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ORIGINAL ARTICLE

Silybin Attenuates Neurobehavioural Impairments and Secondary Brain Damage Following Traumatic Brain Injury in Mice

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Abstract

Background/Aim: Traumatic brain injury (TBI) is a neurodegenerative disorder resulting in temporary or permanent impairment of cognitive, physical and psychosocial functions. Presently no treatment is available to overcome TBI. Flavonoids can be utilised for treatment of TBI due to their anti-oxidant and anti-inflammatory potential. Aim of this study was to investigate the neuroprotective potential of silybin against secondary cascade of TBI.

Methods: TBI induced by weight drop model of trauma has been utilised to assess the neuroprotective effect of silybin at the doses 50 mg/kg and 100 mg/kg in mice. After 30 min of injury, silybin was administered. The effect of silybin was observed on neurobehaviour (Morris's water maze test, beam walk and beam balance), blood brain barrier permeability (BBB), brain oedema and mitochondrial dysfunction after 24 h and 21 days of drug administration. The study of interaction of silybin with N-methyl-D-aspartate receptors (NMDARs) by using PDB: 3QEL was evaluated by molecular docking.

Results: Silybin significantly improved the neurological behaviours such as motor coordination and learning and memory. Silybin significantly decreased the BBB permeability, brain oedema and mitochondrial dysfunction. Molecular docking studies showed the significant interactions of silybin with target residues MET 207, THR174, TYR175, PHE 176, PRO177, GLU236,THR233, GLU106, ALA107, GLN110, ASP136, ALA135, MET134 of PDB: 3QEL.

Conclusion: Silybin provides novel insights into the neuroprotective and therapeutic potential for TBI.

Key words: Silybin; Brain injuries, traumatic; Neuroprotection; Mitochondrial diseases; N-methyl-D-aspartate.

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Introduction

Traumatic brain injury (TBI) causes death and disability, especially for older and younger extremes. The mainstay of current treatment for TBI is optimised intensive care management because there are no effective medicines till date.[1] Numerous clinical investigations have revealed that individual with TBI may be more vulnerable to various psychotropic medication adverse effects.[2] According to Loan and Faden 2010, very complex conditions results, from both primary and secondary damage process.[3] Significant factors in the pathophysiology of secondary injury include, disturbances in the release of excitatory neurotransmitter, mitochondrial dysfunction, overproduction of reactive oxygen species, release of inflammatory cytokines and chemokine

and caspase mediated neuronal apoptosis.[4] For neural synapse functioning, calcium is an important second messenger. N-methyl-D-aspartate receptors (NMDARs), non-selective cation channels that permit a calcium influx into neurons, are closely linked to TBI (NMDAR). GluN1 dimers and two regulatory GluN2A-D subunits combine to form the heterogeneous tetramer known as NMDARs.[5] The crystal structure of amino terminal domains of the NMDA receptor was selected on the basis of previous study.[6]

Silybum marianum is known for traditional Ayurvedic treatment of heartburn and its accompanying health issues.[7] The therapeutic value of this plant for various neurodegenerative disorders (NDs) has also been researched in recent years.[8, 9] Silybin is isolated from the seed extract of this plant. Silybin and its derivatives exhibit various therapeutic and pharmacological activities like hepatoprotective, antioxidant, anti-inflammatory and anti-Parkinson disease.[10, 11] According to Shukla et al, silybin improves energy metabolism, spatial memory and amyloid aggregation in Alzheimer's disease.[12] In presented study, the neuroprotective effect of silybin in TBI was evaluated utilising weight drop model in Swiss albino mice.

Methods

Healthy adult Swiss Albino mice (3-6 months old), either sex, weighing about 25-30 g were housed under normal room temperature at 22 ± 3 °C: 35-55 % humidity with alternating 12h light/dark cycle.

Study design

Mice arbitrarily distributed into 2 sets of 4 groups each. Each group consisted of six mice to examine the effect of silybin.[13, 14]

Grouping of animals for study after 24 h:

Group 1: Control normal (without induction of TBI)
Group 2: Vehicle (traumatic mice without any drug treatment),
Group 3: Treated mice (silybin 50 mg/kg),
Group 4: Treated mice (silybin 100 mg/kg).
Grouping of animals for study on 21st day:
Group 5: Control
Group 6: Vehicle (traumatic mice without any drug treatment),
Group 7: Treated mice (silybin 50 mg/kg)
Group 8: Treated mice (silybin 100 mg/kg).[15]

TBI model

Mice were anaesthetised by using of sodium pentobarbital (50 mg/kg) intraperitoneally (ip). The mice were placed on foam and their skulls were exposed by making a tiny, longitudinal cut across their heads. The mice were positioned beneath the metal pipe with the metallic disk attached in the centre. Then, through the metallic conduit, the metallic spherical weight fell freely on the mice's heads. Disc was removed; the exposed skull was sutured. Following surgery, the animals were observed on a regular basis.[16] Further, behavioural, biochemical and molecular studies were performed.

Morris water maze

For three consecutive days, three trials were given to train the mice. Mouse was placed directly opposite to platform quadrant. Sixty seconds were given to find the hidden platform and were allowed to stay on it for half min. When animal failed to reach the platform, they were placed on platform himself. Further number of crossings over the platform of each mouse, time spent in targeted quadrant (TSTQ) and escape latency were recorded.[17]

Neurological scoring

Neurological score was used for the assessment of posttraumatic neurological impairments.[18] Body proprioception, spontaneous activity, forepaw outstretching, climbing, response to whisker stimulation and symmetry in the movement of all 4 limbs were the criterion of evaluation and the scores were graded 0-3.

Brain oedema

Mice were anesthetised with sodium pentobarbital (50 mg/kg, ip). From the point onward, TBI was induced. Dosing was done with different dose of silybin through (ip) route. After 24 h of injury, mice were sacrificed, whole brain was isolated from every mouse and cleaned with distilled water. Only half brain was used for the estimation of oedema formation. Isolated half brains were put on the weighed glass tubes and weight to get the wet weight of brain. At that point, these brains were dried by placing them in hot air oven at 100 °C for 24 h. After 24 h, reweighed the brain to yield the dry weight of brain[19] by using this formula: % water content = Wet weight – Dry weight/Wet weight

Blood brain barrier permeability

Evans Blue dye (2 %) was injected ip and circulate for 24 h in one day model and for three weeks in 21 days model. Brain was isolated and homogenised in phosphate buffer and centrifuged (15,000 × g × 30 min). Equal amount of supernatant and 50 % TCA was incubated overnight and centrifuged. EB stain was determined at 610 nm.[20]

Biochemical parameters

Tissue preparation of brain

Phosphate buffer pH (7.4) was used to make a homogenate for the assessment of oxidative stress indicators.

Malondialdehyde (MDA): Add tissue homogenate (0.1 mL), acetic acid (1.5 mL), TBA (1.5 mL), SDS (0.2 mL) and heated for 60 min at 100 °C. Add n-butanol: pyridine (5 mL) and distilled water (1 mL). Organic layer was separated after 10-minute centrifugation at 4000 rpm. At 532 nm absorbance was checked against a blank preparation of reaction mixture without brain homogenate. Expressed as nmol/mg protein.[21]

Catalase (CAT): Initially, mixture was prepared to mix brain homogenate (0.1 mL), distilled water (0.4 mL) and phosphate buffer (1 mL). The reaction commenced with addition of 0.5 mL H_2O_2 and incubated for 60 sec at room temp. Mix potassium dichromate acidic reagent (2 mL) with glacial acetic acid to terminate the reaction. Take this on water bath for 15 min. after cooling absorbance read at 570 nm vs control. Control set was obtained from standard plot and outcome was expressed as µm of H_2O_2 consumed mg protein.[22]

Glutathione (GSH): After adding 0.1 mL of 10 % TCA to 0.1 mL of brain tissue homogenate, the mixture was centrifuged for 10 minutes at 300 rpm to de-proteinase it. Add phosphate buffer (2 mL), homogenate (0.1 mL), distilled water (0.4 mL) and Ellman reagent (0.5 mL). Absorbance was measured at 412 nm. The standard curve, represented as µg/mg protein, was plotted against the reduced glutathione concentration.[23]

Mitochondrial dysfunction

Mitochondria were isolated from cortex of brain in Swiss Albino mice by conventional differential centrifugation with minor modification. Brain tissue was homogenised in five volumes of isolation buffer containing EGTA, Sucrose, BSA (0.1 %), Mannitol (215 mM) and HEPES. The homogenate was centrifuged at 13,000 rpm for 5 min. Again added 0.5 mL of isolation buffer in resulting pellets and centrifuged for 10 min. Pellets were washed with isolation buffer and again centrifuged for 10 min at same rpm. Finally, brain mitochondrial pellets were re-suspended in buffer.[24]

Complex-I enzymatic activity: Absorbance was measured at 550 nm after addition of Sample (0.1 mL), glycyl glycine buffer (0.2 M) and NADH (6 mM) in glycyl glycine buffer (2 mM).[25]

$$\frac{\text{nmol NADH oxidised/min/protein (mg/mL)} = \Delta \text{ O.D} \times 0.262 \times 3000}{\text{Protein mg/mL}}$$

Complex-II activity: Sample (0.1 mL), potassium ferricyanide (0.03 M (0.03 M), BSA (1 %), phosphate buffer (0.2 M) and succinic acid (0.6 M) were mixed. Absorbance measured at 420 nm.[26]

$$\frac{\text{nmol succinate oxidised/min/protein (mg/mL)} = \Delta \text{ O.D} \times 1.9 \times 0.435 \times 1000}{\text{Protein (mg/mL)} \times 1000}$$

Complex-IV Activity: A 0.3 mM reduced cytochrome C, 75 mM phosphate buffer and mitochondrial sample were all included in the assay mixture and read at 550 nm.[27]

$$\frac{\text{nmol Cyt C oxidised/min/mg protein} = \Delta \text{ O.D} \times 810 \times 3 \times 1000}{60 \times 29.5 \times \text{protein (mg/mL)}}$$

In silico studies

In silico investigations were conducted using the Schrodinger suite 2016-1. The relationship between silybin and TBI-related core proteins was demonstrated using the Glide module. Based on prior research, protein ligand resolution and crystal structure in association with silybin, the PDB were chosen.[28, 29] Based on the NMDR antagonist PDB mechanism, 3QEL were retrieved from the Protein Data Bank (https://www.rcsb.org/). Prepared target protein using Schrodinger's protein preparation wizard. The OPLS_2005 force field was used to minimise energy while deleting water molecules and adding hydrogen atoms. The grid box was created in the middle of the target protein's active site.[30, 31]

Statistical analysis

By utilising Graph Pad Prism, results were analysed by one-way and two-way ANOVA analysis of variance. Results were presented as mean ± SEM. Statistical significance was denoted as $^{***}p < 0.001$ vs control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs vehicle.[32]

Results

Morris water maze test

Escape latency was significantly increased in vehicle group ($p < 0.001$) as compared to normal mice. Significant ($p < 0.05$) improvement in learning memory was observed after treatment with silybin (50 mg/kg) after 7th day. Significant ($p < 0.01$, $p < 0.001$ and $p < 0.001$) recovery was also exhibited by silybin (100 mg/kg) from day 7th to 21st day, respectively as shown in Figure 1.

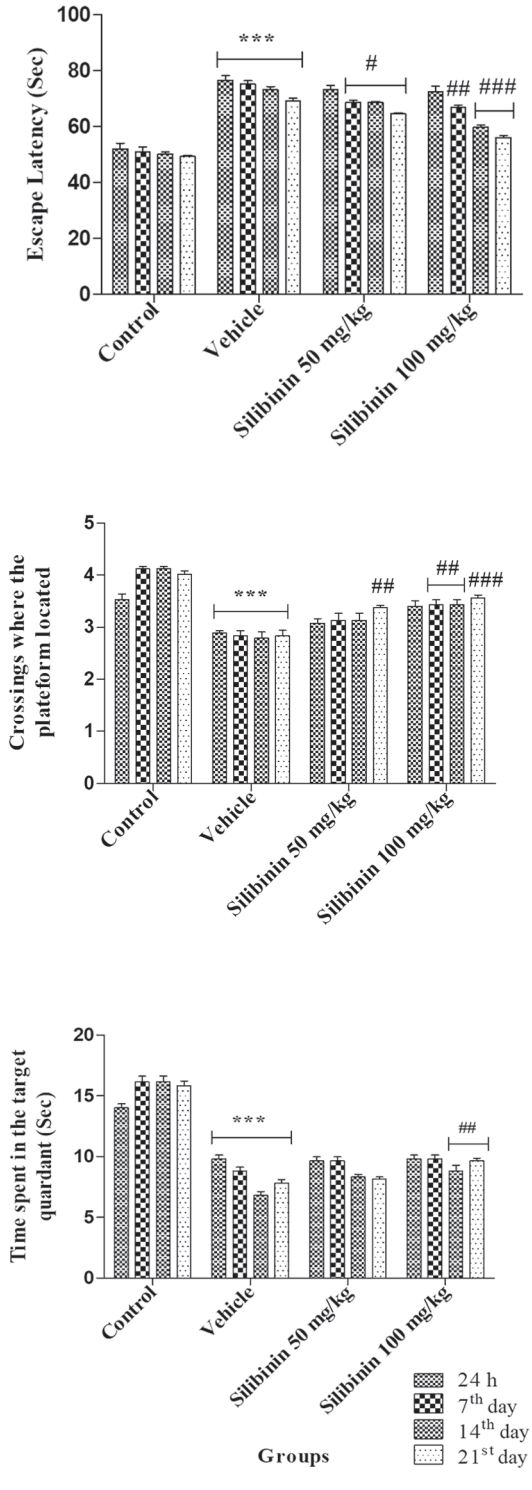

Figure 1: Effect of silybin in learning and memory of traumatic mice. Statistical significance analysed by two-way ANOVA followed by Tukey Post-hoc test

Neurological test

Neurological score of vehicle group was significantly ($p < 0.001$) lower than control group depicted in Figure 2. Silybin (100 mg/kg) exhibited significant ($p < 0.05$, $p < 0.01$ and $p < 0.001$) improvement in the neurological behaviour till 21st day of TBI respectively.

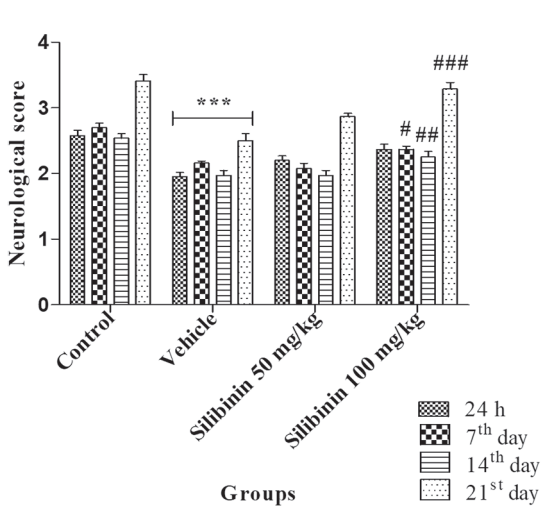

Figure 2: Effect of silybin on neurological scores in injured mice as observed at different time interval. Data was examined by two-way ANOVA followed by Tukey Post-hoc test

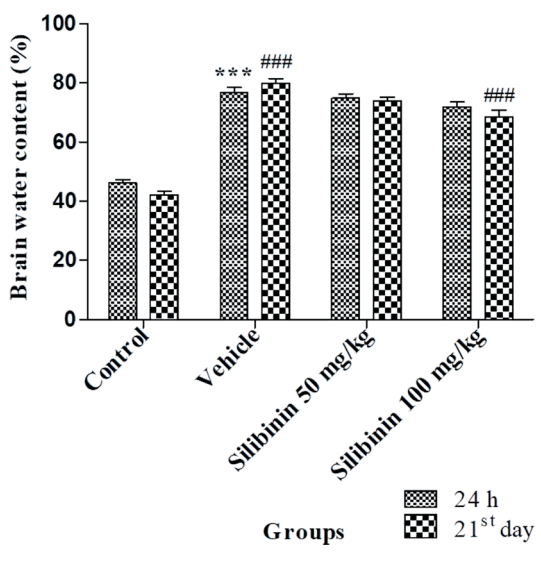

Figure 3: Effect of silybin on brain oedema was observed and analysed by one-way ANOVA followed by Tukey Post-hoc test

Brain oedema

Brain oedema was significantly ($p < 0.001$) augmented in TBI as compared to control group. Silybin (100 mg/kg/bw) exhibited significant ($p < 0.001$) reduction in brain oedema on 21st day shown in Figure 3.

Blood-brain barrier (BBB) permeability

BBB permeability was significantly ($p < 0.001$) augmented in TBI. Silybin (100 mg/kg) unveiled significant ($p < 0.001$) reduction in BBB permeability on 21st day of experiment (Figure 4).

Biochemical assay of oxidative stress markers

After the TBI, significant ($p < 0.001$) increase the level of oxidative stress was observed in vehicle group as compared to normal animal. Silybin (50 mg/kg) on 21st day of injury significantly ($p < 0.05$) normalised MDA and CAT level. Silybin (100 mg/kg) ($p < 0.05$) enhanced GSH level but did not normalise MDA and CAT level after 24 h. Moreover, it exhibited significant ($p < 0.001$ and $p < 0.01$) decrease in MDA but significantly ($p < 0.001$) increased CAT and GSH on 21st day of injury (Figure 5).

Mitochondrial dysfunction

TBI significantly ($p < 0.001$) compromised the mitochondrial enzymatic activity in vehicle group (Figure 6). Silybin (100 mg/kg/bw) ($p < 0.05$) amplified the level of complex II but did not regulate the complex I and complex IV activity significantly after 24 h of TBI. Moreover, it exhibited significant ($p < 0.001$, $p < 0.001$ and $p < 0.05$) improvement after 21 days of injury.

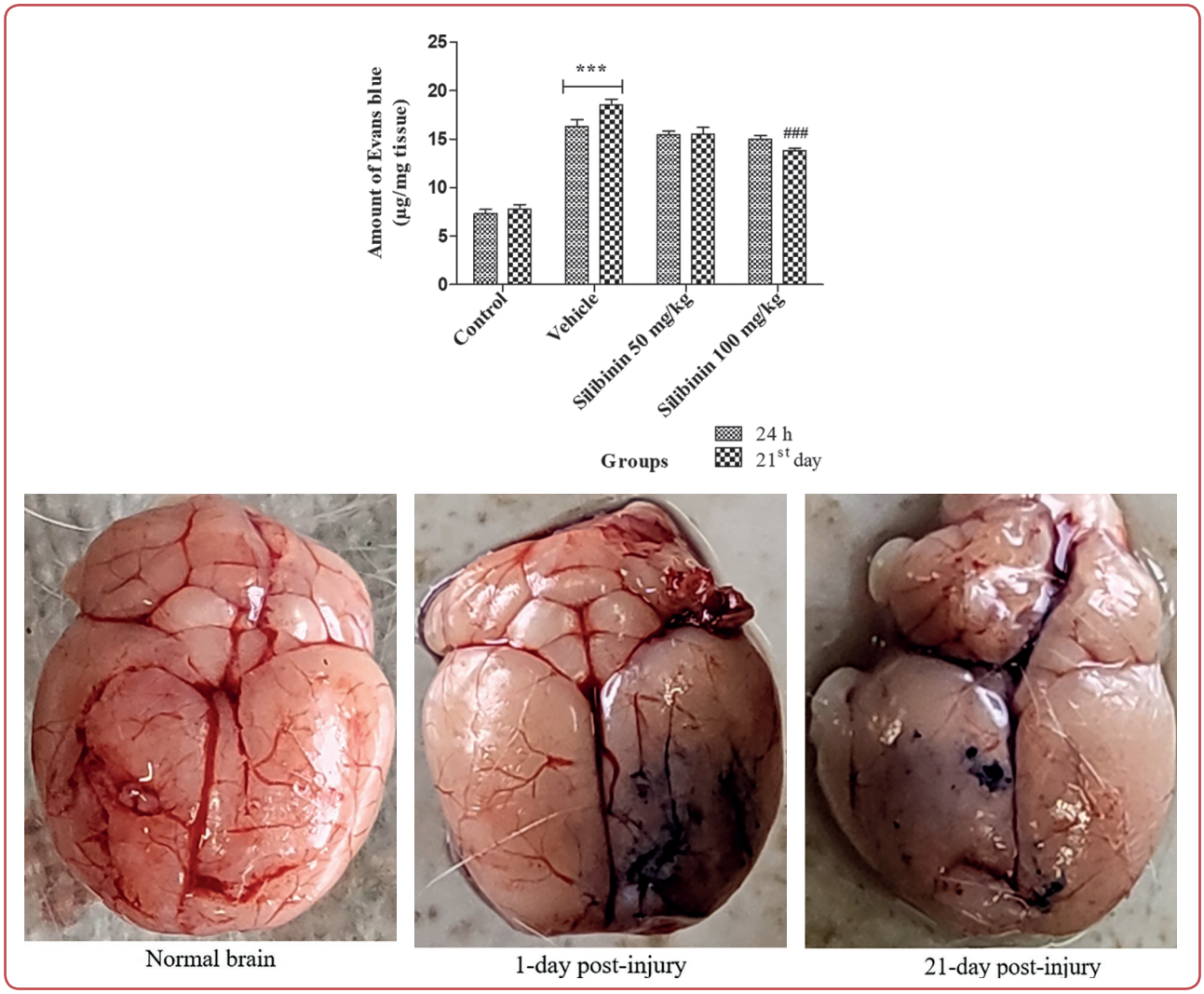

Figure 4: Effect of silybin on blood-brain barrier (BBB) permeability in traumatic mice. Statistical significance analysed by one-way ANOVA followed by Tukey Post-hoc test

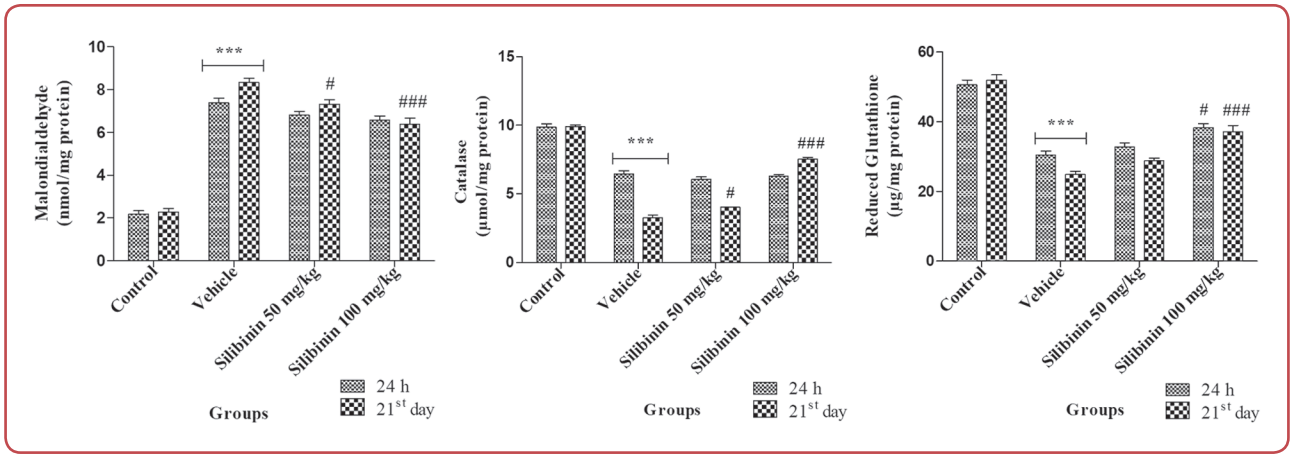

Figure 5: Results of silybin on oxidative markers was detected after injury and analysed by one-way ANOVA

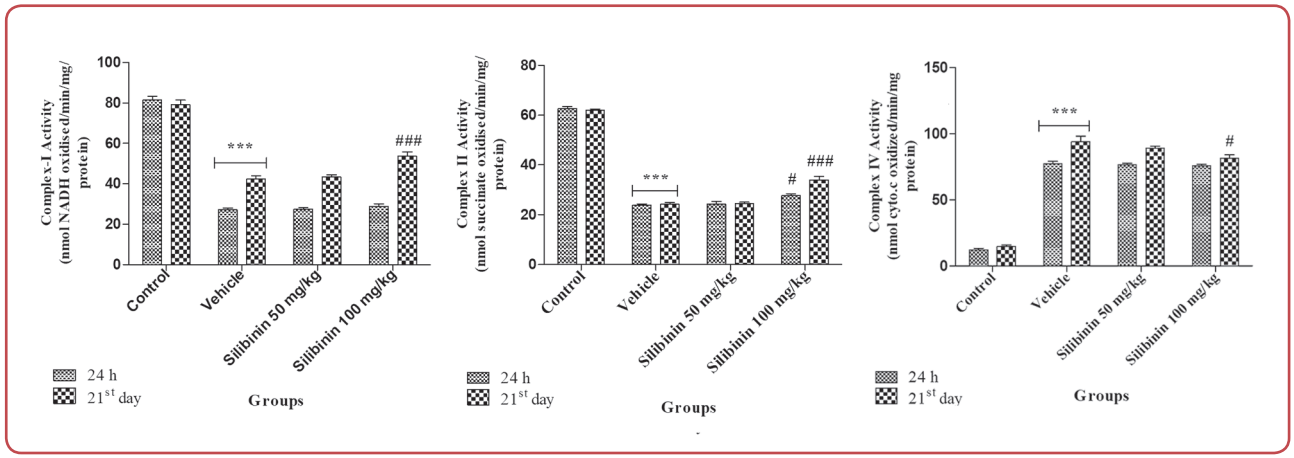

Figure 6: Effect of silybin in mitochondrial dysfunction was observed. Statistical significance analysed by one-way ANOVA followed by Tukey Post-hoc test

In silico studies

Binding pattern of silybin with NMDA receptor is suffused into "aromatic case" enfolded by MET 207, THR174, TYR175, PHE 176, PRO177, GLU236,THR233, GLU106, ALA107, GLN110, ASP136, ALA135, MET134 and aromatic ring of the NMDAR antagonist. GLN110 forms hydrogen bond with electronegative atom of silybin. GLU236, GLU106, ALA135, ASP136 forms Hydrogen bond with aromatic ring shown in Table 1 and Figure 7.

Table 1: Docking outcomes of silybin against PDB: 3QEL

PDB	Ligand	Docking score	Glide energy	Glide score	Glide model
3QEL	silybin	-6.68	-41.72	-6.68	-49.39

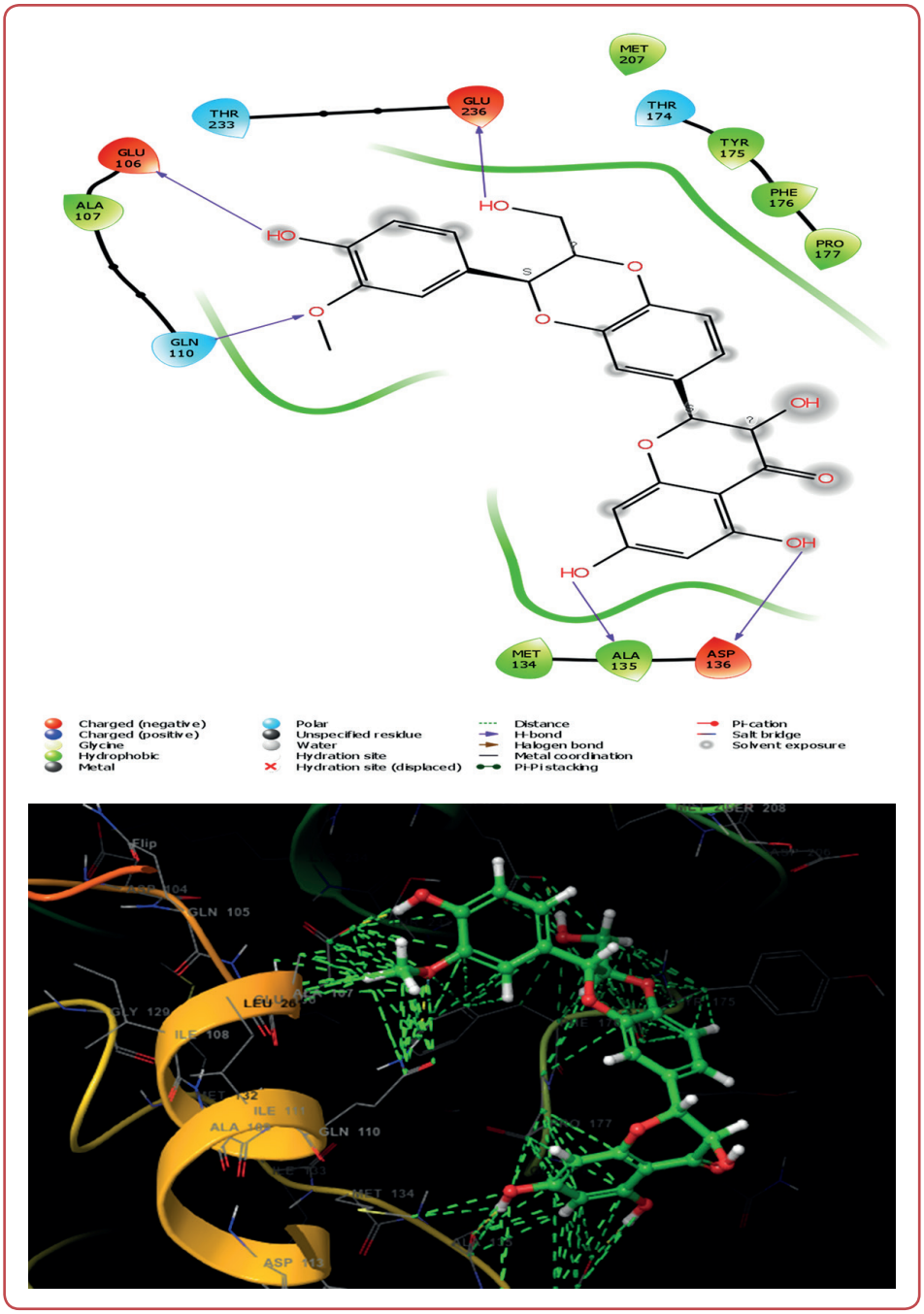

Figure 7: Docking conformation of silybin with PDB: 3QEL

Discussion

TBI is a mechanical event that may happen by indirect or direct impact to the head by vehicles, in sports etc, penetrating source and pressure waves.[8, 9] While primary injury cannot be reversed, secondary injury is a progressive stage that begins on the day of impact and continues for weeks, months and years. It can result in a number of delayed pathological consequences, including inflammation, excitotoxicity, oedema and mitochondrial dysfunction etc.[4] To combat TBI two different approaches are considered using two dissimilar therapeutic agents one targeting the NMDA receptor and the other is voltage gated calcium channel. Both are involved in overloading of calcium mediated widespread neuronal degeneration. Silybin is known to inhibit the glutamate neurotransmission by targeting NMDA receptor.[11]

TBI elucidate the various neurological behaviour in experimental animals such as decreased learning and memory, exploratory behaviour, motor coordination, social interaction, spontaneous and symmetrical movement after injury.[33] In this study, behavioural alterations were observed after traumatic injury and silybin significantly ameliorated the abnormal behaviour of the traumatic mice. Oxidative stress was increased by the formation of excess amount of free radical after TBI results in the neuronal damage by promoting protein breakdown and destruction of BBB.[34] Destruction of BBB permeability was due to the disturbing of tight junction protein which is responsible to increase the albumin content, leakage of fluids, protein, increase water content level which cause oedema. In this investigation silybin at the significantly decreased BBB permeability as exhibited by decreased water content and oedema.

Under normal physiological circumstances, excess reactive oxygen species, also known as free radicals, have a considerable cellular oxidising potential and cause oxidative stress. SOD produces hydrogen peroxide from superoxide anions associated with oxidative damage.[35] Superoxide-mediated lipid peroxidation changes the structure of membranes and impairs the functionality of cellular parts. MDA, GSH, nitrite, catalase and SOD have all been found to be reliable indicators of oxidative stress.[11] The signalling pathways include those for nitrogen metabolism, toll-like receptor signalling and JAK/STAT. Oxidative stress resulting from free-radical scavenging is believed to inhibit the production of adhesion molecules via silybin.[26] In addition, it raises endogenous antioxidant enzyme levels such as GSH, catalase and SOD, as well as oxidative and electrophilic stress reactions.[25] According to earlier studies, silybin restore the permeability of the BBB by preventing MMP activity by reducing inflammation and oxidative stress.[30] In our investigation, TBI raised MDA level and reduced the activity of CAT and GSH, while silybin administration decrease the MDA level whereas increase the level of catalase and GSH in traumatic mice. Thus, the effect of silybin in TBI might be associated with the inhibition of oxidative stress markers.

Due to the accumulation of excess amount of transport protein, level of glutamate was significantly increased.[4, 5] Glutamate binds with the NMDA and AMPA receptor, which is responsible to upregulate the level of intracellular calcium level. Increased amount of calcium further causes mitochondrial dysfunction, release of cytochrome C, activation of apoptosis inducing factor and further cause cell death.[6, 22] Reactive oxygen species have the ability to impede mitochondrial respiration by disrupting the electron transport chain-related activities of complicated mitochondrial enzymes. The sites where electrons from NADH and FADH2 are taken up and moved to complex III are Complex I and Complex II, respectively.[26, 27] These electrons are moved to complex IV and finally assist as electron acceptor. After treatment with silybin enzymatic activity was significantly restored by decreasing the BBB permeability due to which brain oedema was also decreased.

This suggests silybin had a putative target for therapeutic development. Molecular docking studies showed the significant interactions of silybin with target residues MET 207, THR174, TYR175, PHE 176, PRO177, GLU236,THR233, GLU106, ALA107, GLN110, ASP136, ALA135, MET134 of PDB: 3QEL.

In the present study, both doses of silybin (50 and 100 mg/kg) demonstrated neuroprotective effects following TBI; however, the higher dose of 100 mg/kg consistently produced pronounced and sustained benefits. While 50 mg/kg improved learning and oxidative stress markers to some extent, whereas, 100 mg/kg significantly restored neurological function, reduced brain oedema, stabilised BBB permeability, enhanced antioxidant defences and improved mitochondrial enzymatic activity. Moreover, therapeutic effects were modest at 24 h but became robust with continued treatment, particularly by the 21st day, highlighting that silybin exerts its maximum protective efficacy through sustained administration. Thus, 100 mg/kg administered over a prolonged period appears to be the optimal dosing strategy for mitigating secondary injury cascades in TBI.

Conclusion

In conclusion silybin, a natural antioxidant, has therapeutic effect against TBI exhibited through its protective effect by restoring neurological behaviour. It is further supported by the decrease in the brain oedema by regulat-

ing the BBB permeability and decreased mitochondrial dysfunction induced after trauma. Silybin 100 mg/kg is better than 50 mg/kg. Sustained treatment for 21 days post-injury yields the maximum neuroprotective effect, though this effect is visible on 7 day onwards.

Ethics

At the IAEC (Institutional Animal Ethics Committee (IAEC) meeting of Guru Jambheshwar University of Science and Technology, experimental studies on mice were permitted to be conducted in accordance with IAEC decision No IAEC/2021/10-19, dated 19 October 2021.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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Formal analysis: HB
Investigation: HB
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Data curation: HB
Writing - original draft: HB
Writing - review and editing: NV
Supervision: SS, NV
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