

Comparative metabolomics and network pharmacology analysis reveals shared neuroprotective mechanisms of *Bacopa monnieri* (L.) Wettst and *Centella asiatica* (L.) Urb

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Abstract

Bacopa monnieri (L.) Wettst and *Centella asiatica* (L.) Urb., two nootropics are recognized in Indian Ayurvedic texts. Studies have attempted to understand their action as memory enhancers and neuroprotectants, but many molecular aspects remain unknown. We propose that *Bacopa monnieri* (L.) Wettst and *Centella asiatica* (L.) Urb. share common neuroprotective mechanisms. Mass spectrometry-based untargeted metabolomics and network pharmacology approach was used to identify potential protein targets for the metabolites from each extract. Phytochemical analyses and cell culture validation studies were also used to assess apoptosis and ROS activity using aqueous extracts prepared from both herbal powders. Further, docking studies were also performed using the LibDock protocol. Untargeted metabolomics and network pharmacology approach unveiled 3,362 and 2,927 non-redundant metabolites from both extracts, with 2,750 shared metabolites between them, suggesting a potential common neuroprotective mechanism among these drugs. Protein-target prediction highlighted 92.4% similarity among the proteins interacting with metabolites for these extracts. Among them, kinases mapped to MAPK, mTOR, and PI3K-AKT signaling pathways represented a predominant population. Our results highlight a significant similarity in the metabolome of *Bacopa monnieri* (L.) Wettst and *Centella asiatica* (L.) Urb. and their potential protein targets, which may be attributed to their common neuroprotective functions.

1. Introduction

Plant omics is a developing area of systems biology that presents excellent opportunities for new knowledge and clinically relevant translational study. Plant-based formulations have received increased attention for treating several disorders, with accumulating evidence ranging from cell lines to animal models. *Bacopa monnieri* (L.) Wettst (*B. monnieri*), which more commonly refers to *Brahmi* or *Jalabrahmi*, and *Centella asiatica* (L.) Urb. (*C. asiatica*), commonly known as *Mandookaparni*, are two well-known nootropic drugs mentioned in different Ayurvedic texts [1, 2]. Both *B. monnieri* and *C. asiatica* are used as nootropic drugs in traditional Indian medicinal systems. According to a Sanskrit verse mentioned in the *Bhavaprakasha Nighantu*, *Brahmi* is referred to for both *B. monnieri* and *C. asiatica*. However, *B. monnieri* and *C. asiatica* vary taxonomically and in morphological characteristics [3]. *B. monnieri*, or water hyssop, is a perennial creeping plant belonging to the Scrophulariaceae family. *C. asiatica*, also known as Asiatic/ Indian pennywort, is a frost-tender perennial plant that belongs to the Apiaceae family.

Several studies have demonstrated the neuroprotective efficacy of both *B. monnieri* and *C. asiatica* using *in vitro* and *in vivo* models [4–6]. Studies have demonstrated that *B. monnieri* extracts exhibit antioxidant, anti-apoptotic, and anti-inflammatory properties, protect against DNA damage, and enhance mitochondrial functions. [7, 8]. *C. asiatica* extracts, purified Asiatic acid, or its derivative, asiaticoside, have exhibited anti-oxidative and anti-inflammatory profiles and improved mitochondrial dysfunction [9, 10]. Furthermore, *B. monnieri* and *C. asiatica* extracts have been proven to improve learning, memory, behavior, and cognition [11, 10, 12–14]. We recently demonstrated that *B. monnieri* and *C. asiatica*

extracts showed neuroprotective activity in the *Drosophila* model of Alzheimer's disease [15]. Based on this literature, it may be possible that *B. monnieri* and *C. asiatica* may bring neuroprotection via similar mechanisms of action; however, they belong to distant botanical classes.

The metabolites are small molecule entities resulting from intrinsic biochemical pathways and are the end-point players in conferring the various protective effects of *B. monnieri* and *C. asiatica*. Metabolites not only serve as intermediates of metabolism, but they also interact with proteins and modulate biological functions. With the advent of mass spectrometry-based metabolomics, knowledge about metabolite composition is on a steady rise, as it enables better coverage of many unreported metabolites. Several plant-based compounds are approved drugs for regulating proteins, and a steady increase in investigations on small molecules for targeting proteins. In addition, the search for nootropics and neuroprotective compounds is on the rise [16]. Small molecules impart their action by regulating protein-protein dynamics in a host system.

Combining the power of mass spectrometry-based metabolomics and network pharmacology for nootropics classification and identification of target proteins will be invaluable for establishing the efficacy of neuroprotective formulations or purified compounds. Owing to the common neuroprotective functions of *B. monnieri* and *C. asiatica*, we have employed untargeted metabolomics and network pharmacology approaches to compare the metabolite signature and identify the potential protein targets involved in neuroprotection.

We present here a metabolomics and network pharmacology strategy to elucidate the metabolome-level molecular correlates of *B. monnieri* and *C. asiatica*, which lays the path for additional translational clinical research on the neuropharmacology of *B. monnieri* and *C. asiatica*.

2. Materials and Methods

2.1. Procurement of *B. monnieri* and *C. asiatica* powders

B. monnieri (Lot No. 113) and *C. asiatica* (Lot No. 208) processed powders were obtained from SDP Remedies and Research Centre, Puttur, India, a GMP-certified Ayurveda manufacturer. Briefly, the whole plants of *B. monnieri* and *C. asiatica* were collected and authenticated by Dr. Harikrishna Panaje, SDP Remedies and Research Centre. The authentication was done by macroscopic and microscopic methods. A specimen of the same is maintained at the center (*B. monnieri* ID: SDP/BM/001-2019 and *C. asiatica* ID: SDP/CA/001-2019). Briefly, the whole plant of *B. monnieri* and *C. asiatica* were taken, dried in a shade net and vacuum drum, powdered, and pulverized to yield the final fine powder with a yield of 90% was used in the study for further experiments.

2.2. Qualitative chemical analysis of *B. monnieri* and *C. asiatica* extracts

One gram of *B. monnieri* and *C. asiatica* powder was suspended in 10 ml of milli-Q water (concentration, 0.1 g/mL) and incubated overnight at room temperature with continuous rotation. Then, it was centrifuged at 5000g for 10 min. The supernatant was collected in a new tube. The extracts in the respective tubes were dried using SpeedVac (Savant, Thermo Fisher Scientific, USA) and stored at – 20°C until further use. As previously reported, quantitative chemical tests were used for the phytochemical screening of *B. monnieri* and *C. asiatica* extracts [17].

2.2.1. *In-vitro* antioxidant activity

(i) Total antioxidant capacity (TAC)

Phosphomolybdenum assay was used to estimate TAC [18]. Aqueous extracts of *B. monnieri* and *C. asiatica* were used at varying concentrations ranging from 25 to 800 µg/mL. The extract was taken in test tubes and made 1ml using distilled water. Next, 1 ml of Phosphomolybdenum reagent was added and incubated at 95 °C in a water bath for 90 min. At 695 nm, the absorbance was measured against a blank after the contents were cooled to room temperature. The same procedure was used to create a standard graph using L-ascorbic acid. The test was done in triplicate. TAC was expressed in µg of ascorbic acid equivalents/g of extract.

(ii) 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

We have used Brand-Williams [19] method to assess free radical scavenging activity with slight modifications. Different concentrations of the test samples (50, 100, 200, 400, 600, and 800 µg/ml) were made up to 100 µl using ethanol, and to that, 100 µl of a 0.1 mM DPPH solution was added. After 30 minutes of incubation in the dark, the decrease in absorbance at 517 nm was recorded. The test was run in duplicates. The following formula was used to get the inhibition percentage:

$$\text{Inhibition\%} = (\text{Ac} - \text{As}) / \text{Ac} \times 100$$

Where Ac = Absorbance of the control, As = Absorbance of the test

(iii) Reducing power assay:

Reducing power assay was done as previously described [20]. This test is based on the principle that substances in the extract with reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form a ferric–ferrous complex. Here, the antioxidant in the sample reduces the Fe^{3+} /ferricyanide complex to the ferrous form. In the present study, different concentrations of the extract (50, 100, 200, 400, 600, and 800 µg/ml) are taken and made up to 100 µl using distilled water. We have added phosphate buffer (0.2 M, pH 6.6) and Potassium ferricyanide (1%). For 20 minutes, the mixture was incubated at 50°C in a dry bath. After adding 10% TCA, the mixture was centrifuged at 3000 rpm for 10 minutes. The supernatant was mixed with distilled water and 0.1% FeCl_3 , and absorbance was measured at 700 nm against a blank. The same

procedure was used to create a standard graph using L-ascorbic acid. Results were expressed as mg equivalents of ascorbic acid per gram of the extract.

2.2.2 Quantitative analysis of secondary metabolites

(i) Total flavonoid content

The aluminum chloride method was used to assess the total Flavonoid content in the *B. monnieri* and *C. asiatica* aqueous extracts [21]. Different concentrations of extract were mixed with 10% aluminum chloride and 1 M potassium acetate solution. The absorbance was measured at 440 nm with the standard quercetin and a blank after 30 minutes of incubation at room temperature. The Flavonoid concentration was determined by comparing the results to a calibration curve made using quercetin as a reference.

(ii) Total phenolics content

The Bray and Thorpe method was used to determine the total Phenolic compound in the respective extracts [22]. First, different concentrations of the extract were taken and made up to 1ml using distilled water. Next, 20% Na₂CO₃ was added to the extract and thoroughly shaken. Finally, after two minutes, Folin's reagent was added with continuous shaking or vortex. The absorbance at 750 nm was measured after the color had been allowed to develop for 30 minutes. By comparing the total Phenolic content of the extract to a calibration curve made from the reference standard Gallic acid, the total Phenolic content was reported as mg/g extract.

2.3. Metabolite extraction

The metabolite extraction was carried out as mentioned previously [23, 12]. Briefly, 1 mL of the triple solvent mixture containing acetonitrile: methanol: water in the 2:2:1 ratio was used to extract metabolites from 50 mg of the *B. monnieri* and *C. asiatica* powders. First, the extraction mixture was vortexed at room temperature, followed by sonication in an ultrasonic water bath for 10 minutes, and centrifuged at 12,000 g for 15 minutes at 4°C. Then, the supernatant was carefully transferred to a new tube and dried using a SpeedVac concentrator. Finally, before LC-MS/MS data acquisition, the dried metabolite extract was re-suspended in 0.1% formic acid in MilliQ water.

2.4. Untargeted LC-MS/MS-based metabolomics

QTRAP-6500 mass spectrometer (AB SCIEX, USA), controlled with Analyst software (v 1.6.3) as the user interface, was used for metabolomics data acquisition. Sample resolution was carried out using Agilent Infinity II 1290 chromatography system (Agilent Technologies, INC Santa Clara, CA 95051, USA) with reverse-phase ZORBAX Eclipse plus C18, RRHD column (2.1x150 mm, 1.8 microns). Solvent-A, 0.1% formic acid, and Solvent-B, 0.1% formic acid in 90% acetonitrile, used for the following gradient; t = 0–1 min -B = 2%, t = 10 min -B = 30%, t = 11 min -B = 40%, t = 13–17 min -B = 95%, t = 21–30 min -B = 2%. A sample volume of 10 µL was loaded onto the column and resolved at a flow rate of 0.3 mL/min.

Information-dependent acquisition (IDA) built with enhanced mass spectra (EMS) and enhanced product ion (EPI) scan was used to monitor the top 5 most intense ions in the range of 50-1000 Da and was scanned at the rate of 10,000 Da/s. Electrospray ionization with a source probe temperature of 450°C and 4,500 V (or -4,500 V), curtain gas at 30 psi, ion source gas at 30 psi, and a declustering potential of 100 V (or -100 V) was used at the source. In addition, collisionally induced dissociation (CID) was used for the fragmentation of the precursor ions at a collision energy of 40 V (or -40 V) with a spread of ± 25 V and an entrance potential of 10 (or -10V). The data for both *B. monnieri* and *C. asiatica* were acquired in both positive and negative modes in triplicates.

2.5. Data processing and analysis

QTRAP-6500 raw data files were converted to .mzML format using MSConvertGUI, ProteoWizard [24]. MZmine 2.53 open source framework [25] was used for data processing. The processing parameters used for MZmine analysis are described as follows. First, baseline correction was performed with smoothing at 500 units and an asymmetric factor of 0.001. Then, mass detection was done using centroid mode with a noise-level cut-off at $1.0E5$. Chromatogram reconstruction was done with a minimal time span and a peak height of 0.2 mins and $1.0E4$, respectively, with an m/z tolerance of 0.05 Da. Chromatogram deconvolution was performed with local minima search and deisotoping of peaks with isotopic peak grouper algorithm. Alignment was done using the RANSAC algorithm with non-linear modeling and gap-filling with the peak finder algorithm. Metabolite identification at the MS1 level was made with PlantCyc as the back-end database using an in-house tool MS2compound (<https://github.com/beherasan/MS2Compound>) [26], with 0.05 Da m/z error and [M + H], [2M + H], [M + 2H], and [M-H], [M-2H], [2M-H] as the adducts for positive and negative modes respectively. Unassigned m/z after PlantCyc search was filtered and searched against the KEGG (<https://www.genome.jp/kegg/>) database. The metabolites were further subjected to MS/MS analysis using the MS2Compound tool. The output files of relative intensities in the form of “.mgf,” obtained from MZmine 2.35, were searched against this tool. The search parameters for the fragments were precursor tolerance of 0.05 Da and fragment tolerance of 0.5 Da.

2.6. Bioinformatics analysis

Metabolites annotated from PlantCyc and KEGG databases were used for pathway enrichment analysis using MBROLE (<http://csbg.cnb.csic.es/mbrole2/>) version 2.0 [27]. Enrichment for chemical classification of metabolites was performed using MetaboAnalyst 5.0 (<https://www.metaboanalyst.ca/>) [28]. The SMILES IDs of the metabolites were obtained using PubChem Identifier Exchange Service (www.pubchem.ncbi.nlm.nih.gov/idexchange/idexchange.cgi). Protein targets of the metabolites were identified using BindingDB (www.bindingdb.org), a protein-small molecule interaction database based on experimental evidence [29]. UniProt IDs of protein targets from BindingDB were converted to gene symbols using the DAVID gene ID conversion tool and were classified based on the source organism of the gene (<https://david.ncifcrf.gov/conversion.jsp>) tool [30]. Pathway enrichment was performed for the genes belonging to the species *Homo sapiens* using KEGG pathway annotations indexed in the STRING

database [31, 32]. Enrichment identifies several signaling pathways of neuronal regulation, neurotransmitter, and disease-related pathways.

2.7. Cell culture validation and treatment

Neuroblastoma cells IMR-32 (ATCC® CCL-127™) were selected for the cell culture experiments. They were procured from the National Centre for Cell Sciences, Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose and L-glutamine, with 10% fetal bovine serum (FBS) and 1X antibiotic/antimycotic solution, at 37° C with 5% CO₂. 3 x 10⁴ cells per well were seeded on collagen-coated 6-well plates and differentiated using 10 µM retinoic acid in DMEM with 2% FBS for seven days. The differentiated cells were subjected to the following treatment conditions for 48 hours - rotenone treatment (100 nM dissolved in DMSO), 100 µg/ml of *B. monnieri* or *C. asiatica* extracts, Aβ treatment (0.5 µM in DMSO), co-treatment of rotenone with *B. monnieri* or *C. asiatica* extract, and co-treatment of Aβ with *B. monnieri* or *C. asiatica* extracts. Untreated cells were used as control.

2.8. Cell cytotoxicity assay

Cell cytotoxicity of *B. monnieri* and *C. asiatica* extracts were assessed using MTT assay at varying concentrations as previously described (Karthikkeyan et al., 2021). Briefly, IMR-32 cells were seeded in a 96-well plate at 5000 cells/well density, and the cells were treated with different concentrations of *B. monnieri* and *C. asiatica* aqueous extracts (50, 100, 250, 500, 1000, 1500, 2000 µg/mL) for 48h. MTT dye was added, and the IMR-32 cells were incubated for 4h. The resulting formazan crystals were dissolved in ethanol/DMSO solution (50:50), read at 570 nm, and background subtraction at 650 nm. Cell cytotoxicity was calculated and represented in percentage with respect to untreated control cells.

2.8. Detection of cell apoptosis using acridine orange - ethidium bromide (AO-EB) staining in IMR32 Cells

IMR32 cells in the density of 1 x 10⁴ cells per well were seeded in 1X collagen-coated 6-well plates and were differentiated for seven days using 10 µM retinoic acid. In the Parkinson's disease model, the cells were treated with rotenone (100 nM) [33], rotenone (100 nM) + *B. monnieri* / *C. asiatica* extract (100 µg/ml), *B. monnieri* / *C. asiatica* extract alone (100 µg/ml). For the Alzheimer's disease model Aβ (0.5 µM) [34], Aβ (0.5 µM) + *B. monnieri* / *C. asiatica* extract (100 µg/ml), *B. monnieri* / *C. asiatica* extract alone (100 µg/ml) for 48 hours along with the untreated control cells. After treatment, the cells were stained using Acridine orange: Ethidium bromide (2 µg/ml, 1:1) dual stains [35] for 20 min at 37 °C in the dark. Then, the excess stain was removed, and the cells were overlaid with 1X PBS. ZOE™ Fluorescent Cell Imager (BioRad Laboratories, California, USA) was used to image the cells in red and green channels. The experiment is done in triplicate.

2.9. Determination of reactive oxygen species (ROS) by 2',7'-Dichlorofluorescein diacetate (DCFDA)

We carried out ROS assay as previously mentioned [36]. Briefly, IMR-32 cells in the density of 1×10^4 cells per well were seeded in a 6-well plate coated with 1X collagen, and the cells were differentiated using 10 μ M retinoic acid for seven days. After differentiation, the cells are treated with rotenone (100 nM), rotenone (100 nM) + *B. monnieri* / *C. asiatica* extract (100 μ g/ml), *B. monnieri* / *C. asiatica* extract alone (100 μ g/ml) for Parkinson's disease model, and cells are treated with A β (0.5 μ M), A β (0.5 μ M) + *B. monnieri* / *C. asiatica* extract (100 μ g/ml), *B. monnieri* / *C. asiatica* extract alone (100 μ g/ml) for Alzheimer's disease model for 48 hours along with the untreated control cells respectively. The assay is done in triplicate. After 48 hours of treatment, the cells were washed with 1X PBS and stained with DCFDA (25 mM) and HOECHST (5 μ g/mL) for 20 min in the dark. The cells were imaged in ZOE™ Fluorescent Cell Imager (BioRad Laboratories, California, USA) in green and blue channels. The images were analyzed using the ImageJ tool, NIH, USA [37]. The % ROS was calculated relative to the control.

2.10. Molecular docking studies

The molecular docking studies were performed using the LibDock protocol in Discovery Studio 2020 [38] against A2A adenosine receptor (A2AAR) and monoamine oxidase B (MAO-B) target proteins. The selection of these target proteins was further backed up based on previous literature [39–43] and identified metabolites. The X-ray crystallographic structure of target proteins was downloaded from Protein Data Bank (PDB) (A2AAR; PDB ID: 4EIY and MAO-B; PDB ID: 2V61) and further prepared by deleting heteroatoms and non-protein units. The structure of dual inhibitor 8-(3-chlorostyryl) caffeine (PubChem ID: 5353365) [44] and all phytocompounds identified in *B. monnieri* and *C. asiatica* extract were downloaded from the PubChem compound database and then subjected to ligand preparation before performing molecular docking. The target and ligand preparations were done using specific protocols in Discovery Studio 2020. The inhibitor interacting amino acid residues in target proteins were defined as the active sites before running the LibDock protocol. The LibDock score and the intermolecular interactions formed between the phytocompound (ligand) and the target protein were together considered for screening out the best ligand in the ligand library.

3. Results

3.1. Preliminary phytochemical screening for *B. monnieri* and *C. asiatica* extract extracts

3.1.1. In vitro antioxidant activity

(i) Total antioxidant activity

The total antioxidant capacity (TAC) assay was done based on the Phosphomolybdenum method [18], here the sample reduces Phosphate-Mo (VI) to Phosphate Mo (V) and forms a bluish-green colored phosphate/Mo (V) complex. The present study observed that BM aqueous extract showed higher TAC activity, 56.56 ± 1.55 , than CA aqueous extract, i.e., 45.68 ± 0.21 at 800 μ g/ml concentration (Fig. 1A). The

TAC was quantified as ascorbic acid equivalents. The TAC activity is directly proportional to the concentration of *B. monnieri* and *C. asiatica* extract.

(ii) 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

B. monnieri and *C. asiatica* aqueous extracts showed significant DPPH free radical scavenging activity. Here the DPPH nitrogen radical accepts hydrogen from an antioxidant and leads to the disappearance of the DPPH nitrogen radical in the test sample [45]. The change in color from purple to yellow shows the disappearance of the DPPH radical at the wavelength of 517 nm [19]. *B. monnieri* aqueous extract showed the highest free radical scavenging activity at 400 µg/ml concentration (Fig. 1B), i.e., 80.05 ± 0.05 , which remains constant. On the other hand, *C. asiatica* extract showed less free radical scavenging activity when compared to *B. monnieri* aqueous extract, i.e., 54.90 ± 2.34 at 800 µg/ml.

(iii) Reducing power assay:

This assay measures the sample's reductive ability, indicating its potential antioxidant activity. The reducing power of *B. monnieri* and *C. asiatica* aqueous extract increased with the sample concentration. Among the *B. monnieri* and *C. asiatica* aqueous extracts, *B. monnieri* aqueous extract showed the highest reducing power at 800 µg/ml concentration, i.e., 55.35 ± 0.39 , compared to the *C. asiatica*, i.e., 21.65 ± 0.52 . Ascorbic acid was taken as a standard for calculating reducing power. Figure 1C represents the reducing power of the *B. monnieri* and *C. asiatica* aqueous extract.

3.1.2. Quantitative analysis of secondary metabolites

(i) Phenolics

According to Kahkonen et al. [46], phenolic compounds directly contribute to the antioxidant property of plant extract. Therefore, we tested the phenolic content of both *B. monnieri* and *C. asiatica* aqueous extracts in the present study. Our results showed that as the sample concentration increases in µg/ml, the total Phenolic content (µg/ml) also increases. Figure 2A represents the total Phenolic content in the *B. monnieri* and *C. asiatica* aqueous extract. The total Phenolic content of *B. monnieri* and *C. asiatica* aqueous extract at 800 µg/ml is 107.18 ± 1.28 ($P < 0.0001$) and 46.03 ± 0.41 ($P < 0.0001$), respectively, and 1000 µg/ml is 130.64 ± 0.16 ($P < 0.0001$) and 55.39 ± 1.31 ($P < 0.0001$) respectively.

(ii) Flavonoids

Flavonoid is a class of secondary metabolites having a polyphenolic structure. It is associated with health-promoting effects such as antioxidant, anti-inflammatory, anti-mutagenic, and anti-cancer [47]. *B. monnieri* and *C. asiatica* aqueous extracts showed high flavonoid content in µg/ml. For example, the flavonoid content of *B. monnieri* and *C. asiatica* aqueous extract at 800 µg/ml were 4.44 ± 0.19 ($P < 0.0001$) and 7.55 ± 0.22 ($P < 0.0001$), respectively. At 1000 µg/ml concentration, the flavonoid content was 5.52 ± 0.09 ($P < 0.0001$) and 9.09 ± 0.03 ($P < 0.0001$), respectively. Figure 2B represents the total flavonoid content in the aqueous extract. Here, quercetin was taken as a reference standard.

3.2. Metabolite identification for *B. monnieri* and *C. asiatica* extracts

The small molecule metabolites are the players at the cellular level, conferring neuroprotection. We used the untargeted metabolomics approach to unravel and compare the molecular cues using PlantCyc and KEGG databases. The total ion chromatogram of *B. monnieri* and *C. asiatica* from positive and negative modes is given in **Supplementary Fig. 1**. Metabolite identification was performed at the precursor level for *B. monnieri* and *C. asiatica*. Analysis of *B. monnieri* revealed 2,614 and 2,849 metabolites in positive and negative modes, summing to 3,438 non-redundant metabolites. In *C. asiatica*, we identified 2,826 and 778 metabolites in positive and negative modes, respectively, representing 2,927 non-redundant metabolites. Finally, we compared the metabolite profiles across *B. monnieri* and *C. asiatica*, wherein 2,751 (76.1%) metabolites were shared. The mapped metabolite features with respective intensities and polarities from *B. monnieri* and *C. asiatica* are given in **Supplementary Tables 1–4**.

We also identified metabolites at MS/MS level using our in-house tool, MS2Compound [26]. As a result, we identified 1892 and 1794 metabolites from both the polarities for *B. monnieri* and *C. asiatica*, respectively. Among these, 767 and 669 metabolites were unique for *B. monnieri* and *C. asiatica*, respectively, with 1125 metabolites common between them. These metabolite names at MS/MS level are given in **Supplementary Table 10**.

The mapping of metabolites is purely based on the database used. Despite using two different databases, a large amount of data from *B. monnieri* and *C. asiatica* remained unassigned, as provided in **Supplementary Tables 5–8**. For example, at the MS1 level, 5741 and 4161 m/z remained unassigned for *B. monnieri* and *C. asiatica*, respectively.

3.3. Identification of previously reported metabolites from *B. monnieri* and *C. asiatica* extracts

We looked for the previously reported metabolites of *B. monnieri* and *C. asiatica* at precursor and fragment-level from the literature. In the case of *B. monnieri*, we could map phytochemicals such as sitosterol, betulinic acid, and L-aspartate at precursor and fragment levels and D-mannitol, stigmasterol, L-alanine/ α -alanine at MS1 level as reported by Jeyasri et al. [48]. For *C. asiatica*, we could map Asiatic acid at the precursor level as previously reported [49], α -humulene, β -Caryophyllene, and campesterol at the precursor and chavicol [50] at both precursor as well as fragment level (Table 1). The spectra of these metabolites are given in Fig. 3A-B. Identifying previously reported metabolites gives us confidence and validates our data and pipeline used for experiments.

Table 1

List of some previously reported metabolites represented as signature metabolites for *Bacopa monnieri* and *Centella asiatica*

Plant extract	Metabolite name	Molecular formula	Monoisotopic mass	MS1/ MS2/ MS1 & MS2 (both)
BM	Sitosterol	C29H50O	414.38	MS1 & MS2
	Betulinic acid	C30H48O3	455.35	MS1 & MS2
	L-aspartate	C4H5NO4	134.04	MS1 & MS2
	D-mannitol	C6H14O6	182.07	MS1
	Stigmasterol	C29H48O	412.37	MS1
	L-alanine	C3H7NO2	90.05	MS1
CA	Asiatic acid	C30H48O5	488.35	MS1
	Alpha-humulene	C15H24	204.18	MS1
	β -Caryophyllene	C15H24	203.17	MS1
	Campesterol	C28H48O	400.37	MS1
	Chavicol	C9H10O	134.07	MS1 & MS2

3.4. Metabolite chemical classification and pathway analysis

The metabolites identified from the *B. monnieri* and *C. asiatica* extracts were enriched for their chemical classes, and 94.8% of the classes were similar between *B. monnieri* and *C. asiatica* (Fig. 4A). As represented in Fig. 4A, the metabolites mainly comprised alkaloids, flavins and flavonoids, benzenoids, nucleic and organic acids, TCA cycle, phenols, quinones, and carbohydrates. The lipids identified belonged to the classes prenols, sterols, and sphingolipids. Benzoxazines, pyridine carboxylic acids, and glycerophosphates were exclusively present in *B. monnieri*, while pyranones and ketones were exclusively present in the *C. asiatica* extract listed in **Supplementary Table 11**. The enrichment for metabolic pathways resulted in 92.1% similarity. The common and highly enriched pathways between *B. monnieri* and *C. asiatica* extracts were salvage pathways for nucleotides and nucleosides, UTP and CTP dephosphorylation, organic nitrogen assimilation, and tetrahydrofolate biosynthesis, to name a few, given in **Supplementary Table 12**. Enriched pathways related to neuronal functions included choline biosynthesis, tryptophan metabolism, glutamate degradation, MAP kinase cascade, serotonin and melatonin biosynthesis, and dopamine metabolism. The metabolites found in *B. monnieri* and *C. asiatica* extracts shared a significant similarity, suggesting they might interact with related proteins. Therefore,

based on structural similarity, potential protein targets that bind to the *B. monnieri* and *C. asiatica* extract metabolites were retrieved from the BindingDB database.

3.5. Protein targets regulating neuronal functions

B. monnieri and *C. asiatica* are considered neuroprotectants in the traditional Indian medicinal system; we used the metabolomics data to map their protein targets to evaluate their potential interacting partners. Molecular interaction between the identified metabolites and proteins provided us with valuable information on the probable molecular targets of these nootropics. The BindingDB analysis identified 390 and 370 human protein targets for metabolites corresponding to *B. monnieri* and *C. asiatica*, respectively (**Supplementary Table 13**). 92.4% (365) of proteins were common between both metabolite extracts. Further, these proteins were subjected to functional enrichment analysis to find out the pathways regulated by them. Neuroactive ligand-receptor activation was the most enriched pathway for *B. monnieri* and *C. asiatica* protein targets. Other neuronal pathways such as Serotonergic, Cholinergic, and Dopaminergic synapse, Calcium signaling, PI3K-AKT, and MAPK pathway were also enriched (**Supplementary Tables 14 and 15**). The top 20 enriched pathways for *B. monnieri* and *C. asiatica*, respectively, are given in Fig. 4B-C.

3.6 Regulation of neurotransmitter-related signaling pathways

Regulation of neuronal functions

Protein target enrichment enabled us to narrow down important neuronal functions such as calcium signaling, neurotrophin signaling, metabolic pathways, and endocrine resistance. In addition, cellular functions such as cell cycle, autophagy, apoptosis, long-term potentiation, and long-term depression have been implicated in normal physiological functions and various neurodegenerative diseases (**Supplementary Tables 14 and 15**).

Signaling mechanisms related to neuronal activities

Several signaling mechanisms were enriched that may play an important role in the regulation of neuronal activities, such as AMP-activated protein kinase (AMPK), Vascular endothelial growth factor (VEGF), Ras-related protein 1 (RAP1), Mitogen-activated protein kinase (MAPK), RAS, FoxO, cGMP-PKG, Estrogen and ErbB signaling, and longevity regulation pathways (**Supplementary tables 14 and 15**).

Neurodegenerative disease-related pathways

According to an increasing number of studies, the development and control of neurodegenerative disorders are significantly influenced by changes in signaling pathways. Therefore, understanding these pathways might result in better disease management. Several enriched protein targets, including CHRM, KRAS, MAP2K1, GSK3B, AKT1, PRKACA, MAOB, ADORA2A, and SLC6A3, among many others in the present study, mainly belong to the most prevalent neurodegenerative diseases, such as Alzheimer's and

Parkinson's disease, where the cholinergic and dopaminergic neuronal activities are affected. The components of these pathways were identified as potential targets of *B. monnieri* and *C. asiatica* metabolites (Fig. 5A-B.). The present results indicate that *B. monnieri* and *C. asiatica* may have neuroprotective properties in these diseases as their metabolites may regulate cholinergic and dopaminergic synapses. We also identified many classes of neuroactive receptors and their respective ligands, detailed in Table 2.

Table 2
List of neuroactive ligands and receptors as hits of BM and CA

Class-A Rhodopsin-like Amine ligands		
List of neuroactive ligands and receptors as hits of <i>Bacopa monnieri</i> and <i>Centella asiatica</i>	Receptor	Proteins identified (Official Gene symbols)
Acetylcholine	Cholinergic receptors	CHRM5
Epinephrine and norepinephrine	Adrenergic receptors	ADRA1A
		ADRA2A
		ADRA2B
		ADRA2C
Dopamine	Dopamine receptor	DRD2
Histamine	Histamine receptor	HRH1
		HRH2
		HRH3
5-Hydroxytryptamine	Serotonin receptor	HTR1A
		HTR2A
		HTR2B
		HTR2C
Peptide ligands		
Opioids	Opioid receptor	OPRD1
Morphine		OPRK1
Endomorphine		OPRM1
Nucleotide ligands		
Adenosine	Adenosine receptor	ADORA3
Nucleotides	Purinergic receptor	P2RY1
		P2RY14
		P2RY4
		P2RY6
Glucocorticoids		

Class-A Rhodopsin-like Amine ligands		
List of neuroactive ligands and receptors as hits of <i>Bacopa monnieri</i> and <i>Centella asiatica</i>	Receptor	Proteins identified (Official Gene symbols)
Cortisol	Glucocorticoid receptor	NR3C1

3.7. Regulation of kinase signaling

External and internal stimuli elicit many signal transduction pathways involved in normal physiological functions of the nervous system. Several pieces of literature have pointed out the dysregulation of different kinases and their regulation in neurodegenerative diseases or neuroprotection. We came across different kinases implicated in neuronal functions whose regulations confer neuroprotection. It is well-known that signaling pathways do not act alone and often result in cross-talks. In the current study, we have mapped some of the pathways, such as MAPK, PI3K-AKT, RAP1, RAS, mTOR, and AMPK, associated with neuronal regulation, which are targets of *B. monnieri* and *C. asiatica* metabolites shown in Fig. 6.

3.8. Docking of phytocompounds against target proteins:

We further carried out molecular docking studies to identify potential protein targets of metabolites from *B. monnieri* and *C. asiatica* against two selected proteins -A2A adenosine receptor (A2AAR) and monoamine oxidase B (MAO-B). The docking study revealed that in the A2AAR, metabolite Peonidin 3,5-diglucoside showed the highest LibDock score of 175.02 with ten favorable H-bond interactions followed by UDP-B-L-arabinose (164.33), Vicenin-2 (162.755), UDP-alpha-D-sulfoquinovopyranose (158.117), GDP-L-glucose (156.47), respectively. Interestingly we found the phytochemical libraries of both plants docked against A2AAR follow the same docking pattern. Further, we consider the binding site of MAO-B for docking to examine whether any metabolites bind to that site. The results indicated that tetrahydro pteroyl tri-L-glutamate showed the highest docking score of 280.414 with favorable sixteen H-bond interactions and was followed by UDP-alpha-D-sulfoquinovopyranose, Phytofluene, GDP-L-galactose, and dihydrozeatin-9-N-glucoside-O-glucoside with the LibDock score of 225.594, 220.244, 217.511 and 214.563, respectively. Here also, docking studies of both the plant phytochemical libraries showed almost identical order with respect to their docking score. The experimentally determined dual inhibitor 8-(3-chlorostyryl) caffeine (CSC) was used as a standard inhibitor, comparing the docking efficacies of top-ranked phytochemicals. The CSC showed a LibDock score of 105.847 and 123.879 with four and seven H-bond intermolecular interactions, respectively, while docked against A2AAR and MAO-B. This indicates the top-ranked metabolites have more binding affinity than the CSC and can act as dual inhibitors against the targets A2AAR and MAO-B (Fig. 7A-D).

3.9. Validation of neuroprotective activity of *B. monnieri* and *C. asiatica*

3.9.1. *B. monnieri* and *C. asiatica* aqueous extracts prevent neuronal apoptosis in cellular models of Alzheimer's and Parkinson's disease model

IMR-32 cells were differentiated with 10 μ M retinoic acid for seven days. They were treated with rotenone (100 nM) to generate the Parkinson's disease model, 100 μ g/ml of *B. monnieri* and *C. asiatica* extracts, A β (0.5 μ M) to generate the Alzheimer's disease model, co-treatment of rotenone with *B. monnieri* or *C. asiatica* extract, and co-treatment of A β with *B. monnieri* or *C. asiatica* extracts. After drug treatment for 48hrs, cells were subjected to acridine orange - ethidium bromide (AO-EB) dual nuclear staining. The green fluorescence displayed by the control cells indicates its viable nature. The rotenone-treated cells showed early apoptosis with yellowish-green fluorescence, and A β_{42} treated cells showed early and late apoptosis with yellow and light orange fluorescence. Co-treatment with *B. monnieri* or *C. asiatica* aqueous extract significantly decreased the cell apoptosis compared to the rotenone and A β_{42} treated cells (Fig. 8A-B), indicating neuroprotective properties of *B. monnieri* and *C. asiatica*.

3.9.2. *B. monnieri* and *C. asiatica* aqueous extracts prevent excessive reactive oxygen species (ROS) production in cellular models of Alzheimer's and Parkinson's disease model

We tested the ROS production by staining the cells with 2',7'-dichlorofluorescein diacetate (DCFDA). ROS production increased with the rotenone and A β_{42} treatment conditions, which was decreased with the *B. monnieri* / *C. asiatica* aqueous extract co-treatment. Rotenone and A β_{42} treatment increased ROS production (54.68% & 70.96% respectively, $p < 0.0001$) when compared to the control (7.561% (PD) & 9.47% (AD) conditions respectively). On the other hand, co-treatment with *B. monnieri* / *C. asiatica* aqueous extract significantly decreased the ROS accumulation when compared to rotenone (22.03% & 21.32%, respectively, $p < 0.0001$) and A β_{42} (46.60% & 34.72% $p < 0.0001$ respectively) The results are represented in Fig. 9A-D.

Discussion

Neuroprotection is a broad term that signifies the improvement of neuronal activities and functions against degenerative or degradative scenarios. Several plant extracts in the traditional medicinal system have been regarded as neuroprotectants based on experimental and predictive evidence [15, 33, 51, 36, 23, 52]. *B. monnieri* and *C. asiatica* are considered nootropics in Ayurveda, i.e., memory enhancers commonly referred to as Brahmi in India. However, they belong to different botanical classes. Yadav et al. have validated the effects of Brahmi on the memory of a normal rat model, supporting the potential of Brahmi as a memory enhancer by decreasing the transfer latency [53].

Our aim in the present study was to identify the molecular signature responsible for their similar functions, although belonging to distinct botanical classes. Our interest was to delineate the molecular signatures of *B. monnieri* and *C. asiatica* using mass spectrometry-based metabolomics and screening for their protein targets. We looked at the metabolic makeup of *B. monnieri* and *C. asiatica* and found they shared 76.1% of their metabolites. We also identified several metabolites previously reported in the

literature, including saponins such as D-mannitol, sterols such as stigmasterol, L-alanine/ α -alanine in *B. monnieri*, and Asiatic acid, the lead compound of *C. asiatica*. This will further strengthen our mass spectrometry data and be used for validation [49].

In the present study, we carried out phytochemical estimation to identify primary and secondary metabolites in *B. monnieri* and *C. asiatica* extracts. As a result, we identified a significant amount of antioxidant activity, free radical scavenging activity, total phenolic content, and total flavonoid content in *B. monnieri* and *C. asiatica* extracts ([3] [54]. Furthermore, the findings show the similar activity of both extracts, indicating their neuroprotective properties [55–58].

Metabolomics is a powerful tool to understand the metabolite composition and encompass the significance of these metabolites in the system. Our untargeted metabolomics approach was rewarded with identifying several known and unreported metabolites from *B. monnieri* and *C. asiatica* extracts, simultaneously highlighting the void of unknown metabolite features [59, 60].

Some features remain unassigned as they are not identified in the available datasets or databases. For example, even though we used two databases to identify metabolites, there were still unassigned metabolites not entirely mapped by those datasets. These unassigned metabolites are equally crucial as assigned metabolites and require more detailed efforts to characterize them.

We further explored the effect of *B. monnieri* and *C. asiatica* metabolites on the human system using bioinformatics approaches to identify and predict proteins interacting with *B. monnieri* and *C. asiatica* metabolites. We identified hundreds of proteins interacting with the identified metabolites, encompassing important cellular functions such as cell cycle, autophagy, mitophagy, and apoptosis, which are dysregulated in neurodegenerative diseases [33, 36, 52]. Apart from the cellular functions, signaling mediated by neurotrophin, dopamine, serotonin, and acetylcholine was predicted from our analysis, which in part correlates with the inhibition of cholinesterase activity by both *B. monnieri* and *C. asiatica*, potentially alleviating neurodegenerative diseases [61, 62]. Interestingly, kinases from several pathways of importance in neuroprotective and degenerative conditions were mapped from target prediction analysis. Some of the kinases were previously reported to be regulated by *B. monnieri* and *C. asiatica* [63–66]. In addition, several signaling pathways, such as MAPK, PI3K/AKT, and mTOR, are dysregulated in neurodegenerative disease [67, 33, 36, 52]. We have further analyzed proteins interacting with *B. monnieri* and *C. asiatica* metabolites. Our prediction of the relationship between metabolites and proteins identifies many active proteins and kinases in the RAS, MAPK, PI3/AKT, mTOR, and AMPK pathways. All these pathways are essential in regulating neuronal processes such as learning, memory, motor functions, endocrine regulations, and maintaining synaptic plasticity [67–70]. This paves the way for using metabolomics-derived data to identify novel protein targets and explore the pathways regulated by these metabolites. In addition, this opens new avenues for evaluating and validating signaling mechanisms adapted by these metabolites.

Our exploration into establishing the neuroprotective mechanisms by protein-target prediction using the indirect database BindingDB led to rich information on ligand-target binding efficiencies based on the

SMILES ID of the metabolites. The tool predicts the binding of a metabolite and assigns a similarity score based on its shape and the binding efficiency of known small molecules [29]. However, protein identification largely depends on the metabolite input, which relies on identification. The lacunae in metabolite mapping present a grey area that cannot be circumvented for protein-target identification, emphasizing the need for a more comprehensive database for metabolite identification from plant sources. As many of the metabolites remained unassigned, even though we used plant-specific databases for metabolite identification, that was insufficient to annotate all the metabolites. Our work thus represents the tip of the iceberg that can be further explored with improvements in metabolite databases to decrease the number of unidentified metabolites.

It is important to emphasize that the workflow employed here is not restricted only to BM and CA. It can also be extended to other natural product-based extracts to be screened for their efficacies in a given disease model. The study deciphered the metabolite composition and mechanism of action of *B. monnieri* and *C. asiatica* and established a common link between their neuroprotective efficacies. The insights from this study can be used to evaluate their efficacies in cellular and animal models of neurodegenerative diseases, thereby validating their neuroprotection and identification of novel therapeutic targets. Furthermore, it can be used further for clinical trials to treat various neurodegenerative disorders in humans.

Abbreviations

A2AAR: A2A adenosine receptor

AD: Alzheimer's disease

ADORA2A: Adenosine A2a Receptor

AMPK: AMP-activated protein kinase

AO-EB: Acridine orange - Ethidium bromide

A β : Amyloid- β

BM: *Bacopa monnieri* (L.) Wettst

CA: *Centella asiatica* (L.) Urb.

CAM: Complementary and Alternative Medicine

cGMP: Cyclic guanosine monophosphate

CHRM: Cholinergic Receptor Muscarinic

CID: Collision-induced dissociation

CSC: 8-(3-chlorostyryl) caffeine

CTP: Cytidine triphosphate

Da/s: Dalton per second

DAVID: Database for Annotation, Visualization and Integrated Discovery

DCFDA: 2',7'-Dichlorofluorescein diacetate

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EMS: Enhanced mass spectra

EPI: Enhanced product ion

FBS: fetal bovine serum

GSK3B: Glycogen Synthase Kinase 3 Beta

IDA: Information-dependent acquisition

KEGG: Kyoto Encyclopedia of Genes and Genomes

KRAS: Kirsten RAS

LC-MS/MS: Liquid chromatography and tandem Mass spectrometry

MAO-B: Monoamine oxidase B

MAP2K1: Mitogen-Activated Protein Kinase Kinase 1

MAPK: Mitogen-activated protein kinases

MBROLE: Metabolites Biological Role

mTOR: Mechanistic Target Of Rapamycin Kinase

MTT: 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide

NCCIH: National Center for Complementary and Integrative Health

NIH: National Institutes of Health

PBS: Phosphate buffered saline

PD: Parkinson's disease

PDB: Protein Data Bank

PI3K: Phosphoinositide 3-kinase

PKG: Protein kinase G

PRKACA: Protein Kinase CAMP-Activated Catalytic Subunit Alpha

RANSAC: Random sample consensus

RAP1: Ras-related protein 1

RAS: Rat sarcoma virus

ROS: Reactive oxygen species

SLC6A3: Solute Carrier Family 6 Member 3

SMILES: Simplified molecular-input line-entry system

STRING: Search Tool for the Retrieval of Interacting Genes/Proteins

TAC: Total antioxidant capacity

TCA: Trichloroacetic acid

UTP: Uridine-5'-triphosphate

VEGF: Vascular endothelial growth factor

Declarations

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Thottethodi Subrahmanya Keshava Prasad, Ravishankar Pervaje, and Prashant Kumar Modi conceived the idea and designed and planned the experiments. Sakshi Sanjay Parate and Shubham Sukerndeo Upadhyay: data analysis, drafted the manuscript, and prepared figures. Amrutha S: phytochemical estimation and validation experiments. Gayathree Karthikkeyan: mass spectrometry data acquisition and participated in data analysis. Chandran S. Abhinand: involved in docking experiments. Ravishankar Pervaje: a practicing Ayurveda clinician, procured and authentically confirmed the samples. Thottethodi Subrahmanya Keshava Prasad and Prashant Kumar Modi: critically reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

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Availability of data and material

All the data is available in the manuscript and supplementary information.

Ethics approval

This study has been approved by the Institutional Scientific Review Board (YRC SRB/028/2019).

Consent for Publication

All authors have read and approve the manuscript and consent to its publication.

Competing Interests

The authors declare no competing interests.

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Figures

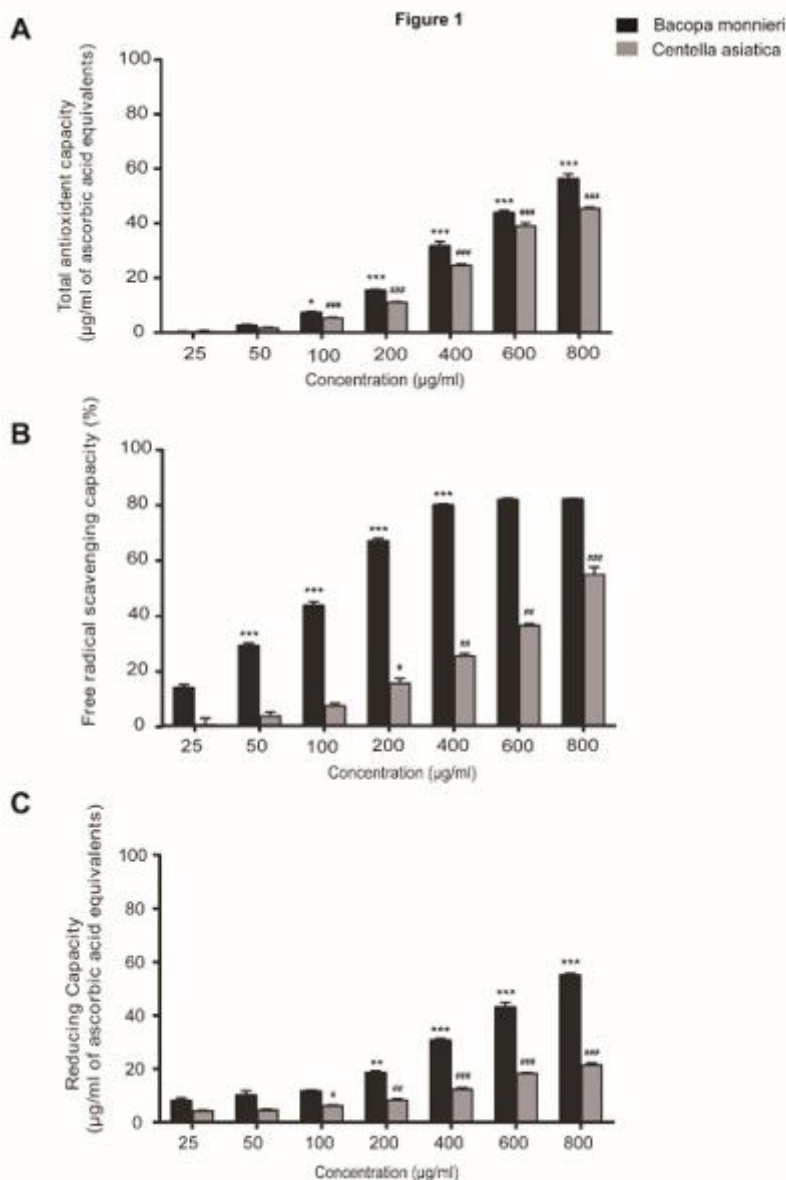


Figure 1

Antioxidant activity of *Bacopa monnieri* and *Centella asiatica* aqueous extracts. *In vitro*, antioxidant activity of *Bacopa monnieri* and *Centella asiatica* was analyzed by using total antioxidant levels, DPPH scavenging activity, and Reducing activity at different concentrations of *Bacopa monnieri* and *Centella asiatica* aqueous extracts. **(A)** Total antioxidant activity of *Bacopa* and *Centella* aqueous extract, **(B)** 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity of *Bacopa* and *Centella* aqueous extract, & **(C)** Reducing power assay of *Bacopa* and *Centella* aqueous extracts (Mean $\pm$ SD, * $p < 0.05$, ** $p < 0.009$, *** $p < 0.001$, # $p < 0.0001$).

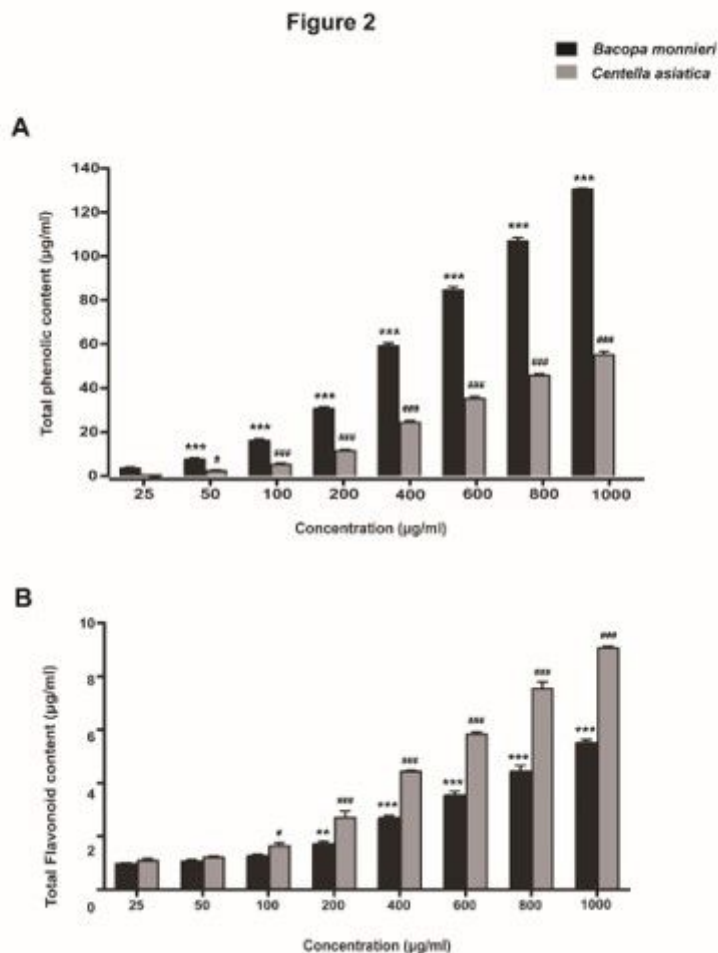


Figure 2

Quantitative analysis of the secondary metabolites in different concentrations of *Bacopa monnieri* and *Centella asiatica* aqueous extracts. **(A)** Total Phenolic content of *Bacopa monnieri* and *Centella asiatica* aqueous extracts **(B)** Total Flavonoid content of *Bacopa monnieri* and *Centella asiatica* aqueous extracts, (Mean $\pm$ SD, * $p < 0.05$, ** $p < 0.009$, *** $p < 0.001$, # $p < 0.0001$).

Figure 3

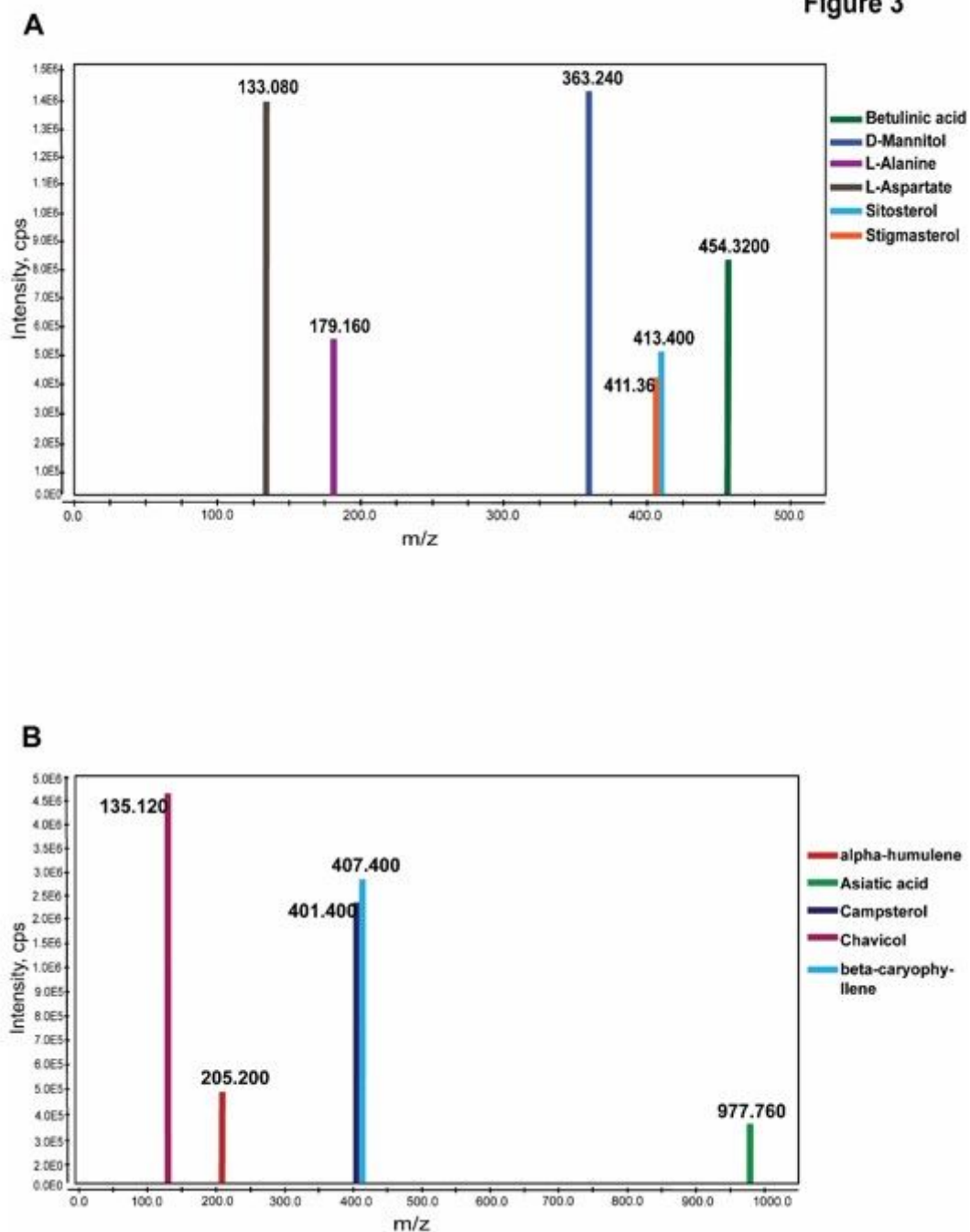


Figure 3

MS identification of previously reported metabolites. (A) Metabolites identified in *Bacopa monnieri* along with the adducts- Betulinic acid (M-H), D-Mannitol (2M-H), L-alanine (2M-H), L-aspartate (M-H), Sitosterol (M-H), Stigmasterol (M-H), **(B)**Metabolites identified in *Centella asiatica* along with the adducts- alpha-humulene (M+H), Asiatic acid (2M+H), Campesterol (M+H), Chavicol (M+H), beta-caryophyllene (2M+H).

Figure 4

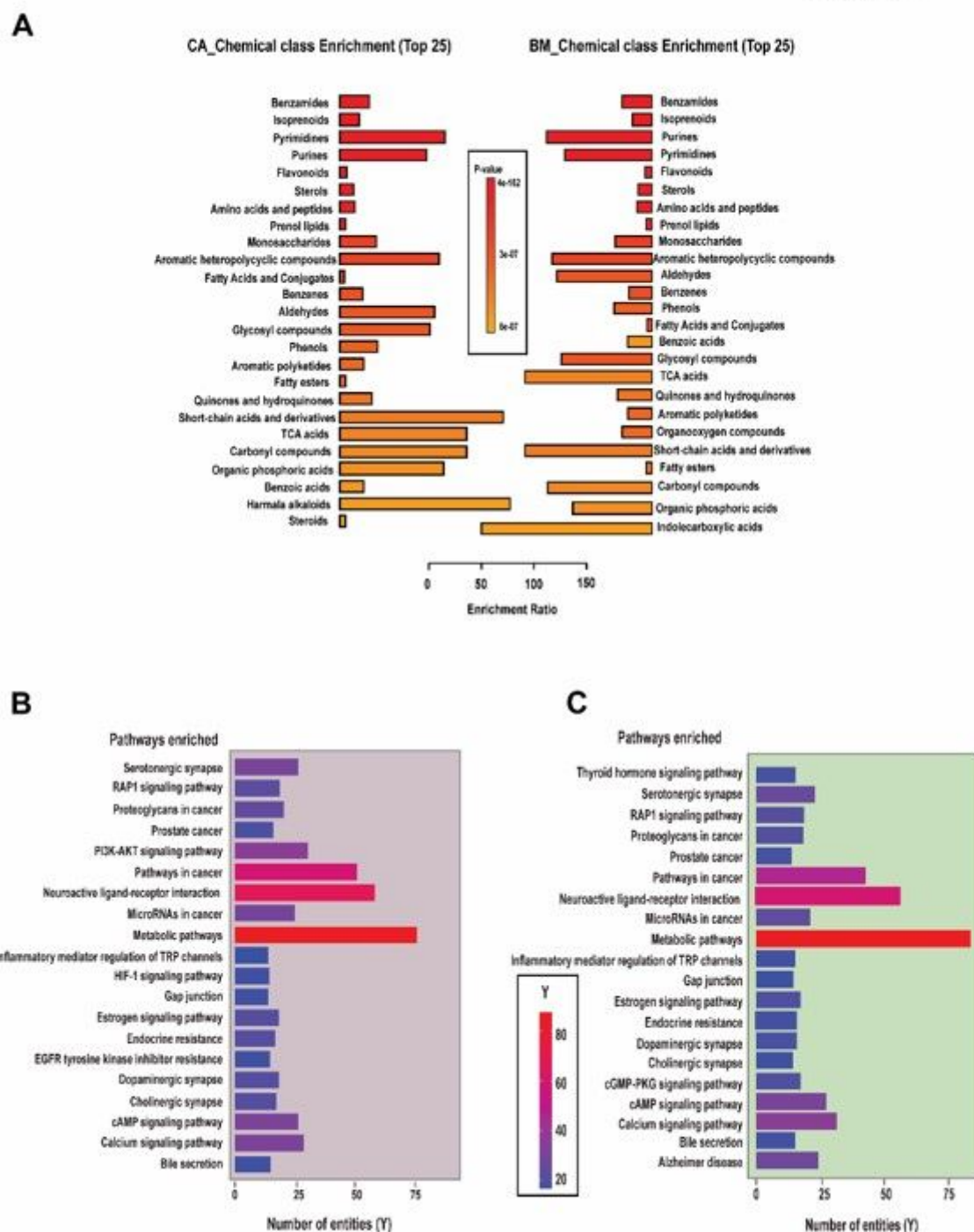


Figure 4

Metabolite and protein targets enrichment analysis. (A) Overall representation of *Bacopa monnieri* and *Centella asiatica* metabolites showing top 25 enriched chemical classes with 94.8% similarity, (B) Top 20 enriched pathways of *Bacopa monnieri* metabolite-protein targets, (C) Top 20 enriched pathways of *Centella asiatica* metabolite-protein targets.

Figure 5

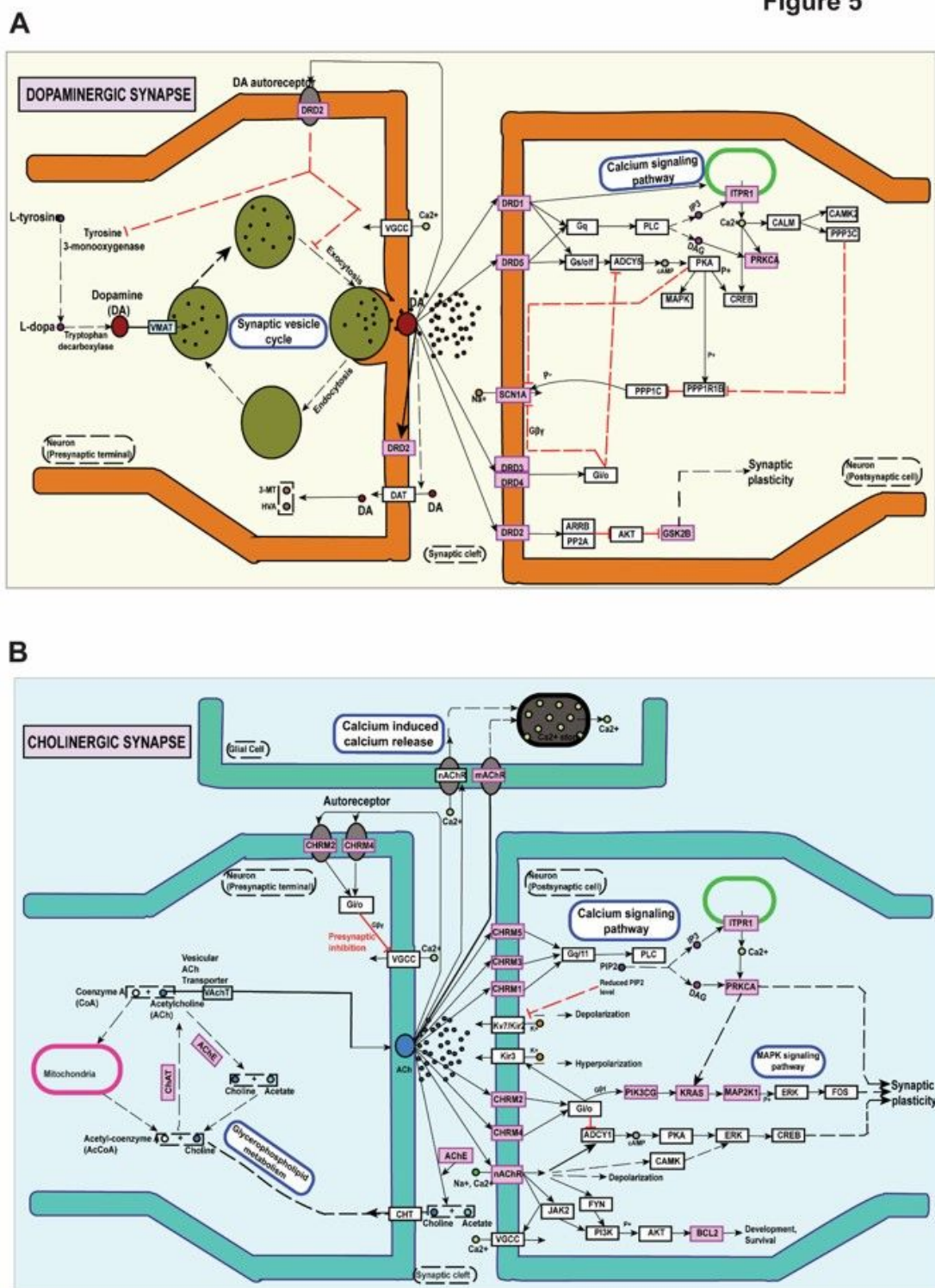


Figure 5

Analysis of predicted proteins interacting with *Bacopa monnieri* and *Centella asiatica* metabolites: The proteins involved in neuroreceptor signaling and synapse modulation are modulated by *Bacopa monnieri* and *Centella asiatica* metabolites. **(A)** Dopaminergic synapse, **(B)** Cholinergic synapse.

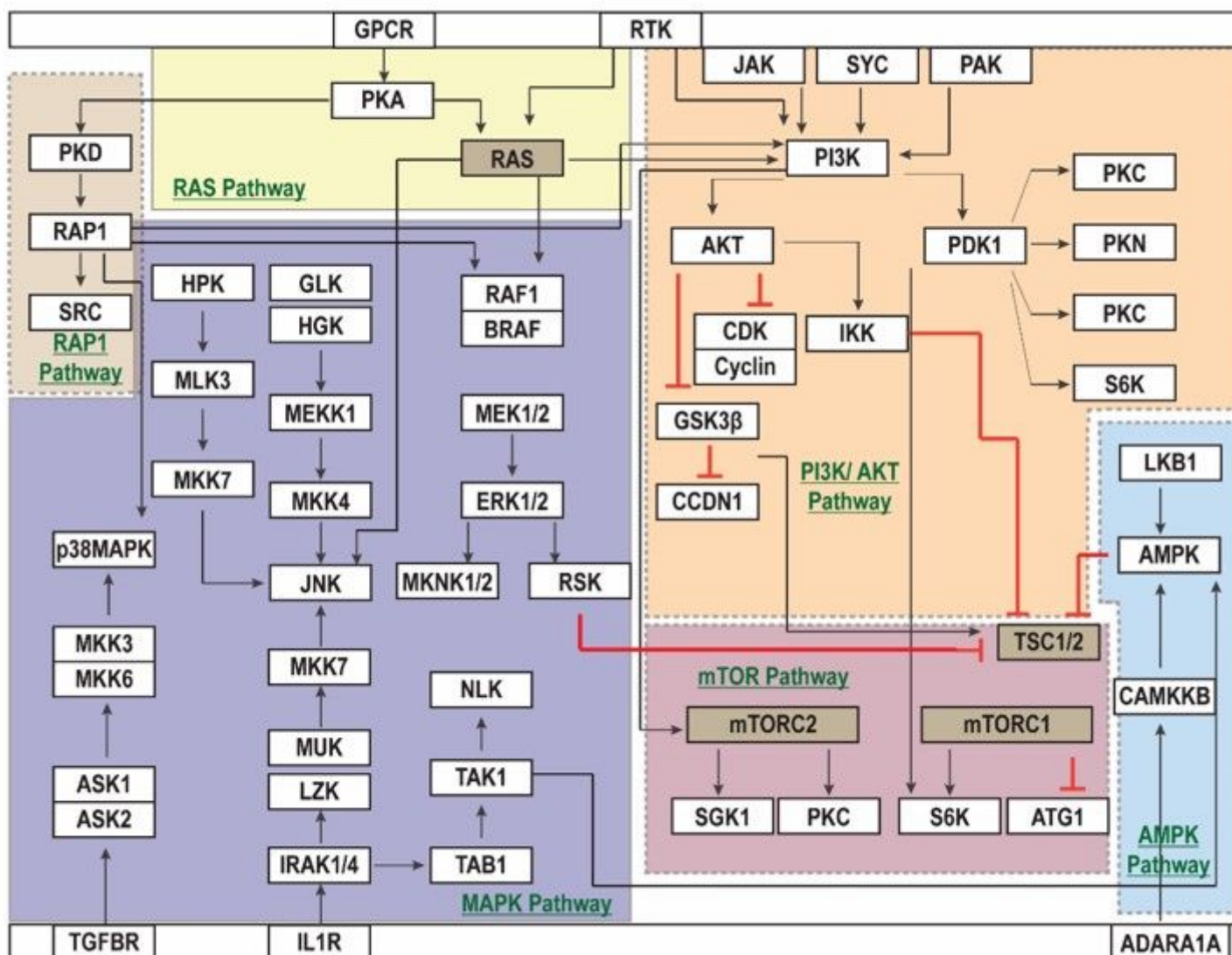


Figure 6

Kinase regulation of *Bacopa monnieri* and *Centella asiatica*. Mapping of kinases from key signaling pathways regulated by common metabolites of *Bacopa monnieri* and *Centella asiatica* and crosstalk of signaling pathways. RAS, MAPK, PI3K/AKT, RAP1, and mTOR pathways are selected for representation purposes.

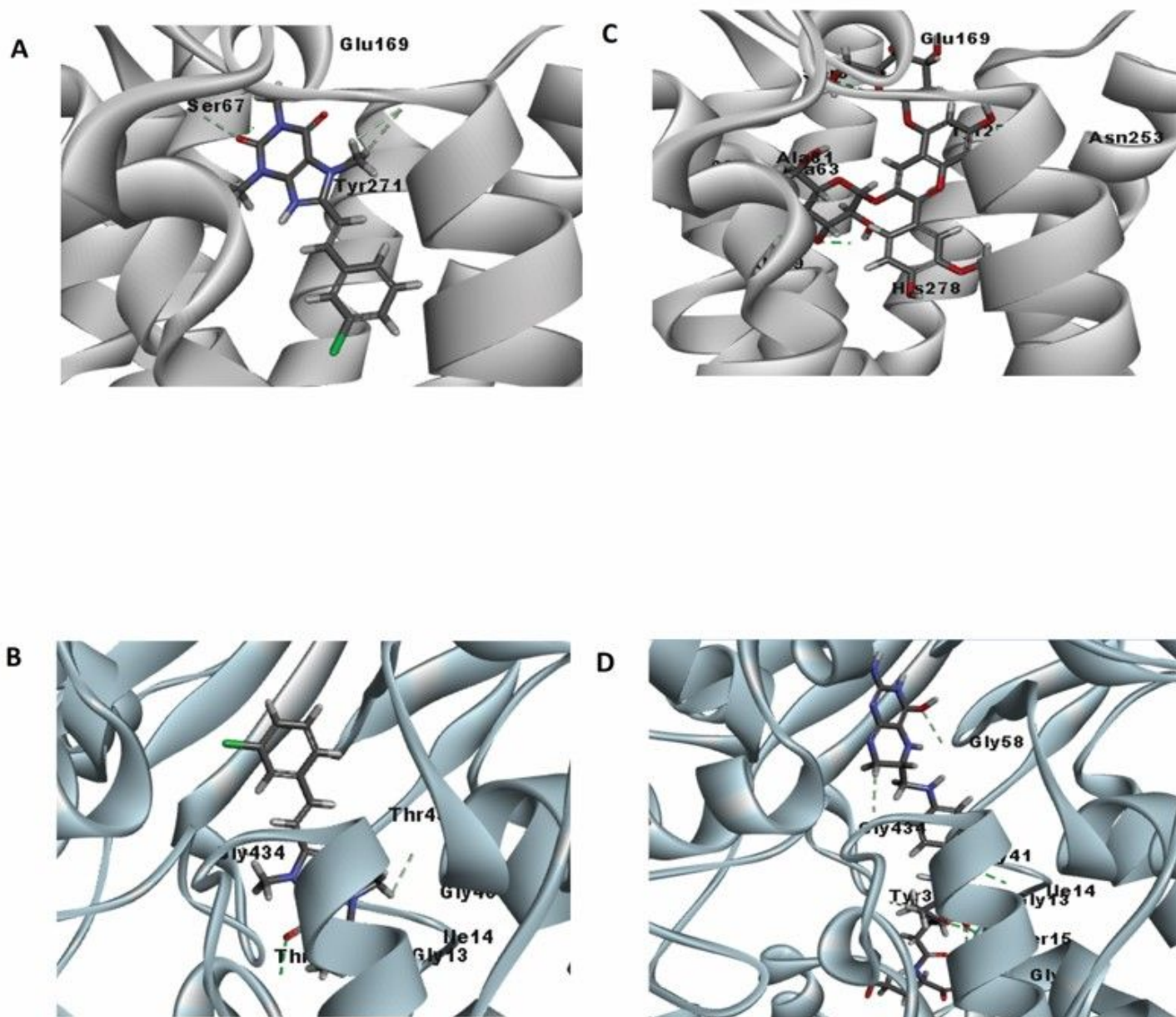


Figure 7

Molecular interactions of the metabolites (ligand) against two potential drug targets, namely A2A adenosine receptor (A2AAR) and monoamine oxidase B (MAO-B). (A) 8-(3-chlorostyryl) caffeine interaction against A2AAR, **(B)** 8-(3-chlorostyryl) caffeine interaction against MAO-B, **(C)** Peonidin 3,5-diglucoside interaction against A2AAR, **(D)** tri-L-glutamate interaction against MAO-B. Key binding site residues are labeled and the ligands are represented in the sticks. The H-Bond intermolecular interactions are shown as dashed lines.

Figure 8

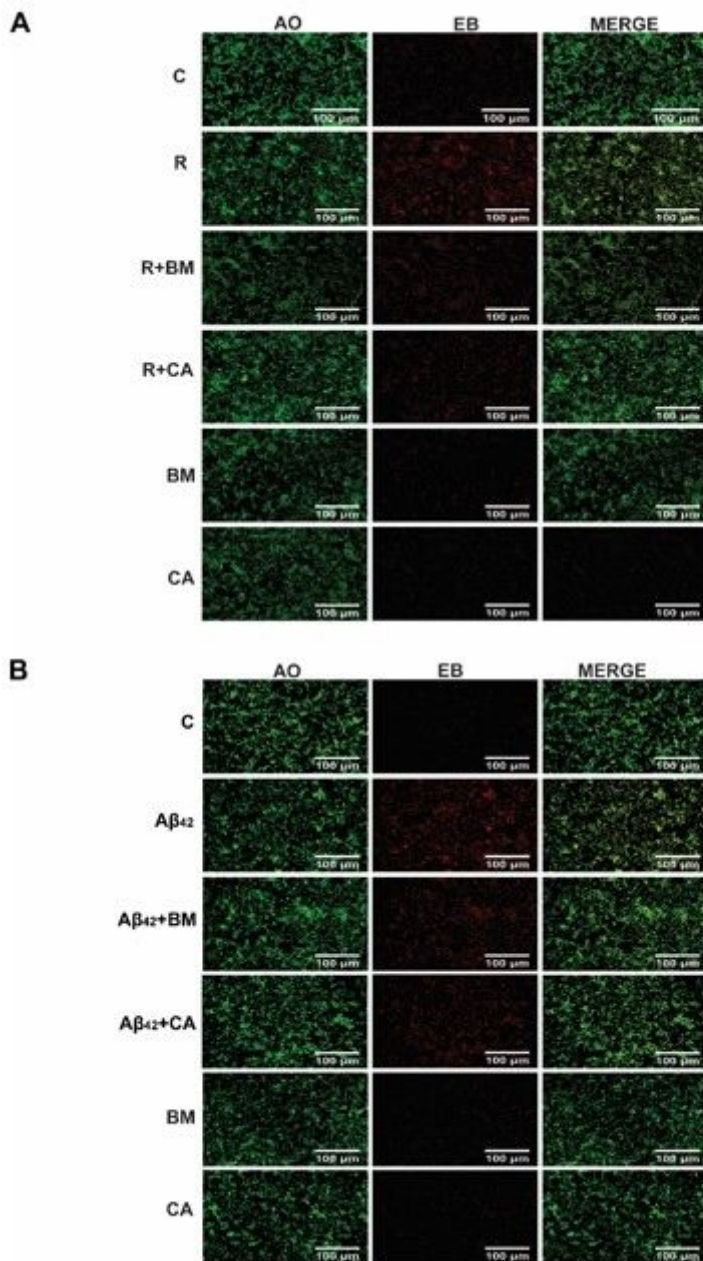


Figure 8

***Bacopa monnieri* (BM) and *Centella asiatica* (CA) prevent neuronal apoptosis in differentiated neuronal cells in cellular models of Parkinson's and Alzheimer's disease.** Differentiated IMR-32 cells were treated with rotenone (100 nM) and Aβ₄₂ (0.5 μM) to generate Parkinson's and Alzheimer's disease models, respectively, BM (100 μg/ml) and CA (100 μg/ml); their co-treatment and untreated cells were taken as control. (A) Rotenone and (B) Aβ₄₂ promote neuronal apoptosis as stained by AO-EB Dual staining. Live cells were stained with green fluorescence (AO stain), whereas apoptotic cells were stained red (EB stain). Live-dead staining shows more apoptosis in rotenone and Aβ₄₂ treated groups. Co-treatment with BM or CA reduced apoptosis, BM: *Bacopa monnieri*, CA: *Centella asiatica*, AO: acridine orange, EB: ethidium bromide.

Figure 9

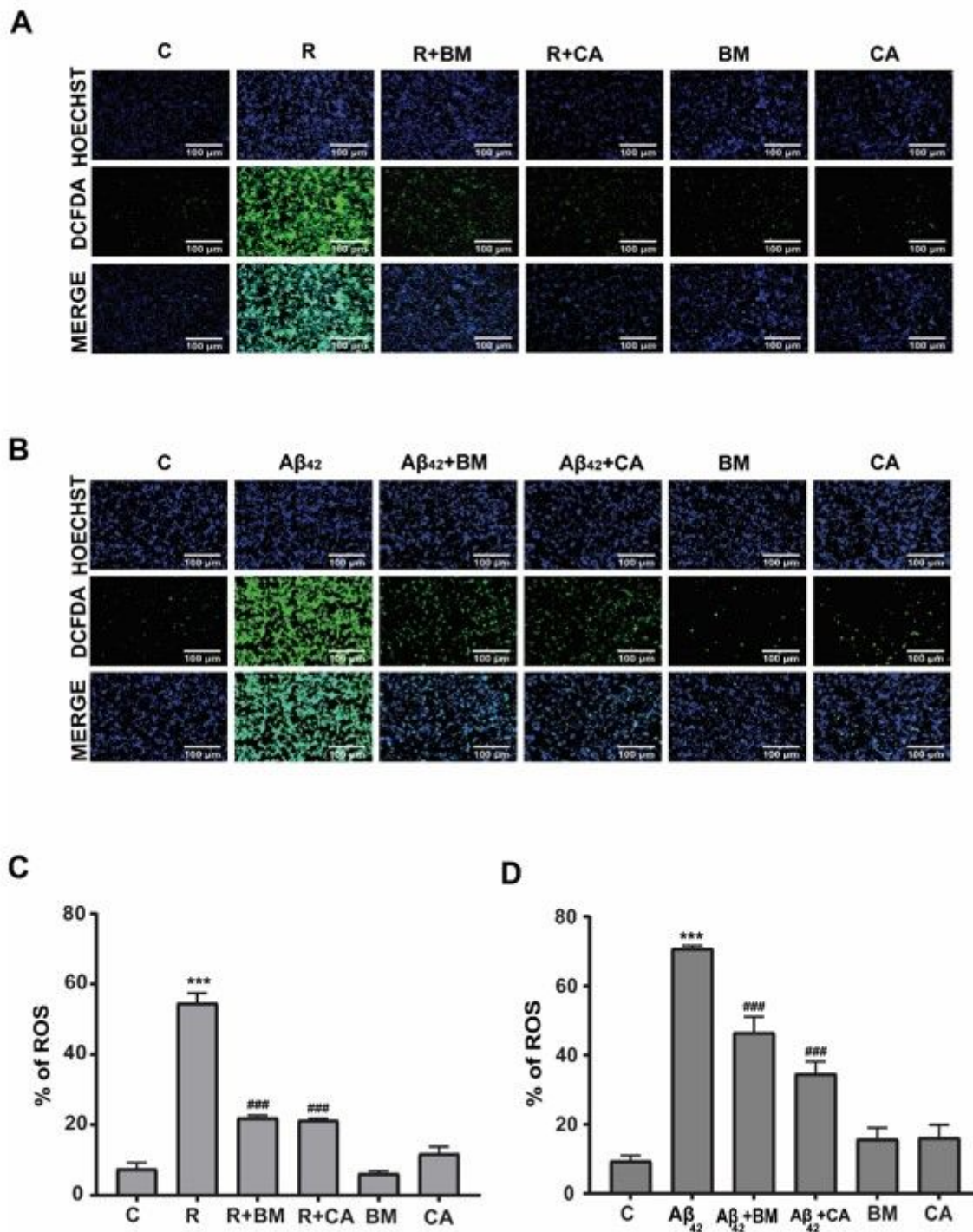


Figure 9

Bacopa monnieri (BM) and *Centella asiatica* (CA) counter aberrant reactive oxygen species (ROS) production in cellular models of Parkinson's (PD) and Alzheimer's (AD) disease. Differentiated IMR-32 cells were treated with rotenone (100 nM, PD)/ Aβ₄₂ (0.5 μM, AD), BM (100 μg/ml), and CA (100 μg/ml); their co-treatment and untreated cells were taken as control. (A) Rotenone (C) Aβ₄₂ promotes ROS production as stained by DCFDA (green) and counterstained with HOECHST (blue). BM/CA co-treatment

reduced the ROS levels. (B, D) Graphical representation of ROS production. * denotes significance with respect to control; # denotes significant with respect to rotenone/ $A\beta_{42}$. *,#, $p \leq 0.05$; **,##, $p \leq 0.01$, ***,###, $p \leq 0.001$. BM: *Bacopa monnieri*, CA: *Centella asiatica*, DCFDA: 2',7'-dichlorofluorescein diacetate.

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