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RESEARCH ARTICLE

Eclipta alba, An Antioxidant-Rich Memory Enhancer: *In-Silico*and *In-Vivo* Evidence with Specific Insight into Alzheimer's Disease

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Abstract

Objectives: *Eclipta alba* (EA), a potent anti-aging drug with unidentified secondary metabolites, is a widely recommended nerve tonic that is used to treat dementia. This study aimed to identify the secondary metabolite profile of EA, followed by their interaction with Alzheimer's disease (AD) related proteins. Therefore, the present study attempted to screen the active constituents of the aqueous extract of EA (aqEA) using liquid chromatography-mass spectrometry (LC-MS) and to identify its effect on spatial learning and memory also understanding its toxicity in *in-vivo*. **Methodology:** Initially**,** Autodocking analysis was performed to analyze the molecular interactions of secondary metabolites with AD-related proteins followed by *in-vivo* investigations using albino Wistar rats, where 150 mg/kg of aqEA was given orally for 28 days and memory was analyzed using 8-arm radial maze(RAM). Blood parameters were screened for toxicity related parameters using an auto analyzer. **Findings:** LC-MS showed a significant proportion of Lilaline, Laccarin, Coriandrone E, Quercetin, Catechin, Wedelolactone, and Luteolin. Autodocking showed a good binding affinity for each phytochemical, with coriandrone E being the most effective. Further aqEA elicited no significant toxic effects in *in-vivo* and interestingly improved memory performance in the RAM. **Novelty:** This study identified unique pharmacologically active metabolites in aqEA that interacted well with AD related proteins with higher affinity thereby providing valuable clue on aqEA as a suitable drug against AD pathology. Interestingly, aqEA observed to influence cholinergic neurons by regulating acetylcholine esterase activity which could replace wide commercial inhibitors available in the market. Thereby the current study paves way for more unidentified pathways that could be addressed by the treatment of aqEA through its active secondary

metabolites.

Keywords: Dementia; Alzheimer's Disease; Aging; Eclipta alba; Neuroprotection

1 Introduction

The pleiotropic potential exhibited by plant extracts has gained major attention in recent times and is considered a reliable choice for drugs because of its herbal origin. This study aimed to investigate the pharmacological effects of one such plant EA - a herbal drug that is widely used among Ayurvedic practitioners to treat dementia. However apart from EA's antioxidant properties there is no other studies to strengthen molecular mechanistic action of EA's on neuroprotection. Therefore, the study focused on screening the components of EA and its neuroprotective properties that could enhance cognitive efficiency in rat models. EA, a well-known Ayurvedic medicine of Indian tradition, was chosen based on its wide range of benefits in the nervous system, including anti-epileptic, nootropic, and anxiolytic actions[(1)]. In addition, EA supplementation in humans was reported to exhibit a reduction in total cholesterol, low-density lipoprotein fraction, triglyceride, and very low-density lipoprotein levels in the blood. It is also reported to reduce oxidative stress-induced complications in hypertensive[(2)]. This provides further insights into the regulation of lipid metabolism by EA in the brain, where abnormal lipid levels are widely considered as a risk factor for Alzheimer disease (AD) progression.

The main objective is to investigate the effect of EA on memory enhancing effects in the *in-vivo* model system by understanding the composition of secondary metabolites in aqEA and its interactive ability on AD related proteins in *in-silico* approach. Further the present study focuses on EA toxicity in *in-vivo* and to identify the possible mechanism that reveals aqEA to exhibit neuroprotective effect in rat model systems.

Initially, LC-MS analysis was performed to investigate the secondary metabolite profile of the plants. The most prevalent metabolites from the LC-MS results were then subjected to molecular docking analysis using auto docking to explore their interactions with AD-related proteins. The binding affinity clearly implies that the EA constituents may interact with AD-related proteins in *in-vivo*. With the knowledge stated above, *in-vivo* toxicity experiments for the liver and kidney were performed by analyzing serum glutamate pyruvate transaminase (SGPT/ALT), alkaline phosphatase (ALP), and serum glutamate oxaloacetate transaminase (SGOT/AST) levels, as well as neurochemical assays, which confirmed that EA does not exhibit any discernible toxicity in liver and kidney functions and increases neuroprotection in the *in-vivo* system. EA also enhances memory and learning, as evidenced by the RAM analysis and AChE assay. Considering the binding affinity and the *in-vivo* analysis of toxicity and memory, it is hypothesized that phytochemicals may directly control apolipoprotein E (ApoE) expression and have an impact on lipid metabolism. In addition, these metabolites may have an effect on the Amyloid Precursor Protein (APP) pathway to ameliorate AD pathology and enhance memory by stabilizing acetylcholine levels in the brain. Overall, EA is a multitarget paradigm for AD pathology.

Despite various traditional practices of EA for different illnesses the exact molecular mechanism behind EA is less explored. Various studies on human and animal models revealed the different beneficial aspects of EA[(3,4)], However, the documentation of Indian traditional medicine like EA remains obscure and needs further mechanistic illustration. This study has taken a step ahead to witness EA as a potential antioxidant containing multi-therapeutic drugs for age-related disorders. Further, this can be extended in different molecular aspects to make EA a safe and reliable drug choice in age-related disorders. In addition, the study proposes a novel hypothesis that EA can

enhance memory by reducing AChE activity in the synaptic junction cognitive centers like the hippocampus.

2 Methodology

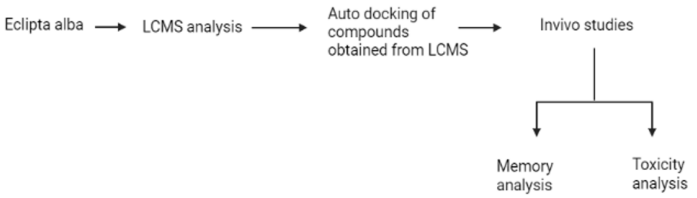

Fig 1. Schematic representation of overall methodology

2.1 Plant Material

EA plant was collected from Kanakapura village, Bangalore. The whole EA plant was cleaned completely with distilled water and dried in the shade at room temperature. Then, the shade-dried plants were reduced to a powder with the help of a mortar and pestle. The crude powder was then weighed and stored in the refrigerator until further use. The EA plant was validated for gene sequence analysis using Random amplified polymorphic DNA (RAPD) and the sequence has been submitted in Pubmed [Accession Number: KJ512896].

2.2 Preparation of plant extract

2.2.1 Extraction by continuous stirring method

The desiccated sample was reconstituted in water at a concentration of 1 part powder to 5 parts water by weight/volume (w/v). Subsequently, this solution was subjected to continuous agitation on a magnetic stirrer, maintaining a constant temperature of 30°C for a duration of 36 hours. Following this incubation period, the resultant aqueous extract was subjected to filtration through Whatman No.1 filter paper and then preserved at a temperature of 4°C for future utilization.

2.3 Liquid chromatography-mass spectrometry (LC-MS) analysis

In order to discern the active secondary metabolites within the extract, it underwent further scrutiny via liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI-MS), employing an Agilent Technologies TOF/Q-TOF Mass Spectrometer (Model G6550A, Q-TOF B.05.01 (B5125.3)) located at the Sophisticated Analytical Instrument Facility (SAIF) within the confines of the Indian Institute of Technology Bombay, Mumbai.

The extract for analysis was prepared using water as the solvent. A high-resolution liquid chromatography and mass spectrometry system, specifically the Agilent Model G6550A with 0.01% mass resolution, was deployed to establish the chemical profile of the extract. The acquisition parameters encompassed a mass range spanning from 100 to 1000 daltons (M/Z), with a scanning rate of 16 spectra per second. A Hip sampler (G4226A-model) was employed with a draw rate of 100 μl/min, an ejection rate of 100 μl/min, a flush out factor of 5 μl, and an injection volume of 8 μl, utilizing an injection mode featuring needle wash for high-resolution liquid chromatography mass spectrometry (HR-LCMS). The acquisition duration amounted to 30 minutes, with the initial 2 minutes allocated for solvent flow. The solvent composition for HR-LCMS ranged from 95% water to 100% acetonitrile. The analysis was executed utilizing an Agilent 1200 series thermostatted Column compartment (Model G1316C). Subsequent to LC-MS analysis, the mass spectral data underwent processing and analysis procedures to generate a comprehensive list of bioactive secondary metabolites.

2.4 Auto-Docking

AutoDock Vina is one of the fastest and most widely used open-source docking engines. It is a turnkey computational docking program that is based on a simple scoring function and rapid gradient-optimization conformational search. The structure-based drug discovery approach completely relies on the three-dimensional structure of target proteins involved in a particular

biological process. The sequence and functional information for all the eight proteins were collected from Protein Data Bank (PDB) maintained by research collaboratory for structural bioinformatics (RCSB) (https://www.rcsb.org) database. Molecules retrieved from PDB usually are not having a complete charge assigned to them. Hence before docking, the polar hydrogens were added to the macromolecules and water molecules were removed then assigned the partial atomic charges using Autodock. The structure of secondary metabolites Lilaline, Laccarin, Quercetin, Coriandrone E, Wedelolacone, Catechin, and Luteolin, were derived from Pubchem. Automated docking software AutoDock vina was used to evaluate the binding affinity of protein with different ligands.

2.5 Experimental Design

All experiments were conducted with the institutional and animal ethical guidelines approved by the Institutional Animal Ethics Committee of Bharathidasan University (Ref No: BDU/IAEC/P03/2021), Tiruchirappalli, India. 9-12-month-old male albino rats of Wistar strain weighing around 250-350g obtained from Biogen, Bangalore confined to three per cage in the temperature 25± 2°C and a light-controlled environment with a 12:12 hrs light-dark cycle. The control and treated animals were fed with a standard ad libitum diet throughout the study. The aqEA extract of 1 ml was administered orally (150 mg/kg) for 30 days.

The animals were divided into two major groups and each group consists of six animals, namely,

Group I: Control Adult Rat

Group II: EA treated Adult Rat

On completion of the experimental period, animals were sacrificed by cervical decapitation. The blood, brain, liver and kidney tissues were excised immediately and washed in several changes of ice-cold saline. Brain (frontal cortex and hippocampus), liver and kidney tissues were separated and stored at -80°C for further analysis. Serum was derived from a blood sample for further biochemical analysis.

2.6 Neurobehavioral analysis

2.6.1 8-arm Radial Arm Maze task (Olton and Samuelson, 1976)

The 8-arm Radial Arm Maze (RAM) which was employed in the current investigation, had 8 arms that were numbered from 1 to 8 and was 48x12cm apiece. The arms extended radially from a central region that was 32cm in diameter. A variety of extra-maze visual signals were positioned in the same location around the apparatus, positioned 40cm above the ground during the trial. There was a meal cup with a single 50mg food pellet at the end of each arm. The animals were kept on a restricted diet for a week prior to the maze task, and their body weight was held at 85% of their free-feeding weight with unlimited access to water. Before the actual training began, three or four rats were placed in the radial maze at the same time and permitted to wander and eat freely for 5 minutes. Food was initially available throughout the maze, but was gradually restricted to the food cup. The animals were trained for four days to rush to the end of the arms and to find the reward. To assess basal activity in a radial 8-arm maze, rats were given 5 consecutive training sessions of running to the end of the arms and swallowing the bait each day. The training experiment proceeded until all four baits were swallowed, or until 5 minutes had passed. The percentage of correct response was calculated by using the following formula [5].

% of correct response = (number of correct response/ number of trials) ×100

2.7 Biochemical analysis

2.7.1 Estimation of Urea

Urea was determined by the method of Geyer and Dabich (1971)

To analyse the toxicity of EA on kidney, urea levels were determined in blood. 0.2 ml of blood was deproteinized with 2.8 ml of Trichloro acetic acid. To 2.0 ml of the supernatant obtained by centrifugation, 1.0 ml of diacetyl monoxime and thiosemicarbazide reagent and 1.5 ml of acid ferric reagent were added and the solution was heated in a boiling water bath for 15 minutes. Aliquots of standard urea and blank containing 2.0 ml water were also treated in a similar manner. After cooling, the colour developed was read at 520 nm in a Shimadzu Ultra Violet (UV) spectrophotometer. The urea levels were expressed as mg/dl blood [6].

2.7.2 Enzymatic analysis

Serum samples, obtained from blood, were subjected to analysis utilizing an automated analyzer (Merilyzer Cliniquant Micro). This analysis was performed employing a biochemical analyzer kit (Labnova and CORAL) following the manufacturer's instructions. To ascertain the suitability of EA as a viable therapeutic option, it is imperative to scrutinize its potential toxicity.

Consequently, a toxicity study was conducted using serum samples to identify functional biomarkers associated with vital organs. The evaluation encompassed the assessment of SGPT/ALT, SGOT/AST and ALP. This comprehensive examination served the purpose of identifying any potential toxicity in major metabolic organs associated with EA administration.

2.7.3 Neurochemical analysis

Assay of Acetylcholinesterase (AChE)

AChE is an enzyme integral to cholinergic neurotransmission, responsible for the hydrolysis of acetylcholine (ACh), thereby terminating the neurotransmission process. The widely employed assay for AChE activity is based on Ellman's method, which utilizes an alternative substrate, acetylthiocholine, in conjunction with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB). The enzymatic reaction yields 5-thio-2-nitrobenzoate, characterized by its yellow coloration resulting from electron transfer to the sulfur atom.

To investigate the potential neuroprotective properties of EA the estimation of AChE activity was conducted following the protocol outlined by Ellman et al. in 1961. Brain tissue, at a concentration of 20 mg per milliliter, was homogenized in 0.1M Tris-phosphate buffer containing 5mM EDTA at pH 8 using a Potter-Elvehjem homogenizer. Subsequently, a 0.4 ml aliquot of the brain homogenate was transferred to a cuvette containing 2.6 ml of Tris-phosphate buffer. To initiate the enzymatic reaction, 100μl of the DTNB reagent was introduced into the cuvette, and the absorbance was measured at 412 nm. Furthermore, 20μl of 0.075M acetylthiocholine iodide was added to the mixture, and the ensuing changes in absorbance were continuously monitored. The rate of change in absorbance per minute was determined, and the enzyme activity was expressed as μmoles of substrate hydrolyzed per minute per gram of tissue[7].

3 Results and Discussion

3.1 *Eclipta alba* constitutes pharmacologically active metabolites

Researchers can gain knowledge more about EA's neuroprotective activity by screening its secondary metabolites. The present study employed LC-MS to screen the constituents of the aqEA. Since the ayurvedic practitioners follow the administration of an aqEA, the current study also used water as solvent and was subjected to LC-MS for screening of dissolved components of aqEA. The total ionic chromatogram (Figures 2 and 3) of the phytochemical screening of aqEA displayed several compounds with maximum peak values that correspond to the vital constituents of the EA. The seven major constituents identified in LC-MS analysis are Lilaline, Laccarin, Coriandrone E, Quercetin, Catechin, Wedelolactone, and Luteolin (Table 1). These plant secondary metabolites are well-known for their potential pleiotropic effects in various pathological and physiological conditions[6,8]. In this aspect, it is clear that the overall biological effects of EA might be contributed by either of the above-mentioned components. There are other major and minor constituents identified through LC-MS analysis, however, the major components that individually possess significant biological effects were chosen for further insilco analysis to support the biological effect of the above.

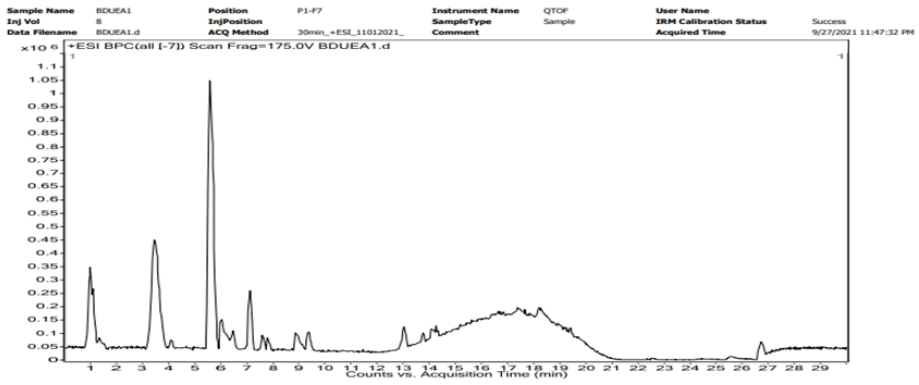

Fig 2. Representative image of chromatogram from LC-MS analysis indicates the presence of various peaks corresponding to different secondary metabolic constituents of EA in LC-MS analysis

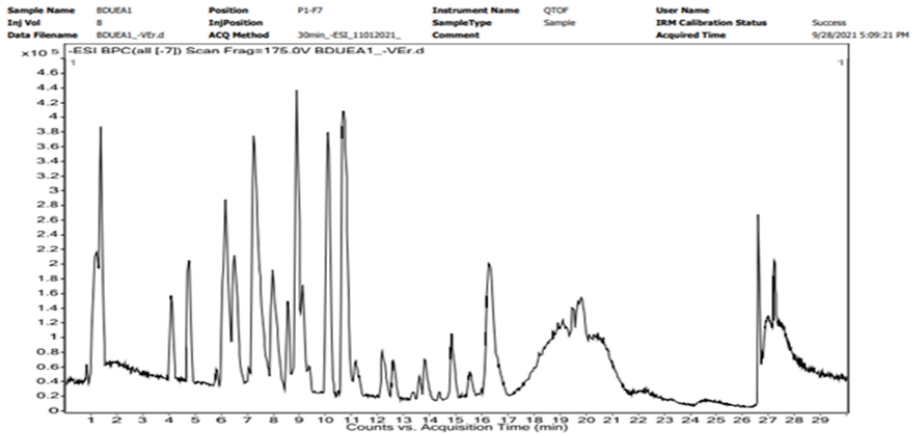

Fig 3. Representative image of chromatogram from LC-MS analysis indicates the presence of various peaks corresponding to different secondary metabolic constituents of EA in LC-MS analysis

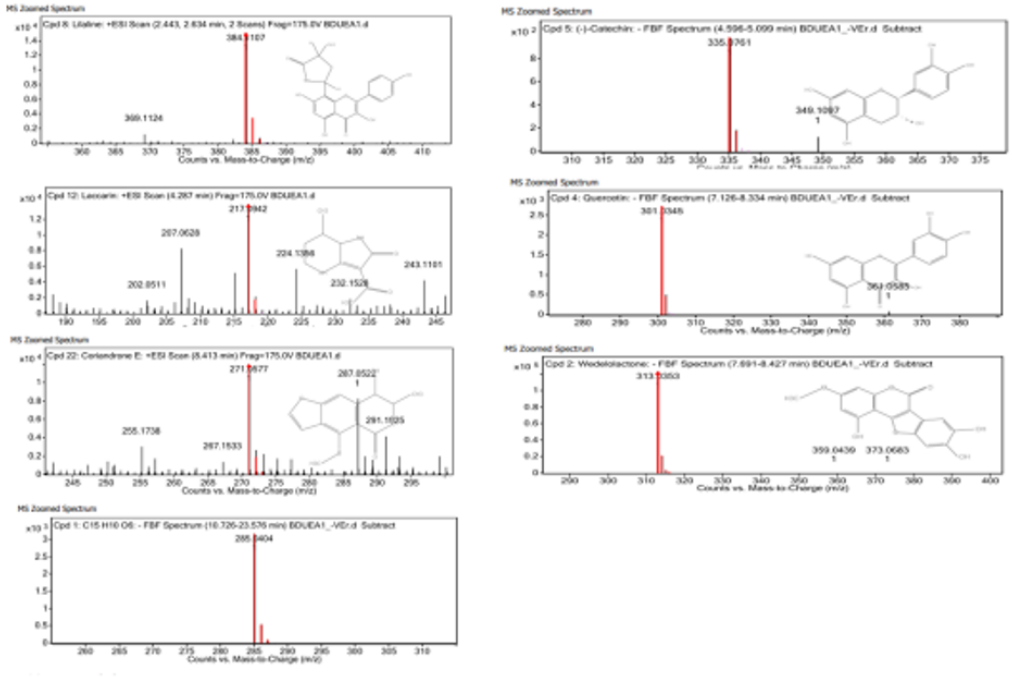

Fig 4. Total Ion Chromatogram of Phytochemical Screening Of Ea By Lc-Ms (Qtof) – X axis and Y axis denotes Counts Vs Mass to charge (m/z) ratio Representative images of chromatogram indicating unique peaks and corresponding structure of different secondary metabolites of EA

Table 1. Major photochemical compounds screened by LCMS aqueous extract of EA

S.No.	Compound	Mass (Da-Dalton)	Abund	RT (minutes or seconds)	MFG formula (Molecular formula generation)
1.	Lilaline	383.1038	15064	2.557	C7H11NO7
2.	Laccarin	194.1051	13913	4.307	C10H14N2O2
3.	Coriandrone E	248.0681	49478	8.499	C13H12O5
4.	Quercetin	302.0419	2718	4.92	C15H10O7
5.	Catechin	290.0778	979	7.0303	C15H14O6
6.	Wedelolactone	314.0426	123084	7.832	C16H10O7
7.	Luteolin	286.0477	3161	15.116	C15H10O6

The table provides information of EA secondary metabolites, including their assigned serial number, compound name, mass (in atomic mass units), abundance, retention time (RT), and molecular formula

3.2 *In-silico* analysis of active secondary metabolites revealed good score with AD associated proteins providing clue on EA's neuroprotective role

In-silico interaction analysis of the major constituents of EA to that of AD associated proteins like beta-site amyloid precursor protein cleaving enzyme-(BACE1), A Disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), Presenillin(PS1), Apolipoprotein E4 (APOE4), Amyloid precursor protein(APP), AChE, N-Methyl-D-Aspartate Receptor (NMDA) and β-hydroxy-β-Methylglutaryl CoA(HMG-CoA) reductase revealed that flavonoid constituents of EA interacted well with the key biomarkers of AD pathology and also yield significant binding affinity. The binding affinity from the table below (Table 2) explains the efficient interaction of the active constituents with AD associated proteins.

Table 2. Docking scores of EA constituents with AD related proteins and the rate limiting enzyme of cholesterol metabolism

Compounds Name	BACE 1	NMDA Receptor	ADAM 10	APOE4	AChE	APP	PS 1	HMG CoA Reductase
	2WJO	4TLL	6BE6	1B68	4EY7	1AAP	6LQG	2R4F
Lilaline	-9.3	-7.8	-8.5	-7.2	-10.1	-7.3	-8.3	-8.8
Laccarin	-6	-6.5	-6.5	-5.2	-8.5	-6.5	-5.9	-6.8
Quercetin	-8.1	-8.6	-8.2	-6.6	-10	-7.7	-7	-8.6
Coriandrone E	-6.8	-7.6	-6.5	-5.5	-7.9	-6.3	-6.0	-6.5
Wedelolactone	-7.9	-8.7	-8.4	-6.4	-9.5	-6.5	-7.1	-8.1
Catechin	-8.1	-8.2	-8.1	-6.2	-9.8	-7.2	-7.2	-8.1
Luteolin	-8.4	-8.2	-7.9	-6.7	-10.3	-7.5	-7.2	-8.3

3.3 No significant biological toxicity was observed in EA administered rats

Based on literature, and preliminary study on dosage fixation of aqEA for animals (data not shown), every day dose administration was fixed at 150 mg/kg body weight dissolved in water and were given to experimental rats. In order to determine the toxic effect of EA at this dosage, present study involved assessment of various biochemical assays. (Represented as Figure 5)

EA is a well-known drug that has been utilized in Ayurveda as a nerve tonic for many years and its phytochemical constituents have proved good for neuroprotective benefits [9,10]. However, it is crucial to analyze its toxicity in *in-vivo* investigations. SGOT and SGPT are typical biomarkers to assess liver and heart function, which may go elevated during any disease condition or injury. Interestingly, the levels of SGOT in the liver and serum of experimental animals displayed lower levels in treated animals compared to controls, however not significant. There was no significant change in the SGPT levels.

Liver and bones constitute higher concentration of ALP than other tissues and any change in its level depicts damage to vital organs like liver, kidney etc. Administration of EA has significantly decreased the ALP levels of liver, kidney and Serum samples depicting beneficial effects of EA in *in-vivo*. Liver produces urea, a waste product that is expelled by the kidney. The presence

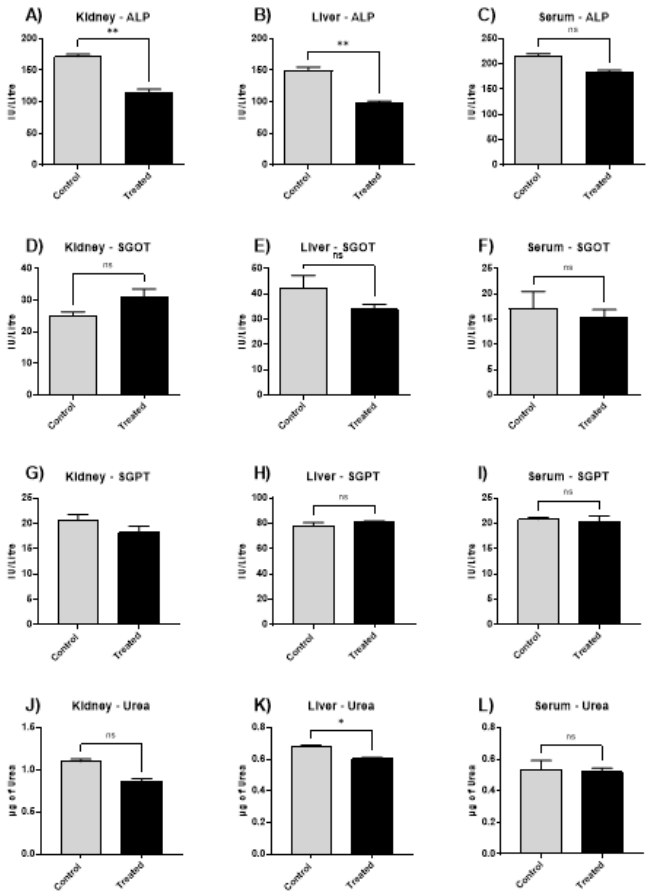

Fig 5. Biochemical analysis of biomarkers to analyse EA toxicity in experimental animals : A) Kidney – ALP B)Liver - ALP C) Serum - ALP D) Kidney - SGOT E)Liver – SGOT F) Serum – SGOT G) Kidney - SGPT H) Liver – SGPT I) Serum – SGPTJ) Kidney – Urea K) Liver – Urea L) Blood -Urea between control and experimental rats. Mean values of these biochemical parameters, along with their respective standard deviations (mean ±SD), were obtained from three independent experiments (n=3). Statistical significance was determined using unpaired t tests, where * indicates significance at $P < 0.05$, ** denotes significance at $P < 0.01$, and"ns" represents no significance

of urea at a normal level in liver and serum and with reduced levels in the kidney indicates healthy renal function. Overall, the graph shows that rats are not adversely affected by aqEA at the prescribed dosage for an *in-vivo* model.

3.4 EA administration alleviated Acetylcholine esterase activity in the hippocampus of experimental animals

Memory is enhanced majorly by retaining acetylcholine levels in the important cognitive centers of the brain viz., hippocampus. As evidenced from the *in-silico* analysis (Table 2), where the phytochemical compounds of EA displayed good binding affinity on interacting with AChE, the same was duplicated with in-vivo analysis by reducing the AChE activity in hippocampus of experimental rats. EA reduced the AchE levels significantly in the rat hippocampus, whereas in the frontal cortex EA does not elicit any effect on AchE. The level of acetylcholine (ACh) activity in different regions of the brain is regulated by the cholinergic system, which includes cholinergic neurons and receptors sensitive to ACh. The cholinergic system plays a critical role in various cognitive functions, including learning and memory. The reason for variations in ACh activity between brain regions, such as the hippocampus and frontal cortex, lies in the specialized functions and regulatory mechanisms of these areas [(11)]

The variation in AChE activity between the hippocampus and frontal cortex is a reflection of the distinct roles these brain regions play in cognition. The hippocampus relies heavily on ACh for memory processes, whereas the frontal cortex's functions are more diverse and involve a complex interplay of neurotransmitters and neuromodulators beyond just ACh. As

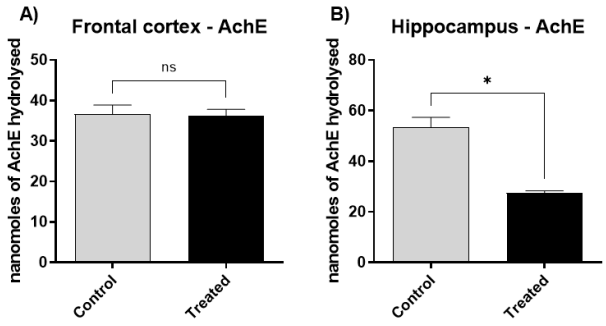

Fig 6. AChE activity in A) frontal cortex and B) hippocampus of control and experimental rat brains. Mean values of these biochemical parameters, along with their respective standard deviations (mean ± SD), were obtained from three independent experiments (n=3). Statistical significance was determined using unpaired t tests, where *indicates significance at $P < 0.05$, ** denotes significance at $P < 0.01$, and "ns" represents no significance.

well the hippocampus is a crucial brain region for the formation and consolidation of new memories. ACh activity is high in the hippocampus because it is involved in the processes of encoding and storing information. The release of ACh in the hippocampus enhances synaptic plasticity, which is essential for learning and memory. The high levels AChE in hippocampus is because of increased number of cholinergic neurons in the hippocampus were reduction in the AChE level favors in retaining memory and cognition.

3.5 EA improves Spatial learning memory in rat models

EA improves the exploration and learning capacity in radial arm maze tests. Number of correct choices was non significantly increased and latency periods were almost similar with no observable difference in the EA treated group when compared with the control group.

Fig 7. The effect of Eclipta alba on spatial learning and memory performance using 8-ArmRAM Maze task in adult rats. A) % of correct choices made in control andexperimental animals. B) Latency period in seconds. Mean values of these biochemical parameters, along with their respective standard deviations (mean ± SD), were obtained from three independent experiments (n=3).Statistical significance was determined using unpaired t tests, where *indicates significance at $P < 0.05$, ** denotes significance at $P < 0.01$,and "ns" represents no significance

AD is a well-known and genetically heterogeneous neurodegenerative disorder with high prevalence and is a familiar cause of dementia around the globe. The amyloid plaque and neurofibrillary tangle formation is considered as the major hallmarks of AD pathology[12] that results in loss of cholinergic neurons and depletion of neuronal population in important cognitive centers like hippocampus[13,14]. Although AD has been characterized in the beginning of the 20th century there is no cure for the disease yet. The chronic level and epidemiology modern medicine system in allopathi is yet to establish a flexible antidote.

Exploring the novel efficacies of Indian traditional plants opens a wide array of research clues. Simultaneously unraveling the intercellular effect of traditional plants used in ayurvedic medicine enables better understanding and authorized documentation of these plants that may help to handle critical disease and disorder arising due to modernization. Numerous traditional ayurvedic plants are investigated so far and have extended extensively to treat many dreadful diseases. Some researchers reported that polyphenolic substances (such that flavonoids and phenolic acids), widely present in plants have been studied to possess multiple biological effects, including antioxidant activity[15,16]. EA is one such medicinal herb known in the Indian subcontinent since ancient times. EA is well documented and practiced for its effect on skin and hair[17–20]. The aqueous and hydrolyzed water extracts of EA at a dose of 300 and 30 mg/kg correspondingly, significantly improved the acquisition and retention time in elevated plus maze under stressed condition[21]. But its exact action on neuroprotection is minimally explored till date. Therefore, this study attempts to explore the potential of EA to manage AD. EA is known to possess diverse phytochemicals, most of which are observed to be of health benefits. In the present study, we tested EA's memory enhancing potential and hypothesized the possible molecular mechanism behind the neuroprotective effect of EA using in-silico, biochemical and behavioral analysis.

Thin Layer chromatography (TLC) analysis of the aqEA revealed significant presence of Flavonoids and Saponins when compared to Tannin (Supplementary data). Flavonoids are plant secondary metabolites with polyphenolic structure; Saponins are glycosides of triterpenoid or steroidal aglycones and tannins that are water-soluble phenolic compounds, both of them being present naturally abundant in plants[22,23]. All these compounds have been well-documented for its neuroprotective effect.

Screening the active components of EA enables investigating the precise mechanism of action in different pathological and physiological conditions. In line with the above, a preliminary work on phytochemical evaluation using LC-MS analysis of the aqEA was performed and revealed the presence of key secondary metabolites that are listed in Table 1. The chromatogram depicts the presence of Lilaline, Laccarin, Coriandrone E, Quercetin, Catechin, Wedelolactone and Luteolin as a major component of aqEA.

These metabolites were well-known for their pleiotropic activities. For example, catechin was proven to improve metabolic disorders like obesity, diabetes and cardiovascular disease as well. Catechin and its derivative epigallocatechin, controls the conversion of APP into the non-amyloidogenic soluble APP (sAPP), preventing the formation of neurotoxic beta-amyloid. Inhibition of the beta-secretase enzyme has also been demonstrated for Epicatechin gallate and other green tea catechins (BACE1)[24]. In addition the anti-hyperlipemdic effect of the EA was reported to have contributed by wedelolactone which regulates obesity by adipose tissue browning through sirtuin (SIRT1)/AMP-activated protein kinase (AMPK)/ Peroxisome proliferator-activated receptor α (PPARα) pathway[25]. The other polyphenol, quercetin, is used to treat arthritis, eye and cardiovascular illnesses, cancer, metabolic and inflammatory disorders, and infectious diseases[26]. Additionally, it has demonstrated effective therapeutic effects against AD. Quercetin has been linked to neuronal protection against Amyloid beta toxicity in *invitro* and *in -vivo*, this substance has been demonstrated to prevent Aβ neurotoxicity, enhance cell survival in a variety of cell types, and attenuates BACE1-mediated cleavage of APP in AD transgenic mice[27]. Notably luteolin was found to reduce inflammation in both in-vivo and invitro condition by interacting with JAK kinase/signal transducers and activators of transcription (JAK/STAT), Nuclear factor kappa B (NF-κB), and other pathways[28]. In specific, luteolin confers cognitive improvement in epileptic rats by upregulating the protein kinase A (PKA), cAMP-response element binding protein (CREB), Brain Derived Neurotrophic Factor (BDNF) pathway in the hippocampus[29]. Luteolin confers neuroprotection by significantly improving glial cell expressions of Glial fibrillary acidic protein (GFAP) and calbindin, with increased dendritic expressions of Purkinje cells in choline cobalt mediated brain impairments[30]. Apart from the above-mentioned compounds some novel flavonoids such as lilaline, coriandrone E and laccarine were also observed by LC-MS analysis, but these compounds are very minimally investigated for their medicinal properties.

Flavonoids being an active constituent of aqEA, exhibit its effect on minimizing cognitive deficits due to cholinergic dysfunctioning. Their profound free radical scavenging action could insulate neuronal tissues from degeneration probably by preserving these areas from stress perturbations. The neuroprotection is apparently resulted by immunomodulatory action of EA. Therefore, EA can be a valuable memory enhancer. In spite of research evidence on various therapeutic roles of these predominant flavonoids of EA, the absolute neuroprotective effect of the listed compound and their combinatorial action in the central nervous system is still a quest.

Analyzing the internal components of medicinal plants represents a promising avenue in the field of molecular drug discovery for addressing neurodegenerative conditions like AD. current study expanded upon an initial approach by utilizing *in-silico* analysis to gain a deeper understanding of how the active compounds in aqEA interact with key AD-related proteins such as AChE enzyme, APOE4, ADAM10, PS1, and NMDA receptors.

Our docking studies involved comparing these AD-related proteins with other proteins like BACE1, APP, AChE, NMDA receptors, and HMG-CoA reductase. Table 2 presents the binding affinities of the major metabolites found in aqEA when interacting with the aforementioned AD-related proteins, revealing strong binding capabilities.

These results suggest that the active constituents of EA, particularly its flavonoids, have the potential to influence or regulate the expression of AD-related proteins, potentially creating a favorable environment for neuroprotection. This outcome underscores the promise of conducting in-vivo experiments to further explore the neuroprotective effects of EA's flavonoid constituents.

Based on the initial investigations outlined above, it becomes evident that EA comprises a diverse array of pharmacologically active compounds. Specifically, the constituents of EA exhibit promising pharmacological actions, as confirmed by docking analysis, which reveals strong binding affinity to both APOE4 and AChE. To gain a deeper understanding of the intermolecular impact of EA, *in-vivo* experiments have been instrumental in shedding light on the underlying pharmacological properties responsible for its nootropic effects.

In the present investigation, aqEA extract was given orally in a dose of 150 mg/kg body weight of control and experimental animals in order to assess the learning and memory. In way of assessing the effectiveness of aqEA, physiological studies were carried out. To understand the efficacy of EA as a potent memory enhancer, the aqEA treated experimental animals were tested for its memory enhancing ability using RAM. RAM analysis performed on rats of control EA treated groups shows improvement in RAM experiments (Figure 7) both in choosing correct choices and latency, which may be due to the effects of different flavonoid constituents of EA. Aqueous extract of *Eclipta prostrata* at the dose of 100 and 200 mg/kg was found to be beneficial on retrieval of learning in *in-vivo* model that was evidenced in elevated plus-maze, and reported to significantly improve the retrieval memory[31]. In correlation with the above findings using similar species of aqEA displayed greater behavioral efficiency in rat model.

Interestingly, at this dose, aqEA did not exhibit any toxicity among experimental rats. Analysis of the neuroprotective effect of aqEA requires study on its effect on overall metabolism and toxicity. A desired characteristic of a drug candidate relies on its safety concern. This study tested the effect of EA on liver and kidney function via enzyme assays which shows EA does not exhibit any toxic effect up to 150 mg/kg of body weight.

Neurochemical analysis was performed by assessing AChE activity in frontal cortex and hippocampus that revealed aqEA enhances cholinergic function by reducing AchE activity in hippocampus. Acetylcholine is an indicator of memory-health; based on *in-silico* observations, we tested the effect of EA on AChE (enzyme involved in cleavage of Acetylcholine) in Frontal cortex and Hippocampus of rat brain (Figure 5). EA reduced AChE activity in the hippocampus of EA treated rats when compared to control. In addition, no noticeable difference in AChE activity was found in the Frontal cortex. Which implicates that hippocampus generally contains large amount of ACh level because of increased cholinergic neuron population when compared to frontal cortex, where AChE reduction improves memory retain. This shows that EA directly elicits its effect on ameliorating AD pathology by stabling cholinergic neurons and also on formation and storage of memory in the hippocampus. Similarly, another plant Rosmarinus officinalis exhibits AChE activity in brains in an in-vivo models[32]. The obtained results strongly support the hypothesis that EA offers robust neuroprotection, particularly in the hippocampal region, exhibiting multifaceted benefits. EA not only demonstrates the ability to mitigate neuronal inflammation during the progression of Alzheimer's disease (AD) but also exerts notable anti-inflammatory and anti-oxidative effects. Furthermore, EA plays a pivotal role in maintaining the normal physiological functioning of neurons[33].

Crucially, EA emerges as a potential preventative measure against dementia by effectively inhibiting Acetylcholinesterase (AChE) activity in AD-like pathological conditions, as demonstrated in our *in-vivo* study. Future investigations, involving the isolation of bioactive compounds from EA and the development of innovative drug delivery formulations tailored for Alzheimer's treatment, hold the promise of unveiling novel insights into its therapeutic effects.

Expanding our understanding of EA's intermolecular interactions with SIRT1 and its potential to induce epigenetic changes opens up a promising avenue for addressing age-related disorders. Given India's status as a developing nation, there is a pressing need for cost-effective pharmaceutical solutions like EA, which can serve as an efficient multitargeted drug to ameliorate the adverse effects of aging on the elderly population and effectively manage age-related dementia.

4 Conclusion

The aqEA demonstrates a notable richness in pharmacologically active secondary metabolites that exhibit an effective binding affinity with proteins associated with AD when assessed through *in-silico* analysis. This intriguing finding underscores the potential therapeutic utility of aqEA in ameliorating cognitive deficits associated with AD and similar dementias that manifest during the aging process. Furthermore, it is novel and noteworthy that aqEA exerts its cognitive-enhancing effects by downregulating AChE activity within the cerebral regions of *in-vivo* model organisms. This reduction in AChE levels is particularly promising, as it aligns with established therapeutic strategies for AD and dementia, wherein the preservation of acetylcholine signaling plays a pivotal role. Importantly, current investigations have demonstrated that aqEA maintains a remarkable safety profile devoid of any discernible toxicological concerns. Additionally, aqEA exhibits a protective effect against

age-related organ damage, further emphasizing its potential as a therapeutic agent for conditions associated with physiological aging. Collectively, the comprehensive body of evidence generated from this study supports the notion that aqEA stands as a viable and dependable pharmacological option for addressing age-related neurodegenerative disorders.

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