Research Results in Pharmacology

Research Results in Pharmacology 8(3): 49–61
UDC: 615.21
DOI 10.3897/rrpharmacology.8.83332

Research Article

Harmine and 7,8-dihydroxyflavone synergistically suitable for amyotrophic lateral sclerosis management: An *in silico* study

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Academic editor: Mikhail Korokin ♦ **Received** 9 March 2022 ♦ **Accepted** 19 July 2022 ♦ **Published** 25 August 2022

Citation: Fatoki T, Chukwuejim S, Ibraheem O, Oke C, Ejimadu B, Olaoye I, Oyegbenro O, Salami T, Basorun R, Oluwadare O, Salawudeen Y (2022) Harmine and 7,8-dihydroxyflavone synergistically suitable for amyotrophic lateral sclerosis management: An *in silico* study. Research Results in Pharmacology 8(3): 49–61. https://doi.org/10.3897/rrpharmacology.8.83332

Abstract

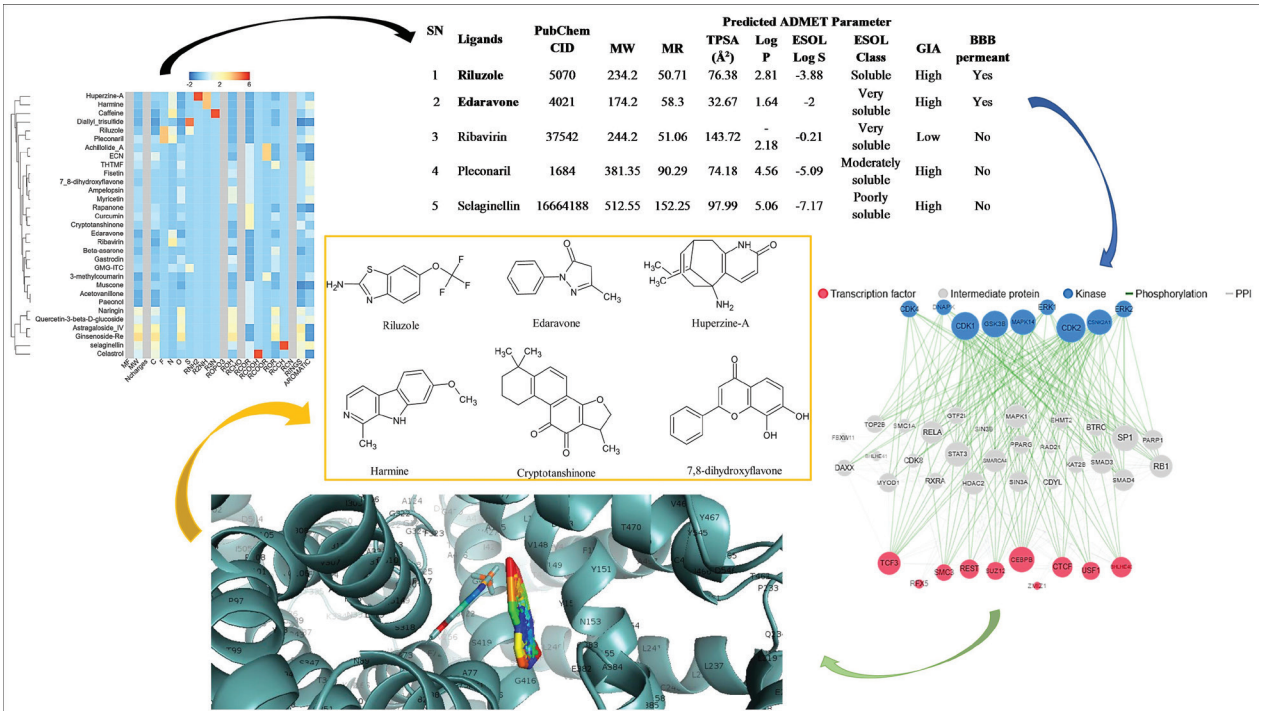
Introduction: Amyotrophic lateral sclerosis (ALS) is a fatal neurological disease characterized by progressive degeneration of both upper and lower motor neurons, resulting in paralysis and eventually leads to death from respiratory failure typically within 3 to 5 years of symptom onset. The aim of this work was to predict the pharmacokinetics and identify unique protein targets that are associated with potential anti-ALS phytochemicals and FDA-approved drugs, by *in silico* approaches.

Materials and methods: Standard computational tools (webserver and software) were used, and the methods used are clustering analysis, pharmacokinetics and molecular target predictions, and molecular docking simulation.

Results and discussion: The results show that riluzole, β-asarone, cryptotanshinone, harmine and 7,8-dihydroxyflavone have similar pharmacokinetics properties. Riluzole and harmine show 95% probability of target on norepinephrine transporter. Huperzine-A and cryptotanshinone show 100% probability of target on acetylcholinesterase. 7,8-dihydroxyflavone shows 35% probability of target on several carbonic anhydrases, 40% probability of target on *CYP19A1*, and 100% probability of target on inhibitor of nuclear factor kappa B kinase beta subunit and neurotrophic tyrosine kinase receptor type 2, respectively. Harmine also shows 95% probability of target on dual specificity tyrosine-phosphorylation-regulated kinases, threonine-protein kinases (haspin and PIM3), adrenergic receptors, cyclin-dependent kinases (*CDK5* and *CDK9*), monoamine oxidase A, casein kinase I delta, serotonin receptors, dual specificity protein kinases (*CLK1*, *CLK2*, and *CLK4*), and nischarin, respectively. Also, the results of gene expression network show possible involvement of *CDK1*, *CDK2*, *CDK4*, *ERK1*, *ERK2* and *MAPK14* signaling pathways. This study shows that riluzole and harmine have closely similar physicochemical and pharmacokinetics properties as well as molecular targets, such as norepinephrine transporter (*SLC6A2*). Harmine, huperzine-A and cryptotanshinone could modulate acetylcholinesterase (AChE), which is involved in ALS-pathogenesis. The impact of 7,8-dihydroxyflavone on several carbonic anhydrases (CA) I, II, VII, IX, XII, and XIV, as well as *CYP19A1*, could help in remediating the respiratory failure associated with ALS.

Conclusion: Overall, harmine is found to be superior to riluzole, and the combination of harmine with 7,8-dihydroxyflavone can provide more effective treatment for ALS than the current regime. Further work is needed to validate the predicted therapeutic targets of harmine identified in this study on ALS model or clinical trials, using *in silico*, *in vitro* and *in vivo* techniques.

Graphical abstract:



Keywords

Amyotrophic lateral sclerosis, ALS, riluzole, harmine, 7,8-dihydroxyflavone, pharmacokinetics, target prediction, molecular docking.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurological disease characterized by progressive degeneration of both upper and lower motor neurons, resulting in paralysis and eventually leads to death from respiratory failure typically within 3 to 5 years of symptom onset (Xue et al. 2018; Saez-Atienzar et al. 2021). ALS can be genetically inherited or occur sporadically in individuals without any apparent family history (Xue et al. 2018). The overall prevalence of ALS in Europe and North America is estimated at about 3–5 cases per 100,000 people and increases with age, with estimated death of 6000 Americans and 11,000 Europeans annually, and the number of ALS cases will increase markedly over the next two decades, mostly due to aging of the global population (Arthur et al. 2016). However, fewer data are currently available for the African population (Osuntokun et al. 1974; Imam and Ogunniyi 2004; Quansah and Karikari 2015), and it has been projected that within the next 25 years, the highest change in ALS cases will be in Africa, with an increase of 116%, followed by Asia and South America, with an increase of 81% and 73% (Arthur et al. 2016; Logroscino and Piccininni 2019).

The 10 steps of ALS disease pathology and mechanisms have been proposed by Van-Damme et al. (2017). Identification of genes underlying ALS has provided critical insights into the cellular mechanisms leading to neurodegeneration, such as protein homeostasis, cytoskeleton alterations, RNA metabolism, endoplasmic reticulum stress, nucleocytoplasmic transport, and autophagy defects (Saez-Atienzar et al. 2021). The genome-wide association study of Western African populations reported that 46 genes out of 165 genes were linked to sporadic ALS in the population (Chaichoompu et al. 2020). A recent study that systematically applied a polygenic risk score analysis to a genomic dataset involving 78,500 individuals to distinguish the cellular processes driving ALS, has reported six differentially expressed genes (*ATG16L2*, *ACSL5*, *MAP1LC3A*, *MAPKAPK3*, *PLXNB2*, and *SCFD1*) within the significant pathways that are relevant to ALS (Saez-Atienzar et al. 2021).

Riluzole (a glutamate release inhibitor) and edaravone (a free-radical scavenger) are the only two FDA-approved drugs for the treatment of ALS, through mechanism that only delaying disease progression and prolonging survival for 2–3 months (Xue et al. 2018). Currently there is no effective therapy, but several

studies have reported the detection of enhanced gene expression of human endogenous retrovirus (HERV)-K and reverse transcriptase activity in the blood and brain tissues of ALS patients and possible use of its inhibitors which include antiviral drug such as Ribavirin and Pleconaril (Schmidtke et al. 2009; Ruller et al. 2012; Li et al. 2015; Liu et al. 2015; Xue et al. 2018). Both ribavirin and pleconaril are used for the treatment of severe lower and upper respiratory tract infections respectively. The aim of this work was to predict the pharmacokinetics and identify unique protein targets that are associated with the potential anti-ALS phytochemicals and FDA-approved drugs, by *in silico* approaches.

Materials and methods

Ligand preparation and clustering analysis

The structures of two FDA-approved drugs for the treatment of ALS (Riluzole and Edaravone) and two antiviral drugs that crosse Blood-brain barrier (BBB), which are Ribavirin and Pleconaril (Xue et al. 2018), were obtained from NCBI PubChem Compound database (http://www.ncbi.nlm.nih.gov/pccompound) in SMILES and SDF formats. We further explored potential anti-ALS phytochemicals (acetovanillone, harmine, fisetin, quercetin-3-*β*-D-glucoside, 7,8-dihydroxyflavone, myricetin, naringin, lactone achillolide A, caffeine, 3,5,4′-trihydroxy-6,7,3′-trimethoxyflavone (THTMF), 3-methylcoumarin, rapanone, 4-(*α*-L-ramnosiloxy)-benzylisothiocyanate (GMG-ITC), 7*β*-(3-ethylcis-crotonoyloxy)-1*α*-(2-methylbutyryloxy)-3,14-dehydro-Z-notonipetranone (ECN), ginsenoside Re, ampelopsin, diallyl trisulfide (DATS), astragaloside IV, β-asarone, huperzine-A, selaginellin, cryptotanshinone, celastrol, curcumin, paeonol, gastrodin, and muscone) based on the reports in literature (Zhang et al. 2014; Silva et al. 2020; Shah and Kim 2021). Clustering analysis with multidimensional scaling was performed on ChemMine server (http://chemmine.ucr.edu/) using the SMILES of the ligands.

In silico pharmacokinetics

The SMILES of each of the ligands were used for *in silico* ADME (absorption, distribution, metabolism, and excretion) screening on SwissADME server (Daina et al. 2017), which was performed at default parameters.

In-silico target prediction

The ligands that could permeate the blood-brain barrier based on the predicted pharmacokinetics were used for target prediction on SwissTargetPrediction server (http://www.swisstargetprediction.ch/), where *Homo sapiens* was designated as a target organism (Daina et al. 2019).

Molecular docking studies

The molecular docking studies were carried out using the selected protein targets that have at least 90% probability and their corresponding ligands based on target prediction results, according to the method of Fatoki et al. (2020). Briefly, the target proteins and ligands were prepared for docking, using AutoDock Tools (ADT) v1.5.6 (Morris et al. 2009) at default settings, and the output file was saved in pdbqt format. Molecular docking program AutoDock Vina v1.1.2 (Trott and Olson 2010) was employed to perform the active site docking experiment. After docking, close interactions of binding of the target with the ligands were analyzed and visualized using PyMol v2.0.7.

Target gene expression analysis

Thirty-two (32) target proteins with at least 90% probability obtained from the target prediction results, which are *SLC62A*, *DYRK4*, *HASPIN*, *ADRA2A*, *CDK5R1*, *ADRA2C*, *ADRA2B*, *MAOA*, *CDK9*, *HTR2A*, *HTR2C*, *DYRK1A*, *CSNK1D*, *HTR7*, *HTR6*, *NISCH*, *CLK4*, *CLK1*, *CLK2*, *DYRK2*, *DYRK3*, *PIM3*, *DYRK1B*, *IKBKB*, *NTRK2*, *HMGCR*, *AKR1B1*, *ACHE*, *BCHE*, *CES1*, *CES2*, and *STAT3*, were used for the analysis. These genes ID were compiled and used for expression network analyses (transcription factor enrichment analysis and protein-protein interaction network expansion and kinase enrichment analysis), using eXpression2Kinases (X2K) Web server https://maayanlab.cloud/X2K/ (Clarke et al. 2018), where the human was selected as a background organism.

Results

Clustering analysis

The result of clustering analysis (Fig. 1) shows that harmine and huperzine-A have physicochemical properties that are closely similar to those of riluzole and pleconaril, while edaravone, ribavirin and β-asarone belong to the same cluster close to cryptotanshinone. The cluster of 7,8-dihydroxyflavone is different from that of riluzole.

In silico pharmacokinetics

Riluzole, edaravone, huperzine-A, β-asarone, diallyl trisulfide, acetovanillone, muscone, paeonol, cryptotanshinone, 3-methylcoumarin, harmine and 7,8-dihydroxyflavone were predicted to be BBB permeant, and thus considered for further analysis in this study (Tables 1–3). These 12 compounds show high gastrointestinal (GI) absorption, out of which riluzole, β-asarone, cryptotanshinone, harmine and 7,8-dihydroxyflavone could inhibit two or more cytochrome p450s (CYPs), and the bioavailability score of cryptotanshinone was the highest among these five compounds (Table 1). Moreover, huperzine-A and cryptotanshinone were predicted to be substrates for p-glycoprotein.

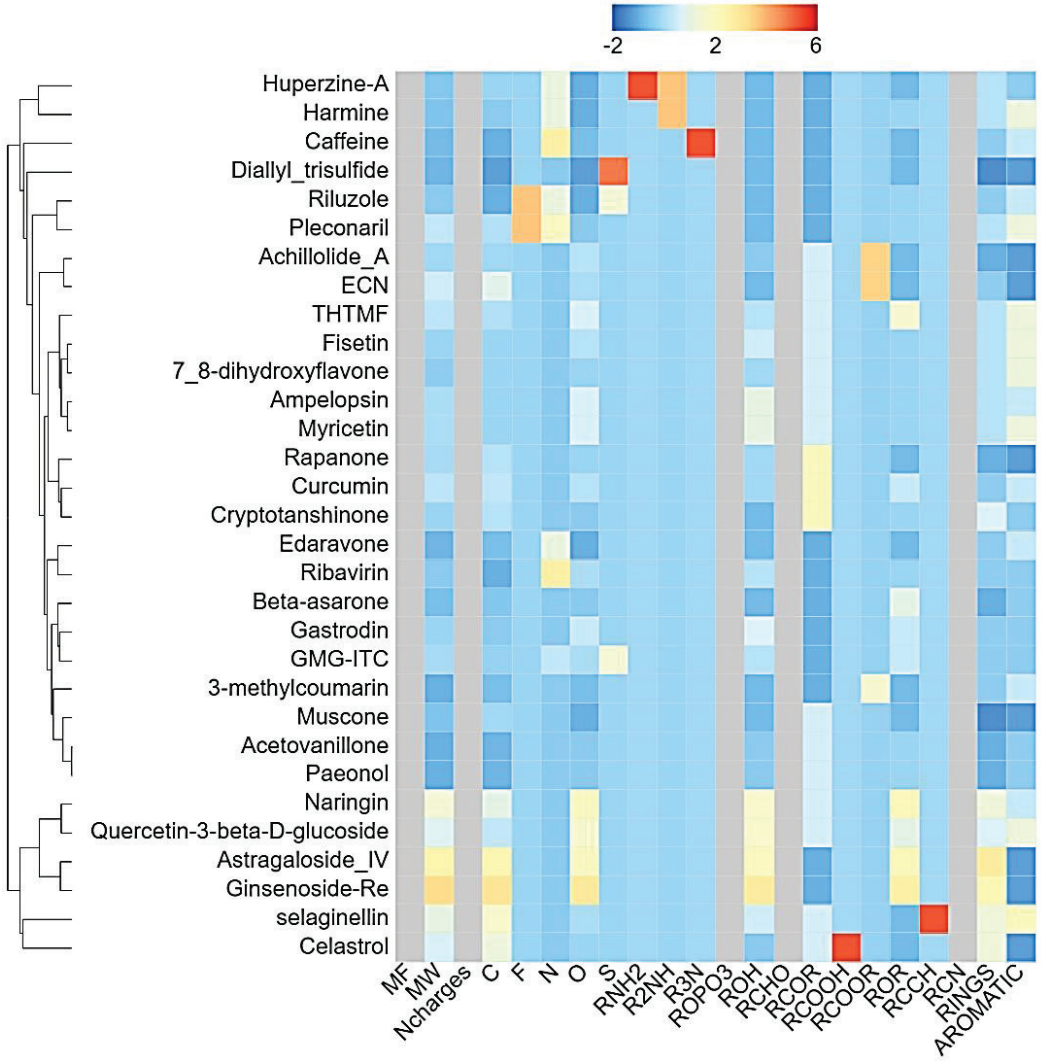


Figure 1. Hierarchical clustering results. Parameter options used are: Heatmap (distance matrix); Linkage Method (single); Physicochemical Properties Heatmap (ChemmineR Properties); Properties Color and Display Values (Z-scores).

In-silico target prediction

The 12 BBB permeant compounds (riluzole, edaravone, huperzine-A, β-asarone, diallyl trisulfide, acetovanillone, muscone, paeonol, cryptotanshinone, 3-methylcoumarin, harmine and 7,8-dihydroxyflavone) show multiples of targets based on prediction probability of at least 20% (Table 2). Riluzole and harmine show 95% probability of target on norepinephrine transporter. Huperzine-A and cryptotanshinone show 100% probability of target on acetylcholinesterase. Huperzine-A also shows 90% probability of target on butyrylcholinesterase. Cryptotanshinone also shows 35% probability of target on aldose reductase, acyl coenzyme A: cholesterol acyltransferase, carboxylesterase 2, and signal transducer and activator of transcription 3, respectively. 7,8-dihydroxyflavone shows 35% probability of target on several carbonic anhydrases, 40% probability of target on *CYP19A*1, and 100% probability of target on nuclear factor kappa B kinase beta subunit and neurotrophic tyrosine kinase receptor type 2, respectively. Harmine also shows 95% probability of target on dual specificity tyrosine-phosphorylation-regulated kinases, threonine-protein kinases (haspin and P*IM3*), adrenergic receptors, cyclin-dependent kinases (*CDK5* and *CDK9*), monoamine oxidase A, casein kinase I delta, serotonin receptors, dual specificity protein kinases (*CLK1*, *CLK2*, and *CLK4*), and nischarin, respectively.

Molecular docking studies

Riluzole, harmine, cryptotanshinone, huperzine-A, 7,8-dihydroxyflavone, and β-asarone (Fig. 2) have targets with at least 90% probability, and these targets were used for molecular docking study (Table 3). The results of molecular docking show that the binding energy of riluzole and harmine to norepinephrine transporter (*SLC6A2*) is equal to -8.5 and -8.2 kcal.mol^{-1} respectively, with active site amino acid residues, which include A73, F74, A77, V79, R81, R83, A145, V148, 149, Y151, Y152, N153, C240, L241, F317, S318, E382, G383, G416, D148, S419, S420, G423, A426, and I428. The binding of harmine to norepinephrine transporter is greater than its binding to monoamine oxidase A (-7.3 kcal.mol^{-1}), and less than its binding to dual-specificity tyrosine-phosphorylation regulated

Table 1. Predicted Pharmacokinetic Properties of Selected Ligands for ALS Treatment

SN	Ligands	PubChem CID	Predicted ADMET Parameter												
			MW	MR	TPSA (Å²)	Log P	ESOL Log S	ESOL Class	GIA	BBB permeant	P-gp	CYPs Inhibitor	Log Kp (cm/s)	BS	SA
1	**Riluzole**	5070	234.2	50.71	76.38	2.81	-3.88	Soluble	High	Yes	No	CYP1A2, CYP2C19	-5.17	0.55	2.24
2	**Edaravone**	4021	174.2	58.3	32.67	1.64	-2	Very soluble	High	Yes	No	CYP1A2	-6.46	0.55	2.25
3	Ribavirin	37542	244.2	51.06	143.72	-2.18	-0.21	Very soluble	Low	No	No	–	-9.1	0.55	3.89
4	Pleconaril	1684	381.35	90.29	74.18	4.56	-5.09	Moderately soluble	High	No	No	CYP1A2, CYP2C19, CYP2C9	-5.35	0.55	3.34
5	Selaginellin	16664188	512.55	152.25	97.99	5.06	-7.17	Poorly soluble	High	No	No	–	-4.97	0.55	4.55
6	**Huperzine-A**	1253	242.32	72.87	58.88	1.91	-1.6	Very soluble	High	Yes	Yes	–	-7.77	0.55	4.26
7	**β-asarone**	5281758	208.25	60.82	27.69	2.7	-3.05	Soluble	High	Yes	No	CYP1A2, CYP2C19	-5.44	0.55	2.39
8	**Diallyl trisulfide**	16315	178.34	52.78	75.9	2.68	-2.21	Soluble	High	Yes	No	–	-5.51	0.55	3.58
9	Ampelopsin	161557	320.25	76.78	147.68	0.22	-2.52	Soluble	Low	No	No	–	-7.83	0.55	3.55
10	Astragaloside IV	13943297	784.97	196.95	228.22	1.23	-5.04	Moderately soluble	Low	No	Yes	–	-10.19	0.17	9.73
11	Achillolide A	132580937	306.31	77.31	89.9	1.23	-1.96	Very soluble	High	No	No	–	-7.69	0.55	4.76
12	**Acetovanillone**	2214	166.17	45.15	46.53	1.28	-1.43	Very soluble	High	Yes	No	–	-6.95	0.55	1.36
13	**Muscone**	10947	238.41	77.11	17.07	4.61	-5.26	Moderately soluble	High	Yes	No	CYP2C9	-3.32	0.55	3.09
14	Gastrodin	115067	286.28	66.72	119.61	-0.86	-0.68	Very soluble	Low	No	No	CYP1A2	-9.05	0.55	4.1
15	**Paeonol**	11092	166.17	45.15	46.53	1.63	-2.36	Soluble	High	Yes	No	CYP1A2	-5.91	0.55	1.28
16	Curcumin	969516	368.38	102.8	93.06	3.03	-3.94	Soluble	High	No	No	CYP2C9, CYP3A4	-6.28	0.55	2.97
17	Celastrol	122724	450.61	131.29	74.6	5.16	-6.31	Poorly soluble	Low	No	Yes	CYP2C9, CYP3A4	-4.83	0.85	6.28
18	**Cryptotanshinone**	160254	296.36	85.13	43.37	3.43	-4.27	Moderately soluble	High	Yes	Yes	CYP1A2, CYP2C19, CYP2C9, CYP3A4	-5.41	0.85	4.11
19	Ginsenoside-Re	441921	947.15	237.03	298.14	0.91	-5.91	Moderately soluble	Low	No	Yes	–	-10.96	0.17	10
20	ECN	78157658	430.58	124.22	69.67	4.94	-5.63	Moderately soluble	High	No	No	CYP2C9, CYP3A4	-4.74	0.55	5.74
21	GMG-ITC	153557	311.35	78.36	123.6	1.31	-2.54	Soluble	High	No	No	–	-7.27	0.55	4.22
22	THTMF	6453535	360.31	93.47	118.59	2.06	-4.02	Moderately soluble	High	No	No	CYP1A2, CYP2C9, CYP2D6, CYP3A4	-6.52	0.55	3.59
23	Rapanone	100659	322.44	93.93	74.6	4.43	-5.14	Moderately soluble	High	No	No	CYP2C19, CYP2C9, CYP2D6,	-3.65	0.85	3.88
24	**3-methylcoumarin**	17130	160.17	47.45	30.21	2.26	-2.89	Soluble	High	Yes	No	CYP1A2	-5.66	0.55	2.63
25	Caffeine	2519	194.19	52.04	61.82	0.08	-1.48	Very soluble	High	No	No	–	-7.53	0.55	2.03
26	Naringin	442428	580.53	134.91	225.06	-0.79	-2.98	Soluble	Low	No	Yes	–	-10.15	0.17	6.16
27	Myricetin	5281672	318.24	80.06	151.59	0.79	-3.01	Soluble	Low	No	No	CYP1A2, CYP3A4	-7.4	0.55	3.27
28	**Harmine**	5280953	212.25	65.06	37.91	2.78	-4.05	Moderately soluble	High	Yes	No	CYP1A2, CYP2D6, CYP3A4	-4.94	0.55	1.66
29	Fisetin	5281614	286.24	76.01	111.13	1.55	-3.35	Soluble	High	No	No	CYP1A2, CYP2D6, CYP3A4	-6.65	0.55	3.16
30	Quercetin-3-*β*-D-glucoside	5280804	464.38	110.16	210.51	-0.25	-3.04	Soluble	Low	No	No	–	-8.88	0.17	5.32
31	**7,8-dihydroxyflavone**	1880	254.24	71.97	70.67	2.35	-4.03	Moderately soluble	High	Yes	No	CYP1A2, CYP2D6, CYP3A4	-5.54	0.55	3.02

Note: Physicochemical properties: Molecular weight (MW), Molar Refractivity (MR), Total polar surface area (TPSA). **Lipophilicity**: Consensus Log P. **Water Solubility:** ESOL Log S, ESOL Class. **Pharmacokinetics:** Gastrointestinal absorption (GIA), Blood-brain barrier (BBB), P-glycoprotein (P-gp) substrate, Inhibition of Cytochrome P450 (CYPs) type CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4, Skin permeation (Log Kp). **Druglikeness**: Bioavailability Score (BS), **Medicinal Chemistry:** Synthetic accessibility (SA). The ligands with acceptable pharmacokinetics chiefly BBB permeant (YES) are in bold.

kinases (-8.9 kcal.mol^{-1}) and serine/threonine-protein kinases (-9.7 kcal.mol^{-1}). Huperzine-A and cryptotanshinone bind to acetylcholinesterase with binding energy of -10.1 kcal.mol^{-1}, respectively. β-asarone shows poor affinity to HMG-CoA reductase with a binding energy of -2.9 kcal.mol^{-1}. Fig. 3 shows the binding pose of riluzole and harmine on norepinephrine transporter (SLC6A2).

Target gene expression analysis

The gene IDs of all the targets with at least 90% probability were used for a gene expression network analysis. The result (Fig. 4) shows the overall interactions of intermediate proteins, kinases and transcription factors with high hypergeometric (-log$_{10}$) p-value. The kinases include CDK1, CDK2, CDK4, ERK1, ERK2, DNAPK, MAPK14, CSNK2A1, and GSK3B, while the transcription factors include TCF3, CEBPB, CTCF, USF1, SUZ12, REST, SMC3, RFX5, ZMIZ1, and BHLHE40.

Discussion

The results of this study show that riluzole and harmine have closely similar physicochemical and pharmacokinetics properties, as well as molecular targets (Fig. 1, Tables 1, 2). These results corroborate the existing documented targets for riluzole which are: sodium channel protein type 5 subunit alpha (UniProt ID: Q14524), cystine/glutamate transporter (UniProt ID: Q9UPY5), ATP-binding cassette sub-family G member 2 (UniProt ID: Q9UNQ0), and cytochromes P450 1A1 and 1A2 (UniProt IDs: P04798 and P05177, respectively) (https://go.drugbank.com/indications/DBCOND0029898#targets). Hierarchical clustering builds collectively a hierarchy of clusters based on pairwise compound similarities defined using the atom pair descriptors and the Tanimoto coefficient, and it has application in drug discovery (Sanni et al. 2017). Permeability glycoprotein, also known as P-glycoprotein (P-gp; *MDR1*; ABCB1), is an efflux transporter, which is present in the BBB, GI tract, kidneys, liver, and placenta of humans, where it actively transports a wide range of structurally and mechanistically diverse endogenous compounds and xenobiotics across the cell membrane at the energy expense of ATP hydrolysis (Fatoki et al. 2020). P-gp efflux and CYPs activity can profoundly implicate the role of BAPs pharmacokinetics by nutritionally altering their efficacy. The partition coefficient (LogP) and solubility coefficient (LogS) contribute to the bioavailability score (Daina et al. 2017; Sanni et al. 2017). Several *in vivo* studies suggest that the expression of P-glycoprotein (P-gp, *ABCB1*, *MDR1*) was elevated in cases of ALS, and

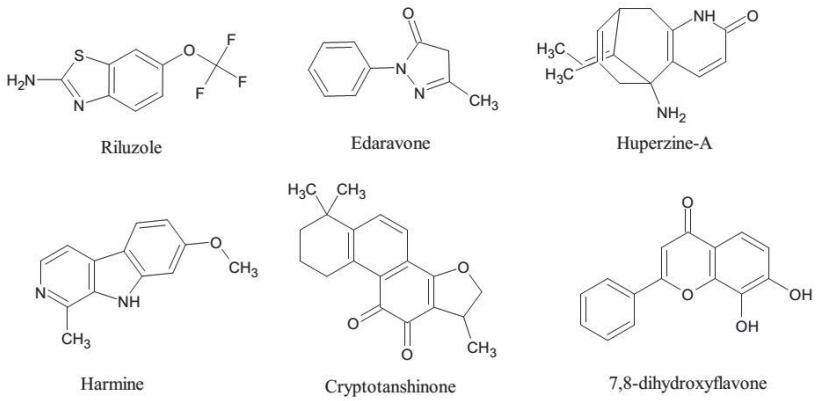


Figure 2. Chemical structures of anti-ALS approved and experimental compounds.

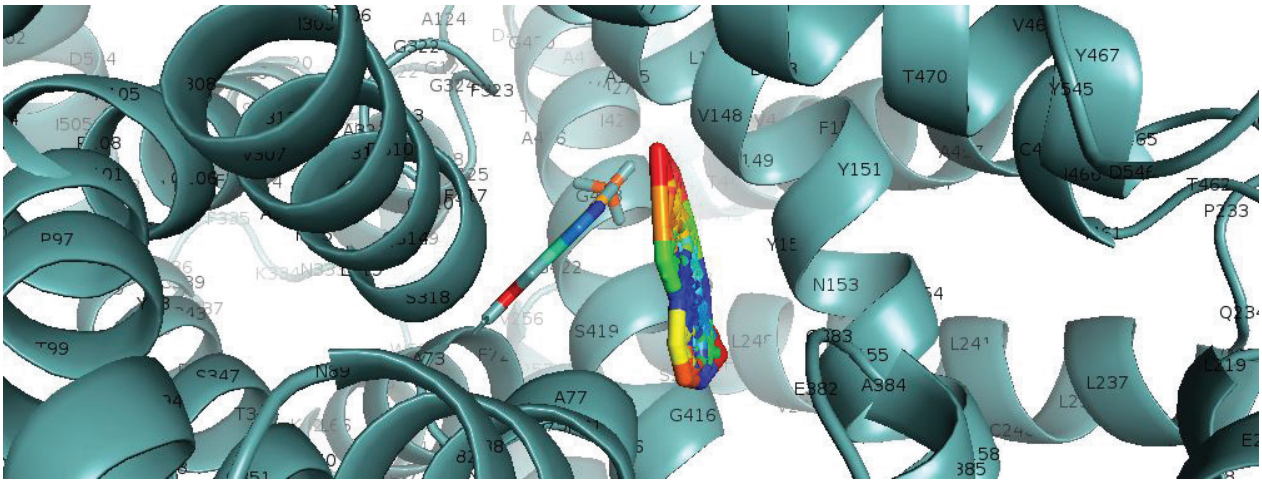


Figure 3. Binding pose of riluzole and harmine on norepinephrine transporter (SLC6A2).

that this increase in *ABCB1* expression could induce progressive pharmacoresistance to riluzole (Robberecht and Philips 2013; Jablonski et al. 2014; Mohamed et al. 2017).

In this study, we predicted that both riluzole and harmine could modulate (induce) the activity of norepinephrine transporter (*SLC6A2*). *SLC6A2* is an amine transporter that terminates the action of noradrenaline by its high affinity sodium-dependent reuptake into presynaptic terminals, and involved in neuron cellular homeostasis, as well as norepinephrine/dopamine:sodium symporter activity (https://www.uniprot.org/uniprot/P23975). Several enzymes and receptors, such as AChE, BChE, MAOA and GABA receptor, have been considered to be pharmacological targets of harmine (Shu et al. 2019). Harmine (7-methoxy-1methyl-9H-pyrido[3,4-b]indole), a tricyclic β-carboline alkaloid, was originally isolated from seeds of *Peganum harmala* L. (Zygophyllaceae) and fruits of *Passiflora incarnata* and *Passiflora edulis* (Mota et al. 2020), *Banisteriopsis caapi* (Callaway et al. 2005), as well as *Melissa officinalis* (Harrington 2012) among others. Studies have shown that harmine have multiple pharmacological activities, such as antimicrobial, antiplasmodial, antiviral, antileishmanial, anti-inflammatory and anticancer effects (Li et al. 2016; Zhang et al. 2016).

Also, we observed that harmine could modulate MAOA (Table 2). MAOA catalyzes the oxidative deamination of various biogenic amines in the brain and peripheral tissues by producing hydrogen peroxide. It is located in the outer mitochondrial membrane and preferentially oxidizes serotonin, norepinephrine, and dopamine (Shih et al. 1999). Thus, dysfunctions of MAOA are involved in a number of neuropsychiatric disorders, such as depression, social anxiety, autism, and attention deficit hyperactivity disorder (Wu et al. 2009). In humans, MAOA breaks down into 5-HT, norepinephrine, and tyramine (Naoi et al. 2016), and a study has shown that that beta-carbolines work as MAO-A inhibitors (McKenna et al. 1984). Harmine could modulate CDK5R1 and CDK9 (Table 2), and implicates CDK2 as one of the kinases associated with ALS. p35 is a neuron specific activator of CDK5, and the complex p35/CDK5 is required for neurite outgrowth and cortical lamination. Cleavage of p35 to p25 may be involved in the pathogenesis of cytoskeletal abnormalities and neuronal death in neurodegenerative diseases (https://www.uniprot.org/uniprot/Q15078). A study that combines *in silico*, *in vitro* and *in vivo* methods has reported that harmine induces mitochondrial membrane depolarization in a dose-dependent manner, induces cell cycle arrest through inhibition of phosphorylation of retinoblastoma protein (pRb) and decreases expression of CDK2, cyclin A and B1, as well as increases animal lifespan at a concentration of 20 mg/kg/day (Mota et al. 2020).

Table 2. Protein Targets of BBB Permeant Ligands

SN	Ligands	% Probability of Predicted Targets																			
		A	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**	**P**	**Q**	**R**	**S**	**T**	**U**	**V**
1	**Riluzole**	95																					
2	Edaravone		20																				
3	**Huperzine-A**			100	90																		
4	**β-asarone**					90	20																
5	Diallyl trisulfide																						
6	Acetovanillone			20		20		20	ALL-20	20	20	20	20	20	20								
7	Muscone								II-20							25							
8	Paeonol					20		30	ALL-20	20		20											
9	**Cryptotanshinone**			100													100	100	100	100	20		
10	3-methylcoumarin								ALL-20													20	20
11	**7,8-dihydroxyflavone**								ALL-35							40							
12	**Harmine**	95																					

		% Probability of Predicted Targets																	
		A2	**B2**	**C2**	**D2**	**E2**	**F2**	**G2**	**H2**	**I2**	**J2**	**K2**	**L2**	**M2**	**N2**	**O2**	**P2**	**Q2**	**R2**
11	**7,8-dihydroxyflavone**	100	100	40	35	30	25	25	25	25									
12	**Harmine**										95	95	95	95	95	95	95	95	95

Note: A: Norepinephrine transporter (P23975). **B**: Beta amyloid A4 protein (P05067). **C**: Acetylcholinesterase (P22303). **D**: Butyrylcholinesterase (P06276). **E**: HMG-CoA reductase (P04035). **F**: Cytochrome P450 1A2 (P05177). **G**: Serine/threonine-protein kinase/endoribonuclease IRE1 (O75460). **H**: Carbonic anhydrase I, II, VII, IX, XII, XIV (P00915, P00918, P43166, Q16790, O43570, Q9ULX7). **I**: Histone acetyltransferase p300 (Q09472). **J**: Catechol O-methyltransferase *(by homology)* (P21964). **K**: Monoamine oxidase B (P27338). **L**: Myoglobin (P02144). **M**: Transthyretin (P02766). **N**: Metabotropic glutamate receptor 5 (P41594). **O**: Cytochrome P450 19A1 (P11511). **P**: Aldose reductase *(by homology)* (P15121). **Q**: Acyl coenzyme A: cholesterol acyltransferase (P23141). **R**: Carboxylesterase 2 (O00748). **S**: Signal transducer and activator of transcription 3 (P40763). **T**: Protein-tyrosine phosphatase 1C, 2C (P29350, Q06124). **U**: Estrogen receptor alpha, beta (P03372, Q92731). **V**: GABA-A receptor; alpha-5/beta-3/gamma-2 (P28472, P18507, P31644). **A2**: Inhibitor of nuclear factor kappa B kinase beta subunit (O14920). **B2**: Neurotrophic tyrosine kinase receptor type 2 (Q16620). **C2**: Arachidonate lipoxygenase -12, 15 (P18054, P16050). **D2**: Telomerase reverse transcriptase (O14746). **E2**: Xanthine dehydrogenase (P47989). **F2**: Lysine-specific demethylase 4D-like (B2RXH2). **G2**: Cyclin-dependent kinase 1 (P06493). **H2**: G protein-coupled receptor kinase 6 (P43250). **I2**: Adenosine A1 receptor *(by homology)* (P30542). **J2**: Dual specificity tyrosine-phosphorylation-regulated kinase 1A, 1B, 2, 3, 4 (Q13627, Q9Y463, Q92630, O43781, Q9NR20). **K2**: Serine/threonine-protein kinase haspin, PIM3, (Q8TF76, Q86V86). **L2**: Adrenergic receptor alpha-2, alpha-2a, alpha-2b (P18825, P08913, P18089). **M2**: Cyclin-dependent kinase 5/CDK5 activator 1 and CDK9/cyclin T1 (Q15078, Q00535 and P50750, O60563). **N2**: Monoamine oxidase A (P21397). **O2**: Casein kinase I delta (P48730). **P2**: Serotonin receptor 2a, 2c, 6, 7 (P28223, P28335, P50406, P34969,). **Q2**: Dual specificity protein kinase CLK1, CLK2, CLK4 (P49759, P49760, Q9HAZ1). **R2**: Nischarin (Q9Y2I1). The ligands with at least 90% probability of target in one or more proteins are in bold.

This study also shows that harmine could modulate adrenergic receptors activity (Table 2). Alpha-2 adrenergic receptor mediates the catecholamine-induced inhibition of adenylate cyclase through the action of G proteins. The rank order of potency for agonists of this receptor is clonidine>norepinephrine>epinephrine=oxymetazoline>dopamine>p-tyramine=phenylephrine>serotonin>p-synephrine/p-octopamine (https://www.uniprot.org/uniprot/P08913). Alpha-2 adrenergic receptors are involved in the negative regulation of norepinephrine secretion and positive regulation of MAPK cascade (Fig. 4). The anticancer properties of harmine have been linked to the suppression of the ERK and AKT/mTOR signaling pathway (Liu et al. 2016; Zhang et al. 2016; Wu et al. 2019), and this also promotes serine/threonine-protein kinase pim-3 and dual specificity tyrosine-phosphorylation-regulated kinases (DYRKs) activities, as well as leads to negative regulation of insulin secretion. Serotonin receptors help in the positive regulation of ERK1 and ERK2 cascade, positive regulation of TOR signaling, regulation of dopamine secretion, activation of phospholipase C activity, and cellular calcium ion homeostasis among others.

Harmine could modulate nischarin and casein kinase I delta (Table 2). Nischarin acts either as the functional imidazoline-1 receptor (I1R) candidate or as a membrane-associated mediator of the I1R signaling. Nischarin binds to numerous imidazoline ligands, which induces initiation of cell-signaling cascades leading to cell survival, growth and migration. It is involved in several biological processes, such as norepinephrine secretion and regulation of GABAergic synaptic transmission (https://www.uniprot.org/uniprot/Q9Y2I1). Casein kinase I delta is an essential serine/threonine-protein kinase that regulates diverse cellular growth and survival processes, including Wnt signaling, DNA repair and circadian rhythms. It triggers down-regulation of dopamine receptors in the forebrain, and regulates fast synaptic transmission mediated by glutamate (https://www.uniprot.org/uniprot/P48730). A study has shown that harmine could increase gene expression levels of the glutamate transporter GLT-1 (EAAT2 in humans) in the cortex of SOD1 mutant mice compared to mice treated with saline, thereby increased the cellular uptake of glutamate (Li et al. 2011).

Furthermore, the results show that huperzine-A and cryptotanshinone could modulate acetylcholinesterase (AChE) as shown in Table 2. AChE is a crucial enzyme for nerve functions, hydrolyzing acetylcholine (ACh) in the synaptic cleft, thus terminating synaptic transmission (Taylor and Radic 1994), while the function of butylcholinesterase (BChE) and its role in the regulation of AChE levels remains unexplored, but it has been discovered that BChE anchored by PRiMA is present on the surface of terminal Schwann cells at mouse neuromuscular junction (Petrov et al. 2014). A study has reported the involvement

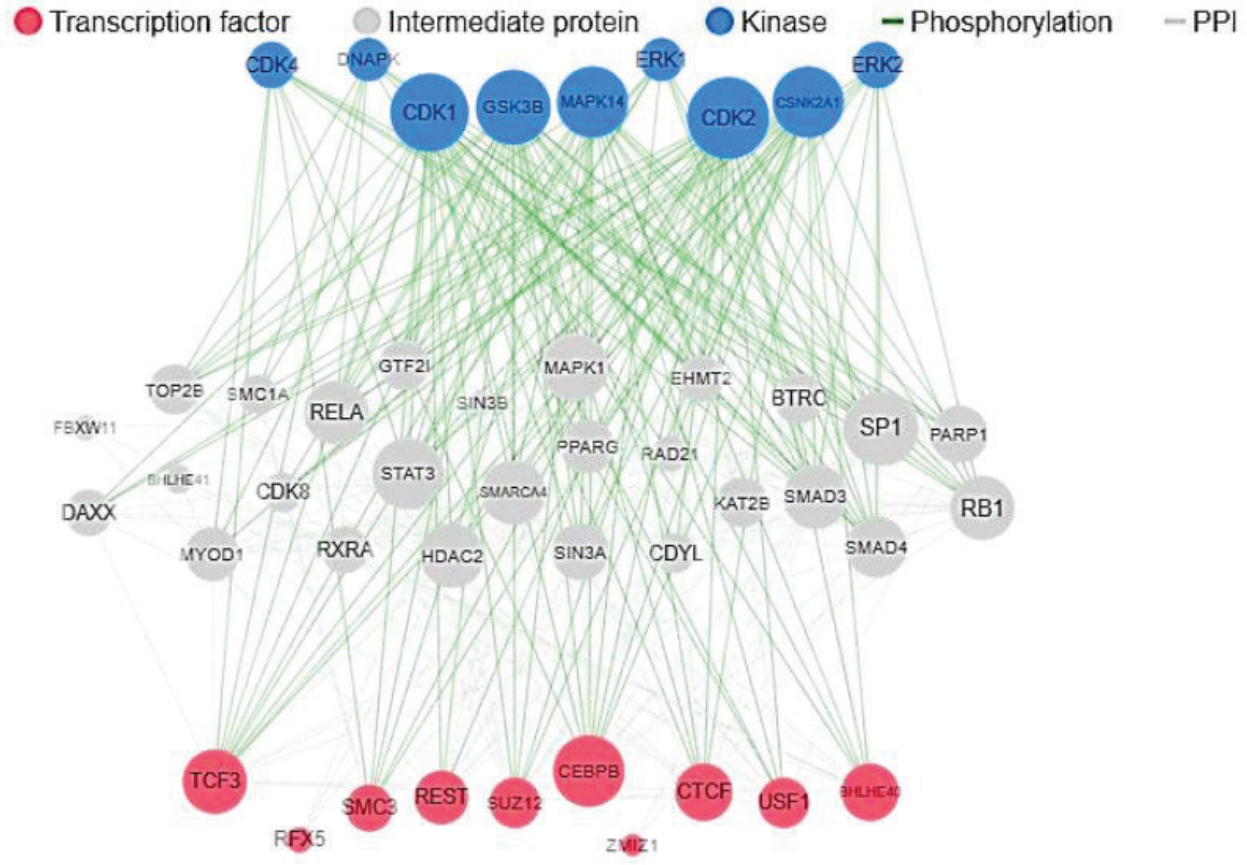


Figure 4. eXpression2Kinases Network. Showing overall interactions of intermediate proteins, kinases and transcription factors with high hypergeometric ($-\log_{10}$) p-value.

Table 3. Docking Parameters and Binding Energy Score of the Interaction Between Ligands and Selected Targets With at Least 90% Probability

SN	Target Protein name	Gene ID	UniProt ID	Alphafold ID	Center grid box (points)	Size (points)	Spacing (Å)	Binding free energy (kcal.mol^{-1}) of anti-ALS targets					
								A	B	C	D	E	F
1	Norepinephrine transporter	*SLC6A2*	P23975	AF-P23975-F1-model_v2	-4.758 × -5.343 × 4.512	126 × 126 × 126	0.625	-8.5	-8.2				
2	Alpha-2a adrenergic receptor	*ADRA2A*	P08913	AF-P08913-F1-model_v2	4.059 × -4.402 × -1.660	126 × 126 × 126	0.675		-6.9				
3	Casein kinase I delta	*CSNK1D*	P48730	AF-P48730-F1-model_v2	0.456 × 5.525 × 1.318	100 × 126 × 100	0.675		-7.4				
4	Cyclin-dependent kinase 5	*CDK5R1*	Q15078	AF-Q15078-F1-model_v2	-2.409 × -2.148 × -11.835	100 × 126 × 126	0.675		-6.6				
5	Dual specificity protein kinase CLK1	*CLK1*	P49759	AF-P49759-F1-model_v2	-0.574 × -13.512 × 3.865	100 × 126 × 126	0.675		-7.4				
6	Dual-specificity tyrosine-phosphorylation regulated kinase 2	*DYRK2*	Q92630	AF-Q92630-F1-model_v2	-6.546 × 1.317 × -1.015	126 × 126 × 126	0.775		-8.9				
7	Monoamine oxidase A	*MAOA*	P21397	AF-P21397-F1-model_v2	-9.622 × 6.254 × -2.780	126 × 126 × 126	0.575		-7.3				
8	Nischarin	*NISCH*	Q9Y2I1	AF-Q9Y2I1-F1-model_v2	11.256 × 3.640 × 5.366	126 × 126 × 126	0.875		-8.1				
9	Serine/threonine-protein kinase haspin	*HASPIN*	Q8TF76	AF-Q8TF76-F1-model_v2	-10.896 × -9.196 × 0.121	126 × 126 × 126	0.875		-9.7				
10	Serotonin 6 (5-HT6) receptor	*HTR6*	P50406	AF-P50406-F1-model_v2	-17.979 × 1.095 × 9.821	110 × 110 × 126	0.775		-8.3				
11	Aldose reductase (by homology)	*AKR1B1*	P15121	AF-P15121-F1-model_v2	1.346 × 2.385 × 0.255	110 × 110 × 110	0.475			-13.3			
12	Acyl coenzyme A: cholesterol acyltransferase	*CES1*	P23141	AF-P23141-F1-model_v2	0.205 × 2.390 × 1.338	100 × 100 × 100	0.775			-10.6			
13	Carboxylesterase 2	*CES2*	O00748	AF-O00748-F1-model_v2	× -0.956 -0.073 × 1.882	126 × 126 × 126	0.575			-9.5			
14	Signal transducer and activator of transcription 3	*STAT3*	P40763	AF-P40763-F1-model_v2	-1.791 × -0.348 × -6.174	126 × 126 × 126	0.775			-10.0			
15	Acetylcholinesterase	*ACHE*	P22303	AF-P22303-F1-model_v2	-3.007 × 1.029 × -4.673	100 × 100 × 100	0.775			-10.1	-10.1		
16	Butyrylcholinesterase	*BCHE*	P06276	AF-P06276-F1-model_v2	11.816 × -6.464 × -1.440	80 × 106 × 116	0.775				-10.2		
17	Inhibitor of nuclear factor kappa B kinase beta subunit	*IKBKB*	O14920	AF-O14920-F1-model_v2	1.674 × -15.158 × 2.813	126 × 126 × 126	0.775					-10.5	
18	Neurotrophic tyrosine kinase receptor type 2	*NTRK2*	Q16620	AF-Q16620-F1-model_v2	0.748 × 8.930 × -8.095	126 × 126 × 126	0.775					-10.3	
19	HMG-CoA reductase	*HMGCR*	P04035	AF-P04035-F1-model_v2	-17.248 × 6.830 × -7.531	126 × 126 × 126	0.775						-2.9

Note: A: Riluzole. **B**: Harmine. **C:** Cryptotanshinone. **D**: Huperzine-A. **E:** 7,8-dihydroxyflavone. **F:** β-asarone

of AChE in ALS-pathogenesis, while raising the possibility of an exacerbating effect of AChE enzyme inhibitors in this disease (Gotkine et al. 2013). AChE has been shown to be present in tissues devoid of cholinergic synapses and to be involved in the process of apoptosis, a process also involved in ALS pathogenesis, by playing a pivotal role in apoptosome formation (Park et al. 2004). Loss of cholinergic synapses was reported in sporadic ALS patients by studying the expression of vesicular ACh transporter (VAChT), involved in the packaging of ACh inside the synaptic vesicles before release, and this suggested a loss of cholinergic inputs as an early event of ALS neurodegeneration (Nagao et al. 1998). However, the cellular origin of the AChE released in the plasma in ALS and the consequences of its absence at the neuromuscular junction remains unexplored (Campanari et al. 2016). Moreover, it has been found that a partially depleted TAR DNA-binding protein 43 (TDP-43) orthologue in zebrafish caused a decrease of AChE expression, and that human AChE overexpression reduced the phenotypic defects in the TDP-43 loss of function model, with amelioration of post- and pre-synaptic deficits at the NMJ (Campanari et al. 2021).

According to the study conducted by Li et al. (2018) to comparatively investigate the effects of harmaline and harmine in memory deficits of scopolamine-induced mice, their results showed that both harmaline and harmine exhibited an enhancement in cholinergic function by inhibiting AChE and inducing choline acetyltransferase (ChAT) activities, and antioxidant defense via increasing the antioxidant enzymes activities of superoxide dismutase and glutathione peroxidase, and reducing maleic dialdehyde production, and anti-inflammatory effects through suppressing myeloperoxidase, tumor necrosis factor alpha (TNF-α), and nitric oxide, as well as modulation of

critical neurotransmitters, such as acetylcholine (ACh), choline (Ch), L-tryptophan (L-Trp), 5-hydroxytryptamine (5-HT), gamma-aminobutyric acid (γ-GABA), and L-glutamic acid (L-Glu).

Insightfully, we found that 7,8-dihydroxyflavone could modulate nuclear factor kappa B kinase beta subunit and neurotrophic tyrosine kinase receptor type 2 (NTRK2). The NTRK2 is involve in the development and the maturation of the central and the peripheral nervous systems through regulation of neuron survival, proliferation, migration, differentiation, and synapse formation and plasticity. It may also play a role in neutrophin-dependent calcium signaling in glial cells and mediate communication between neurons and glia (https://www.uniprot.org/uniprot/Q16620). The impact of 7,8-dihydroxyflavone on several carbonic anhydrases (CAs) I, II, VII, IX, XII, and XIV, as well as CYP19A1, could help in remediating the respiratory failure associated with ALS.

The CAs obtained in this study are both cytosolic (I, II, and VII) and membrane-bound (IX, XII, and XIV). CAs are involve in various physiological reactions, including respiration, pH regulation, Na^+ retention, calcification, tumorigenesis, electrolyte secretion, gluconeogenesis, ureagenesis, and lipogenesis. The excitability of most central neurons and neuronal networks is enhanced by an alkalosis and suppressed by an acidosis, and the cytosolic CA activity in central nervous system (CNS) promotes $GABA_AR$-mediated net HCO_3^- efflux in mammalian CNS neurons (Ruusuvuori and Kaila 2014). Respiratory failure in ALS could have resulted from molecular hypoventilation, leading to increased CO_2 levels, decreased O_2 levels, and can lead to acidosis-induced death. H^+ is one of the most important physiologically-active agents that exert a fundamental modulatory role in neuronal development, plasticity, as well as synaptic and electrical signalling. The awareness that H^+ does act as a modulatory signal in microdomains of the brain extracellular space is supported by the findings showing that transporter-mediated acidification of the synaptic microenvironment is sufficient to enhance GABAergic signaling (Dietrich and Morad 2010; Ruusuvuori and Kaila 2014).

The key concept of molecular docking is to develop an appropriate solution to elucidate the minimum free energy (ΔG) of interaction per mole of ligand (Pagadala et al. 2017). This study shows that harmine can bind DYRK family (Tables 2, 3), and this result corroborates the previous studies which have shown that harmine could inhibit all members of the DYRK family (such as DYRK1A, DYRK1B, DYRK2, and DYRK4), with the highest affinity indicated for DYRK1A (Göckler et al. 2009; Ogawa et al. 2010).

Conclusion

This study has provided insights on the phytochemicals with potential anti-ALS activities. The data revealed that harmine is possibly superior to riluzole, and that combination of harmine with 7,8-dihydroxyflavone and huperzine-A can provide a more effective treatment for ALS than the current regime. This study is the first to predict norepinephrine transporter as one of the key targets of harmine and ALS. Also, we have indicated possible involvement of some molecular targets, such as AChE, CAs, NTRK2, MAOA, DYRKs, CDKs and others, in the positive regulation of CDK2, CDK1, CDK4, ERK1, ERK2 and MAPK14 signaling cascade, and positive regulation of Akt/mTOR signaling. Further work is needed to validate the predicted therapeutic targets of harmine identified in this study on the ALS model or clinical trials, using *in silico*, *in vitro* and *in vivo* techniques.

Conflict of interest

The authors declare no conflict of interests.

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