4.1. Introduction

Oxidative stress, a culprit in many human diseases (Barreiro et al., 2005). In spite of the existence of endogenous defense mechanisms against ROS, it has been observed that whenever either the level of the cellular antioxidant system goes down or when the ROS reach abnormally high levels, oxidative damage to the cells occurs leading to several pathological conditions. Over about 100 disorders like hemorrhagic shock, CVS disorders, rheumatoid arthritis (RA), cystic fibrosis, hepatic disease, neurodegenerative diseases, gastrointestinal ulcerogenesis and AIDS have been reported as ROS mediators. Some specific examples of ROS mediated diseases include Alzheimers disease, Parkinson's disease, Atherosclerosis, Cancer, and ischemic reperfusion injury in different tissues including heart, liver, brain, kidney and GIT.

Since ancient times our ancestors have used phytochemicals found in plants to inhibit the neuroinflammatory process. The emergence of today's pharmaceutical industry, in large part, has been based on natural products.

Reactive oxygen species are yielded by numerous physiological and pathological situations and despite their vital importance to normal cell function including proliferation, growth, signaling and apoptosis they cause continuous impairment to lipids, proteins and DNA (Goto and Nakamura, 1997).

Alternative medicine is another therapeutic approach for treatment of the disease, which include herbal and folklore medicines. Many plants and plant products are under scientific exploration to develop a novel therapeutic agent. Here we adopted LPS induced oxidative stress model in animals.

4.2. Materials and Methods

Table 4.1. List of Equipments

Centrifuge	Remi Scientific Indst; Mumbai
Electronic Balance	AG 135 Mettler Toledo
Incubator	Wirso Instruments, Delhi
Micropipette	J. Micro, New Delhi
Oven	Wirso Instruments, Delhi
Spectrophotometer (Double Beam)	Schimadzu, Japan Model 150-200

Table .4.2. List of Chemicals

Chemicals and reagents	**Manufacturer**
Thiobarbituric acid (TBA)	CDH, Mumbai
Tricholoroacetic acid (TCA)	CDH, Mumbai
Pyrogallol	CDH, Mumbai
Sodium azide (NaN3)	CDH, Mumbai
NADPH	Hi Media Labs
EDTA	CDH, Mumbai
Tris buffer	CDH, Mumbai
Tris HCl buffer	CDH, Mumbai
Hydrogen peroxide ($H_2O_{2)}$	CDH, Mumbai
DTNB	Hi Media, Mumbai
Potassium dihydrogen ortho phosphate	CDH, Mumbai
Disodium hydrogen ortho phosphate	CDH, Mumbai
Methanol	Thermo fischer, Mumbai

Table 4.3. Animals

SD rats	CDRI, Lucknow
Sex	Either sex
Age/Weight	8 weeks /150-180 gm
Strain	SD
Diet	Dayal rats feed

This study was approved by Institutional Animal Ethics Committee (IAEC) of Integral University and CPCSEA No-IU/Pharm/Ph.D/CPCSEA/12/03

4.2.1. Preparation of extract

The plants *A. racemosus and E. hirta* were air-dried and pulverized. 500 g of the powdered material were packed in muslin cloth and subjected to soxhlet extractor for continuous hot extraction with methanol for 72 hrs separately. There after methanolic extract of each plants were filtered through Whatman paper no. 42 and the resultant filtrates were concentrated under reduced pressure and finally vacuum dried. The yield of the methanolic extract was 10.4% and 12.2 % w/w respectively.

4.2.2. Lipopolysaccharide solution- Serotype E. coli 0111:B4 was prepared in pyrogen free saline and administered i.p. at the dose of 4 mg/kg(Barreiro et al., 2005).

4.2.3. Quercetin solution-Quercetin (100 mg/kg) was dispersed in 0.5% CMC suspension and administered orally (Ishisaka et al., 2011).

4.2.4. CMC 0.5 % solution-CMC 0.5 % was administered to control group at the dose of 5 ml/kg p.o

4.2.5. Preparation of homogenate

After transcardially perfusion animals were sacrificed and collect the brain and liver. Tissues of rats were homogenized (10%) in phosphate buffer having pH 7.4 to obtained supernatant, the homogenate was centrifuged at 12000 g at 4°C for 20 min, and it was used for the measurement of biochemical estimation.

4.2.6. Acute toxicity studies

4.2.6.1. Safe dose determination study

4.2.6.2. Selection of animals

SD rats (150-180 g) were selected for this study. The animals were divided into the eleven groups of four animals each. The animals were kept in polypropylene cages and maintained at a temperature of 25 ± 2 ºC. The study has got the approval from the Institutional Animal Ethical Committee (IAEC) of CPCSEA (Committee for the purposes of control and supervision of experiments on Animals).

4.2.6.3. Dose levels

A single dose of extract of methanolic *A. racemosus and E. hirta*, was administered in 0.3% carboxy-methyl cellulose (CMC) suspension at doses of 100, 200,500, 1000 and 2000 mg/kg/b.wt, whereas the control group received the CMC suspension only for each plant extracts. The animals were allowed free access to water and food. However, all the animals were deprived of food for 2 hr before and 4 hr after dosing (OECD, 2001).

4.2.7. Anti-stress studies

- The rats were randomly divided into 12 groups, each group containing 6 rats.
- In this study oxidative stress was induced by LPS (4 mg/kg,i.p.) on 21th day. Quercetin (100 mg/kg, p.o.) were used as a standard drug.
- On 21 day, they were challenged with injection of either saline or LPS (4 mg/kg, i.p.).
- Anxiety were assessed in the elevated plus maze 3 h after the LPS or saline challenge
- Motor activity were assessed in the photoactometer test 4 h after the LPS or saline challenge

Immediately after this with draw blood from rat tail vein for liver function test and all the animals were sacrificed, brain and liver were removed and stored for further studies

Table 4.4. Treatment schedule

Group	Drug Treatment	Dose (mg/kg)
I	Control	CMC (5ml/kg p.o.) once a day then challenged with normal saline i.p. on 21th day
II	LPS	Normal saline (5 ml/kg p.o.) once a day and then challenged with LPS (4 mg/kg i.p.) on 21 day
III	QT	100 mg/kg p.o. daily and then challenged with LPS (4 mg/kg i.p.) on 21 day
IV	AR_1	100 mg/ kg p.o. daily and then challenged with LPS (4 mg/kg i.p.) on 21 day
V	AR_2	200 mg/kg p.o daily and then challenged with LPS (4 mg/kg) on 21 day
VI	EH_1	100 mg/kg p.o daily and then challenged with LPS (4 mg/kg i.p.) on 21 day
VII	EH_2	200 mg/kg p.o daily and then challenged with LPS (4 mg/kg i.p.) on 21 day
VIII	AR_2+QT	200 mg/kg +100 mg/kg p.o. daily then challenged with LPS (4mg/kg i.p.) on 21day
1X	EH_2+QT	200 mg/kg p.o +100 mg/kg p.o.daily then challenged with LPS (4 mg/kg i.p.o.) on 21 day
X	AR_2+EH_2	200 mg/kg+200 mg/kg p.o daily and then challenged with LPS (4 mg/kg i.p.) on 21 day
XI	AR_2 *perse*	200 mg/kg. p.o. daily for 21 day
XII	EH_2 *perse*	200 mg/kg p.o. daily for 21 day

LPS-Lipopolysaccharide, AR_1- Lower dose of *A. racemosus* extract, AR_2- Higher dose of *A. racemosus* extract, EH_1- Lower dose of *E. hirta* extract, EH_2- Higher dose of *E. hirta* extract and QT-Quercetin

4.2.7.1. Parameters investigation

4.2.7.1.1. Behavioural tests

A large variety of rodent behavioral tests are currently being used to evaluate traits such as sensory-motor function, anxiety-like and depressive-like behavior. Most behavioral tests have an inherent complexity, and their use requires consideration of several aspects of particular importance is a test's validity because of its influence on the chance of successful translation of

preclinical results to clinical settings. Charles Darwin may be considered to be the founder of behavioral research. Since then, behavioral testing has been extensively used to gain a better understanding of the central nervous system (CNS) and to find treatments for its diseases (Thierry, 2010).

4.2.7.1.1.1. **Elevated plus maze test**

The elevated plus maze consists of two opposite open arms (50 cm long, 10 cm wide) and two opposite closed arms of the same size, enclosed by 40 cm high walls. The apparatus was elevated 50 cm above the floor and illuminated from the top. Rats were individually placed in the central square facing an open arm and observed for 5 min. The number of entries and the time spent in the open and closed arms were recorded. Rats enter more frequently in the closed arms, The maze was cleaned after each trial to remove any residue or odour of the animals. (Pellow et al., 1985).

4.2.7.1.1.2. **Photoactometer test**

This test measures exploration and voluntary locomotion within an enclosed area. Objective value for spontaneous motor activity was obtained with a photoactometer (Techno electronics, Lucknow, India). Rat was placed individually in a 30x30 cm black metal chamber with a screen floor and a light-tight lid. Six beans of red light will be focused 2 cm above the floor in to photocells on the opposite side. Each beam interruption was registered as an event on the external counter. The floor of the chamber was wiped clean with damp towel before each use. Rat was placed in the chamber one hour after oral administration of the drug. They were allowed to acclimate for 2 min, and then light beam breaks would be counted for the next 10 minutes (Ahmad et al., 2012)

Table 4.5. List of Biochemical estimation

Method	Significance
TBARS	Increased in oxidative stress conditions
GSH	Decreased in oxidative stress conditions
SOD	Decreased in oxidative stress conditions
CAT	Decreased in oxidative stress conditions
Protein	Decreased in oxidative stress conditions
NO	Increased in oxidative stress and inflammatory condition
Cytokines	Increased in oxidative stress and inflammatory condition
Cytokines	Increased in oxidative stress and inflammatory condition
SGOT	Increased in oxidative stress induced hepatic disorders
SGPT	Increased in oxidative stress induced hepatic disorders
ALKP	Increased in oxidative stress induced hepatic disorders
TB	Increased in oxidative stress induced hepatic disorders
ALBUMIN	Decreased in oxidative stress induced hepatic disorders
CHOLESTROL	Decreased in oxidative stress induced hepatic disorders

4.2.7.1.2. Biochemical estimation

4.2.7.1.2.1. Estimation of Thio Barbituric Acid Reactive Substances (TBARS)

Principle:

Lipid peroxidation is a free radical mediated event. The primary products of such damage are a complex mixture of peroxides which then breakdown to produce carbonyl compounds. The malondialdehyde (MDA) is one such carbonyl compound, which forms a characteristic chromogenic adduct with two molecules of TBA. The colorimetric reaction of TBA with

MDA, a secondary product of lipid peroxidation has been widely accepted for measuring lipid peroxidation (Ohkawa et al., 1979).

Preparation of the Reagents:

1. 0.8 % TBA solution: 0.8 gm of TBA was dissolved in distilled water and the volume was made up to 100 ml to make 52 nM.
2. 30 % TCA solution: 30 gm of trichloroacetic acid was dissolved in distilled water and the volume was made up to 100 ml.

Standard TEP reagent - 0.02 g (± 0.004 g) of TEP (1, 1, 3, 3 – tetraethoxy propane) was dissolved in 40% ethanol. A further dilution of 100 ml with distilled water of this solution to 1000 ml was done. A third dilution of 100 ml of the above solution to 500 ml with distilled water was finally done. This dilution contained 4 µ g of reagent per ml

Procedure:

1 ml of suspension medium was taken from the 10% tissue homogenate. 0.5 ml of 30% TCA will be added to it, followed by 0.5 ml of 0.8% TBA reagent. The tubes were then be covered with aluminium foil and kept in shaking water bath for 30 minutes at 80 degree celsius. After 30 minutes tubes were taken out and kept in ice-cold water for 30 minutes. These were then be centrifuged at 3000 rpm for 15 minutes.

The absorbance of the supernatant was read at 540 nm at room temperature against appropriate blank. Blank consist of 1 ml distilled water, 0.5 ml of 30% TCA and 0.5 ml of 0.8% TBA.

Calculation:

The amount of MDA present in a sample was calculated according to the equation:

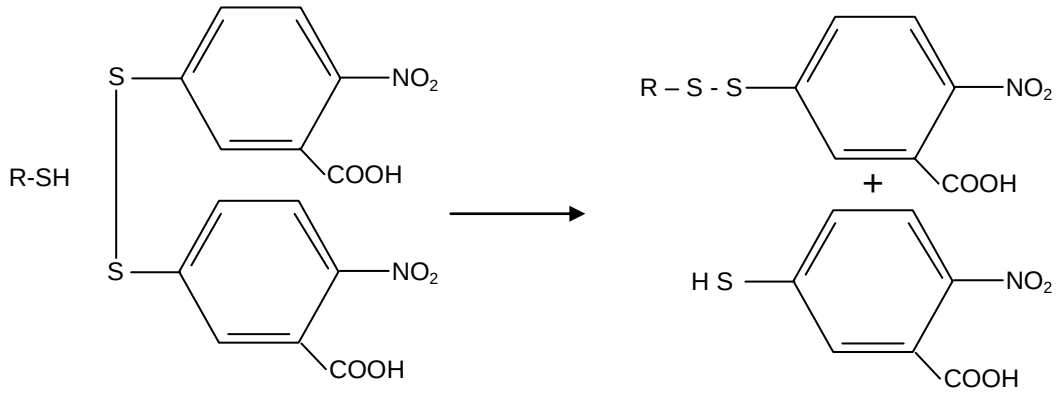
$$\text{nM of MDA} = \frac{\text{O.D. at 540 nm} \times V}{0.156}$$

Where, V is the final volume of test solution.

4.2.7.1.2.2. Tissue Glutathione

Principle

This spectrophotometric procedure is based on the method of Ellman i.e. 5, 5'-dithiobis-(2-nitrobenzoic acid), DTNB, is reduced by –SH groups to form one mole of 2-nitro-5-mercaptobenzoic acid per mole of –SH.



5, 5'- dithiobis- (2- nitro benzoic acid) 2-nitro-5-mercaptobenzoic acid

The nitromercaptobenzoic acid anion has an intense yellow color that is determined spectrophotometrically at 412 nm. (Sedlak and Lindsay, 1968).

Preparation of the Reagents

1. EDTA (0.2 M): 22.3 gm of EDTA was dissolved in 300 ml of warm double distilled water.
2. EDTA (0.02 M): 20 ml of the above solution was diluted to 200 ml with double distilled water.
3. DTNB (0.01M): 99 mg of DTNB was dissolved in 25 ml of absolute methanol.
4. Tris buffer (0.4M, pH 8.9): 24.2 of Tris buffer was dissolved in 100 ml of double distilled

water, 50 ml 0.2 M EDTA was added to it and the volume of the solution was made up to 500 ml with double distilled water. The pH of the solution was adjusted to 8.9 with 6 N HCl.

5. TCA (50%): 50 gm of TCA was dissolved in 100 ml of double distilled water.

Procedure

Test: A known weight of brain tissue ranging from (300-600 mg) was homogenized in 5-8 ml of 0.02 M EDTA and then 4.0 ml of cold distilled water was added to it. After mixing it well, 1 ml of 50 % trichloroacetic acid (TCA) was added and shaken intermittently for 10 minutes using a vortex mixer. After 10 minutes the contents will be transferred to centrifuge tubes (rinsed in EDTA) and centrifuged at 6000 rpm for 15 minutes. Following centrifugation, 2 ml of the supernatant was mixed with 4.0 ml of 0.4 M Tris buffer (pH 8.9). The whole solution was mixed well and 0.1 ml of 0.01M DTNB was added to it. The absorbance was read within 5 min of the addition of DTNB at 412 nm against a reagent blank.

Blank: The method was same as for the test except that 0.02 M EDTA was added in place of tissue homogenate.

Calculation

GSH (tissue) was calculated from the following equation and expressed as µg/mg of protein.

$$\text{GSH (µg/mg of protein)} = \frac{\text{O.D. at 412 nm x 50 x 3.5 x 2.25 x 1}}{\text{0.337 x 2 x mg of protein}}$$

4.2.7.1.2.3. Catalase

Principle:

In the UV range H_2O_2 shows a continuous increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. The difference in absorbance (ΔA) per unit time is a measure of the catalase activity.

Preparation of the Reagents:

1. Potassium di hydrogen phosphate ($KH2PO_4.2H_2O$): 6.81 gm of KH_2PO_4 was dissolved in double distilled water and volume was made up to 1000 ml with double distilled water.
2. Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$): 8.9 gm of $Na_2PO4.2H_2O$ was dissolved in double distilled water and volume was made up to 1000 ml with double distilled water.
3. Potassium phosphate buffer (50 mM; pH 7.4): It was prepared by mixing the above solution (i) and (ii) in the ratio of 1:1.55
4. Hydrogen peroxide 30% (19 mM/L): 187µl of 30% H_2O_2 was dissolved in 100 ml phosphate buffer.

Procedure:

Brain tissue was homogenized in 50 mM/L potassium phosphate buffer with a ratio of 1:10 w/v. The homogenate was centrifuged at 10,000 rpm at 4º C in a cooling centrifuge for 20 minutes. Catalase activity was measured in supernatant obtained after centrifugation. Supernatant (50 µl) was added to cuvette containing 2.95 ml of 19 mM/L solution of H_2O_2 prepared in potassium phosphate buffer. The change in absorbance was monitored at 240 nm wavelength at 1-minute interval for 3 minutes. Presence of catalase decomposes H_2O_2 leading to a decrease in absorbance. Clairbone, 1985).

Calculation:

Catalase activity was calculated as

η moles of H_2O_2 consumed/minute/mg protein

$$\text{Catalase} = \frac{\Delta \text{ A/minute} \times \text{volume of assay}}{0.081 \times \text{volume of homogenate} \times \text{mg of protein}}$$

4.2.7.1.2.4. Superoxide Dismutase

Principle:

Pyrogallol auto-oxidizes rapidly in aqueous solution; higher the pH faster is autoxidation and several intermediate products are formed. Thus the solution first becomes yellow-brown with a spectrum showing a should between 400-425 nm. After a number of minutes the color begins to turn green and finally after a few hours, a yellow color appears. So the auto-oxidation is studied essentially during the first step and the rate is taken from the linear increase in absorbance at 420 nm, which is seen for a number of minutes after an induction period of some 10 seconds. Super oxide anion radical ($O_{2\cdot}^-$) catalyses the auto-oxidation of pyrogallol. A simple and rapid method for assay of SOD is described, based on the ability of the enzyme to inhibit the autoxidation of pyrogallol.

Preparation of the Reagents:

1. Tris HCl buffer (pH 8.5):788 mg of Tris HCl buffer and 186 mg of EDTA were dissolved in 100 ml double distilled water and the and the P^H was adjusted to 8.5 using 1 N NaOH.

2. Potassium di hydrogen phosphate ($KH2PO_4.2H_2O$):6.81 gm of KH_2PO_4 was dissolved in double distilled water and volume was made up to 1000 ml with double distilled water.
3. Disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$): 8.9 gm of $Na_2PO4.2H_2O$ was dissolved in double distilled water and volume was made up to 1000 ml with double distilled water.
4. Potassium phosphate buffer (50 mM; pH 7.4): It was prepared by mixing the above solution (i) and (ii) in the ratio of 1:1.55
5. Pyrogallol (24 mM): 15.1 mg of pyrogallol was dissolved in 5 ml of 10 Mm HCl.

   The soluton was prepared freshly at the time of assay.

Procedure:

The supernatant was assayed for SOD activity by following the inhibition of pyrogallol autoxidation. 100 µl of cytosolic supernatant was added to Tris HCl buffer (pH 8.5). The final volume of 3 ml was adjusted with the same buffer. At least 25 µl of pyrogallol was added and changes in absorbance at 420 nm are recorded at 1 minute interval for 3 minutes. The increase in absorbance at 420 nm after the addition of pyrogallol was inhibited by the presence of SOD (Marklund and Marklund, 1974).

Calculation:

1 unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation per 3 ml of assay mixture and given by the formula:

$$\text{Unit of SOD per ml of sample} = \frac{(A - B) \times 100}{A \times 50}$$

Where, A is the difference of absorbance in 1 minute in control

B is the difference of absorbance in 1 minute in test sample.

Data was expressed as SOD units per mg of protein.

4.2.7.1.2.5. Protein estimation using folin reagent

Principle

Protein reacts with Folin Ciocalteau reagent to give a colored complex, the absorbance of which is determined spectrophotometrically at 750 nm. The reaction involves two steps. The first step is the reduction of copper as in the Biuret test. The second step involves the reduction of the Folin reagent by the tyrosine, tryptophan and cysteine present in the protein (Lowry et al., 1951).

Reagents

1. Standard protein: Bovine serum albumin solution (2 mg/ml)
2. Alkaline Sodium carbonate solution (2% Na_2CO_3 in 0.1 N NaOH)
3. Copper sulfate sodium-potassium tartrate (0.5 % $CuSO_4$ in 1% Na-K tartarate)
4. Alkaline solution: Prepared on the day of use by mixing 50 ml of reagent 2 and reagent 3
5. Folin ciocalteau phenol reagent

Preparation of the reagents

1. Alkaline sodium carbonate: 100 ml of 0.1 N NaOH solution was prepared by dissolving 400 mg of NaOH in distilled water and the volume was made up to 100 ml. Then 2 g of Na_2CO_3 was dissolved in 100 ml of 0.1 N NaOH solution.
2. Copper sulfate: Sodium potassium tartarate solution: 0.5 % copper sulfate solution was mixed with 1 % Na-K tartarate.
3. Folin ciocalteau phenol reagent: The commercial reagent was diluted with 2 volumes of distilled water on the day of use.
4. Standard protein: Bovine serum albumin (2 mg/ml) - 10 mg of bovine serum albumin was

dissolved in 5 ml of distilled water to get a solution of 2.0mg/ml of protein.

Procedure

5 ml of alkaline solution was added to 1 ml of suspension from the supernatant after centrifugation of 10 % tissue homogenate at 10,000 rpm and allowed to stand for 10 minutes. 0.5 ml of diluted folin reagent was added and the tube was shaken to mix the solution. After 30 minutes the extinction against appropriate blank at 750 nm was recorded.

Preparation of the calibration curve for protein

5 ml of bovine serum albumin solution (2 mg/ml) was prepared and different volumes were taken in 6 test tubes. To all tubes distilled water was added to make up the volume in each tube to 1 ml. The protein concentration in the above six tubes was estimated in the same way as for the sample. A graph was plotted between concentration of protein and optical density. The calibration standard plot was used to calculate the concentration of protein in each ml suspension of the sample.

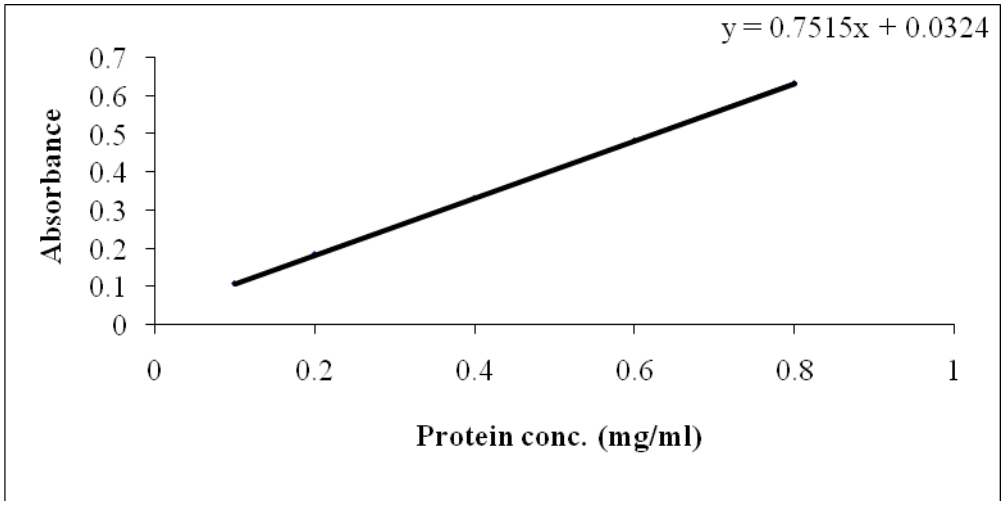


Figure 4.1. Calibration curve for protein

4.2.7.1.2.6. Measurement of Nitric oxide (NO): Griess Reaction

After the experiment, animals were sacrificed and the tissues were washed with PBS (pH 7.4) and placed on ice as method described earlier. Briefly a 50µl sample was added with 100µl of Griess reagent and reaction mixture was Incubate for about 5-10 minutes at room temperature and protects it from light, the optical density was measured at 540 nm in microplate reader according to the reagent manufacturer's protocol. Calculations were done after generating a standard curve from sodium nitrite in the same buffer as used for preparation of homogenate.

4.2.7.1.2.7. Measurement of cytokines level

Levels of inflammatory cytokines TNF-α, IL-1β and IL-6, in the joints were determined by using commercially available cytokine ELISA kits (eBioscience and Cayman Chemical USA). Supernatants were removed and assayed in duplicate according to the manufacturer's guidelines. Tissue cytokine concentrations were expressed as picograms of antigen per milligram of protein (McLaughlin et al., 1990).

4.2.7.1.3. Liver function test in serum

Serum was analyzed for the following parameters as aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphate (ALP), total cholesterol (TC), albumin (ALB) and total billirrubin (TB).

4.2.7.1.3.1. Determination of Serum glutamic oxaloacetic transaminase (SGOT)/ Aspartate transaminase (AST)

The SGOT activity was determined according to the method of Reitman and Frankel, (1957) method using SGOT.

Principle

Transaminase is an enzyme catalyzing the transfer of amino groups from α-amino acid to α-keto acid as follows.

2-Keto glutaric acid + Aspartic acid ⟶ Glutamate + Oxalo acetic acid.

Oxalo acetic acid formed in the reaction is spontaneously converted to pyruvic acid. Rate of reaction is then determined by the estimation of pyruvic acid using dinitrophenyl hydrazine. Dinitropheny hydrazine formed is estimated at 520 nm. The unreacted α-keto glutarate also gives coloured product with color reagent but the intensity is much less than that of pyruvate and hence it is negligible.

Reagents

Phosphate buffer – pH 7.4, standard pyruvate (2 mM), α - keto glutarate-aspartic acid substrate for SGOT, 2,4 – dinitrophenyl hydrazine, and 0.4 M sodium hydroxide.

Procedure

Substrate for SGOT: Place 29.2 mg of α - keto glutarate and 2.66 gm of aspartate in a beaker. Add 1M sodium hydroxide until it is completely dissolved. Adjust the pH to 7.4 with sodium hydroxide. Transfer quantitatively to 100 ml volumetric flask with phosphate buffer and dilute to the mark with buffer. Prepared a standard curve by set up a number of tubes as shown in the following table and added 1.0 ml of dinitrophenyl hydrazine solution to each tube.

Tube No.	Sodium pyruvate (ml)	Substrate (ml)	Water (ml)	SGOT units
1	0.5	0.5	0.2	0
2	0.4	0.6	0.2	22
3	0.3	0.7	0.2	55
4	0.2	0.8	0.2	95
5	0.1	0.9	0.2	150
6	0.0	1.0	0.2	215

Mix well and after 10 min. read the optical density (O.D) in a spectro photometer at 520 nm using green filter.

Solutions	Test (ml)	Control (ml)	Blank (ml)	Standard (ml)
Substrate	1.0	1.0	1.0	1.0
Keep for 5 min. in boiling water bath at 37˚ C				
Serum	0.2	-	-	-
Incubate at 37˚ C for 60 min				
Sodium pyruvate	-	-	-	0.2
Dinitrophenyl hydrazine	1.0	1.0	1.0	1.0
Serum	-	0.2	-	-
Allow to stand for 20 min. at room temperature				
Sodium hydroxide	10.0	10.0	10.0	10.0

Plot the results against the concentration units for SGOT and connect the points by a smooth curve (Reitman and Frankel, 1965).

After 10 min. read the O.D. of test, control and standard against reagent blank at 520 nm using green filter.Mark the O.D. of test on the Y-axis of the standard curve and extrapolate it to the corresponding enzyme activity on X-axis.

Serum glutamic oxaloacetic transaminase was expressed as U/l.

4.2.7.1.3.2. Determination of Serum glutamate pyruvate transaminase (SGPT)/ alanine transaminase (ALT)

The SGPT level was estimated by Reitman and Frankel, (1957) method using SGPT.

Principle

SGPT (ALT) catalyses the following reaction

α -Keto glutarate + L-alanine $\longrightarrow$ L -glutamate + pyruvate

pyruvate so formed is coupled with 2,4-dinitrophenyl hydrazine (2,4-DNPH) to give the corresponding hydrazone, which gives the brown color in alkaline medium and this can be measured colorimetrically.

Reagents

Reagent 1: Buffered alanine α-KG substrate, pH 7.4

Reagent 2: DNPH colour reagent

Reagent 3: Sodium hydroxide 4 N

Reagent 4: Working pyruvate standard, 2mM

Preparation of working solutions

Solution I: Dilute 1 ml of reagent 3 to 10 ml with purified water.

Procedure

Tube No.		**1**	**2**	**3**	**4**	**5**
Enzyme activity (units/ml)		0	28	57	97	100
Reagent 1: Buffered alanine, pH 7.4	(ml)	0.5	0.45	0.4	0.35	0.3
Reagent4: Working pyruvate standard, 2mM	(ml)	-	0.05	0.1	0.15	0.2
Purified water	(ml)	0.1	0.1	0.1	0.1	0.1
Reagent 2: DNPH colour reagent	(ml)	0.5	0.5	0.5	0.5	0.5
Mix well and allow to stand at room temperature for 20 min.						
Solution I	(ml)	5.0	5.0	5.0	5.0	5.0

Mix well by inversion. Allow to stand at room temperature for 10 min. and measure the O.D. of all the five tubes against purified water on a colorimeter using a green filter.

Test procedure

Reagent 1: Buffered alanine, pH 7.4	0.25 ml
Incubate at 37 ^{0}C for 5 min.	
Serum	0.05 ml
Mix well and incubate at 37 ^{0}C for 30 min.	
Reagent 2: DNPH colour reagent	0.25 ml
Mix well and allow to stand at room temp. for 20 min.	
Solution I	2.5 ml

Mix well and allow to stand at room temperature for 10 min and observe the O.D against purified water on a colorimeter using a green filter. Mark the O.D. of test on the Y-axis of the standard curve and extrapolate it to the corresponding enzyme activity on X-axis.

Serum glutamate pyruvate transaminase (SGPT) was expressed as U/l.

4.2.7.1.3.3. Determination of serum alkaline phosphatase (ALP)/ Alkaline phosphatase (ALP)

The alkaline phosphates level was estimated by King, (1965) method alkaline phosphatase.

Principle

Alkaline phosphatase from serum converts phenyl phosphate to inorganic phosphate and phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4- amino antipyrine in presence of the oxidizing agent potassium ferricyanide and forms an orange-red coloured complex, which can be measured at 510 nm.

Reagents

Reagents 1: Buffered substrate, pH 10.0

Reagents 2: Chromogen reagent

Reagent 3: Phenol standard, 10 mg%

Preparation of working solution

Reconstitute one vial of reagent 1, buffered substrate with 4.5 ml of distilled water.

Procedure

	Blank	**Standard**	**Control**	**Test**
Working buffered substrate	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Distilled water	1.5 ml	1.5 ml	1.5 ml	1.5 ml
Mix well and incubate for 3 min. at 37 ^{o}C				
Serum	-	-	-	0.05 ml
Phenol standard, 10 mg%	-	0.05 ml	-	-
Mix well and incubate for 15 min. at 37 ^{o}C				
Chromogen reagent	1.0 ml	1.0 ml	1.0 ml	1.0 ml
Serum	-	-	0.05 ml	-

Mix well after the addition of each reagent and measure the O.D. of blank, test, standard and control at 510 nm against reagent blank (King, 1954).

Serum alkaline phosphatase (KA units): O.D of test – O.D of control / O.D of standard – O.D of blank × 10

Alkaline phosphate was expressed as U/l.

4.2.7.1.2.2.4. Determination of serum bilirubin

The bilirubin level in serum was determined by standard procedure.

Sulfanilic acid reacts with sodium nitrite to produce deoxidized sulfanilic acid. Total bilirubin couples with deoxidized sulfanilic acid in the presence of methylsulfoxide to produce azobilirubin which may be measured at 532-536 nm. In the absence of methyl sulfoxide, only direct (conjugated) bilirubin forms azobilirubin complex.

Reagents

1. Total bilirubin reagent, Sulfanilic acid, Dimethyl sulfoxide, Stabilizer.
2. Direct bilirubin reagent, Sulfanilic acid, Preservative.
3. Activator, Sodium nitrile.
4. Artificial standard – 10 mg/dl.

Procedure

Estimation of total bilirubin: To 1.0 ml total bilirubin reagent, 0.02 ml of activator and 0.1 ml of serum were added, mixed well and incubated for exactly 5 minutes at room temperature.

Sample blank was prepared by mixing 1.0 ml total bilirubin reagent with 0.1 ml of distilled water, mixed well and incubated for exactly 5 minutes at room temperature. The absorbance of each sample blank and test were measured at 532-546 nm against distilled water blank (Dangerfield and Finlayson, 1953).

Total bilirubin and direct bilirubin level in serum was expressed as mg/dl.

4.2.7.1.3.5. Albumin (ALB)

Principle

Proteins bind with copper ions in an alkaline medium of the Biuret reagent and produce a purple coloured complex, whose absorbance is proportional to the protein concentration. Albumin in a buffered medium binds with Bromocresol green (BCG) and produces a green colour whose absorbance is proportional to the albumin concentration.

Reagents

1. Biuret reagent
2. Buffered Dye reagent
3. Albumin standard (4 gm %)

Procedure

Albumin assay

Pipette into three clean dry test tube labeled blank (B), standard (S) and test (T).

Mixed well and measured immediately absorbance of standard and against blank on photocolorimeter with red filter or on a spectrophotometer at 630 nm. (Webster, 1974).

	Blank (B)	Standard (S)	Test (T)
Buffered dye reagent	1 ml	1 ml	1 ml
Distilled water	2 ml	2 ml	2 ml
Albumin standard	..	0.01 ml	..
Serum	..	..	0.01 ml

Calculations

Albumin = Absorbance of test/Absorbance of sample × 4

The total protein and albumin level were expressed as g/dl.

4.2.7.1.3.6. Total cholesterol

The total cholesterol level was estimated by Standard procedure.

Principle

Cholesterol reacts with ferric perchlorate in the presence of ethyl acetate and sulfuric acid when heated in a boiling water bath to produce a lavender colored complex. The intensity of the color produced is proportional to the cholesterol concentration (Zlatkis, 1953).

Reagents

1. Cholesterol reagent
2. Precipitating reagent
3. Standard (200mg %)

Procedure

Pipette into dry test tubes labeled blank (B), standard and test (T).

	Blank (B)	Standard (S)	Test (T)
Cholesterol Reagent (1)	5.0 ml	5.0 ml	5.0 ml
Distilled Water	0.025 ml	..	..
Standard (3)	..	0.025 ml	
Serum/Plasma	..	..	0.025 ml

Mixed well and immediately place in a boiling water bath for exactly 60 seconds. Cooled immediately in running tap water and measured the absorbance of test (T), sample (S) against blank (B) on the photocolorimeter with yellow green filter or on a spectrophotometer 560 nm within 15 min.

Calculations

Total cholesterol = Absorbance of test/Absorbance of sample × 200

The cholesterol level was expressed as mg/dl in serum.

4.2.7.2. Statistical analysis

All results were expressed as mean ± SEM Groups of data was compared with analysis of variance (ANOVA) followed by Tukey-kramer multiple comparison test. Values were considered statistically significant, when $p<0.05$.

4.3. Results

4.3.1. *A. racemosus*

4.3.1.1. Acute toxicity studies

Rat administered with Methanolic extract of *A. racemosus* upto 2000 mg/kg did not show any abnormal behaviour, during initial 4 hour after drug administration. No mortality was observed during 14 days after treatment. Thus two doses (100 mg/kg and 200 mg/kg p.o.) were employed for futher studies.

Table 4.6. Elevated plus maze test and photoactometer test

Group	Drug treatment	Time duration in open arm in sec	No of entries in open arm	Locomotor activity
I	Control	204.6±5.115	6.6±0.509	178±10.677
II	LPS	99.6±3.558***	2.00±0.136***	121±6.403***
III	QT	180.8±3.338$^{###}$	5.2±0.374$^{###}$	170±8.509$^{#}$
IV	AR_1	140.4±3.265$^{###}$	2.4±0.244 ^{ns}	123±5.380^{ns}
V	AR_2	150±3.536$^{###}$	4.00±0.362$^{##}$	169.8±8.777$^{#}$
VI	EH_1	143.4±3.187$^{###}$	3.2±0.372^{ns}	138±6.678^{ns}
VII	EH_2	181.4±2.943$^{###}$	4.2±0.374$^{##}$	168.6±8.471$^{#}$
VIII	AR_2+QT	187.4±4.843$^{###}$	5.6±0.244$^{###}$	171±8.741$^{#}$
IX	EH_2+QT	198.8±3.513$^{###}$	6±0.447$^{###}$	172.4±6.400$^{##}$
X	AR_2+EH_2	179.6±2.315$^{###}$	5.4±0.244$^{###}$	170.4±8.518$^{##}$
XI	AR_2 perse	198.8±3.513	7±0.316	176.4±10.815
XII	EH_2 perse	200.4.4±3.172	6.2±0.200	182.6±11.801

Results were expressed as mean ± SEM. The results were analyzed by Analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test.

n=6, $^{#}$ = p<0.05, $^{##}$= p<.01, $^{###}$ = p<0.001, *** = p<0.001, $^{#}$ Vs. Group II, * Vs. Group I

4.3.1.2. Effect of methanolic extract of *A. racemosus* alone and in combination on Elevated plus maze and Photoactometer test

A. racemosus pretreatment in the doses of 100 mg/kg p.o. and 200 mg/kg p.o. Significantly increased (p<0.001) the locomotor activity and increased time duration in open arm which was impaired by 4 mg/kg i.p LPS treatment. Table: 4.6. shows significant and dose

dependent recovery on LPS induced oxidative stress of animal due to *Asparagus racemosus* pretreatment.

4.3.1.3. Effect of methanolic extract of *A. racemosus* on oxidative stress markers

4.3.1.3.1. Lipid peroxidation / TBARS concentration:

The level of TBARS was found to be significantly increased ($p<.001$) in brain and liver tissue of LPS (4 mg/kg ip) treated animals.

In the rats pretreated with MEAR 100 mg/kg as well as MEAR 200 mg/kg p.o. the level of TBARS were significantly reduced ($p<.001$) as compared to LPS 4 mg/kg i.p. Table 4.2. shows significant and dose dependent recovery on LPS induced elevation of TBARS level in animal due to MEAR pretreatment.

4.3.1.3.2. Reduced glutathione levels:

The level of Reduced glutathione was found to be significantly lower ($p<0.001$) in brain tissue and liver tissue of LPS (4 mg/kg ip) treated animals.

4.3.1.3.3. SOD level:

In the rats pretreated with MEAR 100 mg/kg and 200 mg/kg p.o. the level of SOD were significantly increased ($p<.001$) as compared to LPS 4 mg/kg i.p Table 4.2. and 4.4. Shows significant and dose dependent recovery on LPS induced reduced of SOD level in animal due to MEAR.

4.3.1.3.4. Catalase level:

There was a significant increase ($p<.05$) in the levels of catalase in MEAR pretreated rats as compared to LPS 4 mg/kg p.o. treated rats and here was a significant increase ($p<.001$) in the levels of catalase in MEAR as compared to LPS 4 mg/kg i.p treated rats.

Table 4.7. Effect of oxidative stress markers in brain

Drug treatment	**GSH(µg/mg protein)**	**CAT(nmolH_2O_2/ mg protein)**	**SOD(Units/mg protein)**	**TBARS(nmoles MDA/mg protein)**
Control	3.452±0.080	22.2±0.860	2.604±0.106	3.658±0.076
LPS	1.51±0.050***	9±0.707***	1.46±0.1281***	7.56±0.096***
QT	3.13±0.070$^{\#\#\#}$	19.4±0.500$^{\#\#\#}$	2.42±0.128$^{\#}$	3.79±0.051$^{\#\#\#}$
AR_1	1.894±0.110^{ns}	11.4±0.678^{ns}	1.76±0.114^{ns}	7.06±0.092$^{\#}$
AR_2	1.972±0.037$^{\#\#}$	13.2±0.66$^{\#\#\#}$	2.02±0.073$^{\#\#}$	5.26±0.136$^{\#\#\#}$
EH_1	1.99±0.101$^{\#\#}$	13.4±0.927$^{\#}$	1.92±0.106$^{\#}$	5.48±0.066$^{\#\#\#}$
EH_2	2.20±0.034$^{\#\#\#}$	14.4±0.812$^{\#\#}$	2.16±0.0400$^{\#\#\#}$	4.744±0.068$^{\#\#\#}$
AR_2+QT	3.35±0.065$^{\#\#\#}$	20.4±0.678$^{\#\#\#}$	2.46±0.0812$^{\#\#\#}$	3.704±0.011$^{\#\#\#}$
EH_2+QT	3.45±0.127$^{\#\#\#}$	21.2±0.860$^{\#\#\#}$	2.54±0.107$^{\#\#\#}$	3.646±0.033$^{\#\#\#}$
AR_2+EH_2	3.244±0.861$^{\#\#\#}$	18.2±1.11$^{\#}$	2.33±0.088$^{\#\#\#}$	4.308±0.075$^{\#\#\#}$
AR_2 *perse*	3.516±0.036	22.8±0.583	2.68±0.086	3.746±0.130
EH_2 *perse*	3.578±0.0754	22.4±1.07	2.56±0.103	3.562±0.082

Results are expressed as mean ± SEM. The results were analyzed by Analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test.

n=6,$^{\#}$ = p<0.05, $^{\#\#}$= p<.01, $^{\#\#\#}$ = p<0.001, *** = p<0.001, $^{\#}$ Vs. Group II, * Vs. Group I

Table 4.8. Effect of on cytokines and nitric oxide level in brain

Group	**Drug treatment**	TNF-α Pg/ml	IL-6 Pg/ml	IL-1β Pg/ml	NO(µmol nitrite/mg of wet tissue)
I	Control	33.00±0.707	138.4±2.657	38.4±1.364	3.38±0.086
II	LPS	52.8±1.71***	208±5.762***	79.4±1.503***	8.42±0.0712***
III	QT	37.00±1.09$^{###}$	144±2.429$^{###}$	43.4±1.288$^{###}$	5.866±0.1388$^{###}$
IV	AR_1	42.8±1.715$^{###}$	185.6±1.806$^{###}$	69.8±1.772$^{###}$	7.01±0.135$^{###}$
V	AR_2	40.8±1.68$^{###}$	182.8±5.809$^{###}$	65.4±1.240$^{###}$	6.322±0.066$^{###}$
VI	EH_1	41.6±0.837$^{###}$	183.6±2.694$^{###}$	61.8±1.304$^{###}$	6.73±0.108$^{###}$
VII	EH_2	39.00±1.049$^{###}$	173±2.280$^{###}$	60±1.240$^{###}$	6.33±0.057$^{###}$
VIII	AR_2+QT	35.4±0.927$^{###}$	143.4±2.804$^{###}$	43.6±1.631$^{###}$	5.78±0.105$^{###}$
IX	EH_2+QT	34.00±1.22$^{###}$	139.4±1.806$^{###}$	42±1.414$^{###}$	5.22±0.0815$^{###}$
X	AR_2+EH_2	37.2±0.860$^{###}$	140.8±1.530$^{###}$	45.4±1.077$^{###}$	6.034±0.093$^{###}$
XI	AR_2 *perse*	34.4±1.50	138.6±2.315	38±0.707	3.57±0.129
XII	EH_2 *perse*	34.8±0.969	139.2±2.746	39.2±0.811	3.486±0.101

Results were expressed as mean ± SEM. The results were analyzed by Analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test.

$^{#}$ = p<0.05, $^{##}$= p<.01, $^{###}$ = p<0.001, *** = p<0.001, $^{#}$ Vs. Group II, * Vs. Group I

4.3.1.4. Effect of methanolic extract of *A. racemosus* on proinflammatory cytokines

The level of TNF-α, IL-6 and IL-1β was found to be significantly increased (p<.001) in brain tissue and liver tissue of LPS (4 mg/kg ip) treated animals.

In the rats pretreated with MEAR 100 mg/kg as well as MEAR 200 mg/kg p.o. the level of proinflammatory cytokines were significantly reduced (p<.001) as compared to LPS 4 mg/kg

i.p Table: 4.8 and 4.10 shows significant and dose dependent recovery on LPS induced elevation of cytokines level in animal due to MEAR pretreatment.

4.3.1.5. Effect of methanolic extract of *A. racemosus* on nitric oxide

The level of nitric oxide was found to be significantly increased ($p<.001$) in brain tissue and liver tissue of LPS (4 mg/kg ip) treated animals. (Table: 4.8 and 4.10)

In the rats pretreated with MEAR 100 mg/kg as well as MEAR 200 mg/kg p.o. the level of nitric oxide were significantly reduced ($p<.001$) as compared to LPS 4 mg/kg i.p.

Table 4.9. Effect of oxidative stress markers in liver

Group	Drug treatment	TBARS(nmols MDA/mg protein)	GSH(µg/mg protein)	CAT(nmolH_2O_2/ mg protein)	SOD(Units/mg protein)
I	Control	3.502±0.0596	3.292±0.043	19.625±0.939	2.64±0.086
II	LPS	6.33±0.0743***	1.666±0.035***	9.912±0.919***	1.424±0.067***
III	QT	3.974±0.0812$^{###}$	2.876±0.073$^{###}$	15.716±0.285$^{###}$	2.20±0.087$^{###}$
IV	AR_1	5.614±0.1062$^{##}$	1.794±0.022^{ns}	12.200±0.504^{ns}	1.584±0.058^{ns}
V	AR_2	5.386±0.117$^{###}$	1.942±0.054$^{#}$	13.788±0.359$^{#}$	1.784±0.0508$^{#}$
VI	EH_1	5.56±0.074$^{###}$	1.924±0.053$^{#}$	12.302±0.278^{ns}	1.84±0.039$^{##}$
VII	EH_2	4.792±0.0697$^{###}$	2.032±0.617$^{###}$	14.55±0.264$^{###}$	2.054±0.030$^{###}$
VIII	AR_2+QT	3.802±0.049$^{###}$	3.204±0.0423$^{###}$	17.70±0.475$^{###}$	2.324±0.0305$^{###}$
IX	EH_2+QT	3.662±0.0321$^{#}$	3.24±0.0393$^{#}$	17.916±1.058$^{###}$	2.416±0.0369$^{#}$
X	AR_2+EH2	4.94±0.081$^{#}$	3.012±0.043$^{#}$	15.922±0.2852$^{###}$	2.106±0.057$^{#}$
XI	AR_2 perse	3.598±0.113	3.516±0.036	19.292±1.066	2.616±0.099
XII	EH_2 perse	3.49±0.0781	3.392±0.063	19.416±0.846	2.698±0.103

Results were expressed as mean ± SEM. The results were analyzed by Analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test.

n=6,$^{#}$ = $p<0.05$, $^{##}$= $p<.01$, $^{###}$ = $p<0.001$,*** = $p<0.001$, $^{#}$ Vs. Group II, * Vs. Group I

4.3.1.6. Effect of methanolic extract of *A. racemosus* and on Liver function tests

The present data shows in Table: 4.11 Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), alkaline phosphate (ALP), total cholesterol (CHL), albumin (ALB) and total billirubin (TB) enzymes as markers of liver function were assessed. Data in table-4.11 explain that administration of LPS (4 mg/kg, i.p, once) resulted in marked increase in SGOT, SGPT, ALB, alkaline phosphate (ALP) and total bilirubin activities while total cholesterol and albumin activities decreased campare to corresponding control group. Administration of MEAR daily dose orally (100 mg/ kg and 200 mg/kg) for 21 consecutive days for the group administered LPS (4 mg/kg, i.p, once at day 21) resulted in significant reductions in SGOT, SGPT, ALP and TB while increased the level of total cholesterol and albumin if compared with only LPS-treated group.

4.3.2. *E. hirta*

4.3.2.1. Acute toxicity studies

Rat administered with Methanolic extract of *E. hirta* upto 2000 mg/kg did not show any abnormal behaviour, during initial 4 hour after drug administration. No mortality was observed during 14 days after treatment. Thus two doses (100 mg/kg and 200 mg/kg p.o.) were employed for futher studies.

4.3.2.2. Effect of methanolic extract of *E. hirta* on Elevated Plus maze and Photoactometer

E. hirta pretreatment in the doses of 100 mg/kg p.o. and 200 mg/kg p.o. Significantly increased ($p<0.001$) the locomotor activity and increased time duration in open arm which was impaired by 4 mg/kg i.p LPS treatment. Table: 4.6. shows significant and dose dependent recovery on LPS induced oxidative stress of animal due to *E. hirta* pretreatment.

4.3.2.3. Effect of methanolic extract of *E. hirta* on oxidative stress markers

4.3.2.3.1. Lipid peroxidation / TBARS concentration:

The level of TBARS was found to be significantly increased ($p<.001$) in brain tissue and liver tissue of LPS (4 mg/kg ip) treated animals.

In the rats pretreated with MEEH 100 mg/kg as well as MEEH 200 mg/kg p.o. the level of TBARS were significantly reduced ($p<.001$) as compared to LPS 4 mg/kg i.p Table 4.7 and 4.9 shows significant and dose dependent recovery on LPS induced elevation of TBARS level in animal due to MEEH pretreatment.

4.3.2.3.2. Reduced glutathione level:

The level of reduced glutathione was found to be significantly lower ($p<0.001$) in brain and liver tissue of LPS (4 mg/kg ip) treated animals. In the rats pretreated with MEEH 100 mg/kg and 200 mg/kg p.o. the level of SOD were significantly increased ($p<.001$) as compared to LPS 4 mg/kg i.p illustrated in Table: 4.7 and 4.9

4.3.2.3.3. SOD level:

In the rats pretreated with MEEH 100 mg/kg and 200 mg/kg p.o. the level of SOD were significantly increased ($p<.001$) as compared to LPS 4 mg/kg i.p Table 4.7 and 4.9 shows significant and dose dependent recovery on LPS depleted SOD level in animal due to MEEH.

4.3.2.3.4. Catalase level:

The level of Catalase was found to be significantly decreased ($p<.001$) in brain tissue and liver tissue of LPS (4 mg/kg ip) treated animals.

In the rats pretreated with MEEH 100 mg/kg as well as MEEH 200 mg/kg p.o. the level of Catalase was found to be significantly increased ($p<.001$) in brain tissue and liver tissue as capare to LPS.

4.3.2.4. Effect of methanolic extract of *E. hirta* on cytokines

The level of TNF-α, IL-6, IL-1β and nitric oxide was found to be significantly increased ($p<.001$) in brain tissue and liver tissue of LPS (4 mg/kg ip) treated animals.

In the rats pretreated with MEEH 100 mg/kg as well as MEEH 200 mg/kg p.o. the level of proinflammatory cytokines and nitric oxide were significantly reduced ($p<.001$) as compared to LPS 4 mg/kg i.p

Table-4.10. Effect of cytokines and nitric oxide level in liver

Group	Drug treatment	IL-1β Pg/ml	IL-6 Pg/ml	TNF-α Pg/ml	NO(µmol nitrite/mg of wet tissue)
I	Control	37.4±2.542	136.4±4.377	32.6±1.364	3.24±0.108
II	LPS	73.6±5.115***	196.4±6.964***	51.6±3.027***	7.446±0.198***
III	QT	52.8±3.555$^{\#\#}$	149.31±6.189$^{\#\#\#}$	37.12±2.345$^{\#\#}$	5.488±0.140$^{\#\#\#}$
IV	AR_1	69.6±3.140^{ns}	168.21±5.391$^{\#}$	45.4±4.707^{ns}	6.718±0.093$^{\#}$
V	AR_2	56.8±2.289$^{\#\#\#}$	163.6±6.787$^{\#\#\#}$	38.34±1.732$^{\#}$	6.632±0.087$^{\#\#}$
VI	EH_1	66.6±4.874^{ns}	169.63±2.857$^{\#}$	39.2±1.625$^{\#}$	6.804±0.077$^{\#}$
VII	EH_2	54.2±2.478$^{\#\#}$	159.43±5.958$^{\#\#\#}$	38.8±2.939$^{\#}$	6.46±0.072$^{\#\#\#}$
VIII	AR_2+QT	50.8±2.672$^{\#\#\#}$	140.4±5.636$^{\#\#\#}$	36.6±3.763$^{\#\#}$	4.854±0.145$^{\#\#\#}$
IX	EH_2+QT	48.2±4.363$^{\#\#\#}$	141.6±3.736$^{\#\#\#}$	35.4±0.927$^{\#\#}$	4.684±0.120$^{\#\#\#}$
X	AR_2+EH_2	49.6±1.077$^{\#\#\#}$	156.4±4.400$^{\#\#\#}$	37.53±1.414$^{\#\#}$	6.132±0.062$^{\#\#\#}$
XI	AR_2 perse	37±2.074	137.81±5.187	37.2±0.734	3.106±0.174
XII	EH_2 perse	38.2±2.107	136.4±4.377	37.8±1.530	3.353±0.191

Results were expressed as mean ± SEM. The results were analyzed by Analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test.

n=6, $^{\#}$ = $p<0.05$, $^{\#\#}$= $p<.01$, $^{\#\#\#}$ = $p<0.001$, *** = $p<0.001$, $^{\#}$ Vs. Group II, * Vs. Group I

Table-4.11. Effect of lipopolysaccharide, Quercetin, *A. racemosus* and *E. hirta* extract alone and in combination on liver injury markers

Drug treatment	SGOT U/l	SGPT U/l	ALKP U/l	TB mg/dl	ALB g/dl	CHL mg/dl
Control	138.6±8.599	113.24±7.31	317.4±10.773	0.924±0.058	3.542±0.0737	83.69±4.344
LPS	269.6±12.902***	196.2±8.009***	397.6±8.447***	1.504±0.048***	1.736±0.030***	21.908±4.432***
QT	172.4±8.078$^{###}$	128.8±7.940$^{#}$	322.6±12.428$^{###}$	1.142±0.0731$^{###}$	2.774±0.052$^{###}$	75.626±3.018$^{###}$
AR_1	256.3±9.257^{ns}	179.4±6.337^{ns}	388.6±4.79^{ns}	1.292±0.033^{ns}	1.85±0.0761^{ns}	34.126±4.194^{ns}
AR_2	209.2±10.012$^{##}$	163.2±5.113$^{#}$	331.2±8.691$^{###}$	1.2±0.024$^{##}$	2.066±0.049$^{##}$	42.86±6.344$^{#}$
EH_1	215.34±13.680$^{###}$	160.1±8.515$^{#}$	348.2±13.185$^{###}$	1.196±0.041$^{##}$	2.012±0.064$^{#}$	38.792±4.065^{ns}
EH_2	208.8±12.415$^{##}$	157.31±8.746$^{#}$	329.6±9.026$^{###}$	1.174±0.0710$^{####}$	2.284±0.048$^{###}$	49.32±4.334$^{###}$
AR_2+QT	169.2±7.612$^{###}$	124.8±7.453$^{###}$	320.2±8.628$^{###}$	1.046±0.0710$^{###}$	2.924±0.021$^{###}$	77.482±3.292$^{###}$
EH_2+QT	165.7±6.028$^{###}$	120.6±5.758$^{###}$	317.6±11.016$^{###}$	0.976±0.0529$^{###}$	3.2±0.031$^{###}$	80.366±4.211$^{###}$
AR_2+EH_2	202.4±12.160$^{##}$	151.61±7.235$^{##}$	326.8±9.231$^{###}$	1.086±0.0506$^{###}$	2.476±0.044$^{###}$	56.86±2.200$^{###}$
AR_2 perse	142.61±10.559	105±4.980	320.8±6.946	0.966±0.0403	3.582±0.0582	80.976±3.277
H_2 perse	141.4±8.981	109.2±4.317	322.2±6.560	0.882±.0483	3.498±0.084	82.04±3.810

Results were expressed as mean ± SEM. The results were analyzed by Analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test.

n=6, $^{#}$ = p<0.05, $^{##}$= p<.01, $^{###}$ = p<0.001, *** = p<0.001, $^{#}$ Vs. Group II, * Vs. Group I

4.3.2.5. Effect of methanolic extract of *E. hirta* on nitric oxide

The level of nitric oxide was found to be significantly increased ($p<.001$) in brain tissue and liver tissue of LPS (4 mg/kg ip) treated animals.

In the rats pretreated with MEEH 100 mg/kg as well as MEEH 200 mg/kg p.o. the level of nitric oxide were significantly reduced ($p<.001$) as compared to LPS 4 mg/kg i.p

4.3.2.6. Effect of methanolic extract of *E. hirta* on Liver function tests

The present data shows in Table: 4.11 Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), alkaline phosphate (ALP), total cholesterol (CHL), albumin (ALB) and total billirubin (TB) enzymes as markers of liver function were assessed. Data in table 4.11 explain that administration of LPS (4 mg/kg, i.p, once) resulted in marked increase in SGOT, SGPT, ALB, alkaline phosphate (ALP) and total bilirubin activities while total cholesterol and albumin activities decreased campare to corresponding control group. Administration of MEAR and MEEH daily dose orally (100 mg/ kg and 200 mg/kg) for 21 consecutive days for the group administered LPS (4 mg/kg, i.p, once at day 21) resulted in significant reductions in SGOT, SGPT, ALP and TB while increased the level of total cholesterol and albumin if compared with only LPS-treated group.

4.4. Disscussion

Antioxidants are being investigated for their ability to prevent cardiovascular, hepatic and neurodegenerative disease caused by LPS-induced production of ROS, peroxides, nitric oxide and cytokines (Victor et al., 2000). The present study was therefore designed to study the detailed mechanism of protection offered by *A. racemosus* and *E. hirta* plant against LPS-induced brain and liver damage in rats in terms of behavioural and biochemical parameters.

The Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamate pyruvate transaminase (SGPT), alkaline phosphate (ALP) and total billirrubin (TB) activities, total cholesterol (CHL) and albumin (ALB) show functional activity of liver. An increase or decrease in the activities of these enzymes indicates a generalized effect due to the toxin (McGeer et al., 1998).

The present study showed an increased level of, SGOT, SGPT, ALP and bilirubin, nitric oxide and proinflammatory cytokines while decreased the level of ALB and CHL of the LPS treated animals indicating brain and liver damage.

An increase in the levels of these hepatic marker enzymes in serum is reported due to the leakage of these enzymes from liver as a result of tissue damage. Methanolic extract of both *A. racemosus* and *E. hirta* at doses of 100 and 200 mg/kg respectively brought the serum marker enzyme, oxidative stress markers, nitric oxide and cytokines levels to near normal indicating protection against brain and liver damage.

The methanolic extracts of *A. racemosus* (MEAR) and methanolic extracts of *E. hirta* (MEEH) showed protective effects as they reduce the levels of hepatic markers and oxidative stress markers in liver and brain respectively. Lipid peroxidation can cause changes in membrane fluidity and permeability and increase the rate of protein degradation, which eventually lead to cell lysis. *A. racemosus* and *E. hirta* has been used to prevent oxidative damage by interrupting the propagation of the oxidation of polyunsaturated fatty acids. The quercetin supplementation showed an antiperoxidative effect in the rat brain tissue and liver tissues by significantly decreasing the LPS-induced rise of brain and liver MDA levels (Sekine et al., 1997). This effect of MEAR and MEEH may be explained by its direct free radical scavenging

property, which suggests that by preserving cellular integrity, it can protect against LPS-induced organ damage.

Treatment with LPS often cause distressing side effect like stress, variety of disorders, including neurodegenerative disorder and hepatic disorder. (Gulec et al., 2006). Since LPS induced oxidative stress has underlying pathology of increased oxidative stress. In the present study twenty one days LPS treated animals showed severe stress behaviour, which was analyzed by photoactometer and elevated plus maze test. This result is in agreement with the previous studies of LPS on oxidative stress and markers of oxidative stress (TBARS, GSH, SOD and CAT), thus suggesting the possible induction of free radical generation by LPS treatment. Results of present study are consistent with previous report of LPS induced oxidative stress by which showed its role in anaerobic infection (Ritter et al., 2003). Lipopolysaccharide major factor contributing to the pathogenesis of bacterial infections and it induces the production and release of several cytokines, in response of which, several reactive oxygen species (ROS) are produced from cells such as neutrophils, microglia, macrophages and others phagocytic cells, creating a status of oxidative stress (Laduzek et al., 2005).

As seen in the quercetin treated group and methanolic extract of *A. racemosus* and *E. hirta,* all studied parameters were restored to near normal condition from the abnormal ones. The body has an effective defence mechanism to prevent and neutralize the free radical induced damage. This is carried out by a set of endogenous antioxidant enzymes such as glutathione (GSH), super oxide dismutase (SOD), and catalase (CAT). Suppression of the anti oxidant system in LPS treated rats has been reported earlier.

Whatever be the mechanism of unbalanced production of reactive oxygen species (ROS) and oxidative stress by LPS, *A. racemosus* and *E. hirta* were found to be effective in decreasing the

oxidative stress in LPS treated animals by decreasing cytokines and lipid peroxidation and elevated the cellular defense mechanism such as glutathione, superoxide dismutase.

However, the antioxidant activity of antioxidants (extracts) may be due to various mechanisms, among which is anticipation of chain initiation, binding of transition metal ion catalysts and decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging (Akillioglu et al., 2010).

A. racemosus and E. hirta having anti oxidative properties reduced the anxiety and increased the locomotor activity which was impaired by LPS.

The antioxidant activity of *A. racemosus and E. hirta* could be possibly due to the direct scavenging of the superoxide radicals by the polyphenols or the Flavanoids known to be present in these drugs. From the present study it was concluded that the *A. racemosus and E. hirta* has been proved as beneficial adjuvants in the treatment of LPS-induced oxidative stress related disorders.

4.5. Conclusion

In the acute toxicity studies, *A. racemosus* and *E. hirta* showed neither mortality nor changes in behaviour in rats at maximum dose of 2 gm.

Present study concluded that methanolic extract of *A. racemosus* and *E. hirta* showed significantly ($P < 0.001$) reduced LPS-induced oxidative stress and intensely suggest that *A. racemosus and E. hirta* is newer type anti-stress and hepato-protective agent.

Finding results suggested that 200 mg dose of both drugs showed better protection to restoring the liver markers parameter, antioxidant markers and cytokines towards normal than 100 mg dose. *E. hirta* is more effective than *A. racemosus* in preventing lipopolysaccharide (LPS) induced oxidative stress.

Since selected plants extracts showed significant antioxidant activity, hence this study may offer new prospective for oxidative stress induced diseases viz. hepatic disorder and neurodegenerative disease.

Further efforts may be made to isolate the active from the extracts of selected plants material followed by their exact mechanism of action.