Antioxidant and Cholinesterase Inhibitory Activities of Ethyl Acetate Extract of Terminalia chebula: Cell-free In vitro and In silico Studies

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ABSTRACT
Background: Alzheimer’s disease (AD) is a progressive neurodegenerative disorder clinically characterized by memory loss and impaired cognitive function. Cholinergic enzyme deficiency and oxidative stress are the two major factors implicated in the pathogenesis of AD. The symptomatic treatment, as of now, is the use of cholinesterase inhibitors toward cholinergic “downturn.” Therefore, there is a search for compounds that will be useful in focused therapies. There has been suggestion that Terminalia chebula fruit would be a potential source.

Objective: To assess the anticholinesterase and antioxidant activities of T. chebula fruit which is widely practiced in the Ayurvedic medicines for memory enhancement.

Materials and Methods: Ethyl acetate extract of T. chebula fruit (TCEA) was subjected to phytochemical investigation of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activities and cell-free antioxidant activity. TCEA was further subjected to gas chromatography-mass spectrum (GC-MS) analysis. The bioactive compounds were analyzed for molecular docking with AChE and BuChE proteins.

Results: TCEA exhibited potent AChE and BuChE inhibitory activities comparable to the standard drug donepezil. In vitro cell-free antioxidant assays demonstrated that TCEA possesses excellent free radical scavenging activity, reducing power, and potent metal-chelating activity. Total polyphenolic content of TCEA was 596.75 ± 0.35 μg gallic acid equivalents/mg of extract, which correlates with the antioxidant activity of TCEA. Molecular docking of compounds expounded in GC-MS analysis for AChE and BuChE enzyme revealed that methyl N,N-benzoxycarbonyl-beta-l-aspartyl-beta-d-glucosaminide as the most potent compound with good predicted activities.

Conclusion: Overall, the results revealed that the bioactive molecule methyl N,N-benzoxycarbonyl-beta-l-aspartyl-beta-d-glucosaminide present in TCEA is a potential depressant for the treatment of AD and related neurodegenerative disorders.

Key words: Acetylcholinesterase, Alzheimer’s disease, antioxidant, butyrylcholinesterase, oxidative stress, Terminalia chebula fruit

SUMMARY
• The present study was carried out to assess the neuroprotective effect of Terminalia chebula fruit and its phytoconstituent. Phytochemical analysis of fruit ethyl acetate extract of T. chebula (TCEA) showed the presence of alkaloid, cardiac glycoside, and tannin. TCEA showed potent acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibitory activities when compared to standard drug donepezil. Results of in vitro antioxidant assays revealed excellent free radical scavenging activity, reducing power, and potent metal-chelating activity. Gas chromatography-mass spectrum analysis illustrated the presence of 22 active compounds, among which methyl N,N-benzoxycarbonyl-beta-l-aspartyl-beta-d-glucosaminide exhibited potent AChE and BuChE inhibition analyzed through silico studies.

Abbreviations used: AD: Alzheimer’s disease; TCEA: Ethyl acetate extract of Terminalia chebula; GC-MS: Gas chromatography-mass spectrum; ROS: Reactive oxygen species; RNS: Reactive nitrogen species; AChE: Acetylcholinesterase; BuChE: Butyrylcholinesterase; NFT: Neurofibrillary tangles; Aβ: β-amyloid; NSAIDS: Nonsteroidal anti-inflammatory drugs; FDA: Food and Drug Administration; RT: Room temperature; HCl: Hydrochloric acid; ATCI: Acetylthiocholine iodide; BTOCI: Butyrylthiocholine iodide; BHT: Butylated hydroxytoluene; DPPH: 2,2-diphenyl-1-picrylhydrazyl; TCA: Trichloroacetic acid; GAE: Gallic acid equivalent; NICT: National Institute of Information and Communications Technology; 3D: Three-dimensional; PDB: Protein data bank; OPLS: Optimized potentials for liquid simulations; XP: Extra precision; SD: Standard deviation; ANOVA: Analysis of variance; EDTA: Ethylenediaminetetraacetic acid.

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INTRODUCTION
Alzheimer’s disease (AD) is an irreversible dementia affecting elderly population above the age group of 65 years.[14] Neuropathological hallmarks of AD brains are β-amyloid (Aβ) deposits in the synaptic junctions and neurofibrillary tangles (NFT) in the axons, leading to disconnection of synaptic communication, ultimately resulting in neuronal death.[2] Clinically, AD is characterized by gradual memory loss, decline in cognitive function, and deterioration of physical function. Multiple pathways, including cholinergic dysfunction, inflammation, oxidative stress, alteration in calcium homeostasis, and mitochondrial dysfunction, are implicated in the pathogenesis of AD.[3] Several studies have shown that oxidative stress plays an important role in pathogenesis of AD, leading to neuronal dysfunction and cell death.[5,6] In triple-transgenic mouse model of AD, increased level of oxidative markers such as lipid peroxidation and protein oxidation was observed.[6] In vitro studies have shown that oxidative stress directly promotes tau hyperphosphorylation as well as aggregation of Aβ peptide.[7,8] However, recent studies have shown that Aβ peptide, the key pathogenic factor of AD, generates reactive oxygen species (ROS) and reactive nitrogen species during its aggregation, thereby promoting oxidative stress-mediated neuronal damage. Methionine-35 in the Aβ peptide itself promotes oxidative stress. Recent evidences illustrate that accumulation of transition metal ions such as copper, iron, and zinc during aging facilitates Aβ aggregation and tau phosphorylation and aggregation.[9] These findings suggest that multipotent compounds with anticholinesterase, antioxidant, and metal-chelating properties can act as disease-modifying agents for the treatment of AD and other neurodegenerative disorders.[10] Current therapeutic interventions for AD include cholinergic replacement therapy and neurotophins, antioxidant therapy, β- and γ-secretase inhibitors, anti-aggregators of Aβ peptide, and nonsteroidal anti-inflammatory drugs.[11,12] Current symptomatic treatments for AD include the Food and Drug Administration (FDA)-approved acetylcholinesterase (AChE) inhibitors, namely, donepezil, rivastigmine, galantamine, and tacrine, which are effective only for patients with mild-to-moderate AD and produce severe side-effects.[13] Hence, search for multipotent drugs with diverse AD targets from natural sources is attractive. Terminalia chebula Retz. (Combretaceae) used in the present study is called the “king of medicines” in the Ayurvedic system because of its extraordinary power of healing. Its fruit is the major ingredient of the Ayurvedic drug triphala which is an effective astringent, rejuvenative, nervine, and antihelminthic. [14] Traditionally, T. chebula fruit is used as laxative, digestive, antispasmodic, anti-inflammatory, analgesic, astringent, and dentifrice (to heal gum ulceration). The decoction is useful in the treatment of oral ulcers, sore throat, and nervous weakness.[15] Pharmacologically, T. chebula is also known for its anticancer, antimicrobial, antioxidant, antiviral, cardiotoxic, anti-diabetic, immunomodulatory, memory-enhancing, and anti-inflammatory activities.[16,17] Recent reports have evidenced the AChE inhibitory activity of the crude extracts T. chebula,[18-20] but its bioactive compound(s) with neuroprotective effect have not been yet identified. Hence, the present study was undertaken to identify the neuroprotective compound(s) present in T. chebula fruit so as to apply such compound(s) in the management of AD.

MATERIALS AND METHODS
Preparation of plant material
Fresh fruits of T. chebula Retz. (Combretaceae) were collected from the Western Ghats (Agasthyamalai Hills) of Tirunelveli district, Tamil Nadu, India (8°39’N 77°13’E), taking assistance from the Kani tribals during July 2014. Authentication of sample was done by Dr. S. John Britto, Director, The Rapinan Herbarium and Centre for Molecular Systematics, St. Joseph’s College, Tiruchirappalli, Tamil Nadu, India (Voucher No: NS001). Samples were washed with running tap water, to remove dust and debris, and surface sterilized with ethanol to remove the associated biota. The samples were dried under shade and made into a fine powder. The powder was quantitatively extracted with ethyl acetate in a soxhlet apparatus. The extract was evaporated to dryness under reduced pressure, and the dried extract was stored in airtight containers at −20°C until further studies. The percentage of yield was calculated using the following formula:

\[
\text{Yield of the extract} = \frac{\text{Weight of the beaker with extract} - \text{Weight of the sample in grams}}{\text{Weight of the empty beaker}} \times 100
\]

The dried extract was diluted to desired concentration in sterile distilled water just before use.

Determination of acetylcholinesterase and butyrylcholinesterase inhibitory activities
AChE and butyrylcholinesterase (BuChE) inhibitory activities were determined adopting the method of Ingkaninan et al.[21] with a slight modification to suit spectrophotometric analysis. Donepezil, the standard anticholinesterase drug, was used as the reference. AChE/BuChE (10 U/mL) solution (10 µL) was incubated with various concentrations of ethyl acetate extract of T. chebula (TCEA) (100–500 µg/mL) in 0.05 M Tris-hydrochloride acid (HCl) buffer (pH 8.0) for 45 min at room temperature (RT). The reaction mixture was treated with 3 mM DTNB (125 µL) and the final volume was made up to 300 µL with Tris-HCl buffer (pH 8.0). The enzyme activity was initiated by the addition of 25 µL of 15 mM acetylthiocholine iodide/butyrylthiocholine iodide (ATCI/BTCI). The rate of hydrolysis of ATCI/BTCI was assessed by the degree of formation of the yellow-colored product (5-thio-2-nitrobenzoate anion) at 405 nm in an ultraviolet-visible spectrophotometer (U-2800, Hitachi, Japan). The percentage inhibition of AChE/BuChE was determined by comparison of the rate of reaction of the test samples with the blank (Tris-HCl buffer) using the formula, \((C – T)/C \times 100\), where \(C\) and \(T\) are the activity of enzymes in the presence and absence of test sample.

Antioxidant assay in cell-free system
Free radical scavenging activity
2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is the most widely used method to assess the free radical scavenging activity.[22] DPPH in methanol (0.1 mM) was added to 1 ml of different doses (100–500 µg/mL) of TCEA in water and incubated at RT for 30 min, and the absorbance was measured at 517 nm. Lower the absorbance, higher was the free radical scavenging activity. Butylated hydroxytoluene (BHT) (100–500 µg/mL) was used as positive control. The percent DPPH• scavenging activity was calculated using the equation:

\[
\text{DPPH• scavenging effect (％)} = \frac{A_{\text{cont}} - A_{\text{test}}}{A_{\text{cont}}} \times 100
\]

Where \(A_{\text{cont}}\) and \(A_{\text{test}}\) are the absorbance of the control reaction and test samples, respectively.

Total reducing capacity of ethyl acetate extract of Terminalia chebula
The total reducing capacity of TCEA was determined as described by Oyaizu.[23] Various doses of TCEA (100–500 µg/mL) in 0.25 ml distilled water was treated with 0.5 ml of 0.2 M phosphate buffer (pH 6.6) and 0.5 ml of 1% potassium ferricyanide, and the reaction mixture was incubated at 50°C for 20 min. After incubation 0.5 ml of 10% trichloroacetic acid was added to the
incubating mixture and centrifuged at 1000 xg for 10 min. Upper layer was separated and treated with 0.1 ml of 0.1% FeCl₃ and 0.5 ml of distilled water, and the absorbance was measured at 700 nm with ascorbic acid as positive control. Higher the absorbance, greater was the reducing power.

**Metal-chelating activity**

The metal-chelating activity was determined by adopting the method of Haro-Vicente et al. Briefly, different concentrations (100–500 μg/mL) of TCEA were added to 0.15 mM ferrous sulfate solution, followed by the addition of 0.5 mM ferrozine, the reaction mixture was incubated at RT for 20 min, and the absorbance was measured at 562 nm.

**Phytochemical analysis**

The TCEA was subjected to phytochemical analysis adopting standard procedures as described by Harbome.

**Determination of total phenolic content**

Total soluble phenolic compounds of TCEA were determined using Folin-Ciocalteu reagent as previously described by Singleton and Rossi, using gallic acid as standard. One hundred microliters of the sample (1 g of dry sample in 10 ml of acetone) in duplicate was incubated with 1 ml of diluted Folin-Ciocalteu reagent (1:2 with water) at RT for 5 min. Seven percent Na₂CO₃ (1 mL) was added to the reaction mixture and incubated at RT for 90 min. The absorbance was measured at 750 nm, and the results are expressed as gallic acid equivalent (GAE) in milligram per gram of dry sample.

**Gas chromatography-mass spectrum analysis**

Ten milligrams of TCEA was mixed with 1 mL of ethyl acetate, membrane-filtered (0.22 μm), and fractionated using gas chromatography-linked mass spectrometry (GC-MS; QP-5050, Shimadzu, Japan). Briefly, 2 μL volume of the sample was injected into the 30 m glass capillary column with a film thickness of 0.25 μm (30 m × 0.2 mm i.d. coated with UCON HB 2000) using the following temperature programmer: initial oven temperature of 40°C for 4 min, increased to 175°C at 1°C/min, and then ramped at 250°C for 10 min. The gas chromatography (Shimadzu GC 15A) was equipped with flame ionization detector connected to an integrator. The relative amount of each compound was reported as percent of ion current. Identification of unknown compounds was made by probability-based matching using the computer library built within the National Institute of Information and Communications Technology 12 system.

**Molecular docking studies**

The compounds appearing in GC-MS analysis were further subjected to molecular docking analysis. The three-dimensional structures for all the compounds were downloaded from PubChem database. AChE and BuChE protein structures were retrieved from protein...
data bank (AChE - PDB ID: 1EVE; BuChE - PDB ID: 1P09). Before performing the molecular docking, the protein and ligand structures were prepared using "protein preparation" and "ligand preparation" modules available in Grid-based Ligand Docking with Energetics (GLIDE) software from Schrodinger.\textsuperscript{29,30} AChE and BuChE were subjected to energy minimization to remove the geometric constraints using the optimized potentials for liquid simulations (OPLS) force field. The ligands were processed with the LigPrep program to assign the suitable protonation states at physiological pH (7.0 ± 1.0). Conformer generation was carried out with the ConGen torsional sampling using OPLS_2005 force field. The van der Waals radii were scaled using a default scaling factor of 0.80 and default partial cutoff charge of 0.15 to decrease the penalties. All the compounds were docked into the binding site of AChE and BuChE proteins using Grid GLIDE software. The docking was performed with OPLS_2005 force field using extra precision (XP) module of the Schrodinger Suite. The XP GLIDE score function was used to order the best-ranked compounds and the specific interactions, such as hydrogen bond interaction.

### Statistical analysis
All experiments were carried out in triplicates, and the results are expressed as mean ± standard deviation. Significant of the differences between means were determined by one-way analysis of variance followed by Duncan’s multiple range tests; $P < 0.05$ was considered as significant. $IC_{50}$ value was calculated adopting probit analysis.

### RESULTS AND DISCUSSION
AD threatens to be the scourge of the 21\textsuperscript{st} century as aging population increases worldwide. Therefore, improved management and therapies for AD have become a major health-care challenge of this century. Recent reports have proposed four hypotheses for AD development: (i) loss of presynaptic markers of a cholinergic system; (ii) accumulation of A\textbeta plaques in the brain; (iii) NFT or abnormal tau protein hyperphosphorylation; and (iv) induction of oxidative stress.\textsuperscript{29} Cholinergic hypothesis and A\textbeta plaque formation are the two important platforms for the development of anti-AD drugs. Adverse side effects of FDA-approved drugs diverted the attention of scientists toward natural sources. In the present study, we investigated the antioxidant and cholinesterase inhibitory activities of TCEA fruit in cell-free assays. Percentage yield of TCEA was observed to be 5.78%.

**Effect of ethyl acetate extract of *Terminalia chebula* on acetylcholinesterase and butyrylcholinesterase activities**

Scientific reports have shown that aggregation of A\textbeta peptide is accelerated by AChE leading to formation of A\textbeta-AChE complex at the synaptic region of the hippocampus causing neuronal degeneration.\textsuperscript{30,31} The current symptomatic treatment for AD involves potentiation of cholinergic activity through inhibition of AChE.\textsuperscript{32} Therefore, the present study was conducted to assess the AChE inhibitory activity of TCEA. Figure 1a showed that TCEA inhibits AChE in a dose-dependent manner. At 500 $\mu$g/ml, TCEA produced the highest inhibition to 100.23 ± 0.12% when compared to the same dose of the positive control donepezil (94.30 ± 0.001%). The $IC_{50}$ values of TCEA and donepezil against AChE were found to be 45.44 ± 0.03 and 24.4 ± 0.01 $\mu$g/mL, respectively.

BuChE plays a key role in regulating the cholinergic neurotransmission in the glial cells and subcortical neurons. The level of BuChE increases as AD progresses, which in turn promotes the transformation of “benign” plaques to “malignant” ones leading to neuronal degeneration.\textsuperscript{33} Therefore, inhibition of BuChE not only enhances the level of Acetylcholine (ACh) in the brain but also attenuates the formation of A\textbeta plaques.\textsuperscript{34} Hence, in the present study, the BuChE inhibitory activity of different concentrations of TCEA was evaluated. Figure 1b shows that TCEA inhibits BuChE activity in a dose-dependent manner. At 500 $\mu$g/mL dose, TCEA exhibited a significantly ($P < 0.05$) higher inhibition (100.67 ± 0.07%) than the same dose of positive control, donepezil (92.92 ± 0.05%). The $IC_{50}$ values of TCEA and donepezil were observed to be 52.32 ± 0.04 and 55.63 ± 0.02 $\mu$g/mL, respectively. Recent reports show that FDA-approved drugs (tacrine and rivastigmine) and many naturally derived drugs act as sources of new bifunctional scaffolds targeting cholinesterase as well as A\textbeta aggregation.\textsuperscript{35,36} The results lead us to the conclusion that TCEA, through dual cholinesterase inhibitor activities (AChE and BuChE), restores ACh level, mitigates the inflammatory response, and reduces the aggregation of A\textbeta and its toxicity to neurons, thereby ameliorating the cognitive defect.

**Cell-free in vitro antioxidant assays**

Data generated from experimental and human studies indicate that oxidative stress plays a major role in the pathogenesis of AD. Treatment with antioxidants might prevent tissue damage and improve survival as well as neurological outcome.\textsuperscript{37,38} Preclinical studies provide strong evidence that antioxidants such as Vitamin E, selegiline, lipoic acid, and \alpha-tocopherol prevent or slowdown AD.\textsuperscript{39} Therefore, we aimed at finding

### Table 1: Qualitative phytochemical analysis

| Sample   | *Terminalia chebula* fruit (ethyl acetate extract) |
|----------|--------------------------------------------------|
| Alkaloid  | +                                                |
| Flavonoid | −                                                |
| Cardiac glycoside | +++                                          |
| Terpenoid | −                                                |
| Anthraquinone | −                                              |
| Reducing power | −                                             |
| Tannin   | +++                                              |

←: No response; +: Weak content; ++: Moderate content; +++: Strong content
Table 2: Compounds identified in ethyl acetate extract of *Terminalia chebula* fruit

| Peak number | Name                                                                 | PubChem ID | Molecular weight | Molecular formula          |
|-------------|----------------------------------------------------------------------|------------|------------------|----------------------------|
| 1           | 2-fluoroisoproterenol                                                 | 547892     | 229              | C_{11}H_{14}FNO            |
| 2           | 2-(2-oxo-1,3-oxazolidin-3-yl) 4-pyridinylcarbamate                   | 544634     | 251              | C_{11}H_{12}N_{2}O_{2}     |
| 3           | Benzene, 1-nitro-2-(p-methlyphenoxy)-4-fluoro-                        | 548605     | 247              | C_{8}H_{5}FNO              |
| 4           | Acetic acid, butyl ester                                             | 31272      | 116              | C_{4}H_{8}O                 |
| 5           | Ethylbenzene                                                         | 7500       | 106              | C_{8}H_{10}                 |
| 6           | 1-butanol                                                            | 263        | 74               | C_{5}H_{10}O               |
| 7           | 1,2-ethanediol, monoformate                                          | 69404      | 90               | C_{8}H_{12}O               |
| 8           | Furfural                                                             | 7362       | 96               | C_{8}H_{8}OCHO             |
| 9           | Glycerin                                                             | 753        | 92               | C_{3}H_{8}O                 |
| 10          | Bis (N-methoxy-N-methylamino)methane                                 | 554047     | 134              | C_{8}H_{10}N_{2}O          |
| 11          | 2-butyl-1-octanol                                                    | 19800      | 186              |                            |
| 12          | Methyl N-(N-benzoxycarbonyl-beta-l-aspartyl)-beta-d-glucosaminide     | 562266     | 442              | C_{20}H_{22}N_{2}O_{2}     |
| 13          | 1-Iodo-2-methylenecane                                               | 545590     | 296              | C_{9}H_{17}I               |
| 14          | 3-Eicosene, (E)-                                                    | 5365051    | 280              | C_{22}H_{44}               |
| 15          | N-Chz-glycyglycine p-nitrophenyl ester                               | 273306     | 387              | C_{17}H_{17}NO_{2}         |
| 16          | Levoglucosenone                                                      | 699486     | 126              | C_{9}H_{18}O               |
| 17          | Carbamic acid, phenyl ester                                          | 69322      | 137              | C_{7}H_{11}NO_{2}          |
| 18          | cis-2-methyl-7-octadecene                                            | 6242671    | 266              | C_{16}H_{34}               |
| 19          | 2-Pentadecanol                                                       | 966875     | 228              | C_{16}H_{34}O              |
| 20          | Phenol, 2,4-bis(1,1-dimethylethyl)                                   | 7311       | 206              | C_{12}H_{12}O              |
| 21          | Benzoic acid                                                         | 243        | 122              | C_{7}H_{6}O                 |
| 22          | 5-hydroxymethylfurfural                                              | 237332     | 126              | C_{6}H_{10}O               |

Table 3: Tested ligands with their docking scores and their interactions with the acetylcholinesterase and butyrylcholinesterase receptor site

| PubChem ID  | GLIDE score | GLIDE energy | Amino acid residues | Distance (Å) | GLIDE score | GLIDE energy | Amino acid residues | Distance (Å) |
|-------------|-------------|--------------|---------------------|--------------|-------------|--------------|---------------------|--------------|
| 547892      | −10.13      | −35.12       | PHE 288             | 1.92         | −9.72       | −55.25       | LEU 286             | 1.78, 1.82   |
| 273306      | −7.23       | −55.59       | PHE 288             | 2.03         | −7.23       | −57.84       | GLY 353             | 1.82, 1.72   |
| 7311        | −7.08       | −27.66       | PHE 330             | 2.05         | −7.68       | −38.59       | GLY 353             | 1.82, 1.72   |
| 546805      | −6.83       | −32.97       | ASP 72              | 1.94         | −5.35       | −28.28       | HIP 438             | 1.83         |
| 544634      | −5.81       | −38.56       | Nle                   | Nil          | −5.96       | −34.01       | GLY 197             | 2.56         |
| 69322       | −5.79       | −23.16       | Nle                   | Nil          | −4.68       | −40.32       | TRP 82              | 2.73         |
| 455590      | −5.48       | −26.65       | Nle                   | Nil          | −4.07       | −26.96       | Nil                 | Nil          |
| 96687       | −5.48       | −32.16       | ARG 289             | 1.89         | −3.60       | −30.56       | GLY 197             | 1.86         |
| 5365051     | −5.44       | −32.18       | Nle                   | Nil          | −3.26       | −33.94       | Nle                 | Nil          |
| 6242671     | −5.44       | −30.02       | Nle                   | Nil          | −3.68       | −30.60       | Nle                 | Nil          |
| 237332      | −5.37       | −22.99       | PHE 288             | 2.23         | −4.74       | −22.31       | HIP 438             | 1.85         |
| 7500        | −5.22       | −14.98       | Nle                   | Nil          | −4.42       | −17.25       | Nle                 | Nil          |
| 243         | −5.05       | −17.36       | ARG 289             | 2.40         | −2.95       | −20.61       | GLY 116             | 1.65         |
| 7362        | −4.77       | −16.88       | ARG 289             | 2.21         | −3.58       | −18.09       | HIP 438             | 2.22         |
| 699486      | −4.12       | −18.68       | PHE 288             | 2.08         | −3.10       | −19.94       | TRP 82              | 2.62         |
| 753         | −3.76       | −17.59       | HIP 440             | 1.90         | −3.84       | −19.74       | ASN 83              | 1.79, 1.96   |
| 19800       | −3.59       | −26.56       | TYR 121             | 2.14         | −3.38       | −26.51       | GLY 117             | 2.05         |
| 31272       | −3.54       | −18.10       | PHE 288             | 1.91         | −3.01       | −16.53       | GLY 117             | 2.25         |
| 263         | −2.85       | −12.95       | TYR 121             | 2.31         | −3.22       | −14.20       | SER 198             | 1.83         |
| 116         | −3.59       | −26.56       | TYR 121             | 2.14         | −3.38       | −26.51       | GLY 117             | 2.05         |
| 117         | −3.54       | −18.10       | PHE 288             | 1.91         | −3.01       | −16.53       | GLY 117             | 2.25         |
| 117         | −2.85       | −12.95       | TYR 121             | 2.31         | −3.22       | −14.20       | SER 198             | 1.83         |

Contd...
the antioxidant property of TCEA using a battery of cell-free in vitro antioxidant assays (DPPH, metal-chelating, and reducing power).

**DPPH radical scavenging assay**

Scavenging of the stable DPPH radical is the most widely used method to evaluate antioxidant activities in a relatively short time. The ability of TCEA to scavenge DPPH radical was evaluated by measuring the decrease in its absorbance at 517 nm. The present investigation shows that TCEA inhibits DPPH radicals in a dose-dependent manner with inhibitory activity of 91.05 and 96.6% [Figure 2a], which is lesser than the positive control, BHT (91.05 and 96.6%). The IC50 values of TCEA and BHT were 72.54 ± 0.03 and 70.02 ± 0.02 µg/ml, respectively. The free radical scavenging activity of TCEA is an indication of its hydrogen donating ability, which might be due to the presence of rich source of polyphenols, possessing −OH groups the potent H2 donors.

**Reducing power**

The reducing power assay is useful to evaluate the antioxidant potential of a compound. In this assay, the ability of the extract to reduce Fe3+ to Fe2+ is determined. Compounds with electron donating capacity are called as reductones, which has the ability to reduce the oxidized intermediates of lipid peroxidation indicating it as primary and secondary antioxidants. Figure 2b shows the reducing capacity of TCEA, compared to the standard BHT. TCEA (500 µg/ml) showed significantly higher reducing ability (absorbance 2.7 ± 0.07) than the control (0.053 ± 0.001) and the same dose as the standard BHT (2.42 ± 0.02). The result indicates that the high antioxidant potential of TCEA may be due to its ability to donate electron or hydrogen by the −OH group of polyphenols present in the extract.

**Metal-chelating activity**

Transition metal ions such as Cu2+, Zn2+, and Fe3+ play crucial role in Aβ aggregation and neurotoxicity, leading to AD. In vitro and in vivo studies have shown that increased concentrations of Cu2+ and Zn2+ in amyloid plaques promote the aggregation of Aβ peptide. Furthermore, these metal ions contribute to the generation of ROS including oxidative stress, which are the early events of neurodegeneration. Therefore, metal chelation represents a rational therapeutic approach for attenuation of AD pathogenesis. Results of the present study illustrate that TCEA (500 µg/ml) has higher metal-chelating activity of 93.01% ± 0.82% than standard BHT (76.6 ± 0.20%) and ethylenediaminetetraacetic acid (86.5 ± 0.012%) [Figure 2c]. Metal-chelating activity is one of the antioxidant mechanisms since it reduces the concentration of the transition metal that catalyzes lipid peroxidation. Currently, several lipophilic metal chelators, such as clioquinol and its derivative PBT2, have been subjected to clinical trials, which have shown encouraging results in some AD patients. Overall, the results of antioxidant assay illustrate that TCEA, through its antioxidant property, can effectively attenuate the ROS-mediated neuronal death in AD.

### Table 3: Contd...

| PubChem ID | GLIDE score | GLIDE energy | Amino acid residues | Distance (Å) | GLIDE score | GLIDE energy | Amino acid residues | Distance (Å) |
|------------|-------------|--------------|---------------------|-------------|-------------|--------------|---------------------|-------------|
| 554047     | −2.04       | −23.54       | ARG 289             | 1.98        | −1.84       | −21.69       | Nil                 | Nil         |
| 69404      | −1.84       | −20.33       | GLH 199             | 1.96        | −2.79       | −16.79       | GLY 116             | 2.17        |
|            |             |              | TYR 130             | 2.12        |             |              | GLY 117             | 2.26        |

GLIDE: Grid-based Ligand Docking with Energetics; AChE: Acetylcholinesterase; BuChE: Butyrylcholinesterase

### Phytocellular analysis

Preliminary phytocellular analysis showed the presence of alkaloids, cardiac glycosides, and tannins in TCEA [Table 1]. The total polyphenol content of TCEA was 596.75 ± 0.35 µg GAЕs/mg of extract. Polyphenols are excellent free radical scavengers in view of the presence of OH groups and hence are widely used for the prevention of cardiovascular diseases and neurodegenerative disorders. The antioxidant potential of medicinal plants is essentially due to the redox properties of phenolic compounds, which enable them to act as reducing agents, hydrogen donors, and singlet oxygen scavengers. In the present study, the abundant polyphenols present in the TCEA might be responsible for its potent antioxidant scavenging capacity.

**Gas chromatography-mass spectrum analysis**

GC-MS analysis of the TCEA and comparison of the mass spectra with data in NIST library revealed the presence of 22 different phytocellular constituents [Figure 3]. All 22 compounds were characterized [Table 2].

### Extra precision docking studies on phytoconstituents of ethyl acetate extract of Terminalia chebula with acetylcholinesterase and butyrylcholinesterase

Several drugs and compounds are known to inhibit both AChE and BuChE but are effective only for the treatment of mild-to-moderate AD. Finding potent AChE and BuChE inhibitors for the treatment of AD would be a rewarding aspect of drug discovery. Since drug discovery process is laborious, time-consuming, and expensive, structure-based drug discovery has gained importance. In the present study, molecular docking was carried out to identify novel AChE and BuChE inhibitors using XP docking method of GLIDE (Biologics Suite 2016–4, 2016 Schrödinger, LLC, New York, NY, USA). Sitemap analysis revealed five active sites for both target proteins, and the top best screenshot active site scores of AChE (1.201) and BuChE (1.013) proteins were selected and used for molecular docking [Figure 4a and b]. Based on parameters such as GLIDE score, GLIDE energy, and interaction of hydrogen bonds with protein–ligand complex, the docking results were ranked and illustrated in Table 3. The results of docking showed that all compounds of TCEA fit well in the active sites of AChE and BuChE. Among the 22 compounds docked around the active site of AChE, methyl N-(N-benzylxycarbonyl-beta-l-aspartyl)-beta-d-glucosaminide (562266) displayed a high GLIDE XP score of −10.75 kcal/mol and GLIDE energy of −59.18 kcal/mol. The top-ranked active compound (562266) interacted through the formation of four hydrogen bonds with the active site residues (PHE 288, SER 286, ARG 289, and TYR 121). Furthermore, one arene–arene interaction between the compound (562266) and TRP 84 was also observed [Table 3]. The high affinity of the compound might be due to the three backbone hydrogen bonds with PHE 288, SER 286, and ARG 289 of AChE. In addition, one side-chain hydrogen...
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Figure 4: (a) Three-dimensional structure of docked test ligand Methyl N-(N-benzyloxycarbonyl-beta-l-aspartyl)-beta-d-glucosaminide with AChE. (i) Hydrophobic interactions (green spheres) and (ii) Hydrogen bond interactions of Methyl N-(N-benzyloxycarbonyl-beta-l-aspartyl)-beta-d-glucosaminide with the represented amino acids of the active site. (iii) Methyl N-(N-benzyloxycarbonyl-beta-l-aspartyl)-beta-d-glucosaminide is interacting in the largest binding pocket of AChE. (b) Three-dimensional structure of docked test ligand Methyl N-(N-benzyloxycarbonyl-beta-l-aspartyl)-beta-d-glucosaminide with BuChE. (i) Hydrophobic interactions (green spheres) and (ii) Hydrogen bond interactions of Methyl N-(N-benzyloxycarbonyl-beta-l-aspartyl)-beta-d-glucosaminide with the represented amino acids of the active site. (iii) Methyl N-(N-benzyloxycarbonyl-beta-l-aspartyl)-beta-d-glucosaminide is interacting in the largest binding pocket of BuChE.

bond with TYR 121 of the active site residues of AChE was also observed [Figure 4 ai-iii].

Interestingly, the XP docking results of BuChE protein also showed that methyl N-(N-benzyloxycarbonyl-beta-l-aspartyl)-beta-d-glucosaminide (562266) of TCEA produced high GLIDE XP score of −9.72 kcal/mol and GLIDE energy of −55.25 kcal/mol. BuChE active site residues LEU 286 and GLH 197 were found to strongly interact with the compound (562266) [Table 3]. The most potent compound, 562266, of TCEA interacted with active site residues through two backbone hydrogen bonds of LEU 286 and one side-chain hydrogen...
bond of GLH 197. In addition, one ane–ane interaction between the compound (562266) and TRP 82 was also observed [Figure 4bi–iii]. The results of in silico studies illustrate that the compound 562266 of TCEA inhibits both AChE and BuChE activities very effectively and so it offers potential for use as a drug for AD. Further studies, currently in progress, are expected to throw light on the molecular mechanism(s) underlying the neuroprotective effect of the T. chebula compounds in general and compound 562266 in particular.

CONCLUSION

The present study has brought up experimental evidence that TCEA fruit, rich in polyphenolic compounds, revealed dual cholinesterase inhibitory activity, potent free radical scavenging activity, excellent reducing power, and high metal-chelating activity. GC-MS analysis and in silico analysis illustrated that the compound methyl N-((benzoyl)carbonyl-beta-l-aspartyl)-beta-d-glucosaminide inhibits AChE as well as BuChE activities. Further studies in assessing the neuroprotective effect of the identified compound adopting in vitro and in vivo model systems are underway.

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Conflicts of interest
There are no conflicts of interest.

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