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Evaluation of antioxidant and cognitive improvement activity of aqueous extract of *Raphanus sativus* L. leaves

Syed Ahmedullah Hussaini Quadri◆, Maya Sharma, Khaja Pasha* and Javed Akhtar Ansari.**

Department of Pharmaceutical Sciences, Faculty of Pharmacy, PAHER University, Udaipur-313001, Rajasthan, India

** Department of Pharmaceutical Analysis, Azad College of Pharmacy, Hyderabad-500075, Telangana State, India*

*** Department of Pharmacology, MESCO College of Pharmacy, Karwan-500006, Hyderabad, Telangana State, India*

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Abstract

Cognitive impairment is the major neurological challenge occurring due to degeneration of neurons in the brain. *Raphanus sativus* L. commonly called as radish showed antioxidant effect in hydrogen peroxide (H_2O_2) and hydroxyl radical scavenging assay. Phenytoin, an antiepileptic drug at a dose of 50 mg/kg was used to induce cognitive impairment in experimental animals. Piracetam was used as standard drug for complete study. Cognitive impairment in control group animals was established by decrease in inflexion ratio (elevated plus maze test), recognition index (novel object recognition test), fall off time (rotarod test) and increase in reaction time (cooks pole climbing test), escape latency (morris water maze test), transfer latency (rod walking test). These levels are restored to normal when treated with lower and higher doses of *R. sativus*. Later to explore molecular mechanism, biochemical parameters are estimated in mice brain. Increase in acetylcholinesterase (AChE) activity, lipid peroxidation (LPO) levels, nitric oxide (NO) levels and decreased glutathione (GSH), superoxide dismutase (SOD) levels are the indicators of cognitive impairment in control group animals. Piracetam 100 mg/kg, *R. sativus* 100 and 200 mg/kg are shown to restore all biochemical parameter levels near to normal, indicating cognitive improvement. Histopathological results also showed cognitive impairment, indicated by degeneration of neurons in hippocampus region of cerebral cortex. Piracetam treated animals showed normal morphology of brains without showing any apoptosis of neurons. Mild apoptosis was seen with *R. sativus* lower dose treatment. Whereas, in higher dose, no apoptosis of neurons is seen in cerebral cortex. Thus, *R. sativus* was found to increase learning and memory process by blocking apoptosis of neurons in cerebral cortex region. Effect of *R. sativus* is dose dependent. *R. sativus* 200 mg/kg was found to be statistically more significant than *R. sativus* 100 mg/kg. By taking an account of results of all these parameters, it is concluded that *R. sativus* is proved to possess cognitive improvement activity.

1. Introduction

Free radicals and reactive oxygen species are the fundamentals of various biochemical processes in living organisms (Tiwari, 2001). H_2O_2, hydroxyl, NO, other nitrogen derived free radicals, peroxy nitrite anion and superoxide anion are some of the common reactive oxygen species (Joyce, 1987). Their production and neutralization are under control to maintain normal homeostasis of the human body. Endogenous antioxidants produced by human body plays a vital role in neutralization process and function as defence system of human body against reactive oxygen species (Halliwell, 1994). Oxidative stress occurs when there is an imbalance between their generation and neutralization. This oxidative stress will disrupt homeostasis by causing damage to DNA, lipids and proteins and finally results in different chronic and degenerative diseases (Harman, 1998; Maxwell, 1995). Risk and propagation of these diseases can be controlled either by improving body natural antioxidant defence system or by using external antioxidants as supplements (Stanner *et al*., 2000; Tamanna *et al*., 2020).

Learning is defined as the ability of brain to acquire knowledge through experience whereas memory is ability to store and retrieve this information for short or long period of times. Learning and memory are the two widely studied subjects of the neuroscience. Cognition in broad is defined as processing information related to learning, memory, thinking, and language, *etc*. (Gupta *et al*., 2018). Age, emotion, stress, and trauma are few of many causes which may lead to amnesia, anxiety, dementia which may further lead to severe conditions like schizophrenia and Alzheimer's disease (Vadthya *et al*., 2014). Drugs used for the treatment are capable of only decreasing the symptoms of these diseases but progression of the disease is not controlled. Thus, comprehensive research has to be carried out for the discovery of new plant-based drugs for the treatment of cognitive disorders.

R. sativus is commonly called as radish. This annual herb belongs to family Brassicaceae. It is called as mooli, mullangi, and moolika, in different languages. Other synonyms are white radish, oriental

Corresponding author: Mr. Syed Ahmedullah Hussaini Quadri
Department of Pharmaceutical Sciences, Faculty of Pharmacy, PAHER University, Udaipur-313001, Rajasthan, India
E-mail: sah296@gmail.com
Tel.: +91-7386967786; 9100776347

radish and winter radish, *etc*. (Singh and Singh, 2013; Ahmad *et al*., 2012; Sreelakshmi and Pratibha, 2015). This plant has pinnate leaves, large yellow to white flowers (Kritikar and Basu, 1987). Every part like root, flower, seed, and leaves are reported for therapeutic efficacy in different disorders. This plant is commonly used in Unani medicine for treatment of kidney disorders. Seeds of *R. sativus* was reported for antitussive action, other effects reported were carminative, diuretic and expectorant activities (Bin Sina, 1987). As noted in Chinese Pharmacopoeia, dried ripen seeds are called as raphani semen which have various pharmacological effects like antiasthmatic, antidiarrhoeal and also have noticeable effects in constipation and dysentery (Sui *et al*., 2006). Roots and seeds of radish are reported with antioxidant activity, many phytoconstituents like phenolics, flavones, and anthocyanins present in these parts are reported with antioxidant potential (Goyeneche *et al*., 2015; Koley *et al*., 2017). This plant was also reported to possess hepatoprotective effects by upregulating nuclear factor erythroid 2-related factor-2 and heme oxygenase-1 (Ahn *et al*., 2018). Different cancers like lungs, liver, colon, breast, cervical, and prostate cancer are treated by various extracts of *R. sativus* (Hanlon *et al*., 2007; Pocasap *et al*., 2013; Kim *et al*., 2011; Beevi *et al*., 2010). *R. sativus* was reported to produce antidiabetic effects by upregulating the production of adiponectin (Antonopoulos *et al*., 2015). Moreover, seed extract was also reported to improve cognitive abilities in scopolamine induced memory impairment model (Kim *et al*., 2016). This provides a way to explore cognitive improvement effect on *R. sativus* leaves.

2. Materials and Methods

2.1 Materials

Phenytoin, used as a standard drug was procured from Abbott Healthcare Pvt Ltd, Hyderabad, Hydroxylamine hydrochloride of LR grade was obtained from Accord Labs, Hyderabad. Piracetam obtained from Dr. Reddy's Laboratories Ltd., EDTA procured from Essel Fine Chemicals, Ascorbic acid, Potassium dihydrogen phosphate, TCA were obtained from Finar Chemicals, H_2O_2, Dipotassium hydrogen phosphate, Ferrous ammonium sulphate, DMSO, Ammonium acetate, Glacial acetic acid, Acetyl acetone, Potassium Chloride, NADPH are obtained from SDFCL, Hyderabad, Acetylthiocholine, DTNB, Nitroblutetrazolin are obtained from Sisco Research Laboratories Pvt., Ltd, Hyderabad. All the chemicals are of analytical grade.

2.2 Methods

2.2.1 Plant material collection and extraction

Leaves of *R. sativus*, are collected from in and around areas of Hyderabad and a specimen was deposited in Pharmacognosy Department of MESCO, College of Pharmacy with a reference number as MESCO/PCOG/2020/045. Plant material was authenticated at Botanical Survey of India, Hyderabad, Telangana, India. These leaves are washed under water. They are dried for about 2 weeks in shade. Later on, they are sliced and finally grinded to coarse powder. This leaves powder was subjected to extraction by using Soxhlet apparatus and water as solvent. Process was continued for 2 to 3 days to obtain semi solid extract. This was refrigerated at 4°C.

2.2.2 Evaluation of *in vitro* antioxidant activity of RSWE

2.2.2.1 Hydrogen peroxide scavenging activity

Ruch *et al*. (1989) method was followed to screen H_2O_2 scavenging activity. About 100 µl of *R. sativus* water extract (RSWE) of various concentrations ranging from 100-500 µg/ml was used for the study. Each concentration was taken in to five different eppendorf tubes. 400 µl of 50 mM phosphate buffer (pH 7.4) was added to each tube. Finally, 600 µl of H_2O_2 solution (2 mM) was added to each tube. This is mixed thoroughly and allowed to react for 10 min. At the end of reaction, absorbance was measured at 230 nm. 100-500 µg/ml of ascorbic acid was used as standard. Only phosphate buffer without H_2O_2 was used as blank and control is made up of H_2O_2 solution in buffer. Scavenging activity of ascorbic acid and RSWE was calculated by using the formula:

$$H_2O_2 \text{ scavenging activity percentage} = [(A_0 - A_1)/A_0] \times 100$$

where A_0=Absorbance of control,

A_1=Absorbance of sample. (Bhatti *et al*., 2015).

2.2.2.2 Hydroxyl radical scavenging activity

Klein *et al*. (1981) method was used to explore this scavenging activity. Different concentrations ranging from 100-500 µg/ml of RSWE was prepared. 100 µl of each extract was taken in test tube. To this, add 100 µl of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 500 µl of only EDTA solution (0.018%). To the above mixture, further add 100 µl of DMSO (0.85% in 0.1 mol/l phosphate buffer pH 7.4). Finally, 500 µl of 0.22% ascorbic acid is added to the above reaction mixture. By closing the mouth of tubes, this mixture was heated at 80-90°C. To end the reaction, 100 µl of TCA (17.5%) was added to above mixture. For development of color in mixture 3000 µl of Nash reagent was added. This is incubated at room temperature for about 10-15 min. Intensity of yellow color formed in reaction is measured at 412 nm by using UV visible spectrophotometer. Standard drug for this activity is ascorbic acid. % Inhibition is calculated by using formula mentioned above.

2.2.3 Evaluation of *in vivo* cognitive improvement activity in phenytoin induced cognitive impairment mice model

2.2.3.1 Acute toxicity study

Guideline No. 425 given by Organization for Economic Cooperation and Development (OECD) was followed to conduct toxicity studies. Mice of (swiss albino) body weight ranging from 20-25 g are used. In each step, 5 animals were used in each group. Animals were kept on fasting for 3-4 h prior to dosing. All the animals were evaluated for the signs of toxicity and mortality.

2.2.3.2 Animal grouping

Group I: Normal - Vehicle 1ml//100 g BW

Group II: Control - Phenytoin 50 mg/kg + Vehicle 1ml//100 g. BW

Group III: Standard - Phenytoin 50 mg/kg + Piracetam 100 mg/kg - i.p

Group IV: RSWE - Phenytoin 50 mg/kg + RSWE -100 mg/kg - oral

Group V: RSWE - Phenytoin 50 mg/kg + RSWE -200 mg/kg - oral

2.2.3.3 Animals

Male / Female swiss albino mice weighing 20-25 g were obtained. Animals were housed under standard laboratory conditions with food and water continuously available. A 12 h: 12 h (light: dark) cycle was used with the light on from 7:00 to 19:00 h. All behavioral testing was done during the daylight period between 10:00 and 17:00 h. These are tail marked for identification. Animals were divided into five groups of five animals in each. Phenytoin dissolved in DW was administered intraperitoneally (i.p) in different groups between 10 and 11 a.m. every day for 10 days at a volume of 0.1 ml/100 g body weight. Phenytoin was administered in a dose of 50 mg/kg as this dose was found to produce cognitive decline (Sudha *et al*., 1995). RSWE was administered in IV and V groups orally (per oral, p.o.) by gavage at volumes not greater than 1.0 ml/100 g. RSWE was given at a dose of 100 mg/kg and 200 mg/kg to groups IV and V, respectively as described above, thirty minutes before the administration of phenytoin in the combination group. Mice were weighed every 2ndday before administering the drug. All the drugs were administered for 10 days. The first group served as normal with vehicle only and for control was administered with phenytoin in a dose of 50 mg/kg and the vehicle was administered orally. Satisfactory approval is taken from Institution Animal Ethics Committee of Deccan School of Pharmacy bearing registration number 1773/PO/Re/S/14/CPCSEA. All the ethical guidelines are followed to carry out animal studies.

2.2.3.4 Screening behavioral parameters to assess cognitive improvement activity

All behavioral assessments are performed on 0th day, 1st, 5th day, and 10th day. Before 0th day reading, animals are trained for all parameters for 2 days. For each parameter, training is provided for each animal in 5 sets, with a gap of 30 sec in between each set.

2.2.3.4.1 Elevated plus maze test

Memory and learning capability of experimental animals is evaluated by using elevated plus maze test. The apparatus consisted of two open arms (25 cm × 5 cm) and two covered arms (25 cm × 5 cm ×20 cm). The arms extended from a central platform (5 cm × 5 cm) and the maze was elevated to a height of 25 cm from the floor. Training is provided as mentioned above. On 0th day, transfer latency (TL) was taken as the time taken by the mouse to move into any one of the covered arms with all its four legs. TL was recorded on the first day for each animal. The mouse was allowed to explore the maze for another 2 min and returned to its home cage. Retention of this learned task was examined on 1st day, 5th day, and 10th day. The TL was expressed as inflexion ratio (IR) using the formula given by Jaiswal and Bhattacharya (1992) and (Dhingra *et al.*, 2012).

IR = (L1 – L0)/L0. where L0 =TL after nth day and L1 = initial TL(s).

2.2.3.4.2 Cook's pole climbing test

It is an accepted method for evaluating memory and learning abilities in experimental animals. Apparatus consists of a chamber with iron grid as platform, a centrally placed pole and plexi glass door at front. In this experiment, a tone of 50 Hz was used a buzzer functioning as conditioned stimuli, after buzzer sound an unconditioned stimulus was applied as a foot shock of 1mA current. During training period, animal is trained to jump on to the pole after buzzer sound avoiding foot shock. End point is taken as climbing on pole or after 30 sec of foot shock. Trained animal will avoid foot shock and jump on to the pole after buzzer sound. Retention of this memory was tested on 1st day, 5th day, and 10th day. It was quantified as the percentage of animals avoiding shock by jumping on the pole (Hanumakonda and Maheshwaram, 2016; Kumar and Minshu, 2016)

2.2.3.4.3 Novel object recognition (NOR) test

Ennaceur and Delacour (1988) method with slight modification was followed to conduct NOR test. Apparatus consists of an open field area (40 × 40 × 30 cm). During training, mice was kept in open field for 5 min with two identical objects. During experiment days, same two identical objects were placed in the test area, and the mice were allowed to familiarize with these objects for 5 min than one object was replaced with a new one of different shape and color, and the animals have observed it for 5 min. Time spent by each mouse near to the old (TO) and the new objects (TN) was determined. Recognition index was calculated by using the formula TN/(TO + TN) * 100] where TO and TN are time spent exploring old object and new object, respectively (Batool *et al*., 2016; Antunes and Biala, 2012). Exploration of an object was deemed when a rat sniffed or touched the object with its nose and/or forepaws. This was conducted on 0th day, 1st day, 5th day, and 10th day.

2.2.3.4.4 Morris water maze test

Apparatus is a circular water pool (80 cm in diameter and 40 cm in height) with constant clues external to the maze for spatial orientation of the mice. The water was made opaque by adding black non-toxic colour to prevent animals from seeing the submerged platform. The water temperature was kept at 24-26°C during the whole experiment. An invisible platform (6 cm in diameter and 15 cm in height) providing the only escape from water was placed 1.5 cm below the water surface. The tank is marked off into four quadrants, *i.e.*, North, South, East and West. Animals were placed in the water at a designated starting location and the time to find the hidden platform from the starting point is defined as "Escape Latency". Each animal was given training as mentioned above and then readings were taken 0th day, 1st day, 5th day, and 10th day. (Morris, 1981).

2.2.3.4.5 Skeletal muscle relaxant activity by rotarod test

Phenytoin at higher doses and chronic use causes muscle relaxation. To evaluate muscle relaxant activity, rotarod test is used. Animals were trained for using in test, mice which remained on rotating rod for 1min into successive trials were selected for test. 30 min after administration of drugs as described above animals were placed on rod to note the time taken for mice to fall from rod and this was conducted on 0th day, 1st day, 5th day, and 10th day (Kuribara *et al*., 1977).

2.2.3.4.6 Rod walking test

The ability of mice to balance on a stationary, horizontal rod and walk on it to come in at one end of the rod measures cognitive study and learning activity. Animals were placed in the center of a rod (100 cm long, 5 mm in diameter, and positioned 23 cm above the table surface), parallel to it, and their latency to transfer to its one end is recorded. All mice are trained, and then tested for TL on 0th day, 1st day, 5th day, and 10th day (Kamila *et al.*,2014).

2.2.3.5 Screening biochemical parameters to assess cognitive improvement activity

Preparation of brain tissue samples: After behavioral measurements, all the mice were sacrificed by decapitation; the brain tissues were quickly removed, washed with cold saline solution. 10 % (w/v) homogenate of brain tissue samples was made with ice-cold 0.1 M phosphate buffer (pH 7.4).

2.2.3.5.1 Determination of AChE activity in the brain tissue of mice

Acetylthiocholine is hydrolyzed by AChE to produce thiocholine and acetate. Thiocholine reduces DTNB to 5-thio-2-nitrobenzoate. Yellow color of this compound is measured at 412 nm. Ellman *et al*. (1961) procedure with slight modification was followed to conduct this assay. 2.6 ml of 0.1 M phosphate buffer (pH 8) along with 100 µl of 1.0 mM DTNB was taken in a cuvette. To this, 400 µl of brain homogenate was added. After mixing thoroughly, absorbance was measured at 412 nm in spectrophotometer. Initial stable value is taken as basal reading. Later on, 20 µl of substrate *i.e*., 0.075 macetylthiocholine is added and change in absorbance is recorded after 5 mins.

AChE activity was calculated as follows:

$$R = 5.74 \times 10^{-4} \times \Delta A/CO$$

where, R = Rate in moles of substrate hydrolyzed/minute/mg of tissue

ΔA = Change in absorbance,

CO = Original concentration of the tissue (mg/ml).

Obtained R value is in mole which is converted to µM by multiplying with 10^6. Finally, AChE activity is expressed as µM of acetylthiocholine hydrolyzed/min/mg of tissue (Davies and Maloney, 1976; Appleyard, 1992).

2.2.3.5.2 Determination of malondialdehyde (MDA/TBARS) levels in the brain tissue of mice

A chromogenic product called as MDA is produced during LPO. This compound gives pink color after reacting with thiobarbituric acid (TBA). Intensity of pink color is measured at 540 nm. 500 µl of each trichloroacetic acid (TCA)-30% and TBA-0.8% are taken in a test tube. After adding 1ml of brain homogenate, test tubes are closed with aluminium foil. These are heated for half an hour at 80°C in water bath, followed by cooling in ice-cold water for half an hour. Later on, tubes are centrifuged at 3000 rpm for 15 min Absorbance of the supernatant was measured at 540 nm against appropriate blank (1 ml distilled water, 500 µl of each TCA- 30% and TBA-0.8%) (Okhawa *et al*.,1979). Amount of MDA present in a sample was calculated as follows:

$$\frac{\text{Nanomole of MDA}}{\text{mg protein}} = \left(\frac{V \times 0D_{540}}{0.156}\right) \times \text{mg of protein}$$

where, V = Final volume of the test solution;

OD = Optical density at 540 nm; result was expressed as nanomole of MDA/mg of protein.

2.2.3.5.3 Determination of glutathione in the brain tissue of mice

Sedlak and Lindsay (1968) method is followed. In this test, DTNB reacts with GSH to form yellow colored compound called as 5- thionitrobenzoic acid (TNB). Briefly, 1ml of Tris-EDTA buffer (0.2 M, pH 8.2) and 0.9 ml of EDTA (20 mM, pH 4.7) was taken in a reaction tube. 100 µl of the homogenate or pure GSH was added. Later on, 20 µl of Ellman's reagent (10 mmol/l DTNB in methanol) was added. Mixture is incubated for half an hour at room temperature and finally centrifuged. Absorbance of supernatant was measured at 412 nm (Khynriam and Prasad, 2003). Amount of GSH in the sample is calculated as follows:

$$\frac{\text{GSH}}{\text{mg protein}} = \left(\frac{\text{Dilution factor} \times 0D_{412}}{1.36 \times 10^4}\right) \times 100$$

where 1.36×10^4 = Extinction coefficient; dilution factor = 10

The result is expressed as nanomole/mg of protein

2.2.3.5.4 Determination of superoxide dismutase levels in the brain tissue of mice

In a test tube, take 1 ml of sodium carbonate (1.06 gm in 100 ml of DW). To this, add 400 µl of NBT (24 mmol/l) and 200 µl of EDTA (37 mg in 100 ml of DW). To the above mixture, 100 µl of brain homogenate is added and 0 min was taken at 560 nm. About 400 µl of 1mM hydroxylamine hydrochloride was added to initiate the reaction. This is incubated for 5 min (25°C). Finally, reduction of NBT was measured at 560 nm. SOD level was calculated using the standard calibration curve, and expressed in µg/mg of protein (Misraand Fridovich, 1972). Pecentage inhibition of NBT reduction by SOD is calculated by using the formula:

% inhibition of NBT reduction by SOD

$$= \left(\frac{\text{Control OD} - \text{Treatment OD}}{\text{Control OD}}\right) 100 = \text{X\% inhibition}$$

50% inhibition is equal to 1 unit of enzyme then X%

$$= \left(\frac{1}{50}\right) X = \text{Y unit}$$

2.2.3.5.5 Determination of total nitric oxide levels in the brain tissue of mice

To 1.5 ml of Griess reagent (1:1 solution of naphthalene diamine dihydrochloride (0.1%) and sulphanilamide (1%) in 5%phosphoric acid) add 0.3 ml of brain homogenate. Reaction absorbance was measured at 546 nm. Nitrite concentration was calculated using a standard curve for sodium nitriteand expressed as ng/mg of protein.

2.2.3.6 Screening effects of RSWE on histopathological studies in phenytoin induced cognitive impairment mice model

Brain of one animal from each group is isolated and fixed in 10% formalin. These isolated brains are fixed embedded in paraffin wax and cut in to longitudinal sections of 5 µm thickness. These samples are stained with hematoxylin and eosin dye for histopathological assessment.

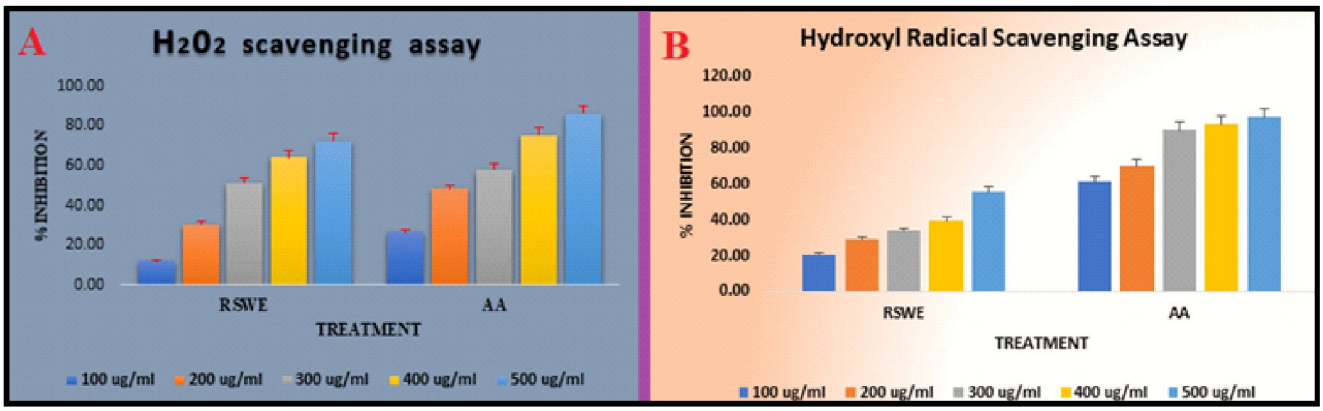


Figure 1: Evaluation of *in vitro* antioxidant activity of RSWE by using ascorbic acid as standard. A-H_2O_2 scavenging assay, B-Hydroxyl radical scavenging assay.

2.2.3.7 Statistical analysis

Results were expressed as mean ± SEM. Statistical comparison was made between treated groups and control group. Statistical difference between two means was determined by one-way ANOVA, followed by Dunnett's multiple comparison test using Graph pad prism 9.0 software. Only those mean values showing statistical difference $p < 0.0001$, $p < 0.001$, $p < 0.01$, $p < 0.05$ were considered as statistically significant and $p < 0.05$ was considered as non-significant. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$ and ns = non-significant.

3. Results

3.1 Plant extraction and *in vitro* antioxidant activity

Extraction of *R. sativus* leaves by using distilled water yielded dark brown coloured semisolid extract. Percentage yield was found to be 10.8 %. Extract obtained was stored in refrigerator at 4°C. *In vitro* antioxidant activity was assessed by using two methods like H_2O_2 scavenging assay and hydroxyl radical scavenging assay. For both assays, ascorbic acid was used as standard. Scavenging effect of RSWE in H_2O_2 assay was found to improve with increase in dose from 100 µg/ml to 500 µg/ml. IC_{50} of RSWE was found to be at 300 µg/ml. Ascorbic acid also showed dose dependent scavenging effect with IC_{50} at 200 µg/ml. Whereas in hydroxyl radical scavenging assay, % inhibition was found to increase dose dependently in both RSWE and standard. IC_{50} was found to be 470 µg/ml and 80 µg/ml for RSWE and standard ascorbic acid, respectively (Figure 1).

3.2 *In vivo* cognitive improvement activity

In acute toxicity study, after dosing animals were observed individually at least once during first 30 min, periodically during first 24 h and daily up to 14 days. It was observed that there were no signs of toxicity and mortality at a dose of 2000 mg/kg. 1/ 20thand 1/10thof 2000 mg/kg, *i.e.*, 100 mg/kg and 200 mg/kg, respectively, a very low doses of plant extract are used for the study. For behavioral assessment, various models like elevated plus maze, cooks pole climbing test, novel object recognition test, morris water maze test, rod walking test and rotarod test are conducted.

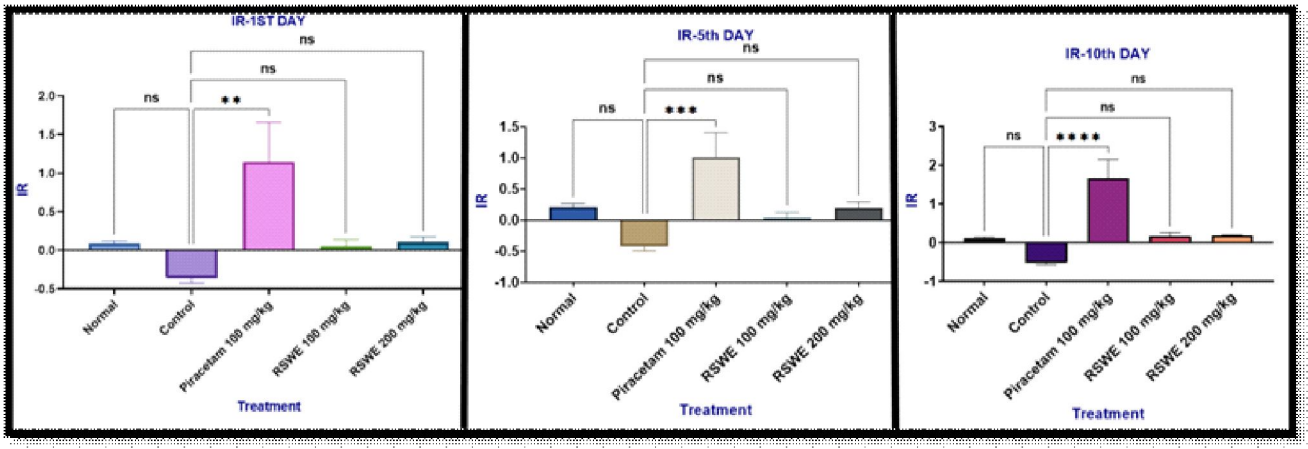


Figure 2: Inflexion ratio calculated from elevated plus maze test. All values are expressed as mean ± SEM, and tested by One -way ANOVA followed by Dunnett's multiple comparison test, n=5, * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$ and ns = non-significant.

In elevated plus maze test, IR was decreased from day 1 to day 10 in control group. Behavior was normal in group I animals which received only vehicle. Whereas, in standard group animals, IR was increased from day 1 ($p<0.01$) to day 10 ($p<0.0001$) and was near to the normal group animals. IR was increased in both treatment groups from day 1 to day 10. RSWE 200 mg/kg was found to be

more effective than lower dose of 100 mg/kg. Results of RSWE are not statistically significant as compared to control group (Figure 2).Another important model to assess learning and memory retention in mouse model is cooks pole climbing test where reaction time is calculated in all groups. Normal group animals showed decrease in reaction time from day 1 to day 10 as they learned to respond to conditioned stimuli. This learning ability was almost compromised in control group animals. As a result, reaction time was increased statistically from day 1 (p< 0.01) to day 10 (p< 0.0001) as compared to normal group. Piracetam treated animals showed statistically significant decrease in reaction time from day 1 (p< 0.01) to day 10 (p<0.0001) as compared to control group. Behavior was almost near to normal group animals. RSWE 100 mg/kg showed non-significant results from day 1 to day 10 as compared to control group. Whereas RSWE 200 mg/kg showed significant decrease in reaction time from day 1 (p < 0.05) to day 10 (p< 0.01). Improvement in reaction time was dose dependent (Figure 3).

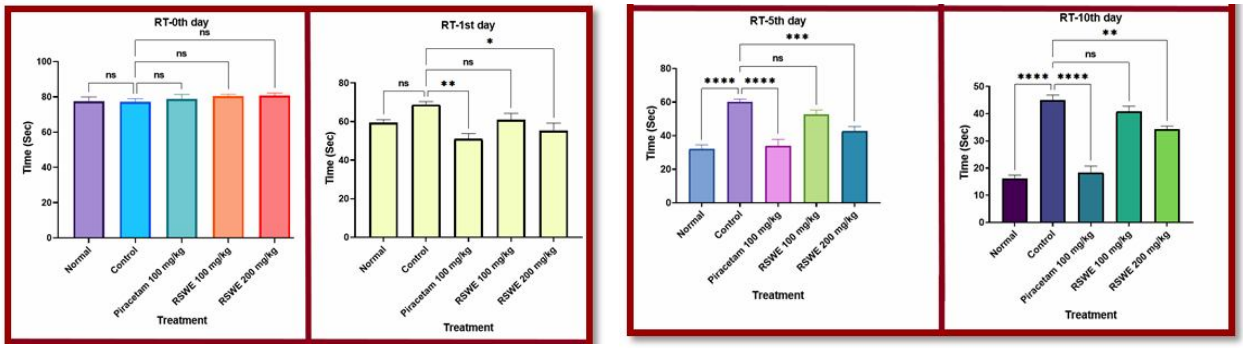


Figure 3: Reaction time calculated in cooks pole climbing test. All values are expressed as mean ± SEM, and tested by One-way ANOVA followed by Dunnett's multiple comparison test, n=5, * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 and ns = non-significant.

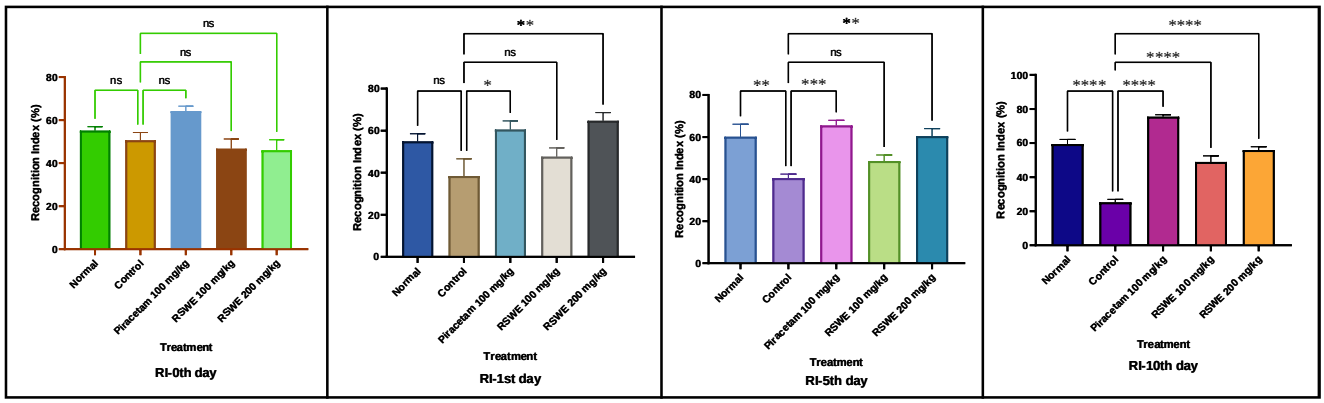


Figure 4: Effect of RSWE on recognition index calculated in Novel object recognition test. All values are expressed as mean ± SEM, and tested by One way ANOVA followed by Dunnett's multiple comparison test, n = 5, * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 and ns = non-significant as compared to control group.

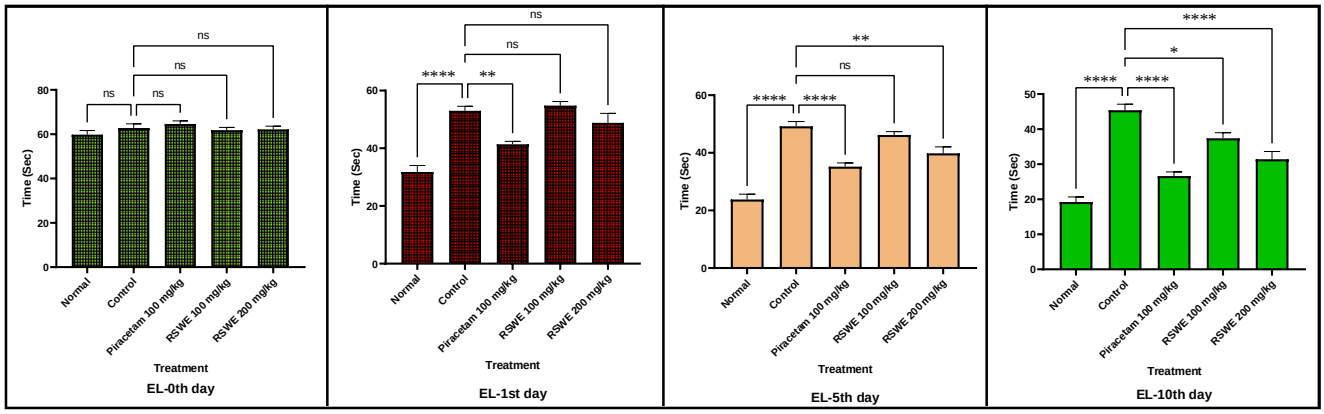


Figure 5: Effect of RSWE on escape latency calculated in morris water maze test. All values are expressed as mean ± SEM, and tested by One-way ANOVA followed by Dunnett's multiple comparison test, n=5, * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 and ns = non-significant as compared to control group.

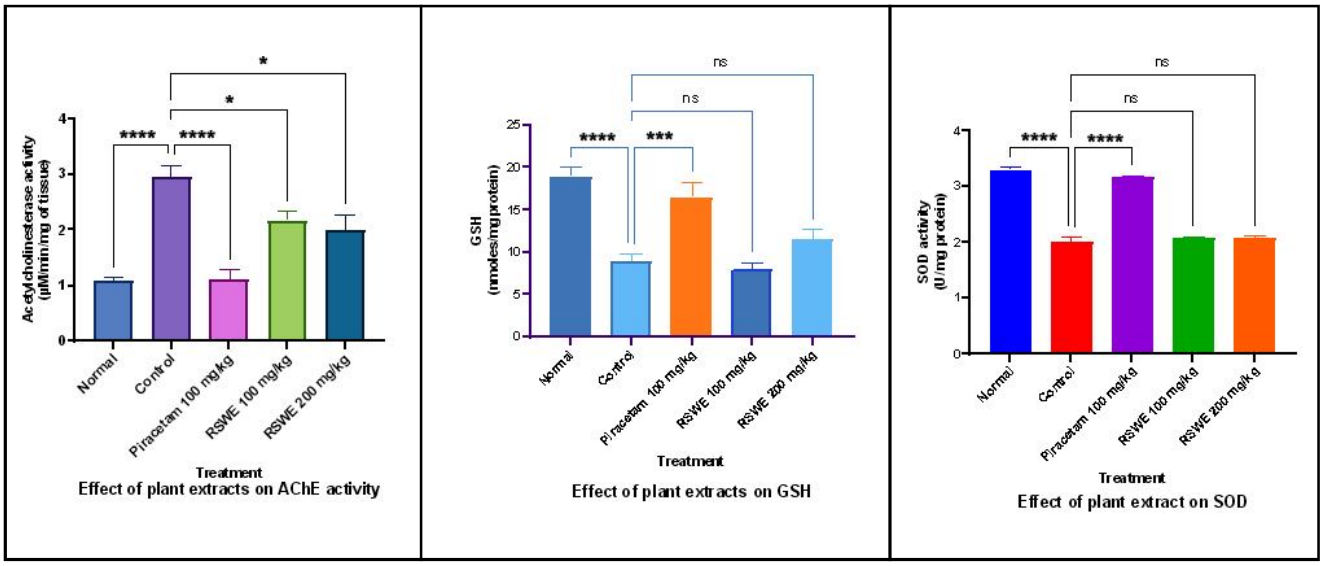
NOR test was used to asses cognitive behavior in mice. Recognition index was calculated for all the animals. Recognition index was significantly reduced in control group animals as compared to normal group. This reduction was seen from day 5 (p<0.01) and was reduced more effectively upto day 10 (p<0.0001). In piracetam treatment group, recognition index increased from day 1 (p<0.05) to day 10 (p<0.0001) as compared to control group. RSWE 100 mg/kg showed significant increase in recognition index only on day 10 (p<0.0001) as compared to control group. Whereas, RSWE 200 mg/kg showed significant increase in recognition index from day 1 (p<0.001) to day 10 (p<0.0001) as compared to control group. This effect is more pronounced with higher dose of RSWE when compared to lower dose (Figure 4). Escape latency is calculated in morris water maze test. Control group showed significant increase in escape latency time as compared to normal group. Piracetam treated group showed significant decrease in escape latency time from day 1 (p<0.01) to day 10 (p<0.0001) as compared to control group. RSWE at both doses showed non-significant result on day 1. Only higher dose of RSWE (200 mg/kg) showed significant decrease in escape latency on day 5(p<0.001). Whereas, on day 10 effects of RSWE are more pronounced as compared to day 1 and day 5 (Figure 5). Among two groups of RSWE decrease in escape latency time is dose dependent. However, effect is less than piracetam.

In rod walking test, TL is calculated. Control group animals showed significant increase in TL time from day 1 (p<0.0001) to day 10 (p<0.0001) as compared to normal group. Piracetam and both doses of RSWE showed significant decrease in TL from day 1 (p<0.0001) to day 10 (p<0.0001) as compared to control group. In rotarod test, fall of time is measured. Control group animals are unable to walk on rotating rod and showed significant decrease in fall off time from day 1 to 10 as compared to normal group. Piracetam and RSWE treated animals showed significant increase in fall off time from day 1 (p<0.0001) to day 10 (p<0.0001) as compared to control group (Table 1).

Table 1: Effect of RSWE on fall off time in rotarod test and transfer latency in rod walking test

Treatment	Day 0	Day 1	Day 5	Day 10
Rotarod test (Fall off time in seconds)				
Normal	168 ± 3^{ns}	171 ± 3.6****	173 ± 2****	150 ± 18****
Control	171 ± 1.3	35 ± 3	26 ± 1.7	11 ± 0.93
Piracetam 100 mg/kg	171 ± 4.1^{ns}	161 ± 5.9****	137 ± 8****	110 ± 3.2****
RSWE 100 mg/kg	168 ± 2.4^{ns}	111 ± 3.9****	83 ± 4.1****	66 ± 6.4**
RSWE 200 mg/kg	169 ± 2.5^{ns}	109 ± 1.7****	100 ± 2****	85 ± 3.1****
Rod walking test (Transfer latency in seconds)				
Normal	8 ± 0.45^{ns}	6.8 ± 0.58****	7.6 ± 0.4****	7.8 ± 0.37****
Control	8.2 ± 0.37	26 ± 1.6	29 ± 1.7	53 ± 3.3
Piracetam 100 mg/kg	6.8 ± 0.8^{ns}	7 ± 0.55****	16 ± 1.8****	20 ± 1.2****
RSWE 100 mg/kg	7.6 ± 0.4^{ns}	16 ± 1.5****	25 ± 1.1***	35 ± 1.3****
RSWE 200 mg/kg	7.6 ± 0.4^{ns}	14 ± 1.5****	18 ± 2.1****	26 ± 1.9****

All values are expressed as mean ± SEM, and tested by One-way ANOVA followed by Dunnett's multiple comparison test, n=5, * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 and ns = non-significant as compared to control group.



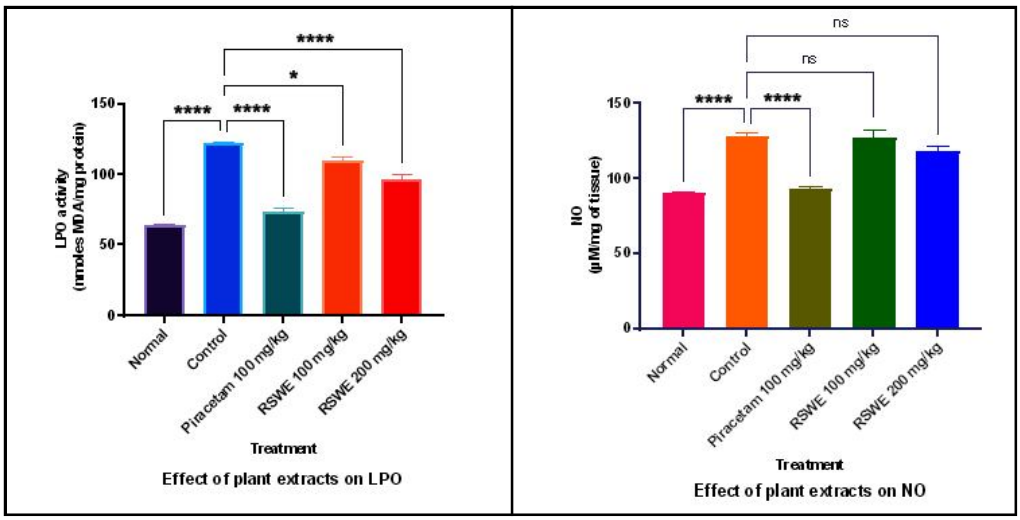


Figure 6: Determination of AChE activity, malondialdehyde (MDA/TBARS), GSH, SOD and nitric oxide levels in the brain tissue of mice. All values are expressed as mean ± SEM, and tested by One-way ANOVA followed by Dunnett's multiple comparison test, n=5, * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$ and ns = non-significant as compared to control group.

In biochemical estimations, control group animals showed significant increase in AChE activity ($p<0.0001$), LPO activity ($p<0.0001$), NO level ($p<0.0001$) and significant decrease in GSH level ($p<0.0001$) and SOD activity ($p<0.0001$) as compared to normal group. Piracetam treated animals showed significant decrease in AChE activity ($p<0.0001$), LPO activity ($p<0.0001$), NO level ($p<0.0001$) and significant increase in GSH level ($p<0.001$) and SOD activity ($p<0.0001$) as compared to control group. Piracetam treatment restored the biochemical measurements to normal group values. RSWE at 100 mg/kg and 200 mg/kg showed mild significant decrease in AChE activity ($p<0.05$), LPO activity ($p<0.0001$) and non-significant effect on GSH level, SOD activity, NO activity as compared to control group (Figures 6, 7). In histopathological studies, brains of control group animals showed apoptosis of neurons in hippocampus region of cerebral cortex. Piracetam treated animals showed normal morphology of brain, RSWE lower dose animals showed apoptosis of neurons in some areas of cerebral cortex. RSWE higher dose treated animals showed normal morphology of brain samples as shown on treatment with piracetam (Figure 8). A graphical abstract explaining detailed outline of the study is represented in Figure 9.

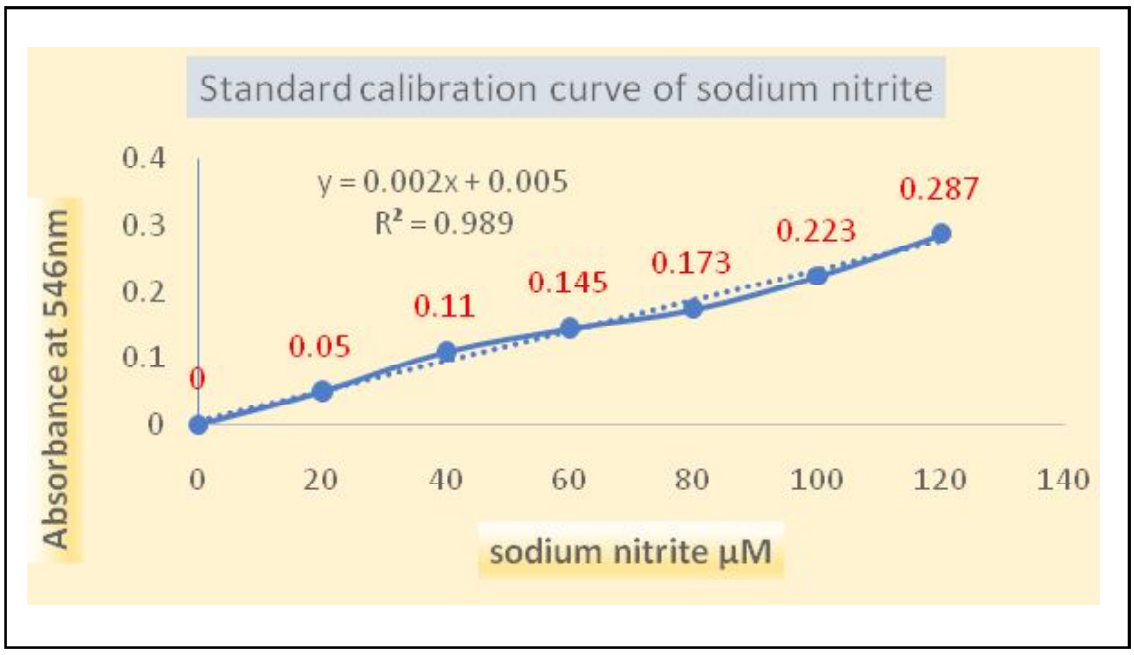


Figure 7: Standard graph of sodium nitrite.

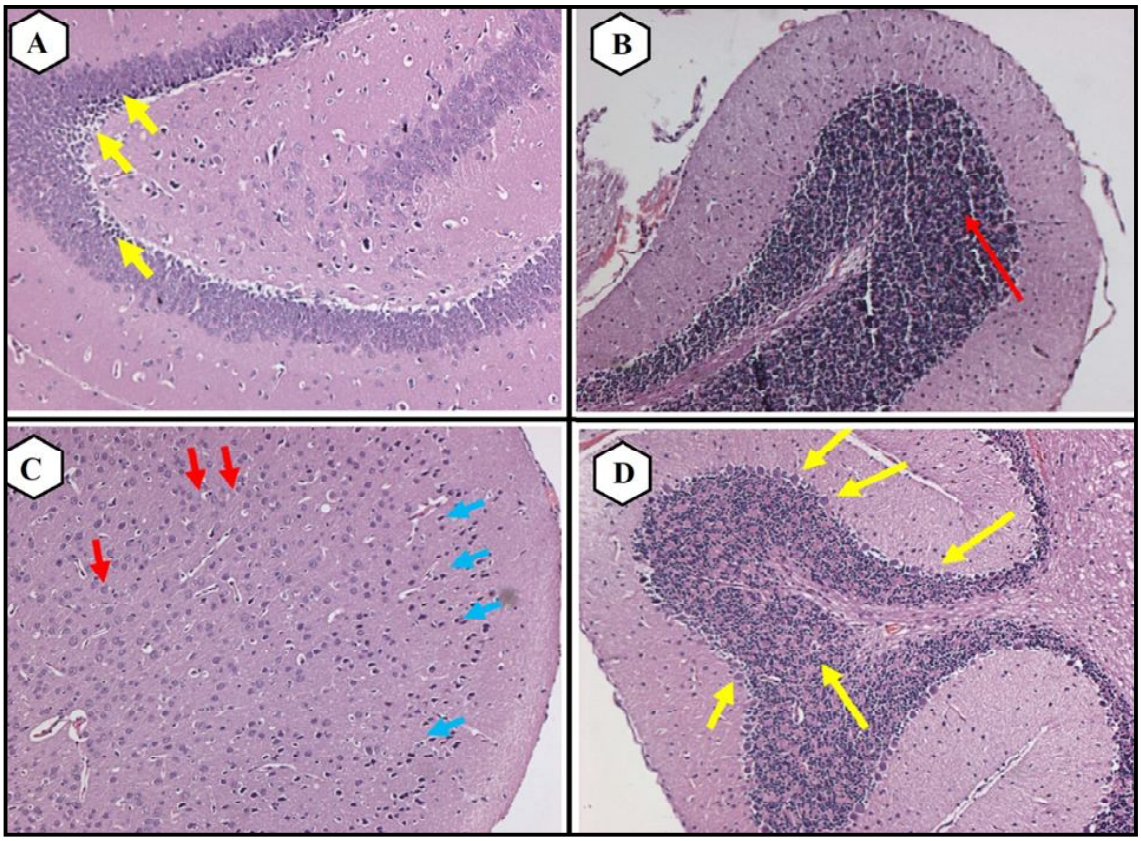


Figure 8: Effect of RSWE on mice brain. A-Control group-Foci of apoptotic neurons were observed in hippocampus of cerebral hemispheres of brain. B-Piracetam treated group showed normal morphology of cerebellum, C-RSWE 100 mg/kg - Foci of apoptotic neurons in cerebral cortex – Blue arrow and normal morphology of neurons - red arrow, D-RSWE 200 mg/kg-normal morphology of purkinjee cells and cerebellum.

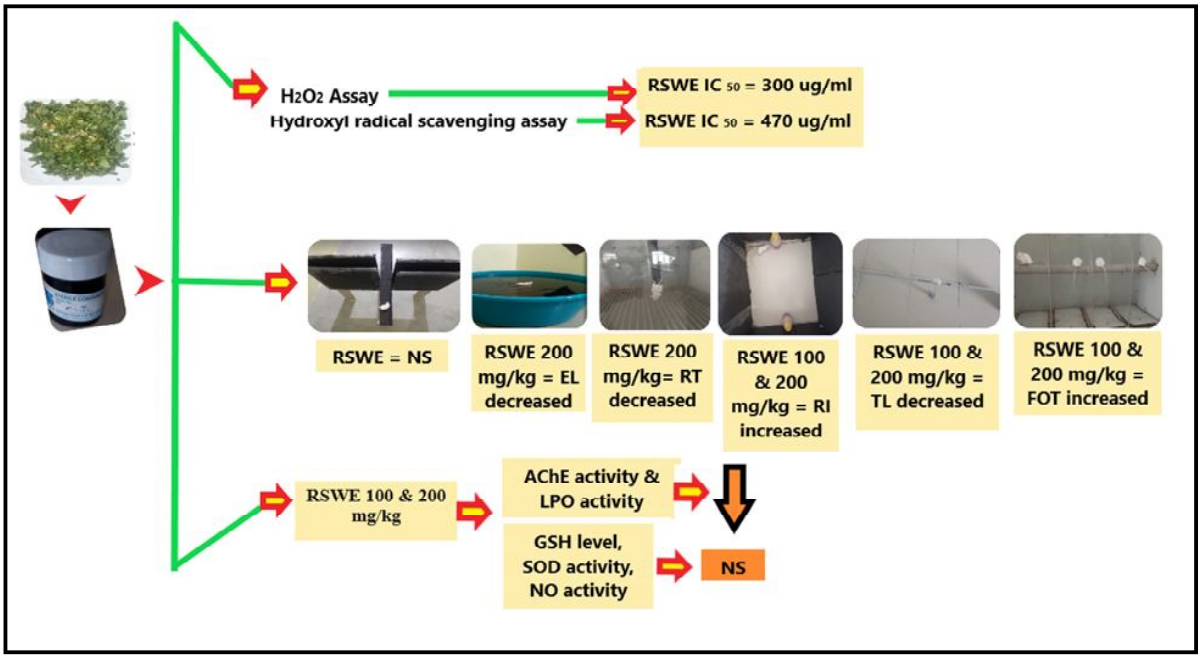


Figure 9: Graphical abstract explaining various aspects of the study.

4. Discussion

During the process of normal metabolism in human body or during disease conditions, free radicals are produced (Yeum *et al*., 2003). These free radicals if not detoxified may cause severe damage to human body by causing damage to biomolecules (Halliwell and Gutteridge, 1998). Many synthetic antioxidants consumed by people in spite of having efficacy in controlling of oxidative stress, may cause severe adverse effects. So, there is a need of safe natural antioxidants (Jerome *et al*., 2011). Till date, many natural antioxidants are proved for their efficacy, so their demand is increasing day-to-day (Das *et al*., 2019). For plant phytoconstituents, it is not recommended to have only one test to prove antioxidant nature (Jovanovic and Simic, 2000). So, in this study, we carried out two methods, *viz*., H_2O_2 scavenging assay and hydroxyl radical scavenging assay. Yongpradoem and Weerapreeyakul (2018) found different phytoconstituents in *R. sativus* like phenolics and isothiocyanates. Phenolics identified are gallic acid, protocatechuic acid, p-hydroxybenzoic acid, vanillic acid, syringic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, and isothiocyanates identified are sulforaphane, sulforaphene. Leaves are found to contain glucoraphasatin, methoxyglucobrassicin, raphanin, glucoraphanin, and glucobrassicin (Shukla *et al*., 2011). During human metabolic processes when oxygen is reduced to produce water hydroxyl radicals are generated. These hydroxyl radicals cause lipid peroxidation. As a result, disulfide bonds of proteins are reduced finally converting them in to non-functional or abnormal functioning molecules which is responsible for many neurological abnormalities (Lipinski, 2011). In the present study, RSWE showed antioxidant activity by inhibiting the production of hydroxyl radicals. This effect is most probably related to phytoconstituents mentioned above. Sulforphane, a phytoconstituent may be responsible for antioxidant action. Fahey and Talalay (1999) reported the antioxidant property of sulforphane. Probable mechanism of action of sulforphane is to induce enzymes involved in antioxidant process. Flavonoids are the other phytoconstituents which are responsible for antioxidant activity. Their efficacy is also reported by Grassi *et al*. (2010). H_2O_2 is another harmful radical produced during metabolic processes in the cells, so it is important to detoxify H_2O_2 produced in the body (Esmaeili and Sonboli, 2010). Many phytoconstituents are proved for their antioxidant effects with respect to H_2O_2 (Kiran *et al*., 2019). These results showed by RSWE are in accordance with the results obtained with leaves extract screened for H_2O_2 radical scavenging effect in MRC-5 cells (human fetal lung fibroblasts cells). This effect is probably due to high concentration of polyphenols in the extract (Luo *et al*., 2018). After obtaining justifying antioxidant results with RSWE, current research is directed to screen *in vivo* cognitive improvement activity in different animal models.

Phenytoin is one of the commonly used antiepileptic drug. It can impair learning abilities and memory at therapeutic doses (Sudha *et al*., 1995). Similar disturbance in cognitive abilities is also found in mice treated with therapeutic doses of phenytoin (Mondadori and Classen, 1984). However, at a dose of 50 mg/kg, phenytoin was found to induce drowsiness. This may also lead to the learning impairment. Phenytoin induced memory impairment is also reported by Reeta *et al*. (2009). In this study, memory was tested by elevated plus maze model. However, elevated plus maze is primarily used for screening anxiety, but it is also reported in many studies to be used for screening cognitive behavior in animals (Kumar *et al*., 2007). TL is the indicator of spatial memory. In this study it is expressed in terms of inflexion ratio. In normal animals, IR increased indicating normal memory. But in animals treated with phenytoin only showed progressive decrease in IR from day 1 to day 10 indicating loss of memory. Increase in IR in treatment groups is an indicator of memory improvement. Improved IR was also reported in elevated plus maze test on treatment with curcumin (Reeta *et al*., 2009). Another study carried out by Retinasamy *et al*. (2019) also showed increase in IR in elevated plus test on treatment with orthosiphon stamineus. Cook's pole climbing test, is another well know method to evaluate learning and memory abilities in rodents. Hearing the sound of buzzer and jumping on to the rod is the measurement of long-term memory. This is measured in terms of reaction time. This reaction time is increased in control group animals indicating week memory after exposing to phenytoin. Shortened reaction time in all treatment groups indicate the retention of long-term memory. These results are in accordance with the results obtained on treatment with *Tridax procumbens* (Ramrao *et al*., 2018).

Similarly, recognition index is measured in novel object recognition test as an indicator of long term non-spatial memory. Its decrease on exposure to phenytoin indicates deficiency in memory. Successful improvement in recognition index of treated animals is an indicator of cognitive improvement. Our results are in accordance with the results obtained on treatment with S-allylcysteine, which showed successful improvement of long term non-spatial memory in senescence-accelerated mice prone 10 (Hashimoto *et al*., 2020). Whereas in morris water maze test, escape latency was measured. It is a widely used method for measuring retention memory and spatial learning abilities. Ability of phenytoin challenged mice to reach hidden platform is decreased, so there is an increase in escape latency time. This indicates compromised condition of retention memory. There is a similarity in results obtained with Li *et al*. (2014), where there is an increase in escape latency time in control group animals. Decrease in escape latency time with all the treatment groups indicate retention of memory. Whereas, TL is the parameter measured in rod walking test. Similar to elevated plus maze test, TL is also used to measure spatial memory. Results of this test are in accordance with elevated plus maze test. Increase in TL indicates decrease in memory, whereas decrease in TL in all treatment groups indicates increase in memory. An attempt was made to measure muscle relaxation effect caused by chronic dose of phenytoin. Fall off time is the parameter measured in rotarod. Loss of muscle coordination, muscle pain and muscle stiffness are some of the side effects caused by chronic dose of phenytoin. Significant decrease in fall off time indicates phenytoin induced muscle complications. Whereas, in treatment groups fall off time was increased representing phenytoin induced muscle abnormalities are controlled.Thus, all the results of treatment groups showed significant cognitive improvement effect. These results are in accordance with the fall off time reported with other studies (Tippu *et al*., 2018).

Chronic dose of phenytoin was also found to disrupt the levels of AChE in brain (Sudha *et al*., 1995). This is one of the possible mechanisms to induce memory impairment. Therefore, memory impairment is associated with increase in the levels of AChE in brain. Ali *et al*. (2011) reported memory impairment caused by decreased acetylcholine level in hippocampus, probably due to increase in levels of AChE in those regions. Therefore, cholinergic

system plays an important role in learning and memory and loss of these neurons will results cognitive impairment (Blake *et al*., 2014). In all the treatment groups, cognitive improvement is achieved by reducing the levels of AChE. Similar results are shown in study conducted by Sharma *et al*. (2009), where AChE level was decreased in different regions of brain on treatment with curcumin. This is one of the possible mechanisms how the *R. sativus* is improving cognitive functions. Chronic exposure of animals to phenytoin resulted in oxidative stress (Bhuller *et al*., 2006). Increase in the levels of MDA in control animal brain homogenates, is an indicator of generation of toxic radicals. On the other side, GSH function as a scavenger and protect cells against oxidative stress. Decrease in GSH levels in control animals indicate oxidative stress caused by chronic dose of phenytoin. These results are in agreement with the results publicized by Reeta *et al*. (2009). Decrease in MDA levels and increase in GSH levels indicate possible mechanism to inhibit cognitive impairment in different treated groups in this study. SOD acting as an important antioxidant enzyme, involved in defence mechanism against oxidative stress. In the present study, decreased levels of SOD in control animals shows phenytoin induced toxicity. Whereas, increased levels indicate inhibition of cognitive impairment in treated animals. These results of present study are in agreement with previously published work, where there is similar increase in levels of SOD, which improved memory in aged mice on treatment with *Polygala tenuifolia* (Li *et al*., 2014). NO level is also increased in control group animals, which is responsible for neutralizing antioxidant enzymes. Thus, responsible for neurodegeneration and finally cognitive impairment (Anadozie *et al*., 2019). Levels of NO increased in all treatment groups indicating improvement in cognitive function. Standard calibration curve of sodium nitrite with R^2 value of 0.9897 is used to estimate the concentration of NO in brain homogenate. Whereas, in histopathological studies control animals showed apoptosis in hippocampus region of the brain, this might be responsible for behavioral and biochemical abnormalities in mice. On treatment with piracetam and higher dose of RSWE morphology of cerebellum is not disturbed. However, in mice with lower dose of RSWE cerebral cortex showed apoptosis of neurons.

5. Conclusion

This study successfully proves the inhibitory effect of *R.sativus* on cognitive impairment effect produced on chronic exposure to phenytoin. Antioxidant activity is also reported and an important correlation is established between antioxidant effect and cognitive improvement effect. Results of this study also justifies the traditional claims of *R. sativus* in cognitive improvement activity. This study put forth an idea to isolate bioactive phytoconstituents from *R. sativus* extract and screen their cognitive improvement effect. Based on the findings of the present study, it is concluded that aqueous extract of *R. sativus* have cognitive improvement effect which is justified by having normal behavior in all *in vivo* screening models, normal levels of all biochemical parameters and also normal morphology of tissue in histopathological studies.

Conflicts of interest

The authors declare no conflict of interests relevant to this article.

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