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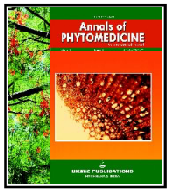

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Screening the protective effect of aqueous leaf extract of *Hibiscus rosa-sinensis* L. against phenytoin induced cognitive impairment in mice

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Abstract

Neurological disorders like Alzheimer's disease, dementia and parkinsonism are associated cognitive impairment. This study aims to evaluate the protective effect of aqueous extract of *Hibiscus rosa-sinensis* L. (HRWE) leaves against phenytoin induced cognitive impairment in mice. Phenytoin chronic treatment (50 mg/kg) for 10 days successfully induced learning and memory impairment in animals. HRWE showed antioxidant effects in hydroxyl radical scavenging assay and hydrogen peroxide scavenging assay. Ascorbic acid was used as standard for these assays. IC_{50} values are found to be 470 µg/ml and 450 µg/ml for H_2O_2 and hydroxyl radical scavenging assay, respectively. Cognition function of animals is assessed by using common behavioural animal models like morris water maze test (MWM), elevated plus maze test (EPM), cooks pole climbing test (CPC), novel object recognition test (NOR), rotarod test (RTR) and rod walking test (RDW). In all these behavioural models, control group animals showed reduced learning and defective memory indicated by, increase in reaction time, escape and transfer latency, and decrease in recognition index, fall off time and inflexion ratio. However, piracetam (standard drug) and HRWE treatment showed significant decrease in reaction time, escape and transfer latency and significant increase in recognition index, fall off time and inflexion ratio; this restoration indicates effective memory and improved learning behavior. At the end of study, brain homogenates are used to conduct biochemical estimations. Increase in levels of acetylcholinesterase (AChE), lipid peroxidase (LPO) and nitric oxide (NO), and decrease in levels of glutathione (GSH) and superoxide dismutase (SOD) in control group animals shows oxidative stress produced on phenytoin challenge. Piracetam is found to be most effective in restoring these enzymes to normal values. However, HRWE 100 mg/kg showed significant effects only in restoration of AChE, LPO and NO levels. In contrast, HRWE 200 mg/kg showed significant restoration of all biochemical enzymes as compared to control group. Important to state that the HRWE effect is less significant than the effect produced by piracetam. Histopathological studies are also conducted to explore morphological changes in brain samples of all the treatments. Apoptosis of neurons in control group animals' correlates to their abnormal behaviour in study. Whereas, normal histoarchitecture of brain examination of piracetam and HRWE treated animals, extrapolates to their cognitive improved behavior in study. All these results justify the protective effect of water extract of HRWE against phenytoin induced cognitive disabilities.

1. Introduction

Phenytoin is a commonly used antiepileptic drug. Even though, it is recognized for beneficial concepts, but it is also noted to induce impairment of cognitive functions. Disturbed memory, impaired learning, altered psychomotor functions are some of the adverse effects of phenytoin. These are produced at both lower and higher doses of phenytoin (Vohora *et al*., 2000; Sudha *et al*., 1995). Cognitive impairment is the major symptom of many degenerative brain diseases like, Alzheimer's disease, dementia, Huntington's disease, Parkinson's disease, multiple sclerosis. All these diseases have a vital impact on moral, financial and social life of not only patients but also family (Domaradzki, 2015; Lauterbach *et al*., 2010). Not only in the disease conditions, cognitive impairment also occurs in aged people. Normally as the individual grows older except few most of the cognitive functions decline (Hazzard, 2009). Therefore, it is an important behaviour of life that has to be controlled. In general, cholinergic drugs are used to alleviate these cognitive problems (Mangialasche, 2010). These drugs usually act by inhibiting AChE enzymes. Many other plants are also explored for this AChE inhibitory activity (Abubucker, 2021). However, effects on cognitive improvement are very mild. As these drugs have short half-life, frequent usage is the major challenge for the patients (Polinsky, 1998). Along with this, usage of cholinergic drugs is also associated with renal failure, vomiting, *etc.* (Rogers, 1998). Another important drug usually studied for this purpose is piracetam (Moran, *et al*., 2002). Piracetam is a nootropic drug, usually act by enhancing cholinergic neurotransmission (Wurtman *et al*., 1981). However, frequent use of piracetam results in dysphoria, amnesia, and psychomotor agitation (Talih and Ajaltouni, 2015). Hence, there is a need of safe and effective

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drugs which can reverse cognitive abnormalities. Since last few decades, plant-based drugs are gaining popularity in treatment of many life-threatening conditions like cancer, diabetes, neurological disease.

H. rosa-sinensis (HRWE) is one of the well-known medicinal plants. It is commonly called as queen of tropics. This plant belongs to family of flowers called as Malvaceae (Braglia *et al*., 2010). Every part of the plant is reported to possess therapeutic activity. Anti-inflammatory (Raduan *et al*., 2013), antimicrobial (Nayak *et al*., 2015), antitumour (Goldberg *et al*., 2017), antioxidant (Masaki *et al*., 1995), antidepressant (Khalid *et al*., 2014), antidiabetic (Afiune *et al*., 2017) are the few important medicinal uses of flowers of HRWE. Leaves and roots are reported to have antibacterial, antifungal (Hemarana *et al*., 2014; Sobhy *et al*., 2017), antidiabetic (Moqbel *et al*., 2011; Kumar *et al*., 2013), antipyretic (Soni and Gupta, 2011; Daud *et al*., 2016) activities. Apart from these, HRWE is also reported for many other activities. All these activities are due to different phytoconstituents like quercetin, quercetin-3-diglucoside, β-sitosterol, cyanidin-3,5-diglucoside (Jadhav *et al*., 2009), cyanidin-3-sophoroside (Senathirajah *et al*., 2017), luteolin-8-C-glucoside (Zaki *et al*., 2017). Flowers and roots of the plant are also reported for neuroprotective activity (Hanumakonda and Maheshwaram, 2016; Begum *et al*., 2016). Scientists now-a-days are focusing towards development of plant-based treatment of neurodegenerative diseases (Imad *et al*., 2020; Abdulrahman, 2019). Use of plant-based medicines is not only effective but also safe. Many drugs in market are derived from plant extracts. Therefore, present study is designed to evaluate protective effect of water extract of leaves of HRWE against phenytoin induced cognitive impairment in mice.

2. Materials and Methods

2.1 Chemicals required for study

Chemicals procured for the study are analytical grade. Piracetam and phenytoin are obtained from Dr. Reddy's Labs, Hyderabad and Abbott Healthcare Pvt. Ltd., Hyderabad, respectively. Acetyl acetone, ammonium acetate, K_2HPO_4, DMSO, ferrous ammonium sulphate, glacial acetic acid, H_2O_2, NADPH, and KCl, are procured from SDFCL Pvt. Ltd., Hyderabad. Other chemicals used in the study are procured as follows, hydroxylamine hydrochloride (Accord Labs, Hyderabad), EDTA (Essel Fine Chemicals), ascorbic acid, KH_2PO_4, TCA (Finar Chemicals), acetylthiocholine, DTNB, nitroblutetrazolin (SRL Pvt. Ltd., Hyderabad).

2.2 Selection, extraction and antioxidant activity of *H. rosa-sinensis*

HRWE leaves are collected in the month of January 2021. Mainly, the plants located in Hyderabad are selected for the study. This plant is subjected to authentication at Botanical Survey of India and a specimen (MESCO/PCOG/2020/046) is deposited in Department of Pharmacognosy, MESCO College of Pharmacy. These leaves are washed under running water to remove dirt and waste, dried in shade for around 8 days. These leaves after grinding to coarse powder, subjected to water extraction by using Soxhlet extractor. This process of extraction was continued for about 1 week to get sufficient amount of extract. This is subjected to *in vitro* antioxidant activity by using hydroxyl radical scavenging assay and H_2O_2 radical scavenging assay. In hydroxyl radical scavenging assay, test tubes containing 0.5 ml of 0.018% EDTA, 0.1 ml of iron-EDTA solution, 0.1 ml of 0.85% DMSO solution and 0.1 ml of HRWE (100 - 500 µg/ml) add 0.5 ml of 0.22% ascorbic acid. Contents are mixed and incubated at 80°C, to this add 0.1 ml of 17.5% TCA, and 3 ml of NASH reagent. Finally, contents are incubated for 15 min for the development of yellow color which is measured at 412 nm (UV-Visible spectrophotometer) Klein *et al*. (1981). Whereas, in H_2O_2 assay, to the test tubes containing 0.1 ml of HRWE (100 - 500 µg/ml) add 0.4 ml of pH 7.4, 50 mM phosphate buffer was added. After adding 0.6 ml of 2 m M H_2O_2 solution, mix it for 15 min and record absorbance at 230 nm (Ruch *et al*., 1989; Bhatti *et al*., 2015). For both assays, ascorbic acid was used as standard and % inhibition was calculated by using the formula:

% inhibition = $[(A_0 - A_1)/A_0] \times 100$. where: A_0= Control absorbance, A_1 = Sample absorbance.

2.3 Methods used for screening protective effect of HRWE against phenytoin induced cognitive impairment

2.3.1 Selection of animals, toxicity studies and grouping

Animal studies are approved by IAEC of Deccan School of Pharmacy (1773/PO/Re/S/14/CPCSEA). Acute toxicity study is conducted on swiss albino mice by using OECD-425 guidelines. After selection of dose, animal grouping is done for main study. Around 25 swiss albino mice of both sexes are selected and divided in to 5 groups with 5 animals in each. Animals receiving only vehicle considered as normal group (Group I), other groups (Groups II to V) received phenytoin 50 mg/kg, i.p for 10 days to induce cognitive impairment (Sudha *et al*., 1995). Animals receiving phenytoin and vehicle served as control group (Group II), whereas, animals receiving piracetam (100 mg/kg, i.p), HRWE 100 mg/kg, p.o. and HRWE 200 mg/kg p.o. are considered as Groups III, IV and V, respectively.

2.3.2 Effect of HRWE on behavioral studies

To screen the cognitive improvement effect of HRWE, different models are used, *viz*., MWM test, EPM test, CPC test, NOR test, RTR test, and RDW test. Before screening, all the animals are trained for 2 days. Readings are taken on 0thday, 1stday 5th day, and 10th day.

In MWM test, a plastic water tank is used which is divided in to 4 quadrants, *viz*., north, south, east and west. It is about 80 cm X 40 cm, with a central platform of 6 cm X 15 cm. Water (25°C) is filled in the tank such that it is 1.5 cm above the platform and it is made opaque by adding non-toxic color. Experimental room is provided with external clues. Animal is placed at the fixed starting point and time taken to find the hidden platform is recorded as "escape latency" (Morris, 1981). Whereas, in EPM test, transfer latency (TL) is calculated. This maze (height = 25 cm from floor) has 2 open and 2 closed arms. Open and closed arms have dimensions like 25 cm X 5 cm and 25 cm X 5 cm X 20 cm, respectively. By keeping the animal on central platform (5 cm X 5 cm), time to taken to enter closed arm is recorded as TL. Total duration of experiment is about 2 min. Inflexion ratio (IR) is calculated as IR = $(L_1 - L_0)/L_0$. where L_0 =TL after nth day and L_1 = initial TL(s) (Jaiswal and Bhattacharya, 1992; Dhingra *et al*., 2012). In pole climbing test, equipment consists of iron grid platform, a central wooden pole. A conditioned and an unconditioned stimulus is provided to animal kept on the grid. A 50 Hz buzzer sound act as conditioned, whereas 1mA shock applied through grid function as unconditioned stimulus. Endpoint of procedure is the ability of an animal to jump on pole after buzzer sound or maximum of 30 sec after giving foot sock. Time taken by an

animal to give this endpoint is recorded as reaction time (RT) (Hanumakonda and Maheshwaram, 2016; Kumar and Minshu, 2016).

In NOR test, open field of dimensions about 40 cm X 40 cm X 30 cm is used. On all sides, field is provided with walls of height 40 cm during training period animal is allowed to explore the field with two similar objects for 5 min on the day of experiment. This is repeated, later on, one old object is replaced with the new object. Time spent by animal near to old (TO) and new (TN) is recorded. Recognition index (RI) is calculated as RI = TN/(TO + TN) * 100] where TO is time spent exploring old object and TN is time spent exploring new object (Batool *et al*., 2016; Antunes and Biala, 2012; Ennaceur and Delacour, 1988). In RTR test, multi compartment apparatus is used. Rod is allowed to rotate on a fixed speed of about 20-25 rpm, and time taken by the animal to fall off from rod is recorded (Kuribara *et al*., 1977). Whereas, in RDW test, ability of an animal to walk on a horizontal stationary rod is recorded. Rod is placed 23 cm above the floor and about 100 cm long and 5 mm in diameter. After placing animal at the center, time taken by the animal to reach the end of rod is recorded as TL (Kamila *et al.*, 2014).

2.3.3 Effect of HRWE on biochemical parameters histopathological studies

At the end of study, on the 11th day, all animals are sacrificed and brain isolated. 0.1 M, pH 7.4 cold phosphate buffer is used to make 10% w/v brain homogenate. This homogenate is used for biochemical estimations. For determination of AChE activity, test tube containing 0.1 ml of 1 mM DTNB, 2.6 ml of 0.1 M phosphate buffer pH 8.0 and 0.4 ml of brain homogenate is mixed thoroughly. Endpoint of reaction is the formation of yellow color compound whose absorbance is measured at 412 nm. After recording basal reading (initial stable value) 20 µl of 0.075 M acetylthiocholine is added to above mixture and incubated for 5 min. Final change in absorbance is recorded (Ellman *et al*., 1961). AChE activity was calculated as follows: R = 5.74×10^{-4} x "A/CO; where, R = Rate in moles of substrate hydrolyzed/min/mg of tissue, "A = 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). Whereas, for determination of amount of malondialdehyde (MDA/TBARS) in brain homogenate, 0.5 ml of 30% trichloroacetic acid and 0.5 ml of 0.8% thiobarbituric acid is taken in a test tube. To this, add 1 ml of brain homogenate and incubate for 1 h at 80°C on water bath. Later on, it is allowed to cool down for 30 min. Mixture is centrifuged for 15 min at 3000 rpm and supernatant is collected. End product of reaction is the formation of pink color compound whose intensity is measured at 540 nm. Blank solution consists of 1 ml of distilled water in place of brain homogenate (Okhawa *et al*., 1979). Nanomole of MDA/mg protein is calculated as $\frac{\text{Nanomole of MDA}}{\text{mg protein}} = \left(\frac{V \times OD_{540}}{0.156}\right) \times$ mg of protein where, V = Final volume of the test solution; OD = Optical density at 540 nm.

For determination of GSH, 900 µl of 20 mM, pH 4.7 EDTA solution, 1000 µl of 0.2 M, pH 8.2 tris-EDTA buffer is taken in a test tube. Test tubes are incubated for 30 min at room temperature after adding 100 µl of brain homogenate or pure GSH and 20 µl of Ellman's reagent (10 mmol/l DTNB in methanol). Endpoint of reaction is the formation of yellow colored compound, 5- thionitrobenzoic acid (TNB). Supernatant is collected after centrifugation and absorbance is recorded at 412 nm. Absorbance reading is used to calculate nanomole of GSH present in mg of protein (Sedlak and Lindsay, 1968; Khynriam and Prasad, 2003).

$$\frac{\text{Nanomole of GSH}}{\text{mg protein}} = \left(\frac{\text{Dilution factor} \times OD_{412}}{1.36 \times 10^4}\right) \times 100.$$

Formula used is where 1.36×10^4 = Extinction coefficient; dilution factor = 10. For determination of SOD, 1.06% of 1 ml of sodium carbonate solution, 0.4 ml of 24 mmol/l of nitro blue tetrazolium, 0.2 ml of 0.37% of EDTA and 0.1 ml of brain homogenate is taken in a test tube. 0 min absorbance is taken at 560 nm. After adding 0.4 ml of 1 mM hydroxylamine hydrochloride above mixture is incubated at 25°C for 5 min and final absorbance is recorded (Misra and Fridovich, 1972). % Inhibition of NBT reduction by SOD is calculated as % inhibition of NBT reduction by SOD = $\left(\frac{\text{Control OD} - \text{Treatment OD}}{\text{Control OD}}\right)$ 100 = X% inhibition 50% inhibition is equal to 1 unit of enzyme then X% = $\left(\frac{1}{50}\right)$ X = Y Unit. NO levels in brain tissue are calculated by using standard curve of sodium nitrite (Figure 1). Reaction mixture consists of 1.5 ml of griess reagent and 0.3 ml of brain homogenate. Absorbance of above mixture is recorded at 546 nm and NO level is calculated as ng/mg of protein. For histopathological studies, brain of one animal from each group is fixed in 10% formalin. Morphological observation is conducted by using brain sections of 5 µm thickness. Staining process is carried out by using hematoxylin and eosin dye.

2.3.4 Statistical analysis

All the values are represented as mean ± SEM. Analysis is carried out by using one-way ANOVA followed by Dunnett's multiple comparison test in Graph pad prism 9.0 software. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$ and $p<0.05$ ns = non-significant.

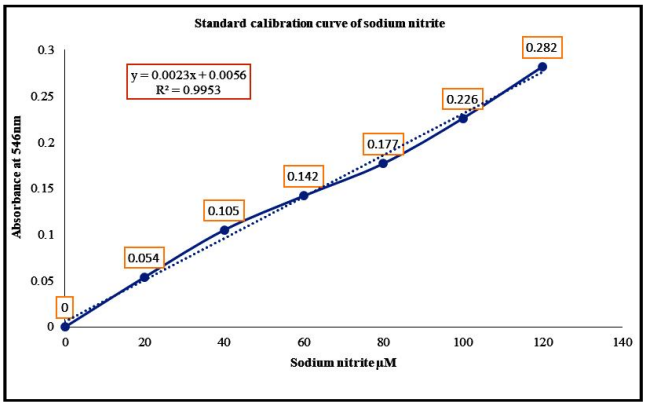

Figure 1: Concentration of sodium nitrite in µM is plotted on X-axis and its absorbance at 546 nm is plotted on Y-axis to obtain calibration graph of sodium nitrite.

3. Results

3.1 Plant extraction and effect of HRWE on *in vitro* antioxidant activity and toxicity studies

Extraction of leaves of HRWE yielded dark coloured semisolid extract with a percentage yield of 11.5%. Scavenging activity of HRWE in both H_2O_2 and hydroxyl radical scavenging assay increased from 100 µg/ml to 500 µg/ml. IC_{50} values are found to be 470 µg/ml and 450 µg/ml for H_2O_2 and hydroxyl radical scavenging assay, respectively. HRWE in both assays is less potent than standard ascorbic acid. IC_{50} values are found to be 200 µg/ml and 90 µg/ml for H_2O_2 and hydroxyl radical scavenging assay, respectively (Figure 2). In acute toxicity studies, no mice showed signs of toxicity throughout the study duration at 2000 mg/kg. Therefore, very low doses 1/20th and 1/10th, *i.e.*, 100 mg/kg and 200 mg/kg are selected for the study, respectively.

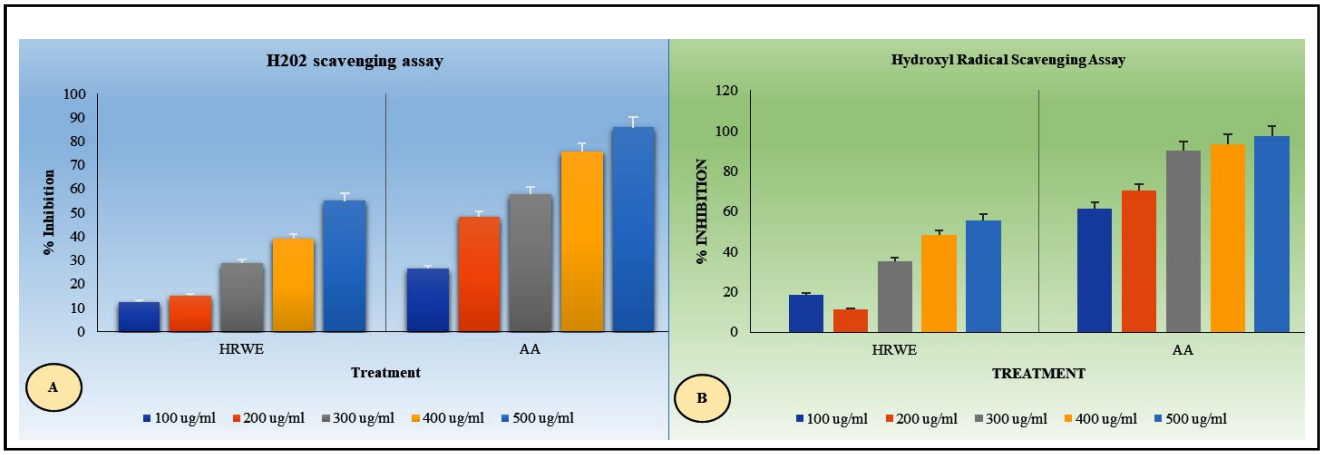

Figure 2: ***In vitro*** **antioxidant activity of HRWE by using ascorbic acid as standard. A: H_2O_2 scavenging assay, B: Hydroxyl radical scavenging assay.**

3.2 Effect of HRWE on *in vivo* cognitive improvement activity

In MWM test, escape latency was found to increase in control group animals from day 1 (p<0.0001) to day 10 (p<0.0001) as compared to normal group. In piracetam treated animals, escape latency decreases from day 1 (p<0.0001) to day 10 (p<0.0001) as compared to control group. HRWE 100 mg/kg has not showed any significant effect, but HRWE 200 mg/kg has showed significant decrease in escape latency from day 1 (p<0.05) to day 10 (p<0.0001) as compared to control group. In EPM test, IR decreased from day 1 to day 10 as compared to normal group. Animals treated with piracetam showed significant increase in IR from day 1 (p<0.01) to day 10 (p<0.001) as compared to control group. Whereas, animals treated with both doses of HRWE has not showed significant increase in IR as compared to control group. RT in seconds is recorded in CPC test. RT significantly increased in control group animals from day 5 (p<0.001) to day 10 (p<0.0001). In piracetam treated group RT significantly decreased from day 1 (p<0.001) to day 10 (p<0.0001) as compared to control group. HRWE 100 mg/kg showed decrease in RT from day 1 to day 10, but changes are not significant. However, HRWE 200 mg/kg showed significant decrease in RT from day 1 (p<0.01) to day 10 (p<0.001) as compared to control group. In NOR test, RI significantly decreased in control group animals from day 1 (p<0.05) to day 10 (0.0001). Piracetam group showed significant increase in RI from day 1 (p<0.01) to day 10 (p<0.0001) as compared to control group. HRWE 100 mg/kg and 200 mg/kg showed significant increase in RI only on day 10 (p<0.001) as compared to control group (Table 1).

Table 1: Effect of HRWE on escape latency (MWM test), inflexion ratio (EPM test), reaction time (CPC test), and recognition index (NOR test).

Treatment	Day 0	Day 1	Day 5	Day 10
Escape latency in sec (Morris water maze test)				
Normal	60 ± 1.9 ^{ns}	32 ± 2.2****	24 ± 1.9****	19 ± 1.5****
Control	63 ± 1.9	53 ± 1.5	49 ± 1.7	45 ± 1.7
Piracetam 100 mg/kg	65 ± 1.4 ^{ns}	41 ± 0.98***	35 ± 1.3****	27 ± 1.2****
HRWE 100 mg/kg	60 ± 0.93 ^{ns}	55 ± 1.2 ^{ns}	45 ± 1.5 ^{ns}	39 ± 0.89 ^{ns}
HRWE 200 mg/kg	60 ± 1.50 ^{ns}	46 ± 1.8*	37 ± 2.3***	31 ± 2.4****
Inflexion ratio (Elevated plus maze test)				
Normal	--	0.084 ± 0.034^{ns}	0.21 ± 0.061 ^{ns}	0.1 ± 0.043 ^{ns}
Control	--	-0.36 ± 0.07	-0.42 ± 0.077	-0.53 ± 0.04
Piracetam 100 mg/kg	--	1.1 ± 0.52**	1 ± 0.39**	1.7 ± 0.5***
HRWE 100 mg/kg	--	0.24 ± 0.12 ^{ns}	0.14 ± 0.16 ^{ns}	0.22 ± 0.097 ^{ns}
HRWE 200 mg/kg	--	0.25 ± 0.037 ^{ns}	0.56 ± 0.39*	0.16 ± 0.067 ^{ns}

Reaction time in sec (Cooks pole climbing test)				
Normal	77 ± 2.5^{ns}	59 ± 1.6^{ns}	32 ± 2.6***	16 ± 1.4****
Control	77 ± 1.8	69 ± 1.6	60 ± 1.8	45 ± 1.8
Piracetam 100 mg/kg	79 ± 2.6^{ns}	51 ± 2.8***	34 ± 3.8***	18 ± 2.5****
HRWE 100 mg/kg	76 ± 1.8^{ns}	63 ± 3.2^{ns}	52 ± 1.7^{ns}	40 ± 2.6^{ns}
HRWE 200 mg/kg	76 ± 1.9^{ns}	54 ± 3.9**	40 ± 2.7****	30 ± 1.6***
Recognition index in sec (Novel object recognition test)				
Normal	55 ± 1.8^{ns}	55 ± 3.7*	60 ± 60***	59 ± 2.7****
Control	51 ± 3.7	38 ± 8.2	40 ± 1.9	25 ± 1.7
Piracetam 100 mg/kg	60 ± 2.3^{ns}	61 ± 4.1**	65 ± 2.5****	75 ± 1.1****
HRWE 100 mg/kg	52 ± 2.1^{ns}	48 ± 1.3^{ns}	49 ± 1.2^{ns}	44 ± 40***
HRWE 200 mg/kg	53 ± 1.8^{ns}	50 ± 1.4^{ns}	53 ± 4.1*	47 ± 5.9***

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.

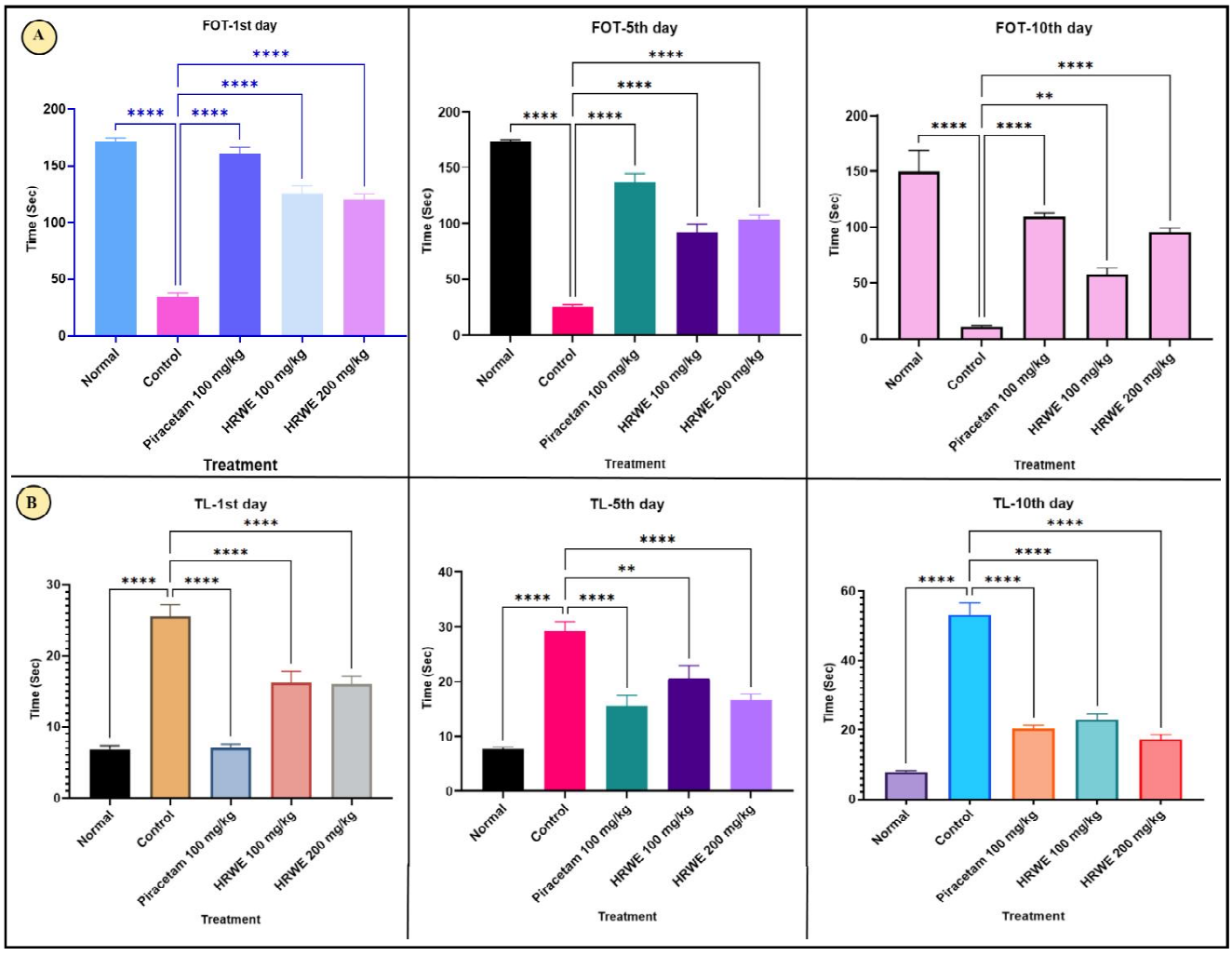

Figure 3: Effect of HRWE on fall off time (FOT) recorded in RTR test and transfer latency recorded in RDW test. All values are expressed as mean ± SEM, and tested by One-way ANOVA followed by Dunnett's multiple comparison test, n=5, * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001 and ns = non-significant as compared to control group.

In RTR test, fall off time significantly decreased in control group from day 1 (p<0.0001) to day 10 (p<0.0001). Fall off time significantly increased in all the treatment groups as compared to control group from day 1 (p<0.0001) to day 10 (p<0.0001) as compared to control group. Effect is more significant in HRWE 200 mg/kg when compared to HRWE 100 mg/kg, however, this is less than piracetam. TL is calculated in RDW test, there is significant increase in TL in control group from day 1 (p<0.0001) to day 10 (p<0.0001). However, TL decreased significantly in all treated groups from day 1 (p<0.0001) to day 10 (p<0.0001) as compared to control group. Piracetam is more effective than both doses of HRWE, whereas, effect of HRWE is dose dependent (Figure 3).

3.3 Effect of HRWE on biochemical parameters and histopathological studies

In biochemical estimations, control group animals showed significant increase in AChE levels (p<0.0001), LPO levels (p<0.0001), and NO levels (p<0.0001). Similarly, these animals showed significant decrease in GSH levels (p<0.0001) and SOD levels (p<0.0001). Piracetam treated animals showed significant decrease in AChE levels (p<0.0001), LPO levels (p<0.0001), NO levels (p<0.0001) and significant increase in GSH levels (p<0.0001) and SOD levels (p<0.0001) as compared to control group. HRWE treatment showed dose dependent effect. Lower dose of HRWE, *i.e.*, 100 mg/kg showed significant decrease in AChE levels (p<0.01), LPO level (p<0.001) and NO levels (p<0.05) as compared to control group. Whereas, higher dose of HRWE, *i.e.*, 200 mg/kg showed significant decrease in AChE levels (p<0.0001), LPO levels (p<0.0001), NO levels (p<0.0001) and significant increase in GSH levels (p<0.05) and SOD levels (p<0.0001) as compared to control group. However, effect of HRWE is less than piracetam (Table 2). In histopathological studies, control group animals showed apoptotic neurons in cerebrum, piracetam treated animals showed normal morphology of cerebrum. HRWE 100 mg/kg and 200 mg/kg showed normal morphology of cerebellum without any signs of toxicity (Figure 4).

Table 2: Determination of AChE activity, malondialdehyde (MDA/TBARS), GSH, SOD and NO levels in the brain tissue of mice

Treatment	AChE (µM of acetylthiocholine hydrolyzed/min/mg of tissue)	LPO (Nanomole of MDA/mg of protein)	GSH (nanomole/mg of protein)	SOD (SOD activity (U/mg protein)	NO
Normal	1.1 ± 0.071****	64 ± 0.94****	19 ± 1.1****	3.3 ± 0.055****	90 ± 1.512****
Control	3.0 ± 0.234	122 ± 1.64	8.9 ± 0.81	2 ± 0.114	128 ± 2.335
Piracetam 100 mg/kg	1.1 ± 0.181****	73 ± 2.82****	16 ± 1.7****	3.2 ± 0.026****	93 ± 1.347****
HRWE 100 mg/kg	2.0 ± 0.152**	95 ± 3.27***	11 ± 1.1^{ns}	2.2 ± 0.045^{ns}	118 ± 3.118*
HRWE 200 mg/kg	1.5 ± 0.172****	86 ± 8.014****	13 ± 0.63*	2.5 ± 0.036****	106 ± 1.427****

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.

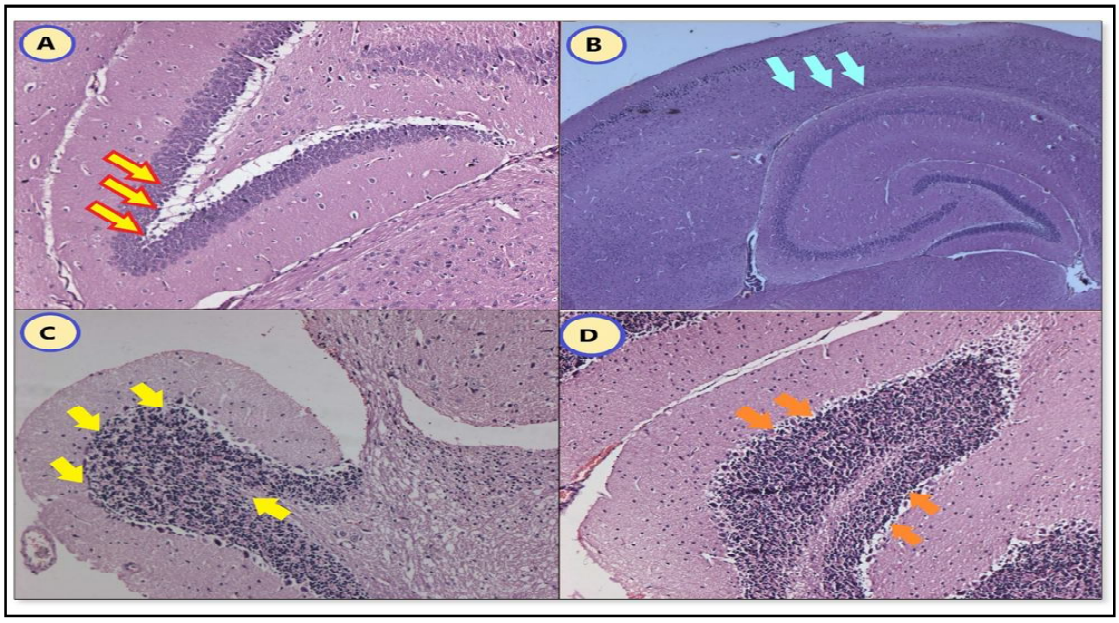

Figure 4: Effect of RSWE on mice brain. A: Control group: yellow arrow marks with red border indicates location of apoptotic neurons in cerebrum of phenytoin challenged mice. B: Arrow marks indicate normal morphology of cerebral cortex of Group II animals. C: Morphology of neurons is normal in cerebellum of HRWE 100 mg/kg treated group. D: Arrow marks represent normal cerebellum without any signs of toxicity.

4. Discussion

Free radicals have an impact on health of human beings. Similarly, oxygen in few forms (reactive oxygen species) have very harmful effects on human body. Free radicals and reactive oxygen species are produced in human body either by normal metabolic process or by exposure to industrial chemicals, smoking, and X-rays, *etc.* (Lobo *et al*., 2010). These are to be detoxified necessarily for cellular survival (Gill *et al*., 2011). They are effectively neutralized by synthetic antioxidants, *viz*., butylated hydroxytoluene and propyl gallate, *etc*., but their regular use is reported with severe side effects (Xiu-Qin *et al*., 2009). In this context, it is mandatory to search for new and safer antioxidants. Plants are good source of regimen for many severe diseases (Imad Uddin and Veeresh, 2020). HRWE is reported to possess many important phytoconstituents like flavonoids, carbohydrates, glycosides, steroids, tannins, phenols, proteins, and triterpenoids (Ghaffar *et al*., 2012; Rajesh *et al*., 2011). Total phenolic content and total flavonoid content of leaves of HRWE was found to be 62.4% (mg/g catechol equivalent) and 73.2% (mg/g quercetin equivalent), respectively. These are majorly responsible for antioxidant activity. Many studies reported similar results, that these phytoconstituents are responsible for antioxidant effects of HRWE leaves (Rengarajan *et al*., 2015). In this study, antioxidant activity of leaves of HRWE can be extrapolated to presence of these phytoconstituents. Hence, establishment of antioxidant activity directs the study to screen cognitive improvement effect of leaves of HRWE.

A commonly used antiepileptic drug, phenytoin at a dose of 50 mg/kg reported to impair cognitive abilities (Mondadori and Classen, 1984). So, it is given to all groups except normal to induce cognitive impairment. To screen protective effect of HRWE, various behavioral models are used. Retention and spatial memory are evaluated by using a very common model of MWM test. This is measured in terms of escape latency. Control group animals showed decrease in ability to reach central platform, indicated by increase in EL, this denotes impaired spatial and retention memory. These results are in accordance with study conducted by Churchill *et al*. (2003), in which phenytoin induced memory impairment is evaluated on MWM test. In piracetam, and HRWE 200 mg/kg treated groups, there is significant decrease in EL, indicating restoration of memory. This effect is not observed with HRWE 100 mg/kg. Similar restoration of learning and memory in water maze test is also reported with ethanolic extract of *Cressa cretica* (Khare *et al*., 2014). Kumar *et al*. (2007), reported the EPM test for screening cognitive abilities. TL represented as inflexion ratio is recorded in EPM test. It is a measure of spatial memory. Control group animals showed decrease in IR representing memory loss. This ratio is increased in HRWE groups, but change is not significant except on day 5 with HRWE 200 mg/kg. Increase in IR is directly related to memory improvement. Similar results are reported with *Orthosiphon stamineus* (Retinasamy *et al*, 2019). CPC test is a very common method to screen cognitive abilities. Conditioned and unconditional stimuli are used to assess long-term memory functions of animals. RT is recorded in this test. Increase in RT in control group animals represents loss of memory. Decrease in RT indicates memory improvement in piracetam, and HRWE 200 mg/kg treated groups. This indicates that HRWE 100 mg/kg is not sufficient to restore memory function in this test. Similar results are reported by Ramarao *et al*. (2018), with water extract of *Tridax procumbens*. Non-spatial long-term memory is assessed by NOR test. RI is recorded for all animals. Decrease in RI indicates disturbed memory in Group II animals, on reverse increase in RI indicates retention of memory. This memory retention effect is shown all the animals treated with piracetam, but in HRWE treated groups, this effect is reported only on 10^{th} day with both doses. Similar results are reported with respect to NOR by Hashimoto *et al.* (2020).

Phenytoin a part form inducing learning and memory impairment, it is also reported with muscle relaxation effect on chronic use. So, fall off time in RTR test is used to measure this effect. Decrease in FOT in control group animals showed muscle relaxation. Significant increase in this FOT in all treated groups indicates normal functioning of muscles. Tippu *et al*. (2018) reported similar effects in swiss albino mice. RDW test is used to record TL of animals. It is used to assess spatial memory of mice. Increase in TL in control group animals show disturbed memory, which is restored in all the treated groups indicated by decrease in TL.

Maurer and Williams (2017) reported the importance of cholinergic system in normal life. Degradation of these neurons severely impairs memory and learning abilities. One important cause for decrease in acetylcholine levels is the increase in activity of AChE in brain (Silveyra *et al*., 2012). Phenytoin is reported to decrease acetylcholine levels in different parts of brain of animals (Agarwal and Bhargava, 1964). In our study, brain AChE levels are increased in control group animals representing deleterious effects on memory and learning process. This level is restored on treatment with piracetam and both doses of HRWE, indicating their potential effect in restoring learning and memory process by improving cholinergic system of brain. Our results are consistent with study conducted by Khan *et al*. (2012). LPO is measured in terms of TBARS, increased in levels of TBARS indicate inhibition of metabolic enzymes (Nobre-Junior *et al*., 2009; Matsunami *et al*., 2010; Tamanna *et al*., 2020). In this study, phenytoin challenged mice showed increase in levels of TBARS indicating enzymatic damage to host cells. Decrease in TBARS level indicate protection provided to metabolic enzymes by piracetam and HRWE. NO and its derivatives are the indicators of oxidative stress, leading to neurological disturbances (Chung and David, 2010). Increase in levels of NO in control group animals represents cognitive disabilities. This level is decreased in all treatment groups indicating their protection against NO stress and retaining cognitive functions.

SOD is well known to convert harmful superoxide in to hydrogen peroxide and oxygen molecule (Buettner, 2011). Whereas, GSH acts by converting hydrogen peroxide into water (Lubos *et al*., 2011). Decrease in their level in group II animals can be extrapolated to their neurological related problems like cognitive impairment. However, increase in their levels in treated animals is extrapolated to cognitive improvement. HRWE 100 mg/kg has not showed significant effect on these enzyme levels. Whereas, HRWE 200 mg/kg is more effective in restoring SOD levels than restoring GSH levels. In histopathological assessment, control group animals showed apoptosis of neurons in cerebrum, this neurotoxicity induced by phenytoin is in consistent with study conducted by Ohmori *et al*. (1992). Normal morphology of cerebral cortex and cerebellum in treated group animals indicate their neuroprotection effect, which can be extrapolated to restoration of behavioral and biochemical parameters in these groups.

5. Conclusion

Current study is clearly justifying the neuroprotective effect of aqueous extract of *H. rosa-sinensis*. Effect of HRWE was dose dependent throughout the study. Animals treated with HRWE showed

normal behavior in all the screening models, indicating their cognitive improvement effects. These effects are well supported by restoration of biochemical enzymes to normal levels in brain homogenate. Normal histoarchitecture of cerebrum and cerebellum of treated animals also supports the results obtained in *in vitro* and *in vivo* studies. Therefore, neuroprotective effect produced by HRWE can be extrapolated to their antioxidant action. Based on these considerations, current study presents an idea to conduct, further research, and also to explore molecular level mechanism involved in neuroprotective effect.

Conflicts of interest

The authors declare no conflicts of interest relevant to this article.

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