CHAPTER 4

BRAIN PENETRATION AND NEUROPROTECTION BY WITHANOLIDE A

Chapter Highlights

- ***Evaluation of Brain penetration ability of Withanolide A after intra-nasal administration in mice model***
- ***Analysis of neuroprotective ability of Withanolide A in bilateral common carotid artery occlusion model of global cerebral ischemia***

ABSTRACT

Cerebral ischemia reperfusion injury is one of the leading causes of global mortality and long-term disability. Due to the heterogeneous and complex nature of the ischemic cascade, very few therapeutic strategies are available to combat this neurodegenerative disorder. Such scenario demands identification of novel neuroprotective molecules and designing of new therapeutics for combating the ischemic pathophysiology. The Ayurvedic herb *Withania somnifera* is a traditionally used nootropic and recent scientific studies have established its ability as a neuroprotectant. One of the major phytochemicals of *W. somnifera* is Withanolide A (WA) which has been previously studied for its neuroprotective ability in amyloid toxicity, dopaminergic injury and hypobaric hypoxia. The present study evaluates the brain penetration ability of WA after intra nasal administration and assesses its neuroprotective ability in global cerebral ischemia. Three different doses of the phytochemical are evaluated and it was observed that WA post treatment significantly ameliorates cerebral ischemic pathophysiology by restoring BBB disruption and brain water content, reducing cerebral edema and lowering the ischemia induced elevated concentrations of neurotransmitters and other biochemical

parameters. The highest dose (10 mg/kg) of WA showed most promising results. Post-treatment with 10 mg/kg revealed marked reduction of morphological damage in cortical region due to cerebral ischemia. The highest dose also reduced apoptotic and necrotic cell death in cortex region. The neuroprotective ability exerted by WA in the global cerebral ischemia model, establishes the phytochemical as a neuroprotectant which can be further exploited as a future neurotherapeutics.

4.1. INTRODUCTION

Cerebral ischemia remains, till date, one of the leading causes of death, long-term disability and morbidity worldwide [1] and contributes to 70% of the cerebrovascular diseases effecting a huge population worldwide [2]. The major cause behind cerebral ischemia is identified as lack of oxygen and nutrient supply in brain tissue, due to obstructed blood flow, which causes neuronal damage as a consequence of energy depletion in the affected brain region [3]. The energy loss further contributes to overproduction of the excitatory neurotransmitter glutamate, thus promoting calcium influx [3] and subsequent free radical generation leading to lipid peroxidation cell membrane and blood brain barrier (BBB) disruption, cerebral edema, DNA damage and neuronal cell death [4-7]. Reperfusion following the ischemic blockage, often causes more severe damage to the brain cells [2, 8].

Presently, there are very few clinical strategies available for treatment of cerebral ischemia [9] and only one Food and Drug Administration (FDA) approved drug which is recombinant tissue plasminogen activator (rtPA) [3]. Though rtPA is clinically used, it has limitations of therapeutic window and poses the risk of transforming the ischemic insult into cerebral hemorrhage [3]. So, the current scenario requires identification of novel pharmacological compounds with neuroprotective ability. Neuroprotective strategies involve the idea of

ameliorating the pathophysiological conditions induced by ischemic insult and thus ensuring the survival of the neuronal cells [10, 11].

Withania somnifera (WS) is an Indian Ayurvedic herb, extract of which has been traditionally used a nootropic agent and a tonic [12]. WS has been also reported to enhance cognition in cases of Aβ and scopolamine induced memory loss [13, 14]. Virtual screening of the constituent phytochemicals of WS for assessing their inhibition potential against Poly-ADP-Ribose Polymerase 1 (PARP-1), a major molecular mediator involved in ischemic cell death, revealed that Withanolide A (WA) shows major binding affinity towards PARP-1. Also, unlike most of the other phytochemicals of WS that showed high affinity towards PARP-1, WA does not show any off-target interaction with the enzyme. A kind of literature study also revealed the ability of WA to exert neuroprotection in neurodegenerative disorders. WA is a steroidal lactone and a major constituent of WS extract [12]. The phytochemical has exhibited neuroprotective ability in Alzheimer's and Parkinson's disease by combating Aβ induced damage and dopaminergic neurodegeneration respectively [15, 16]. WA also augments regeneration of neurites, axons and dendrites [17, 18] and enhance stress resistance in *C. elegans*, thereby increasing its life expectancy [19]. WA also confers neuroprotection in hypobaric hypoxia by modulating glutathione biosynthesis pathway [12]. Our previous *in-silico* study, also predicted WA as a potent inhibitor of PARP-1, a molecular mediator of ischemic cell death pathway. The study also revealed its binding pattern with PARP-1 almost similar to the reported PARP-1 inhibitor Talazoparib. Hence, WA was further studied for its ability to combat cerebral ischemic pathophysiology.

Since our *in-silico* study predicted that WA might be impermeable to Blood Brain Barrier (BBB) an intra-nasal mode of administration was chosen. Intra-nasal administration facilitates

the delivery of BBB imperbeable drugs into the brain via the 'nose to brain' route. Intra-nasal delivery of drug has been recommended as an effective delivery method for studying the neuroprotective potential of the molecules that are not able to cross the BBB. In the present study, we first evaluated the ability of WA to penetrate brain after being administered intra-nasally. Further, the neuroprotective ability of the phytochemical against ischemic injury was assessed in the global cerebral ischemia model of mice. Global cerebral ischemia is induced in mice by using bilateral carotid artery occlusion (BCCAO) method. BCCAO is a simple, reproducible method widely used for studying pathophysiology of cerebral ischemia reperfusion injury and neuroprotection [1, 2 and 21]. Hence, the ability of WA to ameliorate ischemic insult is studied using BCCAO model.

4.2. MATERIALS AND METHODS

Inbred male Swiss albino mice (3-4 months old; weight: 30±2 gm) were used for all of following the experimental procedures. The animals were acclimatized under 12-h light/dark cycle with controlled temperature of 25±2°C and constant humidity for two weeks along with free access to laboratory food and water. Animal handling protocols and experiments were approved by Central Animal Ethical Committee at Institute of Medical Sciences, Banaras Hindu University, Varanasi (Registration No. 542/02/ab/CPCSEA).

4.2.1. Preparation of Withanolide A solutions

Withanolide A (WA) (≥ 95% pure) was purchased from Sigma-Aldrich (Cat No: W2145) and dissolved in methanol (Merck) to obtain standard solutions ranging from 0.05- 50 µg/ml. Different concentrations of WA was prepared in phosphate buffer saline (PBS) for animal experiments. For intra-nasal administration, 15 mg of WA was dispersed in 1ml of PBS to

obtain a concentration of 15 mg/ml and each animal was administered 20 µl of the homogenous dispersion to maintain a dose of 10 mg/kg. To maintain the homogeneity of the dispersion, solutions were well sonicated and vortexed before administration.

4.2.2. Administration of Withanolide A for brain penetration

36 Swiss albino mice (as mentioned in sectioned 4.2.) were divided randomly into 6 groups (n=6) according to different time points, i.e., 15 mins, 30 mins, 1hr, 2 hrs, 4 hrs and 6 hrs. The sample size was decided from literature studies and previous similar experiments conducted in the lab. 10 µl (5 mg/kg) of WA solution was administered intra-nasally in each nostril of the mice via a hollow tubing attached to a 25 µl Hamilton syringe. Administration of 10 µl WA via each nostril of each mouse maintained a dose of 10 mg/kg of WA. The animals were sacrificed at their respective time intervals and the brains were isolated carefully. The brain was further dissociated into cortex and cerebellum and homogenized in methanol. The homogenates are centrifuged and filtered (0.22 µm) to obtain cell free methanol extract.

4.2.3. Determination of WA concentration in mice brain

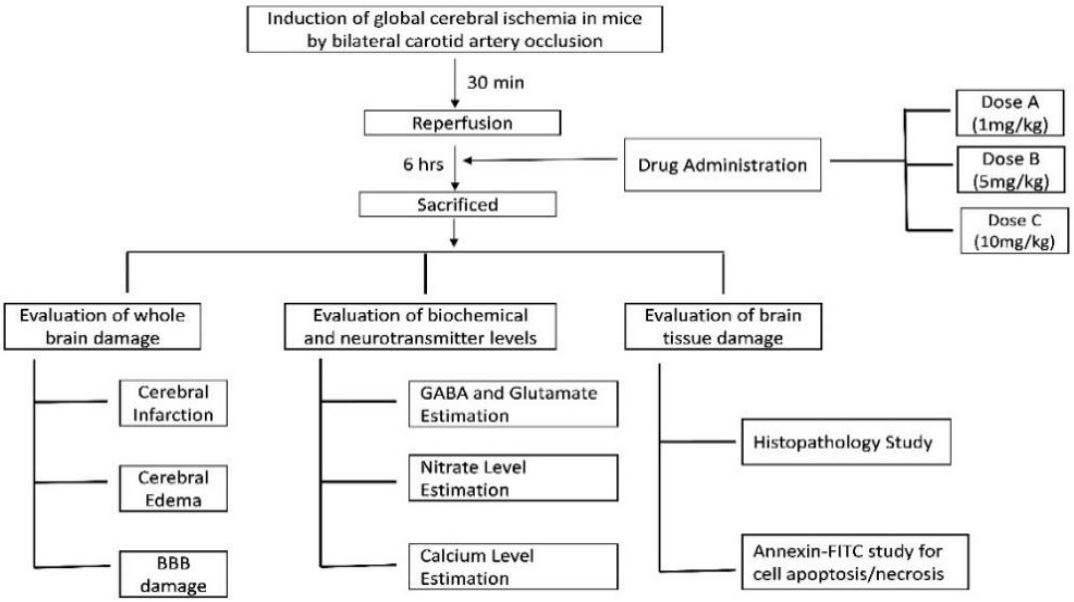
10 µl of WA standard solutions and methanol extracts were injected in HPLC-UV (Shimadzu Scientific Instruments, US) equipped with Kinetex Reversed Phase C18 column for concentration determination. Acetonitrile: water (70:30) was used as mobile phase and flow rate was kept at 1 ml/min. All the samples and standard solutions were analyzed at 227 nm and the concentration of WA in brain was determined from the standard curve obtained.

4.2.4. Global Cerebral ischemia induction

The animals (as mentioned in section 4.2) were anesthetized using intra-peritoneal injection of xylazine (10 mg/kg b.w.) and ketamine (50 mg/kg b.w.) and the common carotid arteries (CCAs) were carefully exposed by a ventral midline cut. The CCAs were occluded by tightening the loops around the arteries using non-absorbable silk sutures (3-0) [1, 2, 21].

Occlusion was continued for 30 minutes, after which the ligature was cut carefully with a sterile surgical blade and reperfusion was allowed for 6 hrs. The incision was stitched and the wound area was sterilized by applying Povidone iodine ointment. The sham group animals were subjected to similar procedures except for ligature of the CCAs. Throughout the surgery body temperature of the animals were maintained at 37±0.5°C and the animals were returned to their cages after surgery at controlled room temperature of 25±2°C with availability to food and water *ad-libitum*. The animals were sacrificed after 6 hrs reperfusion and brain tissue was processed for further studies accordingly. The work plan is illustrated in Fig. 4.1.

A mortality of 15% was associated with BCCAO. The animals which died during the experiment were not included in the experimental study groups.



4.1.: Schematic for study of neuroprotection by WA in mice model of global cerebral ischemia.

4.2.5. Post-treatment by different doses of WA

Three different doses of WA (1 mg/kg, 5mg/kg and 10 mg/kg) was administered intra-nasally in mice after removal of the occlusion. The doses are mentioned as Dose A (1 mg/kg), Dose B

(5mg/kg) and Dose C (10 mg/kg). The vehicle group animals received only PBS solution devoid of drug.

4.2.6. Determination of cerebral infarction

Efficacy of WA post treatment in global cerebral ischemia in mice was evaluated using triphenyltetrazolium chloride (TTC) staining [22]. The mice brains were carefully removed after sacrifice of the animals by cervical dislocation and were sliced into 2 mm coronal sections on an ice pack after rinsing in normal saline. The slices were incubated in 2% TTC (SRL; Cat No: 65599) solution for 30 minutes at 37°C and post-fixed in 10% formalin solution for another 30 minutes. The slices were then scanned at 600 dpi and the obtained images were quantified NIH Image J software. Average infarction of single brain was calculated by measuring percentage cerebral infarction of each 2mm slice (infarct area divided by total brain area). Average cerebral infarction observed in each group was also calculated.

4.2.7. Quantification of blood brain barrier (BBB) damage

Quantification of BBB damage was performed using Evan's Blue (EB) extravasation method as described by Martin et. al., 2010 [23]. EB (TCI Chemicals; Cat. No.: E0197) solution was intravenously injected lateral tail veins of the animals after after 30 minutes of occlusion and 6 hours of reperfusion. After 30 minutes of EB administration, intra cardiac perfusion of the animals were performed with chilled normal saline through the left ventricle. The animals were sacrificed via cervical dislocation followed by careful isolation of brains, which were further chopped into small pieces and dried at 56ºC for 48 hours. Each brain was washed in saline, air dried and weighed before chopping into pieces and weighed again after 48 hours. The pieces were incubated in formamide solution (8ml/1g dry tissue) for 48 hours at 56ºC and the blue colored formamide solutions obtained from different samples were transferred to 96 well plate

and absorbance was measured at 620 nm using multi-mode reader (BioTek Instruments, Inc., USA). The EB concentration was obtained using a previously prepared standard curve of eight exponential concentrations of EB in formamide solution (0.1- 12.8µg/mL).

4.2.8. Measurement of cerebral edema

Cerebral edema for each group was measured using the wet and dry-weight method, using the following formula to obtain the brain water content percent. [24]

$$\%\ of\ brain\ water\ content = 100 \times (wet\ weight - dry\ weight) \div dry\ weight \quad [23]$$

The animals from each group were decapitated and the weight of the freshly isolated brain was noted as wet weight and dried at 100°C for 48 hours to obtain a constant weight, which was recorded as dry weight and the percentage of brain water content was measured using the above formula.

4.2.9. Evaluation of neurotransmitters

Estimation of glutamate and ɣ-Aminobutyric acid (GABA) in different brain regions (cortex, cerebellum and hippocampi) were estimated using HPLC-UV method as reported by Wu et al., 2014 [25]. All the brain parts were homogenized in ice cold saline solution and centrifuged to obtain supernatants, which were further filtered using 0.22 µm filter and 2.5 µl of NBD-F working solution, 20 µl of acetonitrile and 17.5 µl of borate buffer were added per 10 µl sample and mixed well. The solution was incubated for 7 min at 60°C protected from light, cooled and concentration of the neurotransmitters were measured using UV-HPLC system with a mobile phase of 0.02 M phosphate buffer and acetonitrile 84:16 (v/v) with a flow rate of 1ml/min glutamate concentration. The concentration of the neurotransmitters was monitored at 472 nm.

4.2.10. Calcium Concentration Estimation

Estimation of calcium concentration in cortex, cerebellum and hippocampi was performed using calcium detection kit (ab102505; Abcam) according to protocol. Briefly, supernatants were collected after centrifugation of brain tissue homogenates in chilled PBS solution mixed with 0.1% Triton-X and 90 µL of chromogenic reagent and 60µL of Calcium Assay Buffer was added per 50 µL of collected supernatant. The mixed solution was incubated in a 96 well micro plate in dark for 10 minutes at room temperature. Absorbance was measured at 575 nm using multi-mode reader (BioTek Instruments, Inc., USA) and calcium concentration each sample was obtained from a previously prepared standard curve.

4.2.11. Nitrate Estimation

Nitrate level in different brain parts such as cortex, cerebellum and hippocampi were measured to estimate change of nitric oxide (NO) concentration, which is a hallmark of cerebral ischemia [26]. Concentration of nitrate was measured by preparing homogenates of the above-mentioned brain regions in normal saline in normal saline by using UV-HPLC method as reported by Ćurčić-Jovanović et. al., 2007 [27] with slight modifications. An HPLC-UV system (Shimadzu Scientific Instruments, Kyoto, Japan) equipped with SPD-20A detector was used with a mobile phase of 0.3M Sodium Borate Buffer, acetonitrile and methanol (v/v/v; 60:20:20) of pH 8.5 at a flow rate of 1.5 mL/min and the concentration was monitored at 214 nm. The A previously prepared standard curve was used to determine the nitrate concentration in the different brain regions.

4.2.12. Histopathological studies

Hematoxylin and Eosin (H&E) staining was performed to evaluate morphological changes in brain cortical region according to protocol of Cardiff et al. [28]. Animals from three groups, namely, vehicle, sham and Dose C, were used in the study. The brain tissues from were embedded in paraffin wax and cut into 5 µm sections after removing the brains. Before the sectioning brains were removed after transcardial perfusion and fixed in 10% formalin solution and the H&E slides were microscopically observed to determine histological changes.

4.2.13. Tissue damage evaluation with Annexin-FITC/PI

Annexin-FITC/PI staining was performed to evaluate apoptotic and necrotic cell death of brain cortical region using Annexin V Kit: sc-4252 AK (Santa Cruz Biotechnology, USA) according to given protocol for vehicle, sham and Dose C groups (n=4 per group). The brain tissue was dissociated into single cells by trypsinization and a concentration of 1×10^6 cells/mL was taken on slide for staining and visualization under fluorescence microscope at 40X magnification.

4.2.14. Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc tests, with $p < 0.05$ value considered as statistically significant. All data are expressed in mean ± S.D.

4.3. RESULTS

4.3.1. Brain penetration of WA

Distribution of WA in cortex and cerebellum of mice brain was evaluated over a time range of 15 min to 6 hrs. The results show highest accumulation of WA concentration at 1hr (Fig. 4.2.) for both cortex and cerebellum. The C_{max} (maximum concentration of a drug observed in a

specific compartment) of WA in cortex and cerebellum was found to be 11.88 µg/ml and 17.30 µg/ml, respectively (Table 4.1). The results for brain penetration of WA is listed in Table 4.1.

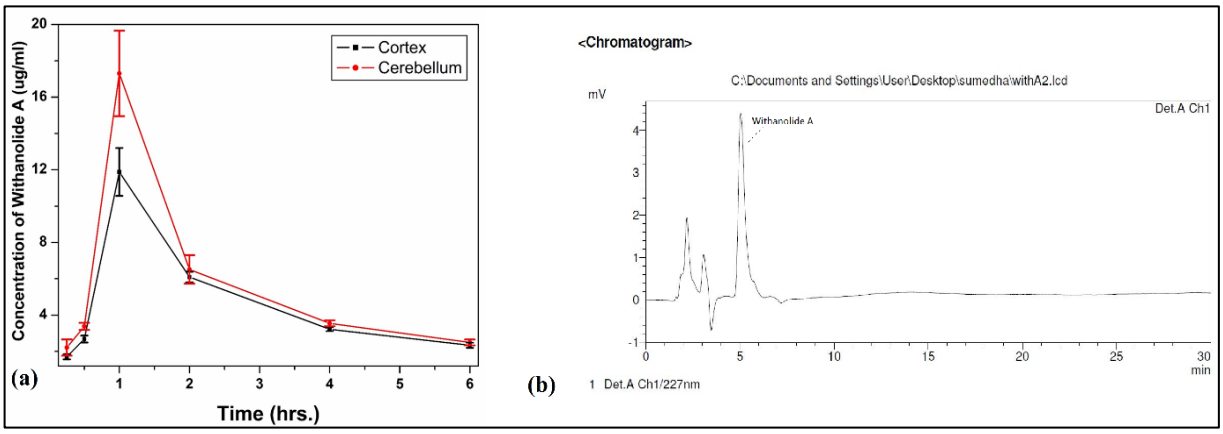


Fig. 4.2. (a) Brain Penetration of WA following intra-nasal administration; (b) UV-HPLC chromatogram of WA obtained from brain homogenate after 1 hr of intra-nasal administration.

Table 4.1.: Brain penetration parameters of WA in cortex and cerebellum

Parameters	Cortex		Cerebellum	
	Mean	S.D. (±)	Mean	S.D. (±)
t1/2 (h)	2.62	0.42	2.97	0.59
Tmax (h)	1	0	1	0
Cmax (µg/ml)	11.88	1.31	17.30	2.35
AUC 0-t(µg/ml*h)	28.24	1.47	34.14	1.25
MRT 0-inf_obs (h)	4.16	0.38	4.18	0.51

4.3.2. Effect of WA on cerebral infarction

The brain sections of vehicle group mice revealed marked cerebral infarction which was reduced significantly ($p < 0.001$) in all three treated groups. Dose C showed highest reduction of cerebral infarction as compared to other groups (Fig. 4.3.).

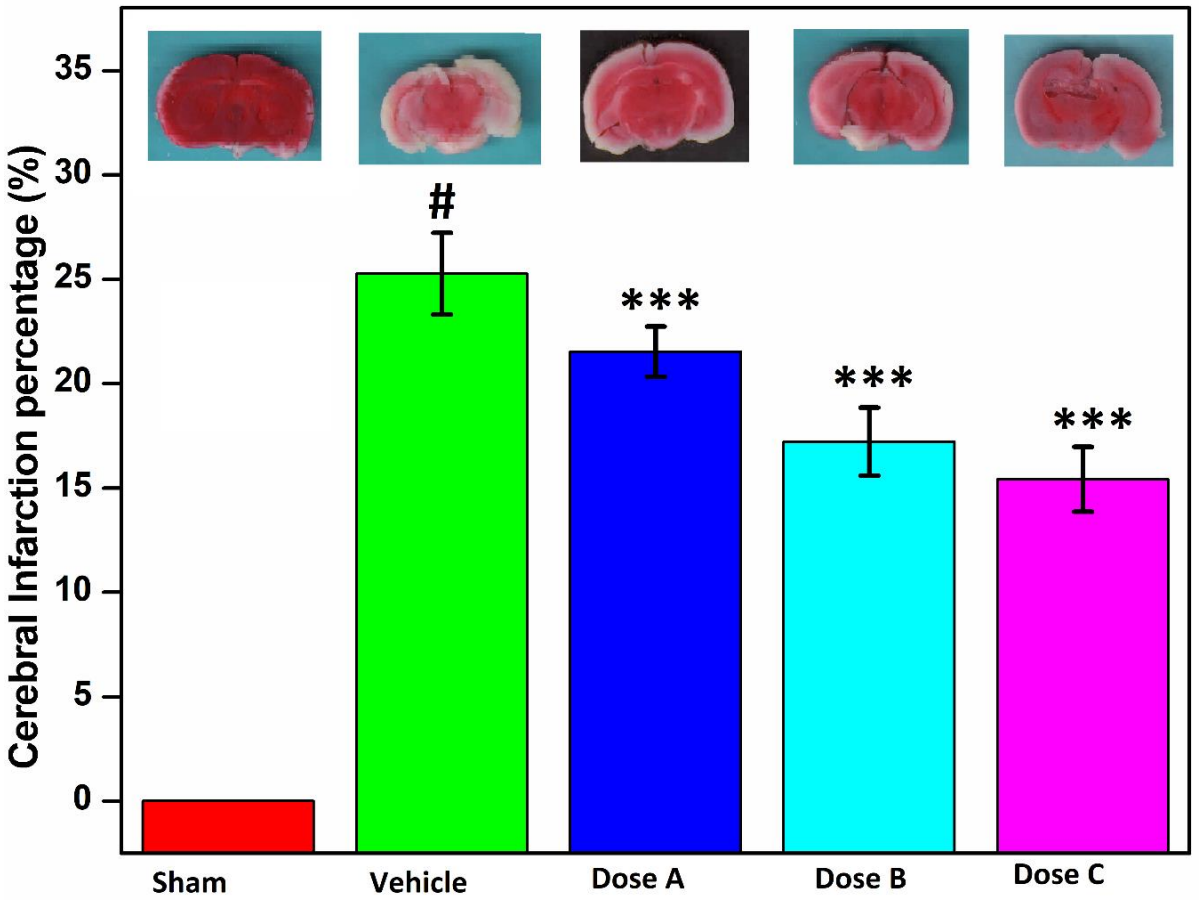


Fig. 4.3.: Effect of WA on cerebral infarction (n=6; ANOVA followed by Bonferroni post-hoc tests for statistical analysis). Bilateral carotid artery occlusion induces significant cerebral infarction in vehicle group animals (p<0.001). All three doses of Withanolide A show significant reduction of brain infarction as compared to animals of Vehicle group. (* signifies p < 0.001 of WA treated groups as compared to the vehicle treated group, # signifies p < 0.001 of vehicle group as compared to sham).**

4.3.3. WA post-treatment restores BBB breakdown

EB extravasation was quantitated to estimate the effect of WA post treatment on BBB. Dose B and Dose C exhibited significant ($p < 0.001$) reduction of EB leakage in post-ischemic brain as compared to vehicle group animals (Fig. 4.4.). Dose A did not have any significant effect on BBB restoration.

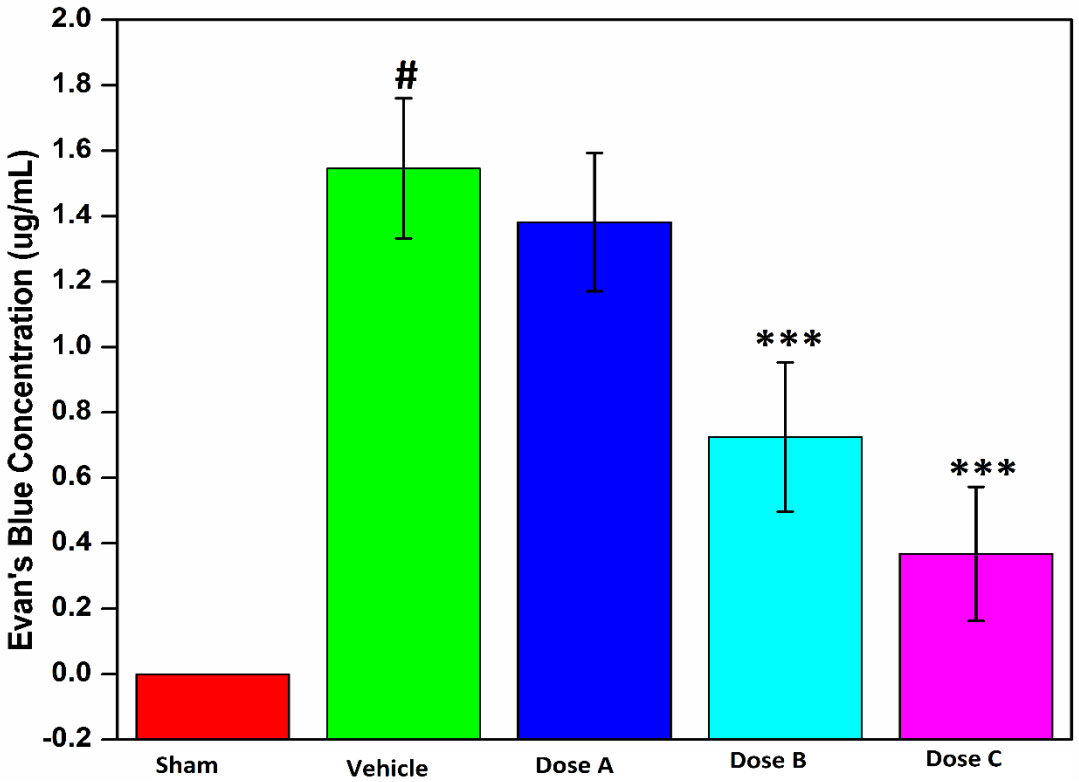


Fig. 4.4.: Effect of WA on BBB disruption (n=5; ANOVA followed by Bonferroni post-hoc tests for statistical analysis). BCCAO induces BBB breakdown in vehicle group animals (p<0.001) as compared to sham group. Dose B (5mg/kg) and Dose C (10 mg/kg) group of animals show significant reduction of BBB damage as compared to Vehicle group (p<0.001). No significant effect is exerted by Dose A (1 mg/kg). (* signifies p < 0.001 of WA treated groups as compared to vehicle group, # signifies p < 0.001 of vehicle group as compared to sham).**

4.3.4. Effect of WA post-treatment on brain water content

Estimation of cerebral edema was performed by calculating brain water content of each group. The vehicle group showed a significant increase in percentage of brain water content as compared to sham group. Dose B and Dose C significantly reduced ($p < 0.001$) increased brain water content but Dose A did not have any significant effect on brain swelling (Fig. 4.5.).

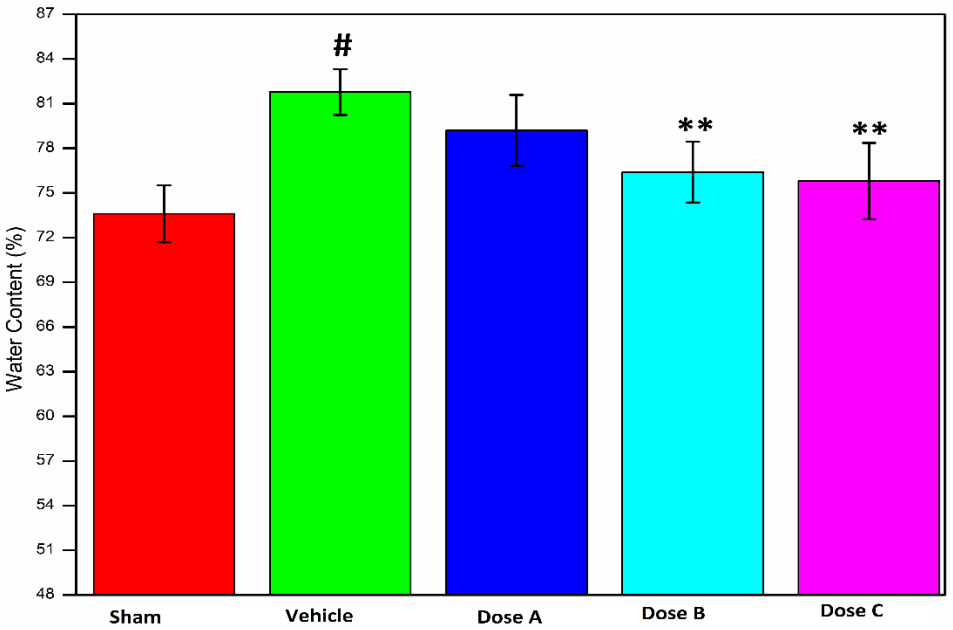


Fig. 4.5.: Effect of WA on cerebral edema (n=5; ANOVA followed by Bonferroni post-hoc tests for statistical analysis). Brain water content is significantly increased in vehicle group as compared to Sham group ($p < 0.001$) signifying cerebral edema. Dose B (5mg/kg) and Dose C (10 mg/kg) significantly reduces brain edema ($p < 0.001$) in comparison to vehicle group. Dose A (1mg/kg) does not have any significant effect. (signifies $p < 0.01$, # signifies $p < 0.001$ of vehicle group as compared to sham).**

4.3.5. WA post-treatment restores neurotransmitter level in brain

A significant increase ($p < 0.001$) in glutamate and GABA levels were observed in vehicle group animals as compared to sham group (Fig. 4.6.). It was observed that Dose C significantly restores glutamate concentration in all three brain compartments ($p < 0.001$) and also lowers the increased GABA levels in cortex, cerebellum ($p < 0.001$) and hippocampus ($p < 0.01$) (Fig. 4.6. a & 4.6.b). Post–treatment with Dose B revealed significant reduction of glutamate levels in cortex, hippocampus ($p < 0.001$) and hippocampus ($p < 0.01$) and significant restoration of GABA levels in cortex and cerebellum region ($p < 0.001$) (Fig. 4.6. a & 4.6.b). Dose B shows no significant effect on hippocampal GABA concentration (Fig. 4.6.b.). Administration of Dose A lowers the glutamate concentration in cortical and hippocampal region ($p < 0.01$ and $p < 0.001$, respectively) and but does not show any significant effect on hippocampal glutamate

concentration (Fig. 4.6.a.). Dose A also significantly restores concentration of GABA in cortex and cerebellum ($p < 0.05$) but has no marked effect on hippocampus region (Fig. 4.6.b.).

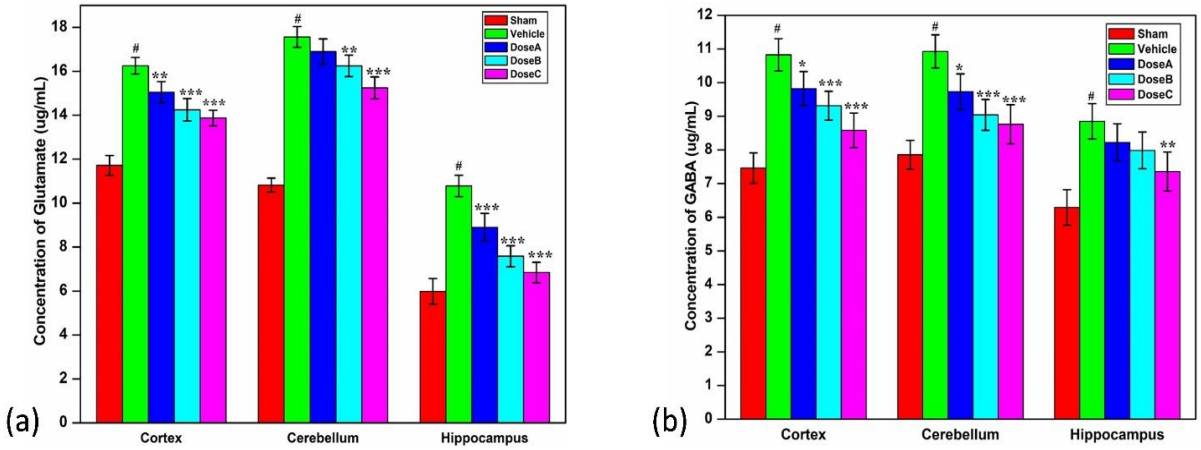


Fig. 4.6.: Restoration of neurotransmitter levels by WA post-treatment (n=5; ANOVA followed by Bonferroni post-hoc tests for statistical analysis). (a) Glutamate and (b) GABA concentrations are increased in all three brain regions in vehicle group animals as compared to sham group ($p<0.001$). Dose C significantly reduces glutamate and GABA concentration in all brain regions ($p < 0.001$ and $p < 0.01$ for hippocampal GABA concentration). Dose B also shows significant restoration of glutamate in all brain compartment and GABA in cortical and cerebellar region. Dose A reduces glutamate in cortex and hippocampus, whereas it reduces GABA in cortex and cerebellum. (* signifies $p < 0.001$, ** signifies $p < 0.01$ and * signifies $p < 0.05$ compared to vehicle group; # signifies $p < 0.001$ of vehicle group as compared to sham).**

4.3.6. WA restores cerebral calcium concentration

A significant increase in calcium concentration was observed in all brain compartments of vehicle group animals in comparison to sham group animals ($p < 0.001$) which was restored upon post treatment by Dose C of WA ($p < 0.001$) (Fig. 4.7.a.). Dose B shows significant restoration of cerebellar, hippocampal and cortical calcium levels ($p < 0.001$ and $p < 0.01$ for cortex), whereas Dose A exhibits significant lowering of calcium levels in cerebellum ($p < 0.001$) and hippocampus ($p < 0.01$) (Fig. 4.7.a.). There is no significant effect of Dose A on cortical calcium levels.

4.3.7. Effect of WA on cerebral nitrate level

Nitrate concentration significantly increases in all three brain compartments ($p < 0.001$), which is reduced by Dose C post-treatment in cortex, cerebellum ($p < 0.001$) and hippocampus ($p < 0.01$) (Fig. 4.7.b.). A significant decrease in nitrate concentration is also observed in cortex, hippocampus ($p < 0.05$) and cerebellar region ($p < 0.01$) (Fig. 4.7.b.). However, Dose A did not have any significant effect on the brain compartments.

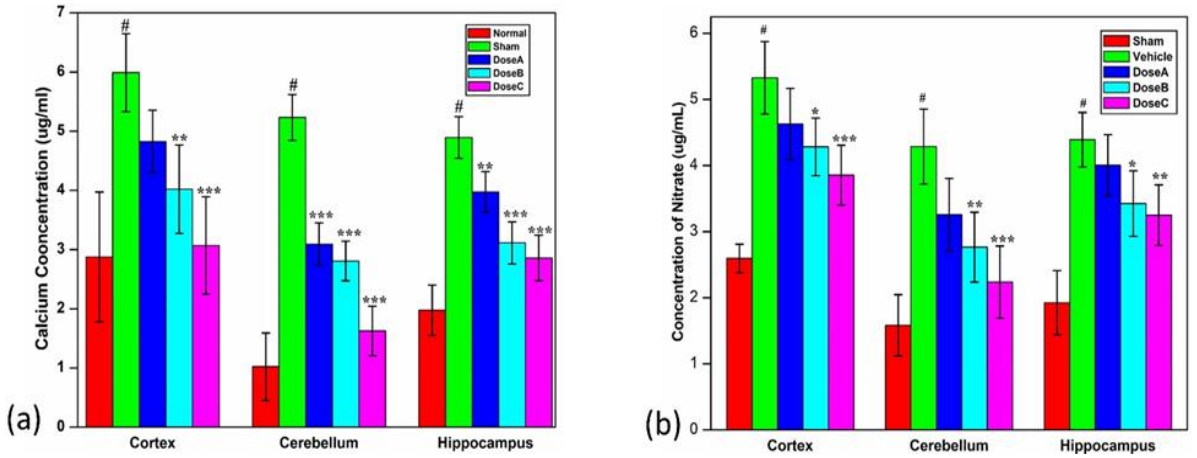


Fig. 4.7.: Effect of WA on cerebral calcium and nitrate levels (n=5; ANOVA followed by Bonferroni post-hoc tests for statistical analysis). (a) Calcium concentration is increased in all three brain regions in vehicle group animals as compared to sham group ($p<0.001$). Dose A shows significant reduction of calcium concentration in cerebellum ($p<0.001$) and hippocampus region ($p<0.01$). Dose B lowers calcium concentration significantly in cortex ($p<0.01$), cerebellum and hippocampus ($p<0.001$)). Dose C significantly reduces calcium concentration in all regions ($p<0.001$). (b) Nitrate concentration is increased in all three brain regions in vehicle group animals as compared to sham group ($p<0.001$). Dose A has no significant effect on nitrate concentration in any of the brain regions. Dose B lowers nitrate concentration significantly in cortex, hippocampus ($p<0.05$) and cerebellum ($p<0.01$)). Dose C also significantly reduces calcium concentration in cortex, cerebellum ($p<0.001$) and hippocampus (0.01). (* signifies $p < 0.001$, ** signifies $p < 0.01$ and * signifies $p < 0.05$ compared to vehicle group; # signifies $p < 0.001$ of vehicle group as compared to sham).**

4.3.8. Effect of WA on brain histopathology

Since Dose C showed significant prospect in combating ischemia induced pathophysiology, the effectiveness of the dose on tissue morphology was studied. It was observed that brain cortical region from animals of Dose C post treated group was characterized by presence of a

number of small nuclei, vacuolization and cracked cytoblasts as compared to that of the vehicle group animals (Fig. 4.8.), confirming the efficacy of Dose C of WA in ameliorating tissue damage.

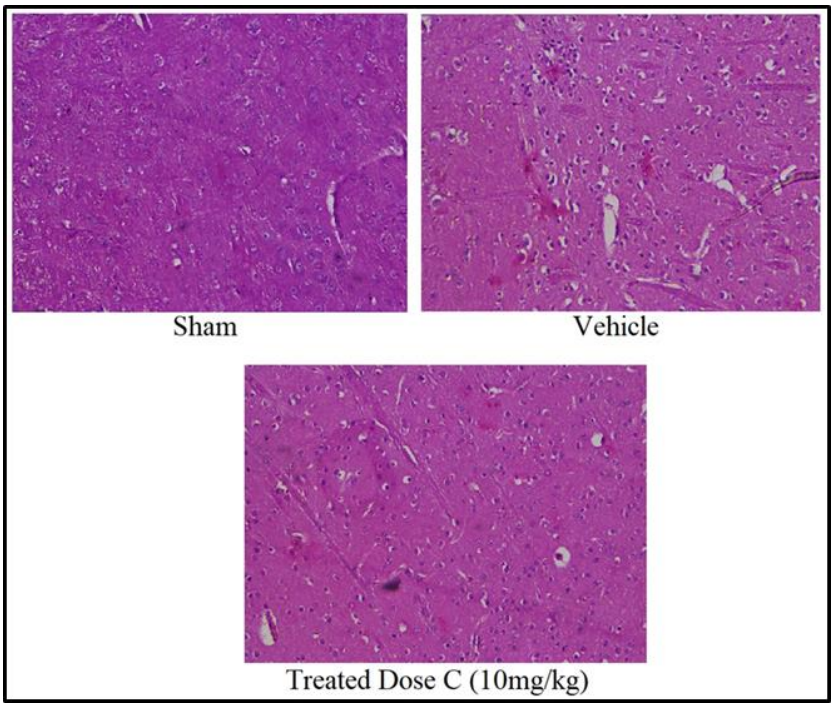


Fig. 4.8.: Effect of WA post-treatment on brain histopathology. Haematoxylin and Eosin staining reveals abundance of small nuclei and vacuoles in vehicle group cortical region as compared to Sham group animals. Treatment with Dose C (10mg/kg) reduces prevalence of vacuoles and small nuclei. (Observed under 40X magnification).

4.3.9. WA post-treatment reduces apoptotic and necrotic cell death

Efficacy of Dose C in combating cell death was further studied using Annexin-FITC/PI staining. Visualization of the cells via florescence microscopy revealed higher number of Annexin-FITC and PI positive cells in vehicle group as compared to the sham group. When compared to vehicle group, a smaller number of Annexin-FITC/PI positive cells were observed in the Dose C group (Fig. 4.9.). The cells were positive for both Annexin-FITC and PI, confirming both membrane and DNA damage.

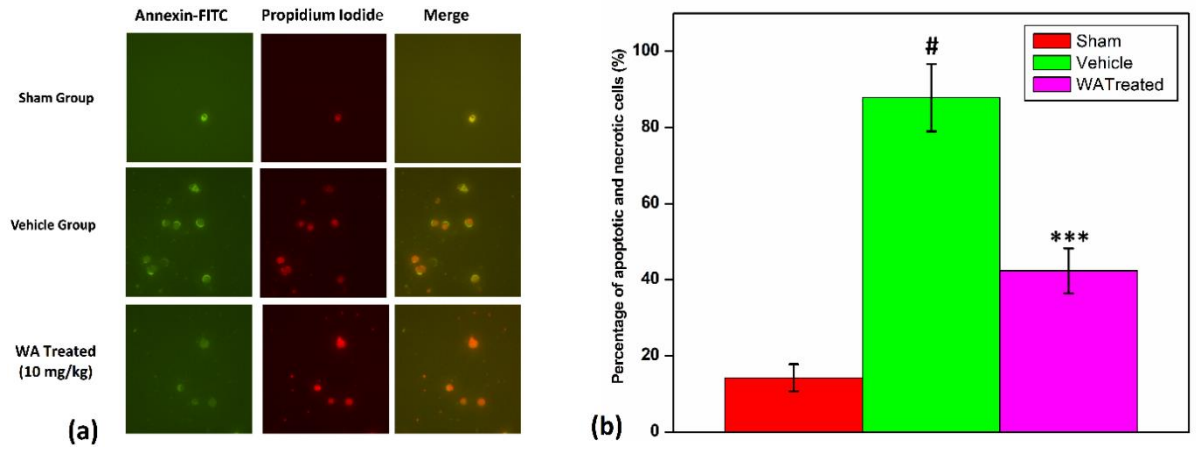


Fig. 4.9. Evaluation of effect of WA on brain cell death (n=4; ANOVA followed by Bonferroni post-hoc tests for statistical analysis). Annexin-FITC/PI staining shows damaged cell membranes and DNA damage in Vehicle group animals signifying apoptotic and necrotic death in cortical region. Less number of damaged cells are observed in Dose C (10mg/kg) treated group. (Observed under 40X magnification).

4.4. DISCUSSION

Several studies have reported neuroprotective effect of WA against neurodegenerative pathophysiology [13-15]. A particular study even confirmed the ability of WA in modulating glutathione biosynthesis in brain and simultaneous protection of hippocampal cells in hypbaric hypoxia situation [12]. But, a clear idea of brain penetration ability of WA has not obtained till date. The present study investigates the fate of WA in brain after intra-nasal administration, since the the present and a previous study by Kumar et al. [29] has predicted that WA might be BBB impermeable. Hence, an intranasal route of administration of the drug was chosen, since this particular administration route overcomes the limitations of crossing the BBB [30,31]. Inability to cross the BBB still remains a major obstacle behind the clinical failure of the neurotherapeutics [31], but intra-nasal delivery enables a drug to be directly transported into the central nervous system (CNS) without having to cross the BBB [32, 33]. Delivery of drugs through the nasal cavity is a non-invasive technique which allows the drug to reach the

brain via the olfactory and trigeminal neural path which innervates the nasal cavity, providing a direct connection to CNS [34, 35].

The present study shows that WA reaches the brain with 15 minutes of administration and reaches highest concentration in both cortex and cerebellum at 1 hr. The maximum concentration accumulated in cortex is 11.88 µg/mL and 17.30 µg/mL in cerebellum when 10 mg/kg is administered via the nasal cavity. These concentrations are comparable to the concentrations of WA reported by Akhoon et al. [36] which exerted neuroprotection in the *C. elegans* model. The AUC_{0-t} is 28.24 and 34.14 µg/ml*h for cortex and cerebellum, respectively and the mean residence time (MRT) for the compound in both cortex and cerebellum is approximately 4 hours, allowing it to exert its neuroprotective ability. Based on the results of brain-penetration studies, the concentrations of WA for neuroprotection studies were determined. Since the concentration of accumulated WA in brain regions after intra-nasal administration of 10 mg/kg was comparable to previously reported concentrations exerting neuroprotection, 10 mg/kg and two lower doses were studied.

Several studies have previously reported induction of global cerebral ischemia and subsequent brain damage by temporary ligation of the CCAs followed by reperfusion [37-40]. Pourheydar et al. reported ischemic damage in the rodent brain due to 30 mins of occlusion and subsequent reperfusion. Formation of cerebral infarction in rodent brain due to occlusion of bilateral CCAs have been also reported [37-39]. Reduced blood flow in the brain due to ischemia can also lead to BBB damage and subsequent cerebral edema [41]. In the present study, induction of global cerebral ischemia in mice brain due to 30 mins occlusion and 6 hrs reperfusion was confirmed from the visible infarction in the vehicle-treated group. A disruption in BBB and an increase in brain water content as an aftermath of BCCAO was also established by quantifying the EB

extravasation and measurement of the brain volume of the vehicle group animals. EB is a high molecular weight (961 Da) dye which is unable to cross the selectively permeable BBB under normal physiological conditions, but it can leak into the brain tissue following BBB disintegration [42]. Subsequent quantification of the dye accumulated in the brain tissue can provide an idea regarding the severity of the BBB damage. BBB disruption is also widely associated with an increase in the water content in the brain [43-45]. Accumulation of EB and an increase in brain water content was observed in the animals of the vehicle group, confirming the loss of BBB integrity due to the induction of cerebral ischemia. It was also found that the post-treatment with the high and medium doses of WA ameliorated the cerebral infarction and alleviated the BBB damage caused due to ischemic insult. BBB disruption due to cerebral ischemia is essentially mediated by the matrix metalloproteinases-2 & 9 (MMP-2 and MMP-9) [46-47]. Though not much data is available regarding the mechanism exerted by WA in neuroprotection, a recent *in-silico* study suggests that phytochemicals of WS, including WA, might have an inhibitory effect towards MMP-2 [48]. In the present study, WA post-treatment preserves the BBB integrity even after exposure to ischemic insult and also ameliorates cerebral edema in accordance with the previously reported hypothesis that WA might be an inhibitor of MMP-2.

N-methyl-D-aspartate receptor (NMDAR) mediated glutamate excitotoxicity is intrinsically linked to ischemia-induced brain damage [3]. The increase in glutamate levels during ischemia also leads to an influx of calcium ions [3] which further causes the generation of free radicals [3,4]. One such free radical is Nitric oxide (NO), which increases in concentration as an aftermath of cerebral ischemic insult [49]. But NO is a highly volatile molecule and quite difficult to quantify, hence, nitrate concentration is often measured for determining NO levels

[50,51]. In the present study, a significant increase in the concentrations of glutamate, GABA, calcium and nitrate was observed in the vehicle-treated group as compared to the sham group. The study also revealed that the high dose of WA (10 mg/kg) successfully restored the concentrations of the neurotransmitters in all three brain regions studied, i.e., cortex, cerebellum and hippocampus, although the low and medium doses showed interspersed effects in different regions. Though the neuroprotective mechanism of WA is not fully understood yet, it has been reported that WA can augment the indigenous glutathione concentration in the brain, which is generally reduced during the hypoxic conditions [12]. An increased endogenous glutathione level can subsequently reduce the hypoxia-induced elevated NO level by forming S-nitrosoglutathione (GSNO) [12,52], thus reducing neurotoxicity induced by an increased NO concentration. Lack of oxygen supply to brain tissues is also characteristic of cerebral ischemia [53]. So, the neuroprotective ability of WA in ischemic pathophysiology may be attributed to its ability to regulate brain glutathione concentrations, since the present study indicates that WA post-treatment combats ischemia-induced nitrosative stress in cortex, cerebellum and hippocampus. It has been also predicted that WA is a potent inhibitor of NMDAR [29]. The current study reveals that WA post-treatment restored glutamate concentration in mice brain, which might be due to the ability of WA in inhibiting NMDAR. An increase in the concentration of the GABA has been considered as a defense mechanism against the glutamate excitotoxicity during cerebral ischemia [54] whereas an increase of brain calcium level is a major characteristic event associated with the glutamate excitotoxicity [3]. So, it could be possible that amelioration of the glutamate excitotoxicity due to WA post-treatment in global cerebral ischemia contributes to the restoration of the GABA and calcium concentrations in mice brain.

The effect of cerebral ischemia-induced morphological damage was also observed in the brain tissue isolated from the infarct region. The infarct area was found to be characterized by vacuolization, small nuclei formation and an abundance of cracked cytoblasts, but a marked reduction of these features was observed upon post-treatment with 10 mg/kg dose of WA. The high dose (10 mg/kg) of WA was chosen for this study because it effectively ameliorated the ischemia-induced biochemical alterations in cortex, cerebellum and hippocampus. Further Annexin-FITC/PI staining of the brain cells also revealed a reduced number of apoptotic and necrotic cells in the cerebral infarction region, when treated with 10 mg/kg of WA.

4.5. CONCLUSION

The present study for the first time reports the ability of WA to reach brain cortex and cerebellum via intranasal administration and to exert neuroprotection in global cerebral ischemia. Among the three doses of WA evaluated, it was observed that Dose C (10 mg/kg) combated the ischemic pathophysiology most significantly. Dose C of WA significantly reduce cerebral infarction and edema, restored BBB disruption, lowered the elevated levels of the neuro-transmitters glutamate and GABA in all the three brain compartments, reduced excessive accumulation of calcium and nitrate ions in brain parts and also ameliorated ischemia induced tissue damage. The study establishes WA as a potent neuroprotectant in cerebral ischemia which confers neuroprotection by lowering glutamate excitotoxicity induced elevated calcium levels in brain. Hence, WA can be potential pharmacological compound for development of future neurotherapeutics.

References

1. Pourheydar B, Soleimani Asl S, Azimzadeh M, Rezaei Moghadam A, Marzban A, Mehdizadeh M. Neuroprotective effects of bone marrow mesenchymal stem cells on bilateral common carotid arteries occlusion model of cerebral ischemia in rat. Behavioural neurology. 2016;2016. doi: 10.1155/2016/2964712.
2. Liu Z, Liu Y, Zhou H, Fu X, Hu G. Epoxyeicosatrienoic acid ameliorates cerebral ischemia-reperfusion injury by inhibiting inflammatory factors and pannexin-1. Molecular medicine reports. 2017 Aug 1;16(2):2179-84. doi: 10.3892/mmr.2017.6831.
3. Xing C, Arai K, Lo EH, Hommel M. Pathophysiologic cascades in ischemic stroke. International Journal of Stroke. 2012 Jul;7(5):378-85. doi: 10.1111/j.1747-4949.2012.00839.x.
4. Brouns R, De Deyn PP. The complexity of neurobiological processes in acute ischemic stroke. Clinical neurology and neurosurgery. 2009 Jul 1;111(6):483-95. doi: 10.1016/j.clineuro.2009.04.001.
5. Jackman K, Iadecola C. Neurovascular regulation in the ischemic brain. Antioxidants & redox signaling. 2015 Jan 10;22(2):149-60. doi: 10.1089/ars.2013.5669.
6. Farhoudi M, Najafi-Nesheli M, Hashemilar M, Mahmoodpoor A, Sharifipour E, Baradaran B, Taheraghdam A, Savadi-Oskouei D, Sadeghi-Bazargani H, Sadeghi-Hokmabadi E, Akbari H. Effect of IMOD™ on the inflammatory process after acute ischemic stroke: a randomized clinical trial. DARU Journal of Pharmaceutical Sciences. 2013 Dec;21(1):26. doi: 10.1186/2008-2231-21-26.
7. Jha SK. Cerebral edema and its management. Medical journal, Armed Forces India. 2003 Oct;59(4):326. doi: 10.1016/S0377-1237(03)80147-8.
8. Wu G, Zhu L, Yuan X, Chen H, Xiong R, Zhang S, Cheng H, Shen Y, An H, Li T, Li H. Britanin Ameliorates Cerebral Ischemia–Reperfusion Injury by Inducing the Nrf2 Protective Pathway. Antioxidants & redox signaling. 2017 Oct 10;27(11):754-68. doi: 10.1089/ars.2016.6885.
9. Case LC, Tessier-Lavigne M. Regeneration of the adult central nervous system. Current biology. 2005 Sep 20;15(18):R749-53. doi:10.1016/j.cub.2005.09.008.
10. Ginsberg MD. Neuroprotection for ischemic stroke: past, present and future. Neuropharmacology. 2008 Sep 1;55(3):363-89. doi:10.1016/j.neuropharm.2007.12.007.
11. Sutherland BA, Minnerup J, Balami JS, Arba F, Buchan AM, Kleinschnitz C. Neuroprotection for ischaemic stroke: translation from the bench to the bedside. International Journal of Stroke. 2012 Jul;7(5):407-18. doi: 10.1111/j.1747-4949.2012.00770.x.
12. Baitharu I, Jain V, Deep SN, Shroff S, Sahu JK, Naik PK, Ilavazhagan G. Withanolide A prevents neurodegeneration by modulating hippocampal glutathione biosynthesis during hypoxia. PloS one. 2014 Oct 13;9(10):e105311. doi: 10.1371/journal.pone.0105311.
13. Dhuley JN. Effect of ashwagandha on lipid peroxidation in stress-induced animals. Journal of ethnopharmacology. 1998 Mar 1;60(2):173-8. doi: PMID: 9582008.
14. Naidu, P.S., Singh, A. and Kulkarni, S.K., 2006. Effect of Withania somnifera root extract on reserpine- induced orofacial dyskinesia and cognitive dysfunction. Phytotherapy

Research: An International Journal Devoted to Pharmacological and Toxicological Evaluation of Natural Product Derivatives, 20(2), pp.140-146. doi:10.1002/ptr.1823.

15. Kurapati KR, Atluri VS, Samikkannu T, Nair MP. Ashwagandha (Withania somnifera) reverses β-amyloid1-42 induced toxicity in human neuronal cells: implications in HIV-associated neurocognitive disorders (HAND). PLoS One. 2013 Oct 16;8(10):e77624. doi: 10.1371/journal.pone.0077624.
16. Prakash J, Yadav SK, Chouhan S, Singh SP. Neuroprotective role of Withania somnifera root extract in maneb–paraquat induced mouse model of parkinsonism. Neurochemical research. 2013 May 1;38(5):972-80. doi: 10.1007/s11064-013-1005-4.
17. Kuboyama T, Tohda C, Komatsu K. Neuritic regeneration and synaptic reconstruction induced by withanolide A. British journal of pharmacology. 144(7). 2005. 961-71. doi: 10.1038/sj.bjp.0706122.
18. Kuboyama T, Tohda C, Zhao J, Nakamura N, Hattori M, Komatsu K. Axon-or dendrite-predominant outgrowth induced by constituents from Ashwagandha. Neuroreport. 13(14). 2002. 1715-20. PMID: 12395110.
19. Akhoon BA, Pandey S, Tiwari S, Pandey R. Withanolide A offers neuroprotection, ameliorates stress resistance and prolongs the life expectancy of Caenorhabditis elegans. Experimental gerontology. 78. 2016. 47-56. doi:10.1016/j.exger.2016.03.004.
20. Andrabi SA, Kim NS, Yu SW, Wang H, Koh DW, Sasaki M, Klaus JA, Otsuka T, Zhang Z, Koehler RC, Hurn PD. Poly (ADP-ribose)(PAR) polymer is a death signal. Proceedings of the National Academy of Sciences. 2006 Nov 28;103(48):18308-13. doi: 10.1073/pnas.0606526103.
21. Pujari RR, Vyawahare NS, Kagathara VG. Evaluation of antioxidant and neuroprotective effect of date palm (Phoenix dactylifera L.) against bilateral common carotid artery occlusion in rats. 2011. 49(08). 627-633. PMID: 21870431.
22. Cheng, C.Y., Tang, N.Y., Kao, S.T. and Hsieh, C.L., 2016. Ferulic acid administered at various time points protects against cerebral infarction by activating p38 MAPK/p90RSK/CREB/Bcl-2 anti-apoptotic signaling in the subacute phase of cerebral ischemia-reperfusion injury in rats. PLoS One, 11(5), p.e0155748. doi: 10.1371/journal.pone.0155748.
23. Martin Y, Avendaño C, Piedras MJ, Krzyzanowska A. Evaluation of Evans blue extravasation as a measure of peripheral inflammation. Protocol Exchange. 2010; 10:1919-31. doi: 10.1038/protex.2010.209.
24. Keep RF, Hua Y, Xi G. Brain water content: a misunderstood measurement?. Translational stroke research. 2012 Jun 1;3(2):263-5. doi:10.1007/s12975-012-0152-2.
25. Wu X, Wang R, Jiang Q, Wang S, Yao Y, Shao L. Determination of amino acid neurotransmitters in rat hippocampi by HPLC- UV using NBD- F as a derivative. Biomedical Chromatography. 2014 Apr;28(4):459-62. doi:10.1002/bmc.3062.
26. O'mahony De, Kendall MJ. Nitric oxide in acute ischaemic stroke: a target for neuroprotection.

27. Jovanović MĆ, Vasiljević I, Ninković M, Jovanović M. Determination of nitrate by the IE-HPLC-UV method in the brain tissues of Wistar rats poisoned with paraquat. Journal of the Serbian Chemical Society. 2007 Apr 1;72(4). doi: 10.2298/JSC0704347C.
28. Cardiff RD, Miller CH, Munn RJ. Manual hematoxylin and eosin staining of mouse tissue sections. Cold Spring Harbor Protocols. 2014 Jun 1;2014(6):pdb-rot073411. doi: 10.1101/pdb.prot073411.
29. Kumar G, Patnaik R. Exploring neuroprotective potential of Withania somnifera phytochemicals by inhibition of GluN2B-containing NMDA receptors: An in silico study. Medical hypotheses. 2016 Jul 1;92:35-43. doi: 10.1016/j.mehy.2016.04.034.
30. Graff CL, Pollack GM. Nasal drug administration: potential for targeted central nervous system delivery. Journal of pharmaceutical sciences. 2005 Jun 1;94(6):1187-95. doi: 10.1002/jps.20318.
31. Erdő F, Bors LA, Farkas D, Bajza Á, Gizurarson S. Evaluation of intranasal delivery route of drug administration for brain targeting. Brain research bulletin. 2018 Oct 1;143:155-70.
32. Hanson LR, Frey WH. Intranasal delivery bypasses the blood-brain barrier to target therapeutic agents to the central nervous system and treat neurodegenerative disease. BMC neuroscience. 2008 Dec;9(3):S5. doi: 10.1186/1471-2202-9-S3-S5.
33. Bors LA, Erdő F. Overcoming the Blood–Brain Barrier. Challenges and Tricks for CNS Drug Delivery. Scientia Pharmaceutica. 2019 Mar;87(1):6. doi: 10.3390/scipharm87010006
34. Chauhan MB, Chauhan NB. Brain uptake of neurotherapeutics after intranasal versus intraperitoneal delivery in mice. Journal of neurology and neurosurgery. 2015;2(1). PMCID: PMC4567259.
35. Dhuria SV, Hanson LR, Frey II WH. Intranasal delivery to the central nervous system: mechanisms and experimental considerations. Journal of pharmaceutical sciences. 2010 Apr 1;99(4):1654-73. doi:10.1002/jps.21924.
36. Akhoon BA, Pandey S, Tiwari S, Pandey R. Withanolide A offers neuroprotection, ameliorates stress resistance and prolongs the life expectancy of Caenorhabditis elegans. Experimental gerontology. 2016 Jun 1;78:47-56. doi:10.1016/j.exger.2016.03.004.
37. Iwasaki Y, Ito S, Suzuki M, Nagahori T, Yamamoto T, Konno H. Forebrain ischemia induced by temporary bilateral common carotid occlusion in normotensive rats. Journal of the neurological sciences.1989 Apr 1;90(2):155-65. doi: 10.1016/0022-510X(89)90098-1.
38. Nagahori T, Nishijima M, Endo S, Takaku A, Iwasaki Y. Ischemic brain damage induced by repeated brief occlusions of bilateral common carotid artery in rats. The Tohoku journal of experimental medicine. 1994;172(3):253-62. doi: 10.1620/tjem.172.253.
39. Shalavadi MH, Chandrashekhar VM, Ramkishan A, Nidavani RB, Biradar BS. Neuroprotective activity of Stereospermum suaveolens against global cerebral ischemia rat model. Pharmaceutical Biology. 2013Aug;51(8):955-60. doi:10.3109/13880209.2013.771685.
40. Pourheydar B, Soleimani Asl S, Azimzadeh M, Rezaei Moghadam A, Marzban A, Mehdizadeh M. Neuroprotective effects of bone marrow mesenchymal stem cells on

bilateral common carotid arteries occlusion model of cerebral ischemia in rat. Behavioural neurology. 2016;2016. doi: 10.1155/2016/2964712.
41. Sadoshima SE, Fujishima MA, Ogata J, Ibayashi SE, Shiokawa OS, Omae TE. Disruption of blood-brain barrier following bilateral carotid artery occlusion in spontaneously hypertensive rats. A quantitative study. Stroke. 1983 Nov;14(6):876-82. doi: 10.1161/01.STR.14.6.876.
42. Yao L, Xue X, Yu P, Ni Y, Chen F. Evans blue dye: a revisit of its applications in biomedicine. Contrast media & molecular imaging. 2018. doi: 10.1155/2018/7628037.
43. Stokum JA, Gerzanich V, Simard JM. Molecular pathophysiology of cerebral edema. Journal of Cerebral Blood Flow & Metabolism. 2016 Mar;36(3):513-38. doi: 10.1177/0271678X15617172.
44. Dharmasaroja PA. Fluid intake related to brain edema in acute middle cerebral artery infarction. Translational stroke res. 2016 Feb;7(1):49-53.doi:10.1007/s12975-015-0439-1.
45. Jiang X, Andjelkovic AV, Zhu L, Yang T, Bennett MV, Chen J, Keep RF, Shi Y. Blood-brain barrier dysfunction and recovery after ischemic stroke. Progress in neurobiology. 2018 Apr 1;163:144-71. doi: 10.1016/j.pneurobio.2017.10.001.
46. Rosell A, Lo EH. Multiphasic roles for matrix metalloproteinases after stroke. Current opinion in pharmacology. 2008 Feb 1;8(1):82-9. doi: 10.1016/j.coph.2007.12.001.
47. Rosenberg GA, Estrada EY, Dencoff JE. Matrix metalloproteinases and TIMPs are associated with blood-brain barrier opening after reperfusion in rat brain. Stroke. 1998 Oct 1;29:2189-94. doi: 10.1161/01.STR.29.10.2189.
48. Kumar G, Patnaik R. Inhibition of gelatinases (MMP-2 and MMP-9) by Withania somnifera phytochemicals confers neuroprotection in stroke: an in silico analysis. Interdisciplinary Sciences: Computational Life Sciences. 2018 Dec 1;10(4):722-33. doi: 10.1007/s12539-017-0231-x.
49. Wahlgren NG. Neuroprotectants in late clinical development–A status report. Cerebrovascular Diseases. 1997;7(Suppl. 2):13-7. doi: 10.1159/000108238.
50. Dawson TM, Steiner JP, Dawson VL, Dinerman JL, Uhl GR, Snyder SH. Immunosuppressant FK506 enhances phosphorylation of nitric oxide synthase and protects against glutamate neurotoxicity. Proceedings of the National Academy of Sciences. 1993 Nov 1;90(21):9808-12. doi: 10.1073/pnas.90.21.9808.
51. Fassbender K, Fatar M, Ragoschke A, Picard M, Bertsch T, Kuehl S, Hennerici M. Subacute but not acute generation of nitric oxide in focal cerebral ischemia. Stroke. 2000 Sep;31(9):2208-11. doi:10.1161/01.STR.31.9.2208.
52. Singh RJ, Hogg N, Joseph J, Kalyanaraman B. Mechanism of nitric oxide release from S-nitrosothiols. Journal of Biological Chemistry. 1996 Aug 2;271(31):18596-603. doi: 10.1074/jbc.271.31.18596.
53. Ogawa S, Kitao Y, Hori O. Ischemia-induced neuronal cell death and stress response. Antioxidants & redox signaling. 2007 May 1;9(5):573-87. doi: 10.1089/ars.2006.1516.
54. Ramanathan M, Babu CS, Justin A, Shanthakumari S. Elucidation of neuroprotective role of endogenous GABA and energy metabolites in middle cerebral artery occluded model in rats. Indian Journal of Experimental Biology. 2012 Jun ;50(6):391-397. PMID: 22734249.