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INTEGRATED NETWORK PHARMACOLOGY AND IN VIVO VALIDATION OF SILYMARIN AS A NEUROPROTECTIVE AGENT AGAINST ALCOHOL WITHDRAWAL-INDUCED COGNITIVE DYSFUNCTION

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ABSTRACT
Abnormal limbic memories result from alcohol abuse, and can contribute to greater alcohol intake and relapse. Those suffering from AWS might suffer from withdrawal symptoms both physically and psychologically, such as memory problems. The therapeutic effects of silymarin on memory dysfunction was investigated in the present study by the network pharmacology method. Nine active compounds were found in silymarin, 69 molecular targets and 12 metabolic pathways were found. The genes PTPN1, DPP4, CA7, CA4, GUS, GALR3 and HSP90AB1 were identified to suppress symptoms of memory impairments and activity of the synapses of the serotonergic and glutamatergic systems. Silymarin was studied for 7 days after 21 days of chronic alcohol therapy for effect on memory dysfunction caused by alcohol withdrawal from male wistar rats. Morris Water Maze Test & New Object Recognition Test employed to measure memory impairments due to alcohol withdrawal. Oxidative Parameters (MDA, NO) & antioxidant enzyme parameters (SOD, GSH, CAT) were performed. A histopathological study of Rat's brain was carried out. The data were analysed by One-way and Two-way ANOVA and Tukey & Bonferroni's post hoc test. The silymarin-treated rats significantly enhanced the learning and spatial memory, as compared to the ethanol-treated rats. But treatment with silymarin led to a decrease of MDA and NO, and an increase of SOD, GSH, and CAT. Histopathologists found that silymarin treated neurons were unaffected in the brain from alcohol withdrawal. Based on the findings of the study, silymarin can be used as an anti-alcohol withdrawal memory dysfunction antioxidant.

KEYWORDS: Alcohol, Alcohol withdrawal symptoms, Flavonoids, Histopathology, Memory Dysfunction, Silymarin.

1. INTRODUCTION
Alcohol is the best acknowledged and most used technique of changing human consciousness due to its long history of production and consumption (Hendriks 2020). Alcohol can have a variety of effects on the brain and on behaviour, ranging from mild anxiolytic and inhibitory effects to drowsiness and motor incoordination, forgetfulness and vomiting, hypnosis and finally unconsciousness (Nutt et al., 2010), The brain plays a role in many functions, and alcohol is a primary drug of world-wide abuse, as is binge drinking. Chronic ethanol use leads to a multitude of consequences, such as autonomic hyperactivity-related symptoms such agitation, tremors, irritability, anxiety, hyperreflexia, disorientation, hypertension, tachycardia, fever, and diaphoresis(WHO., 2014), (Perry 2014)..
Alcohol withdrawal syndrome is the onset of physical alcohol addiction. Following a rapid cessation of heavy/constant drinking, either intentionally or unintentionally, people with alcohol use disorders experience the well-known alcohol withdrawal syndrome (Perry 2014). With the sudden onset of abstinence, the glutamate mediated CNS excitation assumes over-riding control and results in overall CNS excitation as the alcohol mediated CNS inhibition slowly subsides (Liang & Olsen 2014). The alcohol withdrawal symptoms are primarily associated with neurotransmitter release variations, and ETOH has been shown to-induced oxidative stress in the brain in numerous acute and chronic studies. Despite substantial study into the acute and chronic effects of ETOH on oxidative stress, ROS production during the withdrawal period has mostly been disregarded. Long-term use of ETOH in humans produces cognitive deficiencies and brain illness, such as cortical atrophy and ventricular enlargement (Chen et al. 2011). Normally, there is a balance between the excitatory and inhibitory impulses. Regular alcohol use has an effect on the excitatory and inhibitory neurotransmitter systems in the brain. Many neurotransmitters and mechanisms are also thought to be involved in alcohol withdrawal. Alcohol is a depressant of the central nervous system, which causes a rapid release of the amino acid GABA in the brain, particularly affecting the GABA-type a receptor (Wolf et al. 2020).
At the same time it inhibits activation of postsynaptic n-methyl-d-aspartate (NMDA) glutamate receptors. Chronic excessive alcohol consumption leads to downregulation of GABA-A and upregulation of n-methyl-D-aspartate NMDA) receptors and glutamatergic system (Jesse et al. 2017). Sudden drop in BAC produces glutamate mediated excitation

leading to autonomic overactivity and delirium. With the loss of the tonic inhibitory effect of the GABA system, seizure activity is mainly observed in the brainstem. After alcohol withdrawal episodes, epileptiform activity is rarely found in the, probably due to a different trigger zone than is normal in the context of epilepsy. The clinical manifestations of autonomic hyperarousal and hallucinations are caused by an increase of the neurotransmitter dopamine during alcohol withdrawal (Gortney et al. 2016).

Oxidative stress is caused by increase in the levels of reactive oxygen species (ROS) such as hydroxyl radicals (HO), superoxide anion radicals (O2), hydrogen peroxide (H2O2), peroxyl radicals (HOO) and high concentrations of nitric oxide (NO) and its derivatives, reactive nitrogen species (RNS) (Mira et al. 2020). The 3 most important natural antioxidants which scavenge these free radicals are superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH). Ethanol withdrawal in the rat hippocampus led to an increase in extracellular levels of excitatory amino acids.

Alcohol withdrawal seems to have an impact on the hypothalamus-pituitary-adrenal axis. increased levels of Corticotropin-Releasing Factor (CRF) (Kumar et al. 2009; Becker & Mulholland 2014; Yang et al. 2022). Given that it reduces anxiety and seizures, diazepam, a member of the benzodiazepine (BZDs) class of medications, is frequently recommended for the treatment of alcohol withdrawal (Köhnke 2008). But studies indicate that diazepam can be addictive (Owona et al., 2020).

The use of herbs in curing alcoholism dates back to a long period of time (Wang et al. 2016).. The name 'herbal medicine' originates from the word 'herb' which was originally used to refer to the dried and fresh flowering or leafy green parts of plants. Herbal medicines comprise of biological macromolecules including flavonoids, terpenoids, glycosides and alkaloids, and have received a lot of attention because of modulatory effects on inflammasomes that are associated with the onset and progression of chronic diseases such as metabolic and neurodegenerative diseases, which exhibit antioxidative activity (Kurup 2004; Afr, Cam & Traditional 2007; Awuchi 2019; Khan & Ahmad 2019; Sharma et al. 2019).

Flavonoids decrease proinflammatory mediators like cytokines and chemokines, eicosanoids, and adhesion molecules, which has anti-inflammatory effects. They are known as "functional components" or "health promoting biomolecules" in modern literature because they are beneficial for health and prevent chronic diseases. Since flavonoids are found in nature, it was our choice to use them instead of alkaloids, terpenoids and glycosides (Panche, Diwan & Chandra 2016; Kozłowska & Szostak-Węgierek 2019; Prithviraj Karak 2019).

The antioxidant property of silymarin is mainly attributed to its capacity to inhibit free radicals such as reactive oxygen species (ROS) and enhance other antioxidant enzymes such as glutathione (GSH), superoxide dismutase (SOD) and catalase in chronic diseases like cardiovascular and neurodegenerative diseases (Surai 2015). Although silymarin was shown to be useful in cognitive function in animal models of behavioural tasks testing spatial, working and emotional memories, episodic memory deficits are common in clinical settings of neurodegenerative disease, particularly in dementia related to ad. The antioxidant activity of silymarin and its ability to decrease cholinesterase activity in the hippocampus region enhances the cholinergic function (Neha et al., 2014; Wang et al., 2020).

Silymarin has been found to have anti-inflammatory and anti-oxidant properties that may be beneficial in treatment of complications that may arise as a result of alcohol withdrawal (Neha et al.,2016). The current protocol was designed to analyse the impact of silymarin on the management of alcohol withdrawal induced memory dysfunction in rats, because there haven't been any studies on the effect of silymarin on alcohol withdrawal-induced memory dysfunction .

Network pharmacology is a multi-disciplinary research area, which integrates network biology, pharmacology and systems biology for the analysis of drug-target and biological system interaction. A method of generating and analyzing biological networks using various computational and experimental approaches is called network pharmacology (Jiao et al. 2021). Network pharmacology with multiple components of the network as the target of drug action. This can help to enhance therapeutic effects with fewer side effects and drug resistance (Hopkins 2008; Kotadiya 2023). In addition, network pharmacology will help to understand complex diseases and underlying mechanisms (Achenbach et al. 2011). Single focus on molecular targets and pathways can offer important clues, but can be lacking in the complexity and interdependencies of multiple elements in a biological system (Chandran et al. 2017; Jiao et al. 2021).. These drawbacks have led to the advent of a novel approach known as network pharmacology that offers a comprehensive understanding of the molecular mechanisms and overall effects of therapeutic agents. Using network pharmacology, researchers can investigate the intricate interactions of silymarin and better understand its therapeutic potential (Bhatia et al., 2015).

By constructing network models of interactions between the targets of this natural substance, researchers have identified the complex network of biological processes and signalling pathways that are regulated by silymarin. These discoveries have given us a thorough grasp of silymarin's many mechanisms of action and shed insight on its therapeutic potential (Dwivedi et al. 2022; Noor et al. 2022).

In general, network pharmacology can be used to investigate silymarin and reveal a comprehensive and systems-level understanding of its therapeutic potential. By understanding the intricate network of interactions and processes that silymarin influences, researchers can identify potential targets for new treatments, develop more effective treatment strategies, and contribute to the development of precision medicine techniques(Wei et al. 2022).

2. MATERIALS AND METHODS

2.1 Chemicals

Silymarin was purchased from Otto Chemie Pvt. Ltd. India and Memantine (5mg/kg) was purchased from local distributors from Pune. Alcohol and all other required chemicals of analytical reagent grade were obtained from local suppliers of Pune.

Oxidized and reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide (NADPH), potassium dichromate, dithionitrobenzene (DTNB), phosphate buffer (0.1 M, pH 7) Reduced Glutathione (GSH), Thiobarbituric acid (TBA),

Trichloroacetic acid (TCA 20%), physiological normal saline (0.9%), Formalin (10%), SDS 10%, Linalool, Melatonin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents used were of the highest purity commercially available.

2.2 Software's

Table 2.1. List of software's

Sr. No.	Software	Application
1	Open Bable 3.1.1	Convert SDF to PDBQT files
2	BIOVIA discovery studio 2021	Remove hydrogen bonds from structure of macromolecule
3	PyRx – virtual screening tool	Binding affinity of ligands with macromolecules.
4	Cytoscape 3.9.1	Network construction of various bio actives, targets, diseases, genes and pathways
5	Opus version-7.5	Interpretation of IR spectrum.
6	GraphPad prism 5	Statistical analysis of experimental data

2.3 Computational pharmacology

2.3.1 Network pharmacology

1st database construction - Database mining in this step we gathered all the data from literature survey using website PubMed (https://pubmed.ncbi.nlm.nih.gov/) where the chemical composition of plants was discovered, from the website PubChem, along with their individual chemical structures. (https://pubchem.ncbi.nlm.nih.gov/) chemical structures were downloaded in sdf format, software openbable was used to convert the downloaded chemical structures from the sdf format to the pdbqt format. then the RCSB protein data bank was used to download receptors in PDB format. (https://www.rcsb.org/) with the help of the discovery studio 4.5 programme, which included hydrogenation and the removal of crystal water, the target structure was preserved. Each target's binding site was docked with all of its chemical components, and libdock score was used as the yardstick for measuring the degree of docking. Molecular docking was carried out using the discovery studio 4.5 programme for every target site and all chemical components. The target and its conforming chemical constituents that had the highest docking fraction value were chosen (Chester et al. 2019).

PyRx software was used to perform molecular docking on selected chemical components and protein receptors. Using auto dock vina at PyRx, all ligand molecules were docked against receptors. (https://pyrx.sourceforge.io/) platform to determine the binding affinity of flavonolignans, version 0.8. In order to determine the most likely binding mode at 20 exhaustiveness, the grid box size was set to its maximum. To visualize the ligand-protein interface using discovery studio visualizer after docking, the ligand poses with the lowest binding energy and the highest intermolecular interactions were chosen (Chaudhary et al. 2022).

The chosen chemical component is sent to network construction when the maximum binding affinity has been determined. the data necessary for network creation is sourced from a number of databases, including PubChem for 2D or 3D chemical structures, Binding DB for research articles, and PubMed for articles on research. (https://www.bindingdb.org/bind/index.jsp) for various targets for a particular chemical entity, UniProt ID (https://www.uniprot.org/id-mapping) is a database used for target identification that contains information about genes, protein names, and organisms. Using the website DisGeNET, information about the relationship between a gene and a disease is gathered for subsequent use. (http://www.disgenet.org/) After obtaining this knowledge, the KEGG Pathway website is used to gather pathways involved in diseases. (https://www.genome.jp/kegg/mapper/) where all the pathways involved in various diseases are described in detail.

2nd network establishment - Following the collection of all the data from the various websites, excel sheets were created, and the network was constructed. The network diagram of chemical components and action targets was paradigmatized using Cytoscape 3.9.1 software using the relationship between components and potential targets.

3rd network analysis - Gene Ontology (GO) follows. (http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/mapper/) enhancement analysis were carried out to evaluate the screen node attributes (such as protein attributes, etc.) on the network's topological structure. STRING was used to investigate protein-protein interaction gene ontology (GO) (https://string-db.org/) To create a network diagram showing how implicit targets are related to one another, prospective targets were added to the STRING database.

2.3.2 Swiss ADME & TOXTREE online

Canonical SMILES of ligand were downloaded from PubChem website and used on the (http://www.swissadme.ch/) physicochemical properties, lipophilicity, water solubility, pharmacokinetics, and drug likeness were the parameters examined. Following that (https://toxtree.sourceforge.net/) the name of fragments were entered into a fragment search programme, modules were executed, and toxicity profiles for eye irritation, skin irritation, DNA binding, and protein binding were acquired (Mahanthesh MT et al. 2020; Rim 2020).

2.3.3 Analysis of silymarin

Determination of wavelength by UV spectroscopy

Silymarin was accurately weighed out at 10 mg and then transferred to a volumetric flask with a 10 ml capacity. Methanol was utilised to make a final volume of 10 ml with a concentration of 1000 µg/ml. One ml of the mentioned solution was transferred to a 10 ml volumetric flask. The volume was making up to 10 ml using methanol. A standard stock solution of 100 µg/ml was prepared for the UV spectroscopic analysis. For UV spectroscopic analysis, working standards with concentrations ranging from 2 to 12 µg/ml were created from this initial stock solution. From a 100 µg/ml standard stock solution, 1 ml was extracted, and 10 ml of methanol was used to make up the remaining volume. thus, For UV analysis, a working standard with a concentration of 10 µg/ml was used (Moin et al. 2010).

Exact 1 ml of the standard stock solution was taken and placed in a volumetric flask with a 10 ml capacity. It was further diluted with methanol to achieve a concentration of 10 µg/ml. In order to determine the wavelength at which silymarin absorb the most light (max), the UV spectrophotometer was employed to scan this solution from 200 to 800 nm (Moin et al. 2010).

Determination of functional groups by FT-IR spectroscopy

The technique known as fourier transform infrared spectroscopy (FT-IR) is used to get the infrared spectrum of a solid, liquid, or gas's absorption, emission, and photoconductivity. It's employed to find various functional groups in a substance. On an FT-IR spectrometer, silymarin's FT-IR spectra were acquired. The background spectrum is acquired once the FT-IR spectrometer has begun to warm up, after which the sample is placed beneath the probe, and the probe is then fixed in position. Silymarin's FT-IR spectra were recorded, along with absorption frequencies corresponding to the functional groups that were present. Results were also examined (Saiyyad et al., 2017).

2.4 Animal

Male Wistar rats weighing (180-230) gm were housed in groups in opaque polypropylene cages (28×21×14 cm) at 22 ± 2 °C under 12:12 h light/dark cycle and free access to standard rodent feed and tap water. Animals were obtained from the central animal house facility of sinhgad college of pharmacy vadgaon (BK), Pune, India. The animals were acclimatized to standard laboratory conditions (temperature 25±10 ◦C, relative humidity 50±15%) 1 week prior to the actual commencement of the experiment. They were provided with standard food pellets (Hindustan and Lever Ltd., India) and tap water ad libitum. The Committee for the Purpose of Control and Supervision of Experimental Animals approved the study (CCSEA). For the handling and care of the animals, CCSEA guidelines were followed.

2.5 Experimental Design

A balanced liquid diet was given for 7 days. Alcohol dependence was developed in the rat by daily oral administration of ethanol (2.4%, 4.8%, 7.2%) for 21 days (Tayfun 1995; Sharma et al. 2018). Normal control rats served as Group 1 and received a balanced liquid diet (p.o.) without alcohol for 21 days. Ethanol control rats served as Group 2 received 2.4% for 3 days, 4.8% for 4 days, and 7.2% for 14 days. Then Alcohol withdrawal score is observed for the 22nd day and 23rd days. Treatment group 3 contains a low dose of silymarin (50 mg/kg) from day 24th to the 30th day. Treatment group 4 contains treatment of a medium dose of silymarin (100 mg/kg) from the 24th to the 30th day. Treatment group 5 contains treatment of a high dose of silymarin (200 mg/kg) from day 2th to 30th which is for days. Group 6 contains memantine (5 mg/kg) from the 24th to the 30th day. The animals were tested for memory in the Morris water maze test and novel object recognition test on the 3rd, 5th, 7^{th,} and 9th day from the day of withdrawal & withdrawal score is assessed on the 4th, 6th, 8^{th,} and 10th day from the day of withdrawal.

2.5.1 Grouping

All animals included in the study are randomized and then divided into six treatment groups (n = 8).

Group I - Normal control group they were fed by balanced liquid diet (p.o.) without alcohol for 21 days.

Group II - Alcohol control group were fed by alcohol diet (2.4%, 4.8% and 7.2% p.o.) for 21 days.

Group III - Treatment group were fed by alcohol diet for 21 days + Low dose of silymarin (50 mg/kg p.o.) for subsequent 7 days. (on 24th day up to 30th day)

Group IV - Treatment group were fed by alcohol diet for 21 days and + Medium dose of silymarin (100 mg/kg p.o.) for subsequent 7 days. (on 24th day up to 30th day)

Group V - Treatment group were fed by alcohol diet for 21 days + High dose of silymarin (200 mg/kg p.o.) for subsequent 7 days. (on 24th day up to 30th day)

Group VI - Standard group were fed by alcohol liquid diet for 21 days + Std. drug memantine (5 mg/kg p.o.) for subsequent 7 days. (on 24th day up to 30th day)

2.5.2 Blood Ethanol Concentration (BEC)

The tail vein was used to collect blood samples (40 µl). A (160 µl) solution containing 3% perchloric acid was added, vortexed, and then centrifuged at 10,000 g while being cooled. At 4 °C, the supernatant was kept while awaiting analysis. For the test, 3 ml of 0.5 M Tris-Cl buffer (pH 8.8) containing 5.5 µg/ml alcohol dehydrogenase and 1.5 mM β-nicotinamide adenine dinucleotide (β-NAD) were added to 60 µl of supernatant and allowed to incubate for 40 minutes at room temperature. A reading of absorbance at 340 nm was then used to calculate the accumulation of β-NADH. Using a standard calibration curve, the amount of ethanol in the samples was estimated (Bhutada et al. 2010).

2.5.3 Assessment of alcohol withdrawal-induced symptoms

After 21 days, the balanced liquid diet including alcohol was discontinued, and alcohol withdrawal symptoms were noted for 5 minutes at 0 minutes, followed by 2, 4, 6, and 8 hours of the withdrawal period. A rating scale was used to rate stereotypical behaviors, abnormal gait and posture, tail stiffness, and agitation. The parameters' intensity was given as a median value. The behavioral indicators were converted from percent incidence to scores between 1 and 5, which

served as the basis for the calculation of the overall alcohol withdrawal score. The median values of each behavior were then added for each observation period. We did not considered the animals for further studies in which the sign and symptoms were not observed (Uzbay et al. 1997; Kayir & Uzbay 2008).

Table 2.2. Alcohol withdrawal scoring (Uzbay et al. 1997; Kayir & Uzbay 2008).

Signs	**Symptoms**
Stereotyped behavior	1.) Only one behavior
	2.) Two stereotyped behavior
	3.) Three stereotyped behavior
	4.) Four stereotyped behavior
	5.) All stereotyped behavior
Agitation	1.) Mild or moderate irritability
	2.) Very irritable
	3.) Handling vocalization and moderately aggressive
	4.) Handling vocalization and very aggressive
	5.) Spontaneous vocalization and very aggressive
Tail stiffness	1.) Mild tail rigidity
	2.) Moderate tail rigidity
	3.) Tail Rigid but only slightly flexible during ambulation.
	4.) Tail rigid & not flexible
	5.) Tail very rigid & not flexible
Abnormal posture	1.) Mild head down, back hunched
	2.) Moderate head down, back hunched
	3.) Prominent head down, back hunched
	4.) Furthermore, the hind legs are wide apart
	5.) In addition, fore limbs apart
Abnormal gait	1-2.) Mild difficulty ambulating & rearing normally
	3-4.) Moderate difficulty ambulating & rearing
	5.) Prominent difficulty ambulating & no rearing

2.6 Behavioural assessment

2.6.1 Morris water maze test

Morris water maze test was performed according to the methods described by (Uddin & Asaduzzaman 2016). The MWM testing apparatus was a tank-shaped (diameter 1700 mm × depth 450 mm) generally acknowledged tool for assessing spatial learning and memory capacities of rodents. The water maze was divided into four quadrants, each with its own submerged platform (diameter 110 mm × height 250 mm) was placed in one compartment. During testing, the tank was filled to a depth of 260mm with opaque water, placing the platform approximately 1cm below the water's surface and concealing it. During the acquisition trial, a rat was placed in the center of a tank compartment, and the time it took to ascend onto the submerged platform was measured using a stopwatch as escape latency (EL). If the rat did not find the platform within 300 seconds, it was steered towards it. In both of these cases, a rat was permitted to remain on the platform for 30 seconds. Each rat was treated to four consecutive trials while remaining fixed to the platform (East) with a different starting position each day. A stopwatch was also used to record the duration of time spent in the target quadrant (TSTQ). After the fourth trial, the rat was carefully dried with a soft cloth and kept warm under a light in the home case. Following the fourth trial of the day, a probe trial was conducted in which the platform was withdrawn, and the time spent by the rat in the target quadrant (TSTQ) was measured for 60 seconds (Blokland et al., 2004).

2.6.2 Novel object recognition test

The novel object recognition test is based on rodents' proclivity to discover new objects. Rats were trained to differentiate between different shaped objects (cubes, pyramids and cylinders) in an open field Plexiglas box (70 × 60 × 30 cm). On day one, rats were treated to a habituation trial in which they were allowed to freely explore the arena for 6 minutes. On day two, a single session of two trials (T1 and T2) was done, separated by a retention interval of 60 minutes. Specifically, two identical objects were put in two opposite corners of the box during the familiarization session (T1). Each rat had been placed in the center of the box, facing away from the objects, and allowed to study them freely for a maximum of 5 minutes. T1 concluded when the rat spent 20 seconds exploring each object. Exploration is described as directing the nose to an object from a distance < 2 cm and/or touching the object with the nose. During T2, a novel object with an alternate shape and colour was substituted for one of the familiar objects shown in Tl, and rats were placed in the box for 5 minutes. The rats were returned to their home cage following this session. The time spent exploring the familiar

(F) and new (N) objects was recorded separately, and the difference between the two exploration times was used to calculate the discrimination index (DI = N - F/ N + F). Furthermore, to avoid place preference, the role (F or N) and position of the objects during T2 were changed at random. Total distance travelled (cm) was also measured and analyzed for each experimental group to determine locomotor impairment (Uddin & Asaduzzaman 2016; Cassano et al. 2020).

2.7 Determination of antioxidant enzymes

2.7.1 Preparation of Homogenates

The brain was separated for the biochemical studies after separation it was rinsed with ice-cold saline and homogenized with chilled phosphate buffer (PH 7.4). Centrifuged at 4600 rpm for 10 min for 4°C. The supernatant obtained was called "homogenate" and used for the assay's different antioxidant parameters (Nanaware et al. 2017).

2.7.2 Assay of Super Oxide Dismutase (SOD)

A mixture of equal parts of distilled water and brain tissue homogenate (supernatant) was then added, along with 0.25 mL of ice-cold ethanol and 0.15 mL of ice-cold chloroform. Using a cyclomixer, the mixture was thoroughly mixed before being centrifuged at 600×g for 15 min at 4 °C. A 0.5 mL solution of EDTA was added to this. The injection of 0.4 mL of epinephrine started the reaction, and the change in optical density/min was recorded at 480 nm in comparison to a reagent blank (Misra & Fridovich 1972).

2.7.3 Glutathione (GSH) activity in the brain

TCA (20%) and brain tissue homogenate (supernatant) were combined in equal parts. After centrifuging the precipitated fraction at 600×g for 15 minutes at 4 °C, 2 mL of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) reagent were added to 0.25 mL of supernatant. With phosphate buffer, the final volume was adjusted up to 3.0 mL. At 412 nm, the color generated was compared to a reagent blank (Moron et al., 1979).

2.7.4 Assay of Catalase

According to Beers Jr. and Sizer's approach from 1952, the ability of CAT to trigger the disappearance of hydrogen peroxide was used as the basis for measuring CAT activity. The amount of enzyme required to cause the breakdown of 1 mmol of peroxide per minute is equal to one unit of CAT. 40 mL of the brain tissue homogenate's supernatant was mixed with 1960 mL of substrate (a mixture of 1800 mL of 10 mM H_2O_2, 100 mL of Tris-HCl buffer, and 60 mL of D/W). Measurements of the exponential disappearance of H_2O_2 at 240 nm at 25 °C were used to determine activity (Beers Jr. & Sizer 1952).

2.7.5. Malondialdehyde (MDA) levels in the brain

The Wills method was used to measure the amount of lipid peroxidation in the brain quantitatively. Thiobarbituric acid was used to measure the amount of malondialdehyde (MDA) produced in the process at 532 nm. The results were calculated using the chromophore's molar extension coefficient (1.56 × 105 M- 1 cm-1) and expressed as nm of MDA per milligrams of protein. 0.1 ml of tissue homogenate, 0.2 ml of 8% sodium lauryl sulphate (SLS), 1.5 ml of 20% acetic acid, and 1.5 ml of 0.8% thiobarbituric acid (TBA) solution make up the reaction mixture. On a water bath, the mixture was heated for one hour at 95 °C. Then, 5 ml of a 15:1 n-butanol/pyridine combination was added to it. After a quick shake, the mixture was centrifuged at 2200 × g for 5 minutes. At a wavelength of 532 nm, the organic layer's absorbance was measured. The results were calculated using the chromophore's molar extinction coefficient (1.56 × 105 M-1 cm-1) and expressed as nm of MDA per milligrams of tissue (Nade et al., 2020)

2.7.6 Estimation of nitric oxide (NO) levels in brain

In 100 mL volumetric flasks, sample solutions of brain tissue homogenate were produced at various concentrations. This was then mixed with 0.1489 g of sodium nitroprusside (5 mM final concentration) and left for incubation. 5.6 mL was taken at regular intervals, to which 0.2 mL of Griess reagent A was added, and incubated for 10 minutes at 30°C. 0.2 mL of Griess reagent B was added after incubation, maintained at 30° C for 10 minutes, and absorbance measured at 542 nm against a blank (Sreejayan & Rao 1997).

2.8 Histopathology

Animals were sacrificed by decapitation method at the end of the study, and their brains were quickly removed and preserved in 10% formalin. Later, the samples from the brain were processed for histological analysis. Hematoxylin and eosin staining was done, and a light microscope was used to view the pathological alterations (Sivaraman et al., 2015; Chen and Zhou, 2020).

2.9 Statistical analysis

One-way ANOVA followed by Turkey's t-test & two-way ANOVA followed by Bonferroni post hoc test was used to compare the experimental data. All values were reported as mean ± SEM for each group. The significant level was shown as $p<0.05$.

3. RESULTS

3.1 Computational pharmacology

In this study, using network pharmacology, we identified targets for memory dysfunction. 40 targets of memory dysfunction, target disease network was constructed to enrich the target molecules, and multiple pathways were investigated based on the present knowledge of the etiology and pathophysiology of memory dysfunction and the identification of its target molecules. The glutamatergic pathway was revealed to be the disease pathway most associated with memory dysfunction out of the 69 target-enriched disease pathways chosen. To make sure the study's accuracy was within an acceptable range, we used molecular docking to correlate 48 ligands from milk thistle with receptors linked to memory dysfunction. (Table no. 3.1)

Table 3.1. Molecular docking score

Ligand	Binding affinity GABA (5gwm)	Binding affinity NMDA (5gwm)	Binding affinity DOPAMINE (2a3r)	Binding affinity SEROTONIN (5HT2A)
2 3-Dehydrosilychristin	-6.3	-6.3	-8.4	-7.1
5 7-Dihydroxychromone	-6.4	-6.3	-8.6	-5.7
Apigenin	-5	-5	-5.9	-5
Arachidic-acid	-5.9	-5.9	-7.4	-6.3
Aromadendrin	-4.9	-4.9	-5.4	-5
Behenic-acid	-5.8	-5.6	-7.5	-6.3
Beta-Carotene	-4.5	-4.9	-5.7	-4.8
Beta-Sitosterol	-6.5	-6.7	-7.7	-4.5
Betaine	-5.3	-5.4	-7.7	-6.3
Chrysoeriol	-2.9	-2.9	-3.6	-2.7
Cnicin	-6.1	-6.1	-7.5	-6.2
Cosmosiin	-5.6	-5.6	-6.8	-4.2
Dehydroconiferyl-alcohol	-6.7	-6.7	-8.2	-6.5
Dehydrosilybin	-4.9	-4.9	-5.7	-4.8
Eriodictyol	-6.8	-6.8	-6.7	-5.2
Gamma-aminobutyric-acid	-5.9	-5.9	-9	-6.4
Glutamic-acid	-3.1	-3.2	-7	-4.9
Isosilybin	-4	-3.9	-7.6	-6.3
Isosilychristin	-6.4	-6.4	-4.2	-3.6
Kaempferol	-6	-5.7	-5.4	-4.2
Leucine	-5.7	-5.7	-8.5	-7
Linoleic-acid	-3.9	-3.8	-7.7	-5.3
Luteolin-7-Glucoside	-5.9	-5.9	-7.5	-6.1
Luteolin	-4	-3.9	-4.4	-3.8
Myristic-acid	-6.9	-6.7	-7.4	-6.3
Naringenin	-6	-6	-5.3	-4.7
Octadecadienoic-acid	-6.4	-6.1	-8.4	-5.9
Oleic-acid	-3.5	-3.7	-7.6	-6.4
Palmitic-acid	-3.6	-3.6	-8.2	-5
Proline	-5.9	-5.9	-5.1	-3.8
Quercetin	-5.5	-5.5	-4.4	-3.9
Riboflavin	-6.3	-6.3	-7.8	-6.1
Silicon	-6.7	-6.7	-7.2	-5.3
Silybin-A	-7.1	-7.1	-8.4	-5.6
Silybin-B	-6.7	-6.7	-9.1	-6.4
Silybin	-7.1	-7.1	-8.6	-5.9
Silybinin	-6.2	-6.2	-9.1	-6.4
Silychristin	-6.7	-6.7	-8.9	-6.7
Silydianin	-6.4	-6.4	-8.4	-6.1
Silyhermin	-6.6	-7	-8.7	-5.3
Silymarin	**-7.2**	**-6.9**	**-9.2**	**-7.2**
Silymonin	-5	-4.9	-8.9	-6.5
Triamine	-3.7	-3.7	-8.8	-5.2
Tridec-1-Ene-3-5-7-9-11-Pentayne	-4.4	-4.2	-5.8	-4.8

Trideca-1-3-11-Triene-5-7-9-Triyne	-4.4	-4.4	-4.7	-3.9
Tyramine	-6.4	-6.3	-5.2	-4.4
Taxofolin	-5.6	-5.6	-4.8	-4.3
Donepezil	**-7.1**	**-6.7**	**-8.6**	**-7.1**
Memantine	**-7**	**-6.5**	**-6.6**	**-7**
Duloxetine	-7	-6.5	-9	-7.1
Fluoxetine	-7.1	-6.7	-8.9	-7

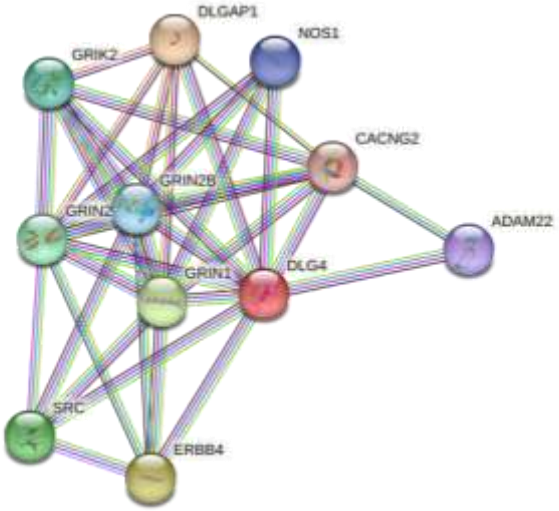

Fig 3.1. STRING PPI Interaction Network

Silymarin has a wide range of target proteins and interactions that work together to treat memory dysfunction. The PPI network diagram (Fig 3.1) reveals that the screened target proteins form a vast network of interactions. The memory dysfunction targets of silymarin mostly involve the glutamatergic pathway and alzheimer disease, which are strongly related to the key processes of memory dysfunction, in addition to several disease-associated pathways, according to the results of the KEGG enrichment analysis. Together, through this analysis, we have established the anti-alzheimer effect of silymarin and identified new research targets for memory dysfunction. The findings, which were based on a theoretical mechanism, required to be further confirmed by additional experiments.

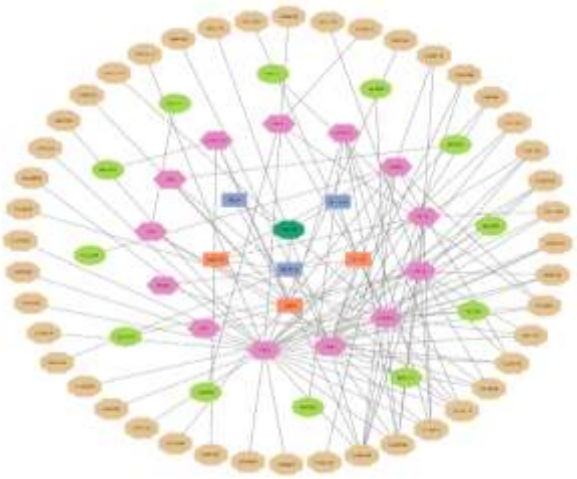

Fig 3.2. Network Pharmacology

Silymarin - Bioactive, Gene, Disease and Pathway network. Bioactive are represented by square nodes, Genes are represented by octagonal nodes, Diseases are represented by oval nodes and Pathways are represented by hexagonal nodes.

3.2 Swiss ADME of silymarin

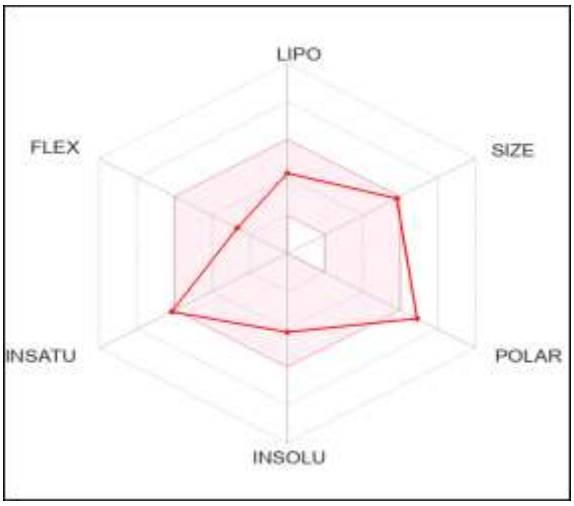

Fig 3.3. ADME Profile of Silymarin

Table 3.2. Physico-Chemical Parameters

Formula	$C_{25}H_{22}O_{10}$
Molecular weight	482.44 g/mol
No. of hydrogen bond acceptors	10
No. of H-bond donors	5
Solubility	Moderately soluble in water
GI absorption	Low
BBB permeant	Yes

As shown in (Table no. 3.2), silymarin demonstrated its pharmacokinetic properties. Silymarin has 10 hydrogen bond acceptors along with 5 hydrogen bond donors. Silymarin is moderately soluble in water; therefore, for solubility, we had used 0.5% CMC for our experimental study. Silymarin crosses the blood-brain barrier.

3.3 TOXTREE online of silymarin

Table 3.3. Physico-chemical parameters

Parameters	**Result**
Eye irritation	Unknown
Skin irritation	Unknown
DNA binding	No DNA binding
Protein binding	No protein binding

In order to conduct the in silico toxicity study, we used toxtree online. As seen in (Table no. 3.3), silymarin uses computer modelling to forecast a number of chemical toxicity features. In which it is demonstrated that silymarin has no DNA and protein binding.

3.4 Determination of wavelength by UV spectroscopy

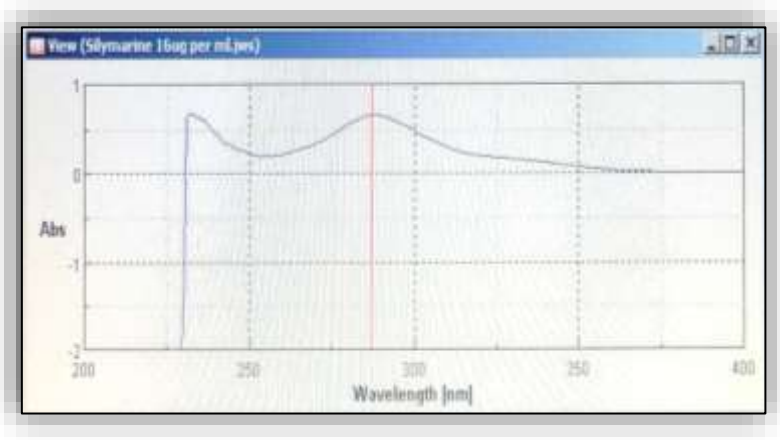

Fig 3.4. Spectra of Concentration Range 16 (µg/ml)

Table 3.4. Absorbance at different concentration 6-16 (µg/ml) at 287nm

Concentration	Absorbance at 287nm
6	0.3751
8	0.4398
10	0.4987
12	0.5441
14	0.6057
16	0.6627

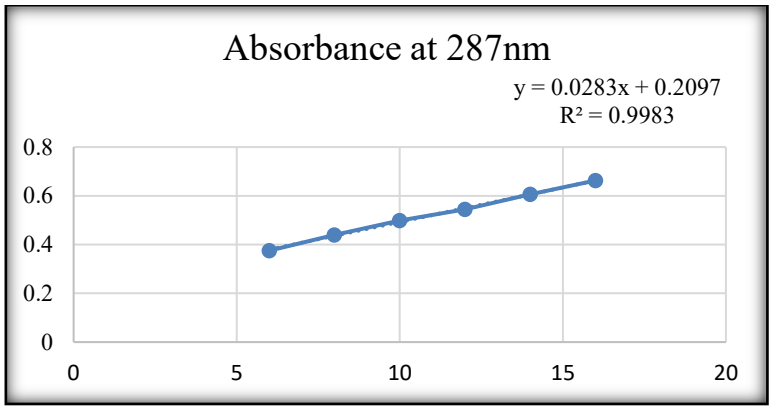

Fig 3.5. Linearity Plot of the silymarin

The correlation coefficient of 0.9983 indicates compliance with the beer lamberts law. Linearity graph. The proposed UV method allows rapid and economical quantification of silymarin. In methanol, absorption maxima of silymarin were detected at 287 nm & spectra of concentration range 6-16 (µg/ml) was recorded (Fig 3.5). Absorbance at different concentration showed in (Table 3.4). The correlation coefficient of 0.9983 indicates compliance with the beer lamberts law. Linearity graph was showed in (Fig 3.5).

3.5 Determination of functional groups by FT-IR spectroscopy

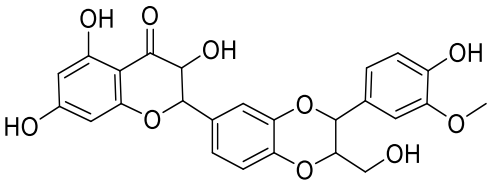

Fig 3.6. Structure of silymarin

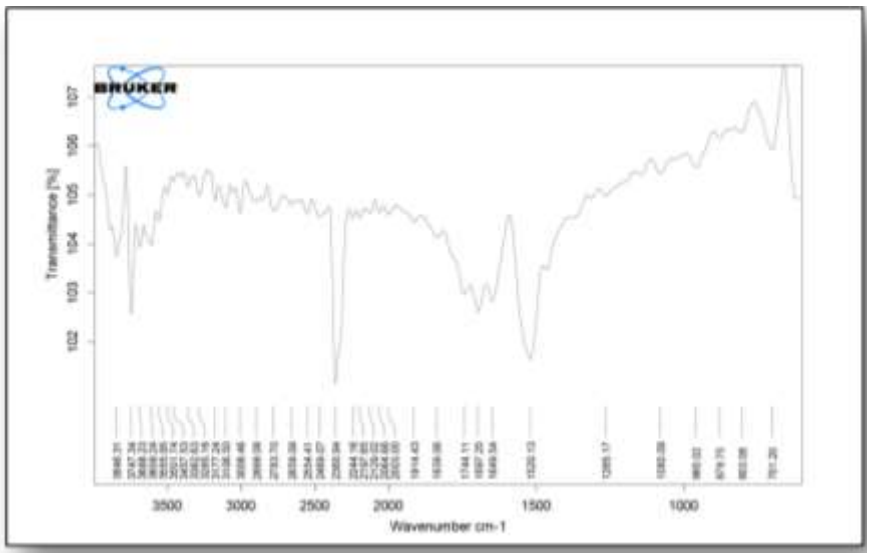

Fig 3.7. FTIR Quantification of Silymarin

The FTIR spectrum of silymarin was recorded and the functional groups were interpreted as per the structure and were found to be appropriate. (Fig. 3.7) gives the FTIR spectra of the silymarin, gives the interpretation of the peaks obtained in the IR spectra along with their corresponding functional groups. The given IR spectra of silymarin shows the principle peaks at 3688.23 cm-1 and 3609.24 cm-1, 3457.53 cm-1 and at 3177.24 cm-1, C=O Stretch at 3555.95 cm-1, 1839.06

cm-1, 1744.11 cm-1, 1649.54 cm-1 & at 3501.74 cm-1, C-H at 2899.08 cm-1 and 2783.70 cm-1 indicate that above IR spectra is of silymarin which is identified and proved.

3.6 Effect of ethanol intake on body weight

Fig 3.8 shows graph with Mean ± SEM (n=8). In the present investigation, two-way ANOVA followed Bonferroni post hoc test showed that, on day 8 we observed a significant increase in the body weight of other groups such as ethanol control, treatment groups (silymarin 50,100, 200) and Std. memantine (5mg/kg) when compared to the body weight of normal control rats (*P< 0.05, **P< 0.01 and ***P < 0.001). After 8th day rats showed symptom like anorexia and due to that there is significant decrease in the body weight. (*P< 0.05, **P< 0.01 and ***P < 0.001).

Table 3.5. Effect of ethanol on body weight [mean ± SEM (n=8)

Time (hrs).	Normal control	Ethanol control	Silymarin (50mg/kg)	Silymarin (100mg/kg)	Silymarin (200mg/kg)	Memantine (5mg/kg)
1	208.50 ± 3.30	219.25 ± 3.05	209.50 ± 3.45	216.50 ± 3.51	210.62 ± 3.94	216.37 ± 4.70***
4	218.25 ± 5.73	224.12 ± 3.35	231.25 ± 5.03	248.12 ± 2.28	241.37 ± 4.11	235.12 ± 4.05
8	217.75 ± 3.13	238.87 ± 3.68***	234.00 ± 3.09**	259.12 ± 1.86***	250.50 ± 4.37***	247.25 ± 3.83***
15	216.50 ± 2.63	219.37 ± 3.25	221.75 ± 2.78	245.37 ± 2.42	241.12 ± 3.62	234.50 ± 4.07
21	214.875 ± 2.14	194.37 ± 3.44***	199.87 ± 2.05*	231.25 ± 2.09**	193.12 ± 2.24***	193.12 ± 2.27***

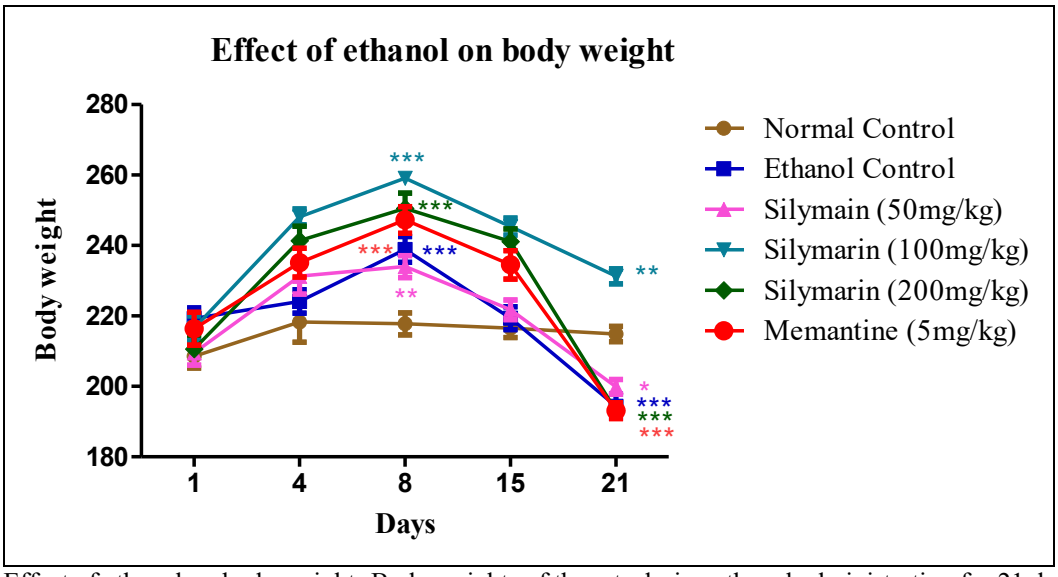

Fig 3.8. Effect of ethanol on body weight- Body weights of the rats during ethanol administration for 21 days. Data are expressed as mean ± SEM (n=8) analyzed by two-way ANOVA followed by Bonferroni post hoc test, where (*P<

3.7 Effect of silymarin on body weight

Fig 3.9 shows graph with Mean ± SEM (n=6). In the present investigation, two-way ANOVA followed Bonferroni post hoc test revels that, after ethanol withdrawal, as compared to normal control, ethanol control group showed decrease in body weight due to anorexia throughout the treatment ($^{###}P < 0.001$). During treatment of 7 days with silymarin (50, 100, 200 mg/kg), as compared to ethanol control, there is significantly increase in body weight throughout the treatment ($^{*}P < 0.05$ $^{**}P < 0.01$, and $^{***}P < 0.001$).

There is similar effect in body weight of animals treated with Std. memantine (5mg/kg) and silymarin (200mg/kg) treated animals vs the normal control animals.

Table 3.6. Effect of silymarin on body weight [mean ± SEM (n=6)].

Time (hrs).	Normal control	Ethanol control	Silymarin (50mg/kg)	Silymarin (100mg/kg)	Silymarin (200mg/kg)	Memantine (5mg/kg)
1	217.50 ± 2.14	197.66 ± 1.68$^{###}$	205.66 ± 2.29	229.33 ± 3.45***	209.16 ± 2.79*	213.00 ± 3.39**
3	226.66 ± 2.65	194.33 ± 1.68	211.00 ± 2.33	233.66 ± 3.63	215.50 ± 2.76	219.16 ± 3.34

5	230.33 ± 2.60	192.50 ± 2.98	217.00 ± 2.74	240.16 ± 3.58	219.16 ± 2.83	224.33 ± 3.53
7	232.83 ± 2.28	190.16 ± 3.04$^{###}$	223.66 ± 3.09***	245.66 ± 3.66***	226.00 ± 3.10***	229.66 ± 3.39***

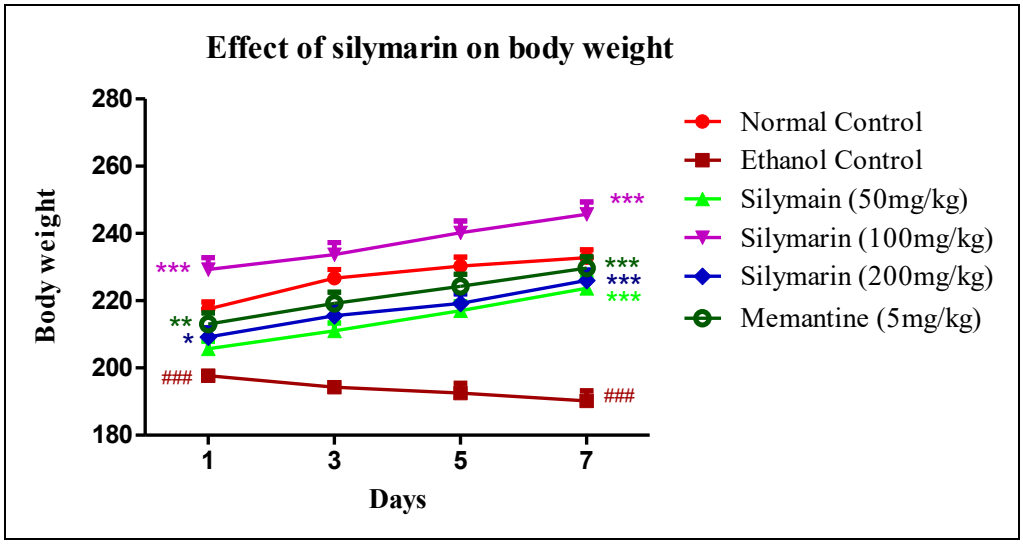

Fig 3.9. Body weight of the rats during drug treatment of silymarin (50, 100 and 200 mg/kg), Std. memantine (5 mg/kg) for 7 days. Data are expressed as mean ± SEM (n=6) analyzed by two-way ANOVA followed by Bonferroni post hoc test, where ($^{###}P < 0.001$) vs normal control group, ($^{*}P < 0.05$ $^{**}P < 0.01$, and $^{***}P < 0.001$) vs the ethanol control group.

3.8 Blood ethanol concentration

Blood ethanol concentrations (BEC) were measured to determine if the ethanol liquid diet would provide accurate BEC during consumption of ethanol and to confirm ethanol clearance during withdrawal period. Two-way ANOVA showed that as ethanol concentration increases i.e. 2.4% for 3 days then 4.8% for next 4 days and 7.2% up to 21days, BEC also significantly increases up to 21 days, after the period of ethanol withdrawal i.e. on 22nd day the rats showed clearance of ethanol from blood (Fig 3.10).

Table 3.7. Blood ethanol concentration [mean ± SEM (n=8)]

Day	**Ethanol control**	**Silymarin (50mg/kg)**	**Silymarin (100mg/kg)**	**Silymarin (200mg/kg)**	**Memantine (5mg/kg)**
3rd	66.55 ± 3.59	94.88 ± 13.43	68.11 ± 10.80	81.69 ± 5.56	68.69 ± 9.22
7th	143.62 ± 11.77	129.58 ± 15.99	125.48 ± 11.04	165.59 ± 21.45	121.51 ± 7.64
21st	304.54 ± 23.77	365.98 ± 9.46	320.58 ± 10.21	345.95 ± 10.96	347.93 ± 10.56
22nd	12.54 ± 1.69	14.20 ± 1.84	13.23 ± 2.23	12.27 ± 1.79	13.09 ± 2.12

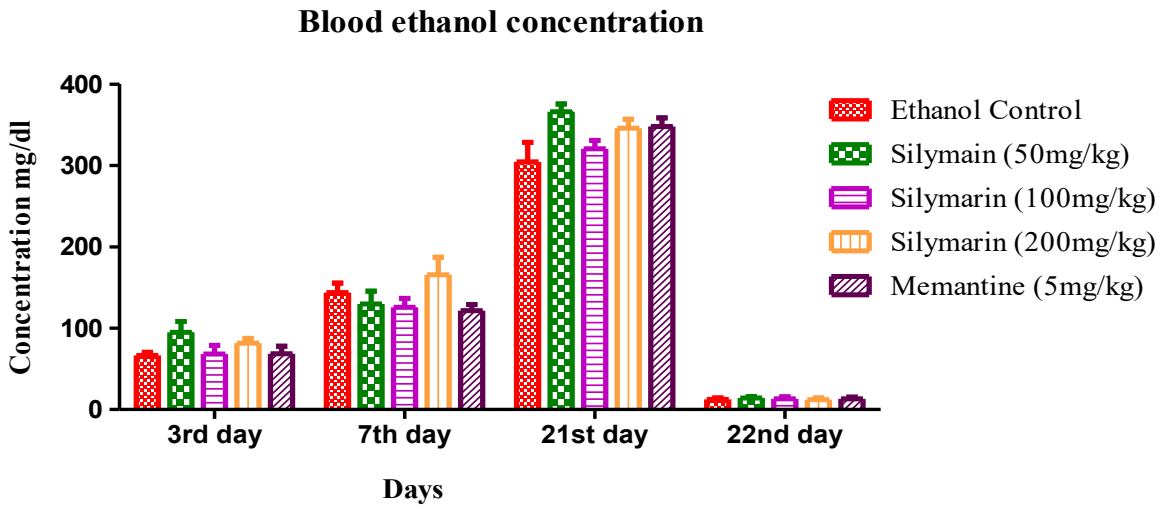

Fig 3.10. Blood ethanol concentration Data is expressed as mean ± S.E.M. (n=8) analyzed by Two-way ANOVA. At 3rd day (2.4%), 7th day (4.8%), 21st day (7.2%) & 22nd day (withdrawal of ethanol).

3.9 Effect of silymarin on ethanol withdrawal-induced symptoms

The development of ethanol withdrawal symptoms was assessed on 22nd day by observing scoring of ethanol withdrawal-induced physical signs of craving at 2, 4, 6 and 8hrs.

3.9.1 Before treatment

Fig 3.11 shows graph with Mean ± SEM (n=8) analyzed by two-way ANOVA followed by Bonferroni's post hoc test discovered that, on 22nd day ethanol withdrawal score of the rats were observed at 2, 4, 6, 8 hrs. After ethanol withdrawal, as compared to normal control, other groups such as ethanol control, treatment groups (silymarin 50,100, 200mg/kg) and Std. memantine (5mg/kg) showed increase in ethanol withdrawal score at 2, 4, 6 and 8hrs (***P < 0.001). Alcohol withdrawal symptoms observed at high peak at 6th hour.

Table 3.8. Ethanol withdrawal scoring before treatment [mean ± SEM (n=8).

Time hrs.	Normal control	Ethanol control	Silymarin (50mg/kg)	Silymarin (100mg/kg)	Silymarin (200mg/kg)	Memantine (5mg/kg)
2	0.75 ± 0.13	2.35 ± 0.41	1.95 ± 0.07	2.17 ± 0.05	1.52 ± 0.07	1.77 ± 0.08
4	0.62 ± 0.15	3.22 ± 0.13	2.97 ± 0.08	3.40 ± 0.12	2.87 ± 0.07	2.30 ± 0.15
6	0.77 ± 0.11	4.10 ± 0.08	3.75 ± 0.19	4.30 ± 0.08	3.57 ± 0.11	3.10 ± 0.17
8	0.85 ± 0.06	4.35 ± 0.09$^{\$***}$	4.30 ± 0.26***	4.57 ± 0.13***	3.95 ± 0.10***	3.90 ± 0.14***

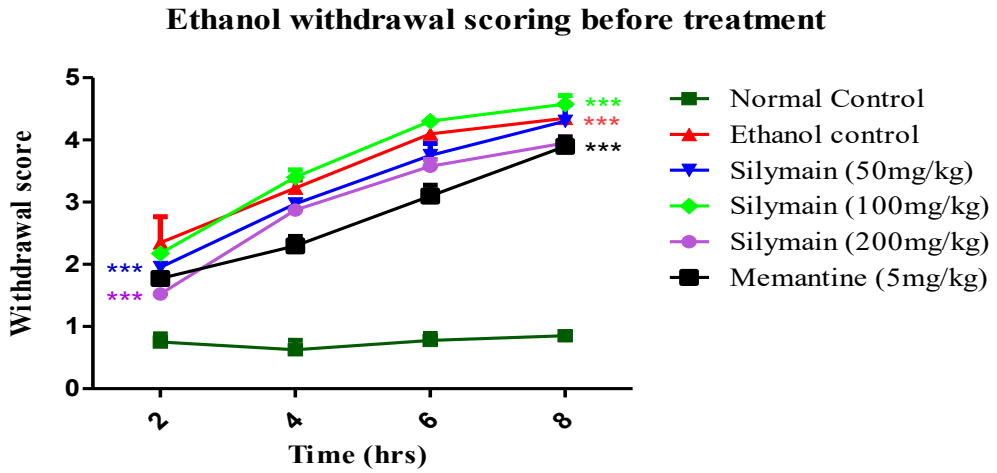

Fig 3.11. Ethanol withdrawal score of the rats before treatment at 2, 4, 6, 8 hrs. Data is expressed as mean ± S.E.M. (n=8) analyzed by two-way ANOVA followed by Bonferroni's post hoc test, where (***P < 0.001) vs normal control group.

3.9.2 During treatment

Development of ethanol withdrawal symptoms was evaluated in ethanol group and treatment groups by scoring the withdrawal-induced physical signs of hyper excitability, graded as per the scale.

Fig 3.12 shows graph with Mean ± SEM (n=6) analyzed by two-way ANOVA followed by Bonferroni's post hoc test revels that, On the 1st day of treatment with silymarin (50, 100, 200 mg/kg) and Std. memantine (5 mg/kg) ethanol withdrawal score was observed at 2, 4, 6 and 8hrs. In ethanol control group, there is significantly increase in ethanol withdrawal score vs normal control rats ($^{###}P < 0.001$) whereas, in treatment groups (silymarin 50,100, 200mg/kg) and Std. memantine (5mg/kg) as compared to ethanol control, there is significantly decrease in ethanol withdrawal score (*P< 0.05 and ***P < 0.001)..

Table 3.9. Ethanol withdrawal scoring during treatment [mean ± SEM (n=6)].

Time hrs.	Normal control	Ethanol control	Silymarin (50mg/kg)	Silymarin (100mg/kg)	Silymarin (200mg/kg)	Memantine (5mg/kg)
2	1.03 ± 0.03	4.20 ± 0.16$^{###}$	4.63 ± 0.20*	4.70 ± 0.19*	4.36 ± 0.13	4.00 ± 0.05
4	0.70 ± 0.06	4.13 ± 0.16	4.10 ± 0.04	3.93 ± 0.09	3.00 ± 0.21	3.86 ± 0.06
6	0.76 ± 0.08	4.50 ± 0.11	3.63 ±0.06	3.33 ± 0.08	2.00 ± 0.17	3.60 ± 0.14
8	0.76 ± 0.06	4.93 ± 0.06$^{###}$	2.73 ±0.15***	2.30 ± 0.11***	1.10 ± 0.04***	3.16 ± 0.09***

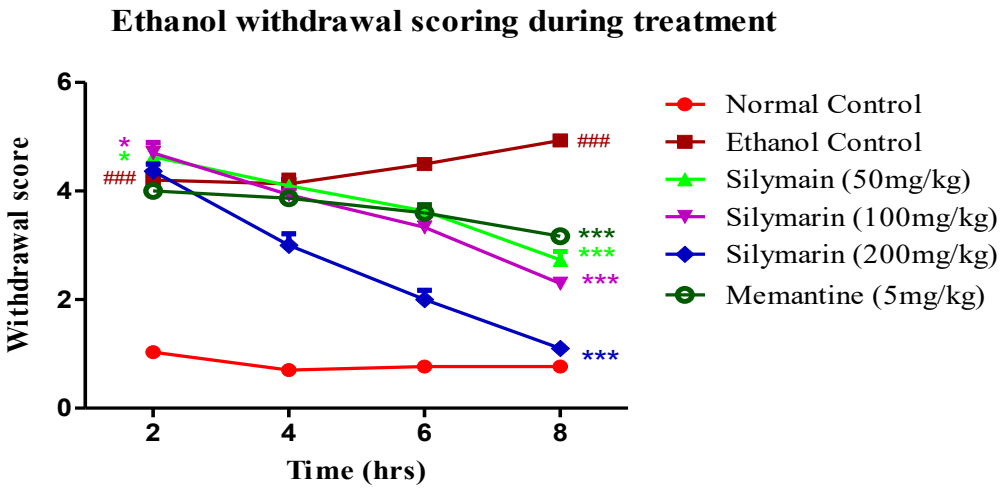

Fig 3.12. Ethanol withdrawal score of the rats during treatment at 2, 4, 6, 8 hrs. Data is expressed as mean ± S.E.M. (n=6) analyzed by two-way ANOVA followed by Bonferroni's post hoc test, where ($^{###}$ P < 0.001) vs normal control group, (*P< 0.05 and ***P < 0.001) vs the ethanol control group.

3.10 Effect of silymarin on ethanol withdrawal-induced memory dysfunction in rats.

3.10.1 Effect of silymarin on time spent in target quadrant in Morris Water Maze test

Fig 3.13 shows graph with Mean ± SEM (n=6). In the present investigation, one-way ANOVA followed by Tukey's post hoc test revels that, effect of silymarin (50, 100 and 200mg/kg p.o. for 7 day) the development of ethanol withdrawal induced memory dysfunction was accessed by measuring the time spent in target quadrant in MWM test on 7th day after 30 minutes. There is significant decrease in time spent in target quadrant in ethanol control rats when compared with normal control rats ($^{###}P < 0.001$).

Treatment groups (silymarin 50,100, 200mg/kg) and Std. memantine (5mg/kg) showed dose dependently increase in time spent in target quadrant vs ethanol control rats (*P< 0.05, **P< 0.01 and ***P < 0.001). There is significant increase in time spent in target quadrant in silymarin (200mg/kg) and Std. memantine (5mg/kg) when compared with silymarin 50mg/kg & 100mg/kg ($^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$) ($^{@}P <0.05$, $^{@@}P <0.01$).

Table 3.10. Effect of silymarin on Morris Water Maze Test [mean ± SEM (n=6)].

Treatment	Mean ± SEM
Normal Control	63.00 ± 2.81
Ethanol Control	28.66 ± 2.17$^{###}$
Silymarin 50mg/kg	40.50 ± 2.75*
Silymarin 100mg/kg	45.00 ± 2.23**
Silymarin 200mg/kg	57.33 ± 2.99$^{***\ \$\$\ @}$
Memantine 5mg/kg	59.66 ± 3.04$^{***\ \$\$\$\ @@}$

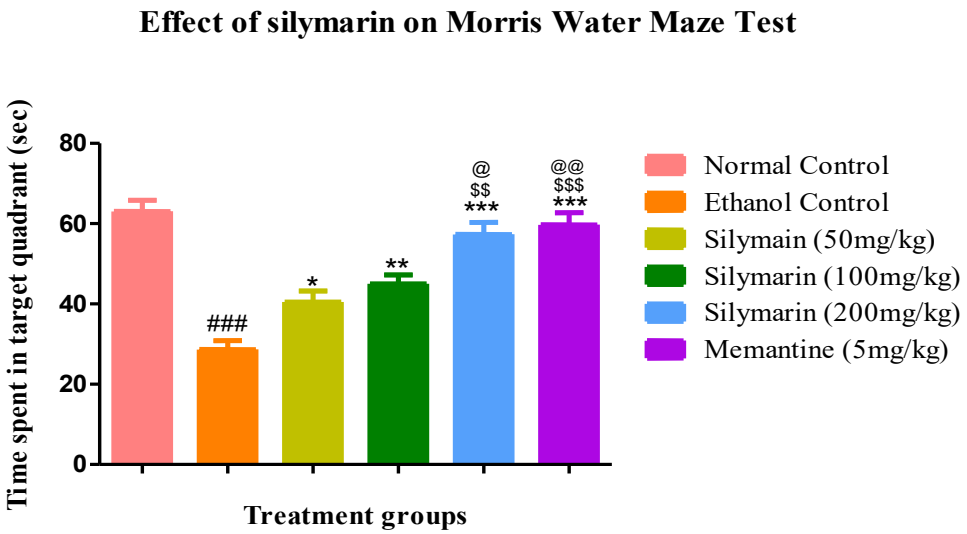

Fig 3.13. Effect of Silymarin (50, 100, 200 mg/kg) on ethanol withdrawal induced memory dysfunction when tested on the rats in Morris water Maze test.

Data are expressed as mean ± SEM (n=6) analyzed by one-way ANOVA followed by Tukey's post hoc test, where ($^{###}P < 0.001$) vs normal control group, ($^{*}P< 0.05$, $^{**}P< 0.01$ and $^{***}P < 0.001$) vs the ethanol control group. ($^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$) vs silymarin 50mg/kg treated group and ($^{@}P <0.05$, $^{@@}P <0.01$) vs the silymarin 100mg/kg treated group.

3.10.2 Effect of silymarin on discrimination index in Novel Object Recognition Test

Fig 3.14 shows graph with Mean ± SEM (n=6). In the present investigation, one-way ANOVA followed by Tukey's post hoc test showed that, effect of silymarin (50, 100 and 200mg/kg oral for 7 days). The development of ethanol withdrawal induced memory dysfunction was accessed by measuring exploration time with familiar and novel object on 7th day of treatment after 30 minutes and then discrimination index were calculated.

There is high significant difference in discrimination index in ethanol control rats when compared with normal control rats ($^{###}P < 0.001$). There is less significant difference in discrimination index in rats treated with silymarin (50mg/kg) when compared to ethanol control group ($^{*}P< 0.05$). In treatment groups Silymarin (100, 200mg/kg) and showed significantly increase in discrimination index vs ethanol control rats ($^{***}P < 0.001$). There is significant increase in discrimination index of rats treated with silymarin (200mg/kg) and Std. memantine (5mg/kg) when compared with silymarin 50mg/kg & 100mg/kg ($^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$) ($^{@}P <0.05$). There is no significant difference observed in discrimination index of rats treated with Std. memantine (5mg/kg) and silymarin (200mg/kg).

Table 3.11. Effect of silymarin on Novel Object Recognition Test [mean ± SEM (n=6)].

Treatment	**Mean ± SEM**
Normal Control	0.33 ± 0.01
Ethanol Control	0.08 ± 0.01$^{###}$
Silymarin 50mg/kg	0.16 ± 0.02*
Silymarin 100mg/kg	0.20 ± 0.01***
Silymarin 200mg/kg	0.27 ± 0.01*** $^{\$\$}$ $^{@}$
Memantine 5mg/kg	0.28 ± 0.01*** $^{\$\$\$}$ $^{@}$

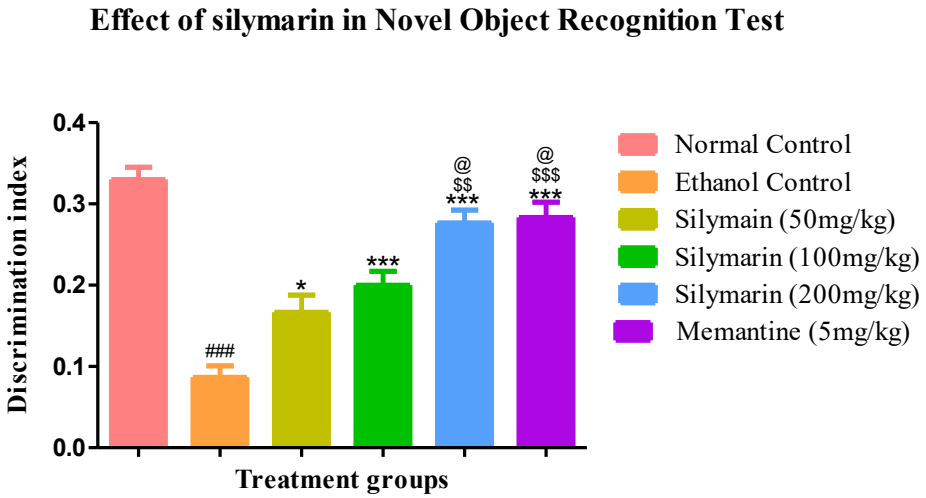

Fig 3.14. Effect of Silymarin (50, 100, 200 mg/kg) on ethanol withdrawal induced memory dysfunction when tested on the rats in Novel Object Recognition test. Data are expressed as mean ± SEM (n=6) analyzed by one-way ANOVA followed by Tukey's post hoc test, where ($^{###}P < 0.001$) vs normal control group, ($^{*}P< 0.05$ and $^{***}P < 0.001$) vs the ethanol control group. ($^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$) vs silymarin 50mg/kg treated group and ($^{@}P <0.05$) vs silymarin 100mg/kg treated group.

3.11 Biochemical estimation

3.11.1 Effect of silymarin on superoxide dismutase level

Fig 3.15 shows graph with Mean ± SEM (n=6) analyzed by, one-way ANOVA followed by Tukey's post hoc test showed that, a significant decrease in the level of SOD in the ethanol control group when compared with the normal control group ($^{###}P < 0.001$). In treatment groups (silymarin 50,100, 200 mg/kg) and Std. memantine (5mg/kg) showed dose dependently increase in reduced level of SOD in comparison with ethanol control rats ($^{*}P < 0.05$, $^{**}P< 0.01$ and $^{***}P < 0.001$). There is significant increase in level of SOD when treated with silymarin (200mg/kg) and Std. memantine (5mg/kg) when compared with silymarin 50mg/kg & 100mg/kg ($^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$) ($^{@}P <0.05$, $^{@@}P <0.01$).

Table 3.12. Effect of silymarin on superoxide dismutase levels [mean ± SEM (n=6)].

Groups	SOD (µmol/ml)
Normal Control	7.73 ± 0.48
Ethanol Control	1.92 ± 0.29$^{###}$
Silymarin 50mg/kg	3.79 ± 0.44*
Silymarin 100mg/kg	4.50 ± 0.47**
Silymarin 200mg/kg	6.47 ± 0.34*** $^{\$\$}$ $^{@}$
Memantine 5mg/kg	6.90 ± 0.47*** $^{\$\$\$}$ $^{@@}$

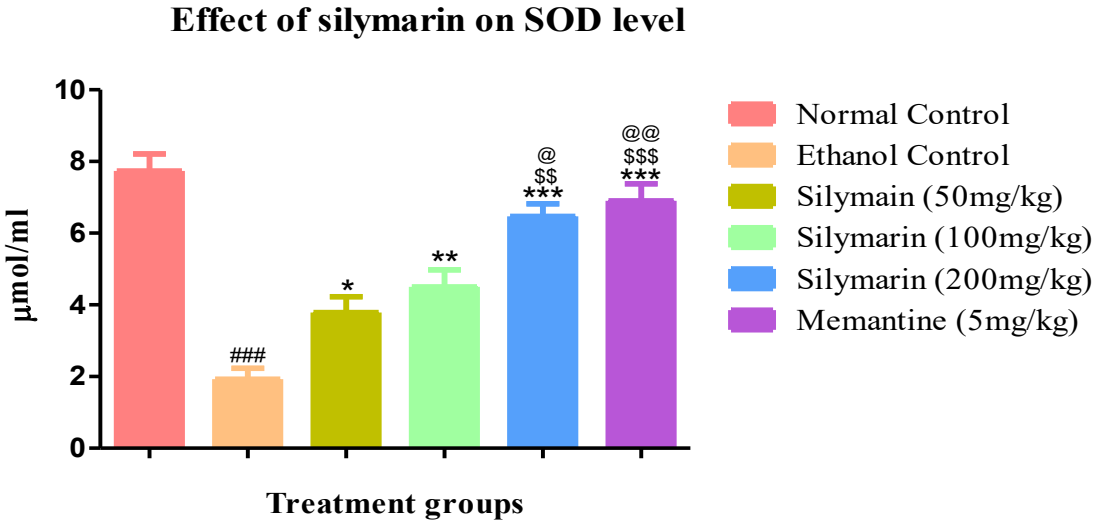

Fig 3.15. Effect of Silymarin (50, 100, 200mg/kg) on SOD level. Data represented as Mean ± SEM (n=6), analyzed by one way ANOVA followed by Tukey's post hoc test, where ($^{###}P < 0.001$) vs normal control group, ($^{*}P < 0.05$, $^{**}P<$

0.01 and ***P < 0.001) vs the ethanol control group, ($$P < 0.01 and $$$P < 0.001) vs silymarin 50mg/kg treated group and (@P <0.05 and @@P <0.01) vs silymarin 100mg/kg treated group.

3.11.2 Effect of silymarin on glutathione level

In the present investigation, effect of silymarin on alcohol withdrawal-induced changes in glutathione (GSH) level was assessed.

Fig 3.16 shows graph with Mean ± SEM (n=6) analyzed by, one-way ANOVA followed by Tukey's post hoc test found that, a significant decrease in the level of Glutathione (GSH) in the ethanol control group when compared with the normal control group (###P < 0.001).

In treatment groups (silymarin 50,100, 200mg/kg) and Std. memantine (5mg/kg) showed dose dependently increase in reduced level of GSH in comparison with ethanol control rats (**P< 0.01 and ***P < 0.001). There is significant increase in level of glutathione (GSH) when treated with silymarin (200mg/kg) and Std. memantine (5mg/kg) when compared with silymarin 50mg/kg & 100mg/kg ($$$P < 0.001) (@P <0.05).

Table 3.13. Effect of silymarin on glutathione levels [mean ± SEM (n=6)].

Groups	GSH (µg/mg of Protein)
Normal Control	65.41 ± 2.74
Ethanol Control	11.95 ± 3.39###
Silymarin 50mg/kg	32.19 ± 2.67**
Silymarin 100mg/kg	43.94 ± 3.41***
Silymarin 200mg/kg	58.34 ± 3.93*** $$$ @
Memantine 5mg/kg	54.43 ± 2.96*** $$$

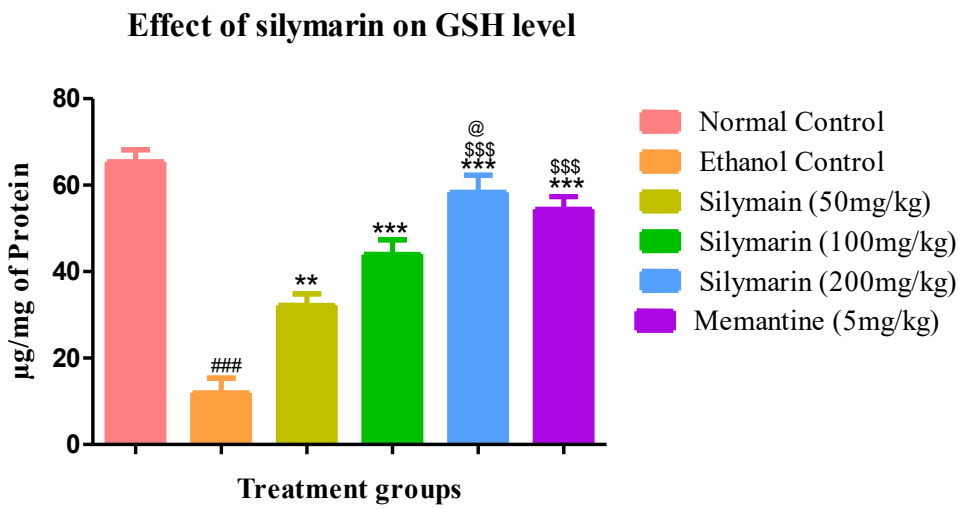

Fig 3.16. Effect of Silymarin (50, 100, 200mg/kg) on GSH level. Data represented as Mean ± SEM (n=6), analyzed by one way ANOVA followed by Tukey's post hoc test, where (###P < 0.001) vs normal control group, (**P< 0.01 and ***P < 0.001) vs the ethanol control group, ($$$P < 0.001) vs silymarin 50mg/kg treated group and (@P <0.05) vs silymarin 100mg/kg treated group.

3.11.3 Effect of silymarin on catalase level

In the present investigation, effect of silymarin on alcohol withdrawal-induced changes in catalase (CAT) level was assessed.

Fig 3.17 shows graph with Mean ± SEM (n=6) analyzed by, one-way ANOVA followed by Tukey's post hoc test revels that, a significant decrease in the level of catalase (CAT) in the ethanol control group when compared with the normal control group (###P < 0.001). In treatment groups (silymarin 50,100, 200mg/kg) and Std. memantine (5mg/kg) showed dose dependently increase in reduced level of CAT in comparison with ethanol control rats (*P< 0.05 and ***P < 0.001). There is significant increase in level of Catalase (CAT) when treated with silymarin (200mg/kg) and Std. memantine (5mg/kg) when compared with silymarin 50mg/kg & 100mg/kg ($P < 0.05, $$$P < 0.001) (@P <0.05).

Table 3.14. Effect of silymarin on catalase levels [mean ± SEM (n=6)].

Groups	CAT (µmol/ml)
Normal Control	12.58 ± 0.44
Ethanol Control	2.24 ± 0.55###

Silymarin 50mg/kg	5.24 ± 0.74*
Silymarin 100mg/kg	8.06 ± 0.72*** $
Silymarin 200mg/kg	11.18 ± 0.69*** $$$ @
Memantine 5mg/kg	10.92 ± 0.68*** $$$ @

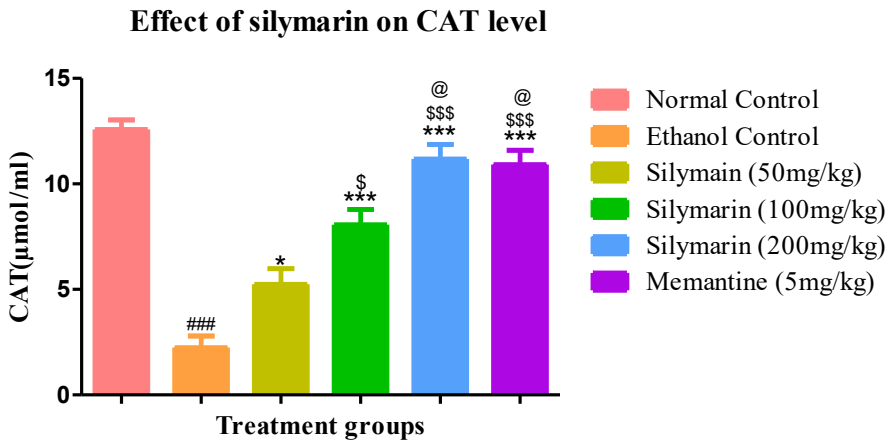

Fig 3.17. Effect of Silymarin (50, 100, 200mg/kg) on CAT level. Data represented as Mean ± SEM (n=6), analyzed by one way ANOVA followed by Tukey's post hoc test, where ($^{###}P < 0.001$) vs normal control group, ($^{*}P< 0.05$ and $^{***}P < 0.001$) vs the ethanol control group, ($^{\$}P < 0.05$, $^{\$\$\$}P < 0.001$) vs silymarin 50mg/kg treated group and ($^{@}P <0.05$) vs silymarin 100mg/kg treated group.

3.11.4 Effect of silymarin on malondialdehyde level

In the present investigation, effect of silymarin on alcohol withdrawal-induced changes in malondialdehyde (MDA) level was assessed.

Fig 3.18 shows graph with Mean ± SEM (n=6) analyzed by, one-way ANOVA followed by Tukey's post hoc test discovered that, a high significance level of malondialdehyde (MDA) in the ethanol control group when compared with the normal control group ($^{###}P < 0.001$).

In treatment groups (silymarin 100, 200mg/kg) showed dose dependently decrease the elevated level of MDA when compared with ethanol control rats ($^{*}P< 0.05$, $^{**}P< 0.01$ and $^{***}P < 0.001$) whereas silymarin (50mg/kg) showed no significant difference Vs ethanol control rats. There is no significant difference observed when silymarin (200mg/kg) compared with memantine (5mg/kg).

Table 3.15. Effect of silymarin on malondialdehyde levels [mean ± SEM (n=6)].

Groups	**MDA (µmol/ml)**
Control	6.67 ± 0.77
Ethanol Control	11.63 ± 0.53###
Silymarin 50mg/kg	9.50 ± 0.64
Silymarin 100mg/kg	8.42 ± 0.71*
Silymarin 200mg/kg	7.67 ± 0.61**
Memantine 5mg/kg	7.17 ± 0.71***

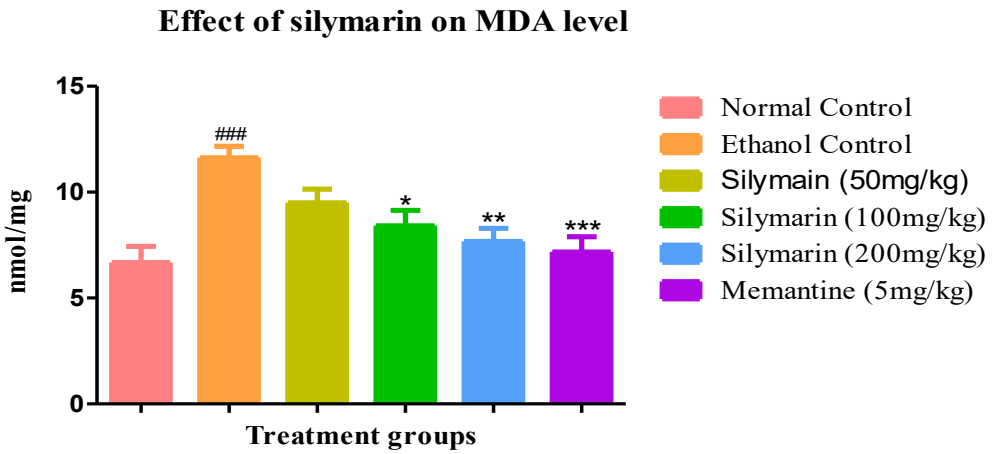

Fig 3.18. Effect of silymarin (50, 100, 200mg/kg) on lipid peroxidation. Data represented as Mean ± SEM (n=6), analyzed by one-way ANOVA followed by Tukey's post hoc test, where ($^{###}P < 0.001$) vs normal control group, ($^{*}P< 0.05$, $^{**}P< 0.01$ and $^{***}P < 0.001$) vs the ethanol control group.

3.11.5 Effect of silymarin on nitric oxide level

In the current investigation, effect of silymarin on alcohol withdrawal-induced changes in nitric oxide (NO) level was assessed.

Fig 3.19 shows graph with Mean ± SEM (n=6) analyzed by, one-way ANOVA followed by Tukey's post hoc test found that, ethanol control group had significantly in the level of nitric oxide (NO) when compared to the normal control group (###P < 0.001). In treatment groups (silymarin 100, 200mg/kg) showed high significant difference in the elevated level of NO when compared with ethanol control rats (***P < 0.001). whereas silymarin (50mg/kg) showed low significant difference in comparison with ethanol control rats (*P< 0.05). There is significant increase in level of nitric oxide (NO) when treated with silymarin (200mg/kg) and Std. memantine (5mg/kg) when compared with silymarin 50mg/kg & 100mg/kg ($$$P < 0.001) (@P <0.05 and @@P <0.01).

Table 3.16. Effect of silymarin on nitric oxide levels [mean ± SEM (n=6)].

Groups	NO (µmol/ml)
Normal Control	78.30 ± 7.44
Ethanol Control	209.2 ± 8.47$^{###}$
Silymarin 50mg/kg	168.3 ± 9.51*
Silymarin 100mg/kg	142.8 ± 9.30***
Silymarin 200mg/kg	90.68 ± 9.40$^{***\ \$\$\$\ @@}$
Memantine 5mg/kg	97.57 ± 11.38$^{***\ \$\$\$\ @}$

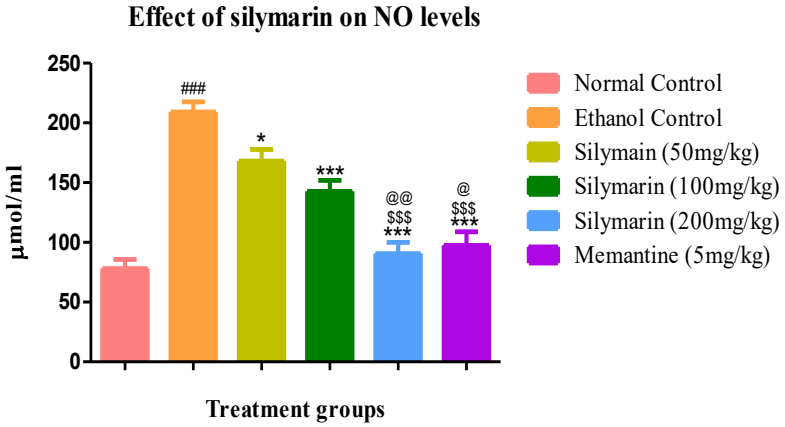

Fig 3.19. Effect of Silymarin (50, 100, 200mg/kg) on NO level. Data represented as Mean ± SEM (n=6), analyzed by one way ANOVA followed by Tukey's post hoc test, where ($^{###}P < 0.001$) vs normal control group, ($^{*}P< 0.05$ and $^{***}P < 0.001$) vs the ethanol control group, ($^{\$\$\$}P < 0.001$) vs silymarin 50mg/kg treated group and ($^{@}P <0.05$ and $^{@@}P <0.01$) vs silymarin 100mg/kg treated group.

3.12. Histopathological examination of the brain

Histopathological examination of stained sections of the brain showed disturbed structural organization and swelling, inflammation, and damage of neurons in the alcohol control group. In the ethanol control group, histopathological analysis of stained brain sections revealed Disturbed structure of hippocampus with severe degeneration of neurons with venules, edema and inflammation. Alcohol also decreases brain levels of glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT), which affects cellular antioxidant status.

Silymarin (50, 100, and 200 mg/kg) administered orally, however, significantly improved the brain's morphology and reduced neuronal degeneration while also reducing swelling and inflammation. Microscopic analysis of the control rats' brains revealed no lesions or pathological significance, as well as shows normal morphology of neurons, regular cell arrangement, and absence of any cells with darkening compacted nucleus. The brains of the rats in the ethanol control group showed moderate multifocal neuronal degeneration, inflammatory cell infiltration, as well as further brain injury. The brains of rats given silymarin at doses of (100 and 200 mg/kg) showed multifocal mild neuronal degeneration and prevented damage to neurons in the brain. Both groups also showed a decline in the number of darkened nuclei and a marked organization of the neurons, which minimized the appearance of damaged, apoptotic neurons. In contrast, the brains of rats given alcohol and silymarin at dose of (50 mg/kg) showed normal structure of hippocampus with moderate degeneration, and some structural disturbances were seen in the brain tissue. However, oral administration of silymarin at doses of (100 and 200 mg/kg) considerably improved the morphology of the brain and minimal degeneration of neurons, as well as reduced swelling and inflammation.

4. Discussion

Table 3.14. Histopathology of rat's brain

Images ID	**Description**
a) Normal	Normal structure of hippocampus with normal architecture
b) Ethanol	Disturbed structure of hippocampus with severe degeneration of neurons with venules, disturbances in the structure and edema
c) Silymarin 50mg/kg	Normal structure of hippocampus with moderate degeneration, some disturbances were seen
d) Silymarin 100mg/kg	Normal structure with mild degeneration of neurons, with less venules
e) Silymarin 200mg/kg	Normal structure of hippocampus with very less degeneration
f) Memantine 5mg/kg	Normal structure of hippocampus with very less degeneration.

The term "alcoholism" refers to a late stage of alcohol dependence when a sizable physical dependence has already formed. According to the stages of alcohol dependence, withdrawal symptoms that range from behavior driven by cravings to severe anxiety, depression, memory loss, and seizures (Edenberg et al., 2019).

Alcohol withdrawal syndrome includes a range of signs and symptoms, from mild (such as trembling and sleepiness) to severe (such as seizures and DTs). According to (Jesse et al. 2017) the symptoms are often influenced by the amount of alcohol ingested, the interval since the last drink, and the number of prior detoxifications. Alcohol withdrawal symptoms are mostly connected to changes in NT release, such as GABA, glutamate, serotonin, dopamine, adenosine, and adrenergic receptors and ETOH has been demonstrated to both abruptly and persistently produce oxidative stress in the brain in numerous studies. Although ROS formation during the withdrawal period has largely been ignored, the acute and long-term effects of ETOH on oxidative stress have been the subject of substantial research.

Alcohol Withdrawal symptoms are linked to the development of CNS neuroadaptations as a result of persistent alcohol consumption (Gupta et al. 2020). Normal conditions include a careful balancing act between excitatory and inhibitory signals. The brain's excitatory and inhibitory neurotransmitter systems are impacted by regular alcohol consumption (Chen et al. 2011). It has been shown that benzodiazepines can safely and effectively treat the symptoms of ethanol withdrawal syndrome, especially when it comes to preventing or treating seizures and delirium. While naltrexone blocks opioid receptors in the brain, reducing cravings and the potential benefits of alcohol consumption, acamprosate is expected to address long-term withdrawal symptoms (Sachdeva et al., 2015) Disulfiram can cause nausea if taken with alcohol, making it undesirable to do so. Topiramate, a

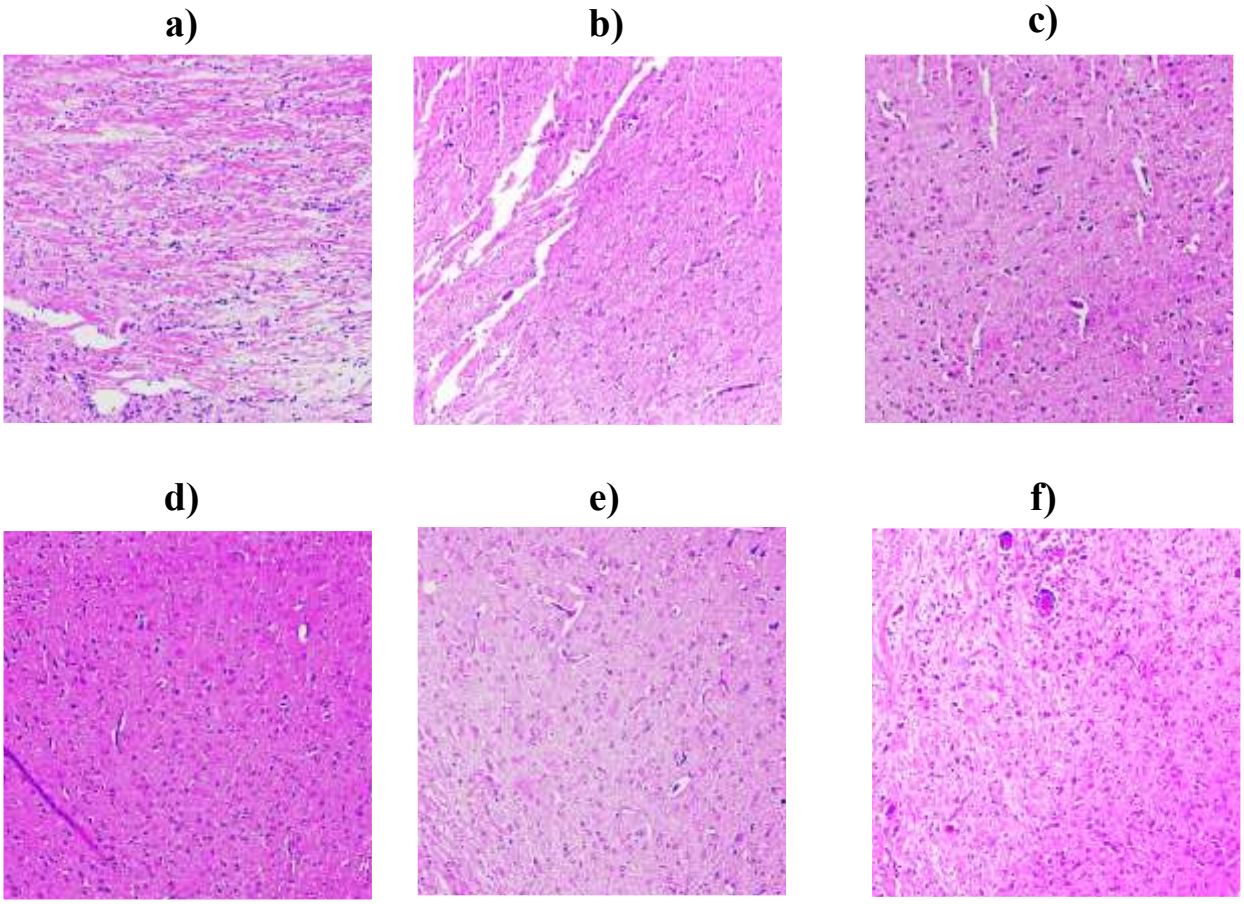

Fig.3.12. Histopathological sections of rats' brains:

Hematoxylin and eosin-stained histopathological sections of the rat's brain a - Normal control, b - Ethanol control c - Silymarin 50mg/kg, , d - Silymarin 100mg/kg, e - Silymarin 200 mg/kg, f - Std. Memantine 5mg/kg **o**bserved alcohol withdrawal induced neurodegeneration under 10X Microscope.

fourth drug, has shown promise for the treatment of ethanol use disorders by perhaps interfering with the way ethanol "rewards" drinkers, according to the Journal Addiction Science and Clinical Practice (Tidwell et al. 2018).

Flavonoids, a category of polyphenolic compounds, have attracted considerable attention on CNS related disorders. It is also effective at preventing and treating oxidative stress, neurodegenerative disorders (Spencer et al., 2009). Silymarin, have been demonstrated to have neuroprotective qualities. These benefits may be mediated through direct interactions with enzymes and receptors; however, additional biological effects, such as anti-inflammatory and antioxidant properties, have been shown to enhance these effects (Wang et al., 2020). Through targeted effects within stress-activated cellular responses, such as the protein kinase signaling cascade, silymarin can also prevent neuronal death. A flavonoid called silymarin also demonstrated some bioactivities that were helpful for the CNS related disorders. Many of these memory-enhancing flavonoid compounds could be employed as lead molecules in the creation of medications for convulsion and other neurodegenerative diseases (Surai 2015)

To induce alcohol dependency, the rats were administered orally 2.4 percent, 4.8 percent, and 7.2 percent alcohol for 21 days. The body weight was measured from day 1 to day 30. In the current investigation, we detected a significant increase in the body weight of the alcohol control rats on day 10 as compared to the normal control rats. On the other hand, we discovered a significant reduction in body weight when comparing alcohol-control rats to normal control rats on day 20. On the 22nd day, the withdrawal-induced physical indicators of hyperexcitability were scored at 2, 4, 6, and 8 hours to measure the progression of alcohol withdrawal symptoms. When compared to the normal control group, rats in the alcohol control group had a high withdrawal score.

In this present study, the effect of silymarin were evaluated in the MWM and NORT, a widely used animal models for memory dysfunction. Silymarin showed dose dependently increase in time spent in target quadrant indicate its improved spatial reversal learning and memory ability (Uddin & Asaduzzaman 2016). In NORT test, discrimination index were evaluated. Silymarin dose dependently increase in discrimination index indicated an elevation in retention and recall aspects of learning and memory.

In ethanol withdrawal rats, there is excess elevation in oxidative stress which disturbs the level of the biochemicals in the brain. In present study, ethanol withdrawal rats showed elevated levels of oxidative stress markers (MDA & NO) and reduced endogenous antioxidant (GSH, SOD & CAT) level in brain tissue. Treatment with silymarin showed significant dose dependent increased in the levels the glutathione, superoxide dismutase and catalase but reduced the level of lipid peroxidation and nitric oxide in the brain when compared with the ethanol control group (Nanaware et al. 2017; NADE et al. 2020).

Histopathological examination of stained sections of brain showed disturbed structure of hippocampus with severe degeneration of neurons with venules, swelling and inflammation of neurons in ethanol control rats. However, oral administration of silymarin at high dose was found to be effective in reversing the brain morphology and minimal degeneration of neurons with reducing swelling and inflammation.
Accordingly, network pharmacology approach facilitates the comprehension of how neurotransmitter alterations, oxidative stress, and memory dysfunction symptoms interact through the utilization of network pharmacology. We might therefore conclude that silymarin has characteristics that are advantageous in the treatment of memory dysfunction. In summary, silymarin prevents ethanol-induced hyperexcitability from manifesting and growing. Additionally, it improves cognitive dysfunction caused by alcohol withdrawal. It has been advised to correlate other study data in order to interpret the treatment's effects Behavioral tests such as MWM and NORT confirmed Silymarin's memory enhancing effect, and histopathological analysis showed its ability to counter alcohol withdrawal-induced Neuronal damage (Sivaraman et al., 2015; Chen and Zhou, 2020)

5. CONCLUSION

Network Pharmacology represents a sophisticated technique in the realm of drug development. It aids in identifying potential therapeutic targets of silymarin in alcohol withdrawal induced memory dysfunction. The intricate interplay between various biological components underscores the importance of a multifaceted approach to managing alcohol withdrawal-induced memory dysfunction. In the broader context of mental health and substance abuse, network pharmacology's integrative nature holds promise for addressing the intertwined challenges of addiction and memory dysfunction.
According to our findings, the hippocampus and cerebral cortex's antioxidant defense and oxidative imbalance can affect on memory. These results showed that rats on alcohol withdrawal exhibit memory impairment and significantly increase in MDA level in the hippocampal tissue and due to treatment with silymarin lowers the elevated amount of MDA level and significantly increases the SOD, GSH and CAT Level.
According to our study results, silymarin have ability to counteract the neuronal damage that leads to memory dysfunction during ethanol withdrawal. As a result, we concluded that silymarin have potential to be a rich source for discovering safer and more effective, potent variety of medicines in alcohol withdrawal induced memory dysfunction.

6. DECLARATIONS

Ethics Approval and Consent to Participate:
All experimental procedures involving animals were conducted in accordance with the applicable institutional and ethical guidelines for the care and use of laboratory animals. The animal experimental protocol was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of the School of Pharmaceutical Sciences, Sandip University, Nashik, under approval number SOPS/IAEC/2022-23/02/310. The study was conducted in compliance with the relevant institutional and regulatory requirements for animal experimentation.

Consent for Publication:
Not applicable.

Availability of Data and Materials:
The data generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing Interests:
The authors declare that they have no competing interests.

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Authors' Contributions:
M.P.D. and R.S.S. contributed to the conceptualization and study design. A.D., S.V.B., and S.K.P. contributed to the experimental work and data collection. S.N. contributed to data analysis and interpretation. P.B.D. contributed to the study supervision, methodology, interpretation of results, and critical revision of the manuscript. S.P.D. contributed to the experimental design, data interpretation, and manuscript review. M.P.D. prepared the initial draft of the manuscript. All authors critically reviewed the manuscript, approved the final version, and agreed to be accountable for all aspects of the work.

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