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Dendrobium nobile alkaloids modulate calcium dysregulation and neuroinflammation in Alzheimer's disease: A bioinformatic analysis

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ABSTRACT

Introduction: Dendrobium nobile Lindl alkaloids, or DNLA for short, are the most active ingredients found in *D. nobile*, a top grade plant in Shen Nong Ben Cao Jing, with an extensive history of medicinal use in Chinese traditional medicine (TCM) as a multifunctional therapeutic agent. Recent evidence has emerged linking the neuroprotective and anti-aging effects of DNLA to their involvement in promoting autophagy of toxic amyloid-β (Aβ) plaques and modulation of key enzymes involved in the hyperphosphorylation of Tau proteins. Although amyloid buildup and the aggregation of Tau proteins are central to the onset of Alzheimer's disease (AD), evidence on how DNLA relate to other overlooked dysregulated AD-associated pathways is still lacking.
Methods: We intend on deciphering the underlying mechanisms driving the anti-AD effect of DNLA, using a combination of network analysis based on differentially expressed genes found in AD patients, target fishing, centrality analysis, enrichment analysis and hub genes identification.
Results: In total, 2069 genes were found differentially expressed in SRP181886 and a PPI network constructed with common targets between DNLA and AD. Five hub genes were identified having a discriminatory power greater than 0.7; *HTR2A, GRIN2B, GABRA1, HTR2C, GRIN2A*, with the former being the top bottleneck node in the network. Enrichment analysis found that DNLA exert an anti-AD effect through the regulation of the calcium signaling pathway and the serotonergic system, by modulating key receptors implicated in excitatory/inhibitory neurotransmission. Additionally, DNLA were found to modulate two subunits of NMDA receptor involved in the release of pro-inflammatory cytokines, underlying the possible involvement of DNLA in neuroinflammation.
Discussion: This further emphasizes the therapeutic value of *D.nobile* and the multi-target, multi-pathway potential of DNLA to counteract the deleterious effects of calcium dysregulation and excitatory toxicity in AD, while providing evidence-based rationale behind the traditional use of *D. nobile* in TCM.

1. Introduction

Dendrobium nobile L. is an epiphytic medicinal plant belonging to the Orchidaceae family [1]. *D. nobile* are widely known for their medicinal properties in Traditional Chinese Medicine (TCM), and have a long-standing history as an anti-inflammatory, anti-tumor, cardio-protective, anti-aging and hypoglycemic agent [2–4].

The earliest record of Dendrobium species' value in traditional medicine can be traced back to Shen Nong Ben Cao Jing [5,6], one of the oldest Chinese pharmacopeia and the foundation of TCM, that classified medicinal herbs into three groups (high, medium and low grade) based on their therapeutic effects, with *D. nobile* listed as high grade [7]. In TCM, *D. nobile* is believed to have a Yin strengthening effect, promoting the production of fluids and clearing heat, with reports of it being the most commonly used Dendrobium species that goes into the preparation of Shih-hu, a tonic traditionally prescribed for rheumatism, menstrual pain, epilepsy, inflammation and other ailments [8,9].

The phytochemical profile of *D. nobile* is mainly made up of alkaloids, polysaccharides, flavonoids, polyphenols and aromatic compounds, with the former being the most active and prominent class with a total content of 79.8% [10]. *Dendrobium nobile* L. alkaloids, or DNLA for short, include dendrobine, with a major content percentage (approximately 92% of total alkaloids), nobilonine and dendroxine having a lower percentage [10].

Growing evidence has alluded to the notion that DNLA are behind the observed anti-aging and neuroprotective effect of *D. nobile* especially

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in the context of Alzheimer's disease (AD). At an intracellular level, AD is marked by the aggregation of β-amyloid (Aβ) proteins and the polymerization of phosphorylated Tau proteins into neurofibrillary tangles (NFTs), accompanied by an avalanche of erratic signaling events including neuroinflammation, oxidative stress, activation of immune mechanisms and mitochondrial dysfunction [11–13].

The sequential cleavage of amyloid precursor protein (APP) produces two major subtypes of Aβ; a 40-amino acid protein $A\beta_{40}$, and a 42-residue protein $A\beta_{42}$, which is a hydrophobic subtype that tends to aggregate into Aβ plaques [14]. Aβ accumulates into senile plaques (SPs), which gives rise to impaired synaptic function and neuronal death [15]. Tau is a microtubule-associated protein, with the primary function of stabilizing microtubules (MTs) in the brain by binding to these structures. In AD, Tau dissociates from MTs and undergoes conformational changes that promote their polymerization into NFTs, leading to neuronal loss [16]. Premature death of neurons is behind the genesis of AD, with the hippocampus being the first brain region to experience atrophy. With the disorder advancing, lesions spread to other parts of the brain, promoting cognitive decline and memory impairment typical of AD [13]. Available drugs only promise transient symptomatic relief, with the objective of either dampening chronically activated receptors (NMDA antagonists; memantine), inhibiting the breakdown of excitatory neurotransmitters (acetylcholinesterase inhibitors; donepezil) or targeting Aβ buildup (monoclonal antibodies; aducanumab) [17]. However, effective disease-modifying therapies are yet to be discovered.

Recent in vivo studies have revealed that DNLA slow down the degeneration of neurons in the CA1 sub-region of hippocampus in animal models of AD. Additionally, DNLA were found to reduce hyperphosphorylation of Tau proteins, it is thought to exert that effect by dampening the activity of GSK-3β and Cdk5, the two major enzymes that phosphorylate Tau proteins [18]. Another study found that DNLA protect against axon degeneration in $A\beta_{25-35}$ treated neurons by promoting autophagy, doubling as neuroprotective [10]. DNLA were also found to improve neurobehavioral performance of AD affected mice, by triggering key proteins in Aβ degradative pathways [19]. In a recent experimental study, Pi and al., reported that DNLA treatment reduces the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ subtypes. This effect was partially mediated by decreasing the expression levels of key enzymes involved in the formation of Aβ fragments, mainly APP, PS1, and BACE-1 [20]. Additionally, a 4-month treatment with two different doses of DNLA (20 and 40 mg/kg) proved to be beneficial on the cognitive decline and neuronal loss of AD mouse models [21] underlying the anti-aging and neuroprotective properties of DNLA.

Although Aβ plaque deposition and NFT formation are the defining hallmarks of AD, the pathology is far more complex to sum up in two dysregulated pathways. Neuroinflammation, especially in glial cells, predates Aβ and Tau pathologies [22], with evidence for the presence of activated microglia in senile plaques in the hippocampus and cerebral cortex of AD brain [23]. Microglia are involved in maintaining the immune balance of the central nervous system (CNS), through the clearance of toxic Aβ plaques and other cellular debris. However, the prolonged over-activation of microglia and constant release of cytokines feed into the loop of neurodegeneration. Additionally, calcium dyshomeostasis has been reported in glial cells (particularly in microglia and astrocytes) and neurons of AD patients, with studies postulating that this imbalance could trigger Aβ plaques formation and Tau hyperphosphorylation [24]. How DNLA relate to these and other dysfunctional pathways in AD is still elusive.

By leveraging concepts from network biology and RNA sequencing data analysis, *viz*. identifying AD-associated genes with altered expression or differential gene expression analysis, protein-protein interaction networks (PPI), modules and hub genes identification and functional enrichment analysis, we intend on uncovering how DNLA may influence other complex AD-associated pathways.

This study addresses the anti-cognitive aging claims of *D. nobile* by uncovering the intermolecular mechanisms by which DNLA exert its effect on AD. Using RNA-sequencing data of normal and AD patients, gene expression analysis, target fishing and network centrality analysis, we intend on identifying dysregulated pathways associated with AD that could be modulated by DNLA, showcasing the true therapeutic potential of *D. nobile* and TCM as a whole.

2. Materials and methods

2.1. Dataset download and preprocessing

RNA-seq data for healthy control and AD samples was downloaded using the Recount3 R package (version 1.14.0) [25]. 289 samples taken from fusiform gyrus tissues were included in the selected study "SRP181886", of which 219 corresponds to samples from AD patients and 70 from neurologically normal controls [26]. The raw counts data was normalized using the variance stabilizing transformation (VST) function implemented in the DESeq2 package (version 1.44.0) [27].

2.2. Differentially expressed genes (DEGs) identification

Differentially expressed genes (DEGs) were identified using the DESeq2 package. Genes with an adjusted P.value $\langle$ 0.01 and $|$log2-FoldChange$|$ $\rangle$ 0.5 were assigned differentially expressed. The screened DEGs were visualized using the R Bioconductor packages ComplexHeatmap and EnhancedVolcano (version 1.22.0) [28,29].

2.3. Target prediction for dnla

Potential targets for DNLA were predicted using SwissTargetPrediction (http://www.swisstargetprediction.ch/) [30], with "Homo Sapiens" set as the target organism. Overlapping targets between DNLA and AD DEGs were identified and visualized using a Venn diagram generated with Venny 2.1.0 web server (https://bioinfogp.cnb.csic.es/tools/venny/) [31].

2.4. Pharmacokinetic evaluation

D. nobile active ingredients were assessed for their bioavailability and CNS-permeability using SwissADME web server (http://www.swissadme.ch/) [32]. Three major parameters were selected in order to evaluate the drug-likeness of DNLA: Oral bioavailibility, human intestinal absorption and blood-brain barrier permeability.

2.5. PPI network construction

Overlapping genes between DNLA and DEGs were further analyzed using STRING (Search Tool for the Retrieval of Interacting Genes) database (https://string-db.org/) [33] to get a better look at the functional interactions of the DNLA-AD-targets network. A confidence score cutoff of 0.4 was applied, and the resulting network was imported to Cytoscape 3.10.1 software [34]. The MCODE plugin (version 2.0.3) was used to screen for potential subnetworks/clusters in the PPI network [35]. Hub genes were selected by overlapping the top 10 nodes obtained by four different algorithms (MCC, MNC, degree, betweenness) computed by cytoHubba plugin [36].

2.6. Centrality analysis

Topological property analysis of the hub genes was carried out using the CytoNCA plugin of Cytoscape [37]. Four centrality metrics were taken into consideration when assessing the topology of selected genes; degree centrality (DC), betweenness centrality (BC), eigenvector centrality (EC) and closeness centrality (CC). These parameters reflect the significance and influence of a given gene in the network.

2.7. Diagnostic efficacy of hub genes

To assess how well the selected genes can distinguish between the two biological conditions (healthy control vs. AD samples), the pROC R package (version 1.18.5) was used to calculate AUC value for each gene [38]. ROC curves for each individual gene were plotted for further analysis.

2.8. GO and kegg pathway analysis

Using ClusterProfiler package, the gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was conducted in order to identify AD-related biological pathways that could be potential targeted by *D. nobile* active ingredients [39,40]. Pathways with a p-value cutoff of 0.01 and q-value cutoff of 0.05 were deemed statistically significant. GO enrichment analysis included biological process (BP), cellular component (CC) and molecular function (MF). The top 10 terms, ranked by significance, were visualized using the bubble plot function from enrichplot R package [41]. Relevant KEGG pathway networks were selected and visualized using pathviewer package [42].

2.9. Statistical analysis

All statistical analysis and interpretation was conducted using R (v4.3.1). Heatmaps and volcano plots were drawn using the R package ComplexHeatmap and EnhancedVolcano, while bubble plots and signaling pathways were established using the packages enrichplot and pathviewer, respectively.

3. Results

3.1. Identification of DEGs between ad and healthy controls

Tissue samples were taken from the superior frontal gyrus (SFG) of 219 AD patients with profiles consistent with BRAAK stages V and VI and 70 neurologically healthy controls with the same sex and age distribution [26].

Count data was transformed using the vst function from DESeq2 package to get a uniform spread of variance across different expression levels. With the adjusted P-value set to 0.01 and log2FoldChange of 0.5, 2069 genes were assigned differentially expressed in SRP181886. 1129 genes were found to be up-regulated and 931 down-regulated genes. Fig. 1 is a heatmap representation of the expression level profiles of identified DEGs across all samples. A different expression pattern can be seen exhibited by the two biological conditions (AD and ctrl) represented by the difference in color and its intensity.

Fig. 2 plots the distribution of log2FC in relation to the statistical significance of DEGs. Genes in red above the threshold with positive fold change value were deemed up-regulated, whereas genes in red dots that have a negative fold change are treated as down-regulated.

3.2. Drug likeness assessment of dnla

The 2D structure of the most prominent alkaloids in *D. nobile* is depicted in Fig. 3. These compounds represent the major sesquiterpenoid alkaloids subgroups found in *D. nobile.* Dendrobine and dendroxine share a picrotoxan skeleton, while nobilonine derives from dendrobine [2].

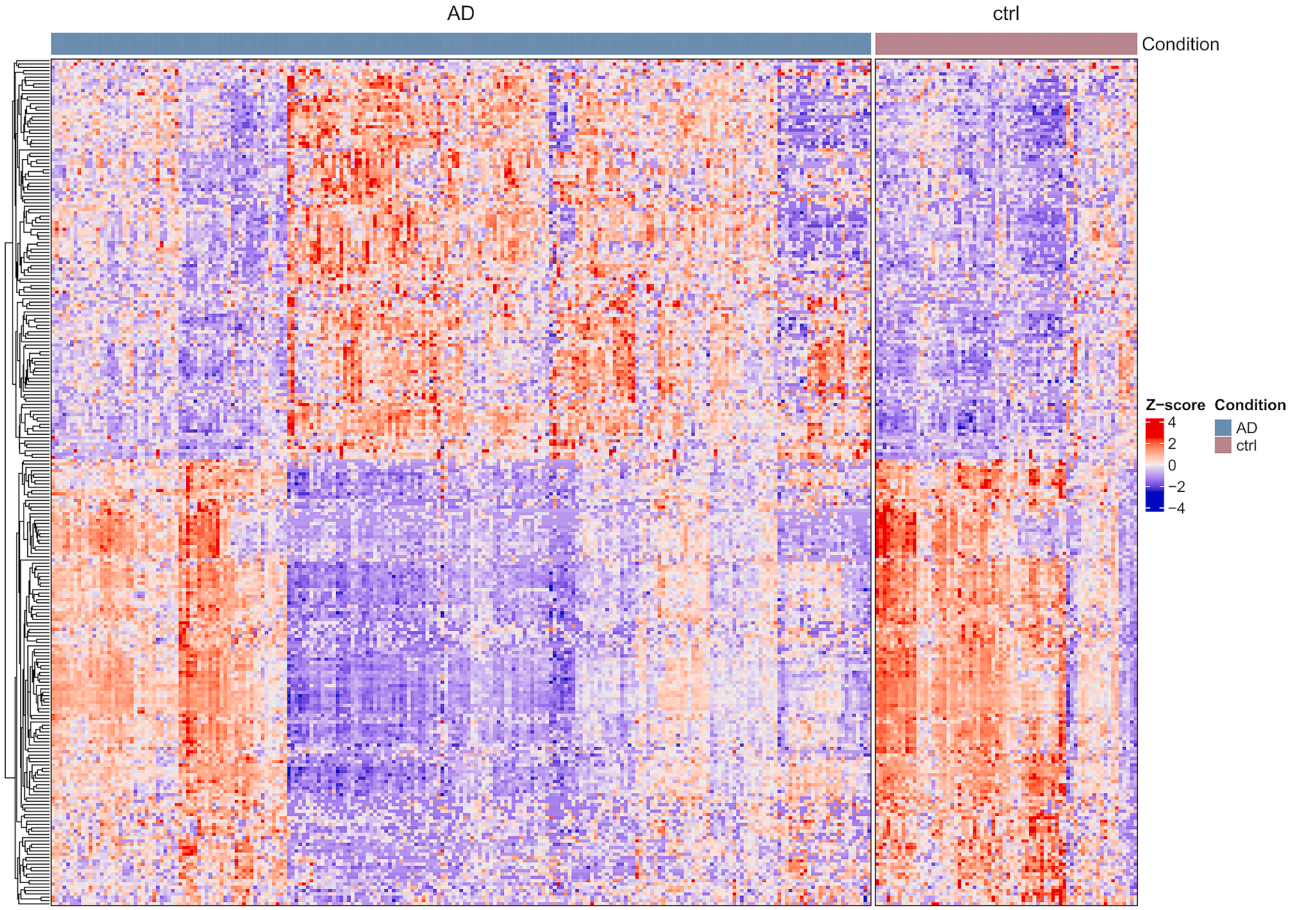

Fig. 1. Heatmap of differentially expressed genes found in SRP181886. Red: Upregulated and blue: downregulated. cutoff: P.value ⟨ 0.01 and |log2FoldChange| ⟩ 0.5. AD: Alzheimer's disease, ctrl: control. Figure created in R (v4.3.1).

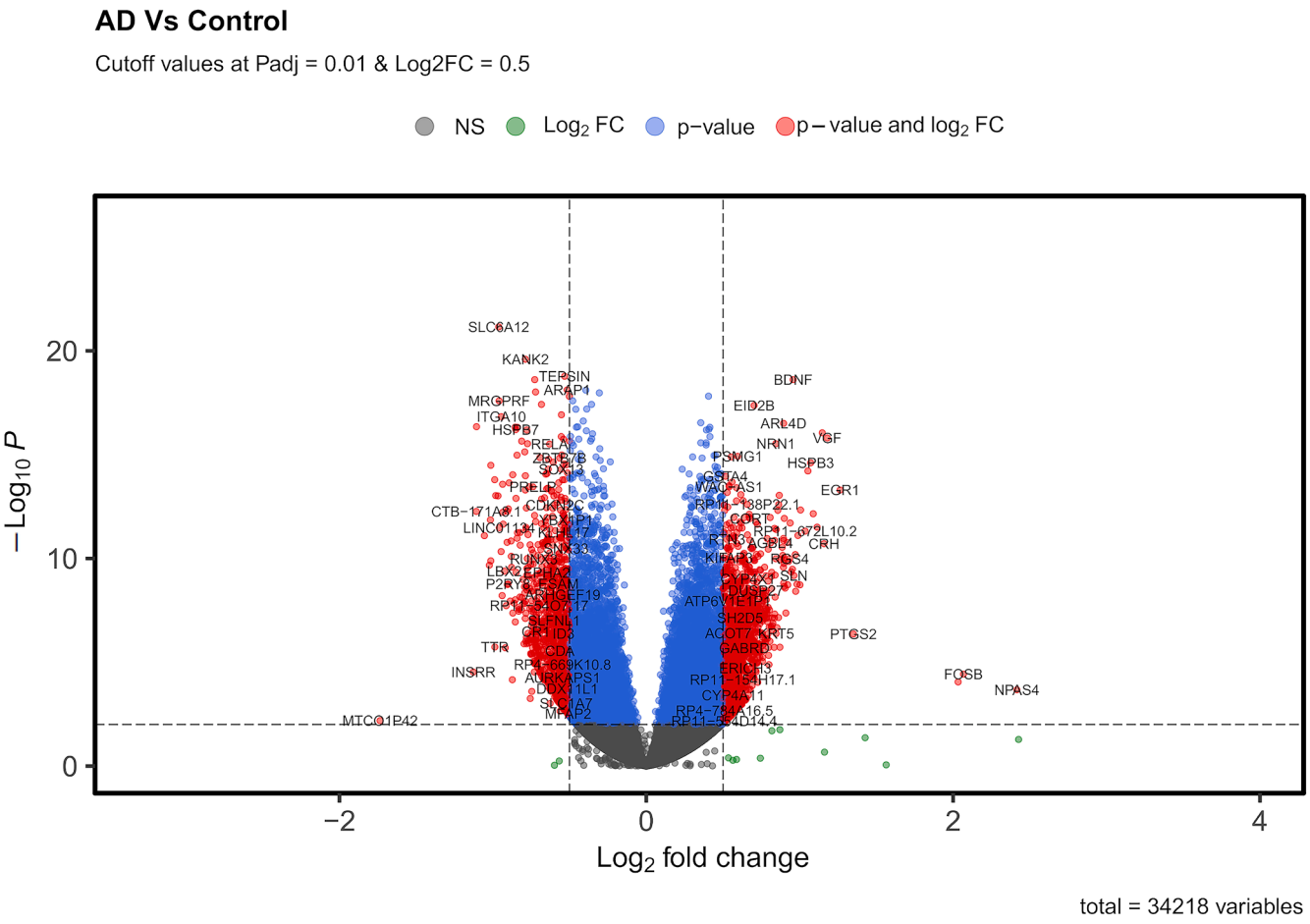

Fig. 2. Volcano plot of DEGs in SRP181886. Red : genes are both statistically significant and biologically relevant, with positive x-axis values indicating up-regulation and negative values representing down-regulation, blue : genes are statistically significant with small changes in expression, green : genes are biologically relevant and statistically insignificant, gray : non-significant. Figure created in R (v4.3.1).

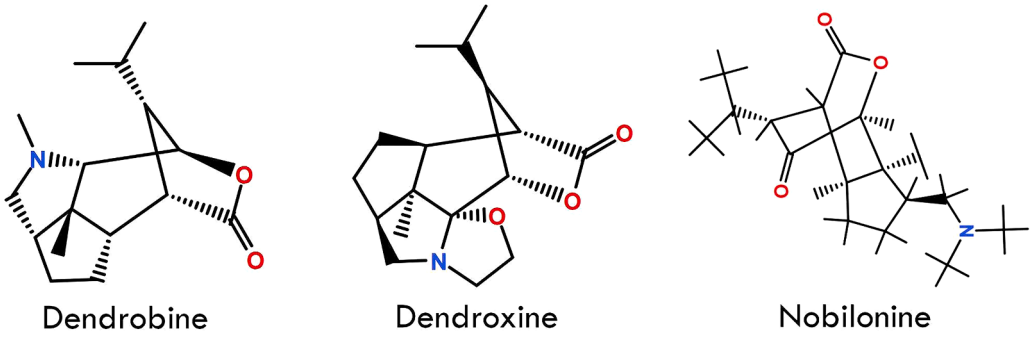

Fig. 3. 2D structure of the most prominent *D. nobile* L. alkaloids (DNLA).

Three physicochemical descriptors were used to assess the bioavailability and overall drug-likeness of *D. nobile* active ingredients. Drug likeness assessment results are summarized in Table 1. According to SwissADME predictions, all compounds exhibit a relatively high bioavailability score and high intestinal absorption. Molecules are also predicted to easily cross the blood-brain barrier, which constitutes a limiting factor when dealing with CNS-targeting therapeutics.

Table 1
Drug-likeness assessment according to SwissADME predictions.

Compound	Bioavailability score	Intestinal absorption	BBB permeability
Dendrobine	0.55	High	Permeable
Dendroxine	0.55	High	Permeable
Nobilonine	0.55	High	Permeable

3.3. *DNLA target prediction*

Based on the Swiss target prediction server, 146 unique proteins were found to be a potential target of DNLA, most of which pertain to the 7-transmembrane receptor family and RIO-like serine/threonine protein kinase family. 38 protein targets were predicted for dendrobine, 53 for dendroxine and 76 were predicted to be targeted by nobilonine, totaling 167 protein targets. Fig. 4 depicts the DNLA-target network constructed

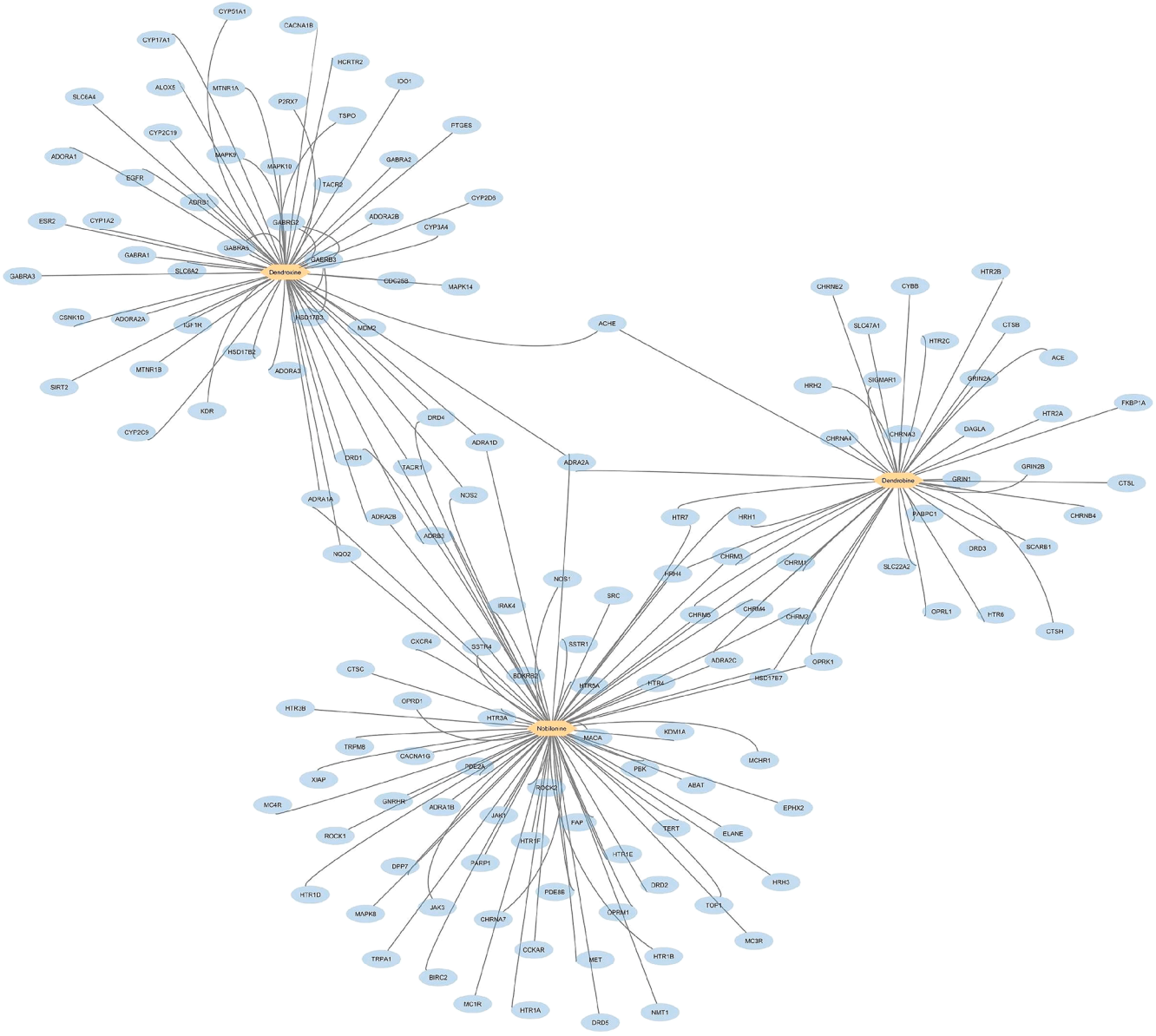

Fig. 4. DNLA-target network. Ellipses present predicted targets, hexagons represent *D. nobile* L. active ingredients. Figure created in Cytoscape (v3.10.1).

using Cytoscape. Upon a closer look, it seems that each network is isolated from the other, with little to no overlap between the three compounds, and ADRA2A being the only common target between the three. Denrobine and dendroxine, although structurally similar, share only one target, AChE, while 11 proteins were found to be common between dendrobine and nobilonine, six of which are different subtypes of muscarinic acetylcholine receptors. Additionally, nine proteins were predicted to be mutual targets for dendroxine and nobilonine. According to results, it seems that DNLA can target an array of therapeutically significant proteins and receptors, owing to the pharmacological and therapeutic relevance of the alkaloid class.

3.4. Common targets between ad and dnla

The Venn diagram in Fig. 5A highlights the overlap found between DEGs and the predicted targets for DNLA. 30 proteins were found to be common in both datasets, with targets belonging either to G-protein coupled receptors (GPCRs) family, ligand-gated ion channels or receptor tyrosine kinase family. Common proteins were imported into Cytoscape to construct the compound-disease-target network (DNLA-AD network). Fig. 5B depicts the DNLA-AD network, where a node signifies a protein and edges are the interactions interconnecting the proteins inside the network. It is evident that the three compounds modulate multiple targets at once, highlighting their multi-target potential, which is very relevant when dealing with a complex and multi-faceted disorder such as Alzheimer's disease.

3.5. PPI network

Common genes between AD and DNLA were used as a query in the STRING database. The database mines protein interactions based on experimental evidence, text mining and gene co-expression. The resulting PPI network is depicted in Fig. 6. The computed network is a tightly connected cluster consisting of 30 nodes and 95 edges, with the exception of one singleton (a node with no interaction) that was removed during the analysis. The network had a mean node degree of 6.33, an average number of neighbors of 6.55 and a network diameter of 6. HTR2A, HTR2C, GABRA1 and GRIN2B exhibited high stress values with an average score of 468, 276, 262 and 258, respectively. Stress assesses how critical and central a node is in a network, determined by

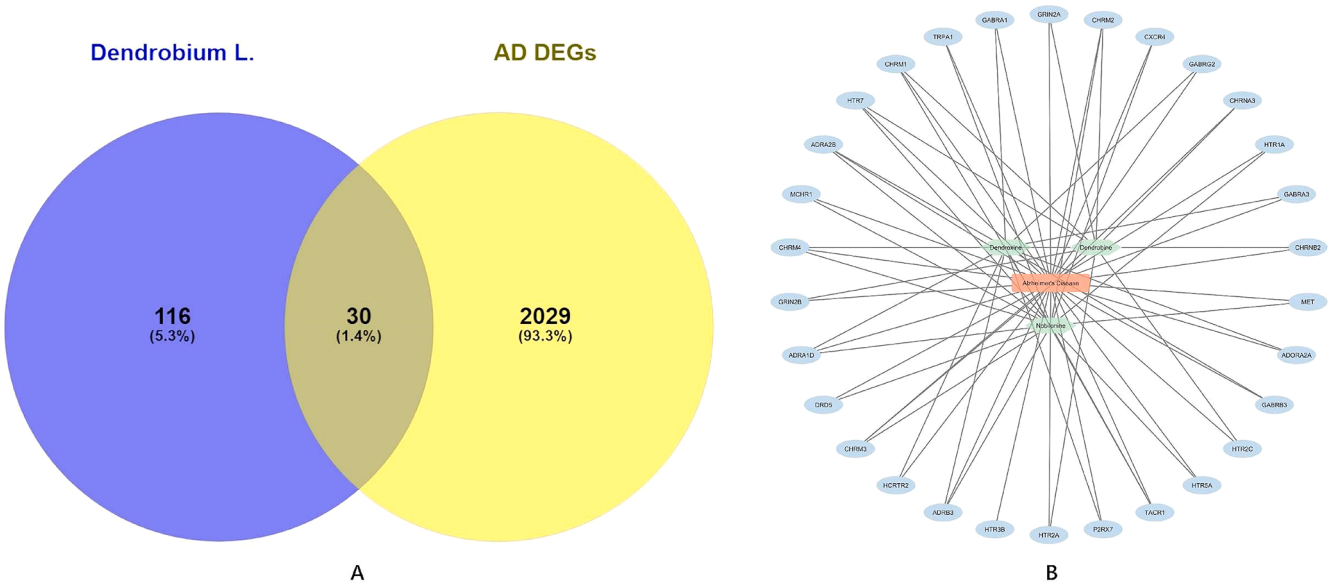

Fig. 5. (A) Venn diagram showing the overlap between AD DEGs & DNLA (B) DNLA-AD network, nodes represent protein targets and edges are the interactions linking the nodes together. Figure created from Venny database (v2.1.0) and Cytoscape (v3.10.1).

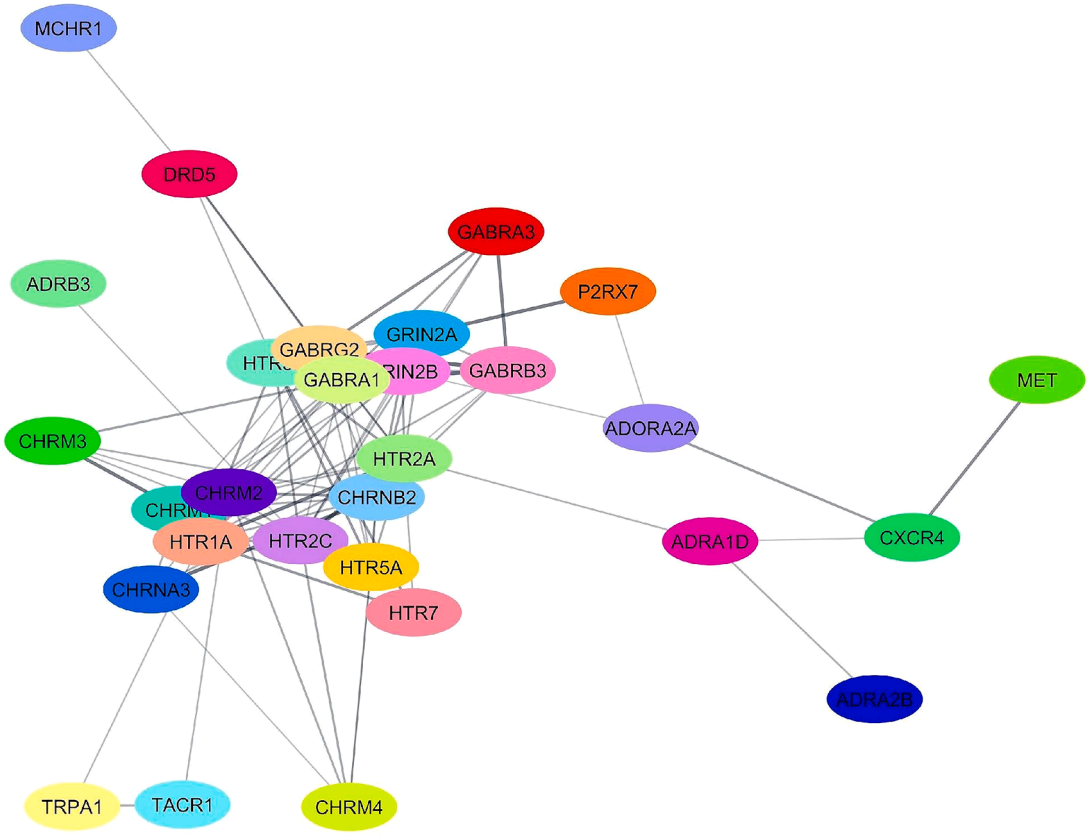

Fig. 6. STRING PPI network of key targets. The thickness of edges indicates the degree of confidence. Figure created from STRING database.

the number of shortest paths that passes through the node [43], this implies that HTR2A is central to the network, and controls the flow of information within the network.

3.6. Hub genes identification

MCODE plugin was used to mine significant sub-clusters that can be found in the PPI network. The cutoff criterion were set as follows: node score cutoff = 0.2, degree cutoff = 2, k-score = 2 and Max depth = 100.

The first cluster, Fig. 7, had 9 nodes and 32 edges, with an 8.5 cluster score. The cluster consisted of the following proteins: CHRNB2, GRIN2B, HTR2A, HTR1A, GABRB3, CHRM1, GRIN2A, GABRA1 and GABRG2, with the latter being the seed of the network with a degree centrality of 12 and a betweenness score of 37. These nodes were found to be mainly

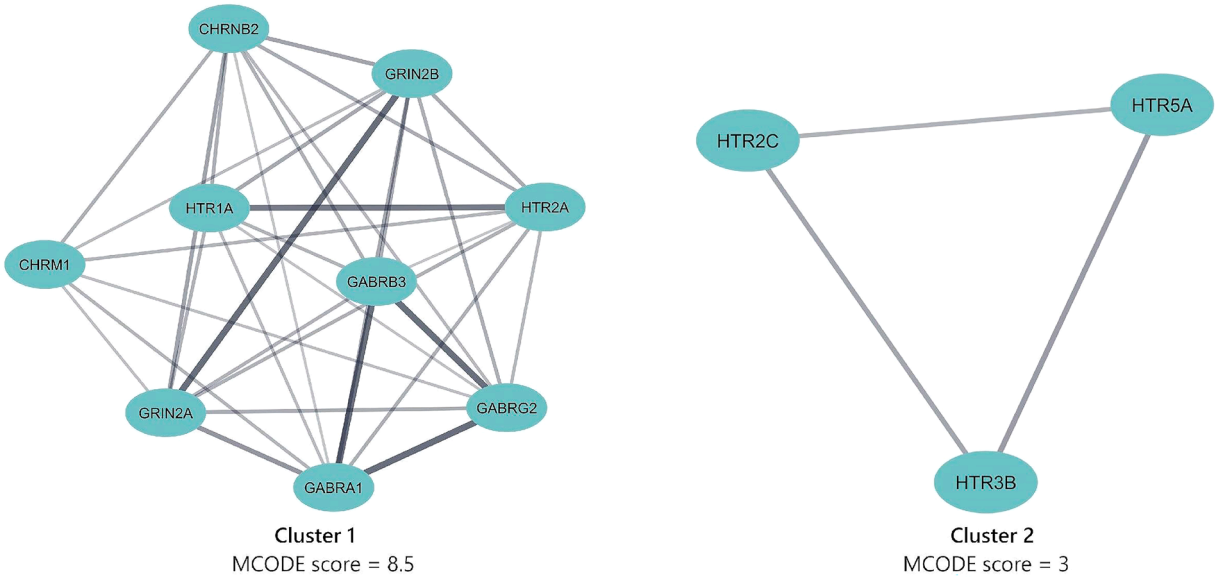

Fig. 7. The two clusters generated using MCODE algorithm. Nodes are represented as ellipses and interactions as edges. Figure created in Cytoscape (v3.10.1).

associated with signal transduction, transmembrane signaling receptor activity and serotonin metabolic process. Cluster 2 (MCODE score = 3), in Fig. 7B, included HTR3B, HTR5A and HTR2C, with HTR3B as the seed gene and a degree and betweenness centrality of 9 and 33, respectively. The nodes are involved in a number of biological processes, of which serotonin receptor signaling pathway, chemical synaptic transmission and neurotransmitter receptor activity were among the most significant.

The PPI network was further analyzed using the cytoHubba plugin to screen potential hub genes. Three local network algorithms (MCC, MNC and degree) and one global algorithm (betweenness) were used to get

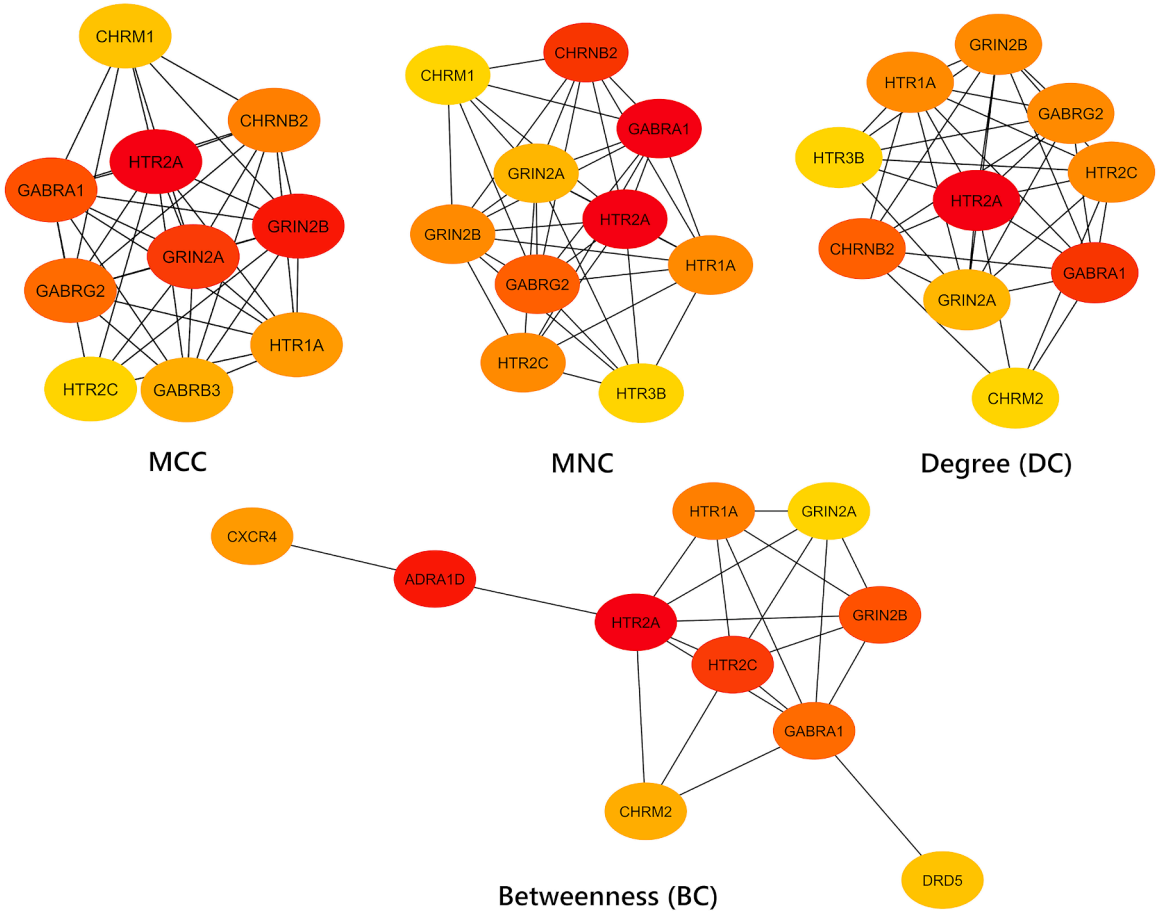

Fig. 8. Hub gene network identified by applying three local and one global network from protein-protein interaction network analysis. Nodes are arranged in decreasing order, where darker colors indicate that the node has a high ranking. Figure created in Cytoscape (v3.10.1).

the top 10 nodes in the network. Degree is a local metric based on the sum of interactions (edges) each node has, it describes how connected a node is to the network [44]. Nodes with high betweenness are regarded as intermediaries between two other nodes. Maximum neighborhood component (MNC), describes how connected the neighboring nodes are [45]. Maximal clique centrality, or MCC for short, is another local algorithm that identifies nodes that are connected to maximal cliques within the network [46]. Nodes with high MCC scores could be involved in a number of biological processes.

The plugin takes the nodes and edges in the network as input and ranks them based on their algorithm score. The top 10 nodes for each algorithm are depicted in Fig. 8. In detail, nodes were not consistent across all algorithms; CHRNB2, GABRG2 were common in MCC, MNC and degree, while CHRM1 was found in both MNC and MCC algorithms. In total, six nodes were common in all four algorithms, namely HTR2A, GRIN2B, GABRA1, HTR2C, HTR1A, GRIN2A. These genes were identified as hub genes.

3.7. Centrality analysis

The topology of the previously identified hub genes was further analyzed using the CytoNCA plugin in Cytoscape. In order to assess the importance of these hub genes in the network, four common metrics were computed: Degree centrality (DC), Eigenvector centrality (EC), Betweenness centrality (BC) and closeness centrality (CC). Eigenvector centrality is a global metric that measures the importance of a node based on the number of interactions it establishes and the centrality of the neighboring nodes [47], while closeness centrality measures how well connected a node is to the network and calculates the length of the shortest path to all other nodes in the network [48].

The mean value of each centrality metric was set as a threshold to identify core nodes: degree mean value = 6.33; eigenvector mean value = 0.14; betweenness mean value = 34.53; closeness mean value = 0.016.

Results of the centrality analysis are tabulated in Table 2. All nodes exhibit high centrality scores and are well above the set threshold, meaning that all selected genes play a crucial and influential role in the network. Interestingly, HTR2A has the highest betweenness score (185.9) across all nodes while the mean betweenness value is 34.53, meaning that HTR2A acts as a bottleneck that greatly regulates the flow of information to the other nodes and is a crucial mediator inside the network.

It is worthwhile to mention that degree centrality for HTR2A and GABRA1 was two-fold greater than the mean value (Degree mean value = 6.33). The same observation can be made for Eigenvector centrality and betweenness, where HTR2A, GABRA1, GRIN2B and HTR2A, GABRA1, GRIN2B, HTR2C are two-times greater than the threshold, respectively. We can infer that these genes are key players in the network.

3.8. Validation of selected hub genes

The expression of all six hub genes showed a significant decrease in AD compared to control cases, as shown in Fig. 9A.

ROC curves were plotted in order to assess the extent selected hub genes can distinguish control cases from AD samples. Results are depicted in Fig. 9B. According to the plots, it seems that the six genes have good discriminatory power, meaning they could correctly stratify healthy controls from AD patients. AUC values for each gene fall within the expected range, with values ranging from 0.74 (HTR2A), 0.7 (GRIN2B), 0.76 (GABRA1), 0.71 (HTR2C) to 0.74 (GRIN2A), suggesting that these genes have good prognostic value except for HTR1A having a moderate AUC value of 0.63.

3.9. GO and kegg pathway enrichment analysis

Gene enrichment analysis showed that the selected hub genes were found to be enriched in a number of neurotransmission related processes. Results are summarized in Fig. 10.

In detail, the top three enriched cellular component (CC) terms were postsynaptic membrane, synaptic membrane and ion channel complex, all related to synaptic transmission. The most significant biological processes were regulation of postsynaptic membrane potential, regulation of membrane potential and excitatory postsynaptic potential. These processes were shown to be related to modulation of neurotransmitter-gated ion channels, neurotransmitter release and overall the modulation of membrane potential at the synapse. While neuroactive ligand-receptor interaction (hsa04080), nicotine addiction (hsa05033), calcium signaling pathway (hsa04020) and serotonergic pathway (hsa04726) were among the most enriched KEGG pathways. Figs. 11 and 12 represent a schematic illustration of the synaptic pathway and calcium signaling targeted by *D. nobile* alkaloids, respectively.

The dysregulation of these pathways and processes are widely known to be closely related to an array of neurodegenerative pathologies, including AD. Neurotransmission related processes are key contributors to the manifestation of AD, whereby any dysfunction in neuroactive receptors or imbalance in neurotransmitters' levels, especially acetylcholine, serotonin and glutamate is at the origin of AD's symptoms [49,50]. Calcium signaling disturbances have been observed in a number of AD affected brain tissue [51]. At the intracellular level, calcium levels are maintained at low concentrations by a number of calcium pumps and channels [52]. Several lines of evidence verified that the dysregulation of calcium homeostasis can lead to neurite degeneration and thereby neurodegeneration and memory dysfunction [53,54]. Nicotinic acetylcholine receptor (nAChR) system is severely compromised in AD brain; evidence has confirmed a significant decrease in the number of nAChRs in AD and PD patients, due to the reduction of nAChR expression [55]. This marked decrease of nAChR density is strongly correlated to memory loss and cognitive symptoms that AD patient's exhibit [55]. As a result, nAChRs have been the focus of several studies in the context of new AD-directed therapeutics [56]. Serotonergic dysfunction is another driving force behind AD pathology. Several studies are supportive of a link between the serotonin system and AD, which stems from the fact that the serotonin system regulates an array of behavioral and cognitive processes, including learning and memory function, both of which are severely impaired in AD [57,58]. Additionally, studies have described extensive serotonin denervation in AD [59]. By targeting these pathways, DNLA could alter several key proteins and pathways at the origin

Table 2
Centrality analysis of the selected hub genes.

Gene symbol	Description	Degree (DC)	Eigenvector (EC)	Betweenness (BC)	Closeness (CC)
HTR2A	5-Hydroxytryptamine Receptor 2A	15	0.33	185.9	0.397
GABRA1	Gamma-Aminobutyric Acid Type A Receptor Subunit Alpha1	14	0.32	71.9	0.381
GRIN2B	Glutamate Ionotropic Receptor NMDA Type Subunit 2B	12	0.29	85.7	0.371
HTR2C	5-Hydroxytryptamine Receptor 2C	12	0.25	91.0	0.367
HTR1A	5-Hydroxytryptamine Receptor 1A	12	0.28	66.7	0.367
GRIN2A	Glutamate Ionotropic Receptor NMDA Type Subunit 2A	11	0.27	49.3	0.358

Baseline: Degree mean value = 6.33; Eigenvector mean value = 0.14; Betweenness mean value = 34.53; Closeness mean value = 0.016.

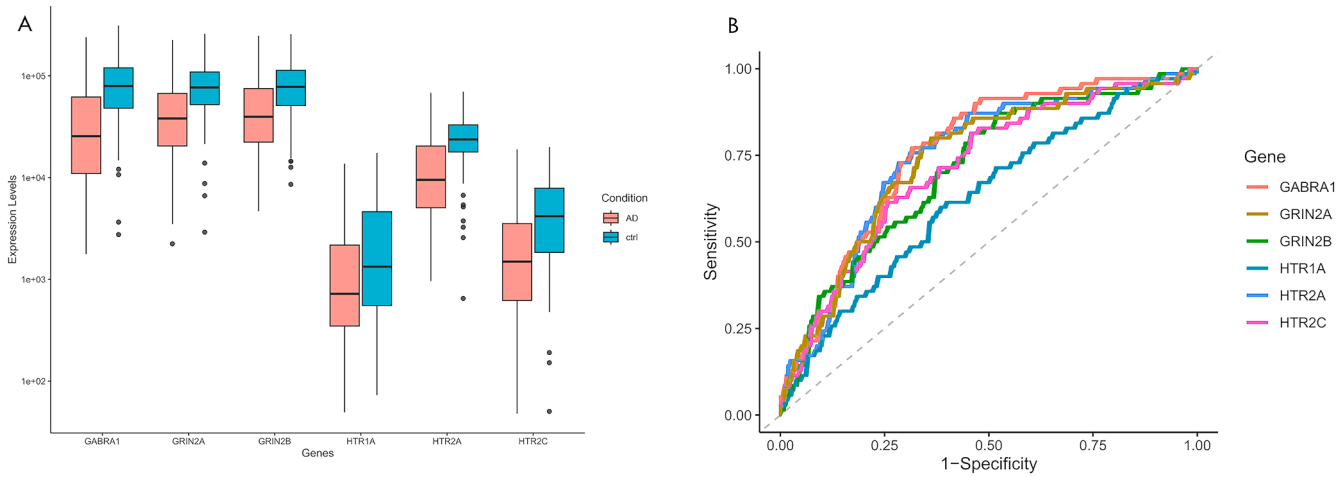

Fig. 9. (**A**) Expression profile of hub genes in control vs. AD (**B**) ROC curves of hub genes. Figure created in R (v4.3.1).

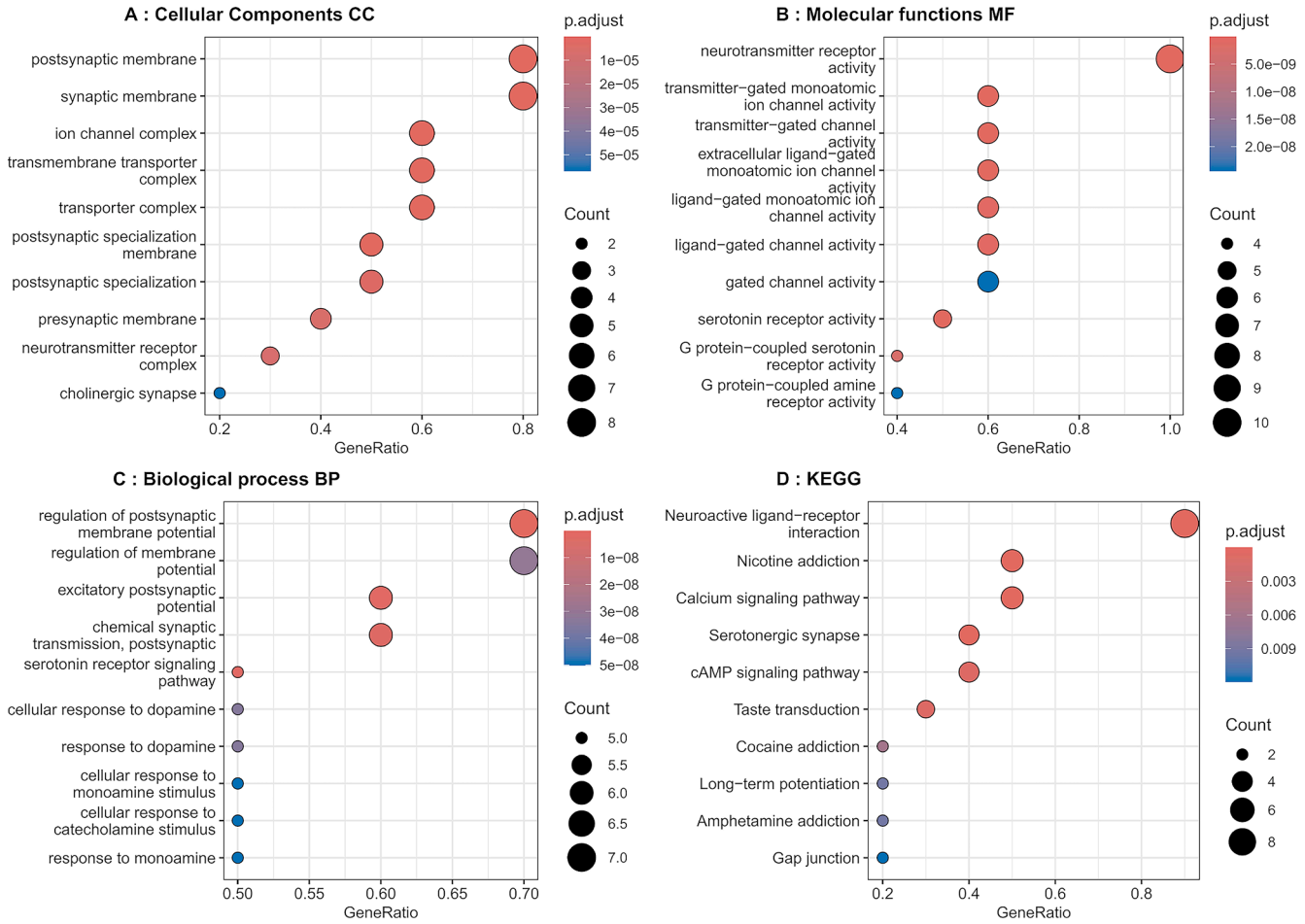

Fig. 10. Bubble plots of the top 10 enriched Gene Ontology (GO) terms (A, B, C) and major enriched KEGG pathways (D). Figure created in R (v4.3.1).

of AD.

4. Discussion

TCM has been the primary healthcare option in several parts of Asia and especially in China, its country of origin, and is considered an effective prevention strategy for an array of disorders, ranging from inflammation, gastrointestinal disorders to chronic disorders like rheumatoid and obesity. TCM has been in practice for over 2000 years with a strong track record in providing medical relief in instances where western medicine was not always accessible or/and affordable. The weight of TCM in relevance to modern medicine has been challenged in multiple instances; *Schisandra chinensis,* prescribed in TCM for hepatitis, served as a blueprint for the development of Dimethyl dicarboxylate biphenyl (DDB) as a treatment for viral hepatitis, the development of anti-cancer drugs based on camptothecin moiety, an active compound

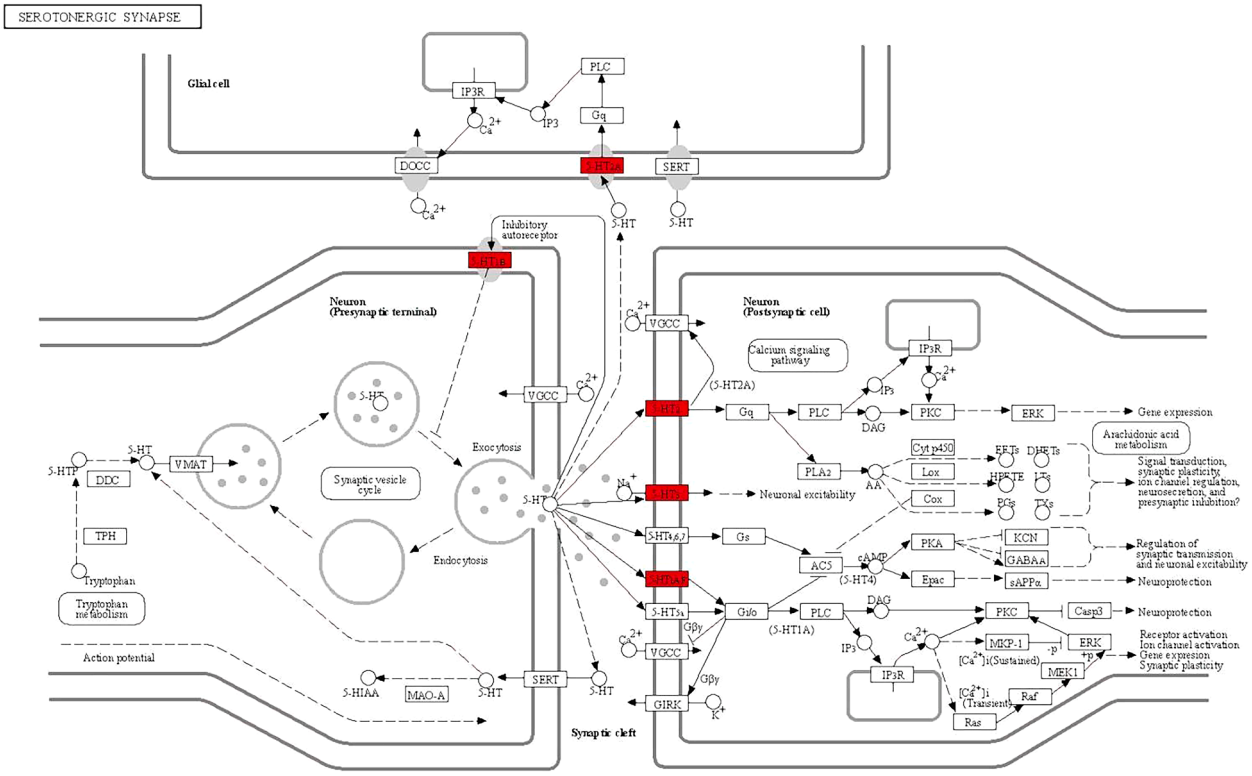

Fig. 11. Schematic depiction of the serotonergic pathway on which DNLA can act on. Nodes in red are potential targets of DNLA. Lines represent activation, dashed lines represent inhibition. Figure created in R (v4.3.1).

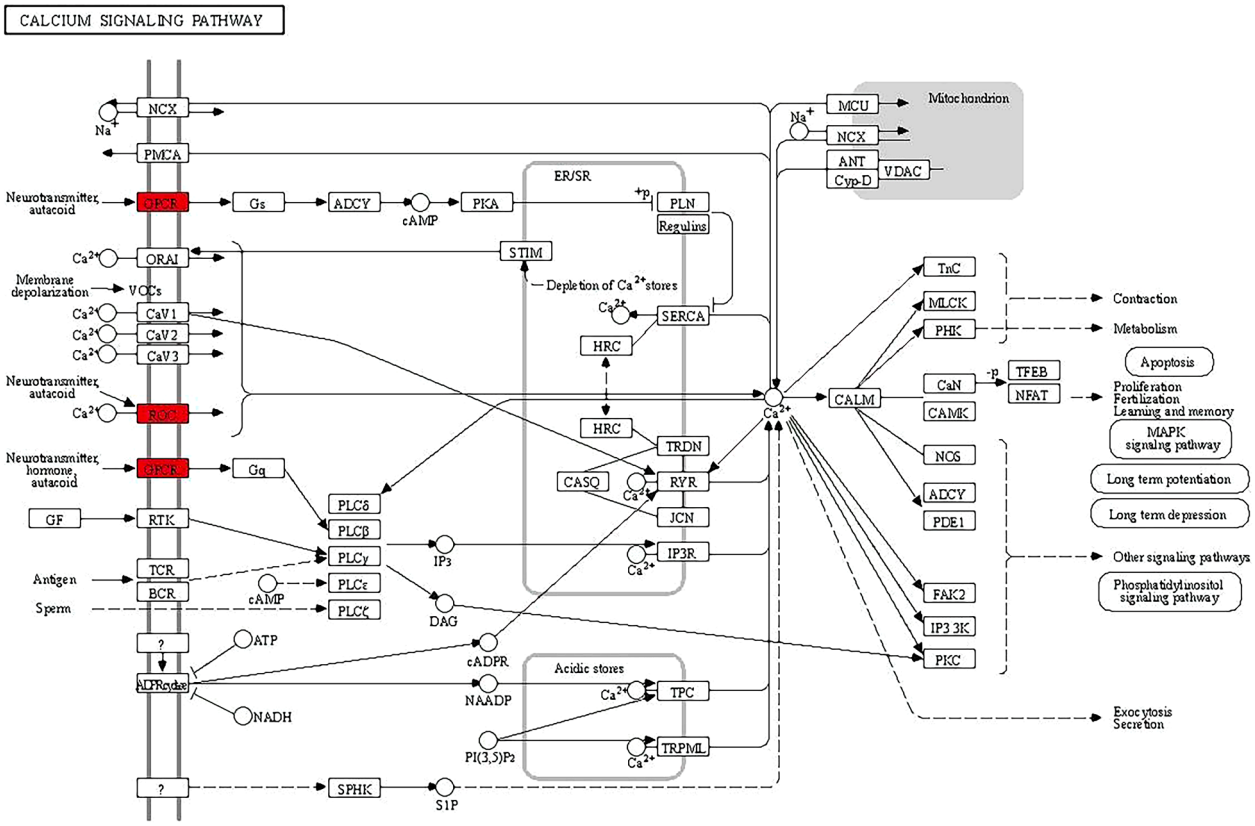

Fig. 12. Schematic depiction of the calcium signaling pathway targeted by DNLA. Nodes in red are potential targets of DNLA. Lines represent activation, dashed lines represent inhibition. Figure created in R (v4.3.1).

found in *Camptotheca acuminata* and the discovery of artemisinin, a sesquiterpine derived from *Artemisia annua* for its action against Malaria [60].

Chinese medicine offers a holistic approach for treating complex and heterogeneous conditions by targeting different facets of the disorder instead of focusing on a single pathway. For instance, in neurodegenerative disorders, TCM has proved its efficacy by promoting neurogenesis, inhibiting neuroinflammation, preventing neuronal apoptosis and triggering the clearance of toxic protein aggregates [61]. This multi-pathway feature gives TCM the advantage over conventional monotherapies that act on a single therapeutic target. Recent evidence has highlighted the potential applicability of TCM in clinical settings; *Curcuma longa*, commonly used in Chinese medicine, was found to promote autophagy in AD mice models, by targeting PI3K/AKT/MTOR pathway [62]. Similarly, in a 24-week randomized controlled trial, Lhl et al. reported that *Ginkgo bilob*, traditionally prescribed for memory loss and cognitive disorders, had positive effects on cognitive functioning and neuropsychiatric symptoms when administered to patients diagnosed with AD and vascular dementia [63]. *Huperzia serrata*, used in TCM for memory loss, was also found to significantly improve memory retention in vivo, have neuroprotective properties against Aβ in vitro, while being more effective in improving memory and behavior of AD patients compared to commercially available AD drugs [64].

This large body of evidence all point to the application of TCM-derived medicinal herbs, in a clinical context, for the management of neurodegenerative disorders, especially AD, while further emphasizing the potential of TCM and herbal drug formulation in providing new templates for the drug development pipeline.

Another medicinal plant that has become the focal point of modern medicine is *Dendrobium nobile* L., a top-grade plant in TCM and is the main ingredient in the preparation of Shihu. It is well documented that *D. nobile* alkaloids exert their neuroprotective effects in AD animal models by counteracting Aβ deposition and attenuating the hyperphosphorylation of Tau proteins. While these two features are the cornerstones of AD etiology, the mechanism underlying the effect of DNLA on other AD-associated dysregulated pathways is still uncharted.

Analysis of SRP181886 revealed a distinct brain expression pattern in AD patients in comparison to their healthy counterparts, with 2069 genes deemed differentially expressed. The six identified hub genes, HTR2A, GRIN2B, GABRA1, HTR2C, GRIN2A and HTR1A had high degree and interconnectivity in the network, with HTR2A as a bottleneck node, signifying its importance in mediating the signaling process and the cross talk between the other nodes inside the PPI network. Additionally, the diagnostic accuracy of the hub genes was examined; all genes had good diagnostic performance (AUC $>$ 0.7, except for HTR1A) and were accurate in distinguishing AD from control samples.

HTR2A and HTR2C are part of the serotonin receptor family, a class of GPCR transmembrane proteins, deeply involved in learning and spatial memory in mammals. These receptors have been extensively studied in the context of age-related disorders, such as AD and dementia. In AD, the serotonergic system undergoes substantial changes; serotonin receptors witness a decrease in number and density, especially in the raphe nucleus, along with a dip in the levels of serotonin [65–67]. This is consistent with the results reported in the hub gene validation section, where the expression profile of HTR1A, HTR2A and HTR2C was significantly lower in AD samples compared to their healthy counterparts.

Several lines of evidence have shown that the serotonin receptor superfamily is involved in the modulation of calcium pathways. According to Fig. 12, upon DNLA binding, activated GPCRs (HTR1A, HTR2A and HTR2C) activate phospholipase C (PLC), which in turn stimulates the production of diacylglycerol (GAP) and inositol 1,4,5-triphosphate ($InsP_3$). The release of GAP promotes the activation of the protein kinase C (PKC) signaling pathway, while $InsP_3$ binds to IP3 receptors (IP3R) which triggers calcium mobilization by releasing $Ca^{2+}$from the endoplasmic reticulum (ER). It is well documented that the dysregulation of calcium signaling triggers the generation of Aβ plaques [68], by modulating calcium influx through the GPCR system, DNLA can rectify the intracellular calcium imbalance seen in AD and indirectly inhibit the accumulation of toxic Aβ plaques. At the synapse level, the serotonin receptors couple to the extracellular-signal regulated kinases (ERK) through the activation of PKC, as described in Fig. 11, which promotes synaptic plasticity and ion channel activation. Additionally, the activation of PKC inhibits the expression of the CASP3 gene, a key player involved in the development of neuronal apoptosis [68]. Recent studies support a causal link between CASP3 and Aβ formation; as stated earlier, Aβ peptides are a byproduct of the proteolysis of APP, this reaction can either be catalyzed by α-secretase, which cleaves APP into neuroprotective soluble sαAPP, or by β-secretase (i.e. BACE-1) and γ-secretase promoting the production of toxic Aβ peptides. It has been shown that the overexpression of CASP3 triggers β-secretase activity, feeding the cycle of toxic Aβ formation [69]. By targeting the serotonin signaling pathway, DNLA can regulate the processing of APP into toxic Aβ fragments.

GRIN2A and GRIN2B genes encode subunit NR2A and NR2B of NMDA receptor (NMDAR), a glutamate-gated ion channel with a high Ca^{2+} permeability, involved in excitatory neurotransmission, synaptic plasticity, learning and memory function. Notably, NMDARs along with GABA receptors are considered key players in fine-tuning the excitatory/inhibitory (E/I) balance, crucial for maintaining normal neuron circuit activity and cognitive function. Aβ peptides have been known to trigger calcium influx through the activation of NMDAR, which in turn promotes mitochondrial dysfunction. This calcium overload can directly stimulate enzymes responsible for cell and membrane damage, leading to neuronal death [70]. Due to their expression in microglia and astrocytes, studies have postulated the involvement of NMDAR in neuroinflammation and excitotoxicity. Evidence has shown that activation of NMDAR stimulate the release of inflammatory mediators by activating microglia, while their chronic activation by glutamate induces mitochondrial Ca^{2+}release and excitotoxic damage as a result [71]. The release of these pro-inflammatory cytokines induces NMDA-mediated currents and causes intracellular calcium increase, which underlie the possible role of NMDAR in neuroinflammation. Some studies have postulated the involvement of DNLA in suppressing neuroinflammation, however the exact mechanism by which DNLA act is still elusive [72, 73]. Our results indicate that this anti-inflammatory effect could be mediated by NMDARs.

Several studies have also highlighted the interplay between NMDARs, Aβ plaques and the ERK pathway; recent evidence has shown that the activation of synaptic NMDARs activate ERK signaling pathways, promoting the processing of APP into neuroprotective sαAPP [74, 75]. Additionally, by activating the ERK signaling pathway, NMDARs can regulate the phosphorylation of Tau proteins and their cleavage [76]. In this sense, DNLA not only can overturn calcium imbalance triggered by Aβ peptides and inflammatory mediators through the regulation of NMDARs, and regulate the protease of Tau proteins by stimulating the ERK pathway, but also hinder the release of pro-inflammatory cytokines, a major driving force of neuroinflammation.

GABRA1 gene encodes one of the major inhibitory neurotransmission systems in the brain; the Gamma-aminobutyric acid (GABA) receptors [77]. The GABAergic system consists of two types of receptors: chloride-gated channels, which include $GABA_A$ and $GABA_C$ and metabotropic G-protein coupled receptors, $GABA_B$, with the former being expressed predominantly in the brain. The GABAergic system along with NMDARs play a pivotal role in maintaining the balance between excitatory and inhibitory synaptic transmission. Any imbalance at the level of certain neurotransmitters (mainly glutamate and GABA), or disruption of the normal functioning of these receptors could overthrow the E/I balance, jeopardizing, as a result, the synaptic function and inducing cognitive and memory impairment. The GABAergic signaling is said to be altered in AD brain, with evidence showcasing GABAergic neurons in

a hyper-activated state with decreasing levels of GABA in the temporal cortex of AD patients and animal models [77]. Other studies have elucidated that Aβ activated astrocytes could also release GABA, further driving the E/I imbalance seen in AD [78]. Aβ plaques prompt E/I disruption, while E/I imbalance has been shown to induce Aβ pathology, suggesting the existence of a feedback loop [79]. DNLA can break that loop by targeting both the GABAergic and glutamatergic system (NMDARs) to restore the E/I balance along with normal neuronal function.

In light of the reported results, it seems that the effect of DNLA extends beyond the clearance of toxic Aβ and decrease in hyperphosphorylation of Tau proteins. As stated earlier, the calcium imbalance has been considered an upstream event in AD pathogenesis, meaning that the dysregulation of the calcium system predates the symptoms of AD. By regulating HTR2A, HTR2C and NMDAR, DNLA can counteract the exaggerated Ca^{2+}release and mitigate excitotoxicity. AD has also been linked to the disruption of glutamatergic and GABAergic systems, wherein the over-stimulation of both systems prompts an imbalance in E/I neurotransmission. DNLA can restore that balance through the modulation of NMDAR and GABA receptors. Aside from excitatory neurotransmission, microglia expressed NMDARs also modulate the release of pro-inflammatory cytokines, underlying the possible involvement of DNLA in neuroinflammation.

This study intends to reinforce the relevance of TCM in reference to mainstream medicine by investigating the mechanisms underlying DNLA in opposing AD progression. Findings reported herein support the rationale behind the use of *D.nobile* in TCM, while revealing the novel multifaceted potential of DNLA in AD therapy. DNLA hold promise in offsetting intracellular calcium overload, one of the most critical upstream events in AD pathology, and can additionally alter E/I imbalance and neuroinflammation. In the absence of an effective therapy that could target different aspects of the pathology, DNLA makes a promising multi-target candidate for the management of AD. Further studies are warranted to assess the tolerability of DNLA in clinical settings.

5. Conclusion

DNLA was found to regulate major signaling events that may predate Aβ formation and Tau polymerization, particularly the calcium dysregulation. Findings suggest that DNLA can enhance calcium release through the glutamatergic and seretonergic systems, which might influence the amyloidogenesis cascade. DNLA also promise to curb erratic excitatoty/inhibitory neurotransmission and neuroinflammation, by regulating NMDAR and GABA receptors.

Limitations

This study has highlighted the therapeutic potential of DNLA in counteracting calcium dyshomeostasis and excitotoxicity, however considering the notorious nature of alkaloids, further studies detailing the toxicity potential of DNLA are needed. Additionally, it is important to note that the samples in SRP181886 were taken from postmortem tissues of patients with late-stage AD (BRAAK stage V and VI). This study was limited to late-stage AD and did not include samples from early stages of the disorder. Considering that gene expression in AD is phase-specific, meaning that the expression pattern in early AD is different from that of later-stages, in this sense, the effects of DNLA on early AD will be different from results reported herein. In addition, the lack of experimental validation is also another limitation to consider.

Ethics statement

The dataset included in this study was downloaded from public repositories and there was no interaction with living human subjects.

Disclosure statement

No potential conflict of interest was reported by the authors.

CRediT authorship contribution statement

Iman Touati: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Yassir Boulaamane:** Writing – review & editing, Conceptualization. **Mohammed Reda Britel:** Resources, Project administration. **Amal Maurady:** Writing – review & editing, Validation, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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