic content was determined from extrapolation of this curve.

Quantitation of total flavonoid content (TFC)

Aluminum chloride colorimetric method was used to measure the total flavonoid content of the extracts of *W. chinensis* as described earlier [20]. To a mixture of methanol (3.0 ml), 10% AlCl₃ (0.2 ml), 1M potassium acetate (0.2 ml) and 5.6 ml of distilled water, plant extract (1.0 ml) was added and left at room temperature for 30 minutes. The absorbance of the reaction mixture was recorded by a spectrophotometer at 420 nm. A standard curve was obtained for catechin and the flavonoid content was determined from extrapolation of this curve.

Antioxidant activity

Reducing power assay

The reducing ability of the extracts of *W. chinensis* was determined by the method of Oyazu et al. (1986) [21]. To a mixture of 0.2 M potassium buffer (2.5 ml) and 1% potassium ferricyanide (2.5 ml), plant extract (1 ml) was added and incubated at 50°C for 20 minutes. Then 10% TCA solution (2.5 ml) was added to the reaction mixture and centrifuged (3000 rpm) for 10 minutes. Finally, 2.5 ml of solution was mixed with 2.5 ml of ultrapure water and 0.5 ml of 0.1% ferric chloride solution. The absorbance of the reaction mixture was recorded at 700 nm. A reference standard catechin was used for comparison.

Total antioxidant capacity assay

The antioxidant capacity of the extracts of *W. chinensis* was assessed by the method as described earlier [18]. To a mixture of sulphuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM), plant extract was added and heated in a water bath at 95⁰C for 90 min. After cooling to room temperature, the absorbance of the mixture was recorded at 695 nm against blank. A reference compound catechin was used for comparison.

DPPH radical scavenging assay

The ability of the extracts of *W. chinensis* to scavenge DPPH radical was measured by the modified method of Choi *et al.* (2000) [22]. A reference compound catechin was used for comparison. Methanolic solution of plant extract or reference compound was mixed with 0.135 mM of methanolic DPPH and left in dark for 30 minutes. The absorbance of the reaction mixture was recorded at 517 nm. The percent scavenging was calculated using the equation:

$$[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where, A_{control} is the absorbance of control and A_{sample} is the absorbance of extract or reference compound. The percentage inhibition was plotted against the compound concentration in order to calculate the IC_{50} values.

Determination of hydroxyl radical scavenging activity

The capacity of the extracts of *W. chinensis* to scavenge hydroxyl radical was assessed by the modified method of Elizabeth *et al.* (1990) [23]. A reference compound catechin was used for comparison. Plant extract or reference compound was added to a 1 ml reaction mixture containing 2.8 mM 2-deoxy-2-ribose, 20 mM phosphate buffer (pH 7.4), 100 μ M $FeCl_3$, 100 μ M EDTA, 1 mM H_2O_2 and 100 μ M ascorbic acid and then incubated at 37°C for 60 minutes. 0.5 ml of the reaction mixture was mixed with 1 ml of TCA (2.8%) and 1 ml of TBA (1%) and heated in a water bath at 90°C for 15 minutes. After cooling to room temperature, the absorbance of the mixture was recorded at 532 nm in a spectrophotometer against an appropriate blank solution. The percent scavenging of hydroxyl radical was calculated as in DPPH radical scavenging assay.

Determination of lipid peroxidation inhibition activity

The ability of the extracts of *W. chinensis* to inhibit the peroxidation of lipid was assessed by the method as described [18]. Brain homogenate was employed for in vitro lipid peroxidation assay. Mice brain homogenates were prepared by homogenizing brain in 50 mM phosphate buffer (pH 7.4) containing 0.15 M KCl using a homogenizer and centrifuged at 10000 *g* at 4°C for 20 min. To a mixture of brain homogenates (0.5 ml), 0.15 M KCl (1 ml) and 10 μ M hydrogen peroxide (100 μ l), plant extract was added and incubated at 37°C for 30 minutes. A solution of 2 ml of HCl (0.25 N) containing TCA (15%), TBA (0.38%), and BHT (5%) was added to the reaction mixture and heated in a water bath at 80°C for 60 minutes. After cooling to room temperature, the mixture was centrifuged to separate the supernatant and then the absorbance was measured at 532 nm by spectrophotometer. The percent inhibition of lipid peroxidation was determined as in DPPH radical scavenging assay. A reference standard catechin was used for comparison.

Cholinesterase inhibitory activities

The assessment of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibiting activities were performed by the colorimetric method of Ellman *et al.* (1961) [24]. Crude AChE enzyme was prepared from mice brain as mentioned above and BChE enzyme was prepared from human blood according to the method as described earlier [18]. The acetylthiocholine iodide and butyrylthiocholine iodide were used as substrates for investigation of AChE and BChE assays, respectively. The hydrolysis of acetylthiocholine and butyrylthiocholine were determined spectrophotometrically. Plant extract or reference compound was added in an enzyme solution and incubated at 37°C for 15 min for interaction. This was followed by the addition of a 50 mM sodium phosphate buffer (pH 8.0) containing 0.5 mM acetylthiocholine and 1 mM DTNB and immediately the absorbance of the solution was recorded against

a blank solution. All the experiments were taken in triplicate. For comparison, a reference compound donepezil was used for AChE activity and galantamine was used for BChE activity. The percent inhibition of cholinesterase activity was computed using the equation:

$$[(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where, A_{control} is the absorbance of control and A_{sample} is the absorbance of extract or reference compound. IC_{50} value could be calculated from the dose response curve obtained by plotting the percent inhibition values against test concentrations of each extract or compound.

Isolation and characterization of an active compound from the bioactive extract

The EAF (5.6 g) of *W. chinensis* was subjected to column chromatography using silica gel 60 (Merck, Germany) as a stationary phase in an open column and sequentially eluted with n-hexane, dichloromethane and methanol stepwise gradient to yield five major subfractions (F1 to F5). Fraction F2 with potent AChE and BChE inhibitory activity was purified on silica gel GF₂₅₄ by preparative thin layer chromatography with n-hexane-acetone (6:4) as the mobile phase to obtain the pure compound **1** (18 mg).

¹H- and ¹³C-NMR spectra of the compound **1** was recorded in DMSO-d₆ on a Jeol-Ex 400 MHz and FT-NMR 100 MHz spectrometers. The chemical structure of the compound **1** was confirmed by comparing its spectral data with the reported values in the literature [27].

Statistical Analysis

All experiments were carried out in triplicate. The results were expressed as mean $\pm$ SD. Graph Pad Prism (version 8.0.1) and Microsoft Excel 2010 were used for the statistical and graphical evaluations. T-test was employed to estimate the statistical significance (P-value $\leq$ 0.05) between the average values. IC_{50} values of different fractions/extractives were calculated using non-linear regression (Dose-Response – Inhibition equation; log₁₀ (inhibitor) vs. normalized response – variable slope) in Graph Pad Prism - 8.0.1. Correlation study was performed using Pearson correlation test.

Results

Phytochemical analysis

A preliminary phytochemical analysis conducted on the CME revealed that the plant contains tannins, phenolics and flavonoids, alkaloids, phytosterols and saponins. Qualitative analysis of the four fractions showed that they all contained phenolics and flavonoids, but higher amounts were found in the EAF and AQF (Supplementary Table S1).

Assays for total phenolic and flavonoid content of the extractives revealed that EAF contained the highest content of phenolics (97.28 $\pm$ 0.49 mg GAE/g dried extract) followed by CME (80.00 $\pm$ 0.62 mg GAE/g dried

extract), AQF (61.05±0.21 mg GAE/g dried extract), CLF and PEF (Table 1). Whereas, AQF contained the highest content of flavonoids (175.78±0.69 mg CE/g dried extract), followed by CME (174.02±1.01 mg CE/g dried extract), EAF (144.35±0.51 mg CE/g dried extract), CLF and PEF.

Table 1. Total phenolic and flavonoid contents and antioxidant activity of the extract and fractions of *Wedelia chinensis*.

TPC (mg GAE/g dried extract)	TFC (mg CE/g dried extract)	DPPH IC ₅₀ (µg/mL)	OH IC ₅₀ (µg/mL)	RP (absorbance at 80 µg/mL)	TAC (absorbance at 80 µg/mL)	LPI IC ₅₀ (µg/mL)
80.00±0.62 ^b 7.34±0.16 ^e	174.02±1.01 ^b 2.68±0.23 ^e	10.13±0.44 ^d 27.46±0.52 ^f	42.59±0.51 ^d 160.33±2.37 ^f	1.783±0.03 ^d 0.942 ± 0.01 ^f	0.456±0.008 ^d 0.347±0.005 ^f	47.18±1.07 ^d 416.20±1.51 ^f
33.52±0.20 ^d	33.46±0.34 ^d	21.53±0.46 ^e	105.47±2.01 ^e	1.383±0.02 ^e	0.365±0.005 ^e	222.60±3.36 ^e
97.28±0.49 ^a	144.35±0.51 ^c	4.78±0.01 ^a	28.21±0.39 ^c	2.245±0.06 ^c	0.529±0.007 ^b	45.20±0.10 ^c
61.05±0.21 ^c	175.78±0.69 ^a	8.60±0.04 ^c	9.65±0.25 ^a	2.542±0.05 ^a	0.556±0.008 ^a	25.53±0.04 ^a
-	-	5.14±0.10 ^b	14.89±0.25 ^b	2.451±0.07 ^b	0.489±0.004 ^c	30.91±0.20 ^b

PEF, petroleum ether fraction; CLF, chloroform fraction; EAF, ethylacetate fraction; AQF, aqueous fraction; CA, catechin. TPC: Total phenolic content, TFC: Total flavonoid content, OH: Hydroxyl radical scavenging, RP: Reducing power, TAC: Total antioxidant capacity and LPI: Lipid peroxidation inhibition. Means in each column with different subscript letters (a b, c, d, e, f) differ significantly (P <0.05).

Cholinesterase inhibitory activity

The CME and its fractions were evaluated for AChE inhibition at different concentration (0-200 µg/ml) using the widely used Ellman method. The percent inhibition of AChE by the extractives has been presented in Fig. 1A. Donepezil was used as the reference AChE inhibitor in this study that showed an IC₅₀ of 9.21±0.45 µg/ml. All the test extract and fractions exerted dose dependent inhibition of AChE enzyme. The IC₅₀ of CME was found to be 93.64±0.28 µg/ml. Among the fractions, high activity was found in AQF and EAF with IC₅₀ values of 40.02±0.16 µg/ml and 57.76±0.37 µg/ml, respectively. CLF and PEF showed less activity with IC₅₀ of 121.97±0.74 µg/ml and 152.60±1.14 µg/ml, respectively (Fig. 1A). Similarly, in BChE inhibitory assay, the CME showed good activity with IC₅₀ of 69.09±0.44 µg/ml. AQF and EAF exhibited high inhibitory activity with IC₅₀ of 31.79±0.18 µg/ml and 48.41±0.05 µg/ml, respectively (Fig. 1B). The IC₅₀ of CLF and PEF were 122.50±0.20 µg/ml and 148.87±0.50 µg/ml. Taken together, the AQF and EAF possess appreciable activity against both AChE and BChE enzymes.

Antioxidant activity

The antioxidant activity of the extractives of *W. chinensis* were assessed by using several *in vitro* models such as DPPH and hydroxyl free radicals scavenging, reducing power and total antioxidant activity.

DPPH is a stable free radical which is widely used for evaluation of scavenging activity of the antioxidant. The percent scavenging of DPPH free radical by different concentration of extract has been shown in Fig. 2A. Catechin was used as the reference antioxidant that showed an IC_{50} of 5.14 ± 0.10 mg/ml. The IC_{50} values of CME, EAF, AQF, CLF and PEF were 10.13 ± 0.44 , 4.78 ± 0.01 , 8.60 ± 0.04 , 21.53 ± 0.46 and 27.46 ± 0.52 mg/ml, respectively, indicating that the EAF possesses the highest radical scavenging activity followed by AQF. EAF was found to be more potent than that of the standard catechin whose IC_{50} was 5.14 ± 0.10 μ g/ml. The CLF and PEF had relatively lower DPPH radical scavenging activity.

Hydroxyl radical is the most harmful radical among the radicals generated in the biological system. Hydroxyl radicals were generated in vitro in Fenton reaction and the ability of the extractives to scavenge the radicals was determined (Fig. 2B). AQF was found to possess the highest scavenging activity followed by EAF with IC_{50} values of 9.65 ± 0.25 μ g/ml and 28.21 ± 0.39 μ g/ml, respectively. It was noted that AQF had higher scavenging activity than that of the reference antioxidant catechin which showed an IC_{50} of 14.89 ± 0.25 μ g/ml. The IC_{50} for CME was found to be 42.59 ± 0.51 μ g/ml. PEF and CLF had relatively lower activity with IC_{50} of 160.33 ± 2.37 and 105.47 ± 2.01 μ g/ml, respectively.

Reducing power assay was used for assessing the reducing ability of the CME and its fractions and the result has been shown in the Fig. 3A. All the extract and fractions were found to possess the reducing activity and the activity was increased with the increase of the concentration of the extract. At high concentration of 80 mg/ml, the absorbance of CME, AQF, EAF, CLF, PEF and catechin were 1.783 ± 0.027 , 2.542 ± 0.05 , 2.245 ± 0.06 , 1.383 ± 0.02 , 0.942 ± 0.01 and 2.451 ± 0.07 , indicating that AQF has the highest activity followed by CAT, EAF, CME, CLF and PEF. Notably, the activity of AQF was found to be higher than that of the standard antioxidant catechin.

The total antioxidant activity of the extractives was assessed based on their capacity to reduce Mo (VI) to Mo (V) and the result has been shown in the Fig. 3B. Similar to reducing power, the AQF and EAF exhibited high total antioxidant activity among the extractives (Figure 3B). At high concentration of 80 mg/ml, CME, AQF, EAF, CLF, PEF and catechin gave an absorbance of 0.456 ± 0.008 , 0.556 ± 0.008 , 0.529 ± 0.007 , 0.365 ± 0.005 , 0.347 ± 0.005 and 0.489 ± 0.004 , respectively. Interestingly, the total antioxidant activity of AQF and EAF were found to be greater than that of the standard antioxidant catechin.

Oxidation of lipid by free radicals results in lipid peroxidation. In this study, lipid peroxidation of the mouse brain homogenate was induced by hydrogen peroxide and the effect of the different extractives of *W. chinensis* in the inhibition of lipid peroxidation were assessed via thiobarbituric acid reactive species (TBARS). As shown in the Fig. 4, incubation of mouse brain homogenate with hydrogen peroxide caused a significant increase of lipid peroxidation. All the extracts inhibited lipid peroxidation in a dose dependent manner. Among the extractives screened, the AQF and EAF exhibited high inhibitory activity with IC_{50} values of 25.53 ± 0.04 μ g/ml and 45.20 ± 0.10 μ g/ml, respectively. The IC_{50} of CME was 47.18 ± 1.07 μ g/ml. The CLF and PEF had little activity with IC_{50} of 222.60 ± 3.36 and 416.20 ± 1.51 μ g/ml,

respectively. These results suggest that the AQF and the EAF can effectively inhibit the peroxidation lipid caused by free radicals.

Table 2. Correlation of total phenolic and flavonoid contents with cholinesterase inhibition and antioxidant activities

Assays	R ² values	
	Total phenolic content	Total flavonoid content
Acetylcholinesterase inhibition	0.6325	0.7748*
Butyrylcholinesterase inhibition	0.7284	0.9036*
DPPH radical scavenging	0.9263*	0.8728*
Reducing power	0.6180	0.7811*
Total Antioxidant Capacity	0.6448	0.8035*
Hydroxyl radical scavenging	0.7711	0.9165*
Lipid peroxidation inhibition	0.8263*	0.9042*

* indicates statistical significance (P<0.05).

Correlation between total phenolic and flavonoid content and the acetylcholinesterase inhibitory and antioxidant activities

Phenolics and flavonoids have been reported to be associated with the antioxidant activity [25, 26]. Since AQF and EAF contained a large amount of phenolics and flavonoids and exhibited high cholinesterase inhibitory and antioxidant activities, we therefore tested their correlations by Pearson's correlation analysis and the result has been given in the Table 2. The content of total flavonoids showed a statistically significant correlation with DPPH radical scavenging activity ($R^2 = 0.8728$, $p < 0.05$), reducing activity ($R^2 = 0.7811$, $p < 0.05$), total antioxidant activity ($R^2 = 0.8035$, $p < 0.05$), hydroxyl radical scavenging ($R^2 = 0.9165$, $p < 0.05$), lipid peroxidation inhibition ($R^2 = 0.9042$, $p < 0.05$), AChE ($R^2 = 0.7748$, $p < 0.05$) and BChE ($R^2 = 0.9036$, $p < 0.05$) inhibitory activities. Whereas the content of phenolics showed a significant correlation with DPPH radical scavenging activity ($R^2 = 0.9263$, $p < 0.01$), lipid peroxidation inhibition ($R^2 = 0.8263$, $p < 0.05$) and good correlation with other antioxidant and cholinesterase activities ($R^2 = 0.62$ - 0.77).

Table 3. ¹H NMR and ¹³C NMR data (δ in ppm and J in Hz) of compound **1**

H/C	¹ H NMR	¹³ C NMR
2	Q	157.96
3 (CH)	6.77 (1H, s)	103.51
4	Q	182.13
4a	Q	105.02
5	Q	161.43
6 (CH)	6.18 (1H, d, <i>J</i> = 2.0 Hz)	99.99
7	Q	165.25
8 (CH)	6.57 (1H, d, <i>J</i> = 2.0 Hz)	95.00
8a	Q	164.26
1'	Q	122.51
2' (CH)	7.93 (1H, d, <i>J</i> = 8.5 Hz)	129.00
3' (CH)	6.93 (1H, d, <i>J</i> = 8.5 Hz)	116.40
4'	Q	161.21
5' (CH)	6.93 (1H, d, <i>J</i> = 8.5 Hz)	116.33
6' (CH)	7.93 (1H, d, <i>J</i> = 8.5 Hz)	128.75

Activity guided isolation of an active compound

Due to potential bioactivity, the EAF was investigated further to isolate and identify the active compounds. Activity guided chromatographic separation resulted in the isolation of a major compound **1** from the EAF and the structure of the compound **1** was established as apigenin by direct comparison of its ¹H- and ¹³C- NMR spectral data with previously reported values (Table 3, Fig. 5) [27]. The compound exhibited inhibition of AChE and BChE enzymes as well as antioxidant activity, which are in accordance with the previous results [28, 29]. Further studies are required to isolate and identify the active compounds present in the AQF.

Discussion

Alzheimer's disease is a progressively developing neurodegenerative disorder of the elderly people and the most common cause of dementia. Until now there is no effective treatment for AD. Approximately 50 million people are affected worldwide in AD which will triple by 2050 [30]. The problem is much more pronounced in the developing countries [31]. Due to the growing population and extended lifespan, AD has become a serious health concern in the elderly people. To date, only three cholinesterase inhibitors namely donepezil, rivastigmine, galantamine and one partial NMDA receptor antagonist memantine are the only approved drugs by the Food and Drug Administration (FDA) to treat AD [32]. These drugs offer the symptomatic relief of the disease and only slow the progression modestly, but does not stop the progression of AD [33]. The handful of drugs and the limitations of their use led us to develop new potent drugs. Plant has already proved to be an important source of different classes of drugs and new candidate drugs for AD has been developed. *W. chinensis* is a potential medicinal herb with multiple biological activities [1, 2, 3]. Herein, we report for the first time the cholinesterase inhibitory and antioxidant activities of the extractives of *W. chinensis* in vitro and apigenin as the major compound contributing to the activity.

The crude methanol extract (CME) of *W. chinensis* was fractionated with petroleum ether, chloroform, ethylacetate and water for investigating their phytochemical profile and biological activity. Qualitative analysis of the CME showed the different classes of phytochemicals present in the extract which were differentially distributed in the different solvent fractions. Interestingly, phenolics and flavonoids were found to be present in each fraction, but high concentration was found in the ethylacetate and AQF (Supplementary Table S-1). Phenolics and flavonoids are an important class of secondary metabolites of plants that display important biological activities. They are known as natural antioxidants due to the ability to scavenge free radical by donating electron or hydrogen. Quantitative analysis of phenolic and flavonoid content revealed that EAF contained the highest content of phenolics (97.28 ± 0.49 mg GAE/g dried extract) followed by CME (80.00 ± 0.62 mg GAE/g dried extract) and AQF (61.05 ± 0.21 mg GAE/g dried extract). Whereas AQF contained the highest content of flavonoids (175.78 ± 0.69 mg CE/g dried extract) followed by CME (174.02 ± 1.01 mg CE/g dried extract) and EAF (144.35 ± 0.51 mg AE/g dried extract). PEF and CLF had lower content of phenolics and flavonoids (Table 1). The presence of large amount of phenolics and flavonoids in the EAF and AQF indicated that they might have potential biological activity.

AChE is still a therapeutic target of choice among other targets for the development of AD drug. Inhibitors of AChE increase the concentration of acetylcholine at the synapse, augment the cholinergic neurotransmission and improve the memory and cognition in animal. Medicinal plants contain a diverse chemical compounds having cholinesterase inhibiting properties and currently used in the treatment of AD [34]. Interest in natural AChE inhibitor has increased due to less toxic effects of the natural compounds. In this study, we report for the first time a substantial AChE inhibitory activity of the CME of *W. chinensis* and its fractions (Fig. 1A). IC_{50} values were calculated for quantitative evaluation of the inhibitory potency of the different fractions. The IC_{50} value of CME was found to be 93.64 ± 0.28 μ g/ml. A large number of plants used in traditional medicine use to enhance memory have been investigated, but only a few of them have been found to possess an acceptable level of AChE inhibitory capacity [35]. In comparison with these plants, *W. chinensis* extract appeared to be a potential AChE inhibitor. When the fractions were evaluated, highest inhibition was found in the AQF and EAF with the IC_{50} values of 40.02 ± 0.16 μ g/ml and 57.76 ± 0.37 μ g/ml, respectively, indicating the polarity of the active compounds. In contrast, CLF and PEF had less activity and their IC_{50} were 121.97 ± 0.74 μ g/ml and 152.60 ± 1.14 μ g/ml, respectively. Pearson's correlation showed a significant association of total flavonoid content with the AChE inhibitory activity ($R^2 = 0.7748$, $p < 0.05$) (Table 2). These results suggest that the extract of *W. chinensis* and its fractions possess an appreciable AChE inhibitory activity which might be attributed to flavonoid compounds.

After AChE, BChE is another drug target of choice for AD. BChE is a cholinesterase involved in the catalysis of acetylcholine and plays a co-regulatory role in the cholinergic neurotransmission that accounts for 20% of cholinesterase activity [36]. The increased activity of BChE in the late stage of pathogenesis suggests it as a target for AD drug. There are reports that dual inhibition of AChE and BChE have been found a better outcome in AD [37]. Of the three approved AChE inhibitor, rivastigmine has dual

activity. In this investigation, the crude methanol extract of *W. chinensis* was found to exert inhibition of BChE with an IC_{50} value of $69.09 \pm 0.44 \mu\text{g/ml}$ (Fig. 1B). This result suggests that the extract has potential BChE inhibitory activity and exhibits similar specificity for BChE as AChE. Among the fractions of CME, EAF and AQF showed the highest inhibition against BChE with IC_{50} values of $69.09 \pm 0.44 \mu\text{g/ml}$, $48.41 \pm 0.05 \mu\text{g/ml}$, $31.79 \pm 0.18 \mu\text{g/ml}$, indicated the polar nature of the active compounds. Pearson's correlation showed a significant association ($R^2 = 0.9036$, $p < 0.05$) of flavonoid content with BChE inhibitory activity (Table 2). Taken together, these results suggest a dual role of *W. chinensis* and hold promise as a source of drug for dual activity.

Oxidative stress plays a crucial role in the development of AD. Oxidative stress results from the excessive production of free radicals by amyloid beta-protein, a major culprit according to amyloid cascade hypothesis [12, 13]. Although a number of biomolecules are affected due to oxidative stress, lipids in neuronal membrane are most vulnerable that lead neuronal dysfunction and death. Lipid peroxidation is thus considered as an important marker for oxidative stress in AD [17, 18, 38]. In this study, we found the potential antioxidant activity of the crude methanol extract and its fractions in all antioxidant assays when compared with the other medicinally important plant. In DPPH radical scavenging, which is a stable synthetic radical, EAF showed the highest activity followed by AQF and CME with IC_{50} values of 4.78 ± 0.01 , 8.60 ± 0.04 , $10.13 \pm 0.44 \mu\text{g/ml}$, respectively (Fig. 2A). The IC_{50} value of catechin was found to be $5.14 \pm 0.10 \mu\text{g/ml}$. In hydroxyl radical scavenging, which is relevant to biological system, AQF showed the highest activity followed by CAT, EAF and CME with IC_{50} values of 9.65 ± 0.25 , 14.89 ± 0.25 , 28.21 ± 0.39 and $42.49 \pm 0.51 \mu\text{g/ml}$, respectively (Fig. 2B). These results suggest that EAF and AQF are potential radical scavenger due to hydrogen donating abilities. Similarly, in reducing power assay, AQF showed the highest activity followed by CAT, EAF and CME (Fig. 3A) and in total antioxidant capacity test, AQF showed the highest activity followed by EAF, CAT and CME (Fig. 3B). At high concentration of $80 \mu\text{g/ml}$, the absorbance of AQF, CAT, EAF and CME were 2.542 ± 0.05 , 2.452 ± 0.07 , 2.245 ± 0.06 and 1.783 ± 0.03 in reducing power assay and 0.556 ± 0.01 , 0.488 ± 0.00 , 0.529 ± 0.01 and 0.456 ± 0.01 in total antioxidant assay, respectively (Table 1). Both the assays indicated the potential ability of the EAF and AQF to donate electron or proton to free radicals in terminating the chain reaction. It is noteworthy that the activity of AQF was superior to the standard antioxidant catechin; whereas the activity of EAF was very close that of catechin. The antioxidant activity of the crude extracts was reflected in the inhibition of peroxidation of brain lipid from mouse. Among the extractives, the AQF showed the highest inhibition followed by CAT, EAF and CME with IC_{50} values of 25.53 ± 0.04 , 30.91 ± 0.20 , 45.20 ± 0.10 and $47.18 \pm 1.07 \mu\text{g/ml}$, respectively (Fig. 4). Pearson's correlation showed a strong association of total flavonoid content with DPPH radical scavenging, reducing power, total antioxidant activity, hydroxyl radical scavenging, lipid peroxidation inhibition, AChE and BChE inhibitory activity (Table 2). While a significant correlation was observed between total phenolic content and DPPH radical scavenging activity, and lipid peroxidation inhibition and a moderate association was found between total phenolic content with hydroxyl radical scavenging & BChE inhibitory activity. These results indicated strong association of flavonoids with both the cholinesterase inhibitory and antioxidant activities.

Because the crude methanol extract of *W. chinensis* and its fractions were found to possess potential cholinesterase inhibitory and antioxidant activity, we explored the bioactive compounds from EAF by bioassay guided chromatography. A major active compound was isolated from EAF and its structure was established as apigenin by ^1H - and ^{13}C - NMR spectral studies (Table-3, Fig. 5) [27]. The compound exerted substantial inhibition of AChE and BChE enzymes and exhibited appreciable antioxidant activity. Apigenin is one of the most naturally occurring flavonoids that are found in edible and medicinal plants. The compound has been reported earlier to inhibit A β aggregation, and neuroinflammation involved in AD [39]. Apigenin can be absorbed in the intestine and cross the blood brain barrier, suggesting its potential as a therapeutic agent in the neurodegenerative pathologies including AD. In the present study, the isolation of apigenin from *W. chinensis* having both cholinesterase inhibitory and antioxidant properties, which according to the previous report, can be useful to prevent or slow down the progression of AD. Taken together, all these evidences suggest that apigenin has neuroprotective potential to prevent or slow down the progression of AD.

Conclusion

Our results demonstrated that *Wedelia chinensis* exerts substantial inhibition of cholinesterase activity and antioxidant properties, which could be useful for the management of Alzheimer's disease. Apigenin was identified as a major compound that contributes to the inhibitory activities. To the best of our knowledge, our studies are the first to report the cholinesterase inhibition and antioxidant properties of this plant. The present findings warrant further evaluation of this plant in *in vivo* animal models.

Abbreviations

AD: Alzheimer's disease; AChE: Acetylcholinesterase; BChE: Butyrylcholinesterase; DPPH: 2,2'-diphenyl-1-picrylhydrazyl; TPC: Total phenolic content; TFC: Total flavonoid content; CME: Crude methanolic extract; PEF: Petroleum ether fraction; CLF: Chloroform fraction; EAF: Ethylacetate fraction; AQF: Aqueous fraction; DON: Donepezil; GAL, Galantamine; CAT: Catechin.

Declarations

Acknowledgments

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Competing interests

The authors declare that they have no competing financial interests.

Authors' contributions

MAI, KB, MKH carried out the collection and extraction of plant materials, isolation of compound and the assays for antioxidant and acetylcholinesterase inhibitory activities; MYA, AHMKA performed the statistical analysis, interpretation of data and drafted the manuscript; GS, SZ, TT has made substantial contribution to conception and design, participated in general coordination of the study, and revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

International ethical guidelines were followed to deal with the animals and the procedures were approved by the Institutional Animal, Medical Ethics, Biosafety and Biosecurity Committee (IAMEBBC), Institute of Biological Sciences, University of Rajshahi, Bangladesh (*Ethical clearance number: 102*).

Consent for publication

Not applicable.

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Figures

Fig. 1

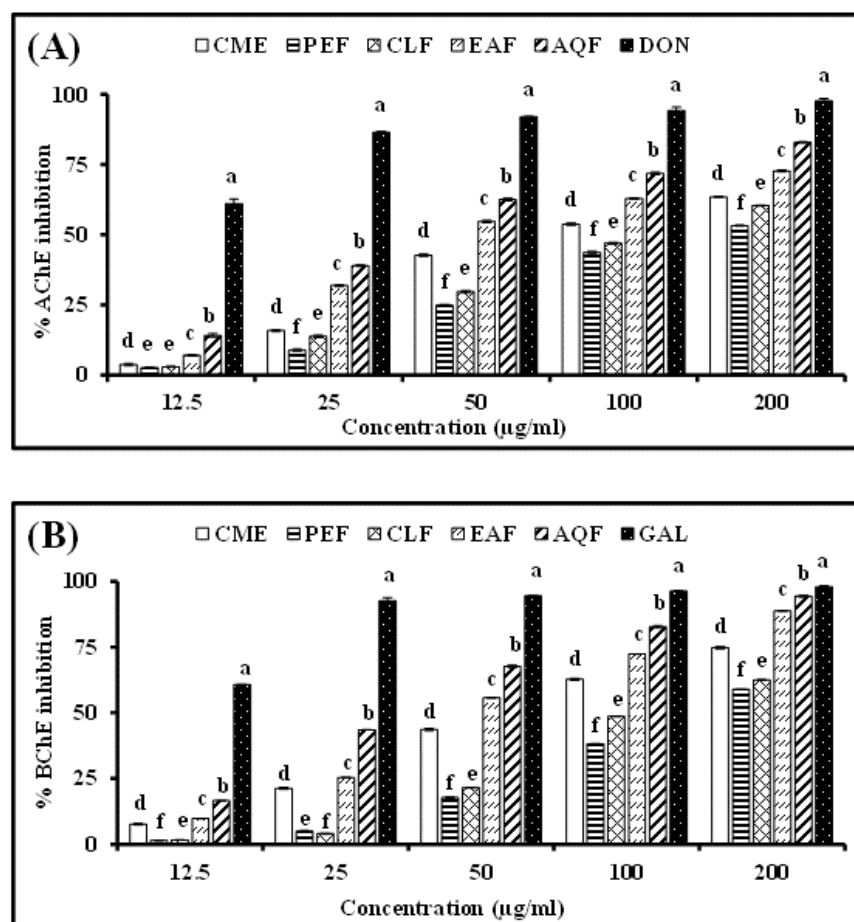


Figure 1

Cholinesterase inhibitory activities of the extract and fractions of *W. chinensis*. (A) Inhibition of acetylcholinesterase (AChE) by extract and fractions of *W. chinensis* and standard donepezil. IC₅₀ (µg/ml): CME, 93.64±0.28; PEF, 152.60±1.14; CLF, 121.97±0.74; EAF, 57.76±0.37; AQF, 40.02±0.16; DON, 9.21±0.45. (B) Inhibition of butyrylcholinesterase (BChE) by extract and fractions of *W. chinensis* and standard galantamine. IC₅₀ (µg/ml): CME, 69.09±0.44; PEF, 148.87±0.50; CLF, 122.50±0.20; EAF, 48.41±0.05; AQF, 31.79±0.18; GAL, 10.62±0.19. Results are expressed as mean±SD (n=3). Means with different letters (a-f) differ significantly (P<0.05). CME, crude methanolic extract; PEF, petroleum ether fraction; CLF, chloroform fraction; EAF, ethylacetate fraction; AQF, aqueous fraction; DON, Donepezil; GAL, Galantamine.

Fig. 2

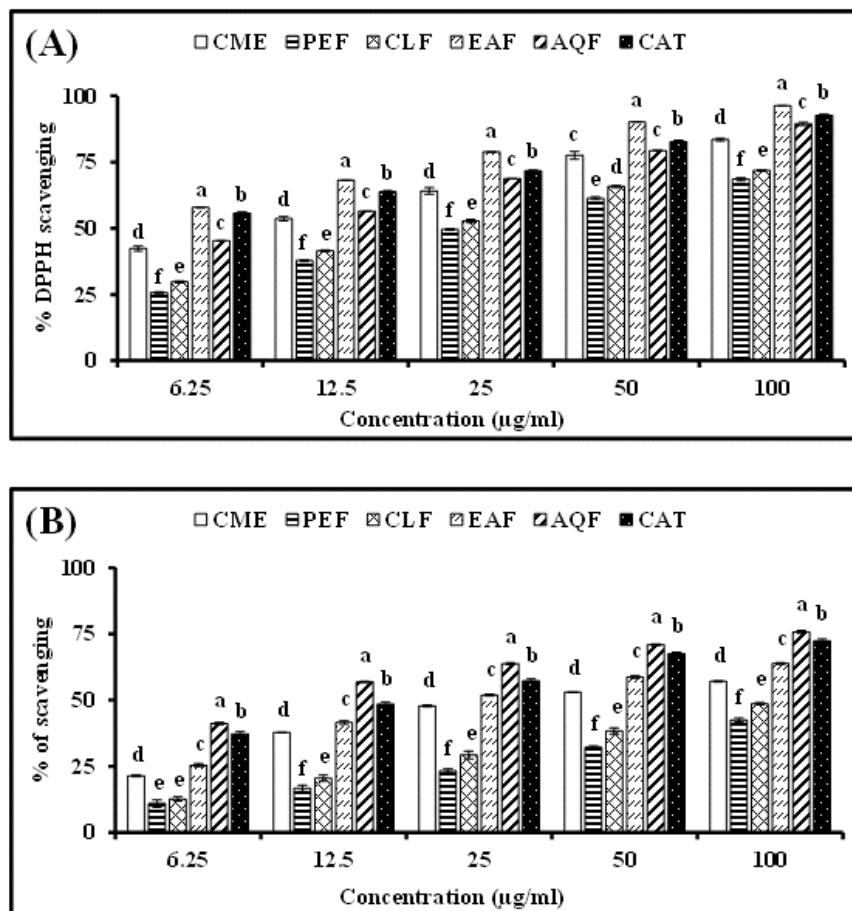


Figure 2

Radical scavenging activities of extract and fractions of *W. chinensis*. (A) DPPH radical scavenging activities of extract and fractions of *W. chinensis* and standard catechin. (B) Hydroxyl radical scavenging activities of extract and fractions of *W. chinensis* and standard catechin. Means with different letters (a-f) differ significantly ($P < 0.05$). CME, crude methanolic extract; PEF, petroleum ether fraction; CLF, chloroform fraction; EAF, ethylacetate fraction; AQF, aqueous fraction; CAT, catechin.

Fig. 3

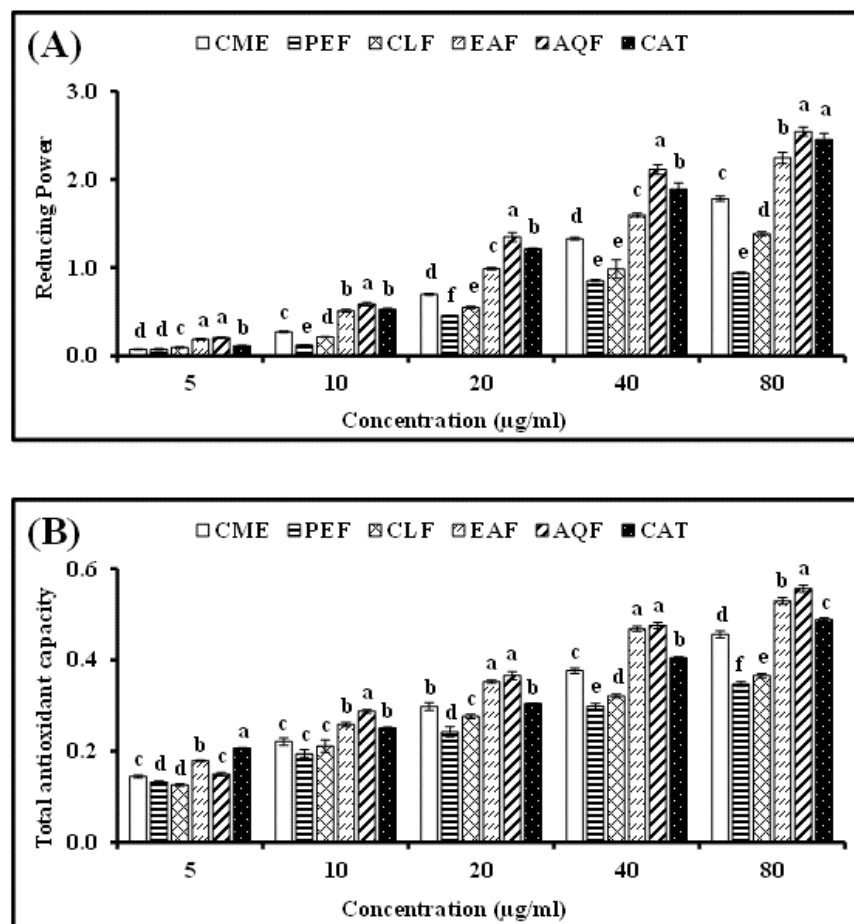


Figure 3

Radical scavenging activities of extract and fractions of *W. chinensis*. (A) DPPH radical scavenging activities of extract and fractions of *W. chinensis* and standard catechin. (B) Hydroxyl radical scavenging activities of extract and fractions of *W. chinensis* and standard catechin. Means with different letters (a-f) differ significantly ($P < 0.05$). CME, crude methanolic extract; PEF, petroleum ether fraction; CLF, chloroform fraction; EAF, ethylacetate fraction; AQF, aqueous fraction; CAT, catechin.

Fig. 4

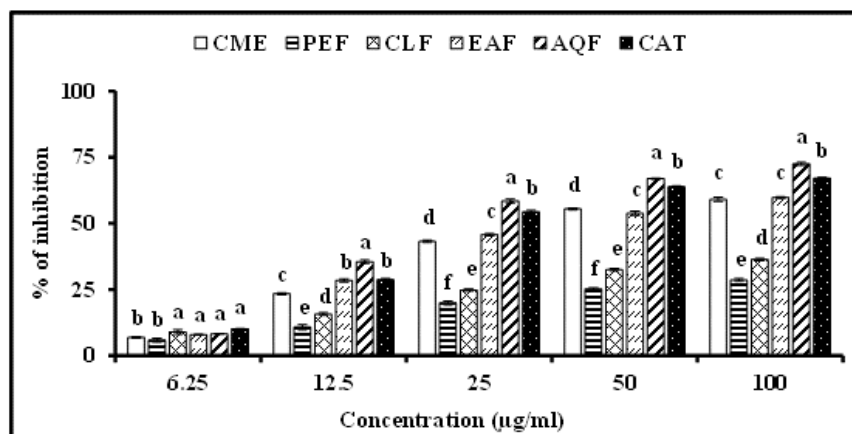


Figure 4

Lipid peroxidation inhibitory activity of extract and fractions of *W. chinensis* and the standard catechin. Results are expressed as mean $\pm$ SD (n=3). Means with different letters (a-f) differ significantly (P<0.05). CME, crude methanolic extract; PEF, petroleum ether fraction; CLF, chloroform fraction; EAF, ethylacetate fraction; AQF, aqueous fraction; CAT, catechin.

Fig. 5

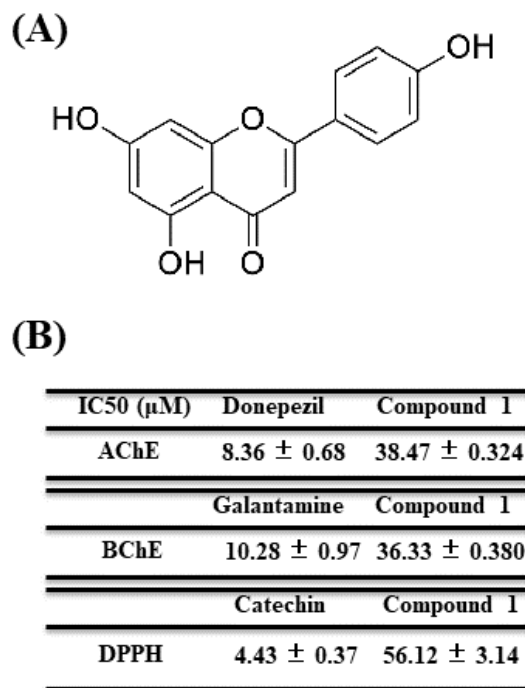


Figure 5

Lipid peroxidation inhibitory activity of extract and fractions of *W. chinensis* and the standard catechin. Results are expressed as mean±SD (n=3). Means with different letters (a-f) differ significantly (P<0.05). CME, crude methanolic extract; PEF, petroleum ether fraction; CLF, chloroform fraction; EAF, ethylacetate fraction; AQF, aqueous fraction; CAT, catechin.

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