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Geigeria Alata- A Potential Source for Anti-Alzheimer's Constituents: *In Vitro* And Computational Investigations

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ABSTRACT

Antioxidants and acetylcholinesterase inhibitors play a key role in the prevention and management of degenerative disorders including Alzheimer's disease in particular. Identifying new anticholinesterases from natural sources may contribute to combating this class of diseases. The present study aimed to evaluate the potential anti-Alzheimer's activity of *Geigeria alata* (DC), a plant used in Sudanese folkloric medicine. Accordingly, the whole DC plant extract including twenty phytoconstituents of phenolic, flavonoid, and tannin types was evaluated *in vitro* as antioxidants and acetylcholinesterase inhibitors. Also, their pharmacokinetics, drug likeliness, and toxicity profiles were assessed. Additionally, the virtual binding of the plant's phytoconstituents with the cholinesterase target was investigated by docking against two AChE X-ray crystallographic structures. The best effective DPPH radical scavenging activity was demonstrated by both ethyl acetate and *n*-butanol fractions with percentages of inhibition of 91±0.02% and 90±0.02% (IC_{50} 22±0.01 and 66±0.02 µg/mL), respectively. The ethyl acetate fraction showed statistically significant and the highest AChE inhibitory activity (78% inhibition, IC_{50} 0.246±0.02mg/mL). Furthermore, the ethyl acetate fraction exhibited the highest total phenolic, flavonoid, and tannin values. Among identified compounds, quercetin and hispidulin showed promising *in silico* anti-AChE activity and hence merit further studies for the isolation and characterization of these active constituents.

Keywords: Alzheimer's disease; *G. alata*; Oxidative stress; Molecular docking; Quercetin; Hispidulin; Heath and wellbeing

INTRODUCTION

Alzheimer's disease (AD) comes in the foremost of causes and signs of dementia. AD-associated dementia counts as one of the leading causes of death as well as one of the major causes of disability in geriatric globally (Gharat *et al.*,

2022; The World Health Organization, 2022). AD is characterized by a progressive impairment of cognitive function along with behavioral manifestations (Slot *et al*., 2019). Globally, about thirty million people are suffering from AD, and this number is estimated to rise to ninety million by 2050 (Prince *et al*., 2016). In 2021, the WHO stated that the psychological and economic impacts of AD are not limited to individuals suffering from AD, but also extended to their caregivers and society. Anticholinesterases especially acetyl-cholinesterase (AChE) inhibitors like donepezil, rivastigmine, and galantamine are considered to be the main class of drugs currently used for the symptomatic treatment of AD (Eissa *et al*., 2023; Prasher, 2004). Nevertheless, due to their mild efficiency and gastrointestinal side effects, there is an unmet need for safer and more effective AChE inhibitors for the symptomatic treatment of AD (Marucci *et al*., 2021). In addition, many studies reported that the oxidative stress characterized by increased oxidative damage to neuronal proteins, depletion of antioxidants, and mitochondrial dysfunction could contribute to neurodegeneration in AD (Guo *et al*., 2013; Venkateshappa *et al*., 2012). Thus, antioxidants could serve as a potential preventive agent for AD-related brain dysfunction and reduce such a disease progression (Marcinkowska *et al*., 2019).

Many plants belonging to the Asteraceae family are traditionally used for their claimed effects on neurological disorders including Alzheimer's disease (Luedtke *et al*., 2003; Sayyah *et al*., 2010). In addition, a review by Murray and co-workers, assigned acetylcholinesterase inhibition activity of seven plants belonging to the Asteraceae family and their promising correlation to Alzheimer's disease therapy (Murray *et al*., 2013). Therefore, the Asteraceae family has long been considered a promising source for the novel anti-AD drug. *Geigeria alata* (DC) Oliv. & Hiern, a weed belonging to the family Asteraceae, is extensively used in Sudanese folklore medicine against epilepsy, pneumonia, rheumatism, hypertension, and diabetes mellites (Sakina and Ahmed, 2018). Phytochemical analysis of *G. alata* revealed the presence of alkaloids, flavonoids, tannins (EL-Kamali and EL-amir, 2010), phenolic acids (Zheleva-Dimitrova *et al*., 2017), and sesquiterpene lactones (Fadul *et al*., 2020).

Considering these documented facts and in a continuation of our recent studies in the identification of potential bioactive molecules (Abul-Khair *et al.*, 2013; Zaki *et al.*, 2020, 2022; Abulkhair *et al.*, 2021; El-Shershaby *et al.*, 2021; Fadol *et al.*, 2021; Khedr *et al.*, 2021), this study is aiming to evaluate the *in vitro* anti-acetylcholinesterase and antioxidant activities of *G. alata* whole plant extract and solvent fractions. Also, phytochemical analysis and *in silico* studies have been conducted to predict the anti-acetylcholinesterase targets, binding modes, pharmacokinetics, and toxicity profile of its reported phytoconstituents.

MATERIALS AND METHOD

Collection and authentication of the plant material

G. alata (DC.) was collected from south Kordofan, Eldebebat area in February 2019. Then, authenticated by Dr. Yahia Suliman at the Medicinal and Aromatic Plants and Traditional Medicine Research Institute, National Center for Research, Khartoum, Sudan, and a voucher specimen was deposited at the herbarium of the institute.

Extraction and fractionation

Repeated maceration with frequent agitation were adopted for extraction of the plant material as described by Trease and Evans (Evans, n.d.). The shade-dried plant was pulverized, then macerated in 80% ethanol at room temperature for 72h at 25 C°. After that, the extract was filtrated, then the solvent was evaporated to dryness under reduced pressure using a rotary evaporator device. The residue was dissolved in 50 mL of 50% aqueous methanol, and fractionated with petroleum ether, chloroform, ethyl-acetate, and *n*-butanol, respectively. Ultimately, the dried crude extracts and solvent fractions were stored in the refrigerator at -4°C.

Acetylcholinesterase inhibitory activity

In vitro AChE inhibition was conducted according to Ellman's method (Ellman *et al*., 1961) with minor modifications to adapt the 96 well micro-plate technique. Each 10 µL extract, ethanol (the negative control) or the positive control eserine (Sigma-Aldrich, Germany) were mixed with 20 µL of enzyme and 130 µL phosphate buffer, pH 8, containing 10 µL of 0.5 mM of 5, 5'-dithio-bis- (2-nitrobenzoic acid) (DTNB, Sigma-Aldrich, Germany) and 20 µL acetylthiocholine iodide (ATCI, Sigma-Aldrich, Germany). In 96-well microplate, each experiment was performed in triplicate. The enzyme inhibition activity was measured at 412 nm every 30 s for 20 min. Then, the inhibition percentages were calculated using

the rates of change in absorbances of the forty readings, adopting the following formula:

$$\%\text{Inhibition} = 100 - \left(\frac{\text{rate of change in the absorbance of the test}}{\text{rate of change in the absorbance of the control}}\right) \times 100$$

***In vitro* antioxidant activity**

Antioxidant activity of the plant extracts was determined by three different colorimetric assays; 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay according to the method of Shimada (Shimada *et al*., 1992). Superoxide scavenging assay as described by Thadhani in 2011 (Thadhani *et al*., 2011), and iron chelating activity assay using the modified method of Dinis and co-workers (Dinis *et al*., 1994). For each assay, the percentage radical scavenging activities of the samples were calculated and compared to DMSO treated control group, and standard one. All tests and analyses were run in triplicate. The results were reported as the mean ± standard error of the mean. IC_{50} was determined by plotting different concentrations of the extracts (0.5, 0.25, 0.125, 0.062, 0.031, 0.0156, and 0.008 mg/mL) versus percentages of inhibition on a Microsoft excel sheet by using EZ-Fit Enzyme Kinetic Program (Perrella Scientific Inc, U.S.A.). This was applied for all extracts that exhibited free radical scavenging activity greater than 50%.

Preliminary phytochemical screening

The ethanolic extract of *G. alata* was subject to preliminary phytochemical screening for major secondary metabolites, according to the standard procedures described by Trease and Evans (Evans, n.d.).

Determination of total phenolic contents

Folin-Ciocalteu colorimetric method was used to determine the total phenolic contents of both the ethanolic extracts and solvent fractions (Ainsworth and Gillespie, 2007). Briefly, 25µL of 1mg/mL extract/fraction, standard or 95% (vol/vol) methanol blank was mixed with 50µL Folin-Ciocalteu reagent in a clean 96-well microplate. The contents were mixed by manual shaking for 15–20 s. Then, 200 µL 700 mM sodium carbonate solution was added. The reaction mixture was incubated away from light at room temperature for 2 h and the absorbance was measured at 765 nm using a microplate reader spectrophotometer. All tests and analysis were run in triplicates. The total phenolic contents were determined using a calibration curve prepared with gallic acid standard (100–0 mg/L) as a reference. The standard curve was calculated from the blank-corrected A765 of the gallic acid standards. Total phenolic contents were expressed as milligrams of gallic acid equivalents per gram dry weight of residues (mg GAE/g of Dried Extract), using the regression equation between gallic acid standards and A765.

Determination of the total flavonoid contents

Total flavonoid contents were measured according to the aluminum chloride colorimetric assay described by Nisa *et al*. in 2017 (Nisa *et al*., 2017). With some minor modifications. First, 25µL of each extract (1mg/mL) or standard was mixed with 15µL of 0.05mg/mL sodium acetate. Then, 15µL of 10% $AlCl_3$ solution was added, followed by 100 µL of 1M NaOH was added to the mixture with 5min intervals between the addition of each reagent. Second, the reaction mixture was incubated in dark at room temperature for 45 min. After that, absorbance was measured at 415 nm using a microplate reader spectrophotometer. The total flavonoid content was determined using a calibration curve prepared with quercetin standard (100-0 mg/L) as a reference. Finally, the total flavonoid contents were expressed as milligrams of quercetin equivalents per gram dry weight of residues (mg GAE/g of Dried Extract).

Determination of the total tannin contents (TTC)

To estimate the total tannin content of the extracts, the modified Prussian blue method with adaptation to microplates was used with minor modifications. Briefly, 25µL of 1% K_3 [Fe $(CN)_6$] solution was added to 25µL of 1mg/mL extract/fraction, standard or 95% (vol/vol) methanol blank, then 25 µL of 1% ferric chloride ($FeCl_3$) and 175µL phosphate buffer (PH8) were added, respectively. The mixture was shaken for 20 seconds, then left to react in a dark place at 25°C for 5 min. The absorbance was recorded at 510 nm using a microplate multi-scan reader. The experiment was conducted in triplicate. The total tannin content was determined using a calibration curve prepared with tannic acid standard (800-100 mg/L) as a reference. The total tannin content was expressed as mg of tannic acid equivalent per gram dry weight of residues.

In silico Studies

Ligands preparation

The chemical structures of the literature reported *G. alata*'s phytoconstituents (twenty compounds) (Fadul *et al*., 2020; Zheleva-Dimitrova *et al*., 2017) were drawn via ChemDraw Professional software version 16.0 and Pub Chem Doc database. The co-crystallized inhibitor was extracted from the protein binding pocket (PDB ID: 4EY7). The 3D structures were generated in mol2 format, then minimized and optimized with Cresset Flare software at the accurate type of calculation method.

Target preparation

The 3D acetylcholinesterase structure with good resolution and validation scores was selected for the study and downloaded from the RCSB protein data bank (Berman *et al*., 2002). In the context of docking results validation, two 3D X-ray crystallographic structures for recombinant human acetylcholinesterase enzyme were downloaded in PDB format (PDB ID: 4EY7, resolution 2.35 Å; and PDB ID: 6WVC, resolution 2.599 Å). Then, both 3D structures were prepared and their energies were minimized at the accurate method of calculation using Cresset Flare software.

Molecular docking

The docking calculations were carried out in Cresset Flare software (Stierand and Rarey, 2010) in normal mode and default settings. The grid box (the active side) was defined according to the co-crystallized ligands. The acetylcholinesterase inhibitor - eserine and the co-crystallized ligand (donepezil) were used as positive controls. The ligands and the targets were prepared in mol2 and PDB format, respectively. Two scoring capacities given by Lead Finder were utilized to score the docked ligand poses which are VS-score: the correct rank ordering of active and inactive compounds in virtual-screening experiments and Rank Score: correct energy ranking of docked ligand poses (El-Adl *et al*., 2021b; Turky *et al*., 2020b). The 2D interaction of each phytoconstituent at the active site of the acetylcholinesterase enzyme was carried out using PoseView software at the Proteins Plus web portal (open webserver) (Fährrolfes *et al*., 2017).

Pharmacokinetics, toxicity, and drug-likeness prediction

Pharmacokinetics properties: intestinal absorption, the apparent volume of distribution, clearance, CYP-450 enzyme inhibition, and the ability to cross the blood-brain barrier of phytochemical constituents were predicted with pkCSM and SwissADME online servers (Daina *et al*., 2017; El-Adl *et al*., 2021a; El-Shershaby *et al*., 2021; Ezzat *et al*., 2021; Pires *et al*., 2015). The major organ toxicity (cardiotoxicity, hepatotoxicity, renal toxicity, and teratogenicity) and maximum tolerated doses were predicted using pkCSM and eMol-Tox webservers (Aljuhani *et al*., 2022; Ji *et al*., 2018). Additionally, the likelihood of phytoconstituents resembling a medication was anticipated through the SwissADME webserver.

Statistical analysis

The results were reported as mean ± standard error of the mean. IC_{50} was calculated using linear regression parameters. A comparison of enzyme activity data was performed by using an unpaired t-test via Graph Pad Prism 5.01 software. P value less than or equal to 0.05 was considered to be significant.

RESULTS AND DISCUSSION

In vitro anti-acetylcholinesterase activity

Owing to the cultural beliefs, accessibility, high safety profile, as well as chemical and bioactivity diversity, the use of plants in medicine has continued to spiral upwards day after day (Verma and Singh, 2008; Yuan *et al*., 2016). However, research in the field of natural products still faces tremendous challenges. Foremost of all, is the need for valid *in vitro*, and *in vivo* studies, besides applying the quality assurance and standardization measures (Heinrich *et al*., 2020). In Sudan, many Asteraceae plants are used traditionally without a scientific rationale. In the current study, the anti-acetylcholinesterase and antioxidant activities of *G. alata* were investigated.

Among ethanol extract and solvent fractions obtained from *G. alata* (Table I), the ethyl acetate extract exhibited 78% AChE inhibitory activity, with IC_{50} value equal to 0.246 ± 0.02 mg/mL in comparison to eserine which was used as a positive control (70% inhibition, IC_{50} 0.00032 ± 0.01 mg/mL). Moreover, the chloroform fraction exhibited 42% AChE inhibitory activity. However, this is considered to be a remarkable result as we compare the activity of crude extract containing a mixture of compounds with the activity of a licensed pure compound.

Molecular docking

Computer-aided virtual screening tools offered great promise for accelerating the drug lead

Table I. *In vitro* acetylcholinesterase inhibition activity of *G. alata* ethanol extract and solvent fractions.

Sample Code	inhibitory% ± SD	P values	IC_{50}
G. alata - ethanol	18 ± 0.01	< 0.0001	-
G. alata - petroleum ether	25 ± 0.02	< 0.0001	-
G. alata - chloroform	42 ± 0.01	< 0.0001	-
G. alata – ethyl-acetate	78 ± 0.00	< 0.0001	0.246 ± 0.02
G. alata - n-butanol	10 ± 0.01	< 0.0001	-
Eserine (Positive control)	97 ± 0.00	< 0.0001	0.00032 ± 0.01

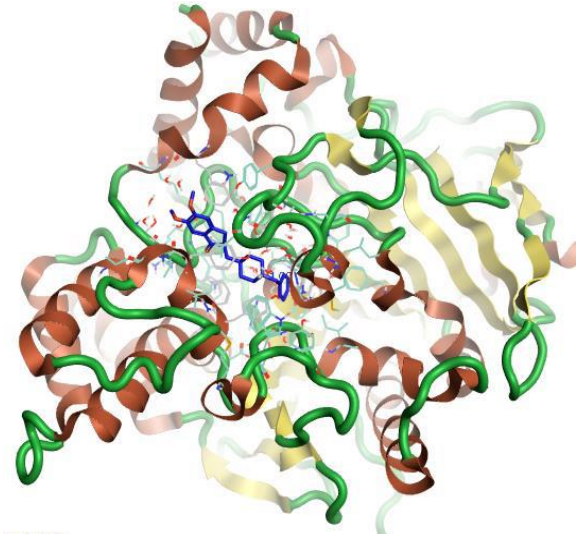

Figure 1. The 3D structure of human acetylcholinesterase (rhAChE PDB ID 4EY7). The structure shows chain A of the enzyme (colored green, yellow and red) in complex with donepezil at the active site. The co-crystalized ligand donepezil is shown in blue-capped stick format.

finding process by predicting drug targets (Kutkat *et al*., 2022; Othman *et al*., 2022; Turky *et al*., 2020b; Turky *et al*., 2020a). In this study, after confirming the *in vitro* anti-AChE activity, *in silico* studies were conducted on *G. alata's* reported phytoconstituents to predict their biological targets that phytochemical constituents exert their Anti-AChE activity through. The X-ray crystallographic structure of the recombinant human acetylcholinesterase enzyme (rhAChE, PDB: 4EY7) was chosen as it is complexed with donepezil (commercially available acetylcholinesterase inhibitor) instead of the snake venom toxin, fasciculin-2, as native ligand to impede the formation of drug complexes. Moreover, the human enzyme is more accurate, convenient, and flexible for the study of drug binding, besides the high resolution of the structure in the unliganded state (2.35 Å). The structure (PDB ID: 4EY7) consists of two independent molecules or asymmetric domains, A and B (Cheung *et al*., 2012). Early investigations showed that the active site of AChE is a highly aromatic, deep, and narrow cavity, about 20 Å long, which infiltrates more than halfway and widens out close to the base of the enzyme (Dvir *et al*., 2010) (Figure 1). The docking result was further validated by docking against another AChE X-ray crystallographic structure which has PDB ID: 6WVC, 2.599 Å resolution and complexed with G-series nerve agent in the state of donepezil (McGuire *et al*., 2021). Out of the twenty screened compounds, seventeen exhibited Vs-scores higher than both, the co-crystalized ligand and the standard positive control Vs-scores. Quercetin was ranked top on the list of poses (LF Rank Score) against both 4EY7 and 6WVC rAChEs (Table II).

Table II. The molecular docking results of the reported *G. alata's* phytoconstituents against the rhAChE (PDB ID: 4EY7 and 6WVC) using docking software Cresset Flare.

Phytoconstituent	LF Rank Score		LF VS Score	
	4EY7	6WVC	4EY7	6WVC
Protocatechuic acid	-6.834	-6.213	-6.061	-5.834
Neochlorogenic	-10.556	-10.034	-10.904	-11.254
Caffeic acid-hexoside	-9.892	-9.524	-12.296	-11.958
Chlorogenic acid	-10.432	-9.633	-10.94	-10.454
Caffeic acid	-6.865	-6.892	-6.758	-6.719
Coumaroylquinic acid	-8.403	-10.153	-9.367	-10.591
4-Feruloylquinic acid	-11.506	-9.807	-11.505	-10.172
5-Feruloylquinic acid	-9.508	-9.374	-9.579	-9.768
3,4-Dicaffeoylquinic acid	-10.85	-11.416	-10.743	-14.576
3,5-Dicaffeoylquinic acid	-12.814	-9.754	-13.249	-14.01
4,5-Dicaffeoylquinic acid	-14.434	-4.353	-14.666	-9.745
3-Caffeoyl-5-sinapoylquinic acid	-11.533	-11.525	-11.818	-13.807
3-Feruloylquinic acid	-10.336	-10.131	-11.365	-10.712
3-Caffeoyl-5-feruloylquinic acid	-13.178	-3.367	-13.012	-9.27
3,4,5-Tricaffeoylquinic acid	-13.194	-3.262	-13.94	-12.544
Geigerianoloide	-4.937	-4.702	-8.207	-7.968
Axillarin	-14.872	-13.626	-10.739	-10.362
Quercetin	-14.96	-15.396	-11.385	-11.777
3-Methoxy-5,7,3`,4`-tetrahydroxy-flavone	-14.238	-15.16	-10.497	-11.341
Hispidulin	-12.4	-12.575	-9.091	-9.226
Donepezil (Co-ligand)	-8.586	-7.275	-10.776	-10.289
Eserine (Standard)	-6.348	-6.479	-8.775	-8.764

Out of twenty *G. alata* phytoconstituents, seventeen have exhibited Vs-scores higher than both, the co-crystalized ligand and the standard positive control Vs-scores' (Table II), among them, quercetin (compound no. 18) ranked top on the list of poses (LF Rank Score) against both 4EY7 and 6WVC rAChEs. The benzyl rings at the two ends of the co-crystalized ligand (Figure 2-a), donepezil, stacks against Trp-86 in the active site, while the indanone ring stacks against Trp-286 in the peripheral anionic site. Notwithstanding, the piperidine ring in the donepezil molecule is flipped over, the situation allows a water-mediated hydrogen connection between N15 of donepezil and Tyr-341 and Tyr-337 of rhAChE. Hydrogen bond interactions between N15 of donepezil and Asp-74, moreover, between the carbonyl group of donepezil's indanone ring and Phe-294 were also observed. Eserine was used as a standard AChE inhibitor in both *in vitro* and *in silico* assays. It has shown almost similar predicted interactions as donepezil with the rhAChE crystal residues at the active site i.e., no new resides added to the interaction zone, yet the bonds with Asp-74 and Trp-296 have run out of eserine's molecule interactions at the active gorge of the enzyme (Figure 2-b). Cheung *et al*. (2012) reported that compounds' interaction with Tyr-337 residue is critical for the inhibition of the human acetylcholinesterase enzyme. Both the co-crystalized ligand and eserine showed strong hydrogen interaction with Tyr-337. Interestingly, among the twenty compounds docked against the rhAChE, only hispidulin bonded with Tyr-337 amino acid resides through a hydrogen bond. Hispidulin interacted with six different residues at the active site of the enzyme (Figure 2c), four out of the six interactions are co-shared with donepezil's interaction, those are: Trp-286, Tyr-341, Tyr-337, and Asp-74, besides two other new hydrogen interactions with Ser-293 and Phe-338. Hispidulin is a methoxy flavone derivative. So, hispidulin resembles donepezil not just structurally, but also on its binding at the active site of rhAChE X-ray crystallographic structure.

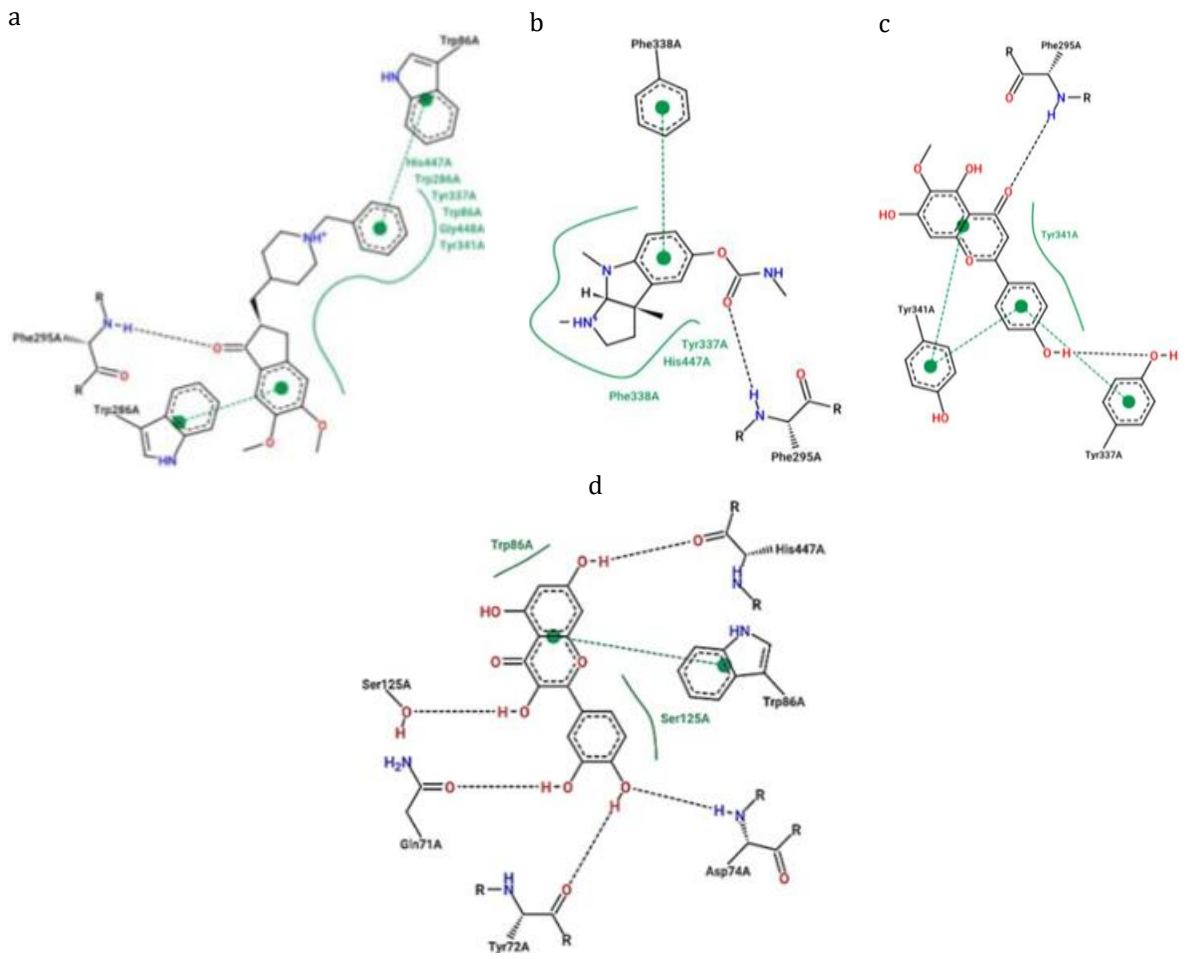

Figure 2. 2D diagram of the active gorge bound ligands. [a–d] Close up perspectives on the crystallized rhAChE active site (PDB ID: 4EY7) with [a] bound donepezil (the co-crystalized ligand), [b] eserine (the standard control), [c] hispidulin (the predicted lead compound), [d] Quercetin (compound showed the highest affinity)

Interaction of hispidulin's benzopyrone two-ring system at the distal end of the active site, which resembles that for indanone ring, but hispidulin interaction with Ser-293 residue gives it the favor of deeper and stronger linkage to the esteratic catalytic subsite at the rhAChE as mentioned above (Figure 3). Moreover, the phenyl ring of hispidulin has quintessentially occupied the same position at the active side as donepezil's piperidine ring. On the other hand, quercetin has shown the highest number of interactions with total eight bonds with eight different residues at the active site (3 aromatic-aromatic bonds and 5 H-bonds) thus recorded the highest Lead Finder Rank Score (Figure 2-d).

Pharmacokinetics, cytotoxicity, and Drug-likeness Prediction

Based on the molecular docking results, the most promising *G. alata* constituents were further subjected to pharmacokinetics, cytotoxicity, and drug-likeness prediction. The activity and safety, molecules also need to be absorbed, reach the active site at the correct concentration, and bind for a specific time to be described as a drug or drug-like compound (Sedahmed *et al*., 2021). Hence, compounds that exhibited a considerable affinity to acetylcholinesterase crystal (Rank score > -6.348, standard eserine score) were subjected to predictive pharmacokinetic properties study (Absorption, distribution, excretion, metabolism, and toxicological studies) via pkCSM, SwissADME,

and eMolTox web servers. The drug-likeness of the phytoconstituents was also conducted utilizing Lipinski′s Rule of five as a determinant for what properties of molecules will reduce or hinder the absorption and permeability of the phytoconstituent (Table S1 and S2).

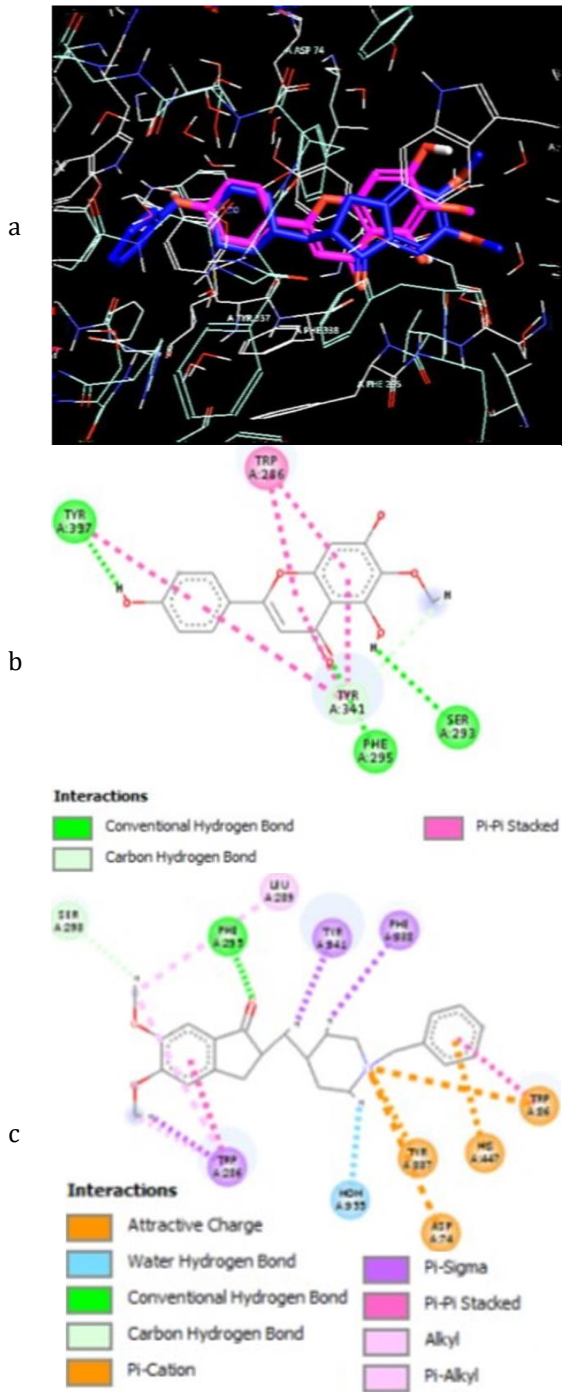

Figure 3. a) The 3D structure of donepezil (the native ligand) and hispidulin (the best test ligand) bound to the active site of human acetylcholinesterase (rhAChE PDB ID 4EY7). Donepezil was shown in blue-capped stick, while hispidulin presented in pink capped stick format. b) The 2D diagram of hispidulin. c) The 2D diagram of donepezil

Molecular weight < 500, Number of hydrogen bond donors ≤ 5, Number of hydrogen bond acceptors ≤ 10, Calculated Log p ≤ 5 and Polar surface area (PSA) <140 Å2 are Lipinski′s rule of five elements used to evaluate drug-likeness. As violating as the compound, it would be poorly absorbed and then less probable to be an orally active drug. Three of the 20 studied compounds violate one rule having NH or OH > 5, while 6 phytochemicals violate 3 rules of MW > 500, N or O > 10, and NH or OH > 5. However, the Lipinski′s rule of five is only considered when the compound shows two or more violations. On the other hand, the phytoconstituents that showed the highest predictive volume of distribution had achieved minimal intestinal absorption, violations from Lipinski′s Rule of five, and hence less likeness to be drugs. 3-methoxy-5,7,3`,4`-tetrahydroxy-flavone showed remarkable predicted intestinal absorption, around 83%. However, it also showed AMES toxicity which reflects its tendency to be a mutagen or carcinogen. Fortunately, the AMES test is not necessarily harmful to humans. The predicted servers assigned our positive control eserine as hepatotoxic compound and CYP3A4 substrate, which is both laboratories and clinically evidenced and reported as the cause lying behind eserine withdrawal from the market. Quercetin showed a considerable volume of distribution, acceptable intestinal absorption, ability to cross the blood-brain barrier, and CYP1A2 inhibitory activity. Promisingly, hispidulin showed a good pharmacokinetic parameter, except for the multiple predicted CYP-450 enzyme inhibition, which increases the risk of drug accumulation, toxicity, and drug-drug interactions when hispidulin is co-administered with drugs affected by the same enzymes.

Antioxidant activity

Many studies owed the neurodegeneration resulting from oxidative stress in Alzheimer's disease brain to the escalated lipid peroxidation and glucose metabolism which consequently leads to the formation of oxidative stressors. Thus, the role of antioxidants in Alzheimer's disease therapy has become a hot area of research (Barbosa *et al*.,

2020; Singh and Devasahayam, 2020). The antioxidant activity of ethanol extract, petroleum ether, chloroform, ethyl-acetate, and *n*-butanol fractions obtained from *G. alata* whole plant, using DPPH, superoxide scavenging, and iron chelating assays (Figure 4). The most polar fractions ethanol, ethyl-acetate, and n-butanol demonstrate the highest DPPH reducing ability around 90% which is nearly similar to standard propyl gallate activity.

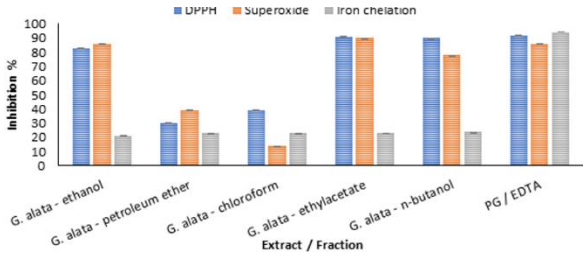

Figure 4. DPPH, superoxide radicals scavenging and iron chelating activities of *G. alata* ethanolic extract and fractions.

Similarly, they exhibited considerable superoxide scavenging ability, even much greater than the standard value regarding the ethyl-acetate activity. On the other hand, since the principle of iron-chelating assay achieves by the catalytic action of ferrous ions (Fe^{2+}) on cell membrane oxidative reactions, it is widely regarded as a secondary antioxidant activity test (Wong *et al*., 2014). Hence, even a negligible iron-chelating activity, as shown in the graph, is highly appreciated; that may later play a significant role in suppressing membrane oxidative stress *in vivo* (Adjimani and Asare, 2015). Fadul and co-workers in 2020 had studied the antioxidant activity of five compounds isolated from *G. alata* using DPPH and superoxide scavenging assays, their results stated that most of the isolated compounds had shown DPPH inhibition activity between 91% to 95% and superoxide scavenging activity between 97% and 99% (Fadul *et al*., 2020). Another Bulgarian group studied the antioxidant and neurodegenerative effect of caffeoylquinic acids isolated from *G. alata* roots using alcohol-induced oxidative stress in a rat. Their findings concluded that 3,4,5-tricaffeoylquinic acid, the main acylquinic acid in the *G. alata* roots, had shown a pronounced antioxidant effect that protect neuronal cells from the deleterious impact of alcohol (Zheleva-Dimitrova *et al*., 2017).

Secondary plant metabolites represent never-ending source of novel compounds for the discovery of therapeutics for treating various human illnesses from the simplest to the most complicated ones like cancer and Alzheimer's disease (Cragg and Newman, 2013) phytochemical analysis indicates the presence of coumarins, flavonoids, tannins, saponins, diterpenes, and alkaloids in the ethanolic extract (**Table S2**), and the highest concentration of the phenolic constituents was associated with the ethyl acetate fraction. Among the detected secondary metabolites, polyphenolic phytoconstituents have caught our interest the most due to their known redox potentials (Piluzza and Bullitta, 2011), secondary antioxidant capacities (Amarowicz, 2007; Heim *et al*., 2002), and boosting abilities to several bioactivities in many biological systems (Aiyegoro and Okoh, 2010). For instance, tannins are known to function as both primary and secondary antioxidants for their ability to chelate metal ions, interfere with some reactions in the Fenton reaction, and also cease lipid peroxidation cascade by inhibiting cyclooxygenase enzyme (Amarowicz, 2007). Flavonoids are phenolic compounds with a protective effect that is exerted by an array of mechanisms in biological systems, for example, their capacity to transfer electrons to free radicals, chelate metal catalysts, activate antioxidant enzymes, reduce alpha-tocopherol radicals and inhibit oxidases (Heim *et al*., 2002). In addition, extensive studies on polyphenols as neuroprotective and treatment options for Alzheimer's disease have shown promising therapeutic results both in animal models and clinical studies (Román *et al*., 2019). The naturally occurring flavonoid, quercetin was found to suppress the incidence of fibril plaque formation of amyloid-β proteins besides its antioxidant and anti-inflammatory effect (Khan *et al*., 2019; Rifaai *et al*., 2020). Another example is polyphenolic phytochemical resveratrol, which showed significant interference with signaling pathways targeted for the prevention and treatment of Alzheimer's disease and other diseases of aging (Sawda *et al*., 2017; Yan *et al*., 2020). So as, total phenolic, flavonoid, and tannin contents of *G alata*s' obtained extracts have been conducted.

The *G. alata* ethanol extract exhibited 83% and 86% DPPH and superoxide antioxidant activity, respectively, compared with the positive the control propyl gallate. Remarkably, the ethyl-acetate fraction demonstrated the highest DPPH and superoxide antioxidant activity with 91% and 90%, followed by 90% and 78%, respectively, for the n-butanol fraction. Low iron chelating

activity was demonstrated for the five fractions compared with the positive control ethylenediaminetetraacetic acid (EDTA).

Phytochemical analysis

The preliminary conducted phytochemical screening on *G. alata* ethanolic extract revealed the presence of a number of secondary plant metabolites, those are, phenols, alkaloids, saponins, diterpenes, triterpenes, coumarins, flavonoids, and tannins. Neither anthraquinone nor cardiac glycosides have been detected in the plant extract. The ethyl-acetate fraction exhibited the highest phenolic, flavonoid, and tannin contents, followed by *n*-butanol, chloroform, and petroleum ether fractions, respectively (Table S3 and Fig. S2a-c).

Ethical consideration

The study was conducted agreeing with the recommendations of the research ethics guidelines. The collection of the plant material was carried out according to guidelines of Medicinal and Aromatic Plants and Traditional Medicine Research Institute, National Center for Research. The study proposal was approved by the Research Ethics Committee of the Faculty of Pharmacy, University of Khartoum (FPEC-01-2019).

CONCLUSION

Geigeria alata extract was evaluated *in vitro* as antioxidants and acetylcholinesterase inhibitor. *G. alata* ethyl-acetate fraction demonstrated promising *in vitro* AChE inhibitory and antioxidant activities. This is suggested to be correlated with its high total phenolic, flavonoid, and tannin contents. Hispidulin reported in the same fraction is presumed to be the lead compound in the fraction with remarkably predicted enzyme inhibition, pharmacokinetic and toxicity profiles. This work provides a promising insight into the importance of further studies on this plants' extracts for the isolation and characterization of natural AChE inhibitors. The current study has some limitations. Additional studies toward isolation and structure elucidation of *G. alata* phytoconstituents should be performed. Then, acetylcholinesterase inhibitory activity and toxicity profile of each isolated phytoconstituent should be investigated to figure out the promising lead compounds for future clinical studies in AD suffering patients. However, the conducted computer aided molecular docking on the previously detected compounds provided some clue of safety and inhibitory potential of these natural moieties against X-ray crystallographic rAchE structure

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for this work.

Authors' contributions

M.A.M., M.Y.A., A.A.: phytochemical experiment, biological assay, writing paper draft. W.O., A.E.S., A.A., M.S.M., H.S.A.: methodology, biological results discussion, Docking. E.F., A.H.A., S.R.M.I., K.F.G., G.A.M.: writing, software, editing and revision.

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