3. Materials and Methods

3.1. Materials

3.1.1. Experimental animals

Swiss albino mice of either sex (equal number of male and female) weighing 35-40 g, 80-90 days old and adult Wistar rats of either sex (equal number of male and female) weighing 200-250 g were procured from Serum Institute of India and National Toxicological Centre, Pune, India respectively. Animals were housed separately (8 mice/per cage) and (6 rats/cage) in a polycarbonate cage (cage size 29 cm × 22 cm × 14 cm) under standard laboratory conditions with food and water were available ad libitum, and lights were on from 07:00 to 19:00 h. Animals were acclimatize to laboratory condition for 1 week prior to experiment. The protocol was approved by the Institutional Animal Ethics Committee with approval no. SIPS/IAEC/2014-15/01 (Annexure III) and conformed to the Committee for the Purpose of Control and Suspension on Experimental Animals (CPCSEA).

3.2. Methods

3.2.1. Dose findings studies

Pilot experiment was performed using acute restraint stress model of depression in mice for dose selection. In this, both, silymarin and PCA were administered at various doses at 50 mg-500 mg/kg body weight by per oral (po) to groups of mice (n=3) and evaluated for immobility time by forced swim test.

3.2.2. Behavioral studies in normal mice

Swiss albino normal mice of either sex (3 male and 3 female) were divided into various groups (n=6), and administered with silymarin (50, 100, 200, 300, 400 and 500 mg/kg,) or PCA (50, 100, 200, 300, 400 and 500 mg/kg,) or vehicle (10 ml/kg) to the respective groups.

3.2.2.1. Locomotor activity in mice (Goyal et al., 2006)

Locomotor activity was performed by the method described by Goyal et al (2006) by using actophotometer (Inco, Ambala, India). Briefly, mouse was kept in center of the
actophotometer and allowed to move, while moving the total crossing of beam of light were recorded as spontaneous locomotor activity.

3.2.2.2. **Rota rod test in mice (Dohare et al., 2008).**

In this test, mice were placed on the rotating rod (speed: 25 rpm) until they learnt to maintain themselves on the rotating rod. After the training, mice were ad received silymarin or PCA or standard drug or vehicle. One hr after drug treatment the fall off the time for from rotating rod was measured by Rota rod. The fall off time was measured for all the mice by Rota rod with cut off time 5 minutes. The Rota rod test was used for the study of effects on motor coordination.

3.2.2.3. **Tail suspension in mice (Steru et al 1985)**

The duration of immobility time in 5 minutes was measured as per the method of Steru et al. (1985). The mice were suspended on the edge of a table 50 cm above the floor by adhesive tape place approximately 1 cm from the tip of the tail. Immobility time, defined as the absence of escape-oriented behavior, scored over 5 min.

3.2.2.4. **Forced swim test (FST) in mice (Porsolt et al, 1977).**

Immobility time in mice was evaluated as per the method previously described by Porsolt et al (1977). Briefly, mice were forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm); containing 19 cm of water (depth) at 25 ± 1 °C; and the immobility time was recorded.

3.2.2.5. **Elevated plus maze (EPM) in mice (Bora and Sharma, 2010).**

The EPM was used to determine the anxiety in mice. In this, mice were placed at the center of EPM and time spent in open arm was measured in 5 minutes of time. Generally normal animal remain in dark place compared to light one.

3.2.2.6. **Effects of PCA or silymarin on cataleptic behavior in mice (Khristiet al., 2002)**

Catalepsy is condition caused by dopaminergic D2 receptor antagonists and documented findings interpreted as evidence for a relationship between inherited catalepsy and depressive behavior (Bazovkina, et al., 2005).The severity of catalepsy in individual mice (n=6) was determined by placing the forepaws of the animal over a wooden bar 0.4 cm in diameter, fixed to a height of 3.5 cm above the table top. The time in seconds until mice bring both forepaws down to the table top was noted, with a maximum cut-off time of 300 s (Khristiet al., 2002).
The silymarin (100 and 200 mg/kg,) or PCA (100 and 200 mg/kg) or vehicle (10 ml/kg) to the respective groups and catalepsy was determined. Catalepsy is a condition in which the animal remains immobile even when placed in an unnatural position.

3.2.2.7. **Maximal electric shock induced convulsions in rats (Swinyard et al., 1952)**

Depressive state seems to be more frequent in patients with epilepsy than in the general population. Comorbid depression has a profound impact upon the quality of life of patients with epilepsy (Mazarati et al., 2010).

The corneal electroshock stimulus was given as per the method described by Swinyard et al., (1952) with minor modification, briefly, rectangular positive pulses of 15 mA at 20 Hz current was given for 0.2 s duration by use of convulsiometer (Inco, Ambala, India). The duration of clonic convulsion were recorded in each group.

3.2.3. **Acute restraint stress (ARS) induced depressive like behavior in mice**

The mice of either sex (3 male and 3 female mice per group) were divided in to various groups. It is to be noted that females are more vulnerable to depression caused by stressful events. However, in the present studies we used animals of either sex (equal numbers) in order to minimize the errors due to differences in animal sex.

Group I- vehicle control, administered with carboxyl methyl cellulose (CMC, 1 % w/v in distilled water) at a dose of 10 ml/kg, body weight, per oral (p.o);
Group II-standard group, administered with fluoxetine (20 mg/kg, p.o.) in distilled water;
Group III and IV-administered with silymarin at 100 and 200 mg/kg, p.o. respectively;
Group V and VI- administered with or PCA at 100 and 200 mg/kg) mg/kg, p.o. respectively;
The vehicle or silymarin or PCA or fluoxetine were administered to their respective group 1hr prior to ARS procedure.

The mice were restraint for stress as per the procedure described by Freitas et al (2010) and Kumar and Goyal (2008). Mice were immobilized for a period of 7 h using an individual rodent restraint device restraining all physical movement without causing pain. The ARS and non stressed mice were evaluated by FST and open field test for depressive behavior mainly determination of immobility time and exploratory behaviors. The behavioral test was performed 8 hours 40 minutes after drugs (PCA and silymarin or fluoxetine) administration. Both the test (FST and OFT) were performed separately.
3.2.4. Olfactory bulbectomized (OBX) induced depression in rats

The OBX was performed as per the procedure described by Van Reizen and Leonard (1990). The rats were fixed in a stereotactic frame (Inco, Ambala, India) and 1-cm rostral-caudal midline incision was made in the skin of the head, and two small burr holes (2 mm in diameter) were drilled into the skull 6 mm rostral of bregma and 1 mm lateral of the midline. Both olfactory bulbs were removed by suction and haemostatic sponge was inserted into the cavity to control bleeding. The incision was then closed with absorbable sutures and rats were treated with sulphadiazine 200 mg and trimethoprim 40 mg) to prevent post-surgical infection.

The OBX rats of either sex (4 male and 4 female rats per group) were divided in to various groups (n=8) and were treated in the following way,

- Group I- sham operated with vehicle treated group (10 ml/kg, po),
- Group II- sham operated with fluoxetine (20 mg/kg, po),
- Group III and IV- sham operated with silymarin 100 and 200 mg/kg, po, respectively,
- Group V and VI- sham operated with PCA 100 and 200 mg/kg, po, respectively.
- Group VII - OBX with vehicle treated group (10 ml/kg, po),
- Group VIII- OBX with fluoxetine (20 mg/kg, po),
- Group IX and X- OBX with silymarin 100 and 200 mg/kg, po, respectively,
- Group XI and XII- OBX with PCA 100 and 200 mg/kg, po, respectively.

In Sham animals, similar procedure was conducted except the removal of the olfactory bulbs. The drug treatment, silymarin or PCA or fluoxetine or vehicle was administered from 16th day to 30th day of OBX surgery once a day every day. All the groups were evaluated by behavioral and biochemical changes after drug treatment.
3.2.5. Chronic unpredictable mild stress (CUMS) induced depression in mice

The mice of either sex (4 male and 4 female mice per group) were divided into various groups (n=8) and treated in the following way,

Group I - non stressed with vehicle treated group (10 ml/kg, po),
Group II - non stressed with fluoxetine (20 mg/kg, po),
Group III and IV - non stressed with silymarin 100 and 200 mg/kg, po, respectively,
Group V and VI - non stressed with PCA 100 and 200 mg/kg, po, respectively.
Group VII - CUMS with vehicle treated group (10 ml/kg, po),
Group VIII - CUMS with fluoxetine (20 mg/kg, po),
Group IX and X - CUMS with silymarin 100 and 200 mg/kg, po, respectively,
Group XI and XII - CUMS with PCA 100 and 200 mg/kg, po, respectively.

PCA and silymarin were prepared in 1% (w/v) Carboxy Methyl Cellulose, fluoxetine in normal saline solution. PCA, fluoxetine and vehicle were administered orally between 9:00 a.m. and 10:00 a.m. once a day for 21 consecutive days.

The CUMS was performed as previously described by Mao et al (2009) with minor modification. Briefly, mice in CUMS groups were exposed to different stressors paradigms for 4 weeks as shown in Table 1 (first week without treatment and next 3 weeks treated with drugs). The non stressed mice were undisturbed except for routine cage cleaning.

The detailed schematic protocol shown in fig3.1.
Table 3.1-CUMS protocol with variety of mild stressors in mice

<table>
<thead>
<tr>
<th>Day</th>
<th>Duration</th>
<th>Stressor paradigms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monday</td>
<td>24 hrs</td>
<td>Food deprivation</td>
</tr>
<tr>
<td></td>
<td>24 hrs</td>
<td>Water deprivation</td>
</tr>
<tr>
<td>Tuesday</td>
<td>01 hr</td>
<td>Empty bottle</td>
</tr>
<tr>
<td></td>
<td>24 hrs</td>
<td>Foreign object</td>
</tr>
<tr>
<td>Wednesday</td>
<td>06 min</td>
<td>Forced swimming</td>
</tr>
<tr>
<td></td>
<td>12 hrs</td>
<td>Overnight illumination</td>
</tr>
<tr>
<td>Thursday</td>
<td>02 hrs</td>
<td>Restraint</td>
</tr>
<tr>
<td></td>
<td>07 hrs</td>
<td>Cage tilt (45°)</td>
</tr>
<tr>
<td>Friday</td>
<td>12 hrs</td>
<td>Food deprivation</td>
</tr>
<tr>
<td></td>
<td>24 hrs</td>
<td>Soiled cage</td>
</tr>
<tr>
<td>Saturday</td>
<td>24 hrs</td>
<td>Water deprivation</td>
</tr>
<tr>
<td></td>
<td>12 hrs</td>
<td>Overnight illumination</td>
</tr>
<tr>
<td>Sunday</td>
<td>01 hr</td>
<td>Empty bottle</td>
</tr>
<tr>
<td></td>
<td>07 hrs</td>
<td>Cage tilt (45°)</td>
</tr>
</tbody>
</table>
Materials and Methods

Pharmacological evaluation of PCA and Silymarin in experimentally induced depression in rodents

Fig 3.1. The detailed schematic protocol of CUMS model
3.2.6. Behavioral evaluation

3.2.6.1. Evaluation of immobility time by Forced swim test (FST)

The FST was used to assess the immobility time. Immobility time in ARS, CUMS subjected mice and OBX rats were evaluated as per the method previously described by Porsolt et al (1977). Briefly, animals were forced to swim in an open cylindrical container (diameter 10 cm, height 25 cm), containing 19 cm of water (depth) at 25 ± 1 °C; and the immobility time was recorded by video tracking system (VJ Instruments, India). Each mouse or rat was judged to be immobile when it ceased struggling and remained floating motionless in the water, producing only those movements necessary to keep its head above water. A reduction in immobility time was considered as indication of an antidepressant-like activity.

3.2.6.2. Evaluation of exploratory behavior by open field test (OFT)

The OFT was performed in ARS, OBX and CUMS animals as per the method described by Freitas et al (2014) and Rodrigues et al (1996). The number of squares crossed with all paws (crossings) and time spent in center of mouse in ARS and CUMS or rearing and ambulatory behavior in OBX were counted in a 6 min session. The apparatus was cleaned with a solution of 10% ethanol between tests in order to hide animal clues.

3.2.6.3. Sucrose preference test in nonstressed and CUMS animal model

At the end of CUMS exposure, on day 29th, sucrose preference test was conducted as per the procedure described by Willner et al (1987). Briefly, 72 h before the test, mice were adapted to 1% sucrose solution (w/v): two bottles of 1% sucrose solution were placed in each cage, and 24 h later 1% sucrose in one bottle was replaced with tap water and kept it for further 24 h. After this, mice were deprived of food for 24 h in order has maximal liquid intake and relative preference in choice paradigms. Sucrose preference test was conducted at 9:00 AM morning in which mice were housed in individual cages and had free to access to two bottles containing 100 ml of sucrose solution (1%, w/v) and 100 ml of water and then the positions of two bottles is switched daily to reduce any confound produced by a side bias. After 1 h, the volumes of sucrose solution and water consumed were recorded and the sucrose preference was calculated as the sucrose preference (%) = sucrose consumption/(sucrose consumption + water consumption).

3.2.6.4. Body weight in nonstressed and CUMS in mice

Body weight of both nonstressed and CUMS stressed mice were determined on every week during the experimental paradigms.
3.2.6.5. Determination of safety measure excitement, convulsion, itching, and tremors in nonstressed and CUMS mice

Various safety measure excitement, convulsion, itching, and tremors were studied with PCA, silymarin or fluoxetine treatment during CUMS paradigms schedule.

3.2.7. Biochemical investigations

Thirty minutes in ARS and 1 hr in OBX and CUMS after the behavioral evaluations, blood was collected between 8.30-9.30 am from direct cardiac puncture and serum was separated and stored (-20°C) until use.

After blood withdrawal, animals were sacrificed by decapitation, brains were opened and the hippocampus and cerebral cortex were identified and isolated, washed with 10 % cold sucrose solution and homogenized (1:10 w/v) in phosphate buffer solution (PBS). The tissue homogenates were centrifuged (REMI, USA) at 16,000×g, at 4°C for 15 min and resultant supernatants were used for neurochemical and biochemical analysis.

3.2.7.1. Serum corticosterone (CORT) estimation

Reagent preparation

Sample preparation

Serum samples from ARS, OBX and CUMS stressed, sham and nonstressed groups were treated with dissociation agent.

The CORT ELISA kit reagents were kept at room temperature for 30 minutes prior to assay.

Assay buffer

Assay buffer concentrate 1:5 diluted by adding one part of the concentrate to 4 parts of deionized water.

Wash buffer

Wash buffer concentrate 1:20 diluted by adding one part of the concentrate to 19 parts of deionized water.

Standard preparation:

Standard CORT was prepared at various concentrations in test tubes. In test tube #1, 450 µl of assay buffer solution was pipette out and remaining 7 test tubes 250 µl of assay buffer were added. Then, 50 µl of CORT stock solution was added to test tube #1 and vortexed. To this, 250 µl of CORT solution from test tube #1 was added to test tube #2 and vortexed it and the serial dilutions for the tubes from #3 to #8 were repeated. The final
concentration of CORT in tubes 1 through 8 were 10,000, 5000, 2500, 1250, 625, 156.25, 78.125 pg/ml.

Serum corticosterone was estimated by ELISA method as per the procedure described by manufacturer’s kit.

- Assay buffer of 75 µl of was added into wells which acts as non-specific binding (NSB) well,
- Assay buffer 50 µl of into wells to act as maximum binding wells (0 pg/ml),
- Serum samples or standard of 50 µl were added into wells in the plate, and then 25 µl of CORT conjugate
- To each well 25 µl CORT antibodies were added except NSB wells.
- The plate was tapped for adequate mixing of the reagents, plate was sealed and shaken and incubated for 1 hour.
- After one hour, the plate was washed 4 times with adding 300 µl of wash buffer solution in each well.
- The plate was dry on clean absorbent towels by firmly tapping, plate was not completely dried.
- Then, to each well, 100 µl of coloring substrate of TMB substrate was added and plate was further incubated for 30 min. the well turned into blue color in 15 minutes.
- The reaction was stopped by adding 50 µl of the stop solution was added to each well, the color changed to yellow from blue within 15 minutes.
- The plate was placed in ELISA reader (Biotek, Germany) and before assuring the ELISA reader was communicating.
- The absorbance was read at 450 nm in duplicate and file was saved and reading were extracted and analyzed statistically.
- The results were expressed as µg/ml of CORT level.

**Oxidative stress studies**

**3.2.7.2. Estimation of malondialdehyde (MDA) formation in hippocampus and cerebral cortex**

The estimation of peroxidation of lipids has been performed by various methods of which thiobarbituric acid reactive substances (TABARs) is selected due to its high sensitivity and simplicity in operation. The TBARs is often used to determine the malondialdehyde
Pharmacological evaluation of PCA and Silymarin in experimentally induced depression in rodents

(MDA) formed in peroxidising lipids system by lipid peroxidation and reacts with TBA under excess temperature (90-100 °C) and acidic condition and that result into pink color MDA-TBA adduct. This colored solution was measured by spectrophotometry at 532 nm.

**Standardization of MDA method**
- The 10 mM stock solution of MDA was prepared by adding 1 mM of 1, 1, 3, 3-tetraethoxypropane to 100 ml of sulphuric acid (1%, v/v).
- The mixture was kept at room temperature for 24 h for complete hydrolysis.
- This stock solution was further diluted to about 10-100 mM. The absorbance of the solution was taken at 245 nm.
- The molecular extinction coefficient of 13000 was used to calculate the MDA concentration in solution.

**Procedure**

The MDA formation as a marker of lipid peroxidation was measured in homogenate samples of hippocampus and cerebral cortex of ARS, OBX and CUMS and nonstressed subjected mice and rats as per the procedure documented and described by Ohkawa et al (1979).

- The MDA solutions 0.1 ml of various concentrations was mixed with 0.2 ml of (8.1 %) sodium lauryl sulphate.
- The equal volumes (2 ml) of the tissue homogenate and acetic acid (10% w/v) and TBA (0.8 % w/v) were mixed.
- The mixture was then centrifuged and supernatant was separated. The volume of supernatant was made to 4 ml by adding distilled water.
- The solution was then heated at 95-100 °C in water bath for 1 h. after this, tubes were allowed to cool at room temperature and final volume was made to 5 ml of each tube.
- To this mixture, 5 ml of n-butanol: pyridine (15:1 v/v) was added and the contents were vortexed for 2 minutes.
- The contents were further centrifuged at 3000 g for 10 minutes. The absorbance was measured against blank at 535 nm on UV spectrophotometer (Shimadzu 1700).
- The standard curve was prepared correlating the concentration of MDA in the solution used and absorbance of colored complex obtained by reaction with TBA.
- The amount of MDA formed was expressed as nM of MDA/g of wet tissue.

The concentration of MDA of sample was calculated from the absorbance by extrapolating on the standard curve.
Calculation

The MDA (mM of MDA formation/g of proteins) = \frac{\text{Absorbance}}{L \times A} \times D

Where,

- L - Light path (1cm),
- A - Extinction coefficient of $1.56 \times 10^5$ M$^{-1}$ cm$^{-1}$
- D - Dilution factor = total volume 10 ml/volume of sample

3.2.7.3. Estimation of superoxide dismutase (SOD) activity in hippocampus and cerebral cortex

Preparation of reagents

Carbonate buffer (0.05M, pH 10.2)

16.8 g of sodium bicarbonate and 22 g sodium carbonate were dissolved in 500 ml distilled water and the final volume was made to 1000 ml.

Ethylenediaminetetra acetic acid (EDTA, 0.1 mM)

37.2 mg of sodium EDTA was dissolved in 100 ml of distilled water. Pipette out 1 ml from this and diluted to 100 ml with distilled water.

Hydrochloric acid (0.1 N)

Concentrated HCL (8.5 ml) was added with 500 ml distilled water and mixed the contents the final volume was made 1000 ml with distilled water.

Epinephrine (3 mM)

Epinephrine of 5.499 g was dissolved in 10 ml of 0.1 N HCL to obtain the 3 mM of final concentration of epinephrine.

Procedure

- To the blank specimen, 1.0 ml of distilled water, 0.25 ml of cold ethanol and 0.15 ml of cold chloroform were mixed
- The homogenates (0.5 ml) of hippocampus and cerebral cortex of ARS, OBX and CUMS and nonstressed subjected mice and rats as were taken, to which 500 µl of distilled water, 250 µl of cold ethanol and 150 µl of ice-cold chloroform were mixed.
- The mixture was then shaken and centrifuged (6000 rpm for 10 min) and the supernatant was separated
To the aliquot of 500 µl supernatant, 1500 µl of carbonate buffer (pH 10.2) and 500 µl of EDTA were added.

At the end, 500 µl ml of epinephrine(3 µM) prior to the absorbance

The change in the absorbance of the same per minute at 480 nm was determined by UV Spectrophotometer (Shimadzu 1700).

One unit of SOD is defined as the amount of enzyme required to produce 50 % inhibition of epinephrineautoxidation.

The SOD activity was expressed as U/g of wet tissue (Mishra and Fridovich, 1972).

Calculation

For % inhibition

\[
\% \text{ inhibition} = \frac{A_{480\ nm/min \ uninhibited} - A_{480\ nm/min \ inhibited}}{A_{480\ nm/min \ uninhibited} - A_{480\ nm/min \ Blank}} \times 100
\]

For Units/ml of enzyme

\[
\text{Units/ml of enzyme} = \frac{\% \text{ inhibition} \times \text{volume of test}}{50 \% \times \text{volume of standard}}
\]

For Units/ mg of protein

\[
\text{Units/ mg of protein} = \frac{\text{Units/ml of enzyme}}{\text{mg protein/ml of enzyme}}
\]
3.2.7.4. Estimation of catalase (CAT) activity in hippocampus and cerebral cortex

Preparation of reagents

Phosphate buffer solution (PBS, 50 mM, pH 7.0)

(a) Potassium dihydrogen orthophosphate (KH$_2$PO$_4$) of 6.81 g was dissolved in 1000 ml distilled water.

(b) 8.9 g of disodium hydrogen phosphate (Na$_2$HPO$_4$) was dissolved in 1000 ml of distilled water.

390 ml of solution (a) are mixed with 610 ml of solution (b), the pH was adjusted to 7.0.

Hydrogen peroxide (H$_2$O$_2$; 30 mM) 

0.34 ml of 30 % hydrogen peroxide was diluted with phosphate buffer to produce 100 ml of final volume.

Procedure

❖ The CAT activity was estimated in tissue homogenate (hippocampus and cerebral cortex) of ARS, OBX and CUMS and nonstressed subjected mice and rats as per the procedure described earlier by Aebi(1974).

❖ The homogenate was diluted 20 times using phosphate buffer

❖ 2 ml of diluted homogenate was taken, and reaction was initiated by addition of 1 ml of hydrogen peroxide (30 mM).

❖ The rate of decomposition of H$_2$O$_2$ as change in absorbance was read at 240 nm against blank on Shimadzu 1700 UV Spectrophotometer.

❖ The CAT activity was expressed as μM of H$_2$O$_2$ formed/min/g of wet tissue.

Calculations

The CAT activity was calculated as

Unit activity (U) = (AT-AS) × 3 ml/0.0436 × volume of sample (ml)

Where,

AT- change in absorbance/min of test

AS- change in absorbance/ min of H$_2$O$_2$

0.436-mM extinction coefficient of H$_2$O$_2$ at 240 nm
3.2.7.5. Estimation of reduced glutathione (GSH) in hippocampus and cerebral cortex

**Preparation of reagents**

**Trichloroacetic acid (10 % w/v)**

Trichloroacetic acid (10 g) was dissolved in sufficient quantity of distilled water and mixed properly. The final volume was made up to 100 ml with distilled water.

**Phosphate buffer (0.2 M, pH 8.0)**

Fifty milliliters of 0.2 M potassium dihydrogen phosphate was mixed in 46.8 ml of 0.2 M sodium hydroxide. The final volume was made to 500 ml distilled water.

**5, 5′-dithiols-2-nitro benzoic acid (DTNB, 0.06 mM)**

60 mg of DTNB was dissolved in 50 ml of buffer and final volume was adjusted to 100 ml with buffer solution.

**Standard GSH solution**

Reduced glutathione, GSH 10 mg was dissolved in 10 ml of distilled water and the final volume was made to 100 ml with distilled water (1 mg/ml) as stock solution.

**Procedure**

- Equal volumes of tissue supernatant from ARS, OBX and CUMS and nonstressed subjected mice and rats and 20% trichloroacetic acid (TCA) were mixed together.
- The resultant solution was then cooled and centrifuged at 6000 rpm for 10 minutes
-Aliquot of 250 µl of supernatant was transfer to test tube and to this 2 ml of DTNB [5, 5′-dithiobis (2-nitro benzoic acid), 0.6 mM] was added.
- The final volume was made up to 3 ml with phosphate buffer (0.2 M, pH 8.0) as the yellow color appeared.
- The absorbance of resultant solution was measured by spectrophotometrically at 412 nm (Shimadzu 1700 UV).

**Calculation**

The non proteins thiol contents (NPSH) were measured and calculated using molar extinction coefficient of chromophore $1.36 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$
3.2.8. Neurobiochemical investigations

3.2.8.1. Monoamines estimation

The monoamines, serotonin (5-HT), norepinephrine (NE) and dopamine (DA) were determined in the homogenates of hippocampus and cerebral cortex of ARS and nonstressed, OBX and sham, CUMS and nonstressed subjected animals as per the method described by Kent Shellenberger and Gordon (1971).

Reagent preparation

Butanol solution (1 %, v/v)
Mixed the 1 ml of butanol in 10 ml of distilled water. The resultant volume was made to 100 ml with distilled water.

Standard 5-HT solution (10 %, w/v)
Dissolved 10 mg of standard serotonin in 50 ml of normal saline solution, mixed properly. The final volume was made to 100 ml with normal saline.

Standard DA solution (10 %, w/v)
Dissolved 10 mg of standard dopamine in 50 ml of normal saline solution, mixed properly. The final volume was made to 100 ml with normal saline.

Standard NE solution (10 %, w/v)
Dissolved 10 mg of standard norepinephrine in 50 ml of normal saline solution, mixed properly. The final volume was made to 100 ml with normal saline.

Procedure

- Hippocampus and cerebral cortex homogenates were acidified with cold butanol solution.
- The homogenates were centrifuged separately at 1000 g for 10 min at 4 °C and supernatant was separated. The portion extract was further processed for fluorescence development.
- The fluorescence of 5-HT was measured by Spectrofluorometrically (Perkin Elmer, USA) with the excitation wavelength of 385 nm and the emission spectra at 490 nm.
- A reading for DA was made at 4 °C at 325 nm activation peaks and fluorescence at 380 nm uncorrected.
- The remaining portion of butanol extract was shaken with 0.1M phosphate buffer (pH 6.5) for 15 minutes.
The mixture then centrifuged and the fluorescence of NE was read with the excitation wavelength at 380 nm and the emission spectra at 495 nm.

The results are expressed as μg/g of tissue of various monoamines.

3.2.8.2. Measurement of BDNF levels in hippocampus and cerebral cortex

Reagent preparation

Preparation of 0.01 M Phosphate Buffer Solution (PBS) as washing buffer

The 0.01 M PBS was prepared as washing buffer by addition of 8.5 g sodium, chloride, 1.4 g Na₂HPO₄ and 0.2 g NaH₂PO₄ to 1000 ml distilled water and pH was adjusted to 7.2-7.6 and final volume was made to 1 liter.

Sample dilution

This is prepared as 1:2 by 25 μl of sample was diluted to 50 μl of sample diluents buffer.

Reconstitution of BDNF standard

BDNF standard solution was prepared 2 h prior to the experiment.

10000 pg/ml of BDNF standard solution:

This was prepared by addition of 1 ml sample diluents buffer into one tube, the tube was kept as room temperature for 10 minutes and thoroughly the solution was mixed.

1000-0.0 pg/ml of BDNF standard solution

This was prepared in Eppendorf tubes with marking of 10000 pg/ml, 5000 pg/ml, 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 152 pg/ml, 62.5 pg/ml 31.2 pg/ml, and 0.0 pg/ml. Aliquot of 0.3 ml of the sample diluents buffer was added to each tube. Then 0.3 ml of the above 2000 pg/ml BDNF standard solution was added to 1st tube and it was mixed. Then 0.3 ml from 1st tube was transferred to 2nd tube and it was mixed again. Similarly, 0.3 ml from tube 2nd to 3rd was transferred and so on for rest of tubes.

Preparation of biotinylated anti-mouse BDNF antibody working solution

The total volume was 100 μl/well was prepared, the biotinylated anti-mouse BDNF antibody was diluted as 1:100 with the antibody diluent buffer i.e. by adding 1 μlbiotinylated anti-mouse BDNF antibody in 99μl antibody diluent buffer.

Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution.

The total volume was 100 μl/well was prepared, the Avidin-Biotin-Peroxidase Complex (ABC) working solution was diluted as 1:100 with the ABC dilution buffer with proper mixing i.e. by adding 1 μl Avidin-Biotin-Peroxidase Complex solution in 99μl ABC dilution buffer.
Procedure

The BDNF levels in hippocampus and cerebral cortex ARS and nonstressed, OBX and sham, CUMS and nonstressed subjected animals were measured by ELISA method as per the procedure described by manufacturer’s kit.

- Aliquot of 0.1 ml per well of the 10000 pg/ml, 5000 pg/ml, 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 152 pg/ml, 62.5 pg/ml 31.2 pg/ml, and 0.0 pg/ml, BDNF standard solution were added into the precoated 96 well plate.
- Sample diluents solution 0.1 ml was added into the control well (Zero well). To each empty well plate, 0.1 ml of diluted supernatant solution was added.
- Then plate was sealed and incubated for 90 min at 37°C. After incubation, seal was removed, plate contents were discarded, and plate was blotted onto paper towels.
- To each well plate, 0.1 ml of biotinylated anti-rat BDNF antibody working solution was added and plate was incubated for 60 min at 37°C.
- Well plate was washed with 0.01 M PBS thrice; washing buffer was discarded from well plate and blotted on paper towels without touching side walls.
- After washing, 0.1 ml prepared solution of avidin-biotin-peroxidase complex working solution was added to each well plate, and incubated for 30 min at 37°C.
- Plate was washed 5 times with 0.01 M PBS, during each wash buffer was stayed in the well for 2 min, washing buffer was then discarded, and plate was blotted on paper towels.
- Ninety micro liters prepared TMB color developing agent was added into each well plate and incubated for 25 min at 37°C, reactions was stopped by adding 0.1 ml of TMB stop solution to each well plate, immediately color was changes into yellow and absorbance was read at 450 nm by using ELISA reader (Biotek, Germany).
- The concentration of BDNF was interpolated from standard curve.
- Results were expressed as ng/g of BDNF content.

3.2.8.3. Measurement of IL-6 and TNF-α in hippocampus and cerebral cortex

Reagent preparation

Wash buffer

Wash buffer was prepared by addition of 5 ml of wash buffer (1X) to 95 ml of deionized water is known as working solution.
Materials and Methods

Assay diluent

Assay diluent was prepared by addition of 10 ml assay diluent (1X) to 40 ml of deionized water.

Detection antibody

Dilution of 1:250 was prepared by addition of 50µl detection antibody solutions to 9950 µl of assay diluent and final volume was 1 ml.

Streptavidin HPP solution

Dilution of 1:250 was prepared by addition of 50µl Streptavidin HRP solution to 9950 µl of assay diluent and final volume was 1 ml.

Standard reconstitution of TNF-α and IL-6

The Standard reconstitution of TNF-α and IL-6 were prepared by addition of 1 ml deionized water to the lyophilized vial, then it was allowed to sand for 15 minutes. The recombinant protein thawed and diluted by adding 267µl standard solution of TNF-α and IL-6 in 1733µl of assay diluent (1X) with resultant standard solution concentration of 2000pg/ml respectively. The final volume was 2 ml.

Procedure

Cytokines, IL-6 and TNF-α were determined in homogenates of hippocampus and cerebral cortex as per the procedure described by manufacturer’s kits.

- 100 µl/well of standard and supernatants of sample were added to the plate.
- The standard concentration were prepared as 2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 120 pg/ml, 62.5 pg/ml 31.25 pg/ml and 0.0 pg/ml separately for rat IL-6 and TNF-α.
- 1X assay diluents served as the zero standards (0 pg/ml).
- Plates were sealed and incubated at 37ºC for 2 hours. Plates were washed 4 times with 1X wash buffer and blotted residual buffer by firmly tapping plates upside down on absorbent paper.
- To this, 100µl of diluted detection antibody solution was added to each well; plates were sealed and incubated again at 37ºC for 1 hour.
- After 1 hthe plates were washed 4 times with 1X wash buffer. Then, 100µl of diluted Av-HRP solution was added to each well.
- The plates were sealed and incubated at 37ºC for 30 minutes in incubator.
- After incubation time, plates were washed 4 times with 1X wash buffer as given above.
The freshly mixed TMB substrate solution 100µl was added and incubated in the dark for 30 minutes. Positive wells were turned in to bluish color.

The reaction was stopped by adding 100µl of stop solution to each well. Positive wells were turned from blue to yellow.

The absorbance for both the cytokines was measured at 450nm on ELISA reader (Biotek, Germany). The files with absorbance reading were saved and extracted for statistical analysis.

The results were expressed as pg/mg of tissue.

3.2.9. Study of silymarin with NMDA receptor antagonist on immobility time by FST

Various groups of mice were treated with silymarin (100 mg/kg), fluoxetine (20 mg/kg) and CPP a competitive NMDA receptor antagonist (10 mg/kg). The CPP was dissolved in physiological saline was administered 1 hr prior to silymarin or fluoxetine treatment.

Training session:

On 1st day, mice were exposed to 15 min of forced swim (training session) and then were removed and kept in their cages.

Test session

Twenty-four hours later, mice were placed in the cylinder again for a 5 min period (test session). Silymarin (200 mg/kg) alone and in presence of CPP was administered three times between these two swim sessions (23.5, 5, and 1 h before the 5 min swim). The FST was performed as similarly described in section 3.2.6.1 of behavioral evaluation in order to measure the immobility time.

3.2.10. Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using a two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test. A value of \( p < 0.05 \) was considered to be statistically significant. The statistical analysis was carried out by using the software Graph Pad Prism trial version 7 (Graph Pad Software, Inc., La Jolla, CA, USA).