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Posted Date: 28 March 2024

doi: 10.20944/preprints202403.1767.v1

Keywords: Verbisina enceloides; Alzheimer's Disease; Carotenoid; Neuro-degenrative; Spirilloxanthin; Phyto-chemicals; Acetylcholinesterase

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Neuroprotective Effects of Carotenoid Rich *Verbesina encelioides* Flower Extract in Scopolamine Induced Memory Impaired Rats Mimicking Alzheimer's Disease

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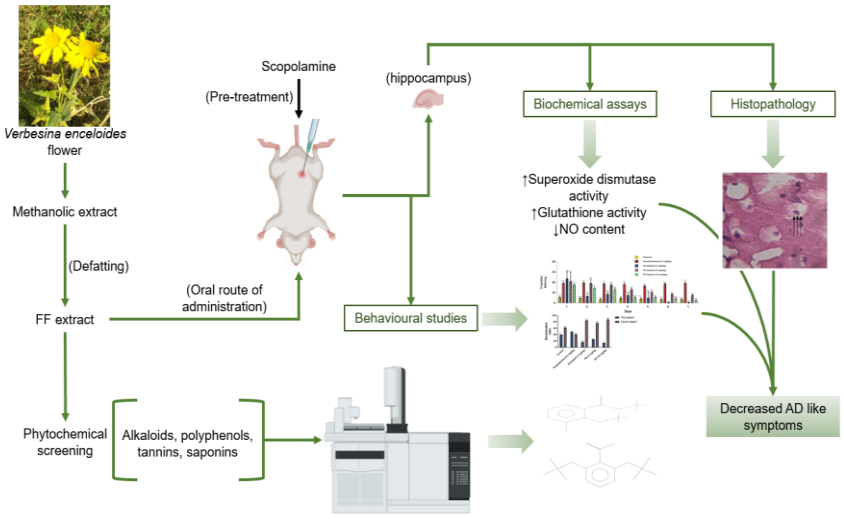
Abstract: Neuron degeneration is the most common old age complications worldwide. Alzheimer's disease (AD) is one of the most common neurodegenerative condition with limited drug options and significant adverse effects. Hence, there is a growing interest in exploring natural products for their potential pharmacological activities against AD. This study investigates the efficacy of *Verbesina encelioides* flower extract (VFE) in alleviating neurobehavioral damage induced by Scopolamine in rat mimicking AD model. GC-MS analysis showed that *V. encelioides* flower extract is rich in carotenoids like spirilloxanthin. Various behavioural models were used to assess the rats' learning and memory capabilities. Additionally, biochemical tests were conducted on the AD rat models to evaluate oxidative stress in the brain, including superoxide dismutase (SOD), nitric oxide (NO), reduced glutathione (GSH), and anti-acetylcholinesterase (AChE) enzyme assays. Findings from this study suggest that the VFE significantly improved the rats' cognitive aptitude and alleviated them from oxidative stress by reducing free radical generation. Based on the results obtained from GC-MS analysis, in silico interaction analysis of gingkolide A and spirilloxanthin with acetylcholinesterase was carried out. In summary, this study suggests that *V. enceloides* flower extract improves learning abilities and mitigates cognitive deficits caused by scopolamine. Thus *V. enceloides* could be potentially seen as natural therapeutic intervention against neurodegenerative disorders likes Alzheimer's disease and dementia.

Keywords: *V. enceloides*; Alzheimer's disease; neuro-degenerative; phytochemicals; spirilloxanthin; acetylcholinesterase

 doi:10.20944/preprints202403.1767.v1

Graphical Abstract



1. Introduction

Alzheimer's disease (AD) is a prevalent neurological disorder that affects millions of individuals globally. This condition leads to an impairment in cognitive and memory functions followed by complete memory loss. According to Alzheimer's Association (AA), approximately 5 million individuals aged 60 or above had AD in 2015, and this number is projected to rise to 13.8 million by 2050. Several studies indicates the deposition of amyloid-beta (Aβ) and neurofibrillary tangles in the brain causing neuro-inflammation as a significant contributing factor to the onset of AD (Fig. 1). Additionally, oxidative stress is also believed to play a role in the pathophysiology and biochemistry of AD by increasing free radical production, leading to excessive lipid peroxidation, and causing neuronal death in specific brain regions (Fig 1).Various studies have reported that most majority of the drugs available against AD target acetylcholinesterase (AChE), the primary enzyme responsible for the breakdown of the neurotransmitter acetylcholine [1]. However, these drugs only provide symptomatic relief instead of completely curing the disease along with decrease in therapeutic efficiency with time.

Utilization of plants and their derivatives has gained significant popularity in recent years due to their effectiveness and safety. Plants contain a wide array of biologically active compounds, which have been traditionally used to treat various illnesses as a substitute for synthetic drugs. The remarkable therapeutic potential of plant-based products has captured the attention of researchers globally (Fig 2), as evidenced by several clinical trials, which have demonstrated their safety and minimal adverse effects [2].

The family Asteraceae (compositeae), commonly known as the sunflower family, has a vast distribution across the globe with over 22,750 species. *Verbesina encelioides* L, an annual herbaceous plant with yellow flowers resembling sunflowers, is a weed that can be found in North and South America, as well as in certain regions of India [3]. Weeds have been acknowledged as a valuable source of medication for local communities [4]. Verbesina species have been extensively studied for their phytochemical composition, which includes sesquiterpene esters, sterols and triterpenes, saponins, flavonoids, alkaloids, and guanidine derivatives, as reported by several researchers [5–10]. Prior research on *Verbesina encelioides* L have unveiled a diverse array of biological activities, notably encompassing antimicrobial, antiviral, anti-tumor, hypoglycemic, and anti-implantation properties [11]. Ginkgolides, are unique terpene trilactones (TTLs) and one of the abundant phytochemicals with diverse benefits. They possess anti-inflammatory, anti-coagulant, antioxidant, anti-apoptotic, and immunomodulatory properties, contributing to neuroprotection. Similarly, carotenoids, prevalent lipid-soluble pigments in plants, microalgae, and more, play a crucial role in

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neuroprotection alongside their varied therapeutic effects like anticancer, anti-inflammatory, and immunomodulatory functions [12,13].

In light of the aforementioned considerations, this study aimed to analyse bioactive compounds in VFE against Alzheimer's disease. In vivo anti-alzheimer's effects of the VFE was assessed, followed by molecular docking to identify potential anti-acetylcholinesterase antagonist bioactives. To the best of our understanding this is the first investigation of the use of *V. enceloides* extract as a neuroprotective agent in Scopolamine induced rats mimicking Alzheimer's disease.

2. Materials and Method

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies [14].

2.1. Plant Material

Healthy *V. encelioides* plants were collected in the month of July, 2019 grown within the premises of Banasthali Vidyapith, Rajasthan, India. Authenticity of the plant was validated by Dr. Afroz Alam at Department of Bioscience and Biotechnology, Banasthali Vidyapith, Rajasthan, India (Accession no- BUR1390/21).

2.2. Preparation of Crude Extracts and Phytochemical Screening

The fresh flowers of *V. encelioides* were collected and air-dried for three days under shade. The dried flowers were then crushed to obtain crude powder, which was subjected to maceration with methanol. The resulting extract was defatted using hexane and petroleum ether. The filtered methanolic extract of the flowers was concentrated and stored in an amber coloured bottle until further use. The flower extract was then subjected to preliminary qualitative testing using standard methods as described by [15].

1. To assess the presence of alkaloids in flower extract Mayer's test; Wagner's test; Hager's test, and Dragendroff's test were performed.
2. Lead acetate test and ferric chloride test were done to confirm the presence of tannins and phenolic compounds in flower extract.
3. Lead acetate test and alkaline reagent test were performed to determine the presence of flavonoids in FF extract.
4. Frothing test was done to confirm the presence of saponins in FF extract.

2.3. GC-MS Analysis of FF Extract

Phytochemical analysis of the VFE was performed using Thermo GC-TRACE ultra ver.: 5.0, Thermo MS DSQ II GC-MS equipment (Thermo Scientific Co.). Prior to the analysis, derivatives were transformed into fatty methyl esters (FAMEs) as described earlier [16]. The TR 5-MS standard non-polar capillary column with dimensions of 30 Mts, 0.25 mm ID, and 0.25 m film thickness was used for the experiment. The carrier gas used was helium with a flow rate fixed at 1.0 ml/min. The gas chromatography section was programmed to increase the temperature from 40°C to 250°C at a rate of 5°C/min, and 1 µl VFE was injected into the instrument. The resultant spectra were compared to a GC-MS library containing known component spectra from NIST database [17].

2.4. In Vivo Anti-Alzheimer Activity

2.4.1. Experimental Animals

Male and female Wistar albino rats weighing between 160-230 g were obtained from Lala Lajpat Rai Veterinary and Animal Sciences, Hisar, India. The rats were housed in groups of four per cage under controlled conditions of temperature (23 ± 2 °C) and a 12-hour light/dark cycle. Standard rodent chow and water were provided ad libitum. All animal procedures were conducted in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of

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Experiments on Animals (CPCSEA), and were approved by the Institutional Animal Ethics Committee (IAEC) (Approval number: 1283/C/09/CPCSEA).

2.4.2. Acute Toxicity Test

Acute oral toxicity study of VFE was carried out in accordance with Organization for Economic Cooperation and Development (OECD) guidelines (OECD guidelines 423). Rats were administered with VFE orally at doses of 5, 50, 300, and 2000 mg/kg, and toxicity and motility were monitored for 24 hours, if any. Based on the results of the investigation, the dose was determined for further pharmacological action.

2.4.3. Experimental Design

Thirty rats (6 in each group) were divided into five groups: Group I served as negative control (administered with 0.9% NaCl), Group II served as positive control (administered with 0.3 mg/kg scopolamine hydrobromide), Group III served as standard (administered with 3 mg/kg donepezil hydrochloride), Group IV and V received VFE at doses 5 and 10 mg/kg, respectively. Rats in groups I, III, IV and V received oral administration of donepezil hydrochloride (TCI), and VFE in varied doses over the course of seven days. Rats in groups II received injections of scopolamine hydrobromide 60 minutes before treatment.

2.4.4. Behavioural Studies

To assess the effectiveness of VFE in negating cognitive impairments in the rat model of AD, multiple behavioural tests were conducted. The novel object identification test and the T-maze test are thought to evaluate cognitive ability by observation of the subject's exploratory behaviours toward a novel object or route, respectively. These tests are used to identify improvements in the rats' cognitive abilities. Rats were sacrificed and their brains were removed once the behavioural tests were completed. Hippocampus was isolated after middle-line brain dissection. The hippocampus was split into two parts; one part was homogenised in phosphate buffer (0.1 M, pH 7.6) and assessed for various biochemical assays, while the other half was stored in 10% formalin solution for additional histological analysis [18,19].

2.4.4.1. Cross Gap Test

Cross gap test was used to assess anti-Alzheimer's activity of VFE in Wistar albino rats. For investigation, a maze apparatus with two arms, white painted plastic, with dimensions of 40*50*12 cm was used. Food was arranged on the ends of arms that were 8 cm apart. For five minutes, rats were free to roam on both maze arms. Before the trial began, rats were fasted for one night after receiving 14 days of training. Each rat received an injection of scopolamine (0.3 mg/kg), and was placed in the unbaited arm and given five minutes to acclimatize. Rat transfer latency was measured from the baited arm to the unbaited arm [20].

2.4.4.2. Novel Object Recognition Test

Novel object recognition test was used to assess anti-Alzheimer's activity of VFE in Wistar albino rats. A grid floor and coloured plywood measuring 70*60*30 cm make up the open field equipment. In the first trail, each animal was exposed to two identical objects for 20 seconds. One of the objects was then replaced with a new one and the process was repeated after an hour. Exploration entails aiming the nose at an object that is no more than two centimetres away from you or reaching out and touching it. Prior to conducting the experiment, rats received seven days of training. Each rat received a scopolamine injection before being released into the field, where they were free to explore both new and old objects while the estimated discrimination index was being calculated [21].

 doi:10.20944/preprints202403.1767.v1

2.4.4.3. T Maze Test

T maze test was done to assess anti-Alzheimer's activity of VFE in Wistar albino rats. For this study, a T maze device made of plastic that has been painted black and measures 40 *13 *3 cm was used. Three mazes were present; one was baited, the others were left unbaited. Each animal was placed at the end of one unbaited arm and given five minutes to freely navigate the maze. Rats tended to systematise their maze exploration, entering each arm one at a time. Prior to conducting the experiment, rats underwent a fourteen-day training period. Each rat was placed in the field and permitted to enter the baited arm after receiving a scopolamine injection. Rat transfer latency was then measured [22].

2.4.5. Biochemical Tests

After the end of study, rats were euthanized, brain was carefully removed and homogenized in phosphate buffer (0.1 M, pH 7.6) using mortar pestle followed by centrifugation at 20,000g at 4 °C for 10 min. Supernatant was collected and stored at -20 °C for further biochemical estimation.

2.4.5.1. Estimation of Thiobarbituric Acid Reactive Substances (TBARS)

Thiobarbituric Acid Reactive Substances (TBARS) are formed as the by-product of lipid oxidation. Quantitative determination of TBARS in the brain homogenates was determine by the method described here. Briefly, 0.5 mL of the homogenate was obtained, and the final volume was brought up to 1.0 mL with distilled water in a test tube. After 0.05 mL of $FeSO_4$ was added, it was incubated for 30 min at 37°C before 1.5 mL of acetic acid and 1.5 mL of TBA were added. The mixture was vortex and then heated for 60 minutes. After cooling, 5 mL of butanol was added, and the mixture was centrifuged for 10 min at 3000 rpm at 4°C. The absorbance of the organic layer was recorded at 532 nm [23].

2.4.5.2. Estimation of Superoxide Dismutase (SOD)

To evaluate SOD activity sodium carbonate buffer (EDTA, xanthine, and nitroblue tetrazolium) was added to rat hippocampal tissue homogenate (2.5 mM NBT). The mixture was additionally added to the tube mentioned above together with xanthine oxidase and absorbance was recorded at 560 nm [24].

2.4.5.3. Estimation of Reduced Glutathione Activity (GSH) Activity

To check for glutathione (GSH) levels within brain, homogenates were combined with the Ellman reagent, which was made in 0.1 M phosphate buffer at room temperature. Absorbance was measured at 412 nm after one hour [25].

2.4.5.4. Estimation of Nitric Oxide (NO) Activity

The Griess Illosvoy reaction was used to determine the nitric oxide activity in the hippocampal tissue. For this , hippocampal tissue homogenate was combined with 10mM sodium nitroprusside that had been dissolved in phosphate buffered saline. 0.5 ml of Griess reagent was added after incubation, and absorbance was measured at 550 nm[26].

2.4.5.5. Estimation of Acetyl Cholinesterase (AchE) Activity

Colorimetric approach was used to evaluate the acetylcholinesterase activity. Homogenate of hippocampus tissue was prepared in 0.1 M phosphate buffer pH 8.0 and used as an enzyme source. In a test tube, 2 ml of supernatant was mixed with 0.5 mM of Ellman reagent prepared in 100 mM Tris buffer (pH.8) and 20 mM acetylthiocholine iodide. The change in absorbance was measured at 412 nm for 3 min at an interval of 30 s using a spectrophotometer [25].

 doi:10.20944/preprints202403.1767.v1

2.4.6. Histopathological Examination of Rat Brain Tissue

Rat hippocampus tissue were collected immediately after etherisation and fixed in a 10% formalin solution for histopathological analysis. Haematoxylin and eosin dye was used to stain the tissue, and a light microscope was used to visualize the tissue's degeneration.

2.5. In Silico Docking Studies

Based on the Gas Chromatography-Mass Spectrophotometry (GC-MS) and biochemical analysis we chose to study the binding of compounds Ginkgolide A and Spirilloxanthin to the enzyme acetylcholinesterase. We used dimeric *mus musculus* acetylcholinesterase (PDB ID: 2JEZ) model for our docking studies. Three-dimensional structure of ginkgolide A and spirilloxanthin were obtained from PubChem (Ginkgolide A: PubChem CID 9909368; Spirilloxanthin: PubChem CID 5366506). AUTODOCK version 4.2.6 was used to probe binding modes of the above compounds to acetylcholinesterase (PDB ID: 2JEZ) [27]. Both the monomeric and dimeric binding sites for *mus musculus* acetylcholinesterase were targeted for this study. Crystallographic water atoms and heteroatoms were removed from the docking template. Polar hydrogen was added, and Kollman charges were assigned to all atoms. Ligands were prepared for calculations by adding Gasteiger charges. For calculations involving the monomer binding site, a 40 × 40 × 40-Å affinity grid was used, whereas for the dimer binding site, a 46 × 44 × 40-Å affinity grid with 0.375-Å spacing was used. The remaining parameters were set to default values. A maximum of 20 poses were sorted based on the scoring function and fitness score implemented in the program. The most stable conformation of the protein-ligand complex was then selected for further analysis.

2.6. Statistical Analysis

All experiments were conducted in triplicate, and the data are presented as mean ± standard deviation. The data were analysed utilizing one-way ANOVA, followed by the Tukey–Kramer multiple comparison test. Statistical analysis and data presentation were performed using Graphpad Prism software (version 8.0.3(263); GraphPad Software, Inc., San Diego, CA, USA). The significance level was set at $p < 0.05$ for all statistical tests.

3. Results

3.1. Acute Toxicity Test

Preliminary phytochemical screening of the methanolic FF extract of *V. enceloides*, revealed the presence of alkaloids, flavonoids, saponins, and phenols in the extract. Please find the details in Table 1.

Table 1. Phytochemicals present in the crude extracts of flowers part.

Sl. No	Metabolite	Methanolic FF extract
1	Alkaloids	+
2	Flavonoids	+
3	Polyphenols	+
4	Tannins	+
5	Saponins	+

3.2. GC-MS Analysis

To characterise the phytochemicals present in *V. enceloides* we performed GC-MS analysis on the methanolic extract of *V. enceloides* flowers. Table 2 provides complete information about the presence of various phytochemicals in *V. enceloides* obtained after GC-MS study GC-MS analysis identified spirilloxanthin as one of the major phytochemical in *V. enceloides* flower extract (Table 2).

 doi:10.20944/preprints202403.1767.v1

Table 2. Compounds identified in V. enceloides flower extract through GC-MS analysis.

Sr.No	Compound	Probability (%)	Nature of compound	Pharmacological activity
1.	1-Methyl-2-phenylbenzimidazole	4.04	Benzimidazole derivatives	antitumor, antibacterial, anti-inflammatory, and anti-hypertensive agents
2.	Propiophenone, 3-phenyl-3-piperidino-	3.51	Acetophenone	antibacterial, antifungal and antitumor activity
3.	Nicotinamide,N-(1-methyl-2-oxo-2-piperidin-1-ylethyl)-	2.99	Nicotinamide derivatives	Anticancer, anti-angiogenic, anti-inflammatory and antinociceptive effect
4.	.psi.,.psi.-Carotene,1,1',2,2'-tetrahydro-1,1'-dimethoxy- (Tetrahydrospirilloxanthin)	12.38	Carotenoids	Antimicrobial, antiviral properties, anticancer, anti-inflammatory, anti-nociceptive, anti-ageing and neuroprotective effects
5.	Ginkgolide A 2TMS	3.37	Diterpene lactones	Wide potential in inflammatory and immunological disorders.
6.	Lycoxanthin	11.20	Carotenol	Neurodegenerative diseases, anti-oxidant, antineoplastic, anti-tumor, antidiabetic, anti-CVD, and anti-aging
7.	á Carotene	15.62	Carotenoids	Antineoplastic, anti-tumor, neurodegenerative diseases, anti-oxidant antidiabetic, anti-CVD, and anti-aging
8.	Lycopene	6.68	Carotenoids	Antineoplastic, antidiabetic, anti-oxidant, anti-tumor, anti-CVD, neurodegenerative diseases, and, anti-aging
9.	.psi.,.psi.-Carotene,3,3',4,4'-tetradehydro1,1',2,2'tetrahydro-1,1'-dimethoxy-2,2'-dioxo- (2,2'-dioxospirilloxanthin)	13.24	Carotenoids	Antineoplastic, antidiabetic, anti-CVD, neurodegenerative Diseases, anti-oxidant, anti-tumor, and anti-aging

3.3. In Vivo Alzheimer's Activity

3.3.1. Acute Toxicity Study

Our acute toxicity studies of VFE did not showed any signs of toxicity and mortality up to the doses 2000 mg/kg on rats (data available on request). Thus, 5 mg/kg (1/400th of 2000 mg/kg) and 10 mg/kg (1/200th of 2000 mg/kg) doses were selected for further studies.

 doi:10.20944/preprints202403.1767.v1

3.3.2. Effect of VFE Extract on Behavioural Parameters

The anti-Alzheimer effects of VFE were evaluated using three different behavioural tests: the cross gap test, novel object recognition test, and T-maze test. In the cross gap test (Fig. 3a), rats treated with the VFE (5 and 10 mg/kg) showed a significant decrease in transfer latency compared to the positive control group induced with scopolamine, indicating improved cognitive function. The positive control group exhibited increased transfer latency, while the group treated with Donepezil showed a remarkable decrease in transfer latency. The novel object recognition test (Fig. 3b) revealed that scopolamine administration reduced the discrimination index, but the VFE treated group at 10 mg/kg displayed enhanced recognition ability in terms of interacting with the object. T-maze test (Fig. 3c) demonstrated that VFE (5 and 10 mg/kg) decreases transfer latency of AD disease mimicking rats, further supporting its anti-Alzheimer effects. Overall, these results suggest that VFE has potential therapeutic benefits in mitigating Alzheimer's disease-associated cognitive impairments.

3.3.3. Effect of VFE on Biochemical Parameters

Exposure of rats to scopolamine led to a significant reduction in the activity of superoxide dismutase in the hippocampus when compared to the rats with no exposure. However, treatment with both donepezil and VFE at 10 mg/kg resulted in a significant increase in superoxide dismutase activity, indicating a potential protective effect of VFE against oxidative stress (Fig. 4a). Scopolamine administration increased brain NO content in rats, which was significantly reduced by VFE at 5 mg/kg and 10 mg/kg dosage (Fig. 4b). Glutathione activity (GSH) was decreased significantly in the scopolamine-administered group, while the administration of 5mg/kg and10 mg/kg VFE reversed this reduction, demonstrating its potential to restore GSH levels (Fig. 4c). Furthermore, scopolamine-induction elevated the levels of thiobarbituric acid reactive substances (TBARs), which was attenuated by VFE at 5mg/kg and 10 mg/kg dosage, indicating a protective effect against lipid peroxidation (Fig. 4d). In the assessment of neurotransmitter metabolic enzyme acetylcholinesterase (AChE), scopolamine treatment increased AChE activity, which was significantly reduced by VFE at 5 mg/kg and 10 mg/kg dosage, suggesting potential role of VFE in modulating neurotransmitter function (Fig. 4e). Overall, these findings highlight the neuroprotective and antioxidant properties of VFE in the context of Alzheimer's disease.

3.4. Histopathological Study

Hippocampal tissues of the rat brain was investigated under light microscope and observations were conducted in order to further validate the neuroprotective effect of VFE in typical Alzheimer's event. Protective effect VEF at doses of 5 and 10 mg/kg on the structure of pyramidal cells, vesicular nuclei, and vacuoles was revealed by histopathological analysis of hippocampal sections. Scopolamine treatment results in the loss of pyramidal cells and darkening of the nucleus (Fig. 5b). Small pyramidal cells were preserved after treatment with donepezil, while granular cells displayed decreased vacuolation and normal cell size (Fig. 5c). Treatment with VFE at a dose concentration of 5 mg/kg resulted in the preservation of small pyramidal cells, along with a reduction in cell disorganization and vacuolation (Fig. 5d). Following treatment with VFE at a dose concentration of 10 mg/kg, there was a significant preservation of small pyramidal cells, a decrease in vacuolation, and a disorganization in cell architecture (Fig. 5e).

3.5. Gingkolide and Spirilloxanthin Binds to Both the Monomer Active Site and Dimer Interface of Acetylcholinesterase

To assess the interactions of both gingkolide and spirilloxanthin to acetylcholinesterase we performed in silico docking of these compounds to its monomeric and dimeric form. For monomer, we assessed the interactions of the above compounds with the amino acids lining the active site of acetylcholinesterase (PDB ID: 2JEZ) (Ekstrom, 2007). Both gingkolide and spirilloxanthin were found to be interacting with the active site of monomeric acetylcholinesterase with the binding energies of 0.20 kcal/mol and -4.21 kcal/mol respectively (Fig. 6A and Fig. 6C). Specifically, both compounds

 doi:10.20944/preprints202403.1767.v1

were hydrogen bonded to Tyr 341 on the active site of acetylcholinesterase, however gingkolide was also found to be interacting with Tyr 124, Trp 286, and Phe 295 of the active site (Fig. 6A and Fig. 6C). Incidentally, the dimer interface of crystal structure of acetylcholinesterase (PDB ID: 2JEZ) also serves as the binding site of a different ligand [27]. Thus, we also tried to study the interaction of the above compounds to this new site. Gingkolide A binds to this dimer interface of acetylcholinesterase with the binding energy of -4.77 kcal/mol (Fig. 6B) and was founded to be hydrogen bonded to the amino acid residues of chain A (His 381, Thr 383, Gln 527, and Thr 528) and amino acid residue of chain B (Gln 527). Similarly, Spirilloxanthin was also found to bind to this dimeric interface with the binding energy of -4.32 kcal/mol and is interacting with the amino acid residues of chain A (His 381, Tyr 382, Arg 534, Phe 535, and Leu 536) and chain B (Gln 527). Overall, this docking study showed that both ginkgolide A and spirilloxanthin can conveniently interact with acetylcholinesterase and thus inhibiting it which can explain their neuroprotective response.

4. Discussion

The present study aims to investigate the potential neuroprotective effects of VFE at dose concentration of 5 and 10 mg/kg against memory impairment induced by scopolamine in rats. Cognitive and memory impairments, such as those seen in Alzheimer's disease, are known to have a significant impact on neurological health and day to day activities. Scopolamine, an anticholinergic agent, has been previously demonstrated to induce severe amnesic effects and spatial memory loss (mimicking Alzheimer's disease symptoms) when administered acutely. This is likely due to its ability to block muscarinic receptors in certain regions of the brain [28,29]. Recent studies have highlighted the importance of phytochemicals being explored as a natural therapeutic intervention against Alzheimer's disease [2]. Crocus sativus L extract has been found to possess antiparasitic, antibacterial, antioxidant, hypotensive, hypolipidemic, anxiolytic, antidepressant, anticonvulsant, antinociceptive, anti-inflammatory, diuretic, and cytotoxic properties, attributed to the presence of crocin, picrocrocin, safranal, phenolic compounds, flavonoids, terpenoids, and saponins [30]. Similarly, a recent study investigated the effects of an aqueous extract of Moringa oleifera Lam leaves on cognitive impairment in scopolamine-induced dementia, attributed to the high content of phenolic compounds, flavonoids, alkaloids, tannins, and saponins [31].

Studies have demonstrated the antioxidant potential of different parts of *V. enceloides* due to the presence of saponins, flavonoids, and alkaloids [32]. In light of these facts, the current study investigate the neuroprotective effects of *V. enceloides* flower in rat models mimicking Alzheimer's disease. *V. encelioides* flowers were collected and air-dried, followed by conversion into powder, maceration with methanol, and defatting using Hexane and Petroleum ether. The phytochemical analysis of the filtered VFE confirmed the presence of alkaloids, flavonoids, saponins, and phenols, which are known for their biological activities. Further GC-MS analysis resulted in the identification of many bioactive biomolecules with known therapeutic importance against various disorders (Table 2). Amongst these, we also identified Ginkgolide as a potential biomolecules with neuroprotective action (Table 2). Ginkgolide is known to increase neuronal cell viability by suppressing the NF-κB pathway in cells mimicking AD disease [13]. Incidentally, VFE was found to be enrich in carotenes family (12%-15%) after GC-MS analysis (Table 2). Spirilloxanthin is one of the member of carotenoid family which was found to have high fraction (12.38% and 13.24%) in the VFE extract (Table 2). Though carotenoids are known to have neuroprotective action [33], the role and exact mechanism of spirilloxanthin in the improvement of cognitive impairment is still unknown.

To assess the effect of VFE on learning and memory, behavioural tests such as the cross-gap, T-maze, and novel object identification were performed. In the current study, the prolonged administration of scopolamine to rats led to an increased latency time for entering the preferred arm, consequently resulting in a reduction in the time spent in the preferred arm. Administration of VFE for seven consecutive days decreased the transfer latency (TL), which is associated with the rat memory process in the cross-gap and T-maze tests. Donepezil is the known FDA approved drug which is widely prescribed for the treatment of Alzheimer's disease symptoms. This medication improves the impeded cognitive ability in the person suffering from Alzheimer's disease. In

 doi:10.20944/preprints202403.1767.v1

comparison to rats treated with donepezil, those administered 10 mg/kg of VFE displayed a noteworthy reduction in transfer latency, signifying an improved ability to recall and enter the closed arm promptly (Fig 3a). Similar to the transfer latency studies VFE showed significant improvement in the cognitive ability of the scopolamine induced rat when compared to the group which were treated with donepezil (Fig. 3b and Fig. 3c)

Several biochemical experiments were conducted on rat hippocampal tissues to investigate the neuroprotective mechanism of VFE. Oxidative stress is recognized as a major contributor to the development of Alzheimer's disease. Due to its high oxygen consumption, high polyunsaturated fatty acids content, and inadequate antioxidant capacity for regular metabolic reactions, the hippocampal region of the brain is particularly susceptible to oxidative stress. Previous preclinical and clinical studies have shown that elevated levels of oxidative stress in the brain leads to neurodegeneration, which results in cognitive impairment, a hallmark of Alzheimer's disease [34,35]irectly contribute to brain health. In order to further elucidate the neuroprotective mechanism of VFE, a variety of biochemical experiments were conducted on rat hippocampal tissues. The current study's results indicates that scopolamine treatment led to a significant reduction in the antioxidant capacity of SOD, NO, and GSH in the rat brain. However, post treatment with VFE significantly improves and restored the levels of these antioxidants in rat brain tissue. This effect was highly pronounced when compared to the rats which were administered with donepezil (Fig. 4). Since our study has proved the potentiality of VFE in improving the cognitive ability of the rats and reduction in free oxygen species and oxidative stress, it could be said that VF has a profound neuroprotective action can be further assessed for the development of natural therapeutic intervention against Alzheimer’s disease.

In the current study, rats treated with Scopolamine exhibited an elevation in hippocampal acetylcholinesterase (AChE) activity, leading to cognitive impairment and memory loss, consistent with previous findings [36]. The treatment with VFE successfully elevated cholinergic activity and reversed cognitive dysfunction by inhibiting hippocampal AChE activity (Fig. 4e). These outcomes suggest that VFE may effectively inhibit cholinergic neuronal loss and mitigate cognitive impairment. The histopathological examination of rat hippocampus tissues sections from the Scopolamine group revealed irregular cell morphology and density, while VFE treatment groups demonstrated significant morphological modifications, such as alterations in the shape of vacuoles, nuclei, and the arrangement of pyramidal cells in the brain tissue of the rats (Fig. 5). In a similar study, administration of Cyperus esculentus L (Tiger nut) extract on scopolamine induced rats results in gliosis reduction in the coronal brain section thus indicating its possible neuroprotective function [37].

Our in silico docking analysis of both gingkolide A and spirilloxanthin with acetylcholinesterase have shown that both the compounds can efficiently interact with the active site of acetylcholinesterase (Fig. 6). Furthermore, our docking analysis has also revealed the very first binding site of the above compounds in the dimeric interface of acetylcholinesterase (Fig. 6B and Fig. 6D). Previous study have shown the effect of Ginkgo biloba as a neurotrophic, neuroprotective, anti-inflammatory, and antioxidant agent [38]. However the molecular mechanism of the same is yet to be known. This study illustrates the mechanistic insights into the neuroprotective action of gingoklide by binding with acetylcholinesterase. This study had also identified spirilloxanthin as one of the novel potential interactor of acetylcholinesterase, however the therapeutic role of spirilloxanthin especially in Alzheimer’s disease is yet to be elucidated. More, biochemical and biophysical studies will be required to ascertain the same.

5. Conclusion

Neurodegenerative disorders, such as Alzheimer's and dementia, have become increasingly prevalent. Due to the lack of effective drugs capable of preventing disease progression, novel therapeutic approaches that target the underlying pathogenic processes involved in AD pathogenesis are urgently required. In this investigation, oral administration of VFE reduces oxidative stress and memory impairment in the scopolamine induced rat mimicking AD. Through, in silico analysis we have determined the interaction between gingkolide and spirilloxanthin to acetylcholinesterase.

 doi:10.20944/preprints202403.1767.v1

Gingkolide is already known to have anti-alzheimer's activity however, the potent therapeutic function of spirilloxanthin in Alzheimer's disease is yet to be known. Nevertheless, this study identifies spirilloxanthin as a novel phytochemical interacting with the monomeric and dimeric interface of acetylcholinesterase and thus can be used explored further as a natural anti-Alzheimer's therapeutic intervention. Furthermore, this study also identifies dimer interface of acetylcholinesterase as a unique inhibitor binding site. More studies will be required to define the role of both acetylcholinesterase dimer interface and spirilloxanthin in containing and curing Alzheimer's disease.

Overall it is plausible that *V. enceloides* may represent a viable option for the treatment of age-related cognitive decline, such as Alzheimer's-type dementia, which could impede the multifaceted progression of AD. Nevertheless, additional research employing *V. enceloides* will be required to understand the exact mechanism of *V. enceloides* extract in the treatment of AD. In general, this study will open up new avenues to explore *V. enceloides* therapeutic potential as neuroprotective agent against neurodegenerative disorder.

Acknowledgments: The authors are thankful to Prof. Inna Aditya Shastri, Hon'ble Vice Chancellor, Department of Pharmacy, Banasthali Vidyapith, Banasthali (Rajasthan), India, for providing necessary facilities for success experimental work, and Director, National Institute of Pharmaceutical Education and Research, Raebareli (NIPER-R), Lucknow, Uttar Pradesh, India for providing library facilities. Dr. Abhishek Dey acknowledges Department of Biotechnology, Govt. of India for the Ramalingaswami Re-entry fellowship (BT/RLF/Re-entry/02/2021). .

Conflicts of Interests: The authors declare that they have no conflict of interests to disclose. This article bears NIPER-R communication number **NIPER-R/communication/518.**

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