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Ex-vivo Acetylcholinesterase and Butyrylcholinesterase Inhibitory Activities Assay of *G. asiatica* and *G. tiliaefolia* (Tiliaceae) Leaves

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Authors' contributions

This work was carried out in collaboration among all authors. Author MKIJ carried out the laboratory tests, prepared the plant extracts and designed the study. Authors MSI prepared the draft of the manuscript and made necessary corrections after peer review process. Authors NA, RR and MRI performed the graphical evaluations. Author MSRA managed the literature searches. Author CVC reviewed the scientific contents of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Our study was carried out to appraise acetylcholinestrase (AChE) and butyrylcholinestrase (BChE) inhibitory activities of *Grewia asiatica* and *Grewia tiliaefolia* leaves extracts.
Study Design: For the purpose of these experiments the extracts were subjected to an ex-vivo study.
Place and Duration of Study: The study was carried out between June 2018 to December 2018 in the Department of Pharmacy, Southeast University, Dhaka, Bangladesh.
Methodology: In this study, cholinesterase inhibitory activities of different fractions of crude

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ethanol extract of both plants were examined using swiss albino mice at 300 mg/kg b.w. dose. We determined anti-acetylcholinestrase (AChE) and anti-butyrylcholinestrase (BChE) activities using slightly modified Elman coupled enzyme assay.

Results: The highest inhibition of bovine brain acetylcholinesterase and human blood butyrylcholinesterase were exhibited by PEF and CLF of *G. asiatica* with the IC_{50} values were found to be 55.88 µg/ml and 26.14 µg/ml respectively whereas the highest inhibition of bovine brain acetylcholinesterase and human blood butyrylcholinesterase were exhibited by CLF of *G. tiliaefolia.*

Conclusion: The result of the present study on various fractions of these plants has a considerable anti-acetylcholinesterase and anti-butyrylcholinesterase activities which suggest its effectiveness against various neurodegenerative disorders.

Keywords: Free radicals; acetylcholinesterase; butyrylcholinesterase; G. asiatica; G. tiliaefolia.

ABBREVIATIONS

IC_{50} : The half maximal inhibitory concentration;
PEFGA : Petroleum ether fraction of G. asiatica;
CEEGA : Crude ethanolic extract of G. asitica;
CLFGA : Chloroform fraction of G. asiatica;
EAFGA : Ethyl acetate fraction of G. asiatica;
AEFGA : Aqueous Ethanolic fraction of G. asiatica;
AD : Alzheimer's Disease;
AChE : Acetylcholinesterase;
BchE : Butyrylcholinesterase;

1. INTRODUCTION

Plants serve various purposes and their usefulness to man is not limited to their role as sources of raw materials for industries; they are also consumed as food and sometimes used as medication. For ages, plants have provided man with diverse means of healing. In fact, many parts of plants such as fruits, seeds, barks, roots, and flowers have been used as medication to provide alternative therapies for various diseases that affect man and animals [1]. Medicinal plants contain potentially useful chemicals that are currently used for the manufacturing of modern therapeutic agents [2]. The evaluation of medicinal plants, used traditionally in treating Alzheimer's disease (AD) is of growing interest. Alzheimer's disease (AD) is one of the major leading causes of mortality after heart disease, cancer and stroke. AD is associated with memory impairment that progressively declines in cognitive abilities and behaviors, which lead to the complete functional dependency that defines the dementia phase of the illness [3]. Therefore, inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes are the two promising strategies in the development of drug for neurological diseases like Alzheimer's and as well as in the treatment of Parkinson's disease, ataxia and dementia [4]. *Grewia asiatica* and *tiliaefolia* belonging to the family Tiliaceae [5] are trees that have been used in traditional medicine for relief of various health problems such as cold, hepatitis, diarrhea, heat stroke, dyspepsia, tuberculosis, sexual debility troubles, rheumatism and also important to promote intellect and enhancing memory, thus supporting its possible anti-Alzheimer's properties [6-7]. Several literature reviews demonstrated that the plants *G. asiatica* and *G. tiliaefolia* possess analgesic, anti-inflammatory, antioxidant, antimalarial, antidiabetic, antiemetic, antipyretic, antifungal, antiviral, antiplatelet, anticancer and immune-modulatory activities [8-16]. Thus, in the present study different extracts of *G. asiatica* and *G. tiliaefolia* (Family-Tiliaceae) available in Bangladesh were evaluated for *ex-vivo* AChE and BChE inhibitory activities.

2. MATERIALS AND METHODS

2.1 Collection of the Plant Materials and Preparation of Extracts

For this present investigation leaves of *Grewia asiatica* & *Grewia tilieafolia* were collected from Moulovibazar, Bangladesh, in April 2018. After collection these plants were thoroughly washed with water and dried. The plants were identified by expert of Bangladesh National Herbarium, Mirpur, and DACB Accession number 73883 for *Grewia asiatica* & DACB Accession number 73882 for *Grewia tilieafoilia.* The whole plant parts were dried and powdered. 100 g powdered material was kept in 500 ml of 90% ethanol for about 14 days at room temperature with occasional shaking. After 14 days the solution was filtered using cotton filter and Whitman's filter paper. An aliquot of the concentrated

ethanolic extract was fractionated by modified Kupchan method and the resultant fractions that is petroleum ether (PEF), chloroform (CLF), ethyl acetate (EAF) and aqueous (AQF) soluble fractions were obtained and used for the experiment purpose.

2.1.1 Drugs and chemicals

5, 5´-dithio-bis-(2-nitro) benzoic acid (DTNB) (Sigma-Aldrich, Japan), Acetylcholine iodide (Sigma-Aldrich, Japan), Tris-HCl buffer (Merck, Germany), Triton X-100 (Sigma chemical company, USA), BCA kit (bicinchoninic acid; Sigma Co., USA), Bovine serum albumin (Merck, India), Donepezil (Sigma-Aldrich, Japan),

2.1.2 Ex-vivo Acetylcholinesterase inhibitory activity assay

2.1.2.1 Principal

The anti-acetylcholinesterase assay was performed according to the colorimetric method of Ellman et al. [17], using acetylthiocholine iodide as a substrate.

2.1.2.2 Procedure

For the enzyme source, the mice brains were homogenised in a homogeniser with 5 volumes of a homogenisation buffer 910 mM Tris-HCl (pH 7.2), which contained 1 M NaCl, 50 mM MgCl2 and 1% Triton X-1000 [18], centrifuged at 10,000 rpm for 15 min. The supernatant was used as an enzyme source. All of the extraction steps were carried out at 4ºC. Protein concentration was determined using the BCA kit (bicinchoninic acid) with bovine serum albumin (BSA) as a protein standard. The rates of hydrolysis by acetylcholinesterase were monitored spectrophotometrically. Each extract or standard (500 µl) was mixed with an enzyme solution (500 µl) and incubated at 37ºC for 15 min. Absorbance at 405 nm was read immediately after adding an 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) to the above reaction mixture. Reading was repeated for 10 min at 2 min intervals to verify that the reaction occurred linearly. The blank reaction was measured by substituting saline for the enzyme [19].

Acetylcholine →Thiocholine + Acetate; Thiocholine + dithiobisnitro→ Benzoate yellow color

2.1.3 Ex-vivo Butyrylcholinesterase inhibitory activity assay

2.1.3.1 Principal

The anti-butyrylcholinesterase (BchE) assay was performed according to the colorimetric method of McShane et al. [20], using butyrylthiocholine iodide as a substrate.

Fig. 1. Accession number -DACB 73883

Fig. 2. Accession number -DACB 73882 for *G. tiliaefolia* for *G. asiatica*

2.1.3.2 Procedure

For the enzyme source, the human blood was homogenised in a homogeniser with 5 volumes of a homogenisation buffer [10 mM Tris-HCl (pH 7.2), which contained 1M NaCl, 50 mM MgCl2 and 1% Triton X-100] [17], centrifuged at 10,000 rpm for 15 min. The supernatant was used as an enzyme source. All of the extraction steps were carried out at 4ºC. Protein concentration was determined using the BCA kit (bicinchoninic acid) with bovine serum albumin (BSA) as a protein standard. The rates of hydrolysis by butyrylcholinesterase were monitored spectrophotometrically. Each extract or standard (500 µl) was mixed with an enzyme solution (500 µl) and incubated at 37ºC for 15 min. Absorbance at 405 nm was read immediately after adding an Ellman's reaction mixture [3.5 ml; 0.5 mM acetylthiocholine, 1mM 5, 5´-dithio-bis (2nitro benzoic acid)] in a 50 mM sodium phosphate buffer (pH 8.0) to the above reaction mixture. Reading was repeated for 10 min at 2 min intervals to verify that the reaction occurred linearly. The blank reaction was measured by substituting saline for the enzyme.

Butyrylcholine →Thiocholine + Acetate; Thiocholine + dithiobisnitro→ Benzoate yellow Color

3. RESULTS

3.1 Acetylcholinesterase Inhibitory Activity Assay of *G. asiatica*

The AChE inhibitory activity of different extractives was determined by Ellman's method. This method estimates AChE using acetylcholine iodide (substrate) and dithiobisnitro benzoic acid (DTNB). The enzymatic activity was measured by the yellow color compound produced by thiocholine when it reacts with DTNB ion. The anti-AChE activity of crude ethanolic extracts different fraction of *G. asiatica* are given in Table 1 and in Fig. 3.

Further, the anticholinesterase activity of all the fractions of crude ethanol extract such as CEEGA, PEFGA, CLFGA, EAFGA and AEFGA have been investigated at 250 µg/ml concentration. Among the fractions the highest inhibition activity was found in PEFGA at 55.88%.

Table 1. Acetylcholinesterase inhibitory activity of the crude ethanol extract, different fractions of ethanolic extract of *G. asiatica,* and donepezil at different concentrations

Name of Sample	Conc. (µg/ml)	% of Scavenging			% of Scavenging Mean ± STD	IC_{50} (µg/ml)±STD
		a	b	C		
	31.25	22.22	22.79	21.65	22.22±0.465	
	62.5	24.50	25.64	25.07	25.07±0.465	286.78±1.77
CEEGA	125	30.77	30.20	84.64	30.77±0.465	
	250	43.87	43.30	43.87	43.48±0.269	
	31.25	57.55	58.12	56.98	57.55±0.465	
	62.5	56.41	54.70	56.70	55.94±0.881	55.88±0.889
PEFGA	125	69.52	68.95	70.09	69.52±0.465	
	250	71.22	70.66	70.94	70.94±0.232	
	31.25	55.27	55.84	54.70	55.27±0.465	
	62.5	51.85	52.42	52.99	52.42±0.465	59.62±0.529
CLFGA	125	62.39	62.68	61.54	62.20±0.484	
	250	63.82	64.39	63.53	63.91±0.355	
	31.25	27.92	28.49	28.77	23.08±0.355	
	62.5	34.19	34.76	33.90	34.28±0.355	250.29±3.58
EAFGA	125	43.30	42.17	43.87	43.11±0.711	
	250	49.00	50.71	50.14	49.95±0.711	
	31.25	23.36	22.79	23.07	23.08±0.232	
	62.5	37.04	37.61	36.47	37.07±0.465	268.66±0.3.37
AEFGA	125	40.46	42.17	41.60	41.41±0.711	
	250	45.87	46.44	47.29	46.53±0.585	
	31.25	54.13	54.70	55.84	54.89±0.711	
Donepezil	62.5	71.23	71.23	71.79	71.41±0.269	28.47±0.150
	125	73.50	73.79	74.07	73.79±0.232	
	250	75.21	75.78	76.07	75.68±0.355	

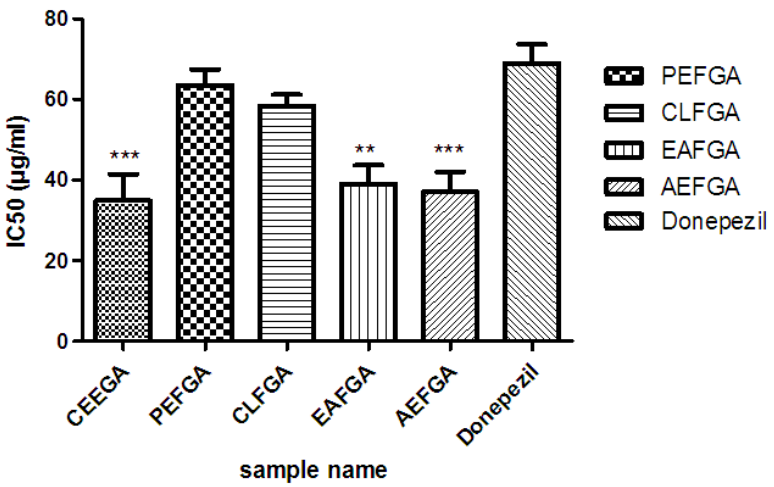

Fig. 3. IC_{50} (µg/ml) values of crude, standard and different extractives of *G. asiatica* of Anti-acetylcholinesterase activity Assay

*Values are presented as the mean ±SD [SD=Standard Deviation]. N=6, *p<0.05 compared with Standard (One-way ANOVA followed by Dunnet's test)*

3.2 Acetyl Cholinesterase Inhibitory Activity Assay of *G. tiliaefolia*

The acetylcholinesterase inhibitory activity of different extractives was determined by Ellman's method. This method estimates acetylcholinesterase (AchE) using acetylcholine iodide (substrate) and dithiobisnitro benzoic acid (DTNB). The enzymatic activity was measured by the yellow color compound produced by thiocholine when it reacts with dithiobisnitro benzoate ion. The results for anticholinesterase activity of the different fractions of the crude ethanol extract of *G. tiliaefolia* are given in Table 2 and in Fig. 4.

Further, the anticholinesterase activity of all the fractions of crude ethanol extract such as CEEGT, PEFGT, CLFGT, EAFGT and AEFGT have been investigated at 250 µg/ml concentration. Among the fractions the highest activity was found in CLFGT (57.13 % inhibition).

3.3 Butylcholinesterase Inhibitory (BchE) Activity Assay of *G. asiatica*

The Butyrylcholinesterase inhibitory activity of extractives was determined by Ellman's method. This method estimates Butyrylcholinesterase (BchE) activity using butyrylythiocholine iodide (substrate) and dithiobisnitro benzoic acid (DTNB). The enzymatic activity was measured by the yellow color compound produced by thiocholine when it reacts with dithiobisnitro benzoate ion. The results for anticholinesterase activity of crude methanol extracts different fraction of *G. asiatica* are given in Table 3 and Fig. 5.

Among the fractions of crude ethanol extract, CLFGA and EAFGA showed the most potent activity with IC_{50} value of 26.14µg/ml and 27.47 µg/ml which is higher than that of DON (Standard) With IC_{50} value of 28.13 µg/ml. On the other hand, (PEFGA), (CEEGA) and (AEFGA) fraction showed free radical scavenging activity with IC_{50} value of 29.54 µg/ml, 30.84µg/ml and 129.33 µg/ml respectively. Our results clearly demonstrate that the extractives of *G.asiatica* possess antiradical activity.

3.4 Butylcholinesterase Inhibitory (BchE) Activity Assay of G. titiaefolia

The Butyrylcholinesterase inhibitory activity of extractives was determined by Ellman's method. This method estimates Butyrylcholinesterase (BchE) activity using butyrylythiocholine iodide (substrate) and dithiobisnitro benzoic acid (DTNB). The enzymatic activity was measured by the yellow color compound produced by thiocholine when it reacts with dithiobisnitro benzoate ion. The results for anticholinesterase activity of crude methanol extracts different fraction of *G.tiliaefolia* are given in Table 4 and Fig. 6.

Table 2. Acetylcholinesterase inhibitory activity of the crude ethanol extract of *G. asiatica* and Donepezil (standard) at different concentration

Name of Sample	Conc. (µg/ml)	% of Scavenging			% of Scavenging Mean ± STD	IC_{50} (µg/ml)±STD
		a	b	C		
	31.25	31.91	32.48	33.05	32.48±0.465	
	62.5	38.18	38.46	37.61	38.08±0.355	272.53±2.73
CEEGT	125	39.32	38.75	39.89	39.31±0.465	
	250	45.58	46.15	46.72	46.15±0.465	
	31.25	56.98	57.83	58.12	57.64±0.484	
	62.5	52.42	51.85	52.99	52.42±0.465	59.61±0.529
PEFGT	125	64.10	63.25	63.82	63.72±0.355	
	250	68.95	68.38	69.23	68.85±0.355	
	31.25	46.15	46.72	48.43	47.10±0.968	
	62.5	54.13	55.27	54.70	54.70±0.465	57.13±0.485
CLFGT	125	56.69	57.83	58.40	57.64±0.711	
	250	60.11	58.12	59.54	59.26±0.839	
	31.25	29.06	29.63	28.77	29.15±0.355	
	62.5	36.75	37.03	34.19	35.99±1.28	277.20±5.01
EAFGT	125	40.17	40.46	42.16	40.93±0.880	
	250	44.16	46.15	45.01	45.10±0.816	
	31.25	26.78	26.21	26.78	26.59±0.268	
	62.5	31.91	32.19	32.48	32.19±0.232	268.66±3.37
AEFGT	125	39.60	39.03	40.46	39.69±0.585	
	250	45.87	46.44	47.29	46.53±0.585	
	31.25	54.13	54.70	55.84	54.89±0.711	
Donepezil	62.5	71.23	71.23	71.79	71.41±0.269	28.47±0.150
	125	73.50	73.79	74.07	73.79±0.232	
	250	75.21	75.78	76.07	75.68±0.355	

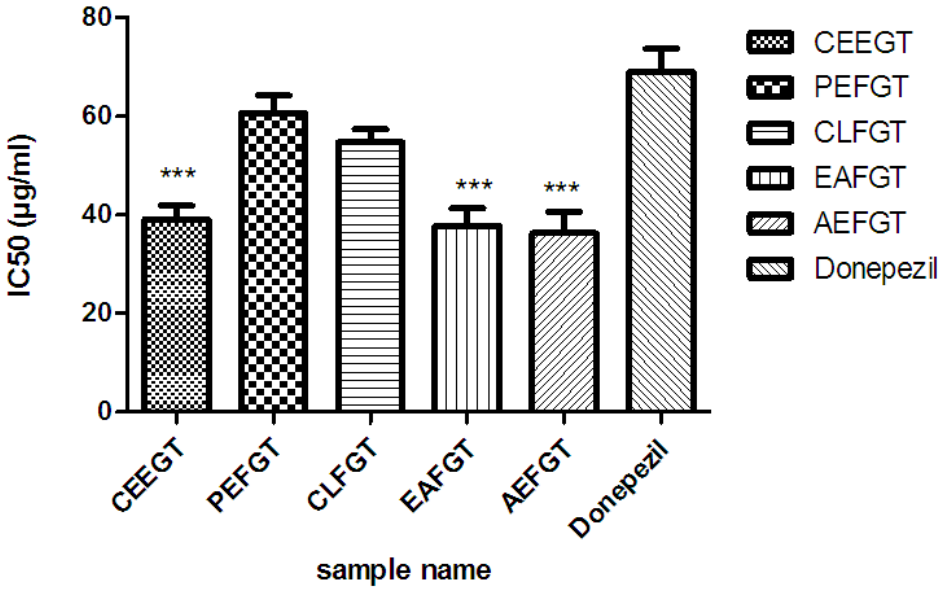

Fig. 4. IC_{50} (µg/ml) values of crude, standard and different extractives of *G. tiliaefolia* of Anti-acetylcholinesterase activity assay

*Values are presented as the mean ±SD [SD=Standard Deviation]. N=6, *p<0.05 compared with Standard (One-way ANOVA followed by Dunnet's test)*

Table 3. Anti-butylcholinesterase activity assay of the crude ethanol extracts different fractions of *G. asiatica* and Donepezil (standard) at different concentrations

Name of Sample	Conc. (µg/ml)	% of Scavenging			% of Scavenging Mean ± STD	IC_{50} (µg/ml) ±STD
		a	b	C		
	31.25	50.33	51.00	52.67	51.33±0.981	
	62.5	53.00	53.67	54.33	53.67±0.544	30.84±0.577
CEEGA	125	55.67	57.00	57.67	56.77±0.831	
	250	58.33	59.67	60.33	59.44±0.831	
	31.25	52.33	53.33	53.00	52.89±0.416	
	62.5	55.33	56.33	57.00	56.22±0.685	29.54±0.233
PEFGA	125	60.33	61.00	61.67	61.00±0.544	
	250	64.33	63.67	64.00	64.00±0.272	
	31.25	59.67	60.00	59.67	59.78±0.157	
	62.5	61.33	62.33	61.67	61.78±0.416	26.14±0.069
CLFGA	125	66.67	69.00	69.67	68.44±1.29	
	250	75.67	75.67	76.33	75.89±0.314	
	31.25	56.33	57.00	57.33	56.89±0.416	
	62.5	58.33	59.00	60.00	59.11±0.685	27.47±0.201
EAFGA	125	61.33	62.33	63.00	62.22±0.685	
	250	65.00	64.67	65.67	65.11±0.415	
	31.25	34.33	33.67	34.33	34.11±0.314	
	62.5	42.67	43.00	43.67	43.11±0.415	129.33±1.47
AEFGA	125	47.67	48.33	49.00	48.33±0.544	
	250	56.33	56.67	57.00	56.67±0.272	
	31.25	55.00	55.67	56.00	55.56±0.416	
Donepezil	62.5	60.00	60.33	61.00	60.44±0.416	28.13±0.170
	125	70.33	71.00	71.67	71.00±0.544	
	250	84.33	85.33	85.00	84.89±0.416	

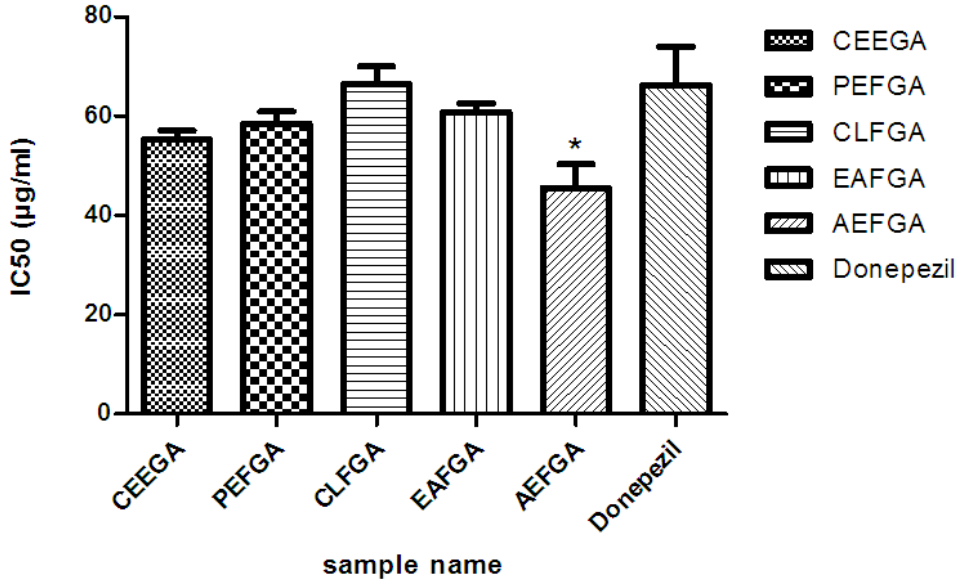

Fig. 5. IC_{50} (µg/ml) values of different extractives of *G. asiatica* of Anti-Butyrylcholinesterase activity assay

*Values are presented as the mean ±SD [SD=Standard Deviation]. N=6, *p<0.05 compared with Standard (One-way ANOVA followed by Dunnet's test)*

Table 4. Anti-butylcholinesterase activity assay of the crude ethanol extracts different fractions of *G. tiliaefolia* and Donepezil (standard) at different concentrations

Name of Sample	Conc. (µg/ml)	% of Scavenging			% of Scavenging Mean ± STD	IC_{50} (µg/ml)±STD
		a	b	C		
	31.25	47.67	49.00	49.67	48.78±0.831	
	62.5	52.33	53.00	53.67	53.00±0.544	32.33±0.550
CEEGT	125	57.33	57.67	58.67	57.89±0.567	
	250	61.00	61.67	59.67	60.78±0.831	
	31.25	54.67	56.00	56.33	55.67±0.720	
	62.5	60.33	61.67	62.00	61.33±0.720	28.07±0.366
PEFGT	125	62.33	63.00	63.33	62.89±0.415	
	250	67.00	67.67	68.33	67.67±0.544	
	31.25	57.67	59.00	59.67	58.78±0.831	
	62.5	63.33	64.33	64.67	64.11±0.567	26.59±0.378
CLFGT	125	66.33	67.67	68.33	67.44±0.831	
	250	73.00	71.67	73.33	72.67±0.720	
	31.25	53.00	54.00	54.33	53.78±0.567	
	62.5	61.67	61.00	60.00	60.89±0.685	29.06±0.308
EAFGT	125	63.67	63.33	64.00	63.67±0.272	
	250	68.00	67.00	65.67	66.89±0.956	
	31.25	32.00	33.00	31.67	32.22±0.567	
	62.5	40.33	39.67	41.00	40.33±0.544	134.25±0.92
AEFGT	125	46.33	47.00	46.33	46.56±0.314	
	250	58.67	57.67	57.00	57.78±0.685	
	31.25	55.00	55.67	56.00	55.56±0.416	
Donepezil	62.5	60.00	60.33	61.00	60.44±0.416	28.13±0.170
	125	70.33	71.00	71.67	71.00±0.544	
	250	84.33	85.33	85.00	84.89±0.416	

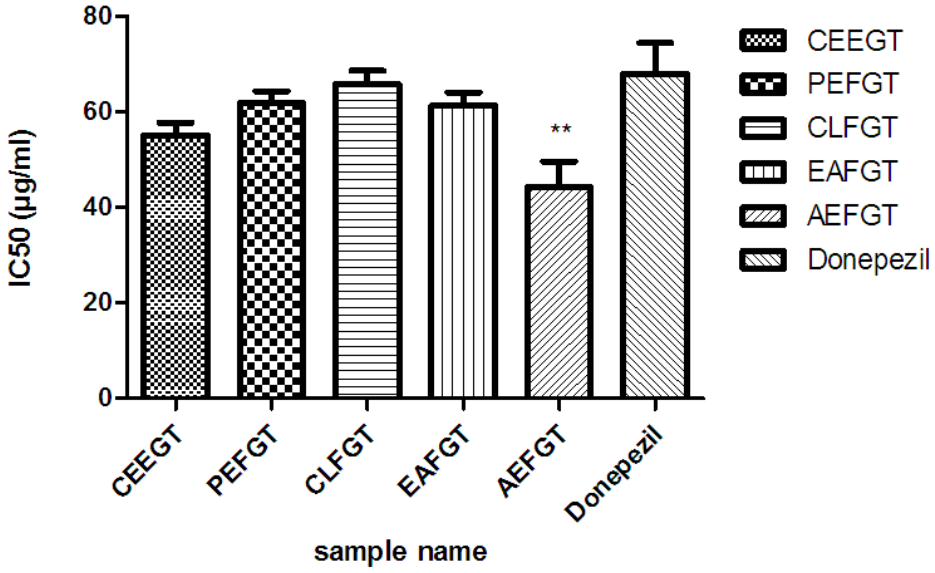

Fig. 6. IC_{50} (µg/ml) values of different extractives of *G.tiliaefolia* of Anti-Butyrylcholinesterase activity assay

*Values are presented as the mean ±SD [SD=Standard Deviation]. N=6, *p<0.05 compared with Standard (One-way ANOVA followed by Dunnet's test)*

Further, the anti-butylcholinesterase activity of all the fractions of crude ethanol extract such as CEEGT, PEFGT, CLFGT, EAFGT and AEFGT have been investigated at 250 µg/ml concentration. Among the fractions the highest activity was found in CLFGT (26.59% inhibition).

4. DISCUSSIONS

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by loss of memory and cognition. Currently, only five medications are approved by the Food and Drug Administration to treat AD. Four of them are acetylcholinesterase inhibitors such as donepezil, galantamine, rivastigmine, tacrine and the fifth is the N-methyl-d-aspartate antagonist memantine [20]. The history of drug discovery showed that plants are highly rich sources of bioactive compounds and lead to the development of drugs for the treatment of neurological diseases including AD [21]. In traditional practices of medicine plants have been used to enhance cognitive function and to alleviate other symptoms associated with the AD. Inhibition of AChE enhances cholinergic transmission by reducing enzymatic degradation of acetylcholine is a promising strategy for the development of AD-drug. AChE inhibitors are the only source of the compound that is currently approved for the treatment of AD. However, our results revealed moderate anti-acetylcholinesterase effect of *G.asiatica* and *G. tiliaefolia.* Also, the activity, the different fractions of the crude extract such as petroleum ether, crude ethanol extract, chloroform, ethyl acetate and aqueous fractions were examined similarly at a concentration of 250 µg/ml. Among the fractions, the CLFGA and CLFGT had the highest % of inhibition on BChE at a concentration of 250 µg/ml. However, our results revealed significant anti-BChE and AChE inhibitory effect of *G. asiatica* and *G. tiliaefolia.*

5. CONCLUSIONS

The present study was undertaken to investigate the *ex vivo* anti-cholinesterase and anti-BChE effects of *G. asiatica* and *G. tiliaefolia.* Inhibition of AChE, which enhances cholinergic transmission by reducing the enzymatic degradation of acetylcholine, is a promising strategy for the development of neurodegenarative disorders like AD drug. *Ex-vivo* effectiveness of *G. asiatica* and *G. tiliaefolia* its components remain to be investigated. The results indicate that *G. asiatica* and *G. tiliaefolia.* may be of value for an effective treatment for AD.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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