ejpmr, 2015,2(5), 342-351.

SJIF Impact Factor 2.026

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 3294-3211

EJPMR

ANTI-OXIDANT AND ANTI-BUTYRYLCHOLINESTERASE ACTIVITY OF AN ETHANOLIC EXTRACT OF *TINOSPORA CORDIFOLIA*.

Suresh Kumar* and Swati Sharma

University School of Biotechnology, Guru Gobind Singh Indraprastha University, Sector 16C, Dwarka, Delhi 110075, India.

Article Received on 21/06/2015 **Article Revised on 15/07/2015** **Article Accepted on 06/08/2015**

***Correspondence for Author**

Dr. Suresh Kumar

University School of Biotechnology, Guru Gobind Singh Indraprastha University, Sector 16C, Dwarka, Delhi 110075, India.

ABSTRACT

Cholinesterase inhibitors are the most sought after drugs for the symptomatic treatment of Alzheimer's disease. The present study, assessed the anti-oxidant and anti-butyrylcholinesterase property of an ethanolic extract of *Tinospora cordifolia's* stem, which is an Ayurvedic plant traditionally used for its several medicinal properties. Our findings demonstrated that an ethanolic extract of *Tinospora cordifolia's* stem significantly inhibited butyrylcholinesterase enzyme and showed maximum inhibition of 73.59±0.02% at 200µg/ml final concentration with IC_{50} value of 37.41 µg/mL calculated from the percentage inhibition curve. The mechanism of enzyme inhibition demonstrated by Lineweaver-Burk plot showed competitive inhibition at higher concentration and mixed non-competitive mode of inhibition at lower concentration of plant extract. Antioxidant activity of the extract was assessed by Trolox equivalent antioxidant capacity assay and Ferric reducing ability of plasma assay, which suggested that this plant also possesses significant antioxidant property. The maximum ferrous sulphate equivalent and trolox equivalent values were found to be 1785.66 ± 0.060 µmol Fe^{2+} E/g of dried sample and 0.72 ± 0.003 mmol Trolox E/g of dried sample respectively. It is concluded that this plant has potential in deriving lead compound for anti-butyrylcholinesterase and antioxidant property which might be beneficial in future for providing symptomatic relief for AD patients.

KEYWORDS: Alzheimer's disease, Butyrylcholinesterase, *Tinospora cordifolia*.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and worldwide it is considered to be the most common cause of dementia, mostly associated with the old aged people. Abnormalities associated with this disease include aphasia, agnosia, apraxia and deterioration of cognitive function, memory, visual-spatial perception, praxis and language.[1, 2] AD is marked by formation of senile plaques of β amyloid protein, gradual loss of brain cells and neurons and disruption of synaptic function. This anomaly that occurs during AD involves that part of neuronal system which is transmitter-specific, such as, brainstem, hippocampus, basal forebrain, neocortex and amygdala.[3] A number of drug development strategies and treatment approaches for AD are based on cholinergic hypothesis. This hypothesis states that the depletion in synthesis of an important cholinergic neurotransmitter acetylcholine (ACh) by the enzyme acetylcholinesterase (AChE) is one of the cause of AD.[4] Traditionally, most of the research related to neurotransmission in brain was based upon AChE. However in recent times, the research for another cholinesterase, Butyrylcholinesterase (BuChE) has gained much momentum. Recent observations delineate possible role of BuChE as a co-regulator of cholinergic neurotransmission, its presence in specific neurons and association with some part of the development of the nervous system.In the case of AD, biochemical properties of BuChE get altered. Also, in the absence of AChE, BuChE compensates for its functions related to cholinergic system.[5,6] BuChE can catalyse the hydrolysis of many choline and non-choline esters, such as, ACh, butyrylcholine (BCh), succinylcholine, acetylsalicylic acid etc. therefore, BuChE is also called as "Pseudocholinesterase" or "non-specific" enzyme.[7] Cholinesterase (ChE) inhibitors play an important role as drugs in treatment of AD by inhibiting the enzymes AChE and BuChE and thereby preventing hydrolysis of neurotransmitter Ach. This results in elevated levels of Ach and thereby improved cognitive function.[8] These inhibitors are therefore beneficial in symptomatic treatment of AD and other types of dementia as well. Amongst many other factors, one of the major cellular features in the pathophysiology of AD is oxidative stress. The oxidative damage affects the membrane properties of cell such as enzyme activities, protein cross-linking, ion transport, and fluidity and eventually leads to neurodegeneration.[9] The currently employed synthetic drugs for the treatment of AD have some limitations and exhibit side effects.[10] So in this view, there is an indispensable need to look for other alternative options to be used as effective drugs so as to substitute or augment those in current use. Exploitation of medicinal plants for discovery of new lead compounds which focus on anti-oxidant property and anti-cholinesterase activity for cognitive enhancement has emerged

as a suitable and safer option in drug discovery for AD. *Tinospora cordifolia* is a traditional Indian medicinal plant. In the Ayurvedic literature it is mentioned as one of the "*Medhya Rasayanas"*, medicinal plants used to improve memory and intellect. It is a large and glabrous plant belonging to the family "Menispermaceae". It is found in tropical regions of India and is commonly known as Giloy or Guduchi.[11] In the present study, the stem of *Tinospora cordifolia* was used. In this study, an ethanolic extract of the plant was screened for anti-cholinesterase activity using Ellman's assay and for anti-oxidant activity using Trolox equivalent antioxidant capacity (TEAC) assay and Ferric reducing ability of plasma (FRAP) assay.

MATERIAL AND METHODS

Chemicals and Reagents

BuChE (Sigma-Aldrich) from equine serum, Butyrylthiocholine iodide (BTChI) (Sigma-Aldrich), 5, 5- dithiobis [2-nitrobenzoic acid] or DTNB or Ellman's reagent (Sigma-Aldrich), ethanol (SRL, India), phosphate buffer, sodium bicarbonate (Sigma, Himedia), acetate buffer, Tripyridyltriazne (TPTZ) (Sigma-Aldrich), ferric chloride (Sigma-Aldrich), ferrous sulphate (Sigma-Aldrich), phosphate buffer solution (PBS), potassium persulphate (Sigma-Aldrich) , 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) or ABTS (Sigma-Aldrich) .

Plant material

The stem of the plant *Tinospora cordifolia* (Voucher no. SS/USBT066) was obtained from a local market and authenticated by botanist. The voucher specimens of this plant sample are stored in a herbarium at USBT, GGSIP University, Delhi, India.

Equipments and instruments

96-well plate (Corning Inc. NY), Pipettes (Biomate and Thermo labsystem), tips (Tarsons products Ltd. India), centrifuge tubes, eppendorf tubes, eppendorf tube stand, measuring cylinder, conical flask, aluminium foil, tissue paper, cotton plugs, needle, spatula, scissors, butter paper, whatman filter paper No.1, parafilm, ice box, blotting paper, vortex machine (REMI), Grinder, -20º refrigerator, hot air oven, rotatory shaker, centrifuge machine, weighing balance, pH meter (EuTech), spectrophotometer (SpectraMax).

Preparation of an ethanolic extract of the plant

Dried stem of the plant sample was powdered using electric grinder and 5g of it was soaked in 50ml of absolute ethanol (1:10 w/v). The flask kept on a rotatory shaker for 48 hours.

After 48 hours the solvent was filtered using Whatman filter paper No.1. Solvent was evaporated by keeping the flask in hot air oven for 24 hours at 40ºC. The sample was stored in the eppendorf tube at -20°C. Using the formula, % yield= (weight of the extract/ weight of plant material) ×100, the percentage yield of an ethanolic extract of *Tinospora cordifolia's* stem was calculated and found to be 1.8% (this value is based on single batch extraction).

Antioxidant Assays

Ferric reducing ability of plasma (FRAP) assay

The FRAP assay measures the ferric reducing ability of plasma. Reduction of ferric ion to ferrous ions results in a formation of blue-colored compound ferrous Tripridyltriazine (TPTZ), which can be measured spectrophotometrically. FRAP values of samples are obtained by comparison of absorbance of TPTZ of test samples with reaction mixtures containing known concentration of ferrous ions i.e. standard.[12]

Ferric-TPTZ^{3+} ⟶ Ferrous-TPTZ^{2+}

(Colorless) (Blue colored)

1mM stock solution of an ethanolic extract of *Tinospora cordifolia's* stem was prepared and from that stock different concentrations ranging from 200 µg/ml to 0.2 µg/ml were made. In a flat-bottom 96-well microtitre plate, 20µl sample was added to 180µl FRAP reagent. The reaction for each concentration was carried out in triplicates. A triplicate control reaction was also carried out, in which 20µl of absolute ethanol was added to 180µl FRAP reagent. The mixture was incubated for 10 minutes. The change in absorbance was measured at 595 nm with the help of a spectrophotometer SpectraMaxM2, 96 well plate reader. The same procedure was followed for standard assay of ferrous sulphate solution.[13]

Trolox equivalent antioxidant capacity (TEAC) assay

TEAC assay is based on the scavenging of the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical and hence converting it into a colorless product. The degree of decolorization induced by an anti-oxidant compound is related to that induced by trolox, giving the TEAC value.[14] 1mM stock solution of an ethanolic extract of *Tinospora cordifolia's* stem was prepared and from that stock different concentrations ranging from 100 µg/ml to 0.78 µg/ml were made. In a flat-bottom 96-well microtitre plate, 10µl sample solution was added to 190µl TEAC reagent. The reaction for each concentration was carried out in triplicates. A triplicate control reaction was also carried out, in which 10µl of absolute

ethanol was added to 190µl TEAC reagent. The mixture was incubated for 30 minutes. The change in absorbance was measured at 734 nm with the help of a spectrophotometer SpectraMaxM2, 96 well plate reader. The same procedure was followed for standard assay of Trolox reagent.[15]

Cholinesterase inhibitory assay

BuChE catalyze the hydrolysis of butyryl-thiocholine, which is a sulfur analog of its natural substrate, acetylcholine. Upon hydrolysis, this substrate analog produces butyrate and thiocholine. Thiocholine reacts with highly reactive dithiobisnitro-benzoate (DTNB) ion to generate a yellow colored 5-thio-2-nitrobenzoate anion. The yellow color can be quantified by taking its absorbance at 412 nm. Cholinesterase activity is recorded as an increase of absorbance at 412 nm.[16] An estimation of cholinesterase inhibition was carried out in flat-bottom 96-well microtitre plate. 1mM stock solution of an ethanolic extract of *Tinospora cordifolia's* stem was prepared and from that stock different concentrations ranging from 200 µg/ml to 0.20 µg/ml were made. The reaction mixture consisted of 5 µl of 0.08 U/ml BuChE solution, 200 µl of 0.1 M phosphate buffer, 5 µl of 0.5mM DTNB and 5 µl of the test ethanolic extract. The reactants were mixed and pre-incubated for 15 min. The reaction was initiated by adding 5 µl of 0.5mM BTChI. As a control the inhibitor solution was replaced with buffer. To monitor any non-enzymatic hydrolysis in the reaction mixture two blanks for each run were prepared in triplicates. One blank consisted of buffer replacing enzyme and a second blank had buffer replacing substrate. The reaction was carried out in triplicates. Change in absorbance was measured at 412 nm with the help of a spectrophotometer SpectraMaxM2, 96 well plate reader over a period of 6 min.[17]

RESULTS

Antioxidant assays

Ferric reducing ability of plasma (FRAP) assay

The results demonstrate that an ethanolic extract of *Tinospora cordifolia's* stem at concentration ranging from 0.2µg/mL to 200 µg/mL displayed concentration-dependent anti-oxidant property. The maximum antioxidant capacity was found to be 1785.66±0.06 µmol Fe^{2+} E/g of dried sample (Figure 1). The FRAP values were calculated using the slope of the standard curve of FRAP assay, the absorbance values of test samples were converted to an equivalent Ferrous sulphate concentration.

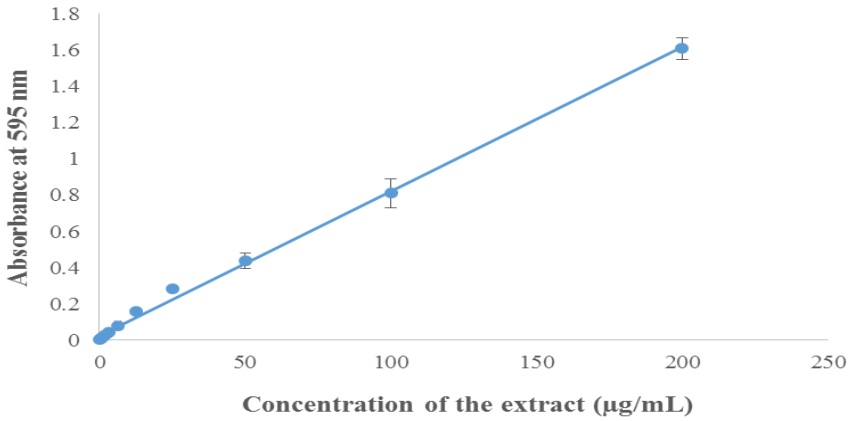

Fig 1: Absorbance values of different concentrations of an ethanolic extract of *Tinospora cordifolia's* stem showing concentration-dependent anti-oxidant property in FRAP assay. [The equation of the line is y = 0.0079x + 0.0278, R^2 = 0.9974].

Trolox equivalent antioxidant capacity (TEAC) assay

Using the slope of the standard curve of trolox assay, the absorbance values of test samples were converted to an equivalent trolox concentration. The results demonstrate that an ethanolic extract of *Tinospora cordifolia's* stem at concentration ranging from 0.78µg/mL to 100 µg/mL displayed concentration-dependent anti-oxidant property. The maximum anti-oxidant property was observed at trolox concentration 0.72±0.003 mmol trolox E/g of dried sample (Figure 2).

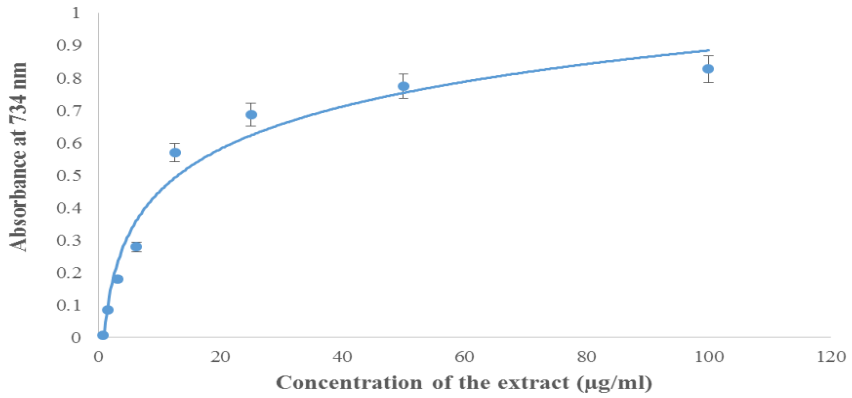

Fig 2: Absorbance values of different concentrations of an ethanolic extract of *Tinospora cordifolia's* stem showing concentration-dependent anti-oxidant property in TEAC assay. [The equation of the line is y = 0.1889ln(x) + 0.0149, R^2 = 0.9665].

Cholinesterase inhibitory assay

The results showed that an ethanolic extract of *Tinospora cordifolia's* stem at concentration ranging from 12.5µg/mL to 200 µg/mL displayed concentration-dependent inhibition of enzyme BuChE. The maximum inhibition of 73.59±0.02% for enzyme BuChE was observed at 200µg/mL final assay concentration. The IC_{50} value calculated from the equation obtained from the concentration versus percentage inhibition curve was 37.41 µg/mL (Figure 3).

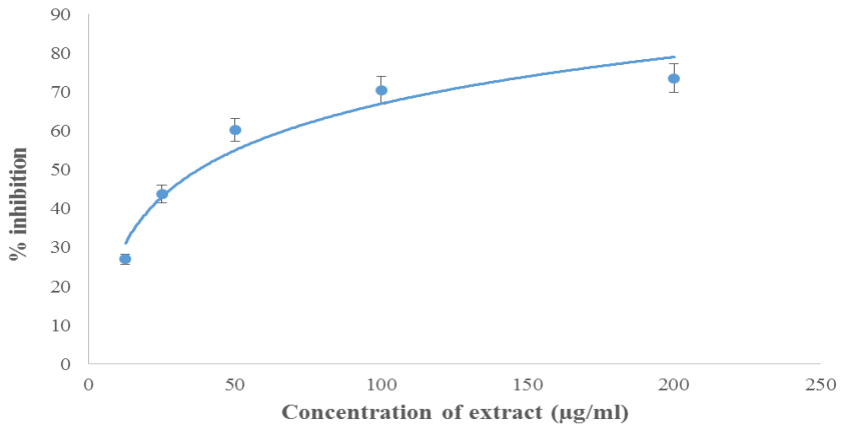

Fig 3: Percentage inhibition of BuChE activity of different concentrations of an ethanolic extract of *Tinospora cordifolia's* stem. [The equation of the line is y = 17.298ln(x) - 12.656, R^2 = 0.9438].

The mode of enzyme inhibition was derived from the Lineweaver-Burk (LB) plot between the reciprocal of substrate concentration on x-axis and reciprocal of velocity on y-axis.[15] The LB plot of an ethanolic extract of *Tinospora cordifolia's* stem showed competitive inhibition at higher concentration and mixed non-competitive mode of inhibition at lower concentration of plant extract as illustrated in Figure 4.

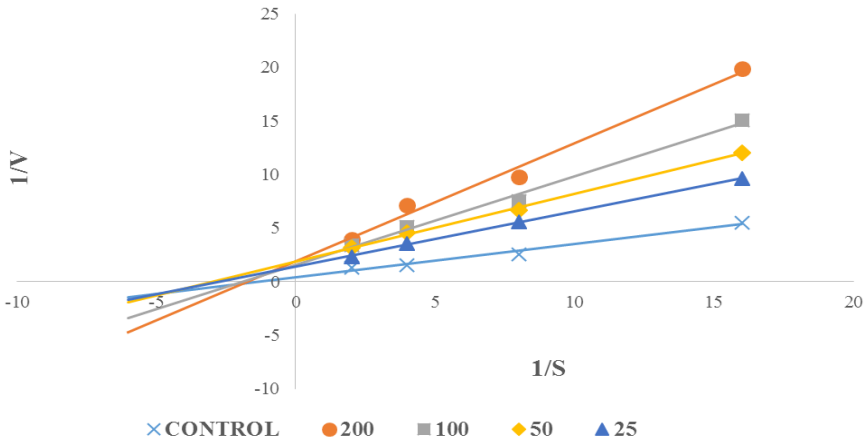

Fig 4: LB plot representing the reciprocal of initial enzyme velocity versus the reciprocal of BTChI concentration in the presence and absence (control) of different concentrations of an ethanolic extract of *Tinospora cordifolia's* stem.

The equation of the line representing extract concentration of: (a) 200µg/mL is y = 1.1042x + 1.9066, R^2 = 0.9874; (b) 100µg/mL is y = 0.8285x + 1.568, R^2 = 0.9922; (c) 50µg/mL is y = 0.6296x + 1.8962, R^2 = 0.9981; (d) 25µg/mL is y = 0.5162x + 1.4064, R^2 = 0.9987; (e) control is y = 0.309x + 0.4284, R^2 = 0.9789.

DISCUSSION

Cholinesterase inhibitors (ChEI) are commonly used drugs for the symptomatic treatment of AD. The currently approved synthetic drugs for the treatment of AD, such as donepezil, rivastigmine and tacrine exhibit side effects such as gastrointestinal disturbances, hepatotoxicity, high cost and low bioavailability.[18] These inhibitors not only reduce the symptoms but also improve cognitive abilities of AD patients. The main drawback associated with these drugs is their inability to delay the progression and provide complete cure for AD. In this view, many researchers are focused on to find new class of ChEI from a number of plants or plant derived compounds, which can be used as a new therapeutic alternative in providing symptomatic relief to AD patients. Compounds from natural sources are expected to have low cost and fewer side effects.

The present study establishes that an ethanolic extracts of *Tinospora cordifolia's* stem inhibited BuChE in a concentration dependent manner and also demonstrated the mode of BuChE inhibition by kinetics study using Lineweaver-Burk (LB) plot for the first time. The mechanism of enzyme inhibition demonstrated by LB plot showed competitive inhibition at higher concentration and mixed non-competitive mode of inhibition at lower concentration of plant extract. The results of present study are complementary to the previous study which shows the role of *Tinospora cordifolia* in improving the memory in normal and cognition deficits animals in behavioural test Hebb William maze task[19] and its neuroprotective role in Parkinson's disease.[20]

The present study exhibited that the high level of anti-oxidant activity present in plant extract of *Tinospora cordifolia* makes it liable for scavenging free radicals, generated during oxidative stress. Oxidative stress and generation of free radicals are also considered one of the causes of AD.[21] Previous study also reported antioxidant activities which further strengthens our result.[22]

One of the advantages of using natural products such as the plant *Tinospora cordifolia* is its plethora of medicinal applications. The notable medicinal properties reported are anti-leprotic, anti-diabetic, anti-spasmodic, anti-malarial, anti-inflammatory, anti-neoplastic, anti-arthritic, anti-stress, anti-allergic, anti-periodic, immunomodulatory, hepatoprotective and anti-oxidant activities.[23]

CONCLUSION

It is concluded that an ethanolic extract of *Tinospora cordifolia's* stem possesses anti-BuChE and antioxidant activity. Further studies are required to identify, isolate and characterize the phytoconstituents from an ethanolic extract of *Tinospora cordifolia's* stem to find the novel molecules which might be useful in mitigating the symptoms associated with AD.

REFERENCES

1. Price D.L. New perspectives on Alzheimer's disease .Neuroscience, 1986; 9: 489–512.
2. Förstl H, Kurz A. Clinical features of Alzheimer's disease. Eur Arch Psy Clinn, 1999; 249(6): 288–90.
3. Shekhar C and Kumar S. Kinetics of butyrylcholinesterase inhibition by an ethanolic extract of *Shorea robusta*. IJPSR, 2014; 5: 480-3.
4. Mann D.M.A. The neuropathology of Alzheimer's disease: a review with pathogenetic, aetiological and therapeutic considerations. Mech Ageing Dev, 1985; 31: 213–55.
5. Darvesh S., Hopkins D.A. and Geula C. Neurobiology of butyrylcholinesterase. Nat Rev Neurosci, 2003; 4(2): 131-8.
6. Mesulam M, Guillozet A, Shaw P and Quinn B. Widely spread Butyrylcholinesterase can hydrolyze acetylcholine in the normal and Alzheimer Brain. Neurobiol Dis, 2002; 9: 88–93.
7. Greig N.H., Lahiri D.K., and Sambamurti K. Butyrylcholinesterase: An important new target in Alzheimer's disease therapy. Int Psychogeriatr, 2002; 14: 77-91.
8. Mukherjee P.K., Kumar V, Mal M, Houghton P.J. Acetylcholinesterase inhibitors from plants. Phytomedicine, 2007; 14(4): 289–300.
9. Nordberg A, Ballard C, Bullock R, Darreh-Shori T, and Somogyi M. A Review of butyrylcholinesterase as a therapeutic target in the treatment of Alzheimer's disease. Prim Care Companion CNS Disord, 2013; 15(2).
10. Shaik AS, Elaya E.R, Vijayalakshmi M. and Devalarao G. Alzheimer's disease – Pathophysiology and treatment. IJPBS, 2010; 1(2).

11. Kulkarni R, Girish K. J., and Kumar A. Nootropic herbs (*Medhya Rasayana*) in Ayurveda: An update. Pharmacogn Rev, 2012; 6(12): 147–53.
12. Huang D, Boxin Ou, Ronald L.P. The chemistry behind antioxidant capacity assays. J Agric Food Chem, 2005; 53(6): 1841–56.
13. Benzie I.F.F., and Strain J. J. The Ferric Reducing Ability of Plasma (FRAP) as a measure of ‘‘Antioxidant Power’’: The FRAP Assay. Anal Biochem, 1996; 239, 70–6.
14. Prior R.L.,Wu X and Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem, 2005; 53: 4290-302.
15. Roberta RE, Pellegrini N, Proteggente A, Pannala A, Yang M, and Evans R. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Bio Med, 1999; 26: 1231–7.
16. A Komersova, K Komers, A Čegan. New findings about Ellman’s method to determine cholinesterase activity. Z Naturforsch C, 2007; 62(1-2): 150-4.
17. Ellman G.L, Courtney K.D, Andres V, JR. and Featherstone R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol, 1961; 7: 88-95.
18. Shekhar C, Kumar S. Kinetics of acetylcholinesterase inhibition by an aqueous extract of *Mentha longifolia* leaves. IJPSDR, 2014; 6(4): 348-50.
19. Agarwal A, Malini S., Bairy K.L., Rao M. S. Effect of *Tinospora cordifolia* on learning and memory in normal and memory deficit rats. Indian J Pharmacol, 2002; 34: 339-49.
20. Kosaraju J, Chinni S, Roy PD, Kannan E, Antony AS, Kumar M.N.S. Neuroprotective effect of *Tinospora cordifolia* ethanol extract on 6-hydroxy dopamine induced Parkinsonism. Indian J Pharmacol, 2014; 46(2): 176-80.
21. Perry G, Cash AD, Smith MA. Alzheimer’s disease and oxidative stress. J Biomed Biotechnol, 2002; 2(3): 120–23.
22. Methew S, Kuttan G. Antioxidant activities of *Tinospora cordifolia* and its usefulness in the amelioration of cyclophosphamide induced toxicity. J Exp Clin Cancer Res, 1997; 16(4): 407-411.
23. Singh S.S., Pandey S.C., Srivastava S., Gupta V.S., Patro B., Ghosh A.C. Chemistry and medicinal properties of *Tinospora cordifolia* (Guduchi). Indian J Pharmacol, 2003; 35: 83-91.