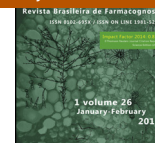




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Original Article

GABAergic effect of valeric acid from *Valeriana wallichii* in amelioration of ICV STZ induced dementia in rats

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ABSTRACT

Valeriana wallichii DC., Caprifoliaceae, is used to have anti-ulcer, anti-spasmodic, anti-epileptic, memory enhancer, anti-anxiety, anti-rheumatic, sedative, anti-asthmatic and diuretic activities. *V. wallichii* is reported to contain valproates, valeric acid, valerianic acid, valechlorine, valerianine, resins and alkaloids. Valeric acid, found in *V. wallichii* appears similar in structure to the neurotransmitter GABA. Valeric acid also acts as an NMDA-receptor antagonist. The aim of present study was to investigate the neuroprotective effect of *V. wallichii* containing valeric acid and its possible mechanism of action in amelioration of intracerebroventricular streptozotocin induced neurodegeneration in Wistar rats. The rhizomes of *V. wallichii* were powdered coarsely and extracted by percolation method using dichloromethane. Wistar rats (220–250 g) of either sex were divided into 5 groups, comprising 6 animals each. Valeric acid was isolated from plant extract and characterized using FT-IR. Picrotoxin (2 mg/kg) was used as GABA-A antagonist. Intracerebroventricular streptozotocin administration caused significant ($p < 0.05$) increase in escape latency, retention transfer latency on morris water maze on 17th, 18th, 19th and 20th day and elevated plus maze on 19th and 20th day respectively, as compared to normal untreated rats. Treatment with *V. wallichii* extract 100 and 200 mg/kg and valeric acid 20 and 40 mg/kg significantly decreased the escape latency and retention transfer latency, as compared to intracerebroventricular-streptozotocin group. Plant extract and valeric acid also decreased the level of lipid peroxidation and restored glutathione level in rat brains. Administration of picrotoxin significantly reversed the effects produced by plant extract and valeric acid in intracerebroventricular-streptozotocin treated rats. The findings may conclude that valeric acid present in *V. wallichii* has significant GABAergic effect in amelioration of experimental dementia.

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Introduction

Dementia is a clinical syndrome which is due to degeneration of neurons in brain and spinal cord. It is associated with deterioration in cognitive ability and capacity for independent living (Fratiglioni et al., 2007). The prevalence of neurodegenerative disorders like Alzheimer's diseases (AD) has increased significantly as global population age (Chen and Zheng, 2012). Currently, the number of deaths caused by AD is severely high and projected to rise by 21.2 million in 2025 (Hirtz et al., 2007; Katzman, 2008). The characteristic feature of AD is vascular dementia, fronto-temporal dementia, and aggregation of amyloid, accumulation of neurofibrillary tangles, lewis body formation and neuronal death. There is the development of multiple cognitive deficits such as disturbances in executive functioning, apraxia, agnosia, aphasia and

memory impairment (Fratiglioni et al., 2007) that interferes with daily social and professional brain outcomes. The most common symptoms of dementia are psychosis, aggression, insomnia, anxiety, depression, delirium, anger and sundowning (confusion in late afternoon or early evening) (Kuller and Lopez, 2008). The treatment of AD includes acetylcholinesterase inhibitor like Tacrine, Donepezil, Rivastigmine and Galantamine (Pokorski, 2002). Moreover, Food and Drug Administration has warned not to use atypical antipsychotics due to their increased mortality risk (Schneider et al., 2005).

AD is characterized by accumulation of amyloid beta peptide (A β) as fibrillary plaques and soluble oligomers in brain regions (Cole and Vassar, 2007). Tau protein is also identified as one of the main component of neurofibrillary tangles in various neurological diseases (Gendron and Petrucelli, 2009). In many areas of CNS, overactivation of NMDAR and subsequent influx or release of excessive Ca²⁺ has been noted to be predominant form of neurotoxicity. Ca²⁺ overload could ultimately lead to production of reactive oxygen species (ROS) and nitric oxide (NO) radicals,

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mitochondrial dysfunction, neuronal excitotoxicity and ultimately neuronal degeneration (Majdi and Chen, 2009). Recent researches have emerged with evidences that some pathogenic receptors are altered with lower levels of GABA_AR-subunits $\alpha 1$, $\alpha 2$, $\alpha 4$, δ , and $\beta 2$, mRNA in prefrontal cortex and $\alpha 1$, $\alpha 5$, and $\beta 3$ mRNAs in hippocampus were observed in AD (Rissman and Mobley, 2011).

Valeriana wallichii DC., Caprifoliaceae, is found abundantly at an altitude of 1300–3800 m in temperate Himalayas from Kashmir to and between 1250 and 1800 m in Khasi hills (Chauhan, 2006). It has got considerable reputation for its traditional use in pain (Vohora and Dandiya, 1992), epilepsy, insomnia, neurosis, sciatica (Nadkarni, 1976; Marder et al., 2003). The plant is widely used in the treatment of anxiety and depression (Panijel, 1985; Ron et al., 2000). It has been used as sedative in the treatment of insomnia and restlessness (Leathwood and Chauffard, 1985). *Valeriana* is reported to have antidepressant, anxiolytic, antispasmodic, anti-inflammatory and anticonvulsant activities (Sah et al., 2010). Valeric acid (VA) or pentanoic acid, a chief constituent of *V. wallichii* (Kokate et al., 2007), is a straight chain alkyl carboxylic acid with chemical formula C₅H₁₀O₂, molecular weight 102.15 and melting point: 34.5 °C. VA appears similar in structure to the neurotransmitter GABA but lacks amine functional group which contributes for the biological activities of GABA. It is reported as GABA-A agonist and is a very good relaxant. It also acts as an NMDA-receptor antagonist and showed anti-epileptic effect (Loeb et al., 1990). GABA-mediated postsynaptic inhibition in cultured mammalian neurons was observed on treatment with VA. It increases availability of synaptic GABA and/or enhances postsynaptic GABA responses, and thus enhances GABAergic activity. Keeping this in view, the present study was hypothesized to investigate GABAergic effect of VA from *V. wallichii* in amelioration of experimental dementia in intracerebroventricular streptozotocin model in rat brain.

Materials and methods

Collection and authentication of plant materials

The rhizomes of *Valeriana wallichii* DC., Caprifoliaceae, were collected from Kufri, Shimla, HP and duly taxonomically authenticated by Dr. R. Raina, Senior Scientist, Professor (Medicinal Plants), Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, HP, India and the sample was linked to UHF-Herbarium with field book number 12425.

Animals

Wistar Albino rats weighing 200–250 g of either sex were obtained from animal house (Reg No. 1541/PO/a/11/CPCSEA), Shoolini University, Solan, HP, India. The animals were allowed to acclimatize to the environment for 7 days prior to the experimental session. The animals were divided into different groups consist of six animals ($n = 6$) in each group. The study was conducted as per the guidelines of Committee for the purpose of control and supervision of experiments on animals (CPCSEA). The experimental study was duly been approved by Institutional Animal Ethical Committee (IAEC) vide protocol no. IACE/SU-PHARM/2/020. The animals were maintained at standard food pellet diet from Ashirwad Industries, Chandigarh, India, water ad libitum, temperature 25 ± 2 °C and humidity $45 \pm 5\%$.

Extraction of plant material

Dried powdered rhizomes of *V. wallichii* (500 g) were taken and extracted by percolation method using dichloromethane as solvent. The powdered material was moistened with 150 ml of

dichloromethane and allowed to stand for approximately 4 h in percolator. After completion of 4 h, 200 ml of dichloromethane was again added to form a shallow layer above the mass, and the mixture was allowed to macerate in closed percolator for 24 h. The outlet of percolator was opened after 24 h and the liquid contained therein was allowed to drip slowly. Additional dichloromethane was added until the percolate measures about three-quarters of required volume of finished product. After completion of extraction, solvent was then recovered and dried in rotary vacuum evaporator.

Isolation of valeric acid

The extract of *V. wallichii* was treated with methanol and sodium bicarbonate, which produces effervescence. Then extract solution was centrifuged, supernatant was separated and the solid mass was dissolved in water. It was completely dissolved in water by stir continuously and dilute hydrochloric acid was added dropwise to maintain pH 2. The extraction was done three to four times with methylene chloride in a separating funnel, concentrated the solution and thus obtained product was subjected to characterization (Houghton, 1988; Barnes, 2002; Gruenwald, 2004).

Fingerprint analysis of valeric acid

The fingerprint analysis of isolated VA in reference to standard sample was done using Agilent Technologies Cary 630 Fourier transform infrared spectrophotometry (FTIR) at range 4000–650 cm⁻¹.

Intracerebroventricular (ICV) administration of streptozotocin (STZ) for dementia

Rats were anaesthetized using ketamine hydrochloride (70 mg/kg *i.p.*) and xylazine (5 mg/kg, *i.p.*). The head of the animal was positioned in stereotaxic apparatus and a midline sagittal incision was made in the scalp. Two holes were drilled through the skull for placement of infusion cannula into lateral cerebral ventricles with the coordinates: 0.8 mm posterior to bregma; 1.5 mm lateral to sagittal suture; 3.6 mm ventrally from the surface of the brain and the tooth bar was set at 0 mm. A Hamilton[®] syringe of 10 μ l was attached through a skull hole to a stereotaxic apparatus and piston of the syringe was lowered manually into each lateral ventricle. STZ (3 mg/kg in two divided doses) dissolved in citrate buffer, pH 4.4, was slowly infused through intracerebroventricular route bilaterally (Tiwari et al., 2009). After ICV administration, the cut skin was sutured and the postoperative care were duly be made by applying povidone–iodine solution on wound.

The behavioral assessments were done using morris water maze test on 17th, 18th, 19th and 20th day and elevated plus maze on 19th and 20th day. On completion of protocol, the animals were sacrificed and brain was isolated.

Drug administration

The plant extract of *V. wallichii* was used in the dose range from 50 to 400 mg/kg in various investigations using rats (Subhan et al., 2009). Therefore, 100 and 200 mg/kg were selected as its submaximal dose to investigate the biological potential against experimentally induced neurodegeneration. The dosage of VA selected was 20 and 40 mg/kg due to its protective action against various experimental diseased conditions in rats (Loeb et al., 1990). *V. wallichii* extract 100 and 200 mg/kg, *p.o.* (suspended in 1% CMC solution) and valeric acid 20 and 40 mg/kg, *i.p.* (suspended in 1% Tween 80 solution) were given to animals treated with ICV-STZ

from day 3rd to 21st days once daily. Picrotoxin (2 mg/kg, *i.p.*, suspended in 1% Tween 80 solution) was given as GABA-A antagonist (Sattigeri et al., 2012) 30 min before valeric acid administration daily.

Morris water maze test

The morris water maze test was conducted for assessment of contextual/spatial memory in a cylindrical pool 120 cm in diameter and 60 cm in depth, filled with water (maintained at $28 \pm 2^\circ\text{C}$ and made opaque using non-toxic paint) to a depth of 40 cm. A circular platform of 9 cm diameter and 38 cm height was placed in water tank 2 cm below the water level for maze acquisition test. The pool is divided in four equal quadrants and a platform is located in the center of target quadrant. The platform remains in the same position during the training. The pool was placed in a large room, the visible cues were provided for development of special memory and remain unchanged throughout the study. Animals were received four daily consecutive training sessions from 17th day to 20th day. In each trial, animal was dropped in water facing its head toward the wall, subjected to swim and the latency to find the platform was recorded up to the maximum of 2 min. The animals reached to the platform were allowed to remain there for 20 s before next trial. The time taken by a rat to reach the platform was recorded as escape latency (EL) from water (Sachdeva et al., 2014; Yang et al., 2014).

Elevated plus maze test

An elevated plus-maze was used to test the cognitive function of animals. Elevated plus-maze consists of two opposite open arms (50 cm \times 10 cm), crossed with two closed arms of the same dimensions with 40 cm high walls. The arms were connected by a central square (10 cm \times 10 cm) and the apparatus was elevated 70 cm from the floor. On day 19th and 20th, rats were placed individually at one end of the open arm, facing away from the central square. The time taken for each rat to move from the open arm and enter into one of the closed arms was recorded as "initial transfer latency" (ITL). The animal was allowed to explore the maze for 30 s after recording ITL and returns to its home cage. Then, 24 h after ITL, the rat was placed again on the open arm and the retention transfer latency (RTL) was noted (Sachdeva et al., 2014).

Tissue biochemical estimations for oxidative stress

On day 21st, animals were sacrificed and their brains quickly removed, washed with ice-cold saline and stored at -80°C . Brain tissue samples (striatum and cortex regions) were thawed and homogenized with ten times (w/v) ice-cold 0.1 mol/l phosphate buffer (pH 7.4). The homogenates were centrifuged at $15,000 \times g$ for 20 min and supernatant was then used to for assessment of level of lipid peroxidation and glutathione as a marker for oxidative stress.

Estimation of lipid peroxidation

The reaction mixture consists of 0.1 ml of tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 20% acetic acid and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid (TBA). The pH of 20% acetic acid was adjusted with 1 N NaOH to 3.5. The mixture was finally made up to 4 ml with distilled water and heated at 95°C for 60 min on an oil bath. After cooling under tap water, 1 ml of distilled water and 5 ml of mixture of butanol and pyridine (15:1, v/v) was added and the mixture was shaken vigorously on a vortex mixer. Then centrifugation at $2800 \times g$ for 5 min was given and absorbance of organic layer (upper layer) was measured at 532 nm

using UV visible spectrophotometer. The level of lipid peroxidation was expressed as nM/mg tissue (Ohkawa et al., 1979).

Estimation of reduced glutathione (GSH)

The tissue homogenate was added with equal volume of 20% trichloroacetic acid (TCA) containing 1 mM EDTA to precipitate the tissue proteins. The mixture was allowed to stand for 5 min prior to centrifugation for 10 min at $1000 \times g$. The supernatant (200 μl) was mixed with 1.8 ml of Ellman's reagent (5,5'-dithio bis-2-nitrobenzoic acid) (0.1 mM) prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution). The absorbance of solution was estimated at 412 nm. The level of GSH was expressed as $\mu\text{M}/\text{mg}$ tissue (Ellman, 1959).

Statistical analysis

All the results were expressed as mean \pm standard deviation (SD) and analyzed by one-way and two-way ANOVA followed by Bonferroni's multi-comparison test as post hoc test using biostatistical software Graph pad prism (version 5). $p < 0.05$ was considered to be statistically significant.

Results

Fingerprint analysis of valeric acid

Fingerprint analysis showed prominent peaks at 1737 cm^{-1} due to stretching frequency of C–O of carboxylic, at $2858, 2929\text{ cm}^{-1}$ due to C–H stretching frequency of C–H and a prominent peak at 12 cm^{-1} due to stretching of C–OH of COOH, along with expected peaks in the finger print region in direction of stretching frequency of $(\text{CH}_2)_n$ and of CH_3 [e.g. 1465 cm^{-1}] (supplementary material).

Effect of drug treatments on behavioral parameters on morris water maze

The acquisition of memory in normal animals was found to be increased as reflected by progressive and significant ($p < 0.05$) decrease in escape latencies of animals from 17th to 19th days. ICV-STZ administration caused significant ($p < 0.05$) increase in escape latency on 18th, 19th and 20th day, respectively, as compared to normal untreated rats. Treatment with *V. wallichii* extract (100 and 200 mg/kg) and VA (20 and 40 mg/kg) significantly decreased the time of escape latency, as compared to ICV-STZ control rats followed on 17th, 18th, 19th and 20th day. Administration of picrotoxin significantly ($p < 0.05$) abolished the response of VA 40 mg/kg and showed increased escape latency, as compared to VA40. The *F* value for each variation factor like interaction, drug and time were found to be 44.95, 453.3 and 3138 respectively (Fig. 1).

Effect of drug treatments on behavioral parameters on elevated plus maze:

No significant change has been observed on elevated plus maze during assessment of ITL after induction with STZ. ICV STZ (3 mg/kg) produced significant ($p < .05$) increase in retention transfer latency, as compared to initial transfer latency and normal control. Treatment with *V. wallichii* extract 100 and 200 mg/kg, VA 20 and 40 mg/kg produced significant ($p < 0.05$) decrease in retention transfer latency, as compared to ICV STZ control. The effect produced by VA-40 (high dose) was significant ($p < 0.05$) in improving memory, as compared to *V. wallichii* extract. The effect produced by administration of picrotoxin and VA-40 concomitantly was

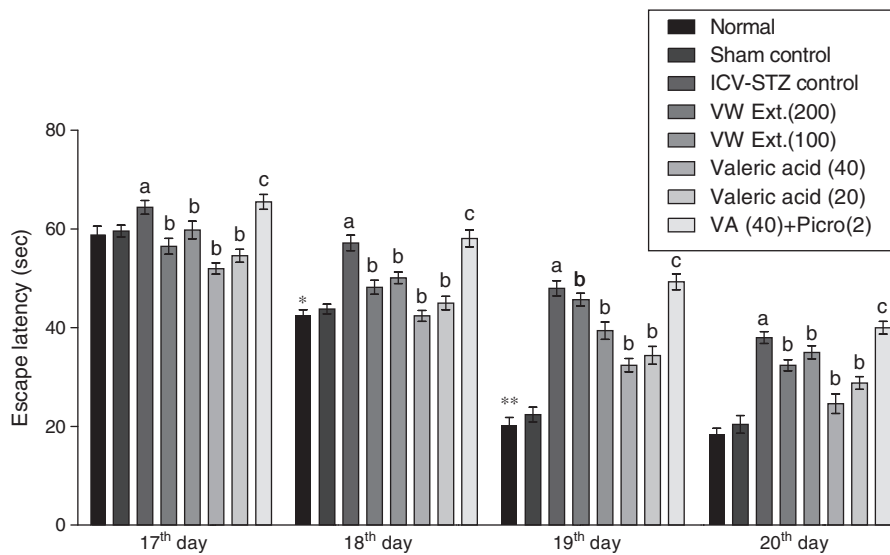


Fig. 1. Effect of drug treatments on behavioral parameters on morris water maze: results: mean \pm SD; ($n=6$); analyzed by two way ANOVA followed by Bonferroni's test whereas ^a $p < 0.05$ vs NC, ^b $p < 0.05$ vs ICV STZ, and ^c $p < 0.05$ vs VA (40); * $p < 0.05$ vs normal on 17th day and ** $p < 0.05$ vs normal on 18th day.

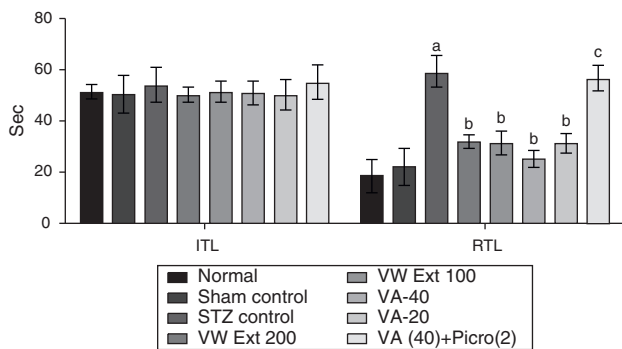


Fig. 2. Effect of drug treatments on behavioral parameters on elevated plus maze: results: mean \pm SD; ($n=6$); analyzed by two way ANOVA followed by Bonferroni's test whereas ^a $p < 0.05$ vs NC, ^b $p < 0.05$ vs ICV STZ, and ^c $p < 0.05$ vs VA (40).

significant, as compared to VA-40 and insignificant to ICV-STZ control (Fig. 2).

Effect of drug treatments on lipid peroxidation level

Induction with ICV STZ (3 mg/kg) produced significant ($p < 0.05$) increase in brain tissue lipid peroxidation level, as compared to normal untreated rats. However, *V. wallichii* extract 100 and 200 mg/kg, VA 20 and 40 mg/kg produced significant reversal by decreasing the level of brain lipid peroxidation, as compared to STZ control ($p < 0.05$). Treatment with picrotoxin (2 mg/kg) showed significant increase in lipid peroxidation reactions characterizing neurodegeneration, as compared to VA40 (Fig. 3).

Effect of drug treatments on tissue glutathione level:

Induction with ICV STZ caused significant ($p < 0.05$) decrease in tissue GSH level, as compared to normal untreated rats. However, *V. wallichii* extract in doses 100 and 200 mg/kg, VA (20 and 40 mg/kg) produced significant ($p < 0.05$) reversal in tissue GSH level, as compared to ICV STZ control. The effect produced by picrotoxin was significant, as compared to VA 40 and showed decrease level of reduced glutathione (Fig. 4).

Discussion

The findings from present investigation revealed the GABAergic effect of valeric acid from *V. wallichii* in amelioration of ICV-STZ induced neurodegenerative dementia in rats. Extraction of dried rhizome of *V. wallichii* was done by percolation method using dichloromethane. Valeric acid, a chief constituent present in *V. wallichii*, identified by FT-IR and yield was found to be 0.8% w/v in present study.

ICV-STZ administration is widely used model for speculating the efficacy of neuroprotective agents and to explore pharmacological interventions underlying neurodegeneration and memory deficits in the form of dementia as both are characterized by progressive deterioration of memory, cerebral glucose and energy metabolism, and production of oxidative stress (Prakash et al., 2015). STZ (3 mg/kg) was given via intracerebroventricular route to induce neuronal damage without altering peripheral blood glucose level. ICV-STZ is reported to cause an increase in tauprotein and amyloid- β in brain, thereby generating inflammatory reactions and free radicals and ultimately results cell death, apoptosis and diminution of learning and memory in experimental animals (Weerateerangkull et al., 2008). The animals of both the sexes were used in present study with an idea that the disease is prevalent in both. The fact that estrogen is known to improve memory is taken care by equally distributing the male and female rats in all groups, including that of control.

The performance of animals during behavioral assessments for spacial memory using morris water maze and for memory cognition using elevated plus maze are well documented to estimate extent of neuronal injury (Sachdeva et al., 2014). The performance in normal and sham control groups were significantly increased as the animals showed progressive decrease in escape latencies during assessments from 17th to 19th day. ICV-STZ treatment to rats showed significant increase in escape latency on morris water maze and retention transfer latency on elevated plus maze characterizing impairment in learning and memory due to neurodegeneration which are in confirmation to earlier literature (Tiwari et al., 2009). This signifies the increased neuronal damage, reactive oxygen species and thereby oxidative stress, amyloid-beta deposition and execution of inflammatory cascades resulting memory deficit (Yang et al., 2014). However, treatment with *V. wallichii* extract 100 and 200 mg/kg and its chief constituent: VA acid 20 and 40 mg/kg significantly decreased RTL and escape

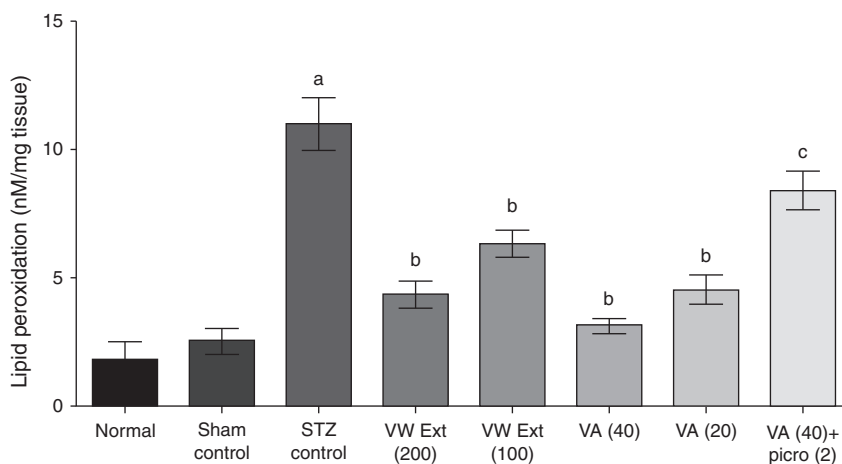


Fig. 3. Effect of drug treatments on lipid peroxidation level: results: mean \pm SD; ($n=6$); analyzed by one way ANOVA followed by Bonferroni's test whereas ^a $p < 0.05$ vs NC, ^b $p < 0.05$ vs ICV STZ, and ^c $p < 0.05$ vs VA (40).

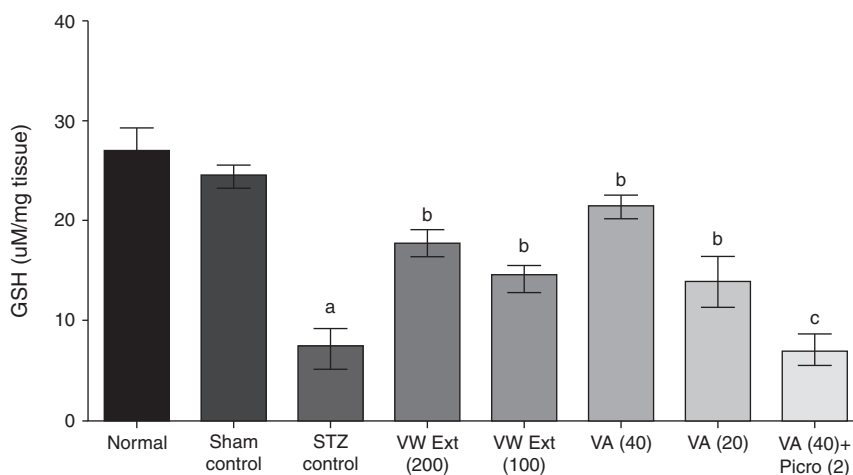


Fig. 4. Effect of drug treatments on tissue glutathione level: results: mean \pm SD; ($n=6$); analyzed by one way ANOVA followed by Bonferroni's test whereas ^a $p < 0.05$ vs NC, ^b $p < 0.05$ vs ICV STZ, and ^c $p < 0.05$ vs VA (40).

latency, attenuated oxidative and inflammatory reactions and thereby ameliorated ICV-STZ induced neurodegeneration. This effect of plant extract may be due to the presence of VA. These findings were confirmed by the results obtained in groups treated with picrotoxin 2 mg/kg, a GABA-A antagonist followed by VA-40 mg/kg administration as it showed increased RTL and escape latency which inferred that blockage of GABA receptors by picrotoxin and makes VA unable to correct ICV-STZ induced neurodegeneration in rats and there was no improvement in memory.

The oxidative stress caused by ICV STZ administration increases lipid peroxidation and decreases GSH level. Lipid peroxidation, a measure of free radical generation was increased on day 21 in brain of rats treated with ICV-STZ. Further production of free radicals caused simultaneous decrease in glutathione levels. Glutathione is an essential tripeptide, an antioxidant found in all animal cells. It reacts with the free radicals, neutralize them and protect the cells from singlet oxygen, hydroxyl radical and superoxide radical damage. Treatment with *V. wallichii* extract (100 and 200 mg/kg) and VA (20 and 40 mg/kg) significantly reduced lipid peroxidation reactions and restored antioxidant defense system of cell. The pathophysiological processes of oxidative stress in chronic conditions are linked with also attributed with the upstream regulation of GABA in neuronal cells which was blocked by administration of picrotoxin and hence showed increased lipid peroxidation reactions and lowered glutathione in rats. The property of *V. Wallichii*

for scavenging free radicals is also reported (Sudhanshu Rao et al., 2012).

GABA is a major inhibitory neurotransmitter in mammalian cerebral cortex. Temporal cortex is an area greatly affected by neuropathic hallmarks of neurodegeneration. It decreases 13–17% of benzodiazepine binding, which suggests that there is a decrease of GABA_AR₅ in AD (Limon et al., 2012). VA elevates GABA levels through the inactivation of α -ketoglutarate dehydrogenase (Luder et al., 1990). It decreases degradation of GABA mediated by GABA transaminase and thus inhibits GABA catabolism. It enhances the response of GABA-A receptor (Monti et al., 2009) and thus acts as GABA-A agonist (Frumkes and Nelson, 1995). The findings from present investigation may conclude that *V. wallichii* and its constituent valeric acid has significant neuroprotective potential to ameliorate memory cognition and retention possibly through GABA receptor modulation in rats.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors have obtained the written informed consent of the patients or subjects mentioned in the article. The corresponding author is in possession of this document.

Author's contribution

SV and RG designed the study. SV and VG have performed the experiments. RG and SV interpreted the research findings. SV and KLD worked for isolation of Valeric acid and characterization. RG and SV drafted the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bjp.2016.02.008](https://doi.org/10.1016/j.bjp.2016.02.008).

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