JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2024/34995

RESEARCH ARTICLE

Preclinical Assessment of *Crocus sativus* Extract Loaded Nano Emulsion for Scopolamine Induced Cognitive Impairment: A Comprehensive Safety and Efficacy Study

Namrata Parikh and Komal Patel*

Pharmacognosy Department, Parul Institute of Pharmacy, Parul University, Vadodara – 391760, Gujarat, India; komalnishit@gmail.com

Abstract

Background: *Crocus sativus* is a popular herb used to treat and enhance memory and cognition related brain function and thereby impose a significant role in preserving brain health. The medicinal important part of *Crocus sativus* is the stigma of the flower, rich in apocarotenoids, crocin, picrocrocin, safranal and many more volatile compounds which have antioxidant, anti-amyloidogenic, anti-depressant, anti-cancer, anti-inflammatory properties. The poor stability, solubility and enzymatic degradation of bioactives have substantially limited their therapeutic application. In this study, a Saffron nanoemulsion (SNE) of carotenoid-enriched *Crocus sativus* extract (CSE) was assessed for its safety and efficacy. **Methodology:** In accordance with OECD guidelines, the single dose acute and sub-acute repeated 28 days oral toxicity of SNE was performed to determine the lethal dose (LD50) and effective dose for the preclinical evaluation. The animals were observed during the entire toxicity experimentation for any toxic signs and changes in hematological, and biochemistry parameters. The efficacy study of SNE was evaluated by two behavioural test models- Passive avoidance test and Morris water maze test using scopolamine induced memory impairment. **Results and Conclusions:** Animals administered 500 mg/kg/day a single dose orally did not cause any signs of toxicity or mortality in rats and results of acute toxicity assessment of SNE have indicated that the NOAEL (No Observed Adverse Effective Level) of SNE was found to be 500 mg/kg. In sub acute toxicity study three different doses of 75, 100 and 125 mg/kg/day for 28 days administered orally in animals revealed no significant changes in body weight, hematological, or biochemical parameters. The histopathological study has indicated no pathological changes observed in the vital organs of rats treated with the 125 mg/kg/day. The efficacy assessment of SNE using two behavioural models has suggested that nanoemulsion at the dose of 10 mg/kg/day significantly reduced the Scopolamine induced memory impairment.

Keywords: Morris Water Maze Apparatus, NOAEL, Passive Avoidance Test, Pharmacodynamic, Saffron Nanoemulsion, Scopolamine

1. Introduction

Brain health is a rising concept that encloses neural development, consistency, functioning and recovery across the life course. Good brain health is a state in which every person can recognize their potential and boost their cognitive, emotional, psychological and behavioural functioning to address life situations[1]. Healthy dietary habits, regular exercise, keeping the brain active, social connections and enough sleep augment brain health[2,3]. Threatening factors for brain health are mental or emotional trauma, stressful lifestyle, disturbed sleep, unhealthy dietary habits, smoking and alcohol. Certain health conditions like diabetes, hypertension, stress, cardiovascular diseases, sleep problems, and vitamin and mineral

*Author for correspondence

Article Received on: 09.09.2023 Revised on: 15.12.2023 Accepted on: 20.12.2023

deficiency negatively affect brain health[4,5]. Impaired brain health invites a spectrum of neurological conditions like headache, multiple sclerosis, autism, dementia, Alzheimer's, Parkinson's disease, neuro-inflammation, epilepsy, cerebral palsy, brain tumors and many more cerebrovascular diseases[6,7]. The WHO predicts worldwide, around 50 million people have cognitive problems, with almost 60% living in low- and middle-income countries and the number of people will almost double every 20 years, i.e., 42.3 million in 2020 and 81.1 million in 2040. Brain health starts worsening at an earlier age around 40's. There is no treatment currently available to stop or modify its progressive course. Quite a few new treatments are being scrutinized in various stages of clinical trials. However herbal remedies are called for continuing health and wellbeing and preventing such types of long-standing disorders[8]. Many traditional herbs having anti-amyloidogenic, anti-oxidant activity such as green tea, turmeric, Ginseng, saffron, *Shunkpushpi*, etc. are a potential source of bioactive compounds, particularly phenolic compounds and well reported for improving brain health[9]. One of the widely approachable herbs *C. sativus* has wider medicinal properties from ancient times due to health-promoting perspectives. The findings from recent research on bioactive constituents present in *C. sativus* such as apocarotenoids, mono terpenoids, flavonoids, volatile compounds, phenolic acids, etc. have demonstrated that the therapeutic application of herb is due to the chemical properties of phytoconstituents. Amongst them the amphophilic nature of Crocin a carotenoid present in saffron has a prominent impact on maintaining brain health by protecting brain cells against oxidative stress, arresting the amyloid formation, neurotoxicity, etc[10]. Crocin interrelates with Aβ peptide and tau protein and arrest the amyloid formation and neurotoxicity[11].

C. sativus is well-reported for improving memory and related functions. Its inhibitory action on AchE and antioxidant potential have been suggested for its pivotal role in memory improvement. Aβ peptide brings neuronal loss and cognitive impairment by reduction of cell viability, induced reactive oxygen species generation and apoptosis[12]. Crocin along with crocetin binds with the phencyclidine binding site of the NMDA receptor that elevates glutamate levels in the brain and thus enhances the neuro-excitation by increasing the production of glutamate which improves learning and memory[13,14]. Still, more detailed research on the mechanism of saffron and its constituents for improving various memory-related functions is under exploration. Crocin is poorly bioavailable at the site of action when taken orally as it eliminates fast through the systemic circulation[15].

To overcome these issues the carotenoid-rich fraction of Saffron extract is encapsulated in the form of nanoemulsion. The benefits of nanoemulsion over the conventional dosage forms are stability towards the chemical, physical and enzymatic degradation and effective delivery of bio-actives. There is a wide difference between the biological interaction of bioactive compounds and novel drug delivery systems. The excipients used for formulating the nanoemulsion produce a very small droplet size which may influence their biological action by altering the solubility, stability, absorption of encapsulated bioactive compounds, etc[16]. It is always beneficial in terms of increasing bioavailability. To establish the safety the developed SNE is subjected to single dose and repeated dose toxicity to identify any signs of dose-related toxicity.

The efficacy of SNE was performed by two behavioural models: Morris water maze apparatus and a passive avoidance test, where memory was impaired by scopolamine- an anticholinergic agent. Morris water maze apparatus is long established model in behavioral neuroscience to investigate the cognitive processes associated with memory and spatial learning. Conceptually it is a useful tool to study the potential impact of novel therapeutic drugs, imbalance of the nervous system etc. using training protocol[17,18]. Passive avoidance helps to exhibit procedural memory by identifying the neuro-modulatory behavioural interaction in brain regions[19]. It evaluates both short-term and long-term memory.

2. Materials and Methods

2.1 Test Substance

The SNE: 83% loading of standardized CSE (dry hydro-alcoholic extract which is prepared from the stigma of *C. sativus* plant, cultivated in India) consists of 67% v/v external phase, 21 % surfactant and 13% internal phase and 94.34 nm globule size. The % total content present in nanoemulsion was identified as 25% with regards to

total crocin, crocetin and picrocrocin by the HPLC-PDA method[20]. To identify any toxic signs related to excipients used in the developed nanoemulsion formulation, a placebo nanoemulsion was prepared and administered without CSE.

2.2 Animal Protocol and Standard Care

The study was carried out on healthy nulliparous and non-pregnant female Wistar rats of age between 6-8 weeks. Protocol No. PIPH05/20 and PIPH06/20 were approved by the Institutional Animal Ethics Committee of the Parul Institute of Pharmacy for toxicity study and efficacy study respectively. The animals were acclimatized for one week to laboratory conditions. The animals were housed as per standard guidelines. The room temperature was maintained at 22°C ± 3°C with NLT 30% humidity and 12 hours of light/dark cycle. The air conditioner provided the controlled temperature and humidity conditions during experiments and was effectively monitored. Animals were randomly allocated to respective groups as per protocol for toxicity and efficacy study. A standard pellet diet and water were provided ad libitum throughout the experiment period (Figure 1).

2.3 Acute Toxicity Study[21]

The acute toxicity study was performed by OECD guideline 420. Individual rats were weighed and categorized into 2 Groups A and B having 6 animals per group. Group A animals were treated as control and Group B rats were

Figure 1. Test substance-Saffron Loaded w/o nano emulsion.

administered a single oral dose of 500 mg/kg body weight of SNE. All rats were observed for 4 hours after dosing, and then daily for 14 days for any clinical signs of toxicity, morbidity and mortality, change in body weight or any other symptoms. On day 15 after completion, animals were euthanized and major vital organs were isolated, weighed and subjected to histopathology study. The detailed study is shown in Figure 2.

2.4 Sub-acute Toxicity Study[21]

The sub-acute toxicity was performed by OECD guideline 407. Animals were divided into six groups and each respective group received oral doses as mentioned in Figure 3. The additional satellite group as the sixth group was kept with a dose of 125 mg/kg/day and was observed for more than 14 days after 28 days without treatment for the recovery period. Body weight was

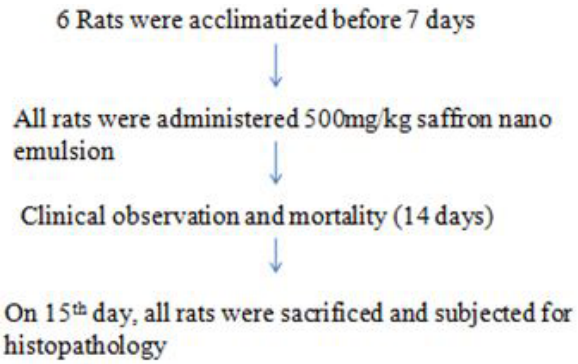

Figure 2. Flow chart of acute toxicity study.

monitored weekly. All rats were observed for any signs of abnormalities during the experiment. The animals of Groups I to V were subjected to haematological, biochemical and histo-pathological measurements after overnight fasting.

2.4.1 Clinical Observation

Clinical observations such as skin, body movements, eyes, mucous membrane changes, breathing patterns etc. were tracked and the body weight of all animals was recorded on 0, 7, 14, 21, and 28 days after the administration of the test substance. During experimentation, the rats were separated from their cages to identify any morbidity or mortality.

2.4.2 Hematological Parameters and Serum Chemistry

The haematological examination was performed for each group of the animals for haemoglobin, erythrocyte count, platelet count, leukocyte count, thromboplastin time, etc. to determine any changes in blood cells or any abnormalities.

Further estimation of total cholesterol, triglyceride, HDL, VLDL serum LDL/HDL ratio. For Liver function tests, serum bilirubin, ALP, and protein content were determined and serum sodium, serum urea, and serum creatinine levels were recorded for normal functioning of the kidney.

2.4.3 Organ Weight and Histopathological Evaluation

After the 28-day treatment period, all animals in the treatment groups were euthanized and separated vital organs of them subjected to observation of any signs of toxicity. The major vital organs such as the brain, heart, kidney, liver, lung, pancreas and spleen were collected, weighed wet organs after properly removing the adhered fats. The relative organ weight was calculated by dividing the organ weight by body weight. After that, the organs were stored in a neutral buffered 10% formalin solution till the scheduled detailed histopathology study.

2.5 Statistical Analysis

The result data were represented as mean value ± standard deviation. All the results for each experiment were compared to the control group. Data were statistically analyzed using GraphPad Prism 5 software for Windows. The statistical method was used to analyze the body weight, relative organ weight, haematological parameters and biochemical parameters. Statistical significance was considered at P-value < 0.05.

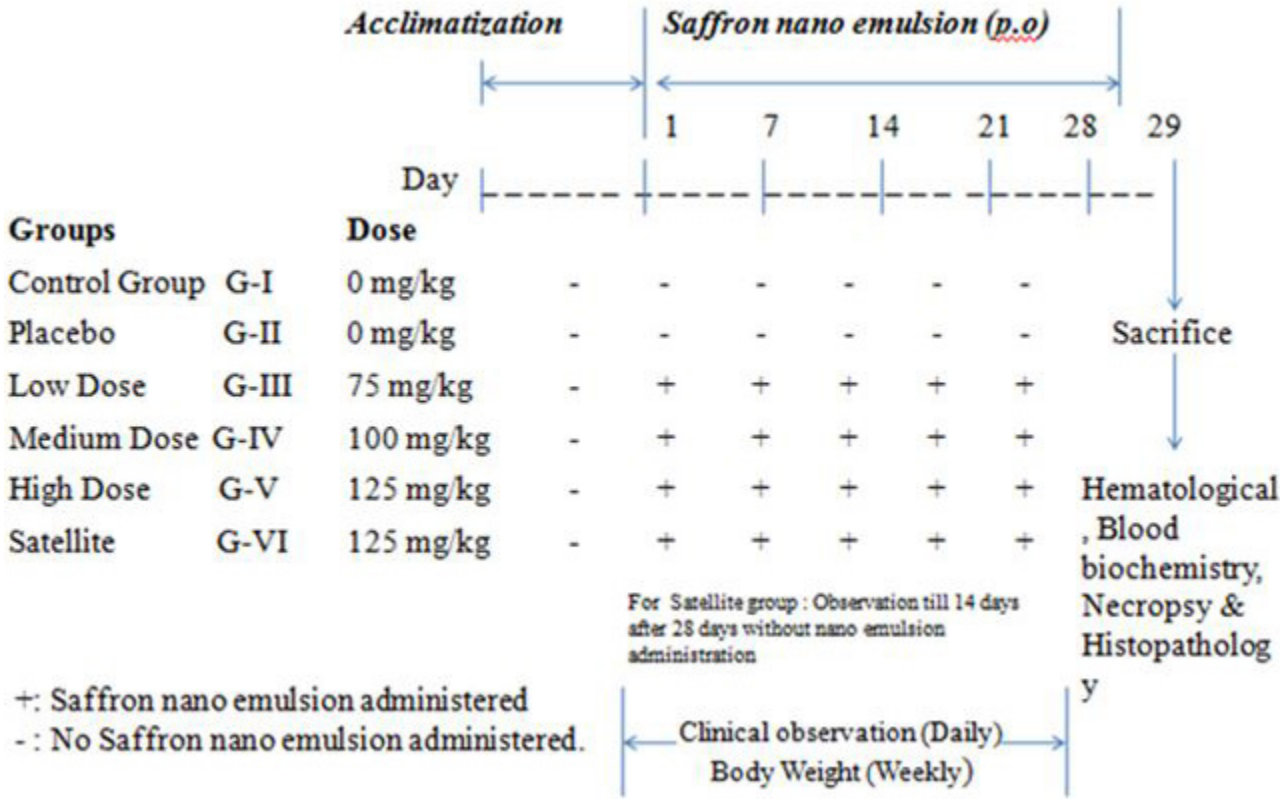

Figure 3. Flow chart of sub acute toxicity study.

2.6 *In Vivo* Efficacy Study

The influence of SNE on learning and memory on scopolamine-induced memory impairment was evaluated by, the Morris water maze test and passive avoidance tests in rats.

Animals were grouped as six animals in each group, and treated with respective doses as mentioned in Table 1. Scopolamine was injected 30 mins before administration of Rivastigmine and nanoemulsion to Groups III and IV.

2.6.1 Morris Water Maze[18,22]

The water maze test was performed using the previously described Morris method[23]. MWM apparatus was divided into four quadrants N, S, E and W. Animals were trained and allowed to swim at the start position. The learning trials were conducted over 5 days with 4 trials per day specifying the spatial learning memory. At the end of the learning last acquisition trial (day 6), a probe trial was given in the absence of a platform and determine the swimming pattern of animals at the target location as indicative of reference memory (Figure 4). The swimming patterns of animals were recorded with the help of the video tracking system of All Maze software.

2.6.2 Passive Avoidance Test in SD Rats[24-26]

Animals were grouped as shown in the water maze test with different ones.

The learning ability of animals to avoid an anxious event is studied by using this behavioural model. The model consists of two compartments light and dark having a grid floor with electrically connected to the sliding door. The total study time duration was six days. All animals were trained before starting the procedure.

Transfer latency from light to dark compartment is noted for all groups of animals and the time for each of them (Figure 5). For statistical analysis, GraphPad Prism software was used.

Table 1. Efficacy study schedule

Study	Groups	Animals	Dose
Morris Water Maze Model	Group I	6	Saline, p.o
	Group II	6	Scopolamine (2 mg/kg), p.o.
	Group III	6	Rivastigmine (2 mg/kg), p.o.
	Group IV	6	SNE 10 mg/kg
Passive Avoidance Test	Group I	6	Saline, p.o
	Group II	6	Scopolamine (2 mg/kg), p.o.
	Group III	6	Rivastigmine (2 mg/kg), p.o.
	Group IV	6	SNE 10 mg/kg

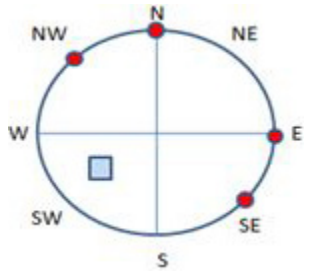

Figure 4. Morrise water maze apparatus.

3. Results

3.1 Acute toxicity

3.1.1 Total Death and Toxic Syndromes

All animals survived during the entire experiment. Administration of SNE at the dose of 500 mg/kg did not cause any toxic effects in rats during the 14-day observation. There was no anomalous behaviour during the first 30 min after dosing and periodically for the first 24 hours and daily up to 14 days. The data of observation of any clinical sign was provided in Table 2.

3.1.2 Body Weight

The measurement of body weight is one of the crucial parameters for predicting the general health condition of animal experiments. A decrease in body weight is an indicator of adverse effects. No significant difference in body weight was observed in the treatment group

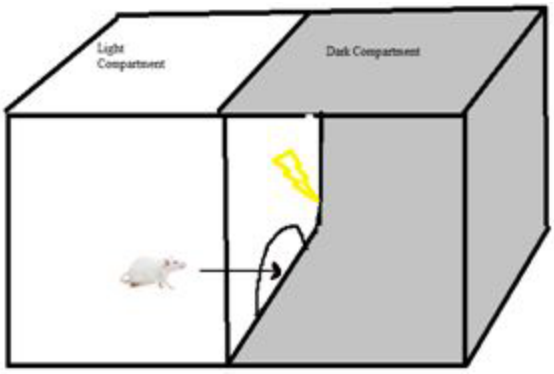

Figure 5. Passive avoidance test apparatus.

compared to in control group. Daily food and water consumption were found to be normal in both the control and treatment groups. The result reflects that SNE does not affect the normal growth of rats. The body weight measurement is shown in Table 3 and Figure 6.

3.1.3 Organ Weight and Histopathological Evaluation

The data of organ weight are expressed as a relative organ weight as shown in Table 4. No significant difference was observed between the control group and the treated group. Histopathological examination revealed normal histopathology of organs. No signs of any morphological disorders like inflammation, necrosis, steatosis, or fibrosis were observed in any of the groups. The images of histopathological observation are shown in Figure 6.

3.2 Sub-Acute Toxicity

3.2.1 Clinical Signs and Mortality

All animals of each group were sustained till the 28th day of repeated dose toxicity study and also satellite group animals survived for more than 14 days after 28 days. The physical and behavioural assessment did not reveal any signs of unwanted effects in any of the groups receiving 75, 100 and 125 mg/kg/day of SNE.

3.2.2 Body Weight

Changes in body weight were measured every week during experimentation. There were no significant changes observed in body weight. The graphical presentation of the body weight is shown in Figure 7.

3.2.3 Food and Water Consumption

As compared to the normal control group, no significant changes such as feed and water consumption were observed in the treatment control group.

3.2.4 Measurement of Blood Parameters

Clinical chemistry or blood biochemical parameters, and haematological parameters of rats exposed to SNE at the dose of 75, 100 and 125 mg/kg were measured. The data was collected and shown in Table 5 and Figure 8. No significant differences were observed for any parameter in the treatment group with the control group.

3.2.5 Organ Weight

At the end of 28 days of repeated dose toxicity study, the rats of each group of animals were sacrificed and weighed the vital organs. The relative organ weights of rats are shown in Table 5. No significant difference in the relative weight of the heart, liver, kidney, brain, lung, spleen and pancreas in comparison to the control group was observed.

Table 2. Clinical signs and mortality of rats in the control group treated with 500 mg/kg SNE for 14 days

Group	Dose (mg/kg)	Total number of rats	Clinical signs	Period (in days)	Mortality
Control	0	6	No undesirable clinical signs were observed	14	0/6
Treatment	500	6	No undesirable clinical signs were observed	14	0/6

Table 3. Body weight measurement of animals of the control group and rats exposed to 500 mg/kg SNE acutely

Groups	Number of Rats	Average body weight (kg)			
		Day 0	Day 1	Day 7	Day 14
Group A- Control	06	0.25 ± 0.32	0.28 ± 0.15	0.24 ± 0.21	0.29 ± 0.17
Group B- SNE	06	0.29 ± 0.28	0.24 ± 0.08	0.30 ± 0.22	0.32 ± 0.31

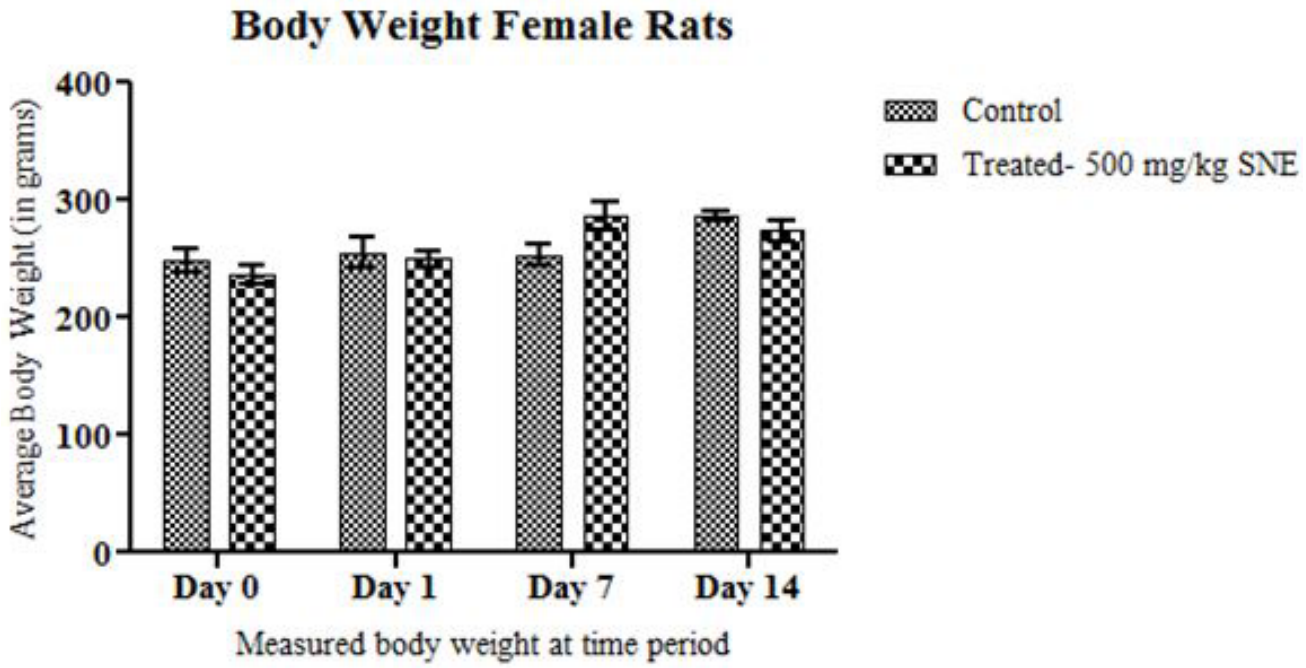

Figure 6. Graphical presentation of body weight measurement of control group rats and saffron nano emulsion treated groups.

Table 4. Average % of relative organ weight of rats exposed to SNE acutely for 14 days and sub-acutely for 28 days

Groups	Number	Average % of relative organ weight of animals (g)						
		Brain	Heart	Kidney	Lung	Liver	Pancreas	Spleen
Control	6	0.61±0.12	0.36±0.11	0.67±0.13	0.64±0.02	2.96±0.06	0.27±0.05	0.32±0.19
SNE- 500mg/kg	6	0.64±0.15	0.31±0.08	0.71±0.09	0.66±0.17	3.33±0.17	0.24±0.15	0.31±0.07

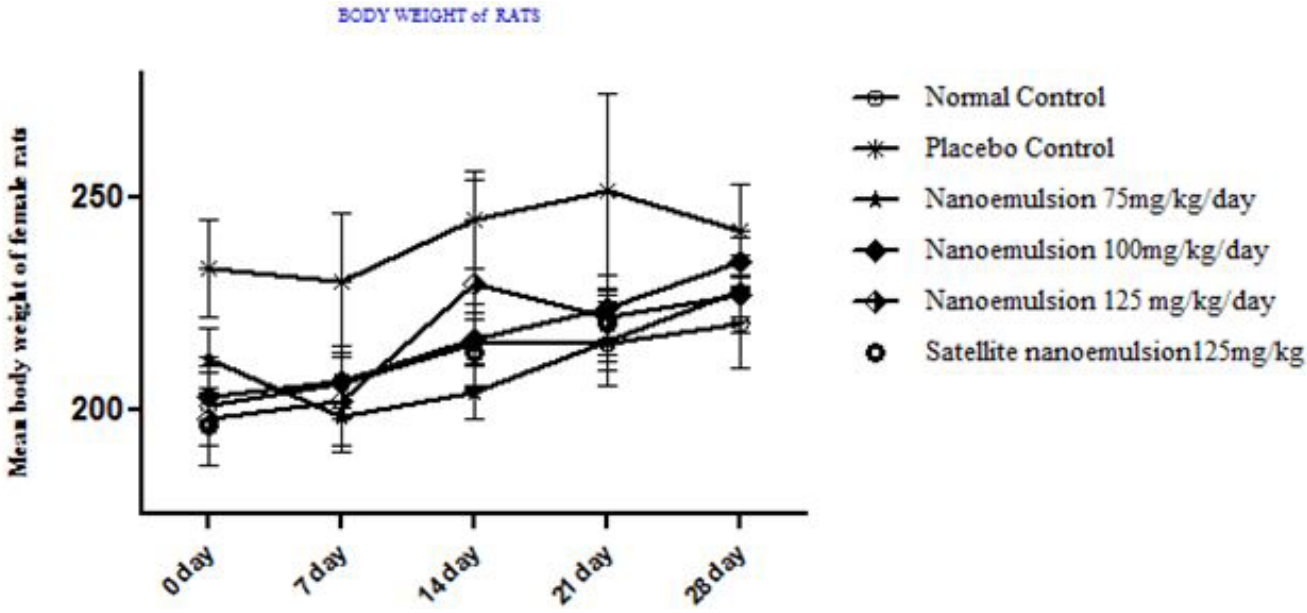

Figure 7. Body weight changes of animals.

Table 5. Average % of relative organ weight of rats exposed to SNE sub-acutely for 28 days for Groups I to V and 14 days more after 28 days for Group IV

Groups	Number	Average % of relative organ weight of animals (g)						
		Brain	Heart	Kidney	Lung	Liver	Pancreas	Spleen
G-I	6	0.63±0.41	0.34±0.19	0.61**±0.**53	0.68**±0.**12	2.67**±0.0**7	0.24**±0.**15	0.33**±0.**29
G-II	6	0.68**±0.15**	0.31±0.08	0.71±0.09	0.66±0.17	2.73±0.37	0.22±0.35	0.31±0.23
G-III	6	0.62**±0.44**	0.29±0.12	0.69±0.19	0.64±0.28	2.83±0.24	0.21±0.25	0.34±0.18
G-IV	6	0.63**±0.1**8	0.32±0.18	0.72±0.07	0.72±0.74	2.88±0.14	0.24±0.36	0.29±0.53
G-V	6	0.65±0.42	0.34±0.24	0.71±0.17	0.69±0.47	2.97±0.23	0.21±0.10	0.31±0.57
G-VI	6	0.66±0.32	0.32±0.15	0.72±0.87	0.71±0.84	2.91±0.74	0.25±0.19	0.29±0.18

3.2.6 Histopathology

Pathological examinations were carried out for the brain, heart, spleen, liver, lungs, kidney, and pancreas of rats. The results showed in Table 6 that administration of SNE at the dose of 125 mg/kg/day up to 28 days to rats has shown no adverse effects on histo-pathological observation and also it did not affect the feed and water consumption. In addition, the satellite group did not show any adverse reaction (Figure 9).

3.3 *In Vivo* Efficacy Study

3.3.1 Morris Water Maze Test

Memory of rats was impaired by scopolamine at the dose of 2 mg/kg. The escape latency time of each group was assessed. The time reached the platform on the 5th day of the experiment whereas time spent on the targeted quadrant without the platform in probe trial on the 6th day was determined to access the learning ability of rats. It was observed escape latency time was found lesser in SNE-treated animals compared to scopolamine-treated rats, saffron emulsion-treated animals spent more time in the targeted quadrant during the probe trial. The result indicated that the SNE improved the scopolamine-induced memory deficit as shown in Figures 10 and 11.

3.3.2 Passive Avoidance Test in SD Rats

In the passive avoidance test, the animals were permitted to explore both compartments without any foot shock during the acquisition trial. On the next day foot shock was given in one of the compartments and normal rats avoided entering the compartment where the previous shock was delivered noted as transfer latency. The animals of scopolamine administration showed decreased transfer latency ensuring memory impairment whereas it was improved in Rivastigmine-treated rats and treatment control of saffron nanoemulsion at the dose of 10 mg/kg as shown in Figure 12.

4. Discussion

Lifestyle, lack of exercise, work stress, sleep disturbances, and human ageing are the leading causes of worsening brain health. Neuronal loss in the brain continuously progresses in neuronal degeneration, in contrast to peripheral neurons, which can self-repair and regenerate. Despite great advancements in technology, modern science has not yet come up with the best remedies to enhance brain health. Brain health generally introduces the concept of brain maintenance, which denotes the process of preserving neurological condition, as well as learning and memory, which are two fundamental brain functions[27]. Preserving memory functioning may be accounted for brain reserve or cognitive reserve[28].

In the recent era of nanotechnology, verifying the safety of all new products is necessary for their therapeutic applications. The safety and toxicity of nanoemulsions depend on their interaction with biological networks. Nanoemulsions contain lipid nanoparticles, they also possess smaller droplet sizes, unique compositions, increased bioavailability, potential accumulation of bioactive molecules in the human cellular system as well interactions with other compounds, which may lead to potential toxic action and adverse effects on health[29-31]. The dose regimen of nanoemulsion should be selected based on the

Table 6. Hematological, biochemical and lipid profile of rats exposed to different doses of saffron nanoemulsion by oral routes

Test	Parameters	Unit	Control (n=6)	Placebo Control (n=6)	SNE-administered Rats (n=6)			
					Low Dose	Medium Dose	High Dose	Satellite
					75 mg/kg	100 mg/kg	125 mg/kg	125 mg/kg
Blood Chemistry	RBC	Millions/µL	6.86 ±0.65	6.21 ±0.57	8.01 ±0.81	7.10 ±0.31	8.53 ±0.17	8.68 ±0.83
	WBC	Number/ml	6.82 ±0.18	7.78 ±0.98	7.39 ±0.45	8.64 ±0.37	8.19 ±0.28	9.09 ±0.51
	Platelets	Number/ mm^3	6.86 ±0.65	6.21 ±0.57	16.4 ±0.527	16.4 ±0.527	16.4 ±0.527	16.4 ±0.527
	Haemoglobin	%	14.45 ±0.527	15.70 ±0.134	14.7 ±0.317	14.93 ±0.514	14.45 ±0.274	14.16 ±0.367
Lipid Profile	S. Cholesterol	mg/dl	146.4 ±0.11	145.5 ±0.16	142.5 ±0.53	148.6 ±0.32	144.8 ±0.74	151.7 ±0.37
	S. Triglyceride	mg/dl	124 ±0.23	125.3 ±0.35	131.5 ±0.29	133.5 ±0.37	136.8 ±0.42	81.33 ±0.69
	S. HDL	mg/dl	70.17±0.36	81.03 ±0.36	80.70±0.17	78.17 ±0.62	69.67 ±0.48	57.17 ±0.32
	S. LDL	mg/dl	51.68 ±0.67	53.2 ±0.47	57.0 ±0.67	51.17 ±0.82	51.83 ±0.39	17.17 ±0.24
	S. VLDL	mg/dl	24.10 ±0.39	17.42 ±0.38	19.83 ±0.38	17.17 ±0.64	17.17 ±0.73	16.4 ±0.527
Liver Function Test	S. Bilirubin	mg/dl	33.33 ±0.13	24.33 ±0.38	24.83 ±0.48	22.00 ±0.14	19.17±0.26	22.33 ±0.94
	S. Alkaline Phosphatase	IU/L	54.33 ±0.51	51.17 ±0.25	49.83 ±0.56	54.50 ±0.24	56.67±0.33	61.50±0.68
	Total Protein	gm/dl	82.83 ±0.32	64.67 ±0.36	85.67 ±0.24	82.67±0.57	80.50±0.48	76.67±0.37
	SGPT	Unit/liter	78.50 ±0.58	78.67 ±0.26	82.00 ±0.64	84.00±0.28	91.33±0.51	89.00±0.61
	SGOT	Unit/litre	156.8 ±0.53	163.3±0.93	142.8 ±0.31	156.5 ±0.96	174.8±0.63	178.3±0.16
Biochemical Parameters	S. Sodium	mEq/liter	147.0 ±0.31	147.2 ±0.43	132.5±0.89	148.3±0.41	155.5±0.68	157.7±0.16
	S. Calcium	10 x mg/dl	44.67 ±0.63	43.17 ±0.16	44.00±0.45	53.17±0.24	60.00±0.59	63.50±0.37
	S. Urea	mg/dl	7.983 ±0.18	8.400±0.29	8.000±0.25	6.517±0.17	5.467±0.43	6.967±0.68
	S. Creatinine	mg/dl	6.800±0.64	6.833±0.72	5.983±0.64	6.683±0.87	6.183±0.29	7.483±0.18

* Values are expressed as the mean ± SD (n=6)

LD50 for safe use. The present work has investigated the preclinical profiling of carotenoid-enriched SNE by conducting single-dose and repeated-dose toxicity studies, along with an efficacy study.

Many studies conducted on rats have indicated that saffron extract is safe up to a dosage of 2000 mg/kg/day. The LD50 value for the oral administration of an aqueous extract of *C. sativus* was found to be 4120 mg/kg[32]. In a more depth study, Hosseinzadeh *et al.* investigated the Crocin at the dose of 15-180 mg/kg by i.p. route and up to 3 gm p.o. did not alter haematological, or biochemical criteria in mice and rats and did not cause any damage to vital organs in the body[33]. With regards to pharmacological action, many types of research indicated that saffron has a remarkable impact on enhancing memory and thus improving brain health. Batarseh *et al.,* studied that Crocin at the dose of 10 mg/kg/day was able to Amyloid β load and tighten the blood-brain barrier[34]. Crocin at the dose of 15 mg/kg and 30 mg/kg attenuated scopolamine (0.2 mg/kg) induced memory impairment[35]. The Morris water maze apparatus is a widely used apparatus for accessing spatial and learning memory in rodents. This test depends on the rodent's natural repulsion to swimming in the water and needs to use visual cues to learn the location of the hidden platform. The performance of the Morris water maze test relies on hippocampal function as its lesions impair acquisition during hidden platform training trials and subsequent probe trial performance[36]. One such behavioural model a passive avoidance test helps study the memory and learning functions that depend on the hippocampus region of the brain[37]. There are

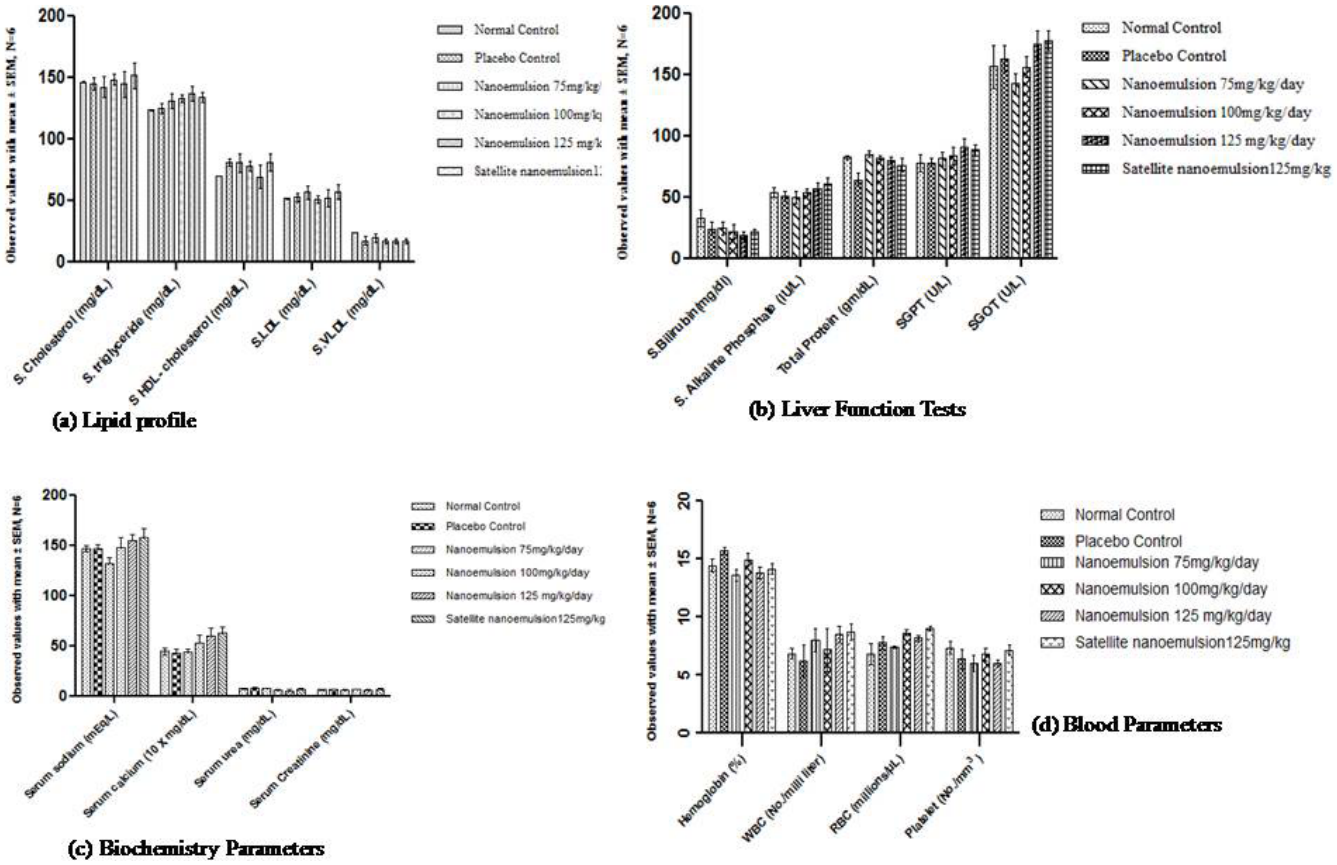

Figure 8. 28 days repeated dose oral toxicity (a) Effect of saffron nano emulsion by oral administration on Lipid parameters of rats (b) Effect of saffron nano emulsion on parameter for liver function (c) Effect of saffron nano emulsion on biochemical parameter (d) Effect of saffron nano emulsion on blood parameters.

various clinical trials conducted on humans for the safety evaluation of Crocin from CSE. Mohamadpour *et al.* has suggested that the Crocin is safe at the dose of 20 mg/day, one month in healthy volunteer[38]. Rao *et al.* investigated the efficacy of Affron®, a commercially available standardized stigma extract of *C. sativus* at the dose of 28 mg/day on improving low mood and stress in healthy volunteers where the standardized extract is consisting 3% Crocin constituent[39].

The safety evaluation of SNE was done by performing the acute dose toxicity study at the dose of 500 mg/kg/day for 15 days and showed no mortality or toxic signs. There was no effect of biochemical/ haematological parameters in rats. Based on the experiment the No Observed Adverse Effect Level (NOAEL) was found to be 500 mg/kg. The repeated dose toxicity study at the dose level of 75, 100 and 125 mg/kg/day for 28 days did not cause any adverse effects. An additional satellite group was also kept for observation of any toxic signs. Histopathological examination showed no observation of any abnormal signs or changes. In recent findings, the pharmacological action of SNE was assessed using two behavioural models, i.e., Morris water maze and passive avoidance tests. In these two behavioural models, the SNE at the dose of 10 mg/kg decreased the scopolamine-induced memory impairment and recovered memory of scopolamine-induced memory deficit in rats ultimately preserving the learning and memory and thereby improving the brain health.

5. Conclusion

The findings of this study suggested that the SNE has been safe at the dose of 500 mg/kg/day. There were no unwanted, undesirable deleterious adverse effects reported for repeated dose toxicity of 75, 100 and 125 mg/kg/day during the experiment. Histopathological examination also indicated no observation of any abnormal signs or changes. On

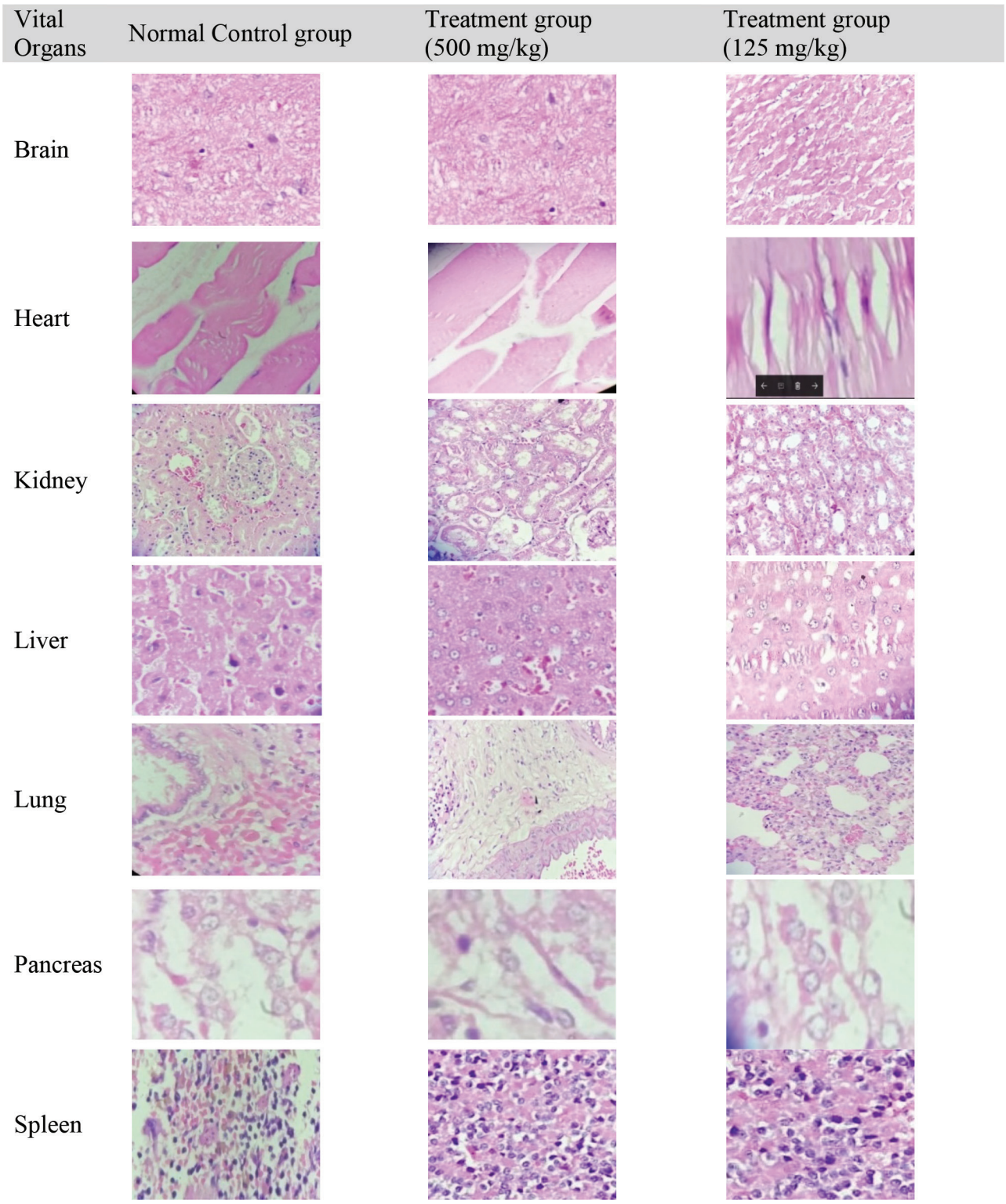

Figure 9. Histological section of Brain, Heart, Kidney, Liver, Lu ng, Pancreas & spleen of contro l group rats, Rats those exposed to 500 mg/kg saffron nano emulsion acutely and Rats those exposed to 125 mg/kg saffron nano emulsion subacutely. No significant alteration was observed in all treatment groups.

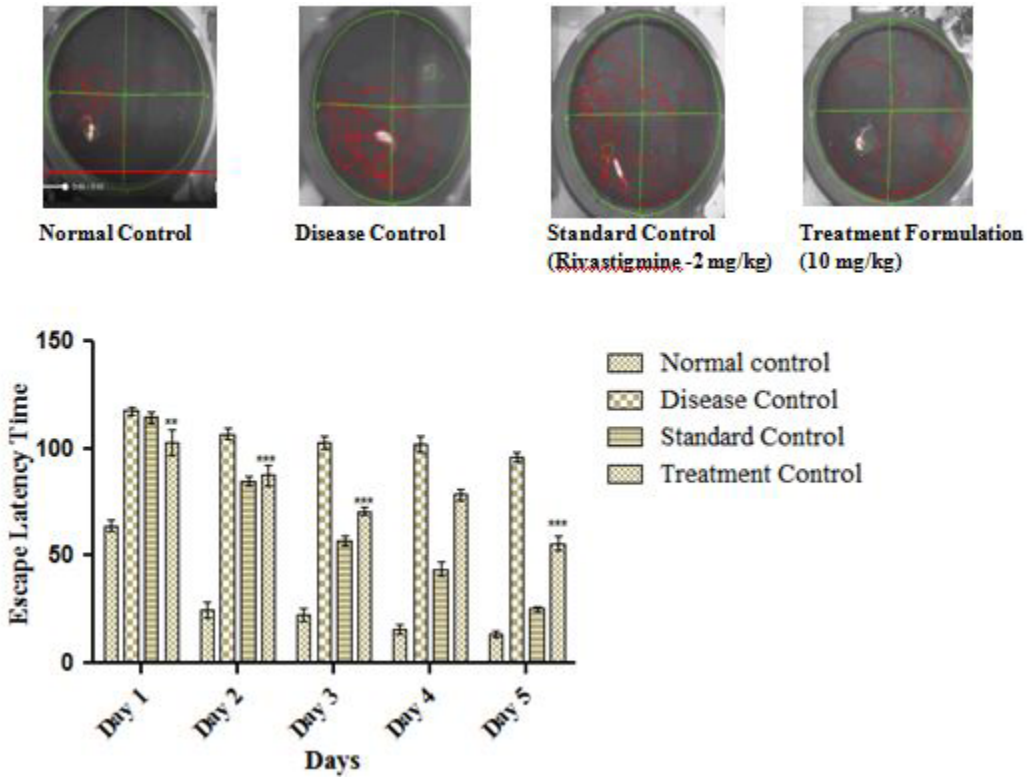

Figure 10. **(a).** Representative tracking of rats captured by Any Maze software in Morris water maze test Images captured by software, and **(b).** Effect of saffron nano emulsion on Escape latency time in Morris Water Maze test.

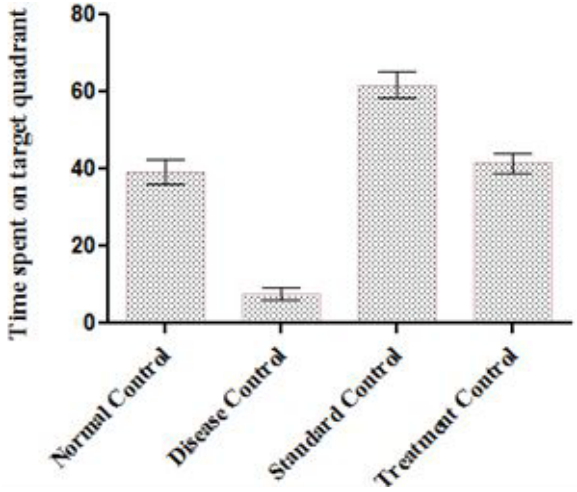

Figure 11. Escape latency time and time spent on target quadrant by Prism Graphpad software.

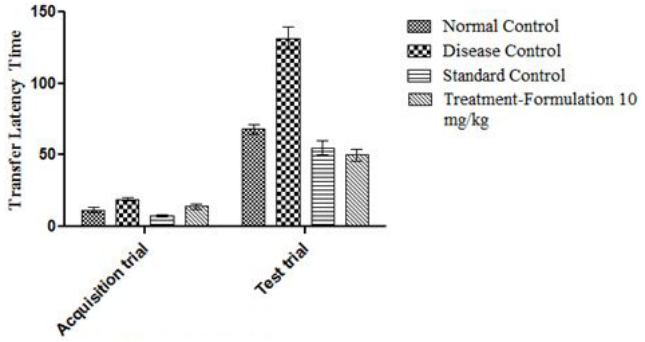

Figure 12. Transfer latency time in Passive avoidance test.

the other hand, the SNE at the dose of 10 mg/kg/day showed significant activity in scopolamine-induced memory impairment by two behavioural models, i.e., Morris water maze apparatus and passive avoidance test. The *in vivo* efficacy study on two behavioural models, i.e., Morris Water Maze and Passive avoidance test demonstrated the SNE was found efficacious to improve brain health by showing the anti-dementia property on scopolamine-induced memory deficit model at very low doses as compared to higher doses of conventional herbal therapy. This finding may increase the interest in promoting further research *in vivo* and *in vitro* toxicity tests to prove the safety of such a novel drug delivery system incorporating standardized herbal extract with effective dose management.

6. References

1. Kessler RC, Ustun T. The WHO mental health surveys. Global Perspectives on the Epidemiology of Mental Disorders. 2008.
2. Benedict C, Brooks SJ, Kullberg J, Nordenskjöld R, Burgos J, Le Grevès M, *et al*. Association between physical activity and brain health in older adults. Neurobiology of Ageing. 2013; 34(1):83-90. https://doi.org/10.1016/j.neurobiolaging.2012.04.013 PMid:22592017
3. Ferris LT, Williams JS, Shen C-L. The effect of acute exercise on serum brain-derived neurotrophic factor levels and cognitive function. Medicine and Science in Sports and Exercise. 2007; 39(4):728-34. https://doi.org/10.1249/mss.0b013e31802f04c7 PMid:17414812
4. Bernick C, Banks S, Phillips M, Lowe M, Shin W, Obuchowski N, *et al*. Professional fighters brain health study: Rationale and methods. American Journal of Epidemiology. 2013; 178(2):280-6. https://doi.org/10.1093/aje/kws456 PMid:23735309
5. Ziedonis D, Hitsman B, Beckham JC, Zvolensky M, Adler LE, Audrain-McGovern J, *et al*. Tobacco use and cessation in psychiatric disorders: National Institute of Mental Health report. Society for Research on Nicotine and Tobacco. 2008; 10(12):1691-1715. https://doi.org/10.1080/14622200802443569 PMid:19023823
6. Gómez-Pinilla F. Brain foods: The effects of nutrients on brain function. Nature Reviews Neuroscience. 2008; 9(7):568-78. https://doi.org/10.1038/nrn2421 PMid:18568016 PMCid:PMC2805706
7. Castelli V, Grassi D, Bocale R, d'Angelo M, Antonosante A, Cimini A, *et al*. Diet and brain health: which role for polyphenols? Current Pharmaceutical Design. 2018; 24(2):227-38. https://doi.org/10.2174/1381612824666171213100449 PMid:29237377
8. Bassett DS, Bullmore ET. Human brain networks in health and disease. Current Opinion in Neurology. 2009; 22(4):340. https://doi.org/10.1097/WCO.0b013e32832d93dd PMid:19494774 PMCid:PMC2902726
9. Huegel HM. Brain food for Alzheimer-free ageing: Focus on herbal medicines. Natural Compounds as Therapeutic Agents for Amyloidogenic Diseases. 2015:95-116. https://doi.org/10.1007/978-3-319-18365-7_5 PMid:26092628
10. Moshiri M, Vahabzadeh M, Hosseinzadeh H. Clinical applications of saffron (*Crocus sativus*) and its constituents: A review. Drug Research. 2014:287-95. https://doi.org/10.1055/s-0034-1375681 PMid:24848002
11. Ghahghaei A, Bathaie SZ, Bahraminejad E. Mechanisms of the effects of crocin on aggregation and deposition of Aβ1-40 fibrils in Alzheimer's disease. International Journal of Peptide Research and Therapeutics. 2012; 18:347-51. https://doi.org/10.1007/s10989-012-9308-x
12. Finley JW, Gao S. A perspective on *Crocus sativus* L.(Saffron) constituent crocin: a potent water-soluble antioxidant and potential therapy for Alzheimer's disease. Journal of Agricultural and Food Chemistry. 2017; 65(5):1005-20. https://doi.org/10.1021/acs.jafc.6b04398 PMid:28098452
13. Asadi F, Jamshidi AH, Khodagholi F, Yans A, Azimi L, Faizi M, *et al*. Reversal effects of crocin on amyloid β-induced memory deficit: Modification of autophagy or apoptosis markers. Pharmacology Biochemistry and Behavior. 2015; 139:47-58. https://doi.org/10.1016/j.pbb.2015.10.011 PMid:26484504
14. Ghadrdoost B, Vafaei AA, Rashidy-Pour A, Hajisoltani R, Bandegi AR, Motamedi F, *et al*. Protective effects of saffron extract and its active constituent crocin against oxidative stress and spatial learning and memory deficits induced by chronic stress in rats. European Journal of Pharmacology. 2011; 667(1-3):222-9. https://doi.org/10.1016/j.ejphar.2011.05.012 PMid:21616066
15. Lautenschläger M, Sendker J, Hüwel S, Galla H, Brandt S, Düfer M, *et al*. Intestinal formation of trans-crocetin from saffron extract (*Crocus sativus* L.) and *in vitro* permeation through intestinal and blood brain barrier. Phytomedicine. 2015; 22(1):36-44. https://doi.org/10.1016/j.phymed.2014.10.009 PMid:25636868
16. Li G, Zhang Z, Liu H, Hu L. Nanoemulsion-based delivery approaches for nutraceuticals: Fabrication, application, characterization, biological fate, potential toxicity and future trends. Food and Function. 2021; 12(5):1933-53. https://doi.org/10.1039/D0FO02686G PMid:33596279
17. Gallagher M, Burwell R, Burchinal M. Severity of spatial learning impairment in aging: Development of a learning index for performance in the Morris water maze. 2015; 29(4):540-8. https://doi.org/10.1037/bne0000080 PMid:26214219 PMCid:PMC5640430
18. Vorhees CV, Williams MT. Morris water maze: Procedures for assessing spatial and related forms of learning and memory. Nature Protocols. 2006; 1(2):848-58. https://doi.org/10.1038/nprot.2006.116 PMid:17406317 PMCid:PMC2895266
19. Ader R, Weijnen J, Moleman P. Retention of a passive avoidance response as a function of the intensity and duration of electric shock. Psychonomic Science. 1972; 26(3):125-8. https://doi.org/10.3758/BF03335453
20. Girme A, Saste G, Pawar S, Ghule C, Mirgal A, Patel S, *et al*. Quantitative determination and characterization of a Kashmir saffron (*Crocus sativus* L.)-based botanical supplement using single-laboratory validation study by HPLC-PDA with LC-MS/MS and HPTLC investigations. ACS omega. 2021; 6(36):23460-74. https://doi.org/10.1021/acsomega.1c03470 PMid:34549144 PMCid:PMC8444316
21. Murwanti R, Nurrochmad A, Gani AP, Sasmito E, Edwina AE, Chandra MK, *et al*. Acute and subchronic oral toxicity evaluation of herbal formulation: *Piper crocatum* Ruiz and Pav., *Typhonium flagelliforme* (Lodd.) Blume, and *Phyllanthus niruri* L. in Sprague-Dawley Rats. Journal of Toxicology. 2023; 2023. https://doi.org/10.1155/2023/7511397 PMid:36636256 PMCid:PMC9831695

22. Hosseinzadeh H, Ziaei T. Effects of *Crocus sativus* stigma extract and its constituents, crocin and safranal, on intact memory and scopolamine-induced learning deficits in rats performing the Morris water maze task. Journal of Medicinal Plants. 2006; 5(19):40-50.
23. Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. Journal of Neuroscience Methods. 1984; 11(1):47-60. https://doi.org/10.1016/0165-0270(84)90007-4 PMid:6471907
24. Gacar N, Mutlu O, Utkan T, Celikyurt IK, Gocmez SS, Ulak G. Beneficial effects of resveratrol on scopolamine but not mecamylamine induced memory impairment in the passive avoidance and Morris water maze tests in rats. Pharmacology Biochemistry and Behavior. 2011; 99(3):316-23. https://doi.org/10.1016/j.pbb.2011.05.017 PMid:21624386
25. Moosavi M, SoukhakLari R, Moezi L, Pirsalami F. Scopolamine-induced passive avoidance memory retrieval deficit is accompanied with hippocampal MMP2, MMP-9 and MAPKs alteration. European Journal of Pharmacology. 2018; 819:248-53. https://doi.org/10.1016/j.ejphar.2017.12.007 PMid:29225190
26. Malikowska N, Sałat K, Podkowa A. Comparison of pro-amnesic efficacy of scopolamine, biperiden, and phencyclidine by using passive avoidance task in CD-1 mice. Journal of Pharmacological and Toxicological Methods. 2017; 86:76-80. https://doi.org/10.1016/j.vascn.2017.04.006 PMid:28412329
27. Nyberg L, Lövdén M, Riklund K, Lindenberger U, Bäckman L. Memory aging and brain maintenance. Trends in Cognitive Sciences. 2012; 16(5):292-305. https://doi.org/10.1016/j.tics.2012.04.005 PMid:22542563
28. Stern Y. What is cognitive reserve? Theory and research application of the reserve concept. Journal of the International Neuropsychological Society. 2002; 8(3):448-60. https://doi.org/10.1017/S1355617702813248 PMid:11939702
29. McClements DJ. Advances in edible nanoemulsions: Digestion, bioavailability, and potential toxicity. Progress in Lipid Research. 2021; 81. https://doi.org/10.1016/j.plipres.2020.101081 PMid:33373615
30. McClements DJ, Xiao H. Potential biological fate of ingested nanoemulsions: Influence of particle characteristics. Food and Function. 2012; 3(3):202-20. https://doi.org/10.1039/C1FO10193E PMid:22105669
31. Salvia-Trujillo L, Martín-Belloso O, McClements DJ. Excipient nanoemulsions for improving oral bioavailability of bioactives. Nanomaterials. 2016; 6(1):17. https://doi.org/10.3390/nano6010017 PMid:28344274 PMCid:PMC5302540
32. Bahmani M, Rafieian M, Baradaran A, Rafieian S, Rafieian-Kopaei M. Nephrotoxicity and hepatotoxicity evaluation of *Crocus sativus* stigmas in neonates of nursing mice. Journal of Nephropathology. 2014; 3(2):81.
33. Hosseinzadeh H, Shamsaie F, Mehri S. Antioxidant activity of aqueous and ethanolic extracts of *Crocus sativus* L. stigma and its bioactive constituents, crocin and safranal. Pharmacognosy Magazine. 2009; 5(Suppl 2):419-24.
34. Batarseh YS, Bharate SS, Kumar V, Kumar A, Vishwakarma RA, Bharate SB, *et al*. CSE tightens the blood-brain barrier, reduces amyloid β load and related toxicity in 5XFAD mice. ACS chemical neuroscience. 2017; 8(8):1756-66. https://doi.org/10.1021/acschemneuro.7b00101 PMid:28471166 PMCid:PMC5559303
35. Pitsikas N, Zisopoulou S, Tarantilis PA, Kanakis CD, Polissiou MG, Sakellaridis N. Effects of the active constituents of *Crocus sativus* L., crocins on recognition and spatial rats' memory. Behavioural Brain Research. 2007; 183(2):141-6. https://doi.org/10.1016/j.bbr.2007.06.001 PMid:17628713
36. Tucker LB, Velosky AG, McCabe JT. Applications of the Morris water maze in translational traumatic brain injury research. Neuroscience and Biobehavioral Reviews. 2018; 88:187-200. https://doi.org/10.1016/j.neubiorev.2018.03.010 PMid:29545166
37. Ishiyama T, Tokuda K, Ishibashi T, Ito A, Toma S, Ohno Y. Lurasidone (SM-13496), a novel atypical antipsychotic drug, reverses MK-801-induced impairment of learning and memory in the rat passive-avoidance test. European Journal of Pharmacology. 2007; 572(2-3):160-70. https://doi.org/10.1016/j.ejphar.2007.06.058 PMid:17662268
38. Mohamadpour AH, Ayati Z, Parizadeh MR, Rajbai O, Hosseinzadeh H. Safety evaluation of crocin (a constituent of saffron) tablets in healthy volunteers. Iranian Journal of Basic Medical Sciences. 2013; 16(1):39.
39. Kell G, Rao A, Beccaria G, Clayton P, Inarejos-García AM, Prodanov M. Affron® a novel saffron extract (*Crocus sativus* L.) improves mood in healthy adults over 4 weeks in a double-blind, parallel, randomized, placebo-controlled clinical trial. Complementary Therapies in Medicine. 2017; 33:58-64. https://doi.org/10.1016/j.ctim.2017.06.001 PMid:28735826