Exploring the neuropharmacological properties of scopoletin-rich *Evolvulus alsinoides* extract using *in-silico* and *in-vitro* methods

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Abstract: Objectives: This study investigates the neuropharmacologic properties of Scopoletin, a bioactive compound in *Evolvulus alsinoides* (EA) extract, for managing cognitive impairment using *in-vitro*, *in-silico*, and zebrafish embryo toxicity assays. Methods: The study estimates Scopoletin concentration in EA extract using HPTLC, assesses antioxidant properties using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing ability of plasma (FRAP) assays, and uses bioinformatic tools for scopoletin targets. Zebrafish embryo toxicity (ZET) is used to assess its toxicological profile. Results: 0.0076% w/w Scopoletin in the samples was quantified using HPTLC, further studies on the DPPH (0.5 mM) and FRAP gave EC\(_{50}\) at 440.0 μg/ml and 84.29 μg/ml respectively. Twelve common targets associated with cognitive impairment (CI) were identified, along with possible pathways and molecular interactions. Our results indicate significant binding affinities of Scopoletin with ERAP1, SCN3A, and COMT. Molecular dynamics simulations further confirm the stability of these interactions. ZET assessment demonstrated mortality after 450 μg/ml concentration of EA extract. Conclusion: The study verifies the presence of Scopoletin in EA along with their targets playing a crucial role in neurogenesis and neuroplasticity. The ZET demonstrated concentration-dependent effects, emphasizing the importance of dosage considerations in developing new formulations or therapeutics. This comprehensive study contributes valuable insight into the therapeutic potential of Scopoletin from EA for cognitive impairment, paving the way for further research.

Keywords: *Evolvulus alsinoides*, scopoletin, cognitive impairment, HPTLC, *in-silico*, FET

Introduction

Medicinal plants have been essential for improving human health and resolving the health issues for a significant portion of the global population throughout history [1]. A plant containing compounds with therapeutic potential or essential components for the development of effective pharmaceuticals is categorized as a medicinal plant. The World Health Organization (WHO) reports that a significant proportion, ranging from 70% to 95%, of individuals in developing nations continue to depend predominantly on medicinal plants to address their primary healthcare needs [2]. This reliance has led to a surge in the global popularity of medicinal plants, evident in both developed and developing countries [3]. In alignment with this principle, the use of herbal remedies holds a pivotal position in ancient Indian healing practices [4].

Several Indian herbal medicinal plants such as *Withania somnifera*, *Centella asiatica*, *Celastrus paniculatus*, and *Bacopa monnieri*, have been utilized for the management of neurological disorders and have demonstrated potential for cognitive improvement [5]. In the
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realm of traditional Indian medicine, four plant species - *Canscora decussata* Schult. (CD), *Clitoria ternatea* Linn. (CT), *Convolvulus pluricaulis* Choisy. (CP), and *Evolvulus alsinoides* Linn. (EA) have been identified as *Shankhpushpi* sources based on their morphologic characteristics mentioned in ancient texts. Notably, EA has exhibited remarkable neuropharmacological effects compared to the other three varieties of *Shankhpushpi* [6]. It belongs to the Convolvulaceae family, and holds significant importance in traditional medicinal practices in India and other regions [7]. This medicinal plant is utilized for its therapeutic properties in treating various ailments such as as anxiolytic, neuroprotective, antioxidant, analgesic, immunomodulatory, antibacterial and anti-inflammatory effects [8-10].

This medicinal herb is known to possess numerous bioactive compounds, including alkaloids (such as convolamine), flavonoids (like kaempferol), phenolics (including β-sitosterol, ceryl alcohol) and bitter (Scopoletin) [9]. Scopoletin identified as the principal active phytoconstituent in *Shankhpushpi*, plays a pivotal role in its bio-potency. It is a bitter compound, and is credited with antioxidant properties and memory enhancement [11]. Furthermore, scopoletin exhibited a mitigating effect on amnesia in animals induced with scopolamine, showcasing its multifaceted neuroprotective potential [12].

The neuropharmacologic effects discussed above primarily result from the cumulative impact of these phytochemicals. However, existing literature struggles to elucidate the precise mechanisms governing the neuroactive functions of these phytochemicals. A comprehensive understanding of these underlying molecular mechanisms necessitates rigorous experimental exploration. Alternatively, network pharmacology emerges as a promising bioinformatic tool capable of forecasting both active phytochemicals and their associated molecular targets, thereby elucidating the pharmacologic actions of plant extracts [12]. Along with the network pharmacology, the evaluation of the toxicity of potential drug compounds has been enhanced in recent years [13]. A toxicology assay is necessary for allopathic medications as well as complementary and alternative therapies to detect any negative effects that may not be fully understood until after excessive use and the onset of symptoms [14]. Nonetheless, the primary goal of toxicity research is to forecast human toxicity through quick and precise testing of several chemicals [15] using model systems [16]. Furthermore, there has been a limited amount of research conducted on scopoletin, with a notable scarcity of in-silico investigations involving molecular docking and simulation, as well as in-vivo studies.

In this study, we initially conducted a quantification of Scopoletin of *EA*, utilizing HPTLC along with phytochemical analysis. These were done in plants obtained from Kanha Shanti Vanam in Mallapur, Telangana, India. This plant has an abundant presence of scopoletin. Recognizing scopoletin as the primary target compound, we employed Network Pharmacology, molecular docking, and simulation techniques to predict its underlying molecular mechanisms and pharmacologic action against cognitive impairment. This study not only sheds light on the therapeutic effects of scopoletin but also advocates for the use of EA and its metabolites for addressing cognitive impairment. Furthermore, antioxidant assays (i.e. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing ability of plasma (FRAP) assays) were employed to find the EC$_{50}$ of their antioxidant potentials. We experimentally validated the toxicological effects of scopoletin-rich EA extract through *in vivo* zebrafish embryo toxicity assessment, thus contributing to a comprehensive understanding of its therapeutic potential.

**Materials and methodology**

**In-vitro analysis**

**Collection of plant samples:** A Fresh sample of *EA* ([Figure 1](#)) was obtained from Heartyculture Nursery, Kanha Shanti Vanam in Hyderabad, Telangana, India. The plant sample was collected from the Ranga Reddy District of Telangana, precisely located at Latitude 17.184442° and Longitude 78.213975°, on 3rd October, 2023. To ensure its credibility, the authenticity of the plant sample was subsequently verified through validation by a taxonomist situated at the same location.

**Preparation and extraction of EA:** The whole plant of *EA* was carefully cleaned and freed
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from dust particles. Subsequently, the plants were dried and pulverized into a coarse powder using an electric grinder. A quantity of 10 g of this plant material was subjected to extraction employing the Soxhlet method at a temperature of 60°C, with methanol serving as the extracting solvent. The resulting solution was then evaporated in a water bath set at 40°C until all traces of solvents were eliminated. The residue was stored at -20°C until needed. Prior to the commencement of the experiment, the dried extract was dissolved in 10 ml methanol.

Preliminary phytochemical screening of EA: Preliminary phytochemical screening entailed analyzing of aqueous extract of EA to determine which classes of compounds they contain. A phytochemical screening procedure was used to find different bioactive compounds such as Alkaloids, Saponins, Tannins, Glycosides, coumarins etc. by using following qualitative chemical test to provide a general understanding of the makeup of the crude drug's constituents [17-20].

Preparation of scopoletin standard: We precisely measured 1 mg of Standard Scopoletin and placed it into a 1 mL volumetric flask. We added 0.5 mL of methanol, sonicated until there was complete dissolution of the standard, and then adjusted the volume to 1 mL with methanol. We utilized the resulting standard solution for HPTLC fingerprinting.

Quantification of scopoletin by HPTLC in EA: A 10 μL sample solution was accurately placed on a silica gel-coated plate using a CAMAG Linomat applicator to ensure consistency. The plate was then dried in the air and put in a glass chamber saturated with a mobile phase containing Toluene, n-Hexane, Ethyl acetate, and Acetic acid. Over a span of 30 minutes, the mobile phase moved along the plate due to capillary action, separating components based on their polarity and their interaction with the stationary phase (silica gel). A CAMAG TLC scanner was used to scan the plate at 366 nm, generating chromatograms that depicted peak intensity against the retention factor (RF), indicating how components moved relative to the solvent front. Bands on the plate were observed under visible light using a CAMAG visualizer, allowing for the examination and recording of band characteristics. Following this, the visionCAT software generated peak tables, which included Rf values, peak areas, and heights. These data aided in identifying and quantifying components by comparing them with reference standards or literature information. An initial analysis of the extract/fractions was conducted to detect the presence of secondary metabolites such as Alkaloids, Saponins, Tannins, Glycosides, Proteins, Coumarins.

In-silico study

Retrieval of SMILES and 3D structure of scopoletin: Initially, we identified the PubChem CID for scopoletin and obtained the corresponding SMILES notation using the “PubChem Identifier Exchange Service” website. Additionally, the 3D structure of scopoletin was downloaded from PubChem in SDF format, subsequently converted to PDB format by using Avogadro Software [21].

Target prediction and identification of genes associated with cognitive impairment: SMILES data retrieved from PubChem was utilized for target prediction using Swiss Target Prediction [22], Superpred [23], SEA [24] and Way2drug.
servers. Predicted targets underwent filtration based on Prediction Score and Model accuracy. This list of predicted target genes was cross-referenced with genes associated with cognitive impairment from the HPO database [26] for identification common targets for subsequent analysis.

**GO pathway enrichment analysis and network pharmacology:** The investigation involved the analysis of pathway enrichment and functional enrichment of common targets through the “GO Pathway Enrichment Analysis” module of SR plot [27, 28]. This encompassed the exploration of Gene Ontology (GO) categories related to biological processes, molecular functions, and cellular components. Additionally, the construction of the scopoletin-target-disease network was accomplished using the Disgenet database [29] in conjunction with Cytoscape [30]. Using the Disgenet server, we initially compiled a list of cognitive impairment diseases associated with common targets. Subsequently, a Scopoletin-Target-disease network was constructed using Cytoscape, where nodes with high degree of connectivity were selectively identified.

**Preparation of targets for docking studies:** The Protein Data Bank (PDB) [31] was utilized to obtain the 3D structures of designated targets, while those lacking 3D structure in the PDB were modelled using the Swiss-model website. Subsequently, extraneous components such as water molecules and ligand groups were eliminated from the PDB structures of the targets using the BIOVIA Discovery Studio visualizer.

**Computational ligand-receptor interaction studies:** In our docking study, we employed Dockey software [32], utilizing scopoletin as the ligand. The PDB structure of scopoletin was systematically subjected to docking with each optimized protein structure through Autodock Vina in Dockey. This systematic methodology ensured a thorough examination of interactions between ligands and protein targets, offering insight into possible binding affinities and crucial molecular interactions. Such findings provide valuable groundwork for subsequent research. Finally, the 2D and 3D visualization of binding interactions between scopoletin(ligand) and the target protein was done using LigPlot+ [33] and Dockey [32].

**MD simulation:** After the docking study, the top 3 complexes with highest binding affinity and ligand efficiency were subjected to 100 ns molecular dynamic simulation using Schrödinger’s Desmond software version 2023.2 [34]. This study employed molecular dynamics simulations to examine the interaction between a Scopoletin and the protein. The simulations were conducted under constant temperature (300 K) and pressure (1 bar) conditions, with the inclusion of a membrane to mimic physiologic settings for membrane proteins. Molecular interactions were modelled using the OPLS_2005 force field, and electrostatic charges were computed using the Ewald method. Trajectories were sampled at 4.0 picosecond intervals to capture detailed information. The objective of this research was to analyse the behavior of both the Scopoletin and the specific protein. For the assessment of the simulation event and validation of the Molecular Dynamics (MD) simulation’s accuracy, Simulation Interaction Diagram (SID) was utilized. To check the stability of the complex, the Root Mean Square Deviation (RMSD) was employed, examining structural variations from a reference state and offering insights into atomic fluctuations within the complex. Additionally, the binding interaction between scopoletin and the specific protein was investigated through protein-ligand contacts (P-L).

**In-vivo study**

**Zebrafish embryo toxicity of EA:** The evaluation of the toxicity of EA extract on zebrafish embryos and larvae was conducted using a 12-well plate setup, following the procedures outlined in [OECD. Test No. 236, 2006]. Healthy zebrafish embryos at 4 hours post-fertilization (4 hpf) were carefully transferred to new polystyrene petri plates and observed using the EVOS FL Auto Imaging microscope. Subsequently, three independent triplicate experiments were performed, each involving five fertilized eggs (n=7) at 4 hpf for each treatment concentration.

2 mL of eight different concentrations of the EA extract (ranging from 1 to 600 µg/ml) prepared in embryo media were added per well in a 12-well plate. The control group had 2 mL of plain embryo media without any treatment. Various developmental endpoints, such as different developmental abnormalities, egg coagulation, mortality, tail development, larvae...
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Table 1. Preliminary phytochemical screening of *Evolvulus alsinoides*

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Phytochemical compound</th>
<th>Test Name</th>
<th>Aqueous extract of EA</th>
<th>Methanolic extract of EA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloids</td>
<td>Mayer’s Test</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Saponins</td>
<td>Foam Test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Tannins</td>
<td>Ferric chloride Test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Glycosides</td>
<td>Fehling’s Test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Proteins</td>
<td>Biurette Test</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Coumarins</td>
<td>NaOH Test</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

heart rate, delay in hatching, and edema in the yolk sac, were assessed over a five-day period. Following this assessment, observations and records were documented using the EVOS FL Auto microscope.

**Evaluations of zebrafish embryo hatch rate:** Assessments of the hatch rate of zebrafish embryos were conducted over a five-day period at various concentrations of EA (1-600 μg/ml) extracts. The hatching process, defined as the chorion rupture leading to the release of larvae, was observed using the EVOS FL microscope.

**Evaluations of zebrafish larvae heart beats:** The pulsation of larvae hearts was investigated after five days of exposure to EA extract (1-600 μg/ml). The counting of heartbeats involved direct visual observation of the zebrafish larval cardiac ventricles using an EVOS microscope connected to a computer and camera device. The heart rate was measured per minute using a stopwatch.

**Antioxidant assay**

The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay is a widely used method to evaluate antioxidant activity of compounds or extracts [35]. It involves dissolving DPPH in methanol, diluting the sample in DMSO, and observing the absorbance at 520 nm using a Bioteck Epoch Elisa Reader after 30 minutes. A ferric reducing ability of plasma (FRAP) working reagent was prepared by mixing acetate buffer, TPTZ, and FeCl3, with DPPH as a sample dilution. Samples and FRAP were incubated at 37°C for 30 minutes [36], and observed at 593 nm.

**In-silico analysis**

Selection of targets associated with cognitive impairment: We conducted an extensive analysis using four distinct target prediction tools to identify potential targets for scopoletin. The retrieved list, consisting of 330 candidate genes, was then compared with a set of 659 genes associated with cognitive impairment sourced from the Human Phenotype Ontology database. Employing Venn diagram analysis through Venny 2.0, we identified 12 common target genes (Table 3) shared between scopoletin and cognitive impairment. These genes were deemed as key targets, underscoring their significance in the context of cognitive...
Enrichment analysis and building a scopoletin-target-disease network: GO analysis and KEGG pathway enrichment was carried out to explore multiple functions of 12 common targets. In GO analysis, 12 potential targets of scopoletin were significantly found enriched in 592 biological processes (BP), 28 cellular components (CC) and 64 molecular functions (MF). Among them the top 3 BP terms are positive regulation of glucose transmembrane transport (GO: 0010828), regulation of glucose transmembrane transport (GO: 0010827), positive regulation of cell cycle process (GO: 0090068). Top 3 CC terms are dendritic spine (GO: 0043197), neuron spine (GO: 0044309), protein-DNA complex (GO: 0032993). Top 3 MF terms are protein serine/threonine kinase activity (GO: 0004674), cytokine receptor activity (GO: 0004896), cytokine binding (GO: 0019955). By employing pathway enrichment analysis, we found 57 pathways that were significantly enriched. Among them the top 3 were FoxO signalling pathway, Hepatitis B, and Kaposi sarcoma-associated herpesvirus infection. The Figure 4 illustrates the top 10 functional categories in Biologic Process (BP), Cellular Component (CC), and Molecular Function (MF) based on Gene Ontology (GO). Additionally, 10 significant pathways have been selected and are visually represented.

The result, as depicted in Figure 5, illustrates significant cognitive impairment diseases associated with the 12 common targets of scopoletin. This network serves as a graphic representation elucidating the intricate interactions between compounds and their respective target proteins. This is instrumental for identifying potential drug targets, providing valuable insight for researchers to strategically select molecules with therapeutic promise tailored to specific diseases. The visualization and analysis of intricate relationships within this network facilitate a comprehensive understanding of the complex landscape associated with cognitive impairment.

Docking study: Utilizing Scopoletin as a ligand, we conducted Molecular Docking Analysis targeting 12 common target proteins (gene product). Employing Dockey software for this investigation, the results revealed noteworthy binding affinities and ligand efficiency between Scopoletin and ERAP1, SCN3A, and COMT as depicted in Table 4. The outcomes suggest a robust interaction between Scopoletin and these specific targets, underscoring its pharmacologic relevance. Moreover, as depicted in Figure 6, Scopoletin establishes hydrogen bonds with amino acid residues Glutamic acid (Glu679), asparagine (Asn641), phenylalanine (Phe644), serine (Ser648), and tryptophan (Trp921) within the ERAP1 protein. In contrast, Scopoletin forms only two hydrogen bonds with SCN3A protein, specifically at amino acid residues isoleucine (Ile408) and glutamine (Gln382). Additionally, Scopoletin interacts with COMT protein through hydrogen bonds at three amino acid residues: asparagine (Asn220), histidine (His192), and aspartic acid (Asp200).
Neuroprotective properties of scopoletin-rich extract

Figure 3. A. Chromatograph of scopoletin standard. B. Chromatograph of scopoletin from *Evolvulus alsinoides* sample at Rf of 0.53.
Neuroprotective properties of scopoletin-rich extract

Molecular dynamic simulation: The results obtained from molecular docking study were validated through 100 ns molecular dynamics simulations. Initially, the root mean standard deviation (RMSD) of both the protein and ligand within individual complexes was computed by comparing their positions with the initial state. In Figure 7A, ERAP1-Scopoletin complex showed a shift in RMSD < 3Å after 80 ns. The stabilization of the SCN3A-Scopoletin complex occurs after 40 nanoseconds, while the ligand undergoes conformational changes twice, first around 30 nanoseconds and then again after 40 nanoseconds, before reaching stabilization (Figure 7B). Initially, the COMT-Scopoletin complex showed a large conformational change, but after 50 ns the complex seemed to get stabilized (Figure 7C). As per Desmond’s documentation, fluctuation in RMSD of Protein-Ligand complex is less than 3Å and under acceptable range, hence, can be called stable. Here in our study, the fluctuation in RMSD values of the 3 protein-Scopoletin complexes were within an appropriate range, that is less than 3Å suggesting a better docking position and no disruption of the protein-ligand structure.

In-vivo toxicity study

To broaden the dataset derived from in-vitro and in-silico investigations, an extensive vertebrate model was assessed in-vivo. 4 hpf Zebrafish larvae were exposed to increasing concentrations of the extract; at concentrations ranging from 1 µg/ml to 600 µg/ml till 120 hpf. By 48 hpf, all embryos had hatched in concentrations ranging from 1 µg/ml to 450 µg/ml. However, at 500 µg/ml and 600 µg/ml, delayed hatching was observed (Figure 8B). At 500 µg/ml, unhatched embryos with bent trunks were observed, while at 600 µg/ml, darkened, unhatched embryos were noted. By 72 hpf, the embryos that persisted in the chorion at a concentration of 600 µg did not develop to the same stage as the control group and found to have morphological abnormalities, including malformed tails, yolk sac edema, and slowed growth, were reported, affecting survival at the 500 & 600 µg/ml concentration (Figure 8A, 8C). The survival rate demonstrated

Table 2. Calculated percentage composition of Scopoletin in sample obtained from Heartyculture Nursery, Hyderabad, Telangana, India

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Standard Scopoletin</th>
<th>EA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>1.3 mg</td>
<td>1254</td>
</tr>
<tr>
<td>Area</td>
<td>34617.9</td>
<td>1940.3</td>
</tr>
<tr>
<td>% Scopoletin</td>
<td>--</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

Table 3. 12 common genes between potential targets of scopoletin and genes associated with cognitive impairment

<table>
<thead>
<tr>
<th>Common Target</th>
<th>Full Protein Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>TERT</td>
<td>Telomerase reverse transcriptase</td>
</tr>
<tr>
<td>EP300</td>
<td>Histone acetyltransferase p300</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol O-methyltransferase</td>
</tr>
<tr>
<td>SCN3A</td>
<td>Sodium channel protein Type 3 subunit alpha</td>
</tr>
<tr>
<td>IL23R</td>
<td>Interleukin-23 receptor</td>
</tr>
<tr>
<td>CCR1</td>
<td>C-C chemokine receptor type 1</td>
</tr>
<tr>
<td>TBK1</td>
<td>Serine/threonine-protein kinase TBK1</td>
</tr>
<tr>
<td>AKT1</td>
<td>RAC-alpha serine/threonine-protein kinase</td>
</tr>
<tr>
<td>BRAF</td>
<td>Serine/threonine-protein kinase B-raf</td>
</tr>
<tr>
<td>PLK4</td>
<td>Serine/threonine-protein kinase PLK4</td>
</tr>
<tr>
<td>ERAP1</td>
<td>Endoplasmic reticulum aminopeptidase 1</td>
</tr>
<tr>
<td>KCND3</td>
<td>Potassium Voltage-gated Channel Subfamily D member 3</td>
</tr>
</tbody>
</table>

The protein-ligand can be seen throughout the 100 ns simulation. Various specific and nonspecific protein-ligand interactions can be tracked throughout the simulation, these interactions can be classified into 4 main types: hydrogen bonds, hydrophobic, ionic, and water bridges. These interaction types further include specific subtypes, which can be investigated using the ‘Simulation Interactions Diagram’ panel of Desmond. Interestingly, throughout 100% of simulation time, scopoletin maintained interaction with glutamic acid (Glu679) amino acid residue of ERAP1 predominantly by hydrogen bonds (Figure 7A). Additionally, Scopoletin exhibited interactions with the Arginine (Arg1624) amino acid residue of SCN3A for more than 30% of the simulation time (Figure 7B), and it consistently interacted with the Aspartic acid (Asp191) amino acid residue of COMT for the entire (100%) of simulation period (Figure 7C).
Neuroprotective properties of scopoletin-rich extract

A. Pathway Analysis

- FoxO signalling pathway
- Hepatitis B
- Kaposi sarcoma-associated herpesvirus infection
- Renal cell carcinoma
- Prostate cancer
- Human papillomavirus infection
- Gastric cancer
- Hepatitis C
- JAK-STAT signalling pathway
- Hepatocellular carcinoma

Enrichment Score (-log10(p-value))

B. Molecular Function

- Protein serine/threonine kinase activity
- Cytokine receptor activity
- Immune receptor activity
- Cytokine binding
- Growth factor receptor binding
- Protein phosphatase binding
- Protein C-terminus binding
- Phosphatase binding
- Voltage-gated ion channel activity
- Voltage-gated channel activity

Enrichment Score (-log10(p-value))

C. Biological Process

- Positive regulation of glucose transmembrane transport
- Regulation of glucose transmembrane transport
- Positive regulation of cell cycle process
- Positive regulation of peptidyl-serine phosphorylation
- Glucose transmembrane transport
- Hexose transmembrane transport
- Monosaccharide transmembrane transport
- Carbohydrate transmembrane transport
- Positive regulation of cell cycle
- Positive regulation of nitric-oxide synthase activity

Enrichment Score (-log10(p-value))

D. Cellular Component

- Dendritic spine
- Neuron spine
- Protein-DNA complex
- Cation channel complex
- XY body
- Telomere cap complex
- Nuclear telomere cap complex
- Chromosome, telomeric repeat region
- Voltage-gated sodium channel complex
- Postsynaptic membrane

Enrichment Score (-log10(p-value))
Neuroprotective properties of scopoletin-rich extract

Figure 4. Top 10 significant results. (A) KEGG pathway, (B) Molecular functions, (C) Biological process and (D) Cellular components associated with scopoletin targets.

Antioxidant assay

The percentage of scavenging was graphed against concentrations (Table 5), and the EC\textsubscript{50} was estimated using exponential regression (Figure 10) and found to be 440.0 μg/ml for the DPPH assay and 84.29 μg/ml for the FRAP assay.

<table>
<thead>
<tr>
<th>Target</th>
<th>Ligand</th>
<th>Affinity</th>
<th>Ligand Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERAP1</td>
<td>Scopoletin</td>
<td>-6.298</td>
<td>0.45</td>
</tr>
<tr>
<td>SCN3A</td>
<td>Scopoletin</td>
<td>-5.886</td>
<td>0.42</td>
</tr>
<tr>
<td>COMT</td>
<td>Scopoletin</td>
<td>-5.877</td>
<td>0.42</td>
</tr>
<tr>
<td>KCND3</td>
<td>Scopoletin</td>
<td>-5.861</td>
<td>0.419</td>
</tr>
<tr>
<td>AKT1</td>
<td>Scopoletin</td>
<td>-5.66</td>
<td>0.404</td>
</tr>
<tr>
<td>TBK1</td>
<td>Scopoletin</td>
<td>-5.614</td>
<td>0.401</td>
</tr>
<tr>
<td>CCR1</td>
<td>Scopoletin</td>
<td>-5.217</td>
<td>0.373</td>
</tr>
<tr>
<td>TERT</td>
<td>Scopoletin</td>
<td>-5.118</td>
<td>0.366</td>
</tr>
<tr>
<td>BRAF</td>
<td>Scopoletin</td>
<td>-4.574</td>
<td>0.327</td>
</tr>
</tbody>
</table>

a clear correlation between increasing EA extract concentration and toxicity. Figure 9 shows morphologic characteristics as indicators of the detrimental effects of EA extract on zebrafish embryos and larvae.

Antioxidant assay

The percentage of scavenging was graphed against concentrations (Table 5), and the EC\textsubscript{50} was estimated using exponential regression (Figure 10) and found to be 440.0 μg/ml for the DPPH assay and 84.29 μg/ml for the FRAP assay.
Cognitive impairment and disorders pose significant challenges, given their irreversible nature even after recovery. These conditions, arising from factors spanning genetics to psychological and physical stressors, present a great obstacle to full recovery [38]. Com-

Discussion

Cognitive impairment and disorders pose significant challenges, given their irreversible nature even after recovery. These conditions, arising from factors spanning genetics to psychological and physical stressors, present a great obstacle to full recovery [38]. Com-

Figure 6. The 2D and 3D binding interactions of top 3 targets, namely (A) ERAP1, (B) SCN3A and (C) COMT with scopoletin. In 2D binding interactions, the amino acid residues of targets which are green in color forms hydrogen bond with scopoletin, while those displaying a black coloration engage in hydrophobic interactions with scopoletin.
Neuroprotective properties of scopoletin-rich extract

Figure 7. The root mean square deviation (RSMD) and protein-ligand contacts histogram of (A) ERAP1-scopoletin complex, (B) SCN3A-scopoletin complex, (C) COMT-scopoletin complex. The scopoletin seems to have a more stable interaction with ERAP1 compared to SCN3A or COMT.
Figure 8. On exposure to increasing quantities of EA extract, zebrafish larvae at 4 hpf did not exhibit any discernible phenotypic alterations until 24 hpf. Morphologic defects including (A) Lower heart rate, malformed tails, edema in yolk sac, slow growth rate and (B) Hatching was reported which affected the (C) Survival at 500 and 600 μg/ml at 48 hpf. The survival rate demonstrated a relationship between increasing concentration and EA extract toxicity.
Neuroprotective properties of scopoletin-rich extract

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Table 5. % Scavenging of free radicals by EA extracts at different concentration

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Subsequently, to elucidate complex interactions of 12 common targets of scopoletin, unravel molecular pathways, investigate ligand-receptor interactions at a molecular level, and gain insights into the stability and behavior of the complex over time, we employed network pharmacology, docking studies, and simulation techniques. The network analysis of scopoletin-target-disease relationships revealed close associations between targets and Alzheimer’s disease, neurodegenerative disorders, as well as presenile disorders (Figure 5). This ‘compound-target-disease’ network serves as a tool to elucidate scopoletin’s potential mechanisms of action against specific diseases associated with cognitive impairment. It is based on the assumption that scopoletin can modulate multiple targets and pathways associated with a given disease, this network representation utilizes nodes and edges to capture these interactions. Through the identification of hub targets and significant diseases related to cognitive impairment, the scopoletin-target-disease network facilitates an understanding of Scopoletin’s therapeutic effects. Moreover, it reveals potential connections suggesting the efficacy of existing drugs in treating diverse diseases, aligning with the concept of drug repurposing. Researchers can leverage this network to strategically prioritize research efforts by focusing on pivotal nodes, such as highly connected targets or compounds, thereby guiding the allocation of resources and research initiatives.

The pathway enrichment analysis reveals that the 12 common targets of Scopoletin are associated with pathways such as the FoxO signaling pathway and JAK-STAT signaling pathway. The FoxO signaling pathway comprises a family of four transcription factors in mammals - namely FOXO1, FOXO3a, FOXO4, and FOXO6 [41]. Notably, FoxO proteins play a pivotal role in regulating the balance between autophagy and apoptosis, impacting neuronal survival and degeneration. In specific contexts, such as autophagy, FoxOs exhibit protective effects by mitigating toxic protein accumulation and Aβ toxicity [42]. The other pathway, namely JAK-STAT signaling pathway, is involved in regulating inflammation, immunity, and various functions in the central nervous system. Research suggests that targeting the JAK/STAT signal pathway may be beneficial for preventing and treating cognitive impairments like Alzheimer’s disease [43]. This underscores the significance of these pathways in the nervous system, implying that Scopoletin may play a substantial role in neuronal growth, survival, and functionality.

The gene ontology analysis shows that the highly enriched molecular function (MF) term was protein serine/threonine kinases activity in which 4 targets of scopoletin namely TBK1, AKT1, BRAF, PLK4 are involved. Serine/threonine kinases facilitate the phosphorylation of intracellular protein targets by transferring a phosphorus group from an adenosine triphosphate molecule to the specific amino acid residues within the target proteins [44]. TBK1 is linked to neurodegenerative diseases like amyotrophic lateral sclerosis and frontotemporal dementia which are associated with cognitive impairment [45, 46]. TBK1 inhibits the activation of RIPK1, a kinase involved in cell death and inflammation in the nervous system [45]. AKT1, also known as protein kinase B, influences cellular survival, growth, and transcriptional regulation, and its deficiency may contribute to abnormal prefrontal cortical structure and function relevant to cognitive disturbances in schizophrenia [47]. BRAF alterations have diagnostic and therapeutic implications in central and peripheral nervous system tumors [48], while Plk4 regulates synaptic function in differentiated neurons [49]. Understanding the involvement of serine/threonine kinases in cognitive function provides insight for developing targeted interventions against cognitive disorders, underscoring scopoletin’s importance for regulating essential aspects of neuro-
Neuroprotective properties of scopoletin-rich extract

Neurodevelopment and function. This suggests the possibility that scopoletin could intervene in the progression of cognitive impairment disorders by modulating these molecular functions.

The molecular docking study revealed that scopoletin showed a significant binding affinity with ERAP1, SCN3A and COMT. ERAP1, a pivotal protein plays a crucial role in activating the Hedgehog signaling pathway. This pathway is implicated in various human cancers, including medulloblastoma-recognized as a primary malignant embryonal tumor of the central nervous system, they manifest primarily in the cerebellum or fourth ventricle, presenting symptoms indicative of heightened intracranial pressure. ERAP1's regulatory function within the Hh pathway involves the modulation of βTrCP, a protein governing the ubiquitylation and proteasomal degradation of the Hh effector Gli1. Consequently, inhibiting ERAP1 emerges as a promising therapeutic approach to disrupt the Hh pathway, offering an innovative strategy for treating Hh-dependent cancers, such as medulloblastoma [50, 51].

SCN3A encodes a sodium channel protein that is important for the development and function of neurons. Pathogenic variants in SCN3A can alter the channel's properties and lead to abnormal brain activity and structure that can give rise to a SCN3A-related neurodevelopmental disorder that can cause different types of severity symptoms such as developmental delay, intellectual disability, seizures, hypotonia, and autonomic dysfunction [52].

COMT encodes an enzyme, catechol-O-methyltransferase which is responsible for the degradation of neurotransmitters including dopamine, predominantly within the hippocampus and prefrontal cortex of the brain. Its pivotal role extends to the regulation of synaptic dopamine levels, neuronal differentiation, as well as the modulation of working memory and executive function. Variations and alterations in COMT, whether in terms of genetic variants or copy numbers, have been linked to various neuropsychiatric disorders, notably including schizophrenia, learning disabilities, and attention deficit hyperactivity disorder (ADHD) [53]. Furthermore, the results of the molecular dynamic simulation indicate that the mentioned complexes namely ERAP1-scopoletin, SCN3A-Scopoletin and COMT-Scopoletin remain stable and hence scopoletin shows significant binding affinity with ERAP1, SCN3A and COMT. This hence shows that the targets of scopoletin are involved in brain related disorders which suggests that scopoletin might play a crucial role in lessening the effects of cognitive impairment.

The herbal extract exhibited potent antioxidant activity in the DPPH and FRAP assays, likely due to the presence of tannins and coumarins. Antioxidants are known to play a role in neuroprotection, and their deficiency can lead to oxidative stress, a key factor in cognitive decline [6, 9]. Plant extracts rich in antioxidants have been shown to improve cognition by inhibiting processes involved in neurodegenerative diseases and regulating the cholinergic system. The antioxidant activity of the herbal extract could contribute to cognitive improvement by protecting neuronal cells from oxidative stress and modulating pathways involved in cognitive function [40]. However, further research is needed to confirm these effects and elucidate the mechanisms involved. While the studies suggest a correlation between antioxidant activity and cognitive improvement, a direct cause-effect relationship has not been established, as the effects of antioxidants on cognition can be influenced by various factors.

Finally, our study included an assessment of the toxicity of scopoletin in zebrafish embryos as an in-vivo vertebrate model, contributing to a comprehensive understanding of the compound's effects. 4 hpf Zebrafish larvae were exposed to increasing concentrations of the extract; at concentrations ranging from 1 µg/ml to 450 µg/ml, the larvae did not exhibit any phenotypic abnormalities till 120 hpf. We investigated morphologic and teratogenic abnormalities as potential markers of the extract's embryotoxic potential on zebrafish at different time points. Morphological abnormalities such as lower heart rate, delayed hatching, and edema were found to affect survival post-72 hpf and 48 hpf at doses of 500 µg/ml and 600 µg/ml, respectively. The embryos that persisted in the chorion at a concentration of 600 µg did not develop to the same stage as the control group. The survival rate demonstrated a clear correlation between growing extract concentration and toxicity.
The study highlights scopoletin in Evolvulus alsinoides extract as a promising natural compound for addressing cognitive impairment. Scopoletin demonstrates affinity for proteins associated with cognitive decline, suggesting therapeutic potential for neurodegenerative disorders. However, toxicity assessment reveals adverse effects at concentrations ≥ 450 µg/ml, emphasizing the importance of safety considerations. Further research is needed to explore mechanisms at lower concentrations. Our findings underscore the multitargeted pharmacologic actions of Scopoletin and its relevance for neurologic disorders, calling for validation in clinical settings to address the pressing need for effective treatment.

Acknowledgements

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Disclosure of conflict of interest

None.

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