"EVALUATION OF NEUROPROTECTIVE PROPERTY OF CITRUS PULP POWDER IN SCOPALAMINE INDUCED ZEBRAFISH MODEL AMNESIA"

Sandeep Chandakavate[1], Shivakumar H[1] , V P Patil[1], Akram Naikwadi[2] & Nanjappaiah H M. [1]

1. Dept. of Pharmacology, BLDEA's SSM College of Pharmacy and Research Centre, Vijayapur, Karnataka, India.

2. Dept. of Pharmacology, Sri B M Medical College, Hospital and Research Centre, Vijayapur, Karnataka, India.

Corresponding author:

Dr. Nanjappaiah H M
Dept. of Pharmacology,
BLDEA's SSM College of Pharmacy and Research Centre
Vijayapur - 586103, Karnataka, India.

ABSTRACT

OBJECTIVES: The present research work was designed to evaluate the neuroprotective activity of Citrus pulp powder (CPP) using scopalamine induced Zebrafish model amnesia.

MATERIALS AND METHODS: In the present study neuroprotective activity was assessed by Novel tank test and Y Maze test.

RESULTS AND DISCUSSION:

Effect of CPP in Novel Tank Test (NTT)

In Novel tank test, the different doses of Citrus pulp powder showed dose dependent increase in the number of entries to the top, time spent in the top, latency to enter the top and total distance travelled whereas decrease in number of entries to bottom and time spent in bottom as compared to scopolamine control group.

Effect of CPP on spatial memory and novelty response in Y-Maze

In Y-Maze test the Citrus pulp powder at doses exhibited dose dependent significant increase in number entries to new arm and total distance travelled in the maze as compared to scopolamine control group. The Citrus pulp powder at different dose showed significant increase in time spent in novel arm compared to scopolamine control.

Effect of CPP on CAT, SOD, MDA and AChE levels in Zebrafish brain homogenate

The different doses of the Citrus pulp powder showed significant increase in the CAT and SOD and significant decrease in AChE and MDA content in brain homogenate of Zebrafish as compared to scopolamine control group.

CONCLUSION: The results of our study conclude that the Citrus pulp powder could effectively improve memory impairments in a scopolamine induced Zebrafish model of amnesia by enhancing the function of the behavioral response and antioxidant enzymes in the amnesic Zebrafish model.

Key words: Neuroprotective, Novel tank test, SOD, CAT, Zebrafish.

I. INTRODUCTION

Alzheimer's disease (AD) is a central neurodegenerative disease that is prominently Age dependent, the proportion of AD patients is gradually increasing as society ages around the world [1]. Alzheimer's disease (AD) is a chronic neurodegenerative disease associated with dementia. AD is characterized by a progressive loss of cognitive abilities and diminished executive function, associated with various degrees of behavioral disturbances [2]. Alzheimer's disease (AD) is a chronic neurodegenerative disease associated with dementia. Zebrafish (*Danio rerio*) is a promising in vivo model for induction of AD as well as to screen drugs to combat AD [3]. Supporting evidence suggested that, in Zebrafish, scopolamine

exhibited memory reducing effects without causing locomotory deficits and is often used with nootropic and memory enhancing drugs to study memory formation [4].

Traditionally, many plants such as *Spondias mombin*, *Quassia undulata*, *Zeamays*, *Moringa oleifera*, *Diosco reabatatas*, and *Bacopa floribunda* have been used to improve memory and cognition in older adults. Plant extracts provide a wealth of bioactive compounds, which helps in alleviation of neurological symptoms of the AD [5]. In current days the research studies focus on herbal drugs and other natural products across the globe for the age related CNS diseases [6].

Citrus limon (L.) is a tree with evergreen leaves and yellow edible fruits from the family Rutaceae. C. limon fruit juice (lemon juice) has traditionally been used as a remedy for scurvy before the discovery of vitamin C. include treatment of high blood pressure, the common cold, and irregular menstruation. The Valuable scientific publications focus on the ever wider pharmacological actions of *C. limon* fruit extract include studies of antibacterial, antifungal, anti-inflammatory, anticancer, hepato regenerating and cardio protective activities [7].

Phenols and polyphenols including flavonoids in Citrus fruits have been reported to exhibit a wide range of biological activities and their effects are mainly attributed to antioxidant properties that prevent free radical mediated cytotoxicity, lipid peroxidation, and oxidation of low-density lipoproteins. Thus, extracts from these fruits could prove to be beneficial for the prevention and treatment of many neurodegenerative disorders [8]. In the present work, we intend to evaluate the neuroprotective property of Citrus pulp powder in scopolamine induced Zebrafish model amnesia.

II. MATERIALS AND METHODS

The Citrus pulp powder was received as a gift sample from Mevive International Food Ingredients. M3, Mayflower Metropolis, Udayampalayam Road, Sowripalayam,

Coimbatore 641028, Tamilnadu, India.

Phytochemical investigation on CPP [9, 10]

The phytochemical investigation on citrus pulp powder was detected based on the procedure described in the C.K. Kokate and Trease & Evans.

Determination of flavonoid in CPP using thin layer chromatography (TLC) [11, 12]

Thin layer chromatography (TLC) was carried out for the presence of flavonoid content in the CPP. The TLC of CPP was performed on 10cm × 1.5 cm precoated aluminium backed silica gel plate GF_{254} plates Merck. CPP samples were spotted using thin capillary tubes on TLC plates. The spotted plate was placed in mobile phase of solvent system (Ethyl acetate: Formic acid: Glacial Acetic Acid: Water 100:11:11:26 v/v/v/v solvent). The plate was placed in a development chamber with a trial solvent. The solvent front was allowed to travel until about 1 cm from bottom to the top end. The derivatization was done by using 1% Ethanolic aluminum chloride solution spraying method. The components were visualized using UV lamp fluorescent at 254 nm. Different spots were observed and its corresponding Rf values were calculated.

Use of Zebrafish [13]

Zebrafish (*Danio rerio*) (1 – 1.2 g) 3 - 4 month old were obtained from an authorized commercial supplier, Vijayapura (Pet Product). Zebrafish were randomly divided into groups of 15/10 L tank with a constant 14:10 h of the light/dark and fed twice daily with commercial flake food. The water parameters such as temperature (26 ± 2°C), pH. (6.0 – 8.0), conductivity (8.2 ± 0.2), and cleaning of the recirculation system will be monitored daily. All fish used in this experiment were observed in quarantine for one week before the conduct of study. Experiments were performed after obtaining Institutional Animal Ethics Committee (IAEC) approval. (BLDEACOP/IAEC/2020/04).

Administration of drug [14]

In the present study, zebrafish was randomly divided into the normal Control, Scopolamine control (100 µM/L), Standard and three different doses of the CPP (50, 100, and 200 mg/L) groups containing 7 zebrafish. The three different doses of CPP were administered by immersion to zebrafish (*Danio rerio*) into 500 ml glass beaker for 1hour. Whereas Scopolamine and Imipramine was administered by immersion into 500 ml glass beaker for 30 min.

Acute oral toxicity study [15]

Acute oral toxicity of CPP was tested in the Zebrafish (*Danio rerio*) as per the OECD guidelines 203 (modified, adopted June 18, 2019). The different concentrations (12.5, 25, 50, 100, 200, 400 and 800 mg/L) of CPP were selected for performing the oral toxicity in Zebrafish. 7 healthy fishes were selected to each concentration and transfer to beakers containing different concentration of CPP solution. The fish were observed after 24, 48, 72, and 96 hours. The mortalities were recorded for every 24 hours and fish were considered dead if there is no visible movement and upon mechanical stimulation could produce no reaction [16]. LC50 was determined based on the concentration of the CPP which killed 50% of fish. Experiments were performed after obtaining Institutional Animal Ethics Committee (IAEC) approval. (BLDEACOP/IAEC/2020/04).

Experimental design

Evaluation of Scopolamine induced amnesia activity

Novel Tank Test (NTT) [5]

The animals were divided into different groups.

Group I : Normal Control

Group II	:	Scopolamine control
Group III	:	Standard, Imipramine 20mg/L
Group IV	:	Received CPP 50 mg/L
Group V	:	Received CPP 100 mg/L
Group VI	:	Received CPP 200 mg/L

Group I served as normal control received water as vehicle, group II served as scopolamine control administered with scopolamine (100 μM/L), group III served as standard received Imipramine (20 mg/L), groups IV, V and VI received different doses of CPP. The different doses of CPP and standard drug was administered individually by immersion to Zebrafish through transferring into 500 ml beaker for 1 hr. once daily for 8 days, whereas scopolamine treatment was given individually by transferring into a 500 ml beaker 30 min before behavioral tests. The behavioral parameters were recorded for 5 min using a mobile camera and analyzed manually.

The behavioral parameters observed were

- ✓ Number of entries to the top
- ✓ Number of entries to the bottom
- ✓ Time spent in top (s)
- ✓ Time spent in bottom(s)
- ✓ Total distance travel in tank (m)
- ✓ Latency to enter the top (s)

Y Maze Test [5]

Once after the completion of assessment of behavioral parameters in Novel Tank Test (NTT) the same fishes on 9^{th} day used for measuring the willingness of Zebrafish to

explore new environments in Y Maze. Spatial memory and the response to novelty in Zebrafish were assessed using the Y- Maze task. The task consisted of two trials to test the response to novelty and spatial memory, separated by 1 hr. between them. During the first trial (training, 5 min), 1 hr. after drug treatment, just two arms of the Y-maze (the start and the other arm) could be explored, while the third arm (the novel arm) was obstructed. For the second trial, each fish was individually introduced in the start arm and had free access to all three arms for 5 min to assess the response to novelty. The time spent in each arm, number of entries to new arm and total distance travelled were evaluated in this test.

Biochemical Estimations [17]

All Zebrafish were euthanized by immersion in ice water of 2 - 4 o C until loss of opercular motions, and their whole brains were isolated for a biochemical parameters assay. The brains were gently homogenized in ice 0.1 M potassium phosphate buffer (pH 7.4), 1.15% KCl with homogenizer. The resulted homogenate was centrifuged at 2000 rpm for 15 min. The supernatant was used for the estimation of acetylcholinesterase (AChE), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) level.

Statistical Analysis

The results were expressed in mean ± SEM. The data obtained from the above study subjected for One way ANOVA followed by Tukey's Kremer Multiple Comparison Test by using Prism Pad 5 software. The $p < 0.05$ was found statistically significant.

III. RESULTS

Preliminary phytochemical screening of Citrus pulp powder

The preliminary phytochemical evaluation of citrus pulp powder extract showed the presence of alkaloids, flavonoids, sterols, tannins.

Determination flavonoid content by TLC

TLC of CPP was carried out for the determination of flavonoid content. The results obtained are tabulated in Table 1.

Table 1 Flavonoid detection by TLC

No. Spot	Rf value	Wave length	Before derivatization	After derivatization
01.	0.78 (A)	264 nm	Whitish	Yellow light
02.	0.82 (B)	264 nm	Whitish	Yellow light

Acute toxicity study

The acute oral toxicity of the CPP was carried out on Zebrafish. In our study the CPP showed mortality in 100, 200, 400 and 800 mg/L. Medial lethal concentration (LC 50) is considered as the most accepted basis to determine the acute toxicity. The LC 50 value with 100 % confidence intervals of different concentrations of CPP were 12.5, 25 and 50 mg/L. for 24, 48, 72 and 96 hours. The CPP was found to be 100% lethal on 1st day of experiment within 24 hours at the dose of 800 mg/L since it killed all the seven fishes in the experiment. Whereas the mortality rate of the Zebrafish found to be 14.28, 28.57 and 71.42 % in acute toxicity study of CPP at doses of 100 mg/L, 200 mg/L and 400 mg/L respectively.

The LC 50 value of the CPP was found to be 312.5 mg/L which was calculated on concentration dependent mortality during 24, 48, 72, and 96 hours of exposure to CPP. The graphical representation was showed in (Figure- 1) Based on the LC 50 value the different doses such as 50, 100 and 200 mg/L were selected for the study. Results are presented in (Table - 2).

Effect of CPP in Novel Tank Test (NTT)

In novel tank test, the different doses of CPP (50, 100 and 200 mg/L) showed dose dependent increase in the number of entries to the top, time spent in the top, latency to enter the top and total distance travelled whereas decrease in number of entries to bottom and time spent in bottom as compared to scopolamine control group. The results are presented in (Table - 3).

Effect of CPP on spatial memory and novelty response in Y-Maze

In Y-Maze test the CPP at doses such as 50, 100 and 200 mg/L exhibited dose dependent significant increase in number entries to new arm and total distance travelled in the maze as compared to scopolamine control group. The CPP at different dose showed significant increase in time spent in novel arm compared to scopolamine control. The results are presented in (Table - 4).

Effect of CPP on CAT, SOD, MDA and AChE levels in Zebrafish brain homogenate

The different doses of the CPP showed significant increase in the CAT and SOD and significant decrease in AChE and MDA content in brain homogenate of Zebrafish as compared to scopolamine control group. Results are tabulated in (Table - 5).

Table 2: Effect of CPP on oral toxicity in Zebrafish

Concentration (mg/L)	% of Mortality	LC50
12.5	0	
25	0	
50	0	
100	14.28	312.5 mg/L
200	28.57	
400	71.42	
800	100	

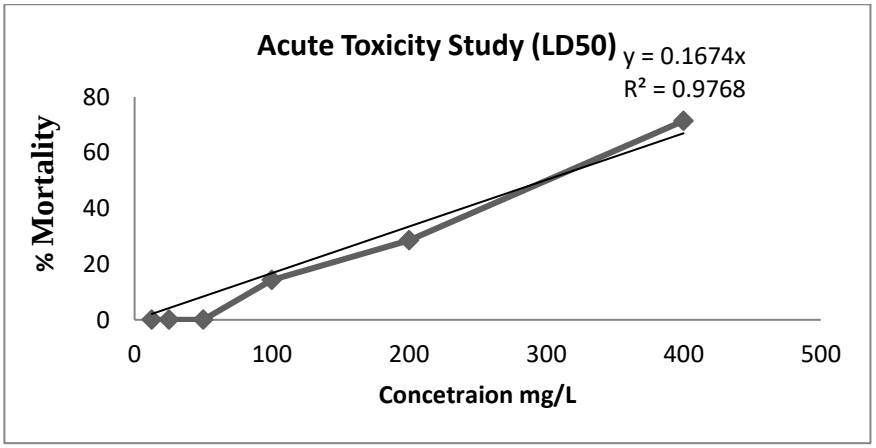

Figure 1: Graph showing the effect of CPP on oral toxicity in Zebrafish

Table 3: Effect of CPP in NTT

	Groups	Number of entries to the top	Number of entries to the bottom	Time spent in top (s)	Time spend in bottom (s)	Total distance travel in tank (m)	Latency to enter the top (s)
I	Normal control	66.2 ± 3.05	55.0 ± 5.10	182.5 ± 5.89	141.3 ± 5.82	36.6± 3.05	39.5 ± 4.55
II	Scopolamine control (100 µM/L)	34.3 ± 2.08$^{@}$	71.3 ± 4.05$^{@}$	122.5 ± 5.86$^{@}$	203.8 ± 6.51$^{@}$	15.5± 2.88$^{@}$	22.0 ± 1.89$^{@}$
III	Standard Imipramine (20 mg/L)	58.6 ± 3.52***	49.1 ± 3.76***	179.5 ± 5.51***	119.8 ± 8.77***	33.6± 2.96***	30.0 ± 2.89***
IV	CPP (50mg/L)	29.3 ± 2.18*	66.5 ± 2.08*	130.1 ± 7.01*	155.5 ± 7.31**	8.3 ± 2.02*	17.2 ± 3.44*
V	CPP (100mg/L)	42.8 ± 3.51**	54.3 ± 2.13**	147.8 ± 4.44**	134.8 ± 5.39**	20.3± 2.33**	23.5 ± 3.13**
VI	CPP (200mg/L)	54.6 ± 3.71***	45.4 ± 5.12***	176.8 ± 6.78***	120.3 ± 6.61***	26.3± 2.03***	26.2 ± 3.75***

Values are expressed as mean ± SEM, n=7, $^{@}p< 0.001$ as compared to normal control group, and ***$p< 0.001$, **$p< 0.01$, *$p< 0.05$ as compared to scopolamine control group

Table 4: Effect of CPP on spatial memory and novelty response in Y-Maze

	Groups	Number of entries in novel arm	Total distance travel (m)	Time spent in novel arm
I	Normal control	33.08± 2.91	48.75 ± 2.21	165.00 ± 5.01
II	Scopolamine control (100 µM/L)	25.75 ± 4.65$^{@}$	24.25 ± 1.65$^{@}$	81.50 ± 3.52$^{@}$
III	Standard Donepezil (10 mg/L)	30.75 ± 2.75***	45.61 ±1.25***	163.01 ± 6.05***
IV	CPP (50 mg/L)	22.50 ± 2.54*	29.06 ± 0.97*	116.22 ± 4.04**
V	CPP (100 mg/L)	27.25 ± 2.90**	31.75 ± 1.96**	136.31 ± 4.02**
VI	CPP (200 mg/L)	31.75 ± 2.59***	42.55 ±2.64***	154.50 ± 5.50***

Values are expressed as mean ± SEM, n=7, $^{@}p< 0.001$ as compared to normal control group, and ***$p< 0.001$, **$p< 0.01$, *$p< 0.05$ as compared to scopolamine control group.

Table 5: Effect of CPP on biochemical parameters

Groups	AChE μg/mg of protein	CAT U/mg of protein	MDA nmol/mg of protein	SOD U/mg of protein
Normal Control	0.13 ± 0.02	0.48± 0.03	0.54 ± 0.04	4.45 ± 0.45
Scopolamine Control (100 μM/L)	0.57 ± 0.05$^{@}$	0.15 ± 0.01$^{@}$	1.50 ± 0.10$^{@}$	1.39 ± 0.26$^{@}$
Standard Donepezil (10 mg/L)	0.15 ± 0.01***	0.44 ± 0.03***	0.51 ± 0.04***	4.21 ± 0.40***
CPP (50 mg/L)	0.34 ± 0.02**	0.10 ± 0.02*	1.17 ± 0.06*	2.15 ± 0.20**
CPP (100 mg/L)	0.23 ± 0.01**	0.28 ± 0.03**	0.99 ± 0.09**	2.77 ± 0.44**
CPP (200 mg/L)	0.15 ± 0.01***	0.36 ± 0.03***	0.55 ± 0.06***	4.13 ± 0.21***

Values are expressed as mean ± SEM, n=7, $^{@}p< 0.001$ as compared to normal control group, and ***$p< 0.001$, **$p< 0.01$, *$p< 0.05$ as compared to scopolamine control group.

IV. DISCUSSION

In the present research work the neuroprotective activity of Citrus pulp powder was carried out using different doses (50, 100 and 200 mg/L) in scopolamine induced Zebrafish model amnesia.

Treatment of scopolamine at 100 μM in group II Zebrafish demonstrated anxiety which was evident by a significant decrease in time spent in the top zone of the tank, entrees to top zone, latency to enter the top and total distance travelled as compared to group I Zebrafish served as normal control in NTT diving test. In our study the pretreatment of CPP in Zebrafish significantly reversed (Increase in the number of entries to the top, time spent in the top, latency to enter the top and total distance travelled NTT diving test) the scopolamine mediated effects in a dose depended fashion.

In Y maze model, the Zebrafish immersed with scopolamine at a dose of 100 μM exhibited significant decrease in number entries to novel arm and total distance travelled in the maze as compared to normal control group. In contrast, scopolamine-treated fish subjected to pretreatment with CPP demonstrated a significant increase in number entries to novel arm and total distance travelled in the Y maze. The effect of CPP at higher dose in novel tank test found to be comparable to the results of the standard drug Donepezil.

Acetyl-cholinesterase is an enzyme known to play a significant role in hydrolysis of Acetyl choline, a crucial cholinergic neurotransmitter [18]. Scopolamine administered Zebrafish exhibited a significant increase in the AChE activity when compared to the normal control group. CPP treated Zebrafish showed a significant decrease in the AChE activity when compared to Scopolamine alone treated Zebrafish and this could be correlated to the improvement of memory parameters, as evidenced in the behavioral approaches.

Scopolamine induced anxiety and amnesia are closely related to increased oxidative stress in the Zebrafish brain. Scopolamine administered Zebrafish clearly showed suppressed antioxidant enzymes SOD and CAT specific activity in the brain along with increased level of lipid peroxidation (MDA) when compared to the normal control group. Alternatively, CPP treatment inhibited in a dose-dependent manner, scopolamine -induced oxidative stress in Zebrafish by enhancing the antioxidant enzyme activity and suppressing the lipid peroxidation levels when compared to scopolamine treated animals.

Many studies have shown that citrus fruits exhibit neuroprotective effect [19]. The citrus flavonoids have been investigated for their effects on cognitive functions in different animal models and improved spatial memory impairments, lipid peroxidation and oxidative stress [20].

Previous studies suggest that administration of flavonoid as dietary supplement have beneficial effects on blood flow to the brain, hence improves learning and memory. Results of present study also reveal favorable effect on memory therefore it may be suggested that flavonoids abundantly present in *Citrus limon* may be responsible for the memory boosting effect of citrus pulp powder.

V. CONCLUSION

The results of our study conclude that the CPP could effectively improve memory impairments in a scopolamine induced Zebrafish model of amnesia by enhancing the function of the behavioral response and antioxidant enzymes in the amnesic Zebrafish model.

Further scope

The findings that were obtained illustrate the possible health benefits from CPP being investigated and indicate its possible use in formulating new medicines for the amelioration of dementia.

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AUTHORS

First author - Sandeep Chandakavate, B.Pharm. BLDEA's SSM College of Pharmacy and Research Centre Vijayapura. schandakavate61@gmail.com M.Pharm, Ph.D. BLDEA's SSM College of Pharmacy and Research Centre Vijayapura.

Second author - Dr.Shivakumar H. M.Pharm, Ph.D. BLDEA's SSM College of Pharmacy and Research Centre Vijayapura. shivakumarhugar@yahoo.com

Third authors - and Dr.V.P.Patil, M.Pharm,Ph.D. BLDEA's SSM College of Pharmacy and Research Centre Vijayapura. patilk183@gmail.com

Corresponding author: Dr.nanjappaiah H, M.Pharm, Ph.D. BLDEA's SSM College of Pharmacy and Research Centre Vijayapura. nanjupharma143@gmail.com