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COGNITION ENHANCING ABILITY OF A POPULAR GREEN LEAFY VEGETABLE-*CHENOPODIUM ALBUM*: *IN-VITRO* AND *IN-VIVO* CORROBORATION

Sanimardeep Kaur, Kudrat Randhawa and Richa Shri *

Department of Pharmaceutical Sciences and Drug Research, Punjabi University Patiala - 147002, Punjab, India.

Keywords:

Acetylcholinesterase inhibition, antioxidant, *Chenopodium album*, Memory disorder, Scopolamine

Correspondence to Author:
Prof. Richa Shri

Professor,
Department of Pharmaceutical Sciences and Drug Research,
Punjabi University Patiala - 147002,
Punjab, India.

E-mail: richashri@pbi.ac.in

ABSTRACT: Oxidative stress and cholinergic deficit are major culprits of cognitive impairment. Intake of green leafy vegetables - rich in antioxidant phytoconstituents- strongly correlates with memory-preserving capacity. Our earlier work showed that hydromethanol (HM) extract of *Chenopodium album* (a common leafy vegetable) had strong acetylcholinesterase inhibitory and antioxidant activity *in-vitro*. The objective of the present study was to isolate the bioactive fraction/constituent from HM extract of *C. album* and substantiate its memory-enhancing capacity with *in-vitro* and *in-vivo* evidence. The HM extract was fractionated by solvent portioning and column chromatography and the antioxidant and acetylcholinesterase inhibition by fractions and sub-fractions thus obtained were assessed by DPPH and Ellman assays, respectively. The fraction F6 showed best potential *in-vitro*. Subsequently, the anti-amnesic capacity of HM extract and F6 was assessed using a scopolamine-induced memory impairment mouse model. Morris Water Maze test was used to determine memory. Biochemical parameters and histopathological studies of brain were also evaluated to elucidate anti-amnesic mechanism. *In-vivo* studies confirmed that F6 protected against scopolamine-induced memory impairment and significantly reduced brain AChE activity, oxidative stress and histopathological anomalies. The bioactive fraction (F6) of *C. album* exhibits marked potential to manage memory disorders via antioxidant and anticholinesterase pathways. This may be attributed to synergistic or additive effect of phenolic compounds present therein. F6 obtained from *C. album* can be developed as a drug or health supplement to ameliorate suffering due to memory disorders.

INTRODUCTION: Dementia involves progressive decline in cognitive ability involving memory loss, communication impairment, disorientation, and impairment of a person's ability to perform daily tasks. Many patients also display behavioral and psychological abnormalities such as psychosis, irritation, mood disorders, depression, *etc* [1, 2]. This causes immense suffering to the patient and is an economic and emotional burden on the caregivers.

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The global prevalence of dementia is estimated to be 50 million and is projected to reach 131.5 million by 2050 (Alzheimer's disease International, 2015) [3]. Alzheimer's disease (AD) accounts for about 60-80% of cases of dementia [4]. The causes are manifold. The loss of cholinergic neurons in the Nucleus Basalis of Meynert (NBM) and hence reduced cholinergic activity, is one of the primary causes of cognitive and memory impairment in AD [10].

Oxidative stress also forms a critical part of the pathophysiological process of disease occurrence and progression [5, 6]. Currently, acetylcholinesterase inhibitors (Tacrine, donepezil, rivastigmine and galantamine) are considered as the standard treatment for cognitive decline associated with

various neurological disorders. Various adverse effects limit their therapeutic success. Hence, there is an increasing need for efficient and safe therapeutic options. Dietary modifications and intake of specific phytonutrients has been widely investigated –preclinicaly and clinically, for their neuroprotective potential. Though not completely conclusive, intake of Mediterranean and ketogenic diets; dietary changes with inclusion of fruits, vegetables, fish, fibre, as well as intake of omega -3 fatty acids, vitamin D, carotenoids, phenolic compounds, resveratrol is recommended for prevention as well as slowing the progression of neurodegeneration [7, 8].

Numerous epidemiological investigations draw attention to the fact that a regular intake of fruits and vegetables rich in phenolic compounds and flavonoids is correlated with preventing as well as slowing the progression of neurodegenerative changes, including cognitive deterioration [9, 10, 11]. Green leafy vegetables are rich sources of diverse phytonutrients and have been designated as "nature's anti-aging wonders" [12]. Studies provide evidence that green leafy vegetables hold great protective potential against cognitive decline [13, 14]. The neuroprotective effects of green leafy vegetables are attributed to the presence of phenolic compounds which are potential candidates for developing therapeutic agents for neurodegenerative diseases [15, 16].

Phenolic compounds are reported to have marked neuroprotective promise [17] mediated *via* their anticholinesterase activity [18] significant anti-oxidant capacity [19] anti-neuroinflammatory activity [20] inhibition of tau phosphorylation and reduction of amyloid deposition and improvement of neural plasticity [21]. *Chenopodium album* Linn (Chenopodiaceae) is a fast-growing annual herb which is commonly called Goose feet (English). It is a popular leafy vegetable valued for its taste and nutritive value. It has been traditionally used in different systems of medicine to treat various ailments [22]. *C. album* contains a wide array of phytoconstituents including phenolic compounds responsible for the various pharmacological actions as analgesic, anticancer, antifungal, antioxidant, antiulcer, anti-inflammatory, *etc*. exhibited by the plant [22, 23]. In our earlier work hydromethanol (HM) extract of *C. album* was evaluated *in-vitro* using DPPH assay for anti-oxidant activity evaluation and Ellman assay for acetylcholinesterase inhibition potential. HM extract had strong AChE inhibitory and antioxidant activity [24]. Hence, the present study was designed to carry out bioactivity guided fractionation of the HM extract to isolate the fraction/constituent responsible for the cognitive enhancing and neuroprotective ability of *C. album* using *in-vitro* assays (AChE inhibition and antioxidant), followed by *in-vivo* study using scopolamine-induced memory impairment in mice as *in-vivo* model. Thus generate pre-clinical evidence for the memory-protective activity of the plant.

METHODS:

Chemicals: Acetylcholinesterase, acetylthiocholine iodide, DPPH (2,2-diphenyl-1-picrylhydrazyl), 5,5′-dithiobis-(2-nitrobenzoicacid) (DTNB), donepezil hydrochloride, reduced glutathione (GSH), 1,1,3,3-tetraethoxypropane, donepezil, p-hydroxy benzoic acid, cinnamic acid, ferulic acid, and sinapic acid were purchased from Sigma. Scopolamine hydrobromide was gifted by Ind-Swift laboratories. All other chemicals and reagents were of analytical grade.

Cultivation and Authentication of Plant Material: *C. album* was cultivated in last week of October 2014 and collected during January 2015. Identification and authentication of the leaves were carried out by Mrs. Sunita Garg, Chief Scientist, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi (Specimen no: NISCAIR/RHMD/Consult/-2015-16/2178/184).

Preparation of Extracts and Fractions: Leaves were shade dried and coarsely powdered. The detailed extraction and fractionation procedure is depicted in **Fig. 1.**

Standardization of Extract and Fractions: The prepared extract and fractions were Total phenol content (TPC) and Total flavonoid content (TFC) of the prepared extract and fractions were determined by Folin-Ciocalteau and aluminium chloride colorimetric procedures, respectively [25].

***In-vitro* Antioxidant Activity:** Antioxidant activity of extract and fractions was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [26].

***In-vitro* AChE Inhibitory Activity:** Inhibitory effect of extract and fractions on acetylcholinesterase (AChE) activity was determined by modified Ellman's method [27].

***In-vivo* Studies:**

Animals: Swiss albino mice (either sex) weighing 20-25 g purchased in February, 2018 from Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar used in the study were housed in the departmental animal house and were exposed to a 12 h diurnal light cycle and access to standard laboratory pellet diet and water *ad libitum*. The animals were acclimatized to the laboratory conditions for 7 days prior to study. The *in-vivo* study was carried out as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India. The experimental protocol was approved by Institutional Animal Ethical Committee (IAEC) (107/GO/ReBi/S/99/CPCSEA/2017-63).

Experimental Protocol: Experimental protocol is presented in **Fig. 2**. Nine groups with 6 animals in each group were made randomly and assigned following treatments.

Group I (Vehicle Treated): Animals were administered 0.5 % w/v CMC (10 ml/kg, i.p) once daily from day 1 to day 7 of the protocol.

Groups II (Scopolamine Treated Group): Animals were administered scopolamine (1 mg/kg, i.p) once daily from day 4 to day 7.

Groups III (Scopolamine + Donepezil treated Group): Animals were administered donepezil (5 mg/kg, i.p) from day 1 to day 7.

Groups IV, V and VI (Scopolamine + HM Extract Treated Groups): Animals were administered HM extract of *C. album* at dose of 100 mg/kg, 200 mg/kg and 400 mg/kg, o.d.; i.p., from day 1 to day 7, respectively. The dose selection was done on the basis of reports in literature on methanol/aqueous extracts of *C. album* [28].

Group VII, VIII and IX (Scopolamine + F6 Treated Groups): Mice were administered F6 at dose of 1 mg/kg, 2 mg/kg and 4 mg/kg, o.d.; i.p., from day 1 to day 7, respectively.

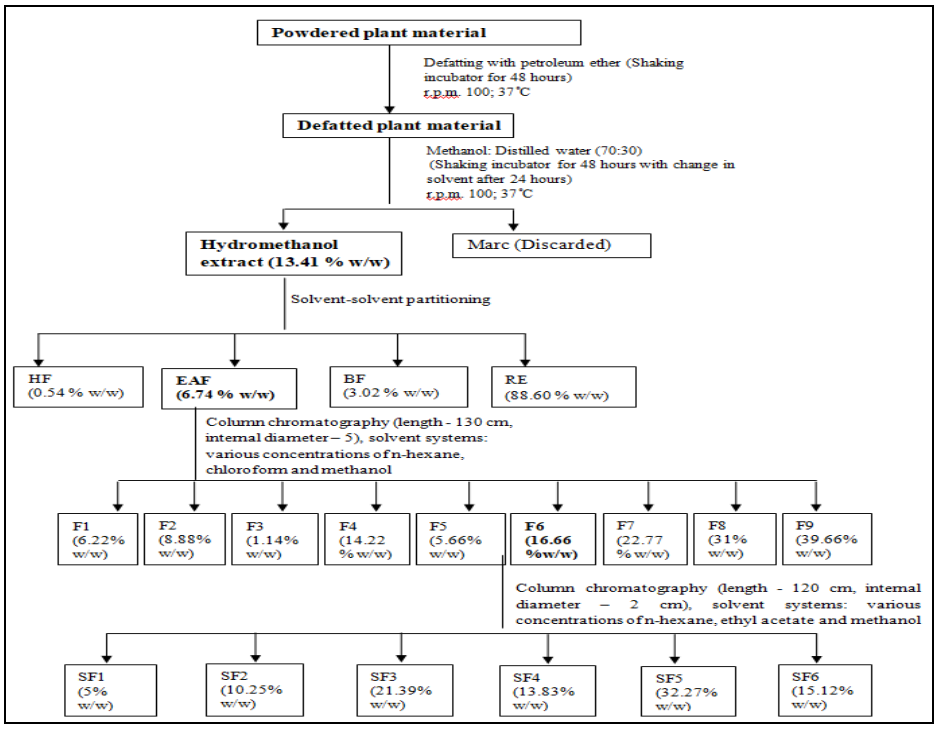


FIG. 1: PREPARATION OF EXTRACT AND FRACTIONS OF *CHENOPODIUM ALBUM*

The dose selection of the fraction F6 was done on the basis of ratio of this fraction in HM extract of *C.album* **Fig. 1.** Memory impairment was induced in the animals by scopolamine injection (1 mg/kg; i.p.) 60 min after administration of standard drug / HM extract/ Fraction F6 from day 4 to day 7. The

behavioral parameters were determined 30 min. after scopolamine administration from day 4 to day 7. The retrieval trial was carried out on day 8 to assess retrieval of memory [29].

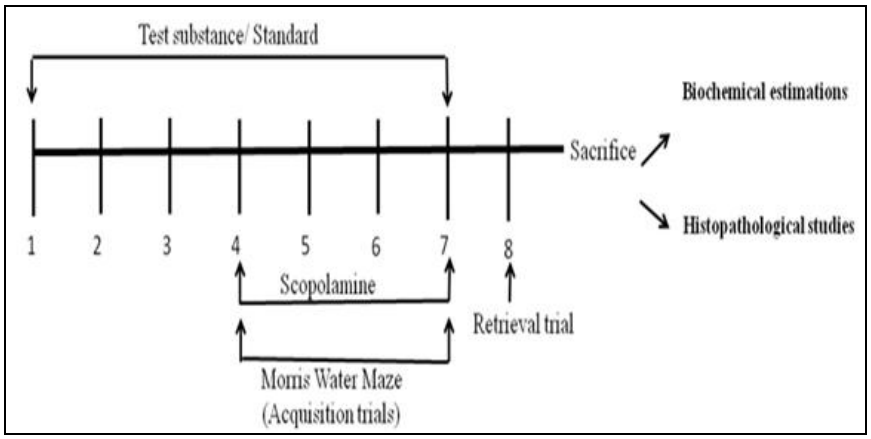


FIG. 2: EXPERIMENTAL PROTOCOL

Assessment of Cognitive Performance:

Morris Water Maze Test: Morris Water Maze (MWM) test was employed to evaluate learning and memory [30, 31]. MWM consisted of a circular pool (150 cm in diameter and 45 cm high) filled with water (25°C) to a depth of 30 cm made opaque with a white-colored nontoxic dye. The tank was divided into four equal quadrants and a white submerged platform (10X10 cm) was placed 1 cm below water surface in one of the quadrants. During the training session, each animal was placed in any of the quadrants and time taken to find the hidden platform, during the 120s trial period, was noted as Escape Latency Time (ELT). Mice were trained over four daily sessions (inter-trial gap: 5 min.) performed on 4 consecutive days (i.e. day 4-7 of the protocol). If the mouse was unable to locate the platform within 120 sec., it was guided to the platform and allowed to remain there for 20 sec. ELT on day 4 of MWM test was considered as the index of learning/acquisition. On the 8th day (i.e. day 5 of MWM test), the platform was removed and each animal was allowed to search the pool for 120 sec. before being removed, the time spent in all four quadrants searching for the hidden platform was noted. The time spent in the target quadrant (TSTQ) *i.e.* the quadrant in which the platform was earlier present, was considered as an index of retrieval/memory.

Biochemical Estimations: At the end of the experiment, animals were sacrificed by cervical dislocation under mild Anaesthesia. Brains were carefully removed and cortex and hippocampus were separated. Homogenates were prepared in ice cold phosphate buffer (pH 7.4) by centrifugation at 14,500 rpm for 15 min. (4 °C). The clear supernatant was subjected to various biochemical estimations. The total protein content, AChE activity, glutathione (GSH) and thiobarbituric acid-reactive species (TBARS) levels in hippocampus and cortex homogenates was estimated using standard procedures developed by [32, 27], Beutler et al (1963) [33] and Ohkawa *et al.* (1979) [34] respectively.

Histopathological Studies: Freshly prepared Bouin's solution was used for preserving intact brains from each group for histopathological examination. Brain sections were stained with hematoxylin and eosin (H & E) as described by Banchroft and Turner (1996) [35].

Estimation of Phenolic Acids in Bioactive Fraction and *C. album* Leaves using TLC-Densitometric Studies: A fixed volume, 10 µl each, of HM extract (10 mg/ml), F6 (1mg/ml) and standard phenolic acids, namely, cinnamic acid, ferulic acid, 4-hydroxybenzoic acid and sinapic acid (1 mg/ml), was applied on a pre-coated TLC plate (E Merck, Mumbai, India; 0.2 mm; aluminium base) using CAMAG LINOMAT 5. The plate was developed using a solvent system – chloroform: methanol (9:1) in a chamber, saturated for 10 min. and visualised under ultraviolet light (254 and 366 nm). The content of phenolic acids in HM extract and F6 was calculated from a standard plot of marker compounds using a regression equation and determined as percentage content with respect to dried weight.

Statistical Analysis: All the results were expressed as mean ± standard error of mean (SEM) for ELT and TSTQ. Data was analyzed using a two-way analysis of variance (ANOVA) followed by Student Newman Keul multiple comparison test. The data for biochemical estimations was presented as mean±standard deviation (S.D.) and analyzed by one way ANOVA followed by Student Newman Keul multiple comparison test. Analyses were carried out using computerized Sigma Stat software (v. 3.5) (Systat Software, San Jose, California). $p<0.05$ was considered to be significant.

RESULTS AND DISCUSSION: In this investigation, HM extract of *C. album* was fractionated based on the polarity of components and the generated fractions and sub-fractions were evaluated for their memory protective potential employing *in-vitro* assays followed by *in-vivo* study of the fraction that demonstrated most significant *in-vitro* activity. The HM extract and most active fraction were studied phytochemically and standardized chromatographically with respect to marker phenolic acids present therein. The results are discussed below.

Standardization of Extract and Fractions: Phytochemical screening of HM extract and fractions showed the presence of phenols and flavonoids. The results were similar to those reported in literature [36, 37]. Literature shows the potential of phenolic compounds for prevention and management of several neurodegenerative disorders through different mechanisms such as acetylcholinesterase (AChE) inhibition, free radical scavenging/antioxidant actions, anti-inflammatory, anti-apoptotic actions, *etc*. [38]. TPC of HM extract was found to be 15.95 ± 0.91 % w/w and TFC was 2.93 ± 0.87 % w/w.

The order of TPC and TFC for the fractions was EAF>BF>RE: TPC of EAF, BF and RE were 25.47±0.75, 6.82±0.17, 1.57±0.54% w/w, respectively and TFC of EAF, BF and RE were 3.48±0.24, 2.60±0.91 and 0.12±0.19% w/w, respectively.

***In-vitro* Antioxidant and AChE Inhibitory Potential of Extracts and Fractions:** Brain oxidative stress and acetylcholine deficiency are major pathogenic factors of neurodegenerative memory disorders. The cholinergic hypothesis states that cholinergic deficit in brain leads to cognitive impairment [39]. Inhibition of AChE activity can help in the restoration of cholinergic function, thereby, improving learning and memory impairment. Oxidative stress hastens neuronal degeneration [40]. Therefore, an agent possessing antioxidant and AChE inhibitory potentials can be ideal in preventing neurodegenerative disorders.

The results (IC_{50} values) of *in-vitro* analyses of antioxidant and AChE inhibitory potentials of the extract and fractions are presented in **Table 1**. Amongst the extract and fractions, F6 exhibited the highest antioxidant and AChE inhibitory activities as depicted by lowest IC_{50} values. The activity might be due to higher content of phenolic compounds present in F6 as compared to HM extract and other fractions.

TABLE 1: IC_{50} VALUES OF EXTRACT AND FRACTIONS IN DPPH AND ELLMAN ASSAYS

Standard/Extract/ Fraction	IC_{50} value (Mean^n±S.D.)	
	Antioxidant activity (µg/ml) (DPPH assay)	AChE inhibitory activity (µg/ml) (Ellman assay)
Ascorbic acid	4.25±0.36	---
Donepezil	---	7.25±0.19
HM extract	133.93±0.89*	75.33± 2.05* (mg/ml)
HF	>1000	>1000
EAF	48.19±1.36*	307.35±1.41*
BF	118.68±2.31*	698.64±1.57*
RE	512.13±1.01*	>1000
F1	>1000	>1000
F2	948.61±1.97*	>1000
F3	308.08±1.32*	987.89±2.62*
F4	83.95±0.95*	202.16±0.48*
F5	32.42±0.55*	66.50±0.29*
F6	3.05±0.18	13.29±0.53
F7	29.54±0.40*	47.83±0.59*

F8	12.21±0.79*	485.46±1.07*
F9	702.26±2.62*	>1000
SF1	358.04±3.01*	707.69±3.94*
SF2	79.35±2.07*	243.61±2.99*
SF3	25.41±1.54*	94.52±2.97*
SF4	11.96±0.98*	33.75±1.84*
SF5	19.49±1.18*	58.02±1.29*
SF6	65.26±1.08*	134.82±1.31*

The data is presented as Mean ± S.D. (n=3) and analysed by one way ANOVA followed by Student Newman Keul's test;*p<0.05 vs Standard

All the sub-fractions of F6 *i.e*. SF1-SF6 showed lower antioxidant and AChE inhibitory potentials as compared to F6. Probably because fractionation of F6 resulted in distribution of phytoconstituents (phenolic compounds) in different fractions, consequently lowering antioxidant and AChE inhibitory potential.

The higher activity of F6 may be attributed to the synergistic effect of phytoconstituents present. Literature provides evidence that synergism/additive effect of various phytoconstituents of an extract/fraction leads to enhanced bioactivity as compared to isolated compounds [41-44]. As is evident from the results of *in-vitro* analysis, F6 shows most significant activity, hence the bioactive extract and F6 were examined *in-vivo*.

***In-vivo* Studies:** In the present study, scopolamine was utilized to induce memory impairment in mice. Scopolamine is a well-known muscarinic receptor blocker that impairs learning and memory in rodents and humans and is widely used for evaluating anti-dementia effects of drug candidates [45]. Post-synaptic binding sites of acetylcholine in brain are blocked by scopolamine, thereby causing extensive damage to the hippocampal neurons and ultimately leading to cognitive impairment [46]. Moreover, scopolamine administration also cause increase in brain oxidative stress levels [47, 48].

Cholinergic deficit and oxidative damage produced by scopolamine lead to progressive learning and memory loss. Therefore, the scopolamine-induced memory deficit model is extensively used for screening. Morris Water Maze (MWM) test was employed in the present study. It is one of the most prevalent animal models for assessing memory and learning potential as it offers cross-species utility, no pre-training requirement and high reliability [49, 50]. During acquisition trials, a significant decline in day 4 escape latency time (ELT) of control group mice depicts normal memory acquisition. During retrieval trials, a significant rise in time spent in target quadrant (TSTQ) to search for missing platform is a measure of normal memory retrieval.

A sharp decline in day 4 ELT compared to day 1 ELT Table 2 observed in control group animals depicted normal learning potential. During the retrieval trial (day 5), the control group animals spent significantly higher TSTQ (Q4) to search the missing platform as compared to non-target quadrants (Q1, Q2 and Q3), indicating normal retrieval of memory **Fig. 3**.

Scopolamine administration leads to a significant cognitive deficit in mice [45, 51]. This was evident in this study by a no change in day 4 ELT along with no increase in TSTQ by the scopolamine-treated animals. Donepezil treatment (5 mg/kg, i.p.) significantly lowered the day 4 ELT and caused a significant rise in TSTQ as expected [52, 53], indicating improved learning potential and memory. The doses of HM extract were selected on the basis of acute toxicity data reported in literature [28, 54].

Administration of extract (100, 200 and 400 mg/kg, i.p.) caused reduction in day 4 ELTs as compared to day 4 ELT of scopolamine treated mice and a significant increase in TSTQ. F6 was evaluated at 1, 2 & 4 mg/kg (the doses were selected on the basis of percentage yield of F6 from HM extract).

F6 treated mice showed a sharp decline in day 4 ELT and increased TSTQ in a dose-dependent manner. Moreover, this reversal of scopolamine-induced memory impairment caused by F6 administration (4 mg/kg) was found to be comparable to that caused by donepezil.

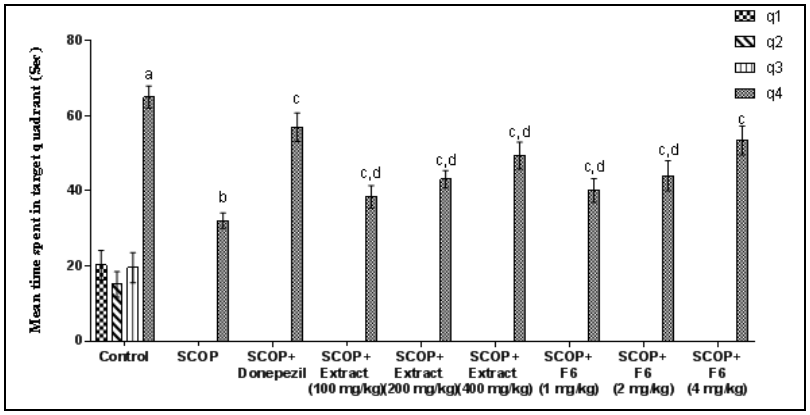


FIG. 3: EFFECT OF VARIOUS TREATMENTS ON TIME SPENT IN TARGET QUADRANT (TSTQ) BY MICE IN MWM TEST AFTER SCOPOLAMINE ADMINISTRATION. The data is presented as Mean^{n} ± S.D. and analyseed by way two way ANOVA followed by Student Newman Keul's test for *post hoc* analysis; ^{a}p<0.05 vs. time spent in Q1, Q2 and Q3 by mice in control group; ^{b}p<0.05 vs. TSTQ by mice in control group; ^{c}p<0.05 vs. TSTQ by mice in scopolamine treated group; ^{d}p<0.05 vs. TSTQ by donepezil treated group.

TABLE 2: EFFECT OF VARIOUS TREATMENTS ON ELT OF MICE IN MWM TEST AFTER SCOPOLAMINE-INDUCED MEMORY DEFICIT

Groups	Day 1 ELT (s)	Day 4 ELT (s)
Control	98.25±3.47	25.19±3.02^{a}
Scopolamine	101.94±6.18	85.56±4.80^{b}
Scopolamine+Donepezil	99.19±3.62	34.87±2.80^{c}
Scopolamine+Extract (100 mg/kg)	99.69±2.85	70.12±3.83^{c,d}
Scopolamine+Extract (200 mg/kg)	101.25±3.55	67.86±3.30^{c,d}
Scopolamine+Extract (400 mg/kg)	99.94±4.06	55.38±3.96^{c,d}
Scopolamine+F6 (1 mg/kg)	97.31±3.90	56.94±3.28^{c,d}
Scopolamine+F6 (2 mg/kg)	99.56±2.90	48.81±2.40^{c,d}
Scopolamine+F6 (4 mg/kg)	98.56±3.05	39.25±3.97^{c}

The data is presented as Mean^{n} ± S.D. (n=6) and analysed by two way ANOVA followed by Student Newman Keul's test for *post hoc* analysis; ^{a}p<0.05 vs. Day 1 ELT; ^{b}p<0.05 vs. Day 4 ELT of control group; ^{c}p<0.05 vs. Day 4 ELT of scopolamine treated group; ^{d}p<0.05 vs. Day 4 ELT of donepezil treated group.

TABLE 3: EFFECT OF VARIOUS TREATMENTS ON BIOCHEMICAL PARAMETERS

Groups	Brain AChE (nM/min/mg of protein)		TBARS (nM/mg of protein)		GSH (nM/mg of protein)	
	HPO	COR	HPO	COR	HPO	COR
Control	0.54±0.03	0.51±0.05	5.04±0.13	5.94±0.49	48.25±1.24	48.53±1.7
SCO	1.42±0.22^{a}	1.08±0.13^{a}	20.32±0.76^{a}	21.28±0.85^{a}	21.56±0.96^{a}	20.03±1.22^{a}
SCO+DON	0.65±0.06^{b}	0.56±0.05^{b}	9.41±0.45^{b}	10.26±0.75^{b}	35.27±1.12^{b}	34.36±1.21^{b}
SCO+Extract (100 mg/kg)	1.11±0.10^{b,c}	0.93±0.07^{b,c}	18.16±0.67^{b,c}	18.34±0.56^{b,c}	24.71±1.04^{b,c}	25.36±0.89^{b,c}
SCO+Extract (200 mg/kg)	0.93±0.06^{b,c}	0.90±0.04^{b,c}	15.31±0.42^{b,c}	16.42±0.46^{b,c}	27.04±0.83^{b,c}	29.67±1.17^{b,c}
SCO+Extract (400 mg/kg)	0.78±0.06^{b,c}	0.73±0.05^{b,c}	13.26±0.59^{b,c}	14.81±0.62^{b,c}	33.37±0.59^{b,c}	33.91±0.21^{b,c}
SCO+F6 (1 mg/kg)	0.89±0.05^{b,c}	0.85±0.06^{b,c}	14.40±0.57^{b,c}	15.98±0.79^{b,c}	32.67±0.94^{b,c}	31.56±1.35^{b,c}
SCO+F6 (2 mg/kg)	0.72±0.03^{b,c}	0.64±0.04^{b,c}	10.60±0.53^{b,c}	11.59±0.50^{b,c}	36.26±0.78^{b,c}	34.69±1.11^{b,c}
SCO+F6 (4 mg/kg)	0.67±0.05^{b}	0.57±0.04^{b}	7.29±0.51^{b}	8.06±0.51^{b}	41.17±0.92^{b}	42.46±1.05^{b}

SCO- Scopolamine; DON-Donepezil; HPO-Hippocampus; COR-Cortex. The data is expressed as Mean^{n} ± S.D. (n=6) and analysed by two-way ANOVA followed by Student Newman Keul's test; ^{a}p<0.05 vs. Control; ^{b}p<0.05 vs. SCO; ^{c}p<0.05 vs. DON.

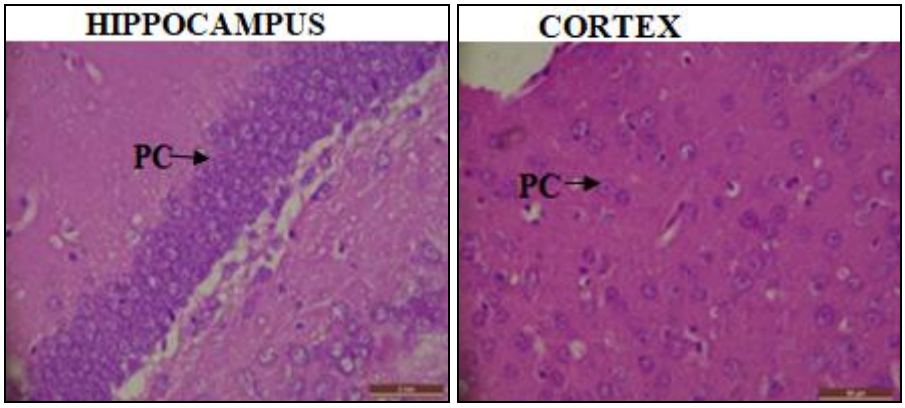
Effect on Biochemical Parameters: On day 8, after the behavioral studies, animals were sacrificed and effect of F6 on biochemical parameters (TBARS, AChE activity, and reduced GSH levels) *ex-vivo* was evaluated in hippocampus and cortex regions of scopolamine-treated mice brain to elucidate the underlying mechanism of action **Table 3**. Scopolamine causes up-regulation of AChE in mice brain, which results in impairment of cognitive functions [55]. A significant increase in brain AChE activity and oxidative stress levels (increased TBARS and decreased GSH) was seen in scopolamine-treated mice compared to the control group mice. Various reports showed that

plants and their constituents that inhibited AChE, effectively reversed the scopolamine-induced memory deficit [29, 56, 59]. The trend of our study was in accordance with these previously reported findings. Donepezil-treated mice and extract (100, 200 and 400 mg/kg, i.p.) showed reduced brain AChE activity and oxidative stress as evidenced by decreased TBARS and enhanced endogenous antioxidant (GSH) levels compared with scopolamine-treated mice.

These effects were most prominent in F6-treated mice and the effects produced by F6 (4 mg/kg) were found to be statistically equivalent to donepezil. Literature provides evidence that scopolamine and AChE cause activation of β-amyloid precursor proteins ultimately leading to formation of β-amyloid plaques (a characteristic feature of AD) [60, 61]. Since, F6 exhibits antagonistic action on scopolamine and inhibits AChE, it might also affect formation and deposition of β-amyloid plaques and may prove to be a beneficial therapy for the management of neurodegenerative diseases like AD.

Histopathological Studies: The cerebral cortex and the hippocampus are the key regions of the brain involved in memory formation. Destruction of hippocampal and cortical neurons in AD is primarily responsible for various memory disorders [62]. Therefore, a histopathological examination of the hippocampus and cortex was carried out to study morphological changes in scopolamine-treated mice brains.

The stained histological sections of hippocampus and cortex **Fig. 4** of the control group mice demonstrated normal brain histology, with cortex showing normal pyramidal cells and hippocampus having a well-defined neuronal area. The scopolamine-treated mice showed alterations reflected by focal gliosis, increased vacuolation and decreased number of pyramidal cells as compared to control group mice. Pretreatment with HM extract and F6 showed marked prevention of scopolamine induced effects indicating reversal of dementia and neuroprotective potential of *C. album*. Similar effects were observed with donepezil.



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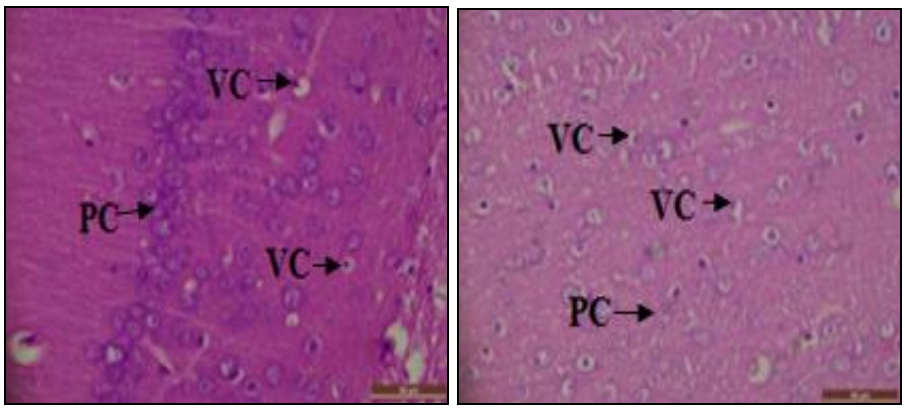

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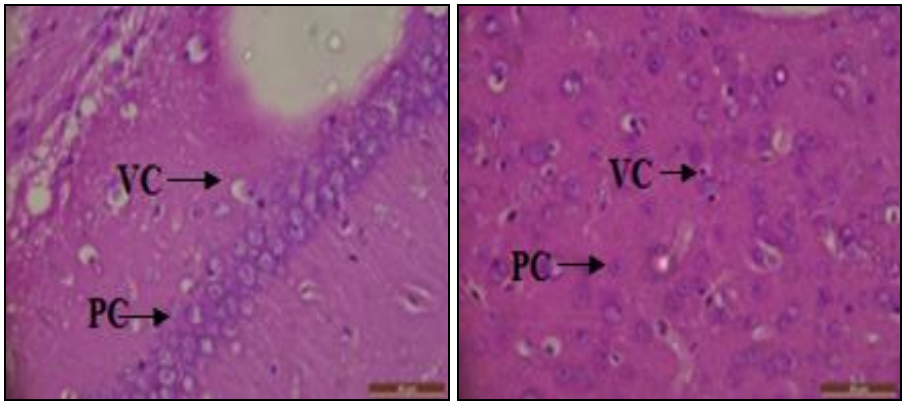

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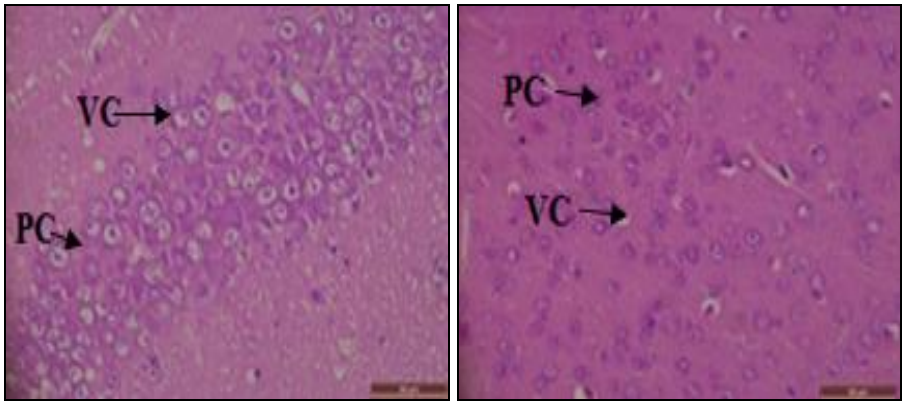

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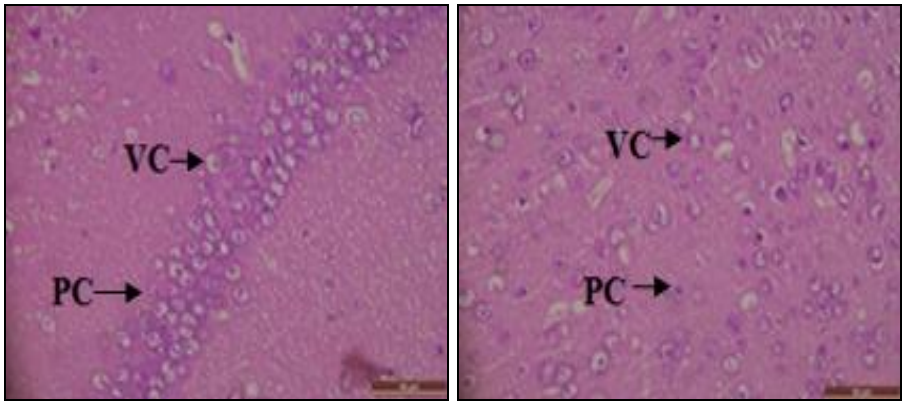

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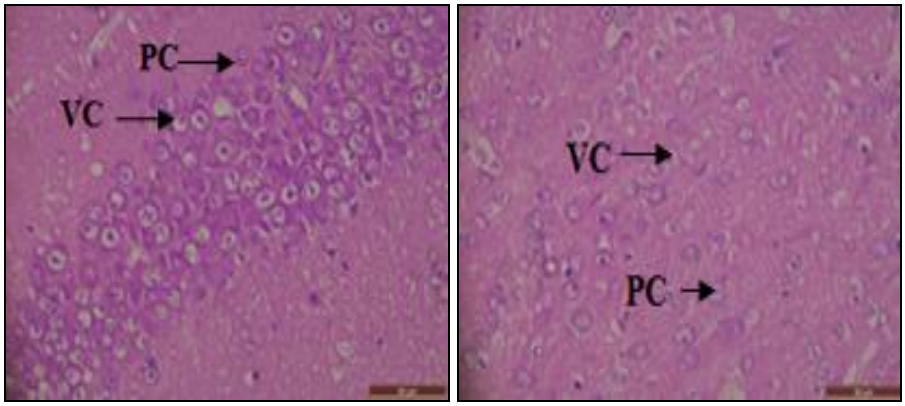

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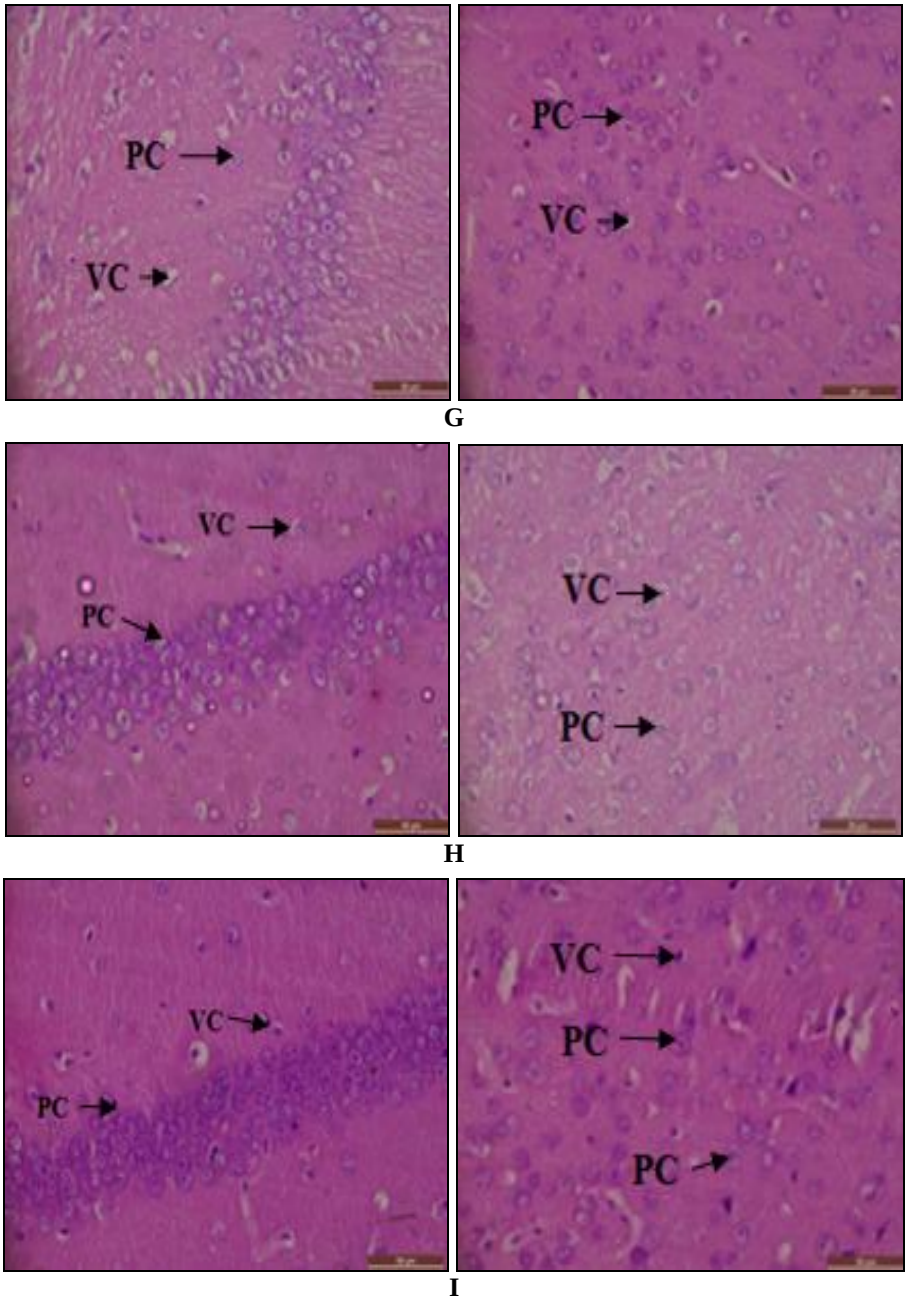

FIG. 4: HAEMATOXYLIN AND EOSIN STAINED HISTOLOGICAL SECTIONS OF BRAIN (40 X); A. CONTROL; B. SCOPOLAMINE TREATED GROUP; C. DONEPEZIL TREATED GROUP; D. EXTRACT TREATED GROUP (100 MG/KG); E. EXTRACT TREATED GROUP (200 MG/KG); F. EXTRACT TREATED GROUP (400 MG/KG); G. F6 TREATED GROUP (1 MG/KG); H. F6 TREATED GROUP (2 MG/KG); I. F6 TREATED GROUP (4 MG/KG). PC- PYRAMIDAL CELLS; VC- VACUOLATED CELLS (MARKERS OF DEGENERATION)

Estimating Phenolic Acids in Bioactive Fraction and *C. album* Leaves using TLC-Densitometric Studies: TLC densitometry is a modern analytical method that enables simultaneous analysis of multiple compounds in complex mixtures in minimal time.

It is a simple, accurate and precise technique offering various advantages such as-selective detection, automation, optimization, minimum sample requirement, *etc*. Therefore, the bioactive constituents of C. album leaves were estimated using this method.

C. album contains phenolic acids like cinnamic acid, 4-hydroxy-cinnamic acid, ferulic acid, methyl ferulate, sinapic acid, 4-hydroxybenzoic acid, 4-hydroxy-3-methoxybenzoic acid, vanillic acid, m-coumaric acid, *etc*. [63, 64]. Four of these, *i.e.*, cinnamic acid, ferulic acid, 4-hydroxybenzoic acid

and sinapic acid were used as markers for standardization of the plant and the bioactive F6 fraction. The presence of marker compounds (ferulic acid, sinapic acid, 4-hydroxy benzoic acid and cinnamic acid) in HM extract and F6 was confirmed by comparative TLC profiling **Fig. 5**.

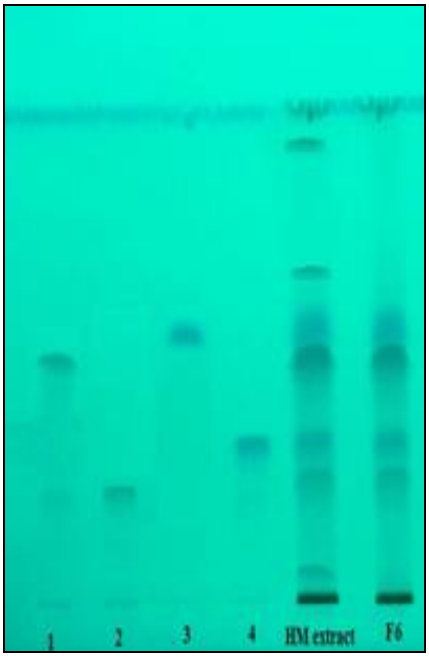

FIG. 5: TLC FINGERPRINT OF HM EXTRACT AND F6 WITH MARKER COMPOUNDS (1: CINNAMIC ACID; 2: FERULIC ACID; 3: 4-HYDROXYBENZOIC ACID; 4: SINAPIC ACID)

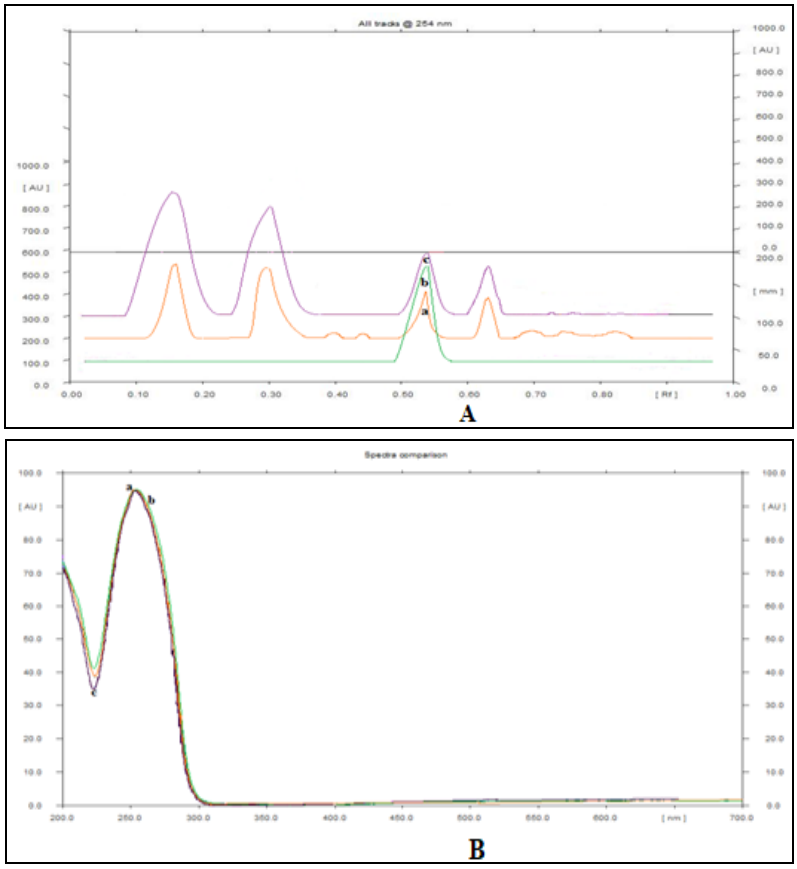

FIG. 6: TL CHROMATOGRAM (A) AND SPECTRA OVERLAY (B) OF HM EXTRACT (A), F6 (C) AND CINNAMIC ACID (B)

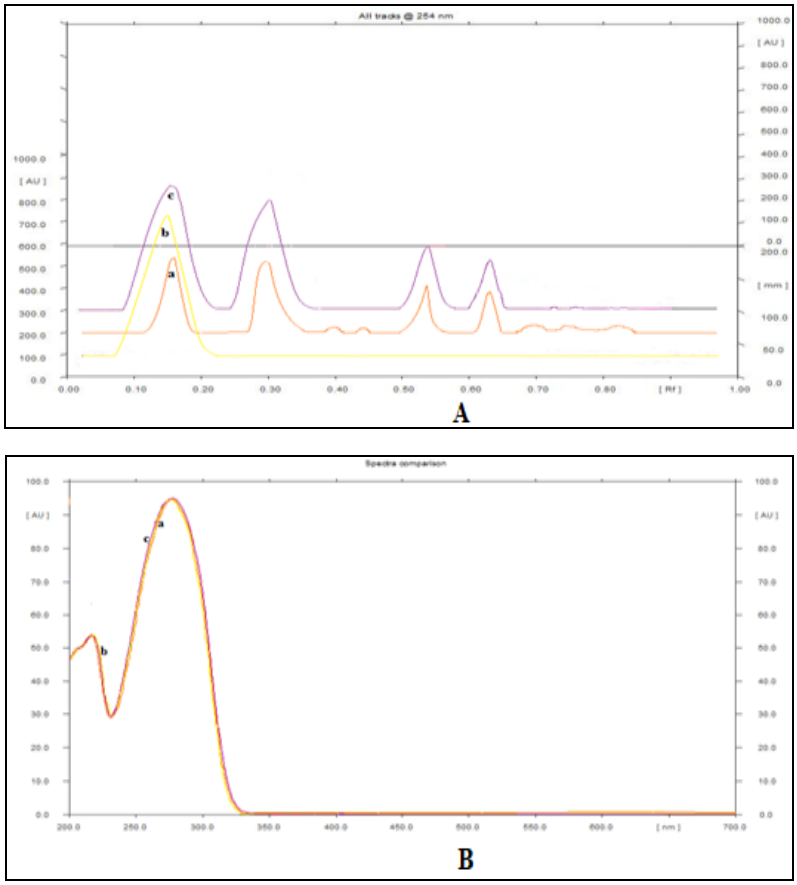

FIG. 7: TL CHROMATOGRAM (A) AND SPECTRA OVERLAY (B) OF HM EXTRACT (A), F6 (C) AND FERULIC ACID (B)

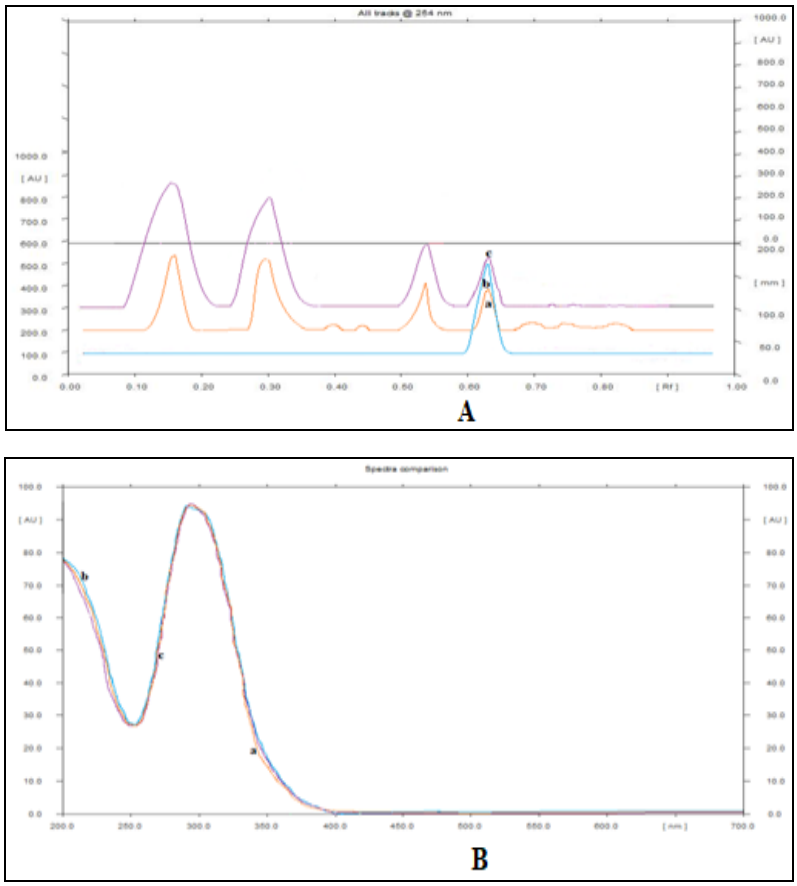

FIG. 8: TL CHROMATOGRAM (A) AND SPECTRA OVERLAY (B) OF HM EXTRACT (A), F6 (C) AND 4-HYDROXYBENZOIC ACID (B)

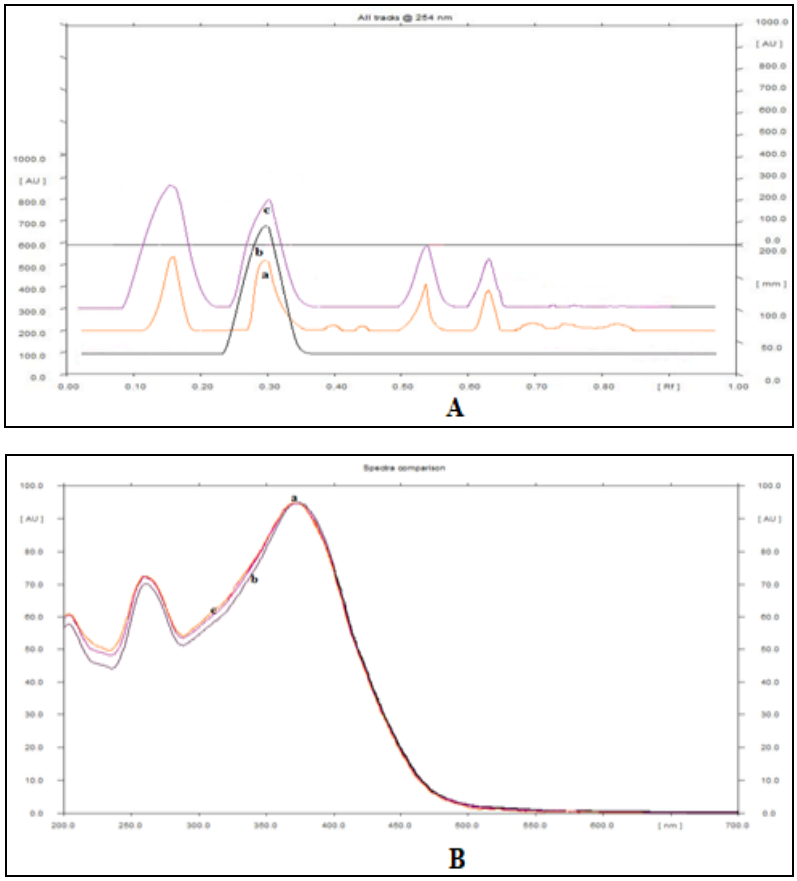

FIG. 9: TL CHROMATOGRAM (A) AND SPECTRA OVERLAY (B) OF HM EXTRACT (A), F6 (C) AND SINAPIC ACID (B)

Hence, using TLC-densitometry, these compounds were used to standardize bioactive fraction (F6) and C. album leaves. The ferulic acid, sinapic acid, 4-hydroxy benzoic acid and cinnamic acid content in F6 was found to be 34.53, 33.13, 5.90 and 7.46% w/w, respectively **Fig. 6-9.** The content of the compounds in *C. album* leaves was found to be 0.0113±0.0002% (cinnamic acid), 0.0542±0.0003% w/w (ferulic acid), 0.0179±0.0001% w/w (4-hydroxy benzoic acid) and 0.0524±0.0006% w/w (sinapic acid), respectively. TLC densitometric analysis revealed that F6 contains four phenolic acids: cinnamic acid, ferulic acid, 5-hydroxybenzoic acid, and sinapic acid. The available literature provides data that phenolic compounds play a significant role in managing several neurological disorders. Antioxidant and neuroprotective activities of phenolic compounds are well documented in literature [65, 66]. Various studies are available in which cinnamic acid, ferulic acid, 5-hydroxybenzoic acid and sinapic acid, found in the bioactive fraction of *C. album*, have shown potential in management of neurodegenerative diseases such as AD [67, 70]. Hence, the neuroprotective activity of F6 may be attributed to these phenolic acids. Several reports in literature provide information about the anti-amnesic mechanism of phenolic compounds. Ferulic acid targets multiple pathways i.e., AChE inhibition, oxidative stress, neuroinflammation and amyloidogenesis involved in pathological processes of learning and memory impairment [71, 72]. Sinapic acid ameliorated Aβ protein-related pathology including neuronal cell death and cognitive dysfunction through antioxidant and antiinflammatory activities [68] (Lee *et al.*, 2012). Moreover, it also acts as an AChE inhibitor, thereby affecting the cholinergic pathway involved in neurodegeneration [73]. Cinnamic acid is reported to serve as a ligand for peroxisome proliferator-activated receptor α (PPARα) and cause induction of lysosomal biogenesis, thereby reducing cerebral amyloid plaque burden and improving learning and memory [74]. p-hydroxybenzoic acid possesses antioxidant potential and protects excitotoxic environment present in various neurodegenerative diseases [75]. However, the combined effect of these phenolic

acids in neurological disorders had not been investigated. This study highlights that phenolic acids are more effective, probably because they act on different targets involved in the complex pathophysiology of cognitive decline. The results of the present study indicate that the bioactive fraction of *C. album, i.e.,* F6 (which has a combination of the phenolic acids) may be an effective treatment strategy to target multiple pathways involved in the neurodegenerative disorders. Future directions for study on this fraction should focus on mechanistic study and drug delivery systems' development. The prospective work should also include the impact of dietary intake of the plant and plant extract as a nutraceutical for prevention and management of neurodegenerative disorders especially related to cognitive decline in experimental models and clinical situations.

CONCLUSION: This study reveals that the bioactive fraction of *C. album* consisting of cinnamic acid, ferulic acid, 4-hydroxy benzoic acid and sinapic acid in combination exhibits strong potential to manage memory disorders. This may be due to the synergistic effect of these phenolic compounds exhibited through antioxidant and acetylcholinesterase inhibitory pathways. The results warrant further studies to develop fraction F6 obtained from *C. album* as a drug or nutraceutical for the management of memory disorders.

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