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Aqueous-ethanolic extracts of *Curcuma longa* and *Withania somnifera* improve memory in the dementia model

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ABSTRACT: Dementia involves impairment in cognitive and behavioral abilities, which leads to disability and dependency, particularly among older people. Alzheimer's disease is the most common form of dementia. Currently, available synthetic drugs against dementia have several side effects and only give symptomatic relief without curing or reversing the pathophysiology of the disease. So, herbal drugs can be good alternatives with a good safety profile. *Curcuma longa* (CL) and *Withania somnifera* (WS) have been reported for neurological activities with individual extracts and select phytoconstituents. However, their aqueous-ethanolic extracts have not been studied in dementia models. This study aimed to find the effect of aqueous-ethanol extracts of CL rhizomes and WS roots in scopolamine-induced dementia in Swiss albino mice using behavior models; Elevated Plus Maze (EPM), Morris Water Maze (MWM) and Spontaneous Alternation (Y Maze). Further, their antioxidant activity was also studied. The findings of the study revealed that both extracts effectively antagonized the detrimental effects of Scopolamine. Comparatively, WS in the dose of 40 mg/kg exhibited more efficacy in EPM and MWM models, while CL at 40 mg/kg was found to be more effective in the Y Maze. Furthermore, both extracts showed higher effects at the dose of 40 mg/kg than 20 mg/kg. They have also exhibited antioxidant activity. It is concluded that the aqueous-ethanolic extracts of CL and WS possess anti-dementia and antioxidant potential like other extracts earlier reported. Their pathophysiology, synergistic effects in different combinations, and influence on the neurotransmitters need further exploration.

KEYWORDS: Dementia; Alzheimer's disease; *Curcuma longa*; Curcumin; *Withania somnifera*; Withanolides

1. INTRODUCTION

1.1. Dementia - an overview

Dementia is a chronic syndrome with progressive deterioration in cognitive functions like learning, orientation, memory, language, comprehension, and judgment due to abnormality in the brain. It has been a reference in historical texts since antiquity. Pythagoras used the concept of 'senium' or old age in the 7th century BC as mental and physical changes in old and advanced age [1,2]. Later the terms'senility' and 'senile dementia' started appearing in ancient literature dating back to the 13th and 14th centuries. In modern times, the more specific pathophysiology of Alzheimer's disease (AD), a common type of dementia, was described based on the microscopic changes in the brain in the year 1907. Many neuropathologic processes may lie beneath dementia; neurodegenerative and vascular causes are most common among them. Increasing age is the leading risk factor for dementia. At such an advanced age, comorbidity is an inevitable part of elderly persons with dementia. Based on pathophysiology, there are various forms of dementia, viz. AD, vascular dementia, frontotemporal dementia, dementia with Lewy bodies, and other minor forms. Dementia mainly affects aged people, and only 2% of cases begin before the age of 65 years. The prevalence of dementia almost doubles every five years after the age of 65. In the late stage of life, dementia is significantly responsible for disability and dependency [3–5].

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1.2. Prevalence and societal impact

According to the WHO's recent report of 2020, around 55 million people are living with dementia worldwide, and around 10 million new cases of dementia are added every year. AD alone contributes around 60–70% of the total cases of dementia [6]. It is estimated that there were about 3. 7 million Indians with dementia in 2010 and 5. 3 million people above the age of 60 have dementia in India in 2020. When it is compared with the population projections for the year, one in 27 people above the age of 60 in India has dementia. The total societal cost is estimated at about USD 2000 million [7–9]. This figure may predictably go as high as 7 to 7. 5 million by 2030. The estimated household costs of caring for a person with dementia are USD 377 to USD 1226 per household per year in rural areas and USD 847 to 3761 per household per year in urban areas in 2019 [10].

1.3. Available treatment options and their risks and benefits

Current drug therapy for dementia involves the use of cholinesterase inhibitors and N-methyl-d-aspartate (NMDA) receptor-blocking agents like memantine [11–15]. However, these drugs are associated with several side effects like vomiting, loss of appetite, frequent stools, headache, constipation, confusion, and dizziness. As per the United States Alzheimer's Association's report, none of the pharmacologic treatments (drugs) available today for Alzheimer's dementia slow or stop the damage and destruction of neurons that cause AD's symptoms and make the disease fatal [16]. However, medicinal plants show promise in AD treatment because of their cognitive benefits and, more importantly, their mechanisms of action concerning the fundamental pathophysiology of the disease [17,18]. Developing scientific trends on their phytoconstituents has consistently inspired the discovery of modern medicine [19].

1.4. *Curcuma longa* L. and *Withania somnifera* (L.) Dunal– possible herbs in dementia

Curcuma longa (CL) and *Withania somnifera* (WS) are among the most commonly used medicinal plants in Indian medicine for many disorders for centuries. CL, commonly known as turmeric or Haldi in Hindi, has been one of the most used spices in India for many centuries. WS, commonly known as Ashwagandha and also called "Indian Winter cherry" or "Indian Ginseng," is also used for millennia in Indian medicine for the treatment of various disease conditions and as a special tonic due to its health benefits [20,21,22,23].

CL contains 3-5% volatile oils like zingiberan, turmerone and other compounds like curcumin, bitter principles, and resins. The active phytoconstituents present in turmeric are collectively termed curcuminoids which mainly include curcumin (diferuloylmethane), demethoxycurcumin, and bisdemthoxy curcumin. It is well documented for antitussive, antifungal, cholesterol-lowering, anticancer, anti-rheumatic and other properties of its different extracts. Curcumin is well studied and found to regulate inflammatory cytokines, cross the blood-brain barrier to target senile plaques and disrupt existing plaques and possess neuroprotective properties [24-29].

WS has over 12 alkaloids, 40 withanolides and several sitoindosides. The main constituents include withanine, withananine, nicotine, somnifer, somniferincine, along with other alkaloids, sugars, ß-sitosterol, etc. It is reported for diverse activities like an adaptogenic, aphrodisiac, brain tonic, diuretic, antihelminthic, astringent, thermogenic and stimulant, antistress, anti-inflammatory, anti-ulcer, anti-aging and anti-rheumatism properties. WS is very frequently used to restore vitality after overwork or nervous exhaustion and is reported as a beneficial effect for the debility associated with long-term stress. Due to its high iron content, it is recommended for anemia and related weakness. Its root extract has been used for centuries as an aphrodisiac, alterative, diuretic, debility, and senile dementia from old age in Indian medicine. Extracts of WS root possess various activities like hypotensive, bradycardic, anti-inflammatory, antimicrobial, antitumor, antistress, antidiabetic, neuroprotective, and cardioprotective activities [30-37]. Its alkaloid stimulates the respiratory centers in the brain stem, and its cardio-inhibitory action seems to be due to ganglion blocking and direct cardio-depressant actions [38].

1.5. Significant role of different extracts and phytoconstituents of CL and WS in dementia models

Both plants have been reported for their effects on cognitive impairment and the underlying mechanisms. CL has been reported to possess antioxidative, anti-amyloidogenic, and anti-inflammatory properties and to decrease Aβ plaques. It is reported to clear the amyloid protein, inhibit the microglial proliferation and differentiation, Aβ-induced expression of Early growth response protein 1 (Egr-1 protein), Egr-1 Deoxyribonucleic acid (DNA) binding activity in a monocytic cell line called Tamm-Horsfall Protein 1 (THP-1) monocytic cells, phospholipases, transcription factor, enzymes involved in metabolizing the membrane phospholipids into prostaglandins and cyclooxygenase (COX-2) [39-41]. WS has been shown to prevent or

suppress free radicals developed during the pathogenesis of AD. It also blocks neuronal cell death triggered by amyloid plaques. Studies show that compounds of WS uniquely bind to the active motif of beta-amyloid (Aβ 25-35) and subsequently prevent fibril formation. WS is also found to increase the acetylcholine content, choline acetyltransferase activity, and cholinergic activity. It has a significant role in AD pathology by enhancing low-density lipoprotein receptor-related protein in the liver [41–44]. Its aqueous extract improved psychomotor and cognitive performance in healthy human participants [45]. Its root extract reversed the behavioral deficits and pathological clues as well as Aβ clearance in AD models by upregulating lipoprotein receptor-related protein in the liver [46].

1.6. Importance of examining aqueous ethanolic extracts of CL and WS in the dementia model

Though there have been many studies found on the role of different extracts of these plants in different solvents, none of them has reported the effects of aqueous ethanolic extracts on cognitive impairments. Water and ethanol are considered to be universal as well as generally recognized as safe (GRAS) solvents [47]. Aqueous ethanolic extracts may have unique therapeutic efficacy, which is different from that of extracts obtained by other solvents. Published studies so far focus either on individual extracts with these solvents or with other solvents like methanol, synthetic analog, etc. For instance, an ethanolic extract of CL [48], a fermented CL powder [49], synthetic analogs of curcumin [50], and nano-encapsulated curcumin [51] have been studied in mouse and zebrafish models of memory and cognition. Konar *et al.* have studied the protective role of the alcoholic extract of WS leaf in mice models [52]. Cholinergic properties of the same extract were further studied by Gautam *et al.* [53]. Likewise, the aqueous methanol extract of Ashwagandha was studied by Vigneswari *et al.* in rats for its cholinergic properties and toxicity [54,55]. Aqueous-ethanolic extracts of these plants have exerted beneficial effects on the cardiovascular, renal, hepatic, immune, and rheumatic systems, but their cognitive effects in behavioral models have not been reported [56-62]. Hence, we envisioned undertaking this study to find out the effect of the aqueous-ethanolic extracts of CL and WS on the behaviors of mice in dementia models.

2. RESULTS

2.1. Elevated plus maze (EPM)

The effect of CL and WS on memory impairment was evaluated using EPM through the Transfer latency (TL) parameter. TL of the first day reflected the learning behaviors of animals, whereas TL of the second day reflected the retention of memory. TL was taken as the time taken by the mouse to move to any of the closed arms with all its four legs.

In acquisition trials, no significant difference was found in the mean initial trial latency between Control, Scopolamine (SCP), CL 20 mg/kg, CL 40 mg/kg, WS 20 mg/kg and WS 40 mg/kg groups (Figure 1, Table 1). However, on Day 2, there was a significant difference in mean TL observed between groups (Figure 2). TL was more in the SCP group than others.

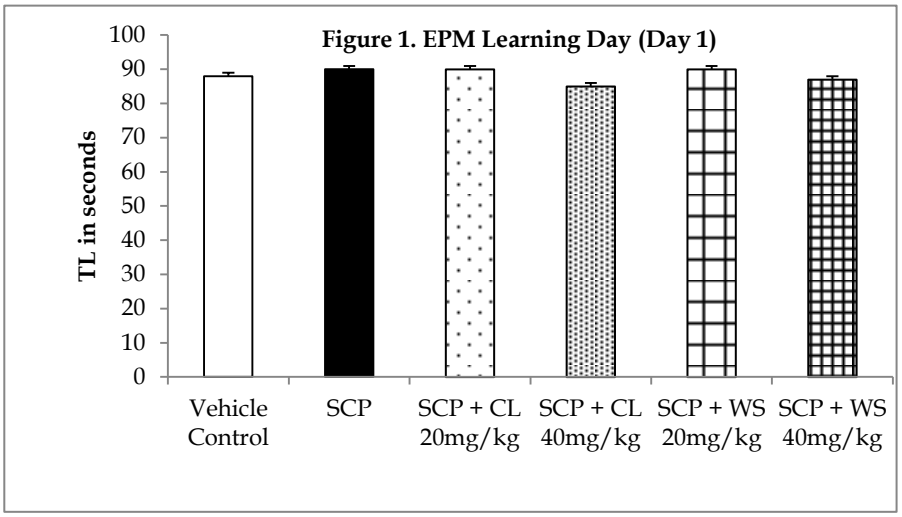


Figure 1. Elevated plus maze on learning day (day 1); means, standard deviation (SD)
TL – transfer latency; SCP – scopolamine; CL – *Curcuma longa*; WS – *Withania somnifera*

Table 1: Elevated Plus Maze (n=6 in each group)

	Day 1		Day 2	
	TL	**SD**	**TL**	**SD**
Vehicle Control	88	6. 9	50	3. 4
SCP	90	8. 6	90	4. 2
SCP + CL 20 mg/kg	90	7. 8	45	2. 4
SCP + CL 40 mg/kg	85	7. 2	40	3. 6
SCP + WS 20 mg/kg	90	9. 2	39	3. 1
SCP + WS 40 mg/kg	87	7	35	4. 5

TL – transfer latency; SD – standard deviation; SCP – scopolamine; CL – *Curcuma longa*; WS – *Withania somnifera*

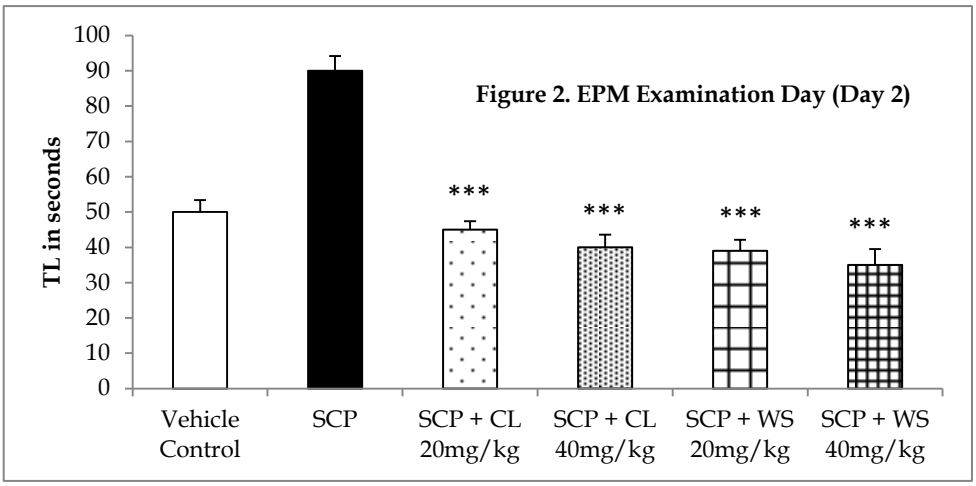


Figure 2. Elevated plus maze Day 2; mean, SD
** P<0. 01 vs control | *** P<0. 001 vs scopolamine
All 4 extracts have shown effectiveness against disease control P<0. 001. However, WS40 has shown more effective than other extracts.
TL – transfer latency; SCP – scopolamine; CL – *Curcuma longa*; WS – *Withania somnifera*

2.2. Morris water maze (MWM)

During the acquisition trial in MWM, all the groups learned almost similarly except the SCP group (Figure 3, Table 2). On the probe trial on day 5, except for the SCP group, all other groups spent in the target quadrant almost equal time (Figure 4, Table 2). This demonstrates that the test drugs could be able to reverse the deleterious effect on cognition induced by SCP.

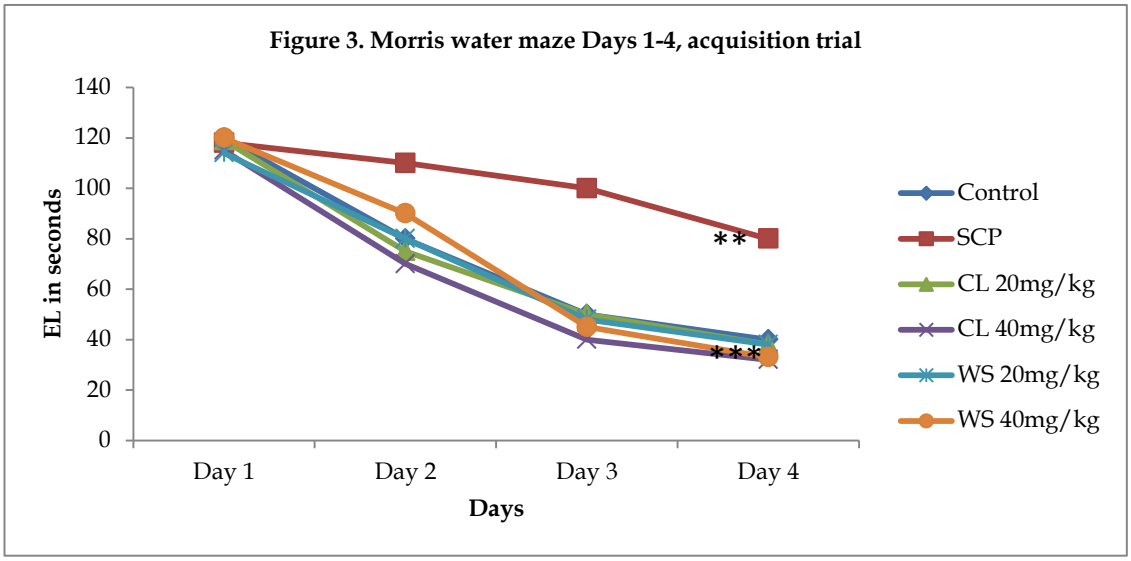


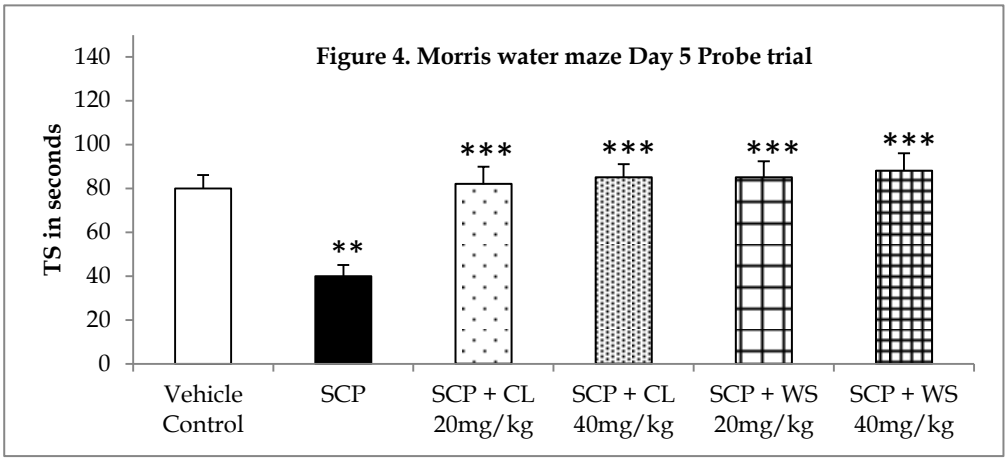
Figure 3. Morris water maze Days 1-4, acquisition trial, mean, SD
** P<0. 01 vs control | *** P<0. 001 vs scopolamine

On probe trial, WS 40 has shown more effectiveness than others. All 4 extracts have shown a statistically significant effect (P<0. 001) than disease control.

Table 2: Morris Water Maze (n=6 in each group)

Control	Day 1 Mean of EL	Day 1 SD	Day 2 Mean of EL	Day 2 SD	Day 3 Mean of EL	Day 3 SD	Day 4 Mean of EL	Day 4 SD	Day 5 Mean of TS	Day 5 SD
SCP	120	7. 4	80	5. 5	50	4. 2	40	2. 1	80	6. 2
CL 20 mg/kg	118	8. 2	110	6. 2	100	7. 4	80	4. 2	40	5. 2
CL 40 mg/kg	119	9. 1	75	7. 4	50	6. 3	38	3. 2	82	7. 9
WS 20 mg/kg	115	6. 8	70	5. 2	40	4. 1	32	2. 8	85	6. 2
WS 40 mg/kg	114	7. 2	80	6. 4	48	5. 9	38	3. 2	85	7. 5
	120	8. 4	90	7. 1	45	6. 6	33	2. 9	88	8. 1

EL – escape latency; TS – time spent on the target quadrant; SD – standard deviation; SCP – scopolamine; CL – *Curcuma longa*; WS – *Withania somnifera*



2.3. Y maze

Y Maze has shown significant differences between the SCP group and others (Table 3, Figure 5). Among the test drugs, CL 40 mg/kg seems more effective than others.

Table 3: Y Maze (n=6 in each group)

	% Novel arm vs. all	SD
Control	48	5. 2
SCP	27	3. 4
CL 20 mg/kg	55	4. 5
CL 40 mg/kg	58	4. 9
WS 20 mg/kg	49	5. 1
WS 40 mg/kg	53	5. 5

SD – standard deviation; SCP – scopolamine; CL – *Curcuma longa*; WS – *Withania somnifera*

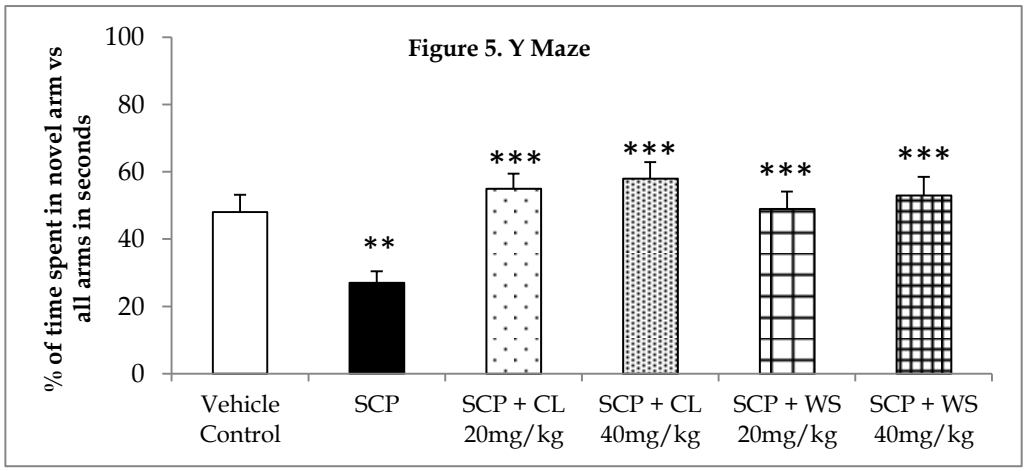


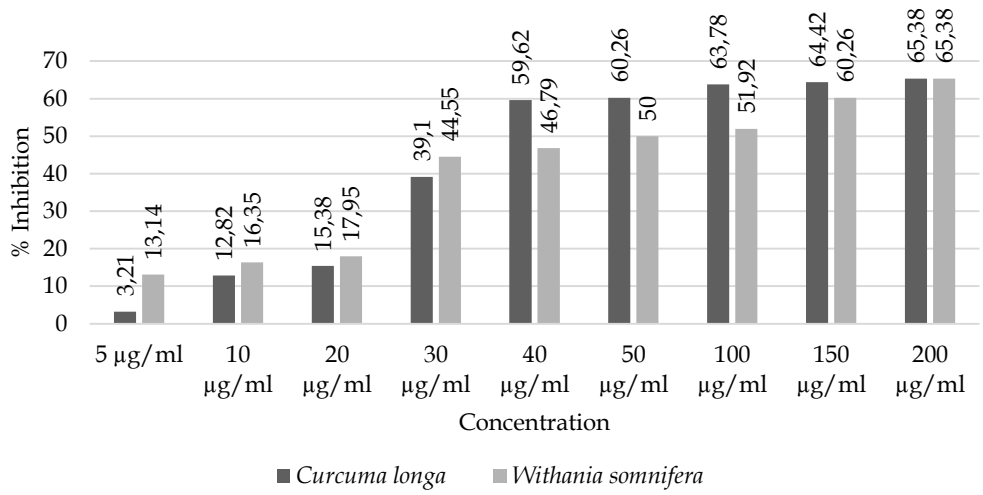
Figure 5. Y Maze % of time spent in novel arm vs all arms; mean, SD
** P<0. 01 vs control | *** P<0. 001 vs scopolamine
Curcuma longa has shown more effective than other extracts. All 4 extracts have shown statistically significant effect (P<0. 001)
SCP – scopolamine; CL – *Curcuma longa*; WS – *Withania somnifera*

2.4. DPPH free radical scavenging activity

Both the extracts exhibited maximum activity at 200 µg/ml, starting from 5 to 30µg/ml. WS has exhibited higher activity than CL, while from 40 to 150 µg/ml, CL exhibited higher activity than WS (Figure 6). Interestingly, at 200 µg/ml, both displayed almost equal activity.

Figure 6. DPPH free radical scavenging activity of CL and WS extracts.

Figure 6. DPPH free radical scavenging activity of CL and WS extracts



3. DISCUSSION

In this study, we have evaluated the cognitive-enhancing effects of CL and WS both in 20 mg/kg & 40 mg/kg body weight doses of aqueous-ethanolic extracts for their anti-dementia effects. Using well-known behavioral models such as the Elevated plus maze, Morris water maze and Y maze, the study was performed. MWM is considered useful compared to the other reported conventional measures of learning and memory because, in this model, training for spatial memory can be easily achieved across several acquisition trials. Further, the task does not require strong motivating conditions such as scent, punishment or food and water deprivation.

This study shows that in 2 out of 3, the tests performed viz. EPM and MWM, WS 40 mg/kg seem more effective than others. In the EPM, the mean TL on day 2 for WS 40 mg/kg was 35 seconds, the lowest of all groups. In MWM, the mean time spent on the target zone for WS 40 mg/kg was 88 seconds, the highest among all, indicating its anti-dementia effect. Only in the case of Y Maze, CL 40 mg/kg tends to be more effective. It indicates that WS and CL could be more effective in the dose of 40 mg/kg than 20 mg/kg.

The findings of this study were analyzed in context with previous reports of CL and WS published on cognitive impairments. Several mechanisms are proposed in the previous studies on curcumin and other curcuminoids for their protective action against dementia and AD. One study revealed that curcumin plays a vital role in the activation of macrophages in clearing the amyloid plaques from the nerve cells [63], while other studies illustrated its role in the reduction of the chronic inflammation of nerve cells [29,57,59,64]. Curcuminoids are also strong antioxidants that hamper the formation of free radicals, which play a prominent role in the pathogenies of AD [26,27,59]. Curcumin is also reported for the induction of neuroprotective proteins like heme oxygenase [65]. In a recent study, Randino et al. performed molecular and computational studies using different curcuminoids to investigate their role in AD [66]. Curcumin and its analogs possessed a high tendency to interact with the lipid bilayer, which reduced the interactions of Aβ–membrane and ultimately inhibited the formation of Aβ fibril formation. All these previous findings show that CL has anti-dementia potential through various mechanisms, and the findings of the current study with aqueous ethanolic extract confirm the same.

The previous clinical report illustrated the role of WS in improving mental and psychomotor performance [45]. The exact underlying mechanism by which WS improves the anti-dementia activity is still not completely revealed. However, previous animal studies showed that it acts by modulating cholinergic neurotransmission [53,54,68]. Effects of the sitoindosides VII-X and withaferin isolated from the roots of WS were studied by Schliebs et al. on glutamatergic, cholinergic, and GABAergic receptors of the Wistar rat brain. The finding of the study suggested that tested constituents mainly enhance the capacity of the muscarinic acetylcholine receptor, particularly in the cortical region, which might be responsible for the improvement in cognitive power [69]. Pingali *et al*. in 2014 performed a double-blind, placebo-controlled

clinical study on healthy volunteers using the dried aqueous extract of roots and leaves of WS. The study reported significant improvement in cognitive and psychomotor performance as compared to the placebo group [45]. Since the current study was with an aqueous ethanolic extract of WS, it shows similar effects as compared to the above studies.

4. CONCLUSIONS

In this study, the aqueous-ethanolic extracts of CL and WS were found to be effective against SCP-induced detrimental cognitive effects. Both plants species have been used for centuries in the Indian system of medicine for many neurological disorders, and current findings also reveal the effectiveness in the same line. So, aqueous-ethanolic extracts of both plants could be potential therapeutic agents in the treatment of dementia. Further studies are warranted to explore its pathophysiology, influence on the neurotransmitters, synergistic effects in combination, and comparison with standard drugs, synthetic analogs, and individual phytoconstituents.

5. MATERIALS AND METHODS

5.1. Extracts

Herbs have been procured from the herbal garden of Dr. Willmar Schwabe India Pvt. Ltd. (Schwabe India). They were identified by the botanist at Schwabe India and sent to the National Institute of Science Communication and Information Resources for confirmation, which was duly confirmed. A specimen herb was submitted at the herbarium of Schwabe India, and the specimen number was obtained. A comparative study of High-performance thin-layer chromatography (HPTLC) and ultraviolet (UV) spectroscopy was also performed with control samples to ensure their standards. CL and WS were extracted using water and ethanol. 100 g of CL fresh rhizome was extracted with 400 ml of water and 635 ml of ethanol using the cold percolation method. The extract was then filtered, and the resulting liquid was concentrated under reduced pressure at 45 °C in a rotary evaporator. This extract was then kept in the incubator at 45 °C for three days to remove the ethanol residue yielding the crude rhizome extract. This extract was then diluted with distilled water at a ratio of 5:1 to prepare the extract stock solution (200 mg/mL). 100 g of WS fresh rhizome was extracted with 250 ml of purified water and 800 ml of ethanol using the methods above for CL. The water-ethanol ratio was defined as per Homoeopathic Pharmacopeia of India [69].

5.2. Animals

The study was conducted using SCP-induced Swiss albino mice (42±2gm) obtained from the animal house of Deshpande Laboratories. Animals were kept in polypropylene cages (29×22×14 cm) with 25±2 °C and 45-55% humidity and a normal day and night cycle. Food and water were kept to be accessed ad libitum. They were handled for at least a week before behavioral examinations. Animal Ethics Committee approval was obtained vi-de approval number CPCSEA/IAEC/01/2021/23 dated 17th January 2021 from the Institutional Animal Ethics Committee, Deshpande Laboratories, Bhopal, India.

5.3. Experimental design and drug treatments

Experiments were carried out during the daytime between 9:00 am to 5:00 pm. The procedure of drug treatment was carried out for 19 days. Institutional guidelines for animal care and use were followed during experiments. Six animals in each group were divided as below:
Group I: Normal saline plus the vehicle was administered orally to mice for 19 days successively. Escape Latency (EL) in Morris Water Maze (MWM) was recorded after 60 min of the vehicle administered from the 15th day to the 18th day, and probe trial was examined after 24 hr (i. e. on the 19th day).
Group II: Negative control SCP was administered orally for 19 days successively. EL in MWM was recorded after 60 min of the drug administration from the 15th day to the 18th day, and the probe trial was examined after 24 hr (i. e. on the 19th day).
Group III and IV: SCP + CL 20 mg/kg (CL 20 mg) and CL 40 mg/kg (CL 40mg) respectively were administered orally for 19 days successively in groups III & IV, respectively. EL was recorded after 60 min of the extract administration from the 15th day to the 18th day, and the probe trial was examined after 24 hrs (i. e. on the 19th day).
Group V and VI: SCP + WS 20 mg/kg (WS 20 mg) and WS 40 mg/kg (WS 40mg) were administered orally for 19 days successively in groups V & VI, respectively. EL was recorded after 60 min of the extract

administration from the 15th day to the 18th day, and the probe trial was examined after 24 hrs (i. e., on the 19th day).
Since it is a preliminary pilot study, the objective is also to find the most beneficial dose of CL and WS; hence two doses were finalized as above under Groups III to VI. The above dosages were defined based on the earlier reports [70-78] and empirical usage by the practitioners of the Indian system of medicine.

5.4. Behavioural studies

5. 4. 1 The elevated plus maze (EPM) used for this study was with two covered arms (16 cm × 5 cm × 15 cm) and two open arms (16 cm × 5 cm) extended from a central platform (5 cm× 5 cm). The total height of the maze is 25 cm elevated from the floor. On the first day, mice were placed at the open arm's end, facing their back toward the central platform. Transfer latency (TL) was noted on the first day for each and every animal. The total time taken by the animal to reach from the open arm into any of the covered arms with all its legs is defined as TL. The animal was gently pushed if it did not enter into one of the covered arms within 90 sec. in such case, TL was assigned as 90 sec. Then for 2 minutes, the mouse was permitted to explore the maze before returning to its cage. It was examined 24 h after the first-day trial to check the retention of this learned task (memory). These procedures, techniques and endpoints for testing memory and learning were as per the standard parameters of psychopharmacology followed by experts in the field [79,80].

5. 4. 2 Morris water maze (MWM) is a circular pool (60 cm in diameter, 25 cm in height, for mice). Water is filled to a depth of 20 cm in the pool, which is maintained at 25◦C. To avoid transparency, water was made opaque with powdered milk. With the help of two threads, the tank was divided into four equal quadrants and named Q1, Q2, Q3 and Q4. The shape of the threads resembled a 'plus' (+) sign. A white platform of 6 cm × 6 cm was sub-merged inside Q4 below 1 cm of the water and designated as the target quadrant. The position of the platform remained the same throughout the training session, and also the observer stood in the same position. The relative location of the water maze was also maintained the same with respect to the objects in the laboratory. Each animal was subjected to two consecutive trials each day with a gap of 5 min for four consecutive days (starting from the 16th day of drug administration to the 19th day). During the trial period, they were allowed to escape onto the hidden platform and remain there for 20 seconds. Animals were placed randomly in the pool between any quadrant facing toward the wall. Each time the location of the dropping changed. They were allowed to locate the submerged platform for 120 seconds. In case the animal did not reach the platform in 120 seconds, they were gently guided to reach the platform and stay there for 20 seconds. The time is taken by the animal to reach the platform from where it was left considered escape latency (EL). During the trial period of 4 days, EL was recorded for each animal. The day after four consecutive trial days, a probe trial was conducted (20th day). On the day of the probe trial, the platform was removed. It was to find out whether the animal could be able to recall and find out the target quadrant by searching the platform for a considerable time or not. Animals were allowed to explore the target quadrant for a maximum of 300 seconds. Finally, the meantime noted the animals' spending (time spent (TS) on the target quadrant) in search of the platform was considered as the index of memory. All these techniques and endpoints for testing learning and memory were as per the established procedures of psychopharmacology [81,82].

5. 4. 3 Spontaneous Alternation (Y Maze) test is used to measure short-term memory in mice using a 'Y' shaped maze. It was conducted to measure the willingness of animals (mice) to explore new environments by retaining the memory of already visited arms. They usually prefer a new area to the usually visited place. Brain areas like the hippocampus, basal forebrain, septum and prefrontal cortex are actively involved in the process of exploring new areas. It also helps to measure the retention of memory by avoiding already visited areas. For this investigation, a 'Y' shaped maze is used with opaque white plastic arms at a 120° angle between each other. The animal is placed in the center of the maze and lets it explores the arms freely. Over a period of time, mice usually show interest in entering the arm, which is not much visited. The percentage of alternation was calculated as the number of alternations divided by the number of triads multiplied by 100. Entry was counted if all four limbs of the animal were placed in the arm. These procedures, techniques and endpoint for testing the behavior were followed as described by the investigators working in the area of psychopharmacology [83-85].

5.5. DPPH free radical scavenging activity

It was prepared based on the procedure described by various investigators [86,87]. In short, a solution of DPPH (2,2-diphenyl-1-picrylhydrazyl) was prepared by dissolving 4 mg DPPH in 100 ml ethanol (0. 004 %

solution). 1ml of this solution was added to 2ml of CL and WS hydroethanolic extracts (for both extracts, separate sets were made) in different concentrations of 5, 10, 20, 30, 40, 50, 100, 150 and 200 µg/ml. Thereafter, all the test tubes were kept in the dark for color development for 20 minutes. After this, the absorbance was read for all the tubes at 517 nm using a UV spectrophotometer. The antioxidant activity was quantified by measuring the change in color of DPPH fading from purple to yellowish. The reduction of DPPH by antioxidant decreases its absorption strength. Finally, the percentage inhibition was calculated using the formula % inhibition= [(Absorbance of control- Absorbance of the sample)/Absorbance of control]. X 100%.

5.6. Statistical Analysis

Statistical analysis was performed using Sigma Stat 2. 0 statistical software. One-way analysis of variance (ANOVA) and paired t-test were used in the comparison between the different treatment groups. In the study, $p<0.05$ was used as statistical significance.

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