Scopoletin: Antiamyloidogenic, Anticholinesterase, and Neuroprotective Potential of a Natural Compound Present in *Argyreia speciosa* Roots by In Vitro and In Silico Study

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ABSTRACT: Alzheimer's disease (AD) is characterized by depositions of amyloid β (Aβ) peptides aggregates resulting in plaques formation in the central nervous system (CNS). This study evaluates the disease-modifying potential of scopoletin against multiple factors associated with AD such as cholinesterase enzymes, Aβ peptides, and neuroprotective properties against Aβ- and H_2O_2-induced cytotoxicity under in vitro conditions. Scopoletin was identified and quantified using UPLC-QTOF (ultra-high performance liquid chromatography-quadrupole time-of-flight) and high-performance liquid chromatography (HPLC), respectively. The antiamyloidogenic potential was evaluated by thioflavin T and congo red binding assay. Inhibition of key enzymes, that is, acetylcholinesterase and butyrylcholinesterase, was investigated by Ellman's assay. UPLC-QTOF analysis showed that most abundant phytoconstituent present in *Argyreia speciosa* hydroalcoholic root extract was scopoletin followed by festuclavine and ergometrine. Scopoletin was further quantified using novel reverse phase (RP)-HPLC method developed in this study. The neuroprotective potential of scopoletin was found to be 69% against Aβ42-induced neurotoxicity and 73% against H_2O_2-induced cytotoxicity in PC12 cell culture at 40 µM final concentration. At the same concentration, scopoletin inhibited Aβ42 fibril formation up to 57%. The IC_{50} concentration for AChE and BuChE enzyme inhibition by scopoletin was 5.34 and 9.11 µM, respectively. The antiaggregation and enzyme inhibition results were complemented with strong molecular interactions of scopoletin with target proteins validated by in silico molecular docking analysis. Based on this study, it can be concluded that scopoletin can be used as a lead for amelioration of symptoms and disease-modifying effects in AD.

KEYWORDS: scopoletin, *Argyreia speciosa*, Alzheimer's disease, amyloid β, neuroprotection, antiamyloidogenic

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Introduction

Alzheimer's disease (AD), a progressive and irreversible neurodegenerative disorder, accounts for more than 80% cases of dementia in elderly people. Worldwide, 47 million patients estimated to be suffering from AD at present, and this number is expected to increase up to more than 130 million by 2050.[1] The present therapies are unlikely to mitigate AD, as they are focused on single specific drug target, that is, cholinesterase inhibitors. Alzheimer's disease is a complex multifactorial etiopathology, which includes depositions of amyloid β (Aβ) protein aggregates in the central nervous system (CNS).[2] These aggregates are formed by mis-cleavage of amyloid precursor protein (APP) that results in sticky insoluble peptide fragments called Aβ fibrils, mostly consisting of oligomers that get deposited extracellularly on cerebrovascular space.[3] Amyloid β fibrils are mainly of 2 types depending on the C-terminal cleavage pattern of APP. Amyloid β 40 is the most prevalent form but Aβ42 is more neurotoxic hydrophobic form of Aβ, as it has faster aggregation kinetics.[4] Amyloid β 42 plaque formation causes neuronal cell damage and also increases oxidative stress in the CNS. As the AD progresses Aβ fibrillation accelerates on binding to AChE.[5] In the later stages of AD, there is an established direct correlation between Aβ aggregation and oxidative stress due to accumulation of reactive oxygen species (ROS).[6] In addition, Aβ fibrils have affinity to reduce redox-active metals and consequently lead to the formation of hydrogen peroxide and oxidized Aβ.[7] Therefore, compounds that could inhibit cholinesterase, having antioxidant potential and prevent aggregation of Aβ peptide could be used for therapeutic intervention. Current treatment only provides symptomatic relief. Presently, there is an urgent need to discover novel drug compounds that could have disease-modifying effects with negligible side effects. Considering this, the current study was undertaken to evaluate multitarget-directed ligand potential (MTDL) of natural compound scopoletin, an isolated coumarin class of compound, from *A. speciosa* roots.

A. speciosa or *vridhadaraka* meaning antiaging, has been mentioned in Ayurvedic material medica for its medicinal properties such as rheumatism, hepatoprotective, immunomodulatory, antioxidant, anti-inflammatory activity, and neurological disorders.[8,9] The identified compound in this study, scopoletin (6-methoxy-7-hydroxycoumarin) is a phytoalexin, low molecular weight compounds that are biosynthesized in response to microbial attack over the plant. Scopoletin

has been used as antibacterial and antifungal compound in traditional medicine.[10] The plants extract *A. speciosa* roots containing scopoletin has been used in folk-lore medicines worldwide in diseases such as convulsions, inflammation, rheumatic pain, leprosy, cardiovascular, and neuromuscular actions.[11] Literature reported studies with anticholinesterase and antioxidative effects of scopoletin.[12-14] This study aims to establish scopoletin as potential lead for therapeutic intervention in AD against multiple targets as MTDL candidate.

Materials and Methods

Zorbex UPLC C_{18} Silica Column, Methanol, Formic Acid, Acetonitrile, Amyloid beta (Aβ42) protein (Link biotech), Acetylcholinesterase (AChE) from electric eel, Butyrylcholinesterase (BuChE) from equine serum, Thioflavin T (ThT), scopoletin and congo red (CR) dye were purchased from Sigma Aldrich, India. Fetal bovine serum (FBS), RPMI 1640 medium, phosphate-buffered saline (PBS) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) salt along with other tissue-culture-grade chemicals were purchased from Himedia, India. All preparations were filtered through 0.45 µm Axiva 25 mm CA filter before use in tissue culture experiments.

Preparation of Hydroalcoholic Root Extract of *A. speciosa*

A certified dried *A. speciosa* root sample (voucher specimen number AS1-USBT was stored in School of Biotechnology, GGS Indraprastha University) was infused in 1:1 ratio of ethanol and freshly boiled distilled (de-ionized) water to prepare the hydroalcoholic extract. The infusion was kept in a shaker at 40°C for 48 h and filtered. The supernatant was freeze-dried (lyophilized) in a lyophilizer for 24 h. The yield obtained was calculated and freeze-dried aliquots were stored at −20°C till further use.

Metabolite Profiling of *A. speciosa* Extract by UPLC-QTOF

Agilent 6545 LC/Q-TOF equipped with autosampler and nanoAcquity column of dimensions 1.8 µm, 100 µm × 100 mm was employed. The mobile phase was (1) water 0.1% formic acid and (2) 70% acetonitrile with flow rate of 500 µL/min. The injection volume was 10 µL. The LC conditions were 0 to 2 min, 5%(2) followed by linear increase during 2 to 8 min up to 50%(2) and finally 95%(2) during 8 to 10 min followed by washing. Quadrupole time-of-flight (QTOF) mass spectrometer equipped with nano electrospray ion source and MassHunter software was used in positive mode of ionization for the MS analysis using automatic switch between mass spectrometry (MS) and tandem mass spectrometry (MS/MS) acquisition modes. The LCMS data were analyzed by MassHunter software developed by Agilent. Peaks generated with ionization above 1800 counts in positive mode of ionization were considered with 0.0075 m/z peak spacing tolerance for a resolved chromatogram. Tandem mass spectrometry fragmentation data were compared with scopoletin data in pubchem to establish the identity.

Novel RP-HPLC Method Development and Validation for Scopoletin

Novel reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed for scopoletin and validated according to International Council for Harmonisation (ICH) guidelines.[15] Reverse phase high performance liquid chromatography (Dionex, Thermo scientific system), autosampler, and ultraviolet (UV)-detector were used for the analysis of Scopoletin. The data were analyzed by Chromeleon 6.8 chromatography data system software (Thermo scientific). Stationary phase as zorbax C_{18} column (250 mm × 4.6 mm, 5 µm) and an isocratic mixture of methanol and water containing 0.1%v/v formic acid in the ration of 40:60 as mobile phase were used in the developed method. The mobile phase was filtered and degassed for 30 min by sonication. The flow rate was adjusted to 1.0 mL/min. Injection volume of scopoletin standard and test fraction was adjusted to 20 µL and detection was made at 366 nm. Parameters of the validated method were calculated as linearity (at 10, 20, 30 ppm), precision, level of detection (LOD), and level of quantification (LOQ) (see supplementary data). Scopoletin present in *A. speciosa* extract was quantified from the pure standards (std) run under the same chromatographic conditions. Six injections of scopoletin standard solution were given with mentioned method followed by 3 injections of test fraction from different samples

$$\text{Quantity}\left(\text{test}\right) = \left[\begin{matrix}\text{Average area}\left(\text{test}\right)\\ /\text{Average area}\left(\text{Std}\right)\end{matrix}\right] * \left[\begin{matrix}\text{weight}\left(\text{std}\right)\\ /\ \text{weight}\left(\text{test}\right)\end{matrix}\right] * \text{Std purity\%}$$

where the average area is the chromatographic area under the peak for standard and test, weight is the measured amount used to prepare dilution. Establishing the identity and purity of scopoletin in the extract, further experimentation was done with pure compound.

Preparation of Aβ42 Fibrils and Antiaggregation Analysis

Amyloid β 42 fibrils were prepared (see supplementary data) and in vitro antiaggregation ThT and CR experiments were conducted using reported protocol.[16] Amyloid β 42 (44 µM, IC50) was dissolved in milliQ with and without various concentrations of compounds. Samples were incubated at 37°C at 260 r/min for 48 h in the shaker incubator. The antiamyloidogenic activity of scopoletin was studied at 11-44 µM concentration and 44 µM tannic acid as positive control. Thioflavin T was excited at 450 nm and spectra were recorded from 400 to 650 nm along

with end-point determination for reduced ThT fluorescence. Observations were further strengthened by CR absorbance spectra (400–650 nm) of the various treatment samples with a UV-Visible spectrophotometer. Percentage inhibition was calculated for inhibition in fibril formation by the formula ([control sample]/control)*100, providing relative inhibition as compared with control. All measurements were performed in triplicates and all experiments were repeated thrice individually.

Aβ42-induced Neurotoxicity on PC12 Cells

The PC12 cells maintained as monolayer adherent culture were used to seed 96-well plate in the log phase of growth at concentration 5×10^5 cells per well (see supplementary data). Preincubation was given with positive control (homotaurine, 40 μM) and scopoletin (10, 20, 40 μM) 3 h before inducing Aβ42 preformed fibrils neurotoxicity. MTT assay was performed and relative survival percentage of PC12 cells was calculated compared with control (PC12 cells only).[16] All measurements were performed in triplicates and all experiments were repeated thrice individually.

Oxidative Stress Cytotoxicity Using H_2O_2

Hydrogen peroxide (IC_{50} 200 μM) was used to induce cytotoxicity in cultured PC12 cells (see supplementary data). Glutathione (GSH, positive control) and scopoletin (10, 20, 40 μM) were used to analyze neuroprotective effects against oxidative damage through MTT assay.[16] Relative percentage survival was calculated against control (PC12 cells only). The experimentation was repeated in a set of 3 technical and biological repeats, respectively.

Ellman's Assay for Anticholinesterase Activity

Ellman's assay was performed to assess the inhibition of the enzyme Acetylcholinesterase (AChE, EC 3.1.1.7) and Butyrylcholinesterase (BuChE, EC 3.1.1.8) by donepezil (positive control) and scopoletin along with hydroalcoholic extract of *A. speciosa*.[17] Enzyme solution (0.03 U/mL), test compound concentrations and DTNB (0.3 mM) probe were preincubated for 15 min at RT. The reaction was initiated by adding the substrate (0.5 mM) and the absorbance was recorded at 412 nm on a SpectraMax M2 reader. Relative absorbance inhibition was calculated against control with whole enzyme activity. Three sets of experiments were repeated each with 3 technical repeats.

Molecular Docking Analysis of Scopoletin With AD Targets by Autodock4.2

In silico docking analysis was performed to study the molecular interactions of scopoletin with selected AD targets (Aβ42, AChE, BuChE, BACE1, and MAO-B) using Autodock 4.2.[18] Receptor protein X-ray crystallographic structures were procured from protein data bank (1YIT for Aβ42, 4PQE for AChE, 2J4C for BuChE, 4D8C for BACE1 and 1S2Q for MAO-B) and processed. Three-dimensional structure of scopoletin, galanthamine and other positive controls were downloaded from the PubChem database and processed as ligand using PyMol.[19] To legitimate the developed docking protocol the root mean square deviation (RMSD) was calculated. Scopoletin was docked with target receptor proteins and molecular interactions, and ligand conformations and binding energies were obtained.

Statistical analysis. Three independent experiments were used in each experimental setup to present data as mean ± standard error mean, for liquid chromatography–mass spectrometry (LCMS) data $n = 4$ runs were used. Statistical analysis was performed using unpaired student's *t*-test. Level of significance was determined as $P \leqslant .01$.

Results

Metabolite analysis of A. speciosa extract by UPLC-QTOF

The UPLC-QTOF analysis for hydroalcoholic root extract of *A. speciosa* revealed the presence of natural coumarins and ergot alkaloids. The most abundant coumarin present was scopoletin (5×10^6 ions) followed closely by festuclavine (4.3×10^6 ions), ergometrine (3×10^5 ions) and psoralen (1.7×10^5 ions; Figure 1). The potent antioxidant flavones, quercetin and kaempferol, were also found to be present along with other phenol and fatty acid, behenic acid and palmitic acid (Table 1).

RP-HPLC method development and validation of scopoletin fraction

A novel reproducible and reliable, RP-HPLC method was developed for rapid and sensitive detection (or quantification) of scopoletin. The method was validated according to "International Council for Harmonisation (ICH) of technical requirements for pharmaceuticals for human use" for stability, linearity, precision, the LOD and LOQ levels. Scopoletin standard was run with 6 injections followed by 3 individual injections of extract fraction samples collected from previous LCMS run. Figure 2A shows scopoletin standard peak at 6.257 ± 0.09 min with area 96.9 mAU/min and scopoletin peak present in *A. speciosa* extract (Figure 2B) at 6.3 ± 0.5 min with area 0.17 mAU/min. Scopoletin amount was quantified as 0.56%w/w in *A. speciosa* extract.

Antiamyloidogenic potential of scopoletin

Amyloid β 42 antiaggregation at monomer, oligomer, or protofibrils stage is an important pathological target to combat AD pathophysiology. Thioflavin (ThT) fluorescence assay was used to analyze the antiamyloidogenic potential of scopoletin along with the evaluation of redshift in CR dye binding assay. Amyloid β 42 protofibril-bound ThT probe fluoresces brightly at 480 nm (emission), whereas free ThT molecules would quench at the same excitation wavelength (450 nm); therefore, the fluorescence intensity would give quantification of amyloid

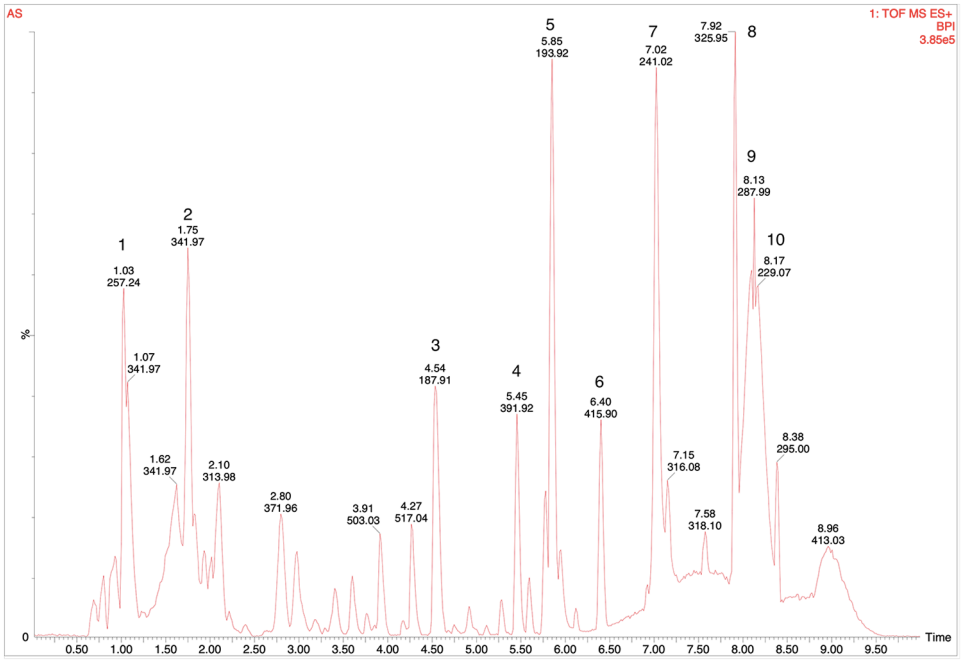

Figure 1. UPLC-QTOF TIC (total ion chromatogram) of hydroalcoholic root extract of *A. speciosa*; peak number represents the order of elution of identified phyto-constituents and retention time along with mass eluted has been labeled.

Table 1. Identified phytocompounds of hydroalcoholic root extract of *A. speciosa* in descending order according to abundance ions percentage.

PEAK NO.	COMPOUND NAME	FORMULA	MONOISOTOPIC MASS	RETENTION TIME (MIN)	M + Z VALUES
5	Scopoletin	$C_{10}H_8O_4$	192.042	5.85	193.92
7	Festuclavine	$C_{16}H_{20}N_2$	240.163	7.02	241.02
8	Ergometrine	$C_{19}H_{23}N_3O_2$	325.18	7.92	325.95
2	Behenic acid	$C_{22}H_{44}O_2$	340.33	1.75	341.97
1	Palmitic acid	$C_{16}H_{32}O_2$	256.24	1.03	257.24
10	Quercetin	$C_{14}H_{28}O_2$	228.209	8.17	229.07
9	Kaempferol	$C_{15}H_{10}O_6$	286.048	8.13	287.99
3	Psoralen	$C_{11}H_6O_3$	186.03	4.54	187.91
6	Beta Sitosterol	$C_{29}H_{50}O$	414.386	6.40	415.386

fibril formation. Scopoletin significantly inhibited the formation of Aβ42 fibrils in a concentration-dependent manner (11-44 μM), with 56% inhibition in fluorescence by scopoletin (at 44 μM; Figure 3A) as compared with control sample (Aβ42 only). The positive control, tannic acid was observed to inhibit Aβ42 aggregation up to 85%.

The emission spectrum (400-650 nm) for ThT fluorescence further reinforces antiaggregatory activity of scopoletin (Figure 3B). Amyloid β 42 fibrils bound to ThT probe showed fluorescence emission maxima at 480 nm, whereas the Aβ42 samples treated with scopoletin concentrations (11-44 μM) showed reduced concentration-dependent fluorescence intensity at 480 nm directly proportional to the less amount of Aβ42 fibril formation. Furthermore, red shift (from 480 to 500-550 nm) in CR dye absorption assay signifies Aβ42 fibril formation. The results of spectral shift assay, also support the ThT determination of Aβ42 fibril inhibition. The typical absorbance of CR assay peaked at 490 nm, whereas when Aβ42 added to CR the absorbance wavelength is shifted to 520 nm. Amyloid β 42 fibrils incubated with scopoletin, demonstrated reduced CR red shift close to 510 nm proving antiamyloidogenic potential of scopoletin.

Neuroprotective effect of scopoletin on PC12 cells against Aβ42-inflicted cytotoxicity

The neurotoxic effect of Aβ42 fibrils along with neuroprotection provided by scopoletin toward in vitro model of PC12

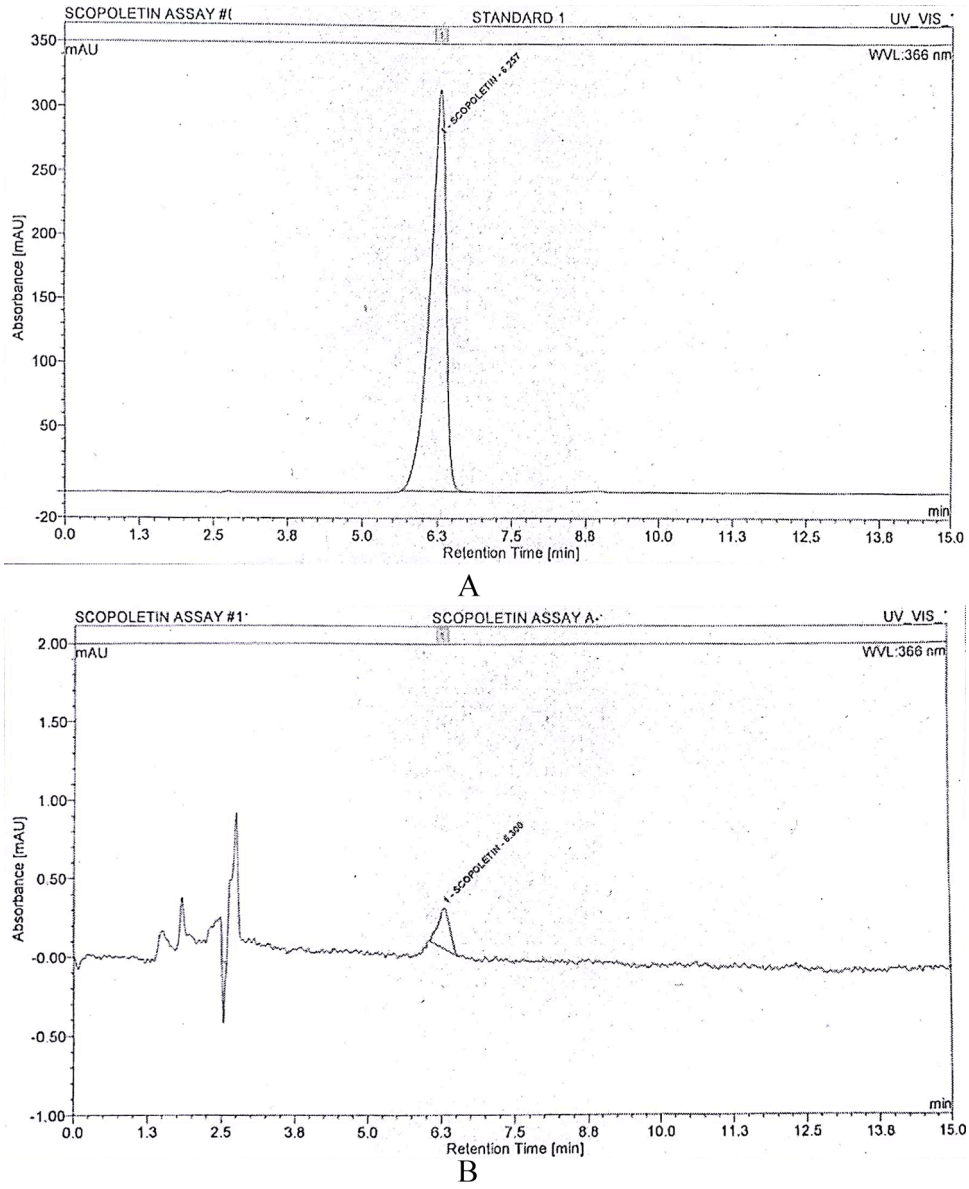

Figure 2. (A) Scopoletin standard peak at 6.257 min retention time and (B) scopoletin peak present in *A. speciosa* test extract at 6.3 ± 0.5 min by developed RP-HPLC method at 366 nm.

cells was studied using MTT assay. A concentration-dependent survival of PC12 cells was observed by scopoletin at an equimolar concentration of Aβ42 cytotoxicity (40 µM). Scopoletin imparted 69% protection against Aβ42 cytotoxicity at 40 µM, which was comparable to the protection demonstrated by positive control, homotaurine (59%), used in the study (Figure 4).

Protective potential of scopoletin against oxidative stress

Scopoletin was found to be imparting protective effect to PC12 cells against H_2O_2-induced oxidative stress. As evident from Figure 5, scopoletin showed 73% protection against H_2O_2-induced cytotoxicity in PC12 cells at 40 µM concentration, whereas the positive control (GSH) gave 91% cell survival as compared with negative control (PC12 cells treated with 200 µM H_2O_2 only).

Evaluation of dual anticholinesterase potential of scopoletin

Scopoletin demonstrated dual anticholinesterase potential in a concentration-dependent manner. At 50 µM, the percentage inhibition for AChE and BuChE was determined to be 85.5 ± 2.2% and 78.9 ± 2.14%, respectively (Figure 6). The hydroalcoholic root extract of *A. speciosa* also inhibited AChE and BuChE to 67 ± 2.8% and 57 ± 1.9%, respectively, at 200 µg/mL concentration. Donepezil used as positive control gave 94% inhibition of AChE and 91% inhibition for BuChE. The IC_{50} value of percentage inhibition by scopoletin, calculated from the inhibition curve was 5.34 and 9.11 µM for

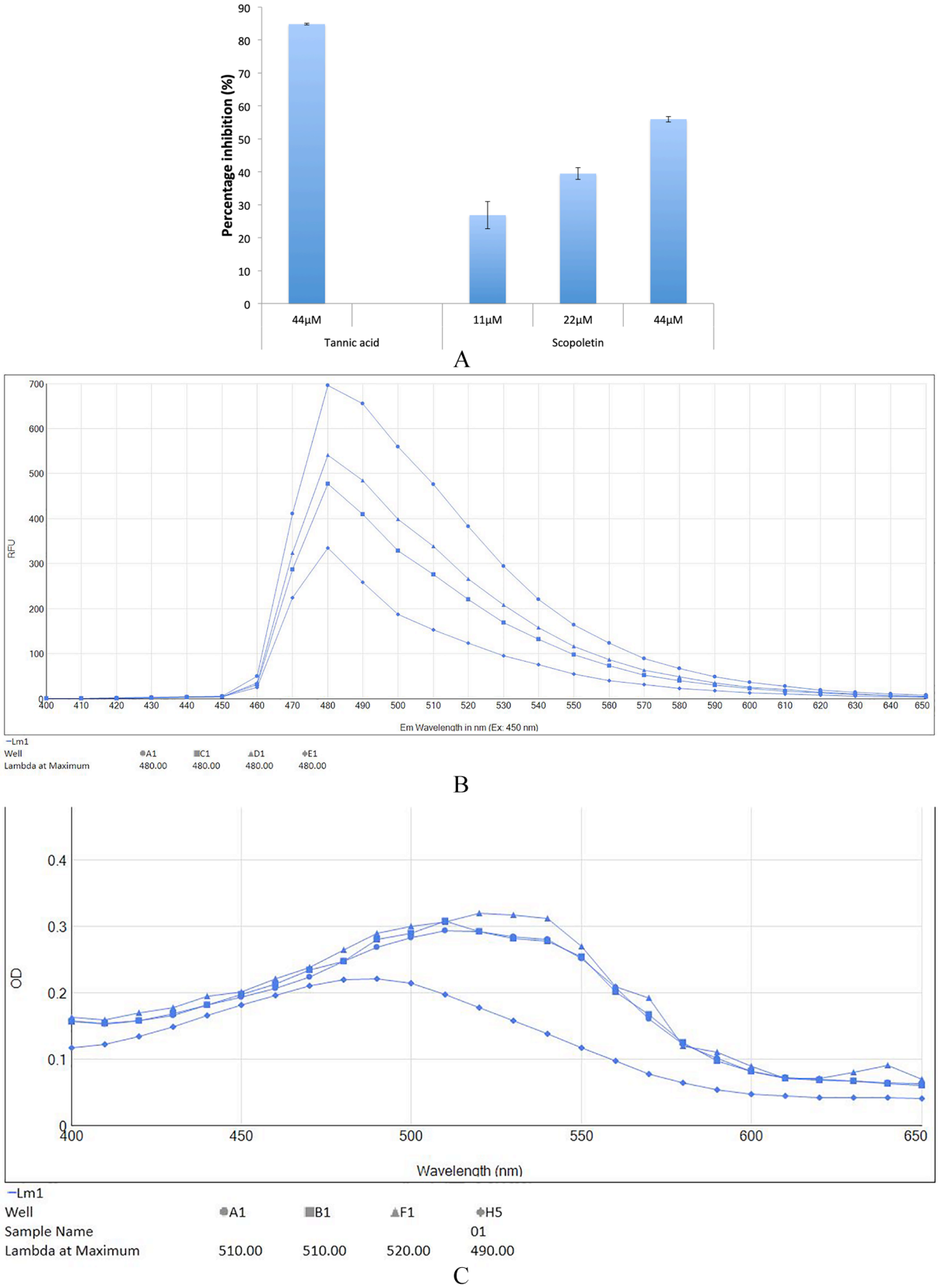

Figure 3. (A) Relative inhibition of Aβ42 fibril formation; scopoletin (442211 μM); PC tannic acid (44 μM), data represented as mean ± SEM, n = 3; difference in mean is statistically significant (*) $P < .01$ as compared with control group; (B) ThT fluorescence emission spectra at 480 nm A1, Aβ42 only; C1, Aβ42 + scopoletin 11 μM; D1, Aβ42 + scopoletin 22 μM; E1, Aβ42 + scopoletin 44 μM; (C) red shift of congo red dye on Aβ42 fibril formation H5, CR only (490 nm); F1, Aβ42 only (520 nm); B1, Aβ42 + SCO 44 μM (510 nm).

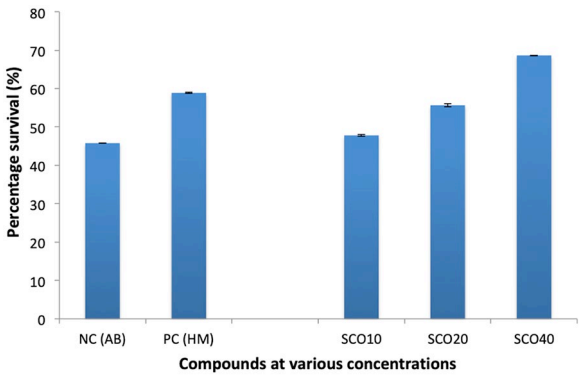

Figure 4. Concentration-dependent percentage survival by MTT assay of Aβ42 fibrils (48 h) induced neurotoxicity on PC12 cells; Scopoletin (40, 20, 10 μM); NC, PC12 cells treated with Aβ42 only (40 μM); PC, homotaurine (40 μM), data represented as mean ± SEM, n = 3.

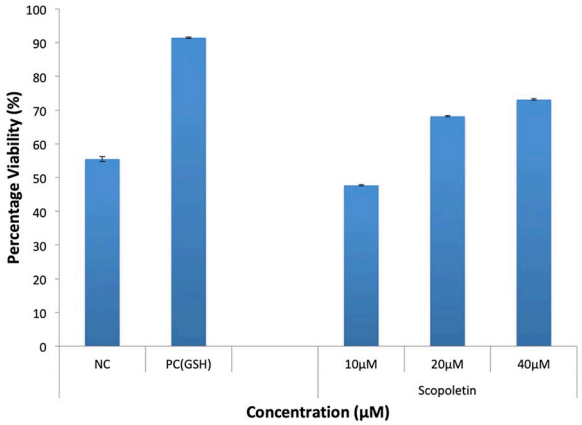

Figure 5. Neuroprotective effect against H_2O_2-induced cytotoxicity in PC12 cells; scopoletin (40, 20, 10 μM); PC: positive control (Glutathione, 40 μM); values represent mean ± SEM, n = 3; the difference in the mean is statistically significant (*) $P < 0.01$ as compared with control group.

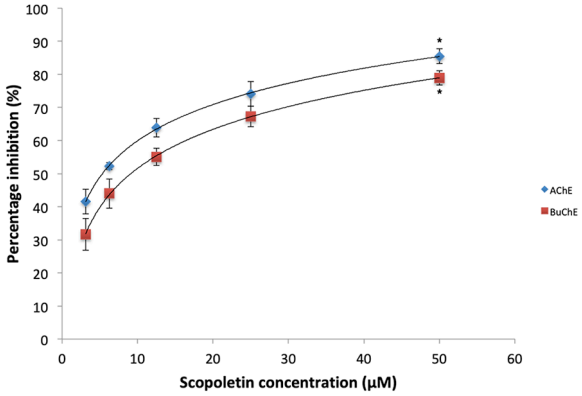

Figure 6. AChE and BuChE percentage inhibition at various tested concentrations of scopoletin (3.125-50 μM); values represent mean ± SEM, n = 3; the difference in the mean is statistically significant (*) $P < 0.01$ as compared with control group.

AChE and BuChE, respectively. Also, scopoletin was observed to inhibit AChE more selectivity than BuChE with selectivity index 1.7 (IC_{50} BuChE/IC_{50} AChE affinity ratio).

Molecular interaction of scopoletin with AD targets

Molecular docking results showed that scopoletin interacts with catalytic site residues of almost all the AD targets (Aβ42, AChE, BuChE, BACE1, and MAO-B). The binding energy analysis revealed that scopoletin has almost equal binding energies as that of positive controls (tacrine and rasagilline, respectively) with BuChE and MAO-B (Table 2). Scopoletin also irreversibly bind to Aβ42 and AChE, as it showed low binding energy and thus high stable protein-ligand complex (Figure 7A and B).

Molecular docking results are in compliance to in vitro experimentation findings, where scopoletin proved as an excellent MTDL capable of inhibiting Aβ42 aggregation and key enzymes implicated in AD, such as AChE and BuChE. Scopoletin occupies the catalytic sites of the target proteins (Figure 7A-E).

Discussion

The cognitive decline, language impairment and memory deficit in AD are characteristic features of disease like AD. Alzheimer's disease's cause is complex, but several factors including mis-cleavage of APP protein, β-amyloid deposits, deficits of acetylcholine, neurofibrillary tangles, and oxidative stress are cardinal features of disease pathophysiology.[20] Although multiple disease targets were known through recent research, no permanent cure is available at present to AD patients, but symptomatic treatment is available through cholinesterase inhibitors with many side effects. The new paradigm shift in AD research has focused on "one molecule, multiple targets" approach, where one compound is sought to bind as many disease targets as possible. With this strategy, the disease-modifying capabilities are expected with much less side effects as compared with multiple drug therapy approach. Natural compounds have always been explored as novel therapeutic option such as huperzine-A, rivastigmine, and galanthamine, are already in use to provide symptomatic relief to AD patients.[21,22] In view of this, this study explored natural phytocompound scopoletin present abundantly in *A. speciosa* roots for its MTDL capabilities, which could sequester Aβ42 and binds with other target enzymes. Scopoletin is naturally occurring coumarin phytoalexin, with medicinal importance as antimicrobial, anti-inflammatory, and blood pressure regulatory compound.[11] *A. speciosa* is abundant in many alkaloids and coumarins such as psoralen, ergometrine, and festuclavine.[23] Scopoletin was found to be the most abundant phytoconstituent in test extract by UPLC-QTOF analysis (0.6%w/w). Secondary metabolite profile of *A. speciosa* by UPLC-QTOF method showed similar identified phytocompounds.[24] A novel RP-HPLC method was developed and validated for detection and quantification of scopoletin present in *A. speciosa* hydroalcoholic extract, using a UV detector. The current RP-HPLC method developed has many advantages over previously reported methods as it offers rapid, reliable and reproducible elusion and detection of scopoletin.[25,26] This method reported

Table 2. Molecular interactions as observed in ligand docked complexes of target receptor proteins obtained from Autodock4.2.

S. NO.	LIGAND	MOLECULAR WEIGHT (G/MOL)	AD TARGET	BINDING ENERGY (KCAL/MOL)	NO. OF H-BONDS	INTERACTING RESIDUES	BOND ANGLE (Å)
1.	Scopoletin	192.17	Aβ42	−4.1	1	Asp23	2.5
			AChE	−7.6	3	Tyr337, Glu202, Tyr133	2.4, 3.4, 3.1
			BuChE	−6.8	2	Trp82	2.3, 3.0
			BACE1	−6.2	3	Phe108, Thr220, Asp217	3.2, 2.2, 3.3
			MAO-B	−7.3	1	Tyr60	2.1
Positive control							
2.	Tannic acid	1701.19	Aβ42	−6.5	2	Asp23, Lys28	2.1, 1.9
3.	Galanthamine	287.354	AChE	−10.8	3	Tyr337, Glu202, Ser203	1.8, 2.2, 1.7
4.	Tacrine	198.264	BuChE	−6.52	1	Trp82	2.3
5.	BXD		BACE1	−10.4	4	Asp32, Gly34, Phe108, Asp217	1.9, 2.2, 2.5, 2.1
6.	Rasagilline	171.238	MAO-B	−7.5	0 (all hydrophobic)	Gln206, Phe343, Tyr326, Leu171	

sensitive detection and quantification of scopoletin with low LOD and LOQ score for trace amount detection. MTDL potential of scopoletin was studied further against disease-modifying targets, Aβ42 peptide aggregation, in vitro neuroprotection against Aβ42 fibrils and oxidative stress, anticholinesterases and other targets such as BACE-1 and MAO-B with in silico approach.

Amyloid β aggregation into insoluble fibrils play pivotal role in triggering a cascade of physiological changes from neurofibrillary tangles (NFT) formation to neuronal dysfunction into AD progression. Amyloid β fibril formation includes the aggregation of soluble monomers or dimers with random α-coil structures into protofibrils which then arrange in insoluble β-sheet cross-structures of senile Aβ plaques.[4] Compounds that could inhibit Aβ42 amyloidogenesis or counter this process of oligomerisation are being explored to have have therapeutic value in treatment of AD. This study showed that scopoletin has the significant antiamyloidogenic potential in concentration-dependent manner. The mechanism insight by molecular-docking studies showed that scopoletin stacks itself in between adjacent β-sheets of Aβ42. Scopoletin interacted with key residues of Aβ42, Asp23 with a hydrogen bond. Amyloid β 42 aggregation requires the formation of hydrogen bond between Asp23 of one monomer and Lys28 of another monomer, therefore by occupying the interacting residue, scopoletin inhibited Aβ42 oligomerisation. Also, scopoletin was observed to orient in a way to occupy the internal sites of the steric zipper composed of residues 16 to 21 (KLVFFA), covering the active site and hence obstruct β-sheet conformation of Aβ42 peptide. Also, it has been reported that Aβ42 amyloidogenesis accelerates on Aβ42 peptide binding to AChE, near the peripheral anionic site (PAS) of AChE.[27] Scopoletin interacts with AChE with 3 hydrogen bonds lying mainly in the catalytic grove of AChE, while covering the PAS residues partially, which is similar to the positive control galanthamine molecular interactions. But scopoletin did not interact directly with any of the main catalytic residues (Ser203, His447, and Glu334) unlike galanthamine. Therefore, scopoletin demonstrated multifarious activity here, as it not only inhibits Aβ42 aggregation but also occupy both the sites of AChE [CAS (catalytic anionic site) and PAS (peripheral anionic site)] thus inhibiting accelerated Aβ42 oligomerisation on AChE binding. These in silico findings are well complemented with in vitro results where scopoletin was demonstrated to significantly inhibit these enzymes in concentration-dependent manner. Cholinergic impairment could be countered with inhibition of cholinesterases (AChE and BuChE). Scopoletin showed similar binding energy for the BuChE inhibition as compared with the positive control, tacrine as it forms dual hydrogen bonds with Trp82 (CAS), thus rendering the enzyme completely inactive. Scopoletin was reported to protect SH-SY5Y cells from neurodegeneration against H_2O_2-induced oxidative stress.[14] In our study, scopoletin gave neuroprotection to PC12 cells against H_2O_2-induced oxidative stress in concentration-dependent manner. To further strengthen the MTDL profile of scopoletin, in silico interactions of scopoletin with BACE-1 and MAO-B targets of AD were studied. Scopoletin was observed to occupy the active site of BACE-1 enzyme by interacting with Asp217, lying adjacent to Asp32 (main interacting residue) but did not interact with prime catalytic residues of the enzyme. Similarly, scopoletin partially occupied the active site of MAO-B (Ile199 and Tyr326) by interacting with

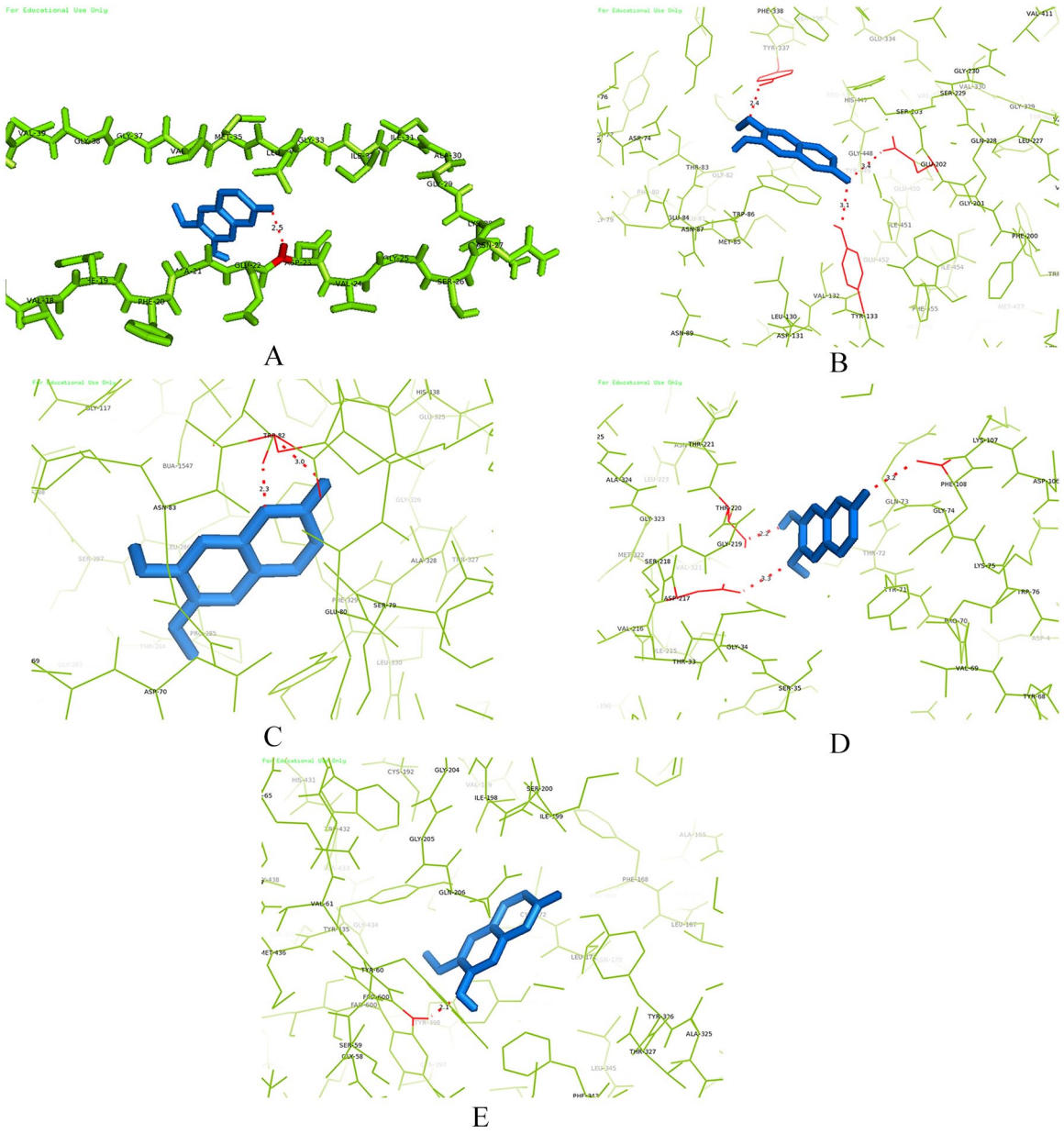

Figure 7. Molecular interactions of scopoletin with multiple targets associated with AD; (A) with Aβ42 (1IYT), (B) with AChE (4PQE), (C) with BuChE (2J4C), (D) with BACE1 (4D8C) and (E) with MAO-B (1S2Q).

non-catalytic residue, Tyr60, and hence gave a binding energy equivalent to the positive control, rasagilline. Yusufzai et al[28] also showed in silico study of scopoletin and scopoletin-derived derivative to inhibit AChE, BuChE, BACE-1, and MAO-B enzymes involved in AD.

In conclusions, the novel study showed that scopoletin could act as MTDL, which can be used against several targets Aβ42, AChE, BuChE, and oxidative stress that are implicated in AD and thus help in the alleviation of symptoms and having a disease-modifying effect in AD.

Author Contributions

SK conceived and designed the study. PK did all the experiments, acquisition of data. SK, PK, HR did result interpretation. SK, PK, HR and SDS written and approved the manuscript.

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Supplemental Material

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