Research Paper

In Silico Studies in Predicting Mechanism of Action of *Amaranthus tricolor* on Alzheimer's Disease

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Patil *et al.*: *Amaranthus tricolor* Modulates Pathways Involved in Alzheimer's Disease

There is a constant demand to develop an effective and affordable new drug entity to manage cognitive impairment. Medicinal herbs serve as natural resources for mining of new drug candidates. We used chemoinformatics to identify the bioactive compounds from *Amaranthus tricolor* L. a common leafy vegetable and often used in traditional medicine to manage cognitive dysfunction. We carried out a series of bioinformatics studies with the phytoconstituents of *Amaranthus tricolor* to elicit their possible molecular functions in cognitive disorders including Alzheimer's disease. We first identified the bioactive phytoconstituents from *Amaranthus tricolor* and predicted their potential protein targets involved in the pathogenesis of cognitive dysfunction using BindingDB (p≥0.7). Gene ontology functional enrichment analysis was performed using search tool for the retrieval of interacting genes/proteins. The pathways that are probably regulated by the identified plant phytoconstituents were analyzed using Kyoto encyclopedia of genes and genomes. Docking studies were carried out with AutoDock4.2v. Molecular dynamics analyses were performed using Schrodinger Desmond 6.1v software for a 50 ns production run. Thirty nine phytoconstituents were identified in *Amaranthus tricolor*, five of which were predicted to modulate eight potential protein targets involved in cognitive impairment. Gene ontology functional enrichment analysis revealed twenty eight biological processes and 10 molecular functions associated with cognitive impairment. Kyoto encyclopedia of genes and genomes pathway identified eight pathways that are directly related to cognitive impairment. Serotonergic and cholinergic synapse was identified as key pathways. Kaempferol exhibited the highest binding affinity with acetylcholinesterase, monoamino oxidase A and monoamino oxidase B. Molecular dynamics simulation demonstrated stable intermolecular interactions between kaempferol and acetylcholinesterase. Our study identified flavonoids from *Amaranthus tricolor* as having benefits in managing cognitive impairment and offers a broader scope to mine for potential drug candidates from this natural resource. Our study was limited to computer simulations and calls for wet lab validation of the predicted molecular functions.

Key words: *Amaranthus tricolor*, cognitive dysfunction, molecular docking, molecular dynamics, gene ontology, network pharmacology

Cognitive impairment is a complex disorder of old age people characterized by changes in cognitive functions like trouble in remembering, concentrating, learning and decision making. A significant increase in life expectancy in the twentieth century has resulted in conditions like Alzheimer's Disease (AD) becoming the most common neurocognitive disorder with high incidence and intricate pathogenesis. AD is characterized by the presence of extracellular deposits of insoluble amyloid-beta (β) plaques, Neurofibrillary Tangles (NFT) and cholinergic deficits[1]. From a clinical point of view, AD has a strong impact on the lifestyle of patients which is characterized by a prodromal phase with a subsequent progressive loss of memory and decline of cognitive functions, leading to the need for continuous medical care[2]. The primary clinical features of AD are loss of memory and memory impairment at the early stage, subsequent topographical difficulties, loss of attention,

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confidence and judgment[3]. The five drugs approved by the US Food and Drug Administration (FDA) that are currently used to treat the cognitive manifestations of AD *viz.* Acetylcholinesterase (ACHE) inhibitors rivastigmine (Exelon), tacrine (Cognex), donepezil (Aricept), galantamine (Razadyne) and N-methyl-D-aspartate (NMDA) receptor antagonist memantine (Namenda)[4]. However, these agents lose effectiveness as the disease progresses due to their actions on a specific protein i.e. donepezil acts on ACHE enzyme and memantine on NMDA receptor. These are also associated with numerous side effects i.e. bradycardia, hypotension, increased respiratory secretion, decreased intraocular pressure and vomiting[5,6].

With increasing life expectancy, the number of geriatric people (>60 y of age) is increasing worldwide. In 2015, about 47 million people were living with dementia and it is expected to triple by 2050[7]. The increase in the number of people with dementia has been exponential in Low Middle-Income Countries (LMICs) like India. Knowledge about the risk factors of developing dementia in LMICs are scanty and very little has been done at ground level for its prevention and treatment. India is going through a significant epidemiological transition with a tremendous increase in non-communicable diseases. There is a strong correlation between the onset of dementia and age. A study carried out in the United States in 2014 estimated a reduction of dementia cases by 2 million in the USA alone by 2020 if any intervention could delay the onset of dementia only by only 2 y[8]. Therefore dementia cases can be delayed and the number of cases decreased by an effective intervention strategy in an aging population. The economic implication of delaying dementia in a resource-limited country like India is tremendous. Since the pathophysiology of dementia starts years before the actual manifestation of symptoms, there is a window of opportunity to intervene and prevent or delay the clinical manifestations[9]. Therefore attempts are being made to 'delay' dementia because there is no ideal drug to prevent it or cure it completely. In the effort to delay dementia/cognitive impairment, several approaches are being tried, some of which rely upon traditional/alternative/complementary forms of medicine and even their combination[10,11].

Ayurveda is an ancient Indian holistic system of medicine that primarily uses plants and minerals in prescriptions that play an important role in managing various diseases, including cognitive disorders, because of their therapeutic effects, often on multiple targets[12]. The introduction of the concept of systems biology into Ayurveda research has opened up a new vista to gain systematic insights into the holistic understanding of the effects of multiple compounds on multiple targets through traditional medicine' complex network analysis. Investigation into the role of traditional herbs for the management of complex diseases like dementia and cognitive disorders has enormous potential and can open up opportunities for the discovery of new drugs for these conditions following the concept of multi-drug, multi-target and multi-pathway approaches.

'Gene Ontology (GO) functional enrichment analysis' and 'network pharmacology' are the two emerging areas of pharmacology that deal with the concept of "multicomponent therapeutics and network targets". These disciplines provide new insights for elucidation of the multi-scale mechanisms of action of herbs in the management of complex diseases like AD[13]. The use of network pharmacology and bioinformatics in research on herbal medicines with classical pharmacognosy and pharmacology has greatly facilitated mechanistic studies on the synergistic/antagonistic actions of herbal phytoconstituents at a molecular level[3,14].

Amaranthus tricolor L. (*A. tricolor*) (tambdi bhaji/lal saag) belongs to the family *Amaranthaceae*[15]. Amaranth (*Amaranthus* spp.) is cultivated and consumed as a leafy vegetable[16]. The leaves of this plant contain flavonoids, alkaloids, cardiac glycosides, phenol, amino acids, saponins, tannins, terpenoids, pterocarpans, steroids, quinones, resins and coumarins as major phytoconstituents. *A. tricolor* also contains amaranthine, kaempferol, ferulic acid, quercetin, apigenin, quercetin 3-o-glucoside, quercetin 3-orutinoside, apigenin 4-o-beta-d-glucopyranoside, feruloylquinic acid, boropinic acid, amarantholidols A, B, C betanin, 4-geranyloxyferulic acid, isoamaranthine, xylofuranosyl uracil, 7-p-coumaroyl, betaxanthin, 7-isopentenyloxycoumarin, etc. The leaves are used as traditional medicine and reported for antioxidant, anti-inflammatory and neuroprotective activity[17-21].

However, there is no credible information on the potential targets regulated by *A. tricolor* for the management of cognitive impairment. GO functional enrichment analysis, network pharmacology and wet lab experiments have not been reported on this potentially important natural herb. In the current study, we used computational tools and public scientific data to investigate the pharmacological interaction of bioactive phytoconstituents from *A. tricolor* with potential protein targets and pathways for the management of cognitive

impairment. The complete workflow of this study is represented in fig. 1.

MATERIALS AND METHODS

Identification of bioactive phytoconstituents from *A. tricolor* and target screening:

Bioactive phytoconstituents of *A. tricolor* were identified from Dr. Dukes Database (DB), Phytochemical Interaction DB (PCIDB)[22] and scientific journals using the keyword "*A. tricolor*". The Compound Identification number (CID), canonical Simplified Molecular Input Line Entry System (SMILES), Molecular Formula (MF), Molecular Weight (MW), Number of Hydrogen Bond Acceptor (NHBA) and Number of Hydrogen Bond Donors (NHBD) were retrieved from the PubChem chemical database[23]. Canonical SMILES were queried for target prediction in BindingDB[24] at the probability score of ≥0.7 (≥70 %) with respect to the known chemical compounds targeting protein molecules. Gene ID of each protein molecule was retrieved from UniProt[25]. The protein molecules associated with cognitive impairment were separated with reference to the successful and approved targets reported in the Therapeutic Target Database (TTD)[26].

GO functional enrichment analysis and network construction:

Search Tool for the Retrieval of Interacting Genes/ Proteins (STRING) is a universally utilized system

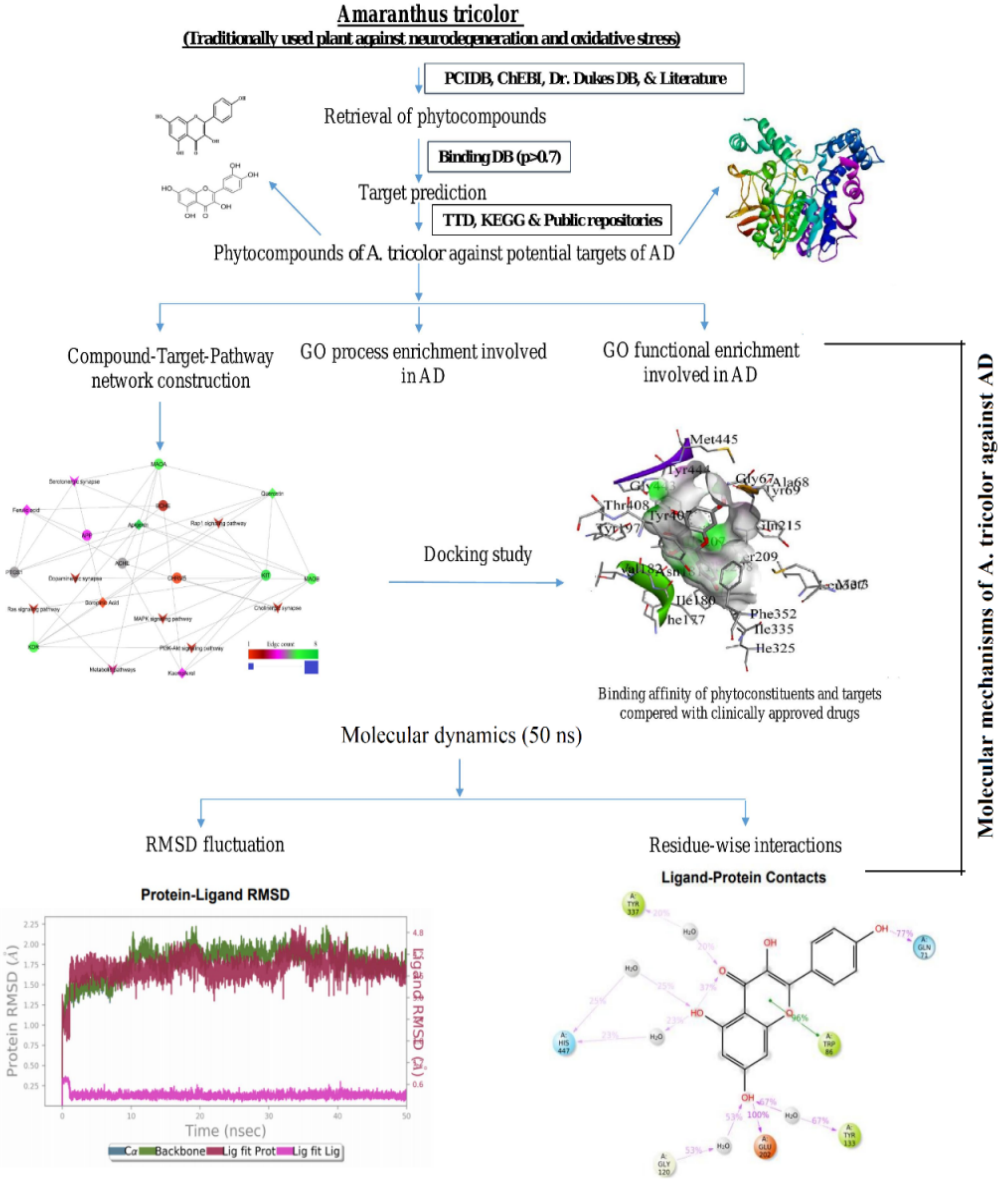

Fig. 1: Detailed workflow of the present study

for retrieval of protein-protein interaction, known interaction, predicted interaction, process and function of genes, etc. To understand protein interactions systematically, predicted targets were provided as input to STRING 11.0 (Search Tool for the Retrieval of Interacting Genes/Proteins, https://string–db.org) to obtain relevant information on protein interaction. The GO method can efficiently identify the process related to biological phenomena and helps to get more meaningful gene functional information. GO functional enrichment analysis was performed using the functional annotation tool of STRING. The Kyoto Encyclopedia of Genes and Genomes (KEGG), (https://www.genome.jp/kegg/pathway.html) database was used to identify the pathways involved in cognitive impairment. Cytoscape v3.6.1[27] was used to construct the network between compounds, protein molecules and pathways. The network was analyzed by choosing the command "network analyzer" and the network was treated as direct. Layout algorithm (degree sorted layout) was applied in constructing the network.

Druglikeness and probable side effects of *A. tricolor* phytoconstituents:

Druglikness property of the phytoconstituents were predicted using Molsoft (http://molsoft.com/mprop/) an online server, which predicts the probable drug-like property based on Lipinski's rule of five, that eliminates compounds having poor absorptivity and bioavailability i.e. if their molecular weight is >500 g/mol, having >5 hydrogen bond donors, >5 log P and >10 hydrogen bond acceptors. ADVERpred (http://www.way2drug.com/adverpred/) an online tool, uses the data of most frequent and severe adverse drug events that have either known or probable relationships to drug consumption and predicts the possible side effects of a compound based on structure-activity relationship. In the current study, we used ADVERpred to predict the probable side effects (Probable activity (Pa) and Probable inactivity (Pi)) of phytoconstituents. The current study utilized the admetSAR2.0 (http://lmmd.ecust.edu.cn/admetsar2/). This online tool contains 2 10 000 experimental data for 96 000 drug candidates. It contains 27 computational models to predict the ADMET profiles i.e. absorptivity, Blood-Brain Barrier (BBB) permeability, oral bioavailability, cytochrome P450 (CYP450) and isoenzyme inhibitory activity, mutagenicity, plasma protein binding affinity and fish aquatic toxicity.

Ligand-protein docking studies:

The ligands were retrieved from the PubChem chemical database in a Three Dimensional (3D) structure data format (.sdf), minimized using the mmff94 force field and saved in protein data bank (.pdb) file by using MarvinSketch[28]. The protein molecules i.e. ACHE (PDB ID: 4PQE), monoamino oxidase B (PDB ID: 1OJD), monoamino oxidase A (PDB ID: 2Z5X) and Prostaglandin G/H Synthase 1 (PTGS1) (PDB ID: 3N8X) were retrieved from the Research Collaboratory for Structural Bioinformatics PDB (RCSB PDB) (https://www.rcsb.org/). The protein structure was prepared by removing heteroatom and water using Discovery Studio Visualizer v2019[29]. The docking of ligands with their respective protein molecules was performed using AutoDock4.2[30]. The ligand protein complex was viewed using Discovery Studio Visualizer v2019.

Molecular dynamics (MD) simulation studies:

MD simulation has become a popular tool in computational biology for analyzing the dynamic behavior of molecular complexes and intermolecular interactions under physiologically realistic circumstances[31]. The stability of intermolecular interactions between kaempferol and ACHE was assessed using MD simulation for 50 ns production run using Schrodinger desmond 6.1v software[32,33]. The complex system was built in a cubic box with 10 Å×10 Å×10 Å dimensions as the periodic boundary, using a preset Simple Point Charge (SPC) water model as the solvent. 5 Sodium (Na^+) counter ions (concentration of 6.533 mM) were added to neutralize the system. Water molecule geometry, bond lengths and bond angles of heavy atoms were all restrained using the SHAKE algorithm. To calculate the long range interactions between the molecules, the Particle Mesh Ewald method was used. The Lennard-Jones interactions cut-off was set to 10 Å. For a 100.0 ps production run, the system was minimized. Finally, the isobaric-isothermal ensemble (NPT) was used to maintain a pressure of 1.01325 bar and temperature of 300 K using the Thermostat "Nose-Hoover chain" method with 1.0 ps relaxation time and the Barostat "Martyana-Tobias-Klein" method with 2.0 ps relaxation time. The short range coulomb cut-off radius was adjusted at 9.0 Å. The entire simulation was analyzed for 5000 frames and recorded at an interval of 10.0 ps. The Root Mean Square Deviation (RMSD) and Root Mean Square Fluctuation (RMSF) were used to check the residue-wise interaction fluctuations and

the complex compactness was analyzed by the radius of Gyration (rGyr).

RESULTS AND DISCUSSION

Thirty nine phytoconstituents were identified in *A. tricolor* from different databases and other open source records. Among them, five compounds were predicted to modulate eight protein molecules involved in cognitive impairment, showing synergistic effect. Apigenin was found to modulate ACHE, Amyloid Precursor Protein (APP), Butyrylcholinesterase (BCHE), KDR, KIT, Monoamine Oxidase A (MAOA), Monoamine Oxidase B (MAOB) and PTGS1; boropinic acid modulated APP; ferulic acid modulated ACHE, APP, BCHE and PTGS1; kaempferol was found to modulate ACHE, KIT, MAOA and MAOB; while quercetin was found modulating ACHE, KDR, KIT, MAOA, MAOB and PTGS1. These phytoconstituents were identified as flavonoids, organoborane and phenolic compounds (Table 1).

The GO functional enrichment analysis result showed 121 biological processes and 18 molecular functions. The peer interpretation revealed 28 biological processes and 10 molecular functions were associated with cognitive impairment (Table 2 and Table 3). The result analyzed from the STRING database showed 19 possible pathways based on the protein-protein interaction. The peer interpretation of the protein-protein interaction using the KEGG pathway identified eight pathways that are directly associated with cognitive impairment. Among 8 pathways, the serotonergic synapse pathway scored the lowest false discovery rate with the highest edge count within the network (Table 4). The constructed network contains 42 edges. Among them, 23 are compound-protein interactions and 19 protein-pathway interactions (fig. 2).

Quercetin, apigenin and kaempferol showed positive drug-likeness scores i.e. 0.93, 0.77 and 0.77 respectively. Ferulic acid and boropinic acid showed negative drug-likeness property i.e. -0.44 and -0.41. Further, quercetin, apigenin and kaempferol were predicted to show hepatotoxicity. Ferulic acid showed myocardial infarction and boropinic acid showed cardiac failure and myocardial infarction. The drug-likeness character and toxicity profiles obtained are shown in Table 5. The predicted probability score for absorptivity, blood-brain barrier permeability, bioavailability, carcinogenicity, isoenzyme inhibition, plasma protein binding affinity and fish aquatic toxicity, etc., of the phytoconstituents of *A. tricolor* is shown in fig. 3.

Molecular docking performed on phytocompounds having drug-like property i.e. kaempferol, quercetin, apigenin and clinically approved drug candidates with ACHE, MAOB, MAOA and PTGS1 showed kaempferol having the highest binding affinity with ACHE, MAOA and MAOB i.e. -7.8 kcal/mol (1.92 μM), -8.28 kcal/mol (0.850 μM) and -8.6 kcal/mol (0.498 μM) respectively. Meloxicam showed the highest binding affinity with PTGS1 i.e. -7.64 kcal/mol (2.52 μM) with four hydrogen bond interactions. However, among apigenin and quercetin, apigenin showed the highest binding affinity with PTGS1 i.e. -4.4 kcal/mol (591.39 μM) with four hydrogen bond interactions. The binding energy, Half-Maximal Inhibitory Concentration (IC_{50}) and hydrogen bond interactions of each compound with their respective protein target are compared with clinically accepted standard drug molecule (Table 6). The interaction of kaempferol with ACHE is shown in fig. 4.

Kaempferol and ACHE complex includes 49 862 atoms with 13 916 water molecules. The system was neutralized by adding 5 Na^+ ions (6.533 mM concentration) and was simulated for 50 ns with a 10 ps recording interval. The RMSD of protein alpha Carbon (Cα) (0.738 Å to 2.219 Å) and backbone (0.764 Å to 2.23 Å) was found to be stable throughout the 50 ns simulation. The ligand RMSD values were within the range of 0.114 Å to 0.818 Å from 0 to 50 ns. The average RMSD of ligand with respect to protein was 3.806 Å and ligand with respect to ligand was 0.297 Å. The rGyr deviation of kaempferol with ACHE was found within 3.588 Å to 3.759 Å, which indicates the higher compactness of the kaempferol-ACHE complex.

TABLE 1: TYPE OF PHYTOCONSTITUENTS WITH THEIR PROBABLE TARGETS INVOLVED IN AD

Phytoconstituents	Compound type	PubChem ID	Targets modulated by phytoconstituents
Ferulic acid	Phenolic	445858	APP, PTGS1
Quercetin	Flavonoid	5280343	ACHE, ADORA2A, MAOA, MAOB, PTGS1, KIT, KDR
Apigenin	Flavonoid	5280443	APP, ACHE, BCHE, ADORA2A, MAOA, MAOB, PTGS1, KIT, KDR
Kaempferol	Flavonoid	5280863	ACHE, ADORA2A, MAOA, MAOB, KIT
Boropinic Acid	Organoboranes	10682896	APP

Note: AD: Alzheimer's Disease

TABLE 2: GO ENRICHMENT ANALYSIS OF PROTEIN TARGETS FOR THEIR BIOLOGICAL PROCESSES INVOLVED IN AD

GO ID	Genes modulated by compounds	Gene count	Biological function	FDR
GO:0001505	ACHE, ADORA2A, APP, BCHE, MAOA, MAOB	6	Regulation of neurotransmitter levels	3.33E-06
GO:0042133	ACHE, BCHE, MAOA, MAOB	4	Neurotransmitter metabolic process	5.96E-05
GO:0042135	ACHE, MAOA, MAOB	3	Neurotransmitter catabolic process	8.55E-05
GO:0007271	ACHE, ADORA2A, CHRM5	3	Synaptic transmission, cholinergic	0.00013
GO:0065008	ACHE, ADORA2A, APP, BCHE, KDR, KIT, MAOA, MAOB, PTGS1	9	Regulation of biological quality	0.00031
GO:0032222	ACHE, ADORA2A	2	Regulation of synaptic transmission, cholinergic	0.0011
GO:0007612	APP, BCHE, KIT	3	Learning	0.0029
GO:0006954	ADORA2A, APP, KIT, PTGS1	4	Inflammatory response	0.0039
GO:0048167	ADORA2A, APP, KIT	3	Regulation of synaptic plasticity	0.0039
GO:0048169	APP, KIT	2	Regulation of long-term neuronal synaptic plasticity	0.0039
GO:0007610	ADORA2A, APP, BCHE, KIT	4	Behavior	0.0043
GO:0042417	MAOA, MAOB	2	Dopamine metabolic process	0.0043
GO:0050877	ADORA2A, APP, BCHE, CHRM5, KIT	5	Nervous system process	0.0069
GO:0008542	APP, KIT	2	Visual learning	0.0072
GO:0061515	APP, KIT	2	Myeloid cell development	0.0085
GO:0040012	ADORA2A, APP, KDR, KIT	4	Regulation of locomotion	0.0137
GO:0007631	ADORA2A, APP	2	Feeding behavior	0.0156
GO:0042391	ADORA2A, APP, KDR	3	Regulation of membrane potential	0.0156
GO:0008360	KDR, KIT	2	Regulation of cell shape	0.0268
GO:0000187	APP, KIT	2	Activation of MAPK activity	0.0278
GO:0007166	ADORA2A, APP, KDR, KIT, MAOA	5	Cell surface receptor signaling pathway	0.0289
GO:0010646	ACHE, ADORA2A, APP, BCHE, KDR, KIT	6	Regulation of cell communication	0.0289
GO:0007399	ACHE, ADORA2A, APP, BCHE, KIT	5	Nervous system development	0.0291
GO:0007626	ADORA2A, APP	2	Locomotory behavior	0.0347
GO:0044237	ACHE, ADORA2A, APP, BCHE, KDR, KIT, MAOA, MAOB, PTGS1	9	Cellular metabolic process	0.0365
GO:0007154	ACHE, ADORA2A, APP, CHRM5, KDR, KIT, MAOA	7	Cell communication	0.0398
GO:0035556	ADORA2A, APP, KDR, KIT	4	Intracellular signal transduction	0.0401
GO:0065007	ACHE, ADORA2A, APP, BCHE, CHRM5, KDR, KIT, MAOA, MAOB, PTGS1	10	Biological regulation	0.0447

Note: GO: Gene Ontology and FDR: False Discovery Rate

TABLE 3: GO ENRICHMENT ANALYSIS OF PROTEIN TARGETS FOR THEIR MOLECULAR FUNCTIONS INVOLVED IN AD

GO ID	Genes modulated by compounds	Gene count	Gene function	FDR
GO:0003990	ACHE, BCHE	2	ACHE activity	0.00021
GO:0008131	MAOA, MAOB	2	Primary amine oxidase activity	0.00025
GO:0042277	ACHE, APP, BCHE	3	Peptide binding	0.0054
GO:0046983	ACHE, ADORA2A, APP, KIT, MAOB	5	Protein dimerization activity	0.0054
GO:0001540	ACHE, BCHE	2	Amyloid-beta binding	0.006
GO:0004714	KDR, KIT	2	Transmembrane receptor protein tyrosine kinase activity	0.0062
GO:0003824	ACHE, BCHE, CHRM5, KDR, KIT, MAOA, MAOB, PTGS1	8	Catalytic activity	0.01
GO:0005178	APP, KDR	2	Integrin binding	0.0145
GO:0004888	ADORA2A, CHRM5, KDR, KIT	4	Transmembrane signaling receptor activity	0.0181
GO:0016491	MAOA, MAOB, PTGS1	3	Oxidoreductase activity	0.0302

Note: GO: Gene Ontology and FDR: False Discovery Rate

TABLE 4: AD PATHWAYS MODULATED BY THE PHYTOCONSTITUENTS

Pathway ID	Pathway description	No. of gene involved in the pathway	FDR	Protein involved in pathways associate with AD
hsa04726	Serotonergic synapse	4	9.33E-06	APP, MAOA, MAOB, PTGS1
hsa04725	Cholinergic synapse	2	0.0051	ACHE, CHRM5
hsa04728	Dopaminergic synapse	2	0.0062	MAOA, MAOB
hsa04015	Rap1 signaling pathway	2	0.0129	KDR, KIT
hsa04014	Ras signaling pathway	2	0.0151	KDR, KIT
hsa04010	MAPK signaling pathway	2	0.0216	KDR, KIT
hsa04151	PI3K-Akt signaling pathway	2	0.0283	KDR, KIT
hsa01100	Metabolic pathways	3	0.0459	MAOA, MAOB, PTGS1

Note: FDR: False Discovery Rate and AD: Alzheimer's Disease

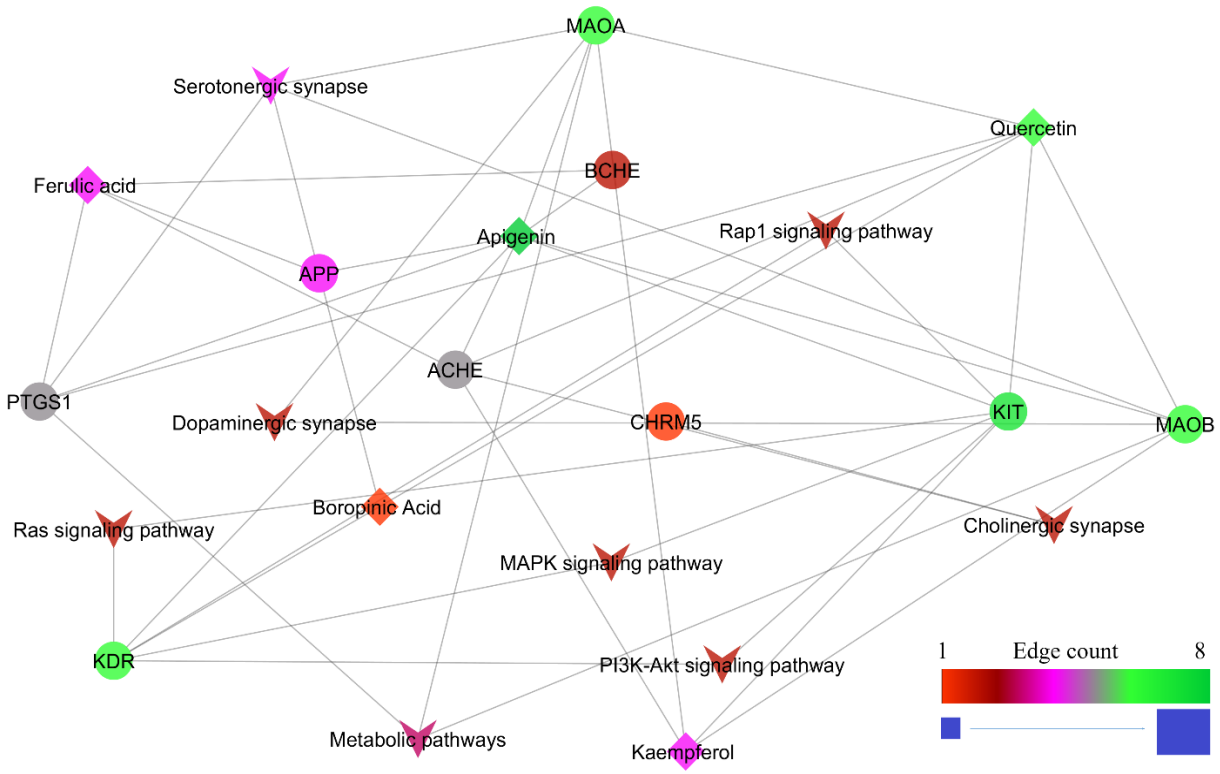

Fig. 2: Network representation of the interaction between phytoconstituents, targets and pathways. Diamond shape represents phytocompounds, round represents protein targets and down arrow represents pathways

TABLE 5: DRUGLIKNESS AND PROBABLE SIDE EFFECTS PROFILE OF PHYTOCONSTITUENTS FROM *A. tricolor*

Phytocompounds	PubChem ID	MF	MW (g/mol)	HBA	HBD	Log P	DL score	Probable side effect(s)
Ferulic acid	445858	$C_{10}H_{10}O_4$	194.06	4	2	2.04	-0.44	Myocardial infarction
Quercetin	5280343	$C_{15}H_{10}O_7$	302.04	7	5	2.11	0.93	Hepatotoxicity
Apigenin	5280443	$C_{15}H_{10}O_5$	270.05	5	3	3.06	0.77	Hepatotoxicity
Kaempferol	5280863	$C_{15}H_{10}O_6$	286.05	6	4	2.49	0.77	Hepatotoxicity
Boropinic acid	1.10E+07	$C_{15}H_{18}O_4$	262.12	4	1	4.05	-0.41	Cardiac failure, myocardial infarction

Note: MF: Molecular formula; MW: Molecular weight; HBA: Hydrogen Bond Accepter; HBD: Hydrogen Bond Donor; Log P: Partition coefficient and DL score: Druglikeness score

Further, kaempferol-ACHE contact analysis concluded that Glu202 and Gln71 to form stable hydrogen-bonded interactions for 100 % and 77 % of the duration with the Hydroxy (OH) group of kaempferol, respectively. Tyr133, Gly120, His447, Tyr337 were found to be involved in hydrophobic and water bridge interactions and showed around 67 %, 53 %, 25 % and 20 % of the time with interaction fraction, respectively. Fig. 5

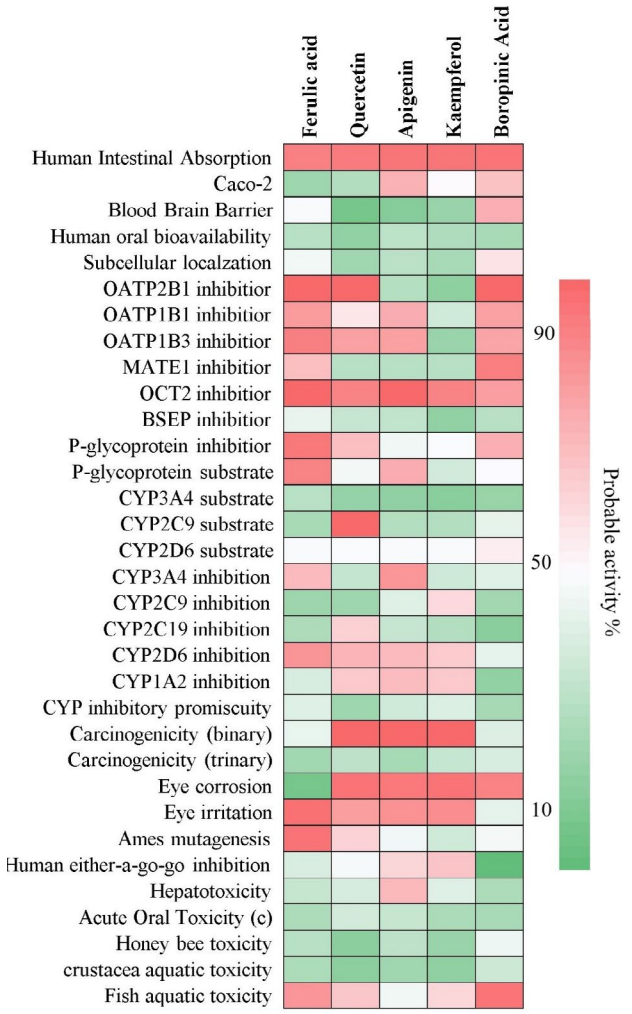

Fig. 3: Heat map representation of ADMET profile of phytoconstituents

TABLE 6: PROBABLE SCORE, BINDING AFFINITY, INHIBITORY CONSTANT AND HYDROGEN BOND INTERACTION OF COMPOUNDS WITH RESPECTIVE TARGETS

Targets	PDB ID	Compound	p	BE (kcal/mol)	IC_{50} (μM)	Amino acid…ligand interactions
ACHE	4PQE	Kaempferol	0.7	-7.8	1.92	Tyr72…OH, Asp74…OH, Glu202…OH
		Apigenin	0.7	-6.76	11.1	Asp74…OH, Phe295…O, Tyr337…=O
		Quercetin	0.7	-6.03	38.27	Pro88…OH, Asp131…=O
		Donepezil*	1	-5.15	168.86	Thr436…O-
MAOA receptor	1OJD	Kaempferol	0.7	-8.28	0.85	Asn181…OH
		Apigenin	0.86	-3.97	1023	Asn133…=O, Ile164…OH
		Quercetin	0.71	-6.09	34.46	Arg109…OH, Asn125…OH, Thr205…=O, Thr205…OH
		Moclobemide*	1	-6.32	23.22	Nil
MAOB receptor	2Z5X	Kaempferol	0.7	-8.6	0.498	Tyr188…=O, Cys172…OH
		Apigenin	0.97	-4.64	393.85	Lys73…=O, Glu466…OH, AspP471…OH
		Quercetin	0.7	-4.31	697.85	Glu366…OH, Lys370…=O
		Safinamide*	1	-6.87	9.16	Arg42…O-, Ala263…NH
PTGS1	3N8X	Quercetin	0.7	-3.87	1044	Leu92…OH, His95…OH
		Apigenin	0.92	-4.4	591.39	Thr60…OH, Arg61…OH, Ile151…OH, Arg469…OH
		Meloxicam*	0.78	-7.64	2.52	Phe210…OH, Thr212…=O, Thr212…S, His386…=O

Note: ACHE: Acetylcholinesterase; MAOA: Monoamino Oxidase A; MAOB: Monoamino Oxidase B and PTGS1: Prostaglandin G/H Synthase 1, *Standard drug of specific protein for AD; BE: Binding Energy and IC_{50}: Half-Maximal Inhibitory Concentration

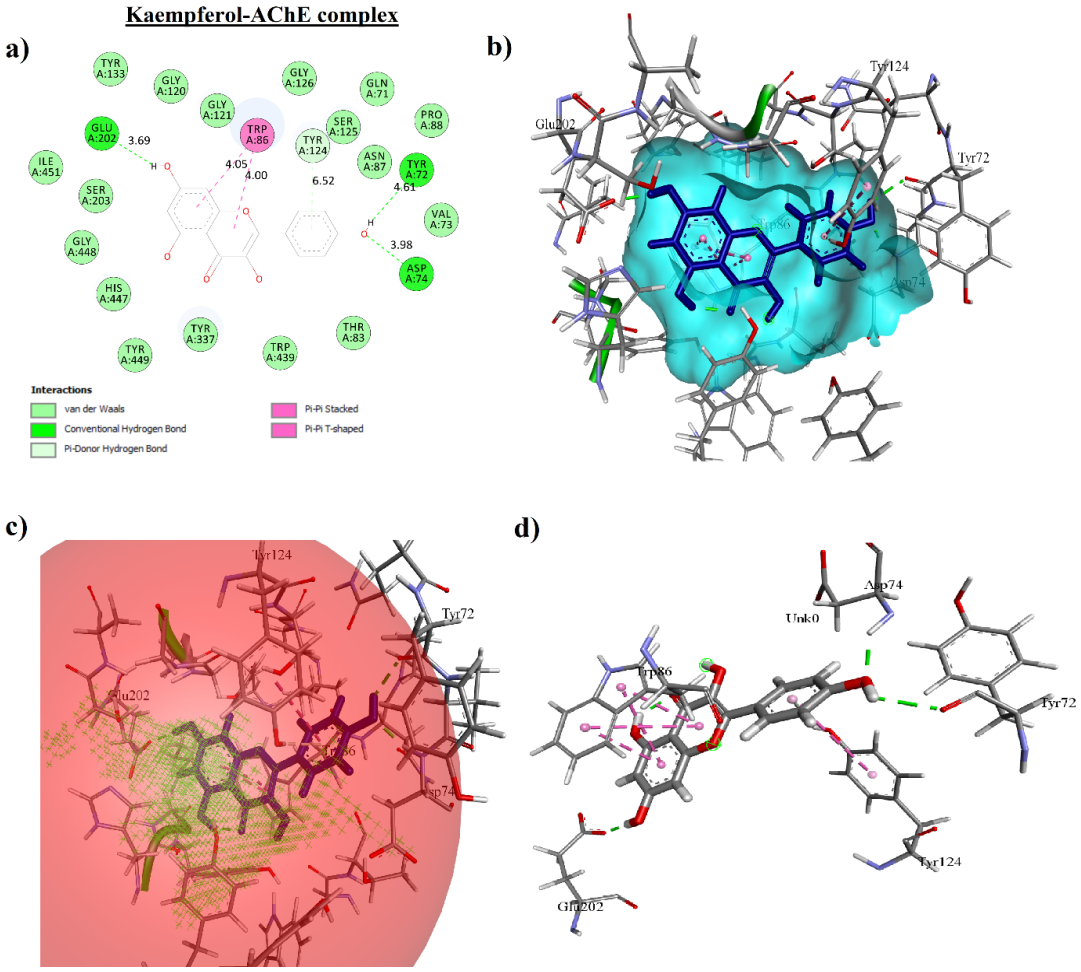

Fig. 4: Intermolecular interactions of kaempferol with ACHE, (a) 2D interaction representation; (b) Kaempferol within protein pocket; (c) Kaempferol at binding site-I of ACHE and (d) 3D interaction representation

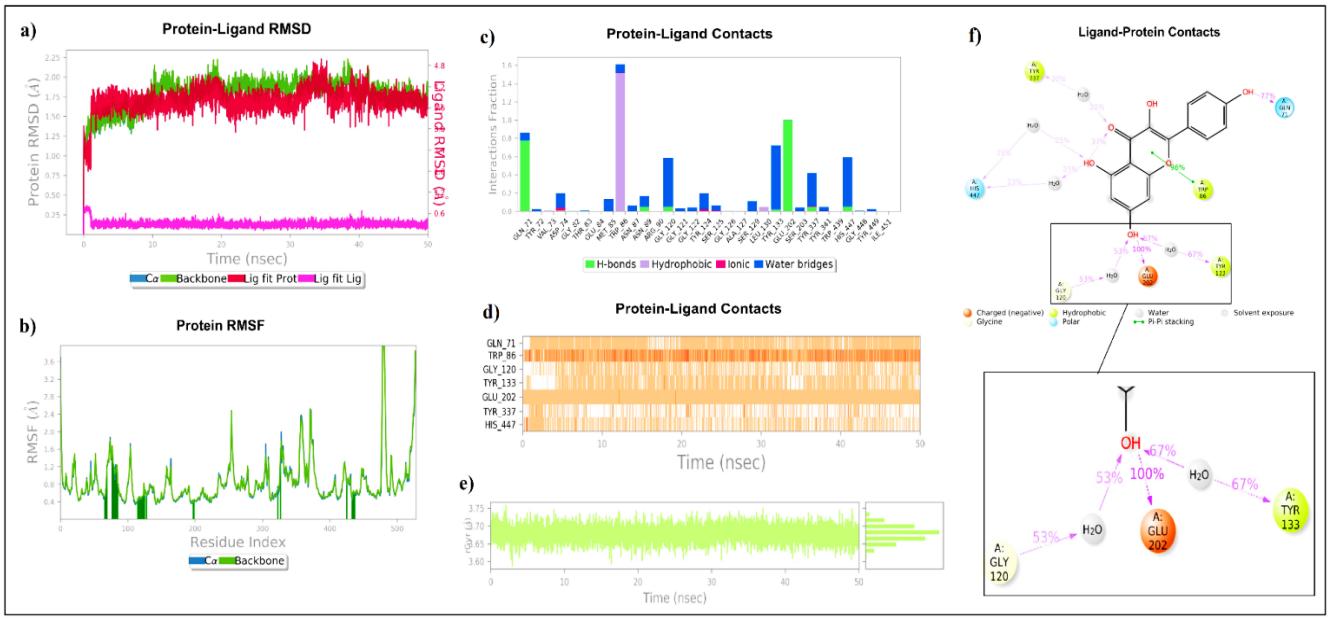

Fig. 5: MD simulation of kaempferol-ACHE complex for 50 ns production run, (a) RMSD; (b) RMSF with ligand contacts; (c) Protein-ligand contractions; (d) Residue-wise contacts and interaction fractions; (e) r-Gyr of ligand molecule and (f) Ligand-protein contractions

represents the ligand-protein complex RMSD, residue-wise contacts and rGyr of the kaempferol-ACHE complex.

Our study showed flavonoids, organoboranes, flavone glycosides and phenolic phytocompounds from *A. tricolor* are likely to interact with numerous protein targets involved in the pathogenesis of AD[34] *viz.*, ACHE [35], MAOA[36], MAOB[36], etc. Further, we identified a number of protein molecules that regulate biological processes, molecular functions and pathways as likely targets of bioactive phytoconstituents of *A. tricolor*. GO functional, process and pathway enrichment analysis was the mainstay of our study. It deals with the interaction and behavior of the biological entities, focus on understanding the relationship between the drug molecules with the biological targets and helps to know the functions of protein complexes, gene regulatory networks and significant pathways associated with disease pathogenesis[37]. The GO analysis identified regulation of neurotransmitter levels, neurotransmitter metabolic process, learning, inflammatory response, behavior, regulation of locomotion, cell communication and many more biological processes associated with cognitive impairment. Results obtained from the GO enrichment analysis in our study revealed various molecular functions *viz.* ACHE activity, primary amine oxidase activity, peptide binding activity, protein dimerization activity, amyloid-beta binding activity, transmembrane receptor protein tyrosine kinase activity, catalytic activity, integrin binding activity, transmembrane signaling receptor activity and oxidoreductase activity are associated with cognitive impairment and AD. We constructed the network interaction between predicted active phytoconstituents of *A. tricolor* with their probable targets and enriched pathways thus identified. Ferulic acid, quercetin, apigenin, kaempferol and boropinic acid were identified as the key bioactive phytoconstituents from *A. tricolor* having modulatory activities on a number of pathways involved in AD e.g. serotonergic synapse (hsa04726)[38], cholinergic synapse (hsa04725)[39], metabolic pathway (hsa01100)[40], dopaminergic pathway (hsa04728)[41], etc. Among five compounds, kaempferol, apigenin and quercetin (flavonoids) showed the highest potential in the pharmacotherapy of AD by targeting ACHE, APP, BCHE, KDR, KIT, MAOA, MAOB and PTGS1 protein molecules within the network. Flavonoids enhance cognitive function at a behavioral stage and attenuate the cognitive decline promoted by brain disorders[42]. The constructed network showed that kaempferol, apigenin and quercetin having the highest edge count (Table 1), which suggests higher probability of these compounds in the modulation of pathogenesis associated with cognitive impairment. Previous literature suggests ACHE as a potential target for AD. It terminates signal transduction at the neuromuscular junction by rapid hydrolysis of the acetylcholine released into the synaptic cleft and plays a role in the neuronal apoptosis and cognitive impairment[43]. The ACHE enzyme degrades the freely available acetylcholine in the cerebral cortex and hippocampus, leading to the impairment of cognitive function in AD patients[35]. PTGS1 converts arachidonate to Prostaglandin H2 (PGH2) in the stomach and platelets, Prostaglandin E2 (PGE2) in gastric epithelial cells and Thromboxane A2 (TXA2) in the plates[44]. Further, these prostaglandin derivatives play a crucial role in cytoprotection, activation, aggregation of platelets, vasoconstriction, proliferation of vascular smooth muscle cells, etc[45]. MAO enzyme catalyzes the oxidative deamination of xenobiotic amines. It has a vital role in the breakdown of neuroactive and vasoactive amines in the peripheral tissues and central nervous system leading to the development of neurodegenerative diseases, anxiety, schizophrenia, mood disorders, depression, migraine and sexual maturation[36].

The affinity of the ligand molecule within the protein pocket is explained by the binding energy and number of hydrogen bond interactions. Docking studies revealed kaempferol to have more affinity towards ACHE, MAOA and MAOB compared to the clinically approved drugs donepezil, moclobemide and safinamide used in the study. Kaempferol showed three hydrogen bonds through the interaction with ACHE i.e. Tyr72…OH, Asp74…OH, Glu202…OH, one with MAOA i.e. Asn181…OH, and two bonds with MAOB i.e. Tyr188…=O, Cys172…OH. Moreover, MD simulation demonstrated the stable interaction of kaempferol with ACHE. Glu202 residue showed 100 % hydrogen bond interaction fraction with the OH group of the kaempferol; which suggests kaempferol as a potential inhibitor of ACHE. Previous studies have demonstrated kaempferol as a potent drug candidate for managing cognitive deficit *via* the modulation of spatial learning and memory, glutathione level, apoptosis marker cytochrome c inflammatory marker Tumour Necrosis Factor alpha (TNF-α), endogenous antioxidants Superoxide Dismutase (SOD) and lipid peroxidation marker Malondialdehyde (MDA)[46,47]. A previous study by Bahrani *et al*. isolated and characterized the ACHE inhibitors from *Aquilaria subintegra* for the treatment of AD and reported kaempferol as a major

ACHE inhibitor (85.8 % inhibition)[48]. Further, a study by Hupparage *et al.* reported that hydroalcoholic extract of *A. tricolor* L. leaves restore the cholinergic system's function, inhibit oxidative stress and improve the memory function in the scopolamine treated rats[49]. Although several researchers reported a decrease in the risk of development of AD with intake of dietary flavonoids. Although, several researchers have reported dietary flavonoids decreases the risk of development of AD[50-52] to the best of our knowledge, despite having high content of flavonoids, such molecular mechanism of action for the leafy vegetable *A. tricolor* L.is not reported so far. An earlier study on *A. tricolor* L. showed a neuroprotective effect on gene expression of Receptor for Advanced Glycation End-Products (RAGE) during oxidative stress in SH-SY5Y cells[15]. Importantly, Ayurveda formulations often contain traditional herbs and dietary plants that are widely used to treat numerous diseases/disorders due to bioactive phytocompounds[53,54]. The current study identified mechanisms of action of phytocompounds in *A. tricolor* and their possible roles in the management of AD mostly attributed to the presence of flavonoids (kaempferol, apigenin and quercetin)[17,20].

Several attempts have been made to understand the molecular mechanisms of action of herbal constituents in the management of complex diseases like AD. Exploration of herbal medicines through GO functional, process and pathway enrichment, network pharmacology and other computational methods for the management of complex diseases like AD is a well-accepted[55-57]. Although, herbs contain a complex mixture of pharmacologically active phytoconstituents, this complexity may up/down-regulate the various protein molecules and may cause the synergistic or additive effect to reduce the complications associated with AD[58]. Identification of herbal toxicity plays a critical role in the minimization of organ damage and severe Adverse Drug Events (ADEs). The current study utilized these approached and available bioinformatics methodologies to identify the toxicity associated with phytoconstituents based on their structure-activity relationships. However, the predicted toxicities have not been found in literature through molecular biological experiments and/or clinical investigations.

In conclusion, the current study utilized the drug-target-pathway relationship to elucidate the molecular mechanism of action of *A. tricolor* L. against AD. Kaempferol, quercetin and apigenin as were identified as major compounds that are likely to interact with the potential protein targets involved in the pathogenesis of AD. Enrichment analysis of genes showed serotonergic synapse, cholinergic synapse, dopaminergic synapse, AD, Ras-proximate-1 (Rap1) signaling pathway, Rat sarcoma (Ras) signaling pathway, Mitogen-Activated Protein Kinases (MAPK) signaling pathway, Phosphatidylinositol 3 Kinase-Protein Kinase B (PI3K-Akt) signaling pathway and metabolic pathways as major pathways regulated by these phytoconstituents, thereby exerting their effects. The probable intermolecular interactions among the predicted therapeutic targets of AD with phytocompounds of *A. tricolor* L. was demonstrated through molecular docking while MD simulation identified stable interactions of the kaempferol-ACHE complex. GO functional enrichment analysis and network pharmacology-based approach offer an easy and reliable strategy for identifying potential therapeutic targets of traditional herbal medicines. The present study should help design wet-lab studies to experimentally validate the findings, which should go a long way in finding one of the multipronged solutions to tackle AD in the future.

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Conflict of interests:

The authors declared no conflicts of interest.

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