Research Article

In vitro antioxidant assessment and a rapid HPTLC bioautographic method for the detection of anticholinesterase inhibitory activity of Geophila repens

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ABSTRACT

OBJECTIVE: Geophila repens (L.) I. M. Johnst. (Rubiaceae), a small, creeping, perennial herb, is claimed to have memory-enhancing property. The goal of this study was to assess its antioxidant and anticholinesterase activity and conduct a rapid bioautographic enzyme assay for screening acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition of G. repens extracts.

METHODS: Antioxidant activity of G. repens extracts was assessed by performing 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide (NO), superoxide (SOD), hydroxyl (OH) and total antioxidant capacity (TAC) assays. Anticholinesterase activity was investigated by quantifying the AChE and BChE inhibitory activities of chloroform (CGR), ethyl acetate (EGR) and methanol (MGR) extract fractions from G. repens leaves. A rapid high-performance thin-layer chromatography (HPTLC) bioautographic method for the detection of AChE and BChE inhibition was performed.

RESULTS: Among all extract fractions, EGR exhibited the highest half maximal inhibitory concentration (IC50) in DPPH, SOD, NO, OH and TAC assays, with IC50 of (38.33 ± 3.21), (45.14 ± 1.78), (59.81 ± 1.32), (39.45 ± 0.79) and (43.76 ± 0.81) µg/mL respectively. EGR displayed competitive, reversible inhibition of AChE and BChE activities with IC50 of (68.63 ± 0.45) and (59.45 ± 0.45) μg/mL, respectively. Total phenolic and flavonoids contents of EGR were found to be 360.42 mg gallic acid equivalents and 257.31 mg quercetin equivalents per gram of extract. Phytoconstituents of the EGR extract that were inhibitors of cholinesterase produced white spots on the yellow background of HPTLC plates in the bioautographic test.

CONCLUSION: The results of this study revealed that phenols and flavonoids could be responsible for the antioxidant, anticholinesterase activities of G. repens.

Keywords: Geophila repens; Alzheimer disease; acetylcholinesterase; butyrylcholinesterase; fingerprint; bioautographic; phenolic, antioxidant


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1 Introduction

Alzheimer’s disease (AD) is a progressive, unrepairable neurodegenerative disease which causes cognitive disorder, decline in memory, behavioural changes and deficits in activities of affected individuals.[1,2] AD is the most common form of dementing illness among middle-aged and older adults. Approximately 10 million people worldwide are affected by AD.[3] Multiple pathological features, such as cholinergic deficiency, deposition of β-amyloid peptide, neurofibrillary tangles, generation of reactive oxygen species and mitochondrial dysfunction, are associated with AD.[4] Acetylcholine (ACh) is a neurotransmitter inhibited primarily by acetylcholinesterase (AChE) and secondarily by butyrylcholinesterase (BChE), and is considered to play a role in the pathology associated with neurofibrillary tangles and neuritic plaques in AD.[5–7] Though the pathogenic cause of AD remains incompletely understood, the leading factor is considered to be the “amyloid hypothesis,” and the most effective tools against AD have been found to be inhibitors of AChE and BChE.[8] However, some synthetic AChE inhibitory drugs, such as tacrine, rivastigmine and donepezil, are only effective for mild-type AD, and their bioavailability and side effects are often problematic. Also, to date, no drug available has been able to inhibit BChE activity.[9] To screen appropriate inhibitors from natural products, an effective and fast assay system is needed.

Ideally this approach will avoid the time-consuming isolation of unknown bioactive molecules from an unexplored medicinal plant. Therefore, for this study, a rapid high-performance thin-layer chromatography (HPTLC) bioautographic assay was chosen for its quick access to the activity and localisation of bioactive molecules in complex plant matrices. The separated bioactive constituents can be directly detected on the HPTLC plate through enzyme activity: AChE enzyme can hydrolyze acetylthiocholine into thiocholine, which can hydrolyze acetylthiocholine into thiocholine, which further reacts with Ellman’s reagent, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), to produce 2-nitrobenzoate-5-mercaptopthiocholine and 5-thio-2-nitrobenzoate. Regions of the HPTLC plate which contain AChE inhibitors appear as white spots against a yellow background. At the beginning of the isolation, this bioautographic assay is required to recognise unknown active compounds. To solve this problem, we used HPTLC silica gel 60 F₂₅₄ for anticholinesterase activity measurement based on the reliable and most widely used method.[10] Free radicals can lead to cognitive disorders, and the cognitive function of brain can be significantly restored by antioxidant potential of plants whose secondary metabolites, like phenols and flavonoids, are responsible for delaying or altering the pathogenesis of AD, by scavenging the free radicals.[12,13] Similarly, galanthamine, a reference drug for AD, derived from Galanthus nivalis, increases ACh level in synapses by inhibiting the AChE.

Geophila repens (L.) I. M. Johnst. (Rubiacaeae), a small, creeping, perennial, prostrate, pubescent herb with long stems and rooting at nodes, can be found in India, China and tropical Africa. Some notable synonyms for plant are Geophila herbacea (Jacq.) K. Schum., and Rondeletia repens L., while its Spanish common name is Corrida yerba de guava and its Sanskrit name is Krishnamandaki. Besides its antifungal activity,[14] G. repens is also used to improve voice, memory, semen quality, and cure oedema, leprosy, piles, fever, inflammatory swellings and enlargements of the spleen.[15] It is also in use for the development of phytomedicine for liver diseases.[16] G. repens is known as Karimuthil in Andhra Pradesh (India), and identified as an endangered species according to the International Union for Conservation of Nature and the Iritty tribes of Kannur District of Andhra Pradesh.[17] Despite its ethnomedical uses demonstrated in literature, the present study is designed to uncover the anticholinesterase and antioxidant potentials of G. repens, and to provide a bridge between traditional and scientific understanding of this plant. A rapid HPTLC bioautographic assay was introduced for the screening of G. repens, providing quick access to bioactivity and localization of molecules of interest within complex plant matrices, before the time- and resource-intensive step of isolation.

2 Materials and methods

2.1 Chemicals and reagents

AChE (1001940206), BChE (1001390738), S-butyrylthiocholine chloride (SBTC; 1001315809), acetylthiocholine iodide (ATCI; 101291297), DTNB (D8130-5G), and galanthamine hydrobromide (101454428) were purchased from Sigma-Aldrich, USA. Bovine serum albumin (BSA; RM9981/500 mL), ascorbic acid, Folin-Ciocalteu reagent, 1,1-diphynyl-2-pirylylhydrayzyl (DPPH), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), thiobarbituric acid, trichloroacetic acid (TCA), sodium nitroprusside, ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, quercetin and gallic acid were purchased from HiMedia, India. HPTLC silica gel 60 F₂₅₄ (Merck, Germany) and all other solvents were analytical grade, and purchased from Sisco Research Laboratories Pvt. Ltd. (SRL), India.

2.2 Plant material collection

The leaves of G. repens were collected in November, 2015 from the Tamana forest, Khurda Forest Division, Odisha (Latitude: 19°52′15.2″N; Longitude: 85°01′37.2″E; Altitude: 386.79 meters above the sea level) and the plant was identified by Dr. P. C. Panda, a taxonomist of...
Regional Plant Resource Centre, Bhubaneswar, India. The voucher specimen was deposited in our centre for future references (No. 7557/RPRC).

2.3 Extraction and phytochemical screening

The leaves of *G. repens* were shade-dried and ground to powder. The powdered materials were subjected to soxhlet (Borosil) extraction with successive solvents, including hexane (HGR), chloroform (CGR), ethyl acetate (EGR) and methanol (MGR). Each fraction was evaporated using a rotary evaporator (Model: HS-2001NS, Hahnshin Scientific Co., South Korea), under reduced pressure ((1.0 ± 0.2) kPa) yielding 5.68% (HGR), 6.23% (CGR), 7.54% (EGR) and 8.38% (MGR) (w/w, with respect to the raw material). Following a standard procedure, qualitative phytochemical analysis was conducted for each of the fractions tested for the presence of various secondary metabolites.[18]

2.3.1 Total phenolic content

Total phenolic content (TPC) of HGR, CGR, EGR and MGR was determined with the Folin-Ciocalteu reagent[20] by using a multimode microplate reader (Synergy H1MF, BioTek, USA). Gallic acid was used as standard and phenolic content was expressed as milligram of gallic acid equivalents per gram of dried extract (mg GAE/g).

2.3.2 Total flavonoid content

Total flavonoid content (TFC) of HGR, CGR, EGR and MGR was assayed according to standard protocol with a slight modification[21] using a multimode microplate reader. Quercetin was used as a reference and the results were expressed as milligram of quercetin equivalents per gram of extract (mg QE/g).

2.3.3 Total alkaloid content

Total alkaloid content (TKC) of HGR, CGR, EGR and MGR was assayed following a standard procedure,[22] where a set of atropine solutions (20–100 μg/mL) was prepared as reference standards. The absorbance of test and standard solutions was measured against the reagent blank at 470 nm with a multimode microplate reader. TKC was expressed as milligram of atropine equivalent per gram of extract (mg AE/g).

2.4 Antioxidant activity of *G. repens*

2.4.1 DPPH free radical scavenging activity

The ability of *G. repens* extract to scavenge DPPH free radicals was assessed using a standard method with minor modification.[23] The antioxidant activity of the samples was determined on the basis of their ability to scavenge the stable DPPH free radical. About 2 mL DPPH solution (5.9 mg in 100 mL methanol) was added to 1 mL different concentrations (0.01 to 0.1 mg/mL) of HGR, CGR, EGR and MGR. The absorbance of the solution was measured at 517 nm after 30-minute incubation (Synergy H1MF, BioTek, USA). Ascorbic acid was used as reference drug. Percentage of inhibition was calculated by the following equation (same equation also in Section 2.4.2 to 2.4.3):

\[
\text{Inhibition (\%)} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

where A is the absorbance of control (A_{\text{control}}) or detect *G. repens* extract (A_{\text{sample}}).

2.4.2 Superoxide free radical scavenging assay

Superoxide dismutase (SOD) assay was carried out according to standard procedure[24] with a minor modification. The reaction mixture consisted of 1 mL of 1 mg/mL sample, 1 mL of 60 μmol/L PMS in phosphate buffer (0.1 mol/L, pH 7.4) and 1 mL of 150 μmol/L NBT in phosphate buffer. Reactions were incubated at ambient temperature for 5 min and the resultant colour was read at 560 nm, against a blank with a multimode microplate reader. Quercetin was used as the reference drug.

2.4.3 Nitric oxide free radical scavenging assay

Nitric oxide (NO) free radical scavenging activity assay followed a standard procedure with a slight modification.[25] Aliquots of HGR, CGR, EGR and MGR across a range of concentrations (10–1 000 µg/mL) were treated with 3 mL of 10 mmol/L sodium nitroprusside in 0.2 mol/L phosphate-buffered saline (pH 7.4) and allowed to incubate at room temperature for 150 min. After the incubation, 0.5 mL of Griess reagent was added and the absorbance of the reaction mixture was read at 546 nm with a multimode microplate reader. Ascorbic acid was used as reference drug.

2.4.4 Hydroxyl free radical scavenging activity

A standard method was followed to measure hydroxyl (OH) radical scavenging activity.[24] The prepared extract and standard solutions of different dilutions from the stock (1 mg/mL) were added to 1 mL iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL EDTA solution (0.018%) and 1 mL dimethyl sulfoxide (0.85% v/v, in 0.1 mol/L phosphate buffer, pH 7.4). In order to initiate the reaction, 0.5 mL ascorbic acid (0.22%) was added, and the reaction was incubated at 80–90 ºC for 15 min in a water bath. After the incubation, 1 mL ice-cold TCA (17.5% w/v) was added to stop the reaction. Then, about 3 mL Nash reagent was added and left at room temperature for 15 min. The absorbance of the reaction mixture at 412 nm was read against a reagent blank to find out the intensity of the colour formation (Synergy H1MF, BioTek, USA). Quercetin was used as a reference drug.

2.4.5 Total antioxidant capacity assay

The total antioxidant capacity (TAC) of CGR, EGR and MGR was determined with the phosphomolybdate method, using ascorbic acid as a reference drug.[26] An aliquot of 0.1 mL sample solution was mixed with 1 mL reagent solution (0.6 mol/L sulphuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate). The tubes were capped and incubated in a water bath at 95 ºC for 90 min. After the samples were cooled to room temperature, the absorbance of the mixture was measured.
at 765 nm against a blank, using a multimode microplate reader. A typical blank contained 1 mL of the reagent solution and the appropriate volume of the solvent and were incubated under the same conditions.

2.5 Anticholinesterase assay

This assay was performed by following the modified method of Rhee et al. In 96-well plates, 25 μL of 15 mmol/L ATCI in water, 125 μL of 3 mmol/L DTNB in buffer C (50 mmol/L Tris-HCl, pH 8, 0.1 mol/L NaCl and 0.02 mol/L MgCl\textsubscript{2}·6H\textsubscript{2}O), 50 μL of buffer B (50 mmol/L Tris-HCl, 0.1% BSA and pH 8), and 25 μL of sample (10 mg/mL in MeOH diluted ten times with buffer A (50 mmol/L Tris-HCl and pH 8), to give a concentration of 1 mg/mL) were added and the absorbance was measured at 405 nm 5 times, at 13-second intervals. After 25 μL of enzyme (0.22 U/mL) was added, the absorbance was again read five times at 13-second intervals. The reaction rate was calculated by the multimode microplate reader’s installed software (BioTek Gen 2.04). Percentage of inhibition was calculated by comparing the rates for the sample to the blank (10% methanol in buffer). In this reaction, the enzyme hydrolyzes the substrate acetylthiocholine, producing thiocholine, which reacts with Ellman’s reagent (DTNB) to produce 2-nitrobenzoate-5-mercaptothiocholine and 5-thio-2-nitrobenzoate, which was be detected at 405 nm. The BChE inhibitory assay was performed by following the same AChE assay protocol. For this assay BChE from Equine serum (Lyophilised powder) was taken for enzyme preparation and SBTC (Crystalline) was used for substrate preparation. Galanthamine was considered as a reference drug for both AChE and BChE inhibition assay.

2.6 Determination of type inhibition of G. repens on cholinesterase

The type (reversible or irreversible) of cholinesterase (AChE and BChE) inhibition was determined by the most potent fraction, i.e., EGR and the restored activity of both enzymes was measured by 10 times dilution of EGR concentration. AChE activity was measured after gentle mixing of 100 μL enzyme and 10 μL EGR with 890 μL of mixture containing 0.1 mol/L sodium phosphate buffer (pH 8), 0.1% BSA, 1.5 mmol/L ATCI (for AChE)/SBTC (for BChE), 3 mmol/L DTNB and 90 μL EGR. For the control, the dilution effect of EGR on AChE or BChE activity was measured after gentle mixing of 100 μL enzyme and 10 μL buffer with 890 μL of the above mixture; the 90 μL EGR was replaced with 90 μL buffer. In reversible inhibition, AChE activity can be restored by dilution of the EGR fraction, while there is no change in AChE activity with dilution of the EGR fraction in irreversible inhibition.

2.7 Mode of cholinesterase inhibitions by G. repens

Kinetic characterizations of AChE and BChE inhibitory activities by the most potent fraction, i.e., EGR, were conducted following the methods of Torre et al. with a slight modification. EGR was selected because the half maximal inhibitory concentration (IC\textsubscript{50}) values of AChE and BChE were comparable with the reference drug galanthamine. The assay contained 20 μL of 0.22 U/mL AChE, 1 mL of mixture of 3 mmol/L ATCI and different concentrations of ATCI (0.05 to 3 mmol/L) in 0.1 mol/L sodium phosphate buffer (pH 8). The final volume was adjusted to 1 mL with 0.1 mol/L sodium phosphate buffer. The assay conditions for measuring the inhibition activity of EGR were the same as IC\textsubscript{50} of anticholinesterase assay for EGR. The kinetic values were obtained by transforming data to Lineweaver-Burk plot (1/V\textsubscript{max} versus 1/ATCI concentration with the presence of EGR as inhibitor). V\textsubscript{max} was the maximum apparent velocity of AChE at the given concentration of EGR (inhibitor) which was obtained from the intersection at coordinate of Lineweaver-Burk plot.

For the BChE kinetic study, the same procedure was followed, with SBTC considered as a substrate in place of ATCI. The kinetic values were applied by transforming data to Lineweaver-Burk plot accordingly. All graphs were plotted in Microsoft Excel.

2.8 HPTLC bioassay

A CAMAG Linomat V automatic sample spotter (CAMAG Muttenz, Switzerland) was made up of a Linomat V sample applicator, a CAMAG glass twin trough chamber (20 cm × 10 cm), the syringe, 100 μL (Hamilton); the densitometer consisted of a CAMAG HPTLC scanner and winCATS integration software (Version 1.4.10, CAMAG) was used. HPTLC aluminium plates of 10 cm × 10 cm with 0.2 mm layers of HPTLC silica gel 60 F\textsubscript{254} were used. EGR and MGR were dissolved in methanol (2–10 μg/mL), applied by means of a Linomat V sample applicator to the plates above 1 cm above the edge using a bandwidth of 6 mm and distance between tracks of 8 mm. Then each concentration was spotted on the silica gel HPTLC plate and developed in the solvent chloroform:ethyl acetate:methanol (1:6:0 v/v) and N-butanol:acetic acid:water (3:0.1:0.2, v/v) for EGR and MGR respectively. After developing the HPTLC plates, they were scanned with a CAMAG HPTLC scanner 4 at 254 and 366 nm. Next enzyme inhibitory activities of the developed spots were detected by spraying the substrate, dye and enzyme, following on Ellman’s method. The plate was sprayed with DTNB/ATCI reagent (1 mmol/L DTNB and 1 mmol/L ATCI in buffer A) until the silica was saturated with the solvent. It was allowed to dry for 5 min and then 3 U/mL of enzyme solution was sprayed onto the plate. A yellow background appeared; white spots indicating inhibiting compounds became visible after 10–15 min. These were observed and recorded within 15 min because they disappeared in 30–45 min.
minimum concentration which could be visually detected was considered to be the detection limit. Retention factor (Rf) values and colour of the resolved bands were noted.

2.9 Statistical analysis

A minimum of five independent experiments were carried and the results are presented as mean ± standard deviation by using Microsoft Excel (STDEV, 2007). Calibration curves of the standards were considered to be linear if $R^2 > 0.99$.

3 Results

3.1 Phytochemical screening and biochemical estimation

The phytochemical screening of HGR, CGR, EGR and MGR indicated the qualitative presence of secondary metabolites. EGR contained substantial polyphenols and flavonoids but alkaloids were absent. TPC, TFC and TKC of all fractions were investigated and EGR exhibited the highest TPCs, and TFCs of 360.42 mg GAEqV/g and 257.31 mg Queqv/g respectively, whereas TKCs were found to be lower in EGR and MGR fractions.

3.2 Antioxidant activity of *G. repens*

3.2.1 DPPH free radical scavenging activity

The method is based on the reduction of alcoholic DPPH solutions in the presence of antioxidant. A direct linear relationship was found between the concentrations of CGR, EGR and MGR in the DPPH solution and percentage of inhibitions was obtained with a correlation factor $R_2 = 0.99$. EGR was found to be a better scavenger ((38.33 ± 3.21) µg/mL) than other fractions; EGR was comparable to the reference drug ascorbic acid as shown in Table 1. The hexane fraction of leaves of *Geophila repens* showed the negligible free radical scavenging activities and hence it was not considered in the comparative study. The results were obtained in triplicate for all assays.

3.2.2 SOD free radical scavenging assay

In the SOD study, EGR showed the strongest ability to neutralise SOD radicals and the IC$_{50}$ value was (45.14 ± 1.78) µg/mL, which was comparable to the reference drug quercetin ((25.10 ± 0.57) µg/mL, Table 1).

3.2.3 NO free radical scavenging assay

EGR (10–150 µg/mL) showed moderately good NO scavenging activity and its inhibition rate was increased with increasing in concentration of the extracts. The IC$_{50}$ value for scavenging of NO by EGR was (59.81 ± 1.32) µg/mL, whereas the IC$_{50}$ of ascorbic acid was (30.76 ± 0.43) µg/mL. The IC$_{50}$ of EGR was higher than those of CGR and MGR (Table 1 and Figure 1).

3.2.4 OH free radical scavenging activity

In the OH radical scavenging activity study, the IC$_{50}$ values of EGR and standard were (39.45 ± 0.79) and (24.53 ± 0.44) µg/mL, respectively, and found to be higher than those of CGR and MGR (Figure 1). The activity of EGR was comparable with quercetin (Table 1).

3.2.5 TAC assay

TAC of CGR, EGR and MGR was measured spectrophotometrically using the phosphomolybdenum method, based on the reduction of Mo (VI) to Mo (V) by the test sample, and the subsequent formation of green phosphate/Mo (V) compounds, with a maximum absorption at 765 nm. The present study demonstrated that EGR had the highest TAC in the phosphomolybdate reduction reaction, at (43.76 ± 0.81) µg/mL, and was comparable to the reference drug ascorbic acid ((29.98 ± 0.87) µg/mL), as shown in Table 1.

Table 1  *In vitro* free radical scavenging and anticholinesterase activities of different fractions of leaves of *Geophila repens* (IC$_{50}$ at µg/mL)

<table>
<thead>
<tr>
<th>Assay</th>
<th>Reference drug</th>
<th>CGR</th>
<th>EGR</th>
<th>MGR</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH</td>
<td>21.16 ± 1.04</td>
<td>312.33 ± 2.56</td>
<td>38.33 ± 3.21</td>
<td>69.01 ± 1.09</td>
</tr>
<tr>
<td>SOD</td>
<td>25.10 ± 0.57</td>
<td>300.29 ± 2.67</td>
<td>45.14 ± 1.78</td>
<td>97.87 ± 2.67</td>
</tr>
<tr>
<td>NO</td>
<td>30.76 ± 0.43</td>
<td>200.98 ± 1.95</td>
<td>59.81 ± 1.32</td>
<td>125.43 ± 1.66</td>
</tr>
<tr>
<td>OH</td>
<td>24.53 ± 0.44</td>
<td>251.33 ± 2.11</td>
<td>39.45 ± 0.79</td>
<td>89.92 ± 1.32</td>
</tr>
<tr>
<td>TAC</td>
<td>29.98 ± 0.87</td>
<td>191.87 ± 1.65</td>
<td>43.76 ± 0.81</td>
<td>92.03 ± 1.61</td>
</tr>
<tr>
<td>Anticholinesterase assays</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AChEi</td>
<td>24.00 ± 0.46</td>
<td>220.33 ± 0.65</td>
<td>68.63 ± 0.45</td>
<td>135.00 ± 0.44</td>
</tr>
<tr>
<td>BChEi</td>
<td>22.86 ± 0.44</td>
<td>204.23 ± 0.74</td>
<td>59.45 ± 0.45</td>
<td>125.55 ± 0.48</td>
</tr>
</tbody>
</table>

Reference drug ascorbic acid was considered for 1,1-diphenyl-2-picrylhydrazyl (DPPH), nitric oxide (NO), and total antioxidant capacity assay (TAC); whereas for superoxide dismutase (SOD) and hydroxyl (OH) assays quercetin was considered as a reference drug to perform the assays. For acetylcholinesterase inhibition (AChEi) and butyrylcholinesterase inhibition (BChEi) activity studies, galanthamine was considered as a reference drug. CGR, EGR and MGR represent the chloroform, ethyl acetate and methanol fraction of leaves of *Geophila repens* respectively. The hexane fraction of leaves of *Geophila repens* showed the negligible free radical scavenging activities and hence it was not considered in the comparative study. The results were obtained in triplicate for all assays. IC$_{50}$: half maximal inhibitory concentration.
3.3 Anticholinesterase assay

The most prominent AChE inhibition activities of CGR, EGR and MGR were recorded. The IC₅₀ values of AChE for EGR and MGR were (68.63 ± 0.45) and (135.00 ± 0.44) μg/mL respectively, whereas the reference drug, galanthamine, had an IC₅₀ of (24.00 ± 0.46) µg/mL. All the experimental groups had concentration-dependent activity (Figure 2).

In the BChE inhibition assay, IC₅₀ values were recorded for CGR, EGR and MGR (Figure 2). Amongst all fractions, the IC₅₀ value of EGR showed the highest activity, at (59.45 ± 0.45) µg/mL, and was followed by MGR, at (125.55 ± 0.48) µg/mL. Both of these fractions’ inhibitory studies were concentration-dependent and were found comparable with galanthamine, which had an IC₅₀ of (22.00 ± 0.43) µg/mL.

3.4 Determination of type inhibition of G. repens on cholinesterase activities

Cholinesterase inhibition by the EGR fraction was greater than 50% as determined by following standard procedures.[27] AChE and BChE activities were restored more than 4 fold by the 10× dilution, indicating that inhibition of AChE and BChE by the EGR fraction was reversible.

3.5 Mode of cholinesterase inhibitions by G. repens

The modes of inhibition of AChE and BChE are shown in Figure 3, as a double reciprocal plot (1/V (where “V” is reaction velocity) versus 1/s (where “s” is substrate concentration)). The modes of inhibition of EGR on AChE and BChE activities were determined by analysis of this plot. The graph shows that EGR inhibited AChE and BChE competitively.[28,29]

3.6 HPTLC bioassay

To determine the AChE and BChE inhibitory activities, the HPTLC bioautographic assay was performed. As shown in Figure 4, seven polyvalent phytoconstituents were identified as distinct absorbance peaks on the scans. The peaks were located between Rᵢ 0.01 and Rᵢ 0.62. The bioactivity staining method identified a region of bioactivity...
Figure 3 Mode of inhibition of *Geophila repens* for anticholinesterase activities
Lineweaver-Burk plots of acetylcholinesterase (\(V_{\text{max}}\) of 0.5 approx.) and butyrylcholinesterase (\(V_{\text{max}}\) of 0.75 approx.) activity over a range of substrate concentrations (0.05 to 3 mmol/L) in the absence (○, +) or presence (▲, ■) of ethyl acetate fraction of *Geophila repens* (EGR). The graph depicts competitive inhibition (reversible) in both the cases.

Figure 4 Fingerprint of the ethyl acetate and methanol fraction of leaves of *Geophila repens*
Fingerprints of the ethyl acetate and methanol fraction of leaves of *Geophila repens* (2–10 µg/mL) were scanned at 254 nm and 366 nm. Sample application for ethyl acetate and methanol fraction of leaves of *G. repens* was at 10 µg/mL where the maximum elutions of compounds were observed at different Rf values. Encircled demarcation of ethyl acetate and methanol fraction of leaves of *G. repens* represented the presence and absence of relevant peaks at Rf values in the range of 0.42 to 0.58 respectively.
on the EGR plates (Rf range 0.42–0.58), which responded positively after spraying DTNB/ATCI reagent with AChE and BChE solution, forming white spots. The bioactivity staining showed this result through the development of white spots, after 10–15 min, on the yellow background of the plate (Figure 5). This region contained 18.89% of total phytochemicals. At 254 and 366 nm, the sensitive bioactive white spots were absent in the given Rf range (0.42–0.58) for MGR. Thus the bioactive compounds which were responsible for cholinesterase inhibitory activities were not present in the MGR fraction (Figure 4).

The range of Rf from 0.42 to 0.58 on EGR plates contained 18.89% of total phytochemicals, and was found to possess cholinesterase inhibitory activities; thus this Rf range is the subject of interest in our present study.

4 Discussion

ACh is an extensively distributed neurotransmitter, especially in the central nervous system (CNS). A decrease in ACh levels results in decline of memory and cognition in AD, because of the reduction of acetyltransferase or increased level of AChE, and these two factors are correlated with dementia and AD.[30,31] Also it is evidenced that free radicals can lead to deterioration and lead to cognitive ageing and CNS disorders.[32] It has already been reported that the cognitive performance and age-related brain performance can be significantly improved by a diet rich in antioxidants, especially the flavonoids, which include effective cholinesterase inhibitors such as Leufolins A and B, isolated from Leucas urticifolia.[33–35] The current investigation demonstrated that TPC and TFC were found to be higher in EGR than in other fractions and had activities of 360.42 mg GAEqv/g and 257.31 mg Queqv/g, respectively.

The current investigation of G. repens extracts demonstrated antioxidant and anticholinesterase activities which are reported here for the first time for this species. We followed a successive extraction process by using a gradient of non-polar to polar solvents; each of these fractions (CGR, EGR and MGR) was tested for antioxidant and anticholinesterase activities in order to refine the extraction of bioactive compounds. Based on the earlier report, plant antioxidant activities were reported to be higher in ethyl acetate extracts because it has the highest phenolic content. In this study, EGR demonstrated greater DPPH, SOD, NO, OH and TAC scavenging activities than the other fractions. The current investigation has found that the activities of hexane extract from G. repens were low in comparison to other extracts; hence it will not be taken into consideration for further studies (data not shown). Phenol and flavonoid contents are responsible for antioxidant activities of medicinal plants and our results showed a positive correlation between the phenolic and flavonoid contents of EGR with DPPH, SOD, NO, OH and TAC scavenging activities (Figure 1).

In the DPPH radical scavenging assay among all the fractions, EGR showed the greatest inhibition, with an IC_{50} of (38.33 ± 3.21) µg/mL; this result was positively correlated with the higher amount of phenolic and flavonoid contents[36] in EGR which are capable of donating hydrogen to a free radical to reduce the potential damage, as shown in Table 1.

Figure 5 Fingerprint of the ethyl acetate fraction of leaves of Geophila repens

Fingerprints of the ethyl acetate fraction of leaves of Geophila repens (2–10 µg/mL; A, B, C, D and E respectively) and the reference drug galanthamine 10 µg/mL (F) were scanned at 254 nm and 366 nm. Plates I and II were scanned at 254 nm and 366 nm. High-performance thin-layer chromatography bioautography of Plates III and IV showed the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitions respectively as both plates developed a yellow background that appeared with white spots in the Rf range of 0.42 to 0.58 becoming visible after 10–15 min. The assay was carried out using silica gel 60 F_{254} plates which was eluted with chloroform:ethyl acetate:methanol (6.0:0.7:0.8). Detail assay protocol may be followed in the experimental section.
The SOD radical is considered a major biological source of reactive oxygen species and it gives rise to the generation of powerful and dangerous OH radicals, as well as singlet oxygen, both of which contribute to oxidative stress. The results of our study revealed that EGR was able to effectively scavenge for SOD radicals, with an IC\textsubscript{50} of (45.14 ± 1.78) µg/mL (Table 1); this result was correlated with total flavonoid content for neutralising the SOD radicals.[36,37]

NO is a key source of reactive oxygen species. In this assay, sodium nitroprusside, in a test solution, at physiological pH, spontaneously generates NO, which interacts with oxygen to produce nitrite ions like NO\textsubscript{3}, N\textsubscript{2}O\textsubscript{3} and peroxynitrite, which can be measured with Griess reagents. Accumulation of large amounts of those radicals may lead to tissue damage through oxidative damage to lipids, proteins, nucleic acids and carbohydrates. EGR was shown to be a good scavenger of NO, through its action against oxygen which may lead to a reduced production of nitrite ions. The IC\textsubscript{50} of EGR was (59.81 ± 1.32) µg/mL, which was comparable to ascorbic acid (Table 1). The activity of EGR may be attributed to the presence of phenolic and flavonoid compounds and these findings are supported by previous work.[36]

The OH radical is a potent reactive oxygen species and it reacts with the polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell.[38] In our study EGR showed appreciable potential to scavenge OH radicals and its IC\textsubscript{50} was found to be (39.45 ± 0.79) µg/mL (Table 1). It is possible that the presence of phenolic and flavonoid compounds in EGR is responsible for scavenging OH radicals.[36,39]

The present study also demonstrated the antioxidant capacity of EGR by reducing phosphomolybdate at (43.76 ± 0.81) µg/mL, which was comparable with ascorbic acid (Table 1). Recent studies have shown that many flavonoid and related polyphenols contribute significantly to the phosphomolybdate scavenging activity of medicinal plants; the antioxidant activity of EGR may be attributed to the presence of flavonoid and polyphenols in the extract.[39,40]

The current investigation also demonstrated the inhibitory activities of all the fractions on both AChE and BChE. EGR inhibitory activity was greater than in other fractions and it exhibited a dependent relationship. The IC\textsubscript{50} values of EGR were (68.63 ± 0.45) and (59.45 ± 0.45) µg/mL, for the inhibition of AChE and BChE respectively, and were comparable with galanthamine (Figure 2). Recent studies have shown that the flavonoids and related polyphenols contribute significantly to cholinesterase inhibition and thus, it is likely that polyphenols and flavonoids in EGR were responsible for AChE and BChE inhibitory activities.[34,41] The kinetics of EGR’s inhibition of AChE and BChE were studied in detail with the graphical analytic tool, the Lineweaver-Burk plot. This tool demonstrated that EGR inhibition of AChE and BChE was competitive and reversible (Figure 3). This mechanism of inhibition indicates that EGR might have competed with ATCI and SBTC for binding sites on AChE and BChE, respectively; this finding is supported by earlier research.[36,37]

HPTLC fingerprint studies were performed on EGR and MGR extracts. The calibration plots for both plates were obtained at 254 and 366 nm (Figure 4). In a given R\textsubscript{f} of 0.01–0.62, different peaks of EGR and MGR were obtained. Of the peaks identified in the EGR extract, only 18.89% (R\textsubscript{f}: 0.42–0.58) contained phytoconstituents that were cholinesterase inhibitors. However, in the fingerprint study of MGR, these same phytoconstituent peaks (R\textsubscript{f}: 0.42–0.58) were absent. The absence of these bioactive phytoconstituents in MGR extract resulted in less cholinesterase inhibition than EGR in the bioactivity assay. In this assay, a yellow background formed over each plate, and white spots emerged, indicating areas of bioactivity. On the EGR plate white spots developed within R\textsubscript{f} of 0.42 to 0.58. In MGR bioautographic plates, these bioactive white spots were less well defined (R\textsubscript{f}: 0.42–0.58; Figure 5). A percentage of the 18.89% of bioactive phytoconstituents responded positively to a specific chemical test conducted for polyphenols.[16] So it may be inferred that the bioactive phenolic and flavonoid compounds were present in higher quantities in EGR extract, and were responsible for its antioxidant and anticholinesterase activities.

5 Conclusion

The thorough literature survey and the results of our current investigation support the potential role of *G. repens* as a memory enhancer in AD. Based on the significant cholinesterase enzyme (AChE and BChE) inhibition and radical scavenging activity of various fractions of *G. repens*, it may be inferred that EGR is a good source of antioxidant and anticholinesterase compounds. We have demonstrated this activity through several antioxidant assays, including DPPH, SOD, NO, OH and TAC. In the present study, we have reported, for the first time, the potential medicinal properties of *G. repens* for its ability to inhibit activities of cholinesterase enzymes in a dose-dependent and competitive reversible manner. A rapid HPTLC bioautographic assay was introduced for the screening of *G. repens*, which gave quick access to information concerning both the activity and the localisation of bioactive molecules (R\textsubscript{f}: 0.42–0.58) in complex plant matrices before isolation. The bioassay guided the isolation of compounds from *G. repens* already in progress in our laboratory, which may lead to a new
compound for use in anti-Alzheimer’s disease and anti-ageing treatments.

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8 Conflict of interests

The authors have declared that there is no conflict of interest.

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