Potent AChE enzyme inhibition activity of *Zizyphus oxyphylla*: A new source of antioxidant compounds

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Abstract: The purpose of this study was to assess the antioxidant potential and enzyme inhibition of various fractions of *Zizyphus oxyphylla*. The plant metabolites were extracted in methanol and partitioned with *n*-hexane, chloroform, ethyl acetate and *n*-butanol successively. Phytochemical screening showed presence of alkaloids, terpenoids and flavonoids in ethyl acetate and *n*-butanol fractions. The antioxidant potential and acetylcholine esterase assay of all these fractions and remaining aqueous fraction was evaluated by using reported methods. The results revealed that chloroform soluble fraction exhibited highest percent inhibition of DPPH radical as compared to other fractions. It showed 95.01±0.37% inhibition of DPPH radical at a concentration of 120µg/mL. The IC_{50} of this fraction was 13.20±0.27µg/mL, relative to butylated hydroxytoluene (BHT, a reference standard), having IC_{50} of 12.10±0.29µg/mL. It also showed highest total antioxidant activity i.e. 1.723±0.34 as well as highest FRAP value (339.5±0.57TEµM/mL) and highest total phenolic contents (142.65±1.20 GAE mg/g) as compared to the other studied fractions. The fractions were also studied for Acetylcholine esterase enzyme (AChE) enzyme inhibition activity and *n*-butanol soluble fraction exhibited maximum inhibition (95.5±0.13mg/mL with IC_{50} =9.58±0.08mg/mL relative to galanthamine (13.26±0.73mg/mL), while *n*- hexane soluble fraction (165.15±0.94mg/mL) showed non-significant. We are still working to isolate pure compounds for active fractions targeting potent inhibition responsible for some activities.

Keywords: *Zizyphus oxyphylla*, phytochemical screening, antioxidant activity, acetylcholine esterase assay.

INTRODUCTION

Medicinal plants are a good source of biologically active compounds known as phytochemicals. Many of these phytochemicals act as antioxidants by scavenging free radicals, and many have remedial potential for disorders caused by free radical (Javanmardia *et al*., 2003;Lee *et al*., 2000). The phenolic compounds present in medicinal plants have strong antioxidant activity and may help to protect the cells against the oxidative damage (Kahkonen *et al*., 1999). They are well known as radical scavengers, metal chelators, reducing agents, hydrogen donors and singlet oxygen quenchers (Proestos *et al*., 2006). Antioxidants from plant materials cease the action of free radicals and protected the body from various diseases (Lai *et al*., 2001). Antioxidants have been shown to prevent the destruction of cells (Slonim *et al*., 1983; Murthy *et al*., 1992) and to prevent or inhibit oxidation processes in human body and food products (Diaz *et al*., 1997; Yen *et al* 2000). In this study, the antioxidant activity of a medicinal plant *Zizyphus oxyphylla* has been investigated and reported.

Acetylcholine, the first identified neurotransmitter is a principal neurotransmitter in the peripheral, central, somatic and the autonomic nervous system. It relays the information across the gap (synapse) between the neuron and its neighboring cells. In the absence of acetylcholine esterase enzyme, acetylcholine accumulates at the synapse, thus paralysis occurs and it also stops the heart beat (Campbell& Reece, 2002). Acetylcholine esterase enzyme (AChE) hydrolyzes the acetylcholine into choline and acetate group and blocks its signaling effect (Luis *et al*., 2006). Inhibitors of AChE are used for the treatment of neurological disorders such as Alzheimer's disease, senile dementia, ataxia and myasthenia gravis (Rahman *et al*., 2001; Ahmad *et al*., 2003).

Zizyphus oxyphylla belongs to family Rhamnaceae. Locally this plant is known as "Mamyanu or Elanai" (Ajaib*et al*., 2010). Ethno botanically, its leaves are used in diabetes; fruits are edible and used in gas trouble. Leaves of *Zizyphus oxyphylla* also reported to have antipyretic and antinociceptive activities and it is used in the treatment of pain and fever (Nisar *et al*., 2007). The various plants of genus *Zizyphus* are reported to have anti-inflammatory, antimicrobial, antioxidant, antitumor, hypoglycemia, hypotensive, liver protective and immune function improvers (Borgi *et al*., 2007; Ahmed *et al*., 2005) and also used in digestive disorders, weakness, liver complaints, diabetes, diarrhea and skin infections (Alves *et al*., 2000; Kirtikar *et al*., 1984). Previous phytochemical studies have established on the different species of the genus *Zizyphus* proved it as a rich source of cyclopeptide alkaloids, triterpenoids, saponins, sterol (Croueour *et al*., 2002). *Zizyphus oxyphylla* as a new natural antioxidant source having antioxidant potential and enzyme inhibition activity.

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MATERIALS AND METHODS

Plant material

The plant *Zizyphus oxyphylla* was collected from Azad Kashmir, and identified by Mr. Muhammad Ajaib (Taxonomist), Department of Botany, GC University, Lahore. A Voucher specimen (GC-Herb-Bot-851) has been deposited in the herbarium of the Botany department of the same University.

Extraction and fractionation of antioxidants

The shade-dried ground whole plant (5kg) was exhaustively extracted with methanol (2.5L×5). The extract was evaporated in rotary evaporator (Laborta 4000-efficent Heidolph) at 40^{o}C under vacuum to yield the residue (748g), which was dissolved in distilled water (1L) and partitioned with *n*-hexane (1L×3), chloroform (2L×4), ethyl acetate (2.5 L×4) and *n*-butanol (2.5 L×4) respectively. These four organic fractions and remaining water fraction were concentrated separately on rotary evaporator and the residues thus obtained were used to evaluate their *in vitro* antioxidant activity and enzyme inhibition.

Chemicals and standards

DPPH˙ (1,1-Diphenyl-2-picrylhydrazyl radical), TPTZ (2,4,6-Tripyridyl-s-triazine), Trolox, Gallic acid, Follin Ciocalteu's phenol reagent, BHT (butylatedhydroxytoluene), acetylthiocholine iodide, 5,5´-dithiobis [2-nitrobenzoic acid] (DTNB) were obtained from Sigma Chemical Company Ltd. (USA) while, erythrocytes (acetylcholine esterase) obtained from the Biochemistry Laboratory, Mayo Hospital Lahore and organic solvents (*n*-hexane, chloroform, ethyl acetate, *n*-butanol), sulphuric acid, sodium phosphate, ammonium molybdate, ferric chloride from Merck (Pvt.) Ltd. (Germany).

Phytochemical screening

Phytochemical screening of all the five crude extracts i.e. *n*-hexane soluble fraction, chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction and remaining aqueous fractions was carried out to identify the phytochemical constituents (alkaloids, terpenoids, saponins, tannins, sugars, phenolics, flavonoids, cardiac glycosides) using the standard procedures (Sofowora *et al*., 1993; Trease *et al*., 1989; Ayoola *et al*., 2008).

Antioxidant assays

Following antioxidant assays were performed on all the studied fractions.

DPPH radical scavenging activity

The DPPH radical scavenging activity of crude extract and fractions were examined by comparison with BHT as standard reference using the reported method (Lee *et al*., 2006). Briefly, different amounts of the samples (1000µg/mL, 500µg/mL, 250µg/mL, 125µg/mL, 60µg/mL, 30µg/mL, 15µg/mL) were mixed with 3 ml of methanolic solution of DPPH (0.1mM). The mixture was shaken vigorously and allowed to stand for one hour at room temperature. Then absorbance was measured at 517 nm against methanol as a blank in the spectrophotometer. The presence of DPPH de-coloration of the samples was calculated according to the formula:

Antiradical activity (%) = $[(A_{control} - A_{sample})/ A_{control}]\times100$

Each sample was assayed in triplicate and mean values were calculated.

Total antioxidant activity by phosphomolybdenum method

The total antioxidant activities of various fractions of plant were evaluated by phosphomolybdenum complex formation method (Prieto *et al*., 1999). 500µg/mL of each fraction was mixed with 4mL of reagent solution (0.6 M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) in sample vials. The blank solution contained 4mL of reagent solution. The vials were incubated in water bath at 95^{o}C for 90 minutes. After the samples had been cooled to room temperature, the absorbance of mixture was measured at 695 nm against blank. The antioxidant activity was expressed relative to that of BHT. All determinations were assayed in triplicate and mean values were calculated.

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was determined according to Benzie and Strain (Benzie *et al*., 1996) with some modifications. The fresh working solution of FRAP was prepared by mixing 25mL acetate buffer (pH 3.6), 2.5mL TPTZ solution, 2.5mL ferric chloride hexahydrate solution and then warmed at 37^{o}C before using. The solutions of plant samples and that of trolox were prepared in methanol (500µM/mL). 50µL of each fraction was taken in separate test tubes and 150µL of FRAP solution was added in each. The plant fractions were allowed to react with FRAP solution in the dark for 30 minutes. The absorbance of the coloured product was checked at 593nm. The FRAP values were expressed as micromoles of trolox equivalents (TE) per mL of the sample solution using the standard curve constructed for different concentrations of trolox. Results were expressed in TE µM/mL.

Total phenolic content

Total phenolics of various fractions of plant were determined by the reported method (Makkar *et al*., 1993). An aliquot of 0.1mL of each fraction (0.5mg/mL) was combined with 2.8mL of 10% sodium carbonate and 0.1mL of 2N Folin-Ciocalteu reagent. After 40 minutes absorbance at 725nm was checked by UV-visible spectrophotometer. Total phenolics were expressed as milligrams of Gallic acid equivalents (GAE) per gram of sample using the standard calibration curve constructed for different concentrations of Gallic acid. Results were expressed in GAE mg/g.

Table 1: Phytochemical constituents of various fractions of *Zizyphus oxyphylla*

Test	*n*-Hexane soluble fraction	Chloroform soluble fraction	Ethyl acetate soluble fraction	*n*-Butanol soluble fraction	Remaining Aqueous fraction
Alkaloids	+	++	++++	++++	++
Terpenoids	++	++	+++	++	+
Saponins	-	+	++	+	-
Tannins	-	-	+	+	-
Sugars	+	-	++	+	+++
Phenolics	+	++	++	+++	-
Flavonoids	-	+	++	+	-
Cardiac glycosides	-	-	+	+	-

("+" Represents presence and "–" represents absence)

Table 2: Free radical scavenging activity and IC_{50} values of the various fractions of *Zizyphusoxyphylla* using 1, 1-Diphenyl-2-picryl hydrazyl radical (DPPH)

S. No.	Sample	Concentration in assay (µg/ml)	% Scavenging of DPPH radical ± S.E.M[a)]	IC_{50} of DPPH Assay (µg/ml) ± S.E.M [a)]
1	Crude Methanolic extract	250 120 60 30	81.15±0.98 62.73±0.17 55.02±0.68 38.07±0.16	68.61±0.48
2	n-Hexane soluble fraction	1000 500 250 120	53.87±0.63 49.14±0.78 31.85±0.52 17.80±0.41	282.5±1.89
3	Chloroform soluble fraction	120 60 30	95.01±0.37 76.01±0.21 53.72±0.14	13.20±0.27
4	Ethyl acetate soluble fraction	60 30 15	73.39±0.65 53.71±0.87 39.94±0.12	38.98±0.54
5	n-Butanol soluble fraction	60 30 15	79.03±0.79 63.74±0.95 36.93±0.86	29.79±1.30
6	Remaining aqueous fraction	500 250 120	67.83±0.43 41.17.±0.97 21.91±0.31	455.21±0.47
7	BHT[b)]	60 30 15	91.49±0.13 75.54±0.07 42.62±0.04	12.10±0.29

a) Standard error mean of three assays. b) Standard reference antioxidant.

In vitro AChE inhibition assay

Acetylcholine esterase inhibitory (AChE) activity was measured by Ellman's (1961) method with minor modification (Shahwar *et al*., 2010a). The reaction mixture contained 2.8 ml of 0.1M phosphate buffer (pH 7.8), 100µl of acetylcholine esterase (erythrocytes), 200µl of each extract/fractions solution at different concentrations (150, 100, 50 and 30mg/mL), 100µl of DTNB and incubated for 15 min (37°C). After 15 minutes the reaction was initiated by the addition of 200µl acetylthiocholine (substrate). The hydrolysis of acetylthiocholine was monitored at 412 nm after 30 min. Galanthamine was used as positive control. All the reactions were performed in triplicate. The percentage inhibition was calculated as follows:

% inhibition = $[(E - S)/ E] \times 100$

Where; E is the activity of the enzyme without sample and S is the activity of enzyme with test sample.

STATISTICAL ANALYSIS

All the measurements were done in triplicate and statistical analysis was executed by Microsoft Excel 2010. All the data were expressed as ±S.E.M. Statistical analysis were determined using one way analysis of variance (ANOVA) followed by post-hoc Tukey's test.

RESULTS

Phytochemical screening

The phytochemical screening was done on all the fractions and results have been shown in table 1. It showed that phenolics, flavonoids and alkaloids are mostly present in chloroform, ethyl acetate and n-butanol fractions. All the results of antioxidant activity are summarized in table 2 and 3.

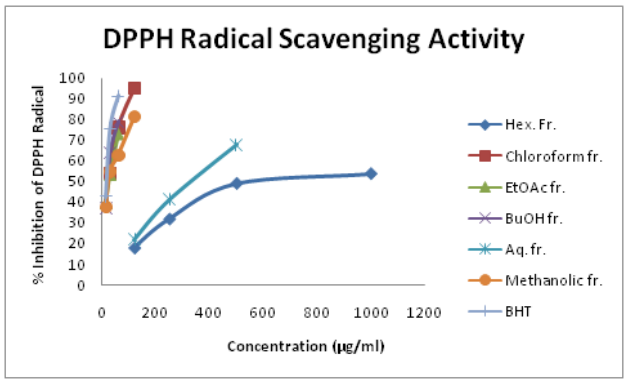

Fig. 1: Graphical representation of results of DPPH radical scavenging activity.

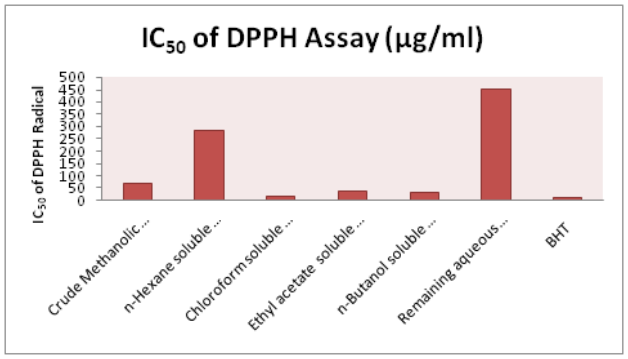

Fig. 2: Graphical representation of results of IC_{50} of DPPH assay.

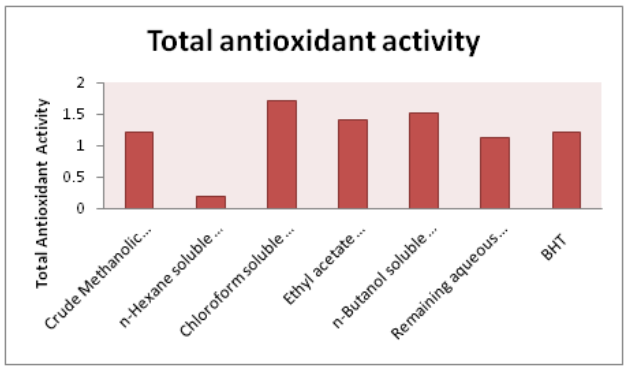

Fig. 3: Graphical representation of results of total antioxidant activity.

Scavenging assay of DPPH free radical

This assay was performed on all the fractions of the plant and the values of percent inhibition of DPPH radical and IC_{50} have been shown in figs. 2 and fig. 3. It was observed that activity was increased by increasing the concentration of the samples. Chloroform fraction showed highest percent inhibition of DPPH radical (95.01±0.37) as than other fractions with IC_{50} value 13.07±0.27µg/mL relative to butylatedhydroxytoluene (BHT), a reference standard (IC_{50}= 12.10±0.29µg/mL).

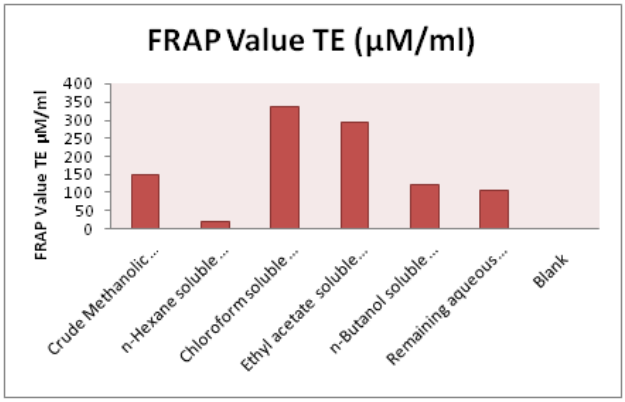

Fig. 4: Graphical representation of results of FRAP value.

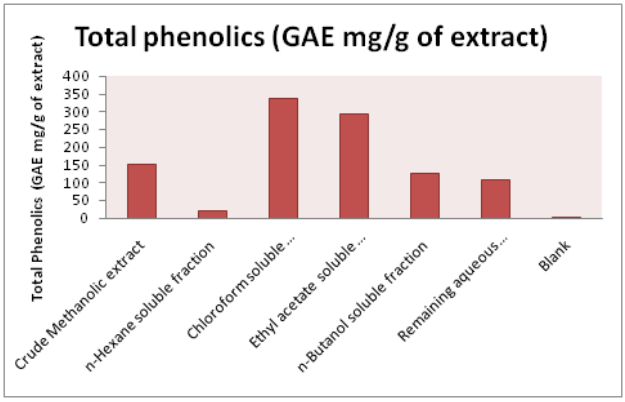

Fig. 5: Graphical representation of results of total phenolics.

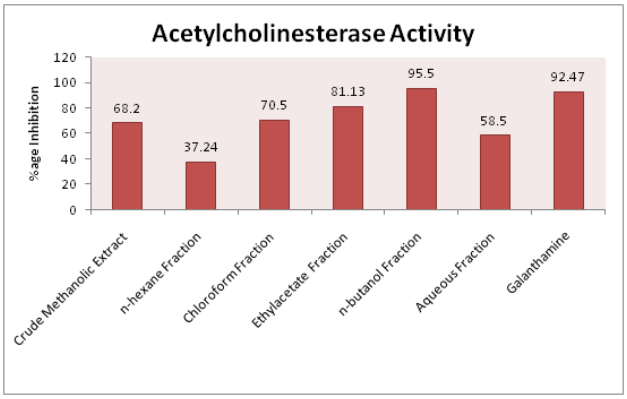

Fig. 6: Graphical representation of results of Acetyl cholinesterase activity

Total antioxidant activity method

Total antioxidant activity of all extracts/fractions were measured by phosphomolybednum method. It was

Table 3: Total antioxidant activities, FRAP values and total phenolics values of different fractions of *Zizyphus oxyphylla*.

S. No.	Sample	Total antioxidant activity ±S.E.M [a)]	FRAP Value TE (µM/ml) ±S.E.M [a)]	Total Phenolics (GAE mg/g of extract) ±S.E.M [a)]
1	Crude Methanolic extract	1.223±0.04	150.53±0.94	86.67±1.45
2	n-Hexane soluble fraction	0.187±0.02	21.5±0.19	14.3±0.31
3	Chloroform soluble fraction	1.723±0.34	339.5±0.57	142.65±1.20
4	Ethyl acetate soluble fraction	1.406±0.41	296.01±0.85	55.06±1.45
5	n-Butanol soluble fraction	1.523±0.07	125±0.49	76.19±1.32
6	Remaining aqueous fraction	1.138±0.013	107.3±0.64	25.33±1.29
7	BHT [b)]	1.2186±0.015		
8	Blank		2.30	1.15

Table 4: Acetylthiocholine Esterase

S. No.	Sample	AChEIC_{50} (mg/mL)	AChE inhibition (%) (10mg/mL)
1	Crude Methanolic extract	92.68±0.38	68.2±0.47
2	n-Hexane soluble fraction	165.15±0.94	37.24±0.59
3	Chloroform soluble fraction	70.59±0.41	70.5±0.71
4	Ethyl acetate soluble fraction	26.48±0.92	81.13±0.81
5	n-Butanol soluble fraction	9.58±0.08	95.5±0.13
6	Remaining aqueous fraction	98.70±0.21	58.5±0.94
7	Galanthamine	13.26±0.73	92.47±0.41

revealed from the results (fig. 3) that the chloroform fraction showed highest total antioxidant activity (1.723±0.34) while the *n*-hexane and remaining aqueous fractions exhibited very less activity (0.187±0.02 and 0.138±0.13 respectively). The results were compared with reference standard BHT (0.96±0.06).

Ferric reducing antioxidant power (FRAP assay)

FRAP assay is used for the determination of the reducing power of various samples which is shown by the colour change of the test solution from yellow to blue and green in proportionate to the reducing power of various samples. From the results (fig. 4) it was revealed that among all the fractions the chloroform fraction showed highest FRAP value (339.5±0.57 TE µM/mL). Ethyl acetate fraction also showed good FRAP value *i.e.* 296.01±0.85TEµM/mL, while *n*-hexane and remaining aqueous fractions showed very less FRAP values *i.e.* 21.50±0.19TEµM/mL 107.3±0.64TEµM/mL respectively.

Determination of total phenolic content (TP)

Fig. 5 showed that the chloroform fraction showed the highest value of total phenolic compounds i.e. 142.65±1.20GAE mg/g while the total phenolic contents of *n*-hexane and aqueous fractions showed the least values *i.e.* 25.33±1.29 and 14.30±0.31GAE mg/g respectively.

In vitro AChE inhibition assay

Acetylthiocholine esterase (AChE) inhibitory potential of the methanolic crude extract and various fractions of plant were determined through spectrophotometer method (Ellman *et al*., 1961; Shahwar *et al*., 2011). AChE inhibition and IC_{50} values of the plant extracts indicating AChE inhibitory activity are presented in table 4. A low IC_{50} value indicated good inhibition of the enzyme. The *n*- butanol fraction had the lowest IC_{50} value (9.58±0.08 mg/mL), indicating that it contained the best inhibition of the enzyme relative to galanthamine (13.26±0.73mg/mL).

DISCUSSION

In this study, chloroform, ethyl acetate and n- butanol contained large amount of phenolics, flavonoids, alkaloids and terpenoids content. Due to the presence of these constituents these fractions showed good antioxidant and acetylcholine esterase activities. The DPPH radical scavenging is thought to be due to their hydrogen donating ability. DPPH is a stable free radical and is paramagnetic molecule, which accepts an electron or hydrogen radical to form a stable diamagnetic molecule (Siddaraju *et al*., 2007). DPPH radical accepts the electron and becomes in reduce from i.e., 1,1- diphenyl-2-picryl hydrazine and is visualized as yellow from purple colour (Meir *et al*., 1995). DPPH radical decolourizes due to the presence of antioxidants. The reduction of DPPH radical was measured at 517 nm when colour changes from purple to yellow. In total antioxidant assay, Mo (VI) reduces to Mo (V) in various fractions of plant, which was detected at 695nm by spectrophotometer for the formation of green phosphate Mo (V) (Miladi *et al*., 2008). In a redox- linked colorimetric method antioxidants are used as reductants in FRAP assay and in stoichiometric excess it provides an easy reduced oxidant system. Ferric form (ferric tripyridyltriazine complex) changes to ferrous form showing intense blue colour. This change was measured at 593nm. In the reaction mixture

the absorption change was linked directly with the total reducing power of electron donating antioxidants, which reduced the ferric form (ferric cyanide complex) to the ferrous form (Shahwar *et al*., 2010b). High FRAP values obtained from polar fractions showed the presence of flavonoid and phenolic contents. Phenolic and flavonoid contents show significant antioxidant action on human health and fitness. These compounds/ antioxidants act through scavenging or chelating process (Kessler *et al*., 2003; Cook *et al*., 1996). The high potential of phenolics to scavenge free radicals may possibly be due to various phenolic hydroxyl groups they possess (Sawa *et al*., 1999). So chloroform fraction showed the highest percent inhibition of DPPH radical (95.01±0.37) with IC_{50} value 13.07±0.27µg/mL, highest total antioxidant activity (1.723±0.34), highest FRAP value (339.5±0.57 TE µM/mL) and highest value of total phenolic compounds i.e. 142.65±1.20GAE mg/g as than other fractions. Acetylthiocholine esterase (AChE) inhibitory potential was based upon the measurement of yellow anion of thionitrobenzoic acid formed by the hydrolysis of DTNB, measured at λ=412nm. AChE inhibitors diminish the rate of acetylcholine (ACh) and increase the concentration of ACh in the brain (Shen., 2004; Samanta *et al*., 2006). So the *n*- butanol fraction had the lowest IC_{50} value (9.58±0.08mg/mL), possessed good AChE inhibitory activity.

CONCLUSION

In this study, the results showed that chloroform fraction exhibited highest % inhibition of DPPH radical with low IC_{50} value, highest total antioxidant activity, FRAP value and total phenolic contents due to the presence of flavonoids and phenolic contents and the % inhibition of acetylcholine esterase of n-butanol fraction showed good result and IC_{50} of this fraction was 9.58±0.08mg/mL, relative to galanthamine (13.26±0.73mg/mL). Therefore, the selected medicinal plants possessing significant antioxidant and AChE inhibition activities may prove of novel values in clinical trials for the treatment of AD patients. Further work on the identification of active principals is proposed.

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